SEMINOMA PATIENTS AS UNTAPPED RESERVOIR OF CANCER-TESTIS ANTIGEN SPECIFIC T CELLS FOR IMMUNOTHERAPY

by

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ABSTRACT

Testicular cancer is a common tumour of younger men and although chemotherapy is effective, tumour-specific immune responses may be important. Previous work from our group demonstrated that patients have activated T cells in blood and functional responses to MAGE proteins.

I analysed the phenotype, checkpoint expression and function of T cells within blood from patients with seminoma or non-seminomatous tumours. No differences in T cell subsets were seen compared to healthy donors but CD27- B cells were increased and CD16 was reduced on $CD56^{Dim}$ NK cells. A distinctive pattern of immune checkpoint expression was observed with increased expression of Tim-3, LAG-3 and CTLA-4. This suggests some degree of T cell exhaustion although functional analysis showed broad cytokine responses after mitogenic stimulation. However, small numbers of T cells that were spontaneously producing IL-17, IL-21 or IL-10 were found, indicating baseline T cell stimulation.

In order to investigate novel approaches to identify and isolate MAGE-specific T cells I utilized cytokine secretion. This showed that TNF-α production was the most sensitive assay and, combined with CD107a, identified strong MAGE responses in two donors. Using a matrix of peptide pools I was able to isolate and define a potential novel epitope from MAGE-A4

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CHAPTER I. INTRODUCTION

General introduction of Cancer and Testicular Cancer

The Hallmarks of Cancer

The WHO predicts that cancer will become the leading cause of death globally within the next few years (de Martel et al., 2020). Cancer arises from the development of transformed cells that develop a range of phenotypic properties (Cooper, 2000). These have been characterised by Pecorino (2012) as disturbed pattern of growth, survival in limited serum and anchorage independence.

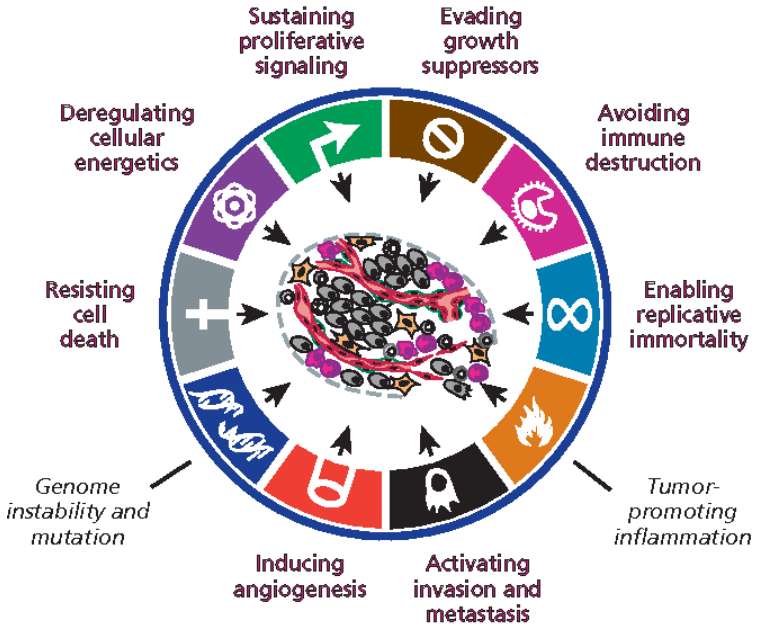

Fig. 1-1. The biological hallmarks of cancer.

Adapted from Hanahan and Weinberg, 2017, Cancer Medicine

In 2000, through fine analysis, Hanahan and Weinberg defined six hallmarks of most, if not all, cancers (Pecorino, 2012). They proposed that the essence of

carcinogenesis relied on the acquisition of several molecular characteristics. These were acquirement for growth signal autonomy, evasion of growth inhibition and cell death signals, unlimited replication, angiogenesis, and the properties of invasion and metastases (Hanahan & Weinberg, 2011). An update of these initial report was published in 2017 and included a range of additional properties such as deregulating cellular energetics and metabolism and avoiding immune destruction (Hanahan et al., 2000). Notably, this included the evasion of host immune defences. In this thesis I undertook an analysis of the immune response against testicular cancer, a disease for which the clinical outlook has improved dramatically in recent years and for which tumour-specific immune responses may play an important role.

Testicular Germ Cell Tumours (TGCT)

The incidence of testicular Cancer

Testicular germ cell tumours (TGCT) are a relatively infrequent cases representing approximately 1% of worldwide malignancies in males. The clinical outlook has improved greatly in recent years such that these now represent only 0.1% of cancer mortality (Khan & Protheroe, 2007). The highest incidence rates are observed in Northern European countries with 12.2 cases per 100,000 men. In contrast, Asian and African males are less affected with only less than 0.7 cases found in 100,000 men population. It seems that racial difference affects genetic susceptibility (Chia et al., 2010). Although small numbers of TGCT cases appeared to affect those elderly with ages around 80 years, this affected vast majority of those whose ages ranged from 25-35 years (Garner et al., 2005). Of note, despite the great clinical outlook,

the incidence of TGCT over time, especially in developed countries, and has doubled over the past 40 years (Oldenburg et al., 2013).

Testicular cancer has a very high cure rate with 5-year survival rate was reported to reach over 96% when treated at an early stage (Stage I) (Hayes-Lattin, 2009). Seminoma, in particular, was often reported to demonstrate the most excellent outlook with survival rate ranges from 97-100% when diagnosed at stage I (Albers et al., 2015).

Classification of Testicular Germ Cell Tumours (TGCT)

Germ cell tumours are a heterogenous group of neoplasms that develop primarily in the gonads. And to less frequent cases, they might also develop from specific extragonadal sites along the midline, the pineal gland, mediastinum, retropritoneum and sacrum (Reuter, 2005). The migration route of the primordial germ cells to the genital ridge is thought to be the main cause of such a particular distribution (Pereda et al., 2006). In males, about 98% of all testicular neoplasms are testicular germ cell tumours (TGCT) (Ghazarian et al., 2015).

It was not until 1946 that Friedmann and Moore proposed that TGCT can be classified into 4 categories namely seminoma (germinoma) and embryonal carcinomas, with the subgroups of choriocarcinoma, teratoma and teratocarcinoma (Mostofi, 1980; Pugh &Parkinson, 1981). Dixon and Moore (1952) refined this classification and divided TGCT into 5 groups as below (Hochstetter, 2002) :

1) Seminoma
2) Embryonal carcinoma
3) Teratoma
4) Teratoma with embryonal carcinoma, choriocarcinoma, and sarcoma
5) Choriocarcinoma

This classification was further simplified by the World Health Organization (WHO) and this is most often used these days. According to the WHO 2016 version TGCT are divided into three major groups as detailed below (Table 1-1):

Table 1-1. Main Histological Types of TGCT Based on WHO Classification

Noninvasive germ cell neoplasia
Germ cell neoplasia in situ (GCNIS; previously termed intratubular germ cell neoplasia unclassified, IGCNU)
Gonadoblastoma
Germ cell tumours derived from GCNIS
Seminoma
Nonseminoma (non seminomatous germ cell tumours)
Embryonal carcinoma
Teratoma
Yolk Sac
Choriocarcinoma and other trophoblastic tumours
Germ cell tumours unrelated to GCNIS
Childhood tumours
Teratoma (prepubertal type)
Yolk Sac tumour (prepubertal type)
Spermatocytic tumour (median of diagnosis is about 50 years of age)

Seminoma and nonseminoma are by far the most common histological subtypes of TGCT (Bahrami et al., 2007a) and were the subject of my thesis. The relative incidence and histological appearances of these two subtypes are discussed and contrasted below.

a) Seminoma

1. Classical seminoma

Classical seminoma affects males after their first decade of age and culminates between 35-45 years. This is the most common subtype of TGCT which contibutes to approximately 50% of TGCT cases (Meyts, et al., 2016) . Large proportion of seminoma is commonly found to constitute mixed TGCT. Less than 10% of patients showed extension pattern of seminoma to the spermatic cord or epididymis is observed and involvement and approximately 2% of cases involved both testes (Albers et al., 2011) .

2. Spermatocytic seminoma

Spermatocytic seminoma arises exclusively in the testis. It has no ovarian equivalent. This affects typically males in their 50-60 but younger patients with this subtype of TGCT were also observed (Pins, 2010). Unlike classical seminoma whose incidence contributes to around 50% of all TGCT cases, spermatocytic seminoma only account for 1-2% (Jha et al., 2018). The cell origin of spermatocytic seminoma appears to be more differentiated

compared to that of seminoma (Raiss et al., 2011), equipped with capability of spermatogenesis (Bostwick et al., 2006).

b) Teratoma

Teratoma tumours are histologically characterised by the presence of typically at least a germinal layer of endoderm, mesoderm and ectoderm. Those arise only from 1 of the 3 germ layers are termed as monodermal teratoma (L. Cheng et al., 2017). Pure teratoma mostly affects children while adults tend to be affected by mixed teratoma. While pure teratoma only accounts for 2-3% of all TGCT cases, teratoma-contained mixed TGCT represent almost 50% of the cases. It is interesting to note that these tumours in prepubertal patients are typically diploid with, often, chromosome 12p loss, whereas in postpubertal patients the tumours are aneuploid and contain isochromosome 12p (Rescorla, 2012).

c) Yolk sac carcinoma

Yolk sac carcinoma is the most common TGCT in pediatric population which incidence represent approximately 80% of prepubertal TGCT with age of onset is at 1.5 years in average and 17-40 years old in postpubertal (Bahrami et al., 2007b). While in prepubertal males this tumour is always seen as a pure yolk sac, in postpubertal this is admixed with other types of germ cell tumours. Yolk sac carcinoma differentiate from the embryonic yolk sac to allantois and end in the extraembryonic mesenchymal zone (Ulbright, 2005).

d) Choriocarcinoma

Choriocarcinoma often present more as clinical systemic symptoms rather than an abnormal terticular mass. This is an infrequent tumour although when it is admixed with other subsets of testicular tumours its incidence rises up to 10% of all TGCT cases. Like majority types of TGCT, choriocarcinoma tends to affect younger male population (Bahrami et al., 2007b).

e) Embryonal carcinoma, with or without teratomatous elements

Embryonal carcinoma is a relatively common testicular germ cell tumour after puberty (Lanzkowsky, 2011). 10% are pure embryonal tumours, and a substantial number of tumours will have a mixed embryonal component (Dicken & Billmire, 2012).

The testis as an immune privileged site

The concept of immune privilege is thought to reflect the observation that some organs such as brain and retina (Forrester & Xu, 2012) require protection from the immune surveillance to suppress immune-mediated tissue damage. In addition to brain and retina as immune privilege sites, the testis is also believed to be one of such site. An additional reason in support of this was the observation that no lymphatic drainage was found within the tissue. However, this thought was subsequently challenged by the discovery of afferent lymphatic vessels. To prevent immune cells from such entering through these afferent lymphatic vessels the testis

is anatomically surrounded by a specific zone called the blood-testis barrier (BTB) (Cheng & Mruk, 2012). The BTB lies between adjacent Sertoli cells in the seminiferous tubules. Furthermore, this compartmentalizes the tubules into an adluminal area, where meiosis, spermiogenesis and spermiation may take place, and basal compartments –where spermatogonial cell division followed by differentiation to preleptotene spermatocytes occur (Jiang et al., 2014). The BTB hence forms an immunological barrier that protects meiotic and postmeiotic cells from circulating blood and external insults (Kaur et al., 2014).

The BTB is formed as a response toward gonadotropic stimulation and the presence of zygotene-pachytene primary spermatocytes in the pubertal period. This formation occurs during the process of spermatogenesis. The BTB is made up of cellular junctions such as adhesion junctions, tight junctions (TJs) and gap junctions (GJs). Interestingly, in men in whom the junctional proteins are impaired due to an inherited dysfunction the immune responses against meiotic and postmeiotic cells can ensue and this causes spermatogenetic failure and infertility (de Kretser et al., 2015).

Cancer Antigens

Proteins that act as antigens for tumour responses in T-cell immunity can be divided into four major groups, based on their expression profile.

1) Differentiation antigens

These proteins are expressed in tumour cells and also in those normal cells from which the tumour develops (Vigneron, 2015). For example, the term ‘melanoma

differentiation antigens' (MDAs) defines proteins that are present in melanoma as well as melanocytes, from which this tumour develops (Davis et al., 2019). Of interest, many MDA proteins work to mediate melanin production and this is presumably a reflection of the most characteristic feature of this cell. MDA protein, such as gp100 ($gp100_{209–217}$) or tyrosinase ($tyr_{369-377}$), often contain peptide epitopes recognized by T-cells (Overwijk & Restifo, 2000).

2) Cancer/testis antigens

The term Cancer/testis antigens (CTAg) describes a family of proteins whose role in tumour immunity reflects their pattern of expression (Yao et al., 2014). CTAg expression is observed in many different subtypes of malignant tumour but these proteins are largely not expressed in healthy somatic cells, except in testis and placenta (Fratta et al., 2011). Due to the immune privileged nature of germ cells it seems that the immune response does not gain access to sites of CTAg expression. This restricted pattern of expression, as well as the potential to reinforce immune responses when on somatic tissue, renders CTAg an ideal target for tumour immunotherapy (Song et al., 2012).

CTAgs are expressed in many subtypes of tumours and serological responses against the antigens have been identified. Results from screening of patients' sera suggests that they are highly antigenic. So far, More than 200 CTAgs have been molecularly charactized, including MAGE, NY-ESO-1, GAGE, AKAP3, SSX, and LAGE (Song, et al., 2016).

3) Overexpressed antigens

These proteins are broadly expressed in the normal tisues as well as in tumours. However, their expressions in tumours are greatly elevated compared to that of normal tissues. They, hence, are capable of inducing immunological responses. Antigens such as sperm protein 17 (sp17) (Schutt et al., 2017), preferentially expressed antigen of melanoma (PRAME) (Hermes et al., 2016), squamous antigen-rejecting tumour (SART-3), and p15 , which are greatly expressed in melanoma and various types of tumours are just few examples of overexpressed antigens that have been well-characterized (Gjerstorff et al., 2015).

4) Tumour-specific antigens

Cancer arises due to acquisition of somatic mutations (Stratton et al., 2009). These mutations lead to coding changes in expressed proteins. When a novel peptide derived from these changed proteins coupled then presented by HLA molecules then these are termed neoantigens (Jiang et al., 2019). In melanoma, neoantigens such as β-catenin is described as a result of either point mutation or translocation-based gene fusions like that of the low-density lipid receptor with GDP L-fucose (Stratton et al., 2009) .

Expression pattern and Biological function of Cancer/Testis Antigens

The present study focused on the immune response to Cancer/Testis Antigens (CTAgs). As alluded to above, these antigens are tumour proteins whose expression

is highly restricted to germ cells and cancer cells. As such, they represent a valid protein target for immune therapy (Whitehurst, 2014).

A classification of cancer has been made, based on the frequency of CTAg expression (Krishnadas et al., 2013) :

1) ***CTAg-rich tumours*** which melanoma and ovarian cancer .
2) ***CTAg-intermediate*** including breast cancer, bladder cancer, and prostate cancer
3) ***CTAg-poor*** including colorectal cancer, and lymphoma/leukemia

Unlike most auto-antigens, CTAgs are highly immunogenic (Caballero & Chen, 2009). This has been widely attributed to the immune-privileged properties of the testis that arise from the fact that Sertoli cells restrict immune cell access into functional spermatozoa and seminal fluid (Kaur et al., 2014). At a physical level this is mediated by the BTB, whilst the molecular basis includes secretion of activin A, granzyme B, FAS ligands and transforming growth factor (TGF-β) (Meinhardt & Hedger, 2011).

Immunological targeting of CTAgs would be expected to be minimally toxic to somatic tissue. Indeed, since CTAgs are normally expressed solely in immune privileged testicular tissue where human leukocyte antigen (HLA) class I molecules expression is also lacking, the immune response is not stimulated. In addition, the presence of the BTB in the testis may help them to stay protected from the exposure to the immune recognition mechanism (Mital et al., 2011).

Despite the fact that the number of studies regarding the expression of CTAgs in the thymus is relatively small, CTAgs-specific T cells appear to have the potential to underpin the robust nature of immune responses against CTAgs in cancer patients. The biological function of the CTAg proteins in tumour development remains poorly understood (Fratta et al., 2011). It is most probably that they play an essential role in cellular transformation and p53 function inhibition or chromatin organisation (Marcar, et al., 2010). MAGE A3 has been reported to play a role in cell cycle regulation. In addition its expression has been associated with impaired treatment outcomes following taxane-based chemotherapy for gastric cancer (Xie et al., 2016).

In addition to the MAGE family, GAGE-7 has also been shown to have a role in cellular transformation (Fratta et al., 2011). GAGE-7C expression was thought to be causing cell resistance to FAS-mediated apoptosis (Caballero & Chen, 2009).

Of note, multiple CTAgs may be expressed in a single tumours at various magnitudes. MAGE A-1, MAGE A-3, NY-ESO-1, SSX-2 and SSX-4 appear to be more frequently expressed compared to BAGE, GAGE-A1 and SCP-1. Genes within a single homologous family also show this pattern and this is characteristically seen for members of the SSX family (Scanlan et al., 2002).

In the previous study from our laboratory, different MAGE-A proteins were seen to be able to stimulate T cells responses in patients with TGCT. Seminoma demonstrated the highest frequency of T cell responses against MAGE-A1 antigen-whereas mixed germ cell tumours (mGCTs) directed their responses at the highest

frequency toward MAGE-A3 and MAGE-A4 antigens (Pearce et al., 2017). These findings led me to focus my research on immune responses to the MAGE A-1/3/4 family of proteins.

CTAg antigens as targets for Cancer Immunotherapy

Fundamental research into the mechanisms of cancer immunology has improved markedly in recent years and has driven improvements in immunotherapy (Yang, 2015). Although conventional therapies, such as chemotherapy and radiotherapy, are the most common initial approach these modalities alone are not sufficient for many patients (Marshall & Djamgoz, 2018). This is particularly true for disease that has relapsed or undergone metastasis. Cancer immunotherapy offers a range of novel opportunities for cancer treatment has holds the potential to provide systemic and long lasting disease control (D'Errico et al., 2017).

Immunotherapy may be delivered in several different forms. Adoptive transfer of autologous cells that have been expanded in the laboratory has been utilised for many years and shown reasonable efficacy in disorders such as melanoma (Wu et al., 2012). The success of allogeneic stem cell transplantation for mediating graft versus leukaemia is also an example of immunological response against tumours. (Dickinson et al., 2017)

Cancer immunotherapy employs the specificity and the strength of the immune system to treat cancer. With a general aim to develop long-lasting tumour-specific immunologic "memory" in patients, it allows the immune rejection over the tumour growth or re-growth to be a lifelong protection system (Locy et al., 2018). The

molecular determination of the MAGE antigens by Boon and colleagues more than two decades ago was a major step forward in peptide-specific therapy (Boon et al., 1997). Nevertheless, adoptive therapy with antigen-specific T cells is technically demanding and carries substantial financial cost (June, 2007). As such, vaccination approaches are also under investigation and DNA and peptide-based approaches have been widely used (Butler & Hirano, 2014).

In order to obtain a comprehensive information about immunogenicity of cancer cells *in vivo*, peptide-specific T cells has been isolated from cancer patients. For instance, following *in vitro* sitmulation with the peptides, Melan-A specific T cells were demonstrable in 87% of melanoma patients compared to 56% of healthy donors and a higher frequency was also seen in the patient group (Parmiani et al., 2002).

In my work I aimed to identify, and potentially isolate, cancer testis antigen-specific T cells, especially those from the MAGE family of proteins, from the blood of men with seminoma. The vaccination approach relies on the knowledge of relevant cancer-associated epitopes and as such this would provide new reagents for immunotherapy.

So far, a range of either HLA-class I-restricted or HLA-class II-restricted CTAgs have been identified. However, immune responses are often weak in cancer patients. In the past decade a total of 44 clinical trials using MAGE-A have been undertaken (https://clinicaltrials.gov); 16 in phase I; 13 in phase I/II; 13 in phase II and 2 in phase III. Many of these studies use adjuvants in order to boost immune responses. More recently, T-cell receptor (TCR) transduced T-cells and expanded cytotoxic T lymphocytes (CTLs) have been exploited in six and two trials, respectively (Zajac et

al., 2017). This level of interest shows that MAGE proteins are considered to highly offer potential as a cancer-specific antigen for immunotherapy. This supports my thesis aims to characterise MAGE-A-specific T cells from the blood and tumour-infiltrating lymphocytes (TILs) of patients with testicular cancer.

The Interaction of Cancer and Immune system: The Concept of Immune surveillance Against Tumours

The concept that the immune system is capable of recognising and eliminating tumours in the absence of therapeutic intervention has existed for almost a century (Pardoll, 2015). Despite this, its validity has been hard to establish. As so little was known regarding the nature of cellular and molecular immune recognition of cancer, the concept was difficult to test experimentally. However, as the field of immunology has developed, this concept, often termed *cancer immunosurveillance*, has gradually been acquired (Ribatti, 2015).

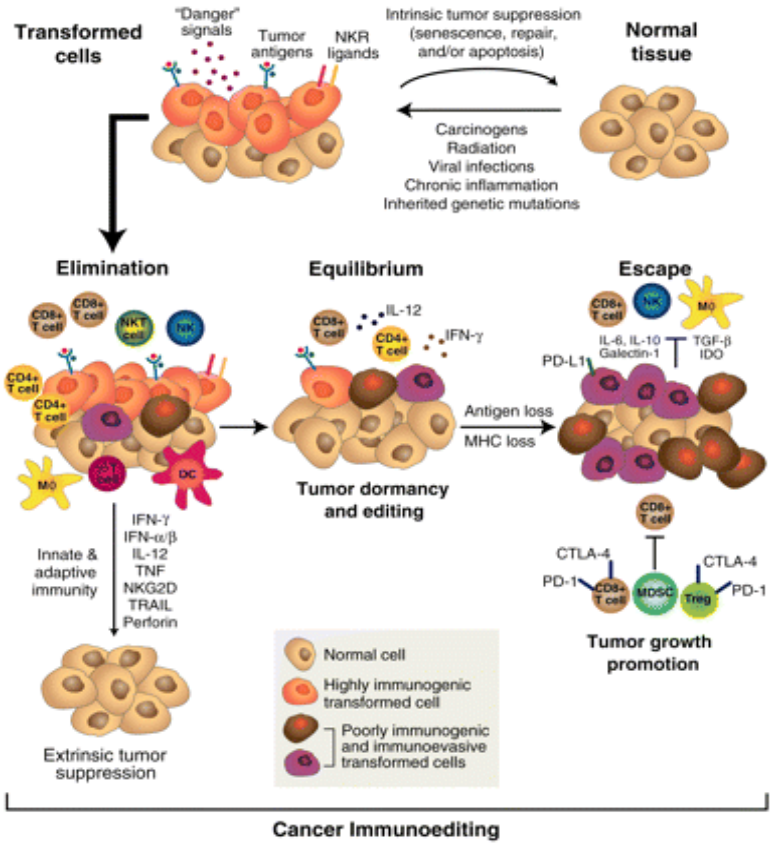

Fig. 1-2. The 3-Es in Cancer Immunoediting (Teng et al., 2013)

Dunn and Schreiber (Fig. 1-2) developed the concept of “cancer immunoediting” which encompassed three phases (Dunn et al., 2002):

1. The Elimination phase where tumour cells are killed by NK, CD4+ and CD8+ cells
2. The Equilibrium phase which corresponds to a balanced state between immune and tumour cells. Only when the immune system fails to combat the tumour cells can the third phase be reached
3. The escape phase that concludes with the clinically detected tumours.

Despite the potential utility of tumour immunosurveillance, it is clear that a huge number of tumours still develop in the presence of an apparently functional immune system (Swann & Smyth, 2007).

In mice evidence for immunosurveillance has been derived from studies of specific gene deleted strains. However in humans there are still those that question the importance of the mechanism. Patients with immunodeficiency or acquired immunosuppression do display an excess of cancer but these are often related to viral infection and a substantial increase in the common subsets of epithelial tumours has been difficult to demonstrate (Dunn et al., 2002).

The importance of immune surveillance can be applied to the study of seminomas. Tumour-infiltrating lymphocytes (TIL) are found abundantly in seminomas and are believed to be of prognostic significance (Hadrup et al., 2006). Indeed, it has been suggested that lymphocytes are able to recognize tumour-specific peptides presented by MHC class I on cancer cells (Comber & Philip, 2014). A study of

tumour-infiltrating lymphocytes in seminoma was performed by Hadrup and colleagues in 2006 and indicated that specific functional T-cell responses were operative which further suggested that the inflammatory infiltrate was indeed involved in the immunological control of the tumour (Hadrup et al., 2006).

Introduction to major cells of the Immune system

NK Cells

Although initially NK cells were recognized as effector lymphocytes encompassing cytolytic functions in the innate immunity, they are now defined as a population of cells with a wide repertoire of activating- and inhibiting-harboring receptors that are well-calibrated to make sure that, while the cells are active against viral infections as well as tumour development, they are tolerant of their host's healthy cells (Boudreau & Hsu, 2018; Orr & Lanier, 2010). Some recent studies revealed that NK cells were capable of mounting a form of antigen-specific memory. They, thus, exert sophisticated function attributable to both innate and adaptive immunity (Vivier, et al., 2011).

In human NK cells are characterized as $CD3^{-}CD56^{+}$ cells. They are present in many peripheral tissues, most notably liver and lung, and represent around 10% of peripheral blood mononuclear cells (PBMCs) (Lee et al., 2017). They develop from a common lymphoid progenitor in the bone marrow and differentiation progresses via several stages (Bozzano et al., 2017). A key feature of NK cells, that discriminates them from T cells, is that they do not express the RAG protein and

therefore cannot mediate somatic recombination of their antigen receptors (Paust et al., 2010). As such, all of their interactions have to be mediated by proteins that are encoded by germline-encoded molecules (Orr & Lanier, 2010).

T- and B-Lymphocytes

T and B lymphocytes are small cells (8–10 microns in diameter) and each has a large nucleus with dense hetero-chromatin. Both, therefore, are morphologically indistinguishable. When they become activated, once they encounter antigenic stimuli, they may enlarge their sizes to accommodate their increasing cytoplasm and organelle numbers (Cano & Lopera, 2013). T and B lymphocytes present TCR and BCR, respectively, on their surfaces for antigen recognition in different specificities. These receptors are encoded from genes that undergo DNA recombination that allows the generation of a considerable amount of diversity in their repertoire (Nemazee, 2000).

Both B and T lymphocytes develop from bone marrow-derived cells. However, while B lymphocytes stay in the site for a further process--gene rearrangement, by which B cell repertoire can be generated, T lymphocytes migrate to the thymus where they undergo maturation (Zhao et al., 2012). The earliest thymic progenitor cells are phenotypically characterized as $CD4^{low}CD8^{-}CD3^{-}$ cells which pass through some stages of maturation through which they become $CD3^{-}CD4^{-}CD8^{-}$ and $CD3^{low}CD4^{+}CD8^{+}$ cells. They eventually differentiate into a fully mature T cells

phenotypically characterized as $CD3^{high}CD4^{+}CD8^{-}$ or $CD3^{high}CD4^{-}CD8^{+}$ T lymphocytes (Roifman & Grunebaum, 2013). There is also a subset called γδ -T lymphocyte that is generated early in this process (Cano & Lopera, 2013).

As alluded previously, B cell repertoire, as a result of gene rearrangement processes, generate a myriad diversity of immunoglobulin (Ig) genes. This leads to the creation of a huge population of B cells with a broad range of specificities for different antigens. Cells with certain specificity of antibody anti-self require inactivation or removal via receptor editing mechanisms (Martin et al., 2016).

γδ- T cells

Differ to conventional T cells that harbor α and β chains on their surface, a subgroup of them carry distinct T cell receptors termed γ and δ chains. They are defined as γδ T cells and initially described in 1987. They account for around 0.5%-5% of the total T lymphocyte repertoire. Despite their circulating number, which is much less than αβ-T cells, γδ-T cells are common as intra-epithelial lymphocytes within the gut (Zhao et al., 2018).

γδ-T cells, unlike αβ-T cells, recognize antigens in a non-MHC restricted manner. They also capable of secreting cytokines abundantly following the antigen recognition (Raverdeau et al., 2019). Attentions toward their potential contributions in tumour immunity increase. Moreover, their promising roles in the clinical trials are now being applied for adoptive transfers into a broad types of cancers (Nussbaumer

& Koslowski, 2019). In general these studies have demonstrated that γδ-T cell infusion to be well tolerated. However, their efficacy remains poorly studied (Zhao et al., 2018).

MAIT Cells

As a subset of innate-like T lymphocytes, mucosa-associated invariant T (MAIT) recognize MHC-related protein 1 (MR1)-restricted stimuli (Xiao & Cai, 2017). Their recognition pattern is somewhat uncertain but appears to be directed primarily towards bacterial antigen. These populations are often localized within mucosal surfaces (Sundström et al., 2019) and may also be localized within tumour tissue (Ling et al., 2016). The cells decrease in frequency with age (van der Geest et al., 2018) They are able to mediate both cytotoxic and cytokine responses but their potential role in the control or support of malignant transformation is currently unclear and needs furher investigation (Garner et al., 2018).

Introduction of Antigen Processing and Presentation

In order to be recognized by T cells proteins must be 'presented' to the immune system by antigen presenting cells (APC). Generally there are two major pathways by which antigens (Ags) may gain access to presentation and so initiate an adaptive immune system, namely the 1) exogenous (endocytic) pathway and 2) endogenous (cytosolic) pathways (Blum et al., 2013).

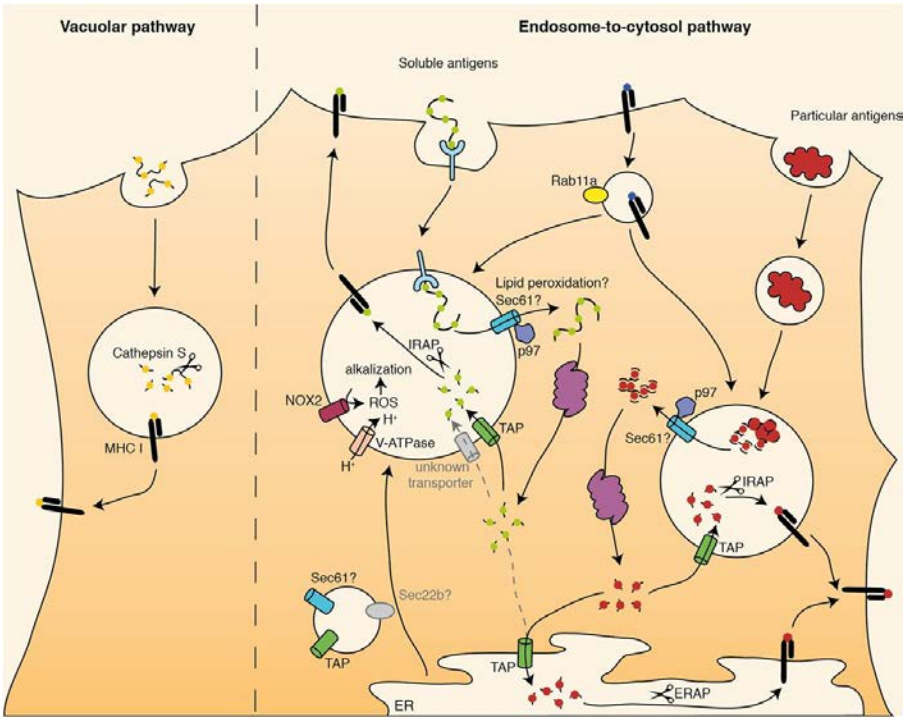

Fig. 1-3. Cross presentation pathways pathways (Embgenbroich & Burgdorf, 2018).

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1) Exogenous (endocytic) pathway

In the exogenous pathway of presentation, antigens that originated external to APC are phagocytosed or pinocytosed prior to endosomal degradation by resident proteases (Mantegazza et al., 2013). Successive compartments display decreasing levels of pH, ranging from 6.0-6.5 at early endosomal processing to 4.5-5.0 in lysosomes. Proteins are fragmented into peptides of 13-18 amino acids in length and presented efficiently on HLA class II proteins to CD4+ T cells (Blum et al., 2013).

2) Endogenous (cytosolic) pathway

Endogenous pathways is initiated when the antigen entering the cytosol is tagged by a tiny protein called ubiquitin (Cruz et al., 2017). This tagging leads the processing of the intact proteins to yield peptides that typically 8-13 amino acids long. The event occurs within the proteasome. The peptides are then transported to the endoplasmic reticulum (ER) through ATP-dependent proteins termed TAP1 and TAP2. Prior to binding to the peptides and the TAPs, the MHC class I molecules, calnexin and β2m form a macromolecular complex in the ER (Hewitt, 2003). Finally, MHC class I molecules loaded with peptide are transported to the cell surface (Blum et al., 2013).

In addition to antigen processing mechanism elaborated above, *Cross Presentation* is a term describing an alternative pathway which is considered to be pivotal in tumour immunity. This pathway permits the presentation of exogenous proteins on HLA class I molecules, a process crucial for the generation of effector CD8+ T cell responses (Fehres et al., 2014). A proposed mechanism for cross presentation includes the exchange of exogenous peptides within an endosomal compartment which already loaded onto HLA-class I molecules in the Endoplasmic Reticulum (ER). However, the mechanism allowing transfer of proteins into the cytosol, or the site at which peptides are loaded onto class I MHC molecules require further investigations (McDonnell et al., 2010). Cross presentation can only takes place within specialised subsets of dendritic cells termed conventional DC (cDC) (Joffre et al., 2012). Therefore, in the tumour antigen vaccination strategy, DCs are seen to be significant as a target (Robson et al., 2010).

Major Histocompatibility Complex (MHC)/Human Leukocyte Antigen (HLA)

As indicated above, for immune recognition by T cells peptides must be presented on the cell surface by the MHC (mice) or HLA (human) complex. The HLA complex is located on chromosome 6 and contains many genes associated with immune regulation. In relation to this thesis, the most notable are the three HLA Class I genes (HLA-A, B and C) which present peptides to CD8+ T cells, and the three HLA Class II genes (HLA-DR, DQ and DP) which present peptides to CD4+ cells. The HLA complex is highly polymorphic and this is thought to have arisen from evolutionary selection for protection against infectious disease.

HLA Class I (HLA-I) antigens are integral membrane glycoproteins that are present on virtually all cells. These molecules bind degraded endogenous proteins and present them to T cell receptor (TCR) on CD8 T cells (Cruz-Tapias et al., 2013). It is important to note that many human tumours express greatly reduced levels of HLA protein at the cell surface. This may be apparent in the expression of individual alleles or in the global expression of HLA class I or class II at the cell surface. These observations have led to speculation that progression of malignant disease could result from the lack/loss of recognition by CTL, allowing tumour to escape immune surveillance (Cruz-Tapias et al., 2013..

Identification of tumour-specific T cell responses *in vitro* using overlapping peptides stimulation

The most commonly used approach to study antigen-specific T cells responses *in vitro* is through the application of *ex vivo* stimulation with relevant protein or a panel of overlapping synthetic peptides (Jiang et al., 2006). The former approach offers an advantage as it does not require *a priori* knowledge regarding the specific immunodominant epitopes in the immunogen. Using this approach, detection of antigen-specific T cell responses irrespective of HLA types is also possible (Zandvliet et al., 2010). On the other hand, since the whole protein needs to be internalized and processed prior to presentation on HLA molecules, this technique ideally requires fresh specimens and is much more effective for analysis of CD4+ responses. The advantage of peptide screening is that it may be pursued using cryopreserved samples and is very effective at eliciting CD8+ T cell responses (Jiang et al., 2006).

The use of overlapping synthetic peptides (OSP) appears to be overcoming the drawback in the use of whole proteins. They have been widely employed to bypass the constraints in the requirement for degradation of intact exogenous protein (Maecker et al., 2001). OSP is a sensitive approach and has been broadly applied to elicit robust peptide-specific responses against immunodominant and subdominant epitopes (Jiang et al., 2006).

An adaptation of the OSP application is the use of overlapping long peptides that have been demonstrated to enable detection of weak peptide-specific responses,

such as against E6 and E7 from HPV. These peptides are, indeed, particularly effective as immunogens. They are, therefore, capable of inducing an immune responses in rabbits as indicated by a reduction in papillomavirus-induced lesions as well as suppressing the number of sites following a latent cottontail rabbit papilloma virus (CRPV) infection (Vambutas et al., 2005).

Within my thesis I focussed on the use of overlapping peptides, and specific defined single epitope peptides, to assess the immune response against cancer testis antigens. This approach was taken as no MAGE proteins were available for study and the OLP approach offers potentially greater sensitivity and ease of use. Moreover, this allowed me to assess the response of both CD4+ and CD8+ T cells, simultaneously.

Cytokine release as a readout for T cell functionality

Intracellular cytokine analysis is commonly used as a method for detection of antigen-specific T cells and NK cell responses (Smith et al., 2015). IFN-γ, TNF-α, IL-2 and GM-CSF are the common cytokines detected in these assay.

Acting as a predominant pro-inflammatory cyokine, IFN-γ is produced by Th1 cells and mature NK cells. Indeed, NK cells harbor epigenetic marks that mediate chromatin opening at the *IFNG* locus and can facilitate rapid cytokine production as required (Stetson et al., 2003; Mah & Cooper, 2016). NK cell activation is initiated by immunoreceptor tyrosine-based activating receptor motifs (ITAMs) that phosphorylate Src family tyrosine kinases, with subsequent activation of MAPK.

Ultimately transcriptional activity is altered through influence on transcription factors such as Fos and Jun (Schoenborn & Wilson, 2007).

The cytokine production by previously differentiated Th1 CD4+ and CTLs cells can also mediated by cytokines such as IL-12 and IL-18 (Nakanishi, 2018). Specifically to the Th1 CD4+ T cells, the cells do not only produce IFN-γ but are also induced to do so in part by IFN-γ itself (Yang et al., 1999). It was reported that, *in vitro*, treatment of Th1-differentiated CD4+ T cells with the combination of IL-12 and IL-18 capable of eliciting IFN-γ even without prior TCR activation by antigen or antibodies against CD3. This suggested that cytokine-mediated T cell activation can occur without engagement of the T cell receptor (Munk et al., 2011).

In the context of tumour, IFN-γ is seen as the main cytokine which functions most effectively to thwart the tumour growth. It, henceforward, is categorized as a pro-inflammatory cytokine. Once the cytokine is secreted, it signals antigen-presenting cells (APCs) activation (Ivashkiv, 2018). Activated APCs, in turn, upregulate the expressions of co-stimulatory molecule CD86, IL-12 and IL18, that together promote Th1 differentiation. As well as activating effector cells, IFN-γ can further promote inflammation by suppressing the activity of T regulatory cells and other cells within the myeloid lineage (Kammertoens et al., 2017; Deligne et al., 2015). This will lead to the tumour destruction. It is now clear that indeed the presence of IFN-γ triggers a variety of signals by which T cells can function effectively whereas when IFN-γ signalling pathway is dampened the T cells' function is diminish. This allows tumour growth into a persistence level (Ni & Lu, 2018).

In addition to IFN-γ, TNF-α is an additional strong pro-inflammatory cytokine that mediates its activity through the TNFR-1 and TNFR-2 receptors. TNFR-1 is expressed on all cell types, and possesses a death domain (DD), whereas TNFR-2 is expressed mainly confined on immune cells and interesting has a much higher affinity for TNF (Yang et al., 2018). The DD mediates some of the physiological outcomes from which TNFR-1 and TNFR-2 can be distinguished. Downstream signaling via TNFR engages a range of intracellular receptors including SODD, TRADD, RIP and FADD (van Horssen, 2006).

IL-2 (interleukin-2) is a key cytokine in T cell development, activation and regulation (Ross & Cantrell, 2018). It is made by T cells on activation and human cells also then express the IL-2 receptor (CD25) such that it can mediate a paracrine effect. Three IL-2 receptor subunits have been identified (IL-2Rα, IL-2Rβ and IL-2Rγc) and together these subunits form an trimeric complex called IL-2Rαβγ with a high affinity (Malek & Castro, 2010). Interestingly, a transduction signal can also be trigerred when IL-2 binds the IL-2Rβγ receptor which occurs at an intermediate affinity (K_d- 10^{-9} M) whilst a lower affinity of K_d- 10^{-8} M is observed when IL-2 binds IL-2Rα but this does not generate transduction signals (Jiang et al., 2016). The Janus family tyrosine kinase members JAK1 and JAK3 are recruited to the cytoplasmic domains of the IL-2R molecules and this leads to phosphorylation of the STAT family, most notably 1, 3 and 5. Phosphoinositide 3-kinase and MAPK-signalling pathways are also initiated (Jiang et al., 2016).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was initially recognized in mice through its capability of stimulating proliferation of bone marrow cells *in vitro* with specific expansion of granulocyte and macrophages colonies (Burgess et al., 1976). GM-CSF shows a heavy glycosylation pattern cytokine and stimulates progenitor cells in a concentration-dependent fashion (Ganguly et al., 2007).

Cytotoxicity assay

Another important T cells function is cytotoxic degranulation. Cytotoxic T lymphocytes (CTLs) kill virally-infected or transformed cells through exocytosis of secretory granules or engagement of death receptors on the surface of the target cell (Trapani & Smyth, 2002). Cytotoxic granules carry diverse perforin and granzymes. The activation of perforin is initiated by the polimerization of perforin monomers on phospholipid membranes in a calcium-dependent manner (Voskoboinik et al., 2005) to form a pore that allow entry of granzymes (Janeway, et al., 2001) . Granzymes are serine proteases capable of inducing apoptosis in the target cell. Blockade of perforin function causes remarkably weakened cellular cytotoxicity. The sequential elimination of several target cells ('serial killing') is a feature of CTLs (Trapani & Smyth, 2002).

The expression of CD107a (also known as Lysosome-associated membrane proteins-1/ LAMP-1) is seen on the cell membrane during cytotoxicity (Krzewski et al., 2013). This has been widely exploited by scientists as a sensitive assay to

assess cytotoxic activity (Betts et al., 2003) and I added CD107a into our flow cytometry assay to evaluate the magnification of degranulation occurred following antigen stimulation.

Aims for my Thesis

Given (1) the defined importance of cancer testis antigens in the immunotherapy of cancer, (2) their expression in testicular cancer and (3) the excellent clinical outcomes for this condition with modern therapy, I was interested to assess the immune response to cancer testis antigens in men with this tumour. This was the primary aim of my thesis.

The objectives of my work were to:

- Study the profile of T cell subsets in patients with testicular cancer
- Assess the range of immune checkpoint expression on T cells in patients with testicular cancer
- Determine if the pattern of cytokine production was modulated in this disease
- Interrogate the immune response to CTAG proteins and attempt to identify new peptide epitopes

CHAPTER II. MATERIALS AND METHODS

Blood samples from Testicular Germ Cell Tumour (TGCT) patients and healthy controls

Up to 36 ml of heparinised whole blood and 6 ml of clotted blood were obtained from TGCT patients (n=57) attending routine appointments at the cancer outpatient clinic at the Queen Elizabeth Hospital, Birmingham. The bloods were collected prior to chemotherapy treatment. Written informed consent and local ethical committee approval (South Birmingham research ethics committee LREC reference 09/H1207/161) were obtained prior to sample collection. Patients recruited in the study were previously confirmed to be HIV-, HBV-, and HCV- free with ages were 18 years old or over and competent to provide full written, informed consent. The ages of recruited patients ranged from 20-69 years with an average age of 41 years (± SD 12.75). The majority were at localized stages (I-II) where distant metastases were not observed. Follow-up blood samples were collected where possible. A summary of patient and histopathological subtype of tumour is given in Table 3-1. Heparinised peripheral blood samples from healthy donors (n=16) were used as controls.

Cell culture media and Buffer recipes

Growth Media (GM) / LCL media

RPMI 1640, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2 mM Glutamine, 10% Foetal Calf Serum

Wash buffer

RPMI 1640, 100 U/ml Penicillin, 100 µg/ml Streptomycin

Freezing Media (FM)

90% Foetal Calf Serum, 10% DMSO

MACS buffer

1 x PBS, 2% FCS, 2mM EDTA

CSA media

RPMI-1640, 10% FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 1µg/ml Cyclosporin A

PBS-T

1 x PBS, 0.05% Tween-20

PBMC Isolation from Peripheral Whole Blood

PBMCs were isolated from heparinised whole blood under sterile conditions by density gradient centrifugation. Whole blood was diluted in wash media at a ratio 1:1. The diluted blood was layered at a ratio of 2:1 over Lymphoprep and centrifuged at 790xg at room temperature for 25 minutes (brake off). The lymphocyte layer was carefully removed with a transfer pipette into fresh RPMI media and centrifuged at 400xg for 10 minutes at room temperature (brake on). The pellet was resuspended in fresh GM media, an aliquot removed for counting and the cells centrifuged at 350xg for 5 minutes at room temperature (brake on). Cells were either used fresh or cryopreserved in Freezing Media for future use.

Flow cytometry - Checkpoint panel

First step was to stain the dead cells with 500 µl ef-506-Fixable Viability Dye at 4ºC for 30 minutes. The PBMCs were washed once with cold MACS buffer and centrifuged at 400xg for 5 minutes. Cell pellets were resuspended in residual

volume, stained with 5ul FcR block for 10 minutes on ice followed by surface staining with antibody cocktail (Table 2-1) for 30 minutes on ice. Cells were washed twice with 3 ml MACS buffer and centrifugated at 400xg for 5 minutes. Supernatant was discarded and cell pellet was resuspended with 200 µl for flow cytometric analysis.

Table 2-1. Checkpoint Panel antibody details

Antibody anti human-	Fluorochrome	Clone	Manufacturer	Volume/ reaction (µl)
CD45	Alexa Fluor ®700	2D1	Biolegend	2.5
CD4	APC/Fire™750	RPA-T4	Biolegend	3.5
CD8	PerCP/Cy5.5	SK1	Biolegend	3
CD3	FITC	UCHT1	Biolegend	5
PD-1 (CD279)	Brilliant Violet 421™	EH12.2H7	Biolegend	4
Tim-3 (CD366)	PE	F38-2E2	Biolegend	5
CD223 (LAG3)	PE/Cy7	11C3C65	Biolegend	5
CD152 (CTLA-4)	PE/Dazzle™594	BNI3/L3D10	Biolegend	5
TIGIT (VSTM3)	APC	A1513G	Biolegend	5
Fc Blocking Solution			Biolegend	5
Total volume				43

Flow cytometry – Immune cell phenotyping panel

First step was to stain the dead cells with 500 µl ef-506-Fixable Viability Dye at 4ºC for 30 minutes. The PBMCs were washed once with cold MACS buffer and centrifuged at 400xg for 5 minutes. Cell pellets were resuspended in residual volume, stained with 5ul FcR block for 10 minutes on ice followed by surface staining with antibody cocktail (Table 2-2) for 30 minutes on ice. Cells were washed twice with 3 ml MACS buffer and centrifugated at 400xg for 5 minutes. Supernatant was discarded and cell pellet was resuspended with 200 µl for flow cytometric analysis.

Flow cytometry was done on a BD LSR II machine and analysed using Kaluza software ®.

Table 2-2. Immune Cell Population antibody panel

Antibody anti human-	Fluorochrome	Clone	Manufacturer	Volume/ reaction (µl)
CD45	Alexa Fluor ®700	2D1	Biolegend	2.5
CD3	FITC	UCHT1	Biolegend	5
TCR Vα7.2	PE	3C10	Biolegend	4
CD16	PerCP/Cy5.5	3G8	Biolegend	5
CD56 (NCAM)	PE/Cy7	5.1H11	Biolegend	5
CD27	Brilliant Violet 421™	O323	Biolegend	4
CD161	APC/Fire™750	HP-3G10	Biolegend	5
CD19	PE/Dazzle™594		Biolegend	5
γδ TR	APC	B1	Biolegend	5
Fc Blocking Solution			Biolegend	5
Total volume				45.5

PMA/Ionomycin stimulation of PBMC from healthy donors and testicular cancer patients

PBMCs were resuspended at $1x10^6$ cells/ml in ImmunoCult (ImmunoCult-XF T Cell Expansion Medium, Stem Cell Technologies) media and either stimulated with 1x cell stimulation cocktail (40.5 µM Phorbol 12-Myristate 13-Acetate (PMA), 670 µM Ionomycin; eBiosciences) or left un-stimulated. Cells were treated with 1x protein transport inhibitor cocktail (eBiosciences) and incubated at 37°C, 5% CO_2 for 4 hours before being washed in MACS buffer. Cells were stained with ef506 Fixable Viability Dye (1:1000, eBioscience) and washed in MACS buffer. Cells were resuspended in the residual volume and stained with surface antibodies listed in Table 2-3a in the presence of 5ul FcR block (Biolegend) for 30 minutes on ice, protected from light. Cells were washed in MACS buffer, resuspended in residual volume and fixed in 4%

paraformaldehyde (Biolegend) for 30-45 minutes at room temperature, protected from light. Cells were centrifuged in 1x permeabilization buffer (Biolegend) at 760xg for 5 minutes and the cells resuspended in 100µl 1x permeabilization buffer. Cells were incubated with the antibodies listed in Table 2-3b for 45 minutes at room temperature, protected from light. Peptides were titrated prior to use. Cells were washed in 1x permeabilization buffer and resuspended in a suitable volume of MACS buffer for analysis by flow-cytometry.

Table 2-3. T-cell functionality flow panel antibodies

Table 2-3a. T cell functionality flow panel antibodies – Surface staining

Antibody anti human-	Fluorochrome	Clone	Manufacturer	Volume/ reaction (µl)
CD45	Alexa Fluor ®700	2D1	Biolegend	2.5
CD4	APC/Fire™750	RPA-T4	Biolegend	3.5
CD8	PerCP/Cy5.5	SK1	Biolegend	3
CD3	FITC	UCHT1	Biolegend	5
FcR Block			Biolegend	5
Total volume				19

Table 2-3b. T cell functionality flow panel antibodies – Intracellular staining

Antibody anti human-	Fluorochrome	Clone	Manufacturer	Volume/ reaction (µl)
IFN-γ	PE/Dazzle™594	4S.B3	Biolegend	2.5
IL-10	APC	JES3-9D7	Biolegend	4.5
IL-13	PE/Cy7	JES10-5A2	Biolegend	4.5
IL-21	PE	3A3-N2	Biolegend	4.5
IL-17a	BrilliantViolet421™	BL168	Biolegend	4
Total volume				20

Overlapping Peptide Stimulation

The overlapping peptide stimulation was performed using either fresh or frozen PBMC. For the latter mentioned, acclimatization in a 5% CO2 incubator at 37ºC overnight was applied immediately after the cells were thawed.

The cells were plated in round-bottomed 96-well plates at concentrations of 1-1.5x10^6/150 ul/well. The overlapping peptides, MAGE A-1, MAGE A-3 and/or MAGE A-4 (JPT Innovative Peptide Solutions, Germany), were added separately at a final concentration of 1 µg/ml/well. Human antibody anti-CD28 (eBioscience) and human antibody anti-CD107a FITC were added to each well at a concentration of 2µg/ml and 1µl/test, respectively. Subsequently, incubation at 37^0 C, for 1-1.5 hours was applied to allow the T cells-peptides interactions occur. Protein Transport Inhibitor (PTI) was added at a concentration of 2µl/ml. Then the cells were incubated for 3-4 hours. Cells were harvested and used for downstream assays such as "Intracellular cytokine staining following overlapping peptide stimulation" outlined below.

Intracellular cytokine staining following overlapping MAGE-A peptide stimulation

Peptide stimulated and control cells were harvested and washed in MACS buffer, then stained with ef-506-Fixable Viability Dye (1:1000) at 4ºC for 30 minutes. Cells were washed in MACS buffer and resuspended in the residual volume and stained with surface antibodies (Table 2-4a) in the presence of 5ul FcR block (Biolegend) for 30 minutes on ice, protected from light. Cells were washed in MACS buffer,

resuspended in residual volume and fixed in 4% paraformaldehyde (Biolegend) for 30-45 minutes at room temperature, protected from light. Cells were centrifuged in 1x permeabilization buffer (Biolegend) at 760xg for 5 minutes and the cells resuspended in 100µl 1x permeabilization buffer. Cells were incubated with the antibodies (Table 2-4b) for 45 minutes at room temperature, protected from light. Cells were washed in 1x permeabilization buffer and resuspended in a suitable volume of MACS buffer for analysis by flow-cytometry.

Table 2-4. MAGE-A overlapping peptide flow panel antibodies

Table 2-4a. MAGE-A overlapping peptide flow panel antibodies – Surface staining

Antibody anti human-	Fluorochrome	Clone	Manufacturer	Volume/ reaction (µl)
CD3	Alexa Fluor ®700	UCHT1	Biolegend	2.5
CD4	APC/Fire™750	RPA-T4	Biolegend	3.5
CD8	PerCP/Cy5.5	SK1	Biolegend	3
Total volume				9

Table 2-4b. MAGE-A overlapping peptide flow panel antibodies – Intracellular staining

Antibody anti human-	Fluorochrome	Clone	Manufacturer	Volume/ reaction (µl)
IFN-γ	PE/Dazzle™594	4S.B3	Biolegend	2.5
TNF-a	PE/Cy7	Mab11	Biolegend	4.5
GM-CSF	PE	BVD2-21C11	Biolegend	4.5
IL-2	BrilliantViolet421™	MQ1-17H12	Biolegend	4
Total volume				15.5

Overlapping peptide-specific T cell clone generation

In this study, antigen-specific T cell lines were generated from the fresh PBMC. The cells were resuspended in sterile T cell line (TCL) media then plated out into wells of a 48 well plate. Each well contained 1-1.5X10^6 cells. Overlapping peptides were added separately at 1.5µl/500 µl cell suspension and incubated at 37^0C, 5% CO2 for 1.5 hours.

The cells were then taken out and placed in 5 ml FACS tubes. To each tube, 3.5 ml TCL media was added. After centrifugation at 400xg for 8 minutes, the cells pellet was resuspended with TCL media containing: IL-7 (25 ng/ml), IL-15 (5 ng/ml), IL-21 (2 ng/ml). This cell suspension was then plated out into wells of a 24 well plate (1 ml per well) and incubated at 37^0C, 5% CO2. On day 3, IL-2 (100 U/ml) was added to each well. To maintain the overlapping peptide-specific T cell clones, IL-2 (100 U/ml) containing TCL media was added to replace the discarded media twice per week. The T cell clones were harvested between day 12 and 14.

Polyclonal T Cell Clones Generation

As many as 2x10^6 PBMCs were resuspended in 1 ml AIM-V media (Thermofisher) supplemented with 7.5% human serum. PBMCs from frozen stock were rested in the same media between 2-4 hours before assay setup. A half fraction of the PBMCs (1x10^6) were taken to be pulsed with peptides at a final concentration of 5 µg/ml. They were incubated in 37ºC for 1 hours. Frozen samples required flicking every 30

minutes to avoid clumping. Then, they were washed with RPMI twice and were added back to remaining half of the sample.

Next, the mixture of peptide-pulsed PBMCs and their other half were resuspended in IL-7 (10 ng/ml) containing AIM-V media and plated out into 24-well plate. The plates were incubated 37ºC, 5% CO_2. Feeding was done at day 3 with 10 ng/ml IL-7 containing AIM-V media. At day 7 the cells were re-stimulated with autologous irradiated (3000 rad) peptide-pulsed PBMCs. The wells that showed significant growth would be analysed further with limiting dilution single cell cloning.

B95.8 LCL generation of TGCT patient B cells for autologous antigen presentation

B95.8 is an EBV-tranformed B cell line. As much as 4 ml supernatant from at least 3 days unfed B95.8 culture was removed and spun down at 900 rpm for 5 minutes. In separate tube, at least $5x10^6$ PBMCs were resuspended in wash media. Patient PBMCs from frozen stock were washed twice with wash buffer to eliminate the toxicity effect of DMSO, then rested for 1 hour in 37ºC. Next, the PBMCs were centrifugated in 400xg for 5 minutes. Supernatant was discarded.

B95.8 supernatant was then filtered onto the cell pellet using 0.45 µm syringe filter then FCS was added dropwise. This was incubated in 37ºC overnight. The following day this mixture (PBMCs diluted in viral supernatant) was spun down at 350xg for 5 minutes and supernatant was discarded. The cell pellet resulted from this step was resuspended in 2 ml CSA medium then plated out into 2 wells of a 24 well plate. The PBMCs were considered to be converted into LCLs completely when the colour of

the diluting media changed from red to yellow. Normally it occurred after 2 weeks of incubation. When the cells seemed too thick, they were split into another well in the same plate and topped up with LCL media.

Next, the LCLs were transferred into 25 cm^2 flask and media was refreshed twice a week. These LCLs were used as antigen presenting cells for both polyclonal and limiting dilution single cell T cell cloning. The remaining LCLs were diluted in 1 ml freezing media and transferred into 2 ml cryovial. Each tube received at least $5X10^6$ cells and stored in liquid nitrogen after being kept overnight at -80ºC via controlled cooling of 1ºC per minute.

Tumour Necrotic Factor Alpha (TNF-α) Capture

PBMCs were divided into 2 fractions: ⅓ part to be pulsed with specific peptide (patient dependent) and ⅔ part to be rested. A single peptide with a final concentration of 10ug/ml was added to the ⅓ fraction and incubated in 37ºC for 30 minutes. These peptide-pulsed cells were then added back to the rested cells. A single peptide at a final concentration of 10 µg/ml was used to pulse ⅓ of PBMC and incubated at 37°C for 30 minutes. After being washed twice, the peptide-pulsed PBMC were mixed with the remaining cells. Antibody anti-human TNF-α-APC (eBioscience) and TNFα-Processing Inhibitor (TAPI-0) (Enzo Life Sciences) were added at a final concentration of 0.5µl/0.5ml and 0.5µg/0.5ml, respectively. Following incubation at 37°C/5% CO2 for 4 hours, cells were washed once with MACS buffer and centrifuged at 400xg for 5 minutes. Cells were prepared for flow cytometric

analysis as follows: Cell viability was first marked by adding 100 µl live/dead fixable molecular probe solution (Life Technologies) and inccubated at RT for 10 min and washed with MACS buffer. Next, surface staining was performed with antibodies listed in Table 2-5. Cells were incubated on ice for 1 hour prior to flow cytometric analysis.

Table 2-5. TNF-α-capture assay antibody details

Antibody anti human-	Fluorochrome	Clone	Manufacturer	Volume/ reaction (µl)
CD4	FITC	RPA-T4	Biolegend	5
CD8	PerCP/Cy5.5	SK1	Biolegend	2.5
CD3	PE/Cyanine7	UCHT1	Biolegend	5
FcR Block			Biolegend	5
Total volume				17.5

HLA Type Identification

In my thesis I was interested to assess donor HLA status in relation to specific alleles that are associated with presentation of immunodominant peptides. As such, genomic DNA was extracted from cell pellet using DNeasy Blood and Tissue Kit (Qiagen). The purity of extracted DNA was measured in ND-100 spectrophotometer. About 140 ng DNA per sample was prepared for HLA-type identification. The amplification primers were adopted from Bunce et al (1995) and purchased from JPT (Germany). The primers' sequences are listed in Table 2-6.

Table 2-6. Primer pairs for HLA typing of patient PBMC

Antigen (HLA-type)	Forward Primer Sequence: 5'-3'	Reverse Primer Sequence: 5'-3'	Product Size (bp)
A1	CGA CGC CGC GAG CCA GAA	AGC CCG TCC ACG CAC CG	629
A2	GTG GAT AGA GCA GGA GGG T	CCA AGA GCGCAGGTCCTCT	489
A3	AGC GAC GCC GCG AGC CA	CAC TCC ACG CAC GTG CCA	628
A11, 6601	ACG GAA TGT GAA GGC CCA G	GAG CCA CTC CAC GCA CCG	552
A1, 11, 36, 80, 3402	TAC TAC AAC CAG AGC GAG GA	CCA CGT CGC AGC CAT ACA TT	300
B7 (inc. B703), B8101	GGA GTA TTG GGA CCG GAAC	TAC CAG CGC GCT CCA GCT	619
B8	GAC CGG AAC ACA CAG ATC TT	CCG CGC GCT CCA GCG TG	606
B44	GGC CGG AGT ATT GGG ACG A	GTC GTA GGC GTC CTG GTC	546/481
	CGC CAC GAG TCC GAG GAA	CGT CGT AGG CGT ACT GGT C	
DR7	CCT GTG GCA GGG TAA GTA TA	CCC GTA GTT GTG TCT GCA CAC	231
DQ6	GGA GCG CGT GCG TCT TGT A	TGC ACA CCG TGT CCA ACT C	249/140
		TGC ACA CCC TGT CCA CCG	
Cw7	CCG CGG GTA TGA CCA GTC	CAG CCC CTC GTG CTG CAT	1062
Control Primer	TGC CAA GTG GAG CAC CCA A	GCA TCT TGC TCT GTG CAG AT	796

PCR amplifications were performed through the following cycling parameters: 1 minute at 96ºC, 5 cycles of 25 seconds at 96ºC, 45 seconds at 70ºC, 45 seconds at 72º, followed by 21 cycles of 25 seconds at 96ºC, 50 seconds at 65ºC, 45 seconds at 72ºC, followed by 4 cycles of 25 seconds at 96ºC, 60 seconds at 55ºC and 120 seconds at 72ºC. Touchdown PCR was not used. The primers were designed such that these temperatures were ideal for all primers. Next, the PCR amplification results were run on 1.5% agarose gel and visualized in UV-Transilluminator using ethidium bromide (EtBr) with a final concentration of 0.5mg/ml.

IFN-γ ELISA

In order to evaluate the response specificity of polyclonal T cell cones, IFN-γ ELISA was performed. As much as 75-100 µl of 2 weeks old polyclonal T cell clones were taken and transferred into 5 ml FACS tubes. They were washed with 3 ml wash buffer and spun down twice at 400xg for 5 minutes each. In separate tubes, autologous previously cryopreserved PBMCs were also prepared. When they were taken from frozen stock, they were rested immediately after thawing at 37ºC 5% CO_2 for about an hour top in 2 ml of GM media. Next, cells were washed twice and irradiated at 40 Gy. After being washed once with wash buffer and resuspended in 2 ml of sterile GM, the irradiated cognate PBMCs were pulsed with corresponding peptide/peptide mix at a final concentration of 10 ng/ml at 37ºC 5% CO_2 for 2 hours. We spared some for negative control in which the irradiated PBMC was not pulsed with peptides.

The peptide-pulsed irradiated PBMCs were added to the corresponding clones with a ratio of 1:10. After being mixed, they were washed once with wash buffer and resuspended in 200 µl GM per peptide to be plated out in duplicate into 96 well V-bottom plates. Each well was treated with peptide at a final concentration of 5 ng/ml accordingly and incubated at 37 ºC 5% CO_2 overnight. Meanwhile, 96 well ELISA plate was coated with IFN-γ capture antibody (clone 1-D1K; Thermofisher) in coating buffer overnight at 4ºC.

The following morning, the coated ELISA plate was washed 6 times with PBS-T (0.05% Triton™X-100 in 1x PBS) and blocked with 5% BSA-containing PBS-T for 2 hours. The blocking agent was removed by washing 6X times using PBS-T. The V bottom plate containing stimulated cells was spun down at 90xg. The supernatant was gently taken and pipetted into the ELISA plate. To measure the concentration of captured IFN-γ, several tubes for standards were prepared with a defined concentration of IFN-γ: 25 µg/ml; 12.5 µg/ml; 6.25 µg/ml; 3.125 µg/ml; 1.56 µg/ml; 0.78 µg/ml; 0.39 µg/ml; 0.195 µg/ml; 0.098 µg/ml; 0.049 µg/ml; 0.024 µg/ml and 0 µg/ml. From each tube, 100 µl was pipetted into ELISA plate. Then, the plate was incubated in the dark at RT for 4 hours. The plate was washed 6 times with PBS-T and then stained with 50 µl of diluted Biotinylated-IFN-γ (mAB-7-B6-1; 1:1000; Thermofisher). The plate was incubated in the dark at RT for 1 hour. The plate was washed 6 times with PBS-T and 100ul/well of SA-HRP (1:1000; Thermofisher) was added to each well, and incubated at RT for 1 hour. The plate was washed 10-times with PBS-T followed by the addition of 100ul of TMB substrate. The plate was incubated for 15minutes then 100ul of Stop solution (1M HCl acid) was added to each well. Absorbance was measured at 450nm in a microplate reader (BioRad).

Statistical Analysis

Prior to the main significance test, the normality of each dataset is evaluated through Saphiro-Wilk's test. Normally distributed data is confirmed when the Saphiro-Wilk's test results in nonsignificance among the 3 compared data. This will be further analysed with one way ANOVA with Tukey's multiple test to check if there is

significance emerges per each comparison. Kruskal-Wallis with Dunn's multiple test is, otherwise, used to obtain significance from dataset that are not normally distributed. Outliers are also identified and omitted to see if these affect significance. If they do, data are re-analysed post outlier-cleanse using one way ANOVA with Tukey's multiple test. All these tests are conducted using Graph Prism8 software.

CHAPTER III. IMMUNE CELL POPULATION IN TGCT PATIENT COHORT

Demography of Testicular Germ Cell Tumour (TGCT) Patient Cohort

A total of 57 patients with histopathologically-confirmed TGCT screening results were recruited and further discriminated, according to their characteristics into 2 major groups: seminoma and nonseminoma as listed in the Table 3-1.

Table 3-1. Patient characteristics

Testicular Germ Cell Tumour (TGCT) Type	Stage	n	Histopathological Characteristic
Seminoma	IA	28	**pT1N0M0**
	IB	7	**pT3N0M0**
	IIA	2	**pT2N1M0**
	IIB	3	**pT3N2M0**
	IIC	2	**pT1N3M0**
	IIIC	1	**TxN2M1b**
	Total	**43 patients**	
Nonseminoma	IA	1	**pT1N0M0**
	IB	4	**pT3N0M0**
	IIA	1	**pT2N1M0**
	IIB	2	**pT3N2M0**
	IIIA	1	**pT2N2M1a**
	IA	1	**pT1N0M0**
	IIB	1	**pT3N2M0**
	IA	2	**pT1N0M0**
	IB	1	**pT3N0M0**
	Total	**14 patients**	

Seminoma groups comprised of 43 patients (75.44%) and nonseminoma comprised of 14 patients (24.56%). Stage IA seminoma dominated the overall cohort. This

constituted about 49.12% (28 out of 57) cases under our investigation. Stage IB seminoma made up a quarter lower cases than stage I (12.28%, 7 out of 57). Stage IB nonseminoma followed as the top 3 with 4 out of 57 (7.02%) of our TGCT cohort belonged to the group. There were 5 patients showed distinctive histopathological features that were attributed for the exclusion of them from the typical nonseminoma. Among those, 2 were categorized as embryonal carcinoma at stage IA and IIB with one patient each stage and the remaining 3 were mixed-germ cell tumor at stage IA and IB with 2 and 1 patient, respectively. Stages in our study spanned from stage IA to IIIC. Stage IV was not observed.

Enumerated on the day of initial blood collection, patients age in seminoma group ranged from 26-69 years old with median value of 44 [IQR 33, 55] whereas nonseminoma group demonstrated slightly younger age which ranged from 20-39 years old with median value of 28 [IQR 24.75, 34.25]. To detect significances of these shown differences, Kruskal-Wallis with Dunn's multiple test, significance at $p<0.05$ was performed and this resulted in a statistical confirmation that overall patients with nonseminoma were younger than patients with seminoma (p-value=0.00007). These ages were not stage-associated since our patients with the more advanced stage were not the eldest in the respective groups.

As control we recuited 13 males who on the day of blood collection were physically healthy. Their ages ranged from 23-43 years old with media value of 32 [IQR 29, 34.5]. This age distribution is not significantly different with those seen for

nonseminoma. But when compared with the age range in seminoma, significance is obtained with p-value is 0.006.

Phenotypic analysis of the peripheral immune repertoire in patients with testicular cancer

Given the excellent clinical outcomes in patients with testicular cancer, even in the setting of metastatic disease, I was keen to begin my research with an analysis of the baseline immune repertoire of patients with testicular cancer. This work was stimulated by previous findings that such patients demonstrated some unusual features within their T cell profile (Pearce et al, 2017). It has been seen that patients with testicular cancer have an increased proportion of T cells within the memory pool with an equivalent reduction in the proportion of naive cells. It had been shown that within healthy donors the proportion of naïve (CD45RA+CCR7+) cells represented 56% and 51% of the CD4+ and CD8+ repertoires respectively. In contrast, in the patient group these had been reduced by 25–40% to values of 38% and 30%. The proportion of CD4+ effector memory cells was also increased from 23% within healthy donors to 32% within TGCT patients ($p = 0.0028$). CD8+ TEM cells were also markedly increased in the patient group, by around 35%, from 32% within healthy donors to 45% within the patient group ($p = 0.0284$). Similar findings were also observed with the CD8+ effector memory subgroup.

These data had suggested that tumor development is associated with the generation of a large pool of memory T cells in the peripheral repertoire. Importantly, this increment had been shown to be corrected by surgery or chemotherapy and

therefore suggests that the T cells may be shortlived. One possible explanation for these findings, and an interpretation that was of great interest to me in my studies, was that these might represent tumour-specific T cells that are circulating within the blood and are primed to kill malignant cells within the periphery.

To date my work had focused on T cells, a major subset of the adaptive immune system. I was also keen to assess if testicular cancer had any influence on the innate immune system as this had not been assessed in previous studies. Innate immune system is the first line of defense to combat and control bacterial infections but is now appreciated to play an important role in the control of malignant disease . As such I included innate-like T cells and NK cells in my study.

In order to undertake this work I went on to develop a flow cytometry panel that allowed evaluation of the magnitude and proportion of major immune cells in TGCT patients in comparison with healthy donors. Relatively little work has been done on this topic in previous studies. Formalin Fixed Parrafin-Embedded (FFPE) archive analysis of tumour-inflitrating lymphocytes (TIL) employing immunohistochemistry (IHC) has recently been undertaken by Fankhauser et. al (2015). They demonstrated that PD-L1 (Programmed Death Ligand-1) was expressed by 73% and 64% of seminoma and nonseminoma samples respectively. Interestingly, PD-L1 positive stromal cells were only present within the seminoma subgroup. However I was not able to find any reports of flow cytometric analysis in this setting. Flow cytometry allows analysis of simultaneous expression of a wide range of membrane proteins. My aim was to utilize this information to gain novel insights into the immune repertoire of patients with testicular cancer.

Flow cytometric analysis of peripheral blood patients with testicular cancer

Blood samples were obtained from patients with testicular cancer and age-matched samples were analysed either fresh or frozen. Blood was collected at the time of diagnosis and before any chemotherapy had been given. Frozen PBMC were incubating in RPMI 1640 at 37°C for 1 hour prior to staining. Cells were then stained with antibodies against a range of membrane proteins prior to flow cytometric analysis.

TGCT samples were classified into 2 groups, as either seminoma (34 samples) or nonseminoma (13 samples) according to histopathology records. The nonseminoma group included embryonal carcinoma, mixed germ carcinoma and teratoma. Blood from 16 healthy donors (HDs), which was age-matched with the TGCT population, was used as a control group (Table 3-2). The gating strategy used in the analysis is illustrated in Fig. 3-1.

Table 3-2. Age Range of Patients and healthy donors

Groups of Samples	Sample Size (n=)	Age (years) Median [IQR Q3, Q1]
Seminoma	34	44 [IQR 32,56]
Nonseminoma	13	25 [IQR 23,35]
Healthy Donor	16	32 [IQR 23,57]

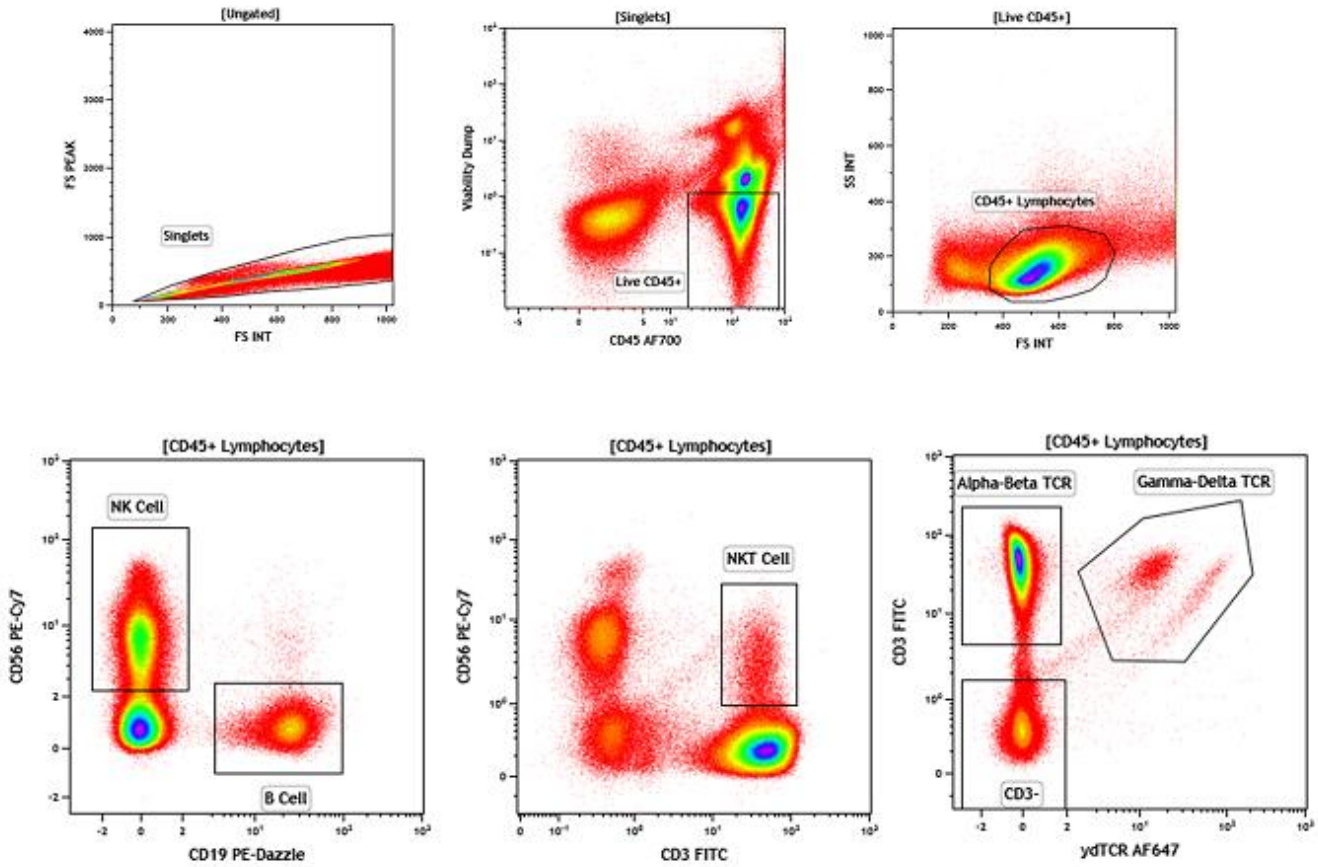

Fig. 3-1. Representative gating strategy to identify immune cell subpopulations in TGCT patients.

Firstly, singlets were obtained on the basis of FS INT (X Axis) and FS PEAK (Y Axis). Secondly, viability dye was used to exclude dead cells from further analysis and live CD45+ lymphocytes were selected. Thirdly, CD45+ lymphocytes were further analysed and divided into distinct (bottom row from left to right) CD3- CD56+ NK cells, CD19+ CD56- B cells and CD3+ T cells to further identify αβ and γδ TCRs.

T cells are the dominant subpopulation in both healthy donors and patients with testicular cancer

As expected, T cells were found to be the major immune subset within peripheral blood in both the control and patient groups. Initial analysis focused on the distribution of the broad immune subsets of T cells, B cells and NK cells within each donor. A subset of CD45+ cells that did not comprise any of these subsets, and is most likely to represent myeloid cells, was also defined.

The distribution of each cell subset did not reveal any major visual differences within the three groups (Fig. 3-2). There was a suggestion of a potential relative increase in the B cells and CD45+ subsets and in order to assess this I next compared the median values for each subset across the patient groups (Table 3-2 and Fig. 3-3).

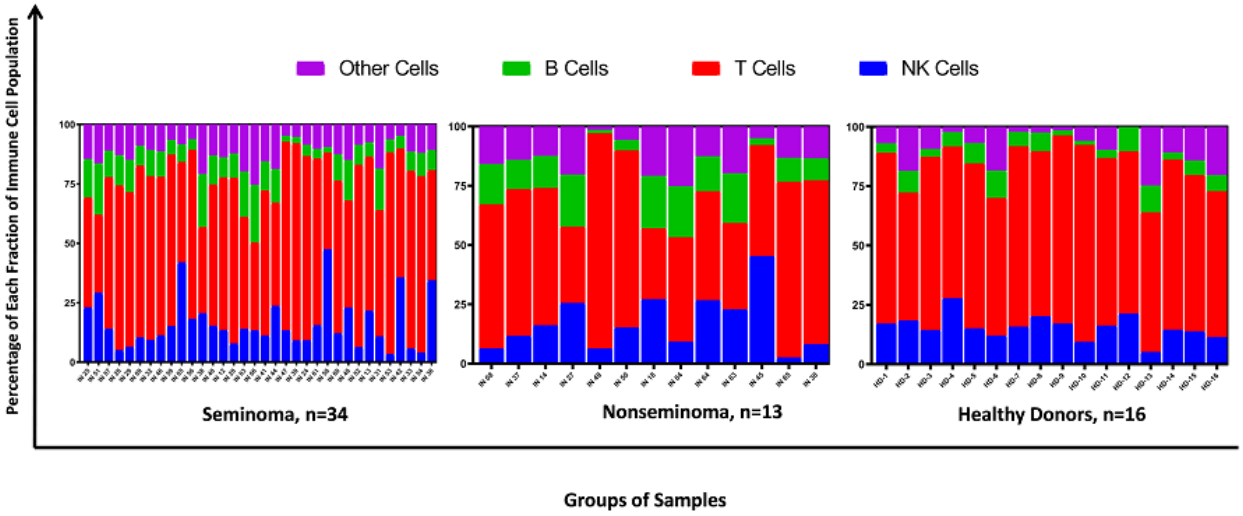

Fig. 3-2. Major immune cell populations within the TGCT patient and healthy donor cohorts

In the three groups immune cells are largely consist of T- (red), NK- (blue), and B-cells (green), respectively. The purple subgroup represents cells that are CD45+ but are not NK, T or B-cells. Each fraction of the defined immune cell types is presented as a percentage of total CD45+ lymphocyte pool.

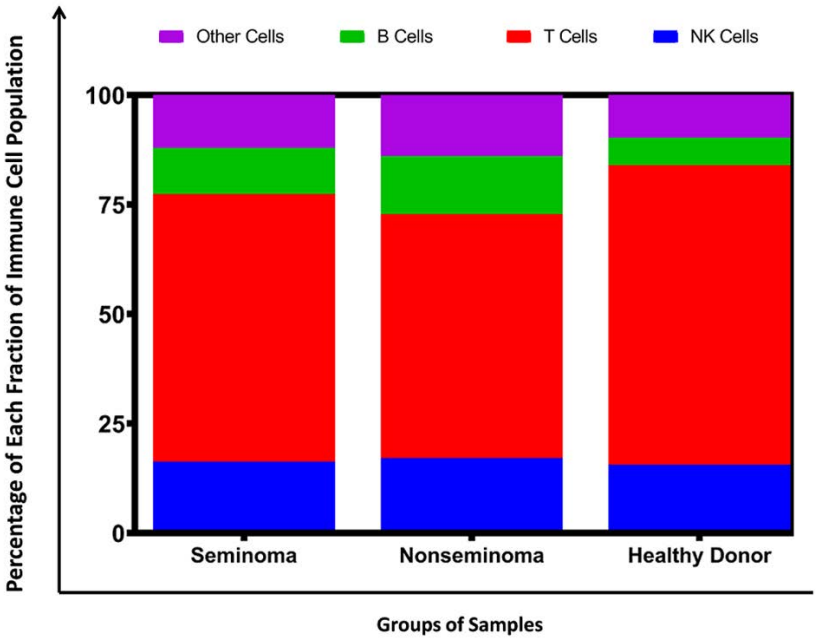

Fig. 3-3. Summary of overall immune cell populations in seminoma, nonseminoma and healthy donors

Immune cell populations in the three groups comprise of T cells (red), NK cells (blue), B cells (green) and other CD45+ cells (purple).

As shown in Table 3-3, in the healthy donor cohort T cells represent the largest immune cell population at 69.6% [IQR 62.03%, 72.62%]. Within the seminoma group this value is somewhat reduced to 64.5% [IQR 46.54%, 72.24%] of immune cells and falls further to 58% [IQR 40.20%, 71.60%] in nonseminoma patients. This decrease frequencies of T cells in seminoma and nonseminoma patients compared with HD, however, are insignificant with adjusted p-values of 0.210 and 0.050, respectively (one way ANOVA with Tukey's multiple test, significance at $p<0.05$).

Table 3-3. The Magnitudes of The Top Three Dominating Immune Cell Fraction

Examined Groups	Sample Size (n=)	Immune Cells Observed in the PBMCs	Mean	Median [IQR Q1, Q3]
Seminoma	34	T Cells NK Cells B Cells	61.1% 16.3% 10.6%	64.5% [IQR 46.31%, 72.20%] 13.5% [IQR 9.2%, 21.9%] 9.9% [IQR 5.8%, 13.7%]
Nonseminoma	13	T Cells NK Cells B Cells	55.7% 17.1% 13.3%	58% [IQR 40.2%, 71.6] 15.2% [IQR 7.24%, 26.1%] 13.5% [IQR 8.85%, 21.3%]
Healthy Donor	16	T Cells NK Cells B Cells	68.4% 15.6% 6.31%	69.6% [IQR 62.%, 72.6] 15.4% [IQR 12.4%, 18.1%] 6.09% [IQR 3.37%, 8.95%]

The total NK cell proportion within HD was measured at 18% [IQR 12.98%, 23.07%] but was somewhat lower in seminoma and nonseminoma respectively at 13.2% [IQR 10.16%, 19.70%] and 16.8% [IQR 8.76%, 21.86%] (Table 3-3). No significance is obtained from the comparisons of these three groups (p-values > 0.999) (Kruskal-Wallis with Dunn's multiple comparison test, significance at $p<0.05$).

Interestingly, compared to HD the percentage of B cells in patients with seminoma and nonseminoma was statistical significantly increased (Table 3-3). The p-values observed for these comparison are 0.049 and 0.004, respectively (one way ANOVA with Tukey's multiple test, significance at $p<0.05$). This may suggest that there may be a peripheral humoral response as a part of the adaptive immune response against testicular cancer. This is an area that has not been thoroughly investigated to date and represents an important area for future study.

In addition, an increase in the CD45+ subset was also seen in the patient group. Unfortunately the composition of these cells was not further examined but it is

possible that they may comprise cells such as myeloid-derived suppressor cells whose numbers have been demonstrated to be increased in many patients with cancers (Gabitass et al., 2011), although this has not yet been addressed in testicular cancer.

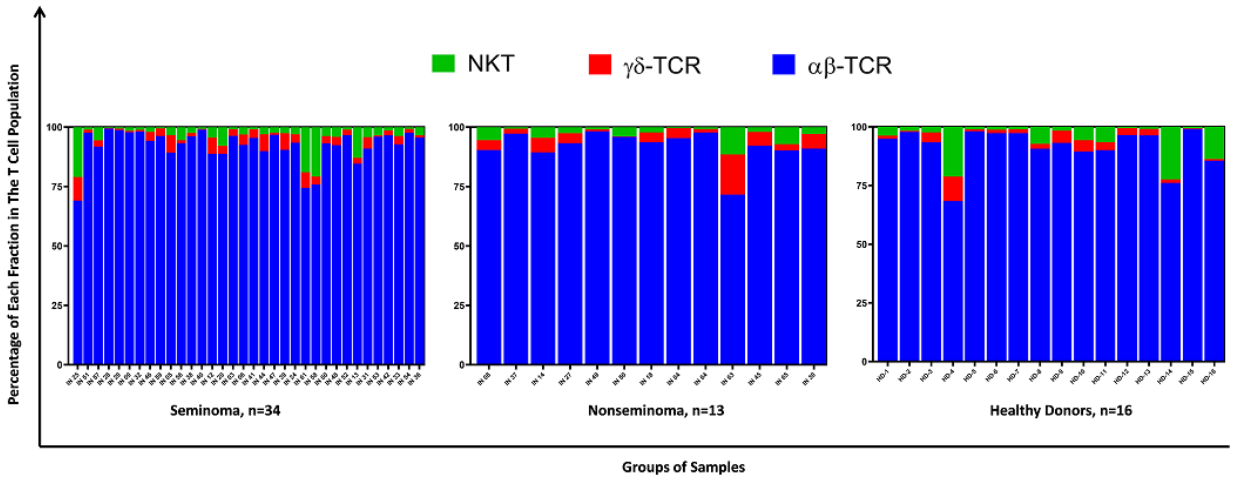

Fig. 3-4. Distribution of major T cell subpopulations in patients with testicular cancer.

T cell populations were delinated according to expression of αβ-TCR, γδ-TCR and CD56 (NKT). The frequencies above are enumerated as a total of the CD3+ pool.

αβ T Cells are the most dominant fraction within the T cell population

T cells were found to be the most common cell subset within the immune repertoire and I next went on to examine the subsets of T cells within this group. In particular I examined the relative proportion of cells that expressed the αβ-TCR or γδ-TCR and also enumerated the NKT subpopulation through co-expression of CD56 and CD3. The individual data of each examined groups is depicted in Fig. 3-4.

αβ-T cells are the predominant cellular subset among all the three groups. On average they are approximately 20 fold more numerous than γδ-T-cells. In seminoma, the median value for the αβ-T cell fraction is 93.9%% [IQR 90.3%, 96.7%] whereas in nonseminoma the value is 93.3% [IQR 90.29%, 96.58%] and the percentage in healthy donors is 94.2% [IQR 89.65%, 97.34%]. All these values are not significant with all observed p-values >0.999 (Kruskal-Wallis with Dunn's multiple comparison test, significance at $p<0.05$).

γδ-T cells are not increased in the blood of patients with testicular cancer

γδ T-cells encompass a well-conserved population of innate lymphocytes whose structures and functions are largely heterogenous. During tumour progression, they participate in diverse immune responses. The cells lately have emerged as an interesting field of study to develop immunotherapy (Wu et al., 2017). In human, γδ-T cells contribute to the immune response against a subset of tumours of haematological and epithelial origin (Hannani et al., 2012). An antibody against the γδ TCR was used to define the presence of this subset in flow cytometric analysis that also included simultaneous staining of CD3 (Fig. 3-5).

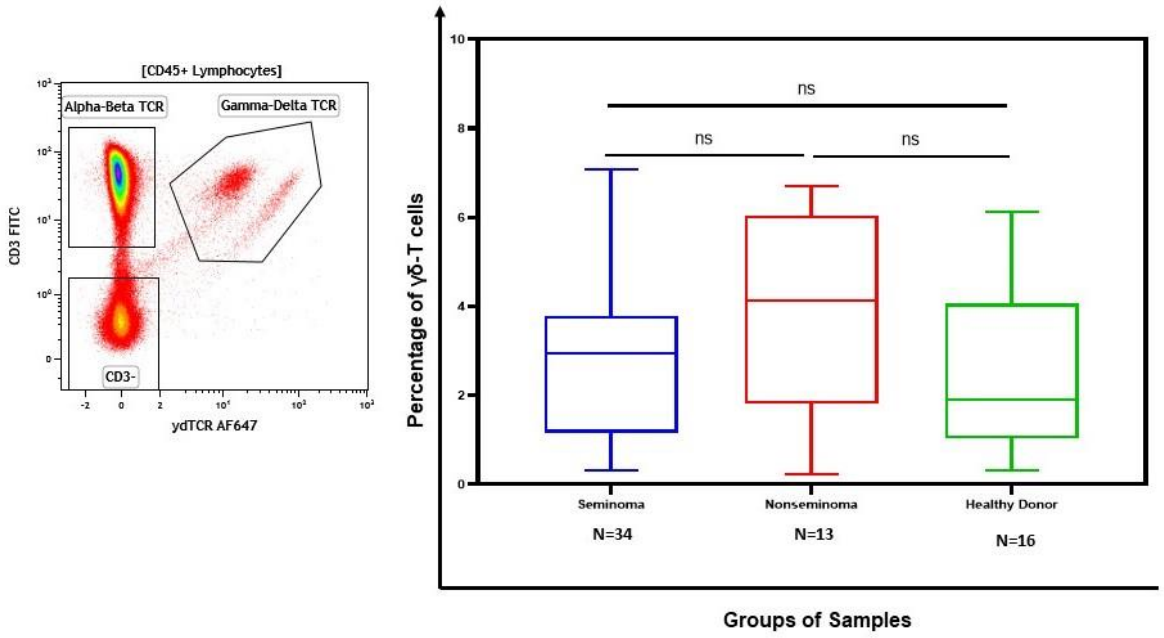

Fig. 3-5. γδ-T cells are detected in seminoma and nonseminoma cohorts with a frequency similar to those in HD

In the left panel γδ-T cells are demonstrated as γδ-TCR-expressing CD3+ T cells. The right panel indicates the percentages of γδ- T cells within the CD3+ repertoire. Two populations of cells are observed by intensity of TCRgd staining. Values were analysed statistically through Kruskal-Wallis with Dunn's multiple comparison to adjust the observed p-values (Graph Prism 8). (Non-significant =ns).

In our samples, γδ- T cells in seminoma, nonseminoma and HD are detected at median values of 2.95% [IQR 1.16%, 3.95%], 4.13% [IQR 1.81%%, 6.04%] and 1.90% [IQR 1.03%, 4.07%] respectively. Although the median and quartile values appear to show a 2 fold and 1.5 fold increase in the nonseminoma and seminoma groups when each is compared with HD, these differences are not significant. These comparison yield p-values of 0.192 and 0.845, respectively. Likewise, γδ- T cell number in seminoma and nonseminoma is insignificant with p-value is detected

at the level of 0.299 (one way ANOVA with Tukey's multiple comparison, significance at $p<0.05$). Despite these statistical insignificance, nonseminoma tend to upregulate more γδ-TCR. One notable observation was the relative heterogeneity in the percentage of γδ cells within the three groups, as indicated in the pattern of distribution within the violin plots. The relative importance of these interesting cells in either the development or control of cancer thus needs considerably more investigation (Zhao et al., 2018).

The relative frequency of total NKT cells is not altered in patients with testicular cancer

Carrying phenotypic characteristic of both T cell and NK cell, NKT cells emerged as a unique cell subset. Their potential role in cancer development and control was noted almost 15 years ago by Tachibana et al who demonstrated that colorectal cancer patients with high NKT-cell infiltration had higher overall and disease-free survival rates (Tachibana et al., 2005).

NKT cells are mainly restricted by CD1d and whilst some populations express a conserved TCR there are also many cells that express a diverse TCR repertoire. In my analysis I defined NKT cells by a CD3+CD56+ phenotype. CD56+ is widely expressed on NKT cells and has been used to assess their profile in previous studies. Alternative approaches could have been to use a CD1d tetramer or potentially antibody staining against the conserved Va24-Vb11 heterodimer on many of these cells.

CD3+CD56+ NKT cells in seminoma, nonseminoma and HD were observed at median and quartile values of 3.02% [IQR 1.10%, 4.25%], 2.53% [IQR 0.84%, 4.95%] and 2.00% [IQR 0.98%, 7.01%] respectively (Fig. 3-6). NKT cell frequencies are therefore not altered in testicular cancer (p-values >0.999) (Kruskal-Wallis with Dunn's multiple test, significance at $p<0.05$). Some outliers are detected in seminoma and healthy donors whose NKT cell frequencies exceed normal distribution of the analysed data. In seminoma, the outliers are identified in IN 28, IN 22, IN 23 and IN 25 with NKT cell frequencies at the levels of 12.9%, 19%, 20.7% and 21%, respectively. Healthy donors with ID HD-4 and HD-14 have NKT cell percentages of 21.1% and 22.4%, respectively, which become the outliers. If these outiers are removed, the p-values yielded by frequency comparison between seminoma and nonseminoma, seminoma and HD and nonseminoma and HD, respectively remain at 0.635, 0.665 and 0.998 and do not improve the significance levels.

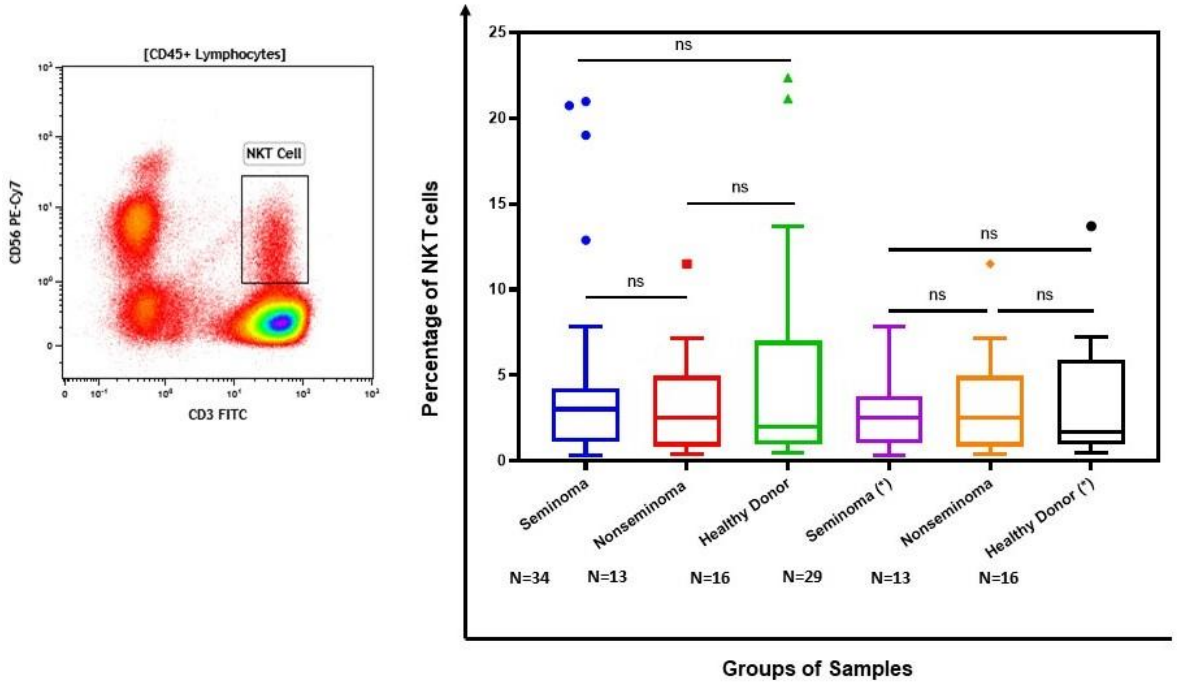

Fig. 3-6. NKT cells are present in PBMCs of seminoma and nonseminoma cohorts

In the left panel, flow cytometry analysis demonstrates that NKT cells are defined as CD45+ lymphocytes which positively co-express CD56 and CD3. In the right panel, NKT cells of each examined group are plotted as percentages of total T cells (CD3+). Asterisk denotes omission of detected outliers. After outliers are excluded from the affected dataset, seminoma and healthy donors have a total of 30 and 16 remaining individual data to be further analysed. Statistical significance is obtained by performing Kruskal-Wallis with Dunn's multiple test and re-testing with one way ANOVA with Tukey's multiple test after the outliers are depleted from the dataset. No significance is observed from the three comparisons either through Kruskal-Wallis or one way ANOVA analysis.

A total of 6 outliers are detected. Of those, 4 outliers are from seminoma where 3 of which are at stage I and one is at stage II TGCT. Both, therefore, did not have metastatic disease. The remaining 2 outliers are found in HD. It worth noting that compared to other members in the corresponding dataset, the outliers are somewhat older (37-69). This suggests that the frequency of NKT may increase with

aging although to confirm this further evalution a much larger sample size is required.

The percentage of circulating B cells is increased in patients with TGCT

The relative importance and contribution of B cells to the adaptive immune response against cancer has been somewhat less well investigated in comparison to tumour-specific T cell responses. B cells constitute approximately 15% of peripheral blood leukocytes and can be defined by a range of different markers such as CD19 or CD20.

I used a combination of CD45+ and CD19+ to define B cells in my flow cytometry panel. CD19 is a biomarker for normal and neoplastic B cells and acts to regulate the intrinsic B cell signaling threshold through modulation of B cell receptor-dependent and independent signaling.

The median proportion of B cells within the peripheral immune repertoire in the seminoma and nonseminoma samples was 9.93% [IQR 5.84%, 13.55%] and 13.5% respectively [IQR 6.85%, 21.34%]. There was no statistically significant difference observed between these numbers (p-value=0.24). In contrast, the values are higher than those seen in HD where the median value was 6.1% [IQR 3.37%, 8.95%]. The p-values for seminoma vs HD and nonseminoma vs HD were seen to be 0.049 and 0.004, respectively (one way ANOVA with Tukey's multiple test, significance at $p<0.05$).

I next went on to assess if this difference might represent an alteration in the relative proportion of naive or memory B cells. In particular, expression of CD27 was used as a marker of B cell memory (Fig. 3-7). Interestingly the proportion of memory B cells in the three groups was very similar at a mean frequency around 2-3% of the CD45+ pool. This failure to see any difference in the frequency of memory B cells in seminoma, nonseminoma and HD suggests that the increased proportion of B cells in the patient group must belong to another subset of B cells, potentially the naïve repertoire. As such it is difficult to interpret the significance of the increased proportion of non-memory B cells in the blood of the patient group. It might potentially imply that this simply reflects a stable residual population that is increased due to a numerical decrease in other leukocyte subsets or it may reflect a systemic activity from the tumour in releasing naïve B cells from the bone marrow into the circulation.

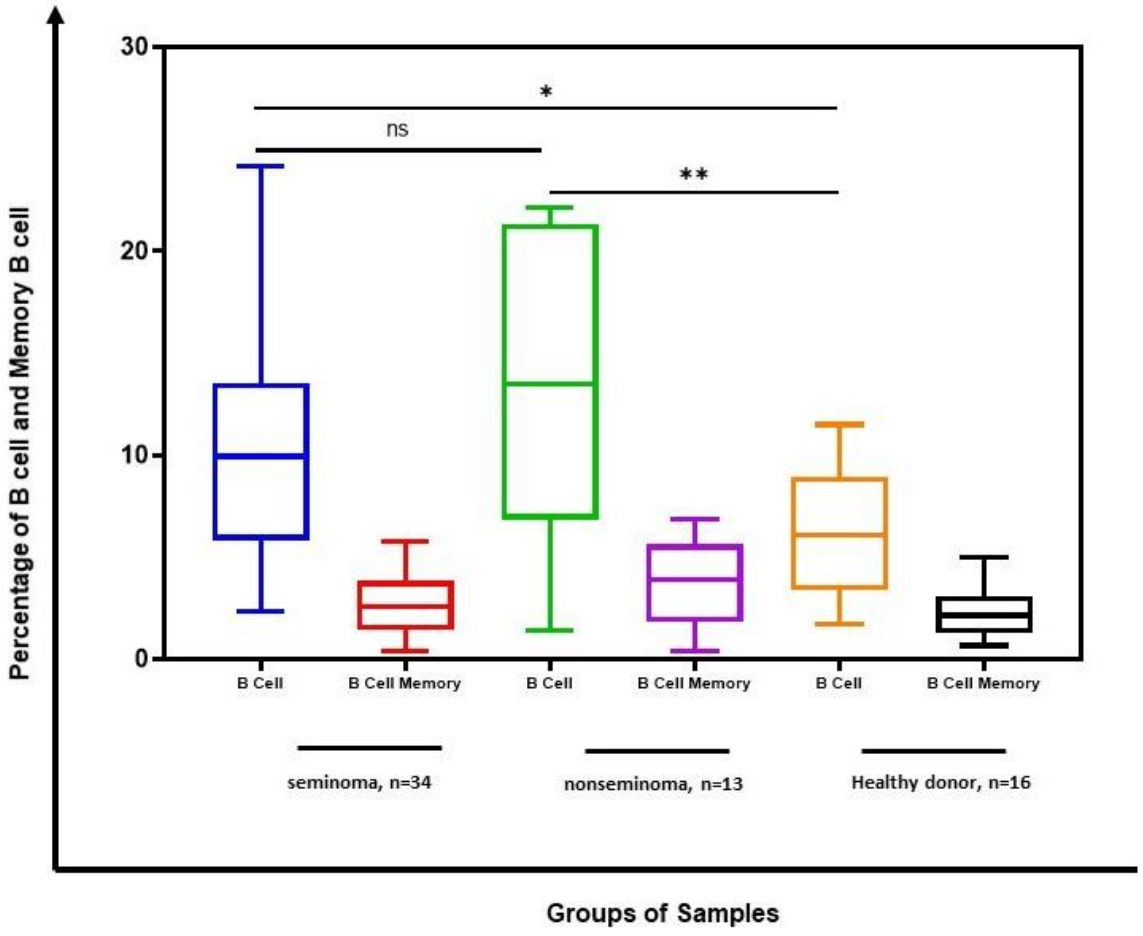

Fig. 3-7. The relative percentage of B cells and memory B cells within patients with testicular cancer or healthy donors

The individual values for percentage of total B cells or B memory cells is presented as percentage of a total CD45+ Lymphocytes.The median of each group is marked by longer dashed-lines in black while the quartile values (lower quartile Q1 and upper quartile Q3) are represented by shorther lines. Colours in red, green and blue represent seminoma, nonseminoma and HD, respectively. The median and the quartile values are determined Statistical significance is obtained by performing ANOVA with Tukey's multiple test after the dataset is confirmed to be normally distributed through Saphiro-Wilk test (Graph Prism 8).

Further, I assessed if there is a disparity in the memory B cell proportion in both TGCT subtypes in comparison with healthy donor group. Phenotypically, memory B

cells are CD45+ cells which co-express CD19 and CD27. In seminoma, the relative proportion is measured at a median level of 27.1% [IQR 21.40%, 32.85%] while in nonseminoma the median is observed at a level of 27% [IQR 15.33%, 44.28%]. As reference group, healthy donor group demonstrates a higher level proportion of memory B cell compared to that of both seminoma and nonseminoma. The median in the group is 39% [IQR 27%, 50.18%].

Compared to values within healthy donors, the proportion of memory B cells in seminoma and nonseminoma patients are significantly lower with observed p-values of 0.003 and 0.046, respectively (Fig. 3-8). Neither age nor stage is seen to be a determinant of this profile. The patients with the highest proportion of B cell memory in seminoma and nonseminoma were diagnosed with stage IA and aged 59 and 34 years old, respectively. Both are not the oldest within the corresponding groups. The pattern of B cell memory proportion in HD is, likewise, not correlated with age because the donor whose the cell frequency is the highest was relatively young, aged only 25 years old.

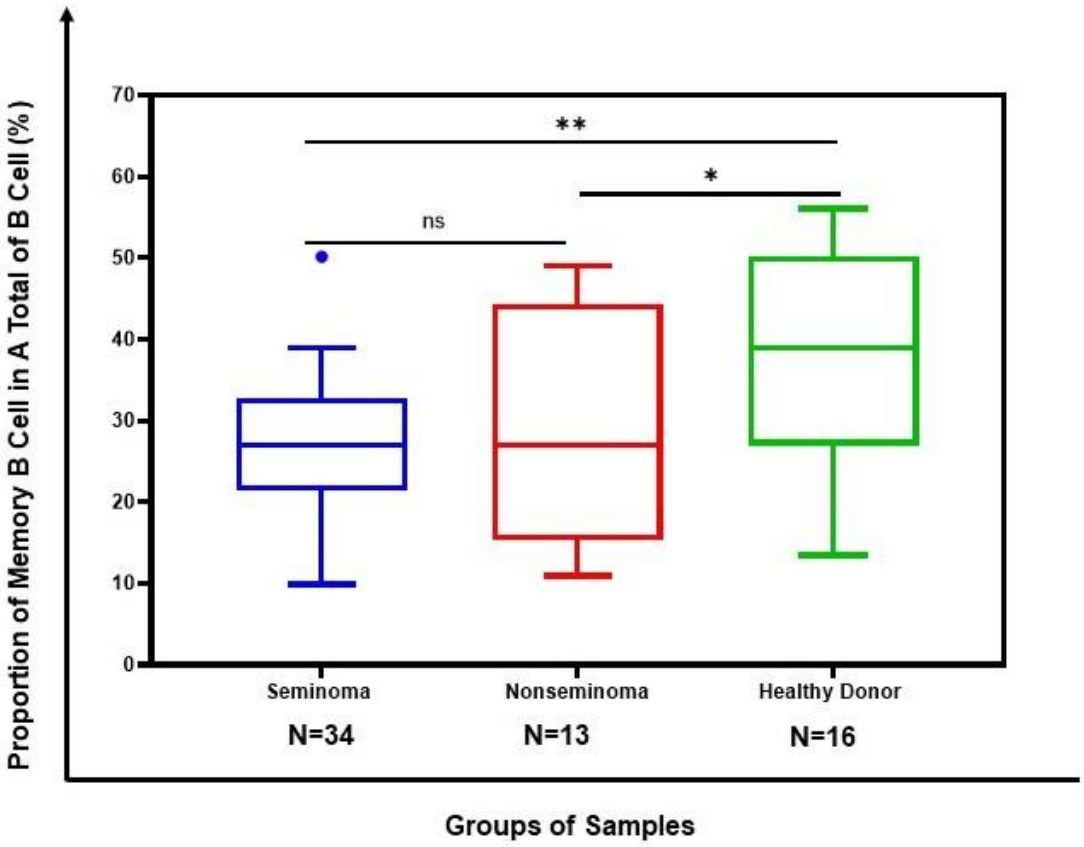

Fig. 3-8. The relative proportion of memory B cells in the total B cell repertoire in seminoma, nonseminoma and healthy donors.

The individual values for proportion of memory B cell is presented as percentage of the cells in the total B cell repertoire. These memory B cells are phenotyped as CD27-expressing CD19+ CD45+ lymphocytes. The data distribution within each group is demonstrated as median (marked by longer dashed-lines), lower quartile Q1 (marked by shorter dashed-lines below the median lines) and upper quartile Q3 (marked shorter dashed-lines above the median lines). Each dataset is confirmed to be normally distributed through Saphiro-Wilk test hence statistical significance is conducted via one way ANOVA with Tukey's multiple test, significance at $P<0.05$. Both seminoma and nonseminoma demonstrate significant lower proportion of memory B cell compared to healthy donor's as indicated by double and single asterisk, respectively.

The percentage of invariant MAIT Cells is not altered in patients with testicular cancer

Mucosa-associated invariant T (MAIT) cells are a class of innate-like T cells that are involved primarily in mucosal immune responses. The cells were identified relatively recently and are recognized phenotypically by their co-expression of TCR Vα7.2 and CD161 (Garner et al., 2018). The median percentage of MAIT cells (expressed as a percentage of the T cell pool) in seminoma, non-seminoma and HD was found to be 1.55% [IQR 0.61%, 3.35%], 1.22% [IQR 0.76%, 2.55%] and 2.77% [IQR 1.05%, 4.71%] respectively. P-values exceed 0.999, 0.695 and 0.527 when MAIT frequencies are compared between seminoma vs nonseminoma, seminoma vs HD and nonseminoma vs HD, respectively. These are all non significant (Fig. 3-9). There are 5 outliers identified from seminoma group whose MAIT cell frequencies range from 5.940%-8.893%. To assess if these outliers alter significance of the analysed dataset, these values were excluded from the analysis and the remaining data re-tested with one way ANOVA and Dunn's test as the post hoc analysis.

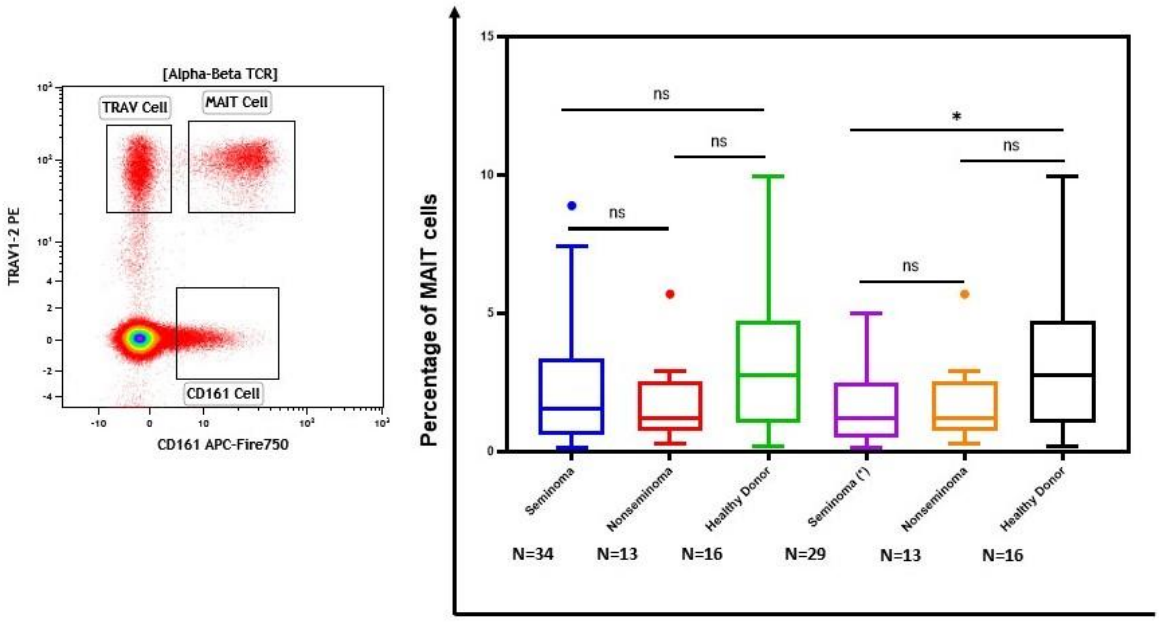

Fig. 3-9. The percentage of expression of MAIT cells within patients with testicular cancer and healthy donors

The percentage of MAIT cells was defined by co-expression of CD161 and TCR Vα7.2 as shown in the left hand panel. This proportion was then calculated as a percentage of the total αβ-T cell population. The data within each study group is shown individually with their respective median and quartiles (lower quartile Q1 and upper quartile Q3). Pre-normality test (Saphiro-Wilk test), significance is determined by performing Kruskal-Wallis with Dunn's multiple test and yield no significance. Since some outliers are detected in seminoma group, the dataset are re-analyzed by one way ANOVA with Tukey's multiple test after the outliers are excluded. The statistical significance emerges from the comparison of MAIT cell frequency in seminoma and HD post outliers exclusion (p-value=0.023) (Graph Prism 8). (Non-significant = NS).

As many as 5 outliers are detected in seminoma group. The highest frequency of MAIT is shown by a patient whose age was 44 and diagnosed with stage IIB whereas the lowest frequency is demonstrated by a 65 year-old patient with stage

IA. This implies that, unlike NKT, MAIT frequency is reflective upon cancer stage instead of age.

I further wondered if these outliers affect significance among the 3 assessed groups. Therefore I conducted outliers data cleanse and re-analyzed the dataset with one way ANOVA. This exclusion results in significant frequency difference between seminoma and HD, suggesting that, in normal data distribution, MAIT frequency is significantly reduced in seminoma (p-value = 0.023). Moreover, the level of such decrease might be positively proportional to the stage. Although this still needs further confirmation, this early finding is seen to be in agreement with a study conducted by Walker et al., that revealed MAIT frequency was negatively correlated with aging (Walker et al., 2014). The study involved patients with severe infectious disease including HBV, HCV and HIV (Walker et al., 2013).

The distribution of peripheral NK cell subsets is markedly altered in patients with TGCT

Based on the co-expression intensity of surface molecules CD56 (neural cell adhesion molecule (NCAM) 2) and CD16 (FcγRIII) , NK cells in human are classified into two major populations being that $CD56^{dim}CD16^{bright}$ which accounts for about 90% of circulating NK cells and $CD56^{bright}CD16^{dim}$ which comprises the remaining 10% (Poli et al., 2009). Of these two, $CD56^{bright}$ NK cells predominate in lymph nodes and sites of inflammation (Chan, et al., 2007). While $CD56^{bright}$ NK cells are generally considered to be more responsive towards cytokine stimulations, $CD56^{dim}$ populations are broadly known to be more cytotoxic (Zamai et al., 2007).

In order to generate IFN-γ, the $CD56^{bright}$ NK population generally requires 2 signals. One of these almost always includes IL-12. The second can be IL-1, IL-2, IL-15 or IL-18, or the binding of an NK-activating receptor such as CD16 (FcγRIIIa) or NKG2D (Caligiuri, 2008). In the context of cytotoxicity, NK cells are more tentative and form transient contacts inducing less profound Ca^{2+} mobilization and cytoskeletal polarization. T cells, in contrast to NK cells, form stable contact with target cells (Chiang et al., 2013).

NK cells express a vast repertoire of germ-line encoded inhibitory- and activating-receptors for target recognitions. Among these receptors, CD16--a low affinity binding Fc receptor, has been thought to be the key. It was demonstrated by Tsukerman et., al (2014) that the tumor-derived expansion of NK cells can lead to

differential loss of CD16 expression in a target-cell dependent manner (Tsukerman et al., 2014).

My data shown above had revealed that the absolute percentage of total NK Cells did not differ between patients with testicular cancer and normal controls. I next analysed the relative contribution of different NK subsets based on the pattern of CD16 and CD56 expression. This approach allowed delineation of 5 different NK cell subsets (Fig. 3-10).

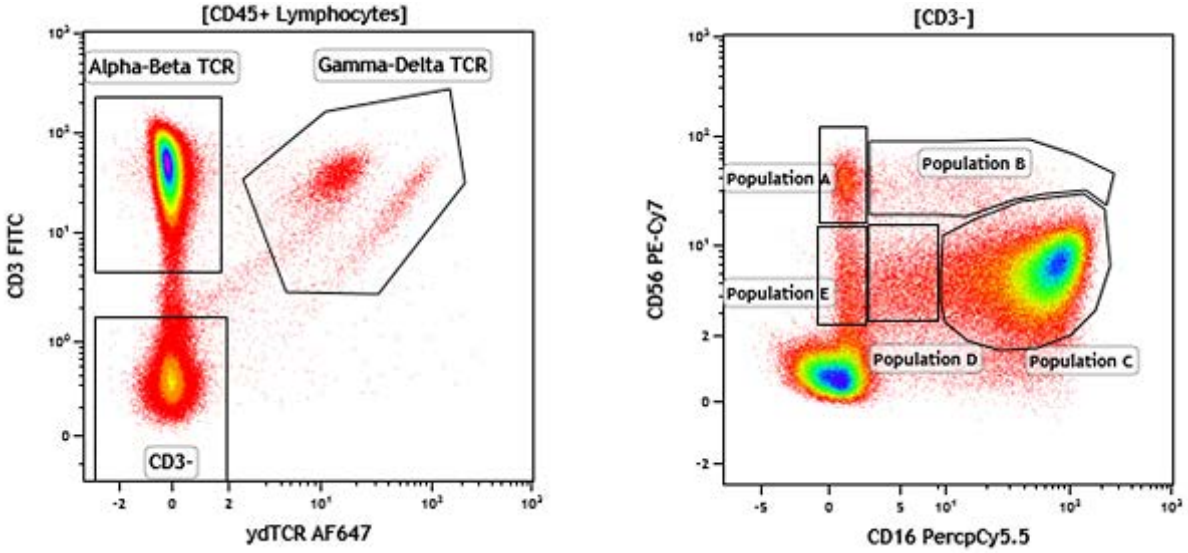

Fig. 3-10. Subset analysis of NK cells as defined by the pattern of CD16 and CD56 expression

NK cells were defined as CD3- and then gated according to relative expression of CD16 (X Axis) and CD56 (Y axis). In this way five distinctive NK cells could be obtained, termed populations A-E.

These 5 NK subgroups were assigned according to the level of expression of CD16 and CD5 at the cell surface:

1. Population A: $CD56^{Bright}CD16_{Neg}$
2. Population B: $CD56^{Bright}CD16_{Pos}$
3. Population C: $CD56^{Dim}CD16_{Pos}$
4. Population D: $CD56^{Dim}CD16_{Intermediate}$
5. Population E: $CD56^{Dim}CD16_{Neg}$

My data demonstrated that there are three populations in which marked differences are observed in relation to their expression in patient or control groups (Fig. 3-11). This was observed most strikingly in population E ($CD56^{Dim}CD16_{Neg}$) which was markedly more common in patients with seminoma (p-value<0.0001) and nonseminoma (p-value=0.003) compared with HD. This is normally a relatively rare NK cell phenotype in normal donors and its functional role is uncertain. Population D ($CD56^{Dim}CD16_{Intermediet}$) was also enriched in both the seminoma (p-value<0.0001) and nonseminoma patients (p-value<0.0007).

Populations D and E were increased in the patient group at the expense of population C ($CD56^{Dim}CD16_{Pos}$) which was markedly reduced in seminoma (p-value=0.0003) and nonseminoma (p=0.0065). Population C is the classic cytotoxic NK phenotype that dominates the peripheral repertoire in healthy donors. These data would suggest that the presence of cancer may act to suppress the expression of CD16 on $CD56^{Dim}$ NK cells, thereby driving cells from population C into the D and

E subgroups. CD16 is a powerful activation marker on NK cells and mediates antibody directed cytotoxicity. As such these data indicate that peripheral NK function is likely to be compromised in patients with TCGT.

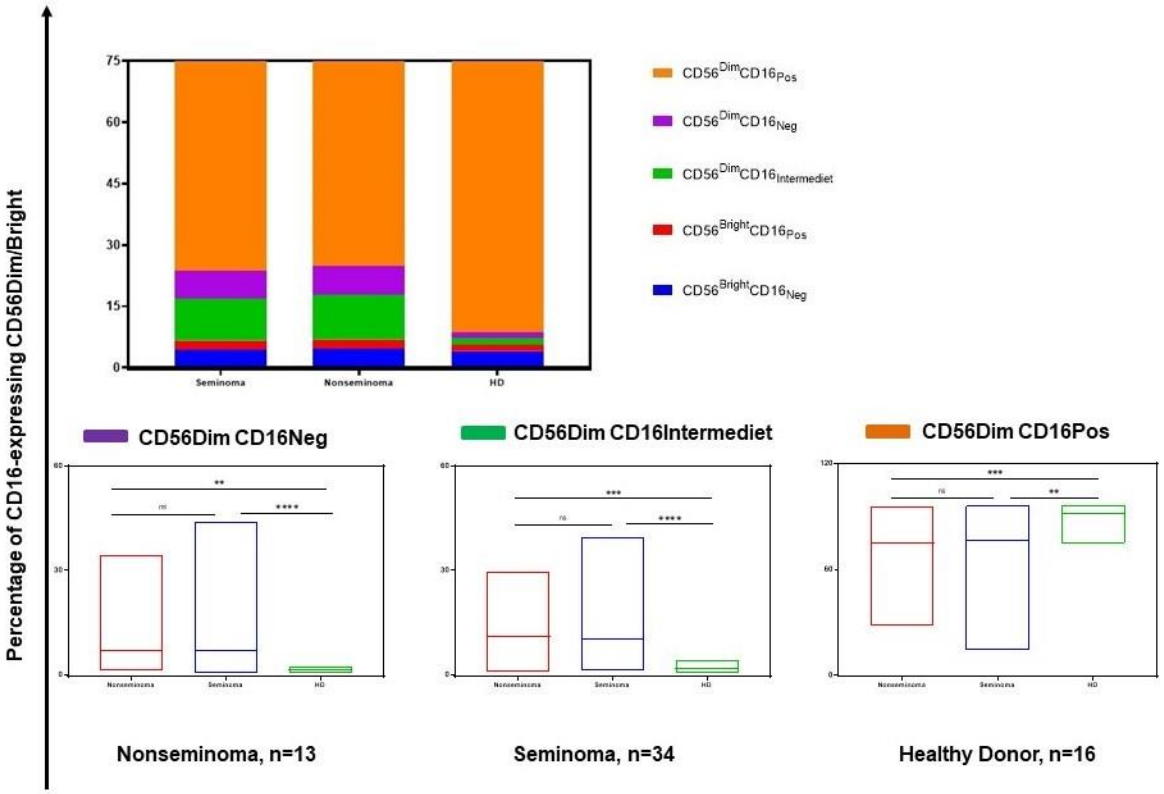

Fig. 3-11. The distribution of NK cell subsets is markedly altered in patients with testicular cancer

(Upper Panel) In the context of CD16 expression, CD56 (NK) cells distinct themselves as five populations being that $CD56^{Bright}CD16_{Neg}$ (blue bar), $CD56^{Bright}CD16_{Post}$ (red bar), $CD56^{Dim}CD16_{Pos}$ (yellow), $CD56^{Dim}CD16_{Intermediate}$ (green bar), $CD56^{Dim}CD16_{Neg}$ (purple bar). The percentages plotted on each are enumerated as a total of CD3- lymphocytes and represent average of each population. (Bottom row) Three out of the five population exhibiting statistically significant dynamics: $CD56^{Dim}CD16_{Pos}$ (yellow), $CD56^{Dim}CD16_{Neg}$ (purple bar) and $CD56^{Dim}CD16_{Intermediate}$. No significance is observed when seminoma is compared with nonseminoma. However, when each of them is compared with the HD, statistical significancies emerge. Statistical analysis was performed using Kruskal-Wallis with Dunn's multiple test (Graph Prism8) with significance is determined at $p<0.05$.

Discussion

Despite the excellent clinical prognosis of TGCT, there has been relatively little investigation of potential tumour-specific immune responses in this tumour subtype. Despite this, PD-1- and CTLA-4 checkpoint-based immunotherapy are beginning to be introduced into patient management (Seidel et al., 2018).

In order to guide introduction of these approaches it will be important to stratify treatment according to the immunophenotype of individual patients. Ideally this would be undertaken by analysis of the primary tumour and assessment of immunological features on the tumour, TIL and stromal populations. However, this is a challenging ambition at the current time and is difficult to perform on patients with enough time to optimize treatment pathways. As such, I undertook a study of the major phenotypic feature of peripheral blood in patients with TCGT. I examined the relative proportion of each fraction of immune cells observed within PBMCs in patients with either seminoma or non-seminomatous lesions and contrasted these results with those seen in healthy donors. Several studies are now indicating that the immunophenotype of blood samples can be used as an important correlate of clinical outcome in patients with cancer (Krijgsman et al., 2019).

My data demonstrated that the major subsets of immune cells within PBMCs of cancer patients were T, NK- and B cells. No gross changes in the overall proportion of T and NK cells were observed in comparison to healthy donors although some differences did emerge when these groups were broken down

further. In contrast, I did detect an increase in the relative proportion of B cells in the patients with TCGT. B cells were defined CD19+ lymphocytes and this is a broad lineage marker that does not discriminate between B cell subsets. As such, I also included CD27 in my panel as this is a reliable marker of memory B cells. This revealed that the proportion of memory B cells was similar in all three groups, suggesting that modulation of antigen-experienced B cell subsets was not the important factor. This suggests that a population of CD19+CD27- B cells is relatively increased in the patient group. At this stage it is not clear what this population might represent although consideration should be given to the immunosenescent IgG+IgD-CD27- subset that is increased in association with ageing (Buffa et al., 2011). At this stage there is no evidence that CD19+CD27+ cells are recruited into tissue. Further analysis should use an extended B cell immune phenotype in order to assess the functional significance of this observation.

γδ T cells are now of considerable interest in cancer immunology (Nussbaumer & Koslowski, 2019). However, a broad overview of the global γδ repertoire did again not reveal any differences between the patient and control groups. These cells can mediate inflammatory or immunosuppressive responses against tumour tissue (Legut et al., 2015) and may also provide help for B cell differentiation. Moreover, the cells are able to control levels of imunoglobulin and influence autoantibody production (Born et al., 2017). Further interrogation of γδ T cells in TCGT will require analysis of the different subgroups of cells and would necessitate incorporation of antibodies against a range of γ and δ receptor V regions or deep sequencing.

NKT cells are a relatively minor component of the immune repertoire but it is believed that they can have profound effects on the rest of the immune system. NKT cells are specific for lipid which is presented on the surface of CD1d, a non-classical member of the HLA gene family. The rationale for 'lipid-sensing' by the immune system is not entirely clear but it is now believed that NKT cells play an important role in determining both the 'quantity and quality' of the immune response to antigen (Terabe & Berzofsky, 2018). Having said that, no differences in NKT cell proportion were observed in my study.

I also examined the profile of MAIT cells in my panel. These cells express a conserved T cell receptor encoced from the TCR α chain Vα7.2-Jα33 which can be paired many different members of the TCRVβ repertoire. Antigenic specificity appears to be directed towards the MR1 protein and a characteristic feature of these cells, and one that is useful for laboratory investigation, is that CD161 is expressed on the majority of the population. The frequency of MAIT cells in seminoma, nonseminoma and HD exhibited no statistical differences between groups.

As described in the Introduction, Natural Killer (NK) cells play a pivotal role in the control of transformed cells and NK cell evasion is emerging as a hallmark of many tumours, especially in metastatic disease (Lorenzo-Herrero et al., 2019). Similar to the findings in relation to T cells, there were no differences observed in the proportion of total NK cells between the three groups of subjects. However NK cells encompass considerable heterogeneity and I therefore went on to assess the relation distribution of cells in relation to the intensity of CD16 and CD56 expression.

Here I discovered significantly reduced expression of CD16 on $CD56^{dim}$ (NK^{dim}) population in seminoma and nonseminoma patients compared with HD. $CD56^{dim}$ NK cells are normally highly cytotoxic and this is strongly mediated through Ab-mediated cell cytotoxicity (ADCC) via expression of the Fc receptor CD16 (FcγRIIIa). Indeed, ADCC is clinically important as a primary mechanism of therapeutic antibodies and binding of Fc receptors has been shown to be critical for their activity *in vivo* (Srpan et al., 2018). As such the loss of CD16 would be expected to significantly compromise their functional activity.

Although the origin and function of the $CD56^{dim}$ $CD16^{-/dim}$ NK population is unclear the population is widely thought as a highly heterogenous population comparising of both maturing and target cell-activated cells. A similar population of $CD56^{dim}CD16^{Neg}$ cells has been observed in pateints with advanced melanoma and here is was also reported that this population is markedly increaed in the tumour microenvironment. Importantly, cytotoxic activity remained strongly associated with retention of the $CD56^{dim}CD16^{Pos}$ subset (Vujanovic et al., 2019).

Amand et al reported that a $CD56^{dim}CD16^{dim}$ population appears to be a relatively immature population of NK cells with a lower level of CD57 and increased NKG2A in comparision to $CD56^{dim}CD16^{bright}$ subsets (Amand et al., 2017). They may represent an intermediate population between the dominant $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{neg}$ subsets but at this stage the pathway for differentiation of these subsets is poorly understood. Chemokines play a dominant role in regulating the

transit of NK subsets and in future work I would have wished to have examine this repertoire in more detail (Wang and Reinherz, 2012).

One of the limitations of my study is that I was not able to investigate the number or phenotype of T regulatory cells. These cells act as an important determinant of cancer outcome and would represent an important area for future investigation (Jørgensen et al., 2019). Also I was not able to assess how these changes were influenced by disease stage and this could be assessed in future studies.

Overall, my findings in this chapter show that no differences were observed in the overall broad phenotype of T cell subsets within peripheral blood in patienst with testicular cancer. In particular, analysis of αβ and γδ subsets, as well as MAIT and NKT subsets did not reveal significant differences. In contrast, the proportion of CD27- B cells was increased in the patient group. The most profound changes were observed in relation to the distribution of NK cell subsets where I detected a significant increase in the proportion of $CD56^{dim}$ cells that had downregulated or lost CD16.

Future studies on immune phenotyping should focus on more detailed characterization of immune subsets, including multiparametric CyTOF or flow cytometry analysis, potentially combined with analysis of sequence data from antigen receptors. My data indicate that the peripheral immune system is significantly altered in patients with testicular cancer although the mechanisms behind this, and its clinical significance, remain unclear.

CHAPTER IV. THE PROFILE OF IMMUNE CHECKPOINT PROTEIN EXPRESSION ON PBMC IN TGCT PATIENT COHORT

Although surgery and chemotherapy are highly effective in the management of testicular cancer it is important to remember that this is not effective in every case and many men die of their disease each year. As such it is important that we continue to aim to develop new treatments for testicular cancer that can achieve cure in every case. In addition, approaches such as immunotherapy may prove to be less toxic than chemotherapy and so may act to reduce morbidity within the patient cohort (D'Souza et al., 2015).

It is unclear why TGCT are so curable by chemotherapy and what lessons may be learnt from this in relation to immunotherapy. Of interest, inactivation of the p53 signalling pathway is observed in almost all solid tumours but p53 is not typically mutated in TGCT. As such, the ability of drugs such as cisplatin to mediate excellent levels of cell death may reflect effective induction of apoptosis pathways. Taken together, these considerations indicate that TGCT can also act as a model system in which to assess optimal molecular determinants of chemotherapy sensitivity in solid tumours (Boublikova et al., 2014). However, modulating common epithelial tumours to obtain the genetic profile of TGCT is currently beyond the scope of gene therapy.

Cancer immunotherapy is aimed primarily at either reinforcing an endogenous anti-cancer immune response in the host or at adoptively transferring anti-tumour

immunocompetent cells in those setting where such a response is not apparent. In the latter case either autologous or third-party immune effector cells are expanded *in vitro* and then infused into the patient (Minato & Honjo, 2016).

Two main approaches that are often considered in cancer immunotherapy are passive and active immunotherapy. *Passive immunotherapy* is principally aimed at enhancing the existing anti-tumour responses through the administration of immunological agents such as monoclonal antibodies, cellular products or cytokines. In contrast, *active immunotherapy* attempts to generate a long-lasting immune response to destroy the tumour cells. This strategy can be implemented through approaches such as immunomodulation or vaccination (Zhang & Chen, 2018).

Immunological products including cytokines (IFN-α and IL-2), monoclonal antibodies (for instance trastuzumab, bevacizumab and ipilimumab) and anticancer cell-based therapy (as in the case of Sipuleucel-T) have received regulatory approval either as single anticancer agents or to be combined with chemotherapy (D'Souza et al., 2015).

Table 4-1. Implemented Passive and Active Immunotherapy

Passive Immunotherapy		Active Immunotherapy	
Immunomodulating antibodies	**Adoptive Immunotherapy**	**Specific**	**Nonspecific**
• Immune checkpoints inhibitors • Immune costimulatory antibodies	• Tumour-infiltrating lymphocytes • TCR gene-modified lymphocytes • Chimeric antigen receptors (CARs)	• Vaccines	• Immune adjuvants • Cytokines

Immune checkpoint blockade

The immune micro-environment within a tumour mediates integration of multiple interactions between tumour cells and reactive cells, including lymphocytes, antigen presenting cells (APCs) and stroma (Rabinovich et al., 2007). The development and activity of the host immune response against tumour tissue will reflect the net balance of inhibitory and stimulatory signals. A further concept that has emerged has been that of activating and inhibitory 'immune checkpoints' that maintain immune homeostasis and preventing auto-immune disease by limiting effector lymphocyte responses. Tumours, however, may co-opt immune-checkpoint pathways. As a consequence, T cell-mediated anti tumour immunity may be supressed and this leads to the promotion of tumour growth (Pardoll, 2015).

Immune checkpoint inhibitors are a novel class of drugs that are designed to enhance immune response by competitively binding the inhibitory checkpoint receptors (Zhang & Chen, 2018; Kareva, 2018). Indeed, immune checkpoint

blockade has been recently seen as one of the most promising approach to activate anti-tumour immunity (Pardoll, 2016). Weakening the inhibitory checkpoint activity can boost inflammatory immune responses and improve patient outcomes in a wide range of tumour settings (Kareva, 2018). CTLA-4, PD-1, T-cell immunoglobulin and mucin domain containing protein 3 (Tim-3) and lymphocyte activation gene-3 (LAG-3) are prototypic checkpoint proteins and antibodies that block their interactions are already in clinic or pre-clinical development. Triggering the activation of these markers by engagement with their ligands on tumour cells is believed to cause hyporesponsiveness and eventually tumour-specific T cell exhaustion. The first successful therapy was the anti-CTLA-4 antibody ipilimumab which was introduced for the treatment of metastatic melanoma in 2011 (Savoia et al., 2016). This marked the beginning of a new era for cancer immunotherapy. Subsequently, pembrolizumab and nivolumab, two antibodies that block PD-1, were found to have remarkable activity in a wide range of cancer settings (Alsaab et al., 2017). Squamous cell lung cancer and melanoma are particularly susceptible to disruption of the PD-1:PD-L1 interaction and this may reflect that fact that these tumours have been exposed to high mutagenic challence in the environment in the form of smoking and sunlight. Atezolizumab is an antibody that can block the major ligand of PD-1, PD-L1, and is finding a role in bladder cancer (Inman et al., 2017).

Despite this success, checkpoint inhibition is also associated with side effects in many tissues which reflect overactive ‘auto-immune’ processes. These may require cessation of therapy or instigation of steroid treatment.

On the basis of these observations, it is of paramount importance to exactly know which inhibitory checkpoint pathway(s) can be harnessed by the different tumour types. I have therefore developed an antibody panel called the 'Checkpoint Panel' which would allow me to examine by flow cytometry the simultaneous expressions of five major checkpoint inhibitors, namely PD-1, TIGIT, Tim-3, CTLA-4 and LAG-3, on peripheral blood cells from patients with testicular cancer.

Table 4-2. Checkpoint-Blockade-Based Cancer Immunotherapy

Target	Drug name	Cancer Type	Current Status
PD-1	Nivolumab	Melanoma, lung cancer	FDA approved
		Multiple cancers	Phase I-III
	Pembrolizumab	Melanoma	FDA approved
		Multiple cancers	Phase I-III
	MED10680	Multiple cancers	Phase I
	AMP-224	Multiple cancers	Phase I
	Pidilizumab	Multiple cancers	Phase I-II
PD-L1	Atezolizumab	Multiple cancers	Phase I-III
	MED14736	Multiple cancers	Phase III
	Avelumab	Multiple cancers	Phase I-III
	BMS-936559	Multiple cancers	Phase I
CTLA-4	Ipilimumab	Melanoma	FDA approved
		Multiple cancers	Phase I-III
	Tremelimumab	Multiple cancers	Phase I-III
LAG-3	IMP321	Multiple cancers	Phase I
	BMS-986016	Multiple cancers	Phase I
B7-H3	Enoblituzumab	Melanoma, prostate cancer	Phase I

Programmed Death-1 (PD-1)

The PD-1 immunoinhibitory receptor is a type-1 transmembrane protein that belongs to the CD28 family (Dong et al., 2017). This is typically expressed by activated immune cells including T cells, B cells and myeloid cells (Nowicki et al., 2017). It may also be present on some tumour cells (Kareva, 2018). PD-1 is delineated as a protein with capability of promoting apoptosis and inhibiting proliferation. Glucose metabolism and cytokine signalling in antigen-specific T cells are also correlated with checkpoint protein expression. PD-1 engagement also appears to reduce apoptosis of regulatory T cells, further increasing the immunosuppressive state of the microenvironment (Gianchecchi & Fierabracci, 2018).

PD-1 suppresses cellular activation and the cytoplasmic tail has two tyrosine residues which may undergo phosphorylation. PD-1 binds to either PD-L1 (CD274) or PD-L2 (CD273) which display moderate homology and whose differential role is not entirely confirmed. Genetic evidence from PD-1 deficient T cells suggests that PD-L1 and PD-L2 may also bind to a still unknown co-stimulatory receptor that is expressed on T cells (Pardoll, 2015).

PD-L1 and PD-L2 are expressed on a range of cells, including lymphoid and non-lymphoid populations, and their expression is increased by inflammatory mediators such as IFN-γ (Dong et al., 2017). This is thought to represent one potential mechanism for inflammation-mediated resistance to cancer immunotherapy. The

PD-1:PD-L1 interaction is very important in immune homeostasis and PD-1-deficient mice can develop auto-immunity, splenomegaly and expanded lymphoid tissues (Mak & Saunders, 2006).

TIM-3 (T Cell Immunoglobulin and Mucin-domain containing-3)

T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) is a negative regulator of T cell activation and expressed on many immune cells, namely NK cells, macrophages (Sakuishi et al., 2013), T cells, regulatory T cells (Tregs), dendritic cells (DCs), B cells and mast cells (He et al., 2018). Tim-3 is also expressed on regulatory T cells and it is of interest that this is seen most strongly within the tumour bed rather than in peripheral sites (Yan et al., 2013). .

Tim-3 is a type I membrane protein and consist of 302 amino acids. Its basic structure encompasses an extracellular IgV domain, a single transmembrane domain, a C-terminal cytoplasmic tail (Monney et al., 2002) and a glycosylated mucin domain with varying length . Despite the absence of either an ITIM and ITAM inhibitory signalling motif, Tim-3 has 5 tyrosine residues in its cytoplasmic tail which may undergo phosphorylation (Meyers et al., 2005). In the absence of ligand binding Bat3 and Lck are recruited to this tail and this promotes T cell signalling. In contrast, when Tim-3 binds to a ligand Bat3 is released, Fyn becomes recruited and T cells are suppressed (Granier et al., 2017). There are four ligands that can bind to Tim-3 including galectin-9 (Gal-9) (Zhu et al., 2005), HMGB1 (Fonslow et al., 2013), carcinoembryonic antigen cell adhesion molecule 1 (Ceacam-1) (Dankner et al.,

2017) and phosphatidylserine (Weber & Zhou, 2017) . The first identified ligand was Gal-9 which binds to the carbohydrate motif on Tim-3 (Zhu et al., 2005). PtdSer binding promotes antigen cross presentation by DCs (Trahtemberg & Mevorach, 2017). Nucleic acid released by dying cancer cells are bound by HMGB1 leading attenuation of the innate immune responses (Fonslow et al., 2013). The most recent ligand characterized was Ceacam-1 whose co-expression with Tim-3 functions as a negative regulator of T cell-mediated immune responses. The interaction between Ceacam-1 and Tim-3 can be either cis or trans, and both allow T cell immune tolerance (Du et al., 2017).

Expression of Tim-3 is highly associated with T-cell exhaustion that leads to anti-tumour immunity inhibition. Blocking this checkpoint molecule allows T cells increase their cytokine production, mainly int the form of IFN-γ. It is therefore enhancing T cell-mediated cancer immunity. In *in vitro* and *in vivo* models, the presence of Tim-3+ CD8+ T cells were seen to be correlated with PD-1 expression (He et al., 2018). Dual expression of Tim-3 and PD-1 is characteristic of highly exhausted CD8+ T cells and dual blockade against PD-1 and Tim-3 appear synergistic in boosting T cell function (Zhou et al., 2011). This does suggest that the molecules have non-redundant functions (Anderson et al., 2016)and this has been shown *in vitro* (He et al., 2018). Currently, the application of one monoclonal antibody against Tim-3 (MBG453) to patients with advanced malignancies (NCT02608268) is being investigated in phase I-II clinical trial. Yet, no clinical results are reported (Marin-Acevedo et al., 2018).

T Cell Immunoglobulin and ITIM Domain (TIGIT)

TIGIT also known as WUCAM, Vstm3 or VSIG9 is a member of the poliovirus receptor (PVR)/nectin family, a subset of the immunoglobulin superfamily (Marin-Acevedo et al., 2018; Manieri et al., 2017). TIGIT expression is restricted to lymphocytes and is seen on a range of cells, including effector, follicular helper and regulatory subsets (Catakovic et al., 2017).

TIGIT indirectly increases the production of immunoregulatory cytokines like IL-10, and on the other hand inhibits the production of IFN-γ and IL-17a, therefore acting to suppress DC maturation (Li et al., 2014). There are two dominant agonists for TIGIT, the poliovirus receptor (PVR;CD155) and nectin-2 (PVRL2; CD112), which are quite widely expressed (Yu et al., 2009). High level expression of TIGIT has been seen in many populations of tumour infiltrating lymphocytes (Li et al., 2019).

T cell functions, generally, are regulated by inhibitory receptors via 3 main mechanisms: (1) cell-intrinsic modulation through intracellular signalling domain of the receptor (Attanasio & Wherry, 2016), (2) indirect effects resulted from competition among T cells and costimulatory receptors for shared ligands on APCs (Chen & Flies, 2013), and (3) function modulation of the ligand-expressing cells (Escors, 2011). Interestingly, in 2014 Johnston et al. discovered that TIGIT's immunomodulatory effects were CD226-dependent. TIGIT is shown to affect CD226 signalling by physically rather preventing its homodimerization than competing for a

ligand, representing a novel mechanism through which inhibitory receptors can elicit their immunomodulatory effects (Johnston et al., 2015).

Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)

The interaction between CD80 or CD86 on dendritic cells, and CD28 on T cells, is a key 'second signal' that facilitates activation of the effector cell during a primary immune response (Beyersdorf et al., 2015). CTLA-4 is a fascinating protein that is expressed on activated T cells and has exceptionally high affinity for CD80/CD86. As such it can act to physically pull CD80 off the surface of the dendritic cell and suppress T cell activation. Ipilimumab is a monoclonal antibody that binds to, and blocks, CTLA-4 and has been shown to have efficacy in the treatment of metastatic melanoma (Savoia et al., 2016). This is attributed by its ability to improve the strength and breadth of primary immune responses, presumably against tumour antigens.

LAG-3 (Lymphocyte Activation Gene-3)

LAG-3 (CD223) is a co-inhibitory receptor that suppresses T cell activation. n physiological condition, it presents to maintain the immune homeostasis. It is expressed on many immune cells but the downstream processes involved in LAG-3 signalling are currently unclear (Andrews et al., 2017) . LAG-3 expresses four IgG loops and as such is somewhat similar to CD4, but there is only around 20% amino acid homology and LAG-3 has an additional loop (Drake & Drake, 2011). This may assist it in binding to MHC-II with greatly increased affinity and subsequently reducing the strength of TCR signaling (Maçon-Lemaître & Triebel, 2005).

The application of LAG-3-targeted immunotherapy started with a LAG-3/Ig fusion protein (IMP321) and there are now many such reagents in development (Isakov, 2018).

To our best knowledge, the inhibitory checkpoint pathway most commonly associated with TGCT is PD-1/PD-L1, seen on around 60-70% of tumours (Fankhauser et al., 2015). PD-L1 expression can also be seen on stromal cells although interestingly this was seen only in seminomas. As such PD-L1 may represent an interesting target in clinical treatment. This study employed IHC technique on archive FFPE TGCT tissues. I used a different method in which flow cytometry was used to detect and quantify the simultaneous expression of the five checkpoint proteins mentioned above. This is important to gain some understanding about possible interplays among these checkpoints (Fig. 4-1).

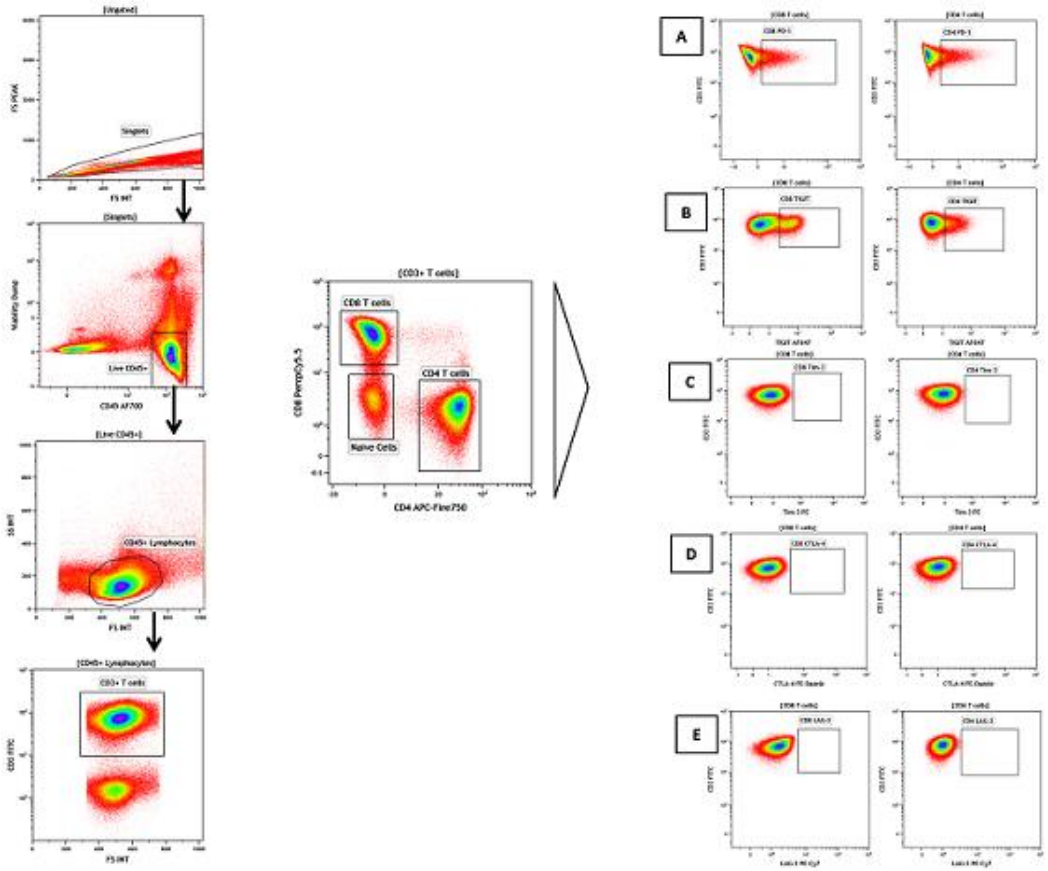

Fig. 4-1. Gating strategy to assess the expression of checkpoint proteins

First, cells were gated for singlets on the basis of FS PEAK (Y Axis) and FS INT (X Axis). Second, live CD45 cells were obtained. Third, live CD45+ cells were gated on the basis of the expressions of CD3. Next, cells were gated for the expression of CD4 or CD8. Finally, checkpoint expression was measured on the two major T cell subsets. Checkpoint markers examined in this study were: A) PD-1, B) TIGIT, C) Tim-3, D) CTLA-4 and E) LAG-3.

Our sample cohort consisted of peripheral blood samples from patients with seminoma (n=37) or non-seminoma (n=14) and also healthy donors (HD) (n=16). I presented my data as a median followed by quartile values. Statistical significance was determined using Kruskal-Wallis with Dunn's multiple test, significance at $p<0.05$. To allow me to measure the simultaneous expression of more than one checkpoint marker on a cell surface, in addition to conventional quadrant gating strategy I also performed a Boolean gating scheme by which as many as 32 different expression combinations could be obtained by consolidating five checkpoint markers for each T cell subset. I therefore had a total of 64 expression combinations.

Furthermore, single expression of a checkpoint protein has been defined in this chapter as a cell that expressed only one checkpoint marker and not the others. For example, PD-1-expressing T cells refers to a population consisting of PD-1+/TIGIT-/Tim-3-/CTLA-4-/LAG-3- T cells (either CD8+ or CD4+), and so forth. To enable this, Boolean gating scheme was used. This approach provided many advantages especially in the sense of multiple-probes/fluorochromes-stained-cell enumeration since they could be discriminated thoroughly according to their expression patterns.

PD-1 alone is expressed at a low level on CD4+ and CD8+ T cells within peripheral blood

PD-1 has been the most studied checkpoint in the context of cancer immunotherapy in TGCT. As discussed earlier, PD-L1 has been shown to be expressed within seminoma tumours and I had therefore anticipated that PD-1+ T cells might be detected at increased frequency in the blood of patients. Indeed, analysis of PBMC

from 25 HD and 42 non-small cell lung cancer (NSCLC) patients found selective increase on CD4+ in the patient group (Zheng et al., 2016).

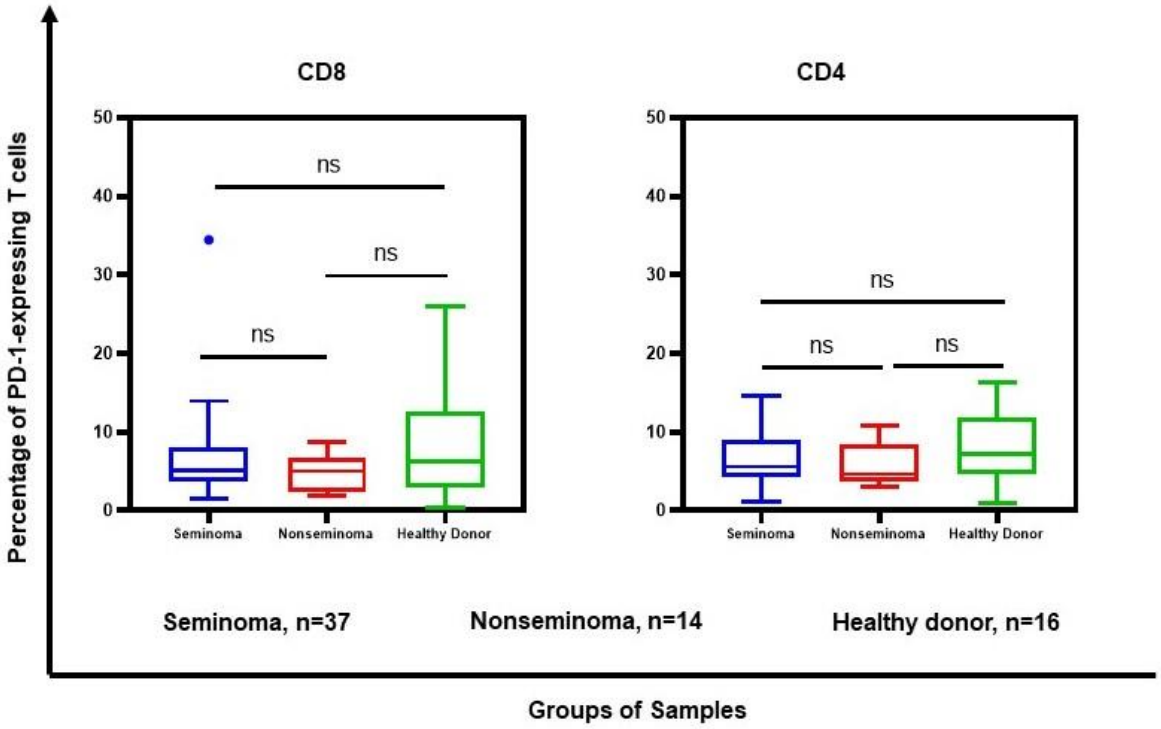

Fig. 4-2. The magnitude of single expression of PD-1 observed in seminoma and non-seminoma in comparison with healthy donor.

However, my data showed otherwise (Fig. 4-2). Indeed, PD-1 alone was expressed at low frequency on both CD4+ and CD8+ T cells, at a median level of around 3-4% and a broad distribution between donors. Of note, whilst the data within the CD4+ T cell subset appeared to be normally distributed, we found some extreme outliers in relation to CD8+ T analysis. In particular one donor in the seminoma group had a frequency of 35% PD-1+ CD8+ T cells. This might be associated with the age and the stage of the patient. Among others, the patient is considerably older as aged 56

years old. Furthermore, the patient was diagnosed with stage IIIC where the stage is the most advanced within the entire dataset.

One of our HD also displayed an exteremely high expression of PD-1 that reached a level of 26%. But this feature seems to be irrelevant with the age as the particular donor was only 25 years old. This abrogates my initial assumption that in physiological condition, PD-1 expression may positively correlated with age.

Further studies will be required to investigate the relationship between PD-1 expression and clinical data such as tumour stage, grade and metastases.

Single expression of TIGIT is observed on many CD8+ T cells within peripheral blood

I next went on to assess the expression of TIGIT on peripheral T cells. The frequencies of CD8+ T cells expressing 'TIGIT alone' in seminoma, non-seminoma and HD were 32.3% [IQR 23.8, 39.8], 25.3% [IQR 19.3%, 29.8%] and 31.1% [IQR 18%, 34.7%], respectively (Fig. 4-3). TIGIT expression of CD4 cells was somewhat lower, typically around 12-14% and did not vary between groups.

Indeed, TIGIT was the checkpoint protein that was expressed most strongly by most cells within my analysis. The frequency of TIGIT expression is approximately 2.5 times greater than that of PD-1 in both T cell subsets in all the groups and this represents the first such analysis that has been reported.

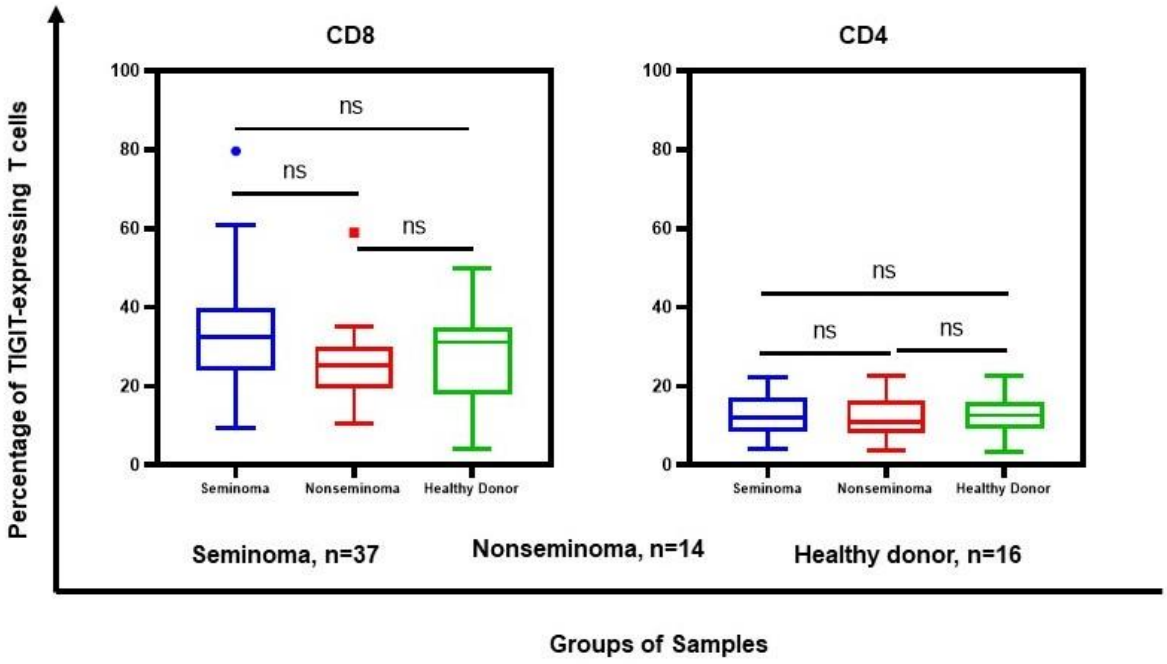

Fig. 4-3. Profile of single TIGIT expression on T cells within peripheral blood

TIGIT is expressed by many cells within the CD8+ (left) and CD4+ (right) T cell subsets. The frequency differences among the 3 investigated groups are not statistically significant (Kuskal-Wallis with Dunn's multiple test, significant at $p<0.05$).

However, even though TIGIT was the most frequent expressed checkpoint in seminoma, non-seminoma and HD, no significant differences were observed between the three groups. The high percentage of TIGIT+ CD8+ T cells in HD suggests that TIGIT might be a marker of senescence/exhaustion. I initially assumed that this might be attributed by age. In order to address this, I picked top 5 highest TIGIT-expressing patients in seminoma and correlated this expression with their corresponding age and stage. The highest TIGIT-expressing patient (68.5%) aged 49 years old with stage IA. On the other hand, a patient who was in stage II C with

features of metastases were observed and aged 56 years old, expressed TIGIT almost a half lower (39.6%). Likewise, a patient who was 54 years old with stage IB seminoma showed 5% lower expression of TIGIT in CD8 population than a 49 year old-patient with stage IB. Similar trends were also observed in nonseminoma and HD. Therefore, speculation that TIGIT expression might be age- and stage-affected is weakened. A bigger size of sample is necessary for further investigation. Song et al (2018) have recently evaluated TIGIT expression in PBMCs collected from HD of different ages by flow cytometry and found results that were comparable to my data. In addition, they also reported that older donors had a higher frequency of TIGIT+ T cells compared with younger donors (Song et al., 2018). However, the majority of these $TIGIT^+$ $CD8^+$ T cells also expressed other inhibitory receptors including PD-1. Furthermore, they exhibited features of exhaustion such as downregulation of CD28 and high susceptibility to apoptosis. It is possible that TIGIT+ CD8+ T cells are undergoing an early stage of T-cell immunosenescence, a phenotype associated with loss of the ability of self-renewal and long-term survival (Kasakovski et al., 2018).

The profile of single Tim-3 expression on peripheral blood T cells

I further went on to determine the profile of Tim-3 expression on T cells within the 3 cohorts. Tim-3 expression was found to be much lower compared to PD-1 and TIGIT with median values that were below 1% of the T cell pool. However, here for the first time I observed statistically significant differences between the findings in the control group and the patient cohorts (Fig. 4-4).

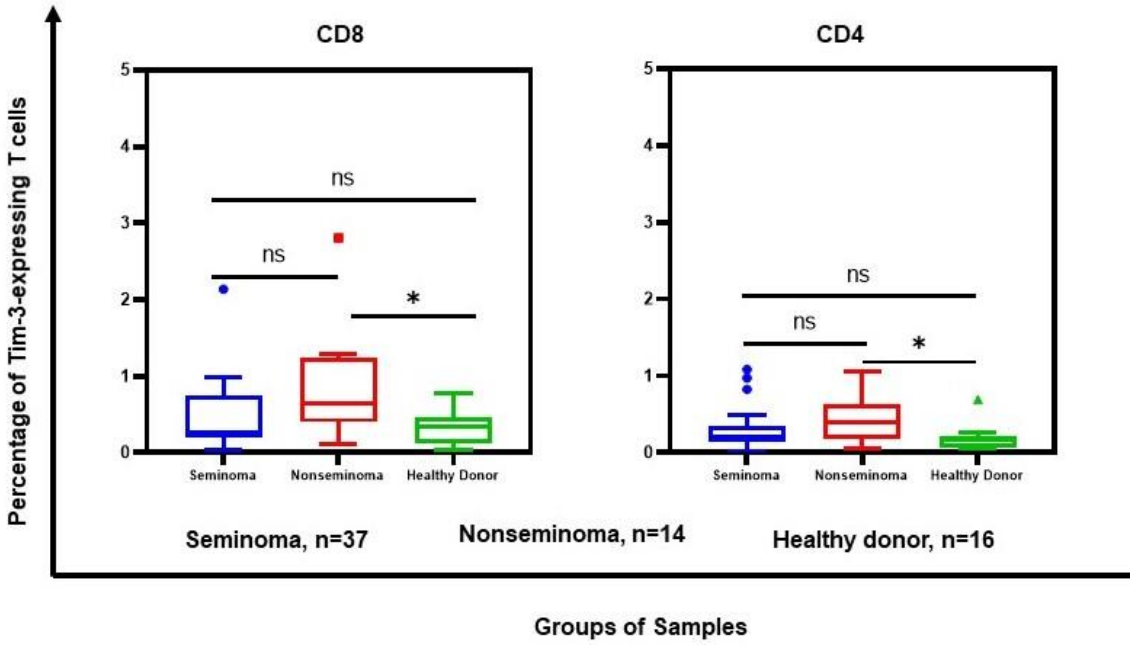

Fig. 4-4. The pattern of single Tim-3 expression on peripheral blood

Tim-3 expression is significantly increased on CD8+ T cells from patients with non-seminoma tumours compared to the other two groups. Within the CD4+ cell subset this value is increased in nonseminoma compared to HD.

In particular, Tim-3 expression on CD8+ T cells from the seminoma, nonseminoma and HD groups are the levels of 0.27% [IQR 0.19%, 0.75%], 0.64% [IQR 0.40%, 1.25%] and 0.35% respectively. Expression frequency on the CD4+ T cell subset was 0.06% [IQR 0.04%, 0.10%], 0.09% [IQR 0.06%, 0.13%] and 0.03% [IQR 0.02%, 0.06%] in these groups respectively [IQR 0.13%, 0.47%]. As such the frequency of expression of this checkpoint marker on CD8+ T cells is 4 to 10-fold higher compared with the values on CD4+ T cell. At this stage it is not clear if this represents the fact that CD8+ T cells experience more intense exhaustion caused by persistent antigen

exposure or if this checkpoint protein has a differential mechanism of action on CD4+ and CD8+ subsets.

In the CD8+ T cell subset, I saw two comparisons that generated statistical significance while in CD4+ cell subset only a comparison turned out to be significant. Significances in CD8+ T cell subset were obtained from non-seminoma vs HD with p-value of 0.002 (Kruskal-Wallis with Dunn's multiple test, $p<0.05$). Within the CD4+ T repertoire, Tim-3 is strikingly enhanced in non-seminoma compared to HD with p-value=0.015 (Kruskal-Wallis with Dunn's multiple test, $p<0.05$).

By exhausting T cells, Tim-3 weakens anti-tumour immunity. Tim-3+ $CD8^+$ T cells exhibit dysfunctional STAT5 and p38 signalling. Blocking of Tim-3 pathway can improve cancer immunity because interferon-gamma (IFN-γ) will be generated more by T cells (Li et al., 2018). The expression of Tim-3+ CD8+ T cells seems to be having some correlation with PD-1 expression in both *in vitro* and *in vivo* models. It can inhibit the effector T cell proliferation and reduce cytokine production such as interleukin-2 (IL-2) (Carter et al., 2002).

At this stage it is not clear why Tim-3 would be increased on T cells from non-seminomatous tumours but not the seminoma subtype. It is certainly true that this category of tumours have a worse clinical outcome and it is possible that the increase of Tim-3 might reflect this, as a reflection of loss of immune control.

The pattern of co-expression of PD-1 and TIGIT on peripheral blood T cells

I next went on to measure the pattern of co-expression of PD-1 and TIGIT on PBMC from TGCT patients and HD (Fig. 4-5). The frequencies of PD-1/TIGIT co-expression on CD8+ T cells were observed at median levels of 10.2% [IQR 6.57%, 13.40%], 7.90% [IQR 5.97%, 15.30%] and 10.8% [IQR 3.45%, 13.805] in seminoma, non-seminoma and HD cohorts, respectively. This co-expression frequency was also quantified in the respective CD4+ T cell subsets at 4.65% [IQR 3.48%, 7.02%], 4.42% [IQR 2.81%, 6.03%] and 6.30% [IQR 3.48%, 7.64%] respectively. As such, the level of PD-1/TIGIT co-expression was typically around 2-fold higher on CD8+ T cells compared to the CD4+ subset.

These data show that the frequency of cells that shows co-expression of PD-1 and TIGIT was larger than PD-1 single expression. However, when compared with TIGIT single expression, PD-1 and TIGIT co-expression was observed at slightly lower frequencies.

In 2018, co-expression of PD-1 and TIGIT was investigated by Li et al (2018). They carried out fluorescence measurements on archived FFPE of Nodular Sclerosis Classical Hodgkin's Lymphoma (NSCHL) tissues. Their results showed that the majority of T cells (68%) in NSCHL had co-expression of TIGIT and PD-1, while single positivity for TIGIT and PD-1 was seen in only 14% and 5% respectively. The remaining 13% T cells had neither TIGIT nor PD-1 expression (Li et al., 2018). These results, from another tumour subtype, are somewhat comparable with my own

observations. However, it was important that I had been able to include control samples in my analysis and this showed that there was no additional increment in the proportion of T cells that exhibited PD-1 and TIGIT co-expression within the cancer patients.

An important consideration is that I chose to focus my studies on the percentage of cells that were positive for cytokine expression. On reflection, it is clear that it would also have been valuable to measure the mean fluorecence intensity (MFI) of cytokine production and these datasets are available for future study.

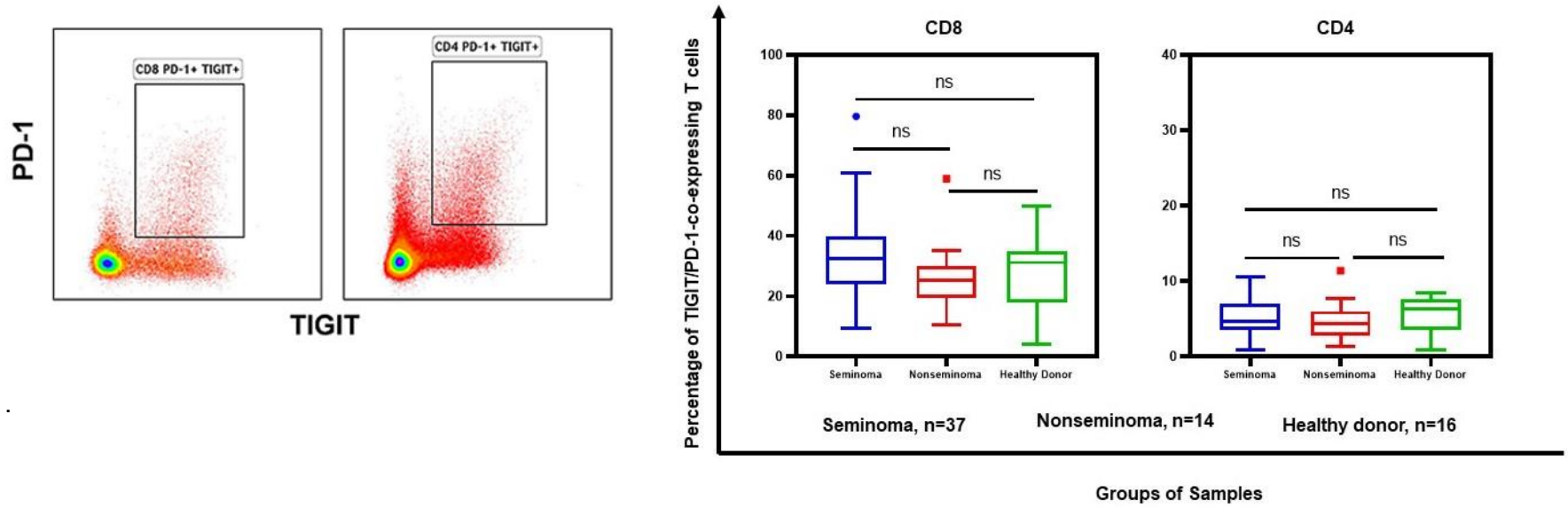

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Fig. 4-5. Co-expression of PD-1 and TIGIT on peripheral blood cells from patients with testicular cancer and healthy donors

(Left panel) Gating strategy to determine and quantify the frequencies of PD-1/TIGIT-expressing T cells. (Right panel) Data of PD-1/TIGIT-expressing T cell is presented individually with median and quartile values (upper and lower quartiles) for each group. The frequencies of the co-expression represent the number of positive T cells within the respective T cell subset

The profile of single CTLA-4 expression on peripheral blood T cells

The next checkpoint protein that I examined was CTLA-4, a protein which binds strongly to CD80/86 and competes with CD28 to suppress T cell activation. The pattern of single CTLA-4 expression on peripheral blood was rather low and indeed somewhat similar to that observed for Tim-3. In particular, CTLA-4 was expressed by either CD8 or CD4 at low frequencies. However, a major difference here was that expression was increased on CD8+ and CD4+ T cells in samples from patients with both seminoma and non-seminomatous tumours (Fig. 4-6).

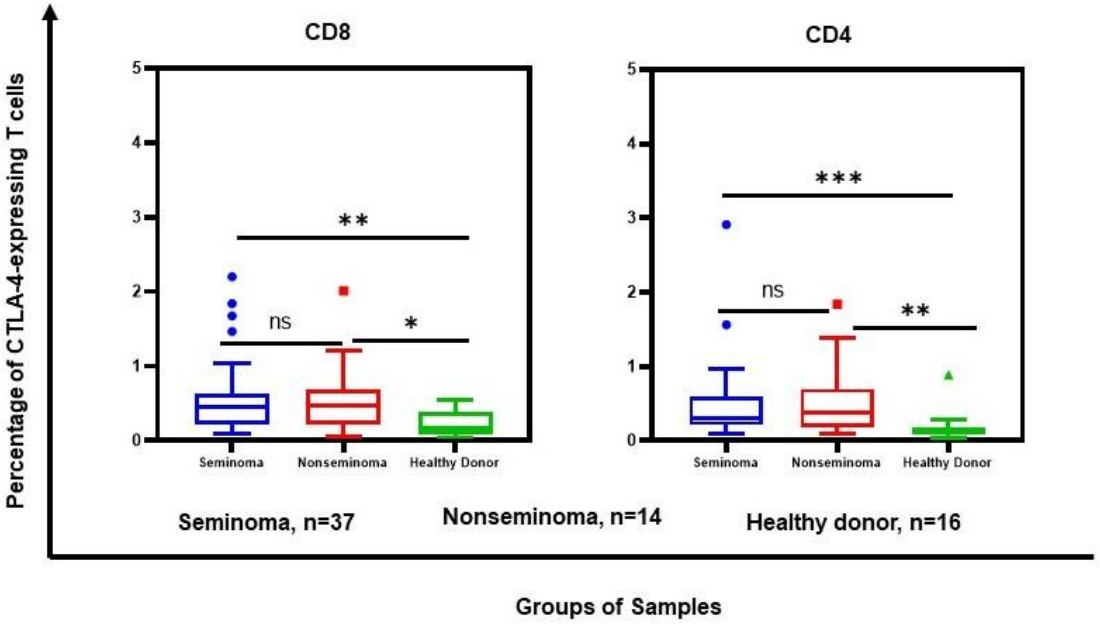

Fig. 4-6. The pattern of CTLA-4 expression on T cells within peripheral blood

CTLA-4 expression frequency is significantly increased in seminoma and non-seminoma groups compared to HD in both CD8+ and CD4+ T cell subset.

The percentage of CTLA-4+ CD8+ T cells in patients with seminoma, non-seminoma and in HD respectively were 0.45% [IQR 0.21%, 0.63%], 0.46% [IQR 0.21%, 0.69%] and 0.16% [IQR 0.09%, 0.39%] respectively. Within the CD4+ T

cell subset, the comparable CTLA-4 expression frequencies were 0.31% [IQR 0.21%, 0.60%], 0.37% [IQR 0.18%, 0.70%] and 0.15% [IQR 0.09%, 0.19%] respectively. As such the frequencies of CTLA-4 expression on CD8+ and CD4+ T cells appear to be broadly comparable. In the CD8+ population, the increase of CTLA-4 yielded p-values of 0.003 and 0.021 for the comparisons of seminoma vs HD and nonseminoma vs HD, respectively (Kruskal-Wallis with Dunn's multiple test, $p<0.05$). For the CD4+ population, the frequencies of CTLA-4-expressing cells in seminoma and nonseminoma resulted in p-values of 0.0005 and 0.005 when each was compared to HD (Kruskal-Wallis with Dunn's multiple test, $p<0.05$). No statistical significance was observed between seminoma compared to nonseminoma.

The profile of single LAG-3 expression on peripheral blood T cells

Finally, I next investigated the expression of LAG-3 on the peripheral blood samples. LAG-3 was the least expressed checkpoint marker, with individual frequencies that varied from very low to undetectable in the majority of samples. However overall expression was higher on CD8+ T cells from both tumour groups compared to the control subjects (Fig. 4-7). A similar profile was observed for the CD4+ subset in relation to the seminoma samples. It should be noted that there were occasional outliers in both these groups. In particular, LAG-3 was expression on 1.4% of CD8+ T cells from one donor whilst another donor showed a value of 9.4% on the CD4+ subset. Despite these outliers it was clear that these did not distort the overall statistical analysis.

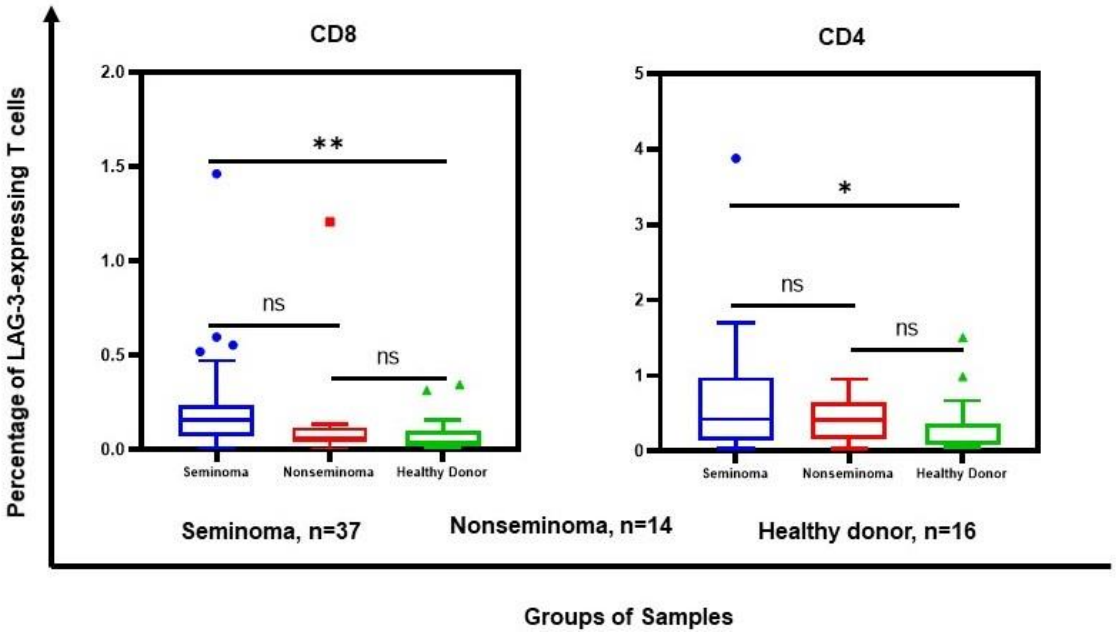

Fig. 4-7. The pattern of LAG-3 expression on T cells within peripheral blood

LAG-3 is expressed at low levels by both CD8+ and CD4+ T cells in HD. Overall expression is increased on blood from patients with either seminoma or nonseminoma. Statistical significance is obtained by both T cell populations in seminoma compared to healthy donor with p-values are 0.006 and 0.043 for CD8+ and CD4+, respectively (Kruskal-Wallis with Dunn's multiple test, significance at $p<0.05$).

The pattern of LAG-3 expression is therefore similar to that observed with Tim-3 and CTLA-4 and rather distinct from that observed with PD-1 and TIGIT. In seminoma, nonseminoma and HD, the LAG-3 molecule is expressed by 0.421% [IQR 0.132, 0.980%], 0.411 [IQR 0.166, 0.644] and 0.104% of T cells [IQR 0.078, 0.368] respectively. Despite the small frequency demonstrated by each tested group, the number of LAG-3-expressing CD4+ T cells in seminoma was higher than HD. On the other hand, no significance was observed in the comparison of LAG-3 expressing CD4+ T cell frequencies in nonseminoma and HD. This suggests that the tumor development within seminoma may activate LAG-3

expression, potentially to avoid immune surveillance. This pattern is also seen for CD8+ T cells where LAG-3 is significantly upregulated within seminoma (p-value=0.006).

Owing to some outliers detected in datasets of seminoma and HD, I wondered if these contributed to the observed significances. I further carried on outliers cleanse and re-analyzed data using one way ANOVA with Tukey's mutilple test. The significance were not affected by the outlier omission (p-value=0.002) suggesting that outliers did not perturb significance in the particular comparison (seminoma vs HD).

In particular, with PD-1 and TIGIT expression, there was no difference in expression between the tumour groups and the HD. As such there is no indication to consider PD-1 and TIGIT as markers of T cells exhaustion due to the testicular tumour. In contrast, LAG-3 joins Tim-3 and CTLA-4 as a potential marker of T cell exhaustion within this patient cohorts. LAG-3 expression has not previously been investigated on PBMC from TGCT patients. A relevant study on LAG-3 was conducted by He et. al (2017) who observed LAG-3 expression on a subset of TILs from 36 patients with NSCLC. LAG-3 was here overexpressed on TILs in nonadenocarcinomas compared to adenocarcinomas and acted as a marker of poor clinical outcome (He et al., 2017). In contrast to the results from He's group we did not find correlation between PD-1 expression and LAG-3.

Discussion

TGCT is an interesting model for studying the role of immune response in relationship with anti-tumour treatment. The tumour demonstrates superior clinical responses to therapy compared to virtually all other subtypes of cancer. A deep and integrative understanding on how this good prognosis is mediated after treatment may help in the design of anti-tumour therapeutic approaches for many patients. The expression of checkpoint proteins on immune cells is now appreciated to play a major role in the regulation of tumour-specific immune responses (Zappasodi et al., 2018). In particular, checkpoint proteins on T cells within the tumour microenvironment has been associated transcriptional and metabolic alterations that induce a profile of 'exhaustion', with incremental loss of effector function. PD-1 is the canonical exhaustion marker. PD-1 is briefly expressed on T cells after activation but when this expression is sustained it it felt to be a reflection of exhaustion due to persistent antigen exposure. This phenotype extends to overexpression of other inhibitory receptors including Tim-3, Lag-3, CTLA-4, and TIGIT (Thommen & Schumacher, 2018). In this chapter I analysed the expression of a range of important checkpoint proteins on peripheral blood T cells from patients with testicular cancer.

I chose to assess the expression of five different checkpoint proteins that are representative of the most important proteins associated with tumour-specific responses. The pattern of expression on the tumour and control samples varied between these different proteins and this is likely to reflect their differential role in immune regulation.

It should be noted that this analysis was performed on peripheral blood samples and not directed on tumour infiltrating lymphocytes within the testicular tumour. This was done as it had proven very difficult to obtain samples of tumour biopsy from men undergoing orchidectomy. In addition, it remains valid to assess peripheral responses as these have been shown to correlate with local analysis in some (Desgrandchamps et al., 2018), but not all studies (Kwiecien et al., 2019). Nevertheless, I did anticipate that my findings in relation to the degree of checkpoint expression would be rather less marked than observed on TIL populations.

One advantage of using peripheral blood samples is that I was able to incorporate the use of healthy donor control samples. These were age-matched with the samples from TGCT patients and this is an important consideration as the expression of checkpoint proteins on peripheral blood lymphocytes can increase during aging.

A number of interesting findings were obtained from my results. Firstly, it was somewhat surprising that the expression of PD-1 did not differ between the patient and control groups. PD-1 is the most heavily studied checkpoint protein and checkpoint therapy that targets the PD-1:PD-L1 interaction is the most effective therapy at the current time. My results did reveal a wide range of PD-1 expression levels on both the control and patient groups. The determinants that regulate the heterogeneous expression of PD-1 on T cells within the blood are not yet known but could include factors such as chronic viral infection or inflammation. It was remarkable that two donors expressed PD-1 on over 15% of T cells although there was no clear association with the TGCT diagnosis.

Expression of TIGIT was also heterogeneous in both patient and control groups but again no associations were observed between this profile and the TGCT diagnosis. Cells with single TIGIT expression were more common than those with PD-1 and represented around 30% and 15% of the CD8+ and CD4+ T cell repertoires respectively. TIGIT was also expressed in association with PD-1 on a considerable number of peripheral T cells. This was enumerated at around 10% of the CD8+ T cell pool and 5% of the CD4+ pool. A similar phenotype has been found in previous studies and shown to reflect a population with senescent phenotype (Song et al., 2018). Importantly, this phenotype increases with age although my data show that considerable numbers of these cells are already present in young to middle aged donors (20-50 years).

In relation to TCGT my results become somewhat more interesting for analysis of Tim-3, CTLA-4 and LAG-3. In particular, expression of these markers was shown to vary in patients with cancer compared to the control group, although the pattern of expression was different in each case.

Tim-3 was unusual in the sense that higher expression levels were observed on CD8+ and CD4+ T cells in patients with non-seminomatous tumours. In contrast there was no such increase in seminoma. Our previous work had shown that cancer testis-specific responses were particularly marked in patients with seminoma and as such I was anticipating that exhaustion might be particularly strong in the seminoma subgroup. Nevertheless, the non-seminomatous group of patients do have an impaired clinical outcome and it will be of interest to see if this is related to T cell exhaustion. The importance of Tim-3 in cancer immunotherapy has been shown in several reports including its potential to be

applied to mark immune responses following nivolumab therapy (Kato et al., 2018).

The expression of CTLA-4 and LAG-3 was found at only low levels in all the samples However, this was clearly increased in the patient subgroups. In particular, CTLA-4 was increased on both CD4+ and CD8+ T cells in patients with either seminoma or non-seminomatous tumours. LAG-3 showed a slightly different profile and was increased only in the seminoma subgroup. LAG-3, Tim-3 and PD-1 co-expression is increased on peripheral T cells in patients with ovarian cancer and further supports the potential importance of my results (Rådestad et al., 2019).

These data reveal that the pattern of peripheral T cell checkpoint expression is heterogeneous in patients with testicular cancer. The findings suggest that PD-1 and TIGIT may not be the most valuable markers in which to define a 'tumour-specific checkpoint phenotype' within peripheral blood in TCGT patients. Indeed, the relatively high expression of these markers may simply reflect physiological regulation of immune function and potentially also a component of aging-associated immunosenescence. However, other groups have shown that PD-1 expression is increased on peripheral T cells in some solid tumours and so there may be a tumour-specific association in this regard (Zgodzinski et al., 2019).

In contrast, CTLA-4 and LAG-3 may represent valuable markers with which to identify relatively rare populations of 'exhausted' cells within blood. This might even allow the isolation of tumour-reactive cells from the vascular compartment and this has been shown for LAG-3+ peripheral T cells in patients with colorectal

carcinoma (Huang et al., 2019). It will be interesting in future work to assess the pattern of co-expression of the Tim-3, CTLA-4 and LAG-3 checkpoint proteins. This might reveal a particularly powerful and specific ‘cancer-associated’ phenotype. Unfortunately, these cells were too rare for detection in my own analyses but represent an important target for future study

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CHAPTER V. THE PATTERN OF CYTOKINE PRODUCTION BY PERIPHERAL T CELLS IN TGCT PATIENT COHORT

Cancer is a very common clinical problem in modern society and as such it is clear that the immune system is not capable of controlling the proliferation of transformed cells in all cases. The mechanisms by which tumours can escape immune control are a major topic in clinical research. This includes the development of antigen-loss variants and the inactivation of antigen processing/presentation pathways through which the T cells lose their ability to recognize tumour cells (Garrido et al., 2016). It is imperative that auto-immune responses are avoided over the lifecourse and as such vertebrates have evolved a range of mechansism to generate immune tolerance to 'self' proteins. As tumour antigens are often subtle modifications of self protein this means that tumour-specific T cell responses are often weak. Evidence of immune escape is seen in many tumour subtypes. This has been used to support the model of immune surveillance as a mechanism to control cancer development (Roufaiel et al., 2015).

As a major effector arm of the immune responses, cytokine production has a profound impact on the nature of the subsequent immune response. Immunological dogma suggests that the immune system facilitate Th1 and Th2 CD4+ polarization in which different CD4+ cells can respond to antigen stimulation by generating cytokines such as IFN-γ and TNF-α, or IL-4 and IL-13 respectively (Mosman *et al,* 1989) . It is now clear that there is a broad spectrum of functional responses within the T cell repertoire and it may also be that T cells

may modulate their cytokine responses over time and exhibit 'plasticity' in their functional response.

Given my previous work on the phenotype and checkpoint protein expression on T cells within the blood of patients with testicular cancer I was interested to assess the functional response of these cells in comparison to healthy donors. Specifically, I undertook an analysis of cytokine production from peripheral blood CD4+ and CD8+ T cells following stimulation with PMA/Ionomycin.

Assessment of cytokine responses in peripheral blood samples from patients and donors

PMA/Ionomycin was used as it is a potent mitogen and is generally acknowledged to be the optimal method for stimulation of T cells prior to assessment of cytokine production (Crawford et al., 2014). PMA activates protein kinase C, whilst ionomycin is a calcium ionophore, and stimulation with these compounds 'bypasses' TCR engagement and leads to activation of several intracellular signaling pathways, resulting in T cell activation and production of a variety of cytokines (Ai et al., 2013).

Blood samples at the time of diagnosis were available from 31 patients with seminoma, 15 with non-seminomatous malignancy and 13 age-matched healthy donors (for details of donors see Chapter III). PBMC from patients and gender-matched controls were stimulated with PMA/ionomycin for 4 hours in the presence of protein transport inhibitor, followed by ICC staining. A non-stimulated control which contained only protein transport inhibitor was used to examine spontaneous production of cytokines. Intracellular cytokine staining was then

performed using antibodies to assess type-1 (IFN-γ), type-2 (IL-10 and IL-13), and type-17 (IL-17a and IL-21) cytokine responses.

IFN-γ production is conserved within T cells from the patient group

Interferons were originally described as a group of molecules with a similar function, that of inducing an immediate defensive response against viral infections. In the mid-1960s, type II IFN or IFN-γ was identified on the basis of its antiviral activities (Lee & Ashkar, 2018). However it is now known to play a much more important role as a more general proinflammatory molecule. It promotes all aspects of the Th1 immune response, while suppressing Th2 and Th17 responses (Martinez et al., 2008). In this study, I found that IFN-γ was the predominant cytokine produced by both CD4+ and CD8+ T cells regardless of disease status.

Without PMA/Ionomycin stimulation, the median proportion of IFN-γ-producing CD8+ T cells in seminoma was only 0.12% [IQR 0.05%, 0.79]. With stimulation, this increased to 35% [IQR 22%, 50%] (left panel, Fig. 5-2). In patients with non-seminoma tumours the proportion of IFN-γ+ CD8+ T cells without and with stimulation were measured at 0.13% [IQR 0.06%, 0.23%] and 44% [IQR 23.54%, 52.88%] (left panel, Fig. 5-2) respectively. Comparative values in the HD cohort were 0.042% [IQR 0.02%, 0.10%] and 44% [IQR 30.78%, 63.14%] (left panel, Fig. 5-2).

Similarly to CD8+ T cells, IFN-γ was the predominant cytokine produced by CD4+ T cells in all groups although the frequency was much less compared to CD8+ T cells. For seminoma patients, IFN-γ was produced by 0.14% [IQR 0.04%, 0.36%]

of CD4+ T cells prior to stimulation compared to 12% [IQR 6.25%, 21.31%] following stimulation. In nonseminoma, these values increased from 0.08% [IQR 0.02%, 0.31%] to 7.9% [IQR 4.02%, 14.38%] after stimulation. Levels of spontaneous secretion were 0.05% in the HD cohort [IQR 0.02%, 0.09%] compared to 14% [IQR 3.27%, 27.35%] following stimulation.

These data show that IFN-γ levels following stimulation were in the range of fifteen to seventy-five fold higher compared to baseline production. It is known that the level of spontaneous cytokine production is very low within circulating T cells and so these results are comparable with other datasets. However it was noteworthy that the level of spontaneous IFN-γ production was around 3 fold high in both the CD4+ and CD8+ T cell subsets in the patient group. This raises the possibility that there are low levels of pre-activated T cells in patients with testicular cancer which may potentially represent tumor-specific clones.

Despite this, my data suggest that the proportion of IFN-γ-producing T cells following stimulation does not differ between patients with different types of TGCT or between health and disease.

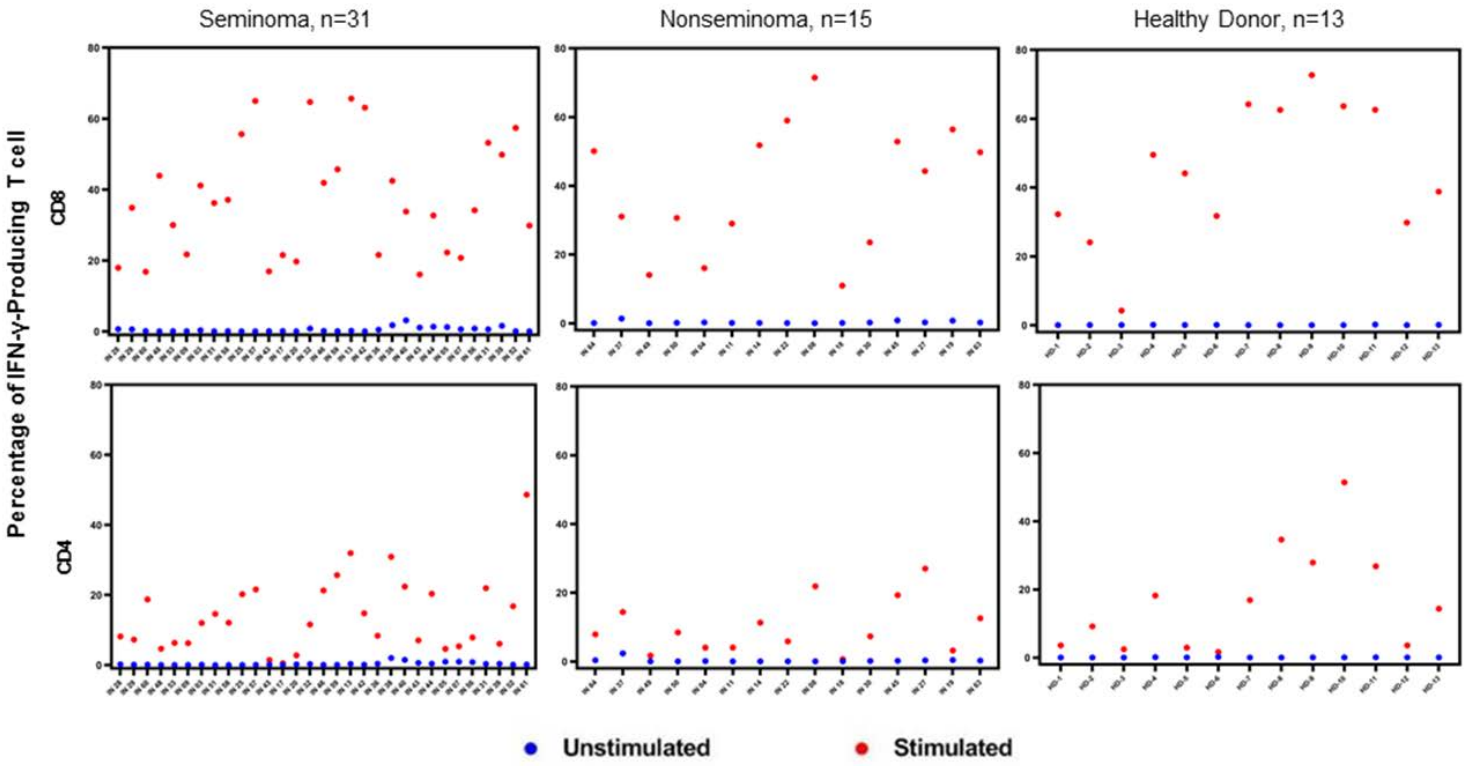

Fig. 5-1. IFN-γ production by CD4+ and CD8+ T cells following PMA/Ionomycin stimulation of PBMC from testicular cancer patients and healthy donors

PBMC from patients and gender-matched controls were stimulated with PMA/ionomycin for 4 hours in the presence of protein transport inhibitor, followed by ICC staining. Graphs represent IFN-γ-producing T cells as a proportion of total CD8+ (top) and CD4+ (bottom) T cells from seminoma patients (left, n=31), nonseminoma patients (middle, n=15) and healthy donors (right, n=13). Each tick along the X axis represents a single patient spontaneous (blue dot) and stimulation induced cytokine release is represented as red dots.

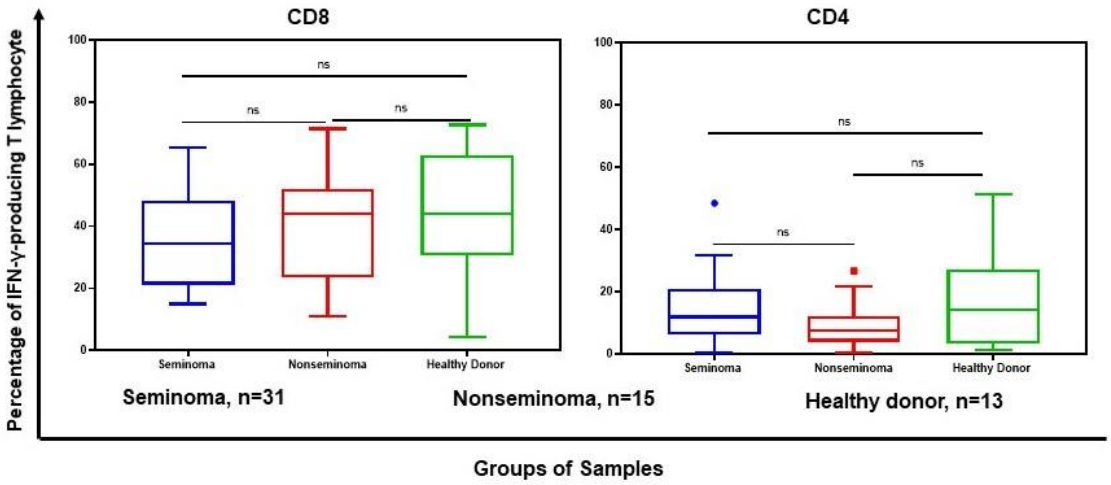

Fig. 5-2. Summary of IFN-γ production by CD8+ and CD4+ T cell subsets following PMA/Ionomycin stimulation

Comparison between the proportion of CD8+ and CD4+ T cells producing IFN-γ following PMA/Ionomycin stimulation in seminoma patients (n=31), nonseminoma patients (n=15) and healthy donors (n=13). Data analysed statistically using Kruskal-Wallis with Dunn's multiple test, significance at $p < 0.05$.

By analyzing the dataset using Kruskal-Wallis and further post-hoc testing with Dunn's multiple test, no significance emerges from the comparisons of the increment of IFN-γ-producing T cell number in the healthy donor and the two subtypes of TGCT (all p-values exceed 0.05) (Fig. 5-2). This suggests that there is no intrinsic impairment in pro-inflammatory T cell function. Since Th1/Tc1 immunity is often associated with anti-tumour responses, this may contribute to the excellent clinical outcome of this disease.

A subset of CD8+ T cells from patients with TCGT demonstrates spontaneous secretion of IL-17a

CD4+ Th17 cells secrete IL-17a, which has been shown to have both anti-tumourigenic and pro-tumourigenic roles and hence may play an important role in regulating anti-tumour immunity. In addition, IL-17-secreting CD8+ T cells

(Tc17) have also been described (Yen et al., 2009) yet little is know of their functional relevance in the cancer setting. Here, we examined spontaneous and mitogen-stimulated production of IL-17a by CD4+ and CD8+ T cells from TGCT patients and healthy controls.

The frequency of spontaneous IL-17a production by CD4+ T cells between seminoma (0.43% [IQR 0.11%, 0.43%]), nonseminoma (0.17% [IQR 0.09%, 0.39%], and healthy donors (0.16% [IQR 0.07%, 0.31%]) was low and not significantly different (right panel, Fig. 5-4). The frequency of IL-17a producing CD4+ T cells following stimulation was 0.86% [IQR 0.28%, 1.85%] in seminoma, 0.48% [IQR 0.22%, 0.86%] in nonseminoma and 0.62% [IQR 0.26%, 2.86%] in HDs (Fig. 5-3).

In general, the proportion of CD8+ T cells producing IL -17a was far lower than that observed from CD4+ T cells (left panel, Fig. 5-4). Interestingly, there was a significantly higher proportion of CD8+ T cells producing IL-17a without prior stimulation *in vitro* in both the seminoma ($p<0.001$) and nonseminoma ($p<0.05$) patients compared to healthy controls. However this difference between groups was lost following functional stimulation and there was no difference in the proportion of CD8+ T cells producing IL-17a following PMA/Ionomycin treatment between seminoma, nonseminoma and healthy donors. Our results show that IL-17a is produced by a higher proportion of CD4+ T cells compared to CD8+ T cells in health and disease although a small proportion of CD8+ T cells demonstrate spontaneous cytokine secretion in the setting of TCGT.

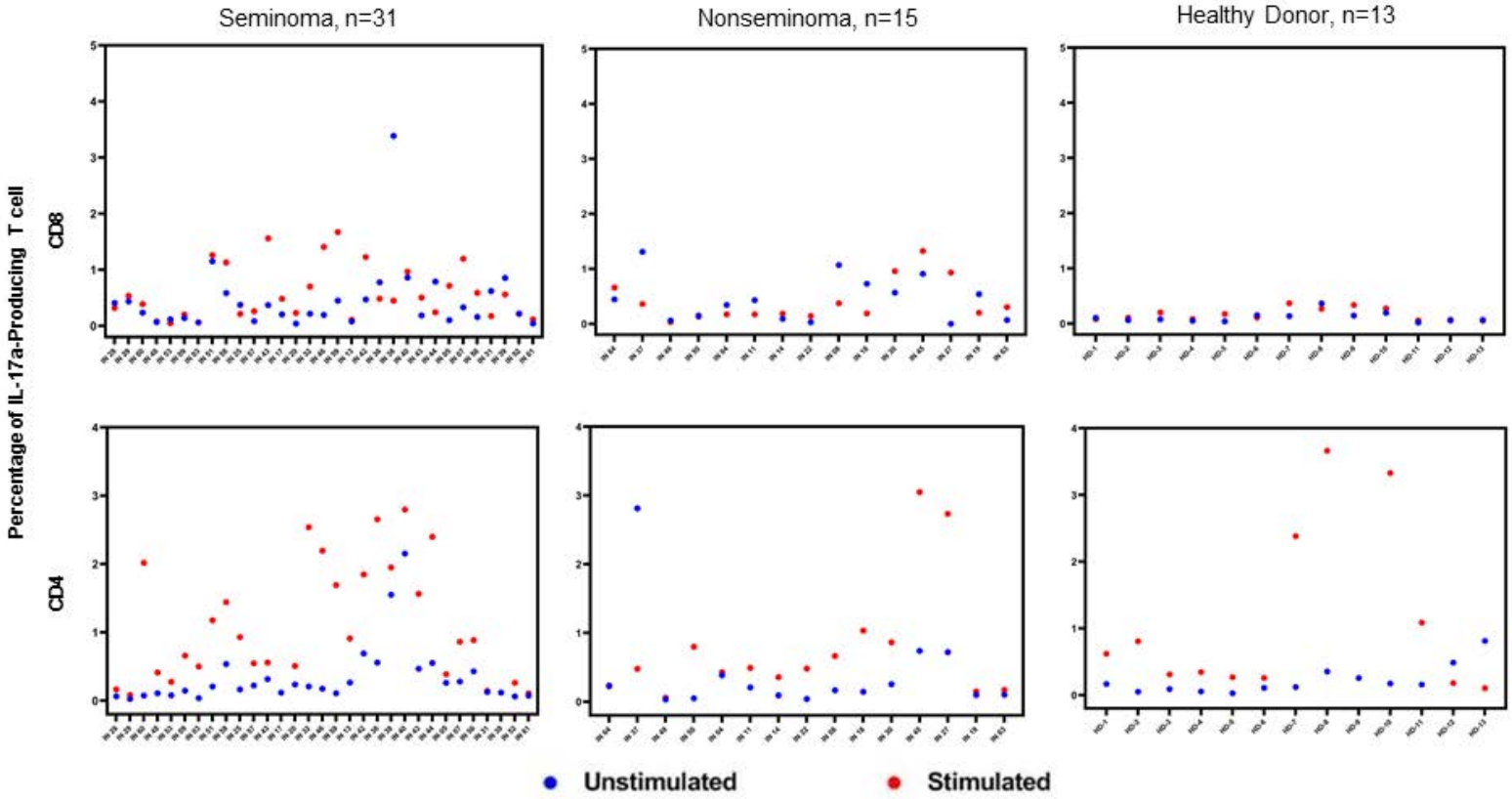

Fig. 5-3. IL-17a production by CD4+ and CD8+ T cells following PMA/Ionomycin stimulation of PBMC from testicular cancer patients and healthy controls

PBMC from patients and gender-matched controls were stimulated with PMA/ionomycin for 4 hours in the presence of protein transport inhibitor, followed by ICC staining. Graphs represent IL-17a-producing T cells as a proportion of total CD8+ (top) and CD4+ (bottom) T cells from seminoma patients (left, n=31), nonseminoma patients (middle, n=15) and healthy donors (right, n=13).

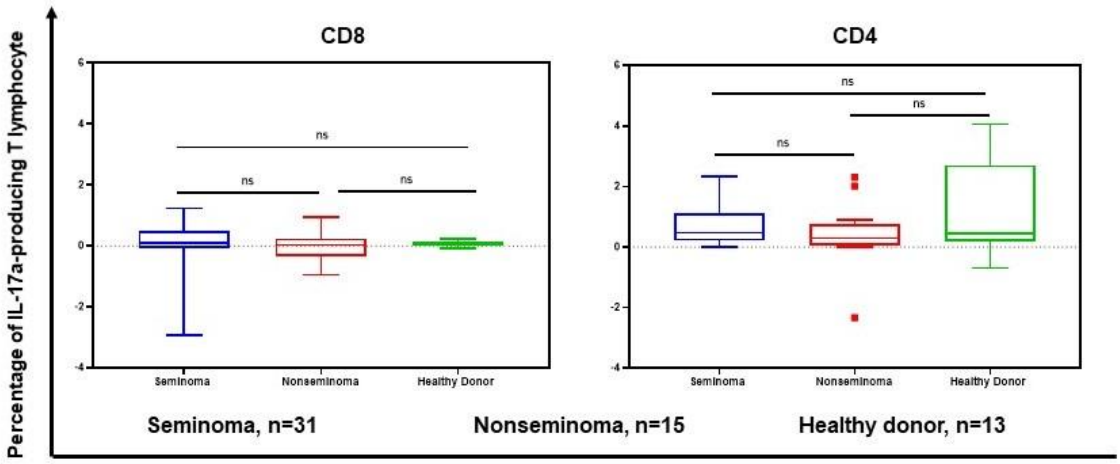

Fig. 5-3. Summary of the IL-17a production patterns by CD4+ and CD8+ T cells in TGCT and HD.

The production of IL-17 was assessed in response to mitogen challenge. No significant differences were observed between the groups (one way ANOVA with Tukey's multiple test, significance at $p<0.05$) (Graph Prism 8).

As demonstrated in Fig. 5-4, without receiving non-specific stimulation of PMA/Ionomycin, both CD8+ and CD4+ T cell populations in both subtypes of TGCTs are able to produce IL-17a at higher frequencies compared with HDs. Some outliers are identified in every tested group but their omissions do not alter significance values. Adjusted p-values remain below $P<0.05$, suggesting that, despite as individual data they are higher than 1.5 Q3 of their respective datasets, the values they present are not by chance.

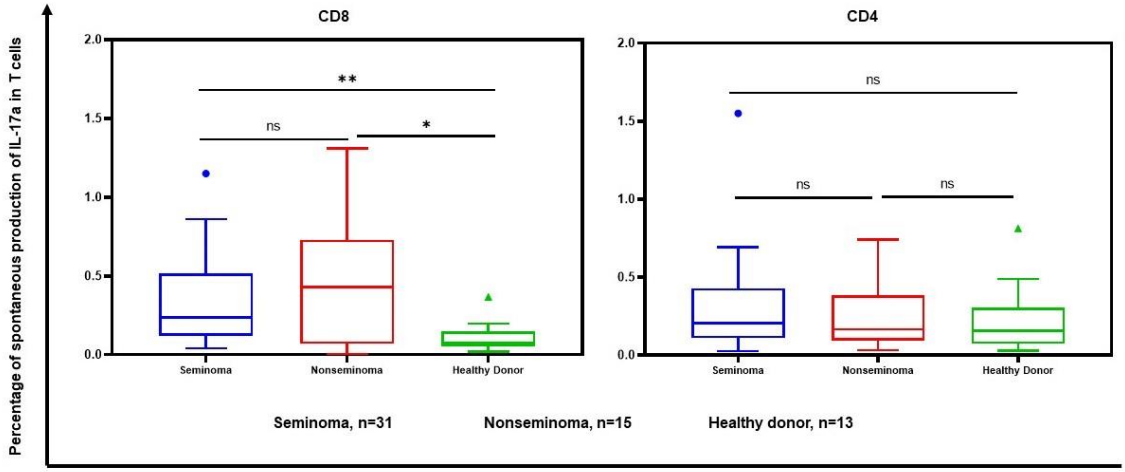

Fig. 5-4. Summary of the proportion of T cells that exhibit spontaneous release of IL-17a from testicular cancer patients and healthy donors

Comparison is made of spontaneous production of IL-17a by CD8+ and CD4+ T cells from seminoma patients, nonseminoma patients and healthy donors. Data analysed statistically using Kruskal-Wallis with Dunn's multiple test; *p-value=0.028, **p-value=0.009.

Production of IL-21 by CD4+ T cells is increased in seminoma patients

IL-21 is a member of the γ_c family of cytokines and is produced by various CD4 T cell subsets including follicular helper T (Tfh) cells and Th17 cells (Leonard et al., 2019). In addition, CD8 T cells have been shown to secrete IL-21 under certain conditions such as during chronic viral infections. IL-21 is able to regulate the function of various T cell subsets including Tregs, Th1 and Th2 cells and may therefore play an important role in balancing pro- and anti-tumour responses.

In my study I found that IL-21 was produced by CD4+ T cells from healthy donors at a frequency of 1.02% [IQR 0.21%, 1.50%] of total CD4+ T cells following PMA/Ionomycin stimulation (Fig. 5-6). Spontaneous IL-21 production was detected at a very low frequency of around 0.08% [IQR 0.03%, 0.53%]. Interestingly, IL-21 was produced by 2.4% of CD4+ T cells from seminoma

patients [IQR 1.18%, 3.06%]) demonstrating a small but significant increase compared to healthy controls ($p<0.001$) (Fig. 5-7). There was no significant difference between nonseminoma and healthy donors suggesting this is a phenomenon restricted to seminoma patients. A similar trend towards increased expression of IL-21 from CD8+ T cells in patients with seminoma was observed but this was not statistically significant (p-value>0.05).

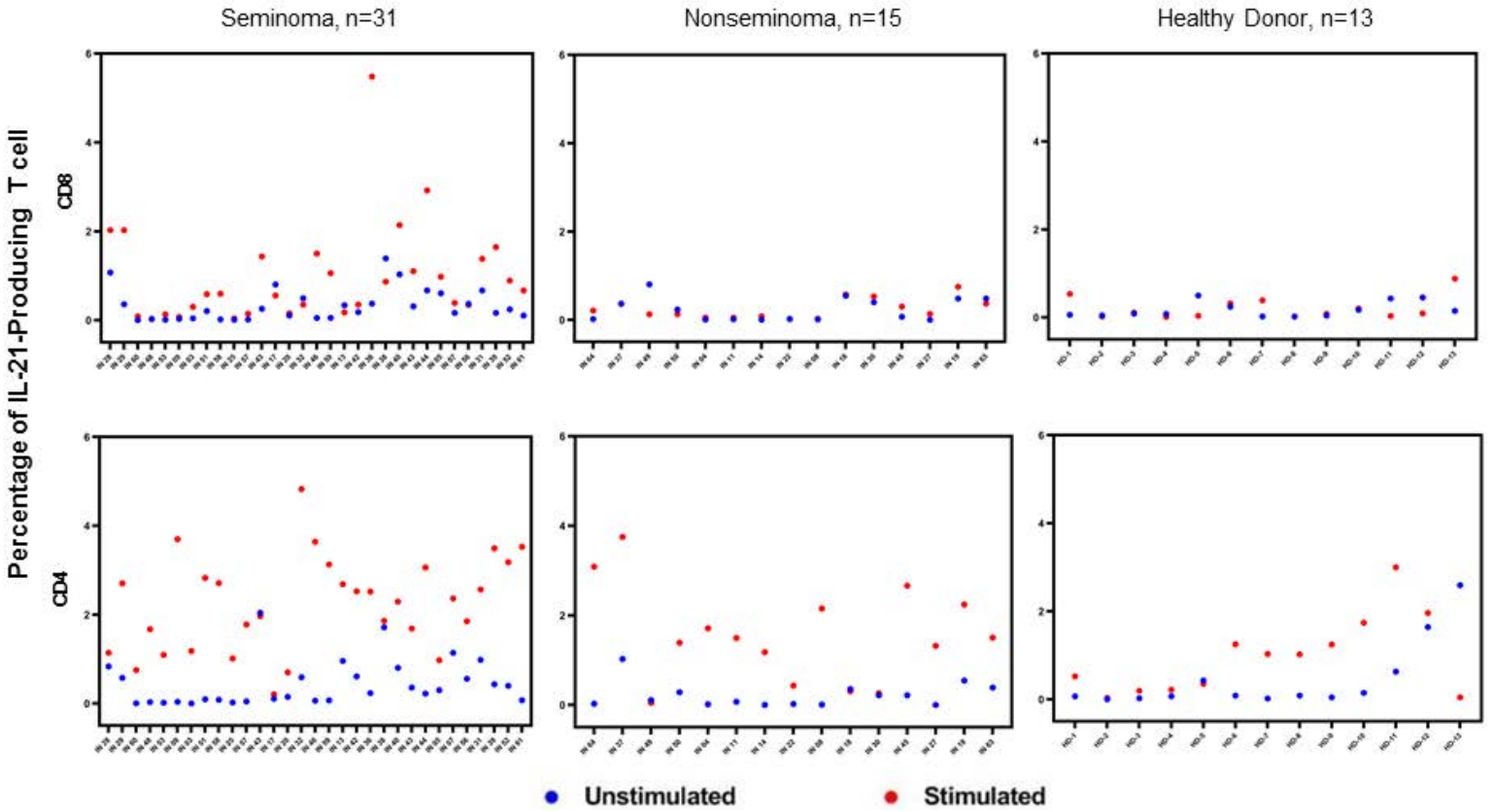

Fig. 5-4. IL-21 production by CD4+ and CD8+ T cells following PMA/Ionomycin stimulation of PBMC from testicular cancer patients and healthy controls.

PBMC from patients and gender-matched controls were stimulated with PMA/ionomycin for 4 hours in the presence of protein transport inhibitor, followed by ICC staining. Graphs represent IL-21-producing T cells as a proportion of total CD8+ (top) and CD4+ (bottom) T cells from seminoma patients (left, n=31), nonseminoma patients (middle, n=15) and healthy donors (right, n=13).

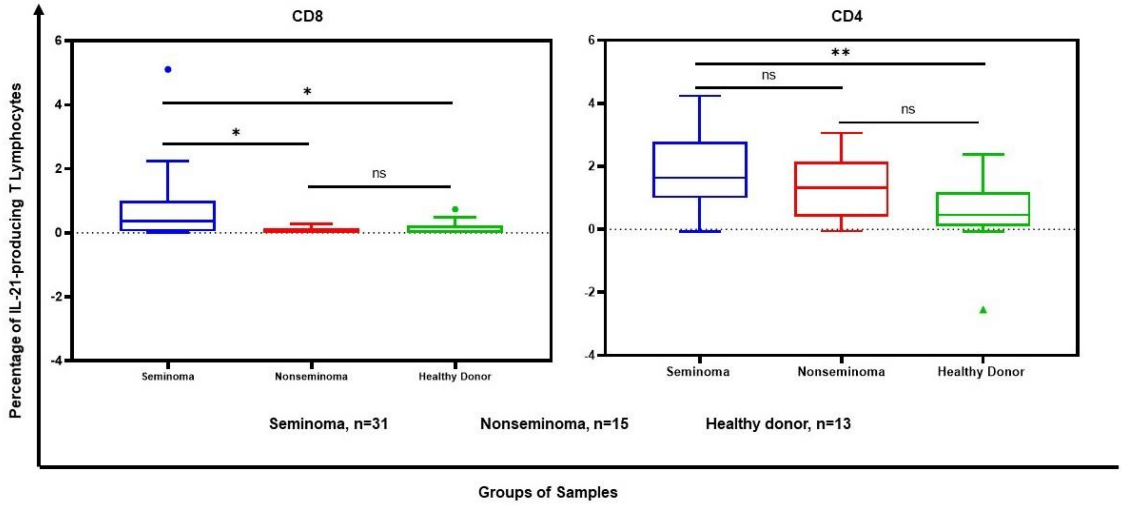

Fig. 5-5. Pattern of IL-21 production by CD8+ and CD4+ T cells following PMA/Ionomycin stimulation of PBMC from testicular cancer patients and healthy controls

Comparison of IL-21 production by CD8+ and CD4+ T cells from seminoma patients , nonseminoma patients and healthy donors. Normality distribution of each dataset is performed via Shapiro-Wilk test. Since the data distribution in CD8+ T cell panel (left panel) is confirmed to be normal, one way ANOVA with Tukey's multiple test is used to detect any significance from each comparison. Data distribution in CD4+ T cell (right panel), in contrast, is not normally distributed and thus is statistically analysed using Kruskal-Wallis with Dunn's multiple test; significance at $p < 0.05$.

This pattern of IL-21 production is similar to that seen for IL-17a where downregulation was observed in some patients across the 3 assessed groups. It is noteworthy that IL-21 can induce IL-17 production whereas generation of Th17 cells is attenuated by blocking IL-21. IL-21 is known to activate STAT3 and its ability to induce Th17 differentiation is abrogated in the absence of STAT3 (Jin & Dong, 2013).

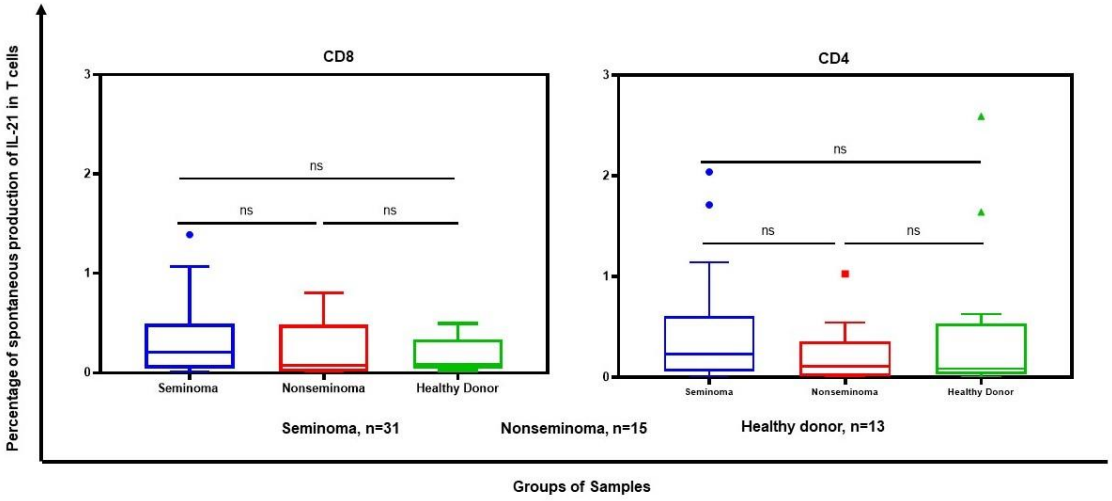

Fig. 5-7. Spontaneous production of IL-21 by CD8+ and CD4+ T cells in PBMC from testicular cancer and healthy donor is detected at very low level and shows no significance among the 3 evaluated groups.

Spontaneous production of IL-21 by CD8+ and CD4+ T cells from TGCT patients in comparison to that of HDs. Due to all datasets are not normally distributed, statistical analysis is performed by using Kruskal-Wallis with Dunn's multiple test, significance at $P<0.05$. This analysis results in insignificant differences of spontaneous IL-21 production among the 3 tested groups.

As illustrated in Fig. 5-7, spontaneous release of the cytokine is also detected, albeit the levels are much lower than that seen for IL-17a. No significance is produced any group comparison either by CD8+ or CD4+ T cell population. This suggests that IL-21 is not a strong feature for immune response against the disease.

A subset of CD4+ T cells from testicular cancer patients spontaneously produce immunosuppressive IL-10

I next evaluated the production of IL-10, a major immunosuppressive factor critical for the induction of tolerance through inhibition of Th1 immune responses and T cell cytotoxic activity. IL-10 hinders the proliferation, cytokine production and migratory capacities of effector T cells and elevated levels obstruct cytolytic

activity in transplant rejection. IL-10 may act either via direct or indirect mechanisms to mediate immune suppression (Dennis et al., 2013).

As expected, my data showed that stimulation with PMA/Ionomycin induced IL-10 production predominantly by CD4+ T cells from healthy and cancer patients (Fig. 5-8). Intriguingly, the frequency of spontaneous IL-10 production from CD4+ T cells was statistically greater in seminoma patients (0.43% [IQR 0.13%, 0.775]; $p<0.01$) and nonseminoma patients (0.37% [IQR 0.11%, 0.60%] $p<0.05$) compared to healthy donors (0.11% [IQR 0.05%, 0.26%]). No difference was observed between seminoma and nonseminoma (right panel, Fig. 5-9). However there was no significant difference in the profile of PMA/Ionomycin-induced production of IL-10 by CD8+ T cells between any of the 3 subject groups (left panel, Fig. 5-9).

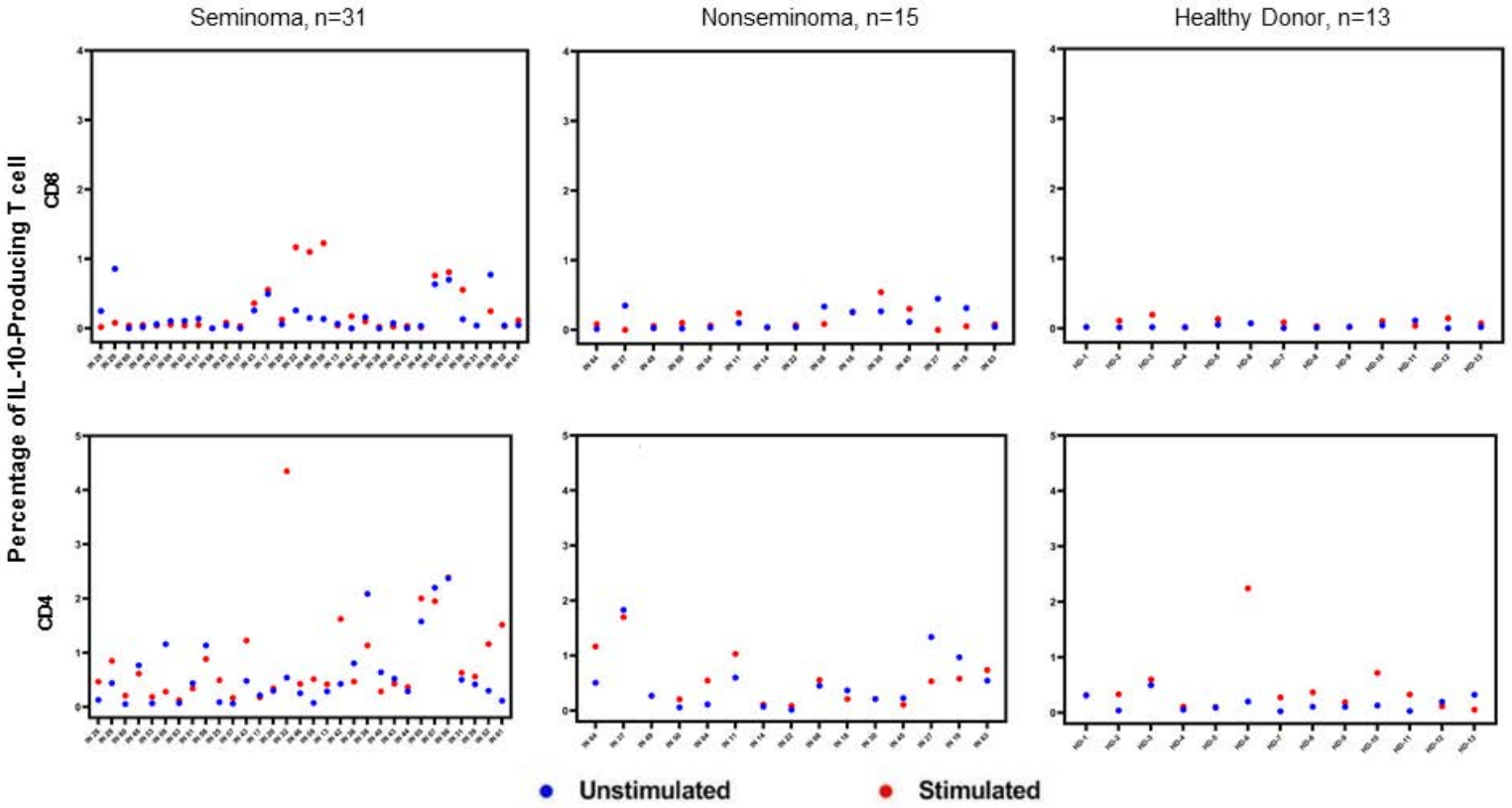

Fig. 5-8 Spontaneous and mitogen-induced IL-10 production by CD4+ and CD8+ T cells from testicular cancer patients and healthy controls

PBMC from patients and gender-matched controls were stimulated with PMA/ionomycin for 4 hours in the presence of protein transport inhibitor, followed by ICC staining. Graphs represent IL-10-producing T cells as a proportion of total CD8+ (top) and CD4+ (bottom) T cells from seminoma patients (left, n=31), nonseminoma patients (middle, n=15) and healthy donors (right, n=13).

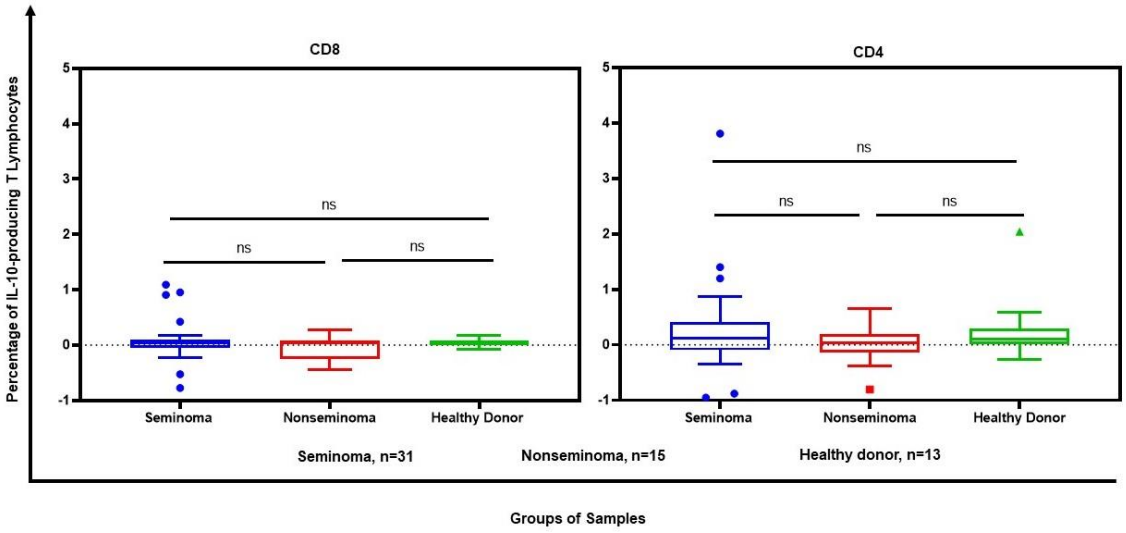

Fig. 5-9. Summary of IL-10 production by CD8+ and CD4+ T cells from testicular cancer patients in comparison with healthy controls following the PMA/Ionomycin stimulation.

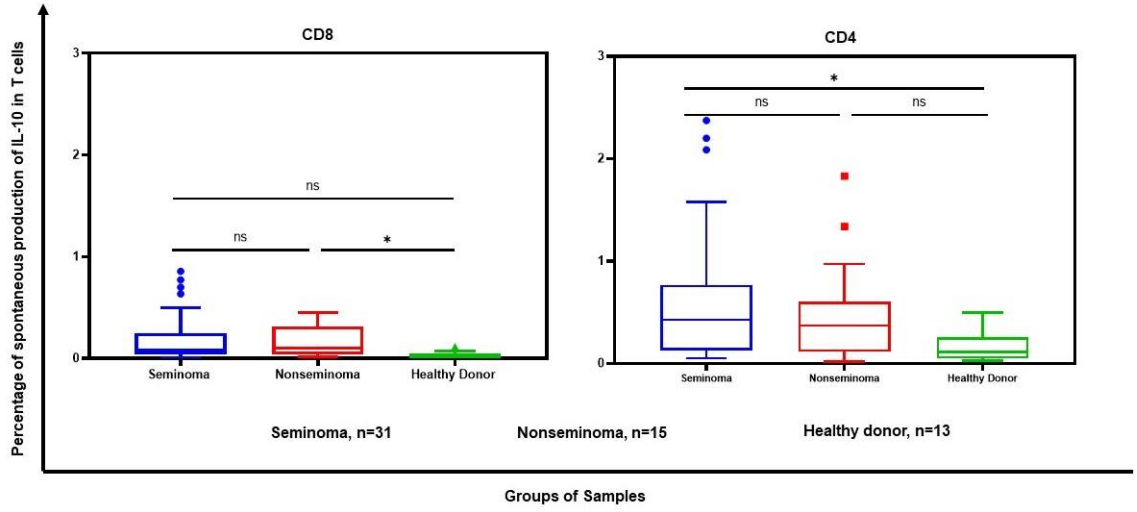

Fig. 5-10 Summary of spontaneous release of IL-10 by CD8+ and CD4+ T cells from testicular cancer patients in comparison with healthy controls

Comparison of spontaneous production of IL-10 by CD4+ T cells from seminoma patients, nonseminoma patients and healthy donors. Data analysed statistically using Kruskal-Wallis with Dunn's multiple test, significance at $p<0.05$.

Some outliers are detected in the spontaneous production of IL-10, mainly within the seminoma group. There are 4 seminoma-derived outliers in the CD4+ subset where the frequency of IL-10-producing cells ranges from 1.58%-2.37%. On the

other hand, CD8+ subsets in 4 patients with seminoma produced extremely high levels of IL-10, ranging from 0.5%-0.77%, compared with those seen for the remainders. A nonseminoma 'outlier' gave a value of 1.830% of IL-10-producing CD4+ and a HD contributed a value of 0.114% IL-10-producing CD8, also an outlier (left panel, Fig. 5-10). All these outliers in the seminoma group were at stage I whereas an outlier in the non-seminoma group was from a 26 years-old patient with stage IIB. It is intriguing that this spontaneous IL-10 production tends to increase by age in the CD4 subset when the disease was diagnosed at early stage (IA). In descending order, IL-10-producing CD4+ T cells are seen in the patients aged 59 (2.373%), 55 (2.200%), 28 (2.086%) and 26 (1.575%) (right panel, Fig. 5-10). This trend, however, is not observed in CD8 subset.

IL-13 production was seen only in CD4+ T cells and was similar between all groups

Finally I went on to examine the profile of IL-13 production from CD4+ T cells following PMA/Ionomycin stimulation. IL-13 is one of the Th2 cytokines that has similar effects on an immune response to IL-4. Recent studies have revealed that IL-13 plays a critical role in many aspects of immune regulation (Terabe, Park and Berzofsky, 2004), and hence may play a role in tumour immune evasion.

IL-13 levels were undetectable without mitogenic stimulation and were also only observed in the CD4+ T cell subset. When the profile of IL-13 production by CD4+ T cells was compared between the three groups no differences were observed.

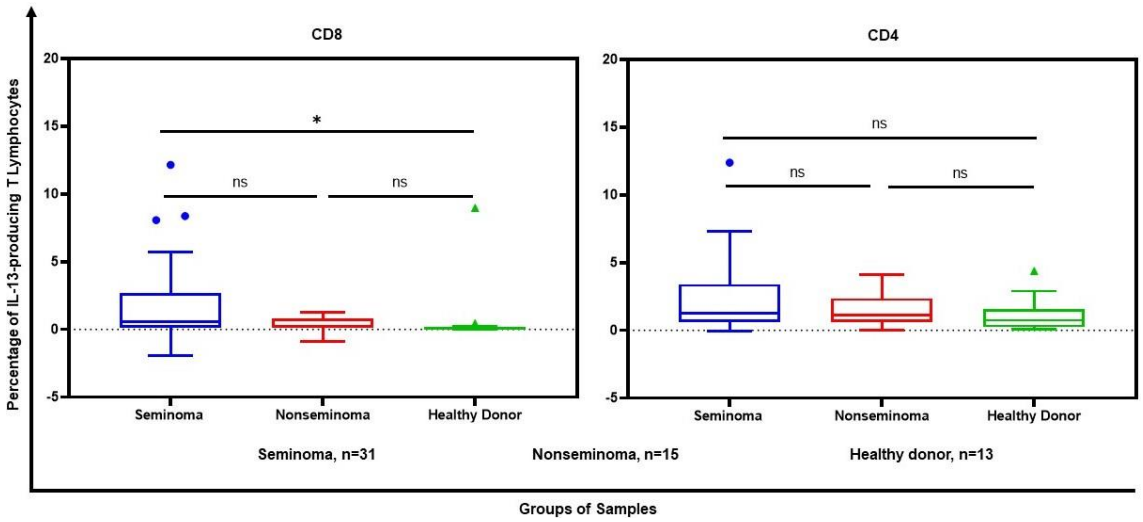

Fig. 5-11. Summary of IL-13 production by CD8+ and CD4+ T cells following PMA/Ionomycin stimulation of PBMC from testicular cancer patients and healthy controls

Comparison of IL-13 production by CD4+ T cells from seminoma patients , nonseminoma patients and healthy donors. Data analysed statistically using Kruskal-Wallis with Dunn's multiple test, significance at P<0.05.

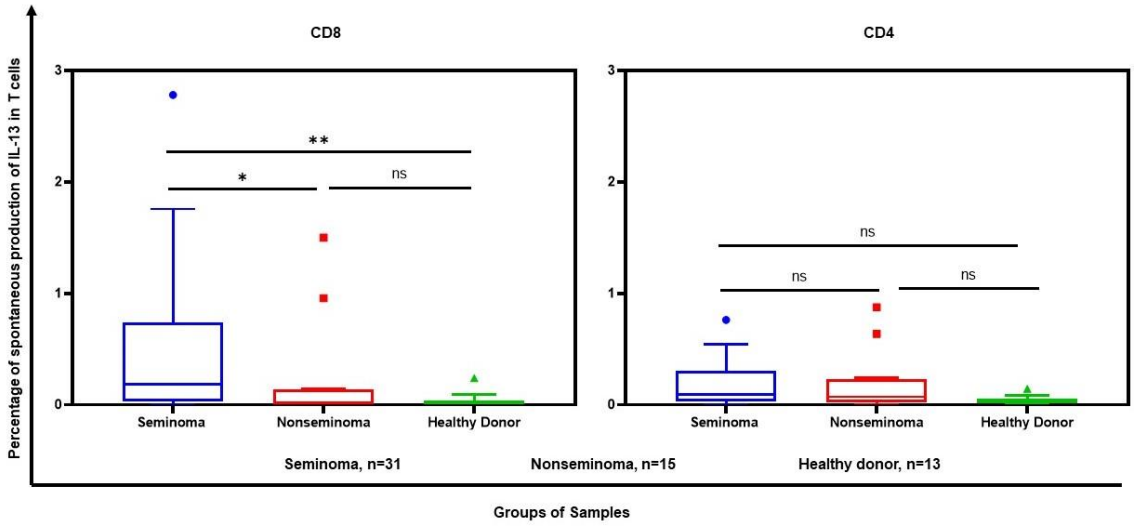

Fig. 5-12. Spontaneous production of IL-13 by CD8+ and CD4+ T cells in PBMC from testicular cancer patients and healthy controls

Comparison of IL-13 production by CD4+ T cells from seminoma patients , nonseminoma patients and healthy donors. Data analysed statistically using Kruskal-Wallis with Dunn's multiple test, significance at p<0.05.

Discussion

My previous work had shown that subtle but clear differences were observed in the phenotype of peripheral T cells in patients with testicular cancer. Whilst these are of interest, their main importance would relate to how they modulate the function of the immune system in the patient group. T cell function can be assessed in a number of ways including proliferative response, cytokine production and cytotoxic activity. In this chapter I chose to focus on the pattern of cytokine production by peripheral T cells as these are established as key regulators of immune activity in malignant disease.

I opted to stimulate T cells with PMA/Ionomycin and one consideration was that this acts through bypassing TCR stimulation. Indeed, PMA/Ionomycin is used routinely to study T cell activation and proliferation as it works in a T cell receptor-independent way. It does this by mimicking the phospholipase C-driven activation of PKC with the subsequent increase of cytosolic Ca^{2+}, PMA/Ionomycin activates the transcription factors: nucelar factor of activated T cell (NFAT1)-1, NF-κB and activator protein-1 (AP-1). This results in downstream gene expression. Under different circumstances, these agents can either activate T cells or initiate activation-induced cell death (AICD) in lymphocytes (Han, et al., 2013). One consideration for future studies is that impairment of T cell receptor signalling is sometimes observed in patients with malignant disease and so parallel studies that incorporate the use of CD3 and CD28-mediated T cell activation would represent a useful comparator assessment.

I chose to select a range of pro- and anti-inflammatory cytokines within my intracellular cytokine analysis. This included IFN-γ, IL-17a and IL-21 as well as the anti-inflammatory cytokines IL-10 and IL-13. A further decision that I made, and which in retrospect proved to be very valuable, was to measure the level of spontaneous cytokine production whenever these were at a detectable level.

IFN-γ was the most abundantly produced cytokine from T cells in all three groups although no differences were observed between healthy donors and the patient groups. This indicates that T cells are broadly functional for Th1 responses and may potentially contribute to the impressive clinical responses seen in this group during chemotherapy.

The pattern of IL-17a production was intriquing as higher levels of spontaneous cytokine production were observed from CD8+ T cells in patients with testicular cancer. The role of Th17 cells in cancer development is not yet defined but they have been suggested to potentially play either a pro- or anti-tumourogenic mechanism in different scenarios. As such the interpretation of this finding is unclear at this stage. Spontaneous cytokine production has not been investigated in any great detail in previous reports and is somewhat difficult to interpret as the number of cells that demonstrate this pattern is very small and large patient groups need to be studied. A similar trend was detected in the profile of spontaneous IL-21 production in CD4+ T cells which were significantly more common in patients with seminoma, but not non-seminoma.

In relation to anti-inflammatory cytokines, IL-13 and IL-10 were both identified only within CD4+ T cells. Again the pattern of spontaneous cytokine production

was of most interest, and this IL-10 expression by CD4+ T cells in seminoma and nonseminoma was significantly enriched compared to that of HD.

Overall my findings show that peripheral T cells from patients with testicular cancer display a full range of cytokine responses following stimulation with mitogens *in vitro*. This profile is compatible with the clinical phenotype of patients with this disorder as they are not known to display any excess susceptibility to infection, inflammatory or auto-immune disease. However, blood from patients with testicular cancer did show increased numbers of T cells that display background levels of 'spontaneous' cytokine production without the need for mitogenic stimulation. This may reflect evidence of ongoing low level immune activation and may potentially represent evidence of a tumour-specific immune response. I was able to detect increased spontaneous production of both pro- and anti-inflammatory cytokines and as such it is not clear what the net effect of this profile would be.

The use of intracellular cytokine analysis necessitates permeabilisation of the cell membrane and therefore the cell is rendered non-viable for further downstream analysis. This is a shame as my research indicates that a pattern of spontaneous cytokine production may represent a marker of immune activation and could potentially offer a tool for the detection and isolation of tumour-reactive T cells. Taken together, the results from my first three chapters suggest that the peripheral immune repertoire of patients with testicular cancer does demonstrate evidence for immune activation and this may reflect an ongoing tumour-specific response. The combination of cellular phenotype and function may represent one

approach to identify these rare cells, even in the absence of knowledge regarding their antigenic specificity.

CHAPTER VI. STUDY OF THE IMMUNE RESPONSE AGAINST CANCER/TESTIS ANTIGENS IN TGCT PATIENT COHORT

As described earlier, the clinical outcome for patient with seminoma is very good and this may partially reflect the induction of an immune response against the tumour.

In the next phase of my thesis I undertook a study to attempt to identify the antigenic target of T cells that might be involved in the immune response against testicular cancer. In particular, I obtained blood from patients with seminoma and undertook an analysis of the immune response against proteins from the MAGE family of cancer testis antigens. This work built on previous studies from the laboratory although these had exploited ELISPOT analyses as a means to identify immune responses (Pearce et al, 2017). Although ELISPOT is a very sensitive technology with which to identify T cell responses it does not allow downstream isolation of antigen-specific T cells, or the examination of multiple cytokines simultaneously. As such, I sought to utilise the application of Intracellular cytokine staining in order to identify peptide-specific responses.

I chose to focus on immune responses against MAGE-A1, MAGE-A3 and MAGE-A4 as these were identified as the major immunodominant proteins in the publication by Pearce et al, 2017. A limitation of my study was that I was not able to confirm expression of these proteins within the tumour sample of the patients studied. This was a shame, as histological sections of tumour would be available within the histopathology archives, but we did not have ethical permission to perform this.

In particular, PBMC were collected from patients with testicular germ cell tumours (TGCT) and stimulated with overlapping peptide libraries followed by intracellular cytokine staining (ICC). Spontaneous immune responses were defined by the release of cytokines. In order to allow the potential identification of a wide range of functional T cell responses I undertook analysis of a range of cytokine responses including IFN-γ, TNF-α, IL-2 and GM-CSF. In addition, the expression of CD107a was identified in order to assess the cytotoxic activity of cells in response to antigen stimulation. IFN-γ and TNF-α are the two major cytokines produced by Th1 and NK cells and these therefore served as the primary focus of interest.

Blood samples were taken from patients with seminoma at the time of diagnostic orchidectomy. These were an unselected subset of samples that had been analysed in previous chapters. PBMC were then isolated by density centrifugation and stimulated separately with 3 different peptideMix™ of MAGE-A1, MAGE-A3 or MAGE-A4. Each peptide mix contained 75 peptides spanning the whole amino acids sequence. Each individual peptide was 15 amino acids (aa) long and an 11 aa overlap was incorporated between each peptide. Peptide mixes were purchased in the form of powder which was then dissolved in DMSO into the appropriate concentration as described in Materials and Methods (Chapter II). A formulation of DMSO alone was used as a negative control for cell stimulation. It was anticipated that the magnitude of the CTAg-specific response would not be intense and I therefore felt it necessary to incorporate a positive control for peptide stimulation. In this regard I used a peptide pool of CEFT. CEFT consists of 27 peptides which correspond to immunodominant epitopes from

Cytomegalovirus, **E**pstein-Barr virus, Influenza (**f**lu) virus and Clostridium tetani (**T**etanus) which are restricted by a range of different HLA class I and class II alleles. The gating scheme for the analysis is shown in Fig. 6-1.

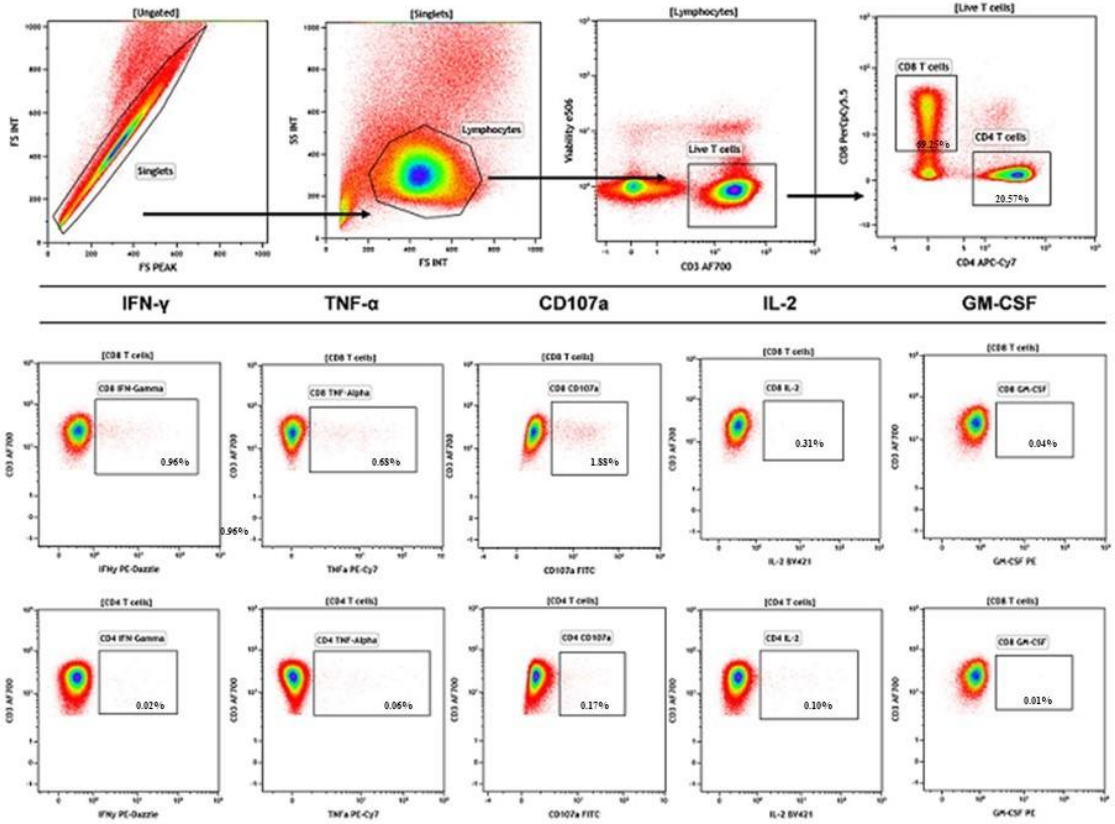

Fig. 6-1. Gating analysis for identification of peptide-specific T cells

Cells were initially gated on the singlets where clumped/doublet cells are excluded. This enables evaluation of cytokine expression on individual cell basis. FS INT (X Axis) and SS INT (Y Axis) gating was used to identify lymphocytes. CD3 expression was used to identify T cells. Further, the cells are divided into 2 primary subsets: CD8+ T cells and CD4+ T cells whose cytokine productions (IFN-γ, TNF-α, IL-2 and GM-CSF) were measured. The cytotoxicity (degranulation) of both T cell subsets was also investigated through their expression of CD107a.

IFN-γ expression allows the identification of MAGE-A family specific T cells in a small percentage of patients with testicular cancer

MAGE-A1 Responses

Blood samples were available from 9 patients with seminoma and 4 with other subtypes of testicular tumour. These were stimulated with the MAGE-A1 overlapping peptide library as previously described.

One sample from patients with seminoma (IN24) showed an increase of IFN-γ production in the CD8+ T cell subsets following stimulation with the peptide pool. The incremental magnitude of the cytokine response was 0.10% following challenge with MAGE-A1 overlapping peptides although there was also a high baseline level of 0.26%. Despite this, the clear increment in cytokine production observed following stimulation with the MAGE peptides was taken to represent a positive response. IN09 also demonstrated an enhanced level of IFN-γ production from CD8+ T cells after MAGE-A1 stimulation but this was very small, an increment of only 0.006% from the baseline level of 0.135% to 0.141%, and so was discounted.

IFN-γ production was also observed in CD4+ T cell subsets. For instance, in sample IN41 this increased by nearly two fold from 0.015% to 0.037%. No such CD4+ response was observed in IN24 which had demonstrated a peptide-specific CD8+ response. The other six samples in the seminoma group, IN36, IN29, IN32, IN33, IN21 and IN12, did not respond to peptide stimulation within either the CD4+ or CD8+ subsets.

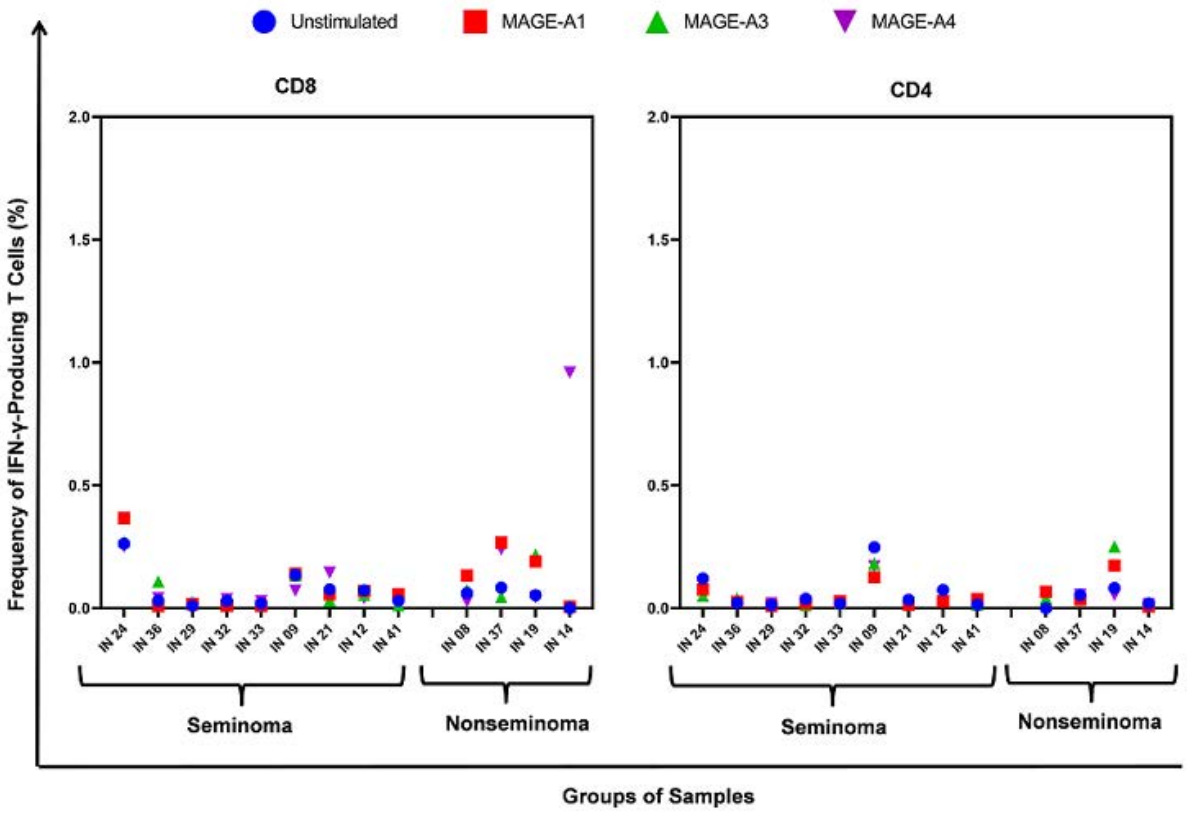

Fig. 6-2. IFN-γ responses of CD4+ and CD8+ T cells from patients with seminoma and non-seminoma tumours following stimulation with MAGE-A family peptide pools

PBMC were isolated from 9 patients with seminoma and 4 patients with non-seminoma tumours. These were stimulated with overlapping peptide pools from MAGE-A1/A3/A4 proteins. Cytokine-positive cells were then identified by expression of IFN-γ as a percentage of total CD8+ (left) or CD4+ (right) T cells.

In the non-seminoma group, MAGE-A1 overlapping peptide stimulation did induce selective production of IFN-γ from CD8+ T cells in two samples, IN08 and IN37. In particular, the baseline cytokine production levels from these samples were 0.062% and 0.084% respectively but this increased to 0.13% and 0.27% following peptide stimulation (left panel, Fig. 6-2). CD4+ responses did not appear to be increasing in these samples (right panel, Fig. 6-2).

Sample IN19 also demonstrated a cytokine response in both the CD4+ and CD8+ T cell subsets against peptide stimulation with MAGE-A1. In particular, the frequency of CD8+ cytokine-producing cells was 0.19% compared to 0.04% in the unstimulated population. A similar response was observed in the CD4+ pool from this sample where the frequency of responding cells was 0.17% with a baseline of only 0.082% (right panel, Fig. 6-2). Overall, the results show a low frequency of T cell response to MAGE-A1 stimulation.

MAGE-A3 Responses

Unlike the MAGE-A1 overlapping peptide stimulation whereby responses were detected in 3 seminoma and 3 non-seminoma patients, the MAGE-A3 overlapping peptide stimulation resulted in fewer responding patients. In the seminoma group, responses were demonstrated by sample IN36 where IFN-γ+ CD8+ T cells were detected at the frequency level of 0.11% (left panel, Fig. 6-2). This value is 3.5 fold higher than that seen in the unstimulated control. The CD4+ T cells in this sample did not seem to produce the cytokine post-stimulation. The other samples in the seminoma cohort did not show an apparent increase on IFN-γ-production either from their CD8+ T cells or their CD4+-counterparts. In the non-seminoma group, a positive response was seen with sample IN19 where the baseline value of 0.053% within CD8+ T cells was increased to 0.218% following MAGE-A3 overlapping peptide stimulation. This specific response was in fact even higher than that of MAGE-A1 stimulation (0.19%) (left panel, Fig. 6-2). CD4+ T cells in sample IN36 and IN19 also responded upon stimulation although their responses were weaker than that of their respective CD8+ T cells. IFN-γ-producing CD4+ T cells in IN36 were at a frequency of 0.039% whilst in IN19 the

value was 0.218% (right panel, Fig. 6-2). The baseline levels were 0.021% and 0.082% in IN36 and IN19, respectively (left panel, Fig. 6-2). MAGE-A3 overlapping peptide could enhance the production of IFN-γ by CD4 approximately 2-folds higher in IN36 and 3-folds higher in IN19. The remaining samples appeared to be irresponsive to the MAGE-A3 stimulation.

MAGE-A4 Responses

Another MAGE-family antigen I employed to induce specific responses from the seminoma and non-seminoma samples was MAGE-A4. Patient IN21 showed a modest CD8+ T cell IFN-γ response of 0.145% compared to unstimulated control (0.073%). In contrast, no CD4+ T cell response to MAGE-A4 was detected in this patient.

Patient IN14 had the greatest IFN-γ response to any MAGE antigen in our cohort which was against MAGE-A4. As marked by the purple triangle, upon stimulation, IFN-γ-producing MAGE-A4 specific CD8+ T cells were observed at a frequency of nearly 1 in 100 (0.96%) CD8+T cells (left panel, Fig. 6-2). Interestingly, this patient did not demonstrate responses to the other MAGE antigens investigated in this study.

The higher percentage of IFN-γ responses in patients with non-seminomatous tumours was something of a surprise based on our previous report. Although, the higher percentage of responses in IN19 might be due to seminoma tissue contained within this mGCT subtype. However, patient IN14 was found to be diagnosed with pure embryonic carcinoma that would not contain seminomatous elements. Further studies on a larger cohort of patients with known expression

levels of MAGE-A antigens by each individual tumour is required to determine if responses are being evoked specifically by the tumour or normal testicular germ cells.

TNF-α expression increases the sensitivity of identification of MAGE-A family specific T cells

My studies to this time had used only IFN-γ as the cytokine readout for a peptide-specific response. TNF-α is an additional cytokine that is produced in Th1 immune responses and in order to increase the potential sensitivity of detection I next went on to examine the TNF-α response to MAGE stimulation (Fig. 6-3).

TNF-α production was found to be a more sensitive approach to the detection of MAGE-specific T cells compared to IFN-γ. In particular, cytokine responses were detectable in the majority of samples except in sample IN33, IN12 (seminoma group) and IN37 (non-seminoma) where the background production of TNF-α was greater than that observed following challenge by either MAGE-A1, A3 or A4 overlapping peptides.

In some samples, the TNF-α production seemed to be consistent with the IFN-γ response. For example, in IN24 the frequency of IFN-γ-producing CD8+ T cells was measured at 0.1% when stimulated by MAGE-A1 overlapping peptides and a similar increment was observed through detection of the TNF-α-specific immune response (left panel Fig. 6-3). Similarly, in sample IN36 the antigen-specific IFN-γ and TNF-α cytokine responses were 3.5- and 2.3-folds higher than the background level.

A particularly strong and TNF-α selective immune response was made following MAGE-A3 overlapping peptide stimulation of sample IN09. Here the frequency of TNF-α-producing CD8+ T cells was 0.5% above background (left panel, Fig. 6-3) whilst the CD4+ response showed an increment of 0.73% (right panel, Fig. 6-3). Interestingly, this profile was not reflected in a similar magnitude of IFN-γ production.

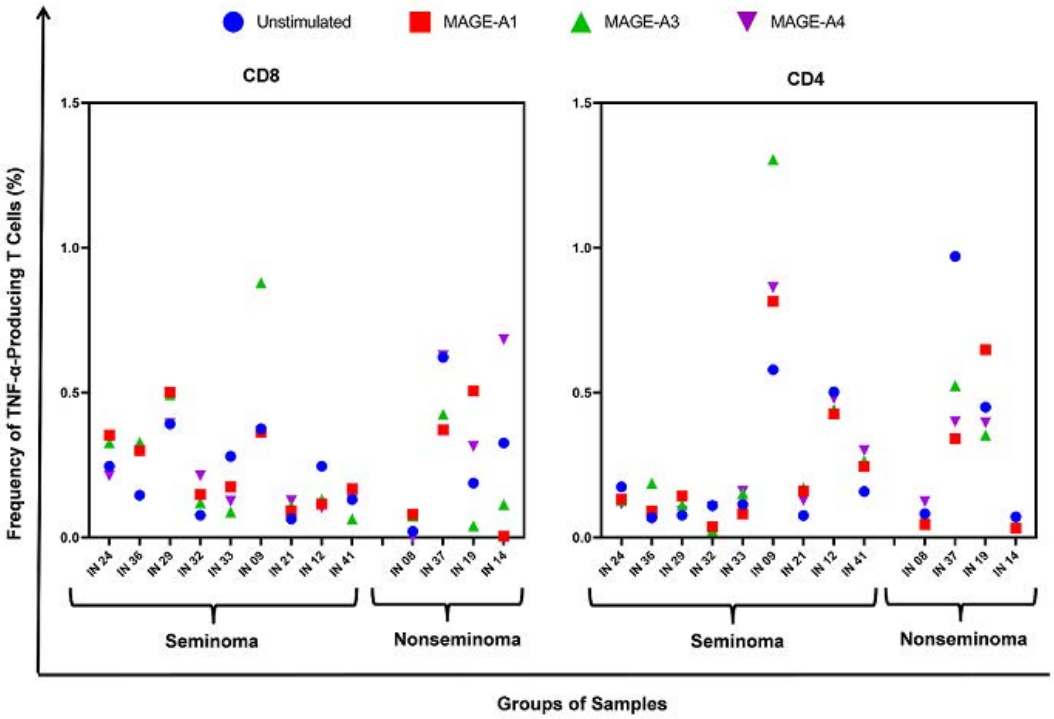

Fig. 6-3. TNF-α responses of CD4+ and CD8+ T cells from patients with seminoma and non-seminoma tumours following stimulation with MAGE-A family peptide pools

PBMC were isolated from 9 patients with seminoma and 4 patients with non-seminoma tumours. These were stimulated with overlapping peptide pools from MAGE-A1/A3/A4 proteins. Cytokine-positive cells were then identified by expression of TNF-α as a percentage of total CD8+ (left) or CD4+ (right) T cells.

The pattern of cytokine production did not correlate with a use of a specific MAGE peptide library. For instance in sample IN09 there was a clear TNF-α CD8+ T cell

response to stimulation from MAGE-A1 or MAGE-A3 but IFN-γ was only generated through use of the MAGE-A3 library. Likewise, CD8+ T cells in sample IN14 consistently responded to MAGE-A4 stimulation only with an increment of 0.36% above background (left panel, Fig. 6-3).

Simultaneous production of IFN-γ and TNF-α (further termed as IFN-γ/TNF-α) was observed most clearly in sample IN24 which demonstrated a strong CD8+ T cell response to stimulation with all three MAGE peptide pools (Fig. 6-4). IN14 also demonstrated increasing frequency of IFN-γ/TNF-α-producing CD8+ T cells although interestingly this was only seen following MAGE-4 stimulation (Fig. 6-4). A similar pattern of co-expression was also detected from sample IN09 in both the CD8+ and CD4+ T cell compartments. However, apart from these three samples there was no clear consistent profile of IFN-γ/TNF-α response to MAGE peptides across the cohort (Fig. 6-5).

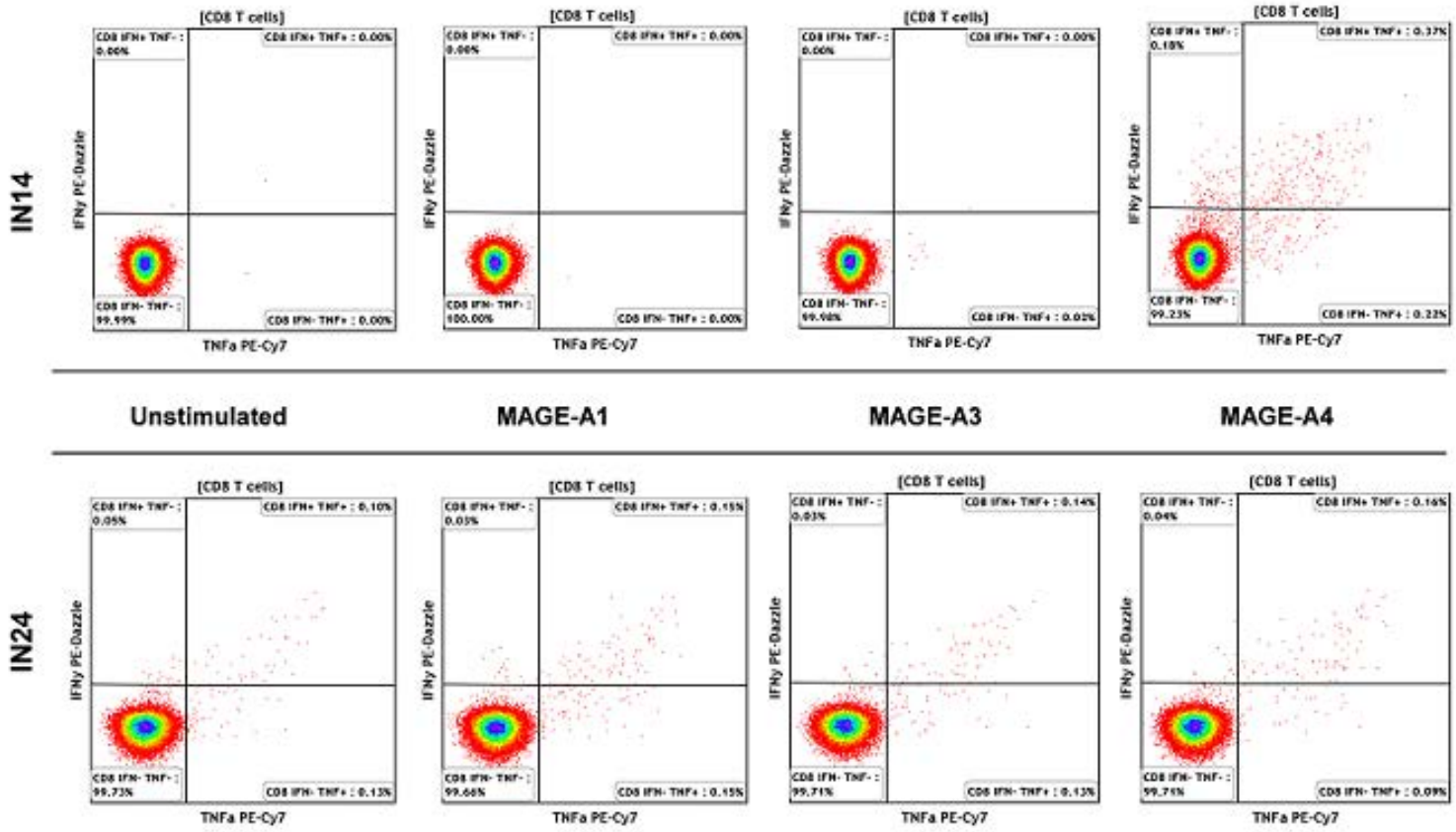

Fig. 6-4. Examples of dual cytokine secretion of CD8+ T cells following MAGE antigen stimulation

Flow cytometry plots showing dual secretion of TNF-α and IFN-γ in CD8+ T cells from patients IN14 and IN24. IN14 exhibited a large CD8+ T cell response to MAGE-A4. IN24 exhibited modest CD8+ T cell responses to all 3 MAGE-A antigens.

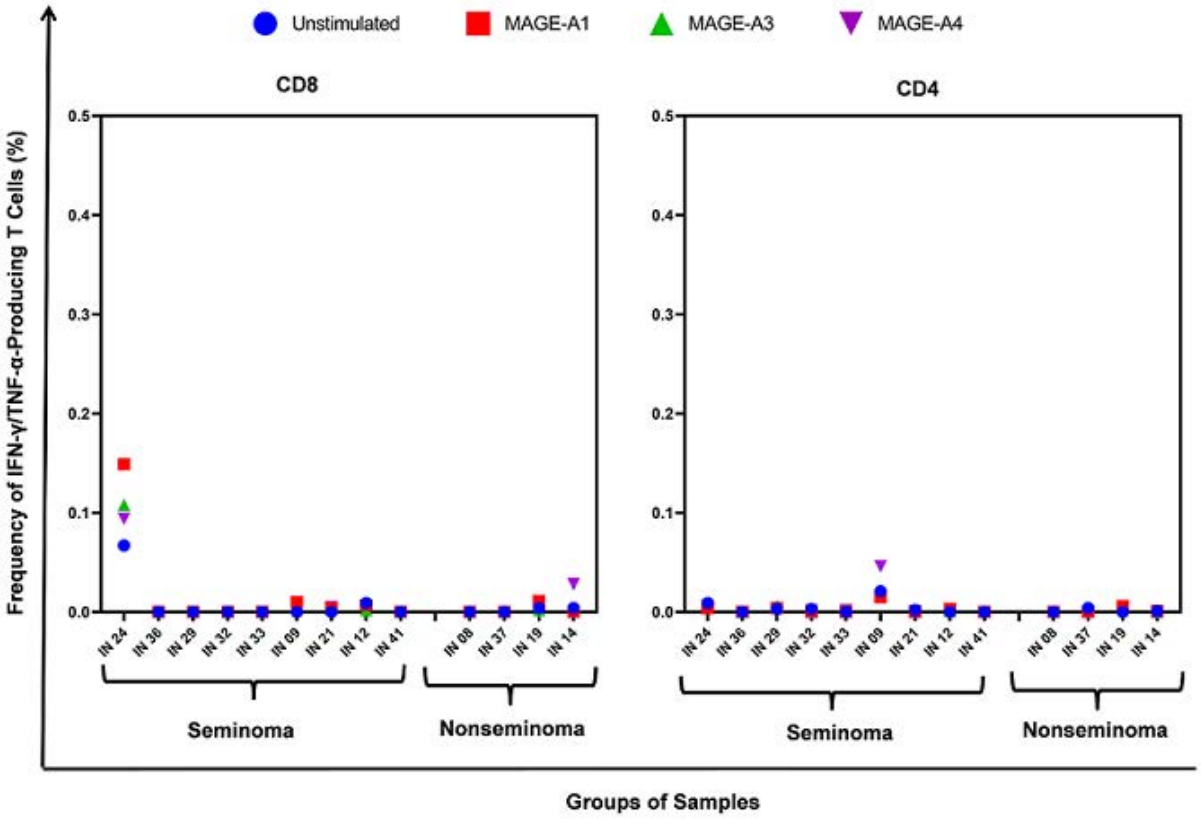

Fig. 6-5 . Overall profile of dual IFN-γ and TNF-α production by CD4+ and CD8+ T cells from patients with seminoma and non-seminoma tumours following stimulation with MAGE-A family peptide pools

PBMC were isolated from 9 patients with seminoma and 4 patients with non-seminoma tumours. These were stimulated with overlapping peptide pools from MAGE-A1/A3/A4 proteins. Cytokine-positive cells were then identified by expression of both IFN-γ and TNF-α as a percentage of total CD8+ (left) or CD4+ (right) T cells.

IL-2 is not produced by T cells following stimulation by MAGE-A peptides

IL-2 is a critical cytokine in T cell survival and differentiation and IL-2-responsive T cell subsets have extremely diverse characteristics including proinflammatory and anti-inflammatory biological roles. Moreover, IL-2 inhibits the differentiation of Th17 and Tfh cells such that IL-2 acts as an important regulator of T cell lineage commitment. In addition, the levels of IL-2 signalling can help to define the differentiation fate of T cells as high levels of IL-2 favour the development of short-

lived effector cells whilst lower levels promote the differentiation of memory T cells (Ross & Cantrell, 2018).

I next decided to use IL-2 production as a further marker of peptide-specific immune response. However, no significant IL-2+ T cell response was observed in any of the samples that were analysed (Fig. 6-6). Of note, donor IN41 expressed quite high levels of spontaneous IL-2 production but these were suppressed following stimulation with antigen. These data indicate that IL-2 is not a useful marker for the detection of MAGE-specific T cells. Furthermore, this raises interesting questions regarding the physiological status of the MAGE-specific T cells. Previous work from our laboratory had shown that MAGE-specific T cell response were not sustained following treatment for testicular cancer (Pearce et al, 2017). IL-2 is an important autocrine survival signal and as such this may go some way towards explaining why these clones do not persist into memory

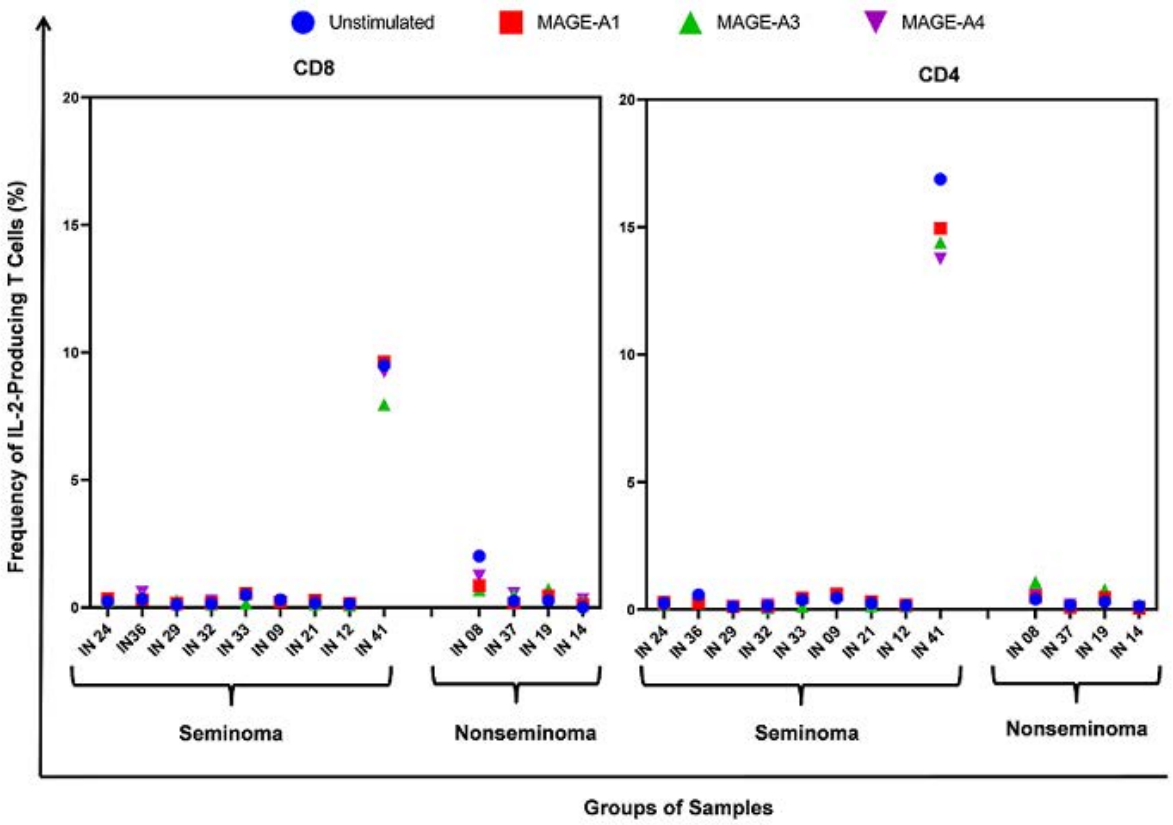

Fig. 6-6. IL-2 responses of CD4+ and CD8+ T cells from patients with seminoma and non-seminoma tumours following stimulation with MAGE-A family peptide pools

PBMC were isolated from 9 patients with seminoma and 4 patients with non-seminoma tumours. These were stimulated with overlapping peptide pools from MAGE-A1/A3/A4 proteins. Cytokine-positive cells were then identified by expression of IL-2 as a percentage of total CD8+ (left) or CD4+ (right) T cells.

GM-CSF production can be used to identify occasional MAGE-specific T cell responses and is independent of Th1 cytokine responses

The next cytokine that I investigated as a potential readout for MAGE-specific T cell response was GM-CSF. Although GM-CSF is primarily considered as a haemopoietic growth factor is also has a role in immune modulation. GM-CSF is produced by a subset of T cells following activation (Shi et al., 2006)and may be associated with the simultaneous production of IFN-γ (Sheng et al., 2014). This production is generally confined to CD4+ T cells (Sheng et al., 2014). GM-CSF is

believed to play a critical role to the maturation of dendritic cells (Shi et al., 2006). In addition to this function, GM-CSF, just recently, was reported to contribute in the mycrobacterial infection control. This was mediated by NKT cell subsets (Rothchild et al., 2017).

The frequency of GM-CSF+ T cells that was detected after MAGE stimulation was relatively low compared to the profile that had been seen following TNF-α or IFN-γ detection (Fig. 6-7). Moreover, we found that the GM-CSF+ T cell response did not coincide with simultaneous production of TNF-α or IFN-γ. For instance patient IN24, whose TNF-α and IFN-γ were highly upregulated following antigen stimulation, did not secrete GM-CSF. No GM-CSF response was observed in donor IN14 following MAGE-A4 stimulation. However an interesting pattern was observed in donor IN09 who demonstrated a strong GM-CSF+ response within both the CD8+ and CD4+ T cell subsets following MAGE challenge.

These findings show that GM-CSF is produced by a small subset of T cells following MAGE stimulation. However, this does not correspond to the subset that produces Th1 cytokines. The strong GM-CSF specific response that was observed in donor IN09 is of particular interest. This would represent an important area for future study if such T cells could be isolated and analysed in downstream functional assays.

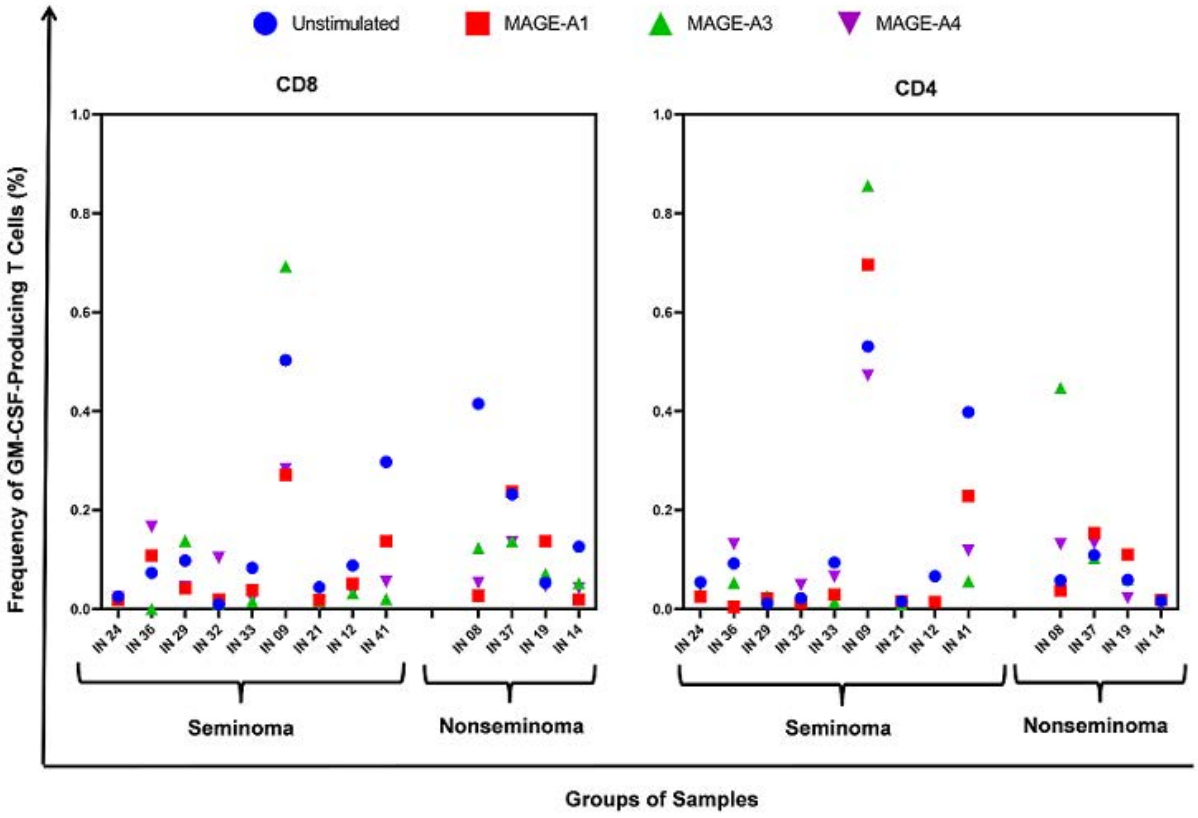

Fig. 6-7. GM-CSF responses of CD4+ and CD8+ T cells from patients with seminoma and non-seminoma tumours following stimulation with MAGE-A family peptide pools

PBMC were isolated from 9 patients with seminoma and 4 patients with non-seminoma tumours. These were stimulated with overlapping peptide pools from MAGE-A1/A3/A4 proteins. Cytokine-positive cells were then identified by expression of GM-CSF as a percentage of total CD8+ (left) or CD4+ (right) T cells.

CD107a upregulation identifies MAGE-A specific CD8+ T cells that fail to secrete inflammatory cytokine

In addition to cytokine production, cytotoxic activity is also a critical determinant of T cell function. Therefore I next went on to examine the upregulation of surface CD107a expression following peptide stimulation. CD107a expression is a reliable marker for cell degranulation which is a prerequisite of T cell mediated cytotoxicity. As such it is used widely as a surrogate for target cell killing which is a more challenging experimental approach.

CD107a expression was observed on CD8+ T cells from patients IN24, IN29 and IN12 within the group of patients with seminoma. These values were measured at 0.42%, 1.7% and 1.7% respectively after MAGE-A1 stimulation although there were also considerable baseline responses at 0.25%, 1.4% and 1.1%, (left panel, Fig. 6-8).

The strongest CD107a response was observed in donor IN14 who was within the nonseminoma group. Here the CD8+ T cell subset exhibited a near 2% increase in surface CD107a expression following stimulation with MAGE-A4, with very little spontaneous upregulation (Fig. 6-8). Interestingly, we also detected a large CD107a response of around 1.75% and 1% from baseline from CD8+ and CD4+ T cells, respectively, from patient IN19. As such this suggests that there are both CD4+ and CD8+ cytotoxic T cell responses to MAGE-A1 within this donor (Fig. 6-8).

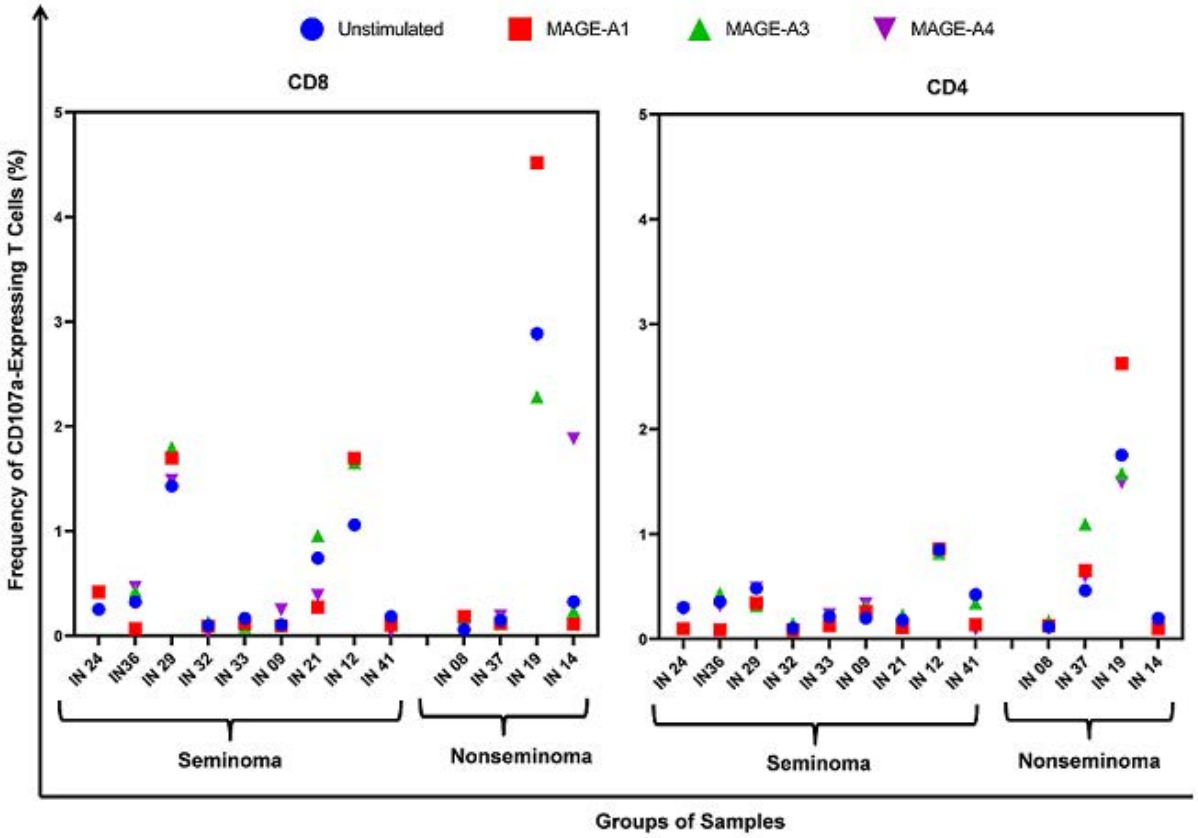

Fig. 6-8 . CD107a surface upregulation on CD4+ and CD8+ T cells from patients with seminoma and non-seminoma tumours following stimulation with MAGE-A family peptide pools.

PBMC were isolated from 9 patients with seminoma and 4 patients with non-seminoma tumours. These were stimulated with overlapping peptide pools from MAGE-A1/A3/A4 proteins. Cells undergoing degranulation were identified by surface expression of CD107a and data represented as a percentage of total CD8+ (left) or CD4+ (right) T cells.

Combined, these data suggest that CD107a expression is able to detect MAGE-A responsive T cells and that this occurs independently of the profile of inflammatory cytokine production. This indicates that there is only a modest overlap in the profile of T cells that can secrete Th1 cytokines and also degranulate in response to challenge with MAGE peptides. For example, MAGE-A4 specific T cells within donor IN14 augmented both TFN-α (Fig. 6-3) and IFN-γ (Fig. 6-2) production but failed to upregulate surface CD107a. The reasons for

this are not entirely clear but may reflect T cells that have differentiated to exhibit differential functional capacity or potentially cells with relative 'exhaustion' of the cytotoxic or cytokine response.

Combinatorial assessment with multiple cytokines and CD107a expression represents a potentially optimal approach to detect MAGE-specific T cell responses

In order to provide an overview of my results I next went on to compare and contrast the profile of cytokine response and CD107a expression within the overall cohort (Table 6-1). A qualitative assessment was used to define the cytokine responses as either weak (**+**, response between 0.02% to 0.1%) or strong (**+++**, response greater than 0.1%). Each response was presented as normalized response where the frequency of each assessed cytokine post stimulation was deducted by that of pre stimulation (considered as background noise).

Overall these data show considerable heterogeneity in the different functional responses both within and between donors. Unfortunately I was not able to isolate, expand and define the potential MAGE-specific responses that I detected with this approach and so it must be considered that not all of these responses represent genuine MAGE-specific T cells. The development of approaches that permit this confirmation represents an important ambition for future studies.

Overall my results do demonstrate the need to examine multiple cytokine responses, as well as a degranulation marker, in order to optimize identification of CTAg-specific T cells. This is reflected in the relative discrepancy between

cytokine production (IFN-γ and TNF-α-production) and degranulation (CD107a) as discussed above.

One of the most convincing responses were observed in donors IN19 where IFN-γ producing CD8+ T cell increased up to 4 times from baseline following MAGE-A3 stimulation whilst the TNF-α response to MAGE-A1 was also strong. MAGE-A1 overlapping peptides also generated a markedly high induction of CD107a expression, although spontaneous degranulation was also high in this patient. A more consistent effector response was found in IN14 whereby CD8+ T cells increased production of both IFN-γ and TNF-α, as well as upregulation of CD107a following MAGE-A4 stimulation. This suggests that immune responses demonstrated by IN14 was more robust and as such this would represent a strong candidate for subsequent isolation of MAGE-A4-specific CD8+ T cells in downstream analyses.

Table 6-1. Qualitative Measurement of T cell responsiveness against MAGE-A overlapping peptides

Samples ID	T Cell subset	MAGE-A1				MAGE-A3				MAGE-A4			
		TNF-α	IFN-γ	IFN-γ/TNF	CD107a	TNF-α	IFN-γ	IFN-γ/TNF	CD107a	TNF-α	IFN-γ	IFN-γ/TNF	CD107a
IN24	CD8	+++	+++	+++	+++	+		+++				+++	
	CD4												
IN36	CD8	+++				+	+		+				+
	CD4	+				+							
IN29	CD8	+			+++	+			+++	+			
	CD4	+				+							
IN32	CD8	+				+				+			
	CD4												
IN33	CD8												
	CD4	+				+				+			
IN09	CD8			+									
	CD4	+++				+++				+++		+	
IN21	CD8								+				
	CD4	+								+			
IN12	CD8				+++				+++				
	CD4												
IN41	CD8	+											
	CD4	+++								+++			
IN08	CD8	+	+										
	CD4		+							+			
IN37	CD8		+++								+++		
	CD4				+				+++				
IN19	CD8	+++	+++		+++		+++			+++	+		
	CD4	+++	+	+	+++		+				+		
IN14	CD8									+++	+++	+++	+++
	CD4												

+ represents a positive response above baseline between 0.02 and 0.1%; +++ represents a positive response >0.1%

Despite the relatively small size of my patient cohort (n=13) I was able to detect potential CTAg-specific T cell responses in the peripheral blood of TGCT patients using ICC staining. The three most convincing such responses, based on TNF-α, IFN-γ or CD107a expression, were observed in one seminoma patient (IN24) and two patients with nonseminomatous diseases (IN14 and IN19). The frequency of MAGE-specific responses is similar to that observed in previous work from our laboratory (Pearce et al, 2017) but the distribution of the positive results in relation to the tissue diagnosis was unexpected as we have previously shown that antigen-specific T cells were focussed within patients with seminoma. This is discussed more fully below.

Attempts to define the minimal immunodominant peptide epitope within the MAGE protein

The work above had shown that MAGE-specific T cell responses were indeed present in a subset of patients with TCGT and that intracellular cytokine secretion was an appreciate methodology for their detection. However, this response was detected against a peptide library that covered the whole of the MAGE protein. As such it does not give any indication as to the nature of the peptide epitope that underlies this response. As such, in the final part of my thesis I attempted to define this minimal epitope using T cells from donors IN14 and IN24 that represented nonseminomatous TGCT (pure embryonal carcinoma) and seminoma, respectively.

This work involved defining the HLA allele status of these donors and then the use of a peptide library 'matrix' that acts to map peptide responses into peptide pools

that share the same short sequence. The ultimate aim was then to define the minimal 9-mers peptide sequence within the 15-mers peptide that were used in the overlapping pool.

Assessment of the HLA genotype of donors IN14 and IN24

Immunogenic peptide epitopes are presented to αβ-T cells bound to an HLA class I or class II molecule. Different HLA alleles present a different spectrum of peptide sequences and as such knowledge of the patients HLA sequence can help to localist the potential sequence of the immunogenic epitope. As such, in initial work I undertook analysis to define the HLA alleles within donors IN14 and IN24, HLA typing was performed by PCR using primer sequences adopted from Bunce, et al that detect common HLA class I and II alleles (Bunce et al., 1995) .

As many as 14 different alleles were examined including: A1 (Lane 1); A2 (Lane 2); A3 (Lane 3); A11, 6601 (Lane 4); A1,11, 36,80, 3402 (Lane 5); B7 (including B703) B8101(Lane 6); B8 (Lane 7); B35, 18, 78, 1522 (Lane 8); B44 (Lane 9); DR7 (Lane 10); DR53 (Lane 11); DQ6 (Lane 12); CW7 (Lane 13) and CW0702,0703 (Lane 14). The results from this HLA typing approach are shown in Fig. 9. From these data patient IN14 was defined as expressing HLA-A11, HLA-B44, HLA-B35 and HLA-DR53 whilst IN24 was typed as HLA-A1, HLA-A2, HLA-DR7 and HLA-DQ6 positive. This information could then be used in the potential definition of individual peptide epitopes from the MAGE library.

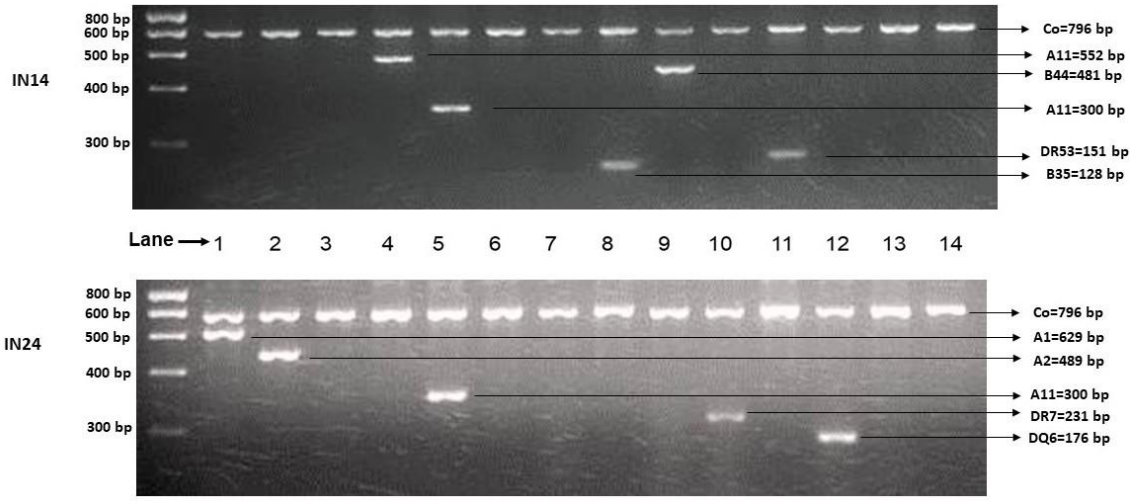

Fig. 6-9. HLA genotyping of patients IN14 and IN24

PCR products were separated by gel electrophoresis on a 1.5% agarose gel. HLA-DRB1 primers were added to each sample as a positive PCR control (+Co). A 100bp DNA ladder was used (far left lane) to confirm size of positive bands.

Screening of MAGE peptide libraries to define minimal MAGE epitopes

I next sought to define the T cell epitopes that were responsible for the positive T cell response in donors IN14 and IN24 following stimulation with the complete MAGE library. In order to do this we obtained two pools of 15-mers long peptides that spanned the entire sequences of MAGE-A1 and MAGE-A4. Unfortunately 3 peptides (p38, p50 and p51) were found not to dissolve in DMSO and so were excluded from the assay (Table 6-2).

The peptides were then utilized in a peptide matrix on 96 well plates such that each peptide was contained within two different pools. Through this approach it should prove possible to detect the peptide that contains the minimal epitope as it would be

present in two different pools, both of which should exhibit a functional response (Table 6-2 and Table 6-3).

Table 6-2. MAGE-A1 Peptide Matrix Pool (IN24)

MAGE-A1	1	2	3	4	5	6	7	8	9	PEPTIDE POOL
1	1	2	3	4	5	6	7	8	9	A
2	10	11	12	13	14	15	16	17	18	B
3	19	20	21	22	23	24	25	26	27	C
4	28	29	30	31	32	33	34	35	36	D
5	37	38	39	40	41	42	43	44	45	E
6	46	47	48	49	50	51	52	53	54	F
7	55	56	57	58	59	60	61	62	63	G
8	64	65	66	67	68	69	70	71	72	H
9	73	74	75							
PEPTIDE POOL	I	J	K	L	M	N	O	P	R	

Table 6-3. MAGE-A4 Peptide Matrix Pool (IN14)

MAGE-A4	1	2	3	4	5	6	7	8	9	PEPTIDE POOL
1	1	2	3	4	5	6	7	8	9	A
2	10	11	12	13	14	15	16	17	18	B
3	19	20	21	22	23	24	25	26	27	C
4	28	29	30	31	32	33	34	35	36	D
5	37	39	40	41	42	43	44	45	46	E
6	47	48	49	52	53	54	55	56	57	F
7	58	59	60	61	62	63	64	65	66	G
8	67	68	69	70	71	72	73	74	75	H
PEPTIDE POOL	I	J	K	L	M	N	O	P	R	

In order to obtain the best possible chance to detect a positive response, and also to 'cross-check' individual assays, I elected to use intracellular cytokine detection of IFN-γ and TNF-α, as well as CD107a expression, as functional assays for response. As shown in Fig. 6-10, relatively weak IFN-γ responses were observed following stimulation of PBMC from the donor.

The results of assessment from donor IN14, who was diagnosed with pure embryonal carcinoma, were somewhat difficult to interpret. Peptide pool M elicited strong functional responses across all three readouts. However, there was no clear positive signal from any other pool that shared individual peptides. Peptide pool B did increase production of TNF-α as well as CD107a expression and therefore I elected to target peptide 14, the only 'shared' peptide between these pools, as potentially containing the minimal epitope. Unfortunately, the use of the peptide matrix with PBMC from donor IN24, a patient with seminoma, did not reveal any specific responses and so I was unable to pursue this further.

Peptide 14 has an amino acid sequence of EVPAAESAGPPQSPQ. Most peptide epitopes that bind HLA class I are 9 amino acids long and therefore I synthesized 7 individual peptide epitopes that commenced at sequential positions in the 15mer. For instance, peptide 1 was **E**VPAAESAG, peptide 2 was **V**PAAESAGP and so on. I then used individual 9-mer peptides in a TNF-α intracellular assay to assess responses for against each peptide (Fig. 6-11).

PBMCs from IN14 were stimulated with peptide-pulsed lymphoblastic cells lines (LCLs) generated from two healthy donors who each shared two alleles with the patient: HLA-A24/B44 and HLA-A11/B35. These were chosen as the patient has been 'tissue typed' as expressing all four of these alleles. The strongest response was observed following challenge with peptide 5 when it was presented by HLA-A24/B44-positive LCL. Indeed, the maximal TNF-α response here was 0.93% of the

CD8+ pool. The same peptide does not induce cytokine production when pulsed onto HLA-A11+/B35+ LCL.

I next went on to try to define if the peptide 5 sequence of AESAGPPQS would be predicted to bind to HLA-A24 or HLA-B44. Use of NetMHC (www.cbs.dtu.dk/services/NetMHC/Pan) revealed that it should indeed bind to HLA-B44 although with relatively weak binding. As such AESAGPPQS seems encouraging for further investigation and may represent a novel peptide epitope from MAGE that is restricted by HLA-B44. Unfortunately at this stage the number of donor PBMCs that I had available for further analysis was limited and I was unable to attempt to derive a peptide 5-specific T cell clone.

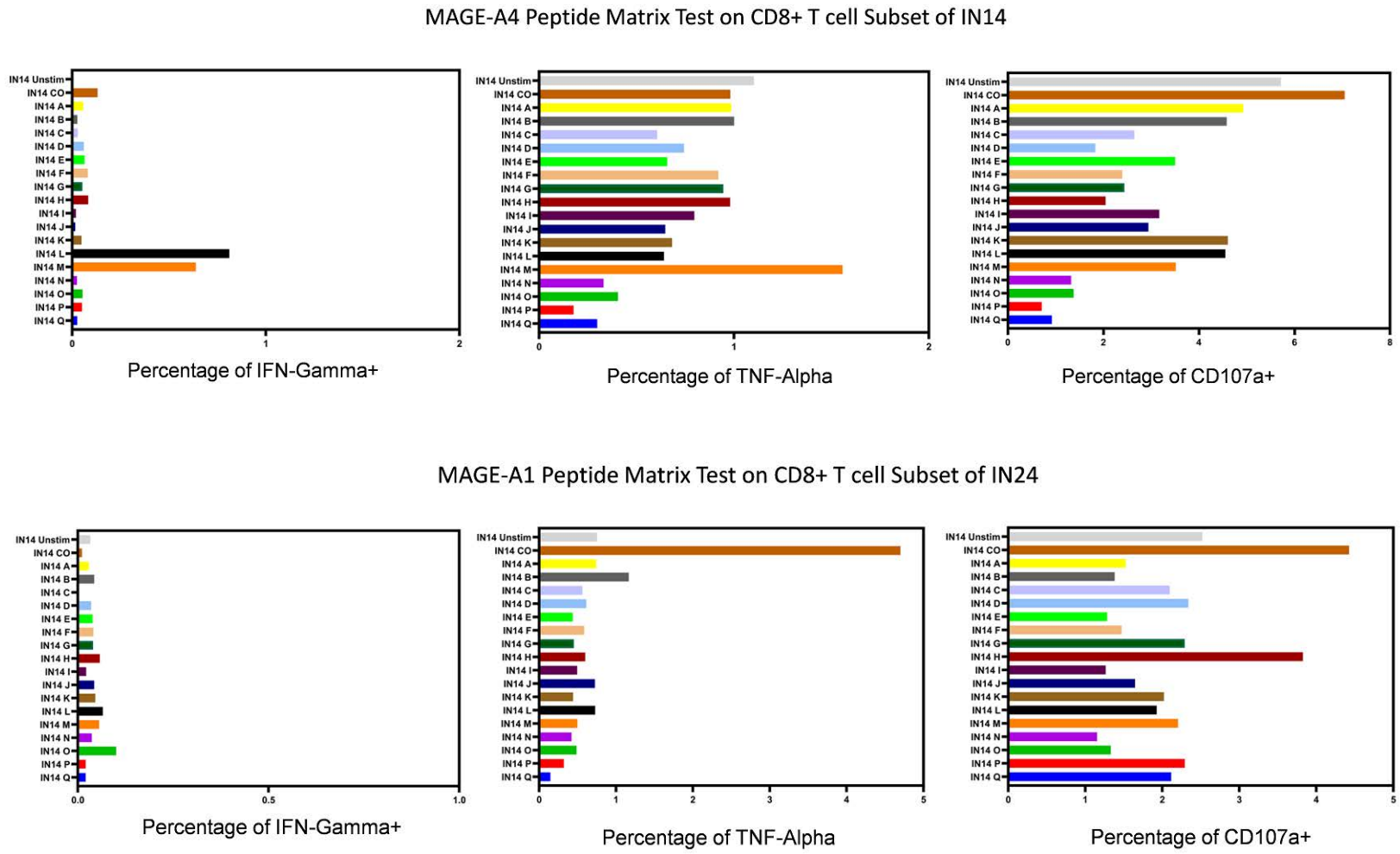

Fig. 6-10. Cytokine and degranulation levels following stimulation with MAGE-A peptide matrices

Responses to peptide matrices using IFN-y (left), TNF-a (middle) and CD107a (right) as readouts following stimulation with MAGE-A4 (NI14; top) or MAGE-A1 (IN24; bottom).

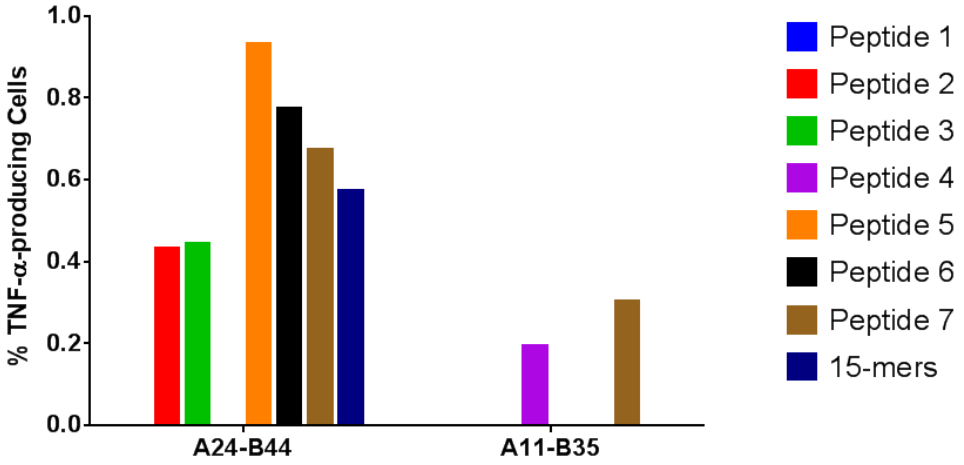

Fig. 6-11. TNF-α production by PBMC from donor IN14 against 7 individual 9-mer peptides derived from the peptide 14's 15-mers

PBMC were stimulated with LCL that had been pulsed with individual 9-mer peptides. The TNF-α production in response to stimulation was then measured after 4 hours. Missing bars indicate no observable TNF-α productions after the T cellsbeing stimulated with corresponding 9-mer peptides.

Next, we attempted to determine the HLA-I restriction of peptide EEVPAAESAGPQSPQ using polyclonal T cell lines we generated. As previously determined, IN14 was HLA-A11, A24, B35, B44 positive, and so we used allogeneic LCLs from healthy donors whose HLA-I shared one common allele. The HLA types of our donors were identified as:

1. A2/**A11**/B7/B8 : shared A11 allele in common
2. A3/A23/B7/**B44**: shared B44 allele in common
3. A2/A68/**B35**/B49: shared B35 allele in common
4. A1/**A24**/B52/B61: share A24 allele in common
5. A2/B27.05 as negative control

We peptide-pulsed the LCLs with peptide pools consisted of seven peptides (each was 9-mers long) spanning the entire amino acid sequence of EEVPAAESAGPQSPQ. Those peptides were: EEVPAAESA, EVPAAESAG, VPAAESAGP, PAAESAGPQ, AAESAGPQS, AESAGPQSP and ESAGPQSPQ.

Each group of peptide pulsed LCLs were irradiated at 40Gy, and used to stimulate polyclonal T cells specific (assumingly) for the 15-mer peptide to help determine what particular epitope the CD8+ T cells responded to. We then did restimulations with peptide-pulsed LCL at day 7 and day 14. At day 21, we performed limiting dilution T cell cloning of these polyclonal populations. Cloning was performed with irradiated peptide pulsed LCL A allele group (bearing LCL A11 and LCL A24) and LCL B allele group (bearing LCLB35 and B44). We plated cells at 3 and 0.3 cells per well. After second restimulation and we observed some potential growth, and therefore perfomed IFN-γ ELISA to measure the clones' capability of secreting IFN-γ following stimulation with the seven 9-mers peptides spanning EEVPAAESAGPQSPQ.

We detected a single response whereby clone 5 in the 0.3 cells/well cloning assay secreted IFN-γ above baseline with the A1/A11 LCLs (Fig. 6-12). Unfortunately, I attempted to bulk up clone 5 to further elucidate the exact peptide to which the clone was responding but the cells failed to proliferate (data not shown).

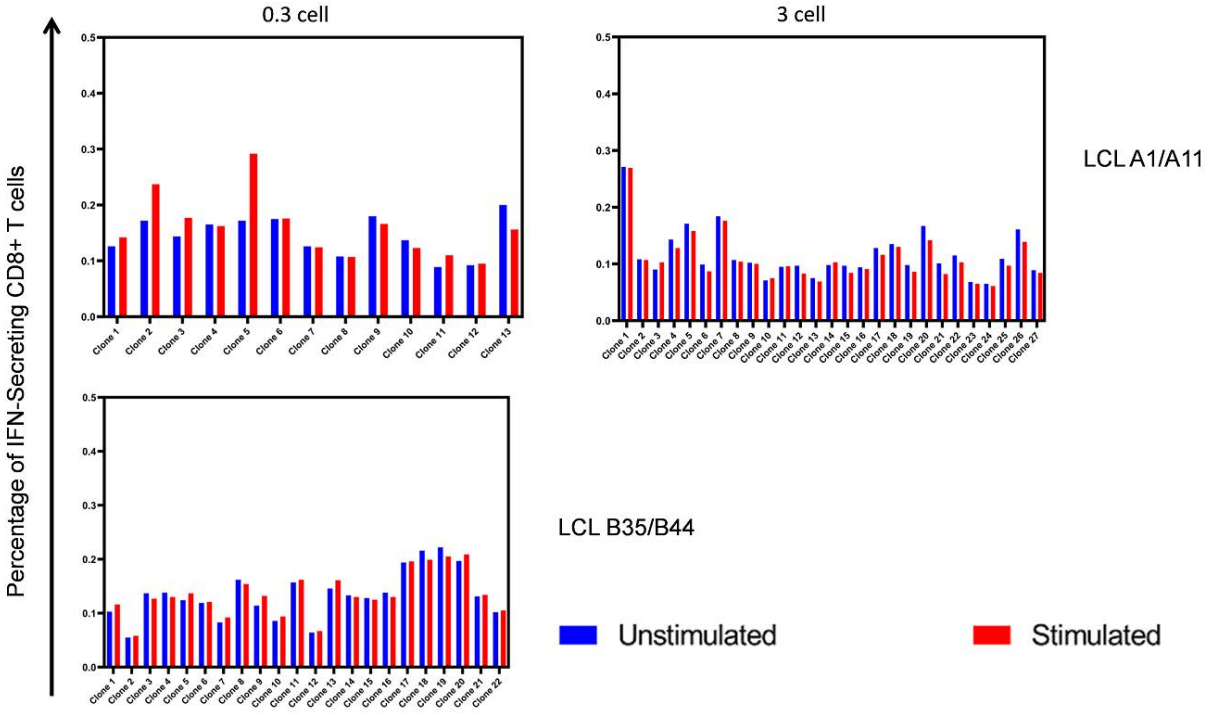

Fig. 6-12. Peptide screening to determine HLA-restriction of CD8+ T cell clones from IN14.

T cell clones were tested for peptide specificity following peptide-pulsed LCL co-culture, and IFN-γ was measured by ELISA. Peptide pulsed LCLs with matched HLA-A alleles (top) and HLA-B alleles (bottom) were tested in clones derived from limiting dilution cloning with 0.3 cells/well (left) and 3 cells/well (right)

Discussion

The first three chapters in my thesis have focused on characterization of the peripheral T cell immune response in patients with testicular cancer. This had shown evidence of low level T cell activation within this patient sub-group, supporting the previous work from our group that this group of patients do indeed show T cell responses against cancer testis antigens. However, in my final chapter I elected to undertake a further screening of these peripheral blood samples against CTAG family members.

A major focus of my chapter was to investigate the potential use of the intracellular cytokine assay (ICC) for the detection of peptide responses as our previous work had used ELISPOT analysis. Despite the high sensitivity that renders it as a widely used tool to assess tumour specific and vaccine-induced T cell responses, ELISPOT has its own disadvantages. Firstly, it is difficult to distinguish between CD4 and CD8 T cell responses. Secondly, it does not allow for the simultaneous cytokines production measurement. And the third disadvantage is that the T cells used for this method are not recoverable thus limits the scope of analysis. With a main consideration to overcome those mentioned disadvantage, I performed intracellular cytokine (ICC) staining and used appropriate age-matched controls. This approach is also a sensitive one. In addition to the sensitivity, it allows delineation of CD4 and CD8 subset as well as additional phenotypic sub-groups. However, like the ELISPOT does, it does have some weaknesses. Firstly, it necessitates a lot more cells to yield reliable staining results. Throughout my study, the safe number of the

running cells are at least 500,000, which is 5 folds higher than that required to conduct ELISPOT analysis. Secondly, it does not allow isolation of viable cells for downstream cloning. However, cytokine capture systems are available to compensate this limitation. The capture system basically functions to facilitate the selection of cytokine-positive T cells for subsequent cell culture. My aim was to use these systems subsequent to detection of positive responses.

My results show that cytokine secretion is indeed a viable approach to detect CTAg-specific T cells. I chose to focus on the MAGE protein family, as this has previously been shown as an immunodominant CTAg family member within this setting. In particular three overlapping peptide pools spanning the whole sequences of MAGE-A1, A3 and A4 were used. These proteins are highly homologous but do show reasonable amino acid variation.

Although compared to ELISPOT, ICC does not seem to be highly sensitive, but I was able to detect responses in around one third of donors. In order to increase sensitivity, I detected a range of cytokines following stimulation but, not surprisingly, the Th1 cytokines of IFN-γ and TNF-α were found to be the most sensitive in this regard. Indeed, TNF-α appeared to identify a greater proportion of responsive T cells compared to (IFN-γ) and this phenotype has been observed in other situations. It was noteworthy that no IL-2-positive T cells were identified and this may be relevant to our previous observation that MAGE-specific T cells are not long lived. GM-CSF was also found to be a relatively unreliable marker for detection of peptide-specific responses.

Since cytokine secretion is only one functional assay, I also therefore included CD107a expression in my analysis. This is considered to be a robust correlation for cytotoxic function. The correlation between cytokine and CD107a responses was moderate but it did allow me to identify 2 donors in which consistent and convincing responses were detected across both of these functional readouts. I then went on to assess these donors in more detail.

The ultimate aim of my work in this chapter was to identify novel peptide epitopes from within the MAGE proteins. Several MAGE immunodominant epitopes have been characterized but these are restricted by individual HLA alleles and are therefore limited in their potential within immunotherapy trials (Yao et al, 2016; Yu et al, 2012). One of the ambitions of my research group has been to use patients with testicular cancer as 'reservoir' for mapping MAGE-specific T cells and I therefore undertook a more detailed assessment of the epitope-specificity of these two donors. I utilized a well described technique of peptide matrices to try and identify the dominant 15-mer peptides. This appeared to work in one of the donors (IN14) whose responses toward MAGE-A4 was deemed to be specific and consistent. After conducting the peptide matrices test where the entire sequence of MAGE-A4 was chopped off into 15-mers with 11 amino acid overlapped, I Fig.d out that 15-mers with a sequence of EVPAAESAGPPQSPQ was able to stimulate cytokine productions at relatively higher frequency compared to that seen for other peptide pools. I then chose to breakdown this 15-mer peptide into individual 9 amino acid peptide fragments in order to assess the specific peptide that gave the optimal response. Once this peptide was indeed identified it was shown to be restricted by

HLA-B44 or HLA-A24. *In silico* analysis showed that it did exhibit moderate binding affinity for HLA-B44 and therefore this peptide does represent a genuine new candidate as a MAGE-A4 specific peptide.

In future studies it will be important to evaluate peptide specific responses patients who express the HLA-B44 allele at various stages of disease, including presentation with testicular cancer, and these results are awaited. One proviso is that not all epitopes presented by HLA class I are 9 amino acid long and peptide lengths between 8 and even 12 amino acids are certainly potential possibilities. However, I was unable to perform further assessment of this possibility due to the expense of generating such a large pool of peptides and a limiting number of patient's PBMCs. Indeed, one of the lessons learned from this chapter was that the number of T cells available from patient blood was limiting to undertake such demanding experiments.

CHAPTER VII. DISCUSSION

Testicular cancer is the most common tumour amongst young men and is increasing in incidence within the Western world. The aetiological factors that drive this disorder are currently unclear although the condition is more common in men of Caucasian origin. The clinical outcomes for men with testicular cancer are now excellent. This is partly because the surgical removal of the tumour (orchidectomy) offers a definitive approach to eliminating the primary tumour, but primarily reflects the fact that systemic chemotherapy, most notably with *cisplatin*, is highly effective at eradicating metastatic disease in almost all cases. Indeed, the clinical response to chemotherapy can be so effective and rapid that the development of necrotic lymph nodes can be a clinical concern and a number of men have to undergo surgical removal of lymph nodes. As such, testicular cancer cannot really be considered as a 'tumour of unmet need' as the number of men who succumb to this diagnosis is now thankfully quite small. However, it should be noted that not all patients do achieve a functional cure hence further comprehensive studies are required to reach the cure rate of 100%. In this context, research within my thesis has a direct relevance to seeking to improve the outcome of men with this disease. However, the major premise of my thesis is that the immune response can play an important role in controlling and curing testicular cancer following treatment intervention. At first sight this may appear contradictory, as chemotherapy is clearly the agent that mediates highly efficient responses. However, it is known that chemotherapy may work, at least partially, by inducing tumour-specific inflammation

and the development of tumour-specific immune responses. Indeed, the use of low dose cyclophosphamide as an approach to reduce the number of T regulatory cells is an area of very active interest. In addition to the remarkable efficacy of chemotherapy in testicular cancer, the anatomical location of this tumour also implicates tumour-specific immunity as potentially playing an important role. In particular, the testis is defined as an immune privileged site where, by the Sertoli cells-built blood-testis barrier (BTB), the immune system is strictly not allowed to invade adluminal compartment of seminiferous tubules, thus the process of spermatogenesis is protected. Once this blood-testis barrier is broken, as in the case of local tumour, the immune system can enter this compartment and would be predicted to generate local immune responses against sperm-based antigens. The systemic dissemination of the response may then be able to control metastatic disease, following priming initiated by chemotherapy.

There remain very few studies of immune response to testicular cancer at the current time. Histological analysis of the immune infiltrate in seminoma has shown that T cells dominate the infiltrate and that this is positively correlated with clinical outcome. Indeed, a major ambition of our research group is to interrogate the immune response locally within the primary tumour site. I contributed to the collection and storage of tumour infiltrated lymphocytes (TILs) from patients with primary testicular tumours. However, I did not carry on further with these TILs since: 1) the samples were uncommon,-- being that the type of TGCT which was teratoma from which the collected tumour inflitrated lymphocytes number was too small, and

2) the samples were of interest yet given at a tiny sizes. This remains an important area for future study.

Previous work from the research group had shown an abnormal profile for the T cell repertoire in patients with testicular cancer (Pearce et al, 2017). In particular, an increased proportion of memory cells is seen in the blood of patients at the time of diagnosis but this disturbance in the memory/naïve ratio was corrected over the first 3-6 months of treatment. As such, this indicated a clear disturbance of the T cell repertoire at the time of disease presentation. The first 3 chapters in my thesis were undertaken on blood samples from patients with seminoma and non-seminomatus tumours and interrogated a range of features.

At the phenotypic level, there were no major disturbances in the T cell subsets within the patient group. This included cells of the innate arm of the immune system including γδ-T cells, MAIT and NKT cells. To some extent this was not surprising, as this phenotypic profile has a very broad scale analysis of the repertoire and more finely detailed assessments should be performed in the future. Our laboratory has now obtained access to a CyTOF machine that allows simultaneous assessment of 35 proteins on the surface of cells. If I were able to continue my study I would be very interested to assess T cell repertoire in patients using this sort of technology.

Perhaps the most interesting results were seen with NK cells within the periphery where there was a clear increase in the number of $CD56^{dim}$ cells that had lost or downregulated CD16 expression compared to $CD56^{bright}$ subset. The differentiation process of NK cells is not entirely clear and so it is difficult to ascertain if this represents downregulation of CD16 or development of a more immature population.

Again, in future studies I would like to assess the phenotype of this population in more detail and ideally assess its transcriptional basis using approaches such as RNA sequencing. Furthermore, assessment of the cytotoxic capacity of these cells, and the relative impairment of antibody dependent cell cytotoxicity, will be important to assess. The relative avidity of the peptide-specific cells would also be valuable to assess.

A somewhat unexpected finding was the increased proportion of B cells in the blood of patients at the time of diagnosis. Unfortunately, a limitation of these studies had been the fact that I did not have access to the absolute count of lymphocytes within the blood of patients and healthy donors. As such, I could not compare the absolute level of discreet cell subsets. Notwithstanding this limitation, this profile does suggest a relative concentration of B cells within the total CD45+ lymphocytes. Somewhat surprisingly, these cells lack CD27 expression and therefore are likely to reflect B cells as not undergone antigen experience. There have been very few studies of the humoral immune response to testicular cancer and my results suggest that this could be a fruitful area of future investigation. One potential explanation is that T cells may have trafficked into the tumour and therefore increased the proportion of B cells in the periphery.

In order to undertake more detailed phenotypic analysis I then went on to assess the expression of five dominant checkpoint proteins on T cells from the patient blood samples. Checkpoint proteins, most notably PD-1 and CTLA-4, are probably the most intensively investigated proteins in biology at the current time. Monoclonal antibodies designed to block their activity have been shown to effectively cure many

patients even those whose cancers are metastased. It has relatively recently been shown that combination antibody therapy against PD-1 and CTLA-4 works more efficaciously compared to antibody blockade directed at either PD-1 or CTLA-4 alone. Despite this, the mechanisms by which this happens have not been totally unveiled. It is thought that antibody blockade releases the 'brake' on partially exhausted T cells and reinforce them to facilitates tumour cell recognition and lysis. However, PD-1 and PD-L1 are also expressed on many other subsets of the immune system and changes in immune regulation may also be equally important. My findings show that PD-1 and TIGIT were expressed on a substantial minority of T cells within the peripheral blood. However, this was matched by a similar proportion within the control group hence the initial assumption that PD-1 and TIGIT, either as single or dual expression, might identify 'exhausted' T cells is not valid in this case. This emphasizes the need to include appropriate age matched control samples in all studies, such as I was able to obtain. In contrast, more interesting results were found from the use of antibodies against LAG-3, Tim-3 and CTLA-4. Here, small populations of checkpoint-positive cells were observed within the patient group alone. This is quite an exciting finding and it suggests that small numbers of T cell have undergone relative phenotypic exhaustion within the periphery and these may represent an important target for future analysis. Indeed, if time allows, I would like to isolate these cells and assess their properties in substantial detail.

The third analysis within peripheral blood was to undertake a functional assessment of T cells. Here I chose to use a strong mitogenic stimulus, in the form of PMA/Ionomycin to see if there was any evidence of gross T cell exhaustion within

the periphery. In fact, my findings showed that T cells from patients with testicular cancer retained a broad profile of cytokine responses, which were comparable with those from healthy donors. This is compatible with the natural history of testicular cancer in which there is no clear susceptibility to infection within the patient sub-group. One surprising, and slightly unexpected, finding within my analysis was the value of assessing the profile of spontaneous cytokine secretion prior to addition of the mitogen. Many investigators elect not to undertake this approach as it is quite costly and increases the amount of data analysis. However, I was able to show that low levels of spontaneous production of cytokines such as IL-21 and IL-17, which are pro-inflammatory cytokines, as well as IL-10 which represents an anti-inflammatory cytokine, were observed selectively within T cells from the patient group. This may again represent a small population of activated T cells within the patient's blood and these certainly are possible candidates for representing tumour-specific T cell responses.

In my final data chapter I was anxious to undertake functional analysis of T cells against cancer testis antigen (CTAg) libraries. Seeking to build on previous data from our laboratory, I elected to use intracellular cytokine analysis. The idea here is that this would allow me to undertake more detailed phenotypic analysis of the nature of the T cells that were responding to MAGE stimulation. Furthermore, I was hoping that a cytokine secretion assay could be incorporated such that we would then be allowed to isolate viable cells for further analysis.

My data did show that this approach was feasible for the detection of MAGE-specific responses although the sensitivity was probably somewhat lower than what had been observed with ELISPOT analysis. Nevertheless, when I combined TNF-α and IFN-γ detection together with a CD107a expression analysis, I was able to detect what appeared to be robust MAGE-specific responses within 2 donors. Interestingly I was able to see responses in patients with tumours other than seminoma, specifically pure embryonic carcinoma, whereas this had previously not been the case in our previous report. One of the major ambitions of the laboratory is to isolate new immunogenic epitopes from the MAGE family of proteins which could potentially be used as immunogens or vaccines in patients with malignant disease. Utilizing the bloods taken from one of my patients, I was indeed able to identify candidate peptide from a patient with B44 and I would be delighted if this were able to be used in the future as such a product.

There is a range of future studies that I would like to address if I were able to continue this work:

1. What is the nature of the T cell immune response to cancer testis antigens?

My own work, and that of the previous report, has shown strong and tantalising evidence of T cell responses against cancer/testis antigens and MAGE-A to be specific. However, it is proving to be challenging to isolate functional clones for a detailed characterization. Some of the data from my work suggests that these T cells may be short-lived and may perhaps not produce IL-2, a cytokine that would be facilitating their proliferation. We, therefore, may need to develop new

approaches to isolate these cells. Potential opportunities include direct isolation from peripheral blood using cytokine secretion followed by sequencing of the T cell receptor of responding cells. This T cell receptor might then be used in a transgenic T cell for the treatment of patients during adoptive therapy. The longevity of these cells could potentially also be mapped *in vivo* by the use of T cell receptor finger printing. It is possible that their half-life is much smaller than would be seen against, for instance, virus-specific T cells.

2. The nature of the humoral response to cancer testis antigens

As discussed above, my work revealed increased proportions of B cells in the blood of patients with testicular cancer. I would now propose to gain evidence of humoral responses in the TGCT by assessing their sera. This could feasibly be performed with either immunohistochemistry or immunofluorescence staining of testicular sections. Many advances have been made in the isolation of antigen-specific B cells with reconstruction of monoclonal B cell products. These may potentially have a use in future immunotherapy.

3. Does the immune response against cancer testis antigens have any role in the control of testicular cancer?

Even if cancer testis antigen-specific T cells are indeed generated in patients with testicular cancer, it is not currently clear if they play in role in eradication of the disease. Indeed, one feature that needs to be addressed is that HLA class I is not thought to be expressed on tumour cells. As such, these will not be expected to present epitopes to the immune system. Nevertheless, the immune response could

still play an important role, potentially through bystander effects within the micro-environment.

4. Can tumour-specific T cell responses that develop within testicular cancer be used in other patients with malignant disease?

Interestingly, although testicular cancer tumour cells may not express HLA class I, the immune responses that are generated from T cells following invasion into the testis may potentially be of benefit in the treatment of other tumours. In particular, if cancer testis antigens are shared on other somatic tumour cells, where HLA class I is retained, such T cells, or the potential transfer of genes-encoding their T cell receptor, could be a potential future immunotherapy of interest. This is something that the group is developing and it will be interesting to follow this over the next few years.

Overall, I have gained substantial experience in investigating this fascinating tumour. Although not a cancer of clinical unmet need, I do believe it represents a disease of immunological unmet need and that the key to understanding why this tumour responds so well to therapy may offer hope for those patients who have tumours which are less responsive to therapy.

REFERENCES

Ai, W., Li, H., Song, N., Li, L., & Chen, H. (2013). Optimal method to stimulate cytokine production and its use in immunotoxicity assessment. *International Journal of Environmental Research and Public Health*, *10*(9), 3834–3842. https://doi.org/10.3390/ijerph10093834

Albers, P., Albrecht, W., Algaba, F., Bokemeyer, C., Cohn-Cedermark, G., Fizazi, K., Horwich, A., & Laguna, M. P. (2011). EAU guidelines on testicular cancer: 2011 update. *European Urology*, *60*(2), 304–319. https://doi.org/10.1016/j.eururo.2011.05.038

Albers, P., Albrecht, W., Algaba, F., Bokemeyer, C., Cohn-Cedermark, G., Fizazi, K., Horwich, A., Laguna, M. P., Nicolai, N., & Oldenburg, J. (2015). Guidelines on Testicular Cancer: 2015 Update. *European Urology*, *68*(6), 1054–1068. https://doi.org/10.1016/j.eururo.2015.07.044

Alsaab, H. O., Sau, S., Alzhrani, R., Tatiparti, K., Bhise, K., Kashaw, S. K., & Iyer, A. K. (2017). PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. *Frontiers in Pharmacology*, *8*(AUG), 1–15. https://doi.org/10.3389/fphar.2017.00561

Amand, M., Iserentant, G., Poli, A., Sleiman, M., Fievez, V., Sanchez, I. P., Sauvageot, N., Michel, T., Aouali, N., Janji, B., Trujillo-Vargas, C. M., Seguin-Devaux, C., & Zimmer, J. (2017). Human CD56dimCD16dimCells As an Individualized Natural Killer Cell Subset. *Frontiers in Immunology*, *8*(JUN). https://doi.org/10.3389/fimmu.2017.00699

Anderson, A. C., Joller, N., Kuchroo, V. K., Hospital, W., & Immunology, E. (2016). Lag-3, Tim-3, and TIGIT co-inhibitory receptors with specialized functions in immune regulation. *Immunity*, *44*(5), 989–1004. https://doi.org/doi:10.1016/j.immuni.2016.05.001.

Andrews, L. P., Marciscano, A. E., Drake, C. G., & Vignali, D. A. A. (2017). LAG3 (CD223) as a cancer immunotherapy target. *Immunological Reviews*, *276*(1), 80–96. https://doi.org/10.1111/imr.12519

Attanasio, J., & Wherry, E. J. (2016). Costimulatory and Coinhibitory Receptor Pathways in Infectious Disease. *Immunity*, *44*(5), 1052–1068. https://doi.org/10.1016/j.immuni.2016.04.022

Bahrami, A., Ro, J. Y., & Ayala, A. G. (2007a). An overview of testicular germ cell tumors. *Archives of Pathology and Laboratory Medicine*, *131*(8), 1267–1280.

Bahrami, A., Ro, J. Y., & Ayala, A. G. (2007b). An overview of testicular germ cell tumors. *Archives of Pathology and Laboratory Medicine*, *131*(8), 1267–1280. https://doi.org/10.1043/1543-2165(2007)131[1267:AOOTGC]2.0.CO;2

Betts, M. R., Brenchley, J. M., Price, D. A., De Rosa, S. C., Douek, D. C., Roederer, M., & Koup, R. A. (2003). Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *Journal of Immunological Methods*, *281*(1–2), 65–78. https://doi.org/10.1016/S0022-1759(03)00265-5

Beyersdorf, N., Kerkau, T., & Hünig, T. (2015). CD28 co-stimulation in T-cell homeostasis: a recent perspective. *ImmunoTargets and Therapy*, 4–111. https://doi.org/10.2147/ITT.S61647

Blum, J. S., Wearsch, P. A., & Peter Cresswell. (2013). Pathways of antigen processing. In *Annu Rev Immunol.* (Vol. 31). https://doi.org/10.1016/0952-7915(91)90068-C

Boon, T., Coulie, P. G., & Van Den Eynde, B. (1997). Tumor antigens recognized by T cells.

Immunology Today, *18*(6), 267–268. https://doi.org/10.1016/S0167-5699(97)80020-5

Born, W. K., Huang, Y., Reinhardt, R. L., Huang, H., Sun, D., & O'Brien, R. L. (2017). γδ T Cells and B Cells. In *Advances in Immunology* (1st ed., Vol. 134). Elsevier Inc. https://doi.org/10.1016/bs.ai.2017.01.002

Bostwick, D. G., Ma, J., & Qian, J. (2006). Chapter 14 - Immunohistology of the Prostate, Bladder, Testis and Kidney. In D. J. B. T.-D. I. (Second E. Dabbs (Ed.), *Diagnostic Immunohistochemistry* ((Second Ed, pp. 509–610). Churchill Livingstone. https://doi.org/https://doi.org/10.1016/B978-0-443-06652-8.50020-X

Boublikova, L., Buchler, T., Stary, J., Abrahamova, J., & Trka, J. (2014). Molecular biology of testicular germ cell tumors: Unique features awaiting clinical application. *Critical Reviews in Oncology/Hematology*, *89*(3), 366–385. https://doi.org/10.1016/j.critrevonc.2013.10.001

Boudreau, J. E., & Hsu, K. C. (2018). Natural Killer cell education in human health and disease. *Curr Opin Immunol*, *50*, 102–111. https://doi.org/doi:10.1016/j.coi.2017.11.003.

Bozzano, F., Marras, F., & De Maria, A. (2017). Natural killer cell development and maturation revisited: Possible implications of a novel distinct Lin-CD34+DNAM-1brightCXCR4+ cell progenitor. *Frontiers in Immunology*, *8*(MAR), 6–13. https://doi.org/10.3389/fimmu.2017.00268

Buffa, S., Bulati, M., Pellicanó, M., Dunn-Walters, D. K., Wu, Y. C., Candore, G., Vitello, S., Caruso, C., & Colonna-Romano, G. (2011). B cell immunosenescence: Different features of naive and memory B cells in elderly. *Biogerontology*, *12*(5), 473–483. https://doi.org/10.1007/s10522-011-9353-4

Bunce, M., O'Neill, C. M., Barnardo, M. C. N. M., Krausa, P., Browning, M. J., Morris, P. J., & Welsh, K. I. (1995). Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens*, *46*(5), 355–367. https://doi.org/10.1111/j.1399-0039.1995.tb03127.x

Burgess, A. W., Camakaris, Ja., & Metcalf, D. (1976). Purification and Properties of Colony-stimulating from Mouse Lung-conditioned Medium *. *The Journal of Biological Chemistry*, *253*(6), 1998–2003.

Caballero, O. L., & Chen, Y. T. (2009). Cancer/testis (CT) antigens: Potential targets for immunotherapy. *Cancer Science*, *100*(11), 2014–2021. https://doi.org/10.1111/j.1349-7006.2009.01303.x

Caligiuri, M. A. (2008). Human natural killer cells. *Blood*, *112*(3), 461–469. https://doi.org/10.1182/blood-2007-09-077438

Cano, L. E. R., & Lopera, D. E. H. (2013). *AUTOIMMUNITY From Bench to Bedside: Chapter 5. Introduction to T and B Lymphocytes*.

Carter, L. L., Fouser, L. A., Jussif, J., Fitz, L., Deng, B., Wood, C. R., Collins, M., Honjo, T., Freeman, G. J., & Carreno, B. M. (2002). PD-1:PD-L inhibitory pathway affects both CD4+ and CD8+ T cells and is overcome by IL-2. *European Journal of Immunology*, *32*(3), 634–643. https://doi.org/10.1002/1521-4141(200203)32:3<634::AID-IMMU634>3.0.CO;2-9

Catakovic, K., Klieser, E., Neureiter, D., & Geisberger, R. (2017). T cell exhaustion: from pathophysiological basics to tumor immunotherapy. *Cell Communication and Signaling*, *15*(1), 1–16. https://doi.org/10.1186/s12964-016-0160-z

Chen, L., & Flies, D. B. (2013). Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature Reviews Immunology*, *13*(4), 227–242. https://doi.org/10.1038/nri3405

Cheng, C. Y., & Mruk, D. D. (2012). The blood-testis barrier and its implications for male contraception. *Pharmacological Reviews*, *64*(1), 16–64. https://doi.org/10.1124/pr.110.002790

Cheng, L., Lyu, B., & Roth, L. M. (2017). Perspectives on testicular germ cell neoplasms. *Human Pathology*, *59*, 10–25. https://doi.org/10.1016/j.humpath.2016.08.002

Chia, V. M., Li, Y., Quraishi, S. M., Graubard, B. I., Figueroa, J. D., Weber, J. P., Chanock, S. J., Rubertone, M. V., Erickson, R. L., & McGlynn, K. A. (2010). Effect modification of endocrine disruptors and testicular germ cell tumour risk by hormone-metabolizing genes. *International Journal of Andrology*, *33*(4), 588–596. https://doi.org/10.1111/j.1365-2605.2009.00975.x

Chiang, S. C. C., Theorell, J., Entesarian, M., Meeths, M., Mastafa, M., Al-Herz, W., Frisk, P., Gilmour, K. C., Ifversen, M., Langenskiöld, C., Machaczka, M., Naqvi, A., Payne, J., Perez-Martinez, A., Sabel, M., Unal, E., Unal, S., Winiarski, J., Nordenskjöld, M., … Bryceson, Y. T. (2013). Comparison of primary human cytotoxic T-cell and natural killer cell responses reveal similar molecular requirements for lytic granule exocytosis but differences in cytokine production. *Blood*, *121*(8), 1345–1356. https://doi.org/10.1182/blood-2012-07-442558

Comber, J. D., & Philip, R. (2014). MHC class I antigen presentation and implications for developing a new generation of therapeutic vaccines. *Therapeutic Advances in Vaccines*, *2*(3), 77–89. https://doi.org/10.1177/2051013614525375

Crawford, T. Q., Jalbert, E., Ndhlovu, L. C., & Barbour, J. D. (2014). Concomitant evaluation of PMA+ionomycin-induced kinase phosphorylation and cytokine production in T cell subsets by flow cytometry. *Cytometry Part A*, *85*(3), 268–276. https://doi.org/10.1002/cyto.a.22444

Cruz, F. M., Colbert, J. D., Merino, E., Kriegsman, B. A., & Rock, K. L. (2017). The biology and underlying mechanisms of cross-presentation of exogenous antigens on MHC I molecules. *Annu Rev Immunol.*, *35*, 149–176. https://doi.org/doi:10.1146/annurev-immunol-041015-055254. The

D'Errico, G., Machado, H. L., & Sainz, B. (2017). A current perspective on cancer immune therapy: step-by-step approach to constructing the magic bullet. *Clinical and Translational Medicine*, *6*(1). https://doi.org/10.1186/s40169-016-0130-5

D'Souza, M. J., Gala, R. P., Ubale, R. V., D'Souza, B., Vo, T. P., Parenky, A. C., Mulla, N. S., D'Sa, S., D'Souza, M., Braz-Gomes, K., D'Souza, N., & Chiriva-Internati, M. (2015). Trends in Nonparenteral Delivery of Biologics, Vaccines and Cancer Therapies. In M. Singh & M. Salnikova (Eds.), *Novel Approaches and Strategies for Biologics, Vaccines and Cancer Therapies* (1st ed., p. 89). Elsevier.

Dankner, M., Gray-Owen, S. D., Huang, Y. H., Blumberg, R. S., & Beauchemin, N. (2017). CEACAM1 as a multi-purpose target for cancer immunotherapy. *OncoImmunology*, *6*(7), 1–16. https://doi.org/10.1080/2162402X.2017.1328336

Davis, L. E., Shalin, S. C., & Tackett, A. J. (2019). Current state of melanoma diagnosis and treatment. *Cancer Biology and Therapy*, *20*(11), 1366–1379. https://doi.org/10.1080/15384047.2019.1640032

de Kretser, D. M., Loveland, K., & O'Bryan, M. (2015). Spermatogenesis. In *Endocrinology: Adult and Pediatric* (Seventh Ed, Vols. 2–2). Elsevier Inc. https://doi.org/10.1016/B978-0-323-18907-1.00136-0

de Martel, C., Georges, D., Bray, F., Ferlay, J., & Clifford, G. M. (2020). Global burden of cancer attributable to infections in 2018: a worldwide incidence analysis. *The Lancet Global Health*, *8*(2), e180–e190. https://doi.org/10.1016/S2214-109X(19)30488-7

Deligne, C., Metidji, A., Fridman, W. H., & Teillaud, J. L. (2015). Anti-CD20 therapy induces a memory Th1 response through the IFN-γ/IL-12 axis and prevents protumor regulatory T-cell expansion in mice. *Leukemia*, *29*(4), 947–957. https://doi.org/10.1038/leu.2014.275

Dennis, K. L., Blatner, N. R., Gounari, F., & Khazaie, K. (2013). Current status of interleukin-10 and regulatory T-cells in cancer. *Current Opinion in Oncology*, *25*(6), 637–645. https://doi.org/10.1097/CCO.0000000000000006

Desgrandchamps, F., LeMaoult, J., Goujon, A., Riviere, A., Rivero-Juarez, A., Djouadou, M., de Gouvello, A., Dumont, C., Wu, C. L., Culine, S., Verine, J., Rouas-Freiss, N., Hennequin, C., Masson-Lecomte, A., & Carosella, E. D. (2018). Prediction of non-muscle-invasive bladder cancer recurrence by measurement of checkpoint HLAG's receptor ILT2 on peripheral CD8+ T cells. *Oncotarget*, *9*(69), 33160–33169. https://doi.org/10.18632/oncotarget.26036

Dicken, B. J., & Billmire, D. F. (2012). Testicular Tumors. In *Pediatric Surgery* (7th ed.). Elsevier Inc. https://doi.org/10.1016/B978-0-323-07255-7.00040-4

Dickinson, A. M., Norden, J., Li, S., Hromadnikova, I., Schmid, C., Schmetzer, H., & Jochem-Kolb, H. (2017). Graft-versus-leukemia effect following hematopoietic stem cell transplantation for leukemia. *Frontiers in Immunology*, *8*(JUN). https://doi.org/10.3389/fimmu.2017.00496

Dong, Y., Sun, Q., & Zhang, X. (2017). PD-1 and its ligands are important immune checkpoints in cancer. *Oncotarget*, *8*(2), 2171–2186. https://doi.org/10.18632/oncotarget.13895

Drake, M. V. G., & Drake, C. G. (2011). LAG-3 in Cancer Immunotherapy. *Curr Top Microbiol Immunol*, *344*, 269–278. https://doi.org/doi: 10.1007/82_2010_114

Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., & Schreiber, R. D. (2002). Cancer immunoediting: From immunosurveillance to tumor escape. *Nature Immunology*, *3*(11), 991–998. https://doi.org/10.1038/ni1102-991

Embgenbroich, M. & Burgdorf, S. (2018). Current concepts of antigen cross-presentation. *Frontiers in Immunology*, 9. https://doi.org/10.3389/fimmu.2018.01643

Escors, D. (2011). On the Mechanism of T cell receptor downmodulation and its physiological significance. *The Journal of Bioscience and Medicine*, *1*(1), 1–6. https://doi.org/10.5780/jbm2011.5

Ewa Rajpert-De Meyts, Katherine A McGlynn, Keisei Okamoto, Michael A S Jewett, C. B., & Lancet. (2016). Testicular Germ Cell Tumours. *Lancet*, *387*, 1762–1774. https://doi.org/http://dx.doi.org/10.1016/ S0140-6736(15)00991-5

Fankhauser, C. D., Curioni-Fontecedro, A., Allmann, V., Beyer, J., Tischler, V., Sulser, T., Moch, H., & Bode, P. K. (2015). *Frequent PD-L1 expression in testicular germ cell tumors*. https://doi.org/10.1038/bjc.2015.244

Fehres, C. M., Unger, W. W. J., Garcia-Vallejo, J. J., & van Kooyk, Y. (2014). Understanding the biology of antigen cross-presentation for the design of vaccines against cancer. *Frontiers in Immunology*, *5*(APR), 1–10. https://doi.org/10.3389/fimmu.2014.00149

Fonslow, B. R., Stein, B. D., Webb, K. J., Xu, T., Choi, J., Kyu, S., & Iii, J. R. Y. (2013). Tumor-

infiltrating DCs suppress nucleic acid–mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. *Nature Immunology*, *10*(1), 54–56. https://doi.org/10.1038/nmeth.2250.Digestion

Forrester, J. V., & Xu, H. (2012). Good news-bad news: The Yin and Yang of immune privilege in the eye. *Frontiers in Immunology*, *3*(NOV), 1–18. https://doi.org/10.3389/fimmu.2012.00338

Fratta, E., Coral, S., Covre, A., Parisi, G., Colizzi, F., Danielli, R., Marie Nicolay, H. J., Sigalotti, L., & Maio, M. (2011). The biology of cancer testis antigens: Putative function, regulation and therapeutic potential. *Molecular Oncology*, *5*(2), 164–182. https://doi.org/10.1016/j.molonc.2011.02.001

Gabitass, R. F., Annels, N. E., Stocken, D. D., Pandha, H. A., & Middleton, G. W. (2011). Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. *Cancer Immunology, Immunotherapy*, *60*(10), 1419–1430. https://doi.org/10.1007/s00262-011-1028-0

Ganguly, D., Paul, K., Bagchi, J., Rakshit, S., Mandal, L., Bandyopadhyay, G., & Bandyopadhyay, S. (2007). Granulocyte-macrophage colony-stimulating factor drives monocytes to CD14low CD83+ DCSIGN- interleukin-10-producing myeloid cells with differential effects on T-cell subsets. *Immunology*, *121*(4), 499–507. https://doi.org/10.1111/j.1365-2567.2007.02596.x

Garner, L. C., Klenerman, P., & Provine, N. M. (2018). Insight Into Mucosal-Associated Invariant T Cell Biology From Studies of Invariant Natural Killer T Cells. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.01478

Garrido, F., Aptsiauri, N., Doorduijn, E. M., Garcia Lora, A. M., & van Hall, T. (2016). The urgent need to recover MHC class I in cancers for effective immunotherapy. *Current Opinion in Immunology*, *39*, 44–51. https://doi.org/10.1016/j.coi.2015.12.007

Ghazarian, A. A., Trabert, B., Devesa, S. S., & Mcglynn, K. A. (2015). Recent trends in the incidence of testicular germ cell tumors in the United States. *Andrology*, *3*(1), 13–18. https://doi.org/10.1111/andr.288

Gianchecchi, E., & Fierabracci, A. (2018). Inhibitory receptors and pathways of lymphocytes: The role of PD-1 in Treg development and their involvement in autoimmunity onset and cancer progression. *Frontiers in Immunology*, *9*(OCT), 1–12. https://doi.org/10.3389/fimmu.2018.02374

Gjerstorff, M. F., Andersen, M. H., & Ditzel, H. J. (2015). Oncogenic cancer/testis antigens: prime candidates for immunotherapy. *Oncotarget*. https://doi.org/10.18632/oncotarget.4694

Granier, C., De Guillebon, E., Blanc, C., Roussel, H., Badoual, C., Colin, E., Saldmann, A., Gey, A., Oudard, S., & Tartour, E. (2017). Mechanisms of action and rationale for the use of checkpoint inhibitors in cancer. *ESMO Open*, *2*(2), 1–8. https://doi.org/10.1136/esmoopen-2017-000213

Hadrup, S. R., Brændstrup, O., Jacobsen, G. K., Mortensen, S., Pedersen, L. Ø., Seremet, T., Andersen, M. H., Becker, J. C., & Straten, P. T. (2006). Tumor infiltrating lymphocytes in seminoma lesions comprise clonally expanded cytotoxic T cells. *International Journal of Cancer*, *119*(4), 831–838. https://doi.org/10.1002/ijc.21894

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Hanahan, D., Weinberg, R. A., & Francisco, S. (2000). *The Hallmarks of Cancer Review University of California at San Francisco*. *100*, 57–70.

Hannani, D., Ma, Y., Yamazaki, T., Déchanet-Merville, J., Kroemer, G., & Zitvogel, L. (2012). Harnessing γδ T cells in anticancer immunotherapy. *Trends in Immunology*, *33*(5), 199–206. https://doi.org/10.1016/j.it.2012.01.006

Hayes-Lattin, B. (2009). Testicular Cancer: A Prototypic Tumor of Young Adults. *Semin Oncol*, *36*(5), 432–438. https://doi.org/doi:10.1053/j.seminoncol.2009.07.006. Testicular

He, Y., Li, X., Cao, J., Zhao, C., Hirsch, F., & Zhou, C. (2018). TIM-3, a promising target for cancer immunotherapy. *OncoTargets and Therapy*, *Volume 11*, 7005–7009. https://doi.org/10.2147/ott.s170385

He, Y., Yu, H., Rozeboom, L., Rivard, C. J., Ellison, K., Dziadziuszko, R., Suda, K., Ren, S., Wu, C., Hou, L., Zhou, C., & Hirsch, F. R. (2017). LAG-3 Protein Expression in Non–Small Cell Lung Cancer and Its Relationship with PD-1/PD-L1 and Tumor-Infiltrating Lymphocytes. *Journal of Thoracic Oncology*, *12*(5), 814–823. https://doi.org/10.1016/j.jtho.2017.01.019

Hermes, N., Kewitz, S., & Staege, M. S. (2016). Preferentially Expressed Antigen in Melanoma (PRAME) and the PRAME Family of Leucine-Rich Repeat Proteins. *Current Cancer Drug Targets*, *16*(5), 400–414. https://doi.org/10.2174/1568009616666151222151818

Hewitt, E. W. (2003). The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology*, *110*, 163–169. https://doi.org/10.1002/eji.201444741

Hochstetter. (2002). The Differential Diagnosis of Testicular Germ Cell Tumors in Theory and Practice. *Pathological Anatomy*, *396*(January), 247–277.

Huang, L., Qiao, G., Wu, J., & Ren, J. (2019). Expression of lymphocyte-activation gene 3 (LAG-3) immune checkpoint receptor identifies a tumor-reactive T cell population in the peripheral blood of patients with colorectal cancer. *Medical Science Monitor*, *25*, 3495–3502. https://doi.org/10.12659/MSM.915741

Inman, B. A., Longo, T. A., Ramalingam, S., & Harrison, M. R. (2017). Atezolizumab: A PD-L1-blocking antibody for bladder cancer. *Clinical Cancer Research*, *23*(8), 1886–1890. https://doi.org/10.1158/1078-0432.CCR-16-1417

Isakov, N. (2018). Cancer immunotherapy by targeting immune checkpoint receptors. *World J Immunol*, *8*(1), 1–11. https://doi.org/doi: 10.5411/wji.v8.i1.1

Ivashkiv, L. B. (2018). IFNγ: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. *Nat Rev Immunol*, *18*(9), 545–558. https://doi.org/doi:10.1038/s41577-018-0029-z

J.Garner, M., C.Turner, M., Ghadirian, P., & Krewski, D. (2005). Epidemiology of testicular cancer: an overview. *Int. J. Cancer*, *116*, 331–339.

Jha, R. K., Mathur, S., & Saidha, N. K. (2018). A case of spermatocytic seminoma in young individual. *Medical Journal Armed Forces India*, *74*(3), 276–279. https://doi.org/10.1016/j.mjafi.2017.01.007

Jiang, S., Song, R., Popov, S., Mirshahidi, S., & Ruprecht, R. M. (2006). Overlapping synthetic peptides as vaccines. *Vaccine*, *24*(37–39), 6356–6365. https://doi.org/10.1016/j.vaccine.2006.04.070

Jiang, T., Shi, T., Zhang, H., Hu, J., Song, Y., Wei, J., Ren, S., & Zhou, C. (2019). Tumor neoantigens: From basic research to clinical applications. *Journal of Hematology and Oncology*, *12*(1), 1–13. https://doi.org/10.1186/s13045-019-0787-5

Jiang, T., Zhou, C., & Ren, S. (2016). Role of IL-2 in cancer immunotherapy. *OncoImmunology*, *5*(6), 1–10. https://doi.org/10.1080/2162402X.2016.1163462

Jiang, X. H., Bukhari, I., Zheng, W., Yin, S., Wang, Z., Cooke, H., & Shi, Q. H. (2014). Blood-testis barrier and spermatogenesis: Lessons from genetically-modified mice. *Asian Journal of Andrology*, *16*(4), 572–580. https://doi.org/10.4103/1008-682X.125401

Jin, W., & Dong, C. (2013). IL-17 cytokines in immunity and inflammation. *Emerging Microbes and Infections*, *2*, 0. https://doi.org/10.1038/emi.2013.58

Joffre, O. P., Segura, E., Savina, A., & Amigorena, S. (2012). Cross-presentation by dendritic cells. *Nature Reviews Immunology*, *12*(8), 557–569. https://doi.org/10.1038/nri3254

Johnston, R. J., Yu, X., & Grogan, J. L. (2015). The checkpoint inhibitor TIGIT limits antitumor and antiviral CD8+ T cell responses. *OncoImmunology*, *4*(9), 1–2. https://doi.org/10.1080/2162402X.2015.1036214

Jørgensen, N., Persson, G., & Hviid, T. V. F. (2019). The Tolerogenic Function of Regulatory T Cells in Pregnancy and Cancer. *Frontiers in Immunology*, *10*(May). https://doi.org/10.3389/fimmu.2019.00911

June, C. H. (2007). *Principles of adoptive T cell cancer therapy Find the latest version : Review series Principles of adoptive T cell cancer therapy*. *117*(5), 1204–1212. https://doi.org/10.1172/JCI31446.1204

Kammertoens, T., Friese, C., Arina, A., Idel, C., Briesemeister, D., Rothe, M., Ivanov, A., Szymborska, A., Patone, G., Kunz, S., Sommermeyer, D., Engels, B., Leisegang, M., Textor, A., Fehling, H. J., Fruttiger, M., Lohoff, M., Herrmann, A., Yu, H., … Blakenstein, T. (2017). Tumour ischaemia by interferon-γ resembles physiological blood vessel regression. *Nature*, *545*(7652), 98–102. https://doi.org/doi:10.1038/nature22311.

Kareva, I. (2018). Cancer as a Systemic Disease That Requires a Systemic Approach. *Understanding Cancer from a Systems Biology Point of View*, 79–90. https://doi.org/10.1016/b978-0-12-813673-7.00006-9

Kasakovski, D., Xu, L., & Li, Y. (2018). T cell senescence and CAR-T cell exhaustion in hematological malignancies. *Journal of Hematology and Oncology*, *11*(1), 1–9. https://doi.org/10.1186/s13045-018-0629-x

Kato, R., Yamasaki, M., Urakawa, S., Nishida, K., Makino, T., Morimoto-Okazawa, A., Kawashima, A., Iwahori, K., Suzuki, S., Ueda, R., Mori, M., Satoh, T., Doki, Y., & Wada, H. (2018). Increased Tim-3+ T cells in PBMCs during nivolumab therapy correlate with responses and prognosis of advanced esophageal squamous cell carcinoma patients. *Cancer Immunology, Immunotherapy*, *67*(11), 1673–1683. https://doi.org/10.1007/s00262-018-2225-x

Kaur, G., Thompson, L. A., & Dufour, J. M. (2014). Sertoli cells- Immunological sentinels of spermatogenesis. *Semin Cell Dev Biol.*, 36–44. https://doi.org/doi:10.1016/j.semcdb.2014.02.011.

Khan, O., & Protheroe, A. (2007). Testis cancer. *Postgraduate Medical Journal*, *83*(984), 624–632. https://doi.org/10.1136/pgmj.2007.057992

Krijgsman, D., de Vries, N. L., Skovbo, A., Andersen, M. N., Swets, M., Bastiaannet, E., Vahrmeijer, A. L., van de Velde, C. J. H., Heemskerk, M. H. M., Hokland, M., & Kuppen, P. J. K. (2019). Characterization of circulating T-, NK-, and NKT cell subsets in patients with colorectal cancer: the peripheral blood immune cell profile. *Cancer Immunology, Immunotherapy*, *68*(6), 1011–1024. https://doi.org/10.1007/s00262-019-02343-7

Krishnadas, D. K., Bai, & Lucas. (2013). Cancer testis antigen and immunotherapy. *ImmunoTargets and Therapy*. https://doi.org/10.2147/ITT.S35570

Krzewski, K., Gil-Krzewska, A., Nguyen, V., Peruzzi, G., & Coligan, J. E. (2013). LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood*, *121*(23), 4672–4683. https://doi.org/10.1182/blood-2012-08-453738

Kwiecien, I., Skirecki, T., Polubiec-Kownacka, M., Raniszewska, A., & Domagala-Kulawik, J. (2019). Immunophenotype of t cells expressing programmed death-1 and cytotoxic t cell antigen-4 in early lung cancer: Local vs. systemic immune response. *Cancers*, *11*(4), 1–18. https://doi.org/10.3390/cancers11040567

Lanzkowsky, P. (2011). Germ cell tumors. *Manual of Pediatric Hematology and Oncology (Fifth Edition): Chapter 27: Germ Cell Tumors*, 776–795. https://doi.org/10.1007/978-1-84628-738-1_23

Lee, A. J., & Ashkar, A. A. (2018). The dual nature of type I and type II interferons. *Frontiers in Immunology*, *9*(SEP), 1–10. https://doi.org/10.3389/fimmu.2018.02061

Lee, H. R., Son, C. H., Koh, E. K., Bae, J. H., Kang, C. D., Yang, K., & Park, Y. S. (2017). Expansion of cytotoxic natural killer cells using irradiated autologous peripheral blood mononuclear cells and anti-CD16 antibody. *Scientific Reports*, *7*(1), 1–13. https://doi.org/10.1038/s41598-017-09259-1

Legut, M., Cole, D. K., & Sewell, A. K. (2015). The promise of γδT cells and the γδT cell receptor for cancer immunotherapy. *Cellular and Molecular Immunology*, *12*(6), 656–658. https://doi.org/10.1038/cmi.2015.28

Leonard, W. J., Lin, J. X., & O'Shea, J. J. (2019). The γ c Family of Cytokines: Basic Biology to Therapeutic Ramifications. *Immunity*, *50*(4), 832–850. https://doi.org/10.1016/j.immuni.2019.03.028

Li, M., Xia, P., Du, Y., Liu, S., Huang, G., Chen, J., Zhang, H., Hou, N., Cheng, X., Zhou, L., Li, P., Yang, X., & Fan, Z. (2014). T-cell immunoglobulin and ITIM domain (TIGIT) receptor/poliovirus receptor (PVR) ligand engagement suppresses interferon-γ production of natural killer cells via β-arrestin 2-mediated negative signaling. *Journal of Biological Chemistry*, *289*(25), 17647–17657. https://doi.org/10.1074/jbc.M114.572420

Li, W., Blessin, N. C., Simon, R., Kluth, M., Fischer, K., Hube-Magg, C., Makrypidi-Fraune, G., Wellge, B., Mandelkow, T., Debatin, N. F., Pott, L., Höflmayer, D., Lennartz, M., Sauter, G., Izbicki, J. R., Minner, S., Büscheck, F., Uhlig, R., Dum, D., … Hinsch, A. (2018). Expression of the immune checkpoint receptor TIGIT in Hodgkin's lymphoma. *BMC Cancer*, *18*(1), 1–8. https://doi.org/10.1186/s12885-018-5111-1

Li, X., Wang, R., Fan, P., Yao, X., Qin, L., Peng, Y., Ma, M., Asley, N., Chang, X., Feng, Y., Hu, Y., Zhang, Y., Li, C., Fanning, G., Jones, S., Verrill, C., Maldonado-Perez, D., Sopp, P., Waugh, C., … Dong, T. (2019). A comprehensive analysis of key immune checkpoint receptors on tumor-infiltrating t cells from multiple types of cancer. *Frontiers in Oncology*, *9*(OCT). https://doi.org/10.3389/fonc.2019.01066

Ling, L., Lin, Y., Zheng, W., Hong, S., Tang, X., Zhao, P., Li, M., Ni, J., Li, C., Wang, L., & Jiang, Y. (2016). Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. *Scientific Reports*, *6*(February), 1–10. https://doi.org/10.1038/srep20358

Locy, H., de Mey, S., de Mey, W., De Ridder, M., Thielemans, K., & Maenhout, S. K. (2018). Immunomodulation of the Tumor Microenvironment: Turn Foe Into Friend. *Frontiers in Immunology*, *9*(December), 2909. https://doi.org/10.3389/fimmu.2018.02909

Lorenzo-Herrero, S., López-Soto, A., Sordo-Bahamonde, C., Gonzalez-Rodriguez, A. P., Vitale, M., & Gonzalez, S. (2019). NK cell-based immunotherapy in cancer metastasis. *Cancers*, *11*(1), 1–22. https://doi.org/10.3390/cancers11010029

Maçon-Lemaître, L., & Triebel, F. (2005). The negative regulatory function of the lymphocyte-activation gene-3 co-receptor (CD223) on human T cells. *Immunology*, *115*(2), 170–178. https://doi.org/10.1111/j.1365-2567.2005.02145.x

Maecker, H. T., Dunn, H. S., Suni, M. A., Khatamzas, E., Pitcher, C. J., Bunde, T., Persaud, N., Trigona, W., Fu, T.-M., Sinclair, E., Bredt, B. M., McCune, J. M., Maino, V. C., Kern, F., & Picker, L. J. (2001). Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *Journal of Immunological Methods*, *255*(1–2), 27–40. https://doi.org/10.1016/S0022-1759(01)00416-1

Mah, A. Y., & Cooper, M. A. (2016). Metabolic Regulation of Natural Killer Cell IFN-γ Production. *Critical Reviews in Immunology*, *36*(2), 131–147. https://doi.org/10.1615/critrevimmunol.2016017387

Malek, T. R., & Castro, I. (2010). Interleukin-2 Receptor Signaling: At the Interface between Tolerance and Immunity. *Immunity*, *33*(2), 153–165. https://doi.org/10.1016/j.immuni.2010.08.004

Manieri, N. A., Chiang, E. Y., & Grogan, J. L. (2017). TIGIT: A Key Inhibitor of the Cancer Immunity Cycle. *Trends in Immunology*, *38*(1), 20–28. https://doi.org/10.1016/j.it.2016.10.002

Mantegazza, A. R., Magalhaes, J. G., Amigorena, S., & Marks, M. S. (2013). Presentation of Phagocytosed Antigens by MHC Class I and II. *Traffic*, *14*(2), 135–152. https://doi.org/10.1111/tra.12026

Marin-Acevedo, J. A., Dholaria, B., Soyano, A. E., Knutson, K. L., Chumsri, S., & Lou, Y. (2018). Next generation of immune checkpoint therapy in cancer: New developments and challenges. *Journal of Hematology and Oncology*, *11*(1), 1–20. https://doi.org/10.1186/s13045-018-0582-8

Marshall, H. T., & Djamgoz, M. B. A. (2018). Immuno-oncology: Emerging targets and combination therapies. *Frontiers in Oncology*, *8*(AUG), 1–29. https://doi.org/10.3389/fonc.2018.00315

Martin, V. G., Wu, Y. C. B., Townsend, C. L., Lu, G. H. C., O'Hare, J. S., Mozeika, A., Coolen, A. C. C., Kipling, D., Fraternali, F., & Dunn-Walters, D. K. (2016). Transitional B cells in early human B cell development - Time to revisit the paradigm? *Frontiers in Immunology*, *7*(DEC), 1–13. https://doi.org/10.3389/fimmu.2016.00546

Martinez, G. J., Nurieva, R. I., Yang, X. O., & Dong, C. (2008). Regulation and function of proinflammatory TH17 cells. *Ann N Y Acad Sci*, *1143*, 188–211. https://doi.org/doi:10.1196/annals.1443.021

McDonnell, A. M., Robinson, B. W. S., & Currie, A. J. (2010). Tumor antigen cross-presentation and the dendritic cell: Where it all begins? *Clinical and Developmental Immunology*, *2010*.

https://doi.org/10.1155/2010/539519

Meinhardt, A., & Hedger, M. P. (2011). Immunological, paracrine and endocrine aspects of testicular immune privilege. *Molecular and Cellular Endocrinology*, *335*(1), 60–68. https://doi.org/10.1016/j.mce.2010.03.022

Meyers, J. H., Sabatos, C. A., Chakravarti, S., & Kuchroo, V. K. (2005). The TIM gene family regulates autoimmune and allergic diseases. *Trends in Molecular Medicine*, *11*(8), 362–369. https://doi.org/10.1016/j.molmed.2005.06.008

Minato, N., & Honjo, T. (2016). *Chapter 29 - Cancer Immunotherapy by Checkpoint Blockade* (B. R. Bloom & P.-H. B. T.-T. V. B. (Second E. Lambert (eds.); pp. 561–580). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-802174-3.00029-1

Mital, P., Hinton, B. T., & Dufour, J. M. (2011). The Blood-Testis and Blood-Epididymis Barriers Are More than Just Their Tight Junctions1. *Biology of Reproduction*, *84*(5), 851–858. https://doi.org/10.1095/biolreprod.110.087452

Monney, L., Sabatos, C. A., Gaglia, J. L., Ryu, A., Waldner, H., Chernova, T., Manning, S., Greenfield, E. A., Coyle, A. J., Sobel, R. A., Freeman, G. J., & Kuchroo, V. K. (2002). Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature*, *415*(6871), 536–541. https://doi.org/10.1038/415536a

Mosmann TR[1], Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989;7:145-73.

Mostofi, F. K. (1980). Pathology of Germ Cell Tumors of Testis: A Progress Report. *Cancer*, *45 Suppl 7*, 1735–1754. https://doi.org/10.1002/cncr.1980.45.s7.1735

Munk, R. B., Sugiyama, K., Ghosh, P., Sasaki, C. Y., Rezanka, L., Banerjee, K., Takahashi, H., Sen, R., & Longo, D. L. (2011). Antigen-independent IFN-γ production by human naïve CD4+ T cells activated by IL-12 plus IL-18. *PLoS ONE*, *6*(5), 1–8. https://doi.org/10.1371/journal.pone.0018553

Nakanishi, K. (2018). Unique action of Interleukin-18 on T cells and other immune cells. *Frontiers in Immunology*, *9*(APR). https://doi.org/10.3389/fimmu.2018.00763

Nassef Kadry Naguib Roufaiel, M., Wells, J. W., & Steptoe, R. J. (2015). Impaired T-cell function in B-cell lymphoma: A direct consequence of events at the immunological synapse? *Frontiers in Immunology*, *6*(JUN), 1–10. https://doi.org/10.3389/fimmu.2015.00258

Nemazee, D. (2000). Receptor Selection in B and T Lymphocytes. *Annual Review of Immunology*, *18*(1), 19–51. https://doi.org/10.1146/annurev.immunol.18.1.19

Ni, L., & Lu, J. (2018). Interferon gamma in cancer immunotherapy. *Cancer Medicine*, *7*(9), 4509–4516. https://doi.org/10.1002/cam4.1700

Nowicki, T. S., Hu-Lieskovan, S., & Ribas, A. (2017). Mechanisms of Resistance to PD-1 and PD-L1 blockade. *Cancer J.*, *24*(1), 47–53. https://doi.org/10.1097/PPO.0000000000000303

Nussbaumer, O., & Koslowski, M. (2019). The emerging role of γδ T cells in cancer immunotherapy. *Immuno-Oncology Technology*, *1*(June), 3–10. https://doi.org/10.1016/j.iotech.2019.06.002

O.Butler, M., & Hirano, N. (2014). Human cell-based artificial antigen-presenting cells for cancer immunotherapy. *Immunological Reviews*, *257*, 191–209.

Oldenburg, J., Fosså, S. D., Nuver, J., Heidenreich, A., Schmoll, H. J., Bokemeyer, C., Horwich, A., Beyer, J., & Kataja, V. (2013). Testicular seminoma and non-seminoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, *24*(SUPPL.6). https://doi.org/10.1093/annonc/mdt304

Orr, M. T., & Lanier, L. L. (2010). Natural Killer Cell Education and Tolerance. *Cell*, *142*(6), 847–856. https://doi.org/10.1016/j.cell.2010.08.031

Overwijk, W. W., & Restifo, N. P. (2000). Autoimmunity and the immunotherapy of cancer: Targeting the "self" to destroy the "other." *Critical Reviews in Immunology*, *20*(6), 433–450. https://doi.org/10.1615/critrevimmunol.v20.i6.10

Pardoll, D. M. (2015). Cancer and the Immune System: Basic Concepts and Targets for Intervention. *Semin Oncol*, *42*(4), 523–538. https://doi.org/doi:10.1053/j.seminoncol.2015.05.003

Pardoll, D. M. (2016). The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*, *12*(4), 252–264. https://doi.org/doi:10.1038/nrc3239

Parmiani, G., Castelli, C., Dalerba, P., Mortarini, R., Rivoltini, L., Marincola, F. M., & Anichini, A. (2002). Cancer immunotherapy with peptide-based vaccines: What have we achieved? Where are we going? *Journal of the National Cancer Institute*, *94*(11), 805–818. https://doi.org/10.1093/jnci/94.11.805

Paust, S., Senman, B., & von Andrian, U. H. (2010). Adaptive Immune Responses MEdiated by Natural Killer Cells. *Immunol*, *235*(1), 286–296. https://doi.org/doi:10.1111/j.0105-2896.2010.00906.x.

Pearce, H., Hutton, P., Chaudhri, S., Porfiri, E., Patel, P., Viney, R., & Moss, P. (2017). Spontaneous CD4+ and CD8+ T-cell responses directed against cancer testis antigens are present in the peripheral blood of testicular cancer patients. *European Journal of Immunology*, *47*(7), 1232–1242. https://doi.org/10.1002/eji.201646898

Pecorino, L. (2012). *The Molecular Biology of Cancer: Mechanisms, Targets, and Therapeutics*.

Pereda, J., Zorn, T., & Soto-Suazo, M. (2006). Primordial germ cells migration: morphological and molecular aspects. *Animal Reproduction*, *2*(3), 147–160. http://www.ncbi.nlm.nih.gov/pubmed/16718662

Pins, M. R. (2010). Male genitourinary system. In *Differential Diagnosis in Surgical Pathology* (Second Edi). Elsevier Inc. https://doi.org/10.1016/B978-1-4160-4580-9.00011-3

Poli, A., Michel, T., Thérésine, M., Andrès, E., Hentges, F., & Zimmer, J. (2009). CD56bright natural killer (NK) cells: An important NK cell subset. *Immunology*, *126*(4), 458–465. https://doi.org/10.1111/j.1365-2567.2008.03027.x

R. C. B. Pugh & Chnstance Parkinson. (1981). The origin and classification of testicular germ cell tumours. *International Journal of Andrology, Supplement*.

R.Stratton, M., Campbell, P. J., & Futreal, P. A. (2009). The cancer genome. *Nature*, *458*(7239), 719–724. https://doi.org/10.1038/nature07943.The

Rabinovich, G. A., Gabrilovich, D., & Sotomayor, E. M. (2007). Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol.*, *25*, 267–296. https://doi.org/doi:10.1146/annurev.immunol.25.022106.141609

Rådestad, E., Klynning, C., Stikvoort, A., Mogensen, O., Nava, S., Magalhaes, I., & Uhlin, M. (2019).

Immune profiling and identification of prognostic immune-related risk factors in human ovarian cancer. *OncoImmunology*, *8*(2), 1–17. https://doi.org/10.1080/2162402X.2018.1535730

Raiss, G. G., Andaloussi, M., Raissouni, S. S., Mrabti, H. H., & Errihani, H. H. (2011). Spermatocytic seminoma at the National Institute of Oncology in Morocco. *BMC Research Notes*, *4*(1), 218. https://doi.org/10.1186/1756-0500-4-218

Raverdeau, M., Cunningham, S. P., Harmon, C., & Lynch, L. (2019). γδ T cells in cancer: a small population of lymphocytes with big implications. *Clinical and Translational Immunology*, *8*(10), 1–15. https://doi.org/10.1002/cti2.1080

Rescorla, F. J. (2012). Teratomas and Other Germ Cell Tumors. In *Pediatric Surgery* (7th ed.). Elsevier Inc. https://doi.org/10.1016/b978-0-323-07255-7.00037-4

Reuter, V. E. (2005). Origins and molecular biology of testicular germ cell tumors. *Modern Pathology*, *18*, 51–60. https://doi.org/10.1038/modpathol.3800309

Ribatti, D. (2015). The concept of immune surveillance against tumors. The first theories. *Oncotarget*, *8*(4), 7175–7180. https://doi.org/10.18632/oncotarget.12739

Robson, N. C., Hoves, S., Maraskovsky, E., & Schnurr, M. (2010). Presentation of tumour antigens by dendritic cells and challenges faced. *Current Opinion in Immunology*, *22*(1), 137–144. https://doi.org/10.1016/j.coi.2010.01.002

Roifman, C. M., & Grunebaum, E. (2013). Primary T-cell immunodeficiencies. In *Clinical Immunology: Principles and Practice: Fourth Edition* (4th ed., Vol. 70). Elsevier Ltd. https://doi.org/10.1016/B978-0-7234-3691-1.00052-0

Ross, S. H., & Cantrell, D. A. (2018). Signaling and Function of Interleukin-2 in T Lymphocytes. *Annual Review of Immunology*, *36*(1), 411–433. https://doi.org/10.1146/annurev-immunol-042617-053352

Rothchild, A. C., Stowell, B., Goyal, G., Yang, Q., Papavinasasundaram, K., Sassetti, C. M., Dranoff, G., Chen, X., Lee, J., & Behar, S. M. (2017). Role of Granulocyte-Macrophage Colony-Stimulating Factor Production by T Cells during Mycobacterium tuberculosis Infection. *American Society for Microbiology*, *8*(5). https://doi.org/https://doi.org/10.1128/mBio .01514-17. Editor

Sakuishi, K., Ngiow, S. F., Sullivan, J. M., Teng, M. W. L., Kuchroo, V. K., Smyth, M. J., & Anderson, A. C. (2013). TIM3+FOXP3+ regulatory T cells are tissue-specific promoters of T-cell dysfunction in cancer. *OncoImmunology*, *2*(4), 1–9. https://doi.org/10.4161/onci.23849

Savoia, P., Astrua, C., & Fava, P. (2016). Ipilimumab (Anti-Ctla-4 Mab) in the treatment of metastatic melanoma: Effectiveness and toxicity management. *Human Vaccines and Immunotherapeutics*, *12*(5), 1092–1101. https://doi.org/10.1080/21645515.2015.1129478

Scanlan, M. J., Gure, A. O., Jungbluth, A. A., Old, L. J., & Chen, Y.-T. (2002). Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunological Reviews*, *188*(3), 22–32. http://www.ncbi.nlm.nih.gov/pubmed/12445278

Schoenborn, J. R., & Wilson, C. B. (2007). Regulation of Interferon-γ During Innate and Adaptive Immune Responses. *Advances in Immunology*, *96*(07), 41–101. https://doi.org/10.1016/S0065-2776(07)96002-2

Schutt, C. A., Mirandola, L., Figueroa, J. A., Nguyen, D. D., Cordero, J., Bumm, K., Judson, B. L., & Chiriva-Internati, M. (2017). The cancer-testis antigen, sperm protein 17, a new biomarker and

immunological target in head and neck squamous cell carcinoma. *Oncotarget*, *8*(59), 100280–100287. https://doi.org/10.18632/oncotarget.22213

Seidel, J. A., Otsuka, A., & Kabashima, K. (2018). Anti-PD-1 and anti-CTLA-4 therapies in cancer: Mechanisms of action, efficacy, and limitations. *Frontiers in Oncology*, *8*(MAR), 1–14. https://doi.org/10.3389/fonc.2018.00086

Sheng, W., Yang, F., Zhou, Y., Yang, H., Low, P. Y., Kemeny, D. M., Tan, P., Moh, A., Kaplan, M. H., Zhang, Y., & Fu, X. Y. (2014). STAT5 programs a distinct subset of GM-CSF-producing T helper cells that is essential for autoimmune neuroinflammation. *Cell Research*, *24*(12), 1387–1402. https://doi.org/10.1038/cr.2014.154

Shi, Y., Liu, C. H., Roberts, A. I., Das, J., Xu, G., Ren, G., Zhang, Y., Zhang, L., Zeng, R. Y., Tan, H. S. W., Das, G., & Devadas, S. (2006). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: What we do and don't know. *Cell Research*, *16*(2), 126–133. https://doi.org/10.1038/sj.cr.7310017

Smith, S. G., Smits, K., Joosten, S. A., Van Meijgaarden, K. E., Satti, I., Fletcher, H. A., Caccamo, N., Dieli, F., Mascart, F., McShane, H., Dockrell, H. M., Ottenhoff, T. H. M., Haks, M., Stenger, S., Kaufmann, S., Maertzdorf, J., Gicquel, B., Tailleux, L., & Sallusto, F. (2015). Intracellular cytokine staining and flow cytometry: Considerations for application in clinical trials of novel tuberculosis vaccines. *PLoS ONE*, *10*(9). https://doi.org/10.1371/journal.pone.0138042

Song, M. H., Choi, K. U., Shin, D. H., Lee, C. H., & Lee, S. Y. (2012). Identification of the cancer/testis antigens AKAP3 and CTp11 by SEREX in hepatocellular carcinoma. *Oncology Reports*, *28*(5), 1792–1798. https://doi.org/10.3892/or.2012.2002

Song, Y., Wang, B., Song, R., Hao, Y., Wang, D., Li, Y., Jiang, Y., Xu, L., Ma, Y., Zheng, H., Kong, Y., & Zeng, H. (2018). T-cell Immunoglobulin and ITIM Domain Contributes to CD8 + T-cell Immunosenescence. *Aging Cell*, *17*(2), 1–12. https://doi.org/10.1111/acel.12716

Srpan, K., Ambrose, A., Karampatzakis, A., Saeed, M., Cartwright, A. N. R., Guldevall, K., De Matos, G. D. S. C., Önfelt, B., & Davis, D. M. (2018). Shedding of CD16 disassembles the NK cell immune synapse and boosts serial engagement of target cells. *Journal of Cell Biology*, *217*(9), 3267–3283. https://doi.org/10.1083/jcb.201712085

Stetson, D. B., Mohrs, M., Reinhardt, R. L., Baron, J. L., Wang, Z.-E., Gapin, L., Kronenberg, M., & Locksley, R. M. (2003). Constitutive Cytokine mRNAs Mark Natural Killer (NK) and NK T Cells Poised for Rapid Effector Function. *The Journal of Experimental Medicine*, *198*(7), 1069–1076. https://doi.org/10.1084/jem.20030630

Sundström, P., Szeponik, L., Ahlmanner, F., Sundquist, M., Wong, J. S. B., Lindskog, E. B., Gustafsson, B., & Quiding-Järbrink, M. (2019). Tumor-infiltrating mucosal-associated invariant T (MAIT) cells retain expression of cytotoxic effector molecules. *Oncotarget*, *10*(29), 2810–2823. https://doi.org/10.18632/oncotarget.26866

Swann, J. B., & Smyth, M. J. (2007). Review series Immune surveillance of tumors. *The Journal of Clinical Investigation*, *117*(5), 1137–1146. https://doi.org/10.1172/JCI31405.antigens

Tachibana, T., Onodera, H., Tsuruyama, T., Mori, A., Nagayama, S., Hiai, H., & Imamura, M. (2005). Increased intratumor Vα24-positive natural killer T cells: A prognostic factor for primary colorectal carcinomas. *Clinical Cancer Research*, *11*(20), 7322–7327. https://doi.org/10.1158/1078-0432.CCR-05-0877

Teng, M. W. L., Kershaw, M. H., & Smyth, M. J. (2013). Cancer Immunoediting: From Surveillance to

Escape. In *Cancer Immunotherapy: Immune Suppression and Tumor Growth: Second Edition* (Second Edi). Elsevier. https://doi.org/10.1016/B978-0-12-394296-8.00007-5

Terabe, M., & Berzofsky, J. A. (2018). Tissue-specific roles of NKT cells in tumor immunity. *Frontiers in Immunology*, *9*(AUG), 1–11. https://doi.org/10.3389/fimmu.2018.01838

Thommen, D. S., & Schumacher, T. N. (2018). T Cell Dysfunction in Cancer. *Cancer Cell*, *33*(4), 547–562. https://doi.org/10.1016/j.ccell.2018.03.012

Trahtemberg, U., & Mevorach, D. (2017). Apoptotic cells induced signaling for immune homeostasis in macrophages and dendritic cells. *Frontiers in Immunology*, *8*(OCT). https://doi.org/10.3389/fimmu.2017.01356

Trapani, J. A., & Smyth, M. J. (2002). Functional significance of the perforin/granzyme cell death pathway. *Nature Reviews Immunology*, *2*(10), 735–747. https://doi.org/10.1038/nri911

Tsukerman, P., Stern-Ginossar, N., Yamin, R., Ophir, Y., Stanietsky, A. M. N., & Mandelboim, O. (2014). Expansion of CD16 positive and negative human NK cells in response to tumor stimulation. *European Journal of Immunology*, *44*(5), 1517–1525. https://doi.org/10.1002/eji.201344170

Ulbright, T. M. (2005). Germ cell tumors of the gonads: A selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. *Modern Pathology*, *18*(SUPPL. 2), 61–79. https://doi.org/10.1038/modpathol.3800310

Vambutas, A., DeVoti, J., Nouri, M., Drijfhout, J. W., Lipford, G. B., Bonagura, V. R., Van Der Burg, S. H., & Melief, C. J. M. (2005). Therapeutic vaccination with papillomavirus E6 and E7 long peptides results in the control of both established virus-induced lesions and latently infected sites in a pre-clinical cottontail rabbit papillomavirus model. *Vaccine*, *23*(45), 5271–5280. https://doi.org/10.1016/j.vaccine.2005.04.049

van der Geest, K. S. M., Kroesen, B. J., Horst, G., Abdulahad, W. H., Brouwer, E., & Boots, A. M. H. (2018). Impact of aging on the frequency, phenotype, and function of CD161-expressing T cells. *Frontiers in Immunology*, *9*(APR). https://doi.org/10.3389/fimmu.2018.00752

van Horssen, R. (2006). TNF- in Cancer Treatment: Molecular Insights, Antitumor Effects, and Clinical Utility. *The Oncologist*, *11*(4), 397–408. https://doi.org/10.1634/theoncologist.11-4-397

Vigneron, N. (2015). Human Tumor Antigens and Cancer Immunotherapy. In *BioMed Research International* (Vol. 2015). https://doi.org/10.1155/2015/948501

Voskoboinik, I., Thia, M. C., Fletcher, J., Ciccone, A., Browne, K., Smyth, M. J., & Trapani, J. A. (2005). Calcium-dependent plasma membrane binding and cell lygis by perforin are mediated through its C2 domain: A critical role for aspartate residues 429, 435, 483, and 485 but not 491. *Journal of Biological Chemistry*, *280*(9), 8426–8434. https://doi.org/10.1074/jbc.M413303200

Vujanovic, L., Chuckran, C., Lin, Y., Ding, F., Sander, C. A., Santos, P. M., Lohr, J., Mashadi-Hossein, A., Warren, S., White, A., Huang, A., Kirkwood, J. M., & Butterfield, L. H. (2019). CD56 dim CD16 − natural killer cell profiling in melanoma patients receiving a cancer vaccine and interferon-α. *Frontiers in Immunology*, *10*(JAN), 1–14. https://doi.org/10.3389/fimmu.2019.00014

W.Mak, T., & Saunders, M. E. (2006). Tumor Immunology. In *The Immune Response Basic and Clinical Principles* (pp. 825–871). Academic Press. https://doi.org/https://doi.org/10.1016/B978-012088451-3.50028-4

Walker, L. J., Marrinan, E., Muenchhoff, M., Ferguson, J., Kloverpris, H., Cheroutre, H., Barnes, E., Goulder, P., & Klenerman, P. (2013). CD8αα expression marks terminally differentiated human CD8+ T cells expanded in chronic viral infection. *Frontiers in Immunology*, *4*(AUG), 1–14. https://doi.org/10.3389/fimmu.2013.00223

Walker, L. J., Tharmalingam, H., & Klenerman, P. (2014). The Rise and Fall of MAIT Cells with Age. *Scandinavian Journal of Immunology*, *80*(6), 462–463. https://doi.org/10.1111/sji.12237

Weber, J. K., & Zhou, R. (2017). Phosphatidylserine-induced conformational modulation of immune cell exhaustion-associated receptor TIM3. *Scientific Reports*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-14064-x

Whitehurst, A. W. (2014). Cause and Consequence of Cancer/Testis Antigen Activation in Cancer. *Annual Review of Pharmacology and Toxicology*, *54*(1), 251–272. https://doi.org/10.1146/annurev-pharmtox-011112-140326

Wu, D., Wu, P., Qiu, F., Wei, Q., & Huang, J. (2017). Human γδT-cell subsets and their involvement in tumor immunity. *Cellular and Molecular Immunology*, *14*(3), 245–253. https://doi.org/10.1038/cmi.2016.55

Wu, R., Forget, M.-A., Chacon, J., Bernatchez, C., Haymaker, C., Chen, J. Q., Hwu, P., & Radvanyi, L. (2012). Adoptive T-cell Therapy Using Autologous Tumor-infiltrating Lymphocytes for Metastatic Melanoma: Current Status and Future Outlook. *Cancer J.*, *18*(2), 160–175. https://doi.org/10.1097/PPO.0b013e31824d4465.Adoptive

Xiao, X., & Cai, J. (2017). Mucosal-associated invariant T cells: New insights into antigen recognition and activation. *Frontiers in Immunology*, *8*(NOV), 12–15. https://doi.org/10.3389/fimmu.2017.01540

Xie, C., Subhash, V. V., Datta, A., Liem, N., Tan, S. H., Yeo, M. S., Tan, W. L., Koh, V., Yan, F. L., Wong, F. Y., Wong, W. K., So, J., Tan, I. B., Padmanabhan, N., Yap, C. T., Tan, P., Goh, L. K., & Yong, W. P. (2016). Melanoma associated antigen (MAGE)-A3 promotes cell proliferation and chemotherapeutic drug resistance in gastric cancer. *Cellular Oncology*, *39*(2), 175–186. https://doi.org/10.1007/s13402-015-0261-5

Yan, J., Zhang, Y., Zhang, J. P., Liang, J., Li, L., & Zheng, L. (2013). Tim-3 Expression Defines Regulatory T Cells in Human Tumors. *PLoS ONE*, *8*(3). https://doi.org/10.1371/journal.pone.0058006

Yang, J., Murphy, T. L., Ouyang, W., & Murphy, K. M. (1999). Induction of interferon-γ production in Th1 CD4+ T cells: Evidence for two distinct pathways for promoter activation. *European Journal of Immunology*, *29*(2), 548–555. https://doi.org/10.1002/(SICI)1521-4141(199902)29:02<548::AID-IMMU548>3.0.CO;2-Z

Yang, S., Wang, J., Brand, D. D., & Zheng, S. G. (2018). Role of TNF-TNF receptor 2 signal in regulatory T cells and its therapeutic implications. *Frontiers in Immunology*, *9*(APR). https://doi.org/10.3389/fimmu.2018.00784

Yang, Y. (2015). Cancer immunotherapy : harnessing the immune system to battle cancer Find the latest version : Cancer immunotherapy : harnessing the immune system to battle cancer. *Journal of Clinical Investigation*, *125*(9), 3335–3337. https://doi.org/10.1172/JCI83871.lt

Yao, J., Caballero, O. L., Yung, W. K. A., Weinstein, J. N., Riggins, G. J., Strausberg, R. L., & Zhao, Q. (2014). Tumor subtype-specific cancer-testis antigens as potential biomarkers and immunotherapeutic targets for cancers. *Cancer Immunology Research*, *2*(4), 371–379.

https://doi.org/10.1158/2326-6066.CIR-13-0088

Yao, X., Lu, Y., Parker, L. L., Li, Y.F., El-Gamil, M., Black, M.A., Xu, H., Feldman, S.A., van der Bruggen, P., Rosenberg, S.A & Robbins, P.F. (2016). Isolation and characterization of an HLA-DPB*04:01-restricted MAGE-A3 T cell receptor for cancer immunotherapy. *Journal of Immunotherapy*, 39(5), 191-201. doi:10.1097/CJI.0000000000000123

Yen, H., Harris, T. J., Wada, S., Grosso, J. F., Getnet, D., Goldberg, M. V, Liang, K., Bruno, T. C., Pyle, K. J., Chan, S., Anders, A., Trimble, C. L., Adler, A. J., Lin, T., Pardoll, D. M., Huang, C., & Drake, C. G. (2009). Tc17 CD8 T Cells: Functional Plasticity and Subset Diversity. *J Immunol*, *183*(11), 7161–7168. https://doi.org/doi:10.4049/jimmunol.0900368

Yu, X., Harden, K., Gonzalez, L. C., Francesco, M., Chiang, E., Irving, B., Tom, I., Ivelja, S., Refino, C. J., Clark, H., Eaton, D., & Grogan, J. L. (2009). The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nature Immunology*, *10*(1), 48–57. https://doi.org/10.1038/ni.1674

Zajac, P., Schultz-Thater, E., Tornillo, L., Sadowski, C., Trella, E., Mengus, C., Iezzi, G., & Spagnoli, G. C. (2017). MAGE-A Antigens and Cancer Immunotherapy. *Frontiers in Medicine*. https://doi.org/10.3389/fmed.2017.00018

Zamai, L., Ponti, C., Mirandola, P., Gobbi, G., Papa, S., Galeotti, L., Cocco, L., & Vitale, M. (2007). NK Cells and Cancer. *The Journal of Immunology*, *178*, 4011–4016. https://doi.org/10.4049/jimmunol.178.7.4011

Zandvliet, M. L., Van Liempt, E., Jedema, I., Veltrop-Duits, L. A., Willemze, R., Guchelaar, H. J., Falkenburg, J. H. F., & Meij, P. (2010). Co-ordinated isolation of CD8 and CD4 T cells recognizing a broad repertoire of cytomegalovirus pp65 and IE1 epitopes for highly specific adoptive immunotherapy. *Cytotherapy*, *12*(7), 933–944. https://doi.org/10.3109/14653240903505822

Zappasodi, R., Merghoub, T., & Wolchok, J. D. (2018). Emerging concepts for immune checkpoint blockade-based combination therapies. *Cancer Cell*, *33*(4), 581–598. https://doi.org/doi:10.1016/j.ccell.2018.03.005

Zgodzinski, W., Grywalska, E., Zinkiewicz, K., Surdacka, A., Majewski, M., Zakoscielny, A., Bury, P., Rolinski, J., & Wallner, G. T. (2019). Peripheral blood T lymphocytes are downregulated by the PD-1/PD-L1 axis in advanced gastric cancer. *Archives of Medical Science*, *15*(3), 774–783. https://doi.org/10.5114/aoms.2018.75092

Zhang, H., & Chen, J. (2018). Current status and future directions of cancer immunotherapy. *Journal of Cancer*, *9*(10), 1773–1781. https://doi.org/10.7150/jca.24577

Zhao, E., Xu, H., Wang, L., Kryczek, I., Wu, K., Hu, Y., Wang, G., & Zou, W. (2012). Bone marrow and the control of immunity. *Cellular and Molecular Immunology*, *9*(1), 11–19. https://doi.org/10.1038/cmi.2011.47

Zhao, Y., Niu, C., & Cui, J. (2018). Gamma-delta (γδ) T Cells: Friend or Foe in Cancer Development. *Journal of Translational Medicine*, *16*(1), 1–13. https://doi.org/10.1186/s12967-017-1378-2

Zheng, H., Liu, X., Zhang, J., Rice, S. J., Wagman, M., Kong, Y., Zhu, L., Zhu, J., Joshi, M., & Belani, C. P. (2016). Expression of PD-1 on $CD4^+$ T cells in peripheral blood associates with poor clinical outcome in non-small cell lung cancer. *Oncotarget*, *7*(35). https://doi.org/10.18632/oncotarget.9316

Zhou, Q., Munger, M. E., Veenstra, R. G., Weigel, B. J., Hirashima, M., Munn, D. H., Murphy, W. J., Azuma, M., Anderson, A. C., Kuchroo, V. K., & Blazar, B. R. (2011). Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood*, *117*(17), 4501–4510. https://doi.org/10.1182/blood-2010-10-310425

Zhu, C., Anderson, A. C., Schubart, A., Xiong, H., Imitola, J., Khoury, S. J., Zheng, X. X., Strom, T. B., & Kuchroo, V. K. (2005). The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nature Immunology*, *6*(12), 1245–1252. https://doi.org/10.1038/ni1271

Zhu, S., van den Eynde, B., Coulie, P.G., Li, Y.F., El-Gamil, M., Rosenberg, S.A. & Robbins, P.F. (2012). Characterization of T-cell Receptors Directed Against HLA-A*01-restricted and C*07-restricted Epitopes of MAGE-A3 and MAGE-A12. Journal of Immunotherapy, 35(9), 680-688. https:// doi:10.1097/CJI.0b013e31827338ea.

APPENDICES

APPENDIX 1. List of 15-mer-containing peptides pools spanning the whole sequence of MAGE-A1

MAGE-A1 :

MSLEQRSLHCKPEEALEAQQEALGLVCVQAATSSSSPLVLGTLEEVPTAGSTDPPQSPQG
ASAFPTTINFTRQRQPSEGSSSREEEGPSTSCILESLFRAVITKKVADLVGFLLLKYRAR
EPVTKAEMLESVIKNYKHCFPEIFGKASESLQLVFGIDVKEADPTGHSYVLVTCLGLSYD
GLLGDNQIMPKTGFLIIVLVMIAMEGGHAPEEEIWEELSVMEVYDGREHSAYGEPRKLLT
QDLVQEKYLEYRQVPDSDPARYEFLWGPRALAETSYVKVLEYVIKVSARVRFFFPSLREA
ALREEEEGV

MAGE-A1 p1	MSLEQRSLHCKPEEA
MAGE-A1 p2	QRSLHCKPEEALEAQ
MAGE-A1 p3	HCKPEEALEAQQEAL
MAGE-A1 p4	EEALEAQQEALGLVC
MAGE-A1 p5	EAQQEALGLVCVQAA
MAGE-A1 p6	EALGLVCVQAATSSS
MAGE-A1 p7	LVCVQAATSSSSPLV
MAGE-A1 p8	QAATSSSSPLVLGTL
MAGE-A1 p9	SSSSPLVLGTLEEVP
MAGE-A1 p10	PLVLGTLEEVPTAGS
MAGE-A1 p11	GTLEEVPTAGSTDPP
MAGE-A1 p12	EVPTAGSTDPPQSPQ
MAGE-A1 p13	AGSTDPPQSPQGASA
MAGE-A1 p14	DPPQSPQGASAFPTT
MAGE-A1 p15	SPQGASAFPTTINFT
MAGE-A1 p16	ASAFPTTINFTRQRQ
MAGE-A1 p17	PTTINFTRQRQPSEG
MAGE-A1 p18	NFTRQRQPSEGSSSR
MAGE-A1 p19	QRQPSEGSSSREEEG
MAGE-A1 p20	SEGSSSREEEGPSTS
MAGE-A1 p21	SSREEEGPSTSCILE
MAGE-A1 p22	EEGPSTSCILESLFR
MAGE-A1 p23	STSCILESLFRAVIT
MAGE-A1 p24	ILESLFRAVITKKVA
MAGE-A1 p25	LFRAVITKKVADLVG
MAGE-A1 p26	VITKKVADLVGFLLL
MAGE-A1 p27	KVADLVGFLLLKYRA

MAGE-A1 p28	LVGFLLLKYRAREPV
MAGE-A1 p29	LLLKYRAREPVTKAE
MAGE-A1 p30	YRAREPVTKAEMLES
MAGE-A1 p31	EPVTKAEMLESVIKN
MAGE-A1 p32	KAEMLESVIKNYKHC
MAGE-A1 p33	LESVIKNYKHCFPEI
MAGE-A1 p34	IKNYKHCFPEIFGKA
MAGE-A1 p35	KHCFPEIFGKASESL
MAGE-A1 p36	PEIFGKASESLQLVF
MAGE-A1 p37	GKASESLQLVFGIDV
MAGE-A1 p38	ESLQLVFGIDVKEAD
MAGE-A1 p39	LVFGIDVKEADPTGH
MAGE-A1 p40	IDVKEADPTGHSYVL
MAGE-A1 p41	EADPTGHSYVLVTCL
MAGE-A1 p42	TGHSYVLVTCLGLSY
MAGE-A1 p43	YVLVTCLGLSYDGLL
MAGE-A1 p44	TCLGLSYDGLLGDNQ
MAGE-A1 p45	LSYDGLLGDNQIMPK
MAGE-A1 p46	GLLGDNQIMPKTGFL
MAGE-A1 p47	DNQIMPKTGFLIIVL
MAGE-A1 p48	MPKTGFLIIVLVMIA
MAGE-A1 p49	GFLIIVLVMIAMEGG
MAGE-A1 p50	IVLVMIAMEGGHAPE
MAGE-A1 p51	MIAMEGGHAPEEEIW
MAGE-A1 p52	EGGHAPEEEIWEELS
MAGE-A1 p53	APEEEIWEELSVMEV
MAGE-A1 p54	EIWEELSVMEVYDGR
MAGE-A1 p55	ELSVMEVYDGREHSA
MAGE-A1 p56	MEVYDGREHSAYGEP
MAGE-A1 p57	DGREHSAYGEPRKLL
MAGE-A1 p58	HSAYGEPRKLLTQDL
MAGE-A1 p59	GEPRKLLTQDLVQEK
MAGE-A1 p60	KLLTQDLVQEKYLEY
MAGE-A1 p61	QDLVQEKYLEYRQVP
MAGE-A1 p62	QEKYLEYRQVPDSDP
MAGE-A1 p63	LEYRQVPDSDPARYE
MAGE-A1 p64	QVPDSDPARYEFLWG
MAGE-A1 p65	SDPARYEFLWGPRAL
MAGE-A1 p66	RYEFLWGPRALAETS
MAGE-A1 p67	LWGPRALAETSYVKV

MAGE-A1 p68	RALAETSYVKVLEYV
MAGE-A1 p69	ETSYVKVLEYVIKVS
MAGE-A1 p70	VKVLEYVIKVSARVR
MAGE-A1 p71	EYVIKVSARVRFFFP
MAGE-A1 p72	KVSARVRFFFPSLRE
MAGE-A1 p73	RVRFFFPSLREAALR
MAGE-A1 p74	FFPSLREAALREEEE
MAGE-A1 p75	LREAALREEEEGV

APPENDIX 2. List of 15-mer-containing peptides pools spanning the whole sequence of MAGE-A3

MAGE-A3: MPLEQRSQHCKPEEGLEARGEALGLVGAQAPATEEQEAASSSSTLVEVTLGEVPAAESPD PPQSPQGASSLPTTMNYPLWSQSYEDSSNQEEEGPSTFPDLESEFQAALSRKVAELVHFL LLKYRAREPVTKAEMLGSVVGNWQYFFPVIFSKASSSLQLVFGIELMEVDPIGHLYIFAT CLGLSYDGLLGDNQIMPKAGLLIIVLAIIAREGDCAPEEKIWEELSVLEVFEGREDSILG DPKKLLTQHFVQENYLEYRQVPGSDPACYEFLWGPRALVETSYVKVLHHMVKISGGPHIS YPPLHEWVLREGEE	
MAGE-A3 p1	MPLEQRSQHCKPEEG
MAGE-A3 p2	QRSQHCKPEEGLEAR
MAGE-A3 p3	HCKPEEGLEARGEAL
MAGE-A3 p4	EEGLEARGEALGLVG
MAGE-A3 p5	EARGEALGLVGAQAP
MAGE-A3 p6	EALGLVGAQAPATEE
MAGE-A3 p7	LVGAQAPATEEQEAA
MAGE-A3 p8	QAPATEEQEAASSSS
MAGE-A3 p9	TEEQEAASSSSTLVE
MAGE-A3 p10	EAASSSSTLVEVTLG
MAGE-A3 p11	SSSTLVEVTLGEVPA
MAGE-A3 p12	LVEVTLGEVPAAESP
MAGE-A3 p13	TLGEVPAAESPDPPQ
MAGE-A3 p14	VPAAESPDPPQSPQG
MAGE-A3 p15	ESPDPPQSPQGASSL
MAGE-A3 p16	PPQSPQGASSLPTTM
MAGE-A3 p17	PQGASSLPTTMNYPL
MAGE-A3 p18	SSLPTTMNYPLWSQS
MAGE-A3 p19	TTMNYPLWSQSYEDS
MAGE-A3 p20	YPLWSQSYEDSSNQE
MAGE-A3 p21	SQSYEDSSNQEEEGP

MAGE-A3 p22	EDSSNQEEEGPSTFP
MAGE-A3 p23	NQEEEGPSTFPDLES
MAGE-A3 p24	EGPSTFPDLESEFQA
MAGE-A3 p25	TFPDLESEFQAALSR
MAGE-A3 p26	LESEFQAALSRKVAE
MAGE-A3 p27	FQAALSRKVAELVHF
MAGE-A3 p28	LSRKVAELVHFLLLK
MAGE-A3 p29	VAELVHFLLLKYRAR
MAGE-A3 p30	VHFLLLKYRAREPVT
MAGE-A3 p31	LLKYRAREPVTKAEM
MAGE-A3 p32	RAREPVTKAEMLGSV
MAGE-A3 p33	PVTKAEMLGSVVGNW
MAGE-A3 p34	AEMLGSVVGNWQYFF
MAGE-A3 p35	GSVVGNWQYFFPVIF
MAGE-A3 p36	GNWQYFFPVIFSKAS
MAGE-A3 p37	YFFPVIFSKASSSLQ
MAGE-A3 p38	VIFSKASSSLQLVFG
MAGE-A3 p39	KASSSLQLVFGIELM
MAGE-A3 p40	SLQLVFGIELMEVDP
MAGE-A3 p41	VFGIELMEVDPIGHL
MAGE-A3 p42	ELMEVDPIGHLYIFA
MAGE-A3 p43	VDPIGHLYIFATCLG
MAGE-A3 p44	GHLYIFATCLGLSYD
MAGE-A3 p45	IFATCLGLSYDGLLG
MAGE-A3 p46	CLGLSYDGLLGDNQI
MAGE-A3 p47	SYDGLLGDNQIMPKA
MAGE-A3 p48	LLGDNQIMPKAGLLI
MAGE-A3 p49	NQIMPKAGLLIIVLA
MAGE-A3 p50	PKAGLLIIVLAIIAR
MAGE-A3 p51	LLIIVLAIIAREGDC
MAGE-A3 p52	VLAIIAREGDCAPEE
MAGE-A3 p53	IAREGDCAPEEKIWE
MAGE-A3 p54	GDCAPEEKIWEELSV
MAGE-A3 p55	PEEKIWEELSVLEVF
MAGE-A3 p56	IWEELSVLEVFEGRE
MAGE-A3 p57	LSVLEVFEGREDSIL
MAGE-A3 p58	EVFEGREDSILGDPK
MAGE-A3 p59	GREDSILGDPKKLLT
MAGE-A3 p60	SILGDPKKLLTQHFV
MAGE-A3 p61	DPKKLLTQHFVQENY

MAGE-A3 p62	LLTQHFVQENYLEYR
MAGE-A3 p63	HFVQENYLEYRQVPG
MAGE-A3 p64	ENYLEYRQVPGSDPA
MAGE-A3 p65	EYRQVPGSDPACYEF
MAGE-A3 p66	VPGSDPACYEFLWGP
MAGE-A3 p67	DPACYEFLWGPRALV
MAGE-A3 p68	YEFLWGPRALVETSY
MAGE-A3 p69	WGPRALVETSYVKVL
MAGE-A3 p70	ALVETSYVKVLHHMV
MAGE-A3 p71	TSYVKVLHHMVKISG
MAGE-A3 p72	KVLHHMVKISGGPHI
MAGE-A3 p73	HMVKISGGPHISYPP
MAGE-A3 p74	ISGGPHISYPPLHEW
MAGE-A3 p75	PHISYPPLHEWVLRE
MAGE-A3 p76	YPPLHEWVLREGEE

APPENDIX 3. List of 15-mer-containing peptides pools spanning the whole sequence of MAGE-A4

MAGE-A4 : MSSEQKSQHCKPEEGVEAQEEALGLVGAQAPTTEEQEAAVSSSSPLVPGTLEEVPAAESA GPPQSPQGASALPTTISFTCWRQPNEGSSSQEEEGPSTSPDAESLFREALSNKVDELAHF LLRKYRAKELVTKAEMLERVIKNYKRCFPVIFGKASESLKMIFGIDVKEVDPASNTYTLV TCLGLSYDGLLGNNQIFPKTGLLIIVLGTIAMEGDSASEEEIWEELGVMGVYDGREHTVY GEPRKLLTQDWVQENYLEYRQVPGSNPARYEFLWGPRALAETSYVKVLEHVVRVNARVRI AYPSLREAALLEEEEGV	
MAGE-A4 p1	MSSEQKSQHCKPEEG
MAGE-A4 p2	QKSQHCKPEEGVEAQ
MAGE-A4 p3	HCKPEEGVEAQEEAL
MAGE-A4 p4	EEGVEAQEEALGLVG
MAGE-A4 p5	EAQEEALGLVGAQAP
MAGE-A4 p6	EALGLVGAQAPTTEE
MAGE-A4 p7	LVGAQAPTTEEQEAA
MAGE-A4 p8	QAPTTEEQEAAVSSS
MAGE-A4 p9	TEEQEAAVSSSSPLV
MAGE-A4 p10	EAAVSSSSPLVPGTL
MAGE-A4 p11	SSSSPLVPGTLEEVP
MAGE-A4 p12	PLVPGTLEEVPAAES
MAGE-A4 p13	GTLEEVPAAESAGPP
MAGE-A4 p14	EVPAAESAGPPQSPQ

MAGE-A4 p15	AESAGPPQSPQGASA
MAGE-A4 p16	GPPQSPQGASALPTT
MAGE-A4 p17	SPQGASALPTTISFT
MAGE-A4 p18	ASALPTTISFTCWRQ
MAGE-A4 p19	PTTISFTCWRQPNEG
MAGE-A4 p20	SFTCWRQPNEGSSSQ
MAGE-A4 p21	WRQPNEGSSSQEEEG
MAGE-A4 p22	NEGSSSQEEEGPSTS
MAGE-A4 p23	SSQEEEGPSTSPDAE
MAGE-A4 p24	EEGPSTSPDAESLFR
MAGE-A4 p25	STSPDAESLFREALS
MAGE-A4 p26	DAESLFREALSNKVD
MAGE-A4 p27	LFREALSNKVDELAH
MAGE-A4 p28	ALSNKVDELAHFLLR
MAGE-A4 p29	KVDELAHFLLRKYRA
MAGE-A4 p30	LAHFLLRKYRAKELV
MAGE-A4 p31	LLRKYRAKELVTKAE
MAGE-A4 p32	YRAKELVTKAEMLER
MAGE-A4 p33	ELVTKAEMLERVIKN
MAGE-A4 p34	KAEMLERVIKNYKRC
MAGE-A4 p35	LERVIKNYKRCFPVI
MAGE-A4 p36	IKNYKRCFPVIFGKA
MAGE-A4 p37	KRCFPVIFGKASESL
MAGE-A4 p38	PVIFGKASESLKMIF
MAGE-A4 p39	GKASESLKMIFGIDV
MAGE-A4 p40	ESLKMIFGIDVKEVD
MAGE-A4 p41	MIFGIDVKEVDPASN
MAGE-A4 p42	IDVKEVDPASNTYTL
MAGE-A4 p43	EVDPASNTYTLVTCL
MAGE-A4 p44	ASNTYTLVTCLGLSY
MAGE-A4 p45	YTLVTCLGLSYDGLL
MAGE-A4 p46	TCLGLSYDGLLGNNQ
MAGE-A4 p47	LSYDGLLGNNQIFPK
MAGE-A4 p48	GLLGNNQIFPKTGLL
MAGE-A4 p49	NNQIFPKTGLLIIVL
MAGE-A4 p50	FPKTGLLIIVLGTIA
MAGE-A4 p51	GLLIIVLGTIAMEGD
MAGE-A4 p52	IVLGTIAMEGDSASE
MAGE-A4 p53	TIAMEGDSASEEEIW
MAGE-A4 p54	EGDSASEEEIWEELG

MAGE-A4 p55	ASEEEIWEELGVMGV
MAGE-A4 p56	EIWEELGVMGVYDGR
MAGE-A4 p57	ELGVMGVYDGREHTV
MAGE-A4 p58	MGVYDGREHTVYGEP
MAGE-A4 p59	DGREHTVYGEPRKLL
MAGE-A4 p60	HTVYGEPRKLLTQDW
MAGE-A4 p61	GEPRKLLTQDWVQEN
MAGE-A4 p62	KLLTQDWVQENYLEY
MAGE-A4 p63	QDWVQENYLEYRQVP
MAGE-A4 p64	QENYLEYRQVPGSNP
MAGE-A4 p65	LEYRQVPGSNPARYE
MAGE-A4 p66	QVPGSNPARYEFLWG
MAGE-A4 p67	SNPARYEFLWGPRAL
MAGE-A4 p68	RYEFLWGPRALAETS
MAGE-A4 p69	LWGPRALAETSYVKV
MAGE-A4 p70	RALAETSYVKVLEHV
MAGE-A4 p71	ETSYVKVLEHVVRVN
MAGE-A4 p72	VKVLEHVVRVNARVR
MAGE-A4 p73	EHVVRVNARVRIAYP
MAGE-A4 p74	RVNARVRIAYPSLRE
MAGE-A4 p75	RVRIAYPSLREAALL
MAGE-A4 p76	AYPSLREAALLEEEE
MAGE-A4 p77	LREAALLEEEEGV