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# Histone Deacetylase 8 is a Novel Therapeutic Target for Mantle Cell Lymphoma and Preserves Natural Killer Cell Cytotoxic Function

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# Histone Deacetylase 8 is a Novel Therapeutic Target for Mantle Cell Lymphoma and Preserves Natural Killer Cell Cytotoxic Function

by

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of the requirements for the degree of
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## DEDICATION

I dedicate this dissertation to my daughter, Hailey Watters. You inspire me every day to have more patience, kindness, and love, toward myself. You are a gift to this world and your happiness will always be the most important thing to me. One day, I hope you look back at this moment and realize that you and I did this together. Always persevere, no matter the obstacles along the way, and remember these words “Just keep moving forward”, or as you would say, “Just keep swimming”. I love you, forever, and always. - Mom (a.k.a. The Honey Badger)

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ADCC | Antibody Dependent Cellular Cytotoxicity |
| AE | Adverse Events |
| AID | Recombination Deaminase |
| AKT1 | AKT Serine/Threonine Kinase 1 |
| ALDHIII | Aldehyde Dehydrogenase III |
| AML | Acute Myeloid Leukemia |
| APL | Acute Promyelocytic Leukemia |
| ARID1A | AT-Rich Interactive Domain-Containing Protein 1A |
| ASCT | Autologous Stem Cell Transplant |
| ATM | Ataxia-Telangiectasia |
| β-ACTIN | Beta-Actin |
| β2M | Beta 2 Microglobulin |
| B7-H6 | Natural Killer Cell Cytotoxicity Receptor 3 Ligand 1 |
| BCL-2 | B Cell Lymphocyte 2 |
| BCL-XL | BCL2 Like Protein 1 |
| BCL2 | B-Cell Lymphoma 2 |
| BCL6 | B-Cell Lymphoma 6 |
| BCR | B-Cell Receptor |
| BIRC3 | Baculoviral IAP Repeat Containing 3 |
| BL | Burkett Lymphoma |
| BLIMP1 | B Lymphocyte Induced Maturation Protein 1 |
| BM | Bone Marrow |
| BM11 | B Lymphoma Mo-MLV Insertion Region 1 Homolog |
| BRAF | B-Raf Proto-oncogene, Serine/Threonine kinase |
| BTK | Bruton's Tyrosine Kinase |
| BTKi | Bruton's Tyrosine Kinase Inhibitor |
| c-JUN | Jun Proto-oncogene, AP-1 Transcription Factor Subunit |
| c-MYC | Myc Proto-oncogene Protein |
| CARD11 | Caspase Recruitment Domain Family Member 11 |
| CBFβ | Core-Binding Factor Subunit Beta |
| CCND1 | Cyclin D 1 |
| CCND2 | Cyclin D 2 |
| CCND3 | Cyclin D 3 |
| CD107a | Lysosomal Associated Membrane Protein 1 |
| CD11a | Integrin Subunit Alpha L |
| CD11b | Integrin Subunit Beta 2 |
| CD132 | Cluster of Differentiation 132 |
| CD16 | Fc Gamma Receptor III |
| CD2 | CD2 Molecule |

| | |
|---|---|
| CD3 | T Cell Antigen Receptor Complex |
| CD5 | CD5 Molecule |
| CD7 | CD7 Molecule |
| CD9 | CD9 Molecule |
| CD94 | Killer Cell Lectin Like Receptor D1 |
| CDC | Complement Dependent Cytotoxicity |
| CDKN1A | Cyclin Dependent Kinase Inhibitor 1A |
| CDKN2A | Cyclin Dependent Kinase Inhibitor 2A |
| CDKN2B | Cyclin Dependent Kinase Inhibitor 2B |
| CFSE | 6-Carboxyfluorescein Succinimidyl Ester |
| cGVHD | Chronic Graft Versus Host Disease |
| CHIP | Chromatin Immunoprecipitation |
| CK2 | Casein Kinase II |
| CLL | Chronic Lymphocytic Leukemia |
| CLP | Common Lymphoid Progenitors |
| cMCL | Classical Mantle Cell Lymphoma |
| CML | Chronic Myelogenous Leukemia |
| CMV | Cytomegalovirus |
| CNA | Copy Number Alterations |
| CR | Complete Response |
| Cr51 | Chromium 51 |
| CREB | CREB Binding Protein |
| CSRP2BP | Cysteine Rich Protein 2 Binding Protein |
| CTCL | Cutaneous T-Cell Lymphoma |
| CTG | Cell Titer Glo |
| DAP10 | DNAX Activation Protein 10 |
| DAP12 | DNAX Activation Protein 12 |
| DAPI | 4', 6-Diamidino-2-phenylindole, dihydrochloride |
| DC | Dendritic Cells |
| DDR | DNA Damage Response |
| DDR | Double Stranded Repair |
| DNA | Deoxyribonucleic Acid |
| DNAM-1 | DNAX Accessory Molecule 1 |
| DSB | Double Stranded Breaks |
| EF1α | Elongation Factor Alpha 1 |
| EGFR | Epidermal Growth Factor Receptor |
| EMT | Endothelial Mesenchymal Transition |
| ERK | Mitogen Activated Protein Kinase |
| ERK2 | Extracellular Signal-regulated Kinase 2 |
| ERRα | Estrogen-related receptor alpha |
| FAS | FAS Cell Surface Death Receptor |
| FASL | FAS Ligand |
| FBS | Fetal Bovine Serum |
| FcγRIII | Low Affinity Immunoglobulin Gamma Fc Region Receptor III-B |
| FDA | Food and Drug Administration |
| FGF18 | Fibroblast Growth Factor 18 |

| | |
|---|---|
| FK228 | Romidepsin |
| FL | Follicular Lymphoma |
| FMO | Fluorescence Minus One |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GC | Germinal Center |
| H2AX | H2A.X Variant Histone |
| HAT | Histone Acetyltransferase |
| HCC | Hepatocellular Carcinoma |
| HDAC | Histone Deacetylase |
| HDACi | Histone Deacetylase Inhibitor |
| HDT-ASCR | High Dose Therapy Followed by Autologous Stem Cell Rescue |
| HLA | Major Histocompatibility Complex |
| HLA-E | Major Histocompatibility Complex, Class I, E |
| HNRNPH1 | RNA Processing Gene Heterogenous Nuclear Ribonucleoprotein H1 |
| HOXA5 | Homobox A5 |
| HRP | Horseradish Peroxidase |
| HSP90 | Heat Shock Protein 90 |
| Hyper-CVAD | Hyper fractionated Therapy-Cyclophosphamide Vincristine Sulfate Doxorubicin Hydrochloride Dexamethasone |
| IC | Intensive Chemotherapy |
| IFNγ | Interferon gamma |
| IgG | Immunoglobulin G1 |
| IGH | Immunoglobulin Heavy Chain |
| IGHV | Immunoglobulin Heavy Chain Variable Region |
| IL-12 | Interleukin 12 |
| IL-15 | Interleukin 15 |
| IL-15Ra | Interleukin 15 Receptor alpha |
| IL-15Rb | Interleukin 15 Receptor beta |
| IL-18 | Interleukin 18 |
| IL-2 | Interleukin 2 |
| IL-2R | IL-2 Receptor |
| IL-2Rb | IL-2 Receptor beta |
| IL-7 | Interleukin 7 |
| IL2Ra | IL-2 Receptor alpha |
| ILR | Interleukin Receptor |
| IMiD | Immunomodulatory Drug |
| IP | Immunoprecipitation |
| IRF1 | Interferon Regulatory Factor 1 |
| IRF2 | Interferon Regulatory Factor 2 |
| IS | Immune Synapse |
| ITAM | Immunoreceptor Tyrosine-based Activation Motif |
| JAK | Janus Kinase |
| JAK1 | Janus Kinase 1 |
| JAK2 | Janus Kinase 2 |
| KIR | Killer Immunoglobulin-like Receptors |

| | |
|---|---|
| KIR2DL3 | Killer Cell Immunoglobulin Like Receptor, two Ig Domains and Long Cytoplasmic Tail 3 |
| KIR2DS | Killer Cell Immunoglobulin Like Receptor, two Ig Domains and Short Cytoplasmic Tail 2) |
| KLKB1 | Kallikrein B (Fletcher Factor) 1 |
| KMT2C | Lysine Methyltransferase 2C |
| KMT2D | Lysine Methyltransferase 2D |
| KRAS | KRAS Proto-oncogene |
| LAK | Lymphokine Activated Killer Cell |
| LAQ824 | Dacinostat |
| LBH589 | Panobinostat |
| LDH | Lactate Dehydrogenase |
| LN | Lymph Node |
| LSC | Leukemic Stem Cell |
| mAb | Monoclonal Antibody |
| MAC | Membrane Attack Complex |
| MAPK3 | Mitogen Activated Protein Kinase 3 |
| MCF-7 | Mammary Gland Adenocarcinoma Cell Line |
| MCL | Mantle Cell Lymphoma |
| MDM2 | MDM2 Proto-oncogene |
| MEF2B | Myocyte Enhancer Factor 2B |
| MEK | Mitogen-Activated Protein Kinase 1 |
| MFI | Median Fluorescent Intensity |
| MICA/B | HLA Class I Polypeptide-related Sequence A/B |
| MLL2 | N-Methyltransferase 2 |
| MLL2 | Histone-Lysine N-Methyltransferase 2D |
| MM | Multiple Myeloma |
| MoA | Mechanism of Action |
| MPN | Myeloid Progenitor Neoplasms |
| MZ | Mantle Zone |
| MZL | Marginal Zone Lymphoma |
| N-CoR2 | Nuclear Receptor Corepressor 2 |
| NAD | Nicotinamide Adenine Dinucleotide |
| NCOA2 | Nuclear Receptor Coactivator 2 |
| NCOA3 | Nuclear Receptor Coactivator 3 |
| NCR | Natural Cytotoxicity Triggering Receptor |
| NFkB | Nuclear Factor Kappa B Subunit |
| NHEJ | Non-Homologous End Joining |
| NHL | Non-Hodgkin’s Lymphoma |
| NK | Natural Killer Cells |
| NKG2A | Killer Cell Lectin Like Receptor C1 |
| NKG2C | Killer Cell Lectin Like Receptor C2 |
| NKG2D | Killer Cell Lectin Like Receptor K1 |
| NKp30 | Natural Cytotoxicity Triggering Receptor 3 |
| NKp44 | Natural Cytotoxicity Triggering Receptor 2 |
| NKp46 | Natural Cytotoxicity Triggering Receptor 1 |

| | |
|---|---|
| NKR | Natural Killer Receptors |
| nnMCL | Non-nodal Mantle Cell Lymphoma |
| NOTCH 1 | Notch Receptor 1 |
| NOTCH 2 | Notch Receptor 2 |
| NOTCH 3 | Notch Receptor 3 |
| ORR | Overall Response Rate |
| OS | Overall Survival |
| PAX5 | Paired Box 5 |
| PB | Peripheral Blood |
| PBMC | Peripheral Blood Mononuclear Cell |
| PCAF | P300/CBP-associated factor |
| PD-1 | Program Cell Death Protein 1 |
| PD-L1 | Program Cell Death 1 Ligand 1 |
| PFS | Progression Free Survival |
| PI | Propidium Iodide |
| PKA | Protein Kinase A |
| PKM2 | Pyruvate Kinase M2 |
| PLCγ1 | Phospholipase C gamma 1 |
| POT1 | Protection of Telomeres 1 |
| PP1 | Protein Phosphatase 1 |
| PRAM1 | ML-RARA Regulated Adapter Molecule 1 |
| PTM | Post Translational Modifications |
| PXD101 | Belinostat |
| R-CHOP | Rituximab-Cyclophosphamide Hydroxy daunomycin Oncovin Prednisone |
| R-DHAP | Rituximab Dexamethasone Cytarabine Cisplatin |
| R/R | Relapsed/Refractory |
| RAD21 | RAD21 Cohesin Complex Component |
| RAF | RAF Proto-oncogene, Serine/Threonine Kinase |
| RAS | Rat Sarcoma Virus |
| RB1 | Retinoblastoma 1 |
| RITUXIN | Monoclonal IgG Antibody Targeting CD20 |
| RNA | Ribonucleic Acid |
| RUNX3 | RUNX Related Transcription Factor 3 |
| SAHA | Superoylanilide Hydroxamic Acid |
| SAMHD1 | SAM Domain and HD Domain 1 |
| SCGM | Stem Cell Growth Media |
| SCL | Small Cell Lymphoma |
| SCT | Stem Cell Transplant |
| SHM | Somatic Hypermutation |
| shRNA | Short Hairpin RNA |
| siRNA | Short Interfering RNA |
| SIRT | Sirtuin |
| SLT | Secondary Lymphoid Tissue |
| SMARCA4 | SWI/SNF Related Matrix-associated Actin-dependent Regulator of Chromatin Subfamily A Member 4 |

| | |
|---|---|
| SMARCB1 | SWI/SNF Related Matrix-associated Actin-dependent Regulator of Chromatin Subfamily B Member 1 |
| SMC1A | Structural Maintenance of Chromosome 1A |
| SMC3 | Structural Maintenance of Chromosome 3 |
| SNAIL | SNAIL Family Transcriptional Repressor |
| SOX11 | SRY-Box Transcription Factor 11 |
| SOX9 | SRY-Box Transcription Factor 9 |
| STAT3 | Signal Transducer and Activator of Transcription 3 |
| STAT5 | Signal Transducer and Activator of Transcription 5 |
| STAT6 | Signal Transducer and Activator of Transcription 6 |
| SUCNR1 | Succinate Receptor 1 |
| TCRβ | T Cell Receptor Beta Subunit |
| TEC | TEC Protein Tyrosine Kinase |
| Th1 | T Cell Helper 1 |
| Thr | Threonine |
| TLR | Toll-like Receptor |
| TNFa | Tumor Necrosis Factor alpha |
| TP53 | Tumor Protein p53 |
| TRAF2 | Tumor Necrosis Receptor Associated Factor 2 |
| TRAIL | TNF Superfamily Member 10 |
| Treg | Regulatory T Cells |
| TSA | Trichostatin A |
| ucNK | Unconventional NK cell |
| ULBP | UL Binding Protein |
| VEGF | Vascular Endothelial Growth Factor |
| VPA | Valproic Acid |
| WHSC1 | Wolf-Hirschhorn Syndrome Candidate 1 |
| WM | WaldenstrÖm’s Macroglobulinemia |
| WT | Wild Type |
| Zn2+ | Zinc |
| ZNF318 | Zinc Finger Protein 318 |

## ABSTRACT

This study demonstrates for the first time that HDAC8 function is critical for MCL survival, and abrogating its activity in human primary NK cells does not interfere with NK IgG antibody directed ADCC therapies *ex vivo*. Human NK cells, isolated from healthy donors, are highly resistant to HDAC8 inhibitor treatment with PCI-34051. Even at the highest concentration, 20uM, no toxicity was observed. Conversely, MCL cell lines representative of the aggressive MCL subtype, classical MCL, were especially sensitive to PCI-34051, an HDAC8 selective inhibitor, treatment. Blocking HDAC8 activity and/or abrogating expression through shRNA silencing induced significant DNA damage, hyperacetylation of SMC3, and apoptosis. Furthermore, DNA damage was an indicator of sensitivity. Drug washout experiments indicated that PCI-34051 can be dosed twice at half the original dose, distributed across 2-sequential days, to achieve the same effect as a single high dose.

Stimulating human NK cells with IL-2, IL-12, and IL-15 or IL-18, leads to a significant upregulation of HDAC8. It was hypothesized that HDAC8 may play a role in NK activity. However, blocking HDAC8 activity did not dampen NK survival, proliferation, cytotoxic responses, cytokine secretion, or NK phenotype. Interestingly, PCI-34051 increased the abundance of $IFN\gamma^+$ NK cells, possibly indicating enhanced activity. There was insufficient evidence that PCI-34051 sensitizes MCL cell lines to NK cytotoxicity. Rather, PCI-34051 may induce a NK memory-like phenotype, indicated by enrichment of $IFN\gamma^+$ NK cells. Therefore, HDAC8 may act as a mediator of memory-like responses in NK cells.

The focus of this study was to identify a novel HDAC protein critical for MCL survival and to evaluate if inhibiting activity interferes with IgG antibody therapies dependent on NK ADCC for therapeutic efficacy. NK cell cytolytic function is critical for anti-tumor response and is dependent on expression of sensors that detect aberrancies. NK cells are capable of sensing abnormally low levels of HLA class I molecules through a plethora of KIR receptors. They are capable of detecting stress signals on the surface of tumor cells through their NKG2D receptor and elicit a robust cytotoxic response. Additionally, the low affinity Fc binding receptor CD16 is imperative for antibody therapies dependent on NK ADCC for clinical efficacy. Thorough investigation was performed to determine if blocking HDAC8 in NK cells caused any attributes not favorable for NK anti-tumor responses. PCI-34051 did not alter NK anti-tumor phenotype. The results presented in this dissertation indicate that NK cells can robustly induce cytotoxic responses during treatment with the *HDAC8 selective inhibitor, PCI-34051, in combination with IgG targeting therapies.*

MCL cell lines representing the highly aggressive SOX11+ MCL subtype, conventional MCL, had the highest sensitivity to HDAC8 inhibition, indicating, a potential therapeutic option for treating this patient population.

# CHAPTER I:

# INTRODUCTION

## 1.1 Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is a rare subtype of B cell non-Hodgkin lymphoma (NHL), accounting for approximately 5-10% of all malignant B cell lymphomas (1). It occurs more frequently in men than women. The median age at diagnosis is 68 years old, and the median survival is approximately 6 to 7 years, which varies and is dependent on stage and aggressive nature (2). In the early 1980s, Weisenburger et al., coined the term Mantle Zone Lymphoma due to the expansion of B cells within the mantle zone surrounding the germinal center in secondary lymphoid organs. It was believed to represent a nodular follicular variant of intermediate lymphocytic lymphoma and was characterized by the outgrowth of slightly irregular atypical small lymphoid cells located in germinal centers (GC) with large mantle zones (3, 4). MCL is considered an aggressive form of NHL and is not curable with current chemo-immunotherapy: Rituximab, Cyclophosphamide, Hydroxy daunomycin, Oncovin (Vincristine Sulfate), Prednisone (R-CHOP). While combination chemo-immunotherapies have improved overall survival, it does not offer a curable option for MCL patients. There is an unmet need to develop targeted strategies to combat the high heterogenetic nature of this disease.

In 95% of MCL cases, the genetic hallmark is the reciprocal chromosome translocation, t(11:14) (q13;q32)/Cyclin D1-Immunoglobulin heavy chain locus (IGH), which occurs during B-cell development in the bone marrow and is considered the primary oncogenic event in the

pathology of this disease (5-7). In approximately 90% of MCL cases, the CCND1 rearrangement is mediated by the Activation-Induced cytidine Deaminase (AID) machinery occurring during pro/pre-B cell stage during the initial step of IGH V(D)J recombination (8). In the other 10% of cases the CCND1 translocation is mediated by the AID machinery and occurs during somatic hypermutation (SHM) or class switching (8).

The chromosomal t(11;14) (q13;q32) translocation places the CCND1 gene (11;q13) next to the IGH enhancer region (14q32), resulting in aberrant overexpression of cyclin D1 activates cyclin dependent kinases (CDKs) 4 and 6, which phosphorylate and inactivate the tumor suppressor protein Retinoblastoma (Rb), promoting G1-S phase transition (6). Cyclin D1 amplification and overexpression in cancer cells allow cells to proliferate in the absence of extracellular growth factors (9). CCDN1 translocations are frequently found in numerous cancers, including oral squamous cell carcinoma, breast, melanoma, and lung cancers (9). CCND1 is a proto-oncogene, and while its overexpression is a hallmark of MCL, it is considered a weak driver for transformation of lymphoid cells and requires a cooperative effort of secondary oncogenic events for cellular transformation (10). Cyclin D1 negative MCL cases often carry rearrangements of the Cyclin D2 and D3 genes (CCND2, CCND3) with Immunoglobulin Kappa (IGK)/Light chain enhancer regions (11) (12). In rare cases, cyclin D1, D2, and D3 are not contributors to the disease; they can be characterized by Cyclin E1/E2 (CCNE1/E2) expression, with no evidence of IGL, IGK, or IGH rearrangements (12). Immunohistochemistry of SRY Box Transcription Factor 11 (SOX11) overexpression is especially helpful to confirm diagnosis in cyclin D1 negative cases.

SOX11 contributes to the aggressive nature of MCL. It is a key transcriptional regulator important for mature naïve B cell development during clonal expansion and expression is

associated with the less the differentiated MCL subtype, with an unmutated IGH which occurs during somatic hypermutation (SHM) and affinity selection of B cell development in the germinal center. Key secondary oncogenic alterations are required for MCL lymphomagenesis, include mutations or deletions in Tumor Protein 53 (TP53), overexpression of the mouse double minute 2 (MDM2) proto-oncogene, amplification of the B lymphoma Mo-MLV Insertion Region 1 Homolog (BMI1) which negativity regulates cyclin dependent kinase inhibitor 2 (CDKN2A) transcription, deletions in the CDKN2A locus encoding p16, a cyclin-dependent kinase inhibitor, and deletions in the ataxia-telangiectasia gene (ATM) (6, 13).

In the last decade, major contributions have helped us understand the molecular cytogenetic aberrations that occur in MCL, responsible for deregulation of pathways involved in cell survival, proliferation, apoptosis, cell cycle progression, DNA damage response, motility, and epigenetics (6). These studies have identified alterations in over 40 driver genes involving eight major pathways that contribute to the disease complexity. The major pathways and genes identified are involved in cell survival, proliferation, DNA damage response, telomere maintenance, gain-of-function mutations in the notch receptor (NOTCH) gene, B-cell receptor (BCR)/Toll-like receptor (TLR)/nuclear factor kappa B subunit (NF-κB), chromatin remodeling, and RNA regulation (8, 14). Several pathways identified are associated with drug resistance and can be detected during subclonal evolution. Drug resistance often results in the development of highly aggressive MCL subtypes difficult to treat with current clinical front-line and second-line FDA approved therapies and often results in the use of salvage therapies when patients stop responding.

The Ann Arbor staging system is used to identify what stage of NHL or Hodgkin's lymphoma are at diagnosis. Stage I indicates a single region is affected by cancer, such as one

lymph node. Stage II indicates 2 separate regions, confined to one side of the diaphragm, are affected. Stage III represents the spreading of cancer to both sides of the diaphragm, involving an organ, area near lymph nodes, or spleen. Stage IV indicates that cancer has metastasized to involve one or more extra-lymphatic organs, including involvement of the BM, liver, and or nodular association of the lung tissue. MCL is a challenging disease to treat due to its complexity, heterogenous clinical behavior, and diagnoses commonly occurring at late-stage, Ann Arbor stage III, IV (15). MCL is considered a chronically relapsing NHL, clinically characterized as an aggressive disease because 80-90% of all cases are diagnosed at the late stage of disease and/or carry mutations that contribute to a highly aggressive disease state, warranting treatment soon after initial diagnosis.

Frontline treatment for younger fit patients is high dose cytarabine plus chemotherapy and autologous stem cell transplants (ASCT). Younger fit patients seeking maximal response can undergo high dose therapy followed by autologous stem cell rescue (HDT-ASCR). Rituximab is used as maintenance therapy post HDT-ASCR, as this has shown patients to have demonstrated improvements in progression free survival (PFS) and overall survival (OS) (16).

MCL is predominately observed in older Caucasian men, >67 years old, often with comorbidities, making it a challenging disease to treat in this patient population. These patients are often unable to tolerate intensive therapies or ASCT. Initially, most elderly >66 years old patients respond to current frontline treatments. Standard front-line treatments include intensive chemotherapy (IC) or the combination of Rituximab-lenalidomide (17). Conventional IC regimens include alternating Rituximab, Dexamethasone, Cytarabine, Platinum (R-DHAP) and, alternatively, Rituximab and Cyclophosphamide, Hydroxydaunomycin, Oncovin, Prednisone (R-CHOP). Importantly, patients who respond to therapy have a high probability of relapse with a

dismal prognosis. Drug induced selection drives clonal evolution and MCL heterogeneity, contributing to drug resistance and relapse (14). For older unfit patients, multiple treatment strategies have been clinically explored; however, there is still a high degree of relapse, often presenting with a more aggressive disease and poor prognosis.

To this end, there is an unmet need for therapeutic treatment options in this patient population. Deciphering molecular mechanisms involved in the MCL pathogenesis will be key in understanding the heterogenic complexity that drives clonal evolution and drug resistance, thus, paving the way for developing novel combination targeting strategies.

### 1.1.1 Classification and Development of Mantle Cell Lymphoma

MCL is divided into two major subtypes based on molecular signatures, leukemic non-nodal MCL (nnMCL) and conventional MCL (cMCL) **(Figure 1.1)** (13). The majority of MCL cases, 95%, are phenotypic of the cMCL subtype. cMCL is considered more aggressive than nnMCL, involving the lymph nodes. While nnMCL cases commonly exhibit an indolent evolution, presenting only with BM involvement and splenomegaly (15). Conventional MCL arises in the GC and retains a naïve-like B cell state, $CD5^{+,}$ and $SOX11^{+}$, with high copy number alterations (CNA) in; CDKN2A (9p21), and RB1 (13q14) genes, ATM deletion (11q22), and a subset with TP53 deletion (17p13). Patients with cMCL commonly present with lymphadenopathy and 90% of patients present with extranodal manifestations, including infiltration into the blood (50%), gastrointestinal tract (~26%), spleen (~50%), and bone marrow (~72%) with an aggressive clinical behavior requiring urgency in treatment (15, 18).

During Pro-B to Pre-B cell development, B cells acquire oncogenic mutations leading to the development of MCL (6). The chromosomal translocation t(11;14) is considered a weak

oncogenic driver and requires somatic mutations in the following genes: TP53, notch receptor 1/2, ATM, histone-lysine N-methyltransfertase-2 (MLL2), baculoviral IAP repeat containing 3 (BIRC3) and/or aberrant activation of the NFκB pathway for transformation into MCL (14, 19, 20). Conventional MCL originates in the MZ, bypassing the germinal center (GC) microenvironment, and is derived from mature B cells with a naïve-like genomic signature, morphologically representing classic/blastoid cells, with SOX11 expression and high chromosomal instability, and presents with an aggressive clinical behavior (20).

Non-nodal MCL is a leukemic form of MCL that has molecular characteristics of memory-like B cells, containing somatic hypermutations in immunoglobulin heavy chain variable region (IGHV). Indicating nnMCL forms during the germinal center reaction, when B cells have undergone somatic hypermutation to increase antigen affinity, representing a "memory"- like B cell. The disease presents with splenomegaly, carrying low genomic complexity and copy number alterations, and usually considered a stable/indolent disease. However, in cases where nnMCL harbor mutations in TP53, patients are at a higher risk due to the aggressive behavior, and it has been shown these patients are resistant: to high-dose chemotherapy cytarabine as well as the high-intensity chemotherapy regimen; Maxi CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) with high dose cytarabine. Indicating there is an unmet need to use targeted therapies to treat patients resistant to current front-line strategies, such as targeting BCR signaling using the irreversible BTKi, Ibrutinib, with an Overall Response Rate (ORR) of 68% and Progression Free Survival (PFS) of 14 months (21).

Patients who fall into the stable/indolent MCL category have normal lactate dehydrogenase (LDH) levels, favorable cytomorphology, low proliferative index measured by

Ki-67% positivity, and present with non-nodal disease (22). A Canadian research group examined the significance of initial management strategies on patients with indolent nnMCL (22). In this low-risk group, they compared patients treated early after diagnosis versus patients not treated and found a significant difference in overall survival (OS), with 72 months in the watch-and-wait group vs. 52.5 months in early treatment group (22). This crucial discovery established that the watch-and-wait strategy was a superior alternative to early treatment of indolent nnMCL, leading to longer OS. However, as scientists and physicians discover more therapeutic strategies to tackle drug resistance using combination drug-targeting approaches, the watch-and-wait strategy may need to be revisited. In some cases, combination therapy could provide an earlier treatment option to tackle disease prior to becoming aggressive.

Leukemic nnMCL is typically considered a stable disease originating in the GC during the GC reaction, harboring a high degree of IGHV mutations, with a memory-like B cell phenotype, and morphologically blastoid with plasma cell differentiation in ~37% of cases (20). The majority of nnMCL cases are indolent and are placed in the "watch and wait" group, having slow disease progression (13). These two subtypes differ in their genomic signature, origin, and expression of SRY-Box Transcription Factor 11 (SOX11). Indolent non-nodal MCL, lacking alterations in TP53, is associated with a favorable prognosis compared to the undifferentiated SOX11 positive mature naïve MCL subtype. TP53 is a paradigm for defining a favorable or unfavorable patient prognosis in both subtypes (23). Genetic abnormalities in TP53 are associated with a poor prognosis.

Conversely, conventional MCL (cMCL) is aggressive and highly heterogeneous. Clonal selection occurs during drug therapy, leading to the outgrowth of drug resistant MCL cells, which are often found to be more aggressive. SOX11 is a transcriptional regulator that influences

many intracellular signals that promote oncogenesis in MCL. In cMCL cases, SOX11 is found overexpressed, compared to nnMCL, which often lacks SOX11 expression. Overexpression of SOX11 promotes MCL pathogenesis by preventing B Cell differentiation, promoting angiogenesis, inducing immunosuppression, and enhancing invasive properties (24). Genetic knockdown of SOX11 causes downregulation of Paired Box Gene 5 (PAX5) (25). SOX11 promotes transcription of the B cell lineage specific activator PAX5. PAX5 acts as a transcriptional repressor, blocking mRNA synthesis of positive regulatory domain I-binding factor 1 (PRDM1), the master regulator of B cell terminal differentiation (26, 27). PAX5 is a critical transcription factor expressed during B-cell development from pro-B cells to GC B cells (28). B-cell lymphoma 6 (BCL-6) is also expressed during GC differentiation and inhibits BLIMP1 expression, the master regulator of terminal differentiation, presenting as a blastoid/classical disease (29). SOX11 promotes adhesion by expressing C-X-C motif chemokine receptor 4 (CXCR4) and focal adhesion kinase (FAK), enhancing its invasive properties. It represses BCL6 through binding to the BCL6 regulatory sequence, located in the non-coding region of exon 1, preventing germinal center transition. SOX11 can enhance angiogenesis through upregulation of platelet derived growth factor (PDGF) protein expression. Furthermore, SOX11 influences the tumor microenvironment through upregulation of CD70, which influences T cell activity via binding to its cognitive ligand CD27 (24). It acts as an immunosuppressor that reduces T cell activity by promoting regulator T ($T_{reg}$) cell survival and expansion. Thus, SOX11 enhances survival of MCL cells and promotes disease aggressiveness. cMCL lacks hyper somatic mutations within IGHV locus, are SOX11 positive, and acquire secondary oncogenic events in the mantle zone (MZ), that lead to disease progression (25). Inversely, nnMCL is

characterized by a hypermutated IGHV locus, lacking expression of SOX11, with secondary oncogenic events occurring in the GC.

### 1.1.2 Molecular Signatures Indicating Disease Progression and Mechanisms Involved in Drug Resistance

In 2013, Campo's group revealed a unique molecular landscape by characterizing 29 MCL primary tumors and 6 MCL cell lines by whole-genome or whole-exome sequencing (14). This study identified 25 genes with reoccurring somatic mutations involving genes that regulate cell homeostasis, cell growth, DNA replication, apoptosis, DNA damage, epigenetic regulation, genotoxic stress pathways **(Table 2)** (14). TP53 mutations were associated with poor overall survival compared to those with wild-type p53 (WT p53) (30). The mutational findings were validated by targeted sequencing of 172 primary tumors and revealed the highest frequency of reoccurring mutations are in the ATM, CCND1, and TP53 genes. Differential distribution of these mutations was associated with SOX11 expression or IGHV mutational status. Thus, CCND1 mutations are preferential to highly mutated IGHV, whereas ATM mutations were observed only in SOX11+ tumors. ATM is a gene involved in the DNA damage repair pathway, and alterations in this gene are associated with shorter telomere length. Consequently, MCL cases with ATM mutations result in a high degree of chromosomal instability compared to WT ATM MCL cases (8, 31). Interestingly, mutational status of TP53 was equally distributed across the MCL subtypes. Furthermore, additional mutations were found in the NOTCH2 gene but not in the NOTCH1 gene. NOTCH regulates cell proliferation, cell death, and activation of specific differentiation programs. MCL subtypes with mutations in NOTCH are associated with blastoid variants and are aggressive with dismal prognosis (14, 32). Additionally, longitudinal samples

taken during drug treatment from different topographical sites within the peripheral blood and lymph nodes, identified intratumoral heterogeneity of MCL, providing evidence of clinical evolution during drug therapy. Advances in understanding key factors involved in MCL transformation and resistance to drug therapy are critical for tackling resistance to current frontline therapies. More recent studies have led to defining novel drug combinations to address the multi-aberrant pathways that cause drug resistance (33, 34).

### 1.1.3 BTK and BCL2 Inhibitors Show Clinical Efficacy for MCL Patients

Bruton's tyrosine kinase (BTK) is an essential component of B cell receptor (BCR) signaling. The BCR pathway is aberrantly active in MCL and is integral for proliferation and survival of MCL cells.

Ibrutinib was the first BTK inhibitor FDA approved to treat chronic lymphocytic leukemia (CLL), relapsed/refractory MCL, marginal zone lymphoma (MZL), WaldenstrÖm's macroglobulinemia (WM), small cell lymphoma (SCL). Clinical trials involving use of Ibrutinib in adult chronic graft-v-host disease (cGVHD) (NCT0219869) and in pediatric cGVHD (NCT03790332) led to FDA approval for these diseases in 2017 and 2022, respectively (35). In the phase 2 clinical trial, PCYC-1104-CA (NCT01236391), with a cohort of 111 patients, single agent Ibrutinib had superior efficacy, with a high overall response rate (ORR) of 68-72%, a complete response (CR) of 20%, and progression free survival (PFS) of 13 months (2). Patients with advanced disease and 2 prior lines of therapy were found to respond better to Ibrutinib, with an overall response rate of 78%, a complete response rate of 37%, and median PFS of 22 months (36).

Ibrutinib is a potent BTK inhibitor; however, it also has off-target binding and is associated with adverse events (AE), specifically cardiovascular toxicities, which can cause abrupt treatment discontinuation. Ibrutinib is known to bind to other kinases such as TEC protein tyrosine kinase (TEC) and epidermal growth factor receptor (EGFR), which likely contributes to cardiovascular adverse events such as atrial fibrillation. Atrial fibrillation is the most common side-effect that causes discontinuation of treatment. Ibrutinib is a first generation irreversible covalent BTK inhibitor, and due to its promiscuity, more selective BTK inhibitors were developed to reduce toxicity. These include covalent irreversible BTK inhibitors: Acalabrutinib and Zanubrutinib, and non-covalent reversible BTK inhibitors: Fenebrutinib and Vecabrutinib. Irreversible BTKis bind to the cystine 481 (C481) residue of BTK, forming a covalent bond that blocks ATP binding and inhibits catalytic activity (37). Resistance to covalent BTK inhibitors is often the result of acquired mutations in BTK residue C481 located within the kinase domain. Vecabrutinib, a non-covalent reversible BTKi, does not bind to the C481 residue. Thus, overcoming acquired drug resistance associated with covalent irreversible BTK inhibitors (37, 38).

ELEVATE-RR, a phase 3 clinical trial, NCT02477696, tested the efficacy of another covalent BTKi, acalabrutinib. It was better tolerated and had lower toxicity issues in patients with Chronic Lymphocytic Leukemia (CLL); however, it was inferior to Ibrutinib (39). Second generation irreversible BTK inhibitors were shown to have higher on-target specificity, with a reduction in off-targeting of TEC and EGFR-family kinases and showed activity against BTK (40). Zanubrutinib, a high affinity BTK inhibitor, was shown to improve outcomes compared to Ibrutinib. In the phase 3 clinical study, ALPINE, NCT03734016, Zanubrutinb demonstrated a superior overall response rate of 78.3% (Zanubrutinib) vs 62.5% (Ibrutinib), 12-month

progression free survival was 94.9% vs 84% and equal to or grade 3 infections were lower, 12.7% vs 17.9%, in patients with CLL or SLL.

Patients with primary resistance to BTKi, all carried loss of chromosome 9p21.1-p24/3, and two-thirds of patients harbored mutations in the SWI-SNF—chromatin-remodeling complex (41). High-risk genetic features are associated with copy number loss of CDKN2A, mutations in KMT2D, NOTCH1, and NOTCH2 and are considered mechanisms of resistance to frontline therapies (42, 43). Patients who do not respond to Ibrutinib therapy rapidly progress with a median PFS of only 4 months (43). Therefore, patients resistant to BTKi's need other therapeutic options to overcome resistance associated with rapid disease progression.

B cell lymphoma 2 (BCL-2) is a member of the BCL-2 family of proteins that regulate apoptosis. BCL-2 localized to the outer mitochondrial membrane where it associates with other members, such as BAD and BAK, to prevent apoptosis and promote cell survival. Often, BCL-2 is aberrantly activated in MCL and contributes to the disease. Therefore, small molecule inhibitors, such as Venetoclax, were designed to target BCL2 and induce apoptosis.

As a single agent, the BCL2 inhibitor venetoclax, inhibits the anti-apoptotic protein B Cell lymphoma 2 (BCL2), which is overexpressed in MCL. Venetoclax therapy achieved a high overall response rate of 75%, complete response rate of 21%, progression free survival of 14 months, and had a lower toxicity profile compared to conventional treatments (44). The highest overall response rate was observed in patients with Relapse/Refractory (R/R) MCL with a 75% ORR compared to Follicular Lymphoma (FL) (ORR, 38%), Mantle Zone Lymphoma (MZL) (ORR, 67%), and Diffuse Large B Cell Lymphoma (DLBCL) (ORR, 18%). MCL patients who relapse during treatment with venetoclax present with a highly aggressive disease, causing death within 1 year of relapse. The phase 2 AIM study, NCI02471391, provided insight into the

genetic signatures responsible for these aggressive resistance mechanisms and established predictive indications of response. Genomic profiling that revealed TP53, NOTCH1/2, and SWI-SNF mutations mediate resistance to ibrutinib and venetoclax (41, 45). Interestingly, most MCL patients at disease progression have treatment acquired TP53 mutations. MCL with CDKN2A mutations did not have Notch 1/2 mutations. Three patients had mutations in Lysine Methyltransferase 2D (KMT2) genes, either KMT2C or KMT2D (45). At progression an increase >2-fold in mutation and copy number variations in the following genes: TP53 (83% vs. 33%), CCND1 (67% vs. 17%), ATM (50% vs. 17%), KMT2C (50% vs. 17%), KMT2D (67% vs. 17%), NOTCH2 (50%), and CDKN2A (67%) (45). Notably, initial complete responders that progressed had mutations in the ATM and in TP53 genes.

Combinational studies targeting BTK and BCL2 were evaluated to look for an enhanced response. In 2019, results from the CLARITY study, ISCRTN13751862, indicated an improvement in response with the combination of Ibrutinib plus Venetoclax, 89% of patients responded to treatment, with 51% achieving a complete response (46). Taken together, this highlights the importance of dual targeting strategies, which will pave the way for combating drug resistance, eventually tailoring treatment based on genetic signatures.

#### 1.1.3.1 Monoclonal Antibody Therapy in Combination with Targeted Therapies

Rituximab, in 1997, was the first monoclonal antibody (mAb) therapy to be FDA approved to treat cancer and the first FDA approved drug in a decade to treat NHL. It was a pivotal therapeutic advancement for MCL therapeutic response. Rituximab is an Immunoglobulin G1 (IgG1) anti-CD20 mAb. There are 4 mechanisms responsible for induction of Rituximab-mediated anti-tumor responses **(Figure 1.4)**. First, Rituximab bound to CD20 on B

cells induces complement dependent cytotoxicity (CDC). CDC induces assembly of the multiprotein complex membrane attack complex (13). CD5 molecule-like convertase catalyzes cluster of differentiation 5 (CD5) into CD5a and CD5b. CD5b binds to CD6, forming the stable intermediate, CD5b6. CD5b6 spontaneously binds to cluster of differentiation 7 (CD7), forming a stabilized MAC precursor, exposing the hydrophobic site on C7, which induces insertion into the lipid biolayer. Subsequently, this leads to C8 forming a complex with CDb67 (CD9). This induces the formation of the MAC ring, consisting of 12-18 CD9 proteins, and causes complement induced cell death. Second, Rituximab triggers crosslinking of CD20—CD20, inducing surface clustering within the lipid raft, which induces the SRC proto-oncogene and SRC kinase mediated apoptosis. Third, the Fc (fragment crystallizable) portion of the Rituximab binds to Fc Receptor (FcR) on macrophages inducing Antibody-Dependent Cellular Cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP) of tumor cells. Fourth, Rituximab's main mechanism of action is to activate NK cytotoxic response by binding the low affinity Fc gamma binding receptor III (FcγRIIIa) to the Fc portion of Rituximab to induce NK-dependent ADCC (47-49).

Understanding the influence of targeted therapies on immune cell function is critical. Considerations should be made to optimize clinical response to mAb therapies. A thorough investigation into the impact a small molecule drug has on immune cell function should be considered to identify good drug candidates and optimal delivery time. For instance, Rituximab taken concurrently with Ibrutinib, a BTK inhibitor, should be taken with caution. BTK inhibitors are known to dampen NK activity and decrease NK ADCC responses against MCL cell lines (50). Lenalidomide, an immunomodulatory drug, does not rescue NK activity from BTK

inhibition. Conversely, as a single agent, lenalidomide enhances NK activity and function. Thus, lenalidomide may be a superior candidate for combination drug therapy with Rituximab.

Lenalidomide is a second-generation immunomodulatory drug (IMiD) derived from Thalidomide, which was originally used to treat pregnancy related nausea. However, Thalidomide was banned due to it causing severe fetal malformation of the limbs. Lenalidomide, designed for higher specificity and lower toxicity, enhances immune responses across multiple cells within the immune compartment.

NK ADCC activity depends on activation of the low Fc affinity binding receptor, cluster of differentiation molecule 16 (CD16, FcγRIIIA). Signaling of the CD16 receptor requires the adaptor molecules, CD3ζ and FcεRγ, which contain ITAM motifs. Lenalidomide plus IL-2 or IL-12 increases NK rate of responsiveness to IgG1 antibodies, enhancing anti-tumor surveillance properties of NK cells (51). However, blocking IL-12 signaling diminishes the ADCC anti-tumor response even in the presence of Lenalidomide, indicating stimulating with IL-12 is critical for enhanced response. Antigen presenting cells (APC) are major contributors of IL-12 production. IL-12 directly influences differentiation into the Th1 lineage, expanding helper $CD4^+$ T cells and enhancing Interferon gamma (IFNγ) production in NK cells (52). Lenalidomide's mechanism of action is multifactorial, enhancing both the activity of T cells and NK cells, depletion of regulatory T cells ($T_{reg}$), and acting as a checkpoint inhibitor by decreasing PD-1 expression in patients with multiple myeloma (MM) (51).

The combination of Lenalidomide and Rituximab has shown clinical efficacy for the treatment of relapsed MCL (17, 53). In the phase 1/2 clinical trial, NCT00294632, in relapsed MCL and DLBCL patients who were treated with lenalidomide plus Rituximab achieved an overall response rate of 57%, progression free survival of 11.1 months and median overall

survival of 24.3 months (53). Lenalidomide likely increases the efficacy of Rituximab because of its multifunctional immune modulatory effects; through enhancing NK activity, helper T cell activity, cytokine secretion, and ability to suppress the immune checkpoint PD-1. In July 2022, results were provided by Celgene (Bristol Myers Squibb), concluding a 6-year follow-up of the clinical trial RELEVANCE (NCT01650701). Patients with untreated advanced follicular lymphoma (FL), dosed with Rituximab in combination with lenalidomide or Rituximab plus chemotherapy, achieved a progression free survival of 60% and overall survival rate of 86% across both cohorts. Median progression free survival and overall response rates have not been reached in either group (54). This provides evidence that non-chemotherapeutic treatments can achieve similar responses as chemotherapy in patients with advanced disease, providing a path forward towards non-chemotherapeutic strategies to treat NHL patients.

### 1.2 Histone Deacetylases and Cancer

Post-translational modification (PTM) of histone proteins occurs in many forms, including acetylation, methylation, ubiquitination, and sumoylation. Histone acetylation is regulated by two families of opposing enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs) (55). These enzymes are responsible for either relaxing or condensing the chromatin structure. Acetylation relaxes the chromatin and occurs via histone acetyl transferases (HATs) which transfer an acetyl group from acetyl-CoA to the amide groups on lysine residues (56). HATs are referred to as “writers” because they add an acetyl group to histone proteins. These PTMs promote relaxation of the chromatin architecture, and increase gene transcription, including anti-tumor genes. Histone deacetylases (HDACs) are enzymes that regulate epigenetic and intracellular messages by the hydrolysis of an acetyl group from acetylated lysines on

histones and non-histone proteins. HDAC post-translational modifications on histone proteins are referred to as “erasers” because they remove acetyl groups, which cause chromatin condensation, and prevent gene transcription (57). In healthy cells, histone acetylation and deacetylation occur harmoniously to achieve proper gene expression levels and maintain cellular homeostasis. Aberrant histone deacetylase activity often leads to global chromatin condensation, contributing to transcriptional repression of key anti-tumor genes that regulate apoptosis, cell division, and cell survival (56). HDAC activity is found to be upregulated across many tumor types. Hyper-deacetylation causes an imbalance in cellular homeostasis, thus contributing to disease progression. HDACs were discovered originally to function on histone proteins. However, it is now known that many HDACs have non-histone substrates and regulate many cellular processes beyond histone modifications. Aberrant deacetylase activity is linked to many diseases, including cancer, autoimmune and inflammatory diseases, neurodegenerative disorders, and metabolic disorders (58).

Histone deacetylases have been shown to upregulate anti-apoptotic gene expression, contribute to invasive properties and cell migration, and alter global transcriptional gene regulation. The first generation non-selective HDAC inhibitor, vorinostat, is a hydroxamic acid that targets class I, II, and IV HDAC proteins (59). Broad-spectrum HDAC inhibitors, Valproic acid (VPA), and vorinostat are highly effective in inducing apoptosis in lymphoma and leukemia cell lines and reducing tumor volume *in vivo* (60). However, clinically broad-spectrum HDACi’s are associated with a high occurrence of adverse reactions and are known for their toxicity issues. They can cause thrombocytopenia, induce tumor lysis syndrome, cause cardiotoxicities and in severe cases results in discontinuation of treatment. These inhibitors are also known to have a negative impact on NK viability and activation receptor expression. NK activity is

essential for clinical efficacy of IgG1 mAb therapy, designed to induce ADCC as its primary mechanism of action. The narrow-spectrum HDAC inhibitor, Entinostat—a class I HDAC inhibitor—was shown to increase NK activity through upregulation of NKG2D and its cognitive stress ligands, which results in synergistically enhancing NK cytotoxicity (61).

VPA and Romidepsin, a narrow spectrum class I and class II inhibitor, increase CD20 antigen expression on low CD20 expressing lymphoma cell lines and causes hyperacetylation of histone proteins (62). However, *in vivo* VPA did not improve response compared to the control and was not well tolerated, as it resulted in neuronal toxicity in mice. Conversely, Romidepsin was well tolerated alone and improved overall response. The combination of Romidepsin and Rituximab synergistically reduced tumor volume compared to single agent therapy alone (62).

### 1.2.1 Histone Deacetylases and Their Role in Tumorgenicity

There are 18 highly conserved HDAC members, divided into 4 classes based on structural homology: class I (HDAC 1-3 and 8), class IIa (HDAC 4, 5, 7, and 9), class IIb (HDAC 6 and 10), class III (sirtuins 1-7), and class IV (HDAC 11) **(Figure 1.2)**. The class III (sirtuins 1-7) are dependent on nicotinamide adenine dinucleotide (NAD), while the remaining HDACs are zinc-dependent ($Zn^2$). Zinc ($Zn^{2+}$) binds to the catalytic core present on the active site of the classical HDAC family members (HDAC1-11) and is responsible for stabilizing the interaction with acetylated substrates. Class I and II HDACs have similar sequences within their core domain and contain a $Zn^{+2}$ within the zinc binding pocket (63, 64).

Class I HDAC proteins 1, 2, and 3 are ubiquitously expressed across multiple tissues and are often found to localize in the nuclease of healthy cells. Class I HDACs have been shown to play a role in tumorigenesis and are often highly expressed in tumor cells. HDAC1/2, HDAC3,

and HDAC8 regulate expression of several known oncogenes. High class I HDAC expression in cancers is associated with upregulation of several oncogenes such as BCL proteins, BCL2 like protein 1 (BCL-XL), NOTCH3, Myc proto-oncogene protein (c-MYC), and T cell receptor beta subunit (TCRβ). Class I HDACs also regulate activity of the tumor suppressor protein TP53. In cutaneous T-cell lymphoma (CTCL), TP53 protein was identified as a substrate for HDAC1 and HDAC8. Baicalein, a dual class I HDACi targeting HDAC1/8, or PCI-34051, an HDAC8 selective inhibitor, causes hyperacetylation of the TP53 protein (65). Interaction with either HDAC1 or HDAC8 reduces TP53 activity through hypoacetylation. Blocking HDAC1 and/or HDAC8 promotes transcription of anti-apoptotic genes, such BCL2 associated X, apoptosis regulator (BAX) and P53-upregulated modulator of apoptosis (PUMA) (65).

HDAC1 function is critical during embryonic development and for immune cell function of B, T, and NK cells (66-68). HDAC1 and HDAC2 enzymatic function are critical for T cell development. During T cell maturation, $CD4^+$ T cells require HDAC1/2 to reduce the formation of RUNX family transcription factor 3 (RUNX3)/Core-binding factor subunit beta (CBFβ), which is involved in commitment towards CD8 differentiation (69, 70). Human primary NK cells stimulated with the proinflammatory cytokines; IL-2, IL-12, and IL-15 or IL-18, upregulate HDAC1, HDAC2, and HDAC8 transcriptionally and at the protein level (71).

HDACs can contribute to cellular transformation and aberrant cell proliferation when there is a loss in functional HDAC regulation. In cutaneous T cell leukemia (CTCL), HDAC1 and HDAC2 were highly expressed in 60% and 32% of cases, respectively (72). Elevated HDAC1 expression was shown to correlate with aggressive disease in patients with CTCL. Irradiation induces DNA damage, during which HATs and HDACs work in concert to regulate

transcription and DNA damage response (DDR) by recruiting DNA damage response proteins to prevent cellular transformation (73).

HDAC inhibitors are potent inducers of sensitizing cancer cells to radiation, indicating that HDACs have tumor promoting roles by enhancing survival of tumor cells (73). In CTCL patients, elevated HDAC1 expression was shown to correlate with aggressive disease (72). HDAC1 expression can be both oncogenic or tumor suppressive, dependent on the tumor type and stage. In the preleukemic stage of acute promyelocytic leukemia (APL), HDAC1 represses PML-RARA regulated adapter molecule 1 (PRAM-1), retarding tumor growth (74). Conversely, in established APL HDAC1 promotes tumor growth (74). Indicating, HDACs functional impact is cell context dependent. Often, HDACs are found overexpressed in late-stage diagnosis, and contribute to tumor growth, invasive properties, angiogenesis, and cancer stemness. HDAC1 and HDAC8 regulate TP53 protein acetylation, which can promote tumor growth in CTCL (65). Constitutive deacetylation of p53 by HDAC8, as seen in Inv(16) AML, results in repression of p53 activity, and accelerates tumor progression (55). In aggressive childhood neuroblastoma, enhanced HDAC8 protein expression correlates with poor prognosis (75). Blocking HDAC8 activity or knocking down HDAC8 protein induces differentiation of neuroblastoma cells, resulting in cell death. In liver cancer, hepatocellular carcinoma (HCC), HDAC8 was found to deacetylate pyruvate kinase M2 (PKM2) at lysine residue 62, directing nuclear localization and glycolysis (76). This indicates that HDAC8 has a regulatory role in glycolytic metabolism, which can result in enhanced tumor growth.

Histone deacetylase 8 activity is regulated by protein kinase A (PKA). PKA reduces the catalytic activity of HDAC8 through phosphorylation of serine 39 (77). This causes structural changes that impact substrate specificity, influence ligand binding, and alters catalytic efficiency

of HDAC8 (78). *In vitro* studies have shown that increasing phosphorylation of HDAC8 serine 39, via PKA, can lead to hyperacetylation of histone H3 and H4 (77). However, to this end histones are not considered significant substrates of HDAC8. As the role of HDAC8 in histone deacetylation has only been described *in vitro*. Non-biased acetylome studies and computer predicted modeling of HDAC8 substrates have identified several novel HDAC8 non-histone protein substrates (79-81).

Class II's major role is to regulate transcription across multiple cell types and is separated into IIa and IIb. Class IIa contains HDAC4, HDAC5, HDAC7, and HDAC9. HDAC6 and HDAC10 are class IIb HDAC proteins. HDAC4 is critical for fetal bone development during embryogenesis, and knockout of HDAC4 in mice causes severe skeletal abnormalities (82). HDAC4 represses endochondral bone formation by suppressing RUNX family transcription factor 2 (RUNX2) (82). In breast cancer, HDAC5 is upregulated and was found to promote stability of the lysine-specific demethylase 1 (LSD1) protein through deacetylation of LSD1, which promoted tumor progression (83). Short interfering RNA mediated knockdown of HDAC5 led to Ubiquitin Specific Peptidase 28 (USP28) dependent ubiquitination of LSD1, resulting in protein degradation. This was associated with an increase in the nuclear level of H3K9ace and H3K4me2 PTMs (83). In triple negative breast cancer, HDAC9 overexpression was associated with pro-tumor attributes, facilitating invasion and angiogenic properties (84). HDAC9 represses micro-RNA miR-206 expression, which results in increased vascular growth factor (VEGF) and mitogen-activated protein kinase 3 (MAPK3) levels, promoting angiogenesis and tumor growth (84). Enhanced HDAC7 activity is associated with poor prognosis in non-small cell lung cancer patients. HDAC7 causes upregulation of fibroblast growth factor 18 (FGF18), which promotes

proliferation and metastasis (85). The interaction of HDAC7 with β-catenin leads to lower levels of β-catenin acetylation, facilitating nuclear transport and increasing FGF18 expression (85).

In class IIb, HDAC6 regulates cell proliferation, invasion, and metastasis through deacetylation of heat shock protein 90 (HSP90), cortactin, and alpha tubulin. It also regulates expression of the checkpoint inhibitor PD-1, and is critical for KRAS Proto-oncogene, GTPase, (KRAS) oncogenic transformation (86). In CLL, blocking HDAC6 activity causes hyperacetylation of HSP90 and leads to a reduction in Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) activity (87). In the mouse model for CLL, euTCL1 (88), sequential dual targeting treatment, HDAC6 inhibitor (ACY738) followed by anti-PD-1 or anti-PD-L1, demonstrated an enhanced efficacy compared to the stand alone treatment groups (87). Programmed cell death 1 (PD-1) receptor is an immune checkpoint inhibitory receptor involved in immunosuppression and is expressed on immune cells to dampen activity. Programmed cell Death 1 Ligand (PD-L1) is often found overexpressed on tumor cells and suppresses immune cell activation. HDAC6 positively regulates PD-1 expression, and blocking activity with ACY738 or genetic knockout of HDAC6 causes down-regulation of PD-1 (87, 89). Clinically, this suggests that selective targeting of the immune checkpoint PD-1 and HDAC6 could prevent immunosuppression and enhance tumor regression. HDAC10 has been indicated as having tumor suppressor function. In a KRAS-driven lung adenocarcinoma model, HDAC10 knockout significantly increased SOX9 expression and increased stem cell properties (90).

HDAC11 belongs to class IV and has been indicated to play a role in regulating the JAK/STAT pathway in myeloid progenitor neoplasms (MPN) with the $JAK2^{V617F}$ mutation; however, it is unknown if it is through direct or indirect targeting (91). In hepatocellular

carcinoma, blocking HDAC11 activity leads to downregulation of Kallikrein B (Fletcher Factor) 1 (KLKB1) protein and lowered glycolysis (66).

### 1.2.2 Clinical Application using Broad-Spectrum Histone Deacetylase Inhibitors to Treat Hematological Malignancies

HDAC inhibitors (HDACi) became an attractive therapeutic in the early 2000s when they were shown to induce apoptosis of NHL and T cell leukemia (92, 93). HDAC inhibitors are classified by their structure. Class selective and broad-spectrum HDACi's include: the Hydroxamic acids; Vorinostat (59), Trichostatin (TSA), Dacinostat (LAQ824), Panobinostat (LBH589), and Belinostat (PXD101); the Carboxylic acid (short-chain fatty acids), Valproic Acid (VPA) and Butyric acid; the Cyclic depsipeptides class I HDAC inhibitors Romidepsin (FK228) and largazole; and Benzamide class I and IV specific inhibitor Entinostat **(Figure 1.3)**. Vorinostat is a hydroximic acid inhibitor targeting class I and II HDAC proteins, with an $IC_{50}$ <86nM *in vitro*. MCL cell lines, acute T cell lymphoma (ATL), and cutaneous T cell lymphoma (CTCL) isolated from patients are sensitive to vorinostat treatment. Vorinostat prevents proliferation in part by reducing STAT6 phosphorylation, upregulating Cycling Dependent Kinase Inhibitor 1A (CDKN1A) and NFκB, causing cell cycle arrest (92, 94). In 2006, vorinostat was approved for treating cutaneous T cell lymphoma (95-97). In clinical trials, relapsed/refractory CTCL patients treated with vorinostat, responded with an overall response rate of less than 30% with a greater than four-month duration of response (97).

#### 1.2.2.1 Broad-Spectrum HDAC Inhibitors Dampen NK Activity, While Selectively Targeting Class I HDACs Enhance NK Anti-Tumor Responses

The following broad-spectrum HDAC inhibitors were FDA approved to treat peripheral and cutaneous T cell lymphoma: belinostat, vorinostat (59), and romidepsin (FK228) (96, 98, 99). Vorinostat and trichostatin (TSA) have been shown *in vitro* to decrease cyclin D1 and induce apoptosis in lymphomas (100, 101). VPA and romidepsin increase CD20 antigen on low expressing tumor cells; CD20 is present on many NHLs (62). VPA and vorinostat demonstrated an additive effect in preclinical models in combination with rituximab, an anti-CD20 monoclonal antibody. However, broad-spectrum HDAC inhibitors can pose a serious health risk to the patient, causing a myriad of toxicity issues, including thrombocytopenia, tumor lysis syndrome, neutropenia, cardiotoxicities, anemia, and adverse neurological and gastrointestinal issues (102, 103). Furthermore, sodium butyrate (104), VPA, and TSA negatively influence natural killer cell survival and cytolytic function by downregulating the cytolytic receptors NKG2D, NKp44, and NKp46 (105, 106). Broad-spectrum HDAC inhibitors can potentially dampen clinical efficacy of immunotherapy. Therefore, it is critical to understand how HDAC inhibitors influence immune cell function, especially in patients who undergo antibody immunotherapy dependent on NK ADCC for efficacy.

There have been significant advancements in the HDAC field since the discovery that broad-spectrum HDAC inhibitors have efficacy across multiple NHL, T cell lymphoma, and solid tumor cell lines. Since then, HDAC inhibitors have been specifically designed to select HDAC proteins to avoid the unwanted toxicity issues associated with broad-spectrum HDAC inhibitors. The class I selective HDAC inhibitor, Entinostat, enhances NK function by increasing NKG2D on NK cells and stress ligands on tumor cells (61).

HDAC8, a class I HDAC enzyme, is involved in survival of T cell leukemia. Inhibiting HDAC8 activity through the small molecule inhibitor, PCI-34051, induces apoptosis of T cell leukemia/lymphoma cell lines (107). HDAC8 is also critical for the survival of several mantle cell lymphoma (MCL) cell lines. Blocking HDAC8 activity in MCL cell lines induces apoptosis, preserves NK antibody-dependent cellular cytotoxicity, and increases the abundance of $IFN\gamma^+$ NK cells (ADCC) (71).

## 1.3 Clinical Implications for Targeting Histone Deacetylase 8 in Cancer

HDAC8 gene is a sex-linked gene located on the X chromosome q13.1 and encodes a 377 amino-acid protein (108). HDAC8 shuttles between the nucleus and cytoplasm and is ubiquitously expressed across many tissues (108, 109). Interestingly, in melanoma, HDAC8 is found localized in the cytoplasm, and expression of both HDAC3 and HDAC8 was associated with improved survival with metastatic disease (110). However, HDAC8 activity in melanoma contributes to B-Raf proto-oncogene, serine/threonine kinase (BRAF) resistance (111). HDAC8 inhibition and knockout sensitizes the cells to BRAF inhibitor treatment. Cytoplasmic localization has also been indicated in smooth muscle tissue, indicating subcellular localization appears to be important for both normal and tumor cell survival (112).

Histone deacetylases are regulated by phosphorylation, either facilitating or preventing enzymatic activity. For instance, the protein kinase casein kinase II (CK2) phosphorylates HDAC1 and HDAC2, which promotes enzymatic activity (113, 114). However, CK2 does not regulate HDAC8 activity, instead the cAMP dependent protein kinase A (PKA) negatively regulates activity by phosphorylating serine 39 (64, 115). Active HDAC8 regulates an array of cellular processes, including energy homeostasis, the cohesion acetylation cycle, muscle

contraction, neurite differentiation, skull formation during embryogenesis, and microtubule integrity (116-120). Thus, HDAC8 is a key regulator of cellular homeostasis; however, constitutive deacetylase activity can negatively impact cellular health and contribute to progressive malignancy.

The best characterized HDAC8 substrate, structural maintenance of chromosome 3 (SMC3), was discovered in patients with Cornelia de Lange syndrome, which is caused by *de novo* mutations and/or deletions in any of the four genes involved in the cohesion acetylation cycle: HDAC8, SMC1A, SMC3 or/and the RAD21 cohesin complex component (RAD21) (119). The disease is associated with malformations to the facial structure in children and contributes to intellectual disabilities due to the disruption in the cohesion acetylation cycle during early mitosis (120-122). HDAC8 controls the development of the cranial neural crest in mice during embryogenesis, and global deletion is embryonically lethal due to skull instability (123). In the normal cellular context, HDAC8 plays a key role in maintaining cellular homeostasis.

HDAC8 facilitates dissemination and tumor development in colorectal cancer by negatively regulating interferon regulatory factor 1 (IRF1). HDAC8 negatively regulates IRF1, which causes overexpression of succinate receptor 1 (SUCNR1) and prevents autophagy (124). Childhood neuroblastoma is an aggressive cancer, and HDAC8 overexpression correlates with poor clinical outcomes (75). Selective inhibition of HDAC8, using PCI-34051, enhances retinoic acid-mediated differentiation of neuroblastoma cells, indicated by the formation of neurite-like structures, which induces apoptosis in tumor cells (118, 125). Expression of HDAC8 in neural crest derived neuroblastoma correlates with advanced disease and poor outcome (125). Moreover, in a preclinical neuroblastoma model, HDAC8 inhibitors reduce tumor burden and exhibit a lower toxicity profile and higher efficacy compared to broad-spectrum inhibitors (126).

In AML, HDAC8 represses acute myeloid leukemia-1 regulated genes, such as p21, by binding to the inv(16) fusion protein (55). The chromosome inversion inv(16)(p13q22) results in expression of the fusion oncogene CBFβ-SMMHC (CM). HDAC8 and TP53 proteins are brought in close proximity to each other, bound to the CM inversion protein, which results in constitutive deacetylation of TP53 and renders it inactive (55). Furthermore, HDAC8 also regulates Leukemic Stem Cell (LSC) growth. Deletion or inhibition of HDAC8 attenuates LSC growth through reactivation of the tumor suppressor protein p53. This indicates that HDAC8 may play a role in maintenance of stem cell hematopoiesis during malignancy. Blocking HDAC8 activity in myeloproliferative neoplasms led to SOCS 1/3 dependent reduction in cell growth (127). Other HDAC proteins are also involved in deacetylation of TP53, such as, HDAC1 which is involved in TP53 protein stability and degradation .

HDAC8 contributes to epithelial-mesenchymal transition (EMT) in breast cancer, increasing cancer invasiveness (128). Association of HDAC8 with AKT serine/threonine kinase 1 (AKT1) leads to deacetylation of the lysine 426 residue located on the C-terminus of the AKT1 protein. This increases the activity of AKT1 and promotes GSK-3 beta phosphorylation on Serine 9, thus potentiating AKT/GSK-3beta/SNAIL Family Transcriptional Repressor (SNAIL) activation and breast cancer metastasis through EMT (128).

As indicated above, aberrant HDAC8 expression has been described across multiple tumor types. Understanding the dynamic role of HDAC8's oncogenic contributions will open up a new path forward to enable scientists and physicians, to define B cell neoplasms that will likely have sensitivity to HDAC8 inhibitors. Its highlighted role in malignancy makes it an attractive therapeutic in the treatment of various types of cancer, including NHLs. In this study, HDAC8 was discovered to play a critical role in MCL survival (71). Disrupting HDAC8 activity through

the selective small molecule inhibitor, PCI-34051, and/or genetic knockdown induces apoptosis of MCL, T cell lymphoma, and leukemia cells (71, 107, 111).

### 1.3.1 Unique Specificity of PCI-34051 to Histone Deacetylase 8

HDAC8 was the first HDAC to be co-crystalized with vorinostat and TSA (64). Providing structural insight into the regulatory mechanism of action and was used to predict the binding site of Vorinostat (59) and TSA in other HDAC proteins. Broad-spectrum HDAC inhibitors contain a hydroxamate moiety that binds to the catalytic $Zn^{2+}$ binding domain, a conserved site within the HDAC family (64). Interestingly, the L1 loop on HDAC8, Serine 30 to Lysine 36, has a unique structure, enabling high flexibility, thus increasing the potential for a variety of substrates to bind to the HDAC8 enzymatic pocket (129). Other HDAC proteins do not have this flexibility, which is attributed to the steric hindrance between the L1 loop and L6 loop. The loop is in a fixed closed position, limiting flexibility. Phosphorylation of Serine 39, located on the C-terminal, disrupts the L1 loop's open confirmation, restricting access to the active site within the binding pocket of HDAC8 and consequently blocking activity. In 2018, Romier's group reported the co-crystal structure of the HDAC8 inhibitor, PCI-34051, bound within a unique selective pocket of HDAC8, not found on other HDAC proteins (129). PCI-34051 interacts with the catalytic zinc within the active site and residues from the L1 and L6 loops contained within the HDAC8 selective binding pocket. Indicating this indole-based derivative is unique and highly selective for HDAC8 with an $IC_{50}$ of 10nM *in vitro* and 200-fold selectivity to HDAC8 compared to other HDAC proteins (107). PCI-34051 has shown activity against the T cell lymphoma cell line, Jurkat, with a $GI_{50}$ (Growth Inhibition) of 11μM within 72hrs of treatment (107)

### 1.3.2 Non-Histone Substrates of Histone Deacetylase 8

HDAC8 is not considered a bona fide histone deacetylase due to the underlying caveats of overexpression studies, which may have altered normal deacetylase activity and lacking genomic knockdown and/or inhibitors studies targeting HDAC8 *in vivo*. Overexpression studies of HDAC8 showed lower levels of histone acetylation on the core histone proteins H3 and H4 and *in vitro* on peptides of histone tails, indicating overexpression can cause deacetylation of histones (108, 109, 115). Chromatin immunoprecipitation (CHIP) experiments identified HDAC8 prefers to deacetylate histone H3 proteins (59). However, targeting HDAC8 activity *in vivo* failed to show hyperacetylation of histone proteins (81, 130). This suggests HDAC8's primary function may be to regulate non-histone substrates, consistent with the profile of HDAC8 targets identified by Choudhary and Scholz, discussed below. Additionally, evidence suggests HDAC8 can act as a scaffolding protein to recruit and promote oncogenesis by inactivating key cell cycle regulators, including p53 (55). HDAC8 can also act as scaffolding platform for CREB and Protein Phosphatase 1 (PP1) (131). This interaction was accompanied by increased PP1 activity, suppressing the transcriptional function of the Cyclic AMP-Responsive Element-Binding protein (CREB) (131).

In 2009, Choudhary and et. al. published a paper that provided evidence that HDAC proteins regulate more than just histone proteins (132, 133). Using an unbiased, mass spectrometry-based approach involving stable isotope labeling using amino acids in cell culture (SILAC), 1,750 proteins with over 3,600 acetylated sites were identified. These included a number of key proteins critical DNA replication, cell cycle DNA damage repair, cytoskeleton reorganization, and RNA splicing. To ensure reproducibility, 3 cell lines (A549, MV4-11, and Jurkat) were labeled and investigated for acetylome changes during treatment with broad-

spectrum HDAC inhibitors, Vorinostat or MS-275. This work provided the foundation for future studies which use selective HDAC inhibitors to determine substrate specificity.

Subsequent to the Chaudhary study, in 2014 Scholz et al. published additional unbiased screening of HDAC targets using HDAC inhibitors to map hyperacetylated proteins and identify novel substrates (130). The following top three observed with the following HDAC and Sirtuin inhibitors produced the highest abundance of acetylated proteins: Tenovin-6 (class III specific), Bufexamac (class IIb HDAC6 and HDAC10 specific), and Nicotinamide (sirtuin 2 specific). While VPA (targets class I HDAC1 and induces proteasomal degradation of HDAC2), Sirtinol (selectively targeting SIRT2), and PCI34051 (class I HDAC8 selective inhibitor) provided the lowest abundance of acetylated proteins. Thus, they concluded HDAC8 as having reduced substrates compared to other HDACs. However, the conclusions are limited by the use of a restricted number of cell lines in the study, as HDACs are known to be differentially regulated across various cell and tumor types. Direct on-target validation through HDAC specific knockdown and with inhibitor studies will be critical to define the spectrum of HDAC8 functional targets.

Alame et al. identified HDAC8 substrates using an *in-silico* based method called Rosetta FlexPepBind (80). Peptide sequences were extracted from proteins with known acetylation sites and used to identify novel HDAC8 substrates (80). The algorithm was calibrated using previously characterized substrates to predict $Zn^{2+}$ binding proteins. This data provides further evidence that HDAC8s primary role is to regulate cellular homeostasis through non-histone regulation. The following proteins: Zinc finger protein 318 (ZNF318), elongation factor 1 alpha 1 (EF1α), structural maintenance of chromosomes 1A (SMC1A), interferon regulatory factor 2 (IRF2), aldehyde dehydrogenase III (ALDHIII), 60S ribosomal protein L7, and nuclear receptor

corepressor 2 (N-CoR2), had some of the strongest peptide substrate interactions. This indicates that HDAC8 may play a role in regulating transcription, cohesion during cell cycle, DNA damage repair, and metabolic stress. The newly identified substrates discovered in this study still need to be validated through *in vitro* and *in* vivo studies.

Olson et al. published a study in 2014 revealing an abundance of potential HDAC8 substrates, mostly involving regulation of proteins involved in cellular homeostasis. Mass-spectrometry revealed a diverse array of novel HDAC8 substrates through enrichment for acetylated proteins by selective and class specific HDAC inhibition (81). Protein substrates were identified through peptide "sequencing" and acetylated residues mapped using stringent cutoff values, $k_{cat}/K_{M,}$ within a factor of 2 or higher than the reference peptide, lysine 106, on SMC3, the previously identified HDAC8 substrate (81). The substrates identified include the cysteine rich protein 2 binding protein (CSRP2BP); the nuclear receptor coactivator 3 (NCOA3); the AT-rich interactive domain-containing protein 1A (ARID1A), a member of the SWI/SNF family; and the tumor suppressor protein histone-lysine N-methyltransferase 2D (MLL2) (81). These proteins all have unique roles in transcriptional gene regulation, providing further evidence for HDAC8 being involved in regulating transcription.

Together these studies have indicated many novel substrate candidates; however, only a small number of substrates have been identified for HDAC8 *in vitro* and *in vivo*. Experimentally defined HDAC8 substrates include estrogen-related receptor alpha (ERRα), jun proto-oncogene (cJun), p53, inv (16) fusion protein, cortactin, and SMC3 (111, 116, 117, 134). ERRα is acetylated by SIRT1 and HDAC8 (117). Deacetylation of ERRα enhances DNA binding activity; conversely, acetylation by P300/CBP-associated factor (PCAF), on lysine residues 266 and 268, reduces DNA binding domain (DBD) activity (117). SIRT1 and HDAC8 were shown to enhance

transcriptional function of ERRα. These findings suggest that HDAC8 is involved in regulating cellular metabolism. In melanoma, high HDAC8 activity leads to deacetylation of Jun proto-oncogene promoting constitutive phosphorylation thus enhancing transcription (111). This causes EGFR activity to increase, resulting in enhanced Ras/Raf/Erk signaling, propagating survival and tumor invasiveness (111). Increased activity of HDAC8 was associated with BRAF and BRAF/mitogen activated protein kinase (MEK) inhibitor resistance in melanoma cells, indicating HDAC8 activity is involved in drug resistance. Targeting HDAC8 sensitizes BRAF resistant melanoma cells to BRAFi treatment. Underscoring the importance of deciphering the dynamic interplay that HDAC8 deacetylation has on facilitating drug resistance.

### 1.4 Natural Killer Cells Ontogenesis, Self-Tolerance, and Maturation

Natural killer (NK) cells serve as our frontline of defense against pathogens and transformed cells. They are cells of the innate immune system with unique memory-like characteristics. NK progenitors are derived from an irreversible commitment of common lymphoid progenitor CLP's, within the bone marrow (BM), into the NK lineage and are marked by expression of CD122 (IL-2Rβ).

During stage 2-3, immature NK cells are educated through HLA class I molecules to recognize "self" and acquire self-tolerance (135). Licensing occurs through recognition of HLA-I class molecules on surrounding cells through the NK receptor plethora of killer-cell immunoglobulin-like receptors (KIRs). Upon binding to HLA class I molecules, the KIR's immunoreceptor tyrosine-based inhibitory motif (ITIMs) transduces intracellular signaling and activates phosphatases to induce self-tolerance. During maturation, NK cells acquire expression of killer immunoglobulin-like receptors (KIRs), which are essential for mitigating NK

cytotoxicity hyper-response. KIR receptors are required for educating NK cells to recognize "self" from "non-self" through the interaction with human leukocyte antigen (136), a process known as "licensing" (137). The "missing-self" response is a critical function of NK cells. For example, malignant cells often downregulate HLA class I molecules to evade T cell recognition. NK cells compensate by recognizing cells that lack HLA class I expression, which triggers robust cytolytic activity (135).

Stage 4-5 constitutes the transition into the $CD56^{bright}$ NK cell phenotype, with an intermediate NK cell phenotype called the unconventional NK cell (ucNK) expressing $CD56^{dim}$ and lacking $CD16^{-}$. This intermediate NK cell subtype is considered to have the highest cytolytic capability of all the NK subtypes, expressing several natural killer receptors (NKRs), lytic granzyme, and perforin, and high expression of NKG2A/CD94. While ucNK cells, $CD56^{dim}CD16$, have powerful cytolytic potential, their anti-tumor effects are limited due to the low abundance of this subtype in peripheral blood (PB). There are two major NK cell subsets within the human body, $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$. The $CD56^{bright}CD16^{-}$ NK subtype's main role is to secrete pro-inflammatory cytokines to prime the recruitment of other immune cells within the surrounding tissues and are primarily found in the secondary lymphoid tissues (SLT), such as the lymph nodes (138). Majority of cytotoxic Natural Killer cells, $CD56^{dim}CD16^{+}$, reside within the PB and make up 7-10% of leukocytes within the PB. They have an intrinsic ability to lysis tumor target cells and mediate robust cytolysis of antibody coated tumor targets after cytokine stimulation. The NK cell subsets, $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$, differ by their functional response to stimulation and homing capability (139, 140). The $CD56^{bright}CD16^{-}$ subtype preferentially migrates to the secondary lymphoid organs, while the $CD56^{dim}CD16^{+}$ subtype migrates to an area of acute inflammation (139, 140). Some

evidence suggests that $CD56^{bright}CD16^{-}$ are the precursor to the $CD56^{dim}CD16^{+}$ subset. Cytokine stimulation causes differentiation of $CD56^{bright}CD16^{-}$ into effector cytotoxic cells, $CD56^{dim}CD16^{+}$.

### 1.4.1 Natural Killer Cell Receptors and Anti-Tumor Responses

NK cells have an array of sensors that allow them to distinguish between a transformed or infected cell versus a normal healthy cell. They lack clonogenic receptors and instead rely on an array of germline encoded inhibitory and activating receptors to recognize normal versus aberrant cells within their microenvironment. The inhibitory family of receptors include: the killer immunoglobulin-like receptors (KIRs), which contain an immunoreceptor tyrosine-based inhibition motif (ITIMs) responsible for recognizing HLA class I molecules; and NKG2A, a member of the NKG2 family which recognize the nonclassical class I HLA glycoprotein HLA-E. Tumor cells can evade NK activation by upregulating expression of HLA-E, thus NKG2A serves as an immune checkpoint on NK cells (141).

Germline encoded NK activating receptors (NKRs) include the natural cytotoxic receptors (NCRs) NKp30, NKp44, and NKp46; the killer cell lectin like receptor 2 (NKG2) family NKG2D and NKG2C; and the low affinity Fc binding receptor FcγRIIIα, CD16, a critical mediator of antibody-dependent cellular cytotoxicity (ADCC). These receptors lack kinase signaling and require adaptor molecules with immunoreceptor tyrosine-based activation motifs (ITAMs) for activation. The following adaptor molecules are required for NKR activity: CD3ζ, FcεRIγ, and DNAX-activation protein 12 (DAP12). NKG2D recognizes the stress signals HLA class I polypeptide-related sequence A/B (MICA/B) and NKG2D ligand UL binding proteins (ULBP) from transformed or infected cells. In human NK cells, NKGD2 uniquely signals

through the adaptor protein DAP10 (142-145) containing an ITAM motif. Activating KIRs also rely on adaptor molecules for signal transduction, such as Killer cell Immunoglobulin like Receptor, Two Ig Domains, and Short cytoplasmic tail 2 (KIR2DS), which contains a charged lysine residue that associates with the signaling adaptor DAP12 for activity (146, 147). KIR2DS receptors have short cytoplasmic tails that lack ITIM motifs on long cytoplasmic tails of inhibitory KIRs. Thus, requiring adaptor molecules to initiate signaling.

### 1.4.2 Priming NK Cells for Cytolytic Response

NK cells are primed through engagement with $\gamma_c$ cytokines and the interleukin receptor (ILR) association with the subunit cluster of differentiation 132 (CD132). ILR's lack kinase activity and instead depend on receptor associated Janus Kinases (JAKs). JAKs phosphorylate and activate signal transducer and activator of transcription (STAT) proteins, mediating transcriptional activity important for NK functional responses and secretion of pro-inflammatory cytokines, interferon gamma (IFN$\gamma$), and tumor necrosis factor alpha (TNF$\alpha$). Interleukins IL-2, IL-12, and IL-15 are key mediators for NK induction of cytolytic granzyme and perforin production, and the proinflammatory cytokine secretion of IFN$\gamma$ and TNF$\alpha$. IL-12 and IL-15 play an integral role in NK maturation. While IL-2 is dispensable for NK cell maturation, it synergizes with IL-15 to produce potent cytotoxic granules and enhance pro-inflammatory cytokine secretion, thus playing an important role in NK cytolytic function. The heterodimeric association of the IL-2R$\alpha$ chain and IL-2R$\beta$ chain form the IL-2R. IL-2 is not required for NK development; however, it is important for NK homeostasis, cytokine production, proliferation, and survival. Both IL-2 and IL-15 promote survival by inducing expression of B-cell lymphocyte-2 (BCL-2), a protein involved in inhibiting apoptosis (148, 149). IL-2, combined

with IL-15 and IL-12, transduce signals through several receptor-associated Janus Kinases (JAK) to activate STATs and exert downstream transcriptional responses responsible for proliferation, cytokine secretion, activity, and survival (150). NK cells lack the high affinity IL-15Rα receptor, and instead IL-15 signals through the low affinity IL-15Rβ/IL-2Rβ/$\gamma_c$ receptor complex on NK cells. Stromal cells and antigen presenting cells stimulate NK cells via transmembrane presentation of IL-15. This initiates the recruitment of JAK1 and JAK3, which activates STAT5 and STAT3 for maturation, proliferation, and activation.

### 1.4.3 The Clinical Importance of Natural Killer Cells in Immunotherapy

Natural killer cells play a vital role in keeping us healthy, free from infection and malignancy. They are equipped with a collection of receptor sensors capable of recognizing warning signals, indicating that cell health has been compromised. They can recognize antibody coated cells, unusually low HLA class I expression, and cellular stress signals. Natural Killer cell function is essential for clinical response to IgG1 mAb therapies. Rituximab, a CD20 directed antibody, induces several anti-tumor mechanisms, including complement-mediated cytotoxicity, antibody-dependent phagocytosis, and NK cell ADCC **(Figure 1.4)**. In 2000, the first report was published, making a direct connection between tumor regression and presence of functional NK cells in mice (151). Clinically, NK cell abnormalities are higher in cancer patients, and dysfunction is associated with an increased risk for cancer (152-154). NK cells and γδ T cells preferentially express the low affinity immunoglobulin gamma Fc region receptor III-B (FcγRIII, CD16). The Fc binding receptor, CD16, is responsible for recognizing the Fc region of antibodies, which initiates antibody-dependent cellular cytotoxicity. Rituximab is an IgG1 antibody, known to promote anti-tumor responses in an NK-dependent manner. Patients with

proportionally higher numbers of circulating NK cells are correlated with therapeutic response and favorable prognosis compared to patients with low circulating NK cell numbers (155, 156). In early-stage multiple myeloma (MM) patients exhibit an increase in circulating NK cells. However, in advanced disease, significantly lower numbers of NK cells are found circulating in the periphery. NK cells from these patients display a reduction in CD16 expression, which impairs ADCC function (157). Collectively, these studies have demonstrated the critical role of NK cells function in mAb therapies. Patients who have been heavily treated with chemotherapy usually have a compromised immune system and limited NK function. Thus it is preferential to treat MCL patients with Rituximab as a frontline therapy in combination with chemotherapy. However, therapeutic efficacy can be short-lived, and these patients are in critical need of non-chemotherapy-based therapies to increase efficacy and promote longer durable response rates.

Importantly, NK cells can acquire memory-like recall, requiring nanomolar levels of cytokine stimulation for activity upon second exposure and self-renewing abilities that could be pivotal in immunotherapeutic treatment of hematological malignancies. For instance, ligand receptor activation and cytokine stimulation can induce memory-like NK responses. Memory-like NK cells have a dynamic recall response that was first identified in mice (158). Cytokines IL-2 and IL-15 can induce cytokine memory-like NK cells, and re-exposure to diminutive doses of IL-2 or IL-15 induce potent cytolytic responses, robust expansion, and are associated with enrichment of IFN$\gamma^+$ NK cells (159).

Pre-activation of CD16 has been shown to promote NK memory-like cytotoxic responses. Re-stimulation of NK cells with Hodgkin's lymphoma and NHL target cell lines coated with the bispecific anti-CD30/CD16 antibody led to a remarkable increase in NK cell expansion, surface expression of CD25 (IL-2R$\alpha$) and CD132 ($\gamma_C$), cytotoxicity, and IFN$\gamma$ production (160).

Indicating that pre-activation of NK cells through the antibody dependent mechanism can induce NK cell memory-like recall responses. Priming NK cells through cytokine stimulation or through activation of CD16 by antibody coated target cells can induce a long-term memory-like NK phenotype which may be pivotal in providing long-term anti-tumor responses in patients treated with antibody therapies (161).

Broad-spectrum HDAC inhibitors have shown clinical efficacy by inducing apoptosis and cell cycle arrest in leukemia and lymphoma (93, 104). Other studies have indicated immune cell toxicity issues related to HDAC inhibition, which can impair NK anti-tumor cytolytic abilities and expansion. HDAC inhibitors can negatively impact immune cell anti-tumor responses during antibody therapy. Therefore, it is important to investigate if targeting HDAC8 in MCL will impact NK cell ADCC during Rituximab treatment. The focus of this dissertation was to investigate the functional consequence of selectively targeting HDAC8 in human primary NK cells. This study examined carefully whether the HDAC8 selective inhibitor PCI-34051 alters the expression of key receptors involved in NK anti-tumor responses, ADCC, survival, and expansion of human primary NK cells stimulated with the pro-inflammatory cytokines, IL-2, IL-12, and IL15.

Table I. Gene List of Frequently Mutated Genes Found in MCL Patients.
The list represented here is the most frequent somatic mutations and mutations related to clonal evolution pre- and post- drug therapy (14).

| Gene | Description |
|---|---|
| CCND1 | Cyclin D1 |
| RB1 | Retinoblastoma 1 |
| TP53 | Tumor Protein P53 |
| ATM | ATM Serine/Threonine Kinase |
| POT1 | Protection of Telomeres 1 |
| NOTCH1 | Notch Receptor 1 |
| NOTCH2 | Notch Receptor 1 |
| CARD11 | Caspase Recruitment Domain family member 11 |
| TRAF2 | Tumor necrosis Receptor Associated Factor 2 |
| CDKN2A | Cyclin Dependent Kinase inhibitor 2A |
| CDKN2B | Cyclin Dependent Kinase inhibitor 2B |
| BIRC3 | Baculoviral IAP Repeat Containing 3 |
| WHSC1 | Wolf-Hirchhorn Syndrome Candidate 1 |
| KMT2D | Lysine Methyltransferase 1 |
| MEF2B | Myocyte Enhancer Factor 2B |
| SMARCA1 | SWI/SNF Related Matrix-associated Actin-dependent Regulator of Chromosome, subfamily A, member 1 |
| SMARCB1 | SWI/SNF Related Matrix-associated Actin-dependent Regulator of Chromosome, subfamily B, member 1 |
| SAMHD1 | SAM and HD Domain Containing Deoxynucleoside Triphosphate Triphosphohydrolase 1 |
| HNRNPH1 | Heterogeneous Nuclear Ribonucleoprotein H1 |

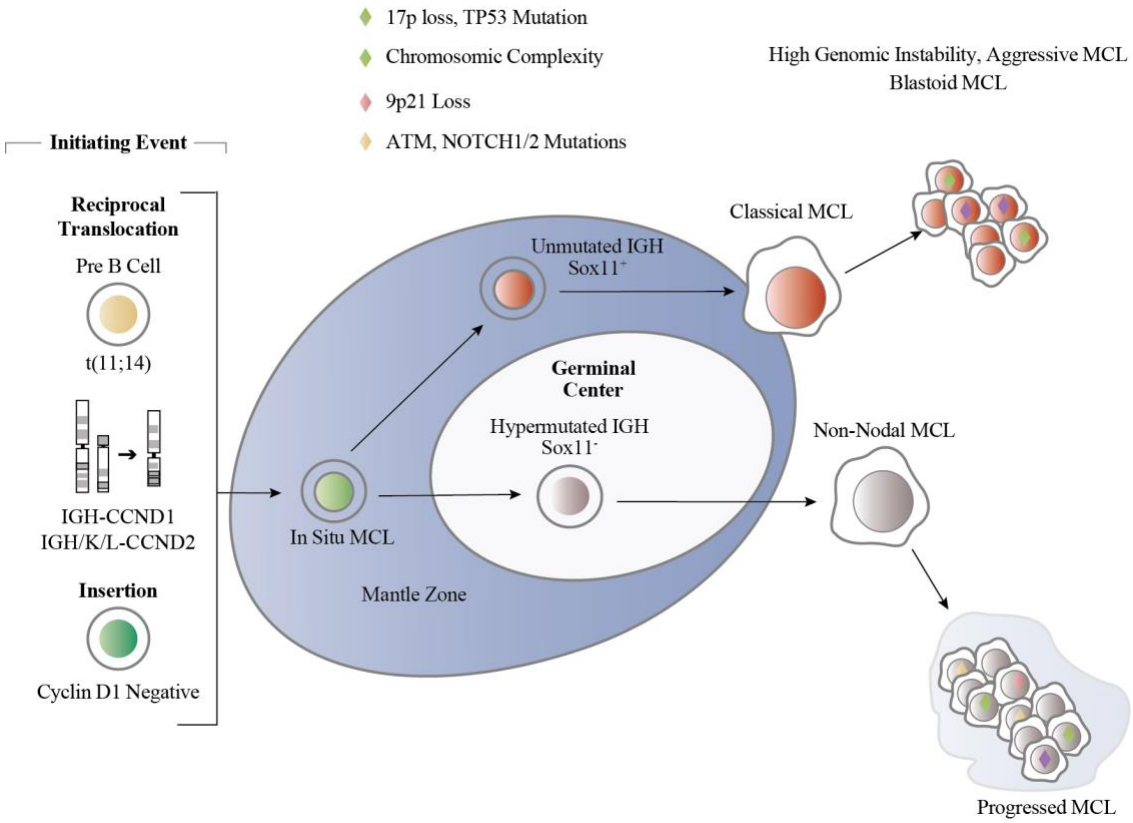


Figure 1.1. Mantle Cell Lymphoma Subtypes: Conventional MCL and Non-Nodal MCL.
Pro-oncogenic events occur early during Pre-B cell development, leading to in situ MCL development. Two major subtypes, classical (cMCL) and non-nodal (nnMCL), MCL arise at different stages of B cell development. cMCL originates in the mantle zone during germinal center reactions, has an unmutated IGH locus, and is $SOX^{+}$. nnMCL originates in the germinal center and is characterized by a hypermutated IGH locus and is $SOX11^{-}$. cMCL is aggressive, with high genomic instability, and is characterized as having a blastoid phenotype. Conversely, nnMCL has an indolent behavior *Adapted from Veloza and et al.* (13)
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Figure 1.2. Basic Structural Similarities Between the Classical HDAC Family Members.
HDACs are grouped into classes based on structural homology and similarities between the $Zn^{+}$ catalytic domains. Class I includes HDAC1-3, and 8; class IIa HDAC4, 5, 7, and 9; class IIb HDAC6 and 10; and class IV HDAC11. *Adapted from Seto et al. and Park et al. (57, 162).*

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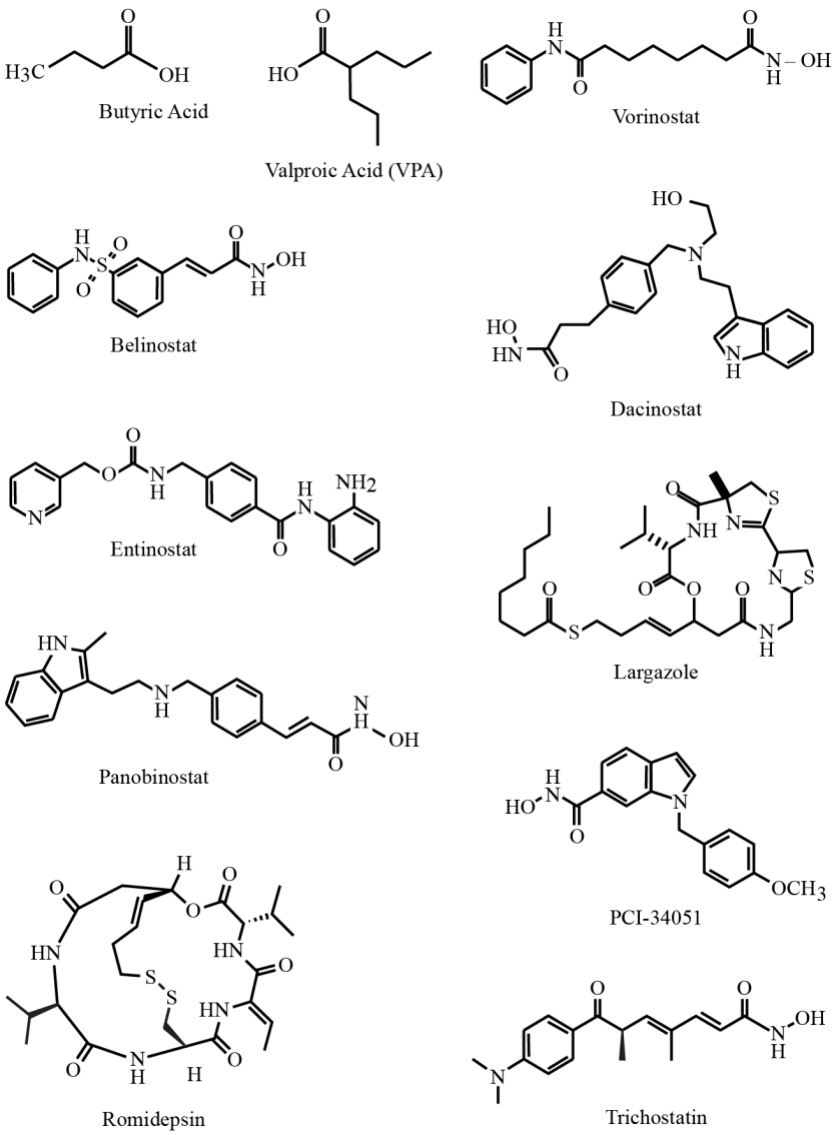


Figure 1.3. Chemical Structure of Broad-Spectrum and Class I Selective HDAC Inhibitors**.**
Hydroxamic acids: Vorinostat (59), Trichostatin (TSA), Dacinostat (LAQ824), Panobinostat (LBH589), PCI-34051, and Belinostat (PXD101): Carboxylic acid (short-chain fatty acids), VPA and Butyric acid; Cyclic depsipeptides class I HDAC inhibitors Romidepsin (FK228) and largazole; and Benzamide; the class specific I and IV HDACi Entinostat. *Adapted from Chun and et al. (102).*
.

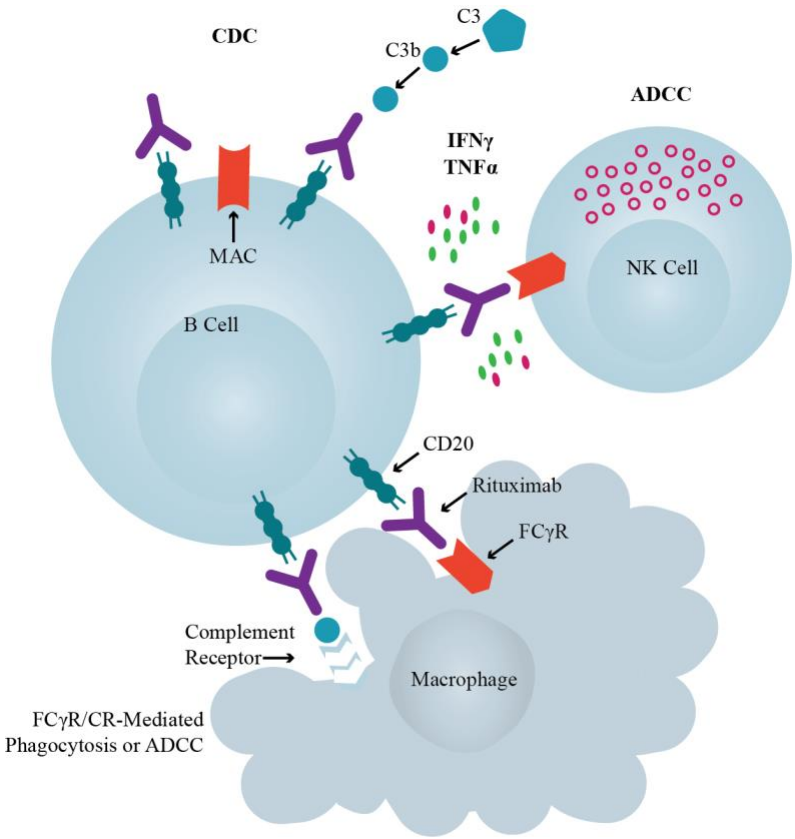


Figure 1.4. Rituximab: Mechanism of Action**.**
Antibody dependent cellular cytotoxicity and antibody dependent cellular phagocytosis is induced via CD16 binding to the Fc portion of the IgG1 anti-CD20 mAb. Rituximab can also induce complement mediated cytotoxicity.

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# CHAPTER II:

# MATERIALS AND METHODS

## 2.1 Cell Lines and Reagents

All cell lines: HEK-293T, Jeko-1, Maver-1, Mino, JVM-2, Rec-1, and Z138 were obtained directly from ATCC (Manassas, VA). Cell lines were grown either in Hyclone RPMI 1640 or DMEM media supplemented with 10% FBS (GE Healthcare Life Sciences, Pittsburgh, PA) and 1% penicillin-streptomycin in a 5% CO2 air incubator at 37oC

## 2.2 Isolation of Primary NK Cells from Peripheral Blood

Lymphocytes were isolated from human peripheral blood mononuclear cells (PBMCs) (One blood, St. Petersburg, FL) by Ficoll-Paque Plus density gradient centrifugation. Phosphate buffered saline (PBS) was used to dilute the blood 1:1 prior to layering with 3 ml Ficoll-Pague Plus (GE Healthcare Life Sciences, Pittsburgh, PA). The tubes were centrifuged at room temperature for 30 minutes at 830 x g with no brake. Lymphocytes were carefully extracted from the middle layer and washed 3 times with 50mls of PBS prior to NK isolation. Human primary NK cells were isolated using the EasySep Human NK Cell Enrichment kit by negative selection per the manufacturer's protocol (STEMCELL Technologies Inc., Vancouver, Canada). NK purity was verified by flow cytometry and routinely found to be >95% CD3- CD56+ CD16+. Isolated NK cells were grown in Stem Cell Growth media (SCGM) (CellGenix) supplemented with 10% FBS and 1% penicillin-streptomycin in a 5% CO2 air incubator at 37oC and

stimulated with interleukin 2 (IL-2) (100U/ml; PeproTech, Rocky Hill, NJ), interleukin 12 (IL-12) (20ng/ml; PeproTech, Rocky Hill, NJ), interleukin 15 (IL-15) (50ng/ml; PeproTech, Rocky Hill, NJ).

2.3 Cytokine Stimulation

NK cells were treated with PCI-34051 for 1 hour prior to being stimulated with IL-2 (100U/ml; PeproTech, Rocky Hill, NJ), IL-12 (20ng/ml; PeproTech, Rocky Hill, NJ), and IL-15 (100ng/ml; PeproTech, Rocky Hill, NJ) for the time indicated.

2.4 RNA Isolation and Quantitative mRNA Analysis

NK cells were rested (no cytokines) or stimulated with IL-2 (100U/ml; PeproTech, Rocky Hill, NJ), IL-12 (20ng/ml; PeproTech, Rocky Hill, NJ), IL-18 (100ng/ml; PeproTech, Rocky Hill, NJ) for 24 hours. RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA), per the manufacturer's instructions. RNA was converted to cDNA as previously described (25) and analyzed by quantitative PCR. Primers for HDAC analysis were generously provided by Dr. Edward Seto (George Washington University), **(Table 1)**.

2.5 Immunoblotting

Immunoblotting was done as previously described (163). Primary antibodies include: anti-HDAC8 (1:1000 dilution) (#17548-1-AP, Proteintech Group, Chicago, IL), anti-HDAC1 (1:1000)(ab7028, Abcam, Waltham, MA), anti-HDAC2 (1:1000)(ab219053, Abcam, Waltham, MA), β-actin (1:12,000 dilution) (#A5441, Sigma Aldrich, St. Louis, MO), GAPDH (1:5000 dilution)(#sc-69778, Santa Cruz Biotechnology), SMC3 (1:6000 dilution) (#A300-060A, Bethyl

Laboratories Inc., Montgomery, TX), acetyl SMC3 (1:1000 dilution) (Lys105/106) (#MABE1073, Millipore Sigma, Burlington, MA), phospho-Histone H2A.X (Ser139) (1:1000 dilution) (#2577, Cell Signaling Technology). Horseradish peroxidase conjugated secondary antibodies (HRP-α-mouse, 1:12,000) (HRP-α-Rabbit, 1:2000) (GE Healthcare Life Sciences, Pittsburgh, PA), and IRDye conjugated secondary antibodies (IRDye®680RD, 926-68071 and IRDye®800CW, 926-32210) (LI-COR Biotechnologies, Lincoln, NE).

2.6 Manufacturing of Lentiviral Particles for shRNA Delivery

Lentivirus particles were manufactured in HEK-293T cells using shRNA vectors and 3rd generation lentiviral packaging construct mixture (Applied Biological materials Inc., Richmond, Canada). The JetPRIME transfection reagent (Polyplus transfection, Illkirch, France) was used to transfect the HEK-293t cells with the 3rd generation lentiviral construct. The supernatant containing the lentiviral particles was collected at 48 hours and 72 hours post-transfection. Supernatant was subjected to ultracentrifugation, for 2 hours at 95,000 x g, at 4ºC in a SW32Ti rotor to pellet the virus particles. The viral particles were resuspended overnight prior to tittering.

2.7 Lentiviral shRNA-Mediated Knockdown

shRNA knockdown was performed using shRNA vectors MISSION® TRC pLKO.5-puro HDAC8shRNA (HDAC8sh-1, TRC0000350469; HDAC8sh-2, TRC0000314872) and MISSON® TRC2 pLKO.5-puro non-Mammalian control shRNA (NTsh; SHC202; Sigma Aldrich, St. Louis, MO). mCherry tagged shRNA vectors were generated by replacing the puromycin resistance gene with the mCherry gene from the pLVmCherry vector (Addgene,

Cambridge, MA). Lentivirus particles were produced and transduced as previously reported (164).

## 2.8 CellTiter Glo Luminescent Cell Viability Assay

NK cells isolated from healthy donors were treated with the vehicle control DMSO; PCI-34051 at 5μM, 10μM, 20μM; Vorinostat at 10μM, and LBH-584 at 0.5μM (#S2012, Selleckchem, Houston, TX) for 1 hour prior to IL-2, IL-12, and IL-15 cytokine addition and viability measured 72hr post inhibitor treatment. Lymphokine activated killer cells (LAKs) were generated from primary human NK cells by crosslinking NKp46 and CD2 using the MACiBead particles and biotinylated antibodies (165 130-094-483, miltenyl Biotec). Cells were cultured with high doses of IL-2 (500U/ml), activated, or rested with low dose IL-2 (100U/ul) for 24 hours. Activated and rested cells were treated with vehicle control DMSO, 10μM, or 20μM PCI-34051 for 72 hours post inhibitor treatment. The CellTiter-Glo viability assay was performed per manufacturer's instructions (Promega). Briefly, cells were mixed thoroughly, and 50uL was aliquoted in a clear bottom black 96 well plate and equilibrated to room temperature before mixing with an equal amount of Cell Titer Glo substrate reagent. Cells were lysed on a plate shaker for 2 minutes, then incubated at room temperature for an additional 10 minutes, protected from light. Luminescence signal was measured to detect intracellular ATP levels and recorded using a standard luminometer at 485-500nmEx/520-530nmEm.

## 2.9 ELISA Assays

From isolated primary human NK cells, supernatant was collected at 24 hours and IFNγ was measured using the human IFN gamma ELISA Ready-Set-Go kit per the manufacturer's

instructions (#88-7316, Affymetrix eBiosciences). ELISAs were performed in triplicate in 100µl volumes. Plates were read at 450nm on a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

## 2.10 NK Cytotoxicity and Antibody Dependent Cytotoxicity Assays

NK cells isolated by negative selection were cultured for 1 hour with 20µM PCI-34051 or DMSO prior to the addition of cytokines. Cells were stimulated with IL-2, IL-12, and IL-15 for 24 hours. After 24 hours, NK cells were washed and resuspended in RPMI with 20% FBS, NK cells were co-cultured with 5000 K562 cells or Jeko-1 cells at 1:10 effector to target (E:T) ratios. For antibody dependent cellular cytotoxicity (ADCC) experiments, NK cells were treated as described above and Jeko-1 cells were treated with 10µg of anti-CD20, Rituximab, for 1 hour (#A1049, BioVision, San Francisco, CA). Jeko-1 cells were washed two times to remove excess Rituximab and resuspended in RPMI supplemented with 20% FBS, plated, and killing activity was assessed 4hr post co-culture addition. All Chromium-51 release assays were performed as previously described (163).

## 2.11 Flow Cytometry Assays

Cell death was assessed by staining with BUV395-Annexin V (#564871) and propidium iodide (#556463) (BD Biosciences) and measured by flow cytometry. Staining was performed per the BD Biosciences instructions. For profiling NK cell activation and inhibitory receptors, the following primary fluorochrome-conjugated antibodies were used: BV510-anti-hNKp46 (#564064), PE-CF594-anti-hNKG2D (#562498), PerCP-Cy 5.5-anti-hCD94 (#562361), BV421-anti-hNKp30 (#563385), BUV395-anti-hCD16 (#563785), BB515-anti-hKIR2DL3 (#564678),

PE-anti-hNKp44 (#558563); all antibodies were purchased from BD Biosciences and stained per the manufacturer's instructions. Antibodies were titrated to determine optimal concentrations. To distinguish the live/dead cell populations, cells were stained with Zombie NIR fixable dye (#423106, BioLegend), and this was done prior to staining surface receptors per the manufacturer's protocol. For accurate gating, fluorescence minus one (FMO) control(s) were used for all experiments. For median fluorescent intensity (138) consistency, Rainbow Fluorescent Particles, 3.0-3.4μm (mid-range FL1 fluorescence) (Sphere, BD Biosciences) were used to set up voltages on the LSRII prior to running samples for acquisition of NK receptors. For compensation of each monoclonal antibody, either cells or BD compensation beads (#552843, BD Biosciences) were used at the same concentration as the experimental samples. To evaluate intracellular IFNγ the following monoclonal antibody conjugates were used; PE-CF594-IFNγ (#562392, BD Biosciences) or BD FastImmune anti-IFNγ (#340449, BD Biosciences) and stained per the manufacturer's protocol. Flow cytometric detection was performed at the Flow Cytometry Core Facility at Moffitt Cancer Center and Research Institute using the LSRII (BD Biosciences, San Jose, CA). For each experiment, the FlowJo V10 cytometry software was used to determine MFI and percent of cell expressing the protein of interest.

### 2.12 Caspase 3/7 Apoptosis Assay

The fluorogenic substrate, Incucyte® Caspase-3/7 Reagent (#4440), which is a four-amino acid peptide (DEVD) conjugated to a nucleic acid-binding dye was used to monitor apoptosis over time per the manufacturer's instructions (Essen Biosciences, Ann Arbor, Michigan). MCL cell lines were plated in a Celigo Imaging Cytometer compatible flat clear bottom black 96-well plate, 1x10^4 cells were seeded per well, and 5μM Caspase-3/7 Reagent

was added to monitor apoptosis in the Celigo Cytometer according to the manufacturer's instructions (Nexcelom Biosciences, Lawrence, MA).

### 2.13 PKA Activity Assay

MCL cell lines were treated with PCI-34051 for 24hrs. The cells were collected, and protein harvested as previously described. The lysate was analyzed for PKA activity. All procedures were done as described in the PKA kinase activity assay kit protocol supplied by the manufacturer (ab139435, Abcam Waltham, MA).

### 2.14 Statistical Analyses

Two-tailed paired t-test was used for statistical analyses; p values less than 0.05 was considered significant. The p-values were indicated as following: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.00001$. The calculations were performed in Microsoft Office Excel (Microsoft, Redmond, WA).

Table II. Primer Sequences for qPCR.
HDAC mRNA levels were examined, using the primers indicated above, through quantitative RT-PCR. *Primers pairs were generously provided by Edward Seto.*

| Primer | Sequence |
|---|---|
| HDAC1-RT-F | 5’-CGAATCCGCATGACTCATAA -3’ |
| HDAC1-RT-R | 5’-CATCTCCTCAGCATTGGCTT -3’ |
| HDAC2-RT-F | 5’-ATGGCGTACAGTCAAGGAGG -3’ |
| HDAC2-RT-R | 5’-ATGAGGCTTCATGGGATGAC -3’ |
| HDAC3-RT-F | 5’-GCAAGGCTTCACCAAGAGTC -3’ |
| HDAC3-RT-R | 5’-CTGTGTAACGCGAGCAGAAC -3’ |
| HDAC4-RT-F | 5’-CGTGGAAATTTTGAGCCATT -3’ |
| HDAC4-RT-R | 5’-CTGGTCTCGGCCAGAAAGT -3’ |
| HDAC5-RT-F | 5’-GGGAACCATCCTTGGAAATC -3’ |
| HDAC5-RT-R | 5’-GAACTGGGCATGGCTCTTG -3’ |
| HDAC6-RT-F | 5’-CCGGAGGGTCCTTATCGTAG -3’ |
| HDAC6-RT-R | 5’-GCGGTGGATGGAGAAATAGA -3’ |
| HDAC7-RT-F | 5’-CTGCATTGGAGGAATGAAGCT -3’ |
| HDAC7-RT-R | 5’-CTGGCACAGCGGATGTTTG -3’ |
| HDAC8-RT-F | 5’-ATACTTGACCGGGGTCATCC -3’ |
| HDAC8-RT-R | 5’-GCGTGATTTCCAGCACATAA -3’ |
| HDAC9-RT-F | 5’- GTACAGAAGTAAAGCAGAAG -3’ |
| HDAC9-RT-R | 5’- AGAGCTTTGATCCAATGATG -3’ |
| HDAC10-RT-F | 5’-CTCGGCTTCACTGTCAACCT -3’ |
| HDAC10-RT-R | 5’-GTCAAATCCTGCCGAGACCA -3’ |
| HDAC11-RT-F | 5’-GCACACGAGGCGCTATCTTA -3’ |
| HDAC11-RT-R | 5’-AAGGAAGTTGGGGAGGAAGA -3’ |

# CHAPTER III:

# CYTOKINE STIMULATION INDUCES EXPRESSION OF HDAC8 IN PRIMARY HUMAN NATURAL KILLER CELLS

## 3.1 Introduction

NK anti-tumor responses are carefully orchestrated by a balance of inhibitory and activation receptor signaling. These receptors are germline encoded and not rearranged for specificity of their antigen target. The C-type lectin like receptors, NKG2, exists as a heterodimer with CD94 which is indispensable for activity. NKG2 receptors recognize HLA-E class I molecules. NKG2A has an inhibitory function, while NKG2C serves as an activation receptor. Both require CD94 for intracellular signaling. Therefore, CD94 surface expression was evaluated to determine if HDAC8 inhibition can alter signaling. NKG2D is activated by the non-classical HLA class I stress molecules (MICA/MICB and ULBP proteins) upregulated on tumor cells. The natural cytolytic receptors (NCRs): NKp30, NKp44, and NKp46, recognize specific tumor antigens. The low affinity Fc binding receptor, CD16 (FcγRIII), is indispensable for NK antibody-dependent cellular cytotoxicity (ADCC). Killer cell immunoglobulin-like receptor 2DL3, KIR2DL3, is an inhibitory receptor that recognizes human leukocyte antigen C alleles. Recognition of this ligand blocks NK cytotoxic responses through phosphatase activation.

Broad-spectrum HDAC inhibitors were originally discovered to cause upregulation of stress signals MICA/B and ULBPs proteins on tumor cells (166). These stress signals are pro-cytotoxic inducing ligands that signal through the NKG2D/DAP10 receptor/adaptor complex on

NK cells. This interaction causes the NK cells to release the potent program cell death cytotoxic enzymes, perforin, and granzyme. However, these studies only focused on the context of tumor cells, not the effect broad-spectrum inhibitors have on NK cell health and effector function. Later studies identified that broad-spectrum HDAC inhibitors, such as LBH-589, can impair primary NK effector functions by causing the loss of key activation receptors on the cell surface and inducing cell death (106).

Broadly, this study focused on identifying if HDAC8 activity is important for NK effector function. The findings in this study highlight HDAC8 as a therapeutic target in MCL and shows that blocking HDAC8 activity will not interfere with NK ADCC response. NK cells are resistant to HDAC8 inhibition compared to MCL cell lines. Exhaustive efforts were made to identify if blocking HDAC8 activity in primary human NK cells impacts their effector functions. This dissertation reports that while blocking HDAC8 in NK cells did not appear to enhance cytotoxicity, it caused an increase in the abundance of IFN$\gamma^+$ NK cells in a dose dependent manner.

## 3.2 Results

### 3.2.1 Cytokine Stimulation Enhances HDAC8 Expression in Human NK Cells

To determine if specific HDAC classes were differentially regulated after cytokine stimulation, NK cells were rested or treated with pro-inflammatory cytokines IL-2, IL-12, and IL-18 for 24 hours to drive up NK effector activity. Interestingly, the class I HDACs; HDAC1, HDAC2, and HDAC8, had a significant increase in expression in transcription, and this was validated at the protein level **(Figure 3.1A and 3.1B)**. At the protein level, HDAC3 expression increased in response to cytokine stimulation compared to *ex vivo* rested NK cells. These results

indicate that class I HDACs may influence NK activity. Conversely, the class II molecules; HDAC4, HDAC9, and HDAC10, are downregulated at the mRNA level. Indicating they are downregulated at the transcriptional and may have regulatory role in NK activation. However, at the protein level, there lacked a clear pattern of HDAC4 expression between donors, thus, suggesting that HDAC4 could be differentially regulated at the protein level in a donor dependent manner **(Figure 3.1B)**. HDAC9 and HDAC10 were not detected by immunoblot (IB). Interestingly, interleukin-2, -12, and -15 induced upregulation of HDAC5 60 hours post cytokine treatment, but not IL-2 alone **(Figure 3.1B)**. Cytokine stimulated NK cells had an increase in HDAC8 expression, which suggested that it may play an important role in NK activity. However, this was not the case, NK cells were highly resistant to PCI-34051, and an EC50 was never achieved even at the highest dose, of 20μM **(Figure 3.1C)**. Human Primary Natural Killer Cells, expanded *ex vivo*, remained viable post PCI-34051 treatment, even at the high dose. **(Figure 3.1D)**. Moreover, HDAC8 activity is not essential for NK proliferation **(Figure 3.1E)**. NK cells were labeled with CFSE, and proliferation was tracked over time. CFSE is diluted out after each cell division, thus the daughter cells express a reduction in CFSE, which is indicated by a subtle drop in MFI. This was monitored every 24 hours across three days to ensure NK cells were healthy during each CFSE assessment. The results indicated an equivalent loss in CFSE with every generation of NK cells compared to treated and untreated samples. Indicating HDAC8 activity is not required for NK cell proliferation. Conversely, the broad-spectrum HDAC inhibitor, LBH-589, was toxic to NK cells at low doses, 50nM **(Figure 3.1C and 3.1F)**. Entinostat and Vorinostat both have a higher selectivity for HDAC1 and HDAC3 than other HDAC proteins. However, at higher concentrations, these inhibitors may become promiscuous

and toxic to NK cell health, indicated by the sharp decline in viability between 500nM and 1000nM **(Figure 3.1F)**.

Lymphokine-activated killer cells (LAKs) were generated from NK cells negatively selected from healthy donor PBMCs to evaluate if HDAC8 activity is critical for NK expansion. This assay mimics *ex vivo* expansion through crosslinking of NKp46 and CD2. LAKs were expanded for 9 days, to reach exponential expansion. Nine days post activation, LAKs were separated into two groups; Rested—with low dose IL-2, 100U, to lower activity levels, or kept at—high dose IL-2, 500U, to achieve maximal activation for 24 hours prior to treatment conditions. Likewise, HDAC8 activity is not required for survival in NK cells that have been expanded *ex vivo* **(Figure 3.1D)**. This is an important finding that will direct preclinical translational studies targeting HDAC8 *in vivo*. One caveat to this study is, to date clinical investigation into selective inhibition of HDAC8 is lacking, and the clinically relevant dosage is unknown. What can be taken from these studies is that even at high doses, PCI-34051, does not impact NK viability or proliferation, compared to class I broad-spectrum HDAC inhibitors. Of note, selectively targeting HDAC8 in human primary NK cells results in a favorable outcome for NK cell survival **(Figure 3.1C – 3.1F)**. Evidence that blocking HDAC8 activity will not interfere with survival or expansion of NK cells during cytokine and antigen stimulated conditions.

Rituximab is a frontline treatment for MCL patients and is used as a maintenance therapy, highlighting the importance of investigating immunotherapy in combination with HDAC inhibitors. It is important to determine if HDAC inhibitors can synergistically enhance immunotherapeutic response, or if they dampen NK response to anti-CD20 directed therapy. For example, dual-targeting HDAC1 and HDAC2 diminishes NK cell effector function, suppressing NK cytolytic activity (167). Entinostat is a class I HDAC inhibitor that targets HDAC1 and

HDAC3 and has an IC50 of 0.51µM and 1.7µM, respectively. A study published by Zhu and et. al, reported that Entinostat enhances NK function through the upregulation of stress ligands on tumor cells and enhances expression of NKG2D on NK cells, synergistically enhancing efficacy (61). However, the study did not investigate if Entinostat affects NK antibody-dependent cytotoxicity, a critical function for clinical efficacy to antibody therapies.

### 3.2.2 NK Phenotype is Stable Post PCI-34051 Treatment

Broad-spectrum HDAC inhibitors can cause downregulation of NKG2D in NK cells. NKG2D is a potent activation receptor that responds to stress signals on tumor cells. Non-selective HDAC targeting, through small molecule inhibitors, can result in deficient NK anti-tumor responses. Contrary to, Entinostat, which has been shown to enhance NKG2D expression on NK cells as well as the stimulatory stress ligands on tumor cells, enhancing NK cytotoxicity. Therefore, it is important to investigate if HDAC8 influences expression of key receptors responsible for NK anti-tumor responses.

NK cells were treated with PCI-34051 at the indicated dose for 1 hour prior to being stimulated by the following cytokines in combination; IL-2, IL-12, and IL-15. To investigate if HDAC8 inhibition can alter expression of key anti-tumor receptors on NK cells, (KIR2DL3, NKG2D, CD94, NKp30, NKp44, NKp46, and CD16) cells were harvested 24h hours post cytokine stimulation, and stained, simultaneously, with a multicolor antibody cocktail mix. All NK receptors evaluated had stable expression levels post PCI-34051 treatment, maintaining equivalent levels of expression as the control treated cells **(Figure 3.2A)**. These results indicate expression levels of these receptors are unaltered and stable after treatment with PCI-34051.

A noteworthy observation suggested PCI-34051 may induce NK cell adhesion. NK cells stimulated with cytokines "cluster" naturally. PCI-34051, in combination with IL-2, IL-12, and IL-15, induced a 2-fold increase in "clustering" size **(Figure 3.2B)** and this prompted the evaluation of key receptors involved in adhesion signaling; CD2, LFA-1, and MAC-1. The cluster of differentiation 2 receptor, CD2, is important for NK activity and proliferation, adhesion signaling, and influences immune cell—cell crosstalk during antigen stimulation. PCI-34051 did not alter surface expression of CD2 **(Figure 3.2C)**. Integrin expression is critical for cellular trafficking to tissues. The β2 integrin receptor, Lymphocyte Function-Associated Antigen 1 (LFA-1), is important for properly forming the immune synapse (IS) during antigen recognition and rapid migration to lymph nodes. Mature NK cells circulating in the blood robustly express LFA-1. Another β2 integrin receptor, Macrophage-1 Antigen Alpha subunit (MAC-1), is also associated with cell migration; however, expression is tissue specific. As anticipated, 100% of peripheral NK cells express LFA-1. PCI-34051 did not alter the abundance of LFA-1 on the cell surface or NK cells expressing LFA-1 **(Figure 3.2C)**. These findings demonstrate that blocking HDAC8 activity in NK cells will not impair activity or interfere with immune synapse (IS) stability. NK cells treated with PCI-34051 express both LFA-1 and MAC-1 molecules, and levels remained unchanged between conditions **(Figure 3.2C)**.

Patients with elevated NK total counts within the peripheral blood are associated with higher response to Rituximab therapy. However, to date, there is a lack of evidence identifying unique NK phenotypic attributes that are important for clinical efficacy with targeted therapies.

One of the goals of this study was to determine if there were any NK phenotypic attributes unique to relapse/refractory MCL patients. However, due to the lower number of MCL patients compared to other NHL's and patients withdrawing from research associated blood

draws, the patient size is too small to draw any conclusions. Two of the MCL donors were relapse/refractory, and one newly diagnosed with MCL. To determine if MCL patients had any significant phenotypic alterations to NK activation receptor expression, all MCL patient data was pooled together and was compared to NK cells isolated from healthy donor PBMCs **(Figure 3.2D)**. The data indicates MCL patients have a lower percentage of NK cells expressing both NKp30 and NKp46 receptors than healthy donors. Furthermore, NKp30 surface expression is downregulated compared to healthy donor NK cells, however this was not found to be statistically significant and needs further evaluation, across multiple MCL patients. NKp30 recognizes B7-H6, an antigen commonly found upregulated on tumor cells, including T cell leukemia and B-cell lymphomas. However, as stated previously, the sample size is too small to determine any trends or correlations and requires further investigation in future studies.

### 3.2.3 PCI-34051 Increases the Abundance of IFNγ$^+$ NK Cells

To establish whether NK cells still retained their ability to execute anti-tumor cytokine response, intracellular levels of IFNγ and secretion were investigated. Interferon gamma is a major proinflammatory molecule secreted by cytokine stimulated and/or antigen activated NK cells. IFNγ is a potent influencer of anti-tumor responses from immune cells. For example, IFNγ activates dendritic cells, induces production of IL-12, and triggers differentiation of T cells (168, 169). It can also cause upregulation of TNF superfamily member 10 (TRAIL) and FAS Cell Surface Death Receptor (FAS) on cancer cells, sensitizing them to NK cytolysis.

Cytokine stimulated primary human NK cells were dosed at various concentrations of PCI-34051, and intracellular levels of IFNγ were investigated at the indicated time points **(Figure 3.3A)**. While there was no indication of enhanced intracellular levels, however, there

was a dose-dependent increase in the abundance of IFNγ$^+$ cells that steadily increased over 24 hours **(Figure 3.3A)**. To confirm these findings, an additional seven healthy NK donors were investigated at the highest dose, 20μM. As anticipated, the results confirm that PCI-34051 treatment induces a significant increase in the abundance of IFNγ$^+$ NK cells stimulated with IL-2, IL-12, and IL-15 **(Figure 3.3B)**. It is important to note secretion was not blocked in these experiments, and the secretion output of IFNγ was uniform, across 8-donors, between both the control and PCI-34051 treated NK cells **(Figure 3.3C)**. Furthermore, IFNγ$^+$ levels were similar between untreated and PCI-34051 treated groups. These results suggest that the increase in the abundance of IFNγ$^+$ NK cells is not due to retention of IFNγ$^+$.

To ensure NK cells treated with PCI-34051 do not retain IFNγ during co-culture, blocking and non-blocking conditions were performed. In blocking conditions, Brefeldin A and Monensin were used to prevent CD107a re-internalization and secretion of IFNγ. As anticipated, in the non-blocking condition, PCI-34051 significantly increased the abundance of IFNγ$^+$ NK cells **(Figure 3.3D)**. Under blocking conditions, there was no significant difference in the frequency of IFNγ$^+$ NK cells between the DMSO and PCI-34051 treated NK cells. The population of CD107a$^+$ NK cells did not significantly increase in either the blocking or non-blocking conditions.

These results confirm attenuation of HDAC8 activity does not cause retention of IFNγ, and NK cells are fully functional to elicit anti-tumor cytotoxic responses. The CD107a mobilization assay confirmed (under non-blocking and blocking conditions) HDAC8 activity does not dampen degranulation **(Figure 3.3D)**. Taken together, HDAC8 activity is not essential for NK cytotoxic function, degranulation, or cytokine secretion, illustrating HDAC8 inhibitors will not attenuate NK effector functions.

### 3.2.4 NK Cells Are Resistant to HDAC8 Inhibition and Retain Full Cytotoxic Function

Co-culture studies were performed to address whether the increase in IFNγ$^+$ NK cells results in enhanced cytotoxic responses. Tumor cells often evade T cell immune surveillance through downregulation of HLA class I molecules. On the contrary, NK cells play a major role in eliminating tumor cells through their ability to recognize a loss in HLA class I expression. Loss of HLA class I molecules or beta-2-microglobulin (B2M) on the cell surface sensitizes them to NK cytolysis. B2M shuttles the HLA class I molecules to the cell surface, thus it is critical for HLA class I expression. The chronic myeloid leukemia cell line, K562, is highly sensitive to NK killing due to lacking HLA class I molecules. This cell line also expresses MICA/B stress ligands which activate cytolytic responses through NKG2D receptor and its adaptor molecule, DAP10.

K562 cells were used to examine if PCI-34051 can enhance NK killing in an HLA class I independent manner. PCI-34051 did not significantly enhance NK cytotoxicity and this was observed across 8 healthy NK donors **(Figure 3.4A and 3.4C)**. Intracellular levels of granzyme B were examined to determine if blocking HDAC8 activity in NK cells interferes with granzyme B production. PCI-34051 does not alter granzyme B expression **(Figure 3.4B)**. This study did not evaluate if HDAC8 inhibitor treatment directly influences expression levels of stress molecules on K562 cells due to lacking evidence of enhanced cytotoxicity, and importantly, K562 cells are resistance to PCI-34051 treatment **(Figure 4.1D)**.

To investigate if the increase in IFNγ$^+$ NK cells enhances secretion of IFNγ during antigen stimulation, supernatants were collected from co-culturing conditions with K562 cells. As previously described, NK cells were pre-incubated with or without PCI-34051 at the indicated concentrations **(Figure 3.4D),** and cytokine stimulated for 24hrs, to enrich the abundance IFNγ$^+$ NK cells prior to co-culture conditions. After several washes, these NK cells

were co-cultured at 10:1 (E:T) with K562 cells, and supernatant was collected after 3hrs post co-culture. Interestingly, there was an increase in IFNγ detected in the rested PCI-34051 treated NK samples, albeit at very low concentrations, but still detectable (**Figure 3.4D)**. The triple combination of IL-2, IL-12, and IL-15 significantly upregulates HDAC8 in human primary NK cells. Blocking HDAC8 activity was described earlier in this study to increase the abundance of IFNγ$^+$ NK cells. However, this does not result in releasing more IFNγ during antigen challenge **(Figure 3.4D and 3.4G)**. High levels of IFNγ are associated with inducing immunosuppressive checkpoints such as upregulation of the program cell death receptor 1 (PD-1) on immune cells. It's important to highlight that PCI-34051 does not affect NK cell effector functions or significantly increase secretion levels of IFNγ.

Further studies are needed to understand the significance of PCI-34051 induced enrichment of IFNγ$^+$ NK cells. Previous studies have indicated that NK cells can differentiate into memory-like NK cells, and one of the key attributes is an increase in the abundance of IFNγ$^+$ NK cells. Natural Killer cells stimulated with cytokines can induce a memory-like imprint, and they respond robustly to low doses of IL-12 and IL-18 after repeated cytokine exposure. Whereas to achieve similar responses, high doses are required at initial exposure compared to NK cells rested and re-stimulated with low doses of IL-12 and IL-18 (170). *In vivo* studies are needed to investigate if HDAC8 induced IFNγ$^+$ NK cells to display memory-like characteristics to overcome the limitations posed by *ex vivo* NK cell studies.

To determine if HDAC8 inhibition can influence activity of NK cells during ADCC, MCL cell lines were labeled with Rituximab and co-cultured with NK cells pre-treated with and without PCI-34051. The results indicate that HDAC8 inhibition preserves NK cell, Rituximab-mediated, antibody-dependent cellular cytotoxicity **(Figure 3.4E and 3.4H)**. This was evaluated

across 6 different NK donors co-cultured with the MCL cell line, Jeko-1, labeled with or without Rituximab **(Figure 3.4E)**. To ensure the results mentioned above are representative across different MCL cell lines, a side-by-side comparison at the indicated E:T ratios was performed with the Jeko-1 and Maver-1 MCL cell lines. As anticipated, no notable changes were observed in ADCC between untreated, and PCI-34051 treated NK cells **(Figure 3.4H)**.

To investigate if PCI-34051 can sensitize MCL cells to NK ADCC, an MCL cell line with sensitivity to HDAC8 inhibitor treatment, Jeko-1, was selected and dosed with a lower concentration of PCI-34051, 5μM, to reduce the spontaneous release of Cr51. The MCL cell line, Jeko-1, was treated with DMSO or PCI-34051 for 24hrs prior to labeling with Rituximab. In parallel, freshly isolated NK cells were treated with DMSO or PCI-34051 and stimulated with cytokines for 24hr prior to being co-cultured with Rituximab labeled Jeko-1 cells. The combined results from both NK donors indicated that HDAC8 inhibition did not appear to sensitize Jeko-1 cells to NK killing or ADCC response. However, the donor variability was high between these two donors; Donor 1 achieved ~80% ADCC and Donor 2 had ~40% ADCC against Jeko-1 treated with DMSO **(Figure 3.4F)**. This was the first time an NK donor had less than 75% ADCC against MCL cell lines coated with Rituximab. If you look at the donors individually, the results indicate that Donor 2 had a two-fold increase in sensitivity when co-cultured with Jeko-1 cells pre-treated with PCI-34051, whereas Donor 1 had no difference **(Figure 3.4F)**. PCI-34051 does not influence NKG2D expression on NK cells; nonetheless, it may influence expression of stress ligands on target cells. Although, PCI-34051 increases the abundance of $IFN\gamma^+$ NK cells it does not result in an increase in IFNγ secretion during co-culture **(Figure 3.4G)**. It is important to note, this was the only instance where NK cells isolated from a "healthy" donor had low cytolytic activity. All other NK donors had robust activity against Jeko-1 and Maver-1 cell lines,

above 75% ADCC at the highest E:T, in the absence of PCI-34051 treatment **(Figure 3.4E and 3.4H)**. Taken together, these results indicate that PCI-34051 preserves robust activity of NK anti-tumor responses.

To address if PCI-34051 influences NK cell phenotype in the presence of PBMCs, freshly isolated PBMCs were rested in IL-15 and treated with or without PCI-34051 for 24 hours. PBMCs were not stimulated with IL-2, due to IL-2s robust effect on T cell proliferation. Expansion of $CD3^+$ T cells would alter the PBMC composition. Blocking HDAC8 activity within the PBMC compartment does not alter the frequency of NK cells or NK subtype composition within the peripheral blood, $CD16^+CD56^+$ **(Figure 3.5A and 3.5B)**. A slight reduction in DNAM-$1^+$ and $CD69^+$ NK cells was observed with PCI-34051 treatment, although this was found to be insignificant **(Figure 3.5C)**. DNAM-1 is an accessory molecule involved in adhesion and regulates IFNγ production (171). CD69 is a marker for activity on immune cells. The decrease in $CD69^+$ NK cells may indicate a lower level of activation. However, these results are likely attributed to monokine stimulation with IL-15 and not the effects of culturing in the presence of autologous peripheral mononuclear cells. IL-2 and IL-12 are necessary to enhance NK activity and upregulate IFNγ. IL-12 significantly induces pro-inflammatory cytokine secretion and granulation, priming them for antigen activation. All NK studies described in this dissertation, showed no indication that PCI-34051 decreases NK activity.

Collectively, these studies demonstrate that NK survival, proliferation, and cytotoxic function is preserved with PCI-34051 treatment. In addition, PCI-34051 increased the abundance of $IFN\gamma^+$ NK cells, indicating it may induce memory-like characteristics.

## 3.3 Discussion

To the best of my knowledge, this is the first study to investigate if selectively targeting HDAC8 can influence NK survival, proliferation, or ADCC effector function. Collectively, this report identifies HDAC8 as a clinically relevant target for treating MCL without disrupting the efficacy of antibody therapies dependent on NK ADCC.

Furthermore, an increase in the proportion of NK cells expressing IFNγ can indicate enhanced cytotoxic function (159). To address this question *ex vivo*, primary NK cell cytolytic responses were assessed against the HLA class I null cell line K562, which also express stress ligands. K562 cells are resistant to PCI-34051 treatment and were not treated prior to co-culture. There was no difference observed between the control and treated groups, indicating PCI-34051 does not directly influence NK cytotoxicity in an HLA independent manner **(Figure 3.4A)**.

Jeko-1 cells were pre-treated with PCI-34051 prior to co-culture, to determine if HDAC8 inhibition sensitizes MCL cell lines to NK killing. When viewed together, there is no observable difference in killing activity of Jeko-1 cells treated with DMSO or PCI-34051 **(Figure 3.4D)**. However, if you look at the donors individually, the NK cells from Donor 2, co-cultured with the PCI-34051 treated Jeko-1 cells, had an enhanced ADCC response compared to being co-cultured with DMSO treated Jeko-1 cells. This was the only instance that a NK donor showed less than 50% ADCC with Rituximab treatment. All other NK donors tested at the highest E:T ratio, of 10:1, had over 80% antibody dependent cytotoxicity, and a 2-fold increase in ADCC between the lowest (1.25:1), and highest (10:1) E:T ratios **(Figure 3.4E)**. Donor 2 demonstrated a marginal decrease in ADCC as the effector to target ratio was diluted out, having only a 1.3-fold increase between the lowest and highest E:T ratios. Therefore, the cellular contributions responsible for enhancing Donor 2 NK ADCC are likely a result of the donor's initial NK

phenotype. This experiment did not include a Rituximab only condition, which is necessary to determine if the killing is due to enhanced CD20 expression or the upregulation of ligands that stimulate NK cytotoxic responses such as the non-classical HLA molecules MICA/B and/or ULBP proteins. These results support investigating expression of stress molecules and CD20 on MCL cell lines that have been treated with PCI-34051. This should be explored with *ex vivo* MCL patient NK cells; through the characterization of each patient's NK cell phenotype, and assessment of ADCC response against MCL cell lines treated with and without PCI-34051. Collectively, the results in this study indicate NK cells treated with PCI-34051 are highly responsive to Rituximab therapy.

In conclusion, these results indicate that HDAC8 function is not essential for NK survival, cytokine secretion, or cytotoxic responses, and does not appear to immunosensitize MCL cell lines to NK killing, in most cases. Activation receptor expression was stable post PCI-34051 treatment, however, HDAC8 may play a role in deacetylation of activation receptors and this should be evaluated, using non-biased approaches, to identify if these activation receptors are substrates of HDAC8.

Interestingly, blocking HDAC8 activity increases the abundance of IFN$\gamma^+$ cells, which may have clinical implications. It has been reported that enrichment of IFN$\gamma^+$ NK cells is correlated with memory-like NK responses *in vivo*. Memory-like NK cells can be stimulated by low levels of cytokine or antigen stimulation resulting in a robust effector function and has been shown *in vivo* to enhance NK function and persistence (159). HDAC8 has been reported to play a role in the stability of proteins. It is plausible that HDAC8 may play a role regulating the stability of IFNγ or if it induces cytokine NK memory like recall responses. Interferon gamma protein levels remained unchanged with HDAC8 inhibition indicating it does not play a role in

expression. Interestingly, an increase in IFNγ$^{+}$ NK cells is an indicator cytokine induced memory-like recall responses. Secretion levels of IFNγ remained unchanged with PCI-34051 treatment, another unique feature of NK memory.

It is important to address why knockdown of HDAC8 in primary NK cells is not reported in this publication. Unfortunately, human primary NK cells are notoriously challenging to infect with virus particles and equally difficult to transiently transfect with siRNA particles. A pool of 4 siRNAs were tested and in different combinations. None of the siRNAs knocked down HDAC8 protein sufficiently enough to study its impact on primary NK cell phenotype. Furthermore, there are limitations on the tools available to study shRNA mediated knockdown in primary NK cells. NK cells start dying around 4-5 days post *ex vivo* isolation. It takes 72-96 hours for shRNA mediated knockdown to observe notable changes in protein expression, which depends on protein turnover. To overcome the short life span issue of primary NK cells *ex vivo*, LAKs were generated, and rested for 3 days to allow for reduction in activity. NK activation is associated with an increased resistance to viral infection. LAKs are generated through ligation of CD2 and NKp46, resulting in robust NK activity and proliferation. Unfortunately, resting LAKs for 3 days did not help transducing conditions, hence HDAC8 knockdown was not achievable in these conditions and was associated with other complications. Due to the significant challenges associated with genetic knockdown in human primary NK cells, on-target validation was not possible to assess if the increase in IFNγ+ NK cells directly resulted from targeting HDAC8. Future experiments utilizing CRISPR may enable more effective ablation of HDAC8 expression and should be explore.

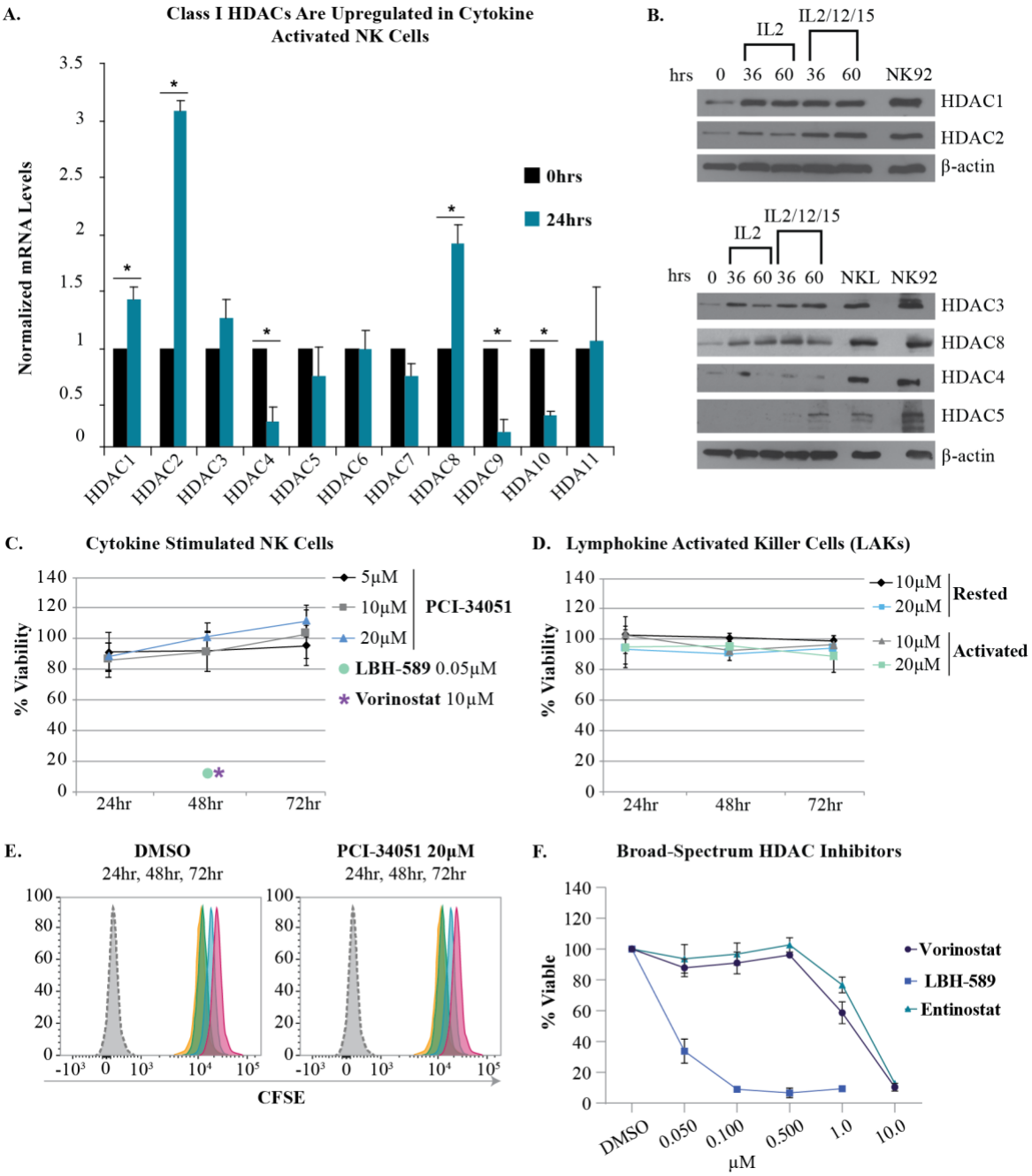


Figure 3.1. Class I HDACs are Upregulated in Human NK Cells Stimulated with Cytokines**.**
**A)** Human primary NK cells isolated from healthy donor leukoPaks, were stimulated for 24 hours with IL2, IL12, and IL18. Cells were harvested and mRNA extracted for to check for transcription of the indicated HDACs. The data is normalized to the rested NK cell control, no-cytokine stimulation, 0 hour. Two-tailed paired t-test was used for statistical analyses; *p<0.05. Data collected across 3 donors. **B)** Human primary NK cells were stimulated with IL2, IL12, and/or IL15 for 24 hours. Total protein was extracted and immunoblotted to check for HDAC1, HDAC2, and HDAC8 class I HDAC proteins. β-actin was used to ensure equal distribution of protein across all lanes. Data representative across 3 donors. **C)** Human primary NK cells were treated with PCI-34051, a selective HDAC8 inhibitor. The pan-spectrum HDAC inhibitors, Vorinostat and LBH-589, were used at 10μM and 0.05μM, respectively. Viability was check via Cell Titer Glo assay by checking ATP levels to determine cellular health. **D)** Lymphocyte activated killer cells were generated and expanded for 9 days to assess the effect of PCI-34051, at 10μM, on LAK expansion. Cells were either rested for 24hr with low cytokine stimulation IL2 (100U) or high dose (500U) IL2 stimulation to assess if inhibiting HDAC8 impacts LAK viability during expansion. **E)** Primary NK cells were stained with CSFE, wash, and treated with PCI-34051 for 1 hour prior to cytokine stimulation with IL2, IL12, and IL15. Samples were collected from treated NK cells incubated with DMSO or PCI-34051 for 0, 24, 48, or 72 hours and formaldehyde fixed. **F)** NK cells were treated with broad spectrum HDAC inhibitors vorinostat and LBH-589 or the class I HDACi Entinostat and stimulated with IL2/IL12/IL15 for 3 days prior assessing viability by Cell Titer Glo. Data collected across 3 donors. Two-tailed paired t-test was used for statistical analyses; *p<0.05 Data collected across 3 donors. *Data is presented in Watters et al. (71).*

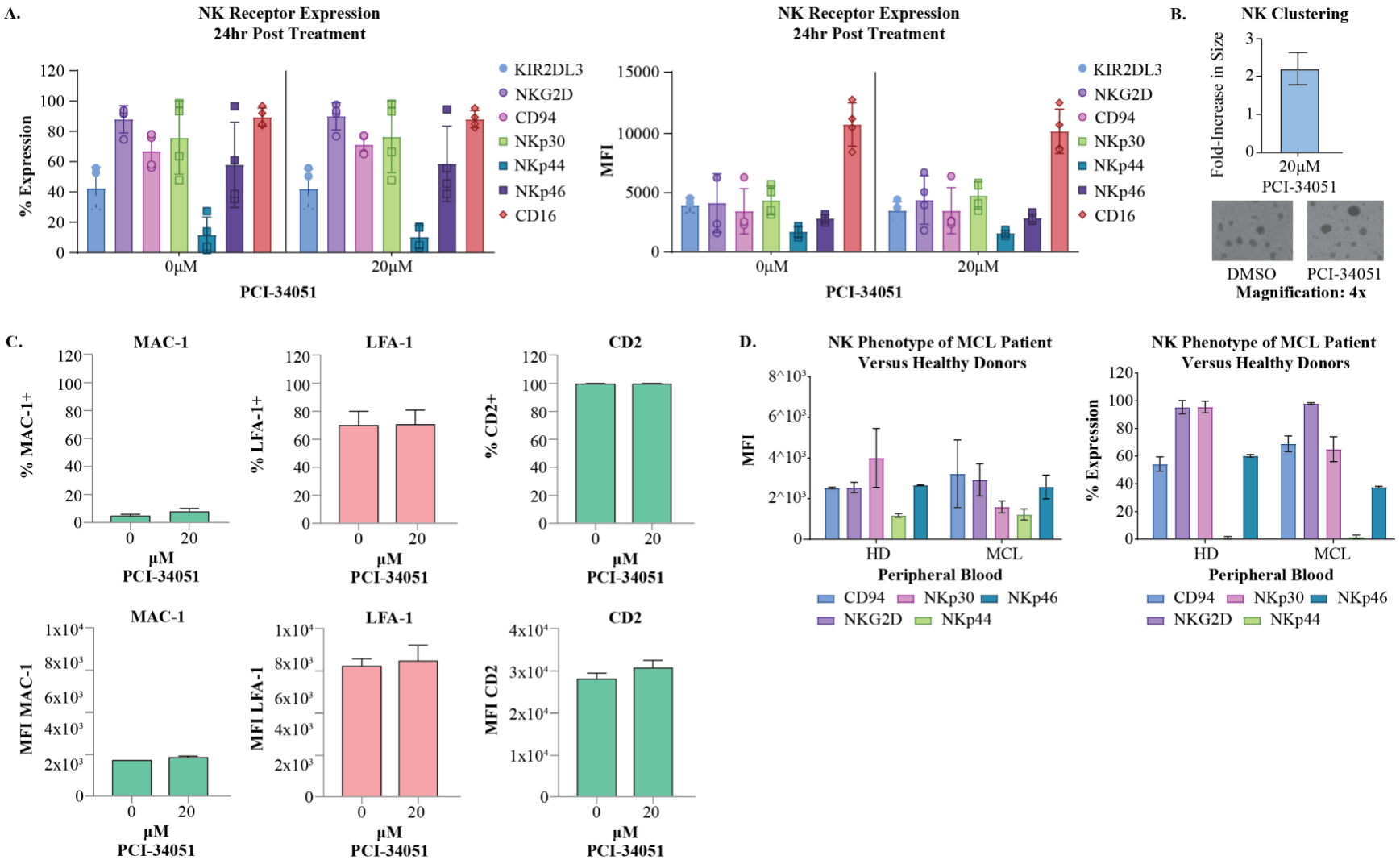


Figure 3.2. HDAC8 Does Not Regulate Expression of NK Activation Receptors.
**A)** NK cells were treated with PCI-34051 20μM for 1 hour prior to addition of cytokines IL2, IL12, and IL15 for 24hrs. Cells were collected, and a panel of surface markers involved in NK anti-tumor activity were stained for to evaluate expression levels and to determine if HDAC8 alters NK phenotype. Data collected across 4 donors. **B)** Microscopy images were taken of cells 24 hours post treatment with either DMSO or PCI-34051 at 20μM plus cytokine treatment. **C)** NK cells were treated with PCI-34051 20μM for 1 hour prior to the addition of cytokines IL2, IL12, and IL15 for 24 hours. Cells were collected and integrin receptor expression levels; CD2, CD11a (LFA-1), and CD11b (LFA-2) were assessed. Data is representative of 2 donors. **D)** NK cells were isolated from the peripheral blood of healthy donor and MCL patients and NK cells subjected to staining with a 5-color multicolor flow cytometry panel. Data is representative of 3 MCL patients and 3 healthy donors *Data is presented in Watters et al. (71).*

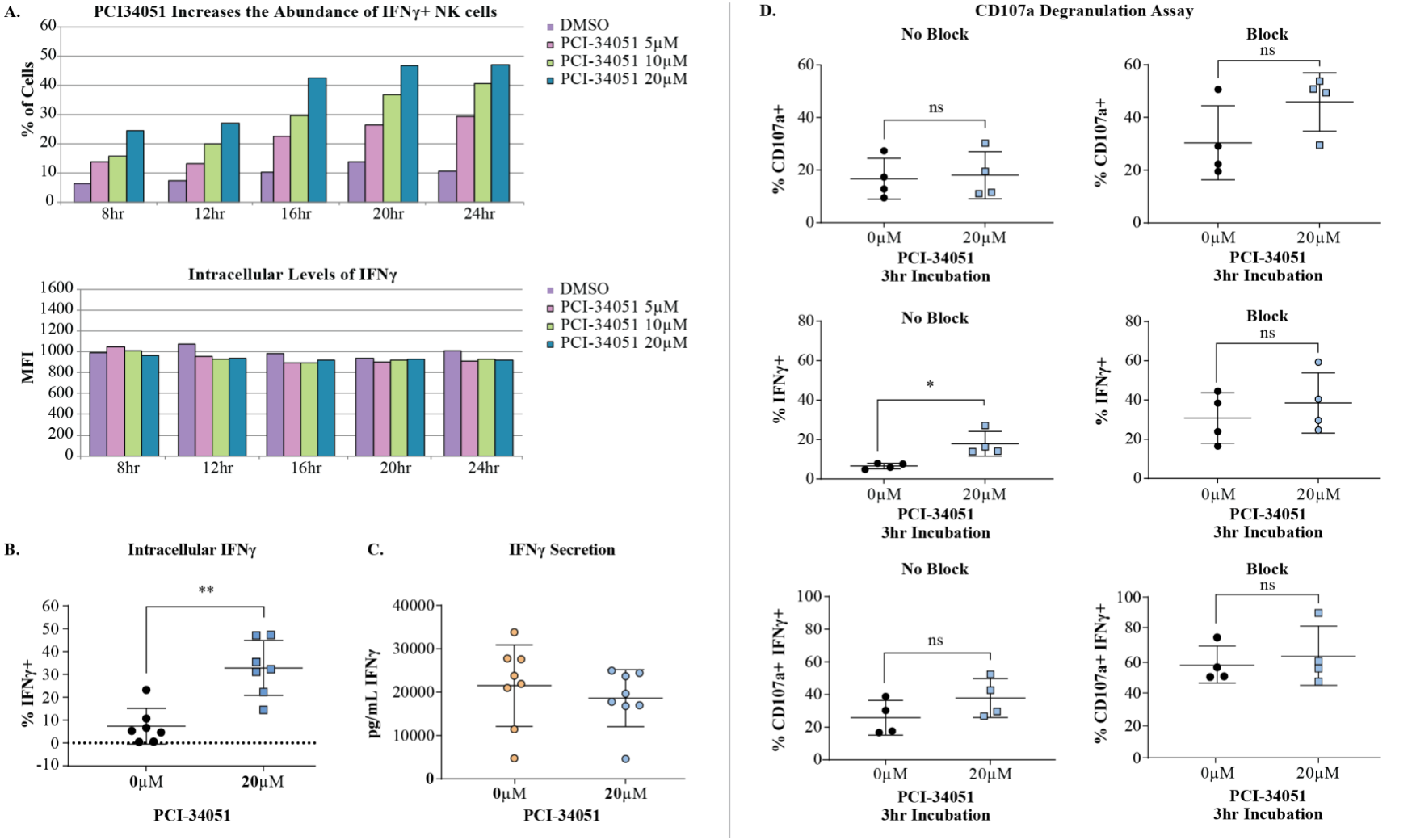


Figure 3.3. PCI-34051 Increases IFNγ$^+$ NK Cells in a Dose Dependent Manner.
**A)** Dose response: human primary NK cells were treated with DMSO, PCI-34051 5μM, 10μM, or 20μM and stimulated for the indicated times. Data is representative of 1 donor and further investigation was performed at 20μM, the dose with the highest increase in IFNγ$^+$ NK cells. **B)** NK cells were treated with DMSO or PCI-34051, followed by stimulation with IL2, IL12, and IL15 for 24 hours prior to intracellular staining for cytoplasmic levels of IFNγ. Cells were permeabilized to evaluate cytoplasmic IFNγ levels. Data was collected across 7 donors. **C)** Supernatants were collected from human primary NK cells 24 hours after treatment with DMSO or PCI-34051 stimulated with IL2, IL12, and IL15 to evaluate secretion of IFNγ. **D)** Cytolytic function was validated using the CD107a degranulation assay and evaluation of IFNγ during blocking and non-blocking conditions to determine if PCI-34051 induces retention of IFNγ. NK cells were either not treated, for no blocking conditions, or for blocking conditions, treated with monensin and brefeldin A to prevent secretion of internalization of CD107a and retention of IFNγ. Data collected across 4 donors. Two-tailed paired t- test was used for statistical analyses; *p<0.05, **P<005.

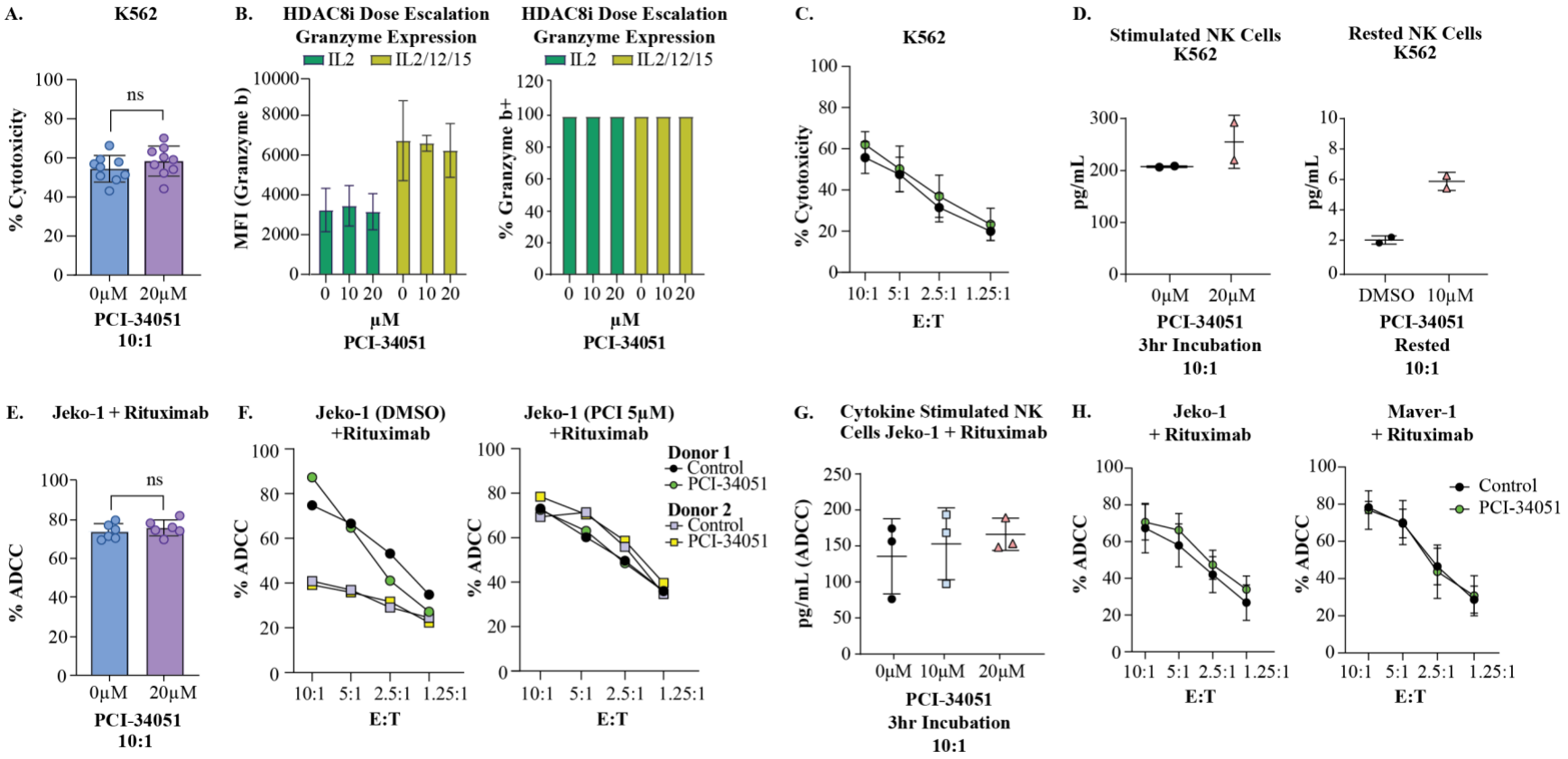


Figure 3.4. PCI-34051 Preserves NK Cell Cytotoxicity and ADCC Responses.
**A)** NK cells were incubated with PCI-34051 20μM and stimulated with IL2, IL12, and IL15 for 24 hours. The cells were washed 2 times prior to cells going into co-culture to ensure removal of cytokines and residual PCI-34051. The target cell line, K562, was used to evaluate the effect of HDAC8 inhibition on target cells null for HLA class I molecules. K562 cells were labeled with Cr51 for 30min, then washed 2 times with media to remove any unbound Cr51 from the cells The NK cells were co-cultured at the indicated effector to target ratios (E:T) 10:1, 5:1, 2.5:1, and 1.25:1 for 4 hours, and supernatants were collected to assess for Cr-51 release. NK cells were stained for granzyme b assess by flow cytometry. Two-tailed paired t-test was used for statistical analyses: no significance was observed across the data sets. Data was collected across 9 donors for the co-culture experiment and 2 donors for granzyme b flow-based assay. **B)** Jeko-1 was pre-coated with 10μg of Rituximab for 1 hour, washed 2-3 times prior to staining with Cr51. Data collected across 6 donors. **C)** NK cells were co-cultured with either: K562, or pre-coated with Rituximab prior to co-culturing with Jeko-1, Maver-1, and Z138. NK cells were pre-treated with DMSO or PCI-34051 20uM for 24 hours prior to co-culture experiments. Data is representative of 3 donors. **D)** The MCL cell line, Jeko-1, was pre-treated with DMSO or PCI-34051 for 24 hours at the lower dose, 5μM, to ensure cells were viable prior to coating with 10ug of Rituximab and Cr51 labeling. Jeko-1 cells were co-cultured with NK cells at the indicated E:T ratios. **E)** The Jeko-1 MCL cell line was pre-coated with 10μg of Rituximab for 1 hour and washed 2-3 times prior to staining with Cr51. NK cells were pre-treated with DMSO or PCI-34051 20uM for 24 hours prior to co-culture experiments. Data collected across 6 donors. **F)** The MCL cell line, Jeko-1, was pre-treated with DMSO or PCI-34051 for 24 hours at the lower dose, 5μM, to ensure cells were viable prior to coating with 10ug of Rituximab and Cr51 labeling. Jeko-1 cells were co-cultured with NK cells pre-treated with DMSO or 5μM of PCI-34051, at the indicated E:T ratios. **G)** Supernatants were collected 3hrs post co-culture setup and IFNγ secretion evaluated by ELISA. Data is representative of 2 or 3 donors. Two-tailed paired t-test was used for statistical analyses *p<0.05. **H)** NK cells were pre-treated with DMSO or PCI-34051 20uM for 24 hours prior to co-culture experiments and were co-cultured with Jeko-1, and Maver-1, pre-coated with Rituximab. Data is representative of 3 donors.

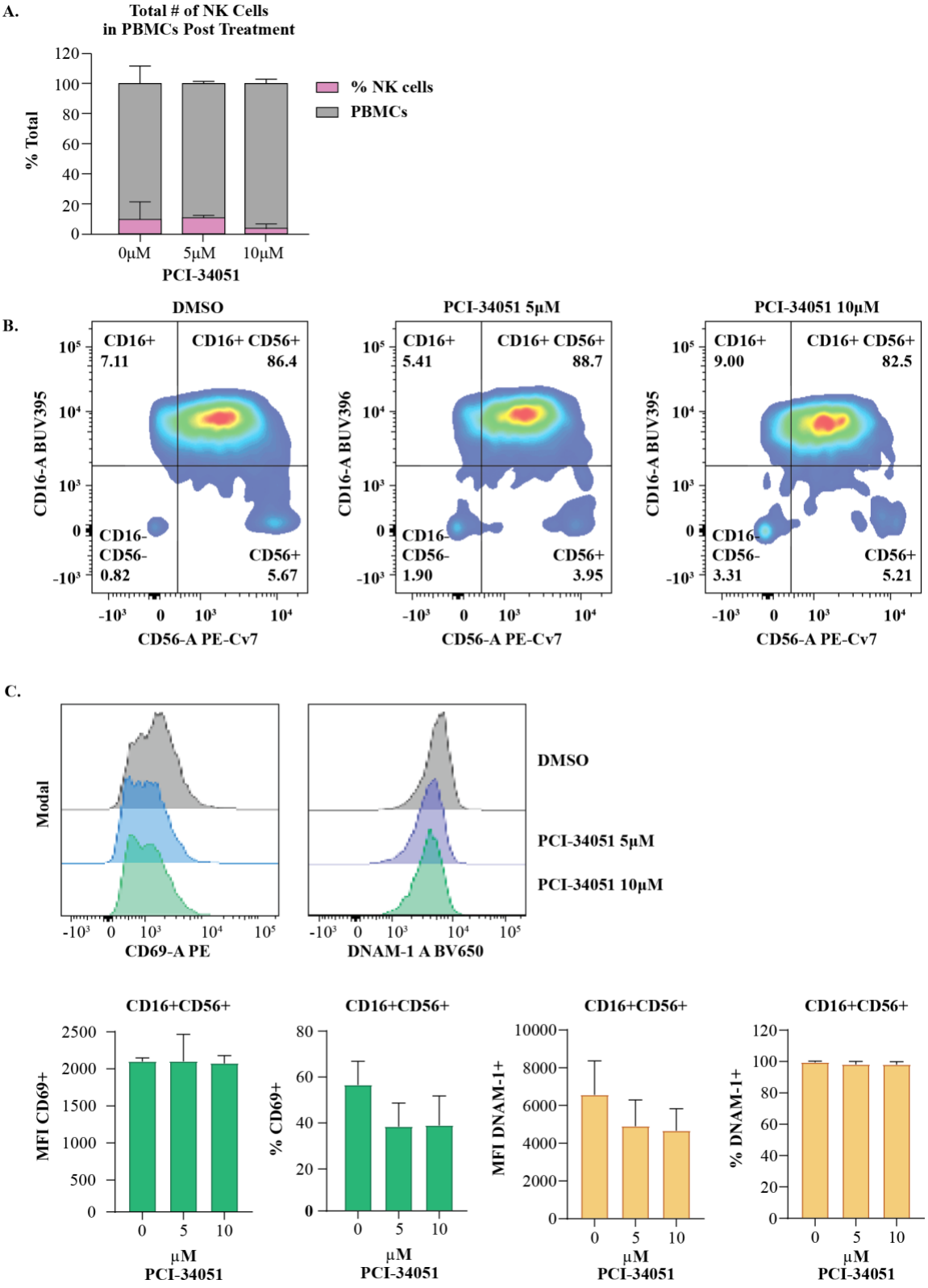


Figure 3.5. HDAC8 Inhibition Does Not Alter the NK Cell Composition of Peripheral Blood Mononuclear Cells.
**A)** PBMCs were treated with 10uM PCI-34051 and stimulated with IL-15 for 24hrs. PBMCs were assessed to determine the % of NK cells in the peripheral blood. **B)** CD3 negative cells, within the PBMC population were gated on, and then NK cells were subtyped by investigating CD16 and CD56 expression. **C)** NK cells were evaluated for CD69 and DNAM-1 expression by flow cytometry. Data is representative of 2 donors.

# CHAPTER IV:

# HISTONE DEACETYLASE 8 SUPPORTS MCL SURVIVAL

## 4.1 Introduction

In the normal cellular context, HDAC8 serves as an important regulator of cellular homeostasis. Loss of HDAC8 in mice is embryonically lethal due to skull instability. HDAC8 controls the skull structure by regulating transcription factors, such as orthodenticle homeobox 2 and LIM homeobox 1, in cranial neural crest cells (123). In humans, mutations in HDAC8 are linked to Cornelia de Lange syndrome and are classified as an X-linked dominant disorder that causes facial malformations and developmental issues (119, 120, 172). The HDAC8 gene is located on the X chromosome, q13.1.

HDAC8 is pro-tumorigenic across multiple cancer types, contributing to survival, growth, and/or invasive characteristics. In neuroblastoma, high expression of HDAC8 is correlated with poor overall survival (125). Knocking down or inhibiting HDAC8 in neuroblastoma cell lines significantly reduced growth, blocked proliferation, and enhanced retinoic acid-mediated differentiation (125, 173). In breast cancer, inhibiting or knocking down HDAC8 reduces growth and invasive properties by altering genes involved in migration (174). Jurkat (an acute T cell leukemia cell line) cells are sensitive to PCI-34051 treatment. Inhibiting HDAC8, using PCI-34051, induces PLCγ-dependent apoptosis, indicating it may play a role in survival of some hematological malignancies. The focus of this study was to identify if MCL cell lines depend on HDAC8 activity for survival.

## 4.2 Results

### 4.2.1 Mantle Cell Lymphoma Cell Lines are Dependent on HDAC8 for Survival

A panel of MCL cell lines were screened for sensitivity to HDAC8 inhibition. The $SOX11^-$ MCL cell line, JVM-2, representing the non-nodal MCL subtype, was resistant to HDAC8 inhibition compared to all the other MCL cell lines, that are $SOX11^+$ and have characteristics of the aggressive conventional MCL subtype. The MCL cell lines with the highest sensitivity to PCI-34051 treatment were the Rec-1, Z138, and Jeko-1 cells, with an $EC_{50}$ of 3.4μM, 4.4μM, and 7.2μM, respectively **(Figure 4.1A)**. The MCL cell lines with the least sensitivity to PCI-34051 treatment were the Maver-1, Mino, and JVM-2 cells with an $EC_{50}$ of 19.2μM, 20.1μM, and 26.9μM, respectively. At 25μM, PCI-34051 is reported to induce histone acetylation (107). This indicates that the MCL cell lines with the highest sensitivity (an $EC_{50}$ less than 8μM) are not contributed to histone acetylation. All 5 cell lines ubiquitously express HDAC8 **(Figure 4.1B)**. Burkett lymphoma (BL) and MCL cells lines were treated with PCI-34051 at 5μM, dosed at 0hrs and 24hrs, with total viable cells counted by trypan blue exclusion. A significant reduction in proliferation over-time was observed in response to HDAC8 inhibition across MCL cell lines compared to the BL cell lines, Namalwa and Ramos **(Figure 4.1C)**. The Maver-1 cell line did not respond to this dosing regimen.

Drug sensitivity was validated by flow cytometry using annexin V and propidium iodide (PI) staining to distinguish between late and early apoptosis. As anticipated, PCI-34051 induced significant apoptosis across all sensitive MCL cell lines, with the exception of the JVM-2 cell line having the highest resistance to HDAC8 inhibition **(Figure 4.1D)**. Notably, the Jeko-1, Z138, and Rec-1 MCL cell lines had the highest % of apoptotic cells **(Figure 4.1D)**. These three cell lines were selected for further evaluation. Jurkat cells have been previously described as

sensitive to PCI-34051 and were used as a positive control for PCI-34051 induced cell death. The K562 cell line was found to be resistant to PCI-34051 treatment and was used as a negative control **(Figure 4.1D)**.

### 4.2.2 Sequential Dosing at a Lower Dose Achieves Similar Toxicity as a Single High Dose

To address the question—if lowering the concentration of PCI-34051 and dosing sequential days could have the same efficacy as one single high dose—each cell line was dosed either with a single high dose 10-20µM or sequentially with a low dose 5-10µM separated by 24 hours. Both cell lines responded similarly to both high and low dose PCI-34051. PCI-34051 induced significant apoptosis after 3 days of incubation **(Figure 4.2A)**. Preliminary results from a dosed time-course indicated maximum apoptosis around 72 hours post treatment, therefore, 72 hours was selected for evaluating drug cytotoxicity. Together, these results indicate a durable cytotoxic response is observed with a lower dose spread across 2 days, equally inducing significant cell death as a single high dose. This suggest that efficacy may be achieved by lowering the dose to reduce any potential toxicity issues.

To ascertain that the results from the sequential dosing experiment are not due to residual PCI-34051, 3-MCL cells lines were dosed, incubated for 24 hours, and washed several times prior to redosing. The absolute cell counts were tracked over time. As anticipated, washing out the PCI-34051 did not affect drug efficacy and was a result of dosing 24 hours apart **(Figure 4.2B)**. Thus, providing evidence that the effect observed by PCI-34051 is a result of low dosing, and is not attributed to accumulation of PCI-34051. Collectively, the effects observed from the

higher dose (10-20μM) does not mean that the drug is around longer. In fact, the higher dose severely compromises MCL survival. The dosing concentration is dependent on cell sensitivity.

Blocking HDAC8 deacetylation is known to cause cell cycle arrest, and exactly where arrest occurs is dependent on the cell type. SMC3 is a well characterized HDAC8 substrate known to regulate deacetylation at the lysine residues 105/106 (120). Deacetylation of SMC3 enables proper release from the cohesion complex on the sister chromatid, which results in correct segregation during division. Failure of this process can cause DNA damage.

MCL cell lines were dosed at the appropriate PCI-34051 concentrations and examined for levels of γ-H2AX levels, a known indicator of DNA damage. As anticipated, PCI-34051 significantly induces DNA damage in MCL sensitive cell lines **(Figure 4.3A)**. The lack of DNA damage correlates with resistance to PCI-34051. PCI-34051 induces barely detectable levels of DNA damage in the Maver-1 cell line compared to the sensitive MCL cell lines; Jeko-1, Z138, and Rec-1. This shows DNA damage is an indicator of sensitivity to PCI-34051 treatment. A dose escalation study was performed to evaluate if SMC3 acetylation corresponds with increasing DNA damage over 24 to 48 hours. Interestingly, DNA damage occurred in Jeko-1 cells at low levels around 24 hours (in both high and low dose scenarios), and significantly increased at 48 hours. However, the cell lines with the highest sensitivity to PCI-34051 displayed an increase in acetylation of SMC3 and DNA damage at both the high and low doses at the earlier 24-hour timepoint **(Figure 4.3A and 4.3B)**, suggesting DNA damage is likely an indicator of sensitivity. Furthermore, SMC3 is a well characterized HDAC8 substrate, and these results validate on target specificity of PCI-34051.

HDAC inhibitors have been indicated to induce cell cycle arrest of leukemia and lymphoma cells. Therefore, the Z138 and Jeko-1 cell lines were treated with PCI-34051 and

stained with DAPI to examine cell cycle phases. PCI-34051 induces a pile up of cells in S-phase, with a significant reduction in G2M phase in both the Jeko-1 and Z138 cell lines **(Figure 4.3C)**. This indicates that blocking HDAC8 activity in MCL cell lines disrupts the cell cycle causing cells to accumulate in S-phase, preventing G2M transition. Collectively, this study reports for the first time that HDAC8 activity is critical for MCL survival, and blocking activity is associated with cell cycle arrest. HDAC8 negatively regulates DNA damage repair (DDR) of double stranded breaks (DSB) through TP53 regulation. HDAC8 can regulate TP53 by both regulating transcription of HOXA5, a transcription factor essential for TP53 expression, or through protein deacetylation. Other class I HDAC proteins are also involved in regulating TP53, such as HDAC1, which cooperates with MDM2 to regulate deacetylation and degradation of TP53 (175, 176).

### 4.2.3 Abrogating HDAC8 Activity Induces Caspase-Dependent Apoptosis and is Directly Related to HDAC8 Loss in MCL Cell Lines

The first hematological malignancy indicated to have sensitivity to HDAC8 inhibition was the Jurkat cell line. PCI-34051 induced apoptosis through caspase activation in a PLCγ-1 dependent manner. To validate that PCI-34051 induces a similar mechanism in MCL cell lines, caspase activity was monitored after treating the MCL cell lines with PCI-34051. As anticipated PCI-34051 induced caspase activity resulting in significant apoptosis **(Figure 4.4A)**. Genetic knockdown studies were performed to ensure apoptosis was directly related to blocking HDAC8 activity and not an off-target effect of PCI-34051. HDAC8 knockdown induced apoptosis of Jeko-1 and Z138 MCL cell lines **(Figure 4.4B)**. Furthermore, apoptosis was induced with a 40% reduction in HDAC8 protein expression, indicating MCL sensitivity to HDAC8 loss. The

complete loss of HDAC8 in Z138's induced a 1.5-fold increase in apoptosis compared to the Jeko-1 cell line, confirming they are more sensitive, and this is a direct result of abrogating HDAC8 activity. Due to protein turnover, it takes 3 to 4 days for shRNA mediated knockdown to occur, therefore apoptosis was evaluated 4 days post transduction.

### 4.2.4 Pre-liminary Results Indicate PCI-34051 Induces Activation of PKA

A previous study indicated that PCI-34051 induced apoptosis of Jurkat cells in a PLCγ dependent manner. PCI-34051 induces calcium release from the ER, which results in cytochrome C release and caspase activation, resulting in apoptosis. Calcium is a key secondary messenger for many cellular processes and initiates the activation of cyclin AMP. Cyclin AMP activates protein kinase A, a key mediator of inducing apoptosis. Therefore, Z138 cells were treated with PCI-34051 and investigated for PKA activity. Blocking HDAC8 activity resulted in an increase in PKA activity **(Figure 4.5A)**. To confirm that PCI-34051 induces PKA activation, phosphorylation of HDAC8 was checked by immunoblot **(Figure 4.5B)**. PKA is known to inactivate HDAC8 by phosphorylating serine 39. HDAC8 inhibition resulted in PKA activation, indicated by an increase in serine 39 phosphorylation. Forskolin, a cyclic adenosine monophosphate (cAMP) activator, was used as a positive control, at a concentration known to increase PKA activity, of 10μM. These results reveal that PCI-34051 is more potent at increasing PKA activity than FSK. Considering, that HDAC8 negatively regulates a downstream effector of the PKA pathway, cAMP response element-binding protein (CREB), blocking HDAC8 activity would enhance CREB transcription and activity. Taken together, a plausible mechanism of action is, PCI-34051 induces significant DNA damage in MCL cell lines, which results in the following:

- *DNA damage induced calcium release from the endoplasmic reticulum (ER), which increases levels of cyclic adenosine monophosphate.*
- *Cyclic adenosine monophosphate activates Protein Kinase A.*
- *PKA activates cAMP-responsive element binding protein (CREB), a key transcriptional regulator of anti-apoptotic genes.*
- *This leads to caspase activation, which results in PKA-mediated apoptosis.*

The study published by Balasubramanian, indicated that PCI-34051 induces PLCγ dependent apoptosis in T cell leukemia. Phospholipase C hydrolyzes the membrane bound phospholipid phosphatidylinositol 4,5-bisphosphat (PIP2) into two intracellular mediators, diacylglycerol (177), and inositol triphosphate (IP3). Soluble IP3 diffuses through the cell and binds to calcium ion channels located on the ER. This causes the release of $Ca^{2+}$ into the cytoplasm. Calcium acts as a secondary messenger and activates PLCγ. Downstream signaling induces caspase activation, and results in apoptosis.

Pre-liminary data suggests that PCI-34051 activates cAMP/PKA dependent apoptosis in MCL cell lines. Furthermore, blocking HDAC8 activity in MCL cell lines induces DNA damage which may sensitize hematological malignancies to chemotherapy and/or other targeted therapies.

## 4.3 Discussion

These studies provide evidence that HDAC8 is functionally important for MCL survival. Knocking down or abrogating HDAC8 activity in MCL cells induces caspase-dependent apoptosis, significantly reducing viability. This finding is consistent with the observations seen in the T cell leukemia cell line, Jurkat (107). Importantly, blocking HDAC8 activity in T cell lymphoma causes activation of PLCγ and $Ca^{+2}$ induced caspase-dependent apoptosis (107). Furthermore, genetic knockdown or inhibition of HDAC8 induces caspase-dependent apoptosis, validating PCI-34051 on-target specificity. Interestingly, all MCL cell lines sensitive to HDAC8

inhibition have aberrancies in TP53 tumor suppressive activity. Either by harboring mutations in TP53 or disrupting proapoptotic gene expression important for TP53-mediated apoptosis. Z138 lack expression of TNF receptor associated factor 2 (TRAF-2), which disrupts TP53 signaling via NF-kB regulation. Clinically, MCL cells that harbor p53 mutations are aggressive, which results in poor clinical outcome compared with patients with functional p53 activity. Additionally, all sensitive MCL cell lines are $SOX11^{+}$. SOX11 is highly expressed in conventional MCL, which is associated with a poor clinical outcome compared to $SOX11^{-}$ MCL, often of which is indolent in behavior. MCL patients with aberrancies in TP53 signaling, are associated with a poor clinical outcome regardless of SOX11 expression. The presence of SOX11 is an indicator of origin during B cell development and is associated with a pre-germinal center B cell phenotype.

Interestingly, PCI-34051 causes an increase in PKA activity which resulted in phosphorylation of serine 39 residue on HDAC8. Indicating that PCI-34051 not only inhibits HDAC8 through binding to the active site but also by influencing its regulatory kinase, PKA, which is known to prevent activity of HDAC8.

Collectively, these results identify HDAC8 as a potential therapeutic target in MCL. HDAC8 activity is nonessential for NK cytolytic function and cytokine secretion. This study reports HDAC8 as an attractive target in MCL. Furthermore, the studies within this dissertation provide evidence that selectively inhibiting HDAC8 will not impede therapeutic response to mAb anti-tumor targeting therapies dependent on NK function for efficacy.

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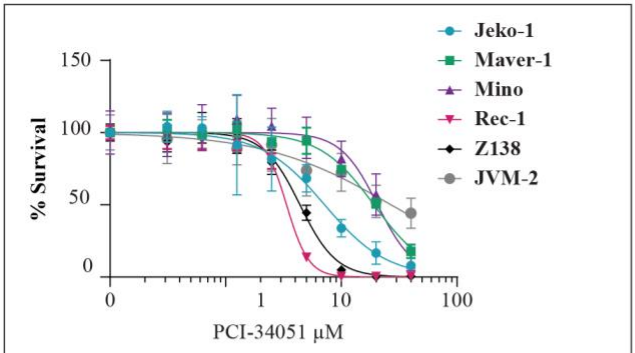
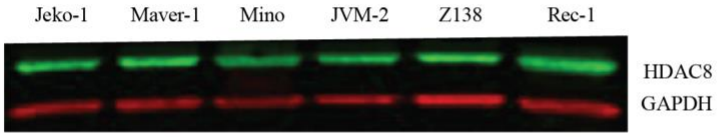
A.




| | Jeko-1 | Maver-1 | Mino |
|---|---|---|---|
| $EC_{50}$ | 7.174 | 19.16 | 20.61 |
| | **Rec-1** | **Z138** | **JVM-2** |
| $EC_{50}$ | 3.362 | 4.391 | 26.92 |

B.

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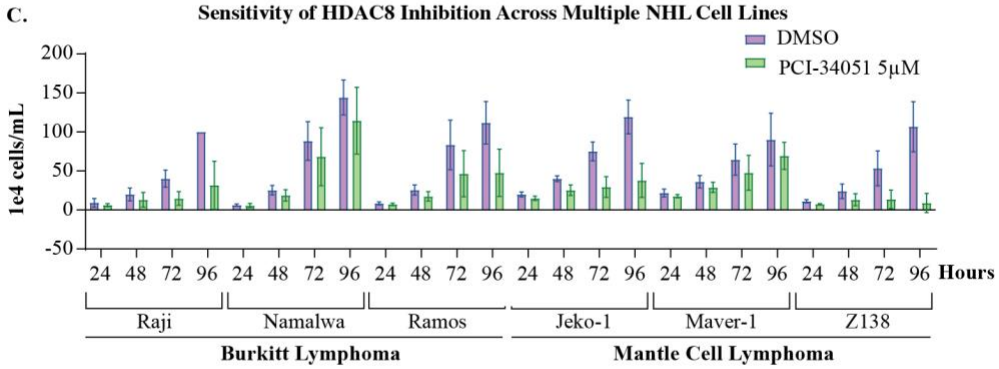


D.

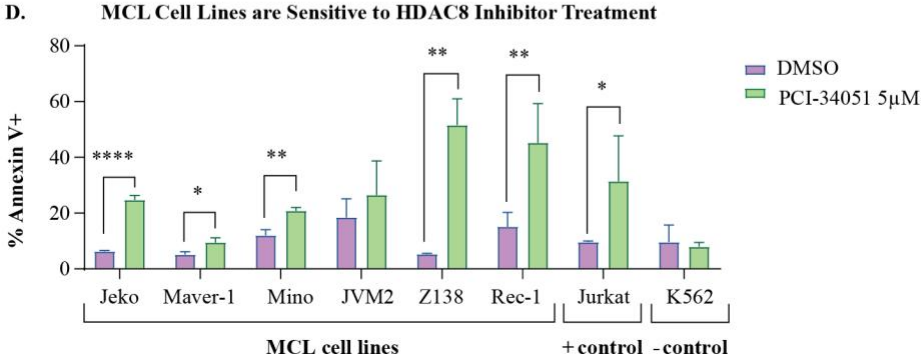


Figure 4.1. MCL Cell Lines are Sensitive to HDAC8 Inhibition.
**A)** A panel of MCL cell lines were treated with low-high doses of PCI-34051 and sensitivity indicated by the EC50. The data is representative across 3 independent experiments. **B)** HDAC8 protein expression was evaluated across all MCL cell lines used in the sensitivity study in a. GAPDH was used as a loading control across all samples and the data is representative of 3 independent experiments. **C)** and **D)** BL and MCL cell lines were treated with 5µM of PCI-34051 at 0 hours and every 24 hours, thereafter, counted and the MCL cell lines were counted to evaluate proliferation, and stained with Annexin V and PI to evaluate cell death. Data was collected across 3 independent experiments. Two-tailed paired t-test was used for statistical analyses; *p<0.05, **p<0.005. *Data is presented in Watters et al. (71).*.

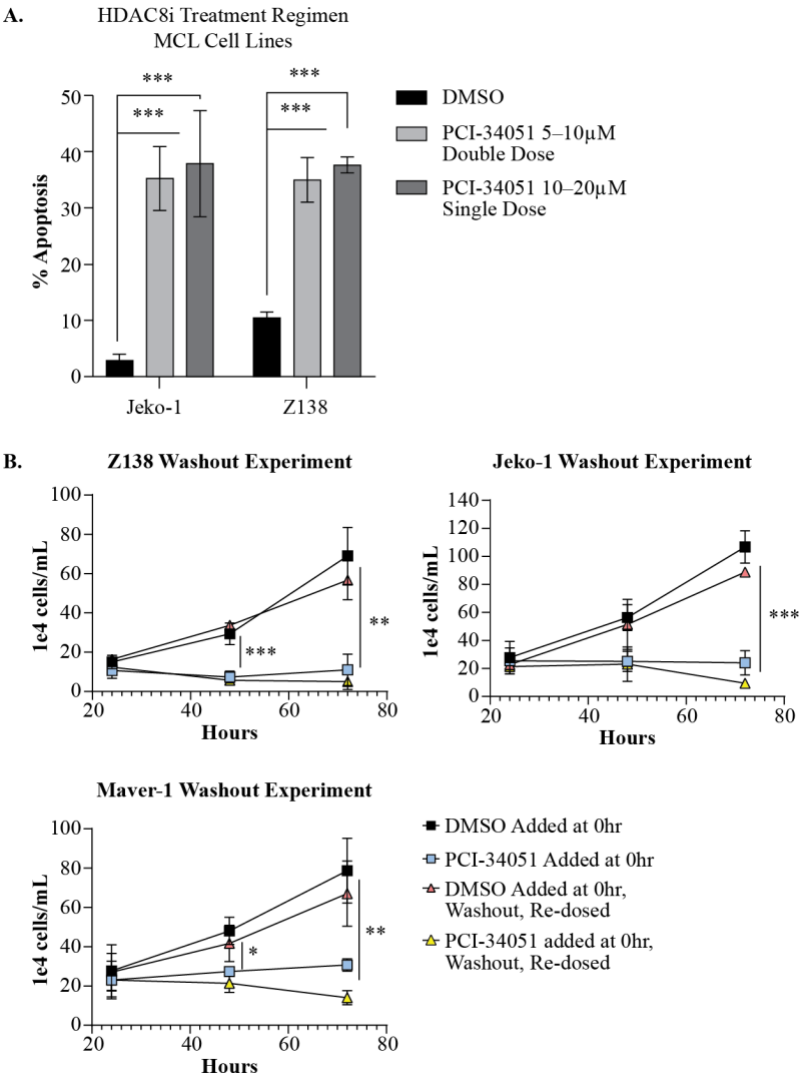


Figure 4.2. Sequential Low Dose PCI-34051 Yields Similar Toxicity as One Single High Dose.
**A)** The Jeko-1 and Z138 cell lines were treated with either high or low dose PCI-34051 and then dosed again with in the low dose conditions 24 hours after the first dose. **B)** Each cell line was dosed with either the high (10-20μM) or low (5-10μM) dose of PCI-34051. In the low dose conditions, another dose was given 24 hours after the first dose. In the re-dosing conditions, the cells were washed to ensure cell death wasn't a result of accumulation of PCI-34051 from the first dose. Data was collected across 3 independent experiments. Two-tailed paired t-test was used for statistical analyses; *p<0.05, **p<0.005, ***p<0.0005.

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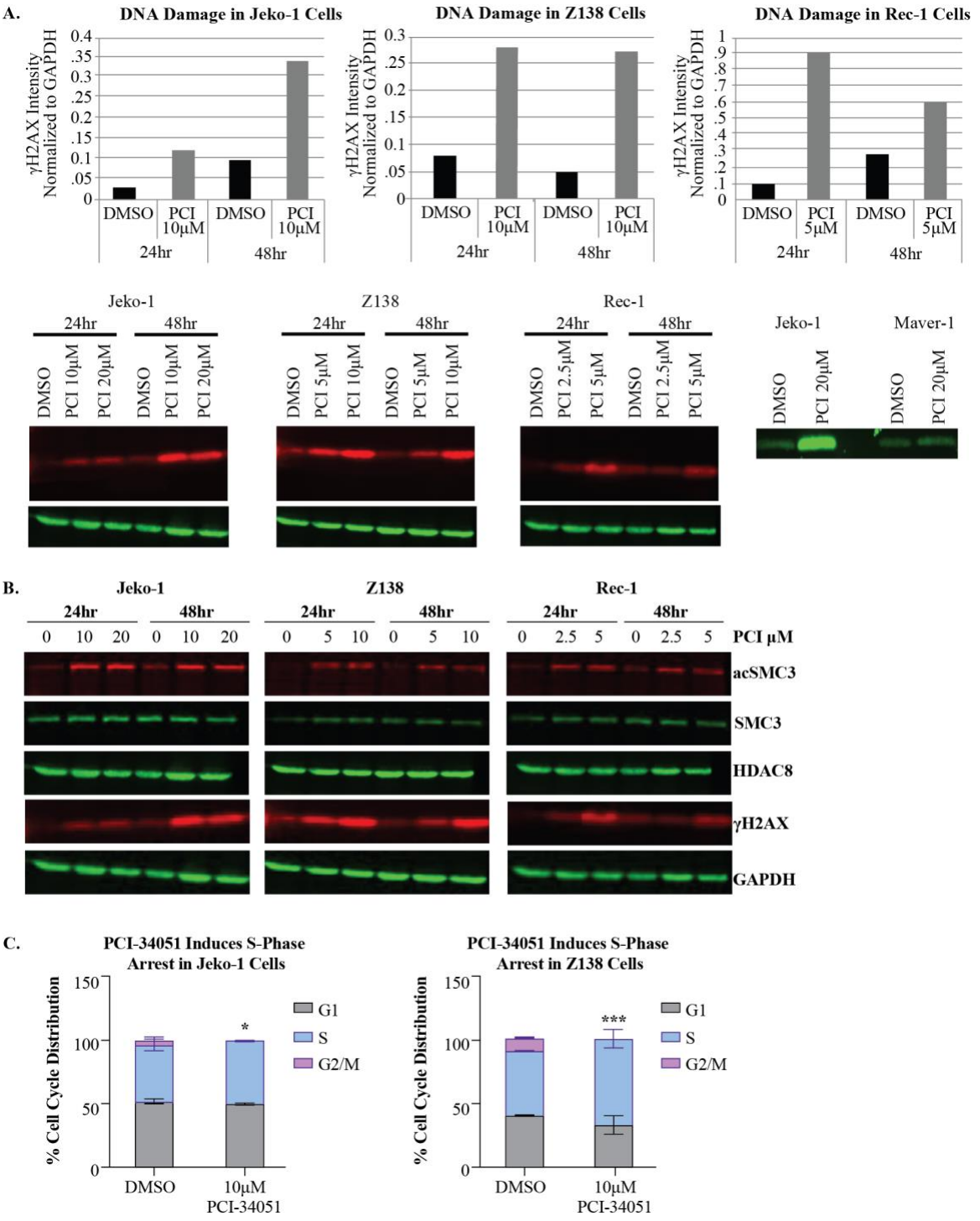


Figure 4.3. PCI-34051 Induces DNA Damage in MCL Cell Lines.
**A)** The most sensitive MCL cell lines were selected (Jeko-1, Z138, and Rec-1) and used to evaluate if PCI-34051 induces DNA damage, γH2AX+, at the concentrations indicated and assessed 24- and 48-hours post treatment. The graphs are representative of the immunoblots and γH2AX intensity was normalized to the GAPDH protein loading control for each lane to evaluate DNA damage. **B)** SMC3, a known HDAC8 substrate, and deacetylation is critical for proper completion of cell cycle. Hyperacetylation causes DNA damage and cell cycle arrest. Jeko-1, Z138 and Rec-1 MCL cell lines were treated with 5μM, 10μM, and/or 20μM for 24-48 hours. Total SMC3, aceSMC3, HDAC8, γH2AX, and GAPDH protein levels were immunoblotted. Data is representative of 2 independent experiments. **C)** To evaluate cell cycle distribution cells were fixed and stained with 4', 6-diamidino-2-phenylindole (DAPI). Jeko-1 and Z138 MCL cell lines were treated with 20μM and 10μM, respectively. Data is representative of 2 independent experiments; cell cycle distribution was evaluated using the ModFit LT 2.0 software. Two-tailed paired t-test was used for statistical analyses; *p<0.05. *Data is presented in Watters et al. (71).*

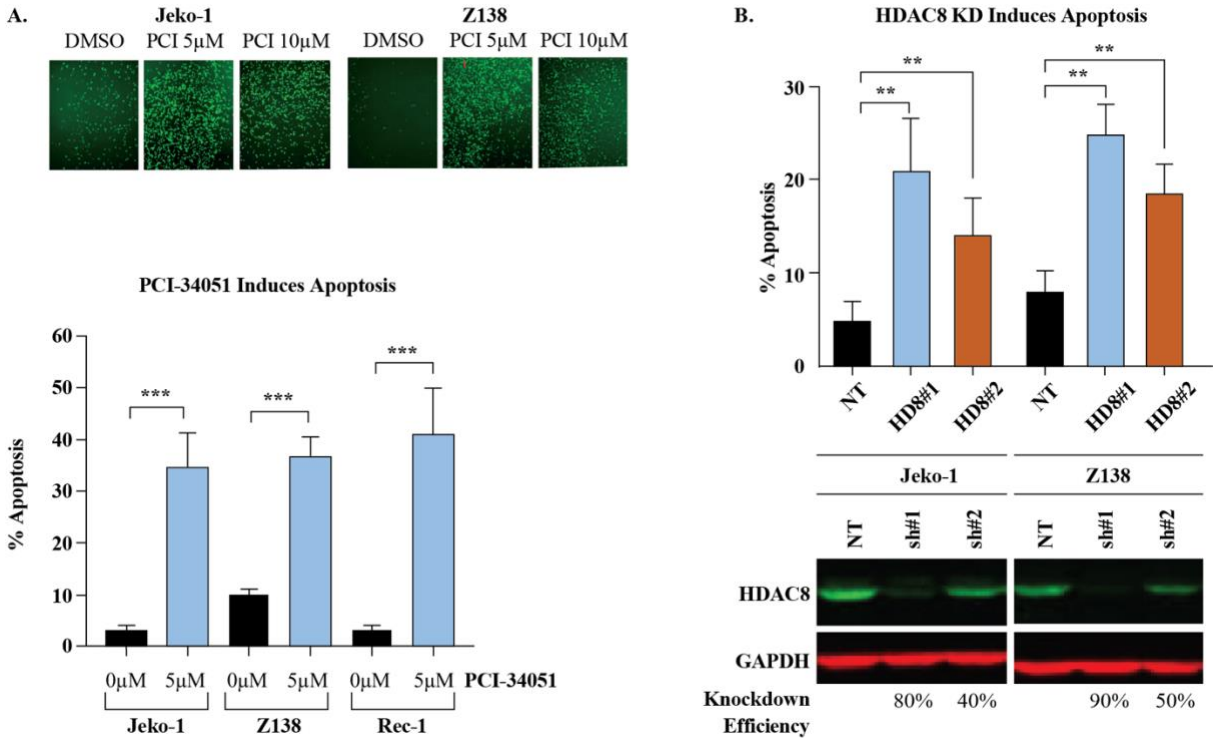


Figure 4.4. PCI-34051 and Genetic Knockdown of HDAC8 Induces Apoptosis.
**A)** The indicated MCL cell lines; Jeko-1, Z138, and Rec-1 were treated with 5μM, every 24 hours for 48hrs prior to evaluating apoptosis via flow cytometry. The MCL cell lines; Jeko-1 and Z138 were treated with PCI-34051 at the indicated concentrations prior to capturing caspase levels after 48hrs via imaging on the Celigo cytometer. Data is representative of 3 independent experiments. Two-tailed paired t-test was used for statistical analyses; ***p<0.0005. **B)** MCL cell lines Jeko-1 and Z138 were transduced with Lenti-virus particles containing non-targeting shRNA, shRNA HDAC8 #1 and shRNA HDAC8 #2. It took 72-96 hours for maximum levels of HDAC8 knockdown to be observed, therefore caspase activity was checked 96 hours post transduction. Percent knockdown of HDAC8 protein levels are indicated below the graph. Data is representative of 3 independent experiments. Two-tailed paired t-test was used for statistical analyses; *p<0.05, **p<0.005, ***p<0.0005. *Data is presented in Watters et al. (71).*

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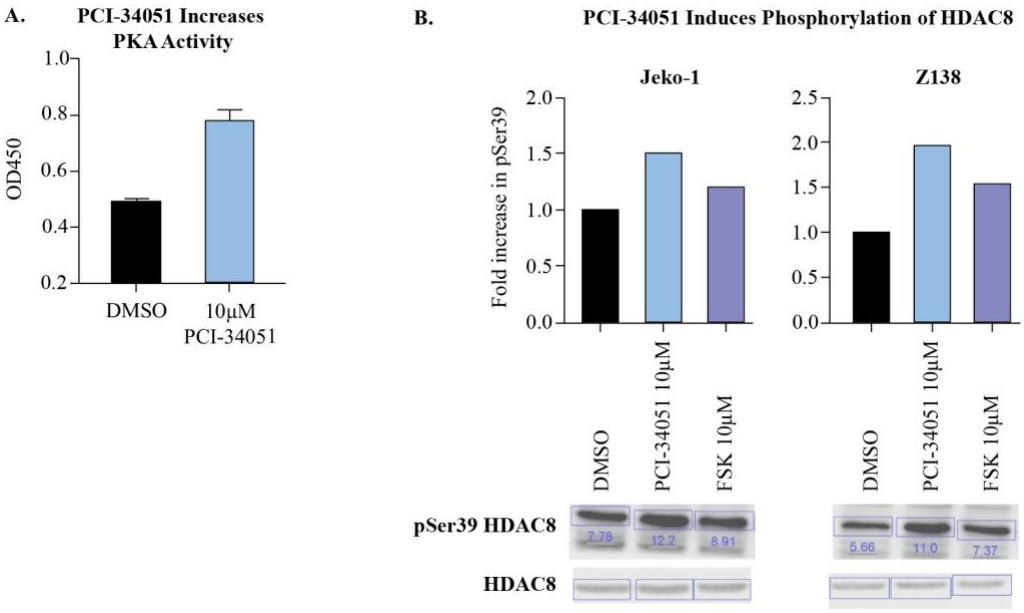


Figure 4.5. PCI-34051 Enhances PKA Activity.
**A)** MCL cell lines were treated with DMSO, PCI-34051 (20-10μM), H89 (PKA inhibitor, 10μM), or Forskolin (FSK, 10μM) (enhances cAMP activity) for 8hrs. Protein was isolated and IB for HDAC8, phosphorylated Ser39, and GAPDH. **B)** Z138 cells were treated with DMSO or PCI-34051 at 10μM, and cell lysate was used to check for PKA activity. Data is representative of 2 independent experiments.

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# CHAPTER V:

# DISCUSSION AND SCIENTIFIC SIGNIFICANCE

The findings in this dissertation indicate HDAC8 plays a pivotal role in supporting survival of MCL cell lines. Genetic knockdown or small molecule targeting induced significant caspase-dependent apoptosis. Interestingly, the MCL cell lines sensitive to HDAC8 inhibition were $SOX11^+$, which is typically considered the more aggressive MCL subtype, cMCL. SOX11 is a neuronal transcription factor that regulates PAX-5 expression (25). In B cells, PAX-5 blocks expression of BLIMP-1, thus preventing terminal B cell differentiation. SOX11 expression is present in ~95% cMCL cases and in the rare cyclin D1-negative MCL subtype, SOX11 is a diagnostic marker for MCL. The significance of reduced sensitivity to HDAC8 inhibition in the $SOX11^-$ cell line JVM-2, was not directly validated in this study, due to limited access to patient samples to compare $SOX11^-$ and $SOX11^+$ MCL. However, clinically $SOX11^+$ MCL cells are more aggressive, while $SOX11^-$ MCL is associated with an indolent behavior, until progression. Importantly, MCL harboring p53 mutations have a poor clinical outcome regardless of SOX11 expression and there is an unmet need in this patient population to provide efficacious therapeutic options and improve overall survival. MCL cell lines sensitive to HDAC8 inhibition were either; wild-type p53, had alterations in p53 function, or deficiencies in p53 regulated pathways, indicating, that sensitivity to HDAC8 inhibition is independent of p53 function. There was an indication that MCL cell lines representing cMCL, $SOX11^+$, had the highest sensitivity to HDAC8 inhibition and knockdown. This should be followed up, ideally by examining sensitivity

of MCL patient tumors to HDAC8 inhibition, that have been evaluated for SOX11 and TP53 expression.

Additionally, this dissertation reports, PCI-34051 may induce another mechanism that has an interplaying role in sensitivity to HDAC8 inhibitor treatment. Preliminary results indicate PCI-34051 influences Protein Kinase A (PKA) activity. The cAMP/PKA/CREB pathway is a pivotal regulator of apoptosis in leukemia and lymphoma (178). Both the Jeko-1 and Z138 cell lines treated with PCI-34051 show an increase in phosphorylation of the Serine 39 residue on HDAC8 which leads to inactivation of HDAC8 **(Figure 4.5A and B)**. This suggests that PCI-34051 not only blocks HDAC8 through binding to the HDAC8 "selective" pocket but also through activation of PKA which phosphorylates HDAC8 inactivating its activity. This suggests that this may increase the potency or duration of response to PCI-34051 treatment.

HDAC8 negatively regulates the PKA pathway through association with protein phosphatase 1 (PP1). Interaction of HDAC8 and PP1 results in loss of cAMP response element-binding protein (CREB) transcription factor activation, and transcription (131). In this case, HDAC8 acts as a scaffolding protein, recruiting the interaction of PP1 with CREB. PP1 dephosphorylates the serine residue 133 on the CREB protein, inactivating its activity, and negatively regulating transcription. This results in repression of the PKA pathway through negative regulation of CREB, a critical transcription factor involved in many cellular processes including regulating apoptosis, IFNγ production, and cell cycle progression. Thus, inhibiting HDAC8 activity can causes upregulation of CREB and enhanced cAMP/PKA signaling (131).

PCI-34051 is also known to induce $Ca^{2+}$ release from the endoplasmic reticulum, in a phospholipase C gamma (PLCγ) dependent manner (107). PLCγ induces hydrolysis of membrane bound phosphatidylinositol-4,5-bisphosphate (PIP2) generating inositol phosphate,

Inositol 1,4,5-triphosphate (IP3). Inositol 1,4,5-triphosphate translocates to the endoplasmic reticulum and binds to the calcium channel receptor, Inositol 1,4,5-triphosphate Receptor 3 (IP3 receptor), forming a ligand-gate ion channel and subsequent release of calcium ions from intracellular stores. Calcium can activate many cellular processes including apoptosis (179).

Calcium ions can also induce the release of soluble adenylyl cyclase, which utilizes ATP as a substrate to convert adenosine monophosphate into cyclic adenosine monophosphate (cAMP). cAMP acts as a second messenger, induces activation of PKA and is critical for many biological processes, including apoptosis. While cAMP is most notably recognized as being activated through G-protein coupled receptor activation, pharmacological inhibitors can induce calcium release which acts as a direct modulator of cAMP activity. Both calcium and cAMP act as secondary messengers. cAMP/PKA pathway can trigger a cascade of events, including transcriptional upregulation of BCL2 family pro-apoptotic proteins, BCL2 Associated X, apoptosis regulator (BAX), and downregulation of anti-apoptotic proteins, BCL2 Associated Agonist of cell death (BAD), resulting in caspase activation and apoptosis. However, the outcome is cell dependent and needs further investigation because activation of cAMP can contribute to disease progression in some cancer types. For example, previous reports have provided evidence that an increase in cAMP can cause apoptosis in lymphoma cells (180). The murine T cell lymphoma cell line, S49, is sensitive to cAMP/PKA activation, as it induces activation of caspase dependent apoptosis, independent of FAS activity (181).

Interestingly, abrogating HDAC8 activity may affect primary immune cells differently. This study reports, NK cells are exceedingly resistant to HDAC8 inhibitor treatment compared to MCL cell lines. PCI-34051 did not impede survival or proliferation of rested, cytokine stimulated, or highly proliferative NK cells (LAKs). Indicating NK cells at rest, stimulated by

cytokines, or antigen stimulation will not be negatively impacted by HDAC8 inhibition. Furthermore, NK cells treated with the HDAC8 selective inhibitor maintain full effector potential, with stable expression of key anti-tumor receptors; NKG2D, NKp44, NKp30, NKp46, and CD16.

It is unknown if HDAC8 is involved in regulating activation of NK cells through PTMs. Future experiments should include investigating if HDAC8 regulates PTMs on NK activation receptors. This should include using an unbiased approach, to identify HDAC8 specific substrates, specifically focusing on acetylation changes on activation receptors. NK cells treated with PCI-34051 retain their intrinsic cytotoxicity and CD16-dependent ADCC function, indicating HDAC8 targeted therapy will not systemically impact immunotherapeutic response with IgG directed therapies. However, HDAC8 may have a regulatory role in activation of these receptors and warrants further studies.

Healthy primary human NK cells are resistant to the cytotoxic effects of PCI-34051 and treatment increases the abundance of IFN$\gamma^+$ NK cells in a dose-dependent manner. IFNγ is a powerful pro-inflammatory molecule, responsible for activating apoptosis of tumor cells, recruiting immune cells, and enhancing immune cell cytotoxic activity. Conversely, IFNγ is also indicated in immunosuppression, causing upregulation of the immune checkpoint inhibitor PD-L1 on tumor cells. Immunosuppression is induced upon association of PD-1 and PD-L1 receptors. This dissertation reports, IFNγ secretion was comparable to the cytokine stimulated NK control, indicating that abrogating HDAC8 activity will not enhance immunosuppressive characteristics associated with IFNγ.

HDAC8 has been reported to play a role in protein stability. Therefore, it is plausible that HDAC8 negatively regulates IFN$\gamma^+$ protein stability. Future experiments should focus on

determining if HDAC8 regulates protein stability of IFNγ$^+$ using the cycloheximide chase assay. Cycloheximide prevents translational elongation, therefore, allowing for interrogation of protein stability.

PCI-34051 significantly increases the abundance of IFNγ$^+$ NK cells. However, this does not increase cytolytic responses *in vitro,* at least in short term assays this was not evident. An increase in IFNγ$^+$ NK cells may have important biological significance *in vivo,* as it has been shown to increase NK persistence and memory recall response (159). Due to the limitations of NK expansion *ex vivo,* challenging NK cells with repetitive antigen stimulation is not possible. However, *in vivo* studies, using a humanized mouse model, should investigate if knocking out HDAC8 in human NK cells leads to memory-like characteristics in the presence of the tumor immunosuppressive microenvironment.

An interesting study would be to investigate if the triple combination: PCI-34051, lenalidomide, and Rituximab, can synergistically enhance NK activity and sensitivity to rituximab therapy. HDAC8 inhibition sensitizes MCL to apoptosis, lenalidomide is known to enhance NK cell activity through its immunomodulatory mechanisms, and Rituximab induces NK ADCC, complement dependent cytotoxicity, and antibody-dependent phagocytosis. Combination therapy including PCI-34051, lenalidomide, plus rituximab could be a potent non-chemotherapy-based therapy where lenalidomide serves to amplify the activity.

NK cell function, in MCL patients, is critical for clinical response with Rituximab. Newer targeting agents such as Ibrutinib have shown some clinical response, however it has been shown to block ADCC cytolytic responses and can lead to clonal outgrowth of MCL resistant to Rituximab therapy (42, 43). Thus, combinational approaches using Rituximab in combination with selective HDAC inhibitors may provide the needed benefit to improve overall response

rates and duration of response in MCL patients. The findings in this report, indicate blocking HDAC8 activity in MCL patients will not affect response to mAb therapies, dependent on NK ADCC. While HDAC8 inhibition may not have immune-sensitize MCL cells to NK cytotoxic responses, MCL cell lines undergo significant apoptosis with HDAC8 targeted treatment.

Notably, this dissertation defines HDAC8 as a novel target in the treatment of MCL. In addition, HDAC8 inhibitor therapy preserves NK ADCC function, and enhances the abundance of IFN$\gamma^+$ NK cells, which may indicate enhanced *in vivo* activity and/or persistence. Lastly, this report provides evidence that targeting HDAC8 in patients will not disrupt response to antibody therapy dependent on NK cells for clinical efficacy.

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# APPENDICES

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