

Curriculum Vitae

Name: Irina Tiper

Email: irinavtiper@gmail.com

Degree and date to be conferred: Doctor of Philosophy, 2016

Education:

- 2011-2016 University of Maryland, Baltimore
Baltimore, MD
Doctor of Philosophy, Molecular Medicine
July 2016
- 2007-2011 University of Maryland, Baltimore County
Baltimore, MD
Bachelor of Science, Biological Sciences
May 2011

Publications:

1. **Tiper IV**, East, JE, Subrahmanyam PB, and Webb TJ. Sphingosine 1–phosphate signaling impacts lymphocyte migration, inflammation, and infection. *Pathogens and Disease*, in press.
2. Shissler SC, Bollino DR, **Tiper IV**, Bates J, Derakhshandeh R, and Webb TJ. Immunotherapeutic strategies targeting Natural killer T cell responses in cancer. *Immunogenetics*, in press.
3. **Tiper IV**, Temkin SM, Spiegel S, Goldblum SE, Giuntoli RL, Oelke M, Schneck JP, and Webb TJ. VEGF potentiates GD-mediated immune suppression by human ovarian cancer cells. *Clin. Can. Res.* 2016
4. Obata F, Subrahmanyam PB, Vozenilek AE, Hippler LM, Jeffers T, Tongsuk M, **Tiper I**, Saha P, Jandhyala DM, Kolling GL, Latinovic O, Webb TJ. Natural killer T (NKT) cells accelerate Shiga toxin type 2 (Stx2) pathology in mice. *Front. Microbiol*, 2015. doi: 10.3389/fmicb.2015.00262
5. Sohn S, **Tiper I**, Japp E, Sun W, Tkaczuk K, Webb TJ. Development of a qPCR Method to Rapidly Assess the Function of NKT Cells. *J. Immunol. Methods*, 2014.
6. Li J, Sun W, Subrahmanyam PB, Page C, Younger KM, **Tiper I**, Frieman M, Kimball AS, Webb TJ. NKT Cell Responses to B Cell Lymphoma. *Med. Sci.* 2014

Abstracts:

1. **Tiper I**, Webb TJ. HDAC inhibitors enhance CD1d-dependent NKT cell responses to lymphoma. *Journal of Immunology* 2016
2. **Tiper I**, Webb TJ. Epigenetic regulation of CD1d-mediated antigen presentation by B cell lymphomas. *Journal of Immunology* 2015, 194(Suppl 1): P211

3. **Tiper I**, Webb TJ. Epigenetic regulation of CD1d-mediated antigen presentation in B cell lymphoma. *Journal for Immunotherapy of Cancer* 2014, **2**(Suppl 3):P177

Oral presentations:

- April 2016 “Confused Identity: Cancer Cells as Antigen Presenting Cells.”
Shepherd University Department of Biology
Shepherdstown, WV
- March 2016 “VEGF potentiates GD-mediated immune suppression by human ovarian cancer cells.”
University of Maryland Graduate Research Conference
Baltimore, MD
- February 2016 “Epigenetic Modulation of CD1d-mediated Antigen Presentation.”
University of Maryland Membrane Biology Student Seminar Series
Baltimore, MD
- June 2015 “Epigenetic Modulation of CD1d-mediated Antigen Presentation.”
University of Maryland Graduate Research Conference
Baltimore, MD
- February 2015 “Epigenetic Modulation of CD1d-mediated Antigen Presentation.”
University of Maryland Membrane Biology Student Seminar Series
Baltimore, MD
- December 2014 “Epigenetic Modulation of CD1d-mediated Antigen Presentation.”
University of Maryland Molecular Medicine Student Seminar Series
Baltimore, MD

Select honors and awards:

- 2015-2016 Great Lakes National Scholar, Great Lakes Higher Education Corporation
2015 Interdisciplinary Honor Cord, UMB
2015 President’s Student Leadership Institute, UMB
2014-2015 American Association of Immunologists Career in Immunology Fellowship, American Association of Immunologists
2014 Outstanding Research Poster Award, Tumor Immunology and Immunotherapy Retreat, UMB
2013 Outstanding Research Poster Award, UMB Cancer Biology Retreat
2013 Outstanding Research Poster Award, UMB Graduate Research Conference

Abstract

Title of Dissertation: Mechanisms of tumor evasion from NKT cell-mediated immunosurveillance.

Irina Tiper, Doctor of Philosophy, 2016

Thesis Advisor: Tonya J. Webb, Associate Professor

Department of Microbiology and Immunology

Natural killer T (NKT) cells are a unique subset of CD1d-restricted T cells that play an important role in mediating anti-tumor responses. NKT cells produce large amounts of cytokines and can directly mediate tumor cell lysis. However, NKT cells are numerically reduced and functionally impaired in cancer patients and the mechanisms by which tumor suppress NKT cell activation are poorly understood. Given that cancer cells possess epigenetic abnormalities, in addition to numerous genetic alterations, we hypothesize that tumors use epigenetic mechanisms to suppress anti-cancer immune responses by dysregulating antigen presentation and secreting soluble inhibitory factors. To test our hypothesis, lymphoma cells were pretreated with histone deacetylase inhibitors (HDACi) and then we assessed their ability to activate NKT cells. Treatment of B cell lymphomas with HDACi enhances anti-tumor immune responses by inducing CD1d-mediated antigen presentation, inhibiting STAT3, and subsequently, decreasing the production of STAT3-associated inflammatory cytokines. Moreover, we found that the enhanced immunogenicity observed following treatment with HDACi was HDAC2-dependent. Specifically, we performed chromatin immunoprecipitation (ChIP) to identify the HDAC that binds to the CD1d promoter. We found that HDAC2 binds to the CD1d promoter and negatively regulates CD1d transcription. In addition, we sought to investigate other mechanisms by which tumors suppress CD1d-mediated NKT cell activation. We found that, similar to lymphomas, ovarian cancers

secrete molecules that can inhibit NKT cell activation. Additionally, we established a link between angiogenic factor secretion and suppression of antigen presentation, suggesting that existing anti-angiogenic therapies can positively impact anti-tumor immune responses. Thus, we identified cell-intrinsic and -extrinsic mechanisms by which tumors suppress CD1d-mediated antigen presentation, which can be targeted in an immunotherapeutic setting. Overall, our studies implicate roles of HDACi and anti-angiogenic therapies in immunotherapeutic approaches.

Mechanisms of Tumor Evasion from NKT cell-mediated Immunosurveillance

By

Irina Tiper

Dissertation submitted to the Faculty of the Graduate School of the University of Maryland,
Baltimore in partial fulfillment of the requirements for the degree of Doctor of Philosophy

2016

To my mentor:

Thank you for helping me become a better scientist and person.

You gave me the guidance to help me grow.

You gave me the freedom to let me grow.

Table of Contents

List of Tables.....	V
List of Figures.....	VI
List of Abbreviations.....	VIII

Chapter 1: Introduction

1.1 Cancer: overview.....	1
1.2 Carcinogenesis: hallmarks of cancer.....	5
1.3 Lymphoma.....	6
1.4 Ovarian cancer.....	10
1.5 HDACs and HDAC inhibitors.....	14
1.6 Epigenetic modulation of antigen presentation.....	26
1.7 Anti-angiogenic therapies in ovarian cancer	30
1.8 The role of the immune system in cancer elimination.....	34
1.9 Innate and adaptive immune responses in cancer.....	38
1.10 NKT cells.....	40

Chapter 2: Materials and Methods

2.1 Cell lines.....	50
2.2 Antigens and inhibitors.....	51
2.3 NKT and T cell assays.....	52
2.4 Western blotting.....	53
2.5 Immunoprecipitation.....	54
2.6 Flow cytometry and LEGENDplex.....	54
2.7 Conditioned medium experiments.....	54
2.8 WST assay.....	55
2.9 Chromatin immunoprecipitation.....	55
2.10 HDAC2 knockdown.....	55
2.11 shHDAC2 JeKo-1 limited dilution assay.....	56
2.12 Mice.....	56
2.13 Statistical analyses.....	56

Chapter 3: Cell-intrinsic effects of HDACi

3.1 Introduction.....	58
3.2 Results.....	60
3.3 Discussion.....	79

Chapter 4: Cell-extrinsic effects of HDACi

4.1 Introduction.....	82
4.2 Results.....	84
4.3 Discussion.....	88

Chapter 5: Effects of tumor-secreted factors on antigen presentation

5.1 Introduction.....	90
5.2 Results.....	92
5.3 Discussion.....	101

Chapter 6: Further discussion and future directions

6.1 Cell-intrinsic effects of HDACi on CD1d-mediated antigen presentation.....	105
6.2 Cell-extrinsic effects of HDACi on CD1d-mediated antigen presentation.....	113

Thesis summary.....	117
----------------------------	------------

References.....	120
------------------------	------------

List of Tables

Chapter 1

Table 1.1: Estimated number of cancer cases and cancer-related deaths in 2016.....	3
Table 1.2: Estimated number of gynecological cancer cases and deaths in 2016.....	3
Table 1.3: Estimated number of hematologic malignancy cases and deaths in 2016.....	4
Table 1.4: The most common pathway alterations in mantle cell lymphoma.....	9
Table 1.5 Five-year survival rates for different types of ovarian cancer, by stage.....	13
Table 1.6 HDAC-mediated regulation of cytokine transcription.....	19
Table 1.7: Summary of findings of the SCR study at the Knight Cancer Institute.....	25

Chapter 3

Table 3.1: Summary of HDACi characteristics and functional effects.....	69
---	-----------

List of Figures

Chapter 1

Figure 1.1: Canonical functions of histone deacetylases.....	16
Figure 1.2: TSA within the histone deacetylase catalytic core.....	24
Figure 1.3: Functions of VEGF in the tumor microenvironment.....	31
Figure 1.4: Signal transduction and biological processes mediated by VEGF receptors.....	32
Figure 1.5: Tumor escape through cell contact-independent mechanisms.....	37
Figure 1.6: NKT cells bridge innate and adaptive immune response.....	44

Chapter 3

Figure 3.1: Human NKT cells mediate tumor regression in NSG mice.....	61
Figure 3.2: Characterization of a new mouse MCL cell line.....	62
Figure 3.3: HDAC inhibitor treatment enhances CD1d- and MHC class II-mediated antigen presentation.....	65
Figure 3.4: TSA enhances antigen presentation by mouse lymphoma cell lines, including a primary mouse MCL line.....	66
Figure 3.5: TSA enhances antigen presentation by human MCL cell lines.....	67
Figure 3.6: Effects of Panobinostat and MC1568 on CD1d-mediated antigen presentation.....	68
Figure 3.7: HDAC inhibitor treatment rapidly induces CD1d mRNA and protein.....	73
Figure 3.8: HDAC2 binds to the CD1D promoter.....	74
Figure 3.9: STAT3 does not bind to the CD1D promoter.....	75
Figure 3.10: HDAC2 is the main HDAC regulating CD1d-mediated antigen Presentation.....	76

Figure 3.11: Slight changes in CD1D levels significantly affect NKT cell activation.....	78
Figure 3.12: HDAC expression and patient prognosis.....	81

Chapter 4

Figure 4.1: HDAC2 and STAT3 exist in a complex and TSA treatment inhibits STAT3.....	85
Figure 4.2: TSA inhibits inflammatory cytokine secretion.....	86
Figure 4.3: IL-10 inhibits CD1d-mediated antigen presentation.....	87

Chapter 5

Figure 5.1: Conditioned medium from ovarian cancer cell lines inhibits CD1d-mediated antigen presentation.....	95
Figure 5.2: VEGF inhibits CD1d-dependent NKT cell function.....	96
Figure 5.3: Avastin treatment restores CD1d-mediated antigen presentation.....	97
Figure 5.4: Inhibiting VEGF blocks GD3 in ovarian cancer cells.....	98
Figure 5.5 Ovarian cancer–associated GD3 inhibits NKT cell responses.....	99
Figure 5.6: Ovarian cancers can present antigen to NKT cells and induce their activation.....	100
Figure 5.7: Proposed model of cross-talk between the VEGF, MAPK, and GD3 signaling pathways.....	102

Chapter 6

Figure 6.1: Effects of HDACi on lymphocyte activation.....	111
Figure 6.2: Ganglioside synthesis pathway.....	113

List of Abbreviations

5' RACE: rapid amplification of cDNA ends

α -GalCer: α -Galactosylceramide

ACTB: beta-actin

AICD: activation induced cell death

AKT: protein kinase B

ANOVA: analysis of variance

APC: antigen presenting cell

ARF: alternative reading frame

ASCO: American Society of Clinical Oncology

ATM: ataxia telangiectasia mutated

Bcl-xL: B cell lymphoma-extra large

CIITA: MHC class II transactivator

CD1d: cluster of differentiation 1 d

CDC: Center for Disease Control

CDK: cyclin dependent kinase

ChIP: chromatin immunoprecipitation

CLL: chronic lymphocytic leukemia

CTCL: cutaneous T cell lymphoma

CR: complete response

CtBP: c-terminal-binding protein

DC: dendritic cell

DLBCL: diffuse large B cell lymphoma

DNA: deoxyribonucleic acid

DNMT: DNA methyltransferase

DNMTi: DNA methyltransferase inhibitor

ELISA: enzyme-linked immunosorbent assay

EMT: epithelial-mesenchymal transition

ERK: Extracellular signal-regulated kinases

FBS: fetal bovine serum

FIGO: International Federation of Gynecology and Obstetrics

FITC: fluorescein isothiocyanate

FL: follicular lymphoma

FMCL: female mantle cell lymphoma

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GFP: green fluorescent protein

GM-CSF: granulocyte-macrophage colony-stimulating factor

HAT: histone acetyltransferase

HDACs: histone deacetylases

HDACi: histone deacetylase inhibitor(s)

HL: Hodgkin lymphoma

HLA: human leukocyte antigen

HSA: human serum albumin

IACUC: institutional animal care and use compliance

IBC: institutional biosafety committee

IFN: interferon

IRF1: interferon response factor 1

IL: interleukin

IB: immunoblot

IP: immunoprecipitation

JAK: Janus kinase

KRAS: Kirsten rat sarcoma viral oncogene homolog

LEF-1: lymphoid enhancer factor

MAPK: mitogen-activated protein kinase

MCL: mantle cell lymphoma

MDM2: mouse double minute 2 homolog

MFI: mean fluorescence intensity

MHC: major histocompatibility

MOI: multiplicity of infection

mTOR: mammalian target of rapamycin

myc: myelocytomatosis viral oncogene homolog

MZL: marginal zone lymphoma

NF- κ B: Nuclear Factor Kappa-B

NHL: non-Hodgkin lymphoma

NK: Natural killer

NKG2D: natural killer group 2D

NKT: Natural killer T

NRP: neuropilins

NSG: non-obese diabetic *scid* common gamma chain deficient

NEU: neuraminidase

ORR: objective response rate

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PD-1: programmed cell death-1

PFS: progression free survival

PGF: placenta growth factor

PI3K: phosphoinositide-3-kinase

PIP₂: Phosphatidylinositol 4,5-bisphosphate

PFS: progression free survival

PMA: phorbol 12-myristate 13-acetate

PTCL: peripheral T cell lymphoma

PR: partial response

RB: retinoblastoma protein

RIME: rapid immunoprecipitation mass spectrometry of endogenous proteins

RNA: ribonucleic acid

RTK: receptor tyrosine kinase

SEER: Surveillance, Epidemiology and End Results Program

sh: short hairpin

SIRTi: sirtuin inhibitor

SLE: systemic lupus erythematosus

SOX11: Sex Determining Region Y Box 11

SP1: specificity protein 1

ST-II: sialyltransferase II

STAT3: Signal transducer and Activator of Transcription 3

SXY: S box, X box, and Y box

TAA: tumor associated antigen

TAP: transporter associated with antigen processing

TCR: T cell receptor

TGF- β : transforming growth factor- β

TIL: tumor-infiltrating lymphocytes

TKI: tyrosine kinase inhibitor

TLR: toll-like receptor

TNF: tumor necrosis factor

TP53: tumor protein p53

TRAIL: TNF-related apoptosis-inducing ligand

Treg: regulatory T cell

TSA: trichostatin-A

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

VPA: valproic acid

WEHI: Walter and Eliza Hall Institute of Medical Research

WHO: World Health Organization

WST: water soluble tetrazolium

Chapter 1: Introduction

1.1 Cancer: overview

Cancer is the leading cause of death worldwide, accounting for over 8 million deaths and 14 million new cases [1]. The World Health Organization (WHO) reports that there will be 22 million cases of cancer within the next two decades [1]. More than 60% of new cases worldwide occur in Africa, Asia, and South and Central America [1]. According to the Surveillance, Epidemiology and End Results (SEER) Program's statistical modeling, rates of new cancer have been falling by an average of 1.0% each year over the last ten years, with death rates falling by an average of 1.5% each year [2].

The most common types of cancer among men are lung, prostate, colon and rectum, stomach and liver [2]. Among women, the most common types of cancer are breast, colon and rectum, lung, cervix, and stomach [2]. Estimated new cases and deaths for the top five leading cases of cancer among both men and women are summarized in Table 1.1. Five-year survival rates vary widely, depending on the tumor type, but SEER reports that 66.9% of patients with cancer of any type survive 5 or more years after diagnosis [2]. In 2016, it is estimated that there will be 1,685,210 new cases of cancer and 595,690 cancer-related deaths in the United States [2]. Thus, while rates have been falling, the magnitude of the cancer burden is unprecedented. In our studies, we focus on a solid and hematologic malignancy with the goal of identifying overarching mechanisms that contribute to decreased anti-tumor immune responses.

Among women, gynecologic cancers affect the cervix, ovaries, uterus, vagina, and vulva (Table 1.2). There are two other types of rare gynecological cancers: fallopian tube and primary peritoneal cancer. The Center for Disease Control (CDC) reports that each year, in the United States, over 70,000 women are diagnosed with gynecologic cancers and over 26,000 die from it

[3]. Each year, approximately 20,000 women are diagnosed with ovarian cancer [3]. Ovarian cancer is discussed in greater detail in Chapter 1.3.

While solid malignancies comprise the majority of the leading new cases of cancer, malignancies arising in hematopoietic and lymphoid cells, termed hematologic malignancies, are high in incidence, relative to the total cancer diagnoses [4]. Hematological malignancies are divided into broad categories: leukemia, Hodgkin lymphoma (HL), non-Hodgkin's lymphoma (NHL), and myeloma [4]. The 2016 statistics are summarized in Table 1.3. NHL is the 7th most common type of cancer [2]. It is expected that there will be 72,580 new NHL cases and 20,150 NHL-related deaths in 2016 in the United States [2]. There are more cases of NHL than of any other type of hematological malignancy (Table 1.3). While the incidence of cancer has been decreasing, the incidence of NHL, including mantle cell lymphoma (MCL), has been falling [5-7]. Thus, further studies of this disease are needed in order to understand the unique trends in NHL rates. NHL, and specifically MCL, will be discussed in Chapter 1.2.

Type of cancer	Estimated new cases in 2016	Estimated deaths in 2016
Breast cancer (female)	246,660	40,450
Lung and bronchus cancer	224,390	158,080
Prostate cancer	180,890	26,120
Colon and rectum cancer	134,490	49,190
Bladder cancer	76,960	16,390
Cancer of any site	1,685,210	595,690

Table 1.1: Estimated numbers of cancer cases and cancer-related deaths in 2016. Data compiled from Surveillance, Epidemiology and End Results Program (SEER).

Type of cancer	Estimated new cases in 2016	Estimated deaths in 2016
Cervical/uterine cancer	12,990	4,120
Ovarian cancer	22,280	14,240
Vulvar cancer	5,950	1,110
Total cases	71,500	26,500

Table 1.2: Estimated number of gynecological cancer cases and deaths in 2016. Data compiled from Surveillance, Epidemiology and End Results Program (SEER) and Center for Disease Control (CDC).

Type of hematologic malignancy	Estimated new cases in 2016	Estimated deaths in 2016
Hodgkin lymphoma	8,500	1,120
Non-Hodgkin lymphoma	72,580	20,150
Leukemia	60,140	24,400
Myeloma	30,330	12,650

Table 1.3: Estimated number of hematologic malignancy cases and deaths in 2016. Data compiled from Surveillance, Epidemiology and End Results Program (SEER).

1.2 The hallmarks of cancer

Carcinogenesis is a complex and multi-layered process that is facilitated by mutations in oncogenes and tumor suppressor genes [8]. Tumors have aberrations in intracellular signaling pathways regulating growth, survival, differentiation, cell death, and metabolism [8]. Cancer cells are able to proliferate uncontrollably due to self-sufficiency in growth signals, limitless replicative potential, and insensitivity to anti-growth signals [8]. Moreover, they can evade apoptosis and metastasize, aided by their ability to induce angiogenesis [8]. These traits are known as the hallmarks of cancer [8]. The hallmarks of cancer were first summarized by Hanahan and Weinberg in 2000, but it was not until 2010 that the role of the immune system in cancer progression was recognized as an emerging hallmark [8]. Thus, our understanding of the role of the immune system in tumor progression is evolving.

One of the enabling characteristics of cancer is acquisition of aberrations within the genome, which may be aided by epigenetic alterations that affect not only cancer cells, but have profound effects on the tumor stroma [8]. Here, we examine epigenetic mechanisms that allow tumor escape from the immune system and link these epigenetic changes to a signaling pathway known to be important for tumor growth. Our emphasis is on the role of Signal Transducer and Activator of Transcription 3 (STAT3) in mediating an inflammatory environment that facilitates immune suppression. Moreover, we investigate how this signaling pathway alters antigen presentation through cell-extrinsic mechanisms. Finally, we define a novel immunosuppressive role for the major angiogenic factor vascular endothelial growth factor (VEGF) by examining its effects on antigen presentation. This work addresses hallmarks of cancer and puts them in the perspective of an emerging hallmark, evasion of immune destruction. Thus, this work intertwines the fields of cancer biology, immunology, epigenetics, and cell signaling.

1.3 Lymphomas

Chapter 1.1 provided an overview of the rates of cancer and highlighted the fact that incidence of lymphoma has been increasing. Thus, elucidation of the mechanisms underlying the pathogenesis of this disease is critical to understanding why the incidence of this disease has been rising.

Lymphoma is a type of cancer under the broad umbrella of hematologic malignancies and is further classified into Hodgkin and non-Hodgkin lymphoma (HL and NHL, respectively). The majority of lymphomas are of B cell origin, arising from germinal center B cells or from B cells that have transited through the germinal center [9]. HL are characterized by the presence of Hodgkin and Reed-Sternberg cells [10]. NHL is a heterogeneous group of both B and T cell neoplasms, which includes many types of lymphomas, including mantle cell lymphoma, follicular lymphoma, and maltoma [11]. Given the heterogeneity of NHL, identification of the etiology and establishment of a clear classification system are crucial for the treatment of these malignancies [11].

Historically, lymphomas have been defined by the clinicopathological behavior and grouped into broad categories [12]. With the advent of novel technologies, our understanding of the molecular mechanisms of lymphoma pathogenesis increased, thus shifting the paradigm of lymphoma diagnosis and treatment. For example, identification of the t(11,14) translocation and overexpression of cyclin D1 definitively distinguishes mantle cell lymphoma (MCL) from other subtypes of aggressive B cell lymphomas [12].

MCL remains a largely incurable disease, with a median survival of 5-7 years [13,14]. Although MCL was previously thought to be a homogeneous disease, its heterogeneity is now better understood, with many pathways discovered to be of prognostic value for tumor growth and

patient outcomes [15,14]. However, the mechanisms by which these pathways contribute to MCL pathogenesis are not well understood. Overexpression of cyclin D1 is thought to be a causative factor for initial oncogenic events in MCL pathogenesis, which is followed by aberrations in a variety of molecular pathways [13]. Cyclin D1 overexpression precipitates subsequent oncogenic events through its ability to cause cell cycle deregulation, promote chromosome instability, and aberrant transcriptional regulation (Table 1.4).

The most common type of MCL is marked by genetic instability and SOX11 (Sex Determining Region Y Box 11) expression [13]. The second and less common subtype of MCL is genetically stable and has low SOX11 expression [13]. SOX11 promotes tumor growth and is one of the common pathways deregulated in MCL. Table 1.4 summarizes the other common pathways found to be deregulated in MCL. For example, STAT3 is one of the pathways found to be upregulated in MCL (Table 1.4). Cross-talk between NF- κ B and STAT3 is discussed later in this chapter. STAT3 has a role in inhibition of apoptosis, enhanced cell proliferation, angiogenesis and inflammation (Table 1.4). Our work examines the role of STAT3 in secretion of immunosuppressive cytokines by MCL cells and the effects of these cytokines on antigen presentation. Importantly, several of the pathways deregulated by MCL (including NF- κ B, STAT3, and PI3K) have been shown to play a role in antigen presentation [16-18].

The biological complexity underlying MCL pathogenesis prompted the search for biomarkers for diagnosis. Immunophenotyping, which compares the MCL cells to the immunophenotype of mantle zone lineage from which they derive [19], and detection of cyclin D1 overexpression or the chromosomal translocation t(11,14), are the classic laboratory approaches used to diagnose the disease [15]. However, a fraction of patients do not have cyclin D1 translocation [15,20] and SOX11 can be detected in both cyclin D1 positive and negative MCL

cases [21]. While SOX11 negativity was initially considered an indicator of indolent MCL [14], a study demonstrated that these patients experienced a more aggressive disease progression, with underlying p53 upregulation serving as a significant molecular marker [22]. Therefore, it is important to determine whether multiple pathways better predict disease outcomes than a single pathway.

Molecular classification is thus paving the way to allow individually optimized treatment strategies [23]. Gene expression profiling may benefit in cases with low cell proliferation, but this approach has not yet been applied in routine clinical practice [24]. In our studies, we sought to identify factors which allow tumor evasion from recognition by the immune system. Profiling of tumors for expression of these factors may help identify patients who would benefit from immunotherapy. We discuss the application of novel methods for identification of potential prognostic factors in Chapter 6.1

Pathway	Function
cyclin D1	Cell cycle deregulation, promotion of chromosome instability, and aberrant transcriptional regulation
SOX11	Promotion of tumor growth
INK4a/CDK4/RB1	Activation of retinoblastoma (Rb) protein and enhanced G ₁ -S cell-cycle progression
ARF/MDM2/TP53	Impaired DNA repair pathways
ATM	Deregulation of the ability to sense DNA damage and DNA repair pathways
WHSK1	Chromatin modifier regulating genes involved in proliferation and cell cycle control
NF-κB	Cell proliferation and protection from apoptosis
PI3K/AKT/mTOR	Enhanced survival, growth and proliferation
STAT3	Inhibition of apoptosis, enhanced cell proliferation, angiogenesis and inflammation

Table 1.4: The most common pathway alterations in mantle cell lymphoma (as reviewed by Campo and Rule [13] and Jares et al. [25]).

1.4 Ovarian Cancer

In the United States, ovarian cancer is the fifth most common cause of cancer death among women. The principal clinical challenge for this disease is that the majority of patients present with late stage disease – 70% of patients present with International Federation of Gynecology and Obstetrics (FIGO) Stage III or IV disease at the time of diagnosis, with various factors contributing to late diagnosis [26]. Despite improvements in treatment, five-year survival rates of patients with advanced ovarian cancer remain less than 50% [27,28]. Ovarian cancer is “not an entirely silent disease” [26], but the non-specific symptoms make diagnosis difficult. The symptoms include abnormal vaginal discharge, pelvic pain, back pain, bloating, and changes in bathroom habits [26]. Given that survival rates decrease drastically as the stages progress (Table 1.5), early detection methods are needed. For example, while patients with stage I epithelial ovarian cancer have of 90% five-years survival rates, only 17% of patients with stage IV cancer survive past five years. Moreover, within a stage, there are drastic differences in survival based on the tumor type (Table 1.5), suggesting that different types of ovarian cancers have different aberrations that are more difficult to target than others. These molecular aberrations are reviewed below.

Early detection of ovarian cancer allows for curative surgery [29]. Platinum-based chemotherapy provides a high response rate in the majority of patients with serous carcinoma [29]. Cisplatin (platinum agent) and paclitaxel (taxane) are offered as the standard of care. Women who have a complete response (CR) lasting at least six months are considered to have “platinum-sensitive” cancer and are thus candidates for re-treatment with platinum-based regimens. Patients with mucinous and clear cell cancers have worse prognosis and standard chemotherapy has modest

activity in these types of cancer [29]. Targeted molecular therapies are thus needed in this patient population.

Epithelial ovarian cancer is now understood to be a heterogeneous disease and this understanding has been guiding treatment strategies. Our work focuses on epithelial ovarian cancer. Importantly, the five-year survival rates of patients with epithelial ovarian cancer decline drastically as the stage progresses. While patients diagnosed with stage I cancer have a 90% survival rate, those diagnosed with stage IV cancer have only a 17% survival rate (Table 1.5). Thus, it is important to understand the molecular aberrations that occur as the cancer progresses. Molecular profiling identified pathways that allow delineation of high-grade serous, low-grade serous, mucinous, endometrioid, and clear cell cancers [30]. The phosphoinositide-3-kinase (PI3K) pathway is mutated in low grade endometrioid tumors, while high grade serous tumors have mutations in tumor protein p53 (TP53) [30]. Early clinical studies showed that mutant p53 contributes to chemoresistance in ovarian cancer patients[31]. Mucinous tumors often have KRAS mutations, with KRAS mutations correlating with histological type of tumors, rather than with stage or grade [30,32].

An important breakthrough in the treatment of ovarian cancer is the application of anti-angiogenic therapies in clinical practice (reviewed in greater detail in Chapter 1.5). Bevacizumab/Avastin has shown single-agent activity in women with recurrent epithelial ovarian cancer [30]. Patients receiving standard-of-care chemotherapy (carboplatin and paclitaxel) and bevacizumab, with bevacizumab given as maintenance, experienced an increase in progression-free survival (PFS) of 3.8 months [30]. In patients with platinum-resistant disease, the addition of bevacizumab significantly improved PFS in patients receiving paclitaxel, topotecan, or

doxorubicin [30]. Thus, anti-angiogenic therapies may benefit patients with platinum-resistant disease.

In our studies, we established a link between anti-angiogenic therapies and immune responses, demonstrating that targeting of angiogenesis enhances immune responses to cancer. Specifically, we show that treatment with bevacizumab restores antigen presentation and that abrogation of angiogenic factor secretion allows ovarian cancer cells to serve as antigen presenting cells.

Stage	Invasive epithelial	Stromal	Germ cell	Fallopian tube
I	90%	95%	98%	87%
II	70%	78%	94%	86%
III	39%	65%	87%	52%
IV	17%	35%	69%	40%

Table 1.5 Five-year survival rates for different types of ovarian cancer, by stage. Data compiled from Surveillance, Epidemiology and End Results Program (SEER). Data is for old (prior to 2014) FIGO staging system and is based on patient diagnoses made from 2004 to 2010. Data based on the new FIGO system are not yet publicly available.

Chapter 1.5 HDACs and HDAC inhibitors

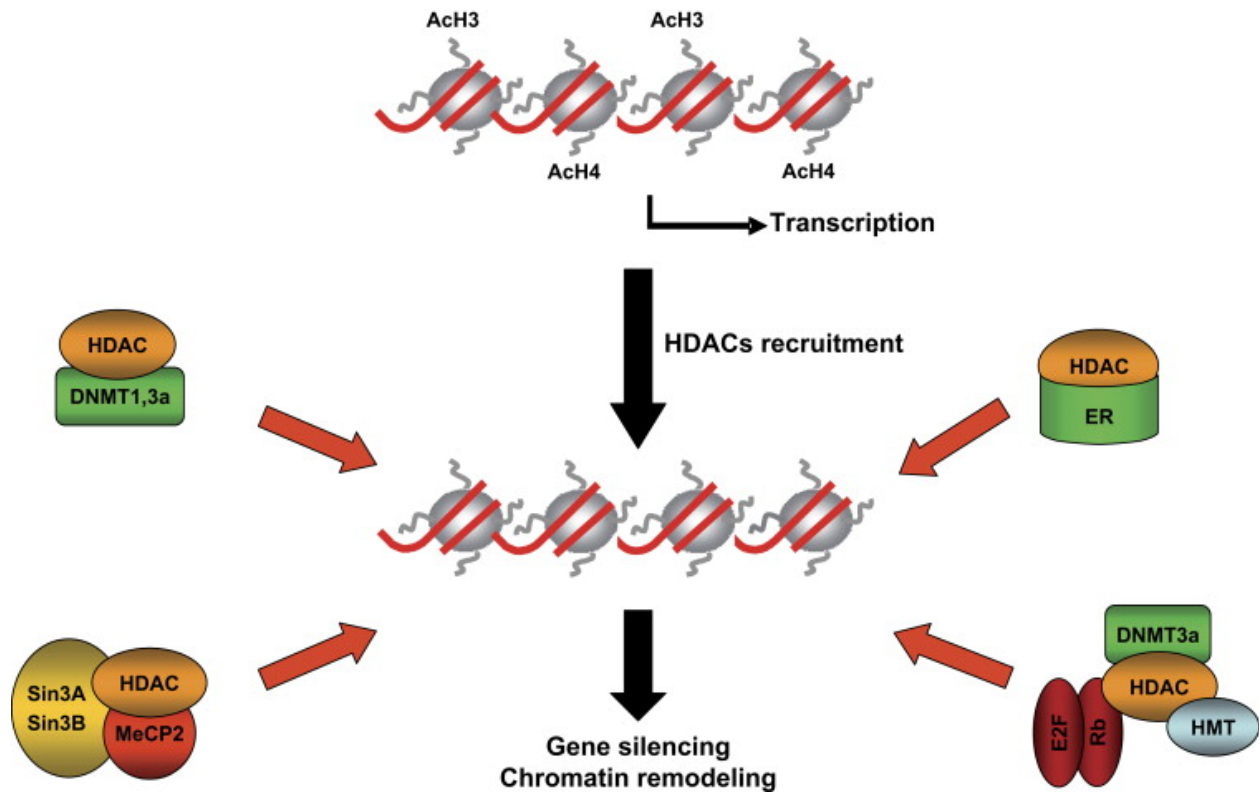
Introduction

Histone deacetylases (HDACs) are a family of enzymes that regulate diverse cellular events such as gene expression, cell proliferation, and immune pathways through deacetylation of their protein targets [33,34]. HDAC activities are frequently dysregulated in cancer and have been implicated not only in tumor onset, but also tumor progression [35]. Moreover, recent evidence demonstrates that HDACs facilitate tumor evasion from immune surveillance [36]. HDAC inhibitors (HDACi) have thus garnered attention as a potential therapy in a variety of cancers [35,33]. Some HDACi have entered the clinic (discussed below), while many others are being developed.

HDACs allow for changes in the epigenetic program of a cell. Epigenetic changes enable modification of genetic information based on gene expression patterns and levels, rather than on the basis of gene sequence [37]. Epigenetic changes – DNA methylation and posttranslational histone modifications – alter gene expression, without changing the sequence of the gene. The field of epigenetics is a relatively new, but it has contributed significantly to our understanding of cancer initiation and progression and revealed the mechanisms underlying tumor heterogeneity [37]. The epigenome, or the collective epigenetic changes within a cell, is now being appreciated for its complexity, with next-generation sequencing and chromatin immunoprecipitation-sequencing (ChIP-Seq) allowing us to understand the role of nucleosome positioning, chromatin conformation, histone localization, and transcription factor binding in cancer [37]. With the advent of these novel approaches, the study of epigenetics has contributed greatly to other fields.

Acetylation of histones, mediated by histone acetylases (HATs), allows for decompression of the chromatin. Acetylation adds negative charges on histone tails, which repels the negatively

charged DNA, thus allowing chromatin decompression. The canonical function of HDACs is deacetylation of histones and initiation of chromatin compression (Figure 1.1). HDACs normally exist as a part of large multiprotein complexes that are then targeted to specific genes through interaction with transcription factors and other epigenetic modifiers, such as DNA methyltransferases (DNMTs) [38].



Reprinted with permission from Elsevier (license # 3857210600568). Ropero and Esteller, *Molecular Oncology*, 1(1) 19-25.

Figure 1.1: Canonical functions of histone deacetylases. Histone acetylases add negatively charged acetyl groups to histone tails, allowing gene transcription to occur. Histone deacetylases remove acetyl groups, thus silencing gene expression.

Cross-talk between HDACs and the STAT3 signaling pathway

Originally, HDACi were developed as pro-apoptotic agents; however, HDACs possess many non-canonical targets and they have been studied in animal models of inflammatory disease, including inflammatory bowel disease, asthma, and septic shock [39-41]. Through deacetylation of non-histone protein targets, they can regulate cell signaling. HDACs can alter expression of a large number of genes by interacting with various transcription factors, including STAT3 [38]. Specifically, HDACs are known to modulate genes involved in inflammation, including IL-10 and STAT3-regulated genes (summarized in Table 1.6) and HDACi have been shown to have anti-inflammatory effects [42]. For example, HDACs have been implicated in regulation of IL-1 gene and the STAT3-inducible gene IL-6 (Table 1.6). In mouse and rat models of arthritis, administration of MS275, a synthetic benzamide HDACi inhibiting class I HDACs [43], led to inhibition of serum IL-1 and IL-6 [41]. In ovalbumin-induced airway inflammation and airway hyperresponsiveness, treatment with trichostatin-A (TSA) reduced eosinophil and lymphocyte infiltration in the bronchoalveolar lavage fluid and decreased levels of IL-4, IL-5, and IgE within the lavage fluid [44]. These changes may be attributed to TSA-mediated inhibition of HDAC1, which was shown to be expressed in most airway cells and infiltrating inflammatory cells [44].

Given the broad anti-inflammatory effects of HDACi, cross-talk between the JAK-STAT signaling pathway and HDACs has gained interest. HDACs not only modulate the JAK-STAT signaling pathway, but are in turn modulated by this pathway [45]. Acetylation of STATs plays an important role in STAT activation: while deacetylation of STAT1 promotes its activation, STAT3 deacetylation impairs STAT3 dimerization because lysine residue acetylation is critical for STAT3 dimerization (Table 1.6) [45,46]. In diffuse large B cell lymphoma (DLBCL), inhibition of HDACs led to a concomitant inhibition of STAT3 tyrosine-705 phosphorylation of STAT3, resulting in

nuclear export of STAT3 [47]. Moreover, HDACi pro-apoptotic activity was dependent on STAT3 constitutive activation, thus justifying selection of patients for HDACi therapy based on STAT3 status [47]. In multiple myeloma, HDACi treatment inhibited STAT3 phosphorylation and activation of STAT3-regulated pathways, including cyclin D1 and Bcl-xL, leading to apoptosis and cell cycle arrest [48]. We were able to demonstrate similar findings in our lab: HDACi treatment of MCL cells inhibited STAT3 tyrosine-705 phosphorylation. Moreover, we confirmed the functional effects of HDACi treatment by ascertaining that the protein products of STAT3-inducible genes were inhibited.

The focus of our work is on HDAC2, which has been implicated in regulating cytokine production and STAT3-inducible genes (Table 1.6). For example, HDAC2 is a negative regulator of STAT3-inducible genes, acting by deacetylation of STAT3 to inhibit its transcriptional function (Table 1.6). In our studies, we demonstrate that HDACi treatment inhibits STAT3 and, consequently, secretion of STAT3-inducible cytokines.

HDAC inhibitors

Due to the wide range of processes regulated by HDACs, they have become candidate targets in cancer treatment [38]. Vorinostat and romidepsin are currently FDA-approved for the treatment of cutaneous T cell lymphoma (CTCL) [37]. Belinostat is approved for the treatment of relapsed or refractory peripheral T cell lymphoma (PTCL) [49]. Currently, these three drugs are being evaluated for efficacy in other hematological malignancies and solid tumors, either as single agents or in combination with other drugs [49]. Design of novel HDACi is informed by the understanding of different classes and mechanisms of action of HDACs. The different classes of HDACi, discussed below, have unique structural characteristics and mechanisms of action.

HDAC	Protein target	Gene target	Comment
HDAC1	Sp1	<i>IL-1</i>	Sp1 recruits HDAC1 to IL-1 promoter
HDAC7, 9	FOXP3	<i>IL-2</i>	HDAC7 and 9 interact with FOXP3 and TIP60 to repress IL-2 expression
HDAC1	ZEB1	<i>IL-2</i>	ZEB1 recruits CtBP and HDAC1 to the IL-2 promoter
HDAC1	—	<i>IL-12</i>	HDAC1 is recruited to IL-12 promoter
HDAC1	GR	<i>IL-5</i>	GR binds to IL-5 promoter and recruits HDAC1, interfering with GATA3 positive regulation
HDAC5	—	<i>IL-8</i>	HDAC5 is recruited to IL-8 promoter in cells infected with <i>Legionella pneumophila</i>
HDAC2	GR	<i>GM-CSF</i>	Glucocorticoid receptor recruits HDAC2 to GM-CSF promoter to antagonize the effect of IL-1b
HDAC1, 6, 8	—	<i>IFN-β</i>	HDAC1 and 8 repress IFN-β expression, and HDAC6 acts as a coactivator to enhance activity
HDAC1, 2	Sin3A	<i>Ifng</i>	Sin3A Recruits HDACs to the <i>Ifng</i> locus for repress its transcription in Th0 cells
HDAC11	—	<i>IL-10</i>	HDAC11 is recruited to IL-10 promoter to repress transcription
HDAC1, 2, 3	—	<i>IL-4</i>	HDACs are recruited to IL-4 promoter to repress transcription
HDAC3	Pro-IL-16	<i>Skp2</i>	HDAC3 binds to pro-IL-16 to repress <i>Skp2</i> expression and subsequently promote cell cycle arrest
HDAC1	STAT5, C/EBPb	<i>Id-1</i>	STAT5 recruits HDAC1 to <i>Id-1</i> promoter for C/EBPb deacetylation, allowing transcriptional activation
HDAC3	STAT3	STAT3 target genes	HDAC3 enhances STAT3 phosphorylation
HDAC1, 2, 3	STAT3	STAT3 target genes	HDACs deacetylate STAT3 to inactivate its transcriptional functions
HDAC3	STAT1	STAT1 target genes	HDAC3 deacetylates STAT1 allowing its phosphorylation and nuclear translocation
HDAC3, 4, 5	GATA-1	GATA-1 target genes	Transcriptional regulation mediated by GATA-1 is diminished in the presence of HDACs
HDAC3, 5	GATA-3	GATA-3 target genes	Phosphorylated GATA-3 recruits HDAC3 and HDAC5 to specific DNA regions
HDAC3	GATA-2	GATA-2 target genes	HDAC3 suppresses the transcriptional potential of GATA-2
HDAC3	NF-κB	NF-κB target genes	RelA is deacetylated by HDAC3 inducing its nuclear export

Reprinted with permission from Nature Publishing Group (license # 3857240110596). Villagra et al, *Oncogene*, 29(2) 157-173.

Table 1.6 HDAC-mediated regulation of cytokine transcription. Abbreviations: granulocyte-macrophage colony-stimulating factor, GM-CSF; histone deacetylase, HDAC; interferon, IFN; interleukin, IL; nuclear factor kappa-B, NF-κB; C-terminal-binding protein, CtBP.

There are six groups of HDAC inhibitors, derived from both natural and synthetic compounds: (i) hydroxamate, such as TSA and SAHA; (ii) aliphatic acid, such as sodium butyrate (NaB) and valproic acid (VPA); (iii) benzamides, such as MS-275 (entinostat); (iv) cyclic tetrapeptides, such as romidepsin; (v) electrophilic ketone; and (vi) sirtuins inhibitors (SIRTi) [50]. Studies of different classes of HDACs revealed structure-function relationships that facilitated the creation of synthetic HDAC inhibitors, with a specific focus on creation of selective HDACi with enhanced clinical utility and decreased side effects. For example, class I HDACs are structurally different than class II HDACs [51]. This observation led to synthesis of hydroxamic class of HDAC inhibitors containing common structural characteristics: a zinc-binding moiety connected to a capping group via an alkyl, vinyl, or aryl group [34]. The aliphatic chain contacts hydrophobic residues in the channel [34], while the capping group is exposed to the solvent and interacts with amino acids near the entrance of the active site [52].

TSA is the most widely studied class I and II HDAC inhibitor, with over 2,600 published studies in the past decade. The hydroxamic acid group of TSA binds to the zinc atom situated at the bottom of the 11Å channel while the aromatic ring neighbors Glu and Asp residues of the active sites of HDACs [51]. The N,N-dimethylaminobenzyl group interacts with a single pocket on the HDAC surface [51,53]. Figure 1.2 depicts a model of TSA bound to the HDAC catalytic core. Elucidation of the mechanisms of HDAC inhibition by TSA has been invaluable in synthesis of novel HDACi, providing the framework for further development of novel HDACi [53]. Furthermore, genome-wide studies of TSA's effects on gene expression have given insights into the biological effects of HDACi in many settings, ranging from cancer, to neurogenesis and infection [54-57].

Studies on the mechanisms of action of TSA and other HDAC inhibitors led to our understanding of how specificity can be achieved through chemical modification of existing inhibitors. Improvement of pharmacokinetic properties arises from incorporation of solubilizing functional groups [34]. Furthermore, derivation of analogues is based on specific interactions between the inhibitor and the enzyme. For instance, the entry channel of class I HDACs is flexible because the amino acids forming the active sites are found in loops [51]. Thus, derived analogues must account for the flexibility of the enzyme itself. Furthermore, class I HDACs are highly homologous in the active site region. Therefore, analogue synthesis approaches focusing on altering the groups that fit within the active site will be very unlikely to achieve specificity. Nonetheless, isoform selectivity can be derived from studying the shape and charges around the opening to the active site [51]. Another approach of analogue synthesis focuses on the chain length, saturation, and presence of rings within the chain. Valproic acid (VPA) features an ethyl group, which allowed improvement of inhibition to the micromolar range, whereas other derivatives with aryl groups within the chain act in the nanomolar range [52].

HDACi in the clinic

Currently, HDACi are in clinical trials as adjuvants to cytotoxic agents and, preclinically, they are studied for their potential use in combination epigenetic targeted therapy. In particular, the combination of DNA methyltransferase and HDAC inhibitors has been proposed [58]. This approach relies on synergistic reactivation of gene expression [58], which would subsequently re-sensitize cells to therapies or turn on transcriptional programs that would be incompatible with cancer cell survival. For example, combination epigenetic therapy of VPA and DNMT inhibitor 5-azacytidine (Vidaza) in patients with intermediate and high-risk myelodysplastic syndrome

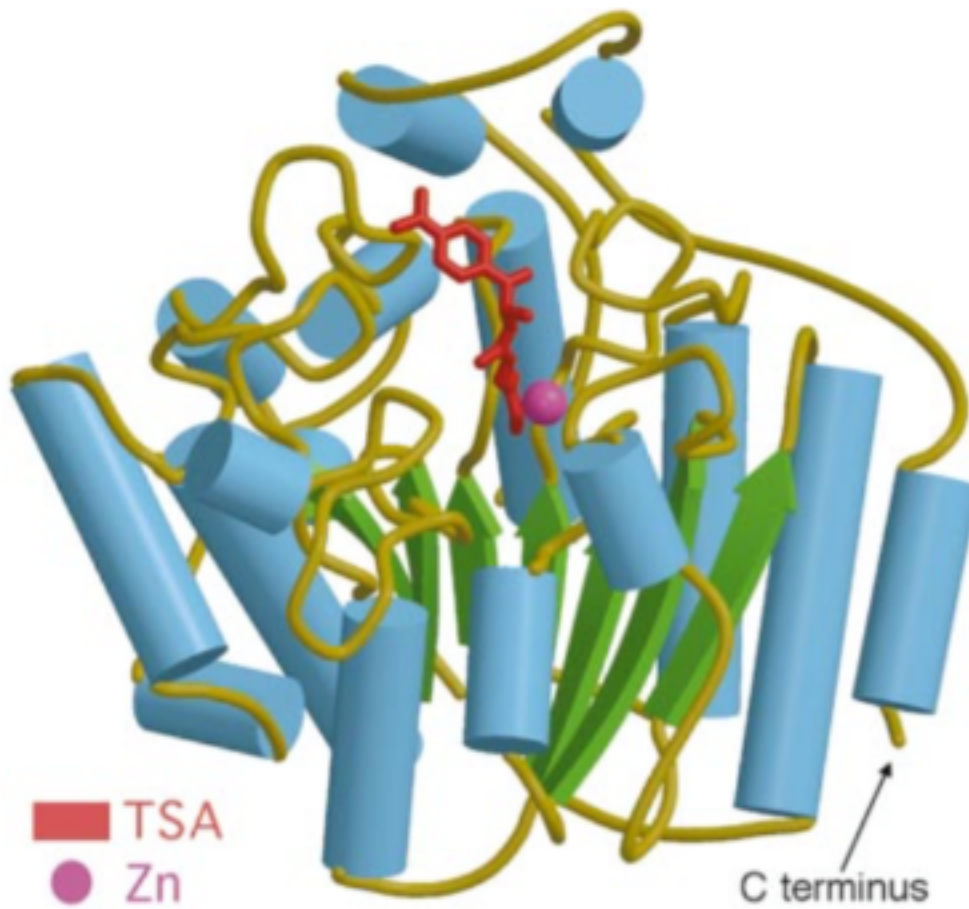
resulted in partial or complete remission in 30.7% of patients, while 15.4% showed hematologic improvements and 38.5% showed stable disease [59]. Moreover, HDAC inhibitors can sensitize cancer cells to DNA-damaging agents, such as topoisomerase inhibitors [50]. This phenomenon became the premise for the use of VPA in conjunction with karenitecin, a topoisomerase I inhibitor. This combination resulted in 47% of myeloma patients achieving stable disease with an overall survival of 32.8 weeks [59].

Other combinations have been explored. In preclinical studies, the STAT3 inhibitor WP1066 synergized with vorinostat in mediating growth inhibition and apoptosis of MCL cell lines [35]. Moreover, Panobinostat was combined with an inhibitor of S-adenosyl-L-homocysteine hydrolase, DZNep, which has been shown to allow re-expression of epigenetically silenced genes. Treatment with DZNep and Panobinostat induced apoptosis of both MCL cell lines and primary MCL cells [60]. Furthermore, the combined treatment synergistically inhibited *in vivo* tumor growth of JeKo-1 xenografts [60]. Importantly, Panobinostat alone inhibited tumor growth, compared to vehicle control [60]. In Chapter 3, we present evidence for NKT cell-mediated regression of JeKo-1 xenografts [60]. Collectively, these data warrant testing of adoptive transfer in combination with HDACi in a humanized mouse model of MCL. While various combinations of HDACi with other chemotherapeutic agents have been widely studied, the combination of HDACi with immunotherapy is a novel avenue, with mechanistic studies helping to build the foundation for the application of this combination in the clinic [61,62].

There are a limited number of clinical trials of HDACi in MCL patients. Kirschbaum et al. performed a phase II clinical trial using vorinostat in patients with relapsed/refractory follicular lymphoma (FL), marginal zone lymphoma (MZL) and MCL [63]. The single-agent activity of vorinostat was poor. There were no responders among MCL patients, median PFS was 5.9 months,

and one patient maintained stable disease for 26 months [63]. The drug was well-tolerated over long periods of time, and, considering the poor outcomes, combination therapy may yield more positive results [63]. A phase I/II clinical trial combining vorinostat, cladribine, and rituximab in patients with previously untreated MCL is currently underway at the Knight Cancer Institute [64]. The last reported overall response rate (ORR) was 100%, with 93% of patients achieving complete response (CR) [64]. Myelosuppression was the primary toxicity of this regimen (Table 1.7), with one case of grade 4 thromboembolic event, and one grade 5 pulmonary hemorrhage [64]. Toxicities were primarily hematologic (Table 1.7) and reversible. Summary of study findings is presented in Table 1.7. The study (NCT00764517) is currently ongoing and is estimated to complete in June 2016.

Given that HDACi have had marginal success as single agents, various combination therapies have been proposed. As discussed in the next chapter, HDACi have been shown to increase immunogenicity of tumor cells. In our studies, we provide evidence of HDACi enhancing antigen presentation by tumors, which warrants future studies of combination of HDACi with immunotherapy.



Reprinted with permission from Nature Publishing Group (license # 3862170835800). Finnin et al., *Nature*, 401(6749) 186-93.

Figure 1.2: TSA within the histone deacetylase catalytic core. The active site pocket contains a zinc cofactor. TSA binds to the zinc.

Clinical Response Rate

Patient population	Number	Response	Response rate
Relapsed NHL			43%
MCL	9	4 (2CR)	
DLBCL	3	0	
FL	1	1 (PR)	
MZL	1	1 (CR)	
Relapsed CLL	7	4 (PR)	57%
Previously untreated MCL	15	14 11 CR	93%

Grade 3 Adverse Events

Event	Number (per 147 cycles of treatment)
Hematologic	
Neutropenia	59 (40%)
Thrombocytopenia	33 (22%)
Non-hematologic	
Infection	12 (8%)
Fatigue	8 (5%)

Table 1.7: Summary of findings of the SCR study at the Knight Cancer Institute. Phase II study evaluated the combination of vorinostat (SAHA), cladribine, and rituximab (SCR) in mantle cell lymphoma, chronic lymphocytic leukemia, and relapsed B cell non-Hodgkin lymphoma. The study is currently ongoing. Findings were presented at the 2011 American Society of Clinical Oncology (ASCO) Annual Meeting [64]. Abbreviations: complete response, CR; partial response, PR; mantle cell lymphoma, MCL; diffuse large B cell lymphoma, DLBCL; follicular lymphoma, FL; marginal zone lymphoma, MZL.

1.6 Epigenetic modulation of antigen presentation

Classical major histocompatibility (MHC) molecules bind peptide antigens and present them on the cell surface for recognition by T cells. There are two classes of MHC molecules, MHC class I and II, which, in humans, are encoded by the human leukocyte antigen (HLA) genes. MHC class I molecules are ubiquitously expressed on all nucleated cells, while MHC class II molecules are expressed on hematopoietic cells and thymic stromal cells. There are three class I α -chain genes in humans, called HLA-A, -B, and -C, and three pairs of MHC class II α - and β -chain genes, called HLA-DR, -DP, and -DQ [65]. The MHC locus encodes many genes whose products are necessary for antigen processing and presentation.

MHC class I genes are regulated by cis-acting regulatory promoter elements: enhancer, interferon-stimulated response element, and the SXY module [66]. Expression of the MHC class II genes is governed by MHC class II transactivator (CIITA), which acts as a transcription co-activator of MHC class II genes in response to IFN- γ [65]. The CIITA is necessary for MHC class II transcription, but it also contributes to the activation of MHC class I gene transcription [65]. CIITA exerts its function through interactions with the MHC class II enhanceosome. Following CIITA interaction with the enhanceosome, numerous co-activators are recruited. CIITA interacts with HDACs, which negatively regulate CIITA's transcriptional transactivation activities [65].

In addition to the highly polymorphic classical MHC class I/II genes, there are many non-classical MHC genes [67,68]. Some MHC class I-like genes are located outside of the MHC region. The CD1 family of genes, including the five human CD1d genes, CD1a-e, are located outside of the MHC locus [69]. In mice, there are only two CD1d genes, CD1d1 and CD1d2, of which only one –CD1d1—is functional [70]. Generally, the mouse product of the CD1d1 gene is referred to as CD1d. CD1 molecules are similar to MHC class I molecules in structure: they have three α

chains and they are associated with β_2 -microglobulin [71]. However, CD1 molecules follow an MHC class II-like route, trafficking via the endocytic pathway [72]. Unlike either MHC class I or class II molecules, CD1 molecules bind lipid antigens, owing to their hydrophobic groove [71].

The basis of transcriptional regulation of CD1d remain poorly understood. In humans, the proximal promoter is regulated by Sp1 [73,74], whereas LEF-1 is a negative regulator of CD1d transcription, exerting its function through binding at the distal promoter and recruitment of HDACs [75]. The murine CD1d promoter is regulated by Ets family transcription factors Elf-1 and PU.1 [76]. Human and mouse CD1d genes share a retinoic acid response element [77]. Finally, two studies have implicated the role of HDACs in controlling CD1d gene expression [74,75]. Our study fills a gap in the field by demonstrating that HDAC2 is a negative regulator of CD1d gene expression.

Tumors frequently dysregulate antigen presentation. MHC class I loss or downregulation has been found to range from 16 to 80% in tumors of different types [78]. Moreover, low MHC class II expression correlates with poor prognosis [79]. CD1d downregulation has been shown to occur during myeloma progression [80]. In contrast, other studies have shown that cells carrying more cytogenetic abnormalities had higher CD1d levels [81], suggesting that upregulation of CD1d may serve to “alert” the immune system and that tumors may utilize various mechanisms to evade NKT cell responses. In CLL, CD1d upregulation was associated with reduced survival [81,82]. Intriguingly, the percentage of CD1d/CD19 double positive cells in patient blood inversely correlated with the percentage of NKT cells [81], suggesting that CD1d upregulation may lead to loss of NKT cell due to overactivation, resulting in activation induced cell death (AICD), or anergy. Ultimately, however, upregulation of CD1d may exhaust NKT cells, inducing anergy. Alternatively, NKT cells may play a more of an immunoregulatory role in CLL. These

findings have important implication for our work because we found that HDACi treatment induces slight induction in CD1d levels. We believe that a slight increase in CD1d levels will help activate NKT cells without inducing anergy.

The molecular mechanisms underlying dysregulation of antigen presentation include structural gene abnormalities, defective transcription/translation regulation, and induction of hypermethylation of gene promoters or aberrant recruitment of HDACs [83,78]. CIITA has been shown to recruit HDACs, leading to dissociation of CIITA from the MHC class II promoter [84].

HDACi induce expression of MHC class I and II genes on the surface of neuroblastoma cells [85]. In neuroblastoma cells, the effects of TSA were dependent on CIITA activation [85]. However, in a CIITA-deficient cell line RJ2.2.5, TSA induced MHC class II, suggesting that HDACi can induce MHC class II independently of CIITA activation [85]. In melanoma cells, HDACi treatment enhanced transcription of genes involved in MHC class I antigen presentation pathway, including transporter associated with antigen processing (TAP) 1 and 2 and tapasin [86].

While there is a scarcity of data on the effects of HDACi on CD1d gene transcription [74], a seminal study by West et al. demonstrated that treatment of E μ -myc lymphoma-bearing mice with vorinostat and α -GalCer led to reduced tumor growth, with concomitant administration of HDACi and α -GalCer causing increased survival, compared to either agent alone [87]. While CD1d levels on the tumor cells were not assessed in this study, these data suggest that CD1d-mediated antigen presentation was enhanced [87].

Although the effects of HDACi on antigen presentation by tumors have been positive, HDACi have been shown to inhibit antigen presentation by professional antigen presenting cells. HDACi suppress expression of costimulatory molecules on dendritic cells (DCs), change their metabolism as well as their secreted cytokine profile, and can impair their antigen uptake

capabilities [88]. Specifically, valproic acid and LBH589 have been shown to reduce the expression of costimulatory molecules CD80, CD40, and CD83 and alter expression of adhesion molecules [88,89]. As a result of these changes, both MHC class I and II-mediated antigen-specific immune responses relayed by DCs were impaired, limiting their ability to activate T cells and NKT cells [90]. The discrepancies in the effects of HDACi may be attributed to the fact that HDACs have normal functions, but due to their overexpression and aberrant recruitment in tumors, HDACi treatment of tumors restores the “normal” epigenomic program by fine-tuning HDAC activities.

Overall, epigenetic agents enhance antigen presentation by tumors through multiple mechanisms. Antigen presentation defects allow escape from immune system recognition and reversal of these defects through epigenetic therapies is a promising approach. Importantly, the functional effects of HDACi on CD1d-mediated antigen presentation have not been studied. We demonstrate that HDACi enhance CD1D-mediated antigen presentation by human MCL cells through induction of CD1D mRNA and cell surface expression. Thus, our studies are the first to implicate a role for HDACs in CD1D-mediated antigen presentation by MCL cells.

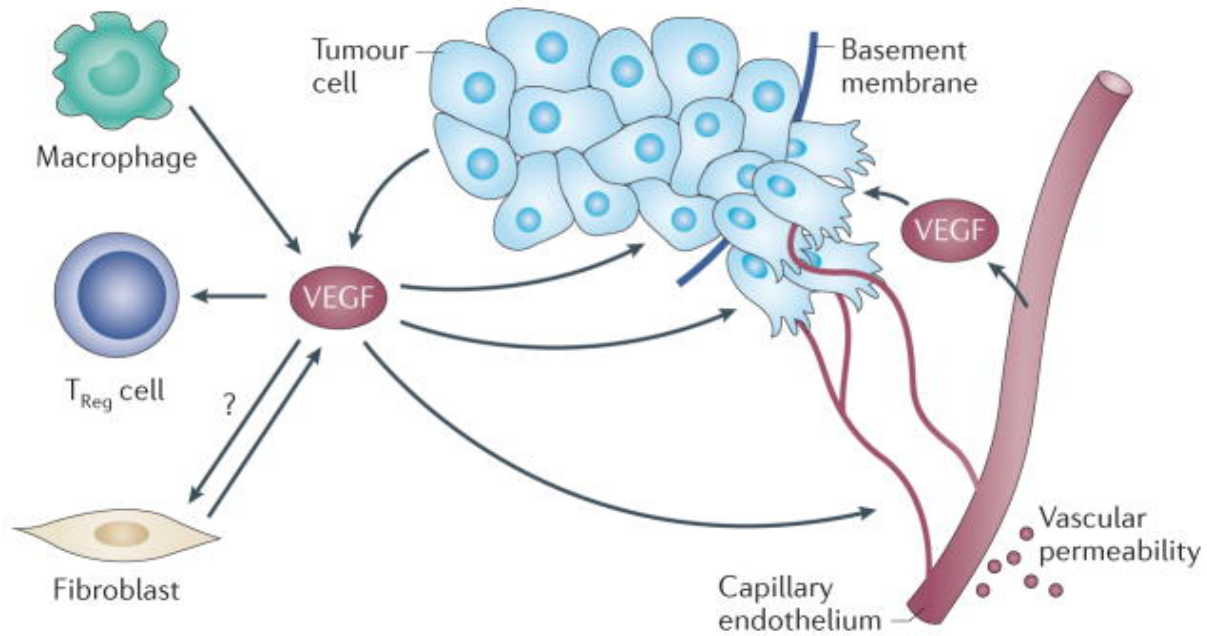
1.7 Anti-angiogenic therapies in ovarian cancer

Introduction

VEGF was first isolated as an endothelial cell-specific mitogen able to induce angiogenesis, or growth of blood vessels [91]. The VEGF family of growth factors includes VEGFA, VEGFB, VEGFC, VEGFD, and placenta growth factor (PGF), which differ in their function, specificity and expression pattern [92]. The most widely studied VEGF family member, VEGFA, commonly referred to as VEGF, includes a number of different variants arising from alternative splicing [91]. VEGF receptor signaling is mediated by receptor tyrosine kinases (RTKs) and neuropilins (NRPs), which can affect the function of multiple RTKs and integrins. Some tumor cells lack one or more VEGF RTKs, but are nonetheless responsive to VEGF signals because NRPs can function as VEGF co-receptors, increasing the binding affinity of VEGF [91,92].

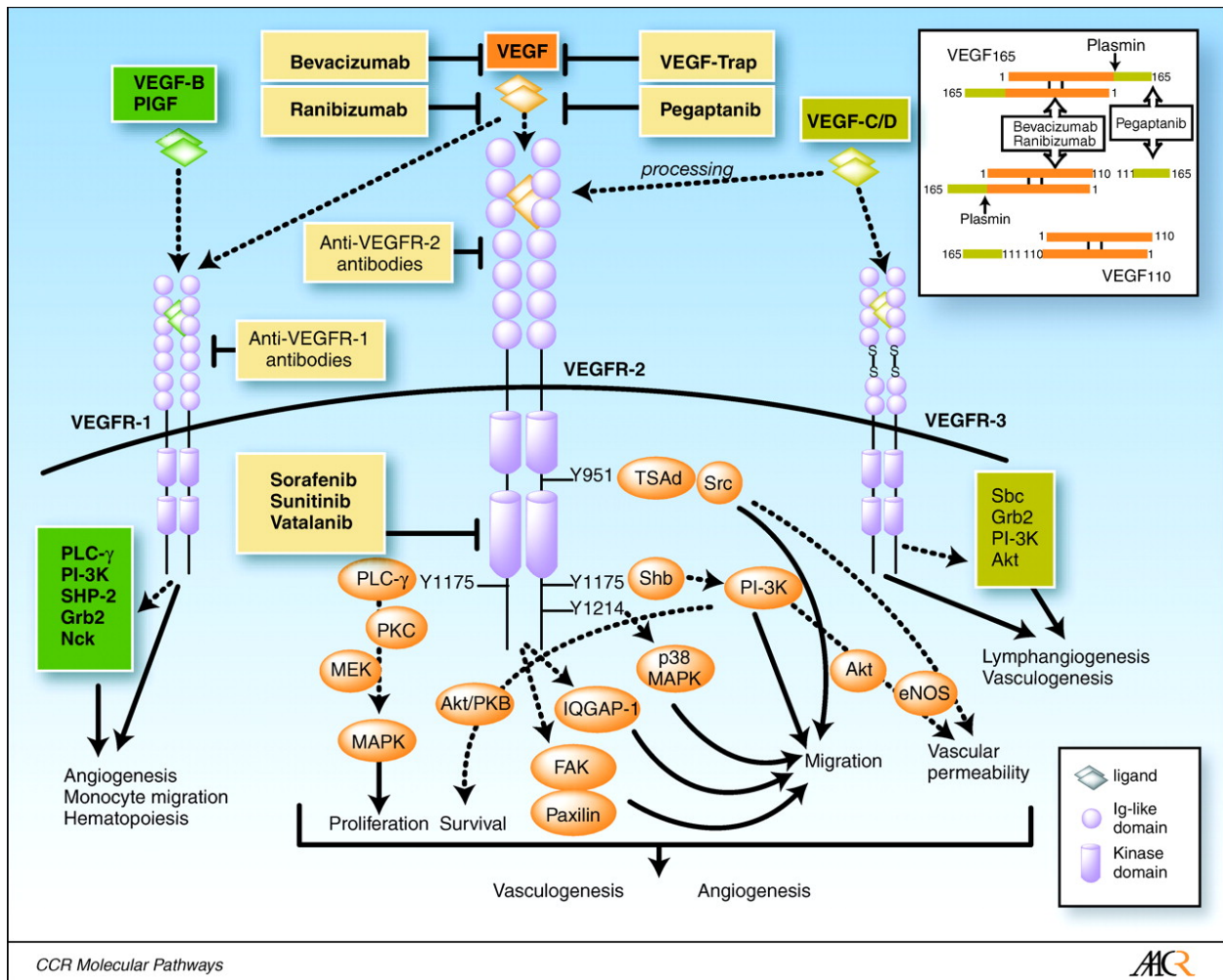
VEGF promotes tumor angiogenesis through multiple mechanisms: enhanced endothelial cell proliferation and survival; increased endothelial cell migration, aided by induced lattice networks; increased vessel permeability; and enhanced homing of vascular precursor cells from the bone marrow [92]. Other described functions include promotion of tumor cell survival, migration, and invasion [92]. VEGF is secreted by tumor and stromal cells and can function in autocrine manner to promote de-differentiation and epithelial-mesenchymal transition (EMT) [91]. The effects of VEGF within the tumor microenvironment are summarized in Figure 1.2.

VEGF activates the PI3K-AKT and MAPK signaling pathways (among others), thus activating tumor cell survival and migration. Moreover, activation of the PI3K pathway by VEGF leads to increased vascular permeability and vasculogenesis. The broad effects of VEGF signaling are summarized in Figure 1.4.



Reprinted with permission from Nature Publishing Group (license # 3857640085632). Goel and Mercurio, *Nat. Rev. Cancer*, 13(12) 871-882.

Figure 1.3: Functions of VEGF in the tumor microenvironment. Tumor cells and stromal cells secrete VEGF, which can function to induce sprouting angiogenesis, increase vascular permeability, and promote de-differentiation and EMT of tumors. Moreover, VEGF can recruit regulatory T cells, which are known to facilitate the creation of an immunosuppressive microenvironment.



Reprinted with permission from American Association of Cancer Research (license # 3857651080839). Kowanetz and Ferrara, *Clin. Cancer Res*, 12(17) 5018-22.

Figure 1.4: Signal transduction and biological processes mediated by VEGF receptors. VEGF receptor signaling activates many signaling cascades, including PI3K-AKT and MAPK. Targeting of VEGF and the VEGFR is accomplished by antibodies, whereas downstream signaling events are inhibited through inhibitors, including sunitinib and sorafenib.

VEGF inhibitors

Due to its potent biological activities, VEGF inhibition has been studied as a potential therapeutic strategy. Antibody-mediated inhibition of VEGF using bevacizumab has shown efficacy in many cancers [91,93]. Moreover, inhibitors of VEGF RTK activity (Figure 1.4), such as sunitinib, sorafenib, and pazopanib are in clinical trials. Sunitinib showed modest response in patients with recurrent and refractory ovarian, fallopian tube, and peritoneal cancers [94]. In another study, sunitinib was used as a single agent and was found to exhibit activity in recurrent platinum-sensitive ovarian cancer, with scheduling proven to be important because of high incidence of ascites and pleural effusions in the intermittent schedule treatment scheme [95]. Sorafenib showed modest activity in a phase II study with recurrent ovarian cancer, while another phase II trial including patients in complete remission demonstrated no significant improvement in PFS, leading the authors of the study to make the recommendation to not use sorafenib as a maintenance therapy [96,97]. Finally, pazopanib demonstrated PFS benefit as a maintenance agent in a phase III trial [98].

Unlike RTK inhibitors, which have given mixed results, bevacizumab was shown to be efficacious in the treatment of recurrent and metastatic disease [99-102]. Bevacizumab has also been used to palliate fluid accumulation in patients with ovarian cancer associated-ascites [103]. Bevacizumab has been combined with conventional therapy (carboplatin and paclitaxel) and used as a maintenance agent [30]. In two similar studies, which combined bevacizumab with carboplatin and paclitaxel, an increased PFS was observed, but there were no differences in OS [100,102]. However, subgroup analysis revealed increased OS in patients with stage IIIC and IV disease [102], suggesting that patients at highest risk for progression may benefit from combination therapy and bevacizumab maintenance [30].

VEGF and immunomodulation

VEGF has been shown to have immunomodulatory activities. For example, VEGF inhibits functional maturation of dendritic cells [104,105]. Moreover, VEGF promotes accumulation of myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg) and macrophages in the tumor microenvironment [106,107]. Low-dose anti-VEGF antibody therapy may facilitate the penetration of immune effector elements into the tumor parenchyma due to vessel “normalization” which generates a homogeneous distribution of tumor vessels, allowing immune cells to infiltrate the tumor [108]. VEGF has also been shown to reduce the cytotoxic activity of T cells leading to increased VEGF receptor expression on T cells [109], thus creating a negative feedback loop that may be able to be targeted in order to achieve improved treatment outcomes or help overcome chemotherapy resistance [103,110]. Importantly, T cells isolated from the ascites of ovarian cancer patients show a significantly reduced proliferation rate in the presence of exogenously added VEGF [109]. Therefore, VEGF can act directly on T cells.

We are the first group to show that VEGF inhibition restores natural killer T (NKT) cell responses. Moreover, we established a link between VEGF and immunosuppressive ganglioside shedding [111]. Given that NKT cells recognize lipid antigens, we provide evidence for existence of two mechanisms by which ovarian tumors suppress NKT cell responses. Finally, as mentioned above, T cells are inhibited by exogenous addition of VEGF and we thus determined whether NKT cells express VEGFR. Given that NKT cells express VEGFR, future studies will help delineate the direct effects of VEGF on NKT cell activation.

1.8 The role of the immune system in cancer elimination

The concept that immune system plays an important role in preventing tumor formation was first proposed by Burnet and Thomas, who put forth the cancer immunosurveillance hypothesis in 1957 [112]. The immune system can 1) protect the host from virus-induced tumors by battling viral infection, 2) control inflammation, and 3) specifically eliminate arising tumor cells in a process called immune surveillance [113]. The immune cells act as sentinels, sensing neoplastic transformation, and destroying malignant cells by initiating a strong anti-tumor response [112]. Early studies of transplant patients who were immunosuppressed and individuals with immunodeficiencies demonstrated that they were at a greater relative risk for cancer development [112,114]. Many epidemiological and laboratory studies offer compelling evidence that the immune system serves as a barrier to tumor formation.

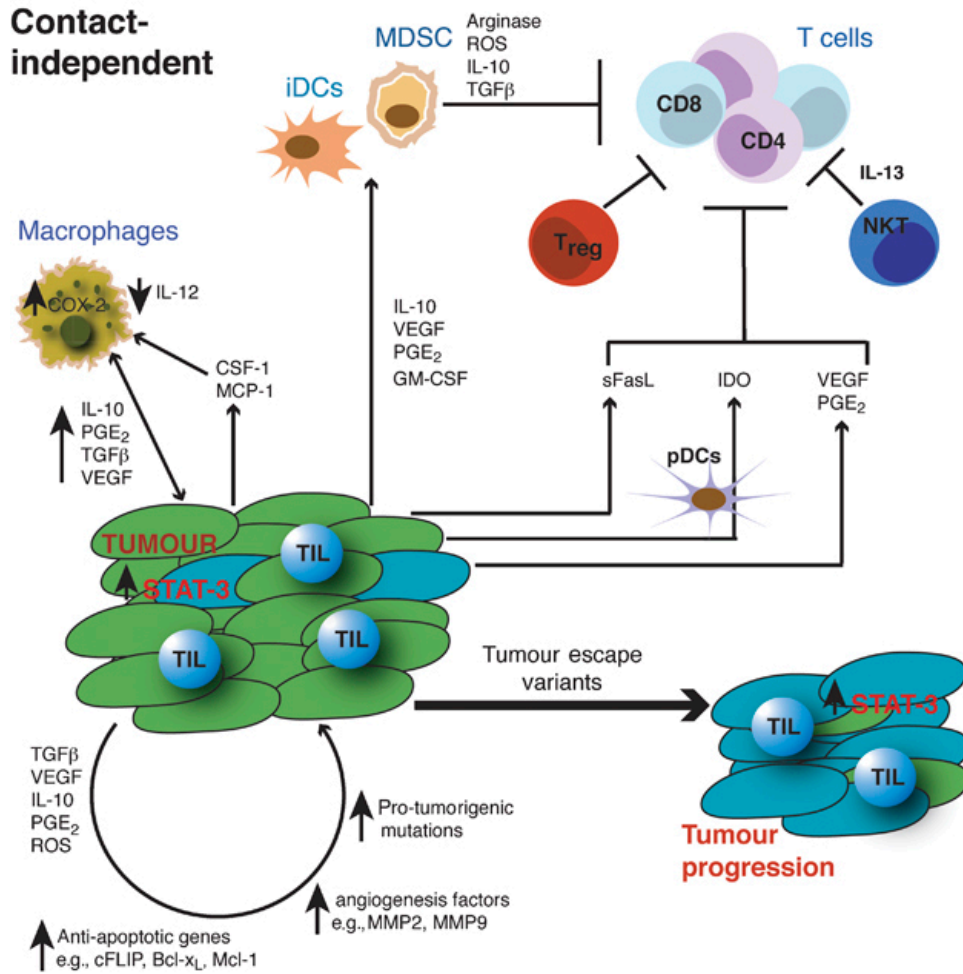
Ultimately, tumors are able to escape immune surveillance by acquiring low immunogenicity [112]. Three phases – elimination, equilibrium, and escape – allow tumor evasion from immunosurveillance [115,113,116]. Tumor cells that escaped the elimination phase will enter the equilibrium phase, remaining either dormant or acquiring mutations and epigenetic changes that allow clones with low immunogenicity to grow [113]. The immune system puts a selective pressure on the tumor, thus allowing only resistant clones to arise [113]. However, at this point, the tumor is kept in check by the immune system and, in mouse studies, this process has been shown to extend for the entire lifetime of the mouse [117]. Some tumor clones are able to escape recognition by the immune system [113,118,116]. Mouse studies demonstrated that tumors derived from immunodeficient mice are more immunogenic than tumors from immunocompetent hosts, suggesting that the immune system sculpts the tumors that are ultimately able to escape [118].

Tumors utilize various mechanisms to achieve escape. Tumors downregulate or completely lose antigens that are able to stimulate immune cells [118,119]. Moreover, tumors can upregulate mechanisms of resistance against cytotoxic effector cells [118,119]. Finally, tumors can establish an immunosuppressive microenvironment by producing cytokines or through upregulation of inhibitory receptors, thus inhibiting the immune system via both cell contact-dependent and independent mechanisms [119]. Factors such as VEGF, IL-10 and transforming growth factor- β (TGF- β) have been shown to induce immune dysfunction, both directly (through suppression of lymphocytes) and indirectly (through suppression of antigen presentation) [119].

A major factor controlling production of immunosuppressive cytokines is STAT3 [119,120,16]. Constitutive activation of STAT3 leads to production of cytokines, such as IL-10 and VEGF, which have been shown to suppress anti-tumor immunity through impaired dendritic cell activation and maturation (Figure 1.4). Our work addresses the role of STAT3 and STAT3-inducible cytokines in antigen presentation.

The mechanisms of tumor escape are varied and complex, including both cell contact-dependent and independent mechanisms. The mechanisms of tumor escape are only beginning to be understood and novel mechanisms may be discovered [119]. Understanding of these escape mechanisms is crucial to the development of successful immunotherapies.

Our work focuses on epigenetic mechanisms of tumor evasion from immune recognition. Moreover, we provide evidence for tumor-secreted factors inhibiting antigen presentation. The scope of our work includes both cell-intrinsic and cell-extrinsic mechanisms of immune suppression.



Reprinted with permission from Nature Publishing Group (license # 3857770398016). Steward and Abrams, *Oncogene*, 27(45) 5894-903.

Figure 1.5: Tumor escape through cell contact-independent mechanisms. STAT3 regulates production of immunosuppressive factors, such as VEGF and IL-10, which can suppress immune cell effector functions.

1.9 Innate and adaptive immune responses in cancer

Natural killer (NK) cells, dendritic cells (DCs), neutrophils, eosinophils, basophils, mast cells and macrophages are the first line of defense against damaged and tumor cells. Innate immune cells (such as NK cells), adaptive immune cells (such as cytotoxic T lymphocytes), and cells displaying both innate and adaptive functions (such as Natural killer T (NKT) and $\gamma\delta$ T cells), work in tandem to orchestrate an anti-tumor response [116]. The innate immune system allows for quick but non-specific responses to pathogens and tumors [116]. On the other hand, the adaptive immune response takes time to develop, but is highly specific and capable of generating immunological memory [116].

NK cells are the primary innate cells that respond to tumors lacking major histocompatibility (MHC) molecules. NK cells can directly kill tumors, through release of perforin and granzymes [121]. Upregulation of stress molecules on the surface of tumor cells allows NK cell activation through engagement of natural killer group 2D (NKG2D) receptors [121]. Macrophages recognize molecules upregulated during tumor cell apoptosis and phagocytose the dying cells. DCs are the most potent professional antigen presenting cells that are able to present antigens to T cells. DC precursors migrate from bone marrow to all tissues where they reside in an immature state to function as sentinels. DC ingest antigens and then migrate to peripheral lymphoid tissue to mature and activate T cells [122]. Neutrophils, eosinophils, basophils and mast cells can significantly impact the tumor microenvironment through the production of cytokines. For example, neutrophil-secreted cytokines and chemokines recruit DCs and macrophages [123].

B and T cells are the major players of the adaptive arm of the immune system. Following activation, these lymphocytes undergo rapid clonal expansion. Activated B cells transform into terminally differentiated plasma cells, which can produce tumor-specific antibodies, along with

other antibody-secreting effector B cells [124]. In addition to antibody generations, B cells can serve as antigen presenting cells and secrete cytokines to modulate the function of other cells [125]. T cells differentiate into subsets that can mediate cytokine production, tumor cell killing, and provide help to B cells.

$\gamma\delta$ T and NKT cells are types of innate-like lymphocytes that have well-established roles in mediating anti-tumor responses [126,127]. Both cell types can directly kill tumors and produce cytokines to activate other immune cells. $\gamma\delta$ T can directly kill tumors via perforin, granzyme, and TNF-related apoptosis-inducing ligand (TRAIL) [126]. Moreover, they can produce interferon- γ (IFN- γ) [126]. CD27⁺ counterparts of $\gamma\delta$ T cells secrete IL-17 and are thought to promote tumor growth and metastasis through cross-talk with myeloid cells [126]. NKT cells can also directly kill tumors and are thought to serve as a bridge between the innate and adaptive immune responses due to their ability to produce cytokines. Importantly, NKT cells are able to respond to tumor-derived lipids, suggesting that alterations of the lipid repertoire within a tumor allow NKT cells to recognize cells undergoing malignant transformation. Just as there is a subset of $\gamma\delta$ T thought to promote tumor growth, there is a subset of NKT cells that also promotes tumor growth. NKT cells are reviewed in more detail in the next section.

1.10 NKT cells

Introduction

The discovery of conserved V α 14 chains across several hybridoma lines was quite unusual [128] because TCRs are highly diverse, with 70-80 α chain genes giving an enormous diversity to TCR α . *In vivo*, a population of thymocytes possessing a single V β chain (V β) was described and posited to “represent a distinct lineage” [129]. It was this discovery that prompted the search for a T cell subtype characterized by an invariant V α 14 chain with V β chains of limited diversity. Koseki et al. discovered that the frequency of the V α 14⁺ cells was much higher than expected, with V α 14⁺ cells comprising up to 10-20% of T cells in the livers of mice [130]. Almost a decade after the discovery of this remarkable population of V α 14⁺ cells, Adachi et al. noted that they were greatly reduced in β 2-microglobulin deficient mice [131]. Later, Lantz and Bendelac ascertained that the V α 14⁺ cells all recognized CD1d and it became clear that they were a distinct population of T cells [132].

NKT subpopulations are highly heterogeneous in both phenotype and function and can be divided broadly into CD4⁺ and CD4⁻ subsets. In humans, CD4⁺ NKT cells produce Th1 and Th2 cytokines, whereas double-negative and CD8⁺ NKT cells primarily produce Th1-type cytokines [133]. Mouse NKT cells also include CD4⁺ and CD4⁻ subsets, with different subsets possessing diverse functional profiles. Mouse NKT cell populations exhibit diversity in their ability to produce cytokines, depending on the organ and subsets. NKT cell subpopulations from thymus, spleen, and liver produced 19 cytokines with differences of 10- to 100-fold in response to CD3/CD28 stimulation, depending on the organ [134]. Liver-derived NKT cells produced equal or higher amounts of IFN- γ , as compared to thymic NKT cells in response to CD3/CD28 stimulation [134]. Moreover, liver-derived CD4⁻ NKT cells mediate tumor rejection more

effectively than CD4⁺ NKT cells [135], suggesting that differential cytokine profiles of each of the subsets play an important role. Production of these cytokines may facilitate conventional T cell differentiation, with NKT cells serving as primary initiators of antigen-specific responses. Studies delineating the differences in NKT cell subtypes are ongoing.

Cytokines produced by NKT cells

The majority of NKT cells found in the periphery express both IL-4 and IFN- γ mRNA [136]. NKT cells are primed to exert their effector function and could thus provide immediate protection at sites of pathogen entry, while conventional T cells are undergoing antigen-specific expansion [136]. Moreover, production of these cytokines may facilitate conventional T cell differentiation, with NKT cells serving as primary initiators of antigen-specific responses [136].

Depending on the type of lipid presented to NKT cells in the context of CD1d, differential cytokine profiles can be induced. The best studied and most frequently used ligand for NKT cells is α -Galactosylceramide, α -GalCer, a glycolipid originally isolated from a marine sponge *Agelas mauritianus* [137]. α -GalCer induces rapid cytokine production and proliferation and has been extensively studied as an adjuvant in cancer. For example, α -GalCer induces IL-4, IL-13 and IFN- γ , but β -GalCer is a poor inducer of IFN- γ , TNF- α , GM-CSF, and IL-4 gene expression [138]. These findings have led to the design of glycolipids that can skew serum cytokine production toward Th1 responses and novel analogs of α -GalCer are currently in development.

IL-12p70 and IL-23 are members of a small family of heterodimeric cytokines predominantly produced by DCs and macrophages. IL-12p70 is involved in the induction and amplification of the Th1 response, while IL-23 mediates inflammatory responses, through induction of expansion of Th17 cells [139]. Uemura et al. demonstrated that when NKT cells are

co-cultured with DCs, NKT cells enhance IL-12p70 production while downregulating IL-23 production by DCs [139]. Moreover, NKT cells can be activated both in antigen-independent and IL-12 and Toll-like receptor (TLR)-dependent manner [140].

Effects of cytokines produced by NKT cells

NKT cells can mediate anti-tumor activity via three mechanisms (Figure 1.6). First, they can directly kill tumor cells [127]. Second, they can induce maturation of dendritic cells, in a CD40-CD40L-dependent manner [141], thus initiating adaptive anti-tumor immunity. Finally, they activate NK cells and T cells by producing pro-inflammatory cytokines, such as IFN- γ and TNF- α . The importance of NKT cells in mediating NK and T cell responses was demonstrated in FBL-3 erythroleukemia and B16 melanoma mouse models. In this model, NK and T cells could not mediate tumor rejection in the absence of NKT cells [142].

In vivo administration of α -GalCer rapidly activates NKT cells to release Th1 and Th2 cytokines, which contribute to the activation of NK cells, dendritic cells, and T lymphocytes [143]. Immature DCs can present antigens to NKT cells, which induce DC maturation, which in turn provides the necessary co-stimulation for NKT cell activation [144].

In addition to playing an important role in activation of DCs, NKT cells can help mount B cell-mediated anti-tumor immune responses. NKT cells induce B cell maturation and expansion of memory B cells, even in absence of α -GalCer, suggesting that endogenous ligands can be presented in the context of CD1d on B cells [145]. Coadministration of α -GalCer and protein vaccines activates NKT cells to help B cells produce primary and memory antibody responses, with CD40-CD40L interaction necessary for optimal antibody responses [145]. In the absence of NKT cells, antibody responses can still be mounted, but the serum antibody titers decrease more

rapidly, suggesting that NKT cells are needed for maintaining plasma cell survival [145]. Since antibody binding of tumor antigens is important to Fc γ receptor engagement-dependent DC activation [146], NKT cells can indirectly facilitate induction of DC maturation by playing a role in allogeneic antibody production.

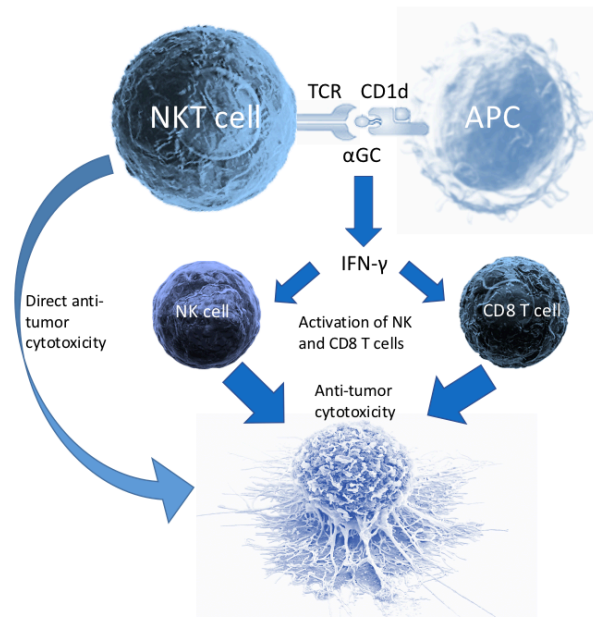


Figure 1.6: NKT cells bridge innate and adaptive immune response. NKT cells have been shown to augment anti-tumor responses due, in part, to their capacity for rapid production of large amounts of IFN- γ , which acts on NK cells to target MHC-negative tumors, and also, to target CD8 cytotoxic T cells to promote killing of MHC-positive tumors.

Co-stimulatory requirements

NKT cells constitutively express cytokine mRNA and can synthesize cytokines in the absence of CD28 signaling, unlike conventional T cells, which require CD28 for cytokine gene transcription [147]. Cytokine production by NKT cells is independent of CD28/CD40 co-stimulatory pathways, and even though CD28^{-/-} mice have NKT cells, CD28 and CD40 signaling is required for optimal expansion of the NKT cells *in vivo*. Thus, NKT cells can be activated without CD28 co-stimulation, while the production of downstream factors such as IL-12 by DCs may be necessary to mount a full NKT cell response [135].

NKT cells express CD28 and CD28-CD80/CD86 co-stimulation is necessary for IL-4 production, which is drastically reduced in the presence of anti-CD80 and anti-CD86 antibodies [148]. Moreover, blockade of CD80 and CD86 resulted in a marked but partial inhibition of IFN- γ production [148]. CD28- and CD40-deficient B6 mice have impaired IFN- γ production, but IL-4 production is impaired in CD28-deficient mice only [148]. In addition, NKT cells from CD28 or CD40-deficient mice have impaired cytotoxic activity. CD154-CD40 blockade polarizes NKT cells toward a Th2 phenotype, with Th2 cytokine production being dependent on CD28 co-stimulation. On the other hand, IFN- γ production is dependent on IL-12 production by DCs and requires CD154-CD40 interaction, while CD28 is able to regulate IFN- γ production independently of IL-12, suggesting that IFN- γ production is dependent on both CD28 and CD40 co-stimulation in C57BL/6 mice [148].

α -GalCer has been shown to promote DC maturation and subsequent antigen-specific T cell responses, with CD40-CD40L necessary for optimal DC-NKT cell interaction [149]. Moreover, TNF- α -induced maturation of DCs led to OX40L upregulation, which, through

engagement of OX-40 on NKT cells, activated NKT cells [144]. IL-12 produced by DCs is critical for IFN- γ production by NKT cells and requires CD154-CD40 interaction.

Due to their ability to rapidly produce a wide variety of cytokines, NKT cells can polarize the immune response toward a Th1 profile, while in other cases, NKT cells drive Th2 responses. In addition, NKT cells play an immunoregulatory or immunosuppressive role in some systems, usually through their production of Th2-type cytokines. In other systems, they appear to promote enhanced cell-mediated immunity via production of Th1-type cytokines. Work focused on delineating the factors that drive cytokine polarity of the NKT cell responses is ongoing. Ultimately, understanding of co-stimulatory and antigen stimulation requirements of NKT cell subsets will help identify the mechanisms of Th1/Th2 polarization.

NKT cells in immunotherapy

NKT cells were shown to be important for effective anti-tumor immunity in several cancer models, with adoptive transfer experiments in mice demonstrating that CD4⁻ NKT cells are the main anti-tumor NKT cell subset [150]. NKT cells can directly lyse CD1d⁺ tumors [137] and ectopic expression of CD1d in tumors can enhance NKT cell-mediated anti-tumor responses [151]. On the other hand, cross-presentation of tumor-derived glycolipids by dendritic cells allows induction of NKT cell-dependent T cell-mediated anti-tumor immunity [152]. NKT cells are reduced in number and function in cancer patients [153] and a correlation between a higher NKT cell frequency and improved prognosis has been demonstrated [154].

The potent anti-tumor functions of NKT cells prompted researchers to examine the efficacy of NKT cell-based therapies. Administration of α -GalCer first showed efficacy in a mouse model of melanoma [155,156]. Moreover, our lab demonstrated that a single dose of α -GalCer reduced

tumor burden and stalled progression of MCL [157]. Similarly, administration of α -GalCer-loaded autologous tumor cells in a transgenic mouse model of myc oncogene-driven B cell lymphoma inhibited tumor growth and enhanced survival [158]. In humans, administration of α -GalCer in a phase I study demonstrated that α -GalCer is well-tolerated and, while no clinical responses were recorded, patients with higher NKT cell numbers exhibited positive biological effects, such as detectable serum IFN- γ [159]. Moreover, administration of DCs loaded with α -GalCer led to expansion of NKT cells, increased serum cytokine levels, and increase in memory T cells [160]. Importantly, there are several clinical trials assessing efficacy and safety of NKT cell adoptive transfer currently underway in China (NCT02562963, NCT02619058, NCT01801852).

Given that NKT cells are reduced in number in cancer patients [153,161] and that correlative studies demonstrated that increased NKT cell numbers have a positive prognostic value, adoptive NKT cell transfer is a rational approach. As mentioned above, the factors governing NKT cell polarization are not yet fully understood. Thus, understanding how NKT cells display differential cytokine profiles will be crucial before adoptive transfer of a specific subset can yield effective results. For example, adoptive transfer of bulk NKT cells may not be efficient because of existence of multiple subtypes of NKT cells within the bulk population that may inhibit the action of Th1-type NKT cells.

Adoptive transfer of NKT cells was demonstrated to have efficacy in a mouse model of CD1d⁺ lymphoma model [162]. In a phase I study of adoptive transfer of NKT cells in patients with advanced and recurrent lung cancer, no partial or complete responses were recorded [163]. Nonetheless, increased numbers of activated NKT cells were observed in peripheral blood [163].

Adoptive transfer of whole peripheral blood mononuclear cells (PBMCs) cultured with IL-

2/GM-CSF and pulsed with α -GalCer in patients with non-small cell lung cancer resulted in an increase in both the number of circulating IFN- γ -producing cells in the peripheral blood [164]. Patients who displayed increased IFN- γ -producing cells had increased median survival time (MST) of 31.9 months, compared to the poor responders, who survived a median of 9.7 months [164]. The authors attributed this stark difference in survival not due to the patients' pre-existing immune system status, but to the intervention itself because the IFN- γ -producing ability of the good responder group was not significantly higher than that of the poor responder group [164]. Moreover, the MST of all patients (including the poor responders) was improved, compared to standard regimens [164]. The authors posited that the administration of α -GalCer-pulsed IL-2/GM-CSF-cultured PBMCs led to endogenous NKT cell expansion. Trials of α -GalCer administration or adoptive transfer of NKT cells have yet to show complete or partial remission [127]. A potential explanation includes the fact that NKT cell numbers are lower in humans than in mice and are more variable from individual to individual [127,165,166]. These studies provided evidence for the role of NKT cells in anti-tumor immunity, but future studies would provide more an explanation for why NKT cells can play an immunoregulatory role in one setting, while mediating tumor regression in another setting.

NKT cells have been exhaustively shown to play a role in anti-tumor immune responses. However, the precise mechanisms that allow tumor evasion from NKT cell-mediated anti-tumor immunity are poorly understood. Further studies will help determine how NKT cells promote tumor rejection. A focus on tumor-intrinsic mechanisms of dysregulation of antigen presentation is crucial to understanding why NKT cells ultimately fail to mediate tumor regression. In our studies, we identified an epigenetic mechanism that allows MCL cells to dysregulate CD1d-mediated antigen presentation. Moreover, we discovered two cytokines previously unknown to

inhibit CD1d-mediated antigen presentation. Finally, we established a link between angiogenesis and immunosuppressive ganglioside shedding. Given the complexity of cancer and the multilayered processes that allow tumor evasion from immune surveillance, it is crucial to study the complex networks that govern anti-tumor immune responses. The mechanisms of tumor immune evasion need to be characterized to identify potential intervention strategies. In this work, we identify several mechanisms of tumor immune evasion that can be targeted in the future.

Chapter 2: Materials and Methods

2.1 Cell lines

NKT cells and T cells

The NKT cell hybridoma cell lines DN32.D3, N38-3C3, and N37-1A12 have been previously described [167,168] and were cultured in IMDM medium supplemented with 5% FBS, 2 mM L-glutamine and Penicillin/Streptomycin.

DR4-restricted 17.9 CD4⁺ T cell hybridomas were generously provided by Dr. Janice Blum (Indiana University School of Medicine, Indianapolis, IN, USA) and cultured in RPMI supplemented with 10% FBS, 50 μ M 2-mercaptoethanol and 2 mM L-glutamine.

L cells

L-CD1dwt cells are CD1d1-transfected mouse fibroblast cells, kindly provided by Dr. Randy Brutkiewicz (Indiana University School of Medicine, Indianapolis, IN, USA), were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 500 μ g/mL G418 as a selection agent, and Penicillin/Streptomycin. L-CD1d-DR4 cells, transfected with CD1d and DR4 were provided by Dr. Randy Brutkiewicz and cultured as above.

Human lymphoma cells

Mantle cell lymphoma lines, JeKo-1 and SP53 were graciously provided by Dr. Raymond Lai (University of Alberta, Edmonton, AB, Canada). The cell lines were authenticated by ascertaining expression of cell surface markers, such as CD19 and CD20, as described [169].

The human B lymphoblastoid cell line transfected with human CD1D, C1R-CD1D, was kindly provided by Dr. Mark Exley (Harvard Medical School, Boston, MA, USA). Burkitt

lymphoma cell lines Raji and Daudi were kindly provided by Dr. Ronald Gartenhaus (University of Maryland Baltimore, Baltimore, MD, USA).

Murine lymphoma cells

Murine B cell lymphoma cell lines, WEHI-231, CH31, and CH33 were graciously provided by Dr. Gregory Carey (University of Maryland School of Medicine, Baltimore, MD, USA). The murine mantle cell lymphoma (FMCL) was established from lymph nodes of a blastoid variant MCL mouse model [157]. Lymph nodes were isolated from a mouse at an advanced disease stage. B cells were isolated using a Mouse B Cell Isolation Kit (Stem cell Technology). The characteristics of the cell line are presented in Figure 2.1.

All lymphoma lines were cultured in RPMI 1640 medium supplemented with non-essential amino acids (Sigma-Aldrich, St. Louis, MO, USA), sodium pyruvate (Gibco, Carlsbad, CA, USA), 2-mercaptoethanol (Gibco), vitamin solution (Gibco), 10% fetal bovine serum (Gibco), and Penicillin/Streptomycin (Gibco).

Ovarian cancer cells

Human ovarian cancer cells, SK-OV-3 (purchased from ATCC), were cultured in McCoy's 5a Modified Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. OV-CAR-3, purchased from ATCC, were grown in in RPMI-1640 medium supplemented with 20% fetal bovine serum, 0.01 mg/mL bovine insulin, and penicillin/streptomycin.

2.2 Antigens and inhibitors

α -Galactosylceramide (α -GalCer), was purchased from Enzo Life Sciences (New York City, NY, USA) and used at a final concentration of 100 ng/mL. GD3 was purchased from Matreya (State College, PA, USA). Human Serum Albumin was purchased from Sigma-Aldrich and used at 10 μ M final concentration.

Trichostatin A was purchased from Cell Signaling Technology and reconstituted in ethanol. MC1568 (Sigma-Aldrich) was reconstituted in DMSO. Panobinostat/LBH589 (Biovision, Milpitas, CA, USA) was reconstituted in DMSO.

Genistein was purchased from Sigma (#G6649) and reconstituted in DMSO. Bevacizumab (Genentech, San Francisco, CA, USA) was supplied reconstituted by the manufacturer in water supplemented with salts.

2.3 NKT and T cell assays

To measure NKT cell responses to lymphoma cells, MCL cells were treated with the indicated amounts of drugs for 4 h, pulsed with α -GalCer (100 ng/mL), washed extensively, and cocultured (1×10^5 cells/well) with primary human NKT cells (2×10^4 cells/well) in triplicate wells in 96-well microtiter plates. In assays using NKT cell hybridomas, MCL cells were pre-treated with drugs for 4 h, pulsed with α -GalCer (100 ng/mL), fixed in 0.05% paraformaldehyde, washed extensively, and cocultured (5×10^5 cells/well) with the NKT cell hybridomas (5×10^4 cells/well) in triplicate wells in 96-well microtiter plates. In human NKT cell assays, after a 48- to 72 h incubation, supernatants were harvested, and IFN- γ was measured by ELISA kit purchased from BioLegend (San Diego, California, USA). In NKT cell hybridoma assays, after

16- to 24 h incubation, supernatants were harvested and IL-2 was measured by standard ELISA kit (BD Biosciences, San Jose, CA, USA).

For the peptide antigen presentation assay, L-CD1d-DR4 cells were loaded with human serum albumin (HSA) overnight, treated with HDACi for 4 h, washed extensively and cocultured with 17.9 T cells hybridomas. Following the co-culture, supernatants were harvested and IL-2 levels were measured by ELISA.

In PBMC assays, NKT cells were stimulated with CD1D-aAPC, as previously described [170]. T cells were activated using CD3/CD28 beads. Supernatants were harvested at the end of a 48- to 72 h incubation and IFN- γ was measured by ELISA kit purchased from BioLegend.

2.4 Western blotting

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich), supplemented with phenylmethylsulfonyl fluoride (PMSF) (Cell Signaling Technology, Beverly, MA, USA). Proteins were resolved by electrophoresis on a 4-12% gradient polyacrylamide gel and transferred to a PVDF membrane using the Bolt Mini Blot Module (Life Technologies, Carlsbad, CA, USA). All polyacrylamide gels, gel boxes, Bolt Mini transfer modules, buffers and other materials were purchased from Life Technologies and were used according to the manufacturer's instructions. Membranes were probed with antibodies to HDACs 1-3, clones 10E2, 3F3, and 7G6C5, respectively, purchased from Cell Signaling Technology. GAPDH levels were assessed on the same blot as the test protein using an antibody (clone 14C10) from Cell Signaling Technology. STAT3 (clone 79D7) and phospho-STAT3 (Y705, clone D3A7) antibodies were purchased from Cell Signaling Technology. DyLight800-conjugated anti-rabbit secondary antibody was purchased from Thermo Scientific (Waltham, MA, USA) and DyLight800-

conjugated anti-mouse antibody was purchased from Cell Signaling Technology. Membranes were scanned using the Odyssey Imaging System from Li-COR Biosciences (Lincoln, NE, USA).

2.5 Immunoprecipitation

STAT3 was immunoprecipitated from JeKo-1 lysates using Protein G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA), following the manufacturer's instructions. Immunoprecipitation was performed with three STAT3 antibodies: D3Z2G (Cell Signaling Technology), 79D7 (Cell Signaling Technology), and C-20 (Santa Cruz). Immunoprecipitates were resolved by SDS-PAGE and HDAC2 detection was performed as described above.

2.6 Flow cytometry and LEGENDplex assay

Cells were stained in PBS containing 0.5% bovine serum albumin and 2 mM EDTA for 30 min at 4°C with a PE-conjugated antibody to human CD1D (clone 51.1) from BioLegend. Intracellular cytokine staining was accomplished using PE-conjugated antibodies for STAT3 (clone M59-50) and phospho-STAT3 (pY705, clone 4/P-STAT3) from BD Biosciences, following the standard protocol by BD Biosciences. GD3 antibody (Abcam) was used at a concentration of 1 µg/mL. ERK and pERK antibodies were purchased from Cell Signaling Technology and used at 1:50 dilution. Secondary antibodies for GD3 (PE anti-mouse IgG; BioLegend) and ERK/pERK (APC anti-rabbit IgG; Life Technologies) were used at 1:50 dilution. Multiplex assay for inflammatory cytokines was performed using LEGENDplex Human Inflammation Panel from BioLegend, following kit instructions. Data were collected on an LSR II from BD Biosciences and analyzed using FCS Express Version 5 from De Novo Software (Los Angeles, CA, USA).

2.7 Conditioned medium experiments

LD1d were treated with the clarified supernatants for 4 hours at 37°C, unless otherwise indicated. The L cells were subsequently washed extensively with PBS, and cocultured with NKT hybridomas for 20 to 24 hours at 37°C. Cytokine release was assessed as an indication of NKT/ T cell activation and was measured by standard sandwich ELISA.

2.8 WST assay

WST (water soluble tetrazolium) assay was purchased from Roche (Basel, Switzerland) and used according to manufacturer's instructions. Absorbances were recorded at 4 and 24 hours post treatment.

2.9 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed by Active Motif (Carlsbad, CA, USA), with a validated and tailored protocol. Promoter probes were designed by Active Motif to span the known sequence of the CD1D promoter [75] and included appropriate positive controls: beta-actin (ACTB) and interferon response factor 1 (IRF1). Primers for both distal and proximal promoters of CD1D were used, spanning the distal promoter at -498 base pairs and the proximal promoter, at +42 base pairs, relative to the transcription start site. At least 2-fold enrichment over the negative control, Untr12, was considered to be a positive signal. Untr12 sequence spans a gene desert on chromosome 12 and does not have transcription factor binding sites.

2.10 HDAC2 knockdown

HDAC2 knockdown was achieved using lentiviral particles purchased from Santa Cruz Biotechnology, at multiplicity of infection (MOI) of 2. Polybrene based transduction was carried out according to the manufacturer's instructions. Stable transductants were cultured in medium containing the selection agent puromycin dihydrochloride at a final concentration of 2.5 $\mu\text{g}/\text{mL}$ (Sigma-Aldrich). HDAC2 knockdown was confirmed by Western blotting.

2.11 shHDAC2 JeKo-1 limited dilution assay

JeKo-1 cells were cloned by seeding an average of 1 cell per well in a 96 well microtiter plate under puromycin selection. Clones were allowed to grow and expand and were selected based on variable expression of CD1D levels using flow cytometry. Low and high CD1d expressing clones were selected for further expansion. HDAC2 knockdown was confirmed by Western blotting.

2.12 Mice

Non-obese diabetic *scid* common gamma chain deficient (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were used at approximately 6 weeks of age. JeKo-1 cells were passaged *in vivo* and injected into the flank. All animals were housed in specific pathogen-free conditions at the University of Maryland, Baltimore. All procedures were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) at University of Maryland, Baltimore. Mice were euthanized when tumors reached maximum IACUC-approved size. After euthanasia, tumors were excised, weighed, and single cell suspensions were prepared using 40 μm filters.

2.13 Statistical analyses

All experiments were performed at least three times. Student's T tests were performed to determine significance. When appropriate, analysis of variance (ANOVA) was performed. A p value of <0.05 was considered significant. All analyses were performed using GraphPad Prism software (La Jolla, CA, USA). * $p<0.05$, ** <0.01 , and *** $p<0.0001$.

Chapter 3: Cell-intrinsic effects of HDACi

3.1 Introduction

A hallmark of cancer cell survival is their ability to evade immune destruction by utilizing various immunosuppressive mechanisms [8]. This is essential for the tumor because the host immune system possesses the potential to eliminate malignancies through a multi-layered process that includes early recognition of transformation events by mediators of innate immunity, followed by the development of a strong and highly specific adaptive immune response [171]. NKT cells have the capacity to mount strong anti-tumor responses and have thus become a major focus in the development of effective cancer immunotherapy, with several studies showing that NKT cells are necessary for appropriate anti-tumor responses [127,172,173]. In this respect, NKT cells have been shown to augment anti-tumor responses due, in part, to their capacity to rapidly produce large amounts of IFN- γ , which acts on NK cells to target MHC-negative tumors, and also, to target CD8 cytotoxic T cells to promote killing of MHC-positive tumors [127]. In good agreement with findings by other groups, studies from our lab have provided evidence that, in a variety of tumor models, treatment of mice with a specific and potent activator of NKT cells, α -GalCer, triggers elimination of both MHC-negative and MHC-positive tumor cells, resulting in complete tumor eradication without detectable tumor recurrence [127].

We posited that tumors utilize epigenetic mechanisms to dysregulate CD1d-mediated antigen presentation. Our studies focus on epigenetic regulation of CD1d transcription. The transcriptional control mechanisms that regulate CD1d gene expression remain to be elucidated. 5' rapid amplification of cDNA ends (RACE) of the CD1D gene identified dual promoters regulating the CD1D gene, with several putative transcription factor binding sites [73]. The transcription factor Sp1 was found to control the function of the proximal promoter [73].

Moreover, lymphoid enhancer-binding factor-1 (LEF-1) negatively regulates CD1D gene expression, through recruitment of HDAC1 [75]. Moreover, previous studies have shown that treatment with HDACi induces CD1D gene expression in solid tumors [74]. We thus postulated that HDACs negatively regulate CD1D gene expression and sought to identify other transcription factors that bind the CD1D promoter. To test our hypothesis, we treated MCL cells with HDACi and assessed antigen presentation capabilities of MCL cells and identified the specific HDAC regulating CD1D gene expression. Our studies are the first to demonstrate a functional effect of HDACi on CD1d-mediated antigen presentation. Moreover, we are the first to demonstrate that HDAC2 is a negative regulator of CD1D gene transcription in MCL.

Chapter 3.2: Results

Establishment of a novel murine MCL cell line and humanize mouse model

Previously, our lab showed that NKT cells can recognize lymphoma cells [157]. Importantly, human MCL cells require addition of exogenous antigen, and while most mouse lymphoma cell lines needed exogenous antigen, one cell line (WEHI-231) did not require addition of exogenous antigen. This suggests that human MCL cells lack an endogenous activating antigen and highlighted the need for the development of a mouse MCL line that better reflected findings in human MCL cell lines (described below).

In vivo, we demonstrated that activation of NKT cells via a single injection of α -GalCer led to decreased tumor burden and improved disease pathology [157]. Furthermore, we tested the ability of NKT cells to mediate tumor regression in a humanized mouse model. We sought to determine whether NKT cells can recognize and kill lymphoma cells *in vivo* in the absence of exogenous antigen or cytokines. JeKo-1, first serially passaged *in vivo* to increase tumor aggressiveness, were injected into NSG mice. Once the tumors were established, primary human NKT cells expanded from healthy donor blood were injected into the tail vein. Tumors were allowed to grow and were then harvested. Tumor weights were recorded. A single injection of NKT cells was able to mediate tumor regression (Figure 3.1).

The use of the JeKo-1 NSG mouse model that we established will help delineate the role of HDACs in NKT cell-mediated responses *in vivo*. The potential applications of this model are described in more detail in Chapter 6.

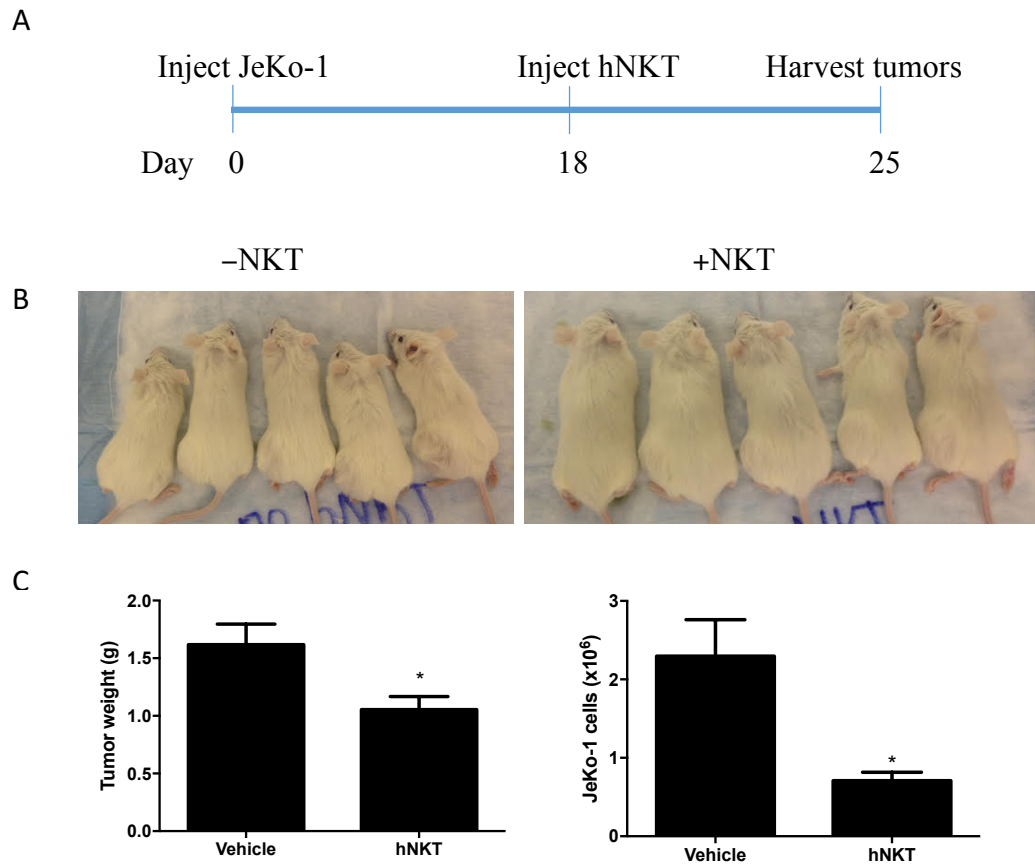


Figure 3.1: Human NKT cells mediate tumor regression in NSG mice. A) Study design. B) NKT cells were injected subcutaneously into the flank of mice. Left photo: no NKT cells. Right photo: NKT cells. C) Tumors weights were recorded and isolated tumor cells were counted. T-tests were performed to determine significance. * indicates a p value of <0.05.

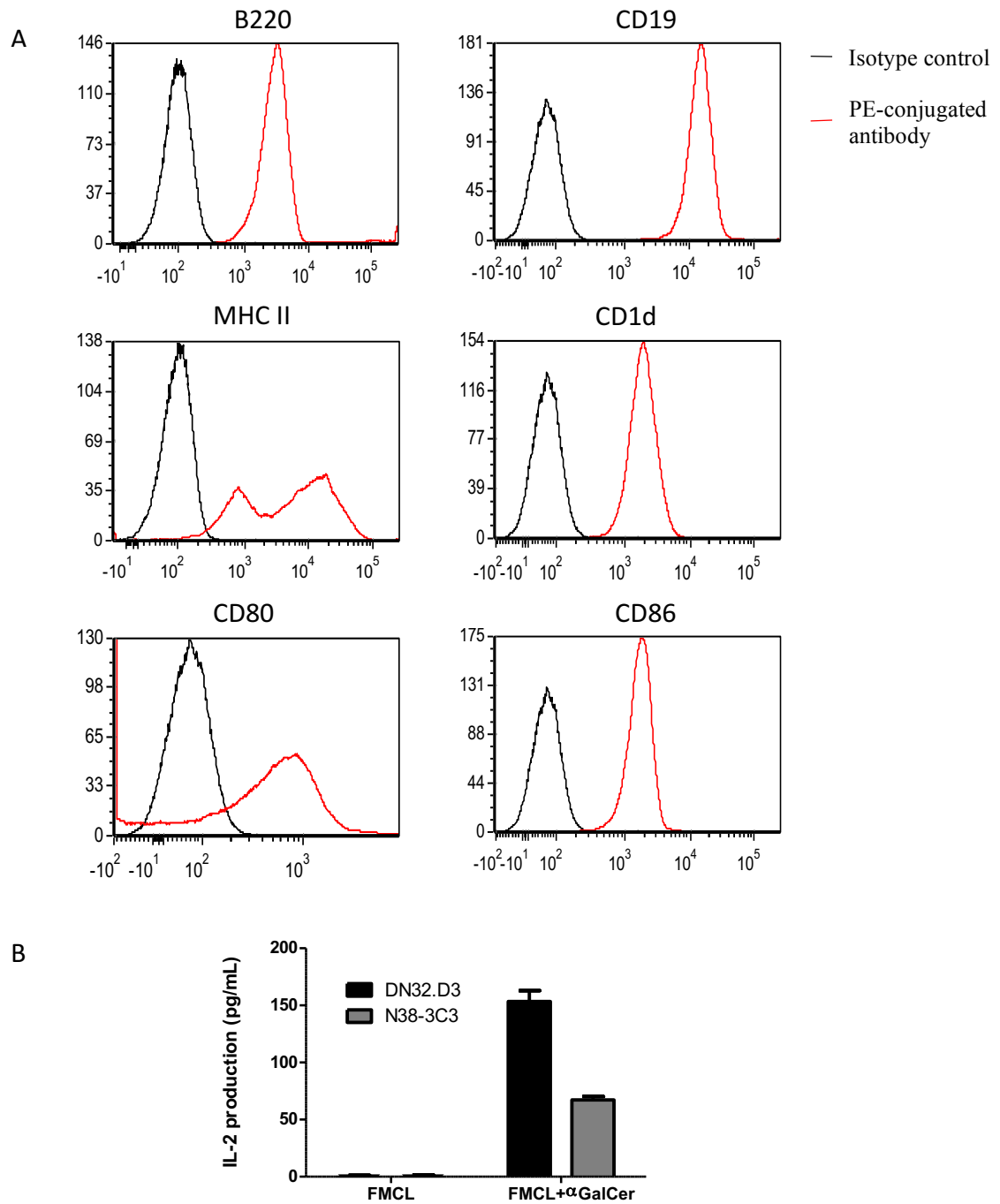


Figure 3.2: Characterization of a new mouse MCL cell line. A) FMCL expresses B cell markers, antigen presenting and co-stimulatory molecules. B) Primary murine lymphoma cells express functional CD1d molecules: FMCL present α -GalCer to NKT cell hybridomas DN32.D3 and N38-3C3.

Since there are no commercially available mouse MCL cell lines, we established a primary mouse cell line (termed FMCL) from a blastoid variant MCL mouse model. We posited that primary murine MCL lymphomas can present antigen to NKT cells and elicit cytokine production. Indeed, we demonstrated that they can serve as antigen presenting cells (Figure 3.2). We used this cell line to demonstrate that our findings on the role of HDACs in CD1d-mediated antigen presentation apply to both human and mouse MCL cells.

The mechanisms that govern NKT cell recognition of tumors remain poorly understood. As seen in Figure 3.2, some tumors can only activate NKT cells in the presence of an exogenous antigen, suggesting that they lack an endogenous activating antigen, dysregulate antigen processing and presentation, or secrete inhibitory molecules. Thus, we hypothesized that tumors utilize epigenetic mechanisms to dysregulate CD1d-mediated antigen presentation.

HDACi treatment enhances antigen presentation

To examine the effects of HDACi on CD1d-mediated antigen presentation, LCD1d cells were pre-treated with TSA, washed extensively, and cocultured with NKT cell hybridomas, DN32.D3, N38-3C3, and N37-1A12. CD1d-mediated antigen presentation was enhanced (Figure 3.3 A, B) as was MHC class II-mediated antigen presentation (Figure 3.3 C). We performed time-courses and found that CD1d-mediated antigen presentation is enhanced in as little as 4 hours following TSA treatment. In these studies, we treated WEHI-231 cells for 0, 2, 4, 6, and 8 hours, fixed them in order to remove confounding variables (such as changes in immunosuppressive cytokine secretion), and co-cultured them with NKT cells (Figure 3.3 D). Moreover, we noted that ability of WEHI-231 to activate NKT cells declined after 4 hours, which can be attributed to cell death-induced aberrations of antigen processing and presentation [174]. We tested whether these

findings are recapitulated in other mouse cell lines, including our MCL cell line, FMCL (described in Figure 3.2). TSA treatment induced antigen presentation by a panel of B cell lymphoma lines and by the primary MCL cell line (Figure 3.4). Thus, the use of the cell line we established was pivotal in demonstrating that HDACi enhance antigen presentation not only in B cell lymphoma lines, but in MCL cells specifically.

Importantly, our data demonstrate that, in as little as 4 hours, pretreatment of JeKo-1 and SP53 (MCL cells) with TSA enhanced antigen presentation to primary NKT cells expanded from healthy donors (Figure 3.5 A, B, C). Moreover, we tested whether TSA-induced changes would be recapitulated in another form of NHL, Burkitt lymphoma, by using Raji and Daudi cell lines (Figure 3.5 D). Comparable findings suggest that these two types of lymphomas utilize similar mechanisms to dysregulate CD1d-mediated antigen presentation. Figure 3.6 shows similar findings with two other HDACi, Panobinostat (10 nM) and MC1568 (50 nM). Panobinostat is a pan-HDACi, while MC1568 inhibits class II HDACs. MC1568 slightly induced CD1d-mediated antigen presentation, suggesting that class II HDACs play a minor role in CD1d-mediated antigen presentation or that the kinetics may be different.

Table 3.1 summarizes the characteristics and the effects of the HDACi tested. Since TSA induced both CD1d and MHC class II expression, we used TSA to ascertain the mechanisms by which HDACi enhance antigen presentation. Moreover, since TSA is the most-widely studied HDACi with well-described biologic effects, it was rational to use it in our mechanistic studies.

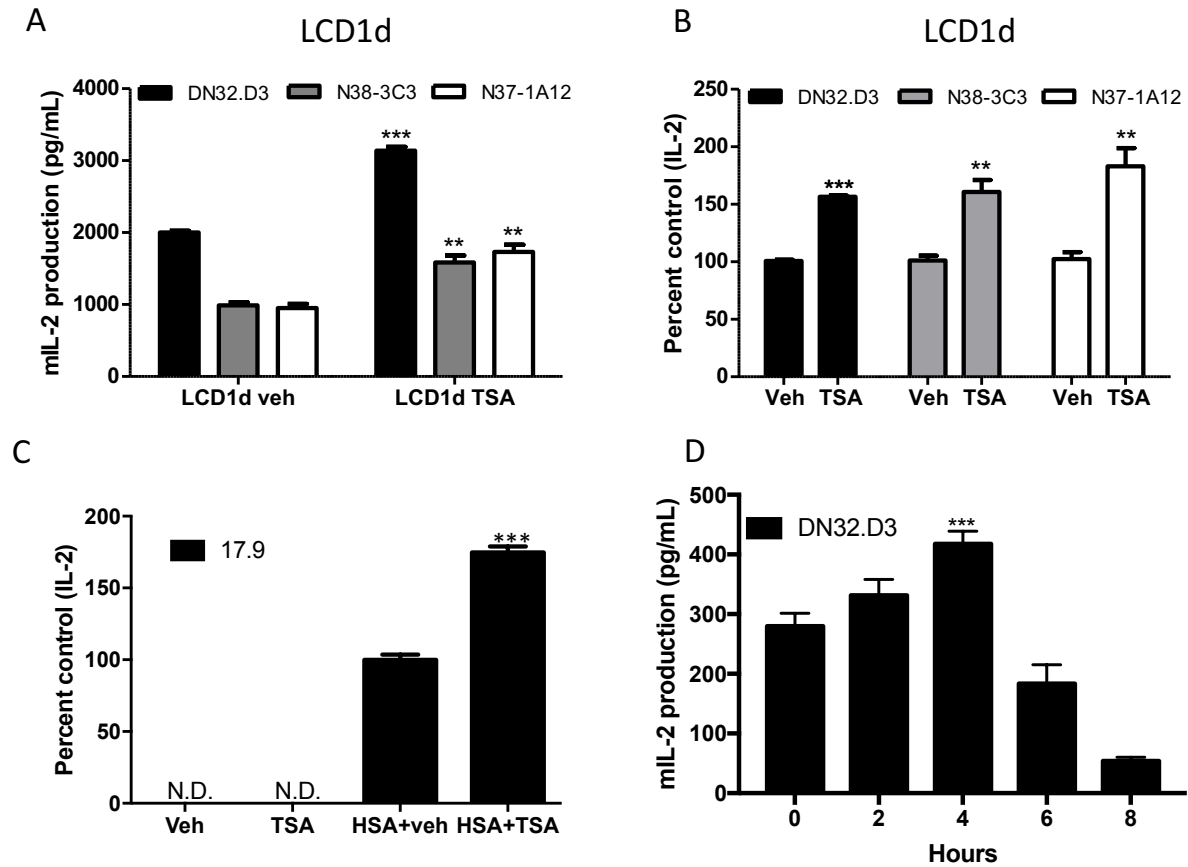


Figure 3.3: HDAC inhibitor treatment enhances CD1d- and MHC class II-mediated antigen presentation. A) LCD1d cells were pretreated with 1 μ M TSA for 4 hours, washed extensively and subsequently co-cultured with NKT cells, DN32.D3, N38-3C3, and N37-1A12. IL-2 levels were measured by standard ELISA. B) Graphical representation showing calculation of percent control of vehicle-treated cells. C) LCD1d-DR4 cells were pretreated with TSA and cocultured with DR4-specific T cells, 17.9, and IL-2 production was measured by ELISA. D) WEHI-231 cells were treated with TSA for 0-8 hours, fixed, and co-cultured with DN32.D3. T-tests were performed to test statistical significance: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$.

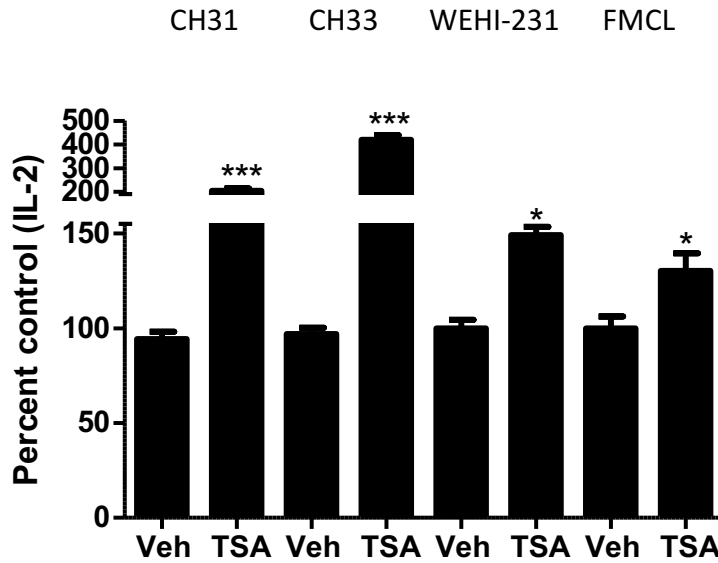


Figure 3.4: TSA enhances antigen presentation by mouse lymphoma cell lines, including a primary mouse MCL line. CH31, CH33, WEHI-231, and FMCL were treated with TSA for 4 hours, washed extensively and co-cultured with DN32.D3. Each of the cell lines was pulsed with α -GalCer (100 ng/mL). T-tests were performed to test statistical significance: * $p < 0.05$ and *** $p < 0.0001$.

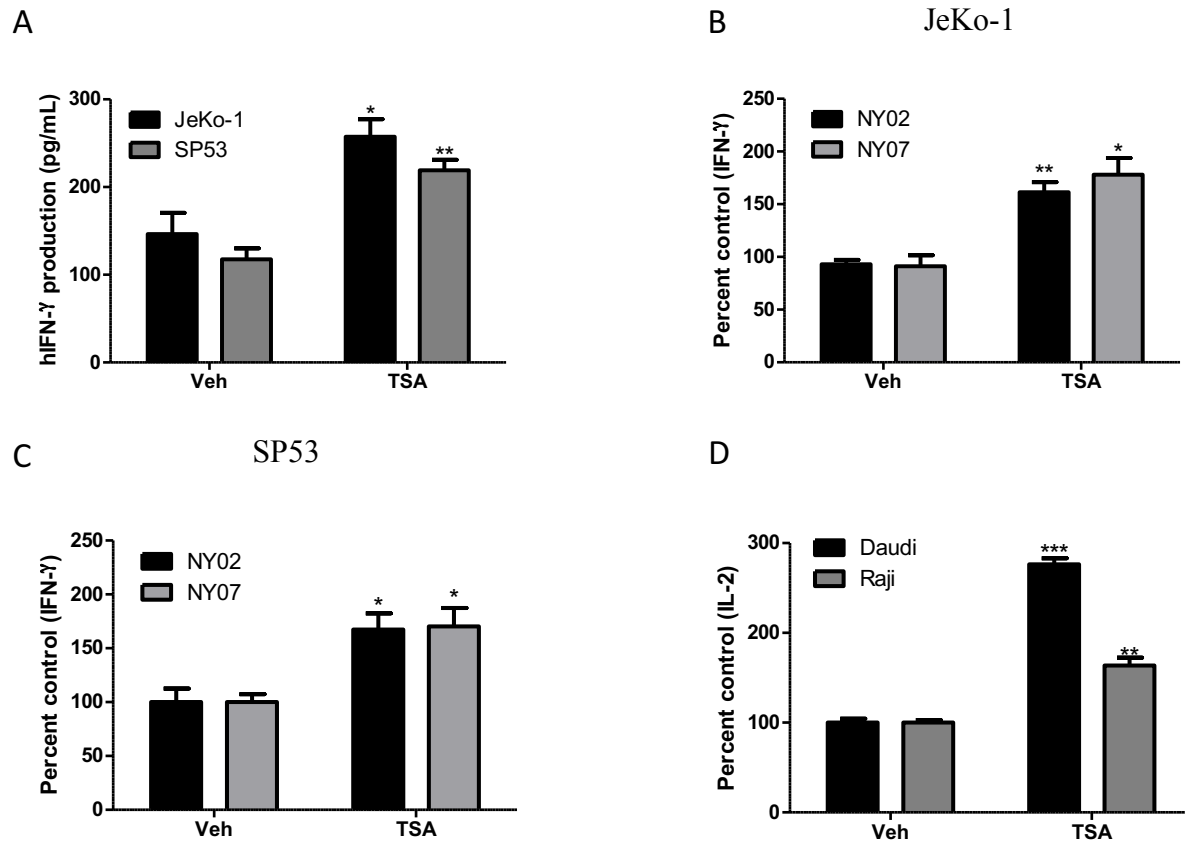
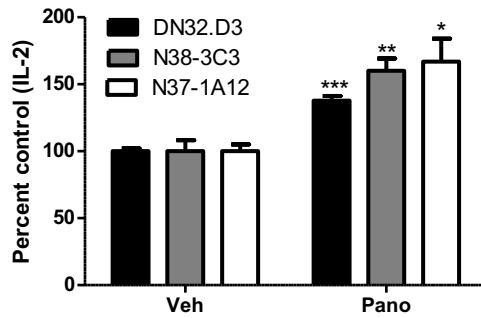


Figure 3.5: TSA enhances antigen presentation by human MCL cell lines. A) JeKo-1 and SP53 were treated with 1 μ M TSA, pulsed with α -GalCer (100 ng/mL), and co-cultured with primary human NKT cells derived from healthy donor blood. B and C) Similar experiments were performed with two other sets of primary human NKT cells; percent control IFN- γ production are shown for a representative experiment. D) Similarly, Daudi and Raji were treated with TSA and co-cultured with DN32.D3. T tests were performed to test statistical significance. * p<0.05, ** p<0.01, and *** p<0.0001.

A



B

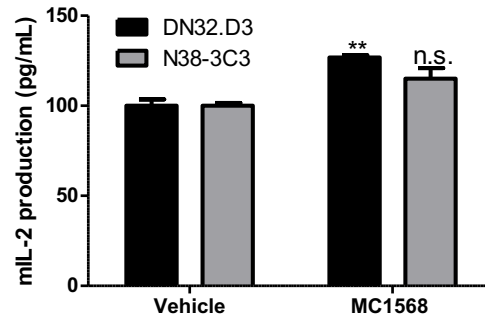


Figure 3.6: Effects of Panobinostat and MC1568 on CD1d-mediated antigen presentation.

LCD1d cells were treated with 10 nM Panobinostat for 4 h, washed, and co-cultured with DN32.D3, N38-3C3, and N37-1A12. B) JeKo-1 cells were treated with 50 nM MC1568 for 4 h, washed, and co-cultured with DN32.D3 and N38-3C3. T tests were performed to test statistical significance. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

	Trichostatin A (TSA)	LBH589 (Panobinostat)	MC1568
Class of HDACi	Hydroxamic acid	Cinnamic hydroxamic acid	(Aryloxopropenyl)pyrrolyl hydroxyamide
HDACs known to be inhibited	Class I and II HDACs: 1, 2, 3, 4, 6 and 10	All class I, II, and IV HDACs: 1-11	Class II HDACs: 4-7, 9, 10
Functional effects	Enhances CD1d-mediated and MHC class II-mediated antigen presentation	Enhances CD1d-mediated antigen presentation	Enhances CD1d-mediated antigen presentation

Table 3.1: Summary of HDACi characteristics and functional effects.

HDACi treatment induced CD1d mRNA and cell surface protein levels

We sought to determine whether the increase in CD1d-mediated NKT cell activation following treatment with TSA was due to increased CD1d expression. In good agreement with Yang et al. [74], who demonstrated that TSA treatment results in an increase in CD1D mRNA levels, we found that CD1D mRNA is induced rapidly following TSA treatment (Figure 3.7 A). Thus, TSA enhances antigen presentation, at least in part, by inducing CD1D transcription. TSA treatment induced an increase in cell surface CD1d expression, as assessed by flow cytometry and evidenced by increase in mean fluorescence intensity (MFI) (Figure 3.7). However, TSA treatment did not alter MHC class II cell surface expression, suggesting that different mechanisms regulate CD1d- and MHC class II-mediated antigen presentation.

To identify the specific HDAC(s) involved in CD1d-mediated antigen presentation, we examined HDAC expression in vehicle and TSA-treated JeKo-1 and SP53 cells by Western blot. We found that TSA treatment inhibited levels of HDACs 1 and 2 without affecting HDAC3 (Figure 3.8 A).

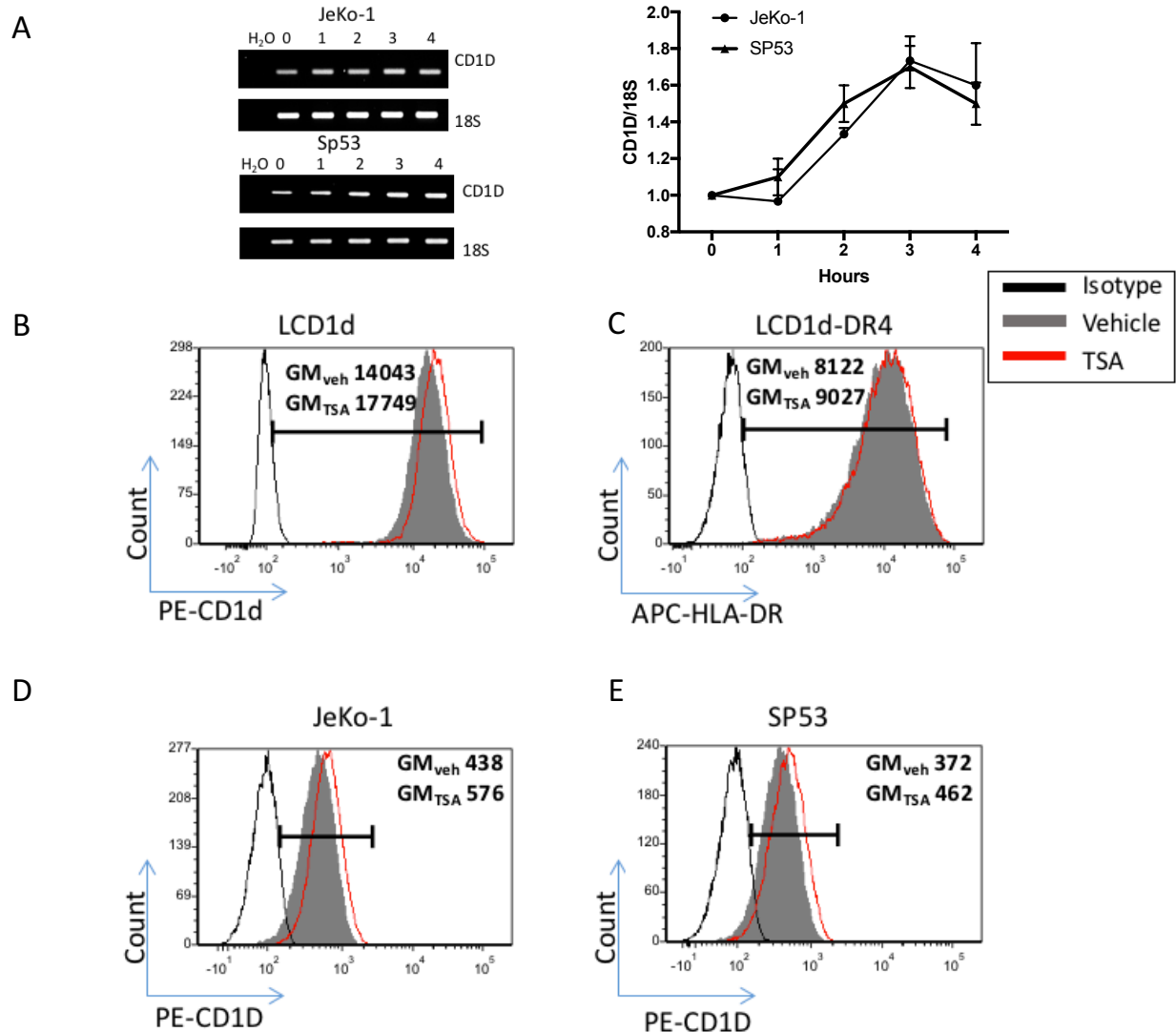
We hypothesized that HDACs bind to the CD1D promoter and negatively regulate CD1D transcription. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP). Primers for the CD1D promoter spanned distal (-498) and proximal (+42) promoters. Beta-actin (ACTB) served as a positive control, while a gene desert on chromosome 12 (Untr12) was the negative control. ChIP identified HDAC2 as binding to the proximal and distal CD1D promoters, whereas HDACs 1 and 3 did not bind (Figure 3.8 B). Bioinformatics analysis of the mouse and human CD1d promoters (Figure 3.9 A) identified putative STAT3 binding sites, but, as determined by ChIP, STAT3 did not bind (Figure 3.9 B). Thus, we knocked down HDAC2 in JeKo-1 (Figure 3.8 C) and assessed antigen presentation (Figure 3.10 A). We found that HDAC2 knockdown

enhances antigen presentation to both NKT hybridomas and primary human NKT cells (Figure 3.10 B, C). Importantly, treatment of shHDAC2 JeKo-1 cells with TSA did not further induce CD1D-mediated antigen presentation (Figure 3.10 D), which prompted us to determine whether HDAC2 is the main HDAC regulating CD1D-mediated antigen presentation. In TSA-treated JeKo-1 control and shHDAC2 cells, CD1D levels were comparable (Figure 3.10 E), suggesting that HDAC2 is the main HDAC regulating CD1D transcription in MCL.

To confirm a role for HDAC2 in regulating CD1D cell surface expression and to demonstrate that minute changes in CD1D expression can have a profound effect on NKT cell responses, we employed limited dilution assay to select HDAC2 knockdown-containing JeKo-1 cells expressing high and low levels of CD1D (termed Low and High clones) (Figure 3.11 B). We confirmed HDAC2 knockdown in the clones (Figure 3.11 A) and assessed their antigen presentation capabilities by co-culturing the clones with NKT cell hybridomas (Figure 3.11 C) and primary human NKT cells (Figure 3.11 D). Importantly, we noted a relationship in CD1D levels and the ability of MCL cells to activate NKT cells. Specifically, the clone expressing higher CD1D levels displayed enhanced antigen presentation capabilities, compared to the clone expressing lower levels of CD1D. Thus, downregulation of CD1D levels via HDAC2 upregulation may serve as a mechanism by which tumors evade recognition by NKT cells. Notably, we showed that NKT cells are sensitive to minute changes in CD1D levels (Figure 3.11 C, D), with a ~50% increase in cell surface expression translating to an approximately 2-fold functional change.

Finally, in order to confirm that NKT cells are sensitive to changes in CD1d levels, we blocked CD1d molecules on the surface of LCD1d cells and co-cultured them with NKT cells. Furthermore, we performed flow cytometry to confirm CD1d blockade. We found that NKT cells were sensitive to changes in CD1d cell surface levels before they were resolvable by flow

cytometry. Specifically, activation of N38-3C3 was inhibited following blockade with 5 $\mu\text{g}/\text{mL}$ blocking antibody (Figure 3.11 D) whereas the changes in CD1d levels were not detectable by flow cytometry (purple histogram, Figure 3.11 E). Importantly, activation of both DN32.D3 and N38-3C3 was inhibited in the presence of 10 $\mu\text{g}/\text{mL}$ blocking antibody (Figure 3.11 D).



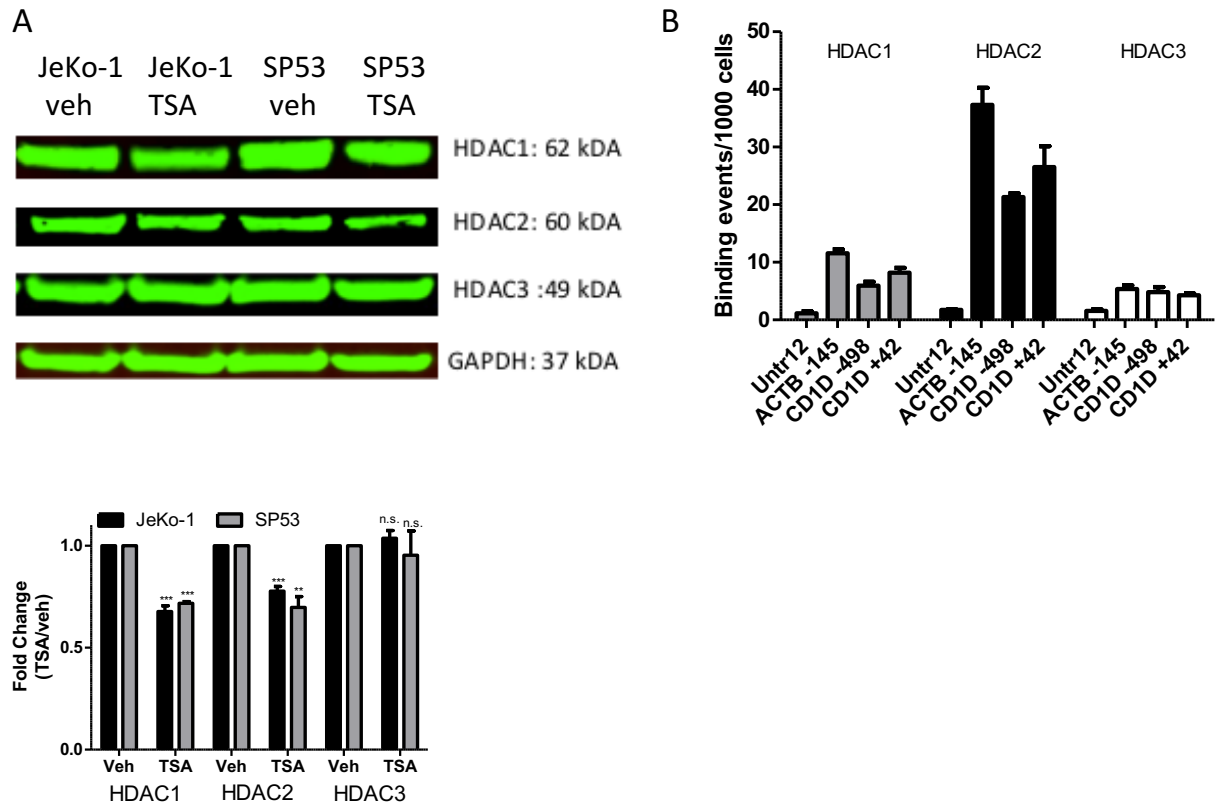


Figure 3.8: HDAC2 binds to the CD1D promoter. A) HDAC1-3 levels in TSA-treated JeKo-1 and SP53 cells were assessed by Western blot. GAPDH serves as a loading control. Lower panel: HDAC1-3 levels were normalized to GAPDH and fold change was calculated relative to vehicle-treated cells. Data are plotted for three representative experiments. B) ChIP was performed in JeKo-1, using primers for proximal and distal CD1D promoter, with beta-actin (ACTB) gene serving as a positive control.

A

	STAT3 binding sequence (TTCxxxGAA) within CD1d promoter	Location
Human	...CGTTAGCAGAAATG... ...CCTTAGTGAAAT...	Chromosome 1, 158149737:158154686:1
Mouse	...ATTTTCGCAAAATC... ...CATTATTTTAAATG...	Chromosome 1, 86995834:86999441

B

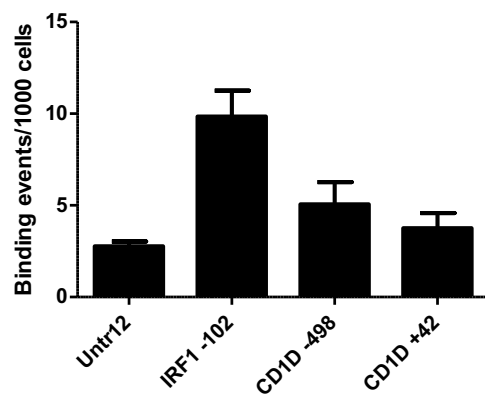


Figure 3.9: STAT3 does not bind to the CD1D promoter. A) Putative STAT3 binding sites within mouse and human CD1d promoters. The STAT3 binding sequence is TTCxxxGAA, with wobble at positions 3 (C, A, and T) and 7 (G, T, and A). B) ChIP for STAT3 binding within the proximal and distal CD1D promoters.

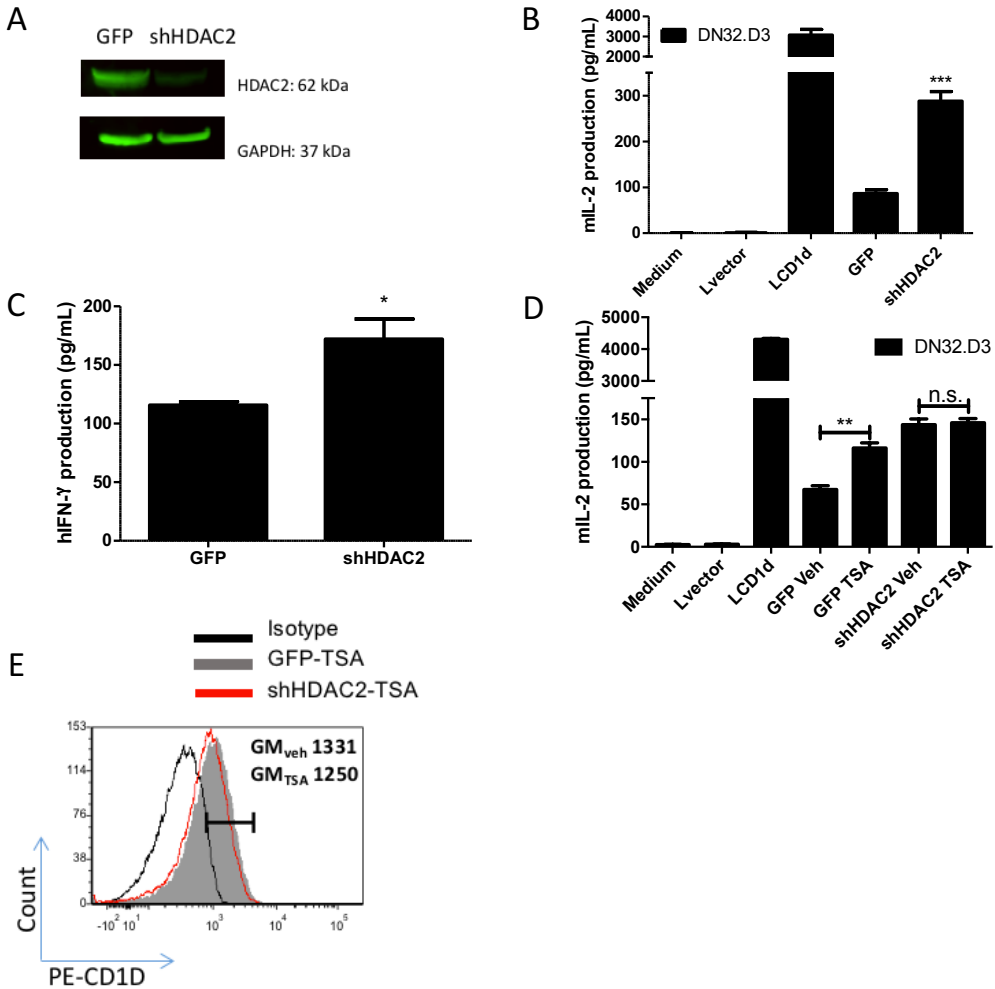


Figure 3.10: HDAC2 is the main HDAC regulating CD1d-mediated antigen presentation. A) HDAC2 knockdown in JeKo-1 cells. B) Antigen presentation of JeKo-1 GFP control and shHDAC2 cells was assessed in a co-culture with DN32.D3. LCD1d is a positive control. IL-2 production is shown. C) In a similar experiment, the GFP control and HDAC2 knockdown JeKo-1 cells were co-cultured with primary human NKT cells. D) Vehicle and TSA-treated GFP control and shHDAC2 JeKo-1 cells were co-cultured with NKT cell hybridomas. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$. E) GFP control and shHDAC2 JeKo-1 cells were treated with TSA and CD1D levels were assessed by flow cytometry. GFP TSA-treated cells are represented by the shaded grey histogram. shHDAC2 TSA-treated cells are represented by the red histogram.

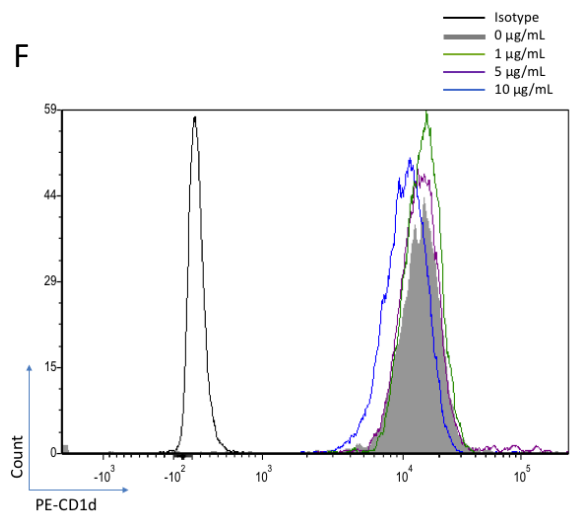
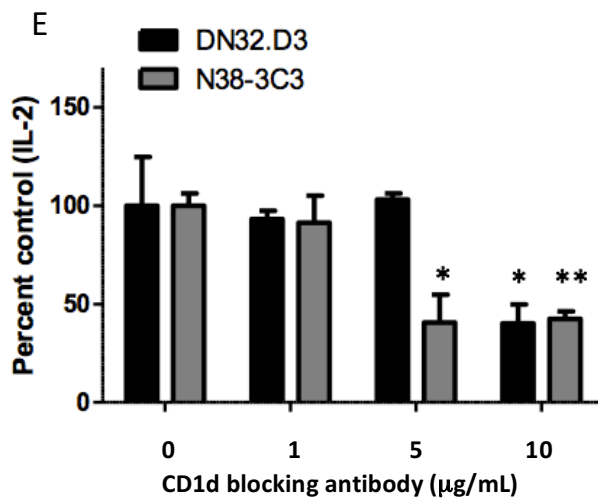
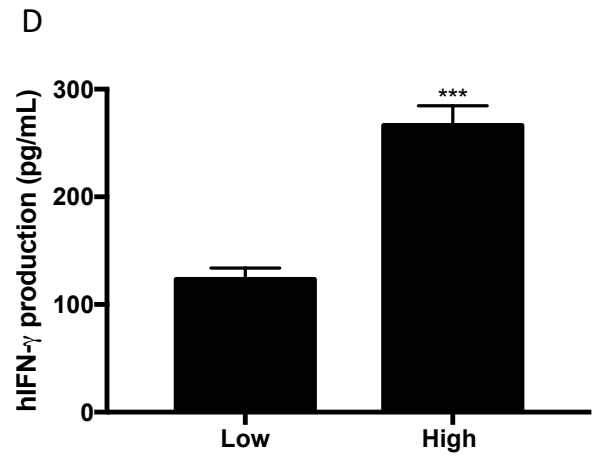
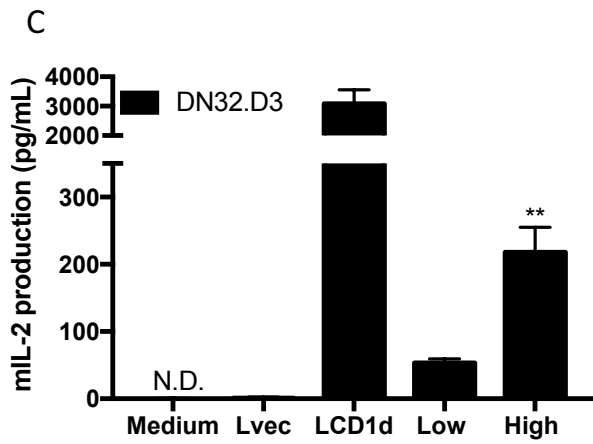
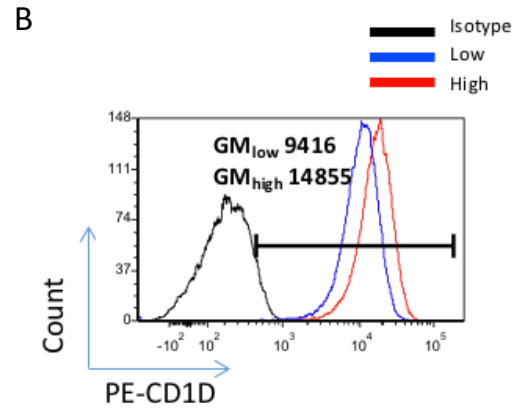
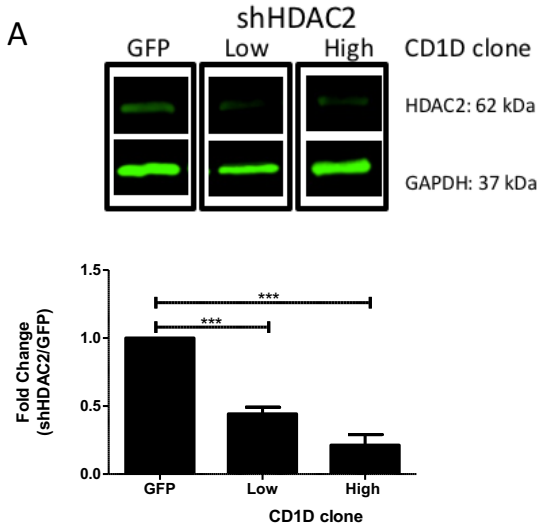


Figure 3.11: Slight changes in CD1D levels significantly affect NKT cell activation. A) HDAC2 knockdown was confirmed in JeKo-1 displaying low and high CD1D levels. Lower panel: Fold change in HDAC2 levels, relative to GFP control, in CD1D clones displaying relatively low and high CD1D levels. B) shHDAC2 clones displaying low (blue histogram) and high (red histogram) levels of cell surface CD1D. B) Antigen presentation capabilities of low and high CD1D level expressing shHDAC2 clones were assessed in a co-culture with NKT cell hybridomas. C) A similar experiment was performed with primary human NKT cells and IFN- γ production was assessed by ELISA. D) Purified CD1d blocking antibody 1B1 was used to block CD1d molecules on the surface of LCD1d cells for 30 minutes at 4°C. Following incubation with the CD1d blocking antibody, LCD1d cells were washed and co-cultured with DN32, D3 and N38-3C3. E) CD1d staining using PE-conjugated anti-CD1d antibody following CD1d blockade on the surface of LCD1d cells. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

3.4 Discussion

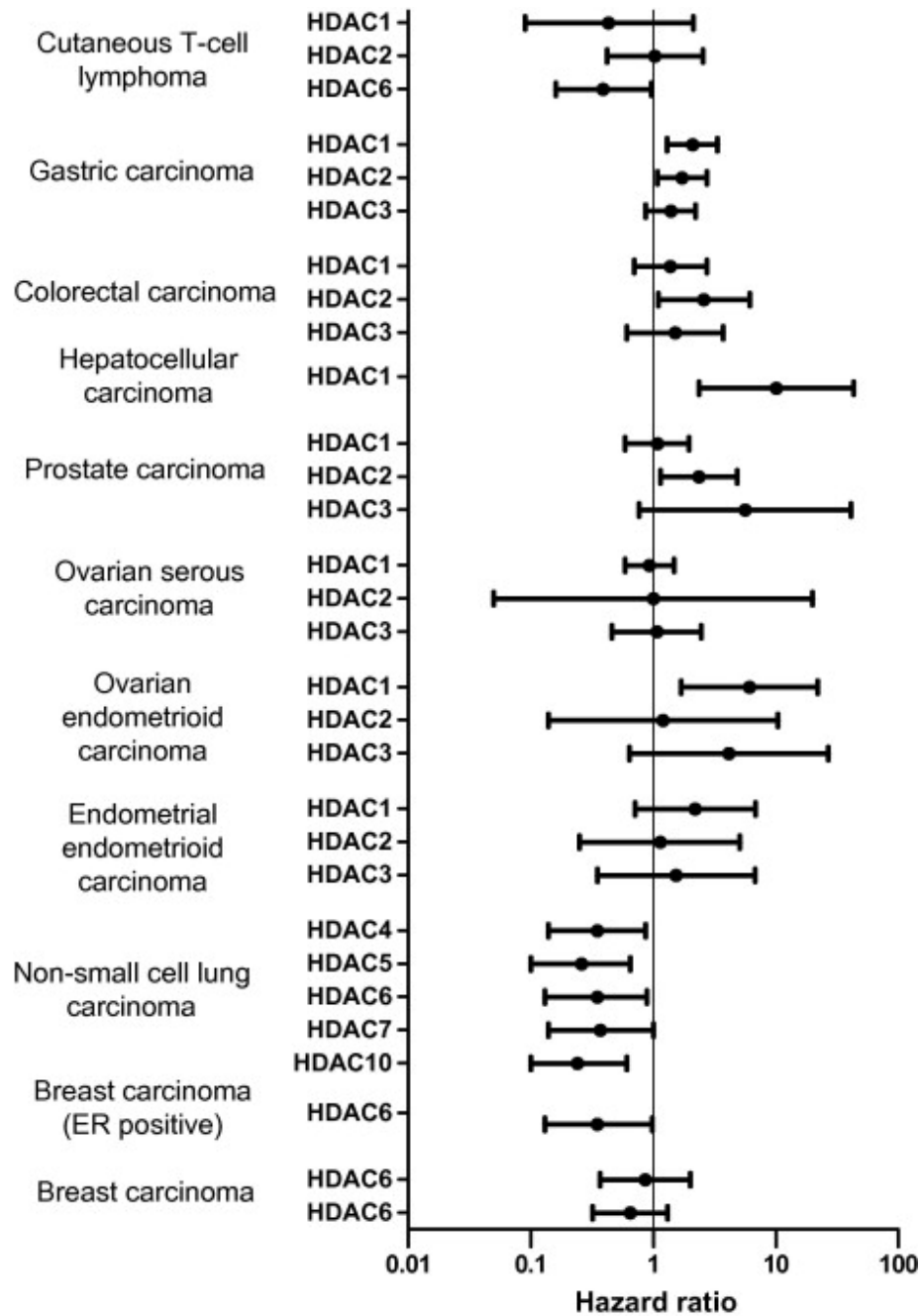
While other groups have ascribed a role for HDACs in regulating CD1d transcription [74,75], our study is the first to demonstrate that HDACi have a functional effect and enhance CD1d-mediated antigen presentation. In our studies, we tested the hypothesis that tumors use epigenetic mechanisms to dysregulate CD1d-mediated antigen presentation. We examined CD1d-mediated antigen presentation to NKT cells following treatment with HDACi. Consistent with previous studies [85,86,175], we found that treatment with TSA, a pan-HDACi, enhanced both CD1d and MHC class II-mediated antigen presentation (Figures 3.3-3.5). Furthermore, we assessed CD1d and MHC class II levels following TSA treatment and found that CD1d levels were modestly increased, but MHC class II levels were unaffected (Figure 3.7), suggesting that different mechanisms are responsible for the functional effects. We found that HDACi enhance antigen presentation due, at least in part, to a rapid increase in CD1D mRNA and, subsequently, CD1D cell surface expression (Figure 3.7).

Tumors may dysregulate epigenetic factors to facilitate growth as well as tumor immune evasion. Since HDACi are currently approved for treatment of cutaneous T cell lymphoma (CTCL), expression patterns of HDACs have been investigated to determine if certain HDACs have a prognostic value in this disease. For example, HDAC2 is upregulated in aggressive CTCL, compared to indolent CTCL [176]. HDAC1 and HDAC2 are ubiquitously expressed in CTCL tumors. Strikingly, HDAC6 upregulation was correlated with improved patient survival [176]. Most studies suggest that levels of HDACs vary between tumors of the same type. In general, class I HDACs are found to be upregulated in tumor tissue compared to the normal tissue from which the tumor arose [177]. Furthermore, in most tumors, class I HDAC levels were higher in late stage

and high grade tumors. On the other hand, class II HDACs are downregulated in tumors and upregulation is linked to better prognosis [177].

Expression patterns of HDACs have been used to calculate the hazard ratio (HR), or the estimated risk of an unfavorable event occurring by a given point in time. As seen in Figure 3.12, some cancers, such as prostate carcinoma and ovarian cancer, have high HR associated with given HDACs. In prostate cancer patients, high levels of HDAC2 expression correlated with significantly reduced disease-free survival and multivariate analysis of HDAC2 expression established its independent prognostic value [177]. HDAC levels are different in different tumor subtypes, with class I HDAC expression having prognostic value in endometrioid subtype of ovarian and endometrial carcinoma, but no statistically significant impact in serous, mucinous and clear cell carcinomas [177,178].

There is a lack of data on the prognostic value of HDACs in NHL. There may be utility in establishing links between HDACs and immune responses. For example, studies have shown that HDACi upregulate genes involved in antigen presentation or co-stimulation, suggesting that HDACs negatively regulate antigen presentation [61]. In this chapter, we provided evidence that HDAC2 is a negative regulator of CD1d-mediated antigen presentation. We posit that a future study of HDAC expression and immune responses may uncover a correlation between HDAC2 levels and NKT cell responses.



Reprinted with permission from Elsevier (license # 3858910869114). Weichert, *Cancer Letters* 280(2) 168-176.

Figure 3.12: HDAC expression and patient prognosis. Hazard ratios and 95% confidence intervals for the expression of different HDAC isoforms in a variety of human tumors.

Chapter 4: Cell-extrinsic effects of HDACi

4.1 Introduction

HDACi have been shown to have anti-inflammatory properties [179]. In fact, a clinical trial of HDACi in graft-versus-host disease following allogeneic hematopoietic stem cell transplantation demonstrated that pro-inflammatory cytokine levels were reduced in the plasma [180]. While plasma levels of IL-1 β , TNF- α , IL-6 were reduced, the levels of IFN- γ were unaffected [180]. The effects were mediated through increased acetylation of STAT3 [180]. Moreover, treatment of T cells isolated from systemic lupus erythematosus (SLE) patients with TSA decreased IL-10, while inducing IFN- γ . Peripheral blood mononuclear cells (PBMCs) from SLE patients were found to produce IL-10 at baseline and TSA treatment completely abolished IL-10 production [181]. Thus, we hypothesized that TSA inhibits IL-10 secretion by MCL. Here, we show that human MCL secrete IL-10 at baseline and that TSA inhibits IL-10 secretion.

STAT3 is known to promote an inflammatory microenvironment, by serving as a transcription factor regulating genes encoding cytokines, chemokines, and growth factors. Moreover, STAT3 is a part of a feed-forward loop, wherein cytokines induced by STAT3 further induce STAT3. For example, IL-10 and VEGF are upregulated by STAT3 and are activators of STAT3 [182]. STAT3 and NF- κ B signaling are highly interconnected, with both transcription factors co-regulating numerous oncogenic and inflammatory genes [40]. Additionally, many NF- κ B-inducible genes are STAT3 activators: NF- κ B activates IL-6, which, in turn induces, STAT3 [40]. In fact, tumors frequently upregulate both transcription factors, and, owing to the fact that they induce the expression of a highly overlapping repertoire of factors, this establishes feed-forward loops, thus creating an immunosuppressive environment.

STAT3 regulates genes whose products mediate inflammation (ex. IL-10) and angiogenesis (VEGF), in addition to other genes [182]. IL-10 has been shown to inhibit IFN- γ production by T cells and T cell proliferation [183]. Exogenous addition of IL-2 did not rescue IL-10-mediated inhibition of IFN- γ production [183]. As reviewed in Chapter 1.6, exogenous addition of VEGF suppresses proliferation and cytotoxicity of T cells [109].

Ablation of the Stat3 gene in tumor cells or immune cells inhibits carcinogenesis [40]. Ultimately, Stat3 ablation leads to autoimmunity and complete blockade of STAT3 leads to severe disease [40]. However, partial STAT3 inhibition for a limited time can potentially be used to facilitate attenuation of tumor-promoting inflammation [40]. Development of STAT3 inhibitors has presented a challenge because STAT3 does not have an enzymatic function and development of small molecules to disrupt protein-protein interactions has been challenging [184]. Thus, focus has been on discovery of inhibitors that block STA3 dimerization or DNA-binding activity. Most STAT3 inhibitors have not shown the desired *in vivo* efficacy and, while the need for a suitable and effective STAT3 inhibitors in the clinic remains high, suitable inhibitors of STAT3 have not entered the clinic [184]. Nonetheless there are several FDA-approved indirect STAT3 inhibitors, including sorafenib and sunitinib [40]. The precise mechanisms of STAT3 inhibition by these drugs remain to be determined.

In this chapter, we present data for HDACi-mediated inhibition of STAT3. Moreover, we examined the effects of a STAT3-inducible cytokine (IL-10) on CD1d-mediated antigen presentation and discovered that HDACi treatment inhibits inflammatory cytokine production via modulation of STAT3 and that IL-10 inhibits CD1d-mediated antigen presentation. Thus, HDACi may fill the need for STAT3 inhibitors.

4.2 Results

In Chapter 3, we found that STAT3 does not bind the CD1D promoter. However, we found STAT3 and HDAC2 to be in complex (Figure 4.1 A). Given that STAT3 levels can be modulated by HDACi treatment [35,185], we examined the effects of TSA on STAT3 expression. Following TSA treatment, both phosphorylated and total STAT3 were reduced (Figure 4.1 B, C). By flow cytometry, total STAT3 was greatly reduced, whereas Western blot showed a decrease in p-STAT3, which may be explained by differences in the antibody clones. Next, we sought to determine whether HDACi treatment inhibits secretion of STAT3-regulated inflammatory cytokines. We found that TSA treatment inhibited secretion of MCP-1, IL-8 and IL-10 (Figure 4.2). We confirmed TSA-mediated inhibition of IL-10 by ELISA (Figure 4.3 A), with PMA/Ionomycin serving as a positive control. We found that both JeKo-1 and SP53 secrete IL-10 even in absence of stimulation and that TSA treatment inhibits IL-10 secretion (Figure 4.3 A).

To elucidate the effects of IL-10 on antigen presentation, we pre-treated LCD1d cells with purified IL-10 and found that IL-10 pre-treatment suppresses antigen presentation to NKT cell hybridomas DN32.D3 and N38-3C3 (Figure 4.3 B). We confirmed that IL-10 activates STAT3 by performing a Western blot for STAT3 and phospho-STAT3 (Figure 4.3 C). Thus, we confirmed that exogenous addition of IL-10 induces STAT3, which then negatively regulates CD1d-mediated antigen presentation. Future studies will determine the precise mechanisms by which STAT3 regulates CD1d-mediated antigen presentation. The potential mechanisms by which STAT3 can modulate antigen presentation are discussed in Chapter 6.

We showed that STAT3 has a role in modulating CD1d-mediated antigen presentation. Overall, these studies demonstrate that HDAC2 regulates STAT3, which in turn modulates inflammatory cytokine secretion, which can suppress CD1d-mediated antigen presentation.

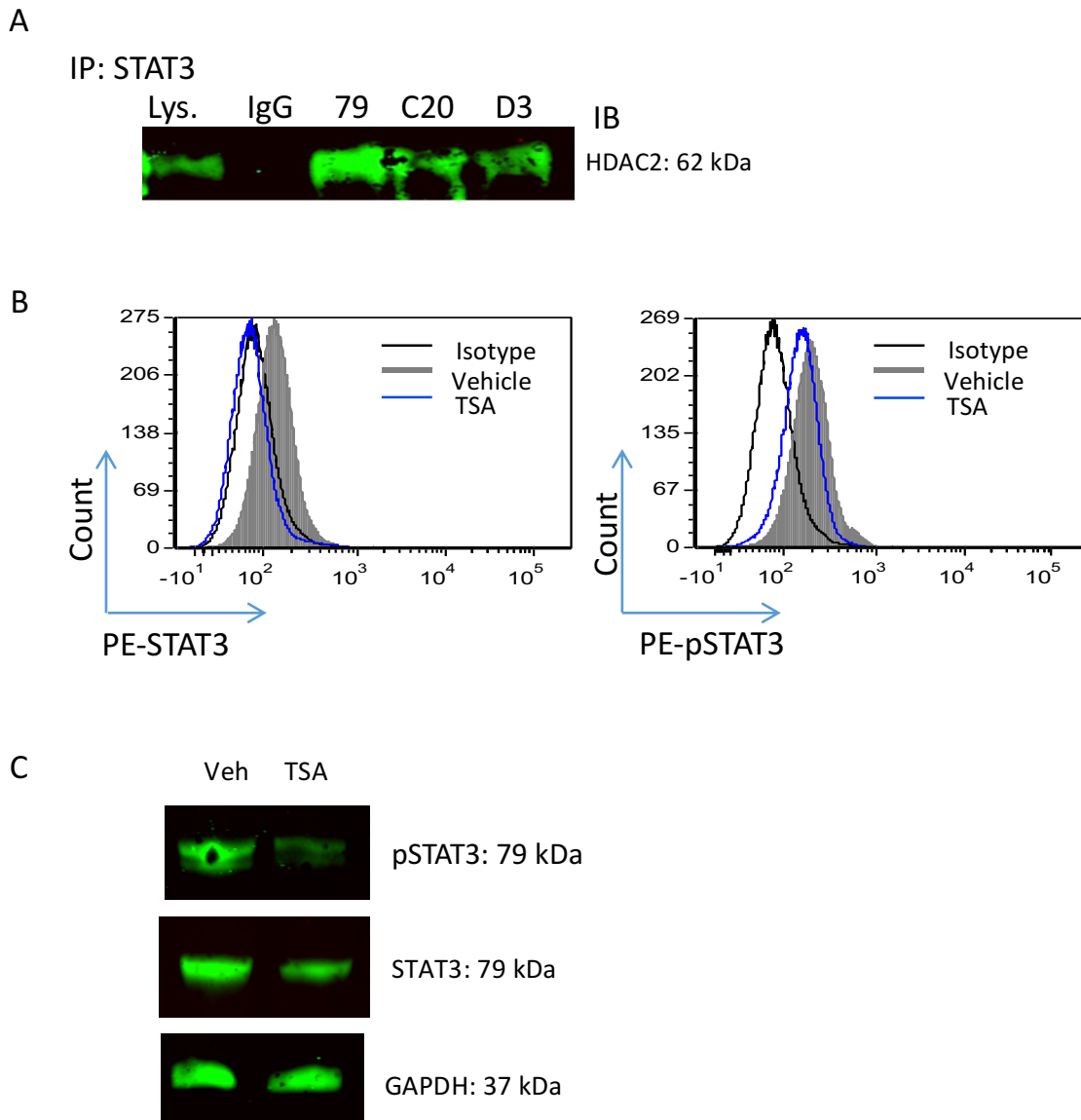


Figure 4.1: HDAC2 and STAT3 exist in a complex and TSA treatment inhibits STAT3. A) STAT3 was immunoprecipitated from JeKo-1 cell lysates using three different STAT3 antibodies and the immunoprecipitates were evaluated for presence of HDAC2. B) JeKo-1 cells were treated with 1 μ M TSA for 4 hours and levels of STAT3 and phosphorylated STAT3 (pY705) were assessed by flow cytometry. Grey histogram: vehicle. Blue histogram: TSA. C) The findings in B were confirmed by Western blot.

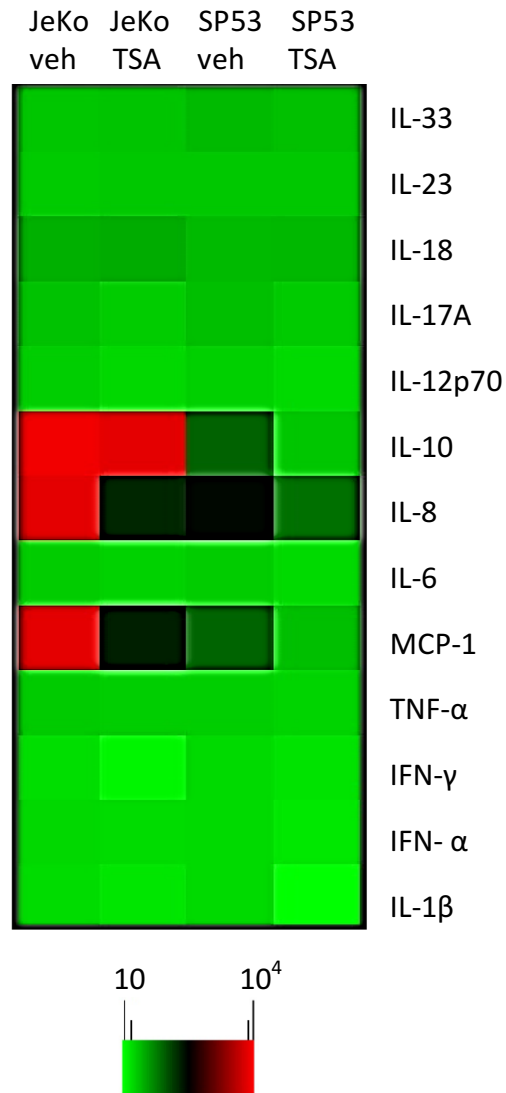


Figure 4.2: TSA inhibits inflammatory cytokine secretion. JeKo-1 and Sp53 were treated with 1 μ M TSA for 24 hours and the cytokine concentrations in supernatants were assessed by LEGENDplex Human Inflammation Panel assay.

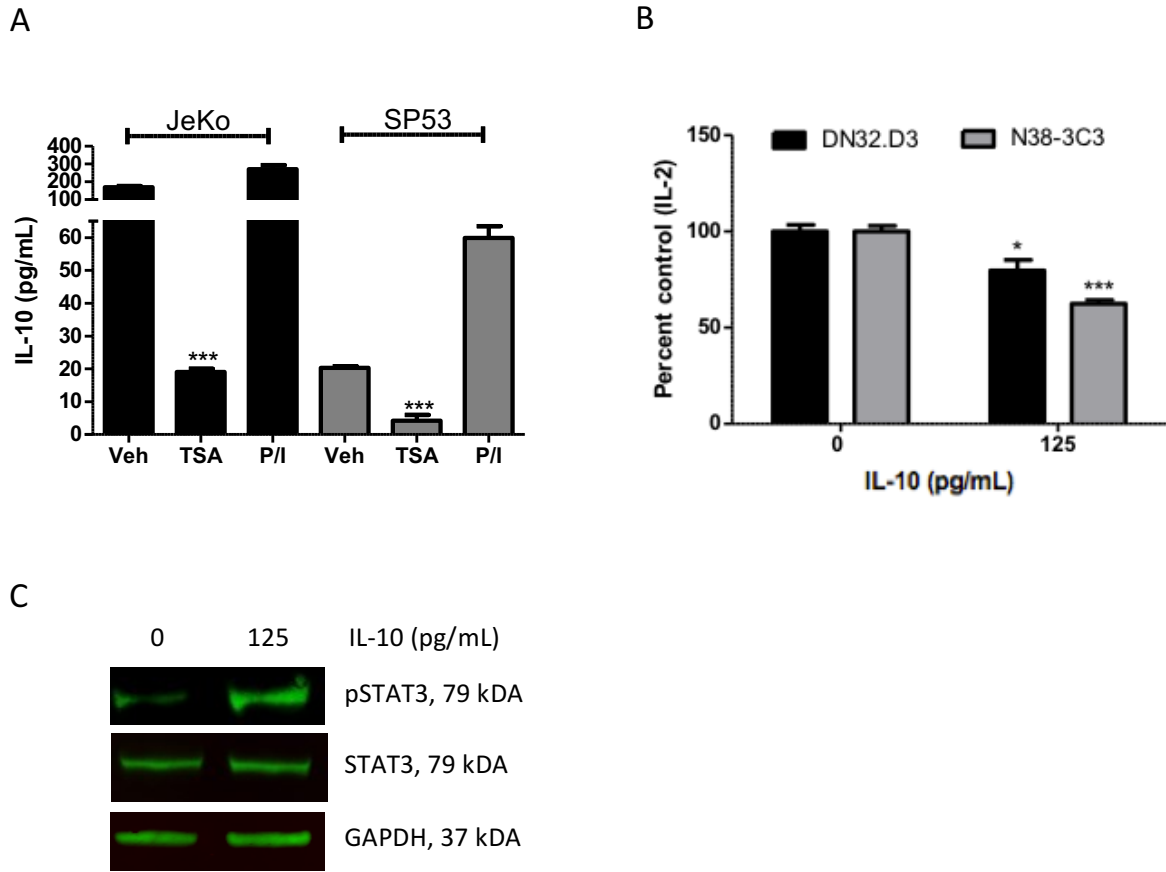


Figure 4.3: IL-10 inhibits CD1d-mediated antigen presentation. A) JeKo-1 and Sp53 cells were treated with 1 μ M TSA for 24 hours and IL-10 levels in supernatants were measured by standard ELISA. P/I is used as a positive control. B) LCD1d were treated with IL-10 to induce STAT3 and co-cultured with DN32.D3 and N38-3C3. T-tests were performed to test statistical significance: * $p < 0.05$ and *** $p < 0.0001$. C) LCD1d cells were treated with IL-10 and induction of STAT3 was confirmed by Western blot.

4.3 Discussion

In these studies, we demonstrate that MCL cell lines produce inflammatory cytokines in the absence of stimuli. We determined that secretion of three inflammatory cytokines, IL-6, IL-10, and MCP-1, is inhibited by TSA treatment (Figure 4.2). Moreover, we are the first to demonstrate that IL-10 inhibits CD1d-mediated antigen presentation. Previously, a study showed that IL-10 treatment inhibits MHC class II-mediated antigen presentation by Langerhans cells, or dendritic cells of the epidermis, through inhibition of co-stimulatory molecule CD80 expression [186]. Given that expression of co-stimulatory molecules is not essential for activation of NKT cell hybridomas, inhibition of antigen presentation by IL-10 is likely mediated through changes in CD1d-mediated antigen processing and presentation, rather than through changes in co-stimulation. Studies utilizing primary mouse or human NKT cells may help delineate whether IL-10-mediated inhibition of antigen presentation by CD1d depends on changes in CD1d alone, co-stimulation, or both.

There are many proposed mechanisms by which IL-10 inhibits antigen presentation. First, IL-10 signaling leads to STAT3-dependent activation of microRNAs that interfere with toll-like receptor (TLR) signaling, which is crucial for antigen endocytosis and assembly of antigen processing components [187,188]. TLR signaling plays a role in production of lipid antigens, which can be presented in the context of CD1d molecules [189,190]. Moreover, TLR signaling is necessary for CD1d-mediated antigen presentation [191]. Thus, future studies can address the effects of IL-10-mediated suppression of TLR signaling on changes in the lipid repertoire and CD1d-mediated antigen presentation.

Furthermore, IL-10 inhibits antigen presentation by inhibiting enzymes involved in antigen presentation, such as cathepsin S. For example, in macrophages, pre-treatment of macrophages

with IL-10 and IFN- γ inhibited IFN- γ -induced expression of cathepsin S, leading to inhibition of MHC class II complex formation [192]. Cathepsin S has been shown to play a role in CD1d intracellular trafficking, with cathepsin S deficiency in DCs leading to accumulation of lipid fragments and thus disrupting normal CD1d trafficking [193]. Thus, IL-10 could dysregulate CD1d-mediated antigen presentation through inhibition of CD1d trafficking, which can be assayed through confocal microscopy.

Finally, IL-10 has been shown to induce expression of an E3 ubiquitin ligase that leads to degradation of MHC class II and CD86 molecules [194]. Thus, as discussed above, the effects of IL-10 treatment on co-stimulatory molecules can be ascertained by assessing CD1d-mediated antigen presentation to primary NKT cells.

Chapter 5: Effects of other tumor-secreted factors on antigen presentation

5.1 Introduction

In Chapter 4, we presented evidence that MCL cells secrete factors that inhibit CD1d-mediated antigen presentation. Here, we examine the role of another factor, VEGF, as a suppressor of CD1d-mediated antigen presentation. VEGF is upregulated in many types of tumors and we sought to determine whether the findings in a hematological malignancy are recapitulated in a solid malignancy. To investigate the effects of VEGF on CD1d-mediated NKT cell activation, a conditioned medium model was established, wherein the supernatants from ovarian cancer cell lines (OV-CAR-3 and SK-OV-3) were used to treat CD1d-expressing antigen-presenting cells (APC) and cocultured with NKT hybridomas. Ovarian cancer-associated VEGF was inhibited by treatment with bevacizumab and genistein. Conditioned medium was collected and CD1d-mediated NKT cell responses were assayed by ELISA.

Ovarian cancer tissue and ascites contain lymphocytic infiltrates, suggesting that immune cells traffic to tumors, but are then inhibited by immunosuppressive molecules within the tumor microenvironment. We sought to establish the link between VEGF secretion and ganglioside shedding. VEGF has been shown to block activation of T cells derived from ovarian cancer patient ascites [109]. Ganglioside GD3 is a factor found in ovarian cancer ascites that inhibits NKT cell activation [195]. *In vivo*, GD3 inhibits α -GalCer-mediated NKT cell activation [195].

In human promyelocytic leukemia cells, PKC/ERK signaling activates synthesis of GM3 synthase, which is an enzyme functioning upstream of GD3 [196]. PKC/ERK pathway activation induces binding of CREB to the GM3 synthase promoter [196]. VEGF induces CREB DNA binding and transactivation activities, in a PKC and p38-dependent manner [197]. We thus sought

to examine the effects of VEGF inhibition on ovarian cancer-associated GD3 levels.

OV-CAR-3 and SK-OV-3 cell lines produce high levels of VEGF and GD3. Pretreatment of APCs with ascites or conditioned medium from OV-CAR-3 and SK-OV-3 blocked CD1d-mediated NKT cell activation. Inhibition of VEGF resulted in a concomitant reduction in GD3 levels and restoration of NKT cell responses. We found that VEGF inhibition restores NKT cell function in an *in vitro* ovarian cancer model. These studies suggest that the combination of immune modulation with antiangiogenic treatment has therapeutic potential in ovarian cancer. Herein, we demonstrate a novel link between immunosuppressive ganglioside shedding and VEGF production by ovarian cancers. By establishing a mechanism through which VEGF impairs anti-tumor immune responses, our studies have the potential to enhance the clinical therapeutic possibilities for women with this disease.

Chapter 5.2: Results

We previously reported that ovarian cancer tumor cells in ascites fluid shed soluble factors that inhibit CD1d-mediated activation of NKT cells [195,198]. In this study, we examined whether treatment with conditioned medium from ovarian cancer cell lines would inhibit the ability of CD1d-expressing cells to stimulate NKT hybridomas. Mouse fibroblasts expressing high levels of CD1d (LCD1dwt) were incubated with cell-free supernatants from ovarian cancer cell lines OV-CAR-3 and SK-OV-3 cultured to confluence, referred to as conditioned medium. Treatment with conditioned medium treatment inhibited NKT cell activation, as evidenced by decreased IL-2 (Figure 5.1A) and IL-4 (Figure 5.1 B) production. These data demonstrate that established ovarian cancer cell lines secrete soluble factors that block CD1d-mediated antigen presentation to NKT cells.

We next assessed whether there were T cells within the tumor microenvironment that could be influenced by the immunosuppressive factors produced by ovarian cancers. We showed that large numbers of lymphocytes infiltrate the tumor microenvironment [111]. Of note, there was a higher percentage of CD4⁻ CD8⁻ double negative T lymphocytes present in NKT cell inhibitory ascites, compared with noninhibitory ascites fluid [111]. This suggests that NKT cells within the tumor microenvironment may be actively suppressed by factors produced by ovarian cancers. Pre-treatment of ovarian cancer-associated ascites with proteinase K resulted in a loss of suppressive activity [111]. These data suggest that in addition to the previously identified factor, ganglioside G3, one or more other soluble factor(s), likely a protein, produced by ovarian cancers modulates CD1d-mediated presentation to NKT cells.

Given the critical role of growth factors (specifically VEGF) in the biology of epithelial

ovarian cancer, we measured VEGF levels in ascites fluid and conditioned medium. In Figure 5.2 A, donors OC1, OC4, OC5, and OC9 had primary disease. Patient OC6 had recurrent disease and another donor was diagnosed as having low malignant potential (NMA). High levels of VEGF were present in the ascites of ovarian cancer patients (Figure 5.2 A) and conditioned medium from ovarian cancer cell lines (Figure 5.2 B). HEC-1-A, an endometrial cancer cell line was included as it has been reported to secrete VEGF and we thus used it as a positive control [199,200]. Moreover, treatment of CD1d-expressing cells with comparable levels of recombinant VEGF resulted in a dose-dependent decrease in NKT cell activation (Figure 5.2 C).

To confirm a role for VEGF in suppressing NKT cell function, we treated ovarian cancer cells with a neutralizing anti-VEGF antibody, bevacizumab/Avastin (Figure 5.3 A). Next, we treated LCD1d cells with conditioned medium from Avastin-pretreated cells. Immunoblockade of VEGF dose dependently restored NKT cell function, indicating that VEGF is directly responsible for NKT cell suppression (Figure 5.3 B). To further establish a role for ovarian cancer-associated VEGF in suppressing NKT cell function, we used a flavonoid known to inhibit VEGF in ovarian cancer cells lines, genistein (Figure 5.4 A) [201]. Similar to Avastin, treatment of ovarian cancer cell lines with genistein restored CD1d-mediated antigen presentation to NKT cells (Figure 5.4 B).

Previous studies showed that the ganglioside GD3 in ovarian cancer ascites was responsible, at least in part, in inhibiting CD1d-mediated NKT cell activation [195]. Accordingly, we examined ovarian cancer cell lines OV-CAR-3 and SK-OV-3 for GD3 expression. As shown in the top panels of Figure 5.5 A, flow cytometric analysis indicated that GD3 is present in these cells. We then asked whether VEGF and ganglioside synthesis pathways might be linked, working in tandem to suppress immune responses. To establish cross-talk between VEGF and GD3, we asked whether VEGF inhibition alters GD3 expression in ovarian cancer cell lines. We found that

GD3 expression was reduced after 72 hours of genistein treatment (Figure 5.5 B).

To establish that genistein-mediated GD3 inhibition is responsible for restoring NKT cell responses, we overexpressed the plasma membrane-associated sialidase neuramidase 3 (NEU3) in ovarian cancer cells. NEU3 has been shown to decrease GD3 [202]. Following infection with adenovirus encoding human NEU3 (AdNEU3), we harvested cell culture supernatants and utilized the conditioned medium. Supernatants from NEU3-overexpressing cells inhibited NKT cell function to a lesser extent than did controls (Figure 5.6 A). In addition, we examined whether there may be a reciprocal regulation between VEGF and GD3 by comparing VEGF levels in conditioned medium from ovarian cancer cells infected with control adenovirus and AdNEU3. However, VEGF levels were similar in the presence and absence of NEU3 ectopic expression (Figure 5.6 B). Taken together, these data suggest that VEGF can modulate GD3 expression and confirm that ovarian cancer-associated GD3 is responsible for suppressing CD1d-mediated NKT cell activation.

We next asked whether ovarian cancers could serve as APCs in the absence of these inhibitory soluble factors. Ovarian cancer cell lines, OV-CAR-3 and SK-OV-3, were fixed with paraformaldehyde and cocultured with NKT cells (Figure 5.7). Importantly, we found that fixation of ovarian cancer cell lines resulted in a >2 fold increase in their ability to activate NKT cells. Taken together, these data suggest that ovarian cancer cells can serve as antigen presenting cells to NKT cells. However, these recalcitrant tumors employ various mechanisms to suppress the host's innate anti-tumor responses.

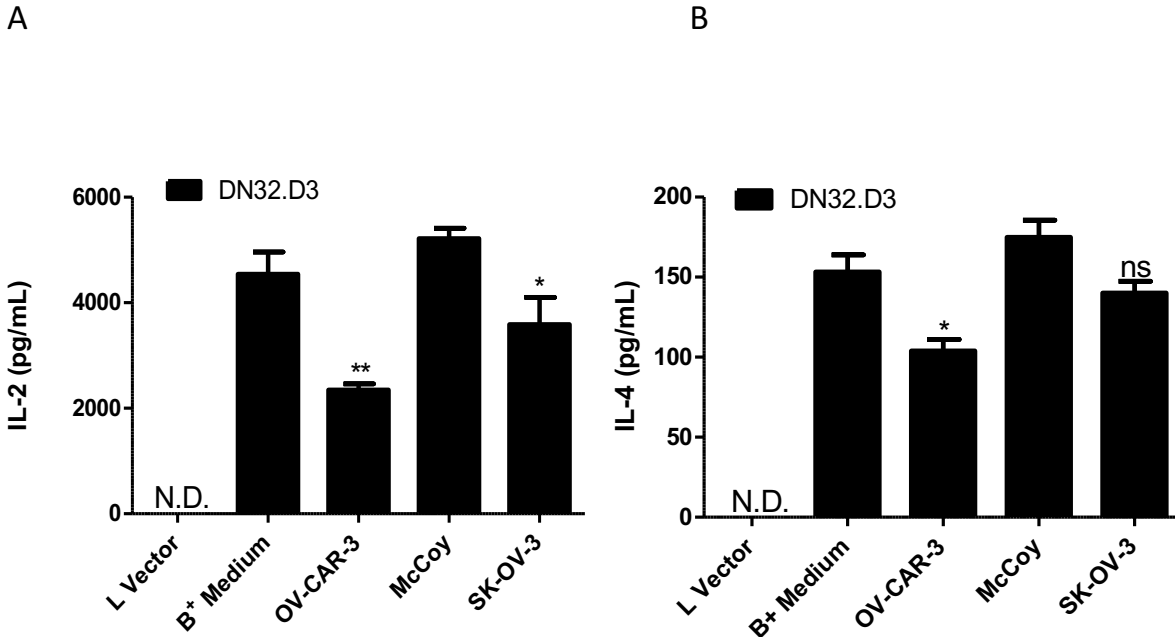


Figure 5.1: Conditioned medium from ovarian cancer cell lines inhibits CD1d-mediated antigen presentation. LCD1d cells were treated with supernatants from confluent ovarian cancer cell lines OVCAR-3 and SK-OV-3 for 4 hours at 37 °C and washed extensively following treatment. Control LCD1d cells were concurrently treated with RPMI (B⁺ medium) and McCoy media. Lvector cells serve as a negative control. N.D., not detectable. Following treatment, LCD1d cells were cocultured with NKT-cell hybridomas, DN32.D3, and incubated for 24 hours at 37 °C. Standard ELISA was performed to measure cytokine production of IL-2 (A) and IL-4 (B). Data are shown as mean ±SEM of one experiment set up in triplicate.

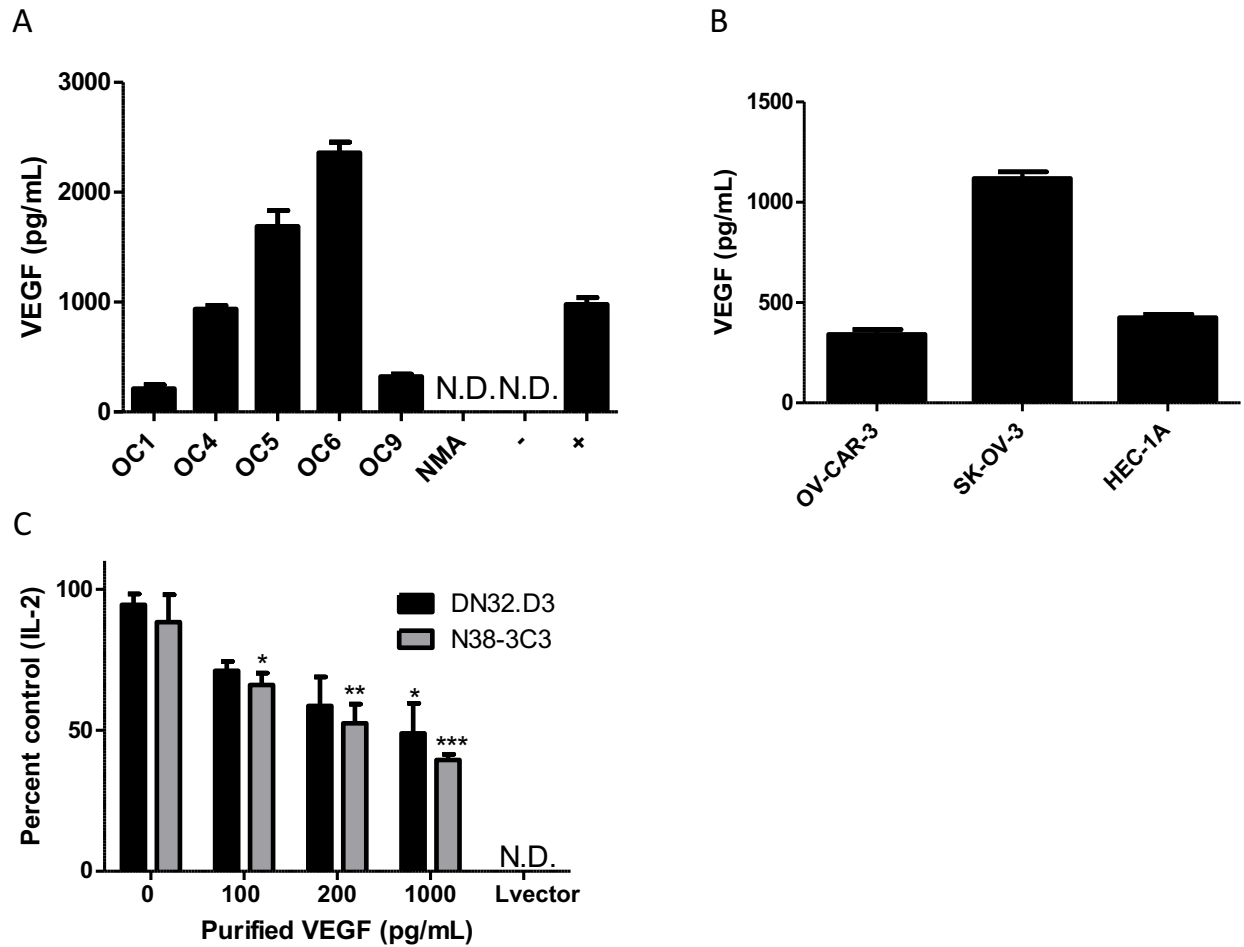


Figure 5.2: VEGF inhibits CD1d-dependent NKT cell function. A) VEGF levels in ovarian cancer patient ascites and B) OV-CAR-3, SK-OV-3, and endometrial cancer cell lines HEC-1 were measured by ELISA. Nonmalignant ascites (NMA) represents VEGF levels in non-cancer-associated ascites. The negative control is the assay diluent used in the ELISA assay, and the positive control was recombinant VEGF (1,000 pg/mL). ND, not detectable. C) LCD1d cells were treated with the indicated concentrations of recombinant VEGF for 4 hours, then washed extensively, and cocultured with NKT cell hybridomas, DN32.D3, and N38-3C3. T-tests were performed to demonstrate statistical significance: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

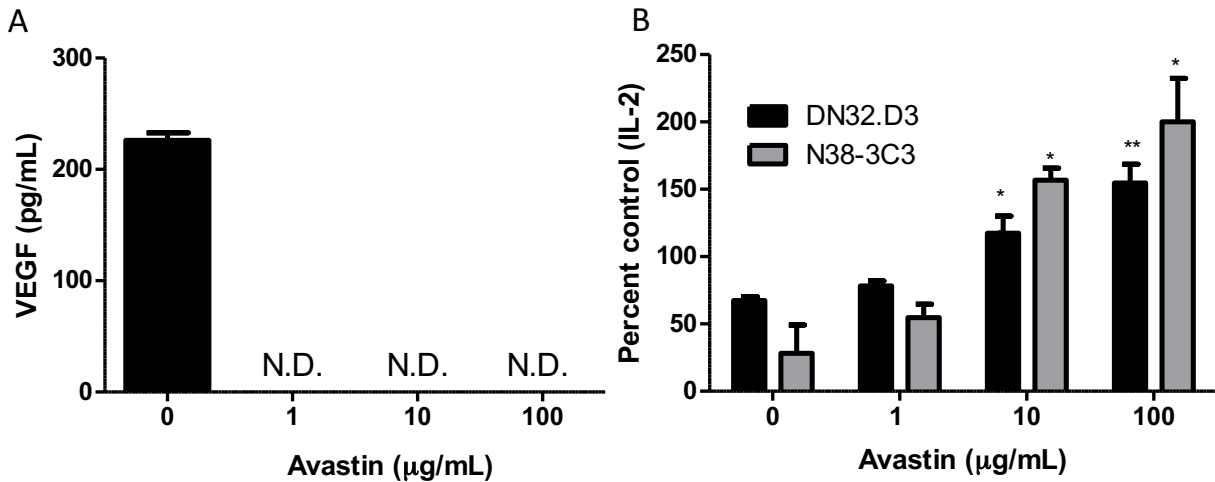


Figure 5.3: Avastin treatment restores CD1d-mediated antigen presentation. A) OV-CAR-3 cells were treated with increasing concentrations of bevacizumab/Avastin and VEGF levels in the cell culture supernatant were assessed after 24 hours by ELISA. B) OV-CAR-3 cells were treated with increasing concentrations of bevacizumab/Avastin for 3 days, following a 1-day recovery period in fresh culture medium. Pretreatment of CD1d-expressing cells with conditioned medium was performed as described above. ANOVA with Bonferroni post-test confirmed significance. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$ for treatment groups compared with control. N.D., not detectable.

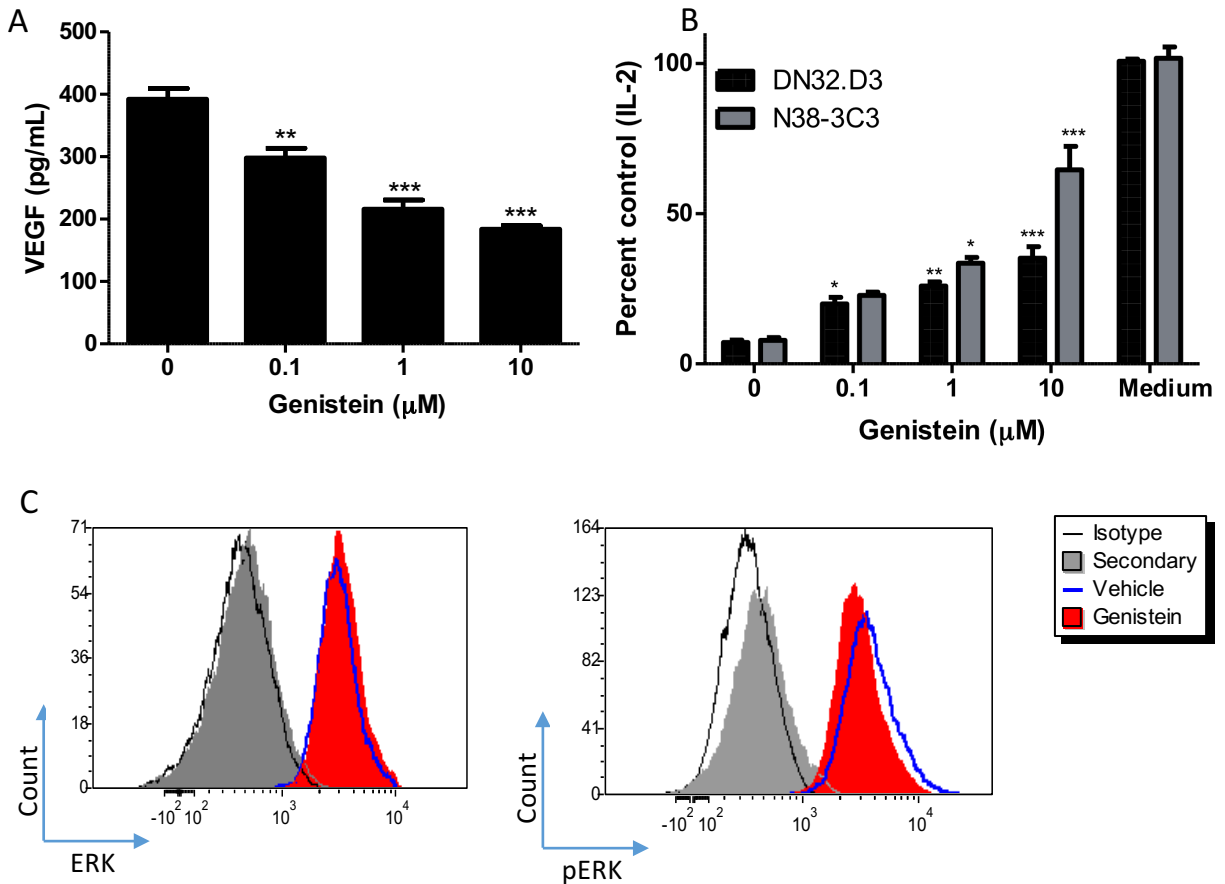


Figure 5.4: Inhibiting VEGF blocks GD3 in ovarian cancer cells. A) OV-CAR-3 cells were treated with genistein and VEGF levels in cell culture supernatants were measured 24 hours post-treatment. B) OV-CAR-3 cells were treated with increasing concentrations of genistein or vehicle (DMSO) for 3 days, followed by a 1-day recovery period. Conditioned media from the vehicle- and genistein-treated cells were used to treat LCD1d cells for 4 hours. Following treatment, LCD1d cells were cocultured with NKT cell hybridomas DN32.D3 and N38-3C3. IL2 production was measured by standard ELISA. ANOVA compared treatment groups with the vehicle-treated group and Bonferroni post-test confirmed significance: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$ for treatment groups compared with control. C) As a control for genistein, ERK and phosphorylated ERK (pERK) were assessed by flow cytometry in OV-CAR-3 cells treated with 10 mmol/L genistein for 24 hours.

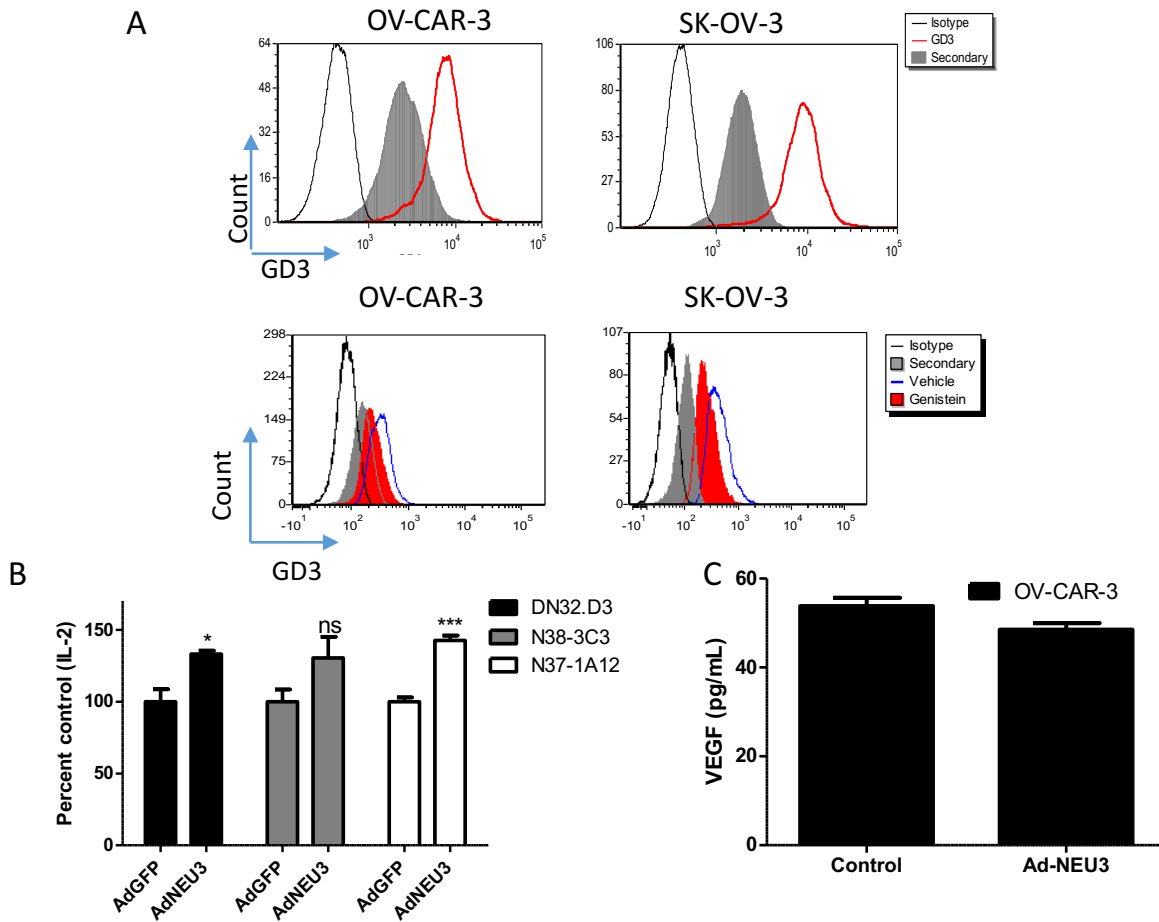


Figure 5.5 Ovarian cancer–associated GD3 inhibits NKT cell responses. A) GD3 expression in ovarian cancer cell lines was assessed using flow cytometry. OV-CAR-3 and SK-OV-3 cells were treated with vehicle or 10 mmol/L genistein for 72 hours. Cells were fixed, permeabilized, and stained either with primary anti-GD3 antibody alone or with primary and PE-conjugated secondary antibodies. B) Adenovirus encoding for human NEU3 (AdNEU3) or GFP (AdGFP) was used to infect OV-CAR-3 cells. Following infection and a 1-day recovery period, supernatants from confluent cells were used in conditioned medium experiments. ANOVA compared treatment groups with the vehicle-treated group, with Bonferroni post-test confirming significance. * $p < 0.05$ and *** $p < 0.001$ for AdNEU3 compared with AdGFP. C) Following infection and a one-day recovery period, supernatants from confluent cells were assessed for VEGF levels.

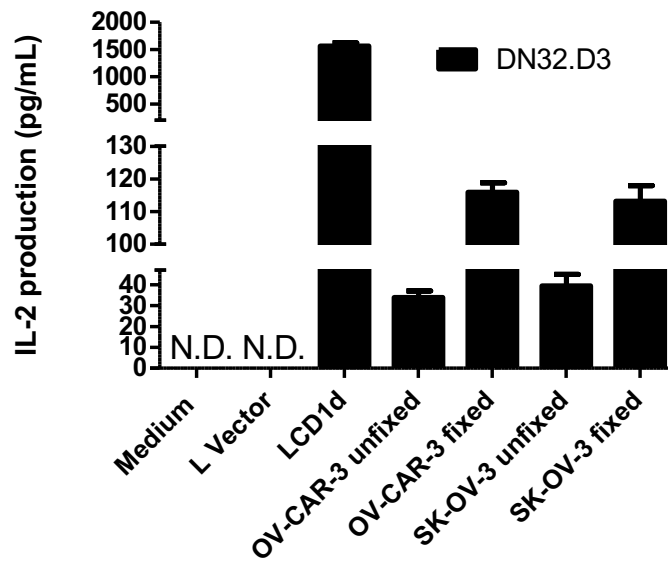


Figure 5.6: Ovarian cancers can present antigen to NKT cells and induce their activation.

Fixation of OV-CAR-3 and SK-OV-3 cells restored their antigen presentation capabilities. The ovarian cancer cells were pulsed with α -GalCer, fixed in 0.05% paraformaldehyde, and cocultured with NKT cells. Supernatants were harvested after 16 h.

5.3 Discussion

Here, we report that treatment of CD1d-expressing cells with conditioned medium from human ovarian cancer cell lines abrogated their ability to activate both canonical and noncanonical NKT cells. Mechanistically, we found that inhibiting VEGF resulted in a decrease in ganglioside GD3 expression and restoration of NKT cell responses. In addition, we addressed the link between VEGF and lipid signaling and demonstrated that tumors may utilize multiple signaling pathways to achieve escape from immune surveillance. We have identified a novel mechanism by which angiogenic signaling pathways contribute to immunosuppression through alteration of the lipid repertoire, with VEGF serving as one of the modulators of the lipid rheostat (Figure 5.7).

Ascites, a clinical hallmark of ovarian cancer, reportedly predicts treatment benefit for bevacizumab in epithelial ovarian cancer [110]. More than one third of ovarian cancer patients present with ascites. Several types of proinflammatory and tumor-promoting factors have been identified in ovarian cancer ascites fluid. For example, IL-6 and IL-10 are detectable in ascites [203,204]. Of the multitude of cytokines present in ascites [205], several have been shown to inhibit T-cell function. Alteration of T cell function by ovarian cancer cells and ascites is known to contribute to poor prognosis [195,203]. On the basis of the well-described relationship between advanced ovarian cancer, ascites, VEGF, and T cell function, we postulated that VEGF plays a central role in mediating this immune response. Our data support the concept that inhibition of VEGF not only affects angiogenesis but also has an unexpected effect on the shedding of GD3, thus altering immune function.

In other disease states, interactions have been reported between VEGF and immune function. A positive correlation between peripheral blood Treg circulating numbers and baseline

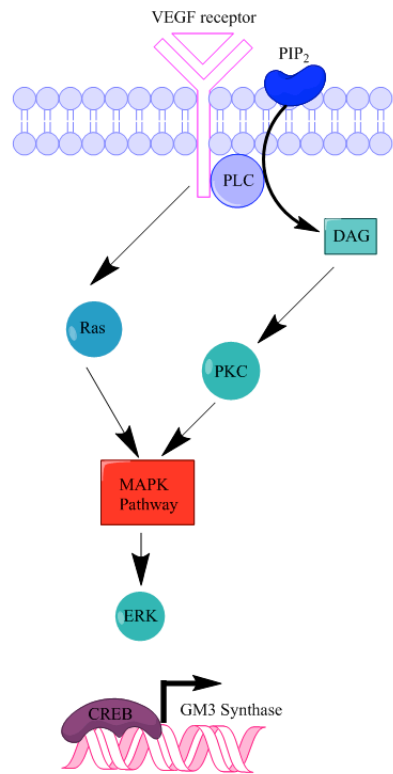


Figure 5.7: Proposed model of cross-talk between the VEGF, MAPK, and GD3 signaling pathways. We postulate that activation of VEGF receptor signaling leads to the activation of MAPK signaling. MAPK signaling can induce GM3 synthase, leading to the production of GD3.

VEGF has been demonstrated in stage IV melanoma patients [206]. In good agreement, a recent study by Farsaci and colleagues showed that using antiangiogenic TKIs in combination with a therapeutic vaccine increased CD3⁺ tumor-infiltrating lymphocytes (TIL) and tumor antigen-specific CD8⁺ T cells [207]. Furthermore, Gavalas and colleagues demonstrated that ascites-derived VEGF directly suppressed T cell activation and reduced T cell proliferation in a dose-dependent manner [109]. Conversely, blockade of VEGF receptor on the surface of T cells restored T cell proliferation. Moreover, T cell-mediated cytotoxicity was suppressed by the addition of VEGF. Taken together, these studies implicate a role for VEGF in directly modulating T-cell responses in ovarian cancer.

To determine whether VEGF might have comparable effects on NKT cells, we ascertained that primary human NKT cells express the VEGF receptor (unpublished data). This finding suggests that VEGF may suppress NKT cell function both directly (via binding to VEGF receptor on NKT cell surface) and indirectly (by altering tumor ganglioside shedding and thus altering CD1d-mediated antigen presentation). Future studies will determine whether VEGF functions through inhibition of NKT cells directly or through alteration of antigen presentation. These studies suggest that targeting of tumor VEGF production is a rational approach to restore anti-tumor immune responses.

It is well known that tumors alter multiple different pathways that can promote tumorigenesis. VEGF receptor engagement activates ERK and may thus be responsible for activation of GM3 synthase-mediated synthesis of the direct precursor to GD3. Chung and colleagues demonstrated that ERK is responsible for activation of GM3 synthase [196]. VEGF inhibition may suppress GM3 activity and thus deplete ganglioside precursor pool, leading to a

decrease in GD3 levels.

Notably, ovarian cancer–associated GD3 may undergo modifications, such as acetylation, that cause shedding of species that are more immunosuppressive. Previously, our lab tested GD3 preparations from different laboratories and companies and have obtained strikingly distinct results with comparable amounts of lipid. This suggests that different sources may possess different modifications of GD3 that cause a difference in its immunosuppressive activity.

In summary, we have found that VEGF inhibition suppresses GD3 and we hypothesize that VEGF receptor–mediated activation of ERK induces ganglioside shedding by ovarian cancer cells. However, further work elucidating the link between VEGF and GD3 axis is needed. Finally, fixation of ovarian cancer cells, which abrogated their VEGF secretion, restored their ability to present antigen to NKT cells. These data demonstrate that VEGF suppresses NKT cell function and that modulation of VEGF secretion via genistein and direct blockade of VEGF with bevacizumab restores NKT cell responses.

Chapter 6: Further discussion and future directions

6.1 Cell-intrinsic effects of HDACi on CD1d-mediated antigen presentation

CD1d expression and NKT cell-mediated anti-tumor responses

In our studies on the role of HDAC2 in CD1d-mediated antigen presentation, we noted slight shifts in CD1D expression (Figure 3.10). We sought to determine whether NKT cells were exquisitely sensitive to minor changes in CD1D cell surface expression. Thus, we were able to clone JeKo-1 cells expressing relatively high and low levels of CD1D and demonstrate that NKT cells were sensitive to minute changes in CD1D levels (Figure 3.10). Given that there are approximately 50,000 CD1d molecules on the surface of splenic B cells and as few as 40,000 molecules on the surface of follicular B cells [211], it is possible that NKT cells are able to recognize minute changes in CD1d levels. Importantly, increased CD1D levels enhance the avidity of APC-NKT cell interaction [212], which suggests that more complex mechanisms than a stoichiometric ratio of CD1D molecules to the number of interactions are at play. Moreover, our current method of detection (flow cytometry) may not be sensitive enough to resolve the differences in CD1d levels. A fluorescent bead method used by Sullivan et al. may be able to resolve these changes [211].

As discussed in the introduction, high levels of CD1D on the surface of CLL cells negatively correlate with NKT cell numbers [81], suggesting that high CD1D levels may induce anergy. Thus, the ability to induce slight changes in CD1D levels may be beneficial in inducing NKT cells without inducing anergy. Future studies using a mouse model and adoptive transfer of NKT cells will determine whether this supposition is correct. In this model, JeKo-1 cells can be engrafted into NSG mice and human NKT cells can be adoptively transferred. Following HDACi

treatment, NKT cells can be assessed for common exhaustion markers, such as PD-1 and LAG3 [213,214].

We established a humanized mouse model, wherein we injected JeKo-1 tumors into NSG mice and adoptively transferred human NKT cells (Figure 3.1). We discovered that adoptive transfer of human NKT cells decreased tumor burden. These results show that transfer of NKT cells can have a significant impact on disease progression. In the future, injection of JeKo-1 containing HDAC2 knockdown will determine whether HDAC2 knockdown enhances NKT cell-mediated immune responses *in vivo*. Moreover, this model will allow us to test whether HDACi treatment facilitates NKT cell-mediated tumor regression. We hypothesize that HDAC2 knockdown in JeKo-1 cells will help NKT cells to clear the tumors. Adoptive transfer of specific NKT cell subtypes will identify the NKT cell type responsible for mediating anti-tumor responses.

RIME and identification of CD1d gene transcription factors

The studies identifying HDAC2 as a regulator of CD1D expression created the foundation for future studies aimed at identifying the mechanisms of CD1D gene regulation. Rapid immunoprecipitation mass spectroscopy of endogenous proteins (RIME) is a novel technique that allows identification of endogenously-bound proteins at a specific promoter [215]. Originally, this technique was used to identify proteins bound to the estrogen receptor (ER). One such protein, GREB1, was found to associate with ER and the GREB1-ER interaction was demonstrated to be predictive of clinical outcome [215]. Our studies identified HDAC2 as a negative regulator of CD1D gene expression. Thus, it is possible that HDAC2 may serve as a correlate for poor NKT cell responses. Further studies of patient biopsies stained for HDAC2 expression and flow cytometric analysis of NKT cell numbers will help establish clinical correlates between HDAC2

expression and NKT cell numbers. Furthermore, the addition of our lab's aAPC-qPCR method [216], which allows to quickly screen patient blood for NKT cell function, will help determine whether HDAC2 overexpression correlates with NKT cell loss of function. Once these studies are completed, RIME can be added to identify whether any particular HDAC2 associations are predictive of anti-tumor immune responses.

Identification of distal regulatory elements regulating CD1d gene

The view that a gene is solely regulated by its promoter is far too simplistic. Current evidence suggests that gene expression is regulated by complex networks of intergenic and intragenic cis-regulatory elements, involving regulatory elements located as far as several hundred of kilobases away from the gene in question. Chromosomes can create higher-level three dimensional networks, bringing together distal regulatory elements, with enhancers not necessarily regulating promoters closest to them [217,218]. The Next Generation Capture-C method allows to capture the chromosomal conformations and to identify cis-regulatory factors that control gene expression [217]. Thus, the conformation of the chromosome itself affects gene expression profoundly. Future studies involving the Capture-C method will allow us to understand the role of distal enhancers in regulating CD1d gene expression.

In Chapter 3, we showed that STAT3 does not bind at the distal or the proximal CD1D promoter. However, it is possible that STAT3 regulates CD1D gene expression through binding at distant enhancer regions. Genome-wide analysis of STAT3 binding sites of the mouse genome revealed over 1.3 million of putative binding sites [219]. Comparative genomics studies will determine which of these binding sites are functional. Thus, future studies will determine whether STAT3 regulates CD1D gene expression through binding at distal enhancers or whether it binds

at the promoter of a gene whose products regulates CD1D gene expression. Moreover, it is possible that STAT3 binds the CD1D promoter indirectly. For example, one study determined that, in a panel of lymphoma cell lines, STAT3 binds the GADD45G promoter indirectly, through association with NF- κ B and that panobinostat inhibits the binding of the NF- κ B-STAT3 complex [220]. Given that STAT3 can bind promoters indirectly and that the limitation of CHIP is that it allows identification of factors binding a gene sequence directly, RIME (as discussed above) will help determine whether STAT3 controls gene expression as a part of a large complex.

It is possible that STAT3 plays a positive role in CD1d-mediated antigen presentation. Iyer et al. demonstrated that STAT3 inhibition reduced endogenous lipid antigen presentation to NKT cells [221]. Knockdown of STAT3 led to decreased levels of UDP glucose ceramide glucosyltransferase, an enzyme involved in glycosphingolipid biosynthesis [221]. Moreover, CD1D levels in HEK-293 cells containing STAT3 knockdown were not deemed significantly different [221]. However, it must be noted that the CD1D gene in these cells is not under the control of an endogenous promoter. Moreover, the studies were performed in fibroblasts, and not cancer cells. The same signaling pathway can have different outcomes in fibroblasts, compared to cancer cells, due to cross-talk with other pathways that are uniquely upregulated in cancer. Overexpression of the human CD1D gene was achieved using pcDNA3.1 vector carrying the CD1D gene under the cytomegalovirus promoter. Thus, STAT3 enhances endogenous lipid antigen presentation through modulation of glycosphingolipid biosynthesis pathways, but future studies are needed to determine whether it controls CD1D gene expression through regulation of distal enhancers.

Effects of HDACi on immune cells

The potential cell-intrinsic effects of HDACi on immune cells stem from their ability to modulate gene expression within immune cells. Beliakova-Bethell et al. defined the differential gene expression changes induced by SAHA treatment [222]. The study aimed to elucidate how CD4⁺ T cells are affected by SAHA treatment to better understand what potential off-target effects could mean for patients receiving HDAC inhibitor treatment [222]. CD4⁺ T cells were isolated from healthy donors receiving SAHA or VPA and a microarray was utilized to identify genes and pathways affected by HDACi treatment. For example, c-myc, a proto-oncogene implicated in regulation of many pathways such as cell growth, proliferation, and apoptosis was downregulated in CD4⁺ T cells of the healthy volunteers receiving SAHA [222].

The off-target effects of TSA on T cells include a reduction in CD4⁺ T cell proliferation, suppression of IL-2 expression, and downregulation of T cell markers such as CD40L and CD25 [210]. Butyrate and TSA were shown to induce anergy to antigens in naive murine CD4⁺ T cells by up-regulating expression of p21 [223]. Lastly, TSA was shown to inhibit proliferation of human CD4⁺ cells and to downregulate CD154 expression, which is required for antigen presenting cell activation.

To test the effects of HDACi on lymphocyte activation, we treated healthy donor blood PBMCs with a panel of HDACi in the presence of stimuli to test NKT and T cell activation (Figure 6.1). NKT cells were stimulated with α -GalCer and artificial antigen presenting cells coated with α -GalCer-loaded CD1D molecules and CD28. T cells were stimulated with CD3/CD28 beads. PMA/Ionomycin serves as a positive control. We found no differences in T cell activation with TSA and panobinostat treatment. However, MC1568 treatment inhibited T cell activation. We performed a WST assay to assess lymphocyte proliferation and found that MC1568 induced proliferation, suggesting that it could induce over-activation of T cells, resulting in AICD. We did

not find that TSA reduces proliferation, but our studies did not determine which specific T cell subsets were affected by TSA treatment. Moreover, we were unable to detect NKT cell activation. Thus, application of our lab's aAPC-qPCR method will allow us to determine the short-term effects of HDACi treatment on NKT cell activation. However, future studies are needed to determine the long-term effects of HDACi on proliferation and effector functions of NKT cells. This can be tested in a mouse xenograft model.

In addition to affecting T cell function, HDAC inhibitors have been shown to impair NK cell activation and function by decreasing IFN- γ production and downregulating expression of NK receptors, thus decreasing NK-mediated immune surveillance [224]. *In vivo* work showed that, although HDACi exert their function on multiple levels, the effects are reversible and NK cell functionality rebounds within 4 days after discontinuation of HDACi therapy [224]. Nonetheless, the authors note that long-term administration of HDACi may lead to a progressive decline in NK cell immune surveillance, resulting in a permissive environment that may facilitate the relapse of the primary malignant disease, induce metastasis, and perhaps lead to the onset of a second malignancy [224]. However, although HDACi have such profound negative effects on NK cells, they open a new avenue of study in which such negative effects can be used to the patients' advantage. For example, valproic acid can be used to allow oncolytic virus infection of glioblastoma cells by downregulating NK response to virus infection [225]. Such work is paramount in determining how HDAC inhibitors can be employed in ways not originally envisioned.

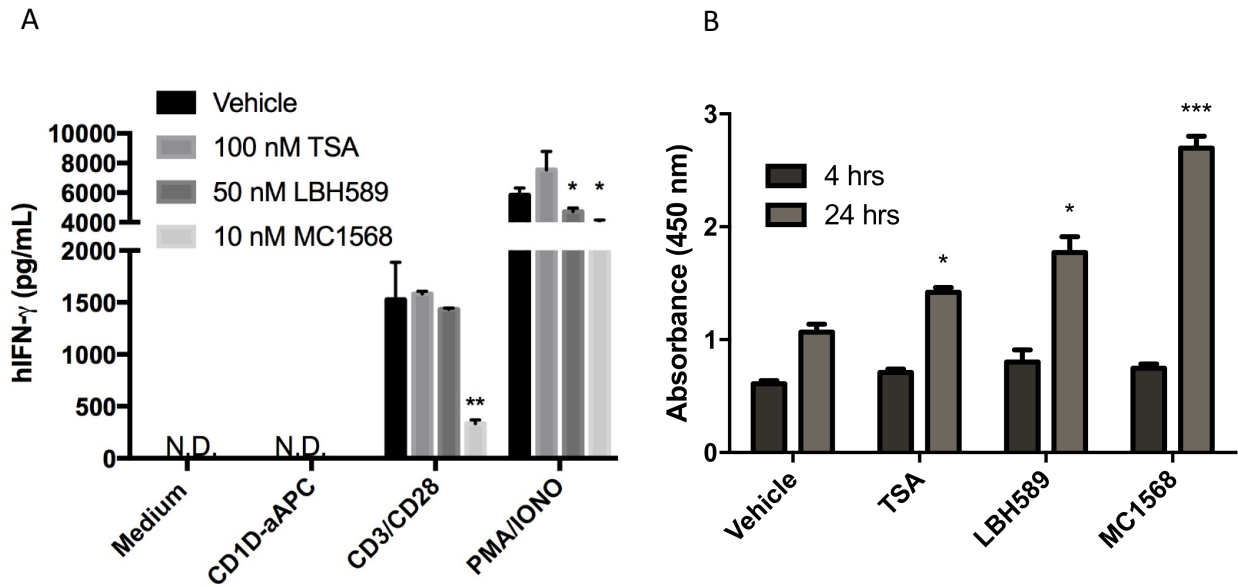


Figure 6.1: Effects of HDACi on lymphocyte activation. A) The effects of HDACi on NKT and T cell activation were examined by treating healthy donor peripheral blood mononuclear cells (PBMCs) with TSA (100 nM), LBH589 (50 nM) and MC1568 (10 nM) and using artificial antigen presenting cells (aAPCs) and CD3/CD28 beads to activate NKT and total T cells, respectively. PMA/Ionomycin treatment is the positive control. Supernatants were harvested and subjected to standard IFN- γ ELISA at the end of 48 h incubation. B) Standard WST assay was performed on PBMCs treated with TSA (100 nM), LBH589 (50 nM) and MC1568 (10 nM) at 4 and 24 h of treatment. T tests were performed to ascertain statistical significance: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$. N.D., not detectable.

Significance and implications

As discussed above, RIME hinges on immunoprecipitation of a known factor using antibodies to a specific factor, followed by identification of interacting proteins by mass spectroscopy. We identified a factor that binds at the CD1D promoter. Thus, RIME can be applied to identify other proteins/co-factors involved in regulating CD1D gene transcription in MCL and identify other HDAC2-regulated genes, with a specific focus on genes involved in antigen presentation. Ultimately, the identification of HDAC2-interacting proteins may help find a protein-protein interaction pair that predicts immune responses to cancer.

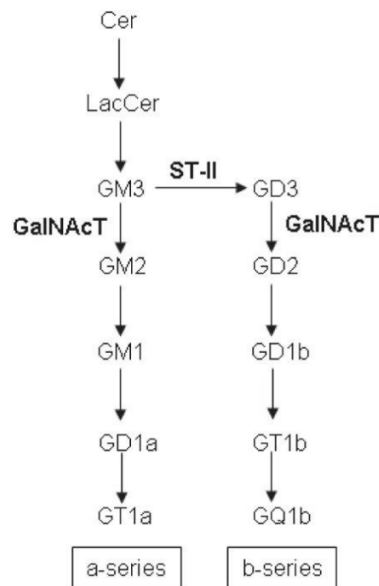
Summary

In Chapter 3, we demonstrated that modulation of HDAC2 leads to enhanced CD1D-dependent NKT cell responses (Figures 3.3-3.6, 3.9). We determined that HDAC2 binds at the CD1D promoter and that HDAC2 knockdown induced CD1D cell surface levels (Figures 3.7 and 3.8). We found that treatment of MCL cells with HDACi enhanced CD1D-mediated antigen presentation (Figures 3.2 and 3.3). Thus, we have identified a novel role for HDAC2 in the regulation of antigen presentation to NKT cells. We also found that antigen presentation by MHC class II to DR-4-specific T cells was induced following HDACi treatment (Figure 3.3). Numerous studies demonstrated increased surface expression of MHC class II [208,85,209,210], but we are the first to demonstrate functional effects of HDACi on CD1d-mediated antigen presentation. Future studies will determine whether other HDACs regulate CD1d expression.

6.2 Cell-extrinsic effects of HDACi on CD1d-mediated antigen presentation

Potential role of HDACi in controlling cell extrinsic mechanisms of immune evasion

In addition to playing a role in regulating cytokine secretion, HDACi can affect lipid synthesis pathways and thus alter immunosuppressive lipid shedding. Specifically, ganglioside GD3 has been shown to inhibit NKT cell activation [195]. N-acetylgalactosaminyltransferase I (GA2/GM2/GD2/GT2-synthase, also known as GalNacT) and sialyltransferase II (GD3-synthase, also known as ST-II) are two key glycosyltransferases that control the balance between a- and b-series of gangliosides, serving as a branching point in the ganglioside synthesis pathway (Figure 6.1) [226]. Multiple studies have implicated a role for HDACi in regulating glycosyltransferases of the lipid synthesis pathway [227,228,226].



Reprinted with permission from the John Wiley and Sons Publishing Company (license # 3862750444063). Suzuki et al. *J Neurochem*, 116(5), 874-880.

Figure 6.2: Ganglioside synthesis pathway. ST-II and GalNacT serve as key enzymes, positioned at branching points of the ganglioside synthesis pathway.

Specifically, in neuroepithelial cells, sodium butyrate was shown to induce GalNacT, without affecting levels of ST-II [226]. Thus, HDACi have the potential to regulate lipid synthesis and alter the balance between a- and b-series lipids. Thin-layer chromatography revealed a decrease in the expression of GM3 and GD3 and an increase in GD1a, GD1b, and GT1b. While GD3 was shown to be inhibitory to NKT cells, GT1b did not affect NKT cell responses [195]. Moreover, HDAC1 and 2 knockdown and valproic acid treatment induces GalNacT [229]. Since ganglioside shedding by ovarian tumors has been shown to inhibit NKT cell activation, future studies are needed to elucidate the effects of HDACi on ganglioside shedding by ovarian cancer cells. In fact, regulation of lipid shedding may be another cell-extrinsic mechanism by which HDACs regulate CD1d-mediated antigen presentation.

Regulation of angiogenesis by HDACi

Under hypoxic conditions, various cell lines exhibit increased expression of HDACs 1-3. Moreover, TSA treatment inhibits hypoxia inducible factor-1 α (HIF-1 α), which regulates expression of various genes involved in angiogenesis, including VEGF [230]. HDACs modulate HIF-1 α activity, both directly and indirectly. Indirect regulation involves HDACi-induced hyperacetylation of factors that target HIF-1 α for degradation. Specifically, TSA inhibited angiogenesis in a Lewis lung carcinoma model [230]. Moreover, HDAC1 and HDAC3 have been shown to directly regulate HIF-1 α stability [231]. *In vivo*, valproic acid inhibits vessel formation in chorioallantoic membrane and matrigel plug assays in mice [232]. Moreover, LBH589 treatment of prostate cancer xenograft-bearing NSG mice resulted in decreased vessel formation around the tumor [232].

While the role of class I, II, and IV HDACs in angiogenesis has been extensively studied, little is known about the role of class III HDACs in angiogenesis. Future studies are needed to determine direct and indirect mechanisms of HDACi-mediated inhibition of angiogenesis. Given that ovarian tumors secrete high levels of VEGF, HDACi treatment may show utility in this setting.

In Chapter 3, we exhaustively demonstrated that HDACi treatment enhances antigen presentation. In Figure 5.6, we showed that fixation (and thus abrogation of inhibitory molecule secretion/shedding) allows ovarian cancer cells to serve as antigen presenting cells. Thus, combination of HDACi with antibodies against VEGF and GD3 may increase immunogenicity of ovarian tumors. Moreover, as discussed above, HDACi have a role in regulating ganglioside synthesis and may thus inhibit GD3 shedding. Thus, HDACi may function to restore antigen presentation through multiple mechanisms. Future studies are needed to determine the effects of HDACi on GD3 shedding, VEGF secretion, and antigen presentation by ovarian tumors.

Significance and implications

HDACi are generally well-tolerated and have shown efficacy in several types of cancers. However, STAT3 inhibitors have shown limited efficacy and yet, STAT3 modulation has been a topic of great interest in cancer research. We identified a potential role for STAT3 in mediating immunosuppressive cytokine secretion by MCL cells through induction of cytokine gene transcription. Thus, we believe that the efficacy of HDACi can be attributed, at least in part, to their ability to inhibit STAT3. In the future, it will be imperative to demonstrate that HDACi-mediated inhibition of immunosuppressive cytokine secretion enhances immune responses *in vivo*.

Summary

We sought to identify cell-extrinsic mechanisms by which tumors evade recognition by NKT cells. We found that MCL cells secrete immunosuppressive cytokines, such as IL-10 and VEGF. Furthermore, we determined that HDACi treatment inhibits STAT3 and immunosuppressive cytokine secretion. Further investigation will be required to determine whether other STAT3-inducible cytokines are immunosuppressive. We identified two other candidate cytokines, IL-6 and MCP-1, which can be tested using our approach to testing the effects of IL-10 and VEGF on CD1d-mediated antigen presentation (Figures 4.3 and 5.2). Furthermore, studies are needed to determine whether these cytokines inhibit CD1d-mediated antigen presentation or co-stimulatory molecule expression. Finally, NKT cells have receptors for VEGF (unpublished data from our laboratory) and further studies are needed to determine whether VEGF inhibits NKT cells directly. These studies will help identify targets for immunomodulation.

Thesis summary

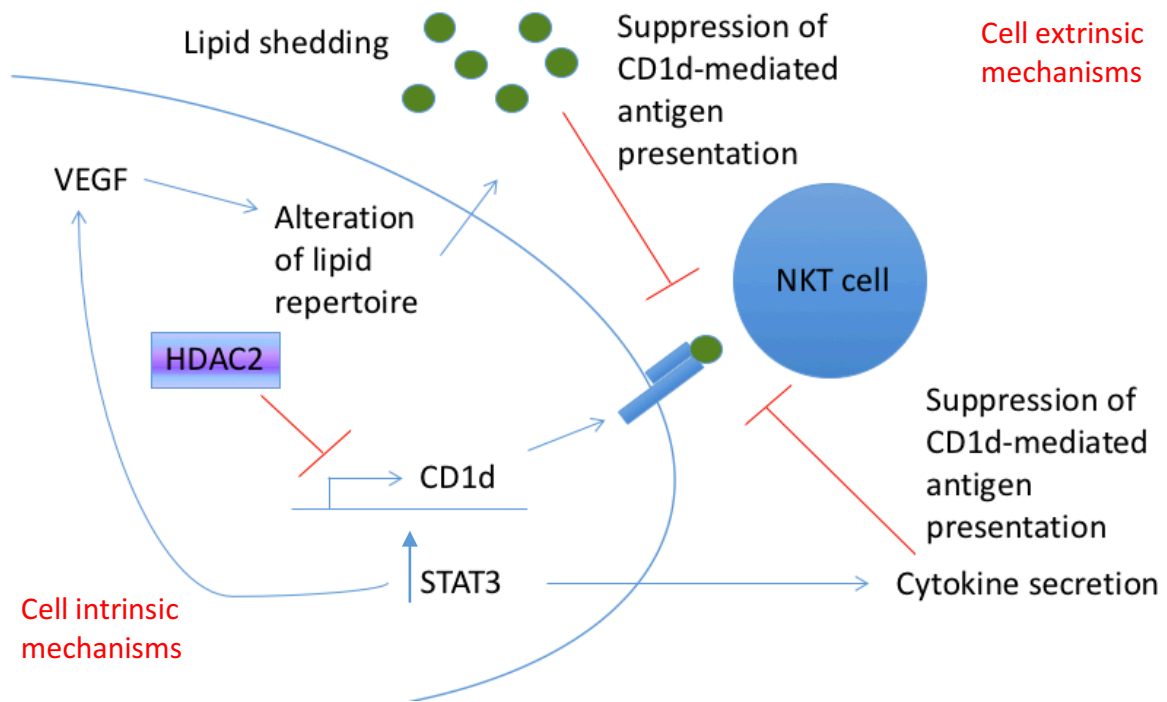
Tumors evade immune surveillance through several mechanisms. In particular, tumors alter antigen processing and presentation by major histocompatibility complex (MHC) proteins and create an immunosuppressive environment. For example, tumors downregulate CD1d, a non-polymorphic MHC class I-like molecule, in order to evade recognition by a subset of T cells called Natural killer T (NKT) cells. NKT cells constitute a population of T cells that is crucial to anti-tumor immunity: NKT cells can not only directly lyse malignant cells, but also serve as pivotal players in modulating other immune cells. Lymphomas, along with several other hematologic as well as solid malignancies, express CD1d and co-stimulatory molecules needed to activate NKT cells. Nonetheless, lymphomas are poorly immunogenic and they can achieve escape from NKT cell-mediated surveillance through downregulation of CD1d. We hypothesized that B cell lymphomas utilize epigenetic mechanisms to dysregulate CD1d-mediated antigen processing and presentation leading to a functional impairment in the ability of NKT cells to recognize tumors. In order to test our hypothesis, we pretreated human lymphoma cells with HDAC inhibitors (HDACi) and we assessed their ability to process and present antigen to primary human NKT cells. Similarly, we utilized a well-characterized mouse antigen presentation model. We found that treatment with trichostatin-A (TSA), a pan-HDACi in the hydroxamic acid class, resulted in an increase in antigen presentation to both human and mouse NKT cells. Moreover, pretreatment with TSA enhanced MHC class II-mediated antigen presentation to CD4⁺ T cells.

These data indicate that HDACi modulate CD1d and MHC class II-mediated antigen presentation and suggests a role for multiple HDACs in regulating antigen processing and presentation. We found that treatment with TSA lead to rapid increase in both CD1D mRNA and protein, suggesting that HDACs are involved in controlling CD1D gene at the promoter level. We

identified HDAC2 as the main HDAC regulating CD1D-mediated antigen presentation. Thus, we identified a cell-intrinsic mechanism by which tumors suppress CD1d-mediated antigen presentation (as summarized in the figure below).

In order to assess global immunomodulatory effects of HDACi, we examined an oncogenic factor frequently constitutively activated in lymphoma, signal transducer and activator of transcription 3 (STAT3). We found that lymphoma cells with constitutively activated STAT3 secrete interleukin-10 (IL-10), which we found to be suppressive to NKT cells. Treatment with TSA inhibited IL-10 secretion. We postulate that HDACi suppress inhibitory cytokine secretion and that cytokine secretion is a cell extrinsic mechanism by which tumors suppress NKT cell-mediated immune responses. These studies demonstrate the efficacy of HDACi in restoring NKT cell-mediated anti-tumor responses and may provide the basis for an NKT cell-based immunotherapeutic strategy that not only enhances the immune response, but also increases the immunogenicity of the tumor itself.

We extended the finding that tumor-secreted cytokines inhibit CD1d-mediated antigen presentation to an ovarian cancer model. We showed that ovarian cancers secrete vascular endothelial growth factor (VEGF) and that VEGF potentiates GD3-mediated suppression of antigen presentation. Moreover, we showed that VEGF inhibits CD1d-mediated antigen presentation. Thus, tumors may utilize multiple mechanisms to evade recognition by immune cells (as summarized in the figure below). Targeting of immunosuppressive factors in combination with immunotherapy may restore anti-tumor immune responses.



Summary Figure: Tumors utilize various mechanisms to evade NKT cell-mediated anti-tumor responses. Cell-intrinsic mechanisms include dysregulation of CD1d gene transcription and aberrations in intracellular signaling pathways, including STAT3. Cell-extrinsic mechanisms include secretion of immunosuppressive cytokines, such as VEGF, and shedding of lipids that inhibit CD1d-mediated antigen presentation.

References

1. Stewart B, Wild CP (2014) World Cancer Report 2014. International Agency for Research on Cancer
2. NIH SEER Stat Fact Sheets: Cancer of Any Site. <http://seer.cancer.gov/statfacts/html/all.html>. 2016
3. CDC Gynecologic Cancers. <http://www.cdc.gov/cancer/gynecologic/>. 2016
4. Smith A, Howell D, Patmore R, Jack A, Roman E (2011) Incidence of haematological malignancy by sub-type: a report from the Haematological Malignancy Research Network. *British journal of cancer* 105 (11):1684-1692. doi:10.1038/bjc.2011.450
5. Fisher SG, Fisher RI (2004) The epidemiology of non-Hodgkin's lymphoma. *Oncogene* 23 (38):6524-6534. doi:10.1038/sj.onc.1207843
6. Boffetta P (2011) I. Epidemiology of adult non-Hodgkin lymphoma. *Annals of Oncology* 22 (suppl 4):iv27-iv31. doi:10.1093/annonc/mdr167
7. Zhou Y, Wang H, Fang W, Romaguer JE, Zhang Y, Delasalle KB, Kwak L, Yi Q, Du XL, Wang M (2008) Incidence trends of mantle cell lymphoma in the United States between 1992 and 2004. *Cancer* 113 (4):791-798. doi:10.1002/cncr.23608
8. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144 (5):646-674. doi:10.1016/j.cell.2011.02.013
9. Kuppers R (2005) Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer* 5 (4):251-262. doi:10.1038/nrc1589
10. Kuppers R, Engert A, Hansmann ML (2012) Hodgkin lymphoma. *The Journal of clinical investigation* 122 (10):3439-3447. doi:10.1172/jci61245
11. Alexander DD, Mink PJ, Adami HO, Chang ET, Cole P, Mandel JS, Trichopoulos D (2007) The non-Hodgkin lymphomas: a review of the epidemiologic literature. *International journal of cancer* 120 Suppl 12:1-39. doi:10.1002/ijc.22719
12. Sehn LH (2015) Introduction to a clinical review series on aggressive B-cell lymphoma. *Blood* 125 (1):1-2. doi:10.1182/blood-2014-09-594580
13. Campo E, Rule S (2015) Mantle cell lymphoma: evolving management strategies. *Blood* 125 (1):48-55. doi:10.1182/blood-2014-05-521898
14. Perez-Galan P, Dreyling M, Wiestner A (2011) Mantle cell lymphoma: biology, pathogenesis, and the molecular basis of treatment in the genomic era. *Blood* 117 (1):26-38. doi:10.1182/blood-2010-04-189977

15. Dreyling M, Ferrero S, Hermine O (2014) How to manage mantle cell lymphoma. *Leukemia* 28 (11):2117-2130. doi:10.1038/leu.2014.171
16. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mule J, Kerr WG, Jove R, Pardoll D, Yu H (2005) Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nature medicine* 11 (12):1314-1321. doi:10.1038/nm1325
17. Granboulan M, Lankar D, Raposo G, Bonnerot C, Hivroz C (2003) Phosphoinositide 3-kinase activation by Igbeta controls de novo formation of an antigen-processing compartment. *The Journal of biological chemistry* 278 (6):4331-4338. doi:10.1074/jbc.M209885200
18. Yoshimura S, Bondeson J, Foxwell BM, Brennan FM, Feldmann M (2001) Effective antigen presentation by dendritic cells is NF-kappaB dependent: coordinate regulation of MHC, co-stimulatory molecules and cytokines. *International immunology* 13 (5):675-683
19. Inamdar AA, Goy A, Ayoub NM, Attia C, Oton L, Taruvai V, Costales M, Lin YT, Pecora A, Suh KS (2016) Mantle cell lymphoma in the era of precision medicine-diagnosis, biomarkers and therapeutic agents. *Oncotarget*. doi:10.18632/oncotarget.8961
20. Narurkar R, Alkayem M, Liu D (2016) SOX11 is a biomarker for cyclin D1-negative mantle cell lymphoma. *Biomarker research* 4:6. doi:10.1186/s40364-016-0060-9
21. Mozos A, Royo C, Hartmann E, De Jong D, Baro C, Valera A, Fu K, Weisenburger DD, Delabie J, Chuang SS, Jaffe ES, Ruiz-Marcellan C, Dave S, Rimsza L, Braziel R, Gascoyne RD, Sole F, Lopez-Guillermo A, Colomer D, Staudt LM, Rosenwald A, Ott G, Jares P, Campo E (2009) SOX11 expression is highly specific for mantle cell lymphoma and identifies the cyclin D1-negative subtype. *Haematologica* 94 (11):1555-1562. doi:10.3324/haematol.2009.010264
22. Nordstrom L, Sernbo S, Eden P, Gronbaek K, Kolstad A, Raty R, Karjalainen ML, Geisler C, Ralfkiaer E, Sundstrom C, Laurell A, Delabie J, Ehinger M, Jerkeman M, Ek S (2014) SOX11 and TP53 add prognostic information to MIPI in a homogenously treated cohort of mantle cell lymphoma--a Nordic Lymphoma Group study. *British journal of haematology* 166 (1):98-108. doi:10.1111/bjh.12854
23. Dreyling M, Ferrero S (2015) Personalized medicine in lymphoma: is it worthwhile? The mantle cell lymphoma experience. *Haematologica* 100 (6):706-708. doi:10.3324/haematol.2015.127472
24. Dreyling M, Geisler C, Hermine O, Kluin-Nelemans HC, Le Gouill S, Rule S, Shpilberg O, Walewski J, Ladetto M (2014) Newly diagnosed and relapsed mantle cell lymphoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 25 Suppl 3:iii83-92. doi:10.1093/annonc/mdu264

25. Jares P, Colomer D, Campo E (2007) Genetic and molecular pathogenesis of mantle cell lymphoma: perspectives for new targeted therapeutics. *Nat Rev Cancer* 7 (10):750-762
26. Argento M, Hoffman P, Gauchez AS (2008) Ovarian cancer detection and treatment: current situation and future prospects. *Anticancer research* 28 (5b):3135-3138
27. Chu CS, Kim SH, June CH, Coukos G (2008) Immunotherapy opportunities in ovarian cancer. *Expert review of anticancer therapy* 8 (2):243-257. doi:10.1586/14737140.8.2.243
28. Heintz AP, Odicino F, Maisonneuve P, Quinn MA, Benedet JL, Creasman WT, Ngan HY, Pecorelli S, Beller U (2006) Carcinoma of the ovary. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics* 95 Suppl 1:S161-192. doi:10.1016/s0020-7292(06)60033-7
29. Raja FA, Chopra N, Ledermann JA (2012) Optimal first-line treatment in ovarian cancer. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 23 Suppl 10:x118-127. doi:10.1093/annonc/mds315
30. Goff BA (2013) Advanced ovarian cancer: what should be the standard of care? *Journal of Gynecologic Oncology* 24 (1):83-91. doi:10.3802/jgo.2013.24.1.83
31. Herod JJ, Eliopoulos AG, Warwick J, Niedobitek G, Young LS, Kerr DJ (1996) The prognostic significance of Bcl-2 and p53 expression in ovarian carcinoma. *Cancer research* 56 (9):2178-2184
32. Dobrzycka B, Terlikowski SJ, Kowalczyk O, Niklinska W, Chyczewski L, Kulikowski M (2009) Mutations in the KRAS gene in ovarian tumors. *Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society* 47 (2):221-224. doi:10.2478/v10042-009-0039-6
33. West AC, Johnstone RW (2014) New and emerging HDAC inhibitors for cancer treatment. *The Journal of clinical investigation* 124 (1):30-39. doi:10.1172/jci69738
34. Marks PA (2010) Histone deacetylase inhibitors: a chemical genetics approach to understanding cellular functions. *Biochimica et biophysica acta* 1799 (10-12):717-725. doi:10.1016/j.bbagr.2010.05.008
35. Lu K, Chen N, Zhou XX, Ge XL, Feng LL, Li PP, Li XY, Geng LY, Wang X (2015) The STAT3 inhibitor WP1066 synergizes with vorinostat to induce apoptosis of mantle cell lymphoma cells. *Biochemical and biophysical research communications* 464 (1):292-298. doi:10.1016/j.bbrc.2015.06.145
36. Heninger E, Krueger TE, Lang JM (2015) Augmenting antitumor immune responses with epigenetic modifying agents. *Frontiers in immunology* 6:29. doi:10.3389/fimmu.2015.00029
37. Dawson Mark A, Kouzarides T (2012) Cancer Epigenetics: From Mechanism to Therapy. *Cell* 150 (1):12-27. doi:<http://dx.doi.org/10.1016/j.cell.2012.06.013>

38. Ropero S, Esteller M (2007) The role of histone deacetylases (HDACs) in human cancer. *Molecular oncology* 1 (1):19-25. doi:10.1016/j.molonc.2007.01.001
39. Ray S, Lee C, Hou T, Boldogh I, Brasier AR (2008) Requirement of histone deacetylase 1 (HDAC1) in signal transducer and activator of transcription 3 (STAT3) nucleocytoplasmic distribution. *Nucleic acids research* 36 (13):4510-4520. doi:10.1093/nar/gkn419
40. Yu H, Pardoll D, Jove R (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer* 9 (11):798-809
41. Halili MA, Andrews MR, Sweet MJ, Fairlie DP (2009) Histone deacetylase inhibitors in inflammatory disease. *Current topics in medicinal chemistry* 9 (3):309-319
42. Adcock IM (2007) HDAC inhibitors as anti-inflammatory agents. *British journal of pharmacology* 150 (7):829-831. doi:10.1038/sj.bjp.0707166
43. Saito A, Yamashita T, Mariko Y, Nosaka Y, Tsuchiya K, Ando T, Suzuki T, Tsuruo T, Nakanishi O (1999) A synthetic inhibitor of histone deacetylase, MS-27-275, with marked in vivo antitumor activity against human tumors. *Proceedings of the National Academy of Sciences* 96 (8):4592-4597. doi:10.1073/pnas.96.8.4592
44. Choi JH, Oh SW, Kang MS, Kwon HJ, Oh GT, Kim DY (2005) Trichostatin A attenuates airway inflammation in mouse asthma model. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 35 (1):89-96. doi:10.1111/j.1365-2222.2004.02006.x
45. Villagra A, Sotomayor EM, Seto E (2010) Histone deacetylases and the immunological network: implications in cancer and inflammation. *Oncogene* 29 (2):157-173. doi:10.1038/onc.2009.334
46. Yuan ZL, Guan YJ, Chatterjee D, Chin YE (2005) Stat3 dimerization regulated by reversible acetylation of a single lysine residue. *Science (New York, NY)* 307 (5707):269-273. doi:10.1126/science.1105166
47. Gupta M, Han JJ, Stenson M, Wellik L, Witzig TE (2012) Regulation of STAT3 by histone deacetylase-3 in diffuse large B-cell lymphoma: implications for therapy. *Leukemia* 26 (6):1356-1364. doi:10.1038/leu.2011.340
48. Zhang S, Suvannasankha A, Crean CD, White VL, Chen CS, Farag SS (2011) The novel histone deacetylase inhibitor, AR-42, inhibits gp130/Stat3 pathway and induces apoptosis and cell cycle arrest in multiple myeloma cells. *International journal of cancer* 129 (1):204-213. doi:10.1002/ijc.25660
49. Mottamal M, Zheng S, Huang TL, Wang G (2015) Histone deacetylase inhibitors in clinical studies as templates for new anticancer agents. *Molecules (Basel, Switzerland)* 20 (3):3898-3941. doi:10.3390/molecules20033898

50. Robert C, Rassool FV (2012) HDAC inhibitors: roles of DNA damage and repair. *Advances in cancer research* 116:87-129. doi:10.1016/b978-0-12-394387-3.00003-3
51. Wang DF, Helquist P, Wiech NL, Wiest O (2005) Toward selective histone deacetylase inhibitor design: homology modeling, docking studies, and molecular dynamics simulations of human class I histone deacetylases. *Journal of medicinal chemistry* 48 (22):6936-6947. doi:10.1021/jm0505011
52. Bieliauskas AV, Weerasinghe SV, Pflum MK (2007) Structural requirements of HDAC inhibitors: SAHA analogs functionalized adjacent to the hydroxamic acid. *Bioorganic & medicinal chemistry letters* 17 (8):2216-2219. doi:10.1016/j.bmcl.2007.01.117
53. Finnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA, Breslow R, Pavletich NP (1999) Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* 401 (6749):188-193. doi:10.1038/43710
54. Heller G, Schmidt WM, Ziegler B, Holzer S, Mullauer L, Bilban M, Zielinski CC, Drach J, Zochbauer-Muller S (2008) Genome-wide transcriptional response to 5-aza-2'-deoxycytidine and trichostatin a in multiple myeloma cells. *Cancer research* 68 (1):44-54. doi:10.1158/0008-5472.can-07-2531
55. Roy S, Jeffrey R, Tenniswood M (2008) Array-based analysis of the effects of trichostatin A and CG-1521 on cell cycle and cell death in LNCaP prostate cancer cells. *Molecular cancer therapeutics* 7 (7):1931-1939. doi:10.1158/1535-7163.mct-07-2353
56. Lopez-Atalaya JP, Ito S, Valor LM, Benito E, Barco A (2013) Genomic targets, and histone acetylation and gene expression profiling of neural HDAC inhibition. *Nucleic acids research* 41 (17):8072-8084. doi:10.1093/nar/gkt590
57. Alberter B, Vogel B, Lengenfelder D, Full F, Ensser A (2011) Genome-wide histone acetylation profiling of Herpesvirus saimiri in human T cells upon induction with a histone deacetylase inhibitor. *Journal of virology* 85 (11):5456-5464. doi:10.1128/jvi.00164-11
58. Juergens RA, Wrangle J, Vendetti FP, Murphy SC, Zhao M, Coleman B, Sebree R, Rodgers K, Hooker CM, Franco N, Lee B, Tsai S, Delgado IE, Rudek MA, Belinsky SA, Herman JG, Baylin SB, Brock MV, Rudin CM (2011) Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. *Cancer discovery* 1 (7):598-607. doi:10.1158/2159-8290.cd-11-0214
59. Wagner JM, Hackanson B, Lubbert M, Jung M (2010) Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy. *Clinical epigenetics* 1 (3-4):117-136. doi:10.1007/s13148-010-0012-4
60. Fiskus W, Rao R, Balusu R, Ganguly S, Tao J, Sotomayor E, Mudunuru U, Smith JE, Hembruff SL, Atadja P, Marquez VE, Bhalla K (2012) Superior efficacy of a combined epigenetic therapy against human mantle cell lymphoma cells. *Clinical cancer research : an*

official journal of the American Association for Cancer Research 18 (22):6227-6238.
doi:10.1158/1078-0432.ccr-12-0873

61. Kroesen M, Gielen P, Brok IC, Armandari I, Hoogerbrugge PM, Adema GJ (2014) HDAC inhibitors and immunotherapy; a double edged sword? *Oncotarget* 5 (16):6558-6572.
doi:10.18632/oncotarget.2289

62. Ribas A, Wolchok JD (2013) Combining cancer immunotherapy and targeted therapy. *Current opinion in immunology* 25 (2):291-296. doi:10.1016/j.coi.2013.02.011

63. Kirschbaum M, Frankel P, Popplewell L, Zain J, Delioukina M, Pullarkat V, Matsuoka D, Pulone B, Rotter AJ, Espinoza-Delgado I, Nademanee A, Forman SJ, Gandara D, Newman E (2011) Phase II Study of Vorinostat for Treatment of Relapsed or Refractory Indolent Non-Hodgkin's Lymphoma and Mantle Cell Lymphoma. *Journal of Clinical Oncology*.
doi:10.1200/jco.2010.32.1398

64. Spurgeon S CA, Okada C, Parekh S, Leshchenko V, Palmbach G, Ratterree B, Subbiah N, Capper C, Epner E. (2011) A Phase I/II Study of Vorinostat (SAHA), Cladribine (2-CdA), and Rituximab Shows Significant Activity in Previously Untreated Mantle Cell Lymphoma. *Blood* 118 (21):203

65. van den Elsen PJ (2011) Expression regulation of major histocompatibility complex class I and class II encoding genes. *Frontiers in immunology* 2:48. doi:10.3389/fimmu.2011.00048

66. van den Elsen PJ, Holling TM, Kuipers HF, van der Stoep N (2004) Transcriptional regulation of antigen presentation. *Current opinion in immunology* 16 (1):67-75

67. Kochan G, Escors D, Breckpot K, Guerrero-Setas D (2013) Role of non-classical MHC class I molecules in cancer immunosuppression. *Oncoimmunology* 2 (11):e26491.
doi:10.4161/onci.26491

68. Gomes AQ, Correia DV, Silva-Santos B (2007) Non-classical major histocompatibility complex proteins as determinants of tumour immunosurveillance. *EMBO reports* 8 (11):1024-1030. doi:10.1038/sj.embor.7401090

69. Blumberg RS, Gerdes D, Chott A, Porcelli SA, Balk SP (1995) Structure and function of the CD1 family of MHC-like cell surface proteins. *Immunological reviews* 147:5-29

70. Chen YH, Wang B, Chun T, Zhao L, Cardell S, Behar SM, Brenner MB, Wang CR (1999) Expression of CD1d2 on thymocytes is not sufficient for the development of NK T cells in CD1d1-deficient mice. *Journal of immunology (Baltimore, Md : 1950)* 162 (8):4560-4566

71. Zeng Z, Castano AR, Segelke BW, Stura EA, Peterson PA, Wilson IA (1997) Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove. *Science (New York, NY)* 277 (5324):339-345

72. Jayawardena-Wolf J, Benlagha K, Chiu YH, Mehr R, Bendelac A (2001) CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. *Immunity* 15 (6):897-908
73. Chen QY, Jackson N (2004) Human CD1D gene has TATA boxless dual promoters: an SP1-binding element determines the function of the proximal promoter. *Journal of immunology* (Baltimore, Md : 1950) 172 (9):5512-5521
74. Yang PM, Lin PJ, Chen CC (2012) CD1d induction in solid tumor cells by histone deacetylase inhibitors through inhibition of HDAC1/2 and activation of Sp1. *Epigenetics* 7 (4):390-399. doi:10.4161/epi.19373
75. Chen QY, Zhang T, Pincus SH, Wu S, Ricks D, Liu D, Sun Z, Maclaren N, Lan MS (2010) Human CD1D gene expression is regulated by LEF-1 through distal promoter regulatory elements. *Journal of immunology* (Baltimore, Md : 1950) 184 (9):5047-5054. doi:10.4049/jimmunol.0901912
76. Geng Y, Laslo P, Barton K, Wang CR (2005) Transcriptional regulation of CD1D1 by Ets family transcription factors. *Journal of immunology* (Baltimore, Md : 1950) 175 (2):1022-1029
77. Chen Q, Ross AC (2007) Retinoic acid regulates CD1d gene expression at the transcriptional level in human and rodent monocytic cells. *Experimental biology and medicine* (Maywood, NJ) 232 (4):488-494
78. Campoli M, Ferrone S (2008) HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene* 27 (45):5869-5885. doi:10.1038/onc.2008.273
79. Chamuleau ME, Ossenkoppele GJ, van de Loosdrecht AA (2006) MHC class II molecules in tumour immunology: prognostic marker and target for immune modulation. *Immunobiology* 211 (6-8):619-625. doi:10.1016/j.imbio.2006.05.005
80. Spanoudakis E, Hu M, Naresh K, Terpos E, Melo V, Reid A, Kotsianidis I, Abdalla S, Rahemtulla A, Karadimitris A (2009) Regulation of multiple myeloma survival and progression by CD1d. *Blood* 113 (11):2498-2507. doi:10.1182/blood-2008-06-161281
81. Bojarska-Junak A, Hus I, Chocholska S, Tomczak W, Wos J, Czubak P, Putowski L, Rolinski J (2014) CD1d expression is higher in chronic lymphocytic leukemia patients with unfavorable prognosis. *Leukemia research* 38 (4):435-442. doi:10.1016/j.leukres.2013.12.015
82. Anastasiadis A, Kotsianidis I, Papadopoulos V, Spanoudakis E, Margaritis D, Christoforidou A, Gouliamtzi S, Tsatalas C (2014) CD1d expression as a prognostic marker for chronic lymphocytic leukemia. *Leukemia & lymphoma* 55 (2):320-325. doi:10.3109/10428194.2013.803222

83. Chang CC, Campoli M, Ferrone S (2005) Classical and nonclassical HLA class I antigen and NK Cell-activating ligand changes in malignant cells: current challenges and future directions. *Advances in cancer research* 93:189-234. doi:10.1016/s0065-230x(05)93006-6
84. Wright KL, Ting JP (2006) Epigenetic regulation of MHC-II and CIITA genes. *Trends in immunology* 27 (9):405-412. doi:10.1016/j.it.2006.07.007
85. Magner WJ, Kazim AL, Stewart C, Romano MA, Catalano G, Grande C, Keiser N, Santaniello F, Tomasi TB (2000) Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. *Journal of immunology (Baltimore, Md : 1950)* 165 (12):7017-7024
86. Khan AN, Gregorie CJ, Tomasi TB (2008) Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. *Cancer immunology, immunotherapy : CII* 57 (5):647-654. doi:10.1007/s00262-007-0402-4
87. West AC, Mattarollo SR, Shortt J, Cluse LA, Christiansen AJ, Smyth MJ, Johnstone RW (2013) An intact immune system is required for the anticancer activities of histone deacetylase inhibitors. *Cancer research* 73 (24):7265-7276. doi:10.1158/0008-5472.can-13-0890
88. Song W, Tai YT, Tian Z, Hideshima T, Chauhan D, Nanjappa P, Exley MA, Anderson KC, Munshi NC (2011) HDAC inhibition by LBH589 affects the phenotype and function of human myeloid dendritic cells. *Leukemia* 25 (1):161-168. doi:10.1038/leu.2010.244
89. Frikeche J, Simon T, Brissot E, Gregoire M, Gaugler B, Mohty M (2012) Impact of valproic acid on dendritic cells function. *Immunobiology* 217 (7):704-710. doi:10.1016/j.imbio.2011.11.010
90. Frikeche J, Peric Z, Brissot E, Gregoire M, Gaugler B, Mohty M (2012) Impact of HDAC inhibitors on dendritic cell functions. *Experimental hematology* 40 (10):783-791. doi:10.1016/j.exphem.2012.06.008
91. Goel HL, Mercurio AM (2013) VEGF targets the tumour cell. *Nat Rev Cancer* 13 (12):871-882. doi:10.1038/nrc3627
92. Ellis LM, Hicklin DJ (2008) VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer* 8 (8):579-591
93. Bottsford-Miller JN, Coleman RL, Sood AK (2012) Resistance and escape from antiangiogenesis therapy: clinical implications and future strategies. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 30 (32):4026-4034. doi:10.1200/jco.2012.41.9242
94. Campos SM, Penson RT, Matulonis U, Horowitz NS, Whalen C, Pereira L, Tyburski K, Roche M, Szymonifka J, Berlin S (2013) A phase II trial of Sunitinib malate in recurrent and

refractory ovarian, fallopian tube and peritoneal carcinoma. *Gynecologic oncology* 128 (2):215-220. doi:10.1016/j.ygyno.2012.07.126

95. Biagi JJ, Oza AM, Chalchal HI, Grimshaw R, Ellard SL, Lee U, Hirte H, Sederias J, Ivy SP, Eisenhauer EA (2011) A phase II study of sunitinib in patients with recurrent epithelial ovarian and primary peritoneal carcinoma: an NCIC Clinical Trials Group Study. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 22 (2):335-340. doi:10.1093/annonc/mdq357

96. Matei D, Sill MW, Lankes HA, DeGeest K, Bristow RE, Mutch D, Yamada SD, Cohn D, Calvert V, Farley J, Petricoin EF, Birrer MJ (2011) Activity of sorafenib in recurrent ovarian cancer and primary peritoneal carcinomatosis: a gynecologic oncology group trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 29 (1):69-75. doi:10.1200/jco.2009.26.7856

97. Herzog TJ, Scambia G, Kim BG, Lhomme C, Markowska J, Ray-Coquard I, Sehouli J, Colombo N, Shan M, Petrenciuc O, Oza A (2013) A randomized phase II trial of maintenance therapy with Sorafenib in front-line ovarian carcinoma. *Gynecologic oncology* 130 (1):25-30. doi:10.1016/j.ygyno.2013.04.011

98. McLachlan J, Banerjee S (2015) Pazopanib in ovarian cancer. *Expert review of anticancer therapy* 15 (9):995-1005. doi:10.1586/14737140.2015.1081383

99. Burger RA, Sill MW, Monk BJ, Greer BE, Sorosky JI (2007) Phase II trial of bevacizumab in persistent or recurrent epithelial ovarian cancer or primary peritoneal cancer: a Gynecologic Oncology Group Study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 25 (33):5165-5171. doi:10.1200/jco.2007.11.5345

100. Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H, Mannel RS, Homesley HD, Fowler J, Greer BE, Boente M, Birrer MJ, Liang SX (2011) Incorporation of bevacizumab in the primary treatment of ovarian cancer. *The New England journal of medicine* 365 (26):2473-2483. doi:10.1056/NEJMoa1104390

101. Cannistra SA, Matulonis UA, Penson RT, Hambleton J, Dupont J, Mackey H, Douglas J, Burger RA, Armstrong D, Wenham R, McGuire W (2007) Phase II study of bevacizumab in patients with platinum-resistant ovarian cancer or peritoneal serous cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 25 (33):5180-5186. doi:10.1200/jco.2007.12.0782

102. Perren TJ, Swart AM, Pfisterer J, Ledermann JA, Pujade-Lauraine E, Kristensen G, Carey MS, Beale P, Cervantes A, Kurzeder C, du Bois A, Sehouli J, Kimmig R, Stahle A, Collinson F, Essapen S, Gourley C, Lortholary A, Selle F, Mirza MR, Leminen A, Plante M, Stark D, Qian W, Parmar MK, Oza AM (2011) A phase 3 trial of bevacizumab in ovarian cancer. *The New England journal of medicine* 365 (26):2484-2496. doi:10.1056/NEJMoa1103799

103. Hicklin DJ, Ellis LM (2005) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 23 (5):1011-1027. doi:10.1200/jco.2005.06.081
104. Gabrilovich D, Ishida T, Oyama T, Ran S, Kravtsov V, Nadaf S, Carbone DP (1998) Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood* 92 (11):4150-4166
105. Oyama T, Ran S, Ishida T, Nadaf S, Kerr L, Carbone DP, Gabrilovich DI (1998) Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa B activation in hemopoietic progenitor cells. *Journal of immunology (Baltimore, Md : 1950)* 160 (3):1224-1232
106. Roland CL, Lynn KD, Toombs JE, Dineen SP, Udugamasooriya DG, Brekken RA (2009) Cytokine levels correlate with immune cell infiltration after anti-VEGF therapy in preclinical mouse models of breast cancer. *PloS one* 4 (11):e7669. doi:10.1371/journal.pone.0007669
107. Voron T, Colussi O, Marcheteau E, Pernot S, Nizard M, Pointet AL, Latreche S, Bergaya S, Benhamouda N, Tanchot C, Stockmann C, Combe P, Berger A, Zinzindohoue F, Yagita H, Tartour E, Taieb J, Terme M (2015) VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. *The Journal of experimental medicine* 212 (2):139-148. doi:10.1084/jem.20140559
108. Huang Y, Goel S, Duda DG, Fukumura D, Jain RK (2013) Vascular normalization as an emerging strategy to enhance cancer immunotherapy. *Cancer research* 73 (10):2943-2948. doi:10.1158/0008-5472.can-12-4354
109. Gavalas NG, Tsiatas M, Tsitsilonis O, Politi E, Ioannou K, Ziogas AC, Rodolakis A, Vlahos G, Thomakos N, Haidopoulos D, Terpos E, Antsaklis A, Dimopoulos MA, Bamias A (2012) VEGF directly suppresses activation of T cells from ascites secondary to ovarian cancer via VEGF receptor type 2. *British journal of cancer* 107 (11):1869-1875. doi:<http://www.nature.com/bjc/journal/v107/n11/supinfo/bjc2012468s1.html>
110. Ferriss JS, Java JJ, Bookman MA, Fleming GF, Monk BJ, Walker JL, Homesley HD, Fowler J, Greer BE, Boente MP, Burger RA (2015) Ascites predicts treatment benefit of bevacizumab in front-line therapy of advanced epithelial ovarian, fallopian tube and peritoneal cancers: an NRG Oncology/GOG study. *Gynecologic oncology* 139 (1):17-22. doi:10.1016/j.ygyno.2015.07.103
111. Tiper IV, Temkin SM, Spiegel S, Goldblum SE, Giuntoli RL, Oelke M, Schneck JP, Webb TJ (2016) VEGF potentiates GD3-mediated immune suppression by human ovarian cancer cells. *Clinical cancer research : an official journal of the American Association for Cancer Research*. doi:10.1158/1078-0432.ccr-15-2518
112. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 3 (11):991-998. doi:10.1038/ni1102-991

113. Swann JB, Smyth MJ (2007) Immune surveillance of tumors. *The Journal of clinical investigation* 117 (5):1137-1146. doi:10.1172/jci31405
114. Gatti RA, Good RA (1971) Occurrence of malignancy in immunodeficiency diseases. A literature review. *Cancer* 28 (1):89-98
115. Dunn GP, Old LJ, Schreiber RD (2004) The three Es of cancer immunoediting. *Annual review of immunology* 22:329-360. doi:10.1146/annurev.immunol.22.012703.104803
116. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ (2011) Natural innate and adaptive immunity to cancer. *Annual review of immunology* 29:235-271. doi:10.1146/annurev-immunol-031210-101324
117. Teng MW, Vesely MD, Duret H, McLaughlin N, Towne JE, Schreiber RD, Smyth MJ (2012) Opposing roles for IL-23 and IL-12 in maintaining occult cancer in an equilibrium state. *Cancer research* 72 (16):3987-3996. doi:10.1158/0008-5472.can-12-1337
118. Teng MW, Galon J, Fridman WH, Smyth MJ (2015) From mice to humans: developments in cancer immunoediting. *The Journal of clinical investigation* 125 (9):3338-3346. doi:10.1172/jci80004
119. Stewart TJ, Abrams SI (0000) How tumours escape mass destruction. *Oncogene* 27 (45):5894-5903
120. Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, Bhattacharya R, Gabrilovich D, Heller R, Coppola D, Dalton W, Jove R, Pardoll D, Yu H (2004) Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nature medicine* 10 (1):48-54. doi:10.1038/nm976
121. Terme M, Ullrich E, Delahaye NF, Chaput N, Zitvogel L (2008) Natural killer cell-directed therapies: moving from unexpected results to successful strategies. *Nat Immunol* 9 (5):486-494. doi:10.1038/ni1580
122. Martin-Fontecha A, Lanzavecchia A, Sallusto F (2009) Dendritic cell migration to peripheral lymph nodes. *Handbook of experimental pharmacology* (188):31-49. doi:10.1007/978-3-540-71029-5_2
123. Gregory AD, Houghton AM (2011) Tumor-associated neutrophils: new targets for cancer therapy. *Cancer research* 71 (7):2411-2416. doi:10.1158/0008-5472.can-10-2583
124. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) *Molecular Biology of the Cell*, 4th edition. Garland Science New York
125. Fremd C, Schuetz F, Sohn C, Beckhove P, Domschke C (2013) B cell-regulated immune responses in tumor models and cancer patients. *Oncoimmunology* 2 (7):e25443. doi:10.4161/onci.25443

126. Silva-Santos B, Serre K, Norell H (2015) $\gamma\delta$ T cells in cancer. *Nat Rev Immunol* 15 (11):683-691. doi:10.1038/nri3904
127. Terabe M, Berzofsky JA (2008) The role of NKT cells in tumor immunity. *Advances in cancer research* 101:277-348. doi:10.1016/s0065-230x(08)00408-9
128. Imai K, Kanno M, Kimoto H, Shigemoto K, Yamamoto S, Taniguchi M (1986) Sequence and expression of transcripts of the T-cell antigen receptor alpha-chain gene in a functional, antigen-specific suppressor-T-cell hybridoma. *Proceedings of the National Academy of Sciences of the United States of America* 83 (22):8708-8712
129. Fowlkes BJ, Kruisbeek AM, Ton-That H, Weston MA, Coligan JE, Schwartz RH, Pardoll DM (1987) A novel population of T-cell receptor alpha beta-bearing thymocytes which predominantly expresses a single V beta gene family. *Nature* 329 (6136):251-254. doi:10.1038/329251a0
130. Koseki H, Imai K, Nakayama F, Sado T, Moriwaki K, Taniguchi M (1990) Homogenous junctional sequence of the V14+ T-cell antigen receptor alpha chain expanded in unprimed mice. *Proceedings of the National Academy of Sciences of the United States of America* 87 (14):5248-5252
131. Adachi Y, Koseki H, Zijlstra M, Taniguchi M (1995) Positive selection of invariant V alpha 14+ T cells by non-major histocompatibility complex-encoded class I-like molecules expressed on bone marrow-derived cells. *Proceedings of the National Academy of Sciences of the United States of America* 92 (4):1200-1204
132. Lantz O, Bendelac A (1994) An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *The Journal of experimental medicine* 180 (3):1097-1106
133. Godfrey DI, Stankovic S, Baxter AG (2010) Raising the NKT cell family. *Nat Immunol* 11 (3):197-206. doi:10.1038/ni.1841
134. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, Berzins SP, Smyth MJ, Godfrey DI (2008) Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proceedings of the National Academy of Sciences of the United States of America* 105 (32):11287-11292. doi:10.1073/pnas.0801631105
135. Uldrich AP, Crowe NY, Kyparissoudis K, Pellicci DG, Zhan Y, Lew AM, Bouillet P, Strasser A, Smyth MJ, Godfrey DI (2005) NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *Journal of immunology (Baltimore, Md : 1950)* 175 (5):3092-3101
136. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, Kronenberg M, Locksley RM (2003) Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells

poised for rapid effector function. *The Journal of experimental medicine* 198 (7):1069-1076.
doi:10.1084/jem.20030630

137. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, Kondo E, Harada M, Koseki H, Nakayama T, Tanaka Y, Taniguchi M (1998) Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proceedings of the National Academy of Sciences of the United States of America* 95 (10):5690-5693

138. Ortaldo JR, Young HA, Winkler-Pickett RT, Bere EW, Jr., Murphy WJ, Wiltout RH (2004) Dissociation of NKT stimulation, cytokine induction, and NK activation in vivo by the use of distinct TCR-binding ceramides. *Journal of immunology (Baltimore, Md : 1950)* 172 (2):943-953

139. Uemura Y, Liu TY, Narita Y, Suzuki M, Nakatsuka R, Araki T, Matsumoto M, Iwai LK, Hirosawa N, Matsuoka Y, Murakami M, Kimura T, Hase M, Kohno H, Sasaki Y, Ichihara Y, Ishihara O, Kikuchi H, Sakamoto Y, Jiao SC, Senju S, Sonoda Y (2009) Cytokine-dependent modification of IL-12p70 and IL-23 balance in dendritic cells by ligand activation of Valpha24 invariant NKT cells. *Journal of immunology (Baltimore, Md : 1950)* 183 (1):201-208.
doi:10.4049/jimmunol.0900873

140. Brigl M, Tatituri RV, Watts GF, Bhowruth V, Leadbetter EA, Barton N, Cohen NR, Hsu FF, Besra GS, Brenner MB (2011) Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. *The Journal of experimental medicine* 208 (6):1163-1177. doi:10.1084/jem.20102555

141. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM (2004) The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *The Journal of experimental medicine* 199 (12):1607-1618. doi:10.1084/jem.20040317

142. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M (1997) Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science (New York, NY)* 278 (5343):1623-1626

143. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y, Bendelac A (1999) Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *Journal of immunology (Baltimore, Md : 1950)* 163 (9):4647-4650

144. Zaini J, Andarini S, Tahara M, Saijo Y, Ishii N, Kawakami K, Taniguchi M, Sugamura K, Nukiwa T, Kikuchi T (2007) OX40 ligand expressed by DCs costimulates NKT and CD4+ Th cell antitumor immunity in mice. *The Journal of clinical investigation* 117 (11):3330-3338.
doi:10.1172/jci32693

145. Galli G, Pittoni P, Tonti E, Malzone C, Uematsu Y, Tortoli M, Maione D, Volpini G, Finco O, Nuti S, Tavarini S, Dellabona P, Rappuoli R, Casorati G, Abrignani S (2007) Invariant NKT

cells sustain specific B cell responses and memory. *Proceedings of the National Academy of Sciences of the United States of America* 104 (10):3984-3989. doi:10.1073/pnas.0700191104

146. Carmi Y, Spitzer MH, Linde IL, Burt BM, Prestwood TR, Perlman N, Davidson MG, Kenkel JA, Segal E, Pusapati GV, Bhattacharya N, Engleman EG (2015) Allogeneic IgG combined with dendritic cell stimuli induce antitumour T-cell immunity. *Nature* 521 (7550):99-104. doi:10.1038/nature14424

147. Wang J, Cheng L, Wondimu Z, Swain M, Santamaria P, Yang Y (2009) Cutting edge: CD28 engagement releases antigen-activated invariant NKT cells from the inhibitory effects of PD-1. *Journal of immunology (Baltimore, Md : 1950)* 182 (11):6644-6647. doi:10.4049/jimmunol.0804050

148. Hayakawa Y, Takeda K, Yagita H, Van Kaer L, Saiki I, Okumura K (2001) Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *Journal of immunology (Baltimore, Md : 1950)* 166 (10):6012-6018

149. Croudace JE, Curbishley SM, Mura M, Willcox CR, Illarionov PA, Besra GS, Adams DH, Lammas DA (2008) Identification of distinct human invariant natural killer T-cell response phenotypes to alpha-galactosylceramide. *BMC immunology* 9:71. doi:10.1186/1471-2172-9-71

150. Berzins SP, Ritchie DS (2014) Natural killer T cells: drivers or passengers in preventing human disease? *Nat Rev Immunol* 14 (9):640-646. doi:10.1038/nri3725

151. Renukaradhya GJ, Sriram V, Du W, Gervay-Hague J, Van Kaer L, Brutkiewicz RR (2006) Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *International journal of cancer* 118 (12):3045-3053. doi:10.1002/ijc.21764

152. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S (2007) Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. *The Journal of experimental medicine* 204 (11):2641-2653. doi:10.1084/jem.20070458

153. Molling JW, Kolgen W, van der Vliet HJ, Boomsma MF, Kruizenga H, Smorenburg CH, Molenkamp BG, Langendijk JA, Leemans CR, von Blomberg BM, Scheper RJ, van den Eertwegh AJ (2005) Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *International journal of cancer* 116 (1):87-93. doi:10.1002/ijc.20998

154. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, Imamura M (2005) Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11 (20):7322-7327. doi:10.1158/1078-0432.ccr-05-0877

155. Kobayashi E, Motoki K, Uchida T, Fukushima H, Koezuka Y (1995) KRN7000, a novel immunomodulator, and its antitumor activities. *Oncology research* 7 (10-11):529-534

156. Nakagawa R, Serizawa I, Motoki K, Sato M, Ueno H, Iijima R, Nakamura H, Shimosaka A, Koezuka Y (2000) Antitumor activity of alpha-galactosylceramide, KRN7000, in mice with the melanoma B16 hepatic metastasis and immunohistological study of tumor infiltrating cells. *Oncology research* 12 (2):51-58
157. Li J, Sun W, Subrahmanyam PB, Page C, Younger KM, Tiper IV, Frieman M, Kimball AS, Webb TJ (2014) NKT Cell Responses to B Cell Lymphoma. *Medical sciences : open access journal* 2 (2):82-97. doi:10.3390/medsci2020082
158. Mattarollo SR, West AC, Steegh K, Duret H, Paget C, Martin B, Matthews GM, Shortt J, Chesi M, Bergsagel PL, Bots M, Zuber J, Lowe SW, Johnstone RW, Smyth MJ (2012) NKT cell adjuvant-based tumor vaccine for treatment of myc oncogene-driven mouse B-cell lymphoma. *Blood* 120 (15):3019-3029. doi:10.1182/blood-2012-04-426643
159. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, von Blomberg BM, Scheper RJ, van der Vliet HJ, van den Eertwegh AJ, Roelvink M, Beijnen J, Zwierzina H, Pinedo HM (2002) A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* 8 (12):3702-3709
160. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, Hutchinson A, Geller M, Liu N, Annable R, Shay J, Kirchhoff K, Nishi N, Ando Y, Hayashi K, Hassoun H, Steinman RM, Dhodapkar MV (2005) Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *The Journal of experimental medicine* 201 (9):1503-1517. doi:10.1084/jem.20042592
161. Nicol A, Nieda M, Koezuka Y, Porcelli S, Suzuki K, Tadokoro K, Durrant S, Juji T (2000) Human invariant valpha24+ natural killer T cells activated by alpha-galactosylceramide (KRN7000) have cytotoxic anti-tumour activity through mechanisms distinct from T cells and natural killer cells. *Immunology* 99 (2):229-234
162. Bagnara D, Ibatici A, Corselli M, Sessarego N, Tenca C, De Santanna A, Mazzarello A, Daga A, Corvo R, De Rossi G, Frassoni F, Ciccone E, Fais F (2009) Adoptive immunotherapy mediated by ex vivo expanded natural killer T cells against CD1d-expressing lymphoid neoplasms. *Haematologica* 94 (7):967-974. doi:10.3324/haematol.2008.001339
163. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, Shimizu N, Horiguchi S, Okamoto Y, Fujii S, Taniguchi M, Fujisawa T, Nakayama T (2006) A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 12 (20 Pt 1):6079-6086. doi:10.1158/1078-0432.ccr-06-0114
164. Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, Hanaoka H, Shimizu N, Suzuki M, Yoshino I, Taniguchi M, Fujisawa T, Nakayama T (2009) A Phase I-II Study of α -Galactosylceramide-Pulsed IL-2/GM-CSF-Cultured Peripheral Blood Mononuclear

Cells in Patients with Advanced and Recurrent Non-Small Cell Lung Cancer. *The Journal of Immunology* 182 (4):2492-2501. doi:10.4049/jimmunol.0800126

165. Lee PT, Putnam A, Benlagha K, Teyton L, Gottlieb PA, Bendelac A (2002) Testing the NKT cell hypothesis of human IDDM pathogenesis. *The Journal of clinical investigation* 110 (6):793-800. doi:10.1172/jci15832

166. Kronenberg M (2005) Toward an understanding of NKT cell biology: progress and paradoxes. *Annual review of immunology* 23:877-900. doi:10.1146/annurev.immunol.23.021704.115742

167. Brutkiewicz RR, Bennink JR, Yewdell JW, Bendelac A (1995) TAP-independent, beta 2-microglobulin-dependent surface expression of functional mouse CD1.1. *The Journal of experimental medicine* 182 (6):1913-1919

168. Roberts TJ, Sriram V, Spence PM, Gui M, Hayakawa K, Bacik I, Bennink JR, Yewdell JW, Brutkiewicz RR (2002) Recycling CD1d molecules present endogenous antigens processed in an endocytic compartment to NKT cells. *Journal of immunology (Baltimore, Md : 1950)* 168 (11):5409-5414

169. Amin HM, McDonnell TJ, Medeiros LJ, Rassidakis GZ, Leventaki V, O'Connor SL, Keating MJ, Lai R (2003) Characterization of 4 mantle cell lymphoma cell lines. *Archives of pathology & laboratory medicine* 127 (4):424-431. doi:10.1043/0003-9985(2003)127<0424:comclc>2.0.co;2

170. East JE, Sun W, Webb TJ (2012) Artificial antigen presenting cell (aAPC) mediated activation and expansion of natural killer T cells. *Journal of visualized experiments : JoVE* (70). doi:10.3791/4333

171. Gajewski TF, Schreiber H, Fu Y-X (2013) Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* 14 (10):1014-1022. doi:10.1038/ni.2703

172. Robertson FC, Berzofsky JA, Terabe M (2014) NKT cell networks in the regulation of tumor immunity. *Frontiers in immunology* 5:543. doi:10.3389/fimmu.2014.00543

173. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L (2012) Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol* 12 (4):239-252

174. Khan MA, Sriram V, Renukaradhya GJ, Du W, Gervay-Hague J, Brutkiewicz RR (2008) Apoptosis-induced inhibition of CD1d-mediated antigen presentation: different roles for caspases and signal transduction pathways. *Immunology* 125 (1):80-90. doi:10.1111/j.1365-2567.2008.02823.x

175. Manning J, Indrova M, Lubyova B, Pribylova H, Bieblova J, Hejnar J, Simova J, Jandlova T, Bubenik J, Reinis M (2008) Induction of MHC class I molecule cell surface expression and epigenetic activation of antigen-processing machinery components in a murine model for human

papilloma virus 16-associated tumours. *Immunology* 123 (2):218-227. doi:10.1111/j.1365-2567.2007.02689.x

176. Marquard L, Gjerdrum LM, Christensen IJ, Jensen PB, Sehested M, Ralfkiaer E (2008) Prognostic significance of the therapeutic targets histone deacetylase 1, 2, 6 and acetylated histone H4 in cutaneous T-cell lymphoma. *Histopathology* 53 (3):267-277. doi:10.1111/j.0309-0167.2008.03109.x

177. Weichert W (2009) HDAC expression and clinical prognosis in human malignancies. *Cancer letters* 280 (2):168-176. doi:10.1016/j.canlet.2008.10.047

178. Weichert W, Denkert C, Noske A, Darb-Esfahani S, Dietel M, Kalloger SE, Huntsman DG, Kobel M (2008) Expression of class I histone deacetylases indicates poor prognosis in endometrioid subtypes of ovarian and endometrial carcinomas. *Neoplasia* 10 (9):1021-1027

179. Grabiec AM, Tak PP, Reedquist KA (2011) Function of histone deacetylase inhibitors in inflammation. *Critical reviews in immunology* 31 (3):233-263

180. Choi SW, Gatzka E, Hou G, Sun Y, Whitfield J, Song Y, Oravec-Wilson K, Tawara I, Dinarello CA, Reddy P (2015) Histone deacetylase inhibition regulates inflammation and enhances Tregs after allogeneic hematopoietic cell transplantation in humans. *Blood* 125 (5):815-819. doi:10.1182/blood-2014-10-605238

181. Mishra N, Brown DR, Olorenshaw IM, Kammer GM (2001) Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-gamma gene and protein expression in lupus T cells. *Proceedings of the National Academy of Sciences of the United States of America* 98 (5):2628-2633. doi:10.1073/pnas.051507098

182. Carpenter RL, Lo HW (2014) STAT3 Target Genes Relevant to Human Cancers. *Cancers* 6 (2):897-925. doi:10.3390/cancers6020897

183. Taga K, Tosato G (1992) IL-10 inhibits human T cell proliferation and IL-2 production. *Journal of immunology* (Baltimore, Md : 1950) 148 (4):1143-1148

184. Yue P, Turkson J (2009) Targeting STAT3 in cancer: how successful are we? *Expert opinion on investigational drugs* 18 (1):45-56. doi:10.1517/13543780802565791

185. Batalo M, Bose P, Holkova B, Grant S (2014) Targeting Mantle Cell Lymphoma with a Strategy of Combined Proteasome and Histone Deacetylase Inhibition. In: Dou PQ (ed) *Resistance to Proteasome Inhibitors in Cancer: Molecular Mechanisms and Strategies to Overcome Resistance*. Springer International Publishing, Cham, pp 149-179. doi:10.1007/978-3-319-06752-0_6

186. Ozawa H, Aiba S, Nakagawa, Tagami H (1996) Interferon-gamma and interleukin-10 inhibit antigen presentation by Langerhans cells for T helper type 1 cells by suppressing their

CD80 (B7-1) expression. *European journal of immunology* 26 (3):648-652.
doi:10.1002/eji.1830260321

187. Mittal SK, Roche PA (2015) Suppression of antigen presentation by IL-10. *Current opinion in immunology* 34:22-27. doi:10.1016/j.coi.2014.12.009

188. Curtale G, Mirolo M, Renzi TA, Rossato M, Bazzoni F, Locati M (2013) Negative regulation of Toll-like receptor 4 signaling by IL-10-dependent microRNA-146b. *Proceedings of the National Academy of Sciences of the United States of America* 110 (28):11499-11504. doi:10.1073/pnas.1219852110

189. Rossjohn J, Pellicci DG, Patel O, Gapin L, Godfrey DI (2012) Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol* 12 (12):845-857. doi:10.1038/nri3328

190. Moody DB (2006) TLR gateways to CD1 function. *Nat Immunol* 7 (8):811-817

191. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, 3rd, Zhou D, Saint-Mezard P, Wang V, Gao Y, Yin N, Hoebe K, Schneewind O, Walker D, Beutler B, Teyton L, Savage PB, Bendelac A (2005) Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434 (7032):525-529. doi:10.1038/nature03408

192. Chan LL, Cheung BK, Li JC, Lau AS (2010) A role for STAT3 and cathepsin S in IL-10 down-regulation of IFN-gamma-induced MHC class II molecule on primary human blood macrophages. *Journal of leukocyte biology* 88 (2):303-311. doi:10.1189/jlb.1009659

193. Riese RJ, Shi GP, Villadangos J, Stetson D, Driessen C, Lennon-Dumenil AM, Chu CL, Naumov Y, Behar SM, Ploegh H, Locksley R, Chapman HA (2001) Regulation of CD1 function and NK1.1(+) T cell selection and maturation by cathepsin S. *Immunity* 15 (6):909-919

194. Thibodeau J, Bourgeois-Daigneault MC, Huppe G, Tremblay J, Aumont A, Houde M, Barte E, Brunet A, Gauvreau ME, de Gassart A, Gatti E, Baril M, Cloutier M, Bontron S, Fruh K, Lamarre D, Steimle V (2008) Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. *European journal of immunology* 38 (5):1225-1230. doi:10.1002/eji.200737902

195. Webb TJ, Li X, Giuntoli RL, 2nd, Lopez PH, Heuser C, Schnaar RL, Tsuji M, Kurts C, Oelke M, Schneck JP (2012) Molecular identification of GD3 as a suppressor of the innate immune response in ovarian cancer. *Cancer research* 72 (15):3744-3752. doi:10.1158/0008-5472.can-11-2695

196. Chung TW, Choi HJ, Lee YC, Kim CH (2005) Molecular mechanism for transcriptional activation of ganglioside GM3 synthase and its function in differentiation of HL-60 cells. *Glycobiology* 15 (3):233-244. doi:10.1093/glycob/cwh156

197. Mayo LD, Kessler KM, Pincheira R, Warren RS, Donner DB (2001) Vascular endothelial cell growth factor activates CRE-binding protein by signaling through the KDR receptor tyrosine kinase. *The Journal of biological chemistry* 276 (27):25184-25189. doi:10.1074/jbc.M102932200
198. Webb TJ, Giuntoli RL, 2nd, Rogers O, Schneck J, Oelke M (2008) Ascites specific inhibition of CD1d-mediated activation of natural killer T cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* 14 (23):7652-7658. doi:10.1158/1078-0432.ccr-08-1468
199. Takahashi K, Saga Y, Mizukami H, Takei Y, Urabe M, Kume A, Suzuki M, Ozawa K (2011) Development of a mouse model for lymph node metastasis with endometrial cancer. *Cancer science* 102 (12):2272-2277. doi:10.1111/j.1349-7006.2011.02099.x
200. Watanabe Y, Shibata K, Kikkawa F, Kajiyama H, Ino K, Hattori A, Tsujimoto M, Mizutani S (2003) Adipocyte-derived leucine aminopeptidase suppresses angiogenesis in human endometrial carcinoma via renin-angiotensin system. *Clinical cancer research : an official journal of the American Association for Cancer Research* 9 (17):6497-6503
201. Yu X, Zhu J, Mi M, Chen W, Pan Q, Wei M (2012) Anti-angiogenic genistein inhibits VEGF-induced endothelial cell activation by decreasing PTK activity and MAPK activation. *Medical oncology (Northwood, London, England)* 29 (1):349-357. doi:10.1007/s12032-010-9770-2
202. Azuma Y, Sato H, Higai K, Matsumoto K (2007) Enhanced expression of membrane-associated sialidase Neu3 decreases GD3 and increases GM3 on the surface of Jurkat cells during etoposide-induced apoptosis. *Biological & pharmaceutical bulletin* 30 (9):1680-1684
203. Bamias A, Koutsoukou V, Terpos E, Tsiatas ML, Liakos C, Tsitsilonis O, Rodolakis A, Voulgaris Z, Vlahos G, Papageorgiou T, Papatheodoridis G, Archimandritis A, Antsaklis A, Dimopoulos MA (2008) Correlation of NK T-like CD3+CD56+ cells and CD4+CD25+(hi) regulatory T cells with VEGF and TNFalpha in ascites from advanced ovarian cancer: Association with platinum resistance and prognosis in patients receiving first-line, platinum-based chemotherapy. *Gynecologic oncology* 108 (2):421-427. doi:10.1016/j.ygyno.2007.10.018
204. Mustea A, Konsgen D, Braicu EI, Pirvulescu C, Sun P, Sofroni D, Lichtenegger W, Sehouli J (2006) Expression of IL-10 in patients with ovarian carcinoma. *Anticancer research* 26 (2c):1715-1718
205. Giuntoli RL, 2nd, Webb TJ, Zoso A, Rogers O, Diaz-Montes TP, Bristow RE, Oelke M (2009) Ovarian cancer-associated ascites demonstrates altered immune environment: implications for antitumor immunity. *Anticancer research* 29 (8):2875-2884
206. Agostino NM, Saraceni C, Kincaid H, Shi W, Nevala WK, Markovic S, Nair SG (2015) A prospective evaluation of the role of Vascular Endothelial Growth Factor (VEGF) and the immune system in stage III/IV melanoma. *SpringerPlus* 4:186. doi:10.1186/s40064-015-0951-5

207. Farsaci B, Donahue RN, Coplin MA, Grenga I, Lepone LM, Molinolo AA, Hodge JW (2014) Immune consequences of decreasing tumor vasculature with antiangiogenic tyrosine kinase inhibitors in combination with therapeutic vaccines. *Cancer immunology research* 2 (11):1090-1102. doi:10.1158/2326-6066.cir-14-0076
208. Gialitakis M, Kretsovali A, Spilianakis C, Kravariti L, Mages J, Hoffmann R, Hatzopoulos AK, Papamatheakis J (2006) Coordinated changes of histone modifications and HDAC mobilization regulate the induction of MHC class II genes by Trichostatin A. *Nucleic acids research* 34 (3):765-772. doi:10.1093/nar/gkj462
209. Cycon KA, Mulvaney K, Rimsza LM, Persky D, Murphy SP (2013) Histone deacetylase inhibitors activate CIITA and MHC class II antigen expression in diffuse large B-cell lymphoma. *Immunology* 140 (2):259-272. doi:10.1111/imm.12136
210. Licciardi PV, Karagiannis TC (2012) Regulation of immune responses by histone deacetylase inhibitors. *ISRN hematology* 2012:690901. doi:10.5402/2012/690901
211. Sullivan BA, Nagarajan NA, Wingender G, Wang J, Scott I, Tsuji M, Franck RW, Porcelli SA, Zajonc DM, Kronenberg M (2010) Mechanisms for glycolipid antigen-driven cytokine polarization by Valpha14i NKT cells. *Journal of immunology (Baltimore, Md : 1950)* 184 (1):141-153. doi:10.4049/jimmunol.0902880
212. Moody B (2007) *T Cell Activation by CD1 and Lipid Antigens: 314 (Current Topics in Microbiology and Immunology)*. 1st edn. Springer Berlin Heidelberg,
213. Wherry EJ (2011) T cell exhaustion. *Nat Immunol* 12 (6):492-499
214. Wherry EJ, Kurachi M (2015) Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 15 (8):486-499. doi:10.1038/nri3862
215. Mohammed H, D'Santos C, Serandour AA, Ali HR, Brown GD, Atkins A, Rueda OM, Holmes KA, Theodorou V, Robinson JL, Zwart W, Saadi A, Ross-Innes CS, Chin SF, Menon S, Stingl J, Palmieri C, Caldas C, Carroll JS (2013) Endogenous purification reveals GREB1 as a key estrogen receptor regulatory factor. *Cell reports* 3 (2):342-349. doi:10.1016/j.celrep.2013.01.010
216. Sohn S, Tiper I, Japp E, Sun W, Tkaczuk K, Webb TJ (2014) Development of a qPCR method to rapidly assess the function of NKT cells. *Journal of immunological methods* 407:82-89. doi:10.1016/j.jim.2014.03.026
217. Hughes JR, Roberts N, McGowan S, Hay D, Giannoulatou E, Lynch M, De Gobbi M, Taylor S, Gibbons R, Higgs DR (2014) Analysis of hundreds of cis-regulatory landscapes at high resolution in a single, high-throughput experiment. *Nature genetics* 46 (2):205-212. doi:10.1038/ng.2871

218. Rusk N (2014) Genomics: Capturing promoter-enhancer interactions in high throughput. *Nat Meth* 11 (3):231-231. doi:10.1038/nmeth.2874
219. Vallania F, Schiavone D, Dewilde S, Pupo E, Garbay S, Calogero R, Pontoglio M, Provero P, Poli V (2009) Genome-wide discovery of functional transcription factor binding sites by comparative genomics: The case of Stat3. *Proceedings of the National Academy of Sciences* 106 (13):5117-5122. doi:10.1073/pnas.0900473106
220. Scuto A, Kirschbaum M, Buettner R, Kujawski M, Cermak JM, Atadja P, Jove R (2013) SIRT1 activation enhances HDAC inhibition-mediated upregulation of GADD45G by repressing the binding of NF-kappaB/STAT3 complex to its promoter in malignant lymphoid cells. *Cell death & disease* 4:e635. doi:10.1038/cddis.2013.159
221. Iyer AK, Liu J, Gallo RM, Kaplan MH, Brutkiewicz RR (2015) STAT3 promotes CD1d-mediated lipid antigen presentation by regulating a critical gene in glycosphingolipid biosynthesis. *Immunology* 146 (3):444-455. doi:10.1111/imm.12521
222. Beliakova-Bethell N, Zhang JX, Singhanian A, Lee V, Terry VH, Richman DD, Spina CA, Woelk CH (2013) Suberoylanilide hydroxamic acid induces limited changes in the transcriptome of primary CD4(+) T cells. *AIDS (London, England)* 27 (1):29-37. doi:10.1097/QAD.0b013e32835b3e26
223. Akimova T, Beier UH, Liu Y, Wang L, Hancock WW (2012) Histone/protein deacetylases and T-cell immune responses. *Blood* 119 (11):2443-2451. doi:10.1182/blood-2011-10-292003
224. Rossi LE, Avila DE, Spallanzani RG, Ziblat A, Fuertes MB, Lapyckyj L, Croci DO, Rabinovich GA, Domaica CI, Zwirner NW (2012) Histone deacetylase inhibitors impair NK cell viability and effector functions through inhibition of activation and receptor expression. *Journal of Leukocyte Biology* 91 (2):321-331. doi:<http://dx.doi.org/10.1189/jlb.0711339>
225. Alvarez-Breckenridge CA, Yu J, Price R, Wei M, Wang Y, Nowicki MO, Ha YP, Bergin S, Hwang C, Fernandez SA, Kaur B, Caligiuri MA, Chiocca EA (2012) The histone deacetylase inhibitor valproic acid lessens NK cell action against oncolytic virus-infected glioblastoma cells by inhibition of STAT5/T-BET signaling and generation of gamma interferon. *Journal of Virology* 86 (8):4566-4577. doi:<http://dx.doi.org/10.1128/JVI.05545-11>
226. Suzuki Y, Yanagisawa M, Ariga T, Yu RK (2011) Histone acetylation-mediated glycosyltransferase gene regulation in mouse brain during development. *Journal of neurochemistry* 116 (5):874-880. doi:10.1111/j.1471-4159.2010.07042.x
227. Taki T, Hirabayashi Y, Kondo R, Matsumoto M, Kojima K (1979) Effect of butyrate on glycolipid metabolism of two cell types of rat ascites hepatomas with different ganglioside biosynthesis. *Journal of biochemistry* 86 (5):1395-1402

228. Datti A, Dennis JW (1993) Regulation of UDP-GlcNAc:Gal beta 1-3GalNAc-R beta 1-6-N-acetylglucosaminyltransferase (GlcNAc to GalNAc) in Chinese hamster ovary cells. *The Journal of biological chemistry* 268 (8):5409-5416
229. Tsai Y-T, Yu RK (2014) Epigenetic activation of mouse ganglioside synthase genes: implications for neurogenesis. *Journal of neurochemistry* 128 (1):101-110. doi:10.1111/jnc.12456
230. Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW, Kim KW (2001) Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nature medicine* 7 (4):437-443. doi:10.1038/86507
231. Kim SH, Jeong JW, Park JA, Lee JW, Seo JH, Jung BK, Bae MK, Kim KW (2007) Regulation of the HIF-1alpha stability by histone deacetylases. *Oncology reports* 17 (3):647-651
232. Qian DZ, Kato Y, Shabbeer S, Wei Y, Verheul HM, Salumbides B, Sanni T, Atadja P, Pili R (2006) Targeting tumor angiogenesis with histone deacetylase inhibitors: the hydroxamic acid derivative LBH589. *Clinical cancer research : an official journal of the American Association for Cancer Research* 12 (2):634-642. doi:10.1158/1078-0432.ccr-05-1132