UTILIZING HUMANIZED MICE TO STUDY HUMAN SPECIFIC
INNATE IMMUNE RESPONSES IN IMMUNO-ONCOLOGY

A Dissertation Presented
By
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University of Massachusetts Graduate School of Biomedical Sciences, Worcester
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DOCTOR OF PHILOSOPHY

JULY 16TH, 2019

INTERDISCIPLINARY GRADUATE PROGRAM
Dedication

I dedicate this body of work to my father,

David Ayitey Aryee

(1945 – 2015)

For always insisting my siblings and I follow our dreams without fear of the unknown, for teaching us to observe, listen and question the world around us, and for supporting and encouraging us through our various paths in life.
Acknowledgements

“Search for the seed of good in every adversity. Master that principle and you will own a precious shield that will guard you well through all the darkest valleys you must traverse. Stars may be seen from the bottom of a deep well, when they cannot be discerned from the mountaintop. So will you learn things in adversity that you would never have discovered without trouble. There is always a seed of good. Find it and prosper.” ~ Og Mandino

I want start by thanking my PhD mentor Dr. Michael Brehm for giving me the opportunity work with him and to pursue PhD in his lab. I joined Mike’s lab with a background in biochemistry and without an inkling of what immunology and humanized mouse research entails. Mike took me in and painstakingly showed the ropes. The first half of my PhD was quite challenging with a lot of false starts, but Mike was always available to help me figure out which direction to take and to shape my work. Thank you for the countless hours of pouring over complex multi-parameter flow cytometry data to make sure our data sets were interpreted correctly, and for always being an open door away to discuss and direct the work. Your many important suggestions on how to view and present data has also been invaluable to me and would continue to be throughout my scientific career. I also wish to thank the other PIs of my larger group, Drs. Dale Greiner, Rita Bortell and Philip Dilorio, who together agreed to have me join the group and Mike’s Lab, for their support over the years and helping me better understand immunology and the humanized mouse platform.
I am very grateful and thankful to my TRAC Committee (literally the best group I could have had) who also became my Dissertation Committee made up of Drs. Ann Rothstein, Lenny Shultz, Lawrence Stern, Rene Maehr and John Harris. They have been very insightful and constructive in helping me shape my graduate career as I muddled through a lot of false starts. Ann as my TRAC Chair always encouraging and constructive through the years and always motivated me to excel in my work. I would also like to thanks Dr. Karolina Palucka for readily agreeing to be my external examiner during my thesis defense and for helping my shape my final work.

I would not have made it through my long stint in graduate school without wonderful co-workers and colleagues and friends here are UMASSMED and beyond. I therefore want to thank past and present members of the Brehm/Greiner Lab for making my graduate school career bearable and even fun. I thank Darcy Reil for putting up with my last-minute need for her help either to set up an assay or to find protocols, reagents and everything else in the lab. I want to also thank Jamie Kady, Jenna Gerrish, Patricia Cannon, Pam St. Louis, Hannah Yensan, Linda Paquin, Drs. Nathalie Jouvet, Laurie Kenney and Nicole Walsh to name a few, for offering helping hands and a warm smile when needed, to carry me through the day. I especially want to thanks Drs. Laurence Covassin, Agata Jurczyk, Sonal Jangalwe and Philip Durost for being amazing colleagues and friends with whom I have had long and interesting discussions about our experiments, challenges, careers and everything in between. I couldn’t have asked for better colleagues to have taken this journey with. I would also like to thank the greater Diabetes Center of Excellence lab
members both past and present and my friends for being a wonderful group of people to have around.

I would also like to thank my family: Roderick, Sheila and particularly Faustina for always being there to help take my mind of the challenging journey that my PhD has been and for putting up with me through the years. I especially want thank Amena Arif for being there to listen to my scientific rumblings, for being the hawk eye needed to scientifically proof my work and for making the years bearable, our conversations kept me going.

Last but most importantly, I would like to offer my heartfelt thanks and appreciation to my parents for their relentless support and sacrifices to enable me pursue and chart my own course and for always being there for me although geographically so far. My accomplishment today is more theirs than mine.
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Transgenic expression of human IL15 in NOD-scid IL2Rγnull (NSG) mice enhances the development and survival of functional human NK cells. *Manuscript in preparation.*

List of publications


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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>B-ALL</td>
<td>B cell acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette–Guérin</td>
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<tr>
<td>BLT</td>
<td>Bone-marrow/liver/thymus</td>
</tr>
<tr>
<td>BM</td>
<td>Bone-marrow</td>
</tr>
<tr>
<td>BRG</td>
<td>BALB/c Rag2-/- IL-2Rγc-/-</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDX</td>
<td>Cell line derived xenografts</td>
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<tr>
<td>cGy</td>
<td>centiGray</td>
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<tr>
<td>CLP</td>
<td>Common lymphoid progenitor cells</td>
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<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
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<td>CRS</td>
<td>Cytokine release syndrome</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Anti- cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
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<tr>
<td>GITR</td>
<td>Glucocorticoid-induced TNFR-related protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<td>GVHD</td>
<td>Graft versus host disease</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HMW</td>
<td>High molecular weight</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic stem cells</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IKB</td>
<td>Inhibitor of nuclear factor kappa-B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor kappa-B kinase</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ILC</td>
<td>Innate lymphoid</td>
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<tr>
<td>ILC</td>
<td>Immunoglobulin-like transcript</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor associated kinase</td>
</tr>
<tr>
<td>IRB</td>
<td>Internal review board</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer-cell immunoglobulin-like receptor</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage</td>
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<tr>
<td>LMP</td>
<td>Latent membrane protein</td>
</tr>
<tr>
<td>LMPP</td>
<td>Lymphoid-primed multipotential progenitors</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Methyl-accepting chemotaxis</td>
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<tr>
<td>mDC</td>
<td>Myeloid dendritic cells</td>
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<tr>
<td>MDSC</td>
<td>Myeloid derived suppressor cells</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MICA</td>
<td>MHC class I polypeptide-related sequence A</td>
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<td>MICB</td>
<td>MHC class I polypeptide-related sequence B</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NHL</td>
<td>Non-Hodgkin’s lymphoma</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NKG</td>
<td>Natural Killer Group</td>
</tr>
<tr>
<td>NKP</td>
<td>Natural Killer progenitor</td>
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<td>NOD</td>
<td>Non-obese diabetic</td>
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<td>NOG</td>
<td>NOD/Shi-scid/IL-2Rnull</td>
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<td>NSG</td>
<td>NOD-scid IL2Rnull</td>
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<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mono-nuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PD-1</td>
<td>Programmed cell death protein 1</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cells</td>
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<td>PDL-1</td>
<td>Programmed cell death protein ligand 1</td>
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<td>PDX</td>
<td>Patient derived xenograft</td>
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<tr>
<td>PLGA</td>
<td>poly-lactic-co-glycolic acid</td>
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<td>poly(I:C)</td>
<td>Polyinosinic:polycytidylic acid</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>Prkdc</td>
<td>Protein kinase, DNA activated, catalytic polypeptide</td>
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<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
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<td>RAG</td>
<td>Recombination activating gene</td>
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<td>RBC</td>
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<tr>
<td>RLR</td>
<td>RIG-I like receptor</td>
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<tr>
<td>s.c.</td>
<td>Sub-cutaneous</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<tr>
<td>SGM3</td>
<td>SCF, GM-CSF, IL3</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<td>SIRPα</td>
<td>Signal regulatory protein alpha</td>
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<td>SRC</td>
<td>SCID repopulating cell</td>
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<tr>
<td>ss</td>
<td>Single stranded</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>TAM</td>
<td>Tumor associated macrophage</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<td>T cell immunoreceptor with Ig and ITIM domains</td>
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<td>Toll-like receptor</td>
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<td>Tumor microenvironment</td>
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<td>TRAF</td>
<td>TNF receptor associated factor</td>
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<td>Tumor necrosis factor-related apoptosis-inducing ligand</td>
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<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<td>TRAP</td>
<td>Tryptophan Regulated Attenuation Protein</td>
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<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>UCB</td>
<td>Umbilical cord blood</td>
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Preface

Amy Cuthbert performed the mouse experiments in Figures 2.1 to 2.9, Pam St. Louis performed the mouse experiments in Figure 3.2 and Carey Zammitti performed the chromium release assay for NK cell killing in Figure 3.4. Besides these, all other experiments in this thesis were performed by me.

Michael Brehm supervised all the research conducted and will be the corresponding author on the manuscripts derived from Chapters II, III and IV.
Abstract

The kinetics of tumor growth and progression are governed by the interaction between tumor cells, the non-malignant stroma and both innate and adaptive immune cell lineages. Innate immunity has a critical role in the control of tumor cell growth and metastasis. The microenvironment of many tumors is populated with innate immune cells, including regulatory natural killer (NK) cells and dendritic cells (DCs), tumor associated macrophages, and myeloid derived suppressor cells, that suppress normal immune function. Much of our understanding of interactions between tumors and the innate immune system is based on experimental studies performed in mouse “syngenic” models. However, there is clear need for a mechanistic understanding of the human innate immune system within the tumor microenvironment.

The goal of my thesis is to characterize the interactions between human innate immune cells and tumors and to define specific pathways and cell lineages that are targets for immune modulation. A central focus of my thesis is the use of cutting-edge humanized mouse models based on the immunodeficient NOD-scid IL2Rγnull (NSG) mouse strain to study human immuno-oncology. In the first section of my thesis I describe studies that evaluate the influence of inflammatory stimuli on innate immune control of tumors. Agents that induce inflammation have been used since the 18th century for the treatment of cancer. The inflammation induced by agents such as toll-like receptor (TLR) agonists is thought to stimulate tumor-specific immunity in patients and augment control of tumor burden. While NSG mice lack murine adaptive immunity (T and B cells), these mice maintain a residual murine innate immune system that responds to TLR agonists. Here I describe a novel NSG mouse strain lacking TLR4 that fails to respond to...
lipopolysaccharide (LPS). NSG-\textit{Tlr4}\textsuperscript{null} mice support human immune system engraftment and enables the study of human specific responses to TLR4 agonists. My data demonstrate that specific stimulation of TLR4 activates human innate immune system and promotes regression of human patient derived xenograft (PDX) tumors. In the second section of my thesis I describe the development of an NSG mouse strain that constitutively expresses human Interleukin 15 (IL15) and supports the development of functional human NK cells. Using humanized NSG-IL15 transgenic mice (NSG-Tg(Hu-IL15), my data clearly demonstrate a critical role for human NK cells in limiting growth of a PDX melanoma. In the third section of my thesis I describe, the use of the bone marrow/liver/thymus (BLT) humanized mouse model to study the interactions between the human immune system and PDX melanoma and to evaluate the response of the melanoma to immunotherapy modalities.

My results collectively suggest that mice engrafted with human immune systems and bearing human tumors can be harnessed as translational models, which are critically needed as tools to study tumor immunotherapy. These humanized mouse models are an ideal translational tool to advance our understanding of human immuno-oncology and for development and testing of novel immune therapies for the treatment of malignancies.
CHAPTER I

Introduction.

The tumor microenvironment and its interaction with the immune system.

An early hypothesis on factors regulating tumor growth was that the main driver for the development, proliferation and progression of cancers were inherent genetic features. Cell-autonomous pathways were therefore believed to be sufficient for controlling cancers (von Hansemann, 1890). In the 19th century, Rudolf Virchow observed the presence of leukocytes within tumors, providing the first connection between the immune system and cancer (Grivennikov et al., 2010). Since then, studies done in both animal models and clinical ex-vivo studies have shown that the immune system can recognize or be manipulated to recognize and reject tumors (Bazhin et al., 2019; Finn, 2018; Liu and Guo, 2018). Therefore, in addition to tumor cells, there are non-tumor components including immune cells that can influence tumor growth and survival. To this end, immuno-oncology tries to understand how components of the immune system are interacting with cancers for tumor immunosurveillance and how, when and why immune control fails in clinical disease.

Immunosurveillance of tumors

Both the innate and adaptive arms of the immune system have important roles in tumor recognition and control (Dranoff, 2004). Detection starts when NK cells sense transformed cells through activating receptors, which stimulate the NK cells causing the lysis of transformed tumor cells (Malmberg et al., 2017). The cellular debris from the lysed tumor cells are phagocytosed and processed by the professional antigen presenting cells...
(APCs), including macrophages and DCs, which are activated by the damage associated molecular patterns (DAMPS) (Janeway and Medzhitov, 2002). Following activation, macrophages and DCs secrete proinflammatory cytokines and present the processed tumor antigens to CD4+ and CD8+ T cells to mediate their activation. The effector T cells control the growth of the tumor through inflammatory mechanisms usually involving the production of interferon-gamma (IFNγ) (Zamarron and Chen, 2011). Together with the activated NK cells, macrophages and DC, the T cells mount an immune response leading to the elimination of the remaining tumor cells (Finn, 2018).

An equilibrium state can occur, such that tumor cells escaping immune recognition and elimination continue to grow. Here, the rate of generation of the new tumor cell variants balances out the eliminated cells, giving the appearance of a dormant tumor. During the equilibrium stage, tumor cells continue to divide, and can accumulate mutational changes that occur due to chance or in response to immune-induced inflammation (Fox et al., 2011). Over time, the acquired mutational burden enable the tumors to escape immune-mediated suppression allowing them to grow. Escape from immunosurveillance often directly involves the immune system itself. The immune system thus plays a critical role in immunosurveillance and tumor development with a dual capacity to both promote and suppress tumor growth: a process termed “immune editing” (Figure 1.1).
Figure 1.1: The process of immune editing. Through immunosurveillance, the immune system can specifically identify and eliminate tumor cells. Nevertheless, a state of equilibrium can develop when the immune system is not fully able to eliminate the tumor. During the equilibrium phase tumor progression does not occur. When the tumor cells suppress the anti-tumor immune response, the tumor escapes and can grow and metastasize.
Methods of immune evasion by tumors.

Immune regulatory cells.
The generation of an immune suppressive tumor microenvironment (TME) by Forkhead box protein 3 (FoxP3)+ CD4 regulatory T cells (Tregs), myeloid derived suppressor cells (MDSC), tumor associated macrophages (TAMs) and immature or functionally impaired DCs is a central mechanism by which tumors escape immune surveillance (Chao and Savage, 2018; Tuccitto et al., 2019). Chemokines produced by the tumor and immune cells such as TAMs and MDSCs within the TME are responsible for Treg migration into the TME (Faget et al., 2011; Li et al., 2013; Menetrier-Caux et al., 2012). In addition, there are data supporting the conversion of CD4+ conventional T cells into Tregs within the TME aided by transforming growth factor-beta (TGFβ) produced by the tumor cells along with immune cell types such as immature DCs (Zou, 2006). In addition to creating a suppressive immune microenvironment, MDSC, and M2-like TAMs can promote angiogenesis and initiate tumor metastasis (Finke et al., 2011; Schmid and Varner, 2010). M2-like TAMs promote tumor growth and often produce high levels of TGFβ, IL10, and vascular endothelial growth factor (Aras and Zaidi, 2017; Sica et al., 2008).

Defects in tumor antigen presentation
Another well recognized mode of immune surveillance evasion by tumors is by down-regulating the antigen processing and presentation machinery, particularly major histocompatibility complex (MHC) class I, the proteosome subunits latent membrane protein 2 and 7 (LMP2 and LMP7) and tapasin (Garrido et al., 1997; Herrera et al., 2017; Rotem-Yehudar et al., 1996) which are important for CD8+ T cell activation. Also MHC II
is downregulated in cancers such as diffuse large B cell lymphoma, where loss of MHC II correlated with poor prognosis (Rimsza et al., 2004). MHC II is important for CD4+ T helper cell activation which assist CD8+ cytotoxic T cells. The reduced expression of antigen processing and presentation machinery results in poor antigen presentation, recognition and activation of cytotoxic CD8+ T cells and promotes tumor survival and growth (Maeurer et al., 1996). The defect in antigen presentation, not limited to MHC downregulation leads to poor or complete absence of leukocyte particularly T cell infiltration in the tumor; a process required for tumor elimination. These tumors that lack T cell infiltration are termed “cold tumors”(Bonaventura et al., 2019).

**Immune tolerance**

Many tumors in addition to the stroma and immune cells within the TME express high levels of inhibitory molecules of the B7 family critical for suppression of T cell response (Zou and Chen, 2008). Programed cell death protein-ligand 1 (PD-L1), a B7 family member, can induce T cell anergy and death on engagement with programed cell death protein 1 (PD-1) expressed on surface of activated T cells (Ahmadzadeh et al., 2009; Crespo et al., 2013; Grosso et al., 2009). Another family member B7-H4, highly expressed on TAMs of ovarian carcinoma, was shown to suppress tumor associated antigen specific T cell immunity (Kryczek et al., 2006). In addition tumors can upregulate the expression of the death ligand FasL which can cause T cell apoptosis once exhausted (a dysfunctional state of T cells characterized by progressive loss of function, changes in transcriptional profiles and sustained expression of inhibitory receptors) (Yajima et al., 2019). Many tumors also lack the expression of costimulatory molecules such as human
leukocyte antigen E (HLA-E) (Huang et al., 2017), needed to complete activation of T cells after the T cell receptor (TCR) has been engaged, a process which also leads to T cell anergy and death (Driessens et al., 2009). Furthermore, studies have also suggested that tumors down regulate death receptors including Fas (Huang et al., 2017) and tumor necrosis factor-related apoptosis-inducing ligand receptor 1 and 2 (TRAIL-R1 & 2)(Shin et al., 2001) to prevent death ligand-mediated killing by both CD8+ cytotoxic T cells and NK cells (Chouaib et al., 2009; French and Tschopp, 2002; Slavin-Chiorini et al., 2004).
Figure 1.2 Methods of immune escape within the TME. A schematic showing how specific immune cell phenotypes and tumor surface expression shape immune surveillance and determine the fate of cancers.
TLRs in innate immunity and tumor immunology

A network of APCs including macrophages, DCs and mast cells which form part of the complex mammalian immune system, use a series of intracellular and transmembrane pathogen recognition receptors (PRRs) that recognize foreign particles associated with pathogens such as bacteria, virus, fungus or protozoa called pathogen associated molecular patterns (PAMPs) and self-derived molecules that may prove dangerous and harmful to the host called DAMPs, to enable the APCs to process and present antigens to naïve T cells in order for their activation (Janeway and Medzhitov, 2002). The activated T cells together with the activated APCs and NK cells can then control and eliminate the source of the antigen (Kumagai and Akira, 2010; Takeuchi and Akira, 2010). TLRs are one of the most comprehensively studied PRRs. TLRs were first described in *Drosophila melanogaster* and thought to be a protein involved in embryonic development (Hashimoto et al., 1988). However TLRs were later observed to function in the detection and response to fungal pathogens (Medzhitov et al., 1997). TLRs are type-I membrane proteins found either in the cell membrane or within endosomes. Each TLR is comprised of an extracellular leucine-rich repeat (LRR) domain for ligand binding and detecting foreign particles, a cysteine-rich region followed by a transmembrane domain and an intracellular toll/interleukin-1 receptor (TIR) domain capable of protein-protein interactions for signal transduction (Meng et al., 2003).

TLRs are located both at the cell surface and intracellularly, where they bind and are activated by different ligands, which are found on different type of organisms and structures as summarized in Table 1.1 (De Nardo, 2015; Savva and Roger, 2013; Vijay,
2018b). TLRs present in the plasma membrane detect bacterial cell wall components in the extracellular compartment. Of these membrane bound TLRs, TLR2 is known to form heterodimers with TLR1, TLR6 and conceivably with TLR10 (which is expressed only by humans and as yet has no defined microbial ligands) and detects triacylated or diacylated lipopeptides, peptidoglycans, lipoglycans, lipoteichoic acid (LTA) present in the cell walls of both Gram-positive and Gram-negative bacteria (Takeuchi et al., 2001; Takeuchi et al., 2002), whereas TLR5 recognizes the bacterial flagellin (Hayashi et al., 2001). TLR8 and TLR13 (mouse only) respond to viral and bacterial RNA (Oldenburg et al., 2012; Tanji et al., 2015). The other mouse only TLRs, TLR11 and TLR12, recognize profilin (Andrade et al., 2013). TLR4 is the only TLR that shuttles between the plasma membrane and the endosome and with the help of MD2 and CD14 can recognize lipoglycans like LPS (Fang et al., 2013). Endosomal TLRs (TLR3, 7, 8 and 9) recognize bacterial or viral nucleic acids within the cytosol; TLR3 detects double-stranded ribonucleic acid (dsRNA), while TLR7 and 8 detect single-stranded ribonucleic (ssRNA) and TLR9 recognizes unmethylated CpG deoxyribonucleic acid (DNA) and DNA:RNA hybrids. All the TLRs except TLR3 signal through myeloid differentiation primary response 88 (MyD88). TLR3 signals through TIR domain containing adaptor inducing interferon beta (TRIF) while TLR4 can signal through both TRIF and MyD88 (O'Neill and Bowie, 2007). Upon TLR engagement, both MyD88 and TRIF signaling results in both nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and mitogen activated protein kinases (MAPKs) activation via tumor necrosis factor receptor associated factor (TRAF6) (De Nardo, 2015). Activation leads to NF-κB dimerization and translocation into the nucleus where it binds DNA and regulates gene expression (Figure 1.3) This process leads to the synthesis of
inflammatory cytokines and chemokines such as tumor necrosis factor alpha (TNFα) and IL6, central to host defense during the early stages of the inflammatory response (Medzhitov, 2001).
<table>
<thead>
<tr>
<th>TLR</th>
<th>Species</th>
<th>Localization</th>
<th>Microbial Ligands</th>
<th>Endogenous Ligands</th>
<th>Signal adaptors</th>
<th>Immune cell expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Human and mouse</td>
<td>Plasma membrane</td>
<td>Tri-acetylated lipopeptides coupled topeptidoglycan</td>
<td>HSP (60, 70), HMGB1, urate crystals</td>
<td>MyD88</td>
<td>monocytes/macrophages, DC, B cells, NK cells, T cells</td>
</tr>
<tr>
<td>TLR2</td>
<td>Human and mouse</td>
<td>Plasma membrane</td>
<td>Lipoarabinomannan, lipoteichoic acid, Zymosan, protozoa and some viruses</td>
<td>HSP (60, 70), HMGB1, urate crystals, ω-(2-carboxyethyl)pyrrole, hyaluronan</td>
<td>MyD88</td>
<td>monocytes/macrophages, mDC, mast cells, T cells</td>
</tr>
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<td>Human and mouse</td>
<td>Endolysosomal membrane</td>
<td>Viral dsRNA, synthetic Poly I:C and Poly A:U</td>
<td>mRNA, statmin</td>
<td>TRIF</td>
<td>DC, B cells, γδT cells</td>
</tr>
<tr>
<td>TLR4</td>
<td>Human and mouse</td>
<td>Plasma and endolysosomal membrane</td>
<td>LPS, fungal mannann and glucuronoxylmannann, protozoa, and some viruses</td>
<td>HSP (22, 60, 70, 72), HMGB1, fibronectin, Defensin 2, oxLDL, Tenascin C, hyaluronan, heparan sulphate</td>
<td>TRIF, MyD88</td>
<td>monocytes/macrophages, mDC, B cells mast cells, neutrophils</td>
</tr>
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<td>Plasma membrane</td>
<td>Flagellin</td>
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</tr>
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<td>Human and mouse</td>
<td>Plasma membrane</td>
<td>Diacyl lipopeptides, LTA, protozoa, and zymosan</td>
<td>ω-(2-carboxyethyl)pyrrole</td>
<td>MyD88</td>
<td>monocytes/macrophages, mast cells, B cells</td>
</tr>
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<td>Human and mouse</td>
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<td>MyD88</td>
<td>monocytes/macrophages, pDC, B cells, CD4+ T cells</td>
</tr>
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<td>Endolysosomal membrane</td>
<td>Viral and bacterial ssRNA</td>
<td>self RNA, siRNA</td>
<td>MyD88</td>
<td>monocytes/macrophages, DC, mast cells, CD4+ T cells</td>
</tr>
<tr>
<td>TLR9</td>
<td>Human and mouse</td>
<td>Endolysosomal membrane</td>
<td>Viral and bacterial CpG DNA, DNA:RNA hybrids</td>
<td>self-DNA, HMGB1, chromatin</td>
<td>MyD88</td>
<td>monocytes/macrophages, pDC, B cells</td>
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<td>Unknown</td>
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<td>Plasma membrane</td>
<td>Bacterial 23S ribosomal RNA(rRNA)</td>
<td>Unknown</td>
<td>MyD88</td>
<td>monocytes/macrophages, DC</td>
</tr>
</tbody>
</table>
Figure 1.3: TLR signaling pathways

The TLR signaling pathway and downstream effector molecules. Depicted are key TLR molecules, their signaling adaptors and downstream mediators that are essential for TLR signaling and function.
**TLRs as targets in immunotherapy.**

TLRs, key members of the PRRs for detecting PAMPs and DAMPs, are ubiquitously expressed on APCs with each member designed to recognize specific conserved epitopes in pathogens (Table 1.1). Within the TME, APCs recognize tumor damage associated antigens, process and present them to immune cells, including T cells, activating them and leading to an attempt at cancer elimination (Wolska et al., 2009). TLR engagement by agonists can therefore be used to activate APCs within the TME that can then activate T cells and NK cells and boosting antitumor immune responses.

**Individual TLR targeting**

After the discovery that ssRNA is detected by TLR3 (Alexopoulou et al., 2001), synthetic agonists have been used to activate DCs, which are critical for activation of CD8+ T cells (Datta et al., 2003), important for immune surveillance. In addition, the activated myeloid dendritic cells (mDCs) can indirectly activate NK cells through IL12 stimulation, demonstrating the potential of TLR3 engagement for cancer vaccines (Perrot et al., 2010). To this end, Poly (I:C) based TLR3 agonists as adjuvants have been evaluated as cancer treatments. These include R-2008 for prostate cancer and oligoastrocytomas and R-2009 for triple negative breast cancer, melanoma and gliomas (Goutagny et al., 2012). Although Poly (I:C) stimulation of TLR3 can lead to tumor killing, it can also hyperactivate the immune system, leading to chronic inflammation (Anders et al., 2005; Lang et al., 2005). Dosage and site of delivery must therefore be carefully controlled with the use of these TLR3 agonists.
The biological active arm of LPS, lipid A, is known to promote antitumor activity on binding to TLR4. Monophosphoryl lipid A (MPL A), one of the many lipid A species available has much lower toxicity than LPS and has been evaluated as a cancer vaccine adjuvant (Goutagny et al., 2012). Forms of MPL A in combination with immunostimulants are therefore being evaluated in clinical trials by Merck (with Stimuvax) and GlaxoSmithKline (with AS)4, ASO2B and AS15) (Goutagny et al., 2012).

The use of agonists targeting TLR9, which recognizes unmethylated CpG (Table 1.1) in cancer therapy, has shown promising results in pre-clinical and early clinical studies (Hanagata, 2017; Suek et al., 2019). TLR9 is believed to activate DCs, which release cytokines and chemokines to stimulate NK cells and expand type 1 helper T (Th1) cells and CD8+ T cells (Krieg, 2006). To this end various companies are running clinical trials for multiple TLR9 agonists for cancer, including CPG 7909 (GSK, UK) (Dreno et al., 2018), CpG-B (Pfizer, USA)(Koster et al., 2017), and CpG-28 (Oligovax, France) (Ursu et al., 2017; Ursu et al., 2015).

**Combination of toll-like receptor agonists and other cancer therapies**

**Combination of immunotherapy and chemotherapy**

The ineffectiveness over time of many chemotherapeutics to improve life expectancy of cancer patients has led to the need for potential combination with other cancer therapies for better treatment outcomes. One such combination is with TLR therapies. A phase III clinical trial that combined the TLR3 agonist poly(A:U) with the chemotherapy drugs 5-fluorouracil and Adriamycin showed extraordinarily enhanced patient survival (Roy et al., 2013), prompting other TLRs and chemotherapy combinations to be tested. For example,
Roy et al. observed a 40% reduction in mean B16 melanoma tumor volume in comparison to that seen with paclitaxel treatment alone when mice were treated with a combination of paclitaxel and a TLR4 agonist using poly-lactic-co-glycolic acid (PLGA)-based nanoparticle preparation. The results showed increase secretion of Th1 cytokines including TNFα, IL12, interferon-gamma (IFNγ) and IL1β providing evidence of Th1 immune response. An additional study evaluated the combination of the TLR7/8 agonist R848 and a conjugate of doxorubicin and hyaluronic acid to simultaneously target immune cells and breast cancer cells. The data showed significant inhibition of tumor growth in 4T1 mammary carcinoma tumor-bearing mice due to the combined effect of the TLR7/8 agonist R848 stimulating the immune system and doxorubicin blocking topo isomerase 2 within the TME (Da Silva et al., 2016).

**Combination of immunotherapy and radiotherapy**

External radiotherapy is used routinely as one of the first treatment option for cancer patients and involves the localized application of ionizing radiation beams to destroy tumor cells (Begg et al., 2011). However, a major drawback for radiotherapy is tumor hypoxia-associated radiation resistance and inability to control tumor metastasis (Song et al., 2016; Vilalta et al., 2016; Zhang et al., 2015). Using hydrophobic imiquimod (R837), a TLR7 agonist and the water-soluble catalase (Cat), which is an enzyme that generates O by decomposing H₂O₂, Chen and colleagues demonstrate the efficacy of combined radiotherapy and immunotherapy. In this study oxygen species eased tumor hypoxia while the tumor-associated antigens generated post-radiotherapy induced immunogenic cell death in the presence of the R837 adjuvant (Chen et al., 2019).
Natural killer cells.

NK cells are cytotoxic innate lymphoid cells that do not require prior antigen exposure to mediate anti-viral or anti-tumor effects and serve as the first line of defense (Hermanson et al., 2016; Kiessling et al., 1975), making them a critical component of the innate immune system. NK cell make up 5 to 20% of circulating human lymphocytes (Langers et al., 2012) and between 2 to 5% in the spleens and bone-marrows (BM)s of inbred laboratory mice (Jiao et al., 2016). Human NK cells are classically defined as mononuclear cells that lack expression of the T cell receptor and the signal-transducing adaptor, CD3ε, but express the activating Fc receptor, CD16 and the neural cell adhesion molecule CD56 (Lanier et al., 1986; Lanier et al., 1989). Human NK cells can be further divided into populations based on expression of CD56 into CD56\textsuperscript{dim} and CD56\textsuperscript{bright} subsets (Melsen et al., 2016).

CD56\textsuperscript{dim} NK cells are the dominant subset in peripheral blood expressing high levels of CD16 (FcγRIII)(Angelo et al., 2015) and represent the more mature and highly cytotoxic phenotype (Cooper et al., 2001; Nagler et al., 1989). CD56\textsuperscript{bright} NK cells are CD16\textsuperscript{−/low} and mediate immune responses by secreting proinflammatory cytokines including, IFN\(γ\) and TNF\(α\), representing the less mature subset mainly found in secondary lymphoid and peripheral tissues (Björkström et al., 2016; Carrega et al., 2014).

NK cell developmental stages.

Human NK cells develop from common lymphoid progenitor cells (CLP) cells (Kondo et al., 1997), which express IL7 receptor-alpha (IL-7Rα, CD127) in lineage (Lin)−CD244+ cells marking the earliest step in the transition of CLPs into the lymphoid lineage. The first
stage in the cellular differentiation process is Lin−CD34+CD33+CD244+ hematopoietic stem cells (HSCs) differentiating into CD45RA+ lymphoid-primed multi-potential progenitor (LMPP) cells (Figure 1.4). LMPPs then transition into CLPs with potential to make lineage commitments into Pro-B, Pre-T, natural killer progenitor cells (NKPs), or other innate-like lymphoid cells (ILCs) (Renoux et al., 2015). The irreversible NK cell lineage commitment by CLPs is marked by the expression of CD122 (IL2Rβ) with expression of CD56 indicating the final transition of immature NK into mature NK cells. (Figure 1.4).
**Figure 1.4:** Development of human NK cells.

Human NK cells develop from Lin−CD34+ HSCs that differentiate into CD45RA+ LMPP also expressing CD38, CD7, and CD127. LMPPs then transition into common lymphoid progenitors with the potential to make lineage committed switching into Pro-B, Pre-T, NKPs, or other innate and ILCs. Expression of CD122 (IL-2Rβ) locks CLPs into an NK lineage.
NK cell receptors and NK cell education.

NK cell function is regulated by two types of receptors: activation receptors which induce cell maturation, maintain survival and function and inhibitory receptors which suppress NK cells activity upon their interaction with the HLA class I. The recognition of target cells with low expression of HLA class I ligands by inhibitory receptors including the killer cell Ig-like receptors (KIR) and CD94/NKG2A is termed “the missing self” phenomenon (Boudreau and Hsu, 2018b) and is central to NK cell education. NK cell education represents a range of NK cell activation and inhibition determined by interactions with HLA molecules. Educated NK cells have high HLA binding voracity and are therefore the most sensitivity for inhibition by ‘self’ HLA class I molecules while uneducated NK cells do not bind “self” HLA molecules, leading to weak ‘missing self’ capabilities and are insensitive to “self” inhibition. NK education thus determines the extent of NK reactivity (Boudreau and Hsu, 2018b). Cells with decreased expression of HLA class I are consequently susceptible to NK cell killing due to failed engagement of inhibitory receptors by HLA class I ligands. The function of NK cells is therefore controlled by the balance between inhibitory and the stimulatory receptor signaling with Inhibitory receptors signals modulating the intensity of NK cell activation and function by the stimulatory receptors. Natural killer receptors (NCR) and CD16 are examples of activation receptors. Table 1.2 summarizes the activating and inhibitory NK cell receptors in humans and their corresponding ligand (Boudreau and Hsu, 2018b; Handgretinger et al., 2016; Malmberg et al., 2008).
**Table 1.2:** Overview of a selection of activating and inhibitory NK cell receptors in humans and their corresponding ligands.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Type of receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16</td>
<td>Fc of antibodies</td>
<td>Activating</td>
</tr>
<tr>
<td>CD7</td>
<td>SECTM-1, Galectin</td>
<td>Activating</td>
</tr>
<tr>
<td>2B4</td>
<td>CD48</td>
<td>Activating</td>
</tr>
<tr>
<td>DNAM-1</td>
<td>CD112, CD155</td>
<td>Activating</td>
</tr>
<tr>
<td>NKp80</td>
<td>AICL</td>
<td>Activating</td>
</tr>
<tr>
<td>CD96</td>
<td>CD155, CD111</td>
<td>Activating</td>
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<tr>
<td>TIGIT</td>
<td>CD122, CD113, CD155</td>
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<tr>
<td>CRTAM</td>
<td>TSLC1</td>
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</tr>
<tr>
<td>CD2</td>
<td>LFA-3</td>
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</tr>
<tr>
<td>NKG2D</td>
<td>MICA, MICB, ULBP-1, ULBP-2, ULBP-3, ULBP-4, ULBP-5, ULBP6</td>
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<tr>
<td>CD94/NKG2C</td>
<td>HLA-E</td>
<td>Activating</td>
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<td>CD94/NKG2E/H</td>
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<td>NKp46</td>
<td>Viral hemagglutinin</td>
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<td>KIR2DS1</td>
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<tr>
<td>KIR3DL2</td>
<td>HLA-A</td>
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</table>
IL15 signaling in NK cells.

IL15 is part of a group of cytokines that signal through the IL2 common γc chain (CD132), a 40 kDa type I transmembrane glycoprotein. Other members of the group include IL2, IL4, IL7, IL9 and IL21 (Wang et al., 2009). The expression of IL15 is very low under homeostatic conditions but is upregulated following inflammation. IL15 is found in three different forms: soluble IL15, membrane-bound IL15, both of which are limited biologically activity and are rarely detected in-vivo, and IL15 complexed with IL15Rα which is the most biologically active (Rautela and Huntington, 2017). Unlike other members of the IL2 receptor gamma chain, IL15 is trans-presented to IL15R β/γ on NK cells by IL15Rα expressing cells in order to confer its full potency (Mortier et al., 2008) (Figure 1.4). A conformational change following IL15 trans-presentation to the IL15R β/γ complex results in the phosphorylation and activation of receptor-associated janus kinase 1 (JAK1) and JAK3, and the subsequent tyrosine phosphorylation of IL15R β/γ itself (Wang et al., 2009). The phospho-tyrosine residues then provide binding sites for signal transduction and activator of transcription 5 (STAT5) molecules which are in turn phosphorylated. The phosphor-STAT5 molecules form dimers and/or tetramers and translocate to the nucleus where they induce target gene expression (Imada and Leonard, 2000) including Mcl1 which is continuously required for maintaining the survival of NK cells. Additional biological responses including priming/activation, increased expression of effector molecules (IFN γ, perforin, granzymes), proliferation and differentiation (Holmes et al., 2014) occur with intensifying IL15 signaling such that IL15 levels in vivo both qualitatively and quantitative tune NK cell responses (Marçais et al., 2014).
Figure 1.4: IL15 requires trans-presentation for functional signaling.

IL15 requires trans-presentation by membrane bound IL15Rα in contrast with the other members of the IL2 R common gamma family of cytokines such as IL2 that binds receptors on the surface of cells to for signaling,
NK cells in immunotherapy

Tumor escape from NK cell immune-surveillance.

NK cells are a major effector population in anti-cancer immunity. However, tumors have evolved many strategies to either impair NK cell-mediated killing or evade NK cell detection with NK cell dysfunction reported in both solid and hematological cancers (Maki et al., 2008; Paul et al., 2016a; Polakova et al., 2009; Reiners et al., 2013; Sun et al., 2015). Tumors upregulate the expression of ligands that bind NK cell inhibitory receptors including HLA-G, a ligand for killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4), immunoglobulin-like transcript 2 (ILT2) and ILT4, which inhibits the proliferation and cytotoxicity of NK cells and reduces the production of IFNγ and TNFα through engagement with ILT2 specifically (Wan et al., 2017). Upregulation of HLA-G thus enables tumors evade NK cell-mediated cytotoxicity (Ibrahim et al., 2001). Tumors also upregulate production of soluble IL2Rα, which binds IL2 needed for NK cell proliferation and activation, and impairs NK activity (Chiu et al., 2018).

The immune suppressive environment created within the TME also contributes to evasion of NK cells by tumors. Tumor cells secrete immnosuppressive factors such as TGFβ, IL10, Indoleamine 2,3-dioxygenase (IDO), and Prostaglandin E2 (PGE2), which suppress NK cell anti-tumor activity (Baginska et al., 2013; Balsamo et al., 2009; Pietra et al., 2012). In addition, TME immune suppressive cells including Tregs, MDSCs, and TAMs also secrete IL10 and TGFβ impairing intra-tumoral NK cells (Cekic et al., 2014; Li et al., 2009). Cells within TME including the tumor cells, APCs, and stromal cells can also express high levels of PD-L1, that binds to PD-1 expressed on NK cells, leading to NK cell dysfunction.
and exhaustion (Bi and Tu, 2017; Sun et al., 2015; Zhang et al., 2016). The exhausted NK cells are characterized by decreased expression of activating receptors CD16, NKG2D and CD226, upregulated inhibitory receptors including natural killer group G2A (NKG2A), T cell immunoreceptor with Ig and ITIM domains (TIGIT), and T cell immunoglobulin and mucin-domain containing-3 (Tim-3), and low secretion of IFN-γ and TNFα (Bi and Tu, 2017; Krmeta et al., 2015; Paul et al., 2016b). To improve NK cell mediated anti-tumor immunotherapy, there is a need to design strategies that overcome NK cell immune evasion by tumors, specifically within immune suppressive TME

**NK cell-based immunotherapies.**

*Autologous or allogeneic NK cells.*

The first attempts at using adoptively transferred human NK cells to treat tumors used IL2 activated autologous NK cells obtained from PB (Sakamoto et al., 2015) since mouse models showed reduced tumor growth and metastasis after transfer of mouse NK cells (Rosenberg, 1984). This approach had limited success in humans due to expression of self-HLA molecules on the tumor cells interacting with KIRs on the autologous NK cells, resulting in their suppression (Sakamoto et al., 2015). Allogeneic NK cells with KIR-HLA mismatch to the tumor were therefore adopted to overcome the observed suppression (Ruggeri et al., 2002). Although multiple clinical trials have indicated that allogeneic NK cell adoptive transfer does not induce graft versus host disease (GVHD), cytokine release syndrome (CRS), or neurotoxicity (Bachanova et al., 2010; Bachanova et al., 2018; Ishikawa et al., 2018; Shaffer et al., 2016), the therapeutic benefits vary immensely between different cancers with objective clinical responses from 25% to 100% for
hematologic malignancies, like refractory non-Hodgkin’s lymphoma (NHL), myelodysplastic syndromes, and acute myeloid leukemia (AML) (Björklund et al., 2018). The variable therapeutic benefits observed in clinical trials were associated with reduced NK cell lifespan in vivo and the increased in Tregs associated with IL2 administration (to promote in-vivo NK cell activation) boosting the immune regulatory environment of the cancers in some cases (Adotevi et al., 2018). Two clinical trials have used IL2-diptheria fusion proteins to deplete Tregs and showed improved rates of disease remission, with improved NK cell survival and expansion (Bachanova et al., 2014).

In addition to peripheral blood (PB) derived NK cells, UCB and iPSCs are also being used as sources of functional NK cells. Umbilical cord blood (UCB) and induced pluripotent stem cells (iPSCs) have added advantages including a wide range of source materials and ease of clinical grade expansion. Evaluation of antitumor activity of UCB- or iPSC-derived NK cells however showed poorer or similar anti-tumor functions compared with PB derived NK cells (Hermanson et al., 2016; Herrera et al., 2017; Knorr et al., 2013; Veluchamy et al., 2017b; Xing et al., 2010). Also, UCB- and iPSC-derived NK cells expressed low levels of KIRs compared to PB-derived NK cells, raising the concerns about NK cell education; the process whereby NK cells acquire effector functions while remaining tolerant to self-HLA (Boudreau and Hsu, 2018a; Boudreau and Hsu, 2018b; He and Tian, 2016). Educated NK cells usually have lower thresholds for activation or inhibitory signaling, while uneducated NK cells have reduced responses to activation or inhibition. Interestingly, a recent study showed that uneducated NK cells isolated from UCB had superior cytotoxicity against HLA-expressing cervical tumor cell lines compared with educated NK cells from peripheral blood mononuclear cells (PBMCs) (Veluchamy et
It therefore remains to be answered whether NK cell education is essential for immunotherapy against cancers.

**NK cell-line-based tumor immunotherapy**

Established NK cell lines are an additional source of NK cells for immunotherapy (Tonn et al., 2001). NK cell lines are easily expanded under GMP conditions compared with allogeneic, iPSC or UCB-derived NK cells, leading to lower treatment costs. In addition, NK cell lines can be efficiently manipulated with viral or non-viral vectors to enhance their targeting, homing, and killing activity (Tonn et al., 2001). Of the several established NK cell lines, NK-92 cells display a robust and broad cytotoxicity against malignant cells, and are the only cell line currently approved for clinical trials by the FDA (Tonn et al., 2013). The effectiveness of NK-92 cell adoptive transfer has been evaluated in melanoma, sarcoma, colorectal cancer, renal carcinoma, lung cancer, and AML patients with improved clinical outcomes observed in lung cancer and renal cell cancer patients (Arai et al., 2008; Tonn et al., 2013). One of the drawbacks for the use of NK-92 cells is their limited persistence *in-vivo*, even with repeated transfusions. As NK-92 cells were derived from an NHL patient (Gong et al., 1994), the cells must be irradiated to abolish their proliferative ability before transfer which severely impairs their survival *in-vivo*, becoming undetectable in circulation within a few days after transfer (Tonn et al., 2013). More frequent cell transfusions are an easy approach to improve the persistence, but this approach raises the concern of evoking a humoral immune response against the HLA antigens expressed on the NK-92 cells.
CAR-NK cells for tumor immunotherapy

Chimeric antigen receptor (CAR)-T cells are an exciting alternative for treating cancer in an antigen-specific manner. CAR-T cells consist of an antigen-binding fragment fused to the TCR signaling components to generate a potent antigen-specific T cell response upon ligand binding (Grigor et al., 2019). CAR-T cell therapies have shown success in the treatment of relapsed NHL, relapsed B-ALL however, considerable drawbacks such as CRS, and neurotoxicity limits wider use of CAR-T cell therapy in clinic (Grigor et al., 2019; Srivastava and Riddell, 2018; Xu and Tang, 2014). In addition, the personalized approach to generate CAR-T cells to avoid GVHD makes this therapy time consuming and costly. CAR-NK cells have a number of advantages over CAR-T cells including allogeneic CAR-NK cells have a low probability of triggering GVHD upon transfusion (Ruggeri et al., 2002). In addition, NK cells do not secrete IL1 and IL6, which are the main cytokines responsible for initiating CRS (Xu and Tang, 2014) and lastly, CAR-NK cells retain their natural receptors, such as natural killer progenitor 46 (NKp46), NKp30, NKp44, NKG2D, and CD226, which recognize stress-induced ligands independent of CARs reducing the likelihood of relapses due to loss of the CAR-targeting antigen like that reported for CAR-T cell therapy (Lee et al., 2015). Preliminary data from CD19-CAR engineered human NK cells showed persistence and antitumor control activity against murine or human CD19⁺ leukemia in mice and humanized mice (Liu et al., 2017). Another study using NK-CAR-iPSC cells in vivo showed efficient control of CD19⁺ leukemia progression in humanized mouse models similar to that observed with CAR T cells in patients, although with reduced toxicity due to CRS (Li et al., 2018b).
Development of humanized mice

Due to ethical and logistical constraints, biomedical research has relied on animal models especially mice and rats. However aspects of the rodent biological systems are dissimilar to humans one of which is the immune system (Lux and Nimmerjahn, 2013; Mestas and Hughes, 2004). Efforts to account for differences in the immune systems have culminated in the development of the humanized mouse models.

The ability to engraft mice with human immune system depends heavily on the mouse strain used which needs to be immunodeficient to permit human cell engraftment.

Immunodeficient mice used in humanized mice generation.

In the quest to develop immunodeficient mice to allow efficient recapitulation of human immune system in mice, three major advances have propagated the field. The first was the discovery of the \textit{Prkdc}^{scid} mutation in CB17 mice (Bosma et al., 1983), which enabled engraftment of human immune cells including human PBMCs, fetal hematopoietic tissues and HSCs (Bristol et al., 1997; Lapidot et al., 1992; McCune et al., 1988; Mosier et al., 1988). The \textit{severe combined immunodeficiency} (scid) mutation in the \textit{Prkdc} gene, which encodes the catalytic subunit of the DNA-dependent protein kinase, led to defects in DNA repair, preventing recombination events in T cell and B cell receptors during their development and essentially leading to the loss of adaptive immunity (Blunt et al., 1996). Engraftment of human immune cells within the CB17-\textit{scid} mice was however very low and the engrafted cells were dysfunctional, an observation that was attributed to spontaneous generation of low levels of mouse T and B cells as the mice aged along with high levels of mouse NK cells and other innate cell immune activity, which limited the engraftment of human immune cells (Greiner et al., 1998). An attempt to overcome these
limitations led to the targeted mutation of the recombination-activating gene 1 (Rag1) and Rag2 loci which prevented the development of mature T and B cells. However the Rag1 and Rag2 mutated mice retained high NK cells and therefore still had limited reconstitution of human immune cells (Mombaerts et al., 1992; Shinkai et al., 1992).

In the mid-1990s, the next major advancement came in the form of NOD-scid mice. These mice, which were generated by crossing the scid mutation onto the NOD mouse strain, allowed for improved levels of human immune cell engraftment (Hesselton et al., 1995). NOD-scid mice also showed lower mouse NK cell activity than the CB17-scid mice (Shultz et al., 1995). Additionally, non-obese diabetic (NOD) -scid mice also have a polymorphism in signal regulatory protein alpha (Sirpα) that is very similar to that of humans. When the SIRP-α protein (expressed by myeloid lineage cells including macrophages and DCs (Barclay and Brown, 2006)) binds to CD47 (expressed on most hematopoietic as well as non-hematopoietic cells), the interaction provides a “do not eat me” signal to the macrophage (Barclay and Brown, 2006; Takizawa and Manz, 2007). The similar polymorphism thus provides a “do not eat me” signal from the engrafting human immune system to the mouse macrophages. The NOD mouse also has additional genetic variations including defects in macrophage cytokine production, NK cell function and C5 complement component, which in NOD-scid mice enabled higher engraftment levels of human PBMC (Hesselton et al., 1995) and HSC (Pflumio et al., 1996) compared to the CB17-scid mice. The NOD-scid mouse still had significant limitations including a shortened life span due to the development of thymic lymphomas (Chiu et al., 2002) and residual mouse NK cells.
The last major advance and perhaps one of the most important events in the timeline for humanized mouse generation came in the early 2000s. This was the targeted mutation in the interleukin-2-receptor \( \gamma \)-chain (IL2rg) locus (also known as \( \gamma_c \) and CD132) (Cao et al., 1995; DiSanto et al., 1995; Jacobs et al., 1999; Ohbo et al., 1996). The IL2r \( \gamma \)-chain is a central component in the high-affinity signaling receptors for IL2, IL4, IL7, IL9, IL15, and IL21 (Sugamura et al., 1996). The loss of the IL2r \( \gamma \)-chain therefore causes severe deficiencies in T and B cell development and function, and completely impairs NK cell development (Cao et al., 1995; DiSanto et al., 1995; Ohbo et al., 1996). These mice supported drastically increased engraftment of human tissue, HSCs and PBMCs when crossed onto NOD-Prkdc\text{scid} and the Rag1\text{null} and Rag2\text{null} strains (Ishikawa et al., 2005), the NOD.Shi.Cg-Prkdc\text{scid} strain (Ito et al., 2002), NOD.Cg-Rag1\text{tm1Mo} strain (Pearson et al., 2008b), and the C;129S4-Rag2\text{tmFwa1} BALB/c.Rag2\text{--} strains (Traggiai et al., 2004). Based on these three major breakthroughs, multiple strains of immunodeficient mice have been developed that support high engraftment of human immune systems from CD34+ HSCs. Table 1.3 provides an overview of available strains of immunodeficient IL2r \( \gamma \text{null} \) mice.
**Table 1.3:** Immunodeficient mouse strains that support high levels of engraftment with human hematopoietic and immune cells (adopted and modified from (Shultz et al., 2019a))

<table>
<thead>
<tr>
<th>Strain and Source</th>
<th>Abbreviation</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charles River <a href="http://www.criver.com">www.criver.com</a></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD-Prkdc&lt;sup&gt;em26cd52&lt;/sup&gt;L2rg&lt;sup&gt;em26cd22&lt;/sup&gt;/NjuCrl</td>
<td>NCG</td>
<td>Lack mature murine T cells, B cells and NK cells, radiosensitive</td>
</tr>
<tr>
<td>Taconic (<a href="http://www.taconic.com">www.taconic.com</a>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD.Cg-Prkdc&lt;sup&gt;scid&lt;/sup&gt;Il2rg&lt;sup&gt;Il2rg&lt;sup&gt;tm1Sug&lt;/sup&gt;&lt;/sup&gt;/JicTac</td>
<td>NOG</td>
<td>Lack mature murine T cells, B cells and NK cells, radiosensitive</td>
</tr>
<tr>
<td>NOD.Cg-Prkdc&lt;sup&gt;scid&lt;/sup&gt;Il2rg&lt;sup&gt;Il2rg&lt;sup&gt;tm1Sug&lt;/sup&gt;Tg(CMV-IL2)4-2Jic/JicTac&lt;/sup&gt;</td>
<td>NOG-huIL2</td>
<td>Expresses hu IL2 driven by CMV promoter</td>
</tr>
<tr>
<td>NOD.Cg-Prkdc&lt;sup&gt;scid&lt;/sup&gt;Il2rg&lt;sup&gt;Il2rg&lt;sup&gt;tm1Sug&lt;/sup&gt;Tg(Alb-Tk)7-2/ShiJic&lt;/sup&gt;</td>
<td>TK-NOG</td>
<td>Transgenic expression of thymidine kinase under control of the albumin promoter. Supports human hepatocyte engraftment</td>
</tr>
<tr>
<td>NOD.Cg-Prkdc&lt;sup&gt;scid&lt;/sup&gt;Il2rg&lt;sup&gt;Il2rg&lt;sup&gt;tm1Sug&lt;/sup&gt;Tg(CMV-IL2/IL15)1Jic/JicTac&lt;/sup&gt;</td>
<td>NOG-huIL15</td>
<td>Transgenic expression of hu IL15 under control of the CMV promoter. Supports human NK cell development</td>
</tr>
<tr>
<td>NOD.Cg-Prkdc&lt;sup&gt;scid&lt;/sup&gt;Il2rg&lt;sup&gt;Il2rg&lt;sup&gt;tm1Sug&lt;/sup&gt;Tg(CMV-IL6)1-1Jic&lt;/sup&gt;/JicTac</td>
<td>NOG-huIL6</td>
<td>Transgenic expression of hu IL6 driven by the CMV promoter</td>
</tr>
<tr>
<td>NOD.Cg-Prkdc&lt;sup&gt;scid&lt;/sup&gt;Il2rg&lt;sup&gt;Il2rg&lt;sup&gt;tm1Sug&lt;/sup&gt;Tg(SV40/HTLV-IL3,CSF2)10-7Jic/JicTac&lt;/sup&gt;</td>
<td>huNOG-EXL</td>
<td>Transgenic expression of hu GM-CSF and IL3 driven by SV40 promoter</td>
</tr>
<tr>
<td>The Jackson Laboratory (JAX) (<a href="http://www.jax.org">www.jax.org</a>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD.Cg-Prkdc&lt;sup&gt;scid&lt;/sup&gt;Il2rg&lt;sup&gt;Il2rg&lt;sup&gt;tm1Wjl&lt;/sup&gt;&lt;/sup&gt;J</td>
<td>NSG</td>
<td>Lack mature murine T cells, B cells and NK cells, radiosensitive</td>
</tr>
<tr>
<td>NOD.Cg-Rag&lt;sup&gt;Il2rg&lt;sup&gt;Il2rg&lt;sup&gt;tm1Wjl&lt;/sup&gt;Wjl/Sz&lt;/sup&gt;</td>
<td>NRG</td>
<td>Lack mature murine T cells, B cells, or NK cells</td>
</tr>
</tbody>
</table>
NOD.Cg-B2m<sup>tm1Unc</sup> Prkdc<sup>−/−</sup> Il2rg<sup>tm1Wjl</sup>/SzJ

NOD.Cg-B2m<sup>tm1Unc</sup> Prkdc<sup>−/−</sup> Il2rg<sup>tm1Wjl</sup>Hprt<sup>−/−</sup> /Esh/J

NOD.Cg-B2m<sup>tm1Unc</sup> Prkdc<sup>−/−</sup> Il2rg<sup>tm1Wjl</sup> Hprt<sup>−/−</sup>/Mvw/J

NOD.Cg-Prkdc<sup>−/−</sup>Il2rg<sup>tm1Wjl</sup>Tg(CMV-IL3,CSF2,KIL)1Eav/MloYSzJ

NOD.Cg-Prkdc<sup>−/−</sup>Il2rg<sup>tm1Wjl</sup>H2- Ab1<sup>tmDai</sup>Tg(HLA-DRB1)31Dmz/Sz

NOD.Cg-Prkdc<sup>−/−</sup>Il2rg<sup>tm1Wjl</sup>Tg (PGK-1 KITL*220)441Daw/SzJ

NOD.Cg-Prkdc<sup>−/−</sup>Il2rg<sup>tm1Wjl</sup>Tg(CAG-EGFP 100/Sb/Sz

NOD.Cg-Rag1<sup>tm1Mom</sup> Il2rg<sup>tm1Wjl</sup>Tg(CMV-IL3,CSF2,KITL)1Eav/MloySzJ

NOD.Cg-Prkdc<sup>−/−</sup>Il2rg<sup>tm1Wjl</sup>Kit<sup>W-41</sup>/ThomJ

NOD.Cg-Rag<sup>tm1Mom</sup> Kit<sup>W-41</sup>Il2rg<sup>tm1Wjl</sup>/EavJ

NOD.Cg- Foxn1<sup>emDvs</sup> Prkdc<sup>−/−</sup>Il2rg<sup>tm1Wjl</sup>/J

NOD.Cg-Prkdc<sup>−/−</sup>II2rg<sup>tm1Wjl</sup>Tg(CSF2)2YgyTg(IL3)1YgyTg(KitL)3Ygy/YgyGckRolyJ

NOD-Cg-Tg(HLADRA 0101,HLADRB-10101DmzPrkdc<sup>−/−</sup>IL2rg<sup>tm1Wjl</sup>Gck/RolyJ

NOD.Cg Hgf<sup>tm1.11</sup> (Hgf)Aveo Prkdc<sup>−/−</sup> IL2rg<sup>tm1Wjl</sup>/J

NOD.Cg-Rag<sup>tm1Mom</sup>Ins2<sup>Akita</sup> /SzJ

NSG-B2M<sup>null</sup> Absence of MHC class I
Relatively resistant to GVHD

NSG-Hprt<sup>null</sup> Lack of Hypoxanthine ribosyl transferase
Permissive for xenograft/human tumor growth

NSG-Hprt<sup>null</sup> Lack of Hypoxanthine ribosyl transferase
Permissive for xenograft/human tumor growth

NSG-SGM3 Transgenic expression of hu IL3, GM-CSF and CSF (KitL)
Enhanced myeloid and regulatory T cell engraftment

NSG-Ab<sup>α</sup> DR4 Transgenic expression of human HLA-DR4 allele in the absence of mouse MHC class II
Useful for CD4 T cell targeted transplantation studies with the absence of GVHD

NSG-Ab0<sup>null</sup>DQ8 Transgenic expression of human HLA-DQ8 allele in the absence of mouse MHC class II

NSG-Hu mbSCF Transgenic expression of human membrane-bound human SCF (KitL)
Supports enhanced human HSC and mast cell engraftment
Wide spread EGFP expression
Allows for visualization of mouse host stromal cells to differentiate from human tumor stromal cells

NSG-EGFP

NSG-EGFP

NRG-SGM3 Transgenic expression of human SCF (KitL), GM-CSF, IL3
Enhanced myeloid, AML and regulatory T cell engraftment
Supports human HSC engraftment without irradiation

NRG-W41 Supports human HSC engraftment without irradiation

NSG-nude Athymic

NSG-porcine cytokine Transgenic expression of porcine G-CSF and SCF (Kit)
Supports porcine cytokine engraftment

NSG-DR1 Transgenic expression of chimeric human MHC class II DR1 transgene

NSG-huHGF Ki Knock in of human Hepatocyte growth factor

NRG-Akita Spontaneously diabetic

NBSGW Supports human HSC engraftment without irradiation

NOD.Cg-Hgf<sup>tm1.11</sup> (Hgf)Aveo Prkdc<sup>−/−</sup> IL2rg<sup>tm1Wjl</sup>/J

NOD.Cg-Rag<sup>tm1Mom</sup>Ins2<sup>Akita</sup> /SzJ

NRG-Akita Spontaneously diabetic

34
NOD.Cg-Prkdc<sup>scid</sup>/Il2rg<sup>tm1Wjl</sup>Tg(HLA/H-2D/B2M)1Dvs/Sz
NOD.Cg-Rag1<sup>tm1Mom</sup>/Il2rg<sup>tm1Wjl</sup>Tg(HLA-DRA,HLA-DRB1*0401)39-2Kito/ScasJ
NOD.Cg-Rag1<sup>tm1Mom</sup>/Il2rg<sup>tm1Wjl</sup>Fah<sup>Em1Mvw/MvwJ</sup>
NOD.Cg-Prkdc<sup>scid</sup> H2Ab1<sup>tm1Doi</sup> Il2rg<sup>tm1Wjl</sup>/Sz /SzJ
NOD.Cg-Prkdc<sup>scid</sup> H2K1<sup>tm1Bpe</sup> H2D1<sup>tm1Bpe</sup> Il2rg<sup>tm1Wjl</sup>/Sz /SzJ
NOD.Cg-Prkdc<sup>scid</sup> H2Ab1<sup>tm1Mvw</sup> H2K1<sup>tm1Bpe</sup> H2D1<sup>tm1Bpe</sup>Il2rg<sup>tm1Wjl</sup>/Sz /SzJ
NOD.Cg- Prkdc<sup>scid</sup> B2m<sup>tm1Unc</sup> Prkdc<sup>scid</sup> H2dilAb1Ea Il2rg<sup>tm1Wjl</sup>/Sz /SzJ
NOD.Cg-Rag1<sup>tm1Mom</sup> Fanca<sup>Em1</sup> Dvs IL2rg<sup>tm1Wjl</sup>/Sz
NOD.CgTg(CD68-EGFP)1Drg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/J
NOD.Cg-Prkdc<sup>scid</sup> Cd274<sup>tm1schr</sup>Il2rg<sup>tm1Wjl</sup>/Sz /SzJ
NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>Tg(CSF1)3Sz/Sz
NOD.Cg-Rag1<sup>tm1Mom</sup> Dysf<sup>med</sup> IL2rg<sup>tm1Wjl</sup>McalJ
NOD.Cg-Gcg<sup>em1Dvs</sup> Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/DvsJ
NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>Tg(SEPIN A1*E342K)#Slcw/SzJ
NOD.Cg-Rag1<sup>tm1Mom</sup> Dmd<sup>mdx-4Cv</sup> IL2rg<sup>tm1Wjl</sup>McalJ
NOD.Cg-Rag1<sup>tm1Mom</sup> Sgca<sup>em1Kcam</sup> Il2rg<sup>tm1Wjl</sup>

NSG-HLA-A2/HHD
Supports development of HLA-A2-restricted T cells

NRG-DR4 (DRAG)
Transgenic expression of chimeric HLA-DR4

NRG-FAH KO
Knockout of fumarylacetoacetate hydrolase (Fah) gene
Supports human hepatocyte engraftment

NSG-IA KO
Lack of mouse MHC class II

NSG (K<sup>b</sup>D<sup>b</sup>) Ko
Lack of mouse MHC class I

NSG-(K<sup>b</sup>D<sup>b</sup>)nullIA null
Lack of mouse MHC class I and II

NSG-B2m<sup>null</sup>|IA IE<sup>null</sup>
Lack of mouse MHC class I and class II

ARGN Fanconi’s anemia
May show enhanced HSC engraftment

NSG-CD68-eGFP
Mouse monocytes/macrophages express eGFP

NSG-PDL1 KO
Knockout of programmed death ligand (Pdl1) gene

NSG-RIP-DTR
Supports depletion of mouse beta cells following low dose injection of Diphtheria toxin

NSG-huCSF1
Supports heightened human macrophage development following human HSC engraftment

NRG-BiaJ
Spontaneous progressive muscular dystrophy (Prmd) mutation
Mice develop limb girdle muscular dystrophy 2D

NSG-Gcg KO
Preproglucagon null allele
Supports human islet alpha cell engraftment

NSG-PiZ
Transgenic expression of the mutant SERPIN A1 mutation
Supports engraftment with human hepatocytes

NRG-Mdx KO
Enu-induced mutation at Duchenne muscular dystrophy (Dmd) locus

NSG-Sgca KO
Model for autosomal recessive limb girdle muscular dystrophy 2D
NOD.CgHcPrkdcscidl2rgtm1Wjl

NOD.Cg-Rag1tm1MomIl2rgtm1WjlTg(SLC10A1)15Mvw/MvwJ

NOD.Cg-PrkdcscidCybbem1Hmamll2rgtm1Wjl/HmamJ

NOD.Cg-PrkdcscidIl2rgtm1WjlTg(IL15)SzSzJ

NOD.Cg-PrkdcscidH2Ab1tm1DoiIl2rgtm1WjlTg(H2-Ea-HLADRB1*0401)1Dv/SvJ

C;129S4-Rag2tm1.1Fv/Il2rgtm1Fv/Tg(SIRPA)1Fv

C;129S4-Rag2tm1Fv/l22rgtm1Fv/J

C;129S4-Rag2tm1Fv/Csf1tm1(CSF1)Fv/Il2rgtm1Fv/J

B6.129S-Rag2tm1FwaCD47tm1Flp/Il2rgtm1Wjl/J

B6.129S-Rag1tm1MomCd47tm1Fpl/Il2rgtm1Wjl/J

Individual Laboratories (Examples)

C;129S4-Rag2tm1.1Fv Csf1tm1(CSF1)Fv Csf2/l3tm1.1(CSF2,IL3)Fv Thpotm1.1(TPO)Fv Il2rgtm1.1Fv Tg(SIRPA)1Fv/J
C.CgRag2tm1Fwail2rgTm1Sug

C57BL/6 NOD Sirpa

MISTRG Hu knock-in MCSF,IL-3,GM-CSF,IL3,GM-CSF,TPO,hu SIRP alpha BAC transgene (Rongvaux et al., 2014)

BRG Improved human myeloid cell engraftment

BRGS (on a C57BL/6 strain background)

B6 background, NOD Sirpa transgene (Yamauchi et al., 2013)

NSG-Hc1 Complement-sufficient NSG strain

Supports determination of hemolytic complement-dependent activity

NRGhNTCP/BAC Lack of T, B, and NK cells

Expresses human solute carrier family10 (SLC10A1) sequence

NSG-Cybb Ko Defective phagocyte function

Model for X-linked chronic granulomatous disease

NSG-huL15 Transgenic expression of human IL15

Supports NK development following human HSC engraftment

NSG-Ab4DR4 Selective transgenic expression of HLA-DR4 in the absence of mouse MHC class II

Enables engraftment with HLA-DR-matched HSC

NSG-DR4 Transgenic expression of HLA-DR4

Enables engraftment with HLA-DR-matched HSC

BRGS Human Sirpa BAC transgene

Improved human HSC engraftment with increased lifespan

BRG BALB/c background Rag2 Il2rg KO

Lack mature murine T cells, B cells and NK cells

BRG,M-CSF KI BALB/c background, Rag2 Il2rg KO CSF-1 KI

Improved human monocyte/macrophages engraftment

B6 Triple KO Triple Ko of Rag2, CD47, and IL2rg on B6 background, Supports human HSC engraftment, resistant to GVHD

BRAGG47 Triple Ko of Rag1, CD47, and IL2rg on B6 background Supports human HSC engraftment, resistant to GVHD

C.CgRag2tm1FwaIl2rgTm1Sug

BRGS BALB/c background Rag2 Il2rg KO (Traggiai et al., 2004)
<table>
<thead>
<tr>
<th>BRG NOD Sirpa</th>
<th>BRGS (on a BALB/c background)</th>
<th>NOD Sirpa transgene backcrossed to BALB/c background (Legrand et al., 2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSIRPA Rag2−/−Il2rg−/−</td>
<td>SRG (on a BALB/c background)</td>
<td>Human Sirpa knock-in backcrossed to BALB/c background Rag2 Il2rg KO (Strowig et al., 2011)</td>
</tr>
<tr>
<td>hSIRPA hIL15 Rag2−/−Il2rg−/−</td>
<td>SRG-15 (on a BALB/c background)</td>
<td>Human Sirpa and IL15 knock-in backcrossed to BALB/c background Rag2 Il2rg KO (Herndler-Brandstetter et al., 2017) Improved human NK cell development</td>
</tr>
</tbody>
</table>
Models of human immune system engrafted mice

Based on the two main types of human immune cells that are engrafted into immunodeficient mice namely PBMCs or HSCs, three forms of humanized mouse models can be generated; the human peripheral blood lymphocyte SCID (Hu-PBL-SCID), the human scid-repopulating cells SCID (Hu-SRC-SCID) and the BLT mice (Figure 1.5), each with their own advantages and limitations (Table 1.4).
Figure 1.5: Schematic for generating humanized mice.

With the Hu-PBL-SCID model, PBMCs are engrafted by intravenous (i.v.) or intraperitoneal (i.p.) injection to an adult immunodeficient mouse. However, with the Hu-SRC-SCID model, CD34+ HSCs derived from mobilized peripheral blood stem cells, bone marrow, fetal liver or umbilical cord blood are injected into irradiated neonatal or adult immunodeficient mice via i.v. or route or directly into the bone marrow (BM) cavity (not shown). The BLT model on the other hand require fetal liver and thymus fragments which are implanted under the renal capsule in irradiated adult immunodeficient mice in addition to CD34+ HSC derived from the same fetal liver are injected i.v..
**Hu-PBL-SCID model**

The simplest and most economic method for establishing a humanized immune system in an immunodeficient mouse is the injection of human PBMCs, known as the Hu-PBL-SCID mouse model (Mosier et al., 1988). The model has been used widely to study human immune responses in immune-oncology, autoimmunity, infectious diseases and transplantation tolerance (Ito et al., 2019; Kenney et al., 2016; Walsh et al., 2017). In this model, CD3+ T cells are the predominant human immune cell population that engrafts and remains functional with very rapid human cell reconstitution observed (Ito et al., 2009; King et al., 2009).

One of the major limitations of the Hu-PBL-SCID model is the development of lethal xenogeneic GVHD due to rapidly expanding xeno-reactive human T cells that are directed against murine MHC I and II (Shultz et al., 2012). Generation of immunodeficient strains lacking mouse MHC I and II are helping overcome this major limitation (Brehm et al., 2018). With these new stains, the Hu-PBL-SCID model can be used to study mature human T cell function in humanized mice without of the effects of GVHD.

**Hu-SRC-SCID model**

Human hematopoietic stem cells were first engrafted into CB17-scid mice and termed SCID repopulating cells or SRC, generating the Hu-SRC-SCID mouse model (Lapidot et al., 1992). The repopulating cells were later identified to be CD34+ HSCs. Human CD34+ cells can be obtained from multiple sources including UCB, granulocyte colony stimulating factor (G-CSF)-mobilized HSCs, bone marrow, or fetal liver with the success and efficiency of engraftment affected by the source of the CD34+ HSC and the age, strain
and sex of the mice used. The highest engraftment efficiency is observed when using fetal liver or cord blood CD34+ cells, (reviewed in (Brehm et al., 2016)) with newborn or up to 4 week old mice showing accelerated T cell development compared with adult mice (Brehm et al., 2010b). The Hu-SRC-SCID model support the generation of all human hematopoietic lineages T cells, B cells, NK cells, myeloid cells, and precursors for red blood cells (RBCs). However, not all lineages are functionally developed. For example due to the need for human-specific factors, myeloid cell development is lackluster (Theocharides et al., 2016). Also, circulating human RBCs, and platelets are minimally present, most likely due their rapid removal by murine macrophages (Hu and Yang, 2012; Suzuki et al., 2007; Willinger et al., 2011). Likewise most of the human B cells generated in Hu-SRC-SCID mice are immature CD5+ B cells unable to undergo efficient class switching (Brehm et al., 2016), as the process of B cell differentiation is blocked at the transition phase, causing the accumulation of B cell precursors (Rossi et al., 2001; Watanabe et al., 2009). Lastly, the human T cells are educated on the murine MHC (Watanabe et al., 2009) limiting physiological interactions between the generated human T cells and HLA-expressing human APCs.

**BLT model**

To generate the BLT mouse model, fetal liver and thymic fragments of 16-22 week gestational age are implanted into the sub-renal capsular space of the immunodeficient mice. The thymic and liver bearing recipient mice are then injected i.v. with CD34+ HSC isolated from the autologous fetal liver tissue (Lan et al., 2006; Melkus et al., 2006). The transplanted thymic fragment develops into an organoid that supports HLA-restricted T cell development with the generation of a robust peripheral human immune system.
including human mucosal immune systems (Denton et al., 2012; Kalscheuer et al., 2012). The BLT model however, eventually suffers from a wasting GVHD-like disease (Covassin et al., 2013; Greenblatt et al., 2012; Lockridge et al., 2013). While these three models of humanized mice have revolutionized the study of human immune systems under multiple contexts including immuno-oncology, there are continued efforts underway to further improve the models. These include introducing immunodeficient strains expressing human factors important for improved, complete and robust engraftment of multiple immune cell subsets including RBCs and platelets, and strains with reduced or non-existent xeno-GVHD upon humanization. **Table 1.4** lists some of the limitations of the 3 main types of humanized mouse models.
<table>
<thead>
<tr>
<th>Humanized mouse model</th>
<th>Common abbreviation</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PBL-engrafted SCID</td>
<td>Hu-PBL-SCID</td>
<td>• Easy to establish</td>
<td>• Predominantly T cells engraft; B cells and myeloid cells do not engraft well</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Good T cell engraftment</td>
<td>• All of the engrafted T cells are activated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Engrafts effector and memory T cell populations</td>
<td>• Human T cell xeno-reactivity directed against mouse MHC class I and II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Provides a model of xenogeneic GVHD</td>
<td>• There is a limited window for experimentation owing to GVHD shortening</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mouse life span</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>It is difficult to generate primary immune responses</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Xenogeneic GVHD confounds induced human immune responses</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Host APCs do not express HLA molecules and do not interact with engrafted T cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Human T cells are educated on mouse thymic epithelium and are H2-restricted and not HLA restricted</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Immature B cells with poor class switching</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Although human-derived polymorphonuclear leukocytes, RBCs and megakaryocytes are present in the bone marrow, only low numbers circulate in the blood</td>
</tr>
<tr>
<td>Human SCID-repopulating cell SCID</td>
<td>Hu-SRC-SCID</td>
<td>• Multiple lineages of hematopoietic cells develop, including B and T cells, APCs, myeloid cells and NK cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Engraftment of newborns leads to higher levels of CD3+ T cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Generates a naive human immune system</td>
<td></td>
</tr>
<tr>
<td>Bone marrow, liver, thymus</td>
<td>BLT</td>
<td>• A complete human immune system is engrafted in the absence of human-specific exogenous cytokines</td>
<td>• Surgical expertise is required</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• T cells are HLA restricted</td>
<td>• Wasting GVHD-like disease limits the lifespan of model</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Higher levels of total human hematopoietic cell engraftment are achieved than in the Hu-SRC-SCID model</td>
<td>• Fetal tissue is required</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• It is the only model that leads to the generation of a human mucosal immune system</td>
<td>• Immune responses to virus infections are strong and in some cases can prevent disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Immature B cells with poor class switching</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Responses to vaccination protocols are predominately limited to IgM antibody production</td>
</tr>
</tbody>
</table>
Challenges of current humanized mouse models and approaches to overcome them.

Humanized mouse models have dramatically improved the ability to study and model human immunity. However, no one model based on any of the immunodeficient mice listed in Table 1.3 is sufficient to support the broad spectrum of immunological research areas of interest. In addition, the models also have numerous limitations that arise from the inherent limitation of either the currently available immunodeficient strains or the model of humanized mouse generated.

The limitations arising from the current stains of immunodeficient mice include the potential for xeno-reactive GVHD, leading to limited lifespan and time frame for usefulness of the mice. To overcome this, immunodeficient mouse stains have been developed that lack both murine MHC class I and class II. The NOG mouse deficient in both MHC class I and class II is one such strain, which shows little GVHD when engrafted with human PBMCs (Yaguchi et al., 2018) and develops antigen-specific human T cells when challenged with HLA-A2 cytomegalovirus peptide. Others are the NSG strains NSG-\(B2M^{null}(IA\ IE)^{null}\) and the NSG-\((K^bD^b)^{null} (IA^{null})\) mouse strains (Brehm et al., 2018) that also readily engraft with mature human T cells from PBMCs and are capable of mediating allograft rejection of human islets without the effects of GVHD.

Other limitations are low levels of human immune system reconstitution of gut-associated lymphoid cells, underdeveloped lymphoid organs coupled with poorly developed lymphoid architecture and lack of cytokines that promote the growth and differentiation of
human HSC and immune systems in mice, as many of the cytokines and factors essential for human hematopoietic and immune cell development are not interchangeable with murine factors due to inter-species variations (Allen et al., 2019). Human factors including cytokines are thus being provided to humanized mice through transgenic expression of constructs, knock-in technology and viral expression vectors (Brehm et al., 2016; Theocharides et al., 2016). A number of these human cytokine producing immunodeficient mice have been developed and some are listed in Table 1.3. These factors include factors that improve human innate immune system development such as the myeloid and NK cells which are otherwise suboptimal in all of the standard humanized mouse models. The MISTRG which has the knock-in of human MCSF,IL-3,GM-CSF,IL3,GM-CSF,TPO,hu SIRPα shows improved human monocytes, macrophages and NK cells development in CD34+ HSC engrafted mice (Rongvaux et al., 2014) and NSG-SGM3 CSF1 which is transgenic for human IL-3,GM-CSF, SCF and CSF1 and shows enhanced human CD33+ myeloid development, improved levels of mature CD14+ macrophages, MAST cells and DCs on human CD34+ HSC engraftment (Shultz et al., 2019b). Others are the introduction of human IL15 to improve human NK cells such as in the NOG-IL-15 Tg (Katano et al., 2017), the SRG-15 which has the added advantage of improving reconstitution of gut-associated lymphoid cells (Herndler-Brandstetter et al., 2017) and the NSG Tg(Hu-IL15) described in this thesis. Figure 1.6 summarizes some of the limitations and challenges of current humanized mouse models (adopted and modified from (Allen et al., 2019))
Figure 1.6: Limitations and challenges of current humanized mouse models.

Aspects of humanized mouse models the need improvement to better recapitulate and/or reflect human immune responses.
Humanized mice in Immune-oncology.

Source of Tumor

For humanized mice to be used for immune-oncology studies, they need to support the growth of human tumors. Two sources are used to generate tumor-bearing humanized mice; cell line derived xenograft (CDX) and PDX. While both sources are currently being used to study the immune system’s interaction with the TME, PDX have been shown to have more predictive value for therapeutic responses in patients with cancer (Izumchenko et al., 2017). PDXs also allow the development of personalized therapies, where treatments can be tailored to a particular patient’s cancer type and stage using pharmacological experiment design to assess the treatment efficacy (Calvo et al., 2017).

Table 1.4 summarizes molecular and experimental variations between CDX and PDX that need to be considered when deciding on tumor sources for tumor-bearing humanized mouse generation.
<table>
<thead>
<tr>
<th>Considerations</th>
<th>Tumor cell line</th>
<th>PDX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroma (including immune cells, endothelium and fibroblasts)</td>
<td>Mouse/Human</td>
<td>Mouse/Human</td>
</tr>
<tr>
<td>Representativity of human cancers</td>
<td>Intermediate</td>
<td>High</td>
</tr>
<tr>
<td>Tumor cell genetic stability</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Evaluation of targeted treatments</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Therapeutic screening (including treatment combinations)</td>
<td>Intermediate</td>
<td>High</td>
</tr>
<tr>
<td>Therapeutic correlation with clinical efficacy</td>
<td>Intermediate</td>
<td>High</td>
</tr>
<tr>
<td>Experimental</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Route of transplantation (s.c., i.v., orthotopic, intraductal)</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Experimental procedures</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Tumor growth kinetics</td>
<td>Good</td>
<td>Variable</td>
</tr>
<tr>
<td>AVATAR approach</td>
<td>Not relevant</td>
<td>Relevant</td>
</tr>
</tbody>
</table>
Use of humanized mice in immuno-oncology.

Humanized mice are currently one of the most attractive in-vivo models for preclinical screening of immunotherapies and for understanding the mechanisms governing their action. For example, Jin et al., showed that anti-CD19 CAR-T cell therapy in Hu-SRC-SCID mice was able to control the tumor burden of autologous primary acute B lymphoblastic leukemia as seen in patients. The CD19 CAR-T cells also lead to rapid production of T cell and monocyte/macrophage derived cytokines and an increase in Tregs frequency as reported in the clinic (Jin et al., 2018), providing validation for the potential use of humanized mice in evaluating and to facilitate development of human CAR-T cell therapies.

Partially HLA matched PDX and CDX tumor-bearing Hu-SRC-SCID mice were also used to study the efficacy and mechanisms of anti-PD-1 immunotherapy. In the study, anti-PD-1 antibody treatment produced significant growth inhibition in both CDX and PDX tumors and the inhibition of growth depended on human CD8+ T cells (Wang et al., 2018).

Following the same idea, another study evaluated the effect of a combination of chemotherapy and immunotherapy in humanized mice. Burlion et al., showed that NSG mice reconstituted with CD34+ HSC and bearing triple negative (progesterone receptor negative, estrogen receptor negative, human epidermal growth factor receptor 2 negative) MDA-MB-231 human breast cancer cell line grafts responded to a combination of anti-Inducible T-cell costimulatory (ICOS) neutralizing monoclonal antibody (mAb) and cyclophosphamide with reduced tumor growth which was associated with improved CD8+ T cell to Treg ratios (Burlion et al., 2019).
Humanized mice have therefore yielded considerable and tangible data in the providing new insight into the mechanisms of immunotherapies and shows promise for the preclinical evaluation of cancer immunotherapies.
CHAPTER II

NOD-scid IL2ry\textsuperscript{null} mice lacking TLR4 support human immune system engraftment and enable the study of human-specific innate immune responses.

Introduction

Toll-like receptors, are evolutionarily conserved pattern recognition molecules on innate immune cells that are vital in activating immune responses, forming the first line of defense against microbial pathogens, tissue injury or cancer. To date, 13 members (both human and mouse) have been discovered (Vijay, 2018a). They are expressed on the plasma membranes (TLR 1, 2, 4, 5, 6, 10, 11, 12 and 13) or endosomal membranes (TLR 3, 4, 7, 8, 9) of APCs and can recognize and bind PAMPs and DAMPs making them an important system for innate immune surveillance. (Kaczanowska et al., 2013). All of the TLRs with exception of TLR3 recruit and signal through MyD88, resulting in the activation of the transcription factor NF-\(\kappa\)B which regulates the expression of multiple genes important of proliferation, proinflammatory cytokine production and survival to help protect against infections (Akira et al., 2001). TLR3 activation exclusively signals through the adaptor molecule TRIF. TLR4 is also able to recruit TRIF through the adaptor bridge, TRIF-related adaptor molecule (TRAM), with both MyD88 and TRIF activation culminating in NF-\(\kappa\)B activation and making TLR4 the only TLR capable of signaling through both MyD88 and TRIF. The activation of proinflammatory signaling cascades upon TLR signaling leads to attempts at pathogen elimination (Pradere et al., 2014). When the pathogens cannot be eliminated, a chronic inflammatory state may result, which may also in part be mediated through TLRs (Newton and Dixit, 2012). Chronic inflammatory states
have recently been documented to promote cancer progression with increased TLR4 expression and signaling suggested to play important roles (Nunez et al., 2012; Rakoff-Nahoum and Medzhitov, 2008; Sepehri et al., 2017). In contract, additional reports have also demonstrated an antitumor role for TLR4 signaling in both immune and tumor cells expressing TLR4 (Awasthi, 2014; Nunez et al., 2012). TLR4 signaling therefore is a significant therapeutic target with the development of both agonistic and antagonistic agents for tumor immunotherapy (Oblak and Jerala, 2011).

Rodent models have been the main-stay for translational medicine in the development and testing of therapeutics including immunological modalities. However, not all aspects of the human and murine biology are synonymous, including immune system development, homeostasis and function (Shultz et al., 2012). To overcome the significant differences between human and mouse immune systems, immunodeficient mouse strains have been developed that enable the efficient engraftment of functional human immune systems to generate humanized mice (Brehm et al., 2010c).

To date however, one of the major limitations in using the existing humanized mice models to study human specific TLR responses including TLR4 is the maintenance of functional components of the mouse innate immune system including dendritic cells and macrophages (Brehm et al., 2013). The mouse innate immune cells thus can respond to TLR4 agonists in a mouse specific manner, rendering generated data difficult to interpret. In this study, we developed and the evaluated human specific TLR4 responses in humanized NSG mice that lack the expression of murine TLR4 also known as NSG-\textit{Tlr4}\textsuperscript{null}(TLR4) mice. NSG-TLR4\textsuperscript{null} mice do not respond to TLR4 agonist challenge, while LPS-treatment of standard NSG mice stimulates a robust mouse inflammatory response. To
determine if NSG-TLR4\textsuperscript{null} mice can support human specific TLR4 responses, these mice together with NSG mice were injected with cord blood derived human CD34+ hematopoietic cells to generate Hu-SRC-SCID mice. Our results show that NSG-TLR4\textsuperscript{null} mice engraft with human CD34+ cells and show similar human immune cell chimerism in the blood and spleen as engrafted NSG mice. LPS treated, HSC-engrafted NSG-TLR4\textsuperscript{null} mice produce human inflammatory cytokines without stimulating the murine innate immune system. Lastly LPS challenge of tumor-bearing NSG-TLR4\textsuperscript{null} mice show a human TLR4 specific reduction in tumor growth kinetics. These findings suggest that HSC-engrafted NSG-TLR4\textsuperscript{null} mice are an important model for studying human specific TLR4 responses and for preclinical testing of TLR4 modulators before advancement to clinic.

**Materials and methods**

*Mice*

NSG mice and NSG-TLR4\textsuperscript{null} mice, which do not express murine TLR4, were obtained from colonies developed and maintained at The Jackson Laboratory (Bar Harbor, ME). NSG mice lacking TLR4 were generated by first crossing NOD/Lt-\textit{Tlr4}\textsubscript{Lps-Del} mice obtained from Sasha Chernovsky (Wen et al., 2008) with NSG mice. Further backcrosses of the F1 offspring to NSG mice were carried out to fix the \textit{Prkdc}^{scid} and \textit{Il2rg}\textsuperscript{tm1Wjl} mutations to homozygosity. The NSG-TLR4\textsuperscript{null} mice were maintained by matings of homozygote sibs. All animals were housed in microisolator cages in a pathogen free facility, given autoclaved food and maintained on sulfamethoxazole-trimethoprim medicated water (Goldline Laboratories, FL) and acidified autoclaved water on alternating weeks. All
animal use was in accordance with the guidelines of the Animal Care and Use Committee of the University of Massachusetts Medical School and The Jackson Laboratory and conformed to the recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

**Engraftment of Mice with Human Hematopoietic Stem Cells**

UCB was obtained in accordance with the Committee for the Protection of Human Subjects in Research guidelines of the University of Massachusetts Medical School. The UCB was provided by the medical staff of the University of Massachusetts Memorial Umbilical Cord Blood Donation Program. Groups of 24 to 72 hour-old (newborn) NSG and NSG-TLR4null mice were irradiated with 100 centiGray (cGy) (Pearson et al., 2008a). Irradiated mice were injected with CD3 T cell-depleted human UCB containing $3 \times 10^4$ CD34+ HSC in a 25-50µL volume via intracardiac injection (Brehm et al., 2010a). After 12 weeks, flow cytometry analyses of the blood of HSC recipients quantified the engraftment of the human immune system. For experimental studies, only mice with >10% peripheral human CD45+ cells and >5% human CD3+ T cells were used.

**Antibodies and flow cytometry**

For analysis of mouse innate immune cells, monoclonal antibodies specific for mouse CD45 (30-F11), PDCA1 (927), CD11c (N418), CD11b (M1/70), CD40 (3/23) and CD86 (GL1) were purchased from BD Biosciences (CA), eBiosciences (CA) or BioLegend, (CA). For analysis of human hematopoietic engraftment, monoclonal antibodies specific for
human CD45 (2D1), CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD11c (B-ly6), CD14 (HCD14), CD20 (2H7) and CD80 (2D10), CD86 (2331) CD123 (AC145) HLADR (L243), and CD33 (WM53), were purchased from BD Biosciences, Inc., eBiosciences or BioLegend. Single-cell suspensions of BM and spleen were prepared from cohorts of engrafted and control mice, and whole blood was collected in heparin tubes. Single cell suspensions of 1x10^6 cells in 50µL or 100µL of whole blood were washed with FACS buffer (phosphate buffered saline (PBS) supplemented with 2% fetal bovine serum, (HyClone, UT) and 0.02% sodium azide (Sigma, MO)) and then pre-incubated with rat anti-mouse FcR11b (clone 2.4G2 , BD Biosciences) to block Fc binding. Specific antibodies were then added to the samples and incubated for 30 min at 4°C. Stained samples were then washed and fixed with 2% paraformaldehyde for cell suspensions or treated with BD FACS lysing solution for whole blood. At least 50,000 events were acquired on LSRII or FACSCalibur instruments (BD Biosciences). Data analysis was performed with FlowJo software(Tree Star, Inc., OR).

**LPS and poly(I:C) Treatment**

Ultra-Pure LPS, *(E.coli* 0111:B4 strain), and poly(I:C), high molecular weight (HMW) were purchased from Invivogen (CA). The indicated mice were injected i.p. with 100 µg of either LPS or poly(I:C).

**Serum cytokine analysis**

Levels of murine IL6, IL10, Methyl-accepting chemotaxis 1 (MCP1), IFN-γ, TNF, and IL12p70 were determined in the serum from the indicated mice using the BD™ cytometric
bead array (CBA) Mouse Inflammation Kit (BD Biosciences (CA)). Levels of human IL8, IL1β, IL6, IL10, TNF, and IL12p70 were determined in the serum from the indicated mice using the BD™ CBA Human Inflammation Kit (BD Biosciences (CA)).

**In-vivo tumor experiments**

Patient derived melanoma tumor was obtained from the Human Avatar Tumor Bank at the University of Massachusetts Medical School and passaged in NSG mice to deplete human leukocytes present within the tumor microenvironment. The PDX melanoma was then processed into 2 x 2 mm³ pieces or a single cell suspension and either one piece or cell suspension (2.5 x 10⁶ viable cells) were transplanted subcutaneously to the right flank of HSC-engrafted and non-humanized NSG and NSG-TLR4null mice. The mice were monitored for tumor growth and were treated with 100 µg of LPS or PBS weekly, when their tumors were between 50 and 100 mm³ in volume. Tumor-bearing mice were taken down when tumor volumes approached 4000 mm³ which was the limit set by the IRB of UMASS medical school. Tumors and spleens were then harvested from the mice and processed for flow cytometry staining and analysis. Tumors were measured by calipers every 3 to 4 days, and volumes (mm³) were calculated by (length X width²)/2.

**Statistical Analyses**

To compare individual pair-wise groupings, we used unpaired t-tests and Mann-Whitney test for parametric and non-parametric data, respectively. Three or more means were compared by one-way ANOVA and the Bonferroni multiple comparison test. Significant differences were assumed for p values <0.05. Statistical analyses were performed using
GraphPad Prism software (version 8.0, GraphPad, CA).
Results

Comparison of mouse innate immune system development in NSG and NSG-TLR4null mice

NSG mice lack T cells, B cells and NK cells but still maintain components of the innate immune system, including dendritic cells and macrophages (Shultz et al., 2005). We compared the percentages and total number of mouse innate immune cell populations in the spleen and BM of NSG mice and NSG-TLR4null mice (Figure 2.1). Mouse PDCA1+ (CD317) plasmacytoid dendritic cells (pDC), CD11c+/CD11b- DC, CD11c+CD11b+ DC, and CD11c-/CD11b+ macrophages were identified by flow cytometric analysis. The percentages (Figure 2.1A and 2.1C) and total number (Figure 2.1B and 2.1D) of mouse innate immune cells in spleen (Figure 2.1A and 2.1B) and bone marrow (Figure 2.1C and 2.1D) were comparable for NSG and NSG-TLR4null mice, with statistically significant differences observed. For example, NSG-TLR4null mice have lower levels of plasmacytoid DC in spleen and bone marrow as compared to NSG mice, while higher levels of CD11b+ macrophages were detected in the spleens of NSG-TLR4null mice. These results demonstrate that the TLR4 mutation results in differences in dendritic cell populations and macrophages in NSG mice, but overall there are not major quantitative changes in these innate immune cell populations.
Figure 2.1: Mouse innate immune cell development in NSG-TLR4null mice and NSG mice

A) Percent in Spleen

B) Number in Spleen

C) Percent in Bone Marrow

D) Number in Bone Marrow
Figure 2.1: Mouse innate immune cell development in NSG-TLR4<sup>null</sup> and NSG mice.

Spleen (A and B) and BM (C and D) from 8 to 12-week old NSG and NSG-TLR4<sup>null</sup> mice were analyzed by flow cytometry for levels of mouse PDCA1+ pDC, CD11c+/CD11b- DC, CD11c+/CD11b+ DC and CD11b+ macrophages, as described in the Materials and methods. The percentage (A and C) and total number (B and D) are shown for each tissue. The data are representative of 3 independent experiments.
To confirm that NSG-TLR4\(^{\text{null}}\) mice are unable to respond to TLR4 agonists, NSG and NSG-TLR4\(^{\text{null}}\) mice were treated with LPS and the ability of innate immune cells to increase expression of phenotypic markers (Table 2.1) and produce cytokines (Figure 2.2) were evaluated. To assess phenotypic changes on mouse innate immune cells, NSG and NSG-TLR4\(^{\text{null}}\) mice were injected i.p. with 100 \(\mu\)g of either LPS or poly(I:C), and 24 hours later expression of CD40 and CD86 was evaluated on the surface of dendritic cells and macrophages recovered from the spleen as shown in Table 2.1. LPS treatment of NSG mice stimulated increased expression of CD40 on CD11c+/CD11b- DC, CD11c+/CD11b+ DC and CD11c-/CD11b+ macrophages and increased expression of CD86 on CD11c+/CD11b- DC as compared to control mice. In contrast, LPS treatment of NSG-TLR4\(^{\text{null}}\) mice did not stimulate increased expression of CD40 and CD86 on mouse innate immune cell populations. Poly(I:C)-induced increases in the expression of CD40 and CD86 on mouse innate immune cells was similar between NSG and NSG-TLR4\(^{\text{null}}\) mice demonstrating normal TLR3-mediated responses in NSG-TLR4\(^{\text{null}}\) mice.
Table 2.1: Mouse innate immune cells from NSG-TLR4null mice do not increase CD40 or CD86 expression following exposure to LPS.

<table>
<thead>
<tr>
<th>CD40 Expression</th>
<th>NSG (MFI)</th>
<th>NSG-TLR4null (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controla</td>
<td>LPS Treated</td>
</tr>
<tr>
<td>CD11c+/-CD11b- DC</td>
<td>6766±753</td>
<td>22170±3076b</td>
</tr>
<tr>
<td>CD11c+/-CD11b+ DC</td>
<td>716.3±101</td>
<td>6087±1099b</td>
</tr>
<tr>
<td>CD11b+ Macrophage</td>
<td>4141±404</td>
<td>6043±517b</td>
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</tbody>
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<table>
<thead>
<tr>
<th>CD86 Expression</th>
<th>NSG (MFI)</th>
<th>NSG-TLR4null (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controla</td>
<td>LPS Treated</td>
</tr>
<tr>
<td>CD11c+/-CD11b- DC</td>
<td>367.3±67.8</td>
<td>1005±71.7c</td>
</tr>
<tr>
<td>CD11c+/-CD11b+ DC</td>
<td>161.3±16.3</td>
<td>188.7±29</td>
</tr>
<tr>
<td>CD11b+ Macrophage</td>
<td>95.98±13.3</td>
<td>24.07±9.84</td>
</tr>
</tbody>
</table>

a. Control animals were treated with PBS and injected with a volume equal to that used for LPS and poly(I:C) injection.
b. $p<0.001$, as compared to untreated
c. $p<0.01$, as compared to untreated
To assess cytokine production, mice were injected i.p. with 100 µg of either LPS or poly(I:C), and 24 hours later blood samples were collected and serum cytokine levels were assessed by CBA (Figure 2.2). Serum samples were evaluated for mouse IL-6, IL-10, MCP1, IFNγ, TNF and IL12p70 and compared to PBS injected mice. NSG mice produced significant levels of IL-6, IL-10 and MCP following challenge with LPS and this cytokine response was absent in NSG-TLR4null mice treated with LPS. Poly(I:C) treatment induced a similar mouse cytokine response in both NSG and NSG-TLR4null mice. Together these data show that NSG-TLR4null mice develop mouse innate immune cells but these cells are unable to mount a functional response to the TLR4 agonist, LPS.
Figure 2.2: LPS treatment does not stimulate production of mouse cytokines in NSG-TLR4null mice.
Figure 2.2: LPS treatment does not stimulate production of mouse cytokines in NSG-TLR4<sup>null</sup> mice. NSG and NSG-TLR4<sup>null</sup> mice were injected i.p. with PBS, LPS (100µg) or poly(I:C) (100µg). Blood samples were collected 24 hours after injections and levels of mouse cytokines (IL6, IL10, MCP1, IFN<sub>γ</sub>, TNF and IL12p70) were quantified by CBA, as described in the Materials and Methods. The cytokine levels shown are an average of 6 mice and error bars indicate the standard error of mean. The data are representative of 3 independent experiments. For statistical analysis, the average cytokine levels for LPS and poly(I:C) treated mice were compared to levels for PBS treated mice; ** p<0.01, *** p<0.001, **** p<0.0001.
Human immune cell chimerism levels are similar in HSC-engrafted NSG-TLR4-null and NSG mice.

The results above show that NSG-TLR4-null mice do not respond to LPS treatment. We next tested if NSG-TLR4-null mice engraft with human HSC and develop a human immune system to the same level as NSG mice. For these experiments newborn conditioned NSG or NSG-TLR4-null mice were engrafted with human HSC as described in the Materials and methods and were evaluated for human cell chimerism in the spleen, bone marrow and blood at 16 weeks (Figure 2.3). Slightly higher percentages of human CD45+ cells were detected in the spleen (Figure 2.3A), bone marrow (Figure 2.3B) and blood (Figure 2.3C) of NSG-TLR4-null mice but this was not significantly different from the levels in NSG mice. There were no significant differences in the number of human CD45+ cells in the spleen (Figure 2.3D) and bone marrow (Figure 2.3E) of NSG-TLR4-null mice as compared to NSG mice. We next compared the levels of human T cells and B cells in spleen, bone marrow and blood of NSG and NSG-TLR4-null mice engrafted with human HSC (Figure 2.4). No significant differences were observed for the percentages of human CD3+ T cells (Figures 2.4A, 2.4B and 2.4C), the CD4:CD8 T cell ratio (Figures 2.4D, 2.4E and 2.4F) or in the percentages of human CD20+ B cells (Figures 2.4G, 2.4H and 2.4I) between the HSC engrafted strains. We next evaluated the development of human pDC, human mDC, and human monocyte/macrophage levels in spleen, BM and blood of NSG and NSG-TLR4-null mice engrafted with human HSC (Figure 2.5). No significant differences were observed for the percentages of human CD123+ pDC cells (Figures 2.5A, 2.5B and 5C) and human CD14 positive monocyte/macrophage (Figures 2.5G, 2.5H and 2.5I) between the HSC engrafted strains. A significantly higher frequency of human CD11c+
mDC were detected in the BM (Figure 2.5E) of NSG-TLR4\textsuperscript{null} mice but no differences were observed in the spleen (Figure 2.5D) or blood (Figure 2.5F). Overall these data indicate that NSG-TLR4\textsuperscript{null} mice engraft efficiently with human HSC as compared to NSG mice and develop both adaptive and innate human immune cells.
Figure 2.3: NSG-TLR4null mice engrafted with human HSC have high levels of human cell chimerism. NSG and NSG-TLR4null newborn mice were engrafted with human HSC, as described in the Materials and methods. Mice were analyzed by flow cytometry for levels of human CD45+ cells in the spleen (A and D), bone marrow (B and E) and blood (C) at 16 weeks of age. The percentages (A, B and C) and total number (D and E) of human CD45+ cells are shown and each point represents an individual animal. The data are from a total of 4 independent experiments.
Figure 2.4: NSG-TLR4null mice engrafted with human HSC develop human T and B cell populations.
Figure 2.4: NSG-TLR4null mice engrafted with human HSC develop human T and B cell populations. NSG and NSG-TLR4null newborn mice were engrafted with human HSC, as described in the Materials and methods. Mice were analyzed by flow cytometry for levels of human T cells and B cells in the spleen (A, D and G), bone marrow (B, E and H) and blood (C, F and I) at 16 weeks of age. The percentage of CD3+ T cells (A, B and C), the ratio of CD4 to CD8 T cells (D, E and F) and the percentage of human CD20+ cells (G, H and I) are shown. Each point represents an individual animal, and the data are from a total of 4 independent experiments.
Figure 2.5: NSG-\textsuperscript{TLR4\null} mice engrafted with human HSC develop human innate immune cell populations.

A. Spleen

B. Bone Marrow

C. Blood

D. Spleen

E. Bone Marrow

F. Blood

G. Spleen

H. Bone Marrow

I. Blood
Figure 2.5: NSG-TLR4null mice engrafted with human HSC develop human innate immune cell populations. NSG and NSG-TLR4null newborn mice were engrafted with human HSC, as described in the Materials and methods. Mice were analyzed by flow cytometry for levels of human innate immune cells in the spleen (A, D and G), bone marrow (B, E and H) and blood (C, F and I) at 16 weeks of age. The percentage of human CD123+ pDC (A, B and C), the percentage of human CD11c+ mDC (D, E and F) and the percentage of human CD14+ monocyte/macrophage (G, H and I) are shown. Each point represents an individual animal, and the data are from a total of 4 independent experiments.
LPS treatment of HSC-engrafted NSG-TLR4null mice increases expression of co-stimulatory molecules on human innate immune cells.

The lack of a mouse innate immune response to LPS in NSG-TLR4null mice enables the study of human-specific TLR4-mediated responses in HSC-engrafted mice. We compared the human response to LPS treatment in newborn NSG and NSG-TLR4null mice engrafted with human HSC. HSC-engrafted NSG and NSG-TLR4null mice were treated with PBS or LPS, and 24 hours later the levels of human CD45+ cells were determined in the spleen, BM and blood. No significant differences were detected in the percentage of human CD45+ cells from the spleen (Figure 2.6A) and BM (Figure 2.6B) for either mouse strain. In contrast, HSC-engrafted NSG mice showed a significant decrease in human CD45+ cells in the blood after LPS treatment and this effect was absent in the NSG-TLR4null mice (Figure 2.6C). Next, we examined human innate immune cell populations (pDC, mDC and monocyte/macrophage) from the blood of HSC-engrafted NSG and NSG-TLR4null mice for changes in the expression of CD80 (Figures 2.6D, 2.6E and 2.6F) and CD86 (Figures 2.6G, 2.6H and 2.6I) at 24 hours after LPS treatment. Treatment of HSC-engrafted NSG-TLR4null mice with LPS stimulated a significant increase in the expression of CD80 and CD86 on human CD123+ pDC (Figures 2.6D, 2.6G), human CD11c+ mDC (Figures 2.6E, 2.6H) and human CD14+ monocyte/macrophage (Figures 2.6F, 2.6I). LPS treatment also stimulated significant increases in CD80 expression on human mDC (Figure 2.6E) and human monocyte/macrophage (Figure 2.6F) and a significant increase in CD86 expression on mDC (Figure 2.6H) in HSC-engrafted NSG mice. These data indicate that the LPS
induces global changes in phenotypic markers on human innate immune cells in HSC-engrafted NSG-TLR4\textsuperscript{null} mice but not in NSG mice.
Figure 2.6: LPS treatment activates human innate immune cells in NSG-TLR4<sup>null</sup> mice engrafted with human HSC.
Figure 2.6. LPS treatment activates human innate immune cells in NSG-TLR4null mice engrafted with human HSC. NSG and NSG-TLR4null newborn mice were engrafted with human HSC, as described in the Materials and methods. HSC-engrafted mice were injected i.p. with PBS or LPS and 24 hours later, percentages of human CD45+ cells were determined in the spleen (A), bone marrow (B) and blood (C). Expression of CD80 (D, E and F) and CD86 (G, H and I) was evaluated on human CD123+ pDC (D and G), human CD11c+ mDC (E and H) and human CD14+ monocyte/macrophage (F and I) recovered from the blood at 24 hours post-treatment. Each point represents an individual animal, and the data are from a total of 3 independent experiments.
**LPS treatment of HSC-engrafted NSG-TLR4null Mice stimulates production of human inflammatory cytokines in the absence of a mouse cytokine response.**

We next compared human cytokine production in HSC-engrafted NSG and NSG-TLR4null mice after LPS treatment (**Figure 2.7**). HSC-engrafted mice were treated with PBS or LPS and serum was harvested 6 hours later. The recovered serum samples were tested for levels of human IL8 (**Figure 2.7A**), IL1β (**Figure 2.7B**), IL6 (**Figure 2.7C**), IL10 (**Figure 2.7D**), TNF (**Figure 2.7E**) and IL12p70 (**Figure 2.7F**). LPS treatment stimulated significant increases in IL8, IL1β and IL6 in both HSC-engrafted NSG and NSG-TLR4null mice. In addition, increased levels of TNF were detected in HSC-engrafted TLR4null mice but not in NSG mice. No significant increases were detected in levels of human IL10 and IL12p70. We next asked if production of human cytokines in HSC-engrafted NSG-TLR4null mice stimulated by LPS would activate murine innate immune cells to produce inflammatory cytokines in a bystander manner. To test this NSG and NSG-TLR4null mice that were either unmanipulated or engrafted with human HSC were treated with LPS and serum was collected 6 hours later for cytokine analyses (**Figure 2.8**). Mouse IL6 (**Figure 2.8A**), IL10 (**Figure 2.8B**), MCP1 (**Figure 2.8C**) and TNF (**Figure 2.8D**) were detected at significantly higher levels in NSG mice as compared to both unmanipulated or HSC-engrafted NSG-TLR4null mice. The levels of mouse cytokines detected in HSC-engrafted NSG-TLR4null mice treated with LPS were not significantly higher than levels in unmanipulated NSG-TLR4null mice treated with LPS. Overall these results indicate that LPS-induced human inflammatory cytokines in HSC-engrafted NSG-TLR4null mice do not stimulate bystander activation of the mouse innate immune system.
Figure 2.7: LPS treatment stimulates production of human cytokines in NSG and NSG-TLR4\textsuperscript{null} mice engrafted with human HSC.

A. Human IL-8 (pg/ml)

B. Human IL-1β (pg/ml)

C. Human IL-6 (pg/ml)

D. Human IL-10 (pg/ml)

E. Human TNF (pg/ml)

F. Human IL-12p70 (pg/ml)

\( p = 0.03 \)

\( p = 0.02 \)

\( p = 0.03 \)

\( p = 0.009 \)

\( p = 0.03 \)
Figure 2.7: LPS treatment stimulates production of human cytokines in NSG and NSG-TLR4<sup>null</sup> mice engrafted with human HSC. NSG and NSG-TLR4<sup>null</sup> newborn mice were engrafted with human HSC, as described in the Materials and Methods. HSC-engrafted mice were injected i.p. with PBS or LPS and 6 hours after treatment serum samples were recovered. Levels of human cytokines (IL8, IL1β, IL6, IL10, TNF and IL12p70) were quantified by CBA, as described in the Materials and methods. Each point represents an individual animal, and the data are from a total of 2 independent experiments. For statistical analysis, the average cytokine levels for LPS treated mice were compared to levels for PBS treated mice.
Figure 2.8: LPS treatment of HSC-engrafted NSG-TLR4null mice does not induce a mouse cytokine response.

NSG and NSG-TLR4null mice that were unmanipulated or engrafted with human HSC as newborns were injected i.p. with LPS (100 µg). Serum

Figure 2.8: LPS treatment of HSC-engrafted NSG-TLR4null mice does not induce a mouse cytokine response. NSG and NSG-TLR4null mice that were unmanipulated or engrafted with human HSC as newborns were injected i.p. with LPS (100 µg). Serum
samples were collected 6 hours after LPS injections, and levels of mouse cytokines (IL6, IL10, MCP1, and TNF) were quantified by CBA, as described in the Materials and methods. Each point represents an individual animal. * p<0.01, ** p<0.01, *** p<0.001, **** p<0.0001.
**LPS treatment of HSC-engrafted NSG-TLR4*null* mice stimulates production of human inflammatory cytokines in the absence of a mouse cytokine response.**

We next performed a kinetic analysis of human cytokine production that was stimulated by LPS treatment of HSC-engrafted NSG-TLR4*null* mice (*Figure 2.9*). HSC-engrafted NSG-TLR4*null* mice were treated with PBS or LPS and serum samples were recovered at 2, 6, 12 and 24 hours. Human IL-8 production was first detectable at 2 hours, peaking at 6 hours and declining by 24 hours (*Figure 2.9A*). Human IL1β was first detectable at 6 hours with the response declining by 12 hours (*Figure 2.9B*). Human IL6 was first detectable at 2 hours with the response declining by 12 hours (*Figure 2.9C*). Human IL10 was first detectable at 6 hours with the response declining by 24 hours (*Figure 2.9D*). Human TNF was first detectable at 2 hours and the response was not detectable at 12 hours (*Figure 2.9E*). Low levels of human IL-12p70 were detectable at the 12-hour time point (*Figure 2.9F*). Together these data indicate that HSC-engrafted NSG-TLR4*null* mice can be used to study human-specific cytokine responses to TLR4 agonists.
Figure 2.9: Kinetics of LPS stimulates human cytokine production in NSG-TLR4null mice engrafted with human HSC.

A. Human IL8 (pg/ml)

B. Human IL1β (pg/ml)

C. Human IL6 (pg/ml)

D. Human IL10 (pg/ml)

E. Human TNF (pg/ml)

F. Human IL12p70 (pg/ml)
Figure 2.9: Kinetics of LPS stimulates human cytokine production in NSG-TLR4\textsuperscript{null} mice engrafted with human HSC. NSG-TLR4\textsuperscript{null} newborn mice were engrafted with human HSC, as described in the Materials and Methods. HSC-engrafted mice were injected i.p. with PBS or LPS and serum samples were recovered at 2, 6, 12 and 24 hours after treatment. Levels of human cytokines (IL8, IL1\(\beta\), IL6, IL10, TNF and IL12p70) were quantified by CBA, as described in the Materials and methods. Each point represents an individual animal, and the data are from a total of 3 independent experiments. For statistical analysis, the average cytokine levels for LPS treated mice were compared to levels for PBS treated mice at each time point; *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\).
**LPS treatment reduces tumor growth kinetics of human PDX melanoma in NSG-\(\text{TLR4}^{\text{null}}\) mice engrafted with human HSC**

Given that we can observe human specific TLR4 responses in HSC-engrafted NSG-\(\text{TLR4}^{\text{null}}\) without bystander activation of the mouse innate system, we next tested the effect of the TLR4 agonist LPS on the growth kinetics of PDX melanoma (**Figure 2.10**). We first tested the effect of LPS on tumor growth in unengrafted NSG and NSG-\(\text{TLR4}^{\text{null}}\) to determine the baseline effect of mouse TLR4 deletion on LPS driven tumor growth rate. A significant reduction in PDX melanoma growth rate was observed in tumor-bearing NSG mice treated with LPS when compared to PBS treated mice (**Figure 2.10B**), however melanoma growth kinetics in LPS treated NSG-\(\text{TLR4}^{\text{null}}\) was not significantly different compared PBS treated tumor-bearing NSG-\(\text{TLR4}^{\text{null}}\) mice (**Figure 2.10C**). Knowing that growth rates for the PDX melanoma do not change following LPS stimulation in NSG-\(\text{TLR4}^{\text{null}}\), we examined the effects of human specific TLR4 challenge on PDX melanoma growth. HSC-engrafted NSG-\(\text{TLR4}^{\text{null}}\) showed a significant reduction in PDX tumor growth kinetics upon LPS challenge (**Figure 2.10E**) albeit more modest to the growth reduction observed in HSC-engrafted NSG mice (**Figure 2.10D**). Taken together these data indicate that PDX melanoma responds to TLR4 agonist stimulation in a human specific manner in HSC-engrafted NSG-\(\text{TLR4}^{\text{null}}\) mice.
Figure 2.10: LPS treatment reduces tumor growth kinetics for a human PDX melanoma in NSG-TLR4<sup>null</sup> mice engrafted with human HSC. Four to eight-week old NSG and NSG-TLR4<sup>null</sup> mice that were left unmanipulated or engrafted with human HSC were transplanted subcutaneously with PDX melanoma and treated with 100 µg of LPS or PBS weekly, as described in the Materials and methods (A). Tumor growth kinetics...
were monitored in unmanipulated NSG (B), unmanipulated NSG-TLR4null (C), engrafted NSG (D) and engrafted NSG-TLR4null (E) mice. The data are representative plots from 2 independent experiments. For statistical analysis the tumor growth curves of LPS treated mice were compared to PBS treated mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.


**LPS treatment of HSC-engrafted NSG-TLR4null mice bearing PDX melanoma reduces the proportions of human innate immune cells within the tumor microenvironment.**

With the observed decrease in tumor growth kinetics following LPS treatment in the PDX melanoma-bearing HSC engrafted NSG-TLR4null mice, we interrogated the effect of LPS on the immune profile in the tumor micro-environment. For these experiments, we harvested tumors from PDX melanoma bearing NSG or NSG-TLR4null mice which were engrafted with human HSC and treated with LPS or PBS as described in the Materials and methods. We used flow cytometry to interrogated the tumor microenvironment for changes in the human immune cell repertoire upon LPS treatment (Figure 2.11). No significant differences in percentages (Figure 2.11A) and numbers (Figure 2.11B) of human CD45+ cells in the tumor microenvironment were observed between LPS and PBS treated NSG-TLR4null or NSG mice. Although slightly higher human CD45+ cells were detected within the tumor of NSG mice, this was not significantly different from the levels in NSG-TLR4null mice (Figure 2.11A and 2.11B). Next, we evaluated the proportions of human T cells, B cells and innate cells within the tumor. We observed no differences in the percentages of human CD3+ T cells between LPS and PBS treated mice from both strains (Figures 2.11C). However, upon LPS treatment, there were significant reduction in the percentage of human CD33+ myeloid cells (Figure 2.11E) compared to PBS treated mice for both strains and a significant increase in the human CD20+ B cells for NSG mice (Figure 2.11D). We then evaluated the proportions of human pDC, human mDC, and human monocyte/macrophage levels in the tumor of NSG and NSG-TLR4null mice to determine which population accounted for the observed reduction
in percentages of CD33+ innate cells following LPS treatment. There was a significant reduction in percentages of human CD14+ monocyte/macrophage cells in the tumor following LPS treatment in both NSG and NSG-TLR4<sup>null</sup> mice (Figure 2.11F). There was also a significant decrease in percentages of both human CD11c+ mDC and human CD123+ pDC in LPS treated compared to PBS treated NSG mice (Figure 2.11G and 2.11H respectively) The trend for decreased levels of human mDC and human pDC in LPS treated NSG-TLR4<sup>null</sup> mice was not significant (Figure 2.11G and 2.11H). Together these data indicate that the LPS induces changes within tumor grafts of HSC-engrafted NSG mice, specifically reducing the levels of human innate immune cells.
Figure 2.11: LPS treatment reduces innate immune cell populations within PDX tumors of NSG and NSG-TLR4\textsuperscript{null} mice engrafted with human HSC.
Figure 2.11: LPS treatment reduces innate immune cell populations within PDX tumors of NSG and NSG-TLR4\textsuperscript{null} mice engrafted with human HSC. Four to eight-week old NSG and NSG-TLR4\textsuperscript{null} mice that were left unmanipulated or engrafted with human HSC were transplanted subcutaneously with PDX melanoma and treated with LPS or PBS as described in the Materials and methods. The tumors were harvested and analyzed by flow cytometry for levels of human CD45+ leukocytes (A, B), human CD3+ T cells (C), human CD20+ B cells (D), human CD33+ myeloid cells (E), human CD14+ monocyte/macrophage (F), human CD11c+ mDC (G), and human CD123+ pDC (H). The percentages (A, C, D, E, F, G and H) and total number (B) of human CD45+ cells are shown and each point represents an individual animal. For statistical analysis the LPS treated mice were compared with PBS treated mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Discussion

Humanized mice have been employed as experimental tools to study human immune system function and for evaluating immune stimulatory compounds (Bryce et al., 2016; De La Rochere et al., 2018; Durost et al., 2017; Wang et al., 2018; Zhang et al., 2018). One of the major limitations in using humanized mice to study innate immune therapeutics however is the presence of mouse innate immune cells that can and do respond to stimulation such as experimental therapy targeting TLRs, making interpretation of experimental data difficult. Here, we have overcome this limitation with regards to TLR4 signaling using a novel strain of NSG mice that lack mouse TLR4. Upon engraftment with UCB-derived human CD34+ cells, NSG-TLR4null humanized mice are able to develop a human immune system with chimerism levels similar to those generated in NSG mice. The human immune cells remain functional as shown by their ability to produce cytokine upon challenge. Moreover, LPS challenge leads to a human-specific response in HSC-engrafted NSG-TLR4null mice without input from the mouse innate immune cells. Importantly, a PDX melanoma tumor responds to LPS modulation in a human specific manner in NSG-TLR4null mice, enabling the testing of TLR4 targeted cancer immunotherapies *in-vivo*.

The NSG mouse strain lacks T, B and NK cells which enables engraftment with human HSC, but these mice still retain mouse innate cells including macrophages and DCs (Shultz et al., 2005). The remaining murine innate cells respond to immune stimuli and generate an inflammatory response that delays the growth of a PDX melanoma. In our present study mouse innate immune cells development in NSG-TLR4null mice was
comparable to those in NSG mice. Of note however, was our observation of significantly lower plasmacytoid DC in the spleen and bone marrow and lower CD11c+/CD11b- DC, CD11c+/CD11b+ DC and macrophages in the bone marrow of NSG-TLR4$^{null}$ mice compared to NSG mice. This observation in mouse innate development in within the NSG-TLR4$^{null}$ we believe is due to the role TLRs signaling including TLR4 play in hematopoietic development, particularly with regards to DCs and macrophages (Griffin et al., 2018; Michelsen et al., 2001). Nagai et al. showed that hematopoietic progenitors expressed functional TLR2 and TLR4, and stimulation of these receptors lead to myeloid progenitors giving rise to monocytes and/or macrophages while lymphoid progenitors becoming DCs (Nagai et al., 2006). In that light, the loss of TLR4 in the NSG-TLR4$^{null}$ mice may be preventing TLR4 signaling in hematopoietic progenitors and negatively impacting mouse innate immune cell replenishment. The NSG-TLR4$^{null}$ mice were unable to respond to LPS treatment, in contrast, poly(I:C) simulated mouse innate immune responses in both NSG-TLR4$^{null}$ and NSG mice similarly with increases in cytokine production (IL-6, IL-10 and MCP) and surface expression of CD40 and CD86 indicting a failure for TLR4 signaling but not TLR3 in the NSG-TLR4$^{null}$ mouse stain.

The ability of NSG-TLR4$^{null}$ mice to engraft human leukocytes was assessed by injecting NSG-TLR4$^{null}$ and NSG mice with UCB-derived CD34+ HSC. Human CD45+ immune cell chimerism was similar between NSG-TLR4$^{null}$ and NSG mice. The two strains also developed similar frequencies of human innate and adaptive immune cells with the exception of CD11c+ myeloid DC which was higher in the bone marrow of HSC-engrafted NSG-TLR4$^{null}$ mice. These human innate immune cells respond to TLR4 specific LPS
challenge with an increase in the mean fluorescence intensity (MFI) of the expression markers CD80 and CD86 and production of IL-8, IL-1β, IL-6 and TNF. Notably, human cytokine production following LPS challenge did not stimulate the bystander activation of mouse innate immune cells, with no observable production of murine cytokine in HSC-engrafted NSG-TLR4<sup>null</sup> mice. These observations suggest that HSC-engrafted NSG-TLR4<sup>null</sup> mice respond to LPS challenge in a human specific manner.

Most importantly NSG-TLR4<sup>null</sup> mice support an in-vivo platform for the development and testing of novel strategies for immunotherapy based on TLR4 stimulation. TLR4 activation has been linked with both cancer progression and regression. The expression of TLR4 has been used as a prognostic indicator in established cancers where TLR4 expression creates an environment to promote cancer cell proliferation (Stevens et al., 2008; Weng et al., 2014; Zhang et al., 2013; Zou et al., 2013). For example, there is experimental data indicating that LPS induced TLR4 signaling promotes hepatocellular carcinoma survival and proliferation (Wang et al., 2013). Also, the blockade of TLR4 signaling through NF-κB inactivation by siRNA reduces the invasive ability of cancer cells, with murine models of colorectal metastasis and hepatic steatosis showing decreased tumor burden (Earl et al., 2009). However, there is also mounting evidence indicating the requirement of TLR4 for a protective immune response. For instance, TLR4 silencing leads to increased breast cancer metastasis (Ahmed et al., 2013) and Paclitaxel (an alternative LPS-like immune stimulatory drug) treatment lead to reduction in murine melanoma and breast cancer tumors growth in a TLR4-dependent manner (Wanderley et al., 2018). In our study LPS treatment was able induce tumor growth regression of a PDX melanoma in HSC-
engrafted NSG-TLR4\textsuperscript{null} mice. The observed impact on tumor growth correlated with a reduction in tumor infiltrating human innate immune cells particularly tumor-associated macrophages and DCs. Tumor associated macrophages have been shown to play a tumor supporting role through production of angiogenesis and growth factors including IL10 and IL-1\textbeta in addition to regulating T cell activity (Aras and Zaidi, 2017; Voronov et al., 2003). The observed reduction in the innate immune compartment within the TME therefore reduces the tumors access the necessary factors for its propagation and potentially stifles the PDX melanoma’s growth.

These results emphasize the ability of NSG-TLR4\textsuperscript{null} mice to be used for the development and evaluation of TLR4 specific modulators both agonist and antagonist. Indeed there are currently a few TLR4 modulators approved for use and more in development including Erictoran (E5564) developed by Eisai (Tokyo, Japan), which prevents TLR4 dimerization and signaling (Kuo et al., 2016), Ibudilast (AV4II), a TLR4 antagonist that suppresses pro-inflammatory cytokines in neuroinflammation (Fox et al., 2018) and MPL A which is a chemically modified and less toxic form of LPS and its derivatives are being evaluated for use as cancer vaccine adjuvants (Wang et al., 2012; Zhou et al., 2015; Zhou et al., 2017).

In summary, we describe here, an NSG mouse model lacking murine TLR4, that can engraft human immune cells with similar chimerism to NSG mice. We also show that NSG-TLR4\textsuperscript{null} mice do not respond to a mouse specific TLR4 agonist, but are able to mount human specific TLR4 response upon human immune system engraftment without bystander activation of murine innate immune cells. Finally, these mice show \textit{in-vivo}
modulation of PDX melanoma by the TLR4 agonist LPS in a human specific manner. This new model of NSG mice will facilitate the development and testing of human specific TLR4 modalities of cancer immunotherapy.
CHAPTER III

Transgenic expression of human IL15 in NOD-scid IL2r<sup>null</sup> mice enhances the development and survival of functional human NK cells

Introduction

The human innate immune system plays critical roles in tumor surveillance and in immunoregulation within the tumor microenvironment (Stolk et al., 2018). NK cells, which are naturally cytotoxic innate lymphocytes with intrinsic and anti-tumor properties (Oldham, 1983) are potential targets for cancer immunotherapy (Bottcher et al., 2018; Fang et al., 2017; Li et al., 2015) with NK cell function being regulated by a balance in activating and inhibitory receptor signaling (Long et al., 2013). NK cells express KIRs such as CD158b, CD158e1 and killer cell lectin-like receptor subfamily C, member 1 (CD94/NKG2A) that can engage self-MHC class I resulting in a negative activation signal the impedes NK cell action (Anthony R French and Yokoyama, 2003; Hans-Gustaf Ljunggren and Kärre, 1990). Thus, cells that have lost expression of self MHC class I are targets for NK cell killing, which is directly relevant to the control of malignancies that have downregulated MHC expression. In addition many transformed cells increase their expression of stress-induced molecules, including MHC class I polypeptide-related sequence A and B (MICA and MICB) which can be recognized by activating NK cell receptors such as NKG2D (Bauer et al., 1999). This balance of positive and negative signaling helps NK cells distinguish between transformed cells such as tumors and MHC class I non-expressing cells like erythrocytes (Waldhauer and Steinle, 2008). Previous studies have demonstrated that, the presence of tumor infiltrating NK cell in humans
correlates with positive prognosis for multiple malignancies including ovarian carcinoma (Webb et al., 2013) squamous cell lung cancer (Villegas et al., 2002), renal cell carcinoma (Schleypen et al., 2006), gastrointestinal stromal carcinoma (Rusakiewicz et al., 2013) and colorectal carcinoma (Coca et al., 1997).

Immunotherapy has revolutionized treatment of human malignancies and has proven to be efficacious against a variety of cancers with improved long-term survival rates (Chen and Mellman, 2017; De La Rochere et al., 2018). However, there are groups of cancer patients that do not benefit from current immunotherapies (Bazhin et al., 2019; Koyama et al., 2016), leading to the challenge of testing and validating new therapies or combination of therapies that can increase response rates and prolong survival in a wider range of individuals. The primary targets of immunotherapies are T cells, B cells, NK cells and macrophages (Greppi et al., 2019; Koury et al., 2018; Maeng et al., 2018; Marshall and Djamgoz, 2018; Patel and Minn, 2018; Yee, 2018). Currently there is a paucity of translatable models to study the effects of immunotherapies on NK cell activation and function (Mestas and Hughes, 2004; Shultz et al., 2012), and preclinical models are therefore needed. Conventional rodent models (one of the main stays of biomedical research), cannot fill this need owing to variations between the murine and human immune systems that prevent murine models from fully recapitulating human immune disease states. Humanized mouse models that have been engrafted with functional human immune systems are thus being used as in-vivo preclinical tools to study human immune system tumor interactions and therapeutics (Walsh et al., 2017; Wang et al., 2018). Nonetheless, human NK cell development and survival are extremely limited in
currently available humanized mice making the study of NK cell in humanized mice limited.

The cytokine IL15 is indispensable in the development, maturation, function and survival of mouse and human NK cells (Cooper et al., 2002; Ranson et al., 2003). Murine IL15 however has poor species cross-reactivity with human, and therefore development of human NK cells is not supported in the standard immunodeficient mouse strains used for humanization. The supplementation of human IL15 into immunodeficient mice and subsequent injection with human hematopoietic stem cells enable the development of human NK cells (Herndler-Brandstetter et al., 2017; Huntington et al., 2009).

In this study we describe a novel NSG based human IL15 transgenic mouse model. The NSG-Tg(Hu-IL15) mouse produces circulating human IL15 at physiological levels. And the engraftment of NSG-Tg(Hu-IL15) mice with human HSC results in improved and sustained NK cell numbers with enhanced maturation and functionality. Most notably, the human NK cells from engrafted NSG-Tg(Hu-IL15) mice kill MHC class I deficient target cells with similar efficacy as compared to NK cells purified from PBMC. Moreover, HSC engrafted NSG-Tg(Hu-IL15) mice show enhanced control of a PDX melanoma tumor growth in an NK cell-specific manner. These data indicate the potential utility of NSG-Tg(Hu-IL15) mice as an in vivo model to study human NK cell biology and as a testing platform for novel human-specific immunotherapeutics targeting NK cells.
Materials and methods

Mice

NSG mice and NSG-Tg(Hu-IL15) mice, were obtained from colonies developed and maintained at The Jackson Laboratory (Bar Harbor, ME). All animals were housed in microisolator cages in a pathogen-free facility, given autoclaved food and maintained on sulfamethoxazole-trimethoprim medicated water (Goldline Laboratories, FL) and acidified autoclaved water on alternating weeks. All animal use was in accordance with the guidelines of the Animal Care and Use Committee of the University of Massachusetts Medical School and The Jackson Laboratory and conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Measurement of human interleukin 15

Human IL15 protein was measured in NSG-Tg(Hu-IL15) plasma by ELISA. NSG-Tg(Hu-IL15) mice at 8-12 weeks of age were bled and peripheral blood was collected in heparin (Sagent Pharmaceutical, IL) tubes. IL15 levels in the plasma were then determined using a human IL15 Elisa Kit (BioLegend, CA).

Human HSC isolation and engraftment of mice

Human UCB was obtained in accordance with the Committee for the Protection of Human Subjects in Research guidelines of the University of Massachusetts Medical School. Then UCB was provided by the medical staff of the University of Massachusetts Memorial
Umbilical Cord Blood Donation Program. Groups of 6 to 8 week old NSG and NSG-Tg(Hu-IL15) mice were irradiated with 200 cGy (Pearson et al., 2008a). Irradiated mice were then injected with CD3 T cell-depleted human UCB containing 1x10^5 CD34+ HSCs i.v. (Brehm et al., 2010a). At the indicated time points, flow cytometry analyses of the blood from HSC recipients were done to quantify engraftment of the human immune system. For experimental studies, only mice with >10% peripheral human CD45+ cells and >5% human CD3+ T cells were used.

**Flow cytometry and antibodies**

For analysis of human hematopoietic engraftment and surface molecules, the following monoclonal antibodies specific for humans were used: human CD45 (2D1), CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD20 (2H7) CD33 (WM53), CD7 (CD7-6B7), CD69 (FN50) CD94 (DX22), NKp30 (P30-15), CD158b (KIR2DL2/L3: DX27), CD56 (5.1H11), CD57 (QA17A04), CD158i (KIR2DS4:179315), CD159c (NKG2C: 134591), CD7 (CD7-6B7), CD314 (NKG2D: 1D11), CD159a (NKG2A: 131411), CD158e1 (KIR3DL1: DX9), CD335 (NKp46: 29A1.4), granzyme B (QA16A02), granzyme A (CB9), perforin (dG9) and CD16 (3G8). Mouse specific CD45 (30F-11) was also used. The antibodies were purchased from BD Biosciences, Inc (CA). or BioLegend (CA). Single-cell suspensions of the spleens were prepared from engrafted mice, and whole blood was collected in heparin tubes. Single cell suspensions of 1x10^6 splenic cells in 50μL or 100μL of whole blood were washed with FACS buffer (PBS supplemented with 2% fetal bovine serum, (HyClone, UT) and 0.02% sodium azide (Sigma, MO)) and then pre-incubated with rat anti-mouse FcR11b (clone 2.4G2, BD Biosciences, CA) to block Fc binding.
Specific antibodies against cell surface markers were then added to the samples and incubated for 30 min at 4°C. Stained samples were then washed and fixed with 2% paraformaldehyde for cell suspensions or treated with BD FACS lysing solution for whole blood. For human granzymes and perforin detection, the cells were lysed, fixed and then incubated with BD Cytofix/Cytoperm Fixation/Permeabilization solution (BD biosciences, CA) for 20 min at 4°C. After washing in BD Perm/Wash buffer, the cells were then stained with antibody against human granzyme A, granzyme B and/or perforin in BD Perm/Wash buffer (BD biosciences, CA) for 30 min in the dark. At least 100,000 events were acquired on LSRII instrument (BD Biosciences, CA) or Aurora (Cytek Biosciences, CA). Data analysis was performed with FlowJo software (Tree Star, Inc., OR).

**Chromium release assay**

The NK cell cytotoxicity assay was performed as described previously (Hatfield et al., 2018). Briefly, the target cells (human leukemia K562), were labeled with 100 μCi of 6 mCi/mL Cr-sodium chromate (Na$_2^{51}$CrO$_4$; Perkin-Elmer, MA) and co-cultured with NK effector cells isolated from human PBMCs or pooled splenocytes from HSC engrafted NSG-Tg(Hu-IL15) by positive selection with magnetic beads conjugated with an antibody specific for human CD56 (Miltenyi Biotec). The target cells (1 × 10$^4$ cells/well) were cultured in round-bottomed microwell plates with various concentrations of effector cells and incubated at 37°C and 5% CO$_2$ for 18 hours. The cells were then centrifuged at 250xg for 5 min and the supernatant added to Optiphase scintillation fluid (Perkin-Elmer, MA) and incubated overnight at room temperature to allow for passive mixing and resolution of sample turbidity prior to reading. The corrected percent lysis for each concentration of
effector cells was then calculated using the mean cpm for each replicate of wells: %
specific lysis = 100 × \[\text{mean sample } ^{51}\text{Cr-release (cpm) – mean spontaneous } ^{51}\text{Cr-}
release]/[\text{mean maximum } ^{51}\text{Cr release (cpm) – mean spontaneous } ^{51}\text{Cr-release (cpm)}].

**In-vivo tumor experiments and treatments**

Patient derived melanoma tumor was obtained from the Human Avatar Tumor Bank at
the University of Massachusetts Medical School and passaged a minimum of 5 times in
NSG mice to deplete human leukocytes present within the tumor microenvironment. The
melanoma PDX was then processed into 2x2 mm³ pieces or into a single cell suspension
and either a piece of tumor or cells (2.5 x 10⁶) were transplanted subcutaneously to the
right flank of humanized and non-humanized NSG and NSG-Tg(Hu-IL15) mice. The mice
were monitored for tumor growth and the indicated mice were treated with OKT8 depleting
antibody (Invivogen, CA), CD335 (NKp46) neutralizing antibody (Perkin Elmer, MA) or
isotype control antibody (Invivogen, CA) i.p. at 100 µg per mouse, when their tumors were
between 50 and 100 mm³ in volume. OKT-8 antibody and its isotype control antibody
were given 3 consecutive days then every 7 days while anti-NKp46 antibody and its
isotype control were given for 2 consecutive days then every 7 days. Tumor-bearing mice
were euthanized when tumor volumes in isotype control mice approached limits set by
the IRB of UMASS medical school of 4000 mm³. The tumors, peripheral blood and
spleens were then harvested from the mice and processed for flow cytometry staining
and analysis. Tumors were measured by calipers every 3 to 4 days, and volumes (mm³)
were calculated by (length X width²)/2. The maximum tumor volume per mouse was not
allowed to get beyond 4000 mm³.
**Statistical Analyses**

To compare individual pair-wise groupings, we used unpaired t-tests and Mann-Whitney test for parametric and non-parametric data, respectively. Three or more means were compared by one-way ANOVA and the Bonferroni multiple comparison test. Significant differences were assumed for p values <0.05. Statistical analyses were performed using GraphPad Prism software (version 8.0, GraphPad, CA).

**Results**

**Human IL15 production by NSG-Tg(Hu-IL15) mice.**

Given the importance of IL15 for NK cell development, survival and function (Campbell and Hasegawa, 2013), the levels of circulating human IL15 was measured in unengrafted NSG, and NSG mice that are either hemizygous (NSG +/Hu-IL15) or homozygous (NSG-Tg(Hu-IL15)) for the human IL15 transgene ([Figure 3.1](#)). The serum human IL15 levels in our NSG-Tg(Hu-IL15) was 18.8 ± 1.7 pg/mL ([Figure 3.1A](#)), which is comparable to physiological levels of IL15 expressed by healthy human donors (Lamana et al., 2010). Upon engraftment with HSC, overall survival of NSG-Tg(Hu-IL15) mice was similar to HSC engrafted NSG mice ([Figure 3.1B](#)), indicating that the presence of the human IL15 transgene does not significantly impact the survival of NSG-Tg(Hu-IL15) mice upon human immune system engraftment.
**Figure 3.1:** Transgenic expression of human interleukin 15 (Hu-IL15) in NSG -Tg(Hu-IL15) mice.

(A) Quantification of human IL15 levels in unengrafted NSG mice, NSG +/IL15 mice (hemizygous for the human IL15 transgene) mice, and NSG-Tg(Hu-IL15), which are homozygous for the human IL15 transgene. Human IL15 was measured in the blood using ELISA and means±SEM are shown (n=3). (B) NSG or NSG-Tg(Hu-IL15) mice 6 to 8 weeks of age were irradiated (200 cGy) and injected i.v. with 100,000 CD34+ HSC derived from umbilical cord blood. The survival of the mice was then followed over time (n=10).
**Improved development of circulating human NK cells in NSG-Tg(Hu-IL15) mice.**

The results above show that NSG-Tg(Hu-IL15) mice constitutively express human IL15 with physiological serum levels. We next tested the consequence of human IL15 expression on human immune system engraftment over time. NSG and NSG-Tg(HuIL15) mice were irradiated and injected i.v. with human HSC, and human immune system development was monitored by flow cytometry (**Figure 3.2 and 3.3**). HSC engrafted NSG-Tg(Hu-IL15) mice show accelerated development of human CD45+ cell in the blood (**Figure 3.2A**) at weeks 6, 8 and 10 as compared to HSC-engrafted NSG mice. We next compared the development of CD3+ T cells, CD20+ B cells, CD33+ myeloid cells and CD56+/CD16+ NK cells between NSG and NSG-Tg(Hu-IL15) engrafted with human HSC. No significant difference was observed for human CD33+ myeloid cells (**Figure 3.2B**) and human CD3+ T cells (**Figure 3.2C**) over time between the two mouse strains. However, the development of human CD20+ B cells was significantly reduced in NSG-Tg(Hu-IL15) mice compared to NSG mice (**Figure 3.2D**). Moreover, the NSG-Tg(Hu-IL15) mice show a significant increase in human NK cell percentages starting at week 4 in HSC engrafted NSG-Tg(Hu-IL15) mice compared to HSC engrafted NSG mice (**Figure 3.3B**) which accounts for the observed reduction in CD20+ B cell proportions. Cell counts of human NK cells between the two stains also showed a significant increase in human NK cell numbers per µL of blood in NSG-Tg(Hu-IL15) mice compared to NSG (**Figure 3.3C**), suggesting that expression of human IL15 improves the development and maintenance of human NK cells in HSC-engrafted NSG mice.
Figure 3.2: Human immune cell chimerism in NSG-Tg(Hu-IL15) mice.

NSG (n=15) or NSG-Tg(Hu-IL15) mice (n=15) 6 to 8 weeks of age were irradiated (200 cGy) and injected i.v. with 100,000 CD34+ HSC derived from umbilical cord blood. Mice were bled fortnightly post injection and blood analyzed by flow cytometry for frequencies of (A) human CD45, (B) human CD33+ innate cells, (C) human CD20+ B cells, (D) human CD3+ T cells, each point represents an individual animal. For statistical analysis HSC engrafted NSG-Tg(Hu-IL15) mice were compared with HSC engrafted NSG mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Figure 3.2: Human immune cell chimerism in NSG-Tg(Hu-IL15) mice. NSG (n=15) or NSG-Tg(Hu-IL15) mice (n=15) 6 to 8 weeks of age were irradiated (200 cGy) and injected i.v. with 100,000 CD34+ HSC derived from umbilical cord blood. Mice were bled fortnightly post injection and blood analyzed by flow cytometry for frequencies of (A) human CD45, (B) human CD33+ innate cells, (C) human CD20+ B cells, (D) human CD3+ T cells, each point represents an individual animal. For statistical analysis HSC engrafted NSG-Tg(Hu-IL15) mice were compared with HSC engrafted NSG mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 3.3: Improved development of circulating human NK cells in NSG-Tg(Hu-IL15) mice.

NSG (n=15) or NSG-Tg(Hu-IL15) mice (n=15) 6 to 8 weeks of age were irradiated (200 cGy) and injected i.v. with 100,000 CD34+ HSC derived from umbilical cord blood. Mice were bled at 2 week intervals post injection and blood analyzed by flow cytometry for frequencies of human NK cells. (A) representative flow plot of human CD56+CD16+ NK cells at 16 weeks post HSC injection. The human NK cells were gated from hCD45+CD3-CD33-CD20-CD7+ cells. (B) frequency of human CD56+CD16+ NK cells and (C) numbers per µL of serum of human CD56+CD16+ CD3- NK cells at 2 week intervals post CD34+ HSC injection. Each point represents an individual animal. For
statistical analysis HSC engrafted NSG-Tg(Hu-II15) mice were compared with HSC engrafted NSG mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
**NK cell specific antigens and functional profiling in NSG-Tg(Hu-IL15) mice.**

We next examined the phenotypic and functional markers of human NK cells in NSG and NSG-Tg(Hu-IL15) mice 12 weeks after reconstitution with human HSC (Figure 3.4 and 3.5). Higher percentages of CD56+CD16+ NK cells were detected in blood and spleen of NSG-Tg(Hu-IL15) mice compared to NSG mice (Figure 3.4A). A significantly higher proportion of human NK cells within the blood and spleen of the NSG-Tg(Hu-IL15) mice stained positive for perforin (Figure 3.4B), granzyme A (Figure 3.4C) and granzyme B (Figure 3.4D) as compared to HSC-engrafted NSG mice. A larger proportion of NK cells from NSG-Tg(Hu-IL15) mice also express the NKG2 family members NKG2A, NKG2D and CD94 with significantly lower expression of NKG2C compared to NSG mice (Figure 3.5B). There was however no difference in expression of the killer cell immunoglobulin receptors (Figure 3.5C) and the natural cytotoxicity receptor (NCR) NKp30 (Figure 3.5A) by NK cells from NSG-Tg(Hu-IL15) mice and NSG mice. Nonetheless, NK cells from the NSG-Tg(Hu-IL15) NK cells have significantly higher expression of NKp46 (Figure 3.5A) and maturation marker CD8 (Figure 3.5D) with lower expression of CD57 (Figure 3.5D).

We next assessed the cytotoxic ability of NK cells generated within NSG-Tg(Hu-IL15) mice to kill NK cell responsive MHC-I deficient K562 cells. Human NK cells were enriched from either spleen of HSC engrafted NSG-Tg(Hu-IL15) mice or human PBMC and evaluated for the ability to lyse K562 target cells using a standard Cr$^{51}$ release assay. After 18 hours of incubation, human NK cells from HSC engrafted NSG-Tg(hu-IL15) mice kill the NK cell sensitive K562 cells with similar capacity as NK cells isolated from human PBMC (Figure 3.4E). Taken together these data suggest that the human IL15 transgene
improves both the numbers and function on NK cells reconstituted in the NSG-Tg(Hu-IL15) mice
**Figure 3.4:** Functional profiling of NK cells in NSG-Tg(Hu-IL15) mice. NSG or NSG-Tg(Hu-IL15) mice 6 to 8 weeks of age were irradiated (200 cGy) and injected i.v. with 100,000 CD34+ HSC derived from umbilical cord blood. At 12 weeks post human HSC engraftment, blood and spleen were analyzed by flow cytometry for the frequency of (A) human CD56+CD16+ CD3- NK cells, the capacity of human CD56+CD16+ CD3- NK cells to produce (B) perforin, (C) granzyme A, and (D) granzyme B. Each point represents an individual animal. (E) Human CD56+ CD3- NK cells purified and pooled from NSG-Tg(Hu-IL15) mice were tested for their ex-vivo ability to kill MHC-I deficient K562 tumor cells by chromium release as described in the Materials and methods, data is a plot of two separate experiments. For statistical analysis HSC engrafted NSG-Tg(Hu-Il15) mice were compared with HSC engrafted NSG mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 3.5: NK cell specific antigens profiling of NK cells in NSG-Tg(Hu-IL15) mice.

NSG or NSG-Tg(Hu-IL15) mice 6 to 8 weeks of age were irradiated (200 cGy) and injected i.v. with 100,000 CD34+ HSC derived from umbilical cord blood. At 12 weeks post human HSC engraftment, blood was analyzed by flow cytometry for human CD56+CD16+ CD3- NK cell surface receptors; (A) natural cytotoxicity receptors, (B) NKG
family molecules, (C) killer cell immunoglobulin-like receptors and (D) maturation markers CD8 and CD57. Each point represents an individual animal. The TSNE 2D scatter plots show the flow cytometry analysis for expression levels (red, high; blue, low) of the surface markers. For statistical analysis HSC engrafted NSG-Tg(Hu-IL15) mice were compared with HSC engrafted NSG mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Growth of patient derived xenograft (PDX) melanoma is delayed in HSC engrafted NSG-Tg(Hu-IL15) mice.

With the improved development and function of NK cells in HSC engrafted NSG-Tg(Hu-IL15) mice, we next evaluated the growth kinetics for PDX melanoma in these mice (Figure 3.6). We observed that tumors transplanted into unengrafted NSG-Tg(Hu-IL15) mice and NSG mice grow with similar kinetics (Figure 3.6A). However, after HSC engraftment there is a significant reduction in tumor growth in NSG-Tg(Hu-IL15) mice compared to the NSG mice. To determine what was driving the observed reduction in tumor growth kinetics, we interrogated the tumors for changes in the human immune cell repertoire and phenotype. There is no significant difference in the percentages and numbers of human CD45+ cells (Figure 3.6C), human CD3+ T cells (Figure 3.6D), and human CD8+ T cells (Figure 3.6E). However, there was a marked increase in NK cell percentages and numbers within the tumors of NSG-Tg(hu-IL15) mice compared to NSG mice (Figure 3.6F). Similar proportions of NK cells isolated from the tumor microenvironment of NSG-Tg(Hu-IL15) express functional markers CD69, CD8 and NKG2D, and produce granzyme A and granzyme B (Figure 3.6G) when compared to NSG mice. However, NK cells from the tumors in NSG-Tg(Hu-IL15) mice showed better capacity to produce perforin compared to NSG mice (Figure 3.6G).
Figure 3.6: PDX melanoma grows with reduced kinetics in HSC engrafted NSG-Tg(Hu-IL15) mice.
Figure 3.6: PDX melanoma grows with reduced kinetics in HSC engrafted NSG-Tg(Hu-IL15) mice. Six to 8 week old NSG (n=11) and NSG-Tg(Hu-IL15) mice (n=10) were left unmanipulated or engrafted with human HSCs were transplanted subcutaneously with PDX melanoma as described in the Materials and methods. Tumor growth kinetics were monitored in (A) non-engrafted (n/e) and (B) HSC engrafted mice. Tumors were recovered from tumor-bearing HSC engrafted mice and analyzed for the frequency and number per gram of tumor of (C) human CD45+ cells, (D) human CD3+ T cells, (E) human CD8+ T cells and (F) CD56+CD16+ CD3- NK cells. (G) the NK cells infiltrating the tumor microenvironment were also monitored for surface production of CD69, CD8, NKG2D and the capacity to produce granzyme A, granzyme B and perforin. Each point represents an individual animal. For statistical analysis HSC engrafted NSG-Tg(Hu-IL15) mice were compared with HSC engrafted NSG mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Representative data of two experiments.
CD8+ T cells are not necessary for the reduced growth rates of the PDX melanoma in NSG-Tg(Hu-IL15).

IL15 is important for NK cell development and function and memory CD8+ T cell maintenance (Patidar et al., 2016). We next investigated the potential role of CD8+ T cell function in the reduced rate of tumor growth observed in NSG-Tg(hu-IL15) mice (Figure 3.7A). HSC-engrafted NSG-Tg(Hu-IL15) mice and NSG mice were implanted subcutaneously with 2.5 million PDX melanoma cells. When the tumor became palpable, CD8+ T cells were depleted from half of the NSG-Tg(Hu-IL15) mice using OKT-8 depleting antibody as described in the Materials and methods. Depletion of CD8+ T cells in the PDX melanoma bearing NSG-Tg(Hu-IL15) mice did not significantly change the tumor growth rates as compared to control treated HSC-engrafted NSG-Tg(Hu-IL15) mice (Figure 3.7B). Within the tumor microenvironment, NSG-Tg(Hu-IL15) mice maintain higher NK cell numbers and percentages than those in the tumors of NSG mice irrespective of CD8+ T cell state (Figure 3.7F). These data suggest that the CD8 T cells are not necessary for the reduced growth rate of the PDX melanoma HSC-engrafted NSG-Tg(Hu-IL15) mice.
**Figure 3.7:** Reduced tumor growth kinetics in NSG-Tg(Hu-IL15) is not driven by CD8+ T cells.

**Figure 7.** Reduced tumor growth kinetics in NSG-Tg(Hu-IL15) is not driven by CD8+ T cells. HSC engrafted NSG and NSG-Tg(Hu-IL15) mice were transplanted subcutaneously with PDX melanoma as described in the Materials and Methods. The NSG-Tg(Hu-IL15) mice were then either left unmanipulated or depleted of CD8+ T cells using an OKT-8 antibody. (A) Experimental design and treatment schedule for OKT-8 depletion of CD8+ T cells. (B) Tumor growth kinetics were then monitored in the mice. The tumors were harvested from the mice and analyzed for the frequency and number per...
gram of tumor by flow cytometry of (C) human CD45+ cells, (D) human CD3+ T cells, (E) human CD8+ T cells and (F) CD56+CD16+ CD3- NK cells. Each point represents an individual animal. For statistical analysis CD8+ T cells depleted NSG-Tg(Hu-Il15) mice were compared with NSG-Tg(Hu-IL15) and NSG mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
Partial depletion of human NK cells from HSC-engrafted NSG-Tg(Hu-IL15) mice results in faster growth kinetics for the PDX melanoma.

To determine if the NK cells were mediated the delay in PDX melanoma growth observed in NSG-Tg(Hu-IL15) mice, NK cells were depleted using an NKp46 depleting antibody. Tumor growth kinetics were compared to HSC-engrafted NSG-Tg(Hu-IL15) and NSG mice that were not treated with the anti-NKp46 antibody (Figure 3.8B). Although the anti-NKp46 antibody did not completely deplete all the NK cell within the NSG-Tg(Hu-IL15) mice, the partial depletion led to partial rescue of tumor killing (Figure 3.8B). Within the tumor microenvironment, the depletion of NK cells did not affect the percentages and numbers of human CD45+ cells (Figure 3.8C) and human CD3+ T cells (Figure 3.8D). The depletion however lowered the percentage and number so NK cells within the tumor microenvironment to levels similar to those in NSG mice (Figure 3.8F), further suggesting that tumor killing in NSG-Tg(hu-IL15) mice was a result of not just an increase in NK cell numbers but also of improved NK cell function.
**Figure 3.8:** Reduced tumor growth kinetics in NSG-Tg(Hu-IL15) partially restored by the depletion of NKp46+ NK cells.

HSC engrafted NSG and NSG-Tg(Hu-IL15) mice were transplanted subcutaneously with PDX melanoma as described in the Materials and Methods. NSG-Tg(Hu-IL15)+anti-NKp46 treatment significantly reduced tumor volume compared to NSG-Tg(Hu-IL15)+Isotype Control. NKp46 depletion was confirmed by a decrease in NKp46+ NK cell count and an increase in CD3+ T cell count of hCD45+ cells.
methods. The NSG-Tg(Hu-IL15) mice were then either left unmanipulated or partially depleted of NK cells using an NKp46 depleting antibody. (A) Experimental design and treatment schedule for NK cell depletion. (B) Tumor growth kinetics were then monitored in the mice. The tumors were harvested from the mice and analyzed for the frequency and number per gram of tumor of (C) human CD45+ cells, (D) human CD3+ T cells, and (E) CD56+CD16+ CD3- NK cells. Each point represents an individual animal. For statistical analysis NKp46+ NK cells depleted NSG-Tg(Hu-IL15) mice were compared with NSG-Tg(Hu-IL15) and NSG mice; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Representative data of two experiments.
Discussion

As the immunodeficient mouse strains used to generate humanize mice continue to advance, these models are becoming invaluable preclinical tools for biomedical research, and more recently useful for immuno-oncology both in drug development and immunotherapy assessment (De La Rochere et al., 2018; Walsh et al., 2017). Although humanized mice have become progressively more sophisticated, the current iteration of humanized mice based on the NSG strain show low frequencies and impaired functional development of circulating human NK cells (Shultz et al., 2012). The lack of efficient human NK cell development in NSG mice was hypothesized to be a result of deficiency in human IL15, which is required for human NK cell development and a lack of species cross-reactivity for mouse IL15 (Pek et al., 2011). We tested this hypothesis by the transgenic expression of human IL15 in NSG mice. The transgene generated using a BAC containing the human \textit{IL15} gene under the human IL15 promoter, lead to the expression of circulating human IL15 at 18.8 ± 1.7 pg/mL which was within the physiological range found in healthy donors (Lamana et al., 2010). NSG-Tg(Hu-IL15) mice engraft with human HSC, show overall good survival post engraftment and support the enhanced development of human NK cells.

IL15 is a key regulator of NK cell biology, including their generation, maintenance and function (Rautela and Huntington, 2017; Waldmann, 2013). Indeed the IL15 receptor complex made up of IL2rβ and IL2rγ chains are assembled on the surface of the NK cell progenitor and are maintained at high levels throughout the NK cell’s life (Carotta et al., 2011; Delconte et al., 2016). Loss of IL15 in mice leads to severe reduction in NK cell
numbers (Huntington, 2014). Also, the administration of recombinant human IL15 into reconstituted Balb/c\(^{c^{-}/c^{-}}\) mice and expression of human IL15 in NOG both lead to a significantly improved human NK cell development and/or survival (Katano et al., 2017; Pek et al., 2011). Similarly, the NSG-Tg(Hu-IL15) show improved NK cell frequency in the periphery and tissues that occur very early post HSC reconstitution and is sustained. In line with enhanced maturation and function within HSC engrafted NSG-Tg(Hu-IL15), the NK cells in the blood and spleen have better capacity to produce granzymes and perforin and improved expression of multiple phenotypic and functional markers including the activation receptors NKp46, NKG2D, and CD94 than those generated in non-transgenic NSG mice. We however observed the up regulation of the inhibitory receptor NKG2A and down regulation of the activation receptor NKG2C. Elpek et al. observed in mice that while transient stimulation by IL15/IL15R\(\alpha\) increased the numbers and enhanced their effector function, sustained stimulation lead to accumulation of mature NK cells which show impaired functionality with an altered balance in activation and inhibitory receptors (Elpek et al., 2010). Certainly, in NOG-IL-15 Tg mice, altered phenotypic receptor expression and reduction in in-vitro cytotoxicity activity were observed (Katano et al., 2017). In our model however, although we see altered activating/inhibitory receptor profile for some receptors when compared to NK cells generated in NSG non-transgenic mice, we also observe improved capacity for cytotoxic activity (with the observed improvement in perforin and granzyme A/B production) and NSG-Tg(Hu-IL15) human NK cells being able to \textit{ex-vivo} kill K562 cells with similar efficiency as those from human PBMC. HSC reconstituted NSG-Tg(Hu-IL15) mice were also able to significantly control PDX melanoma tumor growth than NSG mice. Together, these data suggest that while the
sustained expression of human IL15 may be impacting the phenotypic receptor profile, the effect may not be pronounced enough to drive the NK cells towards senescence and/or anergy with impaired functionality. We believe this observed difference in transgene behavior could be due to the near physiological levels of IL15 produced in the NSG-Tg(Hu-IL15) mice compared to the NOG-IL-15 Tg (Katano et al., 2017) and the human SIRPA and IL15 knock-in mice (SRG-15) (Herndler-Brandstetter et al., 2017).

We also show that NK cell generated in HSC-engrafted NSG-Tg(Hu-IL15) mice were able to infiltrate the tumor microenvironment at a significantly higher level than in tumor-bearing NSG non-transgenic mice. Additionally, the observed reduction in tumor growth was driven primarily by NK cells and not CD8+ T cells, which corresponds to the enhanced NK cell development, maturation, and function in NSG-Tg(Hu-IL15) mice. The observed lack of effect on tumor growth with the depletion of CD8 T cell was unexpected but not surprising. Although they are important for tumor immune surveillance, the CD8 T cells within the TME are dysfunctional (Crespo et al., 2013) and while the presence of circulating IL15 in humanized mice had the potential of improving memory CD8+ T cell development and functional maturation as observed by Herndler-Brandstetter et al., we did not observe any change in CD8 T cell phenotype and function in circulating CD8+ T cell and therefore did not expect improvement in CD8+ T cell function within the TME (Herndler-Brandstetter et al., 2017). The improvement in NK cells within NSG-Tg(Hu-IL15) mice creates a platform within immuno-oncology for the in-vivo study of NK cells in tumor-immune system interactions testing novel immunotherapies targeting NK cells. The reduction in tumor growth though significant was not as robust as would have been
expected which points to potential NK cell dysfunction within the TME. Although we were not able to interrogate this line of thinking in the current study. The NSG-Tg(Hu-IL15) mouse may provide a potential model for understanding NK cell dysfunction within tumors.

Multiple NK cell based therapies are currently approved for use, in clinical trials or under development that would benefit from an in-vivo model that can be harnessed to better understand their mechanisms of action, to evaluate their efficacy before advancement to clinical trial and from personalized medicine to interrogate if a patient would benefit from specific NK cell-based treatment for their particular malignancy. For example, approximately 21% of primary melanoma and 44% of metastatic melanoma samples lack expression of HLA class I antigen, an important component for CD8+ T cell mediated therapies (Kageshita et al., 1993). Additionally it has been shown that check point inhibitor based therapies such as PD-1 and its ligand PD-L1 have a durable response rate of 20 – 50% in metastatic melanoma (Page et al., 2014) owing largely to acquired resistance to the checkpoint inhibitors which is driven by loss of HLA class I (O'Donnell et al., 2017; Zaretsky et al., 2016). NK cells are therefore effective candidates for HLA class I non-expressing melanoma tumors (Besser et al., 2013; Parkhurst et al., 2011; Sottile et al., 2016).

In summary we show here, a novel NSG mouse expressing human IL15 at physiological levels that is able to support better human NK cell development and function creating a
platform for the *in-vivo* study of human NK cell biology and for the development and preclinical evaluation of NK cell targeting immunotherapies.
Chapter IV

NSG-SGM3 mice as a preclinical model for immune oncology

Introduction

Manipulation of a patient’s immune system with the hopes of controlling and/or eliminating tumor burden is a concept that is decades old, but one that has not always been successful (Oiseth and Aziz, 2017). With improved understanding of the human immune system homeostasis, function and regulation, immunotherapies are currently transforming cancer treatment with significant improvements in long-term survival rates among patients with varying tumor types (Greppi et al., 2019; Marshall and Djamgoz, 2018; Nozawa et al., 2018; Weiss et al., 2019). There are however many patients who do not experience durable benefits from current immunotherapies (Pol and Kroemer, 2018; Winder and Viros, 2018) and efforts to identify and develop novel agents and combinations of treatments that are more efficacious to a broader patient population are underway. There is therefore an urgent need for preclinical and predictive models to drive the immunotherapeutic drug discovery and development.

Mouse models and mouse tumors models such as syngeneic mouse tumor models, genetically engineered mouse models (Kersten et al., 2017) and chemically induced mouse models have played pivotal roles in understanding the immune system and its interaction with tumors and for evaluating immunotherapies (Khaled and Liu, 2014; Oiseth and Aziz, 2017). A major disadvantage to mouse models however is that the mouse immune system does not fully recapitulate human immune response with regard to
function, regulation and phenotype (Lux and Nimmerjahn, 2013). Humanized mice, which replicate a functional human immune system, bearing human tumors, are an alternative to traditional mouse models and are highly desirable as preclinical models. The generation of humanized mice bearing human tumors for immuno-oncology experiments requires immunodeficient mice, with impaired NK, T, and B cells so as to prevent rejection of the reconstituted immune system. The NSG mouse model is considered the gold standard for humanization and will support engraftment with human PBMC, fetal liver and thymic tissues, and HSC. Human tumors can be implanted into NSG mice bearing human immune systems to generate the human tumor-bearing humanized mice (Wang et al., 2018). While both human tumor cell lines and PDX tumors have been used in humanized mice for studies, PDX have been associated with higher predictive value for therapeutic responses to oncology treatment in patient including targeted therapies (Rosfjord et al., 2014).

In this study we evaluated the use of NSG-SGM3 BLT mice to interrogate human immune system’s interaction with PDX melanoma tumor and its response to immunotherapies. NSG-SGM3 mice are NSG mice constitutively expressing human stem cell factor (SCF), granulocyte macrophage colony stimulating factor (GM-CSF), and IL3. The presence of the 3 transgenes improves cell frequencies and function of human neutrophils (Miller et al., 2013) and granulocytes (Coughlan et al., 2016), myeloid cells (especially myeloid dendritic cells), and FoxP3+ regulatory T cells (Billerbeck et al., 2011) upon reconstitution with human CD34+ HSC. Furthermore, NSG-SGM3 mice exhibit significant improvement in engraftment of AML cells as well as preleukemic myeloid cell cultures (Wunderlich et
al., 2010). For the studies described below, we implanted NSG-SGM3 with fetal thymic and liver tissues and autologous liver derived CD34+ HSC to generate BLT mice. The NSG-SGM3 BLT mice were then implanted with allogeneic PDX melanoma cells. The NSG-SGM3 BLT mice supported the growth of the PDX melanoma graft. The tumor graft was infiltrated by the reconstituted human immune system and most notably, the graft responded to immunotherapy in a human immune system specific manner with reduction in tumor growth kinetics. Our data indicate that NSG-SGM3 BLT mice bearing PDX tumors are a potential model for studying human immune system interactions and for testing of novel immunotherapies and combinational therapies.

**Materials and methods**

*Mice*

NSG-SGM3 mice were obtained from The Jackson Laboratory (ME). All animals were housed in micro-isolator cages in a pathogen free facility, given autoclaved food and maintained on sulfamethoxazole-trimethoprim medicated water (Goldline Laboratories, FL) and acidified autoclaved water on alternate weeks. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).
**Generation of BLT mice**

Male and female NSG-SGM3 mice between 6–10 weeks of age were irradiated with 100 cGy and implanted with human fetal thymic and liver fragments under the kidney capsule. The fetal tissues (gestational age 16–20 weeks) were obtained from Advanced Bioscience Resources (CA). The thymus and liver tissues were washed with RPMI supplemented with penicillin G (100 U/ml), streptomycin (100 mg/ml), fungi-zone (0.25 mg/ml), and gentamycin (5 mg/ml), and then 2 mm³ fragments of the fetal thymus and liver were implanted under the renal subcapsular space. The mice were then injected subcutaneously with gentamycin (0.2 mg) and cefazolin (0.83 mg) post-surgery. To obtain fetal HSC, fetal liver tissue was processed as previously described (Aryee et al., 2014). Briefly the fetal liver was processed into 1 x 1 mm³ and digested in GIBCO liver digest medium (Thermofisher, MA). The digested liver cells were then gradient separated using Histopaque 1077 (Sigma-Aldrich, MO). The buffy coat representing the human leukocyte population (including CD34⁺ HSCs) was collected and depleted of CD3+ T cells. Post CD3+ T cell depletion, a cell suspension containing 1 X 10⁵ CD34⁺ fetal liver HSC was injected into the tail vein of mice between 4 and 6 h after irradiation.

**Antibodies and flow cytometry**

For analysis of human cells using flow cytometry the following antibodies were purchased from BD Biosciences, Inc. (San Jose, CA), eBiosciences (San Diego, CA), BioLegend (San Diego, CA) or R&D systems (Minneapolis, MN): mouse CD45 (30-F11), human CD45 (2D1), CD3 (UCHT1), CD20 (2H7), CD33 (WM53), CD4 (RPA-T4), CD8 (RPA-T8), CD25 (MA-251 and 2A3), CD127 (A019D5), FoxP3 (206D), CD45RA (HI100), CCR7
(G043H7), PD-1 (EH12.2H7), CTLA-4 (L3D10), CCL22 (IC3361P), GITR (eBIOA1TR),
CD33 (P67.6), CD14 (63D3), PDL-1 (29E2A3) and HLADR (L243). Single cell
suspensions of spleen and PDX tumor were prepared from the tissues harvested from
mice. To process the tumor, it was first minced in a weigh boat containing complete RPMI
media supplemented with 1X penicillin, streptomycin and DNase. Using the plunger of a
5 mL syringe, the minced tumor tissue was pushed through a 70 µm sieve to obtain a
single cell suspension. From the single cell suspensions, 0.5 to 1x10⁶ cells were washed
with FACS buffer (PBS with 2% FBS and 0.02% sodium azide) and incubated with rat
anti-mouse CD16/CD32 (clone 2.4G2) for 5 min at 4°C to block Fc binding. The cells were
then incubated with antibodies for the indicated surface markers for 20 min at 4°C in the
dark. The stained samples were then washed with FACS buffer and treated with red blood
cell lysis buffer for 5 minutes at room temperature using 0.8% NH₄Cl, 10 mM KHCO₃, 0.1
mM EDTA solution in water. The cells were then washed with FACS buffer and fixed with
1% paraformaldehyde. For human Tregs detection, the samples were stained for cell
surface markers as described above and then incubated in eBioscience
fixation/permeabilization buffer for 60 min. The cells were then stained with antibody
against human FoxP3 in eBioscience permeabilization buffer for 60 min and washed with
the appropriate buffers. At least 50,000 events of live gated events were collected on
LSRII flow cytometer (BD Biosciences, CA) using the BD FACS DIVA software. Data
analysis was performed with FlowJo software (Tree Star, Inc., OR).
**PDX tumor transplants**

Patient melanoma tumors were obtained from melanoma surgical specimens after written informed consents were obtained from the patients before tumor excision as part of the Human Avatar Bank at the University of Massachusetts Medical School (Worcester, MA). The PDX was then processed into 2x2 mm³ pieces and implanted subcutaneously at the right flank of NSG mice to expand tumor and delete human immune cell infiltrates: they were passaged at least 5 times to deplete residual patient leukocytes. To generate the PDX bearing NSG-SGM3 BLT model, patient-derived tumors harvested from NSG mice were finely minced and processed into a single cell suspension. The tumor cells were counted and 2.5 X 10⁶ tumor cells were inoculated subcutaneously at the right flank of engrafted NSG-SGM3 mice. Cells from the donor tissues used in BLT generation and the PDX tumor were sent out for HLA typing (ProlImmune, UK).

**Tumor experiments and treatments**

Treatment was started when the tumors reached 50–100 mm³ in volume. Control phosphate buffered saline (PBS; Invitrogen, CA) or corresponding isotype control (BioXcell, NH) was injected intraperitoneally or intravenously depending on the treatment drug. Pembrolizumab (anti-PD-1; Merck, NJ) and its isotype control (human IgG4) were injected intraperitoneally or intravenously at 10 mg/kg for the first dose, followed by 5 mg/kg (i.p. or i.v.) dose every 5 days until the study end point. Ipilimumab (anti- cytotoxic T-lymphocyte-associated protein 4 (CTLA-4); BMS, NY) and its isotype control (human IgG1) were injected i.p. at 3 µg/kg body weight dose every 5 days until the end of study. For CpG-ODN and anti-IL10R antibody treatment, CpG-ODN (2006) (Invivogen, CA) was
injected intratumorally and anti-IL10R antibody were administered i.p. at 50 μg and 100 μg respectively every 5 days until the end of study using PBS as vehicle control. Tumors were measured by calipers every 3 to 4 days, and volumes (mm³) were calculated by (length x width²)/2. The maximum tumor volume per mouse was not allowed to get beyond 4000 mm³.

**Statistical Analyses**
To compare individual pair-wise groupings, we used unpaired t-tests and Mann-Whitney test for parametric and non-parametric data, respectively. Three or more means were compared by two-way ANOVA and the Bonferroni multiple comparison test. Significant differences were assumed for p values <0.05. Statistical analyses were performed using GraphPad Prism software (version 8.0, GraphPad, CA).

**Results.**

*NSG-SGM3 BLT permit the growth and development of melanoma PDX tumors in the presence of partially HLA mismatched human immune system.*

An important consideration for the use of BLT mice with PDX tumors is that in most cases the implanted tumor will not be matched to the engrafted human immune system. Therefore, the tumor will be recognized by the immune system as an allograft, which raised the possibility that the engrafted human immune system will reject the implanted tumors as allografts, as has been demonstrated in the rejection of stromal tissue allografts in humanized mice - reviewed in (Kenney et al., 2016). For humanized BLT mice to be used in immuno-oncology studies, the allogeneic PDX tumor should be able to grow in
the presence of the allogeneic human immune system. To test the ability of a tumor to
grow in BLT mice, PDX melanoma tumors were implanted in engrafted NSG-SGM3 BLT
mice and unengrafted NSG-SGM3 mice (which should permit tumor growth) (**Figure
4.1A**). PDX melanoma was able to grow in both engrafted and unengrafted NSG-SGM3
mice consistently in 4 separate experiments (**Figure 4.1B**), although in the case of
engrafted mice, the reconstituted human immune system was partially mismatched to the
PDX melanoma tumor (**Table 4.1**). These finding are consistent with recent studies from
Wang and colleagues, which demonstrated that human tumor cell lines and PDX tumors
grew in NSG mice that had been engrafted with partially HLA mismatched human UCB-
HSC (Wang et al., 2018).
Table 4.1: HLA match of CD34+ HSC donors and PDX melanoma.

<table>
<thead>
<tr>
<th>CD34+ HSC donors</th>
<th>HLA match with PDX melanoma tumor</th>
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<tbody>
<tr>
<td>1</td>
<td>HLA class I match; HLA-DQB1, HLA-DPB1, HLA-DPA1</td>
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<tr>
<td></td>
<td>HLA class II match; HLA-A, HLA-B</td>
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<tr>
<td>2</td>
<td>No HLA class I match</td>
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<tr>
<td></td>
<td>HLA class II match; HLA-A</td>
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<tr>
<td>3</td>
<td>HLA class I match; HLA-DQA1, HLA-DPA1</td>
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<tr>
<td></td>
<td>No HLA class II match</td>
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Table 4.1: HLA match of CD34+ HSC donors and PDX melanoma. HLA matching between PDX melanoma and CD34+ HSC donors used in generating humanized mice.
Given that the SGM3-BLT mice supported tumor growth, we next interrogated if the PDX melanoma tumor would be permissive to human immune cell infiltration. Staining for human CD45+ cells by flow cytometry (Figure 4.1C and D) and by histology (Figure 4.1E), we observed that PDX melanoma tumor recovered from SGM3-BLT mice were infiltrated with human immune cells and no human immune cells were detected within the tumor microenvironment of the unengrafted NSG-SGM3 mice. Together, these data show that the PDX melanoma is able to grow in the presence of the allogeneic and functional immune system capable of transplant rejection (Brehm and Shultz, 2012). In addition, the immune system infiltrating the tumor microenvironment originates from the CD34+ HSC engrafted into the mice, and not from immune cells from the original patient tumor.
**Figure 4.1:** NSG-SGM3 BLT mice permit the growth of melanoma PDX tumor in the presence of a human immune system.

(A) NSG-SGM3 BLT and unengrafted NSG-SGM3 mice were transplanted subcutaneously with PDX melanoma tumor. (B) The mice were monitored for tumor growth and the tumor measurements were calculated as \((\text{length} \times \text{width}^2)/2\), where \(L\) is the length, and \(W\) is the width of the tumor. The tumors were harvested at the end of the study and analyzed by flow cytometry (C and D) and immunohistochemistry (E) for human CD45+ cell infiltration when the PDX melanoma tumors approached 1000 mm\(^3\). Tumor growth curve is a representative plot of 4
experiments. Each flow cytometry data point represents an individual animal, and the data are from a total of 4 independent experiments. * p<0.01, ** p<0.01, *** p<0.001, **** p<0.0001.
We next tested if the presence of the PDX tumor graft would alter the human immune cell engraftment and chimerism within the periphery of NSG-SGM3 BLT mice. We compared human immune cell chimerism between the spleens of reconstituted NSG-SGM3 mice bearing PDX melanoma or without tumor grafts. We observed a minor decrease in the levels of human CD45+ cells in the spleens of tumor-bearing NSG-SGM3 BLT mice (Figure 4.2A), as compared to non-tumor-bearing NSG-SGM3 BLT mice. However, we observed no differences in the levels of human CD3+ T cells (Figure 4.2B), T cell subsets including conventional CD4+ T cells, CD8 T cells (Figure 4.2D) and regulatory T cells (Figure 4.2E), CD33+ innate cells (Figure 4.2B) and CD14+ monocyte/macrophage cell populations (Figure 4.2F) between NSG-SGM3 BLT mice with or without human PDX melanoma. These data suggest that the presence of the PDX melanoma does not impact the human immune cell chimerism within the periphery of NSG-SGM3 BLT mice.
Figure 4.2: PDX melanoma tumor implantation does not impact human leukocyte chimerism in engrafted SGM3-NSG-BLT mice. BLT mice were generated using NSG-SGM3 mice as described in the Materials and methods. PDX melanoma tumor was then
transplanted subcutaneously into half of the mice after confirmation of human immune system reconstitution. When the PDX melanoma tumors approached 2000 mm$^3$, the spleens of the mice were analyzed by flow cytometry. (A) The gating strategy used to define immune cell subsets in the spleen and tumor is shown for levels of (B) human CD45+ cells, (C) human CD3+ T cells, (D) human CD33+ innate cells, (E) human conventional CD4+ and human CD8+ T cells, (F) human FoxP3+ Treg cells and (G) human CD14+ monocyte/macrophage cells (TAM). Each point represents an individual animal, and the data are from a total of 3 independent experiments. * p<0.01, ** p<0.01, *** p<0.001, **** p<0.0001.
**Immune cell infiltration into PDX melanoma tumors of NSG-SGM3 BLT mice.**

The immune cells infiltrating the tumor microenvironment determine the immune suppressive nature of the tumor and shape the ability of tumor to respond to different immunotherapies. We therefore interrogated the immune cell infiltrates within the tumor using flow cytometry. We observed similar levels of human T cells infiltrating the tumor (Figure 4.3A), however the proportion of CD8+ T cells was significantly higher in the tumor (Figure 4.3B) compared to the spleen of the same NSG-SGM3 BLT mouse. The tumor microenvironment also show a significantly higher frequency of FoxP3+ Treg (Figure 4.3C) and CD14+ TAM (Figure 4.3E). Although the NSG-SGM3 BLT mice have improved Treg reconstitution (Billerbeck et al., 2011), we hypothesize that the increased Treg proportions of CD4+ T cells is due to chemokine gradient and/or conversion of conventional CD4+ T cells within the tumor microenvironment. CCL22 is a chemokine known to attract Tregs (Ishida and Ueda, 2006; Morton et al., 2016). One of the major producers of CCL22 are innate cells particularly monocyte/macrophage cells (Kimura et al., 2019; Wertel et al., 2015). Given that the levels of human macrophages infiltrating the PDX melanoma are increased (Figure 4.3E), and the TAMs ability to produce chemokines important for Treg chemotaxis including CCL22, thereby contributing to Treg immune suppression within the tumor (Anz et al., 2015; Kimura et al., 2019), we determined the expression levels of human CCL22 produced by macrophages within the spleen and tumor of PDX melanoma bearing NSG-SGM3 BLT mice. There was a significant increase in tumor associated macrophages producing CCL22 compared to the macrophages isolated from the spleens of the same mice (Figure 4.3F). The increased production of CCL22 within the TME would indicate the possibility of increased attraction
of Treg into the tumor, potentially accounting for the increase Tregs observed within the TME.
**Figure 4.3:** Human immune cell populations within the spleen and tumors of PDX melanoma bearing NSG-SGM3 BLT mice.

Spleens and tumors were harvested from PDX melanoma bearing NSG-SGM3 BLT mice and processed into single cell suspensions. The cell suspension was then stained for flow cytometry as described in the Materials and methods. The percentages of (A) CD3+ T cells, (B) CD4+ and CD8+ T cells, (C) FoxP3+ Treg cells, (D) CD33+ innate cells, and (E) CD14+ monocyte/macrophage cells within human CD45+ cells are shown. Also shown are (F) the percentage of CD14+ monocyte macrophage cells expressing CCL22. Each data point represents an individual animal, and the data are from a total of 3 independent experiments. * p<0.01, ** p<0.01, *** p<0.001, **** p<0.0001.
We next evaluated the phenotype of conventional CD4+ and CD8+ T cells from the spleen and tumor of NSG-SGM3 BLT mice by staining for markers associated with T cells exhaustion, including PD1, CTLA-4, and glucocorticoid-induced TNFR-related protein (GITR) (Grosso et al., 2009; Leng et al., 2006; Norris et al., 2012). We observed a significant increase in checkpoint marker expression by T cells within the PDX tumor compared to the spleen from PDX melanoma bearing NSG-SGM3 BLT mice. Particularly, we observed significant increases in the expression of CTLA-4 by conventional CD4+ T cells (Figure 4.4E) and also in PD-1 (Figure 4.4A and D), and GITR (Figure 4.4C and F) by conventional CD4+ and CD8+ T cell respectively. Together, these data show that a significant proportion of CD4+ and CD8+ T cells infiltrating a PDX melanoma in NSG-SGM3 BLT mice express immune checkpoint markers, contributing to an immune suppressive and dysfunctional tumor microenvironment.
**Figure 4.4:** Tumor infiltrating T cells express high frequencies of checkpoint inhibitory receptors.

Spleens and tumors were harvested from PDX melanoma bearing NSG-SGM3 BLT mice when the tumor volume approached 2000 mm$^3$ and processed into single cell suspensions which were analyzed by flow cytometry as described in the Materials and methods. The percentage of conventional CD4+ and CD8+ T cells expressing (A and B) PD1, (C and D) CTLA-4 and (E and F) GITR respectively are shown. Each data point represents an individual animal and the data are from a total of 3 independent experiments. * p<0.01, ** p<0.01, *** p<0.001, **** p<0.0001.
PDX melanoma tumors within NSG-SGM3 BLT mice respond to anti-PD-1 immunotherapy.

The infiltration of CD8+ T cells into the tumor microenvironment and the high expression of PD-1 and other markers of T cell exhaustion suggested that the PDX melanoma may be a possible target for checkpoint immunotherapy. Moreover we confirmed that the PDX melanoma expressed PD-L1, which is used as a marker clinically for patient enrollment in PD-1 blockade treatment (Carbognin et al., 2015) (Figure 4.5A). We asked if anti-PD-1 antibody therapy would impact PDX melanoma growth in NSG-SGM3 BLT mice. The PDX tumors were subcutaneously transplanted into mice and when tumor volumes reached 50 to 100 mm$^3$, anti-PD-1 antibody treatment commenced. Anti-PD-1 antibody treatment led to significant reduction in PDX melanoma growth kinetics (Figure 4.5B), as compared to BLT mice treated with an isotype control. To verify if the observed tumor control was acting through the reconstituted human immune system, we transplanted PDX melanoma into unengrafted NSG-SGM3 mice and treated with pembrolizumab. We found no observable reduction in tumor growth between anti-PD-1 treated PDX melanoma bearing NSG-SGM3 mice compared to isotype control treated mice (Figure 4.5C). We also tested another PDX melanoma tumor that showed no human leukocyte infiltration into the TME (Figure 4.6A)- a tumor category term “cold tumors” to further determine impact of human leukocytes on anti-PD-1 antibody therapy. The “cold” PDX-melanoma tumor did not respond to anti-PD1 therapy (Figure 4.6B), indicating the need for human immune cell presence for tumor control action of pembrolizumab. We next evaluated the tumor infiltrating lymphocytes between isotype and anti-PD-1 antibody treatment of NSG-SGM3-BLT mice with tumors. We observed a significant increase in
the proportion and frequency of human CD45+ cell within the tumor microenvironment of mice treated with anti-PD-1 compared to isotype control (Figure 4.5D). The increase in human CD45+ cells was also confirmed by histological staining of human CD45+ cells in tumor sections (Figure 4.5E). We observed a significant increase in conventional CD4+ and CD8+ T cells (Figure 4.5H and I) which reflected an overall increase in T cells within PDX melanoma following anit-PD-1 antibody treatment (Figure 4.5E). In addition, we observed a significant decrease in CD33+ innate cells (Figure 4.5G) specifically CD14+ TAMs and Treg cells (Figure 4.5J) within the tumor upon treatment.
Figure 4.5: Anti-PD-1 antibody checkpoint blockade reduces PDX melanoma tumor growth kinetics in NSG-SGM3 BLT mice.
Figure 4.5: Anti-PD-1 antibody checkpoint blockade reduces PDX melanoma tumor growth kinetics in NSG-SGM3 BLT mice. Tumor-bearing NSG-SGM3 BLT mice and unengrafted NSG-SGM3 mice were treated with anti-PD-1 antibody (Pembrolizumab) at 10 mg/kg, on day 0, followed by 5 mg/kg every 5 days or isotype control as described in the Materials and methods. The mice were monitored for tumor growth and the experiment ended when the isotype control treated mice neared the institutionally allowed limits for tumor growth of 4000 mm³. The spleens and tumors were then harvested and processed for flow cytometry analysis. (A) PDL-1 expression by PDX melanoma (B) tumor growth kinetics of PDX melanoma bearing NSG-SGM3-BLT mice. (C) tumor growth kinetics of PDX melanoma bearing unengrafted NSG-SGM3 BLT mice. The spleens and tumors of NSG-SGM3-BLT mice were analyzed for (D) human CD45+ cell infiltration, (F) human CD3+ T cells, (G) CD33+ Innate cells, (H) conventional CD4+ T cells, (I) CD8+ T cells, (J) FoxP3+ T cells and (K) CD14+ TAMs. (E) Tumors from NSG-SGM3-BLT mice were also processed and analyzed by immunohistochemistry for human CD45+ cells. For flow cytometric analysis, each data point represents an individual animal, and the data is from a total of 3 independent experiments. * p<0.01, ** p<0.01, *** p<0.001, **** p<0.0001.
Figure 4.6: Anti-PD-1 antibody checkpoint blockade permits “cold” PDX melanoma tumor growth in NSG-SGM3 BLT mice.

Cold melanoma tumor-bearing NSG-SGM3 BLT mice were treated with anti-PD-1 antibody (Pembrolizumab) at 10 mg/kg, on day 0, followed by 5 mg/kg every 5 days or isotype control as described in the Materials and methods. (A) immunohistochemistry following tumor harvested for immunohistochemistry. (B) the tumor growth kinetics over time.
Discussion

The impact of immunotherapy in recent years, specifically immune checkpoint therapy, for cancer has set off intense research into other immune-modulatory treatments. There is therefore a need to understand how tumors interact with the immune system, why only some tumors respond to immunotherapies and the mechanisms employed by those that respond. The limited success in the use of mouse models such as GEMM in predicting success of therapies in humans (Rangarajan and Weinberg, 2003; Sanmamed et al., 2016) and the high cost and complexity in designing clinical trials has underscored the need for preclinical models for development and testing of these novel and combinational therapies in a human specific manner, a research area for which humanized mice are becoming invaluable for (Jin et al., 2018; Wang et al., 2018; Williams, 2018; Zumwalde and Gumperz, 2018). Here, we describe the use of the NSG-SGM3 BLT humanized mouse model to study the interactions between the allogeneic human immune system and PDX melanoma, and the response of PDX melanoma to immunotherapy modalities.

Our first goal was to determine if a PDX melanoma tumor would grow in the presence of an allogeneic immune system reconstituted in the NSG-SGM3 BLT mice. The functionality of the human immune systems generated in humanized mice has been documented in experiments detailing immune response against pathogens such as Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV) (Brainard et al., 2009; Joseph et al., 2010; Melkus et al., 2006). In addition, humanized mice have been shown to be able to reject MHC mismatched allografts including human skin (Racki et al., 2010) and Islets grafts (Brehm et al., 2010a; Unger et al., 2012). However, the PDX melanoma
tumor was able to grow in our NSG-SGM3 BLT mice. The ability of the partially HLA
mismatched tumor allogeneic to the engrafted immune system in the NSG-SGM3 BLT
mice to grow strongly indicates the generation of a TME that is immunosuppressing and
recapitulates what is seen within patients TME (Davis et al., 2016; Jie et al., 2013)
indicating the sensitivity of the NSG-SGM3 BLT immune system to regulation by tumors
and enabling its potential use to understand tumor immune-evasive mechanisms and
develop therapies to overcome this immune suppression.

The PDX melanoma that grew in NSG-SGM3 BLT mice allowed human immune cell
infiltration. We confirmed that these leukocytes originated from the reconstituted immune
system and not from residual immune cells from the original patient tumor, as no immune
cells were detected in the PDX tumors isolated from unengrafted NSG-SGM3 mice. This
correlated with work done by other groups who also saw reconstituted immune cell
infiltration into tumor grafts (Capasso et al., 2019; Wang et al., 2018). Of the leukocytes
infiltrating the TME, majority were CD3+ T cells which are skewed more towards CD8+ T
cells compared to the T cell population in the spleen of the same mouse. Given the similar
immune profile between spleens of tumor-bearing and non-tumor-bearing NSG-SGM3
BLT mice, the skewing of the TME immune infiltrates toward more CD8+ T cells appears
to be due to the tumor itself. The T cells within the tumor also had higher expression of
immune checkpoint inhibitors including PD-1, CTLA-4 and GITR, an indication of
exhausted T cell state within the PDX melanoma TME. Exhausted T cells express multiple
inhibitory molecules (including PD-1, CTLA-4, Tim-3, TIGIT and LAG-3)(Ahmadzadeh et
al., 2009; Day et al., 2006) whose expression pattern points to different levels of
exhaustion (Granier et al., 2017). The frequency and level of expression of these immune inhibitory receptors have been shown to be higher in intratumoral T cells than in normal tissue or peripheral blood (Ahmadzadeh et al., 2009), which parallels data from our PDX melanoma bearing NSG-SGM3 BLT mice. The co-expression of many of these receptors on a single type of T cell increases their dysfunctional state (Granier et al., 2017).

The TME of PDX melanoma isolated from the NSG-SGM3 BLT mice also showed increased frequency of FoxP3+ regulatory T cells and tumor associated CD14+ macrophages, both of which contribute to the immune suppressive nature of the TME and tumor proliferation and evasion of immune regulation. The increased Treg correlates with patient TME data and usually signals poor prognosis in cancer due to Treg-mediated suppression of anti-tumor immunity, which benefits the tumor (Bates et al., 2006; Bohling and Allison, 2008; Curiel et al., 2004). Tumor associated macrophages also produce tumor-stimulating molecules such as IFN-γ, angiotensin and IL1b that support tumor growth and metastasis (Wang et al., 2017). Additionally, tumor associated macrophages can recruit Treg into the TME (Wang et al., 2019).

With the higher expression of PD-1 by T cells within TME of our tumor-bearing humanized mice and PD-L1 expression by the PDX melanoma we tested the capacity for PDX melanoma to respond to anti-PD1 checkpoint immunotherapy. Pembrolizumab treatment led to reduction in tumor growth kinetics, suggesting release of immune suppression thus enabling of the immune system to mount an immune response against the PDX melanoma. The observation of immune response is consistent with data from anti-PD-1
antibody treatments in tumor-bearing humanized mice (Capasso et al., 2019; Rosato et al., 2018; Wang et al., 2018). Our data also show that the response to pembrolizumab treatment was dependent on human leucocyte infiltrating the TME as tumor killing effect of pembrolizumab was absent in a cold melanoma tumor that lacked leukocyte infiltration.

As shown in patient data (Ribas et al., 2016), we observed an increase in CD8+ and conventional CD4+ T cells within the TME of our PDX melanoma bearing humanized mice on treatment with pembrolizumab. Consistent with existing literature, we also observed a reduction in Treg and TAM on anti-PD-1 antibody treatment (Li et al., 2018a; Rosenblatt et al., 2011). Lastly, we observed PDX melanoma tumor growth reduction on treatment with Ipilimumab and a combination of Ipilimumab and pembrolizumab and the combinational treatment of the TLR9 agonist CpG and anti-IL10R antibody, highlighting the ability of the reconstituted immune system within our HIS mice to be rejuvenated against PDX tumors by clinically relevant immunotherapeutic agents.

In summary, our data show that PDX tumor-bearing NSG-SGM3 BLT mouse model is a powerful tool for studying the human immune system interactions with tumors. This model may also be useful for testing novel therapies, for understanding the mechanism of action of immune modalities, and equally important, for testing immune response to new therapies before clinical trials.
CHAPTER V

Discussion:
The field of tumor immunology has seen an exponential growth centered around the idea of cancer immune surveillance where as part of its main roles, the immune system serves to protect the host by eliminating cancers. The concept of immune surveillance encompasses three main stages: elimination (where the immune system rejects cancerous cells), equilibrium (where the cancer persists but is controlled by the immune system and therefore in a dormant phase) and escape (where the immune system fails to control cancer growth) (Dunn et al., 2004). The idea of the immune system policing recalcitrant transformed cells was initially hard to accept as the immune system was built around discriminating self from non-self, making it near impossible to identify tumors which arose from self-tissues (Bretscher and Cohn, 1970). With the realization that the immune system recognizes self-danger signs with sensing molecules including TLRs and RLRs, the thinking shifted (Medzhitov et al., 1997; Orange et al., 1995; Salazar-Mather et al., 1996). The importance of the immune system’s role in tumor surveillance was further helped by the advent of mouse models lacking one or more immune functions validating predicted increases in their susceptibility to cancer (Kaplan et al., 1998; Pantelouris, 1968; van den Broek et al., 1996). The identification of human tumor antigens and antigen specific T cells validated the premise of tumor immune surveillance (Gros et al., 2016; Srivastava, 2015).

Following the success of checkpoint inhibitors in treating some cancers, the attempt at discovering additional ways to harness the immune system for tumor elimination have
escalated, creating the need for small-animal models in which to test new therapies and interrogate their mode of action. Studies on human immuno-oncology have historically relied on rodent tumor models including syngeneic mice and on *ex-vivo* characterization of human cells (Khaled and Liu, 2014; Ruggeri et al., 2014). However, these are not without limitations. *In-vitro* studies do not capture the 3D cellular dynamics that may play an important role in shaping the initiation, progression and regulation of the malignancy. Also, rodent models do not fully recapitulate the human immune system and its interactions due to inherent inter-species variation. Failure to account for some of the species variations have resulted in deleterious clinical trial outcomes (Mestas and Hughes, 2004; Shin et al., 2011).

Humanized mice bearing patient derived xenograft tumors fill this niche requirement for a small-animal model that can recapitulate both the 3D cellular microenvironment and human immune system needed for human immune oncology studies. My thesis examined the use of humanized mice to study human immune system PDX tumor interactions. In chapter II, I show the development of the NSG-TLR4<sup>null</sup> mice for interrogating human TLR4 specific immune response. I then show the generation of an NSG mouse transgenic for human IL15 important for human NK cell reconstitution on engraftment with human CD34+ HSCs in chapter III and lastly, I validated the NSG-SGM3-BLT mouse bearing melanoma as a model system for interrogating human immune system PDX tumor interaction and for immunotherapy evaluation.
TLRs play an important part in sensing pathogenic agents and danger signals thereby activating both the innate and adaptive immune system. As a result, multiple TLR agonists are being developed and evaluated as cancer therapeutic agents either individually or in conjunction with other therapeutic modalities. One of the major limitations to the use of the current humanized mice models in evaluating TLRs and more specifically TLR4 agonist for antitumor activity is the presence of murine innate immune cells within the reconstituted immunodeficient mice that also express TLR4 and can confound data obtained from any such studies. I showed that by deleting mouse TLR4 in NSG mice, the resulting NSG-TLR4null mice once reconstituted with human immune system can respond to LPS in a human-specific manner. Additionally, following treatment with LPS the human immune system reduced the tumor growth kinetics of a PDX melanoma tumor providing proof of concept for the use of the NSG-TLR4null model in evaluating TLR4 agonists as immunotherapy agents. For example, in the cases of TLR4 agonist Lipid A and Bacillus Calmette–Guérin (BCG), studies in NSG-TLR4null mice would validate their use as immunotherapeutics and help elucidate the mechanism respectively. Lipid A analog is a synthetically generated TLR4 agonist that has been shown to have antitumor effects in murine mammary tumor models where it promotes the production of proinflammatory cytokines, including IFN-γ, and TNF-α, and induces apoptosis in chemotherapy-resistant tumor cells (Lamrani et al., 2016) while BCG another TLR4 agonist that can also act through TLR2 is one of the most successful biotherapies in use for bladder cancer, although to date there is no clear mechanism of its therapeutic effect in order to improve its mortality rate (Redelman-Sidi et al., 2014).
The data also highlight the possibility of generating NSG mice depleted of other murine TLRs such as TLR2, TLR3, TLR7 and TLR 9 either individually or together or in groups to enable the evaluation of agonist targeting them that are currently being developed and tested for activity against tumors (Ayala-Cuellar et al., 2019; Benbenishty et al., 2019; Liu et al., 2016; Tran et al., 2019). For example Poly(I:C), a TLR3 agonist has been shown to have antitumor activity against glioblastoma, by inducing the production of IFN-β and IL15 in addition to chemokines which increase recruitment of tumor infiltrating T cell through paracrine/autocrine action (De Waele et al., 2018). Also in studies of MDA-MB-231 breast cancer cells and DU145 prostate cancer cells, Poly(I:C) activation of TLR3 results in tumor regression through the upregulation of microRNAs such as miR-29c and miR-152 which in turn lead to the re-expression of the oncosuppressor retinoic acid receptor beta (Galli et al., 2013). In addition, dual activation of TLR3 and TLR7 in MCF-7 human breast cancer cells leads to reduced growth (Kang et al., 2010). The above studies on TLR3 and TLR7 activation would greatly benefit from validation in a more relevant in-vivo system such as humanized mouse lacking the mouse TLRs of interest.

In chapter III, I showed that transgenically expressing human IL15 in NSG mice, led to production of circulating human IL15 at near physiological levels. The observed human IL15 production was closer to physiological levels when compared to the two currently available immunodeficient strains transgenically expressing human IL15 used in humanized mouse generation: the SRG-15 and the NOG-IL-15-Tg. Both the SRG-15 and the NOG-IL-15-Tg mice had circulating human IL15 significantly higher than physiological
levels ((Herndler-Brandstetter et al., 2017; Katano et al., 2017) which had the added effect of expanding memory T cells within the SRG-15 mice upon humanization.

The presence of circulating human IL15 improved human NK cell development and function within the NSG-Tg(Hu-IL15) mice on engraftment with human CD34+ HSCs. Given that NK cells play a major role in immune surveillance (Malmberg et al., 2017; Souza-Fonseca-Guimaraes et al., 2019), I showed that the improved NK cell fitness and frequency was able to reduce the tumor growth kinetics of PDX melanoma transplanted into CD34+ HSC reconstituted NSG-Tg(Hu-IL15) mice. This observation positions NSG-Tg(Hu-IL15) mice as a powerful model for interrogating NK cell function and regulation within the TME and for testing NK cell targeted immunotherapies. The model would therefore be an important tool to test the efficacy of cellular adoptive transfer from various sources including PBMCs autologous to the tumor being transplanted into the mice and allogeneic NK cell mobilized from cord or iPSCs. There is significant effort underway to develop Bi-specific and Tri-specific killer engagers that enhance NK cell tumor targeting while boosting NK cell cytotoxicity multiple folds. With these killer engagers, there are two or three Fab fragments fused against tumor-associated antigens that serve as a cross-link between the tumor cell and the effector cells (Gleason et al., 2014; Steinbacher et al., 2015). The NSG-Tg(Hu-IL15) model would serve as a pragmatic model to test human specific effects of Bi and Tri-specific killer engagers. In this work, although we observe significant improvement in tumor growth on NK cell depletion, the depletion was not complete. Future experiments should completely deplete completely NK cells to test if
tumor growth can be restored to the same kinetics as those observed in the control NSG tumor-bearing humanized mice.

One aspect of NK cell biology that my work did not address is the mode of circulating human IL15 presentation to the NK cells. The most effective method for IL15 signaling is through trans-presentation by the IL15Rα to the IL15βγ dimer. It would be interesting to see if IL15 is being trans-presented or the soluble IL15 is signaling much like the other members of the IL2Rγ chain. Additionally the reduction of tumor growth in the NSG-Tg(hu-IL15) mice, while significant, did not lead to complete regression, indicating a potential lack of fitness or suppressed function of NK cells within the TME. Future studies could look more critically at the functional phenotype of NK cells within the TME. Repression of NK cell function within the TME could potentially leverage HSC engrafted tumor-bearing NSG-Tg(hu-IL15) mice as a model for understanding NK cell regulation and suppression within tumors.

In chapter IV, I showed that the NSG-SGM3 BLT mouse model which engrafts robust human immune system with improved proportions of human myeloid cell lineages and FoxP3+ Treg can be used to interrogate human immune system, PDX tumor interactions and for evaluating immunotherapeutics.

With the strong reconstitution of human immune cell lineages within the NSG-SGM3-BLT mice and their ability to infiltrate permissive tumors and undergo regulation, recapitulating observations in clinic, the NSG-SGM3-BLT mice positions humanized mice as a relevant
model system within which to further investigate how the human immune system interacts intimately with the TME to either eliminate tumor or enable their escape from surveillance. It also shows the potential use of humanized mice in evaluating immunotherapies in a system that closely resembles the patient, while affording personalized and precise evaluation of therapies tailored to specific tumor variants from patient though an avatar system.

Figure 5.1 summaries the uses of humanized mice in immuno-oncology
Figure 5.1: Schematic of humanized mouse use in immuno-oncology. PDXs can be developed from a fresh piece of a patient’s tumor, characterized *in-vivo*, and used for preclinical assessment immunotherapies using humanized mice generated from the autologous patient immune system in an AVATAR approach. Results are then applied to propose the most adapted therapy for the individual patient. Additionally allogeneic tumor and immune system (both engineered and intact) pairs can be set up to characterize the tumor: immune system interaction, assess preclinical immunotherapies and determine mechanism of therapeutic action, which can then be applied large patient populations.
CHAPTER VI

References


gamma-chain molecule is critical for intestinal T-cell reconstitution in humanized mice. *Mucosal immunology* 5:555-566.


Koster, B.D., M. van den Hout, B.J.R. Sluijter, B.G. Molenkamp, R. Vuylstekte, A. Baars, P.A.M. van Leeuwen, R.J. Scheper, M. Petrousjka van den Tol, A.J.M. van den Eertwegh, and T.D. de Gruijl. 2017. Local Adjuvant Treatment with Low-Dose CpG-B Offers Durable Protection against Disease Recurrence in Clinical Stage I-II Melanoma: Data from


Paul, S., N. Kulkarni, Shilpi, and G. Lal. 2016a. Intratumoral natural killer cells show reduced effector and cytolytic properties and control the differentiation of effector Th1 cells. *Oncoimmunology* 5:


patients with hepatocellular carcinoma and are associated with tumor progression. *Cellular And Molecular Immunology* 14:819.


