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An Overview of Acute Myeloid Leukemia and Cancer Immunology: (i) Concepts and Therapeutic Strategies and (ii) RepSox as a Candidate Cell-Engineering Tool

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ABSTRACT

An Overview of Acute Myeloid Leukemia and Cancer Immunology:

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(ii) RepSox as a Candidate Cell-Engineering Tool

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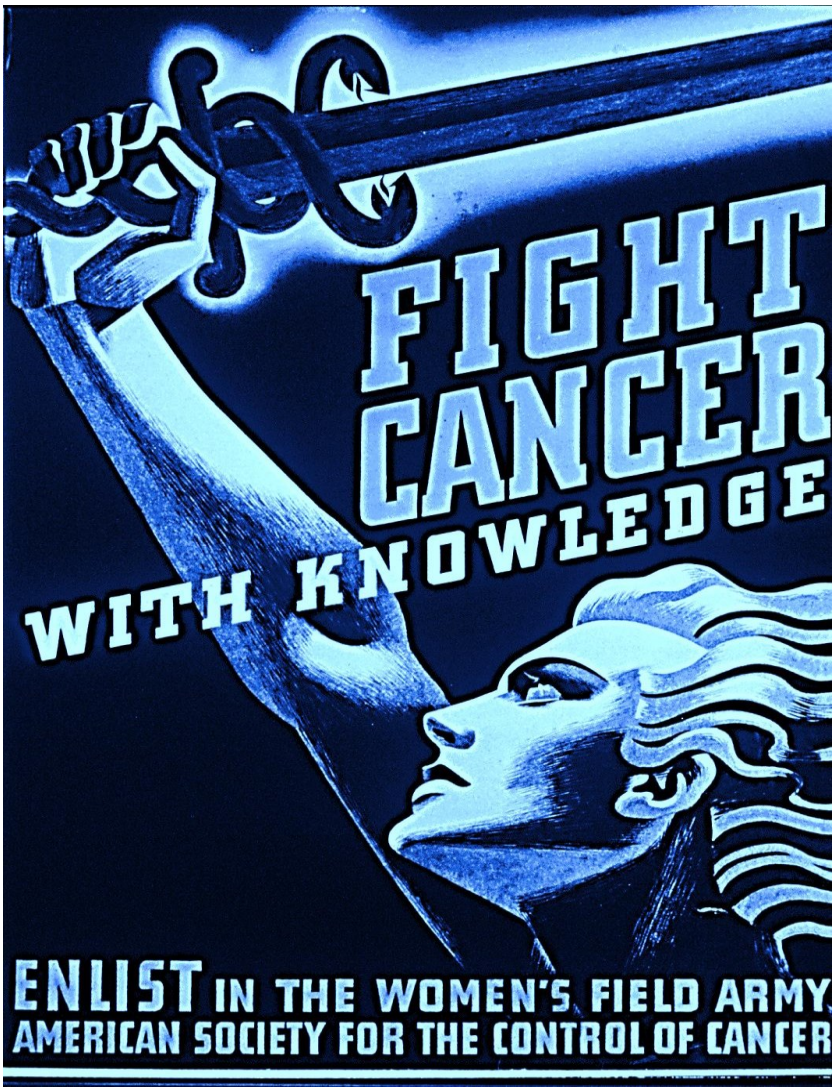
This dissertation is intentionally broad in scope and describes the conceptual frameworks, research advances, and clinical successes that inspired a therapeutic vision that, in turn, prompted specific RepSox experiments (Jajosky, 2014). The goal of this dissertation is to guide and encourage students interested in engineering anti-cancer immune therapies by providing perspective and suggestions. **Chapter I** provides background on acute myeloid leukemia (AML), the cancer stem cell (CSC) theory, and the chemical reprogramming tool "RepSox." **Chapter II** describes how tumor cells passively evade immune recognition and actively suppress immune cells to escape destruction. Immunotherapeutic strategies are described that increase tumor-cell immunogenicity and/or sensitize tumor cells to immune-mediated death. **Chapter III** reviews immune-cell defects induced by cancer-distorted microenvironments. Tumor cells alter local physical and metabolic conditions and distort surrounding stromal cells in ways that impair infiltrating immune cells and promote cancer progression. Strategies are described that can reverse immune-cell defects and improve anti-tumor immunity. **Chapter IV** highlights successful cancer immunotherapies, including patient-specific, FDA-approved, and AML leukemic stem cell (LSC)-targeted strategies. These therapies involve antibodies, activated immune cells, and/or immuno-modulatory agents designed to eradicate tumor cells, repair and activate dysfunctional immune cells, and reduce cancer-induced immune

suppression in tumor microenvironments. **Chapter V** describes the features and rationale of a therapeutic vision that, in combination with recent clinical and research findings, guided this project and identified specific technical obstacles. **Chapter VI** is the published study – entitled "RepSox slows decay of $CD34^+$ acute myeloid leukemia cells and decreases T cell immunoglobulin mucin-3 expression" – which describes how RepSox, a "small molecule" reprogramming tool and TGF-β inhibitor, affects AML cells. The key findings are RepSox (1) slows decay of primary $CD34^+$ AML cells from patients with diverse AML disease, (2) increases *CXCL12* and *MYC*, and (3) accelerates loss of Tim-3, an inhibitory (immune-checkpoint) receptor, from the surface of AML cells. Thus, RepSox may promote *in vitro* engineering of patient-specific AML LSC-targeted therapies by prolonging survival of primitive $CD34^+$ AML cells and increasing AML-cell immunogenicity via Tim-3 reduction. When envisioning the immunologic synapse between antigen-presenting AML cells and T cells, the actions of RepSox suggest RepSox might promote *in vitro* T-cell activation against primitive (relapse-causing) AML cells which represent the most problematic therapeutic target. **Chapter VII** discusses the potential therapeutic applications of these results in the context of AML and other cancers as well as unanswered questions and future research options. For example, the actions of RepSox suggest that its incorporation into pre-existing co-culture methods that exploit TCR agonists may enhance the activation of γδ T cells against a patient's primitive AML cells and, thereby, help generate more effective immune-cell therapies. One goal of this dissertation is to encourage students to integrate new findings and conceptual frameworks from immunology, regenerative medicine, and cancer research when deciding which exciting research agendas to pursue.

DEDICATION

This project was sustained by reviewing, as needed, fascinating stories about past cancer fighters – like those remarkable volunteers in the "Women's Field Army" (1936) immortalized by their "sword of hope" – and by receiving support from the optimistic and creative students and faculty at West Virginia University. In my future endeavors as a physician-scientist, I hope to honor the memory of NCI's Dr. Alessandra Margherita Bini whose death – by cancer – prevented me from thanking her for four years of NIH support.



ACKNOWLEDGMENTS

Patients treated at WVU's Mary Babb Randolph Cancer Center donated their leukemia specimens so this research could be conducted. To them I am indebted, and I intend to "pay their altruism forward." My mentor, Dr. Laura Gibson, members of my PhD committee, Dr. Linda Vona-Davis, Dr. Steven Frisch, Dr. Karen Martin, Dr. Michael Ruppert, Dr. William Tse, and Dr. Fred Minnear were helpful in ways that went beyond insightful suggestions and guidance. Most notably, they fostered a creative environment that inspired me to "have no fear," design my own project, and have it critiqued by experts at the National Cancer Institute (NCI). For four years, a sense of gratitude for the NCI grant award motivated me – especially when working in the lab at 3-am. Cancer researchers at NCI felt I had potential, and I am grateful. At WVU, the examples set by clinicians and scientists showed me how to collaborate and have fun while learning from others. Staff in the Gibson lab, WVU's Pathology Department, and flow cytometry and imaging facilities provided not just technical help but laughs and wonderful memories that make me smile. WVU's "Osborn Program" retreats reminded me that patients are the reason we work as hard as we do and that human suffering engenders a sense of urgency that can drive the creativity that helps us solve technical problems. Daniel Vanderbilt, my MD-PhD classmate, has been a wonderful friend. His constant support, commitment to science, and words of encouragement have inspired and comforted me. Overall, I am impressed by how the MD-PhD community at WVU has succeeded in creating a nurturing and creative intellectual environment. I am grateful to the patients, scientists, physicians, staff, students – and supportive neighbors – for all the fun and laughs in Morgantown, a truly exciting "college town." Go Mountaineers!

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LIST OF ABBREVIATIONS

2i	2 inhibitor
3-D	3-dimensional
DZNep	3-deazaneplanocin A
AhR	aryl hydrocarbon receptor
aka	also known as
ALDH	aldehyde dehydrogenase
ALK5	activin-like receptor 5
AML	acute myeloid leukemia
APC	antigen-presenting cell
ATP	adenosine triphosphate
ATRA	all-trans retinoic acid
Bcl-2	B cell lymphoma 2
BCR/ABL	breakpoint cluster region/abl protein tyrosine kinase
BrHPP	bromohydrin pyrophosphate
CAF	cancer-associated fibroblast
CAR	chimeric antigen receptor
CBF	core binding factor
C/EBP	CCAAT-enhancer-binding proteins
CLIP	class II-associated invariant chain peptide
CLL	chronic lymphocytic leukemia
CPP	cell-penetrating protein
CSC	cancer stem cell
CTLA-4	cytotoxic T lymphocyte antigen 4
CXCL12	C-X-C motif chemokine 12
CXCR4	chemokine C-X-C motif receptor 4
DC	dendritic cell
DMSO	dimethyl sulfoxide
dsRNA	double-stranded RNA
EMT	epithelial to mesenchymal transition
ETO	eight twenty one
FAB	French-American-British
FLIP	FLICE (FADD-like IL-1β-converting enzyme) inhibitory protein
FLT3	fms-related tyrosine kinase 3
FoxP3	forkhead box P3
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GSK3-β	glycogen synthase kinase 3 beta
GVHD	graft-versus-host disease
GvL	graft-versus-leukemia
HDAC	histone deacetylase inhibitor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen

HMGB1	high mobility group box 1
HOXA9	homeobox A9
HOXA10	homeobox A10
HPC	hematopoietic progenitor cell
HRG	histidine-rich glycoprotein
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplant
ICD	immunogenic cell death
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
IL3Rα	interleukin-3 receptor alpha
iPSC	induced pluripotent stem cell
ITD	internal tandem duplication
JAK2	janus kinase 2
LAA	leukemia-associated antigen
LSC	leukemic stem cell
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence 1
MDS	myelodysplastic syndrome
MDSC	myeloid-derived suppressor cell
MEF	mouse embryonic fibroblast
MET	mesenchymal to epithelial transition
MHC	major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
miRNA	microRNA
MLL	mixed lineage leukemia
MRD	minimal residual disease
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NCI	National Cancer Institute (USA)
NIH	National Institutes of Health (USA)
NK	natural killer
NKT	natural killer T
NKG2D	natural killer group 2 member D
NPM1	nucleophosmin
P2RX7	purinergic receptor P2X ligand-gated ion channel 7
PD1	programmed cell death 1
PD1L	programmed cell death 1 ligand
PDGFRβ	platelet-derived growth factor receptor beta
PI3K	phosphatidylinositide 3-kinase
Poly(I:C)	polyinosinic polycytidylic acid
PRAME	preferentially expressed antigen in melanoma

RARα	retinoic acid receptor alpha
RHAMM	hyaluronan-mediated motility receptor
RIG-1	retinoic acid-inducible gene 1
RUNX1	runt-related transcription factor 1
RUNX3	runt-related transcription factor 3
SAH	S-adenosylhomocysteine hydrolase
Sall4	Sal-like 4
SDF-1	stromal cell-derived factor 1
siRNA	small interfering RNA
SIRPα	signal regulatory protein alpha
Smad proteins	homologs of the Drosophila protein “mothers against decapentaplegia” (MAD)
STAT3	signal transducer and activator of transcription 3
STAT6	signal transducer and activator of transcription 6
TAM	tumor-associated macrophage
TAP	transporter associated with antigen processing
Tcl1a	T cell leukemia/lymphoma 1A
TEL oncogene	another name for the ETV6 oncogene that encodes an ETS family transcription factor
TGF-β	transforming growth factor beta
T_h cells	T helper cells
Tim-3	T cell immunoglobulin mucin-3
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
T_{regs}	regulatory T cells
VPA	valproic acid
WBC	white blood cell
WHO	World Health Organization
WT1	Wilms tumor 1
WVU	West Virginia University

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PREFACE

Deciding to write a dissertation very broad in scope is consistent with the decision made by the *Journal for ImmunoTherapy of Cancer* to publish in 2014 an overview entitled "Tumor immunology and cancer immunotherapy: summary of the 2013 SITC primer" authored by Raval, et al. Scientists seem to agree that the exciting anti-cancer breakthroughs and opportunities that have emerged recently should be carefully described and explained. This is an ideal time to encourage students to consider getting involved in the engineering of anti-cancer immunotherapies.

In general, researchers are now extremely optimistic, and are eager to recuit new cancer fighters. Fortunately, advances in cancer immunotherapies are truly fascinating and, when properly explained, usually generate great excitement. The pattern is encouraging: new lab observations, conceptual frameworks, and clinical findings have suggested novel anti-cancer strategies that have, in turn, prompted scientists to identify, and successfully overcome, key technical challenges. While planning this dissertation, it was decided that explaining the reasoning used by cancer researchers could be instructive. Also, to help students fully appreciate current research opportunities, an effort was made to explain the context surrounding new concepts and technical advances.

Although this dissertation is broad, readers should be able to read just the sections of interest since topics were designed to be "logically complete." Thus, redundancy was unavoidable: some of the same concepts, goals, tasks, and findings are repeated in several different locations. Of note, when discussing these topics at meetings, I found that repetition was often needed to eliminate confusion. Some concepts are, apparently, counter-intuitive or "too new or different." For example, some were confused about the

"paradox" of working hard to keep the most lethal, stem-like leukemia cells alive. As might be expected, some immediately think about killing these lethal cells before considering how *in vitro* tasks that keep them alive can be used to engineer anti-LSC therapies. (Coincidentally, to develop anti-ebola therapies, scientists are trying to keep lethal ebola viruses alive *in vitro*.) Five points that often needed to be explained repeatedly included the following: (1) AML cells can directly function as antigen-presenting cells (when, for example, they are co-cultured with γ9δ2 T cells). (2) Spontaneous de-differentiation (of normal cells) is critical for tissue regeneration in primitive animals and for sustaining human (malignant) cancer cells – while de-differentiation artificially induced *in vitro* can repair the dysfunctional (non-malignant) immune cells of cancer patients. (3) At any given time, a cancer patient harbors a distribution of malignant cells that includes a distribution of CSCs that evolves as the cancer progresses. That is, the phenotypes of CSCs evolve: there is not just a single, static CSC phenotype. (4) Human cancer cells are "plastic" and can de-differentiate – as well as differentiate and evolve. Thus, mature cancer cells must be eliminated as well as the CSCs. And, (5) when *in vitro* chemical cell-engineering is used to reprogram mature cells into pluripotent cells, some of the genes/mechanisms involved are the same as those activated during the spontaneous (and limited) cellular de-differentiation that occurs during injury-induced tissue regeneration. Overall, this dissertation tries to provide perspective and explain the rationale for this project and other research agendas. A key premise: to fully appreciate the opportunities that are exciting cancer researchers, the fascinating context for recent successes should be considered.

Chapter I

Background on Acute Myeloid Leukemia, the Cancer Stem Cell Theory, and the TGF-β inhibitor “RepSox”

Acute myeloid leukemia is a white blood cell cancer involving abnormal, immature myeloid cells that rapidly proliferate, continuously evolve, and efficiently disrupt and evade immunologic attack. Accumulation of large numbers of malignant AML cells in the bone marrow suppresses normal hematopoiesis. Due to inadequate numbers of nomal white blood cells (i.e. neutrophils, macrophages), red blood cells, and platelets, AML patients may present with infections, anemia, or easy bleeding. Among the acute adult leukemias in North America and Europe, AML is the most common (90% of cases). Among all cancers of the elderly, AML has one of the highest mortality rates.

Pathogenesis

Leukemia may arise via transformation of normal hematopoietic stem cells or de-differentiation of more committed blood progenitors (Passegué; 2003) as shown in Figure 1.1. Transforming events – initiated by genetic or epigenetic alterations – may bestow progenitors with stem-cell properties such as enhanced self-renewal. Regardless of cellular origin, leukemia is believed to be sustained by self-renewing leukemic stem cells (LSCs) (Lapidot, 1994; Bonnett, 1997).

Chronic myeloid leukemias often involve activating mutations in tyrosine kinases, such as BCR/ABL, TEL/PDGFRβ, TEL/ABL, and TEL/JAK2 (Deguchi, 2002). In contrast, acute leukemias usually involve chromosomal alterations or mutations of transcription factors or transcriptional regulators, such as core binding factor (CBF) comprised of runt-related transcription factor 1 (RUNX1) and CBFβ subunits, retinoic acid receptor α (RARα), and the *mixed lineage leukemia* (*MLL)* gene (Deguchi, 2002). Genetic lesions often occur in transcription factors that regulate hematopoiesis, leading to maturation arrest of myeloid progenitors.

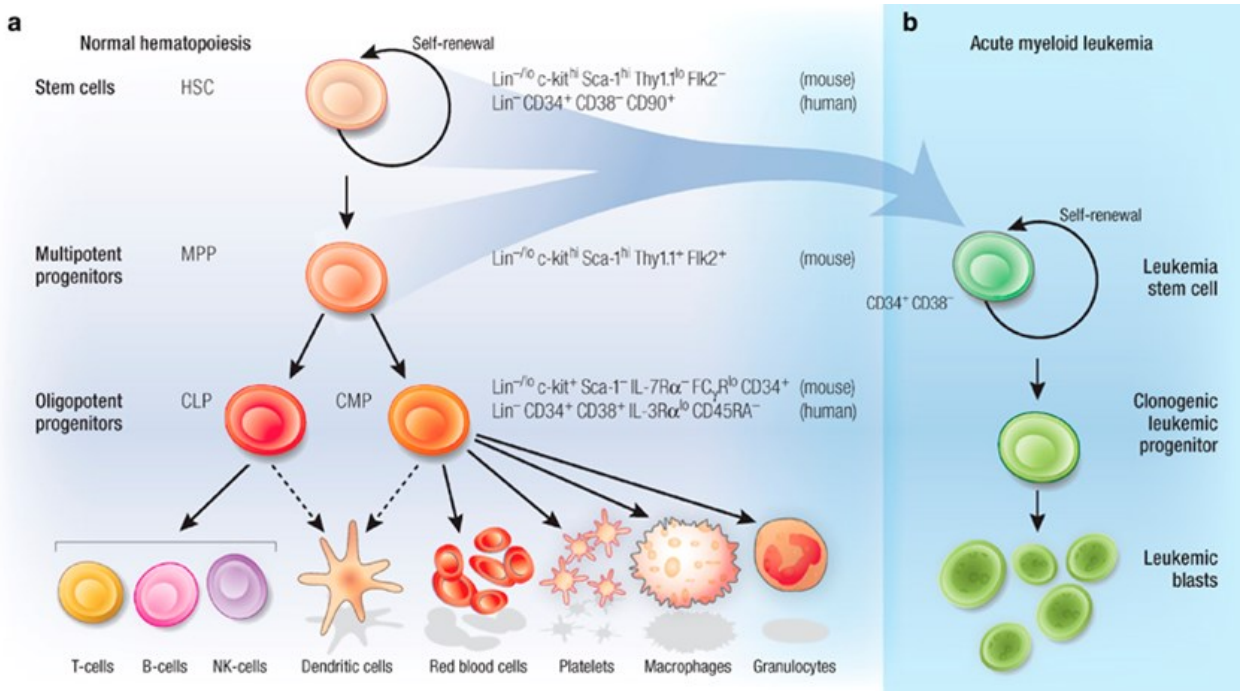

Figure 1.1. Cellular hierarchy within normal hematopoiesis and leukemic disease. During normal blood cell development, self-renewing hematopoietic stem cells (HSCs) give rise to progressively lineage-restricted progenitor cells whose progeny generate mature white blood cells, red blood cells, and platelets. Acute myeloid leukemia (AML) may arise from leukemic transformation of normal HSCs or restricted progenitor cells. According to the cancer stem cell (CSC) theory, AML is sustained by self-renewing leukemic stem cells (LSCs) that ultimately give rise to more mature leukemic blasts. *Reproduced with permission from:* Tan, B. T., Park, C. Y., Ailles, L. E., et al. (2006). The cancer stem cell hypothesis: a work in progress. *Laboratory Investigation*, *86*(12), 1204.

Two-hit model of AML

Gilliland and colleagues proposed the 2-hit model of AML in which leukemic transformation involves cooperation between two types of mutations: class I mutations that promote growth and/or survival and class II mutations that block differentiation and/or enhance self-renewal (Dash, 2001; Gilliland, 2001). Class II mutations generally occur early and are believed to help initiate disease, while class I mutations occur late and likely promote leukemic progression. Class I mutations activate signal-transduction pathways often through constitutive activation of tyrosine kinase surface receptors such as fms-related tyrosine kinase 3 (FLT3) and c-KIT (Gilliland, 2001). In contrast, class II mutations affect transcription factors or other transcriptional regulators. Examples of class II alterations include overexpression of homeobox (HOX) genes, mutations in hematopoietic transcription factors such as C/EBPα, *MLL* gene rearrangements, and creation of fusion genes such as t(8;21) RUNX1-ETO, t(15;17) PML-RARα, and inv(16) CBFβ-MYH11 (Gilliland, 2001). Study of human disease and experimental evidence from mouse models support the 2-hit hypothesis. Combinations of class I and class II mutations, such as internal tandem duplication of the FLT3 receptor (FLT3-ITD) and nucleophosmin (NPM1) mutation, coexist in AML patients (Golub, 1995; Reilly, 2005; Matsuno, 2003). Mouse studies show that hematopoietic progenitors carrying either class I or class II mutations often trigger aberrant myeloid development or myelo-proliferative disorders (Kelly, 2002), while both mutations cooperate to generate leukemia (Schessl, 2005; Grisolano, 2003).

Subtypes

AML is a heterogeneous disease arising from a variety of pathogenic mechanisms. Along with clinical history, AML is diagnosed by examining the morphology, cytochemistry, immunophenotype, and molecular genetics of cells in the bone marrow. According to the French-American-British (FAB) classification system (see Table 1.1), AML is divided into nine subtypes based on the myeloid lineage(s) involved and their degree of differentiation. The newer World Health Organization (WHO) classification system (see Table 1.2) also incorporates cytogenetic and molecular genetic features which provide additional prognostic value (Swerdllow, 2008). The "Immunoscore," another classification system under development, proposes that the immune-cell infiltrate within a patient's tumor can predict prognosis and response to therapy (Galon, 2012; Fridman, 2012).

The French-American-British (FAB) Classification of AML and Associated Genetic Abnormalities

FAB Subtype	Common Name (% of Cases)	Results of Staining			Associated Translocations and Rearrangements (% of Cases)	Genes Involved
		Myeloperoxidase	Sudan Black	Nonspecific Esterase		
M0	Acute myeloblastic leukemia with minimal differentiation (3%)	−	−	−*	inv(3q26) and t(3;3) (1%)	*EVI1*
M1	Acute myeloblastic leukemia without maturation (15–20%)	+	+	−		
M2	Acute myeloblastic leukemia with maturation (25–30%)	+	+	−	t(8;21) (40%), t(6;9) (1%)	*AML1-ETO*, *DEK-CAN*
M3	Acute promyelocytic leukemia (5–10%)	+	+	−	t(15;17) (98%), t(11;17) (1%), t(5;17) (1%)	*PML-RARα*, *PLZF-RARα*, *NPM RARα*
M4	Acute myelomonocytic leukemia (20%)	+	+	+	11q23 (20%), inv(3q26) and t(3;3) (3%), t(6;9) (1%)	*MLL*, *DEK-CAN*, *EVI1*
M4Eo	Acute myelomonocytic leukemia with abnormal eosinophils (5–10%)	+	+	+	inv(16), t(16;16) (80%)	*CBFβ-MYH11*
M5	Acute monocytic leukemia (2–9%)	−	−	+	11q23 (20%), t(8;16) (2%)	*MLL*, *MOZ-CBP*
M6	Erythroleukemia (3–5%)	+	+	−		
M7	Acute megakaryocytic leukemia (3–12%)	−	−	+†	t(1;22) (5%)	Unknown

*Cells are positive for myeloid antigen (e.g., CD13 and CD33).

†Cells are positive for α-naphthylacetate and platelet glycoprotein IIb/IIIa or factor VIII–related antigen and negative for naphthylbutyrate.

Table 1.1. The FAB classification of AML. *Reproduced with permission from*: Lowenberg, B., Downing, J. R., & Burnett, A. (1999). Acute myeloid leukemia. *New England Journal of Medicine*, *341*(14), 1052. Copyright Massachusetts Medical Society.

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13,1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
AML with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated *NPM1*
Provisional entity: AML with mutated *CEBPA*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemias
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis

Table 1.2. The 2008 WHO classification of AML. *Reproduced with permission from*: http://www.cancernetwork.com/articles/acute-leukemias/page/0/2

Treatment

Standard treatment for AML includes induction chemotherapy followed by consolidation therapy. The goal of induction chemotherapy is to eradicate the majority of leukemic blasts and induce remission. With the standard "7 + 3" induction regimen of cytarabine for 7 days along with daunorubicin for the first 3 days, 70-80% of younger (< 60) and 50% of older (> 60) adults achieve complete remission (Lowenberg, 1999). The goal of consolidation therapy – intensive chemotherapy, such as high dose cytarabine, or hematopoietic stem cell transplant (HSCT) – is to eliminate "residual" leukemia cells and, thereby, prevent relapse. If patients cannot tolerate additional rounds of intensive chemotherapy, they may receive a HSCT. Sources of HSCs for transplant include the bone marrow or mobilized peripheral blood of the patient (autologous) or a donor (allogeneic). With allogeneic-HSCT, a post-remission strategy with the lowest rate of relapse, donor T cells attack residual leukemia cells via a graft-versus-leukemia (GvL) immune reaction (Thomas, 1999; Jenq, 2010). A major complication of allogeneic HSCT is graft-versus-host disease (GvHD) (Thomas, 1999). Acute promyelocytic leukemia (APL; also known as M3 subtype), characterized by a translocation between chromosomes 15 and 17 [t(15;17)], is unique in its treatment with all-*trans* retinoic acid (ATRA) which induces APL cells to mature (Tallman, 1997).

Although more than half (50%-70%) of AML patients achieve complete remission after induction chemotherapy, the majority (70%-80%) will relapse and die of disease (Lowenberg, 1999; Döhner, 2010). Patients with poor prognosis, or those who fail to respond to standard therapy, may undergo experimental therapies in clinical trials. Immunotherapies (discussed in **Chapter IV**) may be most effective in the setting of minimal residual disease (MRD) when leukemic disease burden is low. Of note, LSCs

are often enriched during MRD (Gerber, 2012), suggesting they are more likely to escape conventional chemotherapy than bulk leukemic blasts – potentially due to their quiescent nature (Terpstra, 1996) or the activity of drug efflux pumps (Wulf, 2001). Thus, by design, immunotherapies should target both the LSC population that sustains disease and the more mature leukemic blast population which (like melanoma cells) may be able to de-differentiate into stem–like tumor cells when exposed to therapeutic stresses or microenvironmental stresses like chronic inflammation (Landsberg, 2012). Beyond initial control of disease, immune strategies have the potential to provide "cures" via generation of memory T and B cells that can provide long-term protection (Kalos, 2011; Porter, 2011).

Leukemic stem cells (LSCs)

Background

Cancer stem cells (CSCs) were first identified in AML (Lapidot, 1994). Similar to normal hematopoiesis, AML is arranged as a hierarchy originating from LSCs (aka "leukemia-initiating cells") that self-renew and give rise to more mature leukemic blasts (Bonnett, 1997; Hope, 2004). Experimentally, only a small subset of AML cells generates colonies *in vitro* (McCulloch, 1983; Griffin, 1986) and initiates disease when transplanted into immunocompromised mice (Lapidot, 1994; Bonnett, 1997). These findings support a hierarchical organization of AML, similar to normal hematopoiesis (Bonnett, 1997; Hope, 2004). In mice, disease-initiating cells generate the diverse spectrum of AML cells found in patients and can reconstitute disease in secondary recipients, demonstrating a capacity for self-renewal (Lapidot, 1994; Bonnett, 1997). Based on clonal tracking studies that identify cells descending from a single progenitor, the LSC compartment has been shown to contain subsets of LSCs which differ in self-renewal potential (Hope, 2004).

Like normal HSCs, LSCs can display short-term or long-term repopulating capacity/potential (Hope, 2004). Therapeutically targeting LSCs *in vivo* is challenging: LSC populations may be quiescent (Terpstra, 1996), poorly immunogenic (Costello, 1999), immune- and death-resistant (van Stijn, 2003; Barrett, 2010; Lion, 2012), and phenotypically diverse (Eppert, 2011; Goardon, 2011). Furthermore, in response to stresses imposed by the tumor microenvironment, immune system, or cancer therapies, mature leukemia cells may de-differentiate into LSCs. Within any given AML patient, multiple pools of LSCs which differ in molecular and phenotypic traits may coexist (Goardon, 2011). Cancer-cell diversity and plasticity suggest that, for some patients, combination therapies may be needed to (1) attack both LSCs and more mature leukemic blasts, (2) target multiple tumor antigens (to prevent emergence of escape variants), and (3) reverse tumor-cell immune evasion, immune-system suppression, and cancer-induced dysregulations of the tumor microenvironment.

LSCs dynamically interact with the surrounding bone marrow niche, as shown in Figure 1.2. LSCs alter local physical and metabolic conditions, convert stromal cells into cancer-associated fibroblasts (CAFs), suppress infiltrating immune cells, and recruit immunosuppressive cells that support the growth and survival of LSCs by, for example, promoting “immune escape.”

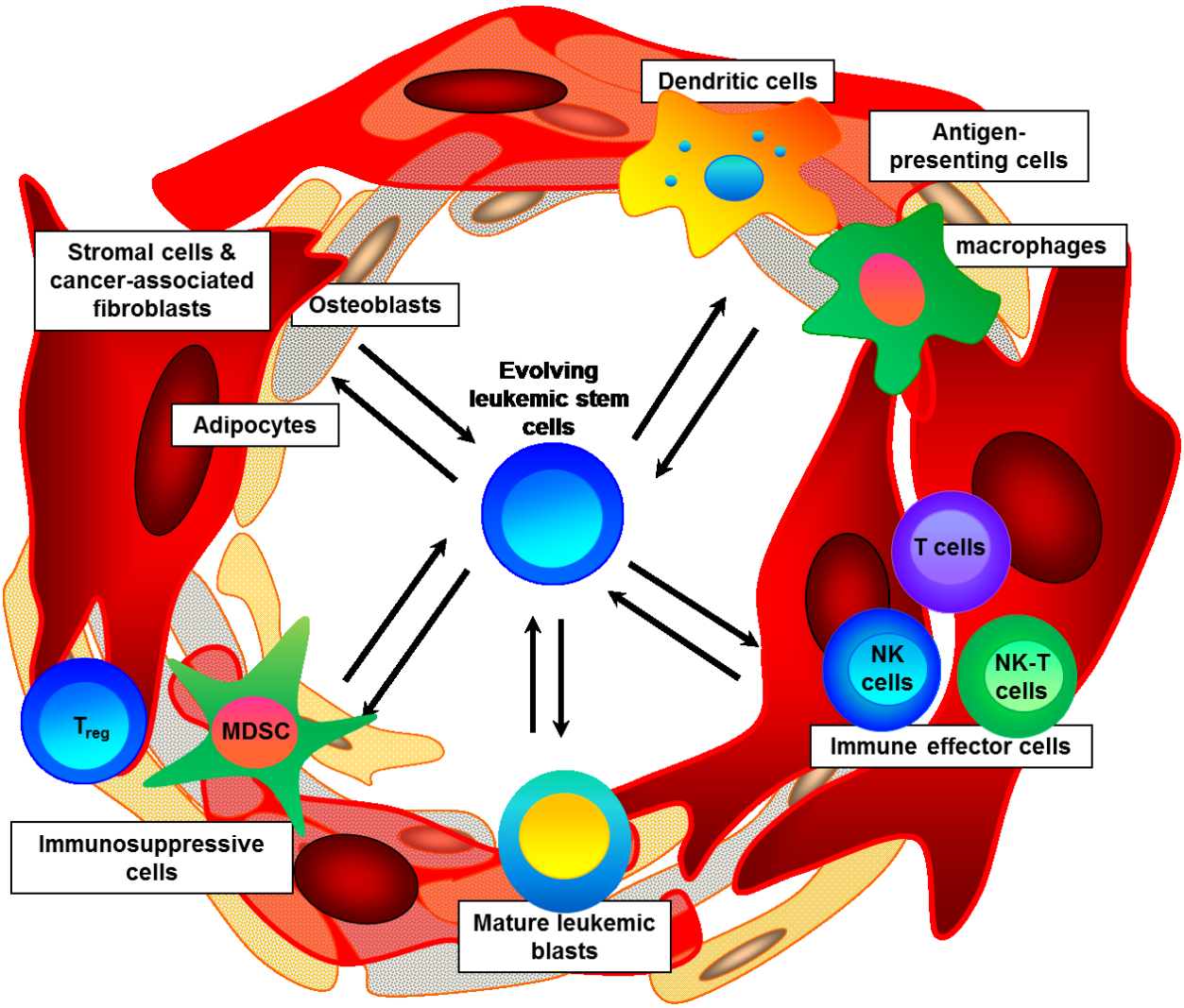

Figure 1.2. Reciprocal interactions between leukemic stem cells and the tumor bone marrow microenvironment. Within the bone marrow microenvironment, leukemia cells alter physical and metabolic conditions as well as interact with surrounding stromal cells and infiltrating immune cells. Leukemic cells deplete local oxygen and nutrients, convert stromal cells into tumor-promoting CAFs, and suppress infiltrating immune cells to promote disease progression. In reaction to microenvironmental stresses like immune selection pressures, more mature leukemia cells may dedifferentiate into LSCs as a pro-survival mechanism. The bone marrow microenvironment also nurtures leukemic cells and protects them from chemotherapy.

Clinical significance

LSCs that survive conventional chemotherapy may trigger relapse of disease. Clinical observations support the CSC theory of AML: $CD34^{+}CD38^{-}ALDH^{int}$ AML cells were enriched during MRD, and persistence of this AML subset after induction chemotherapy was highly predictive of patients who would subsequently relapse (Gerber, 2012). AML LSC gene expression signatures generated by independent research groups correlate with patient outcomes (Eppert, 2011; Gentles, 2010). Using a signature of 52 genes upregulated in $CD34^{+}CD38^{-}$ LSC-enriched fractions (as opposed to more differentiated $CD34^{+}CD38^{+}$ and $CD34^{-}$ fractions), high LSC scores were associated with worse overall, event-free, and disease-free survival than low LSC scores in multiple AML patient cohorts (Gentles, 2010). Rather than relying solely on immunophenotype, Eppert and colleagues generated a 42-gene LSC signature using AML LSC-enriched fractions functionally verified to initiate disease in mice (Eppert, 2011). Likewise, high LSC scores were generally associated with shorter overall and event-free survival as well as lower rates of complete remission than low LSC scores (Eppert, 2011). These studies suggest that AML LSCs are not simply artifacts of xenotransplantation but have clinical relevance. These LSC signatures may also help identify poor-risk cases of AML, drivers of LSC function, and potential LSC targets.

AML LSC markers

Across disease subtypes, disease-initiating AML LSCs were originally reported to reside exclusively within the $CD34^{+}CD38^{-}$ fraction of AML cells when assayed in severe combined immune deficiency (SCID) mice with B- and T-cell defects and non-obese diabetic (NOD)/SCID mice carrying additional NK-cell defects (Lapidot, 1994; Bonnett,

1997). Studies utilizing more immunodeficient mouse strains, such as NOD/SCID/IL-2 receptor γ chain-deficient and NOD/SCID/β2 microglobulin-null mice that lack NK-cell activity, show that disease-initiating LSCs can also reside within the more mature $CD34^+CD38^+$ or $CD34^-$ fractions previously believed to be non-tumorigenic (Taussig, 2008; Taussig, 2010; Sarry, 2011). Because LSC immunophenotypes vary both within (Goardon, 2011) and across (Eppert, 2011) patients, engineering of LSC-targeted therapies may require patient-specific strategies.

The discovery of cellular and molecular differences between AML LSCs and normal HSCs has identified potential therapeutic targets. Surface antigens upregulated on AML LSCs relative to normal HSCs include Tim-3 (Jan, 2011), CD47 (Majeti, 2009), CD123 (Jordan, 2000), and CD96 (Hosen, 2007) among others (Horton, 2012). Of interest, several of these receptors, including CD47 (a phagocyte "do not eat me" signal) and Tim-3 (a negative regulator of anti-tumor immunity), inhibit immune-cell functions and may contribute to the poor immunogenicity of AML LSCs (Costello, 1999; Jaiswal, 2009; Majeti, 2009). Strategies to target surface antigens using antibodies or antigen-specific immune cells, such as chimeric antigen receptor (CAR)-modified T cells, are discussed in **Chapter IV**.

Additional molecular differences between AML LSCs and normal HSCs include distinct patterns of ALDH staining (Pearce, 2005) as well as enhanced NFkB activity (Guzman, 2001) and apoptotic resistance (van Stijn, 2003; Yoshimoto, 2009) within LSCs. Normal HSCs are Aldefluor-bright ($ALDH^{BR}$) cells, while AML LSCs display intermediate levels of Aldefluor staining ($ALDH^{int}$) (Pearce, 2005), providing a convenient way to isolate and/or monitor both populations. Strategies to target LSCs now under

clinical investigation include inhibitors of NFkB activity (Reikvam, 2009) and anti-apoptotic factors of the Bcl-2 family (Bose, 2013).

CD34

CD34 is a single transmembrane surface glycoprotein expressed by a spectrum of normal (Sutherland, 1992; Larochelle, 1996) and leukemic (Lapidot, 1994; Bonnett, 1997) stem and progenitor cells. Experimental studies suggest CD34 can modulate cell-to-cell adhesion, enhance proliferation, and inhibit hematopoietic differentiation (Krause, 1996; Nielson, 2008). CD34 has been proposed to block the differentiation of both normal hematopoietic and leukemic progenitors (Fackler, 1995; Krause, 1996). Of note, during normal hematopoiesis, CD34 levels are highest on immature progenitors and decline with differentiation (Krause, 1996; Stella, 1995). Enforced expression of full-length CD34 on mouse M1 leukemia cells inhibited their cytokine-induced differentiation into macrophages (Fackler, 1995). Compared to wildtype mice, *CD34*-knockout mice showed delayed myeloid and erythroid differentiation and decreased hematopoietic progenitors in embryonic and adult tissues (Cheng, 1996). Despite these defects, *CD34*-knockout mice developed normally and displayed normal mature blood cell counts (Cheng, 1996; Suzuki, 1996). Defects in myeloid and erythroid differentiation within *CD34*-null embryonic stem cells were reversed upon transfection with CD34 (Cheng, 1996). CD34 is expressed by 40-60% of AML patients and is most commonly observed within the more primitive M0, M1, and M2 subtypes (Borowitz, 1989). CD34 can sometimes serve as an “LSC marker” that is linked to the poor prognosis of AML patients (Geller, 1990; Myint; 1992), although conflicting results have been reported.

Tim-3

In cancer patients, Tim-3 has been reported to be expressed by (1) tumor cells (Wiener, 2007; Jan, 2011; Kikushige, 2010), (2) dysfunctional antigen-presenting and immune-effector cells of the innate and adaptive immune system (Zhu, 2005; Anderson, 2012; Chiba, 2012; Da Silva, 2014), and (3) endothelial cells within the tumor vasculature (Huang, 2010; Wu, 2010). With the exception of acute promyelocytic leukemia (M3 subtype), Tim-3 is often expressed by myelodysplastic syndrome (MDS) and AML cells, including the $CD34^{+}CD38^{-}$ subset (Kikushige, 2010; Kikushige, 2013). Tim-3 is generally upregulated upon the progression of MDS to AML, suggesting a potential role for Tim-3 in leukemogenesis (Kikushige, 2013). Because Tim-3 is not expressed by normal $CD34^{+}CD38^{-}$ hematopoietic stem and progenitor cells (Kikushige, 2010; Jan, 2011), Tim-3 receptor is an attractive therapeutic target for eliminating AML LSCs (Kikushige, 2010), restoring the functions of cancer-impaired immune cells (Zhou, 2011; Norde, 2012), and reversing immune suppression mediated by the tumor stroma (Huang, 2010).

The Tim-3 pathway may promote AML-cell survival. Galectin-9 and high mobility group box 1 (HMGB1) are the ligands of Tim-3 on lymphocytes (Zhu, 2005) and dendritic cells (DCs) (Tang, 2012). Upon toll-like receptor (TLR) stimulation, primary AML cells secrete galectin-9, and Tim-3 engagement by galectin-9 may promote survival of AML cells through upregulation of the anti-apoptotic factor myeloid cell leukemia 1 (Mcl-1) (Kikushige, 2013). Tim-3 is also expressed by a variety of solid tumors, including melanoma (Wiener, 2007), osteosarcoma (Shang, 2013), lung cancer (Zhuang, 2012), and cervical cancer (Cao, 2013) where it may promote cancer-cell immune evasion, migration, and invasion. The role of Tim-3 on cancer-impaired antigen-presenting, immune-effector, and stromal cells is reviewed in **Chapter IV**.

TGF-β has been shown to induce Tim-3 expression on mast cells (Wiener, 2007; Kim, 2012). As emphasized in this dissertation, treatment of AML cells with the TGF-β inhibitor RepSox accelerated the decline in Tim-3 expression (Jajosky, 2014). Thus, these findings suggest a mechanism that might explain how TGF-β can function as an immuno-suppressive cytokine in some stages of malignant disease (Gorelik, 2002; Wrzesinski, 2007).

Challenges to developing LSC- targeted therapies

Engineering anti-LSC therapies *in vitro* is challenging because LSCs (1) rapidly die or differentiate in culture (Pabst, 2014), (2) are poorly immunogenic (Costello, 1999; Majeti, 2009), (3) display diverse immunophenotypes (Eppert, 2011; Goardon, 2011), and (4) constitute a "moving target" as they continually evolve *in vivo* under constant immune-selection pressures (Horton, 2012). Furthermore, tumor microenvironments also become progressively dysregulated in ways that promote cancer cells and suppress infiltrating immune cells (Kim, 2006). Thus, LSC-targeted therapies may need to be patient-specific and administered shortly after diagnosis. By engineering therapies *in vitro* – outside of the immunosuppressive tumor microenvironment – it is possible to enhance immune-cell activation and reverse defects induced by cancer-distorted microenvironments. Of note, as purified Tim-3^{+} AML cells were cultured, levels of the immune-checkpoint receptor Tim-3 gradually declined, and RepSox accelerated this loss of Tim-3 (**Chapter VI**, Figure 7). In cell culture, just the removal of AML cells from the tumor microenvironment may eventually eliminate all Tim-3 expression and, in turn, increase AML-cell immunogenicity.

In general, better ways to maintain a patient's LSCs in culture could facilitate a variety of valuable technical tasks: the *in vitro* activation of more relevant (anti-LSC) γδ

T cells and other immune cells (by having LSCs serve as antigen-presenting cells), the identification of therapeutic targets, and the evaluation of candidate therapies preclinically. Also, when LSCs in a patient specimen are rare (or absent), it may be possible to generate LSC "surrogates" via de-differentiation of a patient's more mature ("bulk") leukemic blasts into stem-like tumor cells. By analogy, for tissue regeneration, patient-derived induced pluripotent stem cells (iPSCs) (Takahashi, 2007) may serve as artificial, lab-synthesized substitutes for naturally occurring embryonic stem cells (ESCs). LSC surrogates, chemically or genetically engineered *in vitro*, may serve as relevant therapeutic targets by virtue of immunologic cross-reactivity with a patient's *bona fide* leukemic stem and progenitor cells residing *in vivo*. In general, some of the same antigens that can serve as therapeutically useful targets may be expressed by a wide spectrum of leukemic progenitor cells.

RepSox and the TGF-β signaling pathway

TGF-β influences a variety of cellular processes including differentiation, growth, survival, embryogenesis, immune suppression, tissue fibrosis, and epithelial-to-mesenchymal transition (EMT) (Li, 2006; Watabe, 2009; Wu, 2009; Xu, 2009; Massagué, 2012; Travis, 2014). TGF-β-mediated effects are both context- and cell-specific (Massagué, 2000). Figure 1.3 provides an overview of the TGF-β signaling pathway, including TGF-β superfamily ligands, surface receptors, and Smad proteins that regulate gene transcription (Weiss, 2013). Early in tumor development, TGF-β generally functions as a tumor suppressor by inhibiting proliferation, promoting apoptosis, and limiting inflammation in the tumor microenvironment (Gold, 1998; Ikushima, 2010; Zammaron, 2011). In more advanced disease, however, TGF-β facilitates cancer progression by

promoting tumor-cell EMT, invasion, and metastasis as well as angiogenesis and immune suppression (Gold, 1998; Xu, 2009; Katsuno, 2013).

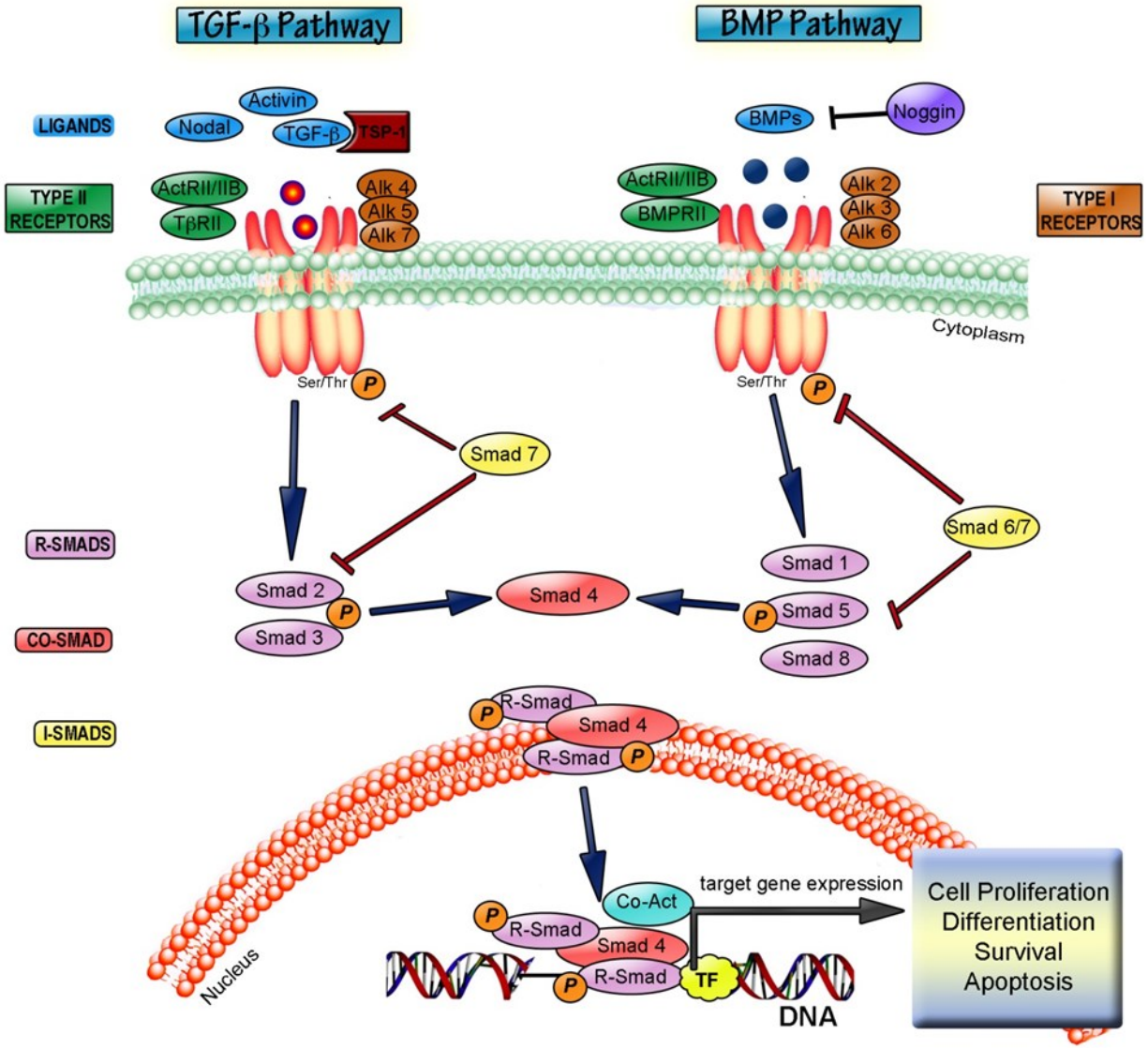

Figure 1.3. The TGF-β signaling pathway. TGF-β superfamily ligands (such as TGF-βs, activins, nodals, and BMPs) bind to type II receptors which recruit, phosphorylate, and activate type I receptors. In the case of TGF-β ligands, activated type I receptors phosphorylate Smad 2/3 which complexes with Smad4 and translocates to the nucleus to regulate gene transcription. RepSox selectively inhibits TGF-β receptor 1/ALK5. © 2013 Sonia Villapol, Trevor T. Logan, Aviva J. Symes. Originally published in: "Villapol, S., Logan, T. T., & Symes, A. J. (2013). Role of TGF-β signaling in neurogenic regions after brain injury, chapter 1, page 5." under CC BY 3.0 license. *Adapted with permission from*: [Derynck, R., & Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-β family signalling. *Nature*, *425*(6958), 577-584. DOI: http://dx.doi.org/10.1038/nature02006] and [Kandasamy M, Reilmann R, Winkler J, et al. TGF-β signaling in the neural stem cell niche: a therapeutic target for Huntington's disease. *Neurology Research International*. 2011. Article ID: 124256. DOI: http://dx.doi.org/10.1155/2011/124256]. Available from: http://dx.doi.org/10.5772/5394.

RepSox, a cell-permeable, small-molecule inhibitor of TGF-β receptor 1 [also known as activin receptor-like kinase 5 (ALK5)] (Gellibert, 2004), promotes cellular reprogramming to pluripotency by replacing Sox2 and c-Myc (Ichida, 2009) and promoting mesenchymal-to-epithelial transition (MET) (Li, 2010). Chemical structures of the other TGF-β inhibitors studied are shown in Figure 1.4. RepSox (also known as "E-616542") is one of the seven small molecules that was used to chemically reprogram mouse fibroblasts to pluripotency – eliminating the need for genetic engineering (Hou, 2013). The other six factors used were (1) histone deacetylase (HDAC) inhibitor valproic acid (VPA), (2) glycogen synthase kinase-3 beta (GSK3-β) inhibitor CHIR99021, (3) monoamine oxidase inhibitor Tranylcypromine, (4) cAMP agonist Forskolin, (5) S-adenosylhomocysteine (SAH) hydrolase inhibitor 3-deazaneplanocin A (DZNep) and (6) "2i treatment" involving dual inhibition of mitogen-activated protein kinase (MAPK) signaling (using PD0325901) and GSK3-β (using CHIR99021) (Hou, 2013).

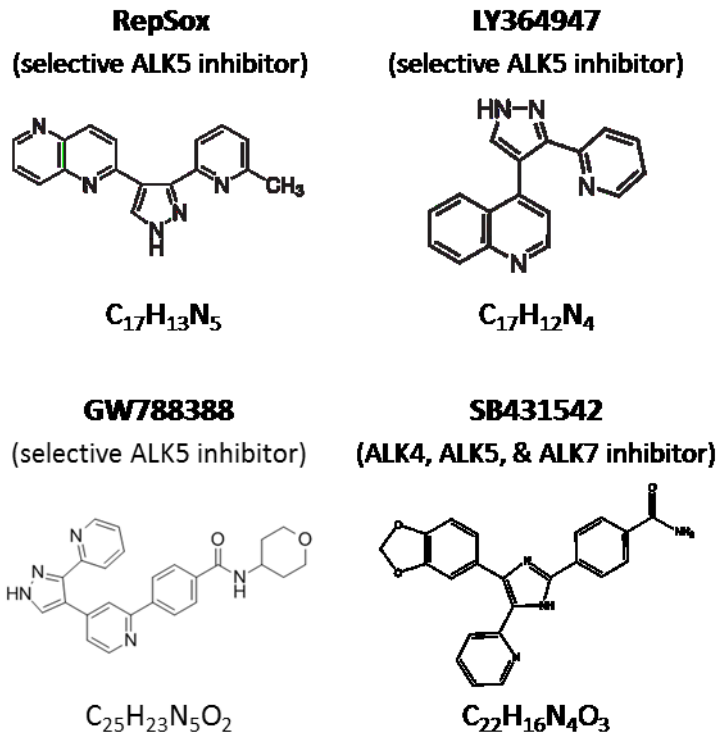

Figure 1.4. Chemical structures of TGF-β inhibitors. RepSox is structurally most similar to LY364947; however, even among selective ALK5 TGF-β inhibitors, we and others (Ichida, 2009) discovered effects unique to RepSox. One unique effect of RepSox's may be its ability to promote *CXCL12*, even across diverse cell types, including AML cells (Jajosky, 2014) and mouse embryonic fibroblasts (Larsen, 2013).

RepSox effects on *CXCL12* and *MYC*

In this project (Jajosky, 2014) and in other studies (Ichida, 2009) key differences between the actions of RepSox and other TGF-β inhibitors were noted which may be due to the unique ways RepSox affects CXCL12 and c-Myc. The RepSox-induced changes in gene expression are conserved across cell types (normal fibroblasts and malignant blood cells) and animal species (mouse and human). Based on gene-expression studies, RepSox-treated MEFs expressed higher levels of *C-X-C motif chemokine 12 (CXCL12)* and *MYC* than DMSO-treated controls (Larsen, 2013). Similarly, in this study, RepSox-treated AML cells more highly expressed *CXCL12* and *MYC* than controls (Jajosky, 2014). Because studies suggest both c-Myc and CXCL12 inhibit differentiation of AML cells (Leon, 2009; Salvatori, 2011; Huang, 2006; Tavor, 2008), upregulation of these two factors may explain how RepSox slows decay of $CD34^+$ AML cells (Jajosky, 2014).

CXCL12 – also known as "stromal cell-derived factor 1" (SDF-1) – is a chemokine that promotes migration and homing of normal hematopoietic progenitors (Aiuti, 1997) as well as metastasis of cancer cells (Müller, 2001) along chemotactic gradients. CXCL12 also promotes survival of AML cells (Tavor, 2014; Kim, 2013) and may help maintain them in an un-differentiated state (Tavor, 2008). Exposing primary AML cells and cell lines to the chemokine C-X-C motif receptor 4 (CXCR4) antagonist AMD3100 induced differentiation as evidenced by changes in morphology as well as expression of myeloid differentiation antigens (i.e. increased CD15, CD11b), differentiation-promoting cytokines (i.e. increased *G-CSF* and *GM-CSF*), and transcription factors (i.e. downregulation of *HOXA9*, *HOXA10*, and *WT1* involved in leukemogenesis and upregulation of *C/EBPε*, *ID1*, and *RUNX3* induced during myeloid differentiation) (Tavor, 2008). Furthermore, the

balance between TGF-β and CXCL12 signaling also influences the cell-cycle status of normal hematopoietic progenitors (Chabanon, 2008).

c-Myc is a transcription factor that facilitates reprogramming of differentiated cells toward pluripotency (Takahashi, 2006) and can inhibit the differentiation of a variety of both normal and malignant cells (Leon, 2009). In transgenic mice engineered to conditionally express c-Myc within blood cells, inactivation of c-Myc triggered differentiation of AML and lymphoma cells, leading to cancer regression (Felsher, 1999). During *all-trans* retinoic acid (ATRA)-induced and 1,25-dihydroxyvitamin D_3-induced differentiation of AML cell lines, c-Myc levels declined (Salvatori, 2011). Furthermore, exposure to the small molecule c-Myc inhibitor "10058-F4" (which inhibits dimerization of c-Myc with transcription factor Max) induced primary AML cells to differentiate (Huang, 2006). Thus, c-Myc might help maintain AML cells in an undifferentiated state.

RepSox is a potent reprogramming tool and the actions of RepSox on normal-cell differentiation (Ichida, 2009) seem relevant for maintaining CSCs. Gene-expression studies revealed RepSox-treated MEFs expressed higher levels of components of the Wnt, Notch, and Hedgehog signaling pathways than DMSO-treated controls (Larsen, 2013). Among TGF-β inhibitors, the unique actions of RepSox may be due to its structure (see Figure 1.4), potency, or selectivity. While RepSox promoted iPSC reprogramming in the absence of the HDAC inhibitor VPA, other TGF-β inhibitors, such as LY364947 (or E-616451), did not (Ichida, 2009). Of note, TGF-β neutralizing antibody did not enhance reprogramming efficiency as well as RepSox (Ichida, 2009). Among the different tools that can be used to inhibit TGF-β signaling, RepSox may be an especially useful way to activate genes involved in stem-cell maintenance, self-renewal, and pluripotency – or to

repress pro-differentiation genes. Comparing TGF-β inhibitors in this project showed that RepSox most effectively (1) increased or maintained *CXCL12* and *MYC* mRNA levels and (2) slowed decay of $CD34^+$ AML cells (Jajosky, 2014). Of note, RepSox substitutes for c-Myc during reprogramming (Ichida, 2009), and both c-Myc and CXCL12 (aka SDF-1) are believed to inhibit the differentiation of AML cells (Huang, 2006; Tavor, 2008).

Interestingly, genes activated during limb regeneration, such as Sal-like 4 (Sall4), are also activated during cellular reprogramming to pluripotency (Neff, 2011). That is, when cell de-differentiation is ("artificially") induced *in vitro*, this may involve some of the same molecular mechanisms that are responsible for the de-differentiation that occurs ("naturally") *in vivo* when primitive animals regenerate their amputated limbs.

As with other cancers that progress, AML persists when the patient's immune system fails to eliminate the malignant cells. A combination of intrinsic tumor-derived factors as well as extrinsic cancer-induced dysfunctions of the immune system and tumor microenvironment promotes AML immune escape and disease progression. **Chapter II** reviews the features of cancer cells (i.e. poor antigen-presentation, low immunogenicity, death-resistance) that enable them to passively escape ("hide") and actively suppress ("combat") immune defenses. *In vitro* cell-engineering strategies are described that may enhance *in vitro* immune-cell activation (i.e. improve presentation of leukemic antigens). Multi-pronged options are described that might, for example, use systemic agents to reduce the death-resistance of leukemia cells *in vivo* (i.e. inhibit generation of the specific anti-apoptotic factors that render a patient's cancer cells resistant to immune-cell attack).

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Chapter II

AML immune evasion and immune suppression;
therapeutic strategies for restoring immunologic function

When used alone, even chemotherapies that induce immunogenic tumor-cell death are unlikely to prevent relapse since the immune sytems of cancer patients are often so dysfunctional by the time treatment is initiated that even when they are stimulated by ICD they cannot eliminate immune-evasive and death-resistant CSCs. Thus, chemotherapies may need to be combined with patient-specific immune strategies that are specifically engineered to (1) eliminate poorly immunogenic CSCs, (2) repair and activate dysfunctional immune cells, and (3) neutralize immuno-suppressive pathways in tumor microenvironments. This chapter describes how malignant cells are able to resist immunologic attack by (1) growing rapidly, (2) avoiding recognition, (3) resisting death, and (4) suppressing anti-tumor defenses – see Figure 2.1 (Leone, 2012). Through immunoediting, the immune system selects for malignant-escape and antigen-loss variants that are poorly immunogenic (Dunn, 2002). As cancer cells evolve, they become poor stimulators – as well as poor targets – of immune cells (Whiteside, 2006). This chapter reviews strategies tumor cells employ to passively "hide" and actively escape from innate and adaptive immune cells (Igney, 2002). Of note, immune evasion has been more extensively studied in solid tumors than in blood cancers.

Avoiding immune detection

Tumor cells can avoid being recognized by the immune system (1) by migrating to immuno-privileged sites, (2) by altering their microenvironments in ways that reduce the survival, and impair the function, of immune cells, (3) by disrupting the presentation of tumor-cell antigens, and/or (4) by blocking costimulatory signals to antigen-presenting and immune-effector cells (Mapara, 2004; Whiteside, 2006; Mellman, 2011; Lion, 2012; Leone, 2012). Tumor cells can successfully grow in immuno-privileged sites such as the

central nervous system and can create “metabolically hostile” microenvironments by altering local oxygen, pH, and amino acid levels in ways that impair the physical entry, survival, and activity of infiltrating immune cells (Calcinotto, 2012; Baginska, 2013). Malignant cells can also induce the generation of physical and molecular barriers to infiltrating immune cells via the synthesis of dense extracellular matrix and/or expression of immuno-suppressive molecules like Tim-3 on tumor-associated vasculature (Huang, 2010) and/or galectins on surrounding stromal cells (Gieseke, 2010; Sioud, 2011).

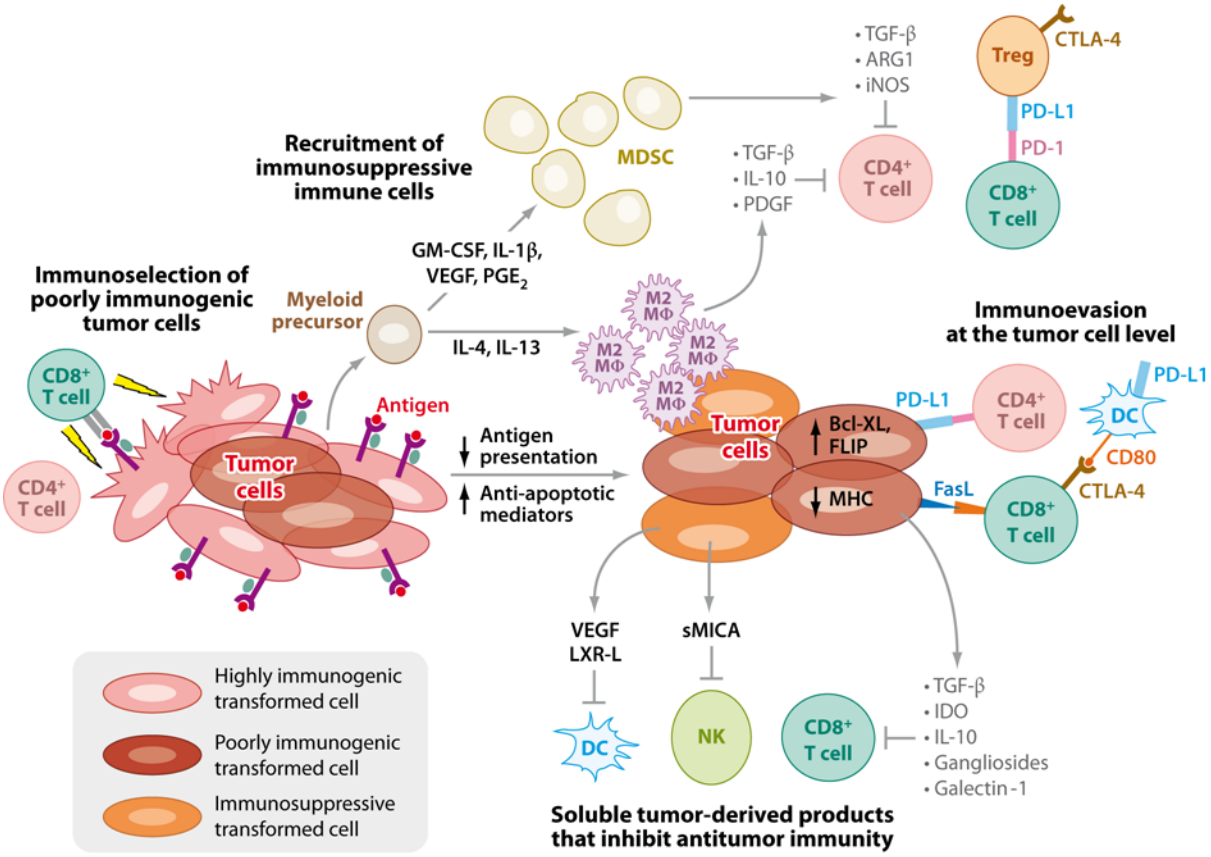

Figure 2.1. Tumor cell immune escape strategies. Tumor cells avoid immune-mediated destruction through a variety of mechanisms, including poor antigen presentation and low immunogenicity, death resistance via high expression of anti-apoptotic factors, expression of inhibitory receptors, release of immunosuppressive factors, and recruitment of immuno-suppressive cell types. *Reproduced with permission from*: Vesely, M. D., Kershaw, M. H., Schreiber, R. D., et al. (2011). Natural innate and adaptive immunity to cancer. *Annual Review of Immunology*, *29*, 252

Impaired antigen-presentation

Steps involved in the processing and presentation of tumor-cell antigens to the immune system via HLA class I molecules are reviewed in Figure 2.2. Normal- and tumor-cell peptides are presented to T-helper (T_h) cells via HLA class I molecules and to cytotoxic T cells via HLA class II molecules. Antigen-presenting cells (APCs), including DCs and monocytes/macrophages, normally present antigens to effector T cells via HLA class II molecules. Because myeloid leukemias arise from the same lineage as DCs and other APCs, AML cells are also able to present leukemic peptides to T cells on HLA class II complexes (van Luijn, 2010). Unfortunately, AML cells may behave like the immature DCs that promote immune tolerance (due to poor antigen-presentation) instead of functioning like the mature dendritic cells that promote immune clearance (Lutz, 2002; Panoskaltsis, 2005; Dudek, 2013). Although converting AML cells into DCs is being explored for the development of therapeutic cancer vaccines, caution must be taken: the differentiation of AML cells into indoleamine 2,3-diosygenase (IDO)-expressing DCs has been shown to impair maturation of normal DCs and inhibit T-cell proliferation, potentially restricting their use (Curti, 2010).

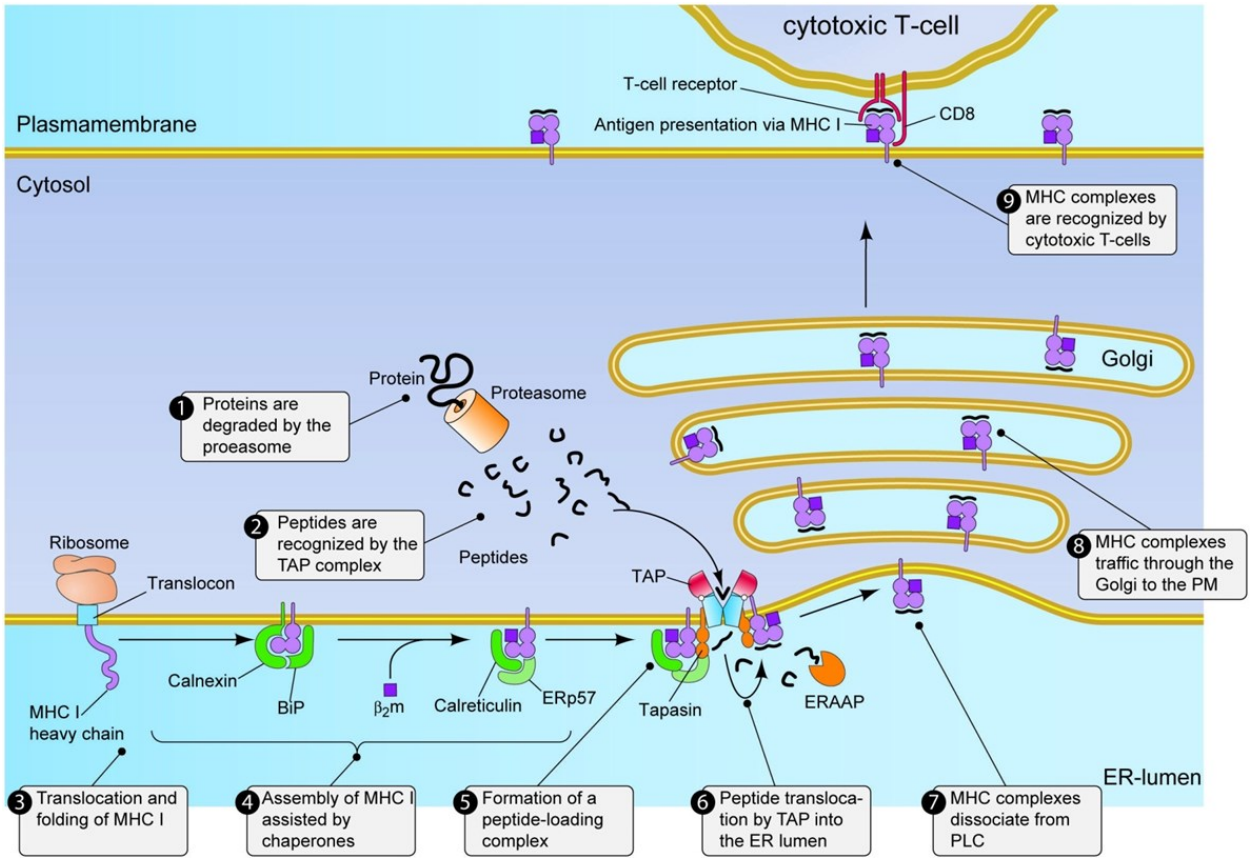

Figure 2.2. Processing and presentation of antigens via MHC class I molecules. Proteins are degraded within cells by the proteasome, loaded onto MHC class I molecules within the endoplasmic reticulum (ER), and translocated through the Golgi apparatus to the plasma membrane where peptide-MHC I complexes can be recognized by cytotoxic T cells. *Reproduced with permission from*: Parcej, D., & Tampé, R. (2010). ABC proteins in antigen translocation and viral inhibition. *Nature Chemical Biology*, *6*(8), 574.

Regarding the ways AML cells can escape anti-tumor immunity, evolving AML cells may acquire the ability to disrupt the presentation and processing of tumor antigens, downregulate expression of immuno-stimulatory receptors and/or their ligands, and/or shed immune-receptor decoys (Hoves, 2009; Lion, 2012; Schmiedel, 2013; Elias, 2014). Evolving molecular changes can alter expression, and disrupt presentation, of tumor antigens which, in turn, can inhibit immune-cell activation (Leone, 2012). Like many other cancer cells, AML cells show reduced expression of HLA class 1 molecules compared to the non-malignant white blood cells (WBCs) from healthy donors which, in turn, impairs effective presentation of leukemia-associated antigens (Garrido, 2010; Vollmer, 2003; Vago, 2009). In addition, tumor-generated peptides may be poorly immunogenic or fail to fit in the HLA class 1 groove. AML peptide-HLA complexes may not be recognized by T cells or may be delivered to T cells in the absence of sufficient costimulatory signals (i.e. CD80 and CD86) which, in turn, promotes T-cell anergy, an unresponsive state (Vollmer, 2003). Persistence of class II-associated invariant chain peptide (CLIP) within HLA class II molecules impairs presentation of leukemic antigens while downregulation of CLIP enhances AML cell immunogenicity which, in turn, promotes activation of T_h cells (van Luijn, 2010). Additional tumor-cell defects in antigen-processing machinery include mutations or deficiencies of proteasome subunits, including LMP2 and LMP7, and proteins that load peptides onto MHC class 1 molecules such as TAP and tapasin (Seliger, 1997; Johnsen, 1999; Leone, 2012).

Promoting immune tolerance

Immunologic tolerance is the inability to mount an immune response against an antigen. Central tolerance – aka “natural” or “self tolerance” which is normal and

protective – is established during development when T cells that can attack normal, self antigens (and potentially trigger autoimmune diseases) are deleted in the thymus. Unfortunately, malignant cells generate a peripheral tolerance to tumor antigens by inducing T-cell anergy, by triggering T-cell apoptosis, and/or by deviating T_h1 cytotoxic T-cell-mediated responses toward T_h2 B-cell-mediated responses that are less effective against cancer cells (Mapara, 2004). Secretion of TGF-β is one way tumor cells distort immune-cell responses by directing T_h1 cytotoxic T-cell responses toward T_h2 humoral responses (Maeda, 1996). AML cells also recruit, or induce formation of, regulatory T cells (T_{regs}) that suppress cytotoxic T cells. Through an IDO-dependent mechanism, AML cells promote conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T_{regs} (Curti, 2007). Finally, tumors can permanently delete tumor-reactive T-cell clones by expression of death-inducing ligands (Mapara, 2004) or by chronic (repetitive) antigen stimulation which triggers activation-induced cell death (Lu, 2008).

Avoiding immune destruction

Resisting death

Tumor cells resist immune-cell-mediated killing via the death receptor (Fas-mediated) and/or granule exocytosis (perforin- and granzyme-mediated) pathways (Igney, 2002; Otten, 2004; Reed, 2003). Tumor cells overexpress anti-apoptotic factors (FLIP, Mcl-1, Bcl-2), resist pro-apoptotic signals, and display mutations or defects in apoptotic pathways (Chiu, 1995; Reed, 2003). Tumor cells also neutralize death-inducing stimuli by shedding soluble receptors that act as decoys for death ligands like TRAIL and FasL (Chamuleau, 2011; Reed, 2003). Strategies that can sensitize AML cells to T-cell or NK-cell killing include targeting anti-apoptotic factors that induce immune resistance.

In a mouse model of colon cancer, treatment with a Nanog inhibitor decreased Mcl-1 levels and sensitized tumor cells to T-cell-mediated lysis (Noh, 2012). Small-molecule inhibitors of Bcl-2, TRAIL-receptor agonists, and IAP antagonists are currently being evaluated as anti-cancer agents in clinical trials (Reed, 2003; Weyhenmeyer, 2012).

Suppressing immune cells

Malignant cells express soluble and membrane-bound immuno-suppressive factors that disable (by inducing defects) or eliminate cancer-cell-reactive immune cells (Whiteside, 2006; Gajewski, 2006). Immune cells can become dysfunctional when cancer cells (1) secrete soluble factors, (2) express immune-checkpoint receptors and/or their ligands, (3) recruit, and/or induce formation of, immuno-suppressive cells, and (4) distort stromal and immune cells within cancer-cell microenvironments (Huang, 2010; Gieseke, 2010; Mellman, 2011; Sioud, 2011). Immuno-suppressive factors can inhibit immune-cell activation, maturation, proliferation, survival, cytotoxicity, and proinflammatory cytokine secretion as well as recruit immuno-suppressive cells and unfavorably polarize immune responses (Spranger, 2013).

Cancer cells can express a variety of immuno-suppressive factors including TNF-family ligands, immune-checkpoint receptors and their ligands (Kikushige, 2010), small molecules, enzymes, and cytokines (Whiteside, 2006; Vesely, 2011). Cancer-cell expression of TNF family ligands (such as FasL, TRAIL, TNF) triggers T-cell apoptosis upon binding to TNF family receptors (Whiteside, 2002). By increasing cAMP, small molecules like prostaglandin E2, histamine, and epinephrine inhibit anti-tumor immune responses by decreasing IL-2 and IFN-γ production by T cells as well as IL-1β, IL-12, and TNFα production by monocytes and macrophages (Uotila, 1996). Expression of the

immuno-suppressive enzymes IDO and arginase I by cancer cells, T_{regs}, and myeloid-derived suppressor cells (MDSCs) impairs T-cell functions. By depleting tryptophan, IDO inhibits T-cell proliferation (Uyttenhove, 2003). Likewise, by depleting local L-arginine, arginase restricts T-cell proliferation and polarizes monocytes toward a suppressive M2-like phenotype (Mussai, 2013). Cancer-cell secretion of cytokines like TGF-β, IL-10, and GM-CSF inhibits the proliferation of T cells and the release of pro-inflammatory cytokines (Bergmann, 1995; Krüger-Krasagakes, 1994).

AML cells also directly inhibit T-cell activity by expressing coinhibitory receptors such as Tim-3 (Kikushige, 2010; Jan, 2011) and CTLA-4 (Laurent, 2007) as well as their ligands such as PD-1 ligand (Norde, 2011). By expressing higher levels of ligands for inhibitory NK-cell receptors than activating NK-cell receptors, AML cells impair NK-cell-mediated lysis (Lion, 2012; Verheyden, 2008; Baessler, 2009; Baessler, 2010). AML expression of CD200 and RANKL inhibits NK-cell activity (Coles, 2011; Schmiedel, 2013) and T-cell activity (Coles, 2012). AML cells also escape NK-cell-mediated killing by secreting MICA and MICB ligands as well as by shedding NKG2D-receptor decoys that absorb activating NK-cell ligands (Weiss-Steider, 2011; Groh, 2002).

Therapies that target immune-checkpoint receptors and immuno-suppressive factors in the tumor microenvironment are reviewed in **Chapter IV**. In addition to tumor-cell-derived and immune-cell-derived factors, CAFs and endothelial cells within the tumor vasculature may also play an important role in immune suppression (Huang, 2010; Gieseke, 2010; Sioud, 2011). Thus, for some cancer patients, effective multi-pronged therapies may need to systemically modulate the tumor-sustaining support cells in addition to targeting the tumor cells.

Strategies to enhance tumor-cell immunogenicity

It is believed most cancers do, initially, trigger adaptive immune responses – evidenced by the presence of circulating tumor-reactive antibodies and immune cells. Over time, however, immunogenic cancer cells are eliminated, favoring the outgrowth of immune-escape variants (Dunn, 2002). When considering how to target AML cells, possible immunologic strategies include increasing AML-cell immunogenicity and/or rendering AML cells less death-resistant (more susceptible to immunologic attack). Increasing the immunogenicity of AML LSCs may be important for eradicating therapy-resistant "minimal residual disease" cells that can trigger relapse (Gerber, 2012). AML LSCs are problematic because they are especially death-resistant and are better able to escape immune recognition and destruction than more mature leukemic blasts (Costello, 2000). As expected, poorly differentiated AML subtypes (M0) have been found to be less immunogenic than the more differentiated subtypes (M4-M5) as evidenced by weak *in vitro* alloimmune recognition of the poorly differentiated AML cells by T cells and their reduced expression of costimulatory molecules (Costello, 1999). In general, CSCs only weakly stimulate immune cells; thus *ex vivo* activation of a patient's immune cells may require manipulation of stem-like cancer cells to improve their immunogenicity. Potential strategies to increase AML-cell immunogenicity – the ability to stimulate an adaptive immune response – include enhancing antigen-presentation by leukemia cells, blocking receptors like the "do not eat me" signals involved in immune escape, and inducing immunogenic tumor-cell death (Majeti, 2009; Lion, 2011; Rosenblatt, 2012).

Improving antigen-presentation by leukemia cells

AML cells can directly activate, and present antigens to, immune cells *in vitro* – at least γ9δ2 T-cells (Gertner-Dardenne, 2012). That is, antigen-presentation and immune-cell activation by AML cells can occur simultaneously. Perhaps AML-cell immunogenicity and antigen-presentation are best envisioned in the context of the immunologic synapse between AML and immune cells. Of note, in co-culture systems, TCR agonists clearly enhance the direct activation of T cells by AML cells (Gertner-Dardenne, 2012). Presumably, a variety of other factors (cytokines, antibodies, immuno-stimulatory compounds, and agents known to modulate antigen-presentation pathways, etc.) can also enhance *in vitro* immune-cell activation by improving antigen-presentation, immune-cell function, and/or tumor-cell immunogenicity.

Of note, cytokine-induced differentiation of leukemia cells into DCs has been shown to improve the presentation of leukemia cell antigens to T cells (Cignetti, 1999; Charbonier, 1999; Harrison, 2001; Woiciechowsky, 2001; Choudhury, 1999). Exposing AML cells to the TLR 7/8 agonist resiquimod, for example, enhanced their expression of MHC molecules, production of pro-inflammatory cytokines, and ability to stimulate allogeneic T cells (Smits, 2010). Silencing STAT3 in AML cells upregulated HLA class II molecules along with costimulatory and proinflammatory mediators (like IL-12) while decreasing expression of the coinhibitory molecule PD-L1 and improving activation of $CD8^+$ T cells (Hossain, 2014). Stimulation with CD40 and IFN-γ increased AML immunogenicity and expression of costimulatory molecules (Costello, 1999). Exposure to the histone deacetylase inhibitor (HDAC) chidamide increased AML expression of the cancer testis antigen PRAME and susceptibility to T-cell-mediated cytotoxicity (Yao,

2013). In addition, inhibiting nonsense-mediated messenger RNA decay improved the immunogenicity of cancer cells by inducing expression of novel immunogenic tumor antigens (Pastor, 2010). Proteasome inhibitors like bortezomib may also improve the immunogenicity of malignant cells by inducing expression of HSP90, a "danger signal," on the surface of myeloma cells (Gavioli, 2002).

Genetic modification of leukemia cells

To increase their ability to activate immune cells, AML cells can be engineered to express danger signals, costimulatory molecules, or proinflammatory compounds (Lion, 2011). Transfection of AML cells with the viral mimetic polyinosinic polycytidylic acid [poly(I:C)], a synthetic non-coding synthetic dsRNA analog, enhanced DC activation and increased IFN-γ secretion by natural killer (NK) cells (Smits, 2007). Following transfection with poly(I:C), AML cells were also more susceptible to killing by NK cells and phagocytosis by DCs, suggesting this dsRNA analog may be a useful vaccine adjuvant (Lion, 2011). Downregulating CLIP expression by AML blasts increased their ability to stimulate $CD4^+$ T-cell proliferation (van Luijn, 2010) consistent with the enhanced ability of CLIP(-) compared to CLIP(+) AML cells to activate and polarize $CD4^+$ T cells toward T_h1 cells (van Luijn, 2011). Modifying AML cells to express the costimulatory ligand CD80 also enhanced their ability to stimulate T-cell proliferation (Mutis, 1998).

Inducing immunogenic tumor-cell death

Cytotoxic chemotherapies are generally believed to suppress the immune system through myelotoxicity. However, radiotherapy and certain chemotherapeutic drugs induce immunogenic forms of tumor-cell death characterized by cell-surface changes that promote the phagocytosis of dying tumor cells and the release of immuno-stimulating

compounds – see Figure 2.3 (Tesniere, 2008; Inoue, 2014). Ionizing radiation (γ-irradiation) – as well as chemotherapies that include anthracyclines (daunorubicin, idarubicin, mitoxanthrone), the platinum derivate oxaliplatin, and the alkylating agent cyclophosphamide – induce immunogenic tumor-cell death (Reits, 2006; Garnett, 2004; Casares, 2005; Obeid, 2007; Panaretakis, 2008). Therapies that induce immunogenic cell death (ICD) can serve as *in situ* "cryptic vaccines" via release of tumor antigens and immune-boosting danger signals (Ma, 2010). After reviewing the chronic lymphocytic leukemia (CLL) trials that exploited adoptive transfer of autologous CAR-modified T cells, Dr. June speculated that pre-treatment of patients with immunogenic chemotherapies may have improved therapeutic outcomes (Kalos, 2011; Porter, 2011). In addition to killing tumor cells, cytotoxic chemotherapies can enhance immune responses by inducing transient lymphodepletion, counteracting immuno-suppressive mechanisms (Lutsiak, 2005), stimulating immune cells, and sensitizing tumor cells to T-cell or NK-cell-mediated killing (Ma, 2010). ICD might explain the "abscopal effect" in which localized radiation can also shrink metastatic tumors far from irradiated areas – presumably by stimulating the immune system (Kingsley, 1975; Robin, 1981; Wersall, 2006; Ohba, 1998).

Unfortunately for cancer research, the immunological effects of anti-cancer drugs cannot be evaluated in immuno-deficient mice transplanted with human tumors. One method to evaluate ICD involves pre-treatment of tumor cells with various chemotherapeutic drugs or cell-damaging agents followed by their subcutaneous injection into syngeneic immuno-competent mice. When inoculated with tumor cells undergoing ICD, mice generate anti-tumor immune responses and establish long-term immunologic memory which prevents tumor growth upon subsequent challenge with live

tumor cells. *In vitro* immune-cell activation assays that measure proliferation, cytokine release, and cytotoxicity are also available.

Features of immunogenic cell death (ICD)

During ICD, tumor-cell antigens are released along with pro-inflammatory stimuli such as “find me,” “eat me,” and “danger” signals that stimulate anti-tumor immunity – see Figure 2.3. The molecular characteristics of ICD have been nicely reviewed (Tesniere, 2008; Kepp, 2011). In general, pro-inflammatory “danger” signals promote tumor-antigen uptake, processing, and presentation – as well as enhance the anti-tumor activity of immune cells. Inducing the ICD of malignant cells is one strategy that can promote “immunogenic conversion” of the tumor microenvironment – see Figure 2.4. Current conceptual frameworks for immunology suggest it is worthwhile to “convert” cancer cells into a “cryptic vaccine” by inducing release of tumor antigens in an immuno-stimulatory context.

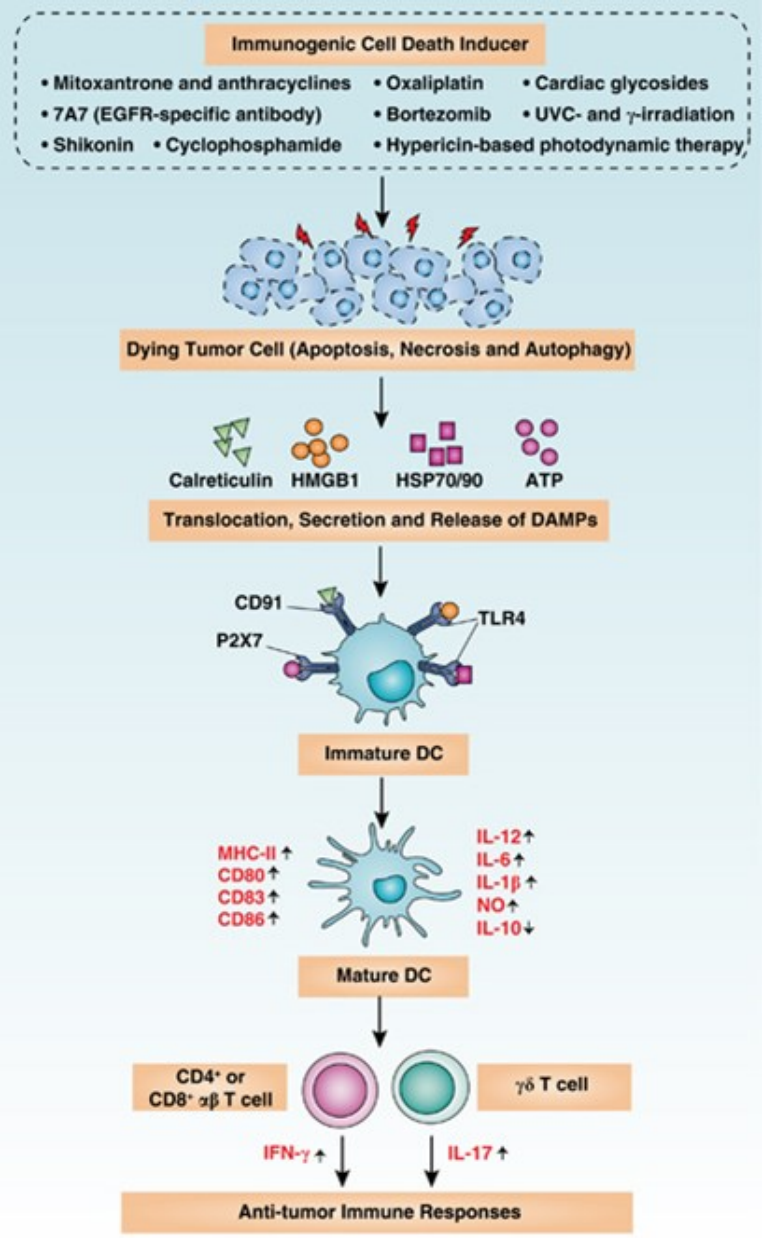

Figure 2.3. Features of immunogenic tumor cell death. A variety of chemotherapeutic drugs, radiotherapy, and targeted anti-cancer agents induce immunogenic forms of tumor cell death. During immunogenic cell death, tumor cells expose and release a variety of danger signals. For example, heat shock proteins (HSP) and calreticulin translocate to the plasma membrane and ATP and HMGB1 are secreted. In response to these danger signals, DCs mature into effective antigen-presenting cells that prime T cells to attack tumors. *Reproduced with permission from*: Hou, W., Zhang, Q., Yan, Z., et al. (2013). Strange attractors: DAMPs and autophagy link tumor cell death and immunity. *Cell Death & Disease*, *4*(12), 5.

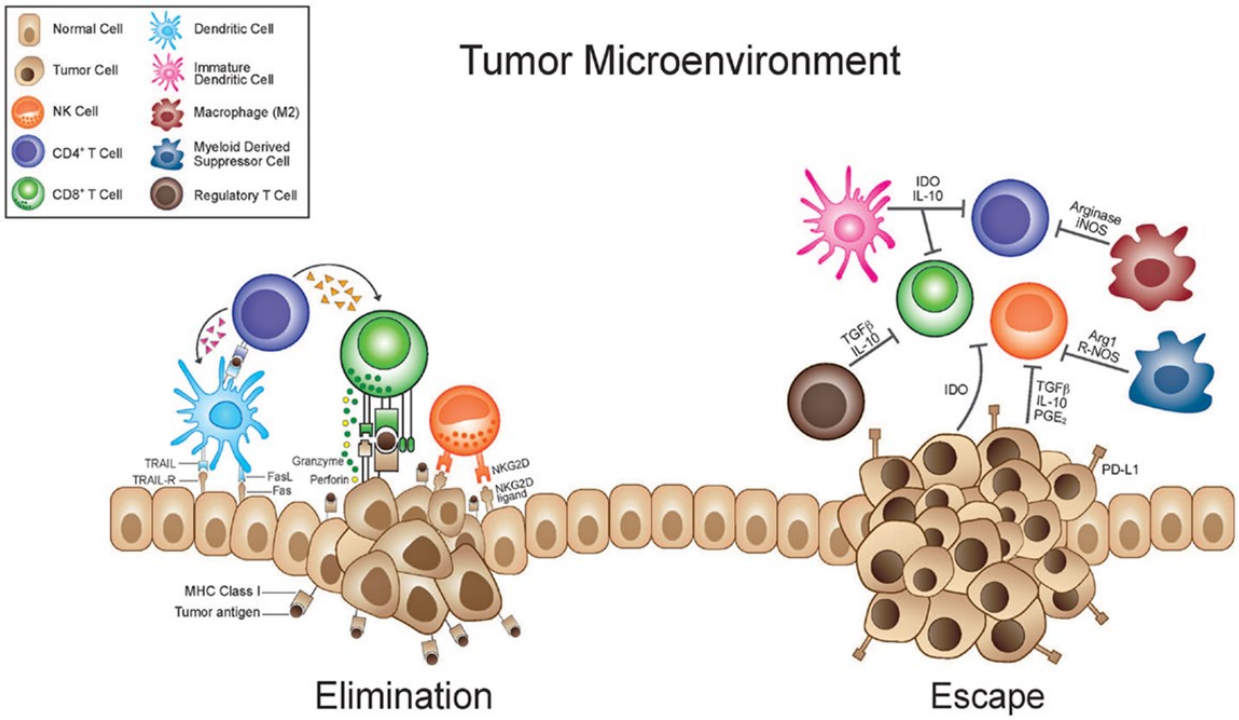

Figure 2.4. Comparison of immunosupportive and immunosuppressive tumor microenvironments. Within immunogenic microenvironments, activated immune cells recognize and eliminate tumor cells. In contrast, within non-immunogenic microenvironments, tumor cells release immunosuppressive factors (such as TGF-β and IL-10) and recruit T_{reg} cells and MDSCs that subvert infiltrating antigen-presenting and immune-effector cells; this leads to immune escape and tumor progression. A major goal of cancer immunotherapy is to convert immunosuppressive tumor microenvironments that promote tumor escape into immunosupportive tissues that eradicate malignancy. *Reproduced with permission from*: Monjazeb, A. M., Zamora, A. E., Grossenbacher, S. K., et al. (2013). Immunoediting and antigen loss: overcoming the Achilles heel of immunotherapy with antigen non-specific therapies. *Frontiers in Oncology*, *3*, 4.

"Find me" signals include small molecules such as HMGB1 and ATP that help recruit and activate infiltrating immune cells like monocytes. HMGB1 binds TLR4 on DCs and inhibits antigen degradation mediated by fusion of phagosomes with lysosomes, thereby favoring the processing and presentation of tumor antigens (Apetoh, 2007). ATP released from cancer cells binds P2RX7 on DCs, leading to inflammasome activation and secretion of IL-1β (Ghiringhelli, 2009). "Eat me" signals stimulate uptake of tumor antigens by antigen-presenting cells like DCs. During ICD, malignant cells express surface calreticulin. When translocated to the plasma membrane, calreticulin functions as a receptor for DC engulfment (Obeid, 2007). During ICD, cancer cells may also downregulate expression of "do not eat me" signals such as CD47. Tumor-cell loss or the redistribution of CD47, which normally inhibits phagocytosis by SIRPα-expressing macrophages, can promote cancer-cell engulfment due to expression of calreticulin (Willingham, 2012). In response to the cellular stress induced by chemotherapy, cancer cells transcriptionally activate heat-shock proteins which, in turn, stimulate DC maturation via CD91 (Somersan, 2001) and facilitate chaperoning of tumor antigens to MHC molecules for presentation to T cells (Zitvogel, 2008; Binder, 2005).

While this chapter focuses on factors mediating immune evasion and suppression on the tumor-cell side of malignant disease, **Chapter III** focuses on the cancer-induced defects of immune cells and the tumor stroma that prevent immune clearance of cancer cells. Strategies are reviewed for (1) the repair of dysfunctional immune cells (*in vitro*) – followed by their activation and expansion and (2) the reversal of the immune suppression induced by cancer microenvironments (*in vivo*). The ways Tim-3 expression can impair

anti-tumor immune responses – when expressed on the surface of tumor or immune cells – are also described.

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Chapter III

Cancer-induced impairments of immune cells and stromal cells; therapeutic strategies for repair

When the immune system mounts an attack against cancer, tumor cells "fight back" by subverting anti-tumor immune defenses (Zitvogel, 2006). While **Chapter II** reviewed cancer-cell-derived factors that mediate immune evasion and suppression, this chapter describes immune-cell defects induced by cancer-distorted microenvironments. Intrinsic immune-cell defects (i.e. anergy, exhaustion, impaired maturation) and extrinsic immune-suppressing factors (i.e. TGF-β, IDO, CAFs) are reviewed. Potential ways to repair dysfunctional immune cells and counteract immuno-suppressive factors in cancer microenvironments are described. Tim-3 is highlighted because it disrupts immune responses and synapses, and both TGF-β and RepSox affect Tim-3 (Wiener, 2007; Jajosky, 2014). *In vivo* Tim-3 blockade and the *in vitro* manipulation of Tim-3 expression by malignant cells warrant consideration when designing anti-cancer immune strategies.

Generation of anti-tumor immune responses

The immune system eliminates malignant cells via a variety of defenses including innate and adaptive immune cells, antibodies, complement proteins, and cytokines. Innate defenses are first to respond, followed by more specialized attack by antigen-specific T and B cells of the adaptive immune system. The key players of the innate and adaptive immune system are outlined in Figure 3.1. Adaptive immune responses, involving antigen-specific T and B cells or antibodies, can be divided into the "activation phase" involving tumor-antigen cross-presentation within lymphoid organs (i.e. lymph nodes) and the "effector phase" involving tumor-cell lysis in the periphery (Dzivenu, 2003; Yao, 2013). Figure 3.2 provides an overview of important soluble factors and cell-to-cell interactions that mediate generation of innate and adaptive anti-tumor immunity.

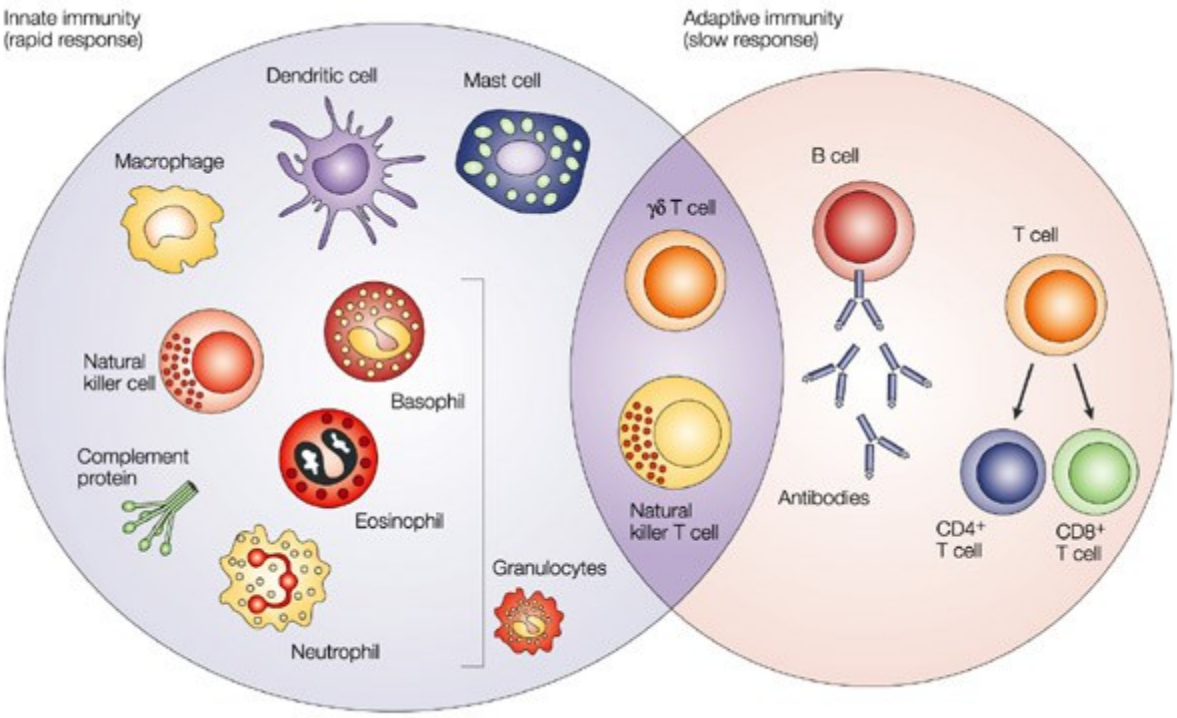

Figure 3.1. Innate and adaptive immune defenses. The innate immune system is the body's first line of defense against invading pathogens and cancer. Components of innate immunity include phagocytes (i.e. macrophages), NK cells, and complement proteins. The adaptive immune system generates antigen-specific anti-tumor responses mediated by antibodies, B cells, and T cells. Unlike innate immunity, the adaptive immune system can generate immunological memory that is capable of protecting against re-emergence of disease. *Reproduced with permission from*: Dranoff, G. (2004). Cytokines in cancer pathogenesis and cancer therapy. *Nature Reviews Cancer*, *4*(1), 13.

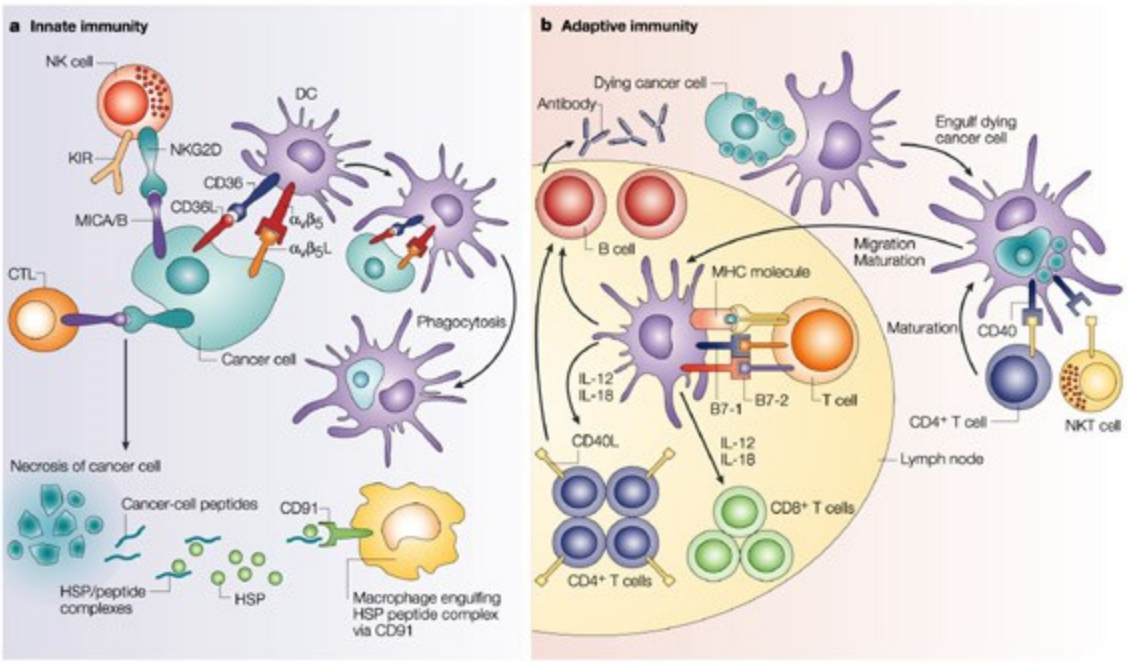

Figure 3.2. Generation of innate and adaptive anti-tumor immune responses. Within NK cells of the innate immune system, the net input of activating and inhibitory signals determines whether or not cancer cells lacking MHC class 1 molecules ("missing self") will be effectively killed. Within the adaptive immune system, DCs engulf tumor antigens and migrate to lymph nodes where they present tumor peptide-MHC complexes to T cells (a process called "cross-priming"). Activated $CD4^+$ T_H cells, in turn, help to activate CD8+ cytotoxic T cells and stimulate B cells to produce antibodies. *Reproduced with permission from*: Dranoff, G. (2004). Cytokines in cancer pathogenesis and cancer therapy. *Nature Reviews Cancer*, *4*(1), 14.

Activation phase

Antigen-presenting cells (APCs), such as macrophages and DCs, engulf cancer cells or capture antigens released by dying cells. Immunogenic tumor-cell death, illustrated in Figure 2.4, activates APCs through release of pro-inflammatory "find me," "eat me," and "danger" signals (Tesniere, 2008; Inoue, 2014). In contrast, non-immunogenic cell death promotes tolerance. APCs process and present captured tumor antigens on their surface via major histocompatibility complex (MHC) class II molecules (Dzivenu, 2003). After migrating to tumor-draining lymph nodes, DCs cross-present malignant peptide-MHC complexes to $CD4^+$ T_h cells (Dzivenu, 2003). T_h cells secrete cytokines that activate other immune cells. T_h cells can be divided into type 1 (T_h1) and type 2 (T_h2) cells based on the cytokines they secrete. By secreting interferon-gamma (IFN-γ) and IL-2, T_h1 cells activate $CD8^+$ cytotoxic T cells and promote cell-mediated immunity (Dzivenu, 2003). In contrast, by secreting IL-4 and IL-5, T_h2 cells stimulate antibody production by B cells (Dzivenu, 2003).

Effector phase

Once activated, cytotoxic T cells recognize malignant cells expressing cancer-specific or cancer-associated antigens bound to MHC class 1 molecules via their T-cell receptor (Dzivenu, 2003). Cytotoxic T cells lyse cancer cells by releasing perforins and granzymes. Once activated, B cells differentiate into antibody-producing plasma cells. Antibodies bind the surface of cancer cells and lead to tumor-cell death through antibody-dependent cellular cytotoxicity (ADCC) or activation of the complement cascade (Dzivenu, 2003). ADCC involves recruitment of immune cells, such as macrophages and NK cells, which engulf or lyse cancer cells (Raval, 2014). Antibodies can also activate

the complement cascade which, in turn, induces tumor-cell lysis by perforating the cell membrane (Raval, 2014).

Cancer immunoediting hypothesis

The cancer immunoediting hypothesis (see Figures 3.3 and 3.4) describes how the immune system affects cancer development through its dual host-protective and tumor-promoting roles (Dunn, 2002; Dunn, 2004). Immunoediting of tumors can be divided into three phases: elimination, equilibrium, and escape – see Figure 3.4 (Dunn, 2002; Dunn, 2004). During the **elimination** ("immuno-surveillance") phase, immunogenic tumor cells are eliminated by innate and adaptive immune cells. If all cancer cells are eradicated, disease is cured. If not, residual "immune-escape variants" remain behind. During the **equilibrium** ("immuno-selection") phase, tumor outgrowth is prevented, but remaining cancer cells continue to evolve under constant immune-selection pressures. During the **escape** ("immuno-subversion") phase, the poorly immunogenic cancer cells that evaded immune destruction grow and expand.

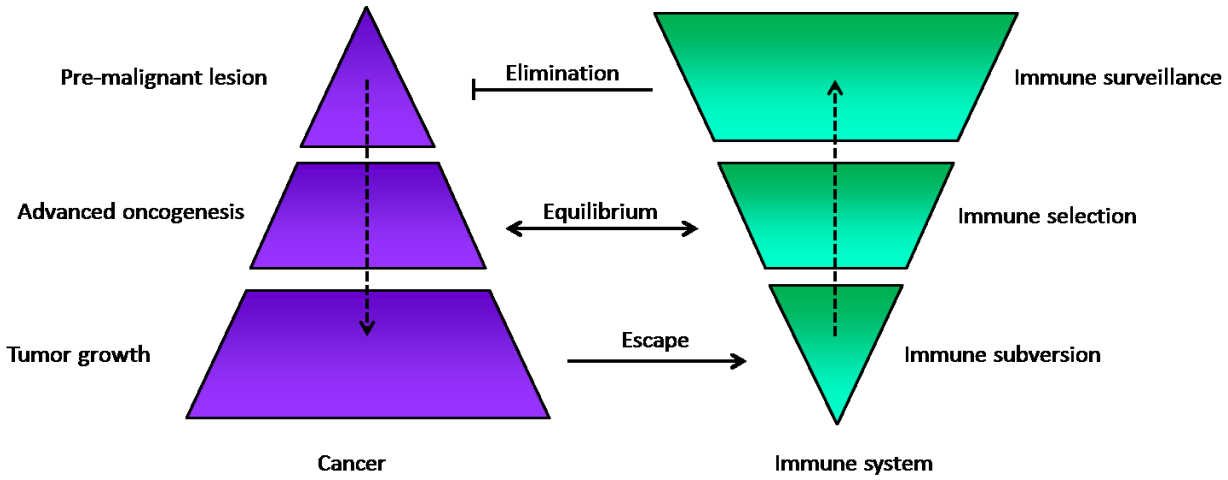

Figure 3.3. Immunoselection and immunosubversion during tumor evolution. By eliminating immunogenic tumor cells, the immune system selects for poorly immunogenic tumor cells that escape immune recognition. These immune escape variants progressively alter ("hijack") their microenvironment to suppress immune defenses and promote tumor growth. *Adapted with permission from*: Zitvogel, L., Tesniere, A., & Kroemer, G. (2006). Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nature Reviews Immunology*, *6*(10), 716.

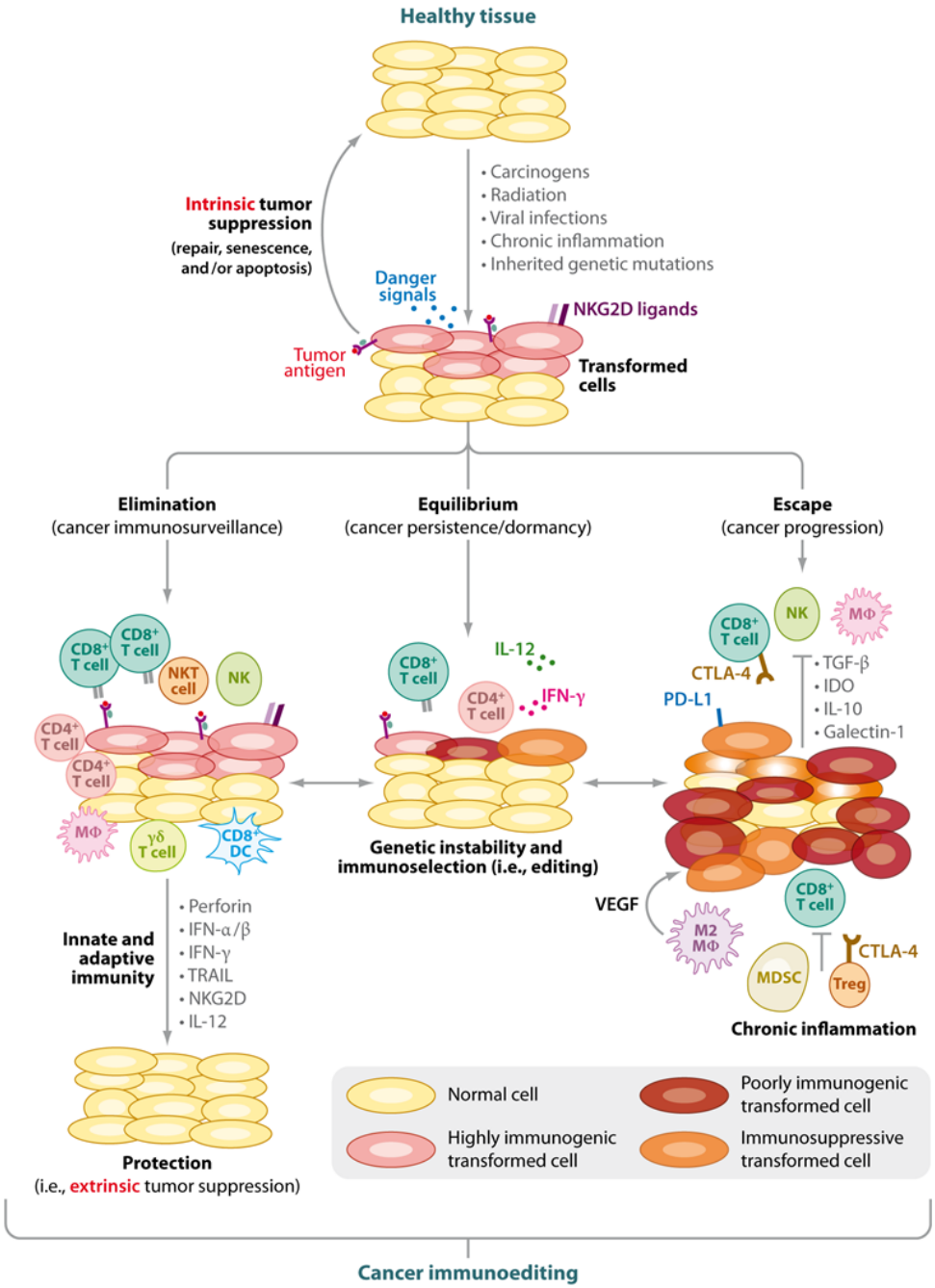

Figure 3.4. Three phases of cancer immunoediting: elimination, equilibrium, and escape. As cancers evolve, tumor cells are recognized and cleared by the immune system. Elimination of all tumor cells cures disease. If malignant disease persists, the immune system may prevent outgrowth of surviving tumor cells for some time. However, tumor cells may continue to evolve and alter surrounding tissues in ways that impair immune defenses. Ultimately, tumor cell "immune escape" variants may emerge and cause overt disease. *Reproduced with permission from*: Vesely, M. D., Kershaw, M. H., Schreiber, R. D., et al. (2011). Natural innate and adaptive immunity to cancer. *Annual Review of Immunology*, *29*, 238.

Cancer-induced dysregulation of the immune system

As cancers progress, immune cells may no longer be recruited to, or activated by, malignant cells; and immune-cell function may be inhibited by tumor microenvironments (Gajewski, 2006; Spranger, 2013). Even when patients do not generate an effective anti-cancer immune response, tumor-reactive immune cells may still exist, but are, unfortunately, overwhelmed or suppressed (Gajewski, 2006; Tran, 2014). Immunotherapies for cancer aim to trigger *de novo* immune responses and enhance ("rescue") pre-existing anti-tumor immune reactions that have become silenced (Mellman, 2011). Conceptually, this involves stimulating the immune-activation phase and rescuing the immune-effector phase. Remarkably, cancer-induced immune-cell defects can be repaired (Hodi, 2010; Reiners, 2013; Vizcardo, 2013), and immuno-suppressive cells, enzymes, and soluble factors can be neutralized or eliminated (van den Boorn, 2013). Thus, it is possible to convert non-immunogenic tumor microenvironments into anti-cancer, immuno-stimulating tissues (van den Boorn, 2013).

Cancer-induced intrinsic immune-cell defects

Unfortunately, dysfunctional immune cells can accumulate in cancer patients (Gabrilovich, 2004; Pinzon-Charry, 2005; Wherry, 2011). Defects include anergy, exhaustion, impaired maturation, deletion, and unfavorable polarization (toward type 2 humoral immunity rather than type 1 cell-mediated immunity). Immune-effector cells become "tolerant" and are unable to eliminate cancer cells (Crespo, 2013; Wherry, 2011). By failing to adequately present antigens and activate T cells, the (immature) DCs actually promote T-cell tolerance (Lutz, 2002; Dudek, 2013). Cancer-induced dysfunctions of T cells, DCs, NK cells and macrophages are reviewed.

T-cell defects

In patients with cancer and chronic viral infections, T cells can become dysfunctional (i.e. anergic, exhausted) or may be deleted. T-cell anergy is due to incomplete activation (i.e. T-cell receptor ligation in the absence of costimulatory signals like CD28 engagement) (Chappert, 2010), while exhaustion arises from chronic over-stimulation (Wherry, 2011). Exhausted T cells often express inhibitory immune-checkpoint receptors (Tim-3 and PD-1) and display impaired cytokine secretion and cytolytic activity upon antigen stimulation (Wherry, 2011). Compared to healthy controls, T cells from AML patients formed defective immune synapses with AML cells and had (altered) gene expressions consistent with impaired cytolytic activity (Le Dieu, 2009).

NK-cell defects

Cancer-cell-induced NK-cell abnormalities include impaired maturation, anergy, decreased cytotoxicity, and defective secretion of cytokines like IFN-γ (Das, 2000; Richards, 2006; Lion, 2012). Compared to patients without cancer, NK cells from AML patients have decreased expression of activating NK-cell receptors and increased expression of inhibitory NK-cell receptors (Costello, 2002; Lion, 2012). This unfavorable balance of stimulatory and inhibitory receptors may prevent effective NK-cell-mediated elimination of AML cells.

DC defects

In cancer patients, incomplete maturation of DCs is one reason why immune responses are impaired (Pinzon-Charry, 2005). Tumor-derived factors, such as gangliosides, VEGF, IL-6, and IL-10, inhibit DC differentiation (Gabrilovich, 2004). This leads to an accumulation of immature DCs and the absence of an adequate number of

functional (mature) DCs as well as an accumulation of immature myeloid progenitors generally – a combination of adverse effects which, collectively, can substantially impair T-cell responses (Gabrilovich, 2004). Of note, immature (dysfunctional) DCs are among the myeloid-derived suppressor cells (MDSCs) that suppress T-cell immunity – see Figure 3.5 (Gabrilovich, 2009; Khaled, 2013; Ostrand-Rosenberg, 2009). That is, MDSCs include a variety of immature myeloid cells that have not fully differentiated into fully functional macrophages, DCs, or granulocytes (Gabrilovich, 2009; Khaled, 2013). Because immature DCs present antigen in the absence of appropriate costimulatory signals (i.e. CD80 and CD86) and/or proinflammatory cytokines, they, unfortunately, induce T-cell tolerance rather than protective anti-tumor responses (Gabrilovich, 2004).

Macrophage defects

Tumor-associated macrophages (TAMs) accumulate in tumors, and high tumor influx of TAMs is associated with poor prognosis in cancer patients (Solinas, 2009; Tang, 2013). TAMs may polarize to type 2 (M2) macrophages that promote tumor growth (Solinas, 2009). While type 1 (M1) macrophages produce IL-12 and enhance T_h1 and NK cell-mediated immunity, M2 macrophages secrete IL-10 and promote T_h2 humoral immunity – see Figure 3.5 (Solinas, 2009). Of note, cell-mediated immunity is believed to be more effective than antibodies in controlling tumor growth.

Strategies to repair dysfunctional immune cells

Remarkably, dysfunctional immune cells can be repaired ("re-educated" or "reprogrammed") and then activated and expanded to promote tumor-rejection. Using a metastatic melanoma patient's exhausted $CD8^+$ T cells, reprogramming to pluripotency – followed by re-differentiation – generated "rejuvenated" cytotoxic T cells that were

functional based on their ability to produce IFN-γ upon encounter with antigen (Vizcardo, 2013). Immature DCs can be differentiated into functional APCs using ATRA (Mirza, 2006; Kusmartsev, 2003). In a mouse model of breast cancer, type 2 tumor-promoting TAMs were re-polarized into type 1 cells with increased tumoricidal activity using a combination of microbial stimulus (TLR9 ligand CpG) and anti-IL-10-receptor antibody (Giuducci, 2005). Here, strategies are reviewed that can repair and activate defective immune-effector cells in order to stimulate anti-tumor immunity. Relevant factors include proinflammatory cytokines, "danger signals" (like TLR agonists), and antibodies that activate costimulatory, and/or block coinhibitory, immune-checkpoint receptors.

Repairing T cells

Several strategies have been developed to reverse T-cell tolerance. Examples include targeting immune-checkpoint receptors, delivering proinflammatory cytokines, and reprogramming T cells to pluripotency (Pardoll, 2012; Vizcardo, 2013).

Modulation of immune-checkpoint receptors

Antibodies that activate costimulatory receptors or inhibit immune-checkpoint receptors can stimulate T cells. For example, inhibiting the PD-1 signaling pathway can rescue exhausted T cells in the setting of cancer and chronic viral infection (Barber, 2006; Zitvogel, 2012). Co-expression of PD-1 and Tim-3 identifies "exhausted" killer T cells in mice with AML (Zhou, 2011), and co-blockade of PD-1 and Tim-3 more effectively stimulates anti-tumor immunity than either therapy alone (Sakuishi, 2010). In clinical trials, PD-1 blockade induced tumor regression in patients with advanced melanoma or lung and kidney cancers (Brahmer, 2012; Topalian, 2012). Treatment with ipilimumab, a CTLA-4 blocking antibody, restored T-cell anti-tumor immunity and improved survival of

melanoma patients (Hodi, 2010). Antibody activation of the costimulatory receptor OX40 (Weinberg, 2011) and blockade of the coinhibitory receptor LAG-3 (Goldberg, 2011) can also promote anti-tumor immunity.

Cytokine stimulation

Exposure to cytokines, including IL-15, IL-17 and IL-2, can prevent, or reverse, T-cell anergy induced by tumors and chronic viral infections (Boussiotis 1994; Gu, 2007; Teague, 2006). Using a mouse model of T-cell tolerance, *in vitro* exposure to IL-15 rescued "tolerant" T cells; furthermore, adoptive transfer of rejuvenated T cells was effective in treating mice with leukemia (Teague, 2006).

Induction of homeostatic proliferation

Lymphopenia-induced homeostatic T-cell proliferation can reverse tumor-induced T-cell anergy and promote tumor rejection (Kline, 2008; Brown, 2006). Lymphopenic conditioning of patients prior to receiving adoptively transferred T cells improves the persistence and expansion of infused T cells (Dudley, 2002; Rosenberg, 2008). Presumably, lymphopenic conditioning depletes T_{regs} and supports engraftment and homeostatic proliferation of therapeutic (functional) T cells.

Repairing NK cells

Following exposure to IL-15, NK cells from AML patients show increased expression of activating receptors and improved cytotoxicity (Szczepanski, 2010). IL-15 may also promote NK-cell maturation (Richards, 2006). Anti-KIR antibodies block inhibitory NK-cell receptors and promote NK-cell-mediated killing of tumor cells (Romagne, 2009; Velardi, 2009; Alici, 2010). NK-cell-mediated cytotoxicity against non-

Hodgkin's lymphoma tumor cells improved with the use of bispecific antibodies that could crosslink the CD16A receptor on NK cells with the CD30 receptor on non-Hodgkin's lymphoma cells (Reiners, 2013). Targeted anti-cancer therapies can also promote the anti-tumor activity of NK cells. For example, imatinib increases DC-mediated NK-cell activation (Borg, 2004).

Role of Tim-3 expression in immune-cell dysfunction

Tim-3 inhibits the pro-inflammatory or anti-tumor activities of a variety of immune cells, including T, NK, dendritic, and monocytic cells. The endothelial and stromal cells that surround tumors also mediate immune suppression. Tim-3-expressing endothelial cells may function as an immunological barrier shielding tumor cells from infiltrating T cells by directly suppressing their activity (Huang, 2010). Tumor cells may convert stromal cells into galectin-9-expressing CAFs capable of suppressing T-cell responses (Gieseke, 2013; Sioud, 2013). Similar to the adverse consequences of Tim-3 expression by immune and stromal cells, Tim-3 expression by tumor cells can also promote immune escape or suppression by, for example, disrupting cancer-cell/immune-cell synapses.

Cytotoxic T cells

Tim-3 promotes T-cell tolerance in the setting of cancer and chronic viral infections (Sánchez-Fueyo, 2003). High Tim-3^+ T cells do not respond to stimulation: they are unable to proliferate or produce cytokines. Thus, high Tim-3^+ T cells are known as "non-effector" or "exhausted" T cells (Golden-Mason, 2009; Hastings, 2009). In lymphoma patients, T-cell dysfunction ("exhaustion") was found to be mediated, in part, by increased Tim-3 expression by T cells (Yang, 2012). In the context of chronic viral infections, Tim-3^+ T cells displayed impaired secretion of the cytokines IFN-γ and TNF-α and were more

prone to apoptosis (Jones, 2008; Golden-Mason, 2009; Jin, 2010; Ju, 2009). Tim-3 expression on the surface of $CD8^+$ T cells impairs their cytotoxicity by inhibiting the degranulation needed for the secretion of perforin and granzymes (Sakhdari, 2012). Cytokine production by T_h1 cells in mice is enhanced following treatment with Tim-3 blocking antibodies (Sabatos, 2003). In the presence of Tim-3 blocking antibodies, $CD4^+$ T cells showed enhanced secretion of the proinflammatory cytokines IFN-γ, IL-2, IL-17, and IL-6 (Hastings, 2009). *In vitro*, the Tim-3 ligand galectin-9 blocks differentiation of naïve T cells into T_h17 cells while inducing their differentiation into suppressive T_{regs} (Seki, 2008). Unfortunately, over-expression of Tim-3 can emerge as malignant disease progresses, and Tim-3 can promote immune tolerance by (adversely) altering the malignant, immune, and stromal cells of cancer patients. Tim-3 blockade can restore anti-tumor immunity by inducing therapeutically beneficial changes in multiple cell types.

Regulatory T cells (T_{regs})

Suppressive T_{regs} inhibit anti-tumor immunity, and in a mouse model of colon cancer, most intratumoral $FOXP3^+$ T_{regs} were found to express Tim-3 (Sakuishi, 2013). $Tim\text{-}3^+$ T_{regs} that co-express PD-1 are highly suppressive (Sakuishi, 2013). As an anti-cancer strategy, the benefit of simultaneously blocking both the Tim-3 and PD-1 signal transduction cascades likely arises not only from the reversal of T-cell exhaustion, but also from the inhibition of T_{reg}-cell function (Sakuishi, 2013). It seems Tim-3 identifies those T_{regs} that very strongly suppress anti-tumor immune responses (Sakuishi, 2013).

Monocytes and Macrophages

Tim-3 expression by monocytes and macrophages has not been well studied in cancer. Blocking Tim-3 receptors on monocytes increased their secretion of IL-12, a

cytokine that promotes T_h1 and NK-cell-mediated immunity (Zhang, 2012). In mice with sepsis, Tim-3 blockade increased macrophage activation (accompanied by increased mRNA expression of proinflammatory cytokines and TLRs) and exacerbated sepsis, suggesting Tim-3 can limit macrophage activity (Yang, 2013). Of note, activation of the Tim-3-galectin 9 pathway has also been shown to promote macrophage bactericidal activity (Sada-Ovalle, 2012).

Dendritic cells

In mice with lung or colon cancer, Tim-3 was over-expressed on tumor-infiltrating DCs compared to the DCs residing within normal tissues and the DCs of healthy mice (Chiba, 2012). These high Tim-3-expressing DCs enable tumors to evade immuno-surveillance by suppressing the sensing of nucleic acids associated with by tumor-induced inflammation (Chiba, 2012; Jinushi, 2012).

Natural killer cells (NK cells)

Although Tim-3^+ NK cells from healthy donors were mature and fully functional, Tim-3 may suppress NK-cell-mediated cytotoxicity upon encounter with target cells expressing Tim-3 ligands (Ndhlovu, 2012). Relative to NK cells from healthy donors, NK cells from melanoma patients displayed elevated Tim-3 expression along with reduced cytotoxicity, IFN-γ secretion, and proliferation (da Silva, 2014). Among melanoma patients, Tim-3 expression on NK cells was generally higher during advanced stages of disease and in patients with poor prognostic factors (da Silva, 2014). Furthermore, Tim-3 blockade reversed NK-cell exhaustion and improved cytotoxicity against melanoma cell lines (da Silva, 2014).

Natural killer-T (NKT) cells

Tim-3$^+$ NKT cells in the liver are functional: they can proliferate and produce cytokines when stimulated. When liver NKT cells are activated by exogenous bacterial antigens, Tim-3 expression is upregulated and IFN-γ is secreted. IFN-γ induces production of galectin-9 by Kupffer cells in the liver which, in turn, induces apoptosis of Tim-3$^+$ NKT cells and reduces the inflammatory response. That is, in some molecular and cellular contexts, Tim-3 expression on NKT cells leads to NKT-cell apoptosis which, in turn, limits the NKT-cell immune response (Kinjo, 2005; Mattner, 2005; Kinjo, 2006; Mengshol, 2010).

Tim-3 blockade to stimulate immune-cell activity

Because Tim-3 restrains anti-tumor immunity, preclinical studies are evaluating the use of Tim-3 blockade to promote immune-mediated tumor-rejection. Along with PD-1 blockade (Sakuishi, 2010) and CD137 activation (Guo, 2013), Tim-3 blockade reverses T-cell exhaustion and promotes anti-tumor immunity in mice. Co-blockade of Tim-3 and Tim-4 also improved vaccine-induced anti-tumor immune responses by stimulating NK and T-cell activity in mice with melanoma (Baghdadi, 2013). Based on these successful animal trials, Tim-3 blocking antibodies are in preclinical development.

Cancer-induced extrinsic immune suppression

Tumors recruit, and induce the formation of, suppressive immune cells. Because inhibitory (aka "regulatory") immune cells normally dampen immune responses, they may be recruited and "hijacked" by tumors to promote immune escape (Zitvogel, 2006). Under normal physiologic conditions, inhibitory cells (like T_{regs}) are useful because they limit chronic inflammation, maintain peripheral tolerance to self antigens, and prevent

autoimmune disease (Hori, 2003; Vignali, 2008). But, in the context of malignant disease, Figure 3.5 shows how T_{regs}, tolerogenic DCs, tumor-associated (M2) macrophages, MDSCs, and CAFs facilitate tumor progression by suppressing the immune system. Regarding the generation of inhibitory immune cells, tumors impair the maturation of myeloid cells into functional anti-tumor effector cells and skew development of immune responses toward those that are antibody-based and away from the more effective, cell-mediated responses (Gabrilovich, 2004; Pinzon-Charry, 2005; Solinas, 2009). Tumors also secrete TGF-β which can convert T cells into T_{regs} by inducing FoxP3 expression (Liu, 2007). Regarding the adverse role played by support cells in tumor-cell microenvironments, cancers distort local stromal and endothelial cells in ways that suppress the infiltrating immune cells (i.e. by inducing expression of Tim-3 or galectins) (Huang, 2010; Mellman, 2011; Gieseke, 2013; Sioud, 2013).

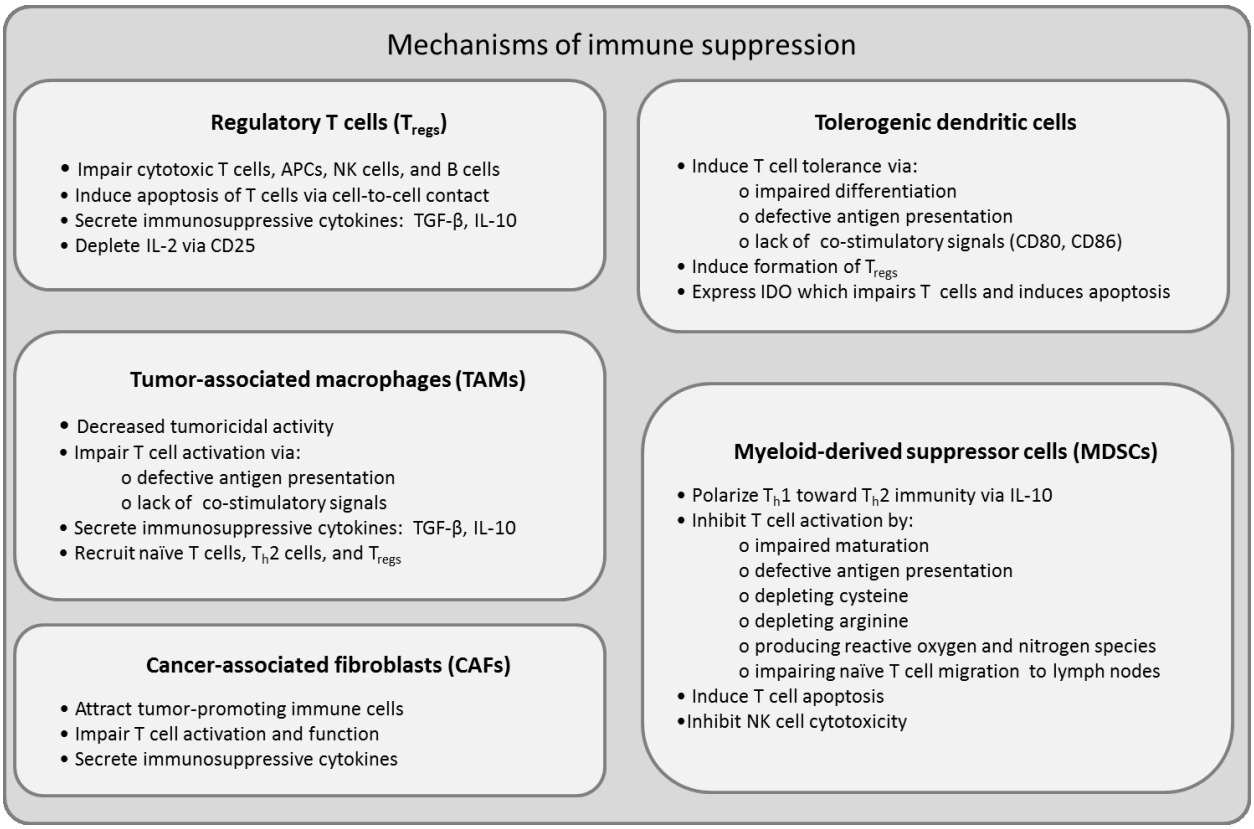

Figure 3.5. Proposed mechanisms of immune suppression by immune cells and cancer-associated fibroblasts. *Adapted with permission from*: (1) Kerkar, S. P., & Restifo, N. P. (2012). Cellular constituents of immune escape within the tumor microenvironment. *Cancer Research*, *72*(13), 3126. and (2) Baitsch, L., Fuertes-Marraco, S. A., Legat, A., et al. (2012). The three main stumbling blocks for anti-cancer T cells. *Trends in Immunology*, *33*(7), 365.

Reversing immune suppression

Strategies to reverse immuno-suppression include eliminating suppressive cells or their secretions, inducing immature ("tolerogenic") cells to differentiate into functional cells, and repolarizing ("re-educating") immune responses from tumor-promoting to tumor-rejecting responses. Inhibitory immune cells can be depleted using targeted therapies or systemic agents with immunological effects (Zou, 2006; Vanneman, 2012).

Targeting T_{regs}

Therapeutic strategies for targeting T_{regs} have been previously reviewed (Zou, 2006). Daclizumab, a CD25-targeting monoclonal antibody, depletes T_{regs} (which are classically $CD4^+CD25^+FoxP3^+$) and improves anti-tumor immunity in preclinical studies (Kline, 2008) and in clinical trials (Rech, 2009; Rech 2012). Denileukin difitox (OnTak), an IL-2-diphtheria toxin fusion protein, reduces T_{regs} and induces tumor regression in patients with melanoma (Telang, 2011). Glucocorticoid-induced tumor-necrosis factor-receptor related protein (GITR)-stimulating antibodies reduced the tumor-suppressor activity of T_{regs} *in vitro* (Shimizu, 2002) and promoted tumor regression in mice (Ko, 2005).

Targeting tolerogenic DCs

Therapeutic targeting of immature tolerogenic DCs can involve eliminating them, inhibiting their formation or expansion, or promoting their differentiation into functional DCs (that can present tumor antigens, secrete proinflammatory cytokines, express costimulatory ligands, and activate T cells). In mice with AML, treatment with agonistic CD40 antibodies – which are known to activate host APCs like DCs – reversed T-cell tolerance and prolonged survival (Zhang, 2013). CD40 ligation also restored T-cell antimicrobial immunity (Tuma, 2002). All-trans-retinoic-acid (ATRA) differentiated

immature myeloid cells into mature DCs (as well as macrophages and granulocytes) and improved anti-tumor immunity in tumor-bearing mice upon vaccination (Kusmartsev, 2003). Because immature DCs are a type of MDSC, see the "**Targeting MDSCs**" section for other ways to therapeutically target these cells.

Targeting tumor-associated macrophages (TAMs)

Therapeutic targeting of TAMs (including strategies to inhibit recruitment to tumors, impair survival, increase M1-like tumor-killing activity, and reduce M2-like tumor-promoting activity) have been extensively reviewed (Tang, 2012). Therapies may seek to reduce the number of TAMs or shift ("repolarize") macrophage behavior from tumor-promoting to tumor-rejecting agendas (Biswas, 2010). Combining the TLR9 agonist CpG with IL-10-receptor-blocking antibodies reprogrammed M1-like TAMs to potent M2-like anti-tumor effectors (Guiducci, 2005). CD40 agonistic antibodies (Buhtoiarov, 2006; Buhtoiarov, 2011; Beatty, 2011) and IL-12 (Watkins, 2007) also enhanced the anti-tumor activity of M1-like TAMs. Exposure to histidine-rich glycoprotein (Rolny, 2011) and inhibition of STAT3 and STAT6 signaling (Kortylewski, 2005; Cheng, 2003) reduced M2-like TAM tumor-promoting activity and inhibited tumor growth.

Targeting myeloid-derived suppressor cells (MDSCs)

Strategies that target MDSCs – by inducing differentiation, preventing expansion, inhibiting function, or eliminating them – have been extensively reviewed (Gabrilovich, 2009). Typically, the goal is to reduce the number of MDSCs or alter their function. ATRA (Mirza, 2006; Kusmartsev, 2003) and 25-hydroxyvitamin D3 (Lathers, 2004) promote MDSC differentiation into mature macrophages or DCs. Nitroaspirin (De Santo, 2005), sildenafil (Serafini, 2006), and biphosphonate (Melani, 2007) inhibit MDSC activity.

Inhibiting stem-cell-factor-mediated signaling reduces MDSC expansion (Pan, 2008). Some chemotherapy drugs like gemcitabine eliminate MDSCs (Suzuki, 2005).

Targeting suppressive metabolic enzymes

Anti-cancer therapies are being developed to not only eliminate inhibitory immune cells, but also to reduce their immuno-suppressive secretions. Enzymes like IDO and arginase impair the anti-tumor activity of NK and T cells by depleting tryptophan and arginine – amino acids needed for T-cell proliferation and function. Inhibiting IDO and arginase activity improves anti-tumor immunity in preclinical models (Uyttenhove, 2003; Liu, 2010; Raber, 2012) and is now being investigated in clinical trials. In normal physiologic settings, IDO limits the activation of T cells and NK cells in the placenta (Frumento, 2002; Munn, 2002).

General non-targeted strategies

The physical removal of malignant cells has been shown to reduce immuno-suppression. Removal of primary tumors restored humoral and cell-mediated anti-tumor immunity in mice with breast cancer despite persistence of extensive metastatic disease (Danna, 2004). Presumably, surgery not only eliminates cancer cells and the immuno-suppressive factors they secrete, but also a variety of inhibitory immune cells that have infiltrated the tumor. Removing both tumor cells and tumor-promoting immune cells may tip the balance toward effective anti-tumor immunity. Perhaps this means leukemia patients are most likely to respond to immunotherapy in the setting of MRD. Of note, lymphodepleting regimens (that reduce the number of T_{regs}) promote expansion of adoptively transferred T cells by inducing their homeostatic proliferation (Dudley, 2002; Rosenberg, 2008). Thus, the numbers of cancer and immune-effector cells – or the

immune-effector/immune-suppressor cell ratio – may determine whether malignant cells can persist or will be eliminated.

Overall, in addition to inducing immunogenic tumor-cell death, some standard ("conventional") anti-cancer therapies may have additional immuno-stimulatory effects (Zitvogel, 2008). For example, chemotherapeutic agents like cyclophosphamide can partially deplete or inactivate T_{regs} (Ghiringhelli, 2004; Lutsiak, 2005).

Cancer-associated fibroblasts

The conceptual frameworks that are driving cancer research include theories and models that integrate diverse scientific findings and viewpoints. This is especially true for the study of cancer microenvironments. Experiments that are clarifying how local surroundings support tumor cells have been inspired by provocative models of tumor-microenvironment interactions (Infanger, 2013). For example, some propose that the physiologic mechanisms that so effectively protect cancer cells had originally evolved to promote wound-healing and regeneration (Oviedo, 2009). In this conceptual framework, some patterns of inflammation are critical. In response to injury, tissue repair in primitive animals occurs in adverse acidic, hypoxic, inflammatory microenvironments that trigger mechanisms that promote the survival and proliferation of critical stem-like (normal) cells that regenerate tissues. Cancer-induced inflammatory states resemble those triggered by injury, and they may trigger the same cell-protection mechanisms that evolved to protect normal stem cells to, unfortunately, promote CSC survival (Landsberg, 2012). In this discussion, (from among many synonyms) the terms "normal stromal fibroblast" and "CAF" are used. As shown in Figure 3.6, tumor microenvironments convert, or reprogram, normal stromal fibroblasts into stromal cells with a CAF phenotype. These CAFs, in turn,

alter the phenotypes of immune cells in ways that promote tumor-cell survival via immune evasion and tolerance. Of note, the terms “mesenchymal stem cells” and “multi-potent mesenchymal stromal cells” are other names that are sometimes used for the cells that give rise to normal stromal fibroblasts. For simplicity, this discussion uses an example involving “the reciprocal-interactions model for CAF creation,” the galectin-9/Tim-3 axis, and the secretion of pro-inflammatory cytokine IL-6 to illustrate one way the interactions of cells and molecular factors may cause immune cells to become dysfunctional in cancer patients. The “reciprocal-interactions model” proposes that tumor cells attract macrophages and, together, these cells convert, or reprogram, normal stromal fibroblasts into CAFs via pro-inflammatory cytokines like IL-6 (Haviv, 2009; Anton, 2012). A study of ovarian cancer cells found that tumor cells can reprogram normal stromal fibroblasts into CAFs by altering fibroblast expression of miRNAs, and these changes could be either duplicated or reversed *in vitro* by transfecting fibroblasts with the appropriate miRNAs and miRNA inhibitors (Mitra, 2012). So, targeting tumor stromal cells with miRNAs and miRNA inhibitors may be a valuable therapeutic strategy. Of note, implicit in both “the reciprocal-interactions CAF model” and the original therapy-engineering vision of this project is the expectation that greater insight into inflammation and regeneration-related mechanisms can help us manipulate cells and engineer effective cancer therapies *in vitro*. Over the course of evolution, the protection of vital stem-like cells has been a priority. Animals that can generate and protect critical (normal) stem-like cells in response to injury and inflammation have a survival advantage. But, for cancer patients, the unfortunate protection of lethal, stem-like tumor cells is a problem that needs to be reversed.

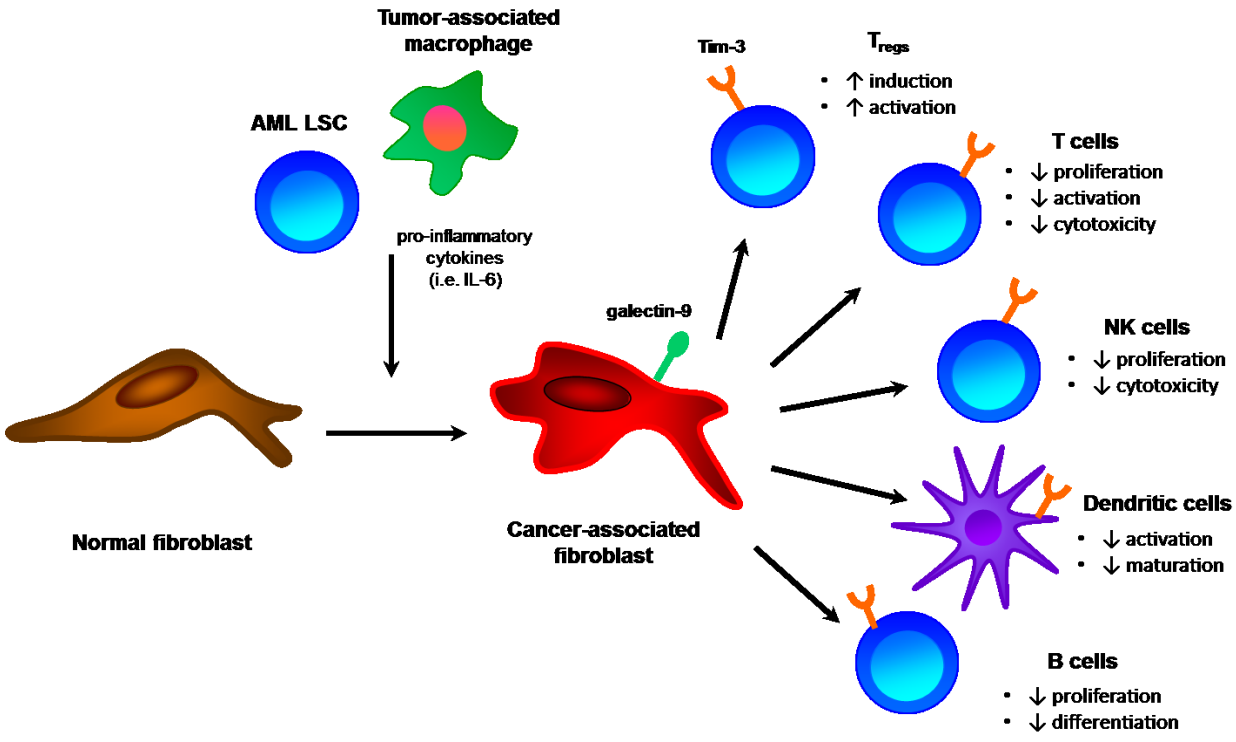

Figure 3.6. Role of AML-impaired stromal cells in immune suppression. A potential model for how AML-distorted CAFs suppress anti-tumor immunity is shown. Leukemia cells recruit immune cells such as tumor-associated macrophages which release pro-inflammatory cytokines (i.e. IL-6). The pro-inflammatory tumor microenvironment triggers conversion of bone marrow stromal cells into CAFs. CAFs may suppress infiltrating immune cells through production and release of immunosuppressive factors and enzymes as well as cell-to-cell contact (i.e. galectin-9-Tim-3 interactions). *Adapted with permission from*: Sioud, M. (2011). New insights into mesenchymal stromal cell-mediated T-cell suppression through galectins. *Scandinavian Journal of Immunology*, *73*(2), 80.

Figure 3.6 is a deliberately simplistic depiction of how CAFs may induce immune-cell defects at the molecular level. IL-6 and Tim-3 are highlighted in this diagram because these two factors have been found to play key roles in AML disease (Sugiyama, 1996; Kikushige, 2010). Furthermore, the IL-6 secreted by AML cells may trigger generation of CAFs in cancer patients (Cirri, 2011). A more realistic model would acknowledge that interactions are likely to be bi-directional and feedback loops may act so quickly it may be difficult to identify cause-and-effect sequences. In addition to IL-6 and Tim-3, many other cytokines (like IFN-γ) and antigens (like MUC-1) may promote immune-system dysfunction. Overexpression of the immune-checkpoint receptor Tim-3 (Kane, 2010) is used here as one potentially informative, and therapeutically relevant, example. Of note, Tim-3 is expressed not only by the malignant cells of diverse cancers, but is also expressed on the surfaces of microglial, mast, monocytic, macrophage, dendritic, and T (cytotoxic, helper, and regulatory) cells (Nakae, 2007; Wang, 2011). Tim-3 ligands include galectin-9 and phosphatidylserine (Zhu, 2005; DeKruyff, 2010).

Tim-3 is considered a critically important factor in AML pathogenesis because it is a surface antigen expressed by highly evolved AML LSCs (Kikushige, 2010). In general, tumor cells can evolve in response to immune-selection pressures, and coexpression of Tim-3 and PD1 increases, for example, as AML disease progresses (Zhou, 2011). Blocking both the PD1/PDL1 and Tim-3/galectin-9 axes can reduce AML tumor burden and mortality (Zhou, 2011). In mice with advanced AML, $CD8^+$ T cells co-expressing PD1 and Tim-3 synthesized less IFN-γ, TNF-α, and IL-2 than T cells which did not express PD1 or Tim-3 when exposed to AML cells expressing the ligands PDL1 and galectin-9 (Tim-3 ligand) (Zhou, 2011). In galectin-9 knock-out mice, AML progressed more slowly

and fewer T_{regs} accumulated compared to wildtype mice (Zhou, 2011). In PD1 knock-out mice, Tim-3 was upregulated on T cells as AML progressed, and Tim-3-expressing $CD8^+$ T cells showed impaired cytokine secretion compared to wildtype mice (Zhou, 2011). Administering a Tim-3 blocking antibody to mice with human AML reduces their tumor burden and eliminates the primitive AML cells that can reconstitute AML in secondary recipients (Kikushige, 2010; Zhou, 2011; Kikushige, 2012).

In general, immunotherapy seeks to eliminate tumor cells – especially the relapse-causing CSCs – and overcome, or reverse, immune and/or stromal-cell defects that prevent effective anti-tumor immunity. **Chapter IV** reviews examples of promising anti-cancer immunotherapies, including adoptive transfer of a patient's *ex vivo*-modified antigen-presenting and immune-effector cells, antibodies, vaccines, and agents that modulate immune-checkpoint receptors such as CTLA-4 and Tim-3. These strategies are designed to target tumor cells, dysfunctional immune cells, and/or the immuno-suppressive tumor microenvironment. Conventional therapies such as radiotherapy and chemotherapeutic agents that induce immunogenic tumor cell death (aka "cryptic vaccines") can also be considered immune therapies. Of course, the ultimate benefit of some therapies is the induction of a protective immunologic "memory" involving immune cells that prevent re-emergence of disease by providing constant tumor-cell surveillance. "Memory T cells," for example, have induced long-term remissions – perhaps lifelong cures – in some leukemia patients.

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Chapter IV

Anti-cancer immunotherapy: strategies and recent successes

Cancer immunotherapy is now a broad and rapidly expanding field (Raval, 2014). This chapter highlights recent technical advances and clinical successes achieved by new patient-specific and FDA-approved anti-cancer therapies, reviews AML immuno-therapies in preclinical development, and discusses rational therapeutic combinations. Key features of effective anti-tumor immune responses involving innate and adaptive immune defenses are reviewed in **Chapter III**. Review articles are recommended that comprehensively address topics that are only briefly summarized in this dissertation.

Introduction

To save some cancer patients, combination strategies may be required that include immunologic components that can overcome cancer-cell immune-evasion and death-resistance, activate and repair immune cells, and neutralize immuno-suppressive factors in the tumor microenvironment (see Figure 4.1). **Chapter II** reviews strategies that increase the immunogenicity of cancer cells (making them more susceptible to immunologic attack), improve the presentation of leukemic antigens, overcome cancer-induced and immune-mediated death-resistance of cancer cells, and induce immunogenic tumor-cell death (ICD). Immunotherapies should (1) unleash pre-existing, tumor-reactive immune cells that are suppressed and/or (2) trigger *de novo* anti-tumor immune responses. Strategies to restore anti-tumor immune cells that have been silenced include removing the inhibitory "brakes" on exhausted immune cells (to restore their function) by blocking immune-checkpoint receptors like PD1 (Pardoll, 2012; Yao, 2013). Strategies to trigger d*e novo* immune responses include inducing immunogenic tumor-cell death which can be considered a form of "vaccination *in situ*" (Tesniere, 2008; Inoue, 2014). Immunogenic – as opposed to tolerogenic – forms of cell death can release

new, previously unexposed leukemic antigens or present previously non-immunogenic antigens in a new, immune-stimulatory context that is enhanced by “danger” signals. ICD can trigger antigen-presenting cells (APCs) to uptake and cross-present leukemia-cell antigens to T cells and, thereby, stimulate anti-tumor immunity. Ideally, therapies should stimulate both the activation and effector phases of the innate and adaptive immune cascades, including both the cellular and humoral arms (Yao, 2013). While the activation phase of adaptive immune responses involves tumor antigen cross-presentation to T cells in lymphoid organs, the effector phase involves tumor-cell lysis in the periphery. **Chapter III** reviews strategies to repair and activate antigen-presenting and immune-effector cells as well as eliminate immuno-suppressive cells in the tumor microenvironment. This chapter reviews cancer immunotherapies involving antibodies, vaccines, adoptive transfer of immune cells, and leukemia-derived APCs. These strategies can be combined with approaches that enhance immune responses in non-specific ways such as immunogenic chemotherapies (Obeid, 2007; Zitvogel, 2008; Vanneman, 2012).

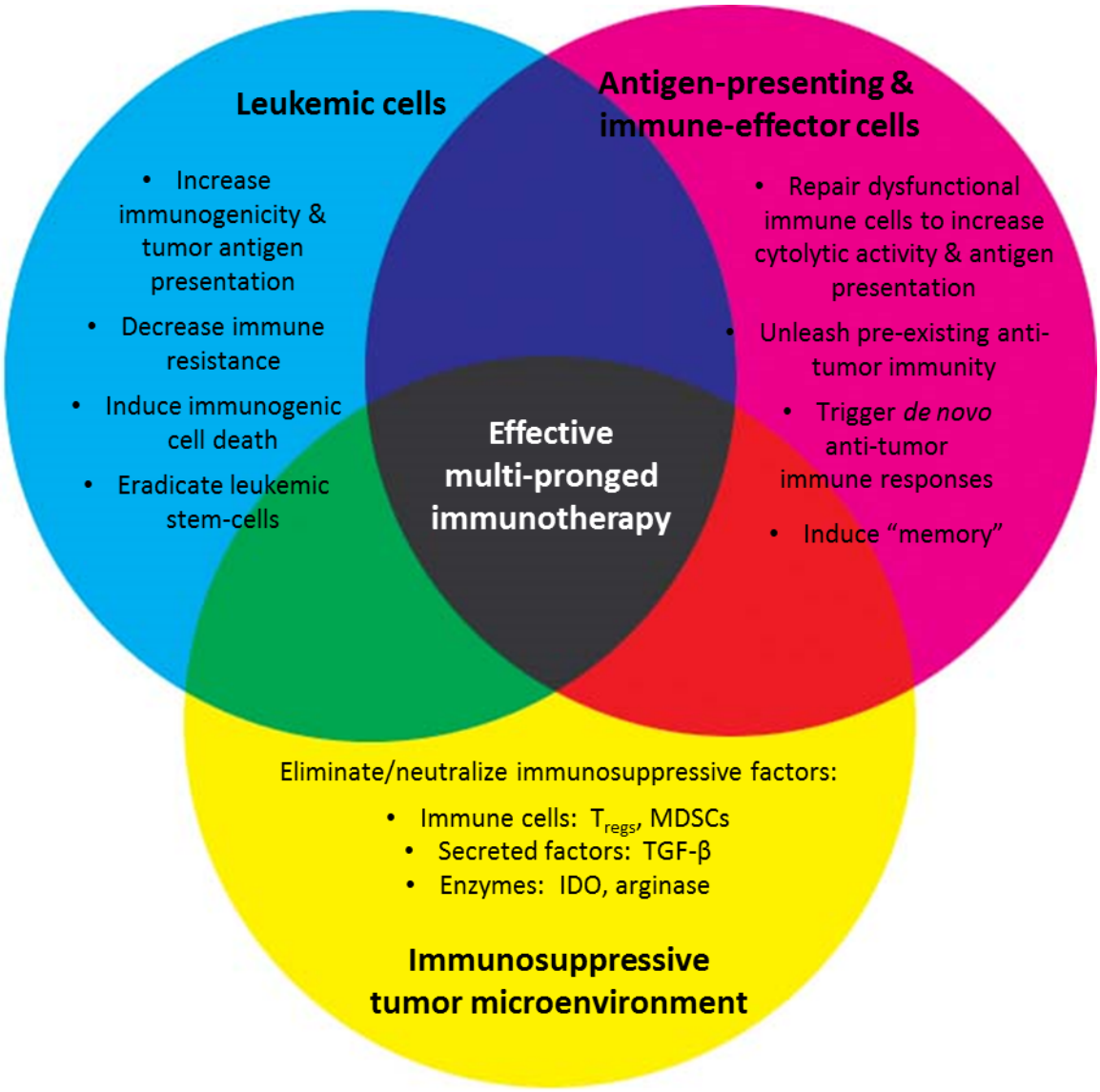

Figure 4.1. Three-pronged anti-cancer immunotherapeutic strategy. To achieve lasting anti-tumor immunity, targeting of (i) tumor cells, (ii) the immune system, and (iii) the immunosuppressive tumor microenvironment may be required. Tumor cells may need to be altered to increase antigen presentation and decrease death resistance. Dysfunctional antigen-presenting and immune-effector cells may need to be repaired to become activated, responsive, and functional. Finally, the tumor microenvironment may need to be targeted to neutralize immunosuppressive factors and eliminate immunosuppressive cell types.

Anti-cancer immunotherapeutic strategies

Adoptive transfer of immune-effector cells

Studies of allogeneic hematopoietic stem cell transplantation (HSCT) show that donor T and NK cells can induce potent graft-versus-leukemia (GvL) immune responses that have cured some patients (Ruggeri, 2002; Bleakley, 2004). However, HSCT carries the risk of graft-versus host disease (GvHD). Using a patient's own NK or T cells ("autologous" cells) eliminates the risk of immune incompatibilities. Recently, cancer patients have experienced dramatic remissions after being treated with their own cancer-reactive, tumor-infiltrating T cells that were isolated, expanded *ex vivo*, and re-infused as therapy (Tran, 2014). Alternatively, a patient's NK or T cells can be genetically engineered to express receptors that target (bind) antigens on the patient's tumor cells (Kalos, 2011; Porter, 2011). Immune cells to be used as therapy may also be genetically modified in other ways to increase their survival, proliferation, trafficking toward malignant cells, activity, and persistence (resistance to immune suppression) (Kershaw, 2013).

CAR-modified T cells: Of historic significance, adoptive transfer of autologous chimeric antigen receptor (CAR)-modified T cells induced rapid and sustained remissions in patients with advanced B cell leukemias (Brentjens, 2011; Brentjens, 2013; Kalos, 2011; Kockenderfer, 2012; Porter, 2011) and lymphomas (Kockenderfer, 2012). In a clinical trial, each chronic lymphocytic leukemia (CLL) patient's T cells were genetically engineered to express "second-generation" CARs composed of an extracellular CD19-targeting domain fused with intracellular CD137 costimulatory and T-cell receptor ζ domains (Kalos, 2011; Porter, 2011). The antibody-binding domain directly recognizes antigens on the tumor-cell surface rather than peptide-MHC complexes, enabling T-cell

activation without the need for antigen-presentation (Gross, 1989; Irving, 1991). Within CLL patients, CAR-modified T cells survived, persisted, killed ~1,000 cancer cells per effector, induced remission (>2 years now), and generated memory T cells (Kalos, 2011; Porter, 2011). Of note, tumor-cell death was so extensive, patients experienced "tumor lysis syndrome" and "cytokine release syndrome" (readily reversed using tocilizumab to block IL-6) along with aplasias of normal B-cells which were managed via immunoglobulin injections (Kalos, 2011; Porter, 2011).

T cells "rejuvenated" through iPSC reprogramming: In patients with cancer (Vizcardo, 2013) and chronic HIV infection (Nishimura, 2013), reprogramming their exhausted T cells to pluripotency – followed by re-differentiation back to T cells – rejuvenated their cytokine-secreting and cytolytic activities. "Rejuvenated" T cells retained their antigen-specificity and acquired high proliferative potential accompanied by elongated telomeres (Nishimura, 2013). Large numbers of functionally "rejuvenated" T cells may be further modified (via genetic engineering, for example) to express CARs prior to use as therapy (Themeli, 2013). Although using a patient's own (autologous) immune cells as therapy eliminates the risk of immune-incompatibilities (such as host rejection of foreign cells or GvHD), sufficient numbers of immune cells may not be available in severely immuno-compromised cancer patients. Therefore, researchers are exploring ways to generate large numbers of reprogrammed tumor-reactive T cells that are universally tolerated by disrupting T-cell receptors or repressing human leukocyte antigens (HLA) (Themeli, 2013). Of note, reprogrammed T cells have not yet been evaluated in clinical trials.

LSC-targeted antibodies

Treatment with antibodies directed against the antigens on AML LSCs (including antigens that emerge on highly evolved AML LSCs) – such as Tim-3 (Kikushige, 2010), CD47 (Majeti, 2009), CD44 (Jin, 2006), and interleukin (IL)-3 receptor α (IL3Rα or CD123) (Jordan, 2000; Jin, 2009) – has eliminated disease and extended survival in mice engrafted with AML. By promoting tumor-cell immune-escape (Jaiswal, 2009), several of these receptors may contribute to the poor immunogenicity of AML LSCs (Costello, 2000). For example, AML LSCs express CD47, a phagocyte "do not eat me" signal (Jaiswal, 2009), and CD47-targeting antibodies promote eradication of AML LSCs by facilitating their uptake and clearance by macrophages (Majeti, 2009). Gemtuzumab ozogamicin (Mylotarg), an anti-CD33 antibody fused to the toxin calicheamicin, is FDA-approved for AML patients with relapsed $CD33^+$ disease (Larson, 2002). Antibodies may facilitate tumor-cell clearance by antibody-dependent cytotoxicity mediated by NK cells or by activation of the complement cascade. To enhance tumor-cell killing, antibodies may be conjugated with drugs, radioisotopes, cytokines, or toxins. Fortunately, leukemia-cell death, in turn, can stimulate anti-tumor immune responses by enhancing the uptake of leukemia antigens and their presentation to immune cells.

Immune-modulating antibodies

Various co-stimulatory and co-inhibitory immune-checkpoint receptors are expressed by T cells and interact with their ligands on APCs and cancer cells (see Figure 4.2). Both (1) agonistic antibodies that activate costimulatory receptors such as CD28 and CD137/4-1BB (Melero, 2007) and (2) antagonistic antibodies that block critical coinhibitory receptors – such as Tim-3, programmed cell death protein 1 (PD-1), and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (Pardoll, 2012) – promote anti-tumor immunity (see Figure 4.3). Depending on the ligand and cell type(s) targeted, immune-modulating antibodies increase the cross-presentation of tumor antigens by DCs or other APCs in the lymph nodes and/or the killing of tumor cells by immune-effector (i.e. NK, T) cells in the peripheral tissues (see Figure 4.3). Of note, ipilimumab – an antibody that blocks CTLA-4 – is the first agent to ever improve the survival of patients with metastatic melanoma (Hodi, 2010). Adverse side effects of anti-CTLA-4 therapy include autoimmune reactions resulting from potent activation of not just tumor-reactive T cells but self-reactive T cells as well (Hodi, 2010). CTLA-4 knock-out mice also suffer from severe autoimmune reactions (Tivol, 1995; Waterhouse, 1995). Co-blockade of the coinhibitory receptors Tim-3 and PD-1 improves anti-tumor immunity in mouse models of melanoma (Fourcade, 2010), supporting the rationale for combination therapies. As with traditional cancer therapies, immuno-modulating strategies can induce both beneficial and undesirable effects that must be carefully considered and anticipated.

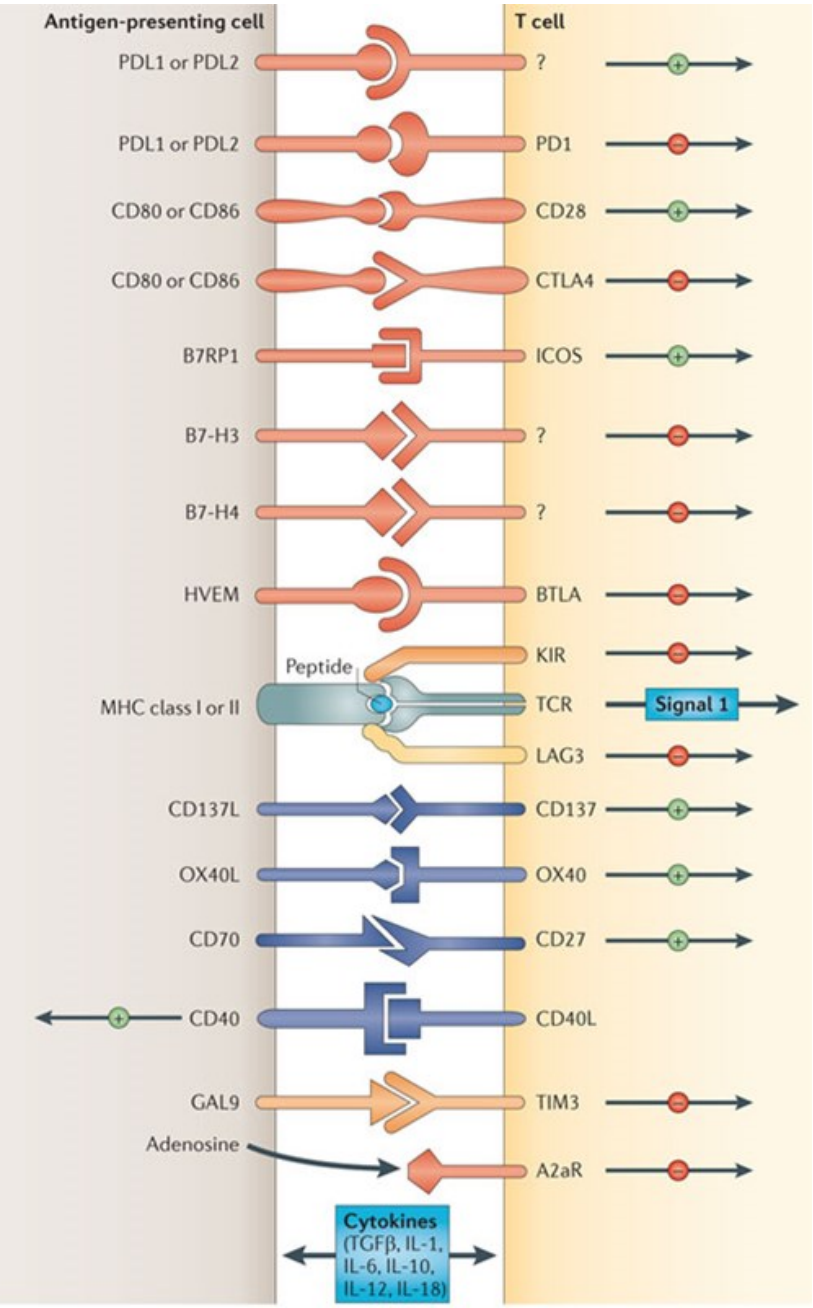

Figure 4.2. Immune checkpoint receptors and their ligands. Immunomodulatory cell surface receptors are emerging as important therapeutic targets for controlling cancer. Numerous co-inhibitory and co-stimulatory ligand-receptor interactions are known. The net effect on immune cell activation or suppression involves integration of all signals. Not only T cells, but also cells of the innate immune system and tumor cells can express immune checkpoint receptors (i.e. Tim-3) as well as their ligands (i.e. PD1-ligand). *Reproduced with permission from*: Pardoll, D. M. (2012). The blockade of immune checkpoints in cancer immunotherapy. *Nature Reviews Cancer*, *12*(4), 254.

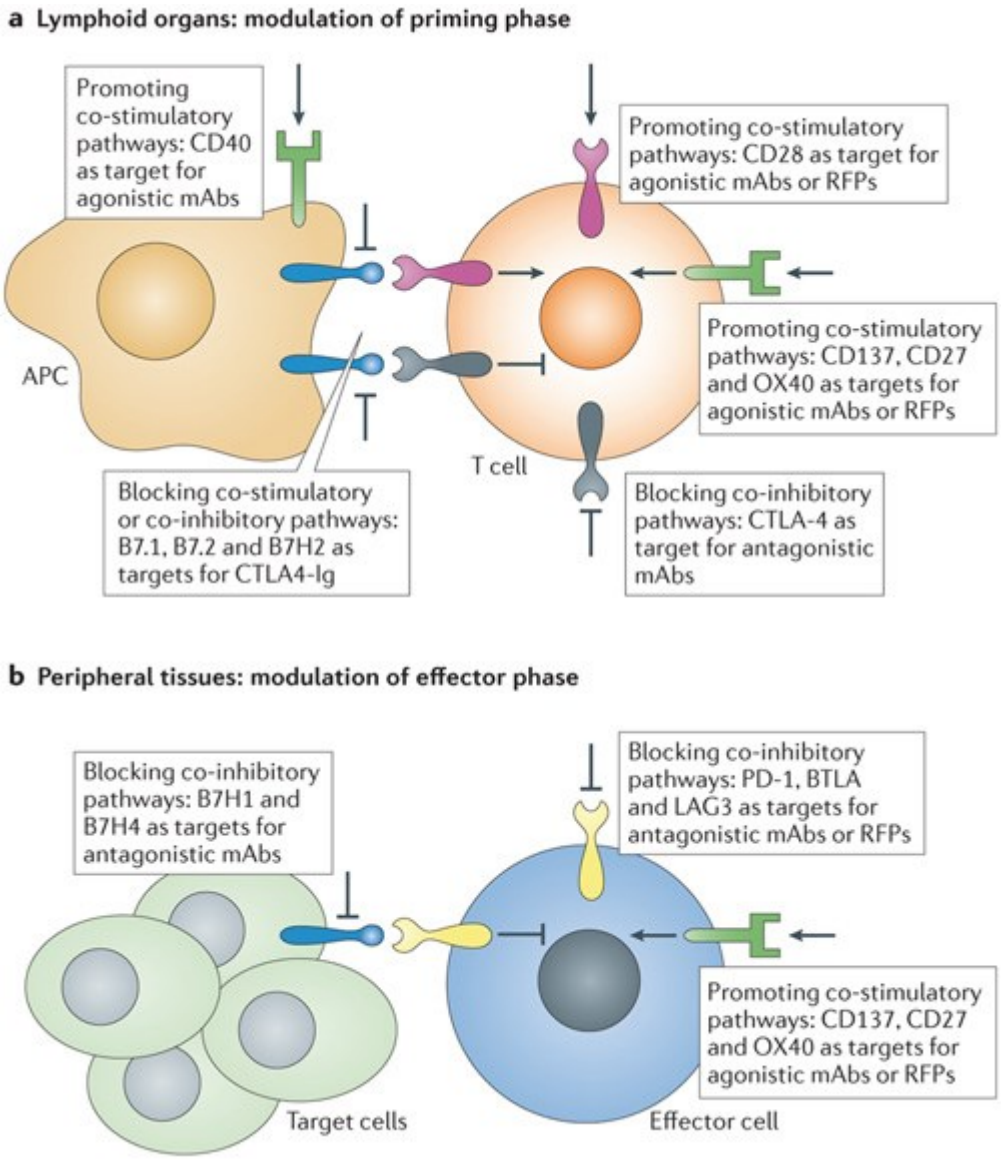

Figure 4.3. Targeting immunomodulatory receptors to improve anti-tumor immunity. Agonistic antibodies to co-stimulatory receptors and antagonistic (blocking) antibodies to co-inhibitory receptors can be used therapeutically to amplify host immune responses against tumors. Potential areas of therapeutic interventions include: ligand-receptor interactions between antigen-presenting cells (i.e. DCs) and T cells within lymph nodes during the "priming phase" (**a**) and ligand-receptor interactions between immune effector cells and tumor cell targets in the periphery during the "effector phase" (**b**). *Reproduced with permission from*: Yao, S., Zhu, Y., & Chen, L. (2013). Advances in targeting cell surface signalling molecules for immune modulation. *Nature Reviews Drug Discovery*, *12*(2), 135.

Vaccines

Therapeutic cancer vaccines – used to treat patients after cancer is diagnosed – may include whole tumor cells, tumor peptides, APCs, viruses, and immune-stimulating adjuvants (Schlom, 2012). Anti-leukemia cell-based vaccines include inactivated whole tumor cells that may be modified *ex vivo* to improve their antigen-presenting or immuno-stimulating capacity (Burkhardt, 2013). Utilizing whole tumor cells (as opposed to a limited number of peptides) may trigger immune responses against multiple leukemia-associated antigens, reduce the likelihood of selecting for "escape variants," and eliminate the need to identify patient-specific immunogenic peptides in advance. To improve their immuno-stimulatory capacity, AML cells may be modified *in vitro* to secrete cytokines like GM-CSF (Ho, 2006; Ho, 2007) and IL-2 (Chan, 2006) or express co-stimulatory molecules like CD80 (Chan, 2006).

For peptide vaccines to be effective, the immunizing peptide must be recognized by the immune system and be able to stimulate anti-tumor immunity. The "ideal target" for an antigen-specific immunotherapy would (1) be expressed by leukemic but not normal cells, (2) be highly expressed by all leukemia cells, including LSCs, (3) be involved in leukemia development, maintenance, and progression, (4) be strongly immunogenic, and (5) when used clinically, be able to stimulate tumor-rejection (Cheever, 2009). Of note, AML vaccines designed to target leukemia-specific antigens – such as mutated nucleophosmin (Greiner, 2005) and leukemia-associated antigens like WT1 (Van Driessche, 2012), proteinase 3 (Rezvani, 2008), and RHAMM/CD168 (Greiner, 2005; Schmitt, 2008) – are currently under development or are being evaluated in clinical trials.

Regarding APC-based vaccines, AML lysates or peptides can be loaded onto DCs by peptide pulsing, loading, or fusion with AML cells. AML cells can also be differentiated into DCs using cytokines or calcium ionophores to improve their antigen-presenting capabilities (Choudhury, 1999). Leukemic DCs maintain expression of leukemia-associated antigens, and their therapeutic potential is being evaluated in clinical trials (Li, 2004; Li, 2006; Hicks, 2003). However, if AML cells are converted into immature or IDO-expressing DCs, they can hinder anti-tumor immunity by promoting T-cell tolerance (Curti, 2012). Of course, vaccines can be combined with adjuvants like TLR ligands, cytokines, and co-stimulators to enhance immune activation.

Provenge (Sipuleucel-T), a patient-specific DC-based vaccine for advanced prostate cancer, is the first FDA-approved cellular immunotherapy (Kantoff, 2010). Provenge is prepared by culturing each patient's peripheral blood mononuclear cells with a fusion protein consisting of prostatic acid phosphatase (PAP) linked to granulocyte-monocyte colony-stimulating factor (GM-CSF) (Kantoff, 2010). Although the precise mechanism of action is unknown, APCs (including DCs) are believed to ingest, process, and present portions of the GM-CSF protein to T cells to stimulate their anti-tumor activity. Provenge improved survival of prostate cancer patients by 4.1 months (Kantoff, 2010), but this survival benefit has been questioned due to possible flaws in the design of the clinical trial (Huber, 2012).

Targeting the immuno-suppressive tumor microenvironment

While some therapies are designed to stimulate immune responses, others seek to reduce immuno-suppression. Strategies to inhibit immuno-suppressive cells, factors, and enzymes within the tumor microenvironment include (1) eliminating T_{regs}, (2) blocking cytokines like TGF-β, and (3) inhibiting immuno-suppressive enzymes such as indoleamine 2,3-dioxygenase (IDO) (Löb, 2009; Qian, 2009) and arginase (Reisser, 2002). CD25-targeting antibodies eliminate T_{regs} in the tumor microenvironment and show promise in the treatment of breast cancer (Rech, 2012). Of note, immune-checkpoint receptors can not only inhibit the activity of cytotoxic T cells, they may also enhance the proliferation and activity of T_{regs} (Pardoll, 2012). Thus, the clinical benefits of immune-checkpoint-receptor inhibitors may be due not only to activation of cytotoxic T cells but also to inhibition of immuno-suppressive T_{regs} (Pardoll, 2012). Competitive inhibitors or "suicide substrates" have been designed to block the enzymatic activity of IDO (Löb, 2009; Qian, 2009) and arginase (Reisser, 2002) which inhibit T-cell activity by depleting local amino acids. Strategies to neutralize immuno-suppressive factors can act synergistically with immune-stimulating agendas since they affect different mechanisms.

Additional strategies

Immuno-therapeutic strategies reviewed elsewhere include the use of cytokines (i.e. IL-2) (Dranoff, 2004), oncolytic viruses (Chiocca, 2002), targeted anti-cancer therapies that enhance immune responses (Vanneman, 2012), and "conventional" radiotherapies and chemotherapies that induce ICD or modulate the immunogenicity of tumor cells (Kono, 2013; Inoue, 2014).

Designing and engineering patient-specific immunotherapies for AML

To save the lives of some cancer patients, multi-pronged therapies may be needed that combine patient-specific immune strategies with "conventional" treatments. An immunologic component may be necessary to eliminate the stem-like, relapse-causing cancer cells and to provide long-term protection via "memory" immune-effector cells. The immunogenic death of mature "bulk" cancer cells induced by radiotherapies and some chemotherapies may act synergistically with immunotherapies.

Technically, the successful engineering of patient-specific immune strategies has been possible because better methods have been developed for maintaining and manipulating a patient's tumor and immune cells *in vitro*. Recently, two small molecules, StemRegenin1 (an aryl-hydrocarbon-receptor antagonist) and UM729 (a pyrimidol indole), were found to inhibit differentiation and maintain AML LSC activity in culture (Pabst, 2014). New approaches and tools are being identified and evaluated on an ongoing basis so patient-specific, anti-CSC immune therapies might, in the future, be developed soon after diagnosis – preferably without genetic engineering. Figure 4.4 provides an overview of diverse tools and conditions that may facilitate *in vitro* cell-engineering. One premise of this project is that any cell-manipulation tasks needed to generate a desired immune therapy – even the de-differentiation of immune and tumor cells – can eventually be accomplished without genetic engineering via the skillful use of molecular tools and physical conditions (Hou, 2013). This optimism is based on the observation that normal cells of primitive animals spontaneously de-differentiate before their amputated limbs regenerate, and malignant human cells can also de-differentiate. That is, even profound cell changes can be induced by physiologic stresses, and these

molecular mechanisms are likely to be clarified and (eventually) exploited *in vitro* for therapy-development purposes.

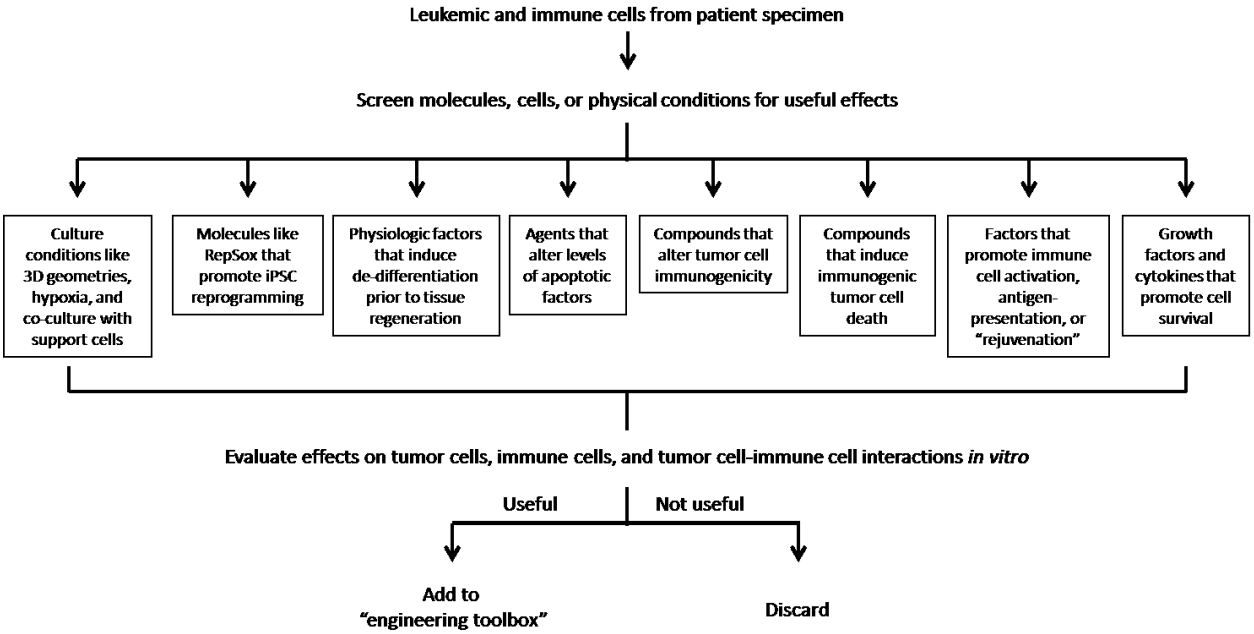

Figure 4.4. Potential "candidate tools" inspired by regeneration and cancer research that may facilitate *in vitro* engineering of cancer immunotherapies.

Regarding antibody therapies, to develop and evaluate antibodies for an AML patient's genetically and immuno-phenotypically unique disease, it may be necessary to identify the specific tumor-associated surface antigens on that patient's stem-like AML cells. The engineering and preclinical evaluation of a patient-specific antibody strategy may require that adequate numbers of the patient's primitive leukemia cells be maintained *in vitro* long enough to complete all the required technical tasks (Pabst, 2014). Currently, finding tools and conditions that promote the survival and expansion of primary leukemia cells remains a priority (Pabst, 2014). Because specimens from some patients contain only small proportions of primitive AML cells, tools may be required to "convert" mature AML cells into stem-like AML cells (Noh, 2012) – see Figure 4.5. When working with the cells available in some patient specimens, de-differentiation may be needed. Fortunately, a variety of cell types can be de-differentiated *in vitro* using just a few defined factors (Takahashi, 2006; Takahashi, 2007; Hou, 2013). As outlined in Figure 4.4, tools and conditions that may improve survival of stem-like AML cells – or convert mature AML cells into more stem-like cells – include both naturally occurring physiologic factors and artificially synthesized compounds. New candidate tools and conditions for cell-engineering are being identified as cell-culture methods improve and new insights are gained into the molecular mechanisms that induce cellular de-differentiation during tissue regeneration and chemical reprogramming. Perhaps, the potent chemicals used for 7-factor reprogramming to pluripotency (Hou, 2013) may be useful for engineering AML immune therapies. Of note, RepSox is one of the seven factors, and, conceivably, the other six chemicals may also help maintain or manipulate primitive AML cells *in vitro* in useful ways. Presumably, better ways to generate, or maintain, primitive AML cells *in*

vitro can help bioengineers design antibodies that target/bind antigens on the stem-like AML cells responsible for disease-relapse.

Of note, because TGF-β can increase cell-surface expression of Tim-3 (Wiener, 2006; Kim, 2012) – which is expressed by the highly evolved LSCs found in patients with advanced AML disease – factors like TGF-β may be useful when there is a need to evaluate, *in vitro*, tumor-cell phenotypes in advanced stages of evolution. In general, AML-cell diversity is substantial, and patient-specific strategies may be necessary since each patient's LSC-associated antigens can have unique molecular and cytogenetic features at disease onset as well as after evolution-dependent phenotypes emerge as disease progresses. Since there is an evolving distribution of cancer cells within any given patient, presumably, any factor that can drive AML cells toward any of the CSC phenotypes (Noh, 2012) is potentially useful when engineering antibodies or cells for the purpose of targeting relapse-causing AML cells.

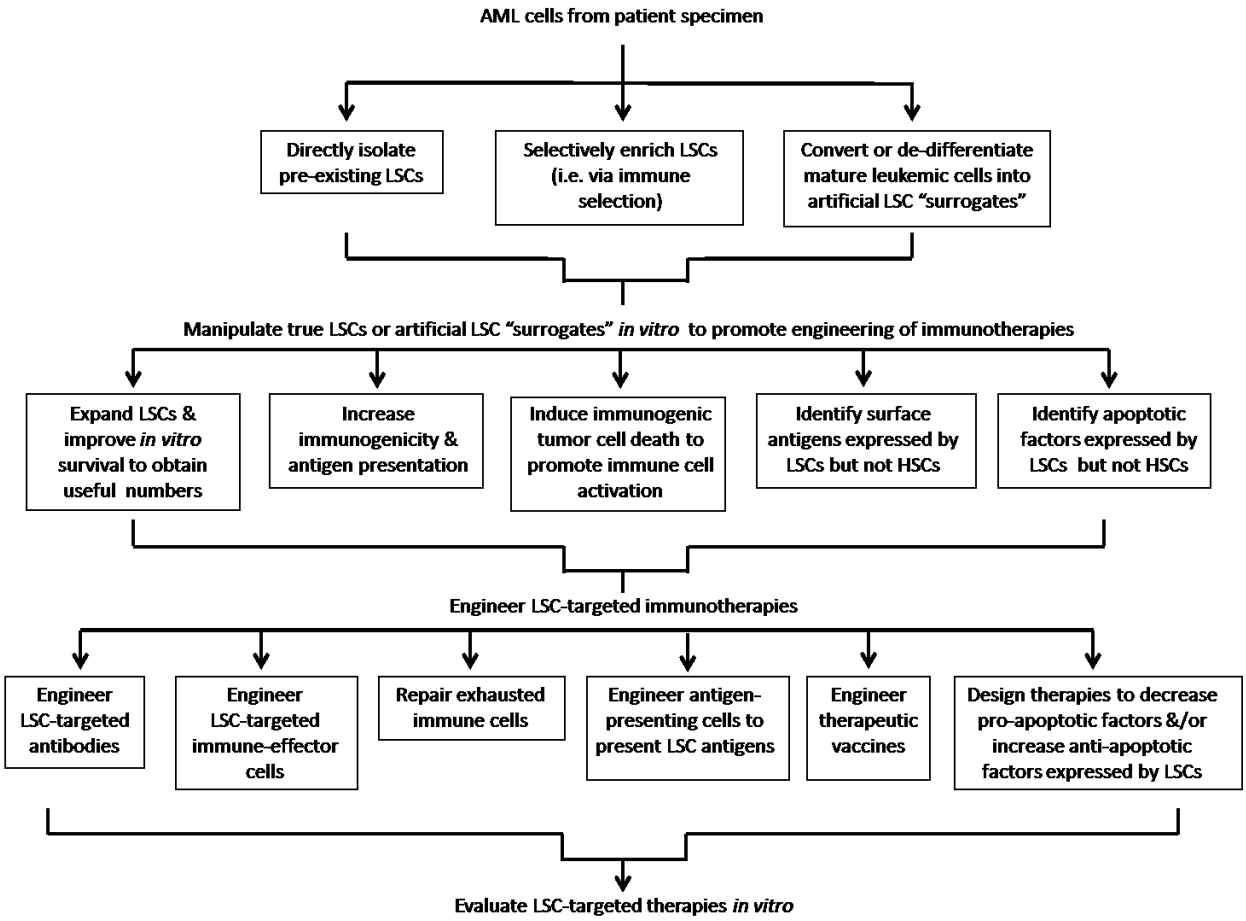

Figure 4.5. Potential ways pre-existing LSCs or *in vitro*-derived LSC "surrogates" may need to be manipulated for the design of LSC-targeted immunotherapies. Overview of ways to isolate, generate, and/or manipulate a patient's pre-existing LSCs or dedifferentiated LSC "surrogates" prior to engineering and evaluating LSC-targeted therapies.

The engineering of patient-specific immune strategies that involve antigen-presenting and immune-effector cells is likely to be more difficult than engineering antibodies – except, perhaps, in the special (and remarkable) context of the spontaneous activation of γδ T-cells in co-culture systems. In general, immune-cell engineering may involve multiple cells (T cells, NK cells, and APCs as well as tumor cells) manipulated individually and during interactions between these cells (like antigen-presentation and

immune-synapse formation). Fortunately, *ex vivo* cell-manipulations provide unlimited options and flexibility. For example, optimal *in vitro* antigen-presentation and immune-cell activation may require using LSC-like cells that are less evolved than those that are used to engineer antibodies since the more evolved LSC phenotypes (that appear in advanced leukemias) may be less immunogenic (Costello, 1999; Costello, 2000; Kikushige; Majeti) and more immuno-suppressive than the less evolved LSCs. That is, different agendas may be facilitated by using cells having specific "task-friendly" phenotypes. Fortunately, factors like TGF-β and RepSox can change the phenotypes of cancer cells into phenotypes that may reflect different stages of tumor-cell evolution. In general, cancer researchers are beginning to use *in vitro* cellular manipulations and observations to invent, develop, and evaluate autologous immune-cell therapies. Advancing these ambitious agendas seems feasible when considering, for example, that even the chemical reprogramming of mature cells to pluripotency has already been achieved (Hou, 2013). Also encouraging, "immune-selection pressures" can progressively "convert" populations of tumor cells into more stem-like populations (Noh, 2012). That is, using methods that have already developed, a spectrum of cancer cells can be generated whose cells exhibit different degrees of "stemness" (Noh, 2012). Similarly, a variety of partially reprogrammed intermediates expressing different degrees of lineage-commitment genes are generated during iPSC reprogramming (Hochedlinger, 2009; Mikkelsen, 2008). Because the underlying "immune-selection" mechanisms that drive cancer cells toward a stem-like phenotype (like the Nanog/Tcl1a/Akt pathway) are being identified (Noh, 2012; Li), it is possible that, when needed, chemical "conversion" of the malignant cells available in specimens into CSCs can be accomplished *in vitro*

(Landsberg, 2012). Partial, chemical de-differentiation of cancer cells for therapy-development purposes seems feasible given that chemicals are able to reprogram mature, differentiated (albeit normal) cells all the way to pluripotency (Hou, 2013).

Although tumor-reactive immune cells are generated in cancer patients, they often become suppressed and dysfunctional through "exhaustion," anergy, or senescence (Wherry, 2011). T-cell exhaustion is a dysfunctional state in which T cells are unable to attack cancer cells and cannot be activated by tumor antigens (Sakuishi, 2010) – perhaps due to ineffective immunologic synapse formation. Before attempting to stimulate an AML patient's immune system, immune cells that are dysfunctional may need to be repaired so they can respond to immune-stimulating agents. In patients with cancer (Sakuishi, 2010) and chronic viral infections (Blank, 2007; Day, 2006; Jin, 2010), exhausted immune cells are often characterized by expression of immune-checkpoint receptors like Tim-3 (Jin, 2010) and PD-1 (Blank, 2007; Day, 2006; Jin, 2010). Of note, the "exhausted" T-cell clones from patients with cancer (Sakuishi, 2010) and chronic viral infections (Blank, 2007; Day, 2006; Jin, 2010) can, *ex vivo*, be restored to normal function – and expanded – by first reprogramming them into pluripotent cells and then re-differentiating them into $CD8^+$ cytotoxic T cells (Vizcardo, 2013; Nishimura, 2013). In addition to reprogramming, blockade of the immune-checkpoint receptors Tim-3, PD-1, and CTLA-4 (when these receptors are expressed) also enhances anti-tumor immune-cell function (Fourcade, 2010; Hodi, 2010; Sakuishi, 2010).

As cancers progress, Tim-3 on the surfaces of a variety of different cells can increase and play key roles in multiple mechanisms that adversely affect a patient's ability to eliminate tumor cells. Remarkably, Tim-3 contributes to immune-cell dysfunction and

cancer-cell immune-evasion when expressed by leukemic stem cells (Kikushige, 2010), endothelial cells (Huang, 2010), and cancer-impaired antigen-presenting (Chiba, 2012) and immune-effector (Ndhlovu, 2012) cells. Because Tim-3 impairs anti-tumor immunity when expressed by tumor cells, Tim-3 expression was evaluated on RepSox-exposed AML cells. Also, because reprogramming to pluripotency restores cancer-impaired immune cells (Vizcardo, 2013), it was reasoned that reprogramming tools (like RepSox) might (used alone or in combination with other factors) "convert" immune cells toward stem-like phenotypes and help restore their functionality. In general, "converting" either immune cells or tumor cells into more primitive phenotypes may be needed at different stages of AML therapy-engineering. In advanced AML disease, expression of Tim-3 on LSCs (Kikushige, 2010) and overexpression of Tim-3 on immune cells (Zhou, 2011) may help explain both the resistance of primitive AML cells to immune attack and the dysfunction of immune cells. However, in view of the diversity of AML cytogenetic and immunophenotypic subtypes, the surface antigens that impair immune responses may vary by patient, and patient-specific approaches may be needed despite the apparently frequent, and consistent, involvement of Tim-3.

The engineering of immunotherapies for AML patients may require not just improving *in vitro* cell-survival (Pabst, 2014) but a variety of other tasks needed to "invent," produce, and evaluate multi-pronged strategies. For example, studies in humans and animals suggest that the inherent death-resistance of primitive tumor cells contributes to their ability to resist immune-cell attack (Costello, 1999; Costello, 2000) and trigger disease-relapse. Thus, a necessary component of a multi-pronged immune strategy might involve administering a (safe) systemic therapeutic agent that directly reduces the

death-resistance of stem-like malignant cells. Prior studies suggest that inhibiting anti-apoptotic factors (like Mcl-1) – that are especially problematic (and elevated) in primitive, highly evolved malignant cells – can promote immune-mediated clearance of cancer cells and improve survival (Noh, 2012). Chemical inhibitors (Weyhenmeyer, 2012) and cell-penetrating proteins (CPPs) (Wang, 2012) that deliver agents like siRNAs or microRNAs inside death-resistant primitive AML cells may be used to reduce levels of anti-apoptotic factors and sensitize tumor cells to cytotoxic T-cell-mediated apoptosis. As a potential treatment for AML patients, a CPP-siRNA conjugate, for example, that can reduce levels of a problematic anti-apoptotic factor must be safe and selectively target the AML cells.

Although therapy-development options for AML patients are unlimited and setting priorities is challenging, researchers agree that a key goal is finding better ways to maintain and manipulate cancer cells *in vitro* (Pabst, 2014). Presumably, molecular tools will simplify and expedite *in vitro* cell engineering so immune therapies might be produced and administered soon after diagnosis. Advances in chemical reprogramming have identified promising candidate tools and methods (Hou, 2013) that seem useful for engineering patient-specific immune therapies. The value of manipulating, activating, and expanding immune cells *in vitro* has been demonstrated: Engineered immune cells have eliminated relapse-causing CSCs in human and animal trials as well as in pre-clinical studies *in vitro*.

Chapter V describes the therapeutic vision that guided this project. This vision emerged after conceptual frameworks for cancer and immunology were integrated with specific research findings and the conclusions drawn from clinical successes. Before designing experiments, it was also considered prudent to envision “worst-case scenarios”

involving (1) leukemia cells that are highly evolved (immune-evasive and death-resistant), (2) dysfunctional immune cells, and (3) distorted cancer microenvironments that support malignant cells and impair immune responses. Technically, the worst possible patient specimen would contain none (or few) of the critically important cells: no CSCs (target cells), no activated immune-effector cells (T and NK cells), and no functional antigen-presenting cells. Thus, attention was directed to the potential need to complete multiple, complex *in vitro* cell-engineering tasks in order to generate patient-specific, multi-pronged immune therapies. This formidable, intimidating possibility, in turn, was juxtaposed with the dramatic cell-engineering advances already achieved by regeneration researchers which, in turn, directed attention to useful small-molecule tools like RepSox. In general, the actions of reprogramming tools suggest they may promote cell "stemness" (and the survival of primitive cells) via their effects on pluripotency genes. Thus, RepSox was screened for its ability to maintain primitive AML cells and/or alter Tim-3 expression (since Tim-3 – when expressed by primitive, highly evolved AML cells that trigger relapse – inhibits immune-cell activation and disrupts immune synapses).

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Chapter V

Guiding therapeutic vision

An intentionally broad, and unassuming, therapeutic vision was used to set experimental priorities. This vision was inspired by advances in cancer immunotherapy and stem-cell technology (noted in Figure 5.1). Described here are key research and clinical findings and the underlying rationale for a multi-pronged, immune-based strategy.

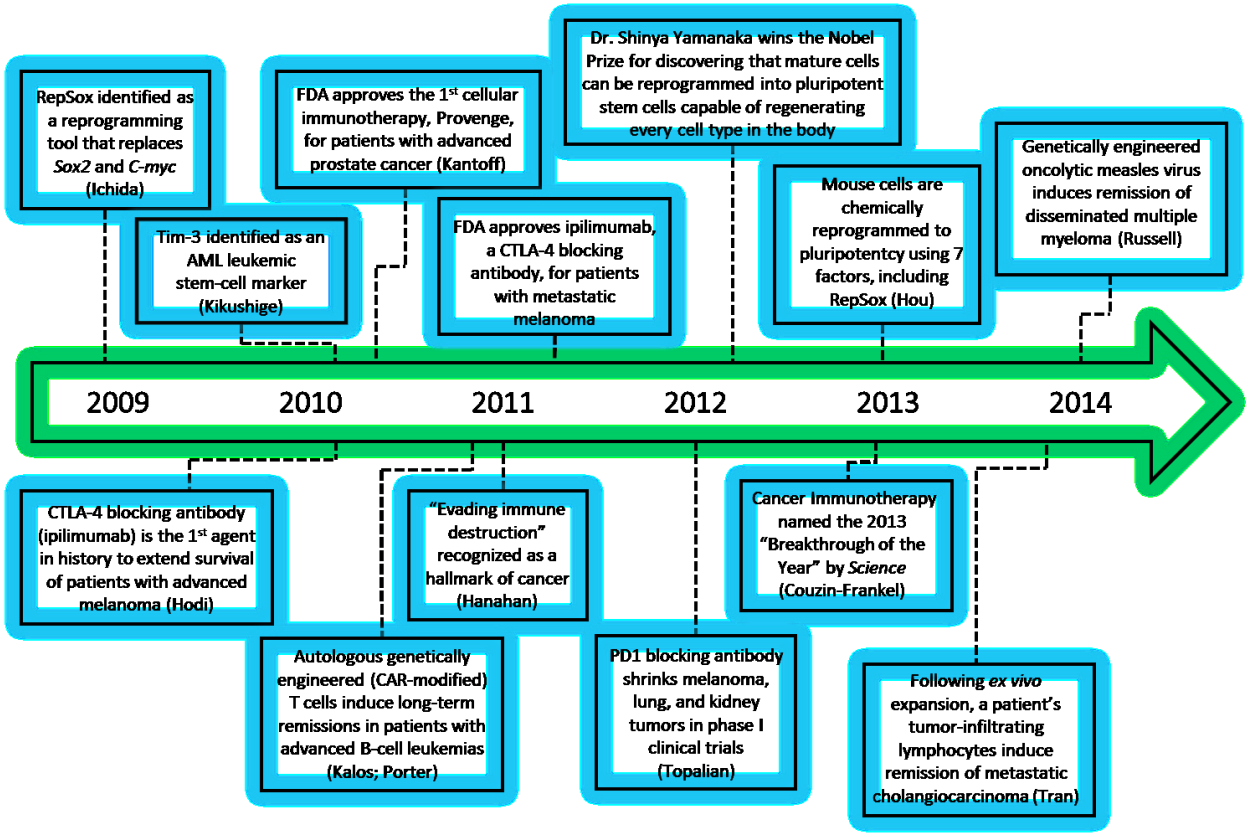

Figure 5.1. Timeline of significant events during thesis.

Key features of an ideal anti-cancer immunotherapeutic strategy and underlying rationale

Utilize a multi-pronged approach

In order to cure leukemia, therapies may require overcoming multiple interrelated mechanisms of immune resistance by (1) increasing the poor immunogenicity of leukemia cells, (2) activating and repairing dysfunctional antigen-presenting and immune-effector cells, and (3) inhibiting immuno-suppressive elements of tumor microenvironments (outlined in Figure 4.1). Of note, perhaps some of these tasks are best visualized as cell-manipulations designed to improve the activation of immune cells *in vitro* by enhancing the immunologic synapse between leukemia and immune cells. A variety of therapeutic interventions involving activated immune cells, antibodies, and agents that increase tumor-cell immunogenicity and/or induce immunogenic tumor-cell death may be needed to generate an effective anti-tumor immune response. Therapies may unleash/reactivate pre-existing immune responses (i.e. tumor-reactive lymphocytes that have become suppressed) or induce new anti-tumor immune reactions (i.e. by releasing previously unexposed tumor antigens or by presenting antigens already being displayed in a new, pro-inflammatory context). The ultimate goal is to eradicate disease-sustaining LSCs and induce immunologic memory that protects against relapse. In clinical trials, skillfully designed immune therapies have eliminated quiescent, stem-like tumor cells that sustain disease and trigger relapse, and "memory" immune cells have been detected that should provide long-term protection by detecting, and responding to, the re-emergence of CSCs.

Personalize therapy for each cancer patient

Patient-specific elements: Technical breakthroughs are suggesting new and diverse ways to repair a cancer patient's dysfunctional immune cells (Hodi, 2010; Kalos, 2011; Porter, 2011; Reiners, 2013). Remarkably, the anti-tumor function of cancer-impaired immune cells can be restored so these cells can be used therapeutically (see Figure 5.2). Defects in antigen-presentation by DCs can be overcome by *ex vivo* loading with tumor peptides (Kantoff, 2010). Cytotoxic killing and/or cytokine release by NK and T cells can be rescued by immune-checkpoint blockade (Hodi, 2010), reprogramming to pluripotency (Vizcardo, 2013; Themeli, 2013), and the physical targeting of immune cells against cancer cells using bispecific antibodies (Reiners, 2013). Fortunately, using the patient's own immune cells as therapy eliminates the risk of host rejection (since autologous immune cells are not "foreign") and graft-versus-host disease (GvHD). Especially encouraging, patient-specific anti-cancer immune therapies involving chimeric antigen receptor (CAR)-modified T cells have induced long-term remissions in patients with advanced leukemia who failed all prior therapies (Kalos, 2011; Porter, 2011). In addition, patient-specific DC vaccines for patients with advanced prostate cancer are now FDA-approved (Kantoff, 2010).

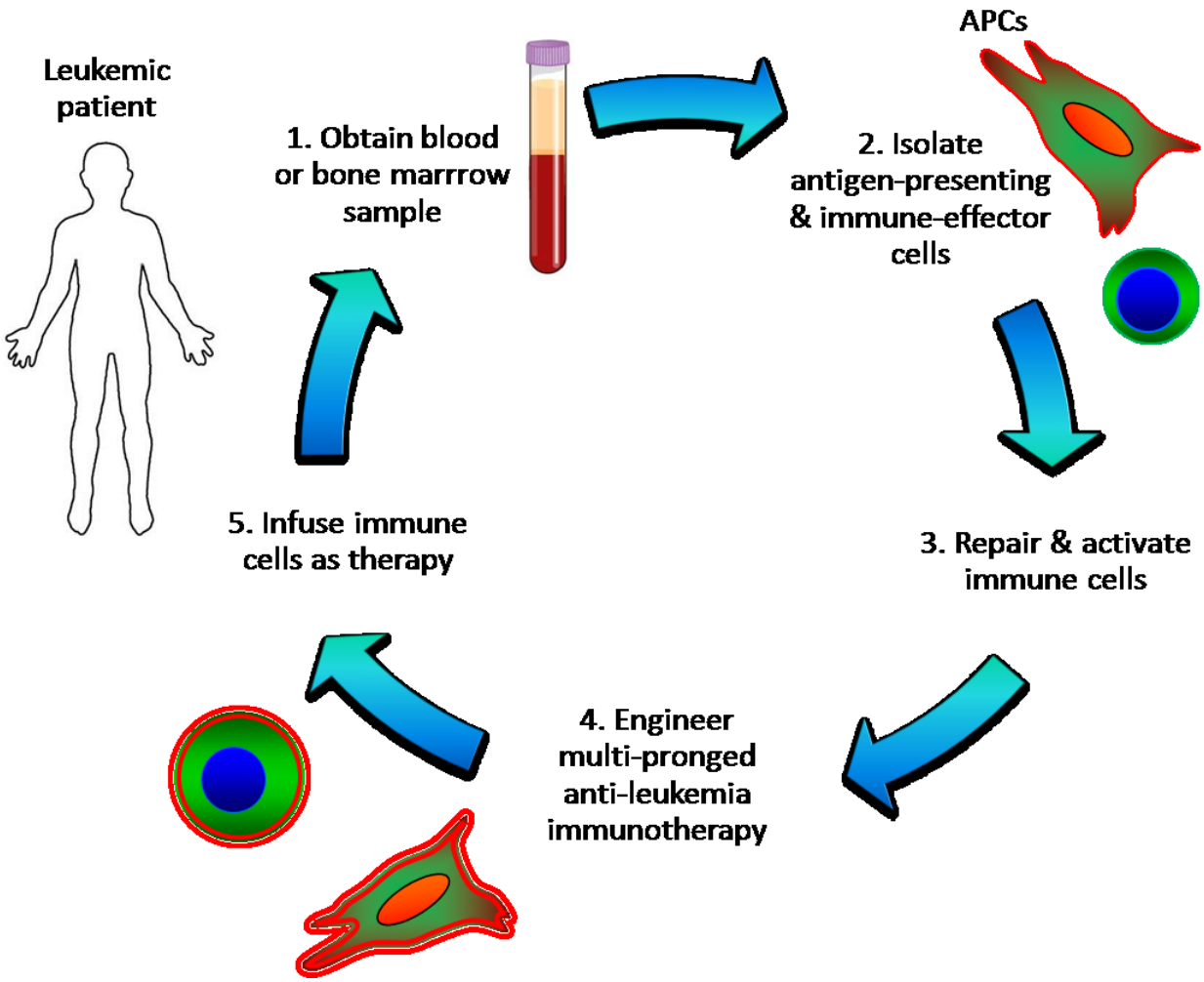

Figure 5.2. Patient-specific cellular immunotherapy. Based on patient-to-patient differences in targetable AML antigens and immune cell dysfunctions, some components of an immune therapy may need to be patient-specific. Following isolation of an AML patient's immune cells (some or all of which may be dysfunctional), they can be repaired, activated, and/or engineered against each patient's genetically unique tumor prior to use as therapy.

Personalized elements: Each patient's disease is genetically unique and has evolved in different settings. Tumor-cell surface-antigens, molecular defects, and mechanisms of immune resistance vary by patient as well as time of diagnosis. For example, cancer-related features can vary in terms of the specific immune-cell defects (i.e. anergy, exhaustion; expression of Tim-3, PD-1, or CTLA-4), immuno-suppressive cell types (i.e. T_{regs}, MDSCs, TAMs), and the presence, or functionality, of tumor-reactive immune cells. Ideally, choosing pre-existing – and/or engineering new – therapeutic antibodies, immunogenicity-altering compounds, inhibitors of anti-apoptotic factors, and immuno-modulatory agents (for *in vivo* or *in vitro* use) should be based on patient-specific technical insights.

In vitro cell-engineering using molecular tools

In some cases, leukapheresis specimens will contain all the cells needed to engineer and evaluate a patient-specific immunotherapy: the LSCs to be targeted, normal HSCs to be spared, and the immune-effector cells and/or APCs to be engineered before being administered therapeutically. Multiple tools (see Figure 4.4) may be needed to alter cancer cells (see Figure 4.5) and repair dysfunctional antigen-presenting and immune-effector cells. Fortunately, immune-effector cells can be activated *ex vivo*, away from immuno-suppressive tumor microenvironments. Small molecules and chemical engineering methods may simplify and expedite the development of patient-specific immunotherapies and, perhaps, completely eliminate the need for genetic engineering.

As noted, to develop patient-specific immunotherapies, engineering both tumor and immune cells may be necessary. Tumor cells may need to be altered to increase immunogenicity and/or maintain or induce "stemness" while immune cells may need to

be repaired and activated. Altering cells by exposure to chemical compounds is simpler and faster than genetic engineering and avoids the fears and safety concerns associated with genetically modified cells. *In vitro*, primary cells can be exposed to environmental conditions and chemical factors that directly alter cells or that select for (via "survival of the fittest") a subset of cells of therapeutic interest. Although tumor and normal cells are different, tools used for tissue regeneration and the de-differentiation of normal cells might also predictably affect cancer cells. For example, tools that induce *Nanog* not only help reprogram normal cells into stem cells, they can also induce a "stem-like" phenotype in some cancer cells (Ichida, 2009; Noh, 2012). Furthermore, the effects of chemical compounds are often reversible, allowing greater flexibility and control over the order/sequence and duration of cellular alterations. To illustrate how cell-engineering tasks can vary by the therapeutic purpose, in order to enhance immune-cell activation *in vitro*, it may be best to increase the immunogenicity of CSCs by changing their phenotype to a less-evolved CSC phenotype. In contrast, when developing antibody therapies, in order to know what new antigens might be expressed by highly evolved CSCs, immune-selection or therapeutic pressures could be used to drive CSCs to more highly evolved CSC phenotypes. Considering the diversity of the cell contents in specimens from different leukemia patients, when only mature leukemia cells are present in a patient's specimen, de-differentiation of the available leukemia cells into LSC-like surrogates may be necessary in order to develop therapies that can target the relapse-causing LSCs.

Administering immunotherapies as soon as possible after diagnosis

Ideally, immune therapies should be administered as soon as possible after initial diagnosis since, via "immunoediting," CSCs can evolve into more problematic CSCs that,

for example, might express Tim-3. That is, as cancers become more advanced, tumor cells become more immune- and death-resistant as the immune system selects for "immune-escape variants" (Dunn, 2002; Noh, 2012). Over time, immune cells become progressively more impaired and exhausted the longer they are exposed to immuno-suppressive factors within tumor microenvironments such as soluble factors (like IL-10, and TGF-β), enzymes (like arginase, IDO), immuno-suppressive cells (like T_{regs}, MDSCs, TAMs), and immune-checkpoint receptors (like Tim-3, PD-1, CTLA-4). As malignant diseases progress, tumor microenvironments also become progressively more distorted as tumor cells disrupt local oxygen and nutrient levels and convert stromal cells into CAFs.

Encouraging research findings relevant to the therapeutic vision

Breakthroughs in the reprogramming of mature (normal) cells to pluripotency – which resembles the de-differentiation that occurs during tissue regeneration in primitive animals – have identified molecular tools that seem useful for replicating complex *in vivo* processes (Feng, 2009; Ichida, 2009; Hou, 2013). Although the stimuli and cues that trigger or coordinate the cellular de-differentiation that precedes tissue regeneration are difficult to dissect and clarify *in vivo*, advances in iPSC reprogramming have identified potent molecular compounds through unbiased chemical screens (Ichida, 2009). RepSox, an inhibitor of TGF-β receptor 1, is a potent chemical reprogramming tool that replaces *Sox2* and *MYC* – two of the four reprogramming factors that previously required viral delivery (Ichida, 2009). Later, a combination of seven small molecules was found to reprogram normal mouse cells to pluripotency without genetic engineering (Hou, 2013). Remarkably, *in vitro* reprogramming of cancer-exhausted T cells to pluripotency – followed by re-differentiation into T cells – was found to restore their anti-tumor activity

and increase their expansion potential, while retaining their antigen specificity (Vizcardo, 2013). These technical breakthroughs in cell reprogramming suggest that the cell transformations needed to generate anti-cancer therapies might (eventually) also be induced *in vitro* using chemical compounds (Hou, 2013).

Animal studies have confirmed how combinations of strategies can cooperate to more effectively eliminate cancer cells and prolong survival. In mice with colon cancer, survival was dramatically extended when an agent that inhibits the generation of an anti-apoptotic factor was administered along with the antigen-specific T-cell therapy (Noh, 2012). Combining the co-blockade of multiple immune-checkpoint receptors with the activation of T-cell co-stimulatory receptors has also improved outcomes (Fourcade, 2010). Combining targeted anti-cancer agents with systemic agents that induce immunogenic cell death, such as radiotherapies and some chemotherapies (like anthracyclines), may also be useful. In addition, the CSC theory (discussed in **Chapter I**) and studies of MRD cells suggest CSCs must be targeted because they trigger relapses as well as initiate disease (Gerber, 2012). Thus, for AML patients, maintaining – or generating – their critical LSC-like cells *in vitro* can be a vital technical priority.

Role and implications of the therapeutic vision for this project

Encouraged by the cell-engineering successes of regeneration researchers, RepSox was screened for its ability to maintain and manipulate the primitive $CD34^+$ AML cells that often contain the LSCs thought to trigger AML relapse. Of course, enthusiasm for this project increased when patient-specific immune therapies were found to save the lives of cancer patients (Kalos, 2011; Porter, 2011; Tran, 2014) and when new chemical tools and *in vitro* methods were developed that could manipulate primary cells in ways

relevant for therapy-development (Hou, 2013; Pabst, 2014).

To plan experiments – and the collection and processing of AML specimens – patient-specific anti-AML therapies considered promising were envisioned. Thinking "backwards" from an aggressive anti-LSC immune strategy was useful because attention was directed to technical obstacles and priorities. One basic challenge seemed to be the need to better maintain and manipulate a patient's AML cells *in vitro*. When considering the engineering of immune therapies, attention is immediately directed to a variety of technical tasks: ways to generate and evaluate therapies involving antibodies, activated immune-effector cells, antigen-presenting cells, and/or agents that can improve the immunogenicity or apoptotic susceptibility of tumor cells. To better activate immune cells, tools may be needed to render tumor cells more immunogenic – perhaps by inducing a less evolved CSC phenotype. In contrast, when investigating antigens on highly evolved CSCs, immune-selection and therapeutic pressures might be used to "convert" CSCs into more highly evolved CSCs that are less immunogenic due to the expression of antigens like Tim-3.

This study was influenced by the CSC theory, new immunologic concepts, recent research findings, and recent patient-specific immunotherapies engineered for leukemia (Kalos, 2011; Porter, 2011) and prostate cancer (Kantoff, 2010). Because patient-specific treatments may be needed to save the lives of some AML patients, AML cells from leukapheresis specimens were used instead of leukemic cell-lines despite the extra time and challenges associated with collecting, processing, and maintaining primary cells. Also, working with cells from diverse AML patients was considered more important than using a cell-line to expedite and simplify the experimental agenda. Thus, the basic

challenge of maintaining and manipulating a patient's primitive cells had to be confronted as well as the challenges highlighted by the CSC theory. Although an AML patient's leukemic stem cells (LSCs) represent the critical target conceptually, in the context of constant tumor-cell "immunoediting," the identity of the most recently evolved CSCs is impossible to know with any confidence. Thus, the frustrating paradox: a patient's CSCs are of utmost importance – but, their most recent identity and locations are unknown. Also, the CSCs capable of inducing cancer relapses may be a distribution of diverse location-specific CSCs with phenotypes ranging from "minimally evolved" to "highly evolved." That is, CSCs may vary by location as they interact locally with different cancer-distorted immune cells and microenvironments. Immune-selection pressures *in vivo* "convert" CSCs into progressively more therapy-resistant and less immunogenic cells. This steady "conversion" of cancer cells (into cells that can engraft cancer with progressively greater efficiency) has been demonstrated *in vitro* as well as in animals (Noh, 2012). Thus, it seems prudent to assume it is impossible to know what are the most recently evolved (and most lethal) CSCs sequestered in protective niches and, therefore, the sooner immune therapies are administered, the better. However, although CSCs are constantly evolving *in vivo*, human and animal studies suggest immune therapies engineered *in vitro* can successfully eliminate relapse-causing cells (Kalos, 2011; Porter, 2011). That is, some leukemia patients have still not relapsed, and this success is consistent with evidence of long-term immunologic memory (Kalos, 2011; Porter, 2011). In one animal study, the observation and manipulation of immune and tumor cells *in vitro* inspired a 2-pronged immune strategy that was effective *in vivo* even though the tumor cells used to design and evaluate the immunotherapy were not

"conventional CSCs," but stem-like surrogates (Noh, 2012). That is, to design, create, and preclinically test immunotherapies *in vitro*, it may not be necessary that the stem-like tumor cells used for therapy-development be identical to the most recently evolved CSCs *in vivo*. Overall, it seems prudent to assume that immune strategies engineered and administered soon after the onset of cancer are more likely to be effective by minimizing the duration of CSC evolution. It seems the CSC and immunoediting theories can provide practical guidance even though, paradoxically, the phenotype of a patient's most recently evolved CSCs may never be known with certainty.

By combining such considerations with what is known about AML disease, a multi-pronged therapeutic strategy was envisioned before specific experiments were planned. This vision identified a need for tools and methods that could better maintain primitive AML cells *in vitro*. Thus, RepSox and other factors were assessed for their ability to prolong survival of primitive AML $CD34^+$ cells. As noted, RepSox had attracted attention because of its known actions on normal cells and its role as a potent reprogramming tool (Ichida, 2009; Hou, 2013). In retrospect, considering the actions of RepSox identified in prior studies, it is not surprising RepSox can slow the loss of primitive AML cells *in vitro*.

Expression of Tim-3 on AML cells was measured because the therapeutic vision suggested it is important to (1) understand, identify, and process stem-like AML cells [Tim-3 is considered a marker of (highly evolved) LSCs (Jan, 2011)] and (2) determine if Tim-3 can be reduced since Tim-3 may be responsible for tumor-cell immune evasion and distortion of immunologic synapses between LSCs and immune cells. If Tim-3 expression by tumor cells can be reduced, immune-cell activation *in vitro* might be enhanced.

Thus, the therapeutic vision and conceptual frameworks directed attention to

specific experiments. **Chapter VI** describes the effects of RepSox on primary AML cells. When viewed in the context of (1) the relationship between TGF-β and Tim-3 (Gellibert, 2004) and (2) the de-differentiation of mature cells to pluripotency (Ichida, 2009), RepSox was considered potentially useful for confronting two key obstacles: LSCs rapidly die or differentiate in culture (making them difficult to study and manipulate) and highly evolved LSCs that express Tim-3 are poorly immunogenic. In the study described in **Chapter VI**, RepSox is found to slow decay of $CD34^+$ AML cells and accelerate loss of Tim-3, an immune-checkpoint receptor that impairs anti-tumor immunity and disrupts immunologic synapses. Thus, used as an *in vitro* cell-engineering tool, RepSox may facilitate the production of patient-specific immune therapies designed to target AML LSCs.

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Chapter VI

RepSox Slows Decay of CD34^{+} Acute Myeloid Leukemia Cells and Decreases T Cell Immunoglobulin Mucin-3 (Tim-3) Expression

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Abstract

Despite initial response to therapy, most acute myeloid leukemia (AML) patients relapse. To eliminate relapse-causing leukemic stem/progenitor cells (LPCs), patient-specific immune therapies may be required. *In vitro* cellular engineering may require (1) increasing the "stemness" or immunogenicity of tumor cells and (2) activating or restoring cancer-impaired immune-effector and antigen-presenting cells. Leukapheresis samples provide the cells needed to engineer therapies: LPCs to be targeted, normal hematopoietic stem cells to be spared, and cancer-impaired immune cells to be repaired and activated. This study sought to advance development of LPC-targeted therapies by exploring non-genetic ways to slow the decay and increase the immunogenicity of primary $CD34^+$ AML cells. $CD34^+$ AML cells generally displayed greater colony-forming and aldehyde dehydrogenase (ALDH) activity than $CD34^-$ AML cells. Along with exposure to bone marrow stromal cells and low (1-5%) oxygen, culture with RepSox (a reprogramming tool and TGF-βR1 inhibitor) consistently slowed decline of $CD34^+$ AML and myelodysplastic syndrome (MDS) cells. RepSox-treated AML cells displayed higher *CD34*, *CXCL12*, and *MYC* mRNA levels than DMSO-treated controls. RepSox also accelerated loss of T cell immunoglobulin mucin-3 (Tim-3), a receptor that impairs anti-tumor immunity, from the surface of AML/MDS cells. Our results suggest RepSox may reduce Tim-3 expression by inhibiting TGF-β signaling and slow decay of $CD34^+$ AML cells by increasing *CXCL12* and *MYC*, two factors that inhibit AML cell differentiation. By prolonging survival of $CD34^+$ AML cells and reducing Tim-3, RepSox may promote *in vitro* immune cell activation and advance development of LPC-targeted therapies.

Introduction

Leukemic stem/progenitor cells (LPCs) are believed to sustain disease, persist after chemotherapy and radiation, and contribute to post-treatment relapses. In any given acute myeloid leukemia (AML) patient, these disease-causing cells may encompass a distribution of diverse immunophenotypes that evolves as disease progresses. Over time, leukemia disrupts the bone marrow (BM), suppresses the immune system, and evolves new "immune-escape" and "growth-advantaged" variants [1, 2]. AML LPCs are often distinctive at the molecular level because of high levels of T cell immunoglobulin mucin-3 (Tim-3) [3, 4] and anti-apoptotic factors [5] as well as low immunogenicity [6]. In the setting of minimal residual disease (MRD), immunotherapy may be needed to eliminate quiescent LPCs spared by conventional therapies. Prompt treatment [7] with antibodies, inhibitors of anti-apoptotic factors, and/or immune cells that target LPCs – while sparing normal hematopoietic stem cells (HSCs) – should improve AML patient survival, especially when combined with immune-modulating strategies and chemotherapies that cause immunogenic tumor cell death.

Regarding development of patient-specific LPC-targeted therapies, recent technical advances are encouraging. Notably, immune cell activity – impaired by leukemic disease states – can be restored in patients after their immune cells have been manipulated *in vitro* [8, 9]. When treated with their own custom-engineered T cells, leukemia patients rapidly entered remission and remain disease-free due to generation of long-lived memory T cells [8, 9]. Because their T cells were genetically engineered against all $CD19^+$ cells, both normal and leukemic B cells were eliminated. Although tumor cells initially trigger an immune response [10], they suppress immune cell activity

by engaging coinhibitory receptors including Tim-3, programmed cell death protein 1 (PD-1), and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) as well as by secreting immunosuppressive factors such as transforming growth factor beta (TGF-β) [11]. Fortunately, cancer-impaired immune cells can be repaired and activated *in vitro* [12] and *in vivo* – without genetic engineering – as demonstrated by potent stimulation of tumor-reactive T cells in metastatic melanoma patients treated with ipilimumab to block CTLA-4 [13].

In the setting of AML, Glaser and colleagues [14] have shown that *in vitro* manipulation of AML cells provides relevant insights and predicts promising therapeutic effects *in vivo*. Furthermore, Noh and colleagues [15] greatly improved survival of mice with colon cancer using a two-pronged immune strategy inspired by *in vitro* study of tumor cells that were not conventional cancer stem cells (CSCs), but stem-like surrogates. By decreasing tumor cell resistance to immune attack, adding a Nanog inhibitor to antigen-specific cytotoxic T cell therapy markedly improved efficacy [15]. In the future, *in vitro* studies of a patient's tumor and immune cells may help predict the effectiveness of candidate therapies if the diversity of AML subtypes requires patient-specific treatments.

Although LPC surface antigens such as CD123 [16], CD47 [17], and Tim-3 [3, 4] are shared by subsets of AML patients, each patient's LPCs are genetically unique and no universal LPC markers have been identified. Thus, patient-specific therapies may be necessary, and some have saved the lives of leukemia patients with no other options [8, 9]. Engineering patient-specific immune therapies may require insight into how similar the primary cells cultured *in vitro* must be to the LPCs evolving *in vivo* in order to serve as relevant therapeutic targets. Presumably, when engineering antibodies and/or

activated antigen-specific immune cells, the greater the similarity, the greater the likelihood of efficacy. Although LPCs are continually evolving *in vivo*, working with stem-like tumor cells (instead of true LPCs) *in vitro* may be adequate for developing immune therapies because of immunologic cross-reactivity.

Unfortunately, the same LPCs that are hard to eliminate *in vivo* (MRD cells) are difficult to maintain *in vitro*. In culture, LPCs tend to rapidly die or differentiate into more mature, less relevant cells with phenotypes that may differ from tumor cells that sustain disease and trigger relapse. For the culture of primitive cancer cells, prior technical advances provide guidance. Physiologic BM oxygen levels (1-5% O_2) [18], hematopoietic cytokines, and BM stromal cells improve survival of AML progenitors [19]. In mammosphere [20] and neurosphere [21] assays, 3-D architecture helps maintain and expand CSCs, illustrating the diversity of signals that influence CSCs.

To address the challenge of maintaining LPCs *in vitro*, we first screened non-genetic factors that might slow decay of $CD34^+$ AML cells. In general, a patient's $CD34^+$ AML cells may include *bona fide* disease-causing LPCs [22], primitive AML cells that are similar enough to LPCs to be useful for engineering immune therapies, or AML cells that need to be reprogrammed toward a more stem-like phenotype in order to serve as relevant therapeutic targets [15, 23]. Unfortunately, a patient's most recently evolved and lethal disease-causing LPCs may be impossible to identify. Despite an evolving therapeutic target, engineering of patient-specific immune therapies will likely advance by extending *in vitro* survival of primitive AML cells as well as normal immune cells and HSCs. Prolonging *in vitro* survival will facilitate exploring how to increase LPC immunogenicity, repair and activate immune cells, improve antigen presentation, and

engineer LPC-targeted therapies. Fortunately, prior studies show *in vitro* results can predict *in vivo* effects despite the differences in these environments [13, 14].

To better maintain primary $CD34^+$ AML cells, we screened factors including low O_2, co-culture with BM stromal cells, and compounds that agglutinate cells, affect TGF-β, or promote stem cell maintenance. Along with low O_2 and co-culture with BM stromal cells, RepSox, a chemical reprogramming tool [24] and ATP-competitive inhibitor of TGF-β receptor 1 (TGF-βR1) kinase [25], attracted our attention because it slowed decline of $CD34^+$ AML and myelodysplastic syndrome (MDS) cells in culture. Of clinical relevance, $CD34^+$ AML cells often contain disease-causing LPCs that may trigger relapse [22].

Materials and Methods

Human WBCs

De-identified leukapheresis specimens were obtained from patients treated at the West Virginia University (WVU) Mary Babb Randolph Cancer Center in accordance with Institutional Review Board guidelines. WBCs were isolated by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient separation and cryopreserved ($5x10^6$ cells/mL) in equal volumes of RPMI 1640 medium/10% fetal bovine serum (FBS) and freezing solution (80% FBS/20% dimethyl sulfoxide or DMSO). $CD34^+$ cells isolated from the mobilized peripheral blood of healthy donors were purchased from AllCells (Emeryville, CA).

Cell culture

WBCs were cultured in RPMI 1640 medium (Mediatech, Manassas, VA) containing 2 mM L-glutamine (Mediatech), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Sigma-Aldrich, St Louis, MO), and 10% FBS (Hyclone, Logan, UT) or serum replacement 1 (Sigma) (also referred to as serum-free) as indicated. Co-cultures included BM stromal cells, adherent cells isolated from the BM of patients without evidence of hematologic disease. BM stromal cells constitutively express VCAM-1, fibronectin, and diverse cytokines that support hematopoietic progenitors, including IL-7 dependent pro-B cells [26]. Cells were cultured at 37°C in 6% CO_2 and 1-5% or 21% O_2 as noted.

TGF-β inhibition and neutralization

TGF-β inhibitors RepSox (E-616452 or SJN 2511; Sigma), SB431542 (Sigma), LY364947, and GW788388 (Tocris Bioscience, Ellisville, MO) were dissolved in DMSO

and added once at initiation of culture. TGF-β neutralizing antibody (anti-TGF-β1/2/3) or matched mouse IgG1 isotype control antibody (R&D systems, Minneapolis, MN) were reconstituted in PBS. Antibodies (10 μg/mL) were added at initiation of culture and subsequently every 3 days.

Surface immunostaining

WBCs were suspended in 100μL phosphate buffered saline (PBS)/1% bovine serum albumin (BSA). Samples were blocked with 1μg human IgG (R&D systems) for 20 minutes at 4°C and incubated with specific or isotype control antibodies for 20 minutes at 4°C in the dark. Samples were rinsed 3 times with PBS/1% BSA and analyzed by flow cytometry within 4 hours of staining. Specific and matched isotype control antibodies directed against the following human antigens were used: CD34-PerCP (Becton Dickinson, San Jose, CA) and mouse IgG1-PerCP (Dako, Carpinteria, CA); HLA-A/B/C-PE and mouse IgG1,κ-PE; CD47-FITC and mouse IgG1,κ-FITC, CD227/MUC1-FITC and mouse IgG1,κ-FITC (BD Biosciences, San Jose, CA); and Tim-3-PE and mouse IgG1,κ-PE (BioLegend, San Diego, CA). Of note, Aldefluor-stained cells were immunostained in Aldefluor assay buffer, which contains ABC-transport inhibitors, to prevent dye efflux.

Isolation of RNA and RT-PCR

RNA was isolated from AML cells using the RNeasy Mini Kit with on-column DNase I digestion (Qiagen, Valencia, CA). One-step RT-PCRs were performed in triplicate using 50 ng of RNA per reaction with the QuantiTect SYBR Green RT-PCR kit (Qiagen) on an Applied Biosystems 7500 thermocycler (Foster City, CA). GUSB served as a loading control. Primers for human *CD34*, *CXCL12*, *MYC*, and *HAVCR2* (encoding Tim-3) were purchased from Qiagen. *GUSB* primers (forward: AAACGATTGCAGGGTTTCAC,

reverse: CTCTCGTCGGTGACTGTTCA) were synthesized by Invitrogen (Carlsbad, CA). Fold changes in relative gene expression were calculated using the $2^{-\Delta\Delta}C_T$ method [27].

Carboxyfluorescein diacetate, succinimidyl ester (CFSE) staining

WBCs were labeled using the CellTrace CFSE cell proliferation kit (Molecular Probes, Eugene, OR) according to manufacturer's instructions. Briefly, WBCs were incubated with 1μM CFSE for 10 minutes at 37ºC. Staining was quenched using ice-cold media, and cells were rinsed and placed in culture. After six days, cells were analyzed by flow cytometry.

Aldefluor assay

Aldehyde dehydrogenase (ALDH) activity was measured using the Aldefluor assay according to manufacturer's instructions (Aldagen, Durham, NC). WBCs were suspended in Aldefluor assay buffer and incubated with Aldefluor reagent, a fluorescent substrate of ALDH. An aliquot of each sample was immediately mixed with diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH, to serve as a negative control. $ALDH^+$ regions were drawn to exclude > 99% of DEAB-treated cells. Samples were incubated for 40 minutes at 37ºC and analyzed by flow cytometry within 3 hours of labeling.

Flow cytometric analysis

Samples were analyzed by fluorescence-activated cell sorting (FACS) using a Becton Dickinson FACSCalibur. Data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA). Dead cells, identified by propidium iodide uptake, were excluded from analysis. The Overton histogram subtraction method was used to calculate the percentage of stained (i.e. $CD34^+$) cells relative to matched isotype controls

[28]. Normalized median fluorescence intensity (MFI) was calculated by subtracting the MFI of isotype control antibody-stained cells from the MFI of specific antibody-stained cells.

Immunomagnetic sorting of CD34$^+$ WBCs

WBCs were co-cultured with BM stromal cells under 5% O_2 for 4-8 hours after thawing. Following recovery, WBCs were labeled with magnetic beads conjugated to anti-CD34 antibody recognizing the QBEND/10 epitope (Miltenyi Biotec, Bergisch Gladbach, Germany). WBCs were separated using a Miltenyi Biotec autoMACS according to manufacturer's instructions. Purity of cellular fractions was evaluated by flow cytometry following immunostaining with anti-CD34 antibody recognizing the HPCA-2 epitope (Becton Dickinson).

FACS of Tim-3$^+$ WBCs

WBCs were co-cultured with BM stromal cells under 5% O_2 for 4-8 hours after thawing. Following recovery, WBCs were stained with anti-Tim-3-PE antibody (BioLegend) and sorted into Tim-3$^+$ and Tim-3$^-$ fractions (> 88% pure) using a Becton Dickinson FACSAria and FACSDiva 6.2 software. Tim-3$^+$ cells were gated against matched isotype control antibody-stained cells. Purity of sorted fractions was evaluated by flow cytometry.

Colony-forming assays

WBCs were cultured in methylcellulose containing recombinant human stem cell factor (SCF), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin-3 (IL-3), and erythropoietin (Epo) (MethoCult Optimum, Stem Cell Technologies, Vancouver, British Columbia, Canada) according to manufacturer's instructions. Colony assays, performed in duplicate, were incubated at

37°C in 6% CO_2 and 21% O_2. Colonies were counted by light microscopy after 14-18 days of culture.

Diff-Quik staining

To evaluate morphology, WBCs were stained using the Diff-Quik Stain Set (Siemens, Newark, DE) according to manufacturer's instructions. Briefly, slides were sequentially dipped in methanol fixative, a solution of Eosin Y, and a solution containing methylene blue and Azure A. Slides were rinsed with deionized water, allowed to dry, and imaged by light microscopy under oil immersion.

Fluorescence *in situ* hybridization (FISH)

WVU Hospital Cytogenetics Laboratory analyzed 100-150 interphase cells for t(8;21), t(15;17), inv(16), del(6q23), and 11q23 chromosomal abnormalities by FISH. Probes were designed by Abbott Molecular (Des Plaines, IL).

Statistical analysis

Differences between groups were compared using paired Student *t* tests (2-tailed) and considered statistically significant when $P < 0.05$. Values are displayed as the mean ± standard error (SE). With the exception of FISH, all data are representative of ≥ 2 independent experiments.

Results

CD34$^+$ AML/MDS cells have greater colony-forming and ALDH activity than CD34$^-$ cells

Leukapheresis specimens (Table S1) from AML/MDS patients (P1-8) displayed variable CD34 expression (Figure 1A). To identify LPC-enriched fractions, we compared the morphology, colony-forming potential, and ALDH activity of CD34$^+$ and CD34$^-$ cells. Following immunomagnetic sorting, CD34$^+$ fractions were 75-99% pure (Figure S1) and 99-100% leukemic by FISH (Figure 1E). CD34$^+$ AML cells (P1) exhibited rounder nuclei and a higher nuclear-to-cytoplasmic ratio than CD34$^-$ AML cells (Figure 1B). CD34$^+$ AML/MDS cells generated 35- to 65-fold more colonies (Figure 1C) and generally displayed greater ALDH activity (Figure 1D; Figure S2) than CD34$^-$ cells. Of interest, the relapsed AML patient (P2) had the highest proportion (85%) of CD34$^+$ cells with ALDH activity. Colonies generated by CD34$^+$ cells were confirmed to be of leukemic origin by FISH (Figure 1F). In culture, CD34$^+$ AML/MDS cells gave rise to CD34$^-$ cells, while CD34$^-$ cells remained CD34$^-$ (Figure S3). CD34$^+$ cells also expanded via self-renewal upon stimulation with SCF, G-CSF, and IL-3 (Figure S4A). In summary, AML/MDS progenitors are enriched within CD34$^+$ fractions of leukapheresis specimens.

RepSox, low O_2, and co-culture with BM stromal cells maintain CD34$^+$ AML cells

Serum-free medium (Figure 2A), co-culture with BM stromal cells (Figure 2B), and low (1-5%) O_2 (Figure 2C) more effectively maintained CD34$^+$ AML cells than serum-containing medium, culture without BM stromal cells, and high (21%) O_2. These conditions mimic *in vitro* assays designed to support LPCs [19] and the BM niche where LPCs reside *in vivo*

[18, 29]. To generate 3-D spheroids, leukemic cells were co-cultured with BM stromal cells or osteoblasts on low attachment plates (Figure S5).

Under all culture conditions examined, RepSox maintained higher proportions of $CD34^+$ cells than DMSO (Figure 2A-C). As expected, RepSox inhibited TGF-β-induced phosphorylation of Smad2/3 (Figure S6). Similar proportions of $CD34^+$ cells were maintained in media and DMSO; therefore, only DMSO controls are shown. In summary, optimal conditions for maintaining $CD34^+$ AML cells include 1-5% O_2, co-culture with BM stromal cells, and exposure to RepSox.

RepSox maintains $CD34^+$ AML cells in a concentration-dependent manner

Increasing concentrations of RepSox maintained higher proportions of $CD34^+$ AML cells (1.6-fold and 2.3-fold increase in the proportion of $CD34^+$ cells, 2μM and 16μM RepSox compared to DMSO) (Figure 3A, B). Enrichment of $CD34^+$ cells plateaued at RepSox concentrations greater than 16μM (data not shown). Effects on CD34 expression were also concentration-dependent (3.0-fold and 12.5-fold increase in CD34 MFI, 2μM and 16μM RepSox compared to DMSO) (Figure 3A, C).

RepSox slows decay of $CD34^+$ AML/MDS cells

Next we investigated the effects of RepSox on diverse AML/MDS specimens. For consistency, all specimens were cultured with 16μM RepSox, 10% FBS, BM stromal cells, and 5% O_2. Inclusion of serum improved viability even though serum-free conditions more effectively maintained the $CD34^+$ subset of cells.

In culture, the proportion of $CD34^+$ AML/MDS cells declined as cells divided asymmetrically and/or differentiated. Culture with RepSox slowed the mean decline in the proportion of $CD34^+$ cells (33% and 54% mean decline, RepSox and DMSO, P1-7)

(Figure 4A, B). That is, RepSox maintained 1.2 to 3.0-fold higher proportions of $CD34^+$ cells than DMSO. RepSox-treated $CD34^+$ cells were confirmed to be of leukemic origin by FISH (Figure 4C). CD34 expression was also 1.9 to 6.5-fold higher on RepSox-treated cells than DMSO-treated controls (CD34 MFIs of 30 and 9, RepSox and DMSO, P1-7) (Figure 4A). RepSox did not induce CD34 expression on $CD34^-$ AML cells (Figure S3). Unlike RepSox, exposure to the TGF-β inhibitors SB431542, LY364947, and GW788388 (Figure S7A) as well as TGF-β neutralizing antibody (data not shown) did not slow decay of $CD34^+$ AML cells. In contrast to AML/MDS cells, RepSox did not slow decay of $CD34^+$ cells from healthy donors (Figure 4A, B).

We investigated whether RepSox slows decay of $CD34^+$ AML/MDS cells by slowing their rate of asymmetric division and/or by inhibiting differentiation. RepSox-treated AML cells retained greater CFSE fluorescence (Figure 4D) and incorporated less EdU (Figure S8) than DMSO-treated controls, consistent with a slower rate of proliferation. Upon stimulation with IL-3, SCF, and G-CSF, RepSox-treated $CD34^+$ cells expanded as effectively as DMSO-treated controls (Figure S4A). We also evaluated how RepSox may affect differentiation of $CD34^+$ cells into $CD34^-$ AML cells. When cultured with DMSO, the proportion of $CD34^+$ AML cells (P1) decreased as the proportion of $CD34^-CD33^-CD14^-$ cells increased (data not shown). In contrast, RepSox maintained a high proportion of $CD34^+$ cells, and no increase in the proportion of $CD34^-CD33^-CD14^-$ cells was observed (data not shown).

RepSox-treated AML/MDS cells display higher *CD34*, *CXCL12*, and *MYC* mRNA levels and similar ALDH activity compared to DMSO-treated controls

Both c-Myc and C-X-C motif chemokine 12 (CXCL12)/stromal cell-derived factor 1 (SDF-1) may inhibit differentiation of AML cells [30, 31, 32, 33]. RepSox-treated AML cells displayed higher *CD34*, *CXCL12*, and *MYC* mRNA levels than DMSO-treated controls (Figure 5A). *CXCL12* and *MYC* mRNA levels were substantially higher following exposure to RepSox than the TGF-β inhibitors SB431542 and LY364947 (Figure S7C).

ALDH is involved in chemotherapy resistance [34] and normal HSC self-renewal [35]. In AML patients, the subset of $CD34^+$ cells with ALDH activity is enriched in long-term culture-initiating cells [36]. Furthermore, persistence of $CD34^+CD38^-ALDH^{int}$ AML cells after induction chemotherapy is associated with disease relapse [37]. Given this clinical relevance, we explored whether RepSox-treated $CD34^+$ cells retain ALDH activity. Similar proportions of RepSox- and DMSO-treated $CD34^+$ cells (gated as shown in Figure S9) displayed ALDH activity (Figure 5B), demonstrating RepSox equally slows decay of $ALDH^+$ and $ALDH^-$ subsets of $CD34^+$ cells. Overall, a subset of $CD34^+$ cells with ALDH activity is maintained in our culture system. The observed pattern of ALDH activity is characteristic of leukemic rather than normal stem/progenitor cells [38].

RepSox reversibly suppresses AML colony-forming activity

In addition to CD34 expression and ALDH activity, we compared the colony-forming activity (CFA) of RepSox- and DMSO-treated AML/MDS cells. Normal and AML $CD34^+$ cells generated fewer colonies after 6-day culture with RepSox than with DMSO (Figure 6A). RepSox did not alter the low CFA of $CD34^-$ AML/MDS cells (data not shown). CFA of normal and AML $CD34^+$ cells was reduced with RepSox compared to DMSO included

in the colony-forming assay (Figure 6B). In contrast, CFA of $CD34^+$ MDS cells was higher with RepSox compared to DMSO included in the colony-forming assay (Figure 6B).

Potential reasons for the reduced CFA of AML cells after RepSox exposure include death, differentiation, or arrest of colony-forming cells in a quiescent stem-like state. To rule out RepSox-induced death or differentiation of colony-forming cells, we measured CFA following RepSox removal. Removal of RepSox partially rescued AML CFA (Figure 6C).

RepSox decreases Tim-3 expression on AML/MDS cells

Increasing tumor cell immunogenicity is one strategy to improve immune cell activation [6]. Therefore, we investigated whether RepSox alters expression of immunomodulatory receptors including Tim-3, CD47, human leukocyte antigen (HLA), and mucin-1 (MUC1) on AML/MDS cells. *HAVCR2* (encoding Tim-3) mRNA levels were consistently reduced in RepSox-treated AML cells compared to DMSO-treated controls (Figure 7A). A smaller proportion of RepSox-treated $CD34^+$ cells expressed Tim-3, a negative regulator of anti-tumor immunity [39], than DMSO-treated $CD34^+$ cells (mean 27% and 53% $Tim\text{-}3^+$ cells, RepSox and DMSO, P2-3) (Figure 7B). To determine whether RepSox promoted selective survival of $Tim\text{-}3^-$ cells or decreased Tim-3 expression, $Tim\text{-}3^+$ and $Tim\text{-}3^-$ fractions were separately exposed to RepSox. $Tim\text{-}3^+$ cells converted to $Tim\text{-}3^-$ cells *in vitro* (Figure 7C). RepSox accelerated loss of Tim-3 from the surface of FACS-purified $Tim\text{-}3^+$ AML/MDS cells (64% and 43% mean decline in $Tim\text{-}3^+$ cells, RepSox and DMSO, P2-3 and P8) (Figure 7 C). A greater proportion of MDS cells expressed Tim-3 following removal from RepSox (Figure S10A) compared to continuous culture with RepSox (Figure S10B), suggesting Tim-3 effects are partially reversible. Exposure to both RepSox and

the structurally distinct TGF-β inhibitor SB431542 [40] accelerated loss of Tim-3 from the surface of AML/MDS cells (81% and 53% mean decline in Tim-3$^+$ cells, SB431542 and DMSO, P3 and P8) without altering viability relative to controls (Figure S11). Expression of CD47 and HLA-A/B/C was not affected by RepSox, while MUC1, an antigen contributing to the poor immunogenicity of AML LPCs [41], was not highly expressed by AML/MDS cells (Figure S12).

Discussion

Engineering patient-specific anti-cancer immune therapies will advance as *in vitro* techniques improve. Because primitive relapse-causing tumor cells must be targeted, a basic challenge is reducing the *in vitro* death and differentiation of cancer progenitors. LPCs must be maintained in culture long enough to study their molecular characteristics, activate immune cells, and/or evaluate potential therapies. Currently, key technical obstacles are being identified and addressed. Glettig and Kaplan have explored ways to better maintain $CD34^+$ hematopoietic progenitors *in vitro* using co-culture with adipocytes [42]. Kellner and colleagues report their "most significant problem" is isolating multiple myeloma stem cells so they can be studied [43]. If relevant stem-like tumor cells can be adequately maintained *in vitro*, another challenge may be increasing low tumor cell immunogenicity that impedes antigen presentation and immune cell activation. In the setting of AML, abnormal Tim-3 expression on tumor cells and dysregulated antigen-presenting and immune-effector cells is an especially attractive target because this one receptor impairs antigen presentation and promotes both immune suppression and immune evasion [44, 45, 46, 47]. Thus, Tim-3 should be considered a potentially serious obstacle when engineering immune therapies *in vitro*.

In light of these technical challenges, the ability of RepSox to prolong $CD34^+$ cell survival and decrease Tim-3 expression seems useful for engineering anti-LPC immune therapies. RepSox is a chemical reprogramming tool with actions linked to maintaining stemness and inhibiting differentiation: two actions relevant for maintaining $CD34^+$ AML cells in culture [24, 48]. In mouse embryonic fibroblasts (MEFs), RepSox increased mRNA levels of components of the Wnt, Notch, and Hedgehog signaling pathways that

promote pluripotency, self-renewal, and stem cell maintenance [48]. Also, as a TGF-β inhibitor, RepSox may mitigate immunosuppressive effects induced by TGF-β during cancer progression [49]. Thus, RepSox may be a valuable tool for manipulating cancer and immune cells because its actions on both normal and cancer cells are potent and consistent [24, 48].

In addition to their poor survival and low immunogenicity, patients' tumor cells may not be stem-like enough to adequately represent the critical *in vivo* target: the most recently evolved LPCs capable of triggering relapse. Thus, primary cells may need to be reprogrammed toward more stem-like phenotypes in order to serve as relevant therapeutic targets. Fortunately, technical breakthroughs are encouraging. The same factors that dedifferentiate normal cells may also dedifferentiate tumor cells [15, 23, 50]. Noh converted tumor cells into a more stem-like phenotype *in vitro* after identifying a mechanism by which immune-selection pressures drove this conversion *in vivo* [15]. Furthermore, mouse cells were chemically reprogrammed to pluripotency using seven small molecules [51].

In this study, the TGF-β inhibitors RepSox and SB431542 reduced Tim-3 expression on AML cells. However, only RepSox-treated AML cells displayed substantially increased levels of *CXCL12* and *MYC* mRNA, and only RepSox slowed decay of $CD34^+$ AML cells. Slowing decay of $CD34^+$ cells is consistent with prior RepSox findings: RepSox replaces c-Myc during reprogramming [24], RepSox upregulates *CXCL12* and *MYC* in MEFs [48], and both c-Myc and CXCL12 promote tumor cell survival [32, 52, 53, 54]. Furthermore, c-Myc is downregulated during AML cell differentiation [31], and exposing AML cells to a c-Myc inhibitor induces differentiation [33].

The CXCL12/CXCR4 and/or CXCL12/CXCR7 axes promote survival of LPCs and normal $CD34^+$ cells [55, 56]. Activation of the CXCL12/CXCR4 axis is thought to inhibit AML cell differentiation because inhibition of CXCR4 by AMD3100 induces AML cells to differentiate [32]. Overall, CXCL12 promotes survival of leukemic progenitors both *in vitro* and *in vivo* [57, 58]. However, the implications of cell surface and intracellular receptor levels are still being clarified. In the case of CXCR7, the CXCL12/CXCR7 axis promotes HSC survival even when CXCR7 surface expression is scarce [56]. Conceivably, when used as a potential *CXCL12* upregulator, RepSox might help clarify the role of intracellular CXCL12 levels. Of note, the balance of CXCL12- and TGF-β-activated pathways affects $CD34^+$ cell cycle status [59]. Therefore, as both a TGF-β inhibitor and an apparent *CXCL12* upregulator, RepSox seems useful for investigating the balance between quiescence and cell cycling.

RepSox may slow decay of $CD34^+$ AML cells by increasing c-Myc which inhibits differentiation. c-Myc blocks differentiation of AML cell lines by increasing microRNA-17 and microRNA-20a levels which, in turn, decrease p21 and STAT3 [60]. RepSox may increase c-Myc by upregulating CXCL12 which signals via CXCR4 to activate NF-κB [61]. NF-κB can increase expression of microRNA binding protein Lin28 [62] which inhibits maturation of the let-7 family of microRNAs [62, 63]. Because let-7 microRNAs repress translation of *MYC* mRNA, reduced let-7 may lead to increased c-Myc expression [63, 64]. Furthermore, RepSox-treated MEFs expressed higher mRNA levels of Wnt signaling components *FZD1*, *FZD4*, *FZD9*, and *LEF1* than DMSO-treated controls [48]. Because *MYC* is a target of Wnt signaling [65], RepSox may upregulate *MYC* via Wnt activation. Upregulation of *MYC* by Wnt/β-catenin signaling has been reported independently by

Zhang [66] and He [65], and He highlighted the T cell factor-4 binding sites in the *MYC* promoter. CXCL12 exposure also increased mRNA levels of the Wnt target genes *CTNNB1* (encoding β-catenin), *CCND1* (encoding cyclin-D1), and *MYC* (encoding c-Myc) in the AML cell line HL60 [67].

In contrast to the "$CD34^+$ effect" unique to RepSox, Tim-3 reduction on AML/MDS cells was reproduced by the structurally distinct TGF-β inhibitor SB431542. Thus, TGF-β inhibition may reduce Tim-3 levels, a mechanism supported by reports that TGF-β induces Tim-3 expression on mast cells [68, 69]. Of note, the ALDH activity of RepSox-treated $CD34^+$ AML cells did not change, despite reduced Tim-3 expression, suggesting these cells were still stem-like. Although Tim-3 is an AML LPC marker, reduced Tim-3 expression may not diminish the disease-initiating or relapse-causing potential of LPCs. Indeed, RepSox-treated AML cells engrafted the BM of NOD-*scid* IL2Rgamma^{null} mice (data not shown).

Tim-3 may be a critical obstacle when engineering AML immunotherapies because it impairs anti-tumor immunity [39]. By increasing Tim-3 expression, tumor microenvironments restrain the anti-tumor activity of dendritic [70], NK [71], T-lymphocytic [39], and monocytic [72] cells. In turn, blocking Tim-3 signaling with antibodies restores the cytotoxicity of $CD8^+$ T cells and improves anti-tumor immunity [12, 73, 74]. Conceivably, reducing Tim-3 expression on AML cells might increase their immunogenicity because Tim-3 binds to, and suppresses, T_H cells when expressed by endothelial cells [47].

Immunotherapies involving antibodies, activated immune cells, and/or inhibitors of anti-apoptotic factors (responsible for tumor cell immune resistance) may be required to

eliminate LPCs that trigger AML relapse. Although a cancer patient's immune-effector and antigen-presenting cells are not malignant, their cancer-induced dysregulations may need to be reversed. Reprogramming exhausted T cells to pluripotency reactivates their function, increases their proliferative capacity, and preserves their antigen-specificity upon subsequent re-differentiation [75, 76]. Because immune cell suppression and tumor cell immune-evasion worsen over time, immune therapies may need to be administered shortly after diagnosis to be effective. Thus, the ability to quickly manipulate a patient's diagnostic cells could be an advantage. Like N-propionylmannosamine [77], resiquimod [78], and the MUC1 inhibitor GO-203 [41], RepSox is a chemical that alters leukemia cells *in vitro* within a week. Prolonging survival of a patient's $CD34^+$ cells in culture by days may be sufficient to chemically engineer cells. Exposing cells to small molecules like RepSox is less labor-intensive than genetic engineering and effects may be reversible. Of note, culture with a leukemic patient's BM stromal cells, rather than normal BM stromal cells, may further enhance maintenance of that patient's $CD34^+$ AML cells because cancer-associated fibroblasts have evolved to support tumor growth *in vivo* [79, 80]. The ability to quickly and reversibly alter the primary cells of AML patients *in vitro* may be valuable if sequential cellular manipulations prove useful for therapy development.

Conclusion

RepSox may be useful for developing immunotherapies as a cell culture additive (to slow decay of $CD34^+$ AML cells which often contain LPCs) and/or as a cell-engineering tool (to decrease Tim-3 expression). Because Tim-3 expression on the tumor and immune cells of cancer patients may adversely affect tumor cell immunogenicity, immune cell activation, and antigen presentation, RepSox may improve the anti-tumor actions of antigen-presenting and immune-effector cells that become dysregulated in cancer patients. Since the effects of RepSox seem potent and predictable, RepSox warrants consideration for manipulating the tumor and immune cells of patients with other cancers. Because molecular tools can chemically reprogram cells, RepSox and other molecules might eliminate the need for genetic engineering when developing patient-specific immune therapies. Characterizing the actions of RepSox and other tools may promote development of immunotherapies by simplifying *in vitro* engineering of tumor, antigen-presenting, and immune-effector cells.

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Figure legends

Figure 1. Enrichment of colony-forming and ALDH$^+$ AML and MDS cells within CD34$^+$ fractions of leukapheresis specimens. **(A)** Flow cytometric analysis of CD34 surface expression (open histograms) on WBCs isolated from leukapheresis specimens. Solid histograms display isotype controls. **(B)** Diff-Quik staining of CD34$^+$ and CD34$^-$ cells (P1). **(C)** Colony-forming activity of CD34$^+$ and CD34$^-$ cells (mean ± high and low counts from duplicate assays). *P < 0.05, 108 ± 29 and 3 ± 1, mean colonies generated by CD34$^+$ and CD34$^-$ cells (P1-4). **(D)** Summary of the ALDH activity of CD34$^+$ and CD34$^-$ cells from the representative experiment shown in Figure S2 (mean difference was NS). **(E, F)** FISH for leukemic alterations in CD34$^+$ and CD34$^-$ cells isolated from leukapheresis specimens as well as colonies generated by CD34$^+$ cells. Abbreviations: NS, not statistically significant; FISH, fluorescence *in situ* hybridization.

Figure 2. RepSox, low O_2, and co-culture with BM stromal cells maintain CD34$^+$ AML cells. Flow cytometric analysis of CD34 surface expression (open histograms) on AML cells (P1) after 10-day culture with 16μM RepSox or DMSO (vehicle control) in the following conditions: **(A)** with 10% FBS or serum replacement (off stroma, 1% O_2), **(B)** on or off BM stromal cells (serum-free, 1% O_2), and **(C)** under 1% or 21% O_2 (on stroma, serum-free). Solid histograms display isotype controls.

Figure 3. RepSox maintains CD34$^+$ AML cells in a concentration-dependent manner. **(A)** Flow cytometric analysis of CD34 surface expression (open histograms) on AML cells (P1) after 10-day culture with 2-16μM RepSox (on stroma, serum-free, 5% O2)

or DMSO (vehicle control). Solid histograms display isotype controls. Summary of the **(B)** proportion of $CD34^+$ cells and **(C)** CD34 MFI of RepSox-treated cells relative to DMSO-treated controls from the representative experiment shown in part A.

Figure 4. RepSox slows decay of $CD34^+$ AML and MDS cells. **(A)** Flow cytometric analysis of CD34 surface expression (open histograms) on normal donor (ND) and AML/MDS $CD34^+$ cells after 6-day culture with 16μM RepSox or DMSO (on stroma, 10% FBS, 5% O_2). Solid histograms display isotype controls. **(B)** Summary of the decline in proportions of $CD34^+$ cells from the representative experiment shown in part A. *P < 0.005, 33 ± 7% and 54 ± 7%, mean decline in $CD34^+$ cells following RepSox and DMSO exposure (P1-7). **(C)** FISH for leukemic alterations in $CD34^+$ cells (P1) purified by sorting after 6-day exposure to RepSox and DMSO. **(D)** CFSE staining profiles of RepSox-treated (open histograms) and DMSO-treated (solid histograms) cells.

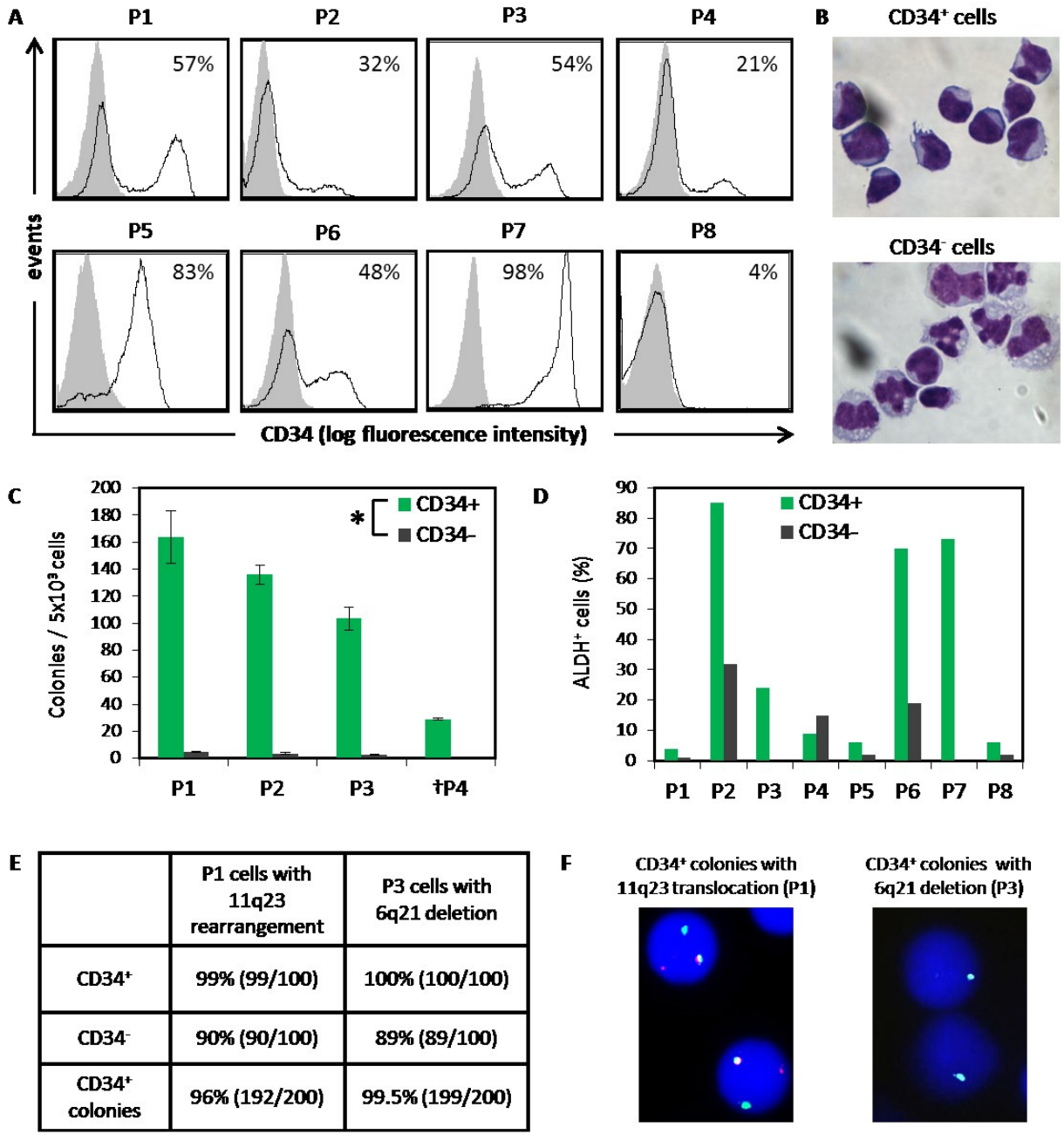
Figure 5. RepSox-treated cells have increased *CD34*, *CXCL12*, and *MYC* mRNA levels and similar ALDH activity compared to DMSO-treated controls. AML/MDS cells were cultured with 16μM RepSox or DMSO for 6 days (on stroma, 10% FBS, 5% O_2). **(A)** qRT-PCR analysis of *CD34* (*P < 0.05), *CXCL12* (*P < 0.05), and *MYC* (*P < 0.005) mRNA levels within RepSox-treated cells relative to DMSO-treated controls. **(B)** ALDH activity of RepSox- and DMSO-treated $CD34^+$ AML/MDS cells measured by flow cytometry (mean difference was NS). $ALDH^+$ regions were drawn to exclude > 99% of DEAB-treated (negative control) cells. Abbreviations: ALDH, aldehyde dehydrogenase; NS, not statistically significant.

Figure 6. RepSox reversibly suppresses AML colony-forming activity. **(A)** CFA of AML and normal donor (ND) $CD34^+$ cells after 6-day culture with 16μM RepSox or DMSO

(on stroma, 10% FBS, 5% O_2). Mean difference between CFA of RepSox- and DMSO-treated AML cells was NS. **(B)** CFA of AML/MDS and ND $CD34^+$ cells grown with 16μM RepSox or DMSO included in the colony-forming assay (added once during initiation). Mean difference between CFA of RepSox- and DMSO-treated AML cells was NS. **(C)** CFA of AML cells (P2) following continuous culture with RepSox for 6 days and removal of RepSox after 3 days. Bar graphs display mean colonies ± high and low counts from duplicate assays. Abbreviations: CFA, colony-forming activity; CFU, colony-forming units; NS, not statistically significant.

Figure 7. RepSox decreases Tim-3 expression on AML and MDS cells. AML/MDS cells were cultured with 16μM RepSox or DMSO for 6 days (on stroma, 10% FBS, 5% O_2). **(A)** qRT-PCR analysis of *HAVCR2* (encoding Tim-3) mRNA levels within RepSox-treated cells relative to DMSO-treated controls (*$P < 0.005$). **(B)** Flow cytometric analysis of Tim-3 surface expression (open histograms) on RepSox- and DMSO-treated $CD34^+$ cells (mean difference was NS). Solid histograms display isotype controls. **(C)** Flow cytometric analysis of Tim-3 surface expression (open histograms) following culture of FACS-purified Tim-3^+ AML/MDS cells with 16μM RepSox or DMSO for 6 days (on stroma, 10% FBS, 5% O_2) (mean difference was NS). Solid histograms display isotype controls. Abbreviations: NS, not statistically significant.

Figures



	P1 cells with 11q23 rearrangement	P3 cells with 6q21 deletion
CD34⁺	99% (99/100)	100% (100/100)
CD34⁻	90% (90/100)	89% (89/100)
CD34⁺ colonies	96% (192/200)	99.5% (199/200)

Figure 1. Enrichment of colony-forming and ALDH⁺ AML and MDS cells within CD34⁺ fractions of leukapheresis specimens.

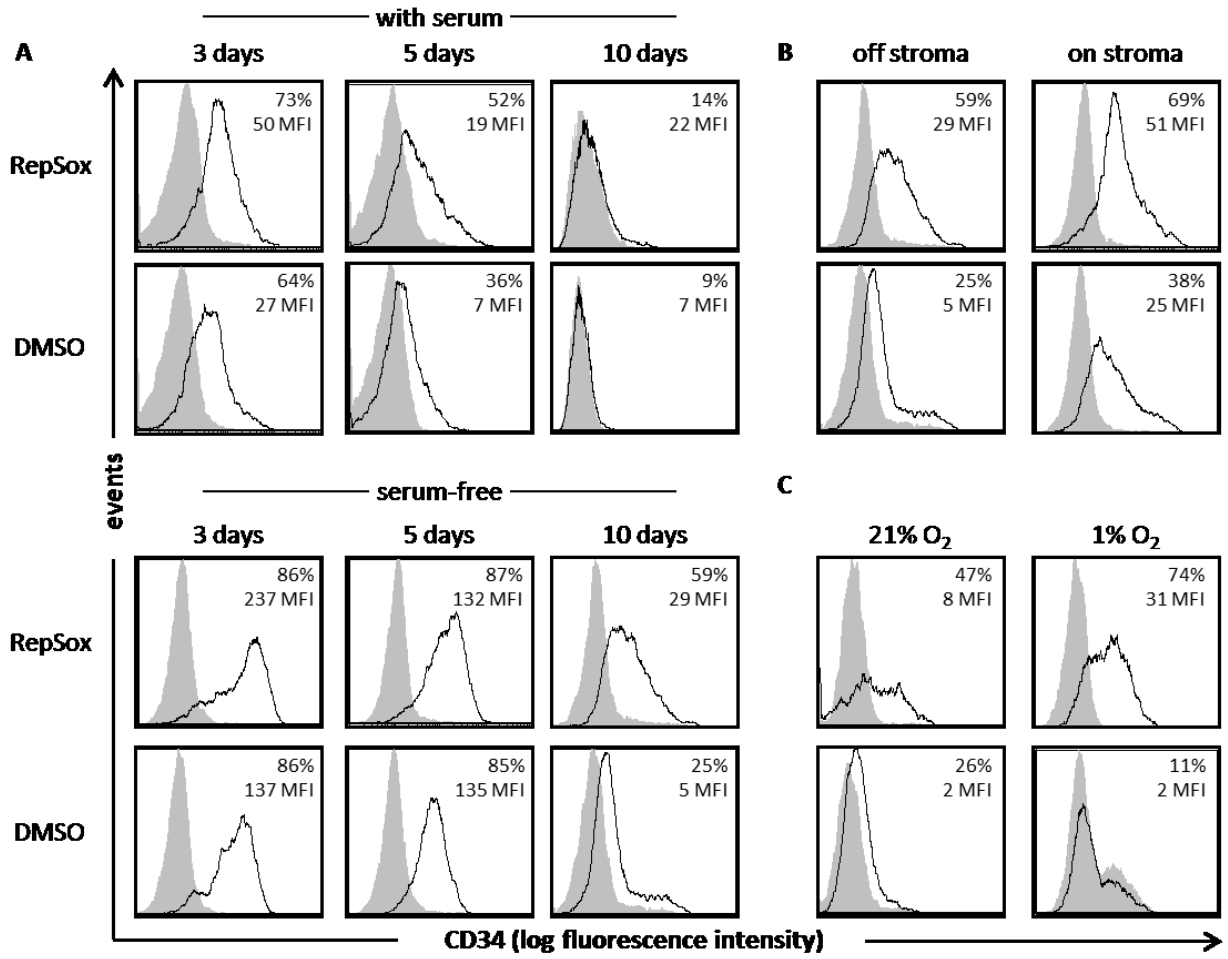


Figure 2. RepSox, low O_2, and co-culture with BM stromal cells maintain $CD34^+$ AML cells.

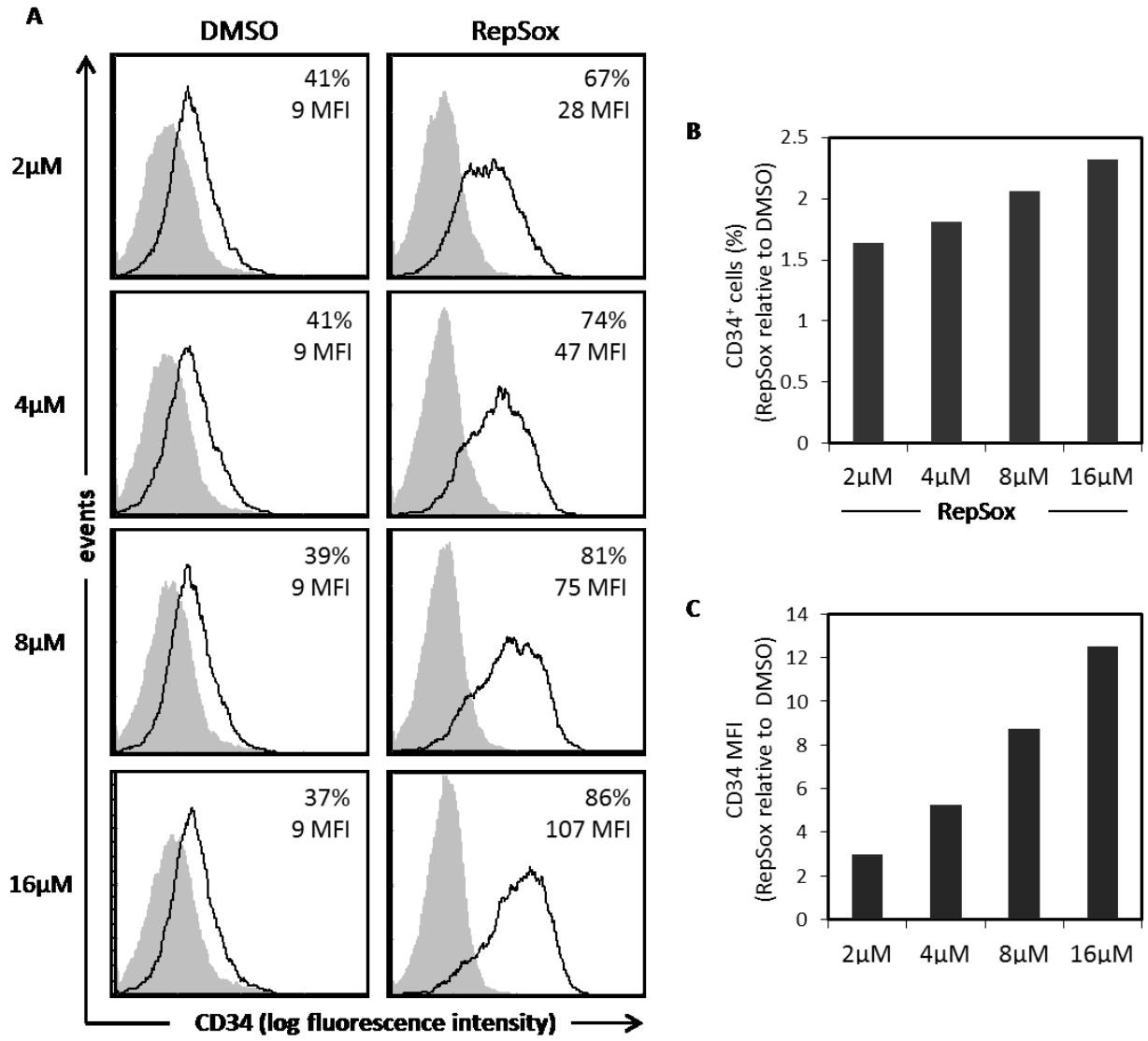


Figure 3. RepSox maintains CD34⁺ AML cells in a concentration-dependent manner.

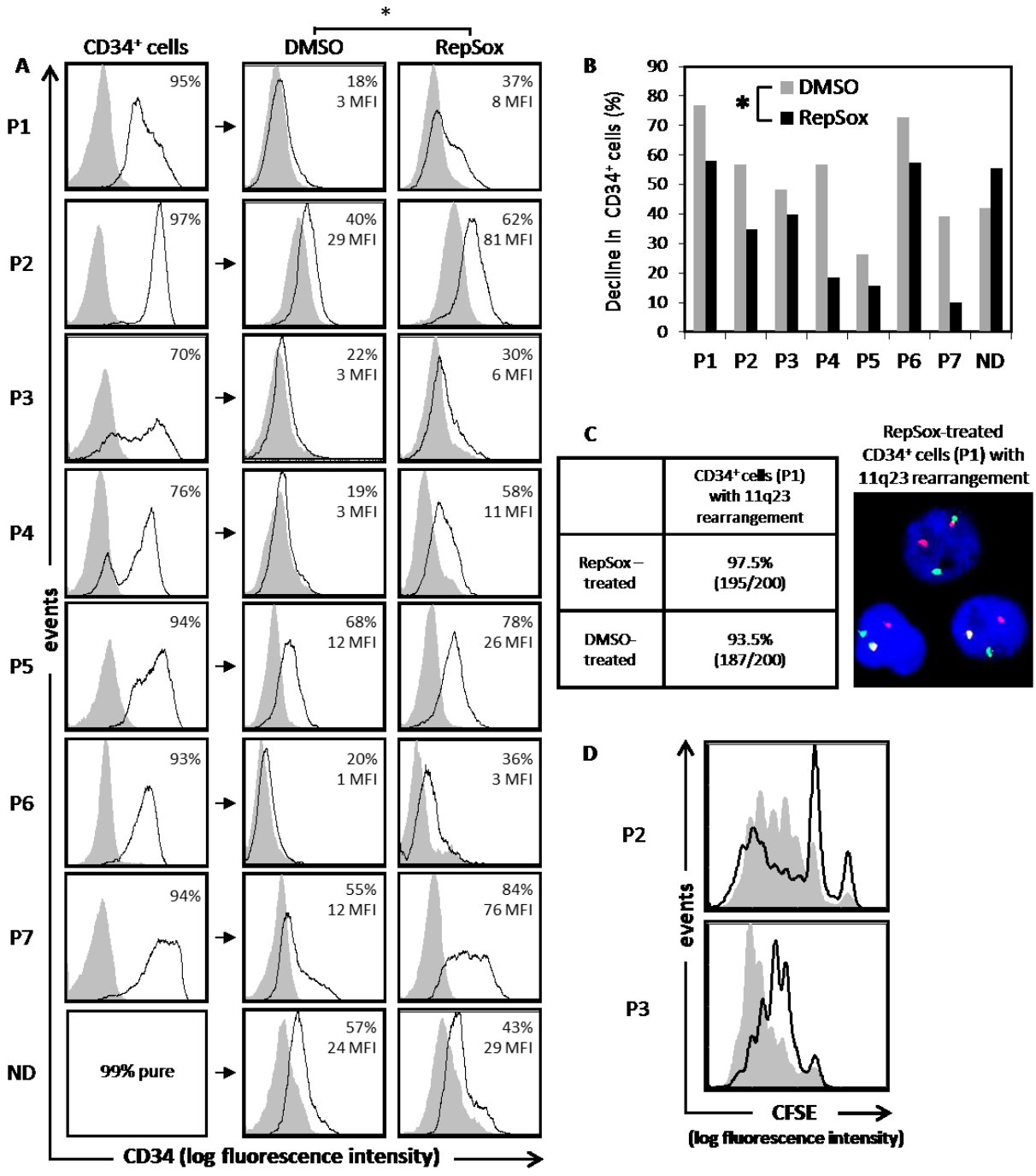


Figure 4. RepSox slows decay of CD34⁺ AML and MDS cells.

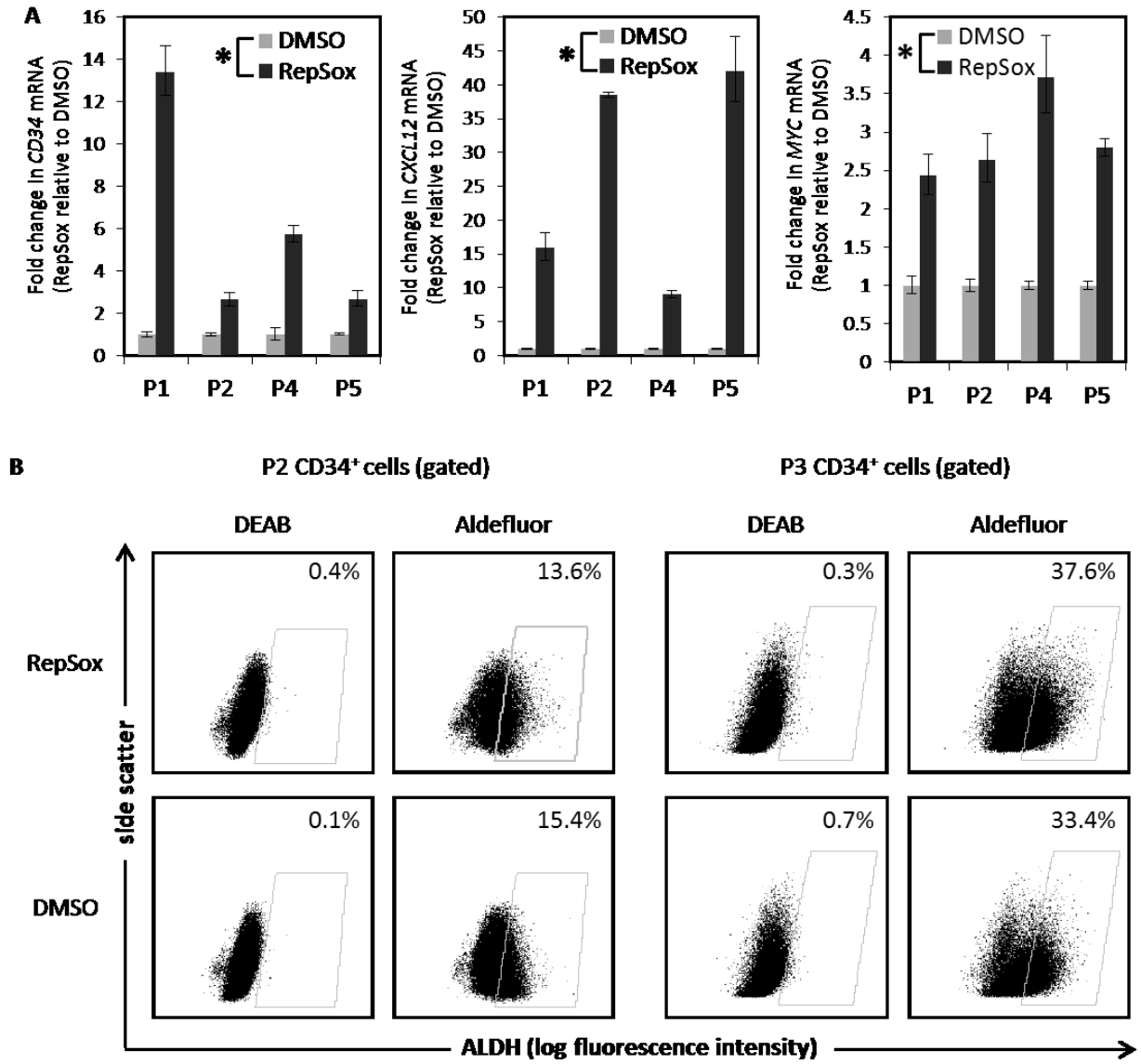


Figure 5. RepSox-treated cells have increased *CD34*, *CXCL12*, and *MYC* mRNA levels and similar ALDH activity compared to DMSO-treated controls.

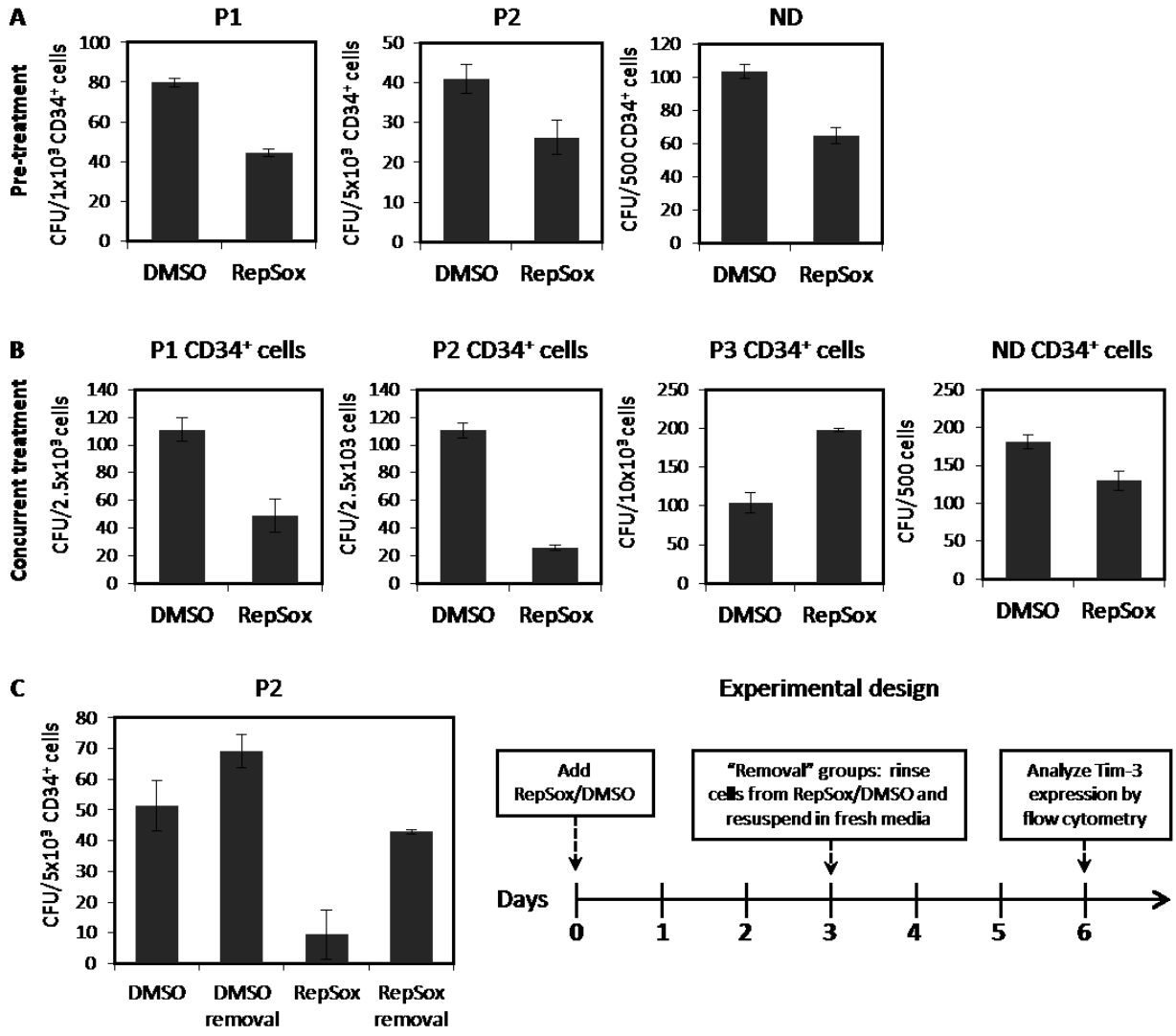


Figure 6. RepSox reversibly suppresses AML colony-forming activity.

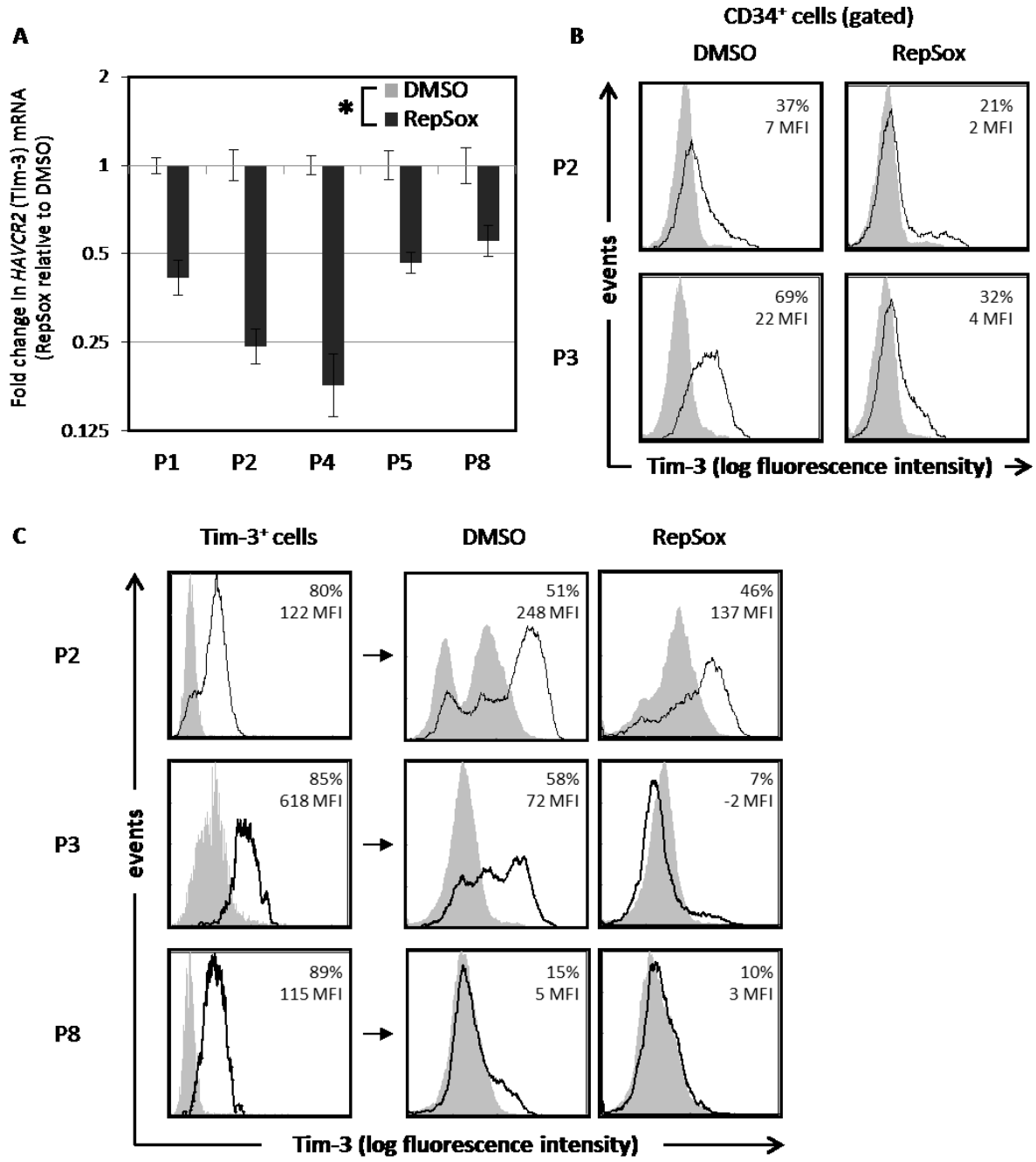


Figure 7. RepSox decreases Tim-3 expression on AML and MDS cells.

Supplemental Methods

Phospho-Smad 2/3 intracellular staining

WBCs were serum-starved for 4 hours in RPMI 1640 medium with serum replacement 1 (Sigma) and subsequently exposed to 10 ng/mL TGF-β (human platelet-derived, R&D systems) for 30 minutes with 16μM RepSox or DMSO. Following stimulation, cells were fixed in 2% paraformaldehyde for 10 minutes at room temperature (RT) and chilled on ice for 1 minute. WBCs were permeabilized in cold 90% methanol for 30 minutes on ice and stored at -20°C. Prior to intracellular staining, samples were rinsed with PBS/1% BSA. Samples were blocked with 1 μg human IgG (R&D systems) for 30 minutes at 4°C and incubated with anti-phospho-Smad-2(S465/467)/3(S423/425) or matched rabbit IgG isotype control antibody (Cell Signaling Technologies, Danvers, MA) for 1 hour at RT. Samples were rinsed with PBS/1% BSA and incubated with AlexaFluor-488 donkey anti-rabbit secondary antibody (Molecular Probes) for 1 hour at RT. Cells were rinsed and analyzed by flow cytometry.

Western blot analysis

Total cellular protein (25 μg/sample) was separated over 4–20% gradient polyacrylamide gels (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose membranes (Whatman, Dassel, Germany) and blocked for 60 minutes in TBS/0.1% Tween-20 (TBS-T) containing 5% non-fat dry milk. Blots were incubated with a 1:1,000 dilution of anti-phospho-Smad2 (Ser465/467) antibody (Cell Signaling) in PBS/5% BSA overnight at 4°C with gentle rocking. Blots were rinsed 3 times with TBS-T for 5 minutes and incubated with a 1:5000 dilution of HRP-linked anti-rabbit IgG (Cell Signaling). Blots were rinsed with TBS-T 3 times for 5 minutes, incubated with Immobilon chemiluminescent HRP

substrate (Millipore, Billerica, MA), and developed. MagicMark molecular weight standard (Bio-Rad) was used to estimate protein size. GAPDH served as a loading control.

Growing and imaging 3-D leukemia spheroids

Leukemia cells and BM osteoblasts were labeled with CellTracker green CMFDA (Molecular Probes) and CellTrace Far Red DDAO-SE (Molecular Probes) fluorescent probes and co-cultured on ultra-low attachment plates (Corning, Corning, NY). For 3-D imaging, 2-week old spheroids were collected in a 40μM cell strainer (BD Biosciences) and imaged by 2-photon microscopy using a 60X water-dipping objective. Images were deconvoluted using AutoQuant software (Media Cybernetics, Inc., Warrendale, PA) and imported into NIS-Elements AR imaging software (Nikon) for 3-D viewing.

WBC expansion

WBCs were co-cultured with BM stromal cells in RPMI 1640 medium supplemented with serum replacement 1 (Sigma) for 10 days. To expand cells, recombinant human IL-3 (20 ng/mL), G-CSF (20 ng/mL), and SCF (100 ng/mL) were added at the initiation of culture and 5 days later.

5-ethynyl-2'-deoxyuridine (EdU) staining

To measure cell proliferation, the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Molecular Probes, Eugene, OR, USA) was used according to the manufacturer's instructions. WBCs were co-cultured with BM stromal cells for 3 days, 1μM EdU (a nucleoside analog to thymidine) was added, and cells were cultured for another 3 days. Cells were fixed with 4% paraformaldehyde and permeablized with saponin. EdU incorporation into DNA was detected by a copper catalyzed covalent reaction.

Surface immunostaining and flow cytometric analysis for RepSox differentiation experiment

Samples were stained according to standard whole-blood lysis procedure using the Becton Dickinson lyse-wash method. Monoclonal antibodies directed against the following human antigens were used: CD19-PE (4G7), CD33-PerCP-Cy5.5 (P67.7), CD3-APC (SK7), CD34-PE-Cy7 (8G12), CD45-APC-Cy7, CD8-FITC (SK1), CD4-PE (DK3), CD3-PerCP-Cy5.5 (SK7), CD14-APC (MϕPg), and CD56-PE-Cy7 (NCAM16.2) (Becton Dickinson). The following 3-tube panel was prepared for each sample: isotype controls and CD45 (tube 1), CD19/CD33/CD3/CD34/CD45 (tube 2), and CD8/CD4/CD3/CD14/CD56/CD45 (tube 3). Samples were analyzed by FACS using a Becton Dickinson FACSCanto II. A minimum of 30,000 events were acquired and data were analyzed using FCSExpress software (De Novo Software). Initial gating was based on forward scatter versus side scatter dot plots to exclude dead cells and debris. Thresholds for positivity were set to exclude > 99% of isotype control antibody-stained cells. Positive events were back-gated to ensure that they constituted a discrete population on CD45 versus side scatter dot plots to confirm specificity of antigen binding.

Supplemental Table and Figure Legends

Table S1. Characteristics of leukapheresis specimens. AML specimens were evaluated for t(8;21), t(15;17), inv(16), and 11q23 alterations, while the MDS/MPS specimen was tested for 4q12, 5q32, 8p12, and t(9;22) chromosomal abnormalities by fluorescence *in situ* hybridization (FISH). All samples were screened for FLT3-ITD, NPM1, and CEBPA mutations by polymerase chain reaction (PCR).

[a] Stage of disease at the time of leukapheresis collection.

[b] Measurements obtained prior to leukapheresis.

[c] Information not available.

Abbreviations: WBC, white blood cell; MLL, mixed-lineage leukemia; ELL, elongation factor RNA polymerase II; MDS, myelodysplastic syndrome; MPS, myeloproliferative syndrome; del, deletion; inv, inversion; FLT3, fms-related tyrosine kinase 3; ITD, internal tandem duplication; NPM1, nucleophosmin; and CEBPA, CCAAT/enhancer binding protein α.

Figure S1. Purity of $CD34^+$ and $CD34^-$ fractions prior to measuring colony-forming activity. AML/MDS leukapheresis specimens were immunomagnetically sorted into $CD34^+$ and $CD34^-$ subsets. Purity of fractions was evaluated by flow cytometry after CD34 surface staining (open histograms). Solid histograms display isotype controls.

Figure S2. $CD34^+$ AML and MDS cells generally display greater ALDH activity than $CD34^-$ cells. ALDH activity of $CD34^+$ and $CD34^-$ cells (selected by gating against isotype controls) measured by flow cytometry. $ALDH^+$ regions were drawn to exclude > 99% of DEAB-treated (negative control) cells.

Figure S3. RepSox does not induce CD34 expression on CD34$^-$ AML cells. Purified CD34$^-$ AML cells were cultured with 16μM RepSox or DMSO (on stroma, 10% FBS, 5% O_2). After 6 days, CD34 surface expression (open histograms) was evaluated by flow cytometry. Solid histograms display isotype controls.

Figure S4. RepSox-treated CD34$^+$ cells expand as effectively as DMSO controls upon cytokine stimulation. AML cells (P1) were cultured with 16μM RepSox or DMSO (on stroma, serum-free, 5% O_2). Recombinant human IL-3 (20 ng/mL), G-CSF (20 ng/mL), and SCF (100 ng/mL) were added at days 0 and 5. After 10 days, **(A)** WBCs were counted (bar graphs display mean counts ± SE from 2 independent experiments) and **(B)** CD34 expression (open histograms) was evaluated by flow cytometry. Solid histograms display isotype controls.

Figure S5. Generation of 3-D spheroids containing leukemic cells and osteoblasts. Primary acute lymphoblastic leukemia (ALL) cells (green) and BM osteoblasts (red) were labeled with fluorescent probes and co-cultured on low attachment plates to generate 3-D spheroids. Spheroids were imaged by 2-photon microscopy (600X magnification) after two weeks.

Figure S6. RepSox inhibits TGF-β-induced phosphorylation of Smad2/3. AML cells (P1) were serum-starved for 4 hours and cultured **(A)** ± TGF-β (10 ng/mL) for 30 minutes and **(B)** with 16μM RepSox or DMSO (vehicle control) for 30 minutes prior to TGF-β (10 ng/mL) exposure for 30 minutes. Phosphorylated Smad-2/3 expression was evaluated by flow cytometry. **(C)** Representative immunoblot of AML cells (P6) serum-starved for 4

hours then cultured with 16μM RepSox or DMSO for 30 minutes prior to TGF-β (10 ng/mL) exposure for 30 minutes. D, DMSO; RS, RepSox.

Figure S7. Unlike RepSox, additional TGF-β inhibitors do not slow decay of CD34$^+$ AML cells. **(A)** CD34$^+$ AML cells (P1) were cultured with TGF-β inhibitors (LY364947, GW788388, SB431542) or DMSO (vehicle control) for 6 days (on stroma, 10% FBS, 5% O_2). CD34 surface expression (open histograms) was evaluated by flow cytometry. Solid histograms display isotype controls. **(B)** Chemical and physical structures of TGF-β inhibitors evaluated. **(C)** qRT-PCR analysis of *MYC* and *CXCL12* mRNA levels following 48-hour exposure of AML cells (P6 and P8) to various TGF-β inhibitors.

Figure S8. RepSox-treated CD34$^+$ cells proliferate more slowly than DMSO controls. CD34$^+$ AML cells were cultured (on stroma, 10% FBS, 5% O_2) with 16μM RepSox or DMSO with and without EdU (added after 3 days). After a total of 6 days in culture, EdU incorporation (open histograms) by CD34$^+$ cells (selected by gating) was evaluated by flow cytometry. Solid histograms display cells cultured without EdU.

Figure S9. Gating strategy used to select CD34$^+$ cells. To select CD34$^+$ cells, regions were drawn on side scatter versus CD34 fluorescence dot plots to include anti-CD34 antibody-stained cells, but exclude > 98% of isotype control antibody-stained cells. Regions were converted to gates for subsequent analyses of CD34$^+$ cells.

Figure S10. Increased proportion of Tim-3$^+$ cells following removal of RepSox. Flow cytometric analysis of Tim-3 surface expression (open histograms) on MDS (P3) cells cultured with 16μM RepSox or DMSO (on stroma, 10% FBS, 5% O_2): **(A)**

continuously for 6 days and **(B)** intermittently for 3 days prior to rinsing and culturing in fresh media for 3 additional days. Solid histograms display isotype controls.

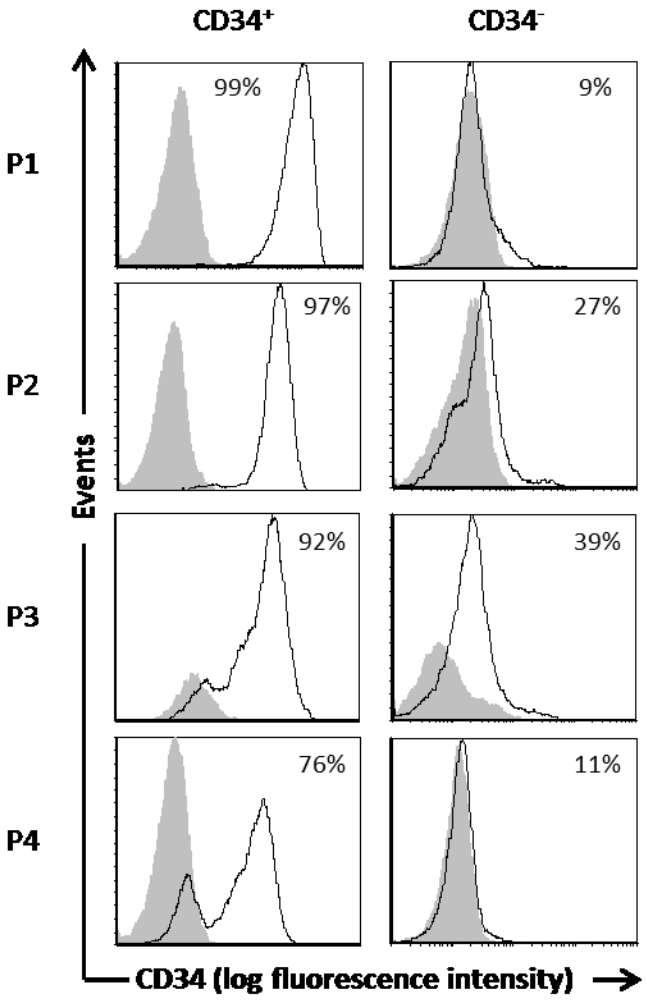
Figure S11. TGF-β inhibitors RepSox and SB431542 reduce Tim-3 expression on AML and MDS cells. FACS-purified Tim-3$^+$ AML/MDS cells (open histograms) were cultured with 16μM RepSox, 2μM SB431542, or DMSO (vehicle control) for 6 days (on stroma, 10% FBS, 5% O_2). Solid histograms display isotype controls. Surface expression of Tim-3 and CD3 (T cell marker) was evaluated by flow cytometry.

Figure S12. RepSox did not drastically alter CD47, HLA-A/B/C, or MUC1 expression on CD34$^+$ AML and MDS cells. AML (P2) and MDS (P3) cells were cultured with 16μM RepSox or DMSO for 6 days (on stroma, 10% FBS, 5% O_2). Surface expression of Tim-3, CD47, HLA-A/B/C, and MUC1 (open histograms) on CD34$^+$ cells (selected by gating) was evaluated by flow cytometry. Solid histograms display isotype controls.

Supplemental Table and Figures

Patient (P)	Age, Gender	Diagnosis	Status[a]	Molecular and cytogenetic abnormalities	Peripheral WBC count[b] ($1x10^3$ cells/µL)	Blasts (%) in peripheral blood, bone marrow[c]	$CD34^+$ cells (%) in leukapheresis specimen
P1	24, M	Acute myeloid leukemia with 11q23 (MLL) abnormalities	Diagnosis	t(11;19)(q23;p13.1), MLL-ELL	52	55%, 76%	57%
P2	48, M	Acute myelomonocytic leukemia (M4)	Relapse	none detected by FISH	102	17%, 40%	32%
P3	66, M	MDS/MPS, unclassifiable in accelerated phase	Diagnosis	t(5;13)(q13;q12), del(6)(q21)	234	12%, 13%	54%
P4	45, F	Acute monoblastic/ monocytic leukemia (M5)	Diagnosis	Inv(16), FLT3 D835 variant+	133	23%, 69%	21%
P5	-	Acute myeloid leukemia	-	t(8;21), AML-ETO	-	-	83%
P6	59, F	Acute myelomonocytic leukemia (M4)	Diagnosis	FLT3-ITD+, NPM1+	45	59%, 84%	48%
P7	-	Acute myeloid leukemia	-	none detected by FISH	-	-	98%
P8	71, F	Acute myeloid leukemia with monocytic differentiation (M5)	Diagnosis	FLT3-ITD+, NPM1+	119	37%, 90%	4%

Table S1. Characteristics of leukapheresis specimens.



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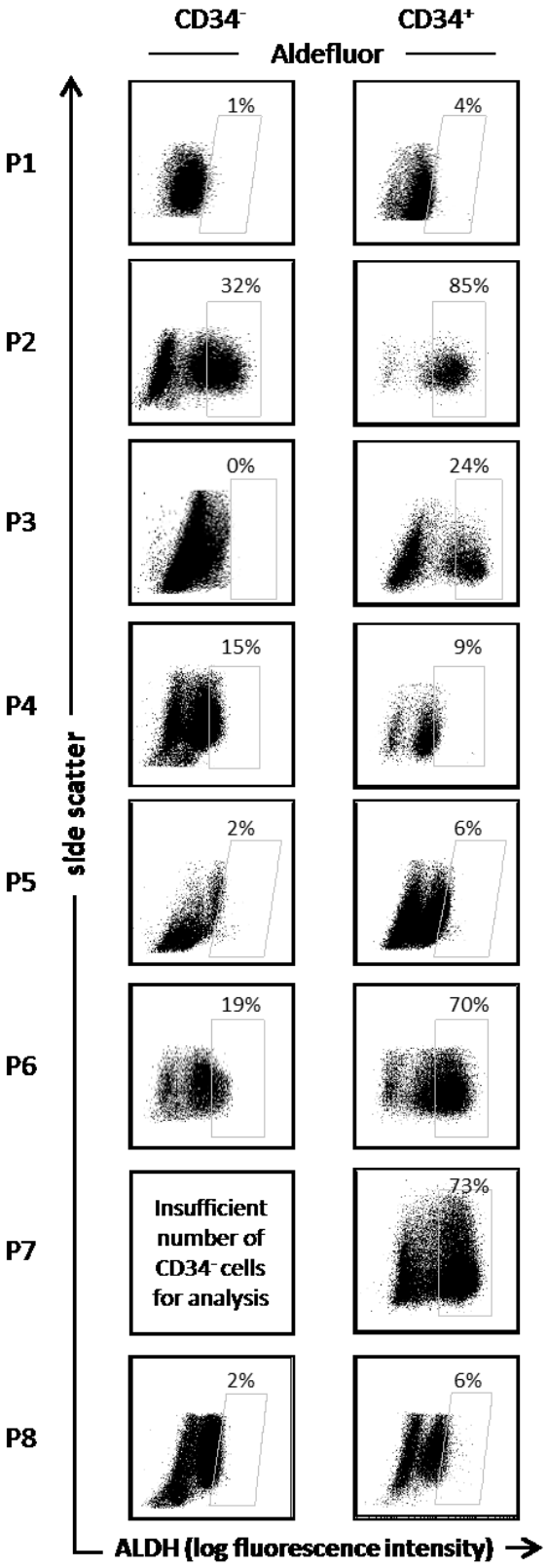

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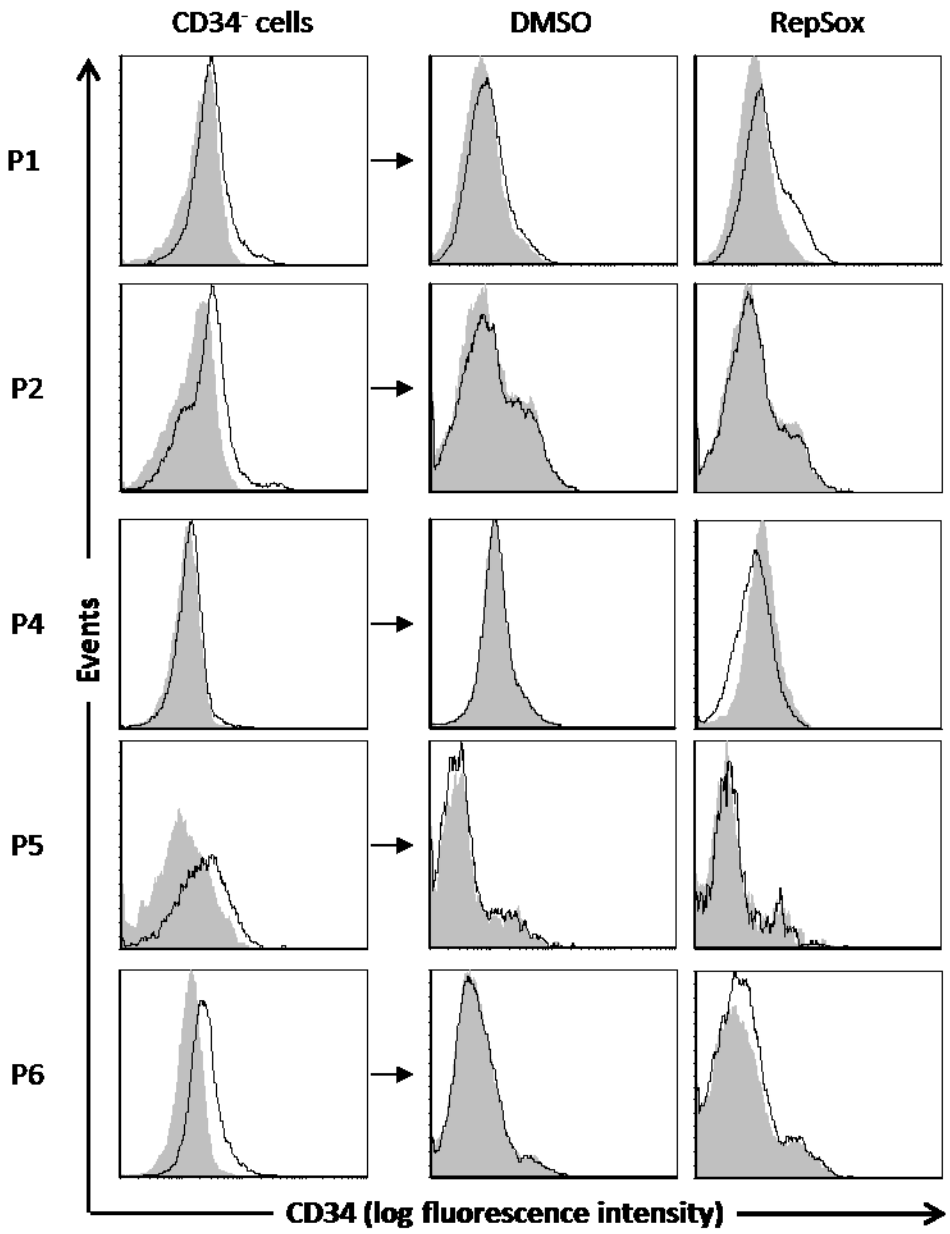


Figure S3. RepSox does not induce CD34 expression on CD34$^-$ AML cells.

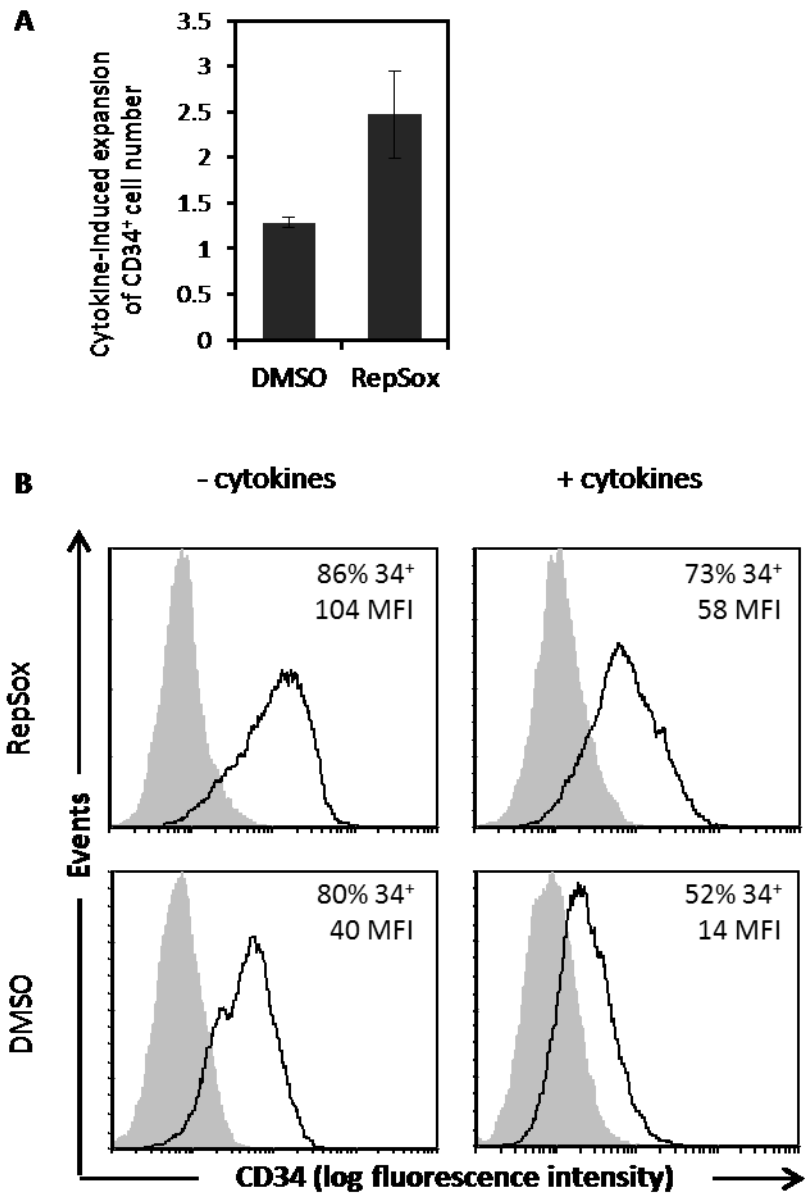


Figure S4. RepSox-treated CD34$^+$ cells expand as effectively as DMSO controls upon cytokine stimulation.

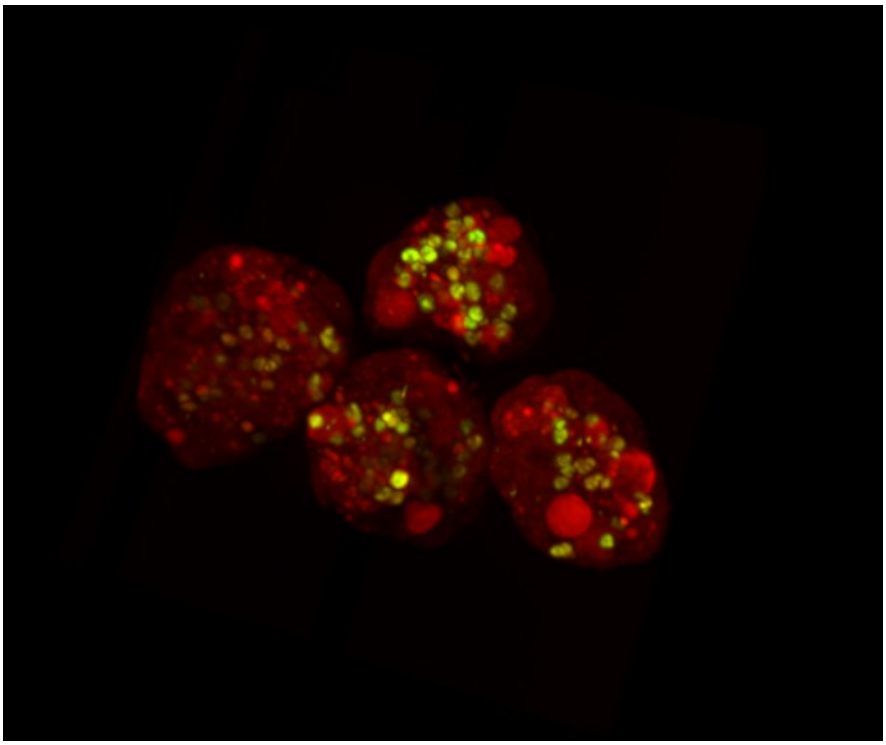


Figure S5. Generation of 3-D spheroids containing leukemic cells and osteoblasts.

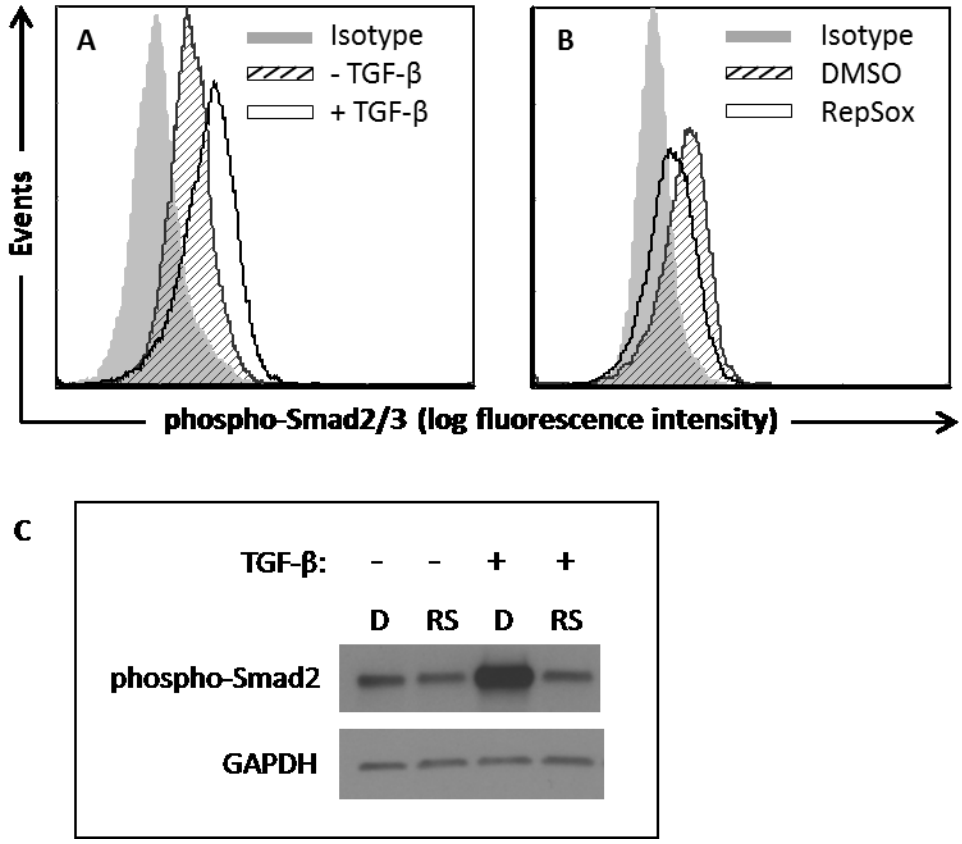


Figure S6. RepSox inhibits TGF-β-induced phosphorylation of Smad2/3.

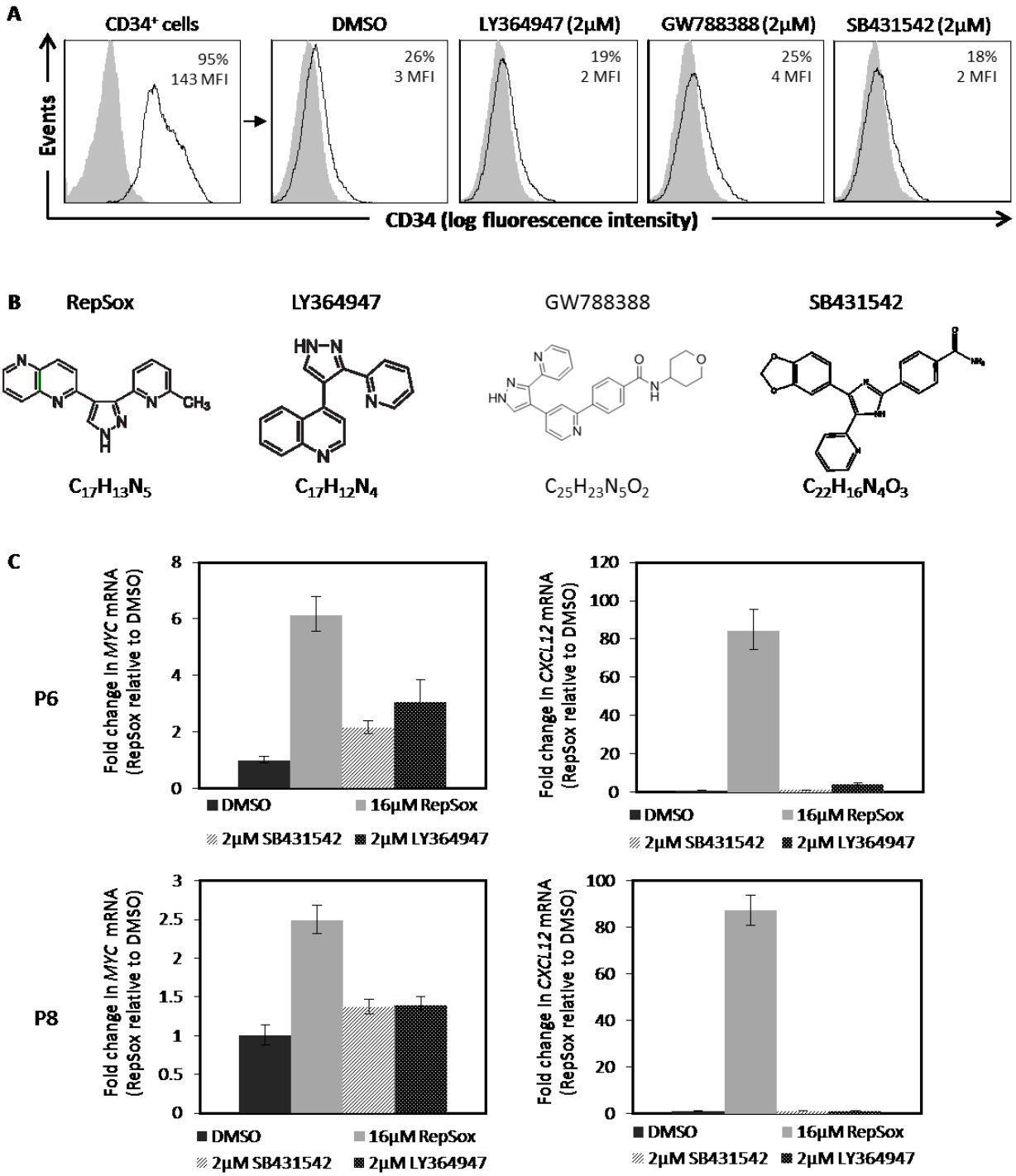


Figure S7. Unlike RepSox, additional TGF-β inhibitors do not slow decay of CD34+ AML cells.

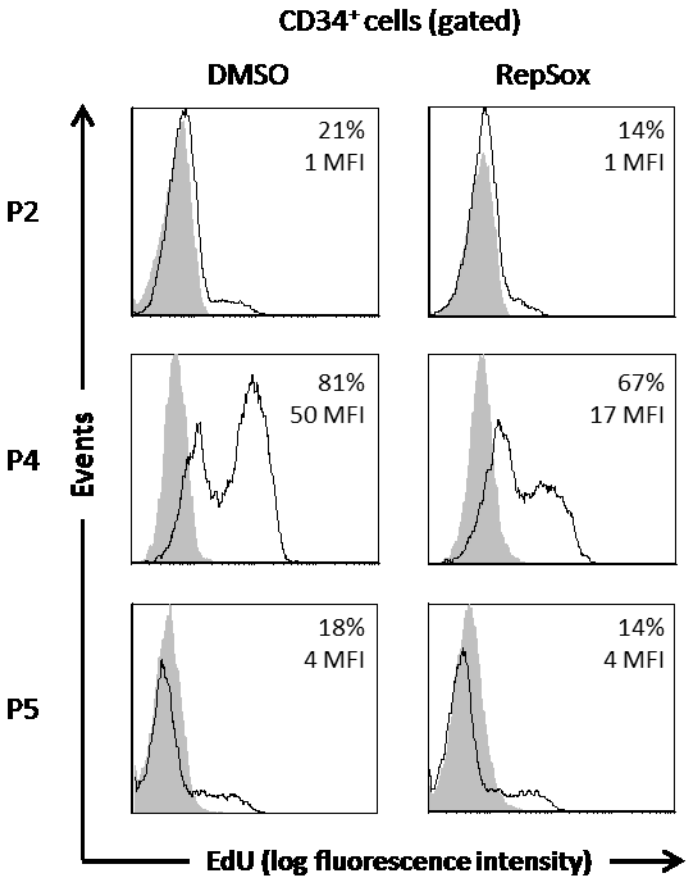


Figure S8. RepSox-treated CD34$^+$ cells proliferate more slowly than DMSO controls.

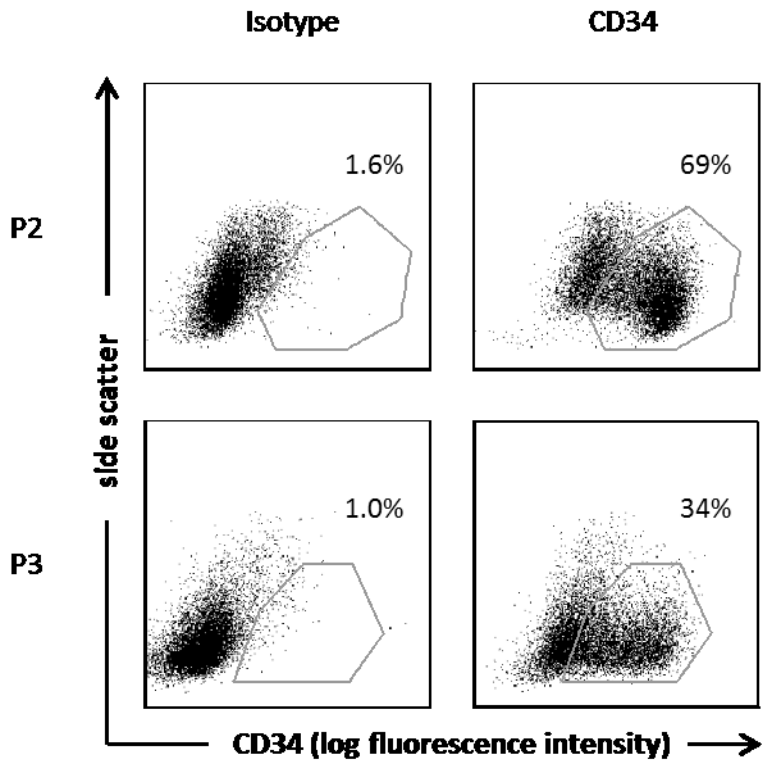


Figure S9. Gating strategy used to select CD34⁺ cells.

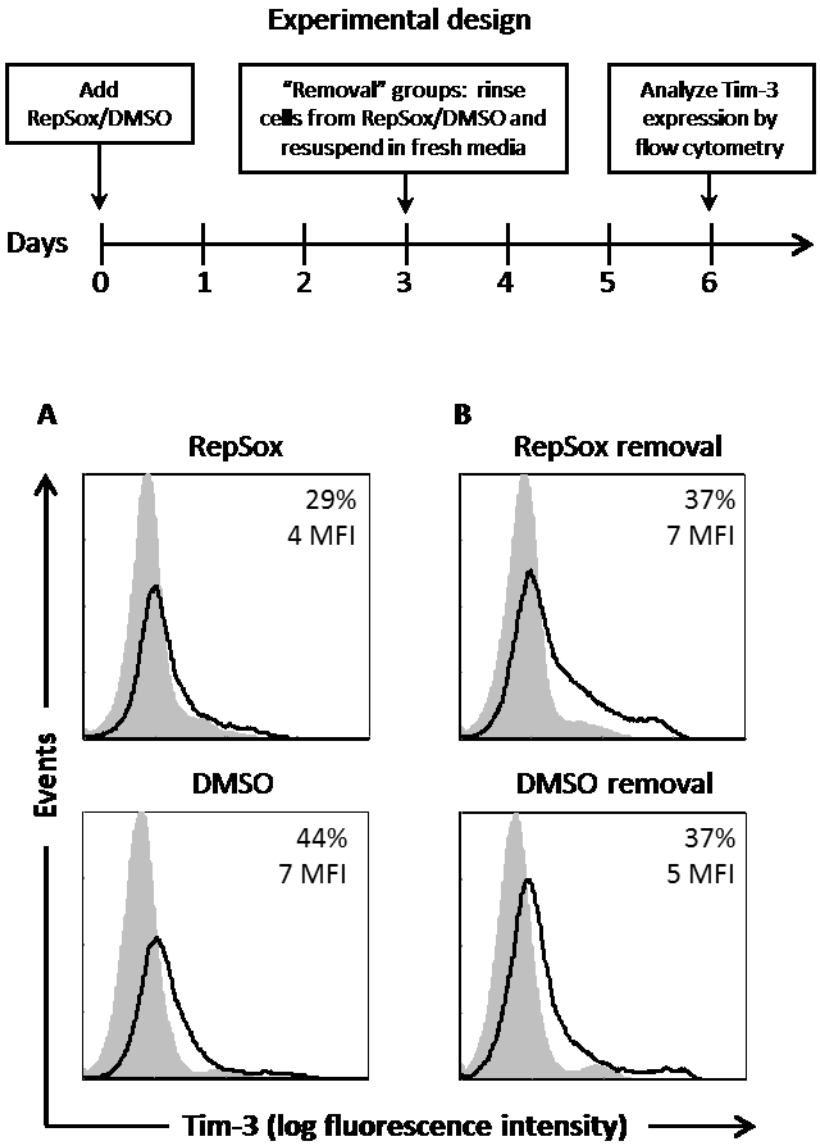
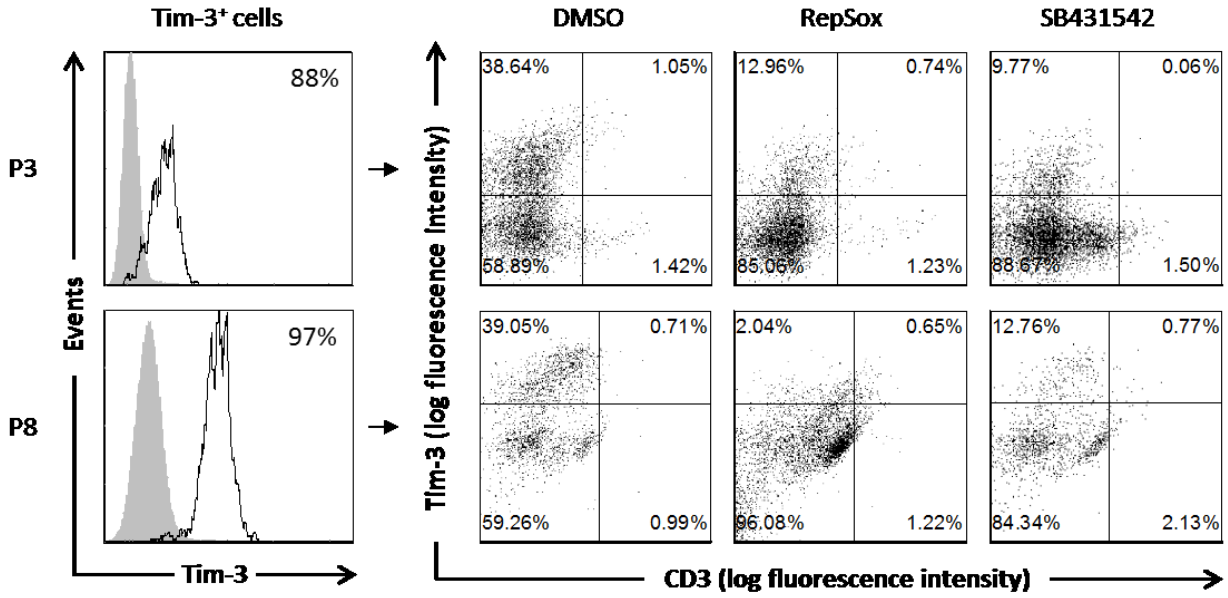


Figure S10. Increased proportion of Tim-3$^+$ cells following removal of RepSox.



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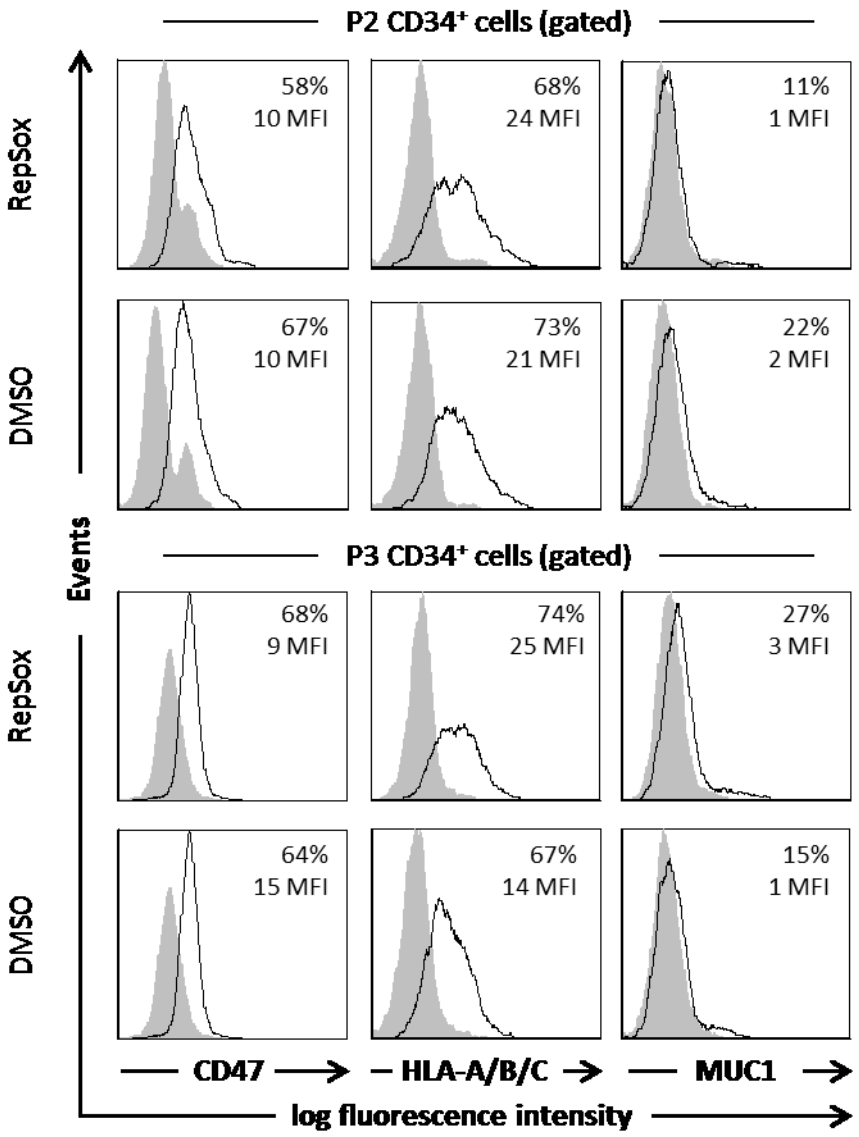

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Chapter VII

Discussion

Purpose

The goal of this dissertation is to give readers interested in anti-cancer immune therapies an overview of historical events, conceptual frameworks, therapeutic options, technical challenges, and research opportunities. Dramatic advances in cell-engineering and reprogramming suggest patient-specific anti-CSC immunotherapies can now be developed even when CSCs in specimens are rare or missing. In general, therapeutic options are unlimited. Perhaps this dissertation can help students choose specific research agendas from the vast array of exciting opportunities.

Because immune therapies engineered *in vitro* have saved the lives of patients with a deadly leukemia, cancer researchers are now extremely optimistic. Historically, the evolution of anti-cancer strategies has been remarkable. Cancer researchers have shifted their attention from the chemical warfare agents of World War II to limb regeneration in salamanders, stem-cell technologies, and the engineering of immune cells as conceptual frameworks for cancer and immunology have evolved. This is truly an exciting time. Just during the course of this project, achievements (see Figure 5.1) have been profound. A Nobel Prize was awarded for clarifying how mature cells can be reprogrammed into pluripotent stem cells capable of regenerating any kind of specialized cell (Takahashi, 2006; Takahashi, 2007). Regarding cancer immune therapies, the FDA has approved Provenge, the first patient-specific cellular immunotherapy, to treat patients with advanced prostate cancer (Kantoff, 2010) and ipilimumab, an antibody that blocks the immune-checkpoint receptor CTLA-4, for the treatment of patients with metastatic melanoma (Hodi, 2010). The lives of leukemia and lymphoma patients have been saved by infusing their own T cells after these cells were genetically engineered *in vitro* to specifically attack their cancer cells (Kalos, 2011; Porter, 2011; Kochenderfer, 2012;

Brentjens, 2013; Grupp, 2013). Major investments in cancer immunotherapy are being announced, including the National Cancer Institute (NCI)'s launch of the Cancer Immmunotherapy Trials Network (http://www.citninfo.org/). These kinds of achievements prompted *Science* to designate cancer immunotherapy as the 2013 "Breakthrough of the Year" (Couzin-Frankel, 2013).

Recently, the skillful use of RepSox and six other biologically potent chemicals was able to reprogram mouse fibroblasts to pluripotency without genetic engineering (Hou, 2013). This groundbreaking advance highlights the fantastic potential of *in vitro* cell manipulations and cell-fate control. Of note, the *in vitro* chemical reprogramming of cells may involve the same de-differentiation mechanisms conserved during evolution to generate and protect the primitive (normal) cells needed for tissue regeneration. These mechanisms, unfortunately, might be exploited to sustain populations of malignant cells when cancer cells are stressed. For example, *Sall4* (Hou, 2013) – and possibly other genes activated during 7-factor chemical reprogramming of (normal) mouse cells to pluripotency – are activated during limb regeneration in salamanders (Neff, 2011). Furthermore, the same four embryonic transcription factors that reprogram mouse cells (Takahashi, 2006) also reprogram human cells (Takahashi, 2007). Diverse stimuli and environmental conditions can activate the same genes and signaling pathways, even across species, suggesting convergence on a few key mechanisms maintained over the course of evolution. Fortunately, critical molecular mechanisms are being clarified, and potent cell-engineering methods are being developed. As conceptual frameworks improve and additional mechanisms are being discovered, new therapeutic options are being identified which, in turn, are directing attention to specific technical obstacles that

need to be overcome to engineer these therapies. Although these technical challenges can seem overwhelming, it is reassuring to know immune therapies are now saving the lives of cancer patients suffering from diseases recently considered "incurable" (Kalos, 2011; Porter, 2011; Kochenderfer, 2012; Brentjens, 2013; Grupp, 2013; Tran, 2014). Frustrated cancer researchers never abandoned their pursuit of immunologic strategies: More than 100 years ago they knew immune responses could (apparently randomly) eliminate tumors (Coley, 1893), and now they are rationally designing immune therapies that are predictably curing cancer patients.

Rationale for studying RepSox

Regeneration research prompted the decision to study how RepSox might affect AML cells. RepSox, an inhibitor of TGF-β receptor 1 (aka ALK5) (Gellibert, 2004), was found to promote the reprogramming of normal cells to pluripotency by replacing two of the four critical factors previously identified: *Sox2* and *c-Myc* (Ichida, 2009). In the context of the CSC theory, this observation seemed relevant for engineering patient-specific anti-cancer therapies. How might RepSox – a potent "reprogramming tool" – alter malignant cells? Fortunately, when a patient's AML cells were exposed to RepSox *in vitro*, two effects were noted that could be useful for developing immuno-therapies: The decline in the proportion of $CD34^+$ cells is slowed, and the decline in Tim-3 surface expression is accelerated (Jajosky, 2014). Thus, molecular tools that alter normal cells (Feng, 2009; Hou, 2013) may also alter tumor cells – and in ways potentially useful for engineering immune therapies.

Perhaps students will find it informative to review the rationale for considering the potential relevance of chemical "reprogramming tools" for anti-cancer therapy-

development. In general, primary cancer cells tend to die or differentiate quickly *in vitro*. The ways RepSox affects normal cells (Ichida, 2009; Larsen, 2013) suggested RepSox might promote survival of primitive AML cells. That is, the reprogramming-associated effects of molecular tools on normal cells might predict their effects on tumor cells. Primitive tumor cells are critical according to the "CSC theory" which suggests stem-like cancer cells must be eliminated – in addition to the more mature and prevalent "bulk" tumor cells – to prevent disease-relapse. Thus, maintaining primitive tumor cells *in vitro* long enough to design, engineer, and test therapies was considered a vital technical goal. Given the ability of RepSox to promote cellular reprogramming, RepSox was evaluated as a "candidate tool" for AML therapy-development (Ichida, 2009). After exposing AML cells from leukapheresis specimens to RepSox, two effects were chosen for initial study: the survival of the $CD34^+$ subset of AML cells and AML-cell expression of Tim-3.

AML stem cells (that can sustain disease and trigger relapse in patients – and engraft disease in immuno-deficient mice) are often enriched within the $CD34^+$ subset (Bonnett, 1997; Eppert, 2011). That is, the $CD34^+$ subset of AML cells in a patient's specimen generally contains the primitive LSCs that must be targeted to prevent relapse. Unfortunately, LSCs are difficult to study because they tend to rapidly die or differentiate in culture. However, the actions of RepSox suggested RepSox, even when used alone, might inhibit differentiation and maintain "stemness" (Ichida, 2009; Larsen, 2013). Thus, RepSox might facilitate engineering anti-AML immune therapies by helping to maintain primitive AML cells *in vitro* long enough so they can be used to identify therapeutic targets or evaluate immune-cell activation, antigen presentation, and candidate therapies.

When expressed on the surface of cancer cells, the immune-checkpoint receptor Tim-3 can promote tumor-cell immune evasion. When expressed on the surfaces of the non-malignant antigen-presenting and immune-effector cells of cancer patients, Tim-3 induces immune tolerance, and Tim-3 levels can increase on these non-malignant cells as they interact with tumor cells, stromal cells, immuno-suppressive factors, metabolic dysregulations, and other components of cancer microenvironments (Huang, 2010; Baghdadi, 2013). In general, Tim-3 expression on cancer cells (Kikushige, 2010; Jan, 2011; Kikushige, 2012), tumor-associated endothelial cells (Huang, 2010), and antigen-presenting (Chiba, 2012) and immune-effector (Anderson, 2012) cells inhibits anti-tumor immune responses in patients with many types of cancers (see Tables 7.1 and 7.2).

Cancer type	Findings	References
Myelodysplastic syndrome (MDS)	Tim-3 is expressed by $CD34^+CD38^-$ MDS cells; Tim-3 is upregulated during progression of MDS into AML	Yuda, 2013
Acute myeloid leukemia (AML)	Tim-3 is expressed by AML LSCs (all subtypes except M3), but not normal HSCs; $Tim\text{-}3^+$, but not $Tim\text{-}3^-$ AML cells generated disease in immunodeficient mice; Tim-3 may promote AML cell survival (by increasing Mcl-1), immune evasion, and disease progression	Kikushige, 2010; Jan, 2011; Kikushige, 2012; Yuda, 2013
Chronic myeloid leukemia (CML) and chronic myelomonocytic leukemia (CMML)	Tim-3 is expressed by primary CML and CMML cells	Yuda, 2013
Melanoma	Tim-3 is over-expressed on melanoma cells and cell lines relative to normal melanocytes; mast cells surrounding melanoma tumors also express Tim-3	Wiener, 2007
Lung cancer	Tim-3 is expressed by non-small cell lung cancer cells (26/30 patients); patients with $Tim\text{-}3^+$ tumors died sooner than patients with $Tim\text{-}3^-$ tumors	Zhuang, 2012
Cervical cancer	Tim-3 is more highly expressed in patients with cervical cancer than patients with cervical intraepithelial neoplasia and chronic cervicitis; high Tim-3 expression correlated with greater metastasis, higher tumor grade, and shorter overall survival	Cao, 2013
Osteosarcoma	$Tim\text{-}3^+$ tumor cells generally co-expressed EMT markers, such as Slug, Snail, and Smad	Shang, 2013
Various cancers of histiocytic and dendritic cell origin	Tim-3 is expressed by neoplastic cells from patients with juvenile xanthogranuloma (3/3), histiocytic sarcoma (6/6), interdigitating dendritic cell sarcoma (3/3), follicular dendritic cell sarcoma (1/4), langerhans cell histiocytosis (6/6), acute monocytic leukemia (6/6), and hematodermic tumors (5/5)	Dorfman, 2010

Table 7.1. Human cancers that express Tim-3.

Disease State	Tim-3$^+$ Immune or Stromal Cells & Key Findings	References
Cancer		
Melanoma	NK cells; Higher Tim-3 expression on NK cells from melanoma patients than NK cells from healthy controls; Tim-3$^+$ NK cells from melanoma patients were functionally exhausted (impaired cytokine production, proliferation, and cytotoxicity), and Tim-3 blockade improved NK cell cytotoxicity against melanoma cell lines	Da Silva, 2014
Acute myeloid leukemia (AML)	CD8$^+$ cytotoxic T cells; mice with AML develop functionally exhausted CD8$^+$ T cells that co-express Tim-3 and PD-1	Zhou, 2011
Follicular B cell non-Hodgkin lymphoma	T cells; IL-12 induces Tim-3 expression on T cells and promotes T cell exhaustion	Yang, 2012
Lymphoma	Endothelial cells dissected from human lymphomas; Tim-3$^+$endothelial cells suppressed activation of CD4$^+$ helper T cells by activating the IL-6-STAT3 pathway and inhibiting T_h1 polarization	Huang, 2010
Melanoma	Endothelial cells; melanoma cell-secreted TLR4 ligand induced endothelial cells to express Tim-3; binding of melanoma cells to Tim-3$^+$endothelial cells promoted tumor cell proliferation and survival	Wu, 2010
Chronic Viral Infection		
HIV	NK cells, T cells, and NKT cells: Tim-3$^+$ immune cells were dysfunctional	Jones, 2008; Finney, 2013
Hepatitis B	NK cells: Tim-3$^+$ NK cells were dysfunctional, and Tim-3 blockade improved NK cell cytotoxicity and IFN-γ production	Ju, 2010
Hepatitis C	CD8$^+$ cytotoxic T cells: Tim-3$^+$ T cells were dysfunctional and associated with viral persistence and disease progression; Tim-3 blockade restored T cell functions, including cytotoxicity against virally-infected hepatocytes	Golden-Mason, 2009; McMahan, 2010; Vali, 2010

Table 7.2. Tim-3-expressing immune and stromal cells in the settings of cancer and chronic viral infections.

Tim-3 expression is linked to AML LSCs (Kikushige, 2010; Kikushige, 2012) and is a useful marker for distinguishing Tim-3^{+} LSCs to be targeted from the Tim-3^{-} HSCs (normal cells) to be spared (Jan, 2011). Tim-3 also highlights a potential confusion that can emerge concerning tumor-cell evolution and plasticity. Most often, CSCs are viewed as being able to give rise to more mature tumor cells. However, it has been found that tumor cells can evolve – even de-differentiate – into more stem-like cells in reaction to stresses like chronic inflammation (Landsberg, 2012) and immune-selection pressures (Noh, 2012b). These same stresses also seem to trigger the de-differentiation of normal cells that precedes the regeneration of tissues in primitive animals after injuries like amputations. Tumor cells have been found to acquire more stem-like phenotypes following serial rounds of "immune selection" *in vivo* (Lin, 2007; Noh, 2012a) and *in vitro* (Noh, 2012b). The acquisition of CSC-like characteristics induced by immune selection was replicated *in vitro* by transfection with Nanog (Noh, 2012b). That is, artificially induced Nanog signaling mimics the signaling induced when pathways are activated in tumor cells when they are attacked by immune cells – signaling that drives the tumor cells toward a stem-like, immune-resistant state (Noh, 2012b). Regarding leukemia cells, Tim-3 expression by AML cells may distort immunologic synapses between immune and AML cells, suppress immune-cell activation, promote immune evasion, contribute to leukemogenesis, and may identify a highly evolved LSC phenotype that can emerge in advanced disease (Kikushige, 2010; Kikushige, 2012). Thus, Tim-3 can be an important immuno-therapeutic target on both (malignant) AML cells and normal, but functionally impaired, immune cells. Thus, molecular tools like RepSox that reduce Tim-3 surface levels may be valuable for engineering immune therapies.

History of leukemia therapy

Hippocrates used arsenic to treat leukemia in ancient Greece; its use continued until the 20th century when it was replaced by radiation therapy. More than 100 years ago it was noted that the cancers of some patients spontaneously disappeared, and this regression of tumors seemed to be linked to fever-inducing infections. Thus, robust immune responses – induced by bacterial infections – were considered responsible for these spontaneous cures. Although radiation could improve the condition of leukemia patients, it caused leukemia in the radiologists who treated them. In 1913, the American Society for the Control of Cancer was founded to educate the public. Malignant diseases were so lethal and feared, cancer diagnoses were often withheld, denied, or ignored (like the taboo now linked to ebola diagnoses in some nations). In the 1930s, the Women's Field Army of volunteers initiated "a war on cancer." Their poster included the "Sword of Hope" – a symbol which remains part of the logo of the American Cancer Society. The handle of the sword consists of two intertwined serpents that symbolize medicine and science: healing and creativity. Regarding chemotherapy, soldiers exposed to the chemical warfare agent "mustard gas" during World War II were found to have low white blood cell counts. That observation essentially invented modern chemotherapy. Study of nitrogen mustard revealed it preferentially attacked rapidly dividing cells, including malignant cells.

In the 1940s, the chemical aminopterin was discovered in leukemia patients experiencing remission. So, Boston's Dr. Farber decided to use it therapeutically. Aminopterin is related to folic acid and blocks DNA replication in tumor cells. By 1950, Hitchings and Elion had carefully designed the compound 6-mercaptopurin specifically to

disrupt DNA synthesis. Although this drug often induced remissions, leukemia patients later relapsed and died. While these classic studies and some early agents are still important, better anti-leukemia treatments are needed to prevent relapse and death. Although many leukemia drugs with diverse mechanisms of action are now available, the survival of residual LSCs that trigger relapse remains a challenge.

The potential of immunologic strategies was demonstrated when some leukemia patients were cured after being treated with monoclonal antibodies or bone-marrow cells from a donor who was not an identical twin of the patient (Thomas, 1999). In the case of allogeneic bone marrow transplants, a donor's genetically distinct immune cells can induce a graft-versus-leukemia immune reaction that blocks re-emergence of the host's LSCs (Thomas, 1999). Elimination of tumor cells by (activated) immune cells is the same mechanism that had been proposed to explain the spontaneous cancer regressions observed in the 19th century (Coley, 1893).

Currently, AML relapses are still lethal, but researchers seem confident they will be able to predictably cure even patients with advanced disease using rationally designed, multi-pronged therapies that include a patient-specific immune strategy. *In vitro* cell-engineering techniques that exploit advances in cell reprogramming (Hou, 2013; Rais, 2013) and methods that repair dysfunctional antigen-presenting and immune-effector cells can now generate immune cells that can be used therapeutically (following activation and expansion) (Hodi, 2010; Vizcardo, 2013; Themeli, 2013). Technical breakthroughs and new insights into cancer immunology are rapidly advancing cell engineering (Hou, 2013; Kalos, 2011; Porter, 2011) and the design of immuno-therapies (Hodi, 2010; Tran, 2014). As needed, even in the most problematic situations, these cell-

engineering advances should enable researchers to manipulate mature cancer cells in routine specimens to generate the "CSC surrogates" that may be needed to develop patient-specific immune therapies. Cancer-induced pluripotent stem cells (iPSCs) have been generated from primary chronic myeloid leukemia cells (Kumano, 2012). Oct-4 has been shown to de-differentiate melanoma cells into CSC-like cells displaying reduced expression of melanocyte antigens, enhanced tumor-spheroid generation *in vitro*, and increased tumorigenicity in mice (Kumar, 2012). The successful reprogramming of tumor cells has been reassuring since patient-specific LSC-targeted immunotherapies may be needed to save the lives of some patients and engineering these therapies may require generating LSCs *in vitro*. That is, because some leukemia specimens contain few or no LSCs, a patient's mature leukemia cells may need to be de-differentiated into LSC-like cells. Thus, this project was influenced by the cell-repgrogramming breakthroughs achieved by tissue-regeneration researchers. Also of great interest, cell-culture advances had demonstrated how tumor microenvironments (Kinugasa, 2014) and bone-marrow factors, such as hypoxia (Yoshida, 2009; Ng, 2014), bone-marrow stromal cells (Sutherland, 1990; Ailles, 1997), and adipocytes (Gletting, 2013), can help maintain stem and progenitor cells. However, cell-culture advances have not yet been fully exploited by, for example, integrating them with new reprogramming methods and molecular tools (Feng, 2009). The therapeutic vision for this project revealed how therapy-development may require not only the manipulation of primitive leukemia cells (to, for example, enhance immunogenicity and immune synapses *in vitro*) but also the manipulation of non-malignant, but cancer-impaired, immune cells (to, for example, reverse defects responsible for immune tolerance). That is, reprogramming may be necessary when

engineering therapies for some patients. Tumor cells may need to be reprogrammed into relevant, LSC-like surrogates (that can be studied, manipulated, and serve as targets *in vitro*), and dysfunctional immune cells may need to be reprogrammed (to repair them before they are activated and expanded). A possible cell-engineering strategy might involve repairing a leukemia patient's immune cells to restore cytolytic and/or antigen-presentation function (via reprogramming), activating effector immune cells against AML LSC antigens in co-culture, and then expanding these tumor-targeted immune cells before infusing them as therapy.

Collectively, experimental findings and conceptual frameworks for cancer and immunology have directed the attention to the potential value of de-differentiation and cell engineering. The technical obstacles encountered when with working with primary tumor cells *in vitro* have prompted cancer researchers to consider how regeneration scientists have successfully de-differentiated mature (normal) cells into pluripotent cells. To develop effective immune therapies for some leukemia patients, we may need to maintain – or generate – LSCs to design patient-specific anti-LSC therapies. That is, in response to the CSC theory and new data, attention has shifted from chemical warfare agents that target rapidly dividing bulk tumor cells to the stem cells and molecules involved in the regeneration of tissues in primitive animals. Thus, although pursuing different agendas, cancer and regeneration researchers now share a strong interest in de-differentiation.

Historically, advances in the conceptual frameworks for cancer seem to have driven progress. However, current cancer-immunology concepts and theories are so reliably predicting outcomes, clinical successes now seem to depend on technical advances. That is, current concepts seem adequate and comprehensive. For example,

recent cancer "cures" induced by engineered T cells are validating the critical importance of "memory" T cells – as predicted. Researchers are envisioning effective therapies but need the methods and tools to engineer these therapies. Progress now seems to be "driven" by the methodical development of new techniques in research labs.

Finally, in retrospect, the history of cancer research is truly admirable and inspiring. It is comforting and reassuring to realize that efforts were not wasted: Many treatments that originally seemed like disappointing failures were actually impressive, rational agendas that are currently being reconsidered. Treatment "failures" have been informative. We now realize that in the context of "immunoediting" and "tumor-cell plasticity" almost any therapeutic strategy has the potential to make matters worse. To be effective long-term, anti-cancer therapies must be aggressive enough to eliminate a broad spectrum of tumor cells so that (1) CSCs do not have the opportunity to evolve into more lethal and death-resistant CSCs and (2) mature ("bulk") tumor cells do not have the opportunity to de-differentiate into CSCs. Thus, treatments initially considered ineffective may prove to be useful in the future as components of broader, multi-pronged strategies.

Of note, many "conventional" or "non-immunologic" treatment options can induce beneficial immunologic effects. For example, physical removal of malignant cells via surgery or leukapheresis can reduce tumor-cell-induced immune-suppression. Radiation and some chemotherapies not only reduce the number of mature tumor cells but induce "immunogenic tumor-cell death" (Inoue, 2014; Ma, 2013) that can stimulate anti-cancer immune responses. Thus, multi-pronged anti-cancer therapies are attractive because, by exploiting the unique actions of diverse agents, they may be able to eliminate both mature and stem-like tumor cells. Of note, it is generally believed that patient-specific

immune strategies may be required to eliminate a patient's CSCs. That is, CSCs are so death-resistant, they can survive even when exposed to radiation and chemotherapeutic drugs. Overall, the history of cancer research is truly inspiring: Each valiant effort has been informative, and past "failures" are being reconsidered for their potential value in the context of more comprehensive conceptual frameworks and multi-pronged strategies.

Conceptual frameworks

As conceptual frameworks for cancer, immunology, and tissue regeneration have evolved, themes and "lessons" have emerged that can provide guidance. For example, advances in diverse fields should be closely monitored so opportunities are not overlooked. As noted, *in vitro* de-differentiation – pioneered by regeneration researchers – may prove useful for engineering anti-cancer immunotherapies. De-differentiating a patient's tumor cells and non-malignant (but dysfunctional) antigen-presenting and immune-effector cells (Vizcardo, 2013; Themeli, 2013) may sometimes be required to engineer effective therapies. Interestingly, in humans, malignant cells may be the only cells that spontaneously de-differentiate – a mechanism that helps cancer cells survive when subjected to therapeutic or immunologic attack (Landsberg, 2012; Reiman, 2010).

Certain genes and signaling pathways seem especially critical for the generation, or survival, of normal stem cells and CSCs – and markedly different factors can induce activation of these genes and signaling pathways. Cell de-differentiation during *in vitro* reprogramming and amputation-induced limb regeneration *in vivo* are linked to the Wnt, Notch, and Hedgehog signaling pathways as well as activation of the *Sall4* gene (Neff, 2011; Hou, 2013). Surface antigens like Tim-3 can be highly expressed on diverse cell types – including tumor (Wiener, 2007; Dorfman, 2010; Kikushige, 2010; Kikushige, 2012;

Cao, 2013; Shang, 2013), endothelial (Huang, 2010), antigen-presenting cells (Chiba, 2012), and immune-effector cells (Anderson, 2012) in patients with a variety of cancers (see Tables 7.1 and 7.2). Furthermore, CAFs may also actively suppress immune cells (Silzle, 2004; Gieseke, 2013) as shown in Figure 3.6. Tim-3 is also expressed by functionally "exhausted" immune cells in patients with chronic viral infections (see Table 7.2) (Jones, 2008; Golden-Mason, 2009; Ju, 2010; McMahan, 2010; Vali, 2010; Finney, 2013). Thus, Tim-3 modulation may be a therapeutic strategy that may be useful for a variety of cancers and (chronic) viral diseases.

As emphasized previously, the most lethal tumor cells are stem-like cells that are hard to kill *in vivo* – but are hard to keep alive *in vitro*. Cancer cells that robustly grow in culture (that are like "HeLa cells") are extremely rare. Thus, researchers are working to find simple ways to maintain, or generate, a patient's lethal tumor stem cells *in vitro* in order to design, and test, strategies that can target them. That is, developing better *in vitro* ways to maintain a patient's CSCs – or generate CSC-like surrogates when necessary – is a major goal. Of note, in response to stress, normal stem and progenitor cells are protected by inflammation- and regeneration-related mechanisms that have been conserved during evolution. These same mechanisms are thought to protect primitive malignant cells that are exposed to chronic inflammation or therapeutic attack (Landsberg, 2013), and may provide guidance for maintaining primitive tumor cells *in vitro*. Of note, cancer's "unholy trinity" refers to three environmental conditions that support cancer cells: chronic inflammation, cytokines, and the transcription factor STAT3 (Li, 2011). These factors are thought to sustain the CSCs that maintain disease and trigger relapse following treatments that predominantly target bulk tumor cells. As the

underlying mechanisms by which the "unholy trinity" (Li, 2011) maintains CSCs are clarified, they may be exploited to improve cell-culture techniques and guide the engineering of CSC-targeted immune therapies. However, the relationship between tumor cells and inflammation may be context-dependent. Chemotherapies that induce ICD are considered valuable components of anti-cancer therapies by inducing inflammation-related anti-cancer immune responses (Inoue, 2014; Ma, 2013).

Although finding better ways to maintain or generate a patient's stem-like cancer cells is still a challenge, recent successes with patient-specific anti-cancer immune therapies have validated current conceptual frameworks, and dramatic advances in cell engineering suggest all technical obstacles can eventually be overcome. Patients have achieved long-lasting remissions following adoptive transfer of their own T cells that have been engineered to express tumor-targeted chimeric-antigen receptors (CARs) *ex vivo* (Kalos, 2011; Porter, 2011; Kochenderfer, 2012; Brentjens, 2013). Following isolation, expansion, and reinfusion of cancer-reactive tumor-infiltrating T cells, tumors regressed in a patient with metastatic cholangiocarcinoma (Tran, 2014). Immune-checkpoint blockade of CTLA4 stimulated immune responses and improved survival of patients with metastatic melanoma (Hodi, 2010). A genetically engineered oncolytic (cancer-lysing) strain of measles virus eliminated tumors in a patient with advanced "incurable" multiple myeloma (Russell, 2014). Although a patient's immune cells can become impaired ("exhausted" or unresponsive) as a cancer progresses, functionality can be restored after de-differentiating these cells (Vizcardo, 2013; Themeli, 2013; Nishimura, 2013), modulating immunoreceptors to unleash anti-tumor activity (Hodi, 2010), or manipulating the cells in other ways described in **Chapter IV**. Following reprogramming to pluripotency

and re-differentiation, T cells were functionally “rejuvenated” and acquired elongated telomeres along with the capacity for significant expansion (Vizcardo, 2013; Themeli, 2013; Nishimura, 2013). Although conceptual frameworks highlight the complexity of malignant disease and the diverse dysregulations of local tumor microenvironments and the global immune system, dramatic clinical and cell-engineering successes have been achieved.

Also encouraging, *in vitro* techniques are effectively completing more tasks. Highly controlled *in vitro* conditions provide the opportunity to manipulate cells in diverse ways not possible *in vivo* where cancer-distorted microenvironments suppress anti-tumor immune responses via recruitment of myeloid-derived suppressor cells, release of soluble factors, and upregulation of inhibitory immune-checkpoint receptors that induce immune tolerance (Rabinovich, 2007). The *in vitro* evaluation and manipulation of cells in different contexts can be highly informative and inspire new treatment strategies. Methods for testing candidate anti-cancer therapies *in vitro* have correctly predicted their success in animals and humans (Peng, 2001; Carpenito, 2009; Milone, 2009; Noh, 2012b; Grosso, 2013). Also encouraging, although a patient’s CSCs may be rare and elusive, *in vitro* engineered stem-like tumor cells that are not “bona fide CSCs” may be similar enough to the CSCs *in vivo* that they can serve as relevant therapeutic targets by virtue of immunologic cross-reactivity (Noh, 2012b).

Worst-case clinical scenario: advanced disease with highly evolved, death-resistant, and treatment-refractory cancer cells

Before planning experiments, it was considered prudent to consider the "worst-case clinical scenario" when envisioning the engineering of a patient-specific anti-cancer immune therapy. What cell types might need to be altered, repaired, or activated? What cell manipulations and which methods might be required? The latest conceptual frameworks for cancer and immunology propose an evolving distribution of cancer cells ranging from death-resistant stem-like cells to their more mature progeny as well as an evolving distribution of dysfunctional antigen-presenting (Gabrilovich, 2004) and immune-effector cells (Radoja, 2000; Frey, 2008; Lion, 2012; Stringaris, 2014). That is, both tumor and immune cells evolve as malignant disease advances, and the immune system becomes progressively dysregulated via a combination of tumor-cell immune evasion and immune-system tolerance that prevents adequate clearance of cancer cells (Kim, 2006). In highly evolved cancers, this emergence of multiple immunologic dysfunctions may require a multi-pronged therapy that combines patient-specific immune strategies with "conventional treatments." That is, a personalized approach may be required to effectively target each patient's unique immune-cell dysfunctions and CSC antigens. The "Immunoscore" cancer classification system proposes carefully examining the immune infiltrate within a patient's tumor to predict outcomes and guide therapeutic decisions (Galon, 2012; Fridman, 2012). As conceptual frameworks have improved, they have identified new mechanisms that explain how non-malignant antigen-presenting and immune-effector cells become dysfunctional in cancer (Radoja, 2000; Gabrilovich, 2004; Frey, 2008; Lion, 2012; Stringaris, 2014). Remarkably, dysfunctional immune cells can

be “repaired” (Reiners, 2013; Vizcardo, 2013), reactivated (Hodi, 2010), and skillfully engineered (Themeli, 2013; Kalos, 2011; Porter, 2011) *in vitro* so they can be used therapeutically. For example, a melanoma patient's exhausted, tumor-infiltrating $CD8^+$ cytotoxic T cells were functionally “rejuvenated” by first reprogramming them to pluripotency and then re-differentiating them back into T cells (Vizcardo, 2013; Themeli, 2013). The successful generation of normal HSCs from iPSCs (Lim, 2013; Suzuki, 2013; Amabile, 2013) suggests a cancer patient’s non-malignant blood cells can be used to repopulate a patient’s bone marrow with normal HSCs as needed. Of note, the latest findings and conceptual frameworks for cancer and immunology not only direct attention to the critical importance of the non-malignant cells of the immune system, but they help explain how these cancer-impaired cells can be repaired via de-differentiation (Vizcardo, 2013; Themeli, 2013). In general, as conceptual frameworks have advanced, they have identified new therapeutic options along with the technical tasks these strategies require. As noted, a “worst-case clinical scenario” (an advanced, highly evolved, and treatment-refractory malignant disease) suggests a cancer patient's antigen-presenting and immune-effector cells may need to be reprogrammed to restore functionality (such as cytolytic activity and cytokine production) using techniques pioneered by regeneration researchers (Vizcardo, 2013; Themeli, 2013; Nishimura, 2013).

Worst-case leukemia specimen: LSCs are rare and immunoevasive and the immune cells are dysfunctional

In general, the blood or bone-marrow specimens taken from leukemia patients who are not severely immuno-compromised should contain the tumor and immune cells that are needed to engineer a patient-specific immunotherapy. However, it seems prudent to

consider “the worst-case cancer specimen” and the challenges associated with maintaining primary cells *ex vivo*. Researchers should be aware, for example, that marked changes in the phenotypes of AML cells can occur as soon as the AML cells leave the bone marrow. Exposure to oxygen concentrations that are higher than the hypoxic levels in the bone marrow can reduce expression of CXCR4 and activate the AhR pathway. This may explain why the maintenance of primitive AML cells *in vitro* is improved by RepSox which may, conceivably (at least in some cells), induce CXCR4 expression via CXCL12 upregulation or via AhR pathway antagonism. In some studies, activation of the CXCL12/CXCR4 axis has been found to activate the PI3K/Akt “survival pathway” and to affect the AhR pathway. In general, the effects of the AhR pathway are cell-specific. Regarding AML, inhibition of the AhR pathway has been shown to improve the survival of primitive AML cells *in vitro* (Pabst, 2014).

Regarding the specimens from different AML patients, there is marked variation in the proportions and functionality of various immune cells as well as the proportions and phenotypes of CSCs. The proportions of LSCs, normal HSCs, antigen-presenting cells, immune-effector cells, and CAFs vary by AML subtype and stage, and by treatment history (Sarry, 2011; Wiseman, 2013; Kasper, 1999). Ideal specimens will contain adequate numbers of the CSCs to be targeted, normal HSCs to be spared, and functional APCs and tumor-reactive immune-effector cells – that is, cells that can simplify the engineering of a patient-specific therapy. In contrast, problematic specimens may contain few or no stem-like tumor cells (and immune cells that are highly dysfunctional). In that case, mature tumor cells may need to be de-differentiated into tumor cells that are sufficiently stem-like (Noh, 2012b; Kumar, 2012) to serve as *in vitro* surrogate targets for

CSCs when engineering and evaluating candidate therapies. Figure 7.1 and Table 7.3 outline strategies and conditions that trigger cell de-differentiation. Immune-selection pressures (Reiman, 2010; Noh, 2012b); tumor-microenvironment conditions such as hypoxia (Sahlgren, 2008), CXCL12, and inflammation (Landsberg, 2012); and exposure to chemotherapy and radiation (Saxena, 2011; Sun, 2012; Chang, 2013) may drive tumor cells to undergo epithelial-to-mesenchymal transition (EMT) and/or acquire stem-like and immune- and death-resistant phenotypes. As immunogenic tumor cells are eliminated by the immune system, immune- and death-resistant cancer cells ("escape variants") can emerge and become predominant via natural selection (Dunn, 2002). Thus, tumor cells may need to be manipulated *in vitro* to increase their immunogenicity (to facilitate immune-cell activation *in vitro*) or modified *in vivo* using a (safe, non-toxic) systemic agent to decrease their death-resistance so they are susceptible to immunologic attack *in vivo*.

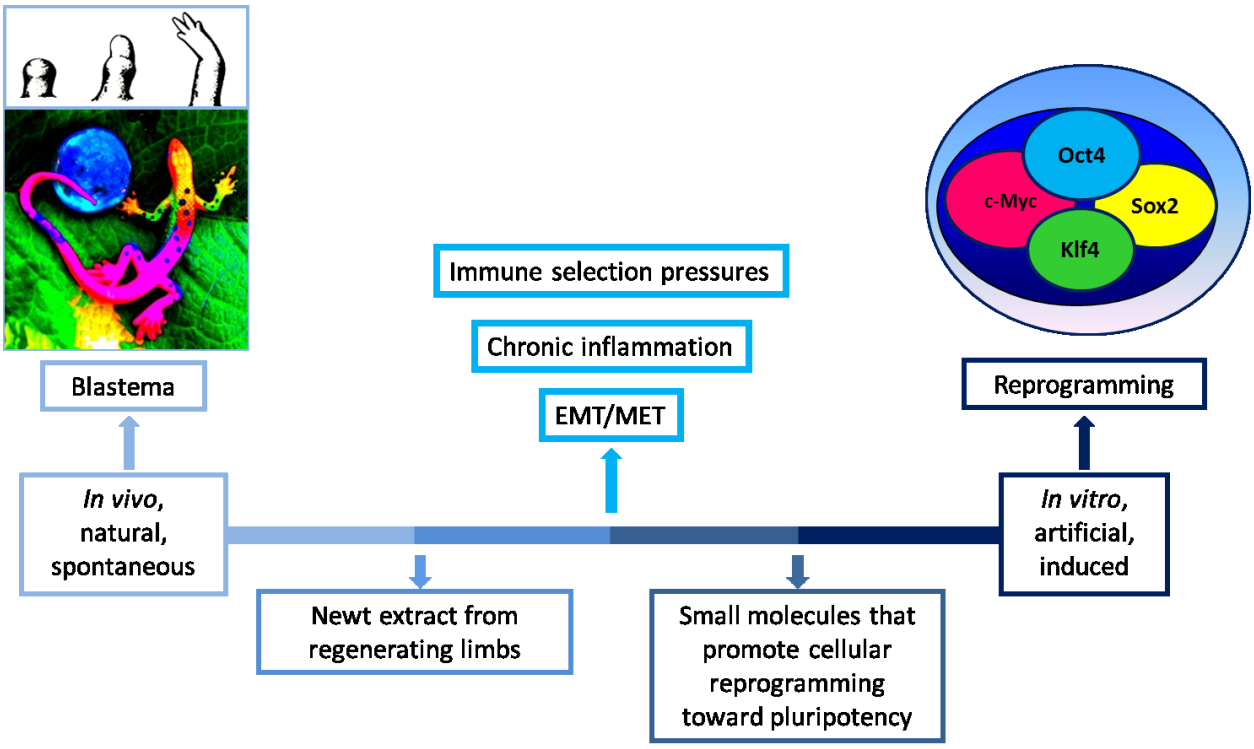

Figure 7.1. Spectrum of ways to induce de-differentiation.

	De-differentiated or "converted" cell types	Factors involved	Degree of de-differentiation or "conversion"
Regeneration post-injury or amputation in primitive animals	Muscle (Nacu, 2014; Echeverri, 2001), skin (Satoh, 2008), Schwann cells (Tweedell, 2010), cartilage (Tweedell, 2010), bone (Sousa, 2011)	*Sall4* gene and other factors linked to inflammation and wound healing (Neff, 2011; Monaghan, 2012)	Partial reprogramming into restricted progenitors (Kragl, 2009; Stewart, 2012)
Immune-selection pressures	Cervical and colon cancer cells (Noh, 2012)	Activated Nanog signaling increases the anti-apoptotic factor Mcl-1 and resistance to T cell-mediated death (Noh, 2012)	Conversion or evolution toward stem-like, immune-resistant tumor cells (Noh, 2012)
Chronic inflammation	Melanoma cells (Landsberg, 2012)	TNF-α (Landsberg, 2012)	Partial de-differentiation into tumor stem-like cells (Landsberg, 2012)
In vitro induced pluripotent stem cell (iPSC) reprogramming	Many cell types including human skin fibroblasts (Takahashi, 2007) and blood cells (Loh, 2009)	Oct4, Sox2, Klf4, and c-Myc transcription factors (Takahashi, 2007); small molecules like RepSox (Feng, 2009; Ichida, 2009; Hou, 2013); Sall4 (Tsubooka, 2009)	A small fraction of cells are completely reprogrammed into pluripotent stem cells; most cells become partially reprogrammed intermediates (Hanna, 2010)
Epithelial-to-mesenchymal transition (EMT)	Cancer cells that undergo EMT acquire stem cell properties (Mani, 2008)	A variety of factors including TGF-β, hypoxia, Snail, ZEB, and immune-selection pressures (Kalluri, 2009; Lamouille, 2014)	Partial conversion into stem-like tumor cells (Mani, 2008)

Table 7.3. Potential ways to induce cellular de-differentiation.

As noted previously, when a patient-specific immune therapy is considered the best strategy, de-differentiation may be needed to repair a patient's defective immune cells (as well as generate CSC-like cells that can serve as surrogate targets *in vitro*). Immune-cell function might also be enhanced by increasing mTOR activity (via upregulation of the PI3K/Akt pathway) since mTOR has been found to improve immune-cell function (Schmitz, 2008; Powell, 2012). Of note, RepSox can upregulate CXCL12, and CXCL12 can activate the PI3K/Akt/mTOR axis (Chen, 2012).

When a problematic cancer specimen contains few or no CSCs, more mature (more differentiated) tumor cells may need to be reprogrammed into stem-like cells. However, it is not clear how similar the CSC-like surrogates (to be used *in vitro*) must be to the actual disease-sustaining CSCs *in vivo*. Presumably, the more closely the CSC-like cells used *in vitro* resemble the CSCs *in vivo*, the more relevant these cells will be when serving as surrogate targets for therapy-development. Of note, Noh found that a spectrum of stem-like cells having different degrees of "stemness" shared the same Nanog-mediated death-resistance to T-cell attack – and the greater their "stemness," the greater their death-resistance (Noh, 2012b). That is, although the "stemness" of these CSC-like cells varied, they shared the same therapeutic vulnerability as the most stem-like CSC. Furthermore, the less stem-like tumor cells could be made more stem-like (and more resistant to immune-cell attack) either through serial rounds of *in vitro* immune-selection pressure (via exposure to activated immune cells) or, more directly, by transfection with Nanog (Noh, 2012b). These observations suggest that the patient's most evolved and lethal CSCs may not be needed to design effective immune therapies – stem-like surrogates engineered *in vitro* may suffice.

In general, if the reprogramming of cancer cells is needed (when only mature cancer cells are available in a specimen), the goal is to de-differentiate them into stem-like (CSC-like) cancer cells that adequately resemble the CSCs that can sustain disease and cause relapse. That is, the goal is not to de-differentiate mature cancer cells all the way to pluripotency. When salamanders regenerate tissues, the de-differentiation of cells is "partial" or "limited" in that the stem-like cells generated (to form the blastema) are "restricted progenitors" – not pluripotent stem cells (Christen, 2010; Garza-Garcia, 2010). Table 7.4 compares the partial, spontaneous (natural) de-differentiation of normal cells that occurs *in vivo* during regeneration with "full" de-differentiation ("artificially") induced *in vitro* by regeneration researchers when they generate pluripotent stem cells. Also notable, when human cancer cells are stressed – such as when exposed to toxic chemotherapeutic agents – they can de-differentiate into more death-resistant, stem-like phenotypes. That is, both normal cells (in primitive animals) and malignant cells (in humans) can spontaneously (and "partially") de-differentiate *in vivo*.

When considering conceptual frameworks and "worst-case scenarios" relevant for therapy-development, the potential need for, and value of, de-differentiating a patient's malignant cells and/or cancer-impaired immune cells *in vitro* becomes apparent. As noted, spontaneous cellular de-differentiation in primitive animals can generate critically important (normal) stem-like cells via mechanisms that can be exploited by human cancer cells to generate death-resistant CSCs. That is, de-differentiation is exploited by human tumor cells stressed by immunologic or therapeutic attack (cancer-cell immunoediting as a survival mechanism) and by normal cells stressed by injury (tissue regeneration as a survival mechanism). As noted, for *in vitro* therapy-development, de-differentiating both

(malignant) tumor and (non-malignant) immune cells of a cancer patient may be required when dealing with problematic clinical scenarios and specimens. When the potential value of de-differentiation for therapy-development was considered, attention was directed to how a reprogramming tool like RepSox (Ichida, 2009) might alter AML cells.

Regeneration in primitive animals	**Cellular reprogramming to pluripotency**
in vivo	*in vitro*
natural, spontaneous	artificial, induced
no genetic manipulation	genetic or chemical engineering
many unknown factors	few defined factors
partial reprogramming into restricted progenitors	complete reprogramming into pluripotent stem cells
cancer resistance	cancer risk

Table 7.4. Comparison of de-differentiation processes: the two extremes.

Most complex, unassuming therapeutic strategy

In general, for patients with advanced cancers, complex, multi-pronged therapies may be needed that combine "conventional treatments" with immunologic strategies. Multi-pronged therapies might involve combinations of radio- and chemo-therapies, antibodies, activated antigen-specific anti-CSC immune cells, immune-stimulating agents, inhibitors of anti-apoptotic factor transcription, and/or many other potentially useful factors. These combination therapies will seek to target the entire spectrum of tumor cell phenotypes (in multiple ways) and immuno-suppressive microenvironments for problematic patients with advanced cancers (see Figure 4.1). Of course, physically removing tumor cells (via surgery or leukapheresis) can reduce immune suppression as well as reduce mechanical and/or hemodynamic problems caused by the presence of large numbers of malignant cells. Creating the patient-specific, anti-CSC immunologic components of multi-pronged strategies is a formidable challenge that may require extensive *in vitro* cell engineering that alters tumor cells and repairs, activates, and expands cancer-impaired immune cells. In the case of leukemias like AML, large numbers of tumor cells can be removed by leukapheresis which reduces the immuno-suppression induced by these malignant cells. Chemotherapies that induce ICD should be beneficial (Zitvogel, 2008; Hodge, 2013; Ma, 2013; Inoue, 2014) via immune stimulation. Regarding cell engineering, developing immune strategies that can eliminate relapse-causing LSCs may require manipulating a patient's cells in different ways during different phases of therapy-development. In the most problematic leukemia scenario, none of the available malignant cells will be primitive enough to adequately represent the lethal disease-sustaining LSCs, and specimens will not contain sufficient numbers of

normal HSCs. Because immune therapies with "immunological memory" must not destroy normal HSCs, LSCs and normal HSCs need to be compared and the status of each should be monitored when evaluating candidate therapies preclinically *in vitro*. These tasks require the ability to maintain pre-existing – or generate *de novo* – (malignant) CSCs and (normal) HSCs which may be the most challenging *in vitro* agendas. Fortunately, advances in cell engineering suggest this is feasible. A remarkable example is a process by which cancer-impaired T cells are functionally "rejuvenated" via reprogramming and then expanded (Vizcardo, 2013; Themeli, 2013). Also, antigen-presenting cells have been successfully repaired and loaded with tumor antigens so they can be used therapeutically (Smits, 2009; Kantoff, 2010). In general, antibodies can be designed once suitable tumor-specific antigens have been identified. As emphasized, recent technical advances suggest that even the most challenging tasks related to immunotherapy-development can eventually be completed.

Despite dramatic advances in the engineering of therapeutic immune cells, studies suggest immune therapies may be inadequate when primitive tumor cells have evolved into extremely death-resistant cells. In that case, systemic agents may need to be administered that target the specific pro-survival mechanisms that protect these highly evolved CSCs (Igney, 2002; Vanneman, 2012). The death-resistance of tumor cells may involve changes in cell-surface antigens or changes in the intracellular transcription of critical molecules. For example, some death-resistant melanoma cells express CTLA-4. Thus, systemic administration of anti-CTLA-4 antibodies may increase the susceptibility of these cancer cells to immunologic attack – as well as enhance the function of immune cells that express CTLA-4 (Laurent, 2013). Regarding the transcription problems inside

cancer cells that increase their death-resistance, systemic agents can reduce the intracellular transcription of those anti-apoptotic factors that block immune-mediated killing, especially the killing of highly evolved CSCs (Vanneman, 2012). Another option is to use systemic agents that increase tumor-cell transcription of proapoptotic factors or factors like RIG-I that promote immune-mediated killing of tumor cells. Fortunately, processes like oligonucleotide production are highly advanced and can be used to produce agents that can be administered systemically to modulate the transcription of tumor-cell factors like RIG-I as well as antiapoptotic and proapoptotic factors. Cancer research now includes the study of intracellular mechanisms and transcription, and such studies are now explaining the anti-cancer actions of some agents for the first time. For example, one herb-derived anti-cancer chemical has been found to modulate transcription of the anti-apoptotic factor XIAP. Some malignant cells are death-resistant via XIAP overexpression, and by down-regulating XIAP, this herb isolate was able to promote the apoptosis of human cancer cells (Fang, 2012). In general, new insights have been gained regarding how herb-derived chemicals improve anti-cancer immunity or reduce tumor-cell death-resistance via studies that assessed cytokine production, immune-cell development, or the transcription of antiapoptotic or proapoptotic factors (Cheng, 2010; Peng, 2013). Herb-derived chemicals that favorably modulate key transcription mechanisms in tumor cells are candidates for systemic use since they have been used safely for centuries. In addition to the targeting of immune and tumor cells, agents have been evaluated that can be administered systemically to counteract a variety of immuno-suppressive factors within the tumor microenvironment (van den Boorn, 2013).

Obviously, the expanding number of systemic options should promote the development of effective multi-pronged therapies.

Considering "worst-case scenarios" and the possible need for complex therapies helped guide this project. Current conceptual frameworks, past treatment failures, and the immunoediting and CSC theories identified potential therapy-development challenges in advance. For example, "working backwards" from a patient-specific therapeutic vision directed attention to the critical importance of maintaining and manipulating primitive tumor cells. CSCs available in specimens need to be maintained. If only mature cancer cells are available, they may need to be converted to CSC-like cells via chemical or genetic engineering or by *in vitro* use of immune-selection (Noh, 2012b) or chemo-therapeutic pressures. Presumably, partial reprogramming of tumor cells is always an option. Furthermore, some specific genetic-engineering tasks might be among the options: for example, Nanog transfection – in the case of some cancers – might render tumor cells more stem-like by inducing the same cell changes induced by immune-cell attack (Noh, 2012b). In general, complex *in vivo* processes that protect and maintain normal and malignant stem cells are being replicated *in vitro* via skillful use of new cell-engineering and cell-culture techniques. Learning how to generate and nurture the most lethal tumor cells may be necessary (so they can be studied) in order to find ways to kill them. Thus, factors and conditions considered "detrimental" because they sustain tumor cells *in vivo* are useful (for therapy-development) if they are also able to maintain cancer cells *in vitro*. For example, because "the unholy trinity" – STAT3, cytokines, and chronic inflammation (Li, 2011) – supports primitive tumor cells *in vivo*, these "unholy" factors may provide clues for how to maintain tumor cells effectively *in vitro*. Also, the same injury-

induced and inflammation-related mechanisms that activate the genes and signaling pathways that protect the (normal) primitive cells (needed for regeneration after injury) may also generate and protect stem-like tumor cells *in vitro*. Thus, tumor necrosis factor (TNF)-α and other pro-inflammatory factors within tumor microenvironments that protect melanoma (and other types of tumor cells) from immune-cell attack by inducing their de-differentiation (Landsberg, 2012) may prove to be useful for *in vitro* cell engineering.

In general, a cancer patients cells may need to be expanded in order to have adequate numbers of cells for *in vitro* studies and for the evaluation of candidate therapies preclinically. When engineered T cells, NK cells, and/or dendritic cells are to be infused as therapy, having sufficient numbers of these cells may require expanding these cell populations *in vitro* (Tran, 2014). In general, when considering multi-pronged immune therapies, both the technical challenges and therapeutic opportunities are numerous.

Future directions related to RepSox

Exploring how RepSox may affect immune-cell activation and Tim-3 expression

As emphasized, the actions of RepSox suggest this reprogramming tool might facilitate the engineering of patient-specific anti-cancer immune therapies. Questions to be answered are straightforward: How does RepSox affect Tim-3 expression on a cancer patient's non-malignant – but cancer-impaired – dendritic, NK, and T cells? Can reducing Tim-3 on AML cells improve immune-cell activation such as the spontaneous activation of γδ T cells via co-culture of these cells? If so, by virtue of immunologic cross-reactivity and the existence of multiple tumor-cell antigens, will immune cells activated against RepSox-treated AML cells (whose Tim-3 expression has been eliminated) also be reactive against (lyse) the original AML progenitors (that were not exposed to RepSox)?

Can RepSox help restore the function of cancer-impaired antigen-presenting and immune-effector cells?

How RepSox affects cancer-impaired immune cells, antigen presentation, and immune-cell activation warrants study for two reasons: (1) combined with other tools, RepSox can induce reprogramming (a process that can be used to restore the function of immune cells) and (2) used alone, RepSox can reduce expression of Tim-3 (a surface receptor that can disrupt the function of immune cells and inhibit immune-cell activation via distortion of immune synapses). As emphasized previously, the function of "exhausted" T cells from cancer patients can be restored ("rejuvenated") *in vitro* following reprogramming to pluripotency (Vizcardo, 2013; Themeli, 2013). Conceivably, combinations of RepSox and other chemicals could be used instead of genetic engineering to improve immune-effector-cell activity and antigen presentation just as combinations of RepSox and other chemical tools can replace genetic engineering when generating pluripotent stem cells (Hou, 2013). Perhaps "7-factor chemical reprogramming" (that fully de-differentiates normal cells) may provide guidance as to how RepSox and other chemicals can be used to alter a cancer patient's malignant cells and non-malignant immune cells in ways useful for therapy-development (Hou, 2013). It seems reasonable to conclude that since chemical engineering (Hou, 2013) and genetic engineering (Takahashi, 2007) have successfully de-differentiated (normal) cells to pluripotency, it should be possible to complete any desired *in vitro* cell-manipulation task. That is, since full reprogramming to pluripotency can be induced *in vitro*, what cell-engineering task is not feasible? To promote cancer therapy-development, RepSox might be combined with some of the same factors that are already being used by regeneration

researchers to manipulate normal cells (Feng, 2009). In general, new culture methods and research findings have identified a variety of potentially useful factors: the chemical reprogramming tools and "boosters" already being used with RepSox (Feng, 2009; Hou, 2013), physical conditions like hypoxia (Yoshida, 2009) and 3-D geometries, and cytokines and growth factors as well as a variety of support cells including adipocytes, osteoblasts, stromal cells, and even CAFs. Guidance is being provided by the steady flow of new advances. Although the possible combinations may seem overwhelming and unlimited, the opportunities are also numerous.

In this project, the effects of RepSox – and other reprogramming tools (Feng, 2009; Hou, 2013) – on antigen-presenting and immune-effector cells and on immune-cell activation were not studied, but warrant investigation. *In vitro* ways to measure immune-cell activation are reviewed in Table 7.5. When evaluating how well immune cells react against tumor cells *in vitro*, adding chemotherapeutic agents like anthracyclines that induce "immunogenic" tumor cell death (Zitvogel, 2008; Ma, 2013; Inoue, 2014) might indicate whether some "conventional" anti-cancer drugs can further activate the immune system and work synergistically with immune strategies.

Immune Cell Activity or Marker	Methods of Evaluation
Proliferation	3H-thymidine incorporation CFSE dilution
Cytokine production	ELISA ELISPOT assay Intracellular cytokine staining
Expression of activation markers	T cell activation markers: CD107a, OX40, 41-BB
Cytotoxicity	Immune-effector (NK or T) cell killing or lysis of tumor cells; measure tumor cell viability or their release of fluorescent dyes or radioactive substrates like ^{51}Chromium
Epitope specificity	Multimeric MHC-peptide complexes

Table 7.5. Measures of immune cell activation.

Regarding Tim-3, functionally exhausted Tim-3$^+$ immune cells can (eventually) appear in advanced malignant diseases and chronic viral infections. Impaired Tim-3-expressing immune cells are associated with many cancers including melanoma (da Silva, 2014), AML (Zhou, 2011), and follicular B-cell non-Hodgkin's lymphoma (Yang, 2012). Exhausted Tim-3$^+$ immune cells have been found in patients with chronic viral infections such as HIV (Jones, 2008; Finney, 2013), hepatitis B (Ju, 2010), and hepatitis C (Golden-Mason, 2009; McMahan, 2010; Vali, 2010). Of note, when expressed by endothelial cells isolated from human lymphoma tumors, Tim-3 was shown to suppress CD4$^+$ helper T-cell activation by stimulating the IL-6/STAT3 pathway (Huang, 2010). Mesenchymal stem cells expressing galectin-9 (a Tim-3 ligand) have been shown to suppress T-cell proliferation (Gieseke; 2013). In general, Tim-3 signaling within tumor microenvironments is believed to promote tumor progression by suppressing the activity of infiltrating immune cells. That is, the Tim-3 receptor is not simply a marker of impaired ("exhausted") immune cells, but directly suppresses their activity. Because Tim-3 expression is a conserved molecular defect on the immune cells of patients with a variety of cancers and chronic infections, the ability of RepSox to reduce Tim-3 receptor levels *in vitro* (and, thereby, potentially restore and activate dysfunctional immune cells) may help researchers engineer therapies against a variety of diseases.

Exploring how RepSox may maintain primitive (normal) hematopoietic stem cells

Chapter VI discusses mechanisms that might explain how RepSox slows decay of CD34$^+$ AML cells via its actions on c-Myc and CXCL12 (see Figure 7.2). Of note, CXCL12 is also known as "stromal cell-derived factor 1" (SDF-1). Considering the actions of RepSox on TGF-β signaling and *CXCL12*, RepSox might also help maintain primitive

(normal) hematopoietic stem cells in specially designed culture systems. For example, regarding the method developed by Khan, et al (Ali, 2014) – that is able to maintain and expand HSCs/HPCs *in vitro* – perhaps RepSox could replace use of "*hKirre* stromal-cell engineering" in their co-culture system. Why consider incorporating RepSox? RepSox seems potentially useful because Khan proposed that *hKirre* may promote the stemness and multipotency of umbilical cord blood cells (when co-cultured with stromal cells) via two *hKirre*-induced effects: promotion of CXCL12 and inhibition of TGF-β. Because RepSox can increase *CXCL12* (Larsen, 2013; Jajosky, 2014) and inhibit TGF-β signaling (Gellibert, 2004), RepSox might replace the *hKirre* manipulation of stromal cells in their co-culture system and, thereby, help maintain and expand HSCs/HPCs more easily.

Regarding RepSox-related mechanisms, exposing cells to RepSox *in vitro* might – via CXCL12 upregulation – inhibit the death and differentiation of stem-like cells (that occurs spontaneously *ex vivo*) by inhibiting the AhR pathway. Of note, hypoxic microenvironments can preferentially sustain stem-like cells *in vivo*. Perhaps RepSox (via CXCL12) can enhance hypoxia inducible factor (HIF) activity *in vitro*, and increased HIF activity, in turn, has been shown to inhibit the AhR pathway. Of note, CXCL12 is a "hypoxia-inducible gene," and the CXCL12 promoter contains hypoxia inducible factor-1α (HIF-1α) binding sites. Also, CXCL12 has been found to activate the PI3K/Akt pathway, and PI3K/Akt signaling contributes to activation of HIF-1α in the context of hypoxia. Research findings suggest CXCL12, HIF, and the AhR pathway are closely related.

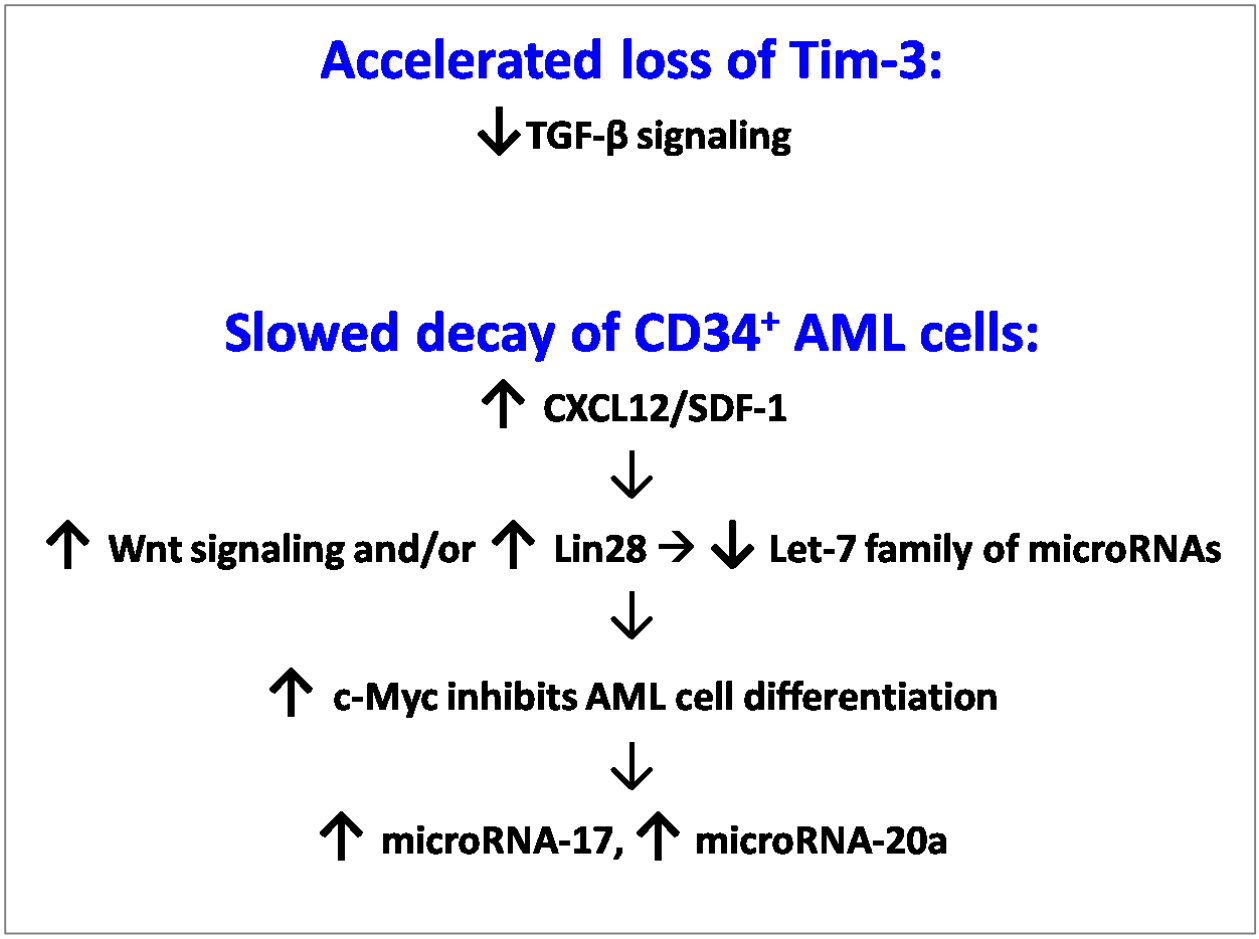

Figure 7.2: Potential mechanisms of RepSox action on AML cells.

Of note, scientists feel "the balance" of CXCL12 and TGF-β (or the "cross-talk" between these factors) controls a "cell-fate switch" that determines whether cells remain stem-like or differentiate. Researchers are interested in the mechanisms for cell-cycle regulation and self-renewal (how "the balance of cycling/quiescence" is controlled) because a more comprehensive understanding of these mechanisms will advance cell engineering (Lataillade, 2002; Chabanon, 2008). The existence of this 2-factor "cell-fate switch" is suggested by experimental observations (Chabanon, 2008) and may explain why RepSox is proving to be an especially useful and potent cell-engineering tool. Perhaps RepSox is unique: RepSox not only inhibits TGF-β, RepSox upregulates *CXCL12* in (normal) mouse embryonic fibroblasts (Larsen, 2013) and in (malignant) AML cells (Jajosky, 2014). Of note, in this AML study, other TGF-β inhibitors did not increase *CXCL12* (Jajosky, 2014). By increasing *CXCL12* mRNA levels, RepSox may be a unique TGF-β inhibitor that can serve as a useful, and convenient, "CXCL12 research tool" that might replace, in some situations, use of expensive recombinant CXCL12 (aka SDF-1).

Clarifying how RepSox affects CXCL12 and c-Myc

One specific, potentially informative RepSox-related study could assess the effects of exposing AML cells to a combination of RepSox and the CXCR4 antagonist "AMD3100" for less than 5 days. Of note, short-term exposure (less than 5-days) to AMD3100 may be critical clinically. A "5-day cut-off" for AMD3100-exposed leukemia cells was identified by Kim, et al who observed two very different net effects induced by AMD3100 *in vitro* depending on whether the duration of AMD3100 exposure was greater than 5-days or less than 5-days (Kim, 2011). Of note, AMD3100 can be used clinically to mobilize primitive (normal or leukemic) blood cells from the bone marrow into the peripheral blood

(Liles, 2003; Broxmeyer, 2005). AMD3100 reduces the ability of the CXCL12/CXCR4 axis to chemotactically retain primitive blood cells within the bone marrow where CXCL12-secreting stromal cells attract CXCR4-expressing cells. AMD3100 reduces CXCL12 binding to CXCR4 by competitively binding to the CXCR4 surface receptor (Donzella, 1998; Rosenkilde, 2004). Thus, AMD3100 reduces the effects of CXCL12 mediated by the CXCL12/CXCR4 axis and is considered a CXCR4 antagonist, although it has some partial agonist activity *in vitro* (Zhang, 2002). However, a second CXCL12 receptor, CXCR7, has recently been discovered, and AMD3100 also binds to this receptor – but at a separate allosteric site not used by CXCL12 (Kalatskaya, 2009). The net effect is that during the first 5-days of exposure, AMD3100 (an "antagonist") may actually enhance the CXCL12-related effects induced by RepSox because AMD3100 can independently (non-competitively) activate the CXCL12/CXCR7 axis. If activation of the CXCL12/CXCR7 axis (vs. the CXCL12/CXCR4 axis) is the key mechanism by which stem-cell maintenance and self-renewal are promoted, then AMD3100 might – during the first 5-days of combined exposure – enhance (instead of reduce) the ability of RepSox to maintain primitive AML cells. Of note, the ability of AMD3100 to potentially promote the maintenance and proliferation of primitive leukemia cells (via CXCL12/CXCR7 axis activation) is an issue – a warning – prudently raised by Kim et al because AMD3100 is used clinically to mobilize leukemia cells. They are concerned because AMD3100 has been assumed to be safe when viewed as a CXCL12 antagonist – before CXCR7, AMD3100-CXCR7 binding, and the actions of the CXCL12/CXCR7 axis had been identified (Kalatskaya, 2009). Based on their insights into CXCR7, Kim, et al have cautioned that use of AMD3100 could, conceivably, promote the maintenance or

proliferation of primitive myeloid leukemia cells (Kim, 2011). If the addition of AMD3100 were to further enhance RepSox-induced maintenance of $CD34^+$ AML cells (during the first 5 days of combined exposure), this would suggest that promoting CXCL12 in AML cells (or in both the stromal and AML cells) helps maintain primitive AML cells *in vitro* via the CXCL12/CXCR7 axis (since AMD3100 inhibits the CXCL12/CXCR4 axis). Of note, studies of normal endothelial cells suggest CXCL12 may maintain normal $CD34^+$ hematopoietic stem/progenitor cells via the CXCL12/CXCR7 axis (Torossian, 2014). Thus, RepSox may promote AML $CD34^+$ cell survival in part, or entirely, via the CXCL12/CXCR7 axis. The extent to which a patient's $CD34^+$ AML cells express CXCR4 and/or CXCR7 may determine how well RepSox slows the decay of these AML cells. Exposing AML cells to combinations of AMD3100 and RepSox could clarify this issue.

To explain how the effects of RepSox on AML cells may be related to c-Myc, the CXCL12/CXCR4 axis, or the CXCL12/CXCR7 axis, normal stromal cells (from a healthy donor) and an AML patient's leukemia cells and CAFs can be exposed to combinations of (1) RepSox and c-Myc inhibitors such as 10058-F4 (Huang, 2006) and (2) RepSox and CXCR4 and CXCR7 agonists and antagonists. Since these research tools are available, it should be possible for interested researchers to clarify, in detail, RepSox-induced effects that are due to changes in CXCL12 and/or c-Myc.

RepSox studies may also be able to clarify how CAFs support tumor cells. Of note, Kojima, et al identified CXCL12 and TGF-β as two factors critical for the evolution of stromal cells into tumor-promoting CAFs (Kojima, 2010): the same two factors believed to control the "cell-fate switch" (Chabanon, 2008). Activation of autocrine TGF-β and CXCL12 signaling promotes conversion of stromal cells into tumor-promoting CAFs

(Chabanon, 2008). Regarding fibroblasts from healthy animals (fibroblasts not exposed to cancer microenvironments), RepSox upregulates *CXCL12* in mouse embryonic fibroblasts (Larsen, 2013) and inhibits TGF-β signaling (Gellibert, 2004; Ichida, 2009). Thus, a potentially informative *in vitro* study might involve comparing how three different populations of stromal cells can support a patient's AML cells *in vitro*: (1) the cancer-altered CAFs obtained from the (same) AML patient, (2) normal stromal cells (from a healthy source) that had been exposed to RepSox, and (3) normal stromal cells that had not been exposed to RepSox. In general, the actions of TGF-β on stromal cells and cancer cells are complex and time-dependent. As noted by Caestecker, et al, during cancer progression, tumor cells often escape from the anti-proliferative effects of TGF-β through acquired mutations or dysregulation of the TGF-β signaling pathway (de Caestecker, 2000). Furthermore, Kojima, et al noted that some interactions between TGF-β and CAFs involve the CXCL12/CXCR4 axis: In stromal fibroblasts, TGF-β promotes TGF-β and CXCL12 autocrine signaling loops (by promoting TGF-β and CXCL12 production and inducing CXCR4 expression) which reciprocally cross-stimulate each other in a positive feedback manner to maintain the CAF phenotype (Kojima, 2010). Thus, experiments that compare how tumor cells are influenced by CAFs, normal stromal cells, and RepSox-treated stromal cells – and that skillfully exploit CXCR4 and CXCR7 antagonists, blocking antibodies, inhibitors, and other tools – may clarify how support cells promote the survival of CSCs. Learning how RepSox affects support cells may be as valuable as learning how RepSox can alter a cancer patient's tumor and immune cells. The evolution of support cells in cancer patients warrants study because

microenvironments protect and sustain the primitive tumor cells that trigger cancer relapses.

Investigating how RepSox might help generate anti-AML immune-cell therapies

Since RepSox maintains primitive AML cells, upregulates CXCL12, and decreases Tim-3, RepSox may promote the activation of immune cells by leukemia-cell antigens that could, under the proper circumstances, directly bind to immune-cell receptors. This seems feasible when the immunologic synapse between immune cells and AML cells is visualized in the context of prior research findings. One way to help readers visualize how RepSox may enhance the direct synapse between AML and immune cells involves the method developed by Gertner-Dardenne and colleagues (Gertner-Dardenne, 2012). In their co-culture system (which includes a "TCR agonist"), a patient's AML cells directly present antigens to immune cells, and this spontaneously activates the patient's γδ T cells against "tumor-associated" AML antigens (Gertner-Dardenne, 2012). This spontaneous activation of T cells triggered by the co-culture of AML and immune cells highlights the structure of the immunologic synapse. AML surface-antigens bind to (and activate) T-cell receptors like TCR and DNAM1 (Gertner-Dardenne, 2012). In the context of this AML-cell/immune-cell synapse, the actions of RepSox seem especially relevant and potentially useful. First, RepSox could promote the activation of immune cells against critically important stem-cell antigens by helping to maintain (*in vitro*) primitive $CD34^+$ AML cells that are more likely to display LSC antigens than more mature AML cells. For those immune cells expressing the CXCR4 receptor, RepSox could enhance the physical aggregation of AML cells and immune cells via upregulation of AML-cell CXCL12 (Smith, 2013). That is, RepSox might promote chemoattraction and increase the number of

immunologic synapses that form during co-culture of AML and immune cells. By decreasing Tim-3 expression, RepSox could reduce the distortion and dysfunction of the immunologic synapse that results when the Tim-3 receptor is present (Clayton, 2014).

Of note, it seems prudent to address a potential confusion that can emerge when Tim-3 is simply called an "LSC antigen." It is suggested here that Tim-3 on leukemia cells should, instead, be viewed as an "antigen on highly evolved LSCs." That is, LSCs that originally did not express Tim-3 may eventually display Tim-3 after leukemic disease has advanced and LSCs have evolved into more problematic and death-resistant LSCs. By simply referring to Tim-3 as an "LSC antigen," some AML cells that do not express Tim-3 might not be viewed as LSCs even though they are LSCs that have not yet evolved substantially and represent "early versions" of the original (disease-initiating) LSCs. It is assumed here that LSCs, like immune cells, evolve as malignant disease progresses, and that the more highly evolved LSCs can display Tim-3 (Zhou, 2011). Of note, the RepSox-treated AML cells whose Tim-3 expression was eliminated in this study still exhibited aldehyde dehydrogenase activity. If it is assumed that only Tim-3-expressing AML cells should be used to activate immune cells (by assuming only these AML cells are LSCs and only these cells express relevant stem-like tumor antigens), this could be counter-productive since Tim-3 can disrupt the immunologic synapse and, thereby, prevent *in vitro* immune-cell activation (Clayton, 2014).

Also for clarity, it is assumed here that the expression of AML cell-surface antigens is, in general, influenced by both the differentiation and evolution of AML cells. Noh and colleagues showed how cancer cells can change in response to "immune-selection

pressures" and that a range of tumor cells with different phenotypes – reflecting different stages of evolution – could engraft disease in mice (Noh, 2012).

Regarding Tim-3 on tumor cells, Tim-3 has been linked to advanced stages of cancer and poor patient prognosis (Zhuang, 2012). This suggests Tim-3 expression emerges via tumor-cell immunoediting (cell plasticity and evolution) as malignant disease progresses (Zhou, 2011). Tim-3 receptors, in turn, can disrupt the immunologic synapse (Clayton, 2014), and this may explain how the more highly evolved cancer cells are better able to evade immune-cell attack.

The RepSox-related research option suggested here assumes that critical tumor-associated antigens on stem-like RepSox-exposed AML cells (that do not display Tim-3 surface receptors) can activate immune cells against primitive relapse-causing AML cells because expression of some LSC antigens should be unrelated to Tim-3 expression. Thus, exposing AML cells to RepSox may enhance immune-cell activation (by reducing AML-cell Tim-3 expression) as well as the aggregation (chemoattraction) of AML and CXCR4-expressing immune cells (by upregulating CXCL12). Of note, it is generally agreed that (1) the immunologic synapse is disrupted by Tim-3 and (2) chemoattraction involving CXCR4-expressing cells is enhanced by CXCL12 (Clayton, 2014; Kumar, 2006; Braza, 2010). Thus, RepSox may simultaneously enhance immune-cell activation and increase the number of immunologic synapses in co-culture systems (Smith, 2013).

As a specific example, RepSox might help generate therapeutic immune cells when incorporated into the co-culture system developed by Gertner-Dardenne and colleagues (Gertner-Dardenne, 2012). When their method is used to co-culture a patient's AML and immune cells – which exploits the "TCR agonist" bromohydrin pyrophosphate

(BrHPP) – AML cells present AML-associated antigens to γ9δ2 T cells and spontaneously activate them (Gertner-Dardenne, 2012). F1F0-ATPase, PVR (CD155), and Nectin-2 (CD112) are some of the AML surface antigens thought to bind to, and activate, the γδTCR or DNAM1 (CD226) receptors on the T cells (Gertner-Dardenne, 2012; Wen-Li, 2012; Scotet, 2005). After expanding and infusing these activated γ9δ2 T cells, survival improved in an animal model of AML (Gertner-Dardenne, 2012). Especially encouraging, these activated T cells had "memory" features which, potentially, can very effectively prevent cancer relapses (Gertner-Dardenne, 2012).

In the study by Gertner-Dardenne and colleagues, the phenotypic profile of the AML blasts was not specified. Thus, it is not known whether the patient-derived AML cells used to present tumor antigens to the immune cells were mature or stem-like. It seems prudent to assume that the phenotype of the AML cells can affect T-cell activation. That is, it seems reasonable to expect that the specific AML cell antigens presented to immune cells (to activate them) will depend on how stem-like the AML cells are. Presumably, the more stem-like the AML cells are, the more likely the activated immune cells will be reactive against those stem-like AML cells (the "minimal residual disease" cells) that can trigger disease-relapse. Thus, it seems prudent to exploit methods that can isolate, generate, and/or maintain stem-like AML cells so LSC-like AML cells are used to present antigens when activating immune cells. By first helping to maintain primitive $CD34^+$ AML cells, RepSox should increase the likelihood that LSC antigens are presented to immune cells (by the RepSox-treated AML cells) when the immune cells are activated during co-culture. That is, RepSox might enhance the γ9δ2 T-cell activation method of Gertner-Dardenne by supplying LSC-like AML cells for co-culture with the patient's immune cells.

Remarkably, the γ9δ2 T cells activated by AML-cell/immune-cell co-culture have the potential, in turn, to present antigens to, and activate, αβ T cells (Brandes, 2009; Anderson, 2012). Exploring this technique for activating αβ T cells – as well as other ways to activate NK cells and other γδ T cells (not just γ9δ2 T cells) – seems especially appropriate for anti-AML therapy-development since AML cells can directly activate γ9δ2 T cells. If RepSox can enhance γ9δ2 T-cell activation, this may, in turn, facilitate the activation of other immune cells in subsequent phases of therapy-development.

Regarding molecular mechanisms, the "tumor-associated antigens" on AML cells thought to spontaneously activate γ9δ2 T cells include Nectin, PVR, and F1F0-ATPase. Of course, when AML cells spontaneously activate γ9δ2 T cells, other unknown AML cell-surface antigens may be involved. By eliminating synapse-disruption by Tim-3, perhaps RepSox can enable AML cells to more directly present AML antigens (like Nectin, PVR, and F1F0-ATPase) to receptors on immune cells.

Overall, it seems reasonable to investigate how RepSox might enhance the spontaneous activation of immune cells by AML cells in co-culture systems. Exploring diverse AML co-culture options might identify ways that a broad spectrum of αβ T cells, NK cells, and other γδ T cells (not just γ9δ2 T cells) can be activated against AML cells. As noted, just as AML cells serve as antigen-presenting cells to activate immune cells, γ9δ2 T cells activated by AML cells can, in turn, serve as antigen-presenting cells that can activate αβ T cells (Deniger, 2013). Perhaps combinations of activated γ9δ2 T cells, AML cells, and chemical factors can be found that are able to activate a broad range of immune cells for therapeutic purposes (a spectrum of NK, γδ T cells, and αβ T cells) without, for example, using genetic engineering to insert chimeric antigen receptors

(CARs). If genetic engineering cannot be avoided, it is reassuring that Deniger and colleagues have successfully activated polyclonal γδ T cells with broad anti-tumor capability using CAR technology and "artificial" antigen-presenting cells (Deniger, 2013). However, in view of the great diversity of AML cells among patients, co-culture methods that can exploit spontaneous immune-cell activation may eliminate the need to identify, in advance, specific AML "tumor-associated" surface-antigens – a task required when, for example, deciding what CAR insertions are to be genetically engineered.

To summarize this research option, the unique actions of RepSox might help generate anti-AML γ9δ2 T-cell therapies by, first, maintaining LSC-like AML cells that could be used to present stem-like AML antigens in co-culture systems that activate a patient's γ9δ2 T cells. Then, either simultaneously or in a separate *in vitro* process, RepSox might improve γ9δ2 T-cell activation when a patient's immune and (LSC-like) AML cells are co-cultured. That is, since RepSox maintains primitive $CD34^+$ AML cells (that may display LSC-like tumor antigens), reduces Tim-3 expression (which disrupts immunologic synapses), and upregulates CXCL12 (that may increase chemoattraction and the number of immunologic synapses), RepSox may promote the co-culture activation of γ9δ2 T cells against those primitive AML cells that trigger AML relapse. RepSox may be especially useful in advanced AML disease when LSCs have evolved to express Tim-3 cell-surface receptors which, in turn, disrupt immunologic synapses and inhibit immune-cell activation.

Future directions not involving RepSox

Students should enjoy and embrace the improvements in conceptual frameworks for cancer, immunology, and tissue regeneration as well as the steady clarification of molecular mechanisms and signaling pathways. This progress is inspiring innovative therapeutic strategies which, in turn, are identifying key technical challenges and exciting research options. Immunology and regeneration research are directing attention to new tools and methods that can be used to engineer immunotherapies *in vitro*.

"7-factor" (chemical) reprogramming to pluripotency directs attention to the six other reprogramming factors that were used with RepSox (Hou, 2013). Perhaps these molecules can alter tumor-cell survival, differentiation status, immunogenicity, or other cellular features in ways useful for therapy-development. Like RepSox, three other chemicals were described as being especially useful: CHIR99021 (a glycogen synthase kinase 3 [GSK3-β] inhibitor), Forskolin (a cAMP agonist), and 3-deazaneplanocin A ("DZNep"; an S-adenosyl-homocysteine hydrolase inhibitor) (Hou, 2013). Perhaps combining one or more of these tools with RepSox would enhance the ability of RepSox to maintain the survival of primary $CD34^+$ AML cells. The remaining three factors included valproic acid (a histone deacetylase [HDAC] inhibitor), tranylcypromine (a monoamine oxidase inhibitor), and "2i" treatment involving dual inhibition of mitogen-activated protein kinase signaling and GSK3-β. "Reprogramming boosters" were also identified including D4476 (an inhibitor of casein kinase I), 2-methyl-5-hydroxytryptamine (closely related to the neurotransmitter serotonin), basic fibroblast growth factor, prostaglandin E2, SRT1720 (a sirtuin 1 activator), and sodium butyrate (a HDAC inhibitor) (Hou; 2013). In general, because these agents and other molecular tools (Feng, 2009) promote cellular

reprogramming (like RepSox), they may also alter the tumor and immune cells of AML patients in ways that can facilitate therapy-development.

Perhaps co-culturing a leukemia patient's tumor cells with support cells such as adipocytes, osteoblasts, stromal cells, or the patient's own CAFs would be helpful – or use of a 3-D cell-culture system. A trial-and-error approach may be unavoidable when trying to identify combinations of factors that might better maintain or alter the cells of AML patients. However, insights into signaling pathways and molecular mechanisms may predict worthwhile combinations. Regarding the activation of immune cells by tumor cells *in vitro*, adding chemotherapeutic agents like anthracyclines that induce immunogenic tumor-cell death (Inoue, 2014) may demonstrate whether or not some "conventional drugs" can enhance immune responses and work synergistically with immunologic strategies. In general, promising research options are unlimited due to the dramatic advances generated by cancer and regeneration research. When evaluating cell-engineering tools, effects will depend on the concentrations and combinations of factors and whether they are used simultaneously or sequentially. The steady discovery of new and exciting tools means setting priorities will be challenging. The ability to more predictably alter tumor and immune cells is increasing as evidenced by the impressive, rational design of "7-factor chemical de-differentiation" (Hou, 2013) along with reprogramming efficiencies near 100% (Rais, 2013). Because mature cells have been chemically reprogrammed to pluripotency (Hou, 2013), any desired *in vitro* cell manipulation now seems feasible. Still, despite dramatic advances, a basic challenge is likely to persist for years: the development of simple techniques that can maintain – or generate as needed – a patient's CSCs *in vitro* for therapy-development purposes.

Practical considerations for students

In addition to describing specific ways RepSox might be used for *in vitro* cell engineering, therapy-development, and the clarification of mechanisms, this dissertation seeks to provide a useful overview. Although interactions between cancer cells and the immune system are numerous, complex, and even intimidating, students should appreciate how each newly identified mechanism provides an opportunity for intervention and therapeutic synergy.

When planning to engineer anti-cancer immune therapies *in vitro*, it seems worthwhile to consider those *in vivo* mechanisms that very effectively support tumor cells. In general, *in vivo* cancer microenvironments promote the maintenance and proliferation of CSCs while inhibiting the activation of immune cells. In contrast, *in vitro* conditions are "the reverse" in that it is difficult to maintain stem-like cancer cells *ex vivo* but effective immune-cell activation is possible in the absence of the immunologic restraints that exist *in vivo*. Understanding these differences can provide guidance for generating and/or maintaining (*in vitro*) the stem-like cancer cells that can serve as relevant "target cells" when designing antibody or immune-cell therapies.

Considering conceptual frameworks – like the CSC and immunoediting theories – is also worthwhile. They explain why the immune-evasive and immune-suppressing CSCs that trigger relapse must be eliminated (as well as the more mature and prevalent "bulk" tumor cells) and that, because CSCs evolve, the sooner the better. To cure some patients, their own immune cells may need to be activated against CSC antigens and administered along with other personalized and conventional therapeutic strategies such as antibodies, immuno-modulating agents, surgery, chemotherapies, etc.

Another worthwhile exercise is visualizing how the immunologic synapse is involved in immune-cell activation. When a cancer is highly advanced and a patient's specimens lack CSCs and immune cells that are functional ("reactive"), generating immune cells *in vitro* that are reactive against CSC antigens may be difficult. In this problematic scenario, it can be instructive to visualize how RepSox might enhance immunologic synapses in the specific context of co-culture systems that use a patient's AML cells to activate γ9δ2 T cells. The actions of RepSox suggest RepSox could facilitate *in vitro* activation of γ9δ2 cells against AML LSCs by enhancing (1) chemoattraction, (2) presentation of LSC antigens, and/or (3) activation of immune cells (by eliminating the adverse influence of Tim-3). That is, RepSox might enhance immunologic-synapse formation between AML and γδ T cells. RepSox seems especially useful because AML cells directly present antigens to, and activate, γδ T cells. Thus, because RepSox (alone or in combination with other culture conditions and factors) may help maintain, or generate, primitive AML cells, RepSox may, thereby, help supply the primitive AML cells that can present (to immune cells) the LSC antigens that need to be targeted to prevent relapse. That is, by helping to provide LSC-like AML cells, RepSox may help activate immune cells so they are reactive against relapse-causing AML cells. During co-culture, RepSox may promote immunologic synapse formation and immune-cell activation by upregulating CXCL12 and/or by decreasing Tim-3 expression. Perhaps RepSox may enhance immune-cell activation against LSC antigens when incorporated into the co-culture method developed by Gertner-Dardenne, et al (Gertner-Dardenne, 2012).

In addition to considering ways to improve the activation of T cells by AML cells, students should consider other challenges. It seems prudent to anticipate "worst-case

scenarios" involving (1) tumor cells that are highly evolved and immune-resistant, (2) antigen-presenting and immune-effector cells that are markedly dysfunctional, and (3) specimens that may not contain adequate numbers of HSCs or primitive tumor cells. Fortunately, many new methods are becoming available, but students may still need to develop their own special techniques to accommodate the novel therapies they envision.

In the case of leukemia, for example, students may need to consider how they can best:

1. Obtain useful patient specimens that, ideally, contain the cells needed to engineer immunotherapies: primitive tumor cells to be targeted, normal HSCs to be spared, and dysfunctional antigen-presenting and immune-effector cells to be repaired. Leukapheresis specimens, obtained from patients with high white blood cell counts, contain billions of cells that are often discarded as waste. Aldefluor staining is a simple technique that distinguishes normal ($ALDH^{bright}$) from AML ($ALDH^{intermediate}$) stem/progenitor cells based on their unique staining patterns (Pearce, 2005; Gerber, 2012).

2. Maintain and manipulate tumor, antigen-presenting, and immune-effector cells as well as isolate normal HSCs for comparison purposes. The bone marrow stromal cells that have evolved in a cancer patient (CAFs) may help to maintain that patient's tumor and immune cells *in vitro*.

3. Develop co-culture methods that use primitive leukemia cells to activate immune cells so these immune cells are reactive against the stem-like leukemia cells that can trigger disease-relapse.

4. Identify those anti-apoptotic factors in a patient's primitive tumor cells that inhibit tumor-cell killing when a candidate immune-cell therapy is tested *in vitro*.

5. Identify therapeutic agents (such as small molecules or oligonucleotides) that could safely be administered to patients to inhibit transcription of problematic anti-apoptotic factors (or promote transcription of apoptotic factors) within tumor cells without harming normal HSCs.

6. Repair, activate, and/or expand cancer-impaired antigen-presenting and/or immune-effector cells (by, for example) reprogramming these cells, altering cell-surface antigens, enhancing tumor-antigen presentation, co-culturing immune and cancer cells with “TCR agonists” or via other strategies reviewed in **Chapter IV**.

7. Compare leukemia cells and normal HSCs to identify tumor-specific (or LSC-specific) cell-surface antigens or even notable intracellular differences.

8. Determine if some immune cells in a patient's specimen already react against tumor antigens. If so, select, or develop, a way to expand these cells. If reactive immune cells are not available, engineer (repair, activate, and expand) immune cells to be reactive against a suitable tumor-specific antigen.

9. Modulate tumor cells so they are more immunogenic or, more generally, improve immunologic-synapse formation *in vitro* to promote antigen-presentation and the activation of immune cells.

10. Identify how tumor cells impair immune-cell activation and acquire death-resistant phenotypes when attacked by immune cells or antibodies.

11. Render tumor cells more stem-like *in vitro*, if needed, so they can serve as suitable *in vitro* therapeutic targets (by resembling the disease-sustaining LSCs that reside *in vivo*). This conversion into stem-like tumor cells may require chemical or genetic engineering or exposure to stresses involving serial rounds of immune-selection or chemotherapeutic "pressure."

12. Design multi-pronged immune therapies: combinations of antibodies, activated immune cells, agents that render tumor cells less death-resistant, factors that enhance immune responses (such as cytokines like IL-2), cytotoxic agents that induce immunogenic tumor cell death, and/or agents that mitigate the adverse impact of cancer-supporting microenvironmental cells and molecular factors that emerge as malignant disease progresses.

13. Evaluate candidate immune therapies pre-clinically *in vitro.*

Finally, students may find it instructive – and motivating – to consider how some therapeutic options that have already been developed might be combined to create rational, multi-pronged anti-cancer therapies. As noted previously, anti-cancer options are diverse and range from traditional *in vivo* tasks (the physical removal of malignant cells or systemic modulation of tumor cells, support cells, and the immune system) to new, more technically complex *in vitro* tasks (such as the skillful engineering of a patient's immune cells). For a blood cancer like AML, bulk tumor-cell removal is accomplished by leukapheresis which reduces the immune suppression induced by malignant cells. "Conventional chemotherapeutic drugs" that induce ICD (cytarabine, daunorubicin,

mitomycin, etc.) not only kill cancer cells, but also enhance anti-cancer immune responses and promote tumor-cell apoptosis (Fredly, 2011). Cytokines like IL-2 can be administered systemically to enhance immunologic responses, and the impact of IL-2 may be enhanced by histamine dihydrochloride (Romero, 2009). Regarding immune-cell strategies, a patient's γ9δ2 T cells can be directly activated against a patient's stem-like AML cells via a co-culture system that exploits a TCR agonist (Gertner-Dardenne, 2012) – an *in vitro* process that might be enhanced by RepSox. After expansion, these T cells could serve as the anti-LSC component of a multi-pronged therapy and provide long-term protection by functioning as "memory effector" immune cells. Ideally, NK cells, DCs and a broad spectrum of γδ and αβ T cells would be exploited. Regarding the death-resistance of stem-like cancer cells, primitive tumor cells might be rendered more susceptible to immune-cell attack by administering systemic agents that reduce the levels of key anti-apoptotic factors. For example, the antisense oligonucleotide "AEG35156" has been shown to reduce the levels of anti-apoptotic factor XIAP and, thereby, promote apoptosis of AML stem cells (Carter, 2011). Regarding systemic immune suppression induced by T_{regs}, IL-2 diphtheria toxin fusion protein has promoted clearance of AML cells via depletion of T_{regs} (Bachanova, 2014). Regarding MDSCs, the agent "sunitinib," a receptor kinase inhibitor, has, in some cancer patients, enhanced the effectiveness of immune-based strategies and reversed immune suppression by reducing the numbers of MDSCs as well as T_{regs} (Ozao-Choy, 2009). An anti-CTLA-4 antibody (MDX-010) improved the ability of AML-derived DCs to activate T cells against AML cells in an autologous culture system (Zhong, 2006). Envisioning such options illustrates how the complexity of malignant disease provides many opportunities for intervention.

Summary

The interactions between cancer cells and the immune system are complex and fascinating. Remarkably, valuable insights into cancer and immunology have been prompted by very diverse and unusual observations. For example, infections, chemical warfare agents, and limb regeneration in salamanders have suggested that immune stimulation, immunogenic tumor-cell death, and cellular de-differentiation are biological phenomena worth considering when treating cancer patients. Perhaps some will find it counter-intuitive that the non-malignant cells of cancer patients are attracting intense attention: current therapeutic cell-engineering agendas involve (non-malignant) antigen-presenting and immune-effector cells, and researchers are also identifying the critical roles played by CAFs and other micorenvironmental factors that so effectively support malignant cells. Immune-cell therapies can be created *in vitro* using genetic engineering techniques (like CAR insertion) or co-culture methods that promote the spontaneous activation of immune cells by cancer cells. Multi-pronged therapies may be required that can simultaneously exploit diverse ways to promote the killing of tumor cells, enhance the immune system, or reverse the cancer-promoting features of cancer microenvironments.

More than 100 years after spontaneous tumor regressions were linked to concomitant infections, cancer researchers are now rationally engineering therapies that are predictably saving the lives of cancer patients (Kalos, 2011; Porter, 2011). Successful therapies include immune-based components (passively administered or actively induced) that eliminates those problematic, primitive cancer cells that can trigger relapse. Despite the complexity of malignant diseases, this is an historic era of cancer cures. Fantastic advances in cell engineering have facilitated the development of life-saving

therapies. Given this historic context, students should feel fortunate. Students should confidently envision innovative therapies, eagerly confront technical challenges, and creatively exploit new tools and methods.

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Zitvogel, L., Tesniere, A., & Kroemer, G. (2006). Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nature Reviews Immunology*, *6*(10), 715-727.

CURRICULUM VITAE

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EDUCATION

West Virginia University (WVU), Morgantown, WV
Ph.D. in Cancer Cell Biology, 2014
MD (pending)

Worcester Polytechnic Institute (WPI), Worcester, MA
B.S. in Biology and Biotechnology with High Distinction, 2007
Concentration in Molecular Biology and Genetics
Minor in Biochemistry

RESEARCH EXPERIENCE

Doctoral Dissertation:

"An Overiew of Acute Myeloid Leukemia and Immunology: (i) Concepts and Therapeutic Strategies and (ii) RepSox as a Candidate Cell-Engineering Tool"
Advisor: Laura F. Gibson, Ph.D., WVU

Undergraduate Projects:

- Sufficiency: "Humor as Medicine"
  Advisor: Daniel G. Gibson, Ph.D., WPI

- Interactive Qualifying Project: "GE-NBC TV Humor-in-Healthcare Initiative"
  Advisor: Lorraine D. Higgins, Ph.D., WPI

- Major Qualifying Project: "Minimal Requirements for Assembly of a Stable Inner Membrane Platform for *Vibrio cholerae* Pilus Biogenesis"
  Advisors: Ronald Taylor, Ph.D., Dartmouth Medical School and Jill Rulfs, Ph.D., WPI

Summer Experiences:

Carnegie Mellon University Scholar, Pittsburgh, PA	2002
Volunteer Breast Cancer Research Assistant in WPI Biology Department	2003
Duke University Biomedical Research Institute, Durham, NC	2004
Medical Enrichment Program at Medical College of Georgia, Augusta, GA	2005
Microbiology/Genetics Research, Dartmouth Medical School, Hanover, NH	2006
MD/PhD rotation in cancer lab of Steven Frisch, Ph.D. Morgantown, WV	2007
MD/PhD rotation in cardiology lab of Mitchell Finkel, MD, Morgantown, WV	2008

WORK

Resident Advisor for students living on WPI campus	2004 -2007
Peer Writing Tutor	2004 - 2007
EMSEP Math & Science Tutor	2005 - 2006

LEADERSHIP

Panelist for NIH Fellowship workshop for WVU graduate students	2011
Host of workshop on how to write an NIH Research Proposal	2010
Treasurer of Phi Sigma (National Biology Honor Society)	2005 - 2007
Member of EMSEP Advisory Board	2004 - 2007

PROFESSIONAL ASSOCIATIONS

American Physician Scientist Association, WVU Chapter	2008 - present
The American Association for Cancer Research	2010 - 2011
The Leadership Alliance	2006 - 2007
Women's Industry Network	2005 - 2007

FELLOWSHIPS & AWARDS

Ruth L. Kirschstein Pre-Doctoral National Research Service Award 2011 - 2014
National Cancer Institute, National Institute of Health

1st-place Poster at Van Liere & WVU Health Sciences Center Research Day	2014
National Cancer Institute Supplemental Funding Award	2010 - 2011
Top-4 Finalist at WV state-wide STaR Science Symposium	2010
"High Distinction" Graduate of WPI	2007
WPI "Medical Professions Scholar"	2003 - 2007
WPI "Excellence in Math, Science, and Engineering" Scholar	2003 - 2007
WPI Student "Woman-of-the-Year" (Ellen Knott Award)	2004

PRESENTATIONS & POSTERS

"Blood cell-spheres: can they help us develop better leukemia therapies?" Presentation, 1st annual Osborn Hematological Malignancy Retreat, Morgantown, WV, August 2010.

Jajosky, A. N., Craig, M. D., Martin, K. H., Zhang, L., and Gibson, L. F. Blood cell-spheres: growth, description, and therapeutic potential. Poster. National MRS-AACR Joint Conference on Metastasis and the Tumor Microenvironment, Philadelphia, PA, September 2010.

Jajosky, A. N., Craig, M. D., Martin, K. H., Zhang, L., and Gibson, L. F. Growing normal and leukemia blood cell-spheres: the quest for stem cells and effective leukemia treatments. Poster, Science, Technology, and Research (STaR) Symposium, Huntington, WV, September 2010.

Jajosky, A. N., Craig, M. D., Martin, K. H., Zhang, L., and Gibson, L. F. Growing normal and leukemia blood cell-spheres: the quest for stem cells and effective leukemia treatments. Poster, 14th Annual Meeting of the Translational Research Cancer Centers Consortium, Seven Springs, PA, February 2011.

Jajosky, A. N., Craig, M. D., Martin, K. H., Zhang, L., and Gibson, L. F. Growing normal and leukemia blood cell-spheres: the quest for stem cells and effective leukemia treatments. Poster, E.J. Van Liere Memorial Convocation and Health Sciences Research Day, Morgantown, WV, March 2011.

"Can we de-differentiate leukemia cells into the stem/progenitor cells we want to target therapeutically?" Presentation, 2nd annual Osborn Hematological Malignancy Retreat, Morgantown, WV, August 2011.

Jajosky, A. N. and Gibson, L. F. The Tgf-β inhibitor "RepSox" slows the decay of leukemic progenitors *in vitro*. Presentation and poster, 15th Annual Meeting of the Translational Research Cancer Centers Consortium, Seven Springs, PA, February 2012.

Jajosky, A. N. and Gibson, L. F. A way to isolate leukemic progenitors? Poster, E.J. Van Liere Memorial Convocation and Health Sciences Research Day, Morgantown, WV, March 2012.

"Evaluating *in vitro* environments that may facilitate the engineering of leukemic stem cell-targeted immunotherapies." Presentation, 3rd annual Osborn Hematological Malignancy Retreat, Morgantown, WV, August 2012.

Jajosky, A. N., Coad, J. E., Vos, J. A., Martin, K. H., Senft, J. R., Wenger, S. L., and Gibson, L. F. Potential utility of RepSox, a cellular reprogramming tool, for engineering "leukemic stem cell"-targeted immunotherapies *in vitro*. Poster. E.J. Van Liere Memorial Convocation and Health Sciences Research Day, Morgantown, WV, February 2014.

PUBLICATIONS

Jajosky, A. N., Coad, J. E., Vos, J. A., Martin, K. H., Senft, J. R., Wenger, S. L., & Gibson, L. F. (2014). RepSox slows decay of $CD34^{+}$ acute myeloid leukemia cells and decreases T cell immunoglobulin mucin-3 expression. *Stem Cells Translational Medicine*, *3*(7), 836-848.

Lewis, J. W., Talkington, W. J., Walker, N. A., Spirou, G. A., **Jajosky, A.**, Frum, C., and Brefczynski-Lewis, J. A. (2009). Human cortical organization for processing vocalizations indicates representation of harmonic structure as a signal attribute. *The Journal of Neuroscience*, *29*(7), 2283–2296.