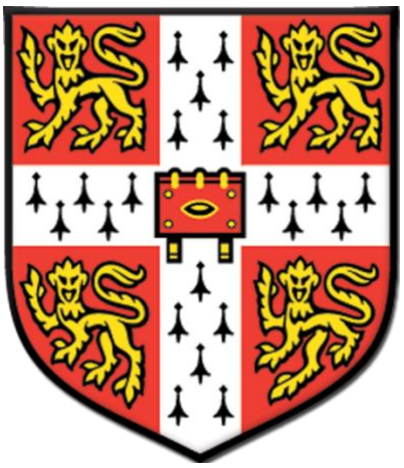
A genetic dissection of cancer cell resistance to immune cell attacks using the CRISPR/Cas9 system



This dissertation is submitted for the degree of

Master of Philosophy

Dimitrios A. Garyfallos

Wellcome Trust Sanger Institute

Wolfson College

University of Cambridge

September 2015

Preface

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

This dissertation does not exceed the word limit of 20,000 words.

Acknowledgements

I would like to thank my supervisor, Dr Pentao Liu, for the opportunity he gave me to work on this exciting project and his support on both my current and future academic endeavours. I would also like to thank Dr Li Li, Dr Yong Yu and Dr Xuefei Gao for their assistance, insight and for answering my many questions, along with all members of Team 18, current and past, for the friendly environment and help throughout this year. I would also like to offer my gratitude to Dr Walid Khaled, for his mentorship, his enthusiasm and the opportunity he gave me to learn from him.

A special thanks to Liliana Antunes for her technical and theoretical expertise, for her constant assistance, friendship and guidance. Also, to Maria Levitin for her friendship, the feedback, the long discussions, the self-analysis and the moral support. To Velislava Petrova, for the feedback on this thesis, for igniting an interest in the world of immunology, and for the understanding and the depth of discussions. To Mari Niemi, for her great feedback, friendship, support and “work club” times.

I would also like to offer my deepest gratitude to Dr Carl Anderson for his advice, time and support. My thanks also to Dr Annabel Smith and Dr Christina Hedberg-Delouka for making every effort to facilitate this year. Moreover, to Dr George Vassiliou for his guidance, feedback and time, along with Dr David Adams for his constructive advice and instructions.

To all the members of “the family”, Fernando, Martin, Veli, Masha, Liliana, Alice, I thank you from the bottom of my heart for the lunch and coffee breaks, the laughs, the stimulating discussions and for making every day unforgettable. Nothing would have been the same without you.

To all members of PhD14, who wholeheartedly accepted an outsider, reading for an obscure, unknown Sanger degree, into their ranks. I deeply thank them for their friendship. To the Sanger community, which showed me that excellent science can be accompanied by a friendly, dynamic and collaborative environment. To the unique community of Wolfson College, Cambridge, for friendships to last and for the highly stimulating environment.

Finally, nothing would have been possible without the endless support of my parents and my family. I owe them the deepest gratitude for encouraging me to pursue my dreams and for always being a pillar of love and care. It is to my parents, Alexandros and Thaleia, that this thesis is dedicated.

Abbreviations

2-ME: 2-mercaptoethanol

bp: base pairs

Cas9: CRISPR-associated protein 9

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CGP: Cancer Genome Project

CRISPR: clustered regularly interspaced short palindromic repeats

CTL: cytotoxic T lymphocyte

DAMPs: damage-associated molecular patterns

DMSO: dimethyl sulphoxide

DPBS: Dulbecco's phosphate buffered saline

EGFP: enhanced green fluorescent protein

EMT: epithelial-to-mesenchymal transition

FACS: fluorescence-activated cell sorting

FBS: foetal bovine serum

GFP: green fluorescent protein

GPI: glycosylphosphatidylinositol

gRNA: guide RNA

HDR: homology-directed repair

IFN-γ: interferon-γ

IL-3: interleukin-3

Indel: insertion or deletion

ITNK cells: induced T-to-NK cells

kDa: kilo Dalton

LAK: lymphokine-activated killer

MDSCs: myeloid-derived suppressor cells

MHC: major histocompatibility complex

NHEJ: nonhomologous end joining

NK cells: natural killer cells

OHT: (Z)-4-hydroxytamoxifen

PAM: protospacer adjacent motif

PAMPs: pathogen-associated molecular patterns

PB transposon: Piggy Bac transposon

PCR: polymerase chain reaction

Pen Strep: penicillin streptomycin

qPCR: quantitative polymerase chain reaction

RAG: recombination-activating gene

RNAi: RNA interference

RSF: Research Support Facility

TGF-β: transforming growth factor-β

T_h cell: helper T cell

TNF: tumour necrosis factor

Treg: regulatory T cell

WTSI: Wellcome Trust Sanger Institute

Abstract

Cancer is undoubtedly one of the main causes of death worldwide that affects individuals indiscriminately. Epidemiological evidence has shown that, in 2012, 14.1 million new cases of malignant neoplasias were reported around the world. Out of all the different carcinomas, the most commonly reported ones are lung, female breast, bowel, and prostate cancer (CRUK). In their journey from normality to higher states of malignancy, incipient cancer cells need to acquire a number of hallmark abilities, as described by Hanahan and Weinberg. Following up current advancements in cancer research, several studies highlighted the importance of the immune system in cancer progression and, most importantly, of tumour immunological evasion (Hanahan and Weinberg 2011). It is this hallmark ability that this project is attempting to shed light on. The main aim is the elucidation of the genetic basis of cancer cell resistance to immunological responses. The Liu group had previously developed a potent cancer-killing cell type, termed "induced T-to-NK (ITNK) cells" (Li, Burke et al. 2010). Taking advantage of ground-breaking advances in genome editing technologies, previous work in the group led to the development of a forward genetic screen based on the CRISPR/Cas9 system. The screen involved the use of genome editing to mediate knockouts in different genes across the genome of the MC38 mouse colon carcinoma cell line. Edited MC38 cells were then co-cultured with the ITNK cells to carry out a phenotypic screening and identify candidate genes, which were suspected to be involved in cancer cell immunological resistance. My work involved the validation of several candidate genes by developing individual knockout cell lines, each carrying a single gene deletion. The cell lines were then screened anew with the ITNK cells in order to verify their ability to evade cytotoxicity. This strategy aimed to identify novel genes which are important for cancer cell-immune cell interactions. These genes could prove to be essential for a better understanding of neoplastic disease and could also be used as clinical markers indicative of cancer progression. Finally, they could form the basis for designing new immunotherapeutic protocols.

Table of contents

1. Introduction

1.1 Aims

The aim of the project is the elucidation of the genetic basis of cancer cell resistance to immunological responses. The highly efficient genome editing tool of CRISPR/Cas9 enables precise targeting of genes at a genome-wide level. Previous work in our group had used this system to design and carry out a forward CRISPR/Cas9-based genetic screen. For the screen, the mouse colon carcinoma cell line MC38 was used to identify genes regulating resistance to "induced T-to-NK (ITNK) cells", a cell type which was developed in our laboratory, and which has robust tumour-killing potential *in vitro*. My work focused on validating the candidate genes identified in that screen. To do so, I developed single knockout cell lines for each of the candidate genes and screened them with ITNK cells individually.

1.2 Cancer: what is it?

Millions of years of evolution and natural selection have created an enormous complexity of tissues. This process allowed the organisms to transcend the boundaries of unicellular structure, reaching towards multicellular organisation of their physiology, by means of tissue morphogenesis. The ability of an organism to differentiate from an initial starting cell into a plethora of cellular subtypes, each with distinct functions, is of paramount importance for achieving higher levels of cellular organisation. These differentiation mechanisms are tightly regulated in normal tissues, following multiple interconnected molecular pathways that operate in tandem. The fact that most cells of the body of metazoans carry a complete copy of the genetic material of the organism ensures their autonomy and adaptability. Moreover, even after the completion of the development of the organism, the ability of a cell to regain access to differentiation mechanisms is retained in most cell types. This quality provides the organism with the ability to repair wounds and replace cells with reduced viability. However, despite its fundamental role in the survival of the whole organism, cellular adaptability creates the potential for dysregulation of cellular processes, giving rise to alternative phenotypes. These phenotypes may be defined by altered and uncontrolled proliferative capabilities, resulting in a cellular population unresponsive to the governing principles of

cellular organisation. These malignant cells could form a multitude of different neoplasias, and they have several distinct characteristics. First of all, they arise from normal tissues, which include a variety of specialised cells in the body. Furthermore, they seem to progress by evolving from a relatively normal to a highly malignant state. Therefore, cancer is the clinical condition that is manifested by the presence of neoplastic growths, which occur when normal cells deviate from the strict control of cellular homeostasis and acquire certain hallmark capabilities (Weinberg 2013).

1.3 Hallmarks of Cancer

In their original work published in 2000 (Hanahan and Weinberg 2000), Hanahan and Weinberg provide the conceptual background for understanding the complexity of neoplastic disease and the characteristics a normal cell needs to acquire in order to progress towards higher states of malignancy (see Fig. 1). Therefore, tumour cells need to be able to maintain a state of constant proliferation, while circumventing protective mechanisms of growth arrest. Furthermore, they need to avoid antiproliferative apoptotic signalling and become immortalised by overcoming the pathways of senescence and crisis. Moreover, they are required to maintain a constant supply of nutrients and disposal of metabolic byproducts by activating proangiogenic signalling. One of the most distinct properties of cancer cells is the ability to relocate from their tissue of origin into new sites and adapt to the new microenvironment. Current advancements in cancer research have revealed several of these mechanisms and have highlighted the importance of new ones. Therefore, in their more current work (Hanahan and Weinberg 2011), Hanahan and Weinberg emphasise the importance of cancer cell mutagenicity and genetic instability, along with the repurposing of cancer cell energy metabolism. They highlight the pivotal role of the immune system and, most importantly, the role of inflammatory responses in promoting cancer progression. Finally, they accentuate the crucial ability of cancer cells to avoid cancer-specific immunosurveillance (see Fig. 2). It is this final emerging hallmark that this project is attempting to elucidate.

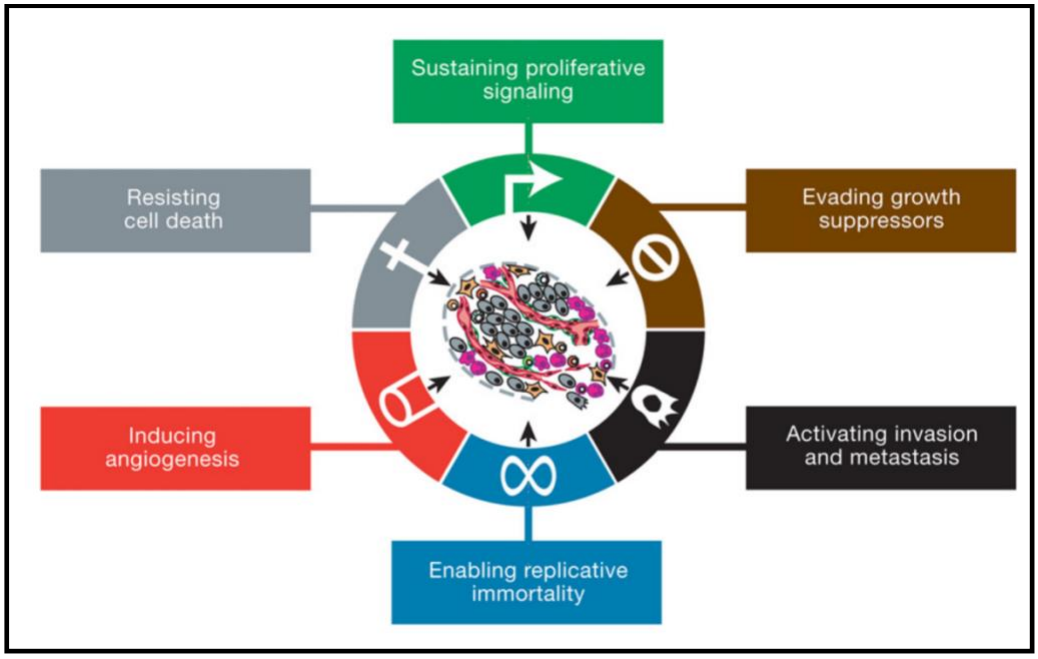


Fig. 1. The original hallmarks of cancer (adapted from Hanahan and Weinberg 2011).

Probably the most characteristic ability of cancer cells is their ability to maintain constant proliferative potential, disregarding the tightly regulated cell fate. This cell fate, in normal cells, controls cellular homeostasis in terms of cell number, morphology, longevity and function, characteristics that are deregulated in neoplasias. Progress in recent years has highlighted the significance of mitogens in promoting tumour progression (Perona 2006, Hynes and MacDonald 2009, Lemmon and Schlessinger 2010, Witsch, Sela et al. 2010, Hanahan and Weinberg 2011). Mechanisms through which cancer cells acquire the ability for unlimited proliferation include production of autocrine signalling that activates proliferation or recruitment of tumour-promoting stromal cells, which, in turn, secrete beneficial growth factors (Bhowmick, Neilson et al. 2004, Cheng, Chytil et al. 2008). Also, they can achieve the same effect by modifying the binding efficiency and sensitivity of their own receptors to signalling molecules. Finally, they can ensure constant firing of certain signals by altering signalling cascades or by deregulating negative-feedback loops (Hanahan and Weinberg 2011).

Cancer cells exhibit the ability to evade suppression of their proliferative signalling. This ability is largely based on genes that act as tumour suppressors, such as genes encoding the retinoblastoma-associated protein (RB) and the TP53 protein (Sherr and McCormick 2002, Deshpande, Sicinski et al. 2005, Hanahan and Weinberg 2011). These two proteins represent an important defence mechanism since they are regulators of cellular proliferation.

Therefore, cancer-promoting mutations that result in their deactivation allow tumour cells to maintain their proliferative potential (Sherr and McCormick 2002, Burkhart and Sage 2008, Hanahan and Weinberg 2011).

Furthermore, the apoptotic pathway is of paramount importance to development, tissue homeostasis, and protection from parasitic and viral infections. A great number of studies have also highlighted its importance in the protection from neoplastic disease, as it constitutes an inhibitory barrier to the excessively proliferating cells (Evan and Littlewood 1998, Cory and Adams 2002, Lowe, Cepero et al. 2004, Adams and Cory 2007). Therefore, cancer cells are able to evade the antiproliferative barrier that apoptosis poses by inactivating TP53-mediated tumour inhibition, by upregulating antiapoptotic or survival signals, or by downregulating proapoptotic molecules (Hanahan and Weinberg 2011).

In normal cells, upon reaching the limit of possible replications, cells become either senescent, a state of non-proliferative viability, or they enter crisis, which results in cell death (Hanahan and Weinberg 2011). The hexanucleotide multiple repeats that protect the ends of chromosomes are termed telomeres. Sequential cell replications result in their continuous shortening. Thus, the chromosomes are left unprotected and exposed to genomic erosion. At this point, cells reach senescence and the ones which manage to survive keep proliferating. Extensive chromosomal erosion results in genomic damage, which leads to crisis. After this stage, only immortalised cells manage to acquire the potential for unlimited replication (Shay and Wright 2000, Blasco 2005, Hanahan and Weinberg 2011). Extension of telomeres is possible through the actions of the DNA polymerase, telomerase. This enzyme negates the effects of telomeric attrition by mediating the addition of telomere repeats at the site of the chromosomal ends. The induction of telomerase activity is an important cancer cell response in order to promote malignant proliferation (Hanahan and Weinberg 2011). Several studies indicated the significance of ephemeral telomere shortening-mediated genomic instability and the evolutionary advantage it provides to cancer progression. More specifically, continuous shortening of telomeres in the premalignant conditions results in chromosomal anomalies and the concomitant acquisition of tumour-enhancing mutations. After that, the activation of telomerase restores genomic chromosomal stability and taps into the unlimited proliferative potential of cancer cells (Chin, de Solorzano et al. 2004, Raynaud, Hernandez et al. 2010, Hanahan and Weinberg 2011).

Cancer progression constitutes an active process of Darwinian evolutionary adaptation, whose driving force is mutation. Cancer cells with enabling mutant genetic traits survive the multitude of molecular and cellular selective pressures and defence mechanisms employed by the organism to halt the proliferation of malignant cells. These subclones with the favourable mutations are able to expand and adapt. The adaptations are permitted by the highly mutagenic nature of the cancer genome (Negrini, Gorgoulis et al. 2010, Hanahan and Weinberg 2011). This increased mutation rate is one of the main sources of cancer adaptability (Sigal and Rotter 2000, Jackson and Bartek 2009, Hanahan and Weinberg 2011).

Another crucial ability of cancer cells is the constitutive activation of angiogenesis, the creation of new vasculature from pre-existing tubes. One of the main limitations for the survival and proliferation of all cell types, including malignant ones, is their ability to ensure a continuous supply of necessary materials for their metabolism, while removing carbon dioxide and metabolic by-products. Therefore, constant firing of angiogenic signalling is a key ability that tumour cells need to acquire (Hanahan and Folkman 1996, Hanahan and Weinberg 2011).

Finally, from early on, it was shown that cancer cells have altered metabolic functions by following the pathway of aerobic glycolysis (Warburg 1956, Warburg 1956, Hanahan and Weinberg 2011).

Arguably, one of the most characteristic traits of cancer cells is their ability to invade neighbouring tissues and metastasise to other parts of the body, adapting to the new environments and forming new tumours there. The processes regulating this hallmark have been termed the invasion-metastasis cascade. The acquisition of the hallmark abilities of invasion and metastasis happens through a developmental programme, named epithelial-to-mesenchymal transition (EMT) (Hanahan and Weinberg 2011).

The site of a tumour is highly infiltrated by cells of both innate and adaptive immunity, which represents an anticancer immunological response. A better understanding of cancer-immune cell interactions has revealed that the recruitment of these immune cells also plays a fundamental role in cancer progression and the acquisition of malignant properties. It has been shown that inflammation positively impacts several stages of cancer progression and provides tumour-promoting molecules to the tumour microenvironment. It also provides signals that regulate developmental programmes through which cancer acquires hallmark abilities (such as EMT) (Karnoub and Weinberg 2006, DeNardo, Andreu et al. 2010,

Grivennikov, Greten et al. 2010, Qian and Pollard 2010). Moreover, it facilitates cancer development to a more malignant state (de Visser, Eichten et al. 2006, Qian and Pollard 2010) and is involved in the secretion of mutagenic chemicals that increase tumour mutagenicity. Thus, it promotes cancer progression (Grivennikov, Greten et al. 2010, Hanahan and Weinberg 2011).

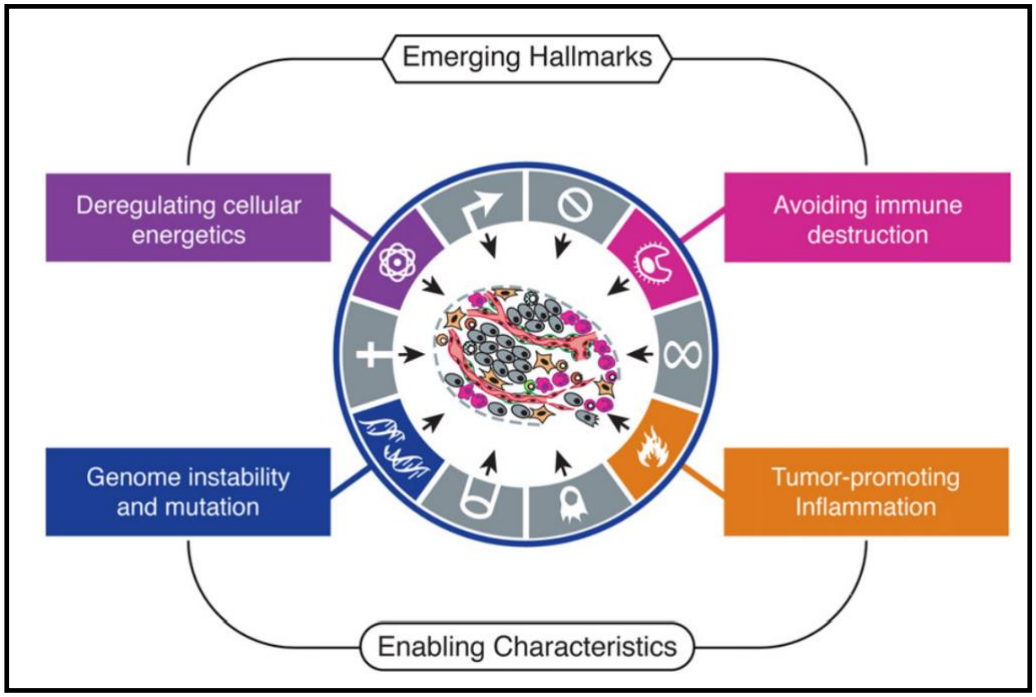


Fig. 2. Emerging hallmarks and enabling characteristics of cancer cells, taking into consideration current scientific advancements in the understanding of cancerous malignancies (adapted from Hanahan and Weinberg 2011).

1.4 Mechanisms of cancer immune evasion

The immune system has exquisitely adapted to combat cancer. Through a series of different mechanisms, cells of the immune system maintain a constant state of surveillance, termed immunosurveillance, in order to identify and eradicate newly arising cancer cells and tumours. Recent data suggest that immunosurveillance is part of a broader mechanism that has been named immunoediting. This mechanism reflects the duality of the nature of the immune system, mediating the direct destruction of tumours as well as the shaping of the immunological profile of cancer cells towards higher stages of immune evasion and malignancy (Dunn, Bruce et al. 2002, Smyth, Dunn et al. 2006, Hanahan and Weinberg 2011).

More specifically, immunoediting has been divided into 3 separate stages: elimination, equilibrium and escape. The elimination stage includes the mechanisms of immunosurveillance, the immune-mediated recognition and eradication of cancer cells. In the equilibrium stage, cancer cells that survived the initial elimination stage are modulated by the immune system's selective pressure towards a less immunogenic stage, a process that might take several years to be completed. These cancer clones with reduced immunogenicity are more likely to evade the cancer-killing mechanisms of the immune system. Finally, these resistant immunomodulated cancer clones, being seemingly undetected by the immune system, are able to escape and lead to the clinical manifestation of neoplastic malignancy (Dunn, Old et al. 2004, Kim, Emi et al. 2007, Teng, Swann et al. 2008). Altogether, this leads to the conclusion that surviving malignant tumour-forming cells have adapted to the immunological pressure and have acquired the ability to evade recognition by the immune cells. Alternatively, they have developed ways to modulate the efficiency of anti-tumour immune responses (Hanahan and Weinberg 2011).

The immunological basis of cancer resistance has been shown by studies in which mice, which had been genetically engineered to lack certain immune cells, were treated with a carcinogen in order to induce tumour formation. Analysing cancer initiation and progression in both immunocompromised and immunocompetent mice, it was found that immunocompromised mice developed malignant tumours in significantly greater numbers, and these tumours grew faster than those observed in the control mice. These studies highlighted the importance of certain immune cell types of both the innate and the adaptive immune system, in particular, $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ T_h1 helper cells, and natural killer cells (NK cells), in intercepting malignant disease (Kim, Emi et al. 2007, Teng, Swann et al. 2008, Hanahan and Weinberg 2011). Clinical evidence on prognostic factors in colon and ovarian cancers shows that patients with tumours which were highly infiltrated by NK cells and CTLs fare better than patients with non-infiltrated malignant growths (Nelson 2008, Pages, Galon et al. 2010, Hanahan and Weinberg 2011).

Other studies, in which cancer cells were transplanted into genetically immunocompatible mice, showed that cancer cells that developed in immunocompromised mice were unable to establish malignant disease in immunocompetent recipients. In contrast, cancers derived from immunocompetent mice easily established tumours in both immunocompetent and immunocompromised mice. The reason for this was that immunocompetent-derived clones

had been selected for their low immunogenicity, which rendered them invisible to immune attacks. On the other hand, selection in immunocompromised mice was inefficient, not enabling cancer cells to reach the necessary level of low immunogenicity (Smyth, Dunn et al. 2006, Kim, Emi et al. 2007, Teng, Swann et al. 2008, Hanahan and Weinberg 2011).

In addition to the previous model, it has been observed that certain cancers which raise a strong immune response are able to evade immune cell-mediated killing. Tumour cells may produce signalling molecules that arrest the cancer-killing immune cells, such as transforming growth factor (TGF)-β (Shields, Kourtis et al. 2010, Yang, Pang et al. 2010). Finally, an alternative approach through which cancer cells achieve immunological evasion is the recruitment of immune cells that have an immunosuppressive effect. For example, regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) are both able to limit cytotoxic lymphocyte-mediated immune responses and are both attracted to the tumour site by tumour-promoting inflammatory responses (Ostrand-Rosenberg and Sinha 2009, Mougiakakos, Choudhury et al. 2010, Hanahan and Weinberg 2011).

1.5 Overview of the immune system

All the different molecules, cells and tissues in the organism working in tandem to confer protection to infectious agents are collectively termed the immune system. Their intertwined mechanisms, which respond to infectious invasions, are called the immune response. Overwhelming data over the last few decades have highlighted the paramount importance of immune responses, not only in the prevention and elimination of infections, but also in protecting from cancer initiation and progression, disposal of cell debris and tissue repair. Moreover, the perturbation of these mechanisms might lead to immunological diseases. Immune responses can be classified into two major complementary categories (see Fig. 3): innate immunity, which is the first to respond, and adaptive immunity, which is raised later on (Abbas, Lichtman et al. 2014).

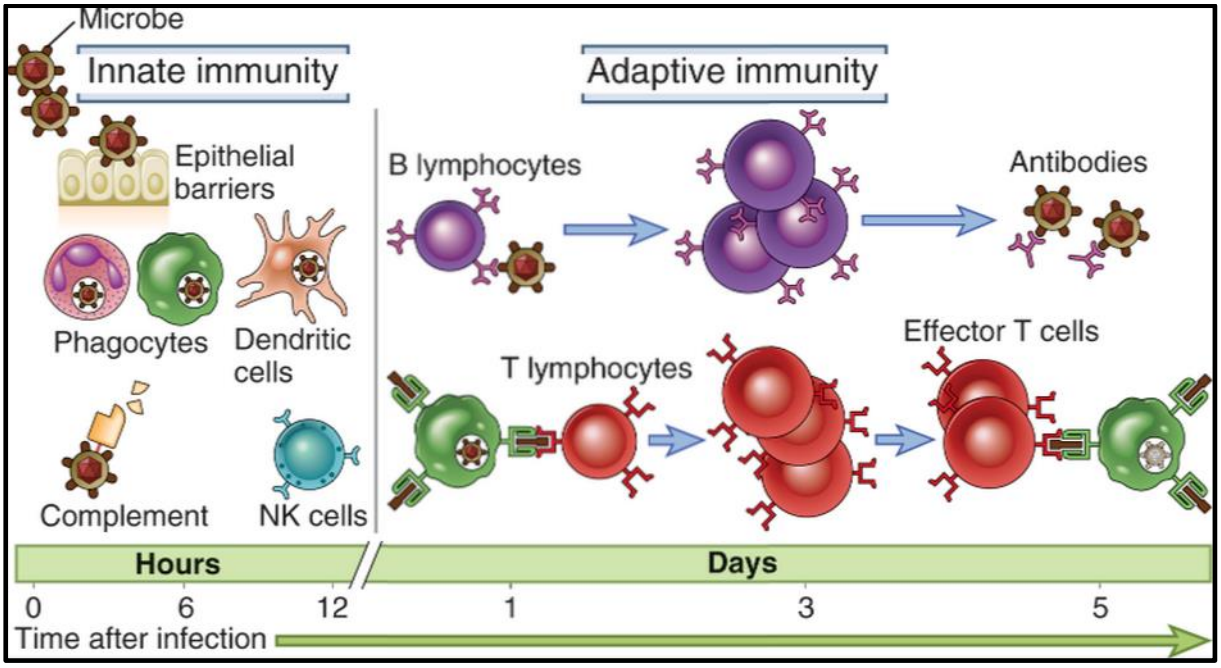


Fig. 3. Overview of the immune system showing the different cellular components of the innate and adaptive immunity. Upon infection, innate immunity responds first to block (epithelial barriers) and eradicate (phagocytes, dendritic cells, complement system and NK cells) incoming infectious agents. Adaptive immunity is raised later and entails the actions of lymphocytes and their products, the antibodies. The antibodies bind to microbes and eradicate them, while T lymphocytes attack cells which have been infected by the infectious agent (adapted from Abbas, Lichtman et al. 2014).

1.6 Innate and adaptive immune system

Innate immunity refers to all the host defence mechanisms mediating the initial and immediate immunological response in the prevention and eradication of infections. These mechanisms are present at all times in healthy individuals and evolutionarily precede the mechanisms of adaptive immunity. The first obstacles to the entry of infectious agents are the epithelial barriers, cells and antibiotics that are naturally present in the epithelium. Apart from these, special cell types, such as phagocytes, natural killer cells, along with proteins circulating in the plasma, such as the complement system, effectively attack microbes that manage to circumvent the initial barriers. Moreover, the components of the innate immune system are also integral in assisting the adaptive immune system to effectively raise a response.

The innate immune responses have several characteristics that differentiate them from the mechanisms of the adaptive immune system. First of all, they have no memory of exposure to a pathogen and respond to any microbial intrusion in a similar manner. The structures they recognise are a limited number of molecules that are common in many classes of microbes

but absent in normal cells. These molecules are termed pathogen-associated molecular patterns (PAMPs), and the receptors of the innate immune cells that recognise and react with them are called pattern recognition receptors. The effectiveness of their recognition stems from the fact that they have evolved to identify integral components of microbes, which are crucial for microbial infectious potential and survival. Also, apart from PAMPs, they are able to identify molecules released upon cell damage or necrosis, termed damage-associated molecular patterns (DAMPs), thus mediating clearance of cell debris and activation of tissue repair. This recognition potential of innate immunity is genetically predetermined, in contrast to adaptive immune responses, for which recognition is the result of somatic genetic recombination and hypermutation. This strict and limited repertoire of molecules and patterns that innate immune cells recognise is the basis of their intrinsic specificity, according to which they do not react against self-antigens (Abbas, Lichtman et al. 2014). Finally, an important non-adaptive response is inflammation, which represents the immune system's response to infection, toxins and cellular damage, in tissues that are vascularised (Abbas, Lichtman et al. 2014).

Adaptive immunity comprises humoral and cell-mediated immunity, and each is controlled by different cell types and molecules (see Fig. 4). In humoral immunity, the main mediators are the antibodies produced by lymphocytes. Antibodies, which are released into the circulation, are able to identify and neutralise microbes and their toxins. They are present in the extracellular space, most importantly in the blood and the lumen of mucosal organs. Their most significant contribution to the adaptive immune response is the fact that they block microbes which have entered the mucosal surface and the bloodstream from entering and proliferating in host cells and connective tissues. Cell-mediated immunity responds against intracellular microbes. These functions are controlled by T lymphocytes, some of which activate other immune cells (helper T lymphocytes), while others kill infected cells through direct cytotoxic effects (cytotoxic T lymphocytes) (Abbas, Lichtman et al. 2014).

Lymphocytes are the main driving force of adaptive immunity, able to produce specific receptors identifying a wide spectrum of antigens. The antibody-secreting lymphocytes, B lymphocytes, produce surface receptors, which are surface-bound forms of antibodies. Cell-mediated immunity is promoted by T lymphocytes, whose receptors recognise antigens displayed by major histocompatibility complex (MHC) molecules. There are several categories: helper T cells (T_h cells) are $CD4^+$ and assist B lymphocytes in antibody production

and phagocytes in destruction of phagocytosed microbes; cytotoxic T lymphocytes are $CD8^+$ and eradicate cells infected with intracellular infectious agents; Treg cells are $CD4^+$ and have the ability to suppress immune responses; finally, natural killer cells (NK cells) are part of the innate immune system (see section 1.8) and also have cytotoxic potential but their recognition patterns are germline encoded (Vivier, Raulet et al. 2011, Abbas, Lichtman et al. 2014).

For the purposes of this study, we will focus mostly on cytotoxic cell types. These cells include cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, natural killer T (NKT) cells and finally induced T-to-NK (ITNK) cells.

1.7 Cytotoxic T lymphocytes

Cytotoxic T lymphocytes (CTLs) are a subtype of T cells, and they belong to the adaptive immune system. They are $CD8^+$ and have cytotoxic effects against cells expressing MHC class I molecules presenting a foreign antigen. They respond to cells which have been infected by microbes and also have anti-tumour properties. These last properties are mediated by dendritic cells, which are involved in cross-presenting cancer antigens to $CD8^+$ T cells. Other cellular events also lead to the activation of the $CD8^+$ T cells, which express their anti-tumour cytotoxic potential by exocytosis of proapoptotic enzymes (Abbas, Lichtman et al. 2014).

1.8 Natural killer cells

Natural killer cells have been considered an integral part of innate immunity (Cerwenka and Lanier 2001, Abbas, Lichtman et al. 2014). Many studies have highlighted the fact that NK cells are characterised by great diversity, moulded by epigenetic and environmental changes (Cerwenka and Lanier 2001). They differentiate from hematopoietic stem cells of the bone marrow (Wu, Li et al. 2014). NK cells represent the initial immune response to infection and cancer and comprise 5-15% of the lymphocytes found in the peripheral blood of healthy individuals (Cerwenka and Lanier 2001, Pahl and Cerwenka 2015). While T and B cells of adaptive immunity gain their antigen recognition repertoire through a recombination-activating gene (RAG)-mediated mechanism, the NK cell source of specificity differs. NK cells have the ability to identify and respond to cancer cells, cells that have been infected by a virus or cells that are under stress, while they do not attack healthy host cells. The basis of their recognition potential is encoded in their germline and is genetically predetermined. It is

mediated by cell surface receptors involved in inhibition, activation and adhesion. However, it has been shown that RAG recombinase is crucial for NK cell genome stability and their sensitivity to apoptosis (Vivier, Raulet et al. 2011, Karo, Schatz et al. 2014, Pahl and Cerwenka 2015). According to their traditional description, NK cells recognise their target cells according to the missing-self hypothesis, identifying cells which have downregulated or express incomplete MHC class I molecules (Karre, Ljunggren et al. 1986, Cerwenka and Lanier 2001, Vivier, Tomasello et al. 2008). NK cell cytotoxicity is mediated by a series of secreted proteins. First of all, the release of perforin induces the formation of pores on the cell surface of target cells. The next step involves the exocytosis of cytotoxic proteins, which belong to the granzyme family of serine proteases. These proteins are encased in cytoplasmic granules and are released into the site of contact between NK and target cells (termed the immunological synapse). These proteins lead to target cell killing via the activation of the apoptotic pathway (Smyth, Cretney et al. 2005, Smyth, Swann et al. 2005, Krzewski and Strominger 2008, Pahl and Cerwenka 2015). Another important aspect of NK cell immunity is their ability to produce interferon-γ (IFN-γ), which results in the upregulation of MHC class I molecules on the cancer cells and of MHC class II molecules on antigen-presenting cells. Thus, NK cells connect the functions of innate and adaptive immunity (Vivier, Tomasello et al. 2008, Pahl and Cerwenka 2015). Finally, it has been shown that NK cells promote anticancer responses of B and T cell-mediated adaptive immunity and memory. Moreover, they have immunomodulatory properties by affecting populations of dendritic cells and CD4/CD8 double-positive T cells (Pahl and Cerwenka 2015). From all these, it becomes obvious that the traditional classification of NK cells as cells of the innate immune system is brought into question, since several studies suggest their adaptive-like properties, such as responses targeted to specific antigens along with selection and expansion of specific clones (Sun, Ugolini et al. 2014, Pahl and Cerwenka 2015).

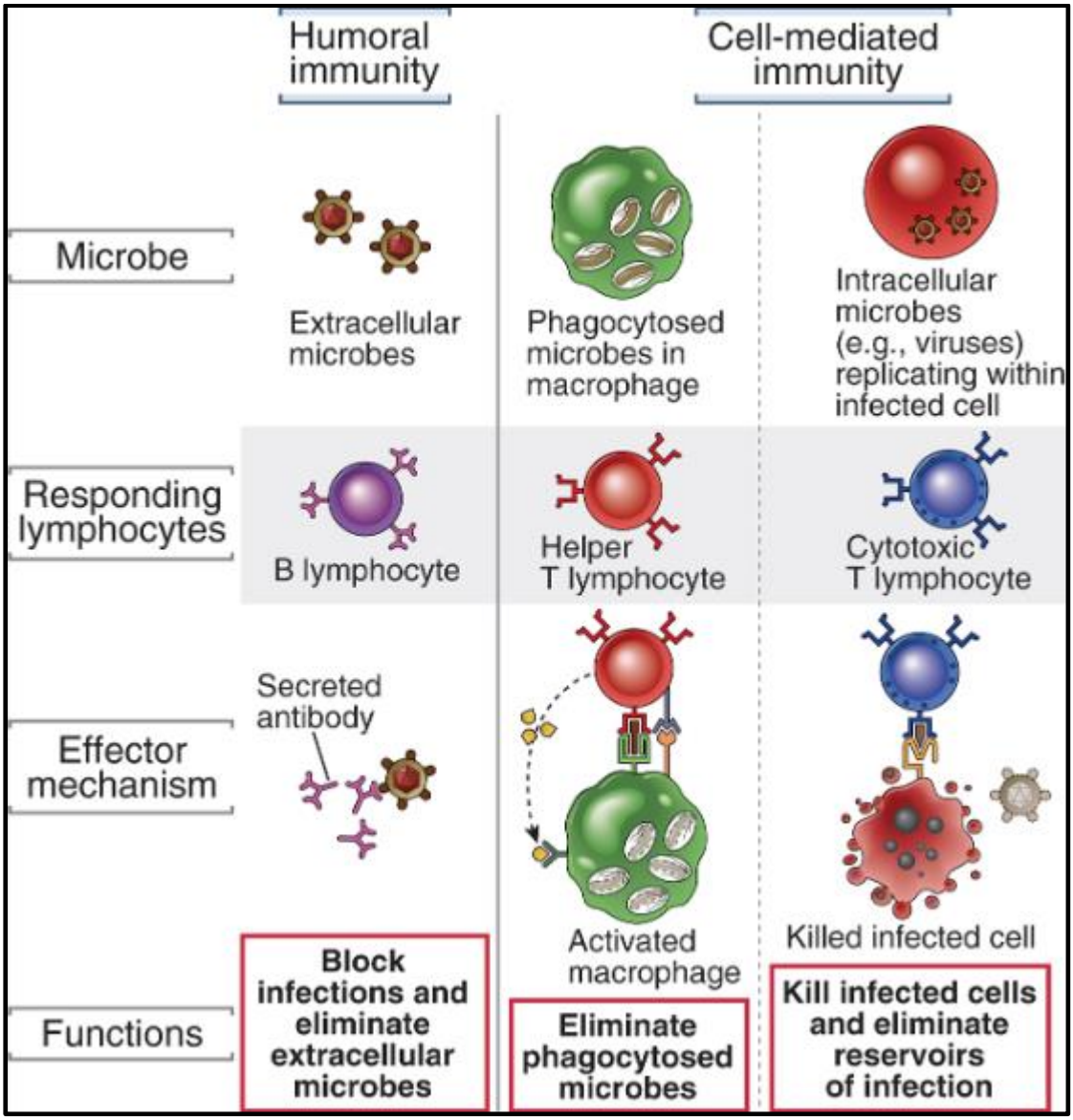


Fig. 4. Outline of humoral and cell-mediated immunity of the adaptive immune system upon infection. In humoral immunity, B lymphocytes produce antibodies, which have the ability to stop infections and eradicate microbes outside the cell. In adaptive immunity, the main mediators are the different categories of T lymphocytes, which result in helper T lymphocyte-mediated killing of microbes that have been phagocytosed, and in cytotoxic T lymphocyte-mediated elimination of infected cellular reservoirs (adapted from Abbas, Lichtman et al. 2014).

1.9 Natural Killer T cells

A small and relatively uncategorised subset of T lymphocytes has been termed natural killer T cells (NKT cells). This cell type expresses certain T cell receptors along with certain surface markers that traditionally characterise NK cells, most importantly NK1.1. Initially, two populations of NKT cells were recognised, one being $CD4^+$ and the other being $CD4^-/CD8^-$ (Godfrey, MacDonald et al. 2004, Abbas, Lichtman et al. 2014). Both of these populations have been observed to express αβ T cell antigens with minimal diversity and recognise

antigens which are presented by CD1 molecules. They seem to have similarities to helper T cells, presenting effector activity. They seem to be a heterogeneous population, while their exact role is largely unknown (Godfrey, MacDonald et al. 2004, Abbas, Lichtman et al. 2014).

1.10 Induced T-to-NK cells

In the mouse, Bcl11b is an integral transcription factor for foetal survival and development of thymocytes. Moreover, it has been shown to play a crucial role in survival and positive selection of CD4/CD8 double-positive thymocytes (Wakabayashi, Watanabe et al. 2003, Albu, Feng et al. 2007, Li, Burke et al. 2010). Apart from this, it has also been shown to be essential for the development of T cells and maintenance of T cell identity. Experimental data have highlighted that loss of *Bcl11b* expression results in loss of T cell commitment from double negative thymocytes (Li, Burke et al. 2010, Liu, Li et al. 2010). Upon conditional deletion of *Bcl11b*, it was found that T cells of all developmental stages downregulated markers associated with a T cell phenotype and upregulated markers characteristic of NK cells (such as NKp46, NK1.1, NKG2A/C/E, TRAIL, perforin and IFN-γ). Furthermore, this cell type was found to be similar to NK cells, from a morphological and genetic perspective. Hence, this novel cell type, developed in our laboratory (Li, Burke et al. 2010), was termed induced T-to-NK (ITNK) cells. They exhibit robust tumour killing potential *in vitro*, along with metastasis prevention potential *in vivo*. In addition, they are able to distinguish healthy tissues since no sign of autoimmunity was detected. ITNK cells also have genetic and morphological similarities to lymphokine-activated killer (LAK) cells, being larger than thymocytes. They have granules, they appear to have elevated protein synthesis, and their endoplasmic reticulum is enlarged. They are also able to kill tumour cells irrespective of whether they express MHC class I molecules or not. ITNK cells can be obtained with 3 different approaches, outlined in Fig. 5. It was found that the cell type has an average life span of 3 months *in vivo*, even though it was shorter *in vitro*. Finally, they are highly proliferative and can be cultured to significant quantities *in vitro*, making them fit for studies for which high cell numbers are necessary. All these qualities make them an ideal candidate for cancer immunotherapy (Li, Burke et al. 2010, Liu, Li et al. 2010) or an immunological selection tool in genetic screening studies.

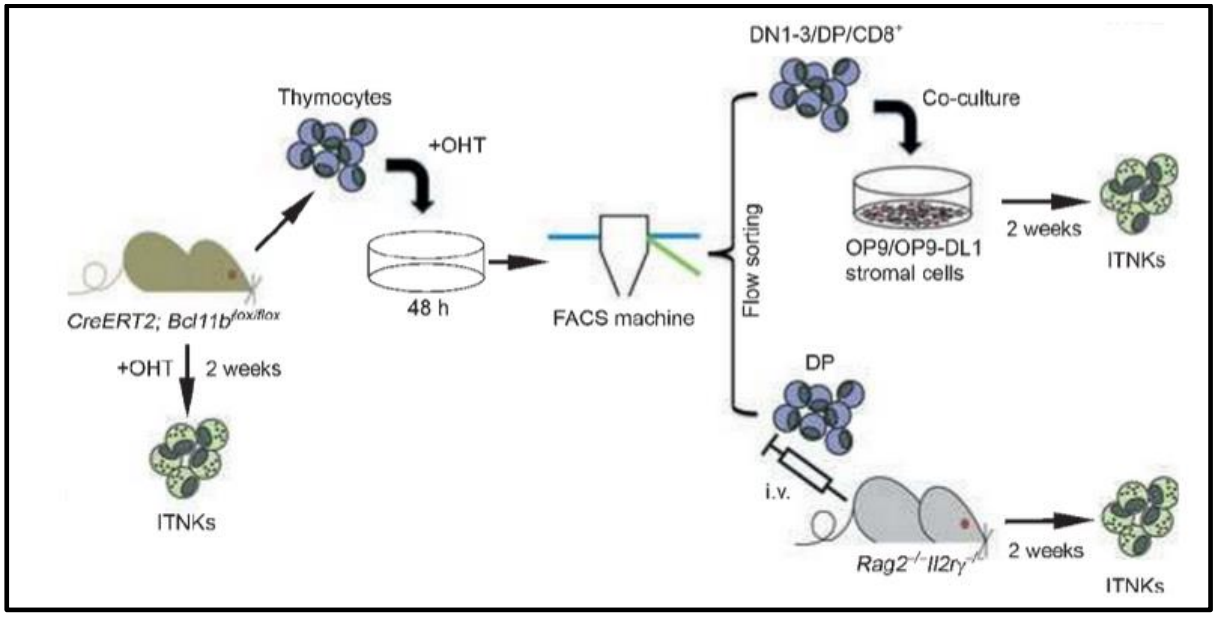


Fig. 5. Obtaining ITNK cells from *Bcl11b*-deficient thymocytes. Three different approaches have been described. For the first approach, *CreERT2;Bcl11bflox/flox* mice were treated with OHT, in order to delete *Bcl11b*, and ITNK cells were observed in the mouse peripheral blood, spleen and thymus, around 2 weeks post OHT treatment. For the second approach, thymocytes from a *CreERT2;Bcl11bflox/flox* mouse were obtained and cultured *in vitro*. The thymocytes were treated for 48 h with OHT and then separated into different subpopulations via FACS. The different populations were then cultured on OP9/OP9-DL1 stromal cells. ITNK cells were obtained approximately 2 weeks post-OHT treatment. Finally, the third approach included the isolation of the double-positive thymocytes, after cell sorting of OHT-treated thymocytes, and their transplantation, by intravenous injection, into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Again, ITNK cells were detected in the recipient mice approximately 2 weeks post-injection (adapted from Liu, Li et al. 2010)

1.11 CRISPR/Cas9 system and lentiviral genome-wide recessive screens

Clustered regularly interspaced short palindromic repeats (CRISPR) were first discovered in *Escherichia coli* cells as short sequential repeats, which are separated by short sequences. They were described by Japanese scientists in 1987. A series of studies have shown that CRISPR, along with CRISPR-associated protein 9 (Cas9), represent a form of bacterial adaptive immune system, protecting host cells from invasions by inducing memory via the use of antisense RNAs (Doudna and Charpentier 2014). Ever since its discovery, the CRISPR/Cas9 system has risen to prominence as a powerful tool of genome editing with countless different applications. The system relies on the nuclease activity of the Cas9 protein, which is targeted to a specific genomic locus by a guide RNA (gRNA) sequence, necessary for Cas9 recruitment (see Fig. 6). This gRNA sequence is composed of a 20 bp guide sequence and a scaffold sequence, and the binding between the 20 bp sequence and the target genomic region is mediated by Watson-Crick base-pairing (Perez, Wang et al. 2008, Garneau, Dupuis et al. 2010, Gasiunas, Barrangou et al. 2012, Jinek, Chylinski et al. 2012, Ran, Hsu et al. 2013). Upon

recruitment, Cas9 mediates a double-stranded break of the targeted sequence, which activates the DNA damage repair machinery. In mammalian cells, the outcome is repair by either nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is activated in the absence of a DNA template and is error-prone. The final outcome of NHEJ is usually the introduction of an insertion or deletion (indel). This last characteristic is the basis of CRISPR/Cas9's gene knockout potential, since indels introduced within the coding region can result in a frameshift, or in the introduction of an early stop codon (Perez, Wang et al. 2008, Ran, Hsu et al. 2013). Given that a DNA template is provided, DNA repair is mediated by high-fidelity HDR. This mechanism operates mostly in dividing cells and has varying efficiency, in relation to the type of cell, the state in which the cell is, along with the template and the genomic locus (Saleh-Gohari and Helleday 2004, Ran, Hsu et al. 2013). The gRNA must bind its target genomic locus right before a sequence, termed protospacer adjacent motif (PAM), which represents the only limitation as far as potential targeted loci are concerned. For the CRISPR/Cas9 system derived from *Streptococcus pyogenes*, the PAM sequence is 5'-NGG, even though CRISPR/Cas systems stemming from different organisms have different requirements (Jinek, Chylinski et al. 2012, Ran, Hsu et al. 2013). The Cas9 protein will mediate the double-stranded break approximately 3 nucleotides upstream of the PAM sequence (Ran, Hsu et al. 2013). Finally, Cas9 allows for multiplex targeting of multiple genetic loci simultaneously (Ran, Hsu et al. 2013).

The implementation of the CRISPR/Cas9 technology in genetics has given rise to powerful new tools for genetic screening with multiple applications. One of these approaches holds great promise as a tool for genetic studies, allowing a genome-wide genetic screen using gRNAs expressed by lentiviral vectors (Koike-Yusa, Li et al. 2014).

Increasing evidence on the importance of the role of the immune system in malignant disease highlights the relevance of the current study. We performed an immunological genome-wide genetic screen using the CRISPR/Cas9 system, attempting to elucidate the genetic basis of cancer immune evasion. The combination of the aforementioned technologies of large-scale genome modifications with our unique cancer-killing cell type (ITNK cells) enabled an *in vitro* recapitulation of the cellular interactions between cancer cells and anti-tumour immunological responses. The project is attempting to identify genes which regulate these interactions, with many potential clinical diagnostic or therapeutic applications.

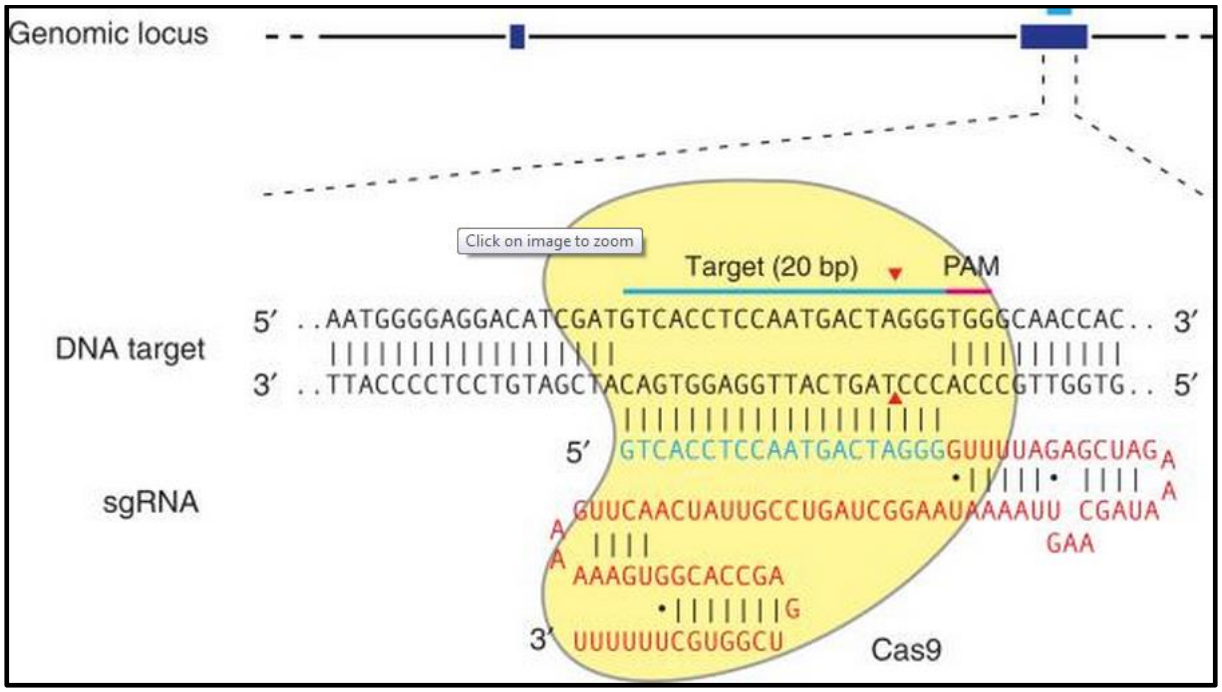


Fig. 6. Schematic of the gene editing tool of the CRISPR/Cas9 system from *S. pyogenes*. Cas9 nuclease is shown in yellow. It is recruited to a specific genomic locus by a gRNA sequence, comprising a 20-nt targeting RNA sequence (in blue) and a scaffold RNA sequence (in red). The targeting sequence pairs with a specific genomic locus, which resides directly upstream of a 5'-NGG PAM sequence (in red). Cas9 mediates a double-stranded break approximately 3 bp upstream of the PAM sequence (red triangles) (adapted from Ran, Hsu et al. 2013).

2. Materials and Methods

2.1 Molecular cloning, bacterial transformation, and plasmid preparation

Transformation of bacterial cells was performed by using One Shot TOP10 Chemically Competent *E. coli* (Invitrogen, cat no: C4040-03), according to the protocol provided by the manufacturer. Mini and maxi preparations were carried out using the QIAprep Spin Miniprep Kit (QIAGEN, cat no: 27104) and the *PureLink* HiPure Plasmid *Maxiprep* Kit (Invitrogen, cat no: K2100-06), respectively, according to the manufacturers' protocols. Sanger sequencing reactions were carried out by the WTSI Capillary sequencing and optical mapping team (team 182) and GATC Biotech.

2.2 Mice

The mice used in the experiments were of a C57BL/6 background and were transgenic for *ERT-Cre/ERT-Cre flox/flox*. They were previously engineered by our group (Team 18) and the Research Support Facility (RSF) of the WTSI, so that exon 4 of the *Bcl11b* gene is flanked by *loxP* sites. This enables the conditional knockout of exon 4, upon treatment with OHT, which induces the expression of Cre recombinase (see Fig. 7).

The housing and breeding of the mice took place at the RSF of the WTSI. All the experimental procedures using these mice were carried out according to the UK 1986 Animals Scientific Procedure Act and local institute ethics committee regulations.

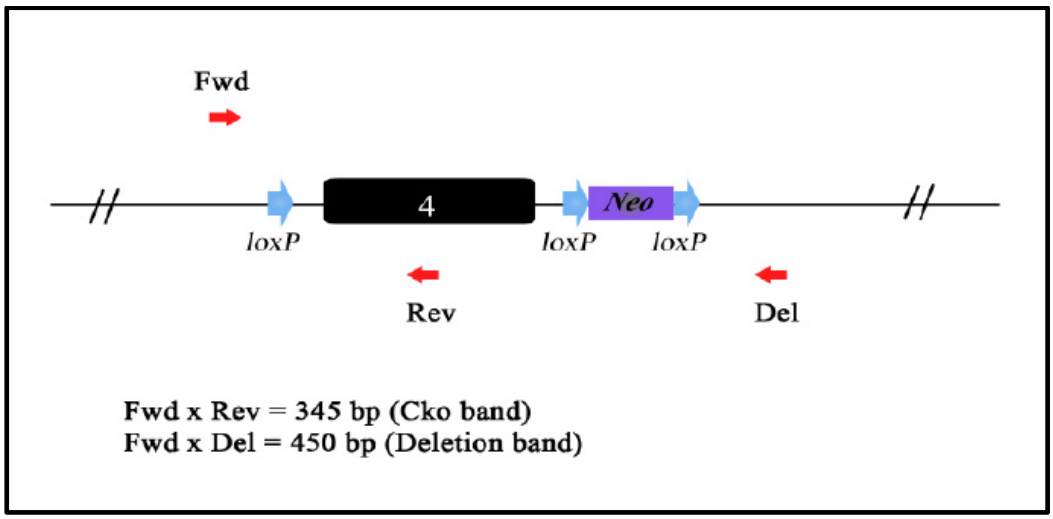


Fig. 7. Schematic of the genotype of exon 4 of the *Bcl11b* gene of *ERT-Cre/ERT-Cre flox/flox* mice. Exon 4 is flanked by *loxP* sites. OHT treatment activates Cre recombinase, which mediates the deletion of exon 4. The OHT-treated thymocytes were genotyped using two different primer sets: Bcl11b_cko and Bcl11b_del. For the first set, Fwd and Rev primers are flanking the *loxP* site, and they amplify a fragment of 345 bp (cko band), which validates the successful incorporation of the *loxP* cassette into the mouse genome. For the latter set, Fwd and Del primers amplify a 450 bp fragment, which validates the successful deletion of exon 4 of *Bcl11b*. Efficient deletion of *Bcl11b*, would completely delete the *loxP* cassette, and there would be no amplification using Bcl11b_cko primers (adapted from Li, Burke et al. 2010).

2.3 In vitro cell culture

All cell types were cultured in polystyrene cell culture flasks (Corning Incorporated). The cells were incubated in humidified incubators at 37 °C and 5% CO_2. All cell lines used in this study tested negative for mycoplasma infection. Cell density was measured by a BioRad TC20 Automated Cell Counter (BioRad, cat no: 1450102). In order to collect the cell pellet, cells were collected by centrifugation at 300 g for 5 min, and the supernatant was discarded.

- **MC38 and OP9-DL1 *in vitro* cell culture**

All adherent cells used in this project (MC38 and OP9-DL1) were passaged at 90-100% confluency. In order to passage the cells, the culture medium was removed and the adherent cells were washed with DPBS (1X) (Gibco, cat no: 14190-094). The cells were trypsinised (Gibco, cat no: 25300-054) and incubated for 2-3 minutes at 37 °C. Trypsin was then neutralised by the addition of media containing FBS (see section 2.4). MC38 cells were normally passaged at a 1:10 dilution, while OP9-DL1 cells were passaged at a 1:5 dilution. MC38 cells were cultured in MC38 cell media throughout the duration of the experiments, except for the final stage of ITNK cell killing, for which T cell media was used (see section 2.4). The MC38 cell line that was used was provided by the laboratory of Dr David Adams at the WTSI. It was developed in the laboratory of Prof Ajit Varki, at the University of California, San

Diego, so that it expresses EGFP constitutively, through the incorporation of the *pEGFP-N1* cassette (see Fig. 9). As described by Borsig, Wong and colleagues (Borsig, Wong et al. 2002), the pEGFP-N1 (CLONTECH) vector was linearised, and MC38 cells were transfected using Lipofectamine, following the manufacturer's protocol. Transfected cells were then selected with medium containing 1 mg/ml G418. The selected EGFP-positive cells were sorted 3 times in total: two initial rounds of bulk sorting of 1000 cells each and one sorting of 10 cells per pool. Afterwards, several pools of 10 cells were expanded and analysed for EGFP expression.

- **Primary culture of thymocytes**

Primary culture of thymocytes was maintained by co-culture of thymocytes on OP9-DL1 stromal cells in the presence of cytokines (see section 2.4). The OP9-DL1 stromal cells were 80-90% confluent at the time of the co-culture with the thymocytes. For details on how to prepare the co-culture of thymocytes with OP9-DL1 cells, see section 2.16.

2.4 Media recipes

All media were sterilised by filtration using 0.45 µM pore size filters (Thermo Scientific, cat no: 166-0045) and stored at 4 ^{o}C.

- **MC38 cell media**
  - 445 ml DMEM (with 4.5 g/L Glucose, w/o L-Glutamine and Na Pyruvate) (Lonza, cat no: BE12-733F)
  - 50 ml FBS (Gibco, cat no: 10270)
  - 5 ml Pen Strep Glutamine (100X) (Gibco, cat no: 10378-016)
- **MC38-Cas9 cell media (puromycin 5 µg/ml)**
  - 500 ml MC38 cell media
  - 250 µl puromycin (10 mg/ml) (Gibco, cat no: A11138-03)
- **OP9-DL1 cell media**
  - 390 ml Alpha MEM (Lonza, cat no: BE12-169F)
  - 100 ml FBS (Gibco, cat no: 10270)
  - 5 ml Pen Strep Glutamine (100X) (Gibco, cat no: 10378-016)
  - 5 ml Hepes Buffer (1M) (SIGMA, cat no: H0997)
  - 500 µl 2-ME (50 mM) (Gibco, cat no: 31350-010)
- **T cell media**
  - 440 ml RPMI 1640 (ATCC modification) (Gibco, cat no: A10491-01)

- 50 ml FBS (Gibco, cat no: 10270)
- 5 ml Pen Strep Glutamine (100X) (Gibco, cat no: 10378-016)
- 5 ml Hepes Buffer (1M) (SIGMA, cat no: H0997)
- 500 µl 2-ME (50 mM) (Gibco, cat no: 31350-010)

2.5 Freezing and thawing cells

In order to freeze down MC38 and OP9-DL1 cells, the cells were cultured in a flask until they reached 80-90% confluency. Cells were harvested by trypsinisation, and cell density was measured. The freezing medium contained 90% FBS and 10% dimethyl sulphoxide (DMSO) (Santa Cruz, cat no: sc-202581). One million cells were collected, resuspended in 1 ml of freezing medium and transferred to CryoTube Vials (Thermo Scientific, cat no: 375353). The vials were placed in a Mr Frosty Freezing Container (Thermo Scientific, cat no: 5100-0001) or enclosed between two Styrofoam racks (used for shipment of 15 ml Falcon tubes). The vials were then placed at -80 ^{o}C for 48 h and then transferred to a liquid nitrogen tank. In order to thaw and recover frozen cells, the CryoTube Vials were thawed in a water bath set at 37 ^{o}C for 1.5 min. The thawed cells were then resuspended in 5 ml of medium and transferred to a new tube. They were collected by centrifugation, and the supernatant containing freezing medium was aspirated. The cell pellets were resuspended in 8 ml of fresh media and transferred to a T25 flask. The following day, the cells were washed with DPBS (1X) and fresh medium was added.

2.6 Transient transfection of MC38 cells

Transient transfection of MC38 cells was performed using the Amaxa Cell Line Nucleofactor Kit V (Lonza, cat no: VCA-1003), following the manufacturer's instructions. In brief, the electroporation was performed using the Nucleofector 2b Device (Lonza, cat no: AAB-1001). MC38 cells were passaged 2-3 days prior to the electroporation. The experiment took place when the cells were 80-90% confluent. Nucleofactor solution V was pre-warmed to room temperature, while 6-well plates were prepared by filling the appropriate number of wells with MC38 culture medium and pre-warming them to 37 ^{o}C. Cells were harvested by trypsinisation, and 1x10^{6} cells were collected for each electroporation reaction. Two micrograms of plasmid DNA were combined with 100 µl of Nucleofactor solution, and the mix was used to resuspend the cell pellet. Incubating the mix for longer than 15 min might reduce

gene transfer efficiency and cell viability. The mix containing cells, plasmid DNA and Nucleofactor solution was transferred to an Amaxa-certified cuvette. For electroporation of MC38 cells, we used Nucleofactor programme H-022, according to instructions provided by the Lonza website. Right after the programme was finished, 500 µl of pre-warmed MC38 medium was added, and all samples were transferred into the appropriate well of the 6-well plates, prepared at the beginning of the experiment. The cuvettes were gently washed twice with medium, which was transferred to the respective wells as well. The cells were left to recover in an incubator for 24 h. Then, the medium was removed, the cells were gently washed with DPBS (1X) and fresh medium was added.

2.7 Carrying out genome-wide CRISPR/Cas9 screen in MC38 cells in co-culture with ITNK cells

- **gRNA library design and lentiviral transduction**

These experiments were carried out by Dr Shannon Burke, Ms Li Ming and Dr Li Li, before I joined the group. The experiments took place in the laboratory of Dr Kosuke Yusa at the WTSI, according to protocols described by Koike-Yusa, Li and colleagues (Koike-Yusa, Li et al. 2014). A genome-wide gRNA library developed by the Yusa group was used. In brief, the library was purchased as a 79-mer oligo pool by Custom Array Inc. The single-stranded oligo sequences were converted to double-stranded by a PCR amplification reaction. The PCR amplification products were purified, digested with BbsI and separated by PAGE. The double-stranded gRNA sequences were gel-purified and cloned into a BbsI-digested and purified lentiviral gRNA expression vector. The vector used was the pKLV-U6gRNA(BbsI)-PGKpuro2ABFP, designed by the laboratory of Dr Kosuke Yusa at the WTSI. A pool of 87,897 gRNA sequences was designed, containing up to 5 different gRNAs per gene. These gRNAs were targeting 19,150 genes in the mouse genome. Thus, the genome-wide gRNA knockout plasmid library was developed. The plasmid region, which contained the gRNA sequence, was then sequenced using primers gLibrary-MiSeq 50-PE-U1 (ACACTCTTTCCCTACACGACGCTCTTC CGATCTCTTGTGGAAAGGACGAAACA) and gLibrary-MiSeq 50-PE-L1 (TCGGCATTCCTGCTGAAC CGCTCTTCCGATCTCTAAAGCGCATGCTCCAGAC). The virus was produced by transfecting 293-FT cells with the plasmid library. Finally, the virus was used to infect Cas9-expressing MC38 cells (previously generated in the group). The infection was carried out in the laboratory of Dr Kosuke Yusa at the WTSI as described by Koike-Yusa, Li and colleagues (Koike-Yusa, Li et al.

2014). The lentiviral gRNA-expressing vector carries the *BFP* gene. BFP^+ cells, which had successfully received gRNAs, were isolated by cell sorting at the WTSI Cytometry Facility.

- **ITNK cell screening: co-culture of edited MC38 cells with ITNK cells**

The library of edited MC38 cells was co-cultured with ITNK cells. These experiments were carried out by Dr Shannon Burke, Ms Li Ming and Dr Li Li, before I joined the group. A detailed protocol for how to develop the ITNK cells is described in section 2.16. The ITNK cell killing assay, used in both the genome-wide screen and in the single knockout validation of the candidate genes, is described in section 2.19.

- **Sequencing of surviving edited MC38 cells after co-culture with ITNK cells**

Sample preparation for sequencing and analysis of the results was carried out by Dr Li Li, before I joined the group. Briefly, after the co-culture with the ITNK cells, individual surviving clones were picked. Genomic DNA was isolated using DNA releasy (Anachem, cat no: 95016898), according to the manufacturer's guidelines. The genomic DNA was used as a template for the sequencing reaction. We used primers to amplify the gRNA cassette of the lentiviral vector. The primers used for the amplification were the gLibrary-MiSeq 150-PE-U1 (ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGAAAGTATTTCGATTTCTTGG) and the gLibrary-MiSeq 150-PE-L1 (TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACTCGGTGCCACTTTTTCAA) (Koike-Yusa, Li et al. 2014). The PCR amplicons were subcloned into the pMD19 (Simple) T-Vector (Clontech) and used to transform *E. coli* cells. Bacterial cultures were grown in 96-well plates, and 2x98 colonies were picked to send for sequencing by Sanger sequencing, using the T7 and SP6 universal primers. The sequencing was performed using the ABI PRISM 3700 Genetic Analyzer, of the Small Sequencing Project Group at the WTSI. The sequencing results were analysed by Mr Stephen Rice of the Systems Support team (Team 0094) at the WTSI. In order to obtain the list of candidate genes, we selected overlapping hits that appeared in more than one resistant colony. The list of hits was compared by BLAST against the list of gRNAs designed by Koike-Yusa, Li and colleagues, in order to identify the genes and the exons that the gRNAs had targeted (Koike-Yusa, Li et al. 2014).

2.8 Flow Cytometry

Cells that were either stained with fluorophore-conjugated antibodies or that constitutively expressed fluorophores were analysed using the LSRFortessa cell analyser (BD), at the WTSI Cytometry Facility. When collecting the pellets, a centrifuge had been pre-chilled to 4 ^{o}C. Approximately 1 million cells were harvested, and cell pellets were washed by resuspension in 1 ml of staining buffer (PBS 1X, FBS 2%). Throughout the duration of the experiment, fluorophore-expressing or stained cells were constantly kept on ice and in the dark. Flow cytometry results were processed using FlowJo software.

- **Flow cytometry analysis of Cas9-expressing MC38 cells constitutively expressing EGFP/mCherry**

If the cells expressed fluorophores, no staining was required. After the wash, they were resuspended in 300 µl of staining buffer and run through the 40 µM filtered cap of a 5 ml Polystyrene Round-Bottom Tube (CORNING, cat no: 352235). The samples were then analysed by flow cytometry.

- **Flow cytometry analysis of OHT-treated thymocytes**

Flow cytometry was used for the analysis of the OHT-treated thymocytes. The expression of certain markers was analysed, one week after the end of OHT treatment (see section 3.6). All antibodies were diluted in staining buffer (PBS 1X, FBS 2%). The cell pellets were resuspended in block solution, which was prepared by diluting anti-mouse FcR Block (BD Bioscience, cat no: 553142) to a dilution of 1:200, in a final volume of 50 µl per sample. The cells were incubated with the block solution for 10 min on ice, and then the cell pellets were collected to continue with incubation with the antibody cocktail. All the antibodies used in the experiment were directly conjugated to their respective fluorophores and used at a 1:50 dilution, for each antibody, in a final volume of 50 µl per sample. Cell pellets were resuspended in that volume and incubated on ice for 30 min. The antibody cocktail contained anti-mouse NKp46-BV421 (BioLegend, cat no: 137611), anti-mouse NK1.1-PerCP-Cyanine5.5 (eBioscience, cat no: 45-5941-82), anti-mouse CD4-APC-eFluor780 (eBioscience, cat no: 47-0041-80), anti-mouse CD8a-PE (BD Bioscience, cat no: 553033), and anti-mouse TCRβ-APC (BioLegend, cat no: 109212). Then, cells were collected and washed three times by resuspension in 1 ml of staining buffer. After that, they were resuspended in 300 µl of staining buffer and run through the 40 µM filtered caps of the FACS tubes described above. Finally, the samples were analysed by flow cytometry.

2.9 Generation of Cas9-expressing MC38 cells

For the purposes of the validation experiments, a new MC38 cell line that constitutively expressed Cas9 was developed. The expression of Cas9 was achieved by the incorporation into the genome of a *Cas9* cassette by a *PiggyBac* transposon (see Fig. 10). The *Cas9* gene was derived from *Streptococcus pyogenes.* The vector that was used (*pPB-LR5.1-EF1a-puroT2Cas9*) was developed in the laboratory of Dr Kosuke Yusa at the WTSI, and the protocol followed was described by Koike-Yusa, Li and colleagues (Koike-Yusa, Li et al. 2014). Incorporation of the *PiggyBac* transposon carrying the *Cas9* gene into the genome was achieved by co-transfection of the *PiggyBac Cas9* vector with a plasmid encoding for transposase. Transfection was performed by electroporation using the Amaxa Cell Line Nucleofactor Kit V (see section 2.6). Briefly, 1 μg of *pPB-LR5.1-EF1a-puroT2Cas9* was combined with 1 μg of the transposase plasmid and transfected into MC38 cells expressing EGFP. The functionality of Cas9 was verified, as described in sections 2.10 and 2.12.

In order to identify the optimal puromycin concentration for efficient selection of MC38 cells carrying the *Cas9* cassette, a titration of puromycin in the transfected Cas9-expressing MC38 cells was performed. The titration was performed in 6-well plates, and cell death was monitored by Trypan Blue staining using a BioRad TC20 Automated Cell Counter. Half a million cells were seeded per well and incubated overnight in an incubator to allow the cells to attach. One 6-well plate was seeded with wild-type MC38 cells and one with the transfected MC38 cells. The following day, the medium was replaced with fresh medium containing a series of increasing concentrations of puromycin: 1 μg/ml, 2 μg/ml, 4 μg/ml, 5 μg/ml and 6 μg/ml. Selection with media containing 5 μg/ml of puromycin for 10 days was chosen.

2.10 Detection of Cas9 expression by Western Blot

To detect Cas9 expression in MC38 cells, 1 million cells were collected and washed once by resuspension in DPBS (1X). The cell pellet was resuspended in RIPA BUFFER (SIGMA, cat no: R0278-500ML), 200 μl per sample, adding Halt Protease & Phosphatase Single-Use Inhibitor Cocktail (1X) (Thermo Scientific, cat no: 78442). The samples were incubated on ice for 10 min and then sonicated for 30 sec ON and 30 sec OFF, for 6 cycles. After sonication, the samples were placed on ice and collected by centrifugation for 15 min at 13,200 rpm at 4 ^{o}C, in a tabletop centrifuge. The protein was soluble in the supernatant, while the pellet was discarded. NuPAGE LDS Sample Buffer (1X) (Novex – Life Technologies, cat no: NP0008) was

added to the samples and mixed by pipetting. The samples were then heated at 70 °C for 10 min. After heating, they were stable to freeze down for storage at -80 °C. The gel electrophoresis apparatus (XCell SureLock Mini-Cell Electrophoresis System) (Invitrogen, cat no: EI0001) was assembled. We used NuPAGE® Novex® 4-12% Bis-Tris Protein Gels, 1.0 mm, 12 well (Thermo Fisher, cat no: NP03322BOX). The tank was filled with around 500 ml of 1X NuPage MES SDS Running Buffer MOPS (Life Technologies, cat no: NP0002). After removal of the combs of the gel, the wells were washed by pipetting running buffer into them using Corning Gel-Loading Pipet Tips (Fisher Scientific, cat no: 07-200-288). Before loading, the samples were heated again for 5 min at 70 °C. Forty microlitres of sample per well, along with 10 µl of protein marker, were loaded (BioRad, cat no: 161-0373). The gel was run at 200 V for 1.5 h.

For the transfer, a 5X stock transfer buffer was prepared by adding 15.1 g Tris (Amresco Inc., cat no: 0826) and 72 g Glycine (SIGMA, cat no: G8898) to ddH_2O, to a total volume of 1 litre. To prepare 1 litre of 1X Transfer Buffer, 200 ml of the 5X stock transfer buffer was mixed with 600 ml ddH_2O and 200 ml methanol. The 1X transfer buffer was pre-chilled to 4 °C before use. Mini Trans-Blot Electrophoretic Transfer Cell (Biorad, cat no: 1703930), Nitrocellulose membrane Amersham Hybond-P (GE Healthcare, cat no: RPN2020F), and Whattman blotting paper (Sigma-Aldrich, cat no: WHA10427810) were used. The Whattman papers were soaked in transfer buffer while the membrane was submerged, first in methanol and then in transfer buffer. The transfer was performed at 395 mA for 70 min, in a cold room on ice.

After the transfer, the membrane was blocked in 6 ml of block solution (1X TBS, 0.05% (w/w) IGEPAL CA-630 (Sigma-Aldrich, cat no: I8896), 10% milk powder). The incubation took place at room temperature for 2 h, with mild rotation. Afterwards, the membrane was incubated with 6 ml of antibody solution (1X TBS, 0.15% (w/w) IGEPAL CA-630 (Sigma-Aldrich, cat no: I8896), 5% milk powder) containing the primary antibody. For Cas9 detection, a mouse monoclonal antibody to CRISPR/Cas9 was used (Abcam, anti-CRISPR-Cas9 antibody [7A9-3A3] (ab191468)) at a dilution of 1:1000. Primary antibody incubation was carried out by adding 6 ml of primary antibody solution to the membrane, at 4 °C, overnight, with mild rotation. The membrane was then washed 3 times, with 50 ml of wash solution (1X TBS, 0.1% Tween (Sigma-Aldrich, cat no: 9005-64-5)) at room temperature, for 15 min each, with mild rotation. The wash solution was replaced after each wash. Then, the membrane was incubated with 6 ml of the antibody solution in which sheep anti-mouse horseradish peroxidase (HRP)-linked

secondary antibody (GE Healthcare, cat no: NA931VS) was added to a dilution of 1:10,000. The incubation was carried out at room temperature for 1 h, with mild rotation. Afterwards, the membrane was again washed 3 times, as before.

Detection of the labelled proteins on the membrane was carried out using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, cat no: RPN2232). The membrane was incubated with 4 ml of Solution A and Solution B (1:1) at room temperature for 5 min, with mild rotation. The excess of liquid was removed, and the membrane was wrapped in Saran Wrap, trying to avoid the formation of bubbles. The detection took place in a dark room using a Hypercassette (Amersham Biosciences, cat no: RPN 13642) and CL-XPosure Film (Thermo Scientific, cat no: 34088). A series of exposures of increasing time was tested: 1 sec, 2 sec, 3 sec and 5 sec. We concluded that exposure of 1 sec was ideal for visualisation of the labelled protein.

2.11 Cloning gRNAs in PiggyBac-gRNA-BsaI expressing vector

The gRNA-expressing vector that was used was the *PiggyBac-gRNA-BsaI* vector (see Fig. 12). The vector was designed in our laboratory by Dr Xuefei Gao. The gRNA expression cassette is flanked by *PiggyBac* repeats. Co-transfection of the *PiggyBac-gRNA-BsaI* vector with a vector encoding for transposase mediates the random integration of the gRNA cassette into the target genome. The gRNA sequences were synthesised by Sigma Aldrich. Upon digestion with BsaI, the gRNA scaffold (428 bp) was released with concomitant creation of sticky ends on the backbone. The sticky ends generated in the backbone after the digestion were 5'-CAAG-3' and 5'-GTTT-3'. Therefore, our gRNA sequences were designed so that the forward had 5'-CTTG-3' at the beginning, while the reverse had 5'-AAAC-3' at the beginning (see Table 2 and Table 5). After digestion, the sticky ends of the backbone are unable to self-anneal, while they are only able to anneal to the sticky ends at the beginning of the gRNA sequences, thus increasing the cloning efficiency. This allows efficient ligation of the gRNA sequences at this site. The forward and reverse gRNA sequences were resuspended to a stock concentration of 100 µM. The *PB-gRNA-Bsa1* vector was digested with BsaI at 37 ^{o}C for 3-4 hours. The digested product was separated by a 1.5% agarose gel, and the backbone (5,998 bp) was purified from the gel, using the QIAquick Gel Extraction Kit (QIAGEN, cat no: 28704), according to the manufacturer's instructions. Single-stranded gRNA sequences were converted to double-stranded, to generate the sticky ends that are compatible with the sticky ends of the BsaI-

digested vector. For this reason, 5 µl of forward was mixed with 5 µl of reverse gRNA sequence and the PCR programme described in Table 1 was used. Then, 5 µl of the annealed gRNA sequences were ligated with 1 µl of the purified backbone, using T4 DNA ligase (NEB, cat no: M0202S). The reaction was incubated either at room temperature for 1 h, or at 16 ^{o}C overnight. Two microlitres of the ligated product were used for transforming One Shot TOP10 Chemically Competent *E. coli* (Invitrogen, cat no: C4040-10), according to the manufacturer's protocol. The transformed bacteria were plated on 10 cm LB agar plates containing ampicillin (100 µg/ml). Mini preparations of the bacterial colonies were performed, using the QIAprep Spin Miniprep Kit (QIAGEN, cat no: 27104). A test digestion was performed by digesting 5 µl of each of the mini preparation plasmid DNA with BglII (NEB, cat no: R0144S) and XhoI (NEB, cat no: R0146S), for 2 h at 37 ^{o}C, and then running the samples on a 1.5% agarose gel. If the ligation was successful, the fragments observed would be: 0.5 kb, 1.7 kb and 3.9 kb. If the ligation hadn't worked, the fragments would be: 0.9 kb, 1.7 kb and 3.9 kb. Maxi preparations of the positive constructs were performed, using the *PureLink* HiPure Plasmid *Maxiprep* Kit (Invitrogen, cat no: K2100-06), according to the manufacturer's protocols. The final product was sequenced using a sequencing primer designed based on the DNA sequence of the U6 promoter, which precedes the gRNA ligation site on the vector (5'-TTGGATTTCTATAACTTCGTATAGC-3').

Table 1. PCR programme for annealing gRNA oligo sequences.

Temperature	Time
96 ^{o}C	5 min
85 ^{o}C	1 min
75 ^{o}C	1 min
65 ^{o}C	1 min
55 ^{o}C	1 min
45 ^{o}C	1 min
35 ^{o}C	1 min
4 ^{o}C	5 min
4 ^{o}C	forever

2.12 Verification of Cas9 functionality by knocking out endogenous EGFP gene

Four gRNAs targeting EFGP were designed using the E-CRISPR website (http://www.e-crispr.org/). Oligos were ordered from Sigma Aldrich and cloned into a *PiggyBac-gRNA-BsaI* vector, as described in section 2.11. Before proceeding with the targeting, EGFP-expressing wild-type and Cas9-expressing MC38 cells were selected by culturing them in MC38 medium containing 1 mg/ml G418. The efficiency of the selection was analysed by flow cytometry to obtain a clear $EGFP^+$ cell population. As a control, B16 mouse melanoma cells were used, which did not express EGFP. The gRNAs targeting the *EGFP* gene (see Table 2) were co-transfected, along with a transposase plasmid, into the EGFP and Cas9-expressing MC38 cells, as described in section 2.6. The transfected cells were maintained in culture and passaged every 3 days. EGFP expression was examined in $mCherry^+$ cells by flow cytometry, 11 days post-transfection, in order to make sure that all residual EGFP activity had disappeared. Cells that had received the *PiggyBac-gRNA-BsaI* vector were $mCherry^+$.

Table 2. List of forward and reverse gRNA sequences used to target the EGFP gene for validating the function of the Cas9 protein.

Name	gRNA Sequence
EGFP_gRNA_7 For	CTTG AGCACTGCACGCCGTAGGTC
EGFP_gRNA_7 Rev	AAAC GACCTACGGCGTGCAGTGCT
EGFP_gRNA_8 For	CTTG GCTGAAGCACTGCACGCCGT
EGFP_gRNA_8 Rev	AAAC ACGGCGTGCAGTGCTTCAGC
PB_BsaI_gRNA_EGFP_9_144 For	CTTG GCCGCTACCCCGACCACATG
PB_BsaI_gRNA_EGFP_9_144 Rev	AAAC CATGTGGTCGGGGTAGCGGC
PB_BsaI_gRNA_EGFP_26_432 For	CTTG CGATGTTGTGGCGGATCTTG
PB_BsaI_gRNA_EGFP_26_432 Rev	AAAC CAAGATCCGCCACAACATCG

2.13 Designing qPCR primers

On the NCBI website, the gene ID for the genes of interest was identified in the “Gene” database. The Primer Bank website (http://pga.mgh.harvard.edu/primerbank/) was used and qPCR primers were retrieved by their gene IDs. The validated primer sets were obtained from

the Primer Bank website, under "Validation Results", as experimental validation data are provided for them. Primer sets were synthesised by Sigma Aldrich.

2.14 Assessing gene expression via qPCR

The expression of 10 of the candidate genes, identified from the initial genome-wide ITNK cell screen, was verified in wild-type and Cas9-expressing MC38 cells. Primers amplifying the beta actin gene (*Actb*) were used as an internal control for the quantification of gene expression. Therefore, two samples (wild-type and Cas9-expressing MC38 cells) and 10 pairs of qPCR primers (see Table 4) were used. Two million cells were collected, and RNA was extracted using the RNeasy Mini Kit (QIAGEN, cat no: 74104). The RNA samples were kept constantly on ice throughout the duration of the experiments and were stored in -80 ^{o}C. The cDNA was obtained using the QuantiTect Reverse Transcription Kit (QIAGEN, cat no: 205310). The qPCR primers were resuspended to a stock concentration of 100 µM. Primer mixes were prepared by diluting both primers of each set in the same tube to a final concentration of 10 µM for each primer. The cDNA of the samples obtained by the RT reaction was diluted 1:10 in ddH_2O. For the qPCR, MicroAmp Optical 96-Well Plate (Life Technologies, cat no: N8010560), MicroAmp Optical Adhesive Film (Life Technologies, cat no: 4311971), and RT SYBR Green qPCR Mastermix (QIAGEN, cat no: 330521) were used. Every reaction was done in triplicate, and a different mastermix for each primer mix was prepared. The volume of each reagent for one reaction is described in Table 3. After pipetting all the reagents, the samples were mixed by vortex, the solutions were collected to the bottom of the wells by centrifugation for 5 min at 1000 rpm and placed in the qPCR machine. The qPCR machine used was the ABI PRISM at the WTSI Microarray Facility.

Table 3. Volumes for preparing mastermix for qPCR reaction to verify gene expression of candidate genes.

Reagent	**Volume (X1)**
cDNA	3 µl
RT SYBR Green qPCR Mastermix	12.5 µl
Primer pair mix (10 µM)	1 µl
ddH2O	8.5 µl
Total	25 µl

Table 4. List of qPCR primer pairs used for verifying gene expression of candidate genes. Primers were designed using the PrimerBank website.

Gene	Gene ID	PrimerBank ID	Primer orientation	qPCR primer sequence
Krt10	16661	52787a1	Forward	CTGGCGATGTGAACGTGGAA
			Reverse	GTCCCTGAACAGTGCGTCTC
Myf6	17878	6678984a1	Forward	AGAGGGCTCTCCTTTGTATCC
			Reverse	CTGCTTTCCGACGATCTGTGG
Pkd2i2	53871	8393965a1	Forward	TCTGAGGCGACTTGGTGGTA
			Reverse	TCCACAAACAGAGACGACATAAC
Trp63	22061	3695092a1	Forward	TACTGCCCCGACCCTTACAT
			Reverse	GCTGAGGAACTCGCTTGTCTG
Ccdc154	207209	294832009c3	Forward	GATGACCAAACTCAACGAGGAA
			Reverse	GCTTTCTGCTATCTGCCGTGTA
Cenpc1	12617	6671740a1	Forward	GATATTGCCGGTCTTCCAGAG
			Reverse	CTGGGCACTTTTTGCTTGATG
Zbtb8b	215627	23956312a1	Forward	TCTTGGGGGAGTTGAATGAGC
			Reverse	GTTGGCGAATAGGATGTTCCTG
Cmtm1	100504164	32526867a1	Forward	ATACGTGCTATACCATCCAGGC
			Reverse	TGACGTGTACTAAAGGTCGCT
Amdhd1	71761	12836736a1	Forward	GTGGGCGGGTGAAAGAGTC
			Reverse	GGAGCAGAACAGTTCCTCCTC
Sugp1	70616	28076967a1	Forward	AACCGGGATGTTGCAGGAAAG
			Reverse	CCCGCTTCTTCTGAGCAATAA
Actb	11461	6671509a1	Forward	GGCTGTATTCCCCTCCATCG
			Reverse	CCAGTTGGTAACAATGCCATGT

2.15 Generation of single knockouts for each candidate gene

Ten candidate genes were selected for validation (see Table 7). For each one, 2-4 different gRNAs, mostly targeting different exons, were chosen from the initial genome-wide library developed by Koike-Yusa, Li and colleagues (Koike-Yusa, Li et al. 2014) and ordered from Sigma Aldrich. The single-stranded gRNA sequences were converted to double-stranded and cloned into the *PB-BsaI-gRNA* expressing vector (see section 2.11). The different gRNA expression vectors (targeting the same candidate gene) were mixed (see Table 5). More specifically, 1 μg of total plasmid DNA was combined and co-transfected with 1 μg of transposase plasmid. The DNA was electroporated into Cas9-expressing MC38 cells according to the protocol described in section 2.6. A control cell line of non-Cas9-expressing MC38 cells was also transfected with 3 different gRNAs: pkd2l2_exon1_gRNA, myf6_exon3_gRNA and krt10_exon1_gRNA (see Table 5). This cell line was used as a control in the ITNK cell killing assay (see section 2.19).

Table 5. List of gRNA sequences targeting different exons of the 10 selected candidate genes. Gene ID: the ID of the gene on NCBI; gRNA ID: the ID of the gRNA from the genome-wide gRNA mutant library developed by Koike-Yusa, Li and colleagues (Koike-Yusa, Li et al. 2014).

Gene	Gene ID	gRNA ID	Exon	Primer orientation	gRNA oligo sequence
Krt10	16661	54648	1	Forward primer	CTTG GAAGAGTCAAACTACGAGC
				Reverse primer	AAAC GCTCGTAGTTTGACTCTTC
		54646	3	Forward primer	CTTG GTGACCCTGCGCCAGAGCG
				Reverse primer	AAAC CGCTCTGGCGCAGGGTCAC
		54645	5	Forward primer	CTTG AATTGAGACGTACTGTTCA
				Reverse primer	AAAC TGAACAGTACGTCTCAATT
Myf6	17878	31428	1	Forward primer	CTTG CTGCTTTCCGACGATCTGT
				Reverse primer	AAAC ACAGATCGTCGGAAAGCAG
		31425	3	Forward primer	CTTG TTCTCTCTTTGATTTAGAC
				Reverse primer	AAAC GTCTAAATCAAAGAGAGAA
Pkd2i2	53871	135357	1	Forward primer	CTTG AGGCGACTTGGTGGTACCG
				Reverse primer	AAAC CGGTACCACCAAGTCGCCT
		135358	4	Forward primer	CTTG AACGTTCTCGTAGTAGATG
				Reverse primer	AAAC CATCTACTACGAGAACGTT
		135360	5	Forward primer	CTTG TTGGCGTATACCGAGATGG
				Reverse primer	AAAC CCATCTCGGTATACGCCAA
		135363	6	Forward primer	CTTG TGAGGAGCCCACCCGTCGC
				Reverse primer	AAAC GCGACGGGTGGGCTCCTCA
Trp63	22061	112252	1	Forward primer	CTTG TGTGGCCACTCTACGTCAA
				Reverse primer	AAAC TTGACGTAGAGTGGCCACA
		112253	2	Forward primer	CTTG CTGAGGAACTCGCTTGTCT
				Reverse primer	AAAC AGACAAGCGAGTTCCTCAG
		112259	5	Forward primer	CTTG CACGGATAACAGCGCCCTG
				Reverse primer	AAAC CAGGGCGCTGTTATCCGTG
Ccdc154	207209	120552	2	Forward primer	CTTG AGGGGCCGTCTGCTGGCTC
				Reverse primer	AAAC GAGCCAGCAGACGGCCCCT

		120553	3	Forward primer	CTTG GCTGAGGTGGTTCGTCTGA
				Reverse primer	AAAC TCAGACGAACCACCTCAGC
		120556	8	Forward primer	CTTG GCCTTGCGGGCACAGCGTG
				Reverse primer	AAAC CACGCTGTGCCCGCAAGGC
Cenpc1	12617	224854	1	Forward primer	CTTG CTAAGCTAGGCTTGTGCGC
				Reverse primer	AAAC GCGCACAAGCCTAGCTTAG
		224851	2	Forward primer	CTTG AAGATATTGCCGGTCTTCC
				Reverse primer	AAAC GGAAGACCGGCAATATCTT
		224848	8	Forward primer	CTTG AAGTTTTTCAAGTCGACTT
				Reverse primer	AAAC AAGTCGACTTGAAAAACTT
Zbtb8b	215627	207398	2-a	Forward primer	CTTG CAACATCGAATTCCACCTT
				Reverse primer	AAAC AAGGTGGAATTCGATGTTG
		207402	2-b	Forward primer	CTTG GTGGAATTCGATGTTGCCA
				Reverse primer	AAAC TGGCAACATCGAATTCCAC
		207391	3	Forward primer	CTTG GGAGCGGATGTGCCGTTTG
				Reverse primer	AAAC CAAACGGCACATCCGCTCC
Cmtm1	100504164	293607	2-a	Forward primer	CTTG CCGCAGGGCGATGGCCCAC
				Reverse primer	AAAC GTGGGCCATCGCCCTGCGG
		293601	2-b	Forward primer	CTTG CCTGTGCTTTGACGACTTC
				Reverse primer	AAAC GAAGTCGTCAAAGCACAGG
		293602	2-c	Forward primer	CTTG ACCCTTGGACCAGTCGAGT
				Reverse primer	AAAC ACTCGACTGGTCCAAGGGT
Amdhd1	71761	30693	1	Forward primer	CTTG GTCAGGAAGCGCTCGCCGC
				Reverse primer	AAAC GCGGCGAGCGCTTCCTGAC
		30692	4	Forward primer	CTTG AGCCTTGCGTTCAGTTGGC
				Reverse primer	AAAC GCCAACTGAACGCAAGGCT
		30684	8	Forward primer	CTTG CCAACTTCCAAAGATCCGT
				Reverse primer	AAAC ACGGATCTTTGGAAGTTGG

		30680	9	Forward primer	CTTG ATCCATGTCCGGGGTTGTC
				Reverse primer	AAAC GACAACCCCGGACATGGAT
Sugp1	70616	287752	2	Forward primer	CTTG GAAAGGCCAATCGGTGGTT
				Reverse primer	AAAC AACCACCGATTGGCCTTTC
		287754	6	Forward primer	CTTG AAGGAAGGTAGCCGAGATC
				Reverse primer	AAAC GATCTCGGCTACCTTCCTT
		287755	7	Forward primer	CTTG GCCAGGTTCATAGCAGACG
				Reverse primer	AAAC CGTCTGCTATGAACCTGGC
		287756	8	Forward primer	CTTG AGGAAGCGGAAGAGCCGGT
				Reverse primer	AAAC ACCGGCTCTTCCGCTTCCT

2.16 Generation of ITNK cells

The procedure followed for the generation of ITNK cells was first described by Li, Burke and colleagues in our group at the WTSI (Li, Burke et al. 2010).

The day before the isolation of thymocytes, x4 10-cm dishes were seeded with $4.8x10^5$ OP9-DL1 cells, along with one 6-well plate with $0.8x10^5$ OP9-DL1 cells. Dishes and plates were 80-90% confluent at the time of the experiment.

The day of the experiment, an *ERT-Cre/ERT-Cre flox/flox* mouse was euthanised and dissected. The thymus was isolated and placed on ice in a 50 ml Falcon tube containing PBS 1X, FBS 2%. A 70 µm diameter filter (FALCON, cat no: 352350) was placed inside a 10-cm petri dish. The thymus was placed in the centre of the filter, adding 1 ml of PBS 1X, FBS 2%. The plunger of a sterile syringe was used to grind the thymus and force it through the filter. The filter was washed with 20 ml of PBS 1X, FBS 2%. Clumps were disrupted by pipetting up and down vigorously, and the cell suspension was moved to a new 50 ml Falcon tube. The cell pellets were collected by centrifugation at 300 g for 5 min at 4 ^oC. They were then resuspended in 20 ml of PBS 1X, FBS 2% and run through a 70 µm diameter filter again. The cell density was measured. Twelve million cells were seeded per 10-cm dish (which was 80-90% confluent with OP9-DL1 cells), while the 6-well plate was seeded with $2x10^6$ cells per well. Thymocytes were resuspended in T cell media (see section 2.4) supplemented with murine IL-7 (Peprotech, cat no: 217-17) and murine Flt3l (Peprotech, cat no: 250-31L), 5 ng/µl

each, and seeded into the dishes and plates. Every 3 days, half the media was removed and replaced with fresh T cell media supplemented with cytokines. Every 7 days, the thymocytes were collected by vigorous pipetting, filtered using 20 μm filters (CellTrics, cat no: 04-004-2325). The cell pellets were then collected by centrifugation, resuspended in fresh media supplemented with cytokines, and seeded in new plates/dishes with OP9-DL1 cells (80-90% confluent).

After seeding, the thymocytes were cultured for 4-6 days. Then, OHT (Sigma, cat no: H7904-5MG) was added. The x4 10-cm dishes were used for OHT treatment, while the thymocytes in the 6-well plate were used as the untreated control. The OHT was diluted to a working concentration of 0.25 μM in T cell media supplemented with IL-7 and Flt3l. The cells were treated with OHT for 48 h at 37 ^{o}C in an incubator. The ideal OHT working concentration was identified as described in section 2.19. At the end of OHT treatment, the thymocytes were collected by vigorous pipetting and filtered using 20 μm filters. The cell pellet was collected and washed once with PBS 1X, FBS 2%, and resuspended in fresh media supplemented with murine IL-7 and murine Flt3l, 5 ng/μl each, and murine IL-2 (Peprotech, cat no: 212-12) 100 ng/μl. The cell suspension was then seeded in new plates/dishes with OP9-DL1 cells (80-90% confluent). In the following days, extensive cell death was observed, and cells were kept in culture to recover, replacing half the media every 3 days (T cell media supplemented with murine IL-7, murine Flt3l and murine IL-2).

2.17 OHT titration

Titration of OHT was performed in order to identify the optimal concentration for effective generation of ITNK cells, minimising the OHT toxicity effects. The plates used for the titrations had been seeded with OP9-DL1 cells and were 80-90% confluent at the time of the experiment. A mouse was euthanised, the thymus was obtained, and the thymocytes were isolated, according to the protocol in section 2.16. One week after OHT treatment, the expression of NK and T cell markers was analysed by flow cytometry, using stained primary bone marrow cells for colour compensation (see section 2.8). The titration was carried out in 6-well plates. Two million thymocytes were seeded per well, cultured in medium with the following OHT concentrations: untreated, 0.1 μM, 0.25 μM, 0.5 μM, and 1 μM. A separate well was seeded with OP9-DL1 alone, to be used as an indication of the viability of the OP9-

DL1 cells. After the end of the treatment, OHT was removed and the cells were washed, as described in section 2.16. Treatment with 0.25 μM of OHT was selected.

2.18 Genotyping ITNK cells

The OHT-treated thymocytes were genotyped one week after OHT treatment, before analysing the samples by flow cytometry, in order to validate whether *Bcl11b* had been efficiently deleted. First of all, genomic DNA was isolated using DNA releasy (Anachem, cat no: 95016898), according to the protocol provided by the manufacturer. Two different primer pairs amplifying different genomic loci were used. The primers were ordered from Sigma Aldrich.

As described in Fig. 7, for the first pair, termed Bcl11b_cko (conditional knock-out), the forward primer (Frw: TGAGTCAATAAACCTGGGCGAC) and the reverse primer (Rev: GGAA TCCTTGGAGTCACTTGTGC) were designed in order to verify whether the *loxP* cassette had been inserted into the mouse genome successfully. The annealing sites of the primers were flanking the *loxP* site. If the *loxP* cassette had not been inserted, the amplicon would be 243 bp, while if it had been, it would be 345 bp. For the second pair, termed Bcl11b_del (deletion), the forward (Frw, which is the same as the forward for Bcl11b_cko) and the reverse (deletion) primer (Del: TCCTGGTAACACACAATTGC), produce a PCR product only if the deletion has happened successfully. If the deletion has not happened, the PCR product would be too long to be amplified using the current PCR amplification programme. On the other hand, if the deletion has happened, the PCR product will be 450 bp, which can be detected by gel electrophoresis, in a 1.5% agarose gel, using Hyperladder I.

The PCR amplification reaction was conducted using Extensor Long PCR ReddyMix Master Mix (Thermo Fisher, cat no: AB-0794/B) using the programme in Table 6.

Table 6. PCR amplification programme using Extensor Long PCR ReddyMix Master Mix for genotyping. Two sets of primers, Bcl11b_cko and Bcl11b_del, were used in order to detect successful incorporation of the *loxP* cassette and successful deletion of exon 4 of *Bcl11b*, respectively.

Temperature	Time
94 °C	2 min
94 °C	10 min
60 °C	30 min
68 °C	26 sec
Go to step 2 for 30 more cycles	
68 °C	7 min
4 °C	forever

2.19 ITNK cell killing assay

The characterisation of the OHT-treated thymocytes as ITNK cells was based on positive genotyping results (see section 2.18), the expression of the NK markers NK1.1 and NKp46 (see Fig. 17), along with morphological changes (see Fig. 20). The killing assay included the co-culture of edited MC38 cells (transfected with gRNA vectors targeting the candidate genes) with the ITNK cells. The co-culture aimed to validate whether the knockout of the candidate genes conferred ITNK cell resistance to MC38 cells. Differences due to knockouts of the candidate genes could be observed, and comparisons were meaningful as the MC38 cells and the ITNK cells have the same genetic background (Borsig, Wong et al. 2002).

The killing assay was performed in 6-well plates. Three to four days prior to the killing assay, the media used for cancer cell culture was switched from DMEM (MC38 cell media) to RPMI (T cell media), so that the cancer cells could adjust to the new media. Six-well plates were seeded with 500,000 cells of each single knockout cell line that was previously generated (see section 0). One set was used for the ITNK cell killing assay, and the other set was a control. In the 6-well plates that were used for ITNK cell killing, two different controls were included: one control well was seeded with 500,000 cells of Cas9-expressing MC38 cells, which had not been transfected with gRNAs, and the other control well was seeded with 500,000 Cas9-negative MC38 cells, transfected with gRNAs targeting three different genes. After seeding, the cells were left to attach to the wells for 24 h.

The following day, the ITNK cells were collected by pipetting up and down, and run through a 20 µm filter (Wolf Laboratories Ltd, cat no: 04-004-2325). Cell concentration was measured, and cell pellets were collected. The ITNK cells were then resuspended in an appropriate volume of fresh T cell media, supplemented by murine IL-7 and murine Flt3l, 5 ng/µl each, and murine IL-2, 100 ng/µl. ITNK cells were added to MC38 cells, approximately at a ratio of 2.5:1 per well. The cancer cells in the 6-well plates were washed once with PBS (1X). After the wash of the control cultures (without ITNK cells), the media were replaced with fresh T cell media containing cytokines.

Every 3 days, half the medium from each well was gently removed and replaced with fresh T cell medium supplemented by cytokines. Killing was observed a few hours after the co-culture of the MC38 cells with the ITNK cells. The control 6-well plates, in which no ITNK cells were added, were passaged every 3-4 days in a dilution of 1:10.

Resistant cells appeared between 2 and 4 weeks after the beginning of the co-culture.

3. Results

3.1 Previous work and outline of experimental strategy

Our lab had previously developed a potent cancer-killing cell type, termed "induced T-to-NK (ITNK) cells" (Li, Burke et al. 2010). These ITNK cells were used to elucidate the genetic background of cancer cell resistance to anti-tumour immunological responses. To this end, a forward genetic immunological screen based on the CRISPR/Cas9 system was developed (see Fig. 8). The experiments involved the co-culture of the ITNK cells with a genome-wide CRISPR/Cas9 knockout mutant MC38 (mouse colon carcinoma cell line) library. The enriched mutant populations were sequenced in order to identify the candidate genes suspected to be involved in cancer cell immunological resistance. This work was carried out by Dr Shannon Burke, Ms Li Ming and Dr Li Li, before I joined the group.

My work involved the validation of Cas9 functionality in Cas9-expressing MC38 cells. These cells were used for the development of different cell lines, each carrying a knockout in one of the selected candidate genes. My work also involved the optimisation of the conditions required for the generation of ITNK cells from *Bcl11b*-/- thymocytes, and the co-culture with MC38 cells. Each of the knockout MC38 cell lines was then co-cultured with the ITNK cells in order to verify their cancer immune evasion potential. Future work will involve sequencing of the surviving cells. Finally, rescue experiments are planned, in which the sensitivity of MC38 cells to ITNK cell attacks will be restored by cDNA overexpression of the candidate genes in the knockout cell lines.

With this approach, novel genes which are important for cancer cell immune evasion mechanisms will be characterised. This can prove to be essential for a better understanding of neoplastic disease. The candidate genes could then be used as clinical markers for cancer progression or could form the basis for designing new immunotherapeutic approaches.

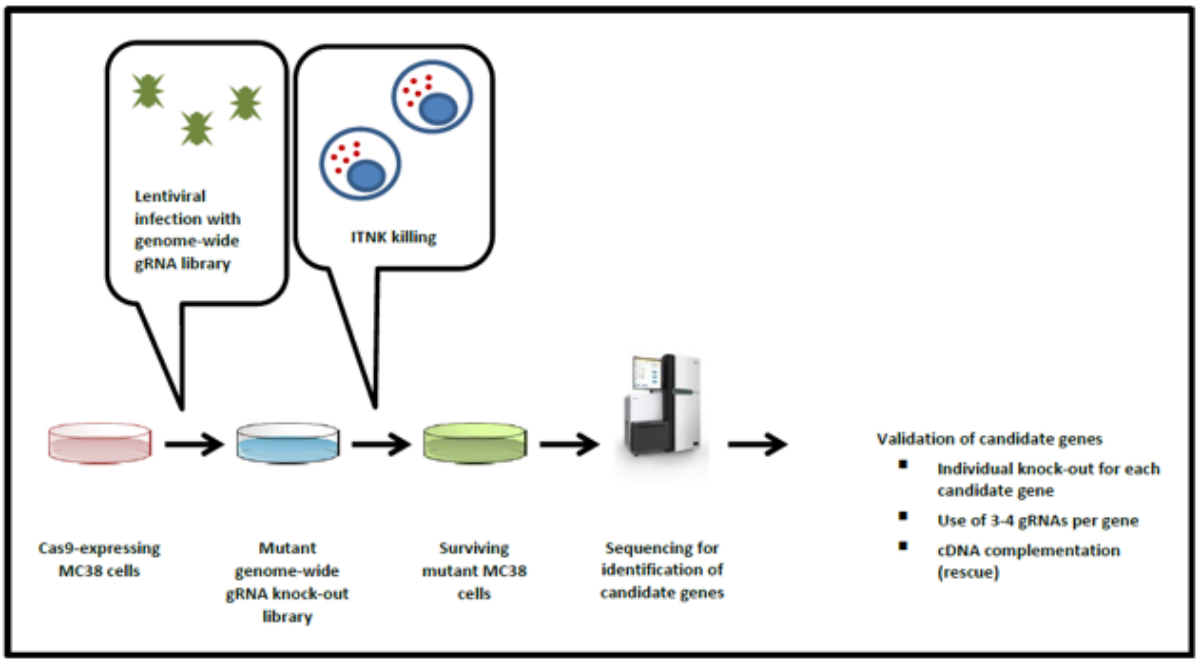


Fig. 8. Schematic of the experimental strategy followed for identifying the genetic basis of cancer cell resistance to immune cell attacks using the CRISPR/Cas9 system. First, a Cas9-expressing MC38 cell line was developed. This cell line was infected with a lentiviral genome-wide gRNA knockout library and then co-cultured with the ITNK cells. The surviving mutant MC38 cells were sequenced in order to identify the candidate genes. Finally, these genes were validated by developing individual knockout cell lines (one for each gene), which were co-cultured with the ITNK cells to validate their cancer-resistance potential. Future work will include to restoration of the sensitivity to ITNK cells in knockout MC38 cell lines by cDNA overexpression of the candidate genes that were knocked out. Image of sequencer was adapted from MIT Technology Review (adapted from Young Rojahn 2014).

3.2 Genome-wide ITNK cell screen in MC38 cells and sequencing of enriched mutants

As described in sections 2.7, Cas9-expressing MC38 cells were infected with the lentiviral genome-wide knockout gRNA library. The infection itself was carried out in the laboratory of Dr Kosuke Yusa at the WTSI. Since the gRNA vector that was used for the screen encoded for BFP, BFP^{+} MC38 cells (which had received a gRNA-expressing lentiviral vector) were isolated by cell sorting and expanded for the rest of the experiments. The library of gRNA-edited MC38 cells was then co-cultured with the ITNK cells. After the co-culture, ITNK cell-selected, surviving MC38 colonies were sequenced. The genes, listed in Table 7, thus represent candidates which were identified. These experiments were carried out by Dr Shannon Burke, Ms Li Ming and Dr Li Li, before I joined the group.

3.3 Generation of Cas9-expressing MC38 cells

For this study, an MC38 mouse colon carcinoma cell line that expressed Cas9 constitutively was generated. The reason for choosing constitutive expression of Cas9 was that, as described by Koike-Yusa, Li and colleagues, Cas9-expressing embryonic stem cell (ESC) lines presented better gRNA targeting efficiency than ESC lines that were transiently transfected with the same vector (Koike-Yusa, Li et al. 2014). The MC38 cell line used expressed EGFP constitutively through random integration of the *pEGFP-N1* cassette (see section 2.3). This was important for validating the functionality of the Cas9 protein (see section 3.4 and Fig. 9). For the permanent integration of the *Cas9* cassette into the genome of EGFP-expressing MC38 cells, a *pPB-LR5.1-EF1a-puroT2Cas9* construct developed in the laboratory of Dr Kosuke Yusa at the WTSI was used (see Fig. 10 and section 2.9). The construct was electroporated into EGFP-expressing MC38 cells, along with the transposase plasmid, according to the protocol described in section 2.6. Since the construct carries a puromycin resistance gene, transfected cells were selected by culturing in puromycin medium. As described in section 2.9, a titration of puromycin was performed in order to identify the ideal concentration for efficiently selecting MC38 cells in which the Cas9 cassette had been integrated. Selection of Cas9-expressing MC38 cells in media containing 5 μg/ml of puromycin for 10 days was chosen.

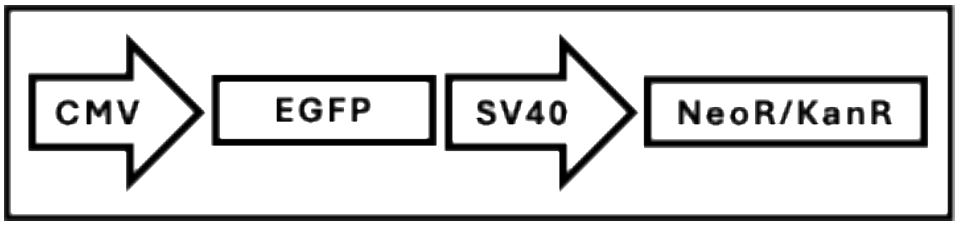


Fig. 9. Vector used for generating a stable EGFP-expressing MC38 cell line. The pEGFP-N1 (CLONTECH) vector carries the neomycin resistance gene under the control of a human EF1α promoter. CMV: human cytomegalovirus promoter; EGFP: enhanced green fluorescent protein gene; SV40: Simian Virus 40 promoter; NeoR/KanR: Kanamycin/neomycin resistance gene.

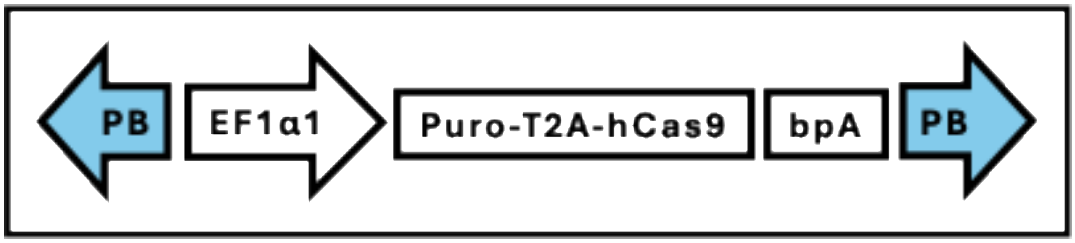


Fig. 10. PiggyBac vector used for generating the Cas9-expressing MC38 cell line. The vector (pPB-LR5.1-EF1a-puroT2Cas9) carries the puromycin resistance gene and a humanised Cas9 cDNA, under the transcriptional control of a human EF1α promoter. PB: PiggyBac repeats; EF1α: human elongation factor-1 alpha gene promoter; Puro: puromycin resistance gene; T2A: T2A self-cleaving peptide; hCas9: humanised Cas9 gene; bpA: bovine polyadenylation signal sequence (modified from Koike-Yusa, Li et al. 2014).

3.4 Cas9 function test

Functionality of the Cas9 protein in the Cas9-expressing MC38 cells was verified by me, firstly by detecting Cas9 protein expression via Western Blot, and secondly, by knocking out the endogenous *EGFP* gene that the MC38 cells constitutively express.

- **Detection of Cas9 expression in Cas9-expressing MC38 cells via Western Blot**

The detection was performed in wild-type (as control) and in Cas9-expressing MC38 cells. A strong expression of the Cas9 protein was observed (see Fig. 11).

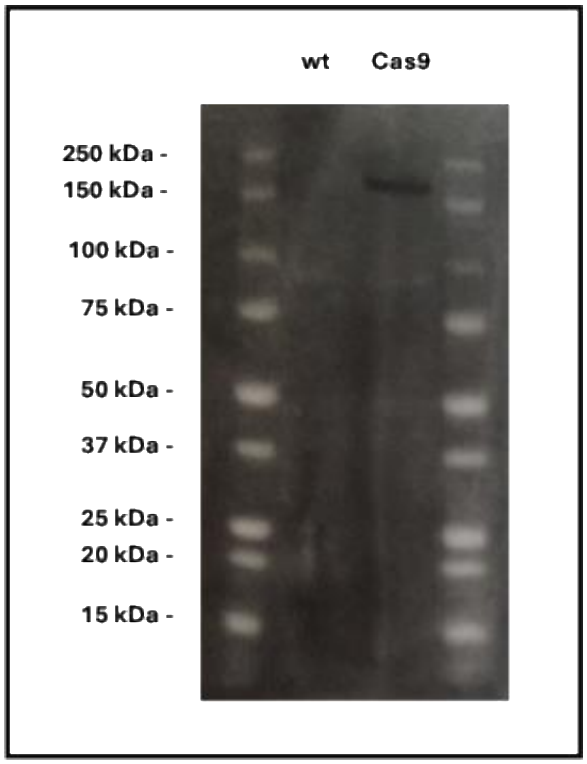


Fig. 11. Western blot of wild-type and Cas9-expressing MC38 cells. A band of molecular weight 160 kDa corresponding to the Cas9 protein is seen in Cas9-stable but not in wild-type MC38 cells.

- **Verifying function of Cas9 in Cas9-expressing MC38 cells by knocking out EGFP gene**

We continued with testing the efficiency of the nuclease ability of the Cas9 protein (see section 2.12). Since the Cas9-expressing MC38 cell line also expressed EGFP constitutively, gRNAs that target the *EGFP* gene were designed. The PiggyBac-BsaI-gRNA expressing vector was used, which carried the *mCherry* gene (see Fig. 12). Therefore, upon transfection of the gRNA constructs into the Cas9-expressing MC38 cells, a decrease in EGFP expression in the mCherry$^+$ cells would indicate good function of Cas9 editing.

In order to ensure that our MC38 cells expressed EGFP uniformly, wild-type and Cas9-expressing/EGFP-expressing MC38 cells were selected in G418 (see section 2.12). Therefore, we can see from Fig. 13 that, after selection, wild-type EGFP-expressing MC38 cells presented an increase in EGFP expression from 65.3% to 94.5% while Cas9-expressing EGFP-expressing MC38 cells presented an increase in EGFP expression from 28.3% to 94.1%.

Four different gRNAs were designed (see Table 2), annealed to obtain the double-stranded oligos, and cloned into the gRNA expression vector (see section 2.11). The constructs were transfected into Cas9-expressing MC38 cells along with a transposase plasmid. G418-selected wild-type MC38 cells (which did not express Cas9) were also transfected with construct PB_BsaI_gRNA_EGFP_9_144, to be used as a control for flow cytometry analysis. The absence of Cas9 activity and targeting in the control cells would result in a double-positive population, expressing both EGFP and mCherry. Untransfected wild-type and Cas9-expressing MC38 cells (only expressing EGFP), which had been selected with G418, were used as single-positive controls. Also, the wild-type MDA-MB-231 cells (negative for both EGFP and mCherry) were used as a negative control. EGFP expression in mCherry+ cells was analysed by flow cytometry, 11 days post-transfection. Results indicated that 99% of the wild-type and 99.3% of the Cas9-expressing MC38 cells were EGFP positive. Wild-type MC38 cells transfected with the gRNA-expressing vector expressed mCherry in a percentage of 40.8%. In Cas9-expressing MC38 cells transfected with EGFP_gRNA_8, EGFP expression was completely knocked out in the mCherry$^+$ cells (see Fig. 14), thus proving the functionality of the Cas9 protein.

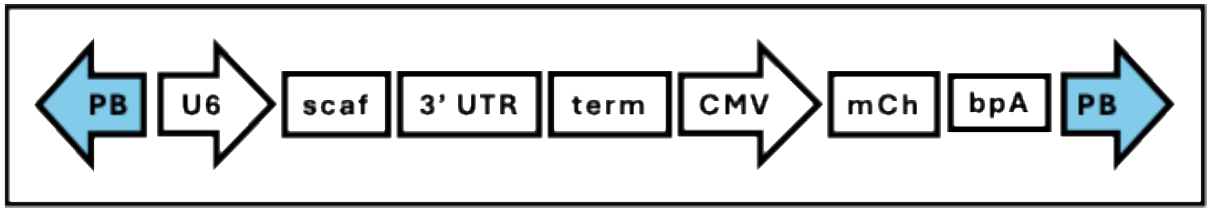


Fig. 12. PiggyBac-gRNA-BsaI vector used for targeted gRNA delivery to MC38 cells. The vector contains a U6 promoter upstream of a directional BsaI cloning site, occupied by a scaffold sequence (scaf). After BsaI digestion, selected gRNAs are cloned into this locus. The individual gRNA-expressing vectors could then be delivered to stable Cas9-expressing MC38 cells to knock out target genes. PB: PiggyBac repeats; U6: U6 promoter; scaff: gRNA scaffold that is replaced by the gRNA sequence upon digestion with BsaI; 3' UTR: chimeric 3' untranslated sequence; term: RNA terminator sequence; CMV: cytomegalovirus promoter; mCh: mCherry gene sequence; bpA: bovine polyadenylation signal sequence. The vector was made by Dr Xuefei Gao at the WTSI.

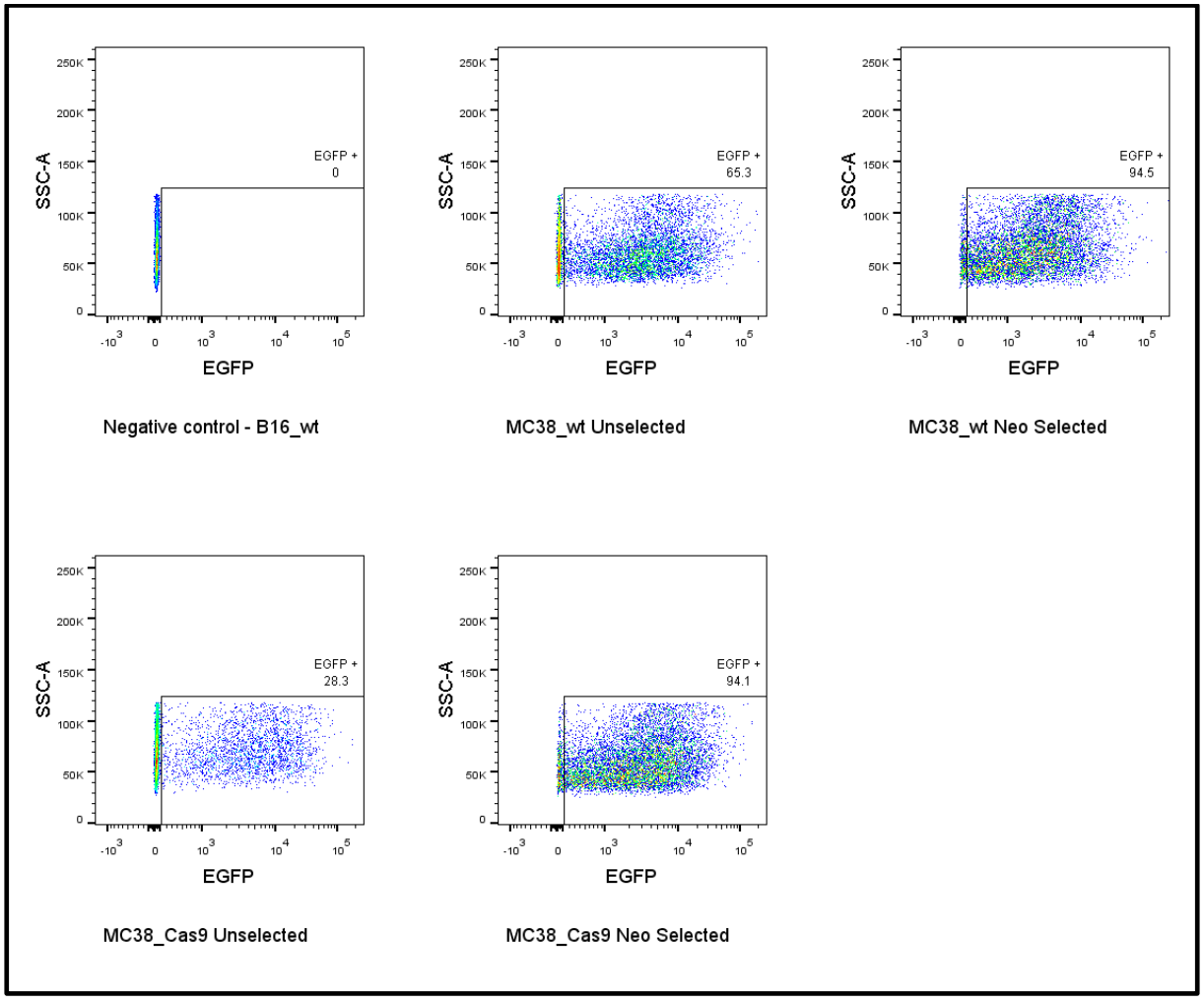


Fig. 13. Flow cytometry analysis of wild-type and Cas9-stable/EGFP-expressing MC38 cells before and after G418 selection. G418-selected MC38 cells show higher EGFP expression compared to unselected MC38 cells. EGFP expression is on the X-axis, and side scatter (SSC-A, indicative of cell size) is on the Y-axis. The gate indicates EGFP-expressing cells. B16 mouse melanoma cells that do not express EGFP were used as a negative control.

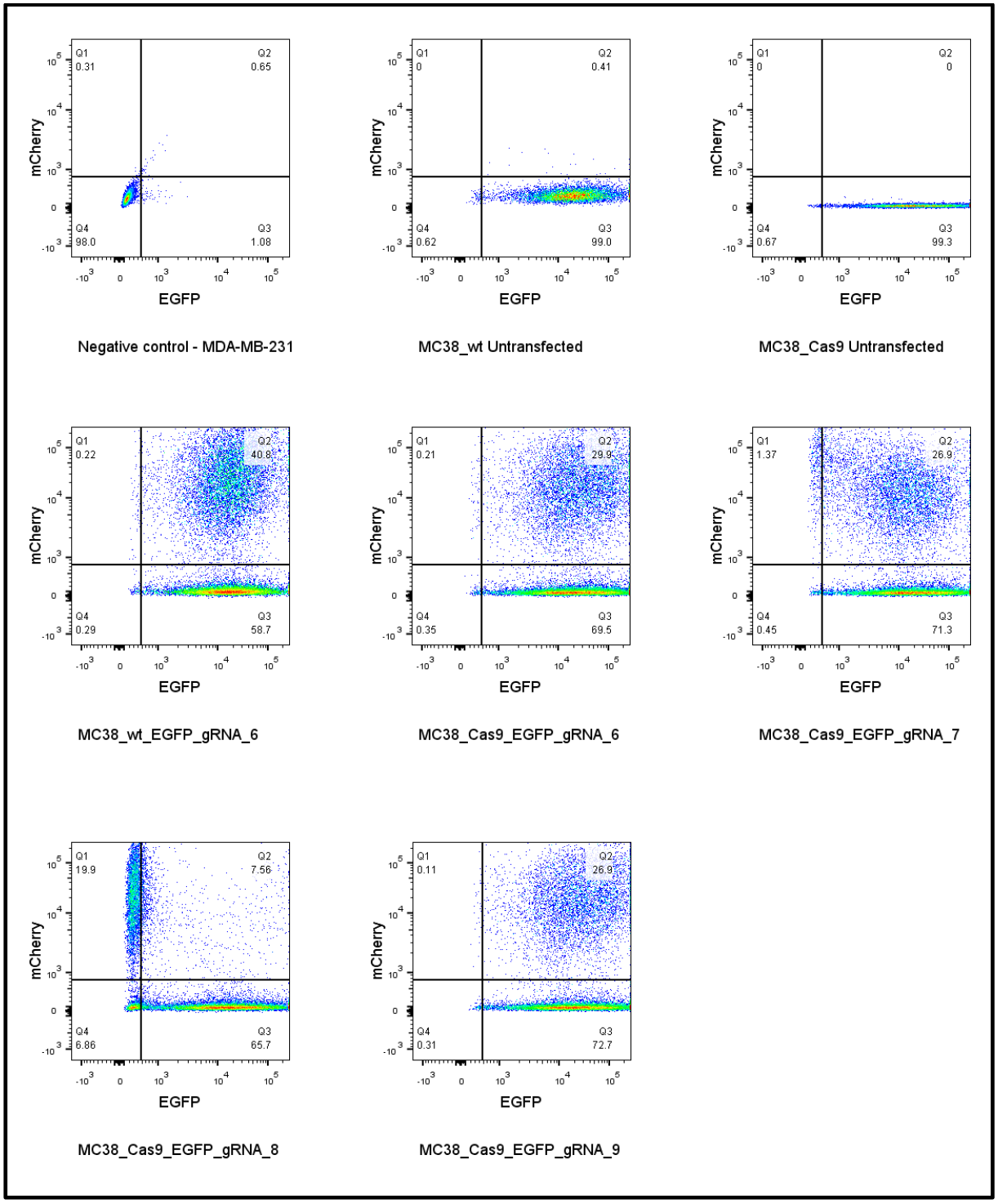


Fig. 14. Test of Cas9 function in stable Cas9-expressing MC38 cells. Flow cytometry analysis of G418-selected, Cas9-expressing and EGFP-expressing MC38 cells transfected with four different PiggyBac-BsaI-gRNA constructs carrying distinct gRNAs against the EGFP gene. mCherry expression is on the Y-axis- and EGFP expression is on the X-axis. Wild-type cells (no Cas9) were transfected with EGFP_gRNA_6 EGFP and expression did not decrease in the mCherry$^+$ cells (i.e. cells taking up the PiggyBac construct). However, in Cas9-expressing MC38 cells transfected with EGFP_gRNA_8, EGFP expression diminished in the mCherry$^+$ cells. This proves the functionality of the Cas9 protein.

Table 7. Table of candidate genes whose knockout confers MC38 cells resistance to ITNK cell-mediated killing. The genes in red were selected for downstream validation.

Candidate genes	
Sugp1	*Srrd*
Ccdc154	*Pot1a*
Cenpc	*Uroc1*
Zbtb8b	*Ppp2r4*
Trp63	*Dgkz*
Amdhd1	*Ptchd1*
Pkd2i2	*1700024P04Rik*
Cmtm1	*Tnrc6b*
Myf6	*Zfp831*
Krt10	*Klhdc10*
Klhdc10	*Ppp6r1*
Dclk2	*4931429L15Rik*
Echdc2	*Gm4841*
Tmem222	*Mpst*

3.5 Verifying expression of candidate genes in MC38 cells by qPCR

Before proceeding with knocking out the candidate genes in MC38 cells, their expression was analysed by qPCR and quantified relative to the expression of beta-actin (*Actb*). Expression of *Amdhd1*, *Cenpc*, *Myf6*, *Sugp1* and *Cmtm1* was higher (see Fig. 15). All 10 genes were selected for validation. The rationale was that the genes which were not highly expressed in MC38 cells might be involved in stress responses. In this case, their expression would be expected to be low when not under the stress of selection, but expression would rise when conditions changed.

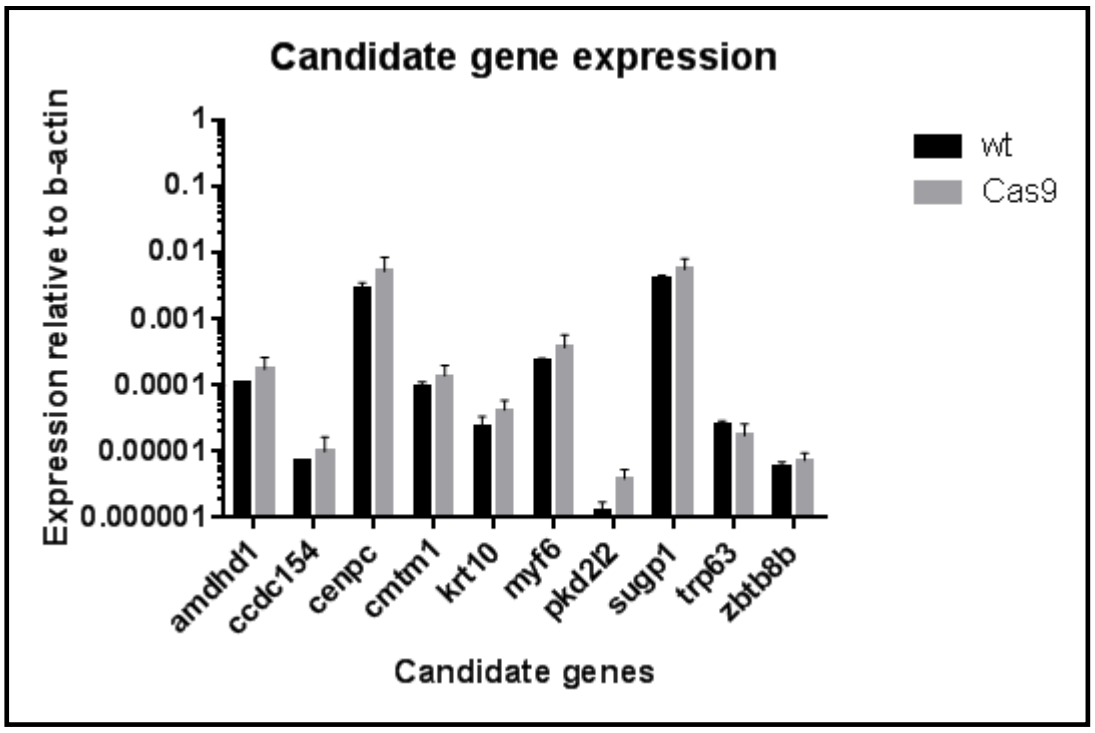


Fig. 15. Expression of candidate genes in wild-type and Cas9-expressing MC38 cells assessed by qPCR. Expression is calculated in relation to beta-actin (*Actb*). *Amdhd1*, *Cenpc*, *Myf6*, *Sugp1* and *Cmtm1* were more highly expressed than the rest. All 10 genes were selected for downstream experiments.

3.6 Optimisation and ITNK cell generation

Initially, thymocytes were treated with OHT 1 µM for 48 h, according to the protocol described by Li, Burke and colleagues (Li, Burke et al. 2010). After trying the protocol 3 times, extensive cell death was observed and a lack of killing potential, as the OP9-DL1 stromal cells remained alive. It was deduced that OHT was exceptionally toxic to the thymocytes.

Thymocytes were then treated with a titration of OHT concentrations for 48 h. One week after the end of OHT treatment, genotyping was performed to verify that the *Bcl11b* gene had been successfully deleted in OHT-treated thymocytes (see section 2.18). As observed in Fig. 16, the successful deletion of *Bcl11b* was observed using Bcl11b_del primers (a 450 bp deletion band was observed in all OHT-treated samples). Amplification with Bcl11b_cko primers indicated that deletion was incomplete, since a 345 bp band was detected in all samples. The 243 bp wild-type band, which was also amplified, may be the result of amplification of the *Bcl11b* locus of the OP9-DL1 cells.

The treated thymocytes were then analysed by flow cytometry, in order to assess the expression of NK cell markers NK1.1 and NKp46, and to evaluate ITNK cell conversion. As observed in Fig. 17, in the untreated sample, 2.18% of the cells are double positive for NK1.1

and NKp46. This can be explained by the characterised population of thymic NK cells that express NK markers (Ribeiro, Hasan et al. 2010, Vargas, Poursine-Laurent et al. 2011) (no further validation was carried out), or by leakage of Cre-recombinase in untreated cells.

Expression of NK1.1 single positive cells remained relatively low, in all conditions, ranging from 3.33% to 6.29%. On the other hand, upon OHT treatment, the percentage of NKp46 single positive cells was increased from 1.45% in the untreated to 18.1% in the sample treated with 1µM. The highest NK1.1 and NKp46 double-positive percentages were observed in samples treated with 0.25 µM of OHT for 48h.

The treated thymocytes were tested for the T cell markers CD4 and CD8 (see Fig. 18). In the untreated sample, 3.17% of the cells were $CD4^+$ but $CD8^-$, a percentage that increases mildly upon OHT treatment, reaching 14.1% in samples treated with 1 µM OHT for 48 h. The expression of CD8 experiences a drop, from 22.2% in the untreated sample to 12.5% in 1 µM OHT. It is worth mentioning that the percentage of CD4/CD8 double-positive cells decreases, from 55.4% in the untreated to 2.66% in 0.25 µM OHT and then increases again, up to 8.46% in samples treated with 1 µM OHT for 48 h.

Finally, the expression of T cell receptor beta chain (TCR-β) was assessed. As we can see in Fig. 19, 70.9% of the untreated cells were TCR-β positive, while the percentage drops in the OHT-treated cells, first to 14.2% in 0.1 µM OHT and then to 33.6% for 0.25 µM OHT, before increasing again to 48.8% for 1 µM OHT.

Both in the case of CD4/CD8 double-positive cells and in the case of TCR-β+ cells, treatment with OHT led to a decrease in the percentages. However, increasing OHT concentration above 0.1 µM resulted in a mild increase in the percentage of positive cells. Thus, treatment of thymocytes with 0.25 µM OHT for 48 h was chosen as the preferred condition. OHT toxicity was not severe using this concentration, and the cells were able to recover. Upon *Bcl11b* deletion, the thymocytes underwent a series of morphological changes, including an increase in size and formation of granules in their cytoplasm (see Fig. 20). These ITNK cells were used for subsequent validations.

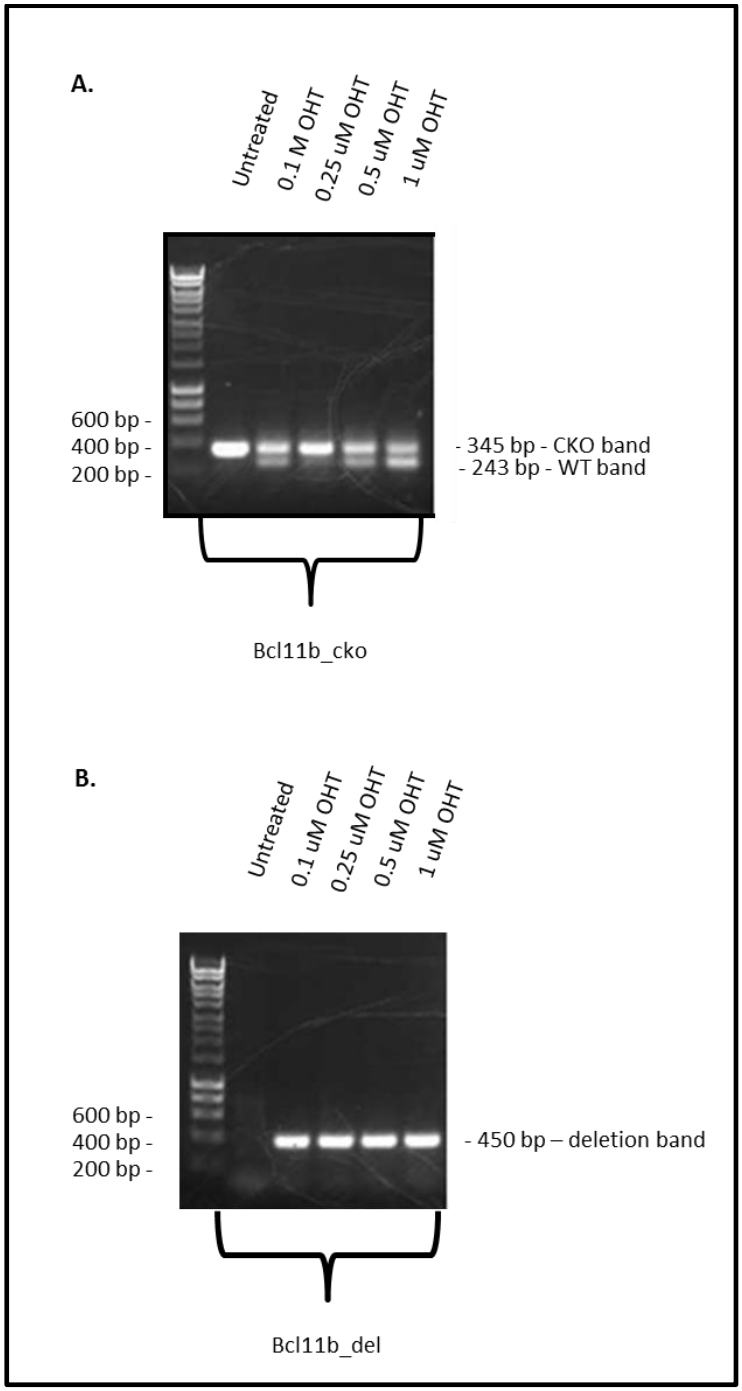


Fig. 16. Genotyping of thymocytes treated with a range of OHT concentrations for 48 h, using different primer sets. A. Conditional knockout primers (Bcl11b_cko), which detect the successful incorporation of the *loxP* cassette into the mouse genome, by amplifying a 345 bp fragment (CKO band). If the *loxP* cassette has not been incorporated, the amplified fragment is 243 bp (wild-type band). B. Deletion primers (Bcl11b_del), which produce a detectable 450 bp fragment only if the deletion of exon 4 of Bcl11b has happened successfully.

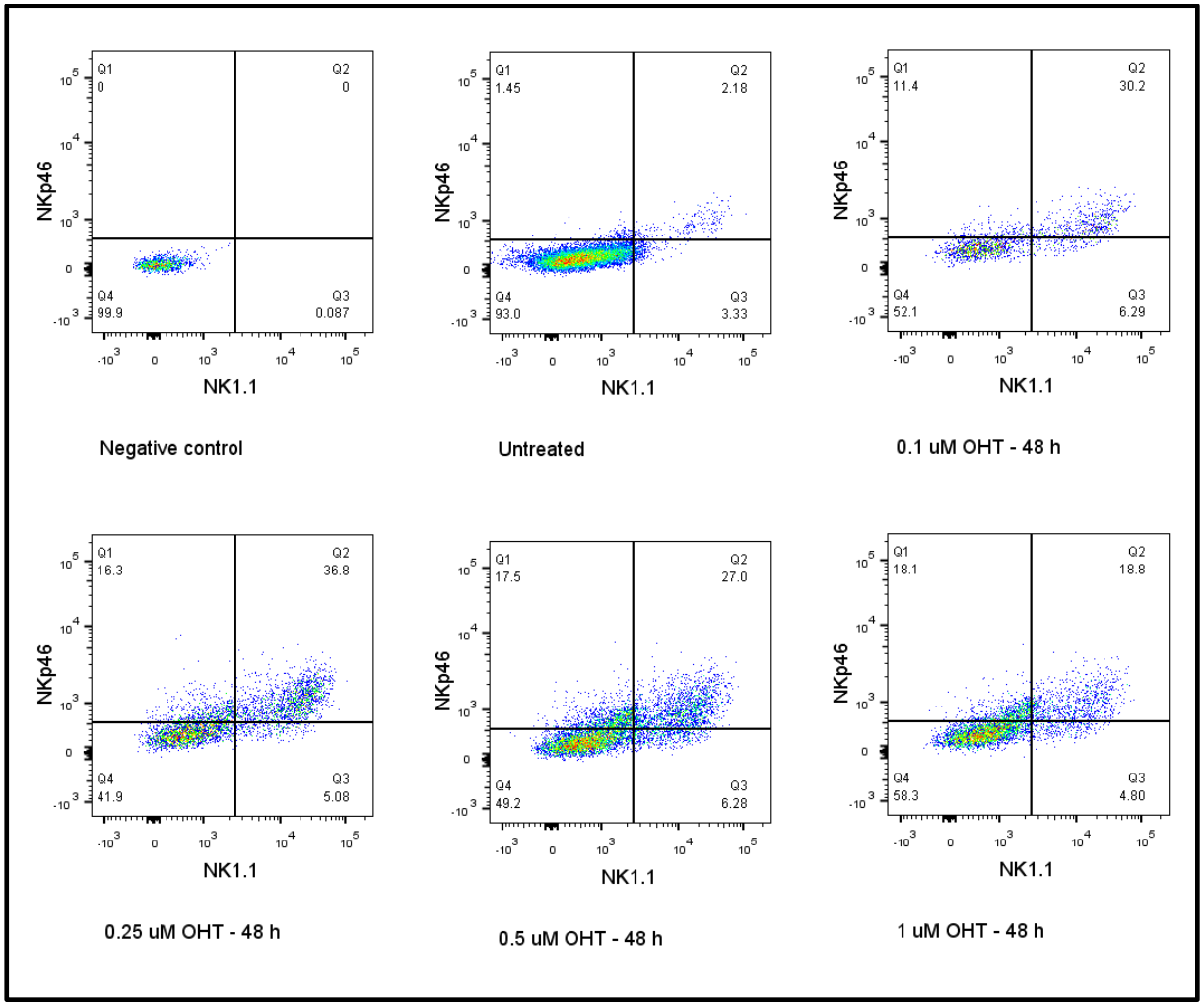


Fig. 17. Expression of NK cell markers NK1.1 and NKp46 in thymocytes treated with a range of OHT concentrations. Thymocytes were treated with OHT for 48 hours and analysed by flow cytometry for the NK markers NK1.1 and NKp46 one week later. NK1.1 expression is seen on the X-axis and NKp46 expression on the Y-axis. NK1.1 and NKp46 double-positive thymocytes appear in the upper right quadrant. Cells treated with 0.25 µM gave the maximum number of NK cells without affecting viability (data not shown). The small NK1.1/NKp46 double-positive population (2.18%) in the untreated sample might be the result of low-level leaky expression of Cre-recombinase.

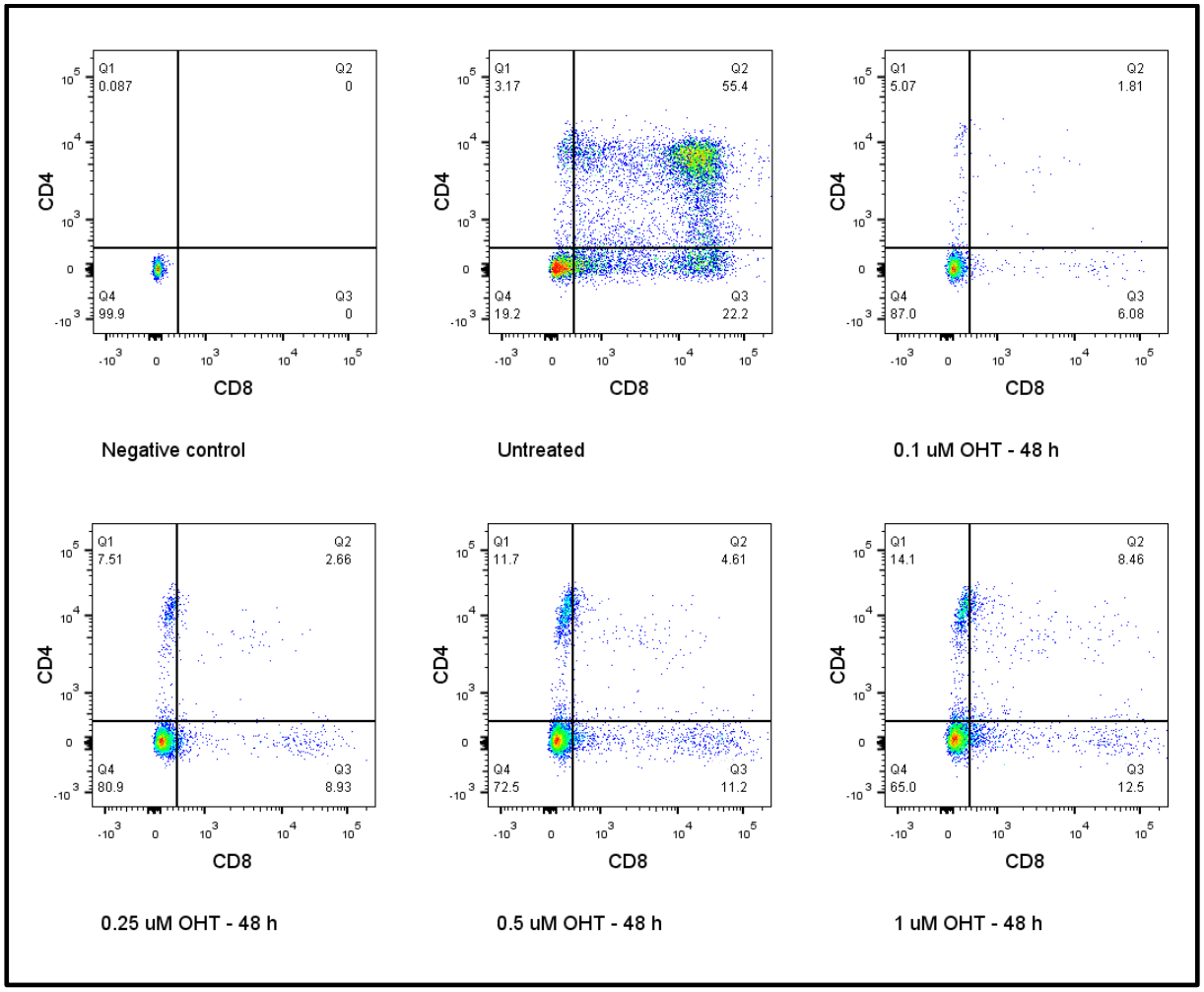


Fig. 18. Expression of T cell markers CD4 and CD8 in thymocytes treated with a range of OHT concentrations. Thymocytes were treated with OHT for 48 h and analysed by flow cytometry for the T cell markers CD4 and CD8. CD8 expression is seen on the X-axis, while CD4 expression is on the Y-axis. The CD4/CD8 double-positive cells appear in the upper right quadrant. The double-positive cells disappear upon OHT treatment. The downregulation of T cell markers and the upregulation of NK markers are characteristics of the conversion of thymocytes to ITNK cells upon Bcl11b deletion.

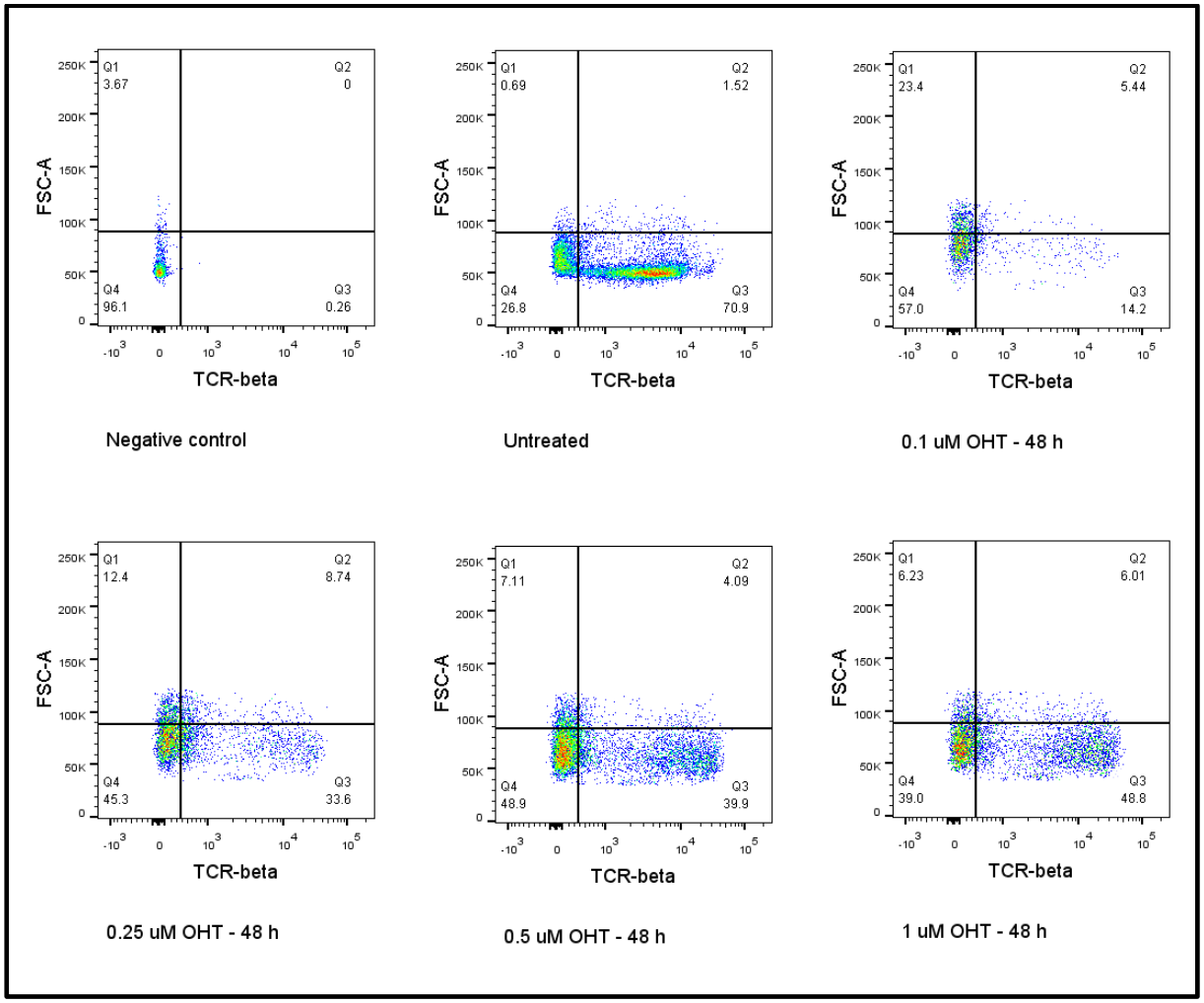


Fig. 19. Expression of TCR-β in thymocytes treated with a range of OHT concentrations. Thymocytes were treated with OHT for 48 hours and analysed by flow cytometry for TCR-β expression one week later. TCR-β expression is seen on the X-axis and forward scatter (FSC-A, indicative of cell size) on the Y-axis. TCR-β+ thymocytes appear in the lower right quadrant. Upon OHT treatment, a drop in TCR-β expression was observed.

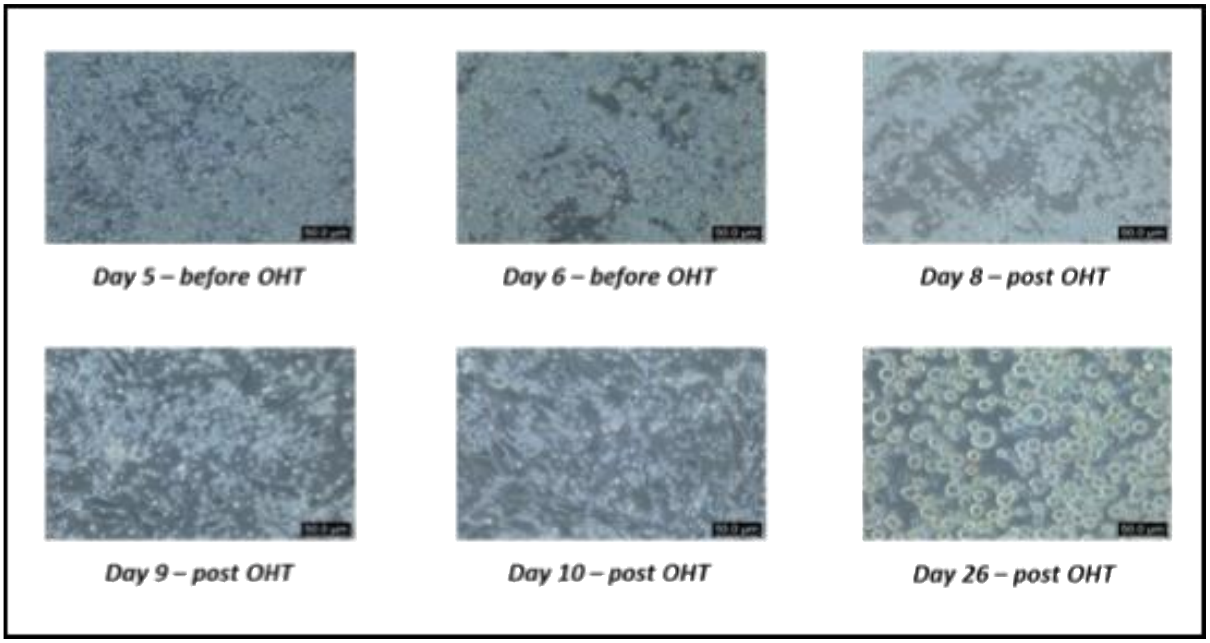


Fig. 20. Morphological changes of thymocytes upon *Bcl11b* deletion, after OHT treatment, towards their conversion to ITNK cells. Thymus collection and thymocyte seeding took place on day 1, while OHT treatment took place on day 7. OP9-DL1 stromal cells are still visually observed on days 5, 6, 8, 9 and 10. On day 26, the thymocytes have been converted to ITNK cells, and no stromal cells are seen. Apart from the killing potential, an increase in the size of the converted cells was also observed.

3.7 ITNK cell co-cultures and validation of candidate genes

In order to validate the findings from the initial genome-wide ITNK cell screen, single knockout cell lines were developed for each of the candidate genes. All the gRNA oligos of Table 1 were annealed and cloned into PiggyBac-BsaI-gRNA expressing vectors and transfected into Cas9-expressing MC38 cells. These cell lines were screened anew with ITNK cells.

So far, out of the 10 single knockout cell lines that were developed, survival colonies are growing in cells with knockouts in the following genes: *Sugp1*, C*cdc154*, C*enpc*, Z*btb8b*, *Trp63* and *Cmtm1*. Observation using a fluorescent microscope was possible, since the MC38 cells are $EGFP^+$. EGFP activity was not observed in our untransfected control MC38 cells, indicating efficient ITNK cell-mediated killing. Cas9-expressing MC38 cells transfected with vectors carrying gRNAs specific for *Sugp1*, *Trp63* and *Cmtm1*, formed distinct clusters (see Fig. 21).

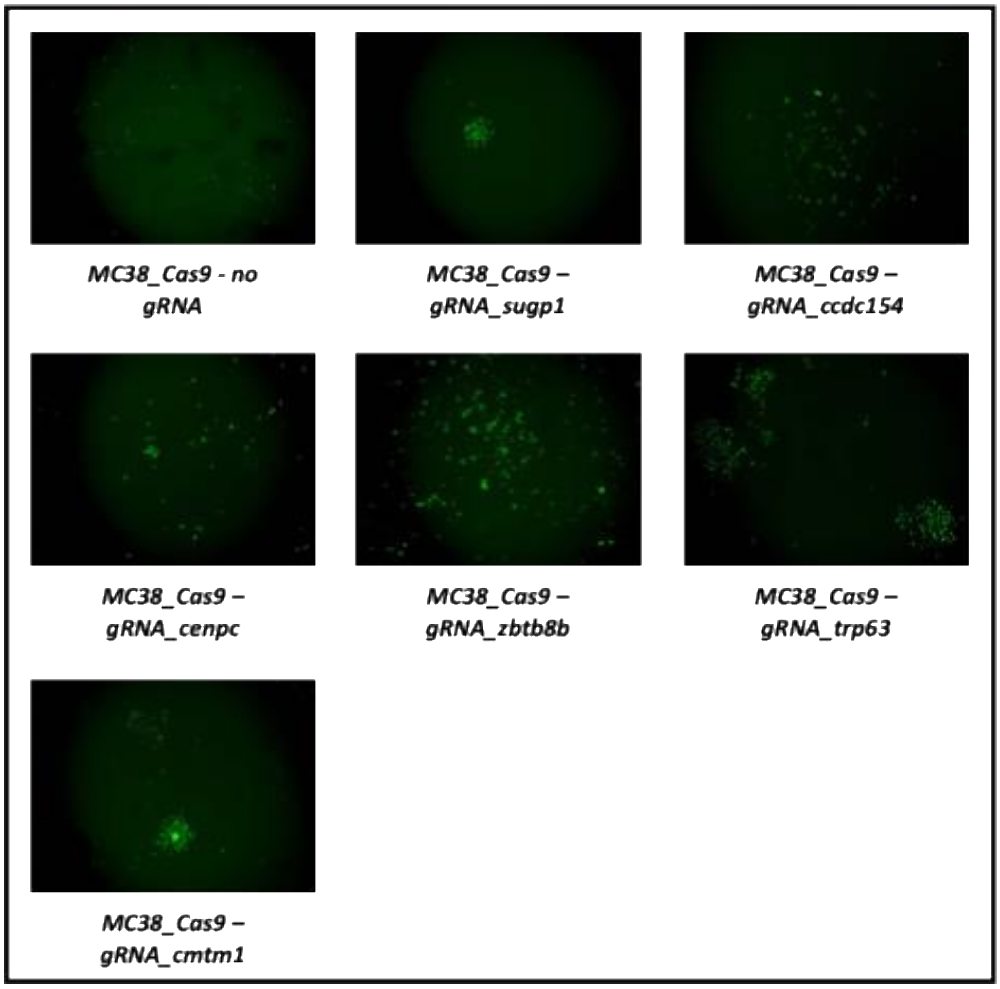


Fig. 21. Individual knockout MC38 cells which survived ITNK cell killing. Cas9-expressing MC38 cells were transfected with gRNAs targeting each candidate gene individually. Cas9-expressing MC38 cells without gRNAs (upper left image) were used as a control. The single knockout cell lines were screened by co-cultures with the ITNK cells. No EGFP expression was observed in the untransfected control condition post-co-culture. In cells transfected with gRNAs targeting *Sugp1*, *Trp63* and *Cmtm1*, the EGFP-positive cells formed distinct clusters. Pictures of EGFP-positive cells were obtained using a 4X magnification.

4. Discussion

4.1 Conclusions and importance of study

Significant progress in the last few decades has highlighted the pivotal role of immune responses in the initiation and progression of malignant disease. Furthermore, a deeper understanding of the immune system and its anti-tumour properties has led to the development of ways of harnessing the immune system for cancer treatment. Our laboratory had previously developed the ITNK cells, a potent tumour-killing cell type generated by conditional deletion of the transcription factor *Bcl11b* in thymocytes at different developmental stages. Previous work in the lab involved the use of the CRISPR/Cas9 technology to carry out a genome-wide genetic screen, in which gRNA-edited MC38 cells were co-cultured with and selected by ITNK cells. The surviving MC38 cells were sequenced in order to identify genes whose knockout was involved in cancer cell resistance to the ITNK cell attacks (experiments carried out before I joined the group). The aim was to use this platform to decipher the genetic mechanisms that govern cancer immune evasion and provide a proof of concept for the applicability of such technologies.

My work involved the validation of the candidate genes that were identified. Using the MC38 mouse colon carcinoma cell line, Cas9-expressing MC38 cells were engineered, and Cas9 protein expression and function were confirmed. To validate the candidate genes, individual MC38 knockout cell lines (one for each candidate gene) were developed in order to be used for ITNK cell co-culture killing assays. The experiments enabled the identification of genes whose knockout conferred MC38 cells resistance to ITNK cell killing. Therefore, it was suspected that their functional state in the cancer genome was partially responsible for cancer cells being sensitive to ITNK cell attacks. Our preliminary results highlight the superiority of the model and the potential it holds for cancer immunology research.

4.2 Optimising a CRISPR/Cas9 genetic screen to investigate cancer cell-immune cell interactions

The development of an MC38 cell line that stably expressed Cas9 was carried out by *PiggyBac*-mediated integration of a *Cas9* cassette into the MC38 genome. The Cas9-expressing cells were selected by puromycin. The expression and function of the Cas9 protein were verified

by Western Blot and by knocking out the constitutive expression of EGFP in the MC38 cells, using *EGFP* gene-specific gRNAs. The outcome of that engineering is a cell population in which the *Cas9* gene has been incorporated randomly, in different parts of the MC38 genome. This could potentially cause problems based on the premise that the incorporation of the *Cas9* cassette in a transcriptionally active or in a silenced part of the genome would result in varying Cas9 expression levels. Alternatively, we could have proceeded with single-cell cloning of the Cas9-expressing MC38 cells by diluting down cell populations and testing their Cas9 expression individually for each clone. Then, the clone with the highest and most stable Cas9 expression would have been expanded and used for the next stages of our strategy. This would result in a high and uniform expression of Cas9 by the MC38 cells, reducing the variability introduced by the random integration of the *PiggyBac Cas9*-carrying cassette into the genome.

Moreover, the strategy that was followed for verifying the function of Cas9 in Cas9-expressing MC38 cells was to transfect the cells with EGFP-specific gRNAs targeting the endogenous EGFP expression of the MC38 cells. Out of the 4 constructs designed using the E-CRISPR website (http://www.e-crispr.org/), only one resulted in efficient knockout of EGFP activity in the cells that received the gRNA (EGFP_gRNA_8). The stable expression of EGFP in MC38 cells was generated through the random integration of an *EGFP* cassette. It is highly possible that more than one copy of the *EGFP* cassette was incorporated into the genome of the MC38 cells. For efficient knockout of EGFP expression, all the *EGFP* copies needed to be knocked out, which posed a difficulty and possibly explained the inefficiency of the other gRNA vectors. Alternatively, another well-expressed surface marker of the MC38 cells could have been chosen, whose expression is based on a single copy of the gene in the MC38 genome. Knockout of the surface marker by gRNA-mediated targeting would be easily detectable by flow cytometry. Another approach would include targeting a glycosylphosphatidylinositol (GPI)-anchor pathway protein, which is used by *Clostridium septicum* alpha-toxin for entry into the cell. Knockout of the protein would provide resistance to alpha-toxin (Koike-Yusa, Li et al. 2014).

The genome-wide lentiviral library was developed by infecting Cas9-expressing MC38 cells with viral vectors carrying gRNAs targeting 19,150 genes in the mouse genome. Even though the ideal viral density was calculated so that each cell receives 1 gRNA, based on stoichiometry, it is difficult to predict how many gRNAs enter each cell. This might potentially

cause problems in identifying which knockout is responsible for which phenotype. Thus, this exemplifies the importance of sequencing the surviving colonies after ITNK cell killing and selecting only the overlapping genes that were identified in different surviving clones, thus maximising the specificity of the screen. This was the strategy that was followed on our screen.

Another way to improve the effectiveness of our screening would be to carry out the lentiviral infection and subsequent ITNK cell killing more times before proceeding to validation. Our candidate genes were identified from one infection of the MC38 cells with lentiviral gRNA vectors and one round of ITNK cell killing. Repeating the process 2 or 3 times could expand the list of potential candidate genes.

Validation of the candidate genes was performed by transfecting Cas9-expressing MC38 cells with gRNAs in *PiggyBac* vectors and developing individual knockout MC38 cell lines (one for each gene). Since the *PiggyBac* gRNA vector carries the *mCherry* gene, cells that received a gRNA could be isolated via cell sorting of the $mCherry^{+}$ cells (see Fig. 12). This would lead to a pure cell population that has received a gRNA, thus increasing the efficiency of the subsequent knockouts and therefore improving the ITNK cell selection.

Moreover, when validating our candidate genes, the individual knockout MC38 cell lines could have been sequenced by MiSeq Illumina next-generation sequencing. The method's speed and accuracy would make it ideal for sequencing modifications of genes that have been the result of genome editing approaches (Liu, Li et al. 2012). This would allow us to validate the efficient inactivation of each of the candidate genes in the knockout cell lines.

For the validations, individual knockout cancer cell lines were co-cultured with the ITNK cells in a ratio of 2.5:1. It would be worth investigating the ideal ITNK cell to cancer cell ratio for efficient killing by carrying out a titration of increasing ratios. Starting with the co-culture of ITNK cells with cancer cells in a ratio of 1:1 and going up to 10:1 would allow us to improve the ITNK cell killing assay and the performance of our screening.

An important aspect to improve the ITNK cell killing would be to specify which developmental stage of thymocytes would result in better tumour-killing potency of the resulting ITNK cells, upon *Bcl11b* deletion. For this purpose, we could sort different thymocyte populations (from naïve to mature T cells), delete *Bcl11b* by OHT treatment and produce ITNK cells. These ITNK cells, derived from different developmental stages, could be assessed for their tumour-killing potential. In this way, it would be possible to increase the ITNK cell killing efficiency. Finally,

a similar approach could be followed to test the cancer-killing properties of different *in vivo*-derived ITNK cells, generated from different thymocyte stages. This would enable the identification of the population that gives rise to more efficient ITNK cells and use these for screening experiments (Liu, Li et al. 2010).

4.3 Future directions

Our strategy provides preliminary results for the feasibility of a CRISPR/Cas9 genetic screening approach in identifying genes involved in the ability of tumour cells to evade immunosurveillance.

In order to complete the validation of our candidate genes, several steps need to be followed. First of all, validations so far involved a single killing of the individual knockout MC38 cell lines by the ITNK cells. The surviving cells need to be expanded, and the ITNK cell killing needs to be repeated 3 more times, using new ITNK cells each time. This would progressively enrich the surviving cancer cell population in ITNK cell-resistant MC38 cells. After the completion of the sequential ITNK cell killings, the surviving MC38 cells could be expanded and sequenced in order to verify that the surviving cells have the inactivating Cas9-mediated mutations. The ITNK cell-resistant MC38 cell lines will then be used in order to rescue the MC38 cell sensitivity to the ITNK cell attacks. This will be performed by introducing the cDNA of the genes that have been knocked out in the resistant cells. This cDNA overexpression assay will result in making the resistant cells sensitive to the ITNK cell attacks, once again. Thus, this would prove that the ITNK cell resistance is the direct result of knocking out the candidate genes and not because of off-target mutations (Koike-Yusa, Li et al. 2014).

Given the different CRISPR/Cas9-based strategies that have been developed, the potential of their application for genetic studies in cancer immunology could be greatly expanded. Recent studies have described protocols for Cas9-mediated gene activation (Gilbert, Horlbeck et al. 2014, Konermann, Brigham et al. 2015). This could work complementarily to the knockout approach we followed and would identify genes whose upregulation leads to cancer immunological resistance. These genes would need to remain in their non-upregulated state in order for cancer to be kept at bay by immune surveillance.

According to a different approach, we could develop a genome-wide lentiviral knockout or activation library to infect the ITNK cells with the viral vectors carrying the gRNAs. This would reveal mutations in the genome of the immune cells that are associated with immune cell-

cancer cell recognition and killing. A limiting factor would be the relatively short life of primary ITNK cells. Therefore, the infection of the ITNK cells with the lentiviral library could happen before the deletion of *Bcl11b*, in order to gain more time for the screening. Developing genome-wide libraries both for the ITNK and for the MC38 cells would allow us to evaluate gene regulation on both interacting elements of our study. Improving our understanding of cancer-enabling genetic abnormalities of immune cells might lead us, to a certain extent, to reconsider malignancy as an immunological disease.

After validating genes involved in ITNK cell resistance, we would examine whether these genes have been observed to be involved in different kinds of malignant diseases. This could be achieved by comparing our validated genes with RNA sequencing data from the Cancer Genome Project (CGP) or other databases. These genes might encode surface markers or intracellular proteins. If they are encoding surface markers, this might indicate that the markers are important for the identification and killing of cancer cells by the ITNK cells. In this case, their absence on the cancer cell surface would render the cancer cell invisible to ITNK cells. If they are encoding for intracellular proteins or transcription factors, they might be involved in cascades of transcriptional regulation. Identification of these genes would be extremely significant for the molecular characterisation of cancer, since the detection of these mutations would indicate that the cancer will most probably be highly malignant and resistant to immunological surveillance or certain NK-based immunotherapies. Therefore, we could design alternative therapeutic protocols. In the case that these mutations might arise before the rise of malignant disease, they could be utilised in clinical practice as pre-cancerous markers, suggesting high risk for developing an immune-resistant malignancy. In the event that the candidate genes are elements of a transcriptional cascade, it would be crucial to identify the specific molecular pathway, and design ways to affect specific molecular events. This could potentially be used as a therapeutic application; since the inactivation of one of these genes leads to cancer cell immune resistance, affecting one of their downstream elements might result in the reactivation of the signalling pathway. Thus, the cancer cells would be rendered sensitive to immune attacks once again. In the case that the specific functions of the candidate genes are unknown, we would proceed with elucidating these functions. First, we could develop *in vitro* reporter cell lines for the unknown genes, in order to test their expression in different conditions and on different genetic backgrounds. This would also allow us to clarify whether these genes are involved in stress response, in the case

that they are not highly expressed normally but are activated under specific conditions and pressures. An *in vivo* approach would include the development of a knockout mouse in order to verify the significance of the gene for development. In the case that the mutation is lethal, we could engineer a conditional knockout mouse to investigate the effects of conditional inactivation of the gene on the phenotype of the mouse, with emphasis on immune system functions. Finally, the function of the gene could be examined in the context of a cancer mouse model. The conditional knockout mouse could be crossed with a hereditary cancer mouse model, in order to evaluate cancer progression, having our candidate gene knockout as the genetic background. Alternatively, cancer might be mediated by the administration of a mutagen.

4.4 Concluding thoughts

In our study, we used the ITNK cells as an *in vitro* recapitulation of innate NK cell immunosurveillance. The reason that NK primary cells were not used instead was that the screening would require high numbers of NK cells, which would be challenging. The ITNK cells are highly proliferative, which makes them ideal for such experiments (Liu, Li et al. 2010). Moreover, selecting the edited cancer cells with ITNK cells in several rounds of killing is theorised to be a good *in vitro* equivalent in order to study the processes of immunoediting, according to which the immune system shapes the level of malignancy and immunogenicity of cancer cells based on the selective pressure it places upon them.

However, this new cell type still has many unknown functions, and more research needs to be done to elucidate its mechanisms. For this reason, the level of the quality of the recapitulation of NK cell-based immunosurveillance by the ITNK cells could be challenged, since it's not a natural cell type, and its exact function is unknown. First of all, we need to clarify the exact mechanism of the ITNK cell cytotoxicity. Even though evidence shows that they express perforin (Li, Burke et al. 2010), it is not known whether they employ a perforin/granzyme-mediated mechanism for cytotoxicity, as NK cells do (see section 1.8). Also, it would be worth comparing the expression profile, the tumour killing efficiency, and the life span of ITNK cells derived from thymocytes at different stages of development (Liu, Li et al. 2010). Employing a single-cell RNA sequencing approach would reveal the heterogeneity of this cell type. Moreover, comparing the same parameters as before, both in ITNK cells, NK cells, NKT cells, and CTLs, would reveal the differences between the ITNK and other cytotoxic

cell types. Furthermore, it is not known how the ITNK cells react to cells that have been infected by a virus and if their infected cell-killing potential is similar to their cancer-killing potential. If that's the case, this would pave the way to developing novel genetic screens that attempt to identify genes involved in cytotoxic effects against cells infected by viruses. It would also be important to know whether there are any naturally occurring ITNK-like cells, in case any thymocyte populations downregulate *Bcl11b* and differentiate to NK-like cells, which are distinct from NK and NKT cells. For this purpose, we could employ a single-cell RNA sequencing approach in infiltrating cells of the tumour microenvironment, in order to examine the presence of $Bcl11b^{-}/NK1.1^{+}/NKp46^{+}$ cells. Finally, the ability to derive human ITNK cells from thymocytes, upon *BCL11B* inactivation, along with addressing the aforementioned questions in the human cell type as well, could be the first step in the development of a novel immunotherapeutic approach for malignant disease (Liu, Li et al. 2010).

The ITNK cells represent a very promising tool for cancer immunotherapy. As mentioned above, they are easily obtained in high numbers from any developmental stage of thymocytes. Moreover, they do not react against self-antigens. Studies in which ITNK cells were either transplanted into wild-type mice or developed by conditional deletion of *Bcl11b in vivo*, concluded that mice did not show any autoimmunity or tumour development. Moreover, the ITNK cells are very efficient in metastasis prevention *in vivo*, while they are potent in attacking tumour cells that express, and tumour cells that do not express MHC class I molecules (Li, Burke et al. 2010, Liu, Li et al. 2010).

Despite the potential for ITNK cell-based cancer immunotherapy, the main evidence our project provides is that cancer resistance to ITNK cell attacks could arise. This would make such a therapy inefficient. Therefore, options for targeting the ITNK cells towards specific antigens should be explored. Experimental data have shown that in ITNK cells generated from $CD8^{+}$ T cells, upon *Bcl11b* deletion, the TCR signalling pathway is seemingly operational. This could be used in order to combine the ITNK cell engineering with chimeric antigen receptor (CAR) technology. In this case, T cells are engineered to express a specific antigen receptor on their surface, giving them the ability to raise a specific immune response against this antigen. This technology has started to be widely used as a tool for cancer immunotherapy (Liu, Li et al. 2010, Lipowska-Bhalla, Gilham et al. 2012). Thus, combining the two approaches would allow CAR-based engineering of a T cell before its conversion to an ITNK cell, which could

increase the specificity of the killing. In a different approach, tumour-infiltrating $CD8^+$ T cells from the tumour microenvironment would be expressing receptors that drive tumour-specific responses. Isolation of this cell population and their conversion to ITNK cells would also result in increased tumour-specific toxicity (Liu, Li et al. 2010).

It becomes evident that genetic screens hold great promise in elucidating the role of genes that regulate cancer initiation and progression. We also demonstrate that the ITNK cells can adequately recapitulate anti-tumour immune responses for *in vitro* genetic studies, while they show potential as a tool for immune-based cell therapies for the treatment of malignant neoplasias.

5. References

1 Abbas, A. K., Lichtman, A. H. & Pillai, S. *BASIC IMMUNOLOGY: FUNCTIONS AND DISORDERS OF THE IMMUNE SYSTEM*. 6th edn, (Elsevier Saunders, 2014).

2 Abbey, J. L. & O'Neill, H. C. Expression of T-cell receptor genes during early T-cell development. *Immunol Cell Biol* **86**, 166-174, doi:10.1038/sj.icb.7100120 (2008).

3 Adams, J. M. & Cory, S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* **26**, 1324-1337, doi:10.1038/sj.onc.1210220 (2007).

4 Albu, D. I. *et al.* BCL11B is required for positive selection and survival of double-positive thymocytes. *J Exp Med* **204**, 3003-3015, doi:10.1084/jem.20070863 (2007).

5 Amaravadi, R. K. & Thompson, C. B. The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clin Cancer Res* **13**, 7271-7279, doi:10.1158/1078-0432.Ccr-07-1595 (2007).

6 Amit, I. *et al.* A module of negative feedback regulators defines growth factor signaling. *Nat Genet* **39**, 503-512, doi:10.1038/ng1987 (2007).

7 Apel, A., Zentgraf, H., Buchler, M. W. & Herr, I. Autophagy-A double-edged sword in oncology. *International Journal of Cancer* **125**, 991-995, doi:10.1002/ijc.24500 (2009).

8 Artandi, S. E. & DePinho, R. A. Telomeres and telomerase in cancer. *Carcinogenesis* **31**, 9-18, doi:10.1093/carcin/bgp268 (2010).

9 Arum, C. J. *et al.* Cancer Immunoediting from Immunosurveillance to Tumor Escape in Microvillus-Formed Niche: A Study of Syngeneic Orthotopic Rat Bladder Cancer Model in Comparison with Human Bladder Cancer. *Neoplasia* **12**, 434-442, doi:10.1593/neo.91824 (2010).

10 Baluk, P., Hashizume, H. & McDonald, D. M. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev* **15**, 102-111, doi:10.1016/j.gde.2004.12.005 (2005).

11 Barral, D. C. & Brenner, M. B. CD1 antigen presentation: how it works. *Nature Reviews Immunology* **7**, 929-941, doi:10.1038/nri2191 (2007).

12 Berdasco, M. & Esteller, M. Aberrant Epigenetic Landscape in Cancer: How Cellular Identity Goes Awry. *Dev Cell* **19**, 698-711, doi:10.1016/j.devcel.2010.10.005 (2010).

13 Bhowmick, N. A., Neilson, E. G. & Moses, H. L. Stromal fibroblasts in cancer initiation

and progression. *Nature* **432**, 332-337, doi:10.1038/nature03096 (2004).

14 Blasco, M. A. Telomeres and human disease: Ageing, cancer and beyond. *Nat Rev Genet* **6**, 611-622, doi:10.1038/nrg1656 (2005).

15 Borsig, L., Wong, R., Hynes, R. O., Varki, N. M. & Varki, A. Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 2193-2198, doi:10.1073/pnas.261704098 (2002).

16 Burkhart, D. L. & Sage, J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer* **8**, 671-682, doi:10.1038/nrc2399 (2008).

17 Carmeliet, P. VEGF as a key mediator of angiogenesis in cancer. *Oncology-Basel* **69**, 4-10, doi:10.1159/000088478 (2005).

18 Cerwenka, A. & Lanier, L. L. Natural killer cells, viruses and cancer. *Nature Reviews Immunology* **1**, 41-49, doi:Doi 10.1038/35095564 (2001).

19 Chavez-Galan, L., Arenas-Del Angel, M. C., Zenteno, E., Chavez, R. & Lascurain, R. Cell death mechanisms induced by cytotoxic lymphocytes. *Cell Mol Immunol* **6**, 15-25, doi:10.1038/cmi.2009.3 (2009).

20 Chin, K. *et al.* In situ analyses of genome instability in breast cancer. *Nature Genetics* **36**, 984-988, doi:10.1038/ng1409 (2004).

21 Cong, L. *et al.* Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* **339**, 819-823, doi:10.1126/science.1231143 (2013).

22 Cory, S. & Adams, J. M. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**, 647-656, doi:10.1038/nrc883 (2002).

23 Davies, M. A. & Samuels, Y. Analysis of the genome to personalize therapy for melanoma. *Oncogene* **29**, 5545-5555, doi:10.1038/onc.2010.323 (2010).

24 de Visser, K. E., Eichten, A. & Coussens, L. M. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* **6**, 24-37, doi:10.1038/nrc1782 (2006).

25 DeNardo, D., Andreu, P. & Coussens, L. M. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metast Rev* **29**, 309-316, doi:10.1007/s10555-010-9223-6 (2010).

26 Deshpande, A., Sicinski, P. & Hinds, P. W. Cyclins and cdks in development and cancer: a perspective. *Oncogene* **24**, 2909-2915, doi:10.1038/sj.onc.1208618 (2005).

27 Dougan, M. & Dranoff, G. Immune therapy for cancer. *Annual review of immunology* **27**, 83-117, doi:10.1146/annurev.immunol.021908.132544 (2009).

28 Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **3**, 991-998, doi:DOI 10.1038/ni1102-991 (2002).

29 Dunn, G. P., Old, L. J. & Schreiber, R. D. The three Es of cancer immunoediting. *Annual review of immunology* **22**, 329-360, doi:10.1146/annurev.immunol.22.012703.104803 (2004).

30 Esteller, M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* **8**, 286-298, doi:10.1038/nrg2005 (2007).

31 Evan, G. & Littlewood, T. A matter of life and cell death. *Science* **281**, 1317-1322 (1998).

32 Feron, O. Pyruvate into lactate and back: From the Warburg effect to symbiotic energy fuel exchange in cancer cells. *Radiother Oncol* **92**, 329-333, doi:10.1016/j.radonc.2009.06.025 (2009).

33 Ferrara, N. Vascular Endothelial Growth Factor. *Arterioscl Throm Vas* **29**, 789-791, doi:10.1161/Atvbaha.108.179663 (2009).

34 Fu, Y. F. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature biotechnology* **31**, 822-+, doi:10.1038/nbt.2623 (2013).

35 Gabhann, F. M. & Popel, A. S. Systems Biology of Vascular Endothelial Growth Factors. *Microcirculation* **15**, 715-738, doi:Pii 794659619
10.1080/10739680802095964 (2008).

36 Gabhann, F. M. & Popel, A. S. Systems Biology of Vascular Endothelial Growth Factors. *Microcirculation* **15**, 715-738, doi:Pii 794659619
10.1080/10739680802095964 (2008).

37 Galluzzi, L. & Kroemer, G. Necroptosis: A Specialized Pathway of Programmed Necrosis. *Cell* **135**, 1161-1163, doi:10.1016/j.cell.2008.12.004 (2008).

38 Garneau, J. E. *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67-+, doi:10.1038/nature09523 (2010).

39 Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **109**,

E2579-E2586, doi:10.1073/pnas.1208507109 (2012).

40 Gilbert, L. A. *et al.* Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* **159**, 647-661, doi:10.1016/j.cell.2014.09.029 (2014).

41 Gilbert, L. A. *et al.* Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* **159**, 647-661, doi:10.1016/j.cell.2014.09.029 (2014).

42 Godfrey, D. I., MacDonald, H. R., Kronenberg, M., Smyth, M. J. & Van Kaer, L. NKT cells: what's in a name? *Nat Rev Immunol* **4**, 231-237, doi:10.1038/nri1309 (2004).

43 Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, Inflammation, and Cancer. *Cell* **140**, 883-899, doi:10.1016/j.cell.2010.01.025 (2010).

44 Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364, doi:Doi 10.1016/S0092-8674(00)80108-7 (1996).

45 Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).

46 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).

47 Horvath, P. & Barrangou, R. CRISPR/Cas, the immune system of bacteria and archaea. *Science* **327**, 167-170, doi:10.1126/science.1179555 (2010).

48 Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature biotechnology* **31**, 827-+, doi:10.1038/nbt.2647 (2013).

49 Hynes, N. E. & MacDonald, G. ErbB receptors and signaling pathways in cancer. *Current opinion in cell biology* **21**, 177-184, doi:10.1016/j.ceb.2008.12.010 (2009).

50 Jackson, S. P. & Bartek, J. The DNA-damage response in human biology and disease. *Nature* **461**, 1071-1078, doi:10.1038/nature08467 (2009).

51 Jao, L. E., Wente, S. R. & Chen, W. B. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 13904-13909, doi:10.1073/pnas.1308335110 (2013).

52 Jiang, B. H. & Liu, L. Z. PI3K/PTEN signaling in angiogenesis and tumorigenesis. *Advances in cancer research* **102**, 19-65, doi:10.1016/S0065-230X(09)02002-8 (2009).

53 Jinek, M. *et al.* A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **337**, 816-821, doi:10.1126/science.1225829 (2012).

54 Jinek, M. *et al.* A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive

Bacterial Immunity. *Science* **337**, 816-821, doi:10.1126/science.1225829 (2012).

55 Jones, P. A. & Baylin, S. B. The epigenomics of cancer. *Cell* **128**, 683-692, doi:10.1016/j.cell.2007.01.029 (2007).

56 Junttila, M. R. & Evan, G. I. p53-a Jack of all trades but master of none. *Nature Reviews Cancer* **9**, 821-829, doi:10.1038/nrc2728 (2009).

57 Karnoub, A. E. & Weinberg, R. A. Chemokine networks and breast cancer metastasis. *Breast Dis* **26**, 75-85 (2006).

58 Karo, J. M., Schatz, D. G. & Sun, J. C. The RAG Recombinase Dictates Functional Heterogeneity and Cellular Fitness in Natural Killer Cells. *Cell* **159**, 94-107, doi:10.1016/j.cell.2014.08.026 (2014).

59 Karre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. Selective Rejection of H-2-Deficient Lymphoma Variants Suggests Alternative Immune Defense Strategy. *Nature* **319**, 675-678, doi:DOI 10.1038/319675a0 (1986).

60 Kazerounian, S., Yee, K. O. & Lawler, J. Thrombospondins in cancer. *Cell Mol Life Sci* **65**, 700-712, doi:10.1007/s00018-007-7486-z (2008).

61 Kennedy, K. M. & Dewhirst, M. W. Tumor metabolism of lactate: the influence and therapeutic potential for MCT and CD147 regulation. *Future Oncol* **6**, 127-148, doi:10.2217/Fon.09.145 (2010).

62 Kim, R., Emi, M. & Tanabe, K. Cancer immunoediting from immune surveillance to immune escape. *Immunology* **121**, 1-14, doi:10.1111/j.1365-2567.2007.02587.x (2007).

63 Koike-Yusa, H., Li, Y., Tan, E. P., Velasco-Herrera Mdel, C. & Yusa, K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nature biotechnology* **32**, 267-273, doi:10.1038/nbt.2800 (2014).

64 Konermann, S. *et al.* Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **517**, 583-U332, doi:10.1038/nature14136 (2015).

65 Krzewski, K. & Strominger, J. L. The killer's kiss: the many functions of NK cell immunological synapses. *Current opinion in cell biology* **20**, 597-605, doi:10.1016/j.ceb.2008.05.006 (2008).

66 Lemmon, M. A. & Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117-1134, doi:10.1016/j.cell.2010.06.011 (2010).

67 Li, P. *et al.* Reprogramming of T Cells to Natural Killer-Like Cells upon Bcl11b Deletion.

Science **329**, 85-89, doi:10.1126/science.1188063 (2010).

68 Lipowska-Bhalla, G., Gilham, D. E., Hawkins, R. E. & Rothwell, D. G. Targeted immunotherapy of cancer with CAR T cells: achievements and challenges. *Cancer Immunol Immun* **61**, 953-962, doi:10.1007/s00262-012-1254-0 (2012).

69 Liu, L. *et al.* Comparison of Next-Generation Sequencing Systems. *J Biomed Biotechnol*, doi:Artn 251364.10.1155/2012/251364 (2012).

70 Liu, P. T., Li, P. & Burke, S. Critical roles of Bcl11b in T-cell development and maintenance of T-cell identity. *Immunol Rev* **238**, 138-149, doi:10.1111/j.1600-065X.2010.00953.x (2010).

71 Lowe, S. W., Cepero, E. & Evan, G. Intrinsic tumour suppression. *Nature* **432**, 307-315, doi:10.1038/nature03098 (2004).

72 Mali, P. *et al.* CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nature biotechnology* **31**, 833-+, doi:10.1038/nbt.2675 (2013).

73 Mali, P. *et al.* RNA-Guided Human Genome Engineering via Cas9. *Science* **339**, 823-826, doi:10.1126/science.1232033 (2013).

74 Mathew, R., Karantza-Wadsworth, V. & White, E. Role of autophagy in cancer. *Nature Reviews Cancer* **7**, 961-967, doi:DOI 10.1038/nrc2254 (2007).

75 Mellman, I., Coukos, G. & Dranoff, G. Cancer immunotherapy comes of age. *Nature* **480**, 480-489, doi:10.1038/nature10673 (2011).

76 Mescher, M. F. *et al.* Molecular basis for checkpoints in the CD8 T cell response: Tolerance versus activation. *Semin Immunol* **19**, 153-161, doi:10.1016/j.smim.2007.02.007 (2007).

77 Mojica, F. J. M., Diez-Villasenor, C., Garcia-Martinez, J. & Almendros, C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiol-Sgm* **155**, 733-740, doi:10.1099/mic.0.023960-0 (2009).

78 Mougiakakos, D., Choudhury, A., Lladser, A., Kiessling, R. & Johansson, C. C. Regulatory T cells in cancer. *Advances in cancer research* **107**, 57-117, doi:10.1016/S0065-230X(10)07003-X (2010).

79 Nagy, J. A., Chang, S. H., Shih, S. C., Dvorak, A. M. & Dvorak, H. F. Heterogeneity of the Tumor Vasculature. *Semin Thromb Hemost* **36**, 321-331, doi:10.1055/s-0030-1253454 (2010).

80 Nagy, J. A. & Dvorak, H. F. Heterogeneity of the tumor vasculature: the need for new tumor blood vessel type-specific targets. *Clin Exp Metastas* **29**, 657-662, doi:10.1007/s10585-012-9500-6 (2012).

81 Nelson, B. H. The impact of T-cell immunity on ovarian cancer outcomes. *Immunol Rev* **222**, 101-116, doi:DOI 10.1111/j.1600-065X.2008.00614.x (2008).

82 Olive, K. P. *et al.* Inhibition of Hedgehog Signaling Enhances Delivery of Chemotherapy in a Mouse Model of Pancreatic Cancer. *Science* **324**, 1457-1461, doi:10.1126/science.1171362 (2009).

83 Ostrand-Rosenberg, S. & Sinha, P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* **182**, 4499-4506, doi:10.4049/jimmunol.0802740 (2009).

84 Pages, F. *et al.* Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* **29**, 1093-1102, doi:10.1038/onc.2009.416 (2010).

85 Pahl, J. & Cerwenka, A. Tricking the balance: NK cells in anti-cancer immunity. *Immunobiology*, doi:10.1016/j.imbio.2015.07.012 (2015).

86 Perez, E. E. *et al.* Establishment of HIV-1 resistance in CD4(+) T cells by genome editing using zinc-finger nucleases. *Nature biotechnology* **26**, 808-816, doi:10.1038/nbt1410 (2008).

87 Perona, R. Cell signalling: growth factors and tyrosine kinase receptors. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico* **8**, 77-82 (2006).

88 Qian, B. Z. & Pollard, J. W. Macrophage Diversity Enhances Tumor Progression and Metastasis. *Cell* **141**, 39-51, doi:10.1016/j.cell.2010.03.014 (2010).

89 Ran, F. A. *et al.* Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity (vol 154, pg 1380, 2013). *Cell* **155**, 479-480, doi:10.1016/j.cell.2013.09.040 (2013).

90 Ran, F. A. *et al.* Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity (vol 154, pg 1380, 2013). *Cell* **155**, 479-480, doi:10.1016/j.cell.2013.09.040 (2013).

91 Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* **8**, 2281-2308, doi:10.1038/nprot.2013.143 (2013).

92 Raynaud, C. M. *et al.* DNA Damage Repair and Telomere Length in Normal Breast,

Preneoplastic Lesions, and Invasive Cancer. *Am J Clin Oncol-Canc* **33**, 341-345, doi:10.1097/COC.0b013e3181b0c4c2 (2010).

93 Ribeiro, V. S. *et al.* Cutting edge: Thymic NK cells develop independently from T cell precursors. *J Immunol* **185**, 4993-4997, doi:10.4049/jimmunol.1002273 (2010).

94 Rothenberg, E. V. Transcriptional control of early T and B cell developmental choices. *Annual review of immunology* **32**, 283-321, doi:10.1146/annurev-immunol-032712-100024 (2014).

95 Saleh-Gohari, N. & Helleday, T. Conservative homologous recombination preferentially repairs DNA double-strand breaks in the S phase of the cell cycle in human cells. *Nucleic Acids Res* **32**, 3683-3688, doi:10.1093/nar/gkh703 (2004).

96 Semenza, G. L. Tumor metabolism: cancer cells give and take lactate. *J Clin Invest* **118**, 3835-3837, doi:10.1172/JCI37373 (2008).

97 Shalem, O. *et al.* Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells. *Science* **343**, 84-87, doi:10.1126/science.1247005 (2014).

98 Shen, B. *et al.* Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nat Methods* **11**, 399-+, doi:10.1038/Nmeth.2857 (2014).

99 Sherr, C. J. & McCormick, F. The RB and p53 pathways in cancer. *Cancer Cell* **2**, 103-112 (2002).

100 Shields, J. D., Kourtis, I. C., Tomei, A. A., Roberts, J. M. & Swartz, M. A. Induction of Lymphoidlike Stroma and Immune Escape by Tumors That Express the Chemokine CCL21. *Science* **328**, 749-752, doi:10.1126/science.1185837 (2010).

101 Sigal, A. & Rotter, V. Oncogenic mutations of the p53 tumor suppressor: The demons of the guardian of the genome. *Cancer research* **60**, 6788-6793 (2000).

102 Smyth, M. J. *et al.* Activation of NK cell cytotoxicity. *Mol Immunol* **42**, 501-510, doi:10.1016/j.molimm.2004.07.034 (2005).

103 Smyth, M. J., Dunn, G. P. & Schreiber, R. D. Cancer immunosurveillance and immunoediting: The roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol* **90**, 1-50, doi:10.1016/S0065-2776(06)90001-7 (2006).

104 Smyth, M. J. *et al.* NKG2D function protects the host from tumor initiation. *J Exp Med* **202**, 583-588, doi:10.1084/jem.20050994 (2005).

105 Strauss, D. C. & Thomas, J. M. Transmission of donor melanoma by organ

transplantation. *Lancet Oncol* **11**, 790-796, doi:10.1016/S1470-2045(10)70024-3 (2010).

106 Sun, J. C., Ugolini, S. & Vivier, E. Immunological memory within the innate immune system. *Embo J* **33**, 1295-1303, doi:10.1002/embj.201387651 (2014).

107 Teng, M. W. L., Swann, J. B., Koebel, C. M., Schreiber, R. D. & Smyth, M. J. Immune-mediated dormancy: an equilibrium with cancer. *J Leukocyte Biol* **84**, 988-993, doi:10.1189/jlb.1107774 (2008).

108 Vajdic, C. M. & van Leeuwen, M. T. Cancer incidence and risk factors after solid organ transplantation. *International Journal of Cancer* **125**, 1747-1754, doi:10.1002/ijc.24439 (2009).

109 Vivier, E. *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44-49, doi:10.1126/science.1198687 (2011).

110 Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat Immunol* **9**, 503-510, doi:10.1038/ni1582 (2008).

111 Wakabayashi, Y. *et al.* Bcl11b is required for differentiation and survival of alpha beta T lymphocytes. *Nat Immunol* **4**, 533-539, doi:10.1038/ni927 (2003).

112 Wang, T., Wei, J. J., Sabatini, D. M. & Lander, E. S. Genetic Screens in Human Cells Using the CRISPR-Cas9 System. *Science* **343**, 80-84, doi:10.1126/science.1246981 (2014).

113 Warburg, O. On the origin of cancer cells. *Science* **123**, 309-314 (1956).

114 Warburg, O. On respiratory impairment in cancer cells. *Science* **124**, 269-270 (1956).

115 Wertz, I. E. & Dixit, V. M. Regulation of death receptor signaling by the ubiquitin system. *Cell Death Differ* **17**, 14-24, doi:10.1038/cdd.2009.168 (2010).

116 White, E. & DiPaola, R. S. The Double-Edged Sword of Autophagy Modulation in Cancer. *Clin Cancer Res* **15**, 5308-5316, doi:10.1158/1078-0432.Ccr-07-5023 (2009).

117 White, E., Karp, C., Strohecker, A. M., Guo, Y. X. & Mathew, R. Role of autophagy in suppression of inflammation and cancer. *Current opinion in cell biology* **22**, 212-217, doi:10.1016/j.ceb.2009.12.008 (2010).

118 Witsch, E., Sela, M. & Yarden, Y. Roles for growth factors in cancer progression. *Physiology* **25**, 85-101, doi:10.1152/physiol.00045.2009 (2010).

119 Yang, L., Pang, Y. L. & Moses, H. L. TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends Immunol* **31**, 220-227,

doi:10.1016/j.it.2010.04.002 (2010).

120 Young Rojahn, S. *Does Illumina Have the First $1,000 Genome?*, <http://www.technologyreview.com/news/523601/does-illumina-have-the-first-1000-genome/> (2014).

121 Yuan, T. L. & Cantley, L. C. PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**, 5497-5510, doi:10.1038/onc.2008.245 (2008).

122 Zee, Y. K. *et al.* Imaging angiogenesis of genitourinary tumors. *Nat Rev Urol* **7**, 69-82, doi:10.1038/nrurol.2009.262 (2010).

123 Zong, W. X. & Thompson, C. B. Necrotic death as a cell fate. *Gene Dev* **20**, 1-15, doi:10.1101/gad.1376506 (2006).

124 CRUK. *Worldwide cancer incidence statistics*, <http://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer/incidence#heading-Zero> (

125 Dvorak, H. F., Flier, J. & Frank, H. Tumors - Wounds That Do Not Heal - Similarities between Tumor Stroma Generation and Wound-Healing. *New Engl J Med* **315**, 1650-1659 (1986).

126 Wang, K., Grivennikov, S. & Karin, M. Innate Immunity, Inflammation and Colorectal Cancer. *Else Kroner Fresen S* **4**, 4-10, doi:10.1159/000346526 (2013).

127 Weinberg, R. A. *The Biology of Cancer*. 2nd edn, (Garland Science, 2013).