

EVOLUTIONARY PRINCIPLES IN HUMAN CANCER IMMUNOLOGY



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General abstract

The immune system plays a fundamental role in driving the evolution of malignant cells. The anti-tumour immune response removes immunogenic tumour cells and preserves immune evasive cells. A clinically detectable tumour arises from a single transformed cell. Through millions of cell divisions, tumour cell populations diversify and acquire mechanisms to avoid destruction by the immune system. Examining the evolutionary outcomes of populations undergoing natural selection can reveal novel adaptive strategies. Evolutionary principles are used to gain insights into different aspects of cancer biology. However, these approaches have not been widely applied to cancer immunology. **Here, we hypothesize that evolutionary principles can be successfully integrated into cancer immunology research to reveal novel findings with clinical potential.**

To explore this hypothesis, we used three approaches taken from evolutionary biology:

1) *conservation of essential genes during evolution*. We performed a gene conservation study on lung tumour tissue to determine essential genes at the interface of tumour and immune cell interactions. Mutations in malignant epithelial cells undergo selection. Deleterious mutations in genes that are essential for survival and proliferation undergo negative selection. Here, we apply this principle to determine the conservation levels of genes expressed in malignant epithelial cells with immune functions. We found several genes involved in antigen presentation conserved in lung cancer, in particular ERAP1/2, which has been identified as a therapeutic target by other approaches. We also found a group of natural killer cell ligands, particularly MICA, PVR, and PVRL2, conserved in wild-type and mutant KRAS tumours. Disruption of antigen presentation on epithelial cells and overexpression of inhibitory molecules are common mechanisms of malignant cell immune evasion. Our gene conservation study demonstrates that probing the outcomes of natural selection can reveal novel immune targets.

2) *evolutionary costs of treatment resistance*. Building on the above model and focusing on the NK ligands identified above, we next examined the evolutionary outcome of radiation resistance. Adaptive strategies that arise under strong selection

pressures often have an associated fitness cost. By evaluating an isogenic cell line model of prostate cancer, we found that radiotherapy resistance increases expression of natural killer cell ligands, MICA/B, PVR, and PVRL2, on tumour cells resulting in increased sensitivity to NK cell-mediated killing. Our pre-clinical study revealed selective targeting of radiation-resistant cells by NK cells.

3) *evolutionary double bind*. A 'double bind' exists when resistance to one treatment increases the sensitivity to a second treatment. Selective targeting of radiation-resistant populations by NK cells is a double bind. Using our isogenic radiation-sensitive and resistant prostate cancer cell lines and novel competition culture models, we show that treatment with NK cells restores the radiation-sensitive cancer cell population. We also found that radiotherapy followed by NK cell therapy reduced the number of tumour cells and controlled the outgrowth of radiotherapy resistance. This effect was amplified in samples with initially high levels of radiation resistance.

We have shown that evolutionary principles applied to cancer immunology research can reveal novel targets with clinical potential. After the target is identified, we demonstrate that evolutionary principles can be used to optimize therapy schedules to exploit treatment-induced evolutionary double binds. We propose that patients will benefit from the integration of evolutionary medicine and cancer immunology to both reduce tumour burden and delay or prevent treatment resistance. We are the first to use gene conservation levels of immune-related genes for novel target discovery in clinical samples and to apply those findings to a pre-clinical model of a treatment-induced immunological double bind.

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Associated Publications

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Further Publications

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Chapter 1: General Introduction

1. Chapter 1: General Introduction

1.1. Cancer Immunology

"There is something unique about a cancer cell that distinguishes it from normal cells, and ... this difference can be recognized by the body's immune system" – Lloyd J. Old 1977

Cancer immunotherapy has revolutionized the way we treat cancer. The success of checkpoint inhibitors in melanoma demonstrates the anti-tumour potential of the immune system. These advances did not arrive overnight. William B Coley conducted the first systematic immunotherapy trial in 1891 (1). Lloyd J. Old's fundamental observation that cancer cell's differed just enough from normal cells that they could elicit an immune response was published over 40 years ago (2, 3). Cancer immunology has progressed since the early days of Coley's toxin. Modern cancer immunology is the culmination of many novel discoveries within immunology and cancer biology.

1.1.1. The changing landscape of cancer immunology

Since the introduction of the 'immunological self' in the 1960s, cancer immunology is in Burnet's self-non-self-framework (4) Normal cells express normal proteins, are recognized as self, and avoid immune destruction. Malignant cells express mutated proteins resulting in a more non-self-appearance. This self-non-self immunological viewpoint in cancer focuses on α/β CD8 T cell recognition of neoantigens bound to MHC

class I molecules on tumour cells. It is central to the success of current immunotherapies and the basis of ongoing immune-oncology research.

Much work has been done since Burnet's time. Bretscher and Cohn developed the two-signal model (5, 6). Followed by Lafferty and Cunningham's discovery of antigen-presenting cells and the importance of co-stimulation (5). The Janeway model of immunology introduced "extended self-non-self," providing critical links between innate and adaptive immunity (7). The discovery of pattern recognition receptors (PRR) highlighted the immune system's reliance on evolutionarily conserved molecules from invading pathogens to trigger the innate immune response (8) (4). Therapeutic cancer vaccines rely on adjuvants that stimulate PRRs (9) (10)

Introduced in 1994, Matzinger's Danger Model theorized that the immune system does not solely recognize exogenous non-self signals. Endogenous signals that indicate danger to the host initiate an immune response (11-13). Danger-associated molecular patterns (DAMPs) are released from damaged tissue and bind pattern recognition receptors triggering immune responses similar to PAMPs (14). The Danger Model and discovery of DAMPs was a paradigm shift demonstrating the requirement for tissue damage to trigger an immune response. For cancer immunology, this meant that quiescent non-invasive tumour cells could hide from the immune system.

The self-non-self framework and the danger model are essential to our understanding of the immune response to cancer. However, the identification of immune regulating checkpoints (CTLA-4 in 1995 and PD-1 in 1999) initiated the breakthrough for clinically

efficacious immunotherapy, leading to the Nobel prize in 2018. Schreiber's contribution of the Cancer Immunoediting concept introduced the significance of natural selection events occurring when the immune system recognizes cancer cells (15). His description of the acquisition of immune-evasive phenotypes will be integral to the future of cancer immunology.

Cancer biology has seen a shift from a tumour cell-centric view to one that focuses on the tumour and its microenvironment. This tissue-centric view highlights the importance of non-malignant cells within the tumour. Additionally, recently introduced concepts, including; the discontinuity theory of immunity, the growth-threshold conjecture, and a call to redefine the immunological self will continue to drive the field (16-18).

1.1.2. Cancer Immunotherapy

Immunotherapy does not directly target tumour cells. Instead, it supports a host immune response or introduces engineered forms of the immune system to control tumour growth. Before the success of checkpoint inhibitors, immunotherapy was used in specialized cases. Sipuleucel-T, an autologous cell therapy, has been used in hormone-refractory prostate cancer since 2010 (19). Immune adjuvants have also been used in limited cancer subtypes, including imiquimod in skin cancer and BCG in bladder cancer (9). Since the introduction of checkpoint inhibitors, immunotherapy has become an important treatment option in many cancer types and transformed clinical responses for melanoma patients (20). While most cancer immunology focuses on CD8 T cell responses, the field is beginning to diversify, and novel treatments are emerging (9).

Challenges for cancer immunotherapy include treatment efficacy, lack of biomarkers, cost of therapy, and the evolution of treatment resistance (21). The tumour microenvironment and small populations of cancer stem cells (CSC) decrease the efficacy of immunotherapy (22, 23). Anti-tumour immune cells are inhibited by the local environment within the tumour, which is often acidic, poorly perfused, and houses a large number of regulatory immune cells (24). CSC are associated with treatment resistance and disease recurrence (22). Immune evasion mechanisms in CSC include decreased antigenicity, increased CD47 expression (macrophage inhibitor), and high PD-L1 expression (22, 25). Interestingly, CSC may be more sensitive to NK cell based immunotherapy due to alterations in NK ligand expression. However, current data is contradictory and more work needs to be done (25).

1.1.2.1. Enhancing anti-tumour T cell responses

Checkpoint inhibitors, including anti-PD1/PD-L1 and anti-CTLA4, are monoclonal antibodies that block the regulatory effects of checkpoint ligand binding to receptors on immune cells (26). Myeloid cells and epithelial cells express PD-L1 during an active immune response. Tumour PD-L1 is expressed on tumour cells, either constitutively or induced by IFN-gamma and other JAK/STAT activating molecules (27). There are currently several antibodies approved for clinical use. The two most widely used checkpoint inhibitors, Pembrolizumab (anti-PD-1) and Nivolumab (anti-PD-1), are approved for use in numerous cancer types, including melanoma, non-small cell lung cancer, head and neck cancer, MSI-high cancers, gastric, cervical cancer, and

hepatocellular carcinoma (26). Inhibition of additional checkpoint pathways are being explored, including anti-TIGIT monoclonal antibodies for advanced NSCLC (28).

Therapeutic vaccines induce tumour-specific T cell responses *in vivo*. Systemic vaccines target known tumour antigens or utilize tumour lysate to trigger anti-tumour T cell responses. *In situ* vaccines elicit immune responses at the tumour (9). These include oncolytic viruses that target cancer cells and behave as adjuvants to enhance the anti-tumour immune response (9). STimulator of INterferon Genes (STING) is an endoplasmic reticulum associated protein activated after detection of cytosolic DNA by cGAS. STING pathway activation induces a type-I interferon response supporting the anti-tumour immune response (29). TLR ligands and STING agonists added directly to the tumour site stimulate the innate immune system feeding into the adaptive anti-tumour response (9). Additionally, chemotherapy and radiation therapy induce cell death and tissue damage releasing DAMPs and tumour antigens (30, 31)

1.1.2.2. Natural killer cell based immunotherapies

Monoclonal antibodies targeting cancer growth pathways bind tumour cells via their antigen-binding fragment (Fab) portion inhibiting cell proliferation. In the absence of immune cells, monoclonal antibodies exert cytotoxic and cytostatic effects on cancer cells (32). Additionally, the fragment crystallizable (Fc) portion of the antibody binds Fc receptors on cytotoxic immune cells, primarily NK cells, via CD16 (32). Linkage of the two cells induces NK cell mediated killing of the tumour cell, known as antibody-dependent cellular cytotoxicity (ADCC). ADCC is critical to the efficacy of monoclonal

antibody treatment in cancer (9). Therapies that enhance ADCC are used in combination with targeted antibody therapy (33).

Adoptive cell therapies using autologous and allogeneic NK cells expanded *ex vivo*, immortalized NK cell lines, and stem cell-derived NK cells are in clinical trials (34). Commonly used cell lines include NK-92, HANK-1, and NKL, although NK-92 is the only line currently approved for use in clinical trials (35). Safety and feasibility studies are ongoing. Additionally, NK cells differentiated from human pluripotent stem cells are autologous, highly proliferative, and are not affected by long-term cryopreservation (34). Once differentiated, they resemble primary NK cells with anti-tumour functions [20]. Cytokine-induced memory-like NK cells generated *ex vivo* via priming with cytokines (IL-2, IL-15, and IL-18) to induce a hyper-responsive state have shown safety in early trials (9, 35). Several engineered NK cells and NK cell engagers are also being tested (discussed in more detail below).

Augmenting the immune-suppressive tumour microenvironment can increase the effectiveness of NK cells *in vivo*. Inhibiting IDO, IL-10, prostaglandin E2, adenosine, reversing hypoxia, and buffering the acidic TME can restore NK cell function (36). Systemic cytokines, IL-2, and IL-15 increase the life span and proliferation of NK cells (36). Recently, alternate forms of IL-2, "super 2" and F42K, that have low affinity for the IL-2R α with higher affinity for IL-2/15R β expressed by NK cells have been developed (37).

IL-15 receptors are present on CD8 memory T cells and NK cells. Infusion with rhIL-15 is not well tolerated. Subcutaneous injection of rhIL-15 is more easily tolerated and induces the proliferation of NK cells and T cells (36). ALT-803, a superagonist of IL-15, has shown early success in clinical trials when combined with nivolumab (PD1/PDL1 inhibitor) in non-small cell lung cancer patients. Many trials are underway investigating combination trials with IL-15 and targeted antibodies (37) (36).

1.1.2.3. Engineered immune cells and immune cell engagers

Bi-specific and tri-specific Killer Engagers (BiKEs and TriKEs, respectively) activate anti-tumour NK cells. BiKEs and TriKEs are small molecules containing a single variable portion of one antibody linked to a second (and third) single variable portion of another antibody (37, 38). For NK cell activation, these typically include a CD16 portion and a tumour target, like CD19. These molecules link NK cells and tumour cells while activating NK cells through CD16 ligation (37).

Car-T cells are cytotoxic T cells with engineered TCRs to target known tumour antigens. The majority of success has been in the more clonal forms of cancer with homogenous antigen expression, including B cell Lymphomas. Genetically modified NK cells have been developed [20]. Chimeric antigen receptor-expressing NK cells (CAR-NK) are genetically engineered from various sources of NK cells, including cell lines and primary cells. Similar to CAR-T cells, CAR-NKs target specific tumour antigens. However, unlike CAR-T, they maintain their native NK cell activating receptors [27, 28]. Additionally, CAR-NK cells do not undergo clonal expansion, thus limiting their potential toxicity compared to CAR-T cells. These NK cell-based therapies are still under investigation, but early

studies indicate their utility in many cancer settings, especially when combined with other treatment modalities [20, 28].

1.1.3. The players: T cells

The adaptive arm of the immune system is activated by antigen-specific receptors leading to effector activity and memory responses. B cells and T cells have differential responses to cancer. Humoral immunity, driven by the bone marrow-derived antibody-producing B cells, has a role in malignant cell growth (39, 40). CD8⁺ $\alpha\beta$ T cells originate in the bone marrow. Following emigration from the bone marrow, T cell progenitors arrive in the thymus, where unique T cell receptors are generated through RAG mediated VDJ gene recombination. Subsequent clones undergo negative and positive selection to ensure appropriate selection MHC restriction and elimination of most self-reactive T cells. Here, they are also assigned to either CD4 helper T cell lineage or CD8 cytotoxic T cell lineage (41). Both play significant roles in anti-tumour activity.

Malignant cells harbour many non-synonymous mutations resulting in mutated proteins with novel amino acid sequences, known as tumour-specific antigens (TSA). Additionally, due to increased chromosome instability and epigenetic alterations, tumour cells express non-mutated proteins at higher levels than normal tissue or an inappropriate stage of development. These tumour associated antigens (TAA) are not unique to tumour cells but are typically not expressed at the same levels elsewhere in the body (42). Both TSA and TAA are often immunogenic, eliciting antigen mediated immune responses that result in tumour infiltrating CD8 T cells and tumour cell death.

Chemotherapies and radiation therapy are non-specific toxic treatments that affect highly proliferative cells through various DNA damaging and inhibitory pathways. Their anti-tumour potential relies on malignant cell dysregulated proliferative signals. Immune cells are also affected by these therapies resulting in neutropenia and lymphopenia in heavily treated patients. Interestingly, chemotherapies and radiation therapy are also capable of inducing antigen mediated immune responses (31). Dying tumour cells release DAMPs and tumour antigens into the microenvironment, causing an *in situ* vaccine effect by stimulating antigen-presenting cells and downstream activation of tumour-specific T cells increasing the effectiveness of these treatments (43). Clinically correlations between numbers of infiltrating T cells and response to chemotherapy have been found in colorectal cancer patients (44).

1.1.4. The Players: NK cells

Innate lymphoid cells (ILCs) lack antigen-specific receptors and are divided into three families. Natural killer (NK) cells are a primary member of group 1 ILCs (ILC1), which utilize the transcription factor T-bet to facilitate the production of IFN-gamma (45-48). NK cells recognize pathogen-infected, stressed, and malignant cells through a diverse array of surface receptors (**Figure 1.1**). NK cells directly kill cancer cells with perforins and granzymes packed into cytolytic granules and through the release of death-inducing ligands, including Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (49, 50). Indirectly, activated NK cells orchestrate and support cytotoxic functions of the adaptive arm through the release of chemokines and cytokines, including IFN-gamma, TNF, GM-CSF, IL6, CCL3, CCL4, and CCL5 (51).

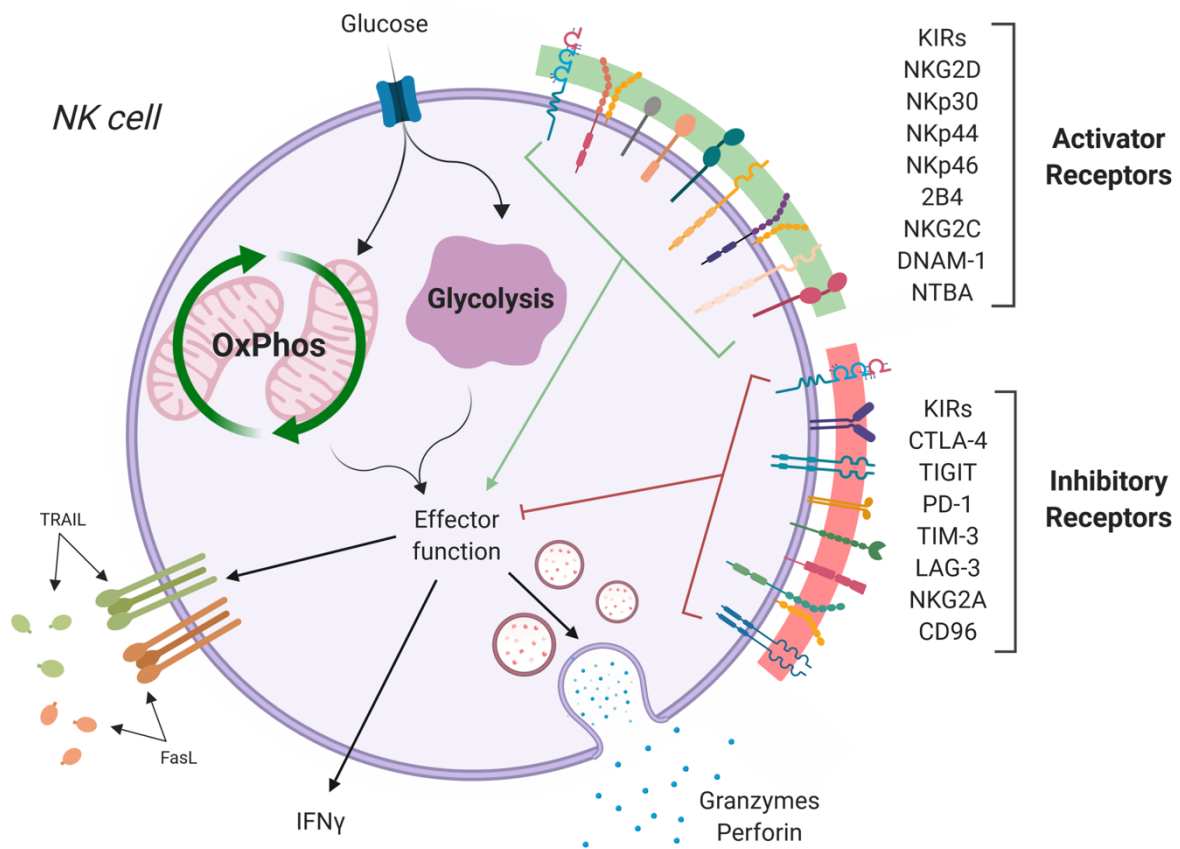


Figure 1-1. NK cell activation is controlled through an array of diverse activating and inactivating receptors.

Figure from Piñeiro Fernández J, Luddy KA, Harmon C, O'Farrelly C. Hepatic Tumour Microenvironments and Effects on NK Cell Phenotype and Function. *Int J Mol Sci.* 2019;20 (17):4131. Published 2019 Aug 24.

NK cell functions are managed by a diverse array of activating and inactivating surface receptors that bind with ligands on target cells (**Figure 1**). Inactivating receptors have an internal motif, immunoreceptor tyrosine-based inhibitory motif (ITIM), that recruits two tyrosine phosphates, SHP-1 and SHP-2, inhibiting cytolytic functions cytokine and chemokine secretion (52). Killer cell immunoglobulin-like receptors (KIR) are a diverse family of activating and inactivating receptors encoded by twelve highly polymorphic genes (52). Inactivating KIRs contain an ITIM domain and are the key receptors for recognition of 'self' via ligation with human leukocyte antigens (HLA) (53). The binding of inactivating receptors is swift and sensitive. Ligation of only one inactivating receptor will halt the effector functions of NK cells, while activation requires ligation of multiple activating receptors (52).

Checkpoint receptors are expressed following activation and during chronic disease limiting over activation of the immune response. These inactivating receptors are involved in cancer cell immune evasion. T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) is a checkpoint receptor expressed by activated T cells and NK cells. Patients with chronic infections and cancer have high levels of TIGIT (54).

Similarly, programmed cell death protein 1 (PD-1) is expressed on subsets of NK cells and is upregulated in cancer (55). The intracellular portion of PD-1 contains an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM). Recent studies highlight the contribution of NK cells in responses to checkpoint inhibitors (37, 55). Pre-clinical models have shown that blocking PD-1/PDL-1 increases the cytotoxic activity of NK cells against multiple myeloma *in vitro*. (53).

Activation of NK cells requires the ligation of receptors lacking an ITIM domain. Classic activating receptors, including; CD16, natural cytotoxicity receptors (NCR), and NKG2D, instead rely upon an intracellular domain containing charged residues that interact with adaptor proteins containing immunoreceptor tyrosine-based activation motif (ITAM) that interacts with downstream proteins resulting in NK cell activation (52). Other, non-classical, activating receptors have more complex and highly regulated intracellular signalling programs. CD226, an activating receptor that competes with TIGIT for its ligands, induces activation independently of adaptor proteins. Instead, CD226 has an intracellular domain with two phosphorylation sites. Once phosphorylated, activation occurs, initiating a complex downstream cascade that is highly regulated and often requires a second activating signal to ultimately induce the cytolytic functions of NK cells (50). 2B4 (CD244) is another non-classical activating receptor with a role in NK cell anti-tumour responses. 2B4 receptors have four immunoreceptor tyrosine-based switch motifs responsible for NK activation. Like CD226, downstream signalling of 2B4 and other related surface molecules are tightly regulated. It has been suggested that additional activating receptors may be required to activate NK cells (56).

Metastatic disease is the primary cause of cancer-related deaths and occurs when malignant cells migrate and disseminate through lymphatic and hematologic vessels and resettle in distant organs. Upon entrance, the arriving cell or cluster of cells must survive and proliferate in the new tissue. Resident tissue NK cells protect against the establishment of new metastatic lesions (52, 57). Increased NK cell numbers correlate with better outcomes in metastatic gastric, renal, and prostate cancer (57). The

depletion of NK cells results in increased lung, liver, and bone marrow lesions in murine models of experimental metastasis (57). Furthermore, epithelial to mesenchymal transition (EMT), often the first step of metastasis, is linked to the increased expression of NKG2D (activating) ligands and loss of cadherin (CDH1 or E-cadherin), an NK cell inactivating ligand (57).

1.1.5. The Players: Myeloid lineage and other cytotoxic cells

In addition to conventional $\alpha\beta$ T cells and natural killer cells, the tumour microenvironment houses a diverse array of immune cells. Regulatory cells, including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSC), immunosuppressive macrophages, and anti-inflammatory neutrophils, suppress the anti-tumour effects of cytotoxic lymphocytes (58). Tregs inhibit antigen-specific T cells. High Treg to effector T cell ratios are associated with poor outcomes in cancer (59). Similar to M1 and M2 macrophages, tumour associated neutrophils elicit pro-tumour or anti-tumour immune responses. Differentiation of both macrophages and neutrophils is mainly dependent on the environment (60).

Tumour infiltrating $\gamma\delta$ T-cells are for their anti-tumour potential. Several strategies to increasing $\gamma\delta$ T cell activity are being investigated clinically (61). Adaptive cell therapy with V γ 9Vd2 TCR–transduced T cells are being examined for safety and efficacy (61, 62)

Natural killer T cells (NKT) are CD1 restricted cells activated by lipid antigens. Type I NKT cells primarily recognize α -galactosylceramide (α -GalCer) (63). Type II NKT cells are a heterogeneous mix of cells that do not recognize α -GalCer. Current evidence suggests

type I NKT cells enhance anti-tumour immune responses and type II NKT cells inhibit. Type II coordinate with Tregs in the tumour. NKT cells are affected by their environment and have diverse tissue-specific functions (63).

1.2. Epithelial cells and the immune response

The current success of checkpoint immunotherapies has inspired a wealth of clinical trials in multiple cancer subtypes. However, success is limited. Epithelial cancers are the most resistant to immunotherapy (64). An understanding of how epithelial cells evade immune destruction is critical for furthering the success of immunotherapy.

Epithelial cells, although not derived from hematopoietic cells, are critical players in the immune response. They express PRRs, secrete chemokines, cytokines, and defensins, and cohabitate with the microbiome (65). Classically referred to as the first line of defence, barrier tissue epithelial cells physically block pathogens from entering the host. They are also directly involved in immune surveillance (66). Epithelial cells interact with the immune system to initiate responses or induce tolerance through a variety of mechanisms. Antigen presentation by MHC molecules alert activated T cells to the presence of foreign or mutated peptides (67). Activating/costimulatory molecules and inhibitory/checkpoint proteins expressed on the surface of epithelial cells directly bind with receptors on immune cells (26). Cytokines and chemokines released from epithelial cells shape immune cell populations by guiding entrance into the tissues and driving differentiation of infiltrating immune cells (68).

Housed within the cytoplasm and endosomes of epithelial cells are sensors for detecting extracellular and intracellular pathogens, danger-associated molecular patterns, and internal and external stresses. Additionally, there are intrinsic mechanisms of immune surveillance occurring within epithelial cells separate from immune cells (59). These pathways can trigger anti-viral immune responses, induce inflammation, initiate proliferation, or cause cell death (69, 70).

1.2.1. Antigen presentation

Professional antigen-presenting cells (APCs), including dendritic cells and macrophages, present short peptide sequences derived from intracellular and extracellular proteins and lipids. Antigen processing and presentation rely on intracellular machinery to break down complex proteins and lipids into peptide pools for presentation on the cells' surface by MHC-I, MHC-II, or CD1 complexes (67, 71, 72). MHC-I is expressed by all nucleated cells. MHC-II and CD1 molecules are traditionally expressed by professional APCs that activate CD4 T cells and NKT cells, respectively (73, 74). However, aberrant expression of both complexes has also been detected on transformed epithelial cells (74, 75). Activated NKT cells enhance the anti-tumour immune response directly through granzyme mediated induction of apoptosis and indirectly through secreting cytokines that stimulate both innate and adaptive immune responses (76). Induction of non-traditional antigen presentation on tumour cells is likely due to inappropriate gene expression, chronic intracellular stress signals, and the highly inflamed microenvironment. Here we explore all three complexes and their related gene families.

1.2.1.1. *Classical MHC-I*

MHC-I is a peptide antigen-presenting complex expressed by all nucleated cells (77). Each heterodimer, composed of a heavy chain (HLA-A, HLA-B, or HLA-C) and an invariant light chain, Beta-2-microglobulin (B2M), is assembled in the endoplasmic reticulum (ER) (77). The majority of peptides presented by MHC-I are derived from intracellular proteins. Once ubiquitinated, proteins are broken down by the proteasome into large peptide pools where they are then transported by Transporter Associated with Antigen Processing 1 (TAP1) proteins into the ER (77). They are then processed by ERAP1/2, further sculpting the peptide pool available to MHC-I molecules in the ER. Once the peptide is loaded, the complete complex is transported to the surface and surveyed by infiltrating CD8 T cells (**Figure 1.2**) (77, 78). Overexpressed in many cancer cell lines, ERAP1 is an IFN-gamma inducible gene that trims peptide precursors after transportation into the ER via TAP. ERAP peptidases sculpt the peptide pool before presentation with MHC-I. Mice lacking ERAP1 present different epitopes in LCMV models and are susceptible to *T. gondii* infections because they no longer present the immunodominant epitope responsible for clearing the infection (79).

Disruptions in antigen processing and presenting are key mechanisms of immune escape and decreased efficacy of checkpoint inhibitors (80, 81). Genetic alterations, including silencing mutations of B2M, have been detected in recurrent melanoma following treatment with immunotherapies (82). Additionally, antigen presentation can be hindered in cancer cells through transcriptional and post-transcriptional regulation of MHC-I associated genes. HLA-A/B/C genes have three transcription binding sites that recognize NF- κ B, Interferon Regulatory Factor 1 (IRF1), and NOD-, LRR- and CARD-

containing 5 (NLRC5). Disruption to these transcription factors results in diminished MHC-I expression (77).

1.2.1.2. Non-classical MHC-I

HLA-E, HLA-G, HLA-F are non-classical MHC-I molecules. HLA-F is a ligand for KIR3DS1, an activating receptor on NK cells and KIR3DL2 and inhibitory receptor. HLA-F is involved in both maternal-fetal tolerance and clearance of HIV infected cells (83). HLA-E was first identified as a ligand for CD94/NKG2C, an activating receptor on NK cells, and CD94/NKG2A, an inhibiting receptor on NK cells. HLA-E binding preference is augmented by the structure of the presented peptide. For example, HLA-E molecules expressing HSP60 peptide can no longer bind the inactivating receptor and preferentially binds the activating receptor leading to NK cell activation and target killing (84).

1.2.1.3. MHC-II

MHC-II complexes are expressed by APC and epithelial cells of the oesophagus and barrier tissue of the respiratory tract (71, 85). MHC-II-peptide complexes are recognized by CD4 T cells. Once activated, CD4 T cells release cytokines and chemokines, coordinating the immune response and inducing differentiation and activation of multiple cell types, including anti-tumour CD8 T cells and NK cells (86). MHC-II are heterodimers composed of a variant chain encoded by either HLA-DR, DP, or DQ, which is regulated by class II transactivator (CIITA), a member of the NOD-like receptor A family (NLRA) (71, 86). While in the ER, MHC-II is bound by an invariant chain, CD74, that serves as a chaperone protein blocking peptide binding. Once MHC-II is transported into the endosome, the invariant chain detaches, and MHC-II is free to bind with the antigen

pool within the endosome typically derived from extracellular proteins (**Figure 1.2**)(71). The expression of MHC-II on non-small cell lung cancer (NSCLC) cell lines is inducible with IFN-gamma (86). Surgical resections in NSCLC show increase MHC-II expression on both immune cells and epithelial cancer cells correlates with positive outcomes (86).

1.2.1.4. *CD1*

CD1 molecules are non-polymorphic MHC-I related proteins with deep hydrophobic binding grooves for presenting lipid antigens (75). There are five isoforms (a-e) in humans. The first four present antigens on the surface, much like MHC molecules, while CD1e remains in the endosome shaping the lipid antigen pool similar to ERAP1/2 in the ER (75, 87). CD1 molecules also bind with B2M prior to surface expression (**Figure 1.2**). CD1d is not limited to hematopoietic cells (88). CD1d is expressed in a variety of cancer types, including constitutive expression in breast cancer and by lung cancer cells following epigenetic modifications (89). Expression of CD1d by tumour cells can elicit an immune response (73). Natural Killer T cells (NKT) are cytotoxic cells with both NK and T cell-like functions. They are CD1d restricted and respond to lipid peptide antigens that can target tumours cells through direct killing and activation of other anti-tumour immune cells. Cancer patients have lower circulating NKT cells compared to healthy donors, and tumour infiltrating NKT cells are associated with better outcomes in patients (90).

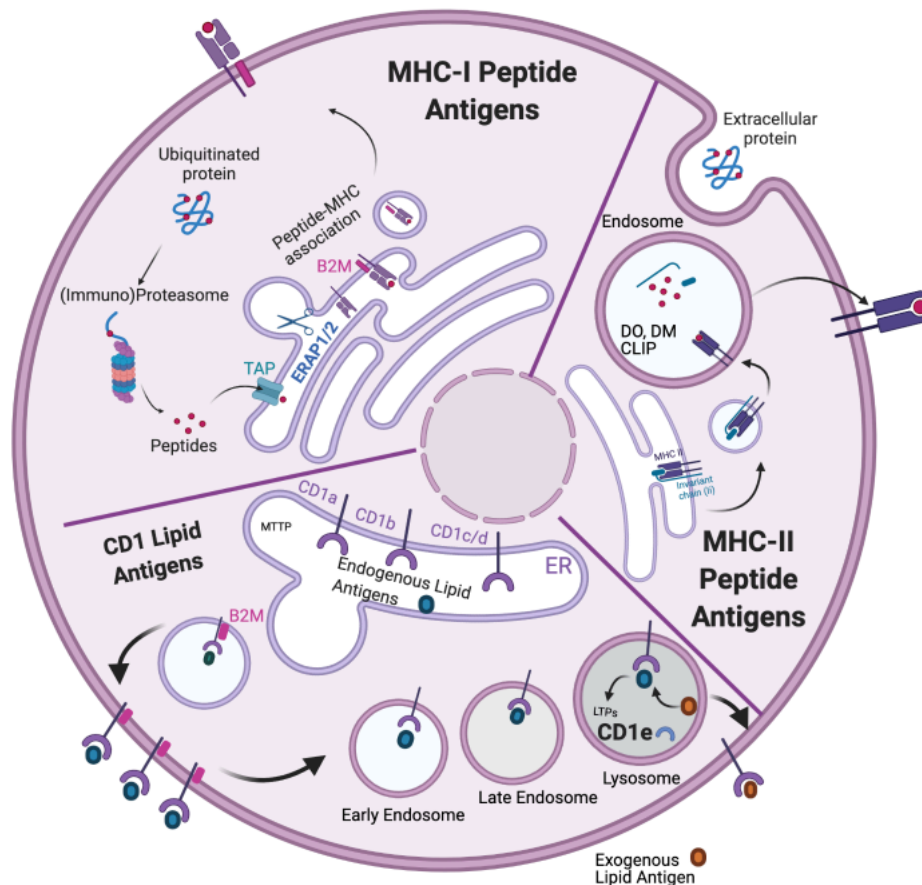


Figure 1-2. Antigen processing and presentation pathways.

MHC-I: endogenous proteins are broken down in the (immuno)proteasome, transferred into the endoplasmic reticulum (ER), cleaved by ERAP1/2, and loaded onto MHC-I. MHC-II: exogenous proteins are broken down in the endosome and loaded onto MHC-II. CD1: endogenous lipid peptides are loaded onto CD1 proteins in the ER, exogenous lipids are loaded onto CD1 proteins in the lysosome.

1.2.2. Immune modulating proteins on epithelial cells

Interactions between cytotoxic immune cells and other host cells are governed by receptor/ligand binding. Target cells express an array of surface and secreted molecules that communicate to nearby immune cells (91, 92). These proteins can inhibit or activate cytotoxic functions. In cancer, many of these communication pathways are altered to favour immune escape (59, 93-95). Increased checkpoint proteins, diminished activating signals, and overactivation of immune regulating metabolic pathways reduce the predator efficiency of cytotoxic cells (**Figure 1.3**). T cells, NK cells, phagocytic macrophages, and other anti-tumour immune cells rely on close interactions with tumour cells to elicit their effects. Checkpoint pathways, including PD1/PDL1, PVR/TIGIT, Gal-9/TIM3, and others, are often exploited by tumour cells leading to immune escape (3, 28, 53, 91, 96). T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) expressed by a variety of immune cells including differentiated CD4 T cells and CD8 T cells inhibits the anti-tumour T cell response (97). Ligation of the TIM3 receptor by Galectin 9 (Gal9) blocks cytokine production and proliferation in T cells. Blocking Gal9/TIM3 interactions reverses inhibits this checkpoint pathway and increases the anti-tumour T cell response (97). Secretion of immunoregulatory factors (soluble MICA/B, TGF-beta, etc.) and metabolic pathways with immune suppressive effects (e.g. tryptophan catabolism and adenosine production) are overexpressed in many cancers (98, 99). Furthermore, cancer cells often downregulate immune-activating surface proteins.

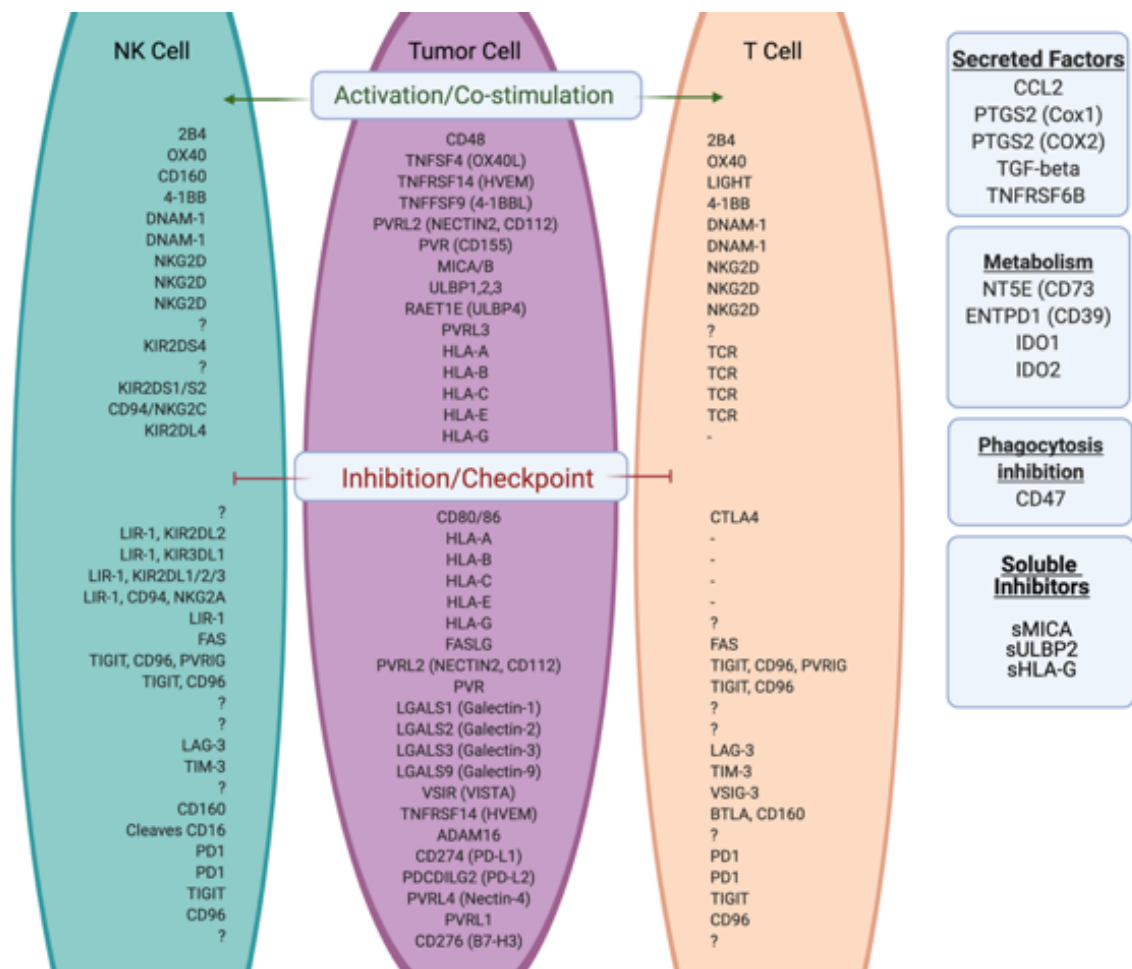


Figure 1-3. Immune modulating proteins expressed by tumor cells.

Checkpoint proteins and activating molecules are expressed by tumor cells (purple). Receptors for various ligands are expressed on natural killer (NK) cells (green) and T cells (yellow). Soluble factors, metabolic enzymes, and secreted factors (blue boxes) contribute to immunosuppression in the tumor microenvironment. CD47 inhibits phagocytosis by macrophages.

1.3. Intrinsic immune responses within epithelial cells

The immunologic functions of epithelial cells can occur independently of immune cells. Lung epithelial cells participate in pathogen clearance through the secretion of anti-microbial peptides, mucus production, and phagocytosis (66). Double-stranded DNA, cytoplasmic nucleic acids, and misplaced adenosine triphosphate (ATP) activate AIM2 and NLRP3 mediated cell death (pyroptosis) independently of immune cells (69, 100). Additionally, epithelial cells secrete cytokines, chemokines, interferons, eicosanoids and other inflammatory cytokines that affect neighbouring epithelial cells (65). Until recently, the role of intrinsic immunity in cancer has garnered limited attention. New insights into intrinsic pathways in epithelial cancers, including inflammasome activation, pyroptosis, and autocrine IFN signalling, have highlighted the importance of epithelial immune functions in cancer development and response to therapy (100-102).

Inflammatory pathways are triggered when inducers of inflammation are released by stressed or damaged tissue (103). These included exogenous sources from invading pathogens as well as endogenous inducers. Molecules with roles in normal cellular functions are endogenous inducers of inflammation if they escape their normal cellular compartments and include nucleic acids, ATP, K⁺ ions, mitochondrial DNA, high-mobility group box 1, uric acid, cellular chaperones and others (103, 104). Sensors for both exogenous and endogenous signals, pattern recognition receptors, are expressed on and within immune cells and epithelial cells. Ligation of these receptors triggers the release of inflammatory mediators, including chemokines and cytokines, vasoactive amines, eicosanoids, and products of proteolytic cascades that control the strength and

direction of the response (103). Alterations in these pathways have been seen in cancer (70).

Innate sensors of cellular stress and damage, pattern recognition receptors (PRR), include Toll-like receptors (TLRs), nucleotide-binding and oligomerization (NOD)-like receptors (NLRs), RIG-I like receptors (RLRs), AIM2-like receptors (ALRs), cGAS, and c-type lectins (105, 106). Toll-like receptors are a well-characterized family of membrane-bound innate receptors with conflicting roles in cancer progression and treatment (107). TLR 1,2,4,5,6, and 10 are expressed on the surface, while TLR 3,7,8, and 9 are expressed on endosomal membranes (106). Ligands for TLRs vary and include danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Once activated, the majority of TLRs recruits MyD88 adaptor protein which binds to IRAK-4, phosphorylates IRAK-1 and results in downstream activation of NF- κ B as well as IRF3, IRF7, MAPK JNK, p38, and ERK. TLR3, an endosomal TLR, functions independently of MyD88, relying instead on adaptor protein TRIF (106). TLR signalling leads to inhibition of cell death, induced proliferation and pro-inflammatory cytokines. In cancer, TLR activation can have either pro- or anti-tumour functions (107, 108) **(Figure 1.4)**.

NLRs, RLRs, cGAS, and ALRs are innate receptors housed in the cytoplasm. Activation of certain receptors in these families (NLRP1, NLRP3, NLRP7, NLRC4, AIM2, RIG-I, MDA5, and Pyrin) will induce inflammasome formation leading to activation of pro-inflammatory cytokines (active IL-1 β and IL-18) and potential pyroptotic cell death (105, 109-113). The inflammasome is a large protein complex composed of the activated innate immune receptors, apoptosis-associated speck-like protein containing a CARD

(ASC) and caspase-1. This results in the cleavage of pro-IL-1 β and pro-IL-18 into their active forms. Additionally, gasdermin D (GSDMD) can also be cleaved, inducing pyroptosis, an inflammation-inducing cell death pathway (105) (FIG 3).

Activation of PRRs induces the transcription and secretion of inflammatory mediators, including cytokines and chemokines, with autocrine and paracrine functions. Type I interferons (IFN α/β) are strong inducers of the inflammatory response and induce transcription of additional PRR in cancer cells. Therefore activation of IRFs further enhances the effects of PRR activation. Furthermore, cytokines and chemokines recruit and activate additional immune cells initiating the secretion of more cytokines and chemokines. Many of the ligands for PRRs, misplaced ATP, cytosolic nucleic acids, cellular chaperones, and HMGB1 are prevalent in cancer cells. While pro-growth signals of the inflammatory response encourage tumour cell growth, overabundant activation of such pathways (including pyroptosis initiators) would be detrimental to tumour cell fitness.

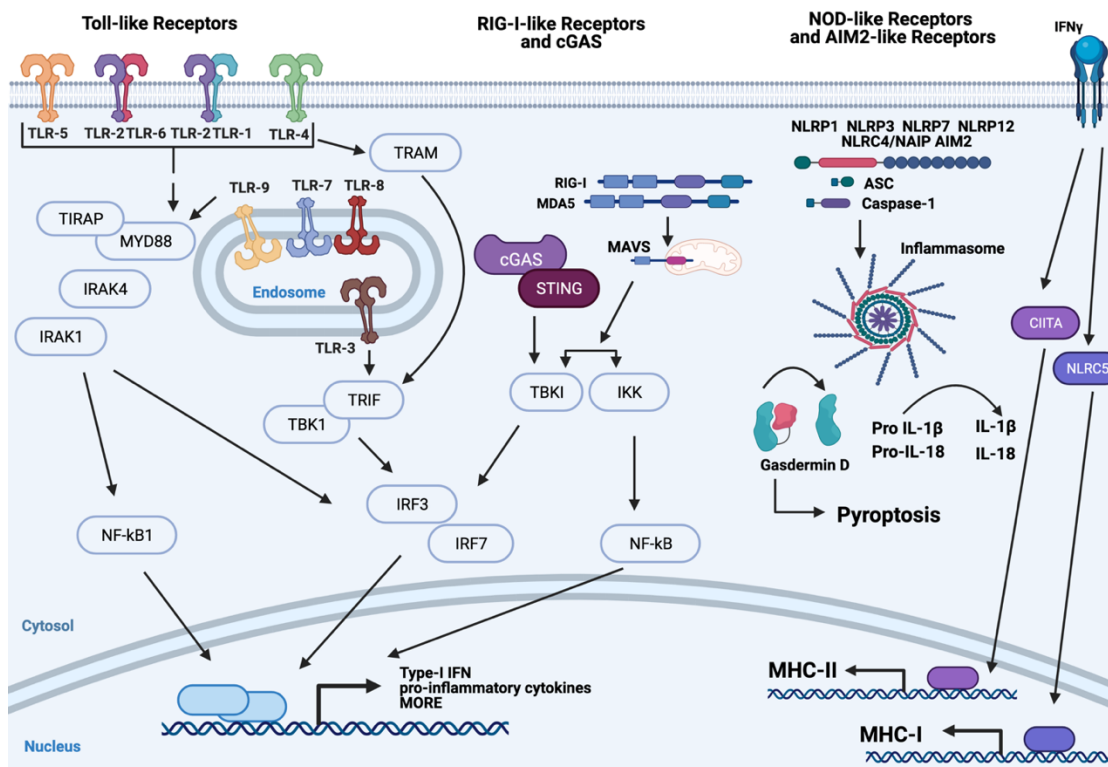


Figure 1-4. Innate receptor signalling in epithelial cells.

Immunological functions occur within epithelial cells independent of the immune system. Activation of intrinsic immunity within epithelial cells induces gene expression of cytokines, chemokines, and other immune modulating proteins. Additionally, innate receptor activation in epithelial cells can induce pyroptotic cell death.

1.4. Ecological and evolutionary principles in cancer immunology

"Nothing in Biology makes sense except in the light of evolution" - Theodosius Dobzhansky 1973

Evolution is the change in a population's heritable traits over time. It is the result of selection for the most fit phenotype within the context of the local environment (114-116). This is a highly dynamic process as the environment is subject to change due to external factors and to local changes caused by the evolving organisms ("niche construction")(117). In cancer, the predator-like activities of the immune system generate strong selection pressures on cancer cells which can, in turn, develop a wide range of adaptive strategies that include both phenotypic changes (e.g., immunoediting) and alteration of the environment to reduce the efficacy of immune cells (118, 119)

1.4.1. Cancer cells are evolving species

Survival, proliferation, and death of normal cells are regulated by internal controls, and signalling received from the tissue. This is necessary to maintain organized, functioning normal tissue that is robust to perturbations, thus optimizing the fitness of the entire organism (120, 121). Tissue structure also prevents the overgrowth of cells within an organ. In an evolutionary framework, this means normal somatic cells do not evolve. They do acquire mutations but, provided the cell's replicative fate remains governed by local tissue control, the mutations do not alter proliferation. In other words, normal cells cannot evolve if mutations and other heritable events do not alter survival or proliferation. Thus, normal cells share a common fitness with the multicellular

organism. In contrast, malignant cells develop a "self-defined" fitness function. Fitness is a measurement of an individual's survival and proliferative potential in relationship to competing individuals. A fitness function is a measure relating the effects of an inherited phenotype on the fitness of the organism and determines the likelihood that the new trait will become fixed within a population (122). Therefore, A "self-defined" fitness function is defined as a fitness function which applies only to the tumour cell and not to the organism as a whole. A tumour cell possess a "self-defined" fitness function when their replicative fate is no longer governed by local tissue controls either because of loss of internal response to these signals or damage to local tissue that prevents generation of these signals (114). As a result of this transformation, cancer cells can evolve because their survival and proliferation are solely governed by interactions of their phenotypic properties with local selection forces (114). Acquiring a "self-defined" fitness function means the fitness of a malignant cell is tied to its own heritable traits.

This distinction is key. Normal cells do not evolve. They do not compete and are not subject to natural selection at the cellular level. (Organism levels of selection are not addressed here but have been covered extensively in the literature (121, 123). Cancer cells, however, similar to interacting species in nature, compete for space and nutrients, they must "forage" for nutrients, evade predation by the immune system, and either avoid or adapt to potentially toxic environmental conditions (81, 124, 125).

The generality of Darwinian principles observed through over a century of theoretical and empirical studies allow their application to oncology to better understand the dynamics of tumour species competing within the temporally and spatially

heterogeneous local tissue environment. Ultimately, these first principles govern the complex dynamics of cancer evolution and ecology leading to improved treatment strategies.

1.4.2. Darwinian dynamics in oncology

An evolutionary view of cancer was first described by Nowell in 1976 (126). Accumulation of mutations and heritable epigenetic changes can increase a tumour cell's fitness resulting in greater proliferative potential and increased survival. Tissue architecture and the surrounding tumour microenvironment exert selection pressures on tumour cells. This includes areas of poor perfusion with low pH and decreased oxygen levels as a result of dysregulated vasculature (127). Additionally, the immune system creates a selection pressure on tumour growth when anti-tumour immune cells participate in immunoediting, which is the selection for immune evasive phenotypes (15, 118, 128). Pro-tumour immune cells increase the fitness of tumour cells able to exploit their wound-healing capabilities (129). Neighbouring normal cells and other tumour cells compete for space and limited nutrients (130). Each of these pressures drives the evolution of the tumour and sculpts the heterogeneous collection of malignant cells (131).

The initial pioneers of cancer as an evolutionary process generally applied a gene-centric view of Darwinian processes described as "mutation-selection." This is correct but somewhat misleading because it implies mutational events are the sole driver of evolution. In particular, this approach ignores the critical role of changes in environmental selection forces in driving evolution. For example, fish stop forming eyes

when living in the constantly dark environment of caves (132). Similarly, the large spatial and temporal variation in environmental conditions in tumours due to chaotic blood flow apply highly different environmental conditions. Thus, while mutations may play a large role in cancer evolution, environmental heterogeneity is equally important in forming the phenotypic and genotypic properties of malignant populations (133).

When cancer treatment is applied, a new selection pressure is created. Sensitive tumour cells die (134). If the population contains any pre-existing resistance or if de novo resistant phenotypes emerge, this new pressure will favour the outgrowth of the resistant phenotype (135). Furthermore, the death of sensitive cells creates a competitive release for resistant clones (130). Before the application of cytotoxic therapy, the sensitive cells dominated the population and suppressed the growth of resistant cells through competition. Once this competition is completely removed, the resistant population is free to proliferate (125, 134).

Fortunately, the evolution of treatment resistance often has evolutionary costs, which can include; diminished proliferation rates increased nutrient needs, and increased predation risk by the immune system (115, 136). Evolution based therapies, including those based on an evolutionary double bind created by costs of treatment resistance, offer potential treatment options in the resistant setting (137)

1.4.3. Darwin and Schreiber: Natural selection by the immune system (immunoediting)

The evolution of cancer cells inevitably requires adaptation to the immune system. Immunoediting selects for alterations in the cancer cell phenotype to avoid lethal

interactions with immune cells. Schreiber described the three e's of cancer immunoediting (15, 118, 128). The initial phase is the elimination phase, in which the anti-tumour immune response is sufficient to eliminate all cancer cells. The second phase is the equilibrium phase. Here the turnover of cancer cells is high. The cytotoxic immune response balances the birth rate of tumour cells, and the tumour does not shrink and does not grow. The last phase, escape, is when tumour cells acquire strong enough evasion mechanisms that they grow faster than the immune system can respond, and the tumour grows (118).

Cancer immunoediting at its foundation is Darwinian selection (81). The immune system is the selection pressure and the tumour cell is the unit of selection. As reviewed previously (114), three conditions must be met for a system to evolve by natural selection: 1) there must be a collection of individuals with phenotypic differences that are heritable, 2) these individuals must occupy a space whereby not all individuals can survive, and 3) the phenotypes in the group must differ enough that the probability of survival differs amongst them. For cancer immunoediting to occur, the tumour must be composed of individual populations of tumour cells with varying immune evasion capacity and fitness. These populations must be competing for space and nutrients and have an immune response capable of inducing selection. Successful immunotherapy will induce selection resulting in the outgrowth of highly immune resistant cancer cells. Clinically this is seen in the emergence of Beta-2-Microglobulin (B2M) mutations, antigen loss, reduced JAK/STAT signalling, and IFN γ insensitivity (9, 138-140).

1.4.4. Evolutionary guided therapies in cancer

Evolution based medicine relies on biological first principles, including gene theory and evolution, to guide treatment regimens (134). In cancer, tumour evolution represents a fundamental barrier to curative treatment strategies. While many highly effective treatments have been developed, the size, phenotypic and environmental heterogeneity, and evolvability of cancer cells nearly always result in the persistence of a surviving, resistant population that eventually expands, resulting in treatment failure and patient death. Currently, cancer drugs are given with little regard to the underlying evolutionary dynamics. For example, cancer treatment drugs are conventionally applied at maximum tolerated doses for extended periods of time with the goal of maximizing cell kill (141). Unfortunately, for the vast majority of cancers, complete eradication of a metastatic cancer population is unlikely (142). Thus, the standard oncologic practice is often evolutionarily unwise because it applies intense selection for resistance while eliminating all competing populations allowing unconstrained proliferation of the resistant population (143). Evolutionary guided therapies utilize currently approved drugs by applying them in a way that considers the underlying evolutionary principles.

Evolutionary principles being utilized include the evolutionary dynamics that result from competition dynamics, population size, and cost of resistance. Competition dynamics occur when two interacting populations are competing for space and nutrients. Interacting species have differential effects on one another; 1) inhibitory, where one population blocks proliferation of another, 2) beneficial, where one population supports the growth of another, or 3) neutral (144). Cancer biology can benefit from

understanding competition dynamics when applying it to two key populations within a tumour, drug sensitive and drug resistant. This is applied in “Adaptive therapy” treatment strategies, discussed in more detail below. Population size is a key indicator of success in ecological systems. When a population falls below a critical threshold the risk of extinction increases. This dynamic is exploited in extinction therapy which looks to eliminate residual disease following a successful first line therapy, discussed in more detail below. Cost of resistance, is a well-studied dynamic in antibiotic resistant bacteria. It refers to the evolutionary consequences tied to acquiring a resistant phenotype. This can be a proliferative cost, where the resistant population grows more slowly than the sensitive, or it can result in collateral sensitivity where resistance to one drug increases the sensitivity to another drug. In cancer drug resistant cancer cells are treated with a second therapy targeted at the resistance mechanisms acquired during first line therapy. We exploit this dynamic in double bind therapies described below. There are currently several evolutionary therapy designs being utilized in clinical trials and tested in pre-clinical models, these include:

- A) adaptive therapy
- B) extinction therapy
- C) double bind therapy

1.4.4.1. Adaptive therapy

Adaptive therapy is a novel treatment strategy that adapts the timing of therapy based on patients' responses with the aim of preventing the emergence of drug resistance (135, 145). It is a personalized dosing strategy that is agnostic to the type of treatment being applied but is dependent on a metric of tumour response that can be measure

often, for example, prostate-specific antigen (PSA) in prostate cancer. Adaptive therapy alters dosing away from extended maximum tolerated dose strategies. Instead, the drug is removed while the tumour is still responding (135, 146). This increases the chances that a proportion of remaining tumour cells will maintain drug sensitivity and be around to compete with the resistant population. Once the tumour begins to regrow, the patient is treated again. This continues until the tumour declines once more, at which time the drug is removed again (135, 146). These shrinking and growing phases create oscillations in tumour size. However, they do not eliminate the tumour. Instead, adaptive therapy prevents progression by shifting the cancer into a chronic but manageable disease. This was largely an academic theory with some pre-clinical success until recently. A current ongoing clinical trial in advanced prostate cancer utilizing adaptive therapy has had early success (135, 146). Two core evolutionary principles must occur for adaptive therapy to be useful: first, resistance must be an inevitable outcome (meaning cure is not possible) and second, there must be competition between the resistant and sensitive populations that favours the outgrowth of the remaining sensitive population over the resistant population. Converting rapidly progressing cancer into a chronic disease has the potential to extend the life of patients who have no current hope for a cure.

1.4.4.2. Extinction therapy

A second evolutionary guided treatment strategy, extinction therapy, applies to patients with a highly effective first-line treatment option. In many cancers, successful first-line treatments will lead to complete remission, but cure is rarely seen because a small clinically-undetectable population survives and may remain dormant for months to

years. When a patient is in remission, clinical practice typically monitors the patient while continuing to apply the same treatment. Thus, treatment is changed only when the tumour progresses, at which point the tumour is usually highly resistant, and the patients are left with few options (147, 148). This strategy may, however, miss an opportunity to completely eradicate any remaining cancer cells that may have survived (149). Based on a common ecological principle coined the 'extinction vortex', a novel evolutionary approach is to treat patients when they have no evidence of disease but in which historical data indicates a chance of recurrence (150) (**Figure 1.5**). Briefly, the extinction vortex is a quantified finding that shows populations undergoing a rapid decline in numbers are highly sensitive to perturbations (150). Meaning, in cancer, there is a period of time following a rapid decline in population size after successful first-line treatment when the tumour cells will be most susceptible to treatment. Indicating the best time to treat patients is during remission instead of waiting for the patient to progress (151). This evolutionary guided treatment design is still in early studies, and the timing for the second strike will likely be highly dependent on disease specifics. Initial trials are being designed in paediatric sarcoma, where the majority of children are cured by first-line therapy, but a small group of patients will recur, and these young patients currently have no option for a cure.

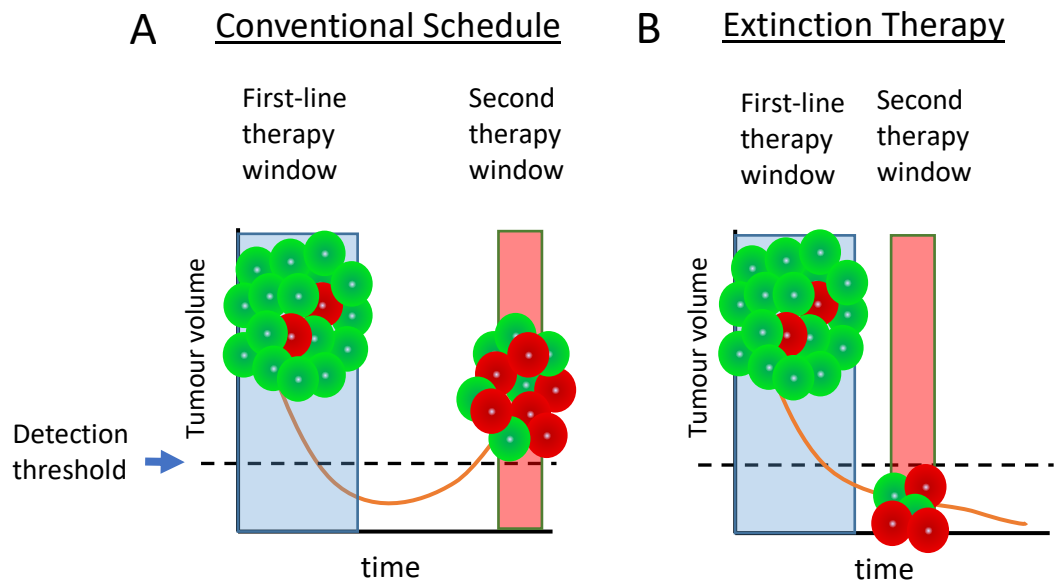


Figure 1-5. Extinction therapy model.

(A) Conventional therapy schedules administer second treatment after the cancer has recurred. (B) The extinction therapy model suggests applying the second treatment while the tumor is below the detection threshold to exploit the sensitive nature of small populations.

1.4.4.3. *Double bind therapy*

The phrase 'double bind' is derived from predator-prey studies in ecology [55]. A double bind occurs when a survival strategy has an unintended consequence that introduces vulnerability to a second perturbation. The classic example is field mice. When an owl is present, field mice hide under a rock or bush to avoid predation. This is successful, however, until snakes are introduced. Since snakes live under rocks and bushes, this survival strategy becomes a liability (**Figure 1.6**). In cancer, many second-line treatments are designed to be the snake [56]. For example, first-generation EGFR targeted therapies often lead to the same gatekeeper mutation, T790M, rendering the drug ineffective but causing the tumour cells to be susceptible to a second therapy targeted at the T790M mutation that was previously not present [57]. Similarly, in hormone receptor-positive breast cancer, resistance to hormonal therapy often occurs through two major pathways, the phosphatidylinositol 3-kinase (PI3K/AKT/mammalian target of rapamycin (mTOR) pathway and the cyclin D1/cyclin-dependent kinase (CDK)4/6 cell cycle pathway. Drugs targeting both of these pro-growth pathways have been developed to exploit this double-bind [58].

While not explicitly described as a double bind therapy, the approval of checkpoint inhibitors in the setting of tumours with high microsatellite instability (MSI) is, at its core, exploiting an evolutionary double bind. MSI is selected in tumours because it grants a fitness advantage, especially during non-immune based therapies, in that high mutation rates increase the chance of developing a resistant phenotype (152). However, while a few of these de novo mutations may grant a fitness advantage in challenging

microenvironments, a large number of mutations creates a wealth of neoantigens with immunogenic potential that is exploited when immunotherapy is given [59].



Evolutionary Double Bind Therapy

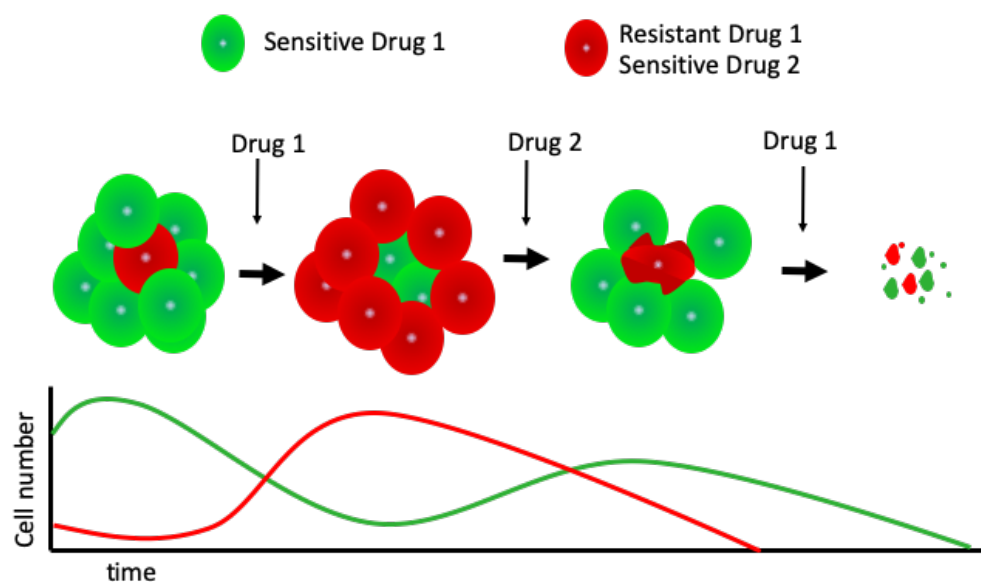


Figure 1-6. Evolutionary double bind model.

Evolutionary double bind occurs when resistance to one therapy increases the sensitivity to a second therapy. The two drugs can be used to reduce tumor burden and prevent the emergence of a fully resistant tumor population.

Evolutionary guided therapies are quite new and will need to be rigorously tested in the clinic. However, early success is promising, and many clinicians are looking for new strategies to optimize cancer treatment. Current trials utilizing evolutionary therapy do not include the use of immunotherapy. This is surprising as immunotherapy is well suited for ecological and evolutionary principled treatment strategies. The utilization of a dynamic and complex system like the immune system to combat an evolving tumour seems intuitive, and cancer immunotherapy typically ignores evolutionary dynamics. **We hypothesize that evolutionary principles can be successfully integrated into cancer immunology research to reveal novel findings with clinical potential.** We used three evolutionary biology principles incorporated into our cancer immunology studies A) gene conservation, B) costs of resistance, and C) an evolutionary double bind to pursue this hypothesis.

In chapter 3, we used normal and tumour tissue from lung cancer patients to determine the conservation levels of immune-related genes. This is based on the evolutionary principle that mutations in genes that are essential for cancer cell fitness will be eliminated from the population so that the *observed* mutation frequency is less than expected (i.e., fewer mutations are observed compared to expressed genes of comparable size). We found several genes involved in antigen presentation and interactions with NK cells to be conserved in our lung cancer clinical cohorts.

In chapter 4, we built on this and examine the same NK cell ligands in a radiation resistance model. Here we hypothesized that radiation resistance would have an evolutionary cost. Based on known biological mechanisms involved in radiation

resistance, we hypothesized that radiation-resistant tumour cells would have differential expression of the stress-induced NK ligands revealed to be essential in chapter 3. We illustrated this in an isogenic cell line model of radiation resistance and show that the cost of resistance is increased predation rates by NK cells.

In chapter 5, we expanded on the finding that radiation resistance increases sensitivity to NK cells to explore the feasibility of an immunological double bind. We applied ecology inspired competition models to test the utility of combining initial radiation therapy (comparable to the owl in our example above) with NK cells that are sensitized to radiation-induced mutations (comparable to the snake) in an evolutionary double bind. We demonstrated that in our *in vitro* system, we can employ NK cells to increase the radiation-sensitive population and restore radiation sensitivity to the tumour as a whole. We showed that this return to sensitivity is the result of two evolutionary costs, increased sensitivity to NK cells and diminished growth rates in the radiation-resistant cells.

Overall, this work demonstrates evolutionary principles, when applied to cancer immunology, can increase understanding of the complex dynamical interactions of the tumour and the immune systems. Collaborations between Trinity's comparative immunology department and Moffitt's evolutionary medicine group have yielded several papers. In addition to the work presented here, we authored a perspective on the effects maximum tolerated dose therapy on anti-tumour immune responses (141, 153), contributed to the immune outcomes of murine evolution of tumour resistance (154), and authored a hypothesis paper on predator-prey dynamics in cancer

immunology (144). These three chapters represent novel theoretically- and empirically-driven collaborations to examine the application of evolutionary principles to cancer immunology.

1.5. Hypothesis

We hypothesize that evolutionary principles can be successfully integrated into cancer immunology research to reveal novel findings with clinical potential. In addition, we believe our interdisciplinary approach will highlight the advantage of using immunotherapy in evolution guided cancer therapies.

1.6. Aims

We have investigated the utility of evolutionary principles in pre-clinical cancer immunology research focusing on the following aims:

- Conservation of immune-related genes in lung cancer tissue
- Hypermutation of immune-related genes in lung cancer tissue
- The expression of immune-modulating proteins in radiotherapy resistance
- The cost of radiotherapy resistance in the context of NK cell sensitivity
- The dynamics of an evolutionary double bind

Conservation and hypermutation analysis of immune related genes was performed to identify essential genes and gene families in clinical tumour samples from lung cancer patients. The genes found through our regression model highlighted important cellular processes under strong evolutionary selection during tumour development and growth. Genes encoding a group of immune modulating proteins (NK cell ligands) was identified in our analysis suggesting a strong survival benefit. Prior studies have found an

association between these proteins and cell survival following radiation. Our aim was to quantify the expression of these proteins and determine the effects of expression on NK cell sensitivity in radiation-resistant tumour cells. From this we determined that resistance to radiation therapy increased sensitivity to NK cell mediated killing and used an *in vitro* cell line model to quantify the dynamics of this evolutionary double bind.

Chapter 2: Materials and Methods

2. Chapter 2. Materials and Methods

2.1. Computational work

2.1.1. Structured literature search

To create gene lists of immune relevant genes with potential functions in epithelial cells an unbiased literature search was performed. The following databases and search engines were searched for relevant studies: MEDLINE, MEDLINEPlus, ScienceDirect, and Google Scholar. Online genetic pathways depositories were used to complete gene lists; KEGG, PathCards, GeneCards, Geneontology, and DAVID.

The searches were not limited by study design or language of publication. Further studies were identified by examining the reference lists of all included articles. Papers were collected covering *in vitro* and *in vivo* murine and human studies. Full gene lists are in the appendix (Appendix 7.1)

2.1.2. Mutation rate model

The mutation rate regression model was built by the bioinformatics core at Moffitt Cancer Center. Somatic mutations for TCGA LUAD samples (n=534 patients) were divided into mEGFR (n=58), mKRAS (n=163), and Wild Type (No identifiable driver gene) (n=313) (data.nci.nih.gov/tcgafiles/ftp_auth/distro_ftpusers/anonymous/tumor/, downloaded March 2016). Coverage files were downloaded from <https://www.synapse.org/#!Synapse:syn1695394> and prepared as described in the Synapse page. Gene-level depth of coverage was calculated by calculating bases covered by sequencing from above files across each of the RefSeq coding genes (with 25 base

pair flanking regions) thereby measuring the fraction of each gene (longest transcript) covered by sequencing data.

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: mc3.v0.2.8.PUBLIC.maf.gz: <https://gdc.cancer.gov/about-data/publications/mc3-2017> and Genome Data Commons: <https://portal.gdc.cancer.gov/repository>

The degree of conservation was calculated based on the mutation rate and size of each gene. This was measured using a robust regression model provided by Moffitt Cancer Center's bioinformatics department. To quantify the mutation rate, we determined the fraction of patients who had a protein altering mutation in a given gene, and then corrected for average depth of sequencing coverage. We then plotted the mutation rate for individual genes vs their size (number of base pairs), and used a robust regression to model the relationship between gene size and mutation rate. Larger genes are more mutated due to the average mutation rate. We then measure the distance of each gene to this model. To quantify the level of hypermutations and conservation for an individual gene we utilize the standard deviation (SD) from the line. Genes with an $SD \geq 1.0$ are hypermutated and genes with $SD \leq -1.0$ are conserved genes.

2.2. Cell lines and primary cells

Isogenic cell lines AMC-22Rv1 and RR-22Rv1 were obtained from Laure Marignol (Trinity St. James's Cancer Institute) and were previously derived from parental line 22Rv1 with either fractionated radiation (30 x 2Gy) or mock irradiation (155). The resulting cell lines,

radiation-resistant (RR-22Rv1) and aged matched control (AMC-22Rv1), have differential sensitivity to X-ray radiation. NKL cells were obtained from internal lab stocks and cultured in RPMI with 10% FBS, 1% pen/strep, and 100 IU recombinant human IL-2. Cell lines were authenticated and mycoplasma tested regularly (Lonza). The isogenic cell lines were used to determine level of protein expression (NKG2D ligands, PVR, and PVL2) and along with the NKL cell line used to determine differential sensitivity to NK cell mediated killing.

All cells were maintained in RPMI (Gibco) with 10% fetal bovine serum (FBS) and 1% pen/step in a humidified incubator with 5% CO₂ at 37°C. Cells were kept in log phase except where specified. All 2D experiments were performed in cell culture treated plates. Hanging drops were used to determine clustering dynamics and GFP and mCherry expression by suspending 100 µl droplets from the cover of sterile 10cm plates. Regular mycoplasma testing was performed (Lonza). Stored cells were frozen down in freezing media (90% FBS with 10%DMSO) in Mr. Frosty freezing containers (Thermo-Fisher) to maintain cooling around -1°C/minute. Routine mycoplasma testing was done for all cultured cells (Lonza).

Stable GFP and mCherry expressing cell lines were created through lentiviral transduction with pLV(Exp)-CAG>3xNLS/EGFP-P2-A-puro and pLV(Exp)-CAG>3xNLS/mCherry-P2-A-puro, respectively. Lentivirus-EGFP and -mCherry were kindly provided by Mursyk Lab (Moffitt Cancer Center). Positively transduced cells were selected with 2 µg/ml puromycin followed by a sterile sort for high GFP and mCherry expression (BD FACS Aria II). Expression was maintained with 1 µg/ml puromycin in all

experiments except where immune cells were needed. GFP expressing AMC-22Rv1 cells and mCherry expressing RR-22Rv1 cells were in the flow based killing assays and incucyte assays.

Healthy donor peripheral blood mononuclear cells were obtained from buffy coats collected and processed by Florida Blood Services (Tampa, FL). Briefly, blood was diluted 1x with PBS and layered on to 15 ml Ficoll-Paque PLUS density gradient media (GE Health care). PBMC layer was recovered following a 30 min spin at 450 g and cells were washed in PBS 3-5 times, counted and plated as needed.

2.3. Flow Cytometry.

2.3.1. Ligand Flow

Following treatment (where applicable) tumor cell lines were stained with Zombie Aqua Fixable viability dye (1:1000 in PBS) and for NK ligand expression in PBS containing 5% FBS and 0.1% sodium azide for 20-30 min at room temperature. Secondary antibody was added for ULBP 1 and 2/5/6 only (R&D Systems). Cells were then washed and fixed in 4% paraformaldehyde. Flow was performed on BD LSR II (Trinity School of Biochemistry and Immunology or Moffitt Cancer Center) and analyzed using FLOWJo software (Treestar). Internal controls were used in all experiments and technical and biological replicates were always performed in the same facility to remove inter-machine variability. Morphology gating, viability gating, and doublet discriminator gates were used prior to calculating mean fluorescence intensity (MFI). Mean fluorescence intensity was determined as MFI stained - MFI unstained. ULBP primary and secondary antibodies were purchased from R&D Systems, all others all others were pre-conjugated

and obtained from Biolegend (Ireland and US). Representative flow diagrams are included in appendix 7.2.

2.3.2. Flow Based cytotoxicity assays

For flow based cytotoxicity assays, tumor cells were tracked with either CellTrace Red, CellTrace violet, or CellTrace blue (Thermofisher) prior to plating. NKL or PBMC were left unstained. NKL cells were added at a 10:1 effector to target ratio with 200 IU IL-2. PBMC were added at a 20:1 PBMC to tumor cell ratio with low dose IL-2 (20ng/ml). At the end of co-culture suspension and adherent cells were collected and stained with Zombie Aqua Fixable viability dye (1:1000 in PBS) or Zombie near IR Fixable viability (Biolegend) (1:1000 in PBS). Data was acquired BD LSR II and analyzed with FlowJo software (Treestar). Percent killing was determined as CellTrace positive and viability dye positive out of total CellTrace positive for each cell line.

2.4. Radiation

In vitro radiation was given in either 2, 6, 10, or 15 Gy doses using XRAD 160 biological irradiator (Precision X-Ray Inc). Prior to RT cells were plated in complete media with 10% FBS and 5% Pen/Strep and left to adhere overnight. Following RT cells were immediately returned to incubator with 5% CO₂ at 37°C.

2.5. Crystal violet assays

Evolved and parental cells were plated at 3,000 cells per well in 96 flat bottom plates. After culturing overnight cells were treated with varying doses of tumour necrosis factor

alpha (TNF-alpha), tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL), Interferon-gamma, and immune conditioned media.

Immune conditioned media was made by treating healthy donor PBMCs with 100ng LPS for 72 hours. Cells were removed by centrifugation and filtration. Conditioned media was frozen down and used as needed. To minimize variability three donors were used. Dilution of conditioned was done with regular cell culture media (described above) to allow for dosing studies.

Crystal violet (CV) assays were used to measure cell mass as an approximation for cell number. Briefly, media was removed from all wells and cells were fixed in 2% glutaraldehyde for 20 min, washed in HBSS three times and stained with crystal violet solution for 1 hour. Plates were then washed with ddH₂O until all CV had been removed and left to dry overnight. Optical density was recorded after dissolving CV in 10% acetic acid. Data was normalized to untreated control wells.

2.6. Competition experiments with Incucyte imager

Cells were counted and mixed at various ratios prior to plating in 96-well flat bottom plates (Corning). Tumour cells were plated at 2,000 cells per well and left to adhere. When NK cells and radiotherapy were given together RT was performed first to avoid RT effects on NK cell function.

Images were taken every 6 hours for a 150 hours on the IncuCyte ZOOM. Cell number was determined by the IncuCyte software. Three technical replicates were used for each run. Normalized cell counts were calculated to allow for growth rate comparison

between the different ratios (time t/time 0). IL18BP and TIGIT blocking antibodies were added at indicated doses prior to treatment with NKL cells.

2.7. Statistical analysis

Prism (version 9, Graphpad software La Jolla California) was used for all statistical analysis. A Student t-Test, One-way ANOVA with Bonferroni correction, and two-way ANOVA was used when the means of more than two groups were compared. All experiments were performed with three technical replicates except for the flow based killing assays. A minimum of 10,000 events were collected for each flow sample. n.s = not significant ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. IC50 calculations were performed with Prism using nonlinear regression (log(inhibitor) vs. response -- Variable slope (four parameters)).

Chapter 3: Conservation of Immune Relevant Genes in the Evolution of Lung Cancer

3. Chapter 3. Conservation of Immune Relevant Genes in the Evolution of Lung Cancer

3.1. Introduction

3.1.1. Immunologic profiles in lung cancer subtypes

Lung cancer is a heterogeneous group of diseases represented by two divergent classes, small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The majority of lung cancers are NSCLC, further divided into squamous cell carcinoma, large-cell carcinoma, and adenocarcinoma, the latter being the largest group (156). Lung adenocarcinoma (LUAD) is driven by diverse risk factors that are modelled in the different clinically targetable driver mutations. Routine mutational analysis is performed to guide treatment decisions. The majority of patients with mutations in epidermal growth factor receptor (mEGFR) are, of East Asian descent, females, and/or never-smokers. In the Caucasian population, mEGFR represents 10-15% of LUAD cases (157). Treatment options include multi-generational tyrosine kinase EGFR inhibitors (Erlotinib, Gefitinib, Afatinib) (158). Kirsten rat sarcoma viral oncogene homolog (KRAS) driven cancers have a new class of small molecule inhibitors targeting allele specific mutations that are effective in smoking related lung cancers (159). EGFR and KRAS mutations are mutually exclusive, with mKRAS representing the largest group. Wild-type tumours have no identifiable driver mutations. Like mKRAS driven cancers, WT tumours are difficult to treat. Surgery and platinum-based chemotherapies are used in early-

stage disease. Advanced or inoperable patients receive chemotherapy. Metastatic NSCLC has a median overall survival of fewer than ten months.

WT and mKRAS lung tumours have a unique immunological history compared to mEGFR tumours. This is primarily due to the increased incidence of smokers in these subtypes (160). Smoking increases the rate of genetic mutations within all bronchial cells; this is magnified in tumour cells with dysregulated DNA repair mechanisms (161). High mutational burdens increase immunogenic proteins within cancer cells resulting in increased adaptive immune responses. Additionally, prolonged smoking exposes lung epithelium to high levels of inflammation. Inflammatory mediators provide pro-growth signals to cancer cells. However, inflammation also drives heightened levels of cytotoxic and anti-tumour immune responses (162). In murine models of mKRAS and EGFR driven lung cancer, tumour tissue has more tumour infiltrating CD45⁺ cells than normal lung tissue. However, EGFR driven tumours were predominately infiltrated by myeloid cells and had poor CD8⁺ T cell responses (160, 163). KRAS driven tumours had more CD8⁺ T cells, regulatory T cells (Tregs), IL-17 producing lymphocytes, and inflammatory cytokines (160, 163). Patients with double mutations in p53 and KRAS had higher levels of PD-L1 expression on tumour cells, and preclinical models showed diminished NKG2D expression by NK cells that resulted in impaired NK cell function systemically. Generally, mEGFR tumours are immunologically 'cold' and have few mutations. WT and mKRAS tumours are immunologically 'hot' with high mutation burdens and better responses to checkpoint inhibitors.

An exception exists in patients with both mKRAS and loss-of-function mutations in LKB1 (Serine/Threonine Kinase 11, STK11). STK11 is a cytosolic protein kinase with multiple functions in DNA repair, apoptosis, and innate immune receptor signalling (164). These tumours are generally immunologically cold, have a worse prognosis, and respond poorly to current checkpoint inhibition.

3.1.2. Conservation of essential genes

Cancer is often described as a disease of the genes. Oncogenic mutations give rise to cancer hallmarks (161). Genetic and epigenetic changes in malignant cells alter gene expression (165). Furthermore, diminished DNA checkpoints and abundant proliferation lead to increased genetic instability and accumulation of somatic mutations within cancer cells (161). Molecular profiling of cancer has revolutionized how cancer is treated. Actionable mutations (e.g. mutations in epidermal growth factor receptor) can be inhibited with targeted therapy, halting tumour growth (166). Molecular sub-typing is utilized to help predict response rates and treatment outcomes. Genomic sequencing, combined with HLA subtyping and computational modelling, has identified tumour antigens with immunogenic potential (167).

Additionally, molecular biology approaches are used to identify essential genes. Essential genes are genes that are required for cell survival and proliferation. They are attractive targets in cancer because inhibition or knockdown of essential genes blocks tumour growth. Experimental approaches using CRISPER-Cas9 libraries and RNA interference using short hairpin RNA screens have been used to measure identify essential genes *in vitro* (168, 169). Additionally, a study performed on clinical specimens

by Pertesi et al. applied computational approaches utilizing the effects of essential genes on limiting homozygous deletions in target genes (170). There are several limitations to these methods including the exclusion of conditionally essential genes, meaning genes which are essential only in the condition of interest, e.g. the patient. New methods are needed to identify essential genes specific to tumour cell fitness within the host.

Here, we applied a strategy used in population ecology to measure gene conservation in lung tumours. In population ecology, essential genes are identified when they are evolutionarily conserved. The knock-out rate hypotheses states that evolutionarily conserved genes should be more essential because any loss-of-function mutation would greatly diminish the organism's fitness (171). This would slow down the rate of evolution for that particular gene leaving it intact for longer than other, less essential genes (172). Tumours are a heterogeneous mix of cancer cells with varying genotypes and phenotypes. Populations of cancer cells are subject to natural selection. Mutations that increase fitness will be selected for (positive selection). Mutations that decrease fitness will be selected against (negative selection). Therefore, essential genes are conserved (172). The evolutionary cost of mutations in these genes would strongly diminish fitness resulting in clonal deletion. In the view of cancer immunology, an essential gene would be one that decreases the probability of being eliminated by the anti-tumour immune response or increases the cell's ability to exploit the pro-tumour inflammatory response.

We used gene sequencing data from The Cancer Genome Atlas (TCGA) database to determine gene conservation levels in lung tumours. Somatic mutations were derived

through comparisons with matching normal tissue. Lung adenocarcinoma (LUAD) samples (n=534 patients) were divided into mEGFR (n=58), mKRAS (n=163), and Wild Type (no identifiable driver gene) (n=313). We assumed the probability of mutation was approximately equal for each base pair in expressed genes. Therefore, the degree of conservation was calculated based on the mutation rate and size of each gene. In prior studies, background mutation rates have been determined based on additional assumptions including gene expression and replication timing (173). While we do not disagree with this premise we did not want to overcorrect based on these features as supporting data was generated in cell lines and may not be reliable. The largest risk for false positives is under-counting mutations. We identified sources of undercounting, including gene annotation errors and poor sequencing coverage. We have adjusted for these potential sources of error. To find the mutation rate, we determined the fraction of patients with a protein altering mutation in a given gene, and then corrected for average depth of sequencing coverage. We then plotted the mutation rate of genes against their size, and used a robust regression to model the relationship between gene size and mutation rate. Larger genes are more mutated due to the average mutation rate. We then measured the distance of each gene to this model. Genes that lie above the line have a higher number of mutations than expected (hypermuted) and genes that fall below the line have fewer mutations than would be expected based on gene size alone (conserved) (**Figure 3.1A-B**). To quantify the level of hypermutations and conservation for an individual gene we utilized the standard deviation (SD) from the line. Genes with an $SD \geq 1.0$ are hypermutated and genes with and $SD \leq -1.0$ are conserved genes (**Figure 3.1C**).

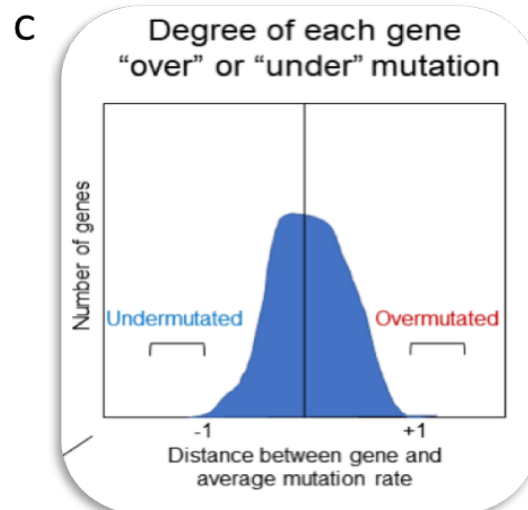
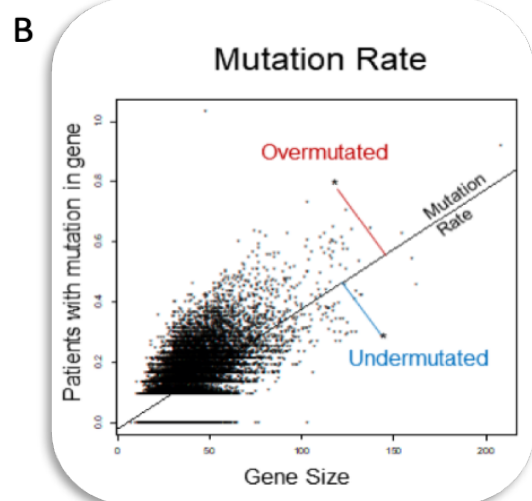
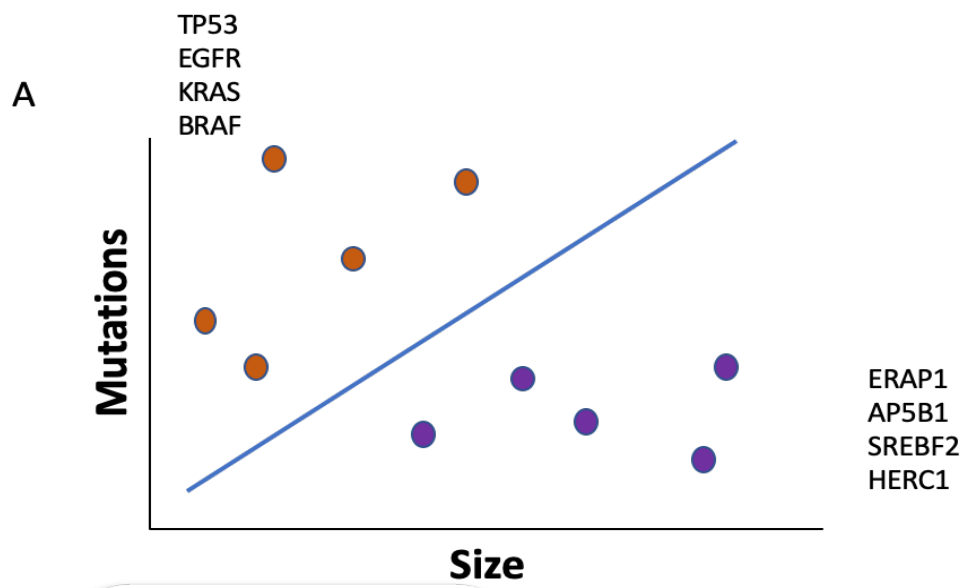


Figure 3-1. Methodology applied to the TCGA data set to identify genes that are mutated more or less frequently than expected by chance alone.

Mutation rate was calculated based on the fraction of patients with a protein altering mutation in a given gene against the size of the gene. Distance from the line for each gene was used to quantify conserved and hypermutated genes. Genes on the regression line have background levels of mutations. (A). Graphical depiction of the model. Hypermutated (brown). Conserved (purple). (B). The robust regression model used to identify the relationship between gene size and mutation rate. (C) Distance from the regression model for each gene. Genes with an $SD \geq 1.0$ are hypermutated and genes with $SD \leq -1.0$ are conserved genes (under mutated). All others have an expected number of mutations (Background mutations).

Malignant epithelial cells evolve. The immune system is a strong selection pressure on a growing tumour. Conservation levels of genes expressed in epithelial cells regulating the immune response provide novel insights into molecular processes and phenotypic properties essential for optimal fitness. Malignant epithelial cells exploit their role as mediators of the immune responses. **We hypothesized that analysis of gene conservation levels in immune-related genes within tumour samples will reveal novel genes that are likely essential to tumour survival in an immune-competent host.**

We compiled genes lists with known or expected immune functions. We divided them into eight categories based on different immune processes (listed below). Evolution occurs in tumour cells. Therefore, we included immunological processes performed by epithelial cells that affect the immune response or involve the intrinsic immune pathways within epithelial cells. Of those conserved, we found a potentially universally essential pair of genes, ERAP1/2. Multiple MHC-II genes were conserved in the WT cohort, and the primary transcriptional coactivator of MHC-II genes, CIITA, was conserved in the mKRAS cohort. Analysis of immune-modulating proteins revealed conservation of poliovirus receptor genes and ligands for NK cell receptor NKG2D. We also defined inflammatory processes conserved within the different subtypes, TLR3

- | | |
|----------------------------------|-------------------------------|
| 1) Complement | 5) IFN signalling |
| 2) Apoptosis/necrosis/pyroptosis | 6) Innate immune receptors |
| 3) Chemokines | 7) Antigen presentation |
| 4) Cytokines | 8) Immune modulating proteins |

pathway in wild type cancers and Type I and Type II IFN signalling in mutant KRAS tumours.

Identification of conserved genes provides novel insights into molecular processes and phenotypic properties that are essential for optimal fitness. Prior investigations have found relatively few genes are under negative selection (114, 174, 175). However, previous mathematical models and recent empirical studies have demonstrated conserved genes are strongly dependent on the tissue of origin and molecular events during early carcinogenesis (176, 177).

Genes are considered hypermutated if they have more mutations than expected based on gene size alone and have a standard deviation greater than or equal to 1.0. Non-synonymous genetic mutations can have differential effects on protein function. Loss-of-function mutations render the protein less effective or completely inactive. Gain-of-function mutations can increase the activity of the protein or grant a new function. Our analysis does not allow us to differentiate between gain-of or loss-of-function in our gene sets. However, some interesting trends should be noted as the accumulation of mutations within a particular gene family of cellular processes indicates an evolutionary investment and can highlight important cellular functions worth further investigation

3.2. Results

3.2.1. General findings and expected mutations

Of all the genes recorded in our study ($n = 17,615$) more genes were conserved (standard deviation (SD) ≤ -1.0) in mKRAS tumours compared to WT (3,011 and 2,587 respectively) (**Table 3.1**). mKRAS and WT tumours had a similar number of hypermutated genes SD ≥ 1.0 (2,581 and 2,398, respectively) (**Table 3.1**). Frequently mutated genes, defined as genes mutated in greater than 10% of patients in each cohort, were observed less in the mEGFR group, 14 genes (data not shown), compared to mKRAS and WT groups, 157 and 208 genes respectively (**Table 3.1**). Due to the limited amount of mutations and the smaller sample size in the EGFR cohort ($n = 58$), reliable gene conservation scores were not quantifiable. Therefore, we focused on WT and mKRAS cohorts.

TP53, which encodes the p53 protein, is a tumour suppressor found to be mutated in over 50% of human cancers (178). TP53 mutations were found in 64% of mEGFR patients, 56.5% of WT patients, and 35% of mKRAS patients. Mutations in liver kinase B1 (LKB1) gene, which encodes STK11 protein, was the 3rd most mutated gene in the mKRAS cohort following KRAS and TP53 (19.6%). Only 1 of the 58 mEGFR patients (1.8%) had a mutation in STK11 and 8.6% of WT patients. STK11 regulates metabolism, energy sensing and modulates STING expression (164). Mutations have been found in immunologically cold tumours and are associated with poor outcomes following chemotherapy and PD1/PD-L1 inhibition (179). Patients in the WT and mKRAS cohorts had 2 to 3-fold more mutations than mEGFR patients. (data not shown). Tumour mutation burden is usually lower in patients with EGFR driven lung cancer (180). This is

likely due to smoking history as EGFR wild-type cancers are associated with prior smoking history and the majority of EGFR mutated tumours occur in never smokers (181).

Table 3-1 The number of total genes conserved or hypermutated varied between mutant KRAS and wild type lung tumours.

Mutation rates of 17,615 genes were calculated in mutant KRAS (n= 163 patients) and wild-type (n=313 patients) lung tumours. Genes with an SD ≥ 1.0 are hypermutated and genes with an SD ≤ -1.0 are conserved genes (under mutated). All others have an expected number of mutations (Background mutations). Standard deviation (SD)

	Mutant KRAS	Wild Type
Conserved genes (SD ≤ -1.0)	3,011	2,587
Highly conserved genes (SD ≤ -2.0)	160	248
Hypermutated genes (SD ≥ 1.0)	2,581	2,398
Highly hypermutated genes (SD ≥ 2.0)	518	593
Frequently mutated genes ($\geq 10\%$ of patients)	157	208
Genes mutated in 0 patients	5,621	2,323

3.2.2. Gene list compilation and conservation among immune processes

We used a combination of literature searches and online gene databases to create custom genes lists. We compiled eight genes lists containing 573 genes with known or suspected functions within epithelial cells relating to the immune response. Gene lists were divided into eight categories related to their immunological function within epithelial cells. Each category has a different number of genes, noted in parenthesis (**Figure 3.2A-B**). Cytokine signalling was the largest gene list, 181 genes. We have included a complete gene list in the appendix (Appendix 7.1).

To distinguish evolutionarily important processes, we examined the frequency of conserved and hypermutated genes within each category. We compared them to the overall percentage of conserved and hypermutated genes for all 17,615 genes examined. We found that 15% and 17% of all genes were conserved in WT and mKRAS (respectively), and 14% and 15% of all genes were hypermutated in WT and mKRAS (respectively) (**Figure 3.2A-B**). Due to the limited amount of mutations and the smaller sample size in the EGFR cohort, reliable gene conservation scores were not quantifiable. Therefore, we focused on WT and mKRAS cohorts.

The antigen presentation group had the highest frequency of gene conservation in both wild type (37%) and mKRAS (23%) (**Figure 3.2A**). Chemokine signalling, complement, and immune-modulating proteins were conserved at a lower frequency than background levels in WT and mKRAS (**Figure 3.2A**). Three immune processes had higher levels of hypermutated genes than background. More than expected complement genes were hypermutated in both subtypes (WT 37%, mKRAS 38%). Innate immune

receptors and antigen presentation pathways had a higher than expected frequency of hypermutated genes in both cohorts (**Figure 3.2B**).

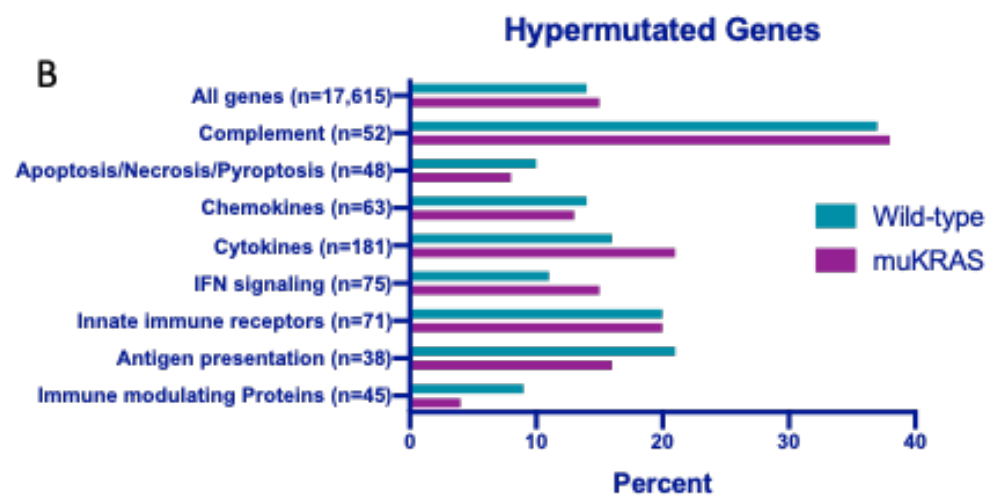
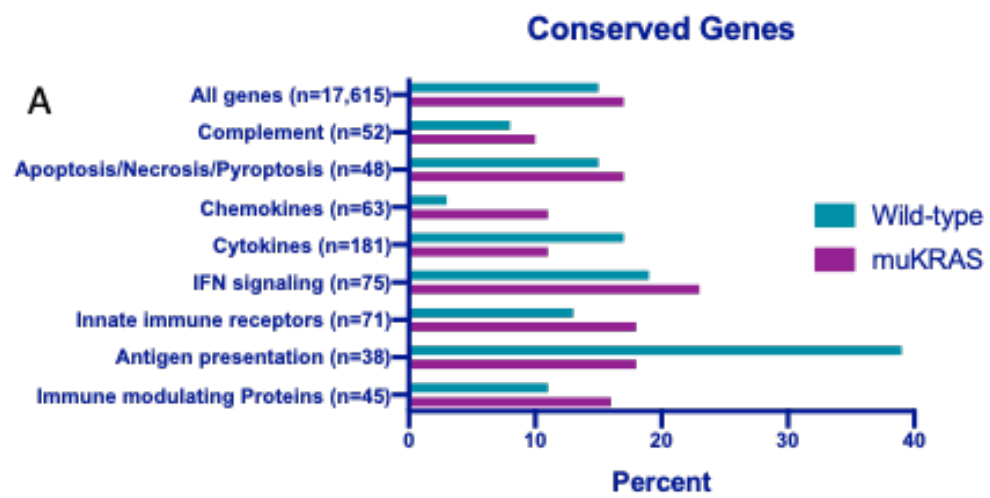


Figure 3-2 The frequency of gene conservation and hypermutation in various immune related functions.

Percent of genes from each gene list that were either conserved (A) or hypermutated (B). Percentage of conservation and hypermutation of all genes was used to establish a baseline. (A) Wild-type and mutant KRAS samples displayed a high level of conserved genes in both antigen presentation and interferon signaling pathways and low levels of gene conservation in complement pathways, chemokine genes, and immune modulating proteins. B) A large percentage of complement genes are hypermutated in both cohorts.

3.2.3. Antigen presentation: Evaluation of antigen-presenting genes highlights the importance of ERAP1/2 and MHC-II genes in tumour evolution

A high frequency of gene conservation was found among the antigen presentation genes compared to all other immune processes we examined in both subtypes (**Figure 3.2A**). WT cancers conserved 39% of genes involved in antigen presentation (15 out of 38). A higher than expected frequency of hypermutated genes were found in both subtypes, suggesting a strong evolutionary investment in antigen presentation (**Figure 3.2B**). Immune evasion by viruses often involves disruption of peptide processing and presentation in both MHC-I and MHC-II pathways and lipid antigen presentation via CD1 proteins (182, 183). In line with previous studies, we found that B2M was hypermutated in WT and mKRAS lung tumours (**Figure 3.3A-B**). We were interested in conserved genes. Therefore, we made Venn diagrams of genes conserved in WT lung cancers (blue), mutant KRAS lung cancers (purple), and both (overlap)(**Figure 3.3C**).

3.2.3.1. *Antigen Presentation: ERAP1 and ERAP2*

Endoplasmic reticulum aminopeptidases, ERAP1 and ERAP2, are polymorphic genes involved in infectious diseases, cancer, and autoimmunity (79). ERAP1 was conserved in WT tumours, and ERAP2 was conserved in mKRAS (**Figure 3.3C**). ERAP1 was found to be one of the most conserved genes with an $SD = -2.8$ in WT cancers, and ERAP2 had an $SD = -2.1$ in mKRAS (**Figure 3.3A-B**). Furthermore, there were no ERAP1 mutations in any of the mEGFR patients, and the same was true in another data set evaluating gene conservation in different melanoma subtypes (data not shown).

3.2.3.2. *Antigen Presentation: MHC-II*

77% of the thirteen MHC-II genes evaluated were either conserved (8/13, 62%) or hypermutated (2/13, 15%) in WT tumours (**Figure 3.3A**). None were conserved or hypermutated in mKRAS cancers. At least one gene from each of the three class II isotypes, HLA-DR -DP -DQ, was conserved in WT samples(**Figure 3.3A**). Both HLA-DMA and HLA-DMB, which are responsible for peptide loading onto MHC-II molecules, were conserved along with HLA-DOB, which regulates these interactions. CIITA was highly conserved in mKRAS tumours (**Figure 3.3B**). CIITA is a gene regulator of HLA-DR and -DM.

3.2.3.3. *Antigen Presentation: MHC-I*

mKRAS conserved genes for HLA-A and HLA-F. HLA-A is the primary heavy chain of classical MHC-I molecules that activates CD8 T cells expressing cognate TCR and inhibits NK cells. HLA-F was conserved in both subtypes (**Figure 3.3C**). HLA-E, conserved in WT tumours, is a non-classical HLA molecule with limited polymorphism that presents a set of self-peptides mainly derived from classic HLA molecules but can also include HSP60 peptides and ATP-binding cassette transporter multidrug resistance-associated protein 7 (MRP7) (84).

MEX3C, conserved in mKRAS and WT, is a post-translational regulator of HLA-A (negative) and the pattern recognition receptor retinoic acid-inducible gene I (RIG-I) (**Figure 3.3A-B**). MEX3C is also associated with lipid metabolism, cell adhesion, invasion, and migration (184). Mutant KRAS tumours, but not WT tumours conserved TAPBP, and NPC1. NPC1 is a cholesterol transporter involved in loading CD1d antigens. TAP Binding

Protein (TAPBP) is involved in peptide loading onto MHC-I. WT lung tumours also conserved PSMB8 and 9 (LMP7, LMP2 respectively), key components of the immunoproteasome.

3.2.3.4. *Antigen Presentation: Hypermutations in CD1 gene family*

All five CD1 genes were hypermutated in the WT cohort (**Figure 3.3A**). Four of the five CD1 isoforms were hypermutated in the mKRAS cohort (**Figure 3.3B**). CD1C had only background levels of mutations (SD = 0.2). CD1A-D present endogenous and exogenous lipid antigens on the cell surface (**Figure 1.2**). Similar to MHC-I molecules, CD1A-D molecules associate with β_2m , which was hypermutated in WT and mKRAS (SD = 1.7, SD = 1.7, respectively) (**Figure 3.3A-B**) (87).

CD1E, the only CD1 protein that does not present antigens, is the most mutated S.D. = 2.5 and SD = 2.3, WT and mKRAS, respectively (**Figure 3.3A-B**). CD1e functions in the endosome shaping the lipid antigen pool similar to ERAP1/2 in the ER. CD1d is expressed in breast cancer and by lung cancer cells following epigenetic modifications (75, 185) Expression of CD1d by tumour cells can elicit an immune response (185, 186). Natural Killer T cells (NKT) are cytotoxic cells with both NK and T cell-like functions. They are CD1d restricted and respond to lipid peptide antigens (186).

A) Antigen Processing & Presenting - Wild Type B) Antigen Processing & Presenting - mKRAS

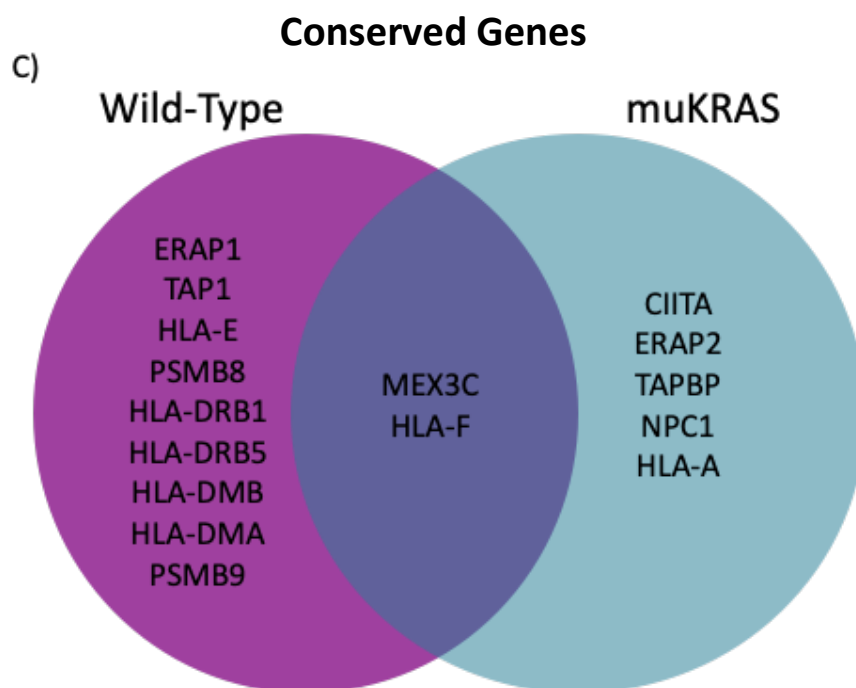
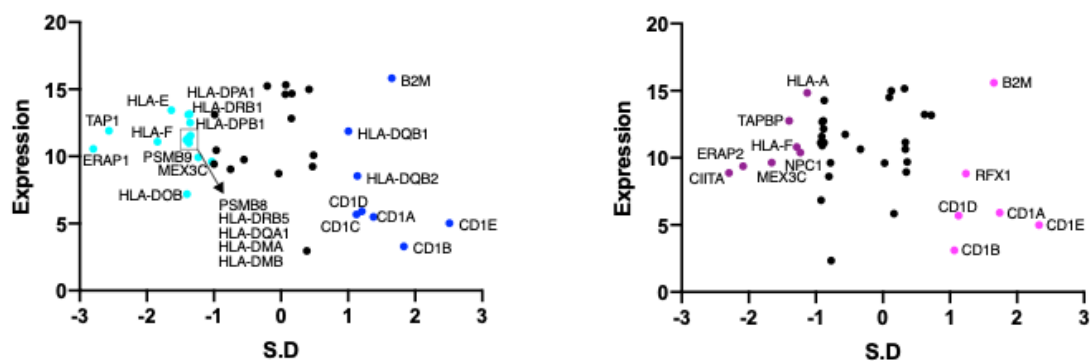


Figure 3-3 Figure 3.3. Gene conservation in antigen presentation genes.

Gene conservation score, measure as the standard deviation from the regression line (S.D) graphed against genes expression levels (Log2) for WT (A) and mKRAS (B). Conserved genes ($SD \leq -1.0$) Hypermutated genes ($SD \geq 1.0$) are coloured and labelled with text. (C) Venn diagram of genes conserved in WT and mKRAS. Standard deviation (SD)

3.2.4. Immune Modulating Proteins: Conservation patterns in genes encoding immune-modulating proteins highlight the importance of NK cells in lung cancer

Tumour cells express proteins that can activate or inactivate (checkpoint proteins) immune cells within the TME. A low percentage of genes encoding immune-modulating proteins were conserved (WT = 11%, mKRAS = 16%) or hypermutated (WT = 9%, mKRAS = 4%) in both cohorts (**Figure 3.2A-B**). Wild-type and mKRAS tumours conserved genes encoding MICA, PVR (CD155), and PVRL2 (CD112) (**Figure 3.4C**). MICA is expressed by stressed or malignant cells and is an NKG2D ligand. NKG2D is expressed on NK cells, NKT cells, gamma-delta T cells, and CD8 T cells (187). Ligation of NKG2D activates NK cells and gamma-delta-T cells and co-stimulates CD8 T cells (98). PVR and PVRL2 induce activation or inhibition of NK cells and T cells depending on the receptors expressed by the immune cells.

Wild-type tumours also conserved genes encoding other NKG2D ligands, UL16 binding protein 2 (ULBP2) and RAET1G (ULBP5) (**Figure 3.4A, C**). NKG2D ligands activate effector cells. Expression is modulated by activation of the DNA repair pathway (via ATM and ATR) triggered by either cell proliferation, DNA damaging agents, cell stress, malignant transformation, or viral infections. HLA-A is conserved in mRAS cancers. Downregulation of HLA-A causes poor recognition by T cells due to lack of antigen presentation. However, lack of HLA-A, a negative regulator of NK cells, induces killing of target cells by NK cells.

NT5E, the gene that encodes ecto-5'-nucleotidase (CD73) responsible for the hydrolysis of ATP and ADP (danger signals) into adenosine, an immunosuppressive molecule, was

also conserved in mKRAS (**Figure 3.4B-C**). The adenosine pathway has been implicated in lung cancer. Similarly, IDO1, also conserved in mKRAS cancers, is a key enzyme in the tryptophan degradation pathway. The immune inhibitory effects of the IDO pathway are caused by the depletion of the essential amino acid, tryptophan, and accumulation of kynurenine, an end product of the tryptophan pathway that inhibits T cell function (188).

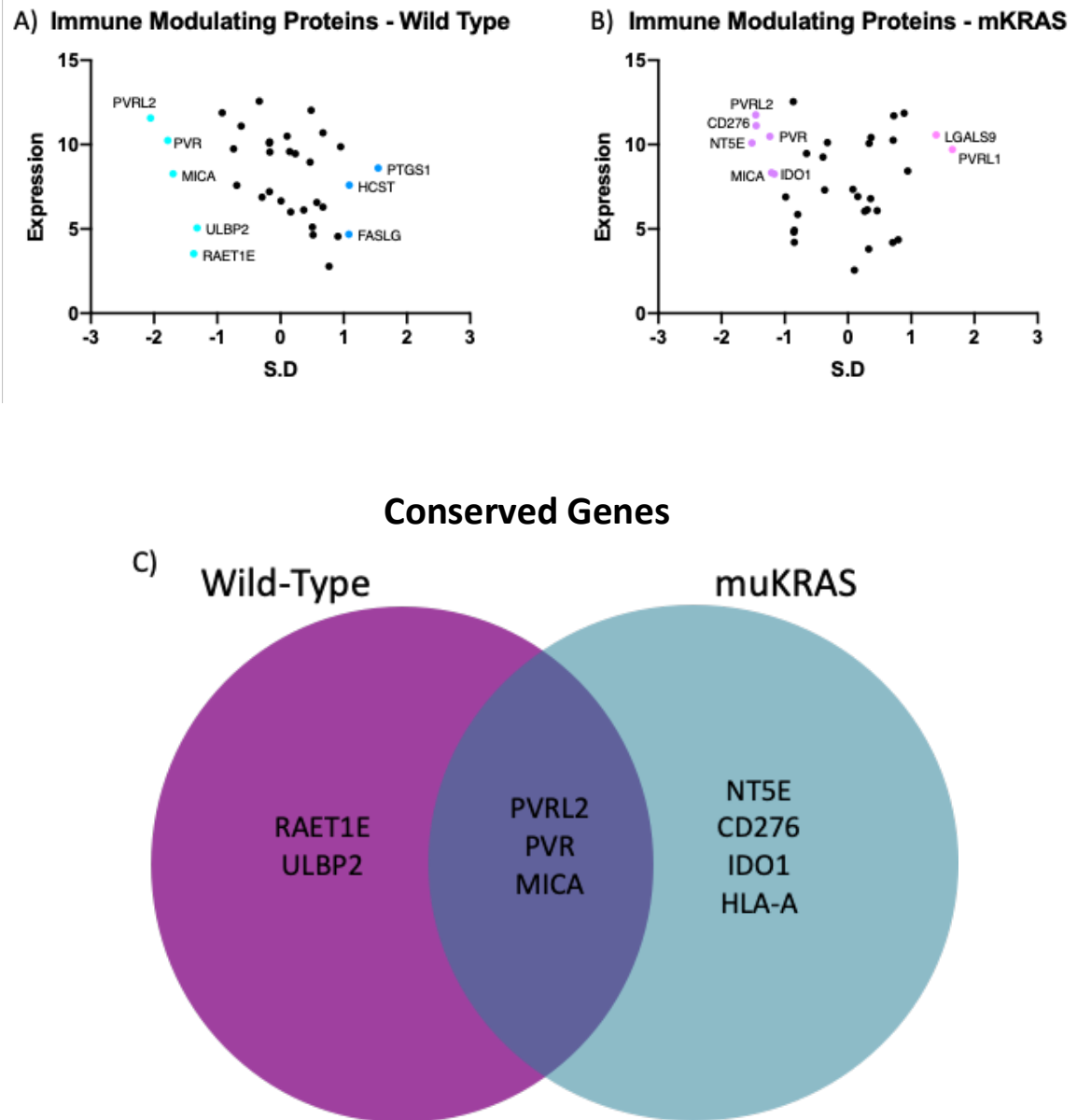


Figure 3-4 Gene conservation in genes encoding immune modulating proteins highlights the important role of NK cells in lung cancer.

Gene conservation score, measure as the standard deviation from the regression line (S.D) graphed against genes expression levels (Log2) for WT (A) and mKRAS (B). Conserved genes ($SD \leq -1.0$) Hypermutated genes ($SD \geq 1.0$) are coloured and labelled with text. (C) Venn diagram of genes conserved in WT and mKRAS. Standard deviation (SD)

3.2.5. Innate immune receptors: Toll-like receptor 3 signalling pathway is conserved in wild-type lung cancers

After examining gene conservation in antigen presentation genes and immune-modulating proteins, we evaluated conservation levels of innate immune receptor signalling genes (**Figure 1.4 and listed in the appendix**). TLR3 pathway genes were conserved in the wild-type cohort. Pattern recognition receptors (PRR) are germline-encoded detectors of PAMPS and DAMPS from diverse pathogen and host stress responses (189). Surface, endosomal, and cytosolic localization of various PRR allows cells to react to stress quickly. TLR3 was conserved in WT lung tumours (SD = -1.3). TLR3 is an endosomal receptor for dsDNA and dsRNA, which, upon ligation, associates with adaptor protein TRIF (TICAM1) and coactivator TBK1. TBK1 phosphorylates IRF-3, all of which were conserved in the WT lung cohort (TRIF SD = -1.4, TBK1 SD = -1.0, IRF-3 SD = -1.9) (**Figure 3.5A,C**) (189-191). Activated IRF-3, conserved in both WT and mKRAS, has a role in signalling induced by activation of RIG-I, cGAS, IFI16 (DAI), and TLR3 and 4. IRF-3 induces expression of type I interferons, TNF, IL6, CCL2 and CXCL1 genes (192). The role of TLR4 in cancer has been well characterized. TLR4 was hypermutated in all lung cancer subtypes (WT SD = 3.6, mKRAS SD = 3.3) (**Figure 3.5A-B**). TLR5 is a surface PRR, conserved in WT lung tumours (SD = -1.2), that recognizes flagellin from beta and gamma-proteobacteria triggering MyD88 activation and proinflammatory pathways (**Figure 3.5A,C**) (193).

3.2.6. Innate Immune Receptors: NLR Family Pyrin Domain Containing gene family is hypermutated in both cohorts

There was substantial variation in conservation and hyper mutational status between the different subfamilies of NOD-like receptor proteins. As discussed above, CIITA, the lone member of the NLRA family, was highly conserved in mKRAS (SD = -2.3). NAIP and NLRX, the only members of their respective subfamilies, were conserved in the WT cohort (S.D = -1.6, SD = -1.0, respectively). Of the five members of the NLRC family, only NLRC3 was conserved in the mKRAS cohort (SD = -2.2), and none were hypermutated. Interestingly, 11 out of the 14 NLRP genes were hypermutated in at least one cohort **(Figure 3.5 A-B)**. NLRP1, NLRP6, and NLRP9 were the only NLRP genes not hypermutated in either cohort. NLRP3 was the most frequently hypermutated gene in the NLRP gene family in both cohorts (S.D. = 3.2 and 3.5 in WT and mKRAS, respectively). The only innate immune receptor with comparative hypermutation scores and expression values was TLR4 **(Figure 3.5 A-B)**.

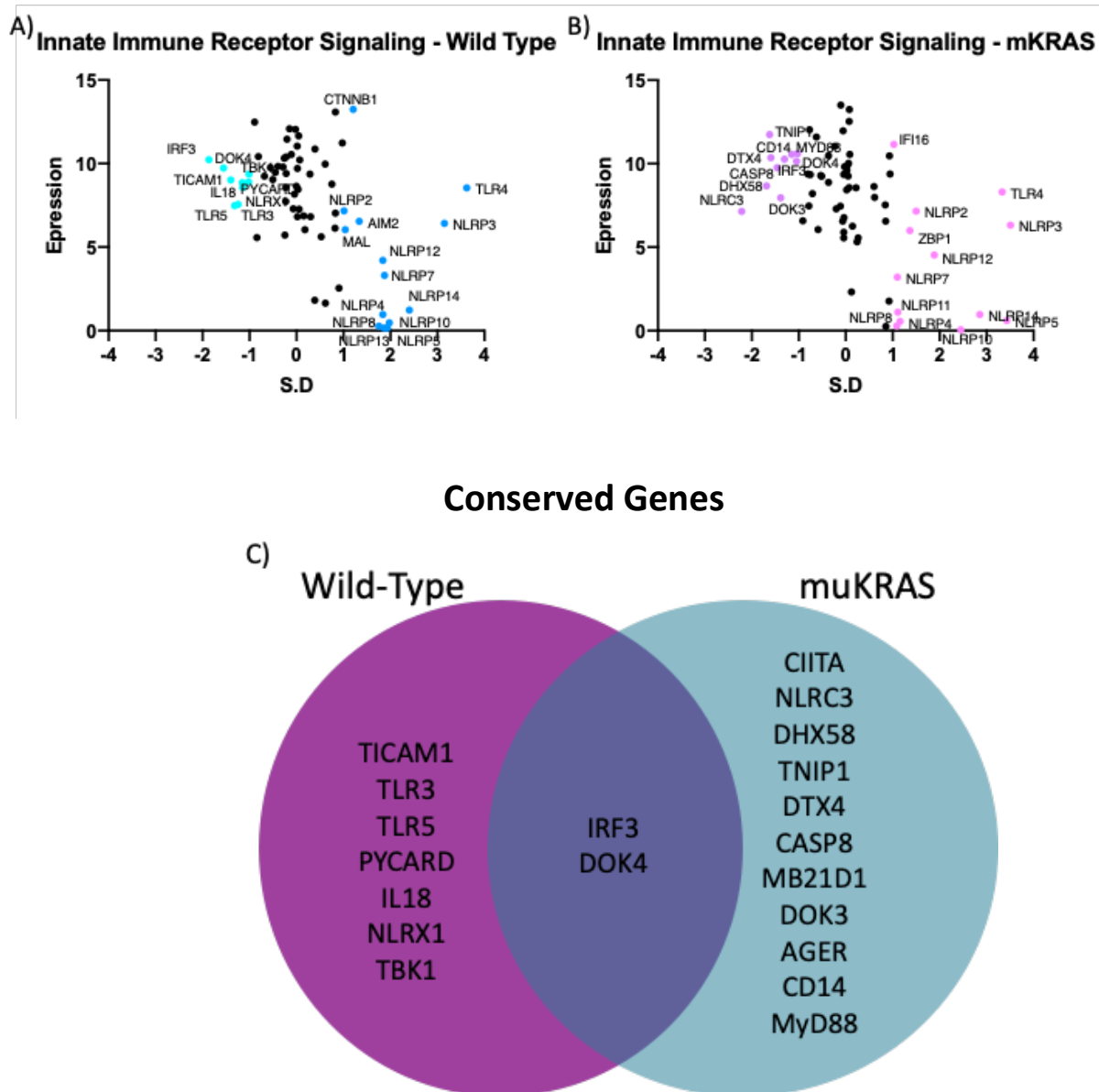


Figure 3-5 Conservation levels of innate immune receptor pathway genes in lung cancer.

Gene conservation score, measure as the standard deviation from the regression line (S.D) graphed against genes expression levels (Log2) for WT (A) and mKRAS (B). Conserved genes ($SD \leq -1.0$) Hypermuted genes ($SD \geq 1.0$) are coloured and labelled with text. (C) Venn diagram of genes conserved in WT and mKRAS. Standard deviation (SD)

3.2.7. IFN Signalling: Type I and Type II IFN receptors are conserved in mKRAS tumours

Both subunits of the type I IFN receptor, interferon-alpha receptor 1 (IFNAR1) and IFNAR2, were conserved in mKRAS samples (SD = -1.5, SD = -1.4, respectively) (**Figure 3.6B-C**). Stimulation of IFNAR1/2 receptor activates Janus kinase 1 (JAK1), not conserved or hypermutated in mKRAS and tyrosine kinase 2 (TYK2), conserved in mKRAS (SD = -1.0). This induces phosphorylation of signal transducer and activator of transcription 1 (STAT1) and STAT2 (neither are conserved in mKRAS), which interact with IRF9 (conserved in mKRAS, SD = -1.2), leading to expression of inflammatory genes. Dysregulation of type I signalling inhibits the expression of IFN-stimulated genes.

Type II interferon receptors were also conserved in mKRAS, interferon-gamma receptor 1 (IFNGR1) and IFNGR2 (SD = -1.4, SD = -1.0, respectively). IFN-gamma (IFN- γ) is a pleiotropic cytokine with pro- and anti-tumour effects. IFN- γ directly kills tumours cells and activates anti-tumour immune cells, including NK cells and CD8 T cells. IFN- γ signalling in epithelial cells induces the expression of multiple genes containing γ -interferon activated sequences. MHC-I, checkpoint inhibitor PD-L1 and immunosuppressive enzyme, IDO are upregulated on cancer cells by IFN- γ treatment (27)}

Four IFN signalling genes were conserved in both WT and mKRAS cancers, Sirtuin 1 (SIRT1), Protein Inhibitor Of Activated STAT 1 (PIAS1), Interferon Regulatory Factor 2 Binding Protein 1 (IRF2BP1) and Interferon regulatory factor 3 (IRF3) (**Figure 3.6C**). The Sirtuin family of proteins play a diverse role in lung cancer and immune cells (194). SIRT1, conserved in both WT and mKRAS (SD = -1.3, SD = -1.8, respectively) inhibits

inflammatory signalling via inhibition of NK-kB. Deletion of both SIRT2, conserved in mKRAS (SD = -1.2) and SIRT3, conserved in WT (SD = -1.7), inhibited inflammatory responses in immune cells. SIRT5 is conserved in mKRAS (SD = -1.0), has a diverse role in cellular metabolism and has been shown to inhibit RIG-I like receptor signalling through MAVS inhibition.

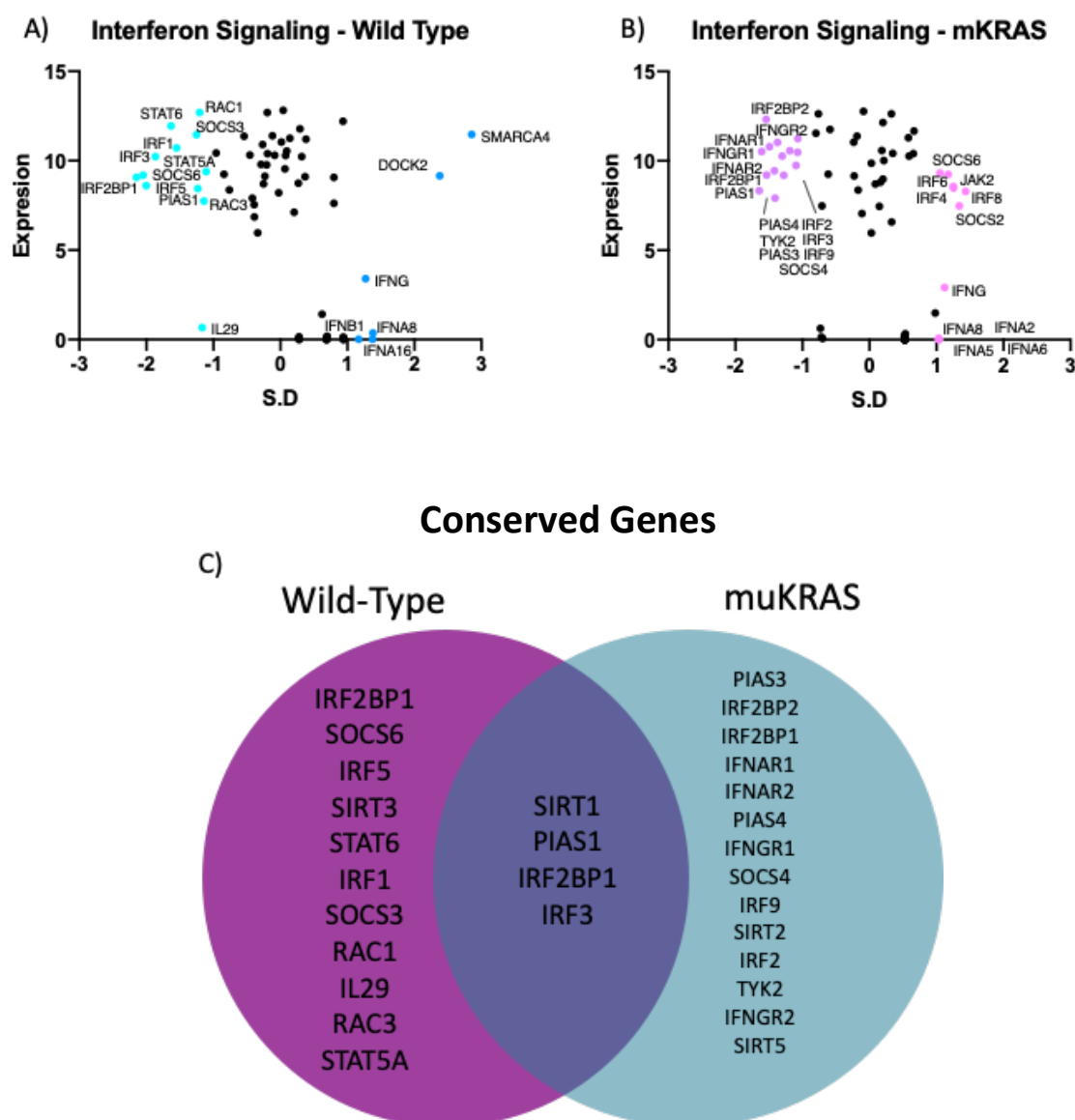


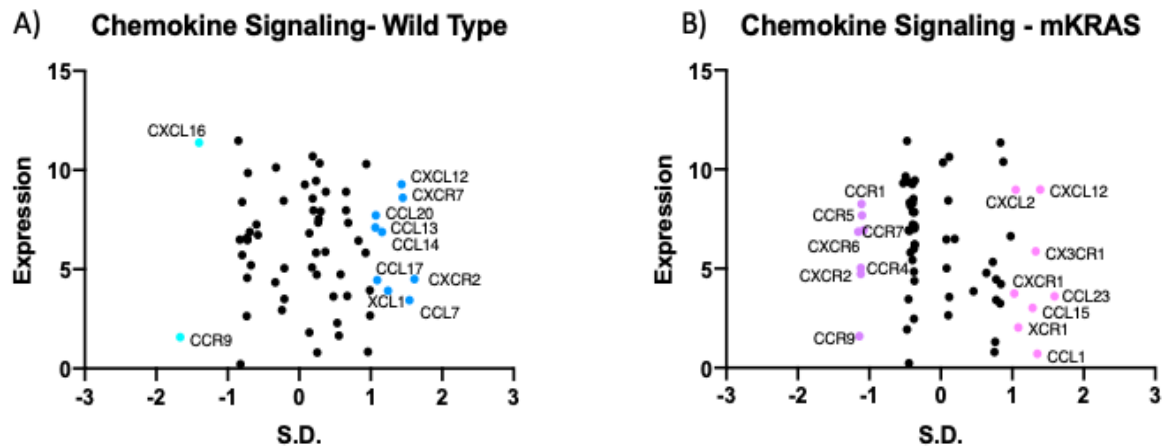
Figure 3-6 Conservation levels of interferon signaling pathway genes in lung cancer.

Gene conservation score, measure as the standard deviation from the regression line (S.D) graphed against genes expression levels (Log2) for WT (A) and mKRAS (B). Conserved genes ($SD \leq -1.0$) Hypermutated genes ($SD \geq 1.0$) are coloured and labelled with text. (C) Venn diagram of genes conserved in WT and mKRAS. Standard deviation (SD)

3.2.8. Chemokine signalling: multiple chemokine receptor genes were conserved in mutant KRAS tumours

More chemokines pathway genes were hypermutated than conserved in both subtypes (**Figure 3.7A-B**). mKRAS lung tumours conserved more chemokine family genes than WT (**Figure 3.7C**). All of the conserved genes encode chemokine receptors and not chemokines themselves. This includes five members of the CC chemokine receptor family and three CXC chemokine receptor family members. WT lung tumours conserved CXCL16 and CCR9. CXCL16 was both highly expressed and highly conserved in WT lung tumours (SD = -1.4). This CXC chemokine family member is traditionally membrane-bound with only one known receptor, CXCR6. However, in cultured meningioma cells, a soluble form of CXCL16 can interact with membrane-bound CXCL16 inducing inverse signalling that diminishes apoptosis and increases proliferation (195).

The majority of hypermutated genes encode chemokines (**Figure 3.7A-B**). While we cannot determine functional outcomes of mutations, CXCL12, a chemokine implicated in the metastatic spread in lung cancer, was hypermutated and highly expressed in both WT and mKRAS tumours (SD = -1.4, SD = -1,4).



Conserved Genes

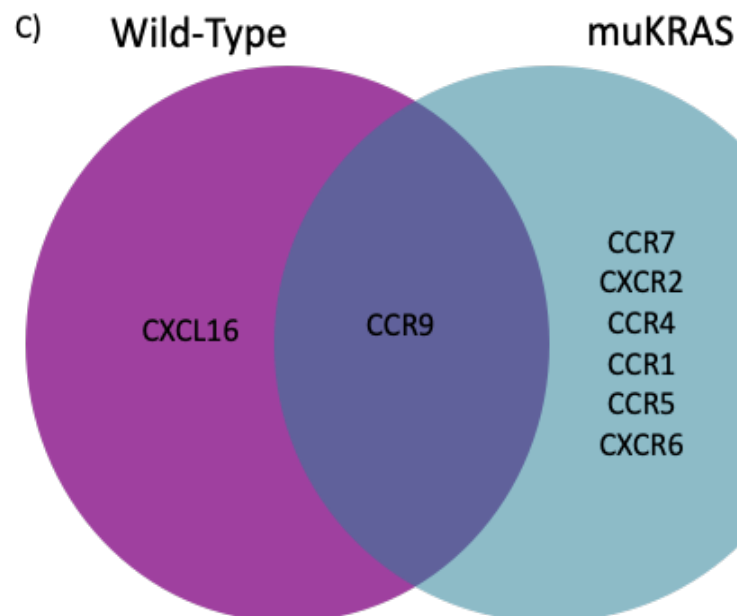


Figure 3-7 Conservation profiles in chemokine genes.

Gene conservation score, measure as the standard deviation from the regression line (S.D) graphed against genes expression levels (Log2) for WT (A) and mKRAS (B). Conserved genes ($SD \leq -1.0$) Hypermutated genes ($SD \geq 1.0$) are coloured and labelled with text. (C) Venn diagram of genes conserved in WT and mKRAS. Standard deviation (SD)

3.2.9. Cytokine signalling: cytokines and cytokine receptors

Cytokine signalling genes made up the largest gene list of the eight we examined. Epithelial cells secrete cytokines that regulate the immune response. The list includes cytokines, cytokine receptors and other signalling molecules involved in regulating the immune response (appendix). Interleukin 1 receptor-1 (IL1R1) was the most conserved among the cytokine signalling genes in WT cancers (SD = -2.1) (**Figure 3.8A**). Following ligation by either IL1A or IL1B, IL1R1 associates with interleukin-1 receptor accessory protein (IL1RAP) (WT SD = -1.1) and activates many downstream proinflammatory pathways including NF- κ B and MAPK (196). IL-17RB was the second most conserved cytokine gene in WT lung cancers and was also conserved in mKRAS cancers (WT SD = -2.0, mKRAS SD = -1.4) (**Figure 3.8C**). Other IL-17 genes, IL17RC and IL17C, were also conserved in WT. IL17RD was highly conserved in mKRAS (SD = -1.8) (**Figure 3.8B**).

3.2.10. Cytokine signalling: Eicosanoids

Leukotrienes are proinflammatory eicosanoids derived from arachidonic acid metabolism that occurs at lipid droplets by various associated enzymes. Briefly, enzymes ALOX5 (5-LO) along with ALOX5AP and COTL1 convert arachidonic acid into leukotriene A₄ (LTA₄) (197). ALOX5 was hypermutated in WT and mKRAS (S.D. = 1.25, SD = 1.05 respectively), ALOX5AP was conserved in WT only (S.D. = -1.22). LTA₄ is broken down into various leukotrienes depending on the enzymes present (197). LTA₄ hydrolase (LTA₄H), an enzyme expressed in most tissues, hydrolyses LTA₄ into LTB₄, a strong chemoattractant and immune activator with important roles in chronic pulmonary inflammatory conditions and cancer. LTA₄H was conserved in both WT and

mKRAS (**Figure 3.8C**) (197). Leukotriene B4 receptor (LTB4R) is the high-affinity receptor for LTB4, while leukotriene B4 receptor-2 (LTB4R2), the low-affinity receptor, was conserved in both WT and mKRAS (198-201). Interestingly, LTB4R2 was revealed as an essential gene in a recent CRISPR/Cas9 dependency screen in lung cancer (202). Prostaglandins, another class of pro-inflammatory eicosanoids, are created when arachidonic acid is metabolized by cyclooxygenases, PTGS1 (COX-1) and PTGS2 (Cox-2). Cox-1 was hypermutated in WT lung samples (SD = -1.5), while Cox-2 was neither conserved nor hypermutated in any subtype.

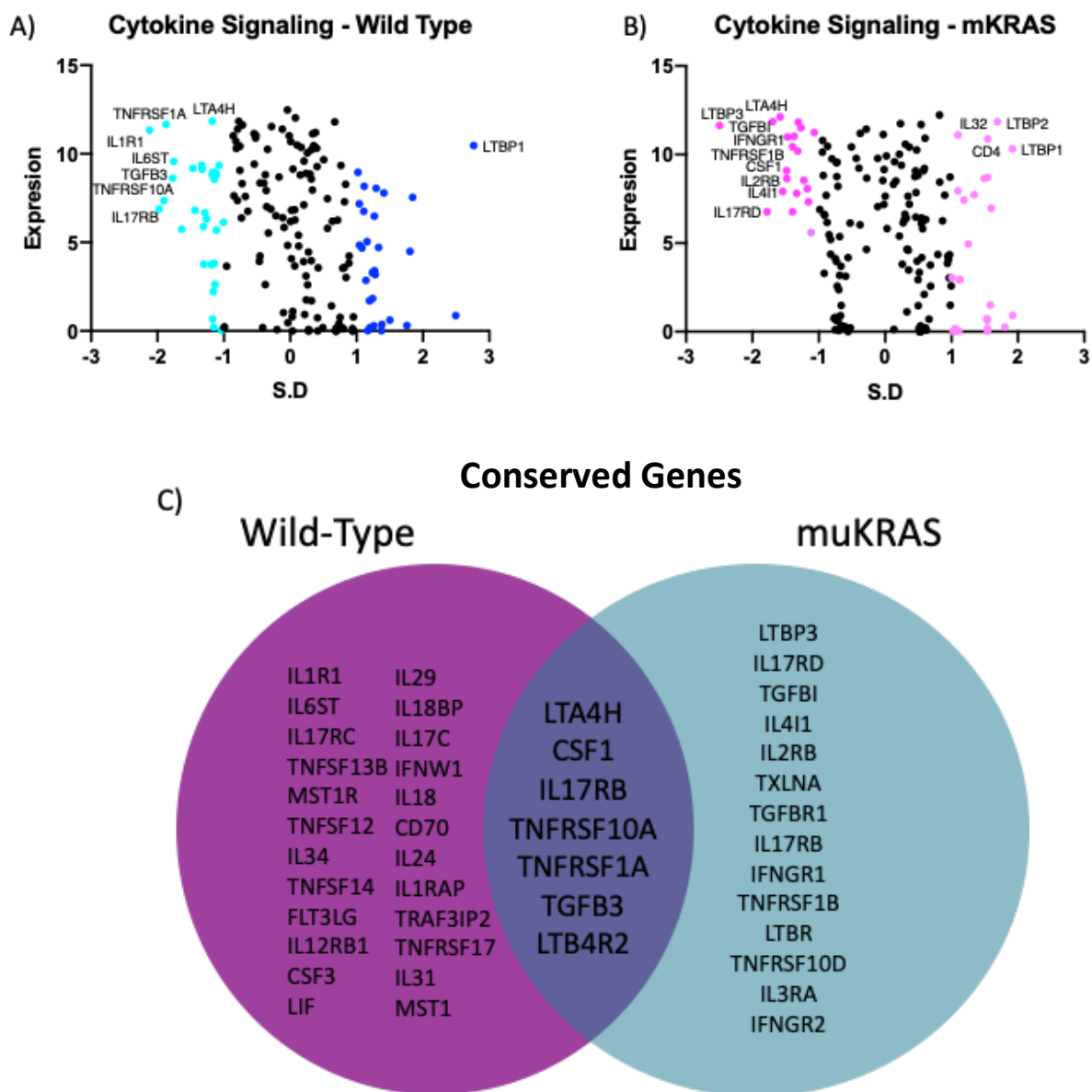


Figure 3-8 Conservation profiles in cytokine genes.

Gene conservation score, measure as the standard deviation from the regression line (S.D) graphed against genes expression levels (Log2) for WT (A) and mKRAS (B). Conserved genes ($SD \leq -1.0$) Hypermutated genes ($SD \geq 1.0$) are coloured and labelled with text. (C) Venn diagram of genes conserved in WT and mKRAS. Standard deviation (SD)

3.2.11. Apoptosis, Necroptosis, and Pyroptosis

Cell death pathways are complex. Cancer cells evolved mechanism to circumvent programmed cell death. Immune cells trigger death pathways in cancer cells through cytolytic granules and soluble death ligands. Additionally, activation of the inflammasome within epithelial cells can induce pyroptosis. TNF Receptor Superfamily Member 10A (TNFRSF10A) was the only gene conserved in both cohorts on the apoptosis, necroptosis, and pyroptosis gene list (WT SD = -1.9, mKRAS SD = -1.3). The TNFRSF10A gene encodes TRAIL receptor -1 (Death receptor-4 (DR4), APO2, TRAILR-1) **(Figure 3.9C).**

There were no pyroptosis genes conserved or hypermutated in mKRAS tumours **(Figure 3.9B)**. Two of the eleven pyroptosis genes examined were hypermutated in the WT cohort (AIM2 and CASP5, SD = 1.3 and 1.6 respectively). Two were conserved, PYCARD and IL18 SD = 1.2, SD = 1.1) **(Figure 3.9A).**

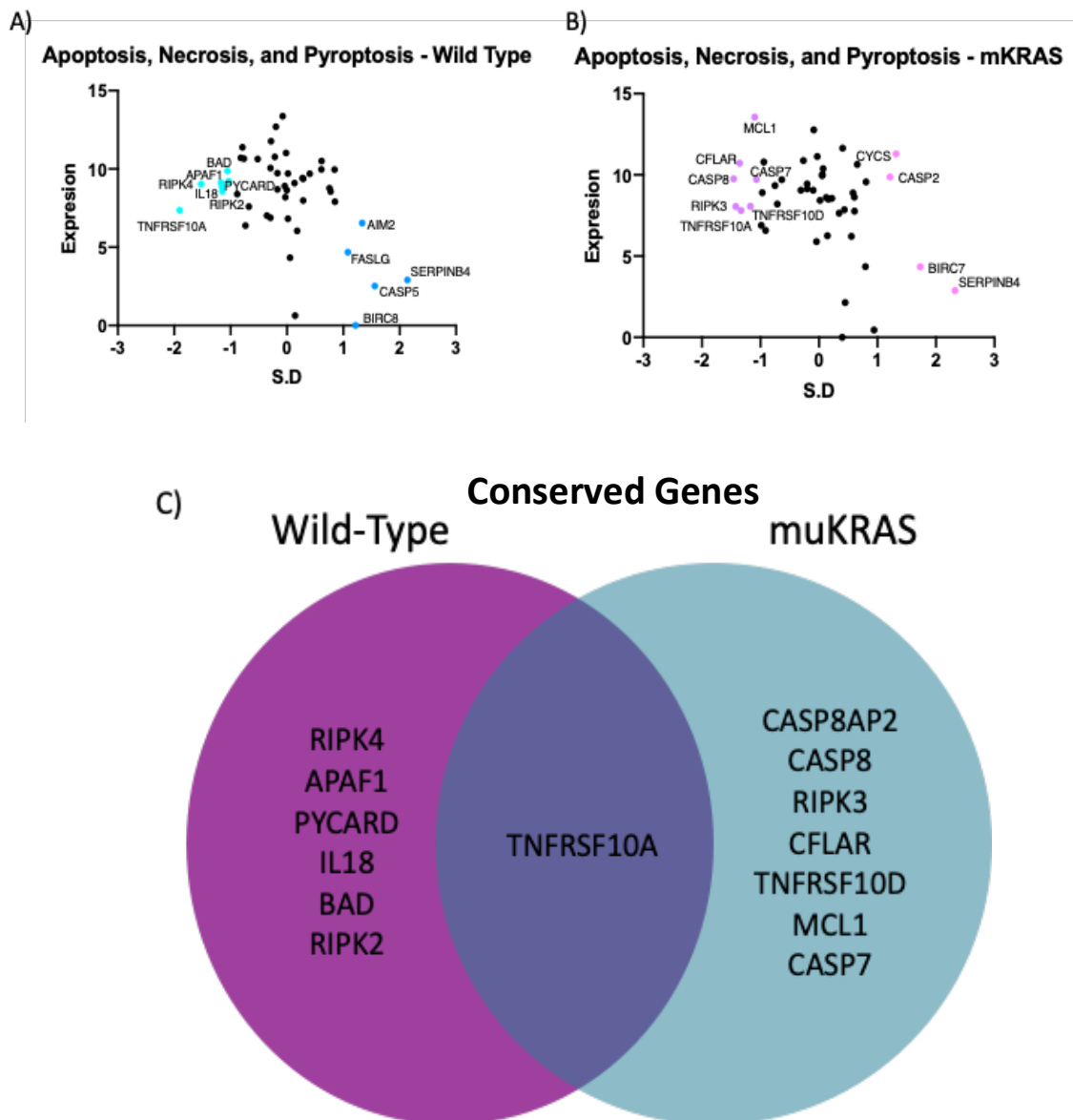


Figure 3-9 Conservation profiles in apoptosis, necrosis, and pyroptosis pathways.

Gene conservation score, measure as the standard deviation from the regression line (S.D) graphed against genes expression levels (Log2) for WT (A) and mKRAS (B). Conserved genes ($SD \leq -1.0$) Hypermutated genes ($SD \geq 1.0$) are coloured and labelled with text. (C) Venn diagram of genes conserved in WT and mKRAS. Standard deviation (SD)

3.2.12. Complement protein genes are highly mutated in WT and mKRAS tumours

A high percentage of genes encoding complement proteins were hypermutated in WT and mKRAS tumours (37% and 38%, respectively) (**Figure 3.2**). CSMD1 and CSMD3 were two of the most highly mutated genes on the entire list. CSMD3 had one of the highest hypermutated scores out of all the genes in the study in WT and mKRAS (SD = 6.2, SD = 5.5, respectively) (**Figure 3.10A-B**). CMSD1 was the second most mutated complement gene (WT SD = 3.7, mKRAS SD = 4.9). C4A, which encodes acidic complement factor 4, was highly conserved in WT (S.D. = -2.9) and conserved in mKRAS (S.D. = -1.7). C5 was also conserved in both subtypes (**Figure 3.10C**)

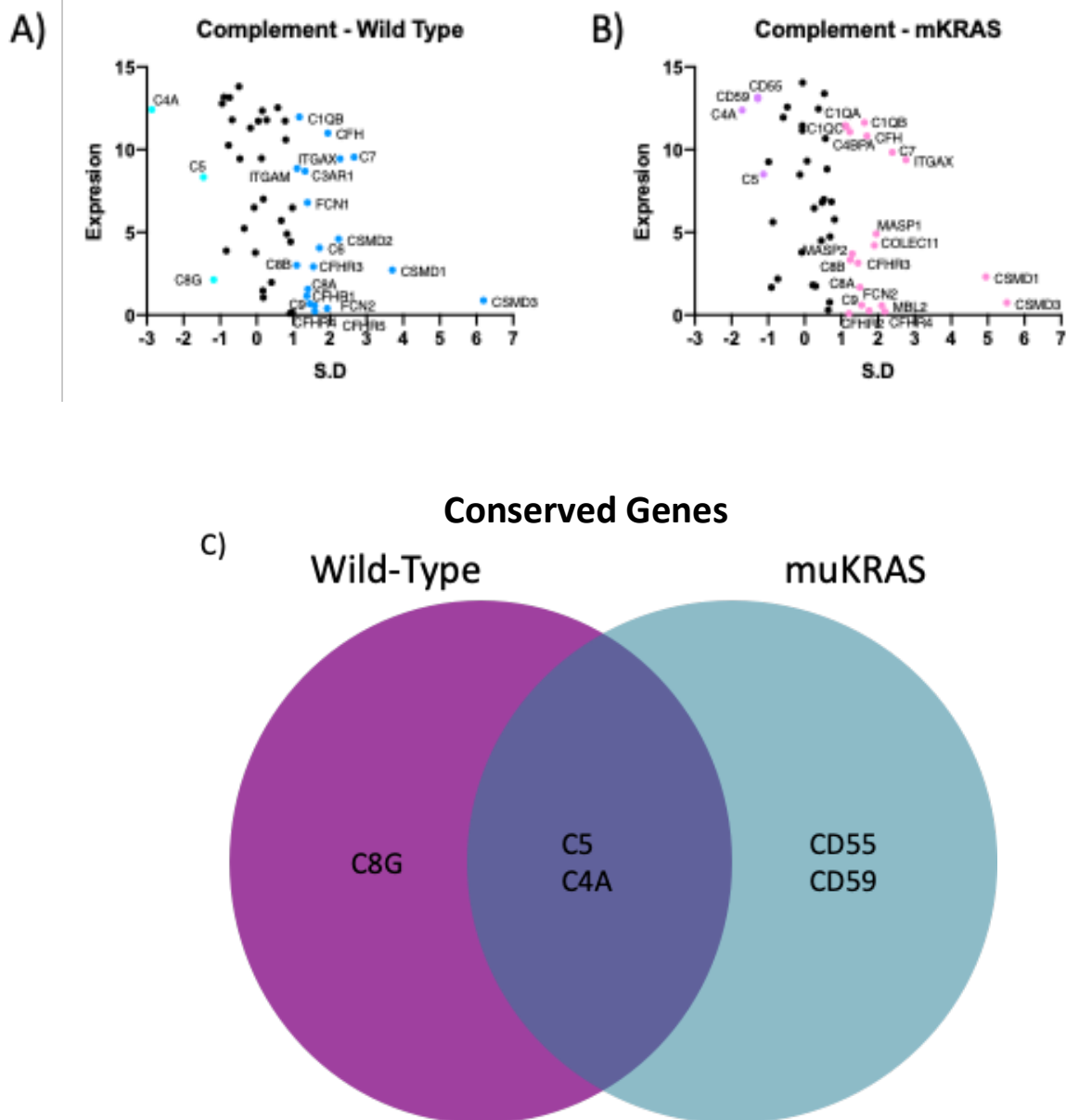


Figure 3-10 Conservation profiles in Complement genes.

Gene conservation score, measure as the standard deviation from the regression line (S.D) graphed against genes expression levels (Log2) for WT (A) and mKRAS (B). Conserved genes ($SD \leq -1.0$) Hypermutated genes ($SD \geq 1.0$) are coloured and labelled with text. (C) Venn diagram of genes conserved in WT and mKRAS. Standard deviation (SD)

3.3. Discussion

Cancer cells evolve immune evasion strategies to escape detection and destruction by immune cells. They also benefit from encouraging a pro-growth inflamed microenvironment. This simple idea, suppress the bad and promote the good, is rooted in a complex web of interacting players involving hundreds of genes and diverse intra- and inter-cell signalling pathways. We let evolution help us unwind some of this complexity and reveal genes that are essential to cell fitness. The mutational status of over 17,000 genes was quantified in tumour tissue from a large cohort of lung cancer patients. We assumed an approximately equal probability of a mutation occurring for each base pair. Comparing gene size with the number of patients harbouring a mutation in each cohort we determine a level of background mutations for each gene. We showed that for the majority of genes, large size yields higher numbers of mutations. Genes with more mutations than expected based on size alone were labelled hypermutated genes. Genes with fewer than expected mutations were labelled conserved genes. Gene conservation typically occurs when the fitness of the cell is impaired by a loss-of-function mutation within that gene. Therefore we utilized gene conservation status to indicate essential genes in lung tumour tissue.

We hypothesized that analysis of gene conservation levels in immune-related genes within tumour samples would reveal novel genes that are likely essential to tumour survival in an immune-competent host. We defined eight gene sets for different immune-related processes occurring within epithelial cells with over 500 genes and found many pathways under evolutionary pressure. While the list of conserved genes is long, we highlight the key findings in our dataset which included ERAP1/2 gene family,

NK ligand genes, and IFN signalling. Of those conserved, we found a potentially universally essential pair of genes, ERAP1/2. Analysis of immune-modulating proteins revealed conservation of poliovirus receptor family genes and ligands for NK cell receptor NKG2D. We also defined inflammatory processes conserved within the different subtypes, TLR3 pathway in wild type cancers and Type I and Type II IFN signalling in mutant KRAS tumours.

ERAP1/2 inhibition has detrimental effects on antigen presentation. Therefore, it seems intuitive to think that tumour cells would benefit from loss-of-function mutations in ERAP genes to avoid detection by the immune system. However, here we saw universal and robust conservation of ERAP genes suggesting they are essential to tumour cell fitness and potentially to all cells. These counterintuitive findings may indicate a host mechanism that has evolved to protect against pathogen mediated inhibition of antigen presentation pathways. The first evidence of this in the ERAP pathway was published in 2012 by Nagarajan *et al.* which demonstrates that ERAP genes are conditionally essential. In immunocompetent mice, inhibition of murine ERAP (ERAAP) initiated a robust CD8 T cell response (203). This was caused by the presentation of a highly conserved peptide, FL9. When ERAAP is fully functional FL9 peptides are digested in the ER and never presented on the cell's surface. When ERAAP is inhibited, FL9 is presented by the MHC class Ib molecule Qa-1 (human analogue HLA-E, (conserved in WT cohort)). Furthermore, they found that naïve mice have abundant antigen-experienced CD8 T cells specific for Qa-1-FL9, which can eliminate ERAAP deficient cells. The essentialness of ERAP1/2 genes is conditional on CD 8 T cells. Therefore it cannot be identified *in vitro* or with immune incompetent murine models. This robust immunosurveillance is likely

a result of co-evolution between pathogens and the host immune response to prevent ERAP inhibition by pathogens.

MHC-II is expressed by professional antigen-presenting cells, group 3 innate lymphoid cells, and epithelial cells in the thymus, intestines, and lungs. Antigen presentation by MHC-II stimulates CD4 T cell-mediated immune responses and shapes the evolution of driver mutations in cancer (204). MHC-II expression is associated with favourable outcomes after immunotherapy (205). However, the role of epithelial MHC-II expression by cancer cells is not well understood. In the intestines and lungs, epithelial antigen presentation by MHC-II has a dual role of immune activation and tolerance induction depending on context (71). MHC-II expression by type 2 alveolar epithelial cells assists with viral clearance but moderates the immune responses demonstrating a potential regulatory role (206).

The effects of immune-modulating ligands PVR and PVRL2 vary depending on the context and type of receptors expressed on immune cells. Binding with DNAM-1 will induce activation however, binding with inhibitory receptors TIGIT, CD96, and PRVIG on immune cells induces immune suppression. TIGIT is upregulated on immune cells following activation and is frequently expressed in NSCLC patients (28, 207).

The role of natural killer cells in tumour immunosurveillance has been documented, and cancer cells are susceptible to NK cells (208). NK cell dysfunction within the TME and tumour cell investment in NK cell evasion highlight many new potential targets. NKG2D ligands, including MICA, ULBP2 and RAET1E (ULBP4), are markers of genomic and

oxidative stress within epithelial cells. Ligation of the NKG2D receptor on NK cells, $\gamma\delta$ T cells, and CD8+ $\alpha\beta$ T cells induces target cell-mediated killing (209, 210). Shedding of surface ligands induces tolerance in NK cells and downregulation of NKG2D receptors resulting in immune evasion (211). Targeting soluble NKG2D ligands is being investigated.

Type I and type II interferons are vital contributors to anti-viral and anti-tumour immune responses. Direct interactions with receptors on tumour cells can initiate cytostatic and cytotoxic pathways within malignant epithelial cells. However, interferons can also have pro-tumour effects. Chronic exposure to type I IFN induces immune suppression during chronic infections (212). Similarly, chronic inflammation in the TME triggers immunosuppression and induces treatment resistance (213). IFNAR2 overexpression in immunohistochemistry analysis of lung tumours was associated with decreased overall survival and shorter progression-free survival in NSCLC (214). Type II IFN, IFN-gamma, effects are also dose and duration dependent. Low dose IFN-gamma within the TME increases the metastatic potential of cancer cells. Ligation of INF-gamma receptors on cancer cells induces the upregulation of tumour cell PD-L1 and the immunosuppressive enzyme IDO. In a murine study, immune cell-derived IFN-gamma increased genetic instability and modulation of DNA editing/repair pathways in cancer cells, suggesting a role for IFN-gamma signalling in cancer evolution and immune escape (215).

We cannot discern if hypermutated genes have gain-of-function or loss-of-function mutations. However, when a family of genes has a high frequency of hypermutated genes, it can indicate an evolutionary investment in the pathway. The role of

complement activation in cancer progression and response to therapy is complex. However, recent studies have demonstrated important connections between the two systems. Clinically the use of complement for biomarker studies and therapeutic effects are ongoing (216). Similarly, lipid antigen presentation by CD1 proteins has also demonstrated important contributions to anti-cancer immunity and lung health (217).

Our analysis was performed on whole tumour tissue, including tumour cells, immune cells, and other host cells. At this time, little is known about mutations in non-malignant cells. The effects of the TME on the induction of mutations in stromal cells has not been examined. DNA from immune cells and non-malignant host cells could contribute to some of our findings. Analysis relying on bulk tumour samples, instead of single cell data, can miss lineage specific mutations that may fall below level of detection. This is particularly important in recent evolutionary events where new mutations are represented in only a small fraction of the tumour cells evaluated. While only non-synonymous mutations were evaluated, it is impossible to know the mutations' effects (activating or inactivating) on protein function. We derived our conservation values assuming that the number of expected mutations is determined by gene size alone. This can lead to misrepresentation of very small and very large genes and does not consider that some genes may be more mutable than others regarding nucleic acid make-up and chromosome localization. A single lesion was measured from each patient. Potentially, multiple lesions from a single patient would have different conservation profiles. The TCGA database has limitations including lack of patient outcomes and treatment responses. Further work is needed to determine the potential for clinical targeting of

the essential genes revealed here including validation studies and analysis of additional cohorts of lung cancer patients from different datasets.

By applying ecology and evolutionary biology approaches, we have shown that careful analysis of conservation in immune-related genes can reveal novel essential genes in lung cancer. Interdisciplinary work has its challenges, however, combining knowledge of the immune system, cancer biology, and eco-evolutionary dynamics has great potential for unlocking new ways to investigate and target cancer. In the following chapters, we build upon this work by using *in vitro* evolutionary models of treatment resistance in combination with ecological concepts, including predator-prey modelling of tumour-immune dynamics and exploitation of evolutionary trade-offs where resistance to first-line treatment increases the sensitivity to immunotherapy.

Chapter 4. Identification of a radiotherapy-induced
immunologic double bind in advanced prostate
cancer

4. Chapter 4. Identification of a radiotherapy-induced immunologic double bind in advanced prostate cancer

4.1. Introduction

Evolution guided therapies (EGT) are novel therapeutic strategies that use biological first principles, including gene theory and evolution, to guide treatment schedules. EGT has two key objectives; 1) to reduce overall tumour burden and 2) to prevent the outgrowth of a fully resistant phenotype (134). The emergence of resistance represents a fundamental barrier to curative treatment strategies. Resistance occurs when a population of tumour cells acquires adaptive traits enabling their survival during therapy. Adaptive strategies that increase tumour cell survival in a harsh environment, such as cancer therapy, are often associated with evolutionary costs. Costs refer to a decline in fitness that arises as a result of the adaptive strategy. These can include a proliferative disadvantage compared to non-resistance cells, an increased sensitivity to a second treatment, or alterations in the tumour cell's vulnerability to the immune system. An evolutionary double bind occurs when an adaptive strategy has an unintended consequence that introduces a vulnerability to a second perturbation (137, 218).

Evolution guided therapy can be applied with most cancer therapies. Radiotherapy (RT) is an attractive treatment modality for the application of EGT. It is widely applicable; more than half of all cancer patients, regardless of cancer type, receive radiotherapy (219). RT can be used in combination with other treatment modalities. Dosing and

timing can be varied and personalized. Furthermore, radiotherapy has immunostimulatory effects (220). Trials combining RT and immunotherapy are ongoing in a variety of cancer types. Here, we aim to identify an evolutionary cost of radiation resistance that immunotherapy can exploit, a radiotherapy-induced immunological double bind.

4.1.1. Radiotherapy and the anti-tumour immune response

Given locally, RT induces double-stranded DNA breaks in tumour cells, initiating cellular stress responses that trigger either cell death or DNA repair and cell survival [62]. RT can cause cell death through multiple pathways including, apoptosis, necrosis, autophagy, and mitotic catastrophe. Tumour cell death alerts the immune system through the release of DAMPs creating an *in situ* vaccine at the radiation site (221). This triggers antigen-presenting cells and the subsequent T cell responses.

Radiotherapy changes the protein expression on surviving cancer cells. Radiosensitivity is related to the DNA repair capabilities of a tumour cell (155). Activation of the DNA damage response pathways, mediated by ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR), triggers stress response pathways within cancer cells. Notably, it has been shown that RT and other DNA damaging agents increase natural killer (NK) cell ligand expression on tumour cells (222).

NK cells directly kill cancer cells with perforins and granzymes packed into cytolytic granules and through the release of death-inducing ligands, including Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (49) (50). Indirectly, activated NK cells

orchestrate and support cytotoxic functions of the adaptive arm through the release of chemokines and cytokines, including IFN-gamma, TNF, GM-CSF, IL6, CCL3, CCL4, and CCL5 (51). NK cell activation is dependent on a combinatorial array of inhibitory and activating signals received from target cells (23). Activation of NK cells only occurs when these signals result in a net activation signal, that is, when activating proteins on the surface of tumour cells achieve a larger signal than regulatory proteins (50). Tumours often exploit this sensitive balance leading to immune evasion. Altering ligand expression on tumour cells reverses this effect [63]. The DNA damage response alters NK cell ligands on tumour cells and radiation resistant cells have increased DNA damage responses. Additionally, altered ligand expression can increase sensitivity to NK cells, therefore, **we hypothesized that radiotherapy resistance will alter the expression of NK ligands on tumour cells, thereby increasing the sensitivity to NK cell-mediated killing and creating an evolutionary double bind.**

4.1.2. Targeting radiation resistance

Radiation resistance is mediated by the activation of various cellular pathways and microenvironmental factors. Detection of DNA damage activates double-stranded break repair pathways, homologous recombination, and non-homologous DNA end joining. Radiation resistant cells express higher levels of repair pathway proteins, DNA-PK, ATM, Rad52, MLH1, and BRCA1 (155). Inhibition of DNA repair re-sensitizes tumour cells to RT. Additionally, cell cycle control proteins including, SHP-1, enhance radiation resistance by arresting cells in G2-M, where they are less sensitive to RT. *In vivo* and *in vitro* models using Gemcitabine to induce cell-cycle arrest in early S-phase, where cells are most susceptible to RT, increases radiosensitivity in cancer cells. Furthermore,

targeting stem-like cancer cells and epithelial to mesenchymal transition can increase the radiosensitivity of cancer cells.

Optimizing therapeutics to increase radiosensitivity in individual cancer cells is a common strategy. Here, we look to identify the evolutionary costs associated with adaptive strategies that cause radiation therapy resistance. To determine if a radiotherapy/NK cell double occurs, we used an isogenic cell line model of radiation resistance derived from an androgen-independent human prostate carcinoma cell line. The radiation-resistant cell line, RR-22Rv1, was selected previously in Laure Marignol's lab at Trinity St. James's Cancer Institute by irradiating with multiple 2 Gy fractions for a total of 60 Gy resulting in a radiation-resistant daughter line (**Figure 4.2**) (155). The control line (AMC-22Rv1) was passaged along with the radiation-resistant cell line (RR-22Rv1) and used as a control for all experiments. The 22Rv1 cell line was chosen for these experiments because it best represents primary prostate cancer compared to other commonly used cell lines (155). 22Rv1 was derived from a CWR22 xenograft after androgen deprivation relapse (223). 22Rv1 cells express prostate specific antigen, p53, and androgen receptor (AR) (224). Different splice variants of the AR gene allows for hormone initiated and hormone independent proliferation (223).

Using multiparametric flow cytometry, we found that the radiation-resistant cell line has differential expression of NK receptor ligands compared to the control line. We focused on specific stress-induced ligands whose expression is regulated by the DNA damage response or previously shown to be essential in our gene conservation study. These include; activating ligands (MICA, MICB, and ULBPs), inactivating ligands (MHC-I

and PD-L1), as well as PVR and CD112 (PVRL2), which can either activate or inactivate NK cells depending on receptor binding (**Figure 4.1**). Following radiation, the surface expression of NK ligands was increased on both cell lines. However, the radiation-resistant line maintained higher levels than the control at all RT doses.

We found that the radiation-resistant line was more sensitive to NK cell-mediated killing in flow cytometry-based cytotoxicity assays using an immortalized NK cell line, NKL. Interestingly, the differential effects between the control line, AMC-22Rv1 and the RT resistant line, RR-22Rv1, were not seen in cultures with soluble death-receptor ligands, TRAIL, FasL, and TNF-alpha or the cytotoxic cytokine, IFN-gamma, suggesting a dependence on interactions with NK cells.

Additionally, we measured the cytotoxic effects of NKL cells when both cell lines were cultured together and found that NKL cells selectively killed the radiation-resistant cells while leaving a greater proportion of radiation-sensitive cells intact. This revealed a true evolutionary double bind that allows us to utilize NK cells to restore the radiation-sensitive population in mixed cultures. Taken together, we demonstrated the potential for combining RT and NK cells in an evolutionary guided treatment strategy in our *in vitro* model of advanced prostate cancer.

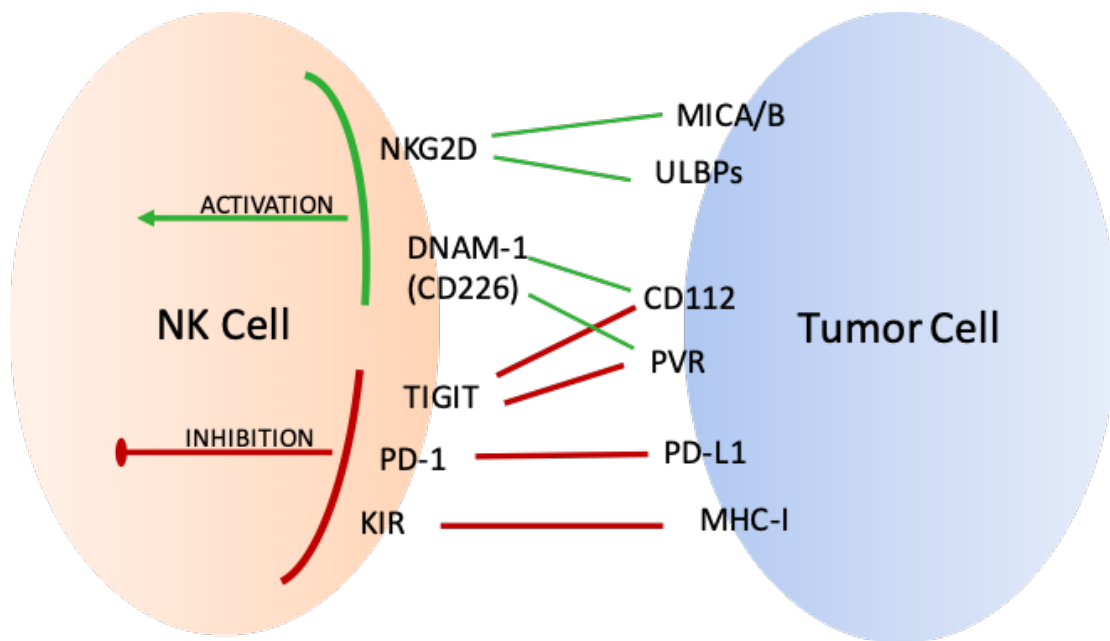


Figure 4-1 Ligands expressed on tumor cells have differential effects on NK cell activation.

Green lines represent activation. Red lines represent inhibition. Natural killer group 2 member D (NKG2D), DNAX Accessory Molecule-1 (DNAM-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), Programmed cell death protein 1 (PD-1), Killer-cell immunoglobulin-like receptor (KIR), MHC class I polypeptide-related sequence A (MICA), MHC class I polypeptide-related sequence B (MICB), UL16 binding protein (ULBP), poliovirus receptor (PVR), programmed death-ligand 1 (PD-L1), major histocompatibility complex (MHC).

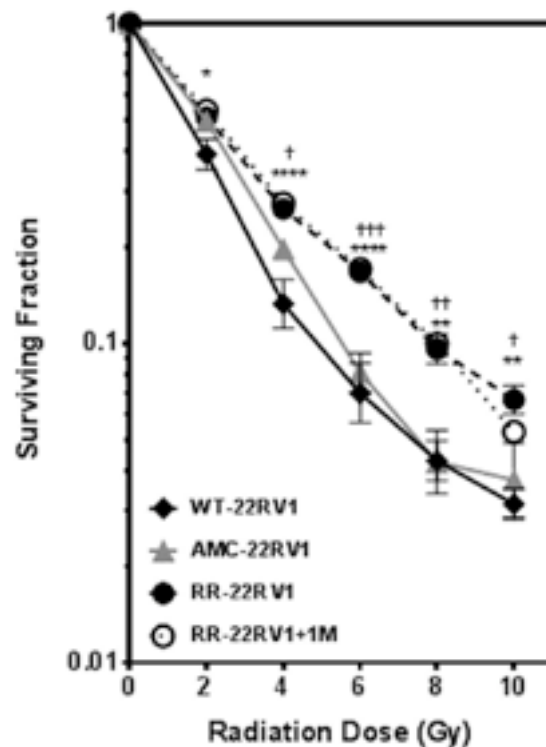


Figure 4-2 Generation of a stable isogenic radiation-resistant prostate cancer cell line.

Surviving fraction, after 60Gy cumulative dose of X-radiation, of the radiation-resistant (RR-22RV1) isogenic cell line model compared with 22RV1 wild type (WT-22RV1) control and 22RV1 age matched control (AMC-22RV1). The isogenic cell line shows consistent resistance to radiation after one month of growth with no treatment (RR-22RV1 + 1M). Survival of irradiated cells was measured by clonogenic cell survival assay after X-radiation with 0–10 Gy. Points are measured as means $\pm$ SEM of at least five individual experiments. Figure and legend from Mcdermott, Nualla et al. “Fractionated radiation exposure amplifies the radioresistant nature of prostate cancer cells.” Scientific reports (2016).

4.2. Results

4.2.1. Radiation resistant cells constitutively express higher levels of NK receptor ligands

Surface expression of NK receptor ligands was measured on the control (AMC-22Rv1) and the radiation-resistant (RR-22Rv1) cell lines. Activating NKG2D ligands, MHC class I polypeptide-related sequence A and B (MICA/B), were upregulated on radiation-resistant cells compared to control (Figure 4.3 A) ($p < 0.01$). Additional NKG2D ligands in the UL16 binding protein (ULBP) family including ULBP1, ULBP2/5/6 were unchanged between the two lines (**Figure 4.3 B-C**). PVR and CD112, two ligands which can bind either activating NK receptor CD226 (DNAM-1) or inactivating NK receptor TIGIT were also upregulated in the radiation-resistant cell line (Figure 4.3 D-E) ($p < 0.01$ and $p < 0.001$, respectively). The mean fluorescence intensity (MFI) for the checkpoint ligand PD-L1 was statistically increased in the radiation-resistant cell line ($p < 0.01$), however, both lines had very low levels (below 400 MFI) of PDL-1 expression and biological relevance has not been established (**Figure 4.3 F**). Human leukocyte antigen ABC (HLA-ABC, MHC class I) is often upregulated following RT, however, constitutive HLA-ABC was not increased in our isogenic cell line model (**Figure 4.3 G**).

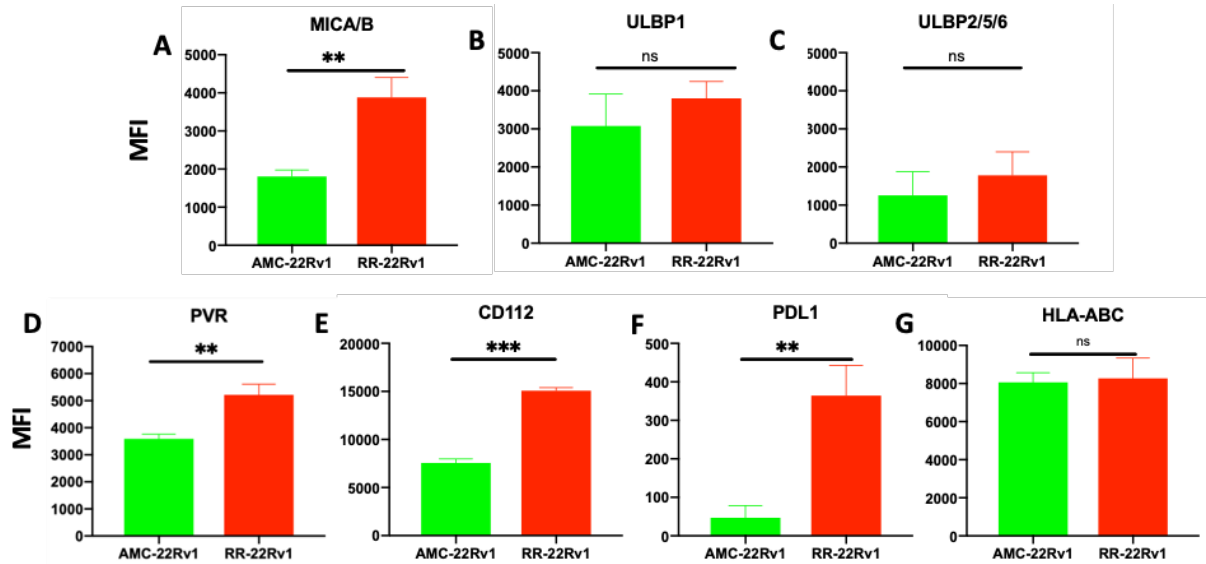


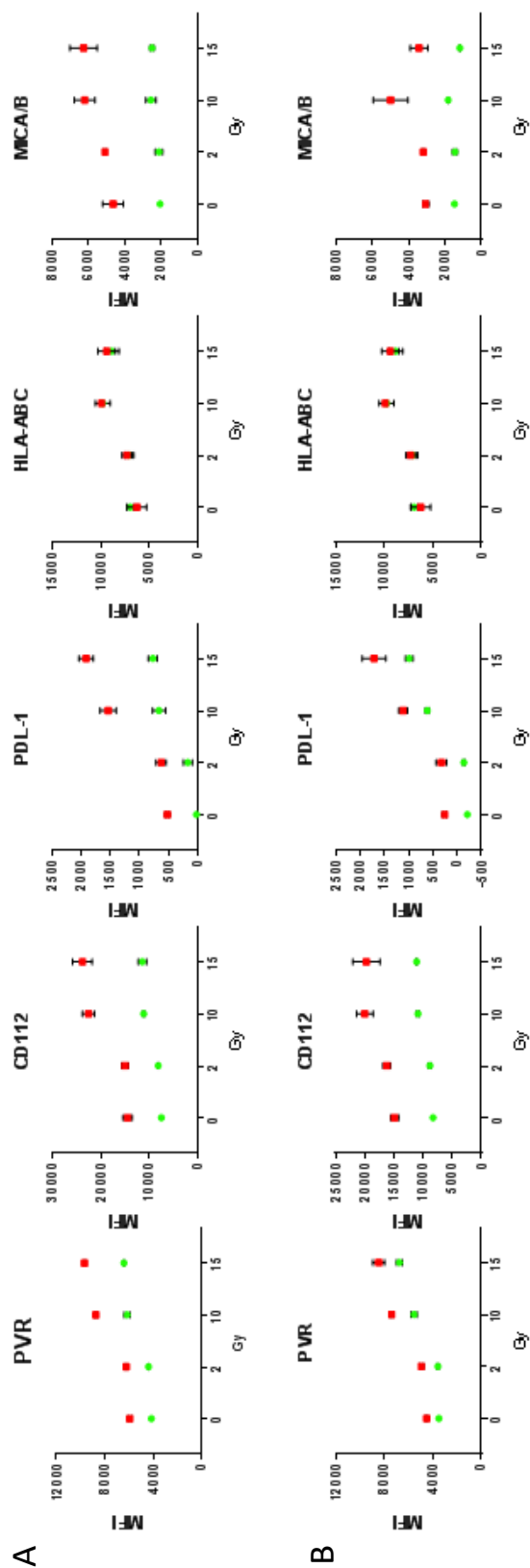
Figure 4-3 Radiation resistant cells constitutively express higher levels of NK receptor ligands

MICA/B, PVR, CD112 and PDL-1 expression are upregulated in the resistant cell line (A,D,E-F). ULBP 1, and 2/5/6 and HLA-ABC are unchanged (B,C, and G). Cells from both the parental line, AMC-22Rv1, and the radiation-resistant cell line, RR-22Rv1, were examined for surface expression of NK ligands by flow cytometry. Cells were stained with predetermined concentrations of antibody cocktail diluted in PBS with 5% FBS and 0.1% sodium azide at room temperature for 20-30 minutes. Cells were then washed and secondary was added where needed. Data was acquired with BD LSR II cytometer and analyzed with FlowJo software. Morphology, viability, and doublet discriminator gates were used prior to determining MFI. MFI was calculated as stained MFI-Unstained MFI for each sample. Three independent experiments, unpaired t test, n.s = not significant **p<0.01, ***p<0.001, ****p<0.0001.

4.2.2. Radiation resistant cells maintain an increased ligand expression profile following additional radiation compared to the radiation sensitive line

Multiparametric flow cytometry was used to measure the effects of radiation on NK ligand expression by our radiation resistant and radiation sensitive cell lines. Radiation increased the surface expression of all NK ligands measured ($p < 0.05$ to $p < 0.00001$) in a dose dependent manner on both cell lines at 24 and 48 hours post treatment (**Fig 4.4 A-B**). The radiation-resistant line maintained increased expression of PVR, CD112, PDL-1, MICA/B ($p < 0.0001$), but not HLA-ABC, above the control line in all conditions.

Interestingly, a two way ANOVA revealed that while the increases were dose dependent and differentially expressed in the resistant and parental cell lines there was no significant interaction between dose and cell line (**Figure 4.4 C**). This is further demonstrated by examining the slopes of the linear regression models (**Figure 4.4 D**). In all instances there was no difference between the slopes indicating that the radiation-resistant (RR-22Rv1) line maintains increased ligand expression compared to the control line (AMC-22Rv1) following radiation, even at very high doses.



C

Interaction	PVR	CD112	PDL1	HLA-ABC	MICA/B
RT Dose (Gy)	ns	ns	ns	ns	ns
Cell line	****	*	****	**	*
	****	****	****	ns	****

AMC-22Rv1 (green square) RR-22Rv1 (red square)

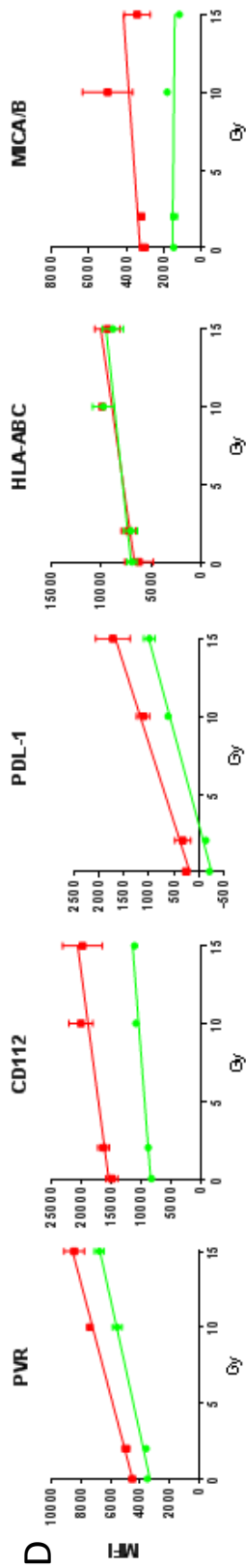
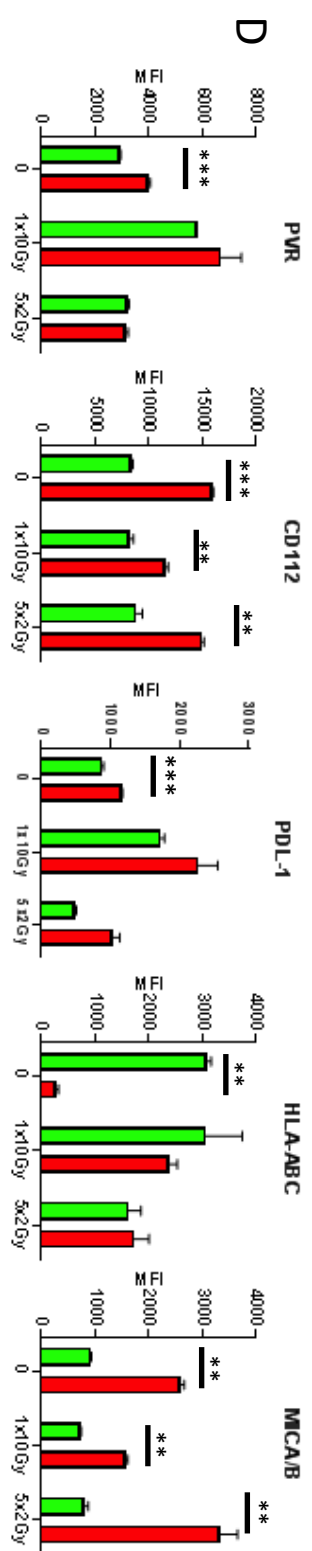
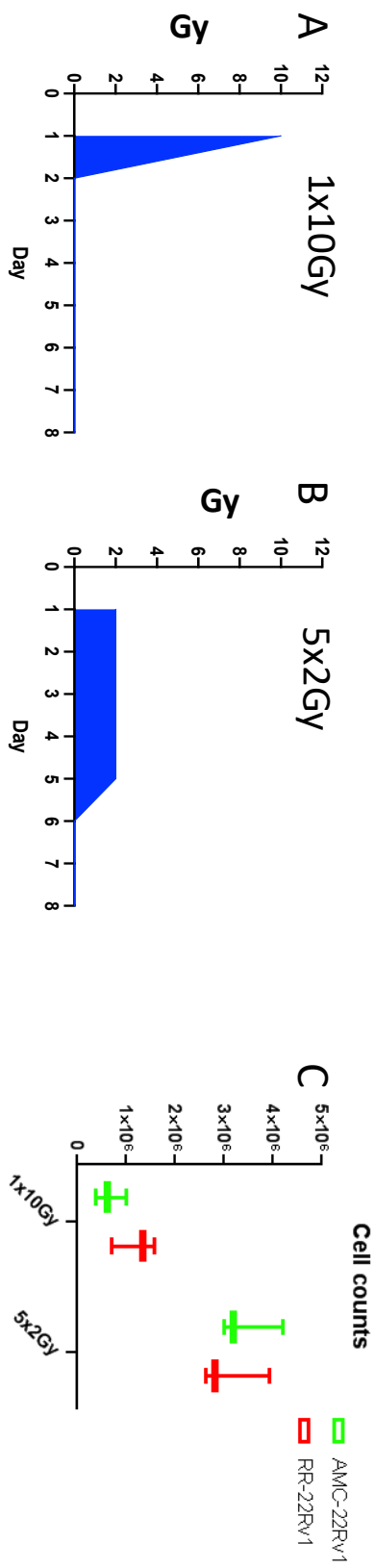


Figure 4-4. Irradiation alters NK ligand expression in vitro.

Radiation increased the surface expression of all measured NK ligands ($p < 0.05$ to $p < 0.0001$) in a dose dependent manner in both cell lines at 24 hours or 48 hours post treatment (0, 2, 10, or 15 Gy RT) (A-B). There are no significant interactions between dose and cell line when analyzed by two-way ANOVA (C), or by examining the slopes of the linear regression models (D). Cells were counted and plated overnight prior to irradiation. 24 and 48 hours after RT cells were harvested and stained with predetermined concentrations of antibody. Data was acquired with BD LSR II cytometer and analyzed with FlowJo software. Morphology, viability, and doublet discriminator gates were used prior to determining MFI. MFI was calculated as stained MFI-Unstained MFI for each sample. Two independent experiments, two way ANOVA, and linear regression (Graphpad). n.s = not significant ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Mean fluorescence intensity (MFI)

4.2.3. Fractionated dosing has differential effects on NK ligand expression

Next, we examined the effects of 10 Gy RT given with different dosing schedules on ligand expression in both cell lines. A single dose (1x10 Gy) was administered on day one (**Figure 4.5A**). Five 2 Gy fractions (5x2 Gy) were given on days 1-5 and ligand expression was measured on day eight for both samples (**Figure 4.5B**). On day 8 single dose was the most effective at reducing cell number compared to 5X2 Gy fractions (**Figure 4.5C**). Single dose fraction increased the expression of PVR and PD-L1 compared to 5x10 Gy fractions. Interestingly, MICA/B, PD-L1, CD112, and PVR were increased in RR cells compared to the control line in all treatment schedules except PVR in 5x2 Gy fractions where there was no difference between the two lines. HLA-ABC expression was inhibited in the untreated RR-22Rv1 cells (**Figure 4.5D**). This is likely related to culturing conditions as the untreated wells were above confluence by day eight. Growth kinetics were likely the driver behind the difference between single dose and fractionated dose. The fractionated dose samples continued to proliferate and reach confluency in the dish. The single dose samples did not reach confluency and were not affected by contact inhibition which can effect protein expression at the cell surface.



AMC-22RV1 RR-22RV1

Figure 4-5 Fractionated radiation has differential effects on NK ligand expression.

Cells were irradiated with hypofractionated (1 x 10 Gy) or hyperfractionated (5 x 2 Gy) schedules in vitro (A-B). Single dose 10 Gy RT inhibited tumor cell growth significantly compared to 5 x 2 Gy (C). PVR, CD112, PDL-1, and MICA/B were increased in the RR-22Rv1 cells in all conditions except PVR treated with hyperfractionated RT (D). On day 8 cells were harvested and stained with predetermined concentrations of antibody cocktail diluted in PBS with 5% FBS and 0.1% sodium azide at room temperature for 20-30 minutes. Data was acquired with BD LSR II cytometer and analyzed with FlowJo software. Morphology and doublet discriminator gates were used prior to determining MFI. MFI was calculated as stained MFI-Unstained MFI for each sample. Mean fluorescence intensity (MFI) **p<0.01, ***p<0.001, ****p<0.0001.

4.2.4. Radiation-resistant cells are more sensitive to NK cell mediated killing

The differential expression of activating and inhibiting NK ligands in the radiation resistant cell line indicated a potential difference in sensitivity to the cytotoxic effects of NK cells. Flow based cytotoxicity assays were performed with the NK cell line, NKL. The radiation resistant cell line RR-22Rv1 was more sensitive to NKL cells compared to the control line (**Figure 4.6A**) ($p < 0.0001$). Additionally, RR-22Rv1 cells were more sensitive to killing in co-culture with freshly isolated allogenic PBMC after 24 hours (**Figure 4.6B**).

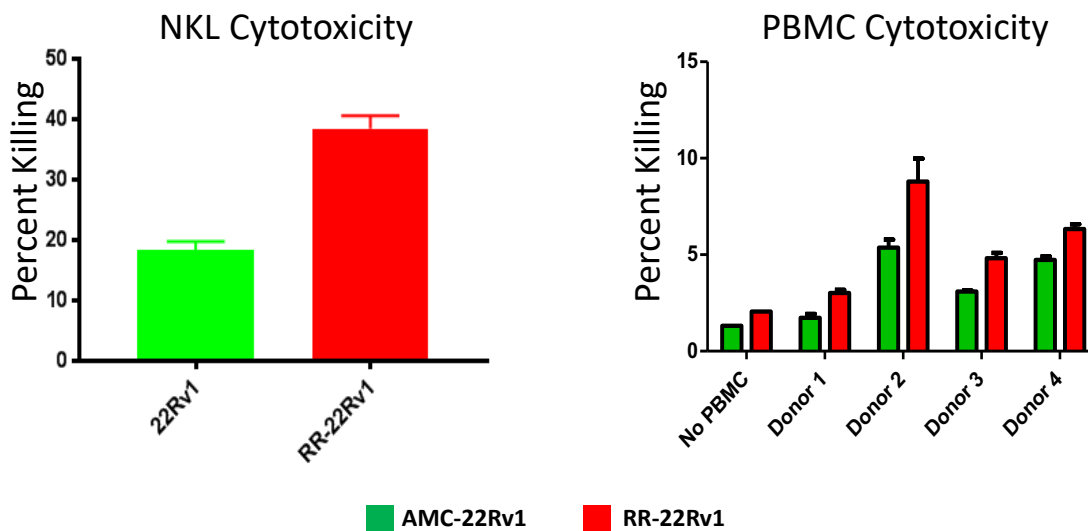


Figure 4-6 Radiation-resistant cells are more sensitive to NK cell mediated killing.

(A) NKL cells kill the radiation-resistant cell line, RR-22Rv1, at a higher rate than radiation-sensitive cell line, AMC-22Rv1 ($p < 0.0001$). (B) RR-22Rv1 cells are more sensitive to killing by healthy donor PBMC after 24 hours co-culture. Tumor cells were tagged with CellTrace prior to plating. NKL or PBMC were left unstained. NKL cells were added at a 10:1 effector to target ratio with 200 IU IL-2. PBMC were added at a 20:1 PBMC to tumor cell ratio with low dose IL-2 (20ng/ml). Viability was measured with Zombie Aqua fixable viability dye. Data was acquired BD LSR II and analyzed with FlowJo software (Treestar). Percent killing was determined as CellTrace positive and viability dye positive out of total CellTrace positive for each cell line. Morphology and doublet discriminator gates were used. Three independent experiments, unpaired t test.

4.2.5. Radiation resistant cells were not more sensitive to soluble death-receptor ligands or IFN-gamma

Next, we measured the effects of soluble death-receptor ligands on the viability of both cell lines. NK cells kill target cells through perforin/granzyme pathways and through release of soluble death-receptor ligands. Previously we found that RR-22Rv1 cells are more sensitive to NK cells, here we wanted to see if this sensitivity is due in part to enhanced susceptibility to TRAIL, FasL, or TNF-alpha. These experiments were performed twice, therefore we cannot extrapolate meaning without additional repeats. However we did find that FasL had little effect on either cell line with no significant difference between the two lines. Both cell lines were highly sensitive to TNF-alpha making IC50 calculations unreliable based on the dosing used in the assay. AMC-22Rv1 cells were significantly more sensitive to TRAIL compared to RR-22Rv1 cells (IC50 = 18.24 and IC50 = 93.26 ng/ml, respectively) (**Figure 4.7**).

We also examined the effects of INF-gamma, a cytokine with known direct anti-tumour effects, and immune cell conditioned media on cell viability. There was no significant difference between the IC50s of the two cell lines treated with IFN-gamma. Immune conditioned media was made by stimulating healthy donor PBMCs with 100ng LPS for 3 days. PBMCs were removed by centrifugation and filtering. The resulting conditioned media (CM) was diluted with normal culture media to achieve a dose effect (10% CM, 25% CM, and 50% CM). Immune conditioned media inhibited the growth of both cell lines AMC-22Rv1 IC50 = 7.683% CM and RR-22Rv1 IC50 = 14.69%CM). The difference between the two cell lines was not significant (**Figure 4.7**). Due to few repeats we cannot

reliably interpret these preliminary findings, however, this data does suggest further study and repeat experiments could provide insight into the mechanisms involved.

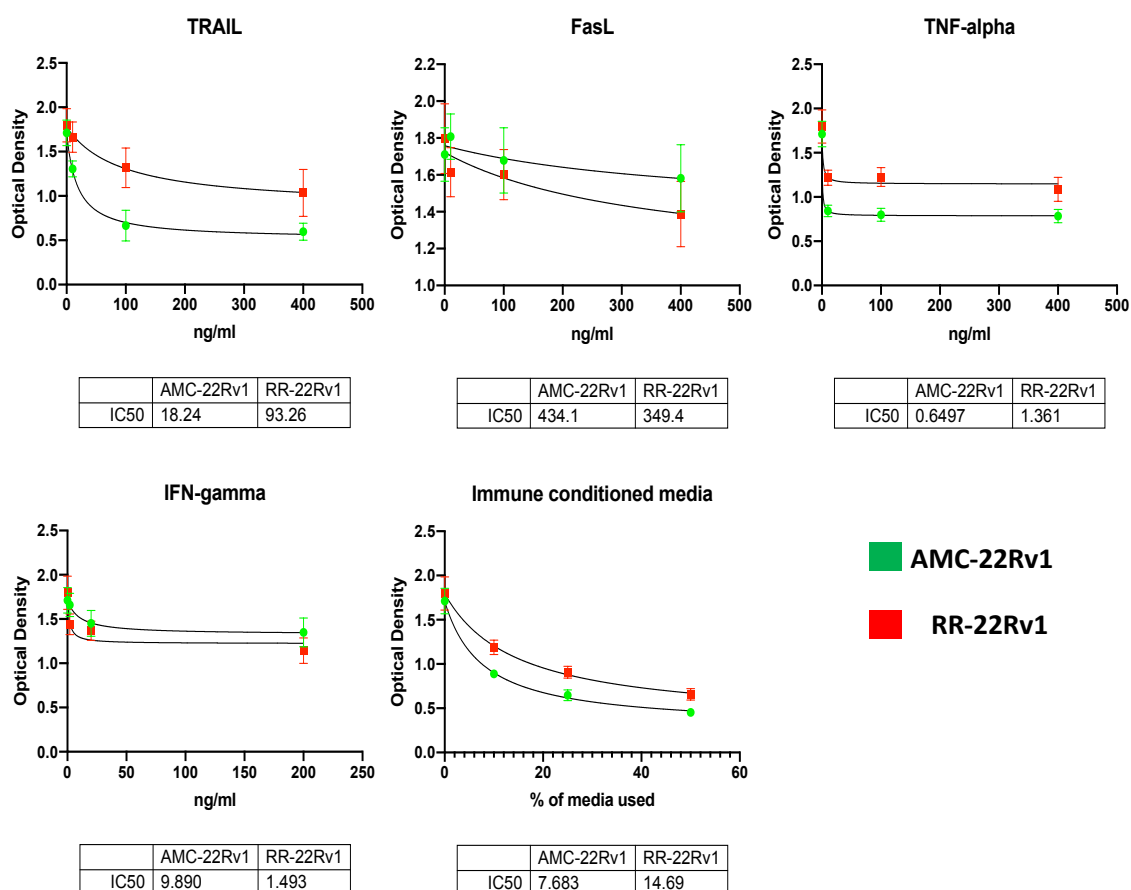


Figure 4-7 Radiation resistant cells were not more sensitive to soluble death-receptor ligands or cytostatic cytokines.

Tumor cells were seeded in 96 well plates and treated with varying doses of TRAIL, FasL, TNF-alpha, IFN-gamma, and immune cell conditioned media for 7 days. Crystal violet was added to each well and optical density was taken to determine cell mass. Two independent experiments.

4.2.6. Radiotherapy sensitizes cells to soluble death-receptor ligands and IFN-gamma

Next, we examined the effects of 6 Gy radiation combined with soluble death receptor ligands or IFN-gamma to determine if RT alters the viability of our cell lines. Others have shown that death receptors on tumour cells increase following RT leading to increased sensitivity to death-receptor ligands. All treatments inhibited the growth of both cell lines following 6 Gy RT (**Figure 4.8A**). We found that FasL sensitivity was increased in both cell lines following 6 Gy RT (**Figure 4.8A**) and that RR-22Rv1 cells were more sensitive (**Figure 4.8C**) than the control line (**Figure 4.8B**).

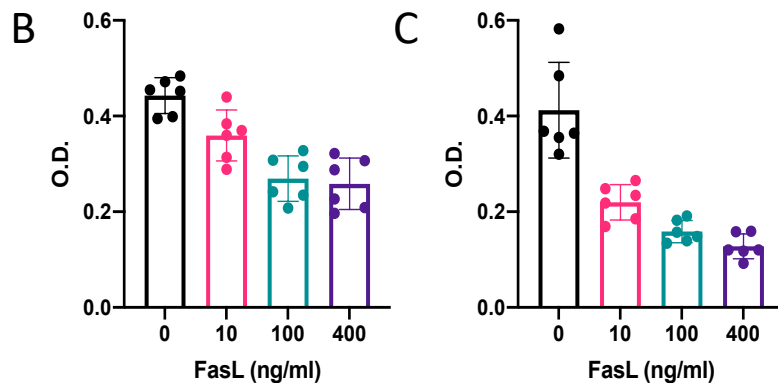
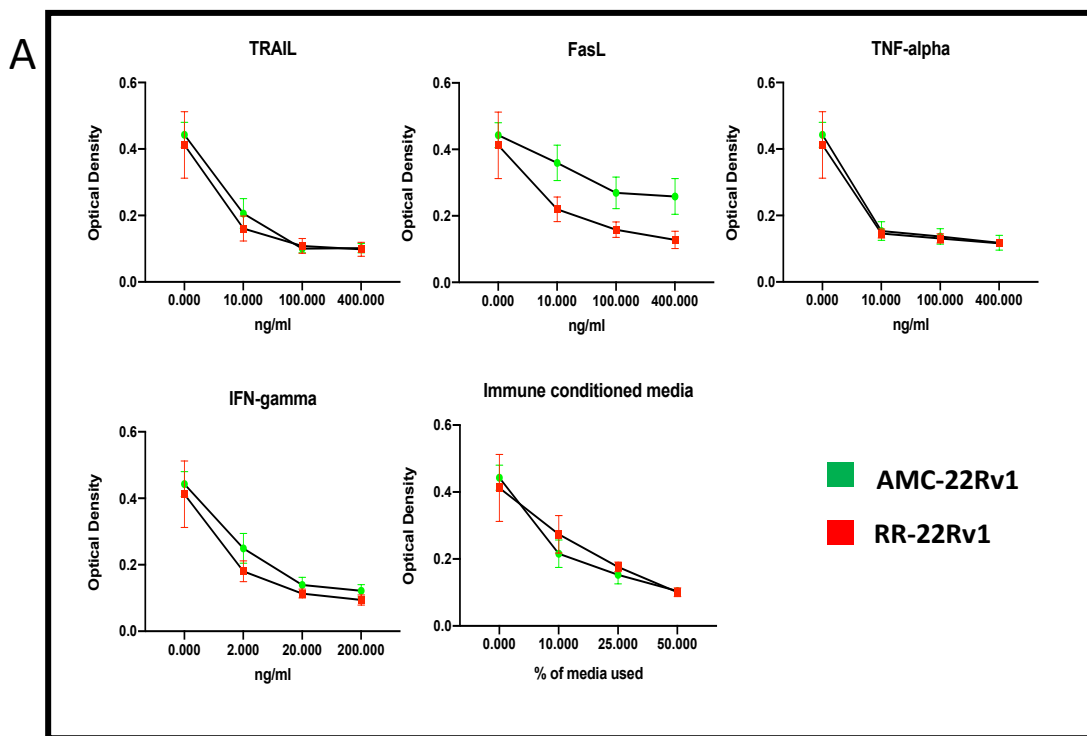


Figure 4-8 Radiotherapy sensitizes cells to soluble death-receptor ligands and cytostatic cytokines.

A) Optical density following 7 days of treatment with TRAIL, FasL, TNF-alpha, IFN-gamma or immune cell conditioned media in combination with 6 Gy radiation. B) FasL and 6 Gy RT in AMC-22Rv1 cells (ANOVA, $p < 0.0001$). C) FasL and 6 Gy RT on RR-22Rv1 cells (ANOVA, $p < 0.0001$).

4.2.7. Preliminary studies show no additional NKL activity with an anti-TIGIT blocking antibody

Live imaging studies were next used to measure NKL cytotoxicity in both cell lines treated with anti-TIGIT blocking antibody or isotype control. PVR binds with both activating DNAM-1 and inhibitory TIGIT on NK cells. Limited anti-TIGIT antibody inventory prevented repeat tests, a single run showed no effect with our antibody, however, we cannot extrapolate meaning from this and more experiments should be performed **(Figure 4.9)**.

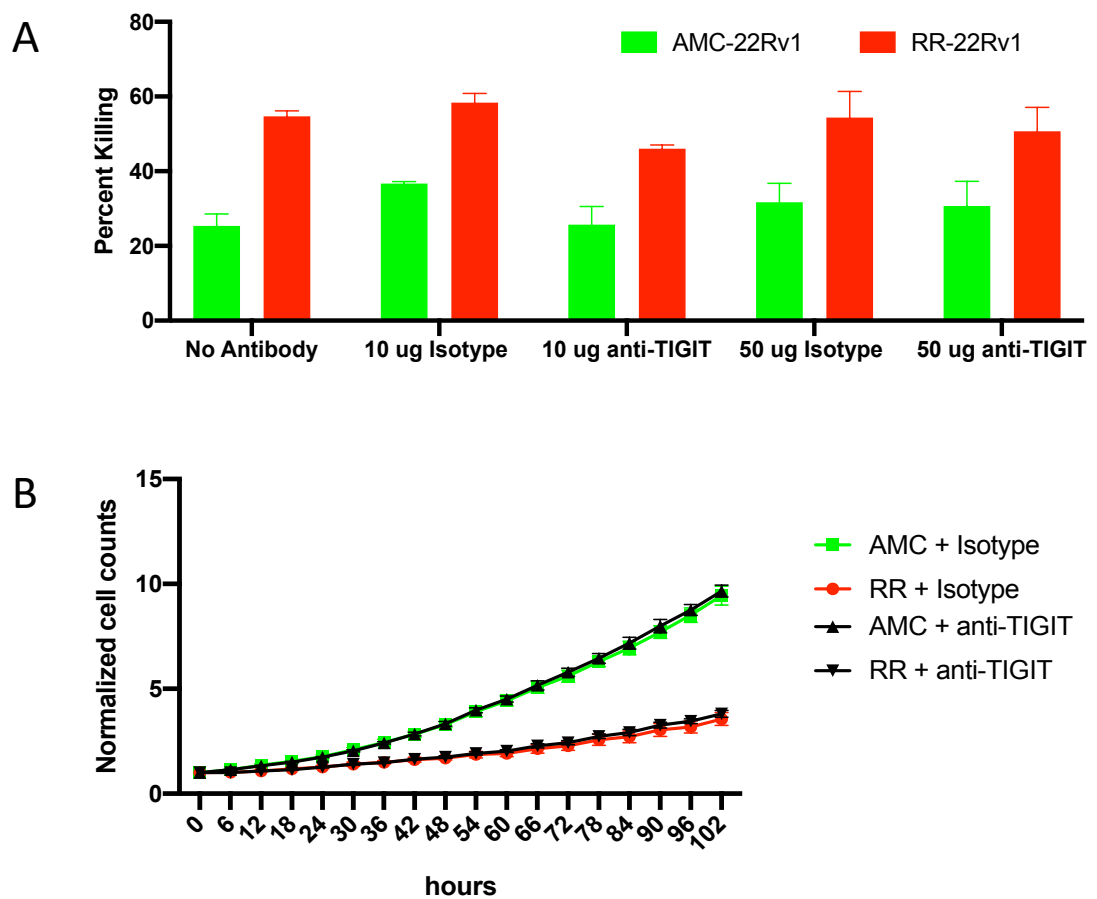


Figure 4-9 TIGIT blocking antibody had no effect on NK cell cytotoxicity in preliminary study.

A) Percent killing by NKs on tumor cells with isotype controls and anti-TIGIT blocking antibody after 96 hours. B) Growth of RR-22Rv1 (Red) and AMC-22Rv1 (green) treated with NK +/– anti-TIGIT (black lines) or isotype control (colored lines). One individual experiment with three technical replicates.

4.2.8. IL18 inhibition did not alter the effects of NKL therapy or radiotherapy

Early in our study we ran full proteomic analysis on the two cell lines (data not shown). Limited availability allowed for only a preliminary study to help guide additional research. The results suggested an increase in IL18 expression in the RR-22Rv1 cells compared to the control. IL18 has been shown by others to increase radiotherapy resistance. Additionally, IL18 can enhance the activation of NK cells. Therefore, we investigated the effects of IL18 inhibition with IL18 binding protein (IL18BP) on NKL therapy and radiotherapy. Live imaging data after 4 days demonstrated no effects of IL18BP on NKL effectiveness (**Figure 4.10A**) on radiotherapy effectiveness (**Figure 4.10B**), or on combination therapy (**Figure 4.10C**).

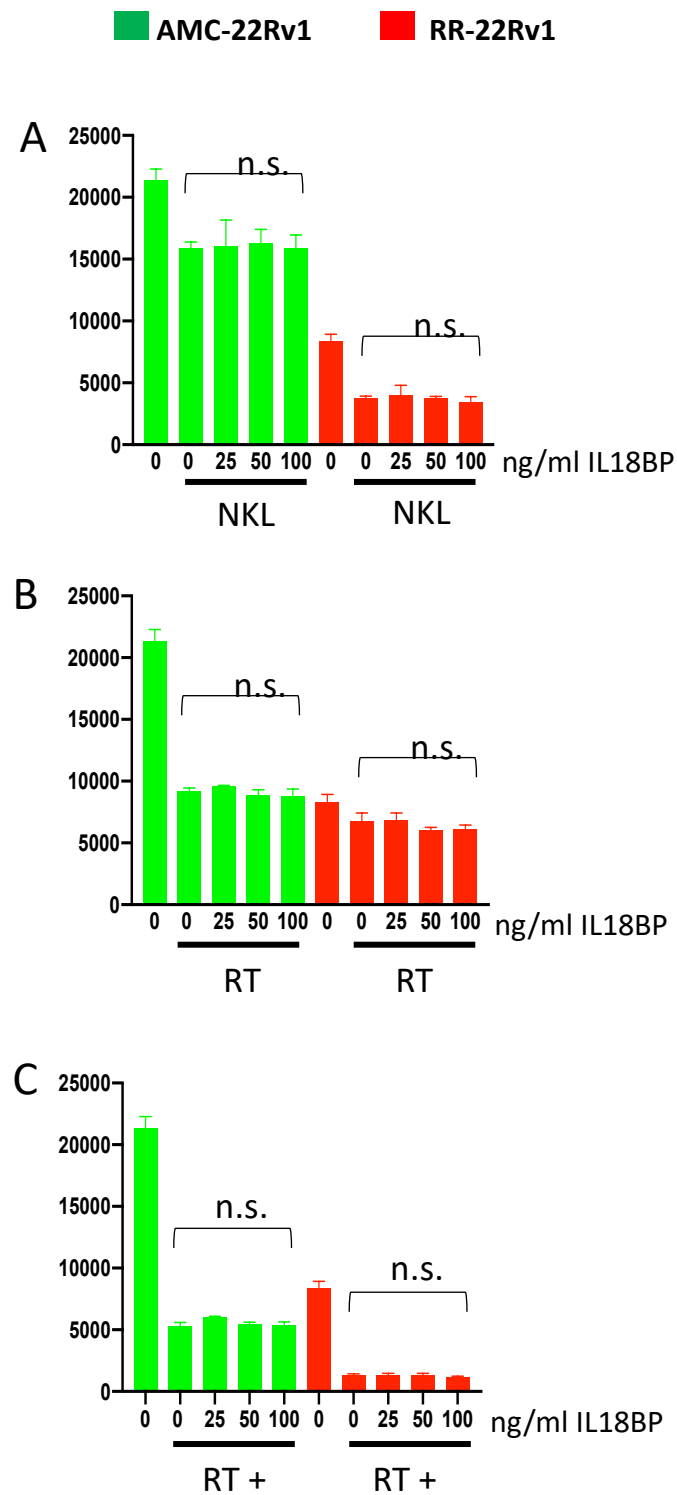


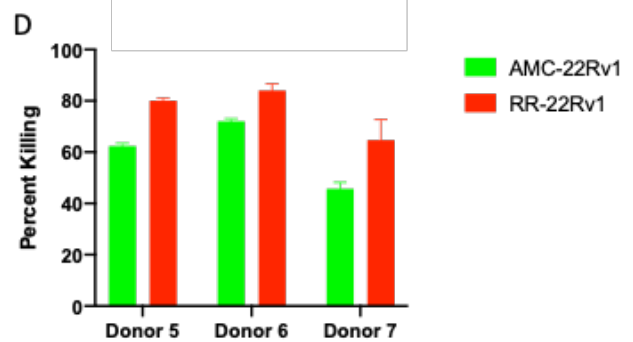
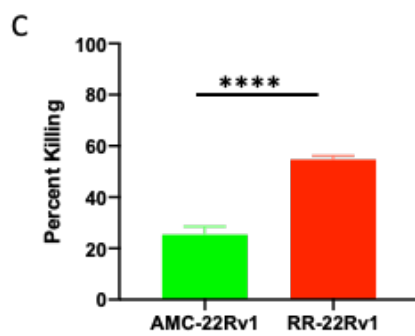
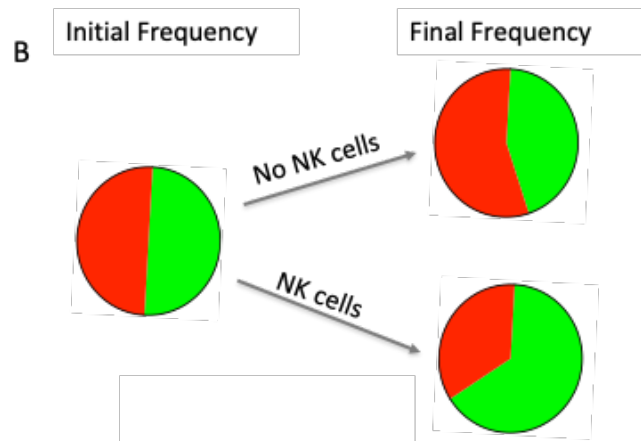
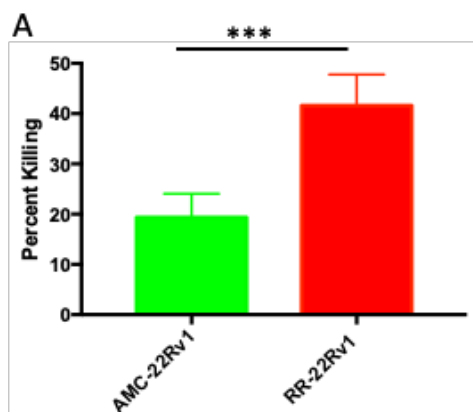
Figure 4-10 IL18 inhibition did not alter the effects of NK therapy or radiotherapy

AMC-22Rv1 (green) and RR-22Rv1 (red) were treated with various amounts of IL18BP and NKL cells (A) or 6 Gy radiation (B) or NKs and radiotherapy (RT) (C).

4.2.9. NK cells selectively kill radiation-resistant cells in dual culture implicating a double bind

The above NK cell cytotoxicity assays were performed by placing a single tumour cell line in a dish and challenging it with NKL cells and then comparing amount of tumour cell kill between the samples. This demonstrated that NK cells have stronger cytotoxic effects against RR-22Rv1 cells compared to the radiation-sensitive control line (AMC-22Rv1). We next wanted to know if this difference would remain when both tumour lines were present in the same dish. Therefore, we tagged each cell line separately and plated both populations at a 50:50 ratio and challenged the mixed cultures with NKL cells and measured amount of cell kill of each line. Again, RR-22Rv1 cells were more sensitive to NKL cells and the control line was more resistant to NKL mediated killing even in the presence of RR-22Rv1 cells after 24 hours ($p < 0.001$) (**Figure 4.11A**). Furthermore, we calculated the change in proportions of the radiation-resistant cell line and the radiation-sensitive cell line after 24 hours without treatment or treatment with NKL cells. In only 24 hours, treatment with NKL increased the portion of radiation-sensitive cells, AMC-22Rv1, from 44% to 65% and decreased to proportion of radiation-resistant, RR-22Rv1, cells from 56% to 35% (**Figure 4.11B**).

Next, we measured the effects after 96 hours with NKL and found the differential NKL sensitivity remained between the two cell lines (**Figure 4.11C**). We also found similar results after 96 hours treated with healthy donor PBMCs stimulated with IL2, IL12, and IL15 (**Figure 4.11D**).



AMC-22Rv1 RR-22Rv1

Figure 4-11 NK cells selectively kill radiation-resistant cells in dual culture implicating a double bind.

Percent killing of AMC-22Rv1 (green) and RR-22Rv1 (red) by NKL cells in mixed cultures containing 50:50 AMC:RR after 24 hours (A) ($p < 0.001$) and after 96 hours (C) ($p < 0.0001$). NKL cells shift the proportion of cells in favor of radiation-sensitive AMC-22Rv1 cells after 24 hours culture (B). Percent killing of AMC-22Rv1 (green) and RR-22Rv1 (red) by healthy donor PBMCs stimulated with IL1, IL12, and IL18 in mixed cultures containing 50:50 AMC:RR after 96 hours (D). AMC-22RV1 and RR-22Rv1 were stained with different cell tracking dyes and co-cultured at a 50:50 ratio with or without NKL cells and 200 IU IL-2 for 24 hours. Viability was measured with Zombie Aqua Fixable viability dye. Percent killing was determined as CellTrace positive and viability dye positive out of total CellTrace positive for each cell line. Morphology and doublet discriminator gates were used (A-B). Three independent experiments, unpaired t test. (C-D) Incucyte was used to count viable cells after 96 hours culture with NKL or PBMCs. Percent killing was calculated as $\frac{\text{untreated} - \text{treated}}{\text{untreated}} \times 100$. t-Test ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.2.10. Proportion of AMC:RR cells is altered by treatment

Since RT selectively kills the radiation-sensitive cells (AMC-22Rv1) and NKL cells selectively kill the radiation-resistant cells (RR-22Rv1) we next tested if NKL cells applied after RT would shift the proportion of tumour cells in favour of radiation sensitivity. Cells were tagged with CellTrace and mixed at a 50:50 ratio *in vitro*. These cultures were either left untreated, treated with NKL cells, irradiated (15 Gy), or both. RT was given on day 1 and NKL cells were given for the last 24 hours of culture on day 4. NKL given alone significantly increased the proportion of the radiation-sensitive population ($p < 0.0001$) (Fig X). RT given alone increased the proportion of radiation-resistant cells, RR-22Rv1 ($p < 0.001$). When NKL were given following RT the proportion of radiation-resistant cells was again reduced ($p < 0.01$) (Fig X). This demonstrates that, in short term cultures, applying NK cells to mixed cultures after RT can restore the radiation-sensitive population suggesting that a tumour treated with NK cells would likely be more susceptible to a second round of RT than if the tumour received no NK cells.

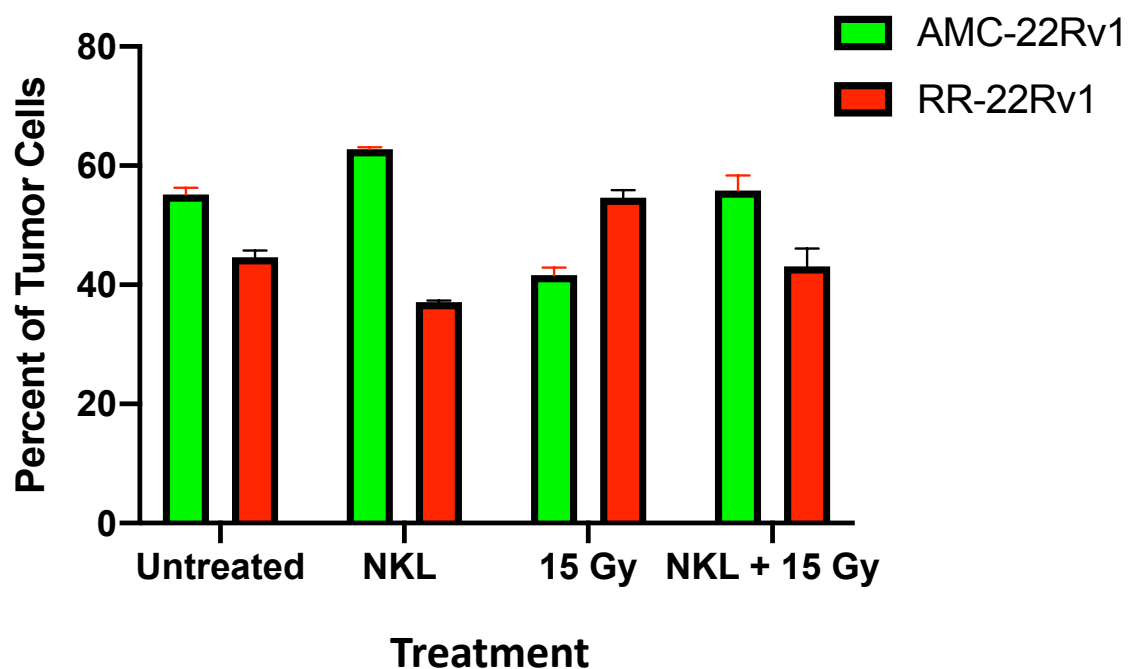


Figure 4-12 Proportion of AMC:RR cells is altered by treatment.

Percent of surviving cells following treatment. Tumor cells were fluorescently tagged and placed in culture at a 50:50 ratio of radiation-sensitive to radiation-resistant cells. Wells were either left untreated, treated with NKL cells, treated with 15 Gy radiation, or with both. Radiation was given on day 1. NKL were given on day 4 and all cells were harvested on day 5. Viability was measured with Zombie Aqua Fixable viability dye. Data was acquired with BD LSR II cytometer and analyzed with FlowJo software. Morphology, doublet discriminator, and viability gates were used.

4.3. Discussion

Prostate cancer patients have a range of treatment options with favourable outcomes for the majority of patients. However, advanced disease is typically metastatic and currently incurable (135). Radiation therapy (RT) is often used in hormone-refractory prostate cancer with initially positive results (155). However, the evolution of radiation resistance is a frequent cause of treatment failure. Our aim in this chapter was to identify an evolutionary double bind in radiation-resistant prostate cancer that can be exploited with immunotherapy. Our previous chapter highlighted the importance of NK receptor ligands on tumour cells during the evolution of the tumour. Additionally, studies have shown that these same stress-induced ligands are increased on tumour cells during genotoxic stress induced by therapy (222, 225). We hypothesized that radiation resistance would alter the expression of NK receptor ligands on tumour cells and render them more susceptible to NK cell-mediated killing.

The DNA damage response alters expression of NKG2D and DNAM-1 ligands in tumour cells (225). Therefore, we explored the first part of our hypothesis, radiation-resistant tumour cells would have altered NK cell receptor expression, in figure 4.3. We showed a significant increase in PVR, MICA/B, and CD112 expression in our radiation-resistant cell line. Additionally, we quantified ligand expression changes in the two cell lines after radiation (and fractionated radiation) in figures 4.4 and 4.5. The second part of our hypothesis, altered ligand expression would render radiation-resistant cells more susceptible to NK cell mediated killing, was demonstrated in figure 4.6 where tumour cells were challenged with NK cells *in vitro*. To explore this finding further we measured sensitivity to soluble death-receptor ligands in the two cell lines (Figures 4.7 and 4.8).

An increased sensitivity to soluble ligands in the absence of NK cells would indicate that radiation-resistant cells were simply more sensitive to soluble factors secreted by active NK cells. The role of TIGIT blockade and IL-18 on the differential effects of NK cell mediated killing (Figures 4.9 and 4.10) was measured in preliminary assays to determine if additional treatments could enhance or alter NK cell killing in our model as these are both used clinically. Killing assays in cultures containing mixed populations of radiation-sensitive and radiation-resistant 22Rv1 cells were performed (Figures 4.11 and 4.12) to determine if activation of NK cells by one cell type could alter the killing rates of the other cell type. A strong double bind requires two treatments that target different cell populations independently. If activation of NK cells by RR-22Rv1 increased killing of AMC-22Rv1 cells the double effect would have been weak.

We determined that NK ligand expression is altered in our isogenic radiation-resistant prostate cancer cell line model and that this difference increases with additional RT. Genotoxic stress induced by chemotherapy and radiation have been shown to increase NK ligand expression in other cells (222). The radiation-resistant line is also more susceptible to killing by an immortalized NK cell line and primary immune cells freshly isolated from healthy donors. This suggests that further exploration into the use of NK cell-based immunotherapy in prostate cancer is warranted. Additionally, we show that when cultures containing radiation-sensitive and radiation-resistant cell lines are treated with NK cells the proportion of radiation-resistant cells is reduced and the radiation-sensitive cells dominate the population.

Inversely, when the same mixed cultures are treated with RT, the radiation-sensitive cell line is reduced, and the radiation-resistant line dominates the population. Interestingly, when NK cells are given after RT, the proportion of radiation-resistant cells is diminished compared to RT alone. This indicates a true double bind (117) where the second treatment targets the resistant population and preserves the sensitive population and supports the use of RT and NK cells in an evolutionary double bind.

Tumour cells express various activating receptors not seen in most normal cells (107) (226). MHC class I chain-related protein A and B (MICA and MICB), and UL16 binding proteins (ULBP) are ligands for the activating receptor NKG2D present on CD8 T cells, NK cells, NKT cells, and subsets of $\gamma\delta$ T cells (227) (228). MICA and MICB are highly polymorphic surface proteins with differential expression in many tumour types. Soluble MICA and MICB can also be cleaved from tumour cells; however, this results in immune evasion (229, 230). ULBP expression is induced in non-tumour cell lines through activation of DNA damage response pathways (187). ULBP expression is blocked by inhibition of ATM or ATR (225). Malignant cells constitutively express many ULBPs due to chronic activation of DNA repair machinery (231, 232) (187). We only measured the constitutive expression of ULBPs on our cell lines and did not measure the effects after radiation, so there is potentially a transient effect by RT.

Major histone compatibility complex (MHC) proteins are a family of surface glycoproteins that present peptide antigens on the cell's surface. MHC class I (MHC-I) molecules, expressed by all nucleated cells and present peptides to CD8 T cells. Additionally, MHC-I molecules are negative regulators of NK cell activity (32). Ligation

of killer cell immunoglobulin-like receptors (KIR) on NK cells by MHC-I inhibits NK cell function. MHC-I expression is modulated in tumour cells following radiation therapy (233) (234). Here, the baseline expression of MHC-I was the same in both cell lines. RT increased the expression of MHC-I in both lines 24 and 48 hours after 10 Gy RT. This was not detected in our hypofractionation study done eight days after RT. Furthermore, MHC-I was highly sensitive to culture conditions. Untreated controls are overgrown in long assays and may affect MHC-I expression unrelated to RT treatment.

Programmed death-ligand -1 (PD-L1) is a negative regulator of the immune response. Expressed by antigen-presenting cells and inflamed epithelial cells, PD-L1 binds with its receptor (PD-1) on NK and T cells, inducing immunosuppression (58). Tumour cells often escape immune surveillance by exploiting this pathway (235). Inhibitors of the PD-1/PD-L1 axis have revolutionized the treatment of many cancer subtypes. Notably, temporal expression of PD-L1 is often inducible and transient. Baseline expression of PD-L1 was low in both cell lines and increased following 10 Gy RT.

Poliovirus receptor (PVR) (CD155) was first identified as the receptor for human poliovirus (236). PVR is a transmembrane protein involved in cell-cell adhesion, immune activation/evasion, and migration. PVR is overexpressed by many cancer cells and is associated with decreased survival [73]. PVR (CD155) and CD112 (Nectin 2) are ligands for activating receptor DNAM-1 (CD226) and inactivating receptor T-cell Immunoreceptor with Ig and ITIM domains (TIGIT) (236). TIGIT, expressed by T cells and NK cells, binds tumour-derived PVR resulting in immune inactivation and tumour escape (237). The PVR/TIGIT axis is an appealing pathway in many cancer subtypes (236).

Interestingly, PVR is upregulated by ATM and ATR, key mediators of the DNA damage response and may enhance proliferation rates in ras-mutated cancer cells [74, 75].

Cytotoxic lymphocytes induce apoptosis in target cells through the perforin/granzyme B pathway and death receptor ligation. We examined the effects of death ligands on our cell lines. RR-22Rv1 were not more sensitive to soluble death ligands than AMC-22Rv1 cells. This indicates the increase in NK sensitivity of the RR-22Rv1 cells is likely dependent on interactions with NK cells. We did not measure differential sensitivity to the perforin/granzyme B pathway. Tumour cells can develop resistance to granzyme B through overexpression of PI-9 (238). RR-22Rv1 cells may have downregulated PI-9 expression.

Fas expression on tumour cells is upregulated by radiotherapy (239). *In vitro* treatment with FasL was more effective in our cell lines after RT. RR-22Rv1 cells had the most increases in sensitivity to FasL after RT. We did not measure Fas expression. However, this may likely result in a further increase in NK sensitivity in the RR-22Rv1 cells.

We explored RT resistance in a single prostate cancer line. Our *in vitro* studies have not tested different sequences of treatment and lack context of the tumour microenvironment. In the future, our study should be expanded to include additional cell lines and tested with additional treatment sequences. The tumour microenvironment can negatively affect NK cell function. To examine the effects of different environmental conditions the culture conditions of our current *in vitro* system can be changed to include, low pH, reduced glucose, or reduced oxygen concentrations.

In vivo options should also be considered as tissue carrying capacity, diverse nutrient loads, and the presence of host stromal cells can alter population dynamics.

NK based immunotherapies are still in the clinical testing phase and have not been approved for wide clinical use. In this study, we used one immortalized NK cell line, NKL. The primary immortalized line being investigated for clinical applications is the NK92 cell line which has some important differences. However, NKs are considered excellent models for *in vitro* (240). NK cell studies NK cell-based immunotherapies are a diverse and promising frontier in immune-oncology (38) (34, 241). Our findings here support further exploration of the radiation-induced NK cell double bind. Our next chapter will expand on these findings using *in vitro* cell competition assays with our radiation-sensitive and radiation-resistant cell lines.

Chapter 5. Exploiting a radiotherapy-induced NK
cell double bind to restore radiation sensitivity in
advanced prostate cancer

5. Chapter 5. Exploiting a radiotherapy-induced NK cell double bind to restore radiation sensitivity in advanced prostate cancer

5.1. Introduction

Cancer therapies are conventionally given at a maximum tolerated dose. This is designed to kill the maximum number of cancer cells. However, the potential for this strategy resulting in a cure requires two things to be true; 1) there are no resistant cells already residing within the tumour and 2) no novel resistant phenotypes will emerge during therapy (151). Unfortunately, in many cases resistance does emerge. However, identifying a novel target in resistant cells offers an additional therapeutic option to delay or prevent a recurrence.

5.1.1. Evolutionary double bind

An evolutionary double bind occurs when resistance to one therapy has a fitness cost that increases the sensitivity to a second therapy. Akin to the owl/snake example outlined in chapter 1, our theoretical model employs radiation as the first therapy (the owl), and NK cells (the snakes) are then used to exploit the evolutionary consequences of radiation resistance (**Figure 5.1**). Currently, when a tumour responds to a therapy, that treatment is used until the tumour stops responding. From an evolutionary perspective, repetition of a single treatment modality will select for 100% resistant cells removing all sensitive cells and any ability for the tumour to respond to the therapy in the future (**Figure 5.2A**). Identification of a resistance mechanism that increases

sensitivity to the immune system creates a double bind that can be used to control the outgrowth of resistant cells and maintain treatment sensitivity (**Figure 5.2B**).

Furthermore, if the two therapies are highly effective at combating resistance and inducing cell death, there is potential to eradicate all cancer cells. This model was motivated by an ongoing clinical trial in prostate cancer designed to exploit the proliferative cost of resistance (135). Zhang et al. employed an adaptive therapy approach that uses patient-specific growth dynamics to determine on/off schedules of Abiraterone to suppress the outgrowth of androgen-independent cancer cells. The adaptive therapy group receives Abiraterone until their prostate-specific antigen (PSA) drops by 50%, then Abiraterone is removed to allow the sensitive cells to remain. Abiraterone is given again when PSA rises. Compared to maximum tolerated dose schedules, patients receiving adaptive therapy had an average 20-month increase in time to progression. At the same time, the cumulative amount of drug given was reduced by half (135, 146).

Other non-continuous dosing strategies including intermittent dosing schedules and treatment holidays have been explored in other cancer types with mixed results (242-244). A large randomized phase II trial in BRAF mutated melanoma with continuous or intermittent (3 weeks off followed by 5 weeks on) BRAF and MEK inhibitors failed to meet its primary endpoint (244). However, interim results presented at the 2022 American Society of Clinical Oncology meeting of a phase II trial of intermittent dosing in metastatic colorectal cancer found an increased progression free survival on treatment of 17.6 months for intermittent dosing compared to 12.6 months for continuous dosing (243). The key difference in the prostate and colorectal cancer trials

compared to the melanoma trial was patient specificity. The melanoma trial used treatment schedules based on successful preclinical mouse models while the other trials relied on individual patient progression to restart therapy (135, 243, 244).

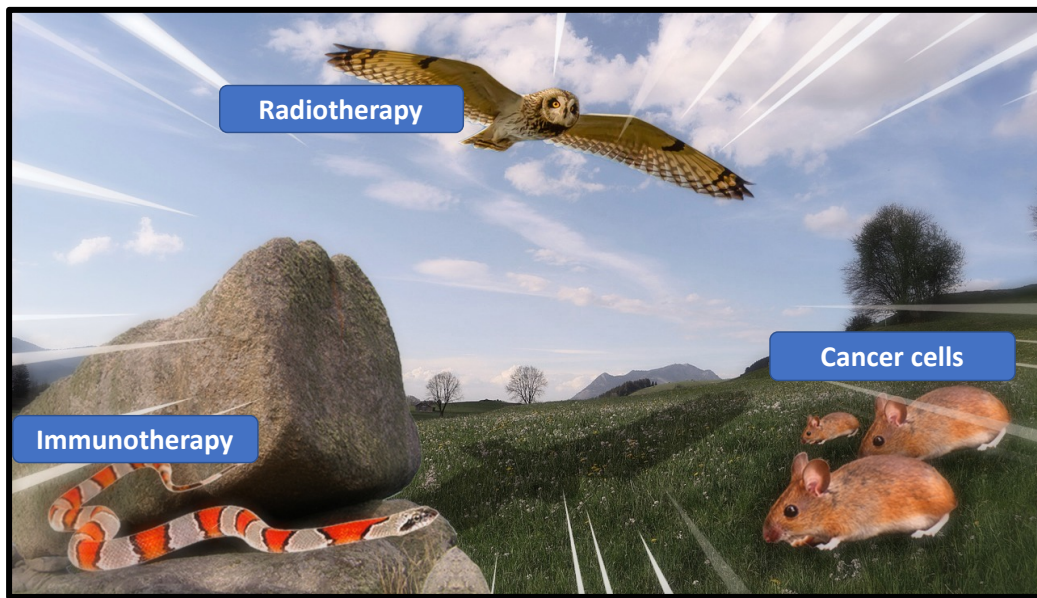


Figure 5-1 Modified evolutionary double bind model

We altered the owl/snake metaphor used to explain an evolutionary double bind in mice to demonstrate how a double bind may be exploited in cancer therapy. The cancer cells are the prey. Radiotherapy, represented by the owl, will kill the radiation-sensitive cells. The radiation-resistant cells will survive but at a cost that makes them more sensitive to immunotherapy.

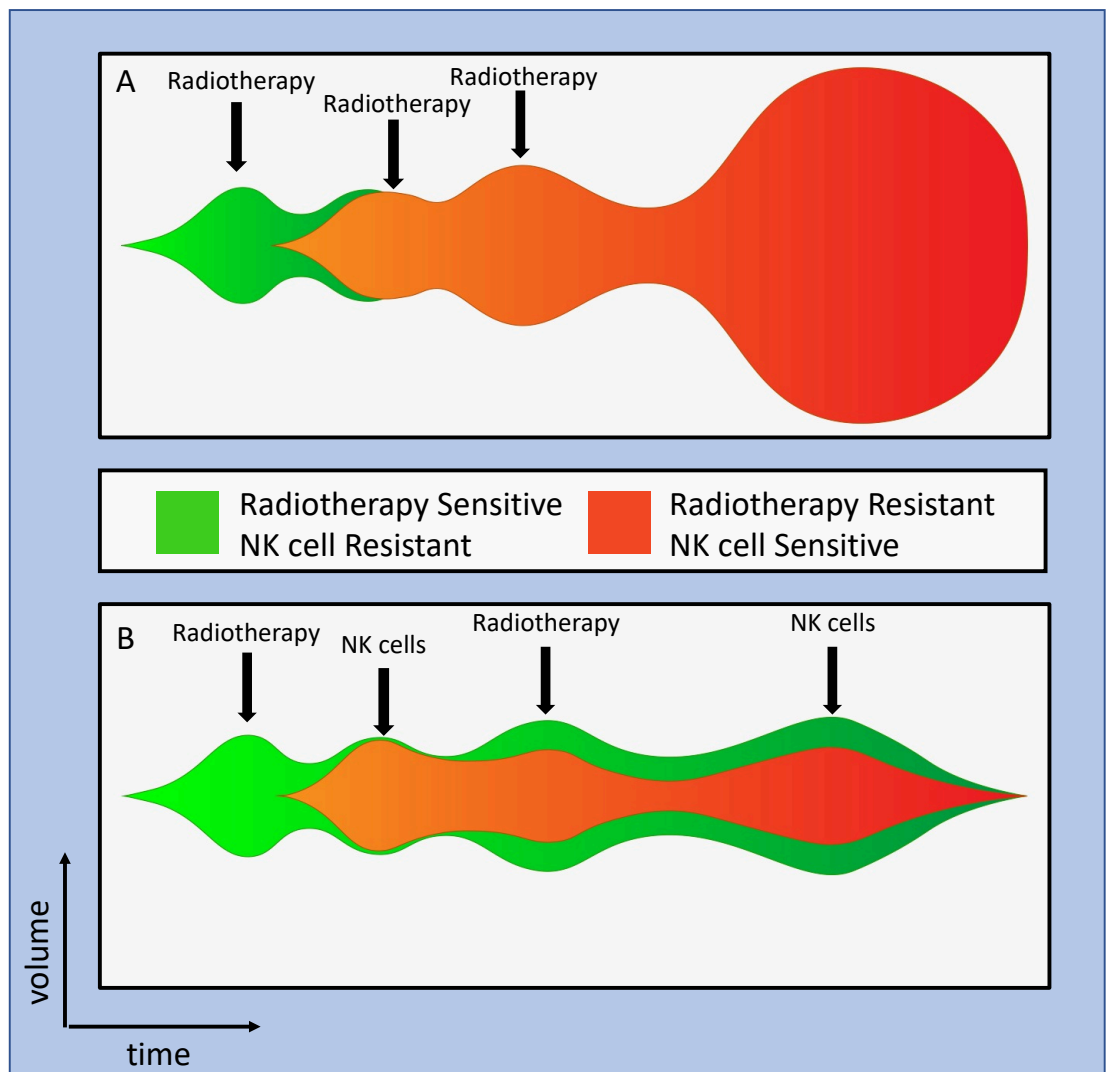


Figure 5-2 Evolutionary guided cancer therapy is designed to suppress the emergence of resistance.

A) Represents standard clinical practice, the same therapy is applied until the tumor no longer responds. This leads to a large tumor with a fully resistant population. B) Evolutionary guided therapy (EGT) exploits the evolutionary double bind between RT resistance and NK cells to both reduce tumor volume and control the outgrowth of resistant populations.

5.1.2. Double bind and the immune system

Immunotherapy is an attractive tool for exploiting the evolutionary consequences of treatment resistance. The key advantages are; 1) immune cells kill targets through a diverse array of mechanisms 2) the fitness costs of adaptive strategies by tumour cells to evade the immune system are likely high, 3) immunotherapy works best when tumour populations are small, and 4) the immune system recognizes stressed cells.

5.1.2.1. *Diversity in killing*

Immune-based therapies activate the immune system by releasing an army of anti-tumour immune cells. In contrast, other cytotoxic therapies, including chemotherapy and radiation therapy, initiate targeted toxic effects directly against cancer cells. Immune cells proliferate, become activated, and signal to other immune cells and tissues to participate in the response; chemotherapy does not. Typically genotoxic therapies have only a few mechanisms of action that contribute to cell death. However, the immune system is reactive and complex, with interacting systems designed to combat evolving species. This offers advantages over other treatment types in combating treatment resistance.

5.1.2.2. *The cost of predator evasion*

Ecological studies of invasive species focus on eradicating an organism that has taken over a niche destroying the native species. A successful eradication strategy often favours the introduction of a predator over the use of toxic chemicals because adaptations to predators, parasites, and pathogens are generally more costly than adaptations to noxious chemicals (218). This is due to the non-lethal effects of predation

on replicative fitness in evasive individuals. For example, the adaptive strategy of prey running under a rock to avoid the owl, even in the absence of the snake, would provide a negative non-lethal effect on fitness as the mouse cannot scavenge for food while hiding under the rock, thereby reducing nutrient uptake and decreasing reproductive output. Costly adaptive strategies decrease the likelihood that a resistant population will thrive. Immune evasive tumour cells will likely have a similar additional fitness cost that can be exploited in a double bind.

5.1.2.3. Effector to target ratios in cancer immunology

Immune effector cells bind with and actively kill target cells. This dependence on cell-cell interactions limits the rate at which a single immune cell can kill target cells. Furthermore, cytotoxic immune cells can kill a finite number of target cells. These limitations set a minimum number of effector cells required to kill a population of tumour cells. The effector to target ratio is the number of cytotoxic immune cells to tumour cells. High effector to target ratios leads to better outcomes in cancer patients.

Using immunotherapy as a second therapy to combat resistance offers the advantage of enhanced effector to target ratios. The first therapy destroys the bulk of the tumour leaving behind a small population of cells resistant to the first therapy. Employing immunotherapy to remove this small population would require fewer effector cells. First, this means immunotherapies that would be ineffective as first-line therapy, when placed up against a large population of cancer cells, would be more effective against a smaller population. Secondly, cancer immunotherapy can cause severe adverse events

in patients. Reducing the dose would limit side effects and allow the patient to stay on therapy longer.

5.1.2.4. Recognizing stress

After the first treatment is applied, resistant cancer cells often display signs of stress that the immune system can recognize. Stress-induced ligands are expressed on the surface of viral infected and malignant cells. Natural killer cells are cytotoxic innate lymphoid cells that lack clone specific variable receptors. Instead, they rely on diverse activating and inhibitory receptors for target recognition (47). Activating receptor NKG2D, constitutively expressed by NK cells, binds with stress-induced ligands, including; MICA, MICB, and ULBPs. Similarly, DNAM-1 on NK cells is activated by ligation with stressed-induced ligands, PVR and PVRL2 (225, 236, 245).

Genotoxic stress caused by chemotherapy and radiotherapy increases the expression of stress-induced ligands on malignant cells (246). Stress-induced ligands are upregulated following activation of the DNA damage repair pathway (247). Additionally, cytosolic DNA caused by DNA damage activates STING/TBK1 and downstream IRF3 inducing NKG2D ligand expression (247). Other cancer therapies, including protease inhibitors and histone deacetylase inhibitors, also upregulate NK activating ligands on cancer cells. This broad response to current cancer therapies makes NK cell-based immunotherapies an attractive tool for exploiting treatment resistance

5.1.3. Optimizing an effective double bind

An evolutionary double bind in cancer allows the oncologist to guide the evolutionary outcomes by choosing which treatments to give and when contingent on the underlying dynamics of the two populations. An ideal double bind would have two key outcomes, 1) reduce tumour burden and 2) control the outgrowth of the treatment-resistant population (**Figure 5.1**). To achieve this, the system must have the following dynamics:

- A successful first treatment option that often leads to resistance
- The resistance comes at a cost
- The second treatment would selectively target the resistant population
- Evolutionarily, it is best if the therapies kill through different mechanisms
- The two populations must compete for space and resources
- The two populations must have differential fitness peaks during therapy

In the previous chapter, we showed that radiation resistance comes at a cost and that we can target this cost with NK cells. We show that NK cells selectively target the radiation-resistant cells and it is known that radiation and NK cells kill through different mechanisms. **Here, we hypothesize that the increased sensitivity to NK cell-mediated killing in the radiation-resistant population can be exploited to restore the radiation-sensitive population by employing an evolutionary double bind treatment strategy.**

We use the same isogenic cell line model from the previous chapter. Briefly, the parental prostate cancer cell line, 22Rv1, was evolved under selection by 2 Gy fractions of RT for a total of 60 Gy. The resulting cell line, RR-22Rv1, is resistant to RT compared to the

control line, AMC-22Rv1. We designed competition experiments based on ecological methods to study cell line interactions under various treatment doses and combinations (RT and NKL cells). We aimed to determine the growth and treatment response dynamics of the two cell lines grown in isolation and co-cultured at various frequencies. We transduced the cells to express nuclear fluorescent trackers throughout the experiments to track the populations. Flow cytometry and live imaging techniques were used to determine population changes over time.

We found that the RR-22Rv1 cells have a reduced proliferation rate when cultured alone compared to the parental line. Interestingly, this proliferative cost of resistance was reduced when RR-22Rv1 cells were cultured with the control line indicating an interaction between the two cell lines. Furthermore, RR-22Rv1 cells suppressed the growth of the control line, AMC-22Rv1.

These interesting growth dynamics were altered when therapy was applied and varied depending on which therapy was given. Radiotherapy was effective at reducing the overall tumour number. However, RT selected for the radiation-resistant cell line. NKL cells were more effective at reducing overall tumour number and selected for the radiation sensitive line. This demonstrates a strong evolutionary double bind between radiotherapy resistance and NK sensitivity.

We also found that when RT was given before NK cells, the effectiveness of NK mediated killing increased leading to a dramatic reduction in overall tumour number. Importantly, RT followed by NK cells selected for the radiation sensitive population in all seeding

frequencies, however, was most effective when there was a high number of radiation-resistant cells present. Our findings demonstrate the utility of NK cell based therapies in the radiation resistant setting even when a patient may have developed near complete RT resistance.

5.2. Results

5.2.1. GFP and mCherry expression can be used for long term culture

To explore the growth dynamics of the isogenic cell lines in various conditions, we performed viral transduction for stable expression of nuclear GFP in the AMC-22Rv1 cell line and nuclear mCherry expression in the RR-22Rv1 cell line. Both cell lines expressed the fluorescent tags well in both 2D (**Figure 5.3 A**) and 3D culture (**Figure 5.3 B**). Interestingly, AMC-22Rv1 cells form tighter spheroids than the RR-22RV1 cells when cultured alone in separate hanging drops and form many small clusters when cultured together in 3D (**Figure 5.3B**). Changes in growth dynamics and integrin expression has been detected in other cell lines following prolonged passaging and development of drug resistance and may contribute to the different clustering effects of the AMC and RR-22Rv1 cell lines. While this finding could be explored to investigate other pathways involved in RT resistance the different clustering dynamics would complicate NK cell killing assays if this 3D model was used, therefore, we performed only 2D assays going further.

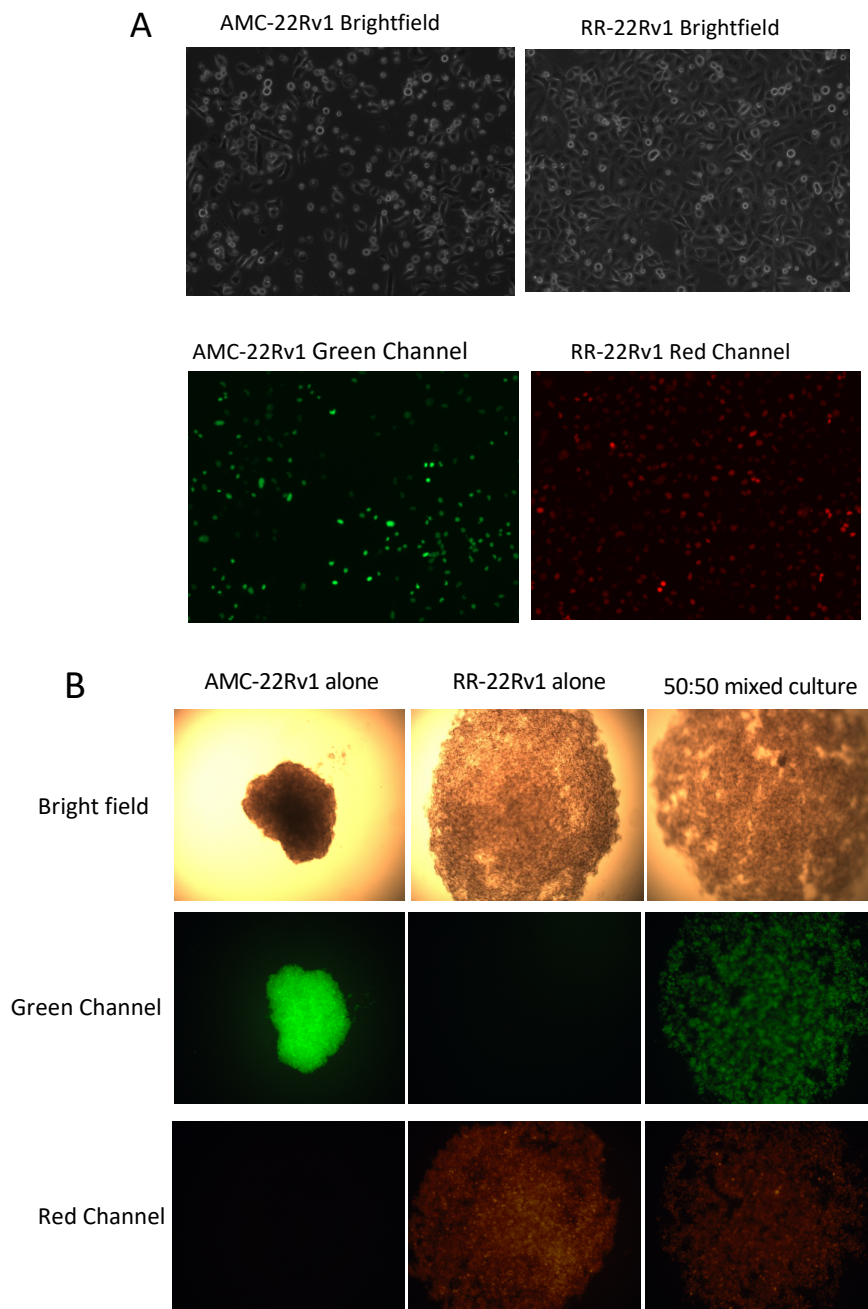


Figure 5-3 Stable expression of nuclear GFP or mCherry in AMC-22Rv1 and RR-22Rv1 cell lines.

AMC-22Rv1 and RR-22Rv1 cells were transduced to express GFP and mCherry (respectively). Cells in 2D (A) as well as 3D culture (B) show positive expression. Viral vectors were a kind gift from Murysky lab (Moffitt Cancer Center). Positive cells were selected with puromycin and maintained in low dose puromycin throughout culturing.

5.2.2. Competition experiments were designed to evaluate the population dynamics of mixed cultures under different conditions

Competition experiments are utilized to simulate the heterogeneity of phenotypes within a tumour. They allow for quantification of any interactions between the populations. If a double bind is robust the fitness of each cell type will be different depending on treatment applied. If there are any interaction effects between the two cell lines the growth dynamics will be different when cultured together. Here we would expect radiation sensitive cells (AMC-22Rv1) to have decreased fitness after RT and increased fitness during NKL treatment (compared to radiation resistant cells, RR-22Rv1). Therefore, competition experiments were designed to measure changes in fitness between the two cell lines when cultured together under various conditions. The conditions were treatment with NKLs and RT. Depending on interaction effects, the competition dynamics between two cell types can be frequency dependent. Therefore, we designed the experiment to include AMC-22Rv1 and RR-22Rv1 cells at various seeding ratios while maintaining a fixed seeding density (**Figure 5.4A**). All ratios are described as AMC:RR. To determine the effects of RT and NKL, four treatment groups were planned; 1) untreated, 2) NKL alone (5 to 1 effector to target ratio), 3) 6 Gy RT alone, and 4) NKL and 6 Gy RT in combination (**Figure 5.4B**). The effects of RT are not immediate. To account for this and allow time for NKL cell mediated killing, longer competition assays were designed (**Figure 5.4C**). Radiation was given prior to adding NKL to avoid radiation effects on NKL cells. Cell counts with CountBright beads and cell frequencies were measured on days 5 and 12 by flow cytometry.

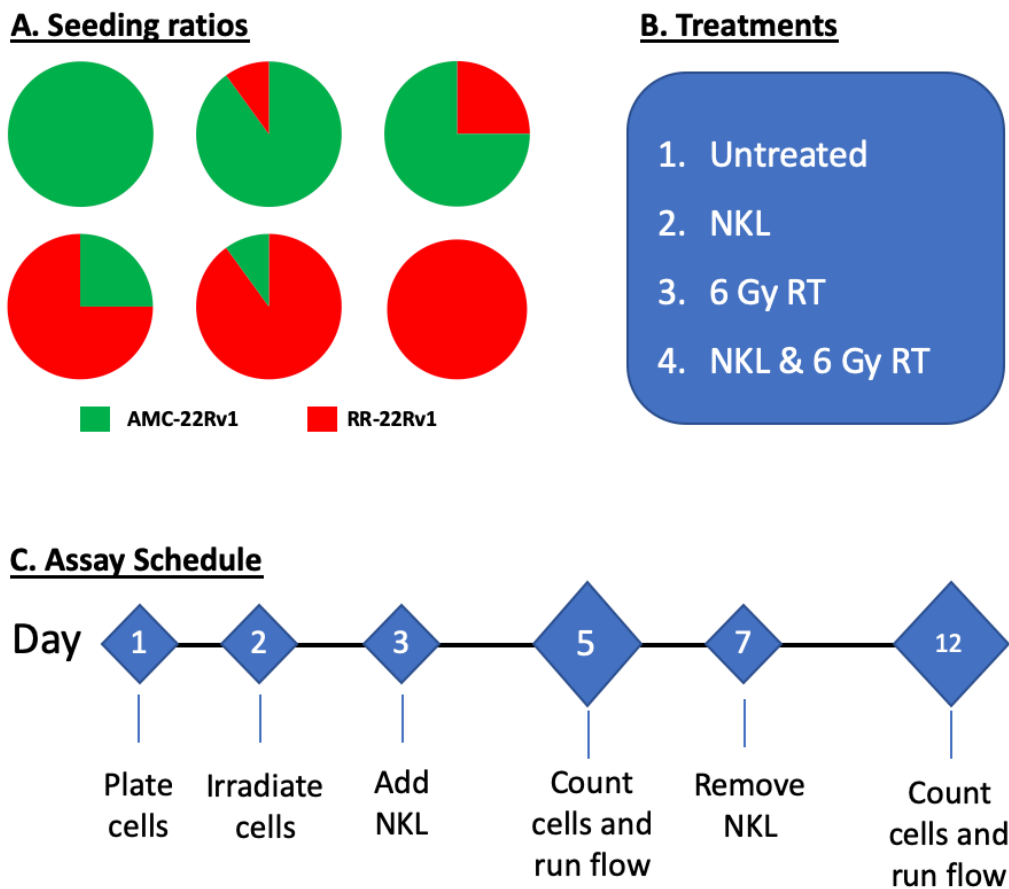


Figure 5-4 Experimental design for flow cytometry based competition experiments

A) Six ratios of cell types were seeded at the same density to determine any frequency dependent effects on fitness. B) Four treatment groups were included, NKL cell line was used at an effector to target ratio of 5:1. Radiation therapy (RT) was given as a single 6 Gy fraction. C) The assay schedule was designed to avoid direct RT effects on NKL cells and to monitor fitness changes following RT and NKL treatments. Four independent experiments were performed.

5.2.3. Two drugs are better than one: Combination of radiation and NKL therapy was more effective than either treatment given alone

Whole population cell counts were performed using CountBright beads on day 5 and day 12 in the assay designed above. The various ratios used are pictured (**Figure 5.5A**). On day 5 radiation therapy (blue bars) was more effective than treatment with NKL (pink bars) (effector to target ratio 5:1). Combination therapy was most effective in all seeding ratios (purple bars) (**Figure 5.5B**). Day 5 data was recorded while NKL cells were still present. NKL cells were removed on Day 7 and tumour cells were given 5 days to regrow. This growth window was used to determine the interaction effects between the two cell lines following therapy. Combination therapy remained the most effective therapy following the regrowth period (**Figure 5.5C**). However, there was a ratio dependent effect on rate of regrowth. Samples initially seeded with higher frequency of RR-22Rv1 cells proliferated more quickly than samples with higher frequency of radiation sensitive cells, AMC-22Rv1, indicating a complex competition effect where the presence of one cell line may be inhibiting the other following combination therapy. Cell counts alone cannot elucidate these interactions, therefore, further analysis using flow cytometry to differentiate between the populations was performed.

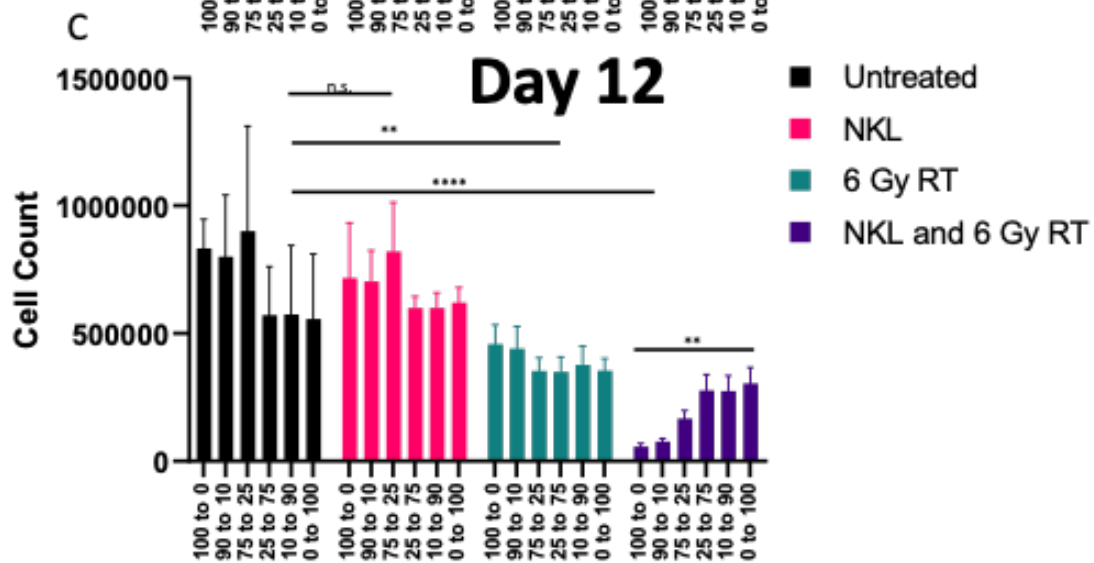
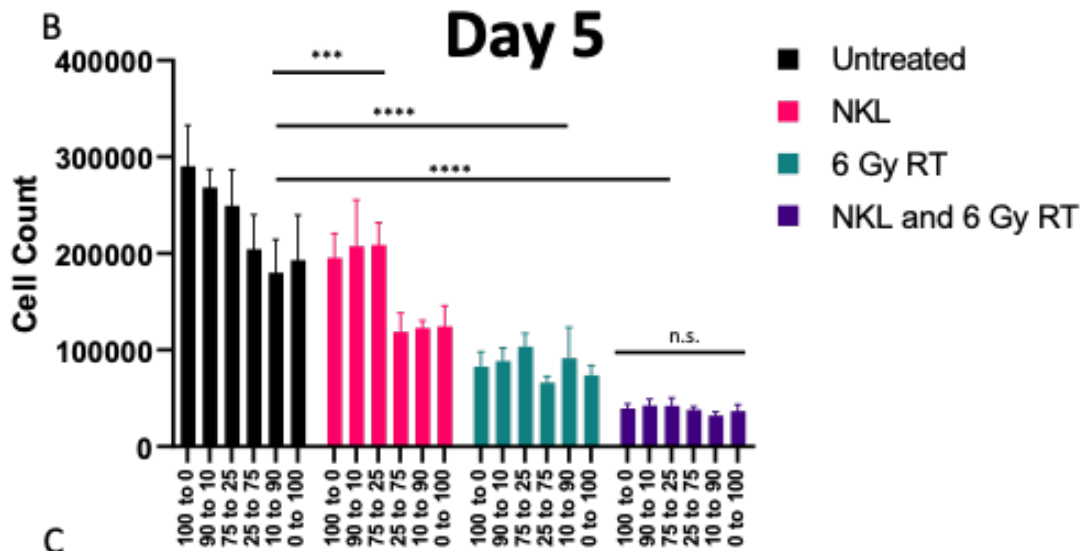


Figure 5-5 Combination therapy was more effective than single treatments at reducing overall tumor size.

Flow cytometry was used to quantify the number of tumour cells. A) Illustration of initial seeding frequencies used. Cells were harvest and counted on day 5 (B) and day 12 (C) Cell counts were measured using CountBright beads. Cell frequencies were measured as GFP positive/mCherry negative for AMC-22Rv1 and GFP negative/mCherry positive for RR-22Rv1 cells. Individual population cell counts were determined by multiplying the frequency by whole tumor counts. Four independent experiments, two-way ANOVA and one-way ANOVA.

5.2.4. Treatment effects the growth dynamics of the two cell lines

In addition to determining the effectiveness of combination therapy on mixed cultures we also wanted to determine the effects of treatment on the different populations of tumour cells. GFP and mCherry expression in our cell lines allowed us to quantify changes in population frequencies during the competition experiments seen in the previous two figures (**Figure 5.6B**). In the untreated condition radiation sensitive cells, AMC-22Rv1, proliferated more quickly than the radiation resistant cells when seeded at a high frequency ($\geq 75\%$) (**Figure 5.6C**). During the regrowth period (Day 7- Day 12) radiation resistant cells grew back more quickly than radiation sensitive cells. At high frequencies, (75% and 90%) radiation resistant cells suppressed the regrowth of radiation sensitive cells following combination therapy. Without treatment AMC-22Rv1 cells growth faster and dominate the population. After treatment AMC-22Rv1 cells are suppressed and RR-22Rv1 cells began to dominate to population (**Figure 5.6C**).

A. Initial seeding frequencies

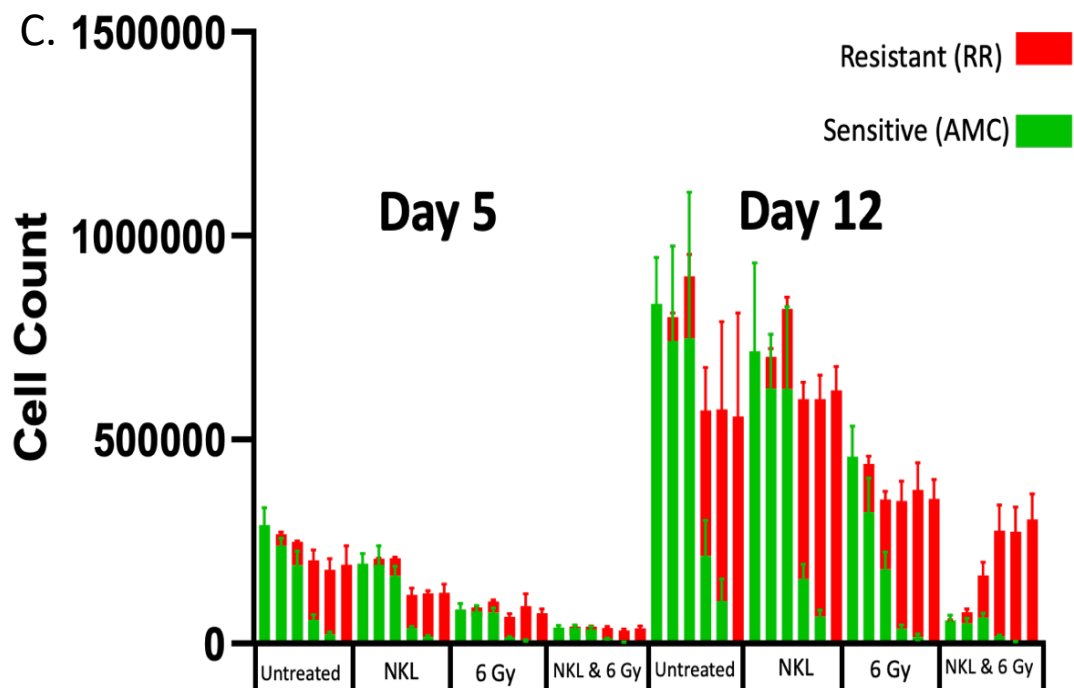
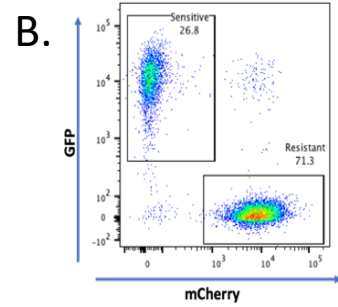
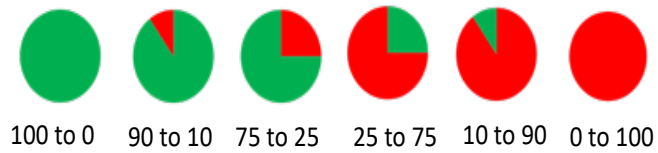


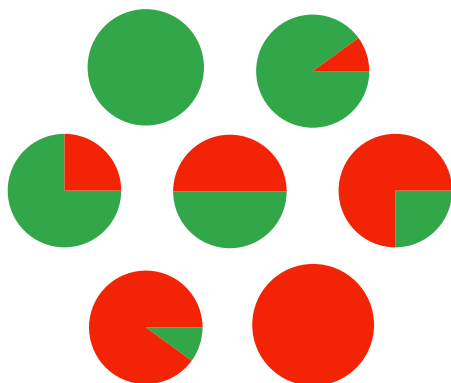
Figure 5-6 Treatment effects the growth dynamics of the two cell lines

Flow cytometry was used to quantify the number and type of tumor cells present following treatment. A) Illustration of initial seeding frequencies used. B) Sample of flow method used to differentiate the two populations. C) Day 12 measurements show that radiation sensitive cells outcompete radiation resistant cells in untreated conditions when seeded at high frequency. Following combination therapy radiation resistant cells outcompete and suppress regrowth of radiation sensitive cells at the doses given (5:1 NKL to tumor and 6 Gy radiation). Cell counts were measured using CountBright beads. Cell frequencies were measured as GFP positive/mCherry negative for AMC-22Rv1 and GFP negative/mCherry positive for RR-22Rv1 cells. Individual population cell counts were determined by multiplying the frequency by whole tumor counts. Four independent experiments

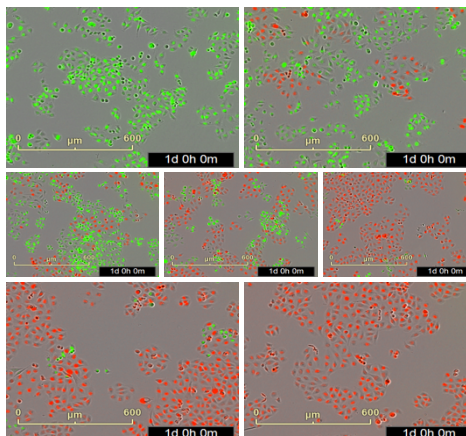
5.2.5. High throughput live imaging was used to quantify population dynamics under multiple treatment doses

The above experiments highlighted two key aspects of the cell lines. First, they respond best to combination therapy. Second, there are interaction effects between the lines that are dependent on seeding frequency. Therefore we needed to design more complex experiments to examine both treatment sensitivity and interaction effects of the two cell lines under various treatment doses. In addition to altered NK ligand expression the above assays hinted there might be an additional proliferative cost to RR-22Rv1 cells. To allow for more time points, more treatment doses, and additional seeding frequencies we designed a second set of competition experiments using live cell imaging (Incucyte). We used 7 seeding frequencies (**Figure 5.7A-B**) and 12 treatments combining 3 doses of RT (0 Gy, 2 Gy, and 6 Gy) with 4 ratios of NK cells (0:1, 1:1, 5:1, and 10:1 effector to target ratio) (**Figure 5.7C**).

A. Seeding ratios



B. Incucyte images



C. Treatments

RT	0	2 Gy	6 Gy
NKL ratio	0	0	0
RT	0	2 Gy	6 Gy
NKL ratio	1 : 1	1 : 1	1 : 1
RT	0	2 Gy	6 Gy
NKL ratio	5 : 1	5 : 1	5 : 1
RT	0	2 Gy	6 Gy
NKL ratio	10 : 1	10 : 1	10 : 1

Figure 5-7 Experimental design for Incucyte competition experiments.

A-B) Seven ratios of cells were seeded at the same density to determine any frequency dependent effects on fitness. C) Twelve treatment groups were included. RT was given prior to administration of NKL cells to avoid RT effects on immune cell function.

5.2.6. Radiation resistant cells inhibit tumour cell growth rates

We then examined the growth rates of each tumour cell population at the 7 seeding frequencies without treatment. Each well was seeded with the same number of total cells, therefore to compare how each line proliferated we normalized each timepoint to the initial number seeded (t_n/t_0). The radiation resistant cell line, RR-22Rv1, had a reduced growth rate compared to the control line when the cells were cultured in isolated wells (**Figure 5.8**). Co-cultures with AMC-22Rv1 increased the growth rate of radiation resistant cells when AMC-22Rv1 was included at high frequencies. Additionally, AMC-22Rv1 cells were inhibited when cultured with high frequencies of RR-22Rv1 indicating a negative interaction effect of RR-22Rv1 on both cell lines *in vitro*.

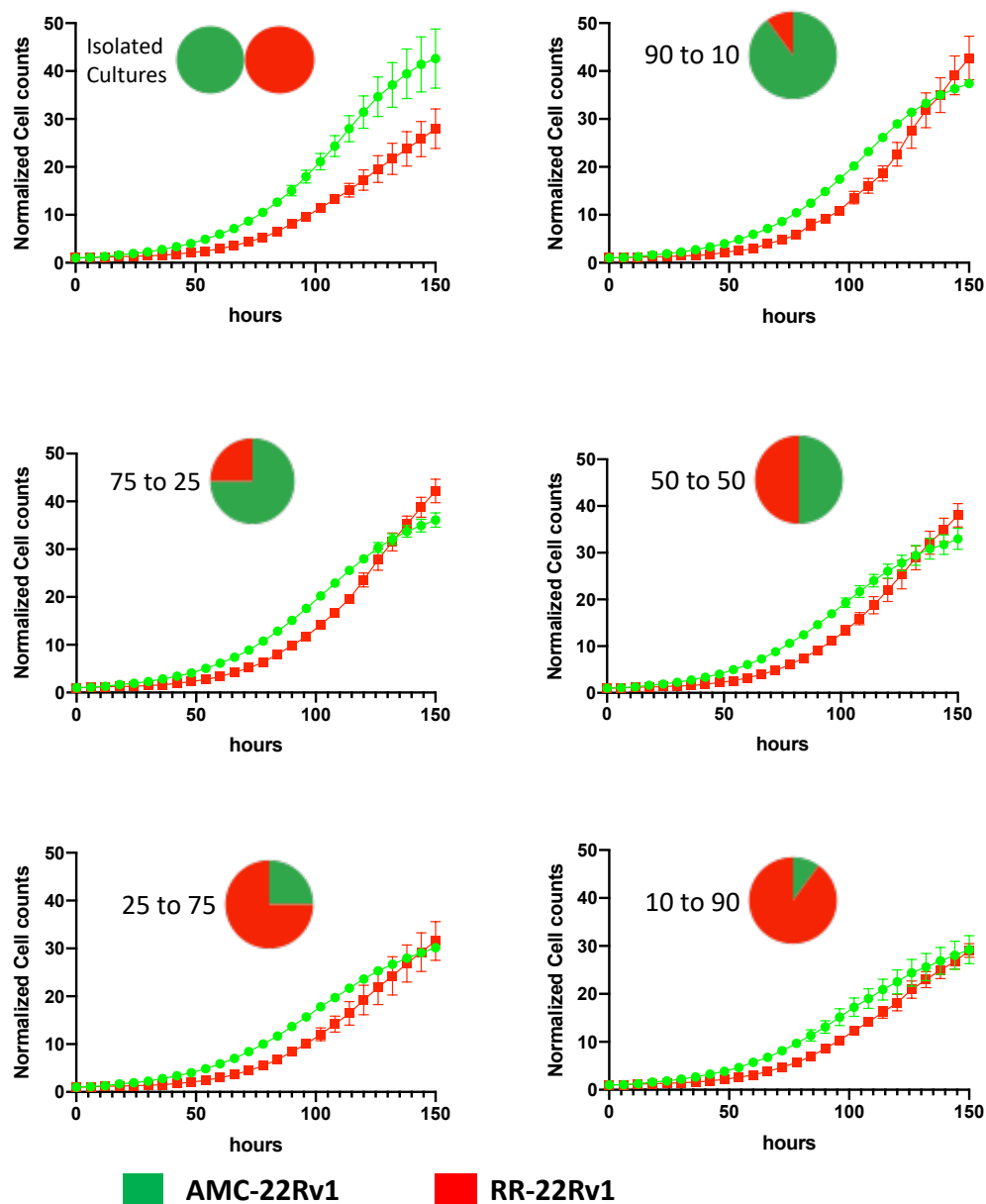


Figure 5-8 Radiation resistant cells inhibited tumor cell growth.

High frequencies of RR-22Rv1 cells inhibits cell growth indicating a negative interaction between the two cell types in untreated conditions. Incucyte data was recorded every 6 hours for 150 hours. GFP positive AMC-22Rv1 and mCherry positive RR-22Rv1 were counted based on nuclear staining. Normalized cell counts were used to account for different seeding numbers (t_n/t_0).

5.2.7. Radiation selects for radiation resistant cells at all seeding ratios

Next, we compared the different growth rates following RT (6 GY). Radiation suppressed the growth of both cell lines, however, AMC-22Rv1 cells were more inhibited than the radiation resistant line (**Figure 5.9**). The differential effects of RT on the two cell lines became apparent 3 days after RT was given. In all seeding ratios RT selected for the radiation resistant cell line. This effect was increased in co-cultures with high frequency of AMC-22Rv1.

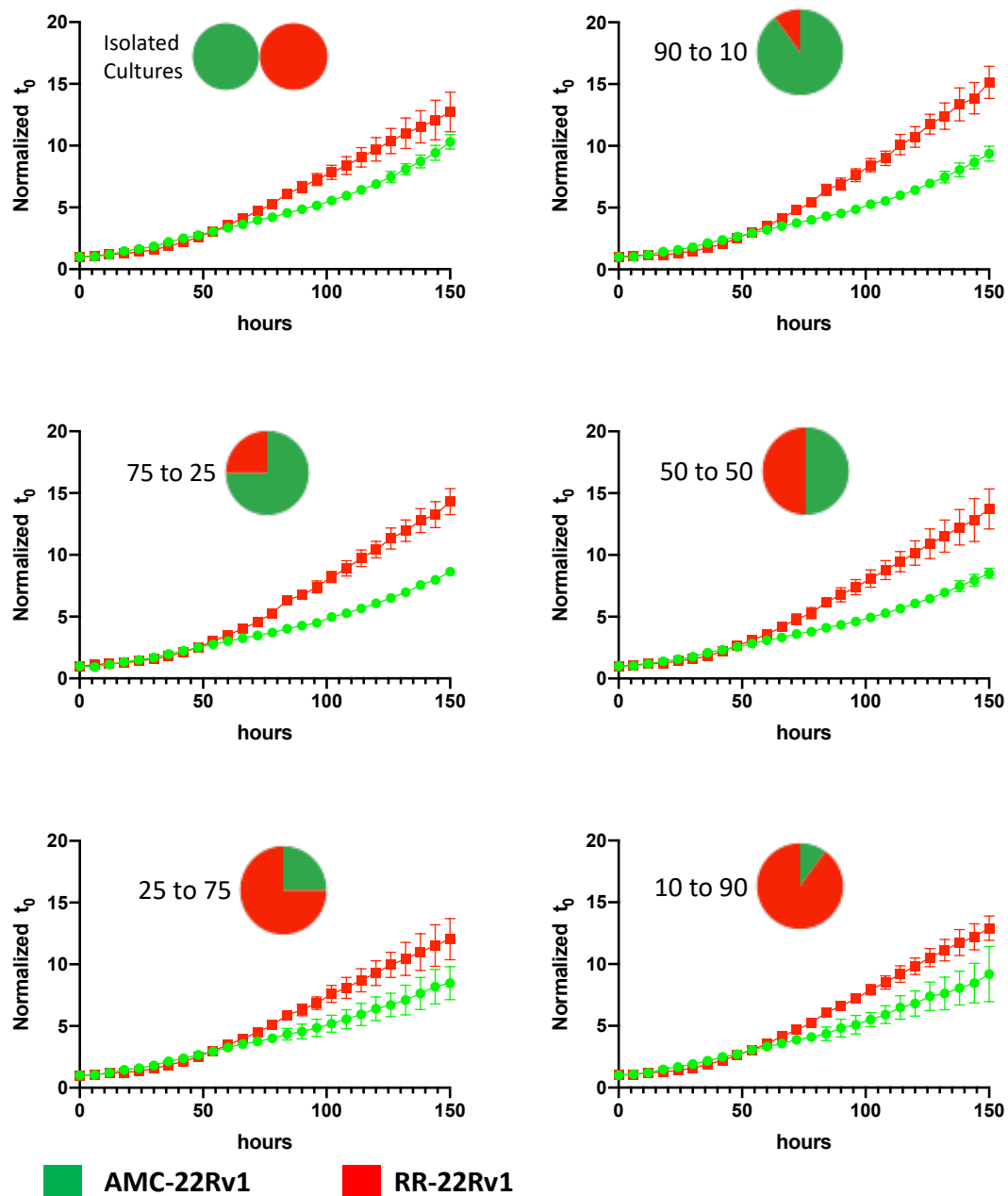


Figure 5-9 Radiation therapy selected for radiation resistant cells in all ratios

Radiation was given in a single 6 Gy dose. Incucyte data was recorded every 6 hours for 150 hours. GFP positive AMC-22Rv1 and mCherry positive RR-22Rv1 were counted based on nuclear staining. Normalized cell counts were used to account for different seeding numbers (t_n/t_0).

5.2.8. NKs selected for radiation sensitive cells

We next investigated the effects of NK therapy in our competition model. NK cells given at a 10:1 effector to target ratio selects for radiation sensitive AMC-22RV1 in all seeding frequencies (**Figure 5.10**). AMC-22RV1 cells continue to grow logistically throughout the assay. RR-22RV1 cells have dramatic decreases in growth rate allowing radiation sensitive cells to dominate the population.

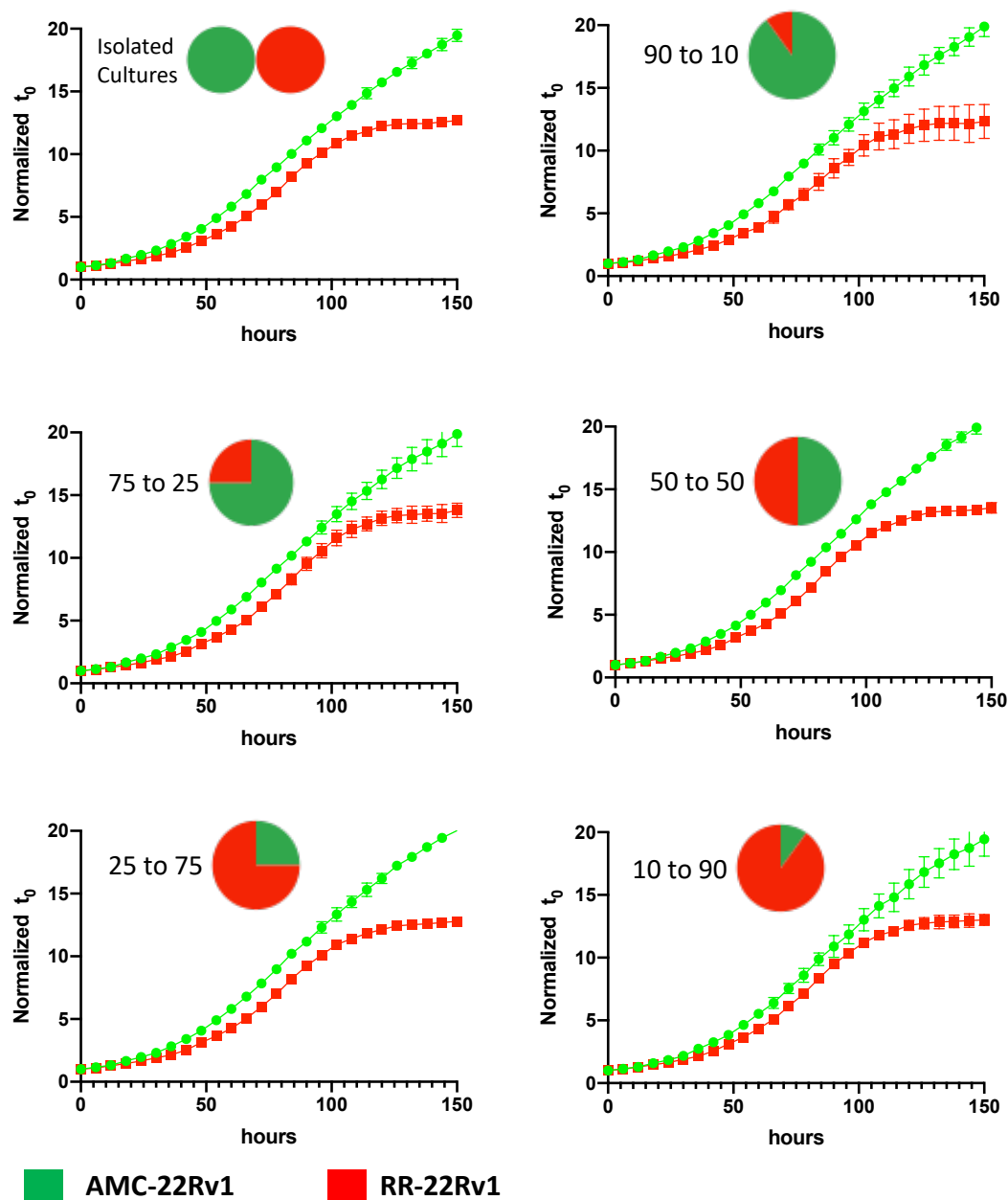


Figure 5-10 NKL cells selected for radiation resistant cells in all ratios

Immortalized NK cells, NKL cells, were given at a 10:1 effector target ratio. Incucyte data was recorded every 6 hours for 150 hours. GFP positive AMC-22Rv1 and mCherry positive RR-22Rv1 were counted based on nuclear staining. Normalized cell counts were used to account for different seeding numbers (t_n/t_0).

5.2.9. NKL therapy was more effective in samples containing a high frequency of RR-22Rv1 compared to those with higher frequencies of AMC-22Rv1 cells

To determine if seeding frequencies affected outcomes after treatment with NKL or RT we compared total population number over time with the different treatments. Following radiotherapy (6 Gy) there was no difference in total cell numbers between different seeding ratios (**Figure 5.11A**). Since NKL selectively kill RR-22Rv1 in mixed cultures and 22-Rv1 suppresses proliferation we found a ratio dependent effect in outcomes following NKL therapy (10:1 effector: target ratio) (**Figure 5.11B**).

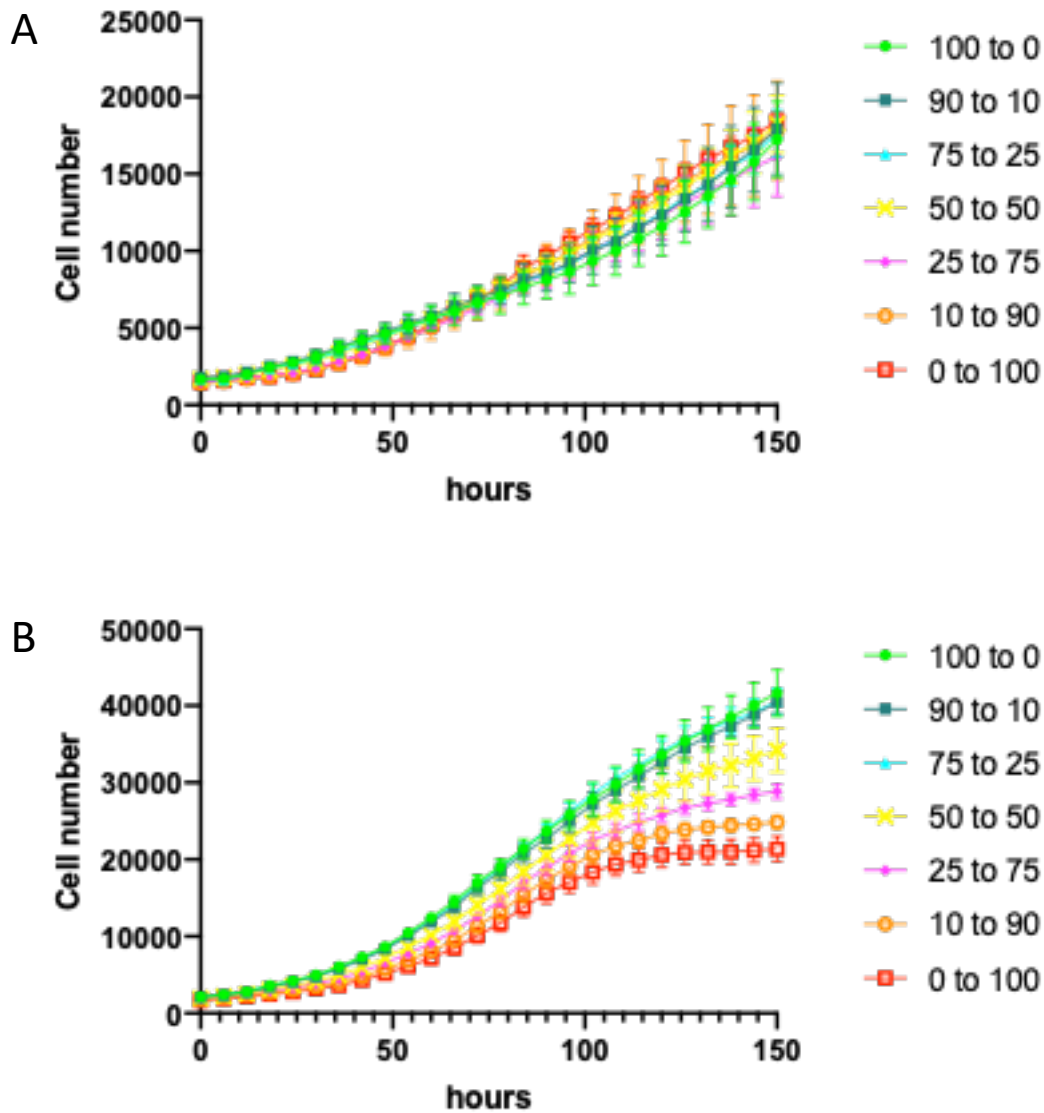


Figure 5-11 NKL therapy was more effective in samples containing a high frequency of RR-22Rv1 compared to those with higher frequencies of AMC-22Rv1 cells.

A) The effects of radiation (6 Gy) were not affected by seeding ratio. B) NKL therapy was most effective in samples with high frequency of RR-22Rv1 cells. Total cell counts of both populations were graphed over time for the seven different seeding ratios. Images were taken every 6 hours.

5.2.10. Radiation prior to NKL treatment increases NKL effectiveness in both cell lines

To determine the effects of sequential treatment we analysed the difference in NKL mediated killing following radiation (2 Gy and 6 Gy). We examined three effector to target (E:T) ratios (NKL:tumor cell) in both prostate tumour cell lines. In all cases RT increased the amount of NKL killing compared to no RT. A 6 Gy dose of RT had the largest impact on NK cell killing when NK cells were given at low E:T ratio in both cell lines (**Figure 5.12 A, D**). At high E:T ratios 6 Gy RT induced the largest increases of NKL mediated killing in AMC-22Rv1 cells, from 36% at 0 Gy to 70% at 6 Gy RT. (**Figure 5.12C**). At high E:T ratios, 6 Gy RT had a positive effect on NKL killing in RR-22Rv1 cells (72% at 0 Gy RT to 91% at 6 Gy RT). However, the amount of increase was less compared to the control line due to the intrinsically high killing percentage of 22Rv1 cells without RT (72%, **Figure 5.12F**). In all settings, except 0 Gy with low E:T ratio and 2 Gy RT with low E:T ratio, RR-22Rv1 cells were more sensitive to NKL cells than AMC-22Rv1.

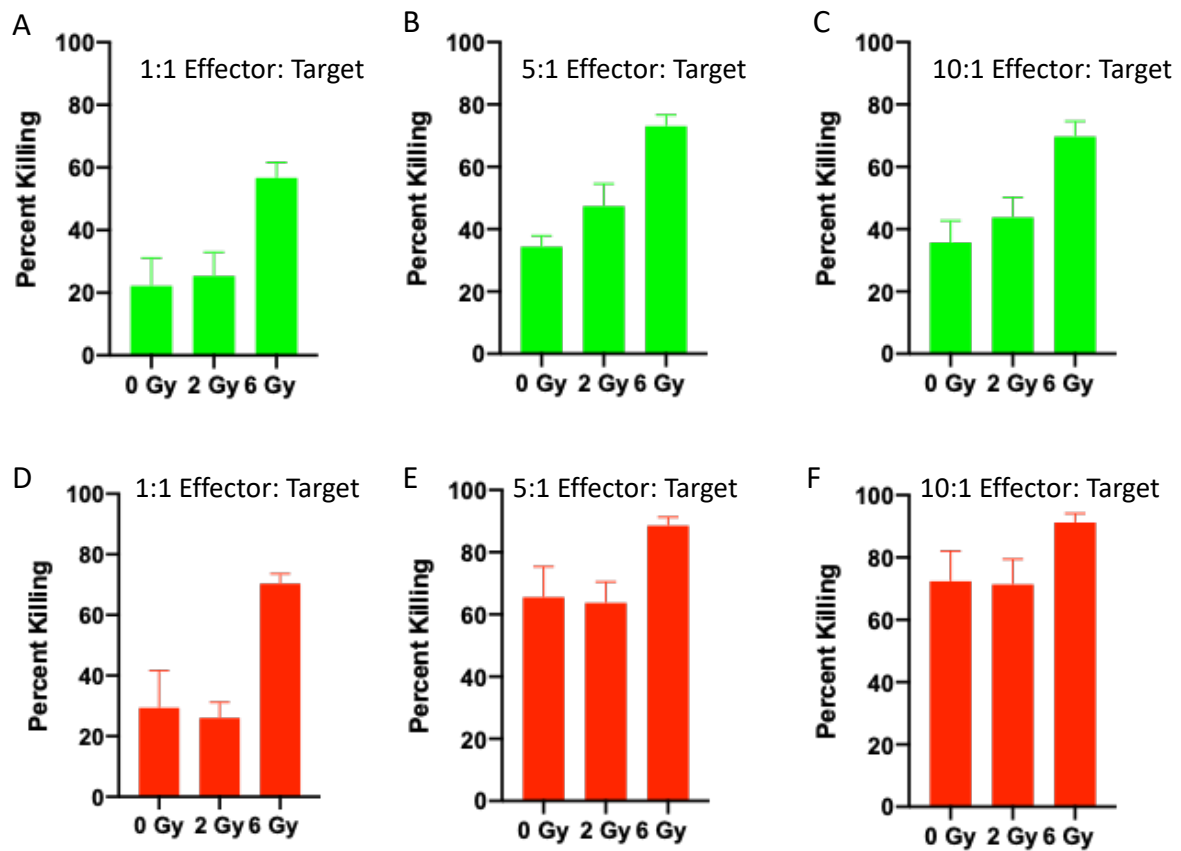


Figure 5-12 Radiation therapy enhances tumor cell susceptibility to NK cells.

Tumor cells were seeded at 50:50 ratio in 96 well plates. Fluorescent images were recorded 150 hours after treatment was applied. Cell counts for each line were then determined and percent killing was calculated as percent change from control wells with same level of RT, $((\text{No NK treatment} - \text{NK treated}) / \text{No NK treatment}) \times 100$.

Two independent experiments. One-way ANOVA, $P < 0.001$

5.2.11. High effector to target ratios enhanced tumour cell susceptibility to NKs

High effector to target ratios in cancer immunology are correlated with better outcomes. We tested three E:T ratios (NK:tumor cells) against 50:50 mixtures of AMC:RR cells. 5:1 and 10:1 E:T ratios killed more tumour cells than the low E:T ratio (1:1) without prior radiation (**Figure 5.13A**) and following 6 Gy RT (**Figure 5.13B**). Again, in all cases except 1:1 E:T ratio RR-22Rv1 cells (red) were more susceptible to NK cells than the control line (green).

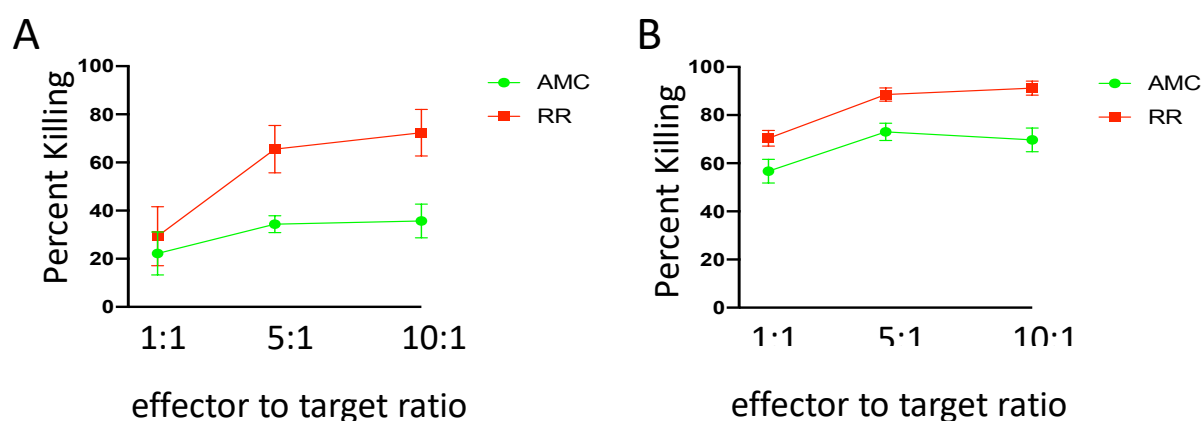


Figure 5-13 High effector to target ratios enhanced tumor cell susceptibility to NKs.

Tumor cells were seeded at 50:50 ratio in 96 well plates and treated with NKs at 3 effector to target ratios (E:T). Fluorescent images were recorded 150 hours after treatment was applied. Cell counts for each line were then determined and percent killing was calculated as percent change from control wells with same level of RT, ((No NKL treatment - NKL treated)/ No NKL treatment x 100)). Two independent experiments. One-way ANOVA, $p < 0.01$

5.2.12. Combination therapy using high dose NKL and 6 Gy radiation was more effective than single treatments with a frequency dependent effect

Next, we sought to determine the effects of the two treatments given together. Our previous findings from the flow based competition assays demonstrated a stronger effect when the two treatments were given in combination with 6 Gy RT and 5:1 E:T ratio. In this high throughput live imaging study using the Incucyte platform we were able to vary the treatment doses. Our results showed that NKs given at an E:T of 10:1 were reduced overall tumour cell numbers in wells with 50:50 mixture of AMC and RR cells (**Figure 5.14A, pink line**). The same was seen after administration of 6 Gy RT (**Figure 5.14A, blue line**). Combination therapy using 6 Gy RT followed by 10:1 NK was the most effective at inhibiting tumour cell proliferation and resulted in a declining population after 96 hours (**Figure 5.14A, purple line**).

We were also interested in the effects of combination therapy on the different seeding frequencies of the two prostate tumour cell lines. Our findings show that combination therapy was most effective in wells with higher seeding frequency of RR-22Rv1 cells (**Figure 5.14B**).

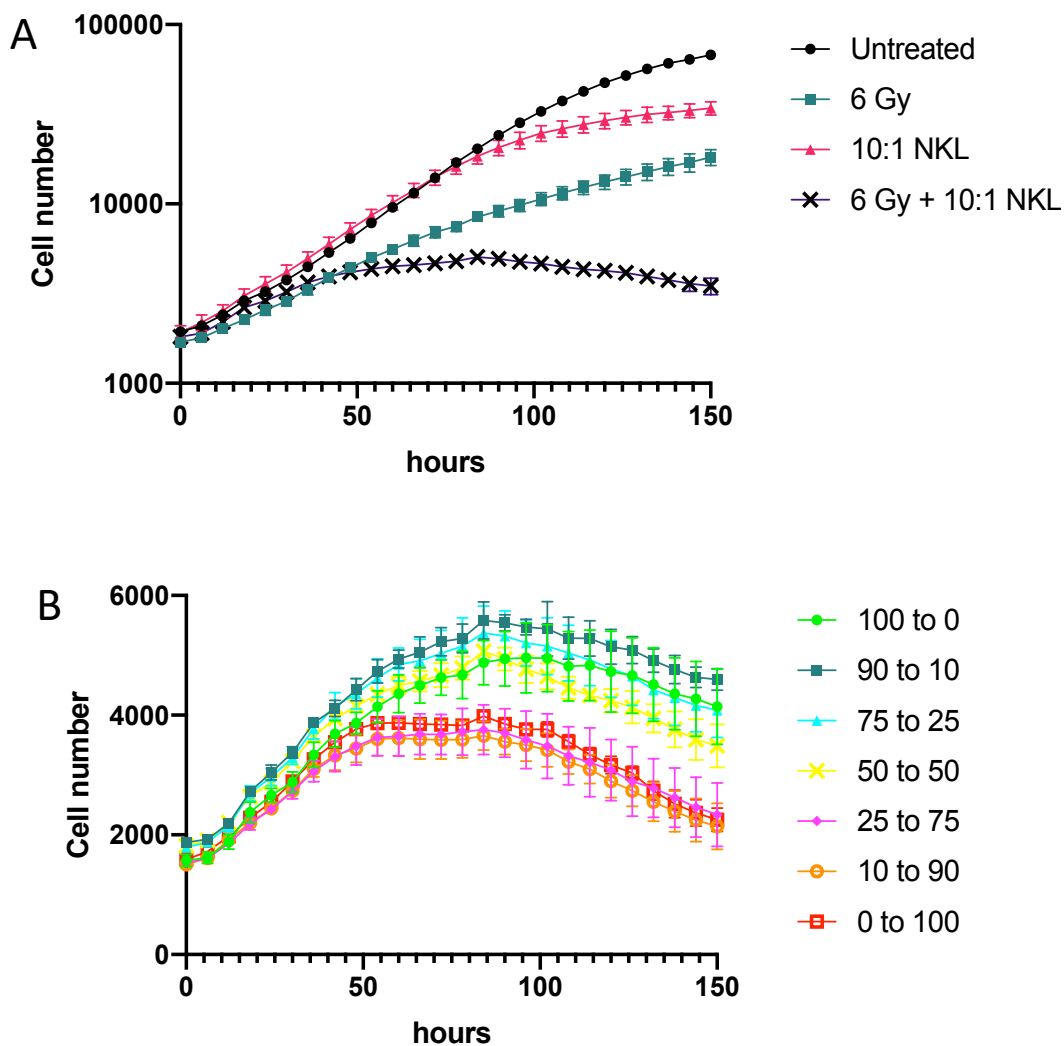


Figure 5-14 Combination therapy using high dose NKL and 6 Gy radiation was more effective than single treatments with a frequency dependent effect.

Cells were seeded in triplicate wells of a 96 well plate. Images were taken every 6 hours for 150 hours. A) total cell counts from samples seeded with a 50:50 seeding frequency, untreated (black line), 6 Gy radiation (blue line), 10:1 NKs (pink line), and 6 Gy followed by 10:1 NKs (purple line) (log scale) B) Cell counts calculated in all seeding frequencies treated with 6 Gy RT followed by 10:1 E:T ratio NKs.

5.2.13. Combination therapy using high dose NKL and 6 Gy RT reduces tumour size while selecting for radiation-sensitive cells – an ideal double bind

Next, we examined the level of selection by treatment with RT and NKLs in combination on the two cell lines. We previously showed that the combination treatment was efficient at reducing overall cell numbers. Here we found that the combination selects for the radiation-sensitive cell line, AMC-22Rv1, in all seeding frequencies (**Figure 5.15A**). Furthermore, our data indicates that the ratio of AMC:RR in each dual culture increased following combination therapy (**Figure 5.15B**) compared to the untreated group.

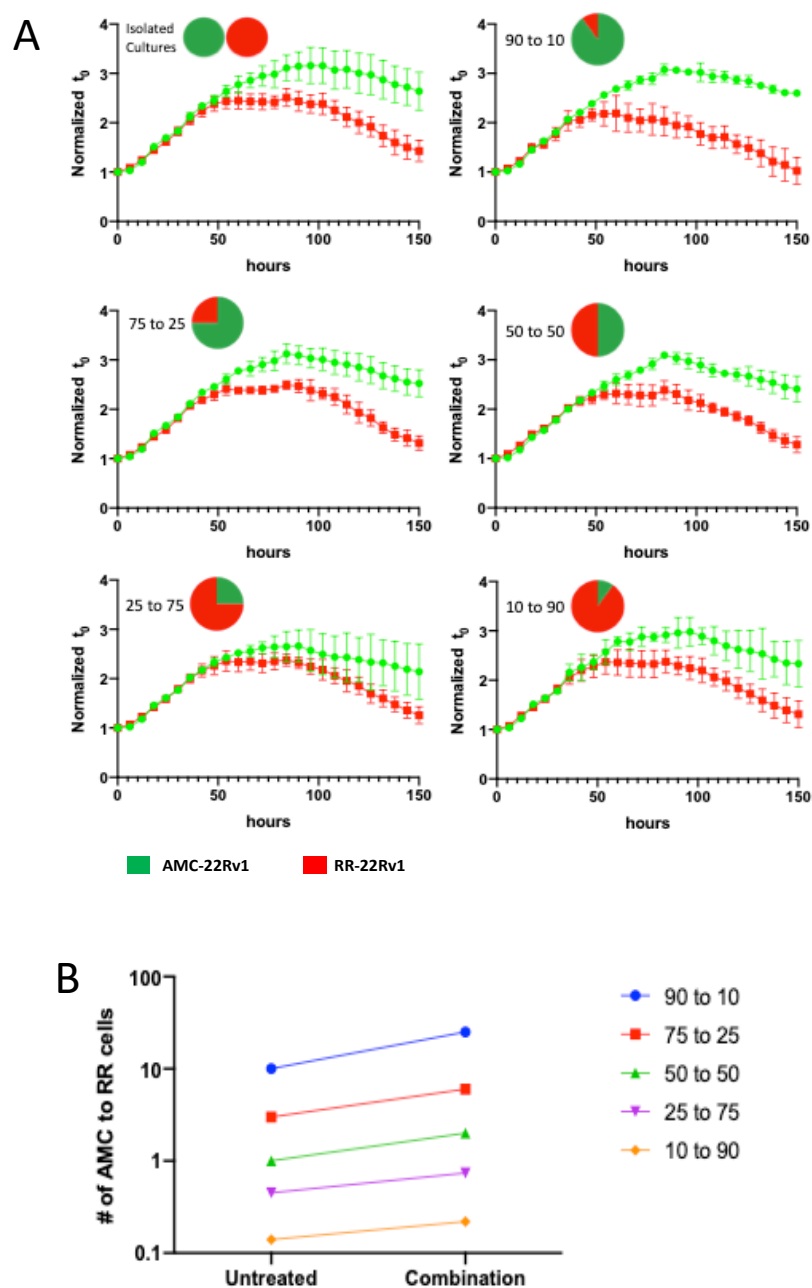


Figure 5-15 Combination therapy using high dose NKL and 6 Gy radiation reduces tumor size while selecting for radiation sensitive cells.

Cells were seeded in triplicate wells of a 96 well plate. Images were taken every 6 hours for 150 hours. A) Normalized cell counts from samples seeded at 7 seeding frequencies. Treated with 6 Gy radiation followed by 10:1 NKLs. B) Changes in number of AMC cells per RR cells following treatment in each initial seeding frequency.

5.3. Discussion

Evolutionary guided therapies have two key objectives; 1) reduce tumour burden and 2) control the growth of the resistant population. In the previous chapter, we show that radiation resistance enhances the susceptibility to NK therapy. Here, we demonstrate that NK cells control the outgrowth of the radiation-resistant population. Growth dynamics in mixed populations are complex. Differential proliferation rates, altered death rates, and interaction effects need to be considered. Therefore, we designed competition experiments to quantify the growth dynamics of the radiation-resistant and radiation-sensitive cell lines in various conditions.

We showed that RR-22Rv1 cells have a slower intrinsic growth rate compared to the control line. This is a common occurrence in treatment resistance (132) (143). Sensitive cells have a higher growth rate and make up the bulk of the tumour in the treatment naïve setting. However, once treatment is applied, their death rate increases, reducing the population. Inversely, resistant cells have a slower intrinsic growth rate. Therefore, even if they evolve before treatment, their frequency would be low until treatment is applied. Fitness is relative to the environment. In the untreated environment, AMC-22Rv1 cells are the most fit, but RR-22Rv1 cells are the most fit when radiation is applied. This relative fitness explains why many tumours initially respond to therapy, but once all the sensitive cells are destroyed, the resistant cells have no competition and dominate the tumour, an outcome termed 'competitive release' (143).

We also found that the proliferation cost is lessened in co-cultures with the control line and increases when the control cells are at a higher frequency than the RT resistant

cells. Interactions between two species occupying the same niche, here two cell lines in a dish, can result from various mechanisms. Cells can secrete factors that increase or inhibit growth in the other cell lines. Cells can also take up space and nutrients, limiting the growth of both cell lines. We did not quantify the mechanism driving growth inhibition by the RR-22Rv1 cells. However, we did note different growth patterns in the two lines. RR-22Rv1 cells grew in a traditional epithelial layer along the plastic and were sensitive to contact inhibition at high densities. They are larger and occupy more space. AMC-22Rv1 cells are smaller and do not rely on adherence to the plastic for growth. These differences may affect growth patterns *in vitro*.

In addition, we showed that radiation selects for RR-22Rv1 cells despite their slower growth rates. This was expected since we and others have previously shown the differential effects of RT on the two cell lines. NKL cells selected for AMC-22Rv1 cells in all seeding frequencies but were most effective at reducing overall tumour cell number in samples containing high frequencies of RR-22Rv1 cells. The increased sensitivity to NKL mediated killing, and the reduced growth rate of the RR-22Rv1 cell line likely contributed to this outcome.

Next, we determine if giving the two treatments consecutively altered these dynamics. We were interested to see if irradiating the AMC-22Rv1 cells would increase their sensitivity to NKs so that their sensitivity would then equal RR-22Rv1 cells. While an increase would help reduce tumour burden, if the two cell lines then had equal sensitivity to NKs, the dual treatment would not be as efficient at controlling the outgrowth of radiation resistance. Interestingly, we found that RT did increase the

susceptibility of AMC-22Rv1 cells to NK cells. However, it also increased the sensitivity in RR-22Rv1 cells, maintaining the differential effects of NK therapy on the two populations.

This *in vitro* system tracks growth in the different cell lines in mixed cultures at multiple timepoints under different treatments. An important limitation to our system is the use of a single *in vitro* model. Additional prostate cancer cell lines including, androgen receptor expressing LNCaP cells which responds to hormone deprivation therapy and PC-3 and DU-145 cell lines that represent more advanced disease and are less sensitive to hormone deprivation would provide further context to our findings. Future experiments should include additional cell lines and *in vivo* work. Specifically, we are applying for funding to determine the robustness of our finding in other radiation resistance cell lines and isogenic cell line models of resistance to other treatment modalities, including chemotherapy and targeted therapy. The utility of murine models will be considered. Our hypothesis could be tested *in vivo* using human cell lines in immune incompetent mice treated with radiotherapy and human NK cell lines. However, an orthotropic prostate cancer model with murine cells lines combining radiotherapy and NK cell-based therapy, including autologous transfer, with and without checkpoint inhibitors would allow for inclusion of the tumour microenvironment, host mediated immune suppression, and appropriate tissue structure. Additionally, the limitations of culture systems hinder our ability to perform long term experiments using both RT and NKL in a repetitive pattern, as we suggest in the theoretical model. Currently, *in silico* mathematical models based on our *in vitro* findings are being developed by the Integrated Mathematical Oncology group at Moffitt

Cancer Center, Tampa, Florida, to facilitate this. Evolutionary game theory models based on classic Lotka-Volterra competition and predation equations will be used to predict outcomes for time points beyond what is possible *in vitro*. Preliminary model outcomes support the use of NK cell therapy as a double bind therapy and suggest a potential for use as an extinction therapy.

In the previous chapters, we identified several immune-related essential genes conserved during cancer evolution. Amongst them was a group of stress-induced NK receptor ligands expressed on cancer cells. These same ligands were also identified in an isogenic cell line model of radiation resistance. We demonstrated that radiation resistance comes at the cost of increased susceptibility to NK cells, a radiation-induced double bind. In this chapter, we show that, in our *in vitro* system, radiotherapy and NK therapy reduce the number of tumour cells and control the outgrowth of resistance. Others have explored combining immunotherapy and radiation therapy which we discuss in more detail in the next chapter. However, we are the first to show the utility of NK cell therapy in an evolutionary guided treatment strategy, demonstrating that evolutionary principles can be successfully integrated into cancer immunology research to reveal novel findings.

Chapter 6: General Discussion

6. Chapter 6: General discussion

We identified several immune-related genes conserved during cancer evolution. Among them was a group of stress-induced NK receptor ligands. We also identified these in an isogenic cell line model of radiation-resistant prostate cancer. We found that radiation resistance comes at a cost, increased susceptibility to NK cells. We then evaluated the effectiveness of exploiting this double bind *in vitro*. We found that radiotherapy followed by NK cell therapy reduced the number of tumour cells and controlled the outgrowth of radiotherapy resistance. Together, these findings demonstrate the utility of applying evolutionary thinking in discovering new therapeutic targets and optimizing treatment schedules. We have developed new ways of studying the interactions between the immune system and malignant cells.

Our gene conservation study was built upon the foundational evolutionary concept of natural selection to uncover potential lung cancer targets. Genetic mutations frequently occur in all populations, be they species or malignant cells (248-250). Mutations that increase fitness are under positive selection. Inversely, genes that decrease fitness undergo negative selection. The majority of mutations, however, do not affect fitness and are under neutral selection (250). Neutral genetic changes can become fixed within a population simply by chance alone, a phenomenon known as 'genetic drift' (251). Determining the effect of a single mutation that occurs within a population is challenging. Beneficial mutations can be hidden amongst the vast number of neutral mutations. Disadvantageous mutations will be lost from the population.

Evolutionary biologists have derived methods to sort through the noise. For example, gene conservation studies are commonly performed in evolutionary biology to identify essential genes within a population. Identification of essential genes in cancer is challenging. CRISPR-Cas9 based and shRNA-based genetic screenings use cell lines and lack selection by the immune system (252). *In vivo* genome-wide CRISPR-Cas9 screens have revealed important essential genes in murine models of breast cancer, leukaemia, and lung cancer (253-255). Here, we described the first method for detecting essential genes in lung cancer patient samples developed by Moffitt's Center of Excellence for Evolutionary Therapy group. Importantly, this method has only been applied to lung cancer samples in the TCGA database which has limited clinical details and no outcomes information. Validation of our findings will be required experimental and clinical evaluation beyond the scope of this thesis. Additionally, other databases that include more detailed clinical information should be used to help identify associations with patient cohorts and predictive value of gene conservation studies. We adapted this method to investigate gene conservation in immune-related pathways within malignant epithelial cells. This interdisciplinary approach highlights the advantage of collaborations between Moffitt's Evolutionary Therapy group and Trinity's Comparative Immunology department.

The frequency of somatic mutations in over 17,000 genes was analysed in tumour tissue samples from lung cancer patients to determine essential immune-related genes in lung cancer. Here, the probability of mutation was approximately equal for every base pair in expressed genes. Therefore, the background mutation rate was determined by regressing each gene's mutation frequency against individual gene size. Genes with

higher than expected mutation rates based on size alone were considered hypermutated, and genes with less than expected mutation rates were considered conserved.

Initially, we validated this approach by looking at the mutations rates of all genes. Key findings in our dataset were consistent with known mutations commonly found in lung cancer. Well-characterized oncogenes, EGFR, Kras, and TP53, were the most hypermutated in our patient cohorts. TP53 is a tumour suppressor gene with loss-of-function mutations in over 50% of all human cancers (178). TP53 encodes the p53 protein, which initiates apoptosis in heavily mutated cells (178). Loss-of-function mutations in TP53 increase cell survival. Therefore, these mutations undergo positive selection and are found in more than 70% of lung adenocarcinoma tumours (156). TP53 was the most frequently mutated gene following driver mutations (EGFR and Kras) in our dataset. Our data was also consistent with the 2008 study by Ding et al., which reported 26 genes significantly mutated in lung adenocarcinoma (156, 256). 21 of the 26 genes they identified were hypermutated in our dataset and 100% of the ten most frequently mutated genes observed in their study were hypermutated in ours. Gain-of-function mutations in driver genes and loss-of-function mutations in tumour suppressor genes have been well characterized (156). Less is known about essential genes in the different tumour environments. Therefore, evidence-based predictions for specific gene conservation was not possible.

Much work has been done to identify genes expressed in epithelial cells that interact with immune cells. These include; antigen presentation, checkpoint proteins, and

inflammatory cytokines. Intrinsic immune processes occurring within epithelial cells independent of immune cells have only recently been recognized. Therefore, comprehensive gene lists for these processes had not been constructed before our study. We employed detailed literature searches and database searches to identify immune-related genes that are important in epithelial defence mechanisms. Although we were diligent in our research, additional genes not included in our lists may be relevant (see appendix for full gene lists). Moreover, intrinsic immune pathways that occur in epithelial cells independent of immune cells are still being explored, and new findings may need to be incorporated in the future (66)

Among the various gene sets we examined, genes involved in presenting exogenous and endogenous antigens were the most frequently conserved immune genes in the wild-type cohort. Antigen presentation by malignant epithelial cells is vital for T cell recognition. The conservation of these genes emphasizes their importance in cancer cell survival and proliferation. Initially, this finding was surprising. The immunoediting hypothesis dictates that cancer cells recognized by the immune system are eliminated (15, 62, 118, 128). Therefore, it is intuitive to hypothesize that antigen presentation would not be conserved in tumour cells following long-term evolution in an immune-competent host.

Conservation in these genes likely indicates the existence of host defence mechanisms not usually considered in cancer immunology. Human host defence mechanisms include safeguards against pathogen and malignant cell immune evasion strategies (257). These evolve as a result of the evolutionary interactions of host and pathogen (258). When

pathogens evolve new mechanisms that subvert the host immune response, the host evolution is affected, selecting new mechanisms. Inhibition or alteration of antigen processing and presenting in epithelial cells is a common method of viral and malignant cell immune evasion (182, 259). The opposing effects of MHC-I binding on cytotoxic T cells and cytotoxic NK cells likely represent a host protective adaptation against viral inhibition of antigen presentation by epithelial cells. Lack of MHC-I molecules on the surface of epithelial cells allows them to evade detection by antigen-specific T cells. However, it also renders cells susceptible to NK cells (260) This dichotomy protects the host from pathogen disruption of MHC-I expression.

The most universally conserved genes in our immune dataset, ERAP1/ERAP2, have been widely studied in infectious diseases. ERAP1 and ERAP2 are enzymes that shape the protein peptidome determining which peptides will be presented by MHC-I complexes. Variants in ERAP genes are prevalent in humans with differing susceptibility to infectious diseases and autoimmune disorders (261, 262). Genome-wide association studies suggest ERAP1 and ERAP2 play a role in multiple autoimmune conditions, including multiple sclerosis, Behcet's disease, and psoriasis. Mice without ERAP1 have increased hepatic NK, NKT, and dendritic cell activation following inflammatory stimuli (79). Additionally, membrane-associated ERAP can cleave cytokine receptors at the cell's surface, including direct binding of IL-6 receptor (257). Human cytomegalovirus microRNA, miR-US4-1, inhibits ERAP1 function and facilitates viral immune evasion (78).

ERAP1 expression levels vary across different tumour types. Interestingly, ERAP1 expression was detected in all 9,125 tumour specimens in The Cancer Genome Atlas

database (TCGA) (263, 264). Genetic alterations were extremely rare, with deep deletions occurring infrequently and only a small percentage of tumours having mutations in ERAP1 or ERAP2 (0.6% - 0.8%) (264). This, along with our findings using a completely different approach, signifies an essential role for ERAP in cancer cell survival and proliferation.

Recent studies have revealed two potential explanations for the conservation of ERAP genes. First, inhibition of murine ERAP1 homolog, ERAAP, leads to the expression of a highly conserved peptide, FYAEATPML (FL9), by the murine functional homolog to human HLA-E, Qa-1 (203). This induced robust CD8⁺ T cell responses specific for Qa-1-FL9 and targeted killing of ERAAP negative cells. Expression of ERAAP breaks down FL9 peptide preventing its expression and all subsequent immune responses (203). Secondly, inhibition of ERAP1 in human cancer cell lines sensitizes tumour cells to NK-mediated killing by preventing the ligation of inactivating receptors on NK cells (265, 266). Targeting ERAP in tumours may increase response to immunotherapy. Specific pharmacological inhibition of the ERAP enzyme is being explored. However, ERAP-specific inhibitors have not been fully developed (264, 267). Moreover, ERAP is likely essential in non-malignant cells and cancer cells, and directed targeting is likely needed. The studies highlighted above, combined with our findings, show tumour dependence on ERAP genes and identify it as an important potential therapeutic target.

Our gene list of immune-modulating proteins included well-known checkpoint inhibitory ligands and other activating and inactivating proteins expressed in epithelial

cells that modulate the immune response. Three of the 45 immune-modulating proteins we examined were conserved in both cohorts,

- A. NKG2D ligand, MHC class I polypeptide–related sequence A (MICA),
- B. DNAM-1 ligand, poliovirus receptor (PVR),
- C. DNAM-1 ligand, poliovirus receptor-like protein 2 (PVRL2).

MICA is not expressed on normal healthy tissue. Expression is upregulated in response to stress-induced by viral infection or molecular changes in transformed cells. NKG2D is an activating receptor on NK cells, $\gamma\delta$ T cells, and CD8⁺ T cells (187, 209). Activation of NKG2D alone initiates effector function on NK cells. However, NKG2D signalling in T cells requires additional T cell receptor (TCR) signals to fully activate CD8⁺ T cells (268, 269). Similar to CD28, NKG2D enhances TCR signalling. Unlike CD28, which induces strong effector functions, NKG2D induces a moderate effector phenotype primed for differentiation into memory cells. This occurs through weaker activation of mTORC1, decreased T-bet, BLIMP, IRF4, and ID2, and increased EOMES and ID3 expression (268, 269).

NKG2D ligands have conflicting roles in tumour immunity. Activation of NK cells by NKG2D ligands is critical for tumour immunosurveillance as NKG2D deficient murine models had increased cancer risk (270). High levels of tumour-derived NKG2D ligands are associated with positive outcomes in colorectal, cervical, and nasopharyngeal cancers (271). Induction of NKG2D ligands by DNA damaging cancer therapies increases the anti-tumour efficacy of NK cells (241, 272) Inversely, NKG2D ligand expression in breast, lung, and ovarian cancer are associated with poor outcomes (271, 273). In a

recent study evaluating the protein expression in tumour tissue from 222 patients, increased MICA was associated with poor prognosis (273). This may be due in part to MICA shedding (274).

NKG2D ligands, including MICA, MICB, and ULBP2, are proteolytically cleaved from the surface of tumour cells. This reduces the amount of activating ligands on the cell's surface. Soluble MICA/B and ULBPs retain the ability to bind NKG2D on distant NK cells. This causes the internalization of NKG2D and hypo-responsive NK cells. Antibodies blocking the shedding or effects of the soluble proteins are being investigated to inhibit their immune-suppressive functions (274). High serum sMICA levels caused downregulation of NKG2D on immune cells and decreased activation. sMICA was significantly higher in serum from cancer patients compared to healthy donors, and these levels correlated with cancer stage and metastasis (275).

PVR and PVRL2 (CD112) were first identified as ligands for activating receptor DNAM-1 on NK cells. TIGIT and CD96 are competitive receptors for PVR and PVRL2 with inhibitory functions. TIGIT is expressed by NK cells and CD8 T cells. Expression levels in lymphocytes vary amongst cancer types (276). Murine models and human colon cancer patients have high levels of TIGIT expression in tumour infiltrating NK cells (46). TIGIT blockade prevents NK cell exhaustion, enhances responses to anti-PD-1 therapy, and increases immune memory in tumour re-challenge studies (46). Similarly, CD96 competes with activating receptors for ligation with PVR. Ligation of CD96 decreases IFN-gamma secretion and cytotoxic functions in NK cells (277). CD96 is predominantly expressed by T cells and has been detected in primary and secondary tumours with

elevated expression on antigen-experienced T cells (278, 279). Blockade of CD96 or TIGIT in pre-clinical models increases anti-tumour immune responses (279). Surprisingly, CD96⁺ NK cells were associated with positive responses in pancreatic cancer. However, CD96⁺CD56^{dim} NK cells were functionally exhausted in hepatocellular carcinoma tissue (280). Several clinical trials are ongoing to determine the efficacy of anti-TIGIT and anti-CD112R antibodies in solid tumours. The first TIGIT inhibitor (Tiragolumab) was granted breakthrough therapy designation by the US Food and Drug Administration in January 2021. However, interim results of two phase III trials, SKYSCRAPER-01, evaluating Tiragolumab in combination with atezolizumab (Tecentriq), for patients with metastatic non-small cell lung cancer (NSCLC) whose tumours have high PD-L1 expression and no EGFR or ALK mutations and SKYSCRAPER-02 in extensive stage small cell lung cancer did not meet their co-primary endpoint of increased progression-free survival (281, 282). The SKYSCRAPER-01 trial is ongoing as overall survival data is not currently available. Tiragolumab was well tolerated and Genentech will continue to test anti-TIGIT therapies in multiple cancer types (281).

Our gene conservation analysis emphasized the importance of NK ligands in tumour evolution. Therefore, we incorporated them when building our *in vitro* models to explore tumour evolution in the context of radiation therapy resistance. While the gene conservation study was performed on lung cancer samples, our *in vitro* work utilized an isogenic prostate cancer cell line model that was previously evolved for radiation resistance experiments. The effects of radiation on lung cancer cells was not tested in our system. New isogenic model using lung cancer cell lines will need to be created to test these effects *in vitro*. we show that radiation resistance increased NK receptor

ligands, MICA/B, PVR, and CD112 (PVRL2). The radiation-resistant cell line was more sensitive to NK cells. Radiation resistance is a common cause of treatment failure in advanced cancers. The resistance mechanisms are complex, and multiple studies have described potential targets to enhance tumour cell sensitivity to radiation therapy (283) (155, 234, 284-286). Here, we introduce a new approach using NK cell therapy to target the radiation-resistant population specifically.

NK cell activity is enhanced following radiotherapy in pre-clinical cancer models. Genotoxic stress activates the DNA repair response triggering increased NKG2D ligand expression on epithelial cells and DNAM-1 ligand expression in multiple myeloma studies (246, 287). Radiotherapy causes genotoxic stress and has been shown to directly upregulate NKG2D ligand expression on cancer cells (222). Here, we are the first to show that NKG2D ligand expression and DNAM-1 ligand expression are increased in an isogenic model of radiation-resistant prostate cancer. This change was durable. RR-22Rv1 cells maintained increased stress-induced ligand expression for over 20 passages after completing radiotherapy. We also show that radiation-resistant cells are more sensitive to NK cell-mediated killing, indicating a radiation-induced immunologic double bind.

Pre-clinical models combining NK cell therapy and radiotherapy demonstrate effectiveness in breast cancer, lung cancer, murine melanoma, and multiple myeloma (246, 288, 289). A contradicting finding was seen in irradiated lung cancer cell lines, A549 and H157, where RT decreased NKG2D ligand expression and increased PD-L1, reducing susceptibility to NK cells (290). Treatment with MEK/ERK inhibitors and PD-L1

inhibitors reversed this effect. There are several differences in study design that may account for the contradictory findings. NKG2D ligand expression was measured as total mRNA using quantitative real-time PCR. Total protein or surface protein was not measured in their model. Importantly, *in vitro* killing assays using isolated primary NK cells were performed after 4 hours co-culture (290). These findings highlight the importance of further studies in various models to fully understand the complexity of radiation responses and NK based immunotherapies. A case study of a patient with inoperable non-small cell lung cancer receiving NK cells stimulated with HsP70 peptide, radiotherapy, and anti-PD1 antibody indicated the treatment was well tolerated and resulted in long-term tumour control (291).

Increased NKG2D ligand expression is the evolutionary outcome of radiotherapy resistance, providing a rationale for combinations with NK cell-based immunotherapy. Here, we show that DNAM-1 ligands, PVR, and CD112 are also increased in the radiation-resistant cell line. PVR and CD112 activate NK cells through ligation of DNAM-1. However, PVR and CD112 also inhibit NK cells by ligating TIGIT or CD96 (277, 292). Therefore, the downstream effects of increased PVR and CD112 are primarily dependent on the condition of the NK cells. We examined the impact of one anti-TIGIT antibody in a single assay with our isogenic cell lines and NKL cells. We did not see an increase in NK cell activity. We did not perform enough repeats of this experiment to extrapolate meaning. Therefore, this maybe the result of poor TIGIT blockade by the antibody, which we did not independently validate, or because we did not repeat our preliminary findings. Further studies should be done to determine if any additive effects inhibiting the PVR/CD112-TIGIT/CD96 axis occur in our system.

Radiation increases PD-L1 expression in AMC-22Rv1 and RR-22Rv1 cells, with the radiation-resistant cells having the highest expression. Others have found increased PD-L1 expression in pre-clinical models by RT (293). NK cells and T cells express PD-L1 receptor (PD-1) in the tumour microenvironment. Ligation of PD-1 induces exhaustion and decreased cytotoxic functions in lymphocytes. Furthermore, activated NK cells secrete INF-gamma enhancing the immune response (260) However, IFN-gamma also upregulates immunosuppressive pathways in tumour cells, including expression of PD-L1 and IDO. PD-L1 expression by tumour cells may hinder the radiation-induced immunologic double. Inhibiting PD-1 and potentially other immune checkpoints (TIM3 and Lag3) may need to be considered.

An important limitation for combining NK-based therapy with RT is the tumour microenvironment. The harsh TME inhibits autologous and allogenic NK cell function. Hypoxia is a common occurrence in solid tumours stemming from dysregulated vasculature. Poor oxygen perfusion limits the effectiveness of radiotherapy, which is largely dependent on oxygen for its cytotoxic effects (285, 294). NK cell function is also inhibited in a hypoxic environment (23). IL2 or IL15 stimulated NK cells upregulate HIF-1 α when exposed to low oxygen. Decreased effector function was seen in NKL cells exposed to hypoxia in an *in vitro* colorectal cancer model (295). Additionally, poor perfusion and highly glycolytic tumour cells cause an increase in extracellular lactate and an acidic TME. Our group has previously shown the negative effects of lactate on tumour infiltrating NK cells in colorectal liver metastasis (296). Radiotherapy normalizes

the tumour vasculature facilitating lymphocyte extravasation (287). TME consideration in the NK-radiotherapy double bind will require *in vivo* studies.

Treatment-induced NK cell double binds may occur in other settings. Similar to radiotherapy, commonly used cancer therapies including, platinum-based chemotherapies, HDAC inhibitors, and proteasome inhibitors activate DNA damage repair pathways inducing NK receptor ligands on cancer cells (246, 285). The duration of increased expression in treatment-resistant populations has not been explored. Here, we did not examine the costs of resistance to other treatments. However, it is reasonable to hypothesize that a similar double bind may occur. NKG2D ligands have also been found on stem-like cancer cells (297). Stem-like cancer cells are a small subset of cancer cells responsible for drug resistance. Targeting this highly proliferative, treatment-resistant population with NK cells could help control the emergence of treatment resistance.

We found that NK cells selectively target the radiation-resistant population when radiation-sensitive AMC-22Rv1 and radiation-resistant RR-22Rv1 cells were cultured together. To address this, we designed novel competition assays motivated by ecological studies of interacting species and modelled after a previous study performed at Moffitt Cancer Center (298).

We found that the radiation-resistant cells, RR-22Rv1, have a reduced intrinsic growth rate compared to the control line when the two were cultured in isolation without treatment. Mechanisms of therapy resistance can reduce proliferation rates (130).

When AMC-22Rv1 and RR-22Rv1 cells were cultured together at various ratios, the growth rate of the resistant population increased relative to the sensitive population, indicating an interaction effect between the two cell lines.

Conventional *in vitro* experiments in cancer biology are performed with standard assay conditions where tumour cell lines are cultured separately. Cancer immunology assays, examining cell-mediated responses, require contact between effector and target cells. If one cell line activates immune cells, it is possible that neighbouring cells can be affected. The inverse is also true; if a cancer cell suppresses the function of an immune cell, it then protects its neighbours. Therefore, creative assays need to be developed to consider these interactions. Additionally, single culture experiments do not account for interaction effects between two cancer cell lines. As reviewed by us previously, interactions between populations include;

- 1) competitive interactions (negative effects between the two populations),
- 2) commensalism (one benefits but causes no good or harm to the other)
- 3) amensalism (one harms the other species at no cost or benefit to itself)
- 4) mutualism (positive effects usually through the exchange of resources or through the production of public goods) (144).

We found a negative interaction effect; RR-22Rv1 cells inhibited the growth of AMC-22Rv1 cells at high density. Interaction effects between isogenic cells lines were previously reported in two radiation-resistant prostate cancer cell line models (299). 3D mixed cultures of the radiation-resistant and radiation-sensitive populations had enhanced growth rates and diminished radiation sensitivity (299). This was driven by

oxygen competition and cellular cross-talk (299). Interaction effects between heterogeneous populations of cancer cells, whether competitive or mutualism, increases resistance to therapy (299-301).

We used different ratios to represent interpatient variability and to determine the direction of interactions occurring between the two cell lines. We found that samples with high frequencies of radiation-resistant cells had the largest decline in overall tumour population size. If this effect directly translated to the clinic, then patients with high levels of radiation resistance would see the most benefit in tumour reduction when treated with NK cells. Furthermore, we showed that RT followed by NK therapy selects for radiation-sensitive cells. If this effect directly translated in the clinic, patients with high radiation resistance but still harbouring a small level of radiation-sensitive cells would see a return to the sensitive phenotype.

In many cases, two drugs have a stronger effect than one. So, the addition of an effective second treatment typically increases response rates. Using evolutionary studies of treatment resistance to determine new targets results in novel drug discovery, for examples drugs targeting T790M mutations in patients recurring following EGFR inhibitors (refs). Once a new target is identified, it is important to continue to think in evolutionary and ecological framing. For an evolutionary double bind, the second treatment restores treatment sensitivity to the population as a whole creating opportunities for strategic evolutionary guided scheduling. Evolutionary biology has shown that if the two selection pressures, the owl (RT) and the snake (NK cells), are applied concurrently, a third mechanism causing resistance to both selection pressures

will emerge. Evolutionary principles could be used in clever ways to delay this process and keep the mice from running into our snake-free homes. This can be done through timing and dosing schedules designed to exploit the underlying dynamics.

Here, we focused on the application of radiotherapy and NKL cells to exploit the double bind as a means to control the outgrowth of the radiation-resistant population. In theory, alternating between the two therapies would control the tumour and inhibit the outgrowth of treatment resistance compared to radiotherapy given repetitively. This strategy would extend the effective treatment window. Due to experimental limitations, we did not run empirical studies to quantify the effects of intermittent dosing with the two treatments. However, scheduling is likely a key factor to success. Mathematical models, informed by the *in vitro* work presented here, are currently being developed to explore the best options for timing and dosing of the two therapies.

One approach being considered is applying the radiotherapy-induced NK double bind as a curative option. The above example, using RT and NKL to suppress resistant populations, is designed for use in patients whose response rates to initial therapy would not result in full tumour clearance. However, although rare in advanced prostate cancer, a subset of patients would have a complete response, with no evidence of disease (NED) following initial therapy. Standard clinical practice is to stop treatment and monitor patients routinely for recurrence. In some patients, the cancer will never recur. In others, the cancer will recur after months or years as microscopic disease which survived therapy begins to grow to detectable levels. Once the tumour is detected, a second therapy is given, 'conventional scheduling' (**Figure 6.1**). Typically, cure is not an

option for these patients. However, new theoretical models based on ecology and evolution dynamics suggest a different treatment schedule, termed extinction therapy (Figure XB). Extinction therapy relies on a first strike / second strike approach to eradicate all tumour cells. The initial therapy is the first strike. It must be effective and render the patient NED. The second strike, given while the patient is still NED, is designed to exploit the ecological principle of small populations (151). In conservation biology, small populations are more sensitive to perturbations and in danger of extinction (151, 302). In cancer, the growth dynamics of tumour cells differ when populations are very small (303). In our model, radiation therapy would be the first strike. Once the patient reaches NED, NK cell therapy would be given to eliminate the remaining cancer cells. Giving NK cells while the tumour is below the detection threshold would exploit the ecological principles of small populations and increase the probability of an extinction event. Additionally, NK cells are most effective at a high effector to target ratio; therefore, NK cell therapy would be more effective against small populations. This theoretical model will be explored further with *in silico* modelling.

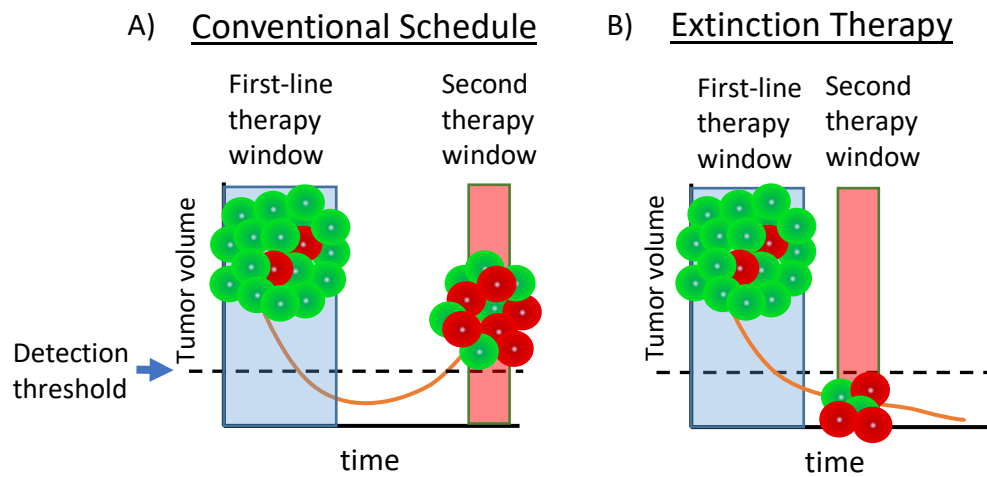


Figure 6.1. Extinction Therapy exploits the sensitivity of small populations to further perturbations in cancer. A) Conventional treatment schedules. B) Extinction therapy model.

In his seminal work, *On the Origin of Species by Means of Natural selection*, Darwin outlined his theory of natural selection as the origin of biological diversity (304). Since Darwin's work on finches, our knowledge of inheritance and the molecular mechanisms driving it has exploded. However, the foundational principle of natural selection continues to direct our understanding of biological outcomes.

Tumours are complex ecosystems with evolving populations of cancer cells. Combating a complex system requires dynamic therapeutic options. Cancer immunology encompasses two highly dynamic and interacting systems. The complexity of the immune system arises from the vast number of distinct cell types with profound temporal and spatial variations. Similarly, the tumour is a dynamic system composed of a heterogeneous mix of normal and malignant cells with varying phenotypes. These intrusive cells invade neighbouring areas disrupting tissue homeostasis and engineering a local microenvironment that favours tumour survival over organ function. The resulting tissue damage and aberrant growth of the malignant cells are capable of eliciting an immune response. When this response is sufficient, the tumour is eradicated. When this response is insufficient, the tumour evolves, and immune-resistant tumour cells thrive.

This work is motivated by the extraordinary complexity and dynamic nature of the anti-tumour immune response. It is further driven by the clinical need to find new ways to treat patients, especially those with few options, and the recent successes in evolution-based medicine. **Therefore, we hypothesized that evolutionary principles can be**

successfully integrated into cancer immunology research to reveal novel findings with clinical potential.

To explore this hypothesis, we used three approaches taken from evolutionary biology:

1) *conservation of essential genes during evolution*. We performed a gene conservation study on tumour tissue to determine essential genes at the interface of tumour and immune cell interactions. Our gene conservation study demonstrates that probing the outcomes of natural selection can reveal novel immune targets.

2) *evolutionary costs of treatment resistance*. Through evaluation of an isogenic cell line model, we found that the evolution of radiotherapy resistance increases sensitivity to NK cell-mediated killing. Our pre-clinical study revealed changes in NK ligand expression on radiation-resistant cells and selective targeting of radiation-resistant cells by NK cells.

3) *evolutionary double bind*. A double exists when resistance to one treatment increases the sensitivity to a second treatment and can be used to combat treatment resistance. Through competition experiments with our isogenic cell lines, we show that treatment with NK cells favours the radiation-sensitive population, re-sensitizing the tumour to radiotherapy.

We will continue to push for more integration of immunological thinking in evolutionary approaches in cancer. Moffitt's Center of Excellence for Evolutionary Therapy works to improve patient outcomes by developing the next generation of cancer therapy through the integration of evolutionary principles. Currently, we participate in Moffitt Cancer Center's Evolutionary Tumour Board. The Evolutionary Tumour Board is composed of an interdisciplinary team with clinicians, radiologists, mathematicians, physicists,

ecologists, and medical oncologists. We are the primary immunological input for the board. Each month we review an individual patient's history who has volunteered for the study. Using historical data and patient-specific information, we design theoretical and mathematical models. These are presented to the treating clinician. Currently, we have participated in 11 case reviews. A manuscript summarizing the first year's outcomes from the tumour board is in preparation.

Additionally, we are exploring formal collaborations between the Immunology MSc program at Trinity College and Moffitt Cancer Center's evolutionary biology department to facilitate cross-training programs. Interdisciplinary work has many challenges, yet the outcomes of cross-collaboration will greatly benefit patients.

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7. Appendix

7.1. Appendix 7.1. Gene lists

Antigen Presentation	Immune modulating proteins
B2M	ADAM17
CD1A	C10orf54
CD1B	CCL2
CD1C	CD274
CD1D	CD276
CD1E	CD40
CIITA	CD47
DAP2	CD48
ERAP1	CD80
ERAP2	CD86
HLA-A	CTLA4
HLA-B	ENTPD1
HLA-C	FASLG
HLA-DMA	HCST
HLA-DMB	HLA-A
HLA-DOA	HLA-B
HLA-DOB	HLA-C
HLA-DPA1	HLA-G
HLA-DPB1	IDO1
HLA-DQA1	IDO2
HLA-DQA2	LGALS1
HLA-DQB1	LGALS2
HLA-DQB2	LGALS3
HLA-DRA	LGALS9
HLA-DRB1	MICA
HLA-DRB5	MICB
HLA-E	NT5E
HLA-F	PDCD1LG2
MEX3C	PTGS1
MTTP	PTGS2
NLRC5	PVR
NPC1	PVRIG
NPC2	PVRL1
PSAP	PVRL2
PSMB8	PVRL3
PSMB9	PVRL4
RFX1	RAET1E
TAP1	TGFB1
TAPBP	TNFRSF14
	TNFRSF6B
	TNFSF4
	TNFSF9
	ULBP1
	ULBP2
	ULBP3

Innate immune receptors		IFN Signaling	
AGER	NLRP4	CREBBP	PIAS1
AIM2	NLRP5	DOCK2	PIAS2
BTK	NLRP6	IFNA1	PIAS3
CASP1	NLRP7	IFNA10	PIAS4
CASP8	NLRP8	IFNA13	PIK3CA
CD14	NLRP9	IFNA14	PTPN11
CIITA	NLRX1	IFNA16	RAC1
CTNNA1	NOD1	IFNA17	RAC2
DDX3X	NOD2	IFNA2	RAC3
DDX41	OAS1	IFNA21	SIRT1
DDX58	PYCARD	IFNA4	SIRT2
DHX58	RIPK1	IFNA5	SIRT3
DOK3	TANK	IFNA6	SIRT4
DOK4	TBK1	IFNA7	SIRT5
DTX4	TICAM1	IFNA8	SIRT7
FADD	TLR1	IFNAR1	SMARCA4
HMGB1	TLR10	IFNAR2	SOCS2
IFI16	TLR2	IFNB1	SOCS3
IFIH1	TLR3	IFNE	SOCS4
IL18	TLR4	IFNG	SOCS5
IL1B	TLR5	IFNGR1	SOCS6
IRF3	TLR6	IFNGR2	SOCS7
IRF7	TLR7	IL10RB	STAT1
LRRFIP1	TLR8	IL28A	STAT2
MAL	TLR9	IL28B	STAT3
MAVS	TMEM173	IL28RA	STAT4
MB21D1	TNIP1	IL29	STAT5A
MyD88	TRAF3	IRF1	STAT5B
NLRC3	TRAF6	IRF2	STAT6
NLRC4	TRAM1	IRF2BP1	TYK2
NLRC5	TRIM56	IRF2BP2	ULK1
NLRP1	TUFM	IRF2BPL	USP18
NLRP10	ZBP1	IRF3	
NLRP11		IRF4	
NLRP12		IRF5	
NLRP13		IRF6	
NLRP14		IRF7	
NLRP2		IRF8	
NLRP3		IRF9	
		JAK1	
		JAK2	
		JAK3	
		MAVS	

Chemokine signaling		Cytokine signaling			
CCL1	CX3CR1	CD27	IL12RB2	LIFR	TNFRSF10A
CCL11	CXCL1	CD4	IL13	LTA	TNFRSF10B
CCL13	CXCL10	CD40	IL13RA1	LTA4H	TNFRSF10C
CCL14	CXCL11	CD40LG	IL13RA2	LTB	TNFRSF10D
CCL15	CXCL12	CD70	IL15	LTB4R	TNFRSF11A
CCL16	CXCL13	CNTF	IL16	LTB4R2	TNFRSF11B
CCL17	CXCL14	CSF1	IL17A	LTBP1	TNFRSF12A
CCL18	CXCL16	CSF1R	IL17B	LTBP2	TNFRSF13B
CCL19	CXCL17	CSF2	IL17C	LTBP3	TNFRSF13C
CCL2	CXCL2	CSF2RA	IL17D	LTBR	TNFRSF14
CCL20	CXCL3	CSF2RB	IL17F	MPL	TNFRSF17
CCL21	CXCL5	CSF3	IL17RA	MST1	TNFRSF19
CCL22	CXCL6	CSF3R	IL17RB	MST1R	TNFRSF1A
CCL23	CXCL9	Epo	IL17RC	NFKB1	TNFRSF1B
CCL24	CXCR1	EpoR	IL17RD	OSM	TNFRSF4
CCL25	CXCR2	FAS	IL17RE	OSMR	TNFRSF6B
CCL26	CXCR3	FASLG	IL27	SPP1	TNFRSF8
CCL27	CXCR4	FLT3	IL27RA	TGFB1	TNFRSF9
CCL28	CXCR5	FLT3LG	IL28A	TGFB1I1	TNFSF10
CCL3	CXCR6	IFNA1	IL28B	TGFB2	TNFSF11
CCL3L1	CXCR7	IFNA10	IL28RA	TGFB3	TNFSF12
CCL4	XCL1	IFNA13	IL29	TGFBI	TNFSF13B
CCL4L1	XCL2	IFNA14	IL2RA	TGFBR1	TNFSF14
CCL4L2	XCR1	IFNA16	IL2RB	TGFBR2	TNFSF18
CCL5		IFNA17	IL2RG	TGFBR3	TNFSF4
CCL7		IFNA2	IL3	TNF	TNFSF8
CCL8		IFNA21	IL31	IL17REL	TNFSF9
CCR1		IFNA4	IL31RA	IL18	Tpo
CCR10		IFNA5	IL32	IL18BP	TRAF3IP2
CCR2		IFNA6	IL33	IL18R1	TSLP
CCR3		IFNA7	IL34	IL18RAP	TXLNA
CCR4		IFNA8	IL36A	IL19	
CCR5		IFNB1	IL36B	IL1A	
CCR6		IFNE	IL36G	IL1B	
CCR7		IFNG	IL36RN	IL1F10	
CCR8		IFNGR1	IL37	IL1R1	
CCR9		IFNGR2	IL3RA	IL1R2	
CCRL1		IFNK	IL4	IL1RAP	
CX3CL1		IFNW1	IL4I1	IL1RAPL1	
		IL10	IL4R	IL1RAPL2	
		IL10RA	IL5	IL2	
		IL10RB	IL5RA	IL20	
		IL11RA	IL6	IL20RA	
		IL12A	IL6R	IL20RB	
		IL12B	IL6ST	IL21	
		IL12RB1	IL7	IL21R	
			IL7R	IL22	
			IL8	IL23	
			IL9	IL23R	
			KIT	IL24	
			KITLG	IL25	
			LIF	IL26	

Apoptosis, Necrosis, and Pyroptosis	Complement
Aim2	C1QA
APAF1	C1QB
BAD	C1QC
Bel-2	C1S
BCL2L1	C2
BIRC5	C3
BIRC6	C3AR1
BIRC7	C4A
BIRC8	C4B
CASP1	C4BPA
CASP10	C4BPB
CASP14	C5
CASP2	C6
CASP3	C7
CASP4	C8A
CASP5	C8B
CASP6	C8G
CASP7	C9
CASP8	CD46
CASP8AP2	CD55
CASP9	CD59
CFLAR	CD93
CYCS	CFB
CYLD	CFDP1
FADD	CFH
FAS	CFHR1
FASLG	CFHR2
GSDMD	CFHR3
IL18	CFHR4
IL1B	CFHR5
MCL1	CFP
Nlr4	COLEC10
NLRP1	COLEC11
PYCARD	CR1
RIPK1	CR2
RIPK2	CSMD1
RIPK3	CSMD2
RIPK4	CSMD3
SERPINB4	ELANE
SERPINB9	F2
STAT3	FCN1
TNFRSF10A	FCN2
TNFRSF10B	FCN3
TNFRSF10C	ITGAM
TNFRSF10D	ITGAX
TNFRSF11B	ITGB2
TNFRSF6B	MASP1
TNFSF10	MASP2
	MBL2
	SERPING1
	VSIG4
	VTN

7.2. Appendix 7.2 Representative flow diagrams.

NK ligand expression in AMC-22RV1 (green) and RR-22Rv1 (red) cell lines.

