Adoptive T Cell Therapy of Breast Cancer: Defining and Circumventing Barriers to T Cell Infiltration in the Tumour Microenvironment

by

Michele Martin
B.Comm., University of Toronto, 1990
DVM, University of Guelph, 2000

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SUPERVISORY COMMITTEE

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ABSTRACT

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In the era of personalized cancer treatment, adoptive T cell therapy (ACT) shows promise for the treatment of solid cancers. However, partial or mixed responses remain common clinical outcomes due to the heterogeneity of tumours. Indeed, in many patients it is typical to see a response to ACT in one tumour nodule, while others show little or no response. Thus, defining the tumour features that distinguish those that respond to ACT from those that do not would be a significant advance, allowing clinicians to identify patients that might benefit from this treatment approach.

The first chapter of this thesis provides the necessary background to understand the principals behind and components of ACT. This chapter also offers selected historical advances contributing to the current state of the field. The second chapter introduces a novel murine model of breast cancer developed to investigate the tumour-specific mechanisms associated with immune evasion in an ACT setting. The third chapter describes the *in vivo* characterization of mammary tumour cell lines derived from our mouse model that reliably showed complete, partial or no response to ACT. Using these cell lines, we were able to characterize *in vivo* tumour-specific differences in cytotoxic T cell trafficking, infiltration, activation, and proliferation associated with response to ACT. In the fourth chapter, we used bioinformatics approaches to develop a preliminary predictive gene signature associated with response to ACT in our mammary tumour model. We used this signature to predict outcome and then test a number of

murine mammary tumours *in vivo*, with promising results, wherein 50% of tumours responded to ACT as predicted based upon gene expression. Thus, using an innovative model for breast cancer, these results suggest that there are tumour-specific features that can be used *a priori* to predict how a tumour will respond to adoptive T cell therapy. Importantly, these findings might facilitate the design of immunotherapy trials for human breast cancer.

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My family and friends have tolerated my ridiculous schedule for the past five years, and deserve acknowledgement for their support. They fed me, housed me, dried my tears in times of frustration and celebrated with me in times of success (even if they didn't quite understand what it was all about). In particular, my father, stepmother, sister and brother have given me encouragement throughout this process. Dr. Duane Robinson has been there every step of the way, and knows all too well the weight of the PhD world on one's shoulders. Andrea Ratcliff has

always been so proud of the work that I do, and has bent over backwards to help me on innumerable occasions. My surrogate parents, Jack and Bev Poulter, have held me up when I could no longer stand on my own. My disappointment is that Bev did not live to see the completion of this work. Christopher Malloy knows better than most what has been sacrificed for this work. I have a deep appreciation for his love and support, and still marvel at how well he was able to time the expression, 'That's science, Michele!'

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DEDICATION

For Dr. Debbie James and the women like her, whose personal battles against breast cancer are humbling and inspiring. Their courage has given new meaning to my work.

CHAPTER 1: Introduction

1.1 Prologue

Adoptive T cell therapy (ACT) is an immunotherapeutic technique that uses one or more infusions of *ex vivo* expanded, autologous, antigen-specific cytotoxic T lymphocytes (CTLs) for cancer patients. The expectation is that these lymphocytes will recognize tumour antigens and effect tumour cell apoptosis through a variety of molecular mechanisms. An advantage to this treatment is in the use of a 'personalized' approach; the patient's own immune cells are harnessed to be used as a treatment for the patient's own tumour, with the potential to provide long lasting protection from recurrence with potentially fewer side effects. This treatment has demonstrated remarkable clinical outcomes, particularly in the setting of malignant melanoma, providing support for the 'personalized medicine' approach to cancer treatment. However, in spite of these clinical successes, there remain significant challenges that must be addressed before this treatment modality can be used more broadly in the setting of solid tumours. This thesis explores the use of ACT in a human epidermal growth factor receptor 2- overexpressing ($HER2^+$) breast cancer setting using a novel preclinical transgenic mouse model, with the specific goal of uncovering and circumventing some of the physical and molecular barriers that might otherwise limit the effectiveness of ACT.

First, this chapter will introduce the components associated with ACT, and put those components in the context of $HER2^+$ breast cancer. This will provide a background for the experimental work described in later chapters and provide perspective for the importance of exploring new treatment options for this particular disease. The second half of this chapter will provide an historical perspective on ACT using selected highlights of important advances in the decades ranging from the 1950s to the present day. This will provide context for the experimental questions and hypotheses explored in the following chapters. Finally, the potential

directions for this work and ACT in general will be discussed as a springboard for future experimental opportunities.

1.2 Adoptive cell therapy: the components

In order to best appreciate the elegance of the ACT technique, it is important to understand the components of the treatment, and the general steps involved in the process.

1.2.1 Cytotoxic T lymphocytes: the critical input

Cytotoxic T lymphocytes (CTLs) are alternatively known as $CD8^+$ 'killer' T cells. They are derived from hematopoietic stem cells, which give rise to a common lymphoid progenitor. From the common lymphoid progenitor, T cell precursors migrate from the bone marrow and mature in the thymus (hence the name 'T' cell). Within the thymus, T cells undergo a series of maturation and selection processes wherein T cell receptors (TCRs) are synthesized and rearranged, and autoreactive ('self'-recognizing) T cells are ideally detected and deleted. Once maturation has completed, T cells destined to be CTLs can be identified using cell surface ('cluster of differentiation' – CD) marker conventions; CTLs are described as $CD3^+CD8^+$. Following maturation in the thymus, T cells migrate to the peripheral lymphoid organs, including the lymph nodes and the spleen (1, 2)

The process of CTL activation within the peripheral lymphoid organs requires contact between the 'naïve' CTL and a professional antigen presenting cell (APC, e.g. dendritic cell) (1, 3). Prior to their arrival in the peripheral lymphoid organs, APCs are found throughout the body's tissues, engulfing the physiological and pathological debris ('antigens') associated with cellular death and turnover. After this debris is engulfed by the APC, it is processed through normal intracellular degradation pathways into small (8-15 amino acid) fragments (epitopes). These fragments are then positioned uniquely into the cleft of a major histocompatibility complex (MHC) molecule within the endoplasmic reticulum (ER). From the endoplasmic reticulum, the

peptide-MHC complex is transported to the cell surface, where it remains for presentation to immune cells, including CTLs. Importantly, CTLs will recognize APCs with specific 8-10 amino acid peptide fragments in the context of MHC Class I (MHC I) molecules on their cell surface (2, 3). As would be expected, a single engulfed protein can be presented to immune cells through a large number of potential epitopes. The term ‘dominant epitope’ is generally used to describe an epitope that is recognized by immune cells during a natural infection, or following immunization with whole intact antigens. Under normal circumstances, these recognition of these epitopes will trigger an immune response (4). By contrast, the term ‘subdominant epitope’ is generally used to describe an epitope to which immune cells will not normally respond, but nevertheless can be immunogenic, and occur as the result of normal antigen processing. (5). If the CTL makes contact with an APC presenting an appropriate, dominant epitope, ligation of signaling receptors (costimulatory factors), downstream signaling and transcription occurs leading to the development of an activated CTL (6). Activation results in both expansion and further differentiation leading to an expanded, clonal population of CTLs bearing the same TCR, and ‘armed’ with the molecular capacity to effect cytotoxicity (1, 2).

In response to a number of activation-induced molecular changes, CTLs will proceed through a cascade of events eventually resulting in a population of differentiated, armed CTLs migrating into the peripheral tissue. Soluble signals including chemokines and cytokines will direct their movement toward the relevant tissue site, dictated by the initial encounter with the APC and cognate antigen (7). Upon encounter with cognate antigen within the appropriate MHC I context, the armed CTL affects cytotoxicity through induction of apoptosis as the result of the release of preformed cytolytic granules (perforin, granzymes), or via ligation of programmed cell death receptors (e.g. Fas/Fas Ligand). Importantly, in contrast to the naïve state, activated CTLs do not require further costimulation to effect cytotoxicity at this point. Notably, the effects of the release of cytotoxic granules can also affect neighboring cells as a so-called ‘bystander effect’,

thereby altering the local population of both normal and abnormal cells. If tumour cells have not thwarted the immune response process by this time, the cycle is then completed as phagocytic cells arrive to clean up the debris, and provide material for inspection by naïve or activated CTLs back in the lymph node.

While this describes the ideal sequence of events that would occur if the CTL recognizes that a tumour is 'foreign', it is clear that there are myriad opportunities for the CTL immune response to fail, resulting in immune evasion by a tumour. Therefore, identifying the potential pitfalls and potential solutions is critical to provide an opportunity to successfully use CTLs for cancer therapy. Early in the immune response in the draining lymph node, failure by APCs to provide sufficient costimulatory signals to naïve CTLs will result in failed activation (8, 9). To circumvent this problem, genetically modified T cells have been developed with the ability to become activated in spite of insufficient costimulatory signaling by APCs. For example, T cells lacking the cbl-b gene (*cbl-b*$^{-/-}$) do not require costimulation through ligation of CD28 to become activated and proliferate (10-12). In these T cells, proliferation is more robust and is sustained longer than in their wild type counterparts, and in preclinical studies in our laboratory, they have been shown to improve tumour response in an ACT model (13). Similarly, T cell chimeric antigen receptors (CARs) have been developed that fuse the antigen recognition component of antibodies at the cell surface to the intracellular signaling component of T cells as a means to circumvent a variety of failures in antigen presentation by APCs (14, 15). There is a great deal of enthusiasm to use genetically modified T cells to augment ACT, however conferring this auto-activating capability to T cells is not without risk. A recent report detailed serious adverse effects and death in a patient following ACT with CAR T cells (16). Clearly, this approach holds promise but it does not necessarily address immune evasion that occurs downstream from the activation point in the lymph node.

If the CTL is successfully activated within the lymph node, it must then navigate to the tumour site to affect cytotoxicity. Physical impedance of CTL trafficking can occur as a result of architectural features unique to the tumour environment (17). First, the finely regulated molecular steps involved in extravasation might be hampered as a result of alterations in the expression of endothelial cell adhesion molecules, including intercellular adhesion molecule-1 (ICAM1) and vascular cell adhesion molecule-1 (VCAM1) within the tumour vasculature (18, 19). Interaction by the T cell with these molecules is required for successful extravasation. Even if CTLs are successful in transendothelial migration, they still must traverse through the interstitium of the tumour. Here they may encounter additional physical obstructions, including an impassable tumour stroma. Several studies investigating the immune response to tumours have documented CTL infiltration failure, and the possible role of stromal cells in this context (20-24). These two examples of post-activation immune evasion present opportunities to target tumour 'support' structures as a means to circumvent tumour-induced local barriers. Antibodies against vascular endothelial growth factor (VEGF) can be used to inhibit the growth of new tumour vasculature and normalize existing aberrant tumour vessels (25, 26). This process might result in more effective CTL extravasation at the site of the tumour. Hans Schreiber's group has demonstrated that targeting non-malignant stroma with CTLs can result in tumour regression in the absence of tumour antigen-specific CTLs, suggesting that tumour stroma may be an unwitting participant in immune evasion (27-29). Thus, there are benefits to directing effort towards addressing immune evasion where it occurs within the tumour infrastructural support.

Upon the successful migration of the activated CTL to the tumour microenvironment, localized immunosuppression can result from the secretion of soluble molecules by immunosuppressive cells, including tumour-associated macrophages (TAMs) and regulatory ($CD4^{+}CD25^{+)}$ T cells (TRegs) (30-35). TAMs are induced to secrete arginase locally, which results in the induction of anergy in CTLs via alterations in the T cell receptor recycling pathway (36-39). Interestingly, this

suppression is reversible and there is interest in investigating this mechanism as a means to augment the patient's immune response in a variety of tumour settings (40-42). Similarly, TRegs secrete the immunosuppressive cytokines transforming growth factor β (TGFβ) and interleukin-10 (IL-10), which locally inhibit CTLs from affecting cytotoxicity (43-45). The influence of TRegs in the settings of melanoma, breast, lung, ovarian, and renal cancer is being studied in pre-clinical and clinical trials investigating the role of anti-CD25 antibodies in restoring CTL activity at the local level (34, 46-49). Because the CD25 molecule forms part of the IL-2 receptor on the surface of TRegs, by binding this molecule, anti-CD25 antibodies inhibit IL-2 signaling within the TReg, thereby inducing apoptosis. Thus, opportunities to address and circumvent tumour-induced immunosuppression at the local microenvironmental level have been identified. Given that each tumour potentially has several unique immune evasion tactics, it is unlikely that a single approach will be successful in ensuring CTLs can find and destroy their targets. Nevertheless, encouraging advances are being made to address known or induced deficiencies in CTL activation, migration and infiltration to improve clinical outcomes using ACT.

1.2.2 An immunogenic tumour: the target

As described above, the function of CTLs is to recognize 'foreign' antigens, then to seek and destroy the cells that express these antigens. As such, if CTLs are to be used as a means to destroy a tumour, that tumour must be recognized as 'non-self' in some way. Ideally, the antigen would be unique to the abnormal tumour tissue and not expressed in any normal tissue ('mutated' antigens). More commonly, tumour antigens are either expressed at varying levels in the normal tissue of origin of the malignancy (so-called 'shared-specific' and 'differentiation' antigens) or normally, but at low levels (so-called 'overexpressed' antigens) (Table 1). Extensive peptide databases exist describing T cell epitopes associated with tumour antigens representing each of these examples (50-52).

Tumour Antigen	Category of Antigen	Example Tumour Sites
Bcr-Abl	Mutated	Chronic myeloid leukemia
MART2	Mutated	Melanoma
TGFβRII	Mutated	Colorectal carcinoma
p53	Mutated	Head & neck squamous cell carcinoma
MAGE-A1	Shared - Specific	Melanoma, lung, colorectal
NY-ESO-1	Shared - Specific	Lung, breast, ovarian
TRP2	Shared - Specific	Melanoma
CEA	Differentiation	Gut carcinoma
mammaglobin-A	Differentiation	Breast
PSA	Differentiation	Prostate
tyrosinase	Differentiation	Melanoma
gp100	Differentiation	Melanoma
HER2	Overexpressed	Breast, ovarian
EphA3	Overexpressed	Melanoma
cyclin D1	Overexpressed	Breast, lung, kidney, leukemia
Bcl-xl	Overexpressed	Prostate, colon, bladder

Table 1. Examples of tumour-associated antigens and typical tumour sites with described antigen expression.

Mutated antigens represent antigens that have tumour-specific expression, and in the mutated form are not expressed in normal tissue. ***Shared – specific*** antigens are expressed only in tumours, placental and testicular germ cells (also known as cancer/testis or **CT** antigens). ***Differentiation*** antigens are expressed in normal tissues from which tumours are derived and hence are not tumour-specific. ***Overexpressed*** antigens are expressed at varying levels in normal tissues, and high levels in tumour tissue. Note: this list is not comprehensive. Adapted from (50).

The immunogenic nature of the tumour is important for a second reason in the setting of ACT. More recently, autologous tumour tissue has become an important and preferred source of activated CTLs to be used in *ex vivo* T cell expansion regimens (53-58). While in early experiments, circulating peripheral CTLs were harvested and expanded to larger numbers, now it is standard practice to obtain tumour-infiltrating lymphocytes (TIL) from tumour tissue harvested surgically for use in *ex vivo* expansion. Thus, this approach provides a potential population of antigen-educated CTLs that have already demonstrated *in vivo* their sensitivity to tumour antigen and the ability to migrate to the site of the malignancy.

A major limitation to using tumours with shared or overexpressed tumour antigens in an ACT treatment regimen is the risk of unintended on-target cytotoxicity. For example, where an antigen is expressed at any level in normal tissue (e.g. HER2 is expressed at low levels in normal lung), targeting a tumour expressing that antigen may result in cytotoxicity in the normal tissue (16). Thus, consideration must be given to the effects of ACT on all tissues that might be expressing the targeted antigen of interest.

1.2.3 HER2 overexpressing breast cancer: the setting

Human epidermal growth factor receptor 2 (HER2, also known as ErbB2, HER2/neu) is one member of the epidermal growth factor (EGF) family of receptor tyrosine kinases. The three other members of this family are HER1 (EGFR/ErbB1), HER3 (ErbB3) and HER4 (ErbB4) (59-63). HER2 is a transmembrane glycoprotein with extracellular, transmembrane, and intracellular tyrosine kinase and regulatory domains (59, 64) (Figure 1). This protein is normally expressed at low levels in the breast as well as in many other epithelial tissues including lung, ovary, gut and skin (65-68). Upon ligand binding, HER2 dimerizes with other members of the HER family (HER1, 3 or 4) as a heterodimer, or with another HER2 molecule as a homodimer (59). Dimerization results in phosphorylation of intracellular tyrosine kinase (TK) domains, activating intracellular signaling pathways associated with cellular proliferation (MAP kinase) and survival

(PI3 Kinase/AKT) via adaptor proteins including Shc and Grb2 (59, 62, 63). The specific physiological ligand that binds HER2 has not been identified, and might not exist since specific HER2 ligation is not required to induce dimerization with its partners (HER1, 3 or 4).

The HER2 gene (proto-oncogene) is located on the long arm of chromosome 17 (17q12-q21) in humans (59, 60, 62, 69). Overexpression of HER2 at the mRNA and protein levels occurs due to gene amplification or dysregulation of transcription (59); and overexpression alone is sufficient to induce carcinogenesis in the breast (62, 63). Increased expression of HER2 leads to an increase in dimerization-associated signaling, and therefore cell proliferation with reduced apoptotic pressure. Where a sufficient density of HER2 molecules occurs on the cell surface, ligand-independent signaling can occur via spontaneous receptor-receptor interactions. Therefore, even with growth factor withdrawal, excessive proliferation and apoptotic resistance can still occur with HER2 overexpression.

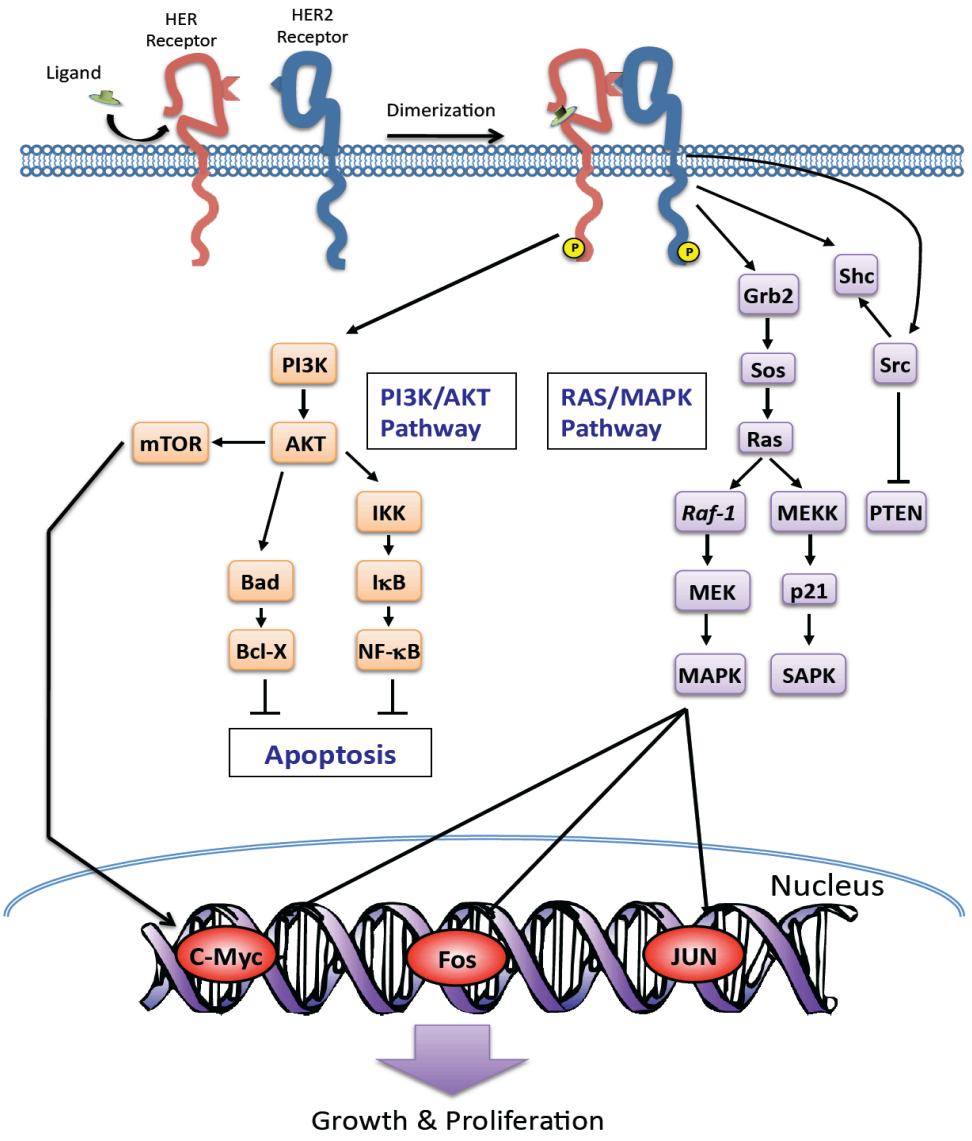

Figure 1. Signaling by the HER2 protein.

Upon ligand binding, HER2 dimerizes with other members of the HER family (HER1, 3 or 4) as a heterodimer, or with another HER2 molecule as a homodimer. Dimerization results in phosphorylation of intracellular tyrosine kinase (TK) domains, activating intracellular signaling pathways associated with cellular proliferation (MAP kinase) and survival (PI3 Kinase/AKT) via adaptor proteins including Shc and Grb2, and nuclear transcription factors including c-Myc, Fos and Jun. (Adapted from (59)).

HER2 overexpression occurs in approximately 20-25% of primary breast tumours and is associated with poor prognosis (59, 70-72). Diagnosis of HER2 overexpressing ($HER2^+$) breast tumours is done using two main methods: fluorescent *in situ* hybridization (FISH), and immunohistochemistry (IHC) for HER2 protein (73). Depending upon other features of the breast tumour (including hormone receptor status, stage and grade), and pre-existing co-morbidities of the patient, treatments for $HER2^+$ breast cancer may include surgery, chemotherapy (anthracyclines e.g. epirubicin, taxanes e.g. paclitaxel, alkylating agents e.g. cyclophosphamide) and/or radiation. Since 1998, patients with $HER2^+$ tumours can additionally be treated with trastuzumab (Herceptin^{TM}), a humanized murine monoclonal antibody that binds the HER2 receptor and inhibits downstream signaling through multiple mechanisms (60, 63). The use of trastuzumab in combination with chemotherapy significantly improves disease-free survival (DFS) and overall survival (OS) in localized and metastatic $HER2^+$ breast tumours (70). Unfortunately, disease recurrence is common, and trastuzumab resistance has been described (60). Thus, a significant opportunity exists to further improve outcomes, particularly in patients for whom trastuzumab is ineffective or for whom anthracyclines or other chemotherapeutics are contraindicated.

T cells specific for the HER2 protein are detectable in patients with $HER2^+$ cancers (5, 74, 75) indicating that HER2 is recognized by the immune system, yet does not induce a curative immune response to the tumour. Generally, these T cells are specific for ‘subdominant’ epitopes associated with the HER2 protein through an immune system developmental selection mechanism designed to prevent an inappropriate autoimmune response to this ‘self’ protein (4, 5, 76). Importantly, an effective cytotoxic response can be elicited from CTLs in response to several HER2-specific ‘dominant’ epitopes (75, 77-79), providing support for the idea of using the immune system to target $HER2^+$ breast cancer using ACT.

1.2.4 Adoptive cell therapy: the method

With the identification of an appropriately immunogenic tumour target, researchers can proceed to extract and expand appropriate CTLs to use therapeutically using the adoptive cell therapy technique. Based on current methods, tumour tissue is excised surgically, and a portion of this is provided to *in vitro* culture technicians and maintained following strict 'Good Manufacturing Practices' (GMP) standards (56). Tumour infiltrating lymphocytes (TIL) are extracted from tumour tissue using one of a number of methods, normally requiring enzymatic and mechanical separation of tumour tissue from other cells, resulting in a heterogeneous population of cells. Depending upon the chosen strategy, the heterogeneous population may be further separated using antibody-conjugated magnetic beads or Ficoll gradients. Additional testing may be undertaken on cell subcultures resulting from these initial steps to identify those with tumour specific activity for further expansion. Once the target population of CTLs has been identified, routine culture methods using (generally) standard T cell culture media are used to expand the populations. A variety of cytokine cocktails are described in the literature (including Interleukin 2 (IL-2), Interleukin 7 (IL-7), and Interleukin 15 (IL-15)), and these are required to sustain T cell growth and activation (54, 80-84). CD3 and CD28 antibodies are used as surrogates for *in-vivo* costimulatory signals and are also included in the mixture (85, 86). The term 'feeder cells' is used to describe an *in vitro* source of growth factors and may also represent a source of antigen presentation cells (87). Often, feeder cells are autologous peripheral blood mononuclear cells (PBMCs), and added to the *in vitro* culture mixture to maintain CTLs in their 'activated' phenotype during the expansion process.

Following an expansion period (ranging from 5-20 weeks), tumour-specific autologous CTLs (on average 10^8-10^{10}) are prepared and transfused intravenously to the patient. The recipient patient may or may not have received lymphodepleting chemotherapy or radiation therapy as part of a preconditioning regimen, depending upon the clinical approach and the patient's condition (85,

88-90). Unless contraindicated, ACT patients usually receive varying doses of systemic IL-2 following transfusion in an attempt to sustain transfused CTLs over an extended period (90-96). The expansion and transfusion procedure may be repeated, depending on the success of the initial expansion process, the condition of the patient, and the clinical approach (Figure 2).

Side effects associated with ACT can range from mild (low grade fever, nausea) to severe (capillary leak syndrome, multiple organ failure, septicemia), and are usually associated with the use of IL-2 (97). As current ACT regimens normally use high dose IL-2, systemic side effects including fever, chills, nausea, vomiting and diarrhea are most common (98, 99). At the highest tolerated doses of IL-2, hypotension and vascular leakage syndrome (potentially resulting in respiratory and liver failure) are of greatest concern. With the exception of the most severe cases, IL-2 related side effects resolve following cessation of treatment. As noted above, on- or off-target effects can also be seen and are associated with CTL activity. For example, melanoma patients can experience post-ACT vitiligo (loss of skin pigmentation) associated with CTLs targeting normal melanocytes (100, 101). Of greater concern, when ocular (uveal) melanocytes are targeted by CTLs, this can result in painful acute uveitis and blindness in post-ACT melanoma patients (85, 102). Therefore, while side effects generally resolve, they can be life threatening and must be considered in the context of a patient's co-morbidities.

It should be noted that patients receiving ACT are usually those with advanced, metastatic tumours for which standard treatments have failed. Usually these patients have experienced one or more surgeries, and multiple rounds of chemotherapy and/or radiation treatments. While the practice of ACT has been in existence for over 30 years, it is not yet considered 'standard of care' in any tumour setting. Additional experience in this field may lead to a more broad application of this modality.

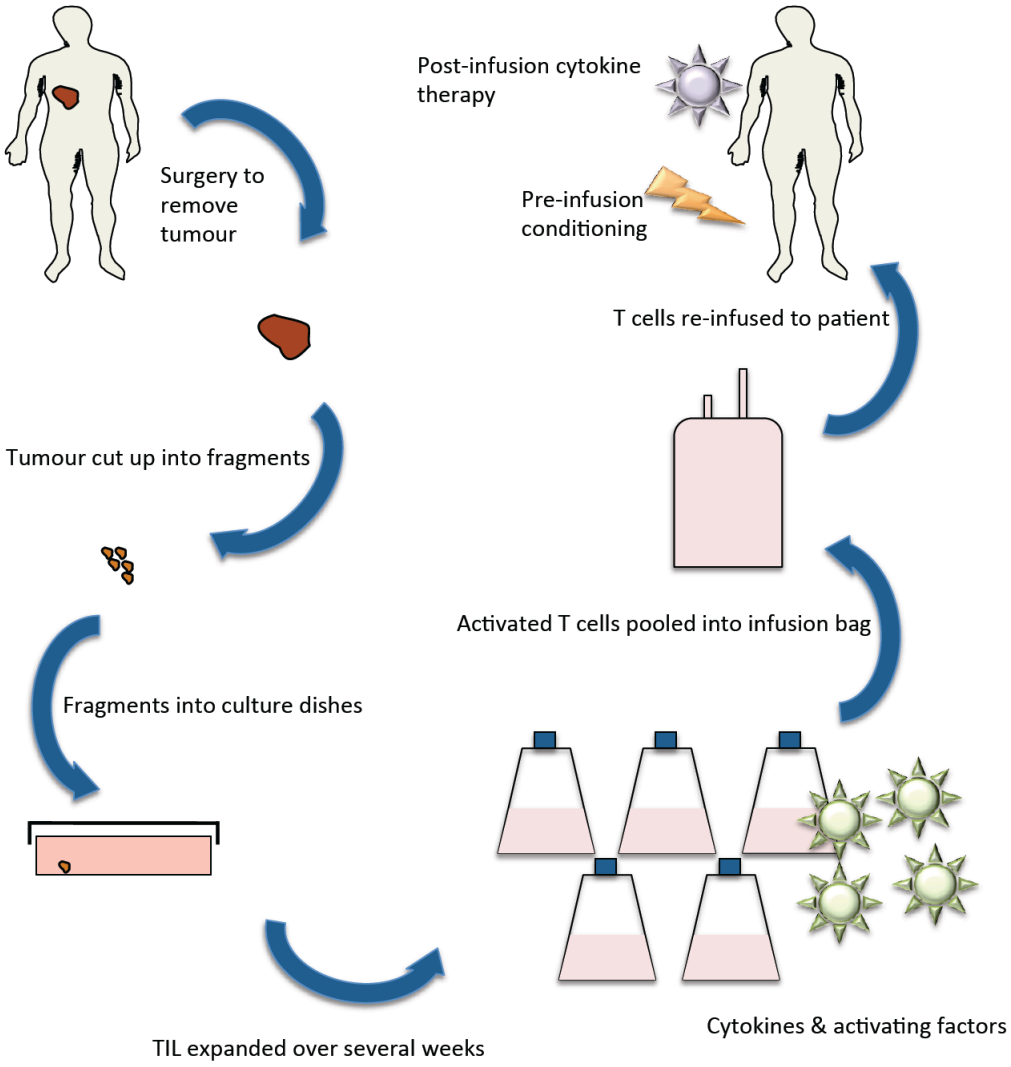

Figure 2. Schematic diagram of adoptive T cell therapy (ACT)

A patient's tumour is surgically removed, and all components (tumour cells, lymphocytes) are dissociated and placed into culture dishes. Over the course of 5-20 weeks, antigen-specific T cells are purified and expanded to very large numbers (10^8-10^{10}). Cytokines (e.g. IL-2, IL-15, IL-7) and costimulating antibodies (CD3, CD28) are used *in vitro* to induce and sustain T cell activation during the expansion phase. Once sufficient T cell numbers have been achieved, T cells are pooled into a single infusion bag and provided intravenously to the patient. The patient is preconditioned with chemotherapy and/or radiation therapy, and treated with IL-2 cytokine therapy following ACT. Adapted from L. Radvanyi (personal communication).

1.3 Adoptive cell therapy: a brief historical perspective

To appreciate the ACT methods currently used clinically and the future opportunities, an historical perspective highlighting specific advances is provided.

1.3.1 Pre-1960s: hints from the tissue transplant experience

The idea of using the immune system to 'reject' cancer in patients was born from the lessons learned in the realm of tissue and bone marrow transplantation. It is well documented that early experimental attempts at solid organ transplant in humans in unrelated donor/recipient pairs commonly resulted in dismal failure: rejection of the organ and death of the patient. To explain this phenomenon, in the early 1950s, Peter Medawar, Rupert Billingham and colleagues performed skin grafting experiments in animals and humans and implicated the host immune system in transplant rejection, the so-called 'host versus graft' effect (103, 104). Mitchison and colleagues similarly used a murine tumour transplantation model to show that lymph node cells contained the immune components that mediated tissue rejection (105). With this knowledge, recipient immune suppression was proposed as a means to improve success rates in skin grafting experiments.

In the late 1950s, Murray and Merril were encouraged by the positive results seen in murine transplantation experiments using total body irradiation (TBI) as a means to immunosuppress tissue transplant recipients. Unfettered by modern human research ethics restrictions, Murray and Merrill used sub-lethal TBI to immunosuppress 10 non-identical human kidney transplant recipients. While 9 of these patients died within a month due to radiation toxicity, the remaining patient (a fraternal twin transplant recipient) survived without rejecting the transplanted kidney (106). This provided support for the host immune response as an agent for allograft rejection. Further, this set the stage for rapid advancements in the field of solid organ and bone marrow transplantation in parallel with critical discoveries in the arena of immunosuppressive pharmacology.

1.3.2 The 1960s: graft versus host disease and anti-tumour immunity

With researchers having learned how to circumvent the remarkable ability of the transplant recipient's immune system to reject 'foreign' tissue using systemic immunosuppression, transplant recipients of the 1960s now had a lower likelihood of rejecting non-identical transplanted tissue. However, a serious, systemic 'secondary syndrome', afflicting transplant recipients, first described in the 1950s, became better understood (107, 108). This syndrome manifested as moderate to severe skin rashes, gastrointestinal disturbances and liver disease, and occurred both early and late in the post-transplantation time frame (108). Intriguingly, in 1965 Mathé and colleagues found that leukemic mice treated with TBI and bone marrow transplantation that survived this 'secondary syndrome' were more likely to be cured from their spontaneous, transplanted, or virally-induced leukemias. To exploit these findings (again unfettered by modern human ethics restrictions), Mathé proceeded immediately to clinical trials using TBI and bone marrow transplantation in human patients with acute lymphoblastic leukemia (ALL). While 7 of 10 evaluable patients did not survive longer than one month post-irradiation and transplant (3 patients died due to radiation toxicity, 4 died due to the 'secondary syndrome'), the remaining patients achieved remission from their disease ranging from 5-11 months (108). From these and subsequent experiments it was concluded that the immune mechanisms associated with the 'secondary syndrome' and the anti-leukemic effect were related, and that immune system components were being 'transplanted' with the graft. 'Graft-versus-host disease' (GVHD) was proposed by Billingham in a landmark paper in 1966, with three requirements for its development: 1) the graft must contain immunologically competent cells; 2) the recipient must express tissue factors that are not present in the donor tissue and; 3) the recipient must be unable to mount an effective immune response against the donated cells (109, 110). While the finer details of the cellular participants and mechanisms involved in GVHD were still to be uncovered, it was clear that the opportunity to capitalize on this phenomenon in the setting of human cancer was being recognized by these and other groups.

During the same time period and building on animal studies in the 1950s and early 1960s (105, 111), Southam and colleagues experimented with terminal cancer patients harboring non-resectable solid tumours (112). Autologous tumour was harvested from the patients, and was either admixed *in vitro* with autologous leukocytes or maintained unaltered in culture for short periods. Cultured tissue was then re-implanted in the patients and tumour growth was monitored. They found that implanted tumour growth was inhibited when implants were admixed *in vitro* with autologous leukocytes, suggesting a tumour suppressive role for autologous leukocytes. Thus, pioneering experiments during these two decades supported the idea that 1) autologous mature immune cells (i.e. adoptive T cell therapy (ACT)) could be used to suppress or eradicate cancer and 2) allogeneic immune cells (e.g. via bone marrow transplantation) could be used to suppress or eradicate tumours.

Many questions surrounding the mechanisms of success and failure still existed, but to this point researchers using animal models for ACT were able to show that, in general, tumours were responsive to T cells in a dose-dependent manner (111). In the context of animal models, increasing T cell inputs simply involved the sacrifice of additional experimental animals; at this time the ability to culture and expand immune cells *in vitro* did not yet exist. Frustratingly, the means to identify the mechanism of the dose response and the means to obtain more T cell inputs from human patients remained as two major stumbling blocks in the way of advancing the use of immune cell therapy in the clinical setting.

Fortunately, the 1960s saw major advances in the understanding of immune cells. In particular, 'lymphocytes' - a subset of the white blood cell (WBC) compartment - were more clearly characterized as having an ability to proliferate *in vitro* in response to a variety of soluble and cellular reagents (e.g. phytohemagglutinin (PHA), tuberculin, allogeneic mixed lymphocytes) (113-115). Additionally, during this period PHA-stimulated lymphocytes were found to have the ability to produce a soluble substance that stimulated proliferation in other lymphocytes, thus

providing the first crude tools that could be used toward obtaining a greater number of immune cells *in vitro* that could be used therapeutically in humans.

1.3.3: The 1970s: providing sufficient T cell inputs

By the early 1970s, it was well known that the immune response to alloantigens seen in the transplant and tumour settings was characterized by the development of effector cells with specificity for those alloantigens. It was also known that those effector cells originated from the thymus-derived ('T') lymphocyte lineage, and these cells were by now referred to as 'cytotoxic T cells' (CTLs) (116-118). Reliable methods had been developed to perform *in vitro* cytotoxicity assays in a variety of contexts, and to keep CTLs in culture and proliferating for short periods of time (~5 days) (119, 120). These advances improved the ability to characterize the nature and kinetics of the CTL response following *in vivo* or *in vitro* sensitization of donor CTLs. By the mid-1970s, it was also well established that T lymphocytes could affect tumour regression *in-vivo* in animals (117, 121), and human tumour cell lysis *in vitro* (122, 123). Several clinical trials in humans described the use of adoptive cell therapy with T cells activated for short periods of time *in vitro* using PHA or autologous tumour with varying (yet generally low) response rates (124-127). By this time, ACT was also being combined with chemotherapy and specific and non-specific vaccination in an attempt to stimulate a greater endogenous immune response (125, 128). Clearly, enthusiasm for the ACT technique was recognized clinically, but limitations associated with CTL numbers for therapeutic use were seen as a roadblock to better clinical responses in humans.

At this time, the limited *in-vitro* lifespan of animal and human T cells limited experimental manipulations. Only Epstein-Barr virus (EBV)-positive or neoplastic T cell cultures could be sustained beyond 5-7 days, and these transformations presented a number of variables that confounded experimental interpretation. This was an important technical challenge that needed to be resolved. A major breakthrough in ACT occurred in 1976 when Morgan and colleagues

described a method of expanding and sustaining untransformed T cells in culture for longer periods of time (>9 months). To sustain and expand relatively pure (>90%) populations of T cells in culture, they used PHA-stimulated pooled human peripheral blood mononuclear cells (PBMCs) to condition T cell culture medium with as yet-undefined growth factors. Importantly, replenishment with this conditioned medium ('Ly-CM') was an absolute requirement to sustain lymphocyte proliferation *in vitro* in untransformed T cells beyond 3-5 days. Exponential growth phases proved to be cyclical, providing the means to systematically explore kinetics and function at various points along the growth curve (129). Thus, by establishing the ability to sustain and expand normal T cells over longer periods of time, it became possible to explore more opportunities associated with the use of CTLs in a human clinical setting.

While the precise nature of the 'growth factor' cocktail required for maintenance of T cells in culture remained uncharacterized, the ability to sustain T cells *in vitro* for longer periods of time would enable researchers to sensitize and expand T cells more intensively. With this in hand, the inevitable goal was to find a more effective T cell. The obvious first choice would likely be a T cell with specificity for a tumour antigen of interest. To this end, achieving CTL antigen specificity was studied by a number of groups in the 1970s. It was found that using a monolayer of antigen-pulsed, adherent 'peritoneal exudate cells' (PECs – now known to represent antigen presenting cells) resulted in high levels of antigen-specific T cell selection (119). This technical advance provided the important foundation for more effective antigen-specific T cell expansion methods in later decades.

1.3.4 The 1980s: seeking the 'optimal' T cell inputs

The 1980s provided more advanced molecular biology techniques for the sequencing of genes of proteins, and thus the mysteries underlying the 'growth factors/activating factors/blast factors' contained in the media derived from PHA-activated CTLs began to be uncovered in the 1980s (130-132). The roles and mechanisms of 'cytokines' (as they were now known) as they related to

T cell activation, proliferation, and cytotoxicity were being described in the setting of cancer and immunity in general (133, 134). Importantly, interleukin-2 (IL-2) was identified as an important T lymphocyte growth factor, and its successful synthesis provided a means to investigate its effects both *in-vitro* and *in vivo* in animal models of cancer as well as in human cancer patients (135).

Indeed, in 1984 Mazumder and Rosenberg used IL-2 - stimulated bulk naïve splenocytes from syngeneic mice to demonstrate the efficacy of ACT in the treatment of metastatic melanoma (136). Using both tumour prevention and tumour treatment approaches, they were able to demonstrate reduced tumour nodule formation and increased survival in a B16 murine melanoma model. Importantly, this study demonstrated that IL-2 stimulation was sufficient to produce cytotoxic T cells with tumouricidal properties from naïve precursors. To build on this, the Rosenberg group soon began studies using IL-2 in the *in vitro* T cell priming phase coupled with post-ACT systemic IL-2 treatment *in vivo*, with promising results in mouse models (137, 138). These studies provided support for the use of IL-2 cytokine therapy as part of the treatment regimen for melanoma, which remains in common use today. Thus, the use of IL-2 *in vitro* and *in vivo* was an important advance in the quest for a more effective T cell in the context of ACT.

While naïve splenocytes could be conditioned with IL-2 to affect tumour cytotoxicity in animal models, in the early-mid 1980s Rosenberg and other groups sought to define whether naïve T cells from peripheral blood (PBMCs) were in fact the best starting materials to use for ACT. To answer this question, tumour infiltrating lymphocytes (TIL) stimulated with IL-2 were investigated as alternative sources of starting materials in the *in vitro* and *in vivo* settings (139, 140), The hypothesis was that TIL were already tumour antigen ‘experienced’, and might provide a more directed and effective attack on the tumour. While in some models, TIL were superior to autologous PBMCs *in vivo* and *in vitro*, others found that TIL were inefficiently activated and had suppressed cytotoxic function (139, 141). These inconsistent findings supported the hypothesis

that the tumour environment might be immunosuppressive in some instances, but less so in others. In spite of these conflicting results, the allure of 'tumour-experienced' TIL was great, and interest in their potential for ACT grew.

Although much was already known about the concept of tumour-associated antigens by this time, a well-characterized armament of antigen–specific T cells was not yet available to use, either experimentally or clinically. In the quest for greater numbers of more effective T cells, Rosenberg and colleagues developed an *in vitro* method of cloning antigen–specific T cells in a mouse model using harvested tumour tissue as a source of antigen (142). These clones showed increased efficacy against established murine tumours, and when combined with IL-2 stimulation, the technique was proposed as a means to provide large numbers of tumour–specific cytotoxic T cells.

In 1987 Rosenberg and colleagues published results from a study building on this technique, wherein human melanomas were excised and used to isolate and expand TIL (up to ~$9x10^4$ fold) with specific cytotoxicity against autologous tumour *in vitro* (140). By marrying the techniques of TIL harvest, autologous tumour pulsing, and IL-2 expansion, Rosenberg and colleagues provided the first description of what became a standard method of selecting and expanding tumour specific lymphocytes used for ACT.

To punctuate the exciting advances of the 1980s, in 1985 Rosenberg and colleagues published results from a small clinical trial detailing the use of these techniques in human patients with metastatic melanoma. Encouragingly, objective responses were seen in up to 44% of patients (11/25 patients) (143). In 1987, the Rosenberg group published interim results from a number of clinical trials using these techniques in human patients with a variety of advanced tumours including melanoma, lymphoma, and renal, colorectal and breast cancers (144). Here, more modest objective responses were seen (33/108 patients ~31%), with the highest response rates

in lymphoma (2/2, 100%), renal cancer (12/36, ~33%) and melanoma (6/26 ~23%). In 1988, by combining ACT, IL-2 and patient preconditioning with cyclophosphamide in a metastatic melanoma setting, response rates were improved, with up to 60% (9/15) demonstrating an objective response (145). Thus, confidence in clinical ACT methods was achieved, with manageable toxicities. Enthusiasm for this immunotherapeutic approach mounted in the 1990s.

1.3.5 The 1990s: building the clinical experience

Moving ACT into the clinic in the 1980s, primarily in the setting of malignant melanoma, provided researchers with a good look at what was to remain a major challenge for ACT. While syngeneic animal models generally could show consistent clinical responses to ACT, in human patients, researchers were seeing variable or mixed clinical outcomes following adoptive transfer of *ex vivo* expanded TIL (146-149). For example, it was not uncommon for patients with several subcutaneous nodules to have some, but not all, nodules respond to ACT. In other cases, patients with subcutaneous or lymph node nodules experienced some regression at these sites, but not in other sites including liver and lung (138, 144, 150, 151). It was easy to speculate about potential reasons for these mixed responses: antigen-loss variants, insufficient number of antigen-specific CTLs, and failure of CTLs to traffic to the tumour site. While these are all valid explanations, the clinical setting with human patients is not amenable to the systematic investigation of any of these mechanisms. Among other questions, issues still needing resolution were a) what was the tumour doing to circumvent the activity of CTLs, and b) what could be done to make T cells more effective in this setting? To answer these questions, a better understanding was required of the interface between the tumour cell and the T cell. Fortunately, in the 1990s a number of novel molecular techniques provided opportunities to study these problems in a systematic way.

Molecular techniques for genetic cloning and expression provided an important platform useful for characterizing the T cell's response to the tumour. In part, this manifested as the

identification of specific, tumour-associated antigenic peptides recognizable by T cell clones *in vitro*. For example, in the melanoma setting, antigenic peptides from the genes encoding MAGE, Pmel and MART were identified (152-154). On the breast and ovarian cancer sides, HER2-encoded antigenic epitopes capable of inducing tumour-specific CTLs *in vitro* were identified for the first time (155, 156). Importantly, this inventory of antigenic peptides could be used to identify immunologically dominant T cell clones responding to a patient's tumour and the magnitude and kinetics associated with that response. Identifying useful antigenic epitopes was a necessary preliminary step required to more fully understand the CTL side of the T cell/tumour cell interface in an ACT setting.

Clarification of the mechanisms involved in the T cell response was another significant advance of the 1990s, particularly in the context of tumours. For example, identifying CD28 as the 'second signal' required for activation and induction of IL-2 expression in CTLs provided the means to enhance *in vitro* activation and expansion methods through the use of CD28 antibodies as part of the T cell culture cocktail (157, 158). In addition, the 1990s saw the identification and characterization of the regulatory role of cytotoxic T lymphocyte antigen-4 (CTLA-4) expression on T cell activation (159, 160). This was important as CTLA-4 ligation inhibits downstream activation signaling in T cells following TCR engagement. These findings would provide the background understanding for future work using CTLA-4 blockade to enhance the immune response.

On the tumour side of the T cell/tumour cell interface, the immunosuppressive microenvironment of the tumour had been described broadly since the dawn of ACT, and was used in part to explain the variable results seen in the clinical ACT setting. Finger pointing at cellular subtypes began to heat up in the 1980s, but the roles and effects of tumour-associated cellular populations on CTLs within the tumour microenvironment became better characterized in the 1990s. For example, several groups explored how tumour-associated macrophages (TAMs) are

recruited by the tumour via transforming growth factor-beta (TGFβ) and other cytokines and are subsequently induced to secrete immunosuppressive cytokines and molecules (such as IL-4 and IL-10, nitric oxide and TGFβ) (161-165). These cytokines and molecules were found to inhibit CTL activity at the tumour site via direct and indirect mechanisms (162). Suppressive $CD4^+$ regulatory T cells (TRegs) also came under heavy discussion in this decade and continue to be an important component of microenvironmental immunosuppression (166-168). Finally, the tumour cells' ability to secrete cytokines and chemokines locally, and to induce the secretion of suppressive cytokines and associated molecules in other cell populations both locally and systemically further rounded out the inhospitable tumour environment. Thus, by defining some of the cellular and soluble factors involved in the T cell/tumour cell interface in the 1990s, further understanding was provided for the mixed clinical responses seen following ACT.

In parallel with the increased understanding of antigen recognition by T cells and the immunosuppressive microenvironment of the tumour, crucial advances in the field of murine genetics were being published in the 1990s. Starting in the 1980s, the ability to genetically modify mice with specifically targeted mutations using blastocyst injection and homologous recombination in murine embryonic stem cells was a revolution for those using mouse models for human diseases, including cancer (169). These tools subsequently allowed researchers to specifically knock out genes associated with molecular pathways involved on the T cell side of the interface, including antigen recognition by T cells (170), T cell signaling (171), activation (172), and cytolytic function (173). Conversely, the manipulation of genes associated with tumour cell function including antigen processing and presentation (174), transcription and translation of cytokines and chemokines (175) as well as their associated receptors provided the means to assess important components of the tumour cell/T cell interface in a step-wise manner. While the breakthrough in murine genetic manipulation was an important advance, it could not provide an all-encompassing means to study each aspect of the interface. It was quickly found

that genetic modification could sometimes result in embryonic-lethal phenotypes, as is the case with TGFβ-1 mutations (176, 177). Further, naturally occurring redundancy in gene expression pathways associated with, for example, T cell signaling and activation, complicated otherwise straightforward cause-and-effect hypotheses. Thus, important components of the T cell/tumour cell interface remained to be uncovered, potentially using alternative molecular tools.

Clinical trials using ACT as a treatment for malignant cancers continued throughout the 1990s in a variety of tumour settings including malignant glioma, lung cancer, renal cell carcinoma, ovarian cancer, and melanoma (146, 148, 178-181). Without exception, mixed clinical responses continued to be the norm. This in spite of a remarkable list of published manuscripts describing optimized approaches for the harvesting and *ex vivo* expansion of T cells (182-186), timing and composition of cytokine therapy (80, 187, 188), and the addition of combination therapy with therapeutic antibodies (189-191) or chemotherapeutic agents (192-195). Unfortunately, mixed responses remained a clinical frustration from the perspective of patients and researchers, and in the face of costly and labour-intensive infrastructural demands associated with this approach, the feasibility of ACT as a viable option came under question.

The essence of the same, vexing questions raised in the early days of ACT remained. Why were some tumours responsive to ACT when others were not? Even within the same patient, reports described one or more tumour nodules regressing or stabilizing, while others progressed. While many of the obvious reasons for immune escape had been described by this time, including antigen loss (196, 197), antigen tolerance (198, 199), and direct and indirect immunosuppression via cellular and soluble mechanisms (200-202), these had mainly been characterized in either *in vitro* or animal models. Based on clinical results in human subjects, it seemed logical that features unique to the tumour-bearing patient were contributing to the mixed responses seen following ACT. Given the complications inherent in clinical trials with human subjects, it is not surprising that researchers in the field were motivated to redouble their efforts

in the next decade to circumvent some of the identified barriers to a more general success of ACT in the setting of solid tumours.

1.3.6 2000 to the present: looking outside the box

Recognizing the technological advances realized in the preceding decades, the basic principles behind successful ACT remained the same: there needed to be a) a tumour antigen that could be recognized by CTLs, b) sufficient and sustained numbers of functional, antigen-specific T cells, and c) the means to infiltrate the tumour and effect cytotoxicity. The first decade of the 21st century saw several important advances associated with each of these essential areas.

In order for an antigen-specific CTL to recognize a tumour, the tumour must express that antigen in the context of MHC Class I; a conundrum in the case of tumours with aberrations in genetic programs associated with antigen processing and presentation. Accordingly, attention was focused on building a T cell that did not require antigen presentation in the context of MHC Class I, thus circumventing this problem. T cells engineered to possess chimeric antigen receptors (CARs) represented one such tool. T cells with CARs are engineered to express a fusion protein composed of a specific single chain variable fragment (scFv) on the extracellular domain, fused to the cytosolic signaling domain (CD3ζ chain) of the T cell (203-205). This approach allows the T cell to become activated and affect cytolysis in the absence of MHC Class I-restricted antigen presentation.

'First generation' CARs have seen Phase I and II clinical testing in a variety of tumour settings in this decade, including lymphoma, renal cell carcinoma, ovarian cancer and neuroblastoma (206-209). Building upon first generation work in the 1990s, pre-clinical ACT models using 'second-generation' CAR T cells incorporating costimulatory molecules (including CD28 and CD137) in addition to the CD3ζ chain gained momentum in the early part of this decade. Second generation CARs will likely see additional clinical testing within the next 10 years (210, 211). The

advantages of CARs are obvious; however the disadvantages would include the technical challenges associated with the use of genetically engineered T cells clinically, and the risk (both real and perceived) of unanticipated on- or off-target effects. Thus, while CARs represent a means to circumvent processing and presentation defects, they are unlikely to represent a universally appropriate tool.

The vital input for ACT, the antigen specific CTL, must be harvested and expanded *in vitro* to sufficient numbers within a clinically acceptable time period to optimize patient benefit. Further, *in vitro* expansion must not result in expansion of poorly functional T cells or T cells with detrimental (e.g. suppressor) phenotypes. Once a reliable method for maintaining T cells in culture was established in the 1980s, Rosenberg's group and others continued to refine the *in vitro* expansion systems in an attempt to optimize the technique for clinical use. However, by the late 1990s, researchers were realizing that in some cases, maintaining T cells in culture under certain conditions resulted in the emergence of CTLs with poor cytolytic activity, or otherwise suppressed functional capacity (212). Indeed, by the early 2000s, it was known that IL-2, the mainstay cytokine for *in vitro* culture of T cells, was implicated in the development of immunosuppressive populations of $CD4^{+}CD25^{+}$ T cells in a mixed culture population (213, 214). Additionally, it was well known that CTLs have a limited lifespan, and anergy and exhaustion were very real risks during the *in vitro* expansion phase (215, 216). These post-infusion challenges were investigated from both *in vitro* and *in vivo* perspectives.

In 2002, Dudley and colleagues described the use of non-myeloablative, lymphodepleting chemotherapy as a means of preconditioning patients prior to being treated with ACT. Using cyclophosphamide and fludarabine, they hypothesized that this approach may result in the depletion of immunosuppressive (TReg) cell populations, changes in normal T cell regulation, and the elimination of the resident 'cytokine sink' represented by pre-transfusion populations of patient lymphocytes (85). Their study found that *ex vivo* expanded tumour-reactive T cells were

persistent, proliferative, and were able to traffic to tumour sites in patients with advanced melanoma. Importantly, objective responses were seen in 6/13 patients (46%), and an additional 4 patients (31%) experienced mixed responses with shrinkage of one or more metastatic nodules. Thus, these promising results using patient preconditioning were seen as a turning point in the field, and this approach has become a standard treatment for patients undergoing ACT.

Encouraged by their *in vivo* studies showing improved persistence of adoptively transferred CTLs, Dudley and colleagues sought to improve the potential for CTLs to perform optimally following ACT. To address this challenge, In 2003 Dudley et al. described a rapid expansion protocol using multiple individual TIL cultures acquired through tumour biopsy samples (58). This technique allowed for the selected expansion of TIL cultures exhibiting the desired properties of rapid proliferation and tumour specific activity. Importantly, these cultures could be delivered to the patient within 6-8 weeks following tumour biopsy, instead of the 12-20 weeks more commonly experienced in standard protocols. While this approach did nothing to address the challenges associated with sustaining T cell activity post-adoptive transfer, it provided progress toward rapidly providing CTLs with demonstrated capacity for proliferation and tumour-specific activity, thus reducing the risk of CTL exhaustion. Nevertheless, much work still remains to be done to optimize the activity and lifespan of the CTL once they leave the comfort of the culture flask.

Once sufficient, functional CTLs have been established *in vitro*, they must reach their final destination with sufficient function for ACT to be successful. The immunosuppressive environment that can develop *in vitro* during the expansion phase also can occur both systemically and locally at the site of the tumour within the recipient patient. To address the ‘host barrier’ problem, Dudley and others returned to the principles learned several decades previously, and revisited the process of preconditioning the ACT recipient with lymphodepletion

(85, 88). Lymphodepletion using chemotherapeutics (cyclophosphamide and fludarabine) followed by ACT has been used clinically in human patients (217), and more recently total body irradiation (TBI) has been used in pre-clinical tumour models in a multi-step process involving varying-intensity TBI and hematopoietic stem cell transplantation (218). Clinical results following this approach reported objective or mixed responses in up to 76% of patients. These reports also detail that patients experienced side effects associated with the use of preconditioning regimens in addition to the anticipated toxicities associated with post-ACT IL-2 (85, 217). Based on outcomes experienced using patient preconditioning regimens, it seems likely that this approach will remain a feature of ACT going forward.

In the year 2010, there are those who will bemoan the state of ACT as a cancer treatment. They insist that, although it has been around for over 30 years, we are still seeing only mixed results clinically following ACT. The method is costly, time consuming and fraught with technical challenges. Realistically, the National Institute of Health (NIH) in Bethesda, Maryland is the only location where this approach is being used clinically on any meaningful scale, and primarily in only one tumour setting: malignant melanoma. Nevertheless, clinical responses are being seen in patients with otherwise completely refractory and terminal disease and side effects of treatment are generally manageable. Laboratory-specific expertise and clinical experience associated with this treatment regimen has grown in the past decades, largely due to collaborations with the NIH by other institutions including MD Anderson Cancer Centre (Houston, TX), and The Fred Hutchinson Cancer Research Centre (Seattle, WA). Unfortunately, infrastructure investments for this approach remain substantial and this inhibits the more widespread implementation of ACT in North American cancer research centers.

Nonetheless, there are a number of large, well organized international associations with members dedicated to the advancement of cancer treatment using ACT, including the International Society for Biological Therapy of Cancer (iSBTc – US based), the Association for

Cancer Immunotherapy (CIMT- Europe based), and the American Association for Cancer Research's (AACR) Cancer Immunology Working Group (CIMM – US based). The molecular tools and pre-clinical models being used to move the knowledge base forward are sophisticated and elegant, and are allowing important questions to be answered sequentially in a way that facilitates bench-to-bedside translation on a reasonable time line. With all this said, there are outstanding technical problems that must be solved, and questions surrounding mixed responses must still be answered. Why are some tumours responsive to ACT, while others are not? What can be done to circumvent tumour-derived barriers to ensure the adoptively transferred CTLs can get to their ultimate destination? Can we use the molecular and diagnostic tools available to us now to predict which tumours might be responsive to ACT in order to optimize patient selection for future clinical trials?

In the chapters that follow, this thesis will describe a novel preclinical mouse model that has been developed to study ACT in the clinically relevant setting of $HER2^+$ breast cancer. This model has been used to investigate the molecular and histological features of individual tumours demonstrating responses to ACT along a clinical continuum, from complete response (CR) to progressive disease (PD). Importantly, this thesis will present data that supports the idea of using gene expression in $HER2^+$ tumours to predict clinical outcome following ACT. In the concluding remarks, the future challenges and opportunities facing this treatment modality will be discussed, both on a general level, and from the perspective of the data presented herein.

CHAPTER 2: Spontaneous mammary tumours differ widely in their inherent sensitivity to adoptively transferred T cells

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2.1 Abstract

Immunotherapy of cancer can lead to the selection of antigen-loss variants, which provides strong rationale to target oncogenes that are essential for tumour growth or viability. To investigate this concept, we tagged the HER2/*neu* oncogene with epitopes from ovalbumin to confer recognition by T cell receptor transgenic $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells. Transgenic mice expressing $neu^{OT\text{-}I/OT\text{-}II}$ developed mammary adenocarcinomas at 6-10 months of age. Adoptively transferred naïve OT-I cells (with or without OT-II cells) proliferated vigorously upon encountering $neu^{OT\text{-}I/OT\text{-}II}$–expressing tumours. This was followed by the complete regression of 37% of tumours, while others showed partial/stable responses (40%) or progressive disease (23%). Those tumours undergoing complete regression never recurred. In mice with multiple primary tumours, simultaneous regressions and non-regressions were often seen, indicating that immune evasion occurred at a local rather than systemic level. The majority of non-regressing tumours expressed $Neu^{OT\text{-}I/OT\text{-}II}$ and MHC class I, and many avoided rejection through a profound block to T cell infiltration. Thus, T cells directed against an essential oncogene can permanently eradicate a subset of spontaneous, established mammary tumours. However, in other tumours, local barriers severely limit the therapeutic response. To maximize the efficacy of immunotherapy against spontaneous cancers, predictive strategies that take into account the heterogeneity of the tumour microenvironment will be required.

2.2 Introduction

Adoptive immunotherapy represents a promising strategy to induce T cell responses against human cancer. In the past two decades, methods have been developed to identify, clone and expand antigen-specific human T cells *in vitro* (219). Upon re-infusion into autologous hosts, such T cells can mount protective responses to Epstein Barr virus and cytomegalovirus in immunocompromised individuals (220). In the allogeneic transplantation setting, donor T cells can mount curative responses against host leukemic cells (graft-versus-leukemia effect) (221). Similarly, adoptively transferred T cells can induce objective tumour responses in advanced melanoma patients (222). Despite many anecdotal successes, however, most immunotherapy trials continue to yield primarily negative results, likely owing to our incomplete understanding of the heterogeneity of human cancer at the immunological level.

It can be argued that the availability of tumour-associated antigens and cognate T cells is no longer a major limitation for cancer immunotherapy. In human breast cancer, for example, a number of attractive target antigens for T cells are available, including HER2/*neu*, MUC-1, NY-BR-1 and MAGE family members (223, 224). Likewise, T cell responses to such antigens can be successfully enhanced through vaccination or adoptive transfer (219, 225). If target antigens and cognate T cells are available, what then limits the therapeutic response? Human cancers demonstrate considerable variability with respect to immunological factors such as (a) cytokine profiles; (b) defects in antigen processing or presentation; (c) the presence of immune-suppressive myeloid cells or regulatory T cells; or (d) permissiveness to lymphocyte infiltration (226). While tumours can easily be typed for expression of target antigens and MHC molecules, these other immunological factors are not understood in a systematic way that allows one to predict whether a given tumour is likely to be sensitive or resistant to T cell therapy. This could explain the unpredictable responses seen in human clinical immunotherapy trials to date.

Our understanding of breast cancer biology and treatment has been greatly accelerated by transgenic mouse models. For example, transgenic mice that express HER2/*neu* under the control of the mouse mammary tumour virus (MMTV) promoter/enhancer (227) have been used extensively to study the molecular genetics of mammary tumourigenesis (228). They have also been subjected to various immune-based therapies, including *neu*-specific monoclonal antibodies (229, 230), *neu*-specific vaccines (231-233), systemic IL-12 administration (234) and combined chemotherapy and vaccination (235). Many of these regimens show efficacy in delaying or reducing the incidence of tumour formation, suggesting these tumours are somewhat susceptible to immune intervention, at least at early stages. However, at late stages, there are few examples of immunotherapeutic approaches eliminating MMTV/*neu* tumours, or any other experimental cancers. From that perspective, mouse models continue to provide similar therapeutic challenges as faced with human cancer.

We describe here a modified version of the MMTV/*neu* transgenic mouse model that allows for the first time precise monitoring of the responses of adoptively transferred $CD8^+$ and $CD4^+$ T cells to spontaneous mammary tumours. Our results demonstrate that the molecular and cellular heterogeneity of cancer is reflected also at the immunological level. After adoptive transfer of tumour-specific $CD4^+$ and $CD8^+$ T cells, spontaneous tumours demonstrated a continuum of responses ranging from complete regression to progressive disease. Responses were largely dictated by inherent factors in the local tumour environment rather than systemic properties. Thus, we provide a new model of breast cancer that will facilitate the development of predictive, personalized immunotherapeutic strategies based on the inherent properties of the tumour environment.

2.3 Materials and methods

2.3.1 Mice

This study followed Canadian Council for Animal Care guidelines and was approved by the University of Victoria Animal Care Advisory Committee. All mice were C57BL/6 (H-2^b). The activated rat *neu* oncogene (227) was tagged at its C terminus with CD8+ (OT-I) and CD4+ (OT-II) T cell epitopes from ovalbumin (OVA). Transgenic C57Bl/6 mice were generated that express $neu^{OT\text{-}I/OT\text{-}II}$ in mammary epithelium under the control of the MMTV promoter (227) (Appendix 1). $neu^{OT\text{-}I/OT\text{-}II}$ mice were bred with mice expressing a dominant negative mutant of p53 (*DNp53*, R172H (236)) under the control of the whey acid protein (WAP) promoter (Appendix 1). TCR transgenic mice included: OT-I, recognizing OVA residues 257-264 on MHC class I (237) (238); OT-II mice, recognizing OVA residues 323-339 on MHC class II (239); and P14 mice recognizing gp33 from LCMV on MHC class I (240). OT-II mice were kindly provided by Eric Butz (Immunex, Seattle, WA), whereas others were from the Jackson Laboratory. Genotyping was by PCR (Appendix 2).

2.3.2 Adoptive transfer and flow cytometry

Single-cell lymphocyte suspensions were stained with 1.5 mM CFSE (Molecular Probes) for 10 minutes at 37°C. Typically, $15x10^6$ each of OT-I and/or OT-II lymphocyte preparations (equivalent to ~$4.5x10^6$ OT-I and/or OT-II T cells) were injected intravenously into tumour-bearing mice. Where indicated, some mice were immunized subcutaneously with 1 mg OVA protein in PBS. To isolate TIL, tumours were pressed through a 40 μm membrane, and lymphocytes in the supernatant were stained with fluorescently labeled antibodies to CD4, CD8a, CD25, CD44, CD62L, CD69, CD90.1 (Appendix 3). Isotype-matched mAb served as negative controls.

2.3.3 Tumour Measurement & Outcomes

Tumour size (length x width) was measured with Vernier calipers. Responses were classified as CR (complete response; no measurable tumour), PR (partial response; >50% reduction), SD (stable disease; <50% reduction or <25% increase), or PD (progressive disease; >25% increase). In some cases, small tumours were discovered at necropsy and hence could not be classified with respect to outcome. Only mice with a total tumour burden less than 350 mm^2 and with no single tumour exceeding 180 mm^2 were used.

2.3.4 Cell Lines

Mammary tumours were dissociated with a 100 μm cell strainer and grown in high glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1x insulin, transferrin, sodium selenite (I1884, Sigma). Fibroblasts were removed by differential trypsinization. Cell lines were assessed by flow cytometry for H-$2k^b$/H-$2D^b$ (28-86), I-Ab (KH74) (BD Biosciences), c-Neu (Ab-4, Oncogene Research), or SIINFEKL/MHC class I (25-D1.15) (241) (kindly provided by Dr. Jonathan Bramson).

2.3.5 Tissue analysis

Tumour tissue was processed following standard methods, and either stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry (IHC) with antibodies to Neu (Cell Signaling, 2242) or CD3 (Sigma, C7930) (Appendix 3). Lung metastases were evaluated by thorough examination of single pan-lobular sections of whole lung tissue. For western blotting, tissues were flash frozen, homogenized and lysed in a Triton X-100 based buffer. Cytoplasmic fractions were probed for Neu (Cell Signaling, 2242), cytokeratin (Sigma, C1801), GAPDH (Abcam) or p70 S6 kinase (Santa Cruz).

2.4 Results

2.4.1 Development and characterization of the neu$^{OT\text{-}I/OT\text{-}II}$ transgenic mouse model

To investigate $CD4^+$ and $CD8^+$ T cell responses to spontaneously arising mammary tumours, we tagged the activated allele of rat HER2/*neu* at its C terminus with $CD4^+$ and $CD8^+$ T cell epitopes from OVA, generating the fusion protein $Neu^{OT\text{-}I/OT\text{-}II}$ (Figure 3). We used epitopes that are recognized in the context of MHC class I and II by the TCR transgenic mouse strains OT-I ($CD8^+$) and OT-II ($CD4^+$), respectively (237, 239). The epitope-tagging strategy ensures that the OT-I and OT-II epitopes are present at 1:1 stoichiometry with Neu, therefore the density of antigen mimics that of a physiological membrane oncoprotein. Moreover, the strategy should theoretically constrain the ability of tumours to evade immune rejection through antigen loss. To test whether the OT-I and OT-II epitopes contained within $Neu^{OT\text{-}I/OT\text{-}II}$ could be processed and presented to T cells, we stably expressed $Neu^{OT\text{-}I/OT\text{-}II}$ in the murine tumour line ID8 (242). As expected, ID8 cells expressing $Neu^{OT\text{-}I/OT\text{-}II}$ induced potent proliferation of splenocyte cultures from both OT-I and OT-II TCR transgenic mice (data not shown).

We next created C57BL/6 transgenic mice that express $neu^{OT\text{-}I/OT\text{-}II}$ in mammary epithelium under the control of the MMTV promoter/enhancer (227). Only ~50% of female transgenic mice expressing $neu^{OT\text{-}I/OT\text{-}II}$ developed mammary tumours by 16 months of age. To accelerate tumour formation, a dominant-negative version of p53 (*DNp53*) was co-expressed under the control of the WAP promoter (243). In contrast to singly transgenic mice, 80% of mice transgenic for both $neu^{OT\text{-}I/OT\text{-}II}$ and *DNp53* developed mammary adenocarcinomas at 6-10 months of age (mean =6.2 months). The majority of mice presented with one or two tumours, although mice with >2 primary tumours were also seen. Primary tumours did not form at sites other than mammary gland.

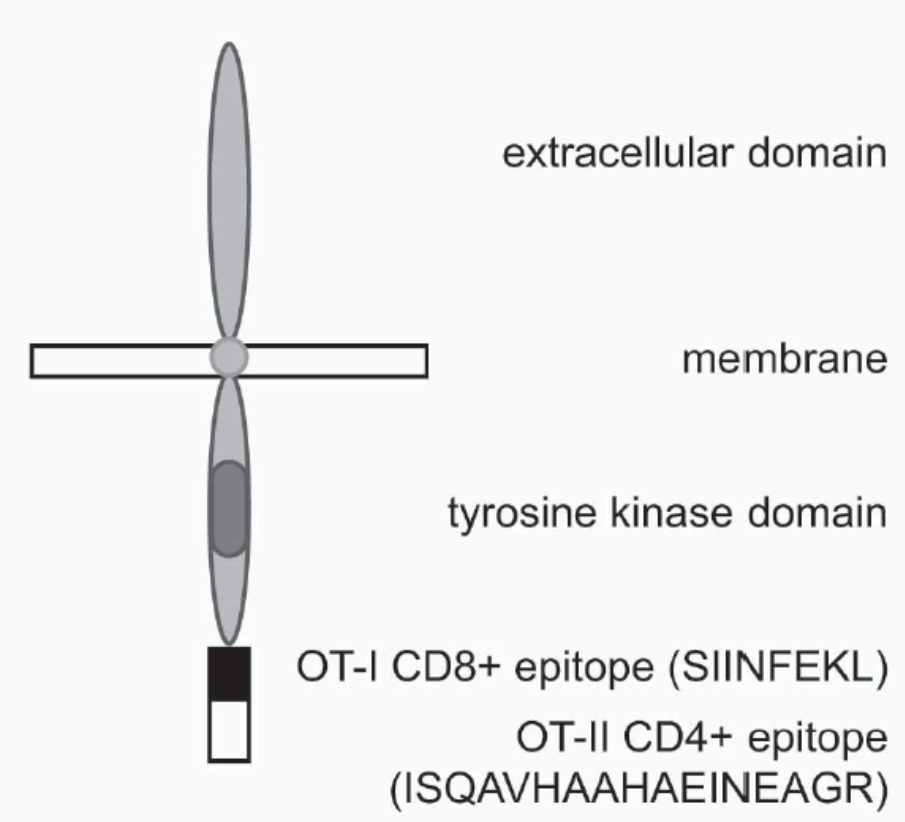

Figure 3. Schematic diagram of Neu$^{OT\text{-}I/OT\text{-}II}$

The activated rat *neu* oncogene (gray) was tagged at the C-terminus with two T cell epitopes from chicken ovalbumin. The OT-I epitope (black) is recognized by $CD8^+$ T cells from OT-I mice, and the OT-II epitope (white) is recognized by $CD4^+$ T cells from OT-II mice.

Consistent with previous reports for MMTV/*neu*-induced mammary adenocarcinomas (244), tumours were high grade, highly mitotic, and showed a solid histological subtype, with glandular differentiation in ~30% of cases (Figure 4A). Most tumours (~90%) had minimal necrosis, consistent with their pronounced vasculature. IHC revealed a range of $Neu^{OT\text{-}I/OT\text{-}II}$ expression in tumours (Figure 4B, Figure 5). In a blinded assessment of 40 tumours on tissue microarrays (TMAs) using standard clinical IHC scoring criteria (245), 3% were negative for $Neu^{OT\text{-}I/OT\text{-}II}$ (0), 20% were marginally positive (1+), and 77% were positive (2+,3+). By Western blot, high-level expression of $Neu^{OT\text{-}I/OT\text{-}II}$ was detected in mammary tumours, whereas all other tissues were negative except for low-level expression in ovary and lung (Figure 4C). Tumours also expressed cytokeratins, consistent with an epithelial origin (not shown).

Expression of the OT-I epitope, MHC class I and class II was assessed by flow cytometry of 26 tumour cell lines derived by short-term culture of spontaneously arising tumours (Figure 6 and 7). Similar to the IHC results, 22/26 lines were positive for $Neu^{OT\text{-}I/OT\text{-}II}$ by flow cytometry and the majority (n=24/26) were also positive for MHC class I. About half of the cell lines (n=14/26) stained positive with an antibody that recognizes the OT-I peptide in the context of MHC class I (241). Few lines (n=4/26) were positive for MHC class II. After exposure to IFN-γ, a large majority of tumour lines expressed MHC class I (n=26/26), the SIINFEKL/MHC class I complex (n=22/26) and MHC class II (n=22/26).

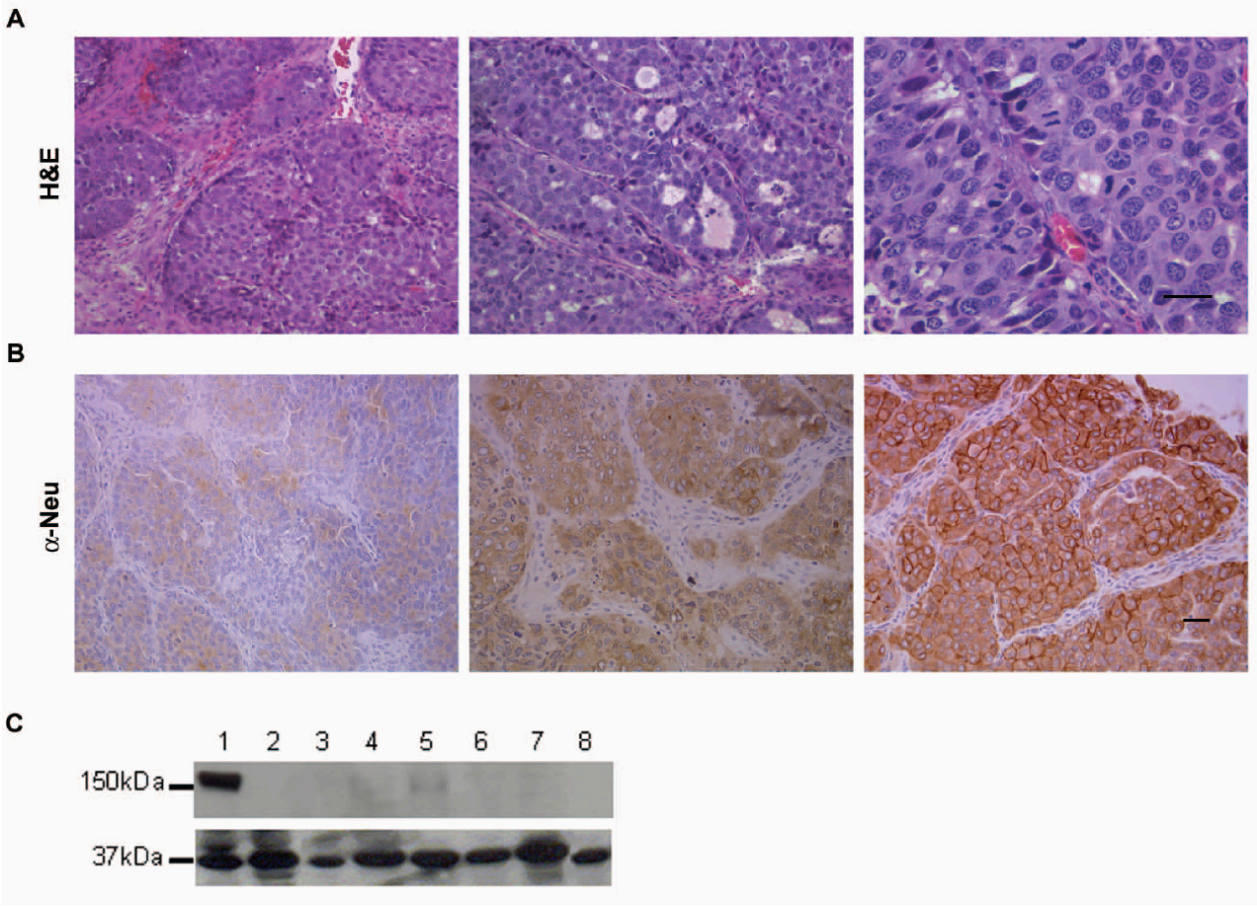

Figure 4. Characterization of tumours and tissues from neu$^{OT\text{-}I/OT\text{-}II}$ x DNp53 mice

(A) H&E staining of three different mammary tumours, showing (*left*) a typical example of the solid histological subtype (200X); (*center*) a solid tumour with glandular differentiation (200X); and (*right*) multiple mitotic figures demonstrating the high mitotic index of most tumours (400X, scale bar = 100 microns). **(B)** Immunohistochemical staining of three tumours with an antibody to Neu, showing examples of (*left*) low, (*center*) moderate, and (*right*) high expression of Neu$^{OT\text{-}I/OT\text{-}II}$. All panels are 200X, scale bar = 100 microns. **(C)** Tissue-specific expression of Neu$^{OT\text{-}I/OT\text{-}II}$ assessed by immunoblot. Various tissues obtained from *neu*$^{OT\text{-}I/OT\text{-}II}$ *x DNp53* mice were probed with an antibody to Neu (upper) or GAPDH (loading control; lower). Lanes contain: (1) mammary tumour, (2) heart, (3) liver, (4) lung, (5) ovary, (6) spleen, (7) thymus and (8) kidney.

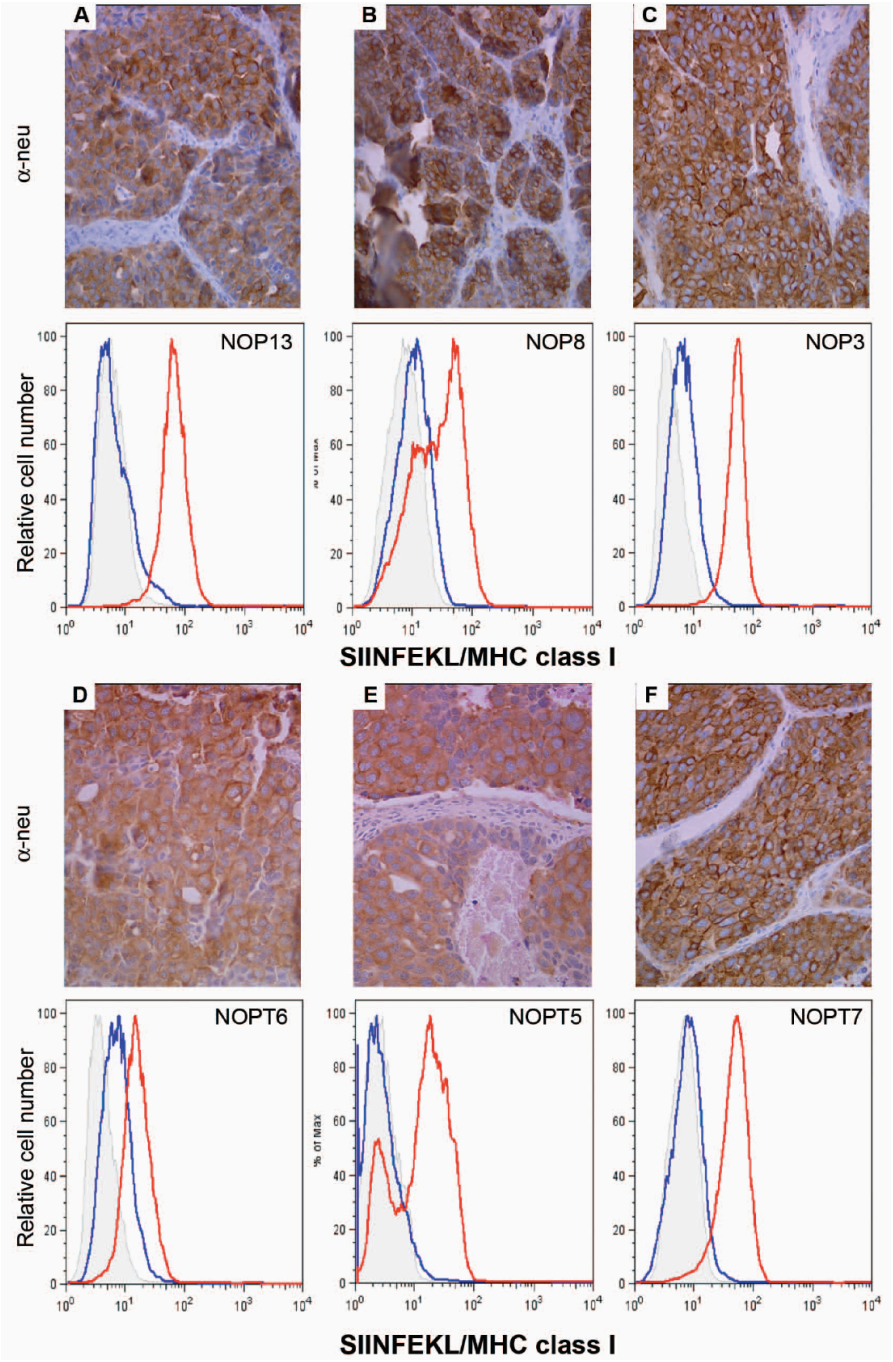

Figure 5. Most non-regressing tumours continue to express Neu$^{OT\text{-}I/OT\text{-}II}$ and present the OT-I epitope in the context of MHC class I

The photomicrographs show Neu$^{OT\text{-}I/OT\text{-}II}$ expression detected by IHC with an antibody to Neu (400x). For each of these tumours, the underlying histogram shows presentation of the OT-I peptide (SIINFEKL) in the context of MHC class I, as assessed by flow cytometry with the

monoclonal antibody 25-D1.15, either before (blue line) or after (red line) exposure to IFN-γ (100 U/ml for 48h). **(A-C)** Three representative untreated tumours (NOP13, 8 and 3). **(D-E)** Three representative tumours that failed to regress after treatment with OT-I + OT-II cells (NOPT6, 5 and 7). The treated tumours were harvested 11, 11 and 61 days after adoptive transfer, respectively.

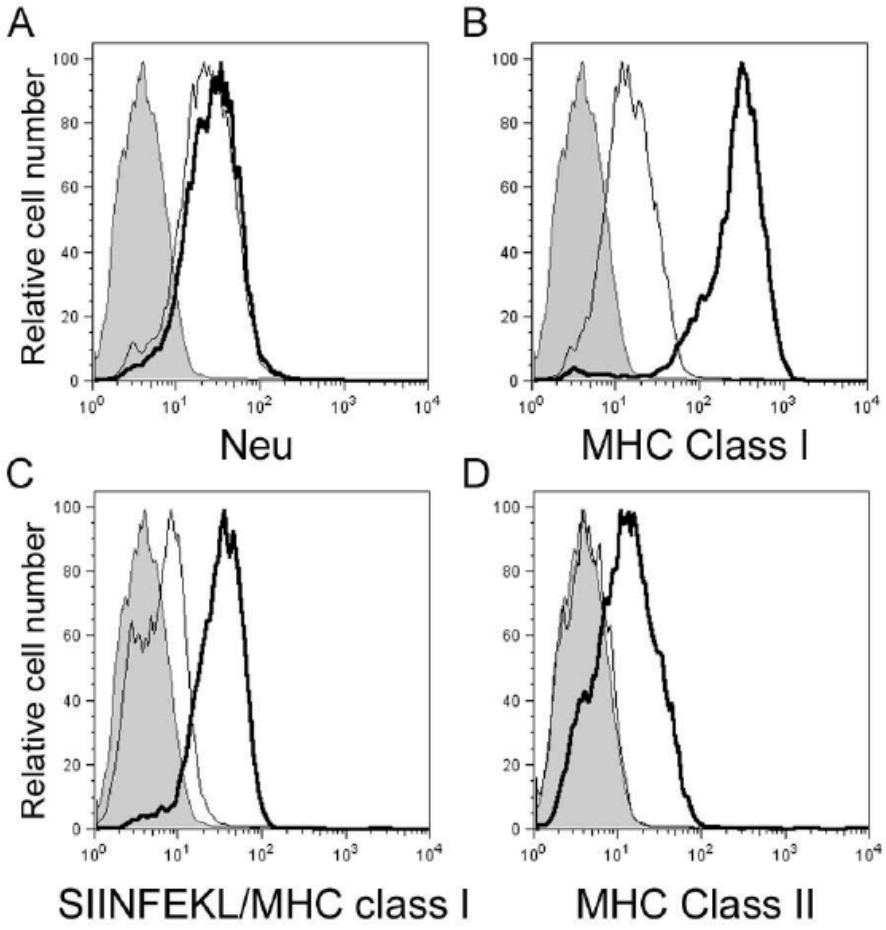

Figure 6. Antigen expression and presentation by neu$^{OT\text{-}I/OT\text{-}II}$ x DNp53 tumour cells

A representative *neu*$^{OT\text{-}I/OT\text{-}II}$ x *DNp53* tumour cell line was grown for 48 hours in the presence (heavy lines) or absence (thin lines) of 100 U/ml IFN-g and analyzed by flow cytometry for expression of **(A)** Neu, **(B)** MHC class I, **(C)** the SIINFEKL epitope from OVA in the context of MHC class I and **(D)** MHC class II. Shaded histograms represent staining with secondary antibody alone.

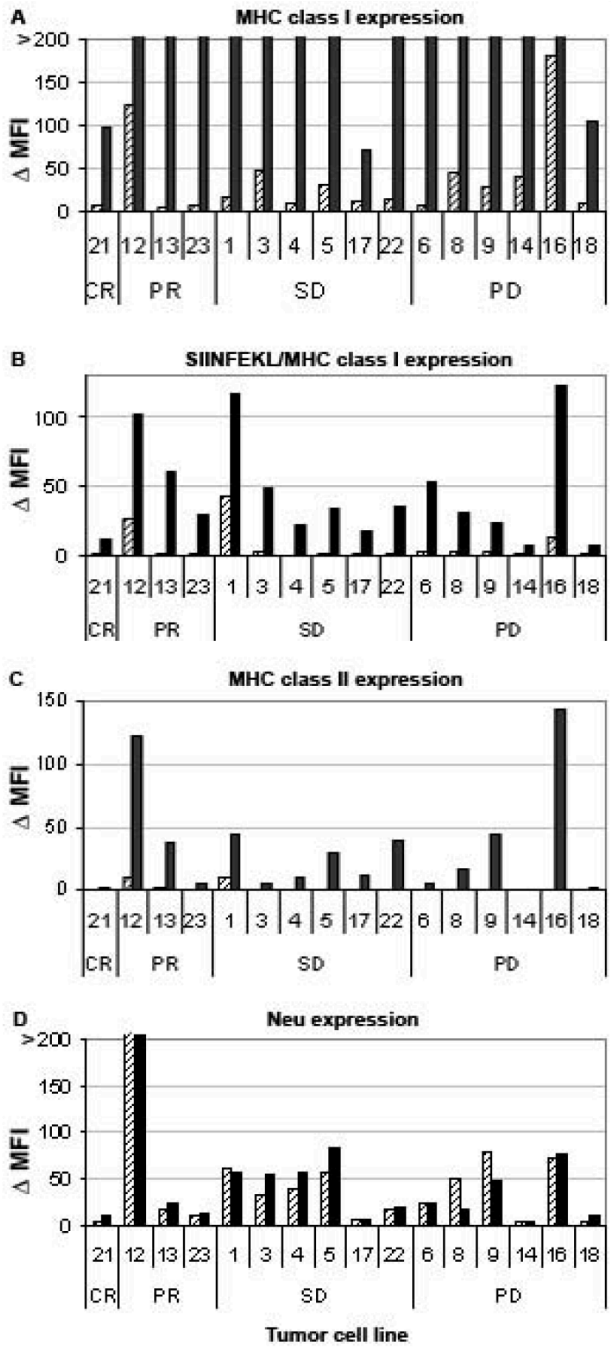

Figure 7. Relationship between antigen expression/presentation and the response to adoptively transferred OT-I + OT-II cells for 16 tumour cell lines

The numeric identifier for each tumour cell line is shown below the X axis (note that cell lines NOP3, 8 and 13 are also represented in Figure 5). Each cell line was derived from a

spontaneous, untreated *neu*$^{OT\text{-}I/OT\text{-}II}$ x *DNp53* tumour. Tumour cell lines were then assessed by flow cytometry for expression of **(A)** MHC class I, **(B)** the SIINFEKL/MHC class I complex, **(C)** MHC class II, and **(D)** Neu$^{OT\text{-}I/OT\text{-}II}$. Results are shown before (hatched bars) and after (black bars) exposure to IFN-γ (100 U/ml for 48h). Expression is presented as change in mean fluorescent intensity (Δ MFI) relative to the negative control (secondary antibody alone). Parallel aliquots of each cell line were implanted into host mice, as described in the text, and the resulting tumours were challenged with adoptively transferred OT-I + OT-II cells. The responses for each tumour cell line are shown below the X axis (CR, PR, SD or PD). Note that there is no obvious correlation between tumour responses and expression of MHC class I, SIINFEKL/MHC class I, MHC class II or Neu$^{OT\text{-}I/OT\text{-}II}$.

2.4.2 In vivo T-cell proliferative responses to mammary tumours expressing neu$^{OT\text{-}I/OT\text{-}II}$

Naïve OT-I and/or OT-II T cells were labeled with CFSE and infused into mice bearing established tumours to achieve a circulating frequency of 1-2 % of total $CD8^+$ or $CD4^+$ T cells, respectively. Adoptively transferred T cells were further demarcated by expression of the congenic cell surface marker Thy1.1, which allows discrimination from $Thy1.2^+$ host T cells. In the majority (83%) of mice, we failed to detect OT-II cell proliferation in the blood, even as late as 13 days post-transfer (Figure 8A,C). In contrast to blood, when lymph nodes (LN) were examined on Day 6, 5-10% of the OT-II cells had undergone multiple rounds of division (8/8 mice) and were $CD44^+$, indicating that a small subset of OT-II cells did indeed respond (Figure 8A). The limited extent of OT-II cell proliferation seen in most mice did not reflect a defect in the T cells themselves, as immunization of tumour-bearing mice with OVA induced robust OT-II proliferation (Figure 8A). Thus, exposure to antigen may be a limiting factor for the OT-II response *in vivo*. Indeed, OT-II cells are known to have a relatively high activation threshold (246).

Analogous studies of the $CD8^+$ T cell response yielded strikingly different results. Whether infused alone or together with OT-II T cells, OT-I cells consistently showed strong proliferation in response to *neu$^{OT\text{-}I/OT\text{-}II}$ x DNp53* tumours (Figure 8B). OT-I cells began proliferating by Day 3, and by Days 4-7 they constituted anywhere from 2% to 43% of the total $CD8^+$ T cell population (Figure 8C). This was invariably followed by a contraction period of several days, after which OT-I cells persisted at detectable levels (0.5-5%) for several weeks. Thus, OT-I cells followed a stereotyped course of expansion and contraction in response to spontaneous mammary tumours, similar to that seen with viral or bacterial challenges (247). The OT-I proliferative response was both antigen- and tumour-specific. Specifically, only negligible OT-I proliferation occurred in tumour-free *neu$^{OT\text{-}I/OT\text{-}II}$ x DNp53* mice, consistent with low expression of the *neu$^{OT\text{-}I/OT\text{-}II}$* transgene in non-malignant tissues (Figure 4C). Furthermore, $CD8^+$ T cells expressing an

irrelevant transgenic TCR (P14 T cells) failed to proliferate in tumour-bearing *neu*$^{OT\text{-}I/OT\text{-}II}$ *x* *DNp53* mice (not shown).

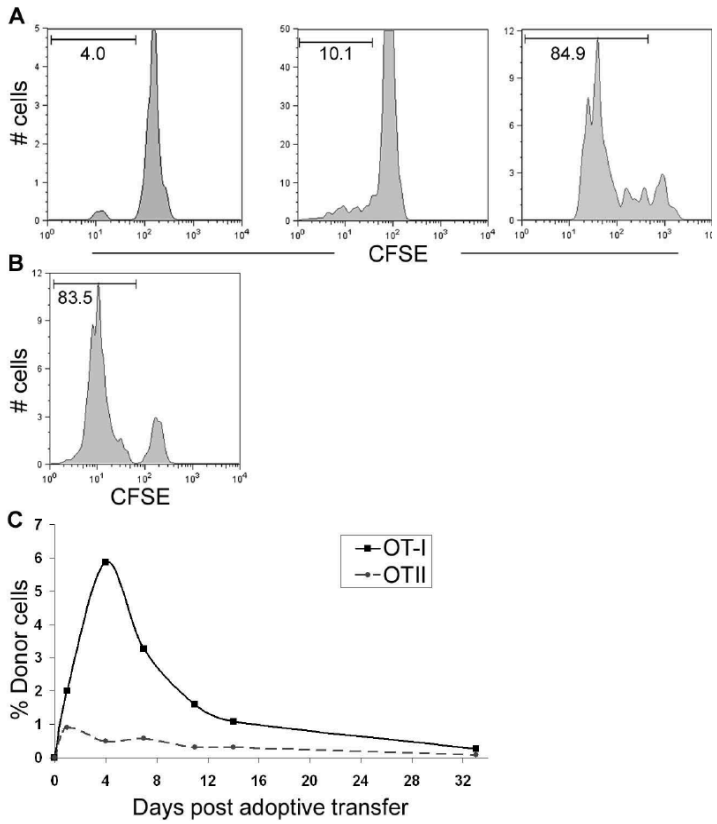

Figure 8. Proliferation of adoptively transferred OT-I and OT-II T cells in mice bearing spontaneous neu$^{OT\text{-}I/OT\text{-}II}$ x DNp53 mammary tumours

(A) (*Left*) OT-II cells assessed in peripheral blood on Day 3. Note the negligible proliferative response, as manifested by retention of CFSE. (*Center*) OT-II cells assessed in LN on Day 6, showing that a subset had undergone several rounds of cell division. (*Right*) OT-II cells assessed in peripheral blood 8 days after adoptive transfer, and 4 days after OVA immunization, showing that weak proliferative responses to tumours can be overcome by immunization. **(B)** OT-I cells assessed in peripheral blood on Day 3, showing a typical robust proliferative response. **(C)** OT-I and OT-II T cells (Thy1.1$^+$) were enumerated by flow cytometry at serial time points after adoptive transfer into a tumour-bearing mouse. The relative number of donor OT-I or OT-II cells in peripheral blood is expressed as a % of total circulating CD8$^+$ or CD4$^+$ T cells, respectively. The results shown are from a single animal, but are representative of 18 experiments.

2.4.3. Mammary tumours demonstrate a range of responses to adoptively transferred T cells

Remarkably, the adoptive transfer of naïve OT-I + OT-II T cells lead to the complete regression (CR) of 37% of tumours. Regressions started within 4-8 days of T cell infusion and were complete by Days 10-17. In other cases, partial responses (PR), stable disease (SD), or progressive disease (PD) were observed. In a study of 68 tumours from 28 mice given similar doses of OT-I + OT-II T cells, the response rates were: CR: 37%, PR: 25%, SD: 15% and PD: 24% (Figure 9A, Table 2). These anti-tumour responses were largely, but perhaps not entirely, attributable to the OT-I cells. Specifically, for 12 tumours (in 4 mice) treated with OT-I cells alone, the response rates were: CR: 8%, PR: 25%, SD: 8% and PD: 58% (Figure 9A). Thus, adoptive transfer of OT-I cells alone can induce anti-tumour responses, but there is a trend toward more frequent regressions in the presence of OT-II cells. By contrast, when OT-II cells were infused alone or with P14 $CD8^+$ T cells, 100% (16/16) of tumours grew progressively.

Tumours that underwent complete regression never recurred, even as late as 14 months after adoptive transfer. Weeks to months later, regressed tumour sites resembled scars and contained fibroblasts, lymphocytes, adipocytes and normal mammary epithelium, without evidence of residual malignancy (Figure 9B). The lymphocytic infiltrates in these scars were often dense but did not extend to adjacent mammary tissue, suggesting the presence of a sustained, localized immune response.

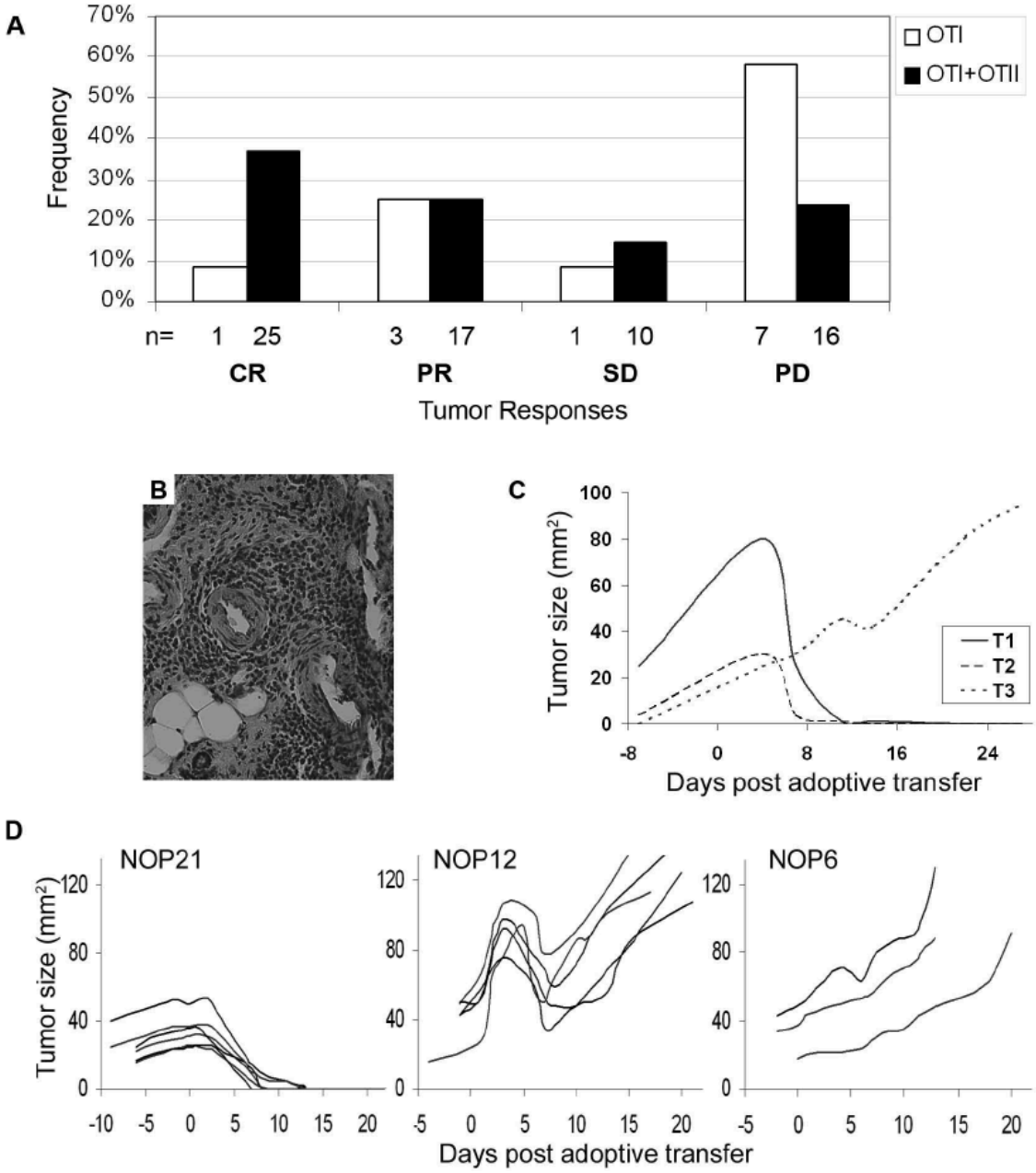

Figure 9. neu$^{OT\text{-}I/OT\text{-}II}$ x DNp53 tumours show a range of responses to adoptively transferred OT-I + OT-II T cells

(A) Mice bearing spontaneous tumours underwent adoptive transfer of either OT-I cells alone (white bars) or OT-I + OT-II cells (black bars), and tumour responses were scored as per Materials and Methods. Numbers below each bar indicate the absolute number of tumours in

each group. **(B)** A scar remaining at a regressed tumour site ~3 months after adoptive transfer. **(C)** Simultaneous regression and progression of established spontaneous tumours in a single *neu*$^{OT\text{-}I/OT\text{-}II}$ x *DNp53* mouse. After adoptive transfer of OT-I + OT-II T cells, two tumours (T1 and T2) regressed completely, while the third tumour (T3) grew progressively. **(D)** Cell lines derived from untreated *neu*$^{OT\text{-}I/OT\text{-}II}$ x *DNp53* tumours and implanted into the mammary fat pads of recipient mice demonstrate reproducible responses to treatment with adoptively transferred OT-I + OT-II cells. Tumour cell line NOP21 underwent a complete response in 6/6 mice. By contrast, NOP12 underwent a partial response in 5/5 mice and NOP6 grew progressively in 3/3 mice.

Mouse	CR	PR	SD	PD	PD*	
1		1				
2		1	2	1		
3		3	1			
4				1		
5		1				
6	1	3				
7	1	2				
8				1		
9	1	2				
10				1		
11	1	2	2			
12				2		
13		1	2	1		
14			1	2		
15				1		
16			2	2		
17	2			1		
18	1			1		
19		1		1		
20				1		
21	3					
22*	1				2	
23*	1				1	
24*	1				1	
25*	2				1	
26**	1					
27**	1					
28**	8					
Total	25	17	10	16		**68**
Response rate	36.8%	25.0%	14.7%	23.5%		

Table 2. Tumour responses for 28 individual mice bearing one or more spontaneous neu$^{OT\text{-}I/OT\text{-}II}$ x DNp53 tumours

Mice underwent adoptive transfer of OT-I + OT-II T cells, and tumour responses were scored according to the criteria in Materials and Methods. Note that some mice had single primary tumours (e.g., Mouse #1), whereas others had multiple primary tumours at the time of adoptive transfer (e.g., Mouse #2). Mixed responses were commonly seen in individual mice in response to the same dose of donor T cells (e.g., Mouse #2). *A subset of mice (22-25) were cured of their primary tumours, but later developed new primary tumours at distinct locations (i.e., these were not recurrent tumours), owing to the continued expression of the *neu*$^{OT\text{-}I/OT\text{-}II}$ x *DNp53*

transgenes. In all cases, these new tumours showed a PD phenotype (PD*) upon adoptive transfer of a second dose of OT-I + OT-II cells. **Three mice (26-28) were cured of their primary tumours and have yet to develop new primary tumours after 257, 348 and 488 days, respectively (as of April 2007).

In addition to primary tumours, we assessed the effect of adoptively transferred T cells on lung metastases. Similar to other MMTV/*neu* transgenic models (228), tumour-bearing mice frequently harbored lung metastases consisting of small (<1 mm) tumour cell emboli growing within blood vessels, with minimal invasion of adjacent lung parenchyma. Most metastatic nodules expressed $Neu^{OT\text{-}I/OT\text{-}II}$ by IHC. To estimate the frequency of lung metastases, we performed a complete microscopic examination of one section of lung tissue (spanning all four lobes) from each animal. By this measure, lung metastases were seen in 37% of untreated mice compared to only 11% of mice treated with OT-I + OT-II cells (p=0.024, Fisher's exact test). Thus, it appears that OT-I and OT-II cells can induce the regression of some, but not all, metastatic lung nodules.

2.4.4 Most non-regressing tumours continue to express and present $Neu^{OT\text{-}I/OT\text{-}II}$

To determine if antigen loss might account for the apparent resistance of some tumours to T cell infusion, expression of $Neu^{OT\text{-}I/OT\text{-}II}$ was assessed by IHC of TMAs containing 40 untreated tumours and 27 tumours that had demonstrated a non-regressing phenotype (i.e., PR, SD or PD) after adoptive transfer of OT-I + OT-II cells (Figure 7). There was no significant difference in the proportion of negative-marginal tumours (i.e., IHC scores of 0 or 1+) between the untreated group and the treated, non-regressing group (23% versus 12%, p=0.26). Moreover, the mean IHC scores for $Neu^{OT\text{-}I/OT\text{-}II}$ expression were 1.98 and 2.29 for untreated and non-regressing tumours, respectively.

Antigen expression and presentation were also evaluated by flow cytometry. Similar to the untreated cell lines described previously, cell lines derived from treated, non-regressing tumours were typically positive for $Neu^{OT\text{-}I/OT\text{-}II}$ (n=5/5) and MHC class I (n=4/5) and showed more limited expression of the SIINFEKL/MHC class I complex (n=1/5) and MHC class II (n=0/5) (Figure 7 and data not shown). Following IFN-γ treatment, increased expression of MHC class I, the

SIINFEKL/MHC class I complex, and MHC class II was observed in 5/5, 5/5 and 2/5 cases, respectively (Figure 7). Thus, 5 of 5 non-regressing tumours retained expression of $Neu^{OT\text{-}I/OT\text{-}II}$ and presented the OT-I epitope on MHC class I after exposure to IFN-γ. Therefore, the majority of non-regressing tumours in this model have not lost antigen expression or presentation.

2.4.5 Tumour-specific factors dictate the outcome of T cell responses

Intriguingly, in mice bearing multiple primary tumours, combinations of CR, PR, SD and PD were commonly observed in response to the same T cell infusion (Table 2). For example, Fig. 9C shows results for one mouse that presented with three primary tumours. After a single infusion of naïve OT-I+OT-II cells, two tumours completely regressed, while the third progressed. Thus, factors in the local tumour environment, as opposed to systemic immunological properties, appeared to dictate the outcome of T cell responses.

To determine whether the different responses of tumours to T cells reflects an inherent, stable property of the tumour cells themselves, we derived several tumour cell lines which were then implanted in host mice and challenged with adoptively transferred OT-I and OT-II cells. We selected cell lines that expressed $Neu^{OT\text{-}I/OT\text{-}II}$, MHC class I and the SIINFEKL/MHC class I complex by flow cytometry. In initial experiments, we attempted to implant the tumour lines into the mammary fat pad of wild type C57Bl/6 mice, but the lines were invariably rejected. By contrast, when tumour-free $neu^{OT\text{-}I/OT\text{-}II}$ transgenic mice were used as hosts, the engraftment rate approached 100%. This presumably reflects tolerance of $neu^{OT\text{-}I/OT\text{-}II}$ transgenic mice to the $Neu^{OT\text{-}I/OT\text{-}II}$ protein, as has been shown for Neu in the conventional MMTV/*neu* transgenic model (248-250). Thus, young, tumour-free $neu^{OT\text{-}I/OT\text{-}II}$ transgenic mice were used as hosts for all subsequent experiments.

Tumour cell lines were implanted in the mammary fat pad, and when tumours reached 50-100 mm^2, mice underwent adoptive transfer with naïve OT-I + OT-II cells as before. As with

spontaneous tumours, strong OT-I cell proliferation and weak OT-II cell proliferation was seen in virtually all cases within 3-6 days of adoptive transfer. Tumour cell lines demonstrated a range of responses, but for each cell line, responses were remarkably consistent (Figure 9D). For example, the cell line NOP-21 underwent complete regression in 6/6 mice. By contrast, the cell lines NOP-12 and NOP-13 each demonstrated a partial response in 5/5 and 3/4 host mice, respectively. Finally, the cell lines NOP-6 and NOP-18 each demonstrated progressive disease in 3/3 mice. Furthermore, when the NOP-13 and NOP-18 cell lines were implanted on opposite sides of the same animal and treated with the same dose of T cells, they responded as if treated alone (data not shown). As with spontaneous tumours, the responses of tumour cell lines could not be predicted based on expression of $Neu^{OT\text{-}I/OT\text{-}II}$, MHC class I, the SIINFEKL/MHC class I complex, or MHC class II (Figure 5). Thus, *$neu^{OT\text{-}I/OT\text{-}II}$ x DNp53* tumours, whether they develop spontaneously or from implanted cell lines, differ in their intrinsic sensitivity to adoptively transferred T cells due to factors other than antigen expression or presentation.

2.4.6 Many non-regressing tumours resist T cell infiltration despite expressing antigen

Having excluded antigen loss as a common mechanism of evasion in this model, we next evaluated the ability of OT-I and OT-II cells to traffic to and infiltrate spontaneous *$neu^{OT\text{-}I/OT\text{-}II}$ x DNp53* tumours. Prior to adoptive transfer, tumours consistently showed minimal lymphocytic infiltrates by H&E staining and anti-CD3 IHC (Figure 10A). Similarly, minimal lymphocytic infiltrates were seen after adoptive transfer of OT-II cells alone or in combination with P14 cells (data not shown). In contrast, when OT-I and OT-II cells were co-infused, 80% (41/51) of tumours on Days 6-7 showed moderate to extensive infiltration by $CD3^+$ T cells (Figure 10B). Flow cytometry confirmed that OT-I was the predominant $CD8^+$ T cell clone in these infiltrates (75% of intratumoural $CD8^+$ cells; range 18-90%; Figure 10D). Consistent with an activated phenotype, intratumoural OT-I cells were $CD44^{hi}$ and $CD62L^{low}$, with subpopulations expressing CD25 and CD69 (Figure 10D). By contrast, the proportion of OT-II T cells never exceeded 3% of

total $CD4^+$ T cells in tumours, consistent with their weak proliferative response. Nevertheless, these intratumoural OT-II cells showed elevated expression of CD44, CD25 and CD69, and downregulation of CD62L, indicating an activated phenotype (Figure 10D). The remaining ~20% of tumours were essentially devoid of $CD3^+$ T cell infiltrates on Days 6-7 (Figure 10C). In many cases, $CD3^+$ T cells were present in the stroma, indicating they had migrated to the tumour site but failed to cross from stroma to malignant epithelium. In other cases, even migration to the stroma had not occurred. Antigen expression was not an issue, as poorly infiltrated tumours showed moderate to strong expression of $Neu^{OT\text{-}I/OT\text{-}II}$ by IHC in 90% of cases. Tumour size was also not an issue, as there was no significant difference in the average size of well- and poorly-infiltrated tumours (67.9 mm^2 vs 71.1 mm^2, p=0.85). Finally, failed T cell activation or proliferation was not an issue, as virtually all mice exhibited robust proliferation of OT-I cells (and sometimes OT-II cells) in peripheral blood. Thus, a significant subset of tumours showed primary resistance to T cell infiltration due to local barriers in the tumour environment, which likely accounts for a large majority of the progressive disease cases.

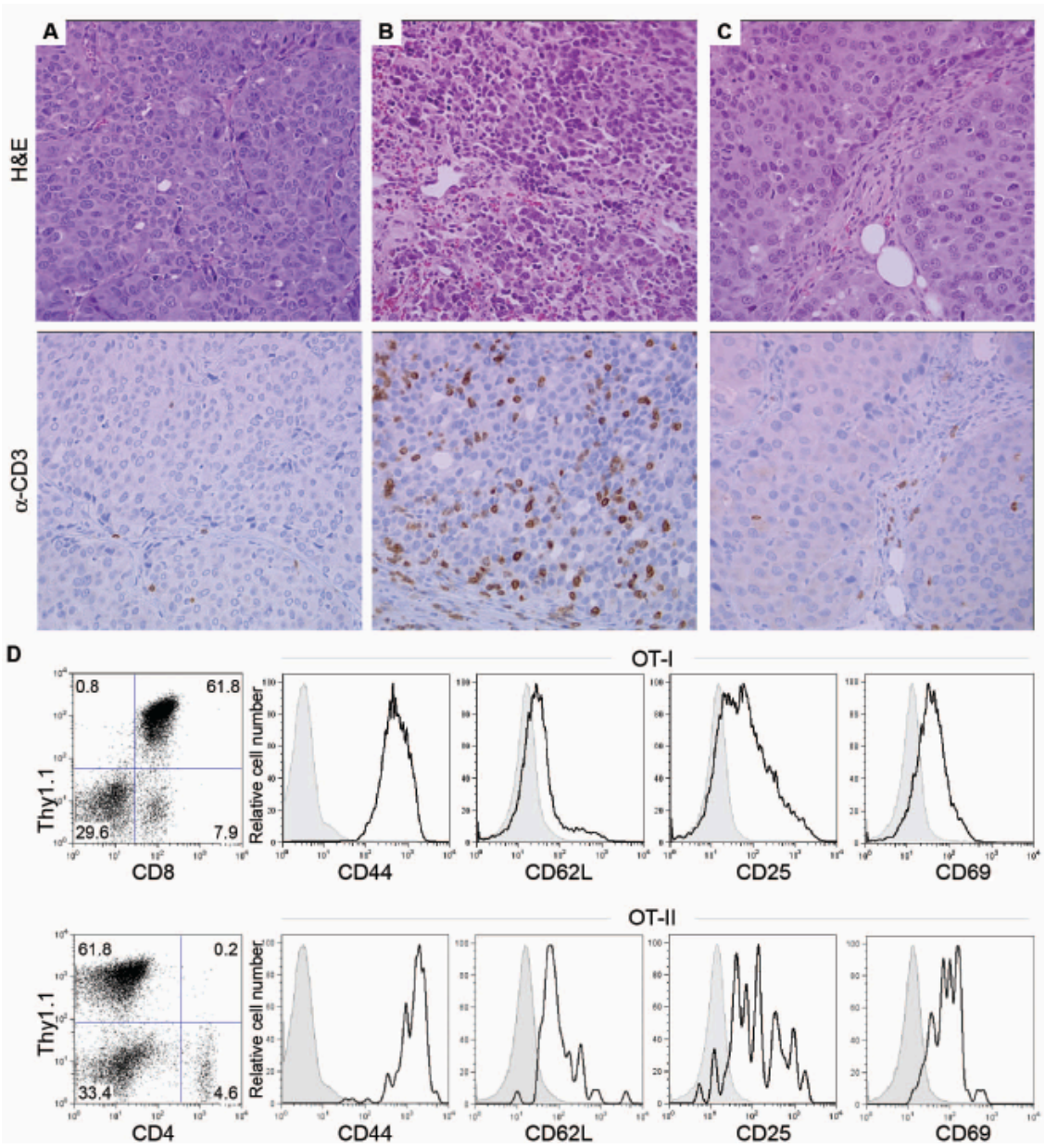

Figure 10. Lymphocyte infiltration of untreated and treated neu$^{OT\text{-}I/OT\text{-}II}$ x DNp53 tumours

(A-C) Three different tumours were stained with H&E (upper panels) or anti-CD3 antibody (lower panels). **(A)** An untreated tumour, showing minimal lymphocytic infiltration. **(B)** A treated tumour harvested on Day 6 after adoptive transfer of OT-I + OT-II cells, showing a heavily infiltrated phenotype. **(C)** A treated tumour harvested on Day 6 after adoptive transfer, showing a poorly infiltrated phenotype. 400X magnification. **(D)** Flow cytometric analysis of tumour-infiltrating lymphocytes harvested on Day 6 after adoptive transfer of OT-I + OT-II cells.

Lymphocytes were gated by forward and side scatter and analyzed for expression of Thy1.1 (which marks all donor T cells), CD4 versus CD8, and the activation markers CD44, CD62L, CD25 and CD69 (black lines) versus isotype-matched controls (gray). (*Upper panels*) Donor OT-I T cells ($CD8^+Thy1.1^+$ cells) were the predominant $CD8^+$ T cell in tumour infiltrates and displayed an activated phenotype. (*Lower panels*) Donor OT-II cells ($CD4^+Thy1.1^+$ cells) were a minor component of tumour-infiltrating $CD4^+$ T cells, but nevertheless displayed an activated phenotype.

2.5 Discussion

Using a novel transgenic mouse model, we have investigated for the first time the response of naive, adoptively transferred $CD4^+$ and $CD8^+$ T cells to spontaneous mammary tumours. The combination of a strong oncogene ($neu^{OT\text{-}I/OT\text{-}II}$) and a disabled DNA repair mechanism (*DNp53*) resulted in mammary tumours displaying a range of molecular, cellular and histological features. After adoptive transfer, $CD4^+$ OT-II cells generally mounted weak proliferative responses, whereas $CD8^+$ OT-I cells proliferated vigorously. This was followed by the complete regression of ~37% of tumours without the need for any other intervention. The remaining tumours showed partial/stable responses or progressive disease, despite retaining antigen expression in most cases. In mice bearing multiple primary tumours, simultaneous regressions and non-regressions were often observed in response to the same dose of T cells. Thus, therapeutic responses were largely (if not entirely) dictated by local, inherent properties of the tumour rather than systemic immunological effects.

Although there are numerous examples in which immune-based interventions can prevent or delay tumour development, or cure early-stage disease (231, 234, 235, 251), to our knowledge this is the first demonstration of advanced, spontaneous tumours being permanently eradicated by adoptive T cell transfer alone. The complete regressions we observed could be attributable to a number of factors, including the use of naïve T cells, the use of a high affinity T cell epitope (SIINFEKL) linked to an essential oncogene, or the use of spontaneously arising tumours rather than implanted cell lines. Previous studies have assessed whether naïve or activated $CD8^+$ T cells have greater anti-tumour activity (252, 253). In general, the evidence favors the use of naïve T cells, which have a greater proliferative and tumour killing capacity than previously activated T cells. However, in unpublished experiments, we found that activated OT-I cells showed equal or greater anti-tumour activity relative to naïve OT-I cells when tested against implanted $neu^{OT\text{-}I/OT\text{-}II}$ x *DNp53* tumour cell lines. While preliminary, this suggests that naïve T

cells are not essential for anti-tumour responses in this model. Furthermore, several other groups have used naïve, TCR transgenic $CD8^+$ T cells and did not observe complete regressions unless other interventions such as irradiation and vaccination were also applied (254, 255). Thus, the use of naïve T cells in our study does not fully explain the striking tumour responses we observed.

A second possibility may stem from our use of a high affinity $CD8^+$ T cell epitope attached to a tumour-initiating oncogene. Expression of $Neu^{OT\text{-}I/OT\text{-}II}$ represents both a benefit to tumours, due to its oncogenic properties, as well as a liability, due to the increased immunogenicity conferred by the epitope tags. The majority of tumours appeared to resolve this dilemma by retaining expression of $Neu^{OT\text{-}I/OT\text{-}II}$, often at high levels, while avoiding immune rejection by other mechanisms. One mechanism may simply be central and/or peripheral tolerance, which reduces the number of tumour-reactive T cells in circulation, as has been documented for $CD4^+$ and $CD8^+$ T cell epitopes from Neu in conventional MMTV/*neu* transgenic mice (248-250). Importantly, tumours relying solely on this mechanism would be highly susceptible to adoptive transfer of T cells, as this would rapidly raise the number of tumour-reactive T cells, resulting in a large, unopposed immune response. This may explain the large subset of tumours that underwent complete regression after adoptive transfer of OT-I + OT-II cells.

A third possible explanation for the complete regressions we observed may be biological differences between spontaneous tumours and implanted cell lines. A bolus of injected tumour cells may trigger inflammatory or immune responses that select for the outgrowth of immune-resistant subclones. By contrast, spontaneous tumours develop in a slow, progressive manner that may evoke less immune recognition and selection. If so, then immunotherapy may prove more effective against spontaneous tumours than implanted tumour cell lines. Indeed, fewer cell lines derived from $neu^{OT\text{-}I/OT\text{-}II}$ x *DNp53* tumours demonstrate complete regressions after adoptive transfer (1/16; Figure 9) compared to the ~37% rate seen with spontaneous tumours.

Non-regressing tumours appeared to resist immune rejection by a number of mechanisms. About 10% of tumours expressed negligible levels of $Neu^{OT\text{-}I/OT\text{-}II}$ by IHC, similar to the antigen-negative variants described in the conventional MMTV/*neu* tumour model (229). A further 20% of tumours resisted T cell infiltration, despite showing moderate to strong expression of $Neu^{OT\text{-}I/OT\text{-}II}$ in the vast majority of cases. Poor lymphocytic infiltration has been reported in numerous other tumour models and has been attributed to diminished leukocyte-vessel wall interactions and adherence (256, 257). Angiogenic factors (vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)) and immunosuppressive cytokines (TGF-β, IL-10) can mediate this effect by down-regulating endothelial adhesion molecules such as ICAM-1 and VCAM-1 (18) . Tumours from conventional MMTV/*neu* transgenic mice reportedly express both bFGF and VEGF but not VCAM-1 (234), which may account for the poor infiltration of some tumours in our model. Importantly, in animal models, several strategies have proven effective in overcoming infiltration barriers, including radiation, chemotherapy, administration of CpG oligonucleotides, CTLA-4 blockade, or infusion of cytokines such as IL-12, GM-CSF or LIGHT (258-263).

The remaining 70-80% of tumours expressed $Neu^{OT\text{-}I/OT\text{-}II}$ and were permissive to T cell infiltration, yet only about half completely regressed. This implies that other factors in the tumour environment can impair the OT-I and OT-II response, resulting in partial or stable responses. Prior work in other models suggests several possibilities. In some tumours, the infiltrating OT-I cells may become anergic due to inadequate co-stimulation or the presence of local immunosuppressive factors (264). If tumour regression does not occur rapidly, OT-I cells could undergo clonal exhaustion or activation-induced cell death due to chronic antigen exposure (265). Other cells in the tumour stroma or infiltrate could suppress the OT-I cells, including myeloid-derived suppressor cells or regulatory T cells (29, 266, 267). Indeed, we have observed infiltration of some tumours by host $FoxP3^{+}$ T cells on Days 6-7 after adoptive transfer (data not

shown). The ability to generate *neu*$^{OT\text{-}I/OT\text{-}II}$ *x DNp53* tumour cell lines with consistent immunological properties will allow future investigation of the different immune evasion mechanisms at play in this model, as well as the most effective countermeasures.

It is noteworthy that T cell-sensitive and -resistant tumours often arose at the same time in the same animals. This implies that evasion of the host immune response during early tumourigenesis occurs at a local level, with different tumours deploying different strategies. Alternatively, it may imply that host immune surveillance does not play a significant role in shaping tumour phenotypes and that immune resistance is instead a by-product of other selective pressures on the tumour.

The concept of predictive and personalized medicine is being applied more broadly in oncological practice, owing to our increasing recognition of the molecular heterogeneity of human cancer. However, with immunotherapy, this concept rarely extends beyond the typing of tumours for antigen and MHC class I expression. We have shown that even in a highly circumscribed experimental system in which the oncogene, antigen, T cell dose and genetic background are all uniform, spontaneous tumours demonstrate a range of inherent immunological phenotypes. It seems likely that these different phenotypes will be sensitive to distinct immunological interventions. With improved understanding of the different immunological environments that develop in spontaneous cancers, it may be possible to prospectively identify the dominant immunological barriers in individual tumours and counteract these with the most appropriate interventions, thereby optimizing both the cost and benefit of immunotherapy.

CHAPTER 3: Density of tumour stroma is correlated to outcome after adoptive transfer of CD4+ and CD8+ T cells in a murine mammary carcinoma model

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KM performed research and edited the manuscript.

PHW designed research, collected data, analyzed and interpreted data, and edited the manuscript.

BHN designed research, analyzed and interpreted data, and edited the manuscript.

3.1 Abstract

Adoptive immunotherapy shows promise for the treatment of cancer, however partial or mixed responses remain common outcomes due to the heterogeneity of tumours. We studied three murine mammary tumour lines that express an ovalbumin-tagged version of HER2/neu and reproducibly undergo complete regression (CR), partial regression (PR), or progressive disease (PD) after adoptive transfer of ovalbumin-specific $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells. The three tumour lines were implanted in immunocompetent C57BL/6 host mice, and established tumours were treated by adoptive transfer of naive OT-I and OT-II T cells. Tumours of the CR and PR classes triggered almost indistinguishable T cell responses in terms of activation, proliferation, trafficking to the tumour site, infiltration of tumour stroma, and intratumoural T cell proliferation; however, tumours of the PR class showed reduced infiltration of tumour epithelium by donor T cells. PD responses were associated with early impairment of T cell activation and proliferation in draining lymph node, followed by negligible infiltration of tumour tissue by donor T cells. Histopathological determinants of outcome were investigated through an unsupervised analysis of 64 untreated tumours representing the three response classes. Tumours of the CR class had proportionately more stroma, which had a looser, more collagen-rich histological appearance. Thus, the amount and composition of tumour stroma distinguished successful (CR) from unsuccessful (PR or PD) outcomes after adoptive T cell transfer, a finding which might facilitate the design of immunotherapy trials for human breast cancer.

3.2 Introduction

Adoptive transfer (AT) of *in vitro* expanded tumour-infiltrating T cells has been pursued as a strategy for the treatment of cancer for over two decades (217, 218, 268-273). While this approach has historically achieved only occasional objective responses, impressive clinical responses have recently been reported in advanced melanoma (85, 88, 274). A key advance has been to pre-condition patients with lymphodepletion prior to AT to remove suppressive and/or competitive T cell populations. Despite this remarkable progress in the systemic aspects of AT, there remain significant barriers in the tumour environment that thwart T cell responses locally. As a result, many patients exhibit mixed clinical responses wherein one or more tumour nodules regress while others do not (88, 218).

Mixed tumour responses after AT can result from impaired T cell trafficking, infiltration or cytotoxic effector function at the tumour site (218, 275-277). Impaired T cell function has been attributed to numerous tumour-associated factors, including immunosuppressive cytokines (e.g., TGF-β and IL-10); inhibitory receptors on T cells (e.g., PD-1 and CTLA-4); regulatory T cells; myeloid-derived suppressor cells; or inadequate expression of adhesion molecules by tumour cells (e.g. ICAM-1 and VCAM-1) (8, 19, 278-285). Notably, while the literature is replete with examples of impaired tumour immunity, far less is known about the features underlying successful anti-tumour responses, especially in the setting of spontaneous tumours.

Recently, attention has been focused upon the various roles played by stromal constituents in the tumour (286-292). Signaling between tumour epithelial cells and stromal cells has been implicated in disease progression (13, 22, 293), metastasis (294-296), and outcome (296). Importantly, some studies have shown that by targeting tumour stroma, adoptively transferred antigen-specific CD8+ T cells can eradicate solid tumours, circumventing established mechanisms of immune escape, including the loss of antigen and MHC Class I expression (289-

292). Therefore, understanding the nature and features of tumour stroma that influence T cell responses is important for the development of effective immunotherapy.

We have developed a murine mammary carcinoma model in which spontaneously arising tumours demonstrate a wide range of clinical responses to adoptively transferred CD4+ and CD8+ T cells. Briefly, we tagged the HER2/*neu* oncogene at its C terminus with CD4+ and CD8+ T cell epitopes from the model antigen ovalbumin (OVA). Mice expressing epitope-tagged Neu ($Neu^{OT\text{-}I/OT\text{-}II}$) as a transgene in mammary epithelium, together with a dominant-negative version of p53, develop spontaneous mammary tumours. These tumours are recognized by OVA-specific CD8+ and CD4+ T cells from TCR transgenic OT-I and OT-II mice, respectively. Remarkably, about one third of spontaneous tumours undergo complete regression (CR) in response to adoptively transferred OT-I and OT-II cells, whereas the remaining tumours demonstrate partial responses (PR), stable disease (SD) or progressive disease (PD) (293). In the present study, we used a panel of tumour cell lines derived from this model to investigate the immunological and histopathological features that dictate the success or failure of AT.

3.3 Materials and methods

3.3.1 Mice

This study followed Canadian Council for Animal Care guidelines and was approved by the University of Victoria Animal Care Committee. All mice were C57BL/6 (H-2^b). Mice expressing the *neu*$^{OT\text{-}I/OT\text{-}II}$ transgene in mammary epithelium under the control of the MMTV promoter have been described (293), as have TCR transgenic OT-I (237) and OT-II mice (236). OT-I mice were bred with CD90.1 (Thy1.1) congenic mice to allow *in vivo* tracking. Genotyping was PCR-based (293), and transgenic TCR expression was confirmed by flow cytometry.

3.3.2 Cell lines, Tumour implantation, measurement & outcomes

Mammary tumour cell lines were derived, maintained and characterized as previously described (13 , 293). Tumour cells (1 x 10^6) were implanted subcutaneously into the inguinal mammary fat pads. Experiments were performed when tumours reached approximately ~50mm^2 (length x width). Responses were classified as CR (complete response; no measurable tumour), PR (partial response; >50% reduction in tumour size), SD (stable disease; <50% reduction or <25% increase in tumour size), or PD (progressive disease; >25% increase in tumour size).

3.3.3 Adoptive transfer and flow cytometry

Lymph nodes and spleens were prepared as previously described (13, 293). Typically, 15 x 10^6 each of OT-I and OT-II lymphocyte preparations (comprising ~4.5 x 10^6 of each OT-I and OT-II cells) were injected via tail vein into tumour-bearing mice. Tumour infiltrating lymphocytes (TIL) were stained with fluorescently labeled antibodies to CD8α (Cat. #553034, Pharmingen), CD4 (Cat. #12-0041), CD90.1 (Thy1.1, Cat. #11-0900-85), CD44 (Cat. #12-0441) (all from BD Biosciences), or for the Vβ segments of the T cell receptor (Mouse TCR Screening Panel; Cat. #557004, Pharmingen). After staining for cell surface markers, intracellular staining was performed for Ki67 expression (Cat. # RM-9106-S, Labvision, Fremont, CA). Appropriately conjugated, irrelevant monoclonal antibodies served as negative controls.

3.3.4 Tissue processing

Tumour tissue was processed following standard methods, and subjected to immunohistochemistry (IHC) using the Ventana Discovery XT autostainer with antibodies to Neu (Cell Signaling, 2242) or CD3 (Labvision, RM9107). Tumour tissue microarrays were constructed using duplicate 1 mm cores (Beecher Instruments).

3.3.5 Histopathological analysis

Immune cell scoring was performed using a Chalkley 25-point grid that defined an area of 0.56 mm^2 at 40X objective magnification. $CD3^+$ T cell infiltration was quantified using five high power fields representative of the overall tumour. The proportion of the field occupied by tumour epithelium was estimated using grid cross hairs, as was the total number of positive immune cells within the area of the grid. The number of grid points that coincided with positively stained immune cells within both epithelial and stromal areas was then determined. Data was expressed as the percent of positively stained cells divided by the total number of enumerated grid points.

The histopathological analysis of untreated tumours was performed in a blinded manner by an experienced breast cancer pathologist (PHW). For mitotic index, areas of proliferation ('hot spots') were identified in H&E-stained whole sections under low power, following which mitotic figures were enumerated in five representative high power fields. For stromal density, the proportion of tumour occupied by stroma (versus tumour epithelium) was estimated and expressed as a percentage. Necrosis was assessed in a similar manner. Graph Pad software was used for statistical analysis.

3.4 Results

3.4.1 Derivation and characterization of mammary tumour cell lines that undergo reproducible responses to OT-I and OT-II T cells

We previously described several primary tumour cell lines derived from *neu*$^{OT\text{-}I/OT\text{-}II}$ transgenic mice (referred to as 'NOP' cell lines, in reference to the components Neu, Ova, and p53) (13, 293). Using flow cytometry, we initially characterized cell lines for the expression of MHC class I; the OT-I SIINFEKL epitope complexed with MHC class I; and Neu$^{OT\text{-}I/OT\text{-}II}$, in the absence and presence of interferon gamma (IFNγ) (Figure 11). Only those tumour cell lines that, in the absence of IFNγ, expressed these molecules at a mean fluorescence intensity (MFI) ¼ log greater than the negative control were advanced for further study. Tumour cell lines were implanted in female recipient mice, and when tumours reached ~50 mm^2, mice underwent adoptive transfer with naive OT-I and OT-II T cells. Tumour responses (CR, PR or PD) showed no clear relationship with the level of expression of MHC class I, the SIINFEKL/MHC class I complex or Neu$^{OT\text{-}I/OT\text{-}II}$ (Figure 11). We selected for further study three lines with distinct, reproducible responses to adoptively transferred OT-I and OT-II cells: NOP21^{CR} (CR in 7/7 mice), NOP23^{PR} (PR in 5/5 mice), and NOP18^{PD} (PD in 6/6 mice) (Figure 12) (13, 293). As before, all three lines expressed MHC class I, the SIINFEKL/MHC class I complex and Neu$^{OT\text{-}I/OT\text{-}II}$ by flow cytometry (Figure 13A). Additionally, all three lines gave rise to tumours that expressed Neu$^{OT\text{-}I/OT\text{-}II}$ by immunohistochemistry (IHC) (Figure 13B). This was true for untreated tumours, as well as recurrent tumours (in the case of NOP23^{PR}) and progressive tumours (in the case of NOP18^{PD}) (data not shown). Thus, the cell lines NOP21^{CR}, NOP23^{PR} and NOP18^{PD} all express antigen in the context of MHC class I, yet differ in the extent of tumour regression after AT of OT-I and OT-II cells.

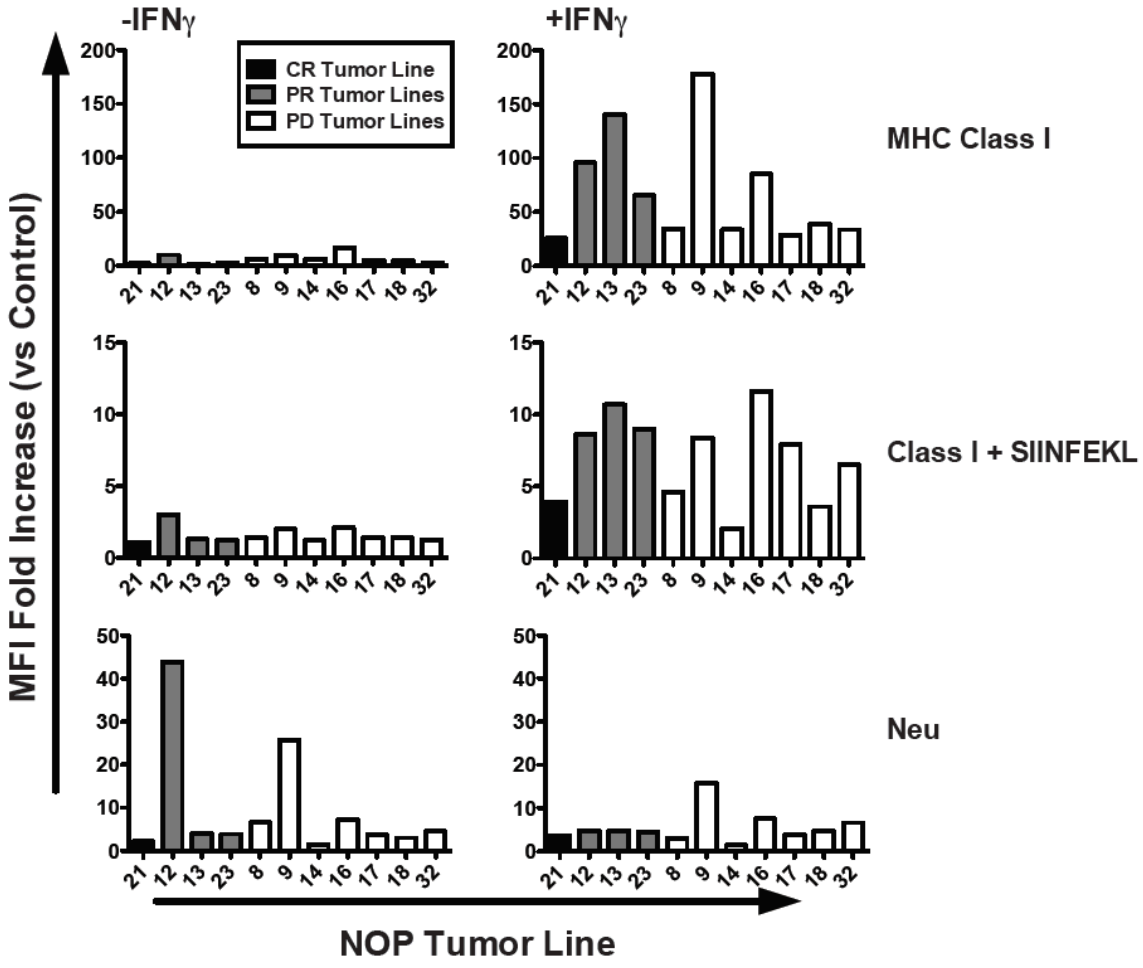

Figure 11. Antigen expression and presentation by mammary tumour cell lines

The indicated tumour cell lines were cultured for 48 hours in the absence (left column) or presence (right column) of 100U/ml IFN-γ and analyzed by flow cytometry for expression of MHC class I (top panels), the SIINFEKL/MHC class I complex (middle panels) and $Neu^{OT\text{-}I/OT\text{-}II}$ (bottom panels). Data is expressed as the fold increase in MFI relative to cells stained with a negative control antibody.

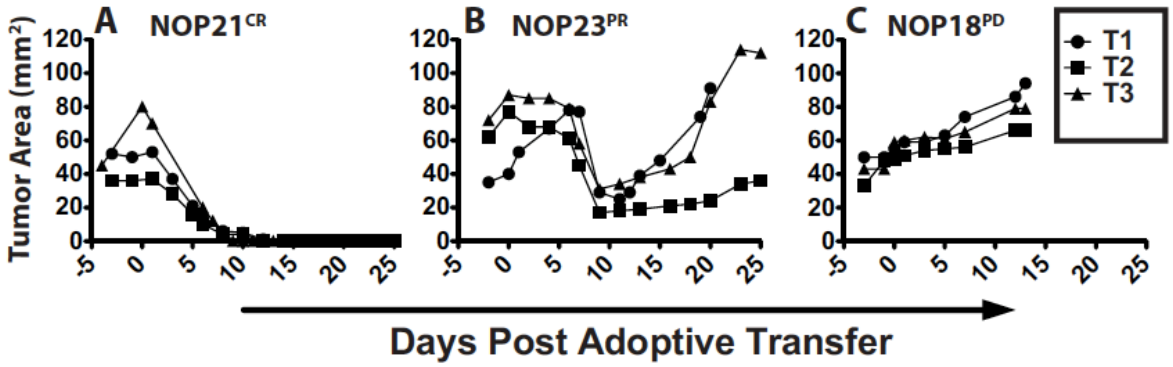

Figure 12. Tumour growth kinetics following adoptive transfer (AT) of OT-I and OT-II cells

The indicated tumour cell lines were implanted in the mammary fat pads of host mice. Once tumours reached a size of approximately 50 mm^2, mice underwent AT with $15x10^6$ OT-I and OT-II splenocytes (Day 0). Tumour size was followed over time. For each tumour line, three representative experiments are shown (T1-T3). (**A**) $NOP21^{CR}$ tumours show rapid, permanent regression; (**B**) $NOP23^{PR}$ tumours regress for a short period of time and then recur; and (**C**) $NOP18^{PD}$ tumours grow progressively. This is a subset of the data shown in Yang et al [13].

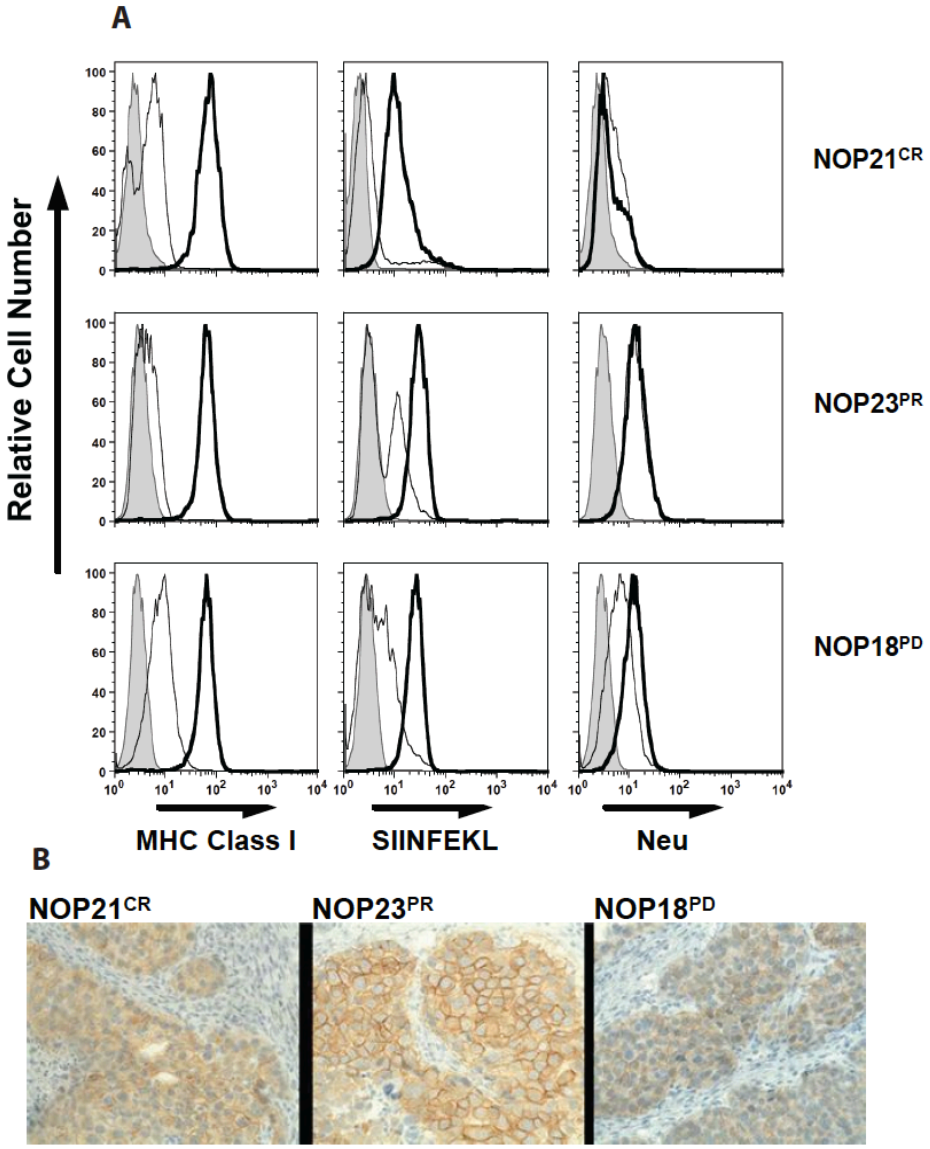

Figure 13. Antigen expression and presentation by selected tumour cell lines

(**A**) NOP21^{CR} (top row), NOP23^{PR} (middle row) and NOP18^{PD} (bottom row) tumour cells were cultured for 48 hours in the presence (heavy lines) or absence (thin lines) of 100U/ml IFN-γ and analyzed by flow cytometry for expression of MHC class I (left panels), the SIINFEKL/MHC class I complex (middle panels) and Neu$^{OT\text{-}I/OT\text{-}II}$ (right panels). Secondary antibody alone served as a negative control (shaded). (**B**) Untreated tumours (~50mm^2) were analyzed by IHC for expression of Neu$^{OT\text{-}I/OT\text{-}II}$ (*200x magnification*).

3.4.2 Activation and proliferation of donor T cells in tumour-draining lymph node

To determine the time and location at which OT-I and OT-II T cell responses first deviate from successful (CR) to unsuccessful (PR or PD) outcomes, we first assessed activation and proliferation of donor T cells in draining lymph nodes (DLNs). Mice bearing established tumours (~50mm^2) derived from NOP21^{CR}, NOP23^{PR} or NOP18^{PD} underwent adoptive transfer with naive OT-I and OT-II T cells. DLNs were harvested 5, 7 or 10 days later, and T cells were analyzed by flow cytometry for expression of the activation marker CD44. NOP21^{CR} and NOP23^{PR} tumours activated OT-I cells to a similar extent, whereas NOP18^{PD} tumours induced delayed, reduced and transient activation (Figure 14A and B). A similar pattern was seen for OT-II T cells, although the percentage of activated cells was generally lower (Figure 14B). At all time points, and regardless of tumour line, host CD4$^+$ and CD8$^+$ T cells showed a modest proportion of CD44^{hi} cells (median = 16%, range = 4-45%), similar to that seen in non-tumour bearing mice (data not shown).

To assess OT-I and OT-II T cell proliferation, parallel aliquots of T cells were stained for expression of the proliferation marker Ki67. While all three tumour lines induced some degree of OT-I cell proliferation, NOP23^{PR} tumours induced markedly greater proliferation than NOP21^{CR} or NOP18^{PD} tumours on Day 7 (Figure 14C). OT-II cells demonstrated a similar pattern of proliferation as seen for OT-I cells, though of lower magnitude (Figure 14D). In general, host CD4+ and CD8+ T cells showed low percentages of Ki67^{hi} cells at all time points irrespective of tumour cell line (data not shown). Similar to the pattern seen for Ki67 expression, the relative number of OT-I and OT-II cells in the draining lymph node was generally higher for NOP21^{CR} and NOP23^{PR} tumours compared to NOP18^{PD} tumours (Figure 15A and Table 3A). Thus, NOP23^{PR} tumours induced equal or greater T cell activation and proliferation than NOP21^{CR} tumours, whereas NOP18^{PD} tumours were significantly impaired in this capacity. Therefore, the

T cell response to PD tumours showed signs of failure as early as Day 5 in DLN, whereas T cell responses to CR and PR tumours were very similar at this stage.

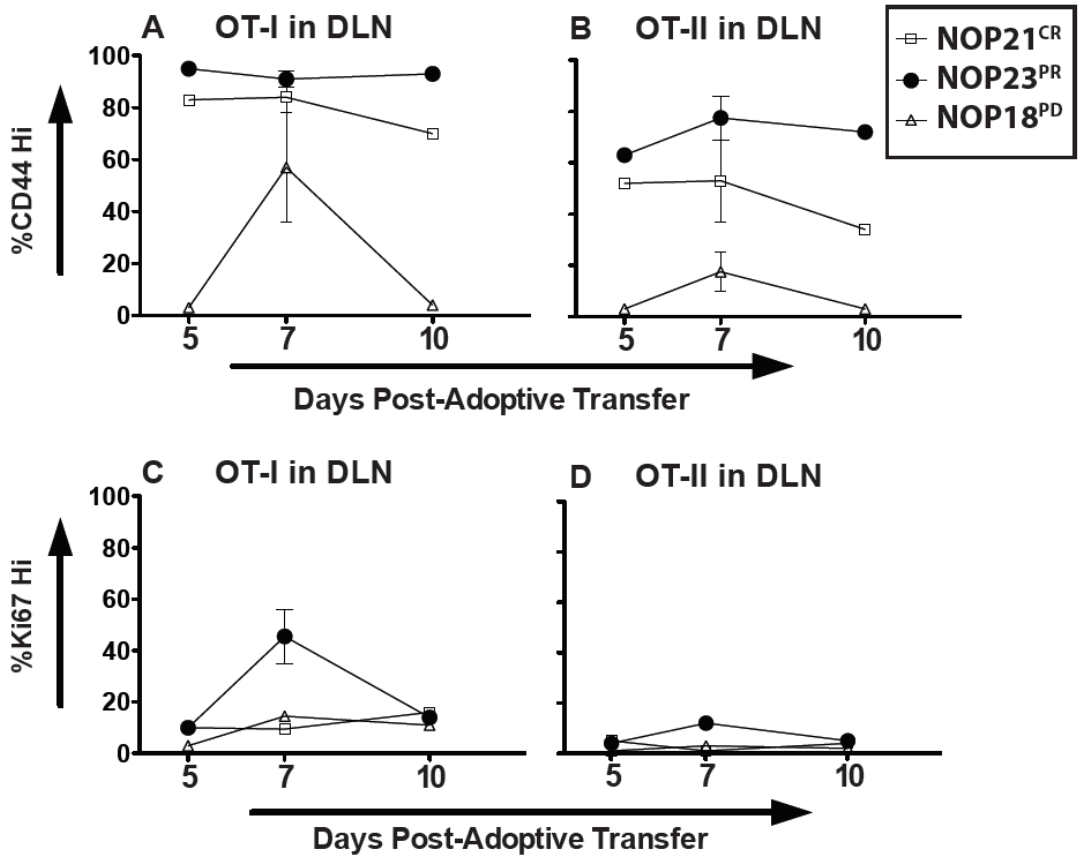

Figure 14: Activation and proliferation of OT-I and OT-II cells in tumour-draining lymph node

Mice bearing established tumours (approximately 50 mm^2) derived from the indicated cell lines underwent adoptive transfer with $15x10^6$ OT-I and OT-II splenocytes on Day 0. Tumour-draining lymph nodes were harvested on Days 5, 7, and 10 and stained with fluorescently labeled antibodies to CD90.1, CD4, CD8 and either CD44 or Ki67. Cells were gated on CD4, CD8, and CD90.1 (which differentiates donor from host cells). **(A,B)** Expression of the activation marker CD44 by (**A**) OT-I cells and (**B**) OT-II cells. $NOP23^{PR}$ tumours induced equal or greater activation of OT-I and OT-II cells compared to $NOP21^{CR}$ tumours, whereas the response to $NOP18^{PD}$ tumours was delayed, reduced and transient. **(C,D)** Expression of the proliferation marker Ki67 by (**C**) OT-I and (**D**) OT-II cells. $NOP23^{PR}$ tumours induced equal or greater proliferation of OT-I and OT-II cells compared to $NOP21^{CR}$ and $NOP18^{PD}$ tumours

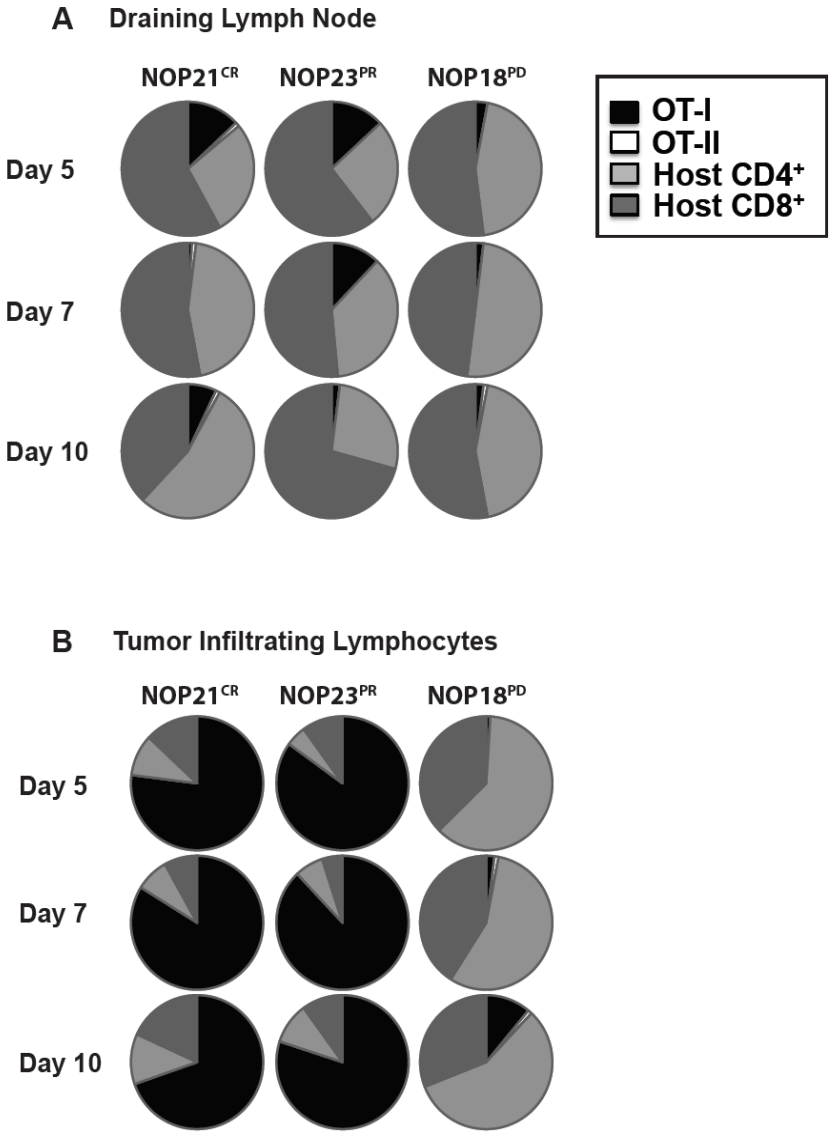

Figure 15. Composition of T cell populations in draining lymph node and tumour following adoptive transfer of OT-I and OT-II T cells

The lymph node and tumour samples from Figure 2 were analyzed for the relative proportions of host and donor CD4+ and CD8+ T cells on Days 5, 7 and 10 after AT. (**A**) *Draining Lymph Node*: Tumour draining lymph nodes from mice bearing NOP18^{PD} tumours had markedly smaller numbers of OT-I cells compared to those from mice bearing NOP21^{CR} and NOP23^{PR} tumours, whereas the number of OT-II cells was consistently low (~1%) in all three cases. (**B**) *Tumour*: In NOP21^{CR} and NOP23^{PR} tumours, a large majority of tumour-infiltrating lymphocytes were OT-I

cells (69-88%) at all time points. In contrast, in NOP18^{PD} tumours, the majority of tumour-infiltrating lymphocytes were host CD4+ and CD8+ cells (>85%), consistent with the values in peripheral blood (data not shown).

A Draining Lymph Node

		NOP21^{CR}		NOP23^{PR}		NOP18^{PD}	
		Number	%	Number	%	Number	%
Day 5	OT-I	5129	13	5120	13	1560	3
	OT-II	482	1	256	<.10	363	<.10
	Host CD4	10535	28	9961	26	23628	45
	Host CD8	22940	58	22941	59.9	27642	52
	Total	39086	100	38278	100	53193	100
Day 7	OT-I	259	1	3173	12	1221	2
	OT-II	82	1	113	<.10	376	<.10
	Host CD4	8022	45	9328	36	28240	50
	Host CD8	9309	53	13125	51	26980	48
	Total	17672	100	25739	100	56817	100
Day 10	OT-I	1439	7	1196	2	472	2
	OT-II	216	1	35	<.10	211	1
	Host CD4	11261	54	13416	27	12780	44
	Host CD8	7818	38	34577	70	15533	53
	Total	20734	100	49224	100	28996	100

B Tumor Infiltrating Lymphocytes

		NOP21^{CR}		NOP23^{PR}		NOP18^{PD}	
		Number	%	Number	%	Number	%
Day 5	OT-I	38151	77	9973	85	8	1
	OT-II	150	<.10	58	<.10	1	<.10
	Host CD4	4931	10	548	5	343	61
	Host CD8	6571	13	1212	10	210	37
	Total	49803	100	11791	100	562	100
Day 7	OT-I	17440	83	12816	88	93	2
	OT-II	88	<.10	23	<.01	42	1
	Host CD4	1784	8	1045	7	2698	56
	Host CD8	1676	8	741	5	1965	41
	Total	20988	100	14625	100	4798	100
Day 10	OT-I	5692	69	19425	80	189	11
	OT-II	16	<.10	80	<.10	21	1
	Host CD4	994	12	2414	10	985	57
	Host CD8	1504	18	2390	10	527	31
	Total	8206	100	24309	100	1722	100

Table 3. Relative number of host and donor T cells in draining lymph node and tumour following adoptive transfer of OT-I and OT-II T cells

The lymph node and tumour samples from Figure 2 were analyzed by flow cytometry for the number of OT-I, OT-II, host CD4+ and host CD8+ T cells on Days 5, 7 and 10 after AT. Bulk tumour specimens were analyzed, and the number of events ranged from ~30,000 – 840,000

per sample. Lymphocytes were identified by staining for CD4, CD8 and the congenic marker Thy 1.1 (which differentiates donor from host cells). Representative examples from **(A)** tumour draining lymph node and **(B)** tumour are shown. In NOP21^{CR} and NOP23^{PR} tumours, a large majority of tumour-infiltrating lymphocytes were OT-I cells (69-88%) at all time points. In contrast, in NOP18^{PD} tumours, the majority of tumour-infiltrating lymphocytes were host CD4+ and CD8+ cells (>85%), consistent with the values in peripheral blood (data not shown).

3.4.3 Infiltration of tumour tissue by donor and host T cells

In parallel with the analysis of tumour-draining lymph nodes described above, we used immunohistochemistry to assess the extent to which $CD3^+$ T cells infiltrated tumour tissue. While all untreated tumours were largely devoid of $CD3^+$ T cells, $NOP21^{CR}$ and $NOP23^{PR}$ tumours showed dense T cell infiltrates 5-7 days after adoptive transfer (Figure 16A). By contrast, $NOP18^{PD}$ tumours showed negligible T cell infiltration at all time points (Figure 16A). At all time points analyzed, donor OT-I cells were by far the predominant T cell constituent in $NOP21^{CR}$ and $NOP23^{PR}$ tumours, representing 69-88% of all tumour-infiltrating $CD3^+$ T cells (Figure 15B and Table 3B). By contrast, in $NOP18^{PD}$ tumours, the few T cells that were present were largely of host origin (85-95%), and comprised a mixture of CD8+ and CD4+ T cells (mean = 37% and 57% respectively; Figure 15B and Table 3B) similar to peripheral blood. Finally, OT-II cells were a minor constituent in all tumours at all time points, representing ~1% of total T cells (Figure 15B and Table 3B). Thus, complete and partial responses were accompanied by dense infiltration of tumour tissue predominantly by OT-I cells, whereas PD responses were characterized by weak infiltration predominantly by host T cells.

We quantified the epithelial versus stromal location of T cell infiltrates by Chalkley grid analysis of $NOP21^{CR}$, $NOP23^{PR}$ and $NOP18^{PD}$ tumours harvested on Days 1, 3, 5, 7 and 10 after AT. $NOP21^{CR}$ tumours became infiltrated earlier and to a greater extent than $NOP23^{PR}$ or $NOP18^{PD}$ tumours (Figure 16B and C). Specifically, $CD3^+$ T cells appeared in the stromal and epithelial compartments of $NOP21^{CR}$ tumours as early as Day 3 after AT, compared to Day 5 in $NOP23^{PR}$ and $NOP18^{PD}$ tumours. Furthermore, the density of $CD3^+$ T cells in tumour epithelium was approximately two-fold greater in $NOP21^{CR}$ tumours compared to $NOP23^{PR}$ tumours ($p<0.001$, 2 way ANOVA). By Day 10, T cell densities were similar in $NOP21^{CR}$ and $NOP23^{PR}$ tumours, but notably $NOP21^{CR}$ tumours had largely regressed by this time and resembled fibrous scar tissue with less inflammatory infiltrate overall. By comparison, T cell infiltrates in $NOP18^{PD}$ tumours

were almost entirely restricted to stromal regions at all time points. Thus, the density of T cell infiltrates in tumour epithelium was strongly correlated with CR, PR or PD outcomes. We also quantified infiltration of $FoxP3^+$ cells (putative regulatory T cells) by Chalkley grid analysis. Regardless of tumour line or time point, fewer than 3 $FoxP3^+$ cells were seen per tumour, indicating that $FoxP3^+$ cell infiltration was weak and not correlated with outcome (data not shown).

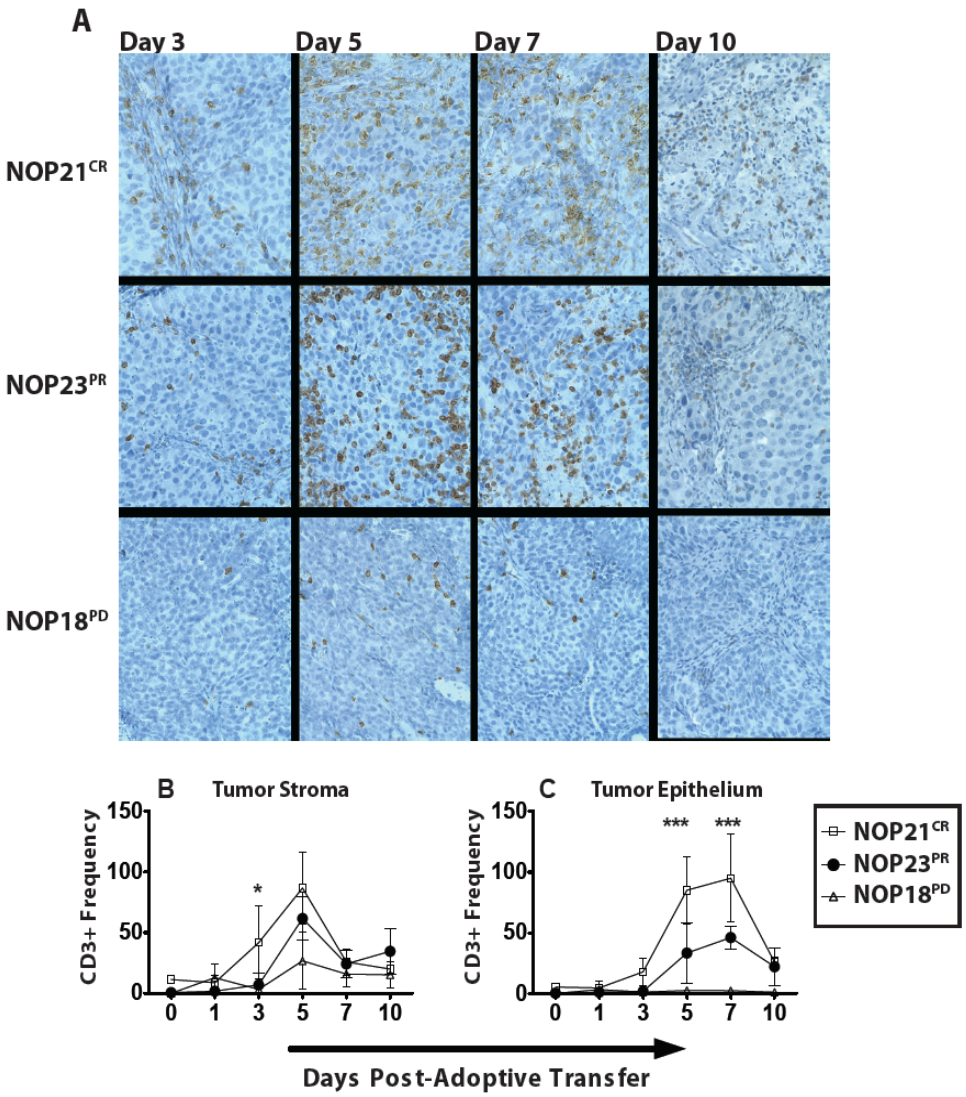

Figure 16. Infiltration of NOP21^{CR}, NOP23^{PR} and NOP18^{PD} tumours following adoptive transfer of OT-I and OT-II

(**A**) Immunohistochemical detection of CD3+ cells in NOP21^{CR} (top row), NOP23^{PR} (middle row) and NOP18^{PD} tumours (bottom row) on Days 3, 5, 7 and 10 after AT of 15x10^{6} OT-I and OT-II splenocytes *(200x magnification).* **(B,C)** Results of Chalkley grid analysis of CD3+ cell infiltrates in the stromal and epithelial compartments of tumours derived from the indicated cell lines. NOP21^{CR} tumours become infiltrated earlier and more densely than NOP23^{PR} and NOP18^{PD} tumours in **(B)** tumour stroma and **(C)** tumour epithelium. *(* indicates $p < 0.05$, *** indicates $p < 0.001$).*

3.4.4 Intratumoural proliferation of donor T cells

The paucity of T cells in NOP18^{PD} tumours could reflect an inherent barrier to T cell infiltration, or a hostile microenvironment for T cell activation and proliferation. To distinguish these possibilities, we assessed TIL for expression of CD44 and Ki67 across the three tumour lines. Similar to what was observed in DLNs, the vast majority (>90%) of OT-I and OT-II T cells were CD44^{hi} in NOP21^{CR} and NOP23^{PR} tumours, whereas CD44 expression was generally lower in NOP18^{PD} tumours (Figure 17A and B). The majority of tumour-infiltrating host CD4+ and CD8+ T cells were CD44^{hi} regardless of tumour cell line (data not shown).

The dynamics of intratumoural T cell proliferation were more complex, but showed a general association with outcome. In NOP21^{CR} tumours, OT-I and OT-II T cell proliferation peaked on Day 5 and declined thereafter (Figure 17C and D). In contrast, OT-I and OT-II proliferation did not peak until Day 7 in NOP23^{PR} tumours, and Days 7-10 in NOP18^{PD} tumours. Indeed, on Day 10, NOP18^{PD} tumours had markedly higher proportions of Ki67^{hi} OT-I and OT-II T cells than either NOP21^{CR} or NOP23^{PR} tumours. Proliferation of host CD4+ and CD8+ cells was roughly equivalent across the different tumours at all time points (mean = 10% and 12% respectively). TCR spectratyping of TIL from NOP21^{CR} tumours revealed that tumour-infiltrating host CD8+ T cells represented oligoclonal populations, consistent with the notion they were undergoing clonal expansion at the tumour site (Figure 18). Thus, despite the presence of modest (NOP23^{PR}) or profound (NOP18^{PD}) T cell infiltration barriers, all three tumour microenvironments supported the activation and proliferation of OT-I, OT-II and host T cells.

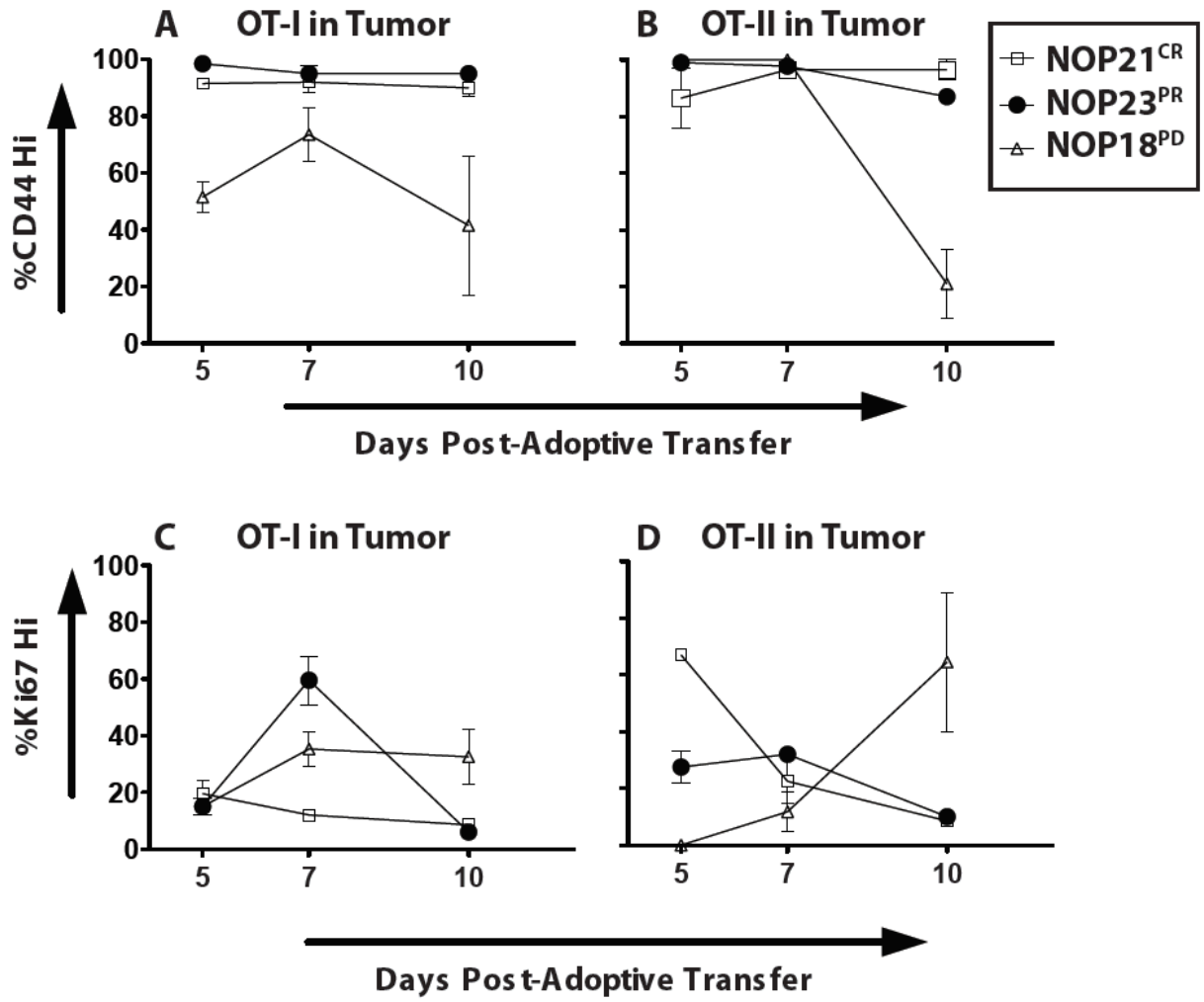

Figure 17. Activation and proliferation of tumour-infiltrating OT-I and OT-II cells

Tumour-infiltrating T cells from the mice shown in Figure 2 were analyzed by flow cytometry for expression of CD44 and Ki67 using the methods described in Figure 2. Cells were gated on CD4, CD8, and CD90.1 (which differentiates donor from host cells). **(A,B)** Expression of the activation marker CD44 by (**A**) OT-I cells and (**B**) OT-II cells. $NOP23^{PR}$ tumours induced equivalent activation of OT-I and OT-II cells compared to $NOP21^{CR}$ tumours, whereas the response to $NOP18^{PD}$ tumours was delayed, reduced and transient. **(C,D)** Expression of the proliferation marker Ki67 by (**C**) OT-I and (**D**) OT-II cells.

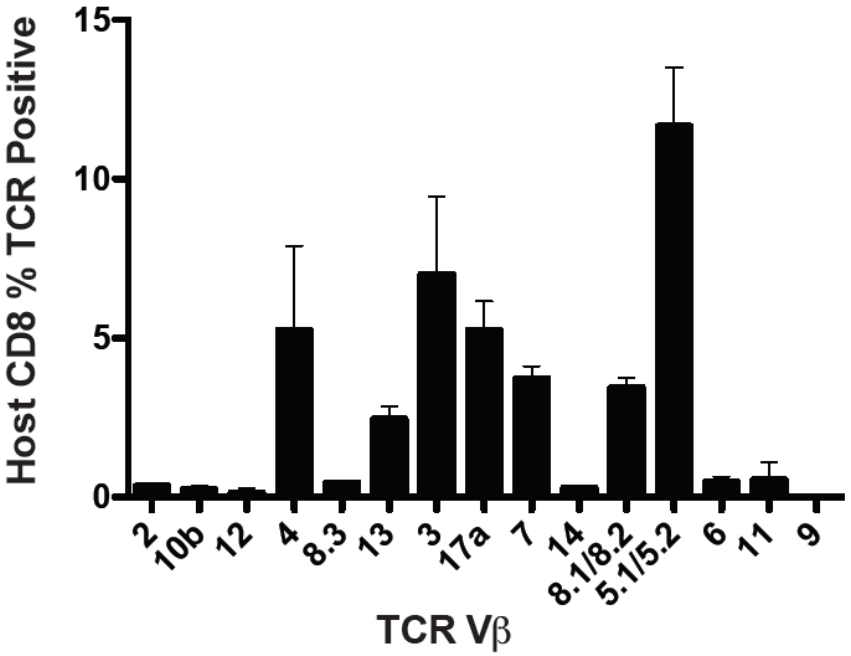

Figure 18. TCR spectratype analysis of endogenous CD8$^+$ NOP21^{CR} tumour infiltrating lymphocytes

Mice bearing NOP21^{CR} tumours were sacrificed 7 days after AT of OT-I and OT-II T cells. Tumours were harvested and tumour-infiltrating CD8$^+$ lymphocytes were stained with antibodies to a panel of T cell receptor beta-chain variable regions (TCR Vβ). The congenic cell marker Thy 1.1 was used to differentiate donor from host T cells. Oligoclonal subpopulations of endogenous CD8$^+$ tumour infiltrating lymphocytes were seen, including a predominant subpopulation expressing Vβ 5.1/5.2. Data is representative of two independent experiments

3.4.5 Histological determinants of T cell responses

Given the impaired T cell infiltration seen in the epithelium of NOP23^{PR} and NOP18^{PD} tumours, we hypothesized they might possess common histological features distinct from NOP21^{CR} tumours. To investigate this, we performed a histological study of untreated, H&E stained mammary tumours representative of the CR, PR and PD outcome categories. In addition to NOP21^{CR}, NOP23^{PR} and NOP18^{PD}, we included two additional tumours of the PR class (NOP12^{PR} and NOP13^{PR}) and four additional tumours of the PD class (NOP9^{PD}, NOP14^{PD}, NOP16^{PD}, and NOP17^{PD}), all of which expressed MHC class I, the SIINFEKL/MHC class I complex and Neu$^{OT\text{-}I/OT\text{-}II}$ (Figure 11). A total of 64 samples were evaluated, consisting of 1-9 biological replicates of each tumour. Tumours were assessed using standard histological criteria, and the reviewing pathologist was blinded with respect to outcome class. There were no reproducible differences in nuclear grade or glandular differentiation between any of the tumours (data not shown). CR tumours (NOP21^{CR}) had a lower necrotic index than any of the PR or PD tumours; however this did not reach statistical significance (Figure 19A). The PR tumours had a higher mitotic index than the PD or CR tumours, and this difference was significant between the CR and PR classes (Figure 19B). The most striking finding was that CR tumours had greater proportional stroma than either PR or PD tumours, meaning a greater proportion of the tumour sample was comprised of stroma rather than tumour epithelium (Figure 19C). Furthermore, the stroma in CR tumours had a looser appearance with less tightly packed collagenous fibrils, whereas PR and PD tumours had tightly packed stromal bundles that were limited primarily to the peripheral capsule of the tumour bed (Figure 19D and E). Thus, the proportion of tumour stroma distinguished tumours of the CR class from those of the PR and PD classes.

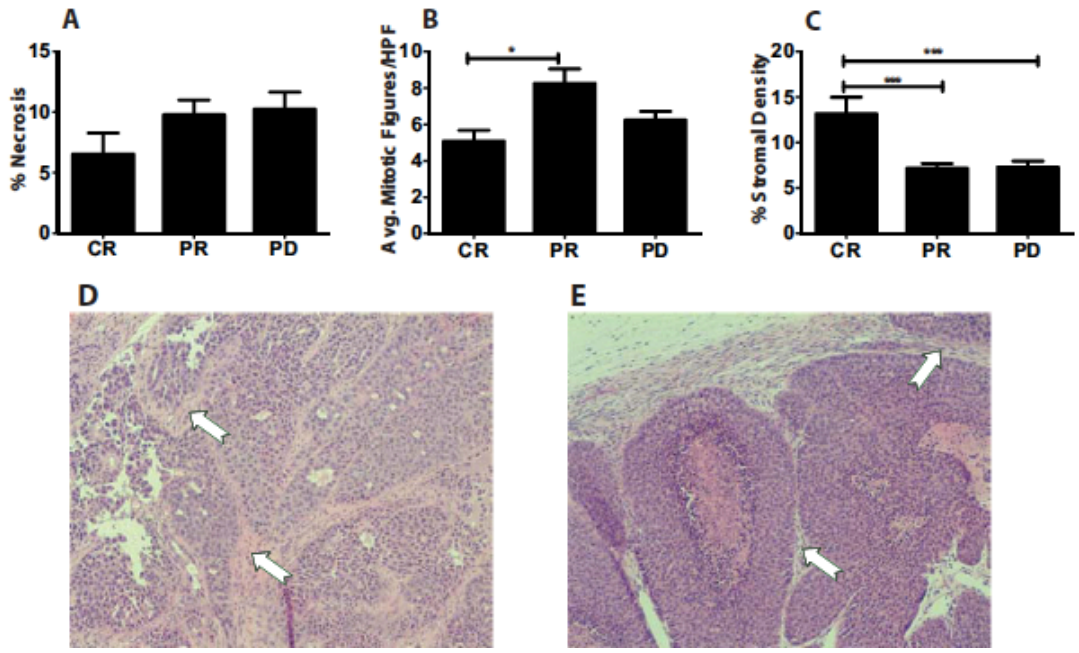

Figure 19. Histologic features of untreated tumours of the CR, PR and PD classes

(**A-C**) A blinded histologic analysis of 64 untreated, H&E stained tumours of the CR, PR, and PD classes revealed that CR tumours had **(A)** a lower necrotic index than PR and PD tumours, though this did not reach statistical significance. (**B**) PR tumours had a significantly ($p < 0.05$) greater mitotic index than CR tumours. (**C**) Untreated CR tumours had a significantly ($p < 0.001$) greater stromal density than PR or PD tumours. (**D,E**) Photomicrographs showing H&E stained tumours of the CR and PD classes. (200x magnification) **(D)** $NOP21^{CR}$ tumours had proportionally more stroma than PR or PD tumours, and the stroma had a looser, more collagen-rich appearance (white arrows). (**E**) PD tumours had proportionately less stroma, which was tightly packed with very few internodular bands (white arrows).

3.5 Discussion

We used a panel of mammary tumour lines that reproducibly undergo CR, PR or PD in response to adoptively transferred OT-I and OT-II T cells to identify the time and anatomical location at which T cell responses deviate from being successful (CR) to unsuccessful (PR or PD). PD responses were associated with early impairment of donor T cell activation and proliferation in draining lymph node, and severely inhibited trafficking of donor T cells to the tumour site. By contrast, CR and PR tumours triggered equivalent levels of donor T cell activation, proliferation and trafficking to the tumour site, but differed in the extent of T cell infiltration of tumour epithelium. Tumours of the CR class had proportionately more stroma than those of the PR or PD classes, suggesting that the density and composition of tumour stroma is an important determinant of outcome after AT.

$NOP18^{PD}$ tumours were characterized by a marked deficiency in the activation, proliferation and expansion of OT-I cells in the DLN (Figures 14 and 15). While $NOP18^{PD}$ tumours expressed $Neu^{OTI/OTII}$ by IHC (Figure 13), impaired antigen presentation by cells within the DLN may have occurred. To effectively activate tumour-reactive T cells, mature, activated dendritic cells (DCs) must be present in the DLN, expressing appropriate molecules supportive of T cell responses (297-299). Tumour microenvironments rich in VEGF, COX-2 or PGE_2 can result in impaired DC maturation and T cell response (298-301). Notably, host T cells in the DLNs of mice bearing $NOP18^{PD}$ $NOP21^{CR}$ and $NOP23^{PR}$ tumours showed similar activation and proliferation profiles, suggesting that the impaired activation of OT-I cells in $NOP18^{PD}$ tumours was restricted to tumour-derived antigens, as opposed to more generalized immune suppression. The impaired activation and proliferation of OT-I cells was also reflected by profoundly inhibited infiltration of $NOP18^{PD}$ tumours. Nonetheless, though few in number, OT-I cells proliferated within the tumour (Figure 17C), suggesting $NOP18^{PD}$ tumours are supportive of T cell proliferation but resistant to infiltration. Collectively, the results suggest that therapeutic responses to $NOP18^{PD}$ tumours at a

minimum need to be enhanced at the systemic level, for example, by vaccination with toll-like receptor agonists such as CpG oligonucleotides (302-304).

In contrast to NOP18^{PD} tumours, the activation and proliferation of OT-I and OT-II cells in DLN of mice bearing NOP23^{PR} tumours was equal to or greater than that seen with NOP21^{CR} tumours (Figure 14). Furthermore, large numbers of OT-I cells trafficked to NOP23^{PR} tumours, and stromal infiltration was as dense as seen with NOP21^{CR} tumours (Figures 15 and 16). However, OT-I cells showed approximately twofold less infiltration of tumour epithelium in NOP23^{PR} tumours compared to NOP21^{CR} tumours (Figure 16C). It is reasonable to speculate that local barriers may be inhibiting intraepithelial infiltration of tumour-reactive T cells, as has been described in other models (24, 262, 305-308). For example, Mrass and colleagues demonstrated that migratory activity of infiltrating OT-I cells required interactions between CD44 and the extracellular matrix (ECM) component hyaluronic acid (HA) (308). Low expression of HA in NOP23^{PR} tumours might hamper T cell infiltration into the epithelial compartment. Others have described deficient expression of the adhesion molecules ICAM1 and VCAM1 on the endothelium of intratumoural blood vessels, thus inhibiting the extravasation of tumour reactive T cells (18, 262), which could potentially be a factor with NOP23^{PR} tumours. Adjuvant treatments such as radiation or chemotherapy have been successfully used in other settings to disrupt local barriers and improve intratumoural T cell infiltration (309-314), and we are investigating these approaches for the treatment of NOP23^{PR} tumours.

NOP21^{CR} tumours present a rare experimental example in which large, established tumours undergo complete, permanent regression in response to AT alone, therefore we sought to identify histological features that may underlie this unique property. By H&E staining, NOP21^{CR} tumours were distinguished from those of the PR and PD classes by greater stromal content with a looser, more collagen-rich appearance. This suggests that the amount and/or composition of tumour stroma influence the sensitivity of tumours to T cells. The greater stromal content seen

in NOP21^{CR} tumours implies they may also have a greater vascular density. While this would be advantageous for tumour growth, it may also provide a conduit for tumour infiltration by T cells. As well, the increased stromal content of NOP21^{CR} tumours might reflect dependency on stromal-derived growth factors such as VEGF, CXCL14 and PDGF (315-318). Since tumour-reactive T cells can also kill stromal cells (289, 290), tumours that are highly dependent on stromal factors might be more sensitive to T cell attack. Finally, the stroma of NOP21^{CR} tumours had less compact collagenous bundles than tumours of the PR and PD classes, suggesting that intermolecular adhesions between collagen fibrils may be weaker, potentially facilitating T cell infiltration. Whatever the precise mechanism, increased stromal content appears to represent an 'Achilles heel' for NOP21^{CR} tumours. Intriguingly, gene expression profiles of tumour stroma are predictive of responses to conventional cancer therapy in human breast cancer, especially with HER2-positive tumours (319). This further highlights the need for improved understanding of the stromal compartment in breast cancer as a determinant of therapeutic outcome. We are currently investigating the relative expression of stroma-related genes in CR, PR and PD tumours, which may provide further insight into the relationship between tumour stroma and sensitivity to immunotherapy.

CHAPTER 4: Gene expression profiles associated with outcome of adoptive T cell therapy in a mouse model of breast cancer

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EHJC analyzed and interpreted bioinformatics data and wrote the manuscript.

DO, JH, HB, JW collected data & performed experiments.

KM collected data.

RJD, ADD & SJJ analyzed and interpreted data and edited the manuscript.

BHN designed experiments, analyzed and interpreted data and edited the manuscript.

4.1 Abstract

Adoptive T cell therapy (ACT) has been used as a treatment for cancer for over two decades, however clinical responses tend to be partial or mixed, largely due the heterogeneity of tumours. More recent approaches combining ACT with immunodepletion regimens and interleukin-2 therapy have shown some clinical benefit, but clinical responses remain largely unpredictable. To investigate potential tumour-specific determinants of clinical outcome following ACT in a breast cancer setting, we used murine mammary tumour lines that express an ovalbumin-tagged version of HER2/neu and reproducibly undergo complete regression (CR), partial regression (PR), or progressive disease (PD) after adoptive transfer of ovalbumin-specific $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells. We used the Affymetrix™ bioinformatics platform to compare gene expression in tumours across outcome classes (CR, PR, and PD). Genes associated with an increased immune response were significantly enriched in tumours with a CR phenotypes and decreased in tumours with a PD phenotype. We developed a preliminary 5-gene signature to predict outcome to ACT in five untested mammary tumour lines. Encouragingly, we were able to successfully predict outcome in 50% (25/50) of previously untested mammary tumours (3/5 tumour lines). These findings provide support for the approach to predictive testing of gene expression as a means to optimize patient selection and preconditioning in advance of ACT.

4.2 Introduction

The use of adoptive cell therapy (ACT) to treat advanced cancers using *in vitro* expanded, autologous cytotoxic T lymphocytes (CTLs) has been aggressively pursued in a number of tumour settings for close to three decades, generally with mixed clinical results (139, 144, 146, 149, 150, 320). Recent approaches using lymphodepleting-preconditioning regimens have resulted in an increased frequency of objective clinical responses to ACT, particularly in the setting of malignant melanoma (89, 222, 321, 322). Nevertheless, durability of responses and overall survival rates remain highly variable amongst recipients of ACT.

In an effort to improve the likelihood of clinical responses, strategies are being explored that use genetically engineered T cells aimed at circumventing common tumour derived escape mechanisms such as deficiencies in T cell costimulation signals (209, 323-326). While encouraging progress continues to be made in the optimization of the experimental T cell inputs, troubling side effects related to on- or off-target effects warrant prudence as these approaches move forward (16). Furthermore, technical and time-intensive techniques may limit the widespread use of these treatments to only a few highly specialized treatment centers. As such, mixed clinical responses are likely to remain a feature of the clinical ACT approach for the foreseeable future. With this in mind, the ability to predict tumour susceptibility to ACT will allow clinicians to more practically apply or combine targeted approaches. In addition, patients will benefit from improved clinical responses following ACT.

At present, predicting clinical response to ACT remains primarily speculative and based largely on *in vitro* characteristics of tumour infiltrating lymphocytes (TIL) pre-infusion and the antigenicity of the targeted tumour (327, 328). However, in addition to these two critical features, a successful immune response following ACT also requires a series of effective immunological events. First, tumour antigens must be taken up and presented by competent antigen presentation cells (APCs) such as dendritic cells and macrophages. This must be followed by

antigen-specific recognition by and activation of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) within the tumour-draining lymph node. Next, activated CTLs must traffic to the tumour site, infiltrate the tumour epithelium and affect direct killing via cytolytic granules, or induce ligand-mediated tumour cell apoptosis. To accomplish this, critical cytokines including granulocyte-macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFNγ), interleukin 2 (IL-2) and interleukin 18 (IL-18) must be secreted locally by a variety of cells (1, 2). Deficiencies in production and secretion of these cytokines at any stage can result in alterations in activation, trafficking, and/or infiltration of CTLs. Thus, insufficiencies in soluble mediators may result in an ineffective cytotoxic response to the tumour and subsequent immune escape (329-334).

To circumvent the immune response, tumours have evolved a number of well-described local and systemic strategies. Important examples include the secretion of suppressive cytokines such as IL-10 and TGFβ by tumour cells, and the recruitment of suppressive cells constituents such as FoxP3+ regulatory T cells and myeloid derived suppressor cells (43-45). Tumour cell or APC-induced expression of T cell regulatory molecules including CTLA-4 can inhibit intratumoural T cell activation and proliferation (285). A microenvironmental milieu that inhibits T cell migration and diapedesis can arise with alterations in the expression of adhesion molecules such as ICAM1 and VCAM1 (19). Alterations in junctional proteins including ZO-1 and Claudins 1 and 4 can affect polarization and cell-cell communication within the tumour itself, or within the supporting normal or neoplastic stroma (335-337). Clinical strategies including cytokine therapy (134) CTLA-4 blockade (285, 338), and targeted radiation therapy (302, 339, 340) have been investigated in an attempt to alter the tumour microenvironment and thus augment the host immune response in the face of tumour-induced immunosuppression.

While the literature is replete with gene expression studies describing predicted tumour behavior to chemotherapeutic or other treatment regimens, the same cannot be said for the ACT setting

(341-346). To address this shortfall, we have used a unique murine mammary carcinoma model developed by our laboratory, in which spontaneously arising tumours demonstrate a wide range of clinical responses to adoptively transferred $CD4^+$ and $CD8^+$ T cells (13, 22, 293). Briefly, we tagged the HER2/*neu* oncogene at its C terminus with $CD4^+$ and $CD8^+$ T cell epitopes from the model antigen ovalbumin (OVA). Mice expressing epitope-tagged Neu ($Neu^{OT\text{-}I/OT\text{-}II}$) as a transgene in mammary epithelium, together with a dominant-negative version of p53, develop spontaneous mammary tumours at 6-10 months of age. Tumours are recognized by OVA-specific $CD8^+$ and $CD4^+$ T cells from TCR transgenic OT-I and OT-II mice, respectively. Remarkably, about one third of spontaneous tumours undergo complete regression (CR) in response to adoptively transferred OT-I and OT-II cells, whereas the remaining tumours demonstrate partial responses (PR; 25% of tumours), stable disease (SD; 15% of tumours) or progressive disease (PD; 24% of tumours). Importantly, we have successfully generated a panel of cell lines from these tumours that, when implanted in the mammary fat pad of host mice, form tumours that reproducibly undergo CR, PR, or PD in response to adoptively transferred OT-I and OT-II T cells. Thus, these tumour cell lines provide a unique experimental system to identify *a priori*, genetic and morphologic features that contribute to outcomes following ACT.

In the present study, we performed gene expression profiling of tumours representing CR, PR and PD classes using this mammary tumour model. This revealed several immune-related pathways that were activated uniquely in CR tumours. Intriguingly, several genes from these pathways showed promise as predictive markers for outcome following ACT. Indeed, we were able to successfully predict outcome to ACT in 3/5 previously untested mammary tumour lines. By comparing our gene expression profiles to publicly available human breast cancer datasets, we found that tumours from different T cell response classes resembled distinct histological subtypes of human breast cancer. Thus, our findings support the hypothesis that features associated with the tumour can be used to predict outcome following ACT in a murine model of

breast cancer. Importantly, these features might translate to the human breast cancer setting, providing clinicians with predictive tools in a clinical immunotherapy setting.

4.3 Materials and methods

4.3.1 Murine mammary tumours

This study followed Canadian Council for Animal Care guidelines and was approved by the University of Victoria Animal Care Committee. Murine tumour samples used in this study originated from cell lines generated from a transgenic mouse tumour model described by Wall and colleagues (293). 'NOP' Cell lines were derived, characterized, and tested *in vivo* as described previously (13, 22, 293). Tumours generated from these cell lines consistently showed distinctive and reproducible responses to ACT, namely: complete regression (CR) (no detectable tumour); partial response (PR) (>50% reduction from the original tumour size); stable disease (SD) ($\leq$50% reduction or $\leq$25% increase in tumour size); and progressive disease (PD) (>25% increase in tumour size). Tumours were implanted *in vivo* as described previously and measured 2-3 times weekly using Vernier calipers. When tumours reached approximately 50mm^2 in size, they were either excised and snap frozen using vaporized liquid nitrogen, or where indicated, tumour-bearing mice were treated with adoptive T cell therapy (ACT) as previously described (13, 22, 293). Importantly, tumours excised and frozen were not treated with ACT prior to excision. The naming convention of the NOP tumour cell lines with outcome in superscript will be used throughout this chapter (i.e. NOP21^{CR} for line NOP21 which showed a complete response (CR)).

4.3.2 Adoptive cell therapy (ACT)

Lymph nodes and spleens were prepared as previously described (13, 22, 293). Typically, 15 x 10^6 each of OT-I and OT-II splenocytes (comprising ~4.5 x 10^6 of each OT-I and OT-II cells) were injected via tail vein into tumour-bearing mice. Tumours were measured regularly to assess response to ACT.

4.3.3 Microarray construction

Ex vivo harvested, flash frozen tumours from six NOP tumour cell lines not previously treated with ACT (NOP21^{CR}, NOP13^{PR}, NOP23^{PR}, NOP6^{PD}, NOP17^{PD} and NOP18^{PD}) were subjected to mRNA expression profiling using microarrays. Two biological replicates from each tumour line were subjected to Affymetrix mouse exon array analysis (MoEx-1_0-st-v1, Santa Clara, USA) following the protocol from the GeneChip® Whole Transcript Sense Target Labeling Assay (Affymetrix, USA). Frozen tumours were processed and RNA extracted by the Centre for Translational and Applied Genomics (CTAG, Vancouver, British Columbia). Using standard methods, 1-2 μg of total RNA was extracted and purified. The poly-adenylated RNA molecules were captured, and subsequently underwent further purification processes to reduce non-mRNAs and concentrate poly-adenylated RNAs in a sample. The purified poly-adenylated RNAs were reverse-transcribed to cDNAs, and then amplified using standard methods. The 5'-to-3' sense strands of the cDNA duplexes were isolated and chemically sheared to shorter fragments whose 3' terminals were chemically bonded with fluorescent labels. The fluorescence-labeled single-stranded cDNA fragments were then hybridized to probes on a microarray, and subsequently scanned by a laser to produce the fluorescence intensity, proportional to the absolute amount of cDNA fragments hybridized to a standardized probe for the mouse transcriptome. The scanned microarray image was stored in an image ('CEL') file and provided by CTAG for further analysis.

4.3.4 Microarray data and cluster analysis

The raw probe intensity values were generated from microarray images in CEL files and analyzed further using the Expression Console software provided by Affymetrix Inc. The raw intensity values were normalized by the sketch-quantile normalization method and then summarized using the median polished Robust Multichip Average (RMA) method (347). All probe values were analyzed at the gene level. The intensity values of the 'core' probes that

were designed from the Refseq full-length mRNA sequences were considered for further analyses, as the core probes were designed with the highest confidence in annotation. Notably, the microarrays were prepared in two separate batches on different dates. The systemic bias, also known as the 'batch effect', which arose from this non-biological variation was corrected using the R script, Combat.R, which was shown to work optimally for a microarray experiment with a small sample size (N < 25) (348).

Genes with differential expression were detected using the Significance Analysis of Microarrays (SAM) methodology (349), which was implemented in the R Bioconductor package, 'siggenes'. As a measure of statistical significance of differential gene expression in tumours from different outcome groups, the false discovery rate (FDR) of 0.10 was set as the significance threshold, except when CR and PR tumour groups were compared. Due to the low number of genes found to be significantly differentially expressed at the FDR 0.10, an FDR threshold of 0.15 was used to define the differentially expressed gene set between CR and PR.

To optimize for clustering analysis, probe intensity values were transformed into ratio-like values by normalizing to the median probe values across all arrays. Hierarchical clustering analysis was performed on log2-transformed ratio-like probe values using Java Cluster (version 3.0) (350), which was adapted from the original Cluster software written by Michael Eisen (351). Clustering of genes and samples were performed based on the iterative distance calculation between two hypothetical clusters by taking all pair-wise distances between data points and subsequently cluster those with the maximum distance. The Pearson correlation coefficient was used to construct the similarity distance matrix. The clustering result was visualized using TreeView software (350). From the clustering analysis, the top 100 differentially expressed genes (ranked by q-values from the SAM analysis) in CR tumours relative to PD tumours were identified.

4.3.5 Outcome-specific differential expression and classifier identification

Genes whose expression profiles were differential exclusively in a single outcome group compared to the others were designated as 'outcome specific' (OS) genes. OS genes were identified by taking the overlap of differentially expressed genes from two different kinds of comparisons: (1) a comparison of two combined outcome groups against a specific outcome group e.g. for CR-specific genes, CR vs (PR and PD); (2) a series of pair-wise comparisons to exclude differentially expressed genes from a specific outcome group that were also differentially expressed in the other outcome groups e.g. for CR-specific genes, (CR versus PD) – (PR versus PD).

Differentially expressed (DE) genes between CR and PD tumours (N = 1,242) were analyzed to identify those that could classify different outcomes following ACT. 'Classifier' analysis was carried out using the Predictive Analysis of Microarray (PAM) method (352) based on a supervised clustering of nearest shrunken centroids, which was implemented in the pamr function in the R bioconductor package. The threshold for the centroid shrinkage, (the delta value), was chosen to represent a FDR rate of <0.01.

4.3.6 Pathway enrichment analyses

To analyze pathway enrichment, DE genes were mapped to well-known canonical pathways using Ingenuity Pathways Analysis software (Ingenuity® Systems, Redwood city, CA, USA) (353). P-values generated from the Ingenuity software were calculated using modified Fisher's exact test and then corrected for multiple hypotheses testing using the Benjamini-Hochberg method. Additional pathway analyses were also done through the use of an on-line bioinformatics tool, DAVID (354), which collated the pathway information from BioCarta and KEGG databases (355, 356). The p-values from DAVID were also corrected for multiple hypotheses testing using the Benjamini-Hochberg method.

4.3.7 Validation of outcome-specific differentially expressed genes

Genes were ranked by fold change difference in expression between CR and PD tumours. Genes found as classifiers according to the PAM analysis were weighted preferentially in ranking. Genes were also considered for their involvement in immune-related pathways that were significantly enriched for outcome-specific genes (corrected p-value < 0.05), the likelihood for therapeutic potential, and potential amenability to *in vitro* and *in vivo* RNA interference according to the literature. A total of 33 genes were selected for empirical validation using real-time quantitative PCR (qPCR) (Table 5).

4.3.8 Real-time quantitative PCR

Whole tumours were homogenized from a snap-frozen state using theTissueRuptor™ instrument (catalogue #9001271) and the QIAzol™ lysis reagent (catalogue #79306, both from Qiagen, Missisauga, Ontario, Canada), following manufacturer's directions. Total RNA was extracted from lysed tumour using the miRNeasy™ kit (Qiagen, catalogue #217004), and the qScript cDNA SuperMix™ kit (catalogue #95048-100, Quanta Biosciences, Gaithersburg, MD) was used to synthesize cDNA, all following manufacturer's directions. Quality and concentration of extracted RNA and synthesized cDNA were measured using the NanoDrop® ND-1000 instrument (Thermo Fisher Scientific, Waltham, MA). QPCR reactions were made in triplicate using the PerfeCta® SYBR® Green SuperMix kit, (catalogue #95053-500, Quanta Biosciences) following manufacturer's directions, and two technical replicates were used for each experiment. Intron-spanning primers from Integrated DNA technologies (Coralville, Iowa) were designed using standard methods and used following manufacture's directions (Appendix 4). QPCR reactions were measured using the MyiQ Thermocycler instrument (Bio-Rad, Hercules CA), and analyzed using the GeneX software program. GraphPad Prism® software was used to compile and analyze cumulative data. QPCR reactions were measured and analyzed to report the

relative abundance of a specific mRNA, which was normalized against the expression level of β-actin.

4.3.9 Outcome-predicting algorithm used for untested NOP tumours

Mean expression was determined for 'putative predictive signature' (PPS) genes in PR and PD tumours and quantified relative to the CR tumour ($NOP21^{CR}$), which was set to a value of '1'. Similarly, mean expression was then quantified for tumours with unknown outcome (NOP7, NOP20, NOP29, NOP37 and NOP38). To normalize gene expression and eliminate the potential bias associated with genes having a larger variation in expression levels (e.g. Reln, Cldn4), the standard deviation for each of the PPS genes was calculated across all tested tumours (known and unknown outcome categories). The relative expression of each PPS gene was then compared between the untested NOP tumour and each outcome class (i.e. CR, PR, and PD), and expressed as a difference in standard deviations. Standard deviations for each gene in a comparison category were then summed and expressed as an overall 'Delta Score'. The category with the lowest Delta Score was used as a predictor of outcome following ACT for each untested tumour (Appendix 5). Statistics were calculated using GraphPad Prism® software.

4.3.10 Gene expression comparison with human breast cancer subtypes

The CEL files of 82 whole-tissue breast tumours from breast cancer patients used in the study by Rouzier and colleagues (357) were obtained from Dr. Lajos Pusztai through a personal correspondence. Rouzier and colleagues used HG-U133A Affymetrix 3' expression array to profile gene expression. In line with our approach in this study, the raw probe intensity values extracted from the CEL files were normalized using the sketch-quantile normalization method and then summarized using the RMA method using the Expression Console software from Affymetrix. Systematic bias arising from separate batches on different microarray platforms for human and mouse breast tumours was corrected using the distance weighted discrimination

(DWD) method, which was shown to work optimally for microarray experiments with a larger sample size (N > 25) (358).

To combine the genes from the mouse and human data sets to compare probe intensities across different microarrays, the Ensembl database was used to pair up the annotated mouse and human orthologous genes. The mouse-human merged gene set was filtered for a one-to-one orthologous relationship between mouse and human genes in the Ensemble database using a web-based cross-databases query program, Biomart (359). The orthologous pairs of mouse and human genes were further filtered to obtain genes targeted by probes annotated from both the human HG-U133A and mouse exon microarrays. Where multiple probes annotated a single gene, the median value was taken to represent overall gene expression. Genes with ambiguous biological annotation, e.g. a single probe annotated to multiple target genes, were excluded from further analyses.

Herschkowitz et al. (360) developed a cross-species (mouse-human) intrinsic breast cancer gene set (N = 106) that demonstrated similarities between mammary tumours from a variety of mouse models and human breast cancer subtypes. This gene set was obtained from Jason Herschkowitz via a personal correspondence. Genes from this list that were not targeted by probes on either human or mouse microarrays were excluded from further analyses.

A total of 82 genes passed the filtering process and were clustered across 94 human and mouse merged microarray datasets. Unsupervised hierarchical clustering on the merged dataset was performed using the Java Cluster (version 3.0) program. The data transformation of the probe intensity values for clustering was carried out in the same process as the mouse microarray data analysis. The parameters used for the clustering algorithm were also the same as the clustering analysis of the mouse microarray data. The pvclust function (361) from the R packages were used to generate bootstrap samplings from the human-mouse merged dataset and calculated

the approximately unbiased (AU) probability values of the bootstrap re-sampling result to assess the statistical significance of the hierarchical clustering result.

4.4 Results

4.4.1 Genes in NOP tumours with responsive outcomes cluster together

Hierarchical clustering analysis that compared differentially expressed genes in all three outcome groups (i.e. CR, PR and PD) revealed that biological replicates clustered tightly together (Figure 20). To establish whether there were significant gene expression differences between tumours with distinct responses to ACT, gene expression profiles were compared amongst the three distinct outcome groups: CR, PR and PD. A two-class unpaired modified t-test for three combinations of pair-wise comparisons among CR, PR and PD was performed using the Significance Analysis Microarray (SAM) tool (349) to identify differentially expressed genes. The pair-wise comparison of CR and PD tumours identified 1,242 differentially expressed genes, whereas the pair-wise comparison of PR and PD tumours identified 1,466 differentially expressed genes at a false discovery rate (FDR) of 0.10. Only 6 genes were differentially expressed between CR and PR tumours, suggesting high similarities between these two tumour groups at the transcriptome level.

This finding was in accordance with the reproducibility in outcomes seen *in vivo* following ACT with mice bearing tumours from each of the cell lines. Further, high similarity between the CR and PR groups was also observed, leaving PD tumours as a separate group (Figure 20). This suggested that CR and PR tumours might differ in gene expression only in very subtle ways. Thus, tumours with an ACT outcome-responsive phenotype (CR and PR) were highly similar to one another from a gene expression perspective, suggesting subtle differences might contribute to later stage immune escape.

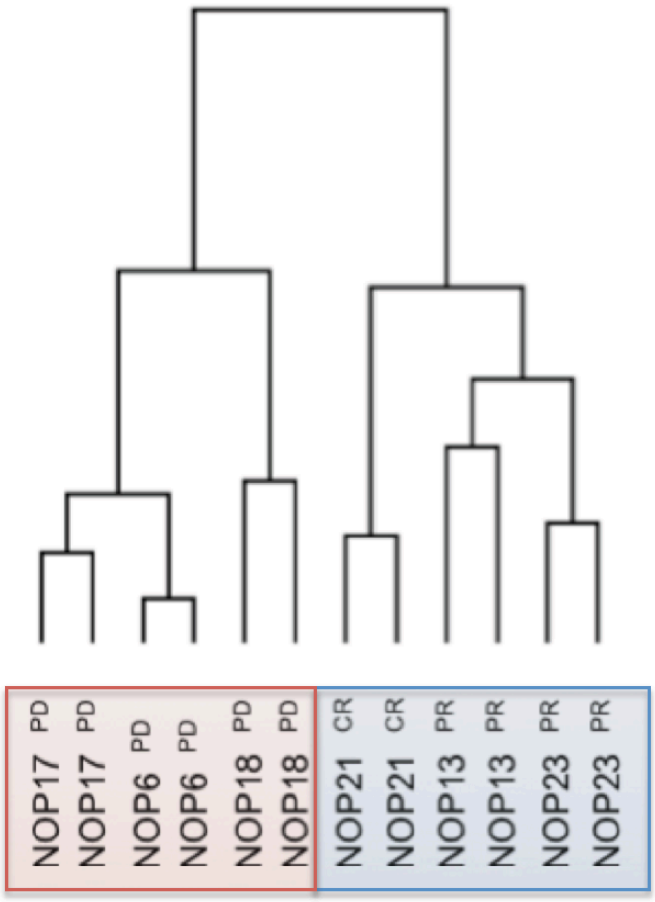


Figure 20. NOP tumours with responsive outcomes cluster together

Dendrogram generated from hierarchical clustering of gene expression profiles from tumours derived from cell lines, NOP21^{CR}, NOP13^{PR}, NOP23^{PR}, NOP6^{PD}, NOP17^{PD} and NOP18^{PD}. Close relationships were observed between biological replicates, and clear distinctions were seen amongst the responding (CR and PR – shown in blue) and non-responding (PD – shown in red) outcome groups.

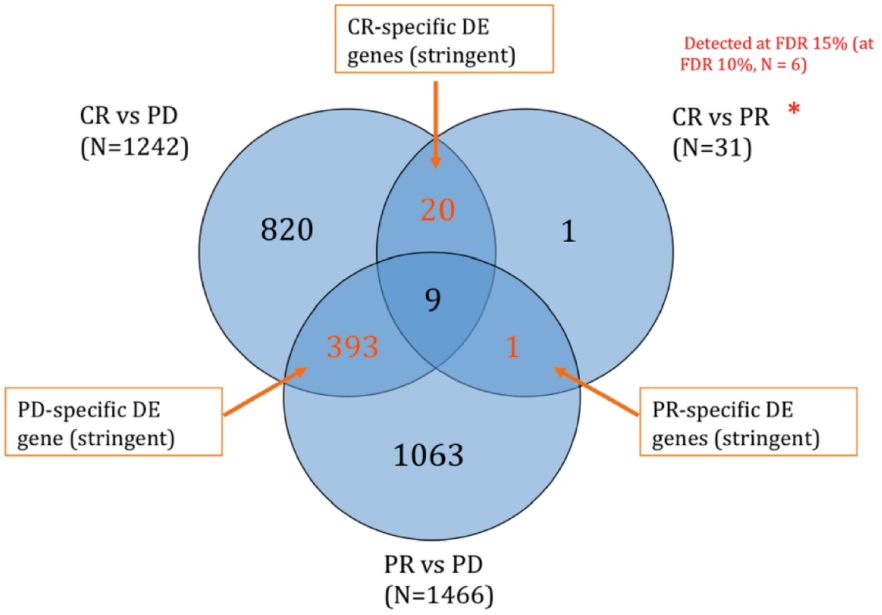
4.4.2 Outcome specific genes identified between ACT-responsive and -unresponsive phenotypes

To identify outcome specific (O/S) genes that may be associated with the biological pathways regulating the response of tumours to ACT, gene expression comparison amongst tumours from each of the outcome groups was first considered. Genes that were differentially expressed specifically in a single outcome group compared to the other two groups were identified in this analysis. Specifically, genes were compared in CR tumours versus PR tumours, CR tumours versus PD tumours, and PR tumours versus PD tumours. Using this approach and an FDR of 0.10, all three pair-wise combinations were evaluated to identify O/S genes. For the CR class, 20 genes were identified, with 393 and 1 genes identified for the PD and PR classes, respectively (Figure 21A). Further analysis revealed that this approach did not result in a gene list sufficient to yield statistical power. Additionally, using an FDR of 0.10 to compare CR and PR outcome classes yielded only 6 differentially expressed genes between these two classes. Therefore, we used an FDR of 0.15 to compare CR and PR outcome classes, which yielded a larger number of genes to consider for further analysis (31 versus 6, respectively). In spite of this minor manipulation, statistical power was still not achieved solely using this comparison technique, thus an additional strategy was applied to increase the gene list for further analysis.

In order to improve statistical power for pathway analysis, O/S genes were further identified by taking the overlap of DE genes using 'lenient two-state' pair-wise comparisons (i.e. CR versus PR + PD, PR versus CR + PD, and PD versus CR + PR) and the combined outcome group comparisons (Figure 21B). By using the more lenient approach, a total of 1,170 genes were identified for further pathway analysis. This consisted of 229 CR-specific, 42 PR-specific and 899 PD-specific genes.

Finally, recognizing the low number of genes discriminating the CR and PR outcome groups, the decision was made to limit further analysis strictly to genes between the CR and PD outcome classes. By limiting our analysis to the extremes of the outcome continuum (i.e. CR versus PD), we hypothesized that we might identify differential expression of a greater fold expression. By contrast, DE between either the CR and PR outcome groups or PR and PD outcome groups might be of a lower magnitude. Thus, our pathway analysis reviewed O/S differential gene expression between only the CR and PD outcome classes using a total of 1,128 genes (229 CR specific + 899 PD-specific).

A



B

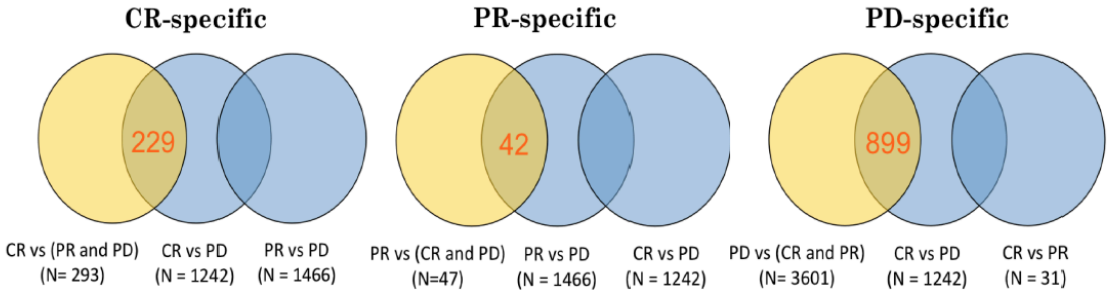


Figure 21. Identification of 'outcome-specific (O/S)' differentially expressed (DE) genes

DE genes were compared in a combination of pair-wise comparisons and the combined outcome group comparisons: **(A)** Stringent comparisons of all three pair-wise comparisons, **(B)** lenient comparisons to find overlapping genes from both two pair-wise comparisons and the combined outcome classes. 229 CR-specific, 42 PR-specific and 899 PD-specific genes were identified. CR-specific and PD-specific genes were used for further pathway analysis.

4.4.3 Outcome-specific genes were significantly enriched within immune-related pathways

To identify whether O/S genes were associated with specific pathways relevant to the immune response, the 1,128 outcome-specific genes were mapped to known pathways using the well-established Ingenuity Pathways Analysis software (Ingenuity® Systems, Redwood city, CA, USA) (353). The majority of the O/S pathways were found to be immune-related pathways. Pathways significantly enriched for CR-specific genes included the complement system, pattern recognition receptor, TREM1 signaling, fibrogenesis, and dendritic cell maturation pathways (Table 4A). Pathways significantly enriched for PD-specific DE genes included interleukin-3 (IL-3) signaling, fibrogenesis, granulocyte macrophage-colony stimulating factor (GM-CSF) signaling, leukocyte extravasation signaling, interferon signaling, and complement system pathways (Table 4B). Intriguingly, overlap between the two O/S gene lists was observed (e.g. complement and cytosolic pattern recognition receptor IRF activation pathways), and further analysis of these pathways revealed that, in general, pro-inflammatory genes were upregulated in the CR tumours, whereas immune response-regulatory genes were upregulated in PD by tumours (data not shown). Thus, pro-inflammatory signals might contribute to a more 'immune-favorable' tumour microenvironment, resulting in improved CTL infiltration and favorable outcome following ACT.

In parallel with our analysis using the Ingenuity® software system, another web-based bioinformatics software, DAVID (354), was used to test the robustness of our pathway analysis. DAVID performs pathway enrichment analysis using multiple pathway databases including KEGG (356) and BioCarta (355). In accordance with our findings using the Ingenuity® system, the complement pathway was significantly enriched for CR-specific genes in KEGG (corrected p-value = 0.0054). The complement pathway was also the top ranked pathway in BioCarta, however the p-value was not significant after multiple hypothesis testing correction (corrected p-

value = 0.409). Interestingly, using KEGG, the extra cellular matrix receptor interaction pathway was also identified as significantly enriched for PD-specific genes, (corrected p-value = 0.00702). Thus, pathway enrichment analyses using multiple databases provided support for our findings that immune-associated pathways were highly relevant for O/S genes.

From our pathway enrichment analysis, O/S genes were reviewed for further validation. Genes were selected based upon magnitude of differential expression (i.e. fold difference between CR and PD) within their specific pathways, relevance in the setting of breast or other cancers based on a literature review, potential for therapeutic intervention, availability of experimental antibodies, and theoretical amenability to *in vitro* and *in vivo* manipulation using RNA interference. Finally, genes identified to be significant classifiers according to Predictive Analysis of Microarray (PAM) analysis with a threshold value of 3.0 (FDR <0.01) were given precedence in ranking. From this review, 33 genes were selected for further validation (Table 5).

A

CR-Specific Pathway Name	p-Value	Example Genes
LXR/RXR	6.92^{-4}	+CD36, +Abca1
TREM1	3.06^{-4}	+Tyrobp, +Trem1
Cytosolic Pattern Recognition Receptor	2.48^{-3}	+Ifih1, +TLR7
Pattern Recognition	1.83^{-5}	+Irf7, +TLR7
Complement	1.06^{-8}	+CD55, +Cfb, +C1q, +Masp2,+C3aR, +C6
Fibrogenesis	0.012	+FIGF
Dendritic Cell Maturation	0.012	+Tyrobp, +IL18

B

PD-Specific Pathway Name	p-Value	Example Genes
Pattern Recognition	4.85^{-5}	-Irf7, -TLR7
IL-3	4.08^{-4}	+Shc1, -Jak1
Cytosolic Pattern Recognition Receptor	2.86^{-3}	-Ifih1, -TLR7
Complement	2.86^{-3}	-CD55, -Cfb
Aminosugars Metabolism	2.55^{-3}	-CHIT1,-PDE4B
Fibrogenesis	1.82^{-3}	-FIGF, -MMP2
GM-CSF Signaling	1.82^{-3}	-Ppp3ca, -Cam2kd
Leukocyte Extravasation Signaling	1.82^{-3}	+Cldn4, +Rapgef3, -Mmp14, -Pik3cg
Interferon Signaling	1.82^{-3}	-Irf9, -Jak1
FcγRIIB	1.82^{-3}	+Shc1, -Inpp5d

Table 4. Significantly enriched biological pathways

Ingenuity® software system-identified biological pathways significantly enriched based on (**A**) CR-specific differentially expressed genes (N=229), and (**B**) PD-specific differentially expressed genes (N=899). Examples of differentially expressed genes within each pathway are shown in the third column of each table; '+' is used if the example gene is relatively upregulated, and '-' is used if the example gene is relatively downregulated.

Pathway Name(s)	Gene Name	Affymetrix Fold Change (PD vs CR)
ECM Receptor	*Npnt	-9.63
Pattern Recognition	Irf7	-6.43
ECM Receptor	Col11a1	-6.04
ECM Receptor	*Reln	-5.72
LXR/RXR, ECM Receptor	Cd36	-5.56
ECM Receptor	Thbs4	-5.19
Pattern Recognition	TLR7	-4.89
Pattern Recognition	Ifih1	-4.43
Complement	Serping1	-4.15
Leukocyte Extravasation	Mmp2	-4.10
Complement	Cfb	-3.92
Leukocyte Extravasation	Cybb	-3.92
Complement	Cd55	-3.86
Leukocyte Extravasation	Cldn1	-3.72
Pattern Recognition	TLR8	-3.49
TREM1	Tyrobp	-3.14
TREM1	*Trem1	-3.13
Leukocyte Extravasation	Mmp13	-2.91
LXR/RXR	Abca1	-2.82
LXR/RXR, TREM1	*IL-18	-2.77
Leukocyte Extravasation	*Pik3cg	-2.75
IL-3	Pak1	-2.70
FcγRIIB, IL-3	Inpp5d	-2.55
Leukocyte Extravasation	*Mmp14	-2.46
FcγRIIB	Blnk	-2.46
GM-CSF, IL-3	*Ppp3ca	-2.31
LXR/RXR	Cd14	-2.11
GM-CSF	Camk2d	-2.11
FcγRIIB, Pattern Recognition	Syk	-2.05
IL-3	Jak1	-1.91
GM-CSF, FcγRIIB, IL-3	Shc1	+2.24
Leukocyte Extravasation	Rapgef3	+2.83
Leukocyte Extravasation	*Cldn4	+4.67

Table 5. Ingenuity® software system-identified genes differentially expressed between CR and PD tumours

Genes were selected based upon magnitude of differential expression within their specific pathways, relevance in the setting of breast or other cancers based on a literature review, potential for therapeutic intervention, availability of experimental antibodies, and theoretical

amenability to *in vitro* and *in vivo* manipulation using RNA interference. A '-' is used if the gene is downregulated in the PD versus the CR, and a '+' is used if the gene is upregulated in the PD versus the CR. Genes identified to be significant classifiers according to Predictive Analysis of Microarray (PAM) analysis are indicated with an asterisk.

4.4.4 Validation of selected outcome specific genes using quantitative PCR

Quantitative PCR (qPCR) was used to experimentally validate the 33 selected O/S genes associated with immune-related pathways. In addition to the six tumours analyzed by Affymetrix (NOP21^{CR}, NOP13^{PR}, NOP23^{PR}, NOP6^{PD}, NOP17^{PD} and NOP18^{PD}; the 'training set'), expression profiles of the selected genes were assessed in four additional NOP tumours exhibiting PR and PD outcomes (NOP12^{PR}, NOP9^{PD}, NOP14^{PD} and NOP16^{PD}; the 'validation set'). Using qPCR, the relative gene expression in all 10 tumours was assessed and compared to gene expression in NOP21^{CR} tumours (i.e. gene expression in NOP21^{CR} was set at 1). Next, the relative gene expression was averaged across the outcome groups for all PR and PD tumours, again using NOP21^{CR} gene expression as a comparator.

Consistency in gene expression patterns predicted by Affymetrix and detected using qPCR in the CR versus PD tumours was used as a preliminary screen to identify genes worthy of further scrutiny. For example, if Affymetrix analysis predicted gene expression to be overexpressed in PD tumours versus CR tumours, and this was validated by qPCR expression, the gene was accepted. As a further benchmark, a gene expression fold difference of 1.5 times in magnitude between CR and PD tumours as determined by qPCR was set as a cut-off to consider the gene differentially expressed to a measurable degree. Thus, 14 genes were validated from the original list of 33 genes, supporting the hypothesis that there were pathway-associated genes with differential expression between tumours with an ACT-responsive phenotype (i.e. CR tumours), and those with an ACT-unresponsive phenotype (i.e. PD tumours) (Table 6).

Gene Name	Pathway(s) involved	Fold difference: Affymetrix (PDvsCR)	Fold difference: qPCR (PDvsCR)	Gene Information
Reln*	Extracellular Matrix Receptor	-5.72	-8.40	Extracellular glycoprotein regulating neuronal migration during brain development. Silencing implicated in gastric & pancreatic cancer.
IL-18*	LXR/RXR, TREM1	-2.76	-3.57	Induces IFNλ secretion in T cells & NK cells. Implicated in inflammatory responses.
Ppp3ca*	GM-CSF, IL-3	-2.31	-3.44	Calcineurin; calcium-dependent serine/threonine protein phosphatase. Activates NFATc, upregulates IL-2 expression.
Ifih1	Pattern Recognition	-4.42	-1.88	MDA5; component of innate immune response (viral RNA sensor). Ligation triggers IFNλ-mediated apoptosis.
Irf7	Pattern Recognition	-6.47	-1.79	Controls expression of IFNα/β genes as well as RANTES; induces cellular senescence.
Abca1	LXR/RXR	-2.81	-1.75	Lipid transporter expressed in epithelial cells, Multi-drug resistance gene.
Blnk	FcγRIIB	-2.46	-1.69	B cell linker protein, tumour suppressor, involved in humoral immune response. Wide range of tissue expression.
Jak1	IL-3	-1.9	-1.68	Cytokine signal transduction, altered expression implicated in invasive breast cancer.
Cldn1	Leukocyte Extravasation	-3.72	-1.66	Tight junction protein, metastasis suppressor, epithelial cell expression.
Cd55	Complement	-3.86	-1.64	Complement regulating factor. If secreted can inhibit T cell function.
Pak1	IL-3	-2.69	-1.56	p21-activated kinase; regulates apoptosis.
TLR7	Pattern Recognition, TREM1	-4.89	-1.50	Induces NK-mediated anti-tumour response.
Shc1	GM-CSF, FcγRIIB, IL-3	2.24	2.737	Signal transduction protein; propagation of mitogenic signals. Wide tissue expression.
Cldn4*	Leukocyte Extravasation	4.67	13.087	Tight junction protein, wide tissue expression, up-and down-regulation implicated in several tumour settings.

Table 6. QPCR validated differentially expressed outcome-specific (O/S) genes

Pathway-enriched O/S genes identified using the Ingenuity pathway analysis system were assessed using qPCR. Consistency between the Affymetrix-predicted expression pattern and qPCR expression was used as an initial indicator of validity. A minimum fold-difference (CR:PD) of +/-1.50 was used as an arbitrary cut off to establish measurable differential expression. A '-' is used if the gene is downregulated in the PD versus the CR, and a '+' is used if the gene is upregulated in the PD versus the CR. An asterisk (*) indicates PAM-identified genes.

4.4.5 Gene expression in NOP tumours can predict outcome to ACT in previously untested tumours

To determine whether genes validated by qPCR could be used as predictors of response to ACT using our NOP model, we selected five genes with the greatest differential expression between CR and PD tumours to serve as 'putative predictive signature' (PPS) genes: Reln (-8.40), IL-18 (-3.57), Ppp3ca (-3.44), Shc1 (+2.737) and Cldn4 (+13.087) [number in parentheses represent fold difference in expression between PD and CR tumours] (Figure 22A). Using established methods, we harvested untreated tumours and used qPCR to assess relative gene expression in 5 NOP tumours previously untested for response to ACT: NOP7, NOP20, NOP29, NOP37, and NOP38. We then compared relative expression of PPS genes in these untested NOP tumours to that in tumours with known outcomes following ACT (Figure 22B). Using our outcome predicting algorithm (Materials and Methods) in which quantitative PPS gene expression in untested tumours was compared to PPS expression in CR, PR and PD tumours, we predicted NOP20, and NOP37 to have a CR, NOP7 and NOP29 to have a PR, and NOP38 to be PD following ACT (Appendix 5). Next, using the established tumour implantation and ACT methods (13, 22, 293), we tested these five tumour lines for response to adoptively transferred OT-I and OT-II T cells, as before.

In accordance with our prediction that NOP29 tumours would partially respond (PR) following ACT, 6/10 tumours were indeed PR, and surprisingly the remaining tumours (4/10) were CR. By contrast, we predicted that NOP7 tumours would undergo PR following ACT, however only 2/10 showed this phenotype, with the vast majority of these tumours (8/10) showing a complete response. Similarly, in contrast to our prediction that NOP38 would show PD following ACT, partial response was seen in 6/10 NOP38 tumours, whereas the remainder (4/10) showed a complete response. Remarkably, as predicted by the PPS, 10/10 and 7/10 tumours showed a complete response (CR) following ACT in NOP20 and NOP37 (respectively) (Figure 23). Thus, the PPS was successful in predicting outcome in 25/50 tumours (50%). Interestingly, in the

majority of the tumours that did not respond as predicted (22/25), outcome was actually better than predicted.

This mixed success may have been the result of the small number of NOP tumour lines that were tested, or the limited inclusion of only 5 validated genes in the PPS. In addition, only a single example of a CR tumour ($NOP21^{CR}$) was represented in the Affymetrix analysis, which may have influenced the overall strength of the PPS. Nevertheless, the 5-gene PPS was successful in accurately predicting a favorable response (either partial or complete) to ACT in 3/5 previously untested NOP tumour lines.

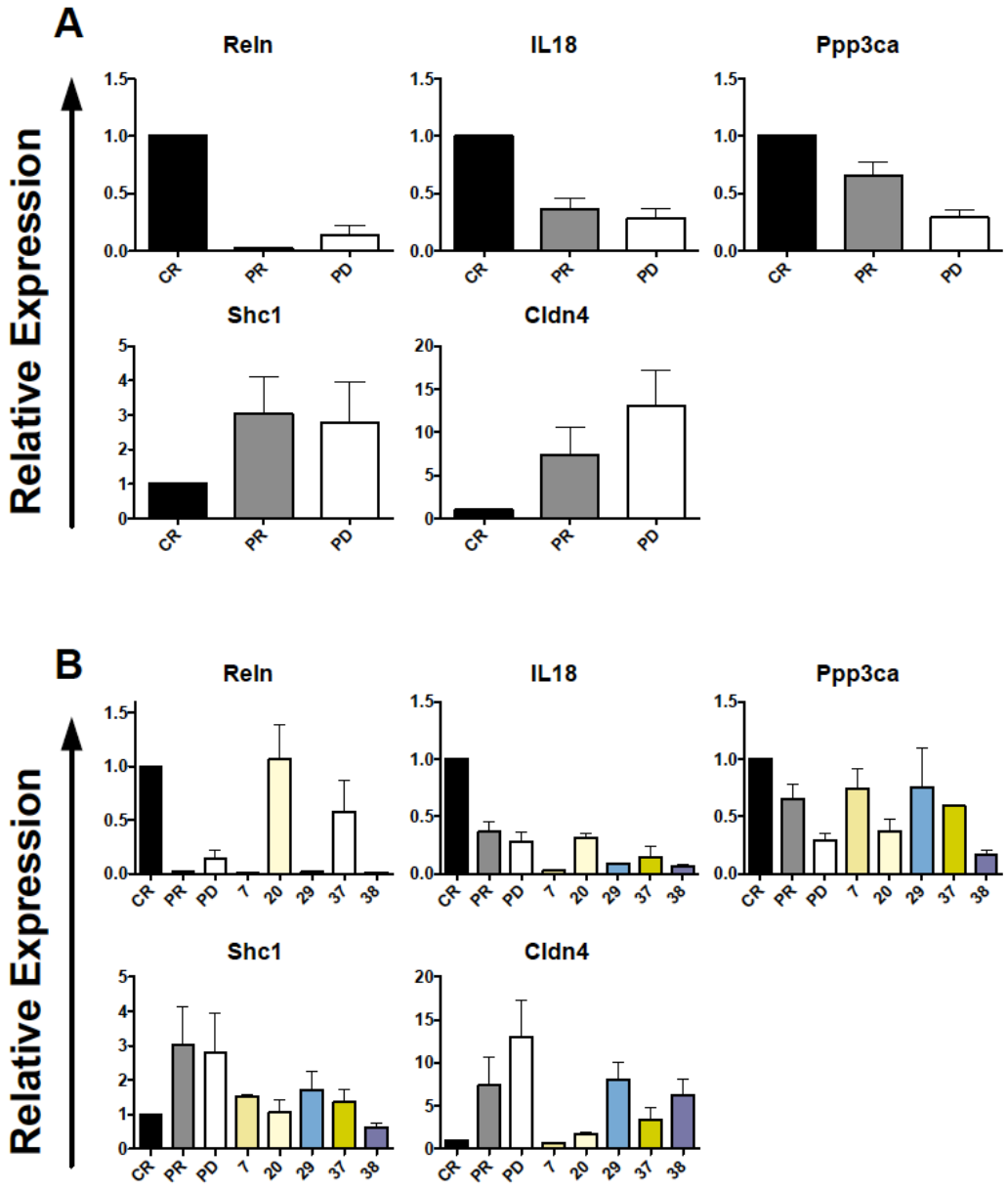


Figure 22. Differential gene expression for genes assigned to the 'putative predictive signature' (PPS)

(**A**) Gene expression levels for the PR (n=3 tumours) and PD (n=6 tumours) outcome classes were calculated as the average expression for all tumours within each class, and were then normalized to that in NOP21^{CR} tumours. Note that scales for relative expression are different for Shc1 and Cldn4. (**B**) As in (A), expression levels in untested NOP tumours (NOP7, 20, 29, 37, 38) were assessed for PPS genes, with NOP21^{CR} tumours used as the comparator. Based upon

similarity to PPS gene expression in NOP tumours with known outcomes, complete response was predicted in NOP20 and NOP37, partial response was predicted in NOP7 and NOP29, and progressive disease was predicted in NOP38.

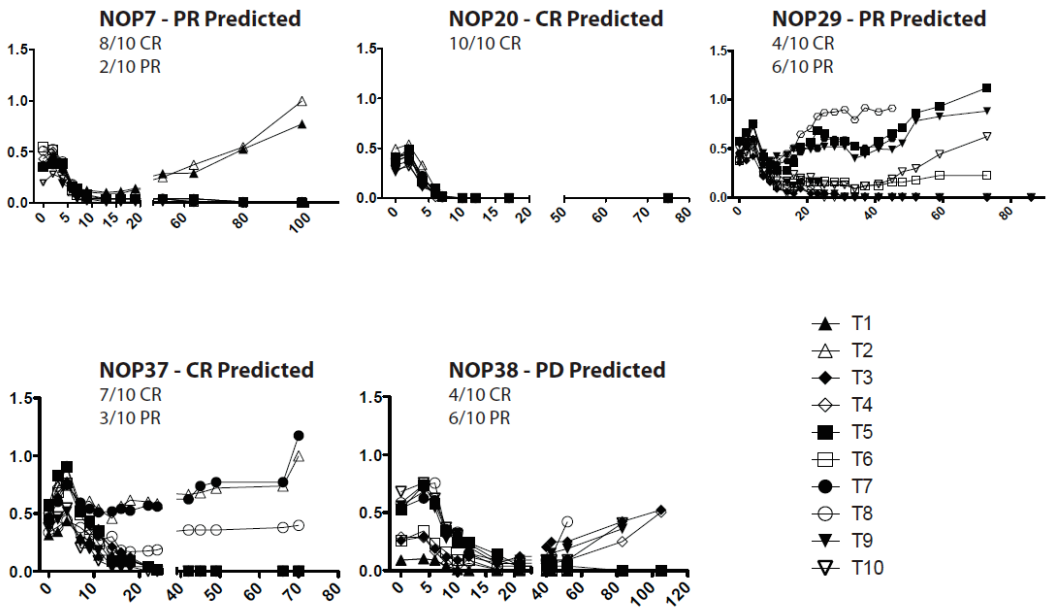


Figure 23. Tumour growth in previously untested NOP tumours following adoptive transfer with OT-I and OT-II T cells

A predictive algorithm (Materials and Methods) compared relative expression of PPS genes in untested NOP tumours to those in NOP tumours with known outcomes following ACT. Complete response **(CR)** was predicted for **NOP20** and **NOP37**, partial response **(PR)** was predicted for **NOP7** and **NOP29**, and progressive disease **(PD)** was predicted for **NOP38**. Outcome following ACT for each NOP line is indicated in each graph. Outcome was accurately predicted in 50% of tumours (25/50). Note that the days post-adoptive transfer for NOP38 are fewer than in other cases (at time of writing (August, 2010).

4.4.6 Gene expression in NOP tumours resembles that in human breast cancer subtypes

In a variety of recent publications, gene expression has been investigated in the context of human breast tumour subtypes, often with associative relationships to prognosis and response to treatment, (341, 362-364). We hypothesized that gene expression patterns in NOP tumours with ACT-responsive (i.e. CR or PR) or –unresponsive (i.e. PD) phenotypes might have gene expression patterns comparable to human breast tumour subtypes with favorable or unfavorable prognoses, respectively. Using described filtering techniques (Materials and Methods), the NOP tumour expression profiles were compared with human tumour expression profiles of pre-chemotherapy whole breast tumour tissues of 82 patients from Rouzier and colleagues (357). We developed a 'cross-species breast cancer intrinsic gene set (CBC)' consisting of 82 genes representing a subset of highly conserved genes between mouse and human. These genes were reasonable classifiers of human breast cancer subtypes, and showed the least intra-subtype variation, but greatest inter-subtype variation. Thus, by using this subset of genes, we reduced false discovery due to technical and biological noise.

To detect transcriptional similarities between NOP tumours and human breast tumours, an unsupervised hierarchical clustering analysis over the merged data of human and NOP microarrays was subsequently performed using the CBC subset of 82 genes (Figure 24). Intriguingly, the clustering analysis repeatedly demonstrated systematic transcriptional differences between the ACT-responsive (i.e. CR and PR) and most of the ACT-unresponsive (i.e. PD) outcome groups (Figure 24). Specifically, all CR and PR (NOP21^{CR}, NOP12^{PR}, NOP13^{PR}, and NOP23^{PR}) tumours clustered together primarily with the human breast cancer samples of the HER2^{+} subtype. By contrast, two out of three PD cell lines (NOP6^{PD}, and NOP17^{PD}) clustered with human breast cancer samples in which the vast majority had estrogen receptor (ER)- progesterone receptor (PR)- and HER2-negative status (100%, 76% and 88% of samples, respectively). This 'triple negative' phenotype most represents the 'basal-like' human

breast cancer subtype. Interestingly, NOP18^{PD} tumours did not cluster within either of these subtypes. Thus, although this murine tumour model over-expresses HER2 by design, only tumours of the ACT-responsive phenotype (CR and PR) have gene expression profiles significantly comparable to the HER2^{+} subset using the CBC data set. This might suggest that genes other than HER2 may be greater contributors to the outcome phenotype in an ACT setting.

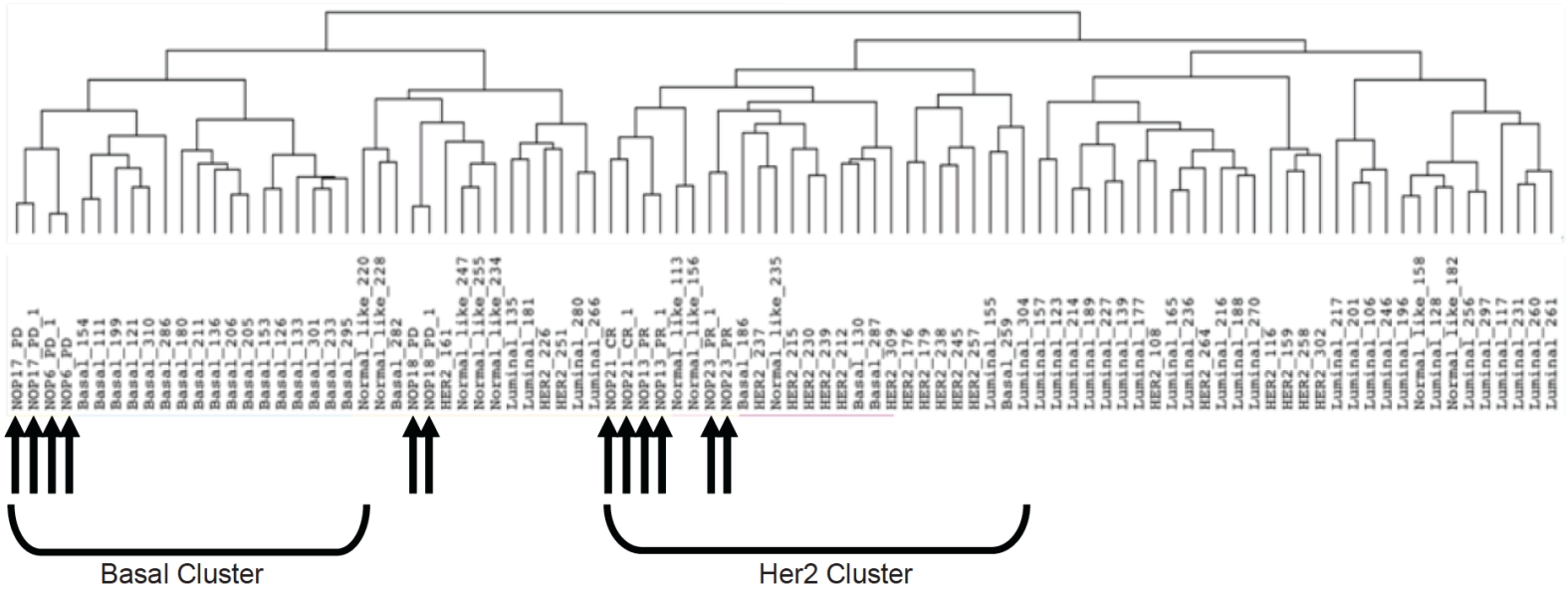


Figure 24. Comparison of the NOP tumour expression profile with human breast cancer subtype expression profiles

An unsupervised hierarchical clustering analysis was performed using an 82 'cross-species breast cancer intrinsic gene set (CBC)' over the merged data from 82 human microarrays (Rouzier et al.) and 12 NOP microarrays. Clustering was based on the complete linkage using a Pearson correlation coefficient as a similarity distance. The dendrogram indicates NOP tumours (arrows) and human breast cancer subtypes determined from estrogen/progesterone receptor (ER/PR), and HER2 over-expression status from the study by Rouzier and colleagues (357).

4.5 Discussion

Here we have described the use of a novel murine pre-clinical model for breast cancer to investigate the development of a predictive signature for the use of adoptive cell therapy in the $HER2^+$ breast cancer setting. Using mouse mammary tumours with known clinical outcomes following ACT, we discovered and validated 14 differentially expressed genes between tumours that completely responded to ACT, and those that had a progressive disease phenotype (Table 3). Using the 5 genes with the greatest differential expression between ACT-responsive and ACT-unresponsive phenotypes, we were able to demonstrate that these genes could predict a favorable response to ACT in 3/5 previously untested mammary tumour lines in follow-up *in vivo* experiments (Figures 22 and 23). Importantly, using a cross-species comparative data set, we have shown that ACT-responsive tumours have a gene expression pattern most similar to human breast tumours of the HER2-overexpressing subtype (Figure 24). While preliminary, these findings provide the basis for promising initial predictors of clinical response to adoptive cell therapy in the setting of human breast cancer.

Our analysis of biological pathways revealed that immune-associated genes were heavily represented in both ACT-responsive and ACT-unresponsive tumours (Table 4A and B). For example, the GM-CSF pathway and IL-3 pathways both contained representative genes on our 14-gene list validated using qPCR. Ppp3ca (also known as calcineurin) is represented on both of these pathways. Ppp3ca a serine/threonine protein phosphatase that can be activated through IL-3 signaling in T cells (365), but perhaps is best known for its role in activating transcription factors associated with the upregulation of IL-2 expression in T cells (366-368). However, Ppp3ca has also been implicated in the TGFβ-mediated regulation of transcription factors associated with the production of the extracellular matrix proteins collagen and fibronectin by a variety of cells (369). Ppp3ca is also involved in the activation of nuclear factor of activated T cells c (NFATc), which induces transcription of genes associated with cell-cell interaction in

endothelial cells (370). This gene was upregulated by CR tumours versus PD tumours, and one could speculate that the role of this gene might be to establish an extracellular matrix with more normalized blood vessels and more loosely bound collagen fibrils, thus more conducive to T cell trafficking and infiltration. Indeed, in our previous studies we found that CR tumours had greater proportional stroma with a more collagenous appearance versus PD tumours (22). Future experiments involving tissue-specific gene expression may reveal cell-associated expression of Ppp3ca that might further elucidate the role of this gene within the mammary tumours.

Our data indicate the leukocyte extravasation pathway was enriched for PD-specific genes (Table 4B). Claudin 4 (Cldn4) was significantly upregulated in tumours with an ACT-unresponsive phenotype (PD) (Table 6, Figure 22). Cldn4 is one of a 23-member family of tight junction proteins that participates in the cell-cell adhesion complex and is expressed in a wide variety of tissues (371-374). Claudin proteins are involved in maintenance of cell polarity, paracellular ion transport, and have the ability to recruit signaling proteins. They are hypothesized to participate in the regulation of cellular functions including proliferation through these and other mechanisms (374). Increased expression of Cldn4 has been described in a number of tumour settings, including ovarian, breast, pancreatic and prostate cancers, and has been associated with a poor prognosis in breast cancer and increased *in vitro* survival of ovarian cancer cells (374, 375). Mechanistically, the advantage of Cldn4 upregulation has not been described however based on our findings, there may be a connection to the immune response via paracellular or secreted soluble factors associated with an immunosuppressive microenvironment. Alternatively, our previous work has found that PD tumours appear more resistant to CTL infiltration following ACT (13, 22, 293). Accordingly, there may be physical features unique to the tight junctions of PD tumours that make them more resistant to CTL infiltration, thus influencing their response following ACT. We are currently undertaking RNA

interference experiments to determine whether Cldn4 knockdown or overexpression can influence outcome in NOP tumours following ACT.

In contrast to our findings with Cldn4, expression of interleukin 18 (IL-18) was markedly downregulated in PD tumours in comparison to those of the CR and PR phenotypes (Table 6, Figure 22). IL-18 is a potent inducer of CTL and T helper$_1$ cell activity through the interferon gamma (IFNγ) pathway (376-378). IL-18 is synthesized by a wide variety of cells including epithelial cells and tissue-specific macrophages. The receptor for IL-18 (IL-18R) is found on immune cells including CTLs and natural killer (NK) cells, as well as epithelial, endothelial and fibroblast cells (377). As a pro-inflammatory cytokine, IL-18 has a controversial role in the setting of cancer. Metastatic disease of the lung and gastrointestinal tract has been associated with elevated serum IL-18 levels (379-381), and tumours are described to induce IL-18 production in normal host cells (382). In other studies, IL-18 synthesis is reduced or abolished in colon cancer cells when compared to normal colonic epithelium (377). It could be argued that elevated expression of IL-18 in CR tumours enhances the activity of CTLs in this tumour microenvironment. Indeed, in our previous *in vivo* studies, we saw enhanced intratumoural activation and proliferation of adoptively transferred CTLs in CR tumours versus PD tumours (22). This may be a function of increased IL-18 expression in CR and PR tumours. Future experiments investigating IL-18 translation and systemic and microenvironmental IL-18 levels in NOP tumours are underway, and should provide an increased understanding about the role of IL-18 in this model.

Although it would be clinically expedient and cost effective to use a very small number of genes in a predictive signature, it is a somewhat naïve goal given what is known about the myriad methods of immune escape within a tumour's armamentarium. For our 5-gene PPS, genes with the greatest magnitude of differential expression as an initial threshold were chosen. This strategy, by definition, excluded genes with a small, yet potentially biologically significant

differential expression. While our predictive results using the 5-gene PPS were mixed, they were encouraging and warrant an expanded investigation using a more inclusive PPS, perhaps with a greater emphasis on biological significance notwithstanding magnitude of differential expression. To this end, we are undertaking further *in vitro* and *in vivo* studies with additional untested NOP tumours.

Remarkably, our initial clustering analysis of gene expression between CR and PR tumours revealed only 6 differentially expressed genes, however none belonged to immune-related pathways (data not shown). This may be yet another reflection of the *in vivo* differences observed between CR and PR tumours in our earlier ACT studies (22). Indeed, in all five of our predictive *in vivo* experiments, we saw instances of partial and complete responses following ACT within the same tumour line (Figure 23). As CR and PR tumours appear to diverge in response after varying intervals following ACT, it is possible that post-translational differences contribute to differences in clinical outcome, even within the same tumour line. Alternatively, CR and PR tumours may respond differently to 'immunoediting', resulting in disease recurrence in the case of PR tumours (332, 383, 384). Future experiments examining post-ACT gene expression in NOP tumours might serve to elucidate differences that might be amenable to clinical intervention.

Providing a platform from which clinicians can predict outcome to various treatments including chemotherapy, immunotherapy, or combination therapies for cancers is a valuable objective and one that has been pursued in a number of tumour settings. To our knowledge, this is the first description of a predictive signature that has its application in the context of ACT. Using data derived from our murine tumour gene analysis experiments, we have successfully predicted outcome in previously untested mammary tumours. Importantly, using our cross-species breast cancer intrinsic gene set, we have found that murine mammary tumours with an ACT-responsive phenotype cluster with human breast tumours of the $HER2^{+}$ subtype. Together, these findings

provide a valuable resource for further validation using an expanded predictive signature and panel of murine mammary tumours. Significantly, a predictive gene expression signature may in future allow clinicians to more judiciously select immunotherapeutic treatments based on the unique features of patients' breast tumours.

CHAPTER 5: Concluding Remarks

Since the 1950s when Medawar, Mitchison and Bellingham first described the immune system's ability to reject 'foreign' tissue, effectively harnessing the immune system to reliably reject tumours has been the goal of those championing ACT. In recent decades, advances in molecular tools and animal models have provided researchers with the means to investigate important questions associated with immune evasion by tumours. Nevertheless, in spite of remarkable advances, proponents of ACT must still answer the question, 'why do some tumours respond to ACT, while others do not?' Critics may see this as a quixotic question; the investment in infrastructure and personnel to implement this treatment are vast in comparison to the seemingly modest results in cancer patients, even after more than 30 years of research. While this is true, these 'modest results' are being seen in patients with very advanced cancers for whom no other options exist, and without exception following exhaustive pretreatments including chemotherapy, radiation and surgery. It is possible that ACT at an earlier stage might offer more convincing results and thus quell the critics for a time. Until that time however, the systematic dissection of immune evasion by tumours will continue, and the promise of powerful bioinformatics tools together with established *in vitro* and *in vivo* models provides optimism throughout the field.

5.1 Summary

Clinical trials in human patients have provided valuable information about not only the efficacy of ACT as a treatment, but also about the feasibility and side effects associated with the regimen. In addition, several studies have been able to glean ancillary information about T cell kinetics and distribution following ACT. Correlative information gathered from these studies has provided some background to address questions around T cell function and patient response following ACT, but the inevitable complications of working with a diverse patient population will continue to

challenge those seeking to tease apart ‘response versus non-response’. Thus, animal models will likely continue to be a mainstay for those in the ACT field.

In Chapter 2 we approached the question of ‘ACT response versus non-response’ with the development and characterization of a novel mouse model that allowed us to study the kinetics and distribution of adoptively transferred antigen-specific T cells in a spontaneous $HER2^+$ mammary cancer context. Using this model, we were able to study tumour responses following a timeline and we observed clinical outcomes along a continuum that reflected those seen in human patients: complete and partial responses, as well as stable and progressive disease, with mixed responses commonly seen. Importantly, we found that outcomes were not correlated with typical features including tumour size, activation status of adoptively transferred T cells, or antigen expression. Rather, we found that tumours that responded (either partially or completely) to ACT were infiltrated by T cells at early time points following ACT, whereas many non-responders were not. Thus, we focused on tumour-specific features associated with T cell infiltration following ACT to further uncover the mechanisms behind immune evasion by tumours.

To further investigate the continuum of responses to ACT that we observed in earlier experiments, in Chapter 3 we detailed the development, characterization and testing of ‘NOP’ cell lines derived from $HER2^+$ murine mammary tumours that arose in our transgenic mice described in Chapter 2. Our hypothesis was that tumour-specific features were associated with clinical outcome following ACT. Using cell lines that reliably showed complete response (CR), partial response (PR), or progressive disease (PD) following ACT, we were able to demonstrate subtle differences in the extent to which adoptively transferred T cells infiltrated tumour stroma and epithelium following ACT. Quantitative differences in T cell infiltration at the level of the tumour epithelium differentiated PR and CR tumours following ACT. Intriguingly, we also found that even though PD tumours were very poorly infiltrated overall, the few T cells that were able

to penetrate the tumour were activated and proliferated, although to a lesser extent than in CR and PR tumours. Finally, using a larger panel of histologically prepared untreated tumours, we discovered that tumour stroma in CR tumours was more abundant, and appeared to have a greater collagenous component than in tumours of the PR or PD outcome class. Thus, this study found that unique physical features of tumours were correlated with infiltration of T cells and clinical outcome following ACT in this mouse model.

To probe the genetic features associated with outcome in NOP tumours with CR, PR and PD phenotypes, in Chapter 4 we reported on a bioinformatics study that compared differential gene expression in NOP tumours with known responses to ACT. Our hypothesis was that there would be unique gene expression patterns that could differentiate between outcome phenotypes, and that these patterns could be used *a priori* to predict response to ACT in untested tumours. We discovered and validated a set of 14 differentially expressed genes that distinguished CR tumours from PD tumours. In addition, we used a 5-gene subset of this list to predict outcome following ACT in 5 previously untested NOP tumours. Remarkably, this subset of genes accurately predicted a response to ACT in 3/5 previously untested mammary tumours. In addition, using a mouse-human cross-species comparative gene list, we were able to show that CR and PR NOP tumours most resembled human $HER2^{+}$ breast tumours, whereas PD tumours most resembled human breast tumours of the 'triple negative' subset. Thus, although preliminary, this work provides an important platform from which larger studies can investigate the biological and mechanistic roles played by differentially expressed genes in this context, and how they might be associated with outcome following ACT in a human breast cancer setting.

5.2 Future opportunities

While the technological advances associated with the molecular biology of the immune response and animal models of cancer in the past 50 years have been impressive, the essence of the questions around mixed responses to ACT remain essentially unchanged:

1) Why do some tumours respond to ACT while others do not?

From our studies, we have identified ACT outcome-associated histological features of $HER2^+$ murine mammary tumours. There are several opportunities to investigate the biological or mechanistic relationships of these features. First, CR tumours had stroma that was more abundant, and was more collagenous in appearance than that of PR or PD tumours. The composition of stroma from these tumours could be studied immunohistologically for stroma-associated proteins to more specifically characterize the constituents. Special immunohistochemical stains exist for collagen (trichrome), blood vessels (CD31, VE-cadherin), and fibroblasts, and could be used to more precisely quantify supporting stromal structures associated with NOP tumours. Further, microdissection of tumour stroma from tumour epithelium could be undertaken to evaluate gene expression in these two compartments separately, with results compared between tumours from each outcome class. This might uncover more subtle distinctions in expression that could contribute to the phenotypic features seen histologically and help explain deficiencies in infiltration seen in PR and PD tumours.

Gene expression data related to cytokines and chemokines exists for several NOP tumours, however, expression at the protein level has not been evaluated *in vitro* (prior to implantation), *in situ* (either prior to or following ACT), or *ex vivo* in this tumour model. Cytokine and chemokine profiles might provide hints related to post-implantation host response, including stromal cell induction and development. Finally, time-line studies investigating microenvironmental chemokine and cytokine synthesis following ACT might provide temporal information to help explain T cell infiltration deficiencies seen in PR and PD tumours.

In our bioinformatics study, we identified and validated a 14-gene panel that differentiated CR tumours from PD tumours. Included in this list were several genes that have been implicated in other tumour settings, including Cldn4, Reln and Jak1. However in general, the literature only speculates about mechanisms relating up- or down-regulation of these genes to prognosis or

treatment response. Using a 5-gene subset of this panel as a putative predictive signature, we completed preliminary experiments in which we were able to predict response to ACT in 3/5 mammary tumour lines. For at least 2 of these genes (Cldn4 and IL-18), RNA interference or blocking antibody experiments (respectively) are of great interest and are imminently planned. Our hypothesis is that by knocking down Cldn4 expression in PD tumours using short hairpin RNA (shRNA) technology, we might influence the architecture of the tumour epithelium, thereby facilitating improved T cell infiltration following ACT. Conversely, we hypothesize that by binding IL-18 using IL-18 binding protein, we might negatively influence the microenvironment of CR tumours in such a way as to inhibit infiltration of T cells following ACT. Therefore, these directions present immediate opportunities to test the biological importance of genes with a relatively large magnitude of differential expression between CR and PD tumours.

2) Can we use the molecular tools available to us to predict which tumours might respond to ACT?

Clearly, our bioinformatics study provided preliminary information that will greatly benefit from testing on expanded panels of $HER2^+$ mammary tumours. Additionally, while implanted tumours provide an important starting point for these types of studies, clinical translation demands that gene expression in spontaneously arising tumours must also be evaluated. Interestingly, using an *in vivo* 'punch biopsy' technique, we have developed the means to sample and assess gene expression of spontaneous $HER2^+$ murine mammary tumours in live animals. Following a short period of recovery, mice are treated with ACT and tumour outcomes can subsequently be evaluated. While these samples could be used to expand our data set for more extensive testing and validation of the 'putative predictive signature', importantly, samples gathered using this technique could potentially be used in the predictive setting to further validate our findings in spontaneously arising mammary tumours. Thus, this approach would closely mirror the normal

clinical approach to a woman's breast tumour at the point of diagnosis: biopsy sampling, followed by molecular characterization and treatment selection.

It is difficult to apply a signature derived from murine studies to human patients if $HER2^+$ breast cancer patients are not proceeding to clinical trials using ACT for their tumours. While ACT is not presently being used to any large degree for breast cancer treatment, there are a few reports of its use in Europe (385) and in North America (386). Recognizing limitations associated with sampling of heavily pretreated tumours, it might be possible to obtain pre-ACT samples of tumour tissue from willing collaborators for comparative gene expression and histological analysis. While sample numbers would be low, they would be human breast tumour samples (as opposed to murine tumour samples), and might provide the materials to start building a body of evidence to support the clinical translation of our murine studies.

Our studies have been focused upon ACT in the context of $HER2^+$ breast cancer, however it is possible that our findings are relevant to other solid tumour settings. Examples would be lung and gastrointestinal cancers (both of which have HER2-overexpressing subtypes) (62), and renal cell carcinoma, for which there is interest in using ACT (387). While these tumours might have substantial phenotypic and behavioural differences, they can all be characterized as solid tumours with supporting stromal architecture. As such, one could assess the expression of putative signature genes, compare histologic features, and evaluate immune response in relevant mouse models in an ACT setting. In human tissue samples from these alternative tumour sites, one could evaluate putative signature gene expression and assess whether any similarities could be seen with data from our studies. Thus, by expanding outside of the breast cancer context, one might uncover alternative examples of tumour sites that exhibit similar phenotypic or genetic features to NOP tumours.

Investigating the use of ACT in larger non-human species might be considered as an alternate resource to advance the field. The use of the canine patient in other immunotherapy-related

contexts has provided invaluable and rapid advances in the fields of bone marrow transplantation (388, 389), and now is being studied in the context of glioblastoma (390), astrocytoma (391), melanoma (392-396), and B and T cell lymphomas (392, 397). The pre-clinical period for reagents associated with veterinary patients is considerably shorter, and restrictions are fewer than those in human cancer patients. The opportunity to investigate therapeutic approaches in the canine patient through large, multi-centered clinical trials might advance the knowledge base associated with ACT more rapidly than is being presently experienced. With this in mind, it is important that researchers involved in both human and veterinary cancers maintain collaborative relationships in which clinical and pre-clinical data are openly reported for the benefit of both.

There is good evidence that ACT has a place in the cancer treatment armamentarium. It is logical to seek the means to arm the immune system with the sustained ability to recognize tumour antigens and reduce the likelihood of local recurrence or metastasis. Ideally, ACT would be provided at earlier time points along the treatment continuum, as opposed to the current approach, wherein only patients with advanced, metastatic, heavily pretreated tumours are eligible for ACT. Providing immunotherapeutic intervention at a point where the tumour is more vulnerable to the immune response, and the patient is less vulnerable to serious complications, should be considered. As the approach moves toward that goal in the human patient, it is reasonable to expect that combination treatments will continue to be the mainstay. Sequential treatment with radiation, chemotherapy, surgery, and targeted immunotherapy will be used in the meantime to maximize the likelihood of success.

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APPENDICES

Appendix 1: Generation of transgenic mice

The *neu*$^{OT\text{-}I/OT\text{-}II}$ construct was made by modifying the activated allele of rat *neu* by splice overlap extension PCR. The sequence of the modified C terminus is as follows: VPVDEVSGLEQLESIINFEKLTEWTSSNVMESGISSAESLKISQAVHAAHAEINEAGREVVGSAEAGV[Stop]. VPV represent the 3 most C-terminal residues of rat *neu*. The remaining residues are all derived from chicken ovalbumin (OVA), with the OT-I and OT-II epitopes underlined. The construct was inserted into a pBlueScript II SK vector (Stratagene) containing the MMTV promoter and enhancer and an SV40 polyadenylation sequence (kindly provided by Tim Lane, UCLA). A *Pvu*II fragment containing the promoter, insert and 3' untranslated region was injected into C57BL/6 oocytes. Of three transgene positive founder lines, line 'C' was selected for all further studies based on mammary-specific expression of *neu*$^{OT\text{-}I/OT\text{-}II}$ and favorable tumor latency and frequency.

neu$^{OT\text{-}I/OT\text{-}II}$ mice were bred with mice expressing a dominant negative mutant of p53 (*DNp53*, R172H (398)) under the control of the whey acid protein (WAP) promoter (Jackson Laboratory). The resultant offspring were backcrossed from a mixed FVB x C57BL/6 background to a C57BL/6 background, and the majority of experimental mice were from the F5 generation or greater. Doubly transgenic *neu*$^{OT\text{-}I/OT\text{-}II}$ *x DNp53* mice were bred at least twice to induce expression of the WAP promoter by lactation.

Appendix 2: Genotyping of transgenic mice

Female *neu*$^{OT\text{-}I/OT\text{-}II}$ *x DNp53* mice were genotyped by PCR. Primers used to detect the *neu*$^{OT\text{-}I/OT\text{-}II}$ transgene were 5'-TGTCCGGCCTGCTGGTGCTACTCTAGAAAG-3' and 5'-TCACACTCCAGCCTCTGCTGACCCTACCAC-3' and for the *DNp53* transgene, 5'-CCGTCGACGGCCACAGTGAAGACCTCCGGCCAG-3' and 5'-GCGTGACACCCTGCTGGGAAGGAGGAGGATGAG-3'.

Appendix 3: Antibodies and histology

A3.1: Antibodies used for flow cytometry

Lymphocytes were stained with fluorescently labeled antibodies to the surface markers CD4 (GK1.5, H129.19), CD8a (53-6.7), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), and CD90.1 (OX-7) (all from BD Biosciences).

A3.2: TMA Construction and Analysis

Tissue microarrays were constructed using the Manual Tissue Arrayer I (Beecher Instruments). A 2mm punch set was used. The microarrays were then sectioned at a thickness of 6μm and attached to Superfrost Plus Slides. The sections were then either stained with Gill's Hematoxylin II (Surgipath 01520) and Eosin (Surgipath 01600) or subjected to immunohistochemistry using the Ventana Discovery XT Autostaining system. The sections were stained for Neu (Cell Signaling Technologies, 2242) and CD3 (Sigma, C7930). Both antibodies required heat-induced epitope retrieval using Ventana's CC1 solution (950-124) following deparaffinization. The standard CC1 protocol was used on the machine followed by blocking of endogenous peroxidase activity using part of the DABMap kit also supplied by Ventana (760-124). Following epitope retrieval and peroxidase blocking, the antibodies were applied to the sections and incubated at 37^{o}C for 60 minutes at a dilution of 1/50 for the anti-Neu rabbit polyclonal and 1/500 for the anti-CD3 rabbit polyclonal. A goat anti-rabbit IgG-biotin antibody (Jackson Immunoresearch, 111-065-144) was used as the secondary antibody at a dilution of 1/500 for 32 minutes. All washes in between were directed by the Discovery XT system. Detection was accomplished using the DABMap kit (Ventana, 760-124). The stained slides were then coverslipped using Cytoseal-60 following passage through ethanol and xylene.

Appendix 4: QPCR primer sequences

Gene	Left	Right
Thbs4	agacgcctgtgatgacgac	tgggacagttgtccaaaatg
Tlr7	tgatcctggcctatctctgac	cgtgtccacatcgaaaacac
Ifih1	tgatgcactattccaagaactaaca	tctgccaaacttgtgtctgatt
Serping1	gaagctgcctagtgaccaaga	cagcaggagggtcagtgg
Mmp2	aactttgagaaggatggcaagt	tgccacccatggtaaacaa
Cfb	actcgaacctgcagatccac	tcaaagtcctgcggtcgt
Cybb	tgagaggttggttcggtttt	gttttgaaagggtgggtgac
Cd55	actgttgattgggacgatgag	tggtggctctggacaatgta
Cldn1	actccttgctgaatctgaacagt	ggacacaaagattgcgatcag
Tlr8	caaacgttttaccttcctttgtc	atggaagatggcactggttc
Tyrobp	tggtgttgactctgctgattg	gtctcagcaatgtgttgtttcc
Trem1	tctggattgtatcgttgtgtga	ggagtgaacacatctgaagaacc
Mmp13	cagtctccgaggagaaactatgat	ggactttgtcaaaaagagctcag
Abca1	gcagatcaagcatcccaact	ccagagaatgtttcattgtcca
Il18	gacaacacgctttactttatacctga	gtgaagtcggccaaagttgt
Pik3cg	gcttagaggacgatgacgtttt	ctgtcgtggtacggttcaaa
Pak1	tgacaatattctgctgggaatg	tgcacagaatccaaagtcagtt
Inpp5d	gagagctggtggtacggttt	ctcggggtcagagataatgg
Mmp14	aacttcgtgttgcctgatga	tttgtgggtgaccctgactt
Blnk	tcccatttaattcgacgtttg	agcataccagggcttaccg
Ppp3ca	aggaacatttcactcacaacaca	tcacacacagctgggtaactg
Cd14	aacctggaagccagagaaca	cagaagcaacagcaacaagc
Camk2d	agttcacagggacctgaagc	cgccttgaacttctatggcta
Syk	ttcaacccctatgagccaac	ggcagggcttctctctgaa
Jak1	tgtgtccgagatgtggaaaa	gtgcatgaagagatccaacg
Shc1	caatacccggactcaggtca	tgagtgggcggctacaag
Rapgef3	cgacaccacaggttggaa	gagccaaacaggtgcattc

Appendix 5: Predictive algorithm

A

	Relative Expn CR	Relative Expn PR	Relative Expn PD	Gene Expn Std Dev
Cldn4	1	7.4	13.000	10.58
Reln	1	0.022	0.139	97.51
Shc1	1	3	2.800	2.52
PPP3ca	1	0.657	0.291	2.46
IL-18	1	0.366	0.280	15.35

B

	Relative Expn NOP7	#SDs from CR	#SDs from PR	#SDs from PD	
Cldn4	0.755	0.030	0.876	1.627	
Reln	0.004	2.560	0.056	0.356	
Shc1	1.528	0.198	0.38	0.329	
PPP3ca	0.747	0.138	0.053	1.041	
IL-18	0.027	2.410	0.879	0.671	
Delta score		5.336	2.244	4.024	Prediction:PR

C

	Relative Expn NOP20	#SDs from CR	#SDs from PR	#SDs from PD	
Cldn4	1.7	0.066	0.411	0.723	
Reln	1.064	0.001	0.495	0.078	
Shc1	1.058	0.023	1.123	1.052	
PPP3ca	0.373	1.090	0.309	0.114	
IL-18	0.314	0.207	0.01	0.002	
Delta score		1.387	2.348	1.969	Prediction:CR

D

	Relative Expn NOP29	#SDs from CR	#SDs from PR	#SDs from PD	
Cldn4	11.3	1.068	0.049	0.014	
Reln	0.02	0.513	0.001	0.071	
Shc1	1.728	0.278	0.277	0.246	
PPP3ca	0.756	0.130	0.061	1.057	
IL-18	0.085	0.766	0.28	0.214	
Delta score		2.755	0.668	1.602	Prediction:PR

E

	Relative Expn NOP37	#SDs from CR	#SDs from PR	#SDs from PD	
Cldn4	3.4	0.321	0.205	0.361	
Reln	0.575	0.008	0.268	0.042	
Shc1	1.359	0.143	0.873	0.817	
PPP3ca	0.594	0.276	0.045	0.829	
IL-18	0.142	0.459	0.168	0.063	
Delta score		1.207	1.559	2.112	Prediction:CR

F

	Relative Expn NOP38	#SDs from CR	#SDs from PR	#SDs from PD	
Cldn4	4.5	0.425	0.057	0.273	
Reln	0.013	0.789	0.007	0.11	
Shc1	0.619	0.246	1.92	1.794	
PPP3ca	0.17	2.390	1.569	0.289	
IL-18	0.064	1.020	0.371	0.285	
Delta score		4.870	3.924	2.751	Prediction:PD

Mean expression was determined for 'putative predictive signature' (PPS) genes in PR and PD tumours and quantified relative to the CR tumour ($NOP21^{CR}$), which was set to a value of '1' (**A, 1st, 2nd and 3rd columns**). Similarly, mean expression was then quantified for tumours with unknown outcome (NOP7, NOP20, NOP29, NOP37 and NOP38) (**B-F, 1st column**). To normalize gene expression and eliminate the potential bias associated with genes having a larger variation in expression levels (e.g. Reln, Cldn4), the standard deviation for each of the

PPS genes was calculated across all tested tumours (known and unknown outcome categories) (**A, 4th column**). The relative expression of each PPS gene was then compared between the untested NOP tumour and each outcome class (i.e. CR, PR, and PD), and expressed as a difference in standard deviations (**B-F, 2nd, 3rd and 4th columns**). Standard deviations for each gene in a comparison category were then summed and expressed as an overall '**Delta Score**'. The category with the lowest Delta Score was used as a predictor of outcome following ACT for each untested tumour (highlighted in yellow). Statistics were calculated using GraphPad Prism® software.