Investigation of the Cancer Testis Antigen Lactate Dehydrogenase C as a CD8 T Cell Target

by

David S Neilson

Bachelor of Science, University of British Columbia, 2012

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Abstract

The infrequency of known T cell targets in high grade serous ovarian carcinoma (HSGC) is a substantial barrier to the development of targeted immunotherapies. Due to their infrequency, antigen discovery is a crucial component of immunotherapeutic design. In our cohort of HGSC cases, the cancer-testis (CT) antigen lactate dehydrogenase C (*LDHC*) is expressed in 76% of tumours (22/29). As LDHC presents with tumour specificity in women, I hypothesize that LDHC is an immunogenic target in HGSC patients, and that LDHC-specific T cells can be activated and expanded for therapeutic purposes. As such, I sought to examine whether endogenous LDHC-specific T cells were present in the ascites of HGSC patients. A standard Rapid Expansion Protocol was used to expand CD8 T cell cultures from patient ascites. These cultures were screened for reactivity to a peptide library encompassing all possible epitopes of the LDHC protein by interferon-γ ELISpot. With this approach, T cell clones from one of five patients were identified that were reactive to minimal peptides contained within LDHC. In this patient, the antigenic LDHC peptide differentiated from LDHA by a single amino acid at its C-terminus (YTSWAIGLSVM versus YTSWAIGLSVA). In recognition assays, tumour cell lines expressing endogenous LDHC, autologous ascites, or autologous B cells transfected with LDHC were unable to elicit T cell responses. Although this study suggests that LDHC is not immunogenic, continued screening of LDHC and other CT proteins will likely provide additional immunotherapeutic targets.

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Dedication

This thesis is dedicated to those who are afflicted and affected by cancers of all origin. May continued research at labs and clinics throughout the world prove to be beneficial to those in need.

Chapter 1: Introduction

1.1 Prologue

This thesis investigates the antigenicity of lactate dehydrogenase C, in order to elucidate its potential role in a targeted immunotherapy of ovarian cancer. The purpose of this introductory chapter is to provide context for this thesis and the work it describes. To begin, in the first section I address the frequency and effects of ovarian cancer, as well as the treatments that are most frequently provided to ovarian cancer patients. To better illustrate the interaction of the immune system with cancer, I provide an overview of numerous T cell subsets, which direct the cell mediated adaptive immune response, as well as a short review of T cell maturation and intracellular antigen processing. I then examine a model which depicts the immune system's interaction with a developing cancer and describe how this interaction engineers a tumour that is able to survive and proliferate despite the presence of a healthy immune system. I go on to discuss immunotherapeutic treatments and their efficacy. Lastly, I discuss a prospective immunologic target: lactate dehydrogenase C. I review its enzymatic activity, its isoforms, and potential reasons for its common expression in cancer.

1.2 Prevalence and Treatment of Ovarian Cancer

1.2.1 The effect of ovarian cancer in Canada

Cancer is an extremely wide reaching and devastating disease. In 2015, over 200 000 Canadians were diagnosed with cancer (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2015). Although often described as a single disease, cancer pathology is diverse and

varies among cases. A cancer may arise from virtually any nucleated cell in the body, and each cell type may promote a cancer with a different phenotype. The most accessible classification for malignant tumours is by the local tissue it has arisen from. A mere 1.4% of all cancer cases are classified as ovarian cancer. While this incidence is proportionally quite low, ovarian cancer represents 15 new cases in every 100 000 women per year. In Canada alone, over 2700 women are diagnosed with ovarian cancer each year, with the majority of them presenting with late-stage advanced disease. The mortality rate of ovarian cancer is higher than that of any other gynecological cancer, with an overall five-year survival rate of approximately 40%. In 2015, ovarian cancer led to the death of 1739 Canadian women (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2015). Its lethality justifies further research into HGSC therapy.

1.2.2 Subtypes of ovarian cancer

Ovarian cancer is further classified into a set of histotypes, which vary in pathology and prognosis. All ovarian cancers are divided into the broad categories of epithelial and non-epithelial, based on cell of origin. Non-epithelial malignancies may be further divided into germ cell tumours and sex cord stromal tumours (Colombo et al., 2012). The rare non-ovarian histotypes are commonly diagnosed at early stages, leading to relatively favourable outcomes and relatively positive prognosis (Colombo et al., 2012; Smith et al., 2006). Epithelial ovarian cancer (EOC) constitutes approximately 90% of ovarian cancer cases and consists of five primary histological subtypes: clear-cell, endometrioid, mucinous, low-grade serous carcinoma, and high-grade serous carcinoma (HGSC) (Sopik, Iqbal, Rosen, & Narod, 2015). The high

mortality rate of ovarian cancer is primarily attributable to HGSC, which at 75% is the most frequently occurring histotype of EOC (George, Garcia, & Slomovitz, 2016). The early stages of HGSC lack observable symptoms, clinically masking the disease and frequently leaving it undetected prior to progression to an advanced stage. When it is detected in its early stages, the five-year survival rate is greater than 90%, while fewer than 20% of advanced stage HGSC patients survive to five years beyond their initial diagnosis (Cuellar-Partida et al., 2016; George et al., 2016; Malpica et al., 2004). Individual histotypes of ovarian cancer must be individually researched so as to provide the best therapy possible.

1.2.3 Treatments of epithelial ovarian cancer

Currently, the standard treatments available for EOC are lacking in efficacy. The front-line standard of care treatment regimen for ovarian cancer is tumour debulking surgery followed by platinum- and taxane-based chemotherapy (Bookman, 1999). There have been no substantial improvements in this standard treatment in the past 25 years (Bookman, 2016). A high proportion of patients (up to 80%) respond well to front-line treatment; however, disease recurs in the majority of patients (Bookman, 1999; Davis, Tinker, & Friedlander, 2014). Tumours that recur prior to six months post-treatment are deemed ‘platinum-resistant’ (platinum resistant ovarian cancer; PROC), while those recurring after six months are deemed ‘platinum-sensitive’. Although this qualification is defined at a relatively arbitrary point in time on a continuum, this division has broadly separated patients in a clinically relevant way. Patients with platinum-sensitive tumours are provided additional cycles of platinum based treatment,

which vary in efficacy. Response rates highly correlate with the previous time to recurrence (Bookman, 1999). As few as 30% of patients with platinum-sensitive tumours which have recurred within 1 year respond well to additional chemotherapy cycles, while up to 90% of those which have recurred beyond 1 year may respond well to second-line platinum therapy (Blackledge, Lawton, Redman, & Kelly, 1989; Markman et al., 1991). Despite varied response rates and therapeutic sensitivity, progression free survival is extremely rare – with few exceptions, all recurrent tumours eventually become platinum resistant.

Once a HSGC tumour has been deemed platinum resistant, alternative chemotherapeutic agents are offered to patients for treatment. While there are numerous options, including paclitaxel, topotecan, and gemcitabine, none offer patients a high probability of tumour regression or increased survival. It is estimated that, at this point, response rates to non-platinum-based chemotherapies have fallen to between 7 and 22% (Thigpen, 2012). Patients may also opt for treatment with tumour targeted agents or enroll in a clinical study – some practitioners believe that some may be better options than non-platinum chemotherapy. Indeed, a select few of these targeted therapies have demonstrated clinical efficacy in HGSC and have been approved by the Food and Drug Administration (FDA) for human use. The agent bevacizumab, an inhibitor of VEGF-angiogenesis, has recently been found to improve response rate, progression free survival, and overall survival compared to standard chemotherapy, in a series of phase III trials (Marchetti et al., 2016). Olaparib, an inhibitor of poly ADP ribose polymerase (PARP), an enzyme involved in DNA repair, has also been approved by the FDA for recurrent HGSC. Studies show that, alone or in combination with paclitaxel and carboplatin,

Olaparib significantly improves progression free survival (Ledermann et al., 2012; Meehan & Chen, 2016; Oza et al., 2015). In addition to the development of targeted cancer therapies, a variety of immunotherapeutic techniques have been explored, some demonstrating efficacy mediated by CD8 T cell activity.

1.3 Immunological T cell Activity

As the primary effector of the cell-mediated immune system, CD8 T cells are responsible for the elimination of infected and malfunctioned cells. All CD8 T cells have unique T cell receptors (TCR) with defined specificity and affinity for protein fragments, called epitopes, which are held by the binding groove of the major histocompatibility complex class I (MHCI), a highly polymorphic surface molecule expressed by all nucleated cells. Endogenous proteins are routinely digested for epitope display by MHCI. As such, these epitopes may be naturally occurring, derived from DNA mutations, or of viral or bacterial origin. Proteins that generate immunogenic epitopes are deemed antigens. In the periphery, naïve CD8 T cells become activated upon TCR engagement by an antigen presenting cell (APC), when accompanied by the appropriate costimulation. To achieve activation, the TCR:MHCI interaction is stabilized by CD8 and is accompanied by engagement of the T cell surface receptor CD28 by the APC ligand B7 (Lenschow, Walunas, & Bluestone, 1996). Activation triggers clonal T cell proliferation and the generation of an antigen specific effector population.

Activated CD8 T cells exhibit a targeted killer phenotype and may eliminate an antigen specific cell population. Upon target recognition, perforin and granzymes are released via

exocytosis of cytoplasmic granules (Harty, Tvinnereim, & White, 2000). Perforin molecules form pores within the target cell membrane, allowing cellular infiltration by granzyme B, which initiates apoptosis in the target cell by caspase activation. Alternatively, CD8 T cell effector function is also mediated by Fas/FasL interaction, whereby T cells expressing FasL bind to Fas on the surface of target cells (Harty et al., 2000). Fas engagement promotes the recruitment of the death-induced signaling complex (DISC), subsequently activating the caspase cascade and causing apoptosis. Additionally, CD8 T cells release a number of cytokines upon TCR engagement. Amongst others, cytokines they release include interferon-γ (IFNγ) and tumour necrosis factor α (TNFα), which have been shown to induce anti-tumour activity (Williams & Bevan, 2007). Indeed, tumour infiltration by CD8 T cells correlates with improved prognosis in various cancer types (Hamanishi et al., 2007; Milne et al., 2009; Sato et al., 2005; Zhang et al., 2003).

1.3.1 Development of TCR specificity during T cell maturation

T cell maturation begins with the generation of TCR variability. This process begins in the bone marrow, where all lymphocytes originate as haematopoietic progenitor cells. Some of these progenitor cells migrate to the cortex of the thymus, where they expand into a population of thymocytes. To generate TCR diversity, early thymocytes undergo random genetic rearrangements at the TCR α and ß loci, which together encode the heterodimeric TCR (Fulton et al., 2015). Due to this genetic rearrangement, each T cell progenitor is committed to one of 10^{15} possible TCR arrangements (Nikolich-Zugich, Slifka, & Messaoudi, 2004). At this

point, thymocytes also express the surface glycoproteins CD8 and CD4, which stabilize interaction of the TCR with either MHCI or major histocompatibility complex class II (MHCII), respectively. Thymocytes are then driven toward either a CD8 T cell lineage or a CD4 T cell lineage. Also known as T helper cells, CD4 T cells recognize epitopes in the context of major histocompatibility complex class II (MHCII), which is expressed only by APCs. As TCR generation is random, it must be determined if the TCR can recognize MHCI, MHCII, or neither. Cortical thymic epithelial cells (cTEC) display self-antigen derived peptides in the context of MHCI and MHCII. While thymocytes migrate through the thymic cortex, they bind any MHC:peptide complexes that have a high affinity for their TCR (Klein, Kyewski, Allen, & Hogquist, 2014). Successful binding of self-antigen provides a survival signal, while a lack of signal leads to death by neglect. Thymocytes that receive a survival signal also commit to a CD4 or CD8 single positive lineage, in accordance with TCR binding to MHCII or MHCI, respectively.

After commitment to a CD4 or CD8 phenotype, thymocytes migrate to the medulla, where tolerance to self-antigen is induced by negative selection. Immunological tolerance of tissue-restricted proteins requires that all potentially autoreactive thymocytes are deleted. To this end, medullary thymic epithelial cells (mTEC) express the transcription factor autoimmune regulator (AIRE), which promotes localized ectopic expression virtually all tissue-restricted transcripts (Klein et al., 2014; Malchow et al., 2016). At this stage, if thymocyte TCRs bind MHC:peptide displayed by mTECs with high affinity, the thymocyte receives an apoptotic signal and is thus deleted (Gallegos & Bevan, 2004; Hinterberger et al., 2010; Oukka, Cohen-Tannoudji, Tanaka, Babinet, & Kosmatopoulos, 1996). As an alternative to deletion, CD4+

thymocytes which exhibit an intermediate affinity for MHCII:peptide receive signals to differentiate into T regulatory (Treg) cells (Apostolou, Sarukhan, Klein, & von Boehmer, 2002; Jordan et al., 2001). Mature T cells then migrate from the thymus into the peripheral blood and lymph where they can initiate their directed effector function.

The efficacy of thymic selection decreases the likelihood of mature T cells recognizing tissue-restricted proteins when they are expressed by tumours. However, despite the vast expression of tissue-restricted antigens within the thymus during negative selection, self-reactive T cells still escape deletion. This is partially evidenced by occurrences of T cell mediated autoimmune disorders in individuals with otherwise healthy immune systems (Dornmair, Goebels, Weltzien, Wekerle, & Hohlfeld, 2003). Within the subfield of antigen discovery for cancer immunotherapy, numerous T cell clonotypes have been isolated which recognize self-antigen expressed by tumours (Wurz, Kao, & DeGregorio, 2016).

1.3.2 Intracellular antigen processing

Antigen specificity of the adaptive immune response relies on the ability of host cells to present a given epitope in the context of MHC. Two primary antigen processing pathways dictate what epitopes may bind to MHCI and MHCII for presentation to CD8 T cells and Th cells, respectively.

Endogenous antigens are presented by MHCI to CD8 T cells

The primary function of MHCI is the presentation of endogenous antigen to CD8 T cells (Figure 1). This molecule is expressed on the surface of all nucleated cells of the human body and is crucial for consistent immune surveillance throughout the body. It consists of an α-heavy chain, ß2-microglobulin, and displays an endogenously-derived short peptide fragment within the extracellular groove (A. C. Goldberg & Rizzo, 2015). Within a given cell, cytosolic proteins, regardless of origin, are routinely tagged with ubiquitin, labeling the protein for proteasomal degradation (Dolan et al., 2012). Peptide fragments are subsequently transported to the endoplasmic reticulum (ER) by TAP (transporter associated with antigen processing), where they are further degraded by endoplasmic reticulum-associated aminopeptidases 1 and 2 (ERAP1 and 2) into short 8-11mer peptides ideal for binding MHCI (Kanaseki, Blanchard, Hammer, Gonzalez, & Shastri, 2006; Serwold, Gonzalez, Kim, Jacob, & Shastri, 2002). Within the ER, chaperone proteins confer stability to the nascent MHCI and facilitate proper entry of the peptide into the binding groove. Once bound, the stabilized MHCI:peptide complex is transported through the Golgi to the cell surface. This process allows CD8 T cells to monitor intracellular protein expression, in order to identify and delete cells harbouring proteins of a foreign or mutated nature (A. C. Goldberg & Rizzo, 2015). In the context of this thesis, this process is notable as it facilitates the presentation of tumour associated antigens (TAA).

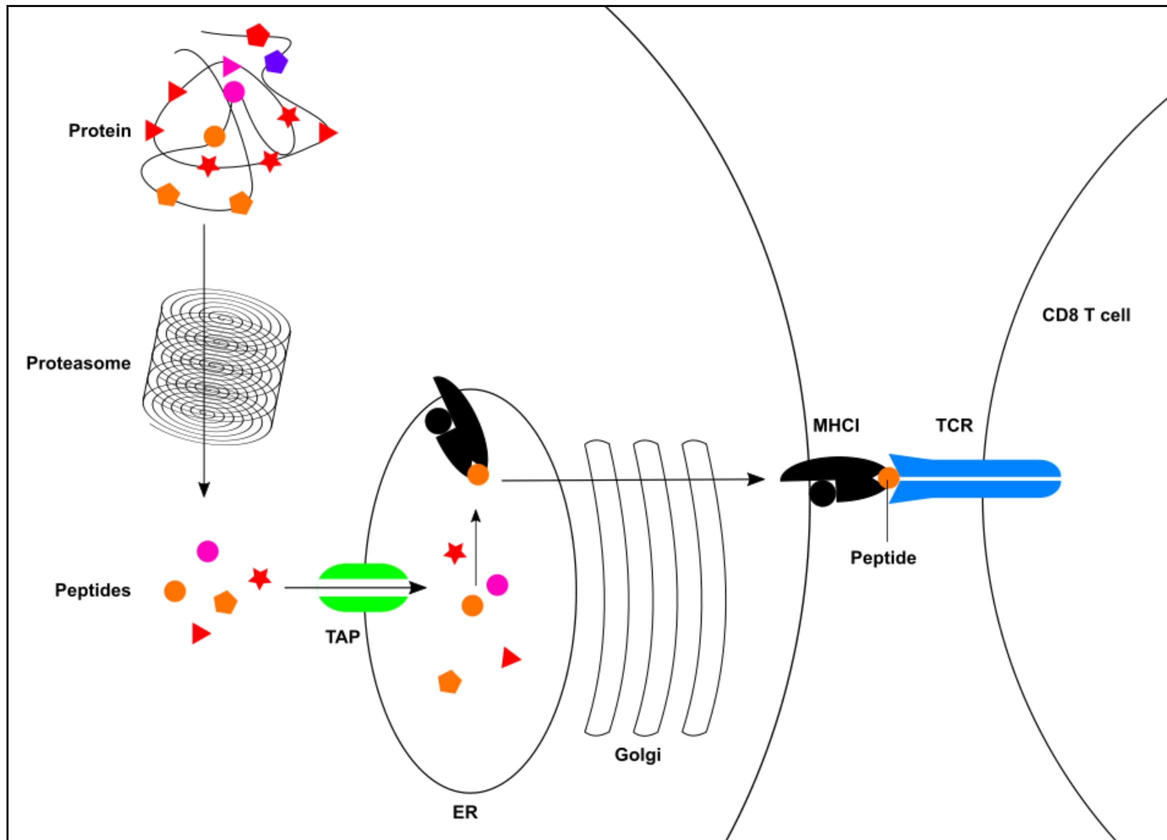

Figure 1. Antigen presentation by MHCI to CD8 T cells. Intracellular proteins are digested by the proteasome and transported into the ER for MHCI loading. Loaded MHCI are shuttled to the cell surface for presentation to TCR of the appropriate specificity.

Exogenous antigens are presented by MHCII to CD4 T cells

The purpose of MHCII is to present of exogenous antigen to CD4 T cells. Unlike MHCI, MHCII is only expressed on professional APCs, such as B cells, dendritic cells (DCs), and macrophages. Exogenous matter is taken up by APCs via endocytosis. Depending on the type of APC, endocytosis may be non-specific, as in innate immune cells such as DCs and macrophages. For B cells, this is triggered exclusively by antigen specific engagement of the B cell receptor. Once endocytosis of the exogenous material has occurred, the endosome fuses with a

lysosome, where the contents are digested into individual peptide fragments prior to being loaded onto MHCII. Due to the unstable structure of MHCII, when it is synthesized in the ER, it is bound to the 'invariant chain', CD74, which offers stability (A. C. Goldberg & Rizzo, 2015). The complex then leaves the ER in the MHCII compartment (MIIC). Within the MIIC, proteases cleave CD74, leaving behind the class II-associated invariant chain peptide (CLIP), which occupies the peptide-binding groove, maintaining its structure. Once MIIC and the peptide-containing endosome fuse, the chaperone protein human leukocyte antigen DM (HLA-DM) facilitates the replacement of CLIP by a foreign peptide, creating the MHCII:peptide complex (A. C. Goldberg & Rizzo, 2015). The complex can then be transported to the APC surface, where peptides of an exogenous origin are displayed to antigen specific CD4 T cells.

Together, MHCI and MHCII presentation of antigenic peptides can lead to the induction of an antigen specific immune response. These responses are directed towards any cell expressing the antigenic protein.

1.3.3 Additional cells involved in the T cell immune response

The CD8 immune response is supported by APCs and CD4 T cells

The cytotoxic activity of CD8 T cells is supported by a series of complex interactions with a variety of other immune cell types. Notably, APCs and CD4 T cells provide stimuli for initiation and maintenance of the cell mediated immune response. Initiation is frequently conducted by DCs, which ingest and degrade cellular debris in order to present antigen for the activation of

both CD8 and CD4 T cells. As mentioned, MHCI is classically reserved for the presentation of endogenous protein; however, cross-presentation pathways allow DCs to display exogenous antigen in order to instigate CD8 T cell activation. Cross-presentation by DCs allows the recruitment of peripheral antigen specific CD8 T cells which have not directly encountered a target cell population. To aid in the activation and maintenance of the CD8 T cell response, DCs also activate CD4 T cells. As professional APCs, DCs express and display MHCII, which facilitates antigen presentation to the CD4 T cell TCR, an interaction which is stabilized by CD4 (Knosp & Johnston, 2012). Activated CD4 T cells may differentiate into a variety of effector phenotypes, including Th1, Th2, Th17, and Treg cells, each of which possess unique functions. Of these, Th1 cells are the most directly related to CD8 directed immunity against intracellular pathogens. The activation of CD8 T cells is promoted by interleukin-2 (IL-2) and IFNγ, which are both secreted by Th1 cells. The differentiation to Th1-type immune responses is favoured in the presence of IL-12, IL-18, and IFNγ (Knosp & Johnston, 2012). Together, APCs and Th1 cells stimulate the immune system to mount a CD8 T cell mediated immune response.

The Th2 lineage of CD4 T cells promotes humoural immunity

The humoural immune response, mediated by B cells, is promoted by CD4 T cell differentiation to a Th2 phenotype. Commitment of CD4 T cells to the Th2 lineage is primarily due to the influence of IL-4. Subsequent IL-4, IL-5, and IL-6 production by Th2 cells serves to function in a positive feedback loop as well as promote B cell activation and B cell differentiation into immunoglobulin-secreting plasma cells (Yang & Ansell, 2012). Plasma cells produce and release antibodies which facilitate antigen-targeted cell killing and inhibition via

phagocytosis, complement activation, or antibody-dependent cellular cytotoxicity (ADCC). Despite this, in anti-tumour immunity, the role of B cells has been controversial. For example, some studies have reported that B cells have immunosuppressive effects by directly inhibiting CD8 T cell responses (Olkhanud et al., 2011; Z. Qin et al., 1998), while others have reported that B and T cell tumour infiltrates can co-localize at tertiary lymphoid structures in order to mount cooperative anti-tumour immune responses that correlate with improved survival (Kroeger, Milne, & Nelson, 2016). Indeed, infiltration of plasma cells has reportedly been associated with favourable prognoses in a number of cancer types (Iglesia et al., 2014; Lohr et al., 2013; Richards et al., 2012; Schmidt et al., 2012). Apart from their classical role in antibody production, B cells also release immunomodulatory cytokines that promote T cell responses, possibly explaining some of the benefits of B and T co-infiltration. B cells also act as APCs, which can supplement DC activity to activate and support a CD8 T cell response. Together, CD4 T cells and B cells provide an additional avenue for the immune system to attack cancerous cells.

An alternate lineage for CD4 cells – Th17

At times in conflict with the other T subsets, CD4 cells may also differentiate into Th17 cells. Differentiation to the Th17 lineage is promoted by numerous cytokines including IL-6, IL-21, IL-23, and TGF-ß (H. Qin et al., 2009; X. O. Yang et al., 2007). Committed Th17 cells are partially defined by their ability to secrete IL-17, but subsets within the Th17 classification have variable effects on tumour immunity. As further discussed in Bailey *et al* (2014), these cells may complement or inhibit an immune response against cancerous cells. In the presence of IL-12, Th17 cells may further differentiate into Th1-like cells to strengthen a CD8 T cell-mediated anti-

tumour response (Wang et al., 2014). This alteration is characterized by a loss of IL-17 production and an initiation of IFNγ secretion. However, in the presence of TGF-ß, Th17 cells may assume a regulatory role, in which they upregulate FoxP3 expression, and also foster the recruitment of Treg cells (Bailey et al., 2014). As such, Th17 cells contribute to the modulation of the cell mediated immune response, playing a variable role between Th1 and Tregs.

Restraining the immune response – T regulatory cells

T regulatory cells are an immunosuppressive class of cells whose classical role involves mediating peripheral tolerance to self-antigens, which is critical to the prevention of autoimmunity and resolving acute inflammatory reactions. Typically defined as CD4+CD25+FoxP3+ T cells, Tregs inhibit the actions of other pro-inflammatory immune responses through mechanisms such as contact inhibition or secretion of immunosuppressive cytokines such as IL-10 and TGF-ß (Ulges, Schmitt, Becker, & Bopp, 2016). Like CD8 or CD4 cells, Tregs typically mature in the thymus; however, regulatory differentiation from naïve CD4 T cells may occur as well. This spontaneous Treg differentiation is induced by the cytokines IL-6 and TGF-ß (Kiraz, Baran, & Nalbant, 2016). While Tregs are critical to the prevention of devastating acute inflammation (e.g. cytokine storm) and autoimmunity, they also frequently infiltrate tumours and antagonize beneficial anti-tumour immune responses. As such, some studies have reported that the presence of intratumoural Tregs correlates with poor prognosis and suppression of tumour immunity (Antony & Restifo, 2005; Bos, 2016). Curiously, other reports have found that Treg infiltration is positively associated with improved overall survival (Fridman, Pages, Sautes-Fridman, & Galon, 2012). While this may be counterintuitive, studies

have shown that Treg infiltration can be a sign of an active anti-tumour immune response – in ovarian cancer, tumours with high ratios of CD8 T cells to Tregs demonstrate superior overall increased overall survival (Sato et al., 2005). Unfortunately, the activity of Treg cells, which is critical during most immune responses, can be quite detrimental for the anti-tumour response.

Overall, the immune system involves a host of cell types which fill specific support and effector roles in order to provide an effective immune response against antigen of tumoural or foreign origin.

1.4 Cancer and the Immune System

1.4.1 Immunoediting – The evolution of a tumour

If the immune system is capable of recognizing and eliminating cancerous cells, why are cancerous cells still able to grow and establish tumours, invade and metastasize? In 2004, Dunn and colleagues proposed their model of cancer immunoediting, which revolves around three concepts: elimination, equilibrium, and escape (Dunn, Old, & Schreiber, 2004). Each concept of this model contributes to a stage of malignant tumour genesis and its interaction with regulatory immune oppression.

Elimination – The immune system kills newly mutated cells

The first phase of this model, elimination, describes the initial generation of a mutating cellular mass. It is triggered by the initial mutations which have led a given cell to circumvent the genetic reins which maintain regular proliferation patterns (Macleod, 2000). Accelerated

proliferation of the mutated cells cause physical tissue disruption of the local environment (Carmeliet & Jain, 2000; Hanahan & Folkman, 1996; Sternlicht & Werb, 2001). This disruption triggers an inflammatory response which recruits an innate immune response against the new tumour (Vicari & Caux, 2002). Through the actions of the innate immune system, macrophages and NK cells cause IFNγ production and subsequent tumour killing (Bromberg, Horvath, Wen, Schreiber, & Darnell, 1996; Coughlin et al., 1998; Kumar, Commane, Flickinger, Horvath, & Stark, 1997; Luster & Leder, 1993; Z. Qin & Blankenstein, 2000). Dead and dying tumour cells provide a source of tumour antigen: proteins to be ingested by DCs to prime T cell responses.

Activated DCs migrate toward the tumour draining lymph node after tumour antigen acquisition for presentation to naïve CD8 T cells (Gerosa et al., 2002). Chemokine gradients then attract newly activated T cells to the tumour site for killing. At the tumour site, cytokines provided by CD4 T cells help maintain the efficacy of tumour-specific CD8 T cells, induce tumour cell killing by mechanisms previously discussed. Dunn *et al* propose that this process repeatedly occurs throughout life (2004), as new adaptive responses are directed toward each antigenically distinct tumour. When the tumour has been successfully eradicated by the immune response, this process ends. However, if the tumour is not killed, it proceeds to a state of equilibrium.

Equilibrium – A balance between tumour proliferation and immunogenic death

The equilibrium phase of tumourigenesis may persist indefinitely and is resolved by either eradication or escape. It is a state of homeostasis in which the immune system places an adaptive evolutionary pressure upon the tumoural cells (Koebel et al., 2007). Throughout,

genetically unstable cells continually proliferate into antigenically distinct generations of potentially malignant progenitors (Lengauer, Kinzler, & Vogelstein, 1998). As in macroevolution, cells that are unfit are unable to survive the attack of the immune system, while those that do survive produce a new generation of daughter cells carrying the mutations of their parental cell. Growing tumours fluctuate between times of increased proliferative rates, when a beneficial mutation occurs, and increased death rates, when the immune system identifies novel tumour antigens. It is only after random mutations accumulate over generations that a tumour cell might have the capability to evade the adaptive immune system – this may take many years (Loeb, Loeb, & Anderson, 2003). When the immune system can no longer identify nor kill tumour cells at a competitive rate, the tumour has escaped the balance between growth and immune directed death, allowing it to proliferate in spite of immunological pressures (Khong & Restifo, 2002).

Escape – The tumour overcomes immune pressure

When the tumour has escaped the immune response, the tumour may proliferate to the point of being clinically detectable (Schreiber, Old, & Smyth, 2011). In the escape stage, a tumour may have acquired various abnormalities that contribute to immune evasion. These may include any of, but are not limited to: loss and down-regulation of MHCI, defective death receptor signaling, cytokine-directed immunosuppression, FasL directed T cell apoptosis. Indeed studies have shown that over 40% of tumours downregulate MHCI, which avoids T cell immune recognition and destruction (Algarra, Collado, & Garrido, 1997; Marincola, Jaffee, Hicklin, & Ferrone, 2000). Additionally, many tumours display defects in the antigen processing

pathway for MHC presentation (Korkolopoulou, Kaklamanis, Pezzella, Harris, & Gatter, 1996; Restifo et al., 1993; Seliger et al., 1997). These alterations inhibit recognition or killing by the immune system, allowing tumour cells to continue proliferating.

1.4.2 Immunotherapy – Helping the immune system fight cancer

The purpose of immune-based therapies is to aid the natural immune system in the conflict with a continually evolving tumoural population. A large portion of the cancer immunotherapy field focuses on improving the presence and activity of T cells in the tumour environment. While tumour infiltrating lymphocytes (TIL) have been identified as early as the 1860s (Virchow, 1863), our understanding of their role in tumour control has only recently appreciated. In many cancer types, including ovarian, T cell infiltration of the tumour is correlated with positive clinical outcomes (Hamanishi et al., 2007; Mariya et al., 2014; Milne et al., 2009; Sato et al., 2005; Zhang et al., 2003). Although this correlation does not mean that TIL directly mediate clinical benefit, it has been found that many of these infiltrative T cells are indeed tumour specific (Lu et al., 2014). Since this discovery, immunotherapy has become a frequent subject of research. Due to the complex nature of cancer immunology, immunotherapeutic strategies are broad and aim to influence the activity of the immune system in diverse ways. The strategies discussed here were selected based on their relevance to this thesis and their prevalence within the field. Each of these strategies has shown some therapeutic promise: vaccines, adoptive cell therapy (ACT), chimeric antigen receptor (CAR) T cells, as well as immune checkpoint blockades.

Vaccines – Providing tumour antigens for immune activation

Therapeutic cancer vaccines aim to direct immune responses by providing tumour antigens to the immune system in order to establish a tumour-specific memory population. Tumour antigens can be provided in a number of different ways: peptides or complete proteins, DCs, recombinant viral vectors, and tumour cells, amongst others (Schlom, 2012). Peptide vaccines typically consist of a set of peptides representing a small number of tumour antigens, and are supplemented with adjuvant on various prime-boost schedules. Dendritic cell vaccines provide a vehicle for the delivery of selected epitopes to the patient, as they are potent antigen presenting cells capable of generating a robust immune response directed towards the tumour antigen. Similarly, viral vector vaccines are tailored to specifically encode the antigen of interest prior to infection of the host. Tumour antigens are then expressed within antigen presenting cells for stimulation of the host immune response. Selection of the target tumour antigen is one of the greatest challenges within the development of any of these strategies. For a given target to give rise to a viable tumour epitope, intracellular processing machinery must naturally process a unique the antigenic epitope from the parent protein, the tumour-specific peptide must have the threshold affinity required to bind MHC, and T cells must exist within the immune repertoire which have sufficient affinity and specificity for the antigen (Martin et al., 2016). In addition, an ideal target would confer a survival advantage to the tumour cells expressing it, thus preventing target-downregulation without consequence.

Identification of tumour antigens for immunological targeting poses a difficult obstacle for vaccination strategies. However, autologous tumour cell vaccines bypass the antigen

selection problem. This strategy has the capacity to apply broad spectrum activation against any tumour antigens present. Autologous tumour cell vaccines are often supplemented with transduced granulocyte-macrophage colony-stimulating factor (GM-CSF) to enhance APC functionality (D. Z. Chang, Lomazow, Joy Somberg, Stan, & Perales, 2004; Simons & Sacks, 2006; Yu, Chueh, Tsai, Lin, & Qiu, 2016), or even fused with autologous DCs directly, in order to bypass the process of initial uptake of the antigen (Garcia-Marquez, Shimabukuro-Vornhagen, Theurich, & von Bergwelt-Baildon, 2013). However, T cells exhibit competitive binding to DCs for activation, which may prevent diverse T cell activation to such a variety of antigen, whereas DCs presenting a single tumour antigen to a single T cell clone may be more efficacious.

Adoptive cell therapy – Providing immune cells for tumour attack

While vaccination aims to target a tumour based on a single or small group of specific antigens, ACT is geared toward broadly targeting any antigens that may be present on the tumour. With this method, TIL are harvested from the tumour and cultured *ex vivo* under conditions which may somewhat vary between studies. For example, they may be stimulated by autologous DCs or allogeneic irradiated mononuclear cells, in conjunction with anti-CD3 antibody and IL-2. In this manner, TIL cultures expand to the order of 10^{11} cells, and are then infused back into the patient after lymphodepletion, which enhances the effector function of infused antigen specific T cells (Gattinoni et al., 2005; Rosenberg & Restifo, 2015). This approach has yielded significant clinical responses by tumours, particularly in melanoma patients (Besser et al., 2013; Dudley et al., 2002; Pilon-Thomas et al., 2012; Radvanyi et al.,

2012). There are now multiple ovarian cancer clinical trials assessing the merits of this technique (NCT01883297, NCT01174121, NCT02482090).

Engineered T cell receptors – Creating antigen specific T cells

Engineered TCR therapy shares many aspects of each of the strategies discussed above. Like vaccination, cloned TCRs focus on single or few known antigens. These antigens must be known in order to generate an antigen specific TCR. A bulk population of antigen specific T cells can then be infused to the patient in a manner identical to ACT. In this technique, TCR specificity is selected and genetically directed for the infused T cell product. These genetic alterations can be made virally with high efficiency (Rosenberg & Restifo, 2015). This can grant the T cell repertoire the ability to recognize antigens which there is existing peripheral tolerance to or to which TCR frequency is undetectably low. In another technique, using chimeric antigen receptor (CAR) T cells, immunoglobulin variable regions may be linked to the intracellular domain of the TCR. This directs T cell cytotoxicity against antigens which are not limited to MHCI:peptide complexes. These new antigens may simply be irrelevant surface proteins expressed uniquely in tumoural tissue (Imai et al., 2004; Song et al., 2011). For example, CD19 CAR T cell therapy has shown remarkable successes in B cell lymphomas (Maude et al., 2014; Porter, Levine, Kalos, Bagg, & June, 2011).

Checkpoint blockade – Taking off the brakes

Debatably the most popular of current immunotherapy tactics, immune checkpoint blockade has shown promising results in various cancer types, including melanoma NSCLC, and

renal cell carcinoma. Within this class of therapy, the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitor ipilimumab and PD-1 inhibitors nivolumab and pembrolizumab have been recently approved by the FDA. As discussed in section 1.3, CD28 binding by the APC molecule B7 is required for CD8 T cell activation. However, T cell activation also causes upregulation of CTLA-4, which intercepts B7 binding to CD28. Engagement of CTLA-4 blocks costimulation and inhibits T cell activation (Chambers, Kuhns, Egen, & Allison, 2001; Teft, Kirchhof, & Madrenas, 2006). Ipilimumab blocks this inhibitory interaction, allowing T cell activation to proceed. Indeed, ipilimumab has demonstrated particular efficacy in the context of melanoma, with rates of response ranging up to 25% (Hodi et al., 2010; Schadendorf et al., 2015). Additionally, studies have revealed significant clinical benefit in a number of other cancer sites, including non-small cell lung cancer, renal cell carcinoma, prostate cancer, and ovarian cancer (Hodi et al., 2008; Lynch et al., 2012; van den Eertwegh et al., 2012; J. C. Yang et al., 2007). Similarly, the receptor programmed cell death-1 (PD-1) and its ligand PD-L1 have also been the target of numerous antibody based blockade strategies. Expression of PD-L1 can be stimulated by IFN-γ in many cell types, including tumour cells, which in turn engage PD-1 receptors on activated T cells. Binding of PD-1 leads to attenuation of the T cell response. Anti-PD-1/PD-L1 therapies have demonstrated significant clinical benefit in many different cancerous settings (Ansell et al., 2015; Brahmer et al., 2015; Gettinger et al., 2015; Le et al., 2015; Motzer et al., 2015), with ongoing trials (NCT02626065, NCT02504372, NCT02853331, NCT02819518, NCT02684292, NCT02702401, and more). Although both checkpoint blockade strategies have shown significant clinical benefit, neither have substantial claims to the mechanism of action behind their effects. However, they have common prognostic predictors, which include a correlation

with mutational load, high TIL frequency, as well as the presence of their respective targets on TIL.

1.5 Lactate Dehydrogenase C as a Tumour Antigen

1.5.1 Cancer Testis Antigens

Despite the efficiency of thymic selection at regulating self-tolerance within the immune system, it is not infeasible for T cells to escape these mechanisms (Dornmair et al., 2003). It is known that autoreactive T cells are present in the periphery. These are the autoreactive T cells we wish to isolate in order to characterize a naturally expressed protein as a TAA. When choosing potential antigens to investigate, tumour specificity is of great importance. For this reason, the upregulation of testis-restricted proteins within cancerous cells is notable. These so called cancer-testis (CT) antigens, of which there are over 200, constitute a significant portion of all identified potential TAA. Many of these CT antigens have been investigated by other groups (Vigneron, Stroobant, Van den Eynde, & van der Bruggen, 2013), and reactive epitopes have been categorized and defined by the MHC allele they were discovered on.

Of these CT antigens, NY-ESO-1 and MAGE-A3 have made a notable impact (Wurz et al., 2016). Studies of NY-ESO-1 targeted immunotherapies, primarily conducted in melanoma patients, have shown significant efficacy, with positive response rates as high as 88%. These clinical trials vary in method, but include adoptive CD4 T cell therapy, adoptive CD8 T cell therapy, NY-ESO-1-expressing viral vaccination, and peptide/adjuvant vaccination.

Immunotherapies based on MAGE-A3 have not shown such promising results. While protein and peptide vaccinations have had promise in early phase clinical trials, no phase III studies have upheld their efficacy. Furthermore, a phase I/II trial of an adoptive cell therapy with engineered TCRs against MAGE-A3, despite generating long-lasting responses in multiple patients, exhibited cross-reactivity with MAGE-A12 (Morgan et al., 2013). This cross reactivity led to substantial neuronal destruction and the death of two patients. As such, it is critical that additional targets continue to be evaluated as potential antigens.

The testis-restricted LDH isoform lactate dehydrogenase C (LDHC), provides an intriguing potential immunotherapeutic target, given its expression patterns. Apart from its testis restriction, one study has observed its ectopic expression in melanoma, breast, colon, lung, ovarian, prostate, renal, thyroid, and cervical cancers (Koslowski et al., 2002). Broad expression between patients across multiple cancer types makes LDHC a tempting prospect for antigen discovery.

1.5.2 Lactate dehydrogenase

Enzymatic activity of lactate dehydrogenase

Lactate dehydrogenase (LDH) is a tetrameric enzyme which catalyzes the conversion between pyruvate and lactate, which is coupled with the conversion between NADH and NAD+. As the catabolic pathway of glycolysis utilizes the reduction of NAD+ to NADH during the breakdown of glucose to pyruvate, it is imperative that NAD+ be regenerated for further energy

production. When sufficient oxygen is available, pyruvate enters the citric acid cycle in the mitochondrion, in order to supply NADH to oxidative phosphorylation. Oxidative phosphorylation utilizes the oxidation of NADH to NAD+ in a series of coupled redox reactions to generate a proton gradient across the mitochondrial inner membrane which in turn is used in the generation of ATP. However, when oxygen is not available to the cell, ATP is solely generated by glycolysis. The reduction of pyruvate by lactate dehydrogenase provides regeneration of NAD+ to be utilized in further glycolytic reactions for additional ATP generation. Utilizing this pathway, a cell can generate ATP at a significantly higher rate than those that rely on oxidative phosphorylation (Pfeiffer, Schuster, & Bonhoeffer, 2001). However it cannot be maintained for long, as it increases the local concentration of lactic acid, as commonly noted in muscles during anaerobic exercise. In the liver, lactate dehydrogenase converts lactate back to pyruvate, which is recycled in gluconeogenesis, to be returned to muscle and other cells as glucose (Kalderon, Korman, Gutman, & Lapidot, 1989). Together, the pathways of anaerobic glycolysis and gluconeogenesis form the Cori cycle.

The isoenzymes of lactate dehydrogenase

The monomer subunits which together assemble the LDH tetramer are encoded by the three genes *LDHA*, *LDHB*, and *LDHC*. Together, subunits A and B may generate any of five unique homo- and hetero- tetramers, which are differentially assembled in various tissues, although general expression of the *LDHA* and *LDHB* genes is fairly ubiquitous throughout the body (Blanco & Zinkham, 1963). The LDH gene *LDHC* encodes a third isoform, which is involved in the assembly of the homotetramer LDH-C_4 (E. Goldberg, Eddy, Duan, & Odet, 2010). For the

purposes of this thesis, it is this gene product which will be here on referred to as LDHC. The expression of LDHC is restricted to male germ cells (E. Goldberg et al., 2010; Koslowski et al., 2002). LDHC, having developed due to duplication of the *LDHA* gene, is structurally extremely similar to both LDHA and B. Where its differences lie, however, are in enzymatic activity, due to select differences in its primary sequence (Figure 2). Amino acid substitutions, strewn throughout the protein and within the active site, provide alterations which to contribute to its unique activity within the testis. These alterations lead to a difference in substrate/product inhibition patterns from LDHA and B (Li, Fitch, Pan, & Sharief, 1983; Li et al., 1989). Each of these isoforms experiences some level of substrate inhibition from pyruvate, LDHC being the most sensitive (Battellino, Jaime, & Blanco, 1968). While catalyzing the reverse reaction, lactate to pyruvate, which is thermodynamically less favourable, LDHC is not inhibited by increased concentrations of lactate, unlike LDHA. However, these studies observed this inhibitory activity at concentrations reaching far beyond that of what would be seen in the body (Battellino et al., 1968; Usher-Smith, Fraser, Bailey, Griffin, & Huang, 2006). This pattern of inhibition has, in the past, led investigators to believe that LDHC preferentially catalyzes the reverse reaction of lactate oxidation. This is, in fact, not the case, as numerous enzymatic studies have shown that each LDH catalyzes this reversible reaction without bias, albeit with different rates (Battellino et al., 1968). A number of studies have also shown that the differential amino acids of LDHC grant it broader substrate specificity – LDHC may additionally catalyze the redox reactions between 2-oxo-butanoate/2-oxo-pentanoate and 2-hydroxy-butanoate/2-hydroxy-pentanoate, whereas the ubiquitous enzyme isoforms cannot (Blanco, 1973). What metabolic advantages this

flexibility may provide spermatozoa in the testis are currently unknown, although LDHC activity is crucial to the development of spermatozoa (Odet et al., 2008).

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LDHC    MSTVKEQLIEKLIEDDENS-QCKITIVGTGAVGMACAISILLKDLADELALVDVALDKLK
LDHA    MATLKDQLIYNLLKEEQTP-QNKITVVGVGAVGMACAISILMKDLADELALVDVIEDKLK
LDHB    MATLKEKLIAPVAEEEATVPNNKITVVGVGQVGMACAISILGKSLADELALVDVLEDKLK

LDHC    GEMMDLQHGSLFFSTSKITSGKDYSVSANSRIVIVTAGARQQEGETRLALVQRNVAIMKS
LDHA    GEMMDLQHGSLFLRTPKIVSGKDYNVTANSKLVIITAGARQQEGESRLNLVQRNVNIFKF
LDHB    GEMMDLQHGSLFLQTPKIVADKDYSVTANSKIVVVTAGVRQQEGESRLNLVQRNVNVFKF

LDHC    IIPAIVHYSPDCKILVVSNPVDILTYIVWKISGLPVTRVIGSGCNLDSARFRYLIGEKLG
LDHA    IIPNVVKYSPNCKLLIVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLG
LDHB    IIPQIVKYSPDCIIIVVSNPVDILTYVTWKLSGLPKHRVIGSGCNLDSARFRYLMAEKLG

LDHC    VHPTSCHGWIIGEHGDSSVPLWSGVNVAGVALKTLDPKLGTDSDKEHWKNIHKQVIQSAY
LDHA    VHPLSCHGWVLGEHGDSSVPVWSGMNVAGVSLKTLHPDLGTDKDKEQWKEVHKQVVESAY
LDHB    IHPSSCHGWILGEHGDSSVAVWSGVNVAGVSLQELNPEMGTDNDSENWKEVHKMVVESAY

LDHC    EIIKLKGYTSWAIGLSVMDLVGSILKNLRRVHPVSTMVKGLYGIKEELFLSIPCVLGRNG
LDHA    EVIKLKGYTSWAIGLSVADLAESIMKNLRRVHPVSTMIKGLYGIKDDVFLSVPCILGQNG
LDHB    EVIKLKGYTNWAIGLSVADLIESMLKNLSRIHPVSTMVKGMYGIENEVFLSLPCILNARG

LDHC    VSDVVKINLNSEEEALFKKSAETLWNIQKDLIF-
LDHA    ISDLVKVTLTSEEEARLKKSADTLWGIQKELQF-
LDHB    LTSVINQKLKDDEVAQLKKSADTLWDIQKDLKDL
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Figure 2. Alignment of LDHC to LDHA and LDHB. Amino acid sequences of LDHC, LDHA, and LDHB. Amino acid residues which are unique to LDHC (62/332) are highlighted.

Lactate dehydrogenase activity in the tumour

In addition to in the testes, LDHC is frequently upregulated in cancer of various types. Within the tumour, while LDHC may specifically confer benefits unique to its particular isoform, general LDH expression does aid in tumour growth and survival. LDH expression allows cancerous cells to generate ATP via anaerobic glycolysis, which is advantageous for a number of reasons. A defining characteristic of cancerous cells is their tendency to proliferate at a

substantially increased rate, which requires a significant amount of biomaterial. That material is provided by mid-cycle substrates in the tricarboxylic acid (TCA) cycle (Vander Heiden, Cantley, & Thompson, 2009). Due to the loss of TCA cycle substrates, oxidative phosphorylation is largely unrealistic; thus, tumours frequently generate ATP by anaerobic respiration. Furthermore, the binary nature of oxygen availability within the tumour lends itself to the requirements of LDH, as hypoxic regions of the tumour can convert pyruvate to lactate, which can be taken up by more oxygenated regions of the tumour for oxidative phosphorylation (Sonveaux et al., 2008). Additionally, the lactic acidosis of the tumour microenvironment is inherently anti-immunogenic, as it compromises the functionality of T cells (Blank, Haanen, Ribas, & Schumacher, 2016). This suppression of function is also evidenced by the strong inverse correlation of serum LDH concentration with the clinical outcomes of PD-1 and CTLA-4 immune checkpoint blockades (Diem et al., 2016). Due to these characteristics which are beneficial to the tumour, LDHC makes a tempting potential antigen: in addition to the immune response being targeted against the tumour, it would be targeted specifically due to a protein which provides a degree of cellular fitness to the tumoural clones expressing it – a quality typically attributed to driver mutations.

1.6 Summary

This chapter has illustrated the interaction between the immune system and cancer in general. The highly evolved adaptive immune system subjects a developing tumour to substantial selective pressure, described as the complex process of immunoediting. Tumour

escape occurs when the immune system has failed to contain tumour development and tumour evolution has developed the ability to grow at rate which has surpassed the rate of immune elimination. The activity of CD8 T cells in immunoediting and tumour control is critical and is supported by and shared with a diverse set of immune cells, of an innate origin as well as an adaptive origin. Immunotherapy posits to bolster the immune response to cancer by acting against some common immune evasion methods, such as antigen escape. In this past chapter I have described some of the methods used with this aim. One recurring challenge that some immunotherapeutic strategies run into is the selection of immunological targets within a given tumour. Here I propose lactate dehydrogenase C as an immunological CD8 T cell target. My hypothesis was that T cells exist within a patient’s T cell repertoire which can recognize LDHC peptides in the context of MHCI, and kill autologous cells expressing LDHC.

Chapter 2: LDHC peptide specific T cells reside in the patient T cell repertoire

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DSN, DAW, and JJL designed the study. DSN, DAW, JLK, SDM, DK, JSN, JW, and JJL were involved in data acquisition and/or interpretation. ZP and LH provided preliminary data.

2.1 Introduction

Interaction between the immune system and cancer is dynamic and complex (Dunn et al., 2004). Throughout life, tumour control is mediated by this interaction. Regardless of the surrounding complexities, studies have shown that CD8 T cell infiltration is associated with improved outcome in most solid cancer types (Fridman et al., 2012; Milne et al., 2012). Recent studies in immune blockade therapy suggest that lymphocytic infiltrates recognize tumour antigens, but are often inhibited through various means within the tumour microenvironment (Blank et al., 2016; Weber, 2010). Despite the knowledge of T cell tumour infiltration and its prognostic relevance, TCR specificities of T cell infiltrates are relatively unknown. However, studies have revealed that T cells specific to known antigens do account for a small portion of all CD8+ infiltrates, and there are indeed numerous accounts of TIL which exhibit tumour specificity. These accounts have shown that subsets of CD8+ TIL can both recognize and kill autologous tumour cells *in vitro* (Andersen et al., 2012; Kelderman et al., 2016).

In the field of cancer immunotherapy, there is a constant need to discover novel antigens. To mount an anti-tumour immune response, CD8 T cells require tumour antigens, derived from mutated or tissue specific proteins. However, on the spectrum of immunogenic cancer types, HGSC ranks relatively low, with a low mutational burden (Alexandrov et al., 2013). In melanoma, proven to be a highly immunogenic cancer, a portion of aggregate TIL are specific to somatic point mutations arisen throughout tumour evolution. In contrast, recent studies of HGSC have revealed that very few cases (12%) are likely to provide point mutation-derived immunogenic neoantigens for therapy (Martin et al., 2016). For these reasons, it is imperative

that projects are undertaken to identify additional tumour targets for immunotherapy. Cancer-testis proteins have shown viability as CD8 tumour targets, as demonstrated in therapeutic studies involving MAGE proteins and NY-ESO-1 (Wurz et al., 2016). These CT antigens are particularly intriguing targets in the context of ovarian cancer, as they are tumour specific and also happen to be expressed in a high proportion of tumours. Antigenic tumour proteins expressed across multiple patients present an opportunity to generate a more widely applicable potential therapy. Notably, the CT antigen LDHC has been found to be expressed in patient tumours at frequencies as high as 100% (lung adenocarcinoma, n=18), through 83% (cervical cancer, n=6), 44% (melanoma, n=16), to as low as 35% (breast cancer n=20) (Koslowski et al., 2002; Yen et al., 2007). In our cohort of patients, preliminary PCR data revealed that 76% of HGSC patients express tumoural *LDHC* (22/29, data not shown). Such high frequency of a potential antigen warranted further investigation. As such, I hypothesized that HGSC patients harbour CD8 T cells which recognize individual MHCI epitopes unique to LDHC.

To investigate this hypothesis, I prioritized the analysis of HGSC patients whose tumours express *LDHC* mRNA. Ascites from *LDHC*+ ovarian cancer patients was utilized for analysis due to the origin of ascites fluid. Ascites is a peritoneal build up of fluid very common in ovarian cancer patients, which contains slough from the tumour. Tumour *associated* lymphocytes (TAL) from the tumour slough may contain antigen specific T cells which have previously infiltrated the tumour.

When mining the T cell repertoire with the aim of identifying a specific T cell clone, it is crucial to consider its availability. While a given activated clone may occupy a substantial

portion of the lymphocytic compartment, naïve antigen specific T cells may be present at frequencies no higher than $1/10^5$. Considering this potential infrequency of a LDHC specific T cell, it had to be ensured that rare T cell populations are detectable. Bulk T cell expansion methods from tumour infiltrate can be used for the assessment of both TIL and TAL; however, these methods provide no degree of enrichment for rare antigen specific T cells. These methods sustain a given T cell clone with a single origin cell at a frequency of $1/10^5$, and the threshold of detection of the methods at our disposal was closer to $1/10^4$. However, selective expansion methods may be and are frequently used in the literature for this reason. Expansion of a select T cell clone may be facilitated by DC stimulation, in which peptide-loaded autologous DCs repeatedly stimulate and expand specific T cells from a bulk T cell culture (Nielsen et al., 2016). However, due to the infrequency of DCs within peripheral blood, maturation of a suitable DC contingent requires autologous peripheral blood mononuclear cells (PBMC) in excess of $2x10^8$ cells. In this instance, these enrichment methods proved to be infeasible for this project, as the accessibility of patient PBMCs was low in comparison.

Patient cells must be efficiently analyzed without using an inappropriate amount of material, as less is typically available from cancer patients. Recent studies have published one such viable method for identifying rare antigen specific T cells (Geiger, Duhen, Lanzavecchia, & Sallusto, 2009; Theaker et al., 2016). In this method, a bulk population of T cells is distributed amongst hundreds of individual cultures. These divided T cell cultures are simultaneously non-specifically stimulated: each culture originates from 2000 autologous T cells and is expanded in a parallel fashion. Provided that individual T cell clones grow at the same relative pace in each

culture, this method generates a series of cultures with a minimal T cell clone frequency of 1/2000. This frequency is well within the bounds of detection for immunological assay. Previous studies have demonstrated this method's efficacy in the assessment of naïve T cell populations directed toward specific antigens such as gp100 in melanoma and cadherin-3 in breast cancer (Theaker et al., 2016). In each of these cases, tumour killing was successfully mediated by these isolated T cells. Given these results, this miniline strategy could be effective for the analysis of LDHC reactivity in HGSC cases.

In order to assess a given immunological target, it is common to evaluate a T cell population's ability to recognize exogenous peptides potentially specific to their TCR. In these assays, synthetic peptides are provided in vitro for extracellular loading onto the MHCI. As such, inquisitor peptides must have a high affinity for the MHCI, which varies between patients in accordance with their human leukocyte antigen (HLA) haplotype – i.e. which set of MHCI alleles that patient carries. Binding prediction algorithms for MHCI such as NetMHCpan can help select specific peptides for assessment. This is an especially beneficial strategy in the assessment of patient-specific mutations. However, in the broad assessment of a shared CT antigen, it was prudent to utilize a method unbiased toward patients possessing any one specific individual HLA allele. With this objective, it was not possible to study just a select few peptides from the 1200+ possible epitopes of LDHC. Fortunately, T cells have been shown to recognize cognate epitopes contained within long 15mer peptides (Fiore-Gartland et al., 2016). Furthermore, a recent study has shown that T cell responsiveness is unaffected by the presence of irrelevant peptides within pools of up to 300 peptides (Chevalier et al., 2015). Thus, a LDHC library of

overlapping 15mer peptides was synthesized for assessment of T cell responsiveness in the context of peptide pools.

In this chapter, CD8 T cells from the ascites of five HGSC patients are screened for recognition of LDHC via a 15mer peptide library. A single CD8 T cell clone which recognizes LDHC peptide was isolated from one of five patients. This T cell clone was isolated by fluorescence-activated cell sorting (FACS) based on CD8 T cell activation marker 4-1BB for further analysis. Four of five patients did not show reactivity to the LDHC peptide library.

2.2 Methods

2.2.1 Patients, biospecimens, and clinical data

The ovarian cancer patient cohort from which biospecimens were drawn has been previously described in reports associated with our laboratory (Castellarin et al., 2013; Wick et al., 2014). In brief, collections occurred through a prospective study, operating in partnership with the BC Cancer Agency's Tumour Tissue Repository. The study was granted ethics approval by the Research Ethics Board of the BC Cancer Agency as well as the University of British Columbia (Certificate REB# H07-00463). Participants provided informed written consent prior to collection of their samples and associated clinical data. Patients were diagnosed with HGSC and underwent the standard treatment consisting of surgery followed by carboplatin-based chemotherapy with or without paclitaxel. To work with these human samples, I have taken the Tri-Council Policy Statement 2: Course on Research Ethics, which is mandatory for Tri-Council funded researchers participating in work with human samples.

Patient ascites samples were collected during primary surgery. Ascites cells were then isolated by centrifugation and cryopreserved in 50% fetal bovine serum (FBS), 40% complete media (Roswell Park Memorial Institute media [RPMI] 1640 [Thermo Fisher Scientific, Nepean, ON, CA] containing 10% FBS, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 2 mM L-glutamine, 50 µM β-mercaptoethanol, and 1 mM sodium pyruvate) and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). Ascites cell preparations were stored in liquid nitrogen vapour.

2.2.2 Peptide library generation

A peptide library of LDHC was designed based on the sequence described in the National Center for Biotechnology Information (NCBI) protein database (NP_059144.1). Known exon splice variants were accounted for as well, provided that mRNA expression had been verified by previous studies (Koslowski et al., 2002), and that variant exon splicing leads to the generation of novel amino acid sequences unaccounted for by the primary library (EAW68393.1, AEW43814.1). The library was designed as a series of overlapping 15mer peptides (Table 1). Each peptide overlapped its adjacent peptides by 11 amino acids. Three additional overlapping 15mer peptides account for each of two unique junctions expressed in the variant sequences. Peptides were synthesized commercially (GenScript, Piscataway, NJ). Lyophilized peptides were reconstituted at 10 µg/mL in DMSO (Sigma-Aldrich).

A minimal peptide library corresponding to peptide 62 of the LDHC library was also designed (Table 2). This library consists of all 8-11mer peptides contained within LDHC peptide 62 as well as the complementary peptides of LDHA, sequence as described in the NCBI protein database (CAG46515.1). Peptides were commercially synthesized (GenScript) and reconstituted at 10 µg/mL in DMSO.

Peptide	Position	Sequence
1	1	MSTVKEQLIEKLIED
2	5	KEQLIEKLIEDDENS
3	9	IEKLIEDDENSQCKI
4	13	IEDDENSQCKITIVG
5	17	ENSQCKITIVGTGAV
6	21	CKITIVGTGAVGMAC
7	25	IVGTGAVGMACAISI
8	29	GAVGMACAISILLKD
9	33	MACAISILLKDLADE
10	37	ISILLKDLADELALV
11	41	LKDLADELALVDVAL
12	45	ADELALVDVALDKLK
13	49	ALVDVALDKLKGEMM
14	53	VALDKLKGEMMDLQH
15	57	KLKGEMMDLQHGSLF
16	61	EMMDLQHGSLFFSTS
17	65	LQHGSLFFSTSKITS
18	69	SLFFSTSKITSGKDY
19	73	STSKITSGKDYSVSA
20	77	ITSGKDYSVSANSRI
21	81	KDYSVSANSRIVIVT
22	85	VSANSRIVIVTAGAR
23	89	SRIVIVTAGARQQEG
24	93	IVTAGARQQEGETRL
25	97	GARQQEGETRLALVQ
26	101	QEGETRLALVQRNVA
27	105	TRLALVQRNVAIMKS
28	109	LVQRNVAIMKSIIPA
29	113	NVAIMKSIIPAIVHY
30	117	MKSIIPAIVHYSPDC
31	121	IPAIVHYSPDCKILV
32	125	VHYSPDCKILVVSNP
33	129	PDCKILVVSNPVDIL
34	133	ILVVSNPVDILTYIV
35	137	SNPVDILTYIVWKIS
36	141	DILTYIVWKISGLPV
37	145	YIVWKISGLPVTRVI
38	149	KISGLPVTRVIGSGC
39	153	LPVTRVIGSGCNLDS
40	157	RVIGSGCNLDSARFR
41	161	SGCNLDSARFRYLIG
42	165	LDSARFRYLIGEKLG
43	169	RFRYLIGEKLGVHPT
44	173	LIGEKLGVHPTSCHG

Peptide	Position	Sequence
45	177	KLGVHPTSCHGWIIG
46	181	HPTSCHGWIIGEHGD
47	185	CHGWIIGEHGDSSVP
48	189	IIGEHGDSSVPLWSG
49	193	HGDSSVPLWSGVNVA
50	197	SVPLWSGVNVAGVAL
51	201	WSGVNVAGVALKTLD
52	205	NVAGVALKTLDPKLG
53	209	VALKTLDPKLGTDSD
54	213	TLDPKLGTDSDKEHW
55	217	KLGTDSDKEHWKNIH
56	221	DSDKEHWKNIHKQVI
57	225	EHWKNIHKQVIQSAY
58	229	NIHKQVIQSAYEIIK
59	233	QVIQSAYEIIKLKGY
60	237	SAYEIIKLKGYTSWA
61	241	IIKLKGYTSWAIGLS
62	245	KGYTSWAIGLSVMDL
63	249	SWAIGLSVMDLVGSI
64	253	GLSVMDLVGSILKNL
65	257	MDLVGSILKNLRRVH
66	261	GSILKNLRRVHPVST
67	265	KNLRRVHPVSTMVKG
68	269	RVHPVSTMVKGLYGI
69	273	VSTMVKGLYGIKEEL
70	277	VKGLYGIKEELFLSI
71	281	YGIKEELFLSIPCVL
72	285	EELFLSIPCVLGRNG
73	289	LSIPCVLGRNGVSDV
74	293	CVLGRNGVSDVVKIN
75	297	RNGVSDVVKINLNSE
76	301	SDVVKINLNSEEEAL
77	305	KINLNSEEEALFKKS
78	309	NSEEEALFKKSAETL
79	313	EALFKKSAETLWNIQ
80	317	KKSAETLWNIQKDLI
81	318	KSAETLWNIQKDLIF
82	Isoform CRA_e	MACAISILLKGLYGI
83		ISILLKGLYGIKEEL
84		LKGLYGIKEELFLSI
85	Variant 4	DCKILVVSNPDSARF
86		LVVSNPDSARFRYLI
87		NPDSARFRYLIGEKL

Table 1. LDHC peptide library. The LDHC peptide library consists of 81 overlapping 15mer peptides which span the LDHC protein. It is accompanied by 2 sets of 3 peptides which account for the alternatively spliced LDHC variant sequences isoform CRA_e (EAW68393.1) and variant 4 (AEW43814.1). Sequences in red designate 15mers which include no amino acids which differ from either LDHA or LDHB.

Position	Length	LDHC sequence	LDHA Complement	Short Hand Name
0	8	KGYTSWAI	KGYTSWAI	
0	9	KGYTSWAIG	KGYTSWAIG	
0	10	KGYTSWAIGL	KGYTSWAIGL	
0	11	KGYTSWAIGLS	KGYTSWAIGLS	
1	8	GYTSWAIG	GYTSWAIG	
1	9	GYTSWAIGL	GYTSWAIGL	
1	10	GYTSWAIGLS	GYTSWAIGLS	
1	11	GYTSWAIGLSV	GYTSWAIGLSV	GSWAG
2	8	YTSWAIGL	YTSWAIGL	
2	9	YTSWAIGLS	YTSWAIGLS	
2	10	YTSWAIGLSV	YTSWAIGLSV	SWAG
2	11	YTSWAIGLSVM	YTSWAIGLSVA	SWAG-M / SWAG-A
3	8	TSWAIGLS	TSWAIGLS	
3	9	TSWAIGLSV	TSWAIGLSV	
3	10	TSWAIGLSVM	TSWAIGLSVA	
3	11	TSWAIGLSVMD	TSWAIGLSVAD	
4	8	SWAIGLSV	SWAIGLSV	
4	9	SWAIGLSVM	SWAIGLSVA	
4	10	SWAIGLSVMD	SWAIGLSVAD	
4	11	SWAIGLSVMDL	SWAIGLSVADL	
5	8	WAIGLSVM	WAIGLSVA	
5	9	WAIGLSVMD	WAIGLSVAD	
5	10	WAIGLSVMDL	WAIGLSVADL	
6	8	AIGLSVMD	AIGLSVAD	
6	9	AIGLSVMDL	AIGLSVADL	
7	8	IGLSVMDL	IGLSVADL	

Table 2. LDHC peptide 62 minimal peptide library and its LDHA complement. The LDHC p62 library consists of 26 8-11mer peptides from LDHC accompanied by their complement sequences within LDHA. Sequences shared between the two proteins are in red text.

2.2.3 T cell expansion

T cells were selectively stimulated towards expansion by Rapid Expansion Protocol (REP) in complete media (Dudley, Wunderlich, Shelton, Even, & Rosenberg, 2003). The REP includes stimulation by 300 IU/mL human IL-2 (Novartis Pharmaceuticals Cancer Inc., Dorval, QC, CA), 30

ng/mL anti-human CD3 OKT3 antibody (16-0037-85; eBioscience Inc., San Diego, CA), and 200:1 lethally irradiated (50 Gy) allogeneic PBMC. Cultures are provided with half-media changes accompanied by fresh cytokines every 3-4 days and split into additional wells/flasks/plates to maintain T cell concentrations at approximately 10^6 cells/mL. All cultures were incubated at 37°C, 21% O_2 and 5% CO_2 in a Water-Jacketed Forma incubator (Thermo Fisher).

For the expansion of CD8 T cells from ascites, CD8 T cells were first purified from ascites samples. Bulk ascites samples were thawed and suspended in phosphate buffered saline (PBS; Thermo Fisher) for CD8 purification. Samples were labeled with phycoerythrin (PE)-conjugated anti-human CD8 (555367; BD Biosciences, San Jose, CA). Labeled cells were tagged with EasySep PE-selection cocktail and Easy-Sep magnetic particles (EasySep PE selection kit, STEMCELL Technologies, Vancouver, BC). Magnetically labeled CD8+ cells were isolated using the EasySep Magnet (STEMCELL Tech). Unmagnetized supernatant containing CD8 negative cellular material was subsequently discarded.

After purification, cells were enumerated by trypan blue exclusion assay (Sigma-Aldrich) or by flow cytometry using the ViaCount (4000-0040; EMD Millipore, Billerica, MA) Assay on a Guava easyCyte 8HT Benchtop Flow Cytometer (EMD Millipore). Then, PBMC from 1-3 healthy donors were irradiated and mixed with patient cells at 200:1. The mixture of cells was seeded into 96-well plates at 2000 patient cells per well for miniline REP expansion. Cultures were divided into additional 96-well plates when the center opaque regions of the culture pellets were greater than 4mm in diameter. Miniline REP cultures continued to be split into new plates over approximately 2 weeks until they occupied 8 plates. During antigen assays, one plate of

cultures was left behind to provide cells from each culture for subsequent expansion and/or sorting.

For expansions of bulk and sorted T cell cultures, cells were expanded by REP in 10 mL complete media in upright Vented Falcon T25 flasks (Corning, Corning, NY). Cultures were maintained at approximately 10^6 cells/mL and split as required over approximately 2 weeks until cell proliferation reached a plateau. At this point they were either cryopreserved, assayed, or REP expanded again.

2.2.4 IFN-γ ELISPOT analysis

Prior to ELISpot assay, T cell cultures were incubated in resting media consisting of complete media with 10 ng/mL IL-7 (PeproTech, QC) and 1 IU/mL IL-2 for 2 days. Nitrocellulose ELISpot plates (MultiScreen$_{HTS}$ IP Filter Plate; EMD Millipore) were coated with 10 μg/mL monoclonal 1-D1K anti-IFN-γ capture antibody (3420-3-250; Mabtech, Cincinnati, OH) and incubated overnight at 4°C. Plates were then washed with PBS and blocked for 2 hours at 37°C with complete media. For miniline assays, 7 miniline plates were combined via repeated centrifugation and then enumerated by ViaCount on a Guava flow cytometer. At random, five cultures were selected to provide an estimated range of concentrations across all 96. T cells were plated at a concentration of 2×10^5 - 8×10^5 cells per well. For ELISpots of single cultures, T cells were confidently plated in at 3×10^5 cells per well. Cultures in ELISpot wells were either left unstimulated, stimulated with test peptides at 10 μg/mL, stimulated with human cytomegalovirus, Epstein-Barr virus, and influenza virus peptides (CEF; AnaSpec EGT Group, San

Jose, CA) at 10 µg/mL, stimulated with 1 µL Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher) or stimulated with the T cell mitogen phytohaemagglutinin at 5 µg/mL (PHA; Sigma-Aldrich). T cell cultures were stimulated for 20 hours at 37°C. After 20 hours, ELISpot plates were washed with PBS and incubated for 2 hours at 37°C with 1 µg/mL monoclonal 7-B6-1 biotinylated anti-human IFNγ antibody (3420-6-250; Mabtech). Plates were then incubated for 2 hours with an avidin:biotinylated-peroxidase complex (Vectastain ABC kit; Vector Labs, Burlingame, CA). After washing, the peroxidase substrate 3-amino-9-ethylcarbazole (AEC; Vector Labs) was added for visual assessment of IFNγ presence. Spots were enumerated using an automated plate reader (Autoimmun Diagnostika GmbH, Straßberg, Germany). Positive results were defined empirically as having three-fold the number of spots as negative control wells as well as a minimum of 30 spots/3x10^5 cells.

2.2.5 Cell Sorting

T cells were incubated in resting media for 2-3 days. After resting, T cells were incubated for 24 hours at 37°C with either no stimulation or with 10 µg/mL of cognate peptide. Cells were washed with PBS containing 3% FBS and stained for 30 minutes with fixable V450 viability dye (0.5 µL/1 mL, 65-0863-18; eBioscience). They were then washed and stained for 20 minutes with PE-conjugated anti-human CD8 (5 µL/100 µL, 555367; BD) and allophycocyanin (APC)-conjugated anti-human 4-1BB (20 µL/100 µL, A14941; Invitrogen, Carlsbad, CA) monoclonal antibodies, or eFluor780-conjugated anti-human CD8 (5 µL/100 µL, 47-0088-41; eBioscience) and PE-conjugated anti-human 4-1BB (5 µL/100 µL, 309810; BioLegend; San Diego, CA)

monoclonal antibodies. Using a BD Influx cell sorter, CD8+ 4-1BB+ cells were isolated by FACS, and sorted cells were expanded via REP. Flow cytometry data was analyzed using FlowJo (vX.0.7r2).

2.2.6 TCR Vß Spectratyping

Patient 2 D4 T cells were cultured at 10^6 cells/mL in resting media overnight or in complete media with SWAG-M peptide at 10 µg/mL. As a control, a bulk REP T cell culture from Patient 2 was also rested overnight. The next day, cells were washed with PBS by centrifugation and suspended in PBS with 3% FBS. Cells from each condition were divided into 8 tubes which were each stained with anti-human 4-1BB APC conjugated antibody, anti-human CD8 BV421 conjugated antibody (1/200; 301036, BioLegend), as well as one of eight anti-Vß TCR cocktails A though H (1/10, IM3497; Beckman Coulter, Brea, CA). Each premixed cocktail contained three anti-Vß TCR antibodies, conjugated to fluorescein isothiocyanate (FITC), PE, or FITC+PE. By expression frequency, 70% of possible Vß are accounted for by these 24 Vß antibodies. Cells were incubated with antibodies in ~100 µL volume for 30 minutes at 4°C. Cells were washed by centrifugation and suspended in 200 µL PBS with 3% FBS for analysis. Flow cytometry was carried out by a FACSCalibur flow cytometer (BD). Results were analyzed using FlowJo.

2.3 Results

2.3.1 Patient selection

Investigations into LDHC immunogenicity had been previously undertaken within our lab. These projects had evaluated LDHC expression of patient tumours by PCR (Data not shown, unpublished). Here, patients were prioritized based upon having positive *LDHC* expression as well as tissue sample availability. This prioritization led to the selection of Patients 1, 3, 4, and 5, which have *LDHC*+ tumours. Additionally, T cells from patient ascites were previously assayed, directly *ex vivo,* for reactivity against two LDHC peptides which had been predicted *in silico* to bind HLA-A*02:01. This preliminary data led to the selection of Patient 2, whose ascites T cells exhibited LDHC reactivity *ex vivo* (Data not shown, unpublished). Due to a lack of Patient 2 tumour tissue sample, this patient's LDHC status remained unknown.

2.3.2 Screening of patient T cells for LDHC reactivity

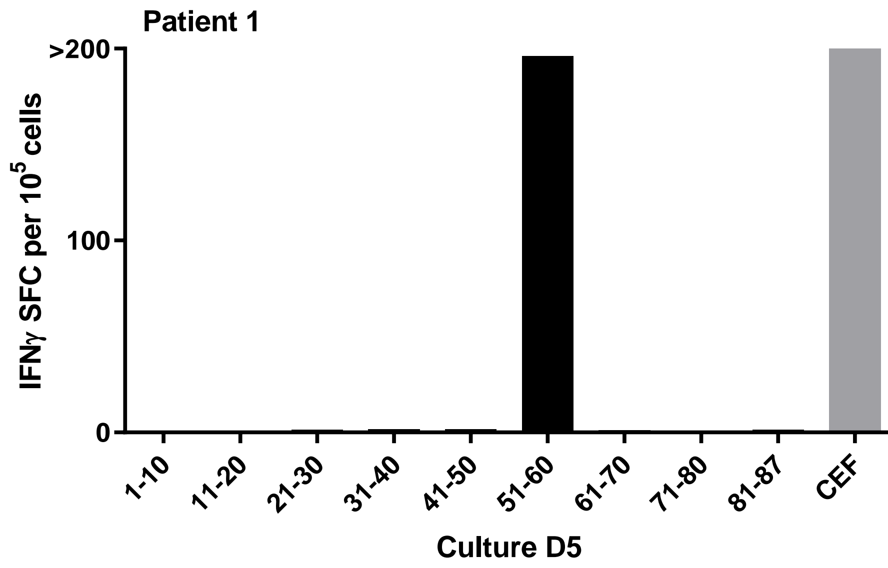
In order to evaluate the immunogenicity of LDHC without bias to any particular MHCI allele, a peptide library of 81 overlapping 15mers was synthesized (Table 1). Adjacent peptides within this library overlap by 11 amino acids, allowing the library to collectively represent all possible 8-11mers contained within LDHC. Additionally, three 15mer peptides were synthesized in order to account for each of two LDHC exon splice variants, bringing the total number of peptides within the library to 87.

The ascites of five HGSC patients was assessed for the presence of LDHC peptide reactive T cells. In the interest of ensuring the detection of low frequency T cells, and preserving patient samples, a 'miniline' approach was utilized in each case. To screen Patient 1, a new vial of ascites was thawed for analysis. CD8 T cells were magnetically purified by positive selection and divided amongst 96 individual REP cultures in a shared 96-well round bottom plate. Each culture originated from 2000 cells, thus overall accounting for 2000 x 96 = 192 000 CD8 T cells. After expansion, these cultures were rested and screened in parallel for reactivity against peptide pools from the LDHC peptide library in 11 individual IFNγ ELISpots. Each culture was plated in its corresponding well throughout each ELISpot: e.g. culture D6 in the wells D6 of each of 11 plates. These ELISpot conditions included: LDHC peptides 1-10, LDHC peptides 11-20, LDHC peptides 21-30, LDHC peptides 31-40, LDHC peptides 41-50, LDHC peptides 51-60, LDHC peptides 61-70, LDHC peptides 71-80, LDHC peptides 81-87, the CEF peptide pool as a positive control, and no peptide as a negative control. Patient 2 miniline expansion and analysis were conducted in an identical fashion. Patient 3 minilines were constructed as both Patients 1 and 2. However, due to unforeseen constraints set by post-expansion cell quantity, Patient 3 T cell cultures were screened against 3 pools of thirty* LDHC library peptides (* - LDHC peptides 1-30, 31-60, 61-87). As studies have shown that the presence of irrelevant peptides within large peptide pools do not cause interference with T cell recognition of cognate peptide (Chevalier et al., 2015), pools of thirty peptides were also used in the screening of Patients 4 and 5. Because these large peptide pools reduced the number of ELISpots required to screen the T cell minilines of these patients, it became logistically feasible to screen an increased number of T cells from each patient: thus, magnetically purified CD8 T cells from the ascites of Patients 4

and 5 were REP expanded across 3 96-well plate minilines. This increase allowed the screening of 288 individual cultures, accounting for 576 000 CD8 T cells.

Of the five patients, neither Patient 3 nor Patient 4 showed any upregulation of IFNγ secretion above background levels under test conditions. However, each of Patients 1, 2, and 5 showed increased IFNγ signals in at least one miniline culture when stimulated with peptide pools. The ELISpots of Patient 1 had one positive result in culture D5 when incubated in the presence of peptides 51-60 (Figure 3a). Patient 2 cultures B7, C10, and F4 were reactive to the peptide pool 61-70 (Figure 3b). Patient 5 had the most varied reaction to screening, displaying IFNγ release upon exposure two individual peptide pools. Patient 5 cultures AG5 and AG9 responded to pool 1-30, CC12 to pool 31-60 (Figure 3c).

A



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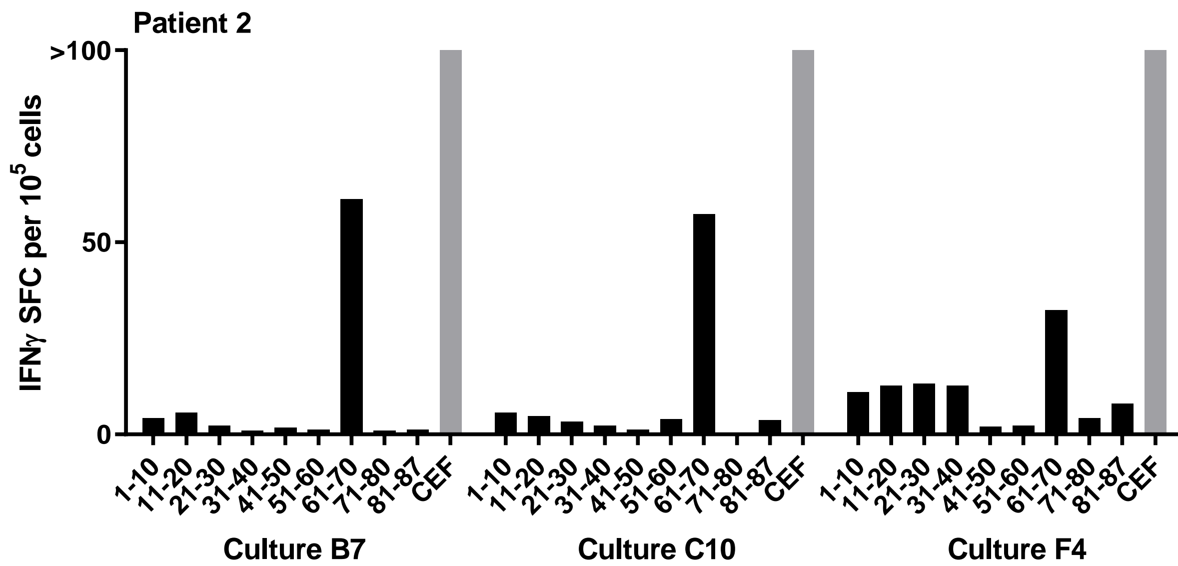

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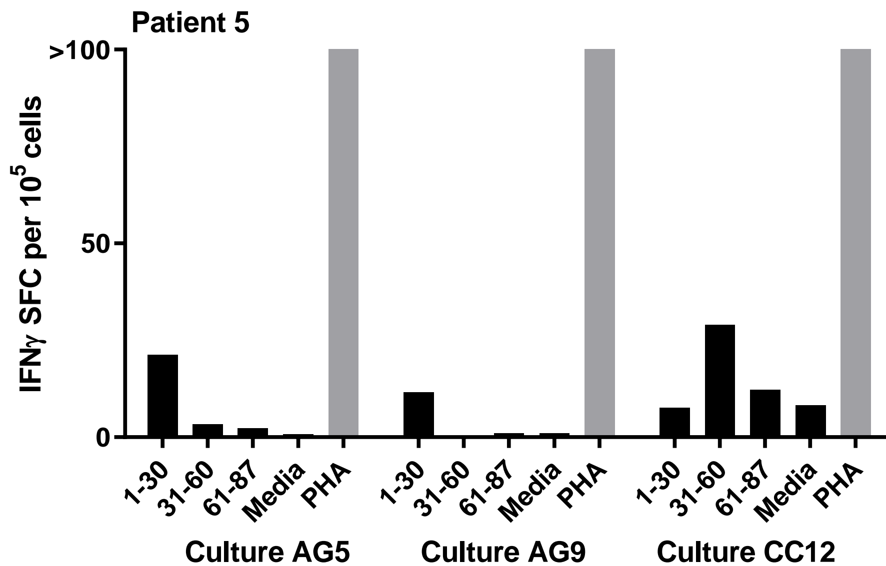


Figure 3. Patient T cell responses to pooled LDHC library peptides as evaluated by IFNγ ELISpot. T cells from individual miniline cultures were plated in singlicate at ~$3x10^5$ cells/well and incubated with exogenous peptides for 20 hours at a total concentration of 10 µg/mL. Positive controls included a CEF peptide pool or PHA. Bars represent the number of IFNγ spot forming cells (SFC) in a given well. (A) Patient 1 IFNγ secretion after overnight incubation with LDHC peptide pools of 10 or a CEF peptide pool (positive control) in the single T cell culture D5. (B) Patient 2 IFNγ secretion after overnight incubation with LDHC peptide pools of 10 in the T cell cultures B7, C10, and F4. (C) Patient 5 IFNγ secretion after overnight incubation with peptide pools of 30 in the T cell cultures AG5, AG9, and CC12, respectively.

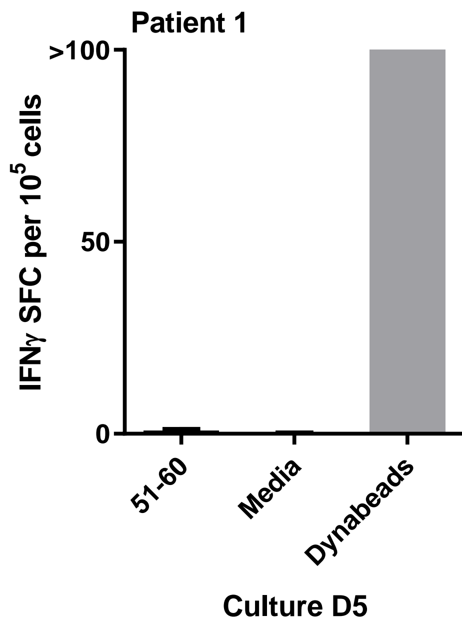
In the investigation of Patients 1 and 2, remaining cells from cultures with positive ELISpot results were harvested from the miniline cultures and expanded by REP for downstream analysis. Due to the eventual discovery of a “REP limit” of T cells, the protocol was adapted to avoid unnecessary expansion steps and thus conserve cells. Additional cells from

positive cultures of Patient 5 were incubated overnight with their respective peptide pools, for confirmation and cell sorting based on 4-1BB upregulation (see section 2.3.4).

2.3.3 Confirmation of LDHC reactivity post-expansion

As dictated by cell numbers, initial miniline ELISpots could be done only in singlicate. Thus, it was necessary to confirm that expanded cultures could indeed recognize LDHC peptide. After bulk REP expansion, cultures were tested against cognate peptide pools by ELISpot in triplicate. The D5 T cell line from Patient 1 proved to be non-reactive to the peptide pool 51-60 (Figure 4a). The previous singlicate response may have been a false positive, or it may be that antigen specific T cells were unable to expand within the REP culture. Expanded cultures from Patient 2 – B7, C10, and F4 – were each confirmed to react to pool 61-70 (not shown). This peptide pool was subsequently deconvoluted in order to identify the specific peptide recognized. Each culture's reactivity to the individual peptides in the pool 61-70 was evaluated in triplicate by IFNγ ELISpot. Each of B7, C10, and F4 showed greatest reactivity to the peptide 62 (p62, KGYTSWAIGLSVMDL) of LDHC (Figure 4b). However the substantial background in these ELISpots was disruptive enough to skew the standard positive definition of three times background. As such, two-tailed T-tests were conducted to determine significant IFNγ secretion by stimulated T cells. In cultures B7 and C10, it was found that p62-incubated wells had significantly more IFNγ than unstimulated wells. There were no statistical increases found in other conditions.

A



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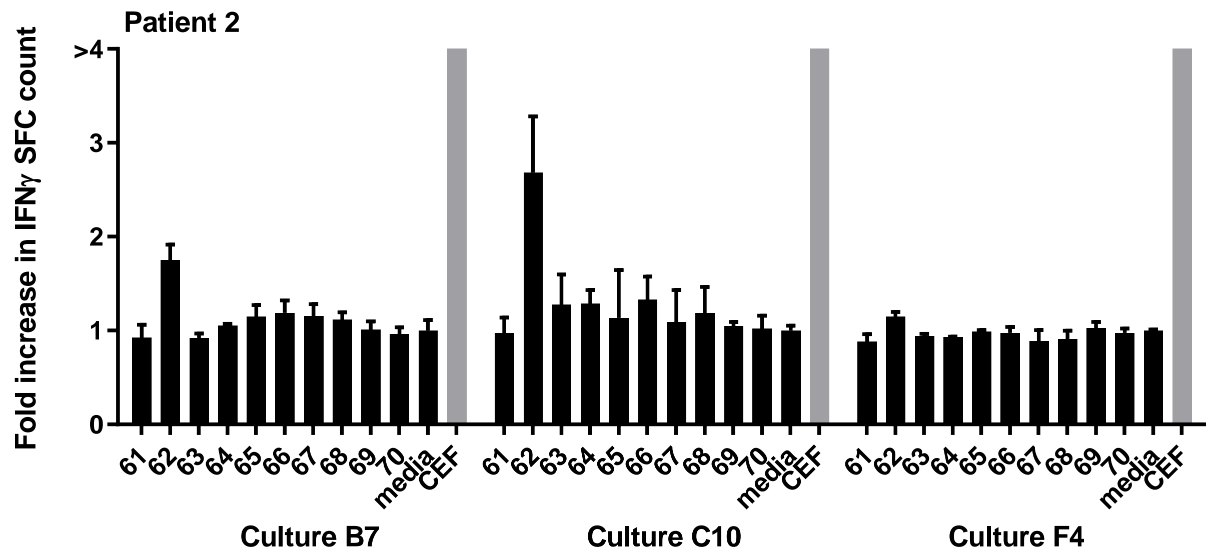
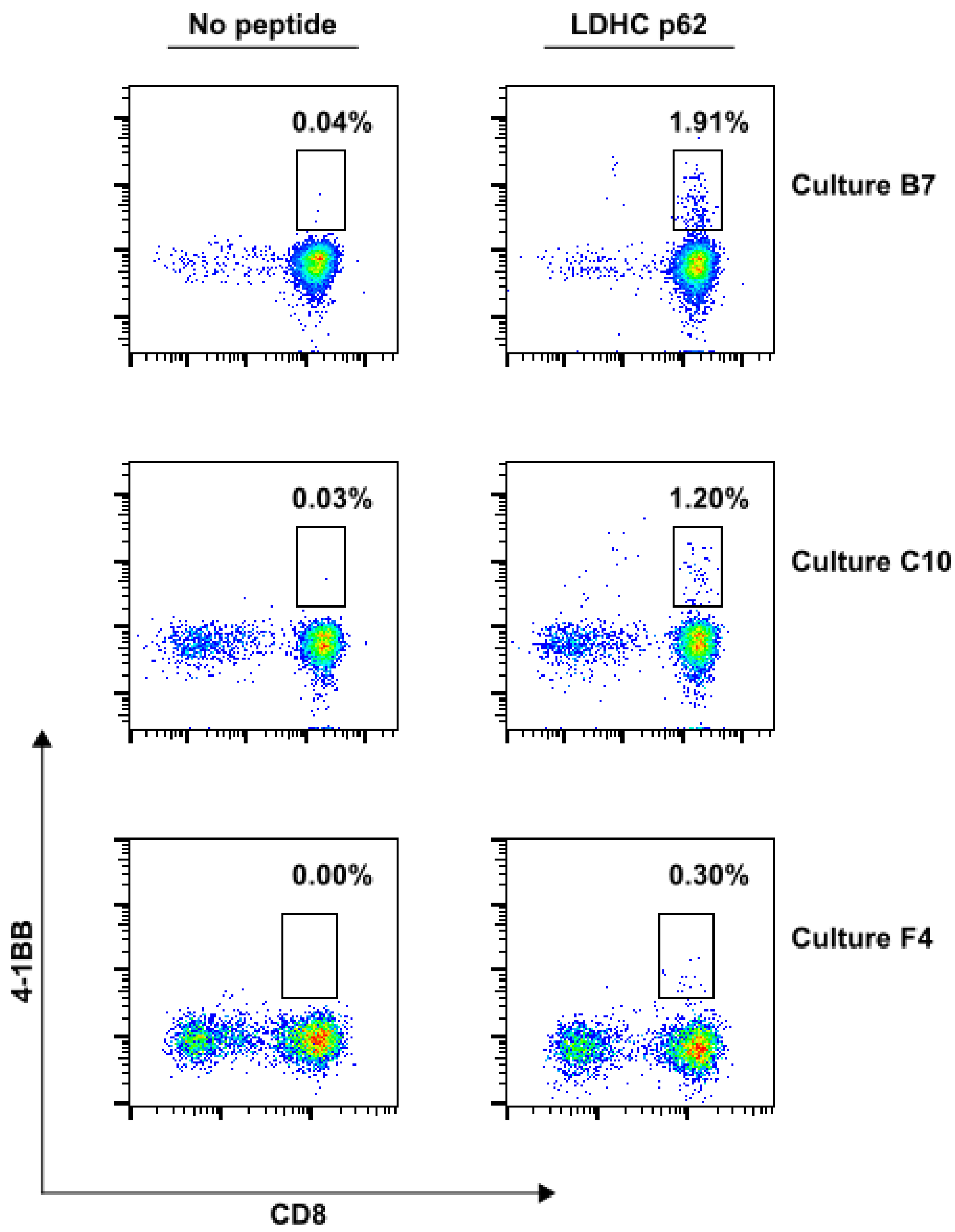


Figure 4. Post-expansion IFNγ ELISpots of first-pass reactive T cells. (A) Expanded T cells from Patient 1 culture D5 were plated in triplicate at $3x10^5$ cells/well and incubated with exogenous LDHC peptides 51-60 for 20 hours at a total concentration of 10 µg/mL. Dynabeads were used as a positive control. (B) Expanded T cells from Patient 2 cultures B7, C10, and F4 were plated in triplicate at $3x10^5$ cells/well and incubated with individual exogenous LDHC peptides from pool 61-70 for 20 hours at 10 µg/mL. The CEF peptide pool was used as a positive control.

2.3.4 Isolation of LDHC reactive T cells by flow cytometry

With the aim of both confirming peptide reactivity by flow cytometry and purifying peptide reactive T cells for further analysis, upregulation of the activation marker 4-1BB was assessed after 20 hours of peptide stimulation. Patient 2 miniline T cell cultures B7, C10, and F4 were stimulated by the cognate LDHC p62. Each culture was stained with APC-conjugated anti-4-1BB and PE-conjugated anti-CD8 antibodies. In each of these cultures, there was a distinct upregulation of 4-1BB by a subset of CD8 T cells. Double positive 4-1BB+ CD8+ cells were sorted by FACS (Figure 5a). Post-sorting, 4-1BB+ CD8 T cells were divided into single cell cultures for REP expansion in an effort to generate a monoclonal culture of LDHC specific T cells. Monoclonal cultures were maintained over 8 weeks in a series of REP expansions. Patient 2's LDHC-specific cultures were unable to sustain a relevant level of proliferation, never grew to the workable stock numbers, and reached a biological endpoint. It was at this point that the reactive T cell was re-isolated from original biological material. As alluded to in section 2.3.2, this led to the realization that the limit of T cell expansion could easily be reached. In an effort to avoid this in future experiments, Patient 5 cultures were not REP expanded and assessed by ELISpot prior to FACS, and future FACS purified cultures were expanded as enriched cultures, rather than attempting to achieve monoclonality through single cell growth. Here, Patient 5's AG5, AG9, and CC12 cultures were stimulated by cognate peptide pools overnight and sorted the next day (Figure 5b). All Patient 5 cultures showed a lack of 4-1BB upregulation on both CD8+ and CD8- cell populations.


A
Patient 2
No peptide
LDHC p62
0.04%
1.91%
Culture B7
0.03%
1.20%
Culture C10
0.00%
0.30%
Culture F4
4-1BB
CD8

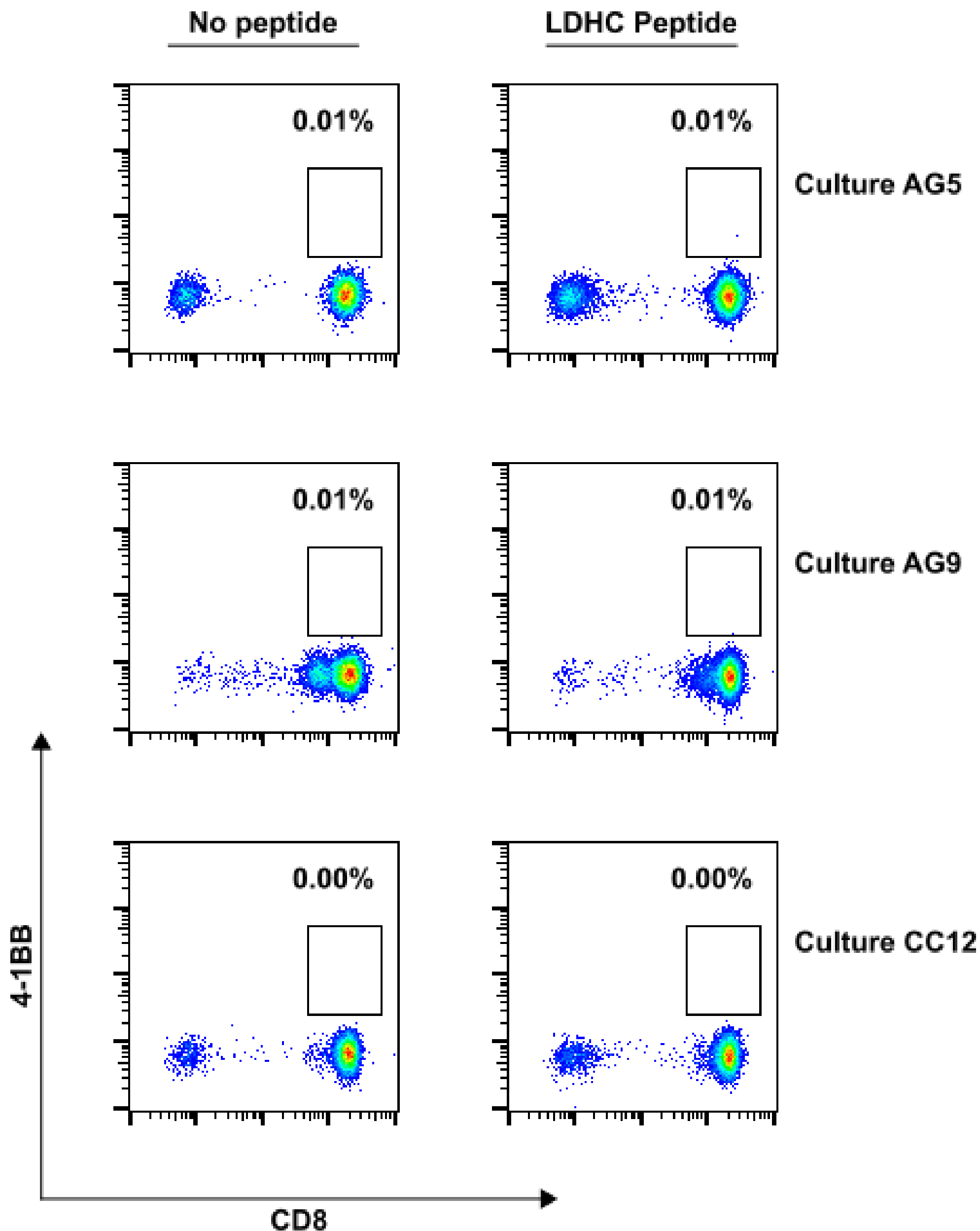


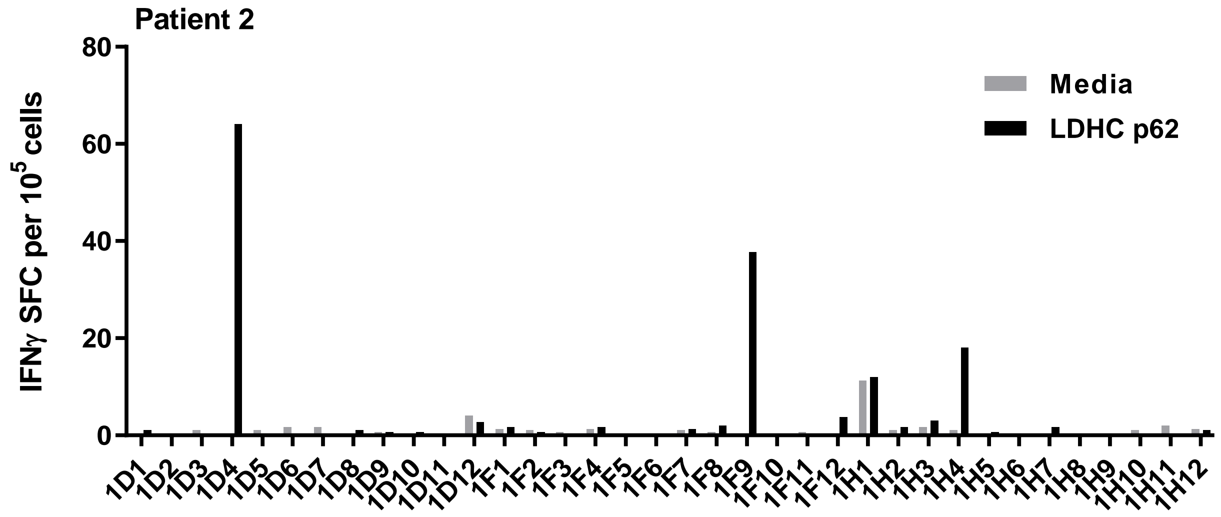
Figure 5. Fluorescence activated cell sorting of CD8+ 4-1BB+ cells after overnight incubation with cognate peptide. Cultures were incubated with or without peptide for 20 hours at 10 µg/mL and stained with anti-CD8 and anti-4-1BB antibodies. Panels in the left columns illustrate flow cytometry analysis of cultures incubated alone, while those in the right column show cell cultures incubated with peptide. (A) Patient 2 cultures B7, C10, and F4, observed in figure 4b, were incubated with LDHC p62. After stimulation, CD8+ 4-1BB+ cells were isolated by FACS.

(B) Patient 5 cultures AG5, AG9, and CC12, first screened in figure 3c, were incubated overnight with peptide pools 1-30, 1-30, and 31-60, respectively.

2.3.5 Re-isolation of LDHC peptide 62 reactive T cells from Patient 2

To re-isolate the LDHC p62 reactive T cell clone from Patient 2, a new set of CD8 minilines was generated from ascites, as before. These minilines were screened by IFNγ ELISpot in singlicate against p62 alone. Wells 1D4, 1F9, and 1H4 showed substantial IFNγ secretion when prompted with p62 (Figure 6a). These cultures were expanded via REP, sorted by 4-1BB upregulation after activation by p62, and again expanded via two REP cycles. In the interest of isolating as many cells as possible, all available cells were stimulated and sorted (Figure 6b). Reactivity would be later confirmed when these cultures had grown to working numbers of greater than 100 million (Figure 6c). During this expansion process, both 1F9 and 1H4 cultures reached a biological endpoint and were unable to further proliferate or sustain life. The subset of p62 reactive cells isolated from the Patient 2 miniline culture 1D4 is here on referred to as culture D4.

A



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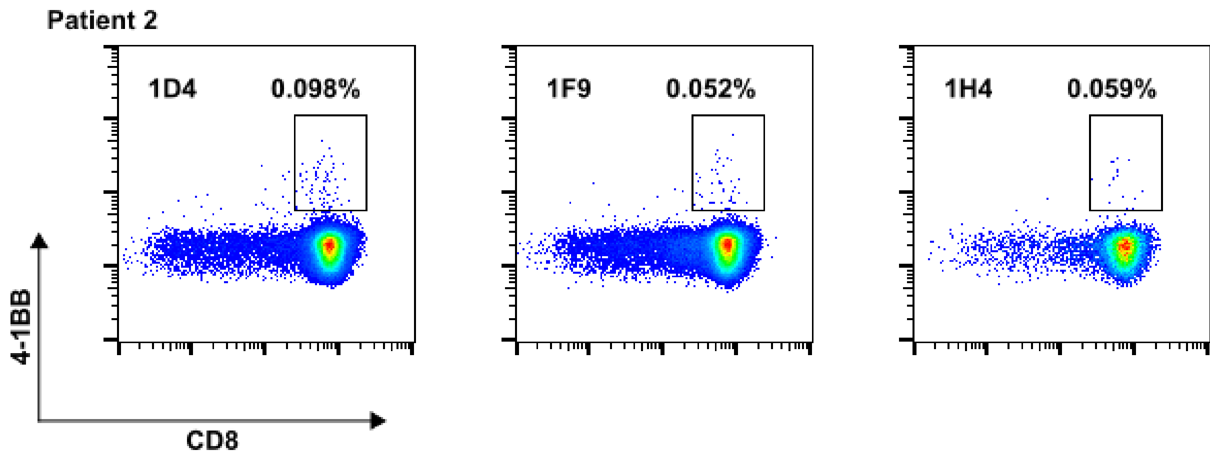

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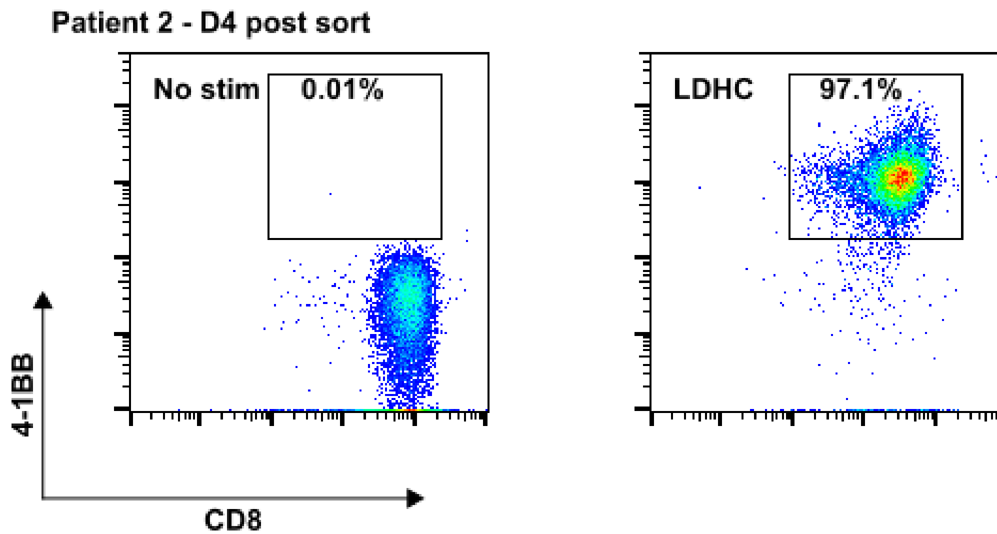


Figure 6. Secondary isolation of Patient 2 LDHC peptide 62 reactive T cells. (A) Individual Patient 2 miniline cultures were assessed for LDHC p62 reactivity by IFNγ ELISpot. Cultures were plated in singlicate at ~$3x10^5$ cells/well and incubated with or without exogenous LDHC p62 for 20 hours at 10 µg/mL. The CEF peptide pool was used as a positive control (not shown). (B) Patient 2 miniline cultures 1D4, 1F9, and 1H4 were incubated with LDHC p62 for 20 hours and stained with anti-CD8 and anti-4-1BB antibodies. After stimulation, CD8+ 4-1BB+ cells were isolated by FACS. (C) After isolation and expansion, expanded D4 cells were incubated with LDHC p62 for 20 hours and stained with anti-CD8 and anti-4-1BB antibodies for analysis by flow cytometry.

2.3.6 Deconvolution of LDHC peptide 62

As a given 15mer represents 26 individual 8-11mer peptides, a small peptide library was commercially synthesized encompassing all possible short peptides from p62, which could be presented in the context of MHCI *in vivo* (Table 2). A complementary library from LDHA was also synthesized, in order to compare reactivity between the tumour-specific protein and the ubiquitously expressed protein. The LDHC p62 reactive D4 culture was assayed by IFNγ ELISpot in duplicate against each individual peptide within the p62 library, along with their peptide

complement from LDHA (Figure 7). The isolated D4 subculture showed the greatest reactivity to the 11mer peptides YTSWAIGLSVA and YTSWAIGLSVM, deemed SWAG-A and SWAG-M.

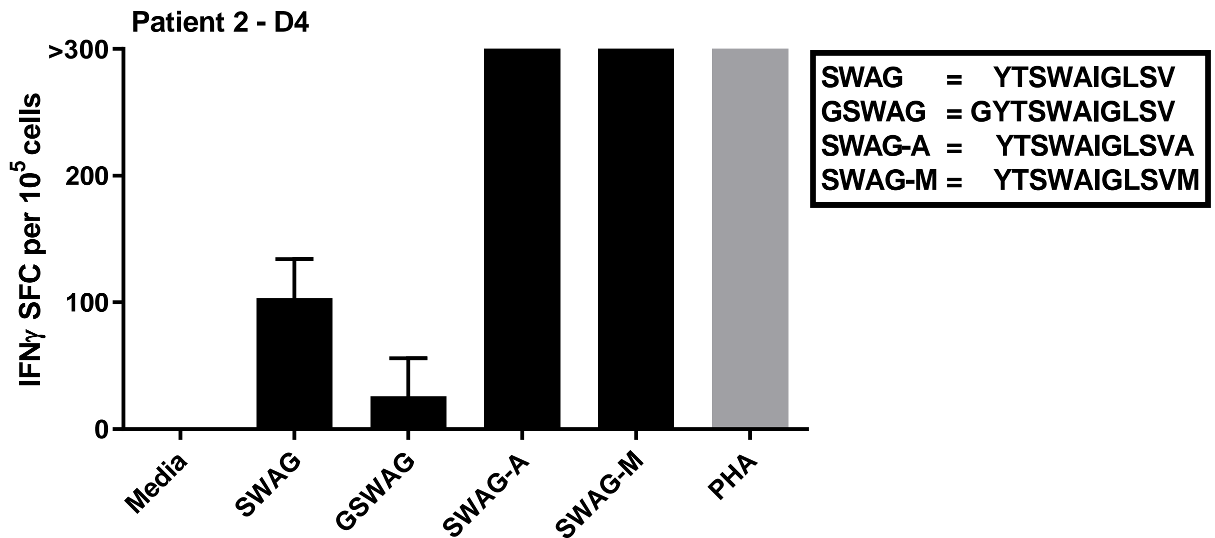


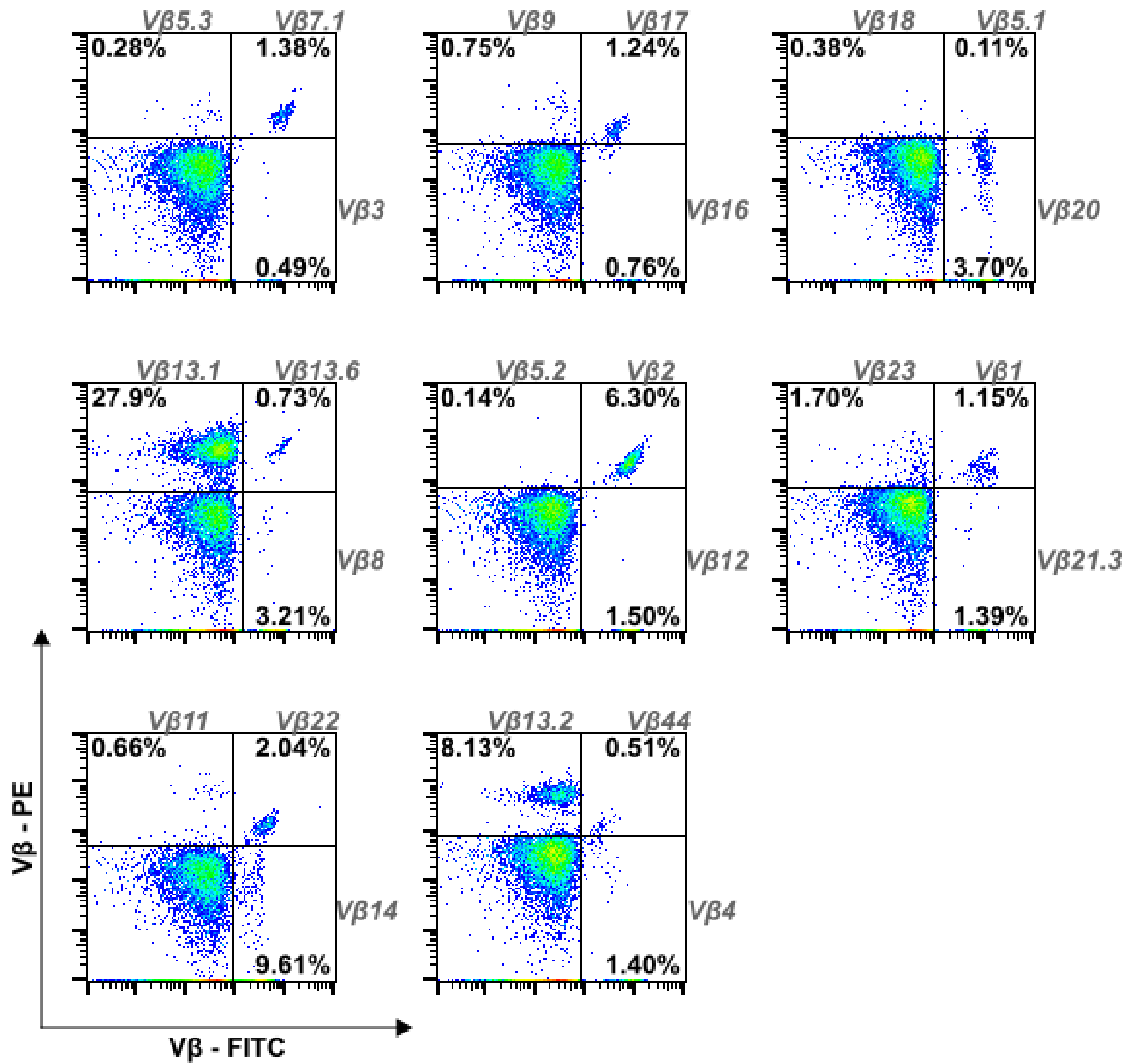
Figure 7. Recognition of LDHC p62 minimal peptides by D4 T cells as evaluated by IFNγ ELISpot. Patient 2 D4 T cells were plated in duplicate at $3x10^5$ cells/well and incubated with individual exogenous 8-11mer peptides from the LDHC/A p62 library for 20 hours at 10 µg/mL. The mitogen PHA was used as a positive control. Here, all wells exhibiting fewer than 5 SFC per 105 cells above media background are excluded from the graph.

2.3.7 Evaluation of the D4 T cell culture purity by TCR Vß

To approximate the clonality of the D4 culture, TCR Vß diversity of the D4 culture was evaluated by flow cytometry. As a given TCR may incorporate only one of the 47 Vß genes, cultures may be divided by Vß surface expression as an estimate of their clonal composition (Hodges, Krishna, Pickard, & Smith, 2003). With this objective, eight individual samples were stained with eight mixtures of three anti-Vß antibodies, each one being conjugated to FITC, PE,

or FITC+PE. This collection of antibodies accounts for ~70% of Vß variability, by frequency, within TCRs. As a control, a bulk REP T cell culture from Patient 2 was used to demonstrate polyclonality. Within the bulk REP culture, 75.46% of CD8 T cells expressed TCRs containing Vß that were accounted for by the anti-Vß antibody collection (Figure 8a). Within the D4 sorted culture, 5.70% of CD8 T cells expressed TCRs containing those same Vß (Figure 8b). By omission, the D4 culture is 94% pure, assuming the culture is not contaminated with additional T cells of other rare Vß chains. Furthermore, as per Figure 6c, 97.1% of cells in the D4 culture upregulate 4-1BB upon activation by incubation with SWAG-M. As such, the SWAG-M response by D4 T cells must be mediated through a TCR which is unaccounted for by the common Vß repertoire.

A



B

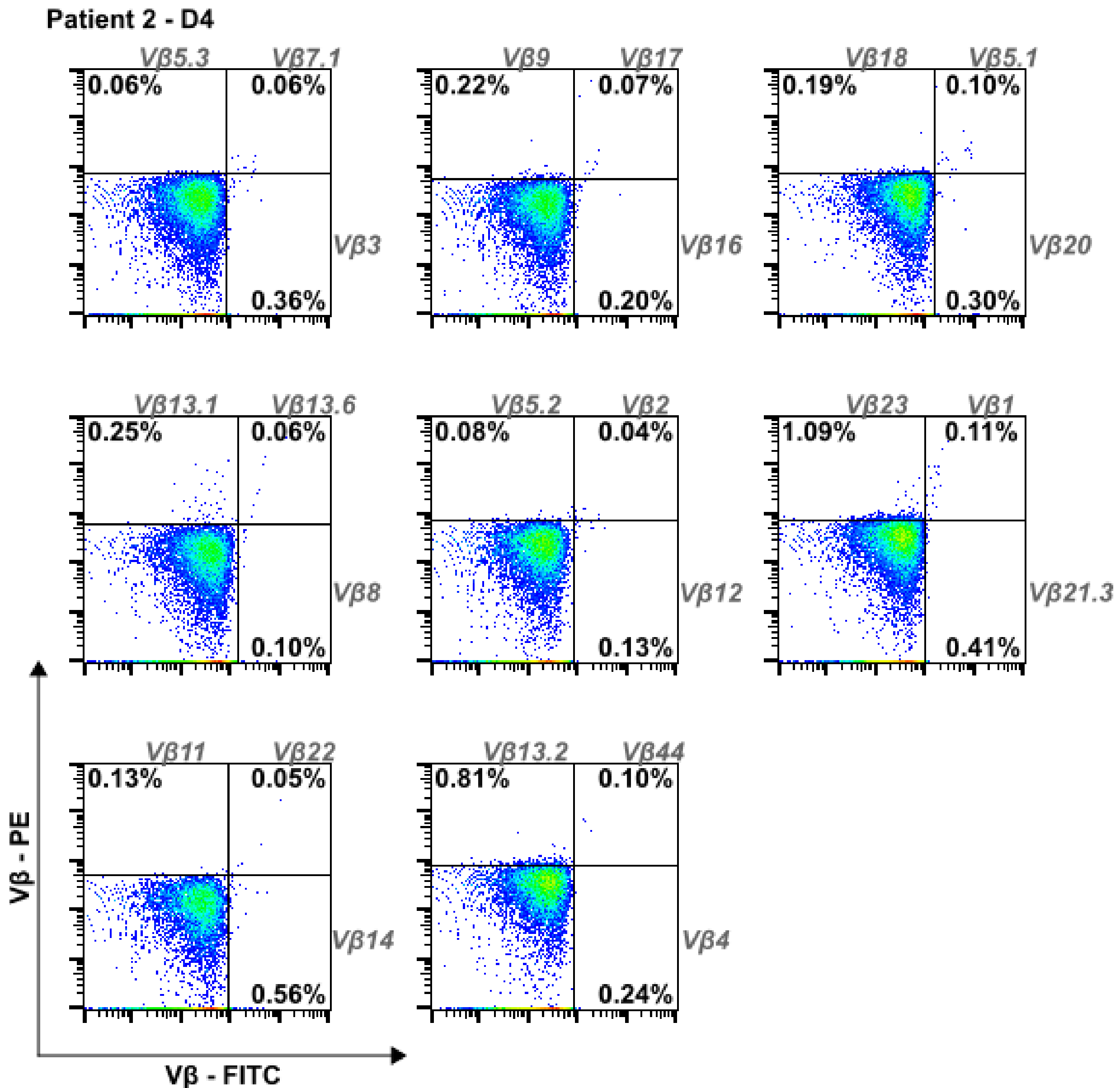


Figure 8. Purity of the Patient 2 D4 culture as assessed by TCR-Vß spectratyping. (A) Patient 2 bulk T cells were stained with viability dye, CD8 antibody, and each of eight Vß antibody cocktails. Cells were examined by flow cytometry (B) Patient 2 sorted D4 T cells were stained with viability dye, CD8 antibody, and each of eight Vß antibody cocktails. Cells were examined by flow cytometry

2.4 Discussion

To assess the presence of LDHC-specific T cells harboured in the T cell repertoire of ovarian cancer patients, CD8 T cell cultures were expanded from the ascites of five HGSC patients. Using a miniline methodology, this screening yielded a single T cell clone which recognizes peptide from LDHC. To my knowledge, this is the first assessment of LDHC as a CD8 T cell target.

Apart from allowing the isolation of this T cell clone, this strategy has additionally offered information about the T cell clone's history. As 3 reactive cultures stemmed from an original 192 000 cells, one may estimate that the frequency of the D4 clone at 1:60 000 within the CD8 population of the ascites. This estimation hinges on two pivotal assumptions: (a) reactive cultures arose from single T cells; and (b) each LDHC p62 reactive T cell identified was part of the same clonal subtype. This frequency is above that of naïve CD8 T cells [1:10^5] (Jenkins, Chu, McLachlan, & Moon, 2010; Jenkins & Moon, 2012), which suggests that it may originate from a previously activated clonal subset of CD8 T cells. Concurrent with this assessment, detection from the ascites also suggests prior tumour infiltration. As CD8 T cells become activated in the periphery prior to infiltration of the immune target site, it is likely that its cognate antigen was present in the tumour. With this knowledge, it is crucial to distinguish between a TCR which recognizes a peptide and a TCR which recognizes a protein. While the D4 clone recognizes a peptide from within the protein LDHC, it does not explicitly prove that the origin of its activation, assuming the T cells were not naïve, is LDHC. It is possible that the activation signal originated from a slightly different peptide of an entirely different origin

protein. Peptide cross-reactivity in CD8 T cells is a common phenomenon which aids in epitope recognition. As the possible permutations of peptides vastly outnumber even the diversity of the TCR repertoire (Arstila et al., 1999), it is essential that TCRs recognize multiple peptides in order to provide a versatile adaptive immune response. However, it is extremely dependent on the specific amino acid differences – a single TCR may differentiate between peptides with a single amino acid substitution while being unable to discern other substantial differences. Considering these arguments, further investigation of this LDHC peptide specific T cell clone are necessary, in order to further elucidate the clone's status of LDHC specificity.

Throughout this portion of the project, one specific challenge continually arose: T cell cultures frequently developed senescence during expansions (Hayflick & Moorhead, 1961). While this disrupted the assessment of LDHC as a *bona fide* antigen, it also illustrated a major roadblock in all CD8 based immunotherapeutic treatments. T cells would frequently expire after 4 rounds of REP expansion, which had varying efficacy. In ideal conditions, this 4-round expansion corresponds to a $1000^4 = 10^{12}$ fold expansion of a given cell. While this could theoretically yield a clonal T cell population of 10^{12}, in practice it does not. Evaluation of reactivity during selection and confirmation of antigen specific cultures between early expansions effectively requires exponentially more cells than final assays. For example, a single T cell (within a culture) has proliferated to 1000 cells; its culture is then assayed for reactivity, requiring 700 of those cells. The remaining 300 cells from that culture may then be expanded to its fullest potential, which is now $3x10^{11}$ cells – a small, inquisitive assay has effectively used 7 trillion antigen specific T cells. When this repeatedly occurs, especially prior to FACS

purification, the effective population of the T cell clone decreases to the point where it is impractical to study the clone. Considering that adoptive cell therapies typically provide T cell transfusions on the order of up to 10^{11} cells, which are required to be efficacious *in vivo*, it is crucial that clinicians and researchers are mindful of these barriers to indefinite proliferation.

While frequent screening reduced the final working population of antigen specific T cells, the miniline approach used in this study was not without its merits. This method allowed the isolation of an antigen specific T cell clone from a single sample of ascites and no autologous material, whereas classical methods of rare T cell isolation, namely DC stimulation, require large amounts of blood for the generation of autologous DCs. The miniline method can be used to detect rare, antigen specific T cells in patients that are unable to provide substantial volumes of blood for analysis. Despite the limits of expansion previously discussed, it may be useful in the determination of antigen viability for immunotherapeutic treatments such as cancer vaccination and ACT.

In summary, a miniline approach was used to screen the tumour associated T cells of the ascites in five HGSC patients for reactivity to LDHC. One patient exhibited reactivity to a peptide library generated from the LDHC protein sequence. A LDHC reactive CD8 T cell clone was purified from this patient by FACS sorting cells based on peptide-induced 4-1BB upregulation.

Chapter 3: Investigation of the intracellular antigen processing of LDHC

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DSN, DAW, and JJL designed the study. DSN, DAW, JLK, SDM, DK, JSN, JW, RR, and JJL were involved in data acquisition and/or interpretation.

3.1 Introduction

The recognition of a given CD8 T cell epitope is moderated through two pathways which intersect at the intercellular synapse of the MHCI and TCR. One path tracks the development of the TCR of a CD8 T cell, which is dictated by the selection events of thymic selection. Barring the complications which arise with cross-reactivity, this TCR has an inherent affinity for a specific, yet unknown, peptide. Another path tracks the intracellular processing of a parent protein, dictated by enzymatic specificity and MHCI affinity. Firstly, this protein is digested by a proteasome and trimmed to the appropriate size in the ER by ERAP1 and 2. Each of these enzymes exhibit preferential degradation favouring specific amino acid sequences. Secondly, the final peptide must be able to bind MHCI with some threshold affinity. As such, if a proposed antigen is to be immunogenic, it must: (a) be processed correctly from its parent protein, (b) bind MHCI, and (c) be recognized by a T cell which survives thymic selection (Martin et al., 2016).

Peptide recognition by a given T cell does not address variability in antigen processing for MHCI epitope display. MHCI is encoded by the HLA gene complex in genes HLA-A, HLA-B, and HLA-C. These genes are highly polymorphic and consist of thousands of differing alleles (10,297 HLA Class I alleles) (Degauque, Brouard, & Soulillou, 2016; Robinson et al., 2015; Robinson, Soormally, Hayhurst, & Marsh, 2016). Alleles are principally differentiated by mutations residing within the peptide-binding cleft, which create a variance in peptide binding affinity. However, in cannot be assumed that all peptides with high-affinity for MHCI are naturally presented in the peptide binding cleft. Intracellular peptide processing plays a

substantial role in the selection of peptides to be bound to MHCI within the ER, prior to transport of the complex to the cell surface. Previous studies estimate that only 14-20% of MHC-binding peptides may be authentically processed and presented in the context of MHCI (Assarsson et al., 2007; Yewdell & Bennink, 1999). In order to confirm the recognition of a protein, rather than a peptide, antigen processing must be specifically evaluated.

To assay antigen processing, a number of different methods may be applied. Amongst these methods are those which involve direct recognition of tumour cells, as well as recognition of RNA/DNA-transfected autologous antigen presenting cells. While tumour recognition is the most clinically relevant measure of cancer antigen-directed immunogenicity, it may be disrupted by various evasion mechanisms. Notably, recognition may be compromised due to MHCI down-regulation or faulty antigen processing (Aptsiauri et al., 2007; Pantel et al., 1991). While this would be a substantial barrier in the evaluation of a personalized mutation, it is crucial that the processing of a shared potential tumour epitope be assessed in a healthy cell, as tumour cells throughout a given cohort will not invariably exhibit compromised antigen presentation.

In this chapter, the cellular processing of LDHC is assessed to determine if the antigenic SWAG-M peptide, previously discussed, is presented at the cell surface by MHCI. With this aim, an enriched LDHC peptide specific T cell line is assessed for recognition of LDHC+ tumour and *LDHC*-transfected B cells. This T cell was unable to recognize any of these cells, except in cases when they were previously pulsed with cognate peptide.

3.2 Methods

3.2.1 Patient 2 HLA haplotyping

DNA from Patient 2 bulk expanded T cells was purified using a QIAamp DNA mini kit (Qiagen, Hilden, Ger.), as per the manufacturer's directions, and sent to ProImmune (Oxford, UK) for commercial HLA haplotype determination.

3.2.2 Cellular enumeration

T cells, B cells, L cells, tumour cells, and PMBC were regularly enumerated for tissue culture and assays by trypan blue exclusion assay (Sigma-Aldrich) and by flow cytometry using the ViaCount Assay on a Guava easyCyte 8HT Benchtop Flow Cytometer (EMD Millipore).

3.2.3 Culturing OVCAR tumour lines

OVCAR8 cells were provided by the OvCaRe program at the Robert Ho Research Centre. OVCAR3 and OVCAR5 cultures were grown from in-house stocks. Cells were incubated in 1640 RPMI supplemented with 20% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 µM β-mercaptoethanol, and 1 mM sodium pyruvate at 37°C. Cultures were routinely divided or passaged when flasks were ≥80% confluent, after trypsinization (HyClone Laboratories, Logan, Utah) at 37°C for 5 minutes.

3.2.4 IFNγ ELISpot analysis

IFNγ ELISpot was performed as per section 2.2.3. ELISpot wells containing target cells were plated at a target to effector ratio of 10^5:10^5 for antigen presentation. Peptide-pulsed targets were incubated with 10 µg/mL peptide for 1 hour. Peptide-pulsed cells were then washed with PBS by centrifugation to a peptide concentration of ≤10 pg/mL in 1640 RPMI without supplement.

3.2.5 CD45 depletion from ascites

As a substitute for tumour, leukocyte depleted ascites was utilized in 'tumour' recognition experiments. Bulk ascites samples were thawed and suspended in phosphate buffered saline (PBS) for CD45 depletion. Samples were labeled with PE-conjugated anti-human CD45 antibody (12-9459-41, eBioscience). Labeled cells were then tagged with EasySep PE-selection cocktail and Easy-Sep magnetic particles (EasySep PE selection kit, STEMCELL Tech). Magnetically labeled cells were removed using the EasySep Magnet (STEMCELL Tech). Unmagnetized supernatant containing CD45- cells was saved for tumour recognition analysis by CD8 T cells.

3.2.6 Culturing B cells

Firstly, CD40L+ lymphoblastoid cell lines were obtained from Julie Nielsen at the Deeley Research Centre. Cells were cultured in 1640 RPMI with HEPES, supplemented with 10% FBS,

1mM sodium pyruvate, 2mM L-glutamine, 50 µM ß-mercaptoethanol, 1x penicillin/streptomycin, and 0.5 mg/mL G418 at 37°C. When cells had proliferated to a substantial number, they were lethally irradiated at 75Gy and cryopreserved in 50% FBS, 40% media, 10% DMSO.

To selectively grow B cells, bulk ascites was first thawed and suspended in RPMI. Lymphocytes from ascites were enumerated and combined with lethally irradiated CD40L L cells at a ratio of 4:1. Mixed cells were incubated in 6 well plates at $5x10^5$ cells/mL in B cell media: Iscove's Modified Dulbecco's Media (IMDM) with HEPES, supplemented with 10% human AB serum, ITS supplement, 2mM L-glutamine, 50 µM ß-mercaptoethanol, 1x penicillin/streptomycin, 2 ng/mL human IL-4 (PeproTech), 1 µg/mL cyclosporine A. Half-media changes were conducted every 2-3 days and B cell concentrations were maintained at $5x10^5$ cells/mL. Every 6-8 days, CD40L L cells were replaced at a ratio of 4:1 B cells to L cells.

3.2.7 IVT Plasmid creation

The LDHC coding sequence (NCBI gene ID: 3948) was commercially synthesized in a gBlock gene fragment, with a preceding CACC sequence and without a stop codon (Integrated DNA Technologies, Coralville, IA). The preceding CACC sequence facilitates site-specific directional insertion into the pENTR/d-TOPO vector. This gene ligation site is flanked by *att*L1/*att*L2 recombination sequences and the plasmid includes a kanamycin resistance gene. To facilitate ligation, 5 µg *LDHC* gBlock was incubated with the pENTR/D-TOPO vector for 30 minutes at 22°C in 5 µL total volume. The ligated vector was transformed into One Shot TOP10

chemically competent *Escherichia coli*: 2 µL of the ligation solution was mixed with 1 vial of TOP10, incubated on ice for 30 minutes, heat-shocked at 42°C for 30 seconds, added 250 µL of Super Optimal broth with Catabolite repression media (SOC; Invitrogen), and shaken for 1 hour at 37°C. Transformed *E coli* were then selected overnight on 50 µg/mL kanamycin Lucia Broth agar plates (LB, Invitrogen; Bacto Agar, BD). Kanamycin-resistant colonies were grown in 50 µg/mL kanamycin liquid LB.

Plasmid DNA was purified using a Miniprep kit as per manufacturer's protocol (Qiagen). To confirm ligation, all plasmids were digested at 37°C over 1 hour using the restriction enzyme Ssp1 in Cutsmart buffer (New England Biolabs, Ipswich, MA). Enzyme activity was ceased by incubation at 65°C over 20 minutes. Digested DNA was run on 5% agarose gel (Invitrogen) in Tris, acetate, EDTA (TAE) buffer.

After ligation, the *LDHC* coding region was recombined into the pDEST-ST1-Rfc1 vector using the LR Clonase II kit (Invitrogen). The pDEST-ST1-Rfc1 vector includes an ampicillin resistance gene, and *att*R1/*att*R2 recombination sequences (complementary to *att*L1/*att*L2). The plasmid also includes a DC-LAMP sequence to promote targeted degradation of LDHC protein (Bonehill et al., 2004). As per manufacturer's directions, 150 ng of the pENTR/d-TOPO/*LDHC* plasmid and pDEST-ST1-Rfc1 destination vector were incubated with the LR Clonase II mix at 25°C for 30 minutes. To stop the reaction, Proteinase K was added, and the solutions were incubated at 37°C for 10 minutes. As described previously, plasmids were transformed into TOP10 *E coli*. Transformed *E coli* were then selected overnight on 100 µg/mL

ampicillin LB agar plates. Ampicillin-resistant colonies were grown in 100 µg/mL ampicillin liquid LB.

Plasmid DNA was again isolated using a Miniprep kit (Qiagen). To confirm recombination, all plasmids were digested at 37°C over 30 minutes using the restriction enzyme XbaI in Cutsmart buffer (NEB). Digested DNA was run on 5% agarose gel.

Commercial sequencing (GenScript) was performed on the plasmid using forward (T7) and reverse (pST2 reverse) primers flanking the *LDHC* insertion to confirm the correct insertion of *LDHC*.

3.2.8 RNA generation

To transcribe mRNA *in vitro*, the pDEST-ST1-Rfc1-gene plasmid was first linearized by the restriction enzyme PmeI in Cutsmart buffer for 1 hour at 37°C (NEB). The digestion reaction was stopped by incubation at 65°C for 20 minutes. An aliquot from the digest was used to confirm linearization by agarose gel electrophoresis. DNA was then concentrated using the MinElute PCR purification kit (Qiagen). To begin transcription, 1 µg of linearized plasmid was mixed with 10 µL of NTP/CAP mix (15 mM ATP, 15 mM CTP, 15 mM UTP, 3 mM GTP, 12 mM cap analog) and 2 µL RNA Polymerase Enzyme Mix in a 20 µL total volume of Reaction Buffer (mMESSAGE mMACHINE T7 Ultra Kit, Ambion Inc., Carlsbad, CA). The reaction was incubated at 37°C for 2 hours, and stopped by adding 30 µL Lithium Chloride Precipitation Solution. Precipitated RNA

was isolated by centrifugation and washed with ethanol, prior to being resuspended in ribonuclease (RNase) free water.

RNA was generated in vitro from: pDEST-ST1-Rfc1-*LDHC*, created as described previously; pDEST-ST1-Rfc1-*LDHA*, provided by Jennifer Kalina (unpublished); and pDEST-ST1-Rfc1-*CEF*, provided by Julie Nielsen (Nielsen, Wick, Tran, Nelson, & Webb, 2010).

3.2.9 Transfection

B cells were transfected by electroporation with the Amaxa Human B Cell Nucleofector Kit (Lonza Group, Basel, Switzerland). Cells were transfected with *LDHC*, *LDHA*, or *CEF* mRNA transcribed *in vitro* or pcDNA3-*LDHC* plasmid. The pcDNA3-*LDHC* expression vector was provided by Edwin Goldberg and prepared by Ryan Ritskes. Upon transfection, this vector provides endogenous expression of LDHC, as directed by a Cytomegalovirus (CMV) promoter. In each case, $3x10^6$ cells were transfected with 3 μg of *in vitro*-transcribed mRNA or 1 μg of pcDNA3 undigested plasmid. To confirm positive transfection, $3x10^6$ cells were transfected with 1 μg of a vector encoding GFP (green fluorescent protein). Expression of GFP by transfected cells was confirmed by flow cytometry.

3.2.10 Lysates

Cells were centrifuged and washed in cold PBS. Cells were then suspended in radioimmunoprecipitation assay (RIPA) buffer containing 150 mM sodium chloride, 1.0% NP-40,

0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, at pH 8.0, and supplemented with 1 ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail tablet (Roche, Mississauga, ON, CA) per 10 mL of buffer. Samples were incubated on ice and periodically vortexed for 20 minutes, and sonicated with a Branson Sonifier 450 (Branson Ultrasonics, Danbury, CT) at output 7, duty cycle 75%, with 2 sets of 10 pulses. Cell debris was removed by centrifugation (Sorvall Legend RT centrifuge, Mandel Scientific, Guelph, ON).

3.2.11 Quantification of protein and nucleic acids

Proteins were quantified using the standard bicinchoninic acid (BCA) assay. Bicinchoninic acid solution and copper II sulfate solution were mixed at 50:1 (Sigma-Aldrich). Lysates to be quantified were diluted in water and mixed with the AB solution at 1:20 in triplicate in a 96 well plate. Plates were incubated at 37°C for 30 minutes. After incubation, they were cooled and analyzed on a VersaMax™ Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA) at 562 nm. Absorbance was compared on a standard curve generated by protein standards to calculate the concentration of protein.

DNA and RNA were quantified by spectrophotometry on a NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE).

3.2.12 Western blotting

Protein lysates were run on 4-12% Bis-Tris or polyacrylamide gels (Invitrogen), transferred onto nitrocellulose membranes (Life Sciences, Pensacola, FL) and blocked for 60 minutes at room temperature in Odyssey blocking buffer (LI-COR, Lincoln, NE). Blots were probed overnight with anti-human LDHC at 1:2500 (EP1746Y; rabbit monoclonal; AbCam, Cambridge, UK), anti-human LDHA at 1:10000 (ab47010; rabbit polyclonal; AbCam), or anti-human GAPDH at 1:10000 (NB300-221; mouse monoclonal; Novus Biologicals) in Odyssey blocking buffer. Blots were washed with TBST and incubated for 1 hour with secondary antibodies anti-rabbit IgG IRDye®800 conjugated at 1:10000 (611-145-002; H&L goat polyclonal; Rockland Immunochemicals Inc., Gilbertsville, PA, USA) and goat anti-mouse IgG IRDye®800 at 1:10000 (610-145-002; goat polyclonal; Rockland Inc.) in 50% TBST 50% Odyssey blocking buffer. Immunoblots were visualized using a LI-COR Odyssey scanner and the corresponding Odyssey software (v3.0.16; LI-COR).

3.3 Results

3.3.1 Evaluation of LDHC+ tumour cell line recognition by Patient 2 D4 T cells

In order to determine if LDHC is intracellularly processed to the peptide SWAG-M on the cell surface in MHCI, multiple of individual methods were used. To start, Patient 2's HLA haplotype was commercially determined. As seen in Table 3, Patient 2's complete HLA haplotype is shared across that of the common tumour cell lines OVCAR5 and OVCAR8 (Adams et al., 2005). Together, this combined coverage made them ideal targets to assess antigen presentation. Indeed, both OVCAR cell lines express *LDHC* (Klijn et al., 2015). Thus, provided that LDHC is processed into the form of MHCI:SWAG-M by either of these cell lines, the D4 T cell culture would recognize it. Patient 2 D4 reactivity to the cell lines was assessed by IFNγ ELISpot in triplicate at a 10^5:10^5 ratio of effectors to targets (Figure 9). As a positive control, MHCI on OVCAR5 and OVCAR8 was loaded with peptide during 1 hour of incubation with SWAG-M at 10 μg/mL. Additional peptide in solution was washed by centrifugation. As an additional positive control, D4 T cells were also incubated with peptide directly. To eliminate other possible confounding factors, negative controls included tumour cells alone, as well as unstimulated T cells alone. However, the purified T cell line was unable to recognize either cell line without peptide pulsing. OVCAR8 pulsed with SWAG-M stimulated the greatest IFNγ secretion. However, when pulsed with SWAG-A, D4 T cells were unable to recognize the tumour cells.

Patient 2 HLA haplotype	OVCAR5 HLA haplotype	OVCAR8 HLA haplotype
HLA-A*02	HLA-A*01	HLA-A*01
HLA-A*02	HLA-A*02	HLA-A*25
HLA-B*08	HLA-B*08	HLA-B*57
HLA-B*57	HLA-B*44	HLA-B*57
HLA-C*06	HLA-C*05	HLA-C*06
HLA-C*07	HLA-C*07	HLA-C*06

Table 3. Compatibility of Patient 2, OVCAR5, and OVCAR8 HLA haplotypes.

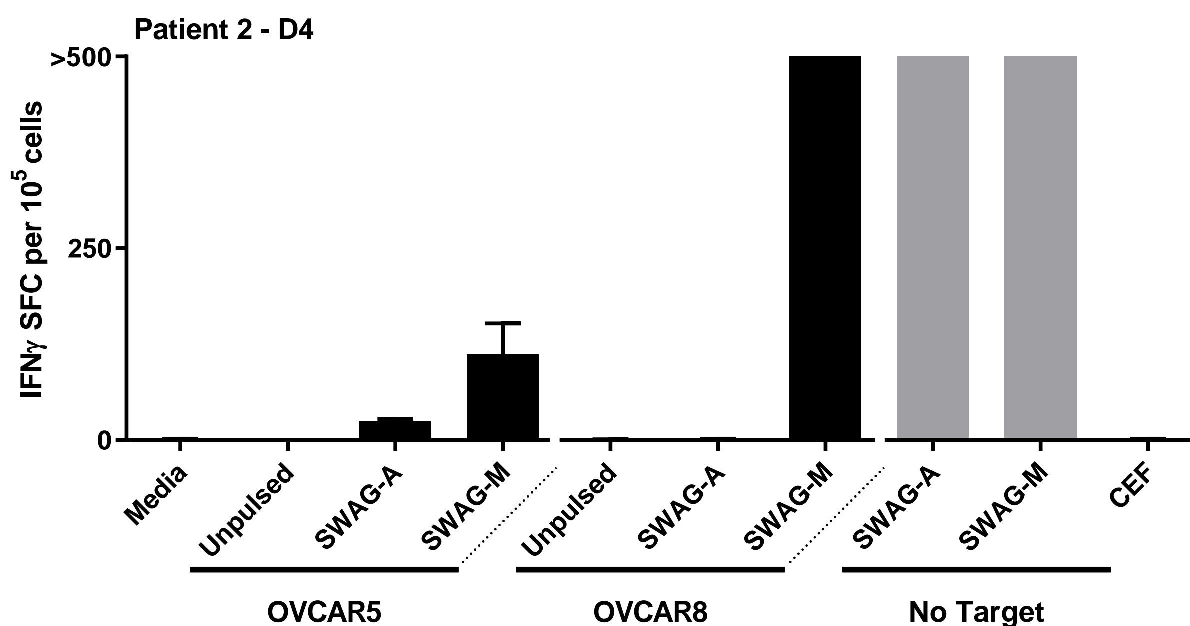


Figure 9. OVCAR recognition assay by Patient 2 D4 T cells as evaluated by IFNγ ELISpot. Patient 2 D4 T cells were plated in triplicate at 10^5:10^5 with OVCAR tumour cells. OVCAR tumour cells were incubated with SWAG-M, SWAG-A, or no peptide for 1 hour prior to ELISpot. Cells were incubated together for 20 hours. Exogenous peptides SWAG-A and SWAG-M were present at 10 µg/mL as positive controls. The CEF peptide pool was used as a negative control.

3.3.2 Evaluation of tumour recognition by Patient 2 D4 T cells

In a parallel effort to determine if LDHC is processed into the epitope SWAG-M, tumour reactivity was assessed by way of ascites slough. To ensure that confounding leukocytes would not interfere in the assessment of D4 reactivity, the ascites was subjected to magnetic CD45 depletion. ELISpot wells were prepared in triplicate with a 10^5:10^5 ratio of T cells to tumour cells, with both unpulsed and peptide-pulsed tumour cells. These cells were peptide loaded as per the methods of loading OVCAR cells. A restriction in cell numbers meant that tumour cells were only able to be pulsed with SWAG-M.

Here, in the most direct way available to assess tumour recognition, D4 was tested by IFNγ-ELISpot against CD45 depleted ascites (Figure 10). Results show that D4 T cells were only able to recognize cells which were previously pulsed with cognate peptide antigen, and did not recognize autologous tumour cells.

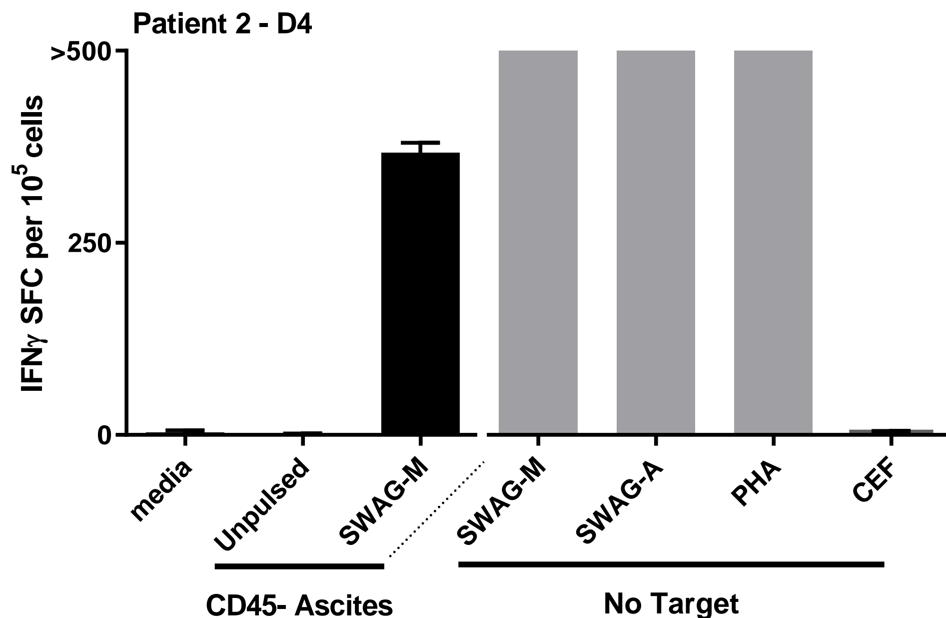


Figure 10. (previous page) **Ascites recognition assay by Patient 2 D4 T cells as evaluated by IFNγ ELISpot.** Patient 2 D4 T cells were plated in triplicate at 10^5:10^5 with CD45 depleted ascites cells. Ascites cells were incubated with or without SWAG-M for 1 hour prior to ELISpot. Cells were incubated together for 20 hours. Exogenous peptides SWAG-A and SWAG-M were present at 10 µg/mL as positive controls. As an additional positive control, PHA was used. The CEF peptide pool was used as a negative control.

3.3.3 Evaluation of LDHC-SWAG-M intracellular processing

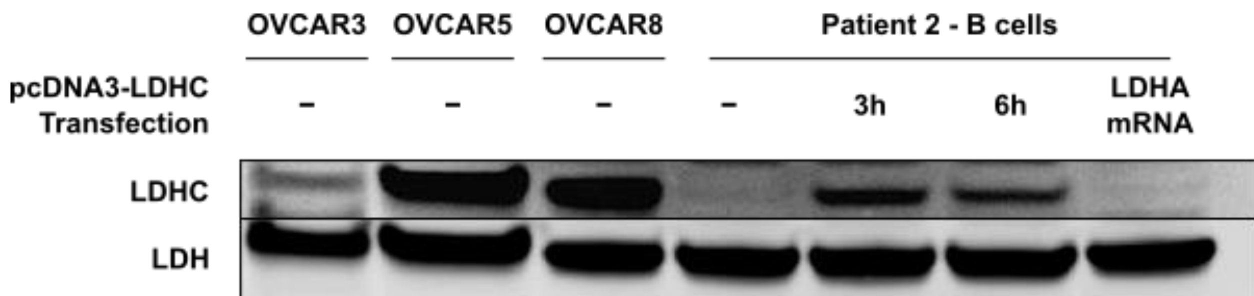
A *LDHC* expression vector was created for *LDHC* mRNA synthesis *in vitro*. Patient 2 B cells were transfected with *LDHC* mRNA in order to mimic endogenous expression of LDHC. However, even 12 hours after transfection, B cells displayed undetectable levels of intracellular LDHC (not shown). B cells were also transfected with a pcDNA3-*LDHC* expression vector. Indeed, LDHC was present at levels detectable by western blot analysis as early as 3 hours post-transfection (Figure 11a). OVCAR5 and OVCAR8 cells were used as positive controls for LDHC expression. As a loading control, expression of LDH was assessed in all cells.

In order to determine if LDHC was processed to SWAG-M and presented by the internal machinery of a healthy antigen presenting cell, IFNγ secretion by D4 T cells was evaluated by ELISpot after incubation with LDHC-expressing autologous B cells (Figure 11b). To simulate LDHC expression, B cells from Patient 2 were transfected with the pcDNA3-LDHC vector. As a negative control, autologous B cells were additionally transfected with mRNA encoding LDHA. To ensure the functionality of the presenting machinery of Patient 2 B cells, autologous B cells were also transfected with mRNA transcribed from a CEF poly-epitope construct. Processing and presentation of the CEF poly-epitope was evaluated by IFNγ production by CEF-reactive

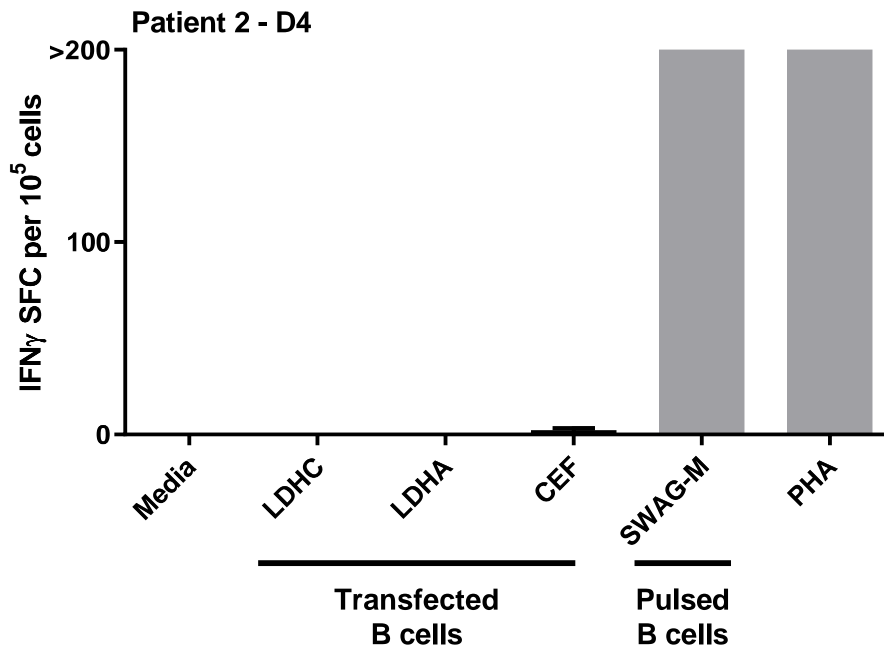
bulk T cell cultures from Patient 2 (Figure 11c). All ELISpot wells were prepared in triplicate with a 10^5:10^5 ratio of T cells to transfected APC as well as peptide pulsed APC.

This ELISpot showed that, while Patient 2 T cells could recognize peptide pulsed cells and autologous B cells could successfully process and present CEF *ivt*-RNA, *LDHC*-transfected B cells were unable to stimulate IFNγ secretion by D4 T cells. LDHC is not processed and presented as SWAG-M in the context of MHCI in healthy cells.

A



B



C

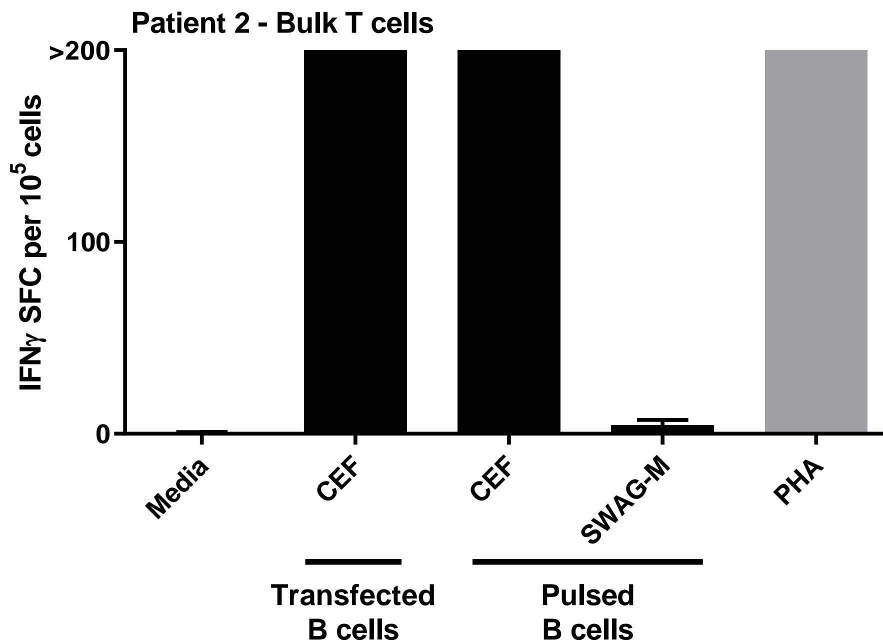


Figure 11. Intracellular LDHC recognition assay by Patient 2 D4 T cells as evaluated by IFNγ ELISpot. (A) Patient 2 B cells were transfected with pcDNA3-*LDHC* plasmid and lysates were analyzed by western blot after 0, 3, and 6 hours. Expression of LDHC by OVCAR5 and OVCAR8 cell lines were used as positive controls. As a negative control, B cells were also transfected with *LDHA* mRNA. To control for protein loading, LDH expression was assessed in all cells. (B) Patient 2 D4 T cells were plated in triplicate at 10^5:10^5 with autologous B cells. B cells were either transfected with pcDNA3-*LDHC* plasmid, *LDHA ivt*-mRNA, or CEF polyepitope *ivt*-mRNA. Cells were incubated together for 20 hours. As a positive control, B cells were also peptide pulsed with SWAG-M for 1 hour prior to ELISpot and plated at 105:105. PHA was used as a positive control. (C) Patient 2 bulk T cells were plated in triplicate at 10^5:10^5 with autologous B cells. B cells were either transfected with CEF polyepitope *ivt*-mRNA or peptide pulsed for 1 hour with CEF or SWAG-M prior to ELISpot. Cells were incubated together for 20 hours. PHA was used as a positive control.

3.4 Discussion

The antigen processing machinery of MHCI expressing cells is complex and its interaction with proteins has not been fully elucidated. While the proteasomes and peptidases involved cut habitually at particular sites within given long peptides (S. C. Chang, Momburg, Bhutani, & Goldberg, 2005; Serwold, Gaw, & Shastri, 2001; Serwold et al., 2002), researchers have thus far been unable to reliably predict how individual proteins may be processed into MHC binding potential epitopes (Singh & Mishra, 2016). As such, it is uncertain whether a given peptide, which is antigenic when supplied exogenously, would be endogenously processed from its parent protein. In this chapter, empirical methods are utilized in order to determine whether LDHC is processed into the SWAG-M epitope for MHC presentation. Secretion of IFNγ by the SWAG-M-reactive D4 T cell line served as a dependent variable to determine whether the D4 cognate peptide was present on cell surface MHCI in a number of LDHC-expressing cells. In each experimental case, there was an absence of IFNγ secretion by the D4 T cells – LDHC is not processed into this particular peptide for antigenic presentation.

Throughout this experimental phase, abandonment of these LDHC epitopes was repeatedly considered due to the cross-recognition of the GSWAG and SWAG peptides, as each are contained within LDHA and LDHC, and the SWAG-A peptide (Table 2). However, since the D4 T cells from Patient 2 do indeed recognize LDHA peptide, it follows that LDHA must not be endogenously processed into the SWAG peptides for presentation. As LDHA is ubiquitously expressed, the lack of self-recognition by the D4 T cells denotes an absence of LDHA processing to SWAG. While one may postulate a similarity in the processing of LDHA and LDHC, this fact

does not explicitly reject the possibility of such LDHC processing. Studies have indeed revealed that antigen processing is not solely dependent on the epitope in question, but rather, is dependent on the flanking sequences of the peptide itself (Steers et al., 2014). As such, due to the differential sequences of LDHA and LDHC, it was feasible that the SWAG peptides may be presented in LDHC+ cells.

While here it is shown that SWAG is not systematically processed (Figure 9-11), it is possible that in some cases it may be. As previously discussed, tumours adopt a number of strategies which are directed toward immunoevasion. These strategies could involve the manipulation of antigen processing. Due to the complexity of antigen processing for MHC presentation, there are numerous points for intervention. Selective pressure may lead tumour cells to manipulate antigen processing to avoid presentation of immunogenic peptides. Similarly, those pathways can be altered in order to favour the presentation of a specific peptide. During the degradation of a given protein, a given epitope might be destroyed by protease or aminopeptidase activity (York et al., 2002). However, protease inhibitor studies have shown that various inhibitors may modulate aminopeptidase activity (Kourjian et al., 2016; Kourjian et al., 2014). By inhibiting degradation of polypeptides, antigenic epitopes may avoid eradication, in favour of MHCI presentation. Furthermore, protease inhibitor activity encourages alternative cleavage of peptide sequences prior to MHCI loading, which generates unique peptides for TCR interrogation. Similar epitope variance has also been demonstrated by ERAP1 knockout experiments in mice: a lack of ERAP1 activity can promote differential MHCI:peptide expression (York, Brehm, Zendzian, Towne, & Rock, 2006). It is possible that

immunomodulatory techniques such as protease and aminopeptidase inhibition lead to the SWAG-M presentation by MHCI.

In this project, it was determined that the SWAG-M peptide from LDHC is not naturally processed in tumour cells or in healthy B cells. However, other discovery studies have revealed new tumour-specific antigens which may be used to the same purposes. In recent years, many new CD8 epitopes have been discovered within the CT antigens Cyclin-A1, MAGE-A1, MAGE-C1, NY-ESO-1, and XAGE-1b, amongst others (Anderson et al., 2011; Eikawa et al., 2013; Ma et al., 2011; Ochsenreither et al., 2012; Ohue et al., 2012; Stroobant et al., 2012). As this project did, these antigenic discoveries faced the identical challenge of evaluating intracellular processing. Throughout, the transfection of healthy APCs with *ivt*-RNA and various expression vectors has been thoroughly vetted as an experimental technique and has yielded confident results (Gros et al., 2016; Tran et al., 2015). Comparisons between studies such as these provide further evidence in favour of the estimation that a given immunogenic peptide with affinity to MHCI has a 14-20% change of being processed and presented in that state (Assarsson et al., 2007; Yewdell & Bennink, 1999). In a cancer setting with a relatively low number of mutational events such as ovarian cancer (Lawrence et al., 2013), antigen discovery generally has a low chance of success. As immunotherapy continues to gain traction, it is crucial that these low odds continue to be challenged.

Chapter 4: Conclusions

4.1 Concluding remarks

The objective of this thesis was to conduct an assessment of LDHC as a CD8 T cell tumour antigen in high grade serous ovarian carcinoma. With this objective, a LDHC peptide library was generated in order to conduct screening against potentially reactive patient CD8 T cells. A series of CD8 T cell cultures were purified from the ascites of five individual patients for LDHC screening. In order to minimize the frequency of false negative errors due to the relative insensitivity of the ELISpot assay, T cell cultures were grown in a miniline format, which theoretically preserves a high frequency of all clones it contains (Geiger et al., 2009). The elucidation of LDHC immunogenicity, as a novel tumour-specific antigen, would provide reasoning for the pursuit of widely applicable T cell mediated cancer therapies directed by LDHC.

Chapter 2 describes the initial evaluation of T cell cultures from five patients. During primary singlicate screening, a number of cultures produced IFNγ in peptide stimulated cultures. Through subsequent confirmation experiments, a single T cell culture was found to genuinely recognize LDHC peptide. After peptide stimulation, this culture was enriched by 4-1BB selection though FACS sorting to generate a functionally monoclonal LDHC peptide specific CD8 T cell culture.

After the initial T cell screening of these five patients, a number of questions are left unanswered. These questions may be collectively asked: *how confidently can we declare that a*

single LDHC peptide reactive T cell resides throughout the T cell repertoire of these five patients? The simplistic answer: *not confidently*. This lack of confidence is primarily an artifact of the experimental preference for complete epitope coverage of the LDHC protein. Using 15mer peptide libraries for broad, efficient, and most importantly high-throughput detection of T cell responses is well researched and established amongst many studies (Betts et al., 2001; Kern et al., 2002; Maecker et al., 2001; Ruwona et al., 2014). The use of 15mers is well-practiced and resource-light and they are indeed "very effective" (Maecker et al., 2001), but they are not the optimal epitope for MHCI expression and TCR binding. The use of these suboptimal peptides has been shown to lead to negative T cell responses to 15mers which would have responded to a peptide of optimal length contained within. This response variance frequently depends on the location of the minimal epitope within the 15mer (Draenert et al., 2004). However, reports have varied on how positioning of the optimal peptide affects recognition – these reports disagree on if recognition is better masked if the optimal sequence is closer to either the N- or C-terminus of the 15mer peptide (Draenert et al., 2004; Fiore-Gartland et al., 2016). Recent studies suggest that these affects may lead to a false negative response rate of up to 25 percent (Fiore-Gartland et al., 2016). However, because of the differential rate of false negativity with respect to the position of the minimal epitope within a given 15mer, this is partially negated: minimal epitopes contained within two adjacent overlapping peptides within a library are less frequently subject to being left unidentified.

Furthermore, the miniline strategy presents a similar complication, albeit of different origin. This method sacrifices the bulk number of T cells one is able to screen in order to

provide a depth of analysis into the rare T cell repertoire. While this technique virtually guarantees the detection of T cells present at frequencies above $1/10^5$, it is feasible that clones of lower frequencies may simply not be included in the sample population. In this study, every 96 original cultures accounted for the evaluation of 196 000 CD8 T cells. Considering this, it is possible that LDHC reactive T cells harboured by these patients have been overlooked. Moreover, there are no antigenic stimuli present to encourage the preferential clonal proliferation of positively reacting T cells. For example, the case of Patient 1 culture D5: a peptide reactive culture, after REP expansion, was found to be non-reactive to LDHC peptide. This was possibly due to competitive proliferation between expanding T cell clones in the culture. Within the miniline approach, REP provides non-specific signals for polyclonal T cell activation, theoretically preserving the original frequencies of the seed cultures (Geiger et al., 2009). However, after stimulation, anergic and exhausted T cells would be unable to maintain competitive rates of expansion when accompanied by unencumbered T cell clones, which could proliferate to the point of occupying a dominant portion of the culture (Willimsky & Blankenstein, 2005). Indeed, variable T cell expansion rates within cultures have been witnessed in many studies utilizing this technique (Geiger et al., 2009; Martin et al., 2016; Wick et al., 2014). Techniques such as DC-stimulation provide reactive T cell clones a competitive advantage through selective engagement, leading to immediate enrichment of antigen specific T cells (Nielsen et al., 2016). This enrichment also avoids the need to purify cultures by flow cytometry, which can be abrasive and traumatic to cells, possibly further inhibiting their proliferative abilities. It could be that LDHC reactive T cells drown in their cultures due to the favourable expansion of irrelevant T cells.

As an aside from logistical preferences, it should be noted that all patient T cells were extracted from ascites samples. While this strategy was theorized to expose previously activated T cells which had encountered LDHC antigen within the tumour microenvironment, it may also have had a negative effect on screening efficacy. While tumour antigen-exposed T cells are more likely to be present in the ascites, antigen-naïve T cells are less likely to be present. To more effectively mine a patient's naïve T cell repertoire, it would be prudent to examine T cells directly from the peripheral blood. To further pursue tumour antigen specific T cells, it would be ideal to examine T cells directly from TIL and TDLN, as they are likely to be tumour specific. However, these resources are scarce, due to the nature of live patient tissue collection.

In Chapter 3, the intracellular processing of LDHC was assessed. The SWAG-M-reactive T cell culture from Patient 2 is used to determine the presence of SWAG-M in the context of MHCI on the cell surface of LDHC+ cells. In order for recognition to occur, LDHC needs to be digested specifically to generate the SWAG-M epitope and have it presented on the cell surface. Firstly, autologous tumour cells and common tumour cell lines were evaluated for D4 recognition. It was found here that these cells would not produce MHCI:SWAG-M. In order to determine if this inability was due to defective intracellular antigen processing, healthy autologous B cells were transfected with an *LDHC* expression vector. The ability of transfected B cells to present SWAG-M to D4 T cells was subsequently assessed by IFNγ ELISpot. It was determined that they were unable to do so; thus, LDHC is not naturally processed into the SWAG-M epitope.

Naturally, this begs the questions: *does the SWAG-M epitope have any relevance in tumour recognition? Could its corresponding TCR serve any anti-tumour purpose?* As discussed in section 3.4, the antigen processing pathways of the cell may be manipulated in order to provide this peptide for presentation. This may be done with protease inhibitors, which have been shown to alter not only proteasome activity, but also aminopeptidase activity within the ER. Indeed, it has been shown that the protease inhibitors saquinavir, nelfinavir, and ritonavir inhibit ERAP1 and 2 activity, which potentially degrade high MHC-affinity epitopes in favour of generating the patient's standard peptide-MHC ligandome (Kourjian et al., 2014). The application of protease inhibitor-integrated treatments utilizing targeted delivery of such drugs could lead to the recognition of LDHC+ tumour cells by the SWAG-M specific TCR.

The negative end result here also points toward one other major concern. This concern is that all truly LDHC-reactive T cells may have been previously deleted during thymic negative selection. Indeed, thymic selection extensively edits the thymocyte population prior to reaching maturity. Knockout studies in mice have revealed that AIRE expression in mTEC causes the epithelial upregulation of 3980 protein-coding genes, including *LDHC* (Sansom et al., 2014). Peptides derived from these proteins are presented within MHCI to maturing thymocytes for negative selection. Furthermore, peptides may also be generated for negative selection from intronic sequences during maturation (Apcher et al., 2013). It suffices to say that the selection process is rigorous and highly developed, accounting for self-tolerance of organs throughout the body. So one must pose the question: *can LDHC be immunogenic?* Previously, studies involving LDHC peptide vaccination have demonstrated the induction of CD4 T cell responses as

well as anti-LDHC antibody mediated responses (E. Goldberg, VandeBerg, Mahony, & Doncel, 2001; O'Hern, Bambra, Isahakia, & Goldberg, 1995). These antibodies recognize, inhibit, and facilitate the killing of autologous spermatozoa. However, to this point, no studies have described an anti-LDHC CD8 T cell mediated response. The viability of CT antigens as immunogenic is denoted by the immunogenicity of sperm. Numerous studies have shown that the body will mount a robust immune response upon the introduction of sperm to the periphery (Tung, Teuscher, & Meng, 1981). Normally, the sperm are protected against such immune responses in the immune privileged environment of the testis (Zhao, Zhu, Xue, & Han, 2014). The necessity of the immunosuppressive environment of the testes and the immunogenicity of spermatozoa suggests that CT antigens are viable immune targets. As such, it is likely that CD8 T cells within the mature lymphocyte repertoire may be able to target cancer cells based upon CT antigen expression. Moreover, as described by the Ludwig Institute for Cancer Research, the CT catalogue includes proteins from over 250 gene families, which encode over 275 individual proteins (Almeida et al., 2009). As previously discussed, a number of these proteins, including NY-ESO-1, have been found to elicit robust CD8 directed immune responses. In the future, though a similar study, it is possible that other antigenic peptides may be identified within LDHC.

4.2 Future Directions

As suggested by the previous discussion, the results presented within this thesis cannot claim that LDHC is not immunogenic. Though no *bona fide* LDHC responses were revealed in the

T cell repertoire of these five patients, they may have been missed, due to the constraints of the screening strategy. Yet, there is still merit to the original objective of the project, and in the pursuit of that goal, it would be beneficial to more extensively screen these patients for the presence of LDHC reactive CD8 T cells. The ascites was specifically selected for assessment as it contains TAL, which have encountered tumour previously. As these cells have encountered tumour, it follows that they were tumour reactive at some time point. As such, in an LDHC+ tumour, it is likely that the ascites may harbour LDHC reactive T cells. However, it is also true that these cells may be more inclined to have assumed an exhausted or anergic phenotype. Moreover, if the tumour had become LDHC+ at a later time point, it is possible that LDHC specific T cells had not yet migrated to the ascites. In order to maximize the chances of detecting T cells specific to this antigen, it would be advisable to screen TIL and PBMC. Indeed, not every naïve T cell encounters antigen, even when cognate peptide is presented by APC. It is possible that naïve LDHC specific T cells exist in the peripheral blood of these patients.

If LDHC is not immunogenic within these patients, investigating other patients could prove to be beneficial. The immune status and HLA haplotype of these specific patients may compromise or even restrict their ability to recognize or present an LDHC epitope. Patients with ongoing infections or similar strains on their immune system may have disproportionate T cell populations, favouring infection-relevant T cell clones over tumour-specific naïve T cell populations. The HLA haplotype of these patients also restricts recognition simply by chance, as LDHC may not contain unique epitopes with adequate affinity for the appropriate MHCI alleles. It would thus be prudent to screen additional patients with variable HLA haplotypes, as

alternative ligands within LDHC will be considered for reactivity. Furthermore, a differential haplotype also provides a new T cell repertoire which has undergone patient-specific thymic selection. While searching for a specific T cell clone, it is crucial that a broad population of T cells be evaluated.

Considering the limitations of the miniline strategy, high throughput methods should be utilized if possible during additional screening. However, the reality of these projects is that patients can rarely afford to provide substantial quantities of blood and tumour tissue. And, as previously mentioned, well-vetted strategies like DC-stimulation of T cell populations are extremely resource intensive. When resources allow it, peptide pulsed DC-stimulation should be used for the discovery of antigen specific rare or naïve T cells. Importantly, this strategy encourages the selective proliferation of antigen specific cells from within large polyclonal T cell populations. It can also be used with large peptide pools in order to assess the presence of T cell clones of multiple specificities simultaneously. DC-stimulation will allow the confident analysis of a T cell culture, while providing a near-clonal antigen specific product.

In addition to the extended assessment of LDHC as a tumour antigen, it is crucial that other CT antigens continue to be assessed. As this report is the first to investigate the immunogenicity of LDHC as a CD8 T cell tumour target, similar reports corresponding to other antigens must also exist. It is only with a broad approach to antigen discovery that novel tumour immunogens will be found. Indeed, there is a constant force within the field searching for new immunogenic cancer epitopes of various origins: tumour point mutations, aberrant protein expression, novel transcription, gene fusions, amongst others. As previously mentioned,

a wide scope for antigenic assessment has led to the elucidation of many novel antigenic epitopes. Indeed, the field of immunotherapy relies on tumour-specific antigens and has flourished with their discovery, as evidenced by the frequency of completed and progressing clinical trials.

Finally, considering that all tumour antigen discovery projects such as this strive towards future clinical trials utilizing varied immunotherapeutic strategies, is it is imperative that T cell limits are considered. In order to combat such limitations, autologous irrelevant T cells can be imbued with tumour specificity via engineered T cell receptors. This strategy can ensure the efficacy of infused T cells via the selection of a specific phenotype: this avoids exhaustion, while guaranteeing antigen specificity. However, genetic and phenotypic heterogeneity, as arisen from varied mutational accumulation throughout tumour development, restricts antigen specific T cell efficacy to spatial regions of the tumour. As such, it is also prudent to maximize the number of target antigens, as they are likely differentially restricted: to attain immunogenic coverage of a tumour, multiple simultaneous antigen specific responses would be required. Thus, it is critical that projects to discover novel tumour antigens continue to be undertaken.

4.3 Outlook

This thesis describes a T cell which can recognize LDHC peptide. However, that peptide is not processed within healthy nor tumour cells for MHCI presentation. Although this is unfortunate, it doesn't end the investigation into LDHC immunogenicity, nor the assessment of other antigens. It is crucial that projects such as this continue to be undertaken, as the cancer

immunotherapy field requires an ever-expanding catalogue of vetted targets to draw upon. While this project has not provided such a target, some portion of like projects may. It is only with a broad scope of discovery projects that discovery will indeed occur.

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Appendix A: Abbreviations

ACT – Adoptive cell therapy

ADCC – Antibody-dependent cellular cytotoxicity

AIRE – Autoimmune regulator

APC – Antigen presenting cell

APC – Allophycocyanin

BCA - Bicinchoninic acid assay

CAR – Chimeric antigen receptor

CEF – Cytomegalovirus, Epstein-Barr virus, Influenza virus

CD – Cluster of differentiation

CMV – Cytomegalovirus

CLIP – Class II-associated invariant chain peptide

cTEC – Cortical thymic epithelial cell

CT – Cancer-testis

CTLA-4 – Cytotoxic T lymphocyte-associated protein 4

DC – Dendritic cell

DISC – Death induced signaling complex

EDTA - Ethylenediaminetetraacetic acid

ELISpot - Enzyme-Linked ImmunoSpot

EOC – Epithelial ovarian cancer

ER – Endoplasmic reticulum

ERAP – Endoplasmic reticulum aminopeptidase

FACS – Fluorescence activated cell sorting

FDA – Food and Drug Administration

FITC - Fluorescein isothiocyanate

GFP – Green fluorescent protein

GM-CSF – Granulocyte-macrophage colony-stimulating factor

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HGSC – High grade serous carcinoma

HLA – Human leukocyte antigen

HLA-DM – Human leukocyte antigen - DM

IFN - Interferon

IMDM - Iscove's Modified Dulbecco's Media

IL – Interleukin

ITS - Insulin, transferrin, sodium selenite

IVT – *In vitro* transcription

LAMP2a – Lysosome associated membrane protein 2a

LB – Lysogeny broth

LDH – Lactate dehydrogenase

MIIC – MHCII compartment

MHC – Major histocompatibility complex

mTEC – Medullary thymic epithelial cell

NCBI - National Center for Biotechnology Information

PARP - Poly (ADP-ribose) polymerase

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate buffered saline

PD-1//PD-L1 – Programmed cell death-1 // Programmed cell death ligand-1

PE – Phycoerythrin

PHA – Phytohaemagglutinin

PROC – Platinum-resistant ovarian cancer

REP – Rapid expansion protocol

RIPA – Radioimmunoprecipitation assay

RNase – Ribonuclease

RPMI – Roswell Park Memorial Institute

SFC – Spot forming cells

SOC - Super Optimal broth with Catabolite repression

TAA – Tumour associated antigen

TAE – Tris, acetate, EDTA

TAL – Tumour associated lymphocytes

TAP – Transporter associated with antigen processing

TCA - Tricarboxylic acid

TCR – T cell receptor

TDLN – Tumour draining lymph node

Th – T helper

TIL – Tumour infiltrating lymphocytes

TNF – Tumour necrosis factor

Treg – T regulatory cell