Importance of specific NK cell subsets for antitumor immunity in HPV+ cancers

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IMPORTANCE OF SPECIFIC NK CELL SUBSETS FOR ANTITUMOR IMMUNITY IN HPV+ CANCERS

A DISSERTATION

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Madison O’Hara, B.S.

Houston, Texas

May 2024
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IMPORTANCE OF SPECIFIC NK CELL SUBSETS FOR ANTITUMOR IMMUNITY IN HPV+ CANCERS

Madison O’Hara, B.S.
Advisory Professor: Jagannadha K. Sastry, Ph.D.

ABSTRACT

High-risk type human papillomaviruses (HPV) are associated with genital and oral cancers, and the incidence of HPV+ head and neck squamous cell cancers is fast increasing worldwide. Survival rates for patients with locally advanced disease are poor and variable after standard of care (SOC) treatment. Identifying the antitumor host immune mediators important for treatment response and designing strategies to promote them are essential for improving clinical outcome. The natural killer (NK) cells are a critical component for antitumor innate effector immunity. Among the multitude of activation and inhibitory receptors on immune cells, HLA-DR is recognized as an important activation marker on both CD8 T and NK cells. Literature studies refer to HLA-DR+ NK cells as pre-mNK. Data from our preclinical HPV tumor models demonstrated that induction of the pre-mNK equivalent NK subset known in literature as natural killer dendritic cells (NKDC), a novel NK subset expressing the DC marker CD11c, via therapeutic HPV peptide vaccination, contribute to tumor-free long-term survival.

In addition to antigen-specific CD8 T cell responses, overall innate immune effector responses, including polyfunctional NKDC, were elevated in mice treated with and HPV peptide therapeutic vaccine containing the combination of QS21 and CpG
adjuvants (TVQC). Further, the CD11c+ NK subset (NKDC) exhibited higher functionality compared to the CD11c- NK subset, further highlighting the importance of NKDC for vaccine mediated immunity. In the clinical setting, we evaluated pre-mNK in HPV+ cancer patients, compared to healthy donors, and detected dysfunctional circulating pre-mNK in terms of increased inhibition indicated by PD-1 expression and reduced cytotoxic potential, indicated by lower levels of granzyme B (GrnzB) and CD16, an NK receptor important for Antibody Dependent Cellular Cytotoxicity (ADCC). In HPV+ head and neck patients, post-SOC, the circulating pre-mNK cells exhibited significant increases in GrnzB and CD16, indicating elevated cytotoxic potential after treatment. In cervical cancer patients, we analyzed the tumor microenvironment (TME) using non-invasive cytobrush sampling and found a significantly increased frequency of cytotoxic pre-mNK cells post-treatment that was not discernible in circulation. Employing clinically applicable cytokines we achieved significant ex vivo expansion of highly cytotoxic pre-mNK populations useful for adoptive cell therapy applications. These results emphasize the importance of pre-mNK subset of NK cells for protection in HPV cancers.
# TABLE OF CONTENTS

Approval page................................................................. Error! Bookmark not defined.

Title page ........................................................................... i

Acknowledgements .................................................................. ii

Abstract ................................................................................ iii

Table of Contents ...................................................................... v

List of Figures .......................................................................... viii

List of Abbreviations ............................................................... x

Chapter 1. Introduction ........................................................ 1

1.1 Innate and Adaptive immunity ........................................... 1

1.2 Innate Immune Mediators .................................................. 2

1.3 NK Cells ........................................................................... 3

1.4 NK Cell Receptors ............................................................ 5

1.5 NK Cell Functions and Killing Mechanisms ............................ 8

1.6 Innate Immunity and Cancer ................................................ 11

1.7 HPV and Cancer ............................................................... 12

1.7.1 Virology and Immune Evasion of HPV ............................... 12

1.7.2 Global Burden of HPV ................................................... 14

1.7.3 Major HPV Cancers ....................................................... 14

1.7.4 Therapeutics for HPV Cancers ......................................... 14
1.8 NK Cells in the Context of HPV Cancers ................................................................. 15

1.9 Defining HLA-DR+ NK Cells .................................................................................... 15

Chapter 2. Materials and Methods ............................................................................. 17

2.1 Animals ...................................................................................................................... 17

2.2 Tumor Cell Lines ...................................................................................................... 17

2.3 *In vivo* Tumor Challenge ....................................................................................... 18

2.4 Intranasal vaccination .............................................................................................. 18

2.5 Magnetic Resonance Imaging (MRI) ....................................................................... 19

2.6 Immune Cell Isolation .............................................................................................. 20

2.7 Flow Cytometry ........................................................................................................ 20

2.8 Patient Samples ........................................................................................................ 27

2.9 Patient Sample Collection and Processing .............................................................. 27

2.10 NK Cell Expansion .................................................................................................. 30

2.11 Statistical Analysis .................................................................................................. 30

Chapter 3. Results ......................................................................................................... 31

3.1 Introduction of Preclinical Studies .......................................................................... 31

3.1.1 Aim I: Evaluate the effectiveness of TVQC in the mEER oral HPV tumor model and determine the immune correlates. ......................................................... 32

3.1.2 Aim II: Investigate whether TVQC would be efficacious in the TC-1 vaginal HPV tumor model and determine the immune correlates. .............................................. 39
3.2 Potential contributions of Innate NK cell immunity in HPV+ cervical and oral cancers and response to standard of care (SOC) treatment ........................................ 42

3.3 Aim I. Determine the changes in frequency and functionality of pre-mNK in HPV+ head and neck squamous cell carcinoma (HNSCC) and cervical cancer patients undergoing standard of care (SOC) treatment. ........................................ 43

3.3.1 Increased Pre-mNK with Dysfunctional Phenotype in HPV+ Cancer Patients .................................................................................................................. 43

3.3.2 Pre-mNK Cells Gain Functionality Post-Treatment in HPV+ H&N Patients .................................................................................................................. 46

3.3.3 Increased Cytotoxic Pre-mNK in the TME of Cervical Cancer Patients After Treatment .................................................................................................. 50

3.4 Adoptive NK Cell Therapies ..................................................................................................................................................................................... 54

3.4.1 Increased Frequency and Functionality of Pre-mNK from Cultures Including IL-18 .................................................................................................. 55

3.4.2 Pre-mNK become the major NK cell subset when expanded with IL-2, IL-15, and IL-18 ................................................................................. 58

Chapter 4. Discussion .......................................................................................................................................................................................... 60

References ........................................................................................................................................................................................................... 67

VITA .............................................................................................................................................................................................................. 77
LIST OF FIGURES

Figure 1. Adaptive and innate lymphocytes. ................................................................. 4
Figure 2. NK cell receptors. .......................................................................................... 7
Figure 3. Mechanisms for NK cell killing. ..................................................................... 9
Figure 4. Mechanism for ADCC. .................................................................................. 10
Figure 5. Flow cytometry gating strategy for NKDC in mice........................................ 25
Figure 6. Flow cytometry gating strategy for pre-mNK cells in human......................... 26
Figure 7. Schema of cytobrush and blood sample collection in cervical cancer patients at sequential timepoints................................................................. 29
Figure 8. TVAC does not provide protection in the oral HPV tumor model................. 34
Figure 9. Efficacy of TVQC in the oral HPV tumor model. ........................................... 35
Figure 10. Adaptive immune responses induced by both TVAC and TVQC. .............. 36
Figure 11. TVQC induces a more robust innate effector response than TVAC in mice with oral HPV tumors. ................................................................. 37
Figure 12. TVQC-mediated protection against HPV vaginal tumors. ......................... 40
Figure 13. Elevated frequencies of circulating pre-mNK in HPV+ cancer patients... 44
Figure 14. Circulating pre-mNK display a dysfunctional phenotype in HPV+ cancer patients. ........................................................................................................... 45
Figure 15. Circulating immune cells in HNSCC patients pre- and post-treatment with standard of care (SOC).................................................................. 47
Figure 16. Circulating pre-mNK gain functionality after SOC treatment in HPV+ head & neck patients. ................................................................. 48
Figure 17. Elevated intra-tumoral cytotoxic pre-mNK levels in the TME of cervical cancer patients during treatment. 52

Figure 18. Addition of IL-18 to the expansion medium induces sustained increase of total and functional pre-mNK. 56

Figure 19. Pre-mNK become the major total and functional subset after expansion with the mixture of IL-2, IL-15, and IL-18 cytokines. 59
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HPV</td>
<td>Human Papillomavirus</td>
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<tr>
<td>NK</td>
<td>Natural Killer cell</td>
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>WBC</td>
<td>White Blood Cell</td>
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<td>MHC I</td>
<td>Major Histocompatibility Complex I</td>
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<td>NCR</td>
<td>Natural Cytotoxicity Receptor</td>
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<td>ITAM</td>
<td>Immunoreceptor Tyrosine-based Activation Motif</td>
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<td>FasL</td>
<td>Fas Ligand</td>
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<tr>
<td>ADCC</td>
<td>Antibody Dependent Cellular Cytotoxicity</td>
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<tr>
<td>LAMP-1</td>
<td>Lysosomal-associated Membrane Protein-1</td>
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<tr>
<td>MDSC</td>
<td>Myeloid Derived Suppressor Cells</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>TAA</td>
<td>Tumor Associated Antigen</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>TME</td>
<td>Tumor Microenvironment</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Rb</td>
<td>Retinoblastoma</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>SOC</td>
<td>Standard of Care</td>
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<tr>
<td>CRT</td>
<td>Chemoradiation</td>
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<td>Pre-mNK</td>
<td>Premature NK cell</td>
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<tr>
<td>IKDC</td>
<td>Interferon-producing Killer Cells</td>
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<td>TLR</td>
<td>Toll Like Receptor</td>
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<tr>
<td>NKDC</td>
<td>Natural Killer Dendritic Cell</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>TIL</td>
<td>Tumor Infiltrating Lymphocyte/Leukocyte</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
</tr>
<tr>
<td>IVIS</td>
<td>In vivo Imaging System</td>
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<tr>
<td>ROI</td>
<td>Region of Interest</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<td>MDACC</td>
<td>MD Anderson Cancer Center</td>
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<tr>
<td>GrnzB</td>
<td>Granzyme B</td>
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<td>HNSCC</td>
<td>Head and Neck Squamous Cell Carcinoma</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>aGalCer</td>
<td>Alpha-galactosylceramide</td>
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<tr>
<td>CpG-ODN</td>
<td>Cytosine and Guanine with phosphodiester backbone Oligodeonucleotides</td>
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<td>Quillaja Saponaria-21</td>
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<tr>
<td>TVQC</td>
<td>Therapeutic Vaccine with QS21 and CpG</td>
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<tr>
<td>PFS</td>
<td>Progression Free Survival</td>
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<tr>
<td>OS</td>
<td>Overall Survival</td>
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<tr>
<td>NIH</td>
<td>National Institute of Health</td>
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<tr>
<td>RARE</td>
<td>Rapid Acquisition with Relaxation Enhancement</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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CHAPTER 1. INTRODUCTION

The present investigation is focused on understanding the potential involvement of innate immune effectors, specifically a subset of natural killer (NK) cells that are less well recognized, particularly in literature related to HPV cancer, but represent one of the potent antiviral and antitumor innate immunity mediators.

1.1 Innate and Adaptive immunity

The immune system serves as a vital defense towards infection and disease in the body. The function of the immune system is driven by a myriad of complex processes, involving multiple organ sites and contributions from numerous cell types. These immune defense processes are largely divided into two major systems, innate and adaptive immunity [1]. The innate immune system is nonspecific and responds immediately to provide the first line of defense from stress or infection caused by foreign substances that have infiltrated the body. In addition to the involvement from several immune cell subtypes and proteins, the innate immune system also includes physical barriers, such as skin and mucosal layers [1, 2]. Unlike the innate immune system, the adaptive immune system is specific, causing a delayed second line of defense [2]. While the innate immune system responds to the initial infection, antigen presenting cells (APCs) known as dendritic cells (DCs) encounter the foreign substance, or antigen, and through a series of intracellular processing events present peptide fragments of the antigen to T cells, which serve as major effectors of the adaptive immune system [1, 2]. The process of antigen presentation allows the T cells to recognize the antigen and differentiate into either helper (CD4+) or cytotoxic (CD8+) T cells, followed by clonal expansion of cytotoxic CD8 T cells that will induce cell death.
by various mechanisms in the target cells [2]. Due to the specificity of the adaptive immune system, the body will form memory cells and antibodies specific to the target cell antigens which will allow an immediate response when exposed to the same pathogen in the future [1]. Thus, while the innate immune system serves as a first line of defense, the adaptive immune system is mobilized for second line of defense.

1.2 Innate Immune Mediators

The innate immune response includes the involvement of several types of immune cells including granulocytes, monocytes, and lymphocytes to immediately respond to infection [1]. Granulocytes are subdivided into neutrophils, eosinophils, and basophils. Eosinophils and basophils are low in frequency (<1% and 5%, respectively) and are involved in allergic inflammation responses [1]. In contrast, neutrophils are the most abundant among the white blood cells (WBC), at approximately 50-70% of WBCs. Neutrophils are key components of the inflammatory response and phagocytosis [1]. These are short-lived, but highly mobile to immediately migrate to the site of infection/inflammation, as one of the first cell types to respond. Monocytes circulate throughout the peripheral blood and recognize “danger signals” and migrate to sites of infection [1]. When monocytes migrate to the tissue, they differentiate to macrophages with diverse functions [1, 3]. Macrophages are longer lived and terminally differentiated phagocytes that produce chemokines to recruit other cells to the infection site [3]. Monocytes are also capable of differentiation into DCs, known as professional APCs, that are the key mediators between innate and adaptive immunity [2, 3]. The primary effector lymphocytes of the innate immune system are natural killer (NK) cells, which function by targeting and killing host cells that have been
compromised or infected [1, 4]. Overall, the interaction from a multitude of immune cells come together to mediate the innate immune response.

1.3 NK Cells

NK cells are the primary effector cells of the innate immune system and are widely known as key mediators for antitumor and antiviral responses. NK cells are lymphocytes, similar to B cells and T cells, derived from a common progenitor, however, they are effectors of the innate immune response, while B and T cells are adaptive lymphocytes (Figure 1) [4]. Within healthy individuals, the NK cells account for 5-20% of circulating immune cells [4, 5]. As their name suggests, NK cells have a natural ability to kill infected or transformed cells in the host without a need to be primed or activated to recognize foreign antigens like the CD8 T cells [1, 6]. NK cells express inhibitory markers that bind to the major histocompatibility complex one (MHC I) alleles, expressed on most healthy cells to help identify them as “self”. This interaction will prevent NK cells from killing healthy cells within the host [2, 7]. Overall, NK cells are critical effector cells for innate immunity and play key roles in immunosurveillance and elimination of foreign invaders, including viruses and cancer cells.
Figure 1. Adaptive and innate lymphocytes. A representative schematic of lymphoid progenitor cells differentiated into adaptive (T and B cells), and innate (NK cells) lymphocytes. Figure created with BioRender.com.
1.4 NK Cell Receptors

NK cells are a heterogenous population with the expression of a wide array of activating and inhibitory receptors for functional activity (Figure 2) [8]. Well known activating receptors include natural cytotoxicity receptors (NCRs), NKp46, NKp44, and NKp30 [8]. The NCRs have an extracellular immunoglobulin domain and are known to cause signal transduction through immunoreceptor tyrosine-based activation motif (ITAM) [4]. Another well-established activation receptor, NKG2D, is generally low in frequency in the homeostatic state, however, when exposed to an external stimulus or stressor, can be abundantly expressed on cytotoxic NK cells [8, 9]. NK cells can also express CD69, a known activation marker on most lymphocytes in general [10]. Other key activating receptors on NK cells include Fas Ligand (FasL) and TRAIL, known for receptor-mediated induction of cell death, or apoptosis. Similarly, the CD16 receptor (FcyRIII), is a widely studied marker for cytotoxicity of NK cells that functions through the mechanism of Antibody Dependent Cell-mediated Cytotoxicity (ADCC) [8, 11].

Several inhibitory receptors expressed on NK cells also modulate their functions. These include KIR, a type I transmembrane domain receptor, and CD94/NKG2A, a type II transmembrane domain receptor [12]. Both KIR and NKG2A belong to a class of HLA-specific receptors and recognize HLA class I molecules, preventing NK cell-mediated attack on HLA class I expressing healthy cells [8, 12]. The NK cells also express other inhibitory checkpoints to maintain homeostasis of immune cells, including PD-1, TIGIT, CD96 TIM-3, and LAG-3 [12, 13]. The PD-1 receptor is well known as a marker for T cell exhaustion, but it has also been described
on NK cells [13, 14]. Similarly, the Immunoglobulin (Ig) superfamily receptors, CD96 and TIGIT, and coinhibitory receptors, TIM-3 and LAG-3, are known for their expression on T cells also [12].
Figure 2. NK cell receptors. A representative schematic of the diverse repertoire of inhibitory and activating receptors expressed by NK cells. Figure created with BioRender.com.
1.5 NK Cell Functions and Killing Mechanisms

Due to their innate nature, NK cells are the first lymphocytes to respond to stress or infection, while the lymphocytes involved in the adaptive immune response, B and T cells, have a delayed response. Because NK cells are the first responders to infection, it is important for them to be potent in their response. They possess a potent killing ability by various mechanisms (Figure 3). These include the release of cytolysis such as perforin and granzyme B, in which perforin will create holes in the target cells and cytotoxic granzymes are released to induce cell killing [1, 4]. Other mechanisms include the expression of FasL or TRAIL to induce death receptor-mediated apoptosis when bound to the target cells that express the corresponding receptors [4]. Additional mechanisms include secretion of immunoregulatory cytokines such as IFNg and TNFa that leads to increased cell signaling for initiation of the inflammatory response, including recruitment of other immune cells, such as APCs [1, 12]. Another critical mechanism for target cell killing involves ADCC, a process mediated by the CD16 receptor (FcyRIII) on NK cells. For this process, the CD16, which is an Fc receptor on the NK cells, will interact with the Fc portion of antitumor antibodies leading to crosslinking of CD16 that will trigger degranulation, leading to target cell death by apoptosis (Figure 4) [8, 11]. When cells release cytolytic granules and the degranulation process occurs, lysosomal-associated membrane protein-1 (LAMP-1, CD107a) becomes expressed on the surface, indicating a degranulation event and activation of the NK cell [4, 15, 16]. These mechanisms for NK cell-mediated killing are critical for the antitumor and antiviral response.
Figure 3. Mechanisms for NK cell killing. A representative schematic of key mechanisms for potent NK cell killing of a target cell. (1) Release of cytolytic granules, granzyme B and perforin to lyse target cells. (2) Death ligand expression of FasL/TRAIL on the NK cell that will bind Fas/TRAIL-R on the target cell to induce apoptosis. (3) Antibody-dependent cellular cytotoxicity (ADCC) with CD16 expression on the NK cell bound to the Fc region of an antibody bound to an antigen on the target cell to trigger cross-linking and tumor cell lysis. (4) Secretion of immunoregulatory cytokines, IFNg and TNF-a, to initiate cell signaling and cytolysis. Figure created with BioRender.com.
Figure 4. Mechanism for ADCC. A representative schematic of key mechanisms for antibody-dependent cellular cytotoxicity (ADCC), a process mediated by the CD16 receptor on NK cells. First, an antibody will bind the antigen on the tumor cell, followed by binding of the CD16 receptor to the Fc portion of the antibody. This interaction will lead to cross-linking to trigger release of lytic granules and tumor cell lysis. Figure created with BioRender.com.
1.6 Innate Immunity and Cancer

The contributions from innate immunity are critical for the antitumor response and clearance of disease. Immune cells, such as NK cells and macrophages, are important for elimination of tumor cells through cytotoxicity and phagocytosis, respectively [17]. Conversely, myeloid-derived suppressor cells (MDSCs) promote tumor progression by suppressing antitumor immune responses, contributing to immune evasion through various mechanisms, such as elevated production of Arg1 and reactive oxygen species (ROS) [17]. High levels of MDSC have been strongly linked to poor prognosis in several different cancer types [18]. DCs are also key modulators of the antitumor innate response due to their ability to recognize and present tumor-associated antigens (TAAs) to T cells, however, these functions can be severely hindered within the immunosuppressive tumor microenvironment (TME) [17, 19]. Thus, a critical balance of protective immune (innate and adaptive) effector functions vs suppressive tumor promoting factors within the tumor and in the host underly the disease status, while effective therapeutic strategies modulate this balance towards control and elimination of the tumors. Innate immune responses being the first line of defense and important to promote the second line of adaptive immunity, are critical players for disease resolution. In general, NK cells equipped with mechanisms to recognize and eliminate infections, are uniquely positioned to impact cancers with viral etiology, such as cervical cancer and some forms of oropharyngeal cancers associated with infection by human papillomaviruses (HPV).
1.7 HPV and Cancer

Human papillomavirus (HPV) stands as the most prevalent sexually transmitted infection globally, contributing to diseases associated with considerable morbidity and mortality [20, 21]. High-risk HPV strains are accountable for nearly 100% of cervical cancer cases. Additionally, they are implicated in various anogenital cancers, such as 90% of anal cancer cases, 70% of vaginal and vulvar cancers, and approximately 60% of both oropharyngeal and penile cancers [20, 21].

1.7.1 Virology and Immune Evasion of HPV

HPV is a small, non-enveloped virus with a circular double-stranded DNA genome. When inside the host cell, the HPV genome is released into the nucleus where it will replicate using host machinery, particularly during the S phase of the cell cycle. HPV utilizes the host cell's transcriptional machinery to transcribe its genes into messenger RNA (mRNA). The viral mRNA is then translated into viral proteins, including early proteins (E1, E2, E4, E5, E6, and E7) and late proteins (L1 and L2). Early proteins are involved in regulating viral replication and modulating the host cell environment, while late proteins form the structural components of the viral capsid. Mature viral particles are released from the host cell, often through cell lysis or exocytosis, to infect neighboring epithelial cells and continue the viral replication cycle [22, 23].

Overall, more than 200 different HPV strains are identified and categorized into high-risk and low-risk groups based on their potential to cause cancer. Among the most common low-risk strains are HPV6 and HPV11, which typically induce genital warts [21]. Conversely, the two predominant high-risk strains are HPV16 and HPV18.
However, there are at least an additional 15 strains linked to carcinogenic potential [23]. It is estimated that 80% of the global population has been or will become infected with HPV at some point in their lifetime [24]. In the majority of cases, HPV infections are asymptomatic, and are successfully cleared by the immune system [23, 25]. However, persistent infection with high-risk strains can lead to chronic infection, resulting in the integration of the viral genome into the host DNA. The primary drivers of HPV-induced cancers are the viral-encoded oncoproteins E6 and E7. The E6 protein functions by inducing the degradation of the host tumor suppressor protein p53, while E7 prevents the binding of retinoblastoma (Rb) to the E2F protein, thus activating the cell cycle and promoting proliferation [22, 23]. Thus, expression of E6 and E7 proteins can induce uncontrolled cell proliferation and ultimately lead to the transformation of healthy cells into cancerous cells.

Additionally, HPV possesses several mechanisms for evading the host immune response. One of its primary mechanisms includes its localization to the epithelium, where it can remain undetected by the host immune system. This localization prevents HPV from being exposed to immune surveillance in the bloodstream or lymphatic system, allowing it to evade detection and clearance. Additionally, HPV has potential to downregulate interferon (IFN) signaling and impair antigen presentation, further evading the host immune response [21, 22]. The IFN signaling serves as a crucial component for the innate immune response, emphasizing the importance of innate immunity for clearance of HPV.
1.7.2 Global Burden of HPV

HPV accounts for the cause of approximately 5% of cancers globally [23]. Its prevalence is highest in women with cervical cancer, the fourth most diagnosed cancer in women. Importantly, the frequency of diagnosis is higher in less developed regions, with Asia and Africa accounting for the most cervical cancer cases and deaths [26]. Despite the FDA approval and availability of prophylactic HPV vaccines (Gardasil 9 and Cervarix), HPV infections remain high [26]. Studies report the high prevalence of disease related to limited access to healthcare resulting in infrequent or absence of screenings for pre-cancerous lesions, allowing progression of disease [27, 28].

1.7.3 Major HPV Cancers

HPV is capable of induction of various types of oral and genital cancers. As described earlier, cervical cancer stands as the most prevalent HPV induced cancer, with HPV16 indicated as the most prevalent strain responsible, followed by HPV18 and HPV45 [26]. Anogenital cancers, including anal, vaginal, vulvar, and penile, are also prevalent but less studied than cervical cancer. HPV also induces oral cancers, where HPV16 is the predominant strain for infection [29]. Factors such as smoking tobacco increase risk of oral HPV cancers [29].

1.7.4 Therapeutics for HPV Cancers

Currently, the standard of care (SOC) for HPV cancers includes a regimen of chemoradiation treatment (CRT). Despite the fact that CRT is an established effective treatment, frequency of mortality is still high [30], indicating a need to identify more effective treatment methods. Immunotherapy has become an increasingly popular strategy for the treatment of cancer in the past few years with reasonable success.
rates [31, 32]. It encompasses checkpoint blockade, therapeutic vaccines, and cellular therapies. Clinical studies with PD-1 blockade, specifically in patients with poor response to SOC, have shown promise for improved survival [33]. The monoclonal antibody for blocking PD-1, pembrolizumab, is approved with or without chemotherapy for metastatic and recurrent head and neck cancers and is approved in advanced cervical cancer if disease progression continues during or after chemotherapy treatment [31]. While there is not an approved therapeutic vaccine, HPV-associated cancer serves as a promising model due to the ability to target the viral antigens, as true foreign antigens. Cellular therapies involve adoptive T cell and NK cell therapies, where cells are infused into the patient to enhance the immune response [31]. Overall, immunotherapy represents a promising therapeutic approach for HPV induced cancers.

1.8 NK Cells in the Context of HPV Cancers

NK cells are well known for their antiviral properties and are therefore crucial effectors for the immunosurveillance of HPV. The combined antiviral and antitumor properties of NK cells make NK cell therapy an attractive subject of study. Additionally, the downregulation of IFNg, a key NK cell activator, by the E6 and E7 oncoproteins indicates a need for replenishing NK cells within the host [34]. The studies related to NK cellular therapy, particularly in HPV cancers, are limited [34].

1.9 Defining HLA-DR+ NK Cells

As discussed earlier in this section, the NK cell population is heterogenous, and the functions depend on their receptor expression. Among these receptors is HLA-DR, a recognized activation marker on CD8 T and NK cells with correlation to patient
outcome in several instances [35-37]. Literature studies characterize HLA-DR+ NK cells as a highly functional subset with a less mature phenotype and are referred to as pre-mature NK cells (pre-mNK) [37, 38]. The antitumor potential for pre-mNK cells has been demonstrated by several mechanisms. In vitro studies have shown HLA-DR expression associated with proliferation activity in NK cells in addition to increased degranulation of target cells [39]. Additionally, the cytotoxic capabilities of pre-mNK can be significantly enhanced by a multitude of manipulations, such as simulation with cytokines, including IL-12, IL-15, and IL-18, and TLR agonists, such as CpG-ODN [38, 40]. In mice, NK cells expressing the dendritic cell (DC) marker CD11c are analogous to the pre-mNK cell in humans and are referred to as natural killer dendritic cells (NKDC), and because these cells produce high levels of IFNg, are also described as interferon-producing killer dendritic cells (IKDC) [40, 41].

There is limited research [37, 38] defining pre-mNK in the context of cancer disease progression or response, and further identifying this activated NK cell subset could provide better insight into their application for antitumor immunity, disease response, and cellular therapies. The present investigation uses preclinical mouse models of HPV cancers and samples from HPV+ cervical and oropharyngeal cancer patients to enumerate and characterize this NK cell subset along with in vivo and ex vivo approaches to harness their antitumor functional potency.
CHAPTER 2. MATERIALS AND METHODS

2.1 Animals

C57BL/6J male and female mice (6-8 weeks) were procured from The Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen-free environment. All animal studies were pre-approved and carried out in accordance with the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC) guidelines. Mice were monitored daily for overall health and euthanized according to IACUC criteria.

2.2 Tumor Cell Lines

The mEER tumor cells are mouse tonsil epithelial cells expressing HPV-16 E6 and E7 along with H-ras and were a kind gift from Dr. John Lee (Avera Cancer Institute, Sioux Falls, SD). These cells were maintained in complete Dulbecco’s Modified Eagle Medium (DMEM)/F12 containing various supplements as previously described [42] and sub-cultured at 80% confluence the day before using for tumor induction in mice. The TC-1-luc tumor cell line is of lung epithelial origin from C57BL/6 mice and express the E6 and E7 oncogenes of HPV-16 as well as firefly luciferase and H-ras. This cell line was a kind gift from Drs. T.-C. Wu and C. Hung (Johns Hopkins School of Medicine, Baltimore, MD). The TC-1luc cells were maintained in complete Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals), 50 units/mL of penicillin–streptomycin and 50 μg/mL gentamycin. Actively growing cells at 80% confluence the day before using for tumor induction in mice as described before [43].
2.3 *In vivo* Tumor Challenge

Syngeneic male C57BL/6 mice were implanted with $4 \times 10^4$ mEER cells in 50 μl phosphate-buffered saline (PBS) into the base of the tongue as described previously [43, 44]. Mice were monitored twice weekly for body weight changes. Mice with oral tumors were euthanized when the mice lost 20% or more of their initial body weight. For the experiments involving analysis of tumor-infiltrating leukocytes (TIL), $1 \times 10^5$ mEER tumor cells in PBS were mixed in a 2:1 ratio with Matrigel (BD Biosciences, San Jose, CA) for implantation. For intravaginal tumor challenge, $2 \times 10^4$ TC-1 cells were implanted in the vaginal tract of diestrus synchronized 6- to 8-wk-old female C57BL/6 mice according to a previously described protocol [45, 46]. Intravaginal TC-1 tumor growth was monitored using *In vivo* Imaging System (IVIS), a small animal imaging system (Perkin Elmer, Waltham, MA) and expressed as average luminescent signal in select Region of Interest (ROI) (p/sec/cm²/sr) as a measure of tumor size. For the TIL experiments, $3 \times 10^4$ TC-1 cells mixed 2:1 with Matrigel was used for intravaginal implantation.

2.4 Intranasal vaccination

The therapeutic vaccine consisted of the following four peptides corresponding to the HPV-16 E6 and E7 oncoproteins; the E6_{43-57} peptide, Q15L (QLLRREVYDFAFRDL); the E6_{49-58} peptide, V10C (VYDFAFRDLC); the E7_{44-62} peptide, Q19D (QAEPDRAHVYNIVFCCKCD); and the E7_{49-57} peptide, R9F (RAHVYNIVTF) [47, 48]. Vaccine grade (>90% pure) peptides were purchased from Elim Biopharma (Hayward, CA, USA), and used at 100 μg per dose of each, along with single or combinations of adjuvants (10 μg CpG-ODN, 5 μg QS-21, or 2 μg
aGalCer). The R9F and V10C peptides represent murine H2b-restricted CD8 T cell epitopes. Vaccine grade ODN 1826 was purchased from InvivoGen (San Diego, CA) and GMP grade QS-21 was from Desert King (San Diego, CA). The ODN 1826 is a type B CpG-ODN specific ligand for mouse TLR9, and is known to induce Th1 response in addition to activation of B cells and is a weak stimulator of IFNα and plasmacytoid DC [49, 50]. The adjuvant aGalCer was purchased from DiagnoCline (Hackensack, NJ, USA). All adjuvants were reconstituted according to the manufacturers’ recommendations. Tumor-bearing mice were randomized and on days 5 and 11 following tumor implantation vaccines were administered via intranasal route as described previously [43, 46]. Untreated mice which received intranasal endotoxin free PBS or those treated with mixture of peptides and individual adjuvants served as control groups.

2.5 Magnetic Resonance Imaging (MRI)

In the mEER oral tumor model, tumor volume was measured using MRI. Mice were imaged under 2% isoflurane vapor anesthesia in 7T small animal MR scanner (Biospec, Bruker Biospin Inc., Billerica, MA) using transmit/receive volume coils with 35 mm inner diameter. The following parameters and settings were used for acquiring image sequences: field-of-view 4x3 cm², matrix 256x192 and spatial resolution 156 microns; a T2-weighted coronal Rapid Acquisition with Relaxation Enhancement (RARE) with T2-Sagital, echo time 38 ms, repetition 1800 ms, slice thickness 0.50 mm and slice gap 0.50 mm. Tumor volumes were calculated based on the analysis of image sequences in three dimension using Image J software (NIH, Bethesda, MD) after defining the region of interest (tumor) on all possible sections.
2.6 Immune Cell Isolation

On day 15 or 16 post-tumor challenge, mice were euthanized and tumors, spleens, tumor draining lymph nodes (LN) were collected to isolate cells that were used to characterize cell-mediated anti-tumor immune responses. Briefly, tumors were digested in complete RPMI media containing Collagenase H (Sigma-Aldrich, St. Louis, MO) and DNase (Roche, Indianapolis, IN) and incubated at 37 °C for 45 min before passing through 70 μm cell strainers. Tumor-infiltrating leukocytes (TIL) were isolated using 67%:44% Percoll (Cytiva, Marlborough, MA) gradient centrifugation. Single cell suspensions from spleens were prepared by mechanical disruption and passing through cell strainers followed by red blood cell lysis. In mice with mEER oral tumors, the cervical lymph nodes were collected and in mice with TC-1 vaginal tumors, the inguinal lymph nodes were collected. In both cases, these tumor-draining lymph nodes were processed by mechanical disruption through 70 μm cell strainers followed by centrifugation and re-suspension in complete RPMI medium.

2.7 Flow Cytometry

Following isolation, aliquots of cells were incubated at 37 °C, 5% CO₂ for 4-6 hours with GolgiPlug/Brefeldin A (ThermoFisher, Waltham, MA). Subsequently, the cells were incubated with the mouse Fc-block (anti-CD16/32) reagent, stained for surface markers, fixed and permeabilized with intracellular/Foxp3 Fix-Perm reagent kit from eBioscience (ThermoFisher, Waltham, MA) followed by staining for intracellular/functional markers (Tables 1-3). All FACS data acquisitions were done on a five-laser Fortessa X-20 flow cytometer (BD Bioscience) and analyzed using FlowJo version 10 (FlowJo LLC, Ashland, OR). Forward and side scatter parameters were
used to set singlets and leukocyte gates. Fixable viability stain 510 included in surface antibody cocktail was used to gate-out dead cells and analyze only viable cells. HPV-16 E7 antigen-specific CD8 T cells were detected using a specific tetramer reagent that was part of the multiparametric panel. The APC-labeled H-2Db epitope E7\textsubscript{49–57} (RAHYNIVTF)-peptide-loaded tetramer was obtained from NIH MHC tetramer core facility at Emory University (Atlanta, GA). For the detection of NKDC, gating strategy involving CD3-NK1.1\textsuperscript{+} CD11b\textsuperscript{(int)} followed by expression of CD11c\textsuperscript{+} (Figure 5) was used as described by Terme \textit{et al.}, [51]. Details of the different antibodies used for analyzing mouse cell are listed in Table 1. For the detection of pre-mNK, gating strategy involving CD3-CD56\textsuperscript{+} (NK cells) followed by expression of HLA-DR\textsuperscript{+} (Figure 6) was used. Details of the different antibodies used for analyzing mouse cell are listed in Tables 2 and 3. Two separate panels were used for more comprehensive review of NK cell receptors than the 18-color maximum allotted for the flow cytometer.
Table 1. Antibodies for flow cytometry analysis of immune cells in mice.

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**Table 2.** Multiparametric 15-color flow cytometry panel for characterization of human NK and pre-mNK cells.

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Table 3. Multiparametric 16-color flow cytometry panel for characterization of human NK and pre-mNK cells.

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Figure 5. Flow cytometry gating strategy for NKDC in mice. Gating shown for the different functional markers (GranzB+, IFNγ+, and polyfunctional GranzB+IFNγ+) and antigen-specific (E7 tetramer+) CD8 T cells assessed.
Figure 6. Flow cytometry gating strategy for pre-mNK cells in human.

Representative gating strategy for the CD56+HLA-DR+ cells (pre-mNK) in human PBMC.
2.8 Patient Samples

The patient sample collection was performed by the collaborating radiation or gynecologic oncologists (for cervical cancer patients) or head and neck surgeons (for the head and neck cancer patients) under intuitionally approved IRB protocols and were supplied for the lab research studies using only coded numbers to protect patient-specific information.

Cervical cancer patient samples: Patients with locally advanced cervical cancer undergoing standard-of-care (SOC) chemoradiation were consented to the study and enrolled on a University of Texas MD Anderson Cancer Center (MDACC) institutional review board (IRB) approved prospective biomarker collection study (MDACC 2014-0543, 2019-1059). As previously described [52, 53], a visible cervical tumor was required for sampling.

Head and neck cancer patient samples: Patients with HPV+ head and neck squamous cell carcinoma (HNSCC) undergoing SOC chemoradiation were consented to the study and enrolled on a University of Texas MD Anderson Cancer Center (MDACC) institutional review board (IRB) approved study (LAB09-0773).

2.9 Patient Sample Collection and Processing

The cervical brushing was collected by an attending radiation or gynecologic oncologist by directly sampling the visible exophytic cervical tumor, as previously described [52, 53]. To ensure sufficient collection of cells from the tumor, the brush was rotated against the visible tumor surface. The cervical tumor cytobrush samples were collected by a physician at sequential time points: T1: baseline, T2: one week, T3: three weeks, T4: five weeks, and T5: first follow-up visit (12 weeks post-treatment),
as previously described [53, 54] (Figure 7A). Brushes were placed into individual conical tubes with saline and immediately transported at room temperature to the lab. In the lab, 10 mLs of sterile complete RPMI-1640 media, containing 1% penicillin-streptomycin and gentamicin antibiotics (HyClone, Corning, Lonza, respectively) and 10% fetal bovine serum (FBS, Corning), were added to each tube, which were then vortexed for 1 min to dislodge and suspend cells. When large amounts of mucus were present, 5 mLs of dithiothreitol solution (1X Hank’s balanced salt solution, 4% bovine serum albumin, 2 mM dithiothreitol; Invitrogen) were added to the cell suspensions and passed through a 70-mm cell strainer into new conical tubes. Cells were pelleted by centrifugation and resuspended in sterile complete RPMI-1640 media for counting. Aliquots of cells were plated in duplicate wells of a 96-well round bottom plate for flow cytometry staining. Representative gating strategy for cytobrush is shown in Figure 7B.

Peripheral blood was also collected at the indicated timepoints (T1-T5) from cervical cancer patients. Similarly, samples were collected at pre-treatment and at 3-month follow-up (post-treatment) from head and neck patients. Flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) was performed as previously described [52, 54].
Figure 7. Schema of cytobrush and blood sample collection in cervical cancer patients at sequential timepoints: T1=Baseline, T2=1 week, T3=3 weeks, T4=5 weeks, and T5=3-month follow-up (A). Representative gating strategy for flow cytometry analysis of tumor infiltrating lymphocytes (TILs) isolated from a cytobrush sample (B).
2.10 NK Cell Expansion

Aliquots of PBMC were incubated with magnetically labeled CD56+ MicroBeads (Miltentyi Biotec Bergisch Gladbach, Germany) and processed for positive selection of NK cells through MACS LS columns as per manufacturer's protocols [55]. Isolated NK cells were counted, re-suspended, and plated in NK MACS Medium (Miltenyi Biotec, Bergisch Gladbach, Germany) and expanded for 14 days according to Miltenyi Biotec protocol, with fresh media with cytokines replenished every 2 days starting at day 5 [56]. The following recombinant human cytokines were added to the expansion medium: 500 IU/mL IL-2 (Peprotech Inc., Waltham, MA), 140 IU/mL IL-15 (Peprotech Inc., Waltham, MA), and IL-18 (R&D Systems, Minneapolis, MN). Flow cytometry staining and analysis was performed at the beginning and on Days 7 and 14 of expansion.

2.11 Statistical Analysis

All graphing and statistical analyses were performed using GraphPad Prism version 8 (San Diego, CA). One-way ANOVA followed by Tukey post-hoc test was used for comparing multiple groups or two-sample Welch’s t-test was applied for comparing two groups. Wilcoxon matched-pairs test was used for analysis of paired samples. Statistical significance between survival curves was calculated using a Log-rank test (Mantel-Cox). Graphs show mean ± standard deviation (SD) for each group unless otherwise indicated. P values of <0.05 were considered significant. Experiments were repeated at least twice to confirm the reproducibility of results.
CHAPTER 3. RESULTS

3.1 Introduction of Preclinical Studies

Previous research in our lab focused on using preclinical mouse models to evaluate the therapeutic efficacy of HPV peptides identified earlier as markers of memory immunity for recurrence-free survival post-excisional treatment in HPV-positive patients with high-grade cervical intraepithelial neoplasia [47, 48]. This HPV peptide-based therapeutic vaccine formulated with aGalCer and CpG-ODN adjuvants (TVAC) when tested in the TC-1 vaginal HPV tumor model afforded long-term tumor free survival for 85% mice and revealed antigen-specific CD8 T cells as a primary contributor to the antitumor efficacy [28]. Based on this impressive protection in the vaginal tumor model, we assessed intranasal delivery regimen for TVAC in the mEER oral HPV tumor model (Figure 8A) and observed it to be ineffective in reducing tumor growth or providing significant survival advantage (Figure 8B).

These results suggest that immune responses in addition to, or in place of, TVAC-induced antigen-specific CD8 T cells may be necessary in this model.

Hypothesis: We hypothesized that changing the adjuvant combination in the vaccine may expand the immune repertoire to include potent innate immune responses, in addition to antigen-specific CD8 T cells, to protect mice with either oral or vaginal tumors.

Rationale: Based on the literature reports showing the effectiveness of the saponin adjuvant QS21 to induce balanced Th1 and Th2 responses, and the fact that QS21 adjuvant is included in FDA approved human vaccines (e.g. Shingrix), we formulated
a modified therapeutic vaccine by replacing aGalCer in TVAC with QS21 for combination with CpG-ODN (TVQC) [57].

Specific aims: To test the hypothesis, we developed the following aims:

- **Aim I.** Evaluate the effectiveness of TVQC in the mEER oral HPV tumor model and determine the immune correlates.
- **Aim II.** Investigate whether TVQC would be efficacious in the TC-1 vaginal HPV tumor model and determine the immune correlates.

3.1.1 **Aim I: Evaluate the effectiveness of TVQC in the mEER oral HPV tumor model and determine the immune correlates.**

Syngeneic male C57BL/6 mice were implanted with $4 \times 10^4$ mEER cells in 50 μl phosphate-buffered saline (PBS) into the base of the tongue as described before [43, 44]. Mice with visible tongue tumors by day 5 were randomized and either untreated or administered TVQC, or the therapeutic vaccine containing single adjuvants QS21 (TVQ) or CpG-ODN (TVC) by the intranasal route on days 5 and 11 after tumor implantation. Tumor volume determined based on MRI measurements of oral tumors on day 18 showed significant reduction in tumor size in TVQC treated mice compared to untreated mice (Figure 9A). Importantly, 72% of mice treated with TVQC showed sustained tumor regression and extended survival for 10 weeks following tumor challenge that was significantly higher when compared to control groups including untreated mice (Figure 9B).

Analysis of tumor infiltrating leukocytes (TIL) in TVQC treated mice, in comparison to that for tumors from TVAC treated mice showed that TVQC was
effective in inducing total CD8 T cells and HPV E7 antigen-specific CD8 T cell responses, that were higher in mice treated with TVQC (Figure 10AB). Thus, these data while clearly demonstrate enhanced effectiveness of TVQC, relative to TVAC to protect against oral HPV tumors, the immunological correlates beyond the adoptive HPV-specific CD8 T cells are unclear.

We therefore carried out multiparametric flow cytometry analyses of TIL from mice treated with the two vaccines in comparison to those in the untreated mice, focusing on the levels of innate immune NK cell responses. The total (Figure 10A) and antigen-specific (Figure 10B) CD8 T cells were elevated in the tumor with both vaccines, with TVQC reaching higher significance. We observed significantly elevated frequencies of total NK cells as well as those expressing the functional markers, granzyme B and/or IFNg in mice treated with TVQC (Figure 11A). Importantly, a subset of total and functional NK cells that express the DC marker CD11c, referred to in the literature as NKDC [40, 58], were significantly higher in mice treated with TVQC compared to TVAC (Figure 11B). Importantly, we observed significantly elevated frequencies of total as well as granzyme B and/or IFNg expressing CD11c+ NK cells (NKDC) compared to the CD11c- subset of NK cells within the tumor microenvironment (TME) of TVQC treated mice (Figure 11C). Together these data highlight the importance of innate effector immunity, specifically NKDC, for the therapeutic efficacy of TVQC in the oral HPV tumor model.
Figure 8. TVAC does not provide protection in the oral HPV tumor model. Syngeneic C57Bl/6 mice were injected with mEER tumor cells into the base of the tongue as indicated and treated with intranasal (IN) delivery of the indicated E6/E7 HPV peptide therapeutic vaccine formulations on days 5 and 11 post-tumor implantation (A). Untreated mice (UNTR) and mice vaccinated with single adjuvants (TVA, TVC) served as controls. Mice in all the groups were monitored overtime and Kaplan-Meier survival curves are shown for mice treated with TVAC (B).
Figure 9. Efficacy of TVQC in the oral HPV tumor model. Syngeneic C57Bl/6 mice were injected with mEER tumor cells as indicated and treated with intranasal (IN) delivery of the indicated E6/E7 HPV peptide therapeutic vaccine formulations on days 5 and 11 post-tumor implantation. Untreated mice (UNTR) and mice vaccinated with single adjuvants (TVC, TVQ) served as controls. The oral tumor volume for mice in the untreated and TVQC treated mice determined using Magnetic Resonance Imaging (MRI) analyses on day 18 showed significant reduction of tumor size in vaccinated mice (A). Mice in all the groups were monitored over time and Kaplan-Meier survival curves are shown for mice treated with TVQC (B). Mantel-Cox log-rank test **p<0.005, ****p<0.0001 (n = 5-25 mice per group) shows significant regression of mEER oral tumors with TVQC vaccine. Welch’s t-test, *p<0.05.
Figure 10. Adaptive immune responses induced by both TVAC and TVQC. Elevated frequencies of total (A) and antigen-specific CD8 T cells (B) were observed in the tumor with both vaccine formulations. Significance determined using Ordinary one-way ANOVA *$p<0.05$, **$p<0.005$ ***$p<0.0005$, ****$p<0.0001$ (n = 5-6 mice per group).
Figure 11. TVQC induces a more robust innate effector response than TVAC in mice with oral HPV tumors. Syngeneic C57Bl/6 mice were injected with mEER tumor cells as indicated and treated with intranasal (IN) delivery of TVAC or TVQC or untreated (UNTR). TVQC induced significantly elevated frequencies of total and functional (granzyme B and/or IFNg expressing) NK cells (A), and NKDC (B) in the TME. Significance determined using Ordinary one-way ANOVA *p<0.05 (n = 5-6 mice per group). Within the TVQC vaccinated mice, the total and functional CD11c-/- NK
cells were compared with the CD11c+ subset (NKDC) exhibiting higher frequency and functionality in the TME (C). Significance determined using Wilcoxon matched pairs test ****p<0.0001, ***p<0.0001 (n = 19 mice).
3.1.2 *Aim II: Investigate whether TVQC would be efficacious in the TC-1 vaginal HPV tumor model and determine the immune correlates.*

We further evaluated whether TVQC, effective against oral HPV tumors in the mEER model would also be useful to treat vaginal HPV tumors using the TC-1 model, commonly used as the immunocompetent preclinical surrogate for HPV+ genital cancers [46, 59]. Syngeneic C57BL/6 female mice were implanted with intravaginal TC-1luc tumor cells as described in Methods and treated with TVQC delivered by the intranasal route on days 5 and 11 post-tumor implantation (Figure 12A). We observed tumor regression (Figure 12B) along with significant survival advantage in 80% of the vaccinated mice (Figure 12C). The therapeutic efficacy of TVQC correlated with significant induction of total and IFNg producing tumor-antigen-specific CD8 T cells (Figure 12D), and NKDC expressing granzyme B or IFNg or both (Figure 12E) in the tumor when compared to untreated controls.
Figure 12. TVQC-mediated protection against HPV vaginal tumors. Syngeneic C57BL/6 female mice were implanted with intravaginal TC-1Luc tumors as described in Methods and treated with intranasal HPV peptide therapeutic vaccine formulations as indicated on days 5 and 11 post-tumor implantation (A). Intravaginal TC-1 tumor size was monitored by luciferase imaging using IVIS bioluminescence imaging system and tumor growth curves for indicated groups are shown (B). Survival curves are shown for intravaginal TC-1 tumor-bearing mice as indicated (C). Mantel-Cox Log-Rank test was used to determine the survival significance between the groups *p<0.05, **p<0.005 (n = 10-13 mice per group). Immune correlates associated with vaccine efficacy were assessed by flow cytometric analysis of TIL performed on day 16 following tumor implantation. Frequencies of total and IFNγ+ E7 tetramer+ CD8 T
cells (D), and NKDC expressing granzyme B or IFNg or both are shown (E). Significance determined by unpaired t-test, *p<0.05, **p<0.01 (representative data from one experiment with n = 3 mice per group, experiment repeated one more time).
Overall, these data demonstrate significant contributions of innate immunity from the NK subset, NKDC, for vaccine mediated protection in the preclinical HPV tumor models and formed the basis for investigating for their potential role in HPV+ cervical and oropharyngeal cancer patients.

### 3.2 Potential contributions of Innate NK cell immunity in HPV+ cervical and oral cancers and response to standard of care (SOC) treatment

The NK cells are a major effector subset of innate immunity, and they comprise approximately 5-20% of circulating immune cells in normal healthy humans [5]. The functions of NK cells depend on a wide array of activating and inhibitory receptors [4, 12]. Among these receptors is HLA-DR, a known activation receptor that is also observed on CD8 T cells [36]. The HLA-DR+ NK cell subset is known as premature NK cells (pre-mNK). There is limited literature on this subset [38, 39, 60, 61], especially in the context of HPV+ cancers, and there is a need to identify its significance in the antitumor response.

**Hypothesis:** We hypothesized that the NK subset expressing HLA-DR in humans (pre-mNK) provides significant contributions for antitumor innate immunity in HPV+ cancers.

**Specific aims:** To test this hypothesis, we developed the following aims:

- **Aim I.** Determine the changes in frequency and functionality of pre-mNK in HPV+ head and neck squamous cell carcinoma (HNSCC) and cervical cancer patients undergoing standard of care (SOC) treatment.
• Aim II. Investigate ex vivo strategies for the expansion of pre-mNK and determine their antitumor activity.

3.3 Aim I. Determine the changes in frequency and functionality of pre-mNK in HPV+ head and neck squamous cell carcinoma (HNSCC) and cervical cancer patients undergoing standard of care (SOC) treatment.

3.3.1 Increased Pre-mNK with Dysfunctional Phenotype in HPV+ Cancer

Patients

Circulating immune cells were isolated from the blood of healthy donors and HPV+ cancer patients and analyzed by multiparametric flow cytometry (Figure 6). We observed comparable levels of CD4+ and CD8+ subsets of T cells between HPV+ patients and healthy donors (Figure 13A). Similarly, the levels of total NK cells were not different (Figure 13B), but significantly higher frequencies of circulating pre-mNK were observed in HPV+ patients compared to healthy donors (Figure 13C). However, the pre-mNK in patients, relative to those in the normal healthy donors, exhibited hindered functionality indicated by elevated frequencies expressing the inhibitory receptor PD-1 (Figure 14A), and reduced functionality in terms of the cytotoxicity marker granzyme B (GrnzB) (Figure 14B), and the CD16 receptor, a marker for antibody-dependent cellular cytotoxicity (ADCC) (Figure 14C).
Figure 13. Elevated frequencies of circulating pre-mNK in HPV+ cancer patients.

The frequencies of CD4 and CD8 T cells (A), as well as total NK cells (B) were similar between HPV+ patients ($n = 36$) compared to healthy donors ($n = 14$). Significantly increased frequencies of HLA-DR+ NK cells (pre-mNK) were observed in HPV+ patients, relative to healthy donors (C). Representative flow cytometry gating strategies from donor and patient are shown. Significant differences for the various subsets in patients and donors were evaluated using the Welch’s t-test $^*p<0.05$. 
Figure 14. Circulating pre-mNK display a dysfunctional phenotype in HPV+ cancer patients. Significantly increased frequencies of inhibitory receptor PD-1 (A), reduced cytotoxicity (Gnrb2+) (B), and decreased frequencies of ADCC receptor, CD16, on pre-mNK cells of HPV+ patients ($n = 36$) compared to those in healthy donors ($n = 14$) (C). Representative flow cytometry plots for indicated populations are shown. Significant differences for the various subsets in patients and donors were evaluated using the Welch’s t-test $**p<0.005$, $****p<0.0001$. 
3.3.2 Pre-mNK Cells Gain Functionality Post-Treatment in HPV+ H&N Patients

To further understand the potential role of pre-mNK cells in HPV+ cancers, we used multiparametric flow cytometry to assess changes in the frequency and functionality of circulating pre-mNK and total NK in HPV+ head and neck patients pre- and post- SOC treatment ($n = 8$). There was no change in the frequency of total NK cells (Figure 15A), but pre-mNK levels were reduced at the post-treatment timepoint (Figure 15B). However, post-treatment, we observed significant increases in the levels of pre-mNK cells exhibiting cytotoxic functionality, in terms of granzyme B expression (Figure 16A) as well as CD16, the marker for ADCC (Figure 16B), while the levels of PD-1 expression were unchanged (Figure 16C). Together, these results support increased functionality of circulating pre-mNK in head and neck patients after SOC treatment, highlighting the potential importance of this innate immune subset in the antitumor immune response. Due to low samples size, specifically in patients with disease progression, we were unable to determine a correlation with protection in terms of clinical outcome.
Figure 15. Circulating immune cells in HNSCC patients pre- and post-treatment with standard of care (SOC). No changes in total NK cells were observed after SOC treatment (n = 8) (A). Frequencies of pre-mNK were reduced at the post-treatment timepoint (B). Representative flow cytometry gating strategy is shown to the left of each panel. Significance of changes were assessed with Wilcoxon matched-pairs test, **p<0.005. The far-right panels show changes in pre-mNK frequencies at matching time points for each patient.
Figure 16. Circulating pre-mNK gain functionality after SOC treatment in HPV+ head & neck patients. A significantly increased frequency of functional pre-mNK is observed in circulation of head and neck patients \((n = 8)\) from pre-treatment (Pre) to post-treatment (Post) in terms of increased expression of GrnzB (cytotoxicity) (A), and CD16 (ADCC) (B). No changes were observed in the frequencies of pre-mNK expressing the inhibitory receptor, PD-1 (C). Representative flow cytometry plots for the indicated populations are shown to the left of each panel. Significance of changes
were assessed with Wilcoxon matched-pairs test, **p<0.005. The far-right panels show changes in pre-mNK frequencies at matching time points for each patient.
3.3.3 Increased Cytotoxic Pre-mNK in the TME of Cervical Cancer Patients

After Treatment

We also assessed changes in the levels of pre-mNK in cervical cancer patients undergoing SOC chemoradiation. For this, we collected blood as well as cytobrush samples, the later a non-invasive procedure for monitoring the tumor microenvironment (TME), as reported earlier [52] (Figure 7A). A representative flow cytometry gating strategy from a cytobrush sample is shown (Figure 7B). Immune cells were isolated from blood and cytobrush samples as previously indicated [52, 53]. No significant differences were observed in the frequencies of total NK (Figure 17A) and pre-mNK cells (Figure 17B) from the baseline (T1) to 5 weeks (T4) treatment time point in both the TME and PBMC. We also did not observe any differences in the levels of pre-mNK cells expressing the inhibitory marker, PD-1 (Figure 17C), and the ADCC marker, CD16, in TME or PBMC (Figure 17D). However, we observed a robust induction of cytotoxic (GrnzB+) pre-mNK cells at the T4 timepoint compared to baseline within the TME, but not in the blood (Figure 17E). A representative flow cytometry gating strategy from GrnzB+ pre-mNK in the TME at T1 and T4 is shown (Figure 17F). Importantly, when separated into subsets of total, HLA-DR+, and HLA-DR- NK cells, only the HLA-DR+ NK subset (pre-mNK) showed significantly increased frequencies of granzyme B at the later treatment timepoint (Figure 17G), which amounted to a 9.3-fold increase from T1 to T4 (Figure 17H). These preliminary data support the hypothesis that treatment-related increases in the levels of functional pre-mNK cells may contribute to antitumor immunity. A limitation of the data presented in cervical cancer patients includes analysis while treatment is ongoing (5 weeks).
instead of the 3-month follow-up post-treatment timepoint due to limited sample size. Furthermore, analysis of the TME at the post-treatment timepoint can be difficult due to limited cell counts caused by tumor regression.
Figure 17. Elevated intra-tumoral cytotoxic pre-mNK levels in the TME of cervical cancer patients during treatment. Cytobrush and blood samples were collected at baseline (T1) and during week 5 of treatment (T4) from cervical cancer patients undergoing SOC chemoradiation treatment. Immune cells were isolated from samples as indicated and analyzed by flow cytometry for the levels of total NK and pre-mNK cells in the PBMC and TME at the T1 and T4 timepoints (A,B), along with the levels of expression of inhibitory marker, PD-1 (C), ADCC marker, CD16 (D), and GrnzB (E) in the TME and PBMC. Representative flow cytometry plots for GrnzB+ pre-mNK from a cytobrush sample at baseline and T4 (F). The increased frequency of GrnzB+ in the TME is observed only in pre-mNK, while there are no significant changes in total NK or the HLA-DR- NK subset (G). The GrnzB+ pre-mNK increase 9.3-fold from the T1 to T4 timepoint (H). Significance of changes were assessed with
Welch’s t-test, ***$p<0.0005$. Cytobrush: T1 ($n = 6$), T4 ($n = 9$). PBMC: T1 ($n = 20$), T4 ($n = 14$).
3.4 Adoptive NK Cell Therapies

Adoptive cell therapies involving *ex vivo* expansion and infusions of autologous T cells for the treatment of cancer patients is a common clinical practice [6, 7]. NK cells are key innate immune effector populations and are amicable for adoptive transfer to patients irrespective of the MHC repertoire expression. Indeed, NK cell-based immunotherapies are increasing in popularity in recent years due to the fast-acting antitumor capabilities of NK cells, relatively simple methods for activation, and established safety profiles [6]. Due to low frequency in circulation (PBMC), a multitude of approaches for preferential expansion of NK cells have been studied [5]. Stimulation with cytokines, including IL-2, IL-12, IL-15, IL-18, and IL-21 in various combinations, is among one of the most common methods for expansion of NK cells and provides a means to generate highly activated NK cells [5, 6]. Of note, the immunoregulatory cytokine IL-18, has been indicated in literature [62] to promote the expansion of NK cells, particularly the HLA-DR+ (pre-mNK) subset.

Therefore, reasoned that such *ex vivo* expansion protocols could potentially be useful to harness the cytotoxic properties of pre-mNK.

**Hypothesis:** We hypothesized that the pre-mNK subset will preferentially increase in frequencies from NK cell *ex vivo* expansion protocols involving the use of mixtures of immune modulating cytokines that include IL-18.

To test this hypothesis, we compared two *ex vivo* NK expansion strategies with the combination of IL-2 and IL-15 in the presence and absence of IL-18.
3.4.1 Increased Frequency and Functionality of Pre-mNK from Cultures Including IL-18

We used PBMCs isolated from healthy donor buffy coat samples to purify NK cells by the MACS separation protocol and subjected to ex vivo expansion employing the combination of IL-2 (500 IU/mL) and IL-15 (140 IU/mL) along with or without IL-18 (100 ng/mL) for culturing over two weeks, as described in the Materials and Methods. Kinetics of changes in the total NK cells and pre-mNK subset were analyzed by flow cytometry on days 7 and 14 (Figure 18A). We observed that the levels of pre-mNK were significantly elevated at the same rate in the presence and absence of IL-18 by day 7, increasing from 2% to ~50% of the total NK population. However, by day 14, cultures containing IL-18 in the medium showed significantly higher frequency of pre-mNK, comprising 89% of total NK compared to 53% in the group without IL-18 (Figure 18BC). In addition to the sustained increase of total pre-mNK in the presence of IL-18, a substantial increase of functional markers expressed on pre-mNK is observed, which included proliferation (Ki67+), ADCC receptor (CD16+), and cytotoxicity (GmzB+) (Fig. 18D-F). These data not only match those in the literature for ex vivo culturing of NK cells from healthy donors, but also reveal significant and specific expansion of the highly functional HLA-DR+ NK subset (pre-mNK) with the cytokine combination of IL-2, IL-15, and IL-18.
Figure 18. Addition of IL-18 to the expansion medium induces sustained increase of total and functional pre-mNK. PBMCs were isolated from a healthy
donor buffy coat samples and MACS separation was utilized for isolation of NK cells to culture with IL-2 (500 IU/mL) and IL-15 (140 IU/mL), in the presence and absence of IL-18 (100 ng/mL) (A). The frequencies of total pre-mNK for the cytokine groups +/- IL-18 are shown for days 0, 7, and 14, along with representative flow cytometry plots (B,C). Frequencies and representative flow cytometry plots are also shown for functional markers, including proliferation (Ki67+), ADCC receptor (CD16+), and cytotoxicity (GranzB+) (D-F). Significance was assessed using a 2way ANOVA Tukey's multiple comparison test, *p<0.05, **p<0.005, ***p<0.0005, samples in duplicates or triplicates.
3.4.2 Pre-mNK become the major NK cell subset when expanded with IL-2, IL-15, and IL-18

To further confirm the effectiveness, we observed in the pilot study described above for the combination of cytokines IL-2, IL-15, and IL-18 in the ex vivo NK cell expansion medium to promote the HLA-DR+ subset (pre-mNK), we extended the study to include NK cells isolated from buffy coat samples of 3 additional healthy donors. Multiparametric flow cytometry analysis was performed on days 0, 7, and 14 of expansion to assess the frequencies of total as well as HLA-DR positive and HLA-DR negative NK cells subsets along with functional markers on each of these populations. Specifically, the analyses included the cytotoxicity marker granzyme B, the ADCC marker CD16, and activation receptors CD69 and NKG2D, all important for strong antiviral and antitumor effector functionalities. On day 0 of expansion, the HLA-DR+ subset (pre-mNK) comprised a minor fraction of only 4% of total NK cells but increased by average across the different donors to 50% by day 7 and 75% by day 14 (Figure 19A, B). Additionally, we observed that by day 7 the HLA-DR positive and HLA-DR negative subsets accounted for a roughly equal proportion of total and functional subsets of NK cells, but by day 14, pre-mNK (HLA-DR positive) cells are the dominant NK subset (Figure 19C) with significantly higher expression of all the functional markers analyzed. The data clearly establish that the ex vivo NK cell expansion protocols employing the clinically relevant cytokine mixture favor the pre-mNK subset to emerge as the dominant population with significant antitumor potential.
Pre-mNK become the major total and functional subset after expansion with the mixture of IL-2, IL-15, and IL-18 cytokines. PBMCs were isolated from healthy donor buffy coat samples (n = 3) and MACS separation was utilized for the isolation of NK cells used for expansion with IL-2 (500 IU/mL), IL-15 (140 IU/mL), and IL-18 (100 ng/mL). The frequencies of total pre-mNK are shown for day 0, 7, and 14 for the 3 different donors (A). The ratio of HLA-DR+/− frequencies along with representative flow cytometry plots are also shown (B). Frequencies of different subsets expressing the different functional markers, including cytotoxicity (GranzB+), ADCC receptor (CD16+), and activation (NKG2D and CD69) (C). Significance was assessed using a 2way ANOVA Tukey’s multiple comparison test, ****p<0.0001, samples in duplicates or triplicates.
CHAPTER 4. DISCUSSION

The data presented here from in vivo preclinical models and in vitro studies with patient samples highlights the potential contributions of NK-mediated innate immune effector responses for antitumor immunity in HPV+ cancers. The novel NK subset expressing the dendritic cell marker CD11c, NKDC, was important for therapeutic HPV peptide vaccine-mediated protection in preclinical HPV+ oral and vaginal tumor models. This is particularly significant because of the superior antitumor functional advantages associated with the CD11c+ NK subset (NKDC) compared to the CD11c- subset of NK cells in vaccinated mice. In addition to the preclinical evidence, we present data from the analyses of HPV+ patient samples in support of the functionally equivalent NK subset in the humans that express HLA-DR and are described as pre-mature NK (pre-mNK) cells. We observed pre-mNK with a dysfunctional phenotype in the circulation of patients with HPV+ oropharyngeal and cervical cancers when compared to healthy donors. Interestingly, we observed gained cytotoxic functional potential of pre-mNK post-treatment with standard of care (SOC). Additionally, we demonstrate that ex vivo culturing of NK cells with clinically relevant cytokines, which is a standard practice for adoptive NK cell therapy approaches, favors preferential and significant expansion of pre-mNK cells with potent cytotoxic functional advantages highlighting their potential importance for effective antitumor immunity. These data strongly support further exploration of strategies to harness the contributions of the pre-mNK cells for the clinical management of HPV+ cancers.
Data from the preclinical studies testing a therapeutic HPV-16 E6/E7 peptide vaccine demonstrated the importance of QS21 and CpG-ODN adjuvant mixture to favor significant induction of NKDC that seem to be specifically relevant for protection in the oral HPV tumor model. Importantly, the CD11c+ subset exhibited significant functional advantages over the CD11c- subset, further indicating the significance of NKDC for antitumor immunity in the orthotopic mEER oral tumors. Overall, robust induction of multiple effector immune responses, including tumor antigen-specific CTLs and polyfunctional NKDC are attributed for the therapeutic HPV peptide vaccine in the current investigation.

When activated, NKDCs are reported to secrete significant quantities of IFNγ and possess potent cytolytic capabilities [58, 63]. It is important to note that in the homeostatic state and in the absence of stimulation, circulating NKDC are low in frequency [63]. This highlights a critical need to identify a means for activation and expansion of NKDC to harness their role for antitumor immunity. In the current investigation, it is evident that the combination of QS21, known to induce IL-1, IL-12, and IL-18 [64, 65], and CpG-ODN, known to induce type I IFNs [49], as adjuvants effectively activated the NKDC. Various mechanisms are likely involved in the unique induction of NKDC by TVQC, however, the precise mechanisms remain unclear. Literature reports describe a phenomenon known as dual licensing of DCs, in which combinations of adjuvants with diverse mechanisms of signaling promote recruitment and activation of multiple immune effector cell subsets [66]. QS21 is reported to induce balanced Th1 and Th2 responses [67], while CpG-ODN is known for TLR-9 mediated signaling [68]. It is possible that the combined signaling from these two adjuvants
underlie effective induction of multiple immune effector responses. Overall, the expansion potential and increased functional activity of NKDCs, induced by cytokines or adjuvants, makes them an attractive subset for cellular immunotherapy to promote antitumor immunity.

In the clinical setting, there is limited literature regarding HLA-DR+ NK (pre-mNK) cell subset, especially in the context of HPV and cancer. We therefore sought out to define this subset in association with HPV+ oropharyngeal and cervical cancers. Although we did not observe a difference in the total NK cell frequency, we did observe higher frequencies of pre-mNK in circulation of HPV+ patients compared to healthy donors. The increase is likely attributed to the infection with HPV causing the NK cells to become activated [34, 39]. However, despite the elevated frequency of pre-mNK, they are dysfunctional as indicated by various markers. Compared to healthy donors, the patient pre-mNK cells exhibit elevated inhibitory receptor, PD-1. The PD-1 immune checkpoint has largely been described for its role in T cell exhaustion, however, it has also been described on NK cells in the context of cancer [14]. Additionally, the pre-mNK exhibited reduced cytotoxicity, shown by lower granzyme B expression, a known effector to induce target cell death [69] and reduced frequencies of CD16, the receptor that mediates antibody dependent cellular cytotoxicity (ADCC).

The antitumor potential of the pre-mNK subset is further supported by the changes in immunophenotype of this subset observed in patients after receiving treatment. Within the head and neck squamous cell carcinoma (HNSCC) patients, post-treatment with SOC, we observed gained cytotoxicity function of pre-mNK in circulation, indicated by elevated levels of granzyme B and CD16. In cervical cancer
patients, we had the opportunity to review pre-mNK within the tumor microenvironment (TME) in addition to PBMC. This is important because the immune effector subsets in circulation may not be similar to that within the tumor. Importantly, we observed significantly increased granzyme B in pre-mNK cells, within the TME at the T4 treatment timepoint compared to baseline (T1), and this was not observed in circulation (PBMC). Additionally, upon comparing HLA-DR+/− cytotoxic NK subsets, it was evident that the HLA-DR+ subset accounted for elevated cytotoxicity from baseline to T4 with a 9.3-fold increase, while the HLA-DR− subset exhibited no change between the two timepoints. This observation further highlights the potential role of pre-mNK for antitumor innate effector immunity. A caveat to the data presented in cervical cancer patients includes analysis of a late-treatment timepoint instead of the 3-month follow-up post-treatment timepoint due to limited sample size. Furthermore, analysis of the TME at the post-treatment timepoint is difficult due to tumor regression and limited cell counts.

A limitation to the current investigation was the inability to correlate patient outcome with the pre-mNK subset, due to numbers of treatment failures too low for appropriate statistical analysis. In the future, it will be important to identify the role of this population as it relates to disease progression and outcome. Chaput et al. [37] report HLA-DR+ NK cells as a correlate for disease outcome in cancer patients with solid refractory tumors receiving a combination of imatinib mesylate and IL-2. They reported levels of HLA-DR+ NK cells positively correlated with progression free survival (PFS) and overall survival (OS).
Our data and that from others show that the HLA-DR+ NK subset (pre-mNK) comprises a minor fraction of the total NK cells within healthy individuals [39, 61]. However, pre-mNK have significant expansion capabilities [38, 39]. It has been reported that IL-18 promotes HLA-DR expression on NK cells [62], and in alignment with this, we observed a sustained increase of total and functional pre-mNK when expanded with the combination of cytokines IL-2 and IL-15, in combination with IL-18 that was superior to the combination without IL-18. Additionally, data from our ex vivo NK cell expansion studies revealed that, by day 14, pre-mNK become the major functional NK subset, indicated by expression of GrnzB, CD16, NKG2D, and CD69. Overall, our studies showed that pre-mNK constitute the predominant population in the expansion cultures and that they represent the most functional subset of the NK cells in general for the effector responses in adoptive cell therapy approaches.

In the future, it would be interesting to explore the effect of other clinically relevant cytokines on the expansion and function of the pre-mNK subset and observe the prevalence and phenotype of pre-mNK in multiple contexts of cytokine stimulation. Studies report stimulation with IL-12, IL-15, and IL-18 induces memory-like NK cells with enhanced antitumor responses [70, 71]. The addition of IL-21 to IL-2 and IL-15 has been reported to increase proliferation and increase cytotoxicity by granzyme B production, while IL-12 is reported to synergize with IL-2 for elevated cytokine production [72]. Interestingly, Choi et al. [73] report the combination of IL-15 and IL-18 with IL-27 increased significant cytotoxicity and proliferation of NK cells compared to other combinations, including the IL-2, IL-15, and IL-15/IL-18. The expansion of NK cells for adoptive transfer techniques is still an emerging therapy and identifying the
most efficacious and potent cytokine combination, and the NK subsets involved, will be crucial for further advancements as a cancer treatment.

Aside from its’ status as a marker for activation [35, 36], the function of the HLA-DR receptor on NK cells is unclear. Senju et al. [62] report expansion with IL-18 promoted an APC-like NK cell phenotype, enhancing the expression of CD80, CD86, HLA-DR, and HLA-DQ. Other studies have also indicated potential for antigen presentation [60, 61], however, research is still limited, and more comprehensive studies are required to determine the function and mechanism of HLA-DR in this context.

The present investigation demonstrated the potential contributions of innate effector immunity from a novel NK cell subset for antitumor immunity in the context of HPV+ oropharyngeal and cervical cancers. It is clear that NKDC played an important role in vaccine-mediated antitumor immunity in preclinical HPV+ tumor models, exhibiting significant polyfunctionality that is superior to that of the CD11c- portion of NK cells. In the clinical setting, we showed pre-mNK exhibited a dysfunctional phenotype in HPV+ cancer patients prior to receiving treatment, however, we were able to identify gain in function and cytotoxic potential, exhibited by granzyme B and CD16, of this subset in patients post-treatment with SOC. The gain of functionally after treatment indicates deficiencies of this subset in patients prior to treatment, highlighting a need to identify a safe and efficient means to activate the pre-mNK subset in HPV patients for potential enhancement of the antitumor response. Importantly, we demonstrated the significant expansion capabilities of pre-mNK with clinically relevant cytokines, indicating pre-mNK become the major functional NK
subset, exhibiting significant cytotoxicity (GrnZB and CD16) and activation (CD69 and NKG2D), when expanded with our protocol. Future studies to further define the function and killing capabilities of pre-mNK and correlations with patient outcome will be important to further define the significance of this novel NK subset and could provide further insight into the implications for antitumor immunity, disease response, and cellular therapies.
References


VITA

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