Integrating immune cell functions and apoptosis-based therapies for the treatment of ovarian cancer

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MEDICINE OF IMPERIAL COLLEGE LONDON IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Abstract

High levels of regulatory T cells (Treg cells) restrict immune responses against cancer cells and are associated with adverse outcomes in patients with ovarian cancer. Treg cell development and function is regulated by the transcription factor Foxp3. Studies into the regulation of Foxp3 may identify therapeutic targets that reduce the suppressive functions of Treg cells and enhance immune responses against cancer cells. Immune cells express TNF-related apoptosis-inducing ligand (TRAIL) which can induce the death of cancer cells, whilst sparing normal tissues. The TRAIL receptor (R) agonistic drug Apo2L/TRAIL/Dulanermin, and the TRAIL-R2-specific antibody, conatumumab (AMG 655), are potential treatments for ovarian cancer. The first part of the study investigated the role of Foxo and Kruppel-like factor 2 (Klf2) transcription factors in the regulation of Foxp3. It was found that the overexpression of Foxo factors promoted TGFβ-mediated induction of Foxp3 expression in activated CD4 T cells. However, the overexpression of Klf2 antagonised the induction of Foxp3 expression by TGFβ and PI3K/Akt/mTOR inhibition. The second part of the study investigated the potential of TRAIL as a treatment for ovarian cancer. Although most primary ovarian cancer cells were resistant to TRAIL-induced apoptosis, the majority could be sensitised to TRAIL by SMAC (second mitochondrial-derived activator of caspases) mimetic treatment or proteasome inhibition. Fcγ Receptors (FcγR) are expressed on immune cells within the ovarian cancer tumour microenvironment. The *in vivo* agonistic, apoptosis-inducing activity of TRAIL-R2-specific antibodies, such as AMG 655, is dependent on FcγR mediated crosslinking. However, FcγR-expressing immune cells were inefficient at enabling AMG 655-induced apoptosis. It was investigated whether AMG 655 could block recombinant forms of TRAIL and thus prevent immune cells from killing cancer cells. However, potent synergy was found between AMG 655 and Apo2L/TRAIL in killing cancer cells via TRAIL-R2. These results suggest that AMG 655 and Apo2L/TRAIL/dulanermin could be combined with a SMAC mimetic drug or proteasome inhibitor to introduce a highly active TRAIL-R agonistic therapy into the cancer clinic. Therapeutic strategies that target Foxo factors or Klf2 in Treg cells; and those that combine AMG 655 and Apo2L/TRAIL/dulanermin have the potential to lead to improved outcomes in patients with ovarian cancer.

Declarations

Declaration of Originality

I, Dr Mark Tuthill, declare that this PhD Thesis is my own work and has not been submitted in any form for another degree at any university or other institute of tertiary education. All information derived from the published and unpublished work of others has been acknowledged in the text and a list of references is provided.

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Conatumumab (AMG 655) Master Materials and Funding Agreement

The research conducted with conatumumab (AMG 655) that is reported within this thesis is subject to a Master Materials and Funding Agreement between Imperial College of Science Technology and Medicine at Exhibition Road, South Kensington, London, SW7 2AZ, together with its Affiliates (the “Institution”) and Amgen Inc. at One Amgen Center Drive, Thousand Oaks, California 91320-1799, together with its Affiliates (“Amgen”). The results from the research conducted with conatumumab (AMG 655) must remain confidential, unless disclosed according to the terms of the Master Materials and Funding Agreement between Imperial College of Science Technology and Medicine and Amgen Inc..

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Chapter 1. Introduction

1.1 Ovarian cancer

Ovarian cancer affects around 6500 women and is responsible for around 4000 deaths in the United Kingdom each year. Patients with ovarian cancer typically present with non-specific symptoms such as abdominal pain, bloating, constipation, urinary symptoms and back pains, which often delays diagnosis and referral for treatment. Around 30% of patients with ovarian cancer are diagnosed with early stage FIGO (International Federation of Gynaecology and Obstetrics) stage I ovarian cancer, that is curable in around 90% of patients with surgery and adjuvant chemotherapy. However, the majority of patients are diagnosed with advanced ovarian cancer (FIGO stage III and IV) where survival is limited to only around 20% of all patients at 5 years after diagnosis.

Outcomes for patients with ovarian cancer have changed little since the introduction of cisplatin-based chemotherapy regimens in the 1980's (Omura et al., 1986), with only 37% of patients surviving 5 years after diagnosis (Coleman et al., 2011). Therefore despite decades of scientific research and clinical studies, there is currently a significant need for new treatments for ovarian cancer.

1.1.1 The pathology of ovarian cancer

The major histological subtypes of epithelial ovarian cancer are high-grade-serous (about 68% of total cases), low-grade-serous (about 3% of total cases), endometrioid (about 11% of total cases), clear cell (about 12% of total cases), mucinous (about 3% of total cases), and rare subtypes (about 1% of total cases) (Seidman et al., 2004, Kobel et al., 2010). For many decades the cellular origins of the different subtypes of ovarian cancer were obscure and all lesions were thought to arise from the ovarian surface epithelium. This was despite differences in the clinical outcomes of the different subtypes of ovarian cancer, different histopathogical, and molecular characteristics or each subtype of the disease, and the lack of precursor lesions within the ovary. This has led to a clinical paradigm in which all patients with ovarian cancer are currently offered the same treatment, regardless of their subtype of ovarian cancer (Kobel et al., 2008).

1.1.2 The origins of ovarian cancer

Recent studies have shown that the majority of the different subtypes of ovarian cancer do not arise from the ovary, but in fact arise from neighbouring tissues. It has now become widely accepted that 'ovarian cancer' is an umbrella term that describes a group of diseases with distinct pathological and molecular characteristics that affect the ovaries of women (Vaughan et al., 2011, Berns and Bowtell, 2012).

High-grade serous ovarian cancer (HGS-ovarian cancer) is the most common and lethal of all the subtypes of ovarian cancer. The cellular origins of HGS-ovarian cancer were determined from pathological and molecular studies of fallopian tubes which were removed from women with familial BRCA1 (breast cancer 1, early onset) or BRCA2 (breast cancer 2, early onset) mutations who were undergoing prophylactic ovarian cancer risk-reduction surgery. Pathological analysis of the surgical specimens led to the identification of ovarian cancer precursor lesions within the fallopian tube. The precursor lesions were characterised by evidence of DNA damage, strong p53 (protein 53 or tumour protein 53) immunostaining within the targeted secretory cells of the fimbriae, and the presence of p53 mutations which are also found in established ovarian cancers (Lee et al., 2007). These p53 driver mutations have been found in all patients with early and advanced HGS-ovarian cancer suggesting that they are an early and defining event in the development of HGS-ovarian cancer (Ahmed et al., 2010).

Other forms of ovarian cancer such as clear cell and endometrioid ovarian cancer are thought to arise from the transformation of endometriosis (Kuo et al., 2009, Wiegand et al., 2010). Mucinous ovarian carcinomas arise from metastasis from the gastrointestinal tract (Kelemen and Kobel, 2011).

1.1.3 The molecular classification of ovarian cancer

Ovarian cancer can be classified into distinct molecular subtypes using gene expression profiling. A recent study identified six different molecular subtypes of ovarian cancer, which have different clinical characteristics (Tothill et al., 2008). Low-grade serous ovarian cancer is characterised by low expression of proliferation markers such as Ki-67. Low-grade endometrioid subtypes of ovarian cancer have overexpression of mitogen-activated protein kinase pathway genes. High-grade serous and endometrial ovarian cancer can be categorised as having a high stromal

response, high immune signature, mesenchymal expression, and low stromal response expression signatures (Tothill et al., 2008). A subsequent study by the Cancer Genome Atlas Research Network described mRNA expression, microRNA expression, promoter methylation, and DNA copy number in 489 cases of ovarian cancer (Cancer Genome Atlas Research, 2011). The study identified four transcriptional subtypes of ovarian cancer, namely 'immunoreactive', 'differentiated', 'proliferative' and 'mesenchymal' which are similar to previous reports of ovarian cancer subtypes (Tothill et al., 2008).

These studies suggest that specific treatments will be required for each different subtype of ovarian cancer. Although there are many new potential drugs in development for ovarian cancer (Perren et al., 2011, Ledermann et al., 2011), tumour heterogeneity makes rational trial design and drug development difficult (Bast et al., 2009). This has led to questions about whether it will be possible to develop treatments that will benefit a wide variety of patients with the different subtypes of ovarian cancer.

1.1.4 The management of ovarian cancer

The standard treatment for ovarian cancer is bilateral salpingo-oophorectomy, hysterectomy, and cytoreductive debulking surgery to remove all visible metastatic deposits of ovarian cancer from the pelvis and peritoneum (Vergote et al., 2010). Surgery is often followed by platinum-based chemotherapy treatment to kill any remaining cancer cells within the abdomen (McGuire et al., 1996b, International Collaborative Ovarian Neoplasm, 2002). Patients can either undergo initial primary debulking surgery before chemotherapy treatment or debulking surgery after 3 or 6 cycles of neoadjuvant chemotherapy. However, there is no overall survival advantage to recommend one approach over another (Vergote et al., 2010).

The current standard first-line chemotherapy treatment for ovarian cancer is platinum-based chemotherapy, which was introduced in the 1980's when the addition of cisplatin, to the then standard chemotherapy regimen of doxorubicin and cyclophosphamide, was shown to improve response rates and overall survival (Neijt et al., 1984, Omura et al., 1986). Subsequent studies showed that the addition of doxorubicin to cisplatin and cyclophosphamide did not offer any significant survival

benefits (Conte et al., 1986, Omura et al., 1989); and cisplatin and cyclophosphamide chemotherapy became the standard therapy for around 10 years.

The next major change in the 1990's was the introduction of paclitaxel and cisplatin combination regimens. The initial landmark studies reported that, when compared to cyclophosphamide/cisplatin chemotherapy, the addition of paclitaxel to cisplatin, improved the overall response rates and overall survival (38 versus 24 months) (McGuire et al., 1996a) and (35.6 versus 25.8 months) (Piccart et al., 2000). These studies lead to the widespread adoption of paclitaxel and cisplatin-based chemotherapy regimens.

However, the addition of paclitaxel to platinum-based chemotherapy, in the setting of first-line treatment for ovarian cancer, remains controversial after a large study of 2074 women with advanced ovarian cancer did not report a survival advantage for the addition of paclitaxel to cisplatin chemotherapy (International Collaborative Ovarian Neoplasm, 2002). In the United Kingdom carboplatin-based chemotherapy is used instead of cisplatin for the treatment of ovarian cancer, as carboplatin is better tolerated and of similar efficacy (du Bois et al., 2003).

Subsequent studies have investigated different first-line chemotherapy regimens (Pignata et al., 2011, du Bois et al., 2010), the timing, route of administration, and scheduling of current chemotherapy regimens without leading to any major changes in clinical practice (Thigpen et al., 2011). However, a recent Japanese phase III study of dose-dense carboplatin based chemotherapy showed a modest improvement in progression-free survival of 28.0 months versus 17.2 months when compared to standard treatment. The dose-dense regimen was associated with an improvement in overall survival at 3 years (72.1%) when compared the standard treatment (65.1%) (Katsumata et al., 2009). Dose-dense carboplatin-based chemotherapy is currently being evaluated in Caucasian patients in an international phase III randomised trial (ICON8) that is currently recruiting patients (ClinicalTrials.gov Identifier: NCT01654146).

The role of the angiogenesis inhibitor bevacizumab (which inhibits vascular endothelial growth factor A (VEGF-A)) as a potential treatment for ovarian cancer is currently under investigation. In the first-line setting, the addition of bevacizumab to carboplatin chemotherapy does not improve overall survival (Perren et al., 2011, Burger et al., 2011). However, a subgroup group analysis has suggested that

bevacizumab may offer a survival benefit for women, who are at high risk of disease progression, of around 36.6 months with bevacizumab versus 28.8 months for women who receive standard treatment (Perren et al., 2011).

1.1.5 Second line therapies for ovarian cancer

The majority of patients with ovarian cancer relapse after first-line platinum-based chemotherapy and require further treatment. Patients whose disease progresses during platinum-based chemotherapy treatment or relapses within 6 months after completion of platinum-based treatment are considered to be platinum-refractory and have a very poor prognosis. Patients who relapse within 6 to 12 months of completing platinum-based therapies are considered partially-platinum-sensitive; and those who relapse after 12 months of platinum-based chemotherapy are considered platinum-sensitive (Markman et al., 1991, Markman and Bookman, 2000, Gore et al., 1990).

The standard second-line chemotherapy for patients with platinum-sensitive ovarian cancer is paclitaxel plus platinum-based chemotherapy (Parmar et al., 2003). Other commonly used combinations include cisplatin and gemcitabine (Aghajanian et al., 2012) and pegylated liposomal doxorubicin and carboplatin (Pujade-Lauraine et al., 2010). The addition of bevacizumab to gemcitabine and carboplatin for relapsed platinum-sensitive ovarian cancer increases response rates (78.5% versus 57.4%) and is associated with a modest increase in progression-free survival of 12.4 versus 8.4 months (Aghajanian et al., 2012).

For patients with platinum-refractory ovarian cancer the therapeutic options include pegylated liposomal doxorubicin and topotecan (Gordon et al., 2001), weekly paclitaxel (Markman et al., 2006), dose-dense paclitaxel, weekly dose-dense paclitaxel/carboplatin (Sharma et al., 2009b), and bevacizumab (Burger et al., 2007). Although many chemotherapy drugs have activity in platinum-resistant ovarian cancer, treatment responses are typically short-lived, and there is an urgent need for new treatments for this group of patients (Thigpen et al., 2011).

Around 14% of all patients with ovarian cancer have germ-line BRCA mutations (Alsop et al., 2012), which leads to defects in DNA repair by homologous recombination (HR). In HR deficient cells, inhibition of Poly ADP-ribose polymerase (PARP) stimulates error-prone non-homologous end-joining which leads to death of

the cancer cells (Patel et al., 2011). The oral PARP inhibitor olaparib (AZD2281) has significant activity against tumours with BRCA1 and BRCA2 mutations, demonstrating that PARP inhibitors are a treatment option for patients with BRCA mutations or dysfunction in ovarian cancer (Audeh et al., 2010).

1.1.6 Ovarian cancer ascites

Ascites is a medical term which refers to the accumulation of excessive fluid within the peritoneal cavity. The development of ascites is one of the most frequent and distressing complications of advanced ovarian cancer. Ascites is usually treated by therapeutic drainage, often requiring frequent visits to hospital (Harding et al., 2012). Currently, the best way manage ovarian cancer ascites is by therapeutic drainage of the fluid and treatment of the underlying ovarian cancer with chemotherapy drugs.

Symptomatic benefits	**Potential therapeutic benefits**	**Research benefits**
Relief of bloating	*In vitro* assessment of drug sensitivity	Reliable source of primary ovarian cancer cells for research
Relief of respiratory distress	The testing of personalised therapies	Permits multiple tissue collections without the need for biopsies during the disease process
Relief of dyspepsia	Easier than taking a biopsy for establishing the diagnosis of ovarian cancer	May allow tumour cell profiling
Relief of pain	Could allow the isolation of immune cells for immunotherapy treatment	May allow immune cell profiling within the tumour microenvironment

Table 1: The advantages of therapeutic drainage of ovarian cancer ascites. Although the relief of symptoms is the primary goal of drainage of ovarian cancer ascites, there are also a number of additional potential therapeutic and scientific benefits.

There are a number of hypotheses about why ovarian cancer leads to the formation of ascites. The seeding of ovarian cancer cells within the peritoneum and the peritoneal lymphatic system may prevent the drainage of peritoneal fluid, which then accumulates (Kipps et al., 2013). This hypothesis is supported by studies in mice which showed that radiolabelled erythrocytes injected into the ascites of tumour bearing mice failed to egress into the blood stream (Feldman et al., 1972). This suggests that obstruction of the lymphatic channels contributes towards the development of ascites (Feldman et al., 1972). However, ascites can be seen in

patients with low tumour volumes, suggesting that other mechanisms contribute towards the development of ascites.

It is possible that ovarian cancer cells increase the vascular permeability of the peritoneum, leading to the accumulation of fluid within the peritoneum. This hypothesis is supported by studies that have shown that higher levels of VEGF are associated with the development of ascites (Zebrowski et al., 1999). Additional studies have shown that ovarian cancer cells that overexpress VEGF are associated with an increase in the production of ascites in murine models of ovarian cancer (Byrne et al., 2003).

Ovarian cancer ascites is a rich scientific research resource (Table 1). Ovarian cancer ascites contains a mixture of immune, cancer, and stromal cells, as well as growth factors, chemokines and cytokines, which may promote or inhibit the growth of tumour cells. It is relatively easy to isolate tumour and immune cells from ascites as the cells are already in suspension within the ascites. As ascites is often a manifestation of active advanced ovarian cancer, its recurrence at different time points during the disease process has allowed investigators to study drug resistance by deriving multiple cell lines from patients after exposure to different lines of treatment (Langdon et al., 1988b).

1.1.7 Harnessing the immune system to treat patients with ovarian cancer

The idea of using the immune system to eliminate established cancers has been around for over 100 years; after William Coley, an American surgeon in New York, began infecting tumours with inoculations of erysipelas, a streptococcus pyogenes bacteria that causes skin infections (Coley, 1991). The toxin, later termed ‘Coley’s Toxin’, could induce tumour necrosis (Nauts et al., 1946, Nauts et al., 1953). Despite interest in this early work, the discovery of radiotherapy and the subsequent development of chemotherapy took centre stage in the race to develop more effective treatments against cancer (Balkwill, 2009).

Interest in immunotherapy was rekindled with the discovery of tumour necrosis factor (TNF); the factor in Coley’s toxin that was later found to induce tumour cell necrosis (Carswell et al., 1975). TNF increases endothelial permeability and improves chemotherapy penetration within tumour tissues, and selectively kills angiogenic endothelial cells (Lejeune et al., 2006). Although TNF is effective at killing tumour

cells, the systemic administration of TNF rapidly leads to the development of endotoxic shock, which has limited its clinical use to isolated limb perfusion for the treatment of sarcoma and melanoma (Cherix et al., 2008).

The discovery of the potential of the immune system to kill cancer cells via molecules such as TNF has promoted the investigation of multiple strategies to harness immune cells to treat cancer. These strategies can be divided into those that stimulate anti-tumour immunity, impair immune tolerance to tumours, administer tumour-specific immune cells, deplete or reprogramme harmful tumour-associated immune cells, or directly administer immune molecules that either kill or stimulate the elimination of the tumour cells.

1.1.8 The effect of immune cells on the prognosis of patients with ovarian cancer

Ovarian cancer is an immunogenic tumour and the ovarian cancer tumour microenvironment contains tumour specific CD8 cytotoxic T lymphocytes that can kill autologous tumour cells (Santin et al., 2000, Peoples et al., 1995). The presence of anti-p53 antibodies within the serum of patients with ovarian cancer is associated with improved overall survival of around 51 months, versus 24 months for patients without anti-p53 antibodies (Goodell et al., 2006). In 2003, a landmark study showed that higher levels of intratumoral CD3 T cells were found in around 55% of patients with ovarian cancer and were associated with a clinically significant improvement in survival of around 38% versus 4.5% of patients at 5 years after diagnosis (Zhang et al., 2003).

High levels of $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells are found within the ovarian cancer tumour microenvironment. Treg cells are found in the ascites, solid tumours, and serum of patients with advanced ovarian cancer. Treg cells within the ovarian cancer tumour microenvironment secrete immunosuppressive cytokines such as transforming growth factor-beta (TGFβ) which promote tumour growth (Woo et al, 2001). Treg cells suppress T cell activation and are associated with a significant reduction in the survival of patients with advanced ovarian cancer (Curiel et al., 2004). On the other hand, a high ratio of intraepithelial CD8 tumour-infiltrating lymphocytes to Treg cells is associated with a favourable prognosis in ovarian cancer (Sato et al., 2005).

Higher expression of *Foxp3* within solid deposits of ovarian cancer is associated with significantly worse overall survival in patients with advanced ovarian cancer (27.8 versus 77.3 months) (Wolf et al, 2005). These data suggest that inhibiting the suppressive functions of Treg cells has the potential to improve outcomes in patients with ovarian cancer. The development of novel therapeutic strategies that specifically target Treg cells in ovarian cancer requires a detailed understanding of Treg cell development and the regulation of Foxp3.

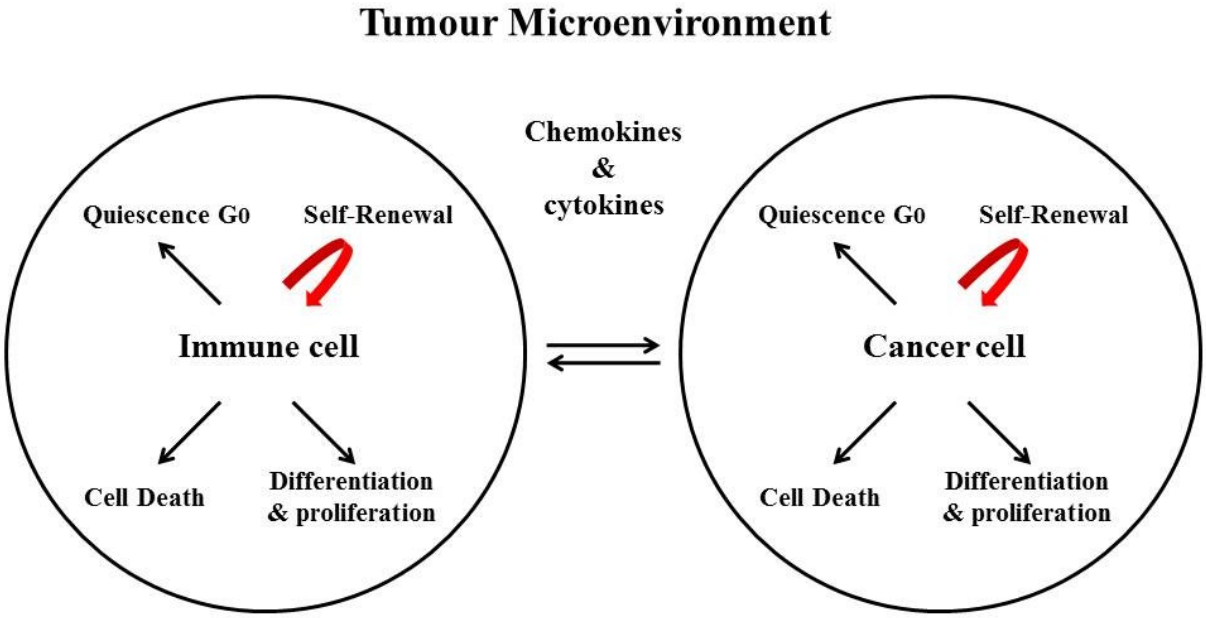

Figure 1: Cell fate decisions within the tumour microenvironment. The fates of immune and cancer cells within the tumour microenvironment are linked. Cancer cells secrete factors that promote their self-renewal, survival and resistance to cell death induction. These same factors can also affect the differentiation of immune cells, proliferation of immune cell subsets, which can be associated with adverse outcomes in patients. Immune cells affect cancer cells by secreting factors, which in turn promote survival, metastasis or even induce the death of cancer cells.

1.1.9 Regulatory T cell development

The adaptive immune system defends against pathogens, but requires regulation by Treg cells to prevent the development of autoimmunity. Treg cells express the lineage-specific transcription factor Foxp3, which is required for their development and function (Fontenot et al., 2003, Hori et al., 2003, Williams and Rudensky, 2007). Foxp3 was initially identified in Scurfy mice where an X-linked mutation in the Foxp3 gene leads to fatal autoimmunity (Brunkow et al., 2001, Bennett et al., 2001). In humans, mutations within the Foxp3 DNA binding domain cause the IPEX syndrome (X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy) (Wildin et al., 2001). Foxp3 represses immune responsiveness by regulating numerous target

genes such as *CTLA-4* (cytotoxic T-lymphocyte-associated protein 4), *IL-2Rα* (interleukin-2 receptor α) and by inhibiting transcription factors such as T-bet (T-box transcription factor TBX21), Gata3 (GATA binding protein 3), and RORγT (RAR-related orphan receptor gamma T) that control the production of proinflammatory cytokines such as IL-2 (interleukin-2), IFN-γ (Interferon gamma), IL-17, and TNF (Zheng and Rudensky, 2007, Zheng et al., 2007).

Effector T cells have unique T cell receptors (TCR) that recognise foreign antigens as short peptide fragments from intracellular proteins, that are displayed at the cell surface on major histocompatibility complexes (MHC). The diversity of the TCR repertoire is generated by the rearrangement of the T cell receptor genes during thymocyte development. TCR gene rearrangement is a random process which generates TCRs with specificities for both self and foreign antigens.

Thymocytes with self-reactive TCRs are removed by apoptosis, which is triggered by the display of self-antigens on thymic stromal and bone marrow derived cells. The mature naive CD4 and CD8 T cells leave the thymus and circulate in the peripheral lymphoid tissues, where upon contact with their specific foreign antigen, they differentiate into immune effector cells. Peripheral tolerance mechanisms, including Treg cells, are required because the thymic negative selection processes are imperfect and self-reactive T cells with the potential to cause autoimmunity escape into the peripheral immune system.

There are two principle types of Treg cells which both play a role in regulating immune responses. Natural Treg cells (nTreg cells) develop within the thymus, whereas induced Treg (iTreg cells) cells develop in the peripheral immune system in response to a variety of stimuli (Figure 2). In the thymus, nTreg cells arise and differentiate from single positive CD4 T cells in a two-step process, where high affinity TCR signalling confers competence for the induction of Foxp3 expression by IL-2 (Burchill et al., 2007, Lio and Hsieh, 2008, Sakaguchi et al., 2006, Wirnsberger et al., 2009) through the binding of STAT5 (Signal Transducer and Activator of Transcription 5) to the Foxp3 promoter (Burchill et al., 2007). nTreg cells have a diverse TCR repertoire that can recognise both self-peptide-MHC complexes and foreign antigen peptide-MHC complexes (Hsieh et al., 2004, Hsieh et al., 2006, Jordan et al., 2001, Pacholczyk et al., 2006).

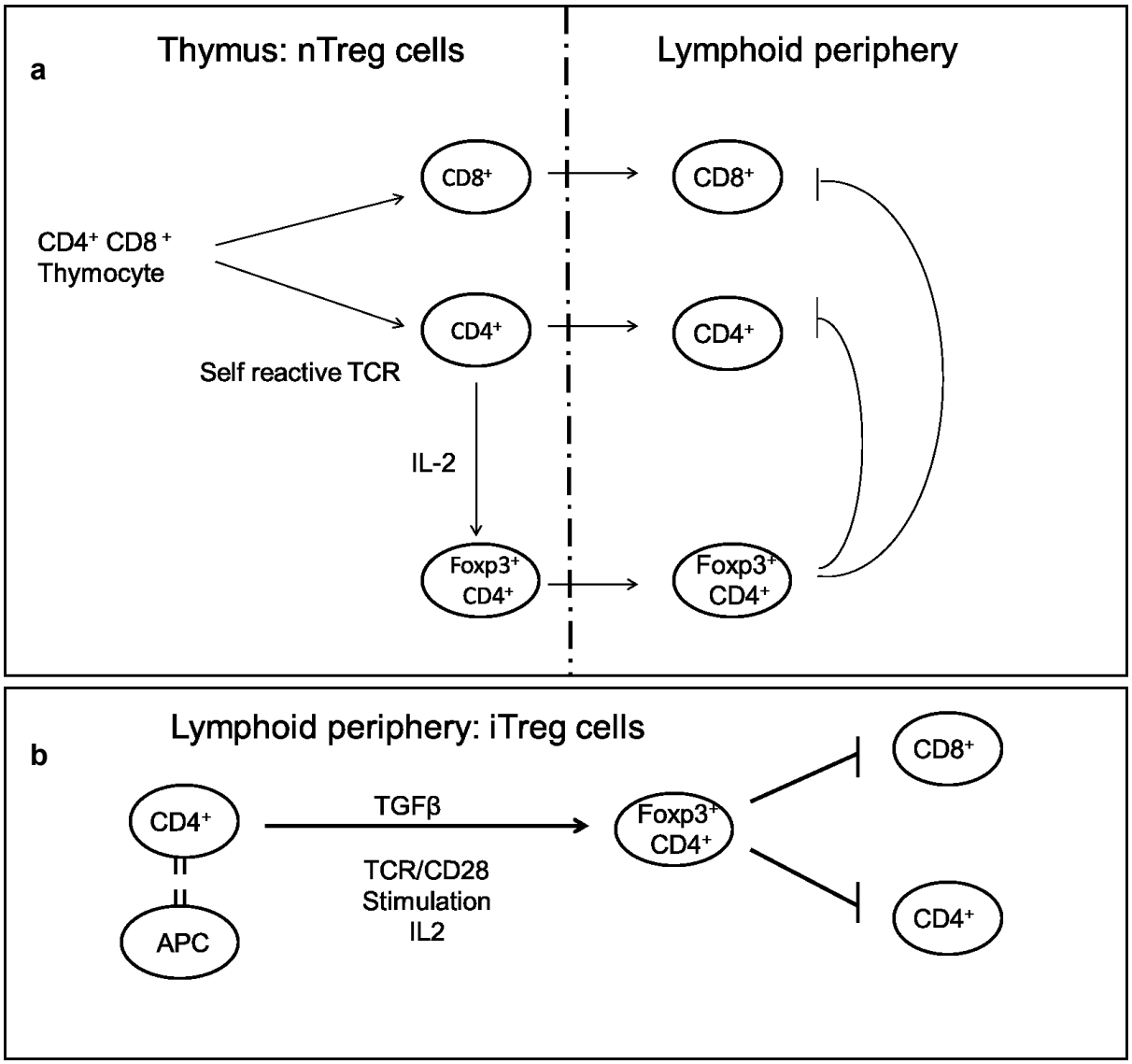

Figure 2: Natural and induced Treg cell development. a) Thymic nTreg cell differentiation starts at the double positive stage with TCR signalling. Double positive $CD4^+CD8^+$ thymocytes that have undergone successful TCR rearrangement can become single positive CD4 or CD8 thymocytes. CD4 thymocytes with a very high affinity self-reactive TCR are eliminated; whereas those with a lower affinity self-reactivate TCR can be induced to differentiate into nTreg cells by IL-2. nTreg cells suppress immune effector cells within the peripheral immune system. b) Peripheral iTreg development: naive CD4 T cells can be induced to become Foxp3 positive Treg cells by TCR stimulation, CD28 costimulation, and by cytokines such as TGFβ and IL-2.

Treg cells can develop in vivo in the peripheral immune system from mature immune cells by low-dose antigen stimulation in the absence of inflammation in tolerogenic microenvironments such as within the gut (Bilate and Lafaille, 2012). TCR stimulation and cytokines such as TGFβ and IL-2 can induce the in vitro differentiation of naive CD4 T cells into Foxp3 positive iTreg cells (Chen et al., 2003, Sauer et al., 2008). Both nTreg cells and iTreg cells contribute towards immune tolerance (Bilate and

Lafaille, 2012). However, iTreg cells are frequently used to study Treg cell development as they can be readily induced in in vitro assays.

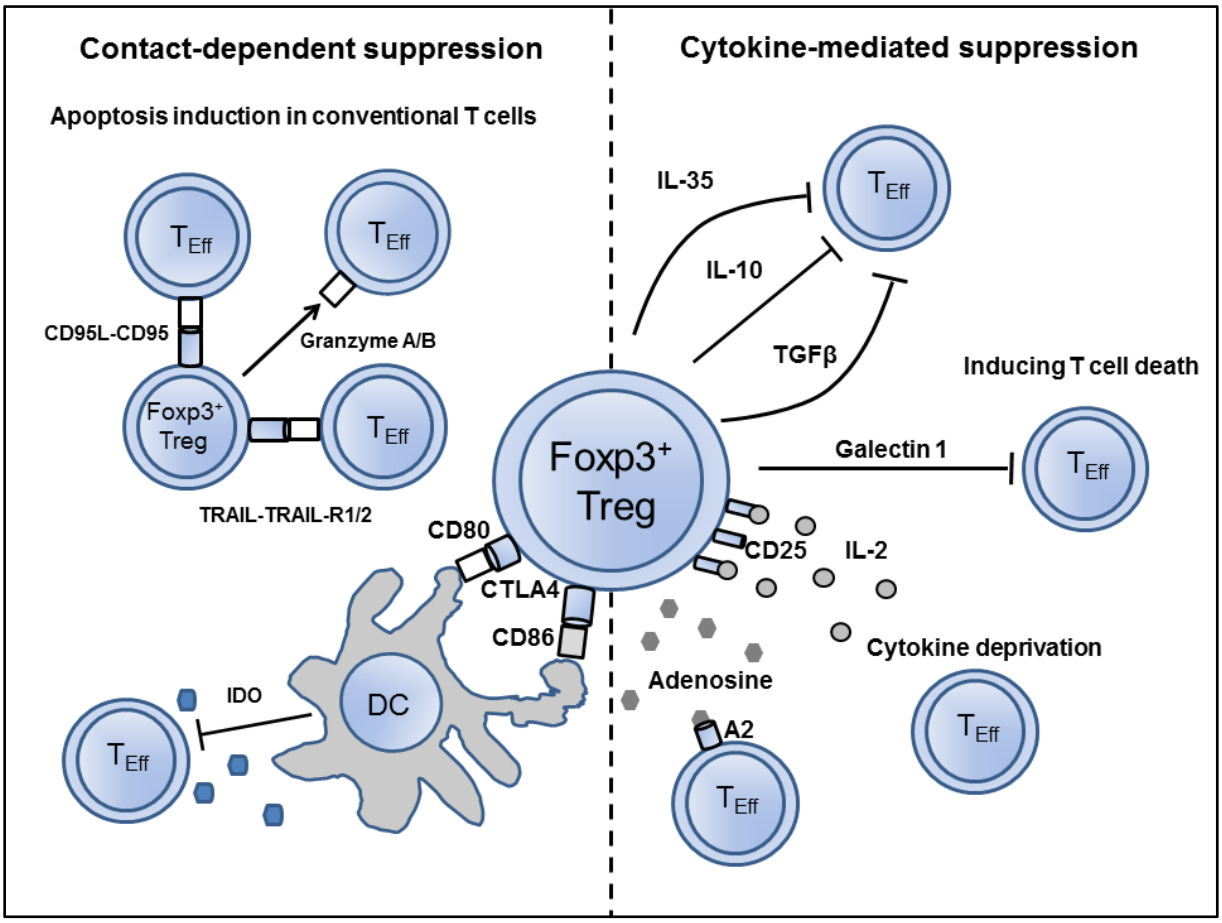

Figure 3: Mechanisms of Treg cell mediated immune suppression. Treg cells express CD25 which binds and sequesters IL-2, which prevents T cell proliferation. Treg cells secrete cytokines such as IL-35, IL-10, and TGFβ and adenosine which supress effector T cells (TEff) (Vignali et al., 2008). Treg cells may directly kill target immune effector cells using TRAIL or CD95L or granzyme-dependent or perforin-dependent mechanisms. CTLA-4 on Treg cells suppresses dendritic cell (DC) activation by binding and removing of CD80 and CD86 from the surface of antigen presenting cells. Treg cells can stimulate DC's to produce indoleamine 2,3-dioxygenase (IDO) by DCs which suppresses T cell activation by tryptophan depletion (Grohmann et al., 2002).

Treg cells have been reported to use a number of different mechanisms to suppress immune cells (Figure 3). Treg cells produce inhibitory cytokines such as TGFβ, IL-10, and IL-35 which suppress the activation of neighbouring immune cells (Vignali et al., 2008). Activated human Treg cells also have cytotoxic activity and can induce target cell killing via perforin and granzyme B (Grossman et al., 2004b). Treg cells have also been shown to induce the death of immune effector cells through the induction of cell death via death ligands, such as TNF-related apoptosis-inducing ligand (TRAIL) and CD95L (Ren et al., 2007). Treg cells also express CTLA-4 (Cytotoxic T-lymphocyte-associated antigen 4) which suppresses T cells activation by binding

CD80 and CD86 which prevents CD28 from activating T cells (Pardoll, 2012). The Treg cell specific deletion of CTLA-4 results in the development of systemic autoimmunity in mice, suggesting that this may be an important mechanism of regulation of autoimmune responses by Treg cells (Wing et al., 2008). Galectin is overexpressed by Treg cells which can induce cell cycle arrest, T cell death and inhibition of proinflammatory cytokine release (Garin et al., 2007).

1.1.10 The regulation of *Foxp3*

TCR stimulation activates multiple signalling pathways which lead to the binding of multiple transcription factors such as NFAT (Nuclear factor of activated T-cells), CREB (cAMP response element binding)/ATF (Activating transcription factors), c-Rel, and AP1 (activator protein 1) to the *Foxp3* promoter and enhancer regions (Kim and Leonard, 2007, Tone et al., 2008, Long et al., 2009, Isomura et al., 2009). Runx (Runt-related transcription factor) transcription factors play an important role in the initiation of Foxp3 in conventional CD4 T cells and in the maintenance of *Foxp3* expression in nTreg cells (Bruno et al., 2009, Rudra et al., 2009, Kitoh et al., 2009, Klunker et al., 2009, Ono et al., 2007). The cytokine TGFβ induces *Foxp3* expression by down-regulating the expression of Smad7 (Mothers against decapentaplegic homolog 7) (Fantini et al., 2004), and activating Smads2/3 which cooperate with NFAT to induce Foxp3 expression (Josefowicz and Rudensky, 2009).

1.1.11 A role for PI3 kinase signalling, Foxo factors and Klf2 in Treg cell development

Several lines of evidence link phosphatidylinositide 3-kinase (PI3K) signalling with Foxp3 expression. The withdrawal of TCR signalling after 18 hours of T cell activation cooperates with the inhibition of PI3K or the mammalian target of rapamycin (mTOR) to induce Foxp3 expression, and a Treg-like gene expression profile in activated CD4 T cells (Sauer et al., 2008). Treg cells require mTOR signalling to maintain both homeostasis and alloantigen-driven proliferation (Wang et al., 2011). Moreover CD4 cells that are deficient in negative regulators of PI3K signalling such as phosphatase and tensin homologue (PTEN) and the E3 ubiquitin ligase Cbl-b, have reduced Foxp3 expression in response to TGFβ signalling (Sauer et al., 2008, Wohlfert et al., 2006).

The retroviral expression of a constitutively active form of Akt substantially reduces TGFβ mediated induction of Foxp3 expression (Haxhinasto et al., 2008). The phosphorylation of Akt (Protein Kinase B) at Serine 473 by mTORC2 (mammalian target of rapamycin complex 2) activates Akt, and the loss mTORC2 activity promotes Treg cell differentiation (Delgoffe et al., 2009). Restoration of Akt activity in human Treg cells via a lentiviral expression system leads to a loss in Treg suppressive function (Crellin et al., 2007).

The Forkhead box O (Foxo) class of transcription factors regulates immune responses, cellular proliferation, metabolism, and cell fate decisions. Foxo factors are canonical targets of Akt-mediated phosphorylation (Burgering, 2008), which leads to their nuclear exclusion and inactivation (Brunet et al., 1999, Huang and Tindall, 2007). In naive CD4 T cells, Foxo1 controls the expression of L-selectin and the chemokine receptor CCR7 (Kerdiles et al., 2009). In mouse models, loss of Foxo factors causes autoimmunity and spontaneous T cell activation (Ouyang et al., 2009). These findings suggest that Foxo factors could have a role in regulating immune tolerance and the expression of Foxp3.

PI3K signalling and Foxo factors have been reported to regulate the expression of the transcription factor Kruppel-like Factor 2 (Klf2) (Finlay et al., 2009, Fabre et al., 2008). Klf2 controls T cell migration by regulating sphingosine-1-phosphate receptor 1 (S1P1) and CD62L (L-selectin) expression (Carlson et al., 2006), and restricts chemokine receptor expression and cytokine production in naive CD4 T cells (Sebzda et al., 2008, Weinreich et al., 2009). S1P1 receptor expression has been reported to regulate Treg cell differentiation (Li et al., 2011b), and other Kruppel-like family members, such as Klf10, maintain Treg cell suppressive function (Cao et al., 2009).

Klf2 expression is reduced in Foxo deficient Treg cells (Ouyang et al., 2010), and in PTEN deficient cells (Finlay et al., 2009). HMG-CoA (3-Hydroxy-3-Methylglutaryl Coenzyme A) reductase inhibitors or 'Statins', which modulate *in vitro* Treg cell differentiation, induce Klf2 expression in T cells (Bu et al., 2010, Kim et al., 2010, Kagami et al., 2009). Genome-wide analysis has shown that Foxp3 binds to the regions that encode *Klf2* and the KLF family members *Klf3*, and *Klf6* (Zheng et al., 2007). These data suggest that Klf2 may have a role in Treg cell development and function.

Although Foxp3 is a specific marker of Treg cells in mice, Foxp3 can be transiently expressed during the activation of human CD4 T cells which does not result in the acquisition of regulatory functions (Walker et al., 2003, Allan et al., 2007, Morgan et al., 2005, Wang et al., 2007, Gavin et al., 2006). The TGFβ mediated induction of Foxp3 expression in naive human CD4 T cells, by T-cell receptor stimulation, does not confer a regulatory phenotype in human T cells (Tran et al., 2007).

Therefore, Foxp3 expression in human CD3 T cells cannot be thought of as a truly specific marker of Treg cells. In humans, the expression of the IL-7 receptor (CD127) is down-regulated in Treg cells and can be used to distinguish Treg cells from other immune subsets (Liu et al., 2006b). Although there is uncertainty as to the best human Treg cell marker, high levels of Foxp3 expressing CD4 T cells are associated with adverse outcomes in patients with ovarian cancer (Curiel et al., 2004). Therefore strategies that reduce the expression of Foxp3 may have therapeutic benefits, even if the effects of targeting Foxp3 are not restricted to Treg cells.

1.1.12 Overcoming the adverse effects of regulatory T cells in ovarian cancer

There is considerable interest in the development of therapies that enhance or inhibit Treg cell function; as such therapies would have wide ranging therapeutic implications for the treatment of autoimmunity and cancer. Recent clinical studies have suggested that therapeutic strategies that reduce immune tolerance to tumours have the potential to improve outcomes in patients with cancer.

The first definitive proof of this concept was recently shown by the demonstration that the treatment of patients with ipilimumab, an anti-CTLA-4 antibody, enhances anti-tumour T cell immune responses, which improves survival in patients with advanced melanoma (Hodi et al., 2010). There is pre-clinical evidence that CTLA-4 blockade may be effective in enhancing immune responses against ovarian cancer cells suggesting that such strategies may be effective in treating ovarian cancer (Hodi et al., 2003, Hodi et al., 2008).

1.1.13 Treg cell depletion

The chemotherapy drug cyclophosphamide has been used to preferentially deplete Treg cells in patients with cancer. However, clinical trials using this approach have

thus far been disappointing (Vermeij et al., 2011). The depletion of Treg cells using an interleukin 2/diphtheria toxin conjugate (DAB/IL-2; denileukin diftitox; ONTAK) has been shown to enhance immune responses to cancer vaccines (Litzinger et al., 2007, Morse et al., 2008), and can cause regression of melanoma metastases in humans (Rasku et al., 2008).

Encouraging clinical results have been obtained from a recently published phase II trial of denileukin diftitox which depleted Treg cells and expanded melanoma-specific CD8 T cells in patients. The treatment was associated with responses in 16.7% of 60 patients with advanced melanoma (Telang et al., 2011). Patients who responded to the treatment survived significantly longer than those with chemotherapy naive disease (Telang et al., 2011). However, Treg cell depletion strategies may remove activated immune effector cells such as CD4 and CD8 T cells which may have unique anti-tumour specificities which may be beneficial for patients with cancer.

1.1.14 Treg cell reprogramming

An alternative to Treg cell depletion is the reprogramming of Treg cells into $Foxp3_{low,}$ so called ‘exTreg’ cells with immune effector functions. The principle advantage of such approaches is that they could harness potentially unique tumour cell-specific T cell receptors on tumour-associated Treg cells. Several studies have shown that Treg cells can be reprogrammed by antigen plus Toll-like receptor 9 ligand into a phenotype expressing proinflammatory cytokines after vaccination (Sharma et al., 2010).

The immunoregulatory enzyme IDO can regulate Treg cell suppressive functions. The inhibition of IDO and an antitumor vaccine was sufficient to cause up-regulation of IL-6 in murine plasmacytoid dendritic cells which was also sufficient to allow the conversion of Treg cells to an inflammatory TH17 phenotype (Sharma et al., 2009a). Given that several IDO inhibitors are currently in early phase clinical trials in patients with cancer, it is possible that this approach could also be tested in patients with ovarian cancer.

An alternative approach to changing the lineage of Treg cells is to directly target key transcription factors that regulate Treg cell function. Recent studies have shown that Runx proteins regulate the initiation and the maintenance of Foxp3 expression (Bruno et al., 2009, Rudra et al., 2009, Kitoh et al., 2009, Klunker et al., 2009). Foxp3

expression can be down-regulated by genetic deletion of the Runx partner protein core-binding factor beta (Cbfβ), which is essential for Runx protein–DNA interaction (Durst and Hiebert, 2004); and by retroviral expression of dominant negative versions of Runx which antagonise the DNA binding of Runx transcription factors (Bruno et al., 2009). As loss of Foxp3 expression in Treg cells has been shown to lead to autoimmunity in mice, dominant negative versions of Runx could reprogramme tumour-associated Treg cells into $Foxp3_{low}$ 'exTreg' cells with immune effector functions such as the production of proinflammatory cytokines such as IL-17, IL-4, or IFN-γ.

It is also likely that other transcription factors and/or Foxo factors or Klf2 may regulate the expression of Foxp3, and therefore regulate the suppressive functions of regulatory T cells. If Treg cells can be successfully reprogrammed into immune effector cells by inhibition of the function of key lineage specific transcription factors, then these cells could potentially generate immune responses against cancer cells. Therefore a deeper understanding of the role of the different signalling pathways and transcription factors that regulate the expression of Foxp3 may identify novel therapeutic targets which have the potential to enhance anti-tumour immunity.

1.1.15 Integrating immune cell functions and apoptosis-based therapies for the treatment of ovarian cancer

The aim of most therapies for ovarian cancer is the induction of cancer cell-specific death. This is usually achieved by the administration of chemotherapy, radiotherapy, or by inhibiting key oncogenic signalling pathways. The ultimate goal is the elimination of all cancer cells and cure of the patient. Although this goal has been achieved in a number of haematological and epithelial malignancies (Savage et al., 2009), cure remains a distant goal for the majority of patients with metastatic epithelial cancers like ovarian cancer.

Most chemotherapy drugs lead to the initiation of apoptosis, a form of regulated programmed cell death, associated with typical morphological changes such as cell shrinkage, nuclear fragmentation, chromatin condensation, and cell membrane blebbing. Apoptosis is a normal cellular process and is essential for the development of multicellular organisms. Metazoans have two pathways of apoptosis induction; the 'intrinsic' or mitochondrial pathway is mediated by cellular damage via a p53

dependent mechanism, and is regulated by the actions of members of the Bcl-2 (B-cell lymphoma 2) family. The 'extrinsic' pathway is triggered by extracellular stress signals that are initiated via the binding of death ligands such as CD95L (Fas Ligand/FasL) or TNF or TRAIL to specific transmembrane death receptors on the cell surface (Galluzzi et al., 2012b).

Both the intrinsic and extrinsic apoptosis pathways are coordinated by caspases, a family of cysteine proteases (Slee et al., 2001). The apoptotic caspases can be divided into initiator caspases such as caspase-2, -8, -9, and -10 and executioner caspases such as caspase-3, -6, and -7 which are responsible for the death of the cell (Slee et al, 2001). After the binding of a death ligand to its receptor, initiator caspases become aggregated at the death receptor signalling complex which leads to the activation of downstream executioner caspases which kill the cell. In the mitochondrial apoptotic pathway, the release of cytochrome C into the cytoplasm is detected by Apaf-1 (apoptosis activating factor 1) which leads to the recruitment and cleavage of caspase-9 at the apoptosome complex. This leads to the activation of executioner caspases-3, -6, and -7 that kill the cell (MacKenzie and Clark, 2012).

Conventional chemotherapy agents that are used for the treatment of ovarian cancer, such as cisplatin or paclitaxel, target DNA and microtubules, leading to DNA damage or mitotic arrest which triggers the induction of apoptosis through the mitochondrial pathway. Cisplatin induces cell death by covalently binding to DNA, forming DNA adducts. This process leads to the activation of DNA-damage checkpoints, the induction of cell cycle arrest, and the induction of apoptosis (Kelland, 2007, Siddik, 2003). Paclitaxel is a tubulin binding agent that stabilises microtubules resulting in prolonged mitotic arrest and cell death (Kavallaris, 2010).

Despite decades of use, it is currently unclear how most chemotherapy drugs induce apoptosis in cancer cells whilst sparing normal tissues. One explanation is that chemotherapy kills rapidly dividing cells whilst sparing normal cells (Skipper and Perry, 1970). However, chemotherapy drugs are active against slowly dividing cancers such as follicular lymphoma and chronic lymphocytic leukaemia suggesting that treatment responses are not restricted to the cell cycle. Although chemotherapy is the most effective treatment for advanced ovarian cancer, the majority of tumours become resistant to chemotherapy drugs. Furthermore, the majority of chemotherapy

drugs for ovarian cancer have significant side effects such as myelosuppression, hair loss, and nerve damage which impair the quality of life of patients.

The integration of an immunotherapy treatment with an apoptosis inducing therapy has the potential to kill the cancer cells, whilst leading to the development of long lasting immunological memory to guard against future relapse. This could be achieved by depleting Treg cells, which could stimulate anti-tumour immunity and enhance the sensitivity of the cancer cells to chemotherapy (DeNardo et al., 2011).

An alternative approach to treat ovarian cancer is to use an apoptosis-inducing therapy, which also stimulates the induction of anti-tumour immunity. Two of the most successful cancer therapies of all time are the monoclonal antibodies herceptin and rituximab (Tuthill et al., 2009), which target HER-2 (Human Epidermal Growth Factor Receptor 2) on breast cancer cells and CD20 on B cells, respectively. The effectiveness of herceptin and rituximab has been shown to partly depend on effector-cell-mediated cytotoxicity for their *in vivo* effects against cancer cells (Clynes et al., 2000). Therefore, therapeutic strategies that target the cancer cells and harness the immune system have the potential to be highly effective anti-cancer therapies.

Immune cells use membrane-bound death receptor signalling molecules such as TNF, CD95L (Fas/APO-1), and TRAIL to induce the death of target cells (Falschlehner et al., 2009). Treg cells have been shown to use both the perforin/granzyme pathway (Grossman et al., 2004b, Grossman et al., 2004a) and TRAIL to kill target cells (Ren et al., 2007). Cytotoxic T cells can secrete performin and granzymes within an immunological synapse with the target cells which leads to the activation of caspases and the induction of cell death (Cullen and Martin, 2008).

1.2 TRAIL

TRAIL is a 281 (human) and 291 (murine) amino acid cytokine, originally identified in 1995, based around its high sequence homology to TNF and CD95L (Wiley et al., 1995, Pitti et al., 1996). The discovery that TRAIL can preferentially kill tumour cells, whilst sparing normal tissues (Walczak et al., 1999) has led to considerable interest in developing TRAIL as a potential cancer therapy (Newsom-Davis et al., 2009).

Although there are five known TRAIL receptors (TRAIL-Rs), only TRAIL-R1 (also known as Death Receptor [DR] 4) and TRAIL-R2 (also known as DR5, KILLER, and TRICK2) are able to induce cell death (Pan et al., 1997, Wu et al., 1997, Walczak et al., 1997, Sheridan et al., 1997, Screaton et al., 1997, MacFarlane et al., 1997). This is because only TRAIL-R1 and TRAIL-R2 contain a Death Domain (DD); a structurally defined motif which is required for the induction of cell death (Lahm et al., 2003).

TRAIL-R3 lacks a cytoplasmic DD and does not induce apoptosis; and is only expressed within peripheral blood lymphocytes and the spleen (Degli-Esposti et al., 1997, MacFarlane et al., 1997). TRAIL-R4 has an incomplete DD and induces NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation and can protect cells against TRAIL-mediated apoptosis (Degli-Esposti et al., 1997). Although it has been suggested that TRAIL-R3 and TRAIL-R4 may act as decoy receptors (Merino et al., 2006), it is not clear whether these experiments accurately reflect the physiological role of these receptors (Pan et al., 1997, Sheridan et al., 1997).

1.2.1 The role of TRAIL within the immune system

Apoptosis has an important role in the normal functioning of the immune system, allowing removal of autoreactive B and T cells during lymphocyte development, viral or bacterially infected cells, and unwanted immune cells (Opferman and Korsmeyer, 2003). TRAIL deficiency in mice leads to a severe defect in TCR induced thymocyte apoptosis and TRAIL deficient mice are hypersensitive to collagen-induced arthritis, streptozotocin-induced type I diabetes and develop heightened autoimmune responses (Lamhamedi-Cherradi et al., 2003a, Lamhamedi-Cherradi et al., 2003b). TRAIL is required for the pathogenesis of thymic-graft versus host disease (Na et al., 2010), which is a common complication after bone marrow transplantation (Hatzimichael, 2010).

Human dendritic cells use TRAIL to kill tumour cell targets (Fanger et al., 1999). Activated Natural Killer (NK) cells express TRAIL (Kayagaki et al., 1999c) and have been shown to suppress tumour metastasis via a TRAIL-dependent mechanism (Takeda et al., 2001). Human monocytes express TRAIL after stimulation with LPS (Lipopolysaccharide) and interferon (IFN)-β (Halaas et al., 2000, Ehrlich et al., 2003),

and acquire the ability to kill tumour cells after activation with IFN-α and CD3 (Griffith et al., 1999).

CD4 T cells have been shown to use TRAIL to induce cytotoxic target cell death (Kayagaki et al., 1999b). TRAIL is rapidly induced on the cell surface of CD4 and CD8 T cells upon stimulation with an anti-CD3 monoclonal antibody and type I interferons, suggesting that TRAIL may play a role in mediating the effects of type I interferons against tumour cells (Kayagaki et al., 1999a). TRAIL has also been shown to delay cell cycle progression in activated lymphocytes (Lunemann et al., 2002, Song et al., 2000). Treg cells can suppress activated CD4 T cells *in vivo* via a TRAIL-R2 dependent mechanism (Ren et al., 2007).

More recent studies have shown that TRAIL prevents autoimmunity in mouse models of autoimmune encephalomyelitis and thyroiditis through the inhibition of T helper (Th) 1 cells and by promoting the proliferation of Treg cells (Hirata et al., 2007, Ikeda et al., 2010, Wang et al., 2009). Treg cells have been reported to regulate the TRAIL-dependent cytotoxicity of tumour-infiltrating dendritic cells in rodent models of colon cancer, suggesting a complex interplay between TRAIL expressing cells within the tumour microenvironment. Interestingly, this effect could be overcome by the depletion of Treg cells or by the addition of BCG (Bacillus Calmette–Guérin), which activates the innate immune system via Toll-like receptor (TLR) signalling (Roux et al., 2008).

1.2.2 TRAIL signalling

TRAIL is a trimeric molecule and its binding to TRAIL-R1 or TRAIL-R2 leads to receptor homo- or heteromeric multimerisation (Mongkolsapaya et al., 1999), resulting in the formation of a death-inducing signalling complex (DISC). TRAIL contains an internal zinc atom at the trimer interface, which is bound by cysteine residues at position 230 of each subunit, which is crucial for trimer stability and biologic activity (Bodmer et al., 2000, Hymowitz et al., 2000). The binding of TRAIL to TRAIL-R1 or to TRAIL-R2 leads to the formation of a stable trimeric complex, which triggers the apoptotic signal through the precise positioning of transmembrane helices and the cytosolic death domains (Mongkolsapaya et al., 1999, Jones et al., 1989).

The TRAIL DISC is comprised of the Fas-associated death-domain containing protein (FADD/Mort1), which recruits caspase-8, caspase-10, and cellular FLICE-like inhibitory protein (cFLIP) (Kischkel et al., 1995, Sprick et al., 2000, Kischkel et al., 2000). Caspases (cysteine-dependent aspartate-directed proteases), the main executioners of apoptosis, are recruited to the DISC via death-effector domains (DED). The recruitment of caspase-8 and caspase-10 to the DISC results in their autocatalytic cleavage, which initiates the activation of 'executioner' caspases 3, 6, and 7, resulting in nuclear fragmentation, chromatin condensation and DNA fragmentation (Figure 4) (Slee et al., 2001, Stennicke et al., 1998).

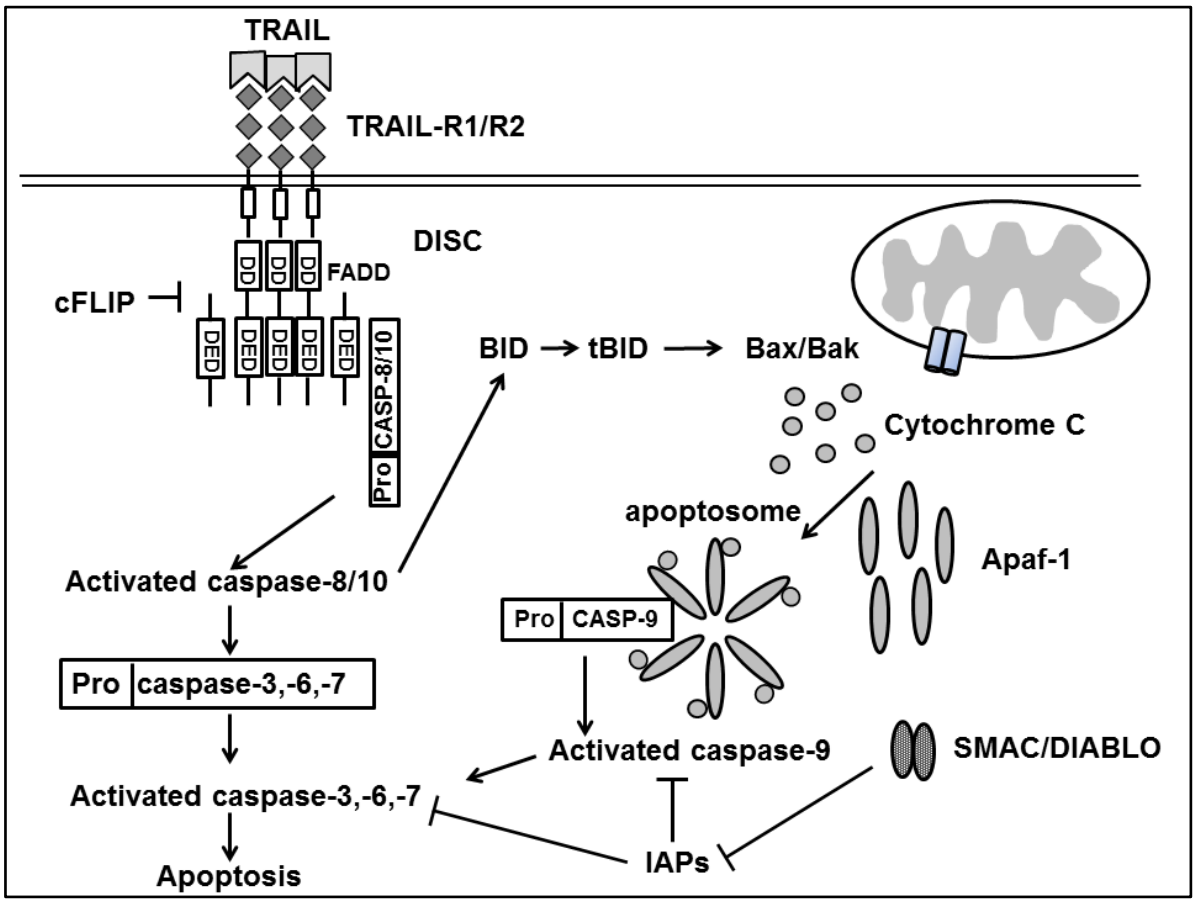

Figure 4: The TRAIL apoptotic signalling pathway. The binding of TRAIL to TRAIL-R1/R2 induces receptor trimerisation and the recruitment of FADD via death domains (DD) and death-effector domains (DED) to the TRAIL DISC. This leads to the recruitment and cleavage of c-FLIP, pro-caspase-8 and pro-caspase-10 (Pro-CASP 8/10) at the TRAIL DISC. In type I cells, the recruitment of caspase-8 and caspase-10 to the DISC results in their autocatalytic cleavage, which initiates the activation of 'executioner' caspases 3, 6, and 7. Type II cells require changes at the mitochondrial membrane to induce apoptosis. Activated caspase 8 cleaves full length BID (Bcl-2 homology domain 3-interacting domain death agonist) into truncated BID (tBID) which activates Bax (bcl-2-like protein 4) and Bak (Bcl-2 homologous antagonist/killer) resulting in the release of cytochrome C, and the pro-apoptotic molecule second mitochondria-derived activator of caspases/direct IAP binding protein with low isoelectric point (SMAC/DIABLO) which antagonises inhibitor of apoptosis proteins (IAPs). Latent Apaf-1 (Apoptotic protease activating factor 1) within the cytoplasm is bound by cytochrome C and forms the apoptosome by recruiting and activating caspase-9.

Although the binding of TRAIL to TRAIL-R1 and TRAIL-R2 can trigger apoptosis, it can also induce the activation of other signalling pathways, suggesting that TRAIL may also exert non-apoptotic functions in cancer cells. The binding of TRAIL to TRAIL-R1, -R2, and -R4 can induce the activation of NF-κB (Schneider et al., 1997), c-Jun N-terminal Kinase (JNK) (Muhlenbeck et al., 2000, Hu et al., 1999), and protein kinase C, which may contribute to resistance of cancer cells to TRAIL-induced apoptosis (Trauzold et al., 2001).

TRAIL-induced NF-κB signalling has been shown to promote the survival and proliferation of TRAIL-resistant cancer cells (Ehrhardt et al., 2003). TRAIL has been shown to induce the migration and invasion of apoptosis-resistant cholangiocarcinoma cells via the NF-κB signalling pathway (Ishimura et al., 2006). TRAIL has also been shown to induce the proliferation of human glioma cells via the c-$FLIP_L$-mediated activation of ERK (extracellular signal-regulated kinases) 1/2 signalling (Vilimanovich and Bumbasirevic, 2008). These data suggest that although TRAIL has the potential to kill cancer cells, particularly at supra-physiological concentrations; however, endogenously expressed TRAIL may have important non-apoptotic functions in cancer cells.

1.2.3 The effect of TRAIL-R and TRAIL on the prognosis of patients with cancer

A large pathological study of 382 cases of ovarian cancer showed that the majority of ovarian cancer cells express both TRAIL-R1 and TRAIL-R2, with about 70% expressing both TRAIL-R1 and TRAIL-R2, and approximately 27% expressing either TRAIL-R1 or TRAIL-R2 (Duiker et al., 2010). The expression of TRAIL is associated with lower grade tumours and better progression-free survival in patients with ovarian cancer (Duiker et al., 2010). The majority of ovarian cancers that express both TRAIL-R1 and TRAIL-R2 also express c-FLIP, which suggests that ovarian cancer cells are intrinsically resistant to TRAIL. These data suggest that the modulation of c-FLIP levels may enhance the sensitivity of ovarian cancer cells to TRAIL (Duiker et al., 2010).

A similar study found that higher TRAIL-R2 expression was associated with more advanced stage ovarian cancer, when compared to tumours that expressed lower levels of TRAIL-R1 and TRAIL. Furthermore higher expression of TRAIL-R2 was

associated with poor survival in grade 3 ovarian cancer (Ouellet et al., 2007). In other cancers, such as renal cell carcinoma (RCC), higher TRAIL expression and low TRAIL-R1 is associated with reduced disease-free survival (Macher-Goeppinger et al., 2009). In breast cancer, higher TRAIL-R1 expression is associated with better differentiation of tumours; however TRAIL-R2 and TRAIL-R4 are associated with higher tumour grade, a higher Ki-67 proliferation index, HER-2 overexpression, and lymph node metastasis. Higher TRAIL-R4 expression is associated with a poor prognosis in patients with breast cancer (Ganten et al., 2009, Sanlioglu et al., 2007). Expression of TRAIL-R1 and TRAIL-R2 is not prognostic in patients with cervical cancer; however higher TRAIL expression is associated with reduced pathological responses to radiotherapy treatment (Maduro et al., 2009).

There have been conflicting results from clinical studies of the effect of TRAIL and TRAIL-R2 expression in colorectal cancer. Studies have reported that $FLIP_{(L)}$, but not TRAIL-R1 or TRAIL-R2 is associated with a poor prognosis in colorectal cancer (Ullenhag et al., 2007). On the other hand, a larger study, which used tissue from 376 patients with stage III colon cancer, treated in a randomised trial of adjuvant chemotherapy, found that higher expression of TRAIL-R1 was associated with a worse disease-free survival and overall survival; and that TRAIL-R2 and TRAIL expression had no effect on the prognosis of patients with colon cancer (van Geelen et al., 2006).

Higher expression of TRAIL-R1 or TRAIL-R2 is associated with longer disease-free survival in patients with bladder cancer (Li et al., 2012). Expression of TRAIL-R1 and TRAIL-R2 is also associated with a better prognosis in patients with acute myeloid leukaemia (Pordzik et al., 2011). Loss of either TRAIL-R1 or TRAIL-R2 expression is associated with a significantly worse outcome in patients with hepatocellular carcinoma (HCC) (Kriegl et al., 2010).

1.2.4 Clinical TRAIL receptor agonistic drugs

Two different approaches have been taken to target the apoptotic TRAIL-R1 and TRAIL-R2 in the cancer clinic. The bioactivity of soluble forms of TRAIL is dependent on the spontaneous oligomerisation of the protomers that permit receptor crosslinking (Wiley et al., 1995, Walczak et al., 1999). Recombinant highly active soluble forms of TRAIL have been developed that promote stable trimer formation by the addition of

leucine zipper (LZ) or isoleucine-zipper (iz) tags to the amino terminus of human trail (iz−TRAIL). Whilst these potently induce tumour cell apoptosis *in vitro* and *in vivo* (Walczak et al., 1997, Walczak et al., 1999), these forms of TRAIL are not suitable for clinical use due the antigenicity of the leucine zipper or isoleucine-zipper which could promote toxicity via antibody-induced hyper-crosslinking.

During the initial clinical development of TRAIL-R agonistic drugs there were significant concerns that highly recombinant forms of TRAIL would be hepatotoxic (Jo et al., 2000). However, subsequent studies have shown that only highly recombinant polyhistidine–tagged or crosslinked FLAG–tagged soluble TRAIL are toxic to primary human hepatocytes (Ashkenazi et al., 1999, Ganten et al., 2006, Kelley et al., 2001, Lawrence et al., 2001).

Dulanermin/Apo2L/TRAIL is a recombinant form of TRAIL which can trimerise both TRAIL-R1 and TRAIL-R2 and induce apoptosis in cancer cells (Ashkenazi and Herbst, 2008). In comparison to highly active recombinant forms of TRAIL such as LZ-TRAIL (Walczak et al., 1999) or iz-TRAIL (Ganten et al., 2006) dulanermin, is a relatively weak agonist of TRAIL-R1 and TRAIL-R2, the two death-domain-containing TRAIL-Rs. However, recombinant proteins such as dulanermin have limitations such as a short *in vivo* half-life of around 1 hour (Herbst et al., 2010a) and can potentially be sequestered by binding to non-apoptotic TRAIL-R.

Therapeutic antibodies against TRAIL-R1 and TRAIL-R2 were developed to address these limitations. They were thought to have the advantages of a long half-life and to only target an apoptosis-inducing TRAIL-R (Newsom-Davis et al., 2009). Their main disadvantage is that the agonistic activity of antibodies to members of the death receptor family requires crosslinking (Wilson et al., 2011, Haynes et al., 2010b). *In vivo*, this activity requires crosslinking by fragment crystallisable region γ receptors (FcγR) on immune cells to enable TRAIL receptor multimerisation and apoptosis induction (Wilson et al., 2011, Haynes et al., 2010b).

1.2.5 Recombinant Apo2L/TRAIL/dulanermin

Dulanermin is a recombinant soluble form of TRAIL which consists of amino acids 114 to 281 of the extracellular domain of the natural TRAIL ligand in an optimised zinc preparation (Soria et al., 2010, Ashkenazi et al., 1999, Lawrence et al., 2001). Unlike anti-TRAIL-R1/2 antibodies, dulanermin is a dual TRAIL-R1 and TRAIL-R2

agonist. In pre-clinical studies dulanermin demonstrated encouraging preclinical activity against glioma, colon cancer, myeloma, lung carcinoma, and lymphoma cells and encouraging synergy with chemotherapy and other targeted agents (Pollack et al., 2001, Gazitt, 1999, Jin et al., 2004, Daniel et al., 2007, Ravi et al., 2004, Ashkenazi et al., 2008).

Highly encouraging pre-clinical data (Ashkenazi et al., 1999) led to the initiation of several large early-phase clinical trials of dulanermin patients with cancer (Table 2). Results from a phase I clinical trial in which 71 patients with heavily pre-treated advanced cancer showed that dulanermin had some evidence of clinical activity, with 31 of 71 dulanermin treated patients (46%) having stable disease after 6 months of dulanermin treatment. There were two sustained partial responses in patients with synovial sarcoma (Herbst et al., 2010a).

However, only 10% of patients completed 8 cycles of treatment (Herbst et al., 2010a). The majority of the patients (61%) discontinued dulanermin treatment due to disease progression. Dulanermin was reasonably well tolerated with the main side effects being fatigue (38%), nausea (28%), vomiting (23%), fever (23%), anaemia (18%), and constipation (18%). Around 47% of the patients suffered from a grade III or IV adverse event. The grade 4 reported adverse events included jejunal perforation, increased blood bilirubin, increased blood lipase, hypokalaemia, and ureteric obstruction which were attributed to progressive metastatic disease. A total of 7 patients died (10%) from progressive disease during the study (Herbst et al., 2010a).

Combination treatment	**Phase**	**Tumour type**	**Trial number**	**Current Status**
FOLFOX/bevacizumab	Ib	mCRC	NCT00873756	active, not recruiting
Cetuximab/irinotecan	I	mCRC	NCT00671372	active, not recruiting
Rituximab	Ib/II	$CD20^+$ Lymphomas	NCT00400764	terminated

Table 2: Current clinical trials of dulanermin. (www.clinicaltrials.gov:database accessed on the 22/02/2013)

There were two notable responses to dulanermin treatment which were associated with rapid tumour involution (Herbst et al., 2010a). The first response occurred in a patient with heavily pre-treated synovial sarcoma. Dulanermin treatment led to intra-abdominal haemorrhage from an abdominal tumour from areas of central tumour necrosis. The second major response occurred in a patient with a poorly

differentiated high-grade sarcoma. Dulanermin treatment led to analgesic-refractory pain from a pelvic mass which had developed a necrotic centre. These data suggest that dulanermin has the potential to induce significant vascular disruption in some patients with cancer.

One explanation for this effect is that tumour-associated endothelial cells within tumour vasculature may express TRAIL-R2 and are thus sensitive to TRAIL. A recent study has shown that crosslinked Apo2L/TRAIL can induce the death of tumour-associated endothelial cells leading to the induction of extensive haemorrhagic tumour necrosis in pre-established fibrosarcoma xenografts (Wilson et al., 2012). Although both patients with tumours that underwent rapid involution and necrosis recovered from their emergency surgery, both of these responses were associated with considerable pain and morbidity. It is therefore difficult to envisage a TRAIL-based therapeutic strategy that would deliberately seek to induce this effect in routine clinical practice.

Another patient with metastatic chondrosarcoma had a sustained partial response to dulanermin treatment (Subbiah et al., 2012). The response to dulanermin treatment was maintained for around 5 years. The patient developed progressive disease within a nodule within the lungs which was resected. An analysis of the nodule was conducted using morphoproteomics; a technique that combines histopathology, molecular biology, and proteomics to identify novel therapeutic targets (Tan, 2008). This revealed that the dulanermin-resistant tumour cells overexpressed Bcl-2, which is associated with resistance to TRAIL (Vogler et al., 2008). There was constitutive activation of the NF-κB and STAT3 pathways, ERK pathway, mTOR pathway, and fatty acid synthase–mediated signalling in the dulanermin-resistant cells (Subbiah et al., 2012).

Pre-clinical studies have shown that dulanermin/Apo2L/TRAIL cooperates with paclitaxel and carboplatin chemotherapy, inhibits tumour growth, and improves overall survival in tumour-bearing mice (Jin et al., 2004). A phase Ib study investigated whether dulanermin could be safely combined with paclitaxel, carboplatin, and bevacizumab in patients with advanced non–small-cell lung cancer (NSCLC) (Soria et al., 2010). The patients were treated with paclitaxel, carboplatin, and bevacizumab on day 1 of each 21-day cycle and then with dulanermin for 5

consecutive days. There were no dose-limiting toxicities for this combination and no patients developed severe complications arising from rapid tumour involution.

A total of 8 out of 24 patients experienced adverse events (perineal abscess, hypercholesterolemia, fever, tachycardia, bowel perforation; and gingival bleeding). About 50% of these adverse events were thought to have been caused by either dulanermin or bevacizumab. The half-life of dulanermin was only 23 to 41 minutes and no dulanermin accumulation was observed despite multiple dosing. Although there was an overall response rate of around 58%, there was no association between dulanermin dose and tumour response. Importantly, the combination of chemotherapy and dulanermin was not associated with hepatotoxicity (Soria et al., 2010). A subsequent randomised phase II study of dulanermin in combination with paclitaxel, carboplatin, and bevacizumab in 213 patients with NSCLC found that the addition of dulanermin did not improve outcomes (Soria et al., 2011b).

It has previously been reported that death-receptor O-glycosylation affects the sensitivity of cancer cell lines to TRAIL. Cells that expressed high levels of the peptidyl O-glycosyltransferase, GALNT14, are more sensitive to dulanermin/Apo2L/TRAIL than low-expressing NSCLC cell lines (Wagner et al., 2007). This observation has led to the development of an immunohistochemistry assay to measure GALNT14 expression in tumours (Stern et al., 2010). The expression of GALNT14 was measured in 84 patients with NSCLC and was elevated in around 33% of the tumours (Soria et al., 2010).

An exploratory analysis was performed to determine if the overexpression of GALNT14 was associated with improved outcomes after treatment with dulanermin. There was a trend towards enhanced progression-free survival (PFS) and overall survival of the patients with GALNT14-positive tumours who received dulanermin versus control. However, the relative difference in overall survival of around 1 month between the GALNT14 positive and negative groups treated with and without dulanermin was small, and did not reach statistical significance. This suggests that GALNT14 expression is not highly predictive of the sensitivity of NSCLC to TRAIL (Soria et al., 2011b). However, this biomarker may have use in other tumour types and could be incorporated in subsequent clinical studies.

It has previously been reported that dulanermin/Apo2L/TRAIL cooperates with the anti-CD20 antibody rituximab against non-Hodgkin lymphoma xenografts (Daniel et

al., 2007). However, a phase II study, which was presented in 2010, found that the addition of dulanermin to rituximab did not improve response rates in patients with relapsed follicular non-Hodgkin's lymphoma (Belada et al., 2010). These data suggest that dulanermin is not a promising treatment in patients with lung cancer or follicular lymphoma cancer.

1.2.6 TRAIL-R1-specific antibodies

The most advanced TRAIL-R1-specific antibody that has been tested in patients is mapatumumab, which is a fully humanised anti-TRAIL-R1 immunoglobulin G1 (IgG1) monoclonal antibody. Mapatumumab has been evaluated in early phase clinical trials in patients with hepatocellular carcinoma (HCC), myeloma, NSCLC and Non-Hodgkin lymphoma (NHL), myeloma, and cervical cancer (Table 3). Pre-clinical studies have shown that mapatumumab (HGS-ETR1) treatment can induce apoptosis in cell lines and enhance cytotoxicity of chemotherapy drugs such as camptothecin, cisplatin, carboplatin, and 5-fluorouracil. Mapatumumab treatment results in the regression or repression of tumour growth in murine xenograft models of human colon, NSCLC, and renal cell carcinoma (RCC), which is enhanced by chemotherapy treatment (Pukac et al., 2005).

TRAIL-R1 agonist	Combination treatment	Phase	Tumour type	Trial number	Current Status
Mapatumumab	Sorafenib	I/II	HCC	NCT00712855	Completed
	Sorafenib	II	HCC	NCT01258608	Active, not recruiting
	Bortezomib	II	Myeloma	NCT00315757	Completed
	Cisplatin and radiotherapy	Ib/II	Cervical cancer	NCT01088347	Recruiting participants
	Paclitaxel and Carboplatin	II	NSCLC	NCT00583830	Completed
		II	NSCLC	NCT00092924	Completed
		Ib/II	NHL	NCT00094848	Completed

Table 3: **Current clinical trials of TRAIL-R1-specific antibodies.**
(www.clinicaltrials.gov:database accessed on the 22/02/2013)

Mapatumumab is well tolerated in patients and the main side effects that were reported in the initial phase I clinical trial were fatigue (36.2%), hypotension (34.1%), nausea (29.3%), and pyrexia (12.2%). No responses were seen in patients within the initial phase I study (Hotte et al., 2008). A subsequent phase II trial of mapatumumab

in patients with refractory colorectal cancer did not lead to any tumour responses, but mapatumumab treatment was associated with disease stabilisation in around 32% of patients for about 2.6 months (Trarbach et al., 2010). A subsequent phase II trial of mapatumumab in patients with advanced NSCLC did not induce responses in any patients (Greco et al., 2008).

One reason for the lack of responses to mapatumumab treatment could have been due to the intrinsic TRAIL resistance of primary NSCLC. Therefore subsequent trials investigated whether the addition of chemotherapy could lead to enhanced responses in patients with cancer. A phase I clinical trial showed that mapatumumab could be safely combined with paclitaxel and carboplatin in patients with advanced solid malignancies, and found that 19% and 44% of patients within the study had either a partial response or stable disease in the study (Leong et al., 2009).

A subsequent randomised phase II trial of mapatumumab in combination with carboplatin and paclitaxel in patients with advanced NSCLC (NCT00583830) did not report any benefit from the addition of mapatumumab to chemotherapy (Von Pawel, 2010). Additional studies have shown that mapatumumab can be safely administered in combination with gemcitabine and cisplatin (Mom et al., 2009), although it is unclear as to whether there is any benefit from this therapeutic combination.

Preliminary results from a phase Ib trial (NCT00712855) in which patients with HCC were treated with mapatumumab and sorafenib (which targets the anti-apoptotic protein Mcl-1 (myeloid cell leukaemia sequence 1)), have been reported that 2 out 19 patients had a response to this treatment and 4 patients had stable disease. The reported side effects included hepatic pain, thrombocytopenia, as well as increased plasma levels of aspartate aminotransferase (AST), lipase, and gamma-glutamyltransferase which were considered manageable by the investigators (Sun, 2011). The results were sufficiently promising to lead to a randomised phase II trial of this combination (NCT01258608).

Pre-clinical studies have reported that the proteasome inhibitor bortezomib (PS-341) sensitises non-Hodgkin's lymphoma cells and myeloma cells to apoptosis (Balsas et al., 2009, Smith et al., 2007). However, a large randomised phase II clinical trial (NCT00315757) which recruited 104 patients with relapsed or refractory myeloma who received either PS-341 or PS-341 and mapatumumab did not find any benefit from the addition of mapatumumab to PS-341.

These disappointing results were offset by results from a phase 1b/2 trial of mapatumumab in patients with relapsed/refractory non-Hodgkin's lymphoma, which showed that 3 patients with follicular lymphoma experienced clinical responses which included two complete responses and one with a partial response to treatment (Younes et al., 2010b). These data suggest that mapatumumab has some activity in follicular lymphoma.

1.2.7 TRAIL-R2-specific antibodies

A number of clinical trials have been conducted with several different TRAIL-R2-specific antibodies (Table 4). Conatumumab (AMG 655) is an investigational, fully humanised IgG1 monoclonal agonistic antibody which was developed by AMGEN (Kaplan-Lefko et al., 2010). AMG 655 has been found to induce apoptosis in tumour cells, both *in vitro* in the presence of a crosslinker and *in vivo* in mouse xenograft models of human cancer (Kaplan-Lefko et al., 2010).

AMG 655 is well-tolerated; and the phase I study found that the adverse effects were mild and there were no dose-limiting toxicities. In the initial phase I study, one patient with NSCLC had a partial response and has remained on treatment for over 4.2 years and 14 patients had stable disease, and also a patient with colorectal cancer had stable disease for around 24 weeks, and subsequently went on to develop a response to the treatment (Herbst et al., 2010b). An additional phase I study in Japanese patients with advanced solid tumours reported additional reassuring safety data, and stable disease rates of around 50% in treated patients. However, there were no confirmed responses after AMG 655 treatment (Doi et al., 2011).

A phase Ib/II trial (NCT00630786) of AMG 655 and panitumumab, an anti-epidermal growth factor receptor antibody used for the treatment of metastatic colorectal cancer (mCRC), found that although the combination was safe, there was no evidence of synergistic activity for this combination (Peeters, 2010). A recently reported study has shown that the addition of AMG 655 to paclitaxel and carboplatin chemotherapy for the first-line treatment of NSCLC does not improve outcomes in unselected patients (Paz-Ares et al., 2013). A study of the combination of AMG 655 with doxorubicin versus doxorubicin alone in patients with metastatic or locally advanced unresectable soft tissue sarcomas found that the addition of AMG 655 led to more early progressions within the first 3.5 months of treatment (Demetri et al., 2012).

A randomised placebo-controlled phase II study of the anti-insulin-like growth factor receptor antibody ganitumab (AMG 479) or AMG 655 in combination with gemcitabine, in patients with metastatic pancreatic cancer, found that the addition of AMG 655 led to a modest improvement in 6 month survival of 57%, versus 50% in the placebo arm (Kindler et al., 2012).

Another recently reported study found that the addition of AMG 655 to fluorouracil (5-Fu) and irinotecan chemotherapy for the second-line treatment of KRAS (Kirsten rat sarcoma viral oncogene homolog) mutant metastatic colorectal cancer (mCRC) was associated with an improvement in the overall response rate of around 13.7% versus 2.0% in the placebo arm of the trial. This was associated with an improvement in the progression-free survival of around 6.5 months, versus 4.6 months in the placebo arm of the trial. Although AMG 655 treatment was not associated with a survival benefit, these findings suggest that AMG 655 may have activity in KRAS mutant mCRC (Cohn et al., 2013). AMG 655 has been safely combined with AMG 479 (Chawla, 2010) and this combination is currently being investigated in a phase II study in patients with KRAS-mutant mCRC (NCT01327612).

Drozitumab (PRO95780) is a fully human IgG1 monoclonal antibody against TRAIL-R2. Drozitumab is well tolerated and the only major side effects reported in the phase I clinical trial were 2 patients with a reversible grade 3 transaminase elevation. There was some encouraging evidence of clinical activity as three minor responses were observed in patients with mCRC, granulosa cell ovarian cancer, and chondrosarcoma (Camidge et al., 2010b). A Phase Ib study is currently investigating whether drozitumab can be combined with first-line chemotherapy and bevacizumab in patients with mCRC. Although this combination appears to be well tolerated it is currently unclear as whether it will lead to improved outcomes in patients (Lima et al., 2012). Drozitumab has also been safely combined with cetuximab plus irinotecan or with fluorouracil and irinotecan with or without bevacizumab in previously treated patients with mCRC. Although it is unclear as to whether the addition of drozitumab has any benefit over standard chemotherapy (Baron, 2011).

Anti-TRAIL-R2 antibody	Combination treatment	Phase	Tumour type	Trial number	Current Status
Conatumumab	Doxorubicin	Ib/II	Sarcoma	NCT00626704	Completed
	Gemcitabine	Ib/II	Pancreas	NCT00630552	Completed
	FOLFOX6, Bevacizumab	Ib/II	CRC	NCT00625651	Active, not recruiting
	Carboplatin, Paclitaxel	Ib/II	NSCLC	NCT00534027	Completed
	Panitumumab	Ib/II	CRC	NCT00630786	Unknown
	AMG 479	1b/2	Solid tumours	NCT00819169	Terminated
	AMG 479	II	KRAS-mutant MCRC	NCT00813605	Active, not recruiting
	Bortezomib or Vorinostat	1b	Refractory lymphoma	NCT00791011	Recruitment suspended
	AMG 480	II	KRAS-mutant MCRC	NCT01327612	Recruiting
Drozitumab	-	II	Chondrosarcoma	NCT00543712	Terminated
	-	II	NSCLC	NCT00480831	Completed
	Cetuximab and Irinotecan or FOLFIRI + Bevacizumab	I	mCRC	NCT00497497	Completed
	Rituximab	II	NHL	NCT00517049	Completed
Tigatuzumab	Abraxane	II	Breast	NCT01307891	Active, not recruiting
	Irinotecan	II	CRC	NCT00969033	Terminated
	Carboplatin, paclitaxel	II	Ovarian	NCT00945191	Completed
	Irinotecan	II	mCRC	NCT01124630	Active, not recruiting
	Sorafenib	II	HCC	NCT01033240	Active, not recruiting
	FOLFIRI	II	mCRC	NCT00969033	Terminated
	Carboplatin/Paclit -axel	II	NSCLC	NCT00991796	Completed
	-	1	Solid/lymphoma	NCT00320827	Completed
	Paclitaxel/Carbop latin/bevacizumab	II	NSCLC	NCT00508625	Completed
	-		Ovarian cancer	NCT00711932	Active, not recruiting
Lexatumumab	–	I	Solid/lymphoma	NCT00428272	Completed
TAS266	–	I	Solid tumours	NCT01529307	Terminated

Table 4: Current clinical trials of TRAIL-R2-specific antibodies. (www.clinicaltrials.gov:database accessed on the 22/02/2013)

Tigatuzumab (TRA-8/CS-1008) is an agonistic humanised IgG1 monoclonal antibody that targets TRAIL-R2; and unlike other TRAIL-R2-specific antibodies, it has been shown to induce apoptosis without crosslinking (Ichikawa et al., 2001). Tigatuzumab is well tolerated and no dose-limiting toxicities have been reported to date. Although, no responses were seen in treated patients, 7 out of 17 patients had stable disease, suggesting that tigatuzumab has some anti-tumour activity (Forero-Torres et al., 2010). Tigatuzumab is currently under investigation in combination with various different chemotherapy drugs as a potential treatment for breast, ovarian, mCRC, HCC, and NSCLC. Although many of the studies have completed recruitment (NCT00320827, NCT00508625, and NCT00945191), the results have not yet been reported in either abstract form or within peer reviewed journals which may possibly be an indication of negative outcomes.

Lexatumumab (HGS-ETR2) is a fully humanised agonistic monoclonal antibody against TRAIL-R2, which has been tested in a phase I clinical trial in patients with advanced cancers (Plummer et al., 2007, Sikic, 2007, Wakelee et al., 2010, Merchant et al., 2012). Like other anti-TRAIL-R2 antibodies, lexatumumab is well tolerated and the major dose-limiting toxicities within the phase I clinical trial were asymptomatic elevations of serum amylase, transaminases, and bilirubin. No responses were seen after lexatumumab treatment; however twelve patients had stable disease which lasted up to 4.5 months (Plummer et al., 2007). Another phase I study reported similar stable disease rates and one patient with chemotherapy-refractive Hodgkin's disease experienced a mixed response (Wakelee et al., 2010).

A phase I trial of lexatumumab in paediatric patients with solid tumours showed that there was some evidence of anti-tumour activity with several patients having stable disease for between 3 to 24 cycles of treatment; and one patient with progressive osteosarcoma had a clinical response to treatment (Merchant et al., 2012). Other studies have reported that lexatumumab can be safely combined with gemcitabine, pemetrexed, doxorubicin or 5-Fu and irinotecan (Sikic, 2007).

Although TRAIL-R2-specific antibodies such as AMG 655 and drozitumab can bind TRAIL-R2, they cannot induce TRAIL-R2 multimerisation and formation of the TRAIL DISC. The *in vivo* activity of TRAIL-R2-specific antibodies such as conatumumab (Kaplan-Lefko et al., 2010) or drozitumab is dependent on an FcγR-dependent mechanism which leads to antibody-mediated-TRAIL-R2-signalling in cancer cells, a

phenomenon first described for anti-Apo-1/CD95 (Wilson et al., 2011, Dhein et al., 1992, Haynes et al., 2010a, Haynes et al., 2010b).

FcγR receptors such as FcγRIIIA (CD16), FcγRIIA (CD32), and FcγRI (CD64) are present on immune cells such as macrophages, neutrophils and NK cells (Hogarth and Pietersz, 2012). FcγRs bind TRAIL-R2-specific antibodies on the surface of cancer cells. This leads to crosslinking of TRAIL-R2, thereby enabling multimerisation of TRAIL-R2 and, consequently, formation of the DISC and induction of apoptosis (Wilson et al., 2011, Dhein et al., 1992, Haynes et al., 2010b).

Previous pre-clinical studies have demonstrated that this mechanism applies to antibodies against CD95 (Fas/APO-1) (Dhein et al., 1992) and TRAIL-R2 (Wilson et al., 2011, Haynes et al., 2010a). However, although TRAIL-R2-specific antibodies can efficiently induce apoptosis in murine xenograft models of cancer (Wilson et al., 2011, Kaplan-Lefko et al., 2010), few tumour responses have been observed in patients treated with TRAIL-R2-specific antibodies in clinical trials (Camidge et al., 2010a, Rocha Lima et al., 2012, Demetri et al., 2012, Doi et al., 2011, Herbst et al., 2010b).

1.2.8 TRAIL as a treatment for ovarian cancer

TRAIL has been considered as a potential treatment for ovarian cancer for over 10 years. Immunohistochemistry studies have shown that ovarian cancer cells express both TRAIL-R1 and TRAIL-R2 (Duiker et al., 2010). Early studies showed that cisplatin, doxorubicin, and paclitaxel chemotherapy synergised with TRAIL to induce the death of chemotherapy-resistant ovarian cancer cell lines (Cuello et al., 2001b, Tomek et al., 2004). Other studies have shown that trastuzumab enhances TRAIL-induced death of HER-2 overexpressing ovarian cancer cell lines (Cuello et al., 2001a). Cisplatin treatment increases the expression of TRAIL-R1 and TRAIL-R2 (Duiker et al., 2009), and decreases the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL (B-cell lymphoma-extra-large) (Siervo-Sassi et al., 2003) which enhances the *in vitro* and *in vivo* sensitivity of ovarian cancer cells to TRAIL (Liu et al., 2006a, Duiker et al., 2011).

Although it is currently unclear as to whether TRAIL-R1 or TRAIL-R2 is the most important inducer of apoptosis, TRAIL-R2 has been reported to make a greater contribution to apoptosis induction in some cell lines (Kelley et al., 2005).

Recombinant TRAIL variants have been developed which exclusively induce apoptosis via TRAIL-R1 or TRAIL-R2 (Reis et al., 2010, van der Sloot et al., 2006). A novel TRAIL-R2-specific variant has been shown to have enhanced efficacy over recombinant TRAIL in both *in vitro* and *in vivo* studies (Duiker et al., 2009). This suggests that therapeutic approaches which specifically target TRAIL-R2 may have clinical utility in ovarian cancer. One explanation for the differential apoptotic effects of TRAIL-R1 and TRAIL-R2 in ovarian cancer is that TRAIL-R1 is hypermethylated in around 27% of patients with advanced ovarian cancer, which leads to the complete loss or down-regulation of TRAIL-R1 in around 10% of patients with ovarian cancer (Horak et al., 2005a).

An important pre-clinical study has evaluated the clinical grade anti-TRAIL-R2-specific antibody, TRA-8, as a potential treatment for ovarian cancer using a novel *ex vivo* tissue-slice model (Estes et al., 2007). The investigators obtained 19 samples of ovarian cancer from patients who were undergoing surgery for advanced ovarian cancer. Unlike other TRAIL-R2-specific antibodies, TRA-8 does not require crosslinking by FcγR expressing immune cells to induce TRAIL-R2 mediated apoptosis (Ichikawa et al, 2001). The investigators studied 5 mm cylindrical cores of tumour which were sliced into 300–600 μm thick sections, which were then cultured and treated with TRA-8. The results showed that TRA-8 reduced proliferation, increased caspase-8 activation, and apoptosis induction within solid deposits of ovarian cancer (Estes et al., 2007).

Several clinical trials of TRAIL-R agonistic drugs have included patients with advanced ovarian cancer. A total of 8 patients with ovarian cancer were treated with dulanermin/Apo2L/TRAIL in an extended dose-escalation phase I clinical trial (Herbst et al., 2010a). Although dulanermin was well tolerated, there were no reported responses in any of the patients with ovarian cancer. However, one of four patients with an ovarian granulosa cell tumour had a partial response in a phase I trial of the TRAIL-R2-specific antibody PRO95780 (Camidge et al., 2010a). A large phase I trial has recently been completed of TRA-8 in combination with carboplatin and paclitaxel in patients with ovarian cancer (NCT00945191). Although the study has been completed, the results have yet to be published which may possibly be an indication of a negative outcome.

1.2.9 The future of TRAIL-R agonistic drugs

Despite promising early results from pre-clinical studies (Walczak et al., 1999, Ashkenazi et al., 1999, Ichikawa et al., 2001), the clinical development of TRAIL-R agonist drugs has been characterised by mostly disappointing results from clinical trials of recombinant forms of TRAIL such as dulanermin, as well as of TRAIL-R1- and TRAIL-R2-specific antibodies.

There could be several reasons behind the failure of TRAIL-R agonists to lead to responses in patients. It could reflect the intrinsic TRAIL resistance of primary human cancers, insufficient agonistic activity of the different clinical grade TRAIL-R agonists, non-suitable combinations with other drugs in clinical trials, or any combination of these factors.

1.2.10 Overcoming TRAIL resistance in ovarian cancer

Although many ovarian cancer cell lines are sensitive to TRAIL, the clinical experience of patients treated with dulanermin/Apo2L/TRAIL and TRAIL-R1/2 antibodies suggests that the majority of *in vivo* primary human cancer cells are resistant to TRAIL. Numerous pre-clinical studies have addressed this question and have reported that many different classes of compounds can sensitise ovarian and other cancer cells to TRAIL-induced apoptosis.

For example the diuretic amiloride has been shown to sensitise SKOV3 ovarian cancer cells to TRAIL-induced apoptosis by decreasing the phosphorylation of HER-2, Akt, PI3K and PDK-1 (Kim and Lee, 2005). On the other hand, dexamethasone, a potent steroid given to patients receiving chemotherapy for ovarian cancer, increases the expression of the caspase inhibitor cIAP2, which can increase the resistance of ovarian cancer cells to TRAIL (Runnebaum and Bruning, 2005).

Curcumin, the active component of turmeric, has been shown to enhance the sensitivity of ovarian cancer cell lines to TRAIL (Wahl et al., 2007), however the effect of curcumin is modest and only seen at concentrations of around 5-15 μM, which is below achievable levels with the oral administration of curcumin to patients with cancer (Baum et al., 2008). Although such studies are interesting, it is difficult to envisage any of these combinations being rationally combined with a TRAIL-R agonistic drug in the clinic.

1.2.11 The combination of TRAIL and Bortezomib as a potential treatment for ovarian cancer

One of the most promising strategies that could be used to overcome TRAIL resistance in ovarian cancer cells is proteasome inhibition. The proteasome regulates the degradation of intracellular proteins that have been targeted for degradation by a ubiquitin tag. The proteasome regulates numerous cellular processes such as cell cycle progression, transcription, antigen presentation, and apoptosis (Jesenberger and Jentsch, 2002). There are a number of different proteasomes which are found in the nucleus and the cytoplasm of cells such as the 20S and 26S proteasomes. The core 20S proteasome contains proteolytic sites which are bound to regulatory complexes at the end of a cylindrical structure. The 26S proteasome is formed from the binding of two 19S ATPase regulatory complexes to a 20s subunit which identifies and induces proteolysis of ubiquitin-tagged proteins (Brooks et al., 2000).

The proteasome regulates many different pathways that are important for the survival of cancer cells. For example cyclins, which regulate the cell cycle, are degraded by ubiquitin-dependent proteolysis (Glotzer et al., 1991). The proteasome regulates the activity of the NF-κB pathway, which is an important regulator of cancer cell growth, proliferation, apoptosis, and resistance to chemotherapy (Nakanishi and Toi, 2005). Inhibition of the proteasome prevents the activation of the NF-κB pathway by preventing the proteasomal degradation of I kappa B-alpha (Traenckner et al., 1994); an important negative regulator of the NF-κB pathway.

Many different types of cancer cells, including ovarian cancer cells, have been reported to be sensitive to the effects of proteasome inhibition (Adams, 2004). The reasons for the relative susceptibility of the cancer cells relative to the normal healthy tissues are unclear. The first licenced proteasome inhibitor was bortezomib (PS-341) a synthetic peptide aldehyde, with an aldehyde substitution to boronic acid to enhance its potency and selectivity of the proteasome inhibition (Adams et al., 1998). PS-341 was selected from a panel of similar proteasome inhibitors as it displayed substantial cytotoxicity against a wide range of human tumour cell lines, in both *in vitro* and *in vivo* assays (Adams et al., 1999).

Several studies have shown that proteasome inhibitors such as PS-341 can sensitise ovarian cancer cell lines and primary ovarian cancer cells to TRAIL-induced

apoptosis (Saulle et al., 2007, Bruning et al., 2009). It has been shown that PS-341 can sensitise primary ovarian cancer cells to mapatumumab and lexatumumab (Pasquini et al., 2010). However, although PS-341 can induce apoptosis in primary ovarian cancer cells (Pasquini et al., 2010), several recently published clinical trials have shown that it has insufficient clinical activity in ovarian cancer to warrant further development as a treatment for ovarian cancer (Parma et al., 2012, Aghajanian et al., 2009).

Despite detailed biochemical studies, it is currently unclear as to how proteasome inhibition sensitises cancer cells to TRAIL. The effect of PS-341 on the sensitivity of HCC to TRAIL appears to be independent of NF-κB signalling. PS-341 treatment leads to enhanced recruitment of $cFLIP_L$ and $cFLIP_S$ and caspase-8 to the TRAIL DISC (Ganten et al., 2005). PS-341 treatment can also up-regulate the expression of TRAIL-R1/TRAIL-R2 as well as down-regulating the expression of $cFLIP_L$ (Koschny et al., 2007a). PS-341 has been shown to increase the sensitivity of glioblastoma cells to TRAIL induced apoptosis by increasing the stability of the pro-apoptotic protein tBid (truncated BH3 interacting-domain death agonist) and through enhancing mitochondrial apoptosis (Unterkircher et al., 2011). However it is not known how PS-341 affects TRAIL sensitivity in ovarian cancer cells.

Pre-clinical studies have reported that PS-341 sensitises NHL cells and myeloma cells to apoptosis (Balsas et al., 2009, Smith et al., 2007). However, a large randomised phase II clinical trial (NCT00315757) which recruited 104 patients with relapsed or refractory myeloma who received either PS-341 or PS-341 + mapatumumab did not find any benefit from the addition of mapatumumab to PS-341. These data suggest that further pre-clinical studies are required before proceeding to a clinical trial of PS-341 and a TRAIL-R agonistic drug in patients with advanced ovarian cancer.

1.2.12 Enhancing TRAIL sensitivity by inhibiting IAP proteins

An alternative approach to enhancing TRAIL sensitivity is to antagonise the function of inhibitor of apoptosis proteins (IAPs) that contribute to the resistance of cancer cells to TRAIL. Apoptosis is a highly regulated process and IAPs prevent the induction of apoptosis by inhibiting caspase activation by directly binding them and indirectly by positively regulating anti-apoptotic signalling pathways. The targeting of

IAPs has the potential to both enhance the sensitivity of cancer cells to death ligands like TRAIL, as well as enhancing their sensitivity to other therapies such chemotherapy or radiotherapy.

IAP family members include X-linked Inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis protein-1 (cIAP1), cellular inhibitor of apoptosis protein-2 (cIAP2), survivin, apollon, melanoma IAP, bruce, neuronal apoptosis inhibitory protein (NAIP), IAP-2 (Vucic and Fairbrother, 2007). All IAP family members contain between 1 and 3 baculoviral IAP repeat (BIR) domains which can directly bind and inhibit caspases; and thereby suppress apoptosis (Deveraux and Reed, 1999). However, not all IAP family members are involved in regulating apoptosis. IAPs are expressed at elevated levels in many human cancers and promote carcinogenesis by inhibiting cell death and activating oncogenic signalling pathways.

Perhaps the most important, and well described IAP is XIAP, which is able to directly suppress apoptosis by binding and inhibiting activated caspases (Holcik et al., 2001). XIAP contains three BIR domains which form a IAP-binding motif (IBM)-interacting groove which allows the BIR_2 domain to inhibit caspase-3, caspase-7; and also allows BIR_3 to inhibit caspase-9 by directly binding and blocking the activity of cleaved caspases (Eckelman et al., 2006). XIAP and other IAPs such as cIAP1 and cIAP2 contain a highly conserved RING (really interesting new gene) domain which confers these IAPs with ubiquitin E3 ligase activity.

Although XIAP deficiency in mice does not lead to an abnormal phenotype, it does lead to an increase in cIAP1 and cIAP2 levels, suggesting that compensatory mechanisms exist for the loss of XIAP (Harlin et al., 2001). In humans, XIAP deficiency causes the X-linked lymphoproliferative syndrome (XLP), which is characterised by lymphohystiocytosis, hypogammaglobulinaemia and lymphomas. XIAP deficiency leads to enhanced T cell activation-induced cell death and increased sensitivity to CD95L and TRAIL. Also XIAP is required for the survival and/or differentiation of NK T cells (Rigaud et al., 2006). XIAP antagonism does not promote the development or survival of regulatory T cells in mice or humans. However, it does promote T cell costimulation and cytokine production, suggesting that therapeutic strategies that target XIAP have the potential to enhance immune responses against cancer cells (Dougan et al., 2010).

XIAP regulates Akt and caspase-3-dependent cleavage when ovarian cancer cells are undergoing cisplatin-induced apoptosis. In cisplatin-sensitive cells, cisplatin treatment decreases XIAP levels, whereas in cisplatin-resistant cells, cisplatin treatment does not affect the expression of XIAP. Overexpression of XIAP leads to increased Akt phosphorylation and activation of PI3K signalling; and knockdown of XIAP leads to Akt cleavage and apoptosis in some cell lines (Asselin et al., 2001). Other investigators have reported that down-regulation of XIAP induces apoptosis in cisplatin-sensitive cells, and to lesser extent in cisplatin-resistant cells, and overexpression of XIAP attenuates the sensitivity of ovarian cancer cell lines to cisplatin (Li et al., 2001).

Akt phosphorylates XIAP at serine-residue 87 which prevents XIAP from undergoing cisplatin-induced auto-ubiqutination and proteasomal degradation. Knockdown of XIAP has been reported to inhibit Akt-mediated survival of ovarian cancer cells (Dan et al., 2004). XIAP has also been reported to regulate the sensitivity of ovarian cancer cell lines and primary ovarian cancer cells to the chemotherapy drug docetaxel. The treatment of docetaxel-sensitive cells, led to the inactivation of XIAP and the induction of apoptosis; however the effect of docetaxel on IAP expression is lost in docetaxel-resistant cells (Sapi et al., 2004). The down-regulation of XIAP has been shown to induce apoptosis in both *in vitro* and *in vivo* models of ovarian cancer and to enhance the survival of ovarian cancer tumour-bearing mice (Shaw et al., 2008).

The anti-apoptotic effects of IAPs are regulated by SMAC (second mitochondrial activator of caspases)/DIABLO (direct IAP binding protein with low pI), a mitochondrial protein that promotes caspase activation by binding the BIR domains and competing with caspases, resulting in the release of inhibition of caspases by IAPs (Du et al., 2000, Verhagen et al., 2000). Under normal physiological conditions SMAC is confined to the mitochondria, but when cells are damaged and undergoing apoptosis, SMAC/DIABLO is released into the cytoplasm, where its amino-terminus binds to the BIR3 domain on IAPs, via a conserved 4 amino acid (Ala–Val–Pro–Ile) domain (Liu et al., 2000, Wu et al., 2000).

This discovery led to the design of small molecule IAP antagonists which mimic the effect of SMAC on IAPs which are active in both *in vitro* and *in vivo* models of cancer (Li et al., 2004, Oost et al., 2004, Zobel et al., 2006, Cao et al., 2009, Sun et al., 2004,

Sun et al., 2008). SMAC mimetics rapidly induce the auto-ubiquitination and degradation of c-IAP1 and c-IAP2, which leads to the activation of non-canonical NF-κB signalling, resulting in the production of autocrine TNF (Varfolomeev et al., 2007, Petersen et al., 2007, Vince et al., 2007).

Compound	Tumour type	Clinical trial	Phase	Combination	Status
Birinapant	Solid Tumours	NCT01573780	2	Gemcitabine	Currently recruiting
Birinapant	AML	NCT01486784	1/2		Currently recruiting
Birinapant	Solid Tumours	NCT01188499	1/2	Chemotherapy	Not recruiting
Birinapant	Ovarian cancer	NCT01681368	2		Currently recruiting
AT-406	Solid Tumours and Lymphoma	NCT01078649	1		Currently recruiting
GDC-0152	Metastatic Malignancies	NCT00977067	1		Completed

Table 5: Current SMAC mimetic compounds in clinical trials in patients with cancer. Data from www.clinicaltrials.gov (database accessed 23/03/2013).

In the absence of cIAPs, TNF signalling does not lead to gene activation but rather to the formation of a cytoplasmic complex (complex II) which contains RIP (Receptor interacting protein) 1, FADD, and caspase-8 which stimulates caspase-8 induced apoptosis. In addition, it has been shown that depletion of cIAPs by SMAC mimetics can promote the formation of a similar complex independently from TRAIL, CD95L, and TNF (Tenev et al., 2011). This cell death inducing complex has been termed the 'ripoptosome', and is negatively regulated by cIAP1 and cIAP2 that constitutively ubiquitinate components of the complex (Tenev et al., 2011).

The ovarian cancer tumour microenvironment contains a dynamic inflammatory 'TNF cytokine network' which is driven by TNF, CXCL12, and IL-6 which leads to angiogenesis and infiltration of myeloid cells (Kulbe et al., 2012, Kulbe et al., 2007). Therefore, SMAC mimetics have the potential to turn the tumour promoting properties of TNF (Balkwill, 2009) against ovarian cancer cells (Wu et al., 2007). A phase II clinical trial of the SMAC mimetic birinapant (TL32711) in patients with advanced

ovarian, fallopian tube, and peritoneal cancer (NCT01681368) is currently enrolling patients. The first results from this study are expected to be presented in early 2014.

Although SMAC mimetic treatments rapidly initially induce the degradation of both cIAP1 and cIAP2, the degradation of cIAP2 requires the presence of cIAP1. The depletion of cIAP1 can lead to the activation of non-canonical NF-κB signalling which induces the expression of cIAP2. In the absence of cIAP1, cIAP2 suppresses TNFα-induced killing of cells, and may suppress TRAIL-induced killing of cancer cells (Darding et al., 2011). These data may have important therapeutic implications for the acquisition of resistance of cancer cells to SMAC mimetic drugs. This suggests that intermittent dosing strategies that temporarily induce the expression of cIAP1 and cIAP2 may have the greatest chance of enhancing the sensitivity of cancer cells to TRAIL and TNF induced apoptosis and sensitivity to SMAC mimetic compounds.

Although ovarian cancer cells do not appear to be dependent on a single IAP for their survival, targeting IAP proteins may enhance the sensitivity of the cancer cells to TRAIL and to chemotherapy. TRAIL-resistant primary ovarian cancer cells can be reliably sensitised to TRAIL-induced apoptosis by SMAC mimetic compounds (Petrucci et al., 2007, Petrucci et al., 2012, Cossu et al., 2012, Lecis et al., 2012). SMAC mimics have been shown to potentiate TRAIL-mediated cell death in *in vitro* and *in vivo* models of ovarian cancer (Petrucci et al., 2007, Petrucci et al., 2012).

A recent study of the SMAC mimetic LBW242 has shown that it enhances the TRAIL-induced death of ovarian cancer cell lines and primary ovarian cancer cells (Petrucci et al., 2012). Recent publications have shown that a potent dimeric SMAC mimetic compound SM-83 (also known as SM 9a) has activity in a peritoneal murine model of ovarian cancer and synergises with TRAIL in killing ovarian cancer cells and melanoma cells (Cossu et al., 2012, Lecis et al., 2010, Lecis et al., 2012). These data suggest that SMAC mimetic drugs are a potential treatment for ovarian cancer and may enhance the sensitivity of ovarian cancer cells to TRAIL-induced apoptosis (Table 5).

1.2.13 Overcoming TRAIL resistance in the ovarian cancer clinic

The main clinical strategy that has been used to overcome TRAIL resistance is to combine standard chemotherapy drugs with TRAIL-R agonistic therapies. Thus far, this strategy has yet to reproduce any of the pre-clinical studies that suggested that

this approach would be useful in patients with cancer. For example, the addition of TRAIL to standard chemotherapy has failed to improve outcomes in patients with lung cancer (Paz-Ares et al., 2013, Soria et al., 2011b), colorectal cancer (Peeters, 2010), and lymphoma (Belada et al., 2010).

A pre-clinical study found that although only 15% of solid deposits of ovarian cancer were sensitive to the TRAIL-R2-specific antibody TRA-8; and there was synergy with carboplatin and paclitaxel chemotherapy in around 77% of patients (Frederick et al., 2009). The same investigators also showed that TRA-8 synergises with docetaxel and carboplatin in a murine intraperitoneal ovarian cancer model. However, the differences in survival of mice treated with chemotherapy and TRA-8 group versus chemotherapy alone was a modest 7 days (Bevis et al., 2011).

One explanation for these differential effects is that current TRAIL-R agonistic drugs are not sufficiently active to induce the death of the cancer cells. However, dulanermin has been shown to induce the death of multiple cancer cell lines *in vitro* and *in- vivo;* and therefore this is probably not the main explanation for the lack of efficacy of TRAIL in the clinic.

Several factors have been shown to contribute the resistance of ovarian cancer cells to TRAIL. The anti-apoptotic protein c-FLIP$_{(L)}$ has been implicated in the resistance of ovarian cancer to TRAIL, and down-regulation of c-FLIP$_{(L)}$ has been shown to enhance the sensitivity of ovarian cancer cells to TRAIL (Lane et al., 2004, Clarke and Tyler, 2007, Li et al., 2011a). Increased c-FLIP$_{(L)}$ is seen in around 40% of ovarian cancers (Horak et al., 2005b). However, proteasome inhibition can circumvent c-FLIP$_{L}$ overexpression, and can enhance the sensitivity of ovarian cancer cells to TRAIL (Syed et al., 2007, Saulle et al., 2007).

Several studies have investigated whether ovarian cancer ascites has an effect on the sensitivity of ovarian cancer cells to TRAIL-induced apoptosis. Ascites has been shown to induce the PI3K/Akt pathway and induce TRAIL resistance (Lane et al., 2007). Cellular detachment modulates the sensitivity of ovarian cancer cells to TRAIL by down-regulating the PI3K pathway (Lane et al., 2008). Another study found that ovarian ascites induces the phosphorylation of focal adhesion kinase (FAK), which is correlated with the phosphorylation of Akt, and can induce protection from TRAIL-induced cell death (Lane et al., 2010).

High expression of crystallin αB is an independent marker for prognosis for patients with ovarian cancer and has been shown to impair TRAIL and cisplatin-induced apoptosis in human ovarian cancer cells (Volkmann et al., 2012). It has been reported that ovarian cancer ascites increases the expression of the anti-apoptotic protein Mcl-1 (Goncharenko-Khaider et al., 2012). Ovarian cancer ascites contains osteoprotegerin (OPG), a secreted factor that acts as a decoy receptor for RANKL (Receptor activator of NF-κB ligand) and TRAIL. OPG is found at high levels within ovarian cancer ascites and attenuates TRAIL-induced apoptosis in ovarian cancer cell lines and in primary ovarian cancer cells (Lane et al., 2012).

The overexpression of the developmental regulator Six1 has been shown to cause ovarian cancer resistance to TRAIL, and is associated with advanced stages of ovarian cancer. Given that Six1 is overexpressed in around 50% and 63% of early and late stage ovarian cancers (Behbakht et al., 2007) it is possible that this protein could be a biomarker for potential TRAIL resistance in patients with ovarian cancer.

There is already significant pre-clinical data that strongly suggests that TRAIL resistant ovarian cancer cell lines and primary ovarian cancer cells can be sensitised to TRAIL-induced apoptosis using either proteasome inhibition (Ganten et al., 2005, Saulle et al., 2007, Bevis et al., 2010) or SMAC mimetic treatment (Fulda et al., 2002b, Petrucci et al., 2007, Petrucci et al., 2012, Allensworth et al., 2013, Mao et al., 2013). However, it is unclear as to which approach is likely to be the most effective in patients with advanced ovarian cancer. Therefore, additional pre-clinical studies are required to determine which approach is most likely to be most effective before proceeding to a clinical trial in patients with ovarian cancer.

Furthermore, although the ovarian cancer tumour microenvironment contains numerous immune cells which express FcγR, it is not known whether these immune cells will be sufficient to induce TRAIL-R2-antibody-mediated cell death in primary ovarian cancer cells. Therefore, it is unclear as to whether a potential clinical trial of a TRAIL-R agonistic drug in patients with ovarian cancer should be conducted with either a recombinant form of TRAIL such as dulanermin/Apo2L/TRAIL or with a TRAIL-R2-specific antibody such as AMG 655. Further studies are required to determine the best approach for targeting the apoptotic TRAIL-R in patients with ovarian cancer.

This thesis will investigate whether Foxo factors and Klf2 have a role in Treg cell differentiation and function. It will also investigate whether TRAIL is a potential treatment for ovarian cancer and whether the TRAIL-R2 specific antibody AMG 655 could be used as a treatment for ovarian cancer, either alone or in combination with TRAIL sensitising agents such as proteasome inhibitors or a SMAC mimetic compound.

AIMS

Part 1: Hypothesis

That Foxo and Klf2 transcription factors regulate Treg cell differentiation.

Part 1: Aims

1. To investigate the role of Foxo factors in Treg cell differentiation and function.
2. To investigate the role of Klf2 in Treg cell differentiation and function.
3. To investigate the mechanisms by which Klf2 regulates Treg cell differentiation and function.

Part 2: Hypothesis

TRAIL is a potential treatment for ovarian cancer. That TRAIL resistance can be overcome by proteasome inhibition or by SMAC mimetic treatment. That the TRAIL-R2-specific antibody AMG 655 is a potential treatment for advanced ovarian cancer.

Part 2: Aims

1. To determine the sensitivity of primary ovarian cancer cells to iz-TRAIL.
2. To determine the effect of proteasome inhibition or SMAC mimetic treatment on the sensitivity of primary ovarian cancer cells to TRAIL-induced apoptosis.
3. To determine whether the presence of tumour-associated immune cells affects TRAIL sensitivity or resistance of ovarian cancer cells.
4. To determine whether immune cells within the tumour microenvironment have the potential to crosslink AMG 655 and induce apoptosis within ovarian cancer cells.
5. To investigate the potential toxicities of any novel therapeutic combinations of TRAIL-R agonistic drugs which are identified within this study.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

All the chemicals and biological reagents were purchased at pA quality unless indicated otherwise from Invitrogen (Gibco; Molecular Probes), Pierce, Roth, Sigma/Aldrich, Merck (Calbiochem), AppliChem, Honeywell or Amersham Biosciences.

2.1.2 Specific inhibitors	Source
Anti-TGFβ	R&D Systems
Bortezomib	Selleck Chemicals
Brefeldin A Solution	eBioscience
Complete Protease Inhibitor Cocktail	Roche
LY-29,4002	Sigma-Aldrich
Rapamycin	Sigma-Aldrich
Necrostatin-1	Sigma-Aldrich
Pan Caspase Inhibitor Z-VAD-FMK	R&D systems
Phosphatase Inhibitor Cocktail	Sigma-Aldrich
TNFR2-Fc - Etanercept (Enbrel®)	Pfizer

2.1.3 Buffers and solutions

Cell freezing medium	90% FCS (v/v) 10% DMSO (v/v)
FACS buffer	1 x PBS 2% FCS (v/v)
HEPES-buffered saline (HEBS; x 2) (pH = 7.05)	50 mM HEPES, free acid 280 mM NaCl 1.5 mM Na_2HPO_4

	12 mM dextrose/glucose
	10 nM KCl
IP-Lysis Buffer 1	0.5% Triton-X
	100 nM NaCl
	40 mM TRIS-HCL
	1 µM $ClCl_2$
	Complete Protease Inhibitor cocktail
	Phosphatase Inhibitor
IP-Lysis Buffer 2	1% Triton-X
	0.1% SDS
	100 nM NaCl
	40 mM TRIS-HCL
	1 µM $CaCl_2$
	Complete Protease Inhibitor cocktail
	Phosphatase Inhibitor
MACS® Running buffer (pH 7.2)	PBS
Miltenyi Biotec	2 mM EDTA
	0.5% BSA (w/v)
	0.09% sodium azide
MOPS running buffer (pH = 7.7)	50 mM MOPS
	50 mM Trizma Base
	3.5 mM SDS
	1 mM EDTA
PBS (pH = 7.4)	137 mM NaCl
(Gibco)	8.1 mM Na_2HPO_4
	2.7 mM KCl
	1.5 mM KH_2PO_4
Western Blot Stripping buffer (pH = 2.3)	50 mM Glycine in H_20
TAE buffer (10x)	400 mM Tris/HCl
	200 mM Acetic Acid
	10 mM EDTA
Transfer buffer	192 mM Glycine

	25 mM Trizma Base
	0.01% EDTA (w/v)
	20% Methanol (v/v)
Wash buffer (Western blotting)	1 x PBS
	0.05% Tween-20 (v/v)
Blocking buffer (Western blotting)	0.05 Tween-20% in PBS
	2% milk powder

2.1.4 Biological agents and chemotherapy

TGFβ	R&D systems
Cisplatin	Sigma
Paclitaxel	Bristol-Myers Squibb

2.1.5 Antibodies

Unconjugated antibodies

(WB: Western blotting; IP: immunoprecipitation; FACS: fluorescence activated cell sorting)

Antibody	**Isotype**	**Source**	**Application**
Anti-caspase-8 (T-16)	goat I	Santa Cruz	IP
Anti-Caspase-8 (C15)	mIgG2b	Axxora	WB
Anti-FLIP (NF6)	mIgG1	Enzo Life Sciences	WB
Anti-FADD (1F7)	mIgG1	Assay Designs Inc.	WB
Anti-Mouse-TCRβ (H57-597)	Hamster	BD Pharmingen™	T cell activation
Anti-Mouse CD28 (37.51)	Hamster	BD Pharmingen™	T cell activation
Anti-TRAIL-R1 (HS-101)	mIgG1	Hybridoma	FACS
Anti-TRAIL-R2 (HS-201)	mIgG1	Hybridoma	FACS
Anti-TRAIL-R3 (HS-301)	mIgG1	Hybridoma	FACS
Anti-TRAIL-R4 (HS-401)	mIgG1	Hybridoma	FACS
Anti-TRAIL-R2/DR5 (2019)	Rabbit	ProSci. Inc	WB

2.1.6 Conjugated antibodies

(WB: Western blotting; IP: immunoprecipitation; FACS: fluorescence activated cell sorting)

Antibody	**Source**	**Application**
Goat-anti-mIgG1-HRP	Southern Biotech	WB
Goat-anti-mIgG2b-HRP	Southern Biotech	WB
Goat-anti-rabbit-IgG-HRP	Southern Biotech	WB
Goat-anti-rabbit-IgG-HRP	Southern Biotech	WB
Anti-human CD11b (ICRF44)-PE/Cy5	BioLegend®	FACS
Anti-human CD14-FITC (MEM18)	AbD serotec	FACS
Anti-human CD14-APC	Miltenyi Biotec	FACS
Anti-human CD16-FITC	Miltenyi Biotec	FACS
Anti-human CD19 (HIB19)-PE	BioLegend®	FACS
Anti-human CD3 (HIT3a)-PE/Cy5	BioLegend®	FACS
Anti-human CD32 (FUN-2)-PE	BioLegend®	FACS
Anti-human CD326/EpCAM (9C4)-PE	BioLegend®	FACS
Anti-human CD33 (WM53)-APC	BioLegend®	FACS
Anti-human CD4 (OKT4)-FITC	BioLegend®	FACS
Anti-human CD4 (OKT4)-PE/Cy5	BioLegend®	FACS
Anti-human CD45 (H130)-PE	BioLegend®	FACS
Anti-human CD45 (H130)-PE/Cy5	BioLegend®	FACS
Anti-human CD56 (130-090-843)-APC	Miltenyi Biotec	FACS
Anti-human CD64 (10.1)-biotin	BioLegend®	FACS
Anti-human CD66 (CD66a-B1.1)-APC	eBioscience	FACS
Anti-human CD68(Y1/82A)-PE	BioLegend®	FACS

Anti-human CD8 (SK1)-FITC	BioLegend®	FACS
Anti-human EpCAM (Ber-EP4)-FITC	Dako	FACS
Anti-human Foxp3 (236A/E7)-PE	eBioscience	FACS
Anti-human IFN-gamma (4S.B3)-APC	BioLegend®	FACS
Anti-human IL-17A (BL168)-FITC	BioLegend®	FACS
Anti-human IL-4 Antibody (8D4-8)-PE	BioLegend®	FACS
Anti-Streptavidin-PE/Cy5	BioLegend®	FACS
Anti-mouse CD4-PE-Cy5	BioLegend®	FACS
Anti-mouse CD25-PE	BioLegend®	FACS
Anti-mouse CD62L-FITC	BioLegend®	FACS
Anti-mouse-Foxp3-PE	eBioscience	FACS

2.1.7 Commercial detection and isolation systems and reagents

Kit	**Source**
BCA Protein Assay	Pierce
ECL Western Blotting Detection	GE Healthcare Life Sciences
Fc-receptor blocking solution	BioLegend®
M65® ELISA	PEVIVA
QIAprep Maxi Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction kit	Qiagen
LDS sample buffer	NuPage®
2×SYBR PCR Master Mix	QIAGEN
Reflotron® AST	Roche
TrypanBlue	Invitrogen

Western Lightning® *Plus*–ECL, Enhanced Chemiluminescence Substrate	Perkin Elmer
Zymoclean™ Gel DNA Recovery Kit	Zymo Research

2.1.8 Instruments

Instrument	**Source**
Blotting equipment X cell IITM	Novex
Chromo 4 TM DNA Engine	Bio-Rad
Cryo 1°C Freezing container	Nalgene Labware, Neerijse; Belgium
Electrophoresis chamber	Bio-Rad
Flow Cytometer FACSCalibur™	Becton Dickinson
Flow Cytometer LSR II	Becton Dickinson
Freezer -20°C	Liebherr
Freezer -80°C	Forma Scientific
Hyper Processor X-Ray film Developer	Amersham Biosciences
Incubator Stericult 2000	Forma Scientific
Light Microscope	Carl Zeiss
Microscope Axiovant 25	Carl Zeiss
Mithras Luminometer LB 940	Berthold Technologies
Multifuge 3S-R	Heraeus
Multiskan Ascent	Thermo Scientific® Labsystems
NanoDrop Spectrophotometer ND-1000	NanoDrop Technologies
Nucleofector™	Amaxa Inc
pH Meter	Mettler, Giessen; Germany
Sonifier	Branson Ultrasonics Corporation
Vortex	Heidolph, Schwabach; Germany

2.1.9 Laboratory materials

Material	Source
Cell Culture Petri dishes	TPP, Techno Plastic Products AG Trasadingen; Switzerland
Cell Sieve (40 µm pore size)	Becton Dickinson, Heidelberg; Germany
Conical tubes (15 ml and 50 ml)	TPP, Trasadingen; Switzerland
Cryogenic vials	Nunc, Wiesbaden; Germany
Falcon 12 x 75 mm 35 µm nylon Cell Strainer	Becton Dickinson
Hybond ECL Nitrocellulose Membrane	Amersham Biosciences
NuPage® 4-12% Bis-Tris Gels	Invitrogen
PCR Tubes	Starlab
Pipetboy	Integra Bioscience
Pipette tips (0.1-10, 1-200, 101-1000 µl)	Starlab
RBC lysis buffer	eBioscience
Round and flat bottom 96-well test plates	TPP, Trasadingen; Switzerland
SDS-Sample buffer	Invitrogen
SeeBlueTM Plus2 Pre-Stained Standards	Invitrogen
Single-Use Syringe (1, 10 ml)	Becton Dickinson
Sterile filter (0.22 µm)	Millipore
Tissue Culture flasks (25, 75 and 150 cm^2)	TPP, Trasadingen; Switzerland
X-Ray film HyperfilmTM ECL	Amersham, Freiburg; Germany

2.1.10 Cell lines

293T cells were generated by the transformation of human embryonic kidney cells using fragments of adenovirus type 5 DNA (Graham et al., 1977).

The PEO4 cell line was originally derived from ascites from a patient with platinum-resistant ovarian cancer (Langdon et al., 1988b).

PEA1 (platinum-sensitive) and PEA2 cells (platinum-resistant) were isolated from a pleural effusion and ascites from a patient before and after treatment with platinum-based chemotherapy (Langdon et al., 1988a).

34957 cells were a gift from Professor David Bowtell; and were recently derived from a patient with grade III serous carcinoma ovarian cancer who had been treated with carboplatin, paclitaxel, and liposomal doxorubicin.

34955 and 34955.2 cell lines were a gift from Professor David Bowtell and were recently derived from a patient with grade III serous carcinoma ovarian cancer who had been treated with carboplatin, paclitaxel, and liposomal doxorubicin.

22846 cells were a gift from Professor David Bowtell and were recently derived from a patient with ovarian cancer who had received treatment with carboplatin chemotherapy.

2.1.11 Cell line culturing conditions

293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS) and 1% penicillin/streptomycin/glutamine.

All cell lines were incubated at 37°C in humidified incubator with 5% CO_2. Ovarian cancer cell lines were cultured in Roswell Park Memorial Institute medium-1640 (RPMI) supplemented with 10% FCS and 1% penicillin/streptomycin/glutamine. Adherent cells were split using a 1 x Trypsin/EDTA solution.

2.1.12 Freezing and thawing of eukaryotic cells

Up to 10 x 10^6 per ml of cells were harvested and suspended in freezing media which contained 90% FCS and 10% DMSO (Dimethyl sulfoxide). The cells were then cooled to -80 ^oC in a freezing container, Nalgene® Mr. Frosty (Sigma-Aldrich) for 2 days before being transferred into liquid nitrogen for long-term storage at -196°C. Frozen cells were thawed by rapidly melting frozen aliquots of cells with warmed cell culture media at 37°C before spinning down the cells, and then transferring the cells into fresh tissue culture media.

2.2 Methods

2.2.1 Transfection of 293T cells and retrovirus production

The night before transfection, a confluent 10 cm plate of 293T cells was split so that the cells were around 30% to 50% confluent on the day of transfection. One hour prior to the transfection, the media was changed and 9 ml of fresh media was added. 293T cells were transfected using calcium phosphate transfection. Then 4 µg of plasmid DNA and 4 µg of pcl-Eco retrovirus packaging vector were added to 500 ml of 0.8 M $CaCl_{2;}$ which was added drop-wise to 500 µl of HEBS Buffer (2 X) which was being vortexed. Then 1 ml of calcium phosphate crystals were added drop-wise to the semi-confluent 10 cm plates of 293T cells with 9 ml of fresh DMEM media whilst slowly swirling the plate. The culture media were changed the following morning, and in the evening; and were replaced with 3.5 ml of fresh media. The retroviral supernatants were collected at 48, 56 and 72 hours after transfection until a total of 10.5 mls of supernatants had been collected. All retroviral supernatants were 0.22 µm sterile filtered and the stored at $4^{o}C$ until use.

2.2.2 Retroviral vectors

The Foxo3-IRES-GFP (IRES – internal ribosome entry site, GFP - Green Fluorescent Protein) and Foxo3-Active-IRES-GFP were a gift and were made by cloning human Foxo3 into the mouse stem cell virus (MSCV). The Foxo3 triple mutant T32A/S252A/S314A was a gift; and was made by site-directed mutagenesis of the nucleotide codons that encode the Akt phosphorylation sites within Foxo3. The GFP-KLF2 vector was a gift from Professor Doreen Cantrell. The GFP-KLF2 vector was made by polymerase chain reaction (PCR) amplification of the RIKEN full length Klf2 clone (2410149J18). The KLF2 PCR fragment was cloned into the EGFP vector, and the EGFP-KLF2 fusion was cloned into the EcoR1 sites of the LZRSpBMN-LacZ construct, which replaced the LacZ gene with EGFP-KLF2. The KLF2-IRES-GFP vector was made by PCR amplification of the Klf2 sequence from the GFP-KLF2 vector. The PCR fragment was cloned in the EcoR1 and Bgll2 sites of the MSCV-IRES-GFP vector. The KLF2-IRES-GFP-G to E vector was made by site directed

mutagenesis and SOEing (Splicing by Overlap Extension) PCR of Klf2 PCR fragment from the GFP-KLF2 vector. The PCR fragment was cloned in the EcoR1 and BgII2 sites of the MSCV-IRES-GFP vector.

2.2.3 Mouse strains, cell sorting, culture and retroviral infections

Animal work was done under the authority of a project license (PPL70/5936) granted to Professor Matthias Merkenschlager under the Animals (Scientific Procedures) Act, UK, 1986. Studies on gene regulation were performed under project license (05/Q0406/145). Mice were sacrificed by dislocation of the neck. Then the mouse lymph nodes were dissected and removed. A single cell suspension of lymphocytes was achieved by passing the lymph nodes through a 40 µm cell strainer using a syringe plunger from a 1ml syringe in T cell media. The cells were then centrifuged (1,500 rpm for 5 minutes) and then stained with anti-CD4, anti-CD62L, and anti-CD25 for 20 minutes on ice in FACS (fluorescence activated cell sorting) buffer. The cells were then washed with 10 ml of ice cold FACS buffer and filtered using a 12 x 75 mm BD Falcon 35 µm nylon Cell Strainer Cap tube, in order to remove all clumps of cells prior to cell sorting in FACS buffer.

Naive CD4 T cells ($CD4^+$, $CD25^-$ and $CD62^{high}$) or regulatory T cells ($CD4^+$,$CD25^+$) were isolated by flow cytometric sorting using BD FACSAria cell sorters from C57BL/6 or BALB/c mouse strains. CD4 T cells were cultured in Iscove's Modified Dulbecco's medium (IMDM), supplemented with 10% heat inactivated fetal bovine calf serum (Bioseara), penicillin [1000 u/ml], streptomycin [1000 µg/ml], gentamycin [25 µg/ml], and beta-mercaptoethanol (0.004%). Cells were activated with anti-TCR (CD3/CD28 Dynabeads®, Invitrogen) at a ratio of 1 bead per cell or plate bound anti-anti-TCRβ (H57-597; 200 ng/ml) and anti-CD28 (2 µg/ml). Exogenous IL-2 [10 ng/ml] was added to Treg cell culture media where indicated.

Activated T cells were retrovirally transduced with retroviral constructs by spin infection (90 min, 2,500 rpm, 37°C) in 24-well tissue culture plates. Before transfection the cells were spun down at 2500 rpm for 2 minutes and then 750 µl of conditioned cell culture media was removed from each well, and replaced with 1.5 mls of retroviral supernatant. The cells were transduced with retroviral constructs by

spin infection (90 min, 2,500 rpm, 37°C). After transduction, the cells were rested in the incubator for 3 hours, and then 1.5 ml of media was removed and replaced with 750 µl of conditioned cell culture media. The T Cell Receptor (TCR) activation signal was withdrawn by the magnetic removal of the anti-CD3/CD28 activation beads. Then TGFβ was added at the indicated concentrations or the PI3K inhibitors LY294002 and rapamycin were added at the indicated concentrations.

2.2.4 SiRNA transfection of mouse lymphocytes

Small interfering RNAs (SiRNAs) against Foxo1 and Foxo3 were purchased from Dharmacon. Murine Treg cells were transfected with 5 nM of siRNA against Foxo1 and Foxo3 using the mouse T cell nucleofector Kit (Amaxa) as per the manufacturer's instructions.

2.2.5 Isolation, purification and culture of $CD45^+$ immune and $EpCAM^+$ tumour cells from ovarian cancer ascites

Ethical approval for use of the patient samples for the study was obtained from Imperial College NHS Research Ethics committee (05/Q0406/178) prior to starting the study. Participants had provided written informed consent for use of their tissue samples for research. Ascites was collected from participating patients who were undergoing therapeutic ascitic drainage at Imperial College NHS Trust. The samples of ascitic fluid from the patients were collected and stored overnight by the ward staff at room temperature. The following day the cells were isolated by centrifugation at 2000 (RPM) for 10 minutes, followed by lysis of red blood cells (RBC) using RBC lysis buffer (eBioscience). $CD45^+$ immune cells were isolated using CD45 MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol.

$EpCAM^+$ tumour cells were enriched from total ascitic cells by depleting $CD45^+$ cells from the total ascitic cells. Primary $CD45^+$ cells were co-cultured with PEO4 cells in RPMI supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin/gentamycin/glutamine. Primary $CD45^+$ cells and primary $EpCAM^+$ cancer cells were cultured in 50% RPMI and 50% 0.22 µm sterile-filtered ascites supplemented with 1% penicillin/streptomycin/gentamycin/glutamine. Ovarian cancer cells and immune cells were isolated from fresh solid deposits of ovarian cancer donated by patients undergoing debulking surgery for advanced ovarian cancer at Imperial College NHS Trust. The tumour cells and immune cells were

dissociated from solid deposits of ovarian cancer using the Tumour Dissociation Kit from Miltenyi Biotec, according to the manufacturer's instructions. After processing the cells were filtered to remove clumps of dead cells and then analysed by FACS.

2.2.6 FACS analysis

The surface expression of proteins was performed using 4 x 10^5 cells. Cells were incubated on ice for 20 minutes with 50 µl of FACS buffer with the primary antibodies and an FcR blocking antibody solution in 96 well round-bottom plates. The cells were then washed with 200 µl of fluorescence-activated cell sorting (FACS) buffer and stained with 50 µl of the secondary antibody for 20 minutes on ice. The cells were then washed twice with FACS buffer and stored on ice until acquisition.

For analysis of immune cells from ovarian cancer ascites, cells were permeablised and fixed according to the manufacturer's protocol using the anti-Foxp3 antibody (eBioscience). For analysis of intracellular cytokine production total ascitic cells were stimulated for 4 hours with PMA [50 ng/ml] (phorbol 12-myristate 13-acetate) plus ionomycin [500 ng/ml] with Brefeldin A Solution, and then fixed and permeabilised with the Foxp3/Transcription factor staining buffer set before staining and acquisition. Retrovirally transduced cells were fixed and permeabilised for 24 hours to improve green fluorescent protein (GFP retention).

Cells were acquired and analysed using the BD FACSCalibur™ and the BD LSR II. Data analysis was carried out using CellQuest software or FlowJo software from Tree star. FcγR expression was determined by staining cells for CD45 and CD16, CD32, and CD64; B cells were identified by staining for CD45, CD3, CD19; macrophages by staining for CD45, CD68, CD14; T cells: CD45 and CD3; NK cells by staining for CD45, CD16, CD56; neutrophils by staining for CD45, CD66. Treg cells were identified by staining for CD4, CD3, and Foxp3.

2.2.7 Statistical analysis

All statistical analysis was performed using GraphPad software.

2.2.8 SMAC mimetics

The SMAC mimetic SM-83 (also known as SM 9a) was synthesised and provided by P. Seneci and L. Manzoni and used a concentration of 100 nM.

2.2.9 Cell death assays

Tumour cell-specific death was measured in mixed populations of tumour and immune cells by the expression of cleaved cytokeratin 18 (CK18), a neo-epitope formed after caspase cleavage of keratin 18 at Asp396, which is specific marker of apoptosis in epithelial cells. Treated cells were fixed and permeabilised for 30 minutes using the Foxp3/transcription factor staining kit (eBioscience), and then stained for cleaved cytokeratin 18 using the M30 antibody (Peviva).

2.2.10 Cell viability assays

Primary $EpCAM^+$ tumour cells and ovarian cancer cell lines were seeded at density of 50000 cells per well, in 96-well plates. After seeding and overnight incubation the primary ovarian cancer cells were treated and cell viability was measured after 48 hours using the CellTiter-Glo® Viability Assay according to the manufacturer's instructions. Each measurement was repeated in triplicate. Plates were read using the Mithras Luminometer LB 940 (Berthold Technologies).

2.2.11 TRAIL-R specific antibodies and synthesis of recombinant forms of TRAIL

AMG 655 was supplied by AMGEN as part of a confidential material transfer agreement between Imperial College London, and AMGEN. For in vitro assays AMG 655 was crosslinked with recombinant protein A/G (Thermo Scientific). A fusion protein with four Fc binding domains that crosslinks AMG 655 and thereby leads to TRAIL-R2 trimerisation. The synthesis and purification of iz-TRAIL was performed as previously described (Ganten et al., 2006). The synthesis and purification of Apo2L/TRAIL was performed as previously described (Ashkenazi et al., 1999, Ganten et al., 2006).

2.2.12 Culture of primary human hepatocytes

Cryopreserved transporter qualified primary human hepatocytes and primary human hepatocyte culture reagents were purchased from GIBCO® Life technologies. The following lots of cryopreserved transporter qualified primary human hepatocytes from GIBCO® Life technologies were used: Donor 1 (lot number: Hu8130), Donor 2 (Lot number: Hu1463), Donor 3 (lot number: Hu8150). After thawing at 37°C, the cells were recovered using CHRM® medium (Cryopreserved Hepatocyte Recovery

Medium). After recovery the cells were counted and seeded as per the manufacture's published recommendations for each individual donor. The cells were seeded in collagen-coated 96-well plates in hepatocyte plating medium which contains 500 ml of Williams medium E without phenol red supplemented with prequalified FCS, dexamethasone, and a cocktail solution of penicillin-streptomycin, bovine insulin, GlutaMAX™ and HEPES. After plating, the primary human hepatocytes were left to attach for 6 hours, then the media was changed to hepatocyte maintenance media: 500 ml of Williams medium E without phenol red supplemented with dexamethasone and a cocktail solution of penicillin-streptomycin, ITS+ (insulin, transferrin, selenium complex, bovine serum albumin (BSA) and linoleic acid), GlutaMAX™ and HEPES. The hepatocyte maintenance media was changed every 24 hours for three days.

After 3 days of culture, the hepatocyte maintenance media was changed and the cells were treated for 24 hours in fresh hepatocyte maintenance media with the different TRAIL ligands. Primary human hepatocyte viability was measured by the CellTiter-Glo® luminescent cell viability assay (Promega), as per the manufacturer's instructions. Changes in the release of the liver enzyme aspartate transaminase (AST) were measured using the Reflovet plus machine (Roche) and the Reflotron AST strips as per the manufacturer's instructions.

2.3 Molecular Biology

2.3.1 DNA digestion and restriction analysis

The sequence-specific cleavage of DNA molecules was performed by incubating samples with restriction endonucleases as per the manufacturer's instructions (New England Biolabs).

2.3.2 Agarose gel electrophoresis of nucleic acids

Plasmids and DNA fragments were visualised using 0.5-1.5% Tris-Acetate Electrophoresis (TAE) agarose gels with either ethidium bromide or SYBR Green (Bio-Rad). The DNA samples were mixed with 6 x DNA loading buffer (New England Biolabs) and loaded onto the agarose gels and were run at 120 volts for 40 minutes.

2.3.3 Gel extraction of DNA fragments

DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions and the DNA Samples were eluted in 30 µL of water.

2.3.4 qRT-PCR

Total RNA was isolated using RNA-Bee (Tel-Test, Inc.). Complimentary DNA was prepared from long RNA using random primers and SuperScript® III reverse transcriptase (Invitrogen). Real-time PCR analysis of *Foxo1*, *Foxo3*, and *Foxp3* mRNA expression was performed on a Chromo4TM DNA engine using Opticon Monitor software (MJ Research Inc). PCR reactions used 2 × SYBR PCR Master Mix (QIAGEN), primers [300 nM], and 2 µl of complementary DNA as a template in a 20 µl reaction volume. The cycle conditions were 94° C for 8 minutes, 40 cycles of 94° C for 30 s, 55° C for 30 s, and 72° C for 1 minute, followed by plate reading. Data were normalised using the comparative threshold (CT) method to the geometrical mean of two housekeeping genes. Primer sequences were the following:

Hprt, forward 5'- TCAGTCAACGGGGGACATAAA-3', and reverse 5'-GGGGCTGTACTGCTTAACCAG3';

Ubc, forward 5'- AGGAGGCTGATGAAGGAGCTTGA-3', reverse 5' TGGTTTGAATGGATACTCTGCTGGA -3';

Foxp3, forward 5'-ACTCGCATGTTCGCCTACTTCAG-3', and reverse 5'-GGCGGATGGCATTCTTCCAGGT-3';

Foxo1, forward *5'*-GAGAGCTCAGCCGAGAAGAG, reverse 5'-CAGATTGTGGCGAATTGAAT-3';

Foxo3, forward 5'-CAAAGCTGGGTACCAGGCTGA-3', reverse 5'-AAGGTGTCAAGCTGTAAACG-3',

Smad7 forward 5'-GCATTCCTCGGAAGTCAAGA-3', reverse 5'-TTGTTGTCCGAATTGAGCTG-3'.

Klf2 forward 5'- ctcagcgagcctatcttgccgt-3', reverse 5'- gcccagaccgtccaatccca-3'

2.3.5 Determination of protein content

The protein concentration of the lysates was determined using the bicinchoninic acid (BCA)-containing protein assay according to the manufacturer's instructions (Pierce). 2 µl of each lysate was incubated with 50 µl of BCA solution at 65°C for between 10 to 15 minutes and then the light absorption was measured using a Multiskan Ascent plate-reader.

2.3.6 Immunoprecipitation of caspase-8 and TRAIL-R2

The complete protease inhibitor cocktail and the phosphatase inhibitor cocktail were used during each lysis. Whole PEO4 cells were lysed in Buffer 1 at 4°C for 20 minutes and then clarified by centrifugation at 14000 rpm for 10 minutes at 4°C. After centrifugation the supernatant was removed and the remaining cell pellet was lysed by sonification in Buffer 2; and then clarified by centrifugation at 14000 rpm for 20 minutes at 4°C. The lysates were then combined and clarified by centrifugation at 14000 rpm for 10 minutes at 4°C. The protein concentration of each lysate was determined using the BCA protein assay. The lysates were pre-cleared with 10 µl sepharose beads at 4°C for one hour.

Caspase-8 was immunoprecipitated by rotation of the tubes for 16 hours at 4°C with 10 µl of protein G sepharose beads coupled with 1 µg/ml of anti-caspase-8. The beads were then washed five times with 1 ml of IP-lysis buffer with 1% Tween; and eluted with 2 × LDS (Lithium Dodecyl Sulfate) buffer (containing a final concentration of DTT (Dithiothreitol) [200 mM] by heating for 10 minutes at 95°C. TRAIL-R2 was immunoprecipitated by rotation of the tubes for 16 hours at 4°C with 10 µl of uncoupled Protein G Sepharose bead (GE Healthcare). The beads were then washed five times with 1 ml IP-lysis buffer with 1% Tween; and eluted with 2 × LDS buffer containing a final concentration of DTT [200 mM] by heating for 10 minutes at 95°C.

2.3.7 SDS-PAGE and Western Blotting

Proteins were separated by SDS (sodium dodecyl sulphate polyacrylamide gel electrophoresis)-PAGE using 4-12 % Bis-Tris-NuPage® gels from Invitrogen at a constant voltage of 100 volts for 10 minutes and then 180 volts for 60 minutes using MOPS NuPage® SDS running buffer. Proteins were transferred onto nitrocellulose

membranes by application of a constant voltage of 30 volts for 1 hour. Membranes were incubated for 1 hour with blocking buffer at room temperature to occupy non-specific protein binding sites. Membranes were subjected to immunoprobing with antibodies against TRAIL-R2, FADD, FLIP, and Caspase-8. Proteins were visualised using the Western Lightning® Plus–ECL, Enhanced Chemiluminescence Substrate (ECL) Western Blotting Detection Plus. Membranes were stripped and then probed with other antibodies.

2.3.8 Enzyme-Linked Immuno Sorbent Assay (ELISA)

Primary human hepatocytes cell death was assessed using the M65® ELISA (PEVIVA) which measures soluble cleaved cytokeratin 18 (CK18) which is released from dying cells into the cellular supernatant, as per the manufacturer's instructions. The microwell absorbance was read at 450 nM with a Multiskan Ascent plate-reader (Thermo Lab systems).

Chapter 3. A role for Foxo and Klf2 transcription factors in the regulation of Foxp3

3.1 A role for Foxo factors in the regulation of Foxp3

Previous studies within the host laboratory had demonstrated that PI3K signalling regulates Foxp3 expression (Sauer et al., 2008). Initial experiments showed that the activation of naive CD4 T cells via CD3 and CD28 leads to the activation of PI3K signalling, which was demonstrated by measuring changes in the phosphorylation of S6 ribosomal protein (pS6) which is a direct target of the mTOR-regulated p70 S6 kinase (Sabatini, 2006, Sauer et al., 2008). The withdrawal of the TCR activation signal led to a decline in PI3K signalling, which was enhanced by treatment with the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin, and the expression of Foxp3 (Figure 5).

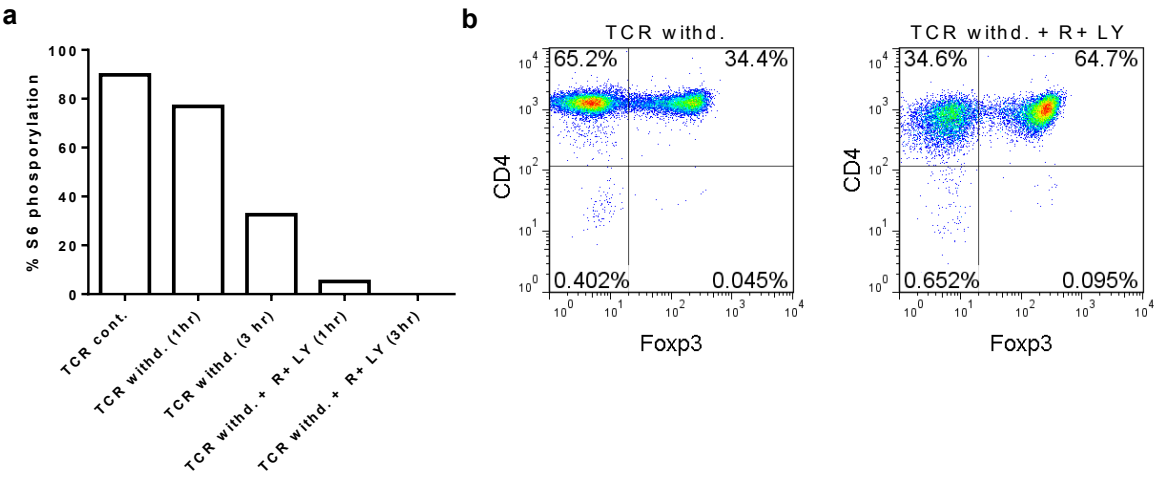

Figure 5: TCR signalling controls the expression of Foxp3 via PI3K/mTOR/Akt. (a) Naive CD4 T cells were stimulated for 18 hours with anti-TCR and anti-CD28 and then the activation signal was either continued (TCR cont.) or withdrawn (TCR withd.) and then the cells were treated with rapamycin (R) [25 nM] and LY294002 (LY) [10 μM]). S6 phosphorylation was determined by intracellular staining at the indicated time points. Data are representative of 2 independent experiments and previously published studies from the host laboratory (Sauer et al., 2008). (b) Naive CD4 T cells were activated for 18 h as in (a) and then cultured for 48 hours without TCR (TCR withd.) or without TCR and R+LY. Foxp3 expression was assessed after 48 hours by intracellular staining. Data are representative of one of 4 independent experiments.

Foxo factors are canonical targets of Akt mediated phosphorylation (Burgering, 2008), which leads to their nuclear exclusion and inactivation (Huang and Tindall, 2007). In

naive CD4 T cells, Foxo1 controls the expression of L-selectin and the chemokine receptor CCR7 (Kerdiles et al., 2009). In mouse models, loss of Foxo factors causes autoimmunity and spontaneous T cell activation (Ouyang et al., 2009). These findings suggest that Foxo factors could have a role in regulating immune tolerance and the expression of Foxp3.

Premature withdrawal of TCR signalling and treatment with the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin has been shown to increase the expression of *Foxo1*, *Foxo3a* and *Foxp3* in activated CD4 T cells (Sauer et al., 2008). To verify that Foxo factor expression is increased following premature withdrawal of the TCR activation signal, naive CD4 T cells were isolated by FACS sorting and were activated with anti-CD3/CD28 coated plates. After overnight activation, the cells were either cultured with the activation signal, or the activation signal was withdrawn and TGFβ was added to induce the expression of Foxp3. *Foxo1*, *Foxo3* and *Foxp3* mRNA expression was measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) at 0, 6, 30, and 54 hours after removal of the cells from the activation signal.

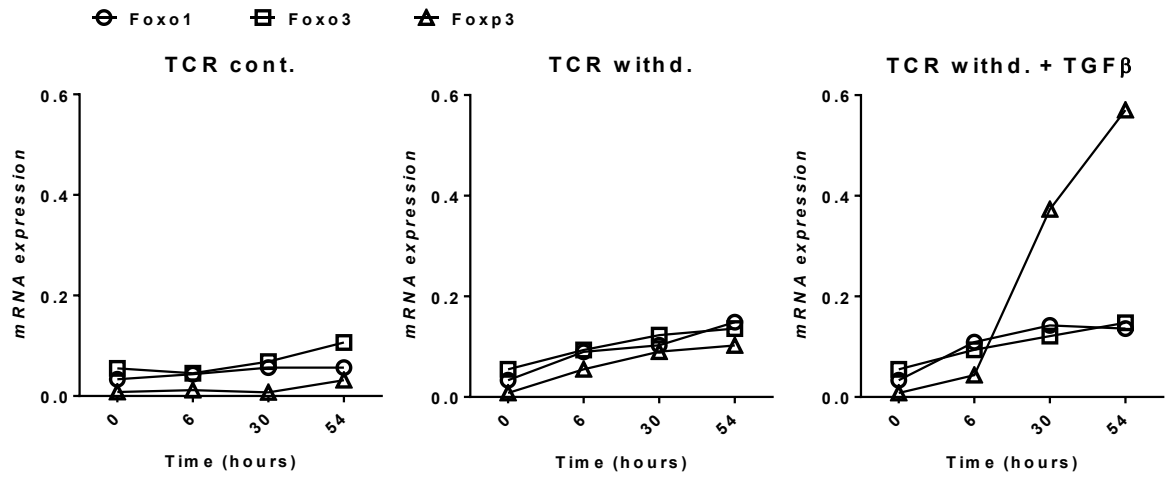

Figure 6: Withdrawal of the TCR activation signal, promotes the expression of Foxo factors and Foxp3. Naive CD4 T cells were isolated by FACS and activated with anti-CD3/CD28 coated plates. After 18 hours of activation the cells were cultured with the TCR activation signal (TCR cont.), or the TCR activation signal was withdrawn (TCR withd.) and TGFβ [1ng/ml] was added. Foxo1, Foxo3, Foxp3 mRNA expression was measured by qRT-PCR at 0, 6, 30 and 54 hours after the TCR activation signal was withdrawn. Data are representative of 1 of 2 independent experiments.

The expression of *Foxo1*, *Foxo3*, and *Foxp3* was not promoted in cells that were cultured in the presence of the activation signal. Whereas the withdrawal of the TCR activation signal increased the expression of *Foxo1*, *Foxo3*, and *Foxp3* (Figure 6).

The addition of TGFβ promoted the expression of Foxp3 but did not change the expression of *Foxo1* or *Foxo3*. This suggests that culture conditions that promote the induction of Foxp3 expression also promote the expression of Foxo factors; and confirmed the findings from previous microarray experiments that were conducted within the host laboratory.

The regulation of Foxo factors is dependent on post translational modifications via Akt mediated phosphorylation, which leads to the nuclear exclusion and transcriptional inactivation of Foxo factors (Tzivion et al., 2011). In order to confirm that the localisation of Foxo factors was regulated by PI3K signalling in activated T cells, CD4 T cells were cultured with and without the PI3K inhibitor LY294002 and rapamycin. The cytoplasmic, versus nuclear localisation of Foxo1 and Foxo3 was determined by confocal microscopy (successful experiments performed by Professor Matthias Merkenschlager). In activated CD4 T cells, over 90% of the Foxo1 and Foxo3 factors were located within the cytoplasm. After withdrawal of the TCR activation signal, and treatment with PI3K inhibitors, over 90% of Foxo factors became localised within the nucleus. This result suggested that the nuclear localisation of Foxo factors is regulated by PI3K signalling in CD4 T cells.

3.1.1 Overexpression of Foxo3a promotes the expression of Foxp3 in activated CD4 T cells

These experiments showed that the expression of Foxo factors is promoted by withdrawal of the TCR activation signal, and that Foxo factors are regulated by signalling pathways that are known to regulate the expression of Foxp3. The effect of overexpressing Foxo3a on the induction of Foxp3 expression was investigated in activated CD4 T cells. Naive CD4 T cells were isolated and activated with anti-CD3/CD28 coated beads. After overnight activation, the cells were transduced with Foxo3-IRES (internal ribosome entry site)-GFP, Foxo3-Active-IRES-GFP (a mutant constitutively active form of Foxo3 which is resistant to Akt phosphorylation and inactivation), or control IRES-GFP (Vector) retroviruses by spin infection. 24 hours after retroviral transduction, the activation signal was withdrawn, and the cells were cultured with TGFβ or rapamycin and LY294002. Foxp3 expression was measured 48 hours later by intracellular staining (Figure 7).

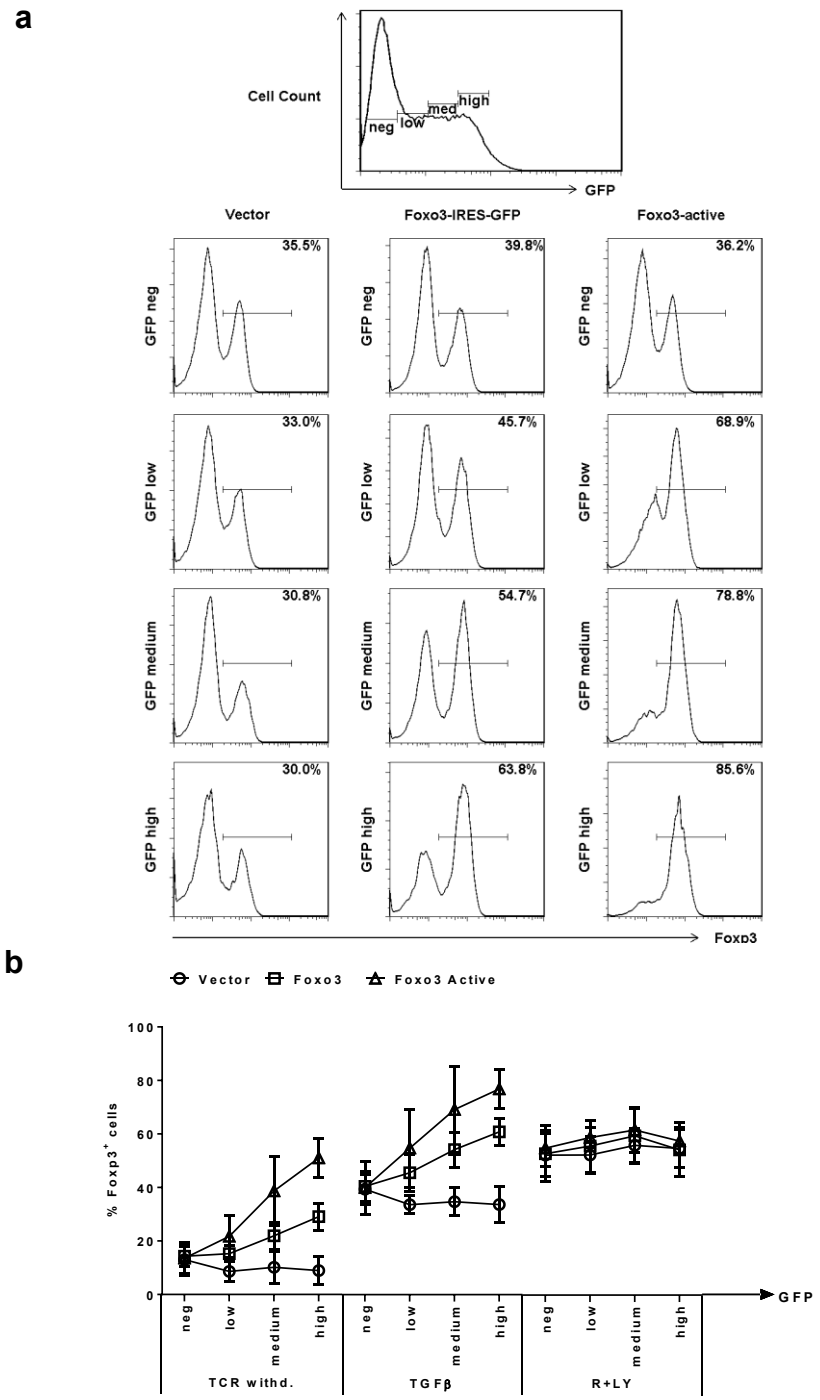

Figure 7: Overexpression of Foxo3 and constitutively active Foxo3 promotes TGFβ-mediated induction of Foxp3 expression. (a) Naive CD4 T cells were isolated and activated with anti-CD3/CD28 coated beads. After overnight activation, the cells were transduced with Foxo3-IRES-GFP, Foxo3-Active-IRES-GFP or control IRES-GFP (Vector) retroviruses by spin infection. 24 hours after retroviral transduction, the activation signal was withdrawn, and the cells were cultured with TGFβ [1ng/ml]. Foxp3 expression was assessed by intracellular staining, gating on cells with negative (neg), low, medium (med) and high GFP expression as an indirect measure of retroviral gene transfer efficiency and transcription factor overexpression. Data are representative of one of three independent experiments. (b) Naive CD4 T cells were activated and retrovirally transduced as in (a) with Foxo3-IRES-GFP, Foxo3-Active-IRES-GFP or control IRES-GFP (Vector) retroviruses by spin infection. 24 hours after retroviral transduction, the activation signal was withdrawn, and the cells were cultured with TGFβ [1ng/ml] or rapamycin (R) [25 nM] and LY294002 (LY) [10 μM]. Data represent the mean ± S.D of 3 biological replicates and are representative of one of 3 independent experiments.

Expression of Foxo3 and constitutively active Foxo3 synergised with TCR withdrawal and TGFβ in promoting the expression of Foxp3 in a dose dependent manner (as quantified by GFP expression) (Figure 7). This suggests that Foxo3 promotes the expression of Foxp3. Higher amounts of constitutively active Foxo3 promoted Foxp3 expression. These data suggested that PI3K/Akt signalling negatively regulates Foxo factor mediated induction of Foxp3 expression. These results were consistent with previous experiments which had shown that the withdrawal of the TCR activation signal, TGFβ, and PI3K inhibitors promote the expression of Foxp3 (Figures 5 and 6).

The overexpression of Foxo factors had minimal effect on the induction of Foxp3 with PI3K inhibitors (Figure 7). This result was surprising, given that overexpression of Foxo factors has been show to promote TGFβ mediated expression of Foxp3. In addition, previous experiments conducted within the host laboratory had shown that overexpression of Foxo factors could promote the induction of Foxp3 with PI3K inhibitors. Therefore these findings could suggest that the model in which TCR signalling inactivates Foxo via PI3K, mTORC2 and Akt is incorrect.

Differences between the induction of Foxp3 expression induced by TCR withdrawal, and TGFβ or PI3K inhibition could partially explain these results. For example, the withdrawal of the TCR activation signal led to a gradual decline in S6 phosphorylation over several hours and a modest induction of Foxp3 expression (Figure 5). Whereas treatment with PI3K inhibitors, lead to a rapid decline in S6 phosphorylation and a stronger induction of Foxp3 expression (Figure 5). It is possible that there were already sufficient Foxo factors within the nucleus or cytoplasm of the activated T cells to maximally induce the expression of Foxp3 after the rapid inhibition of PI3K signalling and nuclear translocation of Foxo factors. If this were the case then the overexpression of Foxo3 would have had a minimal effect on the expression of Foxp3 with PI3K inhibitors (Figure 7).

There are differences between the induction of Foxp3 expression in activated CD4 T cells with TGFβ and PI3K inhibitors. For example, the overexpression of constitutively activated Akt has been shown to impair the de novo induction of Foxp3 expression by TGFβ (Haxhinasto et al., 2008). However, although Akt has a broad effects on the transcriptional signature of Treg cells, it only partially affects the Treg cell transcriptional signature induced by TGFβ (Haxhinasto et al, 2008). Therefore the overexpression of Foxo factors may promote Foxp3 expression by enhancing the

effects of TCR withdrawal on the nuclear translocation of Foxo factors as well as by promoting TGFβ-mediated induction of Foxp3.

3.1.2 The expression of constitutively active Foxo3 partially restores TGFβ mediated Foxp3 expression in PTEN deficient CD4 T cells

CD4 T cells that are deficient in negative regulators of PI3K signalling such as the PTEN (Sauer et al., 2008) and the E3 ubiquitin ligase Cbl-b (Wohlfert et al., 2006) have reduced Foxp3 expression in response to TGFβ signalling. Akt is constitutively active in PTEN deficient cells, which have high levels of phosphorylated and inactivated Foxo factors (Finlay et al., 2009). As Foxo factors are negatively regulated by PI3K/Akt signalling, expression of constitutively active Foxo3 could restore Foxp3 expression in PTEN deficient cells.

Naive CD4 T cells from PTEN deficient and wild-type littermate mice were isolated, activated and transduced with Foxo3-IRES-GFP, Foxo3-Active-IRES-GFP, or IRES-GFP. TGFβ was added 24 hours later. Foxp3 expression was assessed after 48 hours. In wild-type activated CD4 T cells, the expression of Foxo3 and constitutively active Foxo3 synergised with TGFβ in promoting the expression of Foxp3 (Figure 8). Foxp3 expression was minimal in PTEN deficient cells that were transduced with control IRES-GFP and cultured with TGFβ. The expression of constitutively active Foxo3 in PTEN deficient cells partially rescued TGFβ mediated Foxp3 expression; whereas the overexpression of wild-type Foxo3 only had a minimal effect (Figure 8). This data demonstrates that expression of Foxp3 in PTEN cells can be partially restored by the expression of constitutively active Foxo3. This suggests that Foxo3 is regulated by via PI3K, mTORC2 and Akt, and that Foxo3 is essential for TGFβ mediated Foxp3 expression.

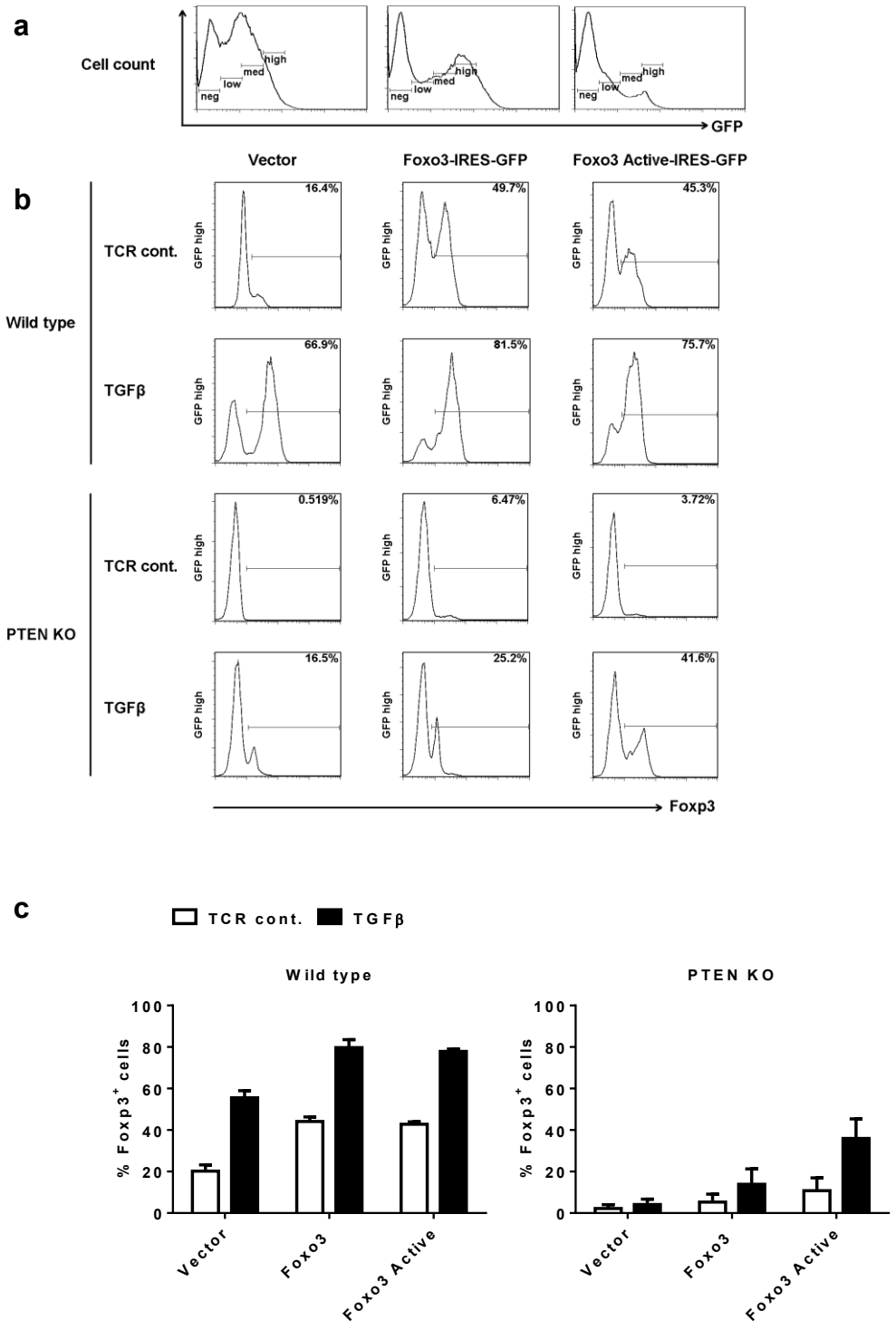

Figure 8: The expression of Foxo3 Active partially rescues TGFβ mediated induction of Foxp3 in PTEN deficient cells. Naive CD4 T cells from wild-type and PTEN deficient (PTEN KO) mice were isolated and activated with anti-CD3/CD28 coated beads. After overnight activation, the cells were transduced with Foxo3-IRES-GFP, Foxo3-Active-IRES-GFP or control IRES-GFP (Vector) retroviruses by spin infection. (a) GFP expression in CD4 T cells 48 hours after transduction with Foxo3-IRES-GFP, Foxo3-Active-IRES-GFP or control IRES-GFP (Vector) retroviruses. (b) TGFβ [1 ng/ml] was added 24 hours after retroviral transduction and Foxp3 expression was assessed 48 hours later by intracellular staining by gating on cells with high GFP expression. (c) Foxp3 expression of high GFP expressing CD4 T cells. Data are representative of the mean of 3 independent experiments (± SD).

There were differences in the expression of Foxp3 between experiments performed with different TCR activation signals. For example the presence of the anti-CD3/CD28 coated beads did not suppress Foxp3 expression (Figures 7 and 8). CD3/CD28 beads were used to activate the CD4 T cells in retroviral transduction experiments as there is a relatively narrow window of opportunity for the induction of Foxp3 expression in CD4 T cells that are activated using CD3/CD28 coated plates for over 24 hours (Sauer et al., 2008).

Cells activated with anti-CD3/CD28 coated beads are not fixed to the activation signal and can come and go from the activation signal; and may receive intermittent activation of the PI3K/Akt/mTOR pathway and thus intermittent nuclear exclusion of Foxo factors. This could permit the expression of Foxp3 in the presence of the TCR activation beads and the endogenous TGFβ that is found within the FCS that is routinely added to T cell culture media. Whereas cells activated by plate-bound anti-CD3/CD28 (Figure 6), may receive a constant TCR activation signal, leading to the continuous activation of the PI3K/Akt/mTOR pathway. This leads to the continuous nuclear exclusion of Foxo factors and the suppression of *Foxp3* gene expression (Figure 6). It would have been interesting to investigate this hypothesis by determining the nuclear versus cytoplasmic location of Foxo factors during T cell activation with anti-CD3/CD28 beads and anti-CD3/CD28 coated plates.

3.1.3 Foxo factors are not required for the maintenance of established Foxp3 expression in natural Treg cells

To investigate whether Foxo3 was required for the maintenance of established Foxp3 expression, activated nTreg cells were transfected with control, or Foxo3 siRNA oligonucleotides. 72 hours later Foxo3 and Foxp3 mRNA expression was measured and Foxp3 expression was assessed by intracellular staining. The knockdown of Foxo3 in nTreg cells had no effect on established Foxp3 expression (Figure 9).

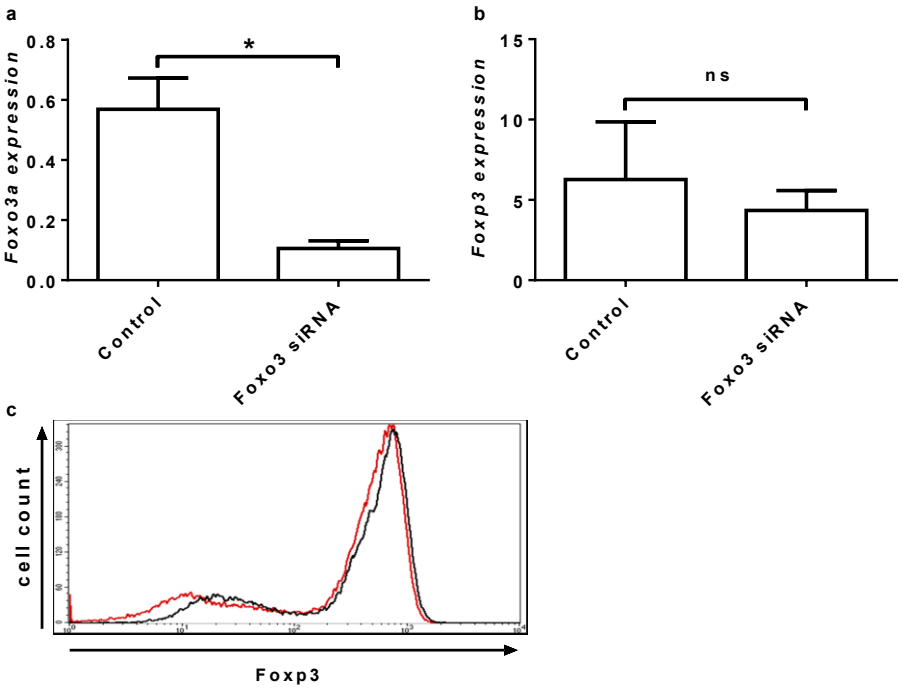

Figure 9: Foxo3 is not required for the maintenance of established Foxp3 expression in Treg cells. (a) nTreg cells were isolated and activated with anti-CD3/CD28 beads and IL-2 [10ng/ml] and transduced with either control siRNA or Foxo3 siRNA. Foxo3 mRNA expression levels were measured after 48 hours. Data are representative of the mean of 3 independent experiments ± SD. (b) *Foxp3* mRNA expression levels in nTreg cells 48 hours after knockdown of *Foxo3*. (c) Foxp3 expression levels in nTreg cells were measured by intracellular staining 48 hours after transduction with Foxo3 siRNA. Data are representative of one of three experiments. Differences in gene expression were compared using student's t-tests. (*p=<0.05, ns=p>0.05).

Additional experiments found that the knockdown of Foxo1 alone or both Foxo1 and Foxo3 did not affect Foxp3 expression in nTreg cells. These findings suggest that Foxo1 and Foxo3 are not required for the maintenance of established Foxp3 expression in nTreg cells. These findings were confirmed by a subsequent study which showed that the Treg cell-specific deletion of both Foxo1 and Foxo3 did not decrease Foxp3 expression in nTreg cells (Ouyang et al., 2010).

3.1.4 A role for Klf2 in the regulation of Foxp3

Foxo factors have been reported to regulate Klf2 (Fabre et al., 2008, Kerdiles et al., 2009). Data generated from within the host laboratory had previously shown that premature withdrawal of the TCR activation signal and PI3K inhibitors increased the expression of Klf2. As Klf2 is regulated by signalling pathways that are known to affect the induction of Foxp3, Klf2 may have a role in the regulation of Foxp3 expression.

To study the effect of Klf2 on Treg cell differentiation, naive CD4 T cells were isolated, activated and transduced with GFP-KLF2 (a retroviral vector which expresses a GFP-Klf2 fusion protein), KLF2-IRES-GFP, Foxo3-IRES-GFP, or IRES-GFP. Increasing concentrations of TGFβ were added 24 hours later. Foxp3 expression was measured after 48 hours by intracellular staining. As seen in previous experiments, the overexpression of Foxo3 synergised with increasing concentration of TGFβ to promote the induction of Foxp3 expression. Whereas overexpression of GFP-KLF2 antagonised TGFβ mediated induction of Foxp3 expression in a dose dependent manner (Figure 10a). However, when compared to GFP-KLF2, the overexpression of KLF2-IRES-GFP had a more modest, negative effect on the expression on Foxp3. These data suggest that overexpression of Klf2 antagonises TGFβ mediated induction of Foxp3 expression and identified important differences in the effects of the different retroviral constructs that were used in the experiments.

When compared to the published Klf2 sequence, the GFP-KLF2 and KLF2-IRES-GFP retroviral vectors contain a Glutamic acid 24 (E) to Glycine (G) substitution within the Klf2 activation domain, which could affect DNA binding and its transcriptional activity. To assess the effect of the substitution on the induction of Foxp3, the Klf2 sequence from the GFP-KLF2 vector was amplified and mutated by SOEing PCR (Horton, 1995) leading to the substitution of Glycine (G) to Glutamic acid 24 (E) within the Klf2 activation domain. The mutated PCR fragment was cloned into the MSCV-IRES-GFP vector and is referred to as KLF2-(G/E).

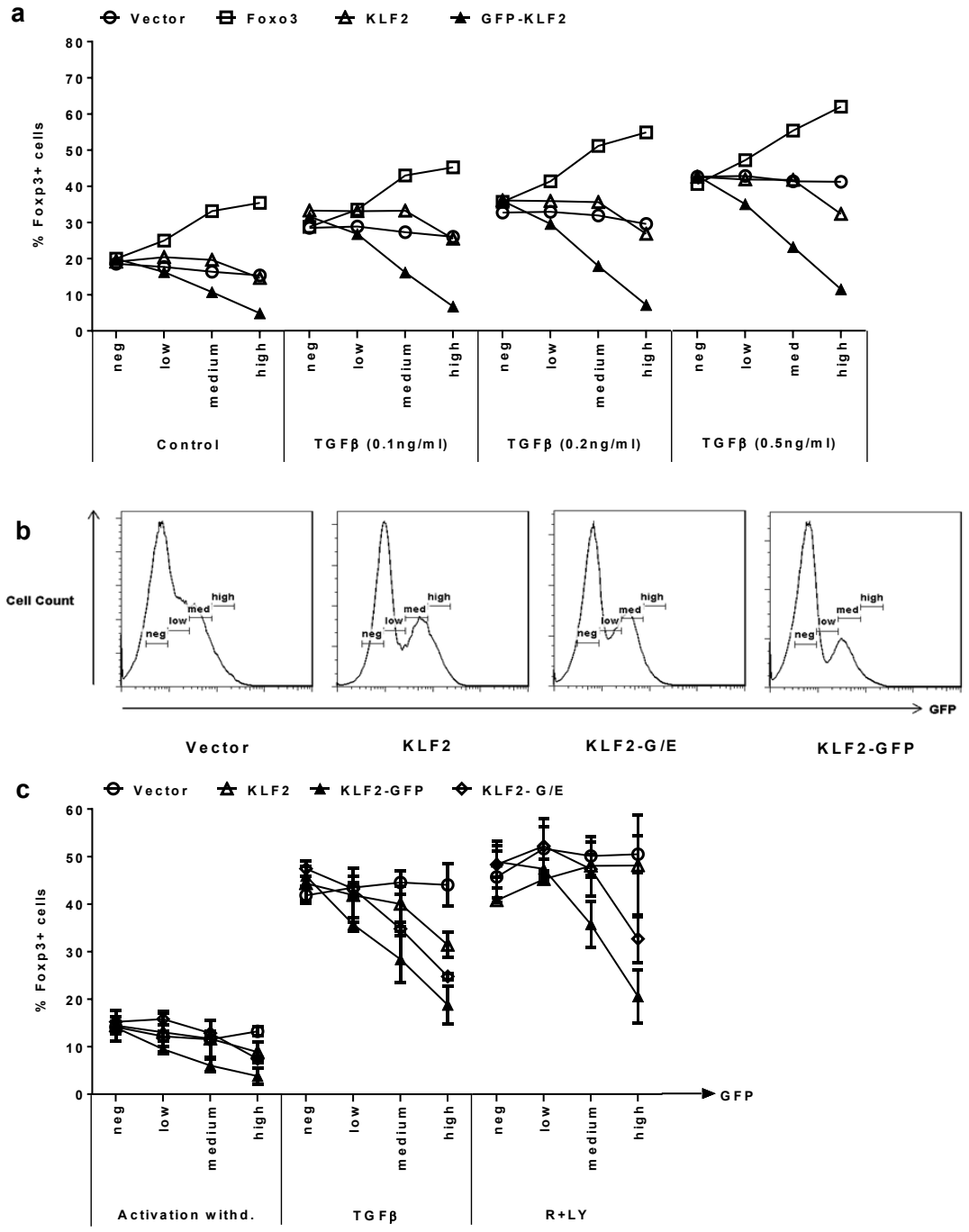

Figure 10: Klf2 antagonises the induction of Foxp3 expression induced by TGFβ and PI3K inhibitors. (a) Naive CD4 T cells were isolated and activated as in figure 5. After overnight activation the cells were transduced with GFP-KLF2 vector, KLF2-IRES-GFP (KLF2), Foxo3-IRES-GFP (Foxo3), or IRES-GFP (Vector). 24 hours after retroviral transduction, increasing concentrations of TGFβ were added. Foxp3 expression was assessed by intracellular staining 48 hours later by gating on cells with negative (neg), low, medium (med) and high GFP expression. Data are representative of 1 of 3 similar experiments. (b) GFP expression in CD4 T cells 28 hours after transduction with KLF2-IRES-GFP (G to E [KLF2-G/E]), GFP-KLF2, KLF2-IRES-GFP, or IRES-GFP retroviruses. (c) Naive CD4 T cells were isolated and activated. After overnight activation, the cells were transduced with KLF2-IRES-GFP (G to E [KLF2-G/E]), GFP-KLF2, KLF2-IRES-GFP, or IRES-GFP. 24 hours after retroviral transduction the activation signal was withdrawn and TGFβ, or the mTOR inhibitor rapamycin [25 nM] and the PI3K inhibitor LY294002 [10 μM] were added (R + LY). Foxp3 expression was assessed by intracellular staining 48 hours later by gating on cells with negative (neg), low, medium (med) and high GFP expression. Data are representative of the mean of 3 independent experiments (± SEM).

Naive CD4 T cells were isolated, activated and transduced with KLF2-IRES-GFP (G to E), GFP-KLF2, KLF2-IRES-GFP, or IRES-GFP. 24 hours after retroviral transduction the activation signal was withdrawn, and TGFβ or the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin were added (Figure 10b). Foxp3 expression was measured 48 hours later by intracellular staining. The overexpression of Klf2 antagonised the induction of Foxp3 with TGFβ and PI3K inhibitors in a dose dependent manner (Figure 10). The Glycine (G) to Glutamic acid 24 (E) substitution within the Klf2 activation domain had modest effect on Foxp3 expression (Figure 10b).

However, overexpression of the GFP-KLF2 fusion protein had the most pronounced antagonistic effect on the induction of Foxp3, although similar but less pronounced effects were seen with the other retroviral constructs. The differences between the different vectors could have been explained by differences in the relative expression of Klf2. It was not possible to perform experiments to assess differences in protein expression as there are no commercially available, effective anti-Klf2 antibodies.

3.1.5 GFP-KLF2 antagonises established Foxp3 expression in nTreg cells

As overexpression of Klf2 appeared to negatively regulate iTreg cell differentiation, the effect of overexpression of Klf2 on established Foxp3 expression was assessed in nTreg cells. nTreg cells were isolated and activated with anti-CD3/CD28 beads and IL-2, and transduced with KLF2-IRES-GFP (G to E), GFP-KLF2, or IRES-GFP. Foxp3 expression was measured 48 hours later by intracellular staining (Figure 11). The overexpression of GFP-KLF2 antagonised Foxp3 expression in a dose dependent manner. The expression of KLF2-IRES-GFP (G to E) did not affect established Foxp3 expression in nTreg cells.

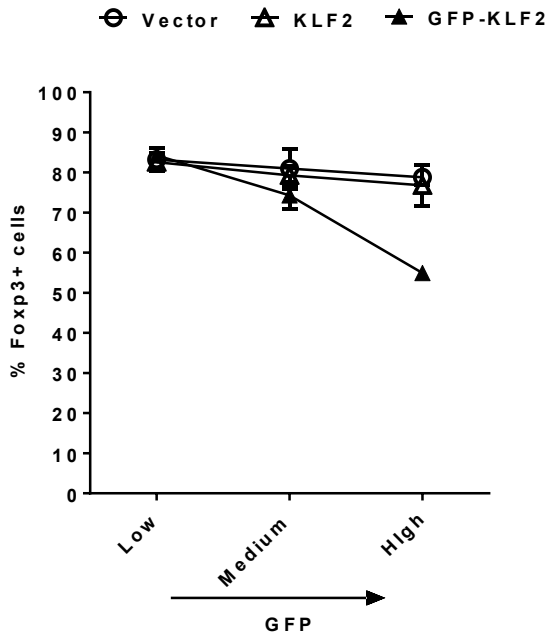

Figure 11: GFP-KLF2 antagonises established Foxp3 expression in nTreg cells. nTreg cells were isolated and activated with anti-CD3/CD28 beads and IL-2 and transduced with KLF2-IRES-GFP, GFP-KLF2, or IRES-GFP (Vector). Foxp3 expression was assessed by intracellular staining after 48 hours, gating on cells with low, medium, and high GFP expression (data represent the mean of three independent experiments ± S.D).

3.1.6 Klf2 antagonises Foxp3 expression through a TGFβ signalling independent mechanism

TGFβ induces the expression of Foxp3 through the binding of activated Smad2/3 transcription factors to the Foxp3 enhancer 1 (Tone et al., 2008). In endothelial cells, it has been proposed that Klf2 antagonises TGFβ signalling by inducing the expression of Smad7, which prevents the activation of Smad2/3 (Boon et al., 2007). To investigate this model in CD4 T cells, naive CD4 T cells were isolated, activated, and transduced with KLF2-IRES-GFP (G to E), GFP-KLF2, or IRES-GFP. Cells expressing high levels of GFP were isolated 24 hours later by FACS sorting; and then Klf2, and Smad7 mRNA expression was measured by qRT-PCR. Overexpression of Klf2 did not promote the expression of Smad7, suggesting that Smad7 does not modulate the effect of Klf2 on Foxp3 expression (Figure 12).

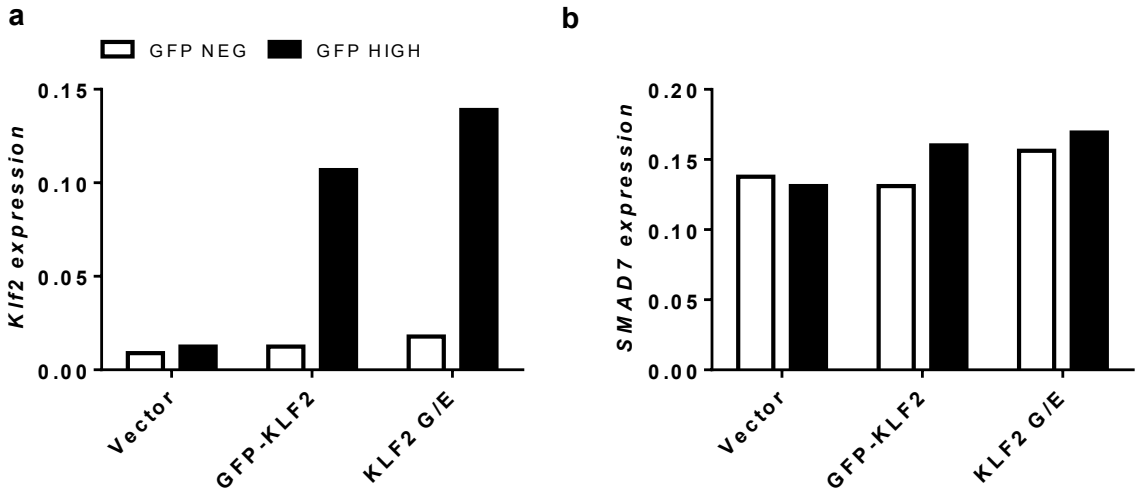

Figure 12: Overexpression of Klf2 does not induce Smad7 expression. a) Naive CD4 T cells were isolated and activated and transduced with KLF2-IRES-GFP (G to E), GFP-KLF2, or IRES-GFP (Vector). 24 hours later the cells were sorted by FACS to isolate cells expressing high levels of GFP. b) Smad7 mRNA levels were measured by qRT-PCR. Data are representative of 2 independent experiments.

Foetal calf serum contains endogenous TGFβ which synergises with PI3K inhibitor mediated induction of Foxp3 expression (Sauer et al, 2008). TGFβ was depleted from the experimental conditions to determine if the antagonistic effects of Klf2 on PI3K inhibitor mediated induction of Foxp3 were dependent on endogenous TGFβ signalling. Naive CD4 T cells were isolated, activated, and transduced with KLF2-IRES-GFP (G to E), GFP-KLF2, or IRES-GFP in the presence of anti-TGFβ. 24 hours after retroviral transduction the activation signal was withdrawn, and the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin were added. Foxp3 expression was measured after 48 hours (Figure 13).

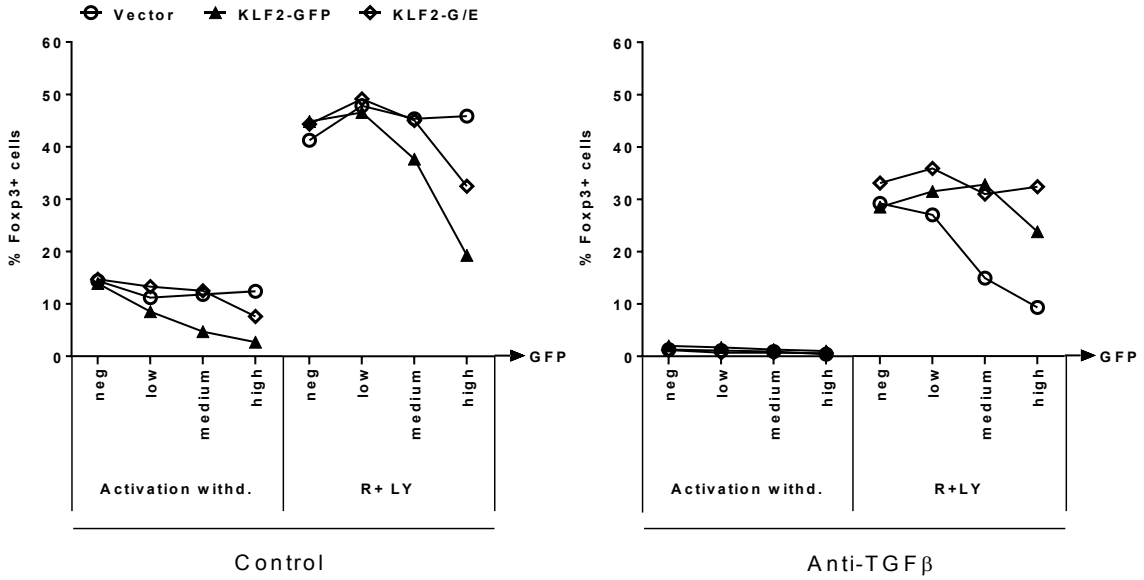

Figure 13: **The effect of overexpression of Klf2 on Foxp3 expression is independent of TGFβ.** Naive CD4 T cells were activated in the presence of neutralising anti-TGFβ and transduced with KLF2-IRES-GFP (KLF2-G/E), GFP-KLF2, or IRES-GFP (Vector). 24 hours after retroviral transduction the activation signal was withdrawn and the mTOR inhibitor rapamycin (R) [25 nM], and the PI3K inhibitor LY294002 (LY) [10 µg/ml] were added (R+LY). Foxp3 expression was assessed after 48 hours by intracellular staining, gating on cells expressing, low, medium, and high levels of GFP. Data are representative of one of 3 independent experiments.

As seen previously, the overexpression of Klf2 antagonised PI3K inhibitor mediated induction of Foxp3. Although the depletion of endogenous TGFβ reduced the efficiency of PI3K inhibitor mediated induction of Foxp3 expression, the antagonistic effects of overexpression of Klf2 on Foxp3 expression were maintained (Figure 13). These data suggest that overexpression of Klf2 antagonises the expression of Foxp3 via a TGFβ signalling independent mechanism.

3.1.7 Klf2 deficient CD4 thymocytes do not express Foxp3 in response to PI3K inhibition or TGFβ

These experiments suggested that Klf2 may function as a negative regulator of the expression of Foxp3, in a model where Foxo factors promote the expression of Foxp3 in activated CD4 T cells, and downstream transcription factors such as Klf2, negatively regulate the expression of Foxp3. If this model was correct, then it would be expected that Klf2 deficient cells would show enhanced Foxp3 expression after activation and PI3K inhibition or TGFβ treatment. Klf2-deficient (KLF2 KO) thymocytes fail to exit from the thymus due to failure to express receptors for thymocyte migration and for peripheral trafficking, such as sphingosine-1-phosphate (S1P) receptor S1P1 and CD62L (Carlson et al., 2006). To address this question,

$CD4^+CD8^-$ thymocytes were isolated from Klf2-deficient mice and were activated and transduced with KLF2-IRES-GFP, Foxo3-IRES-GFP, Foxo3-active-IRES-GFP, or IRES-GFP.

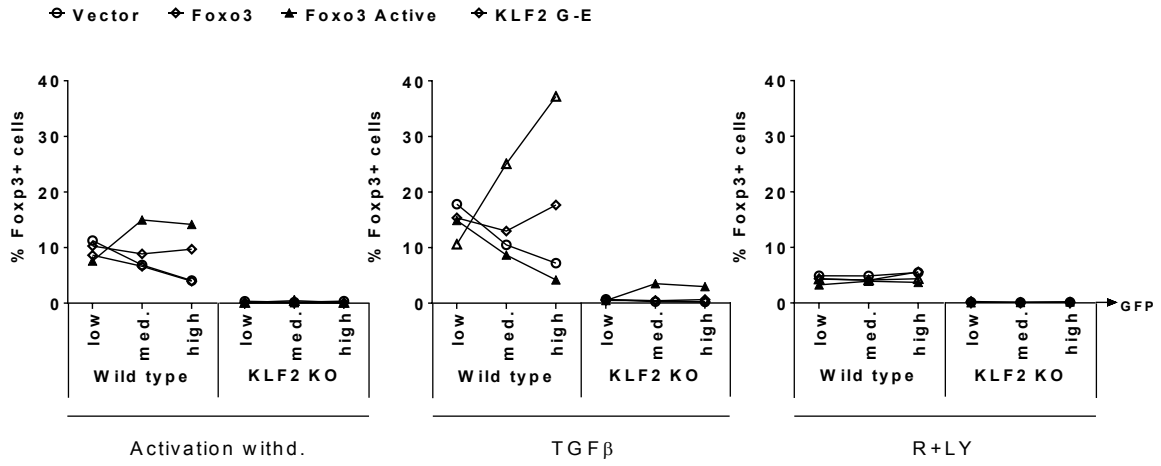

Figure 14: KLF2 deficient CD4 thymocytes do not express Foxp3 in response to PI3K inhibition or TGFβ. CD4 KLF2 KO thymocytes were activated and transduced with KLF2-IRES-GFP (KLF2-G/E), Foxo3-IRES-GFP (Foxo3), Foxo3-active-IRES-GFP (Foxo3 Active), or IRES-GFP (Vector), and 24 hours after retroviral transduction the activation signal was withdrawn, and TGFβ, or the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin were added. Foxp3 expression was measured after 48 hours by intracellular staining.

Unlike wild-type CD4 thymocytes, activated Klf2 deficient CD4 thymocytes did not express Foxp3 in response to TGFβ or PI3K inhibitors (Figure 14). The overexpression of Klf2 antagonised the expression of Foxp3 in wild-type CD4 thymocytes in response to TGFβ. However, the expression of Foxp3, in Klf2 deficient CD4 thymocytes in response to TGFβ or PI3K inhibitors, was not restored when Klf2 expression was reconstituted by retroviral gene transfer (Figure 14).

The inability of the Klf2 deficient thymocytes to express Foxp3 could have been caused by thymocyte developmental abnormalities. It would have been interesting to perform additional studies using an inducible genetic system to deplete Klf2 in activated CD4 T cells and nTreg cells, without pre-existing thymic developmental abnormalities, to further investigate the role of Klf2 in the expression of Foxp3.

Chapter 4. A role for TRAIL in the treatment of ovarian cancer

4.1.1 Ovarian cancer cells express TRAIL receptors

FACS staining of 5 different ovarian cancer lines and tumour cells from ovarian cancer ascites revealed that ovarian cancer cells expressed both TRAIL-R1 and TRAIL-R2, and varying levels of TRAIL-R3 and TRAIL-R4 (Figure 15). All the cell lines, and the patient sample that was tested, expressed TRAIL-R2 which has previous been shown to make a greater contribution to TRAIL-induced apoptosis in some cell lines that express both TRAIL-R1 and TRAIL-R2 (Kelley et al., 2005). The cells from the patient sample expressed relatively low amounts of TRAIL-R1 (Figure 15). However the clinical significance of this is uncertain as the expression of TRAIL-R1 does not correlate with the sensitivity of cancer cells to TRAIL-R1-agonistic therapies (Leong et al., 2009, Younes et al., 2010a).

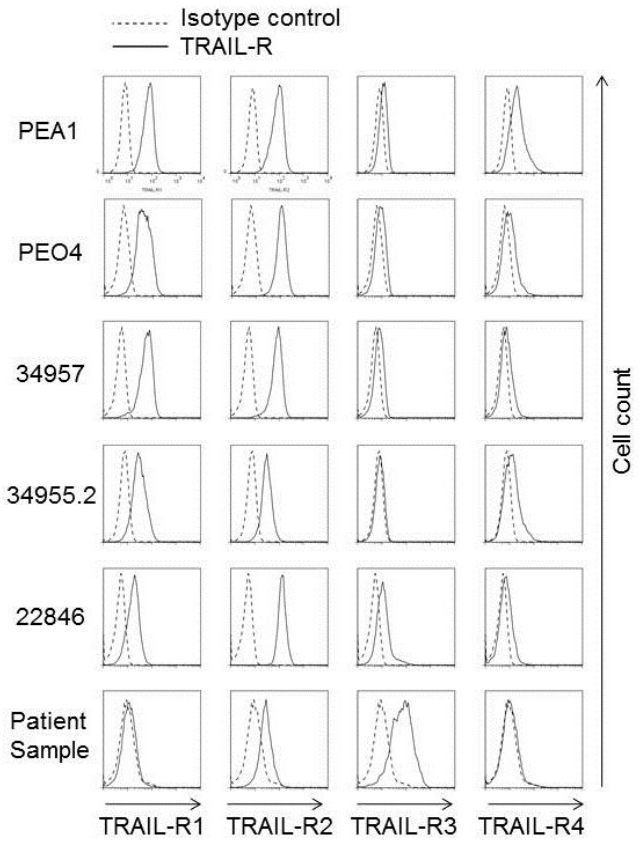

Figure 15: Ovarian cancer cells express TRAIL receptors. TRAIL-R staining is shown of 5 different ovarian cancer cell lines and tumour cells from a patient with ovarian cancer.

4.1.2 A role for SMAC mimetics in the treatment of ovarian cancer cells with TRAIL

SM-83 is a recently synthesised dimeric SMAC mimetic that potently depletes cIAP1, cIAP2 (Figure 16) and inhibits the function of XIAP by binding to the BIR3 domain of cIAP1/2 and the linker-BIR2–BIR3 domains of XIAP (Lecis et al., 2012). SM-83 has potential use as an anti-cancer agent as it has good bioavailability and has been shown to induce apoptosis in murine xenograft models of ovarian cancer, without appreciable toxicity (Lecis et al., 2012).

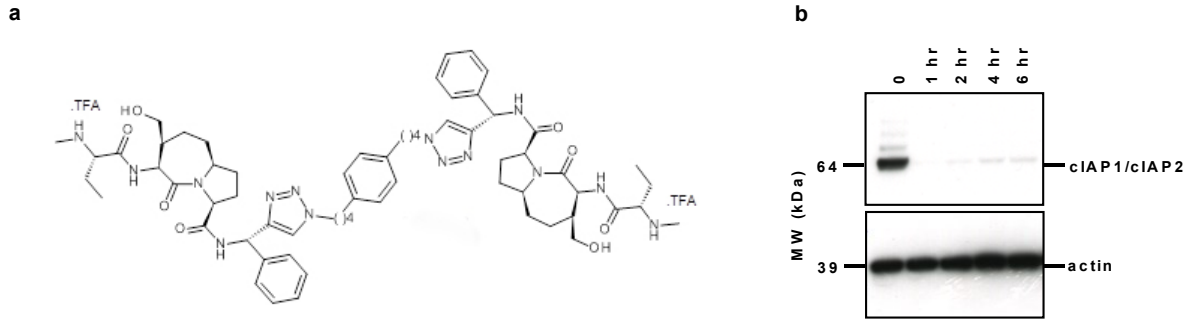

Figure 16: Treatment of ovarian cancer cells with SM-83 leads to a rapid depletion of cIAPs. a) The structure of SM-83 (picture courtesy of Daniele Lecis). b) 34957 ovarian cancer cells were treated with SM-83 [100 nM] for the indicated times and the depletion of cIAP1 and cIAP2 was measured by western blotting using a pan cIAP1/cIAP2 antibody.

The treatment of the 34957 ovarian cancer cell line confirmed that SM-83 leads to the rapid degradation of both cIAP1 and cIAP2 in ovarian cancer cells (Figure 16). In some cell lines, the depletion of cIAP1 and cIAP2 leads to the activation of non-canonical NF-κB signalling and the production of autocrine TNF, which leads to the induction of apoptosis (Darding and Meier, 2012).

The treatment of 34957 and 34955.2 cells revealed that the induction of cell death by SM-83 can be inhibited by blocking TNF (Figure 17). The treatment of ovarian cancer cells with SM-83 also sensitised the ovarian cancer cells to TRAIL-induced cell death. The combination of TRAIL and SM-83 synergised to kill the 34955.2 cells, but not the 34957 cells (Figure 17). One explanation could be that the 34957 cells produce higher amounts of TNF and they are therefore more sensitive to the effects of SM-83. These data suggest that SM-83 may have some activity against primary ovarian cancer cells by stimulating the autocrine production of TNF, and this may sensitise ovarian cancer cells to both TNF and TRAIL-induced apoptosis.

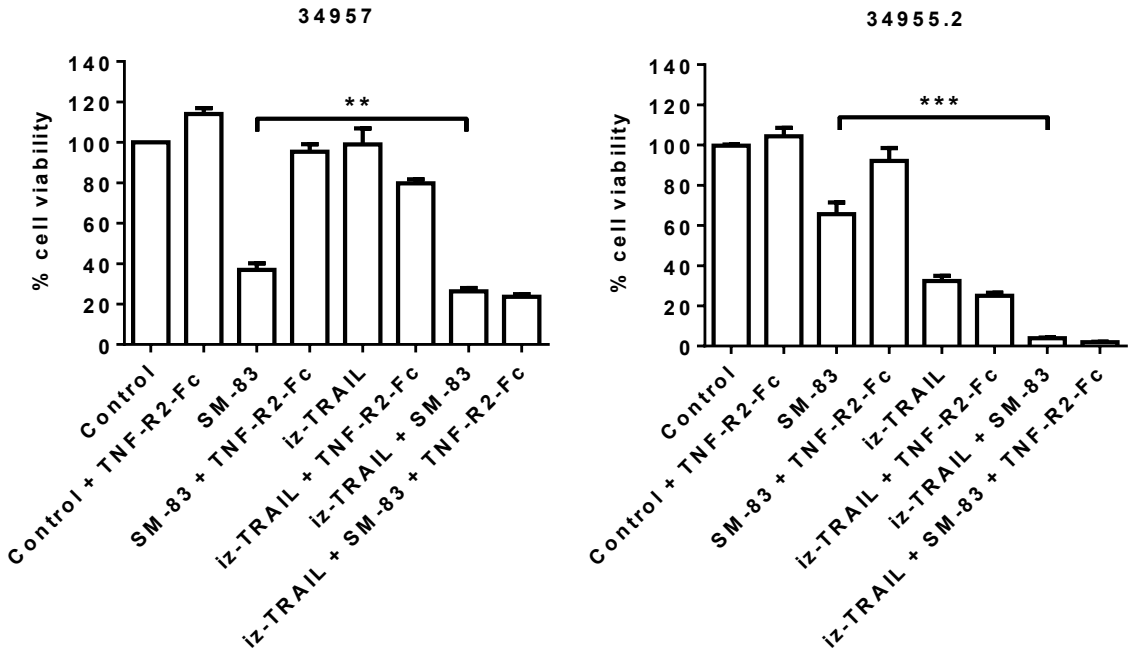

Figure 17: SMAC mimetics induce ovarian cancer cell death in a partially TNF-dependent manner. 34957 and 34955.2 ovarian cancer cell lines were treated with combinations of SM-83 [100 nM] and TNF-R2-Fc (Enbrel, [100 µg/ml]) for one hour, and then iz-TRAIL [100 ng/ml] was added where indicated. Cell viability was measured after 24 hours. Data are representative of 4 biological replicates (± S.D) from 1 of 3 independent experiments. Data were compared using student's t-tests (**p=<0.01, ***p=<0.001).

4.1.3 TRAIL-resistant ovarian cancer cell lines can be sensitised to TRAIL-induced cell death by proteasome inhibition

An alternative approach to using SMAC mimetic drugs to sensitise ovarian cancer cells to TRAIL would be to inhibit the proteasome with PS-341. One of the principle advantages of using PS-341, rather than a SMAC mimetic compound, is that PS-341 is an approved drug which has been extensively used in the cancer clinic. In contrast, SMAC mimetic drugs are only in the early phases of clinical testing in patients (Table 5).

Previous pre-clinical studies performed within the host laboratory have shown that PS-341 can reliably sensitise both TRAIL-resistant ovarian cancer cell lines and primary ovarian cancer cells to TRAIL-induced apoptosis, as well as hepatoma, colon, and pancreatic cancer cell lines (Koschny et al., 2007a, Ganten et al., 2005, Ganten et al., 2006), and also primary human astrocytoma cells (Koschny et al., 2007b).

The treatment of 24683, 34957, and 34955 ovarian cancer cell lines with PS-341 did not affect the viability of cells, but was sufficient to sensitise the cells to TRAIL-

induced apoptosis. The treatment of three different TRAIL-resistant ovarian cancer cell lines with PS-341 or SM-83 revealed that PS-341 and SM-83 treatment can efficiently sensitise each one of these cells to TRAIL (Figure 18). The effect of PS-341 was similar to SM-83 in the 24683 cells that were not sensitive to SM-83 treatment alone.

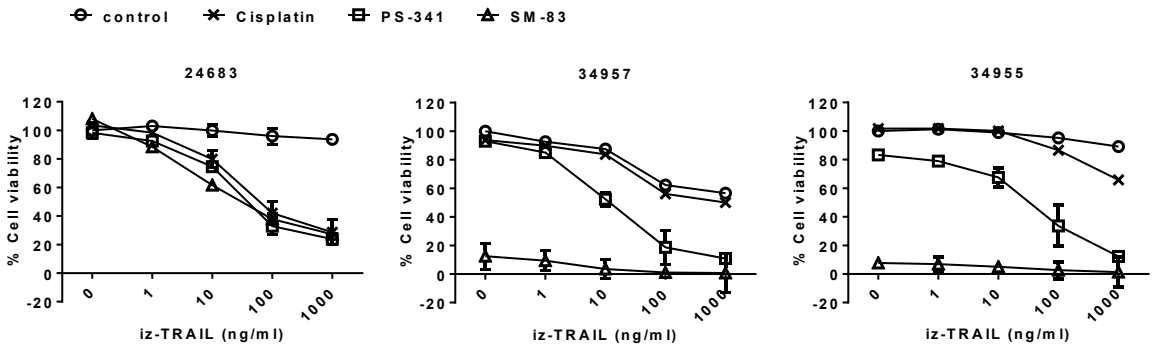

Figure 18: Proteasome inhibition, SM-83 and cisplatin increase the sensitivity of ovarian cancer cells to TRAIL. 24683, 34957, and 34955 ovarian cancer cells were treated with PS-341 [20 nM], cisplatin [10 μM], or SM-83 [100 nM], and then treated with iz-TRAIL. Cell viability was measured after 24 hours. Data are mean (± S.D) of 3 biological replicates from one of 3 independent experiments.

4.1.4 The depletion of $CD45^+$ immune cells leads to the enrichment of $EpCAM^+$ tumour cells from ovarian cancer ascites

Although the data from the cell lines were compelling, one of the aims of this study was to gather sufficient pre-clinical data from patient samples to justify the initiation of a phase I clinical trial of a TRAIL-R agonistic drug in combination with either PS-341 or a SMAC mimetic, in patients with treatment-refractory ovarian cancer. A total of 60 samples of ascites were obtained from 38 patients with advanced ovarian cancer from the oncology wards at Charing Cross Hospital, Imperial College Healthcare NHS Trust, and from patients attending the Constance Wood oncology day unit at the Hammersmith Hospital, Imperial College Healthcare NHS Trust.

The median age of the patients who donated the samples that were used in the study was 68 years old (range 20 to 88 years old). All of the patients had serous ovarian cancer and the majority of these presented with advanced stage III (58%) or stage IV (28%) disease. The majority of the patients (84%) had high-grade serous ovarian cancer (Table 6). Around 50% of the patients had platinum-refractory ovarian cancer, which was defined as ovarian cancer that had progressed within 6 months of

platinum-based therapy. Approximately 39% of the patients had platinum-resistant ovarian cancer, which was defined as ovarian cancer that had progressed within 6 to 12 months after previous platinum-based chemotherapy. Only 17% of the total patients had platinum-sensitive ovarian cancer, which was defined as ovarian cancer that had progressed 12 months after previous platinum based chemotherapy (Table 6).

Patient number	Age (years)	Ovarian cancer subtype	Stage	Grade	Platinum sensitivity	No. lines prior treatment
A1	83	Serous	3	3	Refractory	1
A2*	59	Serous	3	N/A	Refractory	7
A3	72	Serous	3	3	Resistant	1
A4*	61	Serous	3	3	Resistant	6
A5	63	Serous	3	3	Sensitive	0
A6	87	Serous	2	3	Refractory	2
A7	72	Serous	2	3	Resistant	5
A8	81	Serous	2	3	Sensitive	0
A9	52	Serous	4	3	Refractory	>1
A10	78	Serous	3	3	Resistant	1
A11*	88	Serous	3	3	Refractory	4
A12	66	Serous	3	1	Resistant	1
A13	70	Serous	3	3	Resistant	2
A14	59	Serous	3	3	Refractory	1
A15	77	Serous	4	3	Refractory	2
A16	68	Serous	4	3	Resistant	2
A17	68	Serous	3	3	Sensitive	1
A18	66	Serous	4	3	Refractory	3
A19ǂ	20	Serous	4	1	Refractory	1

Table 6: **The clinical characteristics of the patients with ovarian cancer within the study.** Patient data were obtained from the Imperial College Oncology NHS oncology database. *Patients who donated multiple sample of ascites. ǂ Patient only donated immune cells.

The patients donated a mean of 1.41 samples (range 1 to 6 samples) over the 18 month study period. The mean sample volume of ascites per sample that was processed was 1.36 litres (range 0.1 to 3.75 litres), which contained between 5 x 10^6 to 3 x 10^9 cells. Up to 200 x 10^6 of the total cells were used for the isolation of tumour cells, with the remaining cells given to collaborators or frozen for use in later experiments. Tumour cells were identified by the surface expression of epithelial cell adhesion molecule (EpCAM), an epithelial cell adhesion molecule, which is highly expressed across all subtypes of ovarian cancer (Kobel et al., 2008).

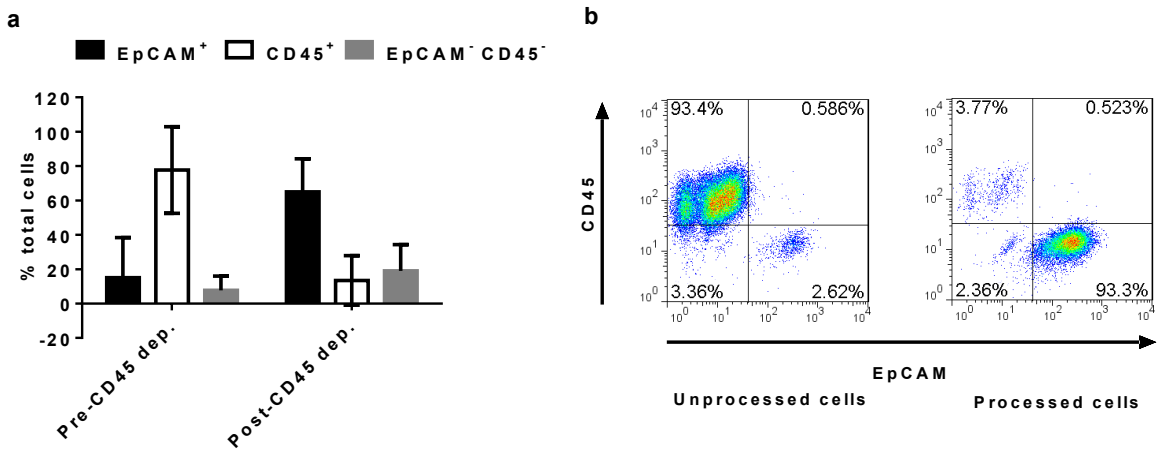

Figure 19: The depletion of CD45$^+$ immune cells leads to the enrichment of EpCAM$^+$ tumour cells from ovarian cancer ascites. a) The mean enrichment of EpCAM$^+$ tumour cells from ascites by depletion of CD45$^+$ immune cells from ascitic fluid from 18 patients with ovarian cancer (± SD). b) Representative FACS plot showing the enrichment of EpCAM$^+$ tumour cells by depletion of CD45$^+$ immune cells from a patient with advanced ovarian cancer.

It was possible to isolate tumour cells from 18 patients and immune cells from 1 additional patient (Table 6) by depleting CD45$^+$ immune cells from ovarian cancer ascites (Figure 19). The mean number of EpCAM$^+$ tumour cells that were isolated from the patient samples was 5.39 x 10^6 cells (range 0 to 28 x 10^6 cells). The failure to isolate tumour cells was most commonly due to a lack of tumour cells or an insufficient total number of cells within the sample. Ovarian cancer ascites contains human serum and growth factors which promote resistance to TRAIL through the activation of Akt by focal adhesion kinase (Lane et al., 2007, Lane et al., 2010), and possibly by other unknown mechanisms. Therefore all tumour and immune cells were cultured in sterile filtered ascites (ascites + 50% RPMI media) in an attempt to closely mimic the *in vivo* ovarian cancer microenvironment.

4.1.5 The sensitivity of primary ovarian cancer cells to iz-TRAIL

There are no published studies that have investigated the sensitivity of primary ovarian cancer cells to iz-TRAIL. Primary EpCAM$^+$ ovarian cancer cells from 18 individual patients were treated with iz-TRAIL [100 ng/ml] and then the viability of the cells was measured after 48 hours. The ovarian cancer cells were considered to be TRAIL-sensitive when they underwent a ≥ 50% decrease in viability after treatment with iz-TRAIL (Figure 20).

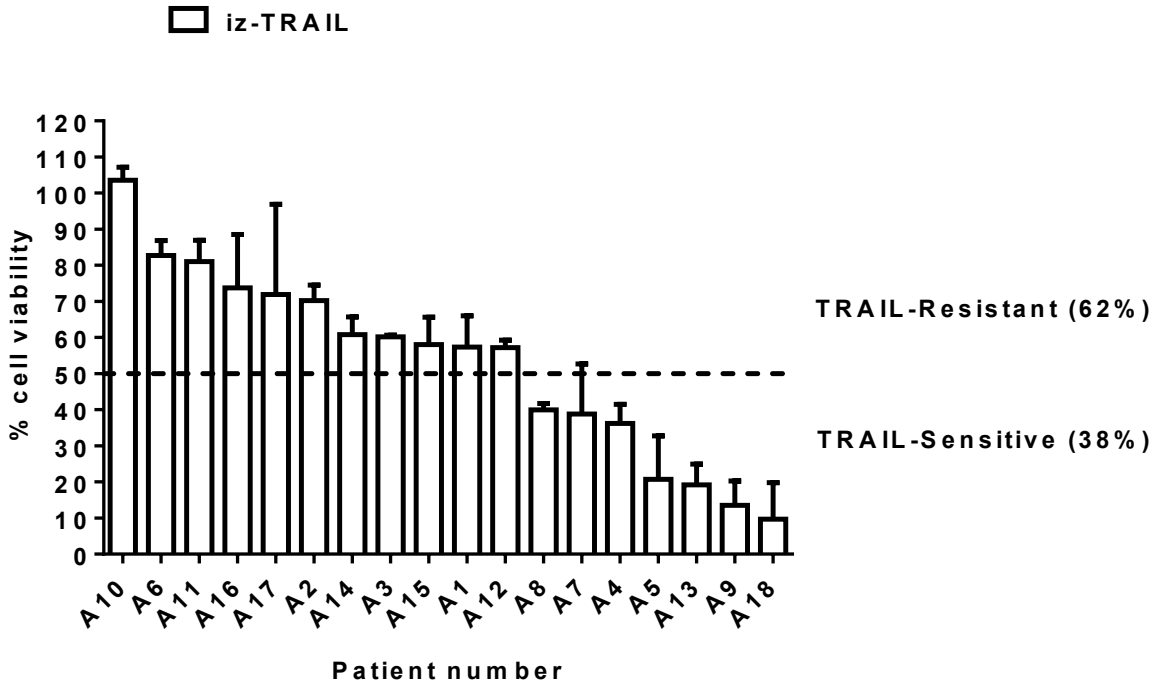

Figure 20: The sensitivity of primary ovarian cancer cells to iz-TRAIL. EpCAM^{+} ovarian cancer cells from 18 different patients were treated with iz-TRAIL [100 ng/ml]. Cell viability was measured after 48 hours. Data are representative of the mean of three biological replicates (± S.D).

The results showed that the majority of the primary tumour cells from ascites from patients with ovarian cancer are resistant to iz-TRAIL treatment. However, the majority of the iz-TRAIL-treated cells underwent some loss of viability (Figure 20). These results suggest that the TRAIL sensitivity of tumours cells is variable. It is important to note that variations in the amount of tumour cells between different patient samples could have affected the results from this analysis. Therefore the only way to formally assess the clinical sensitivity of primary ovarian cancer cells to TRAIL-induced apoptosis would be in a clinical trial in patients.

4.1.6 The effect of the proteasome inhibitor PS-341 on the sensitivity of ovarian cancer cells to TRAIL

Previous pre-clinical studies conducted within the host laboratory have shown that primary ovarian cells could be reliably sensitised to iz-TRAIL by PS-341 treatment. However, these earlier studies were limited by the number of samples that could be obtained from patients. In addition, the ovarian cancer cells were cultured using standard tissue culture conditions (RPMI media + 10% FCS), rather than RPMI media + 50% ovarian cancer ascites, which could have affected the sensitivity of the primary ovarian cancer cells to TRAIL-induced apoptosis.

To investigate the effect of proteasome inhibition on the sensitivity of ovarian cancer cells to iz-TRAIL, primary ovarian cancer cells were isolated from 18 patients and were cultured with PS-341 or iz-TRAIL, or both. PS-341 was used at a concentration of 20 nM in all experiments; as this concentration has been shown to inhibit the proteasome (Adams et al., 1999), to consistently sensitise cancer cell lines to TRAIL-induced apoptosis (Koschny et al., 2007a); and approximately reflects the steady state levels found in patients, 1 hour after intravenous infusions of PS-341 (Papandreou et al., 2004). Higher 50 nM and 100 nM concentrations of PS-341 were evaluated, but these led to a significant loss in viability of the primary ovarian cancer cells without additional TRAIL sensitisation.

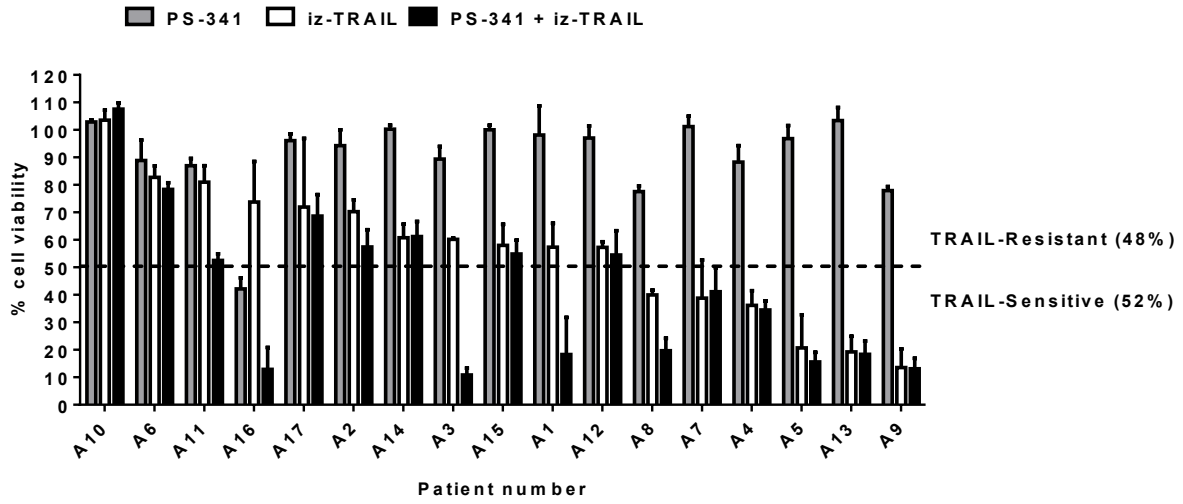

Figure 21: The effect of PS-341 on the sensitivity of primary ovarian cells to iz-TRAIL. Ovarian cancer cells from 17 patients were treated with PS-341 [20 nM] for 2 hours and then iz-TRAIL [100 ng/ml] was added. Cell viability was measured after 48 hours. Data are representative of the mean of 3 biological replicates (± S.D).

PS-341 treatment only had a modest effect on the overall viability of the primary ovarian cancer cells with only one patient showing a >50% loss of viability with PS-341 treatment (Figure 21). These data are similar to the data from clinical trials in patients that have shown that PS-341 either alone (Aghajanian et al., 2009) or in combination with chemotherapy (Parma et al., 2012) is not an effective treatment for ovarian cancer. PS-341 treatment was found to only have a modest effect on sensitivity of the primary ovarian cancer cells to TRAIL-induced apoptosis. PS-341 treatment enhanced the sensitivity of the cancer cells to iz-TRAIL in 24% (4 out of 17) of the patient samples. However, PS-341 treatment increased the overall TRAIL sensitivity from 38% to 52% of the patient samples. Therefore PS-341 treatment

could potentially lead to responses in around 50% of patients treated with a TRAIL-R agonistic drug (Figure 21).

These data were surprising as the majority of TRAIL-resistant cell lines and primary tumour cells that have been tested have been sensitised to TRAIL-induced apoptosis by PS-341 treatment (Ganten et al., 2005, Koschny et al., 2007b, Koschny et al., 2007a). This could have been explained by variations in proteasome inhibition between the samples. However, the limited availability of primary tumour cells did not allow for the measurement of proteasome inhibition in the clinical samples. Another explanation is that ovarian cancer ascites contains factors which antagonise the effect of PS-341 on the sensitivity of primary ovarian cancer cells to TRAIL.

To investigate this concept, primary ovarian cancer cells were cultured in standard tissue culture media or RPMI media + 50% ascites and were then treated with PS-341 and iz-TRAIL. Ascites did not affect the PS-341-induced loss of viability. However, the presence of ovarian cancer ascites antagonised the PS-341-induced sensitisation of primary ovarian cancer cells to TRAIL-induced apoptosis (Figure 22).

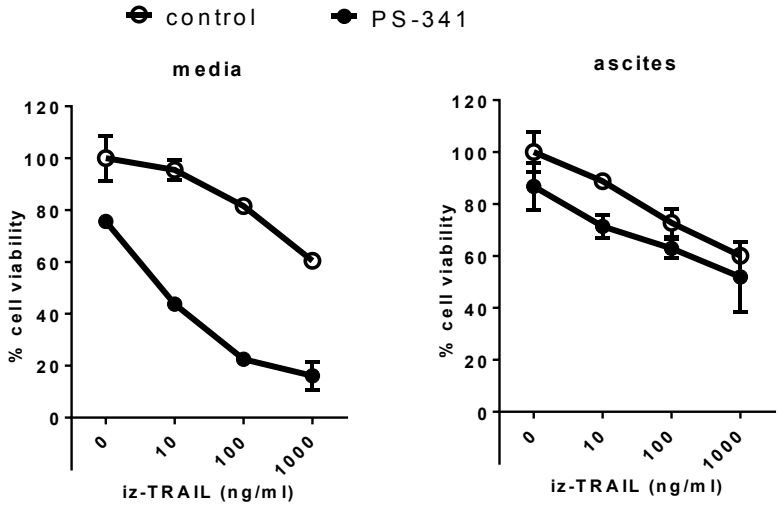

Figure 22: Ovarian cancer ascites antagonises the effect of PS-341 on the sensitivity of primary ovarian cancer cells to TRAIL. Primary ovarian cancer cells from a patient were cultured in the tissue standard culture media (RPMI + 10% FCS) or ascites (RPMI + 50% ascites). Cells were treated with PS-341 [20 nM] and then iz-TRAIL was added. Cell viability was measured after 48 hours. Data are representative of the mean of 3 biological replicates (± S.D) of one of two independent experiments and of similar experiments using ovarian cancer cell lines.

The same effect was also seen in independent experiments with PEA1 ovarian cancer cells that were cultured with and without ovarian cancer ascites. The antagonistic effect of ascites on PS-341-induced TRAIL sensitisation did not appear

to be due to the binding of PS-341 to human albumin within the ascites, as the effect of PS-341 on TRAIL sensitivity could not be restored by increasing the concentration of PS-341 up to 1000 nM. These data suggest that factors within ascites prevent proteasome-inhibition-mediated sensitisation of primary ovarian cancer cells to TRAIL-induced apoptosis.

4.1.7 SM-83 and iz-TRAIL synergise to potently kill primary ovarian cancer cells

To investigate whether SMAC mimetic treatment would synergise with TRAIL treatment, ovarian cancer cells were isolated from 17 patients and treated with the SMAC mimetic SM-83. The results of these experiments demonstrated that the majority of primary ovarian cancer cells were resistant to SM-83 treatment. However, there was substantial variability in the sensitivity of the cancer cells to SM-83 (Figure 23a).

The effect of SM-83 on the ovarian cancer cells could have been induced in both a TNF-dependent and TNF-independent manner, which could have been enhanced by TNF within ovarian cancer ascites. However, experiments showed that the blocking of TNF with TNF-R2-Fc was not able to inhibit SM-83-induced ovarian cancer cell death (Figure 23b). One explanation for this could be that the level of TNF-R2-Fc used in the experiments was insufficient to block the TNF within ascites. Therefore, additional experiments were performed in which the concentration of TNF-R2-Fc was increased up to a concentration 500 µg/ml. However, this also failed to completely block cell death induction by SM-83 in other patient samples.

Another possible explanation is that inhibition of XIAP and depletion cIAP1 and cIAP2 leads to the formation of a cell death signalling platform, referred to as the ‘ripoptosome’, without the involvement of death receptors or the mitochondrial apoptotic pathways (Tenev et al, 2011). The ripoptosome complex contains RIP1, FADD, and caspase-8 and can form after SMAC mimetic treatment and treatment with genotoxic stress inducing drugs such as the chemotherapy drug etoposide which depletes XIAP (Yang et al., 2000), cIAP1 and cIAP2 (Tenev et al, 2011) which suppress the formation of the ripoptosome complex (Darding and Meier, 2012).

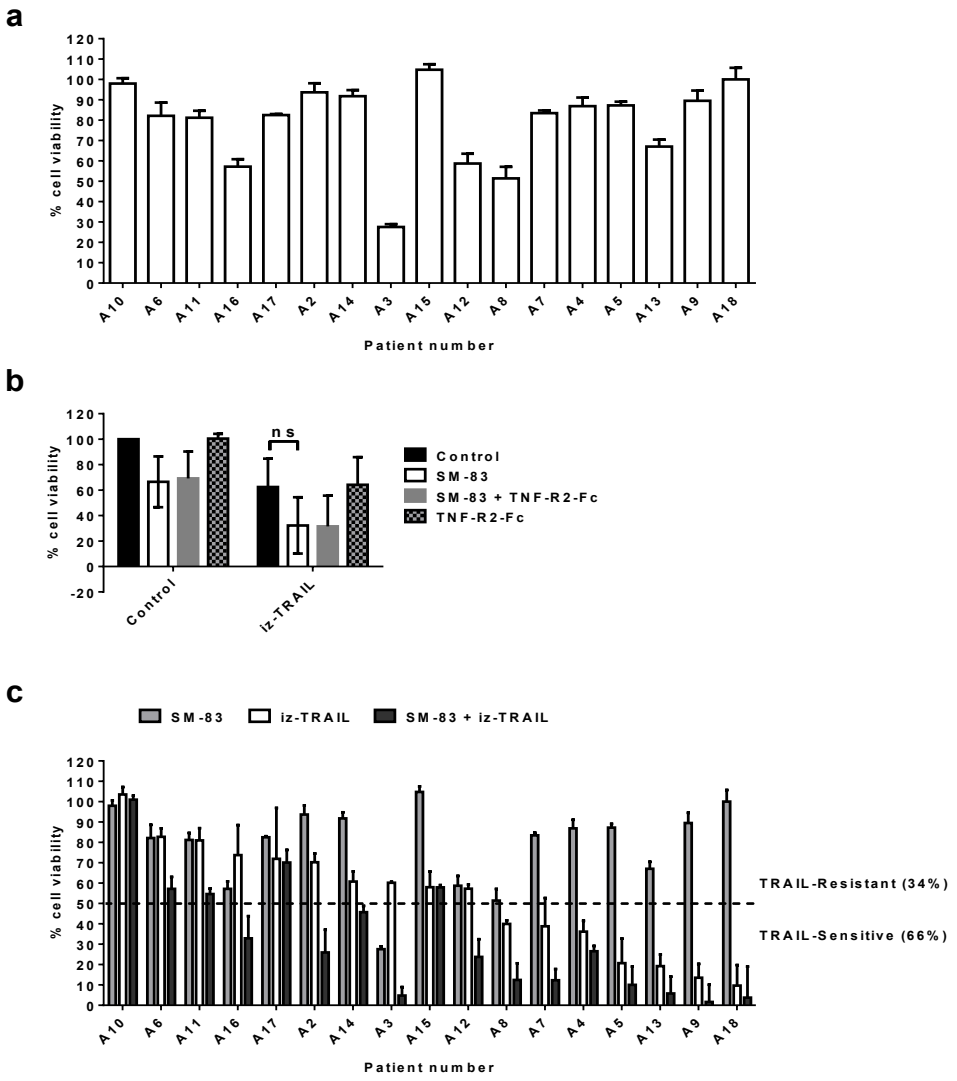

Figure 23: The sensitivity of primary ovarian cancer cells to SM-83 and iz-TRAIL. (a) $EpCAM^+$ tumour cells from 17 patients were treated with SM-83. Cell viability was measured after 48 hours. (b) $EpCAM^+$ tumour cells from 3 patients were treated with combination of SM-83, TNF-R-Fc [Enbrel, 10 µg/ml], and iz-TRAIL [100 ng/ml]. Cell viability was measured after 48 hours. Data shown are the mean of 3 independent experiments using cells from 3 different patients (± S.E.M). Data were compared using student's t-tests (ns= $p>0.05$). (c) Ovarian cancer cells were treated with SM-83 + iz-TRAIL [100 ng/ml] or both and cell viability was measured after 48 hours. Data are representative of the mean of 3 biological replicates (± S.D).

Significant synergy between iz-TRAIL and SM-83 was observed in the majority of the patient samples. Cell viability was decreased by SM-83 and iz-TRAIL treatment in 83% of the patient samples (14 out of 17 patients) that were tested (Figure 23). SM-83 treatment increased the overall sensitivity of the primary ovarian cancer cells to TRAIL-induced apoptosis from 38% to 66% of the patient samples. These data are similar to other reported pre-clinical studies in ovarian cancer (Petrucci et al., 2007, Petrucci et al., 2012) and in other tumour types (Allensworth et al., 2013, Fulda et al.,

2002a, Lecis et al., 2010). This suggests that SM-83 treatment synergises with TRAIL to induce the death of primary ovarian cancer cells.

It would have been interesting to compare the effects of etoposide versus SM-83 on the sensitivity of primary ovarian cancer cells to TRAIL-induced apoptosis. If the results from such studies had suggested that etoposide and SMAC mimetic compounds had similar effects on the TRAIL-sensitivity of primary ovarian cancer cells, then an etoposide and TRAIL therapeutic combination could be an option for clinical development. Etoposide is a cheap, established chemotherapy drug, whereas SMAC mimetic compounds are only in the early phases of drug development. It is currently unclear whether they will ever be licensed for use in patients with cancer.

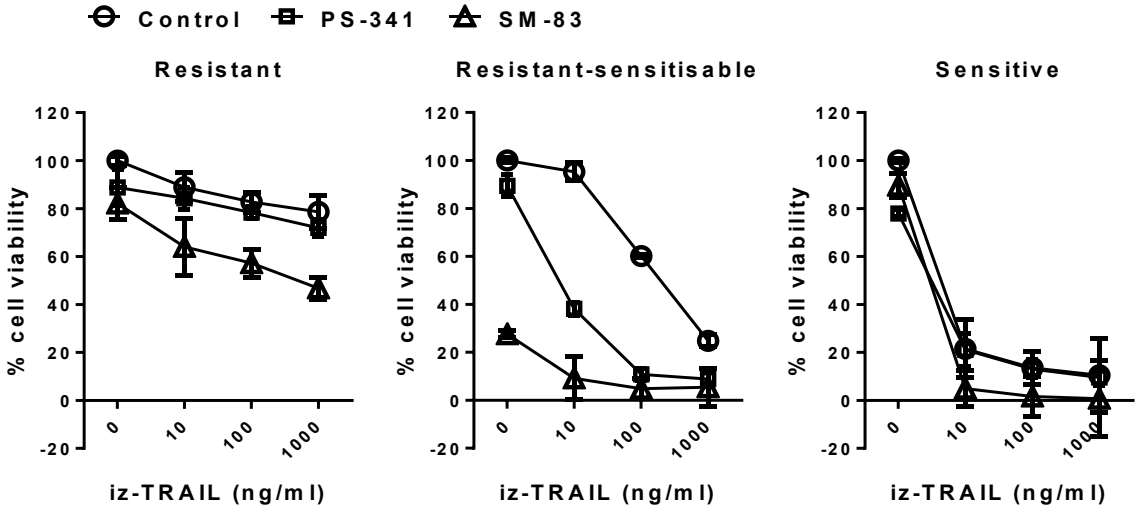

Figure 24: The sensitivity of primary ovarian cancer cells to PS-341, SM-83 and iz-TRAIL varies between patients. Ovarian cancer cells from 3 different patients were treated with iz-TRAIL with either PS-341 or SM-83. Cell viability was measured after 48 hours.

One the most striking findings from this part of the study was the heterogeneity of the responses of the tumour cells to combinations of TRAIL, SM-83, and PS-341. It may be possible to divide ovarian cancers, and probably other tumour types, into three subtypes of TRAIL-resistant, TRAIL-resistant-sensitisable, and TRAIL-sensitive ovarian cancer (Figure 24).

It is currently unclear as to how the different molecular subtypes of ovarian cancer (Tothill et al., 2008) correlate with TRAIL sensitivity and resistance. Although samples of tumour cells were saved for the determination of the molecular subtypes of each patient sample, at present there are no simple ways of reliably determining the

molecular subtype of ovarian cancer without performing complex gene expression profiling techniques such as microarrays. Therefore, this question could be prospectively addressed in a clinical trial in which the molecular subtype of ovarian cancer is established before and perhaps after TRAIL treatment.

4.1.8 SM-83 and PS-341 can sensitise immune cells to TRAIL-induced apoptosis

One of the most common and important side effects of chemotherapy is immunosuppression which can lead to the development of life-threatening infections. Although immunosuppression and infection have not been reported in trials of dulanermin/Apo2L/TRAIL (Herbst et al., 2010a); however, activated immune cells express TRAIL-R. For example, CD8 (Grimm et al., 2010) and CD4 (Barblu and Herbeuval, 2012) T cells, and macrophages express TRAIL-R (Strebel et al., 2002) and can undergo TRAIL-mediated apoptosis. The treatment of patients with the SMAC mimetic TL31211 leads to the sustained depletion of cIAP1 in peripheral blood mononuclear cells (PBMCs) (Graham et al., 2011). Therefore, SMAC mimetic treatments or PS-341 treatment may potentially sensitise immune cells to TRAIL-induced apoptosis and lead to immunosuppression in patients with cancer.

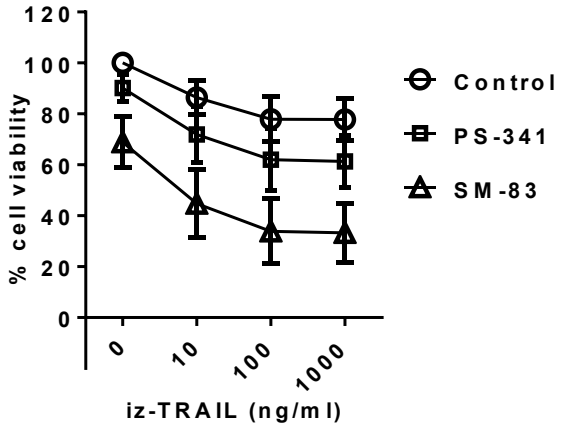

Figure 25: The effect of PS-341 and SM-83 on the sensitivity of CD45$^+$ cells to iz-TRAIL. CD45$^+$ cells from ovarian cancer ascites were treated with PS-341 or SM-83 and iz-TRAIL. Cell viability was measured after 48 hours. Data are representative of the mean of the results from experiments using CD45$^+$ cells from 6 individual patients (± S.E.M).

To investigate the potential effect of TRAIL and SM-83 or PS-341 on the tumour microenvironment, immune cells from ovarian cancer ascites were treated with combinations of iz-TRAIL with SM-83 or PS-341 (Figure 25). The data from these experiments suggest that PS-341 and SM-83 could potentially sensitise immune cells

to TRAIL-induced apoptosis. This could potentially lead to immunosuppression and changes within the tumour microenvironment in patients with ovarian cancer. However, it was not possible to use this assay to determine which immune cells are most sensitive to the combination of SM-83 or PS-341 with iz-TRAIL.

4.1.9 SM-83 or PS-341 with TRAIL induce apoptosis in primary ovarian cancer cells

Although apoptosis is thought to be the principle mechanism of cancer cell death in response to chemotherapy treatment, cells can undergo other forms of programmed cell death such as necroptosis which occurs in a caspase-independent manner. Apoptosis is characterised by caspase activation, DNA fragmentation and the onset of membrane blebbing. Apoptosis is generally not thought to be inflammatory and may even induce tolerance to tumours by generating so called helpless regulatory CD8 T cells (Griffith et al., 2007).

Apoptotic cells express or release multiple factors such as ATP, lysophosphatidylcholine, sphingosine-1-phosphate, which act as ‘find-me’ and ‘eat-me’ signals which promote the recruitment of anti-inflammatory cytokines-secreting phagocytic cells (Zitvogel et al., 2010, Cullen et al., 2013, Kearney et al., 2013). Interestingly, CD95L induced apoptosis is associated with the production of cytokines and chemokines such as IL-6, IL-8, CXCL1, MCP-1, and GMCSF by tumour cells which promotes the chemotaxis of phagocytes towards apoptotic tumour cells (Cullen et al., 2013). TNF treatment also leads to the production of pro-inflammatory cytokines and recruitment of phagocytes to the tumour microenvironment (Kearney et al., 2013). However, it not known whether TRAIL treatment induces cytokine production and recruitment of phagocytes to the tumour microenvironment.

Necroptosis, on the other hand, leads to the swelling of the organelles and the rupture of the plasma membrane which releases the contents of the cytoplasm, including alarmins, into the extracellular spaces which can lead to inflammation (Vandenabeele et al., 2010). When apoptotic cell death is blocked by caspase inhibitors such as viral proteins, cells can switch their mode of death from apoptosis to necroptosis (Dickens et al., 2012). Necroptosis is dependent on the kinase activity of Rip1 and Rip3; and necroptotic cell death is thought to be important in the host

defence against viruses (Kaczmarek et al., 2013), and is considered a potentially immunogenic form of cell death.

In the presence of caspase inhibitors, treatment with TRAIL and SMAC mimetics, could result in necroptosis (Christofferson and Yuan, 2010). To investigate this possibility, primary ovarian cancer cells were treated with SM-83 and iz-TRAIL in the presence of the pan-caspase inhibitor (zVAD-fmk) and/or the RIP1 kinase inhibitor necrostatin-1 (nec-1). Cells treated with PS-341 and iz-TRAIL were included as a control. It was found that the loss of cell viability after the treatment of ovarian cancer cells with SM-83 or PS-341 could be largely prevented by caspase inhibition. There was no significant increase in cell viability with the addition of necrostatin-1. These data suggest that SM-83 and PS-341 and TRAIL induce apoptosis in primary ovarian cancer cells (Figure 26).

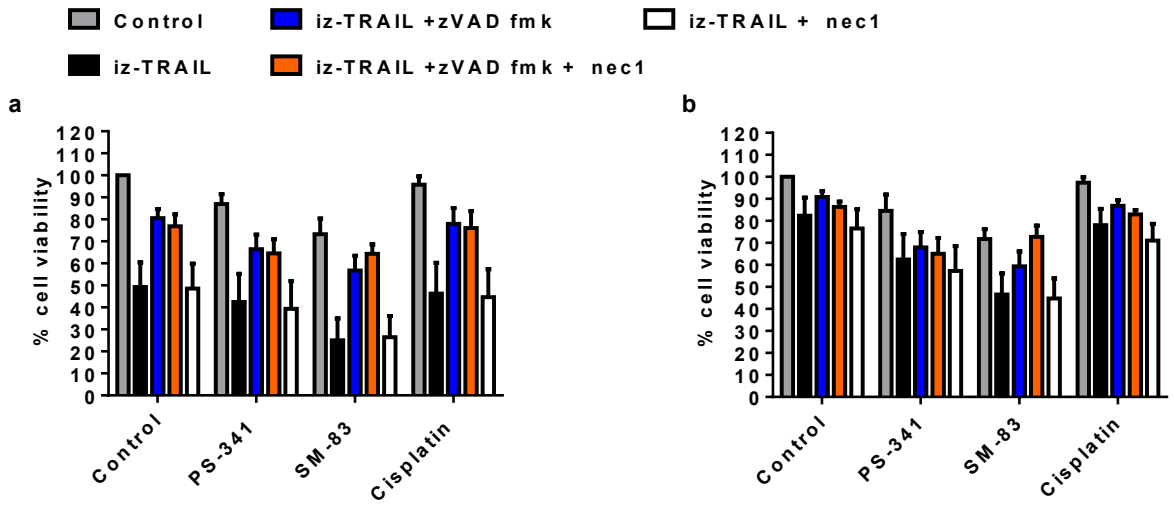

Figure 26: SM-83 and PS-341 and TRAIL induce apoptosis in primary ovarian cancer cells. a) Primary ovarian cancer cells were cultured with the pan-caspase inhibitor zVAD fmK [30 μM], or the RIP1 kinase inhibitor necrostatin (nec1) [10 μM] for 1 hour and then either SM-83 or PS-341 and iz-TRAIL [100 ng/ml] was added. Cell viability was measured after 48 hours**.** b) $CD45^+$ immune cells were treated as in a) and cell viability was measured 48 hours later. Data are representative of the mean (± S.D) of results from experiments using cells derived from 6 patients.

The loss of viability of the $CD45^+$ immune cells after treatment with iz-TRAIL was prevented by caspase inhibition (Figure 26b). PS-341 and SM-83 sensitised the $CD45^+$ cells to TRAIL-induced cell death which could be partially blocked by inhibition of caspases, and was almost completely prevented by the addition of necrostatin. These data suggest that some of the immune cells within ovarian cancer ascites underwent necroptotic cell death upon treatment with the SMAC mimetic and TRAIL.

Previous studies have shown that cIAP1 and cIAP2 limit macrophage necroptosis by inhibiting Rip1 and Rip3 activation (McComb et al., 2012). As macrophages play an important role in scavenging apoptotic cancer cells, it is possible that treatment with a SMAC mimetic and TRAIL could lead to the death of macrophages which had engulfed tumour cells. This could possibly lead to enhanced immune responses against the cancer cells which have been phagocytosed by the macrophages and the release of inflammatory mediators.

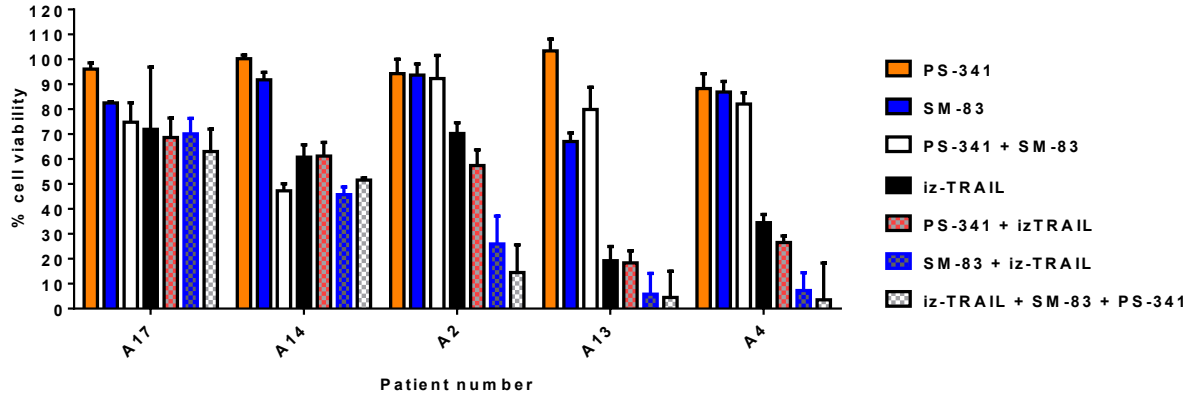

Figure 27: The effect of PS-341 and SM-83 on the sensitivity of primary ovarian cancer cells to iz-TRAIL. Primary ovarian cancer cells were treated with combination of PS-341, SM-83, and iz-TRAIL [100 ng/ml], cell viability was measured after 48 hours. The data are representative of the mean of 3 biological replicates (± S.D) of experiments performed with cells from 5 donors.

A previous study showed that SM-83 and PS-341 synergise with TRAIL to induce apoptosis in melanoma cells (Lecis et al., 2010). Ovarian cancer cells from 5 patients were treated with PS-341, SM-83, and TRAIL to investigate the potential for this combination treatment in ovarian cancer. There was a minor benefit for this combination in 1 of 5 (20%) of the patient samples (A2) (Figure 27). The lack of additional synergy between PS-341 and SM-83 in the study may have been due to the presence of ascites which might have antagonised the effect of the PS-341 on the ovarian cancer cells. The results from these experiments suggest that there is only a modest benefit in combining SM-83 and PS-341 with iz-TRAIL treatment.

4.1.10 The effect of chemotherapy on the sensitivity of primary ovarian cancer cells to iz-TRAIL

It has long been known that chemotherapy drugs can sensitise cancer cell lines to TRAIL-induced apoptosis (Cuello et al., 2001b). However, the effect of cisplatin on the sensitivity of platinum-resistant primary ovarian cancers to iz-TRAIL is unknown.

To address this question, primary ovarian cancer cells from 14 patients were treated with cisplatin and iz-TRAIL. The viability of the cells was determined 48 hours later (Figure 28).

Cisplatin synergised with TRAIL in 20% (3 of 15 patients) which included a patient with BRCA1 mutation (A7) (Figure 28). However, in several patient samples cisplatin treatment appeared to antagonise the effect of the TRAIL on the viability of the cancer cells (patients A4, A6, and A17). An interesting observation from these experiments was that the cisplatin treatment appeared to enhance, rather than decrease the viability of the cancer cells in around half of the patient samples. This effect was seen in patients with platinum-refractory ovarian cancer (patients A2, A15, A6, and A9), in one patient with platinum-sensitive ovarian cancer (A17), and in a patient with platinum-resistant ovarian cancer (A16).

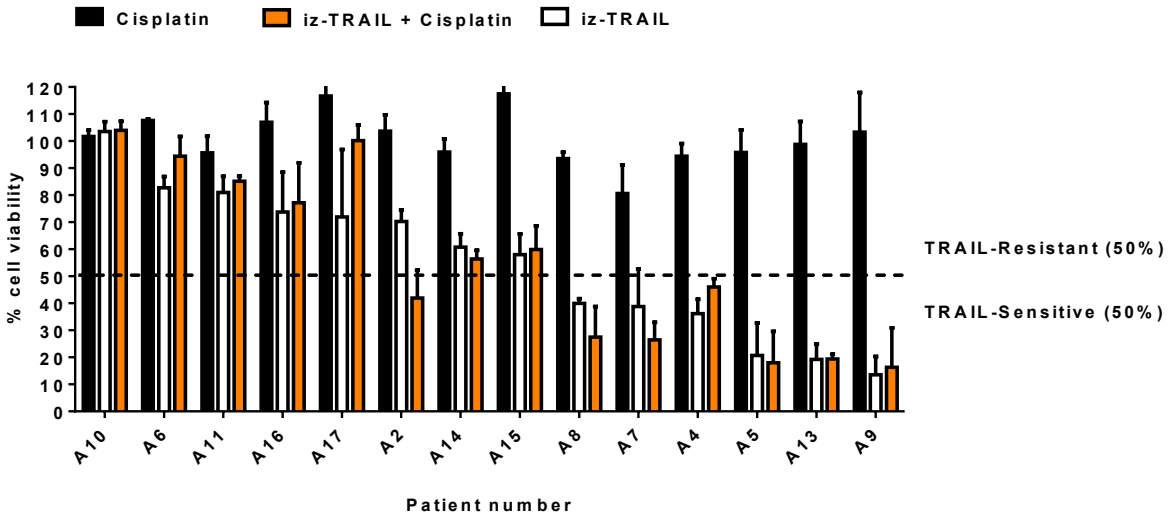

Figure 28: The effect of cisplatin on the sensitivity of ovarian cancer cells to iz-TRAIL. EpCAM$^+$ tumour cells from 14 patients were cultured with cisplatin [25 μM] and iz-TRAIL [100 ng/ml]. Cell viability was measured after 48 hours. Data are representative of the mean of 3 biological replicates (± S.D) of experiments performed using cells from 14 independent donors.

One explanation for this effect is that cisplatin treatment has been shown to lead to enhanced expression of TRAIL-R2 or CD95 by ovarian cancer cells (Duiker et al., 2009). Although this might increase the sensitivity of the cancer cells to TRAIL induced apoptosis, it could also sensitise the ovarian cancer cells to endogenous TRAIL or CD95L; both of which are normally produced by these cells. Endogenous TRAIL and CD95L have been shown to promote the proliferation of cancer cells (Levina et al., 2008, Belyanskaya et al., 2008, Chen et al., 2010). Therefore cisplatin

treatment could have enhanced this non-apoptotic effect of TRAIL on the ovarian cancer cells.

Previous studies have shown that cisplatin leads to activation of JNK/p38 and MAPK pathways which lead apoptosis in ovarian cancer cells (Mansouri et al., 2003, Coltella et al., 2006). However, cisplatin-resistant cells do not activate MAPK after cisplatin treatment (Brozovic et al., 2004, Mansouri et al., 2003), which leads to a failure to induce apoptosis. In cisplatin-sensitive ovarian cancer cells, cisplatin treatment leads to an up-regulation of CD95L expression, which can induces apoptosis in the cancer cells. However, cisplatin-resistant cells do not up-regulate CD95L expression, and do not undergo apoptosis (Spierings et al., 2003, Mansouri et al., 2003). Therefore in cisplatin-resistant cells, cisplatin treatment may inhibit signalling though the CD95/CD95L system (Galluzzi et al., 2012a), and also possibly TRAIL-induced apoptosis.

Paclitaxel is a chemotherapeutic drug that is commonly combined with cisplatin for the treatment of ovarian cancer. Paclitaxel chemotherapy had a modest effect on the sensitivity of the cancer cells to iz-TRAIL with 2 of 7 patient's cancer cells showing a decrease in the viability with combined treatment (Figure 29). The treatment of ovarian cancer cells with both cisplatin and paclitaxel only had a modest effect on the viability of ovarian cancer cells to iz-TRAIL. The addition of paclitaxel appeared to mitigate the effect of cisplatin on enhancing the viability of the cancer cells in some of the patient samples that were tested (Figure 29).

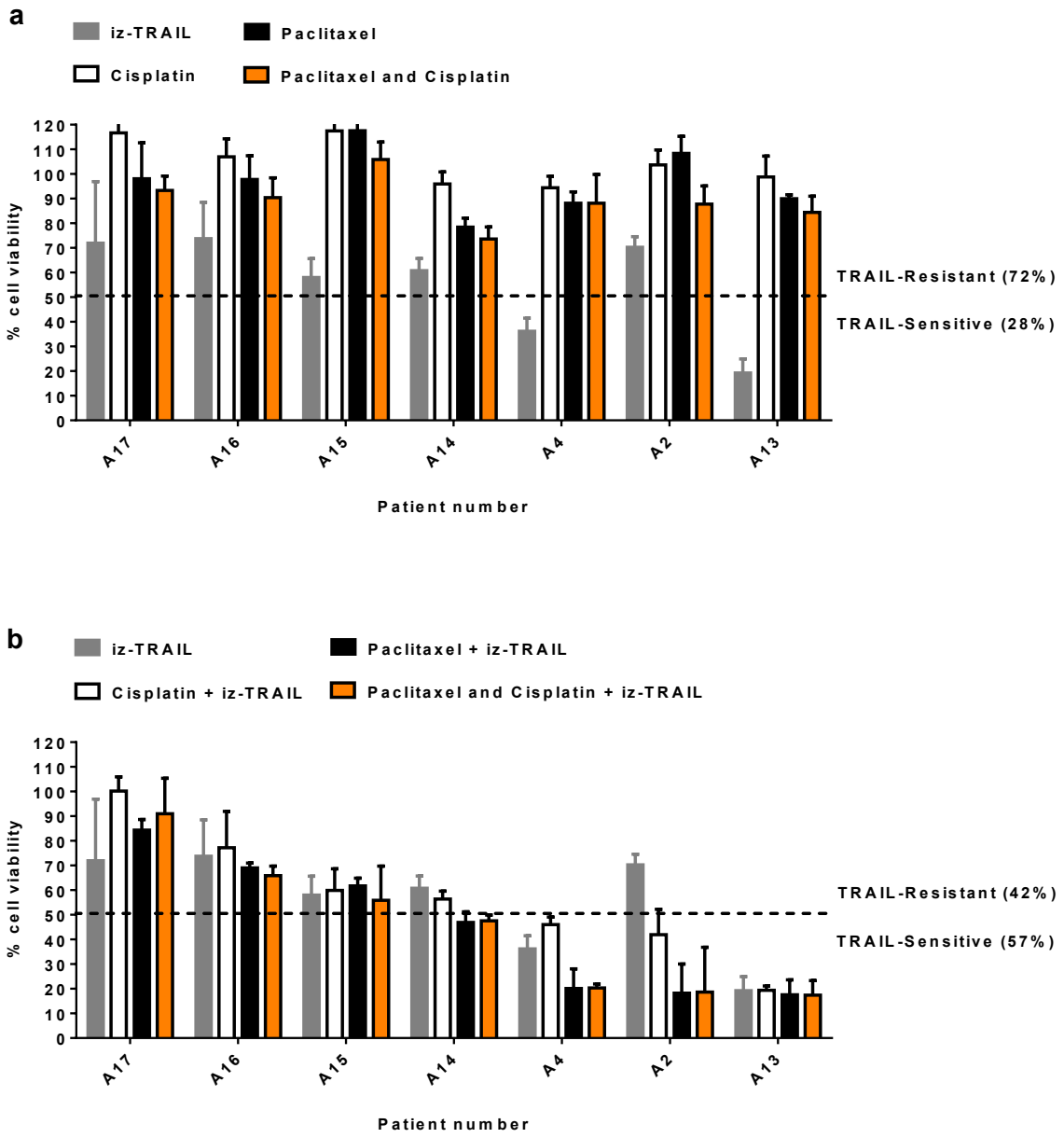

Figure 29: The effect of cisplatin and paclitaxel on the sensitivity of primary ovarian cancer cells to iz-TRAIL. a) EpCAM$^+$ cells were treated with iz-TRAIL [100 ng/ml], cisplatin [25 μM], paclitaxel [20 nM], or both cisplatin [25 μM] and paclitaxel [20 nM]. b) EpCAM$^+$ cells were treated with cisplatin [25 μM], paclitaxel [20 nM] or both for 1 hour, and then iz-TRAIL [100ng] was added. Cell viability was measured after 48 hours. Data are representative of the mean of 3 biological replicates (± S.D) using cells from 5 donors.

These data suggest that cisplatin chemotherapy may have a role as a TRAIL-sensitising drug in patients with platinum-sensitive ovarian cancer (Figure 28). However, the addition of cisplatin to paclitaxel chemotherapy did not appear to provide any additional benefits over paclitaxel treatment alone (Figure 29). However, the number of patients within this cohort was small and therefore it is difficult to draw definitive conclusions from these results. These data suggest that cisplatin and

paclitaxel should probably only be used as TRAIL-sensitising agents in patients with chemotherapy-sensitive ovarian cancer.

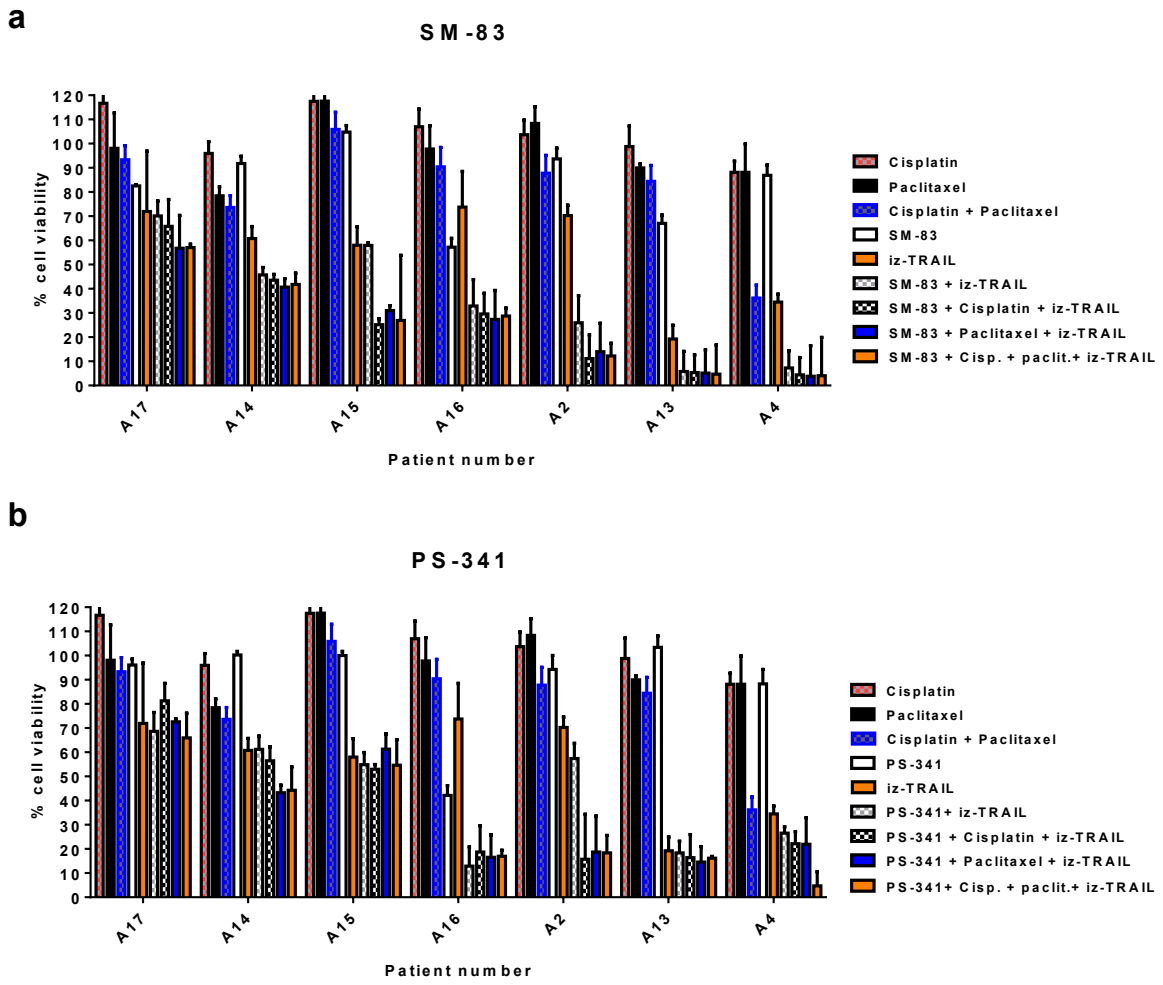

Figure 30: The effect of combining cisplatin and paclitaxel chemotherapy with either PS-341 or SM-83 and iz-TRAIL. (a) Ovarian cancer cells were treated with combination of SM-83, cisplatin, and paclitaxel and iz-TRAIL [100 ng/ml]. (b) Ovarian cancer cells were treated with combination of PS-341, cisplatin, and paclitaxel and iz-TRAIL. Cell viability was measured after 48 hours. Data are representative of the mean of 3 biological replicates (± S.D) of experiments performed using cells from 7 donors.

The traditional practice in oncology is to combine all active agents for any disease together, with the aim of hitting multiple synergistic targets. The addition of chemotherapy only had minor effects on the sensitivity of the ovarian cancer cells to TRAIL (Figure 29). However, if either the combination of TRAIL with PS-341 or with SM-83 was shown to be active in a clinical trial in patients with ovarian cancer; then such combinations would inevitably be combined with chemotherapy in the cancer clinic. Therefore it was investigated whether the addition of cisplatin and paclitaxel

chemotherapy to TRAIL treatment with either PS-341 or SM-83 would potently kill cancer cells (Figure 30).

As expected, there was synergy between cisplatin and paclitaxel treatment on ovarian cancer cell viability. However, TRAIL treatment alone had a greater effect on cancer cell viability than cisplatin and paclitaxel combined in all of the patient samples. There was an additive effect of TRAIL, SM-83, and cisplatin and paclitaxel treatment in some of the patient samples (A17, A15, and A2). There was synergy between PS-341, paclitaxel, and TRAIL in one patient sample (A14); and synergy between PS-341, cisplatin and paclitaxel, and TRAIL in another patient sample (A4) (Figure 30).

Although the number of samples was small and these were *in vitro* studies, the results suggest that these therapeutic combinations may be beneficial. However, the scheduling of a large number of potentially toxic drugs for clinical trials would be complex. In addition, before conducting such clinical studies, it would need to be shown that the combination of TRAIL with either PS-341 or a SMAC mimetic compound was safe and effective in patients with cancer.

4.1.11 The effect of immune subsets within the tumour microenvironment on the sensitivity of ovarian cancer cells to TRAIL

Immune cells within the ovarian cancer tumour microenvironment secrete factors that could potentially influence the sensitivity of ovarian cancer cells to TRAIL. Although the presence or absence of immune cells within ovarian cancer ascites does not reflect the whole ovarian cancer tumour microenvironment, it may give some indication as to which immune cells may have a role in preventing or enhancing TRAIL-induced cell death.

A previous study has shown that TNF cooperates with IFN-γ to repress Bcl-xL expression, leading to the sensitisation of metastatic colon carcinoma cells to TRAIL-mediated apoptosis (Liu et al., 2011). Inflammatory macrophages have been shown to induce Nrf2 transcription factor-dependent proteasome activity in colonic NCM460 cells, which leads to resistance to TRAIL-induced apoptosis (Sebens et al., 2011).

Tumour-associated macrophages have also been shown to protect colon cancer cells from TRAIL-induced apoptosis through an IL-1β-dependent stabilization of Snail in tumour cells (Kaler et al., 2010). Interestingly, vitamin D has been shown to

overcome this effect by interfering with the release of IL-1β from macrophages; thereby restoring the sensitivity of the cancer cells to TRAIL (Kaler et al., 2010).

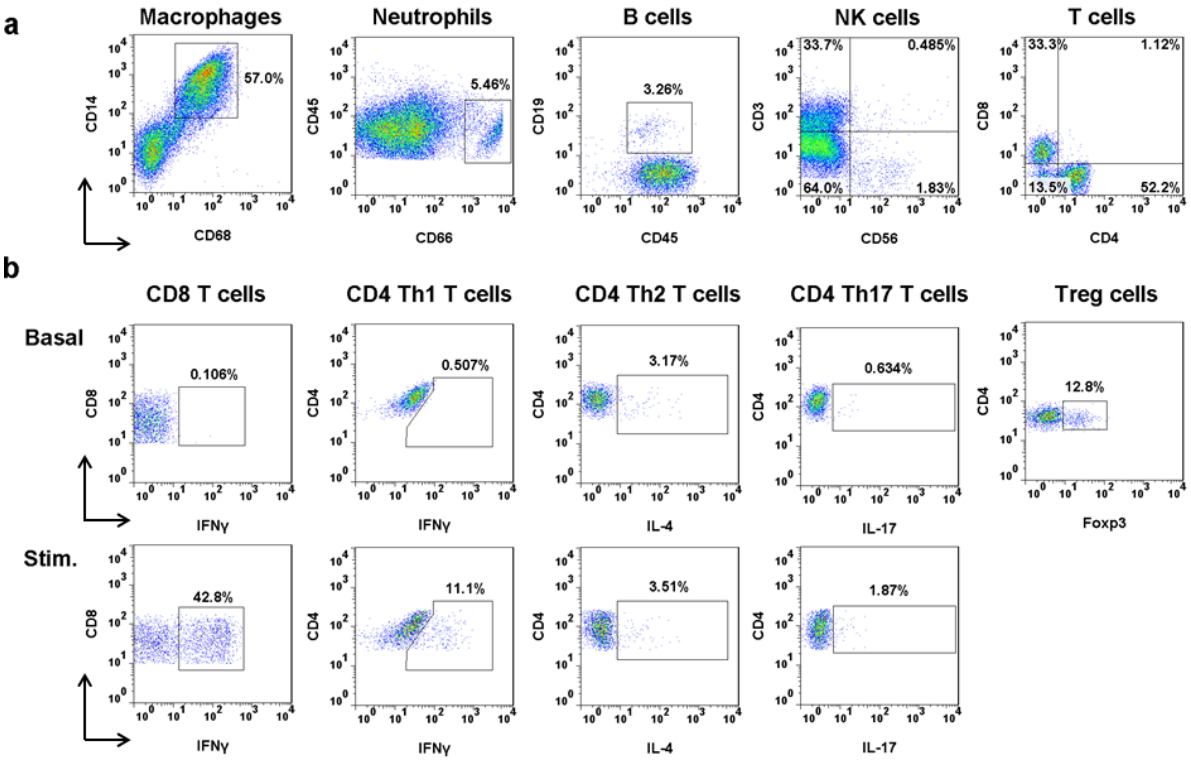

Figure 31: The identification of immune cell subsets within ovarian cancer ascites. Immune cells were isolated from ascites from a representative patient with ovarian cancer. (a) Macrophages were identified by staining for $CD45^+$,$CD14^+$,$CD68^+$, neutrophils by staining for $CD45^+$,$CD66^+$, B cells by staining for $CD45^+$,$CD3^-$,$CD20^+$, NK cells by staining for $CD45^+$,$CD3^-$,$CD56^+$,$CD16^\pm$, and T cells ($CD45^+$,$CD3^+$). (b) T cell subsets were identified in total ascites cells after treatment with golgi block (brefeldin) and with (stim.) or without (basal) PMA and ionomycin for 4 hours to assess intracellular cytokine production. Cytotoxic CD8 T cells were identified by staining for $IFN\gamma^+$,$CD3^+$,$CD8^+$, CD4 Th1 T cells were identified by staining for $CD3^+$,$CD4^+$,$IFN\gamma^+$, CD4 Th2 T cells by staining for $CD3^+$,$CD4^+$,$IL\text{-}4^+$, CD4 Th17 T cells by staining for $CD3^+$,$CD4^+$,$IL\text{-}17^+$, and Treg cells by staining for $CD3^+$,$CD4^+$,$Foxp3^+$.

Several immune cell subsets are potential candidates for mediating resistance to chemotherapy and resistance to apoptosis-inducing therapies such as TRAIL. Macrophages have been implicated in tumour cell migration, invasion, and metastasis (Condeelis and Pollard, 2006); and are associated with adverse outcomes in patients with Hodgkin lymphoma (Tan et al., 2012, Greaves et al., 2013). Macrophages are recruited by the TNF network into the ovarian cancer tumour microenvironment (Kulbe et al., 2007, Kulbe et al., 2012).

FACS analysis of ovarian cancer ascites was performed to investigate whether differences in immune cell subsets within ovarian cancer ascites correlated with TRAIL sensitivity (Figure 31). There were no overall differences in the relative proportions of macrophages or neutrophils in the samples of ascites between the

patients with TRAIL-resistant or -sensitive ovarian cancer cells (Figure 32). Many of the macrophages within ovarian cancer ascites expressed CD206; a marker of alternatively activated, M2, or tumour associated macrophages. However, it is unclear as to whether these macrophages were true tumour-associated macrophages or peritoneal macrophages that were present within the ascites. The proportion of neutrophils in the ovarian cancer ascites did not vary between patients with TRAIL-resistant or –sensitive ovarian cancer (Figure 32).

There were no significant differences seen in the overall percentage of CD8, CD4 or NK cells between the patients with TRAIL-sensitive and TRAIL-resistant ovarian cancer (Figure 32). The presence or absence of Treg cells did not correlate with TRAIL-sensitivity or -resistance (Figure 32). There was a significant difference ($p= 0.01$) in the relative proportions of IL-4^+ Th2 CD4 T cells between the ascites containing TRAIL-sensitive and -resistant ovarian cancer cells and there was also trend towards greater numbers of Th1 CD4 helper T cells in samples from patients with TRAIL-sensitive tumours (Figure 32). This could suggest that IL-4^+ Th2 CD4 T cells are a potential biomarker for TRAIL sensitivity, or that these cells could enhance the sensitivity of ovarian cancer cells to TRAIL-induced apoptosis.

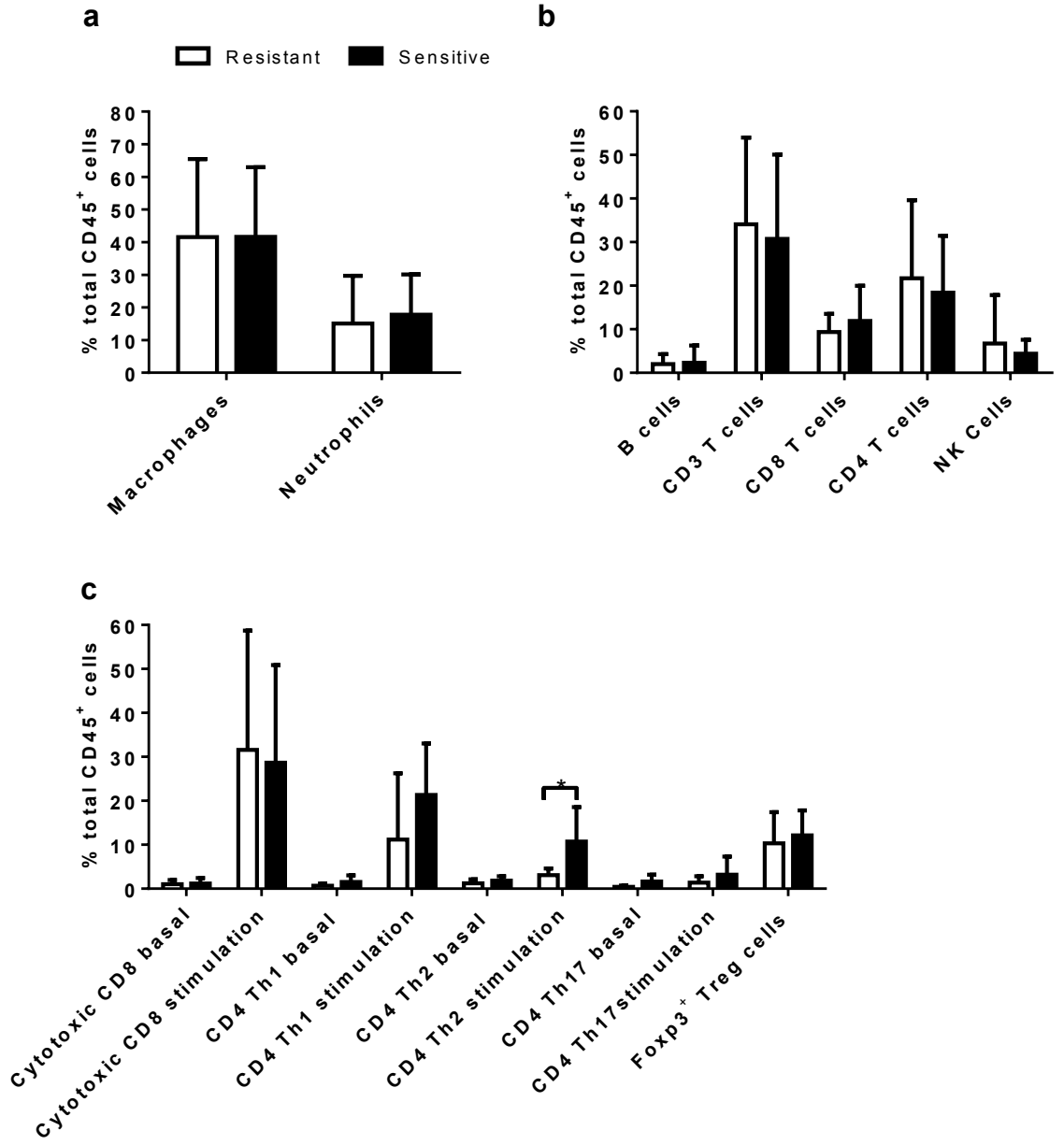

Figure 32: Differences in immune cell subsets within ovarian cancer ascites in patients with TRAIL-sensitive and TRAIL-resistant ovarian cancer. (a) The relative proportions of $CD45^+$ cells within ovarian cancer ascites of macrophages ($CD45^+$,$CD14^+$,$CD68^+$) and neutrophils ($CD45^+$,$CD66^+$). (b) The relative proportions of total $CD45^+$ cells of B cells ($CD45^+$,$CD3^-$,$CD20^+$), T cells ($CD45^+$,$CD3^+$), CD8 T cells ($CD45^+$,$CD3^+$,$CD8^+$), CD4 T cells ($CD45^+$,$CD3^+$,$CD4^+$), and NK cells ($CD45^+$,$CD3^-$,$CD56^+$,$CD16^\pm$) in patients with TRAIL-sensitive and TRAIL-resistant ovarian cancer. (c) The relative proportions of T cell subsets in patients with TRAIL-resistant and -sensitive ovarian cancer. Cytotoxic CD8 T cells ($CD3^+$,$IFN\gamma^+$,$CD8^+$), CD4 Th1 T cells ($CD3^+$,$CD4^+$,$IFN\gamma^+$), CD4 Th2 T cells ($CD3^+$,$CD4^+$,IL-4^+), CD4 Th17 T cells ($CD3^+$,$CD4^+$,IL-17^+), and Treg cells ($CD3^+$,$CD4^+$,$Foxp3^+$) in patients with TRAIL-sensitive ovarian cancer. Total ascites cells were treated with golgi block (brefeldin) and with (stimulated) or without (basal) PMA and ionomycin for 4 hours to assess intracellular cytokine production. Data were analysed using GraphPad and compared using unpaired student's-t test. * p = 0.01.

The results were surprising as other studies have shown that apoptosis resistance in colon, breast and lung carcinomas is mediated by tumour derived IL-4 which

enhances the expression of anti-apoptotic proteins such as cFLIP, Bcl-xL and Bcl-2 (Todaro et al., 2008). Interestingly mast cells can be induced to undergo apoptosis when they have been exposed to culture supernatants from IL-4-stimulated peritoneal cells in a TRAIL, CD95L, and TNF independent manner (Hu et al., 2006). Given that ovarian cancer ascites is a supernatant from peritoneal cells, it is possible that ovarian cancer ascites contains factors which enhance the sensitivity of the ovarian cancer cells to TRAIL.

It would be possible to test this hypothesis by conducting experiments on the TRAIL sensitivity of ovarian cancer cell lines, using ovarian cancer ascites from patients with TRAIL-resistant and TRAIL-sensitive ovarian cancer and with high or low levels of IL-4^+ Th2 CD4 T cells within their ascites. It would also be interesting to determine if IL-4 directly affects the TRAIL-sensitivity of ovarian cancer cells by reducing the expression of anti-apoptotic proteins such as cFLIP, Bcl-xL and Bcl-2.

Chapter 5. **TRAIL-R2-specific antibodies and recombinant TRAIL can synergise to kill cancer cells**

5.1.1 The ovarian cancer tumour microenvironment contains FcγR-expressing immune cells with the potential to crosslink TRAIL-R2-specific antibodies.

TRAIL-R2-specific antibodies, such as AMG 655, bind TRAIL-R2 but cannot alone induce TRAIL-R2 trimerisation and formation of the DISC. The *in vitro* activity of AMG 655 requires the use of recombinant protein A/G which contains four Fc-binding domains; two from protein A and two from protein G. The binding of recombinant protein A/G to AMG 655 results in multimerisation of TRAIL-R2 and induction of cell death (Figure 33).

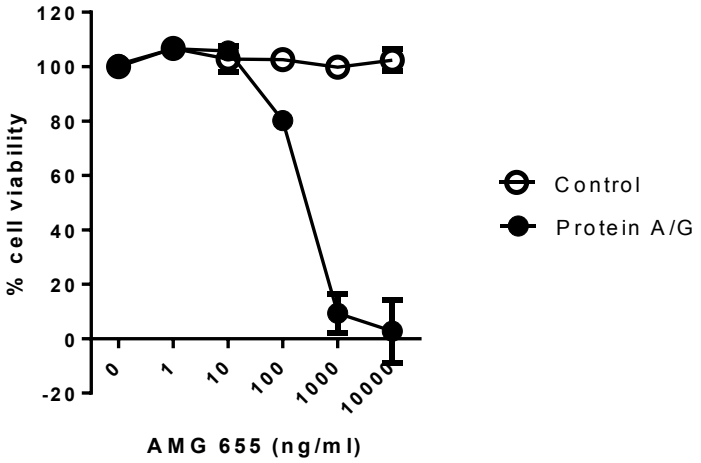

Figure 33: The induction of apoptosis by TRAIL-R2-specific antibodies is dependent on crosslinking. PEO4 cells were treated with increasing concentration of AMG 655 with or without protein A/G. Cell viability was measured after 24 hours. Data are representative of the mean of 3 biological replicates (± S.D) and representative of 3 independent experiments.

The *in vivo* activity of AMG 655 requires crosslinking by FcγR on immune cells to enable TRAIL receptor multimerisation and apoptosis induction (Wilson et al., 2011, Haynes et al., 2010b). FcγR are present on immune cells such as macrophages, neutrophils and NK cells (Hogarth and Pietersz, 2012). FcγRs bind CD95- or TRAIL-R2-bound antibodies on cancer cells, leading to CD95 or TRAIL-R2 multimerisation,

formation of the DISC and induction of apoptosis (Wilson et al., 2011, Dhein et al., 1992).

To investigate whether the ovarian cancer tumour microenvironment contains immune cells that express FcγR, fresh samples of solid deposits of ovarian cancer and ascites were obtained from 14 patients who were undergoing primary debulking surgery or therapeutic ascitic drainage. FACS analysis was performed on the samples to determine the relative proportions of tumour, immune cells, and FcγR expression within the ovarian cancer tumour microenvironment (Figure 34).

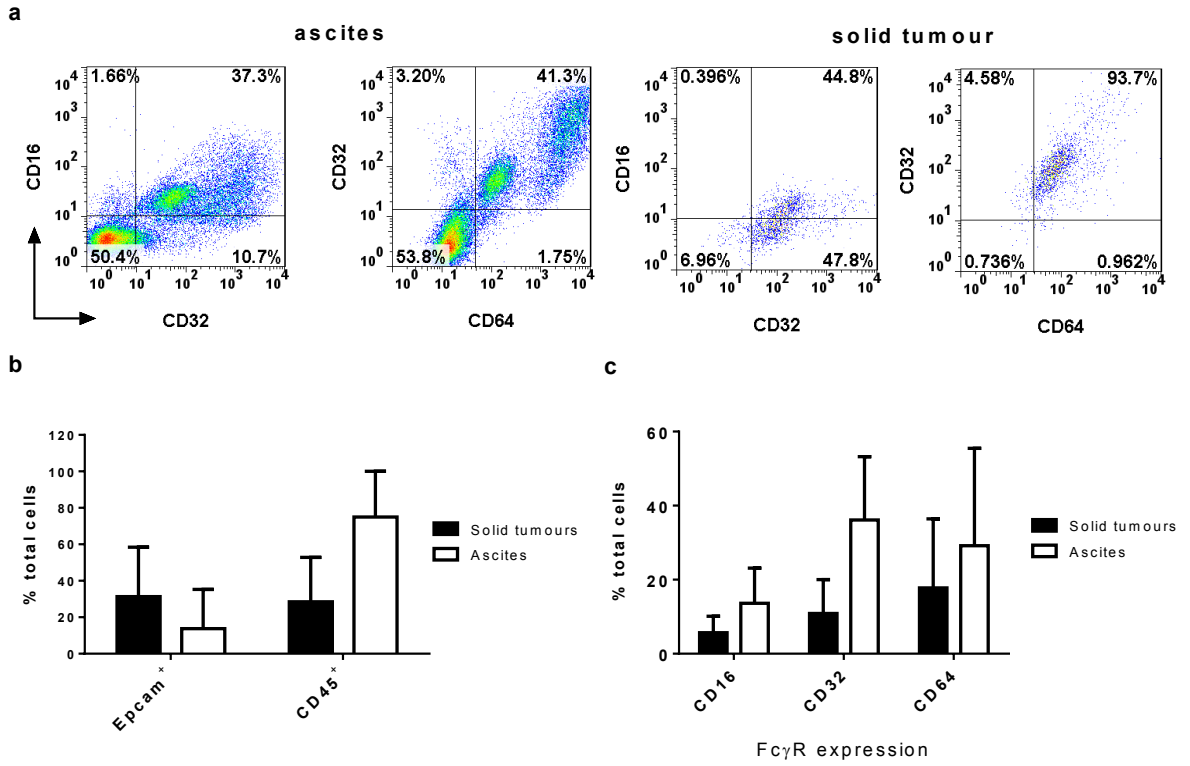

Figure 34: Immune cells and FcγR expression within the ovarian cancer tumour microenvironment. (a) FACS analysis of FcγR expression on immune cells within solid deposits of ovarian cancer and ovarian cancer ascites. Fresh solid deposits of ovarian cancer were digested and then stained with CD45, CD16 (FcγRIIIA), CD32 (FcγRIIA), and CD64 (FcγRIA). Data represent one of 7 samples of solid deposits of ovarian cancer and one of 7 samples of ascites. (b) The percentage of $EpCAM^+$ tumour cells and $CD45^+$ immune cells within the ovarian cancer tumour microenvironment. Data are representative of the mean of 7 samples (± SD). (c) The expression of CD16, CD32, and CD64 within ascites and solid deposits of ovarian cancer. Data are representative of the mean of 7 samples (± SD).

The analysis of solid tumours and the ascites revealed that solid deposits of ovarian cancer contained more tumour cells than ascites. The overall expression of FcγR on

cells within the ovarian cancer tumour microenvironment was higher in ascites. This reflected the higher proportion of immune cells within ovarian cancer ascites. These data show that the ovarian cancer tumour microenvironment contains immune cells that have the potential to induce FcγR-mediated TRAIL-R2 apoptosis (Figure 34).

5.1.2 Ascites-derived immune cells are inefficient enablers of FcγR-dependent TRAIL-R2-mediated apoptosis

Next, it was investigated whether ascites-derived immune cells could crosslink AMG 655 and induce apoptosis in ovarian cancer cells. $CD45^+$ immune cells were isolated from ascites from 11 patients with ovarian cancer. Subsequently these immune cells were profiled, and the expression of FcRγ receptors on their surface was determined by FACS. The majority of the immune cells within ovarian cancer ascites expressed FcγRIIIA (CD16), FcγRIIA (CD32), and FcγRIA (CD64) (Figure 35). The largest population of immune cells within ovarian cancer ascites which expressed FcγRs were macrophages and neutrophils (Figure 35).

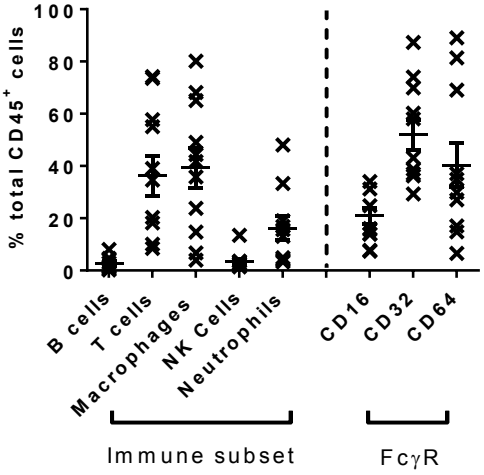

Figure 35: The immune cell profile and FcγR expression within ovarian cancer ascites. FACS analysis of FcγRIIIA (CD16), FcγRIIA (CD32) or FcγRIA (CD64) on the surface of ascites derived immune cells obtained from 11 patients with advanced ovarian cancer. Data are representative of 11 independent experiments.

The highly TRAIL-sensitive PEO4 ovarian cancer cell line was used to determine the efficiency of FcγR-mediated TRAIL-R2-antibody crosslinking by ascites-derived immune cells. Increasing ratios of $CD45^+$ immune cells were incubated with PEO4 cells and then treated with AMG 655. Titration of increasing ratios of $CD45^+$ immune

cells demonstrated that a ratio of 10 $CD45^+$ immune cells to 1 PEO4 cell was sufficient to enable TRAIL-R2-mediated apoptosis induction to occur in PEO4 cells (Figure 36). However, the efficiency of FcγR-dependent TRAIL-R2-mediated apoptosis was significantly lower than that induced by either Apo2L/TRAIL (a recombinant form of TRAIL that is similar to dulanermin), iz-TRAIL, or cells treated with AMG 655 with recombinant protein A/G.

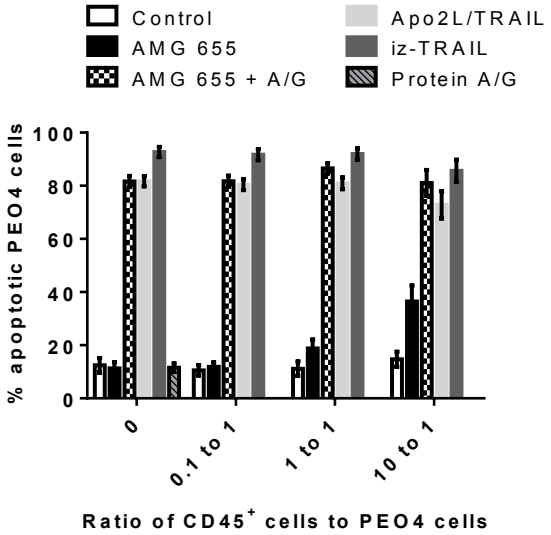

Figure 36: **Immune cells within the ovarian cancer tumour microenvironment can crosslink AMG 655 and induce TRAIL-R2-mediated apoptosis in ovarian cancer cells.** Increasing ratios of $CD45^+$ immune cells were cultured with PEO4 cancer cells and then treated with AMG 655, AMG 655 + protein (A/G), or Apo2L/TRAIL or iz-TRAIL. PEO4 cell death was measured after 24 hours by the expression of cleaved CK18. Data shown are mean (± S.D.) of 11 independent experiments using $CD45^+$ cells from 11 patients.

The results from individual patients were divided into three groups (within the 10 $CD45^+$ cells to 1 PEO4 cells group) of low (<10%), medium (>10% and <25%), and high (>25%) inducers of PEO4 cell apoptosis (Figure 37). This allowed the determination of the relative contribution of the different FcγR and immune cell subsets towards the crosslinking of AMG 655.

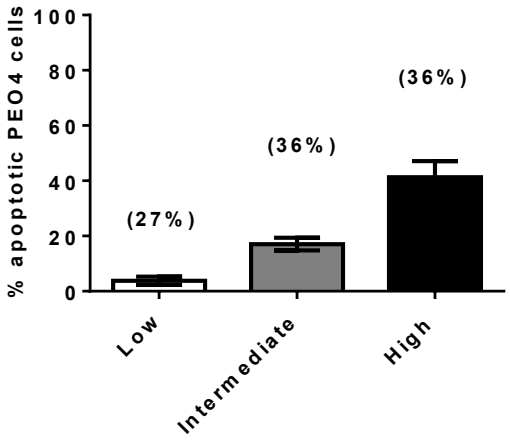

Figure 37: The efficiency of FcγR-dependent TRAIL-R2-mediated apoptosis varies between patients. The results from individual patients within the (10 to 1 $CD45^+$ cell to 1 PEO4 group) were divided into groups of low (<10%), medium (>10% and <25%), and high (>25%) inducers of AMG 655-mediated apoptosis. The percentages above each bar on the graph indicate the percentage of the total number of patients within each group.

Higher expression of FcγRIIIA (CD16) and FcγRIA (CD64) expression and macrophages within ascites were associated with enhanced TRAIL-R2-antibody-mediated apoptosis. There was a trend towards higher levels of T cells being associated with lower PEO4 cell death, but this finding was not statistically significant (Figure 38).

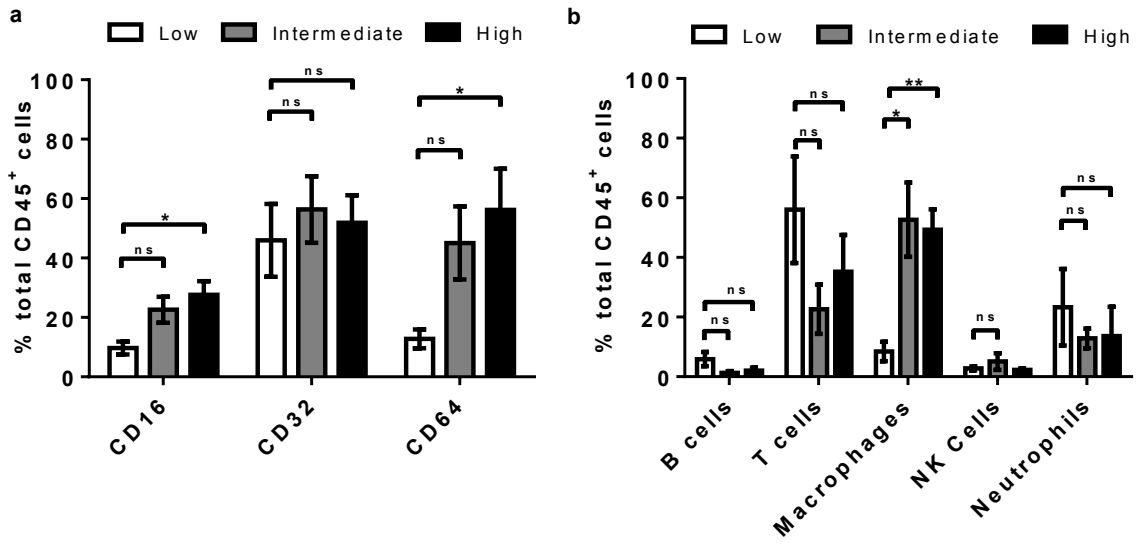

Figure 38: FcγRIIIA (CD16) and FcγRIA (CD64) expression and macrophages within ovarian cancer ascites are associated with enhanced FcγR-TRAIL-R2-antibody-mediated apoptosis. a) $CD45^+$ FcγR expression was compared between low, intermediate and high inducers of FcγR-dependent TRAIL-R2-mediated apoptosis using student's t-tests. b) The relative proportions of immune cells within ovarian cancer ascites were compared between the different groups of patients using student's t-tests. (*p=<0.05, **p=<0.01).

Next, it was investigated whether human FcγR-expressing immune cells were able to enhance anti-TRAIL-R2-induced apoptosis in primary ovarian cancer cells to any significant level (Figure 39). Immune cells were isolated from the ascites from a patient which could crosslink AMG 655 and kill PEO4 cancer cells (Figure 39b). The immune cells were incubated with primary ovarian cancer cells from the same patient in a ratio of 10 immune cells to 1 cancer cell; and then treated with AMG 655.

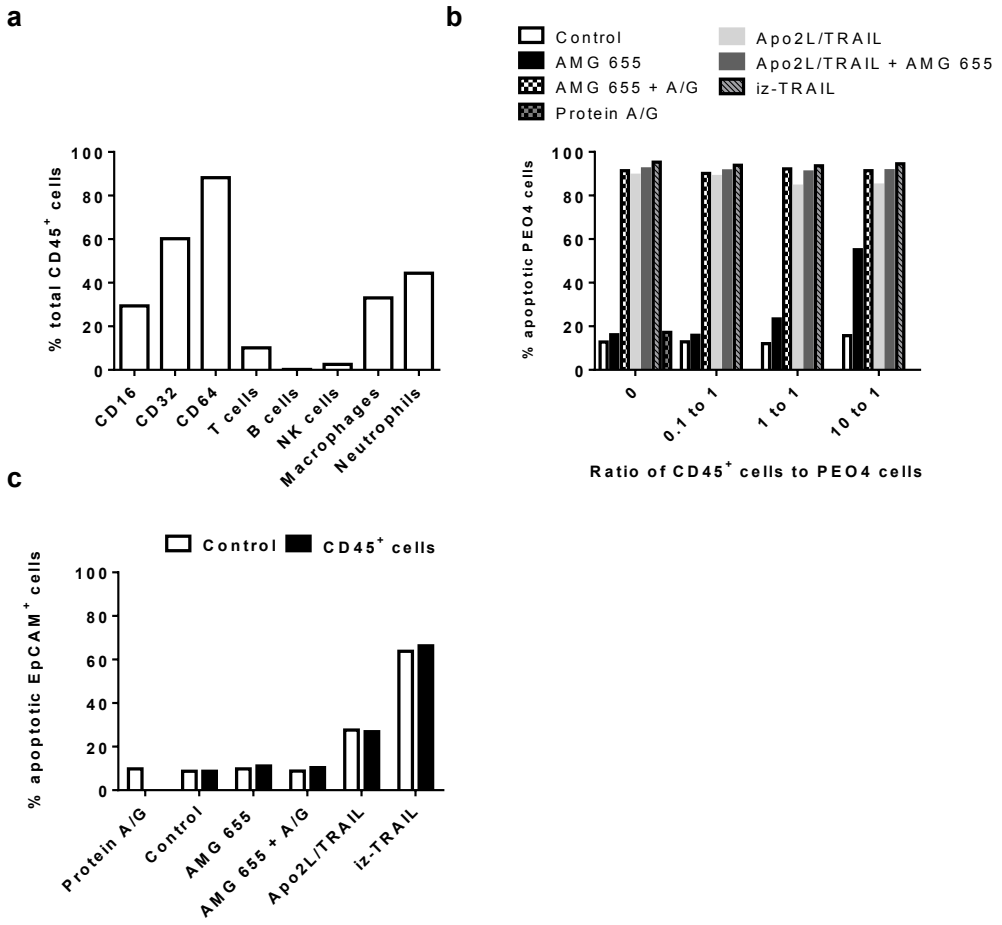

Figure 39: Human immune cells are inefficient enablers of FcγR-dependent TRAIL-R2-mediated apoptosis in primary ovarian cancer cells. a) The immune cell profile of $CD45^+$ cells from ascites from a patient with ovarian cancer. b) $CD45^+$ immune cells from the same patient were co-cultured with PEO4 cells and treated with AMG 655 [500 ng/ml], AMG 655 and recombinant protein A/G, or Apo2L/TRAIL [500 ng/ml] or iz-TRAIL [500 ng/ml]. (c) $EpCAM^+$ ovarian cancer cells from the same patient were cultured with 10 $CD45^+$ immune cells to 1 cancer cell and treated with control, AMG 655, AMG 655 and protein A/G, Apo2L/TRAIL, or iz-TRAIL. Cell death was measured after 24 hours by the expression of cleaved CK18. Data are representative of two independent experiments performed using cells from the same donor and other experiments performed with cells from other donors.

AMG 655 did not induce apoptosis in the primary ovarian cancer cells, even in the presence of immune cells or recombinant protein A/G (Figure 39c). However,

Apo2L/TRAIL and iz-TRAIL were capable of inducing apoptosis in the primary ovarian cancer cells, suggesting that recombinant forms of TRAIL are more efficient at trimerising TRAIL-R1 or TRAIL-R2 and inducing apoptosis than the TRAIL-R2-specific antibody AMG 655, even when crosslinked with protein A/G (Figure 39).

The lack of any significant apoptosis in primary ovarian cancers after treatment with AMG 655 and immune cells could have been due to insufficient *in vitro* concentrations of AMG 655, or other unknown factors within the tumour microenvironment. However, much higher levels (>60 µg/L) of AMG 655 are routinely reached in patients with cancer and are not associated with tumour responses in patients treated with AMG 655 (Herbst et al., 2010b).

Although these are *in vitro* studies, the ratio of immune to cancer cells that was sufficient to drive FcγR-dependent TRAIL-R2-mediated apoptosis was substantially higher than that found in solid deposits of ovarian cancer (Figure 34). Therefore, it appears likely that the concept of enabling TRAIL-R1/2-mediated apoptosis in cancer cells via FcγR-expressing human immune cells is probably not viable for most cancers.

5.2 AMG 655 enhances TRAIL/Apo2L-induced cell death

It is possible that the binding of AMG 655 to TRAIL-R2 could possibly interfere with the binding of endogenous TRAIL to TRAIL-R2. If this were true then AMG 655 treatment might be counterproductive as it may prevent the killing of cancer cells by TRAIL-expressing immune cells such as macrophages (Washburn et al., 2003) and dendritic cells (Taieb et al., 2006).

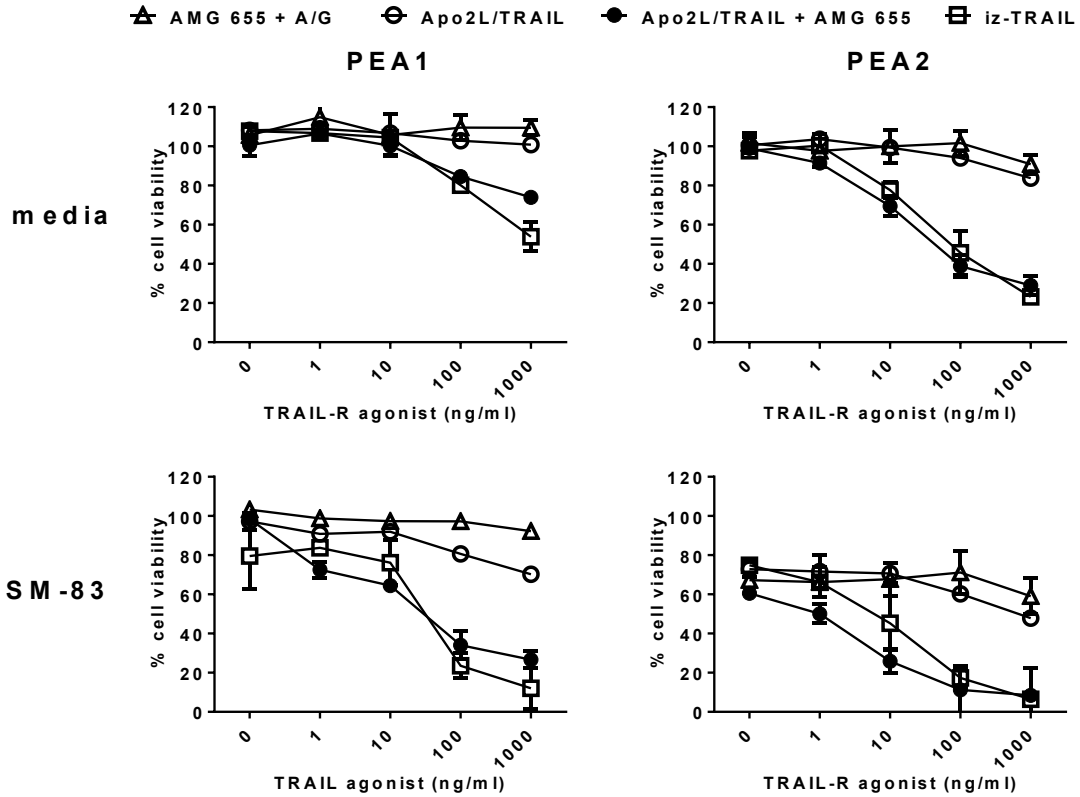

Figure 40: The combination of Apo2L/TRAIL and AMG 655 and SM-83 is highly effective at killing TRAIL-resistant ovarian cancer cell lines. a) PEA1 and PEA2 ovarian cancer cells were treated with the indicated concentrations of AMG 655 + recombinant protein A/G (A/G), Apo2L/TRAIL, iz-TRAIL, or AMG 655 [10 µg/ml] and Apo2L/TRAIL. Cell viability was measured after 24 hours. PEA1, PEA2 cells were treated with SM-83 [100 nM] for 1 hour and then treated with as in (a). Cell viability was measured after 24 hours.

Unexpectedly, however, it was found that AMG 655 did not block, but enhanced Apo2L/TRAIL's capacity to induce apoptosis in cancer cells; together, AMG 655 and Apo2L/TRAIL were as active in killing ovarian cancer cells as iz-TRAIL, a highly active recombinant form of TRAIL (Figure 40).

Interestingly, the activity of iz-TRAIL was not inhibited by AMG 655 but, in contrast to Apo2L/TRAIL, it was also not increased by treatment with AMG 655 (Figure 41). This suggests that there is a maximum agonistic activity that can be achieved by enhanced TRAIL-R-crosslinking; and that combinations of Apo2L/TRAIL and AMG 655 or iz-TRAIL can both achieve this maximum agonistic activity.

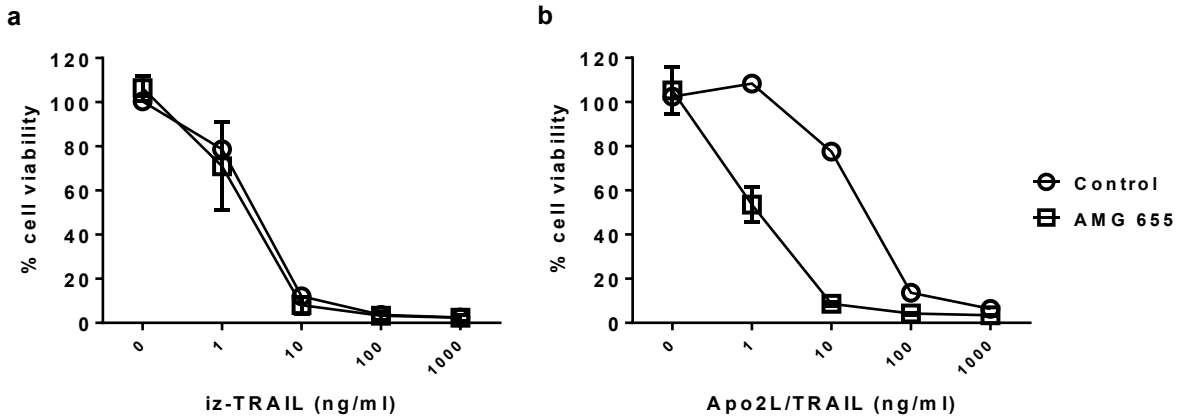

Figure 41: AMG 655 enhances Apo2L/TRAIL but not iz-TRAIL-induced cell death. (a) PEO4 cells were pre-incubated with AMG 655 [10 µg/ml] for 1 hour and treated with iz-TRAIL or Apo2L/TRAIL at the indicated concentrations. Cell viability was measured after 24 hours. Data are representative of the mean of 3 independent replicates (± S.D).

Importantly, the non-tagged Apo2L/TRAIL form that was used in these studies mimics dulanermin (Ashkenazi et al., 1999, Ganten et al., 2006). Therefore the combination of AMG 655 with recombinant Apo2L/TRAIL/dulanermin represents a highly active TRAIL-R agonistic therapy which could be immediately employed clinically in patients.

AMG 655 enhanced Apo2L/TRAIL-induced apoptosis in both TRAIL-sensitive and TRAIL-resistant ovarian cancer cell lines treated with or without SMAC mimetics (Figure 40). The synergistic effect of AMG 655 on Apo2L/TRAIL-induced cell death was observed at concentrations of AMG 655 as low as 10 ng/ml (Figure 42), which is far below levels achieved in patients with cancer (Herbst et al., 2010b). The effect of AMG 655 on Apo2L/TRAIL-induced cell death was unique and not observed with other TRAIL-R1- or TRAIL-R2-specific antibodies that were tested (Figure 42). Thus, not all TRAIL-R2-specific antibodies synergise with Apo2L/TRAIL to kill cancer cells.

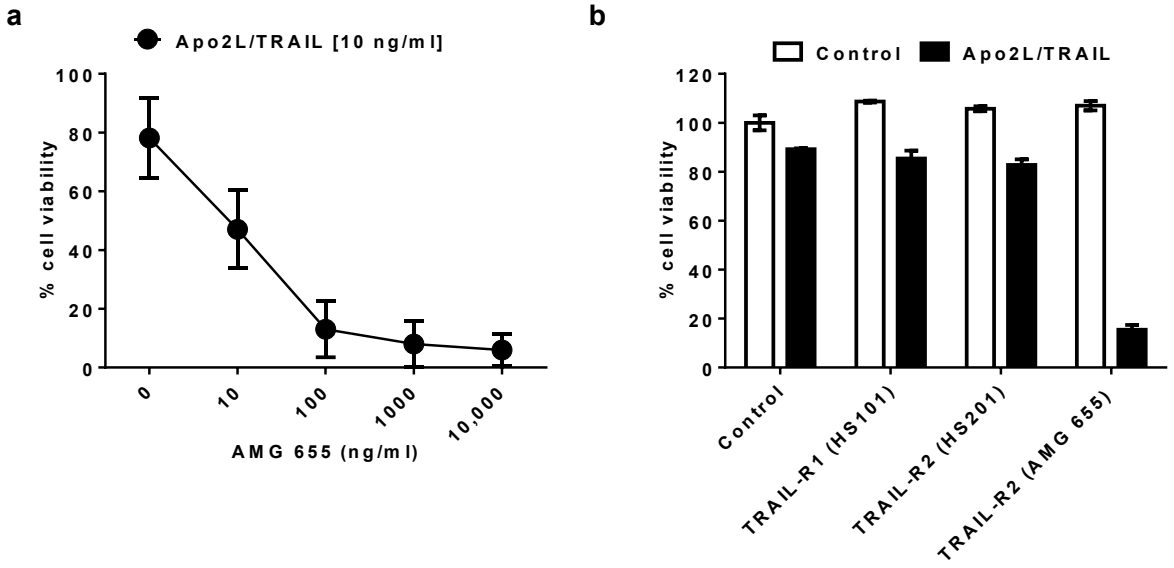

Figure 42: AMG 655 has a unique effect on Apo2L/TRAIL-induced cell death. a) PEO4 cells were treated with Apo2L/TRAIL [10 ng/ml] and increasing concentrations of AMG 655. Cell viability was measured after 24 hours. b) PEO4 cells were treated with anti-TRAIL-R1 (HS101) [10 µg/ml], or anti-TRAIL-R2 (HS201) [10 µg/ml], or anti-TRAIL-R2 (AMG 655) [10 µg/ml] for 1 hour and then Apo2L/TRAIL [10 ng/ml]. Cell viability was measured after 24 hours. Data are representative of the mean of 3 independent replicates (± S.D).

It was hypothesised that the effect of AMG 655 on Apo2L/TRAIL-induced apoptosis could be mediated by concomitant binding to TRAIL-R2, leading to enhanced multimerisation of TRAIL-R2 and increased formation of the TRAIL DISC (Falschlehner et al., 2007). To investigate this, PEO4 cells were stimulated with different combinations of Apo2L/TRAIL and AMG 655 and then caspase-8 was immunoprecipitated. The binding of AMG 655 to TRAIL-R2 enhanced the recruitment of the TRAIL-R2 DISC components FADD, cFLIP, and TRAIL-R2 to caspase-8 (Figure 43).

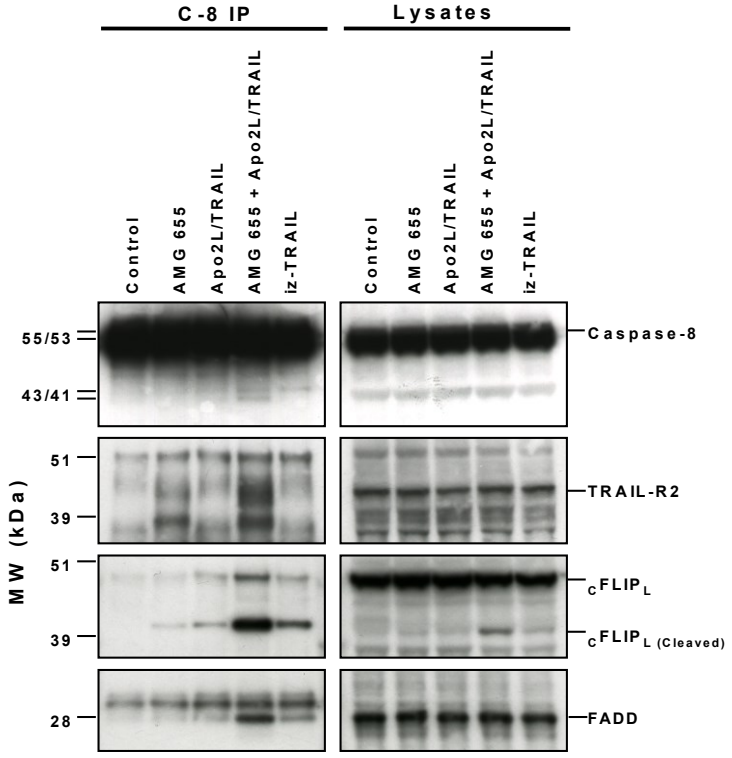

Figure 43: AMG 655 promotes recruitment of TRAIL DISC components to caspase-8. PEO4 cells were treated with either AMG 655 (10 µg/ml), Apo2L/TRAIL (100 ng/ml), Apo2L/TRAIL and AMG 655, or iz-TRAIL for 1 hour and then caspase-8 (C-8) was immunoprecipitated (IP, immunoprecipitation). Molecular weight, killer Daltons (MW (kDa)). Data are representative of one of 3 independent experiments.

To test whether the effect of AMG 655 on Apo2L/TRAIL-induced cell death was dependent on TRAIL-R2 stimulation, PEO4 cells were stimulated with AMG 655, Apo2L/TRAIL and AMG 655, and AMG 655 before lysis of the cells. Then either AMG 655 or Apo2L/TRAIL was added to the lysates of unstimulated cells. TRAIL-R2 was then precipitated using protein A/G beads. The presence of Apo2L/TRAIL or AMG 655 in the lysates of unstimulated cells did not affect recruitment of the TRAIL DISC component cFLIP to caspase-8 showing that the effect is dependent on TRAIL-R2 stimulation (Figure 44).

These data show that the synergistic effect of AMG 655 and Apo2L/TRAIL on TRAIL-induced apoptosis is mediated through enhanced TRAIL-R2 DISC formation. Thus AMG 655 leads to a change in the spatial conformation of TRAIL-R2, which leads to enhanced TRAIL-R2 multimerisation by Apo2L/TRAIL. This indicates that the synergistic apoptosis-inducing activity of AMG 655 and Apo2L/TRAIL is due to

increased TRAIL DISC formation as a consequence of enhanced TRAIL-R2 crosslinking.

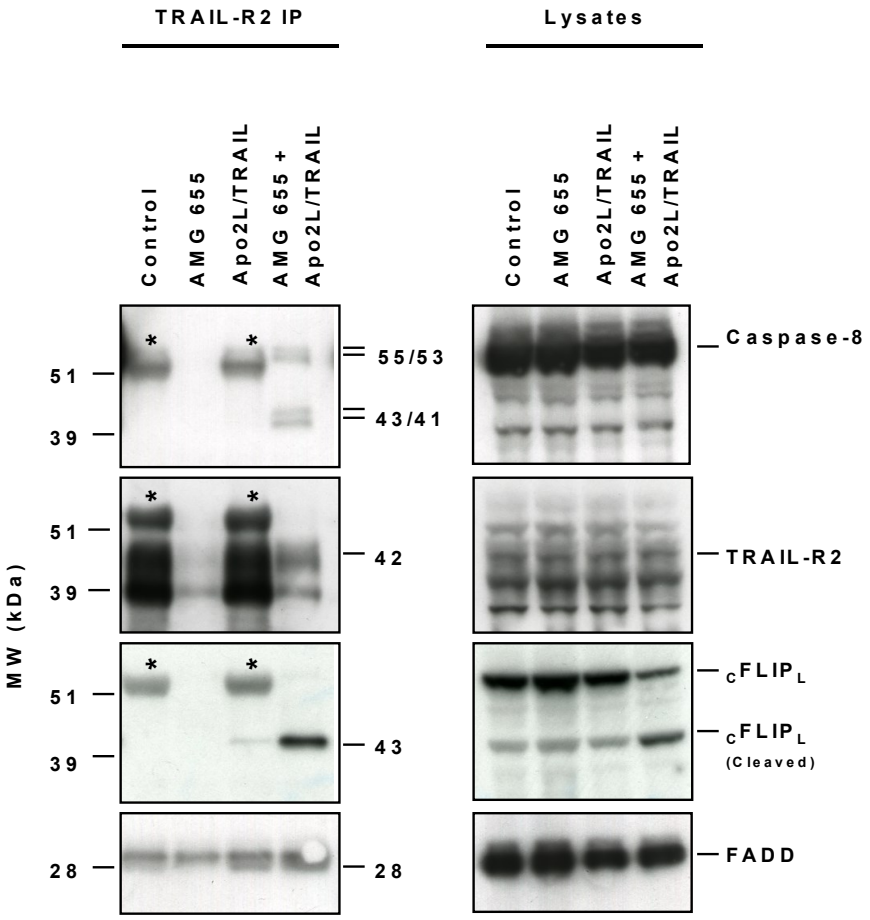

Figure 44: AMG 655 significantly enhances Apo2L/TRAIL-mediated recruitment of FADD, Caspase-8 and cFLIP to the TRAIL DISC. PEO4 cells were treated with either AMG 655 [10 µg/ml], Apo2L/TRAIL [100 ng/ml], or Apo2L/TRAIL and AMG 655 for 1 hour and then lysed. Either AMG 655 [10 µg/ml] or Apo2L/TRAIL [100 ng/ml] was added to the lysates of cells stimulated in the absence of the respective substance before immunoprecipitation of TRAIL-R2 to ensure the observed changes in the TRAIL-R2 DISC were dependent on TRAIL-R2 stimulation (IP, immunoprecipitation; * unspecific bands representing the IgG1 heavy chain from the addition of exogenous AMG 655 to the lysates). Data are representative of 1 of 3 independent experiments.

5.2.1 The combination of AMG 655 and Apo2L/TRAIL is highly efficient at inducing apoptosis in primary ovarian cancer cells

It was investigated whether AMG 655 could enhance Apo2L/TRAIL-induced cell death in primary ovarian cancer cells. Again the combination of AMG 655 with Apo2L/TRAIL was as active as iz-TRAIL in killing primary ovarian cancer cells (Figure 45). These results indicate that the combination of Apo2L/TRAIL with AMG 655 can

kill primary ovarian cancer cells from patients, and suggest that further combination with a SMAC mimetic drug or other TRAIL-sensitising agents may hold therapeutic promise for the treatment of ovarian cancer.

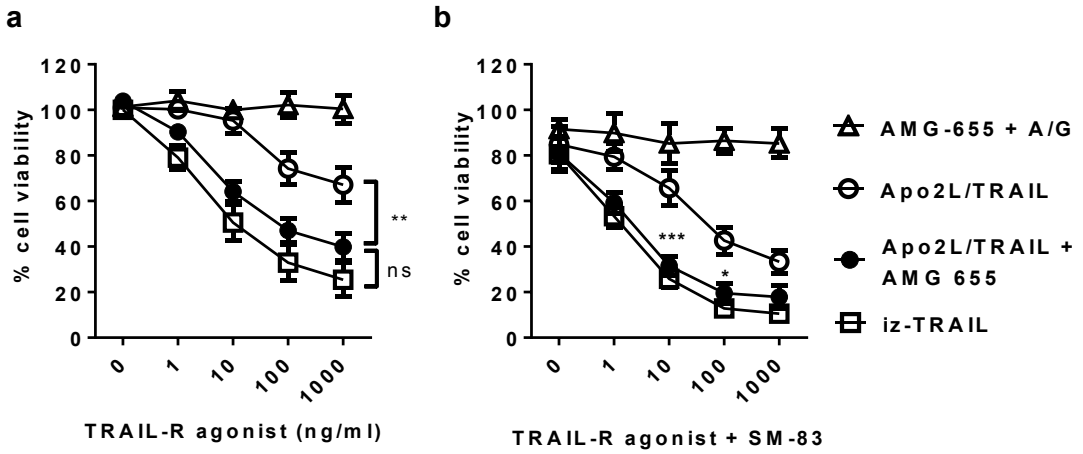

Figure 45: AMG 655 and Apo2L/TRAIL synergise to potently kill primary ovarian cancer cells. (a) Primary ovarian cancer cells were isolated from 6 patients[ł] and treated with AMG 655 + protein A/G, AMG 655 + recombinant protein A/G (A/G), Apo2L/TRAIL, Apo2L/TRAIL and AMG 655 [10 µg/ml], or iz-TRAIL. Cell viability was measured after 48 hours (n=6 ± S.D). (b) Primary ovarian cancer cells were treated with SM-83 for 1 hour and then as in (a). Cell viability was measured after 48 hours (n=6 ± S.D). * = P<0.05, **P=<0.01, *** = P<0.001. ł = 1 of 6 patient samples was processed and treated by Dr Antonella Montinaro.

5.2.2 The combination of AMG 655 and Apo2L/TRAIL is not toxic to primary human hepatocytes

In mice, highly active forms of TRAIL and SMAC mimetic compounds can be combined without causing severe toxicity (Fulda et al., 2002b, Vogler et al., 2008). The potential toxicities of therapeutic combinations of AMG 655 and Apo2L/TRAIL cannot be evaluated in mouse models as AMG 655 binds to human TRAIL-R2, but not to murine TRAIL-R (mTRAIL-R). Also, such xenograft models (Kaplan-Lefko et al., 2010, Ashkenazi et al., 1999) have been shown to be non-predictive of the potential clinical efficacy of AMG 655 and dulanermin.

Because of these factors and previous concerns regarding potential liver toxicity of TRAIL (Lawrence et al., 2001, Ganten et al., 2005, Ganten et al., 2006, Koschny et al., 2007a, Jo et al., 2000), it was investigated whether primary human hepatocytes were sensitive to apoptosis induction by the combination of AMG 655 and Apo2L/TRAIL, either alone or in the presence of SM-83 or PS-341. CD95L, a potent killer of primary human hepatocytes was used as a control (Ganten et al., 2006).

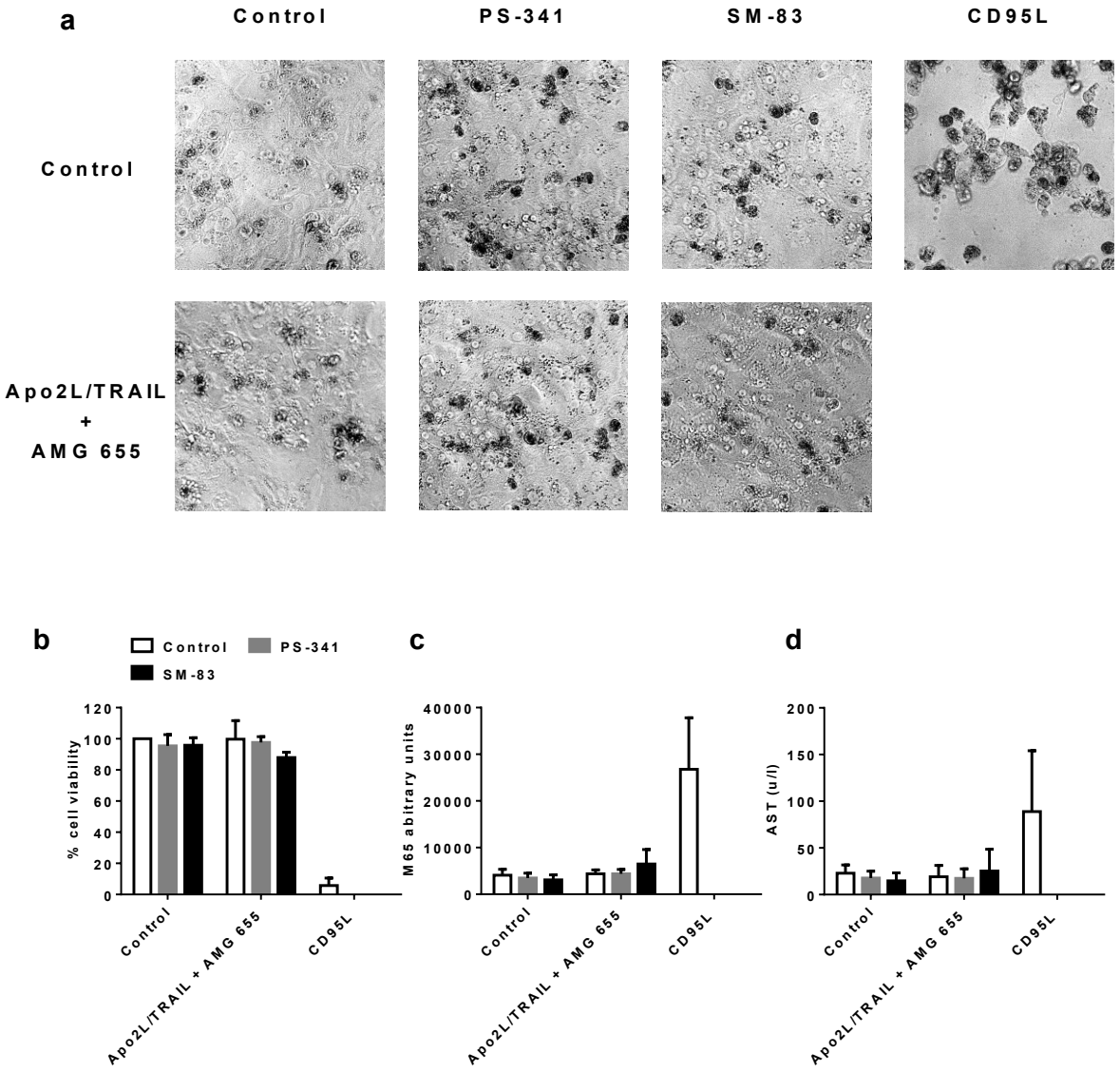

Figure 46: The combination of AMG 655 and Apo2L/TRAIL is not toxic to primary human hepatocytes. a) Primary human hepatocytes from 3 donors* were cultured for 3 days and then treated with Apo2L/TRAIL [10 µg/ml] and AMG 655 [10 µg/ml] ± SM-83 or PS-341. CD95L [1 µg/ml] was used as a positive control. a) Phase contrast microscopy of treated hepatocytes from a representative donor (3) (10 x original magnification). (b) Cell viability of primary human hepatocytes was assessed after 24 hours after Apo2L/TRAIL and AMG 655 ± SM-83 or PS-341 and CD95L treatment (n=3 ± S.D). (c) Primary human hepatocytes cell death was measured 24 hours after Apo2L/TRAIL and AMG 655 ± SM-83 or PS-341 and CD95L treatment by measuring release of soluble cleaved keratin 18 (K18) in the primary human hepatocytes supernatants using the M65 cell death ELISA. (d) Changes in the release of the liver enzyme AST were measured in primary human hepatocyte culture supernatants 24 hours after treatment with Apo2L/TRAIL and AMG 655 (n=3, ± SM-83) and CD95L. * The culture and treatment of cells from 1 of 3 donors was performed by Dr Antonella Montinaro.

Treatment with the combination of Apo2L/TRAIL and AMG 655 did not induce any significant changes in cellular morphology, loss of cell viability, or increase in the death of primary human hepatocytes (Figure 46). Importantly, also further combining

Apo2L/TRAIL with SM-83 or PS-341 did not induce a significant decrease in viability of primary human hepatocytes (Figure 46). None of the AMG 655- and Apo2L/TRAIL-comprising treatments resulted in a significant increase in liver enzyme release. These results are in line with previous findings regarding the effects of highly active recombinant forms of TRAIL on primary human hepatocytes (Ganten et al., 2005, Ganten et al., 2006, Koschny et al., 2007a).

Chapter 6. Discussion and conclusions

6.1 Chapter 3: A role for Foxo3 and Klf2 in the regulation of Foxp3: Discussion and conclusions

The results showed that Foxo factors promote TGFβ-mediated Treg cell differentiation, and that Foxp3 expression in PTEN deficient CD4 T cells can be partially rescued by the expression of constitutively active Foxo3. Whilst this work was in progress, several subsequent studies have confirmed these findings, and have demonstrated the importance of Foxo target genes in Treg cell differentiation, and in mediating the suppressive function of Treg cells (Harada et al., 2010, Kerdiles et al., 2010, Ouyang et al., 2010, Ouyang et al., 2012).

The overexpression of Foxo3 was only able to partially rescue the expression of Foxp3 in PTEN deficient CD4 T cells. This may indicate that PI3K/Akt/mTOR signalling negatively regulates other transcription factors that promote Foxp3 expression or that PTEN deficiency affects other signalling networks that regulate Foxp3 expression.

The overexpression of Foxo factors only had a minimal effect on the induction of Foxp3 with PI3K inhibitors (Figure 7). This may have been due to sufficient Foxo factors within the nucleus or the cytoplasm of the activated CD4 T cells to maximally induce the expression of Foxp3 after TCR withdrawal and PI3K inhibition. This could be investigated further by conducting experiments using an inducible genetic approach to deplete Foxo3, to determine if loss of Foxo3 impairs the expression of Foxp3 in activated CD4 T cells after PI3K inhibition and TGFβ treatment. Whilst this study was in progress, experiments performed by other investigators showed that genetic ablation of Foxo3 expression results in a significant reduction in TGFβ-induced Foxp3 expression in CD4 T cells (Harada et al., 2010).

There were differences between experiments in the expression of Foxp3 after withdrawal of the TCR activation signal and PI3K inhibition (Figures 5, 7, 8, 10, and

13). This could in part be explained by the length of TCR activation signals that the cells received. For example in Figures 5 and 6, the cells were activated using CD3/CD28 coated plates for 18 hours, and then the activation signal was withdrawn and Foxp3 expression was measured 48 hours later. Whereas in Figures 7, 8, 10, and 13, the cells were activated with anti-CD3/CD28 beads for around 36 hours and then the activation signal was withdrawn and Foxp3 expression was measured 48 hours later. However, there were also differences in Foxp3 expression induced by TCR withdrawal and PI3K inhibitors between experiments performed using the same conditions (Figures 7, 8, 10, and 13). This suggests that there were unidentified problems with internal consistency between experiments.

Interestingly, the deletion of Foxo1 and Foxo3 does not extinguish the expression of Foxp3 in CD4 T cells in mice (Ouyang et al., 2010). This suggests that although Foxo factors promote TGF-β-induced Foxp3 expression, Foxo factors are not essential for the expression of Foxp3. Although both Foxo1 and Foxo3 are important for Treg cell differentiation and function, recent studies suggest that Foxo1 appears to have the more important role in regulating Treg cell function (Ouyang et al., 2012).

Chromatin immunoprecipitation coupled to high-throughput sequencing (ChIP-seq) and gene expression profiling studies have shown that around 240 and 70 genes are directly activated or repressed respectively by Foxo1 (Ouyang et al., 2012). Foxo1 directly binds to the promoter of *CTLA-4* and directly controls expression of *CTLA-4*. It is possible that the loss of suppressive functions in Foxo deficient Treg cells are in part explained by the loss of CTLA-4 expression in Treg cells (Kerdiles et al., 2010). However, other studies with Foxo1 deficient mice have questioned this hypothesis, and have suggested that deletion of Foxo1 in Treg cells leads to the production of IFN-γ in Treg cells (Ouyang et al., 2012).

The overexpression of Klf2 was found to antagonise the induction of Foxp3 expression, suggesting that Klf2 may be a negative regulator of Foxp3 expression. The GFP-KLF2 fusion protein had a more pronounced effect than wild-type Klf2 on the induction of Foxp3 and on established Foxp3 expression in nTreg cells. It would

have been interesting to design further experiments to address whether the difference between GFP-KLF2 and KLF2-IRES-GFP was due to differences in protein expression between the different retroviral vectors, or due to the masking of certain Klf2 functions by the GFP-fusion protein. However, there is no commercially available effective anti-Klf2 antibody, which complicates the study of Klf2. This limitation could possibly have been addressed by cloning a short peptide flag tag into either terminus of Klf2 within the different retroviral vectors, which could have allowed the use of an anti-flag antibody to determine Klf2 expression levels from each vector.

GFP is a highly stable protein which has a half-life of around 26 hours (Corish and Tyler-Smith, 1999). The GFP-fusion protein could have stabilised Klf2 and enhanced its transcriptional activity. Klf2 contains an autoinhibitory domain which contains a binding site for the E3 ubiquitin ligase WWP1, which targets Klf2 and other transcription factors such as Klf5, Smad2, Smad4, and TβR-I/I for nuclear export and proteasomal degradation (Conkright et al., 2001). The GFP fusion could prevent the degradation of Klf2 by inhibiting or preventing the binding of WWP1; thus stabilising the protein.

The effect of the glutamic acid to glycine substitution within the Klf2 activation domain had a modest effect on the induction of Foxp3 expression. As Klf2 may regulate the expression of Foxp3, Klf2 polymorphisms could be associated with an increased risk of autoimmunity or other diseases. The Mouse Genome Informatics (www.informatics.jax.org), the Ensemble genome browser (www.ensembl.org), and National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov) databases were searched to identify any common amino acid substitutions between laboratory mouse strains.

Four C57BL/6 mice mRNA sequences were compared with the published Klf2 sequence, and with the RIKEN C57BL/6 Klf2 clone which had been used to generate the KLF-GFP and KLF2-IRES-GFP retroviral constructs. A total of 5 additional nucleic acid substitutions were identified within 4 C57BL/6 mice sequences that did not change the Klf2 protein sequence. One additional Klf2 (D88A) substitution was

identified in a B6CBA (a cross between the C57BL/6 and CBA/J mouse strains) and a C57BL/6 mouse strain sequence. This suggests that the amino acid substitution within the RIKEN clone could have arisen due to a technical error or that it may not be of wider importance. However, no studies were found that associated human KLF2 polymorphisms with human diseases.

Klf2 deficient CD4 thymocytes did not express Foxp3 in response to TGFβ or PI3K inhibitors, even when Klf2 expression was reconstituted by retroviral gene transfer. The inability of Klf2 deficient thymocytes to express Foxp3 could have been caused by thymic differentiation abnormalities, or could suggest that Klf2 has an essential role in Treg cell differentiation. A model that could explain these opposing findings is that low levels of Klf2 promote the expression of Foxp3, whereas high levels of Klf2 antagonise the induction of Foxp3 expression.

It would have been interesting to conduct more experiments with Klf2 deficient thymocytes. However, the thymocytes used in this study were from mice that were sacrificed in the United States, and then live cells were shipped to the UK. If the results from these experiments had been consistent with the results from the different retroviral vectors, then it might have been possible to arrange for the shipment of live animals to the UK for additional, more detailed genetic studies.

Although Foxo factors have been reported to regulate Klf2, a bio-informatics search of the KLF2 gene failed to identify published KLF2 binding sites (Tom Carroll, Lymphocyte Development group, unpublished observations), and deletion of Foxo1 in Treg cells does not appear to significantly affect the expression of Klf2 (Ouyang et al., 2012). Furthermore, overexpression of Foxo3 in activated CD4 T cells did not lead to an increase in *Klf2* expression suggesting that Foxo factors may not directly regulate *Klf2* in activated CD4 T cells.

The discovery that Foxo factors regulate immune tolerance (Ouyang et al., 2012), suggested that Foxo factors may be a therapeutic target for reducing the suppressive functions of Treg cells. However, Foxo factors have tumour suppressor functions and are important for the cytostatic and cytotoxic activity of chemotherapy drugs such as

paclitaxel (Yang and Hung, 2009) which are used for the treatment of ovarian cancer. Therefore it is difficult to envisage a successful therapeutic strategy that would seek to globally reduce Foxo factors within a patient with ovarian cancer that would be helpful. Therefore perhaps the best approach will be to target known Foxo targets, such as CTLA-4, which regulate the suppressive functions of Treg cells (Kerdiles et al., 2010).

In conclusion, the results from this part of the study suggest that Foxo factors promote the expression of Foxp3 in Treg cells, and that Klf2 is a potential negative regulator of regulatory T cell development. Klf2 antagonised Foxp3 expression in a mechanism that was independent of Smad7 and TGFβ signalling. The GFP-KLF2 fusion protein may reveal a previously unseen function of Klf2 in regulatory T cells. These findings suggest that further studies into the role of Klf2 in Treg cell differentiation are warranted.

6.2 Chapter 4: A role for TRAIL in the treatment of ovarian cancer. Discussion and conclusions

The principle aim of this part of the study was to determine whether a TRAIL-R agonist could serve as a potential therapeutic for ovarian cancer. The initial part of the study confirmed the findings of other investigators that SMAC mimetic compounds, PS-341 and chemotherapy can enhance the sensitivity of ovarian cancer cells to TRAIL induced apoptosis. It was possible to isolate tumour cells from 18 patients with ovarian cancer for these studies. The majority of the patients had platinum-resistant or -refractory ovarian cancer, and therefore this group of patients was ideal to study the potential efficacy of novel TRAIL-R-agonistic-comprising drug combinations for the treatment of advanced ovarian cancer.

6.2.1 The effect of PS-341 and SM-83 on the sensitivity of ovarian cancer cells to TRAIL

Although over 60 samples of ascites were collected, it was only possible to isolate tumour cells from 38 samples. The sample size was sufficiently large to allow the investigation of the TRAIL-sensitivity of tumour cells from 18 individual patients with ovarian cancer. One of the limitations of the methods used to isolate ovarian cancer

cells was the low yield of tumour cells from each ascites sample. There were also different numbers of tumour cells within each processed sample, which made direct comparisons between samples difficult. However, the ascites samples contained viable ovarian cancer cells that were already in suspension, and the ascites fluid was used as a tissue culture media. If ascites had not been used to culture the tumour cells, then the effects of PS-341 on the TRAIL-sensitivity of primary cancer cells might have been overestimated.

Approximately 38% of the tumour cells from the ascites samples were resistant to iz-TRAIL treatment. These results indicate that whilst the majority of human ovarian cancer cells are resistant to TRAIL-induced apoptosis, they can be sensitised to undergo TRAIL-induced apoptosis by the proteasome inhibitor PS-341 or a potent dimeric SMAC mimetic compound such as SM-83. Ovarian cancer ascites appeared to antagonise the effect of PS-341 on the sensitivity of ovarian cancer cells to TRAIL-induced apoptosis. This effect was seen in both primary cells and ovarian cancer cell lines. These data suggest that PS-341 is likely to have only a modest effect on the sensitivity of primary ovarian cancer cells to TRAIL. Nevertheless, proteasome inhibition and SMAC mimetic treatment led to over 50% of the ovarian cancer cells being sensitive to TRAIL treatment.

SM-83 had a modest effect on the viability of primary ovarian cancer cells, and only a minority of the primary ovarian cancer cells were sensitive to SM-83 treatment. It was also not possible to block the SM-83-induced loss of viability in primary ovarian cancer cells by blocking TNF. Therefore, a definitive conclusion could not be reached about the role of TNF in ovarian cancer cell death after SM-83 treatment. The results of a phase II clinical trial (NCT01681368) of the potent dimeric SMAC mimetic birinapant (TL32711) in patients with advanced ovarian cancer are expected to be presented in early 2014. This will give the first indication as to whether SMAC mimetic compounds have potential as a treatment for ovarian cancer. Nevertheless the treatment with SM-83 enhanced the sensitivity of the majority of the ovarian cancer cells to TRAIL, even in the presence of ascites fluid, suggesting that this approach has therapeutic potential.

There was significant heterogeneity in the responses of the patients to the different treatments. This could have been due to differences in the amount of tumour cells within the patient samples. Unfortunately it was not possible to isolate sufficient

ovarian cancer cells from each patient sample to undertake any non-candidate-based gene expression, proteomic, or genome-wide sequencing studies. Such studies could identify potential differences in signalling, gene expression, and mutations which may affect TRAIL-sensitivity and TRAIL-resistance. However, such data could be prospectively collected in clinical trials of these therapeutic combinations. This might allow the identification of novel biomarkers that could predict the sensitivity of ovarian cancer cells to TRAIL-R-agonistic drugs.

The addition of cisplatin only had a modest effect on TRAIL sensitivity, which was perhaps unsurprising given that the majority of the patients had platinum-refractory ovarian cancer. The finding that the cisplatin treatment reduced the sensitivity of some of the cancer cells to TRAIL-induced apoptosis, and even appeared to increase the viability of some of the ovarian cancer cells, suggests that cisplatin should only be combined with TRAIL in patients with platinum-sensitive ovarian cancer. The mechanisms behind the effect of cisplatin on the sensitivity of ovarian cancer cells to TRAIL induced apoptosis are unclear and were not investigated within this study.

Paclitaxel chemotherapy appears to have a modest effect on the sensitivity of primary ovarian cancer cells to TRAIL, and further studies of its role as a TRAIL-sensitising drug in ovarian cancer are warranted. However, a study in which dulanermin was combined with paclitaxel-based chemotherapy in patients with lung cancer did not lead to enhanced outcomes (Soria et al., 2011b). These data suggest that although chemotherapy does affect the sensitivity of ovarian cancer cells to TRAIL, chemotherapy should not be considered to be a highly effective TRAIL-sensitising drug in ovarian cancer.

Of note, the results from this part of the study were obtained with a recombinant form of TRAIL, iz-TRAIL, which alone is substantially more effective at killing cancer cells than non-tagged forms of TRAIL such as dulanermin which has been used in clinical studies thus far. The findings suggest that TRAIL alone, even a highly active TRAIL-R agonist, or agonistic combination, is unlikely to become an effective treatment for the majority of patients with ovarian cancer.

In conclusion, although these were *in vitro* studies, the findings are similar to other *in vivo* studies that have combined SMAC mimetics with TRAIL (Fulda et al., 2002b, Vogler et al., 2008, Vogler et al., 2009) and PS-341 with TRAIL (Unterkircher et al., 2011, Shanker et al., 2008) in murine tumour models. Together the results from this

study, and those from other studies, suggest that a TRAIL-R agonistic drug could be combined with a proteasome inhibitor or SMAC mimetic drug in a clinical trial in patients with cancer.

6.2.2 The effect of immune subsets within the tumour microenvironment on the sensitivity of ovarian cancer cells to TRAIL

The presence of immune cell subsets within the tumour microenvironment was correlated with the sensitivity of the ovarian cancer cells to TRAIL. Although the ascites-derived immune cells were from the peritoneum which contains ovarian cancer cells, it does not reflect the tumour microenvironment within solid deposits of ovarian cancer. Therefore, any correlations derived from this analysis can only be regarded as hypothesis-generating. The only significant finding between the different groups of patients was that TRAIL-sensitive patients had more IL-4$^+$ CD4$^+$ Th2 T cells within ovarian cancer ascites. Other immune cell subsets such as macrophages, neutrophils or Treg cells were not correlated with the TRAIL-sensitivity or TRAIL-resistance of primary ovarian cancer cells.

It is known that IL-4 can induce apoptosis in mouse microglial cells by down-regulating the expression of the anti-apoptotic protein Bcl-xL (Soria et al., 2011a). On the other hand, previous studies have shown that apoptosis-resistance in solid tumours such as colon, breast and lung carcinomas is mediated by tumour derived IL-4 (Todaro et al., 2008). However, it was the autocrine production of IL-4 by the tumour cells, rather than tumour associated immune cells, which mediated the effect of IL-4 on the inhibition of apoptosis (Todaro et al., 2008). These data suggest that IL-4 production by CD4 T cells could affect the sensitivity of ovarian cancer cells to TRAIL. It would be possible to investigate this by performing experiments in which exogenous IL-4 was added or depleted during the treatment of ascites derived ovarian cancer cells with TRAIL. These studies could be performed using samples of frozen ascites that were stored during the study.

6.3 Chapter 5: A role for TRAIL in the treatment of ovarian cancer. Discussion and conclusions

6.3.1 Ascites-derived immune cells as enablers of FcγR-dependent TRAIL-R2-mediated apoptosis

The analysis revealed that the ovarian cancer tumour microenvironment contains a significant number of immune cells with the potential to crosslink TRAIL-R2-specific antibodies such as AMG 655. Solid deposits of ovarian cancer contained more tumour cells than ascites, and ascites samples contained more $CD45^+$ immune cells than solid tumours. The relative proportions of FcγR expression were similar between solid deposits of ovarian cancer and within ovarian cancer ascites. However, the overall expression of FcγR was higher in ascites, which reflected the higher proportion of immune cells present within ovarian cancer ascites.

Although human immune cells were found to enable TRAIL-R2 signalling in the highly TRAIL-sensitive PEO4 cell line, this effect was only seen at a ratio of immune cells to cancer cells which was not found in the majority of solid deposits of ovarian cancer. The efficiency of FcγR-dependent TRAIL-R2-mediated apoptosis varied between patients. Only 36% of patients had immune cells within their ascites which enabled significant AMG 655-induced PEO4 cell apoptosis. Higher expression of FcγRIIIa (CD16) and FcγRIa (CD64) and the presence of macrophages within ovarian cancer ascites were associated with enhanced FcγR-TRAIL-R2-antibody-mediated apoptosis.

Although the analysis was limited by the small number of patients that were included within this part of the study; the finding that human immune cells were inefficient enablers of FcγR-dependent-TRAIL-R2-mediated apoptosis in primary ovarian cancer cells is significant. These *in vitro* data mirror the results from the clinical trials with TRAIL-R2-specific antibodies such as drozitumab (Baron, 2011, Lima et al., 2012, Rocha Lima et al., 2012) and conatumumab (AMG 655) (Chawla, 2010, Kaplan-Lefko et al., 2010, Kindler et al., 2012). This suggests that this approach of targeting the apoptosis-inducing TRAIL-R2 has limited clinical potential as a treatment for cancer.

Given that the majority of ovarian cancer cells are resistant to TRAIL, it is likely that most patients with TRAIL-sensitive ovarian cancer and other cancers have

insufficient immune cells in their tumour microenvironment to effectively drive FcγR-dependent TRAIL-R2-mediated apoptosis.

It was not possible to isolate a sufficient number of primary ovarian cancer cells from the patient samples to perform experiments to determine whether increasing the concentration of AMG 655 would lead to significant FcγR-dependent-TRAIL-R2-mediated apoptosis. However, clinical studies have reported that serum levels of 50 to 60 µg/ml of AMG 655 can be routinely reached by the administration of AMG 655 to patients with cancer (Herbst et al., 2010b) and these levels are not associated with responses in patients with cancer. These data suggest that the clinical responses that have been observed in patients treated with TRAIL-R2-specific antibodies such as AMG 655 and drozitumab could have been induced via the induction of T cell specific immunity (Takeda et al., 2004, Stagg et al., 2008) or immune responses mediated by B cells or CD11c dendritic cells (Haynes et al., 2010a).

In conclusion, the findings indicate that there are FcγR expressing immune cells within the ovarian cancer tumour microenvironment that could potentially enable FcγR-dependent TRAIL-R2-mediated apoptosis in patients with ovarian cancer. However, it is currently unclear as to whether these immune cells would be sufficient to enable FcγR-dependent TRAIL-R2-mediated apoptosis in patients with ovarian cancer.

6.3.2 The combination of AMG 655 and Apo2L/TRAIL is highly efficient at inducing apoptosis in primary ovarian cancer cells

The findings that AMG 655 did not become an effective inducer of apoptosis in ovarian cancer cells when crosslinked by FcγRs on immune cells, prompted the investigation of whether AMG 655 would block TRAIL-induced apoptosis. Unexpectedly, however, AMG 655 did not only fail to block apoptosis induction by Apo2L/TRAIL but potently enhanced it. AMG 655 and Apo2L/TRAIL are capable of inducing apoptosis in cancer cells in a highly synergistic efficient manner.

The apoptosis-inducing potential of Apo2L/TRAIL in combination with AMG 655 was as high as that of iz-TRAIL, which is a highly active recombinant form of TRAIL (Ganten et al., 2006). These results provide the basis for combining two TRAIL-R agonists, namely recombinant Apo2L/TRAIL/dulanermin and AMG 655 (conatumumab), both of which have already been tested in the clinic.

Interestingly, AMG 655 did not synergise with iz-TRAIL, suggesting that there is a maximum agonistic activity that cannot be increased further within the context of these assays. However, this result indicates that the combination of non-tagged recombinant Apo2L/TRAIL/dulanermin with AMG 655 can reach high TRAIL-R2 agonistic activity in terms of killing cancer cells via TRAIL-R2.

It was investigated whether this synergistic effect with Apo2L/TRAIL was unique to AMG 655 or whether other TRAIL-R2-specific antibodies could synergise with Apo2L/TRAIL in killing cancer cells. The TRAIL-R2-specific antibody HS201 did not have this effect. This suggests that the TRAIL-R2-specific antibody AMG 655, but not HS201, binds to TRAIL-R2 in a position that permits soluble Apo2L/TRAIL to concomitantly bind to TRAIL-R2. This would lead to enhanced Apo2L/TRAIL crosslinking and thus synergy between the two proteins in crosslinking TRAIL-R2.

These results indicate that the other clinically used antibodies against TRAIL-R2, and possibly against TRAIL-R1 could potentially synergise with Apo2L/TRAIL. It is also possible that the TRAIL-R2-specific antibody TRA-8, which does not require FcγR crosslinking for its activity, has this property (Forero-Torres et al., 2010). It would also be possible to investigate whether the TRAIL-R1-specific antibody, mapatumumab, has this property. Independently of the results from these experiments, it would be possible to generate TRAIL-R1 specific antibodies that enhance Apo2L/TRAIL/dulanermin-induced killing of cancer cells via TRAIL-R1.

There were significant differences in the ability of the different forms of recombinant TRAIL to induce apoptosis in all of the cell lines and in the primary cells that were tested. Apo2L/TRAIL was substantially less efficient at killing primary ovarian cancer cell lines when compared to iz-TRAIL, suggesting that this could be reason for the failure of Apo2L/TRAIL/dulanermin to induce responses in patients within the cancer clinic. However, these disappointing observations were offset by the finding that Apo2L/TRAIL and AMG 655 potently synergise to induce the death of cancer cells. The combination of AMG 655 and Apo2L/TRAIL was as effective as the stably trimerised highly-active TRAIL variant iz-TRAIL at inducing apoptosis in cancer cells, suggesting that AMG 655 and dulanermin could be combined.

The toxicity of the combination of Apo2L/TRAIL and AMG 655 to primary human hepatocytes was investigated because there have previously been concerns about the potential hepatotoxicity of TRAIL (Lawrence et al., 2001, Jo et al., 2000).

Although there have been other studies which have shown that highly active forms of TRAIL are not toxic to primary human hepatocytes (Ganten et al., 2005, Ganten et al., 2006, Koschny et al., 2007a), highly recombinant polyhistidine–tagged or crosslinked FLAG–tagged soluble TRAIL are toxic to primary human hepatocytes (Ashkenazi et al., 1999, Ganten et al., 2006, Kelley et al., 2001, Lawrence et al., 2001).

It was therefore decided to investigate the effects of the combination of AMG 655 and Apo2L/TRAIL on the viability of primary human hepatocytes. The combination of Apo2L and AMG 655 was not toxic to primary human hepatocytes and only induced a small loss in cell viability and a small increase in the release of the liver enzyme AST with co-treatment with SM-83. These data provide additional reassurance that two different clinically developed TRAIL-R-agonistic drugs, namely dulanermin and AMG 655, could be rationally and potentially safely combined to rapidly introduce a highly efficient TRAIL-R2 agonistic therapy into the cancer clinic.

Combining treatment with both Apo2L/TRAIL and AMG 655 would have the advantage of being both highly efficient at targeting TRAIL-R2. It would also have some of the other advantages of AMG 655 such as a long half-life and, potentially, the ability to induce anti-tumour immunity (Takeda et al., 2001, Takeda et al., 2004, Haynes et al., 2010b). The comparably short half-life of Apo2L/TRAIL may be advantageous. AMG 655 could be dosed at a constant high level to patients. Apo2L/TRAIL/dulanermin could be administered to reach pulses of high activity, either alone or in combination with additional TRAIL-sensitising agents such as SMAC mimetics, PS-341 or other drugs. In addition, the short half-life of dulanermin might limit possible unwanted effects of the TRAIL-R-agonistic therapy and would allow the treatment to be rapidly discontinued in the event of unexpected toxicity.

Numerous previous pre-clinical studies conducted with different clinically-used TRAIL-R2-specific antibodies, including AMG 655, as well as with the clinically used form of Apo2L/TRAIL, dulanermin, have demonstrated that each of these drugs are effective at inducing apoptosis in xenograft models of human cancers (Kay et al., 2012, Jin et al., 2004, Pollack et al., 2001, Ashkenazi et al., 1999, Walczak et al., 1999, Fulda et al., 2002a, Ichikawa et al., 2001, Kaplan-Lefko et al., 2010, Jin et al., 2008).

In this study, no *in vivo* experiments using human tumour xenografts into mice or murine autochthonous models of ovarian cancer were performed as thus far, no pre-

clinical studies have predicted the clinical activity of any TRAIL-R-agonistic drug (Kay et al., 2012, Jin et al., 2004, Pollack et al., 2001, Ashkenazi et al., 1999, Walczak et al., 1999, Fulda et al., 2002a, Ichikawa et al., 2001, Kaplan-Lefko et al., 2010, Jin et al., 2008).

Furthermore, although mice are useful for the study of many diseases, it is a limitation to this study that there are no mice that express human TRAIL-R that could be used to evaluate the combination of AMG 655 and Apo2L/TRAIL in a realistic autochthonous cancer model. It would be possible to investigate this concept using the murine anti-TRAIL-R antibody MD5-1 (Haynes et al., 2010b) in combination with murine-TRAIL in a murine autochthonous cancer model. However, given the lack of a suitable definitive murine model of ovarian cancer to assess the *in vivo* effectiveness of the combination of Apo2L and AMG 655; at present the only way to assess the *in vivo* effectiveness of the combination of Apo2L and AMG 655 would be in a clinical trial in patients with ovarian cancer.

Future work arising from this study could include conducting a clinical trial of the combination of AMG 655 and dulanermin either alone or in combination with a SMAC mimetic drug or a proteasome inhibitor in patients with advanced ovarian cancer. These studies could also determine which subtypes of ovarian cancer are sensitive or resistant to TRAIL treatment and identify which patients will benefit from TRAIL treatment. It would also be interesting to investigate the effect of IL-4^+ Th2 T cells on the sensitivity of ovarian cancer cells to TRAIL treatment.

In conclusion, TRAIL-R agonistic therapies are a potential treatment for ovarian cancer. TRAIL-resistant primary ovarian cancer cells can reliably be sensitised to TRAIL-induced cell death using either a SMAC mimetic compound or proteasome inhibitor. The binding of AMG 655 to TRAIL-R2 promotes Apo2L/TRAIL-induced cell death in ovarian cancer cells by leading to enhanced formation of the TRAIL DISC. The combination of AMG 655 and Apo2L/TRAIL was not toxic to primary human hepatocytes. The synergistic effect of AMG 655 and Apo2L/TRAIL on the induction of TRAIL-R2-mediated-apoptosis is a key finding as the form of Apo2L/TRAIL that was used in this study is similar to dulanermin (Belada et al., 2010, Soria et al., 2010, Soria et al., 2011b), which has already been used in clinical trials in patients with cancer. Together, these data suggest that AMG 655 could be rationally combined with dulanermin, and possibly further with a SMAC mimetic drug or other TRAIL-

sensitising drugs, to introduce a highly active TRAIL-R agonistic therapy into the cancer clinic.

Chapter 7. Appendix

7.1 Abbreviations

ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
Apaf-1	Apoptosis activating factor 1
APO-1	Apoptosis antigen 1
AP1	Activator protein 1
AST	Aspartate transaminase
ATF	Activating transcription factors
BCG	Bacillus Calmette–Guérin
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BH3	Bcl-2 homology domain 3
BIR	Baculoviral IAP repeat
BRCA1	Breast cancer 2, early onset
BRCA2	Breast cancer 2, early onset
BSA	Bovine serum albumin
Caspases	Cysteine-dependent aspartate-directed proteases
Cbfβ	Core-binding factor beta
cFLIP	cellular FLICE-like inhibitory protein
CHRM	Cryopreserved hepatocyte recovery medium
cIAP1	Cellular inhibitor of apoptosis protein-1
cIAP1	Cellular inhibitor of apoptosis protein-2
CREB	cAMP response element binding
CTL	Cytotoxic T cells
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DED	Death-effector domains

DIABLO	Direct IAP binding protein with low pI
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EpCAM	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death-domain containing protein
FAK	Focal adhesion kinase
FIGO	International Federation of Gynaecology and Obstetrics
FcγR	Fragment crystallisable region γ receptors
FCS	Foetal calf serum
Fox	Fork head box
Foxo	Forkhead box O3
Foxp3	Forkhead box P3
GALNT14	Polypeptide N-acetylgalactosaminyltransferase 14
Gata3	GATA binding protein 3
GFP	Green fluorescent protein
GVHD	Graft versus host disease
HCC	Hepatocellular carcinoma
HER-2	Human Epidermal Growth Factor Receptor 2
HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
IAP	Inhibitor of apoptosis protein
IBM	IAP-binding motif
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma

IgG1	Immunoglobulin G 1
IL	Interleukin
IP	Immunoprecipitation
JNK	c-Jun N-terminal Kinase
KLF2	Kruppel like factor 2
KRAS	Kirsten rat sarcoma viral oncogene homolog
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
Mcl-1	Myeloid cell leukaemia sequence 1
mCRC	Metastatic colorectal cancer
MSCV	Mouse stem cell virus
mTOR	Mammalian target of rapamycin
mTORC2	Mammalian target of rapamycin complex 2
NAIP	Neuronal apoptosis inhibitory protein
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NHL	Non-Hodgkin lymphoma
NK	Natural Killer
NSCLC	Non–small-cell lung cancer
pA	Pure for analysis
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositide 3-kinase
p53	Protein 53 or tumour protein 53
PFS	Progression-free survival

RANKL	Receptor activator of NF-κB ligand
RPMI	Roswell Park Memorial Institute
RCC	Renal cell carcinoma
RING	Really Interesting New Gene
RIP1	Receptor interacting protein 1
RORγT	RAR-related orphan receptor gamma T
RNA	Ribonucleic acid
RUNX	Runt-related transcription factor
SDS	Sodium dodecyl sulfate
SiRNA	Small interfering RNA
SMAC	Second mitochondrial activator of caspases
Smad5	Mothers against decapentaplegic homolog 5
Smad7	Mothers against decapentaplegic homolog 7
SOEing	Splicing by overlap extension
STAT	Signal Transducer and Activator of Transcription
STAT5	Signal Transducer and Activator of Transcription 5
S1P1	Sphingosine-1-phosphate receptor 1
T-bet	T-box transcription factor TBX21
tBID	Truncated BH3 interacting-domain death agonist
TCR	T cell receptor
TGFβ	Transforming Growth Factor β
Th	T helper
TLR	Toll like receptor
TRAIL	TNF-related apoptosis-inducing ligand
Treg cells	Regulatory T cells
TNFR2-Fc	TNF receptor 2-Fc fusion protein
VEGF	Vascular endothelial growth factor

XIAP X-linked Inhibitor of apoptosis protein

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