



Harnessing the Tumor Microenvironment for the Treatment of Double Hit Lymphoma

Citation

Lossos, Chen. 2019. Harnessing the Tumor Microenvironment for the Treatment of Double Hit Lymphoma. Doctoral dissertation, Harvard University, Graduate School of Arts & Sciences.

Link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:42029642>

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

<https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFIOWI2M2EwYTg>

Accessibility

<https://accessibility.huit.harvard.edu/digital-accessibility-policy>

Share Your Story

The Harvard community has made this article openly available.

Please share how this access benefits you. [Submit a story](#)

Harnessing the Tumor Microenvironment for the Treatment of Double Hit Lymphoma

A dissertation presented

by

Chen Lossos

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

Harvard University

Cambridge, Massachusetts

March 2019

© 2019 Chen Lossos

All rights reserved.

Harnessing the Tumor Microenvironment for the Treatment of Double Hit Lymphoma

Since their inception into clinical practice, antibodies have become a mainstay in the treatment of cancer and other diseases. Antibodies eliminate tumor cells through several mechanisms, including inducing tumor cell death upon binding or immune-mediated killing. Cancer resistance to antibody therapy remains an important therapeutic challenge. Herein we utilized patient-derived xenograft (PDXs) of Double Hit Lymphoma (DHL), which is frequently resistant or refractory to antibody containing chemotherapy regimens, to study mechanisms of acquired lymphoma antibody resistance and ways to overcome them. We demonstrate progressive loss of the ability of alemtuzumab, a monoclonal antibody targeting the CD52 antigen, to clear bone marrow disease in DHL engrafted mice and identify the lymphoma to macrophage ratio as a driver of acquired antibody resistance. Alemtuzumab resistance was successfully overcome by high-doses therapy with alkylating agents like cyclophosphamide (CTX), which demonstrated >80-fold synergy with alemtuzumab to clear bone marrow disease. CTX administration induced a pro-phagocytic phenotypic of DHL cells by decreasing surface expression of CD47, thereby promoting their uptake by macrophages. CTX also induced paracrine secretion of VEGF-A, IL-16 and TNF- α that promoted bone marrow macrophage influx and increased macrophage phagocytosis. Many of these DHL phenotypic changes were caused by CTX-mediated induction of ER stress. Macrophages from lymphoma engrafted CTX-treated mice displayed an increased ability to phagocytose lymphoma cells, including a subset defined by surface CD36/Fc γ R4 that were “super-phagocytic.” Together, these findings

demonstrate a unique mechanism of macrophage dependent tumor clearance by high-dose alkylating agents.

Table of Contents

Acknowledgements	vii
Chapter 1: Introduction.....	1
Non-Hodgkin's Lymphoma and Diffuse Large B-cell Lymphomas	2
Double-Hit Lymphoma	4
Tumor Associated Macrophages.....	10
Monoclonal antibodies in cancer treatment and cancer resistance	11
Cyclophosphamide.....	14
Immunogenic cell death	16
ER Stress.....	17
Bone marrow alemtuzumab resistance in humaneered DHL is overcome by cyclophosphamide.....	20
Chapter 2: DHL bone marrow antibody resistance can be overcome by high-dose alkylating agent therapy	23
Background	24
Results.....	24
Chapter 3: Bone marrow alemtuzumab resistance is primarily driven by a high lymphoma to macrophage ratio	34
Background	35
Results.....	35
Chapter 4: Cyclophosphamide Induces Lymphoma Phenotypic Changes and a Secretory Phenotype to Promote Tumor Clearance by Macrophages	46
Background	47
Results.....	48
Chapter 5: Alkylating Agents Induced Secretory and Phenotypic Changes are Partially Due to ER stress	66
Background	67
Results.....	67
Chapter 6: Cyclophosphamide Treatment in the Presence of DHL Promotes "Super-Phagocytic" Macrophages.....	74
Background	75
Results.....	75
Chapter 7: Discussion.....	86
The Bone Marrow as a Therapy Resistant Microenvironment.....	87
Alkylating Agents overcome Antibody Resistance	91
Alkylating Agent Induced ER Stress.....	96
Alkylating Agent Macrophage Reprogramming in Lymphoma Bearing Mice	99
Summary	103
Chapter 8: Experimental Methods	104
Mouse Models and Therapeutics	105
Bone Marrow-derived Macrophage (BMDM) Generation	106
Cell Culture	106
Phagocytosis Assays (BMDM and In vivo derived Macrophages).....	106

Immunoblotting.....	108
Chromatin Immunoprecipitation and quantitative PCR (ChIP-qPCR)	109
RNA isolation and RNA-Sequencing	110
Cytokine Arrays	110
Flow cytometry.....	111
ELISA	111
Conditioned Media Generation	112
BH3 Profiling.....	112
Single-Cell Whole Transcriptome Amplification.....	113
Preparation of cDNA Libraries for RNA-seq	113
Single-Cell RNA-Seq Expression Quantification.....	114
Single-Cell Filtering and Gene Filtering.....	114
PCA and clustering	114
Differential Expression and Pathway analysis	115
scRNA-Seq analysis Statistics.....	115
XBP-1 Splicing Assay.....	116
Microscopy Phagocytosis Study.....	116
pSYK Phosphorylation Assay	117
Clodronate.....	117
Immunohistochemistry	117
BCL-2 and c-MYC FISH Analysis	119
Statistics	119
Appendix	105
Appendix: Genes comprising single cell macrophage clusters	120
References	122

Acknowledgements

Graduate school is supposed to be difficult but it's hard to fathom how difficult it is until you dive in and experience that first failed experiment. I couldn't have made it through without the support network that I've had. Dad, mom and my sister Shira- your love means more to me than you can ever know. Our constant conversations kept my mood up and allowed me to persevere through 100 mouse take-downs, all night experiments and lots of trials. No words can express how much I love you. Thank you to Iris Trutzer for constant encouragement during the writing process and in life. I love you. A special thank you to my mentor Dr. David Weinstock, who offered constant input, was available for comments and discussion at any time of day and night and who always kept it real. You have made me a better scientist and a better person. To my thesis committee members – Drs. Anthony Letai, Roberto Chiarle and Michael Hemann – who offered thoughtful insight and were available to talk whenever I needed throughout the graduate school process. To my friends and classmates who were there to listen when I vented and to offer support and help in science and life. A special thank you to all the collaborators who have helped with this endeavor- it would not have been possible to complete this otherwise. Your technical expertise is second to none. Lastly a special thank you to the Harvard BBS program for allowing me to pursue my PhD studies- I couldn't think of anything more I would want in a PhD program.

Chapter 1: Introduction

Non-Hodgkin's Lymphoma and Diffuse Large B-cell Lymphomas

Non-Hodgkin's Lymphomas (NHLs) are the sixth most common cancer in the United States, accounting for 4.3% of all new cancer cases with over 70,000 annual diagnoses (Noone et al., 2015). NHLs are highly heterogeneous, with over sixty recognized subsets based on clinical presentation, immunopathology, putative cell of origin, gene expression and mutation patterns (Al-Hamadani et al., 2015). Despite marked improvements in the treatment of NHL in recent years, nearly 20,000 patients are expected to succumb to their disease in 2019, suggesting that identifying patients with poor prognosis and targeting them with novel treatment strategies are pressing clinical needs (Noone et al., 2015).

Diffuse Large B-cell Lymphoma (DLBCL) is the most common subtype of NHL, accounting for ~30-40% of newly diagnosed NHL cases (Grimm and O'Malley, 2019; Li et al., 2018). These lymphomas can present in all anatomical sites in the body, including in immune privileged locations such as the central nervous system and testes (Martelli et al., 2013); accordingly, clinical presentation frequently varies based on the involved disease site. Common presentations include i) enlarged lymph nodes, ii) symptoms related to involvement of extranodal sites, iii) fevers, weight loss and night sweats, so called "B-symptoms"; and iv) elevated blood lactose dehydrogenase (LDH) and hypercalcemia (Martelli et al., 2013). Age, elevated serum LDH levels, involvement of multiple extranodal sites (e.g. bone marrow), the Eastern Cooperative Oncology Group (ECOG) performance status and the stage of disease presentation (specifically Ann Arbor stage III or IV) together define the International Prognostic Index (IPI) of DLBCL patients (Jelicic et al., 2019). High IPI scores are associated with inferior survival to the DLBCL first-line treatment R-CHOP, consisting of rituximab (R), cyclophosphamide (C), hydroxydaunorubicin (doxorubicin, H), oncovin (vincristine, O) and prednisone (P) (Ziepert et

al., 2010). DLBCL patients frequently respond well to R-CHOP, with the five-year overall survival (OS) around 60%, although the clinical course of DLBCL patients is highly variable (Friedberg and Fisher, 2008).

Within DLBCL, several lymphoma subtypes have been characterized based on gene expression and mutation profiling. The first large scale and to this day most commonly used gene expression profiling of DLBCLs stratified patients into two major classes- Germinal center-derived DLBCL (GC-DLBCL, 50-60% of cases) and activated B-cell DLBCL (ABC-DLBCL, 30-40% of cases). These are based on similar expression patterns to normal cell counterparts – germinal center lymphocytes for GC-DLBCL and activated B-cells for ABC-DLBCL, respectively, with the remaining 10% of cases being “unclassifiable” (Alizadeh et al., 2000). Importantly, these distinctions have prognostic implications for patients; specifically, the 5-year OS of GC-DLBCL and ABC-DLBCL patients treated with R-CHOP is 70-80% and 45-50%, respectively (Lenz et al., 2008).

Other gene-expression based characterizations of DLBCLs have been conducted, including a three-part classifier into so-called “Ox-Phos” DLBCLs, which are characterized by higher expression of mitochondrial electron transport genes, “B-cell receptor (BCR) and proliferation” associated DLBCL, and “Host response” DLBCL; the latter are characterized by an inflamed phenotype and overexpression of genes related to stroma and immune cell interactions (Monti et al., 2005). The overlap between the different categorizations is not mutually exclusive (i.e. GC-DLBCLs fall into any of the above three categories) and more recent efforts have gone on to further substratify DLBCLs into five or more categories based on integrated gene-expression profiling, mutations and chromosomal abnormalities (Chapuy et al., 2018; Compagno et al., 2009; Reddy et al., 2017; Schmitz et al., 2018).

Genome sequencing efforts have also demonstrated that GC- and ABC-DLBCLs harbor unique genetic alterations and chromosomal modifications. GC-DLBCLs frequently contain mutations of *MLL2*, *EZH2*, *BCL6* and *GNAI3* while ABC-DLBCLs are characterized by mutations in BCR signaling components (*CD79A* and *CD79B*) and NF- κ B pathway members (*MYD88*, *CARD11* and *TNFAIP3*), respectively (Compagno et al., 2009; Lohr et al., 2012; Morin et al., 2011; Pasqualucci et al., 2011). These distinctions are believed to account in part for their distinct expression profiles and clinical behavior. In addition to targeted mutations, chromosomal translocations are also frequently observed in DLBCLs. These translocations frequently involve *MYC*, *BCL2* and *BCL6* (Akay et al., 2014; de Jonge et al., 2016; Iqbal et al., 2004; Savage et al., 2009; Shustik et al., 2010).

Double-Hit Lymphoma

In 2016, the World Health Organization (WHO) defined a new pathologic lymphoma entity entitled “High-grade B-cell lymphoma, with *MYC* and *BCL2* and/or *BCL6* translocations” (Swerdlow et al., 2016). These “double -hit” lymphomas (DHLs) represent 6-9% of all DLBCL cases and are characterized by *MYC* translocations in concert with *BCL2* (80%) or *BCL6* (20%) (Craig et al., 2018; Riedell and Smith, 2018). “Triple-hit” lymphomas (THLs) that contain translocations of all three genes have also been described and are included in the new WHO entity (Rosenthal and Younes, 2017; Wang et al., 2015). While the etiology of DHLs (from this point forward, the term ‘DHL’ will be used to encompass both double- and triple-hit lymphomas) is unknown, at least a subset arise from transformations from indolent lymphomas such as follicular lymphoma (Witte et al., 2018; Xu et al., 2013).

MYC is a transcription factor known to regulate a vast array of cellular processes including metabolism, cell adhesion and differentiation (Dang, 2012; Eilers and Eisenman, 2008;

Hsieh et al., 2015; Wilson et al., 2004). Cellular levels of MYC are tightly regulated; while low and intermediate levels of MYC are associated with cellular proliferation (Bernard and Eilers, 2006; Lemaitre et al., 1996), high levels of MYC may induce apoptosis (McMahon, 2014). In Burkitt's lymphoma (BL), *MYC* translocations with immunoglobulin gene family members are found in nearly 100% of cases (Schmitz et al., 2014). In contrast, *MYC* translocations are found in approximately 8-12% of DLBCL cases and only about 50% of those are with immunoglobulin gene partners (Chong et al., 2018). Moreover, the chromosomal translocations in DLBCL are heteroclonal, having several different translocation partners within the same tumor (Valera et al., 2016). In B-cells, MYC is involved in regulating germinal center (GC) dynamics. Suppression of MYC is required for GC B-cell entry into the dark zone, the site of somatic hypermutation (SHM) and class switch recombination (CSR), but its expression is required for B-cell entry into the GC light zone, the site of antigen selection (Calado et al., 2012; Dominguez-Sola et al., 2012). Persistent MYC expression prevents B-cell re-entry into the dark zone and inhibits plasma cell differentiation (Shaffer et al., 2002). Therefore, deregulated MYC expression can promote lymphomagenesis by causing increased lymphoma proliferation as well as potentially blocking B-cell maturation and differentiation (Figure 1.1).

BCL6 is a transcriptional repressor that serves as the “master regulator” of the germinal center reaction (Basso and Dalla-Favera, 2010). It functions to promote SHM, CSR and B-cell survival (Bunting et al., 2016; Huang and Melnick, 2015). *BCL6* represses MYC expression in the germinal center dark zone (DZ) as well as genes that promote plasma cell differentiation such as *BLIMP1* (Calado et al., 2012; Crotty et al., 2010; Dominguez-Sola et al., 2012). Thus, *BCL6* translocations, which are found in 10-20% of DLBCL cases

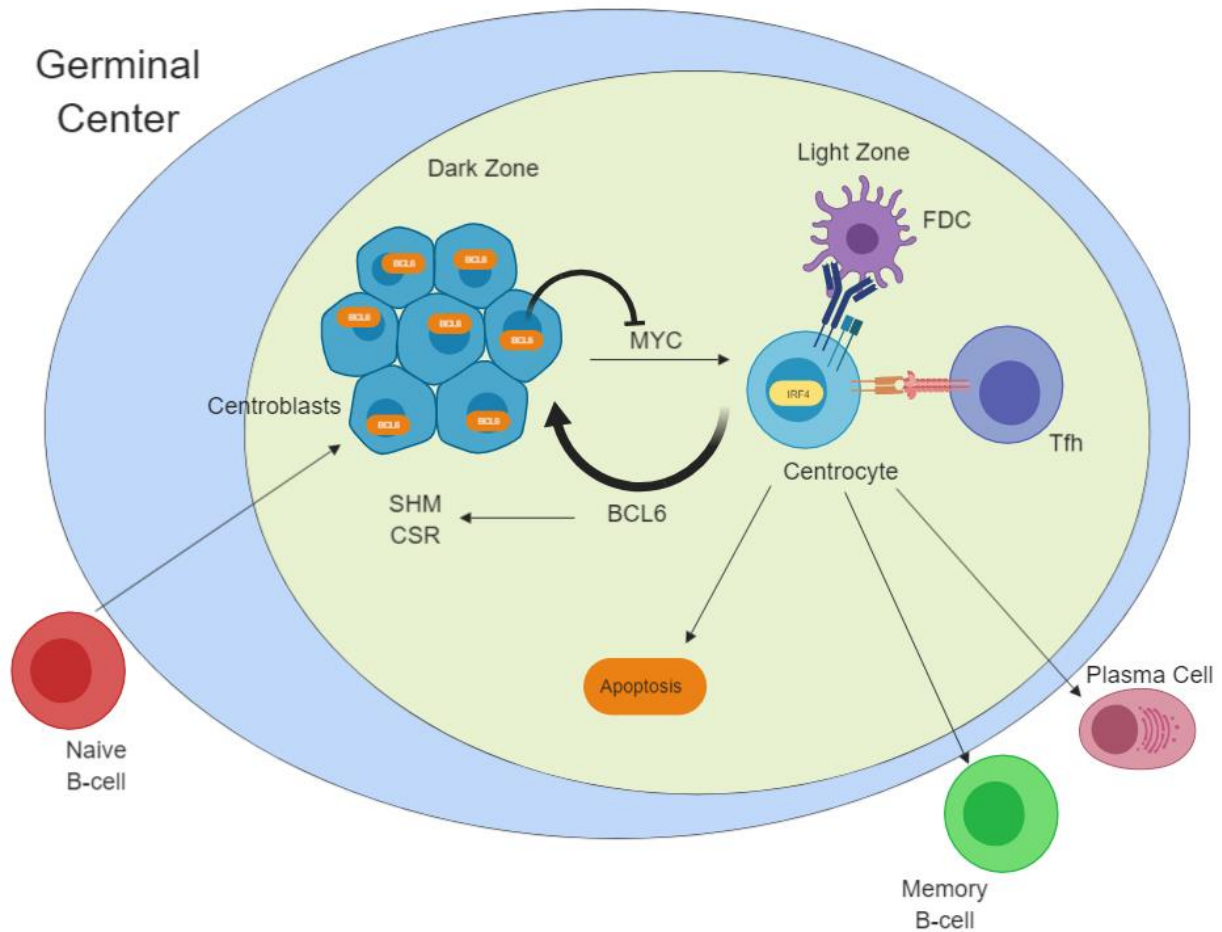


Figure 1.1 Mechanism of BCL-6 and MYC Oncogenesis in Lymphoma. The germinal center (GC) transcriptional program is governed by the “master regulator” BCL-6. BCL-6 expression is turned on in dark zone centroblasts, which are rapidly dividing GC-derived B-cells. In the dark zone, BCL-6 inhibits DNA repair and promotes somatic hypermutation and class switch recombination at immunoglobulin gene loci. Dark zone GC centroblasts migrate to the light zone, where the fitness of their B-cell receptor is checked by antigen-presenting follicular dendritic cells (FDCs). B-cell receptor signaling in the light zone is necessary for survival and B-cell differentiation into plasma and memory cells, respectively, while lack of signaling leads to apoptosis. GC B cells repeatedly cycle between the dark and light zones as part of the affinity maturation process. Entry of dark zone centroblasts into the light zone is dependent on induction of MYC, which in the dark zone is normally repressed by BCL-6. Egress to the dark zone from the light zone is inhibited by constitutive MYC expression. *BCL6* translocations can promote lymphoma pathogenesis by facilitating mutation acquisition by inhibiting DNA repair inhibition and by leading to dark-zone retention. *MYC* translocations, in addition to driving cellular proliferation, conversely lead to light zone retention and block differentiation to plasma and memory B-cells.

(Shustik et al., 2010), can promote lymphomagenesis at least in part by inducing a B-cell differentiation block (Figure 1.1).

BCL2 was originally defined based on its presence as the translocation partner in t(14:18), which occurs in over 90% of follicular lymphomas (Kridel et al., 2012; Leich et al., 2009). It is an anti-apoptotic BH3 domain containing protein that is localized to the mitochondrial outer membrane (Adams and Cory, 1998). Along with other anti-apoptotic proteins (e.g. BCL-xL and MCL-1), BCL-2 antagonizes the activity of pro-apoptotic proteins, including BAK, BAX and BID (Adams and Cory, 1998; Singh et al., 2019). Altering the balance of pro- and anti-apoptotic proteins in favor of apoptosis leads to mitochondrial outer-membrane permeabilization. This causes release of cytochrome C from the mitochondrial membrane and reactive oxygen species (ROS) from the mitochondrial matrix that, through an apoptosome intermediate, lead to caspase cleavage and cellular apoptosis (Dorstyn et al., 2018). Translocations of *BCL2* are found in 20-30% of DLCLs and promote lymphomagenesis through blocking tumor cell apoptosis (Figure 1.2) (Schuetz et al., 2012).

Importantly, DHLs are different than so called “double-expressor” lymphomas (DELs), which are defined as being positive for MYC and BCL-2 proteins expression by immunohistochemistry (Burotto et al., 2016; Riedell and Smith, 2018). Indeed, 95% of DHLs are GC-DLBCL by gene expression profiling while the majority of DELs are ABC-DLBCL (Aukema et al., 2011; Ennishi et al., 2017; Riedell and Smith, 2018; Scott et al., 2018). Notably, most GC-DLBCLs but not DHLs have a favorable outcome with R-CHOP treatment (McPhail et al., 2018; Sarkozy et al., 2015). The poor outcome among patients with DHL (median OS, 1-2 years) may in part be due to a fraction of DHLs expressing lower levels of the CD20 antigen, the target of rituximab, although this is a property not universally observed in all DHLs

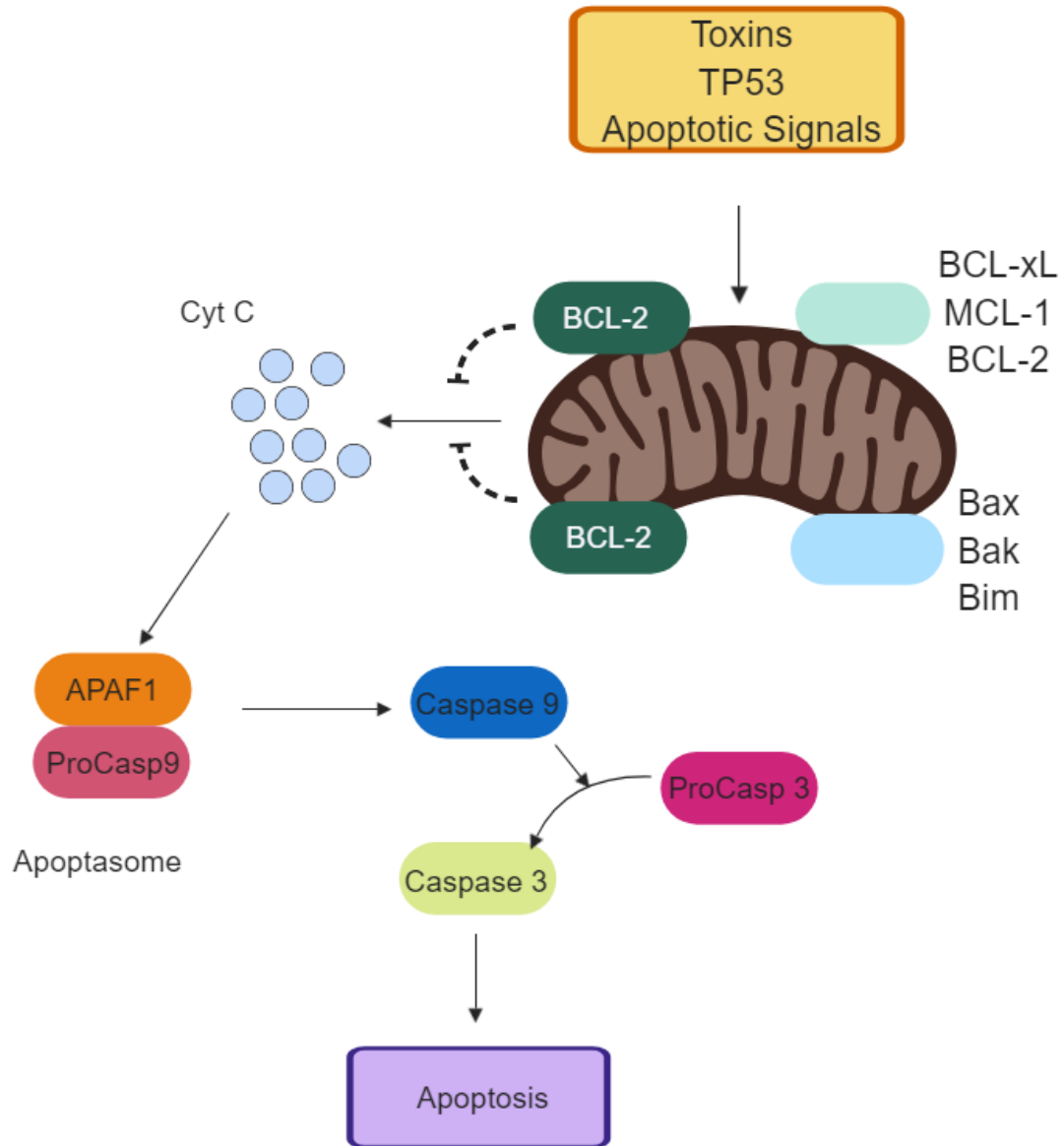


Figure 1.2 *BCL2* translocation-mediated apoptotic resistance. Apoptosis is initiated by cellular or environmental signals that lead to cellular damage or genotoxic stress and are transmitted by proteins such as p53. These apoptotic signals are initiated through cell death receptors (extrinsic pathway) or through cellular caspases (intrinsic pathway) and lead to activation of the BH3 domain-containing proteins BAX and BAK. A net positive balance of pro-apoptotic BH3 proteins compared to the anti-apoptotic BCL-2, BCL-xL and MCL-1, causing permeabilization of the mitochondrial outer membrane and cytoplasmic release of cytochrome C. Cytoplasmic cytochrome C is recognized by APAF1, which can then activate caspase 9. Caspase 9 then activates caspases 3 and 7. Caspase 3 breaks down cellular DNA and leads to apoptotic cell death. In DHL, *BCL2* translocation leading to BCL-2 overexpression, which shifts the balance away from apoptosis.

(Harrington et al., 2011; Wu et al., 2010). Moreover, some DHLs express markers of immature B-cells such as terminal deoxynucleotidyl transferase (TdT), suggesting a potential dedifferentiation phenotype that could contribute to their relative chemoresistance (Moench et al., 2016; Ok et al., 2019).

Several therapeutic strategies have recently been employed in attempts to improve outcomes among patients with DHL. These include more aggressive chemotherapy regimens such as rituximab and dose-adjusted EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin) alone or as part of a bridge to autologous stem cell transplant, the McGrath protocol and R CODOX IVAC (Friedberg, 2015; Merron and Davies, 2018). Despite this, patient outcomes still remain poor (Merron and Davies, 2018). The anti-BCL-2 agent venetoclax (ABT-199) is effective *in vitro* in several DHL cell lines but these results have largely failed to translate to patients (Uchida et al., 2018). Targeting DHLs through MYC-regulating proteins has recently been demonstrated as a potentially promising treatment strategy, as has *in vitro* co-targeting of MYC and BCL2 (Cinar et al., 2015; Ren et al., 2018). However, since these efforts employed non-FDA approved and/or clinically “dead” drugs (e.g. JQ1), immediate translation to patients and clinical trial initiation has been a challenge in the field (Hogg et al., 2016). This has been in part due to a lack of non-cell line models that faithfully recapitulate the biology of DHLs as well as a lack of attempts to incorporate the tumor microenvironment within DHL-targeting strategies.

As can be expected based on their clinical aggressiveness, DHLs have a high Ki-67 proliferation index and frequently involve extranodal sites such as the bone marrow (BM), which is involved in ~60% of patients, and the central nervous system (Aukema et al., 2011; Craig et al., 2018; Dunleavy et al., 2018). Morphologically DHLs appear like other DLBCLs but some

cases can present with features intermediate between DLBCL and BL. Indeed, pathological specimens from DHL cases can sometimes have the “starry-sky” characteristic found in BL that results from foamy macrophages within close proximity to the tumor cells (Ferry, 2006; Ford et al., 2015).

Tumor Associated Macrophages

Multiple tumor types can alter the transcriptional and functional state of macrophages within the tumor microenvironment, thereby promoting tumor growth and metastases. This is accomplished by macrophage secretion of cytokines and angiogenic factors that contribute to an immunosuppressive microenvironment and tumor immune escape, including Arginase-1 and TGF β (Mantovani et al., 2017; Noy and Pollard, 2014). Classically, the tumor-promoting macrophages have been termed “M2” based on their similarity to macrophages differentiated *in vitro* with M-CSF and IL-4 (Italiani and Boraschi, 2014). M2 macrophages are characterized by surface expression of proteins such as CD206. This is in contrast to “M1” macrophages that promote tumor cell clearance via secretion of TNF α and IL-1 β . M1 macrophages highly express surface MHC class II molecules (Mantovani et al., 2017; Noy and Pollard, 2014).

The classification of M1 and M2, while useful, has largely been abandoned in favor of the tumor-associated macrophages (TAM) nomenclature, with TAMs expressing many genes frequently associated with the M2 phenotype (Martinez and Gordon, 2014). In multiple studies, eliminating or reprogramming TAMs leads to tumor regression and/or complete tumor elimination (Guerriero et al., 2017). Mechanisms of macrophage-mediated tumor clearance include tumor-cell killing by release of reactive species such as nitric oxide or antibody-dependent cellular phagocytosis (ADCP) (Mills et al., 2016; Weiskopf and Weissman, 2015).

Monoclonal antibodies in cancer treatment and cancer resistance

Since their introduction into clinical practice, monoclonal antibodies have been a mainstay of cancer therapeutics. Rituximab was the first FDA-approved therapeutic antibody for cancer treatment. Rituximab binds to the CD20 antigen found on the surface of target B-cell tumors and elicits tumor clearance either through direct apoptosis or through immune cell recruitment (Singh et al., 2018). Its broad activity across many B cell lymphoma types, which has translated into meaningful improvements in patient outcome, has led to the development of other clinical-grade antibodies for lymphoma therapy, including CD20-targeting antibody-drug conjugates such as ibritumomab tiuxetan and “second generation” CD20-directed antibodies such as obinutuzumab (Marcus et al., 2017; Mondello et al., 2016). Antibodies targeting other antigens have also been developed for lymphoma therapy such as alemtuzumab (Alem), which binds CD52 and is FDA approved for the treatment of chronic lymphocytic leukemia (CLL) and T-cell prolymphocytic leukemia (T-PLL) (Ravandi and O'Brien, 2006).

Monoclonal antibodies can engage multiple mechanisms to eliminate tumor cells (Figure 1.3). These generally fall into two broad categories: constant (Fc) region-independent and -dependent mechanisms. Fc-independent activity involves direct binding of antibody to its target and either disrupting binding to its natural ligand (e.g. the anti-VEGF-A antibody bevacizumab) or activating (i.e. agonizing) the target (Diaz et al., 2017; Singh et al., 2018). Moreover, some antibodies may directly induce tumor cell death upon binding (Nguyen et al., 2012). Fc-dependent antibody activity involves the binding of the Fc antibody backbone to complement proteins to mediate direct cell lysis or alternatively to Fc-receptor gamma (FcγR)-expressing effector cells, including NK cells and macrophages (Barnhart and Quigley, 2017). These cells can then eliminate the opsonized tumor cells by antibody-dependent cellular cytotoxicity

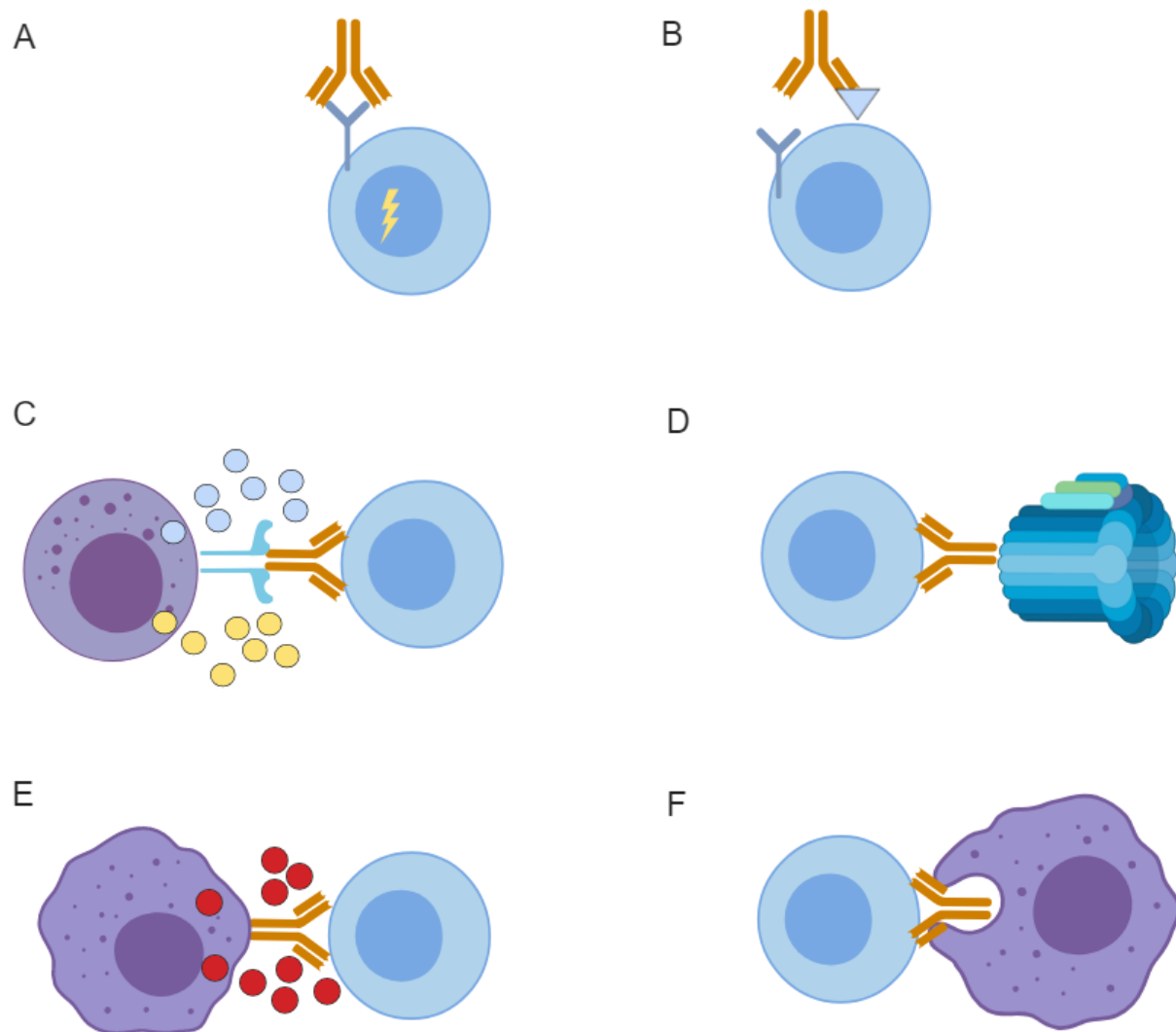


Figure 1.3 Antibody-mediated tumor cell elimination. Monoclonal antibodies can lead to tumor cell death in several ways; these include: (A) direct binding of antibody to a surface target antigen that leads to tumor apoptosis; (B) inhibiting ligand-receptor interactions, either by blocking the receptor (e.g. blocking IL2R by daclizumab) or by sequestration of ligand (e.g. binding VEGF-A by bevacizumab); (C) ADCC, where binding of the antibody Fc region to Fc receptors on NK (or other) cells leads to release of granzyme-B and perforin that lyse tumor cells; (D) binding of the Fc region to serum complement proteins, leading to complement-mediated lysis by the C5 membrane attack complex; (E) binding of the Fc region to Fc receptors on macrophages/neutrophils, leading to the release of reactive oxygen species (ROS) from stored granules and (F) Fc -Fc receptor-mediated ADCP by macrophages and neutrophils.

(ADCC) via direct cell lysis (NK cells, neutrophils and macrophages) or antibody-dependent cellular phagocytosis (ADCP) by neutrophils or macrophages. The importance of FcγRs in the activity of antibodies is underlined by observations that genetic polymorphisms in the gene family are associated with differential success of antibody therapy (Burkhardt et al., 2016; Liu et al., 2016).

In mouse models and in humans, the activity of both rituximab and alemtuzumab at least partially involves NK-cell mediated ADCC (Hu et al., 2009; Weiner, 2010). Complement dependent cytotoxicity (CDC) has also been implicated in the efficacy of rituximab while neutrophil-mediated killing plays a role for alemtuzumab in the skin of SCID mice, which lack B and T cells (Siders et al., 2010; Weiner, 2010). However, in NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ* (NSG) mice, which lack functional B, T and NK cells, macrophages are important mediators of antibody-dependent cellular clearance (Chao et al., 2010a). Both M1 and M2 macrophages have been shown to be capable of ADCP (Zhang et al., 2016). M2 macrophages potentially play a greater role in ADCP due to their higher surface levels of activating FcγRs (Leidi et al., 2009) .

Several mechanisms of resistance to antibody treatment in cancer have been described. These include reduced expression or elimination of target antigen, such as seen with CD20 downregulation/loss in rituximab-refractory lymphomas (Rezvani and Maloney, 2011). It has also been proposed that “epigenetic reprogramming” can modulate alemtuzumab resistance in T-PLL, although the exact mechanisms remain poorly understood (Hasanali et al., 2015). Tumor cell modulation of the microenvironment that promotes antibody resistance can involve higher surface expression of the inhibitory FcγR2B (CD32B) and downregulation of activating Fc receptors on effector cells (Dahal et al., 2017). Indeed, DLBCLs are known to express high

levels of CD32B, which on lymphoma cells serves as an antibody sink (Camilleri-Broet et al., 2004); blocking of this receptor with anti-CD32B antibodies can overcome lymphoma rituximab resistance in pre-clinical models (Roghanian et al., 2015). Antibody efficacy in lymphomas is reduced at sites of bulky disease (Lundin et al., 1998; McLaughlin et al., 1998); while the reason for this is unknown, it may be due to a lack of antibody penetration or alternatively due to an insufficient number of effector cells within large tumor masses.

Cyclophosphamide

Cyclophosphamide (CTX) is a nitrogen mustard family member that is widely utilized to treat various cancers, including NHLs, leukemias, multiple myeloma, pediatric tumors and breast cancer, as well as an agent for suppressing immune responses in patients with autoimmune diseases or those receiving allogeneic stem cell transplantation (Ahmed and Hombal, 1984; Colvin, 1999). Cyclophosphamide functions as an alkylating agent, a class of antineoplastics that induce tumor cell death through a reactive metabolic intermediate.

Unlike other alkylating agents used therapeutically, CTX is a pro-drug and must be activated by the liver *in vivo* for clinical efficacy (Figure 1.4) (McDonald et al., 2003). Once administered, it is metabolized by CYP450 in the liver into 4'-hydroxycyclophosphamide and subsequently to aldophosphamide, which through spontaneous beta-elimination leads to generation of a phosphoramidate mustard that can attach irreversibly to the seventh nitrogen atom of guanine in DNA (Ludeman, 1999). This attachment is believed to be the mechanism of its anti-neoplastic activity, as it causes DNA interstrand and intrastrand crosslinking, thereby inhibiting DNA replication and leading to cell death (Huang and Li, 2013). Aldophosphamide also leads to the generation of acrolein, a highly toxic reactive aldehyde that has been

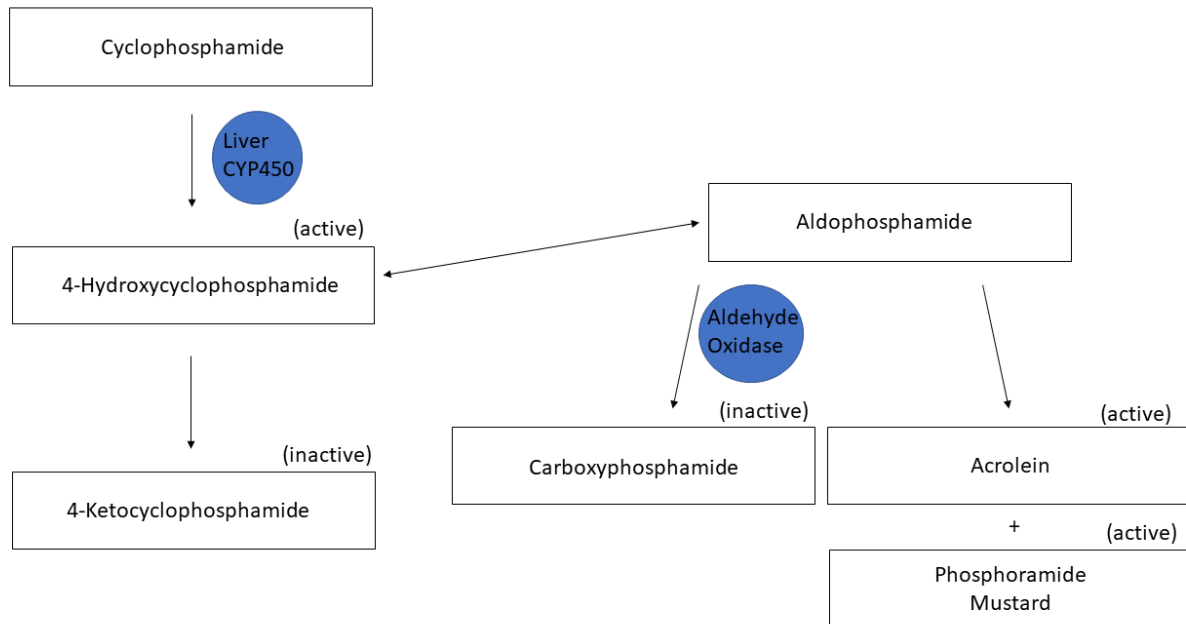


Figure 1.4 Cyclophosphamide Metabolism. Cyclophosphamide is metabolized by liver CYP450 enzymes into the 4-hydroxycyclophosphamide (4-OH CTX). 4-OH CTX is in equilibrium with its tautomer aldophosphamide. Both metabolites can freely diffuse from the liver. 4-OH-CTX that remains in the liver is converted to 4-ketocyclophosphamide, an inactive side product. Aldophosphamide can spontaneously convert to phosphoramidate mustard, which is believed to be the active metabolite that leads to DNA crosslinking and is further metabolized to acrolein, a reactive aldehyde intermediate that confers cyclophosphamide-associated bladder toxicity. Aldophosphamide can also be converted to carboxyphosphamide by the enzyme aldehyde oxidase although this byproduct is not known to have clinical activity.

demonstrated to generate DNA adducts and alkylate substrates such as glutathione and NF- κ B (Lambert et al., 2007; Randall et al., 2013).

Ifosfamide and mafosfamide are alkylating agents that are also believed to work through phosphoramidate mustard and are active *in vitro* (Botzler et al., 1997). Additional alkylating agents can cause DNA alkylation and cell death through a different reactive intermediate, such as the carbonium ion in the case of the alkyl sulfonate busulfan (Myers et al., 2017). However, the role of other metabolic byproducts of alkylating agent metabolism and other mechanisms of alkylating agents (e.g. effects on cellular proteins, lipids and RNA) in causing tumor regression remain poorly understood.

In recent years, it has become apparent that chemotherapies including CTX can modulate tumor microenvironments by affecting both immune- and nonimmune cell constituents (Ahlmann and Hempel, 2016). The extent to which these effects contribute to clinical efficacy is an area of intense investigation. Low doses of CTX can deplete circulating regulatory T cells in various tumor models, leading to enhanced NK- and T-cell mediated tumor killing (Scurr et al., 2017). High-doses of cyclophosphamide are highly lymphodepleting (Miller et al., 2007; Saida et al., 2015). In contrast with both neutrophils and lymphocytes, macrophages and hematopoietic stem cells are largely resistant to cyclophosphamide treatment.

Immunogenic cell death

In addition to altering the immune microenvironment, CTX can also promote “immunogenic cell death,” which makes the tumor cell more easily recognizable by the immune system (Bloy et al., 2017; Kroemer et al., 2013). Features of immunogenic cell death (ICD) include secretion of damage-associated molecular patterns (DAMPs) that then promote immune

recognition, influx into the tumor bed and subsequent tumor clearance (Krysko et al., 2012).

Factors known to be produced and secreted that promote ICD include amphoterin (HMGB1) and ATP (Kepp et al., 2014). Moreover, phenotypic changes to tumor cells have also been observed as part of ICD. These most notably include surface translocation of heat-shock proteins (HSPs) HSP70 and HSP90, which can facilitate immune activation by interacting with CD91 and CD40 on antigen presenting cells, thereby promoting antigen presentation by major-histocompatibility proteins (MHC) to facilitate T-cell responses (Kepp et al., 2014). Increased tumor cell surface expression of calreticulin can also occur (Kroemer et al., 2013). Calreticulin is a major pro-phagocytic signal that promotes phagocytosis by macrophages (Chao et al., 2010b; Feng et al., 2018).

Several cellular mechanisms that lead to ICD have been described. DNA damage induced by alkylating agents and topoisomerase inhibitors (e.g. doxorubicin) can activate tumor-intrinsic NF- κ B signaling, leading to production and secretion of immunomodulators such as interferon- α that can then activate immune cells and promote tumor clearance (Janssens and Tschopp, 2006; Wang et al., 2017). Chemotherapies can also induce tumor cell senescence and associated secretory responses that in certain contexts may induce cytokine release that leads to tumor cell clearance (Hussner et al., 2012; Saleh et al., 2018; Sistigu et al., 2014). Meanwhile, induction of endoplasmic reticulum (ER) stress has been implicated as the driving force for surface calreticulin translocation (Rufo et al., 2017). Cellular stress can lower surface expression of the “don’t eat me” molecule CD47, which is widely expressed on tumor cells and functions to inhibit macrophage phagocytosis (Adkins et al., 2017).

ER Stress

Under normal physiological conditions, mRNA transcripts whose protein product is destined for cell surface expression or secretion are translocated to ER-associated ribosomes for complete translation following the detection of an N-terminus signal sequence initially translated in the cytoplasm by the signal recognition particle (SRP). The SRP can translocate proteins into the ER lumen, where molecular chaperones such as BiP, calreticulin and HSPs can monitor their correct folding, a property that is intrinsic to the amino acid sequence of the translated protein (Nyathi et al., 2013). In addition to facilitating correct protein folding, post-translational modifications can occur in the ER, most notably N-linked glycosylation (Aebi, 2013). Following translation, the proteins are then shuttled by vesicles to Golgi for further processing and ultimately to the cell surface for expression/secretion unless they contain a C-terminus KDEL amino acid sequence that maintains their localization to the ER (van Leeuwen et al., 2018).

In non-homeostatic conditions, cellular insults such as the generation of reactive oxygen species (ROS) or changes in temperature can induce proteins in the ER to misfold (Gu et al., 2016). The accumulation of unfolded ER resident proteins leads to ER stress and the subsequent initiation of the unfolded protein response (UPR) (Figure 1.5). While it is not completely known how unfolded ER-resident proteins are sensed in the ER, hydrophobic residues exposed from misfolded proteins can be bound by BiP, which displaces it from its normal binding partners PERK (protein kinase RNA-like endoplasmic reticulum kinase), IRE-1 (Inositol-requiring kinase 1) and ATF6 (Activating transcription factor 6) (Bernales et al., 2006). Upon BiP dissociation, PERK phosphorylates eukaryotic initiation factor 2 alpha (eIF2 α), leading to the upregulation of ATF4, a transcription factor that can initiate the upregulation of UPR-associated genes (Hetz, 2012). In cases of prolonged ER stress, ATF4 can lead to the transcription of CHOP, which leads to apoptosis (Hetz, 2012). Simultaneously, free IRE-1 homodimerizes and autophosphorylates,

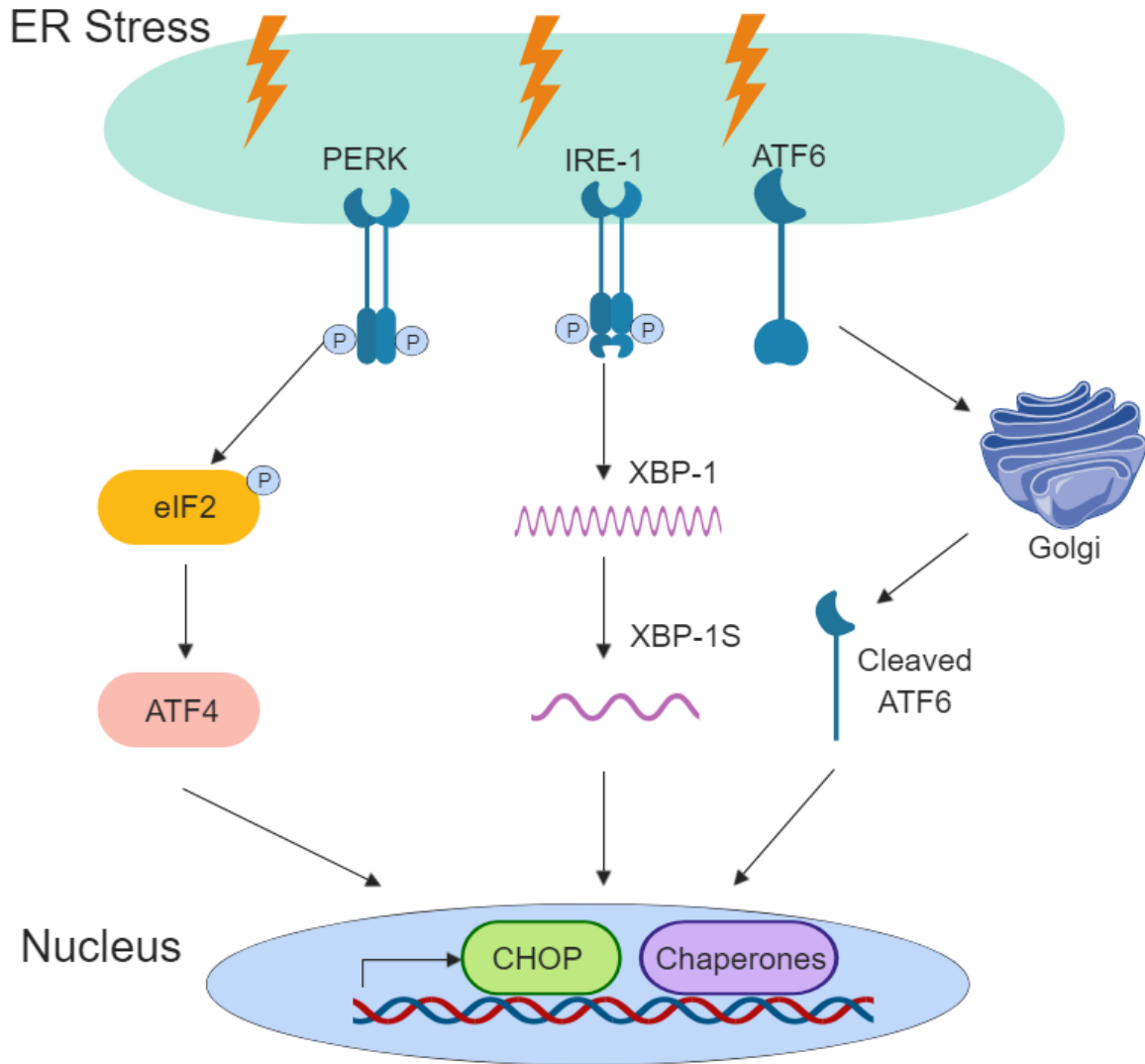


Figure 1.5 The Unfolded Protein Response (UPR). Accumulation of misfolded protein in the endoplasmic reticulum (ER) leads to recruitment of molecular chaperones such as BiP to facilitate proper folding. BiP recruitment releases it from its normal occupancy, where it is bound to PERK, IRE-1 and ATF6. Free PERK and IRE-1 can each homodimerize and auto-phosphorylate while unbound ATF6 translocates to the Golgi apparatus, where it is cleaved to its active state. Phosphorylated PERK leads to phosphorylation of eIF2, which initiates translation of stress response proteins such as ATF4. IRE-1 phosphorylation leads to its localization to the *XBPI* promoter, where it promotes a removal of a 26 base pair intron that leads to alternative splicing of the *XBPI* gene (XBP-1S). ATF4, XBP-1S and activated ATF6 translocate to the nucleus and function as transcription factors to promote the synthesis of chaperone proteins, pro-apoptotic proteins such as CHOP and cytokines such as VEGF-A that drive angiogenesis and alleviate hypoxia.

which induces transcription of *XBPI* that lacks a 26bp intron (Hetz, 2012). This spliced variant of XBP1 (XBP1-S) promotes transcription of ER stress-associated genes. Meanwhile, ATF6 is initially shuttled to the Golgi, where it is cleaved, and then this activated ATF6 translocates to the nucleus, resulting in subsequent transcription of stress-associated genes (Gardner et al., 2013). Transcriptional targets of the UPR are many and include TNF-alpha and VEGF-A (Binet and Sapieha, 2015; Chen et al., 2013; Ghosh et al., 2010). Recent studies have shown that DHLs may have higher levels of ER stress than other DLBCLs, suggesting that targeting ER stress in DHL may be an effective treatment strategy (Ennishi et al., 2019; Sha et al., 2019).

Bone marrow alemtuzumab resistance in humaneered DHL is overcome by cyclophosphamide

To faithfully recapitulate DHL biology and pathogenesis, the Hemann and Chen laboratories generated humaneered DHL (Pallasch et al., 2014). This was accomplished by transfecting human umbilical cord blood (CD34⁺) cells with a lentiviral construct encoding BCL-2 and an activating MYC allele that occurs in human NHLs. Injection of these cells into NSG mice resulted in a “humaneered DHL” that resembled an aggressive leukemia rather than a *bona fide* DHL. Immunophenotyping of this leukemia demonstrated CD20 negativity but CD52 positivity, making it potentially amenable to alemtuzumab treatment. Alemtuzumab treatment of mice fully engrafted with the humaneered DHL (typically upon detectable peripheral blood disease) led to marked reductions in involvement within peripheral blood, spleen and the liver. However, bone marrow involvement was largely unaffected by alemtuzumab. This lack of response was dependent on extensive involvement, as earlier treatment with alemtuzumab was able to successfully reduce bone marrow disease. Importantly, macrophages were the effector

cells of alemtuzumab activity, as their depletion with clodronate led to loss of antibody activity in the spleen.

In attempts to overcome antibody resistance, several different immunomodulatory chemotherapies were tried in synergy with alemtuzumab. Doxorubicin and ionizing radiation were unsuccessful at overcoming bone marrow resistance. Interestingly, high-dose CTX monotherapy (at least 100 mg/kg) was able to induce partial tumor regression. The combination of high-dose CTX with alemtuzumab was >160 fold synergistic in clearing the bone marrow of disease, leading to potential cures in ~60% of tumor engrafted mice treated with a single cycle of the combination. High-dose CTX was associated with an “acute secretory activating phenotype” (ASAP), leading to secretion of VEGF-A, TNF- α , CCL4 and IL-8 that promoted macrophage ADCP.

Important biological questions from this seminal work remained unanswered. Specifically, 1) whether the effects of high-dose CTX extend to *bona fide* human DHL; 2) whether other alkylating agents in the CTX drug class could recapitulate synergy with alemtuzumab; 3) the mechanisms of bone marrow antibody resistance in the “humaneered DHL,” and how this resistance was established; 4) the requirement of macrophages for CTX-mediated killing; 5) whether human DHL exhibits the ASAP and how this is mechanistically promoted by alkylating agents and 6) the transcriptional and phenotypic effects of chemoimmunotherapies on lymphoma-associated macrophages that promote synergy with alemtuzumab.

Herein we use *bona fide* human DHL in the form of patient-derived xenografts (PDXs) to address these questions. We demonstrate that similarly to the “humaneered DHL” model DHL PDXs acquire bone marrow alemtuzumab resistance that is overcome by CTX and other

alkylating agents. Bone marrow alemtuzumab resistance was due to remodeling of the bone marrow microenvironment, with a high lymphoma: macrophage ratio being the key determinant of alemtuzumab efficacy. Alkylating agents induced ER stress in DHL cells, leading to ATF4 mediated VEGF-A production and secretion that enhanced macrophage phagocytosis through the SYK kinase. CTX treatment in lymphoma-bearing mice lead to an expansion of a unique macrophage subset characterized by surface CD36 and Fcgr4 expression that was “super phagocytic” for antibody-coated lymphoma cells. Together these findings illuminate the synergistic activity seen in the bone marrow and provide the rationale for employing the combination of high-dose cyclophosphamide and alemtuzumab in DHL and possibly other diseases with low macrophage: lymphoma cell ratios.

Chapter 2: DHL bone marrow antibody resistance can be overcome by high-dose alkylating agent therapy

Background

While *in vitro* culture systems can be used to assess sensitivity of cancer cells to therapy, *in vivo* drug administration is necessary to study the effect of therapeutics that depend on cell extrinsic induction of apoptosis such as immune mediated clearance. To this end patient derived xenografts (PDXs) in immunocompromised mice (either NOD-SCID or NSG) offer the ability to test therapies on human cancers that depend on the innate immune system. Our laboratory has recently developed a repository of patient-derived xenografts (PRoXe) to establish models of rare diseases that can be grown in NSG mice to study the efficacy of novel cancer therapies (Townsend et al., 2016). This repository includes two PDXs of DHL harboring translocations of both *MYC* and *BCL2* established from patients who were refractory to (DFBL-20954) or relapsed on (DFBL-69487) R-CHOP therapy. Upon engraftment these PDXs home to the spleen and bone marrow and tumor engraftment can be monitored by flow cytometric detection of circulating tumor cells in peripheral blood. Therefore, these PDXs represent *bona fide* human DHL and can serve as representative clinical models to test the efficacy of CTX and alemtuzumab combination therapy.

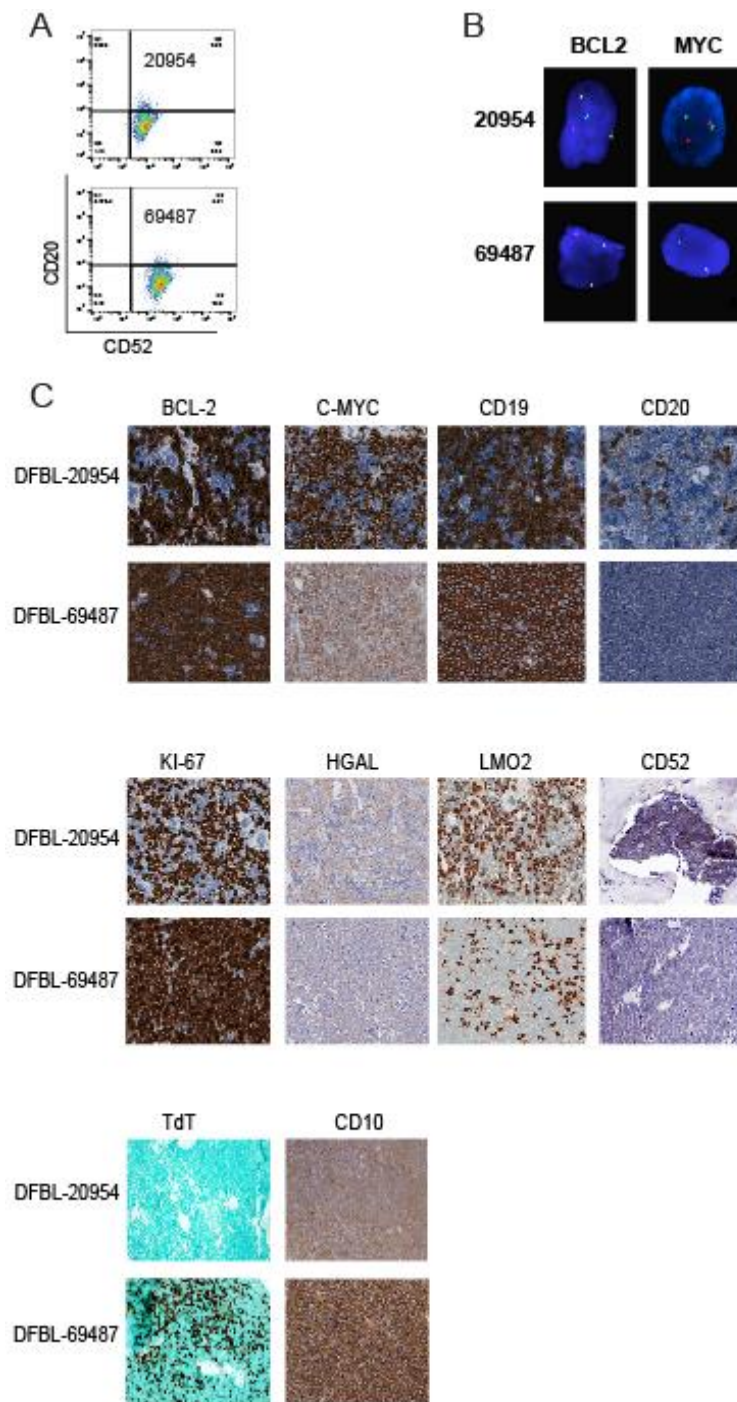
Results

To determine whether the synergy between cyclophosphamide and alemtuzumab persisted in *bona fide* human DHL, we used the two generated DHL PDXs (DFBL-20954 and DFBL-69487). We first sought to confirm that the PDXs were amenable to alemtuzumab therapy by assessing surface expression of CD52, the target antigen of alemtuzumab.

Immunophenotyping of the tumor cells from moribund mice engrafted with the two PDXs revealed that both were positive for CD52 but negative or lowly expressing CD20, consistent with a subset of DHL and/or acquired resistance to rituximab (Figure 2.1A). Importantly, these

Figure 2.1 Characteristics of DHL PDXs. (A) Flow cytometric analysis of surface CD20 and CD52 expression on DFBL-20954 and DFBL-69487. (B) Representative Fluorescence in-situ hybridization (FISH) of DFBL-20954 and DFBL-69487 spleen cells from engrafted NSG mice demonstrating fusion between *IGH@* and *BCL2* (yellow signal; left-sided panels) and break-apart of *MYC* (right-sided panels). (C) Immunohistochemical staining of spleens from tumor bearing DFBL-20954 and DFBL-69487 cells for the indicated markers. CD52 expression was assayed on BM specimens. Images are 40x magnified.

Figure 2.1 (Continued)



features recapitulated the original patient tumor characteristics.

To confirm that the engrafted PDXs represented *bona fide* DHL *in vivo*, we FACS sorted human B-cells from the spleen and bone marrow of moribund mice engrafted with the PDXs and performed fluorescent in-situ hybridization (FISH) for the *MYC* and *BCL2* genes. IGH/BCL2 fusion was detected in 47/50 DFBL-20954 nuclei and in 46/50 DFBL-69487 nuclei (Figure 2.1B). *MYC* rearrangement was observed in 49/50 nuclei in DFBL-20954 and in 40/50 DFBL-69487 nuclei (Figure 2.1B). We further performed immunohistochemistry on spleens and bone marrows to check for lymphoma markers (Figure 2.1C). This revealed that both PDXs were positive for CD10, consistent with DHL of germinal center origin, and expressed other markers of germinal center B-cells such as LMO2 and HGAL (Figure 2.1C). Both xenografts were also positive for MYC and BCL-2 proteins and expressed Ki-67 in more than 80% of cells, consistent with DHL (Figure 2.1C). While DFBL-20954 was negative for TdT, DFBL-69487 had a subset of TdT-positive cells (Figure 2.1C); while consistent with a diagnosis of DHL, this observation suggests that within DHLs the two xenografts may represent different biological entities.

NSG mice were xenografted with these tumors and monitored until >2% peripheral blood hCD19⁺hCD45⁺ cells were detected to confirm disease engraftment, which corresponds roughly to >30% tumor burden in the spleen and >50% tumor in the bone marrow. In DFBL-20954 and DFBL-69487 this corresponded to 21 and 35 days post engraftment, respectively. At these time points DHL engrafted mice were randomized to receive vehicle (PBS), cyclophosphamide (100mg/kg), doxorubicin (5mg/kg), alemtuzumab (2x5mg/kg), doxorubicin plus alemtuzumab or cyclophosphamide plus alemtuzumab. Following treatment mice were assessed at day 8 for tumor burden, the same time point utilized for the previously reported humaneered DHL model.

We also monitored for murine survival following treatment with vehicle, cyclophosphamide, alemtuzumab or the combination.

Alemtuzumab, cyclophosphamide and doxorubicin monotherapies each reduced disease burden in the peripheral blood and spleens of DHL-engrafted mice (Figure 2.2A, B). Akin to the humaneered DHL model, the bone marrow was an alemtuzumab-resistant niche (Figure 2.2B). While CTX was able to induce partial tumor regression, the combination of CTX and alemtuzumab was able to eliminate nearly all disease in the bone marrow in both models (Figure 2.2B), demonstrating 160- and 88-fold synergy in DFBL-20954 and DFBL-69487, respectively, in clearing tumor. Alemtuzumab and cyclophosphamide monotherapies each extended murine survival compared to vehicle-treated mice engrafted with both xenografts (Figure 2.2C). The combination of alemtuzumab and cyclophosphamide was able to significantly prolong murine survival compared to each drug given as a monotherapy; however, in contrast with the humaneered DHL model, all mice ultimately succumbed to disease.

Doxorubicin monotherapy and in combination with alemtuzumab failed to induce bone marrow tumor regression in both models (Figure 2.2B). This was not due to doxorubicin failing to penetrate the bone marrow, as doxorubicin was able to induce DNA damage in bone marrow tumor cells as assessed by gamma-H2AX levels at a similar level to DNA damage induced by high-dose CTX (Figure 2.2D).

We next asked whether the high-doses of CTX were necessary to overcome bone marrow antibody resistance. We engrafted NSG mice with DFBL-20954 and tested the ability of lower doses of CTX (25mg/kg and 50mg/kg, respectively) to overcome bone marrow antibody resistance by assaying bone marrow tumor burden at day 8 post treatment. This revealed a dose-dependent synergistic effect of CTX: 25mg/kg of the drug was unable to induce tumor clearance

Figure 2.2 Alkylating Agents Overcome Bone Marrow Alemtuzumab Resistance. (A) Peripheral blood involvement for both PDX models. Tumor-bearing mice treated with PBS, Cyclophosphamide (CTX), Doxorubicin (Dox) Alem (Alem) or combinations, as indicated. Mice were bled and RBC-lysed viable (7AAD⁻) cells were assessed for hCD19/hCD45⁺ positivity. Two-sided Welch *t*-test, **p*<0.05, ***p*<0.01, ****p*<0.001 (B) On day 8 of treatment, the spleen was harvested and a single femur was flushed from mice treated with PBS, Cyclophosphamide (CTX), Doxorubicin (Dox) Alem (Alem) or combinations, as indicated. Total cells were counted with and analyzed for expression of hCD19⁺hCD45⁺7-AAD⁻. Total tumor cells present are represented as the product of total cells * viable hCD19/hCD45 double positive cells. All comparisons by two-sided Welch *t*-test. (C) Kaplan-Meier curves of overall survival of NSG mice engrafted with DFBL-20954 and DFBL-69487 treated with PBS, CTX, Alem or CTX+Alem. *p* <0.0001 CTX vs Veh, *p*<0.001 CTX+Alem vs CTX for 20954, *p*<0.001 CTX+Alem vs CTX for 69487, *p*<0.001 for CTX+Alem vs. CTX for both. All comparisons by log-rank test. (D) Western blot of gamma-H2A.X levels in BM of mice bearing DFBL-20954 xenograft 16 hours after the indicated treatment. Each lane represents an individual mouse. (E) Bone marrow tumor burden of NSG mice engrafted with DFBL-20954 and treated with PBS, Alem, and CTX in the indicated dose. Total tumor cells present are represented as the product of total cells * viable (7AAD⁻) hCD19/hCD45 double positive cells. BM cells represent tumor cells per mouse femur. Two-sided Welch *t*-test. (F) BH3 profiling of DFBL-20954 cells isolated from peripheral blood, spleen and BM (n=3 per condition) at a time when disease burden was >2% in peripheral blood. (G) Bone marrow tumor burden of NSG mice engrafted with DFBL-20954 and treated with PBS, alemtuzumab, CTX, ABT-263 or IR (4gy). Total tumor burden was assessed from one femur as in (B). All comparisons by two-sided Welch *t*-test. (H) Bone marrow tumor burden on day 8 of DFBL-20954 engrafted mice treated as indicated (mg/kg) when disease burden was >2% in peripheral blood. Total tumor burden was assessed as in (B). (I) Bone marrow tumor burden of NSG mice engrafted with DFBL-20954 and treated with PBS, Alem, CTX, ifosfamide (ifos) or busulfan (bus). Total tumor burden was assessed from one femur as in (B). All comparisons by two-sided Welch *t*-test

Figure 2.2 (Continued)

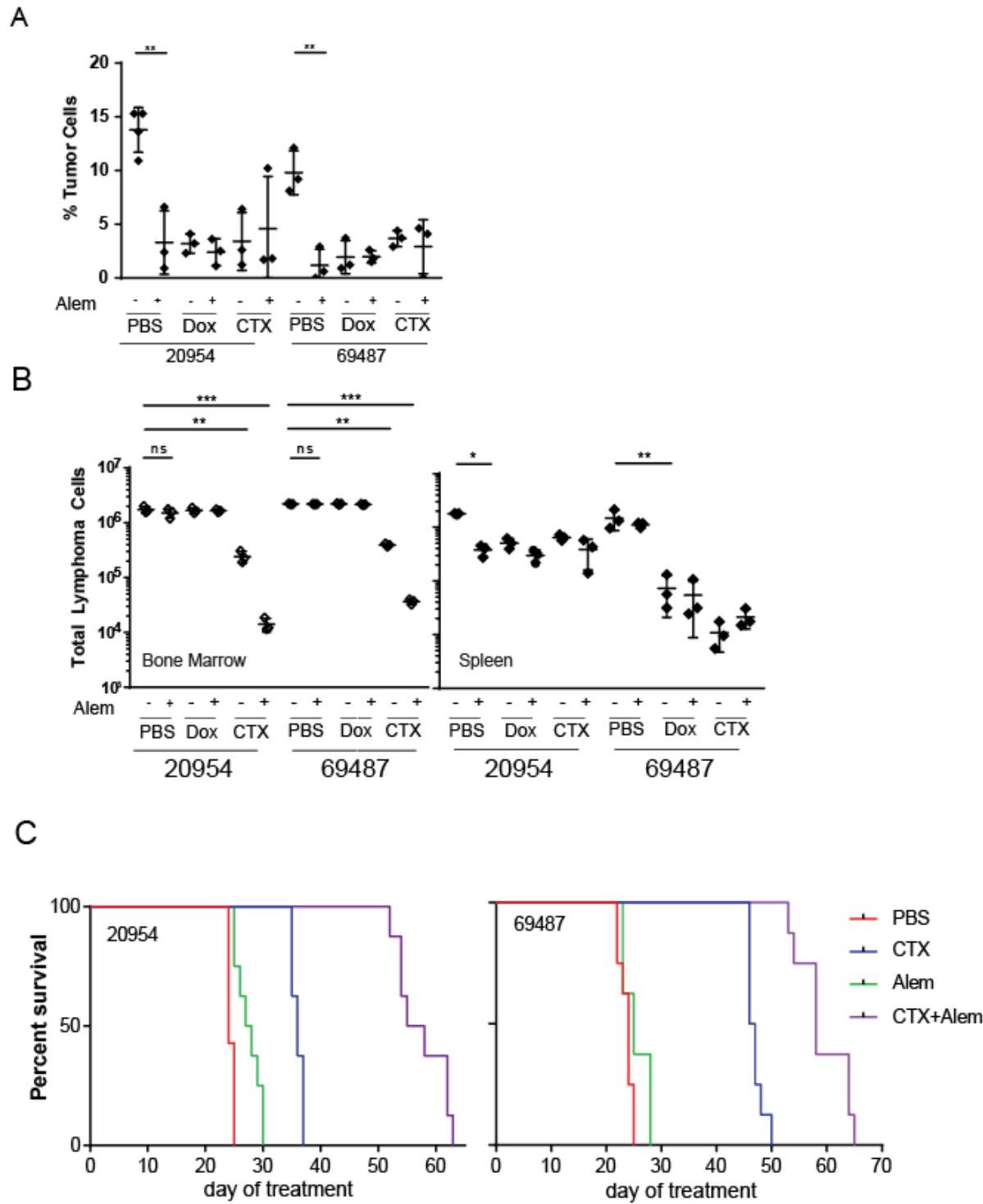
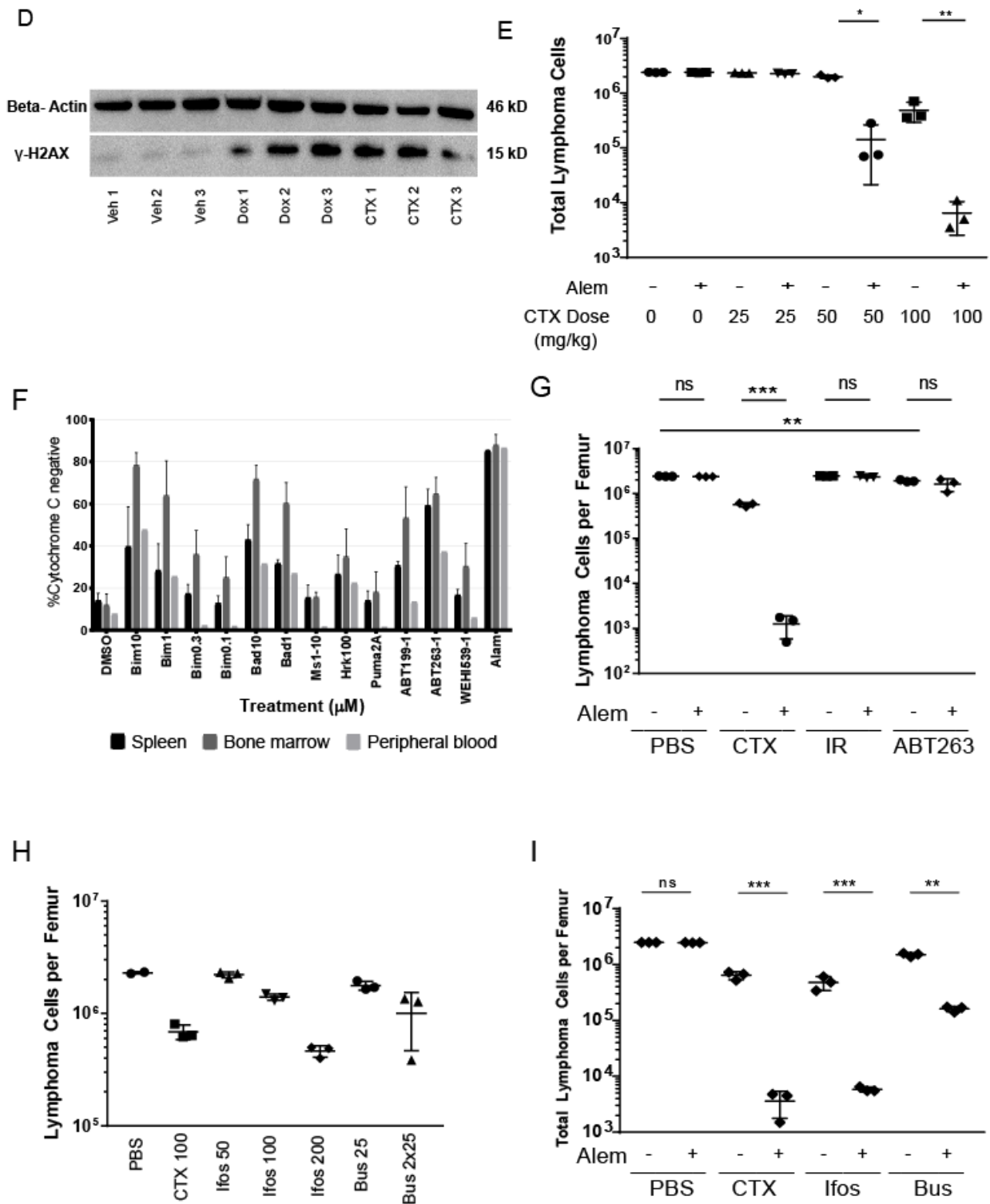


Figure 2.2 (Continued)



as a monotherapy or synergize with alemtuzumab; meanwhile, 50mg/kg of CTX was able to induce partial bone marrow tumor clearance and some synergy with alemtuzumab (10 fold) (Figure 2.2E). However, this was much lower than the synergy observed with the 100 mg/kg dose (160 fold) (Figure 2.2E).

Given these observations, it is possible that the synergy between CTX and alemtuzumab is due to tumor debulking or from the presence of apoptotic debris that could then non-specifically activate macrophages. We therefore sought to find treatment options that could induce DHL tumor clearance from the bone marrow. We reasoned that ionizing radiation, which exhibited partial killing of the humanized DHL, may induce killing of our PDXs. We further performed BH3 profiling of DHL-20954 cells to identify the apoptotic dependencies of the PDX (Figure 2.2F). This revealed dependence on BCL2 and BCL-xL for survival, as seen by cytochrome C release by the Bad and HRK peptides, respectively, and suggested vulnerability to treatment with ABT-263, a dual BCL-2 and BCL-xL inhibitor. Surprisingly, bone marrow DHL cells removed from the microenvironment were more primed to undergo apoptosis than splenic cells, suggesting that bone marrow chemotherapy resistance was likely due to a tumor extrinsic mechanism.

Treatment of DFBL-20954 with ionizing radiation failed to induce tumor cell death and did not synergize with alemtuzumab treatment, consistent with results from the humanized DHL model (Figure 2.2G). While ABT-263 was able to induce a 20% reduction in tumor burden, no synergy was observed with alemtuzumab. Together these results strongly suggest that the synergy between CTX and alemtuzumab were not solely due to a reduced tumor cell burden or nonspecific macrophage activation.

We next asked whether the ability of CTX to overcome bone marrow antibody resistance

was unique to CTX or a general property of alkylating agents. We therefore tested the efficacy of ifosfamide, which is metabolized to the same nitrogen mustard reactive intermediate as CTX, and busulfan, which has alkylating agent activity through a carbonium ion intermediate, in overcoming bone marrow antibody resistance. We initially treated DFBL-20954-bearing NSG mice with different amounts of the two drugs to identify drug doses that recapitulated a similar amount of tumor killing as 100mg/kg of CTX (Figure 2.2H). 200mg/kg of ifosfamide induced similar levels of tumor killing as 100mg/kg of CTX. While 50mg/kg of busulfan (given as two doses of 25mg/kg) induced less cell death than the 100mg/kg CTX dose, this was the administered dose of the drug in all future experiments as the NSG mice did not tolerate higher doses. Treatment of DFBL-20954-bearing NSG mice with either ifosfamide or busulfan in the presence and absence of alemtuzumab demonstrated that both alkylating agents were able to synergize with alemtuzumab in facilitating tumor clearance (Figure 2.2I). Together, these observations demonstrate that the bone marrow of DHL engrafted mice forms an antibody-resistant niche that can be overcome by treatment with high-dose alkylating agents.

Chapter 3: Bone marrow alemtuzumab resistance is primarily driven by a high lymphoma to macrophage ratio

Background

Many factors can promote resistance to antibody therapy, including loss of target antigen, inability of the antibody to access the tumor cell and effector cell exhaustion (Beurskens et al., 2012; Rezvani and Maloney, 2011). Given the complex interplay between tumor cells and their microenvironment, it is also possible that tumor mediated reprogramming of the environment can cause antibody resistance (Dahal et al., 2017). Alemtuzumab can induce direct apoptosis of some lymphoma cells, suggesting that tumors may acquire resistance to directly evade apoptosis (Nguyen et al., 2012). Studies of the humaneered DHL model demonstrated that bone marrow alemtuzumab resistance developed over time and was partly due to intrinsic differences in tumor cells from different microenvironments (Pallasch et al., 2014). We hypothesized that similar factors could mediate bone marrow resistance in our models but sought to determine additional factors that confer resistance.

Results

Given that alemtuzumab is known to induce direct apoptosis of lymphomas, we first asked whether direct alemtuzumab-induced apoptosis was a mechanism of DHL killing. Incubation of DFBL-20954 and DFBL-69487 tumor cells with alemtuzumab failed to induce lymphoma cell death, suggesting that alemtuzumab mediated tumor clearance was dependent on microenvironmental effector cells (Figure 3.1A).

In the humaneered DHL model, alemtuzumab activity was demonstrated to be mediated by macrophage antibody dependent cellular phagocytosis (ADCP). To test whether this was the case with our models we labeled DHL-20954 cells with DAPI and co-cultured them *in vitro* with bone marrow-derived macrophages (BMDMs) in the presence of alemtuzumab. Following two hours of co-culture we labeled macrophages using a FITC conjugated anti- F4/80 antibody and

Figure 3.1 Macrophages are the Effector Cells of Alemtuzumab Treatment (A) Bone marrow cells from untreated mice (n=3 per condition) engrafted with DFBL-20954 and DFBL-69487 were co-cultured in the presence of Alem (30 ug/ml). Apoptosis was assayed 48 hours after co-incubation. (B) Fluorescent microscopy of BMDM (green) phagocytosis of DFBL-20954 cells (blue) in the presence of alemtuzumab. Representative image shown. (C) 1×10^6 DFBL-20954 cells were injected into NSG mice and Alem treatment (daily x 2) was initiated on the indicated days 8, 12 and 16 (base of blue arrows). The tips of the blue arrows correspond to tumor burden of mice treated with Alem at 48 hours after the second treatment dose. N=3 mice for each condition at each time point. (D) NSG mice were treated with clodronate liposomes (200 ul) and assessed for splenic (CD11b⁺F4/80⁺) and bone marrow (CD11b⁺F4/80⁺) monocytes/macrophages. Representative flow cytometry plots shown. (E) On day 10 after transplant of 1×10^6 DFBL-20954 cells, mice were treated with clodronate liposomes (clod, 200ul) or PBS. Alem (Alem) was given as a single dose of 10mg/kg i.v. on day 12. Tumor burden was assessed 48 hours after treatment. Two-sided Welch *t*-test, **p*<0.05, ***p*<0.01, ****p*<0.001. (F) Assessment of DFBL-20954 lymphoma cells after *in vivo* treatment with APC-conjugated Alem. Representative flow cytometry plots shown. (G) Assessment of *in vivo* bound DFBL-20954 lymphoma cells with APC-conjugated Alem. Representative flow cytometry plot shown 5 days after Alem administration. BM stained with hCD19 from mice not treated with Alem is depicted on the left.

Figure 3.1 (Continued)

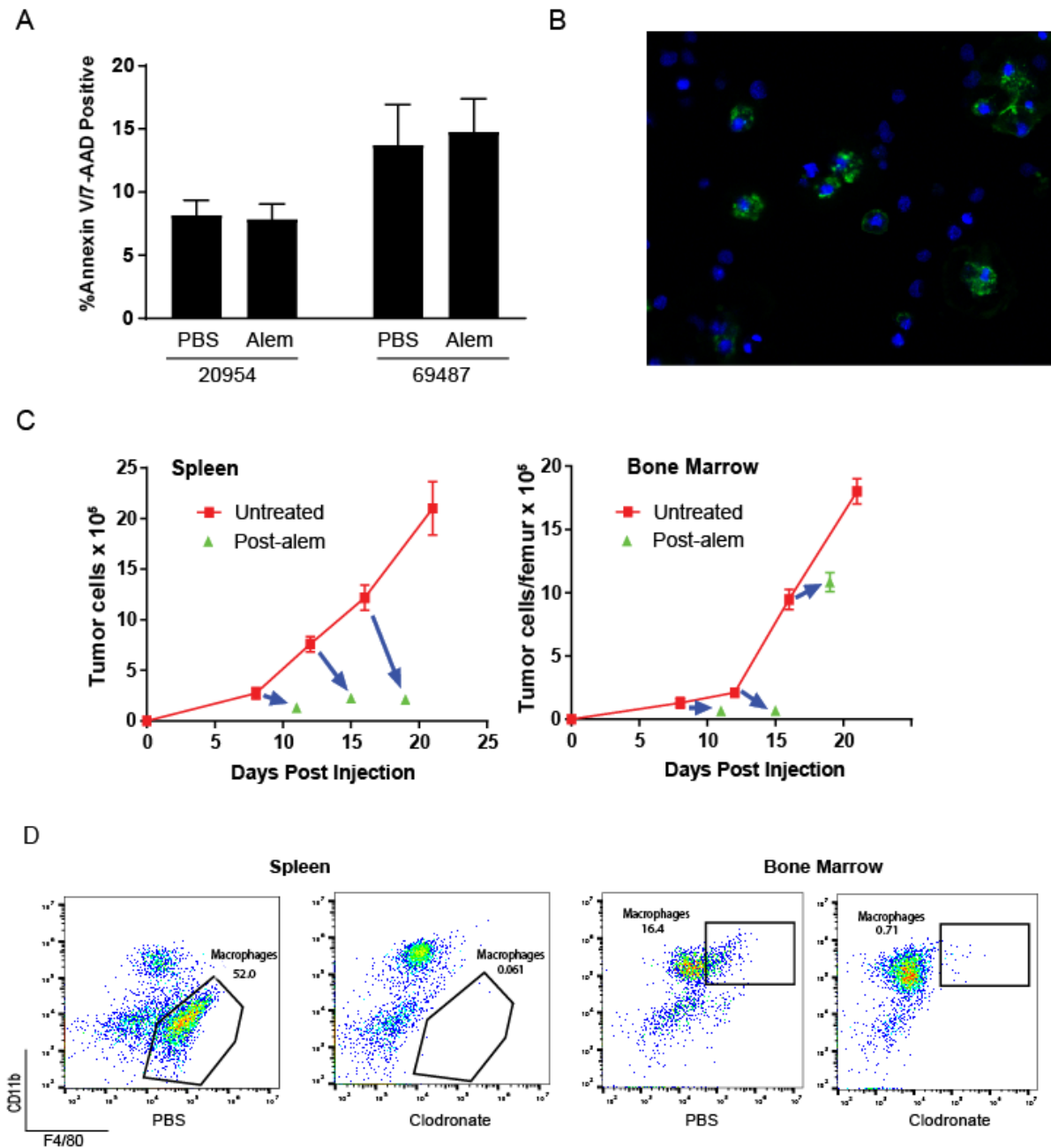
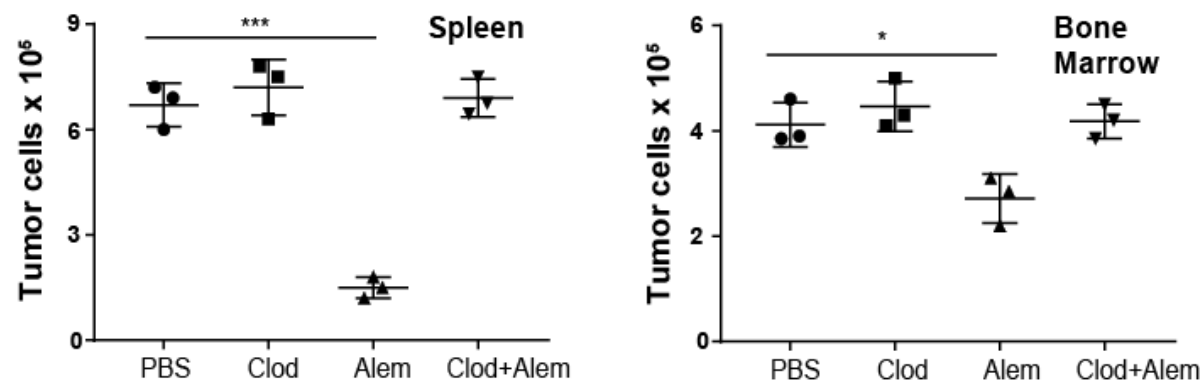
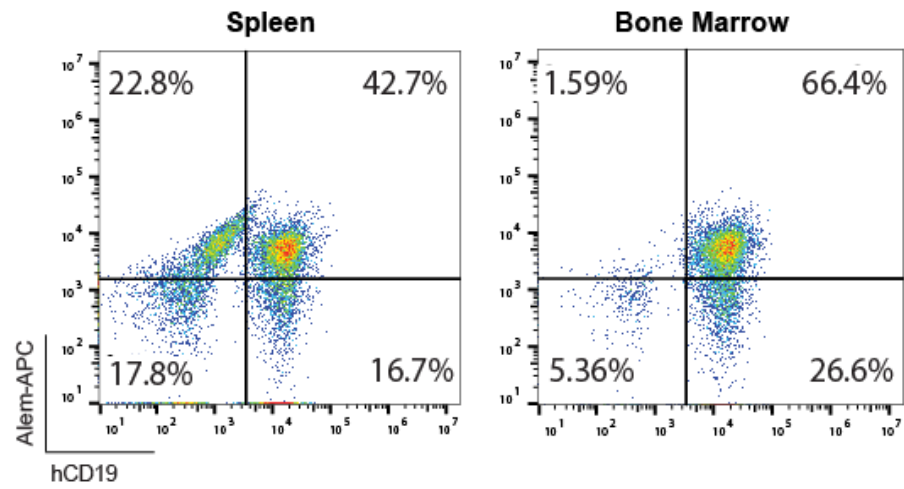


Figure 3.1 (Continued)

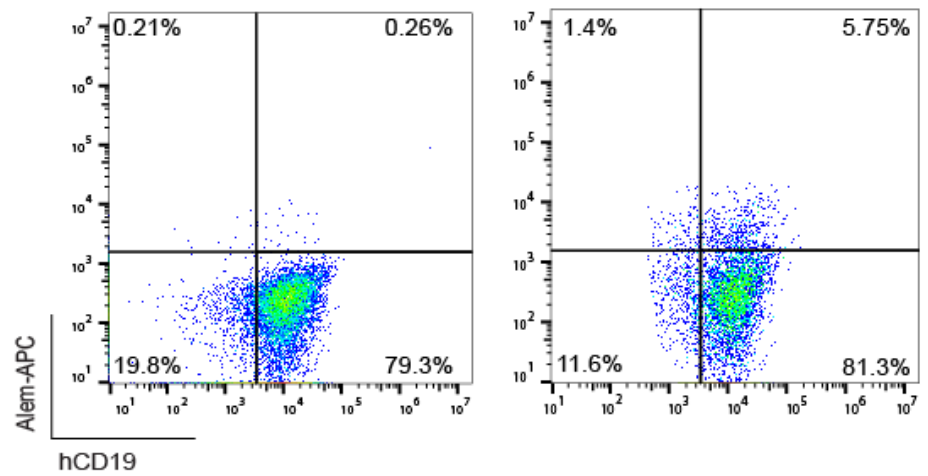
E



F



G



performed fluorescent microscopy looking for tumor cell engulfment. Microscopy confirmed phagocytosis of DHL cells following alemtuzumab administration (Figure 3.1B).

We next asked whether alemtuzumab resistance was an inherent or acquired property of the bone marrow microenvironment. Following injection of DFBL-20954 into NSG mice we treated mice with alemtuzumab over 2 days (5mg/kg each day), initiating treatment at 8, 12 or 16 days post injection and assessed tumor burden in the spleen and bone marrow 48 hours after the second antibody dose. While alemtuzumab consistently led to tumor regression in the spleen, bone marrow response to alemtuzumab was initially present when treatment was initiated on days 8 and 12 but not when initiated on day 16, suggesting that bone marrow alemtuzumab resistance results from extensive engraftment (i.e., acquired) (Figure 3.1C).

In addition to macrophages, *in vivo* alemtuzumab activity in immunocompromised mice has also been attributed to neutrophils. Since both cell populations are present in the bone marrow of NSG mice we asked whether alemtuzumab clearance was solely mediated by macrophages. To test this we injected NSG mice with DFBL-20954 and treated with alemtuzumab on day 12, a time point where both microenvironments responded to treatment. Two days prior to alemtuzumab administration the mice were treated with clodronate (200 ul intravenously) that led to complete macrophage depletion in the spleen and near complete macrophage depletion in the bone marrow (Figure 3.1D). Clodronate pre-treatment led to complete loss of alemtuzumab activity in both bone marrow and spleen, suggesting that macrophages were the alemtuzumab effector cells both microenvironments (Figure 3.1E).

We hypothesized that the lack of alemtuzumab efficacy at later points of engraftment could be due to impenetrance of antibody to the bone marrow or alternatively from rapid bone marrow antibody clearance. To test these hypotheses we generated a fluorophore conjugated

alemtuzumab (alem-APC) and treated NSG mice engrafted with DFBL-20954 21 days post engraftment with the conjugated antibody (5mg/kg). At 4 hours after treatment the vast majority of bone marrow tumor cells were labeled with alemtuzumab (Figure 3.1F). Labeling of bone marrow and splenic tumor cells with alemtuzumab was similar, suggesting antibody penetrance to both microenvironments. Conjugated alemtuzumab was detected bound to bone marrow tumor cells up to five days following injection (Figure 3.1G). Together these observations indicate that bone marrow alemtuzumab resistance is not due to antibody impenetrance or clearance from the bone marrow.

Since macrophages are the effector cells of alemtuzumab therapy, we hypothesized that bone marrow resistance may be due to the lymphoma cells reprogramming or tolerizing macrophages. To test this, we sorted primary bone marrow macrophages from unengrafted NSG mice or NSGs engrafted with the DHL xenografts and assayed their ability to phagocytose bone marrow-derived DHL cells from the same xenograft (aka cells that the macrophages were “experienced” to) or to Raji Burkitt lymphoma cells (aka cells that the macrophages were naïve to) in the presence or absence of alemtuzumab. No differences were observed in macrophage phagocytosis from unengrafted or engrafted mice to clear Raji or xenograft cells (Figure 3.2A), suggesting that macrophage tolerization was not driving alemtuzumab resistance.

Alternatively, it is possible that bone marrow antibody resistance was due to an inherently lower capacity of bone marrow macrophages to phagocytose lymphoma cells as compared to their splenic counterparts. We therefore sorted primary splenic and bone marrow macrophages from unengrafted NSGs or mice engrafted with each DHL xenograft and assayed their capacity to phagocytose alemtuzumab coated Raji cells ex vivo. Macrophages from the spleen and bone marrow from all mice displayed a similar capacity to phagocytose the

Figure 3.2 Bone Marrow Alemtuzumab Resistance is Driven by a High Lymphoma to Macrophage Ratio

(A) Primary, viable BM (7-AAD⁻hCD19⁺CD11b⁺F4/80⁺) monocytes/macrophages were sorted from unengrafted or lymphoma-engrafted NSG mice. Bone marrow tumor cells from corresponding engrafted mice or Raji cells were incubated in the presence or absence of Alem. All comparisons between unengrafted and vehicle under the same conditions (n=3 per condition) were non-significant. (B) Primary viable splenic (7AAD⁻hCD19⁺CD11b⁺F4/80⁺) and BM (hCD19⁺CD11b⁺F4/80⁺) monocytes/macrophages were sorted from unengrafted or lymphoma-engrafted NSG mice and cultured with BM lymphoma cells from the corresponding xenograft that were treated *ex vivo* with Alem. All comparisons between splenic and BM macrophages under the same conditions (n=3 per condition) were non-significant. (C) Ratio of lymphoma (hCD19⁺hCD45⁺) to macrophage (hCD19⁺CD11b⁺F4/80⁺) cells 48 hours after chemotherapy initiation of DFBL-20954 and DFBL-69487 engrafted mice. Two-sided Welch *t*-test, **p*<0.05, ***p*<0.01, ****p*<0.001. Dox vs CTX BM Not significant (D) Characterization of DFBL-20954 tumor burden and tumor to macrophage ratio upon incomplete macrophage depletion with clodronate (25ul). Representative flow cytometry plots shown. (E) Mice were engrafted with DFBL-20954 and given clodronate (25ul) or vehicle on day 19 followed by Alem or vehicle on day 21 and 22 and then sacrificed 48 hours after dosing. Spleen tumor burden shown. Two-sided Welch *t*-test (F) Macrophage BM (hCD19⁺CD11b⁺F4/80⁺) cellularity based on flow cytometry 8 days after initiation of the indicated agents. (G) Representative anti-F4/80 staining (brown) of fixed femurs from mice engrafted with DFBL-20954 and DFBL-69487 harvested 48 hours after treatment with the indicated agents. Images are 1x magnified (H) DFBL-20954-bearing NSG mice were treated with PBS or CTX (n=6 per condition) and BM macrophage cell cycle was assessed 16 hours following chemotherapy administration with Vybrant dye. Bone marrow cells from PBS-treated mice are shown for positive staining control. Representative flow cytometry plots shown.

Figure 3.2 (Continued)

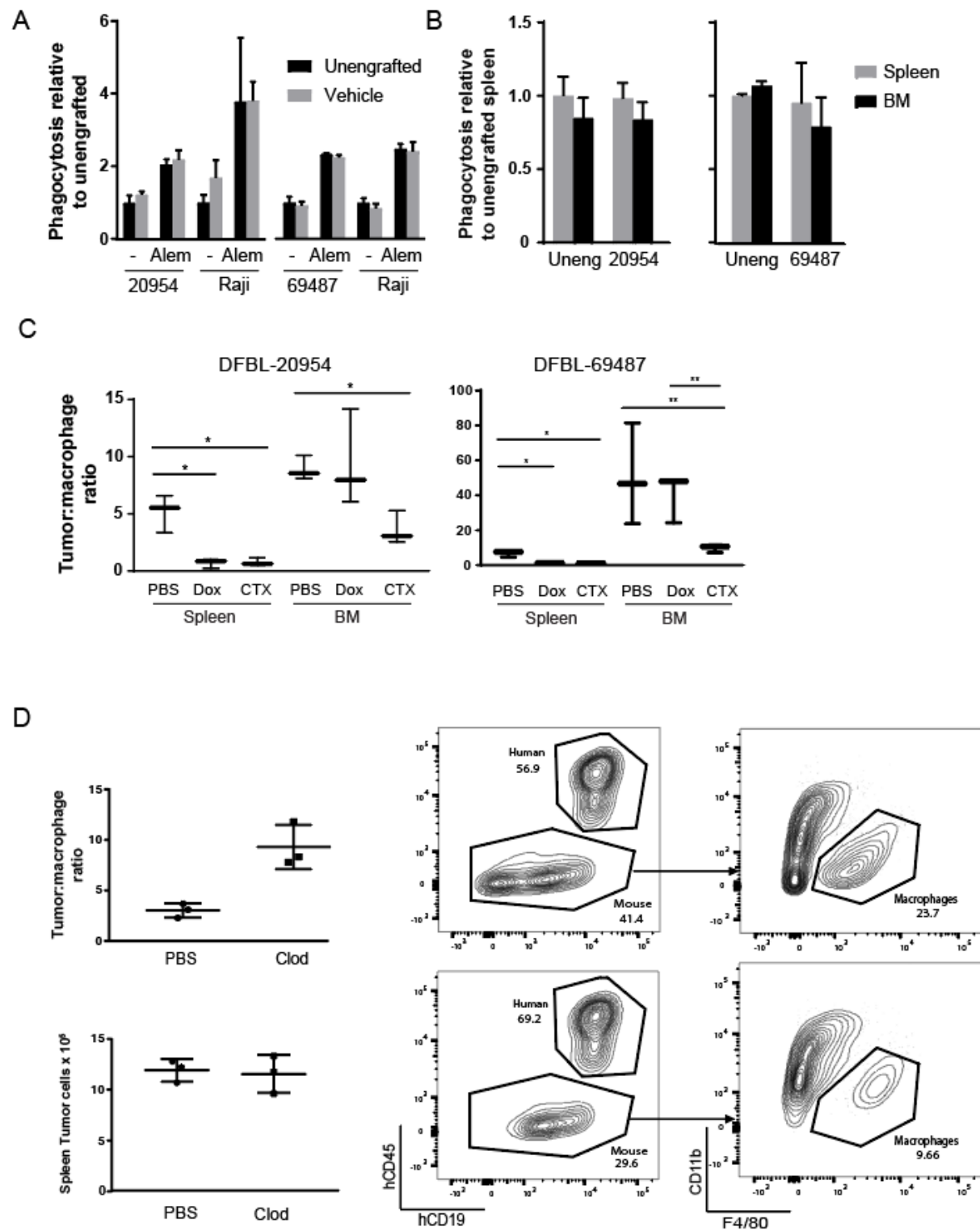
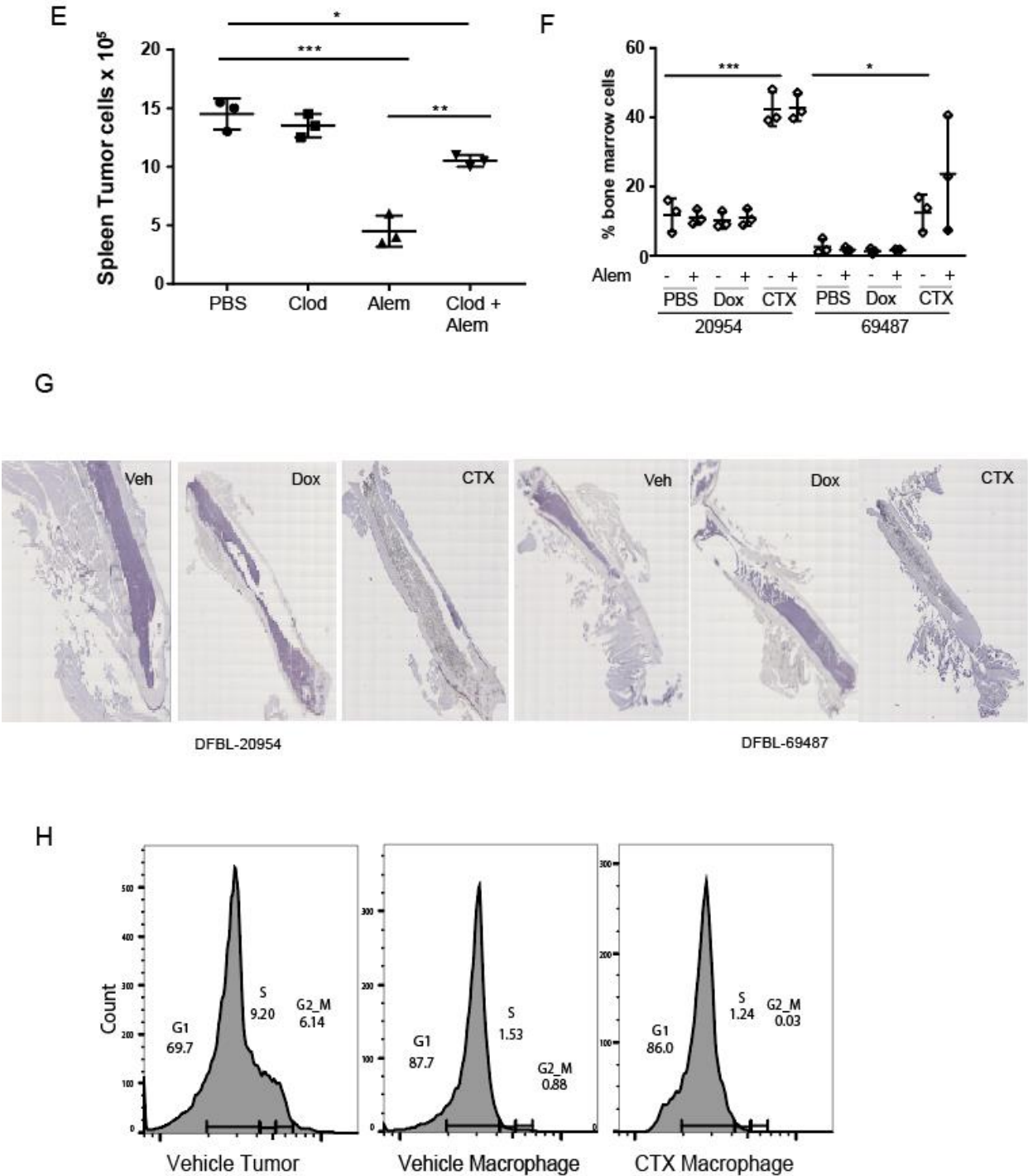


Figure 3.2 (Continued)



lymphoma cells (Figure 3.2B), suggesting that microenvironmental differences in macrophage phagocytosis were not the major drivers of alemtuzumab resistance.

We observed that at the time of alemtuzumab treatment initiation the ratio of lymphoma cells to macrophages was higher in the bone marrow as compared to the spleen of engrafted NSG mice in both xenografts (Figure 3.2C). While both doxorubicin and CTX were able to reduce the tumor to macrophage ratio in the spleens 48 hours after treatment, only CTX was able to decrease the ratio in the bone marrow (Figure 3.2C). We therefore asked whether the lymphoma to macrophage ratio is driver alemtuzumab resistance. We pretreated DFBL-20954 engrafted mice with a dose of clodronate (25 ul intravenously) that led to a partial depletion of macrophages, recapitulating the splenic lymphoma: macrophage ratio observed in the bone marrow at day 21 post engraftment (Figure 3.2D). We then initiated alemtuzumab treatment (2x5mg/kg). Importantly, clodronate treatment did not alter the total amount of lymphoma cells in the spleen or bone marrow (Figure 3.2D). Partial macrophage depletion led to near complete loss of alemtuzumab activity in the spleen (Figure 3.2E), suggesting that bone marrow alemtuzumab resistance was primarily driven by a high lymphoma: macrophage ratio.

The decreased lymphoma to macrophage ratio following CTX treatment was associated with an increased bone marrow macrophage cellularity (Figure 3.2F). This can be caused due to reduced tumor burden, increased macrophage number or both. To test for bone marrow macrophage content we fixed femurs from mice engrafted with DFBL-20954 and DFBL-69487 48 hours after treatment with PBS (Vehicle), doxorubicin or CTX and performed immunohistochemistry for the macrophage marker F4/80. CTX but not doxorubicin therapy led to an increased number of bone marrow macrophages (Figure 3.2G). These macrophages were likely recruited to the bone marrow as cell cycle analysis at 16 hours after CTX treatment of

DFBL-20954 engrafted cells demonstrated a near absence of dividing macrophages (Figure 3.2H).

Chapter 4: Cyclophosphamide Induces Lymphoma Phenotypic Changes and a Secretory Phenotype to Promote Tumor Clearance by Macrophages

Background

Studies utilizing the humaneered DHL model demonstrated that splenic tumor cells were more likely to be phagocytosed than their bone marrow counterparts (Pallasch et al., 2014). Whether this effect was antibody dependent or independent, however, was not analyzed. While the authors demonstrated higher expression of CD32B on the cell surface of bone marrow-derived lymphoma cells, suggesting an antibody dependent anti-phagocytic effect, whether other pro- and anti-phagocytic factors differed between cells from the two microenvironments was unexplored. These pro- and anti- phagocytic markers include the “don’t eat me” CD47 as well the “eat me” VISTA and calreticulin proteins. Other known tumor-derived modulators of macrophage phagocytosis include galectin-1, which is known to be upregulated on lymphomas compared to normal B-cell counterparts and inhibits lymphoma phagocytosis in an antibody dependent manner and prostaglandin E2 (PGE2), which was found to inhibit alemtuzumab-mediated clearance of the humaneered DHL model (Lykken et al., 2016; Pallasch et al., 2014).

CTX treatment of mice engrafted with the humaneered DHL increased phagocytosis of bone marrow-derived cells to levels of untreated splenic cells and further increased phagocytosis of splenic tumor cell by macrophages (Pallasch et al., 2014). The increase in bone marrow-derived DHL cell uptake associated with modulation of pro- and anti-apoptotic factors on the tumor cells, specifically decreased expression of surface CD47 and increased surface calreticulin. CTX also promoted an “acute secretory activating phenotype” (ASAP) that led to tumor secretion of TNF- α , VEGF-A, CCL4 and IL-8. VEGF-A, TNF- α and CCL4 were shown to enhance macrophage phagocytosis of the DHL cells, but the mechanisms by which they promoted tumor clearance were not explored. Whether these changes are observed in *bona fide* human DHL after high-dose CTX remains unknown.

Results

Having established that a higher lymphoma to macrophage ratio in the bone marrow was associated with alemtuzumab resistance, we sought to identify the mechanism by which this ratio was established. Given that modulation of the splenic lymphoma/macrophage ratio to bone marrow levels did not completely abolish alemtuzumab activity, we hypothesized that the residual activity of alemtuzumab in the spleen was due to differences in tumor intrinsic factors that inhibited macrophage phagocytosis in the bone marrow. Moreover, we hypothesized that CTX treatment exerted phenotypic changes to the lymphoma cells that promoted their elimination by macrophages.

To test these hypotheses, we isolated tumor cells from the spleen and bone marrow of mice engrafted with either lymphoma PDX following treatment with PBS, doxorubicin and CTX and assayed their susceptibility to phagocytosis by BMDMs from unengrafted mice. DFBL-20954 splenic cells were significantly more likely to be phagocytosed compared to their bone marrow counterparts in the presence and absence of antibody (Figure 4.1A). While doxorubicin failed to increase the likelihood of cells from either compartment to be phagocytosed, CTX treatment increased the “phagocytic proneness” of tumor cells (Figure 4.1A). In contrast, DFBL-69487 splenic cells were significantly more likely to be phagocytosed than their bone marrow counterparts only in the presence and absence of alemtuzumab (Figure 4.1A). Doxorubicin failed to increase the phagocytic proneness of cells from the two compartments but CTX treatment promoted macrophage uptake of tumor cells (Figure 4.1A).

The above observations suggested that the full activity of CTX in eliminating tumor cells may be macrophage dependent. We therefore treated mice engrafted with DFBL-20954 that were pre-treated either with PBS or a dose of clodronate that leads to near complete loss of

Figure 4.1 The tumor microenvironment and chemotherapy alter the ability of macrophages to phagocytose DHL cells (A) Tumor cells from the spleen and BM of mice (n=3 per condition) engrafted with DFBL-20954 and DFBL-69487 were isolated by mouse cell depletion 16 hours (DFBL-20954) or 48 hours (DFBL-69487) after treatment with PBS, doxorubicin (Dox), or cyclophosphamide (CTX). Dead cells were removed and the remaining live tumor cells were incubated *ex vivo* with bone marrow-derived macrophages in the presence and absence of Alem (Alem). Two-sided Welch's t-test, *p<0.05, **p<0.01, ***p<0.001 (B) On day 19 after transplant of 10⁶ DFBL-20954 cells, mice received clodronate or PBS (-). On day 21, mice received CTX, Alem (10mg/kg i.v. single dose), the combination or vehicle (PBS) and assessed for tumor burden on day 23. Spleens were harvested and bone marrows flushed. Mouse cells were depleted and cell numbers and viability assessed with trypan blue staining. Tumor burden is reported as the number of viable tumor cells. Two-sided Welch *t*-test (C) Surface expression by mean fluorescence intensity (MFI) compared to isotype control or total protein level by ELISA for the indicated proteins in the bone marrow (B) and spleen (S) of DFBL-20954 and DFBL-69487-engrafted mice (n=3-6 per condition).

Figure 4.1 (Continued)

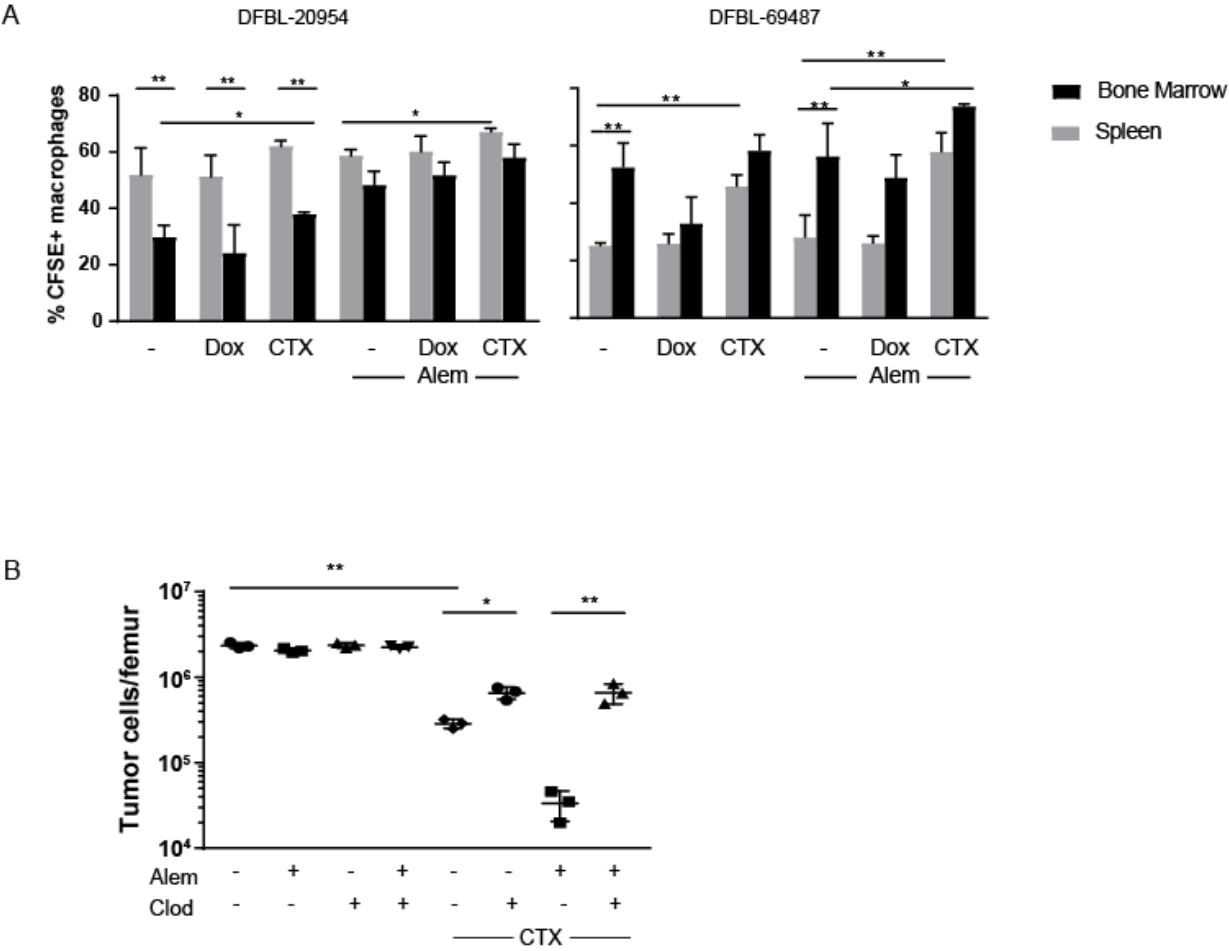
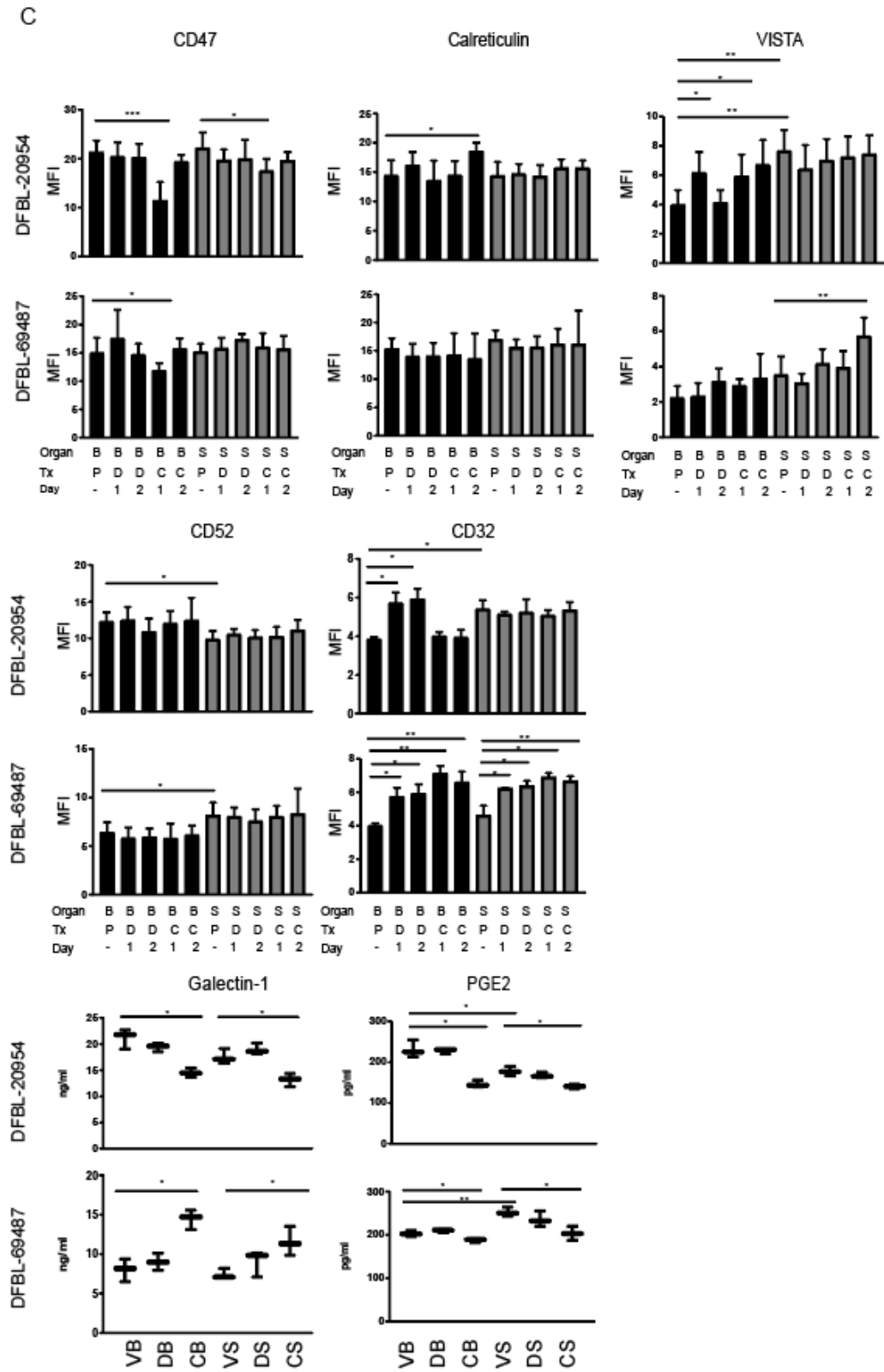


Figure 4.1 (Continued)



macrophages in the bone marrow with PBS, alemtuzumab, CTX or CTX together with alemtuzumab at day 21 post injection. As expected, macrophage depletion led to complete loss of the observed synergy between CTX and alemtuzumab (Figure 4.1B). Consistent with the enhanced proneness of tumor cells post CTX treatment to be phagocytosed, pre-depletion of macrophages led to a partial loss of CTX monotherapy activity (Figure 4.1B).

We next quantified the expression of factors known to affect macrophage phagocytosis from splenic and bone marrow-derived DHL cells by ELISA and flow cytometry from PBS, doxorubicin- and CTX- treated mice. In DFBL-20954, flow cytometric analysis demonstrated that surface expression of the pro-phagocytic molecule VISTA was decreased in bone marrow lymphoma cells compared to splenic lymphoma cells; meanwhile expression of CD32 was elevated on bone marrow-derived lymphoma cells in these same mice (Figure 4.1C). Expression of surface CD47 and calreticulin were not different between tumor cells from the two microenvironments (Figure 4.1C). ELISAs demonstrated statistically significant increased levels of the anti-phagocytic factors PGE2 and galectin-1 in bone marrow-derived lymphoma cells compared to their splenic counterparts in PBS-treated mice (Figure 4.1C).

DFBL-69487 cells had higher expression of PGE-2 and galectin-1 in splenic lymphoma cells as compared to bone marrow cells in PBS- treated mice (Figure 4.1C). No differences were observed in the expression of VISTA, CD32, CD47 and calreticulin of bone marrow and splenic tumor cells. Surface CD52 expression was higher in the bone marrow than the spleen in this model while the opposite trend was observed in DFBL-20954.

Following CTX but not doxorubicin treatment of DFBL-20954 engrafted mice, levels of PGE2 and galectin-1 as well surface levels of CD47 cells were decreased in both bone marrow and splenic cells compared to their counterparts in PBS-treated mice (Figure 4.1C). Surface

calreticulin levels were also increased on DFBL-20954 cells exclusively post CTX administration but only on bone marrow cells. Surface expression of VISTA increased post CTX and doxorubicin administration in bone marrow cells, consistent with surface VISTA expression being regulated by P53 activity (Yoon et al., 2015). No changes in surface CD32 or CD52 were seen following chemotherapy treatment (Figure 4.1C).

CTX but not doxorubicin treatment decreased levels of PGE2 in splenic and bone marrow DFBL-69487 cells compared to their PBS-treated counterparts and, in contrast to observations in DFBL-20954, increased protein levels of galectin-1 (Figure 4.1C). CTX also led to decreased surface expression of CD47 in bone marrow- but not spleen-derived tumor cells (Figure 4.1C). No changes in surface VISTA or calreticulin levels were observed in this model after CTX treatment (Figure 4.1C).

We next asked whether PGE-2 and galectin-1 could affect macrophage phagocytosis of DHL cells. Addition of recombinant PGE2 (2 ng/ml) inhibited BMDM phagocytosis of bone marrow-derived DFBL-20954 and DFBL-69487 cells *in vitro* both in the presence and absence of alemtuzumab (Figure 4.2A). Addition of recombinant galectin-1 (500 ng/ml) inhibited phagocytosis of DFBL-20954 cells by macrophages *ex vivo* only in the presence of alemtuzumab (Figure 4.2B). In contrast, galectin-1 enhanced phagocytosis of DFBL-69487 cells in the presence and absence of alemtuzumab (Figure 4.2B). Galectin-1 mediated effects on phagocytosis were reversed by the galectin-1 inhibitor lactose, suggesting direct galectin-1 effects.

We next asked whether secreted factors from lymphoma cells could also mediate macrophage phagocytosis. To test this, we first generated conditioned media from *ex vivo* cultures of isolated bone marrow and splenic DFBL-20954 cells from mice treated with PBS,

Figure 4.2 CTX Induces Secretory Changes in DHL cells that promote Tumor Clearance by Macrophages (A) DFBL-20954 and DFBL-69487 cells were isolated from BM of untreated mice, labeled with CFSE and co-cultured with bone marrow-derived macrophages in the presence or absence of Alem and Prostaglandin E2 (PGE2, 2ng/ml). Phagocytosis was quantified as percent of CD11b⁺ cells that were CFSE⁺. Representative experiment depicted. (B) As in (A) but with recombinant human galectin-1 (Gal-1, 500 ng/ml) and lactose (20 mM). (C) DFBL-20954 cells were isolated from bone marrow of untreated mice, labeled with CFSE and co-cultured with bone marrow-derived macrophages and conditioned media from mice (n=3 per condition) treated as indicated. MC = media control. Alem specific killing is defined as the % increase in CFSE⁺ macrophages in the presence of Alem compared to no antibody. Unpaired two-sided-*t*-test, **p<0.01, *** p<0.001 (D) Levels of human VEGF-A and IL-16 from bone marrow and spleen of DFBL-20954 and DFBL-69487-engrafted mice collected 16 hours (DFBL-20954) or 40 hours (DFBL-69487) after *in vivo* treatment with vehicle (-), doxorubicin (Dox) or CTX. (E) As in (D), but at 16 hours for DFBL-69487. (F) Fold changes in human VEGF-A expression in conditioned media from DFBL-20954 BM cells treated as indicated (n=3 per condition). Unpaired two-sided *t*-test, *p<0.05 (G) Cytokine array levels of human VEGF-A and IL-16 16 hours from BM of mice engrafted with DFBL-20954 after treatment with PBS (Veh), Ifosfamide (ifos), busulfan (Bu) and CTX. * p<0.05, ** p<0.01 (H) As in G, but treated as indicated with IR (4Gy) or ABT-263

Figure 4.2 (Continued)

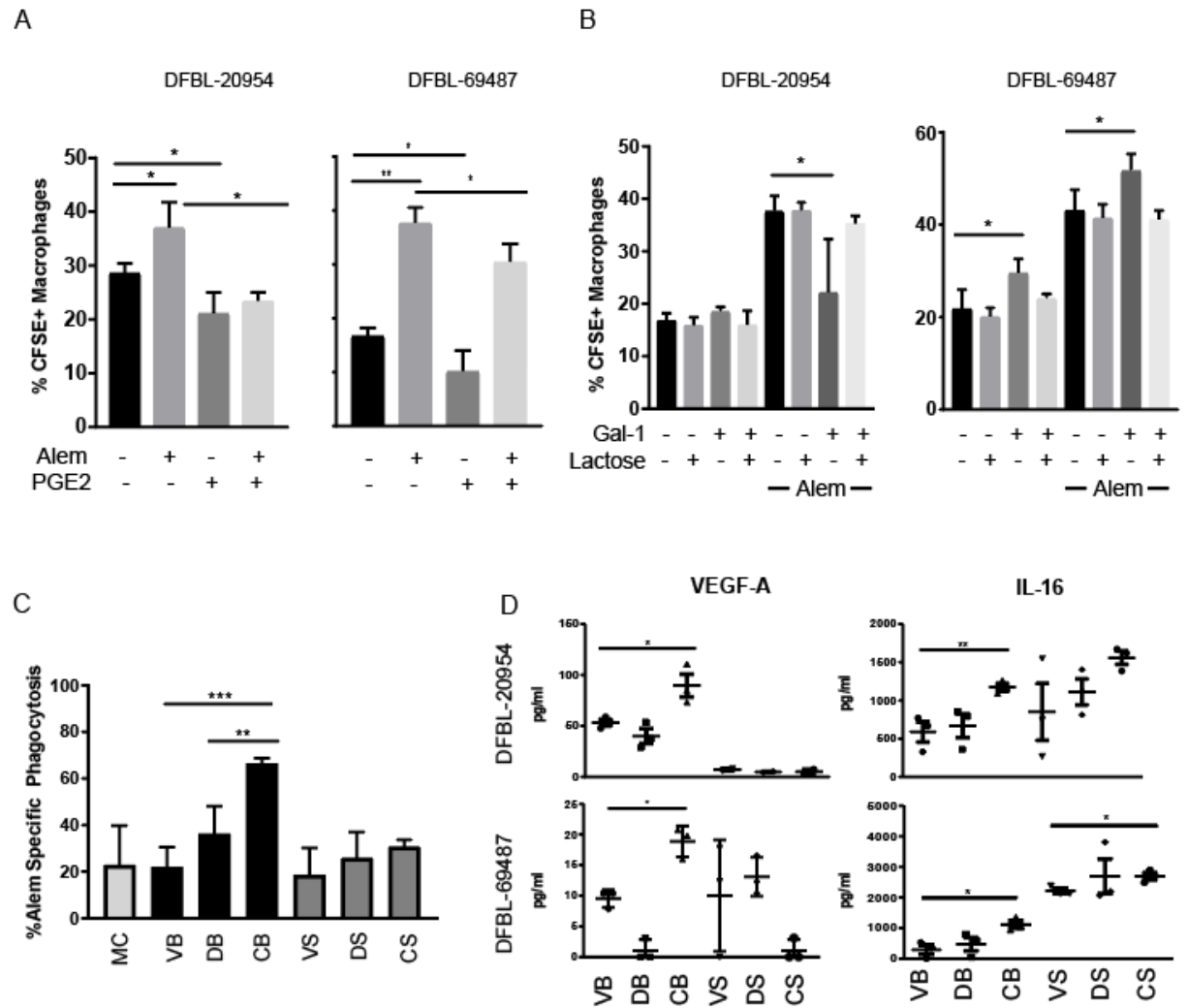
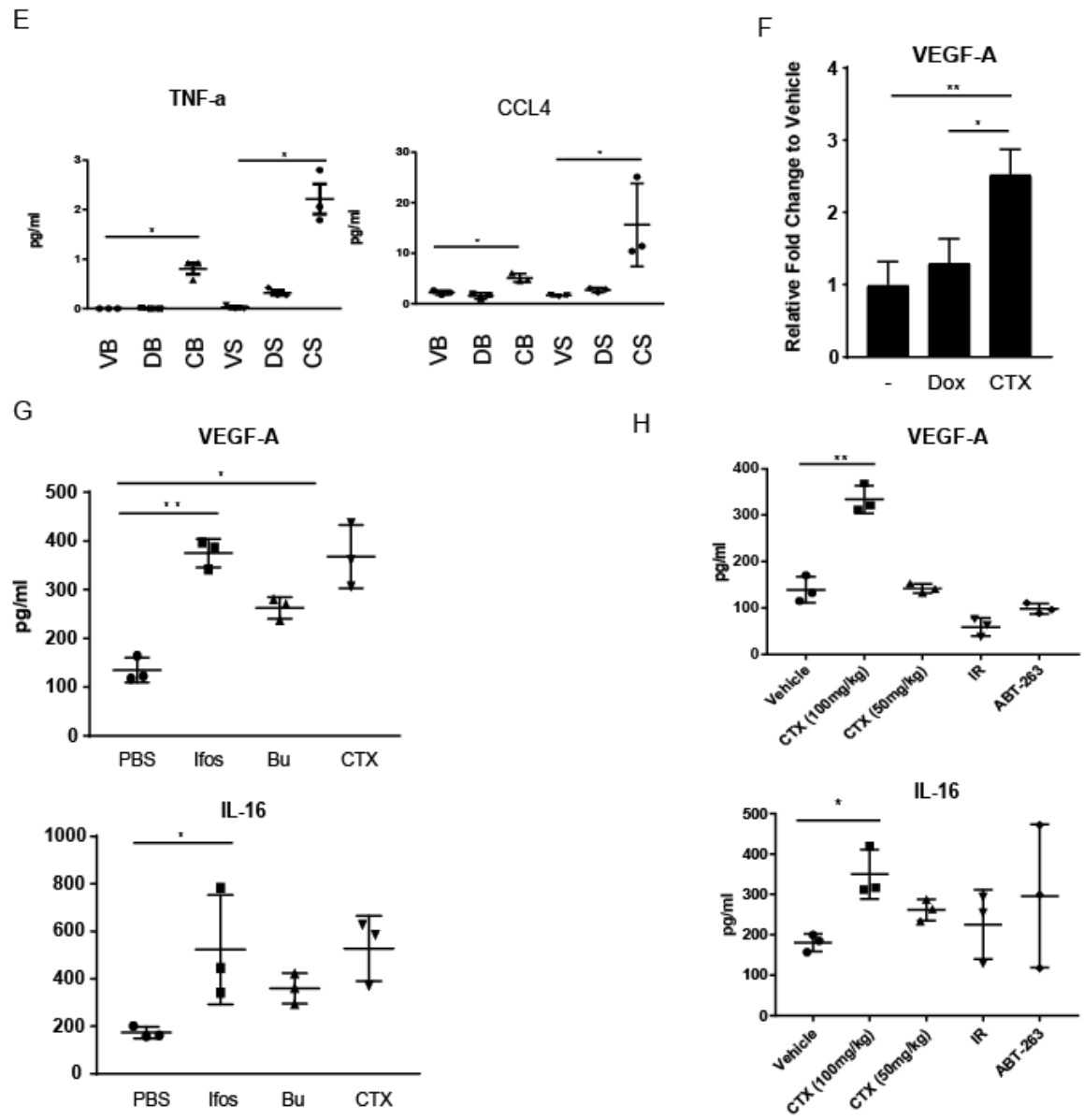


Figure 4.2 (Continued)



doxorubicin or CTX. DFBL-20954 cells isolated from the bone marrow of treatment naïve mice were then co-cultured in the presence of BMDMs from unengrafted mice in the different conditioned medias in the presence and absence of alemtuzumab. Conditioned media from CTX-treated bone marrow cells but not splenic cells increased macrophage phagocytosis of DFBL-20954 cells specifically in the presence of alemtuzumab (Figure 4.2C).

To determine which proteins were secreted post CTX treatment, we generated lysates from the bone marrows and spleens of DHL engrafted mice following PBS, doxorubicin and CTX treatment and performed unbiased human cytokine array analyses. CTX treatment but not doxorubicin induced production of VEGF-A and IL-16 in the bone marrows of both xenografts (Figure 4.2D). Increased IL-16 was also observed in the spleens of DFBL-69487 mice. Moreover, CTX administration induced production of TNF-alpha and CCL4 in DFBL-69487 bone marrow and spleens (Figure 4.2E). VEGF-A secretion was confirmed to be of human origin as murine cytokine arrays of the same lysates registered <1 pg/ml of VEGF-A. We confirmed increased VEGF-A levels in conditioned media generated from CTX-treated bone marrows of DFBL-20954 engrafted mice (Figure 4.2F). Induction of VEGF-A and IL-16 was a general alkylating agent phenomenon as ifosfamide and busulfan treatment of DFBL-20954 cells also induced production of these factors (Figure 4.2G). High-doses of CTX were needed to induce VEGF-A secretion as no increase was observed after administration of a lower dose (50mg/kg) of CTX (Figure 4.2H). Navitoclax and ionizing irradiation also failed to induce secretion of VEGF-A and IL-16 in DFBL-20954 cells (Figure 4.2H).

We next asked whether these secreted factors affected macrophage phagocytosis of lymphoma cells. Addition of recombinant VEGF-A enhanced phagocytosis of DFBL-20954 and

DFBL-69487 cells by BMDMs only in the presence of alemtuzumab (Figure 4.3A). Addition of the anti-human VEGF-A antibody bevacizumab was able to completely block the CTX conditioned media from enhancing phagocytosis of DFBL-20954 cells by BMDMs (Figure 4.3B). Moreover, consistent with their decreased production after CTX treatment, higher levels of recombinant galectin-1 (1 μ g/ml) and PGE-2 (5ng/ml) were needed to be added to the CTX conditioned media than media controls to inhibit BMDM phagocytosis (Figure 4.3B). *In vivo* blockade of VEGF-A by bevacizumab decreased the efficacy of CTX and alemtuzumab combination therapy in promoting bone marrow DHL clearance but increased tumor clearance by CTX in the absence of alemtuzumab (Figure 4.3C).

We next asked how VEGF-A promoted macrophage phagocytosis of tumor cells. VEGF-A has been previously shown to induce phosphorylation of SYK kinase in murine macrophages, a key regulated of Fc γ R dependent phagocytosis (Yang et al., 2014). We confirmed that human VEGF-A and Fc γ R engagement by antibody cross-linking could enhance pSYK levels in BMDM by flow cytometry (Figure 4.3D). The levels of pSYK were higher upon administration of VEGF-A in the presence of simulated antibody cross-linking (Figure 4.3D). The effect of VEGF-A to increase pSYK levels was abolished in the presence of the SYK inhibitor BAY61-3606. BAY61-3606 also inhibited the ability of VEGF-A to promote phagocytosis of lymphoma cells in the presence of alemtuzumab (Figure 4.3E).

TNF- α induced phagocytosis of DFBL-69487 cells by BMDMs both in the presence and absence of alemtuzumab in a dose dependent fashion (Figure 4.4A). TNF- α is a known inducer of apoptosis of MYC-rearranged tumors (Gisslinger et al., 2001; Jurisic et al., 2006). We therefore asked whether TNF- α could promote apoptosis of DFBL-69487. Co-incubation of DFBL-69487 cells with TNF- α induced rapid apoptosis of the tumor cells (Figure 4.4B). The

Figure 4.3 VEGF-A Promotes Macrophage Phagocytosis of Alemtuzumab coated DHL Cells (A) BMDMs were incubated with CFSE-labeled BM lymphoma cells from untreated mice supplemented with Alem, recombinant human VEGF-A or vehicle (-). Representative experiment depicted. Phagocytosis was assessed as the percent of CD11b⁺ cells that were CFSE⁺. Two-sided Welch's *t*-test, * $p < 0.05$. (B) BMDMs were incubated with CFSE-labeled BM lymphoma cells from untreated mice and conditioned media (n=3 per condition) supplemented with vehicle (-), Alem, recombinant Gal-1 (1 μ g/ml), PGE2 (5 ng/ml), and/or bevacizumab (bev, 25 μ g g/ml). Unpaired two-sided-*t*-test, * $p < 0.05$, ** $p < 0.01$. (C) Bone marrow tumor burden of DFBL-20954-engrafted mice on day 8 after treatment with the indicated agents. Two-sided Welch *t*-test, *** $p < 0.001$. (D) Heatmap of total SYK (top row) and phospho-SYK (all other rows) levels in BM-derived macrophages (BMDMs) following the indicated treatments as assayed by flow cytometry. Each column represents an individual mouse. Legend represents Z-scores of mean fluorescent intensities. HFab = anti-human Fab antibody. (E) BMDMs were incubated with GFP⁺ Raji cells and recombinant human VEGF-A (10ng/ml) in the presence and absence of Alem. Representative experiment depicted. The SYK inhibitor BAY61-3606 (20 nM) was added as indicated. Phagocytosis was assessed as the percent of CD11b⁺ cells that were GFP⁺. Unpaired two-sided-*t*-test, * $p < 0.05$, ** $p < 0.01$.

Figure 4.3 (Continued)

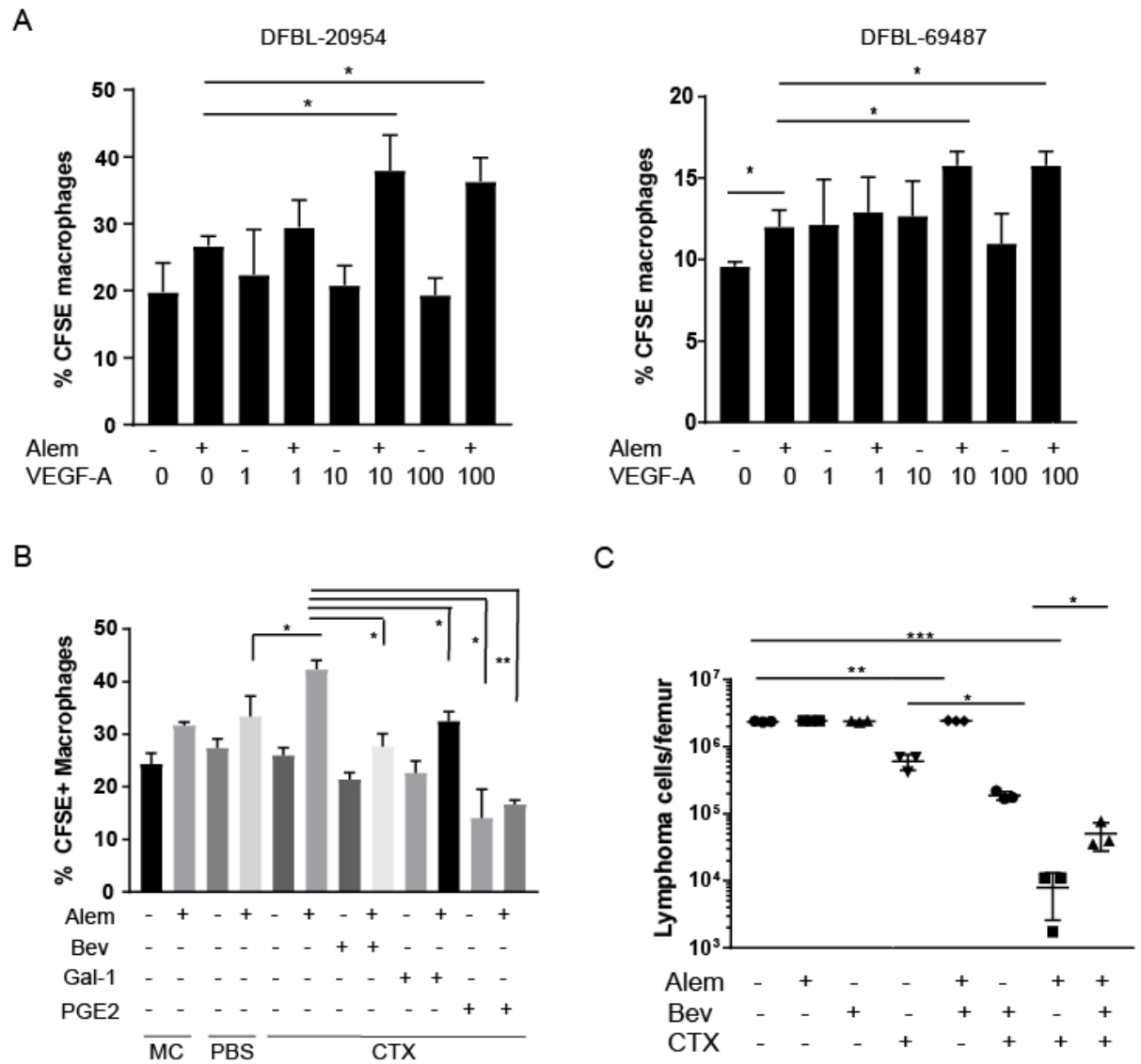
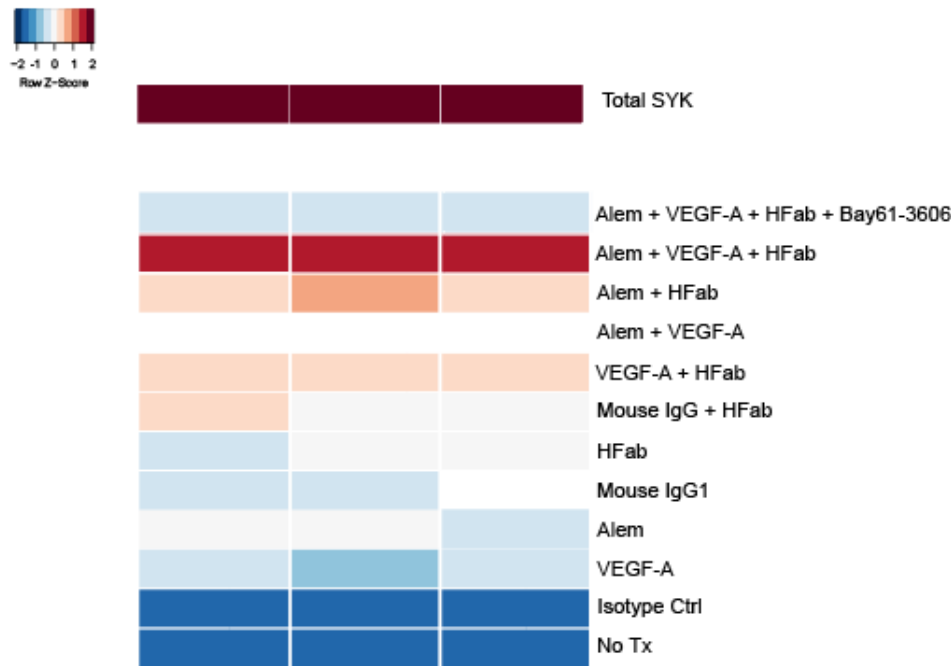


Figure 4.3 (Continued)

D



E

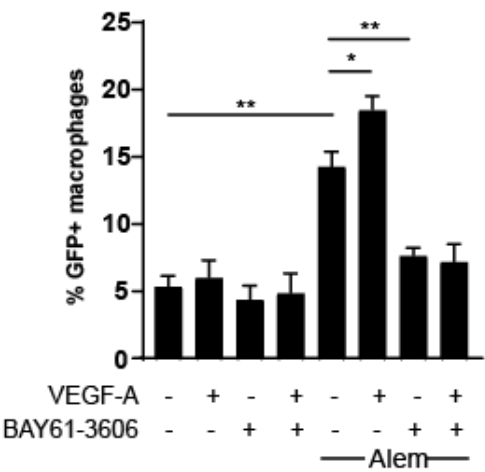
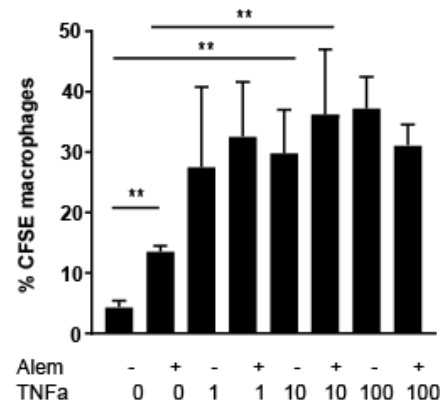


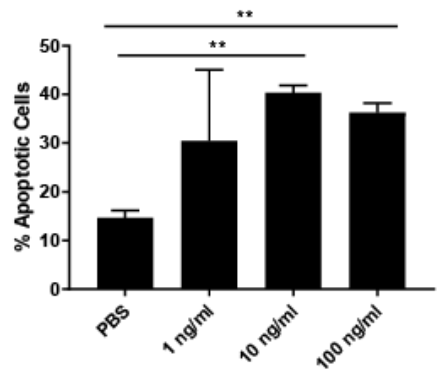
Figure 4.4 TNF- α Enhances clearance of DHL Cells by Inducing Apoptosis (A) BMDMs were incubated with CFSE-labeled bone marrow lymphoma cells from untreated mice supplemented with Alemtuzumab, recombinant human TNF- α or vehicle (-). Depicted are results from 3 independent experimental replicates. Phagocytosis was assessed as the percent of CD11b⁺ cells that were CFSE⁺. Two-sided Welch's *t*-test, * $p < 0.05$. (B) DFBL-69487 bone marrow cells from PBS-treated were treated with TNF- α as indicated. Apoptosis was assessed as the fraction of 7AAD and Annexin V double positive cells. Unpaired two-sided-*t*-test, * $p < 0.05$, ** $p < 0.01$. (C) As in (B) but in the presence of Z-VAD-FMK, Z-IETD-FMK and Z-LEHD-FMK (50 μ M each) administered 30 minutes before TNF- α . Phagocytosis was assessed as the percent of CD11b⁺ cells that were GFP⁺. (D) BMDMs were incubated with CFSE-labeled BM DFBL-69487 bone marrow cells from untreated mice supplemented with TNF- α (10 ng/ml). Caspase inhibitors were given as in (C). (E, F) As in D, but with human IL-16 (100ng/ml) and CCL4 (100 ng/ml) (G) Fraction of BM monocytes/macrophages (hCD19⁻CD11b⁺F4/80⁺) that express CD4 following the indicated treatments. Macrophages were analyzed 48 hours after treatment initiation. Unpaired two-sided *t*-test, * $p < 0.05$

Figure 4.4 (Continued)

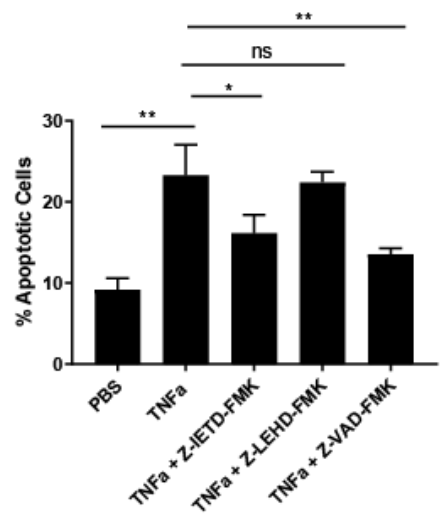
A



B



C



D

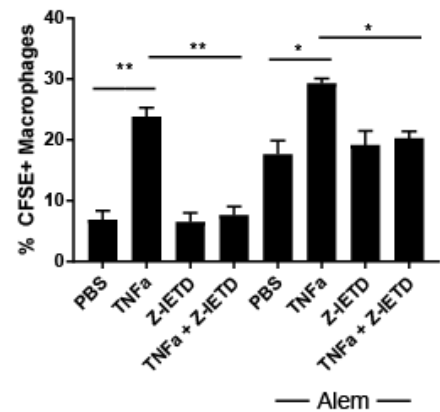
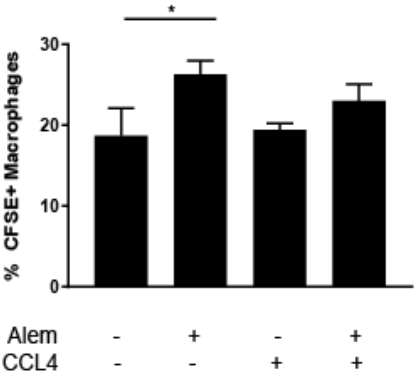
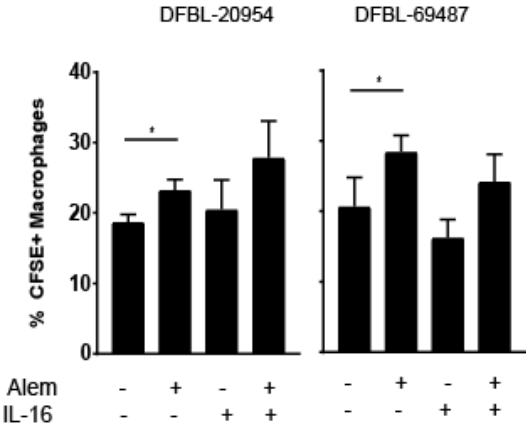


Figure 4.4 (Continued)

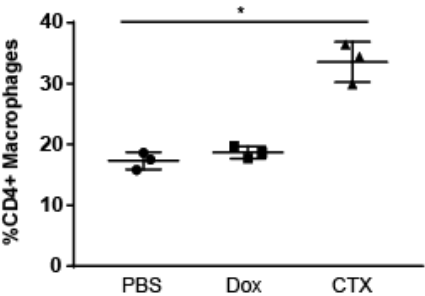
E



F



G



pro-apoptotic effect of TNF- α was abolished by the pan-caspase inhibitor Z-VAD-FMK or the extrinsic apoptosis inhibitor Z-IETD-FMK but not the intrinsic apoptosis inhibitor Z-LEHD-FMK (Figure 4.4C). TNF- α mediated increased phagocytosis of DFBL-69487 cells by BMDMs was inhibited in the presence of Z-IETD-FMK (Figure 4.4D).

The cytokines IL-16 and CCL4 failed to increase phagocytosis of DFBL-20954 and DFBL-69487 cells by BMDMs (Figure 4.4 E, F). Consistent with *in vivo* secretion of IL-16, an increased fraction of bone marrow macrophages from mice engrafted with DFBL-20954 treated with CTX but not doxorubicin were positive for CD4 (Figure 4.4G) (Center and Cruikshank, 1982).

Chapter 5: Alkylating Agents Induced Secretory and Phenotypic Changes are Partially Due to ER stress

Background

The unfolded protein response (UPR) is a coordinated cellular response to sensing unfolded ER proteins. This can be triggered by various genotoxic cellular stresses that promote protein aggregation and/or misfolding (Gu et al., 2016). Misfolded proteins are sensed by ER-resident chaperones such as BiP. The ER stress response is associated with transcription and translation of specific stress-response cytokines that are spared through the general translation block established as part of the response (Bernales et al., 2006). These include genetic elements with internal ribosome entry sites (IRES) such as VEGF-A (Arcondeguy et al., 2013).

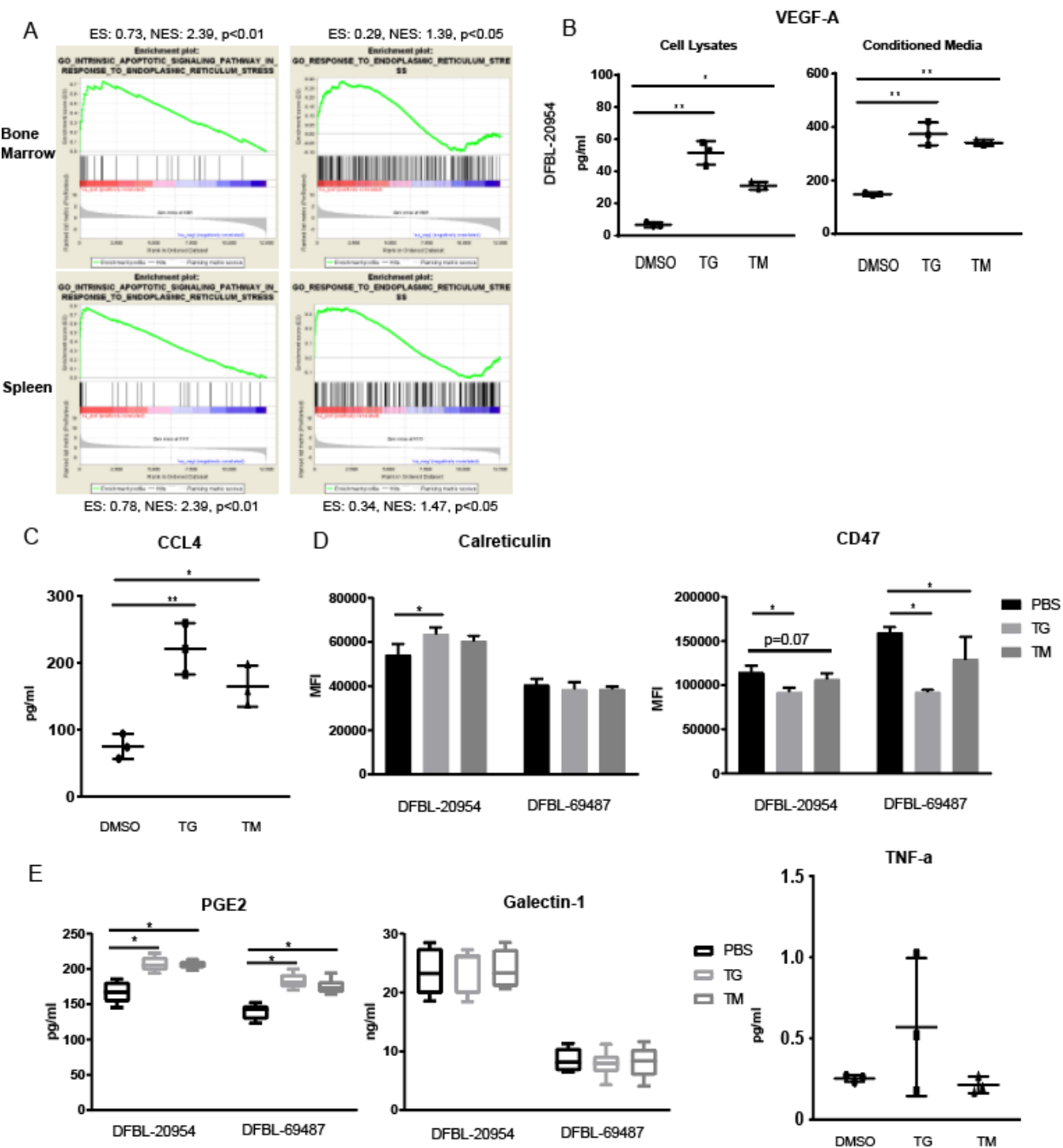
Phenotypic changes in cells undergoing ER stress have been described, including surface translocations of calreticulin (Kroemer et al., 2013). Previous reports have shown that several alkylating agents, including CTX and bleomycin, are known to induce ER stress (Galluzzi et al., 2015). CTX treatment of the humaneered DHL model increased levels of alternatively spliced XBP-1 (XBP-1S), a downstream effector of the UPR (Pallasch et al., 2014). We hypothesized that CTX treatment promoted ER stress in our DHL cells.

Results

We first asked what cellular processes were mediated by CTX treatment. To address this question we initially performed RNA-Seq analysis of spleen and bone marrow purified DFBL-20954 cells collected 16 hours after vehicle, doxorubicin or CTX treatment. We performed gene-set enrichment analyses (GSEA) comparing CTX and doxorubicin induced changes compared to vehicle-treated cells and isolating CTX specific changes. GSEA identified multiple ER stress-related signatures to be significantly enriched in lymphoma cells specifically post CTX treatment (Figure 5.1 A). Several NF-kB related pathways were also significantly upregulated specifically

Figure 5.1 ER stress Mediated Phenotypic Changes in DHL Cells (A) Gene-set enrichment analysis (GSEA) of ER stress signatures from RNA sequencing of DFBL-20954 cells collected from spleens or BM. The comparison reflects genes overexpressed after cyclophosphamide (CTX) treatment as compared to PBS treatment. P-values have a false discovery rate (FDR) adjustment. Abbreviations: ES, enrichment score; NES, normalized enrichment score. (B) Levels of human VEGF-A in cells (left) or media (right) of DFBL-20954 cells isolated from BM and treated *ex vivo* for 24 hours with DMSO, thapsigargin (TG) or tunicamycin (TM). Two-sided Welch *t*-test * $p < 0.05$, ** $p < 0.01$. (C) Levels of CCL-4 of DFBL-69487 cells isolated from bone marrow and treated *ex vivo* for 24 hours with DMSO, TG or TM. (D) Flow cytometry levels of CD47 and Calreticulin from DHL cells acquired and treated as in (B). (E) ELISA for PGE2 and Galectin-1 levels from bone marrow-derived DFBL-20954 and DFBL-69487 cells treated as in (B). TNF- α levels of DFBL-69487 are also depicted.

Figure 5.1 (Continued)



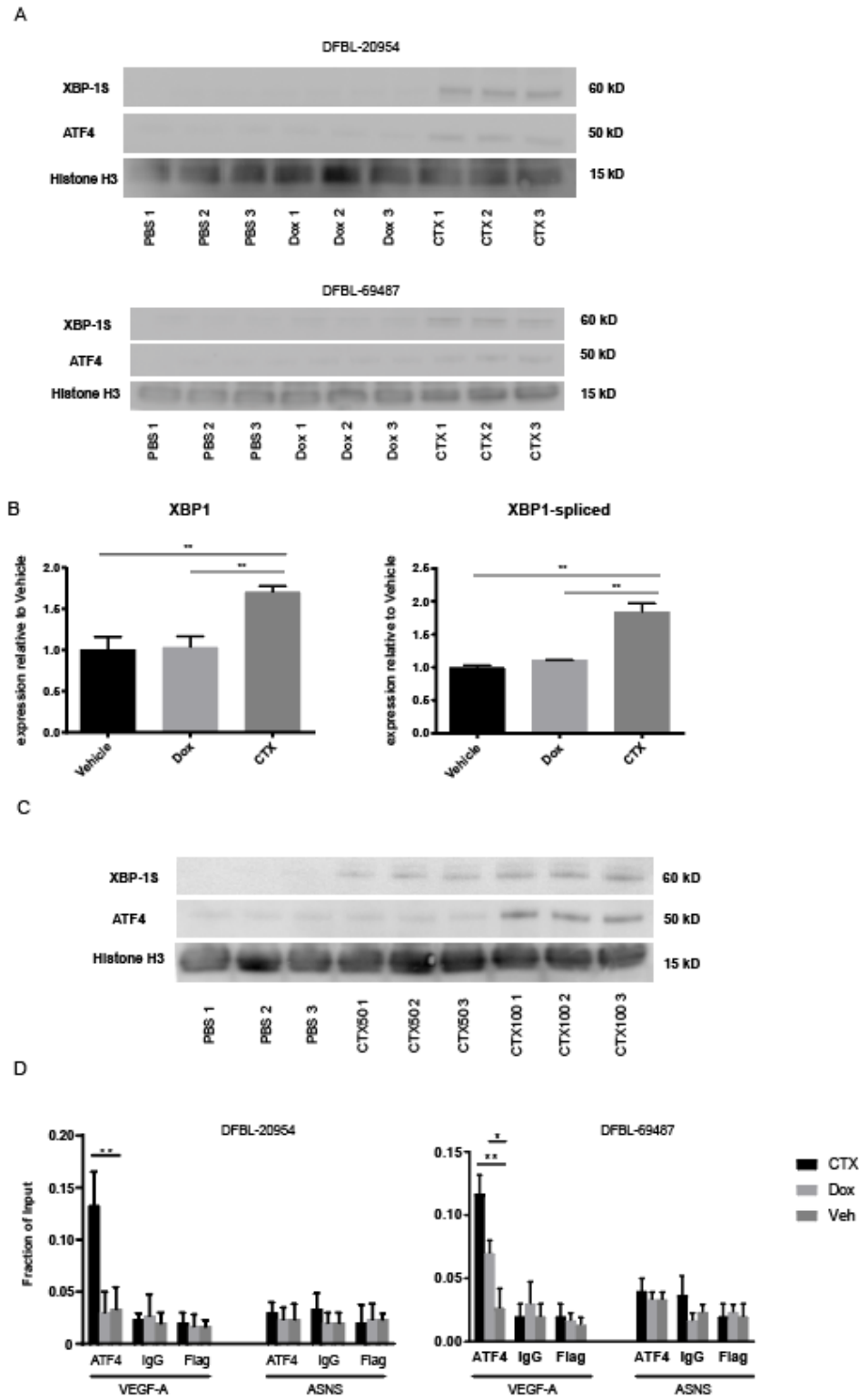
post CTX administration, consistent with known NF- κ B pathway activation by the ER stress response.

We next asked whether ER stress was sufficient to induce production of VEGF-A and the other phenotypic changes seen in the xenograft cells after CTX treatment. Treatment of DFBL-20954 and DFBL-69487 cells *ex vivo* by the ER stress inducing agents thapsigargin (TG) and tunicamycin (TM) induced both production and secretion of VEGF-A (Figure 5.1 B). Moreover, TG and TM administration induced production of CCL4 in DFBL-69487 cells (Figure 5.1 C). TG and TM also led to decreased surface CD47 expression in both xenografts and increased surface calreticulin in DFBL-20954 cells, consistent with observations following CTX treatment (Figure 5.1D). In contrast to what was observed with CTX treatment, TG and TM administration increased levels of PGE2 in both xenografts (Figure 5.1E). Galectin-1 levels in both xenografts and TNF- α levels in DFBL-69487 were not altered by TG or TM (Figure 5.1E).

The ER-executioner proteins ATF4 and spliced XBP-1 have previously been shown to induce VEGF-A transcription and production (Binet and Sapieha, 2015; Ghosh et al., 2010). Levels of both proteins were increased in bone marrow lymphoma cells from both xenografts in CTX-treated mice compared to vehicle- and doxorubicin-treated mice (Figure 5.2 A). Induction of spliced XBP-1 was confirmed in DFBL-20954 cells by qPCR (Figure 5.2B). Lower levels of CTX (50mg/kg) induced spliced XBP-1 but not ATF4 in DFBL-20954 cells (Figure 5.2C). ChIP-qPCR of purified bone marrow lymphoma cells demonstrated significantly increased ATF4 recruitment to exon 1 of *VEGFA* after CTX treatment but not to a negative control region in both xenografts (Figure 5.2D). Enrichment of ATF4 recruitment to *VEGFA* was also observed post

Figure 5.2 CTX Treatment Induces ATF4 Recruitment to *VEGFA* Regulatory Regions (A) Immunoblotting for the indicated targets using lysates from purified, viable bone marrow DFBL-20954 and DFBL-69487 cells collected 16 hours (DFBL-20954) or 48 hours (DFBL-69487) after *in vivo* treatment with PBS (vehicle), doxorubicin (Dox) or CTX. Each lane represents an individual mouse. (B) qPCR of CHOP and XBP-1 variants following treatment of DFBL-20954 cells with PBS (vehicle), doxorubicin (Dox) or cyclophosphamide (CTX). RNA was extracted 16 hours after initiation of chemotherapy (C) Immunoblotting of ATF4 and XBP-1S levels of bone marrow-derived viable DFBL-20954 treated as indicated. Cells were collected 16 hours post treatment. (D) ChIP-qPCR of ATF4 localization to exon 1 of human *VEGF-A* or exon 7 of human *ASNS* in purified, viable bone marrow DFBL-20954 and DFBL-69487 cells collected 16 hours (DFBL-20954) or 48 hours (DFBL-69487) after treatment with PBS (Veh), Dox or CTX (N=3 mice per condition with multiple mice pooled for each sample). Unpaired two-sided-*t*-test, **p*<0.05, ***p*<0.01.

Figure 5.2 (Continued)



CTX induces ER stress within bone marrow lymphoma cells that involves upregulation of ATF4, which directly drives VEGF-A production and secretion.

Chapter 6: Cyclophosphamide Treatment in the Presence of DHL Promotes “Super-Phagocytic” Macrophages

Background

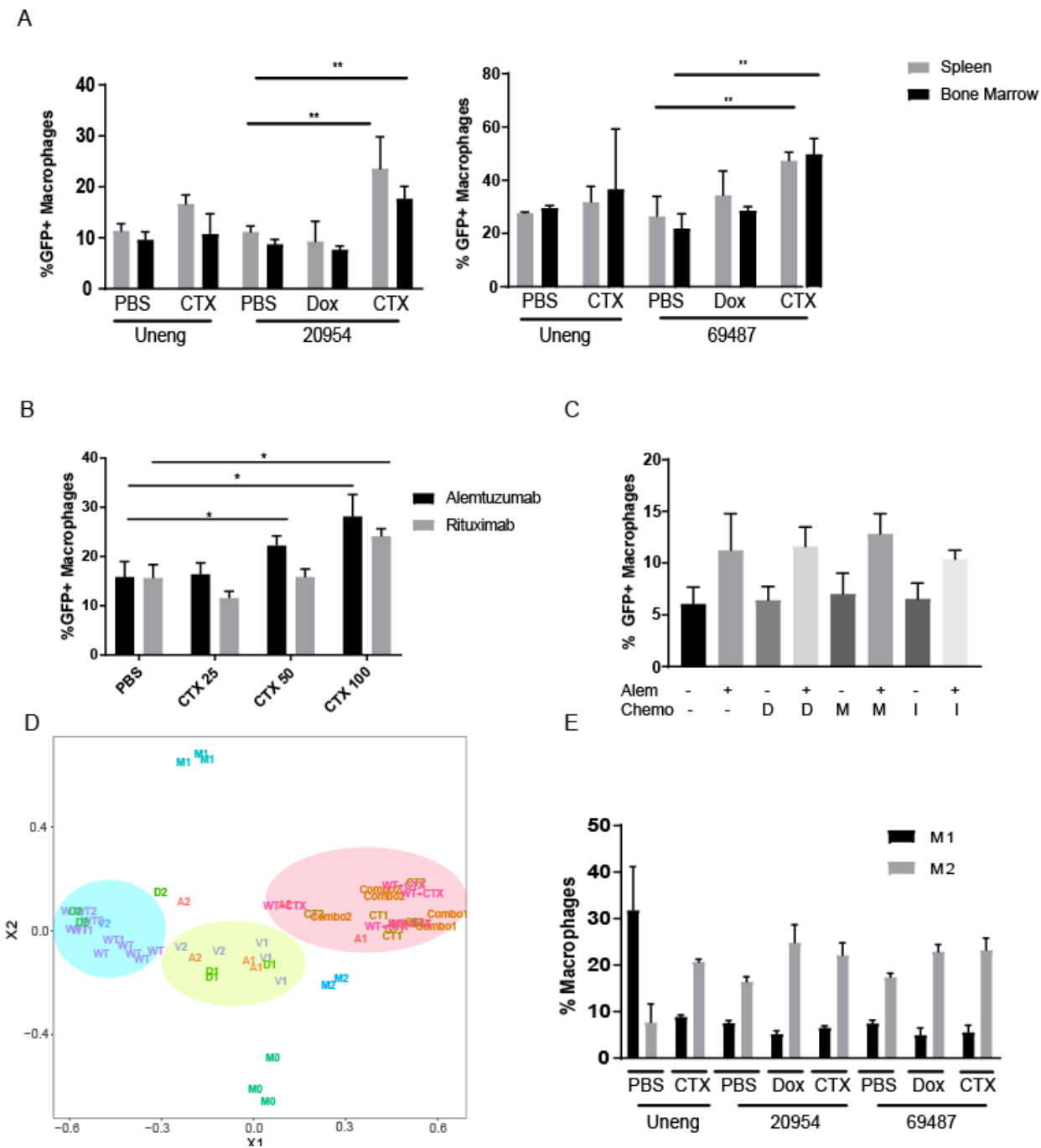
Macrophages are known to be frequently found in the lymphoma microenvironment. This is perhaps best appreciated in Burkitt's lymphoma (Ferry, 2006; Ford et al., 2015). These macrophages are known to be highly phagocytic and are characterized by increased expression of *Fcgr1*, *Fcgr3* and *CCL2*, among other genes (Voss et al., 2017). Previous studies have linked chemotherapies such as doxorubicin to enhancing macrophage clearance of leukemia cells (Mantovani et al., 1979). How these therapies induce macrophage-mediated tumor clearance remains unknown; however, there is evidence that tumor secreted factors may play a role in this activity. Recently, blockade of IAP in multiple myeloma cells was shown to induce interferon production that directly activated macrophage phagocytosis (Chesi et al., 2016). We hypothesized that in addition to directly increasing tumor cell phagocytic proneness to macrophages, CTX administration could activate macrophages by direct reprogramming and/or through activation by tumor-derived factors.

Results

Given that alemtuzumab induced tumor clearance was dependent on ADCP we first sought to address the effects CTX treatment on macrophage phagocytosis. We treated DFBL-20954 and DFBL-69487 bearing mice with PBS, doxorubicin and CTX 48 hours after treatment and sorted primary bone marrow and splenic macrophages and assayed their capacity to phagocytose alemtuzumab-coated lymphoma cells. We also sorted macrophages from unengrafted NSG mice treated with PBS or CTX to tease out direct CTX effects from CTX + lymphoma effects. There was no difference in phagocytosis by macrophages derived from unengrafted mice treated with PBS or CTX (Figure 6.1A). In contrast, phagocytosis was significantly increased among macrophages from lymphoma-bearing mice after treatment with

Figure 6.1 Cyclophosphamide Induces Phagocytic Macrophages in Lymphoma-Engrafted Bone Marrow (A) Primary viable splenic (7AAD⁻CD19⁻CD11b⁺F4/80⁺) and bone marrow (7AAD⁻CD19⁻CD11b⁺F4/80⁺) monocytes/macrophages were sorted from unengrafted DFBL-20954 or DFBL-69487-bearing NSG mice (n=3 per condition) treated with PBS (Veh), doxorubicin (dox) or cyclophosphamide (CTX) and cultured in the presence of GFP⁺ Raji cells that were pre-treated with alemtuzumab. Two-sided Welch *t*-test ** $p < 0.01$ (B) Primary viable bone marrow monocytes/macrophages were sorted from DFBL-20954 mice treated with PBS or the indicated dose of CTX and cultured in the presence of GFP⁺ Raji cells that were pretreated with alemtuzumab or rituximab (n=3 per condition). (C) Phagocytosis assays of CFSE-labeled BM derived DFBL-20954 cells with bone marrow-derived macrophages (BMDM) pre-treated with PBS, doxorubicin (Dox, 200 ng), mafosfamide (M, 10 uM) and ifosfamide (I, 20 uM). Macrophages were treated with chemotherapy for 6 hours and then incubated in drug free media for 12 hours prior to assay initiation (D) Multidimensional scaling (MDS) analysis of RNA-seq from BM macrophages comparing unengrafted (WT) or lymphoma-engrafted (DFBL-69487=1, DFBL-20954=2) mice treated with PBS (V), Dox (D), CTX (C), Alem (Alem) or CTX+Alem (combo). Macrophages were harvested 48 hours after treatment. (E) Macrophage polarization of murine BM macrophages from unengrafted and tumor bearing NSG mice (n=3 per condition) treated with the indicated chemotherapies. M1 macrophages were CD206-MHCII⁺ and M2 macrophages were defined as CD206-MHCII⁻ cells.

Figure 6.1 (Continued)



CTX compared to vehicle (Figure 6.1A). Doxorubicin treatment in lymphoma bearing mice did not increase macrophage phagocytosis (Figure 6.1A). The effect of CTX treatment in the presence of the DHL xenografts on phagocytosis was not due to nonspecific cell death or macrophage cross-species priming, as treatment with doxorubicin induced similar cell death in the spleens of tumor bearing mice while failing to enhance phagocytosis. High-doses of CTX (> 50mg/kg) were necessary to increase macrophage phagocytosis in the presence of DHL (Figure 6.1B). Macrophages from DHL-engrafted CTX-treated mice also had increased phagocytosis of rituximab bound tumor cells, suggesting the enhanced phagocytic phenotype was not restricted to alemtuzumab (Figure 6.1B).

To confirm that enhanced macrophage phagocytosis by CTX treatment depended on the presence of lymphoma cells, we pre-treated BMDMs *ex vivo* with doxorubicin, mafosfamide (a CTX analog that can be used *in vitro*) or ifosfamide in the absence of tumor cells and monitored phagocytic capacity following addition of lymphoma cells to culture. Chemotherapy pre-treatment of macrophages failed to induce lymphoma phagocytosis in the presence or absence of alemtuzumab (Figure 6.1C). Together these observations indicate that *in vivo* treatment with CTX in the presence of lymphoma is required to enhance the phagocytic capacity by macrophages.

We hypothesized that CTX treatment in the presence of lymphoma conferred unique characteristics to the macrophages. To assess these in an unbiased fashion we sorted primary bone marrow macrophages 48 hours after *in vivo* treatment of engrafted mice with vehicle, alemtuzumab, doxorubicin, CTX or CTX plus alemtuzumab and performed RNA-seq. Macrophages were also sorted from the bone marrows of unengrafted NSG mice treated with either PBS or CTX.

Macrophages are commonly categorized by transcriptional signatures into M0 (undifferentiated), M1 (anti-tumor) and M2 (tumor-promoting) subtypes with defined gene-expression changes and functional characteristics. To assess where the sorted bone marrow and splenic macrophages were in “macrophage space” in relation to well characterized macrophage subsets we performed multi-dimensional scaling (MDS) analysis using existing signatures from *in vitro*-differentiated M0, M1 and M2 macrophages as reference groups (see methods).

Macrophages from unengrafted NSG mice treated with PBS clustered farthest from any of the three reference groups (Figure 6.1D). Macrophages from lymphoma engrafted, vehicle-treated mice shifted to a more M2-like signature that was unaffected by treatment with alemtuzumab or doxorubicin (Figure 6.1D). In contrast to the observed difference in their phagocytic properties, macrophages from unengrafted or lymphoma bearing mice treated with CTX clustered together (Figure 6.1D). These macrophages also clustered closely to *in vitro* differentiated M2 macrophages but were clearly different from PBS, doxorubicin or alemtuzumab treated mice. Flow cytometric assessment of M1 and M2 polarization based on surface MHC class II (M1) and CD206 (M2) successfully demonstrated the M2-associated gene expression shifts between macrophages from unengrafted NSG mice and all the other groups (Figure 6.1E). However, it failed to resolve differences between engrafted or unengrafted mice that received CTX as compared to the other treatments.

Given the discrepancy between the observed functional properties of the sorted macrophages and the bulk RNA-seq data, we reasoned that unique macrophage subsets may be present within the CTX-treated lymphoma bearing group that were not being resolved in the bulk analysis. We therefore next performed Smart-Seq2 single-cell RNA-seq (scRNAseq) on bone

marrow macrophages collected 48 hours after treatment with vehicle, doxorubicin or CTX of DFBL-20954-engrafted mice and unengrafted mice treated with PBS or CTX.

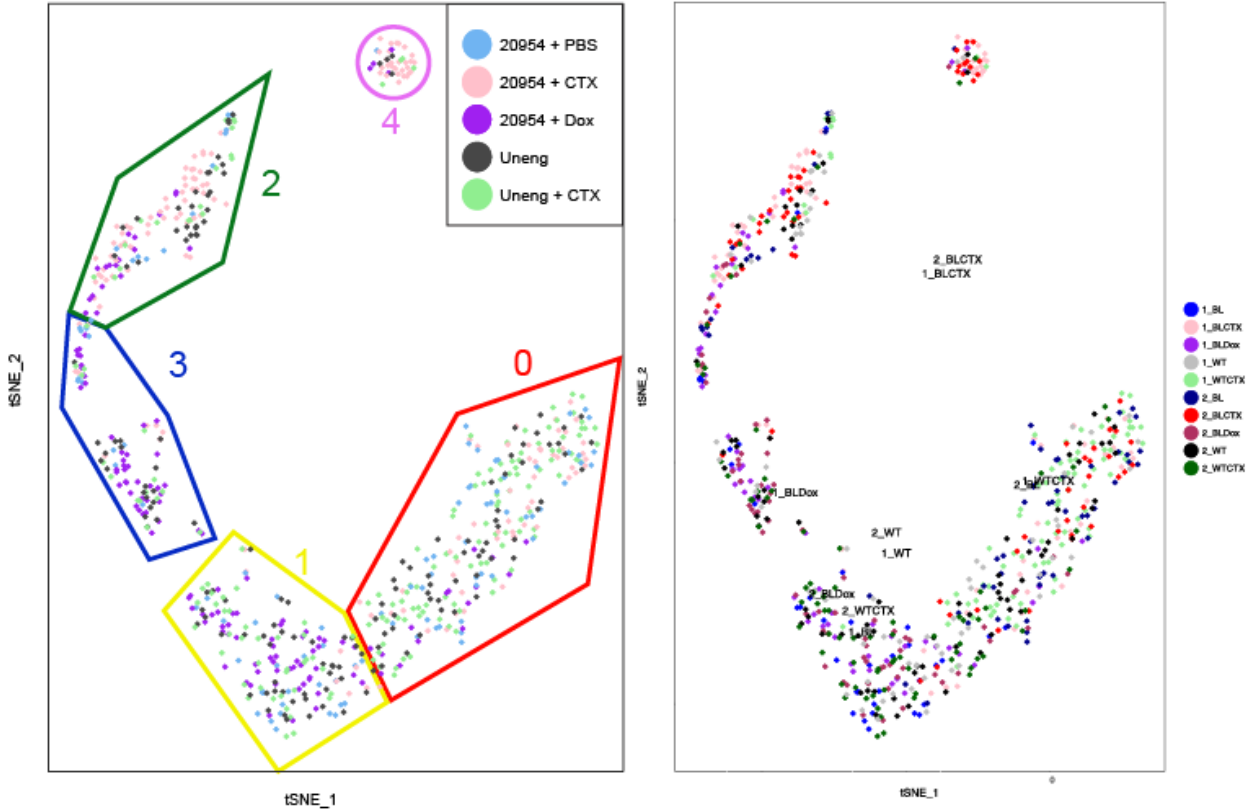
After quality controls, we performed dimensionality reduction by principle component analysis and graph-based clustering on the macrophages by a gene expression matrix and visualized the results with t-distributed stochastic neighbor embedding (t-SNE, see methods). Using this unbiased approach, we identified 5 unique macrophage clusters (Figure 6.2 A). Of these five clusters, which were defined by the top 50 expressed genes that differentiated them from the remaining clusters (Appendix A), two (C2 and C4, respectively,) were enriched for CTX-treated macrophages from lymphoma-bearing mice ($p = 3.7 \times 10^{-8}$ for C2, $p = 1.7 \times 10^{-9}$ for C4, Fisher's Exact Test, Bonferroni corrected for 25 tests). Importantly enrichment of these clusters was not batch effect mediated as enrichment in C2 and C4 macrophages was seen in multiple DFBL-20954-bearing CTX-treated mice (Figure 6.2A).

To understand what characterized the gene-expression profiles of C2 and C4 macrophages, we performed Ingenuity Pathway Analysis (IPA) and an analysis of upstream regulators in comparison to the other clusters. IPA of C2 and C4 cells showed enrichment of genes involved in Fc receptor-mediated mediated phagocytosis (C4 vs all others, $\text{padj} = 0.002$) and phagosome formation (C2 and C4 vs all others, $\text{padj} = 0.0355$) and maturation (C2 and C4 vs all others, $\text{padj} = 3.24 \times 10^{-8}$), consistent with their super-phagocytic phenotype. Upstream analysis also demonstrated that VEGF-A was a regulator of the C4 signature (C4 vs all others, $\text{padj} = 5.91 \times 10^{-8}$). Consistent with this, known curated gene sets from VEGF-A treatment showed higher expression levels in C4 cells as compared to the other transcriptional clusters (Figure 6.2B). Given these observations we hypothesized that C2 and C4 macrophages consisted of gene-expression patterns that could explain the highly phagocytic nature of macrophages from

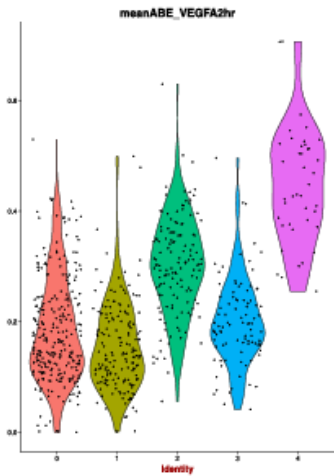
Figure 6.2 CTX Treatment in the Presence of Lymphoma Generates a Unique “Super-Phagocytic” Transcriptionally Defined Macrophage (A) left: t-SNE plot of single-cell RNA-seq of BM macrophages from unengrafted (uneng) or DFBL-20954-engrafted mice treated with PBS, dox or CTX shows five clusters with cluster 4 almost exclusively derived from macrophages in CTX-treated, lymphoma-engrafted mice. right: t-SNE distribution based on mouse of origin. (B) Representative gene list of VEGF-A regulated genes and their relative expression by t-SNE cluster. (C) Expression of Fc-gamma receptors from single-cell RNA-seq of sorted BM macrophages clustered as in (A). (D) Flow-cytometric analysis of Fc-gamma receptor expression on macrophages from unengrafted or DFBL-29054-engrafted mice treated *in vivo* as indicated. Macrophages were collected 48 hours after treatment. Heatmap represents Z-scores of MFIs normalized to isotype controls. (E) Representative flow cytometry of BM macrophages collected from DFBL-29054-engrafted mice treated with PBS or CTX. Unpaired two-sided-*t*-test, ***p*<0.01 (F) Macrophages from (F) were sorted as indicated or unsorted (Bulk), labelled with CFSE and exposed to BM DFBL-20954 cells from mice treated with PBS or CTX that were pre-treated *ex vivo* with Alem (n=5 mice per condition). Unpaired two-sided-*t*-test, **p*<0.05, ***p*<0.01

Figure 6.2 (Continued)

A



B



C

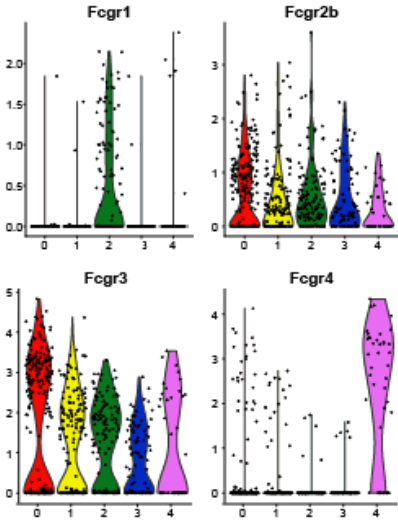
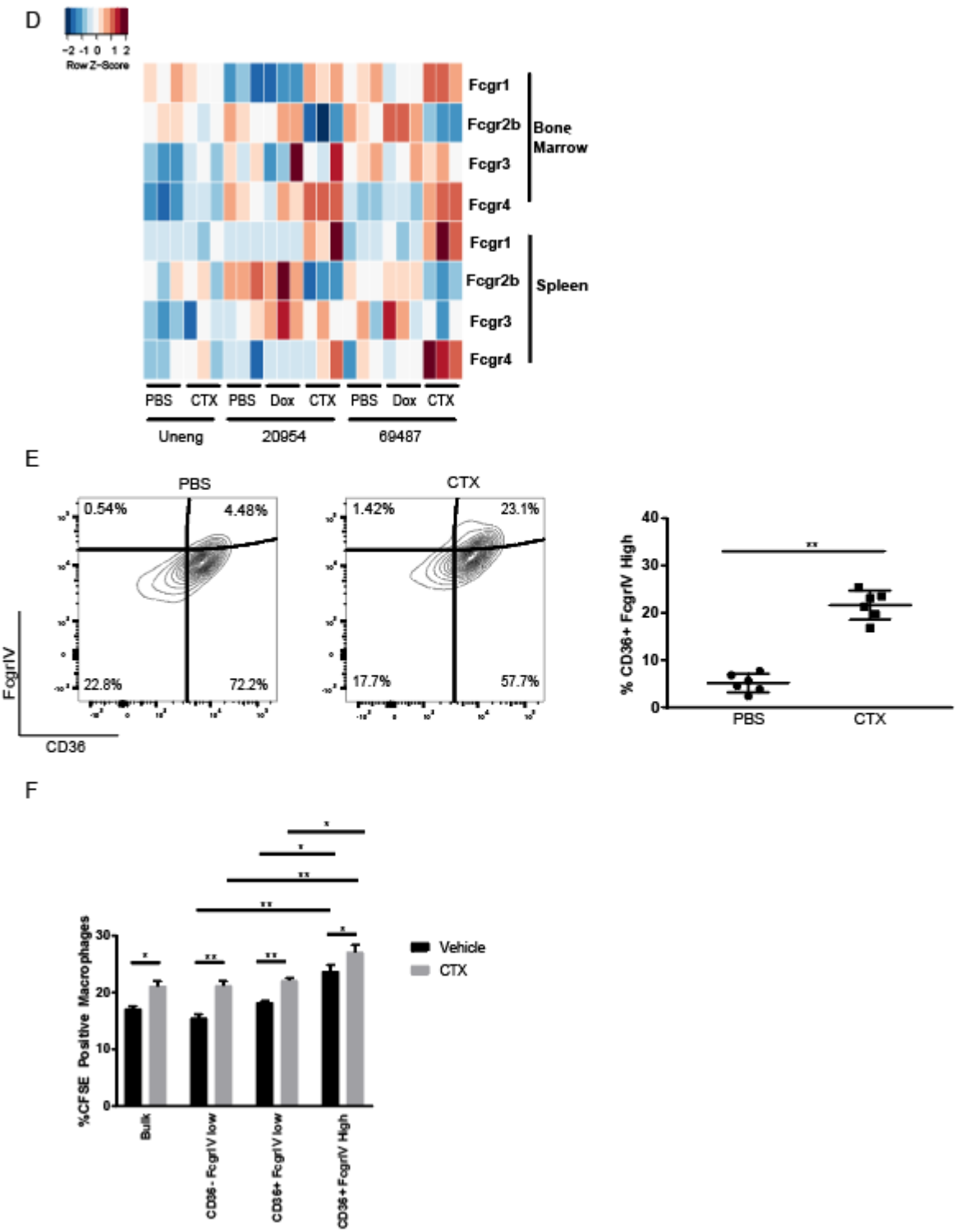


Figure 6.2 (Continued)



lymphoma bearing, CTX-treated mice. Analysis of the genes defining these clusters showed they were enriched for expression of genes known to be important in macrophage-mediated phagocytosis, including CD36 and PPAR γ , which were found in cluster 4 cells (Appendix A). We also noted significantly increased expression of Fcgr1, the high-affinity human IgG receptor, in cluster 2 macrophages ($p = 4.4 \times 10^{-15}$, Bonferroni corrected for 20 comparisons; effect size cohen's $d = 1.29$). At the same time, cluster 4 had markedly higher expression of Fcgr4 ($p = 4.4 \times 10^{-15}$, Bonferroni corrected for 20 tests, effect size Cohen's $d = 2.97$) and lower levels of the inhibitory Fcgr2B as compared to all the other clusters (Figure 6.2C).

Given these observations, we hypothesized that macrophages from lymphoma bearing, CTX-treated mice may have increased phagocytic capacity due to a more favorable balance of surface Fc receptors. We therefore performed flow cytometry assessing levels of these receptors from the five groups on which single cell RNA-seq was performed. Alemtuzumab was not included in this analysis since it can bind non-specifically to macrophage surface Fc by its Fc region and therefore mask expression of these proteins. Macrophages from the spleens and bone marrows of lymphoma bearing CTX-treated mice confirmed increases in surface levels of Fcgr1 and Fcgr4 and decreased expression of the inhibitory Fcgr2B (Figure 6.2D). Fcgr3 levels were not altered by CTX treatment in the presence or absence of lymphoma (Figure 6.2D). We also observed that DHL-engrafted mice treated with doxorubicin had increased Fcgr2B surface expression on both splenic and BM macrophages but observed no modulation of the other Fc receptors.

We observed that cluster 4 macrophages were essentially absent from the bone marrows of all mice except from the lymphoma bearing, CTX-treated mice, where approximately 20% of all macrophages were in this group. Given the expression of many pro-phagocytic markers

within this group, we thus hypothesized that cluster 4 macrophages may define macrophages with a “super-phagocytic” capacity for phagocytosing antibody-opsonized cells.

To test this hypothesis we flow-sorted macrophages from the bone marrows of DFBL-20954-bearing mice treated with PBS or CTX using surface Fcgr4 and CD36 expression as C4 markers (Figure 6.2E). Staining with these 2 markers demonstrated three classes of macrophages: Those not expressing CD36 and having low levels of Fcgr4 ($CD36^{\text{negative}}Fcgr4^{\text{low}}$), those expressing CD36 and having low levels of Fcgr4 ($CD36^{+}Fcgr4^{\text{low}}$) and those expressing CD36 and high levels of Fcgr4 ($CD36^{+}Fcgr4^{\text{high}}$). Consistent with the single cell RNA-seq data, DFBL-20954-bearing CTX-treated mice had an increased percentage of bone marrow $CD36^{+}Fcgr4^{\text{high}}$ macrophages as compared to tumor bearing PBS-treated mice (Figure 6.2E).

We sorted the three macrophage populations based on these markers as well as sorted bulk $CD11b^{+}F4/80^{+}$ macrophages that contained all three clusters and performed *ex vivo* phagocytosis assays using these macrophages and alemtuzumab coated lymphoma cells. Consistent with previous data, bulk macrophages from CTX-treated lymphoma bearing mice had increased phagocytic capacity compared to PBS-treated mice (Figure 6.2F). $CD36^{+}Fcgr4^{\text{high}}$ macrophages from PBS-treated mice had a higher phagocytic capacity compared to bulk sorted macrophages or the $CD36^{\text{negative}}Fcgr4^{\text{low}}$ and $CD36^{+}Fcgr4^{\text{low}}$ sorted macrophages (Figure 6.2F). CTX treatment enhanced the phagocytosis of each of these three macrophage populations (Figure 6.2F).

Chapter 7: Discussion

The Bone Marrow as a Therapy Resistant Microenvironment

Treatment resistant niches pose a challenge for effective cancer treatment. In particular, the bone marrow microenvironment is a well-defined therapy resistant niche. This is in part due to potential poor bone marrow drug access, a unique marrow stromal architecture that can modulate drug response as well as its hypoxic nature, which can lead to cell cycle arrest and thereby decrease the efficacy of cytotoxic chemotherapies (Chen et al., 2016; Das et al., 2016; Karjalainen et al., 2017; van Oosterwijk et al., 2018). Herein using DHL xenografts we were able to model acquired bone marrow resistance to antibody therapy and analyze how this resistance develops by comparison to an antibody permissive niche, the spleen, in the same animals. Using these PDXs provides a unique opportunity to study the efficacy of drugs that depend on the tumor microenvironment for their clinical activity and to understand how tumor-mediated microenvironmental reprogramming affects antibody therapeutic efficacy. Specifically, we were able to look at acquired resistance to antibody treatment dependent on macrophages, the effector cells in our system.

In our models bone marrow antibody resistance was due to an increased lymphoma: macrophage ratio established as a function of lymphoma expansion, as in both models at the time of treatment the lymphoma: macrophage ratio was higher in the marrow than in the spleen. How this ratio is established, however, remains poorly understood. Possible explanations include the expression of chemokine receptors that promote lymphoma homing to the bone marrow (i.e. CXCR4) or due to a decrease in macrophage number in the bone marrow as a function of tumor growth (Mazur et al., 2014; Ratajczak et al., 2012). Indeed, a decrease in the number of bone marrow macrophages during tumor progression, as well as a higher number of phagocytic cells in the spleen than the bone marrow prior to treatment initiation were observed in the

humanized DHL model (Pallasch et al., 2014). These observations may also be true in our two DHL xenografts and merit further investigation.

Another possible explanation of observed lower splenic lymphoma to macrophage ratio may be due to the fact that since splenic macrophages consistently phagocytose cells as part of their natural homeostatic function, they may also take up “bystander” tumor cells, leading to a lower lymphoma to macrophage ratio. This may be strengthened by observations comparing the proximity of tumor cells to bone marrow and splenic macrophages, as distance of target cells from macrophages has been implicated in predicting how likely cells are to be phagocytosed (N et al., 2017). Alternatively, it is also possible that other molecules that affect phagocytosis are differentially expressed by the tumor cells of both xenografts in the spleen and bone marrow and that these drive the different rates of macrophage uptake. Indeed, surface MHC-I has recently been identified as a “don’t eat me” signal and its expression may be higher on splenic tumor cells in both xenografts (Barkal et al., 2018).

While lack of antibody activity in the bone marrow was previously observed in humanized mice and attributed to a lack of macrophages in the bone marrow (Lux et al., 2014), other resistance mechanisms have also been described, including the observation of altered levels of activating and inhibitory Fc receptors on microenvironment effector cells (Dahal et al., 2017). While we observed different levels of activating and inhibitory Fc receptors in bone marrow and splenic macrophages in unengrafted and engrafted vehicle treated mice, these did not translate to differences in macrophage phagocytic capacity *ex vivo*, suggesting that functional studies are necessary to corroborate such observations as antibody resistance drivers. *In vivo* confirmation of equal phagocytic capacity in our models would further strengthen this assertion.

In one of our models, DFBL-20954, we were able to demonstrate that bone marrow-derived tumor cells were less likely to be phagocytosed by macrophages than their splenic counterparts. This was in part due to bone marrow lymphoma cells expressing higher levels of factors that inhibit phagocytosis by macrophages in the absence of antibody, specifically PGE2. Moreover, higher levels of antibody-dependent inhibitors of macrophage phagocytosis such as CD32B and galectin-1 were also more highly expressed on bone marrow tumor cells than their splenic counterparts.

Meanwhile, DFBL-69487 bone marrow-derived cells were more likely to be phagocytosed than splenic derived lymphoma cells. In this model, splenic tumor cells expressed higher levels of PGE2 and galectin-1 than their bone marrow counterparts. The contrast between this model and DFBL-20954 demonstrate marked microenvironmental tumor heterogeneity between lymphomas of the same histological subtype and highlight the uniqueness of each individual model. These observations suggest that individual tumors need to be carefully studied to understand their effects on the microenvironment. How the differential expression of these markers across microenvironments is established remains poorly understood and needs to be analyzed further. The observed differences may be a result of heterogeneity between the two xenografts that cause them to interact differently with the murine stroma. This can be due to xenograft intrinsic factors and/or due to acquired post translational modifications that affect their interactions with the surrounding stroma. In support of these possibilities, the expression of PGE2, galectin-1 and the other phagocytic markers was significantly different between the two xenografts in vehicle treated mice. It is also possible that tumor cells interact differently with macrophages *in vivo* than with bone marrow-derived macrophages in the *ex vivo* setting, thereby

explaining the different propensity to be phagocytosed *ex vivo* between the two microenvironments as compared to the observed lymphoma to macrophage ratio *in vivo*.

Our observations of a large lymphoma: macrophage ratio as a driver of antibody inactivity closely parallels observations in humans, where both alemtuzumab and rituximab have limited efficacy in sites of bulky disease (Lundin et al., 1998; McLaughlin et al., 1998). The composition of bulky disease remains poorly characterized; while it is assumed that it mostly consists of tumor cells, a close dissection of its constituents has not been carried out. Therefore, while it is possible that the lack of antibody activity in bulky disease may be due to a lack of effector cells, it is possible that antibody access to tumor is impeded in these sites or alternatively that conditions associated with tumor bulkiness (i.e. hypoxia) could drive decreased therapeutic efficacy. Further research is necessary to differentiate between these possibilities.

We also observed bone marrow lymphoma resistance to doxorubicin treatment. Importantly, this resistance was not due to intrinsic tumor cell apoptotic resistance as bone marrow cells were more primed to undergo apoptosis than their splenic or peripheral blood counterparts. Moreover, doxorubicin was able to access the bone marrow, as seen by induction of DNA-damage in bone marrow cells via γ -H2AX expression, induction of surface VISTA expression, which is downstream of TP53, and RNA-sequencing which demonstrated lymphoma cell cycle arrest and induction of DNA-damage repair pathways. Previous studies have implicated the stromal architecture in mediating resistance to doxorubicin, specifically with respect to FGF4 dependent signaling between lymphoma cells and endothelial cells (Cao et al., 2014). Further studies are needed to demonstrate whether the same mechanism is observed in our DHL models and whether targeting this axis can improve therapeutic responses to doxorubicin.

Alkylating Agents overcome Antibody Resistance

Our studies also identify alkylating agents as drugs that can overcome bone marrow antibody resistance and synergize with alemtuzumab therapy. Importantly, this ability was not due solely to tumor cell death, as agents that led to partial reduction of tumor burden such as ABT-263 demonstrated no synergy with antibody administration. Using cyclophosphamide, a mainstay of lymphoma chemotherapy as a case study, we identified several methods by which high-doses of alkylating agents can overcome antibody resistance. These include: 1) a reduction of the lymphoma: macrophage ratio; 2) phenotypic changes induced by CTX administration that promoted macrophage phagocytosis; 3) induction of a tumor secretory response due to initiation of ER stress that directly promoted macrophage phagocytosis and 4) an altered macrophage profile that consisted of highly phagocytic macrophages that displayed a favorable balance of activating to inhibitory Fc receptors (Figure 7.1).

Using two DHL xenografts established from patients who were refractory to alkylating agent treatment, albeit at lower doses, we demonstrated that DHL xenografts still exhibited sensitivity to higher doses of alkylating agents (CTX at 100mg/kg) but not to high-doses of other cytotoxic chemotherapies (doxorubicin at 5mg/kg). This tumor intrinsic sensitivity of DHL cells to high-dose alkylating agent-induced apoptosis, coupled with the lack of macrophage depletion by this therapy, can explain the ability of high-doses of alkylating agents to reduce the lymphoma: macrophage ratio from that seen in vehicle treated mice. Treatment with high-dose alkylating agents also led an increased number of bone marrow macrophages as soon as 48 hours after therapy initiation. The lack of observed cell division in these macrophages suggests that they were recruited to the bone marrow but further studies are necessary to confirm this hypothesis.

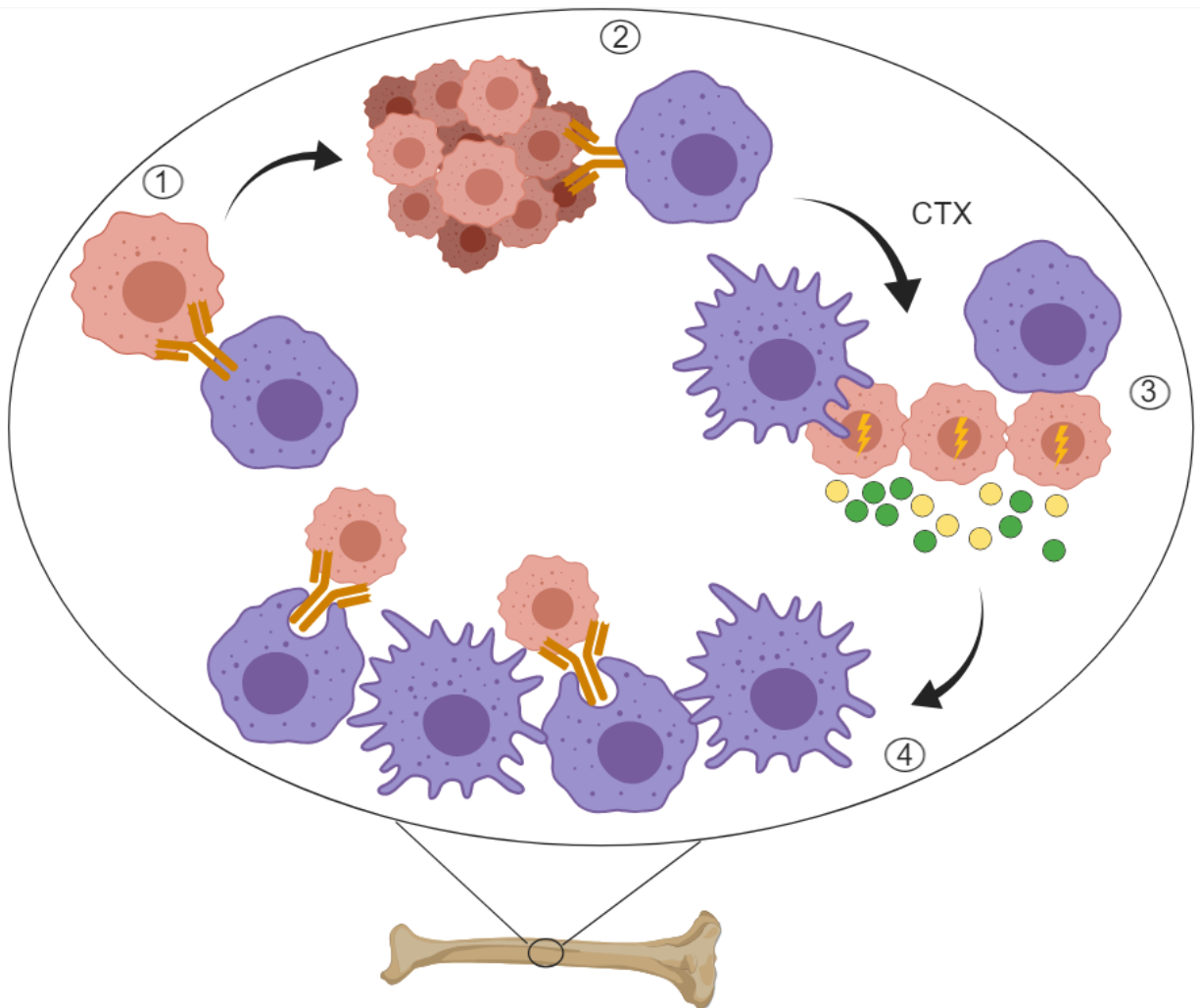


Figure 7.1 Model. Upon initial tumor engraftment (1) the bone marrow niche is responsive to antibody monotherapy. As the tumor progresses there is a higher lymphoma to macrophage ratio (2) that renders the bone marrow refractory to treatment. Treatment with high-doses of alkylating agents such as CTX induces lymphoma ER stress, leading to tumor cell death and release of factors (3) that increase the bone marrow macrophage content and promote a “super-phagocytic” phenotype (4) that can clear antibody coated lymphoma cells (Pallasch et al., 2014).

Consistent with the observed increase in macrophage number post CTX treatment in both xenografts, our studies demonstrate that tumor cell removal by high-doses of alkylating agents were also macrophage dependent, as their ablation with clodronate pre-treatment led to loss of CTX activity in the absence of antibody treatment in DFBL-20954. This observation has important clinical implications: while high-doses of alkylating agents are known to be lymphodepleting, the contribution of macrophages to the activity of high-dose alkylating agents should allow for clinical responses in the absence of T-cells. However, confirmatory studies are needed.

Importantly, a lower lymphoma to macrophage is not the sole mechanism governing antibody resistance or the ability of high-dose alkylating agents to overcome antibody resistance. In the DFBL-69487 model, high-dose CTX administration reduced the bone marrow lymphoma to macrophage ratio to approximately 10:1 at 48 hours after treatment administration. This approximate ratio was observed in the bone marrow at treatment initiation of the DFBL-20954 model, at which point it was resistant to antibody treatment. This observation suggests that while important, the lymphoma: macrophage ratio alone may not fully account for bone marrow antibody resistance and CTX activity.

Consistent with this notion, we observed that high-dose CTX administration was able to alter the balance of pro- and anti-phagocytic markers on tumor cells. In both xenografts CTX but not doxorubicin administration reduced surface levels of the “don’t eat me” CD47 molecule and decreased PGE2 production and secretion. Addition of PGE2 to lymphoma and macrophage co-cultures *ex vivo* inhibited macrophage phagocytosis of DHL cells. In DFBL-20954, high-dose CTX also increased surface expression of the “eat me” calreticulin molecule. Consistent with these observations, CTX-treated tumor cells from both xenografts were more likely to be

phagocytosed by macrophages in comparison to vehicle or doxorubicin treated mice, highlighting an important mechanism by which CTX was able to promote tumor macrophage dependent clearance.

CTX administration differentially altered levels of galectin-1 between the two xenografts. While CTX administration decreased galectin-1 levels in DFBL-20954 cells, galectin-1 levels increased in DFBL-69487 cells after CTX treatment. In DFBL-20954, galectin-1 inhibited phagocytosis in an antibody dependent manner, consistent with previous reports in lymphoma, but increased phagocytosis of DFBL-69487 cells in an antibody independent manner (Lykken et al., 2016). Previous studies have demonstrated that galectin-1 administration was able to enhance macrophage phagocytosis of leukemia cells in the absence of antibody by inducing surface phosphatidylserine expression without causing leukemia apoptosis (Dias-Baruffi et al., 2003). Whether this is the case in DFBL-69487 cells, which express the leukemia marker TdT, needs to be further elucidated but is a possible explanation for the apparent discrepancy between the two xenografts.

In addition to inducing phenotypic changes in the DHL cells that enhanced their uptake by macrophages, CTX administration led to induction of a secretory phenotype in the two xenografts. CTX but not doxorubicin administration led to secretion of VEGF-A and IL-16 in both models as well as secretion of TNF- α and CCL4 from DFBL-69487 cells. Consistent with CTX-induced secretion of VEGF-A, CCL4 and IL-16, all known macrophage chemotactic factors (Barleon et al., 1996; Center and Cruikshank, 1982; Cheung et al., 2009; Zhao et al., 2002), we observed a higher bone marrow macrophage content after CTX but not doxorubicin treatment in both models. Secretion of VEGF-A and IL-16 was also observed following treatment of DFBL-20954 cells with the alkylating agents busulfan and ifosfamide but not

ionizing radiation or ABT-263, suggesting the broad ability of alkylating agents to induce these changes. Together this data demonstrates that high-dose CTX can recruit effector cells to the site of tumor involvement and mediate tumor clearance. Consistent with this, *in vivo* blockade of VEGF-A with bevacizumab decreased DFBL-20954 tumor clearance by high-dose CTX and alemtuzumab treatment. Whether this was due to decreasing bone marrow macrophage content or by directly inhibiting macrophage phagocytosis remains to be explored. The effect of IL-16 in facilitating macrophage influx and tumor clearance also needs to be explored.

VEGF-A administration was able to enhance clearance of antibody bound lymphoma cells of both our DHL xenografts while TNF- α administration enhanced macrophage clearance of DFBL-69487 cells in an antibody independent manner. The activity of the two cytokines to promote tumor clearance was due to different mechanisms: VEGF-A enhanced phosphorylation of the SYK kinase in macrophages, which is downstream of the Fc γ R, while TNF- α induced direct apoptosis of DFBL-69487 cells that promoted their clearance. Therefore, in instances where both are secreted, the two cytokines could potentially enhance tumor cell removal by additive and/or synergistic mechanisms. Both cytokines were identified to be secreted by the humanized DHL and in the DFBL-69487 models, both of which expressed TdT (Pallasch et al., 2014). Further studies are necessary to confirm whether these observations extend to all DHL models and to confirm the ubiquity of these observations in TdT positive DHLs. The mechanism of VEGF-A mediated enhanced macrophage phagocytic function should caution against combining antibody therapy with SYK inhibitors, which are currently in trials in lymphoma (Liu and Mamorska-Dyga, 2017).

Although *in vivo* VEGF-A blockade blocked tumor clearance by cyclophosphamide and alemtuzumab combination treatment, its blockade enhanced tumor clearance by

cyclophosphamide in the absence of antibody. Bevacizumab and cyclophosphamide therapy are frequently used together in ovarian cancer, highlighting the known clinical efficacy of the combination (Chura et al., 2007). It is possible that the mechanism of the additive effect of bevacizumab is to inhibiting nutrient access to tumors through blocking angiogenesis, thereby causing an additional cellular stress that promotes apoptosis (Tamura et al., 2017). In addition to directly blocking macrophage activation/recruitment through VEGF-A inhibition, bevacizumab may also inhibit the efficacy cyclophosphamide and alemtuzumab combination therapy by impeding antibody access to tumors (Arjaans et al., 2013). Further studies are needed to tease the contribution of each of these factors.

TNF- α has previously been shown to induce apoptosis of lymphoma cells, suggesting that CTX mediated TNF- α clearance can act in an autocrine manner to eliminate tumor (Gisslinger et al., 2001; Jurisic et al., 2006). The ability of CTX to induce DHL TNF- α secretion and to assess the sensitivity of other DHL cells to TNF- α needs to be examined in future studies.

The ability of CTX to overcome antibody resistance is not unique to lymphomas, as previous studies in breast cancer and leukemia have demonstrated the ability of alkylating agents to overcome antibody resistance and to display therapeutic synergy and improved survival (Hubert et al., 2011; Pallasch et al., 2014). This suggests that high-doses alkylating agents can be broadly used in combination with antibody treatment to overcome treatment resistance and are not restricted to diseases with bone marrow involvement. However, further studies in other cancers for which there are antibody targets need to be performed to confirm the utility of this treatment approach.

Alkylating Agent Induced ER Stress

Given the shared CTX-induced secretion of VEGF-A in our models and in the humanized lymphoma model, we set out to understand how CTX promoted VEGF-A secretion. Using RNA-seq we observed enrichment of pathways implicated in ER stress uniquely post CTX administration. We confirmed induction of ER stress in *in vivo* treated DHL cells and recruitment of the ER stress induced transcription factor ATF4 to exon 1 of *VEGFA* post CTX treatment at levels higher than vehicle or doxorubicin treatment. Given that our PDX models cannot be efficiently genetically modified with lenti- or retroviruses, we could not confirm the necessity of ATF4 to induce VEGF-A secretion, although ATF4 driven production of VEGF-A has been previously described (Binet and Sapieha, 2015; Ghosh et al., 2010).

Interestingly, we observed higher baseline expression of VEGF-A in the bone marrow compared to splenic cells of DFBL-20954 and induction of VEGF-A only in the bone marrow despite evidence of ER stress in both microenvironments by RNA-seq in both models. Higher baseline VEGF-A production and induction only in the bone marrow may be due to bone marrow hypoxia, as HIF-1a is known to stabilize VEGF-A mRNA and has been shown to play an important role in ATF4 mediated VEGF-A induction (Chipurupalli et al., 2019; Delbrel et al., 2018; Pereira et al., 2014). Further studies are needed to look at induction of ER stress in the spleen at the protein level as well as HIF-1a expression and genomic localization in the two microenvironments.

How alkylating agents can induce ER stress in lymphomas is unknown. From the *in vivo* metabolism of alkylating agents, specifically the generation of acrolein from CTX and ifosfamide and the carbonium ion by busulfan, it is expected that in addition to alkylating DNA substrates they are likely capable of also alkylating protein and lipid substrates, thereby leading to induction of ER stress. Indeed, protein alkylation by acrolein has been observed (Lambert et

al., 2007; Randall et al., 2013). Although no evidence of carbonium ion alkylation exists in the literature, its strong electrophilic properties suggest that it can readily alkylate protein amino groups, although this needs to be experimentally confirmed. The observation of ER stress induction only after high-doses of alkylating suggests that a certain amount of reactive alkylating intermediates need to sufficiently induce enough protein misfolding to observe the UPR but this needs to be corroborated and examined in tumors that were not resistant/refractory to alkylating agents.

In the DFBL-20954 model, while 100mg/kg doses of CTX induced both ATF4 and spliced XBP-1 expression, a lower dose of 50mg/kg was sufficient to induce spliced XBP-1 but not ATF4. These observations suggest that different branches of the ER stress response may be differentially sensitive to stress stimuli and/or other cellular signaling pathways and needs to be studied further. While thrombospondin signaling and metformin administration have been previously demonstrated to induce ATF4 but not XBP-1S, Toll-Like receptor signaling has been shown to induce XBP-1S and inhibit ATF4 expression (Lynch et al., 2012; Quentin et al., 2012; Woo et al., 2009). Illuminating the nature of the cellular stress induced by alkylating agents and the differential sensitivity of the ER stress sensor pathways to different stimuli can provide further insight into the mechanism of alkylating agent induced UPR activation.

Although ER stress induced VEGF-A secretion was able to promote macrophage mediated clearance of antibody bound tumor cells, studies in immunocompetent hosts have demonstrated that ER stress can have immunosuppressive effects on the microenvironment (Chen et al., 2018). These include decreasing antigen presentation by dendritic cells, inhibition of T-cell killing of tumor cells and increased blood supply to tumor cells by promoting angiogenesis (Cubillos-Ruiz et al., 2017; Cubillos-Ruiz et al., 2015). However, the effect of

tumor-intrinsic ER stress on macrophages and antibody therapy has not been studied in these systems. Given the mechanistic insights here, it is possible that antibody therapy could overcome these described negative effects, but further studies are necessary to test this hypothesis.

ER stress induction was sufficient to reduce levels of CD47 in both xenografts and increase surface calreticulin in DFBL-20954 and production of CCL4 in DFBL-69487 cells, respectively, consistent with the observed activity of CTX in our models. However, in contrast to CTX activity, the ER stress inducing agents thapsigargin and tunicamycin failed to induce changes in galectin-1 and TNF- α levels, while leading to increased PGE2 production. These observations suggest that while ER stress can account for many of the observed phenotypic effects of CTX administration, other processes may regulate the altered production of TNF- α , galectin-1 and PGE2 following CTX treatment. Consistent with the activity of high-dose CTX in our models, studies looking at the activity of adding bortezomib to current gold standard therapies in DLBCL have demonstrated a trend towards significance in improving PFS and OS in high grade DLBCLs like DHL by subgroup analysis ($p=0.08$) (Sha et al., 2019). This observation needs to be further confirmed in prospective clinical trials. While using bortezomib may be safer than high-dose alkylating agent administration, which is quite toxic, it may not lead to the entire gamut of beneficial effects of high-dose CTX treatment. Studies in mice, which better tolerate high-dose alkylating agents than humans, may resolve this question though the translational relevance of these experiments remains unknown.

Alkylating Agent Macrophage Reprogramming in Lymphoma Bearing Mice

We extended our observations to look at the functional effects of CTX administration on the ability of macrophages to clear lymphoma cells. Isolated bone marrow and splenic

macrophages from tumor bearing and CTX-treated mice had a significantly enhanced capacity to phagocytose antibody opsonized lymphoma cells, a finding attributed to an altered balance of activating and inhibitory Fc receptors to promote phagocytic intake. Importantly, the presence of lymphoma in the absence of treatment or CTX treatment in the absence of lymphoma failed to induce these phenotypic and functional changes in macrophages, suggesting that both lymphoma and macrophages needed to be present in order for the full effect. How Fc receptor balance is altered in the presence of lymphoma and macrophages needs to be further elucidated.

Analysis of the surface Fc receptors of macrophages demonstrated that levels of the inhibitory Fcgr2B are enhanced on vehicle treated tumor associated macrophages, consistent with previous reports. However, while other groups have reported a loss of activating Fc receptors, we did not observe these changes in our models and consistent with this there was no altered phagocytic capacity of macrophages from unengrafted mice and tumor engrafted vehicle treated mice. Interestingly, administration of doxorubicin increased levels of Fcgr2B in both models. This demonstrates that several members of the treatment regimen R-CHOP which is used in frontline therapy of DLBCL may have antagonistic effects on antibody mediated phagocytosis that would not be seen from *in vitro* administration of the different cytotoxic compounds to lymphoma cells. Therefore, an analysis of any considered chemotherapy should also be performed for *in vivo* efficacy to understand potential additive and/or synergistic future treatment combinations, especially those that depend on the tumor microenvironment for their activity.

We also observed that in the absence of tumor, CTX was able to induce a partial alteration of the balance of activating and inhibitory Fc receptors in bone marrow macrophages but not those in the spleen, while the presence of lymphoma failed to elicit these changes.

Previous studies have demonstrated transcriptional differences between macrophages from different microenvironments, suggesting that bone marrow macrophages may be more “primed” to undergo reprogramming by CTX or alternatively that the reprogramming done by CTX and lymphoma may have a different mechanism in the two microenvironments (N et al., 2017). Indeed, given that we observed macrophage recruitment to the bone marrow after CTX administration in the presence of lymphoma, it is possible that it is the recruited macrophages that undergo the reprogramming or alternatively that they express the different Fc receptors *a-priori*. Future studies are needed to look at splenic macrophage recruitment post CTX and the profile of macrophages in other tissues to address this issue.

Our bulk RNA-sequencing of bone marrow and splenic macrophages demonstrated that macrophages treated with CTX cluster together independently of engraftment with lymphoma. However, single cell RNA-seq profiling was able to resolve a unique transcriptional cluster, which we termed C4, which was present almost exclusively in the bone marrows of tumor engrafted and CTX-treated mice. These C4 macrophages had high expression of Fcgr4 and CD36 and lower expression of the inhibitory Fcgr2B receptor and were highly phagocytic *ex vivo* compared to non-C4 macrophages. Confirmatory studies are needed to ascertain that the sorted CD36⁺Fcgr4^{High} macrophages are in fact transcriptionally defined C4 macrophages and these studies are currently ongoing.

Lymphoma bearing CTX-treated mice were also enriched in C2 cluster macrophages, which possessed high expression of the high affinity Fcgr1. Further studies are needed to determine which macrophage subset mediates the synergistic effect observed with CTX treatment in the presence of lymphoma. Given that C2 macrophages were present in both unengrafted mice and mice with tumor in all treatment conditions, it is possible that C2

macrophages mediate the early activity of alemtuzumab treatment and that subsequent recruitment and/or reprogramming of resident macrophages to C4 macrophages mediates further synergistic effect. Indeed, C4 macrophages are characterized by elevated expression of VEGF-A targets, suggesting that they are programmed/recruited in response to secretion from lymphoma post CTX administration. Further studies are needed to confirm the necessity of VEGF-A for generation/recruitment of C4 macrophages and their importance for mediating synergy with CTX and alemtuzumab.

Our observations suggest that high-dose CTX could synergize with antibody therapy in eliminating tumor cells via phagocytosis. Current trials of CD47 and Sirp-a targeting antibodies, which attempt to eliminate tumor cells by enhancing macrophage phagocytosis, may thus be appropriate to combine with high-dose CTX or other ER stress inducing regimens in lymphoma patients. Sirp-a blocking antibodies may be the more rational treatment target as we observed downregulation of CD47 following ER stress induction in our lymphoma models, although further studies are needed to confirm this hypothesis. On the other hand, high-dose CTX administration could inhibit the efficacy of other therapies. Fc receptors, which are enhanced on macrophages following high-dose CTX administration, have recently been implicated in mediating resistance to checkpoint blockade therapy (Arlauckas et al., 2017). Though this may be of limited concern given the T-cell depleting effects of high-dose CTX, recent studies have demonstrated that NK-cells can also mediate the efficacy of checkpoint blockade inhibitors (Hsu et al., 2018), suggesting the need to rationally design potential synergistic therapies.

In addition to expressing CD36 and Fcgr4, C4 cluster macrophages were also characterized by expression of PPARg and ApoE proteins. PPARg was previously defined as a marker of M2 macrophages, consistent with our MDS analysis, and known to be a marker of

phagocytic macrophages (Bouhlef et al., 2007). These transcription factors and CD36 have previously been demonstrated to be characteristic of “foamy-cell” macrophages that are known to be implicated in cardiovascular disease and atherosclerosis (Marechal et al., 2018; Nicholson, 2004; Park, 2014; Zhu et al., 2014). Interestingly, high-doses (but not low doses) of CTX have been associated with patient cardiotoxicity (Goldberg et al., 1986; Gottdiener et al., 1981; Zver et al., 2007). Whether the C4 macrophages mediate this cardio-toxicity and their role in cardiovascular pathology associated with CTX remains unknown and merits further investigation.

Summary

In conclusion, our studies demonstrate how DHLs modulate the tumor microenvironment to induce antibody resistance and how this can be overcome by high-doses of alkylating agents. Using the bone marrow as a model of alemtuzumab resistance, we show that unchecked lymphoma growth promotes an increased lymphoma to macrophage ratio that leads to loss of antibody efficacy. Additional tumor specific factors at resistant niches, such as higher expression of “don’t eat me” or lower expression of “eat-me” signals may further drive this resistance. In addition to directly leading to DHL cell death, high-dose alkylating agents can modulate the expression of tumor intrinsic factors that can promote macrophage phagocytosis. Moreover, high-dose alkylating agents also lead to macrophage influx into the resistant niches, contributing to a decreased lymphoma: macrophage ratio in these environments and to the presence of lymphoma dependent “super phagocytic” macrophages.

Chapter 8: Experimental Methods

Mouse Models and Therapeutics

DFBL-20954 and DFBL-69487 are double hit lymphoma xenografts available from the Public Repository of Xenografts (PRoXe) (Townsend et al., 2016). 6-8-week old NSG mice (Jackson labs) were injected with 10^6 tumor cells intravenously (i.v.). Mice were bled weekly and treatment was initiated when circulating disease was $>2\%$ as assessed by flow cytometry staining for hCD19 (clone HIB19, Biolegend) and hCD45 (clone HI30, Thermo Fisher). All blood samples were lysed with ammonium chloride red-blood cell buffer (VWR) prior to staining. Mice were assigned to treatment groups based on levels of circulating disease to normalize level of peripheral blood involvement.

Clinical grade Alem (Genzyme-Sanofi) and Rituximab (Roche) were obtained dissolved in PBS at 30mg/ml and 10mg/ml, respectively, from the Dana Farber Cancer Institute Pharmacy and Cologne. Prior to administration Alem was diluted 1:30 in PBS (10mg/kg doses) or 1:60 (5mg/kg doses) and mice were dosed with 10ul/g. Alem was administered 5mg/kg i.p. on day 0 and 5 mg/kg IV on day 1 of treatment to avoid tumor-lysis syndrome unless otherwise specified. Cyclophosphamide (Sigma Aldrich) was dissolved at 10mg/ml in PBS and administered i.p. on day 0 at a dose of 10ul/g of mouse body weight. For studies where cyclophosphamide was administered at 25 and 50 mg/kg, respectively, it was dissolved at 2.5 and 5 mg/ml PBS and dosed at 10ul/g of mouse body weight. Doxorubicin (Tocris Bioscience) was dissolved at 0.5mg/ml of 0.9% NaCl solution and administered i.p. at 10ul/g of mouse body weight. Ifosfamide (Sigma Aldrich) was dissolved at 5 mg/ml (50mg/kg), 10mg/ml (100mg/kg) or 20mg/ml (200mg/kg) in PBS and administered as cyclophosphamide. Busulfan (Sigma Aldrich) was dissolved in DMSO and then in PBS to generate a 2.5 mg/ml solution with 10% DMSO and dosed as 10ul/g mouse body weight for one or two days. Bevacizumab was obtained from the

Beth Israel Deaconess Medical Center pharmacy dissolved at 25mg/ml in PBS and was administered at 25mg/ml at 10ul/g of mouse body weight on days -1, 0 and 1 of treatment. Navitoclax was dosed at 100mg/kg p.o. on days 0-7. All *in-vivo* experiments were conducted in accordance with Dana Farber Cancer Institute Animal Care and use Committee protocol #13-034.

Bone Marrow-derived Macrophage (BMDM) Generation

BMDMs were generated as previously described. Briefly, 8-10-week old NSG mice were euthanized and femurs were flushed with a 27G needle and sifted through a 70 micron filter. The filtrates were Red-Blood Cell (RBC) lysed with ammonium chloride (VWR) and plated in RPMI-1640 media supplemented with 10% L929 cultured cell media (ATCC), 10% Fetal Bovine Serum (Sigma Aldrich) and 1% Pen/Strep solution (Thermo Fisher Scientific). Media was changed every 2-3 days until day 7 when the macrophages were used for functional assays.

Cell Culture

Raji-GFP cells were a generous gift from Dr. Izidore Lossos. Raji cells were maintained in RPMI-1640 media supplemented with 10% Fetal Bovine Serum (Sigma Aldrich) and 1% Pen/Strep solution (Thermo Fisher Scientific). Cells were split 1:10 every 2-3 days and checked for mycoplasma every 3 months.

Phagocytosis Assays (BMDM and In vivo derived Macrophages)

In vitro differentiated macrophages were generated as described above. On day 7 BMDM were plated at 1×10^5 cells per well of a 24 well plate and serum starved for 2 hours in RPMI-1640

media supplemented with 1% Pen/Strep. Target tumor cells (3:1 lymphom to macrophage ratio) were labeled with CFSE (CellTrace CFSE Cell Proliferation Kit, Thermo Scientific) per the manufacturer's instructions and 30 minutes prior to co-incubation with macrophages were pre-coated with Alemtuzumab or rituximab (final concentration 20 µg/mL per ml of total culture media of total plates) at 37° C. Coated and uncoated tumor cells were then co-incubated with macrophages for 2 hours. Following incubation, wells were washed 2X with PBS and macrophages were detached by gentle scraping in ice cold PBS. Cells were then pre-incubated with human (Human TruStain FcX, Biolegend) and murine Fc-Block (BD Biosciences) and then stained with a CD11b (clone M1/M70, APC, Biolegend) for 30 minutes at room temperature and analyzed using a Cytoflex (Beckman Coulter). Phagocytosis was measured as the fraction of CFSE positive CD11b⁺ macrophages. All cytokines used in the assays were purchased from Peprotech and added at the indicated doses immediately following the addition of tumor cells to the macrophage co-culture. Recombinant human galectin-1 was purchased from R&D. Prostaglandin E2 and BAY61-3606 were purchased from Sigma Aldrich. Mafosfamide and lactose were purchased from Santa Cruz Biotechnology. All caspase inhibitors were purchased from R&D. Macrophages were incubated with tumor cells for six hours for phagocytosis assays with TNF-α. For phagocytosis assays using in vivo derived macrophages, 10⁶ xenograft cells were injected into 6-10-week old NSG mice. Once peripheral blood disease reached >2%, mice were treated with PBS, Dox or CTX as described above. Age controlled unengrafted mice were treated with PBS or CTX as above. Mice were euthanized and bone marrows and spleens were processed as described above. Samples were pre-treated with human Fc-block and stained with 7-AAD (Thermo Fisher Scientific), human CD19 (clone HIB19, Biolegend) murine CD11b (clone M1/M70, Biolegend) and F4/80 (clone BM8, BD Biosciences) antibodies and sorted using an

LLC FACSariaII (BD Biosciences). Splenic macrophages were defined as 7AAD⁻CD19⁻CD11b⁻F4/80⁺ cells while BM macrophages were defined as 7AAD⁻CD19⁻CD11b⁺F4/80⁺ cells. Cells were plated at 50,000 cells per well in a 96 well plate in RPMI-1640 media supplemented with 10% L929 media, 10% FBS, 1% Pen/Strep and 50mM 2-ME (Sigma Aldrich). Once cells attached and spinous processes were microscopically visible (12-16 hours after plating), macrophages were serum-starved for 2 hours and co-cultured with CFSE stained tumor cells +/- Ale as described above. Cells were processed and analyzed as above but the murine F4/80 was used to label macrophages instead of CD11b. Phagocytosis was measured as the fraction of CFSE positive F4/80⁺ cells.

Immunoblotting

NSG mice were injected with 10⁶ tumor cells intravenously and treated with PBS, Dox or CTX. 16 (DFBL-20954) or 48 hours (DFBL-69487) after treatment bone marrow or splenic cells were harvested as above and human cells purified using an EasySep Mouse/Human Chimera Isolation Kit (StemCell Technologies). Dead cells were removed by an EasySep Dead Cell Removal Kit (StemCell Technologies). Cells were lysed in RIPA buffer (Cell Signaling Technology) supplemented with Halt protease and phosphatase inhibitors 100x (Life Technologies) and protein amounts quantified (Pierce BCA Protein Assay Kit, Life technologies). Lysates were incubated with NuPAGE LDS Sample Buffer (Invitrogen) supplemented with 2-ME at 95°C, run on an SDS gel and transferred to a PVDF membrane (iBlot2 Transfer Stacks, Life Sciences). Membranes were blocked for 1 hour with 5% dry milk and incubated with ATF4 (D4B8, Cell Signaling Technologies), XBP-1 Spliced (D2C1F, Cell Signaling Technologies), Phospho-Histone H2A.x (Ser 139, Cell Signaling Technology) and Beta-Actin (13E5, Cell Signaling

Technologies) antibodies overnight. All antibodies were diluted 1:1000. Membranes were washed three times with TBS-T solution, incubated with HRP-conjugated Goat Anti-Rabbit IgG (Bio-Rad) and imaged with an ImageQuant LAS-4000 (GE Healthcare Life Sciences).

Chromatin Immunoprecipitation and quantitative PCR (ChIP-qPCR)

NSG mice were injected with 10^6 tumor cells intravenously and treated with PBS, Dox or CTX. 16 (DFBL-20954) or 48 (DFBL-69487) hours after treatment bone marrow or splenic cells were harvested as above and human cells purified using an EasySep Mouse/Human Chimera Isolation Kit (StemCell Technologies). Dead cells were removed by an EasySep Dead Cell Removal Kit (StemCell Technologies). ChIP was performed using a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technologies) per the manufacturer's instructions. Briefly, cells were cross-linked with 4% paraformaldehyde solution and quenched with glycine. Chromatin were digested with Micrococcal Nuclease and sonicated with a QSonica Q500 Sonicator with 8 (DFBL-20954) or 10 (DFBL-69487) cycles 20 seconds on 20 seconds off at 25% amplitude on ice. Input was removed and nuclei were incubated with rabbit anti-ATF4 (Abcam), Rabbit IgG polyclonal isotype control (Abcam) or Anti-Flag (D6W5B, Cell Signaling Technologies). Pulled DNA was isolated with ChIP-grade Protein G Magnetic beads and subjected to washes in increased salt concentrations and cross-linking was undone by incubation with 5M NaCl and Proteinase K and eluted using elution buffer. DNA was purified using the same kit per the manufacturer's instructions. DNA was amplified using a CFX96 Real Time System (Bio-Rad) with SYBR-green (PowerUp Master Mix, Life Technologies). Primers used were VEGF-A (Exon 1, Forward: 5'-CGTCGGGCCTCCGAAACCATGAACT-3', Reverse: 5'-GTGGAGGTAGAGCAGCAAGG-

3'), ASNS (Exon 7, Forward: 5'-GCAGCTGAAAGAAGCCCAAGT-3', Reverse: 5'-TGTCTTCCATGCCAATTGCA-3').

RNA isolation and RNA-Sequencing

NSG mice were injected with 10^6 tumor cells intravenously and treated with PBS, Dox, Alem, CTX or combinations. 16 or 48 hours after treatment bone marrow or splenic cells were harvested. For sequencing of human samples human cells were purified using an EasySep Mouse/Human Chimera Isolation Kit (StemCell Technologies) and total RNA was isolated with an RNEasy Plus Mini Kit (Qiagen). For sequencing of human macrophages bone marrow cells were harvested and macrophages sorted as previously described. Total RNA was isolated with an RNEasy Plus Micro Kit (Qiagen). Mapped read counts for each transcript were normalized using the Bioconductor package DESeq2 in R (Love et al., 2014). Differential expression analysis was also performed using DESeq2. For heatmaps of gene expression, all normalized gene expression values were log2-transformed and scaled to have zero mean and unit standard deviation across samples. To generate a multidimensional scaling (MDS) plot of macrophage expression profiles, reference gene expression data for M0, M1 and M2 macrophages were downloaded from NCBI GEO (Jablonski et al., 2015), and all expression data were quantile normalized to remove batch effects before performing MDS.

Cytokine Arrays

Mouse tibias were flushed. Whole cell suspensions or pelleted cells and the corresponding aqueous portions were incubated with NP-40 lysis buffer with Halt protease and phosphatase inhibitors 100x (Life Technologies) and protein amounts quantified (Pierce BCA Protein Assay

Kit, Life technologies). Thapsigargin and tunicamycin were purchased from Cell Signaling Technology and dosed at 2000 nM and 10 µg/ml, respectively. Samples were diluted to contain equal amounts of total protein and were submitted to Eve Technologies (Ontario, Canada) for Human 65-plex and Murine-41 plex discovery arrays.

Flow cytometry

NSG Mice were injected and cells isolated as above. The following antibodies were used for flow cytometric analyses: human CD19 (clone HIB19, Biolegend), human CD20 (clone 2H7, Biolegend), human CD47 (clone B6H12, Thermo Fisher Scientific), human CD52 (clone HI186, Biolegend), human Calreticulin (clone FMC75, Abcam), human VISTA (clone 730804, R&D Systems), human CD32 (clone AT10, Abcam), murine CD4 (clone GK 1.5, Biolegend), murine F4/80 (clone BM8, BD Biosciences), Zombie-NIR (Biolegend), 7-AAD (Thermo-Fisher Scientific), murine CD64 (clone 290322, R&D systems), murine CD32B (clone AT130-2, eBioscience), murine CD16 (clone 275003, R&D Systems), murine CD16.2 (clone 9E9, Biolegend), human CD36 (clone HM36, Biolegend), murine CD11b (clone M1/M70, Biolegend), murine CD206 (clone MMR, Biolegend) and mouse I-A/I-E (major histocompatibility complex class 2, clone M5/114.15.2, Biolegend). Human (Human TruStain FcX, Biolegend) and murine Fc-Block (BD Biosciences) were used in all instances except for when directly staining for Fc receptor expression. For all mean fluorescent intensity (MFI) stains 10⁴ viable human/mouse cells were used with saturating amounts of antibody.

ELISA

Tibias were flushed and processed as for cytokine arrays. Human galectin-1 ELISA Kit was from Thermo Fischer Scientific. Prostaglandin E2 ELISA Kit was purchased from Abcam. Standard Curves were generated from provided reagents and from recombinant proteins and quantified using SoftMax Pro software on a Spectromax M190 per the manufacturer's instructions.

Conditioned Media Generation

NSG mice were injected and treated as above. Six hours after treatment human tumor cells were purified using an EasySep Mouse/Human Chimera Isolation Kit (StemCell Technologies). Purified cells (10^7) were cultured in 10 ml of X-Vivo 15 media (Lonza Bioscience) for 24 hours post isolation. Cells were pelleted with centrifugation (500g) for 10 minutes and supernatant removed and applied to macrophages following serum starvation for phagocytosis assays. Thapsigargin- and tunicamycin-conditioned media was generated from 10^6 cells.

BH3 Profiling

Cells isolated from the spleen, bone marrow, and peripheral blood were subjected to flow cytometry-based BH3 profiling in accordance with previous methods (Sarosiek et al., 2017). Single cells were resuspended in MEB buffer, permeabilized with 0.001% digitonin, and added to each peptide treatment well and incubated for 60 min at 28°C. Peptide exposure was terminated with 4% formaldehyde. Cells were stained overnight with antibody to Cytochrome C (Biolegend). Cytochrome C negativity was measured on a BD Biosciences LSR II flow cytometer.

Single-Cell Whole Transcriptome Amplification

Single-cell RNA-seq was performed using the Smart-Seq2 protocol as described in Trombetta et al. with minor modification (Trombetta et al., 2014). Briefly, following sorting, 96-well plates of lysed cell samples were cleaned with 2.2x volume AMPure XP SPRI beads (Beckman Coulter). Reverse transcription was performed using SuperScript iii reverse transcriptase (Thermo Fischer Scientific) at a volume of 0.5 μ L per sample in place of the Maxima RT prescribed in the SmartSeq2 protocol. PCR was then performed on the samples as previously described. Following whole transcriptome amplification (WTA), PCR products were cleaned with 0.9x volume SPRI beads and eluted in water. The concentration of the amplified cDNA in the resulting solution was determined using a Qubit 3.0 Fluorimeter (ThermoFischer), and the amplified cDNA was analyzed using a D5000 high sensitivity DNA tape for Tapestation (Agilent).

Preparation of cDNA Libraries for RNA-seq

WTA products were diluted to a concentration of 0.2 ng/ μ L, or used as-is for samples less than 0.4 ng/ μ L, and tagged and amplified using Nextera XT DNA Sample preparation reagents (Illumina). Tagmentation was performed according to manufacturer's instructions, modified to use $\frac{1}{4}$ the recommended volume of reagents, extending tagmentation time to 10 minutes and extending PCR time to 60s. PCR primers were ordered from Integrated DNA Technologies. Nextera products were then cleaned twice with a 0.9x volume of SPRI beads and eluted in water. The library was quantified using Qubit and analyzed using a high sensitivity DNA tape (Agilent). The library was diluted to 2.2 pM and sequenced on a NextSeq 500 (Illumina) to an average depth of 831390 reads using paired-end (PE) 30-base pair reads.

Single-Cell RNA-Seq Expression Quantification

RNA-seq reads were aligned to the UCSC genomeStudio mm10 mouse transcriptome using STAR aligner (Dobin et al., 2013). The resulting transcriptome alignments were processed by RSEM to estimate the abundance (expected counts and TPM) of RefSeq transcripts (Li and Dewey, 2011).

Single-Cell Filtering and Gene Filtering

In sample filtering, we excluded libraries with poor values for number of aligned reads (<200,000) or percentage of aligned reads (<30%). Out of 960 initial samples, we retained 703 after filtering, or about 73% passing filter. We then removed any genes whose expression values summed to 0 following cell filtering, leaving 16,019 genes.

PCA and clustering

We used the Seurat R package (Seurat version 2.3.2, R version 3.5.0) to visualize and cluster our filtered data. We performed log-normalization, scaling and selected the 847 top variable genes for principal components analysis (PCA). Running jackstraw, calculating percent variation captured by each PC, and plotting the standard deviation explained by each PC in an elbow plot suggested somewhere between the top 5 and 9 PCs were significant. After carefully exploring each of the top 9 PCs, we elected to use PC 1 through 5 in our downstream analysis. PCs 1 (11.1% variation) and 2 (7.03% variation) capture differences in cell cycle and separate the BLCTX condition. PCs 3 (3.41% var) and 4 (1.97% var) further separate the BLCTX condition as well as the BLDOX condition, and separate cells by expression of Ly6 genes. PC5 (1.57% var) captures some antigen presentation and phagocytosis related genes. PC6 (1.10% var, not

included in further analysis) is defined by some lowly expressed genes present in only a few cells. Higher PCs had very low signal, both in terms of dynamic range of expression and number of cells expressing top loaded genes. We created a tSNE plot using the top 5 PCs to visualize our dataset. We then performed clustering using the shared nearest neighbors (SNN) algorithm (Waltman and van Eck (2013) *The European Physical Journal B.*) over the top 5 PCs.

Differential Expression and Pathway analysis

We applied the FindMarkers function from the Seurat package, calling the MAST package as the test under test. used to test for differential expression between the different clusters observed in the dataset. Genes differentially expressed with an adjusted p-value >0.1 (Bonferroni corrected), log-fold change >0.5 in either direction, and expressed in a minimum of 25% of the cells in either population were brought into IPA (Qiagen). We ran a core analysis over each DE gene list in IPA to identify pathways possibly activated/inhibited between the different groups.

scRNA-Seq analysis Statistics

Cluster enrichments by sample of origin were calculated by a Fischer's Exact Test with Bonferroni correction for multiple comparisons (Corrected for 25 comparisons, 5 clusters * 5 conditions). Contingency tables were made for each condition tested: number of cells in cluster tested, number of cells not in cluster tested by number of cells from treatment condition tested, and number of cells not from not treatment condition tested.

A Mann-Whitney test calculated p values for expression of FcγR genes between clusters. P values for one cluster vs all others pooled and Bonferroni corrected for 20 comparisons are reported in the text. Pair-wise comparisons for clusters of interest are also reported in

Supplementary Table 7. For sc-RNA Seq expression of *Fcgr* genes, we also report effect sizes for the magnitude of the effect observed in addition to statistical significance (p-value). The calculation was performed as Cohen's d, where effect size d is the number of standard deviations equivalent to the difference in the mean for each population: $d = (\text{Mean1} - \text{Mean2}) / (\text{SD pooled})$.

XBP-1 Splicing Assay

To assay for *XBP-1* mRNA expression and splicing, qPCR primers for total and spliced form of *XBP-1* RNA were used to amplify cDNA generated from 500ng of each RNA sample, and each form of RNA was quantified relatively to beta-actin (*ACTB*) mRNA expression using the delta C_t method. Primer sequences for total *XBP-1* mRNA are as follows: forward 5'-CCTTG TAGTTGAGAACCAGG-3', reverse 5'-GGGGCTTGGTATATATGTGG -3'. Primer sequences for spliced form of *XBP-1* are as follows: forward 5'-TGCTGAGTCCGCAGCAGGTG-3', reverse 5'-GCTGGCAGGCTCTGGGGAAG-3'. Primers for *ACTB* mRNA are as follows: forward 5'-CATGTACGTTGCTATCCAGGC-3', reverse 5'-CTCCTTAATGTCACGCACGAT-3'.

Microscopy Phagocytosis Study

DFBL-20954 cells were stained with DAPI (Thermo Fisher) per the manufacturer's instructions and cultured with BMDMs as in the phagocytosis assays above with alemtuzumab (30µg/ml). Following co-incubation media was aspirated and cells were washed with PBS and stained with a FITC conjugated F4/80 antibody (Biolegend) for 30 minutes and imaged on a Zeiss Axioimager M1 Microscope. Images were taken at 20x.

pSYK Phosphorylation Assay

BMDMs were generated as above. BMDMs were seeded at 10^5 cells per well in a 24 well plate and were treated with Alemtuzumab (20 μ g/mL) or mouse IgG1 isotype control (20 μ g/mL, sc-2025, Santa Cruz Biotechnology) in the presence or absence of human VEGF-A (10ng/ml, Peprotech) and SYK inhibitor BAY61-3606 (Sigma Aldrich). After 5 minutes Anti-Human Fab (I5260, Sigma-Aldrich) was added for 10 minutes (20 μ g/mL). Cells were washed twice with PBS, detached with gentle scraping and fixed and permeabilized with appropriate buffers per manufacturer's instructions (eBioscience IC Fixation and Permeabilization Buffers, Thermo Fisher Scientific). Macrophages were stained with 7-AAD for viability gating and pSYK (Tyr 348, clone mochlct, Life Technologies), Total Syk (clone 5F5, Biolegend) or isotype control IgG.

Clodronate

Clodronate was purchased from clodronateliposomes.com. Mice were injected with 25 (partial depletion) or 200 (full depletion) μ L of liposome solution stored at 4°C and warmed to room temperature i.v. as described in figure legends. Mice were checked 48 hours post injection for depletion.

Immunohistochemistry

NSG mice were injected and treated as previously described. 16 and 48 hours after treatment femurs and spleens were excised and fixed in 4% paraformaldehyde for 48 hours then transferred to 70% ethanol. Immunohistochemistry for c-MYC, Bcl-2, CD20, CD19 and Ki-67 was performed on the Leica Bond III automated staining platform. Antibody c-Myc from Abcam

catalogue # ab32072 clone Y69 was run at 1:100 dilution using the Leica Biosystems Refine Detection Kit with EDTA antigen retrieval. Antibody Bcl-2 from Dako catalogue # M0887 clone 124 was run at 1:2500 dilution using the Leica Biosystems Refine Detection Kit with EDTA antigen retrieval. Antibody CD20 from Dako catalogue # M0755 clone L26 was run at 1:500 dilution using the Leica Biosystems Refine Detection Kit with citrate antigen retrieval. Antibody CD19 from Cell Signaling Technology catalogue # 90176 clone D4V4B was run at 1:800 dilution using the Leica Biosystems Refine Detection Kit with citrate antigen retrieval. Antibody Ki-67 from Biocare catalogue # CRM325 clone SP6 was run at 1:100 dilution using the Leica Biosystems Refine Detection Kit with EDTA antigen retrieval. For HGAL and LMO2 tissue sections were cut from formalin-fixed paraffin-embedded tissue blocks at 4 µm and mounted on positively charged slides. Immunohistochemistry was performed using a standard protocol as previously described (Kim et al., 2009). In brief, all tissue sections underwent heat-induced epitope retrieval in pH 6.0 citrate buffer (Dako, Carpinteria, CA, USA). Endogenous peroxidase was blocked by 3% H₂O₂ solution for 10 min. LMO2 staining was performed using LMO2 antibody (Lossos lab, Miami, FL, USA) using a 1:100 dilution and overnight antibody exposure time and HGAL (Lossos lab, Miami, FL, USA) using a 1:50 dilution and overnight antibody exposure time. CD52 staining was performed with clone YTH34.5 (Serotec, Oxford, United Kingdom) at a 1:2000 dilution as described previously (Craig et al., 2018).

Immunohistochemistry for F4/80, CD10 and TdT was done by Applied Pathology with antibodies CI:A3-1 (F4/80), EPR-5904 (CD10) and 41A (TdT) from Abcam. Antigen retrieval was with Historeveal at a 1:1000 dilution for F4/80 and with Tris for CD10 (1:2000) and TdT (1:50).

BCL-2 and c-MYC FISH Analysis

Five-micron sections were cut from paraffin-embedded tissue onto charged slides and used for fluorescence in situ hybridization (FISH) studies. FISH evaluation for MYC rearrangement was performed on nuclei with the Vysis LSI MYC Dual Color, Break Apart Rearrangement Probe (Abbott Molecular) at 8q24. The normal range established for this probe in the performance laboratory was up to 3%. FISH evaluation for IGH-BCL2 rearrangement was performed on nuclei with the Vysis LSI IGH/BCL2 Dual Color, Dual Fusion Translocation Probe (Abbott Molecular) for IGH at 14q32 and BCL2 at 18q21. The normal range established for these probes in the performance laboratory was up to 1%. Evaluation for rearrangement was performed on 50 nuclei for each probe set; a result was considered abnormal when the percentage of rearranged nuclei exceeded the established normal range.

Statistics

Overall survival was determined by the Kaplan-Meier method using the log-rank test and considered significant at the <0.05 level. Alem and CTX synergy was calculated as previously described. Comparisons of continuous measures between two group was made using a two-sided Welch t-test and were considered significant at the 0.05 level. Differential expression of bulk RNAseq between experimental conditions was determined using raw count data and normalization procedures within the DESeq2 package in R based on a negative binomial distribution. The false discovery rate (FDR) by Benjamini and Hochberg method was used to adjust for multiple comparisons. Ordered lists determined by differential expression analysis were then used in gene set enrichment analysis (GSEA) using tools developed by the Broad Institute and FDR q-values were also reported.

Appendix

Appendix: Genes comprising single cell macrophage clusters

C0	C1	C2	C3	C4
Ccl6	Ltf	Crip1	Hmgn2	Cst3
Retnlg	Ngp	Mndal	H2afz	Clec4a3
Sfxn5	Lcn2	Ctsc	Tubb5	Nr4a1
Mxd1	Camp	Tmsb10	Ptma	Emr4
Csf3r	Anxa1	Ms4a6c	Top2a	Ace
Cxcr2	Ifitm6	F13a1	Tagln2	Gm11711
Msrb1	9030619P08Rik	Rplp0	Hist1h2ao	Gm11710
Fth1	Ly6i	Lgals1	Rrm2	Gm2a
Wdr92	Adpgk	Ccr2	Lta4h	Cd300lh
Sell	Ly6a	Npc2	Stmn1	B2m
Zfp36	Ly6f	Rpl3	H2afx	Abi3
Malat1	Chi3l3	Pld4	Tuba1b	Trf
Gm12505	Cd177	Rps5	Ppia	Ctss
Lep1	Trp53imp2	Ms4a6d	Hmgb1	Cd300ld
Dusp1	1100001G20Rik	Ms4a4c	Serpinb1a	Cd300e
Grina	Plbd1	Ctss	Cd63	Ear2
Mmp9	Ltb4r1	S100a10	Rgcc	Trem14
Ctsd	Dstn	Eef1a1	Ms4a3	Pld4
Il1f9	BC049730	Rps26	Hist1h4d	Ctsb
Trpm2	Lrg1	Rps4x	Hist2h3b	Cd244
Cyp4f18	Pglyrp1	Ctsz	Tuba4a	Pltp
Mmp8	Serpinb1a	Psap	Asf1b	Cx3cr1
Slfn4	Cybb	Prdx1	Tmed3	Gngt2
S100a11	Padi4	Rpl10	Kpna2	Csflr
Cepg1	Syne1	Rps6	Etfb	Fcgr4
C5ar1	St3gal5	Rpl13	Nkg7	Myo1g
Cd33	Ncf1	Vim	Hmgal-rs1	Cd68
Tyrobp	Abca13	Rpl41	Pgl3	Fyb
Trem1	S100a8	Rps28	Igfbp4	Cd36
Srgn	Itgam	Hspa8	Banf1	Ifngr1
Tal1	Prdx5	Rps8	Rps17	Susd3
Junb	Lta4h	Rpl6	Prt3	Smpd13a
H2-D1	Chi3l1	Rpl4	Hist2h3c2	Clec4a1
Gm1821	Mapk13	Rps18	Atp1f1	Eno3
Slc2a3	Arrb2	Gm15772	Chit1	Emr1
Ccr1	C130026I21Rik	Ly86	Prss57	Ctsa
Cxcr4	S100a9	Rps2	Mcm6	S1pr5
Btg1	Zmpste24	Rpl10a	Mpo	Bcl2a1d
Gm16894	Cebpe	Erp29	Elane	Tnfrsf1b
Slfn1	Plaur	Psma7	Snrpd1	Fcgrt
Adam8	A530032D15Rik	Itgb7	Fcnb	Arhgef10l
Lsp1	Trem3	Ly6c2	Hist1h2ap	Hspa1a
Mrgpra2b	Lyz2	Rpl19	Fdps	Cd72
Hp	Tkt	Capg	Npm1	Acp5
Mrgpra2a	Lims1	Ly6c1	Gstm1	Il10ra
Fpr1	Golim4	Ifi30	Mt1	Hfe
Sorl1	Anxa3	H2-DMA	Chi3l3	Hspa1b
Fgl2		Tuba1b	Ctsg	Trex1
Sephs2		Gm5424	Rpl36a	B4galnt1
Cap1		H2-DMb1	Krtcap2	Pparg

References

- Adams, J.M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. *Science* 281, 1322-1326.
- Adkins, I., Sadilkova, L., Hradilova, N., Tomala, J., Kovar, M., and Spisek, R. (2017). Severe, but not mild heat-shock treatment induces immunogenic cell death in cancer cells. *Oncoimmunology* 6, e1311433.
- Aebi, M. (2013). N-linked protein glycosylation in the ER. *Biochim Biophys Acta* 1833, 2430-2437.
- Ahlmann, M., and Hempel, G. (2016). The effect of cyclophosphamide on the immune system: implications for clinical cancer therapy. *Cancer Chemother Pharmacol* 78, 661-671.
- Ahmed, A.R., and Hombal, S.M. (1984). Cyclophosphamide (Cytosan). A review on relevant pharmacology and clinical uses. *J Am Acad Dermatol* 11, 1115-1126.
- Akay, O.M., Aras, B.D., Isiksoy, S., Toprak, C., Mutlu, F.S., Artan, S., Oner, U., and Gulbas, Z. (2014). BCL2, BCL6, IGH, TP53, and MYC protein expression and gene rearrangements as prognostic markers in diffuse large B-cell lymphoma: a study of 44 Turkish patients. *Cancer Genet* 207, 87-93.
- Al-Hamadani, M., Habermann, T.M., Cerhan, J.R., Macon, W.R., Maurer, M.J., and Go, R.S. (2015). Non-Hodgkin lymphoma subtype distribution, geodemographic patterns, and survival in the US: A longitudinal analysis of the National Cancer Data Base from 1998 to 2011. *Am J Hematol* 90, 790-795.
- Alizadeh, A.A., Eisen, M.B., Davis, R.E., Ma, C., Lossos, I.S., Rosenwald, A., Boldrick, J.C., Sabet, H., Tran, T., Yu, X., *et al.* (2000). Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403, 503-511.
- Arcondeguy, T., Lacazette, E., Millevoi, S., Prats, H., and Touriol, C. (2013). VEGF-A mRNA processing, stability and translation: a paradigm for intricate regulation of gene expression at the post-transcriptional level. *Nucleic Acids Res* 41, 7997-8010.
- Arjaans, M., Oude Munnink, T.H., Oosting, S.F., Terwisscha van Scheltinga, A.G., Gietema, J.A., Garbavik, E.T., Timmer-Bosscha, H., Lub-de Hooge, M.N., Schroder, C.P., and de Vries, E.G. (2013). Bevacizumab-induced normalization of blood vessels in tumors hampers antibody uptake. *Cancer Res* 73, 3347-3355.
- Arlaukas, S.P., Garris, C.S., Kohler, R.H., Kitaoka, M., Cuccarese, M.F., Yang, K.S., Miller, M.A., Carlson, J.C., Freeman, G.J., Anthony, R.M., *et al.* (2017). In vivo imaging reveals a tumor-associated macrophage-mediated resistance pathway in anti-PD-1 therapy. *Sci Transl Med* 9.

- Aukema, S.M., Siebert, R., Schuurin, E., van Imhoff, G.W., Kluin-Nelemans, H.C., Boerma, E.J., and Kluin, P.M. (2011). Double-hit B-cell lymphomas. *Blood* 117, 2319-2331.
- Barkal, A.A., Weiskopf, K., Kao, K.S., Gordon, S.R., Rosental, B., Yiu, Y.Y., George, B.M., Markovic, M., Ring, N.G., Tsai, J.M., *et al.* (2018). Engagement of MHC class I by the inhibitory receptor LILRB1 suppresses macrophages and is a target of cancer immunotherapy. *Nat Immunol* 19, 76-84.
- Barleon, B., Sozzani, S., Zhou, D., Weich, H.A., Mantovani, A., and Marme, D. (1996). Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* 87, 3336-3343.
- Barnhart, B.C., and Quigley, M. (2017). Role of Fc-FcγR interactions in the antitumor activity of therapeutic antibodies. *Immunol Cell Biol* 95, 340-346.
- Basso, K., and Dalla-Favera, R. (2010). BCL6: master regulator of the germinal center reaction and key oncogene in B cell lymphomagenesis. *Adv Immunol* 105, 193-210.
- Bernales, S., Papa, F.R., and Walter, P. (2006). Intracellular signaling by the unfolded protein response. *Annu Rev Cell Dev Biol* 22, 487-508.
- Bernard, S., and Eilers, M. (2006). Control of cell proliferation and growth by Myc proteins. *Results Probl Cell Differ* 42, 329-342.
- Beurskens, F.J., Lindorfer, M.A., Farooqui, M., Beum, P.V., Engelberts, P., Mackus, W.J., Parren, P.W., Wiestner, A., and Taylor, R.P. (2012). Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. *J Immunol* 188, 3532-3541.
- Binet, F., and Sapieha, P. (2015). ER Stress and Angiogenesis. *Cell Metab* 22, 560-575.
- Bloy, N., Garcia, P., Laumont, C.M., Pitt, J.M., Sistigu, A., Stoll, G., Yamazaki, T., Bonneil, E., Buque, A., Humeau, J., *et al.* (2017). Immunogenic stress and death of cancer cells: Contribution of antigenicity vs adjuvanticity to immunosurveillance. *Immunol Rev* 280, 165-174.
- Botzler, C., Kis, K., Issels, R., and Multhoff, G. (1997). A comparison of the effects of ifosfamide vs. mafosfamide treatment on intracellular glutathione levels and immunological functions of immunocompetent lymphocyte subsets. *Exp Hematol* 25, 338-344.
- Bouhrel, M.A., Derudas, B., Rigamonti, E., Dievart, R., Brozek, J., Haulon, S., Zawadzki, C., Jude, B., Torpier, G., Marx, N., *et al.* (2007). PPARγ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab* 6, 137-143.
- Bunting, K.L., Soong, T.D., Singh, R., Jiang, Y., Beguelin, W., Poloway, D.W., Swed, B.L., Hatzi, K., Reisacher, W., Teater, M., *et al.* (2016). Multi-tiered Reorganization of the Genome during B Cell Affinity Maturation Anchored by a Germinal Center-Specific Locus Control Region. *Immunity* 45, 497-512.

Burkhardt, B., Yavuz, D., Zimmermann, M., Schieferstein, J., Kabickova, E., Attarbaschi, A., Lisfeld, J., Reiter, A., Makarova, O., Worch, J., *et al.* (2016). Impact of Fc gamma-receptor polymorphisms on the response to rituximab treatment in children and adolescents with mature B cell lymphoma/leukemia. *Ann Hematol* 95, 1503-1512.

Burotto, M., Berkovits, A., and Dunleavy, K. (2016). Double hit lymphoma: from biology to therapeutic implications. *Expert Rev Hematol* 9, 669-678.

Calado, D.P., Sasaki, Y., Godinho, S.A., Pellerin, A., Kochert, K., Sleckman, B.P., de Alboran, I.M., Janz, M., Rodig, S., and Rajewsky, K. (2012). The cell-cycle regulator c-Myc is essential for the formation and maintenance of germinal centers. *Nat Immunol* 13, 1092-1100.

Camilleri-Broet, S., Cassard, L., Broet, P., Delmer, A., Le Touneau, A., Diebold, J., Fridman, W.H., Molina, T.J., and Sautes-Fridman, C. (2004). FcgammaRIIB is differentially expressed during B cell maturation and in B-cell lymphomas. *Br J Haematol* 124, 55-62.

Cao, Z., Ding, B.S., Guo, P., Lee, S.B., Butler, J.M., Casey, S.C., Simons, M., Tam, W., Felsher, D.W., Shido, K., *et al.* (2014). Angiocrine factors deployed by tumor vascular niche induce B cell lymphoma invasiveness and chemoresistance. *Cancer Cell* 25, 350-365.

Center, D.M., and Cruikshank, W. (1982). Modulation of lymphocyte migration by human lymphokines. I. Identification and characterization of chemoattractant activity for lymphocytes from mitogen-stimulated mononuclear cells. *J Immunol* 128, 2563-2568.

Chao, M.P., Alizadeh, A.A., Tang, C., Myklebust, J.H., Varghese, B., Gill, S., Jan, M., Cha, A.C., Chan, C.K., Tan, B.T., *et al.* (2010a). Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell* 142, 699-713.

Chao, M.P., Jaiswal, S., Weissman-Tsukamoto, R., Alizadeh, A.A., Gentles, A.J., Volkmer, J., Weiskopf, K., Willingham, S.B., Raveh, T., Park, C.Y., *et al.* (2010b). Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. *Sci Transl Med* 2, 63ra94.

Chapuy, B., Stewart, C., Dunford, A.J., Kim, J., Kamburov, A., Redd, R.A., Lawrence, M.S., Roemer, M.G.M., Li, A.J., Ziepert, M., *et al.* (2018). Molecular subtypes of diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes. *Nat Med* 24, 679-690.

Chen, O.I., Bobak, Y.P., Stasyk, O.V., and Kunz-Schughart, L.A. (2018). A Complex Scenario and Underestimated Challenge: The Tumor Microenvironment, ER Stress, and Cancer Treatment. *Curr Med Chem* 25, 2465-2502.

Chen, Y., Gao, H., Yin, Q., Chen, L., Dong, P., Zhang, X., and Kang, J. (2013). ER stress activating ATF4/CHOP-TNF-alpha signaling pathway contributes to alcohol-induced disruption of osteogenic lineage of multipotential mesenchymal stem cell. *Cell Physiol Biochem* 32, 743-754.

Chen, Z., Teo, A.E., and McCarty, N. (2016). ROS-Induced CXCR4 Signaling Regulates Mantle Cell Lymphoma (MCL) Cell Survival and Drug Resistance in the Bone Marrow Microenvironment via Autophagy. *Clin Cancer Res* 22, 187-199.

Chesi, M., Mirza, N.N., Garbitt, V.M., Sharik, M.E., Dueck, A.C., Asmann, Y.W., Akhmetzyanova, I., Kosiorek, H.E., Calcinotto, A., Riggs, D.L., *et al.* (2016). IAP antagonists induce anti-tumor immunity in multiple myeloma. *Nat Med* 22, 1411-1420.

Cheung, R., Malik, M., Ravyn, V., Tomkowicz, B., Ptasznik, A., and Collman, R.G. (2009). An arrestin-dependent multi-kinase signaling complex mediates MIP-1beta/CCL4 signaling and chemotaxis of primary human macrophages. *J Leukoc Biol* 86, 833-845.

Chipurupalli, S., Kannan, E., Tergaonkar, V., D'Andrea, R., and Robinson, N. (2019). Hypoxia Induced ER Stress Response as an Adaptive Mechanism in Cancer. *Int J Mol Sci* 20.

Chong, L.C., Ben-Neriah, S., Slack, G.W., Freeman, C., Ennishi, D., Mottok, A., Collinge, B., Abrisqueta, P., Farinha, P., Boyle, M., *et al.* (2018). High-resolution architecture and partner genes of MYC rearrangements in lymphoma with DLBCL morphology. *Blood Adv* 2, 2755-2765.

Chura, J.C., Van Iseghem, K., Downs, L.S., Jr., Carson, L.F., and Judson, P.L. (2007). Bevacizumab plus cyclophosphamide in heavily pretreated patients with recurrent ovarian cancer. *Gynecol Oncol* 107, 326-330.

Cinar, M., Rosenfelt, F., Rokhsar, S., Lopategui, J., Pillai, R., Cervania, M., Pao, A., Cinar, B., and Alkan, S. (2015). Concurrent inhibition of MYC and BCL2 is a potentially effective treatment strategy for double hit and triple hit B-cell lymphomas. *Leuk Res* 39, 730-738.

Colvin, O.M. (1999). An overview of cyclophosphamide development and clinical applications. *Curr Pharm Des* 5, 555-560.

Compagno, M., Lim, W.K., Grunn, A., Nandula, S.V., Brahmachary, M., Shen, Q., Bertoni, F., Ponzoni, M., Scandurra, M., Califano, A., *et al.* (2009). Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. *Nature* 459, 717-721.

Craig, J.W., Mina, M.J., Crombie, J.L., LaCasce, A.S., Weinstock, D.M., Pinkus, G.S., and Pozdnyakova, O. (2018). Assessment of CD52 expression in "double-hit" and "double-expressor" lymphomas: Implications for clinical trial eligibility. *PLoS One* 13, e0199708.

Crotty, S., Johnston, R.J., and Schoenberger, S.P. (2010). Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* 11, 114-120.

Cubillos-Ruiz, J.R., Bettigole, S.E., and Glimcher, L.H. (2017). Tumorigenic and Immunosuppressive Effects of Endoplasmic Reticulum Stress in Cancer. *Cell* 168, 692-706.

Cubillos-Ruiz, J.R., Silberman, P.C., Rutkowski, M.R., Chopra, S., Perales-Puchalt, A., Song, M., Zhang, S., Bettigole, S.E., Gupta, D., Holcomb, K., *et al.* (2015). ER Stress Sensor XBP1 Controls Anti-tumor Immunity by Disrupting Dendritic Cell Homeostasis. *Cell* 161, 1527-1538.

- Dahal, L.N., Dou, L., Hussain, K., Liu, R., Earley, A., Cox, K.L., Murinello, S., Tracy, I., Forconi, F., Steele, A.J., *et al.* (2017). STING Activation Reverses Lymphoma-Mediated Resistance to Antibody Immunotherapy. *Cancer Res* 77, 3619-3631.
- Dang, C.V. (2012). MYC on the path to cancer. *Cell* 149, 22-35.
- Das, D.S., Ray, A., Das, A., Song, Y., Tian, Z., Oronsky, B., Richardson, P., Scicinski, J., Chauhan, D., and Anderson, K.C. (2016). A novel hypoxia-selective epigenetic agent RRx-001 triggers apoptosis and overcomes drug resistance in multiple myeloma cells. *Leukemia* 30, 2187-2197.
- de Jonge, A.V., Roosma, T.J., Houtenbos, I., Vasmel, W.L., van de Hem, K., de Boer, J.P., van Maanen, T., Lindauer-van der Werf, G., Beeker, A., Timmers, G.J., *et al.* (2016). Diffuse large B-cell lymphoma with MYC gene rearrangements: Current perspective on treatment of diffuse large B-cell lymphoma with MYC gene rearrangements; case series and review of the literature. *Eur J Cancer* 55, 140-146.
- Delbrel, E., Soumare, A., Naguez, A., Label, R., Bernard, O., Bruhat, A., Fafournoux, P., Tremblais, G., Marchant, D., Gille, T., *et al.* (2018). HIF-1alpha triggers ER stress and CHOP-mediated apoptosis in alveolar epithelial cells, a key event in pulmonary fibrosis. *Sci Rep* 8, 17939.
- Dias-Baruffi, M., Zhu, H., Cho, M., Karmakar, S., McEver, R.P., and Cummings, R.D. (2003). Dimeric galectin-1 induces surface exposure of phosphatidylserine and phagocytic recognition of leukocytes without inducing apoptosis. *J Biol Chem* 278, 41282-41293.
- Diaz, R.J., Ali, S., Qadir, M.G., De La Fuente, M.I., Ivan, M.E., and Komotar, R.J. (2017). The role of bevacizumab in the treatment of glioblastoma. *J Neurooncol* 133, 455-467.
- Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15-21.
- Dominguez-Sola, D., Vitoria, G.D., Ying, C.Y., Phan, R.T., Saito, M., Nussenzweig, M.C., and Dalla-Favera, R. (2012). The proto-oncogene MYC is required for selection in the germinal center and cyclic reentry. *Nat Immunol* 13, 1083-1091.
- Dorstyn, L., Akey, C.W., and Kumar, S. (2018). New insights into apoptosome structure and function. *Cell Death Differ* 25, 1194-1208.
- Dunleavy, K., Fanale, M.A., Abramson, J.S., Noy, A., Caimi, P.F., Pittaluga, S., Parekh, S., Lacasce, A., Hayslip, J.W., Jagadeesh, D., *et al.* (2018). Dose-adjusted EPOCH-R (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and rituximab) in untreated aggressive diffuse large B-cell lymphoma with MYC rearrangement: a prospective, multicentre, single-arm phase 2 study. *Lancet Haematol* 5, e609-e617.
- Eilers, M., and Eisenman, R.N. (2008). Myc's broad reach. *Genes Dev* 22, 2755-2766.

Ennishi, D., Jiang, A., Boyle, M., Collinge, B., Grande, B.M., Ben-Neriah, S., Rushton, C., Tang, J., Thomas, N., Slack, G.W., *et al.* (2019). Double-Hit Gene Expression Signature Defines a Distinct Subgroup of Germinal Center B-Cell-Like Diffuse Large B-Cell Lymphoma. *J Clin Oncol* 37, 190-201.

Ennishi, D., Mottok, A., Ben-Neriah, S., Shulha, H.P., Farinha, P., Chan, F.C., Meissner, B., Boyle, M., Hother, C., Kridel, R., *et al.* (2017). Genetic profiling of MYC and BCL2 in diffuse large B-cell lymphoma determines cell-of-origin-specific clinical impact. *Blood* 129, 2760-2770.

Feng, M., Marjon, K.D., Zhu, F., Weissman-Tsukamoto, R., Levett, A., Sullivan, K., Kao, K.S., Markovic, M., Bump, P.A., Jackson, H.M., *et al.* (2018). Programmed cell removal by calreticulin in tissue homeostasis and cancer. *Nat Commun* 9, 3194.

Ferry, J.A. (2006). Burkitt's lymphoma: clinicopathologic features and differential diagnosis. *Oncologist* 11, 375-383.

Ford, C.A., Petrova, S., Pound, J.D., Voss, J.J., Melville, L., Paterson, M., Farnworth, S.L., Gallimore, A.M., Cuff, S., Wheadon, H., *et al.* (2015). Oncogenic properties of apoptotic tumor cells in aggressive B cell lymphoma. *Curr Biol* 25, 577-588.

Friedberg, J.W. (2015). Double hit diffuse large B-cell lymphomas: diagnostic and therapeutic challenges. *Chin Clin Oncol* 4, 9.

Friedberg, J.W., and Fisher, R.I. (2008). Diffuse large B-cell lymphoma. *Hematol Oncol Clin North Am* 22, 941-952, ix.

Galluzzi, L., Buque, A., Kepp, O., Zitvogel, L., and Kroemer, G. (2015). Immunological Effects of Conventional Chemotherapy and Targeted Anticancer Agents. *Cancer Cell* 28, 690-714.

Gardner, B.M., Pincus, D., Gotthardt, K., Gallagher, C.M., and Walter, P. (2013). Endoplasmic reticulum stress sensing in the unfolded protein response. *Cold Spring Harb Perspect Biol* 5, a013169.

Ghosh, R., Lipson, K.L., Sargent, K.E., Mercurio, A.M., Hunt, J.S., Ron, D., and Urano, F. (2010). Transcriptional regulation of VEGF-A by the unfolded protein response pathway. *PLoS One* 5, e9575.

Gisslinger, H., Kurzrock, R., Gisslinger, B., Jiang, S., Li, S., Virgolini, I., Woloszczuk, W., Andreeff, M., and Talpaz, M. (2001). Autocrine cell suicide in a Burkitt lymphoma cell line (Daudi) induced by interferon alpha: involvement of tumor necrosis factor as ligand for the CD95 receptor. *Blood* 97, 2791-2797.

Goldberg, M.A., Antin, J.H., Guinan, E.C., and Rapoport, J.M. (1986). Cyclophosphamide cardiotoxicity: an analysis of dosing as a risk factor. *Blood* 68, 1114-1118.

Gottdiener, J.S., Appelbaum, F.R., Ferrans, V.J., Deisseroth, A., and Ziegler, J. (1981). Cardiotoxicity associated with high-dose cyclophosphamide therapy. *Arch Intern Med* 141, 758-763.

- Grimm, K.E., and O'Malley, D.P. (2019). Aggressive B cell lymphomas in the 2017 revised WHO classification of tumors of hematopoietic and lymphoid tissues. *Ann Diagn Pathol* 38, 6-10.
- Gu, S., Chen, C., Jiang, X., and Zhang, Z. (2016). ROS-mediated endoplasmic reticulum stress and mitochondrial dysfunction underlie apoptosis induced by resveratrol and arsenic trioxide in A549 cells. *Chem Biol Interact* 245, 100-109.
- Guerriero, J.L., Sotayo, A., Ponichtera, H.E., Castrillon, J.A., Pourzia, A.L., Schad, S., Johnson, S.F., Carrasco, R.D., Lazo, S., Bronson, R.T., *et al.* (2017). Class IIa HDAC inhibition reduces breast tumours and metastases through anti-tumour macrophages. *Nature* 543, 428-432.
- Harrington, A.M., Olteanu, H., Kroft, S.H., and Esho, C. (2011). The unique immunophenotype of double-hit lymphomas. *Am J Clin Pathol* 135, 649-650.
- Hasanali, Z.S., Saroya, B.S., Stuart, A., Shimko, S., Evans, J., Vinod Shah, M., Sharma, K., Leshchenko, V.V., Parekh, S., Loughran, T.P., Jr., *et al.* (2015). Epigenetic therapy overcomes treatment resistance in T cell prolymphocytic leukemia. *Sci Transl Med* 7, 293ra102.
- Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 13, 89-102.
- Hogg, S.J., Newbold, A., Vervoort, S.J., Cluse, L.A., Martin, B.P., Gregory, G.P., Lefebure, M., Vidacs, E., Tothill, R.W., Bradner, J.E., *et al.* (2016). BET Inhibition Induces Apoptosis in Aggressive B-Cell Lymphoma via Epigenetic Regulation of BCL-2 Family Members. *Mol Cancer Ther* 15, 2030-2041.
- Hsieh, A.L., Walton, Z.E., Altman, B.J., Stine, Z.E., and Dang, C.V. (2015). MYC and metabolism on the path to cancer. *Semin Cell Dev Biol* 43, 11-21.
- Hsu, J., Hodgins, J.J., Marathe, M., Nicolai, C.J., Bourgeois-Daigneault, M.C., Trevino, T.N., Azimi, C.S., Scheer, A.K., Randolph, H.E., Thompson, T.W., *et al.* (2018). Contribution of NK cells to immunotherapy mediated by PD-1/PD-L1 blockade. *J Clin Invest* 128, 4654-4668.
- Hu, Y., Turner, M.J., Shields, J., Gale, M.S., Hutto, E., Roberts, B.L., Siders, W.M., and Kaplan, J.M. (2009). Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model. *Immunology* 128, 260-270.
- Huang, C., and Melnick, A. (2015). Mechanisms of action of BCL6 during germinal center B cell development. *Sci China Life Sci* 58, 1226-1232.
- Huang, Y., and Li, L. (2013). DNA crosslinking damage and cancer - a tale of friend and foe. *Transl Cancer Res* 2, 144-154.
- Hubert, P., Heitzmann, A., Viel, S., Nicolas, A., Sastre-Garau, X., Oppezio, P., Pritsch, O., Osinaga, E., and Amigorena, S. (2011). Antibody-dependent cell cytotoxicity synapses form in mice during tumor-specific antibody immunotherapy. *Cancer Res* 71, 5134-5143.

Hussner, J., Ameling, S., Hammer, E., Herzog, S., Steil, L., Schwebe, M., Niessen, J., Schroeder, H.W., Kroemer, H.K., Ritter, C.A., *et al.* (2012). Regulation of interferon-inducible proteins by doxorubicin via interferon gamma-Janus tyrosine kinase-signal transducer and activator of transcription signaling in tumor cells. *Mol Pharmacol* 81, 679-688.

Iqbal, J., Sanger, W.G., Horsman, D.E., Rosenwald, A., Pickering, D.L., Dave, B., Dave, S., Xiao, L., Cao, K., Zhu, Q., *et al.* (2004). BCL2 translocation defines a unique tumor subset within the germinal center B-cell-like diffuse large B-cell lymphoma. *Am J Pathol* 165, 159-166.

Italiani, P., and Boraschi, D. (2014). From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. *Front Immunol* 5, 514.

Jablonski, K.A., Amici, S.A., Webb, L.M., Ruiz-Rosado Jde, D., Popovich, P.G., Partida-Sanchez, S., and Guerau-de-Arellano, M. (2015). Novel Markers to Delineate Murine M1 and M2 Macrophages. *PLoS One* 10, e0145342.

Janssens, S., and Tschopp, J. (2006). Signals from within: the DNA-damage-induced NF-kappaB response. *Cell Death Differ* 13, 773-784.

Jelicic, J., Larsen, T.S., Maksimovic, M., and Trajkovic, G. (2019). Available prognostic models for risk stratification of diffuse large B cell lymphoma patients: a systematic review. *Crit Rev Oncol Hematol* 133, 1-16.

Jurismic, V., Bogdanovic, G., Kojic, V., Jakimov, D., and Srdic, T. (2006). Effect of TNF-alpha on Raji cells at different cellular levels estimated by various methods. *Ann Hematol* 85, 86-94.

Karjalainen, R., Pemovska, T., Popa, M., Liu, M., Javarappa, K.K., Majumder, M.M., Yadav, B., Tamborero, D., Tang, J., Bychkov, D., *et al.* (2017). JAK1/2 and BCL2 inhibitors synergize to counteract bone marrow stromal cell-induced protection of AML. *Blood* 130, 789-802.

Kepp, O., Senovilla, L., Vitale, I., Vacchelli, E., Adjemian, S., Agostinis, P., Apetoh, L., Aranda, F., Barnaba, V., Bloy, N., *et al.* (2014). Consensus guidelines for the detection of immunogenic cell death. *Oncoimmunology* 3, e955691.

Kim, J.E., Singh, R.R., Cho-Vega, J.H., Drakos, E., Davuluri, Y., Khokhar, F.A., Fayad, L., Medeiros, L.J., and Vega, F. (2009). Sonic hedgehog signaling proteins and ATP-binding cassette G2 are aberrantly expressed in diffuse large B-cell lymphoma. *Mod Pathol* 22, 1312-1320.

Kridel, R., Sehn, L.H., and Gascoyne, R.D. (2012). Pathogenesis of follicular lymphoma. *J Clin Invest* 122, 3424-3431.

Kroemer, G., Galluzzi, L., Kepp, O., and Zitvogel, L. (2013). Immunogenic cell death in cancer therapy. *Annu Rev Immunol* 31, 51-72.

Krysko, D.V., Garg, A.D., Kaczmarek, A., Krysko, O., Agostinis, P., and Vandenabeele, P. (2012). Immunogenic cell death and DAMPs in cancer therapy. *Nat Rev Cancer* 12, 860-875.

- Lambert, C., Li, J., Jonscher, K., Yang, T.C., Reigan, P., Quintana, M., Harvey, J., and Freed, B.M. (2007). Acrolein inhibits cytokine gene expression by alkylating cysteine and arginine residues in the NF-kappaB1 DNA binding domain. *J Biol Chem* 282, 19666-19675.
- Leich, E., Salaverria, I., Bea, S., Zettl, A., Wright, G., Moreno, V., Gascoyne, R.D., Chan, W.C., Braziel, R.M., Rimsza, L.M., *et al.* (2009). Follicular lymphomas with and without translocation t(14;18) differ in gene expression profiles and genetic alterations. *Blood* 114, 826-834.
- Leidi, M., Gotti, E., Bologna, L., Miranda, E., Rimoldi, M., Sica, A., Roncalli, M., Palumbo, G.A., Introna, M., and Golay, J. (2009). M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells in vitro. *J Immunol* 182, 4415-4422.
- Lemaitre, J.M., Buckle, R.S., and Mechali, M. (1996). c-Myc in the control of cell proliferation and embryonic development. *Adv Cancer Res* 70, 95-144.
- Lenz, G., Wright, G., Dave, S.S., Xiao, W., Powell, J., Zhao, H., Xu, W., Tan, B., Goldschmidt, N., Iqbal, J., *et al.* (2008). Stromal gene signatures in large-B-cell lymphomas. *N Engl J Med* 359, 2313-2323.
- Li, B., and Dewey, C.N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12, 323.
- Li, S., Young, K.H., and Medeiros, L.J. (2018). Diffuse large B-cell lymphoma. *Pathology* 50, 74-87.
- Liu, D., and Mamorska-Dyga, A. (2017). Syk inhibitors in clinical development for hematological malignancies. *J Hematol Oncol* 10, 145.
- Liu, D., Tian, Y., Sun, D., Sun, H., Jin, Y., and Dong, M. (2016). The FCGR3A polymorphism predicts the response to rituximab-based therapy in patients with non-Hodgkin lymphoma: a meta-analysis. *Ann Hematol* 95, 1483-1490.
- Lohr, J.G., Stojanov, P., Lawrence, M.S., Auclair, D., Chapuy, B., Sougnez, C., Cruz-Gordillo, P., Knoechel, B., Asmann, Y.W., Slager, S.L., *et al.* (2012). Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. *Proc Natl Acad Sci U S A* 109, 3879-3884.
- Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550.
- Ludeman, S.M. (1999). The chemistry of the metabolites of cyclophosphamide. *Curr Pharm Des* 5, 627-643.
- Lundin, J., Osterborg, A., Brittinger, G., Crowther, D., Dombret, H., Engert, A., Epenetos, A., Gisselbrecht, C., Huhn, D., Jaeger, U., *et al.* (1998). CAMPATH-1H monoclonal antibody in therapy for previously treated low-grade non-Hodgkin's lymphomas: a phase II multicenter study. European Study Group of CAMPATH-1H Treatment in Low-Grade Non-Hodgkin's Lymphoma. *J Clin Oncol* 16, 3257-3263.

Lux, A., Seeling, M., Baerenwaldt, A., Lehmann, B., Schwab, I., Repp, R., Meidenbauer, N., Mackensen, A., Hartmann, A., Heidkamp, G., *et al.* (2014). A humanized mouse identifies the bone marrow as a niche with low therapeutic IgG activity. *Cell Rep* 7, 236-248.

Lykken, J.M., Horikawa, M., Minard-Colin, V., Kamata, M., Miyagaki, T., Poe, J.C., and Tedder, T.F. (2016). Galectin-1 drives lymphoma CD20 immunotherapy resistance: validation of a preclinical system to identify resistance mechanisms. *Blood* 127, 1886-1895.

Lynch, J.M., Maillet, M., Vanhoutte, D., Schloemer, A., Sargent, M.A., Blair, N.S., Lynch, K.A., Okada, T., Aronow, B.J., Osinska, H., *et al.* (2012). A thrombospondin-dependent pathway for a protective ER stress response. *Cell* 149, 1257-1268.

Mantovani, A., Marchesi, F., Malesci, A., Laghi, L., and Allavena, P. (2017). Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol* 14, 399-416.

Mantovani, A., Polentarutti, N., Luini, W., Peri, G., and Spreafico, F. (1979). Role of host defense mechanisms in the antitumor activity of adriamycin and daunomycin in mice. *J Natl Cancer Inst* 63, 61-66.

Marcus, R., Davies, A., Ando, K., Klapper, W., Opat, S., Owen, C., Phillips, E., Sangha, R., Schlag, R., Seymour, J.F., *et al.* (2017). Obinutuzumab for the First-Line Treatment of Follicular Lymphoma. *N Engl J Med* 377, 1331-1344.

Marechal, L., Laviolette, M., Rodrigue-Way, A., Sow, B., Brochu, M., Caron, V., and Tremblay, A. (2018). The CD36-PPARgamma Pathway in Metabolic Disorders. *Int J Mol Sci* 19.

Martelli, M., Ferreri, A.J., Agostinelli, C., Di Rocco, A., Pfreundschuh, M., and Pileri, S.A. (2013). Diffuse large B-cell lymphoma. *Crit Rev Oncol Hematol* 87, 146-171.

Martinez, F.O., and Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* 6, 13.

Mazur, G., Butrym, A., Kryczek, I., Dlubek, D., Jaskula, E., Lange, A., Kuliczowski, K., and Jelen, M. (2014). Decreased expression of CXCR4 chemokine receptor in bone marrow after chemotherapy in patients with non-Hodgkin lymphomas is a good prognostic factor. *PLoS One* 9, e98194.

McDonald, G.B., Slattery, J.T., Bouvier, M.E., Ren, S., Batchelder, A.L., Kalhorn, T.F., Schoch, H.G., Anasetti, C., and Gooley, T. (2003). Cyclophosphamide metabolism, liver toxicity, and mortality following hematopoietic stem cell transplantation. *Blood* 101, 2043-2048.

McLaughlin, P., Grillo-Lopez, A.J., Link, B.K., Levy, R., Czuczman, M.S., Williams, M.E., Heyman, M.R., Bence-Bruckler, I., White, C.A., Cabanillas, F., *et al.* (1998). Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 16, 2825-2833.

McMahon, S.B. (2014). MYC and the control of apoptosis. *Cold Spring Harb Perspect Med* 4, a014407.

McPhail, E.D., Maurer, M.J., Macon, W.R., Feldman, A.L., Kurtin, P.J., Ketterling, R.P., Vaidya, R., Cerhan, J.R., Ansell, S.M., Porrata, L.F., *et al.* (2018). Inferior survival in high-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements is not associated with MYC/IG gene rearrangements. *Haematologica* 103, 1899-1907.

Merron, B., and Davies, A. (2018). Double hit lymphoma: How do we define it and how do we treat it? *Best Pract Res Clin Haematol* 31, 233-240.

Miller, J.S., Weisdorf, D.J., Burns, L.J., Slungaard, A., Wagner, J.E., Verneris, M.R., Cooley, S., Wangen, R., Fautsch, S.K., Nicklow, R., *et al.* (2007). Lymphodepletion followed by donor lymphocyte infusion (DLI) causes significantly more acute graft-versus-host disease than DLI alone. *Blood* 110, 2761-2763.

Mills, C.D., Lenz, L.L., and Harris, R.A. (2016). A Breakthrough: Macrophage-Directed Cancer Immunotherapy. *Cancer Res* 76, 513-516.

Moench, L., Sachs, Z., Aasen, G., Dolan, M., Dayton, V., and Courville, E.L. (2016). Double- and triple-hit lymphomas can present with features suggestive of immaturity, including TdT expression, and create diagnostic challenges. *Leuk Lymphoma* 57, 2626-2635.

Mondello, P., Cuzzocrea, S., Navarra, M., and Mian, M. (2016). 90 Y-ibritumomab tiuxetan: a nearly forgotten opportunity. *Oncotarget* 7, 7597-7609.

Monti, S., Savage, K.J., Kutok, J.L., Feuerhake, F., Kurtin, P., Mihm, M., Wu, B., Pasqualucci, L., Neuberg, D., Aguiar, R.C., *et al.* (2005). Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response. *Blood* 105, 1851-1861.

Morin, R.D., Mendez-Lago, M., Mungall, A.J., Goya, R., Mungall, K.L., Corbett, R.D., Johnson, N.A., Severson, T.M., Chiu, R., Field, M., *et al.* (2011). Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature* 476, 298-303.

Myers, A.L., Kawedia, J.D., Champlin, R.E., Kramer, M.A., Nieto, Y., Ghose, R., and Andersson, B.S. (2017). Clarifying busulfan metabolism and drug interactions to support new therapeutic drug monitoring strategies: a comprehensive review. *Expert Opin Drug Metab Toxicol* 13, 901-923.

N, A.G., Quintana, J.A., Garcia-Silva, S., Mazariegos, M., Gonzalez de la Aleja, A., Nicolas-Avila, J.A., Walter, W., Adrover, J.M., Crainiciuc, G., Kuchroo, V.K., *et al.* (2017). Phagocytosis imprints heterogeneity in tissue-resident macrophages. *J Exp Med* 214, 1281-1296.

Nguyen, T.H., Havari, E., McLaren, R., Zhang, M., Jiang, Y., Madden, S.L., Roberts, B., Kaplan, J., and Shankara, S. (2012). Alemtuzumab induction of intracellular signaling and apoptosis in malignant B lymphocytes. *Leuk Lymphoma* 53, 699-709.

Nicholson, A.C. (2004). Expression of CD36 in macrophages and atherosclerosis: the role of lipid regulation of PPARgamma signaling. *Trends Cardiovasc Med* 14, 8-12.

Noy, R., and Pollard, J.W. (2014). Tumor-associated macrophages: from mechanisms to therapy. *Immunity* 41, 49-61.

Noone AM, Howlader N, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review, 1975-2015, National Cancer Institute. Bethesda, MD, https://seer.cancer.gov/csr/1975_2015/, based on November 2017 SEER data submission, posted to the SEER web site, April 2018.

Nyathi, Y., Wilkinson, B.M., and Pool, M.R. (2013). Co-translational targeting and translocation of proteins to the endoplasmic reticulum. *Biochim Biophys Acta* 1833, 2392-2402.

Ok, C.Y., Medeiros, L.J., Thakral, B., Tang, G., Jain, N., Jabbour, E., Pierce, S.A., and Konoplev, S. (2019). High-grade B-cell lymphomas with TdT expression: a diagnostic and classification dilemma. *Mod Pathol* 32, 48-58.

Pallasch, C.P., Leskov, I., Braun, C.J., Vorholt, D., Drake, A., Soto-Feliciano, Y.M., Bent, E.H., Schwamb, J., Iliopoulou, B., Kutsch, N., *et al.* (2014). Sensitizing protective tumor microenvironments to antibody-mediated therapy. *Cell* 156, 590-602.

Park, Y.M. (2014). CD36, a scavenger receptor implicated in atherosclerosis. *Exp Mol Med* 46, e99.

Pasqualucci, L., Trifonov, V., Fabbri, G., Ma, J., Rossi, D., Chiarenza, A., Wells, V.A., Grunn, A., Messina, M., Elliot, O., *et al.* (2011). Analysis of the coding genome of diffuse large B-cell lymphoma. *Nat Genet* 43, 830-837.

Pereira, E.R., Frudd, K., Awad, W., and Hendershot, L.M. (2014). Endoplasmic reticulum (ER) stress and hypoxia response pathways interact to potentiate hypoxia-inducible factor 1 (HIF-1) transcriptional activity on targets like vascular endothelial growth factor (VEGF). *J Biol Chem* 289, 3352-3364.

Quentin, T., Steinmetz, M., Poppe, A., and Thoms, S. (2012). Metformin differentially activates ER stress signaling pathways without inducing apoptosis. *Dis Model Mech* 5, 259-269.

Randall, M.J., Hristova, M., and van der Vliet, A. (2013). Protein alkylation by the alpha,beta-unsaturated aldehyde acrolein. A reversible mechanism of electrophile signaling? *FEBS Lett* 587, 3808-3814.

Ratajczak, M.Z., Kim, C., Janowska-Wieczorek, A., and Ratajczak, J. (2012). The expanding family of bone marrow homing factors for hematopoietic stem cells: stromal derived factor 1 is not the only player in the game. *ScientificWorldJournal* 2012, 758512.

Ravandi, F., and O'Brien, S. (2006). Alemtuzumab in CLL and other lymphoid neoplasms. *Cancer Invest* 24, 718-725.

Reddy, A., Zhang, J., Davis, N.S., Moffitt, A.B., Love, C.L., Waldrop, A., Leppa, S., Pasanen, A., Meriranta, L., Karjalainen-Lindsberg, M.L., *et al.* (2017). Genetic and Functional Drivers of Diffuse Large B Cell Lymphoma. *Cell* 171, 481-494 e415.

Ren, Y., Bi, C., Zhao, X., Lwin, T., Wang, C., Yuan, J., Silva, A.S., Shah, B.D., Fang, B., Li, T., *et al.* (2018). PLK1 stabilizes a MYC-dependent kinase network in aggressive B cell lymphomas. *J Clin Invest* 128, 5517-5530.

Rezvani, A.R., and Maloney, D.G. (2011). Rituximab resistance. *Best Pract Res Clin Haematol* 24, 203-216.

Riedell, P.A., and Smith, S.M. (2018). Double hit and double expressors in lymphoma: Definition and treatment. *Cancer* 124, 4622-4632.

Roghanian, A., Teige, I., Martensson, L., Cox, K.L., Kovacek, M., Ljungars, A., Mattson, J., Sundberg, A., Vaughan, A.T., Shah, V., *et al.* (2015). Antagonistic human FcγRIIB (CD32B) antibodies have anti-tumor activity and overcome resistance to antibody therapy in vivo. *Cancer Cell* 27, 473-488.

Rosenthal, A., and Younes, A. (2017). High grade B-cell lymphoma with rearrangements of MYC and BCL2 and/or BCL6: Double hit and triple hit lymphomas and double expressing lymphoma. *Blood Rev* 31, 37-42.

Rufo, N., Garg, A.D., and Agostinis, P. (2017). The Unfolded Protein Response in Immunogenic Cell Death and Cancer Immunotherapy. *Trends Cancer* 3, 643-658.

Saida, Y., Watanabe, S., Tanaka, T., Baba, J., Sato, K., Shoji, S., Igarashi, N., Kondo, R., Okajima, M., Koshio, J., *et al.* (2015). Critical Roles of Chemoresistant Effector and Regulatory T Cells in Antitumor Immunity after Lymphodepleting Chemotherapy. *J Immunol* 195, 726-735.

Saleh, T., Tyutynuk-Massey, L., Cudjoe, E.K., Jr., Idowu, M.O., Landry, J.W., and Gewirtz, D.A. (2018). Non-Cell Autonomous Effects of the Senescence-Associated Secretory Phenotype in Cancer Therapy. *Front Oncol* 8, 164.

Sarkozy, C., Traverse-Glehen, A., and Coiffier, B. (2015). Double-hit and double-protein-expression lymphomas: aggressive and refractory lymphomas. *Lancet Oncol* 16, e555-e567.

Sarosiek, K.A., Fraser, C., Muthalagu, N., Bhola, P.D., Chang, W., McBrayer, S.K., Cantlon, A., Fisch, S., Golomb-Mello, G., Ryan, J.A., *et al.* (2017). Developmental Regulation of Mitochondrial Apoptosis by c-Myc Governs Age- and Tissue-Specific Sensitivity to Cancer Therapeutics. *Cancer Cell* 31, 142-156.

Savage, K.J., Johnson, N.A., Ben-Neriah, S., Connors, J.M., Sehn, L.H., Farinha, P., Horsman, D.E., and Gascoyne, R.D. (2009). MYC gene rearrangements are associated with a poor prognosis in diffuse large B-cell lymphoma patients treated with R-CHOP chemotherapy. *Blood* 114, 3533-3537.

Schmitz, R., Ceribelli, M., Pittaluga, S., Wright, G., and Staudt, L.M. (2014). Oncogenic mechanisms in Burkitt lymphoma. *Cold Spring Harb Perspect Med* 4.

Schmitz, R., Wright, G.W., Huang, D.W., Johnson, C.A., Phelan, J.D., Wang, J.Q., Roulland, S., Kasbekar, M., Young, R.M., Shaffer, A.L., *et al.* (2018). Genetics and Pathogenesis of Diffuse Large B-Cell Lymphoma. *N Engl J Med* 378, 1396-1407.

Schuetz, J.M., Johnson, N.A., Morin, R.D., Scott, D.W., Tan, K., Ben-Nierah, S., Boyle, M., Slack, G.W., Marra, M.A., Connors, J.M., *et al.* (2012). BCL2 mutations in diffuse large B-cell lymphoma. *Leukemia* 26, 1383-1390.

Scott, D.W., King, R.L., Staiger, A.M., Ben-Neriah, S., Jiang, A., Horn, H., Mottok, A., Farinha, P., Slack, G.W., Ennishi, D., *et al.* (2018). High-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements with diffuse large B-cell lymphoma morphology. *Blood* 131, 2060-2064.

Scurr, M., Pembroke, T., Bloom, A., Roberts, D., Thomson, A., Smart, K., Bridgeman, H., Adams, R., Brewster, A., Jones, R., *et al.* (2017). Low-Dose Cyclophosphamide Induces Antitumor T-Cell Responses, which Associate with Survival in Metastatic Colorectal Cancer. *Clin Cancer Res* 23, 6771-6780.

Sha, C., Barrans, S., Cucco, F., Bentley, M.A., Care, M.A., Cummin, T., Kennedy, H., Thompson, J.S., Uddin, R., Worriallow, L., *et al.* (2019). Molecular High-Grade B-Cell Lymphoma: Defining a Poor-Risk Group That Requires Different Approaches to Therapy. *J Clin Oncol* 37, 202-212.

Shaffer, A.L., Lin, K.I., Kuo, T.C., Yu, X., Hurt, E.M., Rosenwald, A., Giltane, J.M., Yang, L., Zhao, H., Calame, K., *et al.* (2002). Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 17, 51-62.

Shustik, J., Han, G., Farinha, P., Johnson, N.A., Ben Neriah, S., Connors, J.M., Sehn, L.H., Horsman, D.E., Gascoyne, R.D., and Steidl, C. (2010). Correlations between BCL6 rearrangement and outcome in patients with diffuse large B-cell lymphoma treated with CHOP or R-CHOP. *Haematologica* 95, 96-101.

Siders, W.M., Shields, J., Garron, C., Hu, Y., Boutin, P., Shankara, S., Weber, W., Roberts, B., and Kaplan, J.M. (2010). Involvement of neutrophils and natural killer cells in the anti-tumor activity of alemtuzumab in xenograft tumor models. *Leuk Lymphoma* 51, 1293-1304.

Singh, R., Letai, A., and Sarosiek, K. (2019). Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. *Nat Rev Mol Cell Biol* 20, 175-193.

Singh, S., Kumar, N.K., Dwiwedi, P., Charan, J., Kaur, R., Sidhu, P., and Chugh, V.K. (2018). Monoclonal Antibodies: A Review. *Curr Clin Pharmacol* 13, 85-99.

Sistigu, A., Yamazaki, T., Vacchelli, E., Chaba, K., Enot, D.P., Adam, J., Vitale, I., Goubar, A., Baracco, E.E., Remedios, C., *et al.* (2014). Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nat Med* 20, 1301-1309.

Swerdlow, S.H., Campo, E., Pileri, S.A., Harris, N.L., Stein, H., Siebert, R., Advani, R., Ghielmini, M., Salles, G.A., Zelenetz, A.D., *et al.* (2016). The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood* 127, 2375-2390.

Tamura, R., Tanaka, T., Miyake, K., Yoshida, K., and Sasaki, H. (2017). Bevacizumab for malignant gliomas: current indications, mechanisms of action and resistance, and markers of response. *Brain Tumor Pathol* 34, 62-77.

Townsend, E.C., Murakami, M.A., Christodoulou, A., Christie, A.L., Koster, J., DeSouza, T.A., Morgan, E.A., Kallgren, S.P., Liu, H., Wu, S.C., *et al.* (2016). The Public Repository of Xenografts Enables Discovery and Randomized Phase II-like Trials in Mice. *Cancer Cell* 29, 574-586.

Trombetta, J.J., Gennert, D., Lu, D., Satija, R., Shalek, A.K., and Regev, A. (2014). Preparation of Single-Cell RNA-Seq Libraries for Next Generation Sequencing. *Curr Protoc Mol Biol* 107, 4 22 21-17.

Uchida, A., Isobe, Y., Asano, J., Uemura, Y., Hoshikawa, M., Takagi, M., and Miura, I. (2018). Targeting BCL2 with venetoclax is a promising therapeutic strategy for "double-protein-expression" lymphoma with MYC and BCL2 rearrangements. *Haematologica*.

Valera, A., Epistolio, S., Colomo, L., Riva, A., Balague, O., Dlouhy, I., Tzankov, A., Buhler, M., Haralambieva, E., Campo, E., *et al.* (2016). Definition of MYC genetic heteroclonality in diffuse large B-cell lymphoma with 8q24 rearrangement and its impact on protein expression. *Mod Pathol* 29, 844-853.

van Leeuwen, W., van der Krift, F., and Rabouille, C. (2018). Modulation of the secretory pathway by amino-acid starvation. *J Cell Biol* 217, 2261-2271.

van Oosterwijk, J.G., Buelow, D.R., Drenberg, C.D., Vasilyeva, A., Li, L., Shi, L., Wang, Y.D., Finkelstein, D., Shurtleff, S.A., Janke, L.J., *et al.* (2018). Hypoxia-induced upregulation of BMX kinase mediates therapeutic resistance in acute myeloid leukemia. *J Clin Invest* 128, 369-380.

Voss, J., Ford, C.A., Petrova, S., Melville, L., Paterson, M., Pound, J.D., Holland, P., Giotti, B., Freeman, T.C., and Gregory, C.D. (2017). Modulation of macrophage antitumor potential by apoptotic lymphoma cells. *Cell Death Differ* 24, 971-983.

Wang, W., Hu, S., Lu, X., Young, K.H., and Medeiros, L.J. (2015). Triple-hit B-cell Lymphoma With MYC, BCL2, and BCL6 Translocations/Rearrangements: Clinicopathologic Features of 11 Cases. *Am J Surg Pathol* 39, 1132-1139.

Wang, W., Mani, A.M., and Wu, Z.H. (2017). DNA damage-induced nuclear factor-kappa B activation and its roles in cancer progression. *J Cancer Metastasis Treat* 3, 45-59.

Weiner, G.J. (2010). Rituximab: mechanism of action. *Semin Hematol* 47, 115-123.

Weiskopf, K., and Weissman, I.L. (2015). Macrophages are critical effectors of antibody therapies for cancer. *MAbs* 7, 303-310.

- Wilson, A., Murphy, M.J., Oskarsson, T., Kaloulis, K., Bettess, M.D., Oser, G.M., Pasche, A.C., Knabenhans, C., Macdonald, H.R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes Dev* 18, 2747-2763.
- Witte, H., Biersack, H., Kopelke, S., Rades, D., Merz, H., Bernard, V., Lehnert, H., and Gebauer, N. (2018). Indolent lymphoma with composite histology and simultaneous transformation at initial diagnosis exhibit clinical features similar to de novo diffuse large B-cell lymphoma. *Oncotarget* 9, 19613-19622.
- Woo, C.W., Cui, D., Arellano, J., Dorweiler, B., Harding, H., Fitzgerald, K.A., Ron, D., and Tabas, I. (2009). Adaptive suppression of the ATF4-CHOP branch of the unfolded protein response by toll-like receptor signalling. *Nat Cell Biol* 11, 1473-1480.
- Wu, D., Wood, B.L., Dorer, R., and Fromm, J.R. (2010). "Double-Hit" mature B-cell lymphomas show a common immunophenotype by flow cytometry that includes decreased CD20 expression. *Am J Clin Pathol* 134, 258-265.
- Xu, X., Zhang, L., Wang, Y., Zhang, Q., Zhang, L., Sun, B., and Zhang, Y. (2013). Double-hit and triple-hit lymphomas arising from follicular lymphoma following acquisition of MYC: report of two cases and literature review. *Int J Clin Exp Pathol* 6, 788-794.
- Yang, M., Shao, J.H., Miao, Y.J., Cui, W., Qi, Y.F., Han, J.H., Lin, X., and Du, J. (2014). Tumor cell-activated CARD9 signaling contributes to metastasis-associated macrophage polarization. *Cell Death Differ* 21, 1290-1302.
- Yoon, K.W., Byun, S., Kwon, E., Hwang, S.Y., Chu, K., Hiraki, M., Jo, S.H., Weins, A., Hakrroush, S., Cebulla, A., *et al.* (2015). Control of signaling-mediated clearance of apoptotic cells by the tumor suppressor p53. *Science* 349, 1261669.
- Zhang, M., Hutter, G., Kahn, S.A., Azad, T.D., Gholamin, S., Xu, C.Y., Liu, J., Achrol, A.S., Richard, C., Sommerkamp, P., *et al.* (2016). Anti-CD47 Treatment Stimulates Phagocytosis of Glioblastoma by M1 and M2 Polarized Macrophages and Promotes M1 Polarized Macrophages In Vivo. *PLoS One* 11, e0153550.
- Zhao, Q., Egashira, K., Inoue, S., Usui, M., Kitamoto, S., Ni, W., Ishibashi, M., Hiasa Ki, K., Ichiki, T., Shibuya, M., *et al.* (2002). Vascular endothelial growth factor is necessary in the development of arteriosclerosis by recruiting/activating monocytes in a rat model of long-term inhibition of nitric oxide synthesis. *Circulation* 105, 1110-1115.
- Zhu, X., Ng, H.P., Lai, Y.C., Craigo, J.K., Nagilla, P.S., Raghani, P., and Nagarajan, S. (2014). Scavenger receptor function of mouse Fcγ receptor III contributes to progression of atherosclerosis in apolipoprotein E hyperlipidemic mice. *J Immunol* 193, 2483-2495.
- Ziepert, M., Hasenclever, D., Kuhnt, E., Glass, B., Schmitz, N., Pfreundschuh, M., and Loeffler, M. (2010). Standard International prognostic index remains a valid predictor of outcome for patients with aggressive CD20+ B-cell lymphoma in the rituximab era. *J Clin Oncol* 28, 2373-2380.

Zver, S., Zadnik, V., Bunc, M., Rogel, P., Cernelc, P., and Kozelj, M. (2007). Cardiac toxicity of high-dose cyclophosphamide in patients with multiple myeloma undergoing autologous hematopoietic stem cell transplantation. *Int J Hematol* 85, 408-414.