2014

Immunotherapeutic options for the treatment of neuroblastoma: an analysis of natural killer cell and gamma delta T cell based immunotherapy

Bixby, Catherine Elizabeth

http://hdl.handle.net/2144/14691

Boston University
IMMUNOTHERAPEUTIC OPTIONS FOR THE TREATMENT OF NEUROBLASTOMA: AN ANALYSIS OF NATURAL KILLER CELL AND GAMMA DELTA T CELL BASED IMMUNOTHERAPY

by

CATHERINE E. BIXBY
B.A., Fordham University, 2008
M.S., University of Hartford, 2011

Submitted in partial fulfillment of the requirements for the degree of Master of Science 2014
Approved By

First Reader

Deborah Stearns-Kurosawa, Ph.D.
Associate Professor of Pathology and Laboratory Medicine

Second Reader

Susan Winandy, Ph.D.
Nancy L.R. Bucher Assistant Professor of Pathology and Laboratory Medicine
ACKNOWLEDGMENTS

I would like to acknowledge and sincerely thank those you helped me complete my master’s thesis. Specifically, my first and second readers, Dr. Deborah Stearns Kurosawa and Dr. Susan Winandy for helping me formulate, edit, revise, and structure my thesis. I would also like to thank Dr. Theresa Davies for answering all questions that came up during the process of formulating and submitting my thesis as well as my family for offering their love and support.
IMMUNOTHERAPEUTIC OPTIONS FOR THE TREATMENT OF NEUROBLASTOMA: AN ANALYSIS OF NATURAL KILLER CELL AND GAMMA DELTA T CELL BASED IMMUNOTHERAPY

CATHERINE E BIXBY

ABSTRACT

Neuroblastoma is an aggressive solid tumor that develops from immature cells of the nervous system and is almost exclusively diagnosed in infants and young children. Over the past decade a multitude of immune based therapies have been explored as therapeutic candidates for patients with neuroblastoma. The anti-GD2 monoclonal antibody, 3F8, and more recently, natural kill (NK) cell based therapies have been accepted as hopeful therapeutic options for patients with Neuroblastoma. These options however have many drawbacks including dose limiting pain, the development of tolerance, reliance on MHC mismatch and possible reliance on the invariant NK (iNK) cells population.

γδ T cells, a subpopulation of T cells composed of a T cell receptor (TCR) with a γ and a δ chain instead of an α and a β chain, have been shown to recruit a more robust immune response then both 3F8 and NK cells through their activation of antigen presenting cells (APCs) and non-reliance on MHC mismatch. γδ T cells are also able to recruit NK cells as well as other cytotoxic lymphocytes. For these reasons, it is believed that γδ T cell based treatment alone or in combination with an anti-GD2 monoclonal antibody may have a greater efficacy than either NK cells or an anti-GD2 monoclonal antibody alone. The intent of this thesis is to explore and evaluate the current state of γδ
T cell based immunotherapy against the backdrop of NK cell based immunotherapy for neuroblastoma.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE</td>
<td>i</td>
</tr>
<tr>
<td>COPYRIGHT PAGE</td>
<td>ii</td>
</tr>
<tr>
<td>READER APPROVAL PAGE</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunotherapy and Neuroblastoma</td>
<td>1</td>
</tr>
<tr>
<td>Demographics</td>
<td>2</td>
</tr>
<tr>
<td>Classification</td>
<td>4</td>
</tr>
<tr>
<td>Signs, Symptoms, Differential Diagnosis</td>
<td>5</td>
</tr>
<tr>
<td>Current Immunotherapy Treatment Regimens for Neuroblastoma</td>
<td>6</td>
</tr>
<tr>
<td>Background of Natural Killer Cells</td>
<td>10</td>
</tr>
<tr>
<td>γδ T cells</td>
<td>15</td>
</tr>
<tr>
<td>PUBLISHED STUDIES</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>General Observations-Actions of γδ T Cells Leading to their Investigation as an Anti-Tumor Immunotherapeutic Option</td>
<td>23</td>
</tr>
<tr>
<td>Can γδ T Cells be Manipulated for Immunotherapy?</td>
<td>28</td>
</tr>
<tr>
<td>Activation and Expansion of γδ T Cells through Phosphonates and Phosphorylated Structures</td>
<td>32</td>
</tr>
</tbody>
</table>
Selection of the Vγ1 γδ T Cell Phenotype for Immunotherapy.................37

Inconsistencies in NK Cell Mediated Immunotherapy.........................39

Clinical Trials Investigating γδ T Cell Based Immunotherapy and the Proposed Advantages and Disadvantages of γδ T Cell Immunotherapy...............45

CONCLUSION.................................................................48

LIST OF JOURNAL ABBREVIATIONS........................................51

REFERENCES.................................................................54

CURRICULUM VITAE..........................................................64
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Comparison of Original and New Classification Schemes</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Most Prevalent Cytokines Released by γδ T cells</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>The Advantages and Disadvantages of NK cell and γδ T Cell Immunotherapy</td>
<td>48</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody Dependent Cellular Cytotoxicity</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
<td></td>
</tr>
<tr>
<td>BrHPP</td>
<td>Bromohydrin Pyrophosphate</td>
<td></td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
<td></td>
</tr>
<tr>
<td>COG</td>
<td>Children’s Oncology Group</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
<td></td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-Lymphocytes</td>
<td></td>
</tr>
<tr>
<td>DAMPS</td>
<td>Damage-Associated Molecular Patterns</td>
<td></td>
</tr>
<tr>
<td>DMAPP</td>
<td>Dimethylallyl Pyrophosphate</td>
<td></td>
</tr>
<tr>
<td>DNAM-1</td>
<td>DNAX Accessory Molecule-1</td>
<td></td>
</tr>
<tr>
<td>Fas-L</td>
<td>Fas-Ligand</td>
<td></td>
</tr>
<tr>
<td>Fc-IL-7</td>
<td>Fc-fused IL-7 Cytokine</td>
<td></td>
</tr>
<tr>
<td>γδ</td>
<td>Gamma Delta T Cell</td>
<td></td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
<td></td>
</tr>
<tr>
<td>GVL</td>
<td>Graft Versus Leukemia</td>
<td></td>
</tr>
<tr>
<td>HAMA</td>
<td>Human Anti-Mouse Antigen</td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
<td></td>
</tr>
<tr>
<td>HMP-PP</td>
<td>Hydroxymethylpyrimidine Pyrophosphate</td>
<td></td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic Stem Cell Transplant</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule-1</td>
<td></td>
</tr>
</tbody>
</table>
IDRF.................................International Disease Risk Factors
IFN-γ.................................Interferon-γ
IL-1.................................Interleukin-1
IL-2.................................Interleukin-2
IL-4.................................Interleukin-4
IL-5.................................Interleukin-5
IL-6.................................Interleukin-6
IL-7.................................Interleukin-7
IL-8.................................Interleukin-8
IL-9.................................Interleukin-9
IL-10.................................Interleukin-10
IL-12.................................Interleukin-12
IL-12β2.................................Interleukin-12β2
IL-13.................................Interleukin-13
IL-15.................................Interleukin-15
IL-17.................................Interleukin-17
IL-18.................................Interleukin-18
IL-22.................................Interleukin-22
IL-33.................................Interleukin-33
iNK.................................Invariant Natural Killer Cells
iNOS.................................Inducible Nitric Oxide Synthase
INRG.................................International Neuroblastoma Risk Group
IPP..............................Isopentenyl Pyrophosphate
Jak3..............................Janus Kinase 3
KIR..............................Killer Immunoglobin Receptor
LAK..............................Lymphokine Activated Killer Cells
LFA-1..............................Lymphocyte Function-Associated Antigen-1
LRC..............................Leukocyte Receptor Complex
MCP-1..............................Monocyte Chemoattractant Protein-1
MHC..............................Major Histocompatibility Complex
MIBG..............................Metaiodobenzylguanidine
MIP..............................Macrophage Inflammatory Protein
MIP-1-α..............................Macrophage Inflammatory Protein-1-α
MIP-1-β..............................Macrophage Inflammatory Protein-1-β
OKT3..............................Orthoclone
PAMPS..............................Pathogen Associated Molecular Patterns
PBMC..............................Peripheral Blood Mononuclear Cells
TCR..............................T Cell Receptor
TLR..............................Toll Like Receptor
TNF-α..............................Tumor Necrosis Factor-α
TRAIL..............................TNF-related apoptosis-inducing ligand
VCAM-1..............................Vascular Cell Adhesion Molecule
VIP..............................Vasoactive Intestinal Peptide
Introduction

Neuroblastoma is an extra cranial solid tumor that develops from nervous tissues forming the sympathetic nervous system. The majority of neuroblastoma develop in the adrenal gland, with the remainder developing in other sites related to sympathetic nervous tissues\(^1\). Neuroblastoma makes up 8-10% of all childhood cancers and 15% of all cancer deaths in children with 50% occurring before the age of 2 years and 10.5 per million in children less than 15 years of age\(^1\). Neuroblastoma is more commonly seen in the United States and Europe than in other countries\(^1,2\).

Immunotherapy and Neuroblastoma

The intent of this thesis is to explore and evaluate the current state of gamma delta (\(\gamma\delta\)) T cell based immunotherapy against the backdrop of natural killer (NK) cell based immunotherapy for neuroblastoma. The idea that immunotherapy could be used in the treatment of cancer arose from Dr. Coley’s observation in 1890 that following the contraction of acute bacterial infections patient’s tumors spontaneously regressed \(^3\). Following Dr. Coley’s observation, an increased prevalence of tumors in patients with either congenital or acquired cellular immunodeficiency syndromes was reported \(^3\). The prospect of using immunotherapy to treat cancer is attractive due to the specificity and memory of the immune system enabling a longer more sustained treatment response with reduced toxicity \(^3\). The use of immunotherapy to treat neuroblastoma is particularly attractive since neuroblastoma is a disease of infants, children, and adolescents;
populations especially sensitive to the devastating long term side effects and toxicity of traditional chemotherapy treatments.

Both NK cells and $\gamma\delta$ T cells are being investigated as immunotherapeutic options for neuroblastoma. Currently, NK cell based therapy is more widely accepted, though there are many drawbacks. These include the reliance on major histocompatibility complex (MHC) mismatch and possible reliance on invariant NK (iNK) cells. Additionally, NK cells cannot produce memory cells and have a lower efficacy than $\gamma\delta$ T cells.

$\gamma\delta$ T cells have been shown to recruit a wide immune response through activation of antigen presenting cells (APCs) and are not reliant on MHC mismatch. $\gamma\delta$ T cells additionally recruit NK cells and other cytotoxic lymphocytes. For these reasons, it is believed that treatment with $\gamma\delta$ T cells can have a greater efficacy than that with NK cells.

Demographics

It was noted in a study by the Children’s Oncology Group (COG) on 3539 neuroblastoma patients from the United States that neuroblastoma was more common in Caucasian than in Black infants however this difference disappeared in older children. Despite the lower prevalence of neuroblastoma among Black infants the COG observed that a significantly higher proportion of patients with high-risk disease existed among black infants then between Caucasian, Hispanic, or Asian infants. Furthermore black children and adolescents of all ages showed a significantly worse 5 year event free survival rate and more aggressive disease. COG proposed that these differences arose from genetic
factors, specifically polymorphisms affecting the metabolism of common chemotherapeutic agents used in neuroblastoma treatment regimens, which would leave black patients more resistant to chemotherapy and at an increased risk of developing toxicities from chemotherapy \(^4\). Differences in disease presentation and course in this ethnic and racial group were not attributed to differences in healthcare accessibility since the COG group’s data did not show that tumor stage advanced with time and the status of tumor biomarkers remained stable \(^4\). The highest mortality rate is seen in patients between the ages of 17 and 18 months: 36.4% of Stage 4S and 67.1% of Stage 4 \(^1\). Given the low survival rates of advanced neuroblastoma, an effective therapy is needed to treat these patients. The demographics of the patient population primarily affected by neuroblastoma warrants that alternative forms of therapy be explored to avoid the long term devastating side effects from tradition therapies.

Unfortunately there are many challenges, including large tumor bulk in both the primary tumor and metastases and rapid proliferation capable of overwhelming a child’s under developed immune system that make the delivery of effective immunotherapy challenging \(^5\). Additionally the limited number of unique surface epitopes on neuroblastoma cells and a powerful immunosuppressive microenvironment created by the tumor makes T cell based immunotherapy exceedingly difficult \(^5\). Neuroblastoma tumors frequently manipulate the expression of MHC proteins on their surface in order to evade T cell attack and resist NK cell mediated antibody dependent cell cytotoxicity (ADCC) \(^5\). Neuroblastoma tumors also regularly release proteins capable of inhibiting both T and NK cells and recruit macrophages capable of disabling lymphocytes \(^5\). Although there are
many challenges in delivering immunotherapy to neuroblastoma tumors, the observation
that a significant number of stage 4S tumors, seen exclusively in infants, spontaneously
regress suggests that *de novo* anti-tumor immunity may occur in some patients.

**Classification**

In 2009, the International Neuroblastoma Risk Group (INRG) Project proposed a new
staging system that focused more on radiological findings rather than surgicopathological
findings (Table 1). Table 1 summarizes the previous and current classification systems.

**Table 1: Comparison of original and new classification schemes**

<table>
<thead>
<tr>
<th>Description of Original INSS Tumor Stages</th>
<th>Descriptions of New INRG Tumor Stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Stage</td>
<td>Description</td>
</tr>
<tr>
<td>1</td>
<td>Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically. Nodes attached to and removed with the primary tumor may be positive.</td>
</tr>
<tr>
<td>2A</td>
<td>Localized tumor with incomplete gross excision; representative ipsilateral nonadherant lymph nodes negative for tumor microscopically</td>
</tr>
<tr>
<td>2B</td>
<td>Localized tumor with or without complete gross excision; representative ipsilateral nonadherent lymph nodes positive for tumor; enlarged contralateral lymph nodes negative microscopically</td>
</tr>
<tr>
<td>3</td>
<td>Unresectable unilateral tumor infiltrating across the midline (beyond the opposite side of the vertebral column) with or without regional lymph node involvement, or midline tumor with bilateral extension via infiltration (unresectable) or lymph node involvement</td>
</tr>
<tr>
<td>4</td>
<td>Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/or other organs (except as defined for stage 4S disease)</td>
</tr>
<tr>
<td>4S</td>
<td>Localized primary tumor (as defined for stage 1, 2A, or 2B disease) with dissemination limited to skin, liver, and/or bone marrow (limited to infants younger than 1 year, marrow involvement of less 10% of total nucleated cells, and Iodine-123 metaiodobenzylguanidine (MIBG) scan findings negative in the marrow)</td>
</tr>
</tbody>
</table>

**Signs, Symptoms, Differential Diagnosis**

The most common clinical presentation of neuroblastoma is abdominal distention corresponding to the most common tumor site, the adrenal medulla. The abdominal mass causes swelling which may lead to abdominal pain and/or a feeling of fullness driving the patient to not eat and resulting in marked weight loss. Since neuroblastoma tumors commonly sit on the adrenal medulla they frequently cause an increase in catecholamine secretion. The increased concentration of catecholamines specifically, epinephrine, norepinephrine, and dopamine result in various symptoms associated with hyper-vigilance such as increased blood pressure, tachycardia, significant perspiration, reddening of the skin, and fever. In addition to catecholamines, neuroblastoma tumors on the adrenal medulla frequently secrete vasoactive intestinal peptide (VIP). VIP relaxes the smooth muscle of the digestive system and simultaneously promotes the secretion of water and electrolytes and intestinal motility. Patients with neuroblastoma therefore also frequently present with watery diarrhea. Other common morbidities seen in patients with neuroblastoma are Horner’s syndrome, a syndrome comprised of multiple conditions including a drooping eyelid, pupil constriction, enophthalmos, and anhidrosis all relating to a disturbance of the sympathetic nervous system, and various
paraneoplastic syndromes such as opsoclonus myoclonus syndrome characterized by rapid conjugate eye movements and involuntary muscle twitching\textsuperscript{6}. Differential diagnoses include Wilm’s tumor (in the abdomen), lymphoma (in the neck), and Ewing’s sarcoma and lymphoma (small round cell tumor in the bone) \textsuperscript{1}.

**Current Immunotherapy Treatment Regimens for Neuroblastoma**

For reasons previously explained, research has focused on developing antibody-targeted cytotoxic therapies using monoclonal antibodies directed against oncofetal antigens such as the gangliosides; GD2, GD3 and GM2, which are commonly expressed on neuroblastoma tumors rather then exploring traditional T cell, - based immunotherapies \textsuperscript{5}. Neuroblastoma cells are ideal candidates for monoclonal antibody targeting therapy since they ubiquitously express high levels of the GD2 disialoganglioside, a marker restricted to cells of neuroectodermal origin \textsuperscript{8,9,10,11}. Further evidence supporting the use of monoclonal antibodies targeting the GD2 disialoganglioside on neuroblastoma cells has been provided by Cheung et al who observed that this antigen is not downregulated at the cell surface in response to monoclonal antibody treatment \textsuperscript{11,12}. Additionally it is known that the GD2 oncofetal antigen is T cell independent, within close proximity of the neuroblastoma cell’s membrane, and expressed in high density on neuroblastoma cells \textsuperscript{5}. In addition to GD2, neuroblastoma cells also express the GD3 and GM2 disialogangliosides. Other targets include CD56, a prominent marker on neuroendocrine tumors and an essential component for neurogenesis, neuronal migration, and neurite outgrowth \textsuperscript{13}, and L1-CAM, a neuronal cell adhesion molecule \textsuperscript{8}. GD2 monoclonal antibodies conjugated to toxins, radionucleotides, or powerful growth factors such as
interleukin-2 (IL-2) serve as the cornerstones of immunotherapy for neuroblastoma since they are capable of targeting neuroblastoma cells and recruiting effector cells to carry out ADCC, activating complement, and releasing inflammatory cytokines. GD2 monoclonal antibody based treatment regimens are frequently used in patients with advanced (high-risk) neuroblastoma.

Although anti GD2 monoclonal antibodies are a standard of care in treatment regimens for neuroblastoma because they are effective at targeting and killing neuroblastoma cells they have several limitations preventing them from being an ideal therapeutic option. The GD2 antigen is not exclusively expressed by neuroblastoma cells but is expressed by cells of neuroectodermal origin. Therefore other cells of neuroectodermal origin such as neurons and peripheral pain fibers also expressing GD2 can be targeted by GD2 monoclonal antibodies resulting in bystander cell lysis, inflammation from ADCC and complement activation, as well as dose limiting pain. Dose limiting pain is thought to result both from the binding of anti-GD2 antibodies to peripheral pain fibers and from the activation of the complement system.

The effectiveness of GD2 monoclonal antibodies in treating neuroblastoma is additionally limited by the response of the patient’s immune system. The initial GD2 monoclonal antibodies were first developed using the murine 3F8 antibody. The continual administration of murine 3F8 results in the patient producing human anti-mouse antibodies (HAMA), which neutralize GD2 antibody treatment. Since GD2 antibody treatments are only successful prior to production of HAMA the less immunogenic human-mouse chimeric anti-GD2 monoclonal antibody, ch14.18 was
Although ch14.18 did not provoke the production of HAMA, it did initiate the formation of human anti-chimeric antibodies.

Recently a humanized anti GD2 monoclonal antibody, hu14.18 has been developed. Although hu14.18 is far less immunogenic then either 3F8 or ch14.18, its effectiveness depends on the strength of the patient’s immune system and the ability of the immune system to signal a powerful effector response. Anti-GD2 monoclonal antibodies are not effective enough when used alone to fight Neuroblastoma and thus must be integrated into the third and final maintenance stage of a patient’s treatment protocol. Prior to receiving anti-GD2 monoclonal antibodies the patient must undergo induction chemotherapy and possibly a hematopoietic stem cell transplant (HSCT). Cyclophosphamide, a cytotoxic drug that induces immunodeficiency, in addition to other cytotoxic agents, is the backbone of induction chemotherapy for Neuroblastoma. In addition to cyclophosphamide patients requiring a HSCT must receive myeloablative chemotherapy in preparation for an autologous HSCT rescue. The treatment patients receive prior to the administration of anti-GD2 monoclonal antibodies is chemotoxic to all elements within the bone marrow including immune-competent white blood cells. Patient’s immune systems are suppressed not only by numerous drugs used in the first two stages: induction and consolidation (HSCT) of treatment, but also by the stress imposed by various supportive measures that leave patients highly susceptible to infections: intravascular catheters, ports, prolonged hospital stays, multiple intravascular procedures, and mucosal lesions arising from induction chemotherapy. Since patients with neuroblastoma are immunocompromised the strength and effectiveness of anti-GD2
monoclonal antibody treatment is reduced\textsuperscript{14}. This may explain why a study conducted by Shusterman et al. and the Children’s Oncology Group found that hu14.18 treatment only provided bone marrow remission in patients with minimal disease\textsuperscript{14}. In order to activate leukocytes in immune-compromised patients to carry out a powerful ADCC killing attack of neuroblastoma cells upon anti-GD2 antibody infusion, anti-GD2 antibodies have been combined with interleukin-2 (IL-2)\textsuperscript{5}. IL-2 stimulates T cells, NK cells, and NK T cells thus producing a more robust immune response against neuroblastoma tumors in immune-compromised patients. However in several studies, most notably Shusterman et al. and Yu et al., infusion of IL-2 resulted in grade 3 or 4 capillary leak and abnormal liver function tests\textsuperscript{14,18}. The immunocompromised state of the patient and dose limiting pain from anti-GD2 monoclonal antibody based treatments remain significant barriers hindering this therapy’s efficacy and potential\textsuperscript{5,14}.

It is possible however for anti-GD2 monoclonal antibodies administered in conjunction with either NK or γδ T cells to mount an attack strong enough to eliminate the need for induction chemotherapy. In a study conducted by Tarek et al. the effect of administering the anti-GD2 monoclonal antibody, 3F8, in conjunction with either NK cells or chemotherapy was measured\textsuperscript{19}. Tarek et al. found that patients who received NK cells following 3F8 administration lived on average 32-68 months longer then patients receiving chemotherapy and 3F8 (50 – 114 months verses 18 – 51 months)\textsuperscript{19}. The prolonged survival period of patients receiving NK cells following treatment with 3F8 was attributed to the removal of chemotherapy from the treatment protocol, which reduced the degree of immunosuppression and made it easier for the patient’s immune
system to launch a powerful effector response against neuroblastoma cells. NK cells exhibit a redundant activation pattern when administered with anti-GD2 monoclonal antibodies thus enabling NK cells, along with 3F8, to launch an effector response strong enough to eliminate the need for induction chemotherapy. NK cells are activated by the 3F8 antibody through the CD16 marker expressed on their surface and by neuroblastoma cells through their natural cytotoxicity receptors: NKp30, NKp44, NKp46, and the DNAX accessory molecule-1 (DNAM-1) adhesion receptor.19

Background of Natural Killer Cells

NK cells are lymphoid cells vital to the innate immune response and involved in the adaptor immune response. They are actively involved in the destruction of virally infected and malignant cells. Similar to B-lymphocytes, T lymphocytes, and dendritic cells, they arise from the Common Lymphoid Precursor in the bone marrow.12 They then settle in hematopoietic tissue and peripheral organs lacking B and T lymphocyte markings.12 The NK cell population is defined by the absence of the CD3 marker and presence of either/or the CD56 or CD16 marker.20 The five known populations of NK cells are: CD56dimCD16bright, CD56brightCD16dim, CD56brightCD16−, CD56dimCD16−, and CD56CD16bright.20 The presence or absence of the CD16 marker on the NK cell surface serves as an indication of the NK cell’s principle function. CD16dim or CD16− NK cells typically express the lectin based activating receptors, NKG2A/CD94 and NKG2C/CD94, instead of the CD16 marker or the killer immunoglobulin receptors (KIRs).20 The lectin based activating receptors bind to HLA-E molecules triggering an innate immune response.20 Binding of NKG2A/CD94 and NKG2C/CD94 to HLA-E
molecules additionally enables NK cells to monitor the expression levels of other HLA molecules whose expression levels serve as indicators of disease \(^{20}\). The CD16\(^{\text{dim}}\) and CD16\(^{-}\) NK cells also express the chemokine receptors CCR7, involved in B and T lymphocyte activation, and CXCR3, involved in Th\(_1\) cell migration to the cytoskeleton and the activation of integrin \(^{20}\). CD16\(^{\text{bright}}\) NK cells are involved in ADCC, express KIRs, and migrate to sites of inflammation \(^{20}\). The CD16\(^{\text{dim}}\) and CD16\(^{-}\) NK cells, involved primarily in immune regulation, reside in the tonsils and lymph nodes while the CD16\(^{\text{bright}}\) NK cells reside principally in the peripheral blood and spleen \(^{20}\).

In order for immature NK cell precursors in the bone marrow to mature into fully functional NK cells only the cytokine, interleukin-15 (IL-15) must be present \(^{12}\). Unlike B and T lymphocytes, NK cell maturation does not require the recombination of genes encoding immunoglobulins or the T cell receptor \(^{12}\). NK cells additionally differ from B and T lymphocytes in that they do not require previous antigen exposure or antigen presentation through the MHC to recognize and attack infectious cells \(^{12}\). NK cells are instead activated or inhibited through their surface activating and inhibiting receptors that respond to a variety of ligands and chemical signals. The presence of activating ligands and chemical signals on a tumor cell’s surface fluctuates depending on the state of the tumor cell’s environment and degree of DNA damage \(^{21}\). Significant DNA damage and a stressful microenvironment can cause a tumor cell to upregulate NK receptor ligands leading to the increased activation of NK cells bearing the corresponding activating receptors \(^{21}\). NK cells respond to activating signals by either releasing cytoplasmic granules of perforin and granzyme, expressing Fas-ligand (Fas-L) or TNF-related
apoptosis inducing ligand (TRAIL) to induced death receptor mediated apoptosis, carrying out ADCC through their CD16 surface marker, or producing large quantities of cytokines, most notably, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), and interleukin-10 (IL-10) \(^2\)(23). IFN-γ activates CD8\(^+\) T cells, initiating their transformation into cytotoxic (CTL) T cells and slows tumor growth through signaling to angiostatic chemokines \(^2\). IL-8 attracts neutrophils and IL-10 enhances the survival, proliferation, and antibody production of B lymphocytes \(^2\). NK cells are also capable of secreting cytokines necessary for the growth and development of specific hematopoietic cell lineages such as, granulocyte-macrophage colony stimulating factor (GM-CSF) \(^2\). Thus NK cell activation and cytokine secretion results in the development and activation of CTL T cells along with other immune effector cells for a potent immune response.

Since NK cells carry both activating and inhibiting receptors on their surface they are able to activate and enhance as well as dampen the immune system. The KIR family represents the largest family of activating and inhibiting receptors on NK cells \(^2\). The genes, located on the long arm of chromosome 19 (19q13.4) within the leukocyte receptor complex (LRC), controlling expression of KIRs on NK cells are highly polymorphic at both the haplotype and allelic level \(^2\). Inhibiting and activating KIR receptors, although structurally similar on their extracellular surface, differ structurally on their cytoplasmic surface \(^2\). On the cytoplasmic surface both activating and inhibiting receptors contain immunoreceptor tyrosine based motifs \(^2\). Upon ligand recognition, the inhibitory motif on the inhibiting KIR receptors associates with SHP-1, SHP-2, and SHP
phosphatases leading to the inhibition of phosphorylation and blockage of the cellular
cascade necessary for NK cell activation. The immunoreceptor tyrosine based motif on
activating KIR receptors instead associates with the transmembrane adaptor protein,
DAP10 upon ligand recognition, which results in activation of the P13 kinase pathway
leading to the activation of NK cells. The activation of NK cells results in perforin and
granzyme mediated cell lysis.

The KIR inhibiting receptors on NK cells recognize and respond to MHC I, which
is expressed constitutently on the surface of healthy cells. Since the genes responsible
for encoding the NK cell’s KIR inhibiting receptors are located on chromosome 19 and
segregate independently from the genes encoding MHC I on chromosome 6, it is possible
for an individual to express an inhibiting KIR receptor but not the receptor’s
complementary ligand. The probability of this occurring is increased given that the
decision of which encoded inhibiting KIRs to express is random. It is also possible for
an individual NK cell to express multiple different inhibiting KIR subtypes each with
different ligand specificities.

The NKG2D receptor is the primary activating receptor expressed by all NK cells
and the majority of γδ and CD8+ T cells. The ligands for the NKG2D receptor are
either of MHC I homology (MICA, MICB, and ULBP-4) or glycoprophatidylinositol
anchored proteins (ULBP-1, ULBP-2, and ULBP-3). In contrast to ligands for the
inhibiting KIRs, the NKG2D receptor ligands are expressed almost exclusively on
unhealthy cells and have been found on a variety of carcinomas including (but not limited
to): breast, lung, ovary, brain, and kidney.
The ability of NK cells to elicit an effector response against a target cell is dependent on the licensing status of the NK cell. Although the exact time point and location of NK licensing remains unknown, it is understood that NK cell licensing has a significant protective function by ensuring that only NK cells capable of distinguishing self from non-self MHC ligands are able to provoke an effector response\textsuperscript{26}. If an NK cell’s inhibiting KIR is unable to recognize and interact with self-MHC ligands then the cell is unlicensed and thus inert\textsuperscript{26}. Only after the NK cell’s inhibiting KIR engages with a self-MHC ligand can its activating receptor respond to incoming stimuli by producing and releasing INF-\(\gamma\), TNF-\(\alpha\), IL-8, IL-10, perforin and granzyme\textsuperscript{26}. The number of self-MHC ligands the NK cell’s inhibiting KIR can engage with is positively correlated with the strength of the effector response elicited by the NK cell\textsuperscript{26}. Given that an individual’s NK cells express different inhibiting KIR, different NK cells will require different MHC ligands for licensing yielding a population of both licensed and unlicensed NK cells in an individual. In addition to the ability to recognize and engage with self-MHC ligands, a NK cell’s inhibiting KIR receptor must also have an intact immunoreceptor tyrosine inhibitory motif\textsuperscript{26}.

Since NK cells carry both inhibiting and activating receptors on their surface, their response to different target cells depends upon the integration of inhibitory and activating signals from that cell\textsuperscript{22}. The target cell has the ability to either upregulate or downregulate its’ expression of inhibitory or activator ligands and this affects the NK cells’ response\textsuperscript{22}. 
The immunotherapeutic potential of NK cells lies in the highly polymorphic nature of their inhibiting KIR, which allow them to detect abnormal cells via the “missing self” hypothesis in HSCT and evoke a powerful graft versus leukemia effect (GVL). The “missing self” hypothesis was first observed by Ruggeri et al who demonstrated that the NK cell activation threshold is lowered upon the infusion of donor licensed NK cells into a recipient lacking at least one of the MHC class I ligands for the donor’s NK KIR. The reduced NK activation threshold in this patient led to a significantly more powerful NK cell mediated immune response. Several qualities of NK cells such as their short survival period, limited expansion abilities, and inability to form memory cells make them attractive candidates for immune based therapies, by allowing repeated dosing and the ability to design more patient specific therapies. These qualities however also limit the potency of an NK cell attack. Although the therapeutic use of activated NK cells to treat solid tumors has shown promise there are challenges, discussed further below, which limit their use for neuroblastoma.

γδ T cells

γδ T cells are a heterogeneous population differing structurally from αβ T cells in their T-cell receptor (TCR) element. The majority of T cells are αβ T cells with γδ T cells making up only 10% of the T-cell population. Of this 10%, 1-5% are peripheral γδ T cells residing within the circulation. The remaining γδ T cells are scattered throughout the liver, dermis, spleen, lymph nodes, and reproductive tract. The TCR of both αβ and γδ T cells exists as a heterodimer. The α-chain gene locus for the αβ T cell’s variable chain within the TCR contains both “V” and “J” genes.
while the β-chain gene locus contains “V”, “D”, and “J” genes \(^{30}\). Upon activation of VDJ recombinase, the β-chain gene locus undergoes VDJ recombination and the α-chain gene locus undergoes VJ recombination \(^{30}\). These two recombination events yield a T cell population with highly diverse TCRs \(^{30}\). Each αβ T cell TCR is unique to one particular MHC complex \(^{30}\). This high level of diversity enables the recognition of a wide range of MHC molecules.

γδ T cells differ from the more prevalent αβ T cells in that their TCR is composed of a γ chain and a δ chain \(^{8,15,30}\). The γ chain is located on a separate gene locus from the α-chain and β-chain gene loci and contains the “V” and “J” gene segments \(^{30}\). The gene locus of the δ chain, on the other hand, is located within the α chain gene locus and contains the “V”, “D”, and “J” gene segments \(^{30}\). Although the two populations have different TCRs, both α: β and γ: δ T cells express the CD3 antigen \(^{30}\).

The development of γδ T-cells in the thymus is significantly different then αβ T cell development \(^{30}\). αβ T cells require the thymus for proper development and are subjected to three stages of selection: β-selection, positive selection, and negative selection \(^{8,30}\). γδ T cells, on the other hand, are capable of developing without the thymus as indicated by various studies demonstrating that patients with DiGeorge Syndrome, which results, in thymic hypoplasia, develop fully functional γδ T cells but no αβ T cells \(^{31}\). Key differences in αβ T cell and γδ T cell development occur during β-selection in the thymus \(^{30}\). During β-selection assembly of the pre-TCR commits intrathymic precursors to the αβ T cell lineage \(^{8,30}\). These cells will subsequently undergo positive and negative selection before leaving the thymus \(^{8,30}\). γδ T cells do not undergo β-selection but instead
express a γδ TCR instead of a pre-TCR. Although it is widely believed that the decision to express a pre-TCR or γδ TCR designates whether an intrathymic precursor develops into an αβ or γδ T cell; there is some evidence that suggests high expression levels of the interleukin-7 (IL-7) receptor, independent of the expressed TCR, determines whether a precursor develops into a γδ T cell. γδ T cells, as opposed to αβ T cells, exhibit high expression levels of the IL-7 receptor.

γδ T cells are categorized based on the identity of their γ and δ variable chains. It remains unknown whether the γ and δ variable chain subtypes are determined randomly in the initial development of the γδ TCR or whether the subtypes are intentionally chosen based on the intended tissue location or function of the future γδ T cell. Two significant populations of γδ T cells are the Vγ9Vδ2+ γδ cells and the Vγ2Vδ2+ γδ cells. The majority of peripheral γδ T cells, capable of comprising up to 98% of the total circulating γδ T cell population, are Vγ9Vδ2+ cells. Although the Vγ9Vδ2+ population is more prevalent, it is the Vγ2Vδ2+ population that is believed to be most important for cellular immunity. Upon microbial infection the Vγ2Vδ2+ population expands from composing only 0.5%-5% of the peripheral γδ cell population to composing 50% of both αβ and γδ circulating T cells. The immediate expansion upon microbial infection of the Vγ2Vδ2+ population supports the belief that γδ T cells are heavily involved in cellular immunity and react to infections at a much earlier stage than αβ T cells. Unlike αβ T cells, γδ T cells are widely believed to be members of both the innate and adaptive immune system.
In order for αβ T cells to become activated and evoke an effector response, antigenic peptides must be presented by either MHC I or MHC II complexes located on the cellular surface. \(\gamma:\delta\) T cells are unique T cells in that they do not require MHC presentation of antigenic peptides for activation or to evoke an effector response. Furthermore, it is unnecessary for them to express either the CD4 or CD8 marker. Although \(\gamma:\delta\) T cells do not need to express either the CD4 or CD8 marker, these markers can be found on small subsets within the \(\gamma:\delta\) T cell population. The CD8 marker has been found on \(\gamma:\delta\) T cells following cytomegalovirus (CMV) infection, in patients with human immunodeficiency virus (HIV), and in renal transplant recipients. The majority of \(\gamma:\delta\) T cells expressing the CD8 marker reside within the gut and are identified as \(V\gamma5^+V\delta4^+\). A very small population of \(\gamma:\delta\) T cells carries the CD4 marker. This population is known to secrete increased amounts of Th1 cytokines including, IFN-\(\gamma\) and GMCSF. The population is also known to illicit antibody production and produce Th2 cytokines such as interleukin-4 (IL-4).

It is widely believed that \(\gamma:\delta\) T cells are by default Th1-like cells while CD4\(^+\) αβ T cells differentiate into either Th1 or Th2 cells. Despite this, it's been observed that a small subset of CD4\(^+\) \(\gamma:\delta\) T cells can become Th2-like cells and it is known that the presence of various pathogens and cytokines can influence whether a \(\gamma:\delta\) T cell differentiates into a Th1-like or Th2-like cell. Yin et al. observed that \(\gamma:\delta\) T cells, even when exposed to the Th2 development transcription factor, GATA3, in addition to IL-4 and IFN-\(\gamma\), differentiate into Th1-like cells and secrete interleukin-12 β2 (IL-12 β2). Yin et al. demonstrated that the majority of \(\gamma:\delta\) T cells are destined to become Th1-like.
cells and are thus heavily involved in the primary immune response. From Yin et al.’s observations it can be deduced that γδ T cells play a role in tumor immunology since Th1/Th1-like cells are more important than Th2/Th2-like cells for an antitumor immune response \textsuperscript{32}. Although both Th1/Th1-like and Th2/Th2-like cells can eradicate tumors, Th1/Th1-like cells directly attack tumor cells through the secretion of IFN-γ and generation of CTLs while Th2/Th2-like cells indirectly attack tumor cells by inducing inflammation occasionally leading to necrosis \textsuperscript{32}. The Th1/Th1-like cell mediated attack is additionally superior because it confers immunological memory and results in the secretion of perforin, TNF-α, and Fas/FasL, all highly cytotoxic agents \textsuperscript{32}. Th1/Th1-like cells additionally have strong adhesion properties through their upregulation of leukocyte functional association antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) \textsuperscript{32}.

Although Th2/Th2-like cells can kill tumor cells through promoting inflammation eventually leading to necrosis, they do not confer immunological memory specifically for CTL T cell generation and secrete large amounts of interleukin-6 (IL-6) and IL-10, both strongly associated with cachexia/wasting syndrome \textsuperscript{32}. Furthermore, the inflammation induced by the Th2/Th2-like response can promote tumor growth \textsuperscript{34}. The cytokines, chemokines, and eicanooids released by Th2/Th2-like cells stimulate the proliferation and migration of tumor cells by promoting angiogenesis \textsuperscript{34}. By attracting numerous infiltrating leukocytes, the inflammatory reaction induced by Th2/Th2-like cells helps to remodel the extracellular matrix and thus facilitates tumor cell migration \textsuperscript{34}.
γδ T cells express the chemokine receptor CCR5 and secrete inflammatory cytokines such as IFN-γ and TNF-α, which allows them to enter infected tissues and respond to infection \(^8,16,17,35\). As they mature, γδ T cells acquire characteristics of professional APCs. Some γδ T cells temporarily reduce their expression of inflammatory cytokines and instead upregulate their expression of the chemokine receptor, CCR7 which allows them to present antigens to αβ T cells \(^8,16\).

Similar to NK cells, γδ T cells respond to the stress-induced MIC proteins expressed on the surface of cells from epithelial origins \(^30\). The proteins MICA and MICB resemble MHC I proteins and act as ligands for the NKG2D receptor expressed on NK cells, CD8 αβ T cells, and γδ T cells \(^15,30\). Therefore treatment strategies seeking to activate NK cells through their NKG2D receptors can theoretically also activate γδ T cells. γδ T cells also carry the receptor, FcγR, necessary for ADCC \(^36\). The majority of monoclonal antibody targeting therapies kill tumor cells by inducing ADCC \(^36\). γδ cells could thus serve as a valuable adjuvant to monoclonal antibody targeting therapies particularly in patients with diminished NK cell activity. The ability to activate both γδ T cells and NK cells through one receptor is beneficial because it ensures that a backup population of cells will always be activated and available to evoke an anti-tumor immune response.

In addition to the expression of NKG2D, the NK activating receptor, γδ T cells are linked to the innate immune system through their expression of toll like receptors (TLRs) and ability to activate other TLRs \(^32\). The TLRs are part of the innate immune system and recognize pathogen-associated molecular patterns (PAMPS) and damage
associated molecular patterns (DAMPS) both of which are associated with tumors and carcinogenesis. TLRs respond to PAMPS and DAMPS by signaling the expression of specific genes, through the activation of signal transduction pathways, which help develop antigen specific immunity. The recognition of PAMPS and DAMPS by TLRs on γδ T cells’, results in the secretion of cytotoxic antitumor cytokines by γδ T cells. The presence of TLRs on γδ cells strengths their ability to recognize and react to tumors and supports their potential use in immune based anti-tumor therapies.

Several qualities of γδ T cells suggest that they can generate a more powerful anti-tumor immune response than NK cells, dendritic cells, and αβ T cells. Although the NK cell’s ability to illicit a powerful anti-tumor response has been recognized and harnessed in NK based therapies, Meraviglia et al. and other groups have observed a depletion in the NK cell’s ability to attack and kill tumors following monoclonal antibody therapy. In addition, a study conducted by Maniar et al. suggests that γδ cells may even be able to induce the activation of NK cells and further prompt NK cells to kill NK cell resistant tumors.

Along with the activation of NK cells, γδ T cells promote dendritic cell maturation through the secretion of TNF-α. Dendritic cells are an essential component to the adaptive immune response against tumors. They are able to cross present tumor antigens to CD8+ T cells as well as secrete interleukin-12 (IL-12), which promotes αβ T cell differentiation into anti-tumor Th1 cells. Increased dendritic cell maturation would result in a more powerful anti-tumor attack by the immune system. γδ T cell activation does not require MHC presentation of antigen peptides however when
stimulated, γδ T cells are able to both upregulate and produce MHC II molecules de novo. Dendritic cells, on the other hand when stimulated, are only able to upregulate already existing MHC II molecules. The increased presence of MHC II molecules following γδ T cell mediated de novo production and upregulation increases the activation of CD8+ T cells thus generating more CTL T cells.

The ability to produce MHC II de novo is unique to γδ T cells. In addition to prompting dendritic cell maturation and producing more MHC II molecules, γδ T cells induce the differentiation of CD8+ αβ T cells into alloreactive CTL T cells expanding the CTL T cell population. Alloreactive CTL T cells are a population of activated highly cytotoxic CD8+ T cells that recognize and attack self-antigens, including tumor antigens. γδ T cells are able to help naïve CD8+ T cells differentiate into CTL T cells by secreting interleukin-2 (IL-2). γδ T cells additionally act as APCs and are capable of simultaneously presenting tumor antigens to CD8+ T cells while secreting IL-2 to induce their differentiation to CTL T cells. γδ T cells thus produce CTL T cells directed specifically against tumor cell antigens.

γδ T cells expand the immune system’s anti tumor response by signaling clusters of CD4+ αβ T cells to proliferate and differentiate into Th1 and Th2 cells. Wu at el observed that another unique ability of γδ T cells, not observed in NK or αβ T cells, was their ability, upon licensing, to become phagocytes and serve as professional APCs. As phagocytes, γδ T cells are able to uptake and process tumor antigens to cross present them to tumor specific CD8+ αβ T cells.
Published Studies

General Observations-Actions of $\gamma\delta$ T cells leading to their investigation as an anti-tumor immunotherapeutic option

Previous experiments have shown strong reactions of $\gamma\delta$ cells with *Mycobacterium tuberculosis* $^{41,42}$, *Toxoplasma gondii* $^{43}$, and *Plasmodium falciparum* $^{44}$. More recently, linkage has been shown between $V\gamma 1^+$ $\delta$ T cells and the MHC Class I related molecules, MICA and MICB, which are expressed on cells under stress. Unexpectedly, a relationship between the NK cell activating receptor NKGD2, expressed on the $V\gamma 1^+$ $\gamma\delta$ T cells, and the MICA ligand, led to a strong suspicion that $\gamma\delta$ T cells might play a significant role in not only stress response, but tumor surveillance as well $^{45,46}$. $\gamma\delta$ T-cells can kill cells effectively through ADCC and the secretion of granzyme A, B, perforin, granulysin, Fas ligand, TRAIL, and IFN-$\gamma$ $^{47-49}$. The release of granzyme A induces cell death by inflicting damage directly to cells’ mitochondria. Granzyme A initiates the release of a large amount of reactive oxygen species and causes the mitochondria to lose its transmembrane potential. Granzyme B, on the other hand targets caspase 3 either directly or indirectly resulting in the initiation of the caspase cascade and DNA fragmentation and apoptosis of the target cell. Perforin is a protein accomplice to granzyme A and B and causes cell death by inserting itself into the target cell’s membrane. Granulysin initiates cell death by causing an increase in intracellular calcium resulting in mitochondrial depolarization, cytochrome c release, caspase activation, and eventual apoptosis. Fas and TRAIL are both ligands, which upon binding their appropriate receptors induce apoptosis. Table 2 outlines the various roles, as they relate to tumor immunology, of the most prevalent cytokines released by $\gamma\delta$ T cells.
IFN-γ is an extremely important mediator of the immune system’s antitumor response. The release of IFN-γ triggers nearby leukocytes in the peripheral blood to release several angiostatic chemokines including: IP10, Mig, and I-TAC. These chemokines, in addition to preventing tumor angiogenesis, attract other effector cells from the immune system to the tumor inducing the release of even more IFN-γ. Among the effector cells recruited are NK cells and macrophages, which release immunomodulatory cytokines such as, IL-12 and interleukin-18 (IL-18). As tumor cells are killed the antigen presentation machinery of the immune system is primed so that the adaptive immune response can be activated to help the innate immune response. The release of IFN-γ and IL-12 also induces the differentiation of CD4+ T cells into Th1 antitumor T cells and eventually CD8+ T cells capable of releasing perforin to kill tumor cells.

γδ T cells additionally secrete interleukin-13 (IL-13) after encountering neuroblastoma cells suggesting that these cells are capable of influencing the activation of lymphokine-activated cells (LAK). The population of LAK cells includes NK cells, NK T cells, and T cells. The purpose of this population is to enhance both the adaptive and innate immune system’s response to tumors. LAK cells inhibit IL-10, an immunosuppressant cytokine released by dendritic cells, and secrete large amounts of IL-8. IL-8 is a powerful chemoattractant cytokine, which recruits cells from the immune system with either the CXCR1 or CXCR2 receptor to sites of inflammation therefore expanding the immune system’s response against tumors. LAKs are furthermore able to promote dendritic cell maturation while still enabling dendritic cells to acquire
molecules, such as tumor antigens, from the external environment. The acquisition of tumor antigens, released into the external environment from dying tumors, is essential to the process of dendritic cell cross priming necessary for CD8\textsuperscript{+} T cell activation. LAKs have been associated with both the improvement of dendritic cell CTL T cell priming and the reversal of immunosuppressive environments typically surrounding tumors.

Table 2: Most Prevalent Cytokines Released by γδ T cells

<table>
<thead>
<tr>
<th>Cytokine Released</th>
<th>Action</th>
</tr>
</thead>
</table>
| Interleukin-1 (IL-1) | Inflammation, enhancement of macrophage response, activation of endothelial cells resulting in the upregulation of:  
  1. ICAM-1/CD54: intercellular adhesion molecule that allows activated leukocytes with integrin/LFA-1 on their surface to transmigrate into tissues  
  2. Vascular cellular adhesion molecule-1 (VCAM-1)/CD106: expressed by endothelial cells on blood vessels following cytokine release. It is responsible for enabling the adhesion of lymphocytes, basophils, monocytes, and eosinophils to vascular endothelium.  
  3. E-selectin/CD62: a cell adhesion molecule expressed on endothelial cells upon endothelial cell activation through cytokine secretion. E-selectin recruits leukocytes in inflammation and helps tumor cells adhere to endothelial cells. The presence of increased E-selectin is associated with tumor metastases.  
  4. ICAM-2/CD102: intercellular adhesion molecule similar in function to ICAM-1,  
  5. IL-8: chemokine and neutrophil chemotactic factor. Signals chemotaxis for neutrophils and other granulocytes while inducing phagocytosis and promoting angiogenesis.  
  6. Monocyte chemotactic protein-1 (MCP-1)/ CCL2: a cytokine that recruits monocytes, memory T cells, and dendritic cells to sites of inflammation. (see text for more details) |
| IL-2 | Growth factor for T-cells, promotes differentiation of pCTL, immature cytotoxic T lymphocytes unable to perform cytotoxic functions into fully functional CTL T cells, enhances NK Cell cytotoxicity |
| IL-4 | Promotion of differentiation of mature naïve Th0 cells into Th2 cells. Th2 cells release IL-4, interleukin-5 (IL-5), IL-6, and IL-13. Th2 cells also signal to eosinophils, basophils, mast cells, as well as IgE and promote the release of histamine. Th2 cells are associated with allergy and inflammation. IL-4 also stimulated activated B-cells. |
| IL-5 | Hematopoiesis, chemotaxis, and activation of eosinophils |
| IL-6 | Promotes the development of Th17 cells and production of C-Reactive Protein (CRP). Th17 cells are helper T cells which produce primarily interleukin-17 (IL-17) but also: IL-2, interleukin-9 (IL-9), interleukin-22 (IL-22), GM-CSF, IFN-γ, tumor necrosis factor (TNF) [2#] IL-9 is a cytokine responsible for preventing apoptosis and instead stimulating cell |
proliferation. IL-22 is involved in the innate immune response and a powerful mediator of the inflammatory response. Th17 cells are associated with tissue inflammation and autoimmune diseases. They are suspected to contribute indirectly to antitumor immunity by recruiting T cells, DC cells, and trafficking NK cells. Th17 cells have however also been associated with supporting the tumor’s microenvironment (2#).

<table>
<thead>
<tr>
<th>Interleukin-7 (IL-7)</th>
<th>Hematopoiesis, B and T-cell differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>Chemotactic for neutrophils</td>
</tr>
<tr>
<td>IL-12</td>
<td>Promote NK cell secretion of IFN-γ</td>
</tr>
<tr>
<td>IL-13</td>
<td>Activation LAK</td>
</tr>
<tr>
<td>Interleukin-15 (IL-15)</td>
<td>Recruits NK cells, promotes the proliferation of NK cells, influences NK cell development, and activates NK cells. IL-15 signaling also results in the upregulation of the NKGD2 receptor, maintenance of the memory T cell population, and prevention of apoptosis. In addition to NK cell development, IL-15 promotes the development of macrophages and dendritic cells</td>
</tr>
<tr>
<td>IL-17</td>
<td>Release of pro-inflammatory chemokines and cytokines including: IL-6, IL-8, GM-CSF, granulocyte colony stimulating factor (G-CSF), and metalloproteases. IL-17 is also a chemoattractant for neutrophils.</td>
</tr>
<tr>
<td>Interleukin-33 (IL-33)</td>
<td>Activation of mast cells</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Chemotactic for monocytes</td>
</tr>
<tr>
<td>Macrophage inflammatory protein-1β (MIP-1β)</td>
<td>Chemotactic cytokine also referred to as CCL3/ macrophage inflammatory protein-1α (MIP-1α form) and CCL4 (MIP-1β form). The release of macrophage inflammatory protein (MIP) from monocytes activated by bacterial endotoxins results in synthesis and release of pro-inflammatory cytokines: IL-1, IL-6, and TNF-α. Secretion of MIP-1β also results in the activation of granulocytes.</td>
</tr>
<tr>
<td>G-CSF &amp; GM-CSF</td>
<td>Granulocyte and macrophage colony stimulating factor</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Enhances NADPH Oxidase and activates inducible nitric oxide synthase (iNOS). The activation of iNOS results in the production of nitric oxide and a surge of proinflammatory cytokines including: IL-1, TNFα, and more IFN-γ. IFN-γ has powerful anti-tumor properties through its signaling to angiostatic chemokines, signaling to other immune effector cells, and activation of antitumor CD8+ T cells. (see text for more details)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Alone, enhances NADPH Oxidase, works synergistically with IL-1</td>
</tr>
</tbody>
</table>

Neuroblastoma tumor cells do not typically express MHC I molecules and are thus particularly susceptible to γδ T cell and NK cell lysis\(^{52-56}\). The expression of MHC I molecules by neuroblastoma tumor cells was shown by Schilbach et al to be induced only by exposing neuroblastoma tumor cells to IFN-γ for at least 72 hours\(^{53}\). Upon the upregulation of MHC I molecules, a dose dependent reduction in γδ T cell mediated tumor lysis was observed\(^{53}\). The observed inverse relationship between γδ T cell
mediated neuroblastoma tumor cell lysis and MHC I expression is consistent with previous published observations and further reinforces existing data suggesting that γδ T cells kill neuroblastoma tumor cells in a similar manner as NK cells^53, 57^.

γδ T-cells, as well as NK cells, can upregulate the immune response against tumors through the activation of NKG2D receptors located on their surface^10^. Activation of the NKG2D receptor triggers the upregulation of CD69 and CD25 by γδ T cells and the release of potent cytotoxic granules^10^. CD69 and CD25 are both prominent cell activation markers. γδ T cells’ NKG2D receptor can be stimulated independently by the MICA gene’s protein or the cytokine, IL-15. MICA is a gene responsible for encoding the MHC class I chain related gene A. The MHC class I related protein encoded by this gene differs from a traditional MHC class I protein in that the MICA protein does not associate with beta-2-microglobulin. The MICA protein is known to be a stress induced antigen and ligand to NKG2D. Once MICA activates the NKG2D receptor, the NKG2D receptor can activate DAP-10, an adaptor protein which complexes with the NKG2D receptor in order to activate additional NK and T cells^10^.

Studies done by Parham et al and Rincon-Orozco et al using the RMA mouse tumor cell line provide additional support of NKG2D’s recognition of MICA and subsequent signaling and activation of γδ T cells^8, 10^. The activation of γδ T cells through NKG2D signaling is important because it enables γδ T cell activation upon NKG2D ligand recognition regardless of antigen exposure^10^.

The NKG2D receptor’s downstream signaling pathway can become activated once it is primed by IL-15 signaling^58^. IL-15 signaling activates Janus Kinase 3 (Jak3),
which upon activation phosphorylates a key tyrosine motif within the cytoplasmic tail of the transmembrane adaptor protein, DAP10\textsuperscript{58}. DAP10’s cytoplasmic tail must be phosphorylated for signaling to proceed through the NKG2D receptor’s downstream signaling pathway\textsuperscript{58}. Signaling by IL-15 not only primes the NKG2D receptor-signaling pathway but also stimulates the upregulation of the NKG2D receptor on the cell surface of γδ T cells\textsuperscript{8,10,58}. The discovery that neuroblastoma cells secrete high levels of IL-15 can be used to more effectively direct cytotoxic γδ cells to tumor cells by trafficking γδ cells to those cells producing large amounts of IL-15. This would increase the specificity of the immune response thus reducing possible immunopathology. It is important to mention as well that IL-15 is vital to NK cell development, maintenance and proliferation. A significant finding is that when γδ T cells are cultured with neuroblastoma cells a substantial amount of IL-15 is released\textsuperscript{33}. This suggests that γδ T-cells could be a beneficial immunotherapeutic agent directly and indirectly through the recruitment and proliferation of other immune cells.

**Can γδ T cells be manipulated for immunotherapy?**

Theoretically γδ T cell based immunotherapy is an attractive therapeutic option since γδ T cells are able to directly and indirectly eradicate tumor cells through activating a widespread effector response. Furthermore, γδ T cells, unlike CD8\textsuperscript{+} T cells and NK cells, do not rely on MHC presentation or MHC mismatch. Several obstacles exist however, which have impeded the development of γδ T cell based immunotherapies. Since γδ T cells comprise only 10% of T cells, the population must first be expanded\textsuperscript{48,53}. The *ex vivo* expansion of γδ T cells is difficult and often results in a population of cells
with a significantly reduced cytotoxicity and half-life. Additionally, in the process of \textit{ex vivo} expansion $\gamma\delta$ T cells are very susceptible to microbe contamination. In order for $\gamma\delta$ T cells to become a realistic immunotherapeutic option a means of expanding and protecting this population of cells while maintaining their cytotoxicity must be discovered.

Both Schilbach et al. and Otto et al. have investigated ways of expanding the $\gamma\delta$ T cell population while maintaining or increasing the population’s cytotoxicity. Otto et al, specifically looked at how the half life of $\gamma\delta$ T cells could be increased. In his experiments Otto et al. used NOD.CB17-Prkdc\textsuperscript{scid}/J mice, which he injected (via tail vein) with $1 \times 10^6$ neuroblastoma tumor cells from the 1691 tumor cell line. The neuroblastoma tumor cell injections occurred five to six days prior to five injections, spaced one week apart, of $1 \times 10^6 \gamma\delta$ T cells. The $\gamma\delta$ T cells used in the injections were obtained from human donors and expanded with G-CSF. Prior to the injections, the cytotoxicity of the $\gamma\delta$ T cells was assessed through a europium TDA release assay. One group of mice received 20µg of hu14.18 four times a week along with the $\gamma\delta$ T cells while the other group received a IL-7 fusion cytokine (Fc-IL-7 with human IL-7 sequence) in addition to the 20µg of hu14.18 and $\gamma\delta$ T cells once a week. He found that $\gamma\delta$ T cells when combined with hu14.18 antibody and the IL-7 fusion cytokine had a significantly longer half-life. Otto et al. proceeded to demonstrate that when the combination of $\gamma\delta$ T cells, hu14.18, and Fc-IL-7 were infused into mice injected with $1 \times 10^6$ neuroblastoma tumor cells, the survival time in all of the mice was significantly prolonged (p=0.001). These mice had their survival period extended by an additional
two weeks ($p = 0.005$) $^{48}$. Addition of Fc-IL-7 was found to be responsible for the prolonged survival time ($p = 0.04$). It is interesting to note that when the mice were treated with only the anti-GD2 *hu14.18* antibody or Fc-IL-7, their survival period was not significantly longer than control mice ($p = 0.26$) $^{48}$. This observation suggests that IL-7 alone is not capable of increasing the efficacy of a $\gamma\delta$ T cell based immunotherapy even though its addition is responsible for the prolonged survival time of mice infected with neuroblastoma. It seems paradoxical that the addition of IL-7 to *hu14.18* and $\gamma\delta$ T cells is capable of prolonging the survival period of mice infected with neuroblastoma since it has been widely suggested that IL-7 signaling through the IL-7 CD127 receptor may promote tumor growth $^{59}$. However there are currently no reports of CD127 mRNA expression in pediatric tumor cell lines $^{60}$. There are also no reports of the IL-7-CD127 signaling complex in solid tumors $^{60}$. IL-7 may therefore be capable of changing its role from a tumor growth promoter to a tumor growth inhibitor pending the presence of CD127. The *in vivo* results observed by Otto et al. using mice infected with neuroblastoma were also observed *in vitro* when the group repeated their experiment on the NB1691 neuroblastoma cell line $^{48}$.

Schilbach et al collected peripheral blood mononuclear cells (PBMC) from patients and treated them with Orthoclone OKT3 (OKT3) $^{53}$. OKT3 is an immunosuppressant drug used to reverse acute graft rejection. It inhibits T-cell function by blocking CD3 thus affecting early T-cell activation and cytokine release among other T-cell functions. Schilbach et al found that 12 days post OKT3 treatment the prevalence of $\gamma\delta$ T cells in PBMC increased from 7.6% (SD 4.2%) to 16.5% (SD 9.9%) $^{53}$. On day
22 of culture, 98.9% of PBMC were γδ T-cells and of these 65.9% (SD20.9%) co
expressed the NK cell marker, CD56. Through his experiments Schilbach et al
demonstrates that the proportion of γδ T cells in PBMC steadily increases when αβ T
cells are inactivated. Although it remains unknown why the γδ T cell population expands
upon αβ T cell inactivation it is reasonable to suggest that the observed expansion in γδ T
cells serves a compensatory mechanism. If the process of in vivo αβ T cell inactivation
through OKT3 exposure resulting in the compensatory increase in the γδ T cell
population can be recreated ex vivo, it may serve as a possible way of expanding the γδ T
cell population ex vivo.

Following γδ T cell expansion, Schilbach et al. proceeded to demonstrate the
effect that γδ T cell prevalence has on γδ T cell cytotoxicity. Schilbach et al. incubated
the LS neuroblastoma cell line with human γδ T-cells obtained from healthy donors at
effector: target ratios ranging from 0.3: 1 to 20: 1 and used a BATDA release assay to
asses the γδ T cell’s cytotoxicity. A BATDA release assay measures the degree of cell
lysis by measuring the intensity and scope of a fluorescent signal produced when the
hydrophobic intracellular ligand, TDA combines with an extracellular Eu-solution. The
TDA ligand is formed inside the cell upon hydrolysis of an ester bond present in the
initial cell loading solution, acetooxymethyl (aceto) ester of fluorescence enhancing
ligand. The hydrophobic ligand, TDA, formed following ester cleavage cannot cross the
cell membrane and thus its exit from the cell and combination with Eu-solution to
produce a fluorescent signal indicate cell lysis. Using a BATDA release assay, Schilbach
et al observed that γδ T cells maintained their cytotoxic function at an effector: target
ratio of 20:1 however when the effector: target ratio was reduced to 1.25:1 only 18% showed medium cytotoxicity and 73.8% showed low cytotoxicity. Medium cytotoxicity was defined as specific target cell lysis greater then 30% but less then 50% at an effector: target ratio of 20:1 while low cytotoxicity was defined as specific target cell lysis less then 30% at an effector: target ratio of 20:1. When the group of γδ T-cells at an effector: target ratio of 20:1 showing low cytotoxicity was stimulated with IL-2 for 24 hours, the mean cytotoxicity rose to 81%. Otto et al also observed that when γδ T cells were stimulated with IL-2 68% of the γδ T cells remained viable and maintained their cytotoxicity for more then 10 days in culture.

Schilbach et al additionally observed that all γδ T-cells showed high spontaneous killing at an effector: target ratio of 20:1 regardless of whether they were stimulated with IL-2. When γδ T-cells that had been stimulated with IL-2 for 24 hours were incubated with 6 neuroblastoma cell lines: LS, IMR 5, IMR 32, SK-N-LO, SK-N-SH, and Kelly at effector: target ratios ranging from 10:1 to 0.6:1 a sustained 30 day high degree of cytotoxicity against neuroblastoma cells was observed. Schilbach et al. demonstrates the necessity of expanding the γδ T cell population to maintain cytotoxicity. His work also supports the use of IL-2 during the process of ex vivo expansion.

**Activation and Expansion of γδ T cells through Phosphonates and Phosphorylated Structures**

Observations by Schilbach et al. suggest that γδ T cells target and kill neuroblastoma tumors by binding to phosphorylated structures presented on the surface of neuroblastoma tumor cells. When both the neuroblastoma cell line, LS and neuroblastoma tumor cells were treated with acid phosphatase and then incubated with γδ
T cells, 78% of the plates containing acid phosphatase treated neuroblastoma tumor cells exhibited a significant reduction in tumor cell lysis compared to plates with untreated neuroblastoma tumor cells. Since acid phosphatase treatment is expected to release phosphate structures from the surface of neuroblastoma tumor cells, Schilbach et al’s observations suggest that binding of γδ T cells to phosphorylated structures on neuroblastoma tumor cells is a key aspect of neuroblastoma cell lysis.

In addition to investigating how γδ T cells are able to recognize and bind to neuroblastoma tumor cells, cancer immunologists have focused on learning the mechanism behind γδ T cell stimulation and expansion upon neuroblastoma tumor cell recognition. This is of particular interest to cancer immunologists because until recently, the low prevalence of γδ T cells has been a significant barrier preventing the use of γδ T cells in immunotherapy. Recently it has been widely observed that γδ T-cells can be stimulated and made highly cytotoxic through treatment with phosphonates such as isopentenyl diphosphate (IPP). Unfortunately many phosphonates are toxic which limits the immunotherapeutic use of γδ T cells. As an alternative to the powerful yet cytotoxic phosphonates such as IPP, Schilbach et al investigated the effects of Clondronate, a less toxic phosphonate, currently used to treat hyperkalemia, on γδ T cells. Treatment of γδ T cells with Clondronate and IL-2 resulted in a significant increase in cytotoxicity against neuroblastoma tumor cells at effector: target ratios ranging from 20:1 to 1.2:1. The cytotoxic potential of γδ T-cells treated with Clondronate and IL-2 was 17% higher than controls treated with IL-2 alone even at the lowest effector: target ratios.
Furthermore, Clondronate was able to increase the proliferation of γδ T cells 35.8 to 57.8 times higher.

Another phosphonate studied for its ability to stimulate γδ T cell proliferation is Bromohydrin Pyrophosphate (BrHPP). Chargui et al. examined BrHPP’s ability to expand γδ T cells and induce the secretion of IFN-γ and TNF from γδ T-cells using PBMC from 10 patients with neuroblastoma at varying stages of disease (6 immediately after diagnosis prior to all treatment, 9 following induction therapy, and 4 following autologous transplant). γδ T cells from PBMC of patients immediately following diagnosis proved to be the most susceptible to BrHPP. γδ T cells from these patients showed a 127.63% expansion. γδ T-cells from PBMC of patients following autologous stem cell transplant showed an 82.03% expansion. This data supports the use of BrHPP for the ex vivo expansion of γδ T cells. It was further shown that in 69% of the PBMC samples the prevalence of γδ T cells in the samples increased from 26.07% to 89% following a 14-day treatment with BrHPP. Following 8 days of BrHPP stimulation, samples from neuroblastoma patients were composed of 70% CD3 γδ T cells and less then 2% αβ CD3 T cells. It was also observed that 63% of the γδ T cells in the samples showed a rapid increase in CD25, by day 7. CD25 is the alpha chain of the IL-2 receptor and signifies γδ T cell activity.

γδ T-cells stimulated with BrHPP consistently express CD69, a signal transmitting receptor on lymphocytes that plays an important role in lymphocyte proliferation. γδ T cells also expressed the NK cell markers CD56, CD158, and NKG2D. When γδ T cells from patients with neuroblastoma were cultured overnight with BrHPP, they were able to
kill 80% of neuroblastoma tumor cells at an effector: target ratio of 30:1. Antibody blocking studies on γδ T cells stimulated by BrHPP suggest that γδ T cells interact and kill neuroblastoma tumor cells through their NKGD2 receptor.

One significant finding of Chargui et al. was that γδ T cells are capable of killing neuroblastoma tumor cells without the anti-GD2 antibody and solely through BrHPP activation. The stimulation of γδ T with BrHPP shows promise as a means of expanding and furthermore activating γδ T cells ex vivo to be used for autologous transplantation.

Zoledronic acid is another phosphoantigen that has proven to be a powerful activator of γδ T cells. Three important populations of γδ T cells are the CD45+ CD27+, and CD45− CD27+ memory cells and the CD45− CD27− IFN-γ producing cytotoxic cells. The CD45 marker is also known as the common leukocyte antigen and is a protein tyrosine phosphatase responsible for dephosphorylating phosphotyrosine residues. The CD27 marker is a TNF receptor essential for both the generation and maintenance of T cell immunity, B cell activation, and subsequent immunoglobulin formation. The prevalence of these specific populations within the peripheral circulating γδ T cell population is one way of monitoring γδ tumor surveillance and anti-tumor immune initiation since these populations signify presence and activity γδ T cells carrying out an anti-tumor immune attack. Dieli et al. observed that the prevalence of CD45+ CD27+, CD45− CD27+, and CD45− CD27− as well as the production of IFN-γ by Vγ1 γδ T cells following in vitro stimulation with IPP consistently increased upon zoledronic acid treatment. Dieli et al.’s observations correspond with other evidence demonstrating that
γδ T-cells can be effectively isolated from patients through leukopheresis, expanded and stimulated using zoledronic acid or other phosphonates and then infused back into patients to produce a powerful anti-tumor immune response. Pechhold et al. and other laboratories have demonstrated that phosphonates can be directly infused into patients to produce a potent anti-tumor response. The direct infusion of phosphonates and *ex vivo* phosphonate stimulation of γδ T cells both require the concurrent administration of IL-2. The use of zoledronic acid and phosphonates to stimulate a γδ T cell mediated anti-tumor immune response in multiple myeloma and non-Hodgkin’s lymphoma has also been studied. Wilhelm et al. found that 33% of his patient population with either multiple myeloma or non-Hodgkin’s lymphoma showed partial tumor remission and a corresponding significant increase in the prevalence of activated γ:δ T cells following zoledronic acid infusion.

Currently (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) has proven to be the most potent phosphoantigen capable of activating γδ T cells. HMB-PP is an intermediate of the non-mevalonate pathway used to produce dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), which are necessary for cell membrane synthesis and maintenance, hormone synthesis, N-glycosylation, and protein anchoring. Dunne et al., among others, have observed that if γδ T-cells, treated with HMB-PP, are incubated with immature dendritic cells, the dendritic cells mature and upregulate crucial co-stimulatory adhesion molecules including: HLA-DR, CD11c, CD54, CD40, CD83, CD86 and CCR7. The upregulation of these surface markers on dendritic cells typically occurs following dendritic cell treatment with agents such as,
polyinosinic: polycytidylic acid (poly I: C)\textsuperscript{69}. Poly I: C, along with other similar agents, induces cytotoxic activities in cells with which they interact\textsuperscript{69}. The upregulation of these co-stimulatory adhesion molecules by dendritic cells following γδ T cell exposure further supports the claim that γδ T-cells have a high cytotoxic potential\textsuperscript{69}. In addition to dendritic cell upregulation of key co-stimulatory molecules signifying their maturation and activation following exposure to HMB-PP treated γδ T cells express a similar highly cytotoxic phenotype consisting of the cell activation markers: CD25, CD69, and CD56\textsuperscript{59, 69}. Dunne et al and others, have also demonstrated that γδ T cells are able to function as APCs by showing that γδ T cells express HLA-DR and the co stimulatory molecule CD86 following HMB-PP exposure\textsuperscript{69, 72-74}.

**Selection of the Vγ1 γδ T Cell Phenotype for Immunotherapy**

Schilbach et al. observed that when γδ T cells were exposed to neuroblastoma tumor cells the collection of cytokines secreted by the Vγ1 γδ T cell phenotype differed from those secreted by the Vγ2 γδ phenotype\textsuperscript{49}. The collection of cytokines secreted by Vγ2 γδ T cells includes angiogenin, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1), all of which support tumor growth and angiogenesis\textsuperscript{49}. Vγ1 γδ T cells on the other hand immediately stop secreting angiogenin, VEGF, EGF, IGF-1, platelet derived growth factor (PDGF), and growth regulated oncogene (GRO) upon exposure to neuroblastoma cells\textsuperscript{49}. GRO belongs to the CXCL1 family of chemokines and supports malignant tumor growth, invasive tumor behavior, and is a powerful inflammatory cytokine with growth regulation properties\textsuperscript{49}. Instead of secreting cytokines supporting tumor growth and angiogenesis,
$\text{V} \gamma \text{1} \gamma \delta$ T cells secreted pro-inflammatory cytokines that proved to be significantly more cytotoxic against neuroblastoma tumor cells than those secreted by $\text{V} \gamma \text{2} \gamma \delta$ T cells $^{49}$. Additionally, the microenvironment created by $\text{V} \gamma \text{1} \gamma \delta$ cells was more toxic to neuroblastoma tumor cells than that created by $\text{V} \gamma \text{2} \gamma \delta$ T cells $^{49}$.

Typically, peripheral circulating $\text{V} \gamma \text{2}$ and $\text{V} \gamma \text{1} \gamma \delta$ T cells are present in a ratio of 5:1 ($\text{V} \gamma \text{2}: \text{V} \gamma \text{1}$) $^{49}$. In a 5:1 ratio ($\text{V} \gamma \text{2}: \text{V} \gamma \text{1}$) a $\gamma \delta$ T cell mediated anti-tumor response cannot be observed since the majority of tumor activated $\gamma \delta$ T cells, at this ratio, secrete cytokines, which support tumor growth and angiogenesis. However, when $\gamma \delta$ T cells are treated with Pamidronate, a phosphonate, the ratio of $\text{V} \gamma \text{2}: \text{V} \gamma \text{1} \gamma \delta$ T cells shifts from 5:1 to 1:11 suggesting that phosphoantigens are capable of serving as potent activators of the most effective $\gamma \delta$ T cell phenotype able to illicit an immune response against tumors $^{49}$.

Schilbach et al. also observed that when neuroblastoma tumor cells were incubated with $\text{V} \gamma \text{1} \gamma \delta$ T cells, the $\text{V} \gamma \text{1}$ cells continued to secrete high levels of IL-2 and CCL1 $^{75}$. IL-2 is a powerful cytokine necessary for the activation and proliferation of effector T cells as well as the generation of immunological memory. CCL1 acts as a powerful chemo attractant for monocytes. Additionally, a 3.2 and 1.5 fold increase in the release of TNF-$\alpha$ and monocyte chemotactic factor-2 (MCP-2) and an 8.1 fold upregulation of MCP-1 was observed when $\text{V} \gamma \text{1} \gamma \delta$ T cells were exposed to neuroblastoma cells. MCP-1 and MCP-2 are both powerful chemo attractant molecules for monocytes. Specifically, MCP-1 recruits monocytes, memory T cells, and dendritic cells to sites of inflammation or infection while MCP-2 is capable of binding chemokine receptors to activate cells of the immune system. An increase in MCP-1 and/or MCP-2 is
beneficial because it promotes a more robust immune response. The significant increase in IL-2, CCL1, TNF-α, MCP-1 and MCP-2 following Vγ1 γδ T cell exposure to neuroblastoma tumor cells provides further evidence that the Vγ1 γδ phenotype can also indirectly exert cytotoxic effects against neuroblastoma cells by signaling to other immune effector cells. Vγ1 γδ T cells also secreted significant amounts of IL-4 upon exposure to neuroblastoma tumor cells. IL-4 is a cytokine that induces the differentiation of naïve Th0 cells to Th2 T cells and assists with B cell activation.

**Inconsistencies in NK Cell Mediated Immunotherapy**

While NK cells and monoclonal anti-GD2 antibodies can be activated or designed to target specific tumor cells, they act alone, recruit a transient natural immune response, and in the past have not been as effective on solid tumors such as neuroblastoma. Additionally NK-mediated lysis and neuroblastoma tumor cell susceptibility is prone to environmental and genetic influences making it a less reliable treatment option. Many current studies, such as one performed by Delgado et al. in collaboration with the COG, have tried to demonstrate success using NK cell mediated immunotherapy based on the NK cell’s ability to elicit a powerful graft vs. tumor response. The graft vs. tumor response elicited by NK cells is rooted in the high degree of polymorphism of the KIR inhibiting receptors, which results in a high incidence of mismatched KIR receptor pairs. The high degree of reactivity observed between NK cells with mismatched inhibiting KIR receptor pairs is the foundation of the “missing self” hypothesis used to describe NK cell’s immunotherapeutic potential. Ruggeri et al. has demonstrated that donor licensed NK cells, infused into a recipient lacking at least one MHC I ligand for
the donor’s inhibiting KIR receptors, lead to a lowered NK cell activation threshold which resulted in a more potent immune response. Ruggeri et al. proved that the recipient’s enhanced effector response and increased tumor clearance was due to the infusion of alloreactive NK cells with mismatched inhibiting KIR receptors pairs. He isolated the alloreactive NK cells for three months following HSCT and blocked the immune response initially observed in the recipient by incubating the NK cells with target T cells that expressed matching inhibiting KIR receptor ligands.

The study conducted by Delgado et al. involved 39 patients with neuroblastoma, ranging in age from 1 year to 21 years of age. Delgado et al. separated the patients into two strata depending on their disease status as measured through standard imaging and clinical evaluation at the start of the study. Stratum one contained 15 patients with disease measurable by standard radiological techniques whereas stratum two consisted of 24 patients whose disease could only be measured by MIBG scintigraphy. MIBG scintigraphy uses a radiolabeled molecule, MIBG, which closely resembles noradrenaline and as such localizes within adrenal tissue. The detection of disease through MIBG scintigraphy but not standard radiological techniques indicates non-bulky disease confined to adrenal tissue. Delgado et al. received 38 DNA samples from the 39 participants and determined the KIR/KIR ligand genotype of participants. The seven participants that showed either a complete response (defined as disease free survival lasting longer then 35 months) or stable disease (disease present yet marked clinical improvements and clearing of disease from all marrow) following infusion of the anti-GD2 hu14.18 antibody and IL-2 all had a mismatched KIR/KIR ligand genotype. None
of the study participants with a matched KIR/KIR ligand genotype showed complete recovery \(^2^4\). Instead, 12 of the 14 participants found to have a matched KIR/KIR ligand genotype exhibited progressive disease \(^2^4\). Although a statistically significant correlation (p=0.03) between a mismatched KIR/KIR ligand genotype and hu14.18 treatment response was found, it is important to note that 75% of study participants with a mismatched KIR/KIR ligand genotype were placed in stratum two and therefore were found to have non-bulky neuroblastoma locally confined to the adrenal gland at the start of the study \(^2^4\). Furthermore the correlation between disease status with and without hu14.18 immunotherapy, assessed by the stratum each patient was placed into at the start and conclusion of the study, and KIR/KIR ligand genotype was not found to be statistically significant (p=0.08) \(^2^4\). Therefore, although Delgado et al.’s results suggest that patients with a mismatched KIR/KIR ligand genotype will respond favorably to hu14.18 treatment attaining either complete recovery or stable disease with signs of clinical improvement, it cannot be concluded that all patients with a mismatched genotype, regardless of initial disease status, will manifest a less established disease following treatment with hu14.18 \(^2^4\).

The presence of danger signals, ligands expressed by healthy cells but overexpressed by tumor cells, activates NK cells through activating receptors located on the NK cell’s surface \(^2^9\). In addition to the NKG2D receptor, NK cells also express the NKp46, NKp44, and NKp30 activating receptors \(^2^9\). Aside from the mixed lineage leukemia protein (MLL5) and B7-H6, many of the danger antigens, which bind and activate the NK cell’s activating receptors, remain unknown \(^2^9\). In order for NK cells to
become fully active and orchestrate an attack, NK cells’ activating receptors must either be solely engaged or overwhelmingly engaged with complementary activating ligands. If both activating and inhibiting receptors are engaged, the NK cell will be inhibited.

Therefore tumor cells which express MHC I molecules typically evade attack by NK cells since the MHC I molecules serve as ligands to NK cell’s inhibiting KIR receptors. Neuroblastoma tumor cells in general do not express high levels of MHC I molecules and are thus susceptible to NK cell mediated lysis. Raffaghello et al however observed that when neuroblasts isolated from patients with stage 4 neuroblastoma were treated with activated allogenic NK cells, increased IFN-γ concentrations resulted in increased expression levels of MHC I molecules on the neuroblasts. The increased expression of MHC I molecules lowered neuroblasts’ susceptibility to NK cell mediated lysis.

Raffaghello et al.’s observations correlate with observations by Lorenzi et al who reported that both IFN-γ and TNF-α, secreted by NK cells, activates the transcription of MHC I molecules. Although IFN-γ and TNF-α are also secreted by γδ T cells, their presence does not impinge on γδ T cell’s ability to lyse tumor cells because γδ T cells are not affected by the presence of MHC I or MHC II molecules. An increase in MHC I expression and consequential decrease in NK cell mediated tumor lysis was also observed by Neal et al in mice treated with anti-GD2 monoclonal antibodies that engage the FcγR on tumor cells. The increase in MHC I expression observed in anti-GD2 antibody treatment results from increased secretion of IFN-γ following the engagement between the anti-GD2 antibody and FcγR on neuroblastoma tumor cells.
Additional factors such as the presence or absence of CD155 and the poliovirus receptor on neuroblastoma tumor cells have proven to effect tumor cell susceptibility to NK cell mediated lysis. CD155 interacts with the DNAM-1 receptor on NK cells and signals DNAM-1 receptor activation of NK cell’s NKG2D receptor. In studies using bone marrow aspirated neuroblasts from patients with neuroblastoma it has been observed that an increased presence of CD155 on neuroblasts increased the neuroblast’s susceptibility to NK cell mediated lysis. Neuroblasts from bone aspirates of patients with metastatic disease have shown either limited or absent expression of CD155. These observations all suggest that the expression of CD155 on neuroblastoma tumor cells may be important for optimal NK cell-mediated tumor lysis. The status of CD155 expression on neuroblastoma tumor cells could thus limit the effectiveness of NK cell based immunotherapy in some neuroblastoma patients.

Neuroblastoma tumor cells are capable of evading NK cell mediated attack through a variety of mechanisms. Just as the expression status of CD155 on neuroblastoma tumor cells could limit the ability of NK cells to kill tumor cells, the absence of CD155 ligands on NK cells could impede NK cell mediated tumor lysis. Neuroblastoma cells also tend to release substantial amounts of soluble MICA protein, which decreased NK cell activation. NK cell activity is further dampened by the release of tumor growth factor-β (TGF-β) by neuroblastoma tumor cells which downregulates the expression of the activating NKp30 and NKG2D receptors on NK cell’s surface. Although the levels of TGF-β secreted by neuroblastoma tumor cells are not significant enough to have a drastic impact on the presence of NKG2D receptors on
the NK cell surface, they are significant enough to significantly impact NK cell’s expression levels of various chemokine receptors. The chemokine receptors expressed by NK cells influence NK cell interactions with the endothelium, their recruitment into peripheral tissues, and their movement from the bone marrow. Therefore through influencing the chemokine receptor expression profile of NK cells, TGF-β, secreted by neuroblastoma tumor cells, can significantly impact NK cell mediated tumor lysis.

Another factor that could possibly limit the therapeutic value of NK cells is their potential dependence on the invariant chain NK cell (iNK) population to exert their cytotoxic effects. iNK cells differ from NK cells in that they express a monospecific TCR capable of recognizing foreign and self glycerol or ceramide based lipid antigens presented by the CD1d cell marker. The activity of iNK cells is thus not controlled by KIRs, lectin-like receptors, or natural cytotoxicity receptors (NKp30, NKp44, and NKp46). iNK cells also differ from NK cells in that they undergo somatic recombination and selection in the thymus and thus share some developmental traits with cells of the adaptive immune system. Although their development in the thymus resembles the development or other cells in the adaptive immune system, iNK cells respond in an innate manner by secreting danger signals and proinflammatory cytokines within hours of activation. iNK cells are referred to as preconditioned effector cells due to their unparalleled rapid production and secretion of cytokines upon activation. Because iNK cells are not defined by a single group of markers they are commonly divided into subsets by cell surface markers and behavior resembling other more defined populations of immune cells. Although iNK cells make up only 0.1%-0.2% of T cells
in the peripheral blood, they compose 10% of T cells in the gastrointestinal momentum, the origin of many neuroblastoma tumors. A study conducted by Saito et al. demonstrated that when the iNK cell population was knocked out of mice, a significantly lower amount of NK cells and dendritic cells were able to become activated and exert a cytotoxic attack against tumor cells. Saito et al.’s study suggests that NK cells are not capable of eliciting an anti-tumor immune response on their own but instead require the presence of another sub-population of NK cells. The reliance of NK cells on the iNK population would be an additional factor to consider when using NK cell based immunotherapy. In addition to NK cell dependence on MHC I and CD155 expression status.

**Clinical Trials investigating γδ T cell based immunotherapy and the proposed advantages and disadvantages of γδ T cell immunotherapy**

Clinical trials investigating the effectiveness of γδ T cell based immunotherapy in the treatment of neuroblastoma are limited. Currently Dr. Joseph Pressey is conducting a pilot study at the University of Alabama School of Medicine investigating the efficacy of zoledronic acid and IL-2 treatment to elicit a γδ T cell response in patients with neuroblastoma. Initial results from Dr. Pressey’s investigation have not yet been released. Although the clinical trials on γδ T cell based immunotherapies for neuroblastoma are limited, there have been several clinical trials involving γδ T cell based immunotherapy for other types of cancer, most notably renal cell carcinoma, lung cancer, breast cancer and lymphoid malignancies. These studies showed significant tumor regression when γδ T cells were expanded *ex vivo* with phosoantigens and adoptively transferred into patients. The studies completed on
patients with lung, breast, and renal cell carcinoma were particularly promising because the adoptive transfer of the *ex vivo* expanded γδ T cells had a significant impact on these tumors which in the past have not shown a dramatic response to immunotherapy\(^86-88\).

Although bench and clinical research examining the effectiveness of γδ T cell based immunotherapy for neuroblastoma and other cancers has shown promise there are several potential barriers, which must first be overcome. In order for γδ T cells to exert a significant anti-tumor immune response they must be expanded *ex vivo*. The injection of an extremely large amount of *ex vivo* expanded γδ T cells can induce the secretion of numerous inflammatory cytokines causing deleterious side effects\(^16\). Additionally, although TGF-β does not suppress the activity of γδ T cells through the same mechanism it uses to suppress NK cell activity it promotes the activity of Th17 cells and the upregulation of regulatory T cells\(^47,90,91\). Tumor cells secrete TGF-β in order to escape immunosurveillance. In addition to offering support to Th17 cells and regulatory T cells, TGF-β upregulates a subpopulation of γδ T cells that express the Foxp3\(^+\) phenotype and have immunosuppressant abilities\(^47,90,91\). It is important to note however that the upregulation of Foxp3\(^+\) γδ T cells by TGF-β has only been observed in the mouse and not in humans. Given the complexity of the immune system it cannot be concluded with a high degree of certainty that the molecules secreted by tumor cells and the immune system’s response to these molecules in mice mirrors the response in humans.

γδ T cells exhibit several distinct advantages making them attractive candidate cells for immune based therapies. One of the most significant advantages of γδ T cell based immunotherapy is the ability of γδ T cells to exert a potent anti-tumor immune
response by both directly killing tumor cells and by promoting the maturation, recruitment, and proliferation of other immune cells, specifically dendritic and NK cells, through IL-15 signaling \(^{31, 33, 47, 49, 53, 57, 69, 72-75, 92, 93-96}\). \(\gamma\delta\) T cells additionally secrete IL-12 and Th\(_1\) cytokines, which induce the maturation of immature dendritic cells to mature APC \(^{72}\). By maintaining the balance of Th\(_1\)/Th\(_2\) and Th17 cytokines, \(\gamma\delta\) T cells have an even greater control of the strength and nature of the immune system’s anti-tumor response.

Immunotherapies that employ \(\gamma\delta\) T cells are preferable to those using NK cells because \(\gamma\delta\) T cell activation and attack does not depend on the status of MHC I molecules, or other activators such as CD155 on the tumor cell’s surface. Since \(\gamma\delta\) T cells act independently of MHC I molecules, their activation and attack potency is not hindered by increased levels of IFN-\(\gamma\) as is the case with NK cells \(^{97}\). Both NK cells and \(\gamma\delta\) T cells can be activated through the NKG2D activating receptor expressed on their cell surface. The secretion of soluble MICA protein and TGF-\(\beta\) however can block NKG2D receptor activation. Furthermore, the absence of danger signals such as CD155 or the DNAM receptor can block NKG2D receptor activation. If activation of the NKG2D activating receptor is blocked then NK cells will not be able to elicit an anti-tumor immune response. \(\gamma\delta\) T cell activation and initiation of a powerful anti-tumor immune response does not depend solely on the NKG2D receptor \(^{44, 61, 98}\). Non-peptide phosphoantigens and aminobisphosphate drugs such as zoledronic acid can instead activate \(\gamma\delta\) T cells \(^{44, 61, 98}\). Therefore tumor antigen expression profiles that inactivate NK cells do not affect \(\gamma\delta\) T cells.
Several other important qualities of γδ T cells making them ideal candidates for immunotherapy include the simultaneous secretion of proinflammatory cytokines, proapoptotic molecules, and ability to carry out cell-cell contact lysis, as well as their ability to present tumor antigens to both CD4 and CD8 T cells.97, 99

Conclusion

Table 3 outlines both the advantages and disadvantages of using either NK cell mediated or γδ T cell mediated immunotherapy to treat neuroblastoma.

Table 3: The Advantages and Disadvantages of NK cell and γδ T cell Immunotherapy

<table>
<thead>
<tr>
<th>Immunotherapy Cell</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK Cell</td>
<td>-Doesn’t require previous MHC mediated antigen presentation -Secretes numerous cytotoxic cytokines: TNF-α, granzyme B, perforin, IL-22, CCL3, CCL4, IL-8, IFN-γ -The high degree of polymorphism in NK cell’s KIR receptors increase the probability of mismatched KIR/KIR receptor pairs enabling NK cells to carry through a powerful tumor vs. graft response.</td>
<td>-NK cell activation requires either the absence of MHC expression or MHC/KIR mismatch -Inability to produced memory cells making the immune response more transient which could increase the risk of relapsed disease -Possible reliance on the iNK cell population to achieve full cytotoxicity -NK cell cytotoxicity against tumor cells can be dampened by monoclonal antibody treatment -NK cell immunotherapy is not ideal for solid tumors -NK cell activating receptors must be solely or overwhelmingly engaged to their complementary ligands on tumor cell surface or the NK cell will be inhibited -The high degree of IFN-γ and TNF-α secreted by NK cells upregulates MHC I expression which in turn downregulates tumor cell’s susceptibility to NK cell mediated lysis -NK cell activation status is heavily affected by the presence or absence of danger signals such as...</td>
</tr>
</tbody>
</table>
as CD155 expressed on tumor surface
-NK cells release soluble MICA which decreases NK cell activation
-The release of TGF-β by neuroblastoma cells causes NK cells to downregulate the activating receptors NKp30 and NKG2D

| γδ T cell | -γδ T cell activation does not depend on the absence, presence, or mismatch of MHC
-Since γδ T cell activation is MHC independent, activation and cytotoxicity is not affected by increased levels of IFN-γ, TNF-α
-Ability to acquire characteristics of professional APC
-Expression of CCR7 enabling αβ T cell antigen presentation
-Expression of TLR and the ability to activate TLR enabling γδ T cells to recognize DAMPS expressed on the surface of tumor cells
-γδ T cells promote the maturation of DC cells
-γδ T cells can produce MHC II de novo and upregulate MHC II expression
-The ability to induce the differentiation of CD8+ αβ T cells into CTL cells
-γδ T cells signal for the proliferation and differentiation of Th1 and Th2 cells as well as control Th1/Th2/Th17 cytokine balance
-Ability to become phagocytes that cross present tumor antigens to CD8+ and CD4+ αβ T cells
-γδ T cells secrete numerous potent cytokines: IL1, IL2, IL4, IL5, IL7, IL8, IL12, IL13, IL33, MCP1, CCL1 and Th1 cytokines which kill tumor cells directly and recruit as well as activate other powerful effector cells for an expanded immune response.
-γδ T cells secrete IL-15 which is responsible for NK cell differentiation, maturation,

-Due to the low prevalence of γδ T cells, they must undergo ex vivo expansion. The ex vivo expansion of γδ T cells has been associated with decreased γδ T cell cytotoxicity and half life and an increased risk of microbial contamination
-Risk of cytokine storm following the infusion of an expanded γδ T cell population
-γδ T cells promote Th17 cell activity and upregulate regulatory T cells
-In mice, TGF-β secreted by neuroblastoma cells upregulates the Foxp3+ phenotype of γδ T cells
In the past, γδ T-cells have not been considered a viable therapeutic option due to difficulties in maintaining γδ T cell cytotoxicity following ex vivo expansion. This challenge has been overcome through the use of phosphoantigens with concomitant administration of IL-2 \(^{63, 66-68}\). The primary advantage in using γδ T cell mediated immunotherapy rests in the ability to activate γδ T cells through multiple non-MHC dependent mechanisms and generate a wide immune response involving APCs such as dendritic cells, NK cells, CD8\(^+\) and CD4\(^+\) αβ T cells, and LAK cells \(^{69, 73, 74, 94-96}\). The findings of this thesis suggest that γδ T cells have the potential to become a highly effective immunotherapeutic agent for the treatment of neuroblastoma.
## LIST OF JOURNAL ABBREVIATIONS

<table>
<thead>
<tr>
<th>Journal Abbreviation</th>
<th>Journal Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Am J Pathol</td>
<td>The American journal of pathology</td>
</tr>
<tr>
<td>Annu Rev Immunol</td>
<td>Annual review of immunology</td>
</tr>
<tr>
<td>Biochem Biophys Res Commun</td>
<td>Biochemical and Biophysical Research Communications</td>
</tr>
<tr>
<td>Biol Blood Marrow</td>
<td>Biology of blood and marrow transplantation</td>
</tr>
<tr>
<td>Cancer Chemother Pharmacol</td>
<td>Cancer chemotherapy and pharmacology.</td>
</tr>
<tr>
<td>Cancer Epidemiol Biomarkers Prev</td>
<td>Cancer epidemiology, biomarkers &amp; prevention</td>
</tr>
<tr>
<td>Cancer Immunol Immunotherapy</td>
<td>Cancer Immunology, Immunotherapy</td>
</tr>
<tr>
<td>Cancer Res</td>
<td>Cancer Research</td>
</tr>
<tr>
<td>Cancer Treat Rev</td>
<td>Cancer Treatment Reviews</td>
</tr>
<tr>
<td>Cell Death Differ</td>
<td>Cell death and differentiation.</td>
</tr>
<tr>
<td>Cell Immunol</td>
<td>Cell Immunology</td>
</tr>
<tr>
<td>Cell Mol Life Sci</td>
<td>Cell Molecular Life Science</td>
</tr>
<tr>
<td>Clin Cancer Res</td>
<td>Clinical Cancer Research</td>
</tr>
<tr>
<td>Curr Cancer Drug Targets</td>
<td>Current Cancer Drug Targets</td>
</tr>
<tr>
<td>Curr Mol Med</td>
<td>Current molecular medicine</td>
</tr>
<tr>
<td>Eur J Cancer</td>
<td>European journal of cancer</td>
</tr>
<tr>
<td>Eur J Immunol</td>
<td>European journal of immunology</td>
</tr>
<tr>
<td>Journal Name</td>
<td>Magazine Title</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>FEBS Lett</td>
<td>FEBS letters.</td>
</tr>
<tr>
<td>Front. Immunol</td>
<td>Frontiers in immunology</td>
</tr>
<tr>
<td>Hum Immunol</td>
<td>Human immunology</td>
</tr>
<tr>
<td>Immunol Res</td>
<td>Immunologic research.</td>
</tr>
<tr>
<td>Int Immunol</td>
<td>International Immunology</td>
</tr>
<tr>
<td>Int Rev Immunol</td>
<td>International reviews of immunology</td>
</tr>
<tr>
<td>J Clin Invest</td>
<td>The Journal of clinical investigation</td>
</tr>
<tr>
<td>J Clin Oncol</td>
<td>Journal of clinical oncology</td>
</tr>
<tr>
<td>J Immunol</td>
<td>Journal of Immunology</td>
</tr>
<tr>
<td>J Immunother</td>
<td>Journal of Immunotherapy</td>
</tr>
<tr>
<td>J Intern Med</td>
<td>Journal of internal medicine</td>
</tr>
<tr>
<td>Nat. Biotechnol</td>
<td>Nature biotechnology</td>
</tr>
<tr>
<td>Nat Med</td>
<td>Nature medicine</td>
</tr>
<tr>
<td>Nat Rev Cancer</td>
<td>Nature reviews. Cancer</td>
</tr>
<tr>
<td>Nat Rev Immunol</td>
<td>Nature reviews. Immunology</td>
</tr>
<tr>
<td>PLoS Pathog</td>
<td>PLoS pathogens</td>
</tr>
<tr>
<td>Proc Natl Acad Sci U S A</td>
<td>Proceedings of the National Academy of Sciences of the United States of America</td>
</tr>
<tr>
<td>Journal Name</td>
<td>Natural Text</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Reprod Biomed Online</td>
<td>Reproductive biomedicine online</td>
</tr>
<tr>
<td>Trends Immunol</td>
<td>Trends in immunology</td>
</tr>
<tr>
<td>Yale J Biol Med</td>
<td>The Yale journal of biology and medicine</td>
</tr>
</tbody>
</table>
REFERENCES


British Journal of Cancer, 105, 787-795


CURRICULUM VITAE

CATHERINE E. BIXBY

cebixby@gmail.com

1985

Education

Boston University School of Medicine
Masters of Science in Medical Sciences
August 2012 – present (expected graduation 05/2014)

St. George University School of Medicine
M.D.
August 2011 – August 2012

University of Hartford
M.S. Neuroscience
Dec 2009-May 2011

University of Hartford
Pre-Medical Post Baccalaureate Program
June 2009-Dec 2009

University of Connecticut
Non-Degree Summer Sessions
June 2008 - July 2009

Fordham University
Bachelors of Arts
Major: Communications
Minor: Psychology
Sep 2003-May 2008

Employment and Volunteer Experiences

Student Researcher: Department of Surgery Division of Otolaryngology
University of Connecticut Health Center
Principle Investigation/ Supervisor: Dr. Kourosh Parham M.D./ Ph.D.
November 2013 – present
Farmington, CT

In my position with UCHC’s Department of Surgery Division of Otolaryngology I am
working on several projects. One of my projects investigates the association between idiopathic benign paroxysmal positional vertigo (BPPV) and disorders of bone turnover, specifically osteoporosis. In order to begin exploring this association a biomarker for inner ear diseases, specifically BPPV is needed. Currently however, there are no reports in the literature of biomarkers for inner ear disease. Dr. Parham and I are investigating whether the scaffolding protein, Otolin, expressed exclusively by support cells of the vestibular maculae, semicircular canal cristae, organ of Corti and marginal cells of the stria vascularis could serve as a biomarker for inner ear disease.

I am additionally working on a project led by Dr. Jeffery Marino, a 5th year resident in the University of Connecticut’s Otolaryngology Residency Program. This project seeks to better understand diabetes and its influence on posterior glottis stenosis (PGS). For the project Streptazocin-induced diabetic rats were used. PGS was induced in both the Streptazocin-induced diabetic rats and in non-diabetic control rats. I am currently working on the histopathological studies to compare stenosis in diabetic rats to stenosis in non-diabetic rats at the histological level.

**Assistant Clinical Researcher: Department of Pediatric Hematology/Oncology**
Connecticut Children’s Medical Center
April 2011 – August 2012
Hartford, CT

I helped lead a quality improvement research study in association with the National Association of Children’s Hospitals and Related Institutions (NACHRI) on reducing central line associated blood stream infections in pediatric hematology/oncology patients. I collaborated with patients and families to create an educational brochure providing essential information about central lines, CLABSI contributing factors, and ways to reduce the risk of CLABSI. I also wrote a quiz to assess patient’s and their family’s knowledge about central lines, risks, and the importance of preventing CLABSI. The quiz was used to evaluate how well the brochure served as an educational tool for families. Everyday I met with patients and their families in both the outpatient and inpatient unit at CCMC to deliver the quiz and brochure as well as discuss ways through which they could become more active participants in their medical care. To evaluate whether the brochure encouraged families to become active participants, I attended Family Centered Rounds every morning and recorded the frequency of patient/family and doctor/nurse discussion on reducing line entries. Family Centered Rounds entails the healthcare team traveling to each patient’s room and discussing the patient’s medical case with the patient and their family. Patients are included in any discussion and asked to clarify or correct any incorrect statements. They are also asked if they have any questions or concerns. Following Family Centered Rounds, I visited with each patient separately and discussed any concerns they had regarding their role as active participants in their medical care. I also spoke with them about how the healthcare team at CCMC could more effectively communicate and reach out to patients.
University of Connecticut School of Medicine Summer Research Fellowship Program
Immunology Department: Leo Lefrancois Ph.D. Lab
March 2011 – August 2011
Farmington, CT

I examined different \textit{in vivo} sources of IL-15, an essential immune protein, that has the potential to enhance anti tumor immunity and looked at the impact of deleting IL-15 production from individual subsets of immune cells in models of infection. Through my project I gained experience in Flow Cytometry, murine bone marrow transplantations, mouse handling, models of infection, and the Cre-lox technique.

University of Hartford Laboratory Instructor
August 2010-May 2011
West Hartford, CT
-Taught BIO 212: Anatomy and Physiology Laboratory
-Taught BIO 273: Genetics Laboratories

University of Hartford Genetics Laboratory Preparation
August 2010-May 2011
West Hartford, CT

Prepared, maintained, and managed all model organisms used in the genetic laboratory. I was also responsible for maintaining/ caring for all primary tissue culture used by the Genetics Research Department.

Biochemistry/Genetics Research
University of Hartford
June 2009-March 2011
West Hartford, CT

-Examined p53 regulation of cyclin B in breast epithelial cell lines that had undergone DNA damage.
-Performed cell cloning, PCR, and cell transformations to develop a cyclin B promoter driven green fluorescent reporter to monitor cell cycle perturbation
-Performed chromatin immunoprecipitation assays to find p53 binding regions within the cyclin B promoter
-Ran Western blots and gel electrophoresis to assess p53 and cyclin B expression levels within different breast epithelial cell lines treated with SN38 or UCN-01
-Performed glutaraldehyde cross-linkage to assess the tetramerization status of p53 in different cell lines following DNA damage
Connecticut Children’s Medical Center
Pharmacy Technician (per diem)
Sep. 2008 – August 2011
Hartford, CT
- Prepared IV fluids, chemotherapy, and total parenteral nutrition bags for patients
- Checked and filled anesthesia kits
- Filled and delivered PO medications to patients

Memorial Sloan Kettering Cancer Center (MSKCC)
Pediatric Volunteer
Sept. 2007 – August 2011
New York, New York

- Provided companionship and support to inpatients, day patients, siblings and parents while children receive treatment
- Assisted the Child Life Specialists in the Pediatric Day Hospital, Inpatient Unit, and Pediatric Observation Unit
- Helped organize and led different activities for the patients and their families including mock clinic, cooking group, and arts and crafts projects

Publications/Poster Presentations


5th International Conference on Patient and Family Centered Care
Washington D.C.
June 4th – 6th 2012
Poster Presentation and Abstract Publication


Awards/Honors/Recognitions/Additional Experience

- Recognized June 10th 2010 at Memorial Sloan Kettering Cancer Center’s 37th Annual Volunteer Recognition Ceremony for my hours of service and dedication as a volunteer in pediatrics

Certifications

- American Heart Association BLS Certification