The role and treatment potential of natural killer T (NKT) cells in patients with upper gastrointestinal cancers.

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by

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Declaration

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__________________________________
Ashanty Maggyvie Melo Rodriguez    July 2019
Abstract

Oesophageal adenocarcinoma (OAC), squamous cell carcinoma (SSC) and gastric cancers (GAC), collectively cause over 1.3 million deaths worldwide reported in 2018. Current therapeutic regimens focus on chemo-radiotherapy prior to surgery, however, only 20-30% of patients respond to treatment. Therefore, new treatments are urgently required. Invariant natural killer T (iNKT) cells are innate T cells with semi-invariant T cell receptors that recognise glycolipids presented by CD1d. iNKT cells have antitumour activities that are currently being tested as cellular therapies. iNKT cell frequencies were quantified in pre- and post-treatment blood and omentum from 152 patients with GAC, SCC or OAC by flow cytometry. They were found to be depleted in peripheral blood from GAC, SCC and OAC patients compared to controls. Omentum had higher frequencies of iNKT cells in the cancer patients compared to blood. These findings suggest that iNKT cells may mediate immunity against GAC, SSC and OAC and that the omentum could be a source of cells for use in adoptive cell therapy. Since clinical trials involving iNKT cells in other cancers have to date shown limited clinical efficacy, we investigated if the antitumour activities of these cells could be improved by coordinating treatment with current chemotherapy and radiotherapy regimens or by the use of alternative glycolipid ligands. iNKT cells were isolated from healthy donor blood samples and expanded \textit{in vitro}. They were treated with various concentrations of cisplatin, carboplatin, paclitaxel, 5-fluorouracil for 24 or 48h or irradiated with a single dose of 2 Gy or 10 Gy or five fractionated doses of 2 Gy. Cells were then assayed for viability, apoptotic markers, or co-cultured with CD1d transfected HeLa cells pulsed with the iNKT cell agonist ligand, \(\alpha\)-galactosylceramide (\(\alpha\)-GalCer), for assays of cytolytic degranulation and intracellular cytokine, granzyme B and perforin production. Cisplatin, 5-FU, carboplatin, paclitaxel and radiation exhibited a dose-dependent inhibition of iNKT cell viability. Cisplatin also inhibited degranulation and IFN-\(\gamma\), but not IL-4, production by viable iNKT cells. While 5-FU, carboplatin, paclitaxel and radiation increased apoptosis of iNKT cells, it did not affect activation. A number of synthetic glycolipids analogues of \(\alpha\)-GalCer were also tested for their ability to bind to CD1d-transfected HeLa cells and activate the antitumour activities of iNKT cells. One novel glycolipid, XZ7, induced cytolytic degranulation and IFN-\(\gamma\) production by CD8\(^+\) and double negative iNKT cells, suggesting that it may be superior to \(\alpha\)-GalCer as a lead compound for activating the antitumour activities of iNKT cells. We also tested for the presence of non-invariant (type II) NKT cells reactive against a number of glycolipids that were previously shown to bind to CD1d and stimulate T cells, in OAC patients and control subjects. OAC patients had higher frequencies of sulfatide and tetramyristoyl-cardiolipin (TO CL)-specific T cells compared to controls. Most of these cells expressed the V\(\delta\)1 T cell receptor. TO CL induced the production of transforming growth factor-\(\beta\) by expanded V\(\delta\)1 T cells \textit{in vitro}. Low iNKT cell and high type II NKT cell numbers may predispose individuals to upper gastrointestinal cancers and boosting iNKT cell numbers may have therapeutic value. However, exposure to systemic chemotherapy can negatively affect their functions and should be considered when developing iNKT cell-based immunotherapies.
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<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>7AA-D</td>
<td>7-aminoactinomycin D</td>
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<tr>
<td>APAF-1</td>
<td>Activating factor 1</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>Bid</td>
<td>BH3-interacting domain death</td>
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<td>Chimeric antigen receptors</td>
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<td>CEA</td>
<td>Carcinoembryonic antigen</td>
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<td>CFSE</td>
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<td>EBABO</td>
<td>Ethidium bromide/acridine orange</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Authenticated Cell Cultures</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked imunosorbent assays</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial cell adhesion molecule</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibroblast activating protein</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GAC</td>
<td>Gastric cancer</td>
</tr>
<tr>
<td>GD2</td>
<td>Disialoganglioside</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPC3</td>
<td>Glypican-3</td>
</tr>
<tr>
<td>Gray</td>
<td>Gy</td>
</tr>
<tr>
<td>GSL-1</td>
<td>Glycosphingolipid-1</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HMB-PP</td>
<td>(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IBTS</td>
<td>Irish blood transfusion service</td>
</tr>
<tr>
<td>ICAD</td>
<td>Inhibitor of the caspase-activated DNase</td>
</tr>
<tr>
<td>ICAM-I</td>
<td>Intracellular adhesion molecule I</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>iGb3</td>
<td>Isoglobotrihexosylceramide</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IPP</td>
<td>Isopentenyl pyrophosphate</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer immunoglobulin-like receptors</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
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<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>LSM</td>
<td>Lysosphingomelin</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAIT cells</td>
<td>Mucosal-associated invariant T cell</td>
</tr>
<tr>
<td>MCA</td>
<td>Methylcholanthrene</td>
</tr>
<tr>
<td>MDSCs</td>
<td>Myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>LSMN</td>
<td>Mesothelin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MUC1</td>
<td>Mucin 1</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Killer cell lectin-like receptor subfamily K</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural Killer T cell</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung carcinoma</td>
</tr>
<tr>
<td>OAC</td>
<td>Oesophageal adenocarcinoma</td>
</tr>
<tr>
<td>OC</td>
<td>Oesophageal cancer</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death-1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHA-P</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI9</td>
<td>Granzyme B inhibitor proteinase inhibitor 9</td>
</tr>
<tr>
<td>PPRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SJH</td>
<td>St James’s Hospital</td>
</tr>
<tr>
<td>SSBs</td>
<td>Single strand break</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumour associated macrophages</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIGIT</td>
<td>T cell Ig and ITIM domain</td>
</tr>
<tr>
<td>TILs</td>
<td>Tumour infiltrating lymphocytes</td>
</tr>
<tr>
<td>TIM-3</td>
<td>T cell immunoglobulin mucin-3</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor- α</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TRG</td>
<td>Tumour regression grade</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>α-GalCer</td>
<td>α-galactocylceramide</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1 Introduction

1.1.1 Upper gastrointestinal cancer

Upper gastrointestinal (GI) cancers are the second most common and lethal cancers worldwide. Upper GI cancers include all the cancers in the organs in the upper section of the digestive system, including the gallbladder, liver, pancreas, duodenum, oesophagus and stomach (MacCalum 2017).

1.1.2 Oesophageal and gastric cancer

Oesophageal and gastric cancer are further defined by their origin site. Oesophageal cancer (OC) has two major subtypes: oesophageal adenocarcinoma (OAC) and squamous cell carcinoma (SCC). OAC develops from the glandular cells at the junction of the oesophagus and the stomach. SCC arises from the squamous epithelial cells in the upper part of the oesophagus (Nabil F. Saba 2015; WCRF 2016). The most common cancer in the stomach is adenocarcinoma, constituting 90% of the cases. Gastric adenocarcinoma arises from a single cell (Zali, Rezaei-Tavirani, and Azodi 2011). GAC is divided based upon anatomical location in cardia (near the gastro-oesophageal junction) which is associated with gastrooesophageal reflux, and non-cardia (lower portion of the stomach) associated with patients with *Helicobacter pylori* (Figure 1.1) (Mukaisho et al. 2015).
Risk factors differ for OAC, SCC and GAC. One of the main risk factors for developing OAC is Barrett’s oesophagus, a pre-malignant lesion that develops in 6% to 14% of patients with gastroesophageal reflux disease. 0.5-1% of patients with Barret’s oesophagus will develop OAC (Domper Arnal, Ferrández Arenas, and Lanas Arbeloa 2015). Obesity has also been reported to be a risk factor for OAC and GAC, but not SCC (Domper Arnal, Ferrández Arenas, and Lanas Arbeloa 2015; Zali, Rezaei-Tavirani, and Azodi 2011). Increased secretion of inflammatory markers by adipocytes due to gastroesophageal reflux diseases has been suggested as a mechanism to explain the association of obesity with OAC (Domper Arnal, Ferrández Arenas, and Lanas Arbeloa 2015). Smoking is a known a risk factor for OAC, SCC and GAC (Zali, Rezaei-Tavirani, and Azodi 2011;
Smokers have a 5-fold increased risk of developing SCC compared to non-smokers and smoking contributes to 18%-45% of the risk for developing GAC (Domper Arnal, Ferrández Arenas, and Lanas Arbeloa 2015; Zali, Rezaei-Tavirani, and Azodi 2011). Alcohol intake has also been shown to be a risk factor for SCC but not OAC, the risk depends on the amount of alcohol ingested per week (Domper Arnal, Ferrández Arenas, and Lanas Arbeloa 2015). Alcohol intake/consumption is not associated with the development of GAC, but it is associated with disease progression (Zali, Rezaei-Tavirani, and Azodi 2011). Diet also influences susceptibility to developing OC and GAC. High consumption of processed meat and low consumption of fruits and vegetables are associated with development of the cancer, due to low levels of vitamins and minerals (Zali, Rezaei-Tavirani, and Azodi 2011; Domper Arnal, Ferrández Arenas, and Lanas Arbeloa 2015). Helicobacter pylori infection results in a 3-6-fold increased risk of developing GAC compared to those without infection (Zali, Rezaei-Tavirani, and Azodi 2011).

The symptoms of OC and GAC are similar and non-specific, include: dysphagia, weight loss, chest pain, worsening indigestion, hoarseness, persistent nausea, vomiting, swollen abdomen and tiredness (Harris, Croce, and Munkholm-Larsen 2017; Correa 2014).
1.1.3 Epidemiology of oesophageal and gastric cancer

OC is the seventh most common cancer in terms of incidence, with 572,034 new cases reported in 2018 and the sixth in terms of morality worldwide (Bray et al. 2018). Approximately 70% of OC cases occur in men, with a 2-3-fold difference in incidence and mortality between sexes (Bray et al. 2018). OC is more common in Eastern and Southern African countries, and it is the number one cancer related mortality in Kenyan men. In Malawi, OC has the highest incidence rates in both men and women, whereas in developed countries the mortality rates rank in the fifth place (Bray et al. 2018). The majority of OC patients are diagnosed with SCC, however, in recent years OAC has become more prevalent than SCC in Western countries (Abbas and Krasna 2017).

GAC is the fifth most frequently diagnosed cancer, with nearly 1 million new cases diagnosed annually and the third in terms of mortality worldwide (Ibrahim et al. 2018). GAC has a 2-fold higher incidence in males than females over 40 years old (Bray et al. 2018; Zali, Rezaei-Tavirani, and Azodi 2011). In several Western Asian countries such as Iran, Turkmenistan, and Kyrgyzstan, GAC is the leading cause of cancer related deaths in men. In contrast, rates are 6-fold lower in North America and Northern Europe than in Western Asia (Bray et al. 2018).
1.1.4 Tumour stages

OC and GAC are divided in four stages according to the progression. At stage T1, the cancer has formed and spread from the innermost layer of tissue to the lamina propria, muscularis mucosae, or the submucosa, which are the inner layers of the oesophagus and stomach. At T2, cancer invades the muscularis propria. In T3, cancer has spread to the outer wall of the oesophagus to nearby tissue and lymph nodes. T4, is subdivided into T4a and T4b, where in T4a, cancer is invading adjacent structures like pleura, pericardium or diaphragm in case of OC and into the serosa in case of GAC; at stage T4b, cancer has already spread to distant lymph nodes and to other organs Figure 1.2 (Rice 2015; Fukuda, Sugiyama, and Wada 2011).

1.1.5 Therapies for upper GI cancer

Current treatment for upper GI cancers normally involves a combination of surgery, chemotherapy and radiotherapy, and it is related to the stage and type of the cancer. The most common chemotherapy drugs used for OC and GAC are cisplatin, 5-fluorouracil (5-FU), carboplatin and paclitaxel (Donohoe and Reynolds 2017). Chemotherapy in combination with radiotherapy helps to shrink the tumour prior to surgery and relieve some symptoms, but it does not clear the tumour in many cases. Due to the non-specific symptoms, tumours are frequently at an advanced stage at diagnosis, leaving a significant proportion of patients with no treatment
options and as a result they are referred for palliative care (Russell 2016). OC claimed 508,585 deaths worldwide in 2018, whereas GAC claimed 782,685 (Bray et al. 2018).

The limited efficacy, adverse side effects, costs, and contra-indications of the current standard of care modalities limit the therapeutic options (Enzinger and Mayer 2003). Therefore, new treatments are urgently needed.

1.1.6 Tumour immunology

The immune system plays an important role in the tumour control but also in its development. The antitumour immune response is carried out mostly by cytotoxic T cells (CTLs) and natural killer (NK) cells, by a process called immunosurveillance.
immunoediting consists of three phases: 1) elimination 2) equilibrium and 3) escape (Figure 1.3) (Schreiber, Old, and Smyth 2011).

In the elimination phase, eradication of the premalignant and malignant tumour cells occurs. CTLs recognise peptides presented by the major histocompatibility complex (MHC) class I on the surface of target cells by the T cell receptor (TCR) (Lebedeva et al. 2004). Once activated, CTLs produce and release perforin, granzyme B and interferon-γ (IFN-γ) causing cytotoxic effects on epithelial cells (Pennock et al. 2013).

Granzyme B induces apoptosis of target cells by the indirect activation of the caspase pathway. Granzyme B binds to target cell membrane by
electrostatic interactions and by the mannose-6-phosphate receptor (Chowdhury and Lieberman 2009). This activates the pro-apoptotic agonist BH3-interacting domain death (Bid). After activation Bid and Bax induce the transport of the pro-apoptotic mitochondrial mediator, cytochrome C, into the cytosol. Cytochrome C activates pro caspase-9 which binds to activating factor 1 (APAF-1), converting pro caspase-9 into the mature form caspase-9, activating caspase-3. Caspase-3 cleaves the inhibitor of the caspase-activated DNase (ICAD), which translocates to the nucleus to fragment DNA.

Alternatively, harsh conditions, such as nutrient deprivation and hypoxia results, in the expression of danger signals (oncogenic signals) by tumour cells, in the form of soluble or plasma membrane associated molecules, known as danger associated molecular patterns (DAMPs) (Luo, Solimini, and Elledge 2009). DAMPs bind to pattern recognition receptors (PPRs) on dendritic cells (DCs) initiating an immune response by NK cells and CD4⁺ cells in a process known as cross priming (Brenner et al. 2013; Chow, Franz, and Kagan 2015; Joffre et al. 2012). CD4⁺ T cells recognise peptides presented by MHC class II molecules on the surface of DCs, monocytes, macrophages, B lymphocytes and cancer cells, once activated these cells produce immunostimulatory cytokines, including IFN-γ and interleukin-2 (IL-2), further activating the immune system (Fridman et al. 2017). IFN-γ has a variety of functions including the promotion of macrophage activation, enhancing antigen presentation by inducing MHC molecule
expression, activating the innate immune system, regulating Th1/Th2 balance, controlling cellular proliferation and apoptosis (Tau and Rothman 1999). IL-2 contributes as a growth and death factor for T cells and also plays a key role in the survival and expansion of CD4+CD25+ T reg cells in the periphery (Malek 2003).

Figure 1.4. Granzyme B apoptosis pathway. Perforin and granzyme cause apoptosis of target cells via the caspase pathway.

On the other hand, NK cells recognise MICA/B or ULBP1-6 by the killer cell lectin-like receptor subfamily K, member 1 (KLRK1, best known as NKG2D), HLA-E by NK2C, CD112 or CD155 by CD226 (best known as DNAM-1), B7-H6 by NKp30a,b, CD48 by 2B4 on tumour cells promoting the antitumour response (Vivier et al. 2011) (Figure 1.5). CTLs and CD4+ T lymphocytes are known to act in local immunosurveillance, whereas NK
cells contribute to the control of metastatic dissemination (Vivier et al. 2011).

![Figure 1.5. NK cell receptors and ligands. Inhibitory and activation receptor on NK cells (Chan, Smyth, and Martinet 2014).](image)

The equilibrium phase is described as failure of the immune system to eliminate all tumour cells and this is where editing occurs (Schreiber, Old, and Smyth 2011), however the mechanisms leading to editing is poorly understood. The equilibrium phase is a balance between antitumour cytokines such as IL-12 and IFN-γ and protumour cytokines like IL-10 and IL-23 (Mittal et al. 2014).

The escape phase occurs when cancer cells are not recognised by immune cells resulting in the clinical manifestation of a tumour. This phase is characterised by the absence of or dysfunational effector tumour
infiltrating lymphocytes (TILs), and the accumulation of cells such as tumour-associated macrophages (TAMs) and regulatory T (Treg) cells (Finn 2008; Gabrilovich, Ostrand-Rosenberg, and Bronte 2012). Tumour cell escape phase is mediated by different immunosuppressive mechanisms, such as inhibition of effector cells through their expression of immunosuppressive or immunoregulatory receptors including cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed cell death-1 (PD-1), prostaglandin-endoperoxide synthase 2 (PTGS2, best known as COX-2), and IDO1 (Godin-Ethier et al. 2011; Pardoll 2012) by soluble mediators (IL-10, VEGF, TGF-β, IL-6) (Shimabukuro-Vornhagen et al. 2012) or by the inhibition of essential metabolic substrates, like tryptophan and arginine (Munder 2009).

1.1.7 Pro tumour immunology

The immune system can also contribute to the tumour development. By the formation of TAMs and MDSCs. TAMs are monocytes that perform immunosuppressive functions producing cytokines that promotes proliferation of solid tumours (Sica and Bronte 2007; Solinas et al. 2009). TAMs promote tumour growth by secreting vascular endothelial growth factor (VEGF)-A, VEGF-C, IL-8 and MMPs promoting angiogenesis (Zhu et al. 2016), whereas MDSCs accumulate at tumour sites and inhibit both adaptive and innate immunity by the production of TGF-β and the further stimulation of Tregs and suppression of the immune system (Ostrand-Rosenberg and Sinha 2009). Tregs downregulate the function of immune
cells by three different mechanisms: 1) by cell-to-cell contact, where Tregs express inhibitory molecules on their surface like indoleamine 2,3-dioxygenase (IDO). IDO stimulate Treg and induces T cell apoptosis, reduces T cell proliferation and T cell expansion, via tryptophan depletion (Soliman, Mediavilla-Varela, and Antonia 2010; Fujimura, Kambayashi, and Aiba 2012). 2) By secreting immunosuppressive cytokines, such as transforming growth factor beta 1 (TGF-β), IL-13 and IL-10, tumours can influence T cell differentiation and regulation (Jutel et al. 2003), or 3) suppression by cytolysis through granzyme (Grossman et al. 2004).

1.1.8 Conventional and unconventional T cells

The majority of peripheral T cells are termed ‘conventional T cells’ expressing αβ TCRs that recognise peptide antigens presented by MHC molecules and co-stimulatory receptors CD40 and CD28 (Pennington et al. 2005). A second class of T cells, termed ‘unconventional T cells’ express αβ or γδ TCRs, which recognise non-peptide antigens presented by other antigen-presenting molecules, such as CD1, MR1 and butrophilin 3A1 (Lepore, Mori, and De Libero 2018).

Activation of conventional T cells requires three activation signals, 1) recognition of a peptide presented by the MHC molecule to the TCR; 2) co-stimulatory molecules (CD40:CD40L, CD80/86:CD28), and 3) cytokines that will determine cell polarisation (Curtsinger et al. 1999). CD8+ T cells respond by specifically by killing cells that express MHC class I complex presenting antigenic peptides. In contrast CD4+ T cells recognise MHC
class II presenting peptides and respond by the release of cytokines. The presence of IL-12 and IFN-γ drive Th1 polarisation. Th1 cells are CD4+ effector cells that produce IFN-γ, IL-2 and lymphotoxin-α (LTα), which contribute to the activation of other immune cells, such as macrophages, NK cells and cytotoxic T cells to clear intracellular pathogens. The presence of IL-4 and various ligands on APCs leads to Th2 cell differentiation. Th2 cells regulate other immune cells, including B cells, mast cells and eosinophils by the secretion of IL-4, IL-5 and IL-13, which contribute to the clearance of large multicellular parasites, and also the development of allergies and atopy. TGF-β is an immunoregulatory cytokine, which in the presence of IL-6, polarises naïve CD4+ T cell differentiation into Th17 cells that secrete IL-17, IL-21 and IL-22. Th17 cells recruit neutrophils and macrophages, playing an important role in host defence against extracellular bacteria and fungi. Th17 cells are also responsible for promoting and inflammation and autoimmunity. Lastly the presence of IL-2 and IL-10 cells differentiate T cells into Treg cells. Treg cells secrete TGF-β, IL-10 and IL-35, which regulate immune responses by suppressing the differentiation and actions of Th1, Th2 and Th17 cells (Figure 1.6) (Barr, Gray, and Gray 2012; Awasthi, Murugaiyan, and Kuchroo 2008).

Unconventional T cells recognise non-peptide antigens presented by monomorphic antigen-presenting molecules. These cells recognise lipids, presented by CD1, small metabolites presented by MR1 or small
phosphorylated metabolites recognised by cells expressing the TCR Vγ9Vδ2 (Lepore, Mori, and De Libero 2018).

Figure 1.6. CD4+ T cell activation and polarisation. T cells activation depends on three signals. 1) Recognition of peptides presented by MHC molecule; 2) co-stimulatory molecules CD28, CD86; 3) the presence of cytokines that will determine T cell polarisation into Th1, Th2, Th17 or Tregs.

CD1d is a molecule that belongs to the CD1 family. The CD1 family is divided in two groups based on amino acid homology. Group 1 includes CD1a, b, and c, group 2 only consists of CD1d. Whereas CD1a, b and c can present antigens to a variety of T cells, CD1d mostly presents antigen to NKT cells (Brutkiewicz 2006). CD1d atomic structure is similar to MHC-class I, it consists of an α-chain that folds into three domains α1, α2 and α3 associated by noncovalent bonds to β2-microglobulin (Figure 1.7) (Brigl and Brenner 2004).
1.1.9 Innate T cells

Innate T cells are not MHC-restricted but recognise non-peptide antigens presented by MHC-like antigen-presenting molecules, such as CD1 and MR1. Natural killer T (NKT) cells, γδ T cells and mucosal-associated invariant T (MAIT) cells are the best characterised innate T cells. These cells differ from conventional T cells in that they possess primed/effector phenotypes and are capable of rapid expansion without the need for prior antigen exposure (LaMarche, Kohlgruber, and Brenner 2018).

1.1.9.1 MAIT cells

MAIT cells are innate T cells that comprise up to 10% of circulating T cells in humans (Dusseaux et al. 2011). MAIT cells are characterised by the expression of a semi-invariant T cell receptor Vα7.2-Jα33 chain, and high expression of the C-type lectin CD161 (NKR-P1A) (Porcelli et al. 1993; Treiner et al. 2005). MAIT cells recognise microbe-derived vitamin B
metabolites and small organic molecules, drugs and drug metabolites presented by MR1, an MHC-Ib-related protein (Tilloy et al. 1999; Kjer-Nielsen et al. 2012; Keller et al. 2017). When activated, MAIT cells produce granzymes and cytokines such as IFN-γ, tumour necrosis factor-α (TNF-α) and IL-17A (Dusseaux et al. 2011).

MAIT cells have been detected in tumours of patients with gastric, lung, breast, liver, thyroid, colorectal, kidney, brain and oesophageal cancer, and multiple myeloma (Peterfalvi et al. 2008; Sundstrom et al. 2015; Zabijak et al. 2015; Gherardin et al. 2018)(Melo et al. 2019, in press). However, their role in cancers is poorly understood. It has been shown that activated MAIT cells inhibit the growth of colorectal and oesophageal cancer cell lines (Ling et al. 2016)(Melo et al. 2019, in press). However, high frequencies of MAIT cells in colorectal tumours and in blood of patients with mucosal cancers is associated with poor survival outcomes (Zabijak et al. 2015; Won et al. 2016). Whether MAIT cells act as anti-tumour effectors or pro-tumour is still to be determined.

1.1.9.2 γδ T cells

γδ T cells comprise up to 5% of circulating T cells and are more predominant in the gut mucosa, characterised by the expression of heterodimeric TCRs composed of γ and δ chains (Allison et al. 2001). As mentioned previously, the majority of γδ T cells are activated in an MHC-independent manner. γδ T cells produce perforin, granzymes, TNF-related
apoptosis-inducing ligand (TRAIL) and a range of cytokines including IFN-γ, TNF-α and IL-17 (Chien, Meyer, and Bonneville 2014).

Human γδ T cells are subdivided based on their TCR δ chain usage into Vδ1, Vδ2 and Vδ3 T cells. Vδ1 and Vδ2 T cells also express toll-like receptors (TLR) and NK receptors, such as NKG2D and CD94 (Chien, Meyer, and Bonneville 2014). Vδ1 T cells can recognise MICA and MICB expressed by virus-infected cells, and a number of self-glycolipids presented by CD1c and CD1d (Groh et al. 1998), whereas Vδ2 T cells recognise phosphorylated metabolites such as microbial (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) or eukaryotic isoprenoid precursor isopentenyl pyrophosphate (IPP) bound to butrophilin 3A1 (Moser and Eberl 2007). Once activated Vδ1 and Vδ2 T cells rapidly secrete cytokines, chemokines, antiviral and antimicrobial factors stimulating monocytes, neutrophils, DCs, B cells and other T cells (Chien, Meyer, and Bonneville 2014; Conti et al. 2005; Eberl et al. 2009; Petrasca and Doherty 2014). To date, no Vδ3 T cell specific antigen has been identified, but Vδ3 T cells that recognize HLA-A2 (Allison et al. 2001) and CD1d (Mangan et al. 2013; Petrasca et al. 2018) have been reported. Subsets of Vδ2 and Vδ3 T cells have also been shown to be capable of inducing maturation of DC into cytokine producing antigen-presenting cells (APC) and maturation and IgM secretion by B cells (Petrasca et al. 2018; Mangan et al. 2013).
The cytokine secretion profiles of γδ T cells suggest that they mediate antitumour immunity. Vδ2 T cells can inhibit cell proliferation, angiogenesis, lymphangiogenesis and promote apoptosis of tumour cells (Sengupta 2019). Numerous clinical trials have been carried out using adoptively transferred Vδ2 T cells, in cancer such as renal cell carcinoma, malignant leukaemia, and advanced lung cancer, as well as others. Vδ2 T cells have been shown to be safe and tolerated for the patients (Vantourout and Hayday 2013; Yamasaki et al. 2011). Vδ1 T cells have protumour and antitumour functions. Vδ1 T cells have shown to kill melanoma cell lines by the production TNF-α and IFN-γ (Cordova et al. 2012). However, Vδ1 T cells can reduce susceptibility to activation-induced T-cell death and can exhibit immunosuppressive and regulatory properties (Sengupta 2019). T17 γδ T cells have shown to promote tumour progression by the production of IL-17. IL-17 supports angiogenesis in gall-bladder cancer, gastric cancer, non-small cell lung carcinoma (NSCLC), as well as other cancers (Patil et al. 2016; Wu et al. 2016; Pan et al. 2015). T17 γδ T cells also promote proliferation of myeloid-derived suppressor cells (MDSCs) that facilitate cancer progression by the inhibition of T cells and NK cells (Wu et al. 2014).

1.1.9.3 NKT cells

NKT cells are a population of regulatory and effector T cells, characterised by the expression of TCRs that recognise glycolipid antigens presented by CD1d and NK cell receptors (CD161/NK1.1) (Carreno,
NKT cells are divided into two subsets based on the TCR expression: type I NKT cells, also known as invariant (iNKT) cells, express a semi-invariant TCRα-chain (Vα24Jα18 in humans and Vα14Jα18 in mice), whereas type II NKT cells have more variable TCR-α and -β chains repertoires (Tard, Rouxel, and Lehuen 2015; Singh, Tripathi, and Cardell 2018).

Studies have shown that both iNKT cells and type II NKT cells play important roles in the immune system. They contribute to activation and regulation of other immune cells, and have a role in tumour immunity, autoimmunity, and infectious diseases (Matsuda et al. 2008). However, type II NKT cells appear to predominantly have immunoregulatory activities, whereas iNKT cells are more inflammatory (Singh, Tripathi, and Cardell 2018).

1.1.9.3.1 Invariant natural killer T cells

iNKT cells account to up to 0.1% of human peripheral blood, however, they are often localised in tissue with low circulation like the omentum, where they comprise up to 10% of T cells (Lynch 2014). iNKT cells recognise glycolipid antigens presented by CD1d, by a restricted set of TCR rearrangements generated by somatic recombination in the thymus (Kohlgruber et al. 2016; Godfrey et al. 2015). CD1d deficient mice fail to develop iNKT cells and have an impaired immune response to many bacteria, viruses and fungi, demonstrating the role of iNKT cells in host defence (Brigl and Brenner 2010; Chandra and Kronenberg 2015; Cohen,
Garg, and Brenner 2009). Although iNKT cells are activated by signals through their TCRs, they can also be activated through receptors for cytokines such as IL-12, IL-18, IL-25 and IL-23 (Figure 1.8) (Watarai et al. 2012; Raufi and Klempner 2015). Therefore, iNKT cells can be activated independently of the iNKT cell TCR (Kohlgruber et al. 2016). iNKT cell activation is followed by the rapid secretion of a diverse array of Th1, Th2, Th17 and Treg cytokines, such as IFN-γ, TNF-α, IL-4, IL-5, IL-13, IL-10, IL-17 and IL-22 (Carreno, Saavedra-Avila, and Porcelli 2016; O’Reilly et al. 2011) leading to activation and regulation of other immune cells including DCs, T cells and B cells (Matsuda et al. 2008). Their ability to respond rapidly strongly indicates a crucial role in the interaction between innate and adaptive immune response (Carreno, Saavedra-Avila, and Porcelli 2016; Kain et al. 2014).

Figure 1.8. TCR-dominated and cytokine-dominated activation of iNKT cells. iNKT cells can be activated in two different methods; a) by a strong TCR signal, where glycolipids are presented by CD1d to TCR, and has little dependence on APC-derived-cytokines; or b) where pattern-recognition receptors (PRRs) activate APC, leading to the generation of IL-12. In this case, a TCR signal is still required, but can be of a much lower affinity (Brennan, Brigl, and Brenner 2013).
1.1.9.3.2 Type II NKT cells

Type II NKT cells also recognize lipid antigens presented by CD1d. Type II NKT cells are thought to be more abundant than iNKT cells in humans, however these cells have not been studied extensively, due to the lack of phenotypic markers to define them. It has been shown that type II NKT cells do not recognize α-GalCer, but recognize mammalian glycolipids, such as sulfatides and lysophosphatidylcholine (Arrenberg et al. 2010; Marrero, Ware, and Kumar 2015). Similar to iNKT cells, type II NKT cells can secrete IFN-γ, IL-4, IL-10, IL-13, IL-17, and IL-22, TNFα, and GM-CSF, modulating NK, T and B cell responses (Godfrey et al. 2015; Kumar and Delovitch 2014). Studies in healthy mice have shown that type II NKT cells can be activated by autologous glycolipids such as sterol carrier protein 2 (SCP2), leading to a release of immunosuppressive cytokines, preventing excessive inflammation (Nishioka et al. 2017).

1.1.9.4 iNKT cells in cancer

The functions of iNKT cells can also be harnessed for the treatment of cancer. Cui et al. reported for the first time that iNKT cell-deficient mice were unable to mediate tumour rejection, indicating that iNKT cells have an essential role in IL-12-mediated rejection of tumours (Cui et al. 1997). Jα18 knockout mice exhibited significantly increased susceptibility to methylcholanthrene (MCA) induced sarcomas and B16F10 melanoma tumours (Smyth et al. 2000). Crowe et al. demostrated that this can be
reversed by the administration of liver-derived iNKT cells during the early stages of tumour growth (Crowe, Smyth, and Godfrey 2002).

Tumour rejection by iNKT cells depends mostly on IFN-γ production (Seino and Taniguchi 2005). IFN-γ and other cytokines promote host response to tumours by stimulating innate and adaptive cytotoxic lymphocytes, including T cells, NK cells, DCs and innate lymphoid cells (Parker, Rautela, and Hertzog 2016). IFN-γ also inhibits proliferation, and modulates apoptosis, differentiation, migration and cell surface antigen expression of tumour cells (Parker, Rautela, and Hertzog 2016) (Figure 1.9). The mechanism that leads to tumour rejection involves enhancing IL-12p70 while inhibiting IL-23 cytokine production by DCs leading to the activation of NK and T cells. iNKT cells also directly kill target cells by secreting cytolytic molecules from cytotoxic granules, such as granzyme B and perforin, and expression of FasL and TRAIL inducing apoptosis of tumour cells by caspase-dependent pathways (Figure 1.10) (Voskoboinik, Whisstock, and Trapani 2015).

In addition to the activation of other immune cells and direct killing of tumour cells, iNKT cells regulate tumour growth by affecting the tumour microenvironment by killing CD1d-positive TAMs (Sica and Bronte 2007). TAMs express CD1d allowing recognition and killing by iNKT cells in an IL-15 dependant manner (Song et al. 2009).
Indirect cytotoxicity. APCs activate iNKT cells via TCR:CD1d and CD40:CD4L interactions, resulting in the release of several cytokines that secondarily activate and promote the antitumour cytotoxicity of other effector lymphocytes including NK cells, T cells and dendritic cells.
MDSCs are another potential target for iNKT cells immunotherapy. It has been reported that MDSCs accumulate at tumour sites and inhibit both adaptive and innate immunity. iNKT cells eliminate the suppressive activity of MDSCs in a CD40-CD40L-dependent manner, restoring the immune response (Figure 1.11) (Ostrand-Rosenberg and Sinha 2009).
1.1.9.4.1 Glycolipids that activate iNKT cells

\(\alpha\)-galactocylceramide (\(\alpha\)-GalCer) is a glycolipid present in marine sponges which causes the rapid production of IL-4 and IFN-\(\gamma\) by human and murine iNKT cells. *In vitro*, \(\alpha\)-GalCer activates iNKT cells promoting an antitumour response, following presentation by DC (Kawano et al. 1998). At the same time, activation of DCs by iNKT cells leads to the activation of effector T cells via tumour antigen presentation, IL-12 production and upregulation of costimulatory molecule expression. The upregulation of CD40, CD80 and CD86 by DCs leads to a positive feedback loop that boosts expression of CD40L on iNKT cells inducing a further production of IFN-\(\gamma\) (Kitamura et al. 1999; Fujii et al. 2003). It has been shown the IFN-\(\gamma\) production by iNKT cells activated by \(\alpha\)-GalCer provides the signal that skews the IL-12p70/IL-23 balance (Uemura et al. 2009; Yang et al. 2000). These in combination, lead to activation of antitumour immune cells including: NK cells, cytotoxic CD8\(^+\) cells and CD4\(^+\) cells (Fujii et al. 2003; Metelitsa et al. 2001). Activated iNKT cells also produce TNF-\(\alpha\), which activates \(\gamma\delta\) T-cells. NK cells, cytotoxic T cells and \(\gamma\delta\) T-cells have direct effector activities against tumours (Schneiders et al. 2012). Shin *et al.* reported that CD1d\(^{-/-}\) mice exhibited a stronger immune response, with an increase in cytokine production than CD1d\(^{+/+}\) mice when CD4\(^+\) T cells were transferred. Thus, iNKT cells are critical for the regulation CD4\(^+\) T cells in the secondary immune response (Shin et al. 2010).
Figure 1.11. Modulation of the tumour microenvironment. TAMs and MDSCs produce IL-10 and IL-6 inhibiting the immune response. iNKT cells suppress tumour-supporting cells, such as TAMs and MDSCs in a CD40:CD40L dependent manner, restoring the antitumour response.
Several other glycolipids are known to activate iNKT cells. Exogenous glycolipids from bacteria such as glycosphingolipid-1 (GSL-1) derived from *Sphingomonadaceae*, galactosyl diacylglycerols (DAGs) from *B. burgdorferi* and cholesterol-based glycolipid (PI57) from *H. pylori* can bind to CD1d and activate iNKT cells. Additionally, iNKT cells can recognise autologous glycolipid antigens. Isoglobotrihexosylceramide (iGb3) was the first endogenous iNKT cell antigen reported (Sanderson et al. 2013; Zhou et al. 2004). Lysophosphatidylcholine (LPC) and lysosphingomelin (LSM) are also endogenous glycolipids that bind to CD1d and stimulate iNKT cells to a lesser extent. Several groups have worked on creating α-GalCer analogues to activate iNKT cells. Miyamoto *et al* designed OCH a glycolipid that showed a Th2 profile (Miyamoto, Miyake, and Yamamura 2001), whereas Li *et al* designed 7DW8.5. 7DW8.5 actives Th1 and Th2 responses (Li et al. 2010). Lastly, by modifying the sphingoside base and the glycosidic linkage, Sun *et al* demonstrated that the galactose is crucial for TCR activation (Anderson et al. 2013).

**1.1.10 Immunotherapy for cancer**

The development of immunotherapies for cancer is based on the premise that progressive mutations are monitored by the immune system, a process called immune surveillance (Mahoney, Rennert, and Freeman 2015). Recently, immunotherapy has become more accepted and successful as treatment for cancer patients. The main types of
immunotherapy now being used to treat cancer patients include the use of monoclonal antibodies and cellular immunotherapies.

1.1.10.1 Monoclonal antibodies

Many recent immunotherapies for cancer are based on the development of inhibitory antibodies which modulate immune checkpoints (Raufi and Klempner 2015). Monoclonal antibody can target specific antigens on cancer cells. Rituximab was the first monoclonal antibody approved to treat lymphoma patients. It binds to the CD20 antigen on the surface of the leukaemia and lymphoma cells making them susceptible to be recognised by immune cells (Grillo-Lopez et al. 2000).

CTLA-4 and PD-1 proteins are two immunomodulatory receptors expressed by T cells (Figure 1.12). Ligation of CTLA-4 by CD80 or CD86 stimulates regulatory T cells and inhibits activated CD4+ T cells (Peggs et al. 2009). Similarly, ligation of PD-1 by its ligands PD-L1 and PD-L2 result in T cell inhibition (Freeman et al. 2000; Topalian, Drake, and Pardoll 2015), resulting in reduced tumour immunosurveillance and tumour recognition (Pardoll 2012). 43.9% of SCC patients and 42.2% of GAC patients have either PD-L1 or PD-L2 overexpressing tumour cells (Ohigashi et al. 2005; Wu et al. 2006).

CD28, CD27, CD137L, T cell immunoglobulin mucin-3 (TIM-3), T cell Ig and ITIM domain (TIGIT), CD160, CD200, CD137 and CD158, are other immune checkpoints dysregulated in OC patients, where TIM-3 and TIGIT are upregulated in TILs (Xie et al. 2016). The combined expression of PD-1,
TIGIT, PD-L1, and PD-L2 are associated with a poor prognosis in patients with SCC (Zhao, Zhou, et al. 2018). The presence of TiGT, TIM-3 and PD-1 drives decrease functions of CD8+T cells in GAC patients. Monoclonal antibodies targeting CTLA-4 and PD-1 have shown significant clinical benefits in patients with different malignancies (Gubin et al. 2014).

Two anti PD-1 monoclonal antibodies have been approved by the U.S. Food and Drug Administration (FDA) for GAC (Wang and Shen 2018). Several other monoclonal antibodies targeting PD-1 and CTLA-4 are been carried out in early-phase clinical trials for GAC and OC patients, alone or in combination with chemotherapy (Mimura et al. 2018). Preliminary clinical data showed that 22-27% of patients with metastatic gastrointestinal cancer with PD-L1+ tumours responded to PD-1/PD-L1 treatment (Kelly 2017). However, several gastrointestinal side effects have been observed furthermore optimization of these treatments is much needed.
Figure 1.12. Immunomodulatory receptors expressed by T cells. Several molecules have been described to regulate T cells. Tumour cells can express immune checkpoints modulating the T cell immune response. Monoclonal antibodies bind to these checkpoints allowing T cells to recognise and kill tumour cells (Pardoll 2012).
1.1.10.2 Cellular immunotherapy

Cellular immunotherapy is based in the concept of *ex vivo* expansion of autologous immune cells and transferring them back into patients. *Ex vivo* manipulation, expansion and reinfusion of T cells that target viruses has been used for many years (Rooney and Leen 2012; Heslop et al. 2010). CTLs have a central role in cell-mediated cancer immunotherapy. CTLs recognize tumour cells via their TCR, killing tumour cells directly. Tumour-antigen specific T cells can be isolated, cultivated, activated and expanded *ex vivo*, and reinfused into the patients (Kvistborg et al. 2012).

T cells can also be selectively expanded *in vitro* with APCs expressing the tumour antigen and transferred into the patient (Steinman and Cohn 1973; Inaba et al. 1992). This method has been used to present the tumour antigen and stimulate CTLs and CD4+ T cells *in vivo* in melanoma patients (Diamond et al. 2011; Fuertes et al. 2011; Mukherji et al. 1995). Hsu *et al.* reported the first clinical trial using DCs in adaptive transfer therapy. Four patients with follicular B cell lymphoma were treated with three or four infusions of DCs, pulsed with a tumour-specific antigen expressed by non-Hodgkin’s lymphomas. All patients developed cellular immune responses. In addition, one patient experienced complete tumour regression, the second patient had partial tumour regression, and the third patient resolved all evidence of disease (Hsu *et al.* 1996). In 1998, Nestle *et al.* published the first clinical trial using monocyte-derived DCs. Sixteen melanoma patients were treated with 6-10 injections of
autologous monocyte-derived DCs pulsed with autologous tumour lysate every 1–4 weeks. Tumour regression in skin, soft tissue, lung, and pancreas was seen in 5 of the 16 patients (Nestle et al. 1998). MHC class I-restricted cells can successfully present GAC antigens isolated from primary tumours. However, preliminary data showing a limited proportion of T cell population in tumours and the long time required to grow these cells proved the limited applicability of the use of TIL cells (Dolcetti, De Re, and Canzonieri 2018).

NK cells directly kill tumour cells by releasing perforin and granzymes and indirectly by activating DCs, macrophages and T cells (Vivier et al. 2008; Bryceson et al. 2006). Early studies administrated systemic cytokines including, IL-2, IL-13, IL-15, IL-18, IL-21 and type I IFNs to activate NK cells (Kobayashi et al. 2005). Expanded NK cells in the presence of IL-15 and hydrocortisone reduced tumours in a lung metastasis mouse model, but high doses of IL-15 were necessary to observe a meaningful antitumour response (Kobayashi et al. 2005). In patients where IL-2 was administrated limited antitumour responses were observed, with severe life-threatening toxic side effects (Smyth et al. 2004). The use of cytokines like IL-12 and IL-15 for cancer treatment is limited by the high toxicity when administrated systemically, a short half-life and by cytokine-induced NK-cell apoptosis (Zamai et al. 2007). On the other hand, ex vivo expansion of NK cells with IL-2 resulted in improved clinical response in metastatic renal carcinoma, malignant glioma and breast cancer patients (Ishikawa
et al. 2004; deMagalhaes-Silverman et al. 2000; Burns et al. 2003; Schadendorf et al. 2009; Vivier et al. 2012). Several phase I clinical trials have been carried out using NK cells in GAC patients, these studies have shown good tolerability with no severe adverse events (Dolcetti, De Re, and Canzonieri 2018).

Cytokine-induced killer cells (CIK) are mainly expanded CD3*CD8*CD56- cells differentiated into CD56*NKT cells. CIK cells recognise tumour cells in the presence or absence of antibodies and MHC. Once activated CIK cells release perforin and several cytokines and chemokines. The induction of cytokine-induced killer cells (CIK) in combination with chemotherapy have shown longer disease-free survival and overall survival in post-operative GAC patients (Zhang et al. 2015; Jiang et al. 2010; Liu, Song, Yang, et al. 2013). Therefore, finding new ways to direct autologous NK cells to kill cells are needed.

1.1.10.3 Chimeric antigen receptors (CARs)

T cells can also be genetically manipulated to express protein-fusion-derived chimeric antigen receptors (CARs), allowing higher antigen specificity (Sharpe and Mount 2015). CARs are composed of an antigen-binding region (single-chain variable fragment (scFv) for antigen binding), a transmembrane domain and intracellular signalling domains, and one, two or three signalling domains from CD28 and/or 4-1BB (Bridgeman et al. 2010; Heczey et al. 2014).
Most of CARs therapies have been developed to target CD19 in B cell cancers. Several clinical trials have shown complete remission of 70-90% of patients with leukaemia, with continue remission for up to 2 years after treatment with CARs (Lee, Kochenderfer, et al. 2015; Maude et al. 2014). Tisagenlecleucel by Novartis is the first CAR-T cell therapy approved. Tisagenlecleucel is a CAR-T cells that target CD19 and it is used to treat refractory/relapsed acute lymphoblastic leukaemia and lymphoma (Zhao, Song, and Liu 2019). Recombinant TCR expressing T cells that target peptides are reported to result in a complete response of 80% in patients with myeloma with a median progression free survival of 19.1 months (Rapoport et al. 2015). Several clinical trials are ongoing at the moment targeting different tumour associated antigens, including carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), glypican-3 (GPC3), human epidermal growth factor receptor 2 (HER2), mesothelin (MSLN), mucin 1 (MUC1), epithelial cell adhesion molecule (EpCAM), fibroblast activating protein (FAP), GPC3 overexpressed in lung cancer, OC and pleural mesothelioma (Kiesgen et al. 2019). So far, no benefit has been shown using EGFR or HER2-targeted CAR T cells in patients with lung cancer, with less than 3% of patients showing a partial response (Ahmed et al. 2015; Feng et al. 2016). A phase I/II clinical trial (NCT02713984) treating GAC patients with HER-targeting CAR-T cells in ongoing. MSLN and MUC1 are been studied as a target in lung cancer patients, whereas EpCAM is associated with poor survival in SCC patients, and it is been studied as a target in OC patients (Matsuda et
A phase I/II study (NCT03706326) has been carried out looking to compare the efficacy of MUC1 CAR T cells and PD-1 knockout engineered T cells in patients with advanced OC. Even though, CAR T cells have shown promising results, the use of these cells have several disadvantages, loss of specific tumour associated antigens, loss of MHC molecules and absence of co-stimulatory molecules lead to tumour resistant to T cell cytotoxicity (Mirzaei et al. 2017), and have shown serious side effects like cytokine release syndrome and lethal neurotoxicity (Wolf, Choi, and Exley 2018). The development of CAR-γδT or CAR-NK cells has been suggested to overcome these limitations (Mirzaei et al. 2017).

The ability to release cytokines, prime αβ T cells, induce DC maturation, potent MHC-unrestricted cytotoxicity and recognition of cancerous ligands make γδT cells a good target for CAR cell therapy (Mirzaei et al. 2017). Targeting disialoganglioside (GD2) with CAR-γδT cells has been tested. In patients with neuroblastoma CAR-γδT cells increased the antigen-specific tumour reactivity in vitro (Schulz et al. 1984). In the presence of GD2, CAR-γδT cells produce IFN-γ, TNF-α, macrophage inflammatory proteins (MIP)-1α and MIP1β (Rischer et al. 2004). MIP1α and MIP1β regulate leukocyte activation and trafficking (O’Grady et al. 1999). When co-cultured with a huCD19+ murine cell line, CAR-γδT cells produce RANTES, a chemokine that plays a role in homing and migration of memory and effector T cells in acute infections (Crawford et al. 2011;
Fisher and Anderson 2018). No clinical trials are been carried out at the moment using CAR-γδT cells are listed in clinicaltrials.gov.

CAR-NK cells may be superior to CAR-T cells for the treatment of solid malignancies where the target antigen is unknown. CAR-NK cells do not undergo clonal expansion or immune rejection within days to weeks, furthermore they do not induce cytokine release syndrome as CAR-T cells. CAR-NK cells do not require strict HLA matching suggesting that allogenic CAR-NK cells can be used for immunotherapy (Rezvani et al. 2017). When autologous NK cells were used, a lower response against the patient’s own tumour was observed in acute myeloid lymphoma, B cell lymphoma and neuroblastoma patients compared to allogenic NK cells (Rezvani et al. 2017). This indicates that allogenic CAR-NK cells may be better targets than CAR-T cells. At the moment, more than a dozen pre-clinical trial are been carried out using CAR-NK cells in haematological and solid tumours, including glioblastoma, prostate cancer, and ovarian cancer (Rezvani et al. 2017). Construction of human induced pluripotent stem cells (iPSCs) into functional NK cells significantly inhibited tumour growth in an ovarian cancer mouse model. NK-CAR-iPSC-NK cell had similar efficacy as CAR-T cells, but mice treated with NK-CAR-iPSC-NK cells exhibited longer survival and did not show weight loss or increase cytokine levels compared to mice treated with CAR-T cells (Hermanson and Kaufman 2015). NK cell lines have also been tested. The most common cell line studied is NK-92 cells, a cell line originally from a patient with non-Hodgkin’s lymphoma. Several
studies are been carried out using NK-92 cells expressing various CARs targets in both solid malignancies and hematologic tumours, including HER-2 for ovarian and breast cancer (Schonfeld et al. 2015), EGFR for breast cancer metastasis and glioblastoma (Han et al. 2015; Chen et al. 2016), CD138 and CS-1 for multiple myeloma (Chu et al. 2014; Jiang et al. 2014), CD19 and CD20 for B cell malignancies (Boissel et al. 2013; Muller et al. 2008) and GD2 for neuroblastoma (Esser et al. 2012). These alterations overcome resistance to NK cells, by restoring NK cell mediated killing of tumour cells in vitro and in vivo. Twenty two clinical trials using CAR-NK cells are registered on ClinicalTrials.gov, with eleven recruiting at the moment. CAR-NK cells are a potential immunotherapy for solid and hematologic malignancies.

1.1.10.4 iNKT cell immunotherapy

Although, extensive studies have been performed in mice to confirm the anti-tumour potential of iNKT cells, clinical trials in humans have been unsuccessful. Three main iNKT cell-directed therapeutics have been studied so far, intravenous (i.v.) injection of α-GalCer, administration of APCs pulsed with α-GalCer and transfer of ex vivo expanded and activated iNKT cells. Direct i.v. injections of α-GalCer were first tested in patients with solid tumours. The aim of the trial was to find the maximum tolerated dose and the optimum activating dose of α-GalCer. Although, no dose-limiting toxicity was found, immunological effects of α-GalCer were limited to patients with high pre-treatment numbers of iNKT cells, rather
than to the doses of α-GalCer (Giaccone et al. 2002). Giaccone et al. reported elevated levels of iNKT cell-associated cytokines including TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) in 5 of 24 patients, only 7 of the 24 patients showed stable disease for a median of 123 days after α-GalCer administration (Giaccone et al. 2002). While α-GalCer is well tolerated in patients, more studies are necessary to validate the anti-tumour effects in humans.

α-GalCer-pulsed APCs have also been used as a therapeutic strategy. Fujii et al. observed that administration of DCs pulsed with α-GalCer in mice induced prolonged cytokine production compared to α-GalCer administrated alone (Fujii et al. 2002). Monocyte-derived DCs pulsed with α-GalCer were first tested in patients with metastatic tumours (Nieda et al. 2004). IFN-γ and IL-12 production and trans-activation of T and NK cells by iNKT cells were observed, however, only 2 of 12 patients exhibited a decrease in serum tumour markers (Nieda et al. 2004). Two independent studies showed that weekly i.v. injections of α-GalCer-pulsed DCs into patients with non-small-cell lung cancer (NSCLC) caused expansions of iNKT cells and an increase in IFN-γ production. IFN-γ levels decreased prior the second injection, and no partial or complete clinical response was observed (Ishikawa et al. 2005; Motohashi et al. 2009). More recent studies have reported that i.v. injections of α-GalCer-pulsed APCs stimulate antitumour responses at the tumour site and at sites of metastasis, where half of the patients showed disease stabilisation or
reduction in their tumour mass (Nicol, Tazbirkova, and Nieda 2011). These findings suggest that α-GalCer-pulsed APCs are a possible therapeutic strategy to improve the antitumour response.

The third iNKT cell based therapeutic strategy involves the expansion of autologous iNKT cells in vitro. In patients with NSCLC, transfer of in vitro-activated iNKT cells resulted in the further activation of NK cells and IFN-γ release (Ishikawa et al. 2005). The combination of this strategy and α-GalCer-pulsed DCs has been reported to induce antitumor immunity in patients with head and neck squamous cell carcinomas (Yamasaki et al. 2011; Kunii et al. 2009). Recently, a phase 1 clinical trial carried out by Exley et al. showed the potential anti-tumour effects of iNKT cells in 9 patients with advanced melanoma (Exley et al. 2017). Patients received 3 infusions of autologous in vitro expanded iNKT cells, with granulocyte-macrophage colony-stimulating factor (GM-CSF) in the second and third infusions. Evidence of T cell and myeloid cell activation was observed. Three of the patients were progression-free at 58, 60 and 65 months (Exley et al. 2017). These prove that iNKT cells have an important value in antitumour therapeutics, but further studies of optimization are required.

Lastly, Heczey et al. targeted GD2 antigen expressed by neuroblastoma cells with CAR-iNKT cells, transfer of GD2-specific CAR-iNKT cells was shown to induce antitumour activity, prolonging the survival of the mice (Heczey et al. 2014). Another study using CD19-specific CAR-iNKT cells expressing CD62L showed a predominant CAR-iNKT cell population to be
the mediators of tumour regression in a mice B cell lymphoma model (Tian et al. 2016). An overview of clinical trial for iNKT cells as immunotherapy for cancer are shown in Table 1.1.

**Table 1.1. Overview of iNKT cells in Clinical Trials**

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Treatment</th>
<th>Phase</th>
<th>Estimated patients</th>
<th>Trial</th>
<th>Status</th>
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</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>Autologous infusion of iNKT cells</td>
<td>Phase I</td>
<td>300</td>
<td>NCT01801852</td>
<td>Ongoing (recruiting)</td>
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<td>NCT02562963</td>
<td>Ongoing (recruiting)</td>
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<tr>
<td>Squamous cell lung cancer</td>
<td>Autologous infusion of iNKT cells</td>
<td>Phase I</td>
<td>20</td>
<td>NCT02619058</td>
<td>Ongoing (recruiting)</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Autologous infusion of iNKT cells</td>
<td>Phase I</td>
<td>24</td>
<td>NCT00909558</td>
<td>Suspended</td>
</tr>
<tr>
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<td>20</td>
<td>NCT00631072</td>
<td>Completed</td>
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<tr>
<td>Prostate cancer</td>
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<td>Phase I</td>
<td>24</td>
<td>NCT02820584</td>
<td>Ongoing</td>
</tr>
</tbody>
</table>
It is known now that iNKT cells play an important role in the antitumour immune response, making iNKT cells of potential value for cancer immunotherapy, but further optimisation studies need to be done.

The combination of therapies has become more popular in recent years. Treatment with PD-1 or PD-L1 checkpoint inhibitors at the time of iNKT cell activation with α-GalCer have shown an increase in cytokine production and cytotoxicity *in vitro* and *in vivo* in a melanoma mouse model. On the contrary, the use of CTLA-4 seems to be more efficient in the absence of iNKT cells (Shissler, Lee, and Webb 2017). Combination of chemotherapy and iNKT cell immunotherapy has also shown potential, for example cyclophosphamide or gemcitabine do not altered iNKT cell activation but enhance recruitment into primary tumours (Gebremeskel et al. 2017). In the present study iNKT cell are been suggested as treatment for GAC and OC patients, furthermore the use of this immunotherapy has been tested in combination with chemotherapy and radiotherapy commonly used for upper gastrointestinal cancer.
1.2 Hypothesis

Cancer mortality has been estimated to increase to 15 million by 2030. Current treatments for OC and GAC involve surgery, chemotherapy and chemoradiation therapy (CRT). Only 30% of patients respond to current treatment, therefore novel therapeutics for these cancers are required. Immunotherapy is an attractive potential option for OC and GAC patients. However, to date no studies have examined iNKT cells or iNKT cell-based therapies in patients with OC or GAC. We hypothesised that, like in other human cancers, NKT cells are deficient or impaired in patients with OC and GAC and that iNKT cell-based immunotherapies will benefit patients. Having found that they are depleted, making them potentially amenable as cellular therapies, we then asked why iNKT cell-based therapies have shown limited efficacy in humans with other cancers, and sought to pre-clinically optimise conditions for the use of iNKT cells to treat OC and GAC. We also investigated the potential role of type 2 NKT cells in the development of OC and GAC.

1.3 Objectives

The main objectives of this study are:

1. To evaluate if frequencies of peripheral and omental iNKT cells are altered in patients with OAC, SCC and GAC compared to control subjects in order to predict if immunotherapy involving iNKT cells
might benefit patients and if the omentum is a good source of iNKT cells for immunotherapy.

2. To investigate if cisplatin, 5-FU, carboplatin, paclitaxel and radiotherapy affect viability, cytotoxicity and cytokine secretion of iNKT cells \textit{in vitro} to assess the potential of combining iNKT cell-based immunotherapy with conventional therapies.

3. To investigated if a number of novel synthetic glycolipids show better antitumour activities than α-GalCer as potential antigen for human iNKT cells.

4. To identify type II NKT cells in peripheral blood of patients with OAC and characterise their function in cancer immunology.
Chapter 2

Materials and methods
2.1 Materials

Equipment and reagents used in the present study are listed in Tables 2.1-2.11.

Table 2.1. Equipment and software used in study

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Model</th>
<th>Company</th>
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<td>Adventurer Pro</td>
<td>Ohaus, NJ, USA</td>
</tr>
<tr>
<td>Cell sorter</td>
<td>Moflo XDP</td>
<td>Beckman Coulter, CA, USA</td>
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<tr>
<td>Cell sorter</td>
<td>BD FACS Melody™</td>
<td>BD biosciences, Oxford</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Eppendorf 5810</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Eppendorf 5415 D</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Cytospin</td>
<td>Shandon 3</td>
<td>Shandon Thermodisher</td>
</tr>
<tr>
<td>Haemocytometer</td>
<td>Kova slides</td>
<td>Hycor Biomedical, IN, USA</td>
</tr>
<tr>
<td>CO₂ Incubator</td>
<td>Heracell 150i</td>
<td>Thermo Fisher Scientific, MA, USA</td>
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<td>Flow cytometer</td>
<td>FACS Canto II</td>
<td>Becton Dickinson, Oxford</td>
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<td>Flow cytometry software</td>
<td>FACSDiva</td>
<td>Becton Dickinson, Oxford</td>
</tr>
<tr>
<td>FlowJo</td>
<td>v10.2</td>
<td>Treestar Incorporated, NJ, USA</td>
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<tr>
<td>Freezing container</td>
<td>Nalgene Cryo</td>
<td>Thermo Fisher Scientific, MA, USA</td>
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<td>GraphPad Prism</td>
<td>v6.0</td>
<td>GraphPad Software Incorporated, CA, USA</td>
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<td>Hot plate and stirrer</td>
<td>AGB 1000</td>
<td>Jenway, UK</td>
</tr>
<tr>
<td>Laminar air flow class II</td>
<td>CleanAir MSC BSS6-2</td>
<td>Thermo Fisher Scientific, MA, USA</td>
</tr>
<tr>
<td>Laminar air flow class II</td>
<td>MSC 1.2</td>
<td>Thermo Electron LED GmbH, Germany</td>
</tr>
<tr>
<td>MACS Magnet</td>
<td>LS magnet</td>
<td>Miltenyi Biotech, Bergisch-Gladbach, Germany</td>
</tr>
<tr>
<td>MACS Magnet stand</td>
<td>MACS Multi Stand</td>
<td>Miltenyi Biotech, Bergisch-Gladbach, Germany</td>
</tr>
<tr>
<td>Microscope</td>
<td>Inverted; NAO 30</td>
<td>Olympus Corporation, Japan</td>
</tr>
<tr>
<td>Microscope</td>
<td>Inverted: IX51</td>
<td>Olympus Corporation, Japan</td>
</tr>
<tr>
<td>Microscope software</td>
<td>Olympus cellSense Standard</td>
<td>Olympus Corporation, Japan</td>
</tr>
<tr>
<td>Automated Microscope</td>
<td>Lionheart FX</td>
<td>BioTek, USA</td>
</tr>
<tr>
<td>Item</td>
<td>Source</td>
<td>Catalogue #</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------</td>
<td>--------------------</td>
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<tr>
<td>Autoclave tape</td>
<td>Fisher Scientific</td>
<td>11-889-5</td>
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<tr>
<td>Biohazard bin liners</td>
<td>Fisher Scientific</td>
<td>BAJ-560-050U</td>
</tr>
<tr>
<td>Cardboard freezer boxes 10x10</td>
<td>Sarstedt</td>
<td>95.064.997</td>
</tr>
<tr>
<td>Cell strainers, 40 µm</td>
<td>Fisher Scientific</td>
<td>FB35180</td>
</tr>
<tr>
<td>CellTrics disposable filters 30uM</td>
<td>Partec</td>
<td>04-0042-2316</td>
</tr>
<tr>
<td>Containers, 60 ml</td>
<td>Fisher Scientific</td>
<td>FB51806</td>
</tr>
<tr>
<td>Containers, 120 ml</td>
<td>Fisher Scientific</td>
<td>FB51808</td>
</tr>
<tr>
<td>Containers, 180 ml</td>
<td>Fisher Scientific</td>
<td>FB51810</td>
</tr>
<tr>
<td>Cryovials (Nalgene)</td>
<td>Fisher Scientific</td>
<td>CRY-100-025U</td>
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<tr>
<td>Eppendorf tubes, 1.5 mL</td>
<td>Eppendorf</td>
<td>T9661-1000EA</td>
</tr>
<tr>
<td>Flow cytometry tubes – FACS</td>
<td>Fisher Scientific</td>
<td>12650366</td>
</tr>
<tr>
<td>Filter,0.2µM Minisart Plus</td>
<td>Satorius</td>
<td>17823K</td>
</tr>
<tr>
<td>Gloves, small</td>
<td>Shield</td>
<td>GD055</td>
</tr>
<tr>
<td>Gloves, nitrile, small</td>
<td>Fisher Scientific</td>
<td>FB69262</td>
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<tr>
<td>LS columns</td>
<td>Miltenyi Biotec</td>
<td>130-042-401</td>
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<tr>
<td>Maxisorp ELISA plates</td>
<td>Thermo-Scientific</td>
<td>442404</td>
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<tr>
<td></td>
<td>Nunc</td>
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<tr>
<td>P10 pipettor tips</td>
<td>Sarstedt</td>
<td>70.1130</td>
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<tr>
<td>P200 pipettor tips</td>
<td>Sarstadt</td>
<td>70.760.002</td>
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<tr>
<td>P1000 pipettor tips</td>
<td>Fisher Scientific</td>
<td>FB78084</td>
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<tr>
<td>Parafilm 75 m 100 mm</td>
<td>Fisher Scientific</td>
<td>SEL-400-060G</td>
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**Table 2.2. Consumables and plasteware**
<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR plates, 96 well</td>
<td>Applied biosystems</td>
<td>N8010560</td>
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<tr>
<td>Plastic pipettes, 3 mL</td>
<td>Sarstedt</td>
<td>86.1171.010</td>
</tr>
<tr>
<td>Pipettes, 5 ml</td>
<td>Corning</td>
<td>PN5E1</td>
</tr>
<tr>
<td>Pipettes, 10 ml</td>
<td>Sterlin®</td>
<td>47110</td>
</tr>
<tr>
<td>Pipettes, 25 ml</td>
<td>Corning</td>
<td>4489</td>
</tr>
<tr>
<td>Pre-separation filters, sterile, 30 μM</td>
<td>Miltenyi Biotec</td>
<td>130-041-407</td>
</tr>
<tr>
<td>Sharps bins</td>
<td>Fisher Scientific</td>
<td>SAT-641-060M</td>
</tr>
<tr>
<td>Syringes, 10 mL</td>
<td>Terumo Europe N.V.</td>
<td>SS+10ES1</td>
</tr>
<tr>
<td>Syringes, 50 ml</td>
<td>Terumo Europe N.V.</td>
<td>SS+50ES1</td>
</tr>
<tr>
<td>Syringe filters, 0.2 μM</td>
<td>Fisher Scientific</td>
<td>FDP-635-010M</td>
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<tr>
<td>Tissue culture flasks, 75 cm²</td>
<td>Thermo-Scientific Nunc</td>
<td>156499</td>
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<tr>
<td>Tissue culture plates, 6 well</td>
<td>Sarstedt</td>
<td>83.8920</td>
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<tr>
<td>Tissue culture plates, 24 well</td>
<td>Cellstar®</td>
<td>E13090334</td>
</tr>
<tr>
<td>Tissue culture plates round bottom, 96 well</td>
<td>Costar®</td>
<td>3799</td>
</tr>
<tr>
<td>Tissue culture plates flat bottom, 96 well</td>
<td>Costar®</td>
<td>3596</td>
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<td>Tubes, 0.5 ml</td>
<td>Sarstedt</td>
<td>72.730.006</td>
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<td>Tubes, 2 ml</td>
<td>Sarstedt</td>
<td>72.694.006</td>
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<td>Tubes, 5 ml</td>
<td>Sarstedt</td>
<td>62.558.201</td>
</tr>
<tr>
<td>Tubes, 15 ml</td>
<td>Sarstedt</td>
<td>62.554.502</td>
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<tr>
<td>Tubes, 30 ml</td>
<td>Fisher Scientific</td>
<td>FB55151</td>
</tr>
<tr>
<td>Tubes, 50 ml</td>
<td>Sarstadt</td>
<td>62.547.254</td>
</tr>
<tr>
<td>Tissue paper</td>
<td>Fisher Scientific</td>
<td>CMC-716-021F</td>
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<tr>
<td>Weighboats polystyrene 100 ml</td>
<td>Fisher Scientific</td>
<td>FB61504</td>
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<tr>
<td>Waterbath treatment</td>
<td>Sigma-Aldrich</td>
<td>S5525</td>
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Table 2.3. General reagents & kits
<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
<th>Catalog Number</th>
</tr>
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<tbody>
<tr>
<td>Conjugation kit lightning-link PE (for PI9 conjugation)</td>
<td>Innova Biosciences</td>
<td>703-0005</td>
</tr>
<tr>
<td>Cytometer setup &amp; tracking research beads</td>
<td>BD Biosciences</td>
<td>655050</td>
</tr>
<tr>
<td>Duolbenco’s Minimun Essential medium (DMEM)</td>
<td>Gibco</td>
<td>61965026</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Fisher Scientific</td>
<td>BP231-100</td>
</tr>
<tr>
<td>Ethanol 95%</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>Ethidium Bromide (EB)</td>
<td>Alfa Aesar</td>
<td>1239-45-8</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Sigma-Aldrich</td>
<td>E7889</td>
</tr>
<tr>
<td>Fixable viability dye eFluor-506</td>
<td>eBioscience</td>
<td>65-0866-18</td>
</tr>
<tr>
<td>Foetal Bovine serum, Hyclone</td>
<td>Thermo scientific</td>
<td>SH3 071.03</td>
</tr>
<tr>
<td>Hepes</td>
<td>Gibco</td>
<td>15630056</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>Sigma-Aldrich</td>
<td>IO634</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Sigma-Aldrich</td>
<td>34486</td>
</tr>
<tr>
<td>Lymphoprep</td>
<td>Biosciences</td>
<td>1114547</td>
</tr>
<tr>
<td>Methanol</td>
<td>Sigma-Aldrich</td>
<td>34860</td>
</tr>
<tr>
<td>Monensin</td>
<td>Biolegend</td>
<td>420701</td>
</tr>
<tr>
<td>Mycoplasma-Off</td>
<td>Minerva Biolabs</td>
<td>15-5000</td>
</tr>
<tr>
<td>OneComp beads</td>
<td>eBioscience</td>
<td>01-1111-42</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA, 4% in PBS)</td>
<td>Santa Cruz, USA</td>
<td>sc-281692</td>
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<tr>
<td>Penicillin/Streptomycin</td>
<td>Gibco</td>
<td>15140122</td>
</tr>
<tr>
<td>Phytohaemagglutinin (PHA-P)</td>
<td>Sigma-Aldrich</td>
<td>L-9132</td>
</tr>
<tr>
<td>Phosphate buffered saline, sterile</td>
<td>Gibco</td>
<td>14190094</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate (PMA)</td>
<td>Sigma-Aldrich</td>
<td>P1535</td>
</tr>
<tr>
<td>Prolong gold antifade reagent with DAPI</td>
<td>Life Technologies</td>
<td>P36935</td>
</tr>
<tr>
<td>Poly-L-lysing</td>
<td>Sigma-Aldrich</td>
<td>P4707</td>
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<tr>
<td>RPMI medium 1640 (1x) + Glutamax™-I</td>
<td>Gibco</td>
<td>61870010</td>
</tr>
<tr>
<td>RNase</td>
<td>Roche</td>
<td>10 109 169 001</td>
</tr>
<tr>
<td>Saponin</td>
<td>Sigma-Aldrich</td>
<td>S7900</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>Sigma-Aldrich</td>
<td>S8032</td>
</tr>
<tr>
<td>Sodium captothecin</td>
<td>Fluorochem</td>
<td>516596</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>Gibco</td>
<td>11360039</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>Fisher Scientific</td>
<td>S/3120/65</td>
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<tr>
<td>Sulphuric acid (H2SO4)</td>
<td>Sigma-Aldrich</td>
<td>339741</td>
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<tr>
<td>Tetramethyl benzidine (TMB)</td>
<td>Sigma-Aldrich</td>
<td>T0440-1L</td>
</tr>
<tr>
<td>Thiazolyl Blue tetrazolium bromide (MTT)</td>
<td>Sigma-Aldrich</td>
<td>M5655-16</td>
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<tr>
<td>Total cytotoxicity and apoptosis assay kit</td>
<td>Immunochemistry technology</td>
<td>971</td>
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### Table 2.4. Cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Source</th>
<th>Medium</th>
</tr>
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<tbody>
<tr>
<td>Mock and CD1d-transfected C1R cells</td>
<td>Gift from Prof. Steven Porcelli, Albert Einstein College Institute</td>
<td>RPMI 1640 containing 10% HyClone FCS, 1% penicillin-streptomycin</td>
</tr>
<tr>
<td>Mock and CD1d-transfected HeLa cells</td>
<td>Gift from Prof. Steven Porcelli, Albert Einstein College Institute</td>
<td>DMEM containing 0.05 mM L-glutamine, 10% HyClone FCS, 1% penicillin/streptomycin, 25 mM HEPES</td>
</tr>
<tr>
<td>iNKT cells</td>
<td>Enriched from PBMCs from the Irish Blood Transfusion Service (IBTS)</td>
<td>RPMI 1640 containing 0.05 mM L-glutamine, 10% HyClone FCS, 1% penicillin/streptomycin, 25 mM HEPES, 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids mixture and 1% essential amino acids mixture</td>
</tr>
<tr>
<td>Vδ1T cells</td>
<td>Enriched from PBMCs from the Irish Blood Transfusion Service (IBTS)</td>
<td>RPMI 1640 containing 0.05 mM L-glutamine, 10% HyClone FCS, 1% penicillin/streptomycin, 25 mM HEPES, 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids mixture and 1% essential amino acids mixture</td>
</tr>
</tbody>
</table>
OE33 cells
European Collection Authenticated Cell Cultures (ECACC)
RPMI 1640 containing 10% HyClone FCS, 1% penicillin-streptomycin

OE19 cells
European Collection Authenticated Cell Cultures (ECACC)
RPMI 1640 containing 10% HyClone FCS, 1% penicillin-streptomycin

**Table 2.5. Cell isolation kits**

<table>
<thead>
<tr>
<th>Cell separation kit</th>
<th>Company</th>
<th>Catalogue#</th>
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<tr>
<td>Anti-iNKT TCR Microbead kit (positive selection)</td>
<td>Miltenyi Biotec</td>
<td>130-094-842</td>
</tr>
<tr>
<td>Anti- TCRγ/δ Microbead kit (positive selection)</td>
<td>Miltenyi Biotec</td>
<td>130-050-701</td>
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</tbody>
</table>

**Table 2.6. Recombinant human cytokines**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Stock concentration</th>
<th>Company</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>2x10^6 U/mL</td>
<td>Miltenyi Biotec</td>
<td>130-097-748</td>
</tr>
</tbody>
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**Table 2.7. Glycolipids**

<table>
<thead>
<tr>
<th>Glycolipids</th>
<th>Stock concentration</th>
<th>Company</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-GalCer</td>
<td>1 mg/mL reconstituted in DMSO</td>
<td>Funakoshi Co. Ltd</td>
<td>KRN7000</td>
</tr>
<tr>
<td>7DW8.5</td>
<td>1 mg/mL reconstituted in DMSO</td>
<td>Funakoshi Co. Ltd</td>
<td>7DW8-5</td>
</tr>
<tr>
<td>Sulfatide</td>
<td>50 mg/mL reconstituted in DMSO</td>
<td>Matreya LLC</td>
<td>1049</td>
</tr>
<tr>
<td>Lyso-sulfatide</td>
<td>1 mg/mL reconstituted in DMSO</td>
<td>Matreya LLC</td>
<td>1904</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>1 mg/mL reconstituted in DMSO</td>
<td>Avanti Polar Lipids, Inc.</td>
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</tr>
<tr>
<td>Tetramyristoyl-cardiolipin</td>
<td>1 mg/mL reconstituted in DMSO</td>
<td>Avanti Polar Lipids, Inc.</td>
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</table>
### Table 2.8. Chemotherapy drugs

<table>
<thead>
<tr>
<th>Chemotherapy drugs</th>
<th>Stock concentration</th>
<th>Company</th>
<th>Catalogue#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>3.3 mM reconstituted in 0.15M NaCl</td>
<td>Sigma-Aldrich</td>
<td>PHR1624</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>100 mM reconstituted in DMSO</td>
<td>Sigma-Aldrich</td>
<td>F6627</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>10 mM reconstituted in DMSO</td>
<td>Sigma-Aldrich</td>
<td>T7402</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>100 mM reconstituted in DMSO</td>
<td>Sigma-Aldrich</td>
<td>C2538</td>
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### Table 2.9. ELISA kits

<table>
<thead>
<tr>
<th>ELISA kit</th>
<th>Company</th>
<th>Catalogue#</th>
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<tr>
<td>IFN-γ Duoset</td>
<td>R&amp;D Systems</td>
<td>DY285</td>
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<tr>
<td>IL-4 Duoset</td>
<td>R&amp;D Systems</td>
<td>DY204</td>
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### Table 2.10. Fluorochrome-conjugated monoclonal antibodies for flow cytometry

<table>
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<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Company</th>
<th>μL per 1x10^5 cells</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin V</td>
<td>FITC</td>
<td></td>
<td>Santa Cruz Biotechnology</td>
<td>3</td>
<td>sc-4252</td>
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<tr>
<td>CD1d</td>
<td>PE</td>
<td>51.1</td>
<td>BioLegend</td>
<td>2</td>
<td>350305</td>
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<tr>
<td>CD3</td>
<td>APC-Cy7</td>
<td>HIT3a</td>
<td>BioLegend</td>
<td>1</td>
<td>300318</td>
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<tr>
<td>CD3</td>
<td>Pacific Blue</td>
<td>HIT3α</td>
<td>BioLegend</td>
<td>1</td>
<td>300330</td>
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<tr>
<td>CD3</td>
<td>PerCP</td>
<td>UCHT1</td>
<td>BioLegend</td>
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<td>CD4</td>
<td>PE-Cy7</td>
<td>SK3</td>
<td>BioLegend</td>
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<td>CD8a</td>
<td>PerCP</td>
<td>HIT8α</td>
<td>BioLegend</td>
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<td>300922</td>
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<td>CD19</td>
<td>Pacific Blue</td>
<td>HIB19</td>
<td>BioLegend</td>
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<td>CD45</td>
<td>FITC</td>
<td>HI30</td>
<td>BD Biosciences</td>
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<td>555482</td>
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<td>CD107a</td>
<td>FITC</td>
<td>H4A3</td>
<td>BD Biosciences</td>
<td>2</td>
<td>555800</td>
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<tr>
<td>CD107a</td>
<td>PE-Cy7</td>
<td>H4A3</td>
<td>BioLegend</td>
<td>2</td>
<td>328618</td>
</tr>
<tr>
<td>Rabbit anti-human antiphosphor-histone2ax</td>
<td>Alexa Fluor 647</td>
<td>ser139</td>
<td>Cell signalling technology</td>
<td>2577</td>
<td></td>
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<tr>
<td>Granzyme B</td>
<td>Fluorochrome</td>
<td>GB11</td>
<td>BioLegend</td>
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<td>Antibody</td>
<td>Color</td>
<td>Catalog Number</td>
<td>Supplier</td>
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### 2.2 Methods

#### 2.2.1 Ethical approval

This research was carried out in accordance with the Declaration of Helsinki ethical guidelines for medical research involving human subjects. Ethical approval was granted from the St James’s Hospital and Adelaide, Meath and National Children’s Hospitals Research Ethics Committee (SJH/AMNCH). All specimens were collected with prior informed written consent, from patients attending St James’s Hospital, or from healthy age- and sex-matched donors.
2.2.2 Subjects

Peripheral blood and omentum samples were obtained from patients with OAC, SCC or GAC at St. James’ Hospital, Dublin, Ireland. Peripheral blood was collected in EDTA tubes, while omentum was kept in transfer buffer (PBS containing 0.1% gentamicin and 0.22% glucose 5.5 mM) and kept in the incubator at 37°C until processed (omentum was processed within 30 mins).

2.2.3 Analysis of iNKT cells in omentum

Frequencies of iNKT cells were measured in omental samples from patients undergoing surgery for OAC, SCC and GAC. 5 g of omentum were mechanically-digested with a sterile scalpel on a petri dish and transferred into a 50 mL falcon tube to be enzymatically digested for 20 min at 37°C rotating at 180 rpm with collagenase II (2mg/mL). Collagenase II was previously reconstituted in Tesca buffer (50 mM TES, 0.36 mM calcium chloride, pH was adjusted to 7.4) to a stock concentration of 100g/mL. After the enzymatical digestion, cells were flushed through a polypropylene filter and washed with 10 mL of compete RPMI (cRPMI) medium (RPMI 1640 containing 10% HyClone FCS, 1% penicillin-streptomycin). Cells were centrifuged at 1500 rpm for 7 min and floating adipocytes and supernatants were removed. The cell pellets were washed twice with cRPMI, transferred into a flow cytometry tube and stained with fixable viability die (eFlour-506 diluted 1:1000 in PBS) for 15 min in the
dark. Cells were then washed with PBA buffer and then stained with mAb specific for CD3, Vα24Jα18 TCR and CD45 for 15 min in the dark, followed by washing with 1 mL of PBA and analysing by flow cytometry (Figure 2.1).

**Figure 2.1. Gating strategy for iNKT cells in omental samples.** Stromal vascular fraction (SVF) was obtained from omentum and stained with mAb specific for CD3, CD45 and Vα24Jα18 TCR, and fixable viability dye (eFlour-506). A) Expression of CD45 and side scatter are used to define the lymphocyte gate. B) Cells expressing the fixable viability dye are excluded from the analysis. C) Only single cells were included in the analysis. D) T cells were selected by gating on CD3 positive cells. E) Vα24Jα18 TCR positive CD3 positive cells were considered to be iNKT cells.

### 2.2.4 Cell culture

All cell culture work was carried out aseptically in a class II laminar air-flow cabinet. All equipment and materials were decontaminated with 70% ethanol before entering the safety cabinet. Lab coat and gloves were worn and aseptic technique was used. The cabinet was wiped down before and after use.
2.2.4.1 Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were prepared from buffy packs obtained from the Irish Blood Transfusion Service (IBTS) in St. James’s Hospital Dublin. Blood was diluted 1:1 in PBS with 5% FCS and layered over the density gradient solution Lymphoprep at a ratio of 3:2. Tubes were centrifuged at 400 x g for 25 min without break and with minimum acceleration. Using a Pasteur pipette, the buffy coat layer which contains the PBMCs was transferred into a 50 mL falcon tube to be washed twice with 40 mL of PBS containing 1% FCS, centrifuging at 400 x g for 10 min to pellet the cells. The PBMCs were then resuspended in 5 mL of cRPMI to be counted.

2.2.4.2 Counting of viable cells

Cells were counted using a Glasstic slide and a UV microscope. The cells were diluted in 1:20 ethidium bromide/acridine orange (EBAO, 100X EBAO was made 15 mg AO, 50 mg EB, 1 mL 95% ethanol and 49 mL dH2O, a working 1X solution was made up in 100 in PBS). 10 μL of stained cells were transferred into the glass slide. Viable cells were identified by a green fluorescent colour, while non—viable were orange. Viable cells present in 2 big (3x3) squares, holding a 2 mm x 1 mm x 0.1 mm volume, were counted and an average was taken. The number of cells were then multiplied by the dilution factor, and by a factor of 10^4 to obtain the cell count/ mL of the original cell suspension.
2.2.4.3 Cryopreservation and recovery of cells

Cells were cryogenically frozen for long-term storage using a freezing solution of 10% dimethylsulphoxide (DMSO) with 90% FCS. The freezing solution was freshly made and allowed to cool to 4°C before adding to the cell suspension. Cells were placed in cryovials and frozen at -80°C in the freezing container (Nalgene) which reduces the temperature of the sample at a rate of 1°C per minute. The following day cells were taken out of the freezing container and transfer to -80°C for short term storage before being transferred to liquid nitrogen for long term.

Cells were removed from cryopreservation and thawed rapidly in the water bath at 37°C with continuous shaking. Cell-appropriate media were added (Table 2.4). Cells were transferred to a sterile 50 mL falcon tube and 10 mL of medium was added. Cells were centrifuged at 1500 rpm for 7 min, and supernatants were discarded. Cells were re-suspended in cell appropriate media and added to tissue culture flasks or 96 well sterile plates. All cells were incubated at 37°C, 5% CO₂.

2.2.5 Principles of flow cytometry

Flow cytometry can detect physical characteristics such as size, shape and properties of individual cells, allowing for counting and sorting the cells, by passing single cells in front of a laser. Thousands of particles can be analysed per second. As each cell passes through the laser beam, the light will scatter in multiple directions, the flow cytometer scatters white light in a forward scatter, which is proportional to the size of the cell, and in a
side scatter which is proportional to the shape and internal complexity of the cell. A flow cytometer can detect light emitted from excited fluorescent molecules, as fluorescent labelled antibodies or dyes. When labelled cells are exposed to a laser beam, causing the fluorescent molecules to elevate to a higher energy state. After excitation, the light emitted by the fluorochrome is received by emission filters that allow the detection of multiple fluorochromes in a cell. A flow cytometer can detect the presence of the fluorochrome, but also quantify the relative amount of the fluorescence emitted from a cell.

2.2.6 Antibodies and flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human CD3, the Vα24Jα18 TCR used by iNKT cells (6B11), CD8, CD4, IL-4, perforin, granzyme B, CD45, CD1d, Vδ1, CD107a, IFN-γ, IL-13, TGF-β, Annexin V, granzyme B inhibitor proteinase inhibitor 9 (PI-9), 7-aminoactinomycin D (7AA-D), fixable viability dye (eFlour-506) and propidium iodide (PI) were used. Cells were acquired on a FACSCanto II flow cytometer and analysed using FlowJo Version 10 software (Table 2.10).

2.2.7 Analysis of iNKT cells in whole blood

The proportions of iNKT cells in whole blood from patients with OAC, SCC or GAC and age matched healthy controls were measured by flow cytometry. 100 μL of total blood was stained with CD3, CD45 and
Va24Jα18 TCR mAb for 15 min. Red cells (100 μL) were lysed with 1 mL of BD lysis buffer (1X diluted in water) for 10 min. Cells were washed twice with 1 mL of PBA (5% of 30% bovine serum albumin, 0.1% of 10% sodium azide, in 1X PBS), and analysed by flow cytometry. The gating strategy used for flow cytometry is shown in Figure 2.2.

**Figure 2.2. Gating strategy for iNKT cells in blood samples.** Peripheral blood from patients was stained with mAb CD3 and Va24Jα18, red cells were lysed using the BD lysis buffer, and analysed by flow cytometry. **A** Flow cytometry dot plot showing forward scatter (FSC) and side scatter (SSC) of total blood showing gated lymphocytes. **B** Doublets were excluded by gating on single cells in FSC-H and FSC-A flow cytometry dot plot. **C** Flow cytometry dot plot showing gated CD3 positive cells. **D** Va24Jα18 TCR positive CD3 positive cells were considered as iNKT cells.
2.2.7.1 Generation of iNKT cell lines

Lines of iNKT cells were generated from 40 mL of blood from healthy donors. PBMC were prepared from human buffy coat packs obtained from the IBTS (St. James’s Hospital, Dublin) and enriched for iNKT cells by magnetic bead separation using anti-iNKT Microbeads. Cells were washed with 2 mL of Miltentyi buffer (PBS containing 30% BSA and 500 mM of EDTA), pelleted by centrifugation and the pellet was resuspended in 400 μL of Miltentyi buffer and stained with 100 μL of anti-iNKT cell microbeads per 1x10^8 cells and incubated for 15 min at 4°C in a rotator. Cells were then washed with 1 mL of Miltentyi buffer and the pellet was resuspended in 500 μL of Miltentyi buffer and placed in a LS Miltentyi separation column, previously rinsed with 3 mL of Miltentyi buffer. Cells in the column were washed three times with 3 mL of Miltentyi buffer. 5 mL of Miltentyi buffer were used to flush out the iNKT cells using the plunger. This was followed by labelling the cells with mAb specific for CD3 and the Vα24Jα18 TCR, washing them with 1 mL of PBS and resuspending them in 200 μL of PBS. CD3^+Vα24Jα18^+ cells were sorted using a MoFlo™ XDP Cell Sorter or a BD FACSMelody Cell sorters into a 15 mL falcon tube with 2 mL of iNKT medium (RPMI 1640 containing 0.05 mM L-glutamine, 10% HyClone FCS, 1% penicillin/streptomycin, 25 mM HEPES, 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids mixture and 1% essential amino acids mixture). Sorted iNKT cells were expanded by culturing a minimum of 100 iNKT cells in 100 μL of iNKT cell medium in a 96 well round bottom plate and stimulating them with α-GalCer (100
ng/mL). The 1 mg/mL stock of α-GalCer was vortexed for 1 min, heated for 2 min at 80°C, and sonicated for 10 min before diluting it in RPMI. Cells were incubated in the presence of irradiated allogeneic feeder PBMC prepared from two donors (2×10⁶ cells/mL each). Medium was replaced with fresh iNKT cell medium containing 250 U/mL IL-2 after 24 and 48 h. Medium containing 250 U/mL IL-2 was changed twice a week for maintenance of the cells. Cells were expanded for a minimum of 2 weeks before use (Figure 2.3). iNKT cells were counted before and after expansion.

![Flow cytometry dot plots showing iNKT cell purity in peripheral blood and following in vitro expansion.](image)

Figure 2.3 Flow cytometry dot plots showing iNKT cell purity in peripheral blood and following in vitro expansion. Left and centre panels show the percentage of iNKT cells within the lymphocyte population on day 1 before and after magnetic bead cell-sorting, respectively. Right panel shows percentage of iNKT cells after using a BD FACSMelody Cell sorter and after 14 days expansion with IL-2.

### 2.2.7.2 Generating Vδ1 T cell lines

Lines of Vδ1 T cells were generated from healthy donors. PBMC were prepared from human buffy coat packs obtained from the IBTS (St. James’s Hospital, Dublin) and enriched for γδ T cells by magnetic bead separation using anti-γδ T cells Microbeads. Cells were washed with 2 mL
of Miltenyi buffer (PBS containing 30% BSA and 500 mM of EDTA), and the pellet was resuspended in 400 μL of Miltenyi buffer and stained with 10 μL of anti-TCRγδ hapten- antibody per 1x10^7 cells and incubated for 10 min at 4°C in a rotator. Cells were then washed with 1 mL of Miltenyi buffer and pellet was resuspended in 30 μL of Miltenyi buffer and 20 μL of anti-hapten microbeads per 1x10^7 cells for 15 min at 4°C using a rotator. Cells were then washed with 1 mL of Miltenyi buffer and pellet was resuspended in 500 μL of Miltenyi buffer and place in a LS Miltenyi separation column, previously rinsed with 3 mL of Miltenyi buffer. Cells in the column were washed three times with 3 mL of Miltenyi buffer. 5 mL of Miltenyi buffer were used to flush out the iNKT cells using the plunger. This was followed by labelling the cells with mAb specific for CD3 and the Vδ1 T and sorting the CD3^+ Vδ1 T^+ cells using a BD FACSMelody Cell sorter. Sorted Vδ1 T cells were expanded by culturing a minimum of 100 Vδ1 T cells in 100 μL of iNKT cell medium in a 96 well round bottom plate (RPMI 1640 containing 0.05 mM L-glutamine, 10% HyClone FCS, 1% penicillin/streptomycin, 25 mM HEPES, 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids mixture and 1% essential amino acids mixture) and stimulation with phytohaemagglutinin (PHA-P; 2 μg/mL) overnight. Cells were incubated in the presence of irradiated allogeneic feeder PBMC prepared from two donors (2x10^6 cells/mL each). Medium was replaced with fresh iNKT cell medium containing 250 U/mL IL-2 after 24 and 48 h. Cells were expanded for a minimum of 3 weeks before use.
2.2.7.3 Dendritic cell generation

Human monocyte-derived dendritic cells were a kind gift from Maria E. Morrissey (Trinity College Dublin, Ireland). Cells were generated from PBMC obtained from buffy coat preparations (IBTS, St. James’s Hospital, Dublin) using Lymphoprep. Monocytes were isolated by positive selection using CD14 microbeads and seeded at a density of 1 x 10^6 cells/mL in 6-well plates in 3 mL of RPMI-1640 medium containing 10% defined HyClone FBS, 25 mM HEPES, 1% penicillin, streptomycin and fungizone, in addition to human granulocyte macrophage colony-stimulating factor (50 ng/mL), and human IL-4 (70 ng/mL) in a humidified atmosphere with 5% CO₂ in air at 37°C. Cells were fed at day 3-4 by replacing half the medium made up with fresh cytokines. At day 6, the cells could express dendritic cell maturation markers in response to LPS (CD80, CD86, CD54 and HLA-DR).

2.2.7.4 Maintenance of cell lines

Oesophageal adenocarcinoma (OE33) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and maintained in cRPMI in T75 flasks in a humidified atmosphere with 5% CO₂ in air at 37°C. 5 mL of trypsin was used to detached cells from the flask, and this was deactivated by adding 10 mL of cRPMI to the flask, cells were transferred into a 15 mL Falcon tube and centrifuged at 1500 rpm for 7 min. Cells were split twice a week in a 1:5 ratio. Mock-transfected and CD1d-transfected HeLa cells were a gift from Prof. Steven Porcelli, Albert
Einstein College of Medicine, New York. Cells were cultured in complete DMEM (cDMEM) medium (DMEM containing 0.05 mM L-glutamine, 10% HyClone FCS, 1% penicillin streptomycin, 1% fungizone 25 mM HEPES) in T75 flasks and split 1:5 twice per week. Mock transfected and CD1d-transfected C1R cells were also obtained from Prof. Porcelli and were maintained in cRPMI in T75 flasks, suspension cells were split 1:7 twice a week. CD1d expression by HeLa cells and C1R cells was assessed routinely by flow cytometry (Figure 2.4). Cells were mycoplasma tested by PCR regularly. 1 mL of supernatant from a confluent T75 flask was centrifuged for 1 min at 2000 rpm to pellet any cell debris. PCR reaction was set up in a PCR tube by adding 25 μL of Green GoTaq, 1 μL of FWD and REV primers, 22 μL of PCR sterile water and 1 μL of the cell culture supernatant, or negative control (medium or sterile water), or positive control (contaminated medium) to a total of 50 μL. Cells with mycoplasma were discarded. Samples were heated at 95°C for 5 min, followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 min. samples were heated at 72°C for 10 min for the final step. Samples were loaded into a agarose gel (2% agarose, 5 μL of ethidium bromide in a electrophoresis system), once the gel was formed, TBE was added into the electrophoresis system, and 20 μL of samples and 2 μL of DNA ladder mixed with 6X of loading dye were loaded. Power source was set at 80 W and ran for approx. 40 min. The expected product size is 270 bp.
Figure 2.4. CD1d expression by HeLa cells and C1R cells. CD1d expression was assessed routinely by flow cytometry. The upper plots show CD1d expression on CD1d transfected and mock HeLa cells, whereas the lower plots show CD1d on mock and transfected C1R cells.

2.2.8 Chemotherapy drugs

The clinically relevant chemotherapy drugs for GI cancer, cisplatin, 5-FU, carboplatin and paclitaxel were purchased from Sigma-Aldrich (UK). Stocks were made following the manufacture instructions. Cisplatin was reconstituted in 0.15 M sodium chloride (NaCl) to a final concentration of the stock of 3.3 mM. 5-FU was reconstituted in DMSO to a final concentration of the stock of 100 mM. Paclitaxel was reconstituted in DMSO to a final concentration of the stock of 10 mM. Carboplatin stock
was resuspended in distilled water to a final concentration of the stock of 100 mM. Aliquots were stored at -20°C.

2.2.9 Radiotherapy

iNKT cells were irradiated using the X-Ray generator RS 225 system (Gulman Medical, UK) or CIX2 Cabinet X-Ray (Xstrahl life science, UK). Cells were mock irradiated (0Gy), irradiated with single doses of 2Gy or 10Gy X-rays, or five accumulative 2Gy doses. Mock irradiated cells were treated and transported the same as the cells irradiated with 2Gy or 10Gy but were not irradiated.

2.2.10 Glycolipids

Glycolipids stocks were prepared by resuspending the solid in DMSO to a final concentration of 1 mM. Stocks were stored at -20°C. Further dilution in RPMI were made after vortexing for 1 min, followed by heating for 2 min at 80°C, and sonicating for 10 min. Diluted glycolipids were stored at -20°C. All the previous steps were carried out every time the glycolipids were used.

2.2.11 MTT cell viability assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay is based on the ability of metabolic cells to convert MTT into a purple coloured formazan product with an absorbance maximum near 570 nm. When cells die, they
cannot convert MTT into formazan, thus colour formation is used as a marker for viable cells (van Meerloo, Kaspers, and Cloos 2011). Cells were starved with serum depleted medium (0.5% FCS) overnight and seeded in a 96-well flat plate at a density of 1x10^5 cells per well. Cells were treated with different concentrations of clinically relevant chemotherapy drugs for 24 and 48 h. 100 μL of 2.5 mg/mL of MTT was added into each well for 3 to 4 h until purple formazan crystals were taken up by the cells. Crystals were dissolved with DMSO, and absorbance was read at 595 nm on a VersaMax microplate reader (Molecular Devices) and analysed using SoftMax Pro software. DMSO and medium alone were used as negative control and medium with cells as positive control.

2.2.12 Cell death analysis

Toxicity of the different chemotherapy drugs was tested by flow cytometry. iNKT, OE33 and OE19 cells were plated at densities of 1x10^5 cells per well and starved overnight with serum depleted medium. Cells were treated for 24 and 48 h with different concentrations of each drug. After treatment cells were stained for Annexin V and PI to measure apoptosis and necrosis respectively. Apoptotic cells externalise phosphatidylserine to the surface of the extracellular membrane. Annexin V binds to phosphatidylserine (Schutte et al. 1998). In order to stain for Annexin V and PI, cells were centrifugated at 1500 rpm for 7 min. Cells were washed with 1 mL of 1X of binding buffer (10 mm HEPES, 150 mm NaCl, 2.5 mm calcium chloride (CaCl_2), pH7.4. Prepared in distilled water).
3 μL of Annexin V was added to each sample and incubated for 15 min in the dark. Cells were then washed with 1X of binding buffer. The pellets were resuspended in 250 μL of binding buffer. Immediately before flow cytometry acquisition, 1mg/mL of PI was diluted 1:4000 in binding buffer. 250 μL was added to the appropriate tube giving a final concentration of 1:8000. Incubation with 1000mM of cisplatin for 24 h was used to create an apoptosis-positive control. Cells were incubated at 56°C for 6 min to generate the necrosis positive controls.

2.2.13 CD1d-dependent activation of iNKT cells

Cytolytic degranulation of iNKT cells was analysed by flow cytometric analysis of CD107a expression by Vα24Jα18 TCR positive cells. iNKT cells were stimulated for 4 h using CD1d transfected HeLa cells loaded with glycolipids or vehicle control (DMSO). To load the glycolipids to HeLa cells, 1x10^5 CD1d-transfected or mock-transfected HeLa cells were cultured in round bottom 96-well tissue culture plates in 100 μL of medium and incubated in the presence of various concentrations of glycolipids, vortexed, heated and sonicated as above for 18 h at 37°C with 5% CO₂. After 18 h medium was removed and iNKT cells (1x10^5/well) were added in 100 μL of medium for 4 h in the presence of anti-CD107a mAb. 1X monensin was added after 1 h to each well, to prevent reinternalization of CD107a. Cells were then transferred into a flow cytometry tube (3mL tube) and stained for extracellular markers (CD3, CD8, CD4 and V
α24Jα18) for 15 min in the dark. Cells were washed with 1 mL of PBS and analysed by flow cytometry.

2.2.14 Detection of intracellular cytokines, perforin and granzyme B

IL-4 and IFN-γ production by iNKT cells was assessed by flow cytometric analysis. 1x10^5 iNKT cells were stimulated with CD1d transfected HeLa cells, previously pulsed with different concentrations of glycolipids for 4 h in the presence of 1X monensin as described in section 2.2.13. After stimulation cells were stained with mAb specific for CD3, Vα24Jα18 TCR, CD8 and CD4, washed with 1 mL of PBS and fixed with 1mL 4% paraformaldehyde (PFA) for 15 min in the dark. The membrane was permeabilized with 1 mL of 0.2% saponin for 15 min in the dark, cells were pelleted by spinning them at 1500 rpm for 7 min and antibodies against IL-4, IL-13, TGF-β or IFN-γ in 02% saponin were added for 30 min in the dark. Cells were then washed and analysed by flow cytometry. The same protocol was used to stain for the cytotoxic molecules, granzyme B and perforin.

2.2.15 Antibody conjugation

Purified anti- PI9 mAb for flow cytometry was labelled using a 100 μg PE conjugation kit (Lightning Link, Expedon). The kit recommended using 100-150 μg of antibody for 100 μg of PE. 25 μl of the anti-PI9 antibody (5.12 mg/ml), corresponding to 128 μg, was added to the 100 μg of PE. The antibody was diluted to a total volume of 100 μl with sterile PBS and
the PBS-diluted antibody was then added to the vial containing solid PE and mixed gently. 1 µl of the modifier solution provided in the kit was added per 10 µl of antibody-PBS mixture (i.e. 10 µl) and the mixture was incubated at room temperature, protected from light, for 3h. 1 µl of quencher solution provided in the kit was added per 10 µl of antibody mixture (i.e. 10 µl) and mixed gently and incubated for 30 min. The PE-labelled PI9 antibody was diluted with sterile PBS to a total volume of 5 ml and a titration assay was carried out to determine the recommended staining concentration for cell samples Figure 2.5. Experiments were carried out using 5 µL of the labelled antibody. The labelled antibody was aliquoted and stored at 4°C protected from light.

![Figure 2.5. PI9 titration](image)

*Figure 2.5. PI9 titration.* Different concentrations of PE-labelled PI9 antibody were tested on iNKT cells to determine the optimal staining concentration for cell samples.
2.2.16 Examination of iNKT cell proliferation

The ability of iNKT cells to proliferate after treatment with chemotherapy drugs was tested by flow cytometric analysis using CellTrace™. A vial of CellTrace™ reagent (Component A) was dissolved in 20μL DMSO (Component B) to obtain a 5 mM stock solution. 1μL of CellTrace™ stock solution in DMSO was added to each mL of cell suspension in PBS (cells 1x10^6 per mL). Cells were incubated for 20 min at 37°C, protected from light. Five times the original staining volume of iNKT medium was added and incubated for 5 min. Cells were pelleted by centrifugation and resuspended in iNKT medium. Cells were incubated for 3 days before analysis. Celltrace violet was detected on FL7 on the violet laser.

2.2.17 Total cytotoxicity of iNKT cells assay

iNKT cell cytotoxicity was tested by Total Cytotoxicity and Apoptosis Detection Kit purchased from Immunochemistry technologies (MN, USA) and analysed by flow cytometry. C1R-CD1d cells were pulsed with α-GalCer, as described above. 2X10^6 cells/mL of target cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, signal in FL-1) for 30 min at 37°C. Stained target cells were adjusted to 4X10^4 per well. 100 μL of 3 different effector:target (E:T) ratios (1X, 10X, 25X; 1x10^4, 1x10^5 and 2.5x10^5 cells/100 μL) were used. Cells were co-cultured for 5h at 37°C. Annexin V was used to identify apoptotic cells and 7-aminoactinomycin D (7-AAD) was used to detect dead cells. 7-AAD is a dye that signal in FL-3 in
the FACs Canto II flow cytometer. It binds to the DNA intercalating between cytosine and guanine bases (Falzone, Huyser, and Franken 2010). 3µL of Annexin V were added to 1x10^5 cells for 10 min in the dark, and washed with binding buffer, 2µL of 7AA-D was then added, and cells were immediately analysed by flow cytometry. Incubation with 4 µg/mL camptothecin for 5h was used to create an apoptosis-positive control. For the generation of necrosis positive controls, target cells were incubated at 56°C for 6 min. Target cells were labelled with CFSE, and apoptosis and cell death were measured with 7-AAD and Annexin V by flow cytometry. Percentage of specific lysis was calculated using the formula:

\[
\% \text{ of specific lysis} = \frac{\% (\text{dead}) \text{target cells} - \% \text{ spontaneous (dead) target cells}}{100 - \% \text{ spontaneous (dead) target cells}} \times 100\%
\]

2.2.18 Cell cycle arrest analysis by PI staining

Cell cycle arrest of the cells was measured by PI staining. PI is a dye typically used for cell cycle stain. It passes through the disrupted membrane and intercalates with the DNA (Shen, Vignali, and Wang 2017). Cell cycle is divided in different phases, in the G1 phase if the conditions are favourable cells divide, if the extracellular conditions are not favourable, cells go into a resting stage known as G0. If conditions are favourable and signals to divide are present, cells then progress into the S phase, here the DNA replicates. After S phase, G2 and M phase start with chromosome segregation and cell division (Alberts et al. 2002). The
intensity of the PI signal is directly proportional to DNA content, as cells in S phase will take up more dye and will be brighter than G1, G2 will be twice as bright as cells in G1 (Cecchini, Amiri, and Dick 2012). iNKT cells were fixed in 2.5 mL of cold ethanol (-20°C, 70% in PBS) added in a drop-wise fashion for 30 min at room temperature. Cells were wash twice with 1 mL of PBS. 1 mL of 1:40 dilution of PI was added with 100 mL of 100μg/mL of RNAse for 30 min at 37°C. Cell cycle arrest was measured by flow cytometry (Figure 2.6). Four controls were used for this assay, untreated cells plus PI and RNAse, untreated cells with PI in the absence of RNAse, untreated cells with RNAse and untreated cells without PI or RNAse.

**Figure 2.6. Cell cycle arrest.** Representative histogram of cell cycle by PI staining in iNKT cells. Tallest pick on the left represents G0/G1 phase, followed by the S phase and the farthest pick on the right represents the G2/M phase.
2.2.19 Analysis of CD1d expression

Venous blood samples were obtained from 5 healthy donors. PBMC were isolated by density gradient solution with Lymphoprep as described in section 2.2.4.1 and washed twice with 1 mL of PBS. The PBMCs were then resuspended in cRPMI. 1x10^5 PBMCs or OE33 cells in 100 µL of medium were plated in a 96-well round bottom plate and treated with cisplatin, carboplatin, paclitaxel or 5-FU. CD1d expression by B cells (CD19^-CD3^- cells), monocytes (CD14^+ cells) and OE33 cells was analysed by flow cytometry (Figure 2.7).

![Flow cytometric histogram of CD1d positive cells](image)

*Figure 2.7. Flow cytometric histogram of CD1d positive cells.* Cells were stained with mAb CD1d and analysed by flow cytometry. Representative plot showing OE33 cells unstained (CD1d^-) and stained (CD1d^+) with mAb CD1d.
2.2.20 Analysis of associations between CD1d mRNA expression and overall survival of gastric cancer

Gastric cancer patient groups in the KMPlot database (URL http://kmplot.com/analysis/index.php?p=service&cancer=gastric) were analysed to determine whether high CD1d mRNA expression is associated with overall survival. Patients were divided into high and low CD1d mRNA expression groups, with the best performing threshold being used as the final cut-off in a univariate Cox regression analysis. Basic univariate analysis was performed to assess overall survival in gastric cancer patients of all stages. The analysis was considered significant if \( p < 0.05 \).

2.2.21 Cytotoxic potential of iNKT cells by immunofluorescence microscopy

Immunofluorescence analysis of granzyme B was performed using the Lionheart FX Automated Microscope. \( 1 \times 10^5 \) iNKT cells were seeded on a 96-well flat bottom plate and stimulated with CD1d transfected HeLa cells, previously pulsed with \( \alpha \)-GalCer for 4 h. Cells were transferred into 15 mL tubes and blocked with 1% BSA for 10 min at room temperature, cells were then washed with PBS and centrifuged at 1500 rpm for 7 min. Cells were labelled with mAb anti CD45 for 10 min at room temperature in the dark, washed with PBS and fixed with 1mL of 4% PFA for 10 minutes at in the dark. The membrane was permeabilized with 1 mL of 0.2% saponin for 10 minutes at room temperature and centrifuged at 1500 rpm for 7 min. Cells were labelled with anti-granzyme B mAb for 30 min in the
dark. iNKT cells were attached to previously coated poly-L-lysing slides using a Shandon Cytospin 3. 10 μL of cells were attached to the slide by spinning them for 3 min at 700 rpm in a Shandon Cytospin 3. One drop of prolong gold antifade reagent with DAPI was used to mount cells with coverslip. Slides were sealed using clear nail varnish and left at 4°C until analysed. Images were taken with Lionheart FX Automated Microscope, at 10X magnification, fluorescence intensity was measured using ImageJ software.

2.2.2.2 Analysis of DNA damage by γH2AX detection

γH2AX was used to identify double strand breaks in DNA. γH2AX was detected in iNKT cell lines by immunofluorescence microscopy. 1x10^5 iNKT cells were fixed with 1 mL of PFA (4% v/v in PBS) for 15 min and washed three times with PBS. Cells were attached to cover slips previously coated with poly-L-lysine for 5 min and washed with water cover slip, using a Shandon Cytospin 3 at 700 rpm for 3 min. Each cover slip was placed in a well of a 6 well plate. A volume of 1 mL of blocking buffer (5% FCS, 0.3% Triton X-100 in PBS) was added to each well and incubated for 2 h at room temperature. The blocking buffer was removed, and cells were washed 3 times with 2 mL of PBS for 5 min each. Cells were incubated with 10 μL rabbit anti-human anti-phosphor-histone2ax (ser139) antibody at a dilution of 1:100 in antibody buffer (1% BSA, 0.3% Triton X-100 in PBS), except negative control where cells were incubated in antibody buffer in the absence of the antibody, plates were sealed with parafilm and
incubated overnight at 4°C. The primary antibody was then removed, and cells were washed 3 times with 2 mL of PBS for 5 min each time. Cells were then incubated with 1 mL of Alexafluor 555-labelled donkey anti-rabbit secondary antibody at a dilution of 1:1000 in antibody buffer. Cells were washed with PBS as previously describe before placement onto labelled slides using 1 drop of prolong gold antifade reagent with DAPI. Slides were sealed using clear nail varnish and left at 4°C until analysed. Slides were visualised using the IX51 Olympus inverted microscope, at 100X magnification. Foci of at least 25 cells were counted per slide.

2.2.23 Cytokine release from cell cultures

A sandwich enzyme-linked immunosorbent assays (ELISA) was used to quantify cytokines present in cell culture supernatants. 1x10^5 iNKT cells were co-cultured with CD1d-HeLa cells previously pulsed with glycolipids for 4 h. The supernatants were harvested and levels of IFN-γ and IL-4 were quantified by ELISA. Nunc immunoplates were coated with mouse anti-human capture antibody, which bound any cytokine present. The capture antibody was diluted in PBS to to 4 μg/mL for IL-4 and 2 μg/mL for IFN-γ and plated in triplicate at 50 μl per well plates were sealed with parafilm and incubated overnight at room temperature to allow the antibody to coat the plate. The following day the plates were washed in wash buffer (PBS with 0.05% Tween) three times. Antibody standards were diluted to create a 7-point curve using 2-fold serial dilutions in PBS, with the highest standard being 2000 pg/mL for IL-4 and 600 pg/mL for IFN-γ. The
standards and supernatants were plated in triplicate at 50 μl per well and left to incubate at 4°C overnight. The plates were washed to remove unbound antigen and coated with 50 μL of 75 ng/mL of IL-4 and 125 ng/mL goat anti-human biotinylated detection antibody for 2 h. After washing the plates, horseradish-peroxidase conjugated streptavidin was added (5 μL/mL) for 15 min, followed by washing. Colour substrate tetramethylbenzidine (TMB) was added at 50 μL per well and left to incubate in the dark for 15 min. Stop solution (2N H₂SO₄) was added at 25 μL per well to stop the reaction, and the plates were read spectrophotometrically at 450 nm using a VersaMax plate reader. Optical density values were transferred into an Excel 2013 (Microsoft) file, where a standard curve was computed to determine the cytokine levels (ng/mL).

2.2.24 Tetramer loading

A 0.2 mg/mL solution of glycolipids in 0.2% saponin was prepared. Glycolipids were heated at 80°C for 2 min and sonicated for 10 min before diluting them in 0.2% saponin in PBS. Glycolipids were added in a 12 molar excess to the CD1d tetramer and incubated overnight at room temperature. After incubation loaded tetramer with glycolipids were diluted with PBS and used immediately to stain cells for analysis by flow cytometry.
2.2.25 **Statistical analysis**

Statistical analysis was done using GraphPad Prism Version 6.0 (GraphPad Prism, San Diego, California). *p* values between groups were obtained using the unpaired Mann-Whitney *U* test for comparison between patient samples and controls, one-way ANOVA for comparison within groups, Wilcoxon matched pairs test for matched samples from patients, and paired *t* test for cell lines. *P* values of <0.05 (*) were considered statistically significant.
Chapter 3

iNKT cell frequencies and CD1d expression are altered in patients with OAC, SCC and GAC.
3.1 Introduction

iNKT cells has been suggested as cellular immunotherapies for breast, liver, pancreatic, colon, prostate, lung, gastric, colorectal cancer, glioblastoma, myeloma and neuroblastoma (Wolf, Choi, and Exley 2018). α-GalCer, a glycolipid present in marine sponges, induces IFN-γ production by iNKT cells when presented by DC, promoting an antitumour response in vitro (Kawano et al. 1998). Extensive studies have been carried out in mice using iNKT cells for cancer immunotherapy. Administration of α-GalCer to mice with a T cell lymphoma and lung and liver metastasis enhanced IFN-γ production by iNKT cells and NK cells causing the generation of tumour-specific T cell (Nishimura et al. 2000; Smyth et al. 2002). The most recent study showed a better reduction of polyps in a murine colorectal cancer model when treated with α-GalCer C26:0 or α-GalCer C20:2 for a short- term treatment compared to long-term treatment (Wang et al. 2019).

So far, clinical trials have used α-GalCer alone, α-GalCer-pulsed DC or α-GalCer-expanded iNKT cells as cellular therapies in patients with breast, liver, pancreatic, colon, prostate, lung, gastric, colorectal cancer, glioblastoma, head and neck carcinoma, myeloma and neuroblastoma (Kunii et al. 2009; Waldowska, Bojarska-Junak, and Roliński 2017). Alternatively, some studies have looked at the ability of iNKT cells to recognize α-GalCer loaded on CD1d-expressing tumour cells. These experiments lead to inhibition of metastasis, without the need of
additional co-stimulation (Shimizu et al. 2007). However, in all studies, the clinical efficacies were modest. It is possible that the iNKT cell-based immunotherapy trials in humans demonstrated poor efficacy because of advanced disease, inappropriate iNKT cellular subset being used or because their cellular immune responses are impaired by previous or ongoing use of conventional cytotoxic chemotherapies or radiotherapy.

Functions of iNKT cells are tissue specific (Watarai et al. 2012). iNKT cells are often localized within tissues with limited recirculation, rather than in lymph nodes (Lee, Wang, et al. 2015; Lynch 2014). Lynch et al. showed that 10% of T cells in omentum are iNKT cells, higher than in any other organ (Lynch et al. 2009). Omental iNKT cells produce less IFN-γ than iNKT cells in any other part of the body and produce more IL-4, IL-13 and IL-10 than splenic iNKT cells. However, omental iNKT cells are depleted in obese individuals and in patients with many cancers. Even though, omental iNKT cells have Th2/Treg cytokine profiles, they are still capable of killing CD1d+ targets (Lynch et al. 2009). However, iNKT cells are depleted in peripheral blood of patients with different malignancies, including rectal, breast, renal, prostate and lung cancers, malignant melanoma and chronic lymphocytic leukaemia (Molling et al. 2008; Motohashi et al. 2009).

Overweight and obesity are directly related to the increase risk of 13 cancers, including OAC, GAC, meningioma, thyroid, breast, multiple myeloma, liver, kidney, gallbladder, endometrium, pancreas, ovary and colon cancer (NIH 2019; Stone, McPherson, and Gail Darlington 2018).
The World Cancer Research Fund reported that 14% of tumours are related to obesity, specifically, 35% of OC cases are associated with obesity (Donohoe et al. 2014). Centrally located or visceral fat is associated with increased risk of developing gastro-oesophageal reflux, Barrett’s oesophagus and OAC (Corley 2007; Lagergren, Bergstrom, and Nyren 1999). In obese patients, even after regulating gastro-oesophageal reflux, the rates of conversion to OAC remained increased compared to non-obese subjects (Edelstein et al. 2007). OAC accounts for approximately 12% of cases of OC globally, while SCC accounts for approximately 88% (Nabil F. Saba 2015). However, as a consequence of the rise in obesity, gastro-oesophageal reflux disease and Barrett’s oesophagus, in Western countries OAC has become more prevalent than SCC (Abbas and Krasna 2017). Distal gastric carcinoma was the most frequent type of GAC, however, in recent years, the incidence of proximal adenocarcinoma has surpassed the incidence of distal cancer (Zali, Rezaei-Tavirani, and Azodi 2011).

3.2 Objectives

The aim of this chapter was to determine if peripheral and omental iNKT cell numbers and CD1d expression are altered in patients with OAC, SSC and GAC, in order to predict if immunotherapy involving iNKT cells might benefit patients and if the omentum is a good potential source of iNKT cells for immunotherapy.
The specific aims of this chapter were:

1. To determine the iNKT cell frequencies in blood and omentum from patients with OAC, SCC and GAC.
2. To evaluate if iNKT cell frequencies are altered by treatment, sex, or TRG.
3. To study if CD1d expression in patients with GAC correlates with survival.

3.3 Specific methods

3.3.1 Patient specimens

Peripheral blood and omentum samples were obtained from 152 patients with OAC, SCC or GAC undergoing surgery at St. James’s Hospital, Dublin, Ireland. Pre-treatment blood was taken at diagnosis, and post-treatment blood was taken on the day of surgery. The demographics of the patients are shown in Table 3.1. Blood samples were also obtained from 17 age-matched non-cancer control subjects (10 male; mean age 63).

3.3.2 Antibodies and flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human CD3, Vα24Jα18 (clone 6B11), CD45 death cell stain (eFlour-506) were used (Table 2.10).
Table 3.1. Demographic information of the patients.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>OAC (n=86)</th>
<th>SCC (n=33)</th>
<th>GAC (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.73 (43-96)</td>
<td>64.16 (46-80)</td>
<td>67.87 (49-87)</td>
</tr>
<tr>
<td>Sex</td>
<td>91.86% male</td>
<td>60.60% male</td>
<td>75.75% male</td>
</tr>
<tr>
<td>Treatment</td>
<td>Chemotherapy 38 patients</td>
<td>7 patients</td>
<td>19 patients</td>
</tr>
<tr>
<td></td>
<td>CRT 22 patients</td>
<td>13 patients</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Other 31 patients</td>
<td>13 patients</td>
<td>14 patients</td>
</tr>
<tr>
<td>TRG</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Mean BMI at diagnosis</td>
<td>27.00±4.6</td>
<td>24.79±4.12</td>
<td>26.51±3.85</td>
</tr>
</tbody>
</table>

*Other, surgery alone or palliative treatment.
**TRG, tumour regression grade. TRG for 40 patients was not available.
***BMI, body mass index. BMI for 27 patients was not available.

3.3.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6.0 (GraphPad Prism, San Diego, California). P values between groups were obtained using the unpaired Mann-Whitney U test, paired Wilcoxon test and one way-ANOVA. P values of <0.05 (*) were considered statistically significant.
3.4 Results

3.4.1 Frequencies of circulating iNKT cells are significantly diminished in patients with upper GI cancers compared to non-cancer controls.

Peripheral blood samples were obtained from 152 patients with OAC, SCC or GAC at St. James’s Hospital, Dublin, Ireland. The frequencies of iNKT cells, as percentages of T cells, in peripheral blood from patients with OAC, SCC or GAC were analysed by flow cytometry. Whole blood was stained with mAb specific for CD3, CD45 and Vα24Jα18 (Figure 3.1A). The frequencies of iNKT cells were significantly lower in blood samples from patients with OAC (0.073±0.01%. \( p=0.006 \)), SCC (0.08±0.03%. \( p=0.03 \)) and GAC (0.07±0.02%. \( p=0.02 \)) compared to non-cancer controls (0.1±0.02%. Figure 3.1B). No significant differences were observed in the proportions of iNKT cells between patients with OAC, SCC or GAC.

3.4.2 iNKT cells are found at low frequencies in omentum from patients undergoing surgery for upper GI cancers.

Stromal vascular fraction of omental adipose tissue was digested and stained with mAb specific for Vα24Jα18, CD3 and CD45 and analysed by flow cytometry (Figure 3.2A). Because of the invasive nature of omental sample collection, it was not possible to obtain omentum from healthy donors for comparison. No significant differences were observed in frequencies of iNKT cells between samples from OAC (1.2±0.3%), SCC (1.7±0.6%) and GAC (1.5±0.6%) patients (Figure 3.2B).
Figure 3.1. Frequencies of circulating iNKT cells are reduced in patients OAC, SCC and GAC. Whole blood from non-cancer age matched controls and patients with OAC, SCC and GAC was stained with mAb specific for the Vα24Jα18 TCR, CD3 and CD45. A) Representative flow cytometry dot plot showing Vα24Jα18 TCR expression by circulating CD3+ cells from a patient with OAC. B) Graphs showing mean (± SEM) frequencies of iNKT cells, as percentages of T cells, from non-cancer donors (n=17) and patients with OAC (n=91), SCC (n=30) and GAC (n=36). (*p<0.05, **p<0.01 using the Mann-Whitney test compared to controls).
Frequencies of iNKT cells in post-treatment blood samples and omentum were compared. Significant differences were observed between samples from patients with OAC ($p<0.001$. Figure 3.2C), SCC ($p=0.01$. Figure 3.2D) or GAC ($p=0.02$. Figure 3.2E) with higher frequencies of iNKT cells in omentum compared with blood. These differences were also observed in the proportion of iNKT cells in the omentum and blood samples from matched patients with OAC ($p=0.0036$. Figure 3.2F), SCC ($p=0.0167$. Figure 3.2G) or GAC ($p=0.04$. Figure 3.2H).

3.4.3 Frequencies of iNKT cells are similar in treatment-naïve OAC patients and patients after receiving chemotherapy or CRT.

OAC patients in St. James’s Hospital undergo one of two different treatments. One group of patients receives chemotherapy prior to and after surgery, while the other receives chemotherapy and radiotherapy prior to surgery. Patients in this study were stratified according to these treatment groups. No significant differences were observed in frequencies of iNKT cells in post-treatment blood ($p=0.3$, Figure 3.3A) or omentum ($p=0.3$, Figure 3.3B) between chemotherapy, CRT or untreated patients (straight to surgery).

Frequencies of iNKT cells were also compared between pre-treatment and post-treatment samples within the same cancer type. The proportions of iNKT cells was similar in pre-treatment and post-treatment blood of patients with OAC (Figure 3.4C), SCC (Figure 3.4D) and GAC (Figure 3.4E). No significant differences were observed in the frequencies
Figure 3.2. Frequencies of iNKT cells in omentum and peripheral blood from patients with OAC, SCC or GAC. Blood and stromal vascular fraction from omentum from patients with OAC, SCC and GAC were stained with mAbs specific for Vα24Jα18, CD3 and CD45 and analysed by flow cytometry. A) Representative flow cytometry dot plot showing Vα24Jα18 TCR expression by omental CD3+ cells from a patient with OAC. B) Graph showing mean (± SEM) percentages of iNKT cells among T cells from omentum in OAC (n=21), SCC (n=14) and GAC (n=15). C-E) Graphs showing mean (± SEM) frequencies of iNKT cells, as percentages of T cells, from blood and omentum of patients with OAC (C, n=21, 24), SCC (D, n=9, 14) and GAC (E, n=15). (*p<0.05, ***p<0.001 using the Mann–Whitney test). F-H) iNKT frequencies in peripheral blood and omentum from matched patients with OAC (F, n=16), SCC (G, n=8) and GAC (H, n=11). (*p<0.05, **p<0.01 using the Wilcoxon matched pairs test).
Figure 3.3. Comparison of frequencies of circulating and omental iNKT cells from OAC, SCC and GAC patients after receiving chemotherapy, CRT or treatment naïve (straight to surgery). Whole blood and stromal vascular fraction from omentum from patients with OAC, SCC or GAC were stained for Vα24Jα18, CD3 and CD45 and analysed by flow cytometry. A and B) Graphs show mean (± SEM) iNKT cell frequencies as percentages of CD3⁺ cells in blood (A, n=31, 11, 7) or omentum (B, n=26, 12, 10) of patients receiving chemotherapy, CRT or treatment naïve (straight to surgery).
of iNKT cells between matched samples of pre-treatment and post-treatment blood from patients with OAC (Figure 3.4F), SCC (Figure 3.4G) or GAC (Figure 3.4H). This suggests that their overall frequencies are not affected by chemotherapy, but their functions may be.

3.4.4 Frequencies of iNKT cells are not altered by tumour regression grade.

Tumour regression grade (TRG) is the pathological categorization of the regressive changes post cytotoxic treatment, by estimating the percentage of residual tumour in relation to percentage of tumour pre-treatment. TRG of 1 and 2 represent a positive response to treatment, whereas TRG3-5 represent little to no response to CRT. No difference was observed in the frequencies of iNKT cells in pre-treatment, post-treatment blood, or omentum, between the different TRG in patients with OAC (Figure 3.5A), SCC (Figure 3.5B) and GAC (Figure 3.5C).

3.4.5 Frequencies of iNKT cells are similar in male and female patients.

As men tend to deposit more fat viscerally, the proportions of iNKT cells in peripheral blood and omentum of male and female patients with OAC, SCC and GAC were compared. No differences were observed in the frequencies of iNKT cells in pre-treatment, post-treatment blood, or omentum, between male and female patients with OAC (Figure 3.6A), SCC (Figure 3.6B) and GAC (Figure 3.6C).
Figure 3.4. Frequencies of iNKT cells are similar in peripheral blood from patients with OAC, SCC and GAC before and after treatment. Whole blood from healthy controls and patients with OAC, SCC and GAC was stained with mAb specific for the Vα24/Jα18 TCR, CD3 and CD45. **A-C** Graphs showing mean (± SEM) frequencies of iNKT cells, as percentages of T cells in pre-treatment and post-treatment patients with OAC (A, n=63, 28), SCC (B, n=9, 21) or GAC (C, n=15, 21). **D-F** iNKT frequencies in pre-treatment and post-treatment blood from matched patients with OAC (D, n=11), SCC (E, n=5) and GAC (F, n=5).
Figure 3.5. Comparison of frequencies of iNKT cells in pre-treatment, post-treatment blood and omentum between different TRG of patients with OAC, SCC and GAC. A-C) No difference was observed in proportion of iNKT cells in pre-treatment (A, n=1-5), post-treatment blood (B, n=1-5) or omentum (C, n=1-7) according to the different TRG. Graphs show mean ± SEM percentages of iNKT cells.
Figure 3.6. Comparison of iNKT cell frequencies in pre-treatment (A, n=21, 39), post-treatment (B, n=14, 24) blood and omentum (C, n=14, 21) from women and men with OAC, SCC and GAC. Graphs show mean (± SEM) percentages of iNKT cells among T cells.
3.4.6 Higher intratumoural CD1d correlates with improved survival in gastric cancer

CD1d mRNA expression in tumours from datasets on 882 gastric cancer patients was analysed using the KMplot database. Univariate analysis of mRNA expression determined that high expression of CD1d is associated with significantly improved overall survival compared with those who had low levels of CD1d mRNA expression ($p=0.02$. Figure 3.7A).

To determine whether gender can affect overall survival in GC patients, CD1d mRNA expression was assessed in the same dataset (Figure 3.7). Using univariate analysis, high CD1d mRNA expression is associated with improved survival in males ($p=0.02$. Figure 3.7B). There was no significant difference in overall survival between CD1d-high and CD1d-low females (Figure 3.7C), this could be explained by the lower number of patients in the female group compared to the male group. These results show that low CD1d expression is associated with poorer survival rates in GC. No information for CD1d was available for OAC.

3.5 Discussion

CD1d-restricted iNKT cells promote anti-tumour immunity in mice and humans, however, iNKT cells are frequently depleted from the blood and tissues of cancer patients (Molling et al. 2008; Motohashi et al. 2009). In the present study, iNKT cell frequencies in peripheral blood from 148 patients with OAC, SCC or GAC were analysed by flow cytometry. Frequencies of iNKT cells were significantly depleted in peripheral blood
from patients with OAC, SSC and GAC compared to non-cancer controls. Obesity is associated with the development of various cancers, such as: OAC, GAC, kidney, gallbladder, liver, prostate, ovarian and pancreatic cancers (WCRFI 2017). Research from our lab have reported that 35% of oesophageal cancer cases are associated with obesity (Donohoe et al. 2014). According to the BMI at diagnosis most of the patients that participated in the present study fall in the category of overweight. Adipose tissue is a dynamic tissue with multiple functions, it regulates satiety, storage capacity, insulin sensitivity and glucose handling (Lynch 2014). Immune cells are found in the vascular fraction of adipose tissue. Lynch et al. reported that omentum has the highest frequencies of iNKT cells in humans, however, average omental iNKT cell frequencies, as percentages of T cells, were 19% in healthy controls but reduced to 5.5% in obese subjects and 4.5% patients with colorectal cancer (Lynch et al. 2009). In the present study, iNKT cells were found to be more frequent in omentum than blood from patients with OC and GAC. However, omental iNKT cells only accounted for approximately 2% of adipose tissue T cells. The invasiveness of the procedure to obtain omentum made it impossible to obtain healthy omentum as control for comparison.
Figure 3.7. High expression of CD1d in gastric cancer is associated with significantly improved overall survival. **A** Kaplan-Meier plot analysis of CD1d mRNA expression in 882 patients with gastric cancer demonstrating that high expression of CD1d mRNA is associated with significantly better overall survival (p=0.02). Patients were then stratified by gender. **B-C** Univariate analysis of CD1d mRNA expression on a dataset containing 545 male (B) and 236 female (C) patients with gastric cancer. HR, hazard ratio. These data were generated using KMPlot.
Crough et al. quantified iNKT cells in peripheral blood of 109 cancer patients (colorectal, breast, melanoma, lung cancer, RCC and others). No significant differences were found in iNKT cell frequencies between patients with prior and no prior radiation treatment or chemotherapy. However, iNKT cell frequencies where lower in patients with colorectal, melanoma and lung cancer that received chemotherapy compared to healthy controls. On the contrary, patients with breast cancer that received radiotherapy were the only group to show a reduction of iNKT cells compared to healthy controls (Crough et al. 2004). As mentioned before iNKT cell frequencies were found to be lower in peripheral blood of patients with OC and GAC compared to non-cancer controls in this study. Frequencies of iNKT cells were then stratified according to the treatment. No difference in iNKT cell frequencies were observed in peripheral blood or omentum in pre-treatment and post-treatment samples, or between patients that received chemotherapy, CRT or treatment naïve (patients went straight to surgery).

Tumour regression grade (TRG) is the categorization of the tumour change to treatment, based on the percentage of residual tumour in relation to percentage of tumour pre-treatment (Zhu et al. 2017). Grade 1 and 2 are associated with good prognosis, whereas grade 4 and 5 are associated with bad prognosis. It has been reported that higher numbers of iNKT cells prior treatment is associated with better response in clinical trial (Giaccone et al. 2002). In this study, no difference in peripheral iNKT
cell frequencies were observed between the different groups. However, more samples are needed for proper statistical analysis.

A study carried on in peripheral blood of healthy donors showed a tendency of iNKT cell frequencies to be higher in women than men, however, the difference was not statistically significant (Bernin et al. 2016). In the present study, the same tendency of higher iNKT cell frequencies were observed in peripheral blood of women than men in patients with OAC and SCC, but this was not significant.

iNKT cells respond to glycolipid antigens presented by CD1d by releasing cytokines and directly killing tumour cells. Some tumours such as prostate cancers, myelomonocytic leukaemia and some neurologic tumours, express CD1d and as such can be targeted for NKT-mediated cell killing (Metelitsa et al. 2003). In the last few years evidence has suggested that tumour cells can downregulate MHC class I molecules to evade immunosurveillance (Algarra et al. 2004). Downregulation of tumour expression of CD1d is also associated with increased malignancy in breast (Hix et al. 2011), cervical (Miura et al. 2010) and lung (Dockry et al. 2018) carcinoma and chronic lymphocytic leukaemia (Ghnewa et al. 2017). We hypothesized that CD1d expression is downregulated in upper GI cancers as a mechanism to evade the actions of iNKT cells. We therefore investigated if low expression of CD1d is associated with poor survival using the KMPlot database. CD1d mRNA expression in 882 patients with gastric cancer revealed that low CD1d expression in gastric cancer
patients is associated with significantly lower overall survival. Therefore, it is likely that CD1d downregulation is an effective mechanism employed by upper GI tumours to avoid killing by iNKT cells. Several studies have been carried out trying to increase CD1d expression on tumour cells to facilitate recognition by iNKT cells. It has been shown that molecules that regulate cell cycle progression, cellular proliferation, inhibitors of histone deacetylases, all-trans retinoic acid and certain chemotherapeutics, such as gemcitabine and cyclophosphamide analogues, are all able to induce CD1d expression levels (King et al. 2018; Chen and Ross 2007; Gebremeskel et al. 2017; Ghnewa et al. 2017; Dockry et al. 2018). Therefore, it is of interest to combine chemotherapy with iNKT cell-based immunotherapy. Future iNKT cell-based cellular therapies for GAC may benefit from simultaneous treatment with drugs that induce CD1d expression on tumour cells (Ghnewa et al. 2017; Dockry et al. 2018).

In conclusion, iNKT cells are depleted from the circulation of patients with OAC, SCC and GAC and CD1d expression is reduced in patients who had poorer survival rates. These observations suggest that iNKT cells may play a role in antitumour immunity in patients with OAC, GAC and SSC, implying that cellular therapies involving iNKT cells may benefit patients with these cancers.

Whereas therapeutic activation of iNKT cells can prevent and cure cancer in mice, cellular therapies involving iNKT cells have shown limited clinical efficacy in trials in humans with cancer. A factor that contributes to this
clinical failure may be the ongoing or previous exposure of patients to chemotherapies and radiotherapy, which frequently have toxic effects on cells of the immune system. The impact of commonly-used chemotherapies and radiotherapies for OAC, SSC and GAC on iNKT cell viability and functions will be explored in the next two chapters. The abilities of a number of natural and synthetic glycolipids to bind to CD1d and activate the antitumour activities of type 1 and type 2 NKT cells will be tested in chapters 6 and 7.
Chapter 4

Chemotherapies for upper gastrointestinal cancers inhibit the antitumor activities of invariant natural killer T cells
4.1 Introduction

Peripheral iNKT cells account for 0.1% of lymphocytes in humans. The low amount of peripheral iNKT cells may be the reason why iNKT cell-based immunotherapies in humans showed clinical efficacy compared to the successes in mice. Lynch \textit{et al.} have previously described that omentum is the organ with the highest amount iNKT cells in humans (10%) (Lynch 2014), therefore, this organ could be a good source of iNKT cells for immunotherapy for patients who are undergoing surgery. However, as described in the previous chapter iNKT cells are present in very low numbers in both peripheral blood and omentum from patients with OC and GAC. It is possible that iNKT cellular responses are impaired by previous or ongoing chemotherapies or radiotherapy.

Treatment for upper GI cancers normally involves a combination of surgery, chemotherapy and radiotherapy. The most common chemotherapy drugs used to treat OC and GAC are cisplatin, 5-FU, carboplatin and paclitaxel (Donohoe and Reynolds 2017). In general, chemotherapy drugs induce apoptosis in rapidly dividing cells (Bagnyukova \textit{et al.} 2010). Cisplatin and carboplatin are platinum analogues that bind to the guanine of the purine bases in the DNA, interfering with replication, transcription and DNA repair resulting in cell apoptosis (Dasari and Tchounwou 2014). 5-FU interferes with the synthesis of DNA by inhibiting thymidylate synthase (Longley, Harkin, and Johnston 2003). Paclitaxel induces the polymerization and further
stabilization of microtubules, leading to cell cycle arrest in the G2/M phase, inhibiting normal mitosis of the cells (Horwitz 1994).

As mentioned before chemotherapy drugs interfere with DNA metabolism, disrupting function and viability of cells. Unfortunately, these effects are not target specific; chemotherapy drugs also affect other cells in the patient (Penn and Starzl 2011). Chemotherapy is associated with lymphodepletion affecting the adaptive immune response of the patient, it also affect myeloid cells killing DCs directly (Rebe and Ghiringhelli 2015). These drugs affect both protumour and antitumour T cells. It has been reported that cisplatin and 5-FU also kill MDSC and increase percentages of DC in tumour bearing mice (Chen et al. 2012; Vincent et al. 2010), whereas paclitaxel is known to kill Treg cells (Zhang et al. 2008).

4.2 Objectives

The aim of this chapter was to evaluate the effect of cisplatin, 5-FU, paclitaxel, and carboplatin on iNKT cell may be the reason why iNKT cell-based immunotherapies in humans showed limited clinical efficacy compared to the successes in mice.

The specific aims of this chapter were:

1. To test if cisplatin, 5-FU, carboplatin or paclitaxel affect expression of the antigen presentation molecule CD1d on B cells, monocytes and oesophageal adenocarcinoma cell lines.
2. To compare the cytotoxic effect of cisplatin, 5-FU, carboplatin and paclitaxel against iNKT cells and an oesophageal adenocarcinoma cell line OE33 \textit{in vitro}.

3. To evaluate the effect chemotherapy drugs on the antitumour activities of iNKT cells including cytolytic degranulation, direct tumour cell killing, and the production and release of IFN-\gamma, granzyme B and perforin.

4.3 Specific methods

4.3.1 Generating iNKT cells

Lines of iNKT cells were generated from healthy donors as describe in sections 0 and expanded for 2 weeks.

4.3.2 Antibodies and flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human CD3, Vα24Jα18 (clone 6B11), IL-4, perforin, granzyme, CD19, CD14, CD1d, CD45, CD107a, IFN-\gamma, PI9, Annexin V, the dye PI and death cell stain (eFlour-506) were used (Table 2.10).

4.3.3 Chemotherapy drugs

The commonly used chemotherapy drugs for upper GI cancer, cisplatin, 5-FU, carboplatin and paclitaxel were purchased from Sigma-Aldrich (UK). Stocks were made following manufacturer’s instructions. Cisplatin was
reconstituted in 0.15 M NaCl to a stock concentration of 3.3 mM. 5-FU was reconstituted in DMSO to a concentration of the stock of 100 mM. Paclitaxel was reconstituted in DMSO to a concentration of the stock of 10 mM. Carboplatin was resuspended in distilled water to a concentration of the stock of 100 mM.

4.3.4 MTT cell viability assay

The concentrations of drugs used to treat cells were based on the half maximal inhibitory concentration (IC\textsubscript{50}) of iNKT cells. IC\textsubscript{50} was calculated using the MTT cell viability assay as described in section 2.2.11. The IC\textsubscript{50} of iNKT cells after treatment for 48 h was 42.63 μM for cisplatin, 243.3 μM for 5-FU, 62.08 μM for carboplatin and 54.74 μM for paclitaxel.

4.3.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6.0 (GraphPad Prism, San Diego, California). \( P \) values between groups were obtained using the paired \( t \) test. \( P \) values of <0.05 (*) were considered statistically significant.

4.4 Results

4.4.1 Significantly decreased CD1d surface expression on upper GI cancer cells following cisplatin treatment

The effect of chemotherapy treatment on CD1d expression by B cells and monocytes within PBMC from 5 healthy donors, and oesophageal cancer
cells (OE33 cells) was assessed by flow cytometric analysis (Figure 4.1A), to elucidate whether these first line chemotherapies might effect iNKT cell activation. Treatment with cisplatin resulted in a dose-dependent downregulation of CD1d on OE33 cells (\( p=0.01 \). Figure 4.1B). No significant differences were observed in the MFI of CD1d in cisplatin-treated B cells (Figure 4.1C) or monocytes (Figure 4.1D) compared to untreated control samples. No significant differences were observed in CD1d expression by OE33 cells, B cells or monocytes after treatment with 5-FU (Figure 4.2A), carboplatin (Figure 4.2B) or paclitaxel (Figure 4.2C). These data suggest that cisplatin makes OE33 cells less capable of presenting lipid antigens through the downregulation of CD1d, making them less likely to be recognized and killed by iNKT cells.

4.4.2 Chemotherapies induce apoptosis in human iNKT cells in vitro.

Next, the effect of the clinically relevant chemotherapy drugs used in the treatment of upper GI cancer on iNKT cell survival and functions was determined. Expanded iNKT cell lines or OE33 cells were cultured in the absence or presence of cisplatin, 5-FU, carboplatin or paclitaxel for 24 or 48 h and cell viability was measured by MTT assay to obtain the IC\(_{50}\) of the drugs. OE33 cells were used to compare sensitivities between transformed epithelial cells and iNKT cells, as previous studies from our group showed that OE33 cells are sensitive to the different chemotherapy drugs. Based on doses previously reported, three doses of drug were tested to calculate the IC\(_{50}\) (Barr et al. 2013). These concentrations span
the range of plasma concentrations of the drugs found in patients, measured in a number of publications, which vary according to dose and time after administration. Cisplatin at a concentration of 100 µM reduced iNKT cell viability by 66±7.4% (p=0.01) and 62±3.5% (p=0.003) after 24 and 48 h respectively, whereas OE33 cell viability was reduced by 53±2.01% (p=0.001) and 67.6±12.4% (p=0.1) after 24 or 48 h. 1000 µM cisplatin reduced iNKT cell viability by 70±5.02% (p=0.02) and OE33 cell viability by 75±1.5% and 91±1.8% (p=0.003) after 24 and 48 h (Figure 4.3A and B). In contrast, only the highest doses of 5-FU (46.9±4.8%, p=0.01. Figure 4.3C), carboplatin (40±11.1%, p=0.04. Figure 4.3F) and paclitaxel (39±2.2%, p=0.003. Figure 4.3H) significantly decreased cell viability of iNKT cells, whereas a reduction of 77±7.9% (p=0.009) with 5-FU (Figure 4.3D), 89±2.6% (p=0.0009) with carboplatin (Figure 4.3G) and 69±7.8% (p=0.01) with paclitaxel (Figure 4.3I) were observed in OE33 cells after 48 h. The IC_{50} of iNKT cells obtained after treatment with cisplatin, 5-FU, carboplatin and paclitaxel were 42.63 µM, 243.3 µM, 62.08 µM and 54.74 µM. Viability was not altered by the drug vehicles NaCl and DMSO.
Figure 4.1. **Significantly decreased CD1d surface expression on upper GI cancer cells following cisplatin treatment.** The effect of cisplatin on CD1d expression was tested in OE33 OAC cells, and B cells and monocytes from 5 healthy donors. Isolated PBMCs or OE33 cells were treated with two doses of cisplatin (10 μM and 100 μM) for 24 and 48 h. Cells were stained for mAbs specific for CD1d, CD19, CD3 and CD14. CD1d expression was analysed by flow cytometry. 

A) Representative plot of CD1d expression in untreated and cisplatin-treated OE33 cells. 

B) Graphs show mean (± SEM) MFI of CD1d after 24 or 48 h treatment with cisplatin on OE33 cells (B), B cells (CD19^+CD3^−; C) and monocytes (CD14^+; D). (*p<0.05 using the paired t test compared to untreated).
Figure 4.2. CD1d surface expression by B cells, monocytes and OE33 cells was not altered after treatment with 5-FU, carboplatin or paclitaxel. The effect of 5-FU, carboplatin and paclitaxel on CD1d expression was tested in OE33 OAC cells, and B cells and monocytes from 5 healthy donors. Isolated PBMCs or OE33 cells were treated with two doses of the drugs (5 μM-100 μM) for 24 and 48 h. Cells were stained for mAbs specific for CD1d, CD19, CD3 and CD14. CD1d expression was analysed by flow cytometry. A-C) Graphs show mean (± SEM) MFI of CD1d after 24 or 48 h treatment with 5-FU (A), carboplatin (B) and paclitaxel (C) on OE33 cells, B cells (CD19+CD3-) and monocytes (CD14+).
To test if cell division altered cytotoxicity by the drugs, iNKT cells were treated with PHA-P (2 μg/ml), a lectin that binds to the membrane of T cells and stimulates cell division (Movafagh et al. 2011). Only the highest dose of carboplatin showed a significant difference in viability between treated iNKT cells in the absence or presence of PHA-P ($p=0.04$. Figure 4.4C). Since no significant difference was observed in cell viability, after treatment in the presence or absence of PHA-P, from now on experiments were carried out in the absence of PHA-P.

The induction of iNKT cell apoptosis by the chemotherapy drugs was also examined by staining the cells for the apoptotic marker Annexin V and marker of necrosis PI and analysed by flow cytometry (Figure 4.5A). Figure 4.5C and D show apoptosis (Annexin V‘PI’ cells) of 58.9±7.0% ($p=0.0004$) of iNKT cells and 50±8.02% ($p=0.03$) of OE33 cells after 24 h treatment with 100 μM cisplatin. No significant difference was observed in the percentage of apoptotic iNKT cells between untreated and treated with 100 μM cisplatin after 48 h. Only the highest dose of 5-FU (15.8±4.6%, $p=0.04$. Figure 4.5E) and carboplatin (20.7±8.7%, $p=0.05$. Figure 4.5G) resulted in a significant increase in apoptotic iNKT cells after 48 h. Apoptotic OE33 cell frequencies increased over time, when treated with cisplatin (31±5.4%, $p=0.06$. Figure 4.5D), 5-FU (34±5.4%, $p=0.03$. Figure 4.5F), carboplatin (35±5.0%, $p=0.03$. Figure 4.5H) and paclitaxel (41±6.8%, $p=0.0286$. Figure 4.5J).
Chemotherapies reduce the viability of human iNKT cells in vitro. Lines of expanded iNKT cells and OE33 cells were cultured for 24 or 48 h with medium alone or 10-1000 μM of cisplatin, 10-1000 μM 5-FU, 10-500 μM carboplatin or 5-50 μM paclitaxel. Cell viability was calculated by MTT assay. Results show mean (± SEM) percentages of iNKT cells (A, C, F, H; n=3) and OE33 cells (B, D, G, I; n=3) that remained viable after incubation for 24 and 48 h with cisplatin (A-B), 5-FU (C-D), carboplatin (F-G) and paclitaxel (H-I). (*p<0.05, **p<0.01, ***p<0.001 using the paired t test compared to untreated at the same time point).
Figure 4.4. PHA-P did not alter iNKT cell sensitivity to chemotherapy drugs. Lines of expanded iNKT cells from three donors were cultured for 24 or 48 h with medium alone or 10-1000 μM of cisplatin, 10-1000 μM 5-FU, 10-500 μM carboplatin or 5-50 μM paclitaxel in the presence of absence of PHA-P. Cell viability was calculated by MTT assay. Results show mean (± SEM) percentages of iNKT cells that remained viable after incubation for 24 and 48 h with cisplatin (A-B), 5-FU (C-D), carboplatin (F-G) and paclitaxel (H-I). (*p<0.05 using the paired t test compared between presence or absence of PHA-P).
An increase of 11.23±2.2% (p=0.02) in cell death, identified as double positive for Annexin V and PI, was observed for iNKT cells after 48 h treatment with 10 µM cisplatin, and 26.1±3.0% (p=0.002) and 64±8.8% (p=0.002) when treated with 100 µM cisplatin for 24 and 48 h (Figure 4.6A), but this increase in cell death was not observed in OE33 cells (Figure 4.6B). 10% (p=0.4) of dead OE33 cells were observed with 100 µM cisplatin after 24 and 48 h. No significant killing of iNKT cells or OE33 cells was observed after treatment with 5-FU (Figure 4.6C and D), carboplatin (Figure 4.6E and F) or paclitaxel (Figure 4.6G and H).

A non-significant dose dependant increase of necrotic iNKT cells (Annexin V-PI+) was observed when treated with cisplatin (Figure 4.7A), 5-FU (Figure 4.7C), carboplatin (Figure 4.7E) and paclitaxel (Figure 4.7G). 14.5±2.4% (p=0.007) of OE33 cells were necrotic after 48 h of treatment with 100 µM cisplatin (Figure 4.7B) and 12.4±3.3% with same dose of 5-FU (Figure 4.7D).
Figure 4.5. Chemotherapies induce apoptosis in human iNKT cells in vitro. Lines of expanded iNKT cells or OE33 cells were cultured for 24 or 48 h with medium alone or 10-100 μM of cisplatin, 10-100 μM 5-FU, 10-50 μM carboplatin or 5-50 μM paclitaxel. A-B) iNKT cells (A) and OE33 cells (B) were then stained with Annexin V and PI and analysed by flow cytometry. C-J) Graphs show mean (± SEM) percentage of apoptotic iNKT or OE33 cells (n=5) after 24 or 48 h treatment with cisplatin (C-D), 5-FU (E-F), carboplatin (G-H) and paclitaxel (I-J). (*p<0.05, **p<0.01, ***p<0.001 using the paired t test compared to untreated).
Figure 4.6. Cisplatin kills human iNKT cells in vitro. Lines of expanded iNKT cells or OE33 cells were cultured for 24 or 48 h with medium alone or 10-100 μM of cisplatin, 10-100 μM 5-FU, 10-50 μM carboplatin or 5-50 μM paclitaxel. iNKT cells and OE33 cells were then stained with Annexin V and PI and analysed by flow cytometry. (A-H) Graphs show mean (± SEM) percentage of dead iNKT or OE33 cells, as detected by positivity for Annexin V and PI, after 24 or 48 h treatment with cisplatin (A-B), 5-FU (C-D), carboplatin (E-F) and paclitaxel (G-H) (n=5). (*p<0.05, **p<0.01 using the paired t test compared to untreated).
iNKT cell proliferation after treatment with cisplatin was tested using Cell Trace Violet and analysed by flow cytometry (Figure 4.8A). Cells were treated with 10 or 100 µM of cisplatin. 24 or 48 h later cells were washed, and cells were stained with cell trace violet for 3 days. Since cells had been re-stimulated 3 to 4 weeks prior the experiment, 80.7% of cells were already proliferating in the absence of PHA-P. No difference in proliferative cells was observed when cells were treated with 10 µM of cisplatin for 24 h, 3 days after removing the drug. When cells were treated for 48 h, only 47.4% ($p=0.04$) of the cells were proliferating 3 days after removing the drug. This effect was rescued with PHA-P, when cells were incubated with cisplatin and PHA-P, up to 90.4% ($p=0.04$) of the cells proliferated (Figure 4.8B). All cell treated with cisplatin 100 µM were dead at this point.
Figure 4.7. Cisplatin and 5-FU induce necrosis in OE33 cells, but not iNKT cells in vitro. Lines of expanded iNKT cells or OE33 cells were cultured for 24 or 48 h with medium alone or 10–100 μM of cisplatin, 10–100 μM 5-FU, 10–50 μM carboplatin or 5–50 μM paclitaxel. iNKT cells and OE33 cells were then stained with Annexin V and PI and analysed by flow cytometry. A-H) Graphs show mean (± SEM) percentages of necrotic iNKT cells or OE33 cells detected as Annexin V/PI+ after 24 or 48 h treatment with cisplatin (A-B), 5-FU (C-D), carboplatin (E-F) and paclitaxel (G-H) (n=5). (*p<0.05, **p<0.01 using the paired t test compared to untreated).
Figure 4.8. INKT cell proliferation was reduced in the presence of cisplatin but rescued by PHA-P. A) Lines of INKT cells were treated with cisplatin for 24 and 48 h, after this time drug was removed, and cells were allowed to recover for three days, proliferation of the surviving cells was measured by Cell Trace Violet assay by flow cytometry. B) Graphs show mean (± SEM) percentages of proliferative cells after treatment with cisplatin, in the presence or absence of PHA-P (n=5). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 using the paired t test compared to untreated).
4.4.3 Chemotherapies significantly inhibit cytolytic degranulation by viable iNKT cells

The above experiments indicate that cisplatin, 5-FU, carboplatin and paclitaxel induce apoptosis in a proportion of iNKT cells. The effect of these drugs on the function of the remaining viable iNKT cells was investigated. Cell degranulation was measured by treating lines of iNKT cells with medium alone, cisplatin (10 or 100 μM), 5-FU (10 or 100 μM), carboplatin (10 or 50 μM) or paclitaxel (5 or 50 μM) for 24 and 48 h then co-cultured for 4 h with CD1d-transfected HeLa cells or C1R cells that were previously pulsed with 100 ng/mL α-GalCer, and staining for CD107a and analysed by flow cytometry (Figure 4.9A). Whereas less than 10% of unstimulated iNKT cells expressed extracellular CD107a, a mean of 45.4±9.0% expressed CD107a expression after stimulation with α-GalCer (Figure 4.9). Expression of CD107a by viable iNKT cells was significantly reduced to unstimulated levels after treatment with 100 μM cisplatin for 24 and 48 h (6.7±5.6%, p=0.03. Figure 4.9B). 100 μM 5-FU significantly reduced CD107a expression by viable iNKT cells to 31±9.6% (p=0.003) and 25±8.1% (p=0.006) when treated for 24 and 48 h, respectively (Figure 4.9C). 5μM paclitaxel significantly reduced CD107a expression to 36.7±9.0% (p=0.04) after 24 h, and 50 μM to 37±6.1% (p=0.05) and 31.4±8.8% (p=0.002) when cells were treated for 24 and 48 h (Figure 4.9E). Treatment with carboplatin did not alter CD107a expression (Figure 4.9D). Degranulation was not altered by the drug vehicles NaCl or DMSO.
No degranulation was observed in the presence of the drugs in the absence of α-GalCer.

Direct cytotoxicity by iNKT cells after treatment with the platinum analogues cisplatin and carboplatin was also examined using the Total Cytotoxicity and Apoptosis Detection Kit. The percentage of CD1d-transfected C1R cells specifically lysed by α-GalCer stimulated iNKT cells was 86.4%. No significant difference was observed in the percentage of specific lysis of target cells by viable iNKT cells after the different chemotherapy treatments, was observed when compared to the untreated control (Figure 4.10B). This suggests that even though cytolytic degranulation was reduced, cytotoxic function of the cells is still intact.

4.4.4 Granzyme B and perforin production by iNKT cells is increased following cisplatin treatment, but not following 5-FU, carboplatin or paclitaxel treatment.

Expanded iNKT cell lines were treated with medium alone, cisplatin (10 or 100 μM), 5-FU (10 or 100 μM), carboplatin (10 or 50 μM), or paclitaxel (5 or 50 μM) for 24 or 48 h, and co-cultured for 4 h with equal numbers of CD1d-transfected HeLa cells, previously loaded with medium alone or 100 ng/mL α-GalCer. The proportions of iNKT cells producing granzyme B (Figure 4.11A) and perforin (Figure 4.12A) were quantified by flow cytometry. Up to 16% of iNKT cells produced granzyme B and 17% expressed perforin in the presence of CD1d-transfected HeLa cells pulsed
Figure 4.9. Cisplatin, 5-FU and paclitaxel, but not carboplatin inhibit cytolytic degranulation by viable iNKT cells in vitro. Lines of expanded iNKT cells were cultured for 24 or 48 h with medium alone or 10-100 μM cisplatin, 10-100 μM 5-FU, 10-50 μM carboplatin or 5-50 μM paclitaxel. Cells were then co-cultured for 4 h with CD1d transfected HeLa cells, previously pulsed with α-GalCer. A) Cells were then stained with mAbs specific for CD107a, CD3, CD45 and Vα24-Jα18, and analysed by flow cytometry. B-E) Graphs showing mean (± SEM) percentages of viable iNKT cells from 5 donors expressing cell-surface CD107a after 24 or 48 h culture with cisplatin (B), 5-FU (C), carboplatin (D) or paclitaxel (E). (*p<0.05, **p<0.01, using the paired t test).
Figure 4.10. Cisplatin and carboplatin do not inhibit cytotoxicity of iNKT cells. To test direct killing by iNKT cells after treatment, iNKT cells were co-cultured for 5 h with CD1d transfected C1R target cells, previously pulsed with α-GalCer in three E:T ratio (25:1, 10:1, 1:1). A) Target cells were stained with CFSE, apoptotic cells were positive for Annexin V and dead cells for 7-AAD by flow cytometry. B) Graph showing mean (± SEM) percentages of specific lysis of CD1d-transfected C1R cells by iNKT cells. Results are means of 5 independent experiments.
with α-GalCer. After treatment with 100 µM cisplatin, viable iNKT cells exhibited a significant increase of granzyme B expression 24 h (27±4.0%, \( p=0.03 \)) and 48 h (38.9±4.0%, \( p=0.02 \). Figure 4.11B) and an increase in perforin expression (33.9±5.5%, \( p=0.001 \). Figure 4.12B) 24 h after treatment, compared to cells stimulated with α-GalCer in the absence of cisplatin. This increase in granzyme B expression in response to cisplatin treatment was later confirmed by immunofluorescence analysis (Figure 4.13A). An increased in fluorescence intensity on iNKT cells was observed after 24 h treatment with 100 µM cisplatin (\( p=0.06 \) when compared with α-GalCer stimulation alone, Figure 4.13B). No significant difference in granzyme B or perforin production by activated iNKT cells were observed when treated with 5-FU (Figure 4.11C and Figure 4.12C), carboplatin (Figure 4.11D and Figure 4.12D) and paclitaxel (Figure 4.11E and Figure 4.12E). The effect of cisplatin treatment on PI9 expression, a serine protease inhibitor that protects NK, CD8⁺ T cells and NKT cells from self-damage by granzyme B (Ansari et al. 2010) was investigated. Expression of the intracellular serine protease inhibitor of granzyme B, PI9 after treatment with cisplatin was measured by flow cytometry (Figure 4.14A). While there was no difference in PI-9 expression following treatment with 10 µM cisplatin, a significant reduction of 10% (\( p=0.0394 \)) in PI-9 expression by iNKT cells was observed following treatment with 100 µM cisplatin (Figure 4.14B). These results combined with the reduction observed on CD107a expression suggest that cisplatin-, 5-FU-, carboplatin- and paclitaxel-treated iNKT cells are still capable of producing
granzyme B and perforin but are unable to release them. The reduction in PI9 expression suggests that iNKT cells are being killed by the increased granzyme B production and the inability to protect themselves through PI-9 from self-damage by granzyme B. Granzyme B and perforin expression were not altered by the drug vehicles NaCl or DMSO. No degranulation was observed in the presence of the drugs in the absence of α-GalCer.
Figure 4.11. Cisplatin increases granzyme B production by viable iNKT cells in vitro. Lines of expanded iNKT cells were cultured for 24 or 48 h with medium alone or 10-100 μM cisplatin, 5-FU, 10-50 μM carboplatin or 5-50 μM paclitaxel. Cells were co-cultured with CD1d transfected HeLa cells, previously pulsed with α-GalCer or medium. A) Cells were then stained with mAb specific for granzyme B, CD3, CD45 and Vα24Jα18, and analysed by flow cytometry. B-E) Graphs showing mean (± SEM) percentages of viable iNKT cells from 5 donors producing granzyme B after 24 or 48 h treatment with cisplatin (B), 5-FU (C), carboplatin (D) or paclitaxel (E). (*p<0.05 using the paired t test).
Cisplatin increases perforin expression by viable iNKT cells in vitro. Lines of expanded iNKT cells were cultured for 24 or 48 h with medium alone or 10-100 μM cisplatin, S-FU, 10-50 μM carboplatin or 5-50 μM paclitaxel. Cells were then co-cultured with CD1d transfected HeLa cells, previously pulsed with α-GalCer. A) Cells were then stained with granzyme B, perforin, CD3, CD45 and Va24α24, and analysed by flow cytometry. B-E) Graphs showing mean (± SEM) percentages of viable iNKT cells producing perforin after 24 or 48 h treatment with cisplatin (B), S-FU (C), carboplatin (D) or paclitaxel (E) (n=5). (**p<0.01 using the paired t test).
Figure 4.13. Increase in granzyme B expression by iNKT cells by microscopy after treatment with cisplatin. Cells were treated for 24 h with cisplatin and stained for immunofluorescence microscopy. A) Representative immunofluorescence images, blue: nuclei, green: CD45, red: granzyme B. B) Graph showing fluorescence intensity of granzyme B on iNKT cells after 24 h treatment with cisplatin. (*p<0.05 using the paired t test).
Figure 4.14. Cisplatin reduces the intracellular serine protease inhibitor of granzyme B, PI9 on iNKT cells in vitro. A) iNKT cells were treated with 10-100 μM cisplatin and PI9 expression was measured by flow cytometry. B) Graphs showing mean (± SEM) percentages of viable iNKT cells expressing PI9 (n=5). (*p<0.05 using the paired t-test).
4.4.5 Chemotherapies significantly reduce IFN-γ production by viable iNKT cells

We next investigated if these chemotherapy drugs affected cytokine production by viable iNKT cells. Lines of iNKT cells were treated with medium alone, cisplatin (10 or 100 μM), 5-FU (10 or 100 μM), carboplatin (10 or 50 μM) or paclitaxel (5 or 50 μM) for 24 and 48 h then co-cultured for 4 h with CD1d-transfected HeLa cells that were previously pulsed with 100 ng/mL α-GalCer. The proportions of viable iNKT cells producing intracellular IFN-γ and IL-4 were quantified by flow cytometry (Figure 4.15A). CD1d transfected HeLa cells pulsed with α-GalCer induced significant IFN-γ expression by iNKT cells (42.6±7.6%). A significant inhibition of IFN-γ expression by stimulated viable iNKT cells after 24 h (5.2±2.0%, p=0.007) and 48 h (4.1±1.5%, p=0.006) treatment with 100 μM cisplatin (Figure 4.15B) was observed. No significant decrease in IFN-γ expression by viable iNKT cells was observed following 5-FU (Figure 4.15C), carboplatin (Figure 4.15D) or paclitaxel (Figure 4.15E) treatment when compared with untreated iNKT cells. IL-4 was not significantly altered after treatment with cisplatin, 5-FU, carboplatin or paclitaxel (Figure 4.16). Intracellular cytokine production was not altered by the drug vehicles NaCl or DMSO. No cytokine production was observed in the presence of the drugs in the absence of α-GalCer. This result suggests that cisplatin inhibits Th1 production, but not Th2 cytokines by iNKT cells, which might result in inhibition of the antitumour response.
iNKT cells treated for 24 or 48 h with 10 μM cisplatin were washed and left to recover for 3 days to investigate if cytotoxicity and cytokine production remained the same after treatment. No difference in CD107a, IFN-γ, granzyme B and perforin was observed between iNKT cells stimulated and treated with 10 μM of cisplatin for 24 and 48 h (Figure 4.17). However, when iNKT cells were treated with 100 μM of cisplatin all iNKT cells were dead by 72 h.

4.5 Discussion

Cui et al. first reported that iNKT cell-deficient mice were unable to mediate tumour rejection and that iNKT cells have an essential role in IL-12-mediated rejection of tumours (Cui et al. 1997). Several phase I trials in humans using i.v. injection of α-GalCer, administration of APCs pulsed with α-GalCer and transfer of ex vivo expanded and activated iNKT cells have shown little therapeutic benefit (Ishikawa et al. 2005; Motohashi et al. 2009; Nieda et al. 2004; Crowe et al. 2003). Recent studies have reported that i.v. injections of α-GalCer-pulsed APCs stimulate anti-tumour responses at the tumour site and at sites of metastasis, where half of the patients showed disease stabilisation or reduction in their tumour mass (Nicol, Tazbirkova, and Nieda 2011). These findings suggest that α-GalCer-pulsed APCs are a possible therapeutic strategy to improve anti-tumour immunity. The combination of transfer of in vitro-activated iNKT cells and α-GalCer-pulsed DCs has been reported to induce anti-tumour immunity in patients with head and neck squamous
Figure 4.15. **Cisplatin inhibits IFN-γ production by viable iNKT cells in vitro.** Lines of expanded iNKT cells were cultured for 24 or 48 h with medium alone or cisplatin, 5-FU, carboplatin or paclitaxel. Cells were then co-cultured for 4 h with CD1d transfected HeLa cells, previously pulsed with α-GalCer. A) Cells were then stained with mAbs specific for cell-surface CD3, CD45 and Vα24Jα18, intracellular IFN-γ and analysed by flow cytometry. B-E) Graph show mean (± SEM) percentages of viable iNKT cells that produced IFN-γ after 24 and 48 h treatment with cisplatin (B), 5-FU (C), carboplatin (D) and paclitaxel (E). Results are means of 5 independent experiments (**p<0.01, using the paired t test compared to stimulated with α-GalCer).
Figure 4.16. *Chemotherapy drugs do not affect IL-4 expression by viable iNKT cells in vitro.* Expanded iNKT cell lines were cultured for 24 or 48 h with medium alone or cisplatin, 5-FU, carboplatin or paclitaxel. Cells were co-cultured with CD1d transfected HeLa cells, previously loaded with α-GalCer. Cells were then stained with mAbs specific for cell-surface CD3, CD45 and Vα24Jα18 and intracellular IL-4 and analysed by flow cytometry. A-D) Graphs show mean (± SEM) percentages of viable iNKT cells that produced IL-4 after 24 and 48 h culture with cisplatin (A), 5-FU (B), carboplatin (C) or paclitaxel (D). Results are means of 5 independent experiments.
Figure 4.17. Three days after treatment with 10µM cisplatin iNKT cells still degranulate, produce IFN-γ, granzyme B and perforin. Lines of iNKT cells were treated with cisplatin for 24 and 48 h, after this time drug was removed, and cells were left to recover for three days, cells were stained with mAb for CD3, Vα24Jα18, CD107a, IFN-γ, granzyme B and perforin, and analysed flow cytometry. A-D) Graphs show mean (± SEM) percentages of iNKT cells expressing CD107a (A), IFN-γ (B), granzyme B (C), perforin (D). (*p<0.05, **p<0.01, using the paired t test compared to stimulated with α-GalCer).
cell carcinomas (Yamasaki et al. 2011; Kunii et al. 2009). Recently, a phase 1 clinical trial carried out by Exley et al. showed the potential anti-tumour effects of iNKT cells in 9 patients with advanced melanoma. Evidence of T cell and myeloid cell activation was observed. Three of the patients were progression-free at 58, 60 and 65 months (Exley et al. 2017). This proves that iNKT cells have an important role in anti-tumour therapeutics, but further studies and optimisation are required.

We hypothesised that the failure of human clinical trials involving iNKT cells may be due to iNKT cell function being compromised by the chemotherapeutic regimens administrated to patients. The most common chemotherapy drugs used for OC and GAC are cisplatin, 5-FU, carboplatin and paclitaxel (Donohoe and Reynolds 2017). Chemotherapy in combination with radiotherapy helps to shrink the tumour prior to surgery and relieve some symptoms, but it does not clear the tumour in many cases. Due to the non-specific symptoms, tumours are frequently at an advanced stage at diagnosis, leaving a significant proportion of patients with no treatment options and as a result they are referred for palliative care (Russell 2016).

MHC class I molecules present peptides on the cell surface to T cells, leading to cytotoxic CD8+ T cell-activation (Ohtsukasa et al. 2003). Several studies have shown that cisplatin, 5-FU and paclitaxel can increase MHC class I expression on mouse and human tumour cells (Nio et al. 2000; Ohtsukasa et al. 2003; Wan et al. 2012; Gelbard et al. 2006; Gameiro,
The regulation of the MHC class I-like molecule, CD1d, is still largely unknown. We tested the effect of chemotherapy drugs on CD1d expression on immune and tumour cells. No effect was observed on CD1d expression on B cells or monocyte; however, cisplatin induced a dose-dependent downregulation of CD1d on an OAC cancer cell lines. These results may impact treatment design, since cisplatin induces a reduction in CD1d expression on tumour cells, the use of cisplatin as first line chemotherapy may not be optimal for use with iNKT based immunotherapies.

Conflicting results have been reported about the effect of chemotherapies on the immune system. While some report that 5-FU impairs NK cell functions, other showed higher numbers of infiltrating CD8+ T and NK cells with enhanced cytotoxicity in breast cancer patients (Sewell et al. 1993; Brenner and Margolese 1991; Wijayahadi et al. 2007). Others showed that paclitaxel inhibits human NK cell-mediated killing without affecting its viability, but are not altered with carboplatin and 5-FU (Markasz et al. 2007). Combination of carboplatin and paclitaxel lead to a decrease in numbers of CD4 and double positive T cells (Hatziveis et al. 2012). Cisplatin enhances the efficacy of NK cells to better suppress hepatocellular carcinoma (Shi et al. 2016), NSCLC in mice (Okita et al. 2016) and uterine endometrial cancer (Zhou 2017) by upregulating the NKG2D ligand, and enhances cytokine production by T cells in a murine melanoma model (Chen et al. 2012). Fallarini et al. found iNKT cell
treatment enhanced cisplatin-induce osteosarcoma cell death in a dose dependent manner (Fallarini et al. 2012). We describe for the first time the direct effect of chemotherapy on iNKT cell viability and function. A significant dose-dependent reduction in the numbers of viable iNKT cells was observed when expanded iNKT cells were cultured in the presence of cisplatin, 5-FU, carboplatin and paclitaxel. IFN-γ production was reduced to unstimulated levels after treatment with cisplatin, suggesting that cisplatin inhibits Th1 cytokine production by iNKT cells, but not Th2 cytokines which might result in the inhibition of anti-tumour responses, while promoting the tumour by the release of IL-4, IL-5 and IL-13 by inhibiting cell-mediated immunity and promoting angiogenesis (O'Reilly et al. 2011). The ability of iNKT to directly kill target cells was similar to those untreated activated with α-GalCer, suggesting that even though iNKT cells are unable to produce IFN-γ and activate other immune cells, their cytotoxic functions are still intact.

Conventional chemotherapy increases sensitivity to granzyme B and perforin mediated cell death via upregulation of mannose-6-phosphate receptors on the surface of tumour cells (Ramakrishnan et al. 2010). Interestingly, in contrast with the decrease of cytolytic degranulation by iNKT cells after treatment with cisplatin, iNKT cells expressed more perforin and granzyme B intracellularly. This suggests that iNKT cells can produce the cytotoxic molecules, granzyme B and perforin. Reduction of PI9 expression in the presence by cisplatin was then investigated. PI9 is a
serine protease inhibitor that protects NK and NKT cells from self-damage by granzyme B (Ansari et al. 2010). PI9 expression is induced by lipopolysaccharide, IFN-γ, and IL-1β, and enhanced by oestrogen and hypoxic conditions (Chowdhury and Lieberman 2009). Univariate analysis shows that absence of PI9 is associated with poorer outcomes in patients with NK/T-cell lymphoma (Bossard et al. 2007). A reduction of PI9 expression was observed after treatment with the highest dose of cisplatin, suggesting that iNKT cells are being killed by the increase in granzyme B production and the inability to protect themselves from self-damage by granzyme B.

In conclusion, upper GI cancers may be amenable to iNKT cell-based therapies. However, current treatment for OAC, SSC and GAC should be considered when developing iNKT cell-based immunotherapies.
Chapter 5

Radiotherapy for upper gastrointestinal cancers does not inhibit the antitumor activities of invariant natural killer T cells
5.1 Introduction

Ionising radiation is an important component of cancer treatment (Liauw, Connell, and Weichselbaum 2013). Radiotherapy affects cellular proliferation, morphology and induces cell death, leading to tumour shrinkage (Maier et al. 2016). Radiotherapy uses X-rays or gamma rays and the dose is expressed in Gray (Gy), which is the energy deposited per unit of mass in kilograms (Schlesinger et al. 2017). Radiation targets rapidly dividing cells, and results in DNA damage by causing single strand breaks (SSBs), double strand breaks (DSBs), sugar moiety modifications, and modifications to purine and pyrimidine bases, such as deaminated adducts (Redon et al. 2010; Aparicio, Baer, and Gautier 2014). When DNA is damaged, the cell undergoes cell cycle arrest, giving time to repair the damaged DNA. However, the failure to repair DSBs can lead to cell death and radiosensitivity correlates closely with DSB repair capability of the cell (Mladenov et al. 2013).

DNA double strand breaks (DSBs) are considered the most lethal event, since DSBs lead to apoptosis and cellular senescence (Eriksson and Stigbrand 2010; McMillan et al. 2001). Immediately after radiation with 1Gy, approximately 1000 SSBs, and 25 DBSs can be detected (Olive 1998). Radiation directly causes approximately 35% of the DNA damage, whereas the other 65% is caused by the creation of reactive oxygen species (ROS) (Figure 5.1) (Desouky, Ding, and Zhou 2015).
Patients with oesophageal cancer receive 23 fractions of 1.8Gy daily to a total of 41.4Gy (Donohoe and Reynolds 2017). Radiation is applied over several fractions over several weeks to reduce the normal cell toxicity (Bentzen 2006). Multiple fractionated doses increase tumour cell killing, and reducing normal tissue toxicity, by allowing reoxygenation, redistribution, repair and repopulation (Brown, Carlson, and Brenner 2014).

Radiation is generally thought to be an immunosuppressive agent, since most lymphocytes are very radiosensitive, however, it is subset specific (Schaué and McBride 2012). Radiation will have a direct cytotoxic effect on circulating lymphocytes, but the effect will depend on the tissue, size
of the field, delivery method and dose, and activation status of the cells. For instance, non-proliferative cells and activated lymphocytes are more radioresistant than rapidly dividing cells (MacLennan and Kay 1978; McBride et al. 2004).

Radiotherapy can also boost different aspects of the immune system. It causes the release of tumour antigens by damaged cells and the expression and release of DAMPs causing the activation of tumour antigen specific T cells, leading to an anti-tumour effect (Lee et al. 2009). Radiation enhances the expression of the co-stimulatory immune checkpoint CD137 and the inhibitory checkpoint PD-1 on T cells (Rodriguez-Ruiz et al. 2016), while also enhancing expression of the stimulatory T cell and NK cell ligands; MHC class I, NKG2D and NKp30 (Gasser et al. 2005; Matta et al. 2013; Kwilas et al. 2012; Rosental et al. 2012). Radiotherapy also induces trafficking of NK cells, Treg cells and CD11b⁺ cells into the tumour by the induction of the intracellular adhesion molecule I (ICAM-I) (Ni et al. 2012). Therefore, radiotherapy in combination with immunotherapy may offer a new strategy to overcoming some of the current limitations with immunotherapy.

In chapter 3, iNKT cells were shown to be depleted in patients with upper GI cancers, implying that immunotherapies involving these cells may benefit patients. However, the effect of radiation on human iNKT cells is unknown. It has been previously shown that murine iNKT cells are more
radioresistant than T cells and B cells *in vivo* (Kajioka et al. 2000). Here the effect of radiation on human iNKT cells was investigated.

### 5.2 Objectives

The aim of this chapter was to determine if therapeutic doses of X-ray radiation affect viability and function on iNKT cells *in vitro*.

The specific aims of this chapter were:

1. To compare the cytotoxic effects of radiation on iNKT cells, OE33 cells and OE19 cells.
2. To examine the ability of iNKT cells to repair DNA damage after radiation by the γH2AX expression a biomarker used to measure DNA damage.
3. To determine if radiation alters the cell cycle progression in iNKT cells, since the position and progression of the cycle will affect the radiosensitivity.
4. To examine cytotoxic and cytokine secretion activities of viable iNKT cells post radiation.

### 5.3 Specific methods

#### 5.3.1 Generation of iNKT cell lines

iNKT cell lines were isolated from PBMC from human buffy coat packs and sorted by magnetic beads and MoFloTM XDP Cell sorter and expanded for 2 weeks before use as described in section 0. Purity of the cell lines was at least of 92%.
5.3.2 Radiotherapy

iNKT cells were irradiated with X-rays using a biological irradiator RS 225 system (Gulman Medical, UK) or CIX2 Cabinet X-Ray (Xstrahl life science, UK). Cells were mock irradiated (0Gy), irradiated with single doses of 2Gy or 10Gy X-rays, or five cumulative 2Gy doses every 24 h, to reach a total of 10Gy.

5.3.3 Cell cycle arrest analysis by PI staining

Cell cycle arrest of the cells was measured with PI staining by flow cytometry. The ability of PI bind to the DNA makes it a good dye for cell cycle arrest stains. 1x10^5 iNKT cells were radiated with 0Gy, 2Gy, 10Gy or five fractionated doses of 2Gy. Cell cycle was measure 6 h, 10 h and 24 h post radiation.

5.3.4 Analysis of DNA damage by γH2AX detection

Double strand breaks were detected by γH2AX expression by iNKT cells. γH2AX is the first histone recruited after double strains breaks of the DNA form. γH2AX was detected 20 min, 6 h and 24 h post radiation with 0Gy, 2Gy or 10Gy, by immunofluorescence microscopy using an anti-γH2AX antibody.
5.3.5 Antibodies and flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human CD3, the Vα24Jα18 TCR used by iNKT cells (6B11), IL-4, perforin, 7-aminoactinomycin D (7-AAD), granzyme B, CD107a, IFN-γ, Annexin V, and PI and death cell stain (eFlour-506) were used. Flow cytometry was performed using a FACSCanto flow cytometer and FlowJo Version 10 (Tree Star, Ashland, Oregon) software was used for analysis (Table 2.10).

5.3.6 Statistical analysis

Statistical analysis was carried out using GraphPad Prism Version 6.0 (GraphPad Prism, San Diego, California). p values between groups were obtained using the paired t test. P values of <0.05 (*) were considered statistically significant.

5.4 Results

5.4.1 iNKT cell numbers decrease 5 days post single doses of radiation and after the third dose of fractionated doses.

The effect of therapeutic doses of radiation on iNKT survival and function was investigated. 1x10^5 iNKT cells were mock-irradiated (0Gy) or irradiated with single doses of 2Gy or 10Gy or five fractioned doses of 2Gy. iNKT cells were stimulated with PHA-P and incubated in the presence of feeder cells for 15 days. After 15 days, iNKT cells were irradiated with 2Gy, 10Gy or fractions of 2Gy and cells were counted every 24 h for up to 10
days after every treatment. Numbers of iNKT cells in the mock-irradiated group increased exponentially for 72 h (7.7±0.7x10^5 cells/mL, p=<0.001), cells were found to keep expanding to a lesser degree for up to 12 days (13.41±3.25x10^5 cells/mL, p=0.05 compared to 72 h). iNKT cells irradiated with single doses of 2Gy (5.7±2.2x10^5 cells/mL, p=<0.05 compared to baseline) or 10Gy (4.8±1.6x10^5 cells/mL, p=<0.05 compared to baseline) showed an increase in iNKT cell numbers for up to 96 h post irradiation. iNKT cell frequencies decreased on day 5 (3.1±0.2 cells/mL when irradiated with 2Gy and 2.8±0.79 cells/mL when irradiated with 10Gy). iNKT cells numbers decreased to 1.18±0.6x10^5 cells/mL (p=0.05) day 12 post radiation with 2Gy and 1.65±03x10^5 cells/mL (p=0.05) with 10Gy (Figure 5.2A). iNKT cells were also irradiated with clinically relevant five 2Gy fractionated doses. iNKT cell numbers showed a significant difference after the third dose of radiation (2.4±0.7x10^5 cells/mL) compared to control (7.7±1.05 cells/mL. p=0.009). 6 days post treatment with five fractionated doses (day 12) of radiation (0.8±0.32 cells/mL) significant lower frequencies of iNKT cells were noted compared to the controls (13.4±3.25 cells/mL, p=0.05) and to day 2 post treatment with five fractions of 2Gy (day 6, 3.7±0.85 cells/mL, p=0.05. Figure 5.2B). Considering that iNKT cells are innate T cells that rapidly produce cytokines after activation and that iNKT cells were still able to expand up to 96 h post radiation, 24 and 48 h timepoints were chosen for the rest of the experiments.
Figure 5.2 Radiation inhibits proliferation by iNKT cells in vitro. **A** Lines of iNKT cells from three different donors received a single dose of 0Gy, 2Gy or 10Gy radiation and were counted using EBAO every 24 h, for 9 days. **B** Expanded iNKT cell lines from three donors were irradiated with five fractionated doses of 2Gy and cells were counted after 24 h after every dose, and every 24 h for 6 days after the fifth accumulative dose. Media was replaced on day 2, day 5 and day 9. Graphs shows mean (± SEM) of numbers of iNKT cells. (*p < 0.05, **p < 0.01 using the paired t test comparing numbers of radiation iNKT cells to mock irradiated control on the same day. (*p < 0.05, **p < 0.001 using the one way-ANOVA test comparing iNKT cell numbers within the same treatment).
5.4.2 Radiotherapy induces apoptosis in iNKT cells \textit{in vitro}.

The effect of therapeutic doses of radiation on iNKT survival and function was investigated. Expanded iNKT cells, OE33 cells and OE19 cells were mock-irradiated (0Gy) or irradiated with single doses of 2Gy or 10Gy. OE33 cells and OE19 cells were used to compare sensitivities between transformed epithelial tumour cells and iNKT cells, as previous studies have reported that OE33 cells are more radiosensitive than OE19 cells (Hotte et al. 2012). Toxicity was examined by staining the cells with the apoptotic markers Annexin V and PI and analysed by flow cytometry 24 and 48 h after irradiation (Figure 5.3A). Figure 5.3B shows an increase in early apoptotic (Annexin V\(^+\) PI\(^-\) cells) iNKT cells 24 h (19.2±6.4\%, \(p=0.02\)) and 48 h (14.7±3.8\%, \(p=0.02\)) post radiation with 10Gy compared to 0Gy (1.4±0.3\%). Similar percentages of early apoptosis in OE19 cells was observed after 48 h with 2Gy (24.3±1.4\%, \(p=0.004\) compared to 7.9±1.0 when mock irradiated) and 10Gy (21.7±1.2\%, \(p=0.002\) compared to 0Gy. Figure 5.3C). Only 10Gy radiation induced apoptosis in OE33 cells after 24 h (22.3±1.7\%, \(p=0.003\)) and 48 h (32.8±1.6\%, \(p=0.0009\). Figure 5.3D).

No significant cell death (Annexin V\(^+\)PI\(^+\)) was observed in iNKT cells after radiation (Figure 5.3E). Figure 5.3F show an increase in cell death in OE19 cells 48 h after 10Gy treatment (15.1±1.5\%, \(p=0.03\) compared to 8.7±1.3 with 0Gy). Figure 5.3G shows an increase of cell death in OE33 cells 24 h (7.7±1\%, \(p=0.002\)) and 48 h (16.9±1.4\%, \(p=0.0009\) post 10Gy radiation compared to 0Gy (3.9±0.7%). No significant increase in necrotic iNKT cells
and OE19 cells was observed when radiated with 2Gy or 10Gy (Figure 5.3H and J). Radiation with 10Gy induced necrosis in 3.9±0.2% of OE33 cells 48 h post radiation (p=0.002) compared to 0Gy (1.5±0.3%, Figure 5.3J).

To analyse the cytotoxic effect of cumulative doses, cells were irradiated with five cumulative doses of 2Gy for 5 days. A dose dependent increase in apoptotic and dead cells was observed when iNKT cells were irradiated with fractionated doses of 2Gy. 23±5.5% of iNKT cells were early apoptotic 24 h after the fifth fractioned dose of 2Gy (p=0.01. Figure 5.4B) and 27.6±8.7% were dead (p=0.05 when treated with five doses of 2Gy Figure 5.4C). This suggests that iNKT cells cannot repair the cumulative DNA damage when irradiated for five consecutive days. No significant increase in necrotic iNKT cells was observed after the fifth fractionated dose of radiation.

Since radiation targets dividing cells, to confirm that iNKT cells were still expanding at the time of the experiments, iNKT cells were counted daily with EBAO. iNKT cells were still proliferating 30 days after restimulation (Figure 5.5).
Figure 5.3. Radiation induces human INKT cell death in vitro. A) Lines of expanded INKT cells from 6 healthy donors, OE33 cells and OE19 cells were mock irradiated (0Gy) or irradiated with single doses of 2Gy or 10Gy. INKT cells, OE33 cells and OE19 cells were then stained with Annexin V and PI and analysed by flow cytometry. B-J). Graphs show mean ± SEM percentages of early apoptotic (Annexin V+PI-) (B-D), dead (Annexin V+PI+) (E-J) and necrotic (Annexin V-PI+) cells (H-J) for INKT cells (B,E,H), OE19 cells (C,F,I) and OE33 cells (D,G,J) 24h or 48h after radiation. (*p<0.05, **p<0.01 using the paired t test compared to 0Gy).
Figure 5.4. Cumulative doses of radiation induce human iNKT cell death in vitro. Lines of expanded iNKT cells from 6 healthy donors were mock irradiated (0Gy) or irradiated with five cumulative fractions of 2Gy. A) iNKT cells were then stained with Annexin V and PI and analysed by flow cytometry. B-D) Graph shows mean (± SEM) percentages of early apoptotic iNKT cells (Annexin V+PI−, B), dead cells (Annexin V+PI+, C) and necrotic (Annexin V−PI+, D). Results are from 6 independent experiments (*p<0.05, **p<0.01 using the paired t test compared to 0Gy).
Figure 5.5. iNKT cells proliferated for up to 30 days after stimulation. iNKT cells from 5 different donors were isolated with magnetic beads and MoFloTM XDP Cell sorting and expanded with α-GalCer and feeders for 15 days. 15 days after stimulation, cells were counted using EBAO. Graph shows number of iNKT cells counted for five days and up to 30 days after stimulation. Cells received IL-2 on days 15, 19, 22, 26 and 30.
5.4.3 iNKT cells can repair double strain breaks in DNA 24 h after radiation.

Lines of expanded iNKT cells were mock irradiated (0Gy) or irradiated with 2Gy or 10Gy. γ-H2AX was used as a marker for double strand breaks in the DNA and analysed by microscopy (Figure 5.6A). Double strand breaks were counted 20 min, 6 and 24 h after radiation. γ-H2AX foci of at least 25 cells per treatment were counted. A significant increase in DNA double strand breaks was observed 20 min post radiation. An average of 16±2 foci (p=0.002) of γ-H2AX per cell were counted after 2Gy radiation and 30±3 (p=0.002) after 10Gy. Number of foci per cell was reduced, but still significantly higher 6 h after radiation (5±1 foci, p=0.009 when radiated with 2Gy and 12±2 foci, p=0.008 when radiated with 10Gy) compared to control (1±0.6 foci). An even further reduction in γ-H2AX foci was observed after 24 h, with no significant difference was observed with 2Gy radiation. 10±2 foci were counted (p=0.012) when radiated with 10Gy. iNKT cells irradiated with 2Gy were able to completely repair their DNA 24 h after radiation (Figure 5.6B). Double strand breaks in the DNA are considered the most lethal damage of the DNA, inability of cells to repair the damage leads to apoptosis and cell death. The inability of iNKT cells to repair double strand breaks mimics with the percentage of cell death observed when irradiated with 10Gy.
5.4.4  iNKT cell cycle was not altered 24 h post radiation with 10Gy.

Cell cycle can determine sensitivity of a cell to radiation. Cells in G2-M phase are more radiosensitive than cells in G1, and in S phase (Pawlik and Keyomarsi 2004). To determine if iNKT cells enter cell cycle arrest after radiation, iNKT cells were stained with PI and cell cycle arrest was analysed by flow cytometry (Figure 5.7A). PI passes the membrane and intercalates with the DNA (Shen, Vignali, and Wang 2017). The intensity of the PI signal is directly proportional to DNA content. Cell cycle arrest of expanded iNKT cell lines was tested 6, 10 and 24 h post radiation with 0, 2 and 10Gy and 24 h after every dose of the five 2Gy consecutive doses. Cell cycle was not altered after 6 h (Figure 5.7B) or 10 h (Figure 5.7C). In contrast, a decrease of 12.5±3.5% (p=0.02) of iNKT cells in G0/G1 was observed 24 h after 10Gy radiation (Figure 5.7D). This is reflected by an increase in apoptotic cells in SubG1. No difference was observed when cells were irradiated with five cumulative doses (Figure 5.7F).

5.4.5  Effects of radiation on cytolytic degranulation by viable iNKT cells

Lines of iNKT cells were mock-irradiated (0Gy), irradiated with single doses of 2Gy or 10Gy, or five cumulative doses of 2Gy. 24 and 48 h post radiation iNKT cells were co-cultured for 4h with CD1d-transfected HeLa cells previously pulsed with 100 ng/mL α-GalCer and cytotoxic potential of iNKT cells was measured by the expression of the degranulation marker CD107a expression by viable iNKT cells using flow cytometry (Figure 5.8A).
Figure 5.6. Radiation induces double strand breaks in iNKT cell DNA in vitro. Lines of expanded iNKT cells were irradiated with mock radiation (0Gy), single doses of 2Gy or 10Gy. 20 min, 6h or 24h later cells were stained with γ-H2AX a marker for double strand breaks in the DNA and analysed by microscopy. A) γ-H2AX is red and nuclei (blue) stained with DAPI. Original magnification 100X. B) Graph shows mean γ-H2AX foci per cell 20 min, 6 and 24h after radiation. Results are means of 25 cells from 5 independent experiments (*p<0.05, **p<0.01, using the paired t test compared to 0Gy).
Figure 5.7. Cell cycle of iNKT cells was altered 24 h after radiation with 10 Gy. A) Cell cycle arrest was assessed by PI staining by flow cytometry. Lines of expanded iNKT cells from 6 healthy donors were mock irradiated (0 Gy), given single 2 Gy or 10 Gy doses, or given five cumulative 2 Gy fractions. Cells were stained with PI after irradiation. B-E) Graphs show percentages of iNKT cells in different phases of the cell cycle 6 h (B), 10 h (C), or 24 h (D) after single doses of 2 Gy or 10 Gy or 24 h after five fractionated doses of 2 Gy (E). Results are means of 6 independent experiments (*p<0.05, using the paired t test compared to 0 Gy).
A significant increase in CD107a expression was observed between unstimulated and α-GalCer-stimulated cells (48.0±7.7%). No significant differences were observed in CD107a expression between stimulated non-irradiated and irradiated viable iNKT cells (Figure 5.8B). A significant decrease in CD107a expression was observed after the fifth fractioned dose of 2Gy when compared to control (34.3±5.0%, p=0.02. Figure 5.8C).

Direct cytotoxicity by iNKT cells after radiation was also examined using the total cytotoxicity and apoptosis detection kit by flow cytometry (Figure 5.9A). CD1d transfected-C1R cells pulsed with α-GalCer were used as target cells. iNKT cells were mock-irradiated (0Gy) or irradiated with 2Gy or 10Gy, and then co-cultured in three different effector:target ratios (25:1, 10:1, 1:1) for 5 h at 37°C.

CD1d-transfected C1R cell viability was reduced by 15% when co-cultured with iNKT cells prior to stimulation but the frequencies of viable target cells was more significantly decreased when α-GalCer was present, with stimulated iNKT cells directly killing 73% of CD1d-transfected C1R. No significant differences in the percentage of specific lysis of target cells by iNKT cells after radiation was observed when compared to the untreated control (Figure 5.9B) suggesting that this function of iNKT cells is still intact post radiation.
Figure 5.8. Radiation does not inhibit cytoltyic degranulation by viable iNKT cells in vitro. Lines of expanded iNKT cells were irradiated with 0Gy, single 2Gy or 10Gy doses, or five cumulative 2Gy fractions. 24 h and 48 h later, cells were co-cultured for 4h with CD1d transfected HeLa cells, previously pulsed with α-GalCer. A) Cells were then stained with a dead cells stain (labelled with eFlour-506) and mAbs specific for cell-surface CD3, Vα24Jα18 and CD107a and analysed by flow cytometry. B-C) Graphs show mean (± SEM) percentages of viable iNKT cells that expressed CD107a 24 and 48h after single doses (B) or fractioned doses (C) of radiation. (*p<0.05 using the paired t test compared to 0Gy irradiation in the presence α-GalCer).
Figure 5.9. Radiation does not alter total cytotoxicity by iNKT cells in vitro. A) Direct killing by iNKT cells after radiation was tested by co-culturing 0Gy, 2Gy or 10Gy irradiated iNKT cells with CD1d transfected C1R target cells previously pulsed with α-GalCer for 5 h. Target cells were stained with CFSE. Apoptotic cells were positive for Annexin V and dead cells for 7-AAD by flow cytometry. B) Graph showing mean (± SEM) percentages of specific lysis of CD1d-transfected C1Rs by iNKT cells. Results are means of 6 independent experiments.
5.4.6 iNKT cell production of the cytotoxic molecule perforin, but not granzyme B, was increased following radiation.

Lines of iNKT cells were mock-irradiated (0Gy) or irradiated with single doses of 2Gy or 10Gy. 24 or 48 h after irradiation, the iNKT cells were co-cultured for 4h with equal numbers of CD1d-HeLa cells, previously loaded with medium alone or 100 ng/mL α-GalCer. The production of intracellular granzyme B (Figure 5.10A) and perforin (Figure 5.10B) were measured by flow cytometry. No significant differences were observed in granzyme B production between mock-irradiated, 2Gy or 10Gy radiation after stimulation (Figure 5.10C). Perforin was significantly increased 48 h after radiation with 2Gy (33.8±5.3%, \( p=0.01 \)) and 10Gy (32.5±6.5%, \( p=0.05 \)) compared to control (16.7±5.7%. Figure 5.10D). These results further show that viable iNKT cells post radiation have retained the potential to kill target cells.
Figure 5.10. **Perforin expression by viable INKT cells in vitro increased 48 h after radiation.** Lines of expanded INKT cells from 6 healthy donors were cultured with 2Gy, 10Gy or mock radiation (0Gy) for 24 or 48 h. Cells were then co-cultured with CD1d transfected HeLa cells, previously pulsed with α-GalCer. A-B) Cells were then stained with dead cells stain (labelled with eFlour-506) and mAb specific for granzyme B (A), perforin (B), CD3, and Vα24-Jα18 and analysed by flow cytometry. C-D) Graphs show mean (± SEM) percentages of viable iNKT cells from 6 donors that produced granzyme B (C) and perforin (D) 24 h or 48 h after radiation. (*p<0.05 using the paired t test compared to 0Gy plus α-GalCer).
5.4.7 IFN-γ expression by iNKT cells was inhibited by radiotherapy.

iNKT cell lines were exposed to 0, 2 or 10 Gy radiation or five fractioned doses of 2 Gy. Cells were then co-cultured with CD1d transfected HeLa cells previously loaded with 100 ng/mL of αGalCer. The proportions of viable iNKT cells expressing intracellular IFN-γ and IL-4 was quantified by flow cytometry (Figure 5.11A). CD1d-HeLa cells pulsed with α-GalCer induced 58±7.3% of iNKT cells to express IFN-γ and 56.1±14.2% IL-4. No significant decrease in IFN-γ and IL-4 expression by viable iNKT cells was observed following single doses of radiation (Figure 5.11B and C) and in IL-4 expression after the cumulative doses of 2 Gy (Figure 5.11E). However, a significant inhibition of IFN-γ, but not IL-4, expression by stimulated viable iNKT cells was observed after every dose of the cumulative regimen, reaching the lowest point 24 h after the fifth fractioned dose (10.5±3.7% p=0.0003. Figure 5.11C).

5.5 Discussion

Radiotherapy is an essential element in the treatment program for upper GI cancers, with approximately two-thirds of all cancer patients receiving radiotherapy at some point during the treatment of the malignancy (Guo et al. 2013). Radiation targets rapidly dividing cells. It not only kills tumour cells, but also normal tissues including immune cells within the target field (Barnett et al. 2009).

A few studies have been carried out enumerating iNKT cells in peripheral blood of cancer patients after radiation. Crough et al. demonstrated that
Figure 5.11. Radiation decreased IFN-γ expression by viable iNKT cells in vitro. Lines of expanded iNKT cells were exposed for 24 or 48 h to mock radiation (0Gy), single doses of 2Gy, 10Gy or five cumulative doses of 2Gy. Cells were then co-cultured with CD1d transfected HeLa cells, previously pulsed with α-GalCer. A) Cells were then stained with dead cells stain (labelled with eFlour-506) and mAbs specific for cell surface CD3 and Vα24Jα18 and intracellular IFN-γ and IL-4 and analysed by flow cytometry. B-E) Results show mean (± SEM) percentages of viable iNKT cells (n=6) producing IFN-γ or IL-4 after single doses of 2Gy or 10Gy (B-C) or five cumulative doses of 2Gy (D-E) after 24 and 48h radiation. (*p<0.05, **p<0.01, ***p<0.001, using the paired t test compared to 0Gy plus α-GalCer).
the frequencies of iNKT cells, but not conventional T cells, were similar in patients with different solid tumours such as, colorectal, breast, melanoma, lung, renal cell carcinoma with prior history of radiation and no radiation history (Crough et al. 2004). Other studies quantified iNKT and CD3+ T cells in patients with advanced head and neck cancer before and after radiation, even though a reduction of CD3 T cells was reported, no difference in iNKT cell numbers were observed (Kobayashi et al. 2010). However, these studies did not investigate the direct effect of radiation on iNKT cells, and mechanisms of iNKT cell radioresistance are poorly understood. Radiation causes cell death of rapidly divided cells, for this study iNKT cells were freshly isolated and expanded for two weeks and all experiments were carried out within a month after expansion to simulate cell activation and division in the human body. Cell were counted daily for up to 30 days to confirm that the cells were still dividing the day of the experiment. In the present study, a dose dependent increase in iNKT cell death after radiation with therapeutic doses levels of X-rays was observed. Previous studies have shown that the oesophageal cancer cell line, OE19 cells is more radioresistant than another oesophageal cancer cell line, OE33 cells (Hotte et al. 2012). iNKT cells had similar radiosensitivity to OE19 cells suggesting an inherent level of radioresistance in iNKT cells.

Double strand breaks in the DNA are considered the most lethal type of damage of the DNA and inability of cells to repair the damage leads to
apoptosis and cell death. When DNA damage forms double strand breaks, these breaks are followed by the phosphorylation of the histone, H2AX. γ-H2AX is the first step in recruiting and localizing DNA repair proteins. γ-H2AX foci can be used as a biomarker for DNA damage (Kuo and Yang 2008) and expression following different doses of radiation and different time points has been reported in total lymphocytes. γ-H2AX expression reaches a maximum 30 min post radiation and returns to baseline levels 24 h post exposure (Redon et al. 2009; Sak et al. 2007; Scarpato et al. 2013). CD4+, CD8+ T cells and B cells display a similar dose response relationship of γ-H2AX expression, however, CD4+ and CD8+ T cells express more γ-H2AX than B cells (Andrievski and Wilkins 2009). On the contrary, total lymphocyte expression of γ-H2AX is lower than that of CD4+, CD8+ T cells and B cells, likely due to other subsets, such as NK cells (Andrievski and Wilkins 2009). γ-H2AX expression by iNKT cells after radiation was here examined for the first time and was found to reach maximum expression 20 min post radiation, returning to baseline after 24 h. Radiation can directly damage normal tissue or induce bystander effects in adjacent cells. Radiosensitivity is predominantly determined by the DSB repair capability of a cell (Mladenov et al. 2013). It must be considered that high doses of radiation can lead to toxicity, reducing the overall prognosis for the patient (Brown, Mutter, and Halyard 2015). DNA repair capability should be a consideration when planning the appropriate treatment for cancer patients. DNA repair in iNKT cells suggests that iNKT
cells can be used in combination with radiotherapy without compromising cell functions.

iNKT cells are known to direct anti-tumour immunity by the release of Th1 and Th2 cytokines that activate and modulate other immune cells such as T cells, NK cells and macrophages (Matsuda et al. 2008). Low doses of radiation can enhance T cell responses, including IFN-γ production and specific lysis (Spary et al. 2014). On the contrary, IFN-γ production by iNKT cells has previously been described as impaired in patients with advanced head and neck cancer after a cumulative dose of 50Gy radiation compared to iNKT cells prior to radiation from the same patients, but IFN-γ production was recovered 7 days post radiation (Kobayashi et al. 2010).

iNKT cells are known to directly kill target cells by the release of perforin and granzyme B. In combination these cytotoxic molecules lead to apoptosis of target cells by the activation of the caspase pathway (Voskoboinik, Whisstock, and Trapani 2015). No difference was observed in direct killing of target cells by non-irradiated and irradiated iNKT cells. We observed an increase in iNKT cells expressing perforin after treatment with both 2Gy and 10Gy irradiation, confirming the ability of iNKT cells to kill target cells after radiation. When IFN-γ and IL-4 expression was tested no difference was observed after single doses of radiation. On the contrary, IFN-γ, but not IL-4 was significantly reduced when cells received cumulative doses of radiation. A reduction in IFN-γ production indicates that iNKT cells are not able to activate other antitumour immune cells when treated with high doses of radiation, however, the presence of
granzyme B and the increase on perforin indicates that iNKT cells are still able to kill target cells directly.

Several clinical trials combining radiotherapy and immunotherapy are ongoing at the moment (Ko and Formenti 2018; Bhalla, Brooker, and Brada 2018). However, the dose of radiotherapy, timing and the immune status of the patient can be limiting factors to the efficacy of immunotherapy and radiotherapy in combination. It has been shown that patients with lower tumour-infiltrating MDSCs and high frequencies of T cells respond better to immunotherapy combined with radiotherapy (Seung et al. 2012; Finkelstein et al. 2012).

In the previous chapter, it was shown that iNKT cells are depleted in OC and GAC patients, making iNKT cells a target for cellular immunotherapy. Restoring iNKT cell numbers in patients to normal levels might be a mechanism of inducing an antitumour response. However, iNKT cell-based immunotherapy should consider the effect of concurrent treatment. Here, iNKT cells were shown to be susceptible to cisplatin, a common chemotherapy drug used in patients with OC and GAC. In this chapter we demonstrated that iNKT cells are been killed by radiation, but their direct cytotoxic activities are intact suggesting that iNKT cell-based immunotherapy should be used carefully in combination with radiotherapy. Here we suggest the use of radiotherapy prior to the iNKT cell treatment. However, future studies are required to test the combination of iNKT cells and radiotherapy in vivo.
Chapter 6

Novel thioglycoside analogues of α-galactosylceramide stimulate cytotoxicity and preferential Th1 cytokine production by human invariant natural killer T cells
6.1 Introduction

T lymphocytes kill pathogen-infected and tumour cells directly by releasing perforin and granzymes, which induce apoptosis of target cells and indirectly by releasing soluble factors (cytokines) that selectively activate and regulate other immune cells. iNKT cells respond to lipid-based antigens presented by CD1d molecules expressed on APCs (Bendelac, Savage, and Teyton 2007; Salio et al. 2014). A number of self (Brennan et al. 2011; Facciotti et al. 2012) and microbial (Kinjo et al. 2005; Mattner et al. 2005) glycosphingolipids have been shown to bind to CD1d and stimulate iNKT cells, but most of our understanding of iNKT cells comes from studies using the xenogeneic glycolipid, α-galactosylceramide (α-GalCer). α-GalCer is a natural product isolated from the Agelas genus of marine sponges (Carreño, Kharkwal, and Porcelli 2014), which has been recognised for its anti-tumour activities. α-GalCer has been widely used as the key glycolipid to elucidate the role of iNKT cells (Bendelac, Savage, and Teyton 2007). Upon activation with α-GalCer, iNKT cells kill target cells and secrete a diverse range of growth factors and cytokines, allowing them to contribute to the activation of T cells (Gumperz et al. 2002; O'Reilly et al. 2011), NK cells (Carnaud et al. 1999) and macrophages (Lynch et al. 2015). Activated iNKT cells can also interact directly with other cells of the immune system and can induce the maturation of dendritic cells (DC) into APC (Kitamura et al. 1999; Vincent et al. 2002) and of B cells into antibody secreting plasma cells (Galli et al. 2003; Zeng et al.)
Thus, immune recognition of glycolipids plays a central role in the activation and regulation of innate and adaptive immune responses.

As mentioned above, iNKT cells are thought to play a central role in immunity against tumours and numerical and functional iNKT cell deficiencies have been reported in a number of human cancers (Molling et al. 2008; Kenna et al. 2003; Berzins, Smyth, and Baxter 2011). Clinical trials involving the adoptive transfer of α-GalCer-pulsed autologous DC and/or ex vivo expanded iNKT cells are ongoing for a number of human cancer types (Nieda et al. 2004; Kunii et al. 2009; Chang et al. 2005). However, these trials have to date shown limited clinical efficacy.

When human iNKT cells are activated with α-GalCer they have the ability to simultaneously release Th1 and Th2 cytokines (Gumperz et al. 2002; O'Reilly et al. 2011), including IFN-γ that can activate an antitumour response and IL-4, IL-10 and IL-13, which can attenuate antitumour immunity and promote tumour growth (Bricard et al. 2009; Lynch et al. 2015; Terabe et al. 2005). Th1/Th2 cytokine production differs among subsets of human iNKT cells, with CD8+ and double negative iNKT cells being the predominant producers of Th1 cytokines and a more cytotoxic profile, whereas CD4+ iNKT cells predominantly produce Th2 cytokines, showing a more immunoregulatory profile (Gumperz et al. 2002; O'Reilly et al. 2011). The efficacy of α-GalCer as an antitumour drug may be limited by inhibition of antitumour immune responses exhibited by Th2 cytokines.

To optimize the antitumour properties of human iNKT cells, a number of α-GalCer derivatives and analogues have been synthesized to develop
compounds, which can selectively induce particular responses in iNKT cells. Previous studies by Xing et al. show that the α-anomeric configuration is a key antigenic property of α-GalCer (Xing et al. 2005), this was observed after modifications to the galactose residue. Over the years, several modifications to the α-GalCer molecule has been made, assessing the preferential stimulation of Th1 cytokine secretion by iNKT cells. Shortening of the acyl chains (Miyamoto, Miyake, and Yamamura 2001; Goff et al. 2004) and the introduction of double bonds (Yu et al. 2005) resulted in iNKT cell ligands that preferentially stimulated Th2 cytokine secretion. In contrast, the addition of aromatic groups resulted in Th1-inducing glycolipids (Fujio et al. 2006; Chang et al. 2007). Specifically, addition of an aromatic moiety at the 6'-position of the galactose led to Th1 cytokine secretion in vitro, taking advantage for the first time the flexibility of CD1d in accommodating galactose-modified glycolipids inducing an enhanced lipid binding to CD1d (Aspeslagh et al. 2011). It has also been shown that depletion of the glycosidic oxygen allowing the methylene group link alone to the sphingoid chain with the carbohydrate moiety increases the antitumour response (Lu et al. 2006). Similarly, substitution of the O-glycosidic linkage in α-GalCer with a C-glycosidic linkage resulted in a Th1-inducing compound with 100-fold greater antimetastatic activity in mice (Schmieg et al. 2003). Li et al. identified that E-alkene-linked C-glycoside analogues are potent human iNKT-cell stimulant, which increased Th1 responses (Li, Chen, et al. 2009). Replacement of the 5α'-oxygen atom of the D-galactopyranose ring of
alpha-GalCer by a methylene group also led to a Th1 polarization of the cells (Tashiro et al. 2010). Previous studies from our group synthesized and studied a thioglycoside analogue of α-GalCer (α-S-GalCer) and found that it was similar to α-GalCer in its ability to bind to CD1d and stimulate human iNKT cells, but had increased stability in biological systems and enhanced flexibility around the anomeric linkage (Hogan et al. 2011). Analogues of α-S-GalCer were designed and biologically tested in the present study.

The CD1d binding cleft is characterized by two hydrophobic pockets, the A’-pocket and the F’-pocket. The crystal structure of CD1d/α-GalCer/iNKT TCR interaction shows that both the mouse and human iNKT TCRs adopt a tilted and parallel docking mode over CD1d, with the acyl chain of α-GalCer buried in the A’-pocket and the phytosphingosine chain buried in the F’-pocket (Figure 6.1A) (Borg et al. 2007; Pellicci et al. 2010). In contrast, the α-galactosyl head group is exposed from the cleft (Figure 6.1B), making it directly available to contact the iNKT TCR. The 2-hydroxyl (2-OH) of the galactose ring, the 3-OH of the sphingosine chain, and the anomeric oxygen of α-GalCer form hydrogen bonds with residues on CD1d, which serve to anchor α-GalCer in the lipid-binding groove of CD1d. The 2-, 3- and 4-OH groups of the galactose ring interact with the invariant TCR α-chain making hydrogen bonds with the corresponding amino acids, which are responsible for the fine specificity that the NKT TCR exhibits for α-GalCer. Only the 6-OH group of the sugar ring is not involved in any hydrogen bond formation during α-GalCer binding and recognition and
modifications of the 6-OH group can be made without losing antigenicity (Kawano et al. 1997; Prigozy et al. 2001; Zhou et al. 2002). Based on this premise, a number of groups have synthesized novel α-GalCer analogues with substitutions in the 6-OH position of the galactose, which resulted in ligands that could bind to CD1d and stimulate iNKT cells (Liu et al. 2006; Trappeniers et al. 2008).

Based on our recent work on α-S-GalCer (Hogan et al. 2011; Murphy et al. 2013) and the above literature, glycolipids XZ7, XZ8, XZ9, XZ10 and XZ11 were designed and chemically synthesized. Glucosylation of 6-OH of α-S-GalCer gives disaccharide ceramide XZ7 (Zhang et al. 2017), while thioglucosylation of the same group gives target molecule XZ11. Their ability to bind to human CD1d and activate human iNKT cells was tested.
using set of iNKT cell lines generated from blood samples from healthy donors.

6.2 Objectives

Since iNKT cells have been shown to have antitumour activities, the aim of this chapter was to test the ability of novel synthetic glycolipids to activate the antitumour activities of human iNKT cells.

The specific aims of this chapter were:

1. To examine the effect on cytolytic degranulation of iNKT cells after stimulation with the novel synthetic glycolipids when presented by HeLa cells expressing transfectant CD1d and dendritic cells, which naturally express CD1d.

2. To analyse cytokine production of iNKT cells after stimulation with the novel synthetic glycolipids when presented by HeLa-CD1d and dendritic cells.

3. To investigate if the novel glycolipids preferentially activates subsets of iNKT cells.

6.3 Specific methods

6.3.1 Glycolipids

α-GalCer or 7DW8-5 were purchased from Funakoshi Co. Ltd (Tokyo, Japan). The chemical synthesis of XZ7, XZ8, XZ9, XZ10 and XZ11 has previously been described (Zhang et al. 2017).
6.3.2 Glycolipids

Glycolipids stocks were prepared by resuspending the solid in DMSO to a stock concentration of 1 mM. Stocks were stored at -20°C. Further dilution in RPMI were made after vortexing for 1 min, followed by heating for 2 min at 80°C, and sonicating for 10 min. Diluted glycolipids were stored at -20°C. Glycolipids were vortexed for 1 min, heated for 2 min at 80°C, and sonicated for 10 min prior to every use. The structures of the glycolipids used are shown in Figure 6.2.

6.3.3 Antibodies and flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human CD3, Vα24Jα18 (clone 6B11), CD8, CD4, IL-4, CD107a, IFN-γ were used (Table 2.10). Flow cytometry was performed using a FACs Canto flow cytometer, and FlowJo Version 10 software was used for analysis.

6.3.4 Glycolipid activation of iNKT cells

iNKT cells were stimulated in vitro with HeLa-mock and HeLa-CD1d cells or DC presenting α-GalCer or 7DW8-5, or XZ7, XZ8, XZ9, XZ10 and XZ11. CCD107a, IFN-γ and IL-4 by iNKT cells was examined by flow cytometric analysis.

The release of IL-4 and IFN-γ by iNKT cells following stimulation with glycolipid was measured by ELISA using antibody pairs purchased from R&D Systems.
6.3.5 Statistical analysis

iNKT cell responses to different glycolipids were compared using the paired t test. p values of <0.05 (*) were considered statistically significant.

6.4 Results

6.4.1 Phenotypic analysis of iNKT cell lines.

iNKT cell lines were sorted and expanded from human blood as described in section 0 and phenotypically examined by flow cytometry. Purity of iNKT cell lines was at least 92% (Figure 6.3A). All the cell lines contained significant numbers of CD4+ (24.9±5.6%), CD8+ (34.8±5.8%) and double negatives (40.1±4.0%) iNKT cells (Figure 6.3B), except for cell line 2 that only expressed CD8+ and double negative, but not CD4+ T cells.

6.4.2 The novel glycolipids XZ7 and XZ11 stimulate cytolytic degranulation by iNKT cells in vitro

Expanded iNKT cell lines were co-cultured for 4 h with equal numbers of CD1d-transfected or mock-transfected HeLa cells (hereafter referred to as HeLa-CD1d and HeLa-mock) that were previously loaded with medium, 100 ng/mL α-GalCer, 10 ng/mL 7DW8-5 or 10–10,000 ng/mL of XZ7, XZ8, XZ9, XZ10 or XZ11, concentrations of α-GalCer and 7DW8-5 were previously optimised. Cytolytic degranulation was assessed by flow cytometric measurement of cell-surface CD107a expression (Figure 6.4A). iNKT cells exhibited significant CD107a expression after stimulation with
Figure 6.3. Purity and phenotype of iNKT cell lines. iNKT cells were generated from PBMC by magnetic bead enrichment followed by MoFlo™ cell sorting of CD3⁺Vα24Jα18⁺ cells and expanded using PHA and IL-2 in the presence of irradiated feeder cells. After 3-4 weeks, cells were stained with mAbs specific for CD3, CD4, CD8 and Vα24Jα18 and analysed by flow cytometry. **A and B** Representative flow cytometry dot plots of CD3 and Vα24Jα18 expression by gated viable lymphocytes within and iNKT cell line (**A**) and CD4 and CD4 and CD8 expression by gated iNKT cells (**B**) The numbers show the percentages of cells in each gate. **C** Graphs show percentage of CD8⁺, CD4⁺ and CD8⁻CD4⁻. Cells within iNKT cell lines generated from 7 donors. Each donor is represented by different symbols.
α-GalCer and 7DW8-5. A weaker but dose-dependent response to XZ7 (Figure 6.4B) and XZ11 (Figure 6.4C) was also observed. 26±3.05% (p=0.0116) and 22.33±5.41% (p=0.0271) of iNKT cells expressed CD107a when stimulated with the highest dose of XZ7 and XZ11, respectively. Little or no degranulation occurred in the absence of glycolipids or in the presence of XZ8, XZ9 or XZ10 (Figure 6.4D).

6.4.3 The novel glycolipids XZ7 induces IFN-γ production, whereas XZ11 induces IL-4 by iNKT cells.

Expanded iNKT cell lines were co-cultured for 4 h with equal numbers of CD1d-transfected or mock-transfected HeLa cells that were previously loaded with medium, 100 ng/mL α-GalCer, 10 ng/mL 7DW8-5 or 10–10,000 ng/mL of XZ7, XZ8, XZ9, XZ10 or XZ11. The proportions of iNKT cells that produced intracellular IFN-γ and IL-4 were quantified by flow cytometry (Figure 6.5A). 2.62±1.22% of iNKT cells expressed IFN-γ and 9.97±1.4% produced IL-4 in response to CD1d-transfected HeLa cells in the absence of stimulation, potentially due to residual activation following expansion. 65.7±9.7% and 59.3±11.8% of iNKT cells produced IFN-γ in the presence of CD1d-transfected HeLa cells pulsed with α-GalCer or 7DW8-5 respectively, and 16.7±1.2% and 28±5.0% produce IL-4 in the presence of the same glycolipids. Results from seven different iNKT cell lines showed that XZ7 presented by CD1d induced weak but significant IFN-γ (17±5.19%, p=0.0117. Figure 6.5B) but not IL-4 production (Figure 6.5D), compared to
Figure 6.4. Cytolytic degranulation by iNKT cells in response to the novel glycolipids XZ7 and XZ11.

Lines of expanded iNKT cells were co-cultured with CD1d transfected HeLa cells, previously pulsed with medium alone, 100 ng/mL of α-GalCer, 10 ng/mL of 7DW8-5, or 10, 100, 1000 or 10,000 ng/mL of XZ7, XZ8, XZ9, XZ10 or XZ11 (summarized by black triangles). Cells were stained with antibodies specific for CD107a, CD3 and the Va24Ja18 T cell receptor (TCR) and analysed by flow cytometry. A) Flow cytometry dot plots showing the expression of cell-surface CD107a by iNKT cells after stimulation with medium, α-GalCer, XZ7 and XZ11. B-D) Graphs showing mean (± SEM) percentages of iNKT cell lines generated from seven donors that expressed CD107a after stimulation with XZ7 (B), XZ11 (C), XZ8, XZ9 and XZ10 (D). (*p < 0.05, **p < 0.01 compared to medium-only controls using the paired t test).
unstimulated controls when used at 10,000 ng/mL. In contrast, XZ11 induced IL-4 (16.74±1.92%, p=<0.0285. Figure 6.5C), but not IFN-γ (Figure 6.5E), production by a minority of iNKT cells. Little or no cytokine production was observed in the absence of glycolipids or in the presence of XZ8 (Figure 6.5B), XZ9 (Figure 6.6C) or XZ10 (Figure 6.6D) by flow cytometry. Cytokine release by the cells was also quantified, stimulated as above using ELISA. The results show again that iNKT cells stimulated with 10,000 ng/mL XZ7 produced IFN-γ (776.68±333.70 pg/mL. Figure 6.7A) and cells stimulated with similar amounts of XZ11 produced IL-4 (282.65±276.44 pg/mL. Figure 6.7B). Of note, the amounts of both cytokines were 3–4-fold lower than were produced by the same cells in response to stimulation with 100 ng/mL α-GalCer or 10 ng/mL 7DW8-5.

6.4.4 Activation of iNKT cells by XZ7 presented by DC.

Since XZ7, presented by HeLa-CD1d cells, was capable of stimulating the antitumour functions (cytolytic degranulation and IFN-γ production) of iNKT cells without inducing IL-4 production, thought to have protumour effects (Terabe et al. 2005; Bricard et al. 2009), the effect of the glycolipids when presented by DCs was investigated, as they naturally express CD1d (Gerlini et al. 2001). Expanded iNKT cell lines were co cultured for 4 h with equal numbers of monocyte-derived DC that were previously loaded with medium, 100 ng/mL α-GalCer, 10 ng/mL 7DW8-5 or 10–10,000 ng/mL of XZ7. Cells were stained with mAbs specific for cell surface CD107a, CD3 and the Vα24Jα18 TCR or mAbs specific for cell-surface CD3 and Vα24Jα18
Figure 6.5. Cytokine production by iNKT cells in response to the novel glycolipids XZ7 and XZ11. Expanded iNKT cells were co-cultured with CD1d-transfected HeLa cells, previously loaded with medium alone, 100 ng/mL of α-GalCer, 10 ng/mL of 7DW8-5, or 10, 100, 1000 or 10,000 ng/mL of XZ7 or XZ11 (summarized by black triangles). A) Cells were stained with mAbs specific for cell-surface CD3 and Vα24Jα18 and intracellular IFN-γ and IL-4 and analysed by flow cytometry. B-G) Graphs showing mean (± SEM) percentages of iNKT cells producing IFN-γ (B and C) or IL-4 (D and E) or both IFN-γ and IL-4 (F and G) after stimulation with XZ7 (left panels; n = 4) or XZ11 (right panels; n = 7). (*p < 0.05, **p < 0.01 compared to medium-only controls using the paired t test).
Figure 6.6. No cytokine production by iNKT cells in response to the novel glycolipids XZ8, XZ9 and XZ10. Expanded iNKT cells were co-cultured with CD1d-transfected HeLa cells, previously loaded with medium alone, 100 ng/mL of α-GalCer, 10 ng/mL of 7DW8-5, or 10, 100, 1000 or 10,000 ng/mL of XZ8, XZ9 or XZ10 (summarized by black triangles). A) Cells were stained with mAbs specific for cell-surface CD3 and Vα24Jα18 and intracellular IFN-γ and IL-4 or both and analysed by flow cytometry. B-D) Graphs showing mean (± SEM) percentages of iNKT cells producing IFN-γ or IL-4 after stimulation with XZ8 (B), XZ9 (C) or XZ10 (D) from seven independent experiments. (*p < 0.05, **p < 0.01 compared to medium-only controls using the paired t test).
Figure 6.7. Cytokine release by iNKT cells in response to the novel glycolipids XZ7 and XZ11. Expanded iNKT cells were co-cultured with CD1d-transfected HeLa cells, previously loaded with medium alone, 100 ng/mL of α-GalCer, 10 ng/mL of 7DW8-5, or 10, 100, 1000 or 10,000 ng/mL of XZ7 or XZ11 (summarized by black triangles). The release of IFN-γ and IL-4 into the cell supernatants was measured by ELISA. A and B) Graphs show mean (± SEM) levels of IFN-γ (A) or IL-4 (B) after stimulation of iNKT cells with XZ7 (left panels; n = 4) or XZ11 (right panels; n = 4). (*p < 0.05, **p < 0.01 compared to medium-only controls using the paired t test).
and intracellular IFN-γ and IL-4 and analysed by flow cytometry. DC pulsed with α-GalCer and 7DW8-5 induced significant CD107a, IFN-γ and IL-4 expression by iNKT cells, whereas DC pulsed with medium alone did not activate iNKT cells, confirming the absence of endogenous iNKT cell stimulatory glycolipids. In contrast, DC pulsed with XZ7 induced a dose-dependent induction of CD107a (26.18±2.83% expressed CD107a in the presence of 10,000 ng/mL of XZ7, p=0.0051. Figure 6.8A) and IFN-γ (20.75±8.88%, p=0.0313. Figure 6.8B), but not IL-4 (Figure 6.8C), expression by iNKT cells. Thus, glycolipid XZ7 appears to stimulate cytotoxicity and Th1 cytokine production, but not Th2 cytokine production by iNKT cells.

6.4.5 Cytolytic degranulation by iNKT cells in response to XZ7 is restricted to the CD4-negative subset

XZ7, presented by HeLa-CD1d cells or monocyte-derived DC, induced cytolytic degradation and Th1 but not Th2 cytokine production by iNKT cells. Since CD4+, CD8+ and DN iNKT cells differentially exhibit cytotoxicity, Th1 and Th2 cytokine production (Gumperz et al. 2002; O’Reilly et al. 2011), the activation of specific subsets of iNKT cells by the novel glycolipids was also investigated. Expanded iNKT cell lines were co-cultured for 4 h with equal numbers of HeLa-CD1d cells or monocyte-derived DC that were previously loaded with medium, 100 ng/mL α-GalCer, 10 ng/mL 7DW8-5 or 10–10,000 ng/mL of XZ7. Cytolytic degranulation was measured by flow cytometric analysis of CD107a
Figure 6.8. Cytolytic degranulation and cytokine production by iNKT cells in response to XZ7 presented by dendritic cells. iNKT cell were stimulated by monocyte derived dendritic cells previously pulsed with medium alone, 100 ng/mL of α-GalCer, 10 ng/mL of 7DW8-5 or 10, 100, 1000 or 10,000 ng/mL of XZ7 (summarized by black triangles). A-D) Cells were stained with mAbs specific for CD107a, CD3 and the Vα24Jα18 TCR (A) or mAbs specific for cell-surface CD3 and Vα24Jα18 and intracellular IFN-γ and IL-4 (B and C) and analysed by flow cytometry. Graphs showing mean (± SEM) percentages of total iNKT cells that expressed CD107a (A, n = 5), IFN-γ (B, n = 6) and IL-4 (C, n = 6) after stimulation with the glycolipids. (*p < 0.05, **p < 0.01 compared to medium-only controls using the paired t test).
expression. CD4+, CD8+ and DN iNKT cells exhibited significant CD107a expression after stimulation with the agonist glycolipids α-GalCer or 7DW8-5 when presented with CD1d-transfected HeLa cells or DC. However, 32.9±6.99% ($p=0.0375$. Figure 6.9A) of CD8+ iNKT cells and 29.4±5.37% ($p=0.0276$. Figure 6.9B) of DN iNKT cells expressed CD107a when XZ7 was presented by CD1d-transfected HeLa cells, but not by CD4+ iNKT cells (Figure 6.9C) and 30.5±5.3% CD8+ of CD8+ iNKT cells expressed CD107a when stimulated by XZ11 presented by CD1d ($p=0.0183$. Figure 6.9D) but the frequencies of DN and CD4+ iNKT cells that expressed CD107a were similar to those of unstimulated cells (Figure 6.9E and F).

When DC were used as APCs, XZ7 induced degranulation in a dose-dependent manner by CD8+ iNKT cells (27.46±4.25%, $p=0.0071$. Figure 6.9G) and DN iNKT cells (47.66±6.78%, $p=0.0034$. Figure 6.9H), but not by CD4+ (Figure 6.9I). Limited or no degranulation was observed in the absence of glycolipid or in the presence of XZ8 (Figure 6.10A), XZ9 (Figure 6.10B) or XZ10 (Figure 6.10C).

It is well known that activation of iNKT cells by glycolipids can vary between mouse and human (Hogan et al. 2011; Li, Shiratsuchi, et al. 2009). In collaboration with Raul Castaño in Universidad de Autonoma de Barcelona we tested the ability of the five novel glycolipids to expand iNKT cells in vivo, the glycolipids were intraperitoneally injected into mice at two different doses (0.5 µg or 2µg) and relative numbers of iNKT cells in peritoneum and spleen were analysed 4 days post administration by flow cytometry. An increase of iNKT cells reactive to XZ11, but not XZ7 was
observed in peritoneum (Figure 6.11A) and spleen (Figure 6.11B) of 3 mice 4 days after administration. The different response found in mouse and human highlights the importance of using human systems to test glycolipids.

6.5 Discussion

iNKT cells play critical roles in antitumour immunity in murine models and can be activated therapeutically to prevent or reverse tumour growth (Cui et al. 1997; Kawano et al. 1997; Crowe et al. 2005). These observations have led to a number of clinical trials assessing the utility of α-GalCer, α-GalCer-pulsed DC or α-GalCer-expanded iNKT cells as cellular therapies in humans (Nieda et al. 2004; Chang et al. 2005; Kunii et al. 2009). The results have shown that iNKT cell directed therapies were well-tolerated and have resulted in elevated serum levels of proinflammatory cytokines and persistent expansions of IFN-γ-producing cells. However, in all studies the clinical efficacies were modest.

The divergent responses to iNKT cell-based therapies in mice and humans may also reflect numerical or functional differences in murine and human iNKT cells. iNKT cells are found at 100-fold lower frequencies in humans compared to mice at most body locations examined (Kenna et al. 2003; Berzins, Smyth, and Baxter 2011). Furthermore, mice and humans have different subset distributions of iNKT cells, with most murine iNKT cells expressing CD4+ or DN phenotypes, whereas humans have significant populations of CD4+, CD8+ and DN iNKT cells which differ in their effector activities (Gumperz et al. 2002; O'Reilly et al. 2011).
Figure 6.9. Cytolytic degranulation by iNKT cells in response to XZ7 and XZ11 is restricted to the CD4-negative subset. iNKT cells were stimulated by HeLa-CD1d cells or monocyte-derived dendritic cells that were previously pulsed with medium alone, 100 ng/mL of α-GalCer, 10 ng/mL of 7DW8-5 or 10, 100, 1000 or 10,000 ng/mL of XZ7 or XZ11. Cells were stained with mAbs specific for CD107a, CD3, CD4, CD8 and the Vα24Jα18 TCR, and analysed by flow cytometry. A-F) Graphs show mean (± SEM) percentages of CD8+ (left panels), CD4+CD8− (DN; centre panels) and CD4+ (center panels) iNKT cells that expressed cell-surface CD107a after stimulation with XZ7 (A-C, n = 4) or XZ11 (D-F, n=7) presented by HeLa-CD1d cells. G-I). Graphs show mean (± SEM) percentages of CD8+ (left panels), CD4+CD8− (DN; centre panels) and CD4+ (center panels) iNKT cells that expressed cell-surface CD107a after stimulation with XZ7 presented by dendritic cells (n = 6). (*p < 0.05, **p < 0.01 compared to medium-only controls using the paired t test).
Figure 6.10. XZ8, XZ9 and XZ10 do not induce cytolytic degranulation by iNKT cells in vitro. Lines of expanded iNKT cells were co-cultured with CD1d transfected HeLa cells, previously pulsed with 100 ng/mL of α-GalCer, 10 ng/mL of 7DW8-5, 10, 100, 1000 or 10,000 ng/mL of XZ8, XZ9 or XZ10. Cells were stained with CD107a, CD3, CD4, CD8 and Vα24Jα18 TCR, and analysed by flow cytometry. A-C) Graphs showing mean (± SEM) percentages of CD4+ (A), CD8+ (B) and DN (C) iNKT cells expressing CD107a after stimulation with XZ8 (n=6), XZ9 (n=6) or XZ10 (n=7). (*p<0.05, **p<0.01 using the paired t test).
Figure 6.1. Intraperitoneal administration of XZ11 resulted in iNKT cells expansion in peritoneum and spleen. 2 ng of XZ7, XZ8, XZ9, XZ10 and XZ11 were intraperitoneally administrated to mice in collaboration with Dr. Raul Castano. A-B) 4 days after administration, relative numbers of iNKT cells in peritoneum (A) and spleen (B) were obtained by flow cytometry. (**p<0.01, ***p<0.001 using the unpaired t test).
Given the pleiotropy of iNKT cell functions, it is likely that differences in iNKT cell subset numbers and distributions between mice and humans and between different humans may underlie the divergent outcomes observed after therapeutic activation. Additionally, differences in the fine specificities of TCR recognition of CD1d/glycolipid complexes by murine and human iNKT cells (Wun et al. 2012) will also contribute to divergent outcomes of iNKT cell activation, and in this regard, α-GalCer may not be the ideal iNKT cell agonist for therapeutic use in humans.

In the present study, a number of novel glycolipid analogues of α-GalCer that were predicted to bind to CD1d and activate human iNKT cells were synthesized and biologically tested. Our group previously reported that replacement of the glycosidic oxygen atom in α-GalCer with a sulfur atom created a glycolipid that could bind to CD1d and induce simultaneous Th1 and Th2 cytokine production by iNKT cells (Hogan et al. 2011). Since thioglycosides have more flexibility around the glycosidic linkage compared to the corresponding O-glycosides, owing to the longer C–S bond and weaker stereoelectronic effects (Witczak 1999), after binding to CD1d, the sugar head of α-S-GalCer may orientate in a different angle from that of α-GalCer, which could result in differential recognition by TCR of iNKT cells. Analogues of α-S-GalCer with an extra glucose or thioglucose connected to the hydroxyl groups of the galactose moiety were synthesized. Two of these compounds, in which the 6-OH group of the galactose moiety of α-S-GalCer was substituted with a glucose group, one with a 6-O-glucosyl group (XZ7) and the other with a 6-thio-glucosyl group
(XZ11), could bind to CD1d and displayed agonist activity for human iNKT cells. Both glycolipids, in the presence of CD1d+ APC, were capable of inducing cytolytic degranulation by iNKT cells, albeit with weaker potency than α-GalCer and 7DW8-5, requiring 100-fold higher concentrations. Both glycolipids activated cytokine production by proportions of iNKT cells, but while XZ7 predominantly stimulated intracellular IFN-γ production and secretion, XZ11 stimulated IL-4 production and secretion. Again, the stimulatory capacities of XZ7 and XZ11 were weaker than those of α-GalCer and 7DW8-5, which at 100–1000-fold lower concentrations activated higher proportions of iNKT cells resulting in the secretion of 3–4-fold higher levels of cytokines than XZ7 and XZ11.

Since glycolipid XZ7 was capable of stimulating antitumour activities of iNKT cells (cytolytic degranulation and IFN-γ production) without stimulating IL-4 production, this compound was selected for further analysis. Stimulation of iNKT cells with XZ7 presented by DC was first tested, which constitutively express CD1d (Bendelac, Savage, and Teyton 2007; Salio et al. 2014), and has the same functional profile as iNKT cells stimulated with XZ7 presented by HeLa-CD1d cells. The results showed that XZ7 presented by DC induced degranulation and IFN-γ production, but not IL-4 production, by iNKT cells, confirming the potential antitumour activity of this novel glycolipid in vitro. Since, only a small fraction of iNKT cells were activated by XZ7 in all experiments, levels of activation of CD4+, CD8α+ and DN iNKT cells was next investigated. The results indicate that, while α-GalCer and 7DW8-5 stimulated degranulation by all subsets of
iNKT cells, XZ7 only stimulated CD8α⁺ iNKT cells when DC were used as APC and CD8α⁺ and DN iNKT cells when HeLa-CD1d cells were used. No degranulation was observed in CD4⁺ iNKT cells within any of six lines of iNKT cells that were stimulated with HeLa-CD1d or DC presenting XZ7. The failure of XZ7 to activate CD4⁺ iNKT cells may explain why only a small proportion of total iNKT cells were activated and may underlie the inability to of this glycolipid to stimulate IL-4 production, an effector function that is associated with CD4⁺ iNKT cells (Gumperz et al. 2002; O'Reilly et al. 2011). This lack of IL-4 production is convenient in the context of cancer, since IL-4 downregulates the antitumour functions of NK, NKT and T cells, contributing to tumour growth.

IFN-γ and other Th1 cytokines promote host immunity to tumours by stimulating tumour antigen presentation and promoting tumour cytotoxicity by innate and adaptive lymphocytes. They also inhibit proliferation and modulate apoptosis, differentiation and migration of tumour cells (Ikeda, Old, and Schreiber 2002; Parker, Rautela, and Hertzog 2016). On the contrary, IL-4 and Th2 cytokines are regulatory cytokines for cytotoxic T cells and NK cells and they can permit tumour growth through the inhibition of cell-mediated immunity (Brown and Hural 1997). Production of the Th2 cytokines IL-4, IL-10 and IL-13 by iNKT cells has been reported to inhibit antitumour immunity and promote tumour growth (Terabe et al. 2005; Bricard et al. 2009; Lynch et al. 2015). Since human iNKT cells simultaneously produce Th1 and Th2 cytokines upon activation with α-GalCer, this glycolipid may not be an optimal ligand for stimulating
the antitumour immune responses of iNKT cells. The partial agonist effect of glycolipid XZ7, inducing cytotoxicity and IFN-γ production, but not IL-4 production, indicates that the protumour activities of iNKT cells can be prevented, while preserving their antitumour activities, by introducing a second glycosyl group to α-S-GalCer, which was previously found to have similar activity to that of α-GalCer. Dr. Raul Castano in the Universidad Autonoma de Barcelona, also showed that XZ11 can activate murine iNKT cells, by injecting mice with this glycolipid and showing expansions of iNKT cells in the peritoneum and spleen. Interestingly, administration of mice with XZ7 did not result in iNKT cell expansions, indicating that antigen presentation by CD1d differs in mice and humans. A similar finding was obtained using the α-S-GalCer analogue of α-GalCer, which stimulated cytokine production by human iNKT cells in vitro but not by murine iNKT cells in vivo (Hogan et al. 2011).

Even though XZ7 are not as potent iNKT cell agonist as α-GalCer, its ability to stimulate only a Th1 cytokine profile in iNKT cells makes them a good therapeutic agent for cancer. It may serve as a parent compound for the development of structural analogues that display similar cytokine profiles but with greater potency.
Chapter 7

Type 2 NKT cells in patients with OAC
7.1 Introduction

NKT cells are innate lymphocytes that recognise lipid antigens presented by CD1d molecules. There are 2 subsets of NKT cells – type 1 NKT cells (iNKT cells) and type 2 NKT cells. In contrast to iNKT cells, which possess invariant TCR α-chains, type II NKT cells have a broader TCR repertoire. Type II NKT cells also differ from iNKT cells in that they do not recognise α-GalCer. Sulfatide was the first lipid antigen reported to be recognised by type II NKT cells (Jahng et al. 2004). Sulfatide is a molecule derived from galactosylceramide (GalCer) via esterification of a sulphate group to 3-hydroxyl of the galactose moiety (Figure 7.1) (Takahashi and Suzuki 2012). Sulfatide is present in the membranes of various tissues such as myelin, pancreas, kidney and liver (Takahashi and Suzuki 2012).

![Figure 7.1. Sulfatide structure. Sulfatide is derived from the GalCer molecule by the esterification of a sulphate group to 3-hydroxyl of the galactose moiety. Ceramide is transformed into galactosylceramide by the ceramide galactosyltransferase (CGT) and into sulfatide by the cerobroside sulfotransferase (CST) Adapted from: Eckhardt et al. (Eckhardt et al. 2007).](image)

It has been shown that sulfatide is not the only lipid that can activate type II NKT cells. Cerebroside sulfotransferase (CST)^− and UDP-galactose
ceramide galactosyltransferase (CGT)−/− mice still have sulfatide-CD1d specific T cells, CST and CGT are two key enzymes in the production of sulfatide, suggesting that self-ligands other than sulfatide can potentially select type II NKT cells (Arrenberg et al. 2010; Jahng et al. 2004). Isoforms with long fatty acid chains (over C24) are better stimulators of T cells than those with C16, such as the first intermediate in the synthesis of glycopbingolipids, β-glucosylceramide (βGlcCer) and a precursor of sulfatide present in the pancreatic beta-cell, β-galactosylceramide (βGalCer) (Rhost et al. 2012). In general, isoforms of glycolipids which lack the fatty acid chain are more potent activators of type II NKT cells, such as lyso-sulfatide (Blomqvist et al. 2009) (Figure 7.2). Diphosphatidylglycerol (also known as cardiolipin) is another phospholipid that has been found to activate murine type II NKT cells hybridomas (Tatituri et al. 2013). Cardiolipin consists of 2 phosphate residues and 4 kinds of fatty acyl chains and it is found in mitochondrial membranes, and alterations in concentration and structure of cardiolipin are associated with cardiac diseases (Dudek 2017; Nakagawa 2013).

When activated with sulfatide or by the bioactive proinflammatory ligand lysophosphatidylcholine (LPC), type II NKT cells produce IL-4 and IL-13 (Singh, Tripathi, and Cardell 2018), and inhibit iNKT cells, DCs, Th1 cells and B cells (Bandyopadhyay, Marrero, and Kumar 2016; Shah et al. 2012), having an impact in autoimmune diseases, inflammatory liver disease and cancer (Terabe et al. 2005; Jahng et al. 2004; Maricic et al. 2015).
Type II NKT cells are known to respond better to lipids without a fatty acid chain (Blomqvist et al. 2009).

In contrast with iNKT cells, the main activation pathway for type II NKT cells is through the TCR. In type II NKT hybridomas, CD1d was able to activate type II NKT cells by the presentation of lipid to the TCR but did not induce TLR signalling (Roy et al. 2008; Tatituri et al. 2013). However, type II NKT cells express receptors for different cytokines such as IL-18, IL-2rβ (CD122) and to a lower degree IL-2rα (CD25) (Rolf et al. 2008), suggesting type II NKT cells can be activated by cytokines independently of the TCR.

Type II NKT cells have an important role in tumour, by the suppression of other immune cells. Terabe et al. showed CD1d-mediated suppression of antitumour immune responses in murine models of colon carcinoma, fibrosarcoma, mammary carcinoma colorectal and renal cancer (Terabe et al. 2005). This suppression was maintained in Jα18−/− mice, leading to the conclusion that immunosuppression was led by type II NKT cells.
In murine colon cancer and lung metastasis, the administration of sulfatide enhanced tumour growth by the activation of sulfatide-reactive type II NKT cells (Ambrosino et al. 2007). Moreover, in B cell lymphoma, type II NKT cells were shown to produce TNF-α in combination with IL-13/STAT6 upregulated IL-13Rα2 expression which led IL-13 to induce TGF-β production by MDSCs (Terabe et al. 2003). TGF-β regulates CD8⁺ T cells and stimulates Treg cells (Terabe et al. 2003) (Figure 7.3). This cross talk between type II NKT cells and MDSCs has been shown to impact on B cell lymphoma mice model (Renukaradhya et al. 2008). Studies have been carried out targeting TGF-β in patients with metastatic malignant melanoma using an antibody that blocks all three isoforms of active TGF-β which has shown clinical benefits (Morris et al. 2014; Lacouture et al. 2015).

Figure 7.3. Type II NKT cells promote tumour growth. Type II NKT cells produce IL-13 activating MDSCs to produce TGF-β activating Treg cells and regulating CD8⁺ T cells, contributing to the tumour (Kato, Berzofsky, and Terabe 2018).
Type II NKT cells can also promote carcinogenesis by the recognition of lipid produced by organs damaged by inflammation. In Gaucher’s disease, a genetic disorder characterised by the deficiency of glycosphingolipid enzyme and the storage of β-glucosylceramide and glucosylsphingosine. In this disease, β-GlcCer and glucosylsphingosine accumulate in the lysosome, and type II NKT cells can react to these lipids and promote plasma cell differentiation. The chronic B-cell activation may be a risk factor for plasma cell tumour formation in Gaucher’s disease (Liu et al. 2012; Nair et al. 2015). Obesity is also viewed as a chronic inflammatory disease associated with cancer (WCRFl 2017). It has been shown that type II NKT cells exacerbate diet-induce obesity that may lead to carcinogenesis by mediating adipose tissue inflammation, steatohepatitis and insulin resistance (Satoh et al. 2012). Another approach for type II NKT cell immunotherapy could be the development of antagonistic antigens for type II NKT cells. However, studies of lipids recognised by type II NKT cells in cancer patients would first need to be carried out.

Several different models are currently used to study type II NKT cells, however, they all have limitations. The creation of type II NKT cell hybridomas are limited to in vitro experiments and are not representative of all subsets of type II NKT cells. Another common technique is the comparison of WT mice, with Jα18−/− mice and CD1d KO−/−, however this model only provides indirect evidence of type II NKT cell functions. The use of 24αβ-TCR transgenic mice, a TCR transgenic mouse line that
expresses Vα3.2, Vβ9, can provide a model for the study of in vivo behaviour of type II NKT cells, but only for one TCR repertoire. Lastly, the use of CD1d tetramers loaded with lipid antigens can provide direct identification of type II NKT cells, but no reagent can identify all types of type II NKT cells (Kato, Berzofsky, and Terabe 2018; Liao, Zimmer, and Chyung-Ru Wang 2013). In the present study, four different glycolipids were used to identify and study the role of type II NKT cells in OAC.

7.2 Objectives

The aim of this chapter was to identify and characterise type II NKT cells in peripheral blood of patients with OAC, with a long-term objective of developing these cells as therapeutic targets. Type II NKT cells were identified using a CD1d tetramer loaded with glycolipids which are known to bind to CD1d and activate T cells.

1. To optimize the use of a CD1d tetramer to identify type II NKT cells
2. To compare the frequencies of type II NKT cells differ in patients with OAC and control subjects.
3. To determine the cytokine profiles of type 2 NKT cells in the presence of sulfatide, lyso-sulfatide, cardiolipin and tetramyristoyl cardiolipin (TO CL).
7.3 Methods

7.3.1 Generating iNKT cells and Vδ1 T cell lines

Lines of iNKT cells and Vδ1 T cells were generated from healthy donors as described in sections 0 and 2.2.7.2.

7.3.2 Antibodies and flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human CD3, the Vα24Jα18 TCR used by iNKT cells (6B11), Vδ1, IL-13, IL-4, IFN-γ, TGF-β and fixable viability dye (eFlour-506) were used.

7.3.3 Samples

Venous blood samples were obtained from 9 treatment naïve OAC patients and 9 non-cancer controls (Table 7.1). The non-cancer controls were sex and age matched (8 males and 1 female, mean age 68.7 years, age range 50-78 years). The patient group included 8 males and 1 female, representative of the male predominance in oesophageal adenocarcinoma. PBMC were isolated by density gradient solution with Lymphoprep. 1X10⁶ cells were stained with CD1d tetramer, mAb CD3, and analysed using a FACSCanto flow cytometer, and FlowJo Version 10 software was used for analysis.

Table 7.1. Demographic information of the patients

<table>
<thead>
<tr>
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<th>OAC pre-treatment patients (n=9)</th>
<th>Non-cancer controls (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>66.4 (45-78)</td>
<td>68.7 (50-78)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>88% male</td>
<td>88% male</td>
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7.3.4 Glycolipids

Sulfatide, lyso-sulfatide, cardiolipin and TO CL were prepared in DMSO to a stock concentration of 1mM. Glycolipids were further diluted to a 0.2 mg/mL concentration in saponin after vortexing for 1 min, followed by heating for 2 min at 80°C, and sonicating for 10 min and loaded to the tetramer immediately.

7.3.5 CD1d tetramer loading

A 12-molar excess of 0.2 mg/mL of glycolipid were added to the CD1d tetramer and incubated overnight at room temperature. After incubation tetramer loaded with glycolipids were diluted with PBS (13 μL of loaded tetramer in 87 μL of PBS) and analysed by flow cytometry.

7.3.6 Cytokine production

Lines of Vδ1 T cells were stimulated with CD1d transfected HeLa cells pulsed with 10 μg/mL of sulfatide, lyso-sulfatide, cardiolipin and TO CL and stained for IFN-γ, IL-13, IL-4 and TGF-β as described in section 2.2.14 and analysed by flow cytometry.

7.3.7 Statistical analysis

p values between groups were obtained using the unpaired Mann-Whitney U test, and paired t test where appropriate. P values of <0.05 were considered statistically significant.
7.4 Results

7.4.1 Protocol optimization

To optimize the tetramer loading protocol, lines of iNKT cells were stained with mAbs specific for CD3 and Vα24Jα18, similar amounts of unloaded CD1d tetramer (containing saponin only) and α-GalCer-loaded CD1d tetramer (Figure 7.4) and an FMO control consisting of anti-CD3 mAb only. Tetramer binding was analysed by flow cytometry. No positivity was observed when iNKT cells were stained with the unloaded tetramer or the FMO. The α-GalCer-loaded tetramer detected 97.4% of iNKT cells, when 0.25 μl of tetramer (original volume of tetramer before adding ligands) was used, similar to the frequency detected using anti-Vα24Jα18 mAb. This experiment showed that the use of 0.25 μl of α-GalCer-loaded tetramer is optimal for detecting type 1 NKT cells.

Even though, most type II NKT cells are thought to express TCR α and β-chains, Bai et al. showed that 0.5% of human Vδ1 T cells recognise sulfatide presented by CD1d (Bai et al. 2012). Hepatic γδ T cells have also been shown to respond to cardiolipin in a CD1d dependant manner in mice (Dieude et al. 2011). We therefore used lines of Vδ1 T cells to optimise CD1d tetramer loading with glycolipids for detection of type II NKT cells.

Lines of Vδ1 T cells were generated from three donors and expanded with PHA-P and IL-2 in the presence of irradiated feeder cells, as described in section 2.2.7.2 (Figure 7.5A). Vδ1 T cells did not stain positive for the
Figure 7.4. iNKT cells stain positive for α-GalCer-CD1d loaded tetramer. iNKT cell lines were stained with a mAb specific for CD3 and either a mAb specific for the Vα24Jα18 TCR or a CD1d tetramer loaded with α-GalCer and analysed by flow cytometry. A) Dot plot showing the α-GalCer-CD1d tetramer FMO. B) Representative dot plot showing iNKT cells stained with a mAb specific for CD3 and 0.025, 0.1 and 0.25 μl unloaded-CD1d tetramer as control. C) Dot plot showing the same cells stained with anti-Vα24Jα18 and CD3 mAbs. D) Dot plot showing the same cells stained with anti-CD3 mAb and 0.025, 0.1 and 0.25 μl α-GalCer-loaded CD1d tetramer.
α-GalCer-loaded CD1d tetramer (Figure 7.5B). Vδ1 T cells were also stained for mAbs specific for CD3 and 5 different amounts of glycolipid-loaded-CD1d tetramer and analysed by flow cytometry. No positive cells were observed when cells were stained with FMO (Figure 7.6B) or unloaded tetramer (Figure 7.6C). Cells that stained positive for the CD1d tetramer was observed when 1 µL of the loaded tetramer was used loaded with sulfatide (Figure 7.6D), the lysoform of sulfatide, lacking the fatty acid chain, lyso-sulfatide (Figure 7.6E), cardiolipin (Figure 7.6F) or the synthetic standard for cardiolipin tetramyristoyl cardiolipin (TO CL, Figure 7.6G). These results show that T cells that recognise CD1d presenting all 4 glycolipids are present among Vδ1 T cells. They also show that, whereas 0.25 μl of α-GalCer-loaded tetramer was optimal for detecting type 1 NKT cells (Figure 7.4), 1 μl of loaded tetramer was required for optimal for detection of type 2 NKT cells.

Stax et al. reported that human iNKT cells can recognise sulfatide presented by CD1d, however the iNKT cell affinity to sulfatide-CD1d is lower than the CD1d-αGalCer (Stax et al. 2017). We therefore tested if lines of iNKT cells would stain
Figure 7.5. Generation of Vδ1 T cells and analysis of their ability to recognise α-GalCer. Lines of Vδ1 T cells were generated from 3 healthy donors. Cells were stained with mAbs specific for CD3 and the Vδ1 TCR before and after magnetic bead enrichment of the γδ T cells and after expansion. The left and centre panels show the percentage of Vδ1 T cells within the lymphocyte population on day 1 before and after magnetic bead cell-sorting, respectively. The right panel of figure A shows the percentages of Vδ1 T cells obtained after using a BD FACSMelody Cell sorter and after 14 days expansion with IL-2. Expanded Vδ1 T cell lines from three donors were stained with a mAb specific for CD3 and α-GalCer-loaded CD1d tetramer. B) Plots on the left show Vδ1 T cells stained with unloaded CD1d tetramer, plots on the right show Vδ1 T cell stained with α-GalCer-CD1d tetramer.
Figure 7.6. Flow cytometric detection of Vδ1 T cells using CD1d tetramers loaded with sulfatide, lyso-sulfatide, cardiolipin and TO CL-CD1d tetramer. Lines of Vδ1 T cells were generated from 3 healthy donors and stained with various amounts of glycolipid-loaded tetramers. 

A) Graphs showing percentage of Vδ1 T cells stained with unloaded CD1d tetramer and CD1d tetramer loaded with sulfatide, lyso-sulfatide, cardiolipin and TO CL. 

B) Dot plots showing Vδ1 T cell FMO and unstained control. 

C) Dot plots showing the same cell line stained with a mAb specific for CD3 and 0.025, 0.1, 0.25, 0.5 and 1 μl unloaded CD1d tetramer as negative controls. 

D-G) Dot plots showing the Vδ1 T cells stained with the same volumes of sulfatide-CD1d tetramer (D), lyso-sulfatide-CD1d tetramer (E), cardiolipin-CD1d tetramer (F) or TO CL-CD1d tetramer (G). Results are representative of findings using 3 Vδ1 T cell lines.
positive for CD1d tetramers loaded with sulfatide, lyso-sulfatide, cardiolipin or TO CL. When iNKT cell lines were stained with 1 μL tetramer, no positive population was observed with the FMO or unloaded tetramer (Figure 7.7A and B), but a small positive population was observed when stained with sulfatide (Figure 7.7C), lyso-sulfatide (Figure 7.7D), cardiolipin (Figure 7.7E) and TO CL (Figure 7.7F). This result confirms that iNKT cells, like Vδ1 T cells, display a degree of promiscuity in glycolipid recognition.

### 7.4.2. OAC patients have increased numbers of sulfatide and tetramyristoyl cardiolipin positive T cells

Samples from 10 pre-treatment patients with OAC were collected from St. James’s Hospital. Frequencies of CD1d-sulfatide specific T cells, as percentages of T cells, in peripheral blood from patients with OAC were analysed by flow cytometry using a sulfatide-loaded CD1d tetramer and an mAb specific for CD3.

4.1±1.2% of sulfatide-CD1d specific T cells were observed in blood samples from patients with OAC, significantly higher than 1.4±0.8% in non-cancer controls (p=0.04. Figure 7.8). Cells positive for lyso-sulfatide (0.4±0.2% in patients and 0.1±0.03% in controls. Figure 7.9) and cardiolipin (0.4±0.2% in patients and 0.06±0.02% in controls Figure 7.10) were barely detectable in patients and non-cancer controls. Frequencies of TO CL-CD1d specific T cells were higher in blood samples from patients...
Figure 7.7. A minor subset of iNKT cells stain positive for CD1d tetramer loaded with sulfatide, lysosulfatide, cardiolipin or TO CL. A-F) Dot plots showing iNKT cell lines stained with mAb specific for CD3 and CD1d tetramer FMO (A), unloaded-CD1d tetramer as negative control (B), sulfatide-CD1d tetramer (C), lyso-sulfatide-CD1d tetramer (D), cardiolipin-CD1d tetramer (E) or TO CL-CD1d tetramer (F). G) Graphs showing percentage of iNKT cells stained with CD1d tetramer. Results are representative of data using iNKT cell lines generated from 3 healthy donors.
Figure 7.8. Frequencies of sulfatide-CD1d specific T cells are higher in peripheral blood from OAC patients than blood from non-cancer controls. PBMCs were isolated from OAC patients and non-cancer control subjects and stained with a mAb specific for CD3 and unloaded or sulfatide-loaded CD1d tetramer and analysed by flow cytometry. A-B) Flow cytometry dot plots showing unstained, FMO and unloaded and sulfatide-loaded CD1d tetramer staining by CD3⁺ cells from a non-cancer control (A) and an OAC patient (B). C) Scatterplot showing mean (± SEM) sulfatide-CD1d reactive T cell frequencies as percentages of CD3⁺ cells in blood from 9 non-cancer controls and 9 untreated patients with OAC. (*p<0.05 using the Mann-Whitney test compared to controls).
Figure 7.9. **Lyso-sulfatide-CD1d specific T cells were barely detectable in peripheral blood from OAC patients and non-cancer controls.** PBMCs were isolated from OAC patients and non-cancer control subjects and stained with a mAb specific for CD3 and unloaded or lyso-sulfatide-loaded CD1d tetramer and analysed by flow cytometry. **A-B** Flow cytometry dot plots showing unstained, FMO and unloaded and lyso-sulfatide-loaded CD1d tetramer staining by CD3+ cells from a non-cancer control (A) and an OAC patient (B). **C** Scatterplot showing mean (± SEM) lyso-sulfatide-CD1d reactive T cell frequencies as percentages of CD3+ cells in blood from 9 non-cancer controls and 9 untreated patients with OAC.
Figure 7.10. Cardiolipin-CD1d specific T cells were barely detectable in peripheral blood from OAC patients and non-cancer controls. PBMCs were isolated from OAC patients and non-cancer control subjects and stained with a mAb specific for CD3 and unloaded or cardiolipin-loaded CD1d tetramer and analysed by flow cytometry. A-B) Flow cytometry dot plots showing unstained, FMO and unloaded and cardiolipin-loaded CD1d tetramer staining by CD3+ cells from a non-cancer control (A) and an OAC patient (B). C) Scatterplot showing mean (± SEM) cardiolipin-CD1d reactive T cell frequencies as percentages of CD3+ cells in blood from 9 non-cancer controls and 9 untreated patients with OAC.
with OAC (4±1.3%) compared to non-cancer controls (1.1±0.3%), however this was not significant (p=0.07. Figure 7.11).

The frequencies of sulfatide, lyso-sulfatide, cardiolipin and TO CL reactive T cells were also studied as subpopulations of Vδ1 T cells in patients with OAC and non-cancer controls. PBMCs were stained with mAb specific for CD3, Vδ1 and unloaded or glycolipid-loaded CD1d tetramers and analysed by flow cytometry. No significant difference in the frequencies of Vδ1 T cells was observed between OAC patients (1.8±0.4%) and controls (0.8±0.2%, Figure 7.12).

Frequencies of sulfatide-reactive Vδ1 T cells and TO CL-reactive Vδ1 T cells were significantly higher in blood samples from patients with OAC compared to non-cancer controls. 31±6.3% of Vδ1 T cells from peripheral blood of OAC patients were sulfatide-reactive whereas 8.5±3.0% of Vδ1 T cells from non-cancer controls were detected by the sulfatide-loaded tetramer (p=0.003. Figure 7.13). Furthermore, 41.5±9.6% of Vδ1 T cells from OAC patients were TO CL-reactive, in comparison with 15±5.3% of Vδ1 T cells in controls (p=0.02. Figure 7.16). No significant differences were observed in frequencies of lyso-sulfatide-reactive Vδ1 T cells (Figure 7.14) and cardiolipin-reactive Vδ1 T cells (Figure 7.15) between OAC patients and non-cancer controls. These results show that type II NKT cells are expanded in patients with OAC.
Figure 7.11. No significant difference was observed in the frequencies of TO CL-specific T cells in peripheral blood of OAC patients and controls. PBMCs were isolated from OAC patients and non-cancer control subjects and stained with a mAb specific for CD3 and unloaded or TO CL-loaded CD1d tetramer and analysed by flow cytometry. A-B) Flow cytometry dot plots showing unstained, FMO and unloaded and TO CL-loaded CD1d tetramer staining by CD3+ cells from a non-cancer control (A) and an OAC patient (B). C) Scatterplot showing mean (± SEM) TO CL-CD1d reactive T cell frequencies as percentages of CD3+ cells in blood from 9 non-cancer controls and 9 untreated patients with OAC.
Figure 7.12. Vδ1 T cell frequencies are similar in peripheral blood from OAC patients and non-cancer controls. PBMCs were isolated from OAC patients and non-cancer controls and stained with mAb specific for CD3 and the Vδ1 TCR and analysed by flow cytometry. **A-B** Flow cytometry dot plots showing Vδ1 T cells from a non-cancer control subject (A) and an OAC patient (B). **C** Scatterplot showing mean (± SEM) Vδ1 T cell frequencies as percentages of CD3+ cells in blood from 9 OAC patients and 9 non-cancer controls.
Figure 7.13. Frequencies of sulfatide-CD1d-specific Vδ1 T cells are higher in peripheral blood from OAC patients than in blood from non-cancer controls. PBMCs were isolated from OAC patients and non-cancer controls and stained with mAbs specific for CD3 and Vδ1 TCR and a sulfatide-loaded CD1d tetramer and analysed by flow cytometry. A-B) Flow cytometry dot plots showing staining by the FMO, unloaded-CD1d tetramer and sulfatide-loaded CD1d tetramer by Vδ1+ T cells from a non-cancer cancer control subject (A) and an OAC patient (B). C) Scatterplot showing mean (± SEM) sulfatide-CD1d reactive T cell frequencies as percentages of Vδ1 T cells in blood from 9 controls and 9 OAC patients. (**p<0.01 using the Mann-Whitney test compared to controls).
Figure 7.14. No difference in the frequencies of lyso-sulfatide-CD1d-Vδ1 T cells in peripheral blood from OAC patients than non-cancer controls. PBMCs were isolated from OAC patients and non-cancer controls and stained with mAbs specific for CD3 and Vδ1 TCR and a lyso-sulfatide-loaded CD1d tetramer and analysed by flow cytometry. A-B) Flow cytometry dot plots showing staining by the FMO, unloaded-CD1d tetramer and lyso-sulfatide-loaded CD1d tetramer by Vδ1+ T cells from a non-cancer control subject (A) and an OAC patient (B). C) Scatterplot showing mean (±SEM) lyso-sulfatide-CD1d reactive T cell frequencies as percentages of Vδ1 T cells in blood from 9 controls and 9 OAC patients.
Figure 7.15. No difference in the frequencies of cardiolipin-CD1d-Vδ1 T cells in peripheral blood from OAC patients than non-cancer controls. PBMCs were isolated from OAC patients and non-cancer controls and stained with mAbs specific for CD3 and Vδ1 TCR and a cardiolipin-loaded CD1d tetramer and analysed by flow cytometry. A-B) Flow cytometry dot plots showing staining by the FMO, unloaded-CD1d tetramer and cardiolipin-loaded CD1d tetramer by Vδ1+ T cells from a non-cancer cancer control subject (A) and an OAC patient (B). C) Scatterplot showing mean (± SEM) cardiolipin-CD1d reactive T cell frequencies as percentages of Vδ1 T cells in blood from 9 controls and 9 OAC patients.
Figure 7.16. Frequencies of TO CL-CD1d-Vδ1 T cells are higher in peripheral blood from OAC patients than non-cancer controls. PBMCs were isolated from OAC patients and non-cancer controls and stained with mAbs specific for CD3 and Vδ1 TCR and a TO CL-loaded CD1d tetramer and analysed by flow cytometry. A-B) Flow cytometry dot plots showing staining by the FMO, unloaded-CD1d tetramer and TO CL-loaded CD1d tetramer by Vδ1+ T cells from a non-cancer control subject (A) and an OAC patient (B). C) Scatterplot showing mean (± SEM) TO CL-CD1d reactive T cell frequencies as percentages of Vδ1 T cells in blood from 9 controls and 9 OAC patients.
7.4.2 Tetramyristoyl cardiolipin induces pro-tumour response in Vδ1 T cells

A number of studies have provided evidence that type II NKT cells can release immunoregulatory cytokines that suppress antitumour immunity and promote tumour progression (Terabe and Berzofsky 2014). To test if the CD1d-restricted, glycolipid reactive Vδ1 T cells, identified above, release antitumour or protumour cytokines when activated with sulfatide, lyso-sulfatide, cardiolipin or TO CL, lines of Vδ1 T cells, generated from 3 healthy donors, were co-cultured with CD1d-transfected HeLa cells previously loaded with 1 or 10 μg/mL of the lipids for 18 h. Cells were stained with mAbs specific for cell-surface CD3 and the Vδ1 TCR and intracellular IFN-γ, IL-13, IL-4 and TGF-β and analysed by flow cytometry. No changes in the frequencies of Vδ1 T cells IFN-γ (Figure 7.17) or IL-13 (Figure 7.18) expression were observed when they were stimulated with sulfatide, lyso-sulfatide, cardiolipin or TO CL or the vehicle control DMSO. A non-significant induction of IL-4 production was observed in the presence of all glycolipids tested compared to the unstimulated controls. However, we found that DMSO, which was used as vehicle, non-significantly induced IL-4 expression by Vδ1 T cells (10.9±5.4%) compared to unstimulated controls (0.7±0.4%). No significant differences were observed in TGF-β expression in the presence of sulfatide (Figure 7.20B), lyso-sulfatide (Figure 7.20C) or cardiolipin (Figure 7.20D). On the contrary, 11.4±2.4% of Vδ1 T cells expressed TGF-β in the presence of the highest
Figure 7.17. Vδ1 T cells do not express IFN-γ response to sulfatide, lyso-sulfatide, cardiolipin or TO CL presented by CD1d. Lines of Vδ1 T cells from three different donors were co-cultured with CD1d-transfected HeLa cells, previously loaded with medium alone, 1 or 10 μg/mL of sulfatide, lyso-sulfatide, cardiolipin or TO CL or DMSO as vehicle control. A) Cells were stained with mAbs specific for CD3 and Vδ1 and intracellular IFN-γ and analysed by flow cytometry. B-E) Bar graphs show mean (± SEM) percentages of viable Vδ1 T cells that expressed IFN-γ in the presence of sulfatide (B), lyso-sulfatide (C), cardiolipin (D) or TO CL (E).
Figure 7.18. Vδ1 T cells do not express IL-13 in response to sulfatide, lyso-sulfatide, cardiolipin or TOCL presented by CD1d. Lines of Vδ1 T cells from three different donors were co-cultured with CD1d-transfected HeLa cells, previously loaded with medium alone, 1 or 10 μg/mL of sulfatide, lyso-sulfatide, cardiolipin or TO CL or DMSO as vehicle control. A) Cells were stained with mAbs specific for CD3 and Vδ1 and intracellular IL-13 and analysed by flow cytometry. B–E) Bar graphs show mean (± SEM) percentages of viable Vδ1 T cells that expressed IL-13 in the presence of sulfatide (B), lyso-sulfatide (C), cardiolipin (D) or TO CL (E).
Figure 7.19. Vδ1 T cells do not express IL-4 in response to sulfatide, lyso-sulfatide, cardiolipin or TO CL, presented by CD1d. Lines of Vδ1 T cells from three different donors were co-cultured with CD1d-transfected HeLa cells, previously loaded with medium alone, 1 or 10 μg/mL of sulfatide, lyso-sulfatide, cardiolipin or TO CL or DMSO as vehicle control. A) Cells were stained with mAbs specific for CD3 and Vδ1 and intracellular IL-4 and analysed by flow cytometry. B-E) Bar graphs show mean (± SEM) percentages of viable Vδ1 T cells that expressed IL-4 in the presence of sulfatide (B), lyso-sulfatide (C), cardiolipin (D) or TO CL (E).
concentration of TO CL, significantly higher than the unstimulated control (1±0.6%, \( p = 0.03 \)). This confirms the previous results where Vδ1 T cells can recognise TO CL and further show that TO CL-stimulated Vδ1 T cells produce TGF-β. TO CL-CD1d-specific Vδ1 T cells were found at higher frequencies in OAC patients than in non-cancer controls, indicating that Vδ1 T cells could be contributing to tumour growth by the production of the pro-tumour cytokine TGF-β that activates Tregs, and regulates CD8+ T cells (Kato, Berzofsky, and Terabe 2018).

### 7.5 Discussion

NKT cells recognise both self and foreign lipid antigens presented by CD1d. Activation of type 1 NKT cells (iNKT cells) in response to α-GalCer has been widely studied, however little is known about glycolipid presentation and activation of type II NKT cells. The lack of a specific marker to detect type II NKT cells makes this task difficult. In the present study, frequencies of type II NKT cells in peripheral blood from patients with OAC were detected for the first time using CD1d tetramers loaded with sulfatide, lyso-sulfatide, cardiolipin and TO CL.

In 2004, sulfatide was the first lipid to be described to bind to CD1d and activate type II NKT cells (Terabe and Berzofsky 2014). Sulfatide is present in different organs in the human body, including kidney, gastrointestinal tract, islet of Langerhans, trachea and mainly in the brain (Takahashi and Suzuki 2012). Sulfatide has several functions in normal tissue. It plays an important role in the nervous system in myelin formation and
Figure 7.20: TO CL induces TGF-β expression by Vδ1 T cells. Lines of Vδ1 T cells from three different donors were co-cultured with CD1d-transfected HeLa cells, previously loaded with medium alone, 1 or 10 μg/mL of sulfatide, lyso-sulfatide, cardiolipin or TO CL or DMSO as vehicle control. A) Cells were stained with mAbs specific for CD3 and Vδ1 and intracellular TGF-β and analysed by flow cytometry. B-E) Bar graphs show mean (± SEM) percentages of viable Vδ1 T cells that expressed TGF-β in the presence of sulfatide (B), lyso-sulfatide (C), cardiolipin (D) or TO CL (E). (*<0.05 using the paired t test).
maintenance (Takahashi and Suzuki 2012; Marcus et al. 2006). Sulfatide has been found to be present at higher concentrations in cancer tissue in humans, such as lung adenocarcinoma, renal carcinoma, ovarian carcinoma, colorectal cancer and gastric cancer (Takahashi and Suzuki 2012). In the present study, higher frequencies of type II NKT cells reactive to sulfatide presented by CD1d were found in OAC patients compared to non-cancer controls. Sulfatide can bind to CD1d and activates type II NKT cells, including Vδ1 T cells, inducing the production of IL-13 and TGF-β, which can enhance tumour growth (Terabe et al. 2000). Sulfatide can also promote tumour metastasis by inducing P-selectin expression and integrin αVβ5-mediated signalling, resulting in adhesion of cancer cells to P-selectin-expressing platelets in vitro in humans and in murine models (Suchanski et al. 2018; Garcia, Callewaert, and Borsig 2007; Cao et al. 2018). UDP glycosyltransferase 8 (UGT8) is an enzyme that catalyses the transfer of galactose to ceramide and it is also a marker for breast cancer malignancy and lung metastasis (Dziegiel et al. 2010). UGT8 expression triggers the sulfatide biosynthetic pathway (Cao et al. 2018). Cao et al. showed that inhibition of UGT8 suppresses breast cancer progression by the attenuation of sulfatide activation of integrin (Cao et al. 2018). Sulfatide is not the only glycolipid known to bind to type II NKT cells, Fuss et al. reported that lyso-sulfatide reactive type II NKT cells are present in patients with ulcerative colitis and unlike liver type II NKT cells, these cells produce IL-13 and not IFN-γ (Fuss et al. 2014).
Lyso-sulfatide-CD1d positive cells were barely detectable in peripheral blood from OAC patients and non-cancer controls. This suggests that the frequencies of this subset of type II NKT cells are very low in peripheral blood in humans, but they may be higher in other organs. No activation of expanded Vδ1 T cells was observed in the presence of sulfatide or lyso-sulfatide contrary to what has been reported (Dhodapkar and Kumar 2017). This might be because of the pH of the medium, since Roy et al. demonstrated that acidification (pH 5 or 6) enhances type II NKT cell activation (Roy et al. 2008). More experiments should be carried out controlling the pH to optimise activation of peripheral type II NKT cells with sulfatide or lyso-sulfatide.

Cardiolipin is a lipid present in the inner mitochondria and it is associated with the membrane design to generate the electrochemical gradient responsible of the production of ATP (Paradies et al. 2014). Cardiolipin also interacts with different proteins playing a role in the respiratory chain, and it is part of the mitochondrial apoptosis process (Paradies et al. 2014). To date, no tumour has been found with a normal content of cardiolipin (Kiebish et al. 2008). Murine Vδ1 T cells that recognise cardiolipin have previously been reported (Tatituri et al. 2013). Here we show that human Vδ1 T cells do not recognise cardiolipin. However, a high frequency of cells recognise the cardiolipin standard TO CL in peripheral blood of OAC patients and controls. TO CL is a synthetic glycolipid derived from cardiolipin. The ability of type II NKT cells to recognise this synthetic
glycolipid and our finding that TO CL-specific T cells are found more frequently in OAC patients compared to healthy donors suggests that the human body may have an endogenous glycolipid similar in structure to TO CL. Murine γδ T cells (which differ substantially from human Vδ1 T cells) are able to promote tumour progression via the production of IL-17 in mice (Kimura et al. 2016). Human Vδ1 T cells can also produce TGF-β (Kuhl et al. 2009) and IL-10 (Mao et al. 2016) in response to treatment with of IL-4. TGF-β is synthesized and secreted as a large protein complex TGF-β and LAP (Khalil 1999). LAP noncovalently binds to TGF-β to prevent its activity (Khalil 1999). LAP must be released from TGF-β or undergo conformational changes allowing TGF-β to bind to its receptor (Khalil 1999). TGF-β production in the presence of TO CL, suggests that these cells can promote tumour growth by the regulation of effector cells.

In conclusion, higher frequencies of sulfatide and TO CL reactive T cells in OAC patients than controls suggest that these cells play an important role in the tumour progression, potentially through the production of TGF-β and the further regulation of CD8+ T cells and iNKT cells, furthermore type II NKT cells should be taken into consideration when planning immunotherapies using iNKT cells.
Chapter 8

General discussion
8.1 General discussion

With an estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018, cancer in general is one of the leading causes of death worldwide (Bray et al. 2018). Gastric cancer (GAC) and oesophageal cancer (OC) account for 5.7% and 3.2% of new cancers diagnosed. GAC and OC are in third and sixth place of cancer related death, claiming over 1.3 million deaths in 2018 worldwide (Bray et al. 2018). Incidence of OC and GAC is higher in men than women, being more prevalent in men over 40 years of age (Bray et al. 2018).

Current treatments for upper GI cancers normally involve a combination of surgery, chemotherapy and radiotherapy. Cisplatin, 5-FU, carboplatin and paclitaxel are the most common chemotherapy drugs used for upper GI cancers (Donohoe and Reynolds 2017). Due to the non-specific symptoms such as dysphagia, weight loss, chest pain, worsening indigestion, hoarseness, persistent nausea, vomiting, swollen abdomen and tiredness (Harris, Croce, and Munkholm-Larsen 2017; Correa 2014), patients are frequently diagnosed at a late stage, leaving them with limited treatment options and many patients only receiving palliative care (Russell 2016). Even though several advances in surgical techniques, preoperative care and neoadjuvant treatment strategies have been made, OC and GAC are still associated with poor survival, with a long-term survival beyond 5 years considered uncommon (Ghaly et al. 2016). Therefore, new improved treatments are urgently needed.
Natural protection against cancer is largely mediated by the immune system. Most immunotherapies for cancer are based on the development of inhibitory antibodies which modulate immune checkpoints (Raufi and Klempner 2015). Overexpression of HER-2 is related with poor prognosis in cancer. 30-80% of OAC patients exhibit overexpression of HER-2 (Milano et al. 2010). Trastuzumab is a monoclonal antibody that targets HER-2 and was the first molecularly targeted agent to improve outcome in OC and GAC (Bang et al. 2010). Ramucirumab and pembrolizumab have also been approved for GAC targeting VEGF and PD-1 respectively (Casak et al. 2015; Fashoyin-Aje et al. 2019). Several clinical trials in phases I to III using monoclonal antibodies, alone or in combination with chemotherapy drugs, are currently being carried out in patients with upper GI cancer (Procaccio et al. 2017). Clinical trials are also being carried out using CAR-T cells genetically manipulated to express protein-fusion-derived chimeric antigen receptor in OC and GAC targeting molecules such as HER2, EPCAM, MUC1 and PD1 (Bridgeman et al. 2010; Heczey et al. 2014; Zhao, Chen, et al. 2018). However, thus far in most of these trials the primary endpoint is toxicity (Procaccio et al. 2017).

iNKT cells are a rare population of T lymphocytes, characterised by the expression of NK markers and an invariant TCR that recognizes glycolipids presented by the MHC class I-like molecule, CD1d. iNKT cells are able to kill, prevent and reverse tumour growth (Cui et al. 1997). Cui et al. showed for the first time that iNKT cells play an important role in
antitumour immunity, where iNKT cell-deficient mice were unable to reject tumours via IL-12 (Cui et al. 1997). When iNKT cells were transferred from wild type into Jα18-KO mice, protection against cancer was restored (Bellone et al. 2010). Following presentation by DCs, α-GalCer activates iNKT cells, resulting in a release of Th1 and Th2 cytokines (Kawano et al. 1998).

In contrast to the successful response in mice, human immunotherapy using iNKT cells has shown little therapeutic benefit (Exley and Nakayama 2011). Even though the use of α-GalCer has no toxic effect in patients, only 7 out of 24 patients with solid tumours showed disease stabilisation in response to α-GalCer in a phase I trial (Giaccone et al. 2002), whereas no patients with NSCLC achieved either partial or complete response when α-GalCer-pulsed DCs were used (Ishikawa et al. 2005). Three of nine patients with advanced melanoma were progression-free when autologous iNKT cells were expanded in vitro in the presence of GM-CSF (Exley et al. 2017). The difference in the response between mouse and human may be the result of many factors including the difference in iNKT cell frequencies between the two species, the complexity of human tumours or the advance stage of the disease.

Another reason for the low response to iNKT cells therapy in humans may be the use of the incorrect iNKT subtypes. Lynch et al. reported that iNKT cells make up to 10 of the cells in omentum compared to 0.1% in peripheral blood (Lynch et al. 2009). The first aim of this study was to
investigate the frequencies of iNKT cells in peripheral blood and omentum from patients with OAC, SCC and GAC to determine if they are depleted in these patients, implying that they may benefit from iNKT cell-based therapies, and to ascertain if omentum could be a potential source of iNKT cells for therapeutic use. iNKT cells were found to be significantly depleted in peripheral blood from cancer patients compared to controls, similar to what has been previously reported in other cancers such as rectal, liver, breast, renal, prostate and lung cancers, malignant melanoma and chronic lymphocytic leukaemia (Molling et al. 2008; Motohashi et al. 2009). Percentages of omental iNKT cells from patients were higher than in peripheral blood, but not as high as previously reported by Lynch et al. iNKT cell frequencies were not altered by treatment, sex or TRG. Lower numbers of IFN-γ-secreting iNKT cell have been reported in cancer patients independent of tumour type or stage of the cancer and viable iNKT cells exhibited impaired IFN-γ production but released similar levels of IL-4 compared to healthy controls (Tahir et al. 2001; Molling et al. 2005). Low numbers of iNKT cells in patients with cancer, such as acute myeloid leukaemia, head and neck squamous cell carcinoma have been associated with poor survival (Najera Chuc et al. 2012; Molling et al. 2007). Low iNKT cell frequencies in patients with OC and GAC observed in this study suggests that iNKT cells may play a role in antitumour immunity in OC and GAC. Restoring iNKT cell frequencies in patients with OC and GAC may have a beneficial response against the tumour.
Tumour rejection by iNKT cells can occur in one of two ways, 1) by indirectly killing tumour cells when activated by APCs expressing CD1d, leading to the trans-activation of NK and T cells (Parker, Rautela, and Hertzog 2016), or 2) by direct recognition and killing of CD1d-bearing tumour cells (Parker, Rautela, and Hertzog 2016). Myelomonocytic and lymphoma cells, prostate cancer, breast cancer, renal cell carcinoma are the most common cancers to express CD1d, and can be targeted for iNKT cell-based immunotherapy (Nowak et al. 2010; Hix et al. 2011; Chong et al. 2015; Dhodapkar et al. 2004; Liu, Song, Brawley, et al. 2013).

Tumour cells can downregulate MHC class I molecules and evade immunosurveillance (Algarra et al. 2004). Using the KMPlot data base, we showed that low CD1d expression in GAC is associated with lower overall survival. In general, CD1d mRNA expression in gastric cancer cells is found at similar levels to most other cancers (Uhlen et al. 2015). Downregulation of CD1d expression may be an escape mechanism used by tumour cells to avoid killing by iNKT cells. Molecules such as all-trans retinoic acid and certain chemotherapies are able to induce CD1d expression (King et al. 2018; Ghnewa et al. 2017), which makes it of interest to combine these molecules with iNKT cell-based immunotherapies to boost responses.

Another reason for the failure of iNKT immunotherapy may be due to the first line treatment the patients receive. Current treatment for patients with upper GI cancers involves radiotherapy and chemotherapy (Donohoe
and Reynolds 2017). The effect of chemotherapy on CD1d expression was investigated in this study. CD1d expression by the oesophageal cancer cell, OE33, was significantly reduced when treated with cisplatin, but not 5-FU, carboplatin or paclitaxel, making OE33 cells less likely to be recognised and killed by iNKT cells. Further studies are required to determine if cisplatin, 5-FU, carboplatin and paclitaxel can inhibit CD1d expression by primary tumour cells. Additionally, we observed no effect of these drugs on CD1d expression by traditional APCs such as B cells and monocytes.

Chemotherapy and radiotherapy are known to cause lymphopenia in patients, affecting the adaptive immune response of the patient (Rebe and Ghiringhelli 2015). Even though immunotherapy for cancer has showed some success, there are still some limitations regarding response rates and duration of therapy. Several studies have shown the potential synergy of immunotherapy with other treatments (Tseng et al. 2011; Joshi and Durden 2019; Ramakrishnan et al. 2010; Spiotto, Fu, and Weichselbaum 2016). Some chemotherapies including, cyclophosphamide, cisplatin, 5-FU, gemcitabine, carboplatin and paclitaxel induce upregulation of MHC class I expression, recruitment and proliferation of effector T cells and macrophages, downregulation of the immunosuppressive microenvironment and increases in tumour cell-susceptibility to granzyme B (de Biasi, Villena-Vargas, and Adusumilli 2014; Weir, Liwski, and Mansour 2011). Radiation boosts the immune
response by increasing antigen cross presentation, enhancing expression of the co-stimulatory immune checkpoint molecule CD137 (Rodriguez-Ruiz et al. 2016) and releasing cytokines such as IL-6, IL-8, IFN-γ and IL-1α from endothelial cells, fibroblasts, immune cells and parenchymal cells (Spiotto, Fu, and Weichselbaum 2016; Diegeler and Hellweg 2017). Radiation also induces the expression and secretion of danger associated molecular patters (DAMPs) such as HMGB1 and calreticulin and induces trafficking of immune cells to the tumour site (Spiotto, Fu, and Weichselbaum 2016; Ni et al. 2012; Gasser et al. 2005; Rodriguez-Ruiz et al. 2016; Ahmed et al. 2013). Currently, only 30% of OC patients and 25% of GAC patients respond to CRT (Ferlay, Shin et al. 2010, Achilli, De Martini et al. 2017). It is of major clinical relevance to optimise and understand the effect of standard and novel therapies in combination.

The direct effect of chemotherapies and radiotherapy for upper GI cancers on iNKT cells was showed for the first time in this present study. These drugs induced apoptosis of iNKT cells, similar to what has been previously reported for CD4 and double positive T cells (Hatziveis et al. 2012). However, contrary to what has been previously reported in the literature where the mixture of 5-FU, epirubicin and cyclophosphamide or 5-FU, doxorubicin and cyclophosphamide increased T cell and NK cell numbers (Wijayahadi et al. 2007), we found a decrease in iNKT cell numbers following treatment with 5-FU. Interestingly, iNKT cells were found to be more resistant to cell death when treated with 5-FU, carboplatin and paclitaxel than OE33 cells.
The function of the viable iNKT cells post chemotherapy treatment was also tested. In the present study, 5-FU and paclitaxel reduced CD107a expression. 5-FU and paclitaxel are known to increase ROS production by cells (Focaccetti et al. 2015; Alexandre et al. 2006). ROS increases iNKT cell susceptibility to apoptosis by inducing oxidative stress (Kim et al. 2017). High levels of ROS reduce IFN-γ and increase IL-4 production in hepatic iNKT cells (Kim et al. 2017). Therefore, it is possible that iNKT cell function is diminished by the presence of high levels of ROS as a consequence of treatment with 5-FU and paclitaxel (Figure 8.1C).

Lower numbers of NK cells have previously been reported in patients treated with cisplatin (Kubota et al. 2001). In the present study, cisplatin induced apoptosis of iNKT cells, potentially through the reduction of PI9 expression, a serine protease that protects lymphocytes from autologous and allogenic granzyme B (Ansari et al. 2010). We also found that cisplatin induced an increase in granzyme B expression and a decrease in PI9 expression, making iNKT cells susceptible to killing by granzyme B. In addition to a reduction in viability, cisplatin also reduced expression of CD107a and the Th1 cytokine IFN-γ (Figure 8.1B). The increase in cell death and inhibition of functions of iNKT cells by cisplatin suggests that patients would not benefit from iNKT cell-based immunotherapy if they are receiving cisplatin as first-line treatment, but iNKT cells treated with 5-FU, carboplatin and paclitaxel remained functionally viable, showing that different chemotherapies have different effects on immune cells. The
results observed in this study suggest that the combination of these drugs with iNKT cell-based immunotherapy could be a plausible course of treatment, but viability and number of iNKT cells should be considered.

Around 75% of cancer patients will receive radiotherapy as part of their treatment (Guo et al. 2013). Radiation is split into several fractions to reduce the normal cell toxicity (Bentzen 2006). Patients with oesophageal cancer receive 23 daily fractions of 1.8Gy to a total of 41.4Gy (Donohoe and Reynolds 2017). Radiation induces double strand breaks in the DNA of rapid dividing cells including lymphocytes (Maier et al. 2016). In this study, the direct effect of radiation on iNKT cells was studied for the first time. Single doses of 2Gy and 10Gy radiation caused double strand breaks in the DNA of iNKT cells inducing cell death. However, iNKT cells were able to repair the DNA when treated with 2Gy radiation. Degranulation, IL-4 and granzyme B production were not altered when radiated with single doses of 10Gy. However, IFN-γ expression was reduced when treated with single doses or five fractionated doses of 2Gy. Similar to 5-FU, radiation induces the production of ROS (Desouky, Ding, and Zhou 2015) and interferes with IFN-γ, but not IL-4 production. iNKT cells were still able to produce granzyme B and perforin post radiation indicating that the direct antitumour activity by iNKT cells was still intact, however iNKT cells did not reach same levels of expansion and eventually died after radiation (Figure 8.1D). Patients with OC and GAC receive different therapeutic regimens depending on disease type, stage and previous response to
Figure 8.1. Effect of chemotherapy and radiotherapy on iNKT cells. A) Upon activation iNKT cells produce IFN-γ activating an immune response against the tumour, while iNKT cells also produce cytotoxic molecules killing tumour cells directly. B) Cisplatin reduces viability of iNKT cells, impairs IFN-γ production by iNKT cells and the immune response by NK cells and CD8 T cells, however, it increases granzyme B and perforin production. C) Paclitaxel, carboplatin and 5-FU reduce viability of iNKT cells, but do not affect functions. D) Radiation reduces viability of iNKT cells and impairs IFN-γ production, but do not alter granzyme B or perforin expression.
treatment. Further studies are warranted to investigate the effects of combination therapies, mostly chemotherapies, radiotherapies and immunotherapies on host immunity.

Most trials using iNKT cells have been carried out in patients with advanced disease where no standard therapy was available or given simultaneously with iNKT cell-treatment. Even though 5-FU, carboplatin, paclitaxel and cisplatin have relatively short half-lives, the lowest cell blood count in patients is detected around 20 days after treatment with these drugs (MacDonald 2009; Chemocare.com 2019). Radiation has a longer effect, with cells dying for weeks or months after the therapy ends (Saito et al. 2017). In this study we showed the negative effect of these therapies on iNKT cells, suggesting that autologous cells derived from the patients to be used as immunotherapy have been previously damaged by the treatment, and may explain why the iNKT cell immunotherapy has not been successful. Therefore, iNKT cells isolated and manipulated from healthy controls may be a better option. Although cisplatin, 5-FU, carboplatin, paclitaxel and radiotherapy are known to boost the immune system. This study showed that these treatments have a temporary negative effect on iNKT cells suggesting that the use of iNKT cell-immunotherapy in combination with chemotherapy, especially cisplatin or radiotherapy would not be the optimal course of treatment. Therefore, further studies are required before treatment with both therapies can be combined.
α-GalCer was the first glycolipid discovered to activate iNKT cells and has been used to elucidate the role of iNKT cells (Bendelac, Savage, and Teyton 2007). Upon activation with α-GalCer, iNKT cells kill target cells and secrete a diverse range cytokine including IFN-γ, IL-4, IL-10 and IL-13 (Gumperz et al. 2002; O’Reilly et al. 2011). Wang et al demonstrated that α-GalCer reduced iNKT cell frequencies and proliferative responses in mice, as a result of over activation and anergy (Wang et al. 2019; Parekh et al. 2005). This feature and the ability of α-GalCer to induce both Th1 and Th2 cytokine responses make this molecule a less than an ideal therapy for cancer treatment. Several α-GalCer analogues have been synthesized to selectively induce a Th1 response in iNKT cells. Our group previously synthesized and studied a thioglycoside analogue of α-GalCer, denoted α-S-GalCer, which binds to CD1d and stimulate human iNKT cells but did not skew iNKT cell responses towards Th1 (Hogan et al. 2011). In the present study four novel glycolipids analogues of α-GalCer were synthesized and biologically tested. Only two, XZ7 and XZ11 activated iNKT cells inducing cytokine production. XZ7 induced degranulation and intracellular IFN-γ but not IL-4, production and secretion by CD8α+ T cells, confirming the potential antitumour activity of this novel glycolipid in vitro (Melo et al. 2018). The ability of XZ7 to stimulate only a Th1 response makes this glycolipid a good compound for the development of more analogues that induce a similar cytokine profile, but with more potency. The release of the cytotoxic molecules granzyme B and perforin and other cytokines should be tested when future analogues of XZ7 are used to
confirm the polarization of the cells. These novel analogues could also be used to expand and examine iNKT cell antitumour functions in vitro as a replacement of α-GalCer (Figure 8.2B-C).

iNKT cells are not the only CD1d-restricted, glycolipid specific T cells found in humans. Type II NKT cells recognise glycolipids presented by the CD1d molecule, however, these cells do not carry invariant TCR or recognise α-GalCer. Sulfatide was the first lipid discovered to activate type II NKT cells (Terabe and Berzofsky 2014). After activation with sulfatide, type II NKT cells produce IL-13 and TGF-β, which promote tumour progression. Low frequencies of type II NKT cells is associated with the development of autoimmune diseases including multiple sclerosis (MS), type 1 diabetes, ulcerative colitis, inflammatory bowel disease (IBD) and Gaucher's disease (Singh, Tripathi, and Cardell 2018). On the contrary higher levels of type II NKT cells have been reported in colon, ovarian and gastric cancers (Singh, Tripathi, and Cardell 2018; Terabe et al. 2000). Type II NKT cells are not widely studied due to the lack of specific antibodies that can be used to detect these cells by flow cytometry. CD1d tetramers loaded with different glycolipids have helped to overcome this limitation. In the present study, type II NKT cells were enumerated in OAC patients for the first time using a CD1d tetramer loaded with sulfatide and other glycolipids thought to activate type II NKT cells, including lyso-sulfatide, cardiolipin or tetracyrystoyl cardiolipin (TO CL). A subpopulation of Vδ1 T cells is also known to be type II NKT cells, where at least 0.5% of human Vδ1 T cells recognise sulfatide presented by CD1d (Bai et al. 2012).
Sulfatide is known to be in higher levels in cancer patients, including GAC patients. Sulfatide helps in the adhesion of *Helicobacter pylori* to GAC cells (Kamisago et al. 1996; Takahashi and Suzuki 2012). Activation of type II NKT cells in the presence of sulfatide in combination with lower levels of iNKT cells in GAC and OC patients suggests that type II NKT cells have a role in tumour development.

In the present study, the cytokine profile of type II NKT cells was measured by stimulating Vδ1 T cells using CD1d-expressing APC presenting these lipids *in vitro*. Higher frequencies of type II NKT cells reactive to sulfatide and TO CL, presented by CD1d, were found in peripheral blood of OAC patients compared to controls. Previous studies have reported that sulfatide expressed on cancer cells binds to P-selectin, promoting metastasis in breast cancer patients (Cao et al. 2018). It would be of interest to compare type II NKT cell frequencies in these patients with the TRG and follow the response to treatment. Previous reports have shown the presence of cardiolipin reactive type II NKT cells in mice (Tatituri et al. 2013). Cells reactive to lyso-sulfatide and cardiolipin were barely detectable in both OAC patients and controls in this study. No cardiolipin reactive cells have been reported in humans. When these lipids were used to activate type II NKT cells, an increase of TGF-β, but not IFN-γ, IL-4 or IL-13 production was observed in the presence of TO CL. Higher frequencies of TO CL reactive cells in OAC patients and TGF-β expression in the presence of this glycolipid suggest that endogenous glycolipids similar to TO CL may activate type II NKT cells promoting a protumour
response (Figure 8.2D). This glycolipid may in future be used to study the role and target of type II NKT cells in cancer, and as a possible therapeutic target for autoimmune diseases.

Type II NKT cells are thought to regulate iNKT cells and vice versa (Kato, Berzofsky, and Terabe 2018). Mechanisms of cross regulation between type II NKT cells and iNKT cells have yet to be elucidated but it is believed that type II NKT cells inactivate iNKT cells possible by the indirect inactivation of other myeloid cells and neutrophils (Dhodapkar and Kumar 2017). Lower frequencies of iNKT cells and higher frequencies of type II NKT cells in peripheral blood from patients with OAC, suggests that type II NKT cells may inhibit iNKT cells, promoting cancer development. iNKT cells and type II NKT cells recognise glycolipids presented by CD1d, so it is of interest to study if novel glycolipids known to activate iNKT cells can also be recognised by type II NKT cells leading to the regulation of iNKT cells. Currently sulfatide is the only glycolipid known to be recognised by both iNKT cells and type II NKT cells (Stax et al. 2017). In the present study, limited or no iNKT cells were detected to recognise sulfatide, presumably because iNKT cells were expanded using α-GalCer, making these cells specific for α-GalCer. The design of a novel glycolipid capable of only activating iNKT cells and inhibiting type II NKT cells would be desirable for cancer treatments.
Figure 8.2 Activation of NKT cells by glycolipids. A) Upon activation by α-GalCer iNKT cells produce different cytokines and cytotoxic molecules leading to tumour rejection. B) XZ7 induces IFN-γ production, but not IL-4 by iNKT cells promoting the activation of other immune cells. C) iNKT cells produce IL-4 in the presence of XZ11, but not IFN-γ. D) Sulfatide and TO CL are recognised by type II NKT cells, but not iNKT cells, these glycolipids induce TGF-β production by type II NKT cells.
9.1 Conclusion

Decreased frequencies of circulating iNKT cells and increased numbers of type II NKT cells characterise upper GI cancer, suggesting a role for these cells in the pathogenesis of these cancer types. Restoring and expanding iNKT cells frequencies may help boost antitumour immunity. Type II NKT cells should be considered when planning clinical trial and treatment with iNKT cells, as well as the negative effect of chemotherapy and radiation on iNKT cell viability and function. Glycolipids others than α-GalCer should be considered and the novel synthesised glycolipid XZ7 could be used as a parental glycolipid for the design of more glycolipids. These results explain some of the reasons why iNKT cell immunotherapy has not been successful in humans to date but should be considered in future clinical trials as viable therapies.

10.1 Future directions

In recent years the use of CAR T cells as possible treatment has gained recognition. CAR-NK and CAR-iNKT cells have several advantages over CAR-T cells, particularly in solid tumours where the target antigen is unknown, as CAR-NK and iNKT cells are not MHC dependent. A common immune-escape mechanism by tumour cells is the downregulation of HLA molecules, making MHC-dependent killing of tumour cells by T cells ineffective. Therefore, CAR-iNKT cells may be able to limit the risk of tumour clonal escape (Rotolo et al. 2019). Since CD1d does not exhibit polymorphic variation, iNKT cells may be used in the allogeneic setting,
allowing for ‘off the shelf’ therapies, rather than personalised treatments with autologous cells (Heczey et al. 2014). Allogenic iNKT cells can be easily expanded using anti-CD3 mAb and IL-2 and engineered into CAR-iNKT cells targeting specific cancer cells (Exley et al. 2017; Heczey et al. 2014). CAR-iNKT cells modified to target specific cancers cells, could be the future for upper GI cancer treatment. The results of the present study show that, before this can be achieved, the effects of first line treatments, the choice of antigenic glycolipid, and the potential regulatory effects of type II NKT cells require careful consideration.
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Appendix