STRESS AND PROBIOTICS IN OVARIAN CANCER

Maysa AL-Natsheh

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Abstract

Ovarian cancer (OC) is the deadliest type of gynaecologic cancer predominantly of the epithelial subtype (EOC). Despite advances in treatments, relapse and dissemination are very likely to occur after remission. The heterogenous nature, complex immunosuppressive environment and the chronic inflammation promoting dysbiosis/oncobiosis are characteristics of OC and imply poor prognosis. Disparities in the outcomes of current therapies warrant new therapeutic strategies that are clinically effective and less invasive.

Diagnosis, relapse, and treatment of cancers are often stressful experience associated with mental and physical comorbidities. Cortisol is released in response to stress and strong evidence shows that prolonged release of cortisol can influence tumour biology and immunity. Although these effects are currently under debate as to whether cortisol promotes or inhibits ovarian tumour growth.

Our enhanced understanding of the gut microbiome and its pivotal role in the immune and brain development and function led to growing interest in their role in treating or preventing immune and neuroendocrine-mediated pathologies such as cancer and chronic stress. Therefore, probiotics are of interest because of their potential anti-tumour effect and their role in promoting positive mental health outcomes. Hence the name 'psychobiotics'.

This study aims to first examine the effects of cortisol and psychological stress on ovarian cancer immunity, and to probe underlying mechanisms. Secondly, to investigate the anti-tumour effect of the probiotic *Escherichia Coli Nissle 1917 (EcN)* as a possible complementary therapy in OC metastasis. A 3D-ovarian cell culture (spheroids) model and a syngeneic female C57BL/6j mouse model for OC were used to investigate the impact of stress on anti-tumour immunity. Metastatic nodules, tumour burden and immune signature were assessed to probe an underlying mechanism. Gamma-H2AX immunofluorescence assay was applied to examine DNA damage in splenocytes of stressed mice.

Restraint stress (RS) significantly reduced overall mice weight at 4 weeks and the activation of splenic dendritic cells (DCs) at 2 weeks. Furthermore, restraint stress

significantly increased DNA damage in splenocytes of stressed mice as large and compact spheroids were observed in the spheroids splenocytes co-cultures. This was verified *in vitro* using *ex vivo* T lymphocytes treated with pharmacological concentration of cortisol (10µM). *EcN* reduced tumour burden potentially through downregulation of Toll Like Receptor 4 (TLR-4) and upregulation of IL-23. But it did not reduce tumour burden in stressed mice. In summary, stress negatively impacted the anti-tumour immunity at an early stage of OC and *EcN* did not provide protection against the stress-induced negative effects in OC metastasis.

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List of the Main Abbreviations

APCs: Antigen presenting cells

ACTH: Adrenocorticotrophic hormone

AVP: Arginine vasopressin

C GAS: Cyclic GMP-AMP synthase

CRH or CRF: Corticotrophin releasing hormone/factor

CTLA-4: Cytotoxic T lymphocyte antigen 4

DAMP: Damage associated molecular pattern

PAMP: Pathogen associated molecular pattern

DCs: Dendritic cell

DSB: Double strand break

END: Endorphin

E: Epinephrine

EOC: Epithelial ovarian cancer

EcN: Escherichia coli Nissle 1917

GCs: Glucocorticoids

GRs: Glucocorticoid receptors

GRE: Glucocorticoid response elements

HGSOC: High grade serous ovarian cancer

HPA: Hypothalamus pituitary Adrenal axis

HR: Homologous recombination

ICI: Immune checkpoint inhibitors

IF: Immunofluorescence

IFNs: Interferons

LAB: Lactic acid bacteria

IL: Interleukin

LGSOC: Low grade serous ovarian cancer

MHCII: Major histocompatibility complex molecule

MMPs: Matrix metalloproteinases

Myd-88: Myeloid Differentiation primary response gene 88

NOS: Nitrous oxide synthase

NE: Norepinephrine

OC: Ovarian cancer

OS: Overall survival

PAMP: Pathogen associated molecular pattern

PARP: poly ADP-Ribose polymerase

PMA: phorbol myristate acetate

PRRs: pattern recognition receptors

PCR: Polymerase chain reaction

PFS: Progression free survival

ROS: Reactive oxygen species

RS: Restraint stress

SCFA: short chain fatty acids

SNS: Sympathetic nervous system

Sph-SP- Spheroid-splenocytes

STING: Stimulator of Interferon Genes

TAM: Tumour associated macrophages

TILs: Tumour infiltrating lymphocytes

TLRs: Toll like receptors

TME: Tumour microenvironment

TP53: Tumour suppressor gene 53

VEGF: Vascular endothelial growth factor

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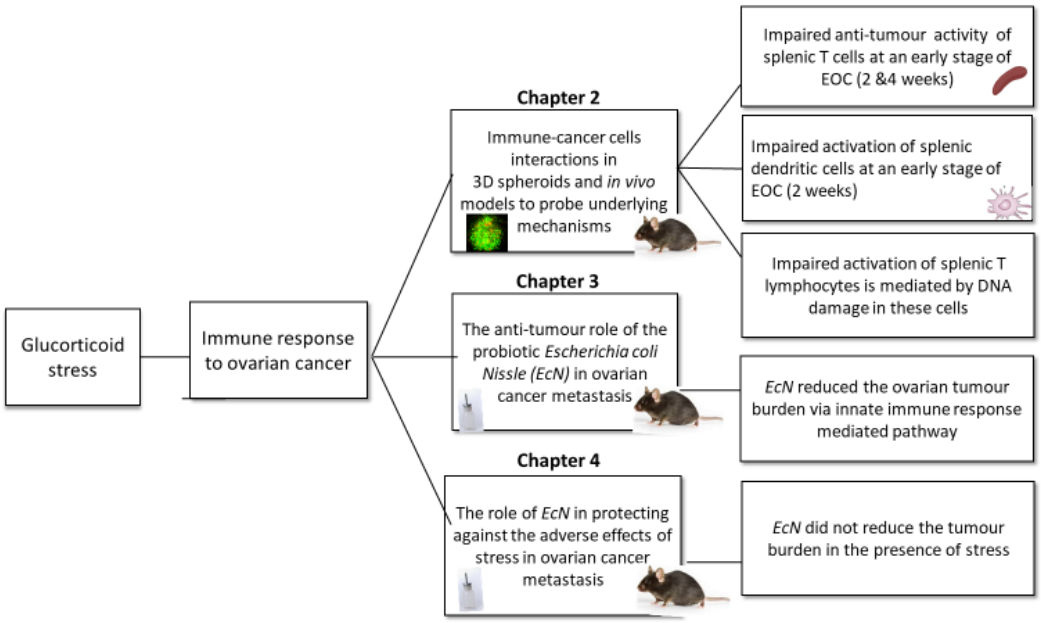
Declaration I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

Signed

Date

Chapters Overview



1. Chapter 1 – Introduction

1.1 Ovarian cancer

1.1.1 Epidemiology

Ovarian cancer (OC), predominantly of the epithelial type (EOC), is the deadliest gynaecological cancer (1-3) . OC is the sixth most common female cancer in the UK; there are 7400 new cases of OC each year (20 every day) and 4100 annual deaths (11 each day) with the highest incidence rate being recorded in women aged 75-79 years as reported by Cancer Research, UK (2015-2017) (4). Globally, OC is the third leading cause of gynaecologic cancer amongst women (5). The incidence rate is expected to exceed 300,000 in 2020 with more than 190,000 expected deaths (6, 7).

The high mortality is thought to be due to firstly, the absence of specific signs and symptoms (OC is referred to as the `silent killer`) and secondly, due to the lack of robust and reliable screening tests with high sensitivity and specificity to allow for early detection and effective treatment. Indeed, only 30-45% of OC cases can be detected at an early stage with the available blood screening tests for biomarkers such as CA125 protein (8). However, there are some promising developments in this regard, for instance, glycodelin (an essential glycoprotein for the maintenance of normal reproductive activities in human) is expected to become a useful prognostic biomarker in serous carcinoma OC (9-11).

Recently, the use of the CA125 antigen test combined with transvaginal sonography (TVS) based on Risk of Ovarian Cancer Algorithm (ROCA) to prompt for laparotomy has been a promising approach. This screening is allowing for adequate specificity at an early-stage detection, as demonstrated by two clinical studies conducted in asymptomatic postmenopausal women at average risk. However, there is still a need to improve the sensitivity to panels of biomarkers to achieve early detection of OC (10, 11). Ultimately, 63% of EOC are diagnosed at a late III-IV stage (International Federation of Gynaecology and Obstetrics [FIGO]) when the disease is widely metastatic within the abdomen and their five-year survival is 28-16% respectively (12).

A novel approach for early EOC detections and prevention is emerging, involving the detection of microbiota (the useful microorganisms that reside on mucosal surfaces

to maintain their state of health) and their metabolites in the tumour and body's fluids such as plasma, saliva, blood, urine, faeces and cervicovaginal discharge (13).

1.1.2 Risk factors

Unlike other cancers, OC does not have clear identifiable risk factors. Hereditary factors such as family history of breast or ovarian cancer are prominent factors contributing to 5-10% of OC, genes such as *p53* and *BRCA1/2* were found to be significantly mutated in ovarian carcinoma irrespective of the germline status (14). Indeed, *BRCA1/2* genes are found to play a vital role in homologous recombination (HR) repair of DNA double strand breaks (DSBs). Up to half of the ovarian tumours which were analysed were found to have a mutation related to a gene involved in HR repair (14). Recently, testing for homologous recombination repair deficiency (HRD) has been approved for use as a predictive/prognostic biomarker in OC as a standard of care, ESMO Guidelines 2021(15).

Reproductive factors such as age at menopause, infertility and parity are considered to play a pivotal role in the development of OC. Additionally, lifestyle factors such as diet, obesity and smoking may also affect OC risk. On the other hand, hormonal factors including pregnancy, breastfeeding and oral contraception are likely to protect against OC (16, 17). The American Institute of Cancer Research and the World Cancer Research Fund estimate that 30-40% of all cancers could be prevented by appropriate diet, physical activities, and maintenance of appropriate body weight. It was reported that increased intake of Flax seed especially its lignans, abundant portions of fruits and vegetables as well as high fibre intake have been reported to lower cancer risk. Also, supplementary intake of digestive enzymes and probiotics have been shown to have a potential for a protective anti-cancer effect (18).

Deepening our understanding of the aetiology of EOC would enable prediction of those at high risk and consequently open an avenue for early detection and diagnosis or successful preventative measures.

1.1.3 Ovarian cancer classification

Over the years OC has undergone different classifications to identify the tumour site and its profile (including: histopathology, morphology and molecular-genetic characteristics) in addition to the extent of the disease or disease stage to anticipate response to treatment (19). Based on the type of cells of origin within the ovary, ovarian tumour has been divided into the following subtypes: epithelial, mesenchymal, germ and stromal. Nine out of ten of patients who are diagnosed with OC are of the type 'epithelial ovarian cancer' (EOC), which is the most lethal/invasive type (20). Thanks to the advancement in the understanding of the biology of the tumour, EOC is now identified as heterogenous disease with diverse type of tumours different in cellular origin, morphology, hereditary pathologies, molecular alterations, and potential for targeted therapy (20, 21).

This consequently has an impact on prognosis and treatment by enabling prediction of response. Therefore, stage 1 OC is up to 90% curable, stage II up to 70% curable but this drops rapidly to only 20-25% in stage III and IV cancers (16). The WHO has taken into account the current enhanced understanding of the origin, pathogenesis and prognosis of the different OC subtypes and classified EOC based on histology and morphology as observed through microscopy into five subtypes, namely: 'high-grade serous' (HGSOC) accounting for ~70 % of subtypes, followed by 'endometrioid' & 'clear cell carcinoma' (~20%), then 'mucinous' and 'low-grade serous carcinoma' (LGSOC) (21, 22). Clinical observation of tumours revealed two very rare types; malignant transitional cells (Brenner), mixed types and undifferentiated carcinoma (Table 1).

Ovarian carcinoma is further subdivided clinically based on the degree of differentiation (tumour grade/stage), which gives a better understanding of the metastatic pattern. Two systems have been used for staging OC. Firstly, FIGO (International Federation of Obstetrics and Gynaecology) defines four stages: in stage I the tumour is confined to the ovary, stage II involves one or two ovaries with pelvic extension or primary peritoneal cancer, stage III involves one or both ovaries with confirmed spread outside the pelvis and/or metastasis to the lymph nodes and

stage IV involves distant metastasis excluding peritoneal metastasis. The alternative TNM staging system by AJCC (American Joint Committee on Cancer) is the most used system for many cancers, including colorectal cancer, where N is followed by a number to describe the size and extent of the primary tumour (tumour burden). N refers to the number and locations of the involved lymph nodes and M refers to the extent of metastasis to secondary organs. Both staging systems have important implications on disease prognosis and therapy outcomes (22).

Shih and Kurman (23) proposed a dualistic model for carcinogenesis based on molecular/ genetic changes, pattern of tumour progression and clinical behaviour, which was later recognised and adopted by the WHO. This dualistic model consequently led to a fundamental shift in the classification of EOC beyond histology and morphology. It was categorised into two main types: Type I and type II. Type I is found to develop from a premalignant or borderline region, where tumours are confined to the ovary (stage 1), harbour gene mutations (different than the *TP53* mutation), genetically stable and have excellent prognosis. Type 1 includes low-grade serous, clear-cell and mucinous.

Whereas type II is generally aggressive present in advance stage, harbour high frequency *TP53* mutations, genetically unstable, has poor prognosis and predominantly of the type HGSOC (21, 24, 25). This has improved our understanding of carcinogenesis and revolutionised the conventional understanding of the origin and development of tumour (mesothelial ovarian epithelial origin theory) (22). The dualistic model demonstrates an independent molecular pathway for the two types; both develop outside the ovary predominantly from the fallopian and endometrium but involve it secondarily. Consequently, this has an impact on prognosis and treatment by enabling prediction of response which is of important clinical significance for early detection, prevention, and treatment (1, 23, 26-28) (Table 1).

Surface Epithelial Ovarian Cancer					
Cellular origin of epithelial ovarian cancer (EOC) (histology)	Fallopian tube epithelium	endometriosis	endometriosis	Fallopian tube epithelium	? unknown
Cellular morphology of EOC	High-grade serous carcinoma (HGSOC) from serous tubal intraepithelial carcinoma	Endometroid carcinoma	Clear cell carcinoma	low-grade serous carcinoma (LGSOC) from benign or borderline serous neoplasms	mucinous
% Of subtypes of EOC	~70	~10	~10	<5	<5
Common mutation and molecular aberrations	*TP53* *BRCA1/2* and genetic instability				
Potential molecular targeted therapies	-PARP inhibitors -Immune checkpoint inhibitor				

Table 1: Epithelial ovarian cancer subtypes
Based on histology, morphology, molecular /genetic aberrations, and therapy. Epithelial ovarian cancer is the most common type of ovarian cancer, predominantly of the high-grade serous carcinoma (~ 70%). Adapted from (29).

1.1.4 Treatment

The standard first line treatment of EOC comprises cytoreductive surgery with platinum and taxane-based cytotoxic chemotherapy (2, 30). It is a very aggressive treatment with severe side effects, especially when used in high doses. There is also a potential for residual resistant cancer cells to escape this primary treatment (28). For patients who relapse within 6 months of treatment, life expectancy does not exceed 1 year, targeted treatment with inhibitors to poly ADP-Ribose polymerase (PARPs, proteins involved in DNA repair and maintenance of genomic stability), showed a significant positive impact in the outcome of patients who harbours the breast cancer gene *BRCA1/BRCA2* mutation and recurrent high-grade serous OC (31). Recently 3 PARP inhibitors were approved for women with recurrent EOC: Olaparib (32), rucaparib (33) and niraparib (34).

Other targeted therapies such as monoclonal antibody inhibitors to immune checkpoints; Programmed Death 1 (PD-1), Programmed Death ligand 1(PD-L1), Cytotoxic T lymphocyte Antigen 4 (CTLA-4) are collectively known as Immune Check Point Inhibitors (ICIs). Also, antibody inhibitors to angiogenesis growth factor (known as vascular endothelial growth factor, VEGF) such as bevacizumab in combination with standard treatments are showing some promise by delaying reoccurrence and prolonging overall survival (OS) in EOC.

Despite advancements in the surgical management and the systemic therapeutic developments for the last four decades, the 5-years survival rate for advanced stages of EOC is only 47% (2, 35). There is a poor improvement in long-term survival at late stage and no substantial decrease in the death rate has been observed (23, 28). PARP inhibitors showed a significant increase in the progress free survival (PFS) particularly in women with BRCA mutation but not in the overall survival (OS) (15). Indeed, 80% of cases with advanced stage would relapse within 18 months (12) then progress into chemo resistance and eventually abdominal obstruction which is the most frequent cause of death. The disparity in the outcome of current treatments urges for novel therapeutic strategies.

1.2 Psychologic stress

1.2.1 Introduction to psychological stress

Stress is one of the essential survival mechanisms in nature. Organisms have always been subject to environmental pressures or stressors such as natural disasters which threaten their homeostasis. This leads to physiological, biochemical and cognitive behavioural responses to support adaptation, restore homeostasis and enhance survival (36).

Hans Selye (1907-1982) , the founder of the stress theory, was the first to link stress with health and defined the stress as “nonspecific response of the body to any demand for a change”(37). He divided stress into three stages: alarm reaction, resistance or adaptation and exhaustion and called the three stages ‘General Adaptation Syndrome’ which is later known as the ‘stress response’. In 1929 Walter Cannon called it the ‘fight and flight’ response (38).

Currently, stress is known as any extrinsic or intrinsic force or stressor that impair the body’s state of balance or homeostasis physiologically and psychologically (39). Stress could be induced by physical, mental, or emotional pressure, and manifests in a form of negative emotions or altered mood and behaviour such as anxiety, irritability and depression depending on the individual’s susceptibility. Persistence of these behaviours can negatively impact other organs and systems causing morbidities such as impaired immune function, disturbed microbiota balance, increased proinflammatory cytokines production, cardiovascular diseases, and cancer (40).

Based on its duration and effect on an individual’s health, stress can be divided into two types, acute and chronic, manifesting beneficial or detrimental physiological effects respectively (41). The former helps to promote healthy survival but the latter can contribute to physiological pathologies such as cardiovascular diseases and tumour pathogenesis (42).

It has been shown that the stress response is mediated through the neuroendocrine system via two main branches: First, the hypothalamus pituitary adrenal (HPA) axis, which is activated slowly in response to stress and continue working for a long time. Second, the sympathetic nervous system (SNS) which is involved in rapid/immediate response to stressor and switches off immediately following the removal of stressor (43). The HPA axis normally maintains the basal and stress-related homeostasis of the central nervous system (CNS), cardiovascular system (CVS), immune function and metabolism (41, 44).

Stressors, either emotional or physical, stimulate the meta paraventricular nucleus of the hypothalamus to release Corticotrophin, releasing hormone/factor (CRH) or (CRF) and arginine vasopressin (AVP), which stimulate the pituitary gland to release adrenocorticotrophic hormone (ACTH), encephalin and endorphin (END). ACTH in turn stimulates the adrenal cortex to release glucocorticoids (GC) (cortisol) and the adrenal medulla to release the catecholamine hormones epinephrine (E) and norepinephrine (NE) (41). The SNS, which originates from the brain stem, stimulates the adrenal medulla and the sympathetic ganglia to release epinephrine (45). The secretion of E and NE stimulate physiological functions and alertness through adrenergic receptors which form part of the fight and flight response (38, 39).

Dopamine is another catecholamine that is released in response to stress as well as oxytocin and prolactin (46) (figure 1). In this study, the effect of the stress hormone cortisol on the anti-tumour immune response in OC is going to be investigated.

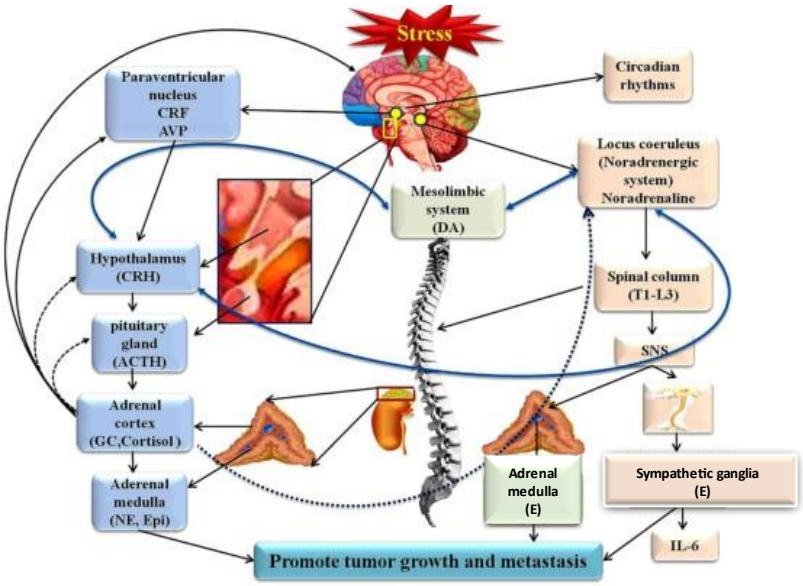


Figure 1: The stress hormones glucocorticoids and catecholamines are released through the stimulation of HPA and SNS axis
In response to stress, the hypothalamus secrets CRH which stimulates the pituitary gland to secrete ACTH. ACTH in turn stimulates the adrenal gland to secret glucocorticoids and catecholamines (epinephrine and norepinephrine). Excess circulating cortisol provides negative feedback to hippocampus and hypothalamus to stop cortisol production. Persistent secretion of the stress hormones in response to stress, dysregulate the feedback mechanism and the HPA-Axis becomes unresponsive to the negative feedback. This can promote tumour growth and metastasis. *CRH-corticotrophin hormone, ACTH-adrenocorticotrophin hormone*. Adapted from (41).

1.2.2 Cancer and stress

Diagnosis and treatment of cancer is often a stressing experience associated with mental and physical comorbidities. About 29% to 38% of women diagnosed with OC experience a significant level of anxiety (47). Watts and colleagues conducted a meta-analysis to assess the prevalence of depression and anxiety in OC patients across the treatment's stages (before, during and after treatments). They reported that women with OC showed a significant increase in prevalence of depression and anxiety compared to healthy females population (48).

The psychologic stress response in humans can be assessed by observation, interviews, and self-reports, which are completed by patients based on their personal experience. Knowing that people vary in their susceptibility to stress, expression of

symptoms, their perception, recalling of events and coping ability, these methods of assessments are subjective and thus unreliable (38). Therefore, to assess the relationship between psychologic stress and cancer to probe the underlying mechanisms effectively, it is important to choose a model that resembles stress response in human in a way that can be used to apply physiological stress to promote anxiety or fear. These models include social isolation, immobilisation or confinement to small space and intimidation. They can be used to mimic the different types of stressors including physical and emotional with different duration of time to reflect the acute and chronic status of stress (49).

Chronic restraint stress (immobilisation) is one of the most used models to study the impact of stress on the immune response to infectious diseases. It is used to recapitulate physical restraint stress (RS) by inserting a mouse in 50 ml perforated conical tubes for varying lengths of time. The observed outcomes are increase in the stress hormones levels (E, NE, and corticosterone) and significant decrease in the inflammatory response to influenzas virus (50). The RS model can be used to apply daily restraint stress from 1-12 h (51, 52). In C57BL/6J mice a 50% increase in serum corticosterone was observed after 3h of restraints stress and a 100% increase after 6h (53, 54). Further RS has shown a decrease in anti-tumour effect of cyclophosphamide in the same mouse model (53).

Cortisol, a glucocorticoid released in diurnal cycle, peaks up in the morning and drops to the lowest level in the second half of the night (figure 2). However, individual variations are observed in these levels amongst healthy individuals (flattened cycles) (55, 56). Excess circulatory levels of cortisol induced by chronic stress can impair the HPA-Axis negative feedback and consequently disturb the diurnal cortisol rhythm. Dysregulation of the circadian rhythm of cortisol is found to be the first observed harmful effect from long term stress (57, 58). RS for 2 h daily in the morning can be applied to mice to mimic the circadian rhythm of cortisol production.

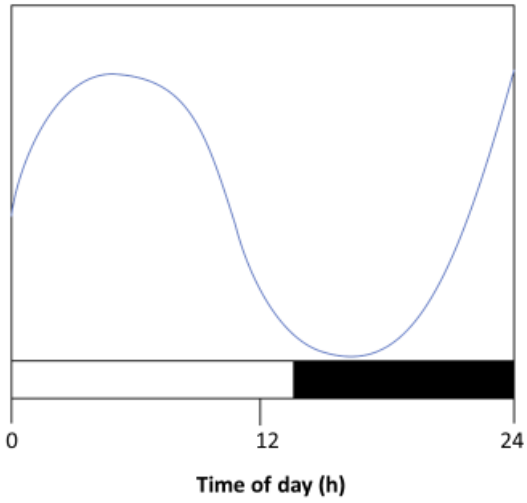


Figure 2: Schematic diagram illustrating the diurnal cycle for plasma cortisol
It peaks up in the morning and drops to the lowest level in the second half of the night. The black bar represents the sleep/dark period (59).

Strong evidence from epidemiological and clinical studies suggests a link between psychosocial behaviours such as chronic stress, depression, social isolation and cancer development and progression (60, 61) whereas only limited and contradictory evidence is supporting a link between these psychosocial behaviours and cancer initiation (62, 63).

Studies from restraint stress (RS) of xenograft and metastatic mouse models of oral and colon cancer respectively showed that chronic RS promoted cancer angiogenesis and metastasis. This was observed through an increase in circulatory level of stress hormones such as GC and catecholamines and in tumour infiltration with tumour associated macrophages (TAM) (64, 65). Further, it was shown in colorectal cancer model that chronic RS severely weakened the effect of sunitinib (antiangiogenic therapy (anti VEGF), mainly through the activation of β-adrenergic signalling pathway which in turn promotes angiogenesis. It was suggested that using β-adrenergic antagonist may enhance the efficacy of sunitinib (66). It was also found in a retrospective clinical studies of breast cancer patients that early blocking of β-adrenergic receptors limited skeletal metastasis and tumour recurrence (67)

suggesting a pivotal role for the neuroendocrine system in chronic stress mediated cancer progression and metastasis.

Furthermore, it was demonstrated in a RS mouse model that chronic stress could induce a change in the phenotype and properties of T regulatory (Tregs) and DCs driving intestinal inflammation which was thought to be mediated through prolactin (68), highlighting a modulating role for stress on the immune response to promote cancer development and growth.

In ovarian cancer patients, it was found that increased in ovarian tumours' TAMs and increased number of peripheral blood monocytes were associated with tumour growth and worse overall survival (69). A study using orthotopic xenograft models with SKOV3ip1 and HeyA8 ovarian carcinoma cell lines, demonstrated an increase in tumour growth and metastasis in response to chronic stress. The observed increase in tumour growth and metastasis was linked to upregulation of interleukin 8 (IL-8) resulting from prolonged stimulation of the SNS. Using β-blocker or silencing the gene for IL-8 expression decreased tumour growth and angiogenesis (70). This evidence highlights a role for the immune response and inflammation in promoting ovarian cancer growth and metastasis during chronic stress.

In a meta-analysis study evaluating the association between stress and cancer, 165 studies showed correlation between stress-psychosocial behaviours and higher cancer incidence in initially healthy people. Also, poorer survival was observed in patients with diagnosed cancer from 330 studies and higher cancer mortality was seen in 53 studies. Further, a meta-analysis was conducted to study the relation between stressful life events and breast cancer incidence found a slight increase in breast cancer incidence with history of stressful life events (63). Recently, Flacinelli and team reviewed the clinical relevance of psychological stress on cancer risk and proposed potential mechanisms that may link the stress response with early stages of malignant cell transformation (71).

In summary, persistent long-term stress has the potential to activate the physiological stress response constantly resulting in detrimental biological effect of which cancer initiation, progression, and metastasis. The stress effect on cancer is

found to be exerted by different mechanisms: non-immune-mediated, circadian rhythm-mediated and immune-mediated (72). The latter is observed through suppression of the protective immunity or enhancing the regulatory immunity and chronic inflammation (73). Therefore, there is a potential for patient-specific interventions based on the identification and management of persistent changes in behaviours to prevent or protect against cancer (74). In this study, the effect of RS on OC metastasis is going to be evaluated.

1.2.3 Glucocorticoids (GCs)

Natural GCs (cortisol in humans and corticosterone in rodents) are steroid hormones derived from cholesterol in the adrenal glands and play a key role in many physiological processes, including homeostasis, development (cellular proliferation, differentiation, and apoptosis), metabolism and control of immune and inflammatory response. GCs synthesis and release in the body is regulated in a circadian/ultradian (time integrated release) and stress-related manner to maintain their homeostatic functions that are necessary for life. This is maintained by a negative feedback loop suppressing ACTH levels in the anterior pituitary and CRH levels in the hypothalamus once the systemic concentration of cortisol has been reached (43). Only 5% of GCs is free in the circulation and can enter the cells, the remaining 95% is bound to corticosteroid-binding globulins in the blood.

Cortisol has diurnal release pattern where a circadian clock (an endogenous biological timekeeper that set the organisms for the daily changes in their environment (46) consists of central clock in the hypothalamus and peripheral one in all other tissues. Where the central clock controls the HPA axis through CRH/AVP to regulate the diurnal oscillation of circulating ACTH and cortisol hormones, the HPA axis adjusts the rhythmicity of the peripheral clock in response to different stressors. Dysregulation of the circadian clock and HPA axis have been reported to result in detrimental physiological effects such as obesity, metabolic disorders, and cardiovascular diseases, like the physiological impact observed from chronic stress (38, 43).

Synthetic glucocorticoids such as dexamethasone and prednisolone not only have different potencies, metabolism, and clearance from natural GC but also have higher bioavailability (43, 75). Since 1940, Phillip Hench has used synthetic GCs successfully in clinics as therapeutic agents for their immunosuppression and anti-inflammatory effects. GCs have been effective in treating patients undergoing organ transplants and in patients with chronic inflammation. Their anti-proliferative and antiangiogenic effects have been exploited in treating cancer (using high pharmacological doses) (76).

Therefore, they have been at the for front of the scientific research to further explore these characteristics for more advanced therapeutic applications.

1.2.4 Glucocorticoid Receptors

Many of the effects of both natural and synthetic GCs in almost all tissues of the body such as immune and cancer cells are mediated through glucocorticoids receptors (GRs) which are members of the superfamily of nuclear receptors. GRs are shown to exist in two different isoforms: GRα and GRβ, where the former is the most expressed form that binds to GCs to induce the signalling pathways for targeted genes, and the latter does not bind to GC and its function is yet to be elucidated (77). Researchers are starting to reveal the complexity of GCs signalling pathway. It is found that GCs exert their actions through a genomic (a prolong onset) (figure 3a) or non-genomic (rapid onset) (figure 3b) signalling pathways. It is considered though that the non-genomic pathways are the most important determinant of GCs action (43, 77).

GRs resides in the cytoplasm and is associated with accessory proteins such as heat shock proteins (HSPs). Upon binding to its GCs ligands, the cytoplasmic GR becomes activated and dissociates its accessory protein to form GC-GR complex. The ligated GRs then translocate to the nucleus to exert their effect via genomic mechanisms of transactivation and transrepression which consequently determine the GC function. The ligated GR either binds directly to the DNA via positive glucocorticoid response elements (GRE) (figure 3a) resulting in genes transactivation (upregulation) of

immunosuppressive genes, such as IκB kinase, annexin-1, mitogen-activated protein kinase (MAPK) phosphatase 1, and interleukin 10 (IL-10). On the other hand, when GR binds to negative GRE on the DNA or binds to a transcription factor on the DNA (Tethering-Protein to protein binding), it results in genomic transrepression (downregulation of inflammatory genes) such as activator protein-1 (AP-1), nuclear factor-kB (NF-kB), nuclear factor of activated T cells (NFAT) or signal transducers and activator of transcription (STAT) and interferon regulatory factor 3 (IRF-3), leading to suppression of proinflammatory cytokines such as IL-12 & IL-6, cyclooxygenase 2 (COX 2) and many others. Different GREs are thought to be responsible for the diverse GR signalling (77, 78). The tethering mechanism is thought to illustrate the immunosuppressive function of GC and it is believed that stimulating this pathway with a selective GR agonist would maintain GCs immunosuppressive function (43, 77-80).

Evidence suggests that GCs can also act rapidly (within minutes) through a non-genomic signalling pathway either by a specific interaction with cytosolic GR or membrane-bound GR or by no specific physiochemical interactions with the cell membrane (77, 78). The former action is shown to take place through changing the activities of different kinases such as Src, extracellular signal-regulated kinase (ERK1/2), phosphatidylinositol 3-kinae (PI3K) (85), whereas the latter is mediated by rapid interference with cellular processes such as calcium level and phosphorylation events. This rapid effect of GC has been observed in clinical and pre-clinical studies in systems such as the immune, cardiovascular, and neuroendocrine and it was not reversed by GR antagonists (86).

Therefore, deepening our understanding of the molecular pathway underlying this rapid, non-genomic effect could be exploited for more effective GCs therapies with fewer side effects.

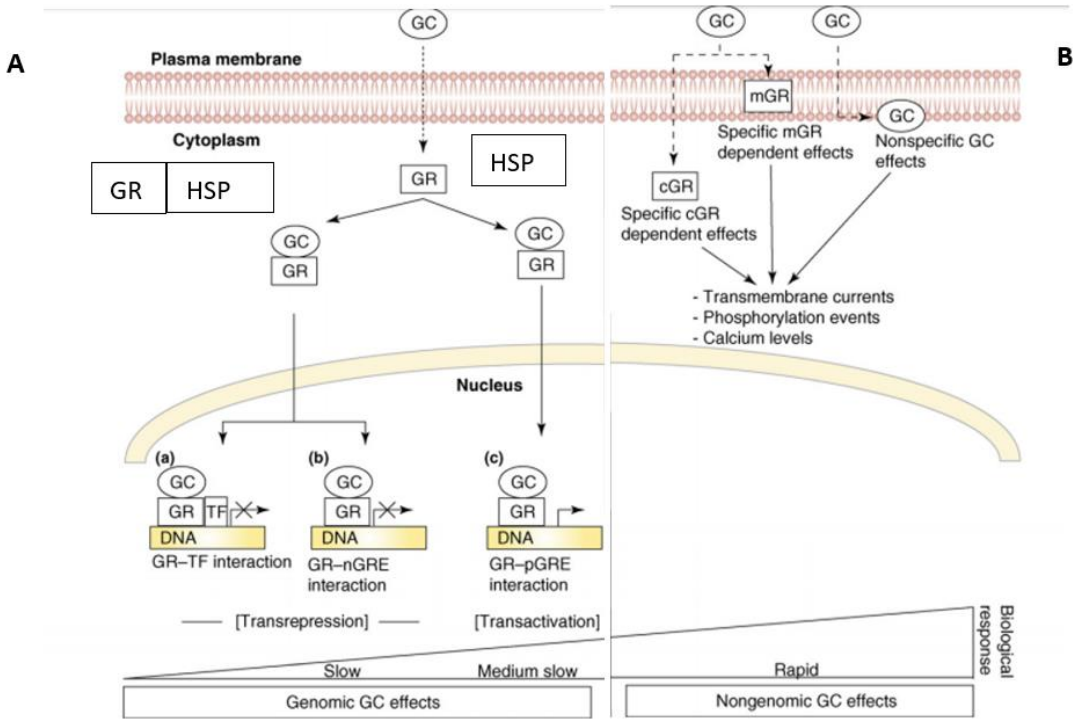


Figure 3: The genomic and non-genomic immunoregulation by glucocorticoids (GRs)
(A) GCs passively enter the cytoplasm, dissociate the GR-HSP complex and bind to its receptor (GR) in the cytoplasm. The ligated GC-GR translocate to the nucleus and bind directly to the DNA via positive GRE to upregulate expression of immunosuppressive genes (transactivation). On the other hand, when GC-GR complex binds to a negative GRE on the DNA or indirectly to TF on the DNA (Tethering), it downregulates the proinflammatory genes (transpression). *GC Glucocorticoids, GR Glucocorticoids receptors, HSP Heat Shock Protein, TF Transcription Factor, GRE Glucocorticoid Response Element, p Positive, n Negative.*
(B) GCs can also act rapidly (within minutes) through a non-genomic signalling pathway either by a specific interaction with cytosolic GR (cGR) or membrane-bound GR (mGR) or by no specific physiochemical interactions with the cell membrane resulting in: Change in the activities of different kinases, phosphorylation events and calcium levels. Adapted from (78).

1.2.5 Role of GC in immune cells function

GCs affect major effector immune cells in different ways including apoptosis, change in differentiation, inhibition of inflammatory cytokines release and change in migration. A GR knock out and functional selective mouse models have been previously used to investigate these effects of GCs on various immune cells, including dendritic cells and lymphocytes (80). It was found that dexamethasone both *in vitro* and *in vivo* severely impairs differentiation and maturation of dendritic cells (DCs)- the sentinel antigen presenting cells necessary for the induction of primary immune response. This effect was observed through a reduction in the major histocompatibility complex class 2 (MHCII), co-stimulatory factors and cytokine production (81). Furthermore, GCs inhibit the migration of DCs to the lymph nodes

(82) and potently induce apoptosis of DCs (43, 81). The GC effect on T cells is dependent on the T cells subtype and it is found to induce apoptosis in proinflammatory T cells and pro survival in Treg stimulating intestinal inflammation (41, 80). GCs caused a suppression of cytotoxic activity of natural killer cells, phagocytosis of macrophages and neutrophils activities which consequently impaired the immune response against tumours (80, 83).

In this study, the underlying mechanism for the impact of cortisol and RS on immune cells trafficking and tumour infiltration as well as their impact on the maturation and activation of DCs pulsed with OC tumour lysate will be investigated.

1.2.6 Glucocorticoids and cancer

GCs have a complex mechanism of action dependent on the type of cancer, whereby they can either promote or suppress the tumour (44). In haematopoietic malignancies mainly of the lymphoid lineage, GCs exert pro-apoptotic effect, hence its valued role in clinics where synthetic GCs such as dexamethasone are routinely included in the chemotherapy protocols for lymphoid malignancies, such as Acute Lymphoblastic Leukaemia (ALL) (77). The mechanism for GCs apoptotic effect seems to be complex involving several signalling pathways but still not fully understood (84). However, in solid tumours, the role of GCs is controversial. Only in breast and prostate cancer, modest benefit was observed when GCs was administered as a monotherapy or combined with cytotoxic drugs and no change in long term survival was observed in advanced breast cancer despite the enhanced response rate (85, 86). On the other hand, an anti-apoptotic effect for dexamethasone was observed in breast cancer epithelial cell lines, which diminished the effect of paclitaxel, an effect which was found to be GR-mediated MAPK- inactivated pathway. Inhibition of this pathway restored the cytotoxic effect of paclitaxel (87). Further, GR antagonism in GR^+ high grade serous ovarian carcinoma cell lines (HGS-OVCA) and xenograft models increased the sensitivity of the cancer cells to chemotherapy (88). GC' role in breast cancer (BC) therapy varies depending on the subtype of cancer. GCs have been shown to suppress the tumour growth in oestrogen receptor positive breast cancer (ER-positive BC) (89). Conversely, it was shown to increase metastasis in triple negative

breast cancer (TNBC) by reducing cell adhesion and increasing their motility (90, 91). Further, activation of GR has been associated with increased proliferation, escape of apoptosis and disease progress.

Despite the contradictory role of GCs in cancer, GCs have widely been used as adjuvant therapy with chemotherapy and radiation to alleviate their severe side effects (92). Interestingly, GR expression on ovarian cancer cells is found to be associated with higher grade, and advanced stage which correlates with decreased progression-free survival (PFS), but not overall survivor (OS), 39% of invasive epithelial ovarian cancers express GR (93-95). These controversies in the function of GCs could be attributed to the cancer type, GRs level or the dose of GCs.

1.2.7 Glucocorticoids and ovarian cancer

The role of GCs in EOC is controversial. One group found that GCs administration to patients is associated with rapid up-regulation of anti-apoptotic genes (*SGK1* and *MKP1*) expression in ovarian tumours (96). This finding supports the hypothesis that pharmacologic doses of glucocorticoids may decrease chemotherapy effectiveness in ovarian cancer patients (96). Therefore, blocking the GR receptor using a (GR) antagonist could inhibit GR' anti-apoptotic signalling pathway and restore the cytotoxic effect of chemotherapy (88). On the other hand, others have showed that GCs have a novel role in metastatic ovarian cancer mediated by the upregulation of the metastasis suppressor gene MicroRNA-708 (*miR-708*).

Researchers have further investigated doses of GCs in an immunocompetent mouse model and found that low dose of GCs as low as 5μg/kg body weight can suppress ovarian cancer progression and metastasis. This is believed to be mediated via several mechanisms: upregulation of *miR-708*- anti metastatic gene, downregulation of pro-inflammatory cytokines (interleukins IL-1β and IL-18), decrease in the number of tumours associated macrophages (TAMs) and myeloid-derived suppresser cells (MDSCs) in the tumour microenvironments. Conversely, Sood and team showed a significant increase in ovarian tumour potential invasion by cortisol *in vitro* as

demonstrated in the cell line SKOV3; this effect is found to be likely mediated by stimulation of Matrix metalloproteinases (MMPs) (93).

Further, an altered circadian rhythm was observed amongst women with ovarian cancer prior to treatment, evidenced by elevated nocturnal cortisol, flattened diurnal cortisol slope, and reduced cortisol variability. Each of these effects were associated with poor overall survival (OS) and increased intratumoural inflammation (97). This suggests more data is needed to clarify the role of GCs in metastasis of ovarian cancer (98, 99). In this study the impact of cortisol on the splenic T lymphocytes and bone marrow generated DCs activation and subsequently their anti-tumour activity in EOC will be studied to probe an underlying molecular mechanism. the impact of to enable translations of clinical benefits.

1.2.8 Psychologic stress and DNA damage

Studies suggest significant DNA damage in cells and tissues can occur on daily basis because of exposure to different intracellular and extrasellar agents such as free radicals and reactive oxygen species (ROS) which are by-products of cell replication and oxidative stress. A possible mechanism that is most likely to cause the highest damage in DNA lesions is Double Strand Breaks (DSBs). The induction of DSBs is attributed either to physical, chemical, or biological factors. If DSBs are not repaired immediately via DNA repair mechanisms, they can have a detrimental effect on cell survival resulting in chromosomes defects, genetic instability, apoptosis, and the initiation of pathologies such as cancer (100-102).

DNA repair mechanisms are usually activated in response to DNA damage. A damaged DNA in a cell activates DNA damage response (DDR) pathways which in turn stimulate a repair pathway to delete or physically remove the damage to restore genomic stability and ensure survival of the cell. DSBs can result in early cellular activation of different factors including phosphorylation of the histone protein H2AX to produce gamma phospho-histone (γ-H2AX) (103, 104), which is necessary for the assembly of proteins required for DNA repair to maintain cellular homeostasis in addition to activation of check points proteins necessary for cell cycle arrest. Because

γ-H2AX correlates well with each DSBs, it is considered the most sensitive and specific marker for analysing the initiation and resolution of DNA damage (107,108).

Sometimes the DNA damage can be tolerated, and the cell continues proliferating. However, when the damaged DNA persists, and the DNA repair mechanism fails to repair the damage, the cell will be programmed to undergo apoptosis. Otherwise, if apoptosis is resisted, somatic mutations build up and may result in cell transformation and /or cancer initiation and progression (105).

DNA DSB repairs takes place through two main mechanisms: non-homologous end joining which involves re-joining of the broken DNA ends, usually it lacks precision due to the loss/gain of some nucleotides at the joins and takes place at all phases of the cell cycle. Second is homologous recombination which is more precise and only takes place at the S and G2 phases of the cell cycle, it maintains genomic stability and supress tumorigenesis. Mutations in genes encoding homologous recombination (HR) proteins such as *BRCA1/2* play key role in the abnormal cellular development and tumorigenesis associated with ovarian cancer. Indeed, half of the ovarian tumours which were analysed were found to have a mutation related to a gene involved in HR repair (14).

DNA damage by stress hormones in pre-cancerous cells was evaluated *in vitro* by Flint and her team using the murine fibroblast cell line (3T3). Acute exposure to physiological doses of the stress hormones: Cortisol, NE & E resulted in a 5-fold increase in DNA damage in comparison with untreated cells. This effect was found to be mediated by an upregulation of genes involved in DNA damage/repair signalling pathway such as *PARP 1, BRCA 1, TP53* (106). Further a study was conducted by Flaherty and colleagues (107) to evaluate the impact of acute exposure to cortisol and NE on DNA damage in breast cancer cell lines by monitoring reactive oxygen species (ROS) and reactive nitrogen species (RNS). They showed a significant increase in DNA damage mediated by (RNS/ROS) suggesting that stress hormones induce ROS leading to DNA damage. The study also investigated the impact of stress hormones

in vivo in a mouse model and observed an increase in DNA damage in breast tumours mainly caused by cortisol. This effect is thought to involve inducible nitric oxide synthase (iNOS) pathway to produce detrimental levels of NOS in a non-genomic pathway.

1.2.9 Stress and DNA damage in immune cells

A study showed impaired DNA damage repair and cell apoptosis in unstimulated (resting) T lymphocytes when they were subjected to irradiation and chemotherapeutic agents. It was found that DNA damage fails to induce γ-H2AX formation and DNA repair. This was reported to be due to a defect in the ataxia-telangiectasia mutated (ATM)/ATM-and Rad3-Related (ATR), and DNA- dependent protein kinases (DNA-PKcs) that play a key role in the phosphorylation of H2AX in response to DSBs DNA damage. However, when activated T cells (proliferating) were subjected to the same stressors, DNA damage was efficiently repaired. Further, when mice were subjected to a DNA damaging stressor, they displayed fewer T cells in their peripheral blood, lymph nodes and spleens (108).

Although DNA repair studies investigated the impact of stress on T lymphocytes repair, the impact of acute and chronic exposure to stress on DNA damage in immune cells has been poorly elucidated to date. Exploring this effect can lead to advancement in the reduction in cancer progression and enhanced patient survival. In this study, the impact of stress and cortisol on DNA damage in splenocytes and T lymphocytes will be investigated.

1.3 Immune system

1.3.1 Introduction to the immune system

The immune system consists of cellular and humoral (fluid) components which work together to preserve the integrity of the body by protecting it from hazards, such as pathogens, as well as helping the body to maintain tolerance to self-antigen (109). It branches into two different but interacting arms: the innate and adaptive (acquired) immunity. The former provides rapid and non-specific protection against hazards such as microbes and transformed cells through inflammation and phagocytosis, the

latter provides latent, specific protection mediated by antibodies and lymphocytes. Adaptive immunity provides the host with immune memory for subsequent invasion by the same invader. Phagocytes and lymphocytes collectively known as leukocytes and stored in lymphoid organs, which include thymus, bone marrow, spleen, and lymph nodes.

1.3.2 Innate immunity

The innate immunity plays a key role in maintaining a healthy tissue environment by rapidly acting upon microbial infections and danger signals from tissue injuries (110). The function of the innate immune system at steady state is to maintain tolerance to self-antigens. When phagocytic macrophages encounter invaders such as bacteria in the tissues through surface receptors (e.g., pattern recognition receptors (PRR) of which the most important are the Toll Like Receptors (TLR), they firstly release cytokines which cause vasodilation and consequently the release of fluid, proteins, and inflammatory cells into the tissues. Secondly, they release chemokines which recruit and direct the inflammatory cells such as neutrophils to the site of infection to eradicate it, consequently causing redness, swelling, pain and heat, common signs of inflammation. Complement proteins can also stimulate phagocytosis and inflammation through the release of small peptides which triggers surface receptors on macrophages and neutrophils**.** Thus, innate immunity provides the body with rapid, non-specific and effective first line defence then prepare the adaptive immune system by presenting the engulfed pathogen (antigens) to the lymphocytes by the DCs at the regional lymph nodes (110, 111).

1.3.3 Antigen presentation by DCs

Dendritic cells (DCs) are immune cells discovered in 1973 by Cohn and Steinman(112). DCs are professional antigen presenting cells (APCs) because they play a prominent role at initiating and modulating specific immune responses. DCs are considered as the core of anti-tumour immune responses (80, 113, 114). They originate from the bone marrow then migrate to peripheral and lymphoid tissues where they reside in an immature phenotype (111). Immature DCs are characterised

by high endocytic capacity and low expression of co-stimulatory molecules such as: CD40, CD86 and surface markers such as MHC I and II. DCs are deployed throughout the body to monitor for pathogen associated molecular pattern (PAMP) such as lipopolysaccharide (LPS) and damage associated molecular pattern (DAMP) such as damaged and transformed cells through surface receptors or through non-surface receptors-dependent (intracellular) process. Therefore, they are the sentinels of the immune system. (111, 115).

Once DC capture antigens and in the presence of activating stimulus from present inflammatory signals such as cytokines and adhesion molecules, they mature into highly effective APCs to present their antigen and migrate to a secondary lymphoid organ namely: lymph node and spleen. Maturation of DCs involves essential morphological and functional changes that facilitate T cells activation characterised by the expression of surface markers (MHC I&II) and costimulatory or adhesion molecules in addition to the production of specific cytokines and chemokines (111, 116, 117) (figure 4).

Upon presentation of antigen to naïve T cells, they activate, differentiate, and proliferate into different effector subtypes to initiate and modulate antigen specific immune response. Conversely, immature DCs (iDCs) present self-antigens to T cells to maintain immunological tolerance either through the induction of anergy T cells or the differentiation of regulatory $CD4^{+}CD25^{+}FoxP3^{+}$ T cells (Tregs). This will prevent the body from attacking its own antigens at a steady state and protect it against autoimmunity (118). Tolerogenic DCs phenotype develops when DCs are unable to mature, this is believed to happens in the presence of immune modulatory factors such as IL-6, IL-10 and VEGF, exosomes, tumour derived soluble factors and the activation of ER stress response, subsequently they inhibit the adaptive immune response. For instance, differentiation of human DC in the presence of the GC dexamethasone resulted in more immature DCs (119). Further, to investigate the innate immunity receptors for tolerogenic DCs It was found that triggering TLRs on

these cells results in upregulation of TLR2, enhanced production of IL-10 and reduced production of IL-12 mediated cytokines (120, 121).

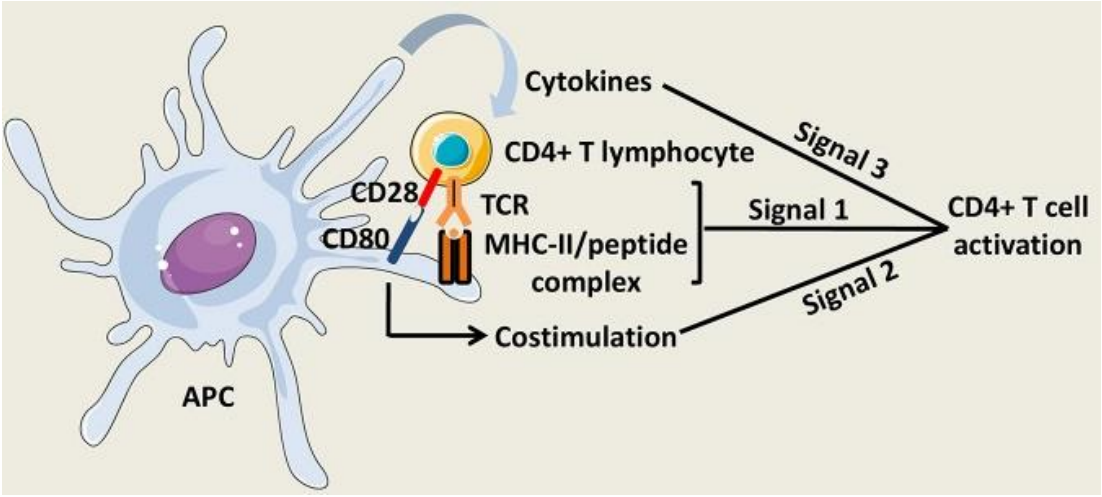


Figure 4: Activation of CD4⁺T cells
Three signals are required to fully activate CD4⁺T lymphocytes: First signal result from the interaction between T cells receptors (TCR) and the antigens presented on MHCII expressed by APC. The second signal result from the binding of costimulatory molecules such as CD80 on APC and CD28 on CD4⁺T cells to enable cell to cell adhesion and the third signal results from the release of soluble factors such as cytokines from APC. *APC- Antigen Presenting Cell, MHCII-Major Histocompatibility Class II, TCR-T cells receptor*. Taken from (117).

1.3.4 Adaptive immunity

The adaptive immune system consists of B and T lymphocytes. B lymphocytes are produced and matured in the bone marrow to provide antibody-mediated, antigen-specific immunity Meanwhile, T lymphocytes are processed by the thymus gland to become differentiated, matured, and functional to deliver cell-mediated, antigen-specific immunity. Mature T lymphocytes express on their surface either the glycoprotein CD8 and are known as cytotoxic $CD8^+$ T cells which actively kills any infected or abnormal cells upon activation, or the glycoprotein CD4 and known as helper $CD4^+$ T cells. Once $CD4^+$ T cells are primed, they differentiate into different T helper cells (Th) subsets with characteristic effector functions dependent on the cytokines they release (80, 111).

Once naive Th cells are primed by DCs, they differentiate into different lineages mainly: Th1, Th2, Th17 and T regulatory cells (T reg) to perform an immune response

against pathogenic microorganisms, damaged and transformed cells or self-antigen (117). The different subsets of $CD4^{+}$T cells in turn release specific cytokines to perform different functions (111, 122). Th1 cells are activated by the pro inflammatory cytokines: Interleukin 12 (IL -12) and interferon-γ (IFN-γ) to release pro- inflammatory cytokines such as IFN-γ, IL-2 and tumour necrosis factor (TNF) to assist the activation of effector memory T cells, natural killer (NK) cells and macrophages against intracellular pathogens and tumour cells.

Th2 cells are activated by IL-4, then they release anti-inflammatory cytokines: IL-4, IL-5, IL-10 and IL-13 to induce immunity against parasites, inflammatory functions and production of antibodies by B cells (123). Th-17 cells are activated by the cytokines: IL-1, IL-6, IL-23, TGF- β to release cytokines IL-17, IL-22, IL-25 to induce immunity against exogenous pathogens (124) and maintain tissues homeostasis. Thus, a controversial pro and anti-inflammatory role for Th17.

Finally, Tregs cells are identified by FoxP3 (a very specific marker that Tregs express intracellularly) and activated by IL-2 &TGF-β to release IL-10, TGF-β which suppress the immune response to maintain homeostasis and self-tolerance. Therefore, they play a key role in protecting the host against autoimmunity. Modulating Tregs is a potential therapeutic target for treating inflammatory disorders such as allergy and allograft rejection (117, 125). Contrarily, the presence of Tregs in the EOC tumour microenvironment was associated with tumour progression and poor prognosis (126). It was demonstrated that Tregs get recruited and have their function altered by the cancer tumour under the influence of chemokines specific for Tregs receptors (127). Subsequently, Tregs release potent immunosuppressive molecules such as the interleukins: IL-10, TGF-β, IL-35 which in turn suppress the cytotoxic activity of effector T cells additionally, IL-10 and TGF-β are reported to induce the formation of tolerogenic phenotype of DCs (128). Tregs also stimulate the release of VEGFA which is vital for the induction of angiogenesis and promotion of tumour progression (figure 5). Moreover, it has been reported that Tregs in the ovarian TME inhibits the function of effector T cells by modulating DCs maturation and function. This inhibition was found to be mediated through the increased catabolism of tryptophan by the enzyme indoleamine 2,3-dioxygenase (IDO) resulting in immune suppression (129), (table 2).

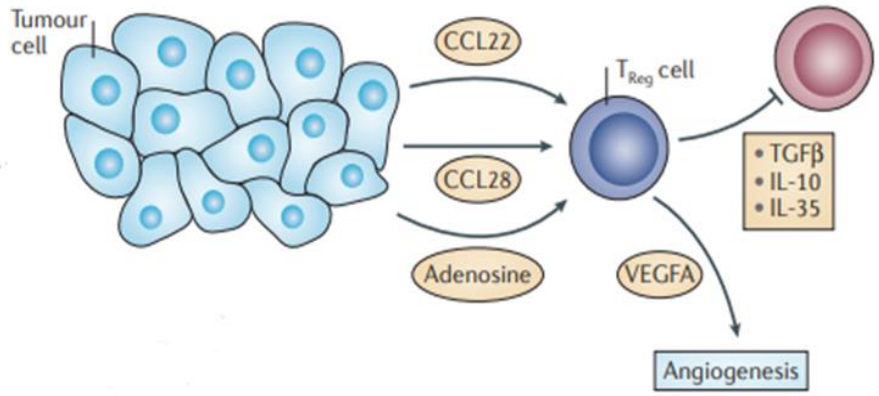


Figure 5: Recruitment of T regs by the tumour cells
Tumour cells secrete chemokines such as CCL22& CCL28 which attract Treg cells and stimulate them to secrete potent immunosuppressive cytokines such as: IL-10, TGF-β & IL-35: to promote tumour growth and metastasis through suppression of T effector cells and induction of angiogenesis. *CCL - Chemokine ligands, IL- Interleukins, VEGFA- Vascular endothelial growth factor A, TGF β- Transforming growth factor-β.* Adapted from (127).

Subsets of naïve $CD4^+$ T helper cells	cytokines involved in the differentiation of naïve $CD4^+$ T helper cells	Cytokines released by the differentiated T cells to enable their function	Transcription factor	Function of T cells subsets
Th1	IL-12, IFN-γ (pro inflammatory cytokines) and anti-tumour function	IFN-γ, TNF-α	T bet	▪ Induction of immune response against intracellular pathogens (bacteria and viruses) & cancer immunosurveillance ▪ generation of effector and memory CD8+ T cells ▪ & Activation of macrophages
Th2	IL-4	IL-4, IL-5 & IL-13	GATA3	▪ Induce immunity against parasites ▪ Antibodies (IgE) production by B cells ▪ inflammatory function
T regs	IL-2 &TGF-β	IL-10, TGF- β	FOXP3	▪ Supress the immune response and ▪ regulate self-tolerance
Th-17	IL- 1β, IL-6, IL-23, TGF- β	IL-17, IL-22, IL-25	RORγt	Enable host defence against exogenous pathogens, tissue homeostasis, cancer immunosurveillance & inflammation pro & anti-inflammatory. A controversial role?

Table 2: Subsets of naïve CD4+ T cells upon activation with antigen presented by DCs
They differentiate into different lineages mainly: Th1, Th2, Th17 and T regulatory cells (T regs) to perform an immune response against pathogenic microorganisms, damaged and transformed cells, regulate tolerance and immunosurveillance. Adapted from (122).

1.3.5 Local immunity at the gut mucosa

Local immunity is triggered by (PAMP) such as lipopolysaccharide and flagella when detected by pattern recognition receptors (PRR) expressed on some immune cells in the gut like dendritic cells (DCs) as well as the intestinal epithelial cells (IECs). Short chain fatty acids (SCFAs) are metabolites of fibre, which is fermented by commensal bacterial, further play a role in enhancing local immunity by stimulating the production of antibodies IgG by plasma cells (130). Once DCs are triggered by PAMPs at the LP (lamina propria), they mature by expressing receptors and excreting cytokines, then become activated and migrate to the mesentery lymph node (m LN) of the small bowel and colon (where all the gut LNs drain) to present their antigens to $CD4^+$T cells or sometimes directly to $CD8^+$. $CD4^+$T cells differentiate into regulatory T cells (Tregs) or Th17 (131). Tregs maintain homeostasis at the gut mucosa by developing tolerance to mucosa's commensals. This is achieved by keeping the commensal microbiota confined to the mucosa and by secreting immunosuppressive cytokines such as IL-10 and TGF-β to prevent prolonged activation (132, 133). Meanwhile, Th17 cells play a key role in protecting the epithelium against bacterial and fungal infections through secretion of inflammatory cytokines such as IL-17 (134) to maintain tissue homeostasis (117).

1.3.6 Cancer immunoediting and immune surveillance

The idea of cancer immune surveillance was only accepted in the 1990s when experimental models of knockout mice were used to validate the existence of cancer Immune surveillance. Using chemically induced and spontaneous tumours, the key roles of different effector cells such as B and T cells, NK and NKT cells in the immune surveillance was identified (135).

Immune-surveillance is an extrinsic cell process whereby transformed cells that have escaped the cell intrinsic tumour-suppressor mechanisms including apoptosis and senescence are identified and destroyed before they manifest clinically. A competent immune system will protect the host against carcinogenesis and maintain cellular homeostasis (136, 137). This protection could be achieved by detecting and

destroying viruses that induce tumours. Protection could also occur by suppressing prolonged inflammation which is likely to induce tumorigenesis and finally by destroying transformed cells expressing specific antigens to establish long lasting antigen-specific immunity (136). Despite that, tumours can escape immune surveillance, progress, and become malignant (135, 138).

Later, the immune editing theory was developed to better describe the process of tumour immunogenicity which involves three phases: elimination, equilibrium, and escape (known as the 3 E's'), each phase involves several effector cells and cytokines (135, 138). When the elimination of the tumour is only partially complete, a temporary state of equilibrium develops between the tumour (transformed cells) and the immune system. During this stage, the tumours either remain functionally dormant and clinically unapparent under the control of the immune system or continue to evolve and progress by undergoing structural changes such as DNA mutations and genetic instability or by developing variants that are resistant to the immune effector cells and then escape because of their low immunogenicity (137). Escape can occur via two possible pathways: firstly, the loss of surface antigen expression and the recruitment/infiltration of immunosuppressive cells and secondly, block off immune enhancing (effector) cells. This leads to immunological resistance and evasion then invasion, intravasation, dissemination and colonisation at secondary sites such as the lung, liver and peritoneum resulting in a clinically apparent disease (139). Indeed, metastasis is the major cause of death in cancer and responsible for 90% of cancer mortality (140). Evasion of immunosurveillance is considered as the seventh hallmark of cancer (141). Cancer hallmarks including core and emerging are illustrated below (figure 6a & b)

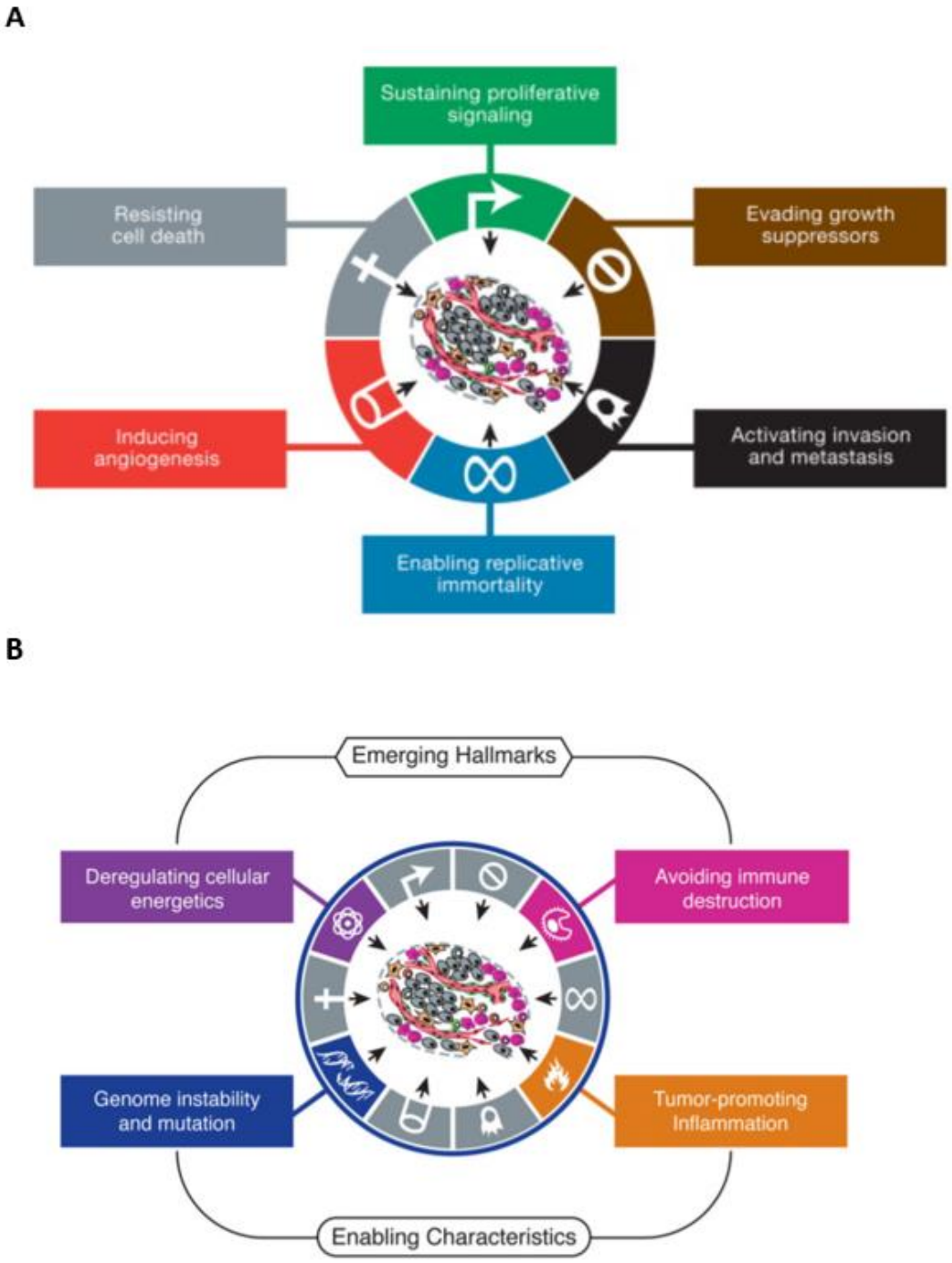


Figure 6: Hallmarks of cancer as illustrated by Hanahan and Weinberg
It illustrates the biological capabilities acquired by the cell during the development of a tumour. (A) illustrates the 6 core hallmarks and (B) describes the 2 emerging hallmarks (they have not yet been generalised and validated to all cancers) and the underlying enabling characters which facilitate the acquisition of both the core and emerging hallmarks Taken from (141).

1.3.7 Ovarian cancer is immunogenic

Advancement in technology and molecular cell biology have enhanced our understanding of the nature of EOC and some mechanisms for its pathogenicity. It is a heterogenous disease with diverse types of tumours different in cellular origin, morphology, hereditary pathologies, molecular alterations, and potential for targeted therapy (23). This contributes to the resistance of these tumours to both chemo and other targeted therapies. Further, ovarian cancer has a complex, immune-suppressive microenvironment (142). Altogether they explain the slow progress in successful treatments and the importance of targeting the tumour micro environment as a potential to restore immune surveillance (143).

Evidence from epidemiologic and clinical data (144-148) showed that there is an association between Tumour-infiltrating T lymphocytes (TILs) in the EOC tumour microenvironment (TME) and improved patients' overall survival (OS) (149). Survival at 5 years was 38% in patients whose tumours had TIL cells (n=102) as opposed to only 4.5% (n=72) in patients lacking them. In another large, pooled cohort of patients with HGSOC treated with platinum-based chemotherapy, the presence of multiple immune cells such as macrophages and $CD8^+$ T cells in their tumours was associated with increased survival (5). Furthermore, a meta-analysis of 2903 EOC patients observed a strong association between intraepithelial $CD3^+$ $CD8^+$ TILs and improved progress free/ disease free survival (PFS/DFS). Moreover, when 186 frozen specimens of advanced stage EOC were analysed for TILs distributions, a correlation was found between intratumoural T cell and improved clinical outcome (146). This further supports the role of intratumour TILs as an accurate predictor for clinical outcome and prognosis in EOC (150).

Meanwhile, an association between tumour immune evasion and poor prognosis was identified (25, 28). Taking this evidence together, EOC is intrinsically immunogenic, and patients are likely to benefit from the alternative immunotherapies interventions that have revolutionised the treatment of other kind of immunogenic tumours such as melanoma, lung, and kidney cancers (151). These therapies include Immune checkpoint blockade, cancer vaccines and adoptive cell therapy (148, 151).

The aim of immunotherapy is to initiate a prolonged tumour-specific immune response involving both the innate and adaptive immune system to permanently destroy the tumour and prevent any future relapses. Thus, shifting the balance from immunosuppression to immune surveillance or moving the immune response from the escape phase to the elimination or equilibrium (144, 152, 153) (figure 7). Successful initiation of long lasting, tumour specific effector immune response requires: First, effective presentation of the tumour associated antigen (TAA) and homing of the pulsed APC to the lymph nodes to prime T cells. Second, expansion of the primed cytotoxic T cells into large number to undertake a long lasting TAA specific attack (154-156). Third, enhancing trafficking of TAA-primed cytotoxic T cells & infiltration into the TME. Finally, overcoming tumour microenvironment (TME) immune suppression (28). The residual resistant cancer cells following chemotherapy are the ideal target for immunotherapy.

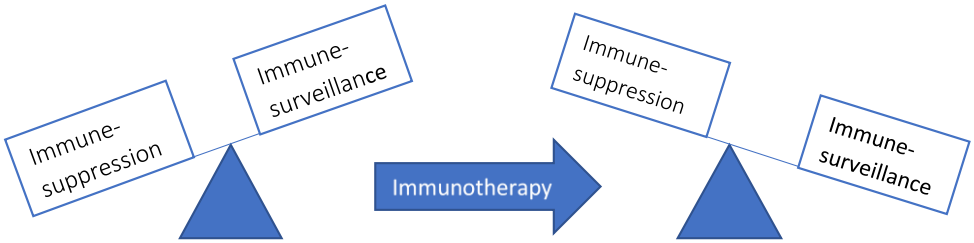


Figure 7: Schematic illustration of the immune balance during cancer development and after immunotherapy
Immunotherapy in cancer is hoped to shift the balance from immune suppression to immune surveillance (152).

1.3.8 Approaches in immunotherapy and their role in Ovarian Cancer

Immunotherapy is undergoing testing in epithelial ovarian cancer and there have been modest responses observed in the clinics so far (144). Cytotoxic T cells were found to be exhausted in a tumour environment which could either be induced by a chronic infection or cancer. Exhausted T cells start overexpressing inhibitory receptors such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and program death-1 (PD-1). Also, they produce less effector cytokines such as IL-2, IFN-γ and TNF-α, subsequently they become hyporesponsive and exert less cytotoxic activity which dampen their ability to identify and eliminate cancer leading to cancer evasion.

Therefore, restoring these exhausted T cells (to turn the immune cold ovarian tumour into immune hot) is a key target for therapies in cancer (157). Evidence is showing that TILs-infiltrated ovarian TME is correlated with enhanced overall survival, while immunosuppressant TME is associated with poor prognosis (149).

Unexpectedly, immune therapy has only showed a moderate success clinically in EOC patients. For instance: patients with platinum-resistant ovarian cancer showed a 17% complete response rate when they received weekly intraperitoneal infusion of the effector cytokine Interleukin 2 (IL-2) (158, 159). Different types of immunotherapies have been tried in EOC.

Immune checkpoint inhibitors (ICI)

ICI involve the blockade of PD1/PD-L1 or (CTLA-4) which are the most studied immune check points. When PD1 binds to its ligand PD-L1, the activation and proliferation of T cells is inhibited (160). It was found that administration of ICIs as monotherapies in EOC patients controlled the disease in less than half of the patients and only a median response rates of 10–15% were achieved overall (149). However, combinations of PARPIs and PD-1/PD-L1 blockade is showing an enhanced anti-tumour immunity without an increased toxicity in EOC. This promising combination is currently under investigations in few phase III clinical trials illustrated in (149).

Recently, the gut microbiome and their associated immune modulation have been identified as contributary factors to the inter-individual variation in response to anti-cancer treatments. It was observed in preclinical models and patients with metastatic melanoma that higher abundances of the commensals *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* stimulate more $CD4^+$ $CD8^+$ effector T cells resulting in a more favourable T cell-mediated response to anti-PD1 therapy (161). 112 patients on immunotherapy with PD1 inhibitor for melanoma underwent assessment for their oral and gut microbiota, microbiota analysis of responder patients showed higher diversity and composition compared to the non-responders. Immune profiling of responders and of germ-free mice received faecal transplant of responder's patients showed an enhanced systemic and antitumour immunity in response to PD1 therapy (162). Similar observations were reported in patients with

advanced epithelial tumour where faecal abundance of *Akkermansia muciniphila* was associated with better response to PD1 blockade, this was correlated with enhanced $CCR9^{+}CXCR3^{+}CD4^{+}$ T lymphocyte recruitment to the tumour mediated by increased IL-12 secretion (163). Also, enhanced response to CTLA-4 blockade was shown in both mice and patients to be associated with distinct gut microbiome signature (164).

It was further shown in both mice and human that ICI toxicity is associated with the gut microbiota signature. Vetizou and colleagues showed that colitis (an immune - mediated complication of CTLA-4 blockade) is microbiota dependent and has the potential to antagonise the anti-tumour effect of CTLA-4 blockade. Prophylactic treatment with Bacteroidales probiotic protected against colitis and enhanced the efficacy of CTLA-4 inhibitor (164). Others also demonstrated in human cohort of metastatic melanoma that the use of patients stools at the onset of ICI therapy could be used to identify biomarkers that are likely to predict ICI toxicity (162). Altogether, the evidence suggests a potential valuable role for microbiota in predicting not only the response to ICI but also susceptibility to toxic complications such as colitis which make the gut microbiota targeted as valued diagnostic and therapeutic tool in ICIs recipients.

Adoptive cell transfer (ACT)

ACT is another promising personalised approach to activate effector immunity in the TME. ACT involves the transplant of autologous or allogeneic TILs or genetically engineered T cells to express novel receptors (TCR) that can recognise tumour specific antigens, though, they can only recognise major histocompatibility complex (MHC)-expressed antigens. Or Chimeric antigen receptors (CAR)-T cells which on the other hand can recognise tumour antigens independent of MHC. These T cells are first expanded *in vitro* before they are infused into the patients (165). ACT provides a promising improvement in clinical outcomes for patients with solid tumours. Nonetheless, it is still facing a lot of challenges such as the manufacturing process and the cost; good manufacturing practice is complicated and expensive. Also, only limited data is available in EOC (166, 167).

Vaccines

Vaccines are another personalised cell therapy in cancer, they have been the main approach to OC immunotherapy because they elicit a tumour specific immune response. The ability of dendritic cells to initiate a profound primary immune response that is specific to tumour antigens and persistent make them an ideal target for vaccination. DCs are pulsed *ex vivo* with tumour antigens (autologous or allogenic antigens) then infused into patients to elicit a tumour antigen specific attack (figure 8). In fact, tumour-pulsed DCs vaccines have been a very promising personalised form of immunotherapy particularly in late-stage melanoma (which has a strong immune component) and prostate cancer. Stage III and IV melanoma patients were vaccinated with autologous DCs pulsed with allogenic lysate from 3 melanoma cell lines showed increased survival and disease stability, implying that the lysate provided sufficient antigens to provoke very effective anti-melanoma immune response (168).

Further, DCs pulsed with prostate cancer tumour lysate showed biochemical and memory immune response in castration-resistant prostate cancer patients. Unfortunately, due to the enormous variation at each stage of the vaccine preparation including: preparation of immature DCs (iDC) and tumour associated antigens (TAA), maturation of iDC, co-culturing (DCs-TAA) time and route of injecting the pulsed DCs that enables the migration of TAA-pulsed DCs to the draining lymph nodes. Therefore, variations in the results were observed. In addition to the lack of well characterised immunodominant antigen on the ovarian tumour, poor clinical success has been observed (113, 152). In this thesis bone marrow generated DCs will be pulsed with ovarian tumour as a source of known and unknown antigens.

The recent improvements in vaccines development along with the identification of different tumour associated antigens in ovarian tumour such as *P53*, mucin antigen 1 (*MUC 1*), *NY-ESO-1* (it is the most immunogenic antigen to date, expressed only in cancer tissues but not in somatic cells except for the germ cells of the testis (169) and others have enhanced the clinical benefits of ovarian cancer patients (170). For instance, expression of NY-ESO-1 on ovarian tumour was linked to aggressive

phenotypes and predicted poor prognosis (169). When a clinical trial was conducted in EOC patients, an OS of 2 years was observed in patients with NY-ESO-1 positive tumours who received vaccines compared with those how did not have it (171). Despite the observed clinical benefits from vaccines, there are still challenges facing the development of safe and effective vaccines for OC patients. The molecular heterogeneity of the ovarian tumour and its immune suppressive nature are by far the most significant challenges (170).

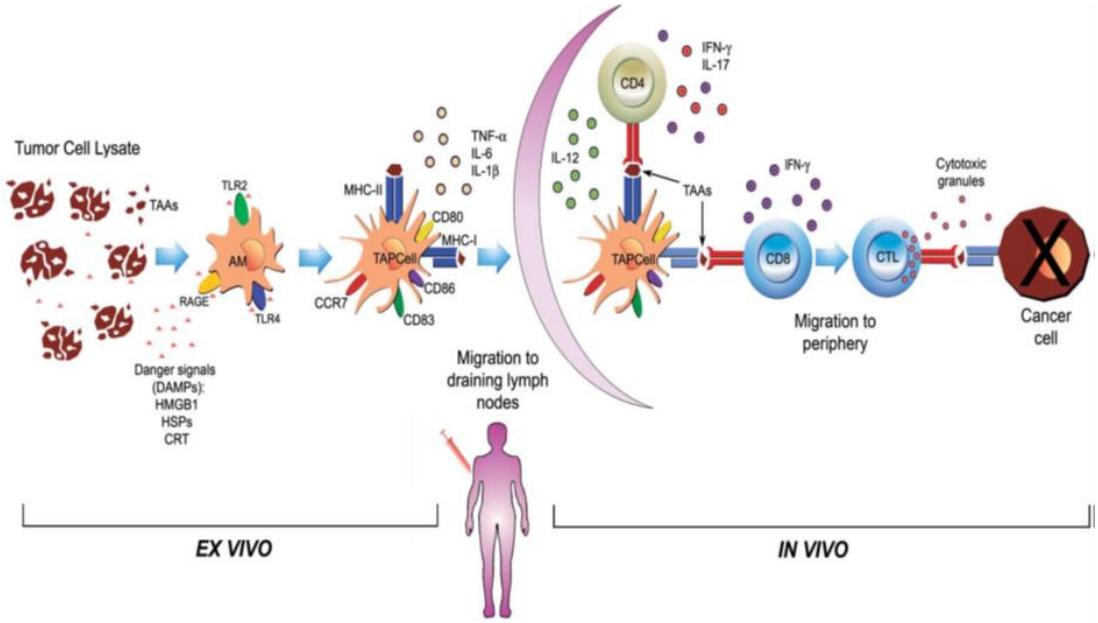


Figure 8: Illustration of the immune-stimulatory role of dendritic cells when pulsed with whole tumour lysate
When DCs are pulsed with tumour lysate, they mature by expressing the major histocompatibility complex (MHC) class I/II, co-stimulatory factors such as CD80 and other activation markers. In addition, they release cytokines including IL-6, TNF-α and IL-1β. Subsequently, they migrate to the nearest lymph nodes to present the tumour associated antigens (TAA) to both: CD4 and CD8 and prime them. Primed T cells in turn release effector cytokines such as IL-12, TNF-α and IFN- γ then migrate to the peripheries to identify and perform a tumour-specific attack. *IL-6 Interleukin 6, TNF-Tumour necrosis factor, IFN-Interferon, IL-1β- Interleukin 1 beta, IL-12 Interleukin 12*. Taken from (172).

Tumour microenvironment and resistance to treatment

The correlation between T cells infiltrates into TME and clinical outcome in solid tumours has been extensively studied. Indeed, the quality, quantity, and location of the cytotoxic and memory cells (known as immune contexture) within the solid tumour have been accurate predictor of both clinical outcome and immune response to ICI. In a large cohort of colorectal cancer patients, the immune contexture analysis gave better prediction of survival than the traditional histopathological methods that

are currently used to stage colorectal cancer. The results were validated in 2 more patients' population (173). Also, when 186 frozen specimens of advanced stage OC were analysed for TILs distributions, correlation was found between intratumoural T cell and improved clinical outcome (146). Meanwhile, the density of TILs has been associated with the genomic characteristic of the tumour; the more genetically immune heterogenous the tumour is, the less T cells infiltration is observed (174).

Importantly, the availability of 'omics' technology (genomic, proteomic and transcriptomic) unravelled the molecular signature of EOC tumour microenvironment (TME) (175). This enabled the identification of effector immunity-suppressive components such as: Tregs, vascular endothelial growth factor (VEGF), myeloid derived suppressive cells (MDSC), IL-10, IL-6, prostaglandin E2 (PGE2), tumour associated macrophages (TAM) and IDO which is found to be the dominant immune suppressant molecule in TME. The presence of these molecules in the TME of EOC is correlated with tumour progression and poor prognosis. (28, 141, 175). Additionally, the enhanced understanding of the ovarian TME architecture which is found to be composed of cancer, stromal and immune cells that cross talk through secretory molecules such as cytokines, chemokines, and receptors such as MHC and programmed death-ligand 1 (PD-L1). This communication system within the heterogenous environment is regulated by cellular and molecular network resulting in either tumour progression or suppression. Therefore, ovarian TME has been a potential target for immunotherapy with prime focus on targeting: TAMs, angiogenesis and immune checkpoints (175). However, the immune suppression and tumour heterogeneity have been major obstacles for TME targeted therapies.

In summary, cancer immunotherapies are emerging as promising treatments in EOC. Unfortunately, their toxic side effects, the short period of remission and modest response associated with immunotherapies, in addition to the interindividual variations in response or susceptibility to toxicity hinder their efficacy. There is a need for therapies that are not toxic or provide less toxicity, effective and can help to improve ovarian cancer patient's quality of life. Immune signature and gut

microbiome are found to be contributing factors to the interindividual variations observed (176).

1.4 Microbiota and probiotic

1.4.1 Gut microbiome and homeostasis

The microbiome-host form a diverse complex ecosystem, composed of one hundred trillion organisms of more than thousand different species (177). Human microbiota consists of mostly bacteria and some fungi, viruses, and protozoa, inhabit different niches of the epidermal and mucosal tissues of the host including: the lung, skin, mucous membrane of the oral cavity, vagina, and gastrointestinal tract which is the most highly colonised organ (177, 178). While the term microbiome is a collective name used to describe the genetic material of the microbiota and their interactions with the environment (179). Research studies exploring the microbiome along with culture-independent genomic techniques are showing an active role for the microbiota on the function of human's immune, metabolic, and neurobehavioral systems which impact the health and fitness of the individual (178, 180). Therefore, gut microbiota is often described as the 'virtual organ' and their gene catalogue is known as 'our other genome' (181).

The microbiota has co-evolved with their host towards mutualism and homeostasis, whereby an equilibrium is maintained and benefits are exchanged between commensal microbiota and the immune system at steady state (182). While the microbiota provides the host with energy (through degradation of plant's polysaccharides), protect it against pathogenic microorganisms and barrier damage, in addition to shaping the function, and development of its immune system. The host in return, provides the microbiota with shelter, nutrition, and protection from the immune system. Germ free (GF) animal models have enhanced our understanding of the relationship between the commensal microbes and immune function.

In a pre-clinical mouse model, severe destruction to the lymphoid tissues structure and impaired mucous production were observed in GF mice, highlighting the importance of gut microbiota in maintaining the immune functions (183, 184). The

gut microbiota and the host-immune system crosstalk at the intestinal mucosa (a single layer of epithelial cells separating the intestinal lumen from the underlying tissues), particularly at the Lamina propria (LP) which is the richest source of lymphocytes at the human mucosa. Though, evidence is showing a crosstalk is taking place at further extraintestinal sites (189). Beneath the intestinal mucosa lie Peyer's patches which maintain symbiosis and homeostasis at the mucosa where tolerance is developed to commensal microbiota and the immune response is reserved to pathogens (163, 190).

For instance, Tregs are generated in response to signals from commensal microbial such as their metabolites (e.g. short chain fatty acids) to maintain gut homeostasis. Also, the host gut associated lymphoid tissues (GALT) which is the largest lymphoid tissue in the body and consist of the immune cells: antigen presenting cells (APC), $CD4^+$ & $CD8^+$ T cells, B cells and innate lymphoid cells (ILC). Altogether, constitute a large part of the body's immune system and play a prominent role in the immune response at a local and systemic level (191).

The immune response to the microbiota at steady state is strictly by compartmentalization that minimises their direct contact with the mucosa (185). This is achieved through the development of mucous layers, tight junctions, secretory IgA antibodies and antimicrobial peptides (186) to restrict trans-epithelial permeability of commensals and maintain immune surveillance (176, 187). DCs are the key cells in compartmentalisation as they constantly sample gut bacteria for antigen presentations. Commensal-loaded DCs present their antigens at the mesenteric lymph nodes to induce a local immune response.

Studies are now showing that perturbation of the microbiome-immune equilibrium can be driven by modifiable factors such as: diet, disease, medications and infections or none-modifiable factors such as: age and genetics (182, 183) or by defects in the regulatory mechanism controlling homeostasis (172) resulting in disruption to the composition and function of the microbiota (dysbiosis), loss in the barrier integrity and function. Consequently, translocation of commensals and pathobiont

(commensals with pathogenic potentials) occurs which is likely to activate the mucosal and systemic immunity (127). All of which are likely to increase the propensity for gastrointestinal immune related pathologies such as inflammatory bowel disease (IBD) or extra-intestinal pathologies such as, carcinogenesis or cancer progression, rheumatoid arthritis, and others (127, 178). The mechanisms underlying the microbiome-immune dysregulation and the precipitation of immune-mediated disorders in humans are not fully elucidated.

Our enhanced understanding of the gut microbiome complex composition and its essential role in human's health and disease have stemmed from advances in technologies such as second-generation sequencing (16S ribosomal RNA and shotgun metagenomics) and computational techniques, which enabled thorough characterisation of microbes down to strains, unravelled their metabolites and functional pathways (176). Consequently, possible mechanisms have been probed for their roles in cancer (183, 193). The gut microbiome has been correlated with cancer initiation, progression, anti-cancer treatments 'efficacy and toxicity mainly check points inhibitors (127, 176). Therefore, growing interests have risen in developing new approaches to manipulate the microbiome composition and function to treat or prevent cancer and optimise anti-cancer therapies and minimise their toxicity.

1.4.2 Gut dysbiosis and cancer development

The bacterial microbiome has the potential to promote carcinogenesis locally at the intestine or at extra intestinal site such as liver, brain, and ovaries. It is believed that infectious diseases attribute to over 15% of cancer malignancies worldwide (188). Oncogenic bacteria have been implicated in carcinogenesis either through interference with the genes regulating the cell cycle, apoptosis, and other tumour-suppressor mechanisms or through modulation of inflammation and immunity. For instance, Helicobacter pylori has been associated with induced gastric inflammation leading to gastric cancer or mucosa-associated lymphoid tissue (MALT) lymphoma (189).

Further, potent chronic inflammation secondary to dysbiosis of the gut can drive carcinogenesis through 3 different mechanisms: firstly, translocation of the gut microbe and stimulation of Toll Like Receptors (TLRs) via microbe-associated molecular pattern (MAMPs) initiating potent inflammations. TLRs play a pivotal role in innate immunity and are estimated to be one of the most powerful pro-inflammatory stimuli (212). They are expressed by different type of cells mainly immune cells. when TLRs are triggered by MAMPs, pro-inflammatory cytokines such as TNF, IL-1β and IL-23 are released. These cytokines in turn induce Th17 which activates transcription factors: nuclear factor kappa B (NF-kB) and Signal Transducer and Activator of transcription (STAT). Consequently, causing proliferation and an anti-apoptotic effect (a hall mark of cancer).

Secondly, some bacterial strains promote carcinogenesis through the production of genotoxins, increased Reactive Oxygen species (ROS) and hydrogen sulphide (H2S) which act directly on DNA causing its damage. The production of the genotoxin colibactin from E. coli was shown to promote colon tumour growth (190). Third, the production of tumour-promoting metabolites which lead to genomic instability and aberrant proliferation (figure 9).

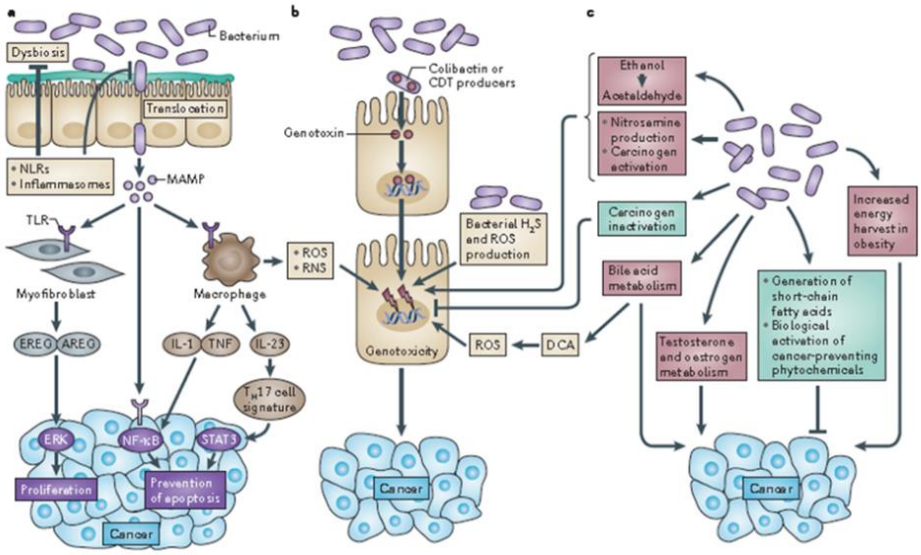


Figure 9: illustrates the possible mechanisms for microbiome mediated carcinogenesis

Dysbiosis and translocation of the gut microbiome stimulate potent inflammation mediated by TLRs activation (A). Genotoxicity mediated by carcinogens and genotoxins such as Reactive Oxygen species (ROS) and hydrogen sulphide (H_2S) causing DNA damage (B). The metabolic action of the microbiome result in the production of tumour-promoting metabolites which lead to genomic instability and aberrant proliferation (C). Taken from (127).

1.4.5 Microbiome and metastasis

A significant amount of research has investigated the role of the microbiome in tumour initiation. However, little has been done to investigate the impact of the microbiome on cancer metastasis. It has been shown in a recent study that microbes from primary tumours travel with metastatic cells to distant sites. Biopsies from patients with colorectal cancer have *Fusobacterium nucleatum*, the most prevalent bacteria of the gut microbiome in colorectal cancer tissues. The same bacteria are found in biopsies from liver metastasis of the same patients suggesting that these bacteria have travelled from the primary tumour to a distant metastatic site and maintained stability. This result was validated by mouse xenograft of human primary colorectal adenocarcinomas which was found to retain viable Fusobacterium on successive passages. It was demonstrated that treating the mouse with the antibiotic metronidazole reduced Fusobacterium load, proliferation of cancer cells and tumour growth (191).

Furthermore, others have demonstrated in a Hormone Receptor-Positive (HR^+) mammary cancer mouse model a role for the gut microbiome dysbiosis in promoting breast cancer metastasis (192). Conversely, Zahra Nouri and his colleagues observed an anti-metastatic role for lactic acid bacteria (LAB) when their supernatant was added to human colon adenocarcinoma and cervical cancer cell lines HT-29 & Hela respectively. This anti-metastatic effect was found to be mediated though the manipulation of Matrix Metalloproteases (MMPs) (193).

The variation in experimental results from different studies (194, 195) and the lack of successful reproducibility and translation of promising pre-clinical results clinics limit their successful use in the clinics (196, 197).

1.4.6 Dysbiosis and OC

Dysbiosis can cause depletion of the good microbes such as Lactobacillus and the dominance of anaerobic species (the virulent microbes) which is correlated with gynaecological and obstetric pathologies of which gynaecological cancers (184). In a human xenograft mouse model of ovarian cancer, it was shown that intestinal

disturbances induced by daily feeding of water supplemented with antibiotics was associated with the release of inflammatory cytokines such as (TNF-α and IL-6) in OC tumour. Consequently, the cytokines promote epithelial-mesenchymal transition of OC cells and tumour progress (198). In a population-based cohort study, pelvic inflammatory disease (PID) is found to be correlated with a modest increase in risk of serous OC but not in other histotypes (199). Another population-based case-control study showed increased risk of EOC with history of PID further supporting the evidence for a role for inflammation in the aetiology of OC (200).

Further a notable increase in the ratio of the two phyla Proteobacteria/Firmicute was observed when samples from OC tissues and normal distal fallopian tubes tissues (control) underwent 16s rRNA high-throughput sequencing for composition and diversity of microbiota, suggesting a decrease in richness and diversity indexes in OC tissues. Thus, a role for microbiota composition in the development of OC (201).

Furthermore, when plasma samples from women undergoing surgery for suspected ovarian pathology were analysed for the presence of antibodies for some upper genital tract infections such as C. trachomatis and chlamydial Heat Shock Protein. The infections were found to increase the risk of EOC development (202).

Other studies were conducted and showed a role for chronic infections and inflammation in the development and pathogenesis of EOC (203-205). Finally, in a case control study comparing women with EOC, healthy women and women diagnosed with benign gynaecological conditions, the presence of the community type O cervicovaginal microbiota was associated with the presence of EOC or factors known to increase the risk of OC such as germline BRCA1 mutation and older age (13).

Altogether, this suggests a role for microbial infection in carcinogenesis of OC. However, little is still known about the underlying mechanisms and the identity of the microorganisms involved in OC metastasis.

1.4.7 Microbiome and stress

Studies from RS animal models also show a role for the gut microbiome (65) in the postnatal development of the HPA stress response and a relationship between dysbiosis of the gut, neuroendocrine responses, and the development of stress-related behaviours such as anxiety and depression (66, 67). It was further shown that chronic stress may have a potential effect on the composition and diversity of the gut microbiota which could contribute to disease aetiology such as cancer (e.g. colon cancer). Administration of bioactive dietary supplements: probiotic & prebiotic resulted in a decreased risk in colon cancer through shaping functional gut microbiota (68). This section will be discussed in more detail in chapter 4.

1.5 Probiotics

1.5.1 What are probiotics?

Probiotics have recently been more valued and scientifically evaluated because of the increased understanding of their beneficial role not only locally on the gastrointestinal system but extending to systemic effect mediated by modulation of the immune and inflammatory response to improve human's overall health (206, 207). Therefore, restoring the gut microbiota composition and function is likely to enhance the immune response via innate immune signalling mainly mediated by TLR s or by activation or differentiation of immune cells such as T lymphocytes ($CD8^+$, Th1/Th17) and DCs (208). Probiotics can be bacteria, yeast or mould, collectively known as lactic acid bacteria (LAB) (209) and defined as "non-pathogenic microorganisms which when administered in adequate amounts confer health benefits on the host by improving its microbial balance" (210).

Probiotics have been well known for many years to have various health-promoting effects like anti-pathogenic, antidiabetic, anti-inflammatory, and anti-obesity (209). They are shown to have the potential to down regulate the gene expression for pro-inflammatory cytokines and upregulate the ones for anti-inflammatory cytokines, promote the integrity and function of the epithelial barrier and consequently reinstate homeostasis and immune surveillance (214). For these benefits to persist,

probiotics should be ingested regularly from the outside in a form of yogurt or any fermented food (215). However, these effects may vary depending on the strain studied (217), the state of the disease, dose, and duration of treatment. Therefore, studies illustrating the clinical efficacy of probiotics have been inconclusive, consequently, probiotics have not yet been utilised optimally in the clinics (217, 218).

1.5.2 Escherichia coli Nissle 1917

Probiotics are mainly anaerobic, gram positive none-pathogenic bacteria, though gram negative bacteria have also been used as probiotics; of which *Escherichia coli Nissle 1917* (ECN, serotype O6:K5:H1). *EcN* lacks any defined virulence genes, does not produce toxins, has a semi-rough lipopolysaccharide on its cell wall which increase its serum sensitivity and has flagellum which enables efficacious colonisation of the gut (211, 212). *EcN* was first isolated by Alfred Nissle in 1917 based on its potential to protect from infectious gastroenteritis (213) since then, its genotype, phenotype and clinical efficacy have been well characterised (214). It is now the active component of a pharmaceutical product which has a license in some countries *Mutaflor*® (Ardeypharm GmbH, Herdecke, Germany and *EcN*, Cad group, In Italy). Indeed, it is found to be of comparable efficacy to mesalazine (one of the gold standard treatments for maintaining remission in Ulcerative Colitis; an intestinal auto immune disease) (211).

Further, *EcN* was shown to exert beneficial an anti-inflammatory role which extends beyond the intestine, a study demonstrated using two experimental models of altered immune response: the trinitrobenzenesulphonic acid (TNBS) model of rat colitis and the lipopolysaccharide (LPS) model of systemic septic shock in mice, the inflammatory impact of *EcN* locally and systematically was evaluated. Both models showed reduced production of the TNF-α in the intestine for the colitis rat and in the plasma and lungs for LPS treated mice (215).

Adding to the long valued therapeutic benefits of *EcN* in treating inflammations, infection, and chronic constipation (214, 216, 217), it has been used recently as a vector to deliver drugs, RNA, and immune factors to tumour because of its tumour

targeting properties. Interestingly, Lian et al observed restrained growth of a murine melanoma tumour by *EcN* engineered to express the anti-angiogenic active fragment (Tum-5) (218). More recently, engineered *EcN* (SYNB1891) were injected intratumorally to stimulate a complementary innate immune activation of APCs (activation of the STING-pathway) in the tumours of murine cancer models. A robust activation of APC s and a profound anti-tumour immunity with immunological memory were observed (219). SYNB1891 is currently undergoing phase 1 clinical trial in human

1.5.3 Probiotic in cancer prevention and progression

One of the most controversial effect of probiotics is their anti-tumour effects. The mechanism for their anti-tumour effect is not fully understood. However, the availability of germ-free mice and advances in genomic sequencing, as well as the increased understanding of the microbiota's composition and characteristics, have resulted in studies demonstrating their safety and possible mechanisms for their anti-tumour action (table 3).

These actions are likely to be exerted through different mechanisms. Firstly, by changing the intestinal microbiota diversity and enrichment mediated by suppression of bacteria that convert procarcinogens into carcinogens. This has been evaluated in intestinal carcinogenesis such as colorectal cancer where Interventional treatment with probiotic showed an increase in the density and diversity of mucosal microbes and altered the mucosa associated microbiota. A study showed from 16S r RNA gene sequences of colonial and faecal microbiota samples, an increase in butyrate-producing bacteria such as *Faecalibacterium* and *Clostridiales spp*. in the tumour, the surrounding non -tumour mucosa and stool following the consumption of the probiotics *L. acidophilus* and *B. lactis* strains consequently, a decrease in the CRC-associated strain *(Fusobacterium)* which is associated with tumour was observed (220, 221).

Secondly, probiotics are shown to induce cellular apoptosis by lowering the pH resulting from butyric acid release (a short chain of fatty acids which is the by-product

of fibre metabolism). This effect was evaluated in a colon cancer Caco-2 cell line (222). Thirdly, through modulation of the immune response at the mucosa or systemic level by decreasing the inflammatory response and the pro-angiogenic cytokines IL-6 and increasing regulatory anti-inflammatory cytokine namely IL-10 and TGF β. This effect was evaluated using milk fermented with lactic acid bacteria in chemically induced colon cancer and hormone-dependent breast cancer model. Data from breast cancer animal studies showed an increase in survival time by inducing Th1 mediated anti-tumour immune response as well as stimulating NK cytotoxicity in the probiotic groups which consumed the probiotics *L. acidophilus and Lactobacillus casei* respectively (223-227). Finally, probiotics can work by diminishing procarcinogen enzymes. This was demonstrated in a study using *B. polyfermenticus KU3* which was isolated from a Korean dish of fermented vegetables and showed that this probiotic did not generate the carcinogenic enzymes: β-glucosidase, N-acetyl-β-glycosaminidase, and β-glucuronidase, and consequently inhibited the proliferation of cancer cells from different cell lines but did not affect the proliferation of the normal cells (228).

Mechanisms for cancer occurrence	Mechanisms for probiotic anti-tumour effect
Intestinal flora translocation leading to persistent chronic inflammation	Maintain barrier integrity, reduce bacterial translocation, and prevent inflammation
Increased pathogens producing toxins and carcinogens	Regulate intestinal flora, inhibit colonisation and proliferation of pathogenic bacteria
Reduction of beneficial probiotic in the intestine	Increase the number and diversity of probiotics
Impaired intestinal microenvironment	Reduce pH value and improve the intestinal microenvironment
Dysfunctional immune response	Regulate the immune response by enhancing the anti-inflammatory and suppressing the pro-inflammatory response

Table 3: Postulated mechanisms for cancer initiation and probiotic anti-tumour function
Reproduced from (229)

1.5.4 Safety and side effects of probiotics

It is important to mention however that those probiotics are likely to become opportunistic and cause sepsis when taken by an immunocompromised people such as those on chemotherapy and undergo radiotherapy. Therefore, it is important to be used under oncologist supervision, but generally they are safe and are becoming more valued in medical use as they are increasingly more evaluated scientifically (209). The wide variations in probiotics formulations, compositions and stability, authenticity warrant thorough investigation of these probiotics before advocating their use in cancer patients. The possible anti-tumour role for *EcN* in ovarian cancer metastasis and the mechanism for this role has not been elucidated to date. This is also a goal for this current study.

1.6 Hypothesis

We hypothesise that stress has negative impact on anti-tumour immunity at an early stage of cancer (2 & 4 weeks). Also, we hypothesise that the probiotic *Escherichia coli Nissle 1917 (EcN)* has a protective effect against tumour in epithelial ovarian cancer (EOC) metastasis and this effect can protect against the negative impact of stress in ovarian cancer metastasis.

1.7 Aims of the thesis

To examine the effects of stress on the anti-tumour immunity in ovarian cancer and to assess the use of the probiotic *EcN* as a complementary therapy to enhance the anti-tumour immunity and reduce the tumour burden in ovarian cancer.

1.8 Objectives

- To assess the impact of cortisol on the anti-tumour effect of the immune cells splenocytes and splenic T lymphocytes in the absence of any underlying pathology in co-cultures of 3D-ovarian cell culture model (spheroids) and *ex vivo* splenocytes.
- To probe the underlying mechanism for the effect of cortisol on the immune cells-ovarian tumour interactions.
- To evaluate the impact of restraint stress (RS) on the anti-tumour effect of the antigen presenting cells (dendritic cells) at an early stage of ovarian cancer (2& 4 weeks) using immunocompetent syngeneic mouse model for RS.
- To generate ovarian tumour lysate-pulsed dendritic cells *in vitro* and assess the impact of cortisol on the maturation and activation of these cells (DCs). Then, to probe underlying mechanisms for this effect.
- To investigate the immunomodulatory role of the probiotic *Escherichia Nissle 1917 (EcN)* on EOC metastasis for an anti-tumour effect. We aim to confirm our previous finding regarding the anti-tumour effect of *EcN* in ovarian cancer metastasis (unpublished data) to demonstrate reproducibility and probe a signalling pathway for this effect.
- To evaluate the impact of RS on tumour metastasis and the role of the probiotic *EcN* in protecting against the negative effect of stress on tumour burden.

2. Chapter 2- Stress and its impact on the Anti-tumour immune response in epithelial ovarian cancer

Aim: To investigate the impact of stress on the anti-tumour activity of antigen presenting cells and effector lymphocytes in ovarian cancer.

2.1 Introduction

2.1.1 Ovarian cancer and psychological stress

Ovarian cancer (OC) is ranking as the 7th most common cancer worldwide as reported in GLOBOCAN for 2018 (98). It is predicted that the incidence rate will increase by 47% in 2040 (*World ovarian cancer coalition)*. Despite advances in treatments for the last four decades, relapse and dissemination are still very likely to occur after remission.

Stress starting from diagnosis of cancer, going through treatment, prognosis and even once in remission is associated with mental and physical comorbidities (230). It starts with a stressor or stimulus, which leads to reactions that are perceived and processed by the brain and results in physiological response (231, 232). Stress activates the neuroendocrine system via two pathways: the hypothalamus-pituitary-adrenal (HPA) axis and the sympathetic-adrenal medullary (SAM) axis, both resulting in the release of stress hormones, namely glucocorticoids and catecholamines, respectively.

These hormones exert their physiological effects through receptors which are expressed in almost all cells of the body. The former is a ligand for the nuclear receptor glucocorticoids (GRs) and the latter is the ligand for the cellular beta-adrenergic receptors. Eventually, the stress hormones affect the function of different physiological systems including the immune system. Recent research has shown that the magnitude and duration of stress-induced elevation in the stress hormones affects the trafficking, maturation and functioning of immune cells such as dendritic cells, macrophages, and lymphocyte (232-234). In this study, the focus will be on glucocorticoids (GCs).

2.1.2 Immune response: Effect of stress on innate and adaptive Immunity

The immune system branches into two arms functioning cooperatively to provide effective immune response: the innate (general) and adaptive (specialised). The former provides the first line of defence against pathogens, foreign substances and injuries and responds quickly, within minutes to hour, to all pathogens in the same way hence the name ‘nonspecific’. The adaptive immune system on the other hand

provides specific attacks mediated by antibodies and surface receptors, normally takes longer (days) to work but results in a long-lasting immunity (235) (figure 10).

The innate immune response is exerted by the effector immune cells (e.g. neutrophils, macrophages, dendritic cells, and mast cells) along with complement proteins and cytokines. Effector cells are stimulated in response to foreign bodies/pathogens that escape the physical barriers such as skin and mucous membranes. Whereas the adaptive immune response is delivered by B and T lymphocytes which identify unique antigens through surface receptors. Lymphocytes develop in the primary lymphoid tissues (bone marrow and thymus), then transfer to the secondary lymphoid tissues, such as the lymph nodes (LN), to present any encountered antigens.

Dendritic cells and macrophages are the professional antigen presenting cells bridging the innate and adaptive immune response. Upon uptake of antigens, dendritic cells mature and express markers such as MHCI/MHCII, co-stimulatory factors such as CD40 and release cytokines, they then migrate to the nearest draining lymph nodes to present their antigens to T lymphocytes and prime them to undertake a specific immune response. T cells are the main key players in the adaptive immune response, once they are activated in response to antigens, they differentiate into alternative T cells lineages: $CD4^+$T helper cells (Th), cytotoxic $CD8^+$ T cells, Th17 and T regulatory (T regs) cells to undertake different functions (235) (figure 11). The immune-protective response provided by the innate and adaptive immune system mediates immuno-surveillance which enables rapid elimination of infections and transformed cells, efficient wound healing, and successful resolution of inflammation (236).

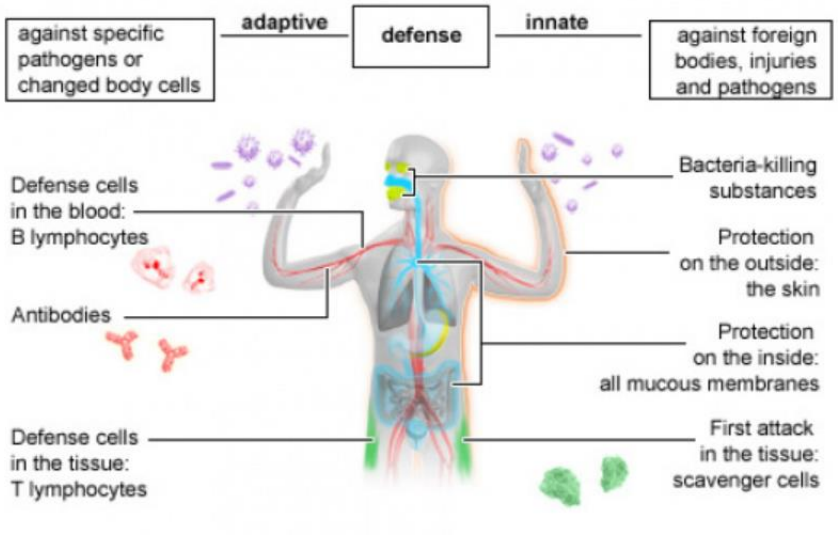


Figure 10: Illustrates the branches of the immune system and their functions
The innate immune system provides nonspecific first line response to protect against foreign bodies, injuries, and pathogens. The adaptive immune system on the other hand provides antigen-specific response mediated by antibodies and activation of lymphocytes to deliver antigen associated long lasting immunity**.** Reproduced from (236).

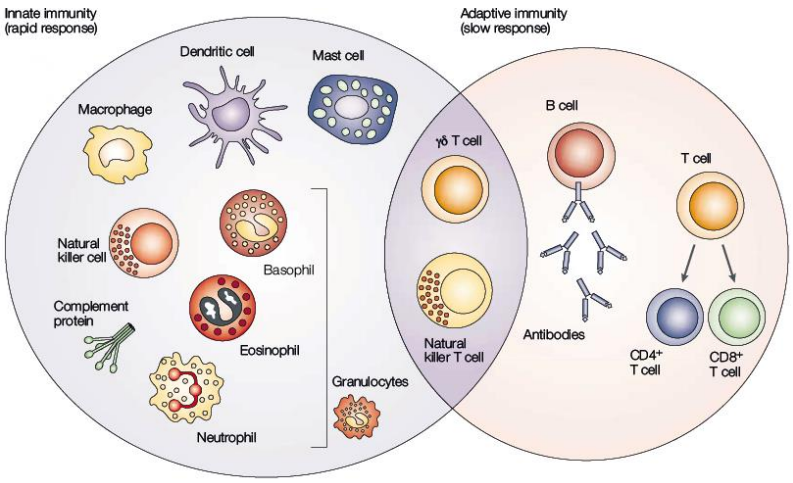


Figure 11: Illustration of the effector immune cells mediating the innate and adaptive immune response
Macrophages, dendritic cells, granulocytes, and mast cells are the main mediators of the innate nonspecific immune response, while lymphocytes B cells and T cells mediate the adaptive specific immune response. Once the lymphocytes are activated by antigens, B cells produce antibodies and T cells activate to CD4+ and CD8+ effector T cells. Natural killer T cells and gamma delta T cells (γδ T cells) are overlapping cells between the innate and adaptive immune systems. Reproduced from (235).

The availability of inflammatory models using conditional knock out and function selective mice enhanced the understanding of the effect of GCs on key immune cell types such as dendritic cells and T lymphocytes (80). Although GCs action on DCs is not yet fully understood, they are shown to affect all stages of DCs differentiation and activation. GCs selectively induce apoptosis in mature but not immature DCs, this effect was found to be dependent on the expression pattern of GR isoforms and observed in DCs from healthy mice treated with Dexamethasone (a potent GCs) and confirmed in bone marrow derived DCs (81). GCs can also supress the activation markers expressed upon antigen uptake such as (MHCI/MHCII, co-stimulatory factors (e.g. CD40) and supress the production of cytokines. (237-239). Further, GCs are shown to inhibit the migration of DCs to lymph nodes (LNs) (240) and they are a potent inducer of tolerogenic DCs phenotype characterised by enhanced IL-10 production and phagocytosis. This phenotype results in T cells anergy whereby effector T cells are suppressed and Tregs are generated (241). DC tolerance mediates the GCs' powerful anti-inflammatory function (121).

GCs affects thymocytes maturation and Th cells differentiation. Endogenous GCs induce potent apoptosis in double positive (DP) thymocytes with high affinity to self-antigen but not in single positive $CD28^{+}$ and peripheral T lymphocytes which are more resistant cells because of the presence of surface T cells receptors (TCR) (242). However, the role of GCs in the selective process of thymocytes requires further exploration due to the controversy in the data available (243, 244). Further, at physiological level GCs can cause shift in immunity from Th1 (cellular immunity) to Th2 (humoral antibodies mediated) through TCR signalling (245) whereas at pharmacological doses they exert anti-inflammatory function on all differentiated lineages of Th cells including Th1, Th2,Th17 and Tregs via suppression of proinflammatory cytokines and regulation of transcription factors (246).

The biological stress responses can be classified based on their cellular and molecular components into innate or adaptive (e.g., Th1, Th2, Th17 or Tregs) driven responses. Alternatively, they can be classified based on their functional effects into: Immune

protective, immune pathologic/inflammatory and immune regulatory/suppressive (247). All these responses may exist with various dominance.

2.1.3 Mechanisms mediating the effect of stress on ovarian cancer

Based on evidence from the literature, it is postulated that stress exerts its effect in OC through different mechanisms. Firstly, through immune-independent pathways. For instance, Lutgendorf and colleagues demonstrated a link between chronic stress, depression, low social support and increased tumour proliferation and metastasis. They showed that the link between chronic stress and increased metastasis is associated with increased levels of the enzyme Matrix Metalloproteinase-9 (MMP-9) in tumour associated macrophages (TAM) (stromal cells in the tumour microenvironment and play a key role in promoting tumour growth and angiogenesis) of OC patients resulting in an increased level of angiogenic cytokines such as IL-6 and vascular endothelial growth factor (VEGF) which can promote metastasis (248). Increased social support was shown to reduce the level of TAM's MMP-9. The study further demonstrated *in vitro* an increase in TAM's MMP-9 following direct exposure to cortisol and other stress hormones (249).

Secondly, through modulation of the circadian rhythm (57). An altered circadian rhythm was observed amongst women with ovarian cancer prior to treatment, evidenced by elevated nocturnal cortisol, flattened diurnal cortisol slope, and reduced cortisol variability. Each of these effects were associated with poor overall survival (OS) and increased intratumoural inflammation (97). This circadian cortisol rhythm dysregulation is often one of the early negative effects of prolonged stress and thought to be associated with impaired HPA-axis negative feedback and cortisol resistance (57, 250). HPA-axis mediated immune response is essential for coordinating the circadian rhythm to maintain healthy physical and mental functions. Disruption of the circadian rhythm could either be a physical response from the host to a present tumour or an endocrine response to the physical and emotional demands caused by the disease (250).

Finally, through immune-dependent pathways by suppression of the protective immunity or induction of chronic inflammation or enhancement of the regulatory/suppressive immunity. Cell mediated immunity (CMI) is very important component of the protective immunity, characterised by the production and release of the immune protective cytokines IL-12 and IFN-y from T helper 1 cells (Th1). It maintains immune surveillance and prevent tumour progression, invasion, and metastasis (74).

Evidence from studies of patients with advanced stage of OC and other cancers showed suppressed protective immunity mediated mainly by GCs and catecholamines (74) and by reduced/altered distribution of immune cells between different body compartments (232). The studies further showed a decrease in natural killer cells cytotoxicity (NKCC) and T-cell cytokines production in the tumour microenvironment (TME) of depressed OC patients. This cytotoxicity was increased following social support to counteract the effect of chronic stress (251). Additionally, social support correlated with increased level of natural killer T cells (NKT) in TME and peripheral circulation of OC patients (252). Therefore, harnessing the protective immunity by controlling the stress levels is important for the health and survival of cancer patients and for the success of cancer treatments.

Stress can also promote inflammation in OC patients. Ovarian tumours of distressed patients showed more pro-angiogenic cytokines (IL-6 and VEGF) along with an increased expression of macrophages compared to controls, and this increase was associated with decreased survival (74, 251).

Finally, stress can enhance the regulatory/suppressive immunity in OC patients. George Coukos and team demonstrated *in vivo* a role for T regs cells in promoting tumour growth by supressing the effector anti-tumour immunity in OC patients. The research further showed an increase in mortality and reduction in survival associated with the presence of these cells in the ovarian tumours and ascites (253).

2.1.4 C GAS-STING pathway a pivotal innate immune response driving anti-cancer immunity

C GAS-STING pathway is an inflammatory innate immune response. It has been recently demonstrated by different studies that the STING pathway might play a vital role in anticancer immunity by upregulating multiple steps in the cancer-immunity cycle (254, 255). C GAS-STING pathway can be activated in both tumour and DCs in response to cytosolic sensing of DNA from bacteria, viruses, damaged or dying tumour cells resulting in downstream signalling and the production of type 1 interferons (IFNs). These in turn promote the maturation, activation, and migration of DCs, enhance the cytotoxic activity of T lymphocytes and natural killers and protect effector cells from apoptosis (114, 255, 256). On the contrary, evidence is showing a dampened DCs expansion and an induction of suppressed phenotype of DCs in response to prolonged release of type 1 IFNs. For instance, *In vivo* stimulation of splenic DCs with IFN-β resulted in fewer $CD11c^{+}$ DCs and a decrease in DCs expansions (257, 258).

Cyclic GMP-AMP synthase (cGAS) is a cytosolic DNA sensor/enzyme that catalyses the formation of 2',3' -cGAMP. C GAS senses and binds to double strand DNA either from exogenous source such as bacteria, virus, or endogenous source such as damaged cells or dying tumour cells. cGAMP will then bind and activate the Stimulator of Interferon Genes (STING) which in turn traffics between endoplasmic reticulum (ER) membrane and Golgi apparatus. STING under certain conditions recruits and activates Tank Binding Kinase1 (TBK1) to phosphorylate the transcription factor Interferon Regulatory Factor 3 (IRF3). IRF3 translocate to the nucleus and activates the expression of innate immune response inflammatory genes and type 1 interferons (IFNs). STING under certain conditions also activates the NF-kB pathway which collaborates with the TBK1-IRF3 to increase the production of type 1 IFNs (figure 12). In this study, cGAS-STING pathway will be examined in DCs activated with ovarian tumour lysate as a possible innate immune sensing of cancer and the impact of cortisol on this pathway will be explored.

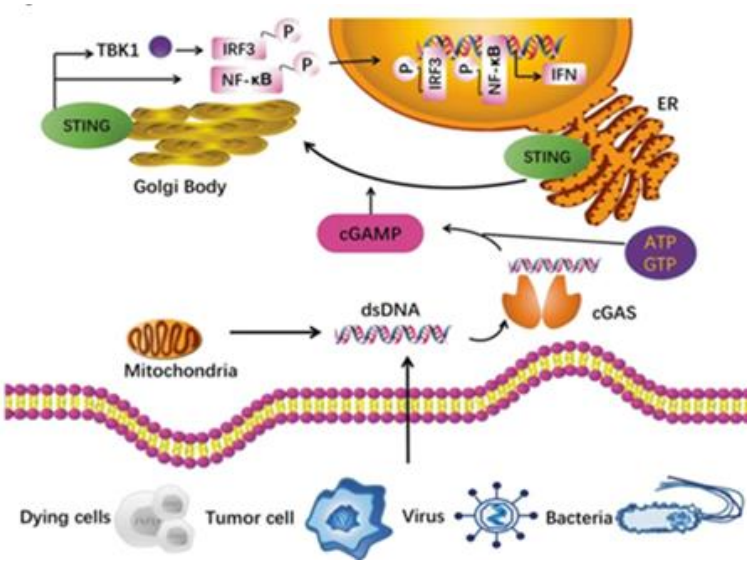


Figure 12: Illustration of the c GAS-STING pathway
The STING (stimulation of interferons gene) pathway is activated in response to Exogenous DNA from dying cell, tumour cell, virus and bacteria, and endogenous DNA leakage from mitochondria. The DNA interact with the cytosolic DNA sensor cGAS which catalyse the formation of 2',3'-cyclic GMP-AMP (cGAMP). The cGAS activation as well as cGAMP synthase activate protein STING, in which the STING undergoes endoplasmic reticulum (ER)-to-Golgi trafficking. STING could recruit and activate Tank Binding Kinase1 (TBK1) to phosphorylate the transcription factor Interferon Regulatory Factor 3 (IRF3). P-IRF3 translocate to the nucleus and activate the expression of innate immune response inflammatory genes and type 1 interferons (IFNs). STING could also activate the NF-kB pathway which collaborate with the TBK1-IRF3 to increase the production of type 1 IFNs. Taken from (255).

2.2 Animal model to study stress

Because the stress outcome varies depending on the intensity, duration and on the individual susceptibility to stress, it is important to account for all these variables in the choice of the animal model. C57BL/6J mice have a functioning immune system and are one of the most widely used mice strains because a wide range of genetically modified lines are available. ID8 is the C57BL/6J-derived epithelial ovarian cancer cell line which is used throughout this study. 3D spheroids were developed to better mimic the biological ovarian tumour.

2.3 Restraint stress

Restraint stress has consistently been shown to induce behavioural and immunological changes that mimic those alterations commonly observed in the chronic stress response (259). Therefore, this is the type of stress applied throughout

this study. Research clearly shows that stress exerts an immunomodulatory role in EOC, which is considered an immunogenic tumour, i.e. disease prognosis is closely related to competent immune system.

Little is known about the impact of stress on antigen presentation, a prerequisite for the activation of cytotoxic T lymphocytes at an early stage of ovarian cancer (2 weeks) and at a later stage (4 weeks) during tumour progression. Also, there is a lack of data on the impact of stress on T cell infiltration into ovarian cancer tumours. Therefore, the focus of this chapter is to investigate the immunopathology underlying stress in EOC by exploring the interactions between ovarian cancer cells and the immune cells, using appropriate models (*in vitro* and *in vivo*). This will help to identify possible molecular mechanisms for potential pharmacological and/or behavioural interventions that can be targeted to improve the clinical benefits of ovarian cancer patients.

2.4 Hypothesis

We hypothesise that stress will inhibit the antigen presenting action of dendritic cells on ID8 cancer cells at an early stage of ovarian cancer. Activation of DCs is a prerequisite for developing adaptive immune response. Also, we hypothesize that stress will impair the interactions between the immune cells (splenocytes) and ovarian cancer spheroids (ID8 cells).

2.5 Objectives

- To prepare and optimise 3D ovarian cell culture model (spheroids), to assess the effect of cortisol and restraint stress (RS) on immune cells (splenocytes) trafficking and their ability to disintegrate the tumour spheroids as a sign of anti-tumour activity.
- To examine the effect of gold standard and novel glucocorticoid receptor (GR) antagonists mifepristone (RU486), relacorilant (c134) and CORT12528 (c281) on reverting the impact of stress on splenocytes trafficking.
- To identify underlying mechanisms for the effect of stress on the activities of splenocytes

- To use immune functioning syngeneic mouse model of ovarian cancer for restraint stress (RS) to investigate the impact of stress on the activation of the antigen presenting cells (DCs). This is a prerequisite for developing adaptive immune response and effector lymphocytes (a component of splenocytes) at the early and later stages of cancer (2 & 4 weeks)
- To generate DCs *in vitro* from *ex vivo* tissues and pulse them with whole ovarian tumour lysate as a source of known and unknown tumour antigens to elucidate underlying mechanism for the action of cortisol on DCs maturation and activation.
- To examine the ability of RU486 to revert the effect of cortisol on tumour lysate pulsed DCs.

2.6 Materials and methods

2.6.1 Preparation of fluorescently stained 3D culture (spheroids) from a murine (C57BL/6J) ovarian surface epithelial cell line (ID8)

ID8 cells were donated from Dr Premal Thaker, Washington University, St. Louis, USA (Material Transfer Agreement (MTA) in place). The cells were cultured in T-75cm^2 filtered tissue flasks in Dulbecco modified Eagle Medium (DMEM, Gibco) media with 4% FBS (Foetus Bovine serum) and ID8 supplement (5µg/ml insulin, 5µg/ml transferrin, 5µg/ml sodium selenite). When the cells were 80-90% confluent, the media was aspirated, and the cells were washed with PBS and scraped with cell scraper to maintain surface markers then centrifuged for 5 min at 4500g. Cells were counted for viability using trypan blue and a haematocytometer. A total of 1x10^6 cells were resuspended in 1 ml of serum free media then incubated with 5 µl of green, fluorescent, lipophilic carbocyanine-DiO dye (excitation 484nm & emission 501nm) (D7778, Fisher scientific) dye for 2h at 37°C and 5% CO_2. Cells were centrifuged and resuspended in PBS first then resuspended in ID8 media and seeded at a density of 2500 cells in 30 µl per well in a 96 well plate (using Costar 7007 with round ultralow attachment surface to enable uniform and reproducible spheroid formation) and incubated at 37° and 5% CO_2 for 7 days. Extra media (30µl per well) was added on day 3 (260).

2.6.2 Animals

Mice were allowed to rest after transport then handled for two weeks before any experimental procedures to acclimatise to researchers (myself, PPL: I81347B) and reduce their stress level. Ten female C57BL/6J mice 10-12 weeks old, weighing 18±2g were obtained from Charles River (C.R) laboratories (Margate, Kent, United Kingdom), divided into two groups: stress (S) and no-stress (NS) (n=5). The mice were kept in cages at a constant 12-hour light-dark cycle, temperature of 19-21°C and humidity of 40-60%.

2.6.3 Stress Procedure

To evaluate stress-related immunobiological changes induced by acute or chronic exposure to stressor, restraint stress model was used as it is efficient and the most used stress model in laboratories to induce physiological responses. It has a relatively low cost and technical requirements (10). The mice in the stress group (n=5) underwent restraint stress (RS) by placing each mouse in a 50 ml perforated conical falcon tube for two hours daily from 10:00 am to 12:00 noon to mimic the *in-vivo* physiological stress hormones release. Cortisol is released in diurnal pattern; it peaks during the day and drops down at night. The no stress (NS) group (n=5) were kept in their cages for this period. The restraint stress continued daily for 21 consecutive days (261). All procedures were in adherence with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments).

2.6.4 Isolation of spleens from both groups for splenocytes.

Mice from S and NS groups were euthanized by cervical dislocation, the spleens were extracted and placed in complete media (RPMI-Gibco + 10%FBS) then transferred to a laminar flow hood and mashed through 70 µm plastic strainer (Fisher Scientific) to recover cells. Splenocytes were then centrifuged at 300g for 10 min as per manufacturer's company protocol (262) (Miltenyi Biotec, UK), resuspended in RBCs lysis buffer (Sigma) to remove red blood cells, centrifuged, and washed twice with

PBS. It is finally re-suspended in complete media and viability counts were performed using trypan blue and a haematocytometer.

2.6.4.1 Staining of splenocytes with florescent stain before co-culturing with ID8 spheroids

Splenocytes from both groups of mice were stained with pharmacologically inert red lipophilic carbocyanine-DiL dye (excitation 549 nm & emission 565 nm) (D7777, Fisher scientific). $1x10^6$ cells were resuspended in 1 ml of serum free media then incubated with 5 µl of red fluorescent stain for 2 h at 37°C. Cells were centrifuged and resuspended in PBS first then resuspended in complete media for splenocytes.

2.6.4.2 Co-culture of ID8 spheroids with splenocytes to assess the effect of splenocytes on spheroids area

To optimise the cells ratio for the co-culture, splenocytes were co-cultured with ID8 spheroids cells at 3 different ratios: 1: 1 (2500: 2500), 1: 3 (2500: 7500) & 1: 5 (2500: 12500) cells. The ratios were ID8: Splenocytes. Each concentration was performed in duplicate for each mouse.

2.6.4.3 Isolation of T lymphocytes from splenocytes

T lymphocytes constitute roughly 30-35% of all splenocytes, of which $CD4^+$T cells account roughly for 70% and $CD8^+$T cells account for the remaining 30% (263). Splenocytes were extracted from spleens as described in section 2.6.4, T lymphocytes were then extracted by magnetic labelling using Pan T Cell Isolation Kit II mouse as detailed in the company's protocol (Miltenyi Biotec, UK) (264).

2.6.5 Flow cytometry for assessment of T lymphocyte early activation

$1x10^6$ cell/ml splenocytes from each mouse were resuspended in wash buffer (PBS+BSA 1%) and stained with the optimal concentration of murine fluorescent-labelled monoclonal antibodies. The antibodies were specific for cell surface markers (fluorescein isothiocyanate (FITC) - $CD3^+$ 0.75 ng/µl of wash buffer) and Allophycocyanin (APC) - CD69 0.75ng/µl of wash buffer) (Miltenyi Biotec, UK).

Following incubation for 30 min at 4°C in the dark, cells were washed twice with PBS and were assessed by a flow cytometer (BD Accuri C6, BD Biosciences). The cells were first run with single fluorochrome-stained preparations for colour compensation. For each analysis 20,000 events were acquired and gated on forward and side scatter properties.

2.6.6 Restraint stress and co-culture of *ex vivo* splenocytes with ID8 spheroids

C57BL/6J female mice were stressed as described in section 2.3, (n=4) for 6 weeks. At the end of 6 weeks, all mice from S and NS groups were euthanized by cervical dislocation and their spleens were harvested. Splenocytes (SP) were isolated from the spleens and stained as described in sections 2.4 and 2.4.1 respectively, then co-cultured with ID8 spheroids as in 2.4.2 to assess SP infiltration into ID8 spheroids.

2.6.7 Activation of splenocytes with PMA & ionomycin to stimulate the production of effector cytokines such as IL-2 & IFN-γ

Splenocytes were extracted from a spleen which was isolated and stained as described in sections 2.6.4 and 2.6.4.1 respectively, then activated with 25ng/1ml Phorbol 12-Myristate 13-Acetate (PMA), a protein kinase c activator (Sigma Aldrich, UK) and 1µg/ml ionomycin, calcium ionophore (Sigma, Aldrich, UK) to stimulate cytokines production. Cells were seeded in a 12-well plate at a density of $1x10^6$/well, PMA & ionomycin were diluted in media to the required concentrations and added to cells which were then incubated at 37°C in 5% CO_2 for 3h. Cells were then centrifuged, washed with PBS, cell pellets resuspended in media, and assessed for viability to be co-cultured with previously prepared ID8 spheroids as it will be described in the next section (2.6.7.1).

2.6.7.1 Co-culture of ID8 spheroids and ex vivo splenocytes activated with PMA & ionomycin in the presence and absence of cortisol

ID8 spheroids were prepared as described in section 2.1. Splenocytes were extracted from a spleen which was isolated and stained as described in sections 2.4 and 2.4.1 respectively then activated with 25ng/1ml Phorbol 12-Myristate 13-Acetate (PMA),

(Sigma Aldrich, UK) and 1µg/ml ionomycin, calcium ionophore (Sigma, Aldrich, UK) to stimulate cytokines production as described in section 2.7. Subsequently it was incubated at 37°C in 5% CO_2 for 3 h. Cells were then centrifuged, washed in PBS, cell pellets resuspended in media, and assessed for viability. The samples were divided into 2 groups: Cortisol group (Cort) treated with 5µM cortisol (the concentration was chosen based on a dose response optimisation) and control group (cont) without cortisol. Cortisol (Sigma Aldrich, UK) was diluted in media from a stock concentration daily for 5 days. Spheroids with unstimulated splenocytes (U) were used as a negative control and spheroids alone were used to exclude any background effects. Images from both light and confocal microscopes were captured daily for 4 days. Images were compared for spheroids area and splenocytes infiltration (265).

2.6.7.2 Co-culture of ID8 spheroids and ex vivo splenocytes activated with PMA & ionomycin and treated with cortisol and glucocorticoids receptor (GRs) antagonist RU486

Co-culture of ID8 spheroids and PMA/ionomycin activated splenocytes was prepared and treated with 5µM cortisol as described in section 2.6.7.1. Co-cultures were treated with 5µM RU486 (a gold standard non-selective GR antagonist (266)) (Sigma Aldrich, UK) for 30 min prior to cortisol exposure. The RU486/cortisol treatment continued for 4 days, and images were captured as in section 2.6.7.1.

2.6.8 Syngeneic mouse model of ovarian cancer was used to investigate the cancer- immune interactions at the antigen presenting sites, the spleens and lymph nodes, and at the tumour microenvironment

An immune competent syngeneic mouse model was used to study the immune signature and the interactions between immune cells and tumour of OC. *In vivo* experiments were conducted under a Project License and the procedures were carried out as per (PiL: I81347B) under the project licence (Ppl) PP6508061.

To explore the impact of RS on the expression of maturation and activation markers of the antigen presenting cells (DCs) at an early stage of OC, 2 and 4 weeks, twenty 8 weeks old C57BL/6J females weighing 18±2g were purchased and handled as in

section 2.6.2. All mice were injected with $7x10^6$ ID8 cells in 200ul of PBS IP and stratified into 4 groups of n=5 with 2 stress groups (S) and 2 no stress groups (NS) which were left in their cages all the time. The 2 stress groups underwent RS as described in section 2.6.3 for one week prior to the cancer injection (to stimulate protective immunity and enhance resistance to cancer). The mice from one stress group underwent restraint stress for 2 weeks. Whilst mice from the second stress group underwent RS for 4 weeks.

Weekly weight was recorded for all groups to monitor tumour development. The 2 weeks stressed mice and their associated control group were scarified at the end of 2 weeks period and the 4 weeks stressed mice along with their associated control group were sacrificed at the end of 4 weeks. Gross necropsy was performed to observe tumour development and progression. Blood was collected for cytokines analysis in plasma. Spleens and lymph nodes were collected to isolate DCs by Pan DCs isolation kit (Mitenyi Biotec, UK), to look at maturation and activation markers. The 2 weeks RS was repeated using another 10, 8 weeks old C57BL/6J females mice weighing 18±2g to reproduce results. Mice were purchased and handled as described in section 2.6.2 (figure 13).

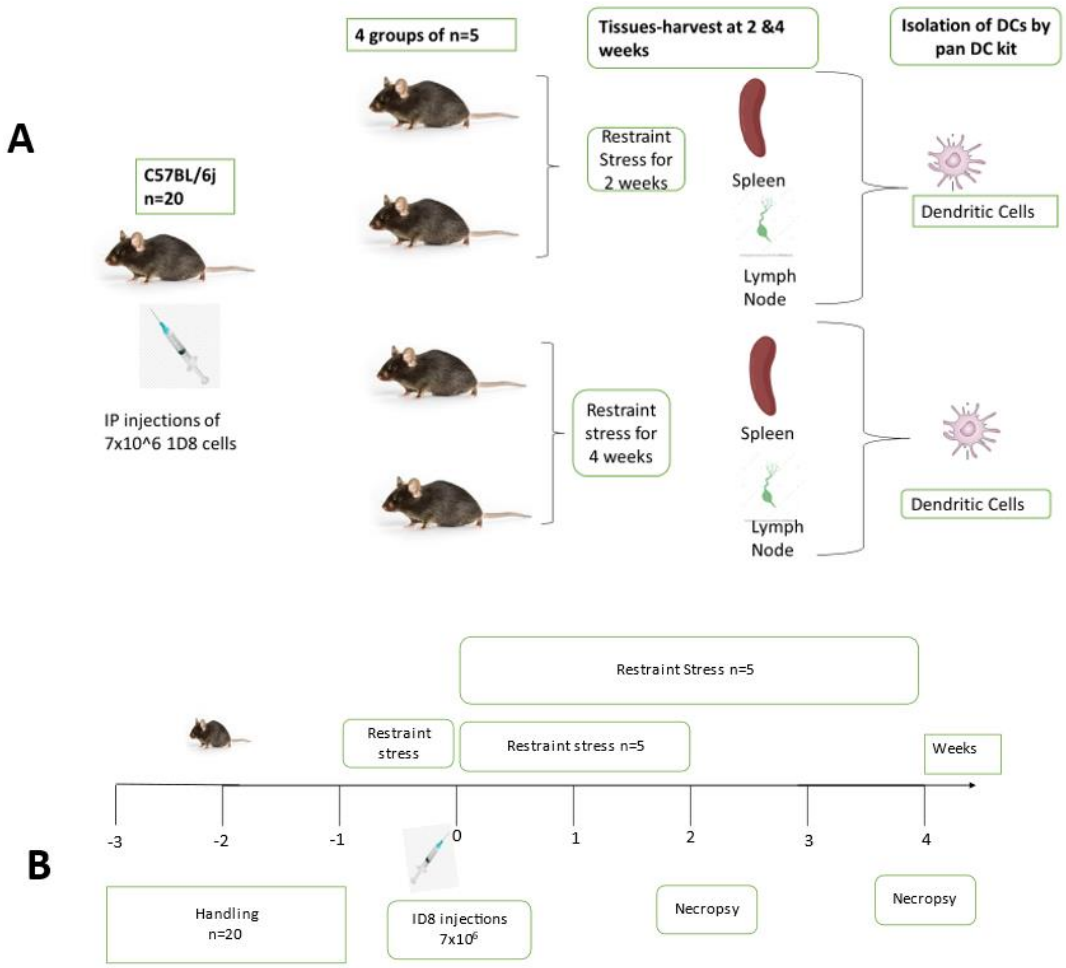


Figure 13: Schematic illustration of the cancer-stress *in vivo* study with a timeline
(A) 20 C57BL/6J female mice were handled for 2 weeks then injected with 7x10 ^{6} ID8 cells and stratified into 4 groups. They were named based on the procedure they were subjected to stress (2 groups) and no stress (2 groups). The stress groups were subjected to restraint stress one week before the cancer injections and continued for 2 weeks for one group and 4 weeks for the second group. The non-stressed mice were kept in their cages. At the end of 2 weeks, 2 groups were sacrificed (stress and no stress; n=5) and the other 2 groups were sacrificed at the end of 4 weeks. Spleens and lymph nodes were harvested to isolate DCs for immune analysis by flow cytometry.
(B) is a schematic timeline for A.

2.6.9 Generation of dendritic cells (DCs) *in vitro* from *ex vivo* tissues and the use of OC tumour lysate as an immunogenic source of tumour-associated antigens to mimic the observed *in vivo* effect of cortisol on DC ability to express the activation markers MHCII/CD40 and probe an underlying mechanism

2.6.9.1 Preparation of whole tumour lysate as a source of known and unknown antigens from ex vivo freshly frozen murine ID8 ovarian tumour tissues to activate DCs generated from ex vivo tissues

The frozen ovarian tumour tissues (10mg) were washed with chilled PBS to remove any blood, treated with 500µl Radioimmunoprecipitation assay (RIPA) buffer then homogenized thoroughly for 30 min while on ice and vortexed, sonicated for 2-3 mins at a power of 180 watts in rounds of 10 seconds sonication/ 10 seconds rest for each cycle to break the tissues up. The tissues were then centrifuged at 10000g for 20 min at 4°C to pellet any debris. The supernatant was transferred to a fresh microfuge tube without disturbing the pellet to determine the protein concentration by Bradford protein assay. All samples were then stored at -80°C (Proteintech).

An alternative method was used to prepare the tumour lysate through mechanical disaggregation where lesions of ovarian tumour from syngeneic animal model (C57BL/6J) were collected, heated in a water bath for 1 h at 42 °C and further heated for 2 h at 37 °C in 5% CO_2. Tumour cells were then digested in cell dissociation solution (Gibco, Thermo Fisher Scientific, UK) and washed with PBS. Cells were counted, resuspended at $1x10^6$ and lysed by 3 cycles of freeze-thaw in dry ice. Finally, the preparations were centrifuged at 12000 rpm for 15 minutes, and the supernatant was stored at -80 °C (153).

2.6.9.2 Isolation of murine bone marrow (BM) and splenocytes (SP) to co-culture and generate immature dendritic cells (BMSP DCs)

One of the common techniques for generating DCs *in vitro* for immunotherapy is to culture bone marrow (BM) progenitor cells in the presence of granulocyte-macrophage-colony stimulating factor (GM-CSF) and interleukin (IL-4) for 7 to 10 days (267). However, this method can only generate a limited number and purity of

DCs. Therefore, an alternative method has been employed to overcome the above problems where BM-derived cells are co-cultured with *ex vivo* splenocytes to generate DCs with large number and high purity (268).

A C57BL/6J female 12 weeks old mouse (20-25g) was euthanised by cervical dislocation, after which the hind legs were cut just above the pelvic/hip joint (while the epiphysis remained intact) after removing the skin covering them to expose the muscles. The tibias and femurs from both sides were purified from muscles while both ends of the bones were kept intact then kept in ethanol and washed with PBS to be disinfected. The bones were transferred to a petri dish with culture media (RPMI-1640) and both ends were trimmed with sharp sterile scissors to expose the interior marrow shaft. The content of the bone marrow was then flushed with PBS using 1-ml insulin syringe with a 21G x ½ needle into a sterile 50-ml centrifuge tube until the bone appeared white ensuring all the marrow has been expelled out.

The bone marrow suspension cells were diluted with PBS and clusters cells were disintegrated by vigorous pipetting then centrifuged at 450g for 5 minutes, resuspended in RBCs lysis buffer then washed twice with PBS and viable cell count was performed using trypan blue. The BM cells were then co-cultured with splenocytes (prepared as described in section 2.4 from a spleen) at a ratio of 1:7 respectively as previously optimised by researchers (268) in sterile, non-TC Petri dishes. The cell suspensions were suspended in complete culture media (RPMI-1640, 10%FBS, 2mM L-Glutamine & 10ng/ml GM-CSF). Petri dishes were swirled gently and incubated at 37°C, 95% humidity and 5% CO_2 for 9 days. Fresh media was added every 3 days to each petri dish and the cells with the media were transferred to fresh petri dishes as macrophages attach to the surface and the generated DCs remain floating or loosely attached. On day 9 the cells (BMSP DCs) were harvested and centrifuged then counted for viability (153, 267).

2.6.9.3 Antigen pulsing of DCs with whole ovarian tumour lysate

BMSP generated DCs were harvested on day 9 and $2.2x10^5$ cells were seeded in 96 well (Costar 7007 plate with ultralow attachment). DCs were then incubated with a whole ovarian tumour lysate (prepared as described in section 2.9.1) at a ratio of 1:5 respectively for 24 h (153) to become pulsed and matured. Non loaded DCs were used as a negative control and the toll like receptor agonist LPS-Lipopolysaccharide (1µg/ml) was used as a positive control. DCs were either treated with 5 µM cortisol to assess the impact of cortisol on the maturation of DCs (by looking at expression of activation surface markers) or treated with glucocorticoid antagonist RU486.

2.6.9.4 Flow Cytometry to evaluate the activation of BMSP DCs or DCs isolated from ex vivo spleens and lymph nodes by looking at activation surface markers

Following the 24-h co-culture, $2x10^4$ DCs were collected from each well, resuspended in 1% BSA in PBS (wash buffer) and stained with fluorescent labelled monoclonal murine antibodies at optimal concentration directed towards specific surface markers on the DCs. These are fluorescent isothiocyanate (FITC) CD11c (a marker for DC^+ cells), phycoerythrin (PE) CD40 (co-stimulatory factor) and Allophycocyanin (APC) MHCII (for antigen presentation) (Miltenyl Biotec, United Kingdom). The cells were incubated with the conjugated antibodies for 15 min in the dark at 4°C then washed twice with the wash buffer for cytometric analysis using BD Accuri C6 (BD Biosciences) equipped with 4 lasers: 3 blue and 1 red. Single stained samples were prepared as well as a no stain sample for colour compensation. For each analysis 20,000 events were acquired and gated on CD11c expression and side scatter properties. Green fluorescence from FITC (FL1) was collected through 530/30 nm band pass filter, orange red fluorescence from PE (FL2) was collected through 574/26 nm band pass filter and red fluorescence APC (FL4) was collected through 661/16 nm band pass filter. Data collected from side and forward scatters were linearly amplified whereas data from filters 1-4 were amplified at a logarithmic scale.

2.6.10 Detection and quantification of gamma phospho-histone (У-H2AX) foci using immunofluorescence to assess for double strand break (DSB) of DNA in splenocytes

The Gamma H2AX assay is a sensitive and specific marker for DNA double strand break (DSB). Phosphorylation of the histone protein H2AX to Gamma H2AX (У-H2AX) occurs in response to DNA double strand breaks (DSB) to recruit DNA repair proteins to the site of DNA damage and thus maintain genomic stability of the cells. У-H2AX foci which can be detected and quantified by immunofluorescence (269).

2.6.10.1 In vivo stress exposure

Mice (n=3) were used as described in section 2.6.2 were stressed for 6 weeks as mentioned in section 2.6.3. They were then sacrificed by cervical dislocation and the spleens were isolated, mashed and splenocytes were isolated and counted as described in section 2.4.

2.6.10.2 Evaluation of DNA double strand break by gamma phospho-histone У-H2AX assay in splenocytes extracted ex vivo from the S and NS groups

Cells were seeded at a density of $3x10^5$ per well on sterile glass cover slips in a 12-well plate and incubated overnight at 37 °C and 5% CO_2 to allow the splenocytes to adhere on the cover slips. Cells were then fixed in 3% paraformaldehyde for 10 min, washed with PBS and then permeabilized with 0.2 % Triton X-100 in PBS for 5 min at room temperature (RT). Subsequently, cells were blocked with 2% BSA in PBS for 30 mins at RT and incubated with the primary antibody; anti-phospho-histone У-H2AX (1:200 in 2% BSABPS) (Cell Signalling) at 4 °C in the dark overnight. Samples were then washed and incubated with the secondary antibody; anti-mouse /rabbit IgG fluorescein isothiocyanate (FITC) (1:200 in 2% BSAPBS) (Sigma Aldrich, UK) at RT for 1 h followed by 3 washes in PBS. The slides were then mounted using mounting media (Fluoroshiel with DAPI-histology, Sigma), sealed, stored overnight at 4°C in the dark then visualised using confocal microscopy (Leica, Germany) to detect fluorescent foci. Cells with > 4 foci were considered У-H2AX positive and expressed as a percentage of the total cells which was 50 cells per slide. Hydrogen peroxide was

used as a positive control and negative controls with no primary antibodies were prepared for each slide to eliminate any background interferences.

2.6.10.3 Evaluation of DNA double strand break by ɣ-H2AX assay in ex vivo T lymphocytes treated with cortisol and RU486

Splenocytes were extracted from a spleen of C57 BL/6J female mouse as described in section 2.6.4 then seeded on sterile glass coverslips in 12-well plate as in section 2.6.10.2. Cells were treated with 10 µM RU486 and incubated for 30 min at 37°C in 5% CO_2, followed by a treatment of 10 µM cortisol (optimal concentration following a dose response curve) and incubated at 37°C in 5 % CO_2 for 2 h. The plates were then centrifuged at 800 rpm for 8 min then underwent procedures for immunofluorescence as in section 2.6.10.2. Three technical replicates were prepared.

2.7 Statistical analysis

Data was analysed statistically using Graph Pad prism software version 9. For normally distributed discrete data, paired t tests were used to compare 2 groups. For non-normally distributed data, non-parametric paired tests (Wilcoxon test) were used to compare 2 groups. For multiple-group comparisons, 2-way ANOVA for continuous data and one way ANOVA for column analysis with Sidak's or Tukey's multiple comparison corrections were used, as indicated in the figures. Levels of significance are as follows, *P ≤ 0.05, **P ≤ 0.01, and ***p ≤ 0.001 unless specified differently in individual figures.

2.8 Results

2.8.1 Spheroids (3D-culture) from a murine ovarian cancer cell line (ID8) were successfully formed over a period of 7 days to model the ovarian tumour

This series of experiments are designed to show how splenocytes interact with ID8 ovarian cancer cells and how this interaction is modulated by stress. First, basic culture protocols were developed to demonstrate that ID8 cells can form reproducible spheroids in culture to better mimic the biological tumour. Spheroids were irregular in shape, small, compact and had a consistent average area of 0.03-0.1mm^2 (measured by the software ImageJ) (figure 14).

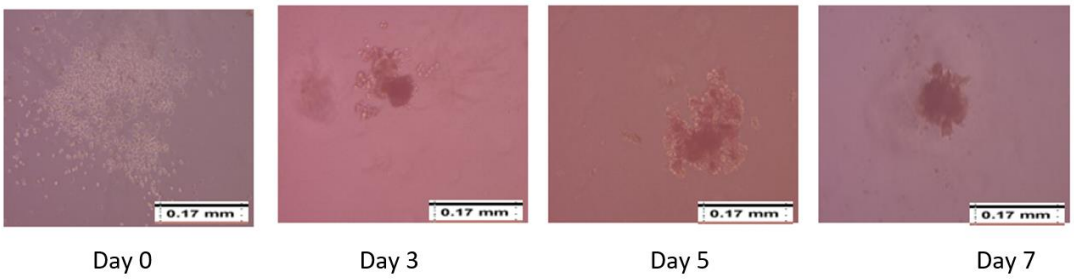


Figure 14: A representative sample of ID8 epithelial ovarian cancer cell line spheroids
Spheroids were successfully formed over a period of 7 days in 96-well corning low attachment plate. Ovarian cancer cells (C57BL/6J ID8 cells) were stained with lipophilic membrane fluorescent green stain DIO (for confocal imaging) then seeded at a density of 2500 cell per well in 30 µl of media. Light microscope images were captured on days 0**,** 3, 5 and 7. Magnification: X100. Spheroids were irregular in shape and had an average area of 0.03-0.1 mm^2 as measured by ImageJ.

2.8.2 Restraint stress and activation of T lymphocytes

2.8.2.1 Decreased activation marker on splenic T lymphocytes was observed in stressed mice

The effect of restraint stress (RS) on the activation of T lymphocytes isolated from splenocytes was first investigated. Mice were restraint stressed for 21 days, whilst control mice received no restraint stress. Restraint stress did not affect the percentage of $CD3^+$ T Lymphocytes (figure 15). However, RS significantly decreased the percentage of $CD3^+$ $CD69^+$ T lymphocytes, $p<0.01$, where CD69 is the standard marker for early activation of T lymphocytes**.**

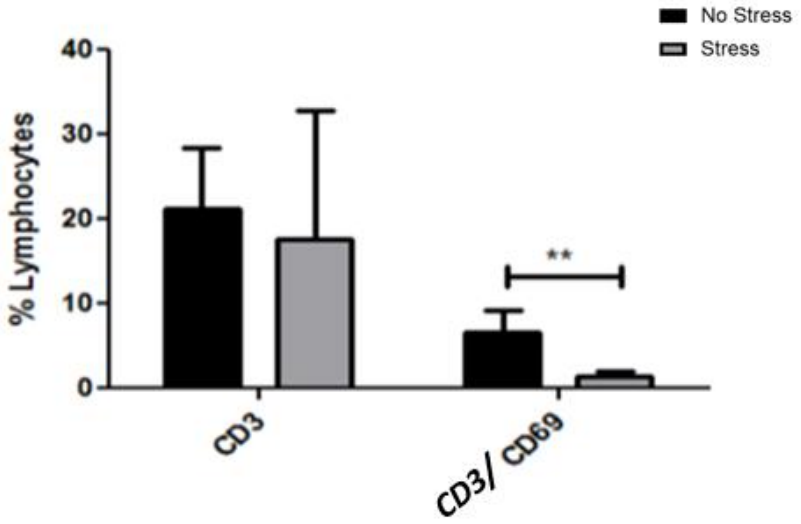


Figure 15: Restraint stress effectively decreased the activation of T lymphocytes
T lymphocytes were isolated from spleens isolated from both groups, S & NS (n=5). Mice from the S group were subjected to restraint stress for 2 h over 21 days. Cells were stained for flow cytometry analysis to detect the surface markers CD3 (a marker for T lymphocytes) and CD3/CD69 (a marker for early activation of T lymphocytes) by the fluorescently conjugated antibodies FITC- CD3 & APC-CD69, respectively. 20,000 events were acquired and gated at forward and side scatter. Each reading is the average of 3 technical replicates ± SEM. A significant decrease in T lymphocytes activation was observed in the stress group **$p<0.01$.

2.8.2.2 Optimisation of the spheroids-splenocytes co-culture

ID8/splenocyte co-cultures were developed to investigate the effects of stress on the interaction between immunocompetent cells and ovarian cancer cells. Splenocytes from stressed and non-stressed mice were co-cultured with previously fully formed ovarian cancer spheroids. To optimise this, isolated splenocytes and ID8 spheroid cells were seeded at a range of ratios, 1:1 (2500:2500), 1:3 (2500: 7500) and 1:5 (2500:12500) as illustrated in figure 16. The optimal ratio was found to be a 1:3. This is the ratio at which splenocytes are seen close to spheroid which show loss of integrity, suggesting that T cells within the splenocytes are cytotoxic against the tumour. Splenocytes contain T lymphocytes which undertake tumour associated attacks.

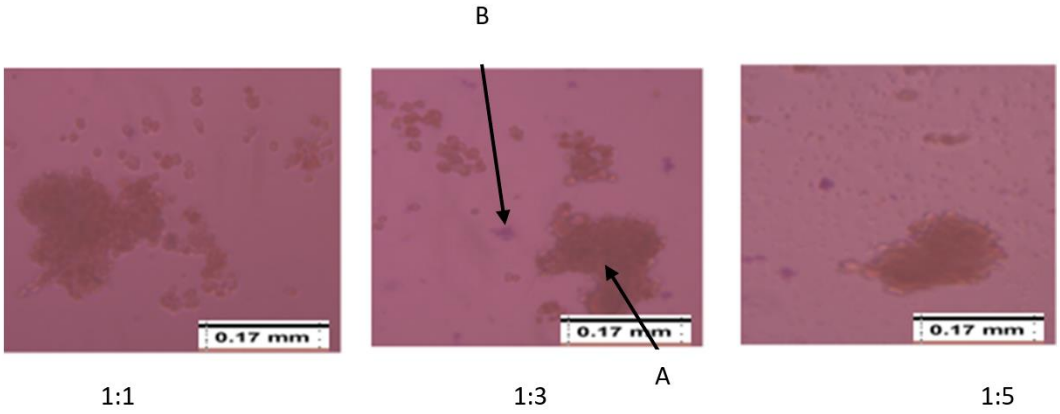


Figure 16: Spheroid cells-splenocytes co-culture of 1:3 is found to be the optimal ratio
Splenocytes were extracted from spleens isolated from both C57BL/6J mice groups, Stress (S) and no stress (NS). SP were stained with the lipophilic membrane fluorescent red stain DIL for 2 h and co-cultured with previously formed C57BL/6J ID8 ovarian tumour spheroids cells (green) over 7 days. The co-culture was performed at three different ratios of spheroid: splenocytes: 1:1 (2500:2500), 1:3 (2500: 7500) and 1:5 (2500:12500*). A- ID8 spheroids (large brown) and B-splenocytes (small pink)*. Light microscope images were captured 24 h post co-culture. Magnification X100.

2.8.2.3 Splenocytes from the stressed mice did not infiltrate effectively into the ID8 ovarian cancer spheroids

The ID8/splenocyte co-culture was developed to investigate the effects of stress on the interaction between immunocompetent cells and ovarian cancer cells. Splenocytes from stressed and non-stressed mice were co-cultured for five days with previously fully formed ovarian cancer spheroids. Splenocytes from non-stressed animals were able to infiltrate into ID8 spheroids, supporting a role of T cells to directly compromise ID8 spheroid integrity. Splenocytes from mice stressed for 21 consecutive days did not infiltrate into the ID8 spheroids as shown by confocal microscopy (figure 17). These experiments demonstrate that stress alone (in the absence of any underlying pathology) can reduce the responsiveness of splenic T Lymphocytes. The mechanisms and characteristics of these phenomena will be investigated in subsequent sections.

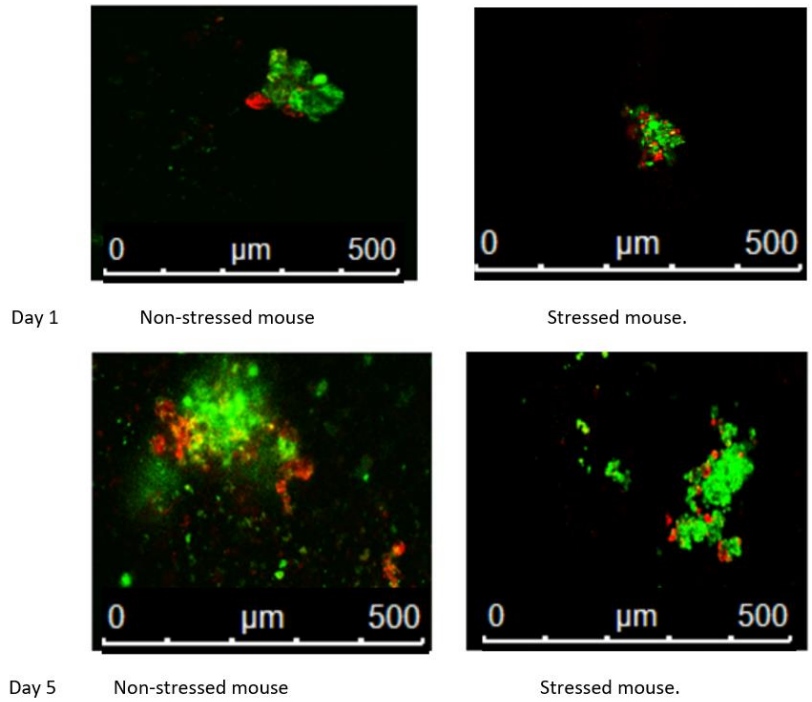


Figure 17: Splenocytes from restraint stressed mice did not infiltrate effectively in the ovarian cancer spheroid cells
Splenocytes from both S and NS C57BL/6J mice groups were extracted from the spleens following 2h daily RS (for the S group) over 21 consecutive days. The splenocytes were fluorescently stained with lipophilic membrane fluorescent red stain DIL and co-cultured with the previously formed ID8 spheroids, and fluorescently stained with green lipophilic membrane stain DIO at a ratio of 3:1 respectively. Confocal images of spheroids-splenocytes co-culture were captured on days 1 and 5. Magnification X100. The green cells are the ID8 spheroids, the red are the splenocytes and yellow/orange represents splenocytes infiltrated in the spheroid. Stress impairs the ability of splenic T lymphocytes to infiltrate the tumour spheroids.

2.8.3 Stress results in larger tumour spheroids-splenocytes (Sph-Sp) area.

Restraint stress seemed to affect the infiltration of splenocytes in OC spheroids (figure 17), directly affecting their anti-tumour activity. Based on morphological analysis of images taken by light microscopy, it was observed that the area of spheroids increased when co-cultured with splenocytes from stressed mice compared to the control unstressed mice. Images from light microscopy were captured daily for 4 days (figure 18). When the area of spheroid-splenocytes were measured, a significant increase in the areas of Sph-Sp from stressed mice was observed on days 2, $p<0.05$, day 3, $p<0.05$ and day 4, $p<0.001$ (figure 19). This increase in Sph-Sp areas could be due to SP surrounding the ID8 spheroids but unable to disintegrate them. To explore this, images were also captured by confocal microscopy daily for 4 days to study splenocytes trafficking and infiltration.

Representative images are presented in (figure 20). Splenocytes from stressed mice were unable to infiltrate into the ovarian cancer spheroids suggesting that the SPs are unable to disintegrate ID8 spheroids either because their anti-tumour activity is impaired by the stress, or they did not recognise the ID8 antigens because they have not been exposed to them previously.

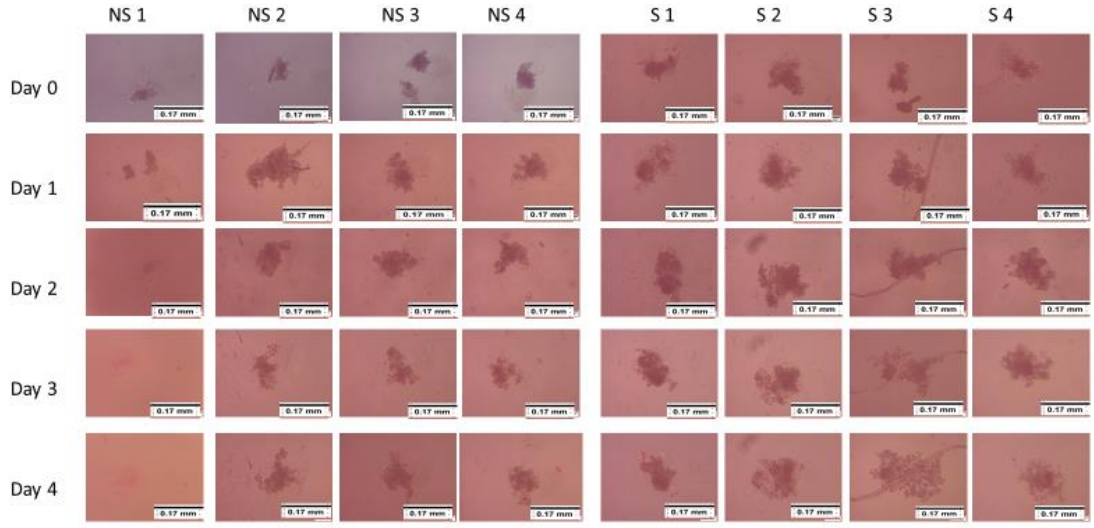


Figure 18: Co-cultures with splenocytes from stressed mice showed larger area of spheroid-splenocytes compared to non-stressed ones as observed from light microscopy images captured over the course of 4 days following the co-culture

ID8 spheroids were co-cultured with splenocytes extracted from spleens of NS and S C57BL/6J groups (n=4) at a ratio of 1:3, respectively. Mice from the stressed group were subjected to restraint stress for 2h daily over a period of 6 weeks. Light microscope images were captured daily for 4 days. Magnification X100. 3 images were captured for each sample daily.

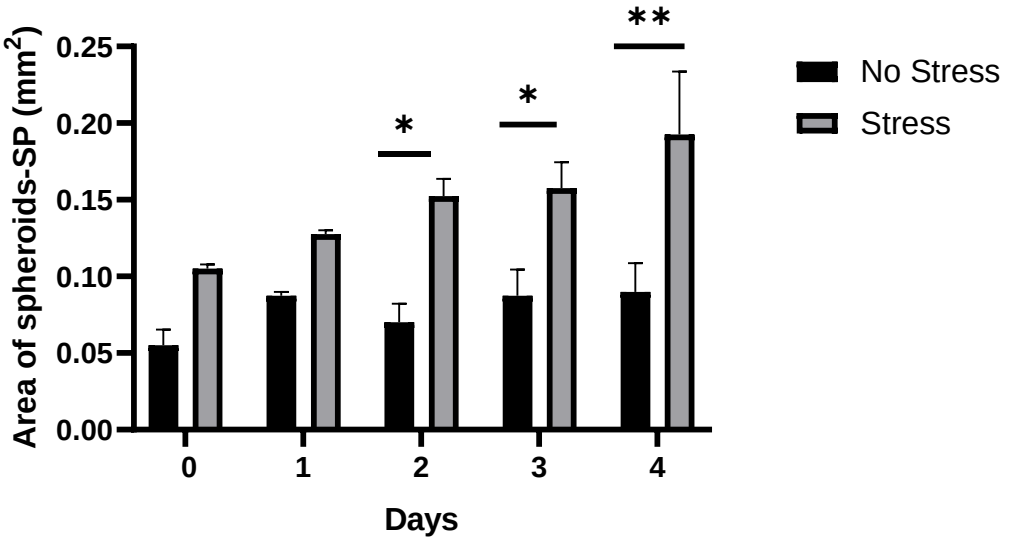


Figure 19: A significant increase in the spheroids-splenocytes area was observed in co-cultures with splenocytes from stressed mice

Previously prepared ID8 spheroids were co-cultured with splenocytes extracted from spleens of NS and S groups (n=4) at a ratio of 1:3 respectively. Mice from S group were subjected to restraint stress for 2h daily over a period of 6 weeks. 3 images were captured by light microscopy for each sample daily. Data are expressed as mean ± SEM. 2-way analysis of variance followed by Tukey's multiple comparisons test was used for the statistical analysis. There was a significant statistical difference between the groups on days 2 & 3, * $p<0.05$ and on day 4, ** $p<0.01$. *S-Stress, NS-No stress.*

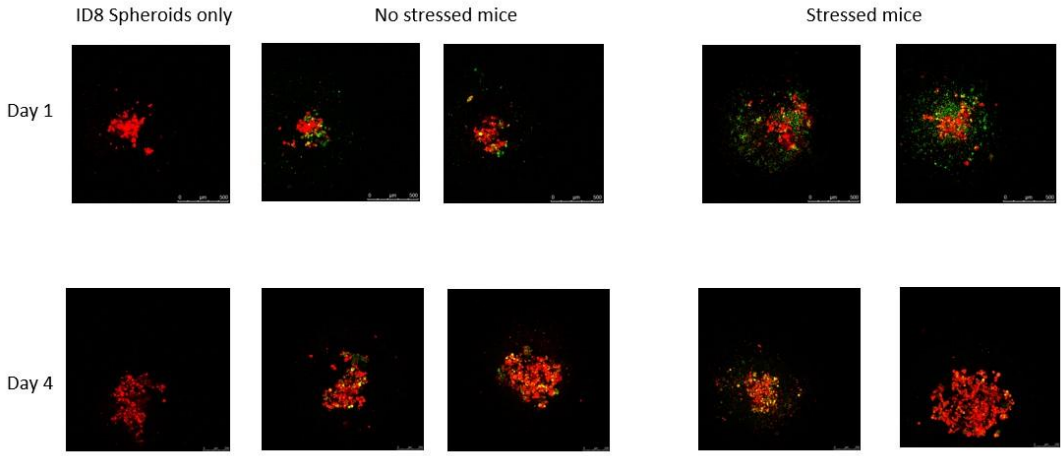


Figure 20: Splenocytes from stressed mice were unable to infiltrate into the ovarian cancer spheroids, increased the area of the spheroids by making the spheroids appear like a bigger cell cluster

Previously prepared ID8 spheroids were fluorescently stained with lipophilic red stain then co-cultured with fluorescently stained splenocytes (green) extracted from spleens of NS and S groups (n=4) at a ratio of 1:3, respectively. Mice from S group were subjected to restraint stress for 2 h daily over a period of 6 weeks. Confocal images were captured over a course of 4 days. Representative confocal images are presented, magnification X100. *Green -splenocytes, red- ID8 cells spheroid, yellow/orange-infiltrated splenocytes*

2.8.4 Activation of splenocytes with PMA/ionomycin and treatment with cortisol

2.8.4.1 Microscope analysis of spheroids-splenocytes 3-D co-cultures.

To mimic the observed effect of restraint stress on T cells activation and infiltration into ID8 tumours to identify the underlying mechanism, splenocytes were activated *in vitro* and treated with pharmacological concentration of cortisol.

Because restraint stress reduced T cell activation by reducing CD69 expression (figure 15), It was next decided to examine if this was mediated by the stress hormone, cortisol. The ability of cortisol to affect PMA/ionomycin activated splenic T cells infiltration in ID8 spheroids was investigated. To probe this mechanistically, splenocytes were isolated from a spleen of C57BL/6J mouse then either activated with PMA/ionomycin and named activated splenocytes (ASP) or left without activation and named unstimulated splenocytes (USP). Subsequently, both ASP and USP were co-cultured with previously formed and fluorescently stained ID8 spheroids and the ASP were treated with cortisol for 4 days.

Light microscope images were captured and analysed, and representative samples can be seen in figure 21. Spheroids co-cultured with PMA/ionomycin activated splenocytes were larger, less compact, and more irregular compared to spheroids with unstimulated ones, (compromised integrity of the spheroids is a sign of cytotoxic activity of splenic T lymphocytes). The areas of the Sph-Sp were measured and illustrated in figure 22, where a significant increase in the Sph-Sp areas was observed on day 4, $p<0.01$. Pharmacological concentration of cortisol (5muM) resulted in smaller areas and more compact spheroids-splenocytes co-cultures. This effect was also significant on day 4, $p<0.05$. To determine if the observed results from ASP and ASP/cortisol co-cultures were caused by splenocytes infiltration, confocal images were captured on day 1 and 4 of the co-cultures as illustrated in figure 23. It is not clear from the confocal images if the cortisol is allowing the spheroid to stay intact or preventing the splenocytes from dispersing the ID8 spheroid.

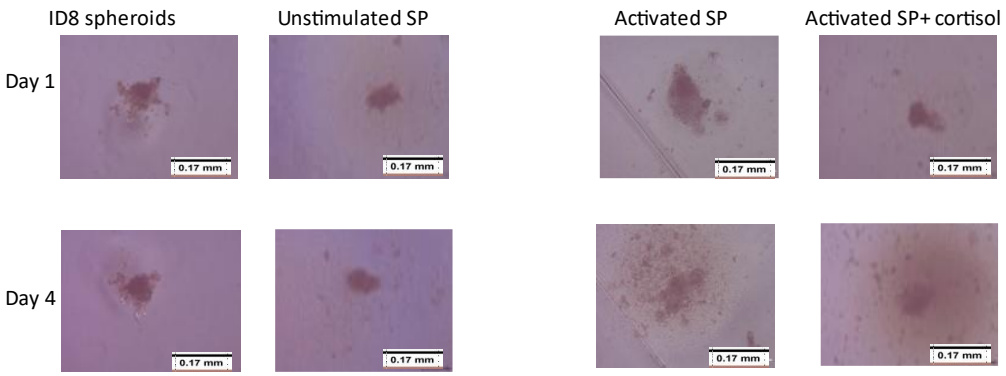


Figure 21: Larger, less compact, and more irregular Sph-Sp were observed from PMA/ionomycin activated SP and smaller, more compact Sph-Sp were observed from cortisol treatment
Previously formed ID8 spheroids were co-cultured with splenocytes or PMA & ionomycin activated SP extracted from C57BL/6J female mouse at a ratio of 1:3 respectively, either treated with cortisol (5µM) or left without treatment. Co-cultures of unstimulated splenocytes and spheroids were used as a control. Light microscopy images were captured over a period of 4 days, and 3 images were taken for each sample. Magnification X100.

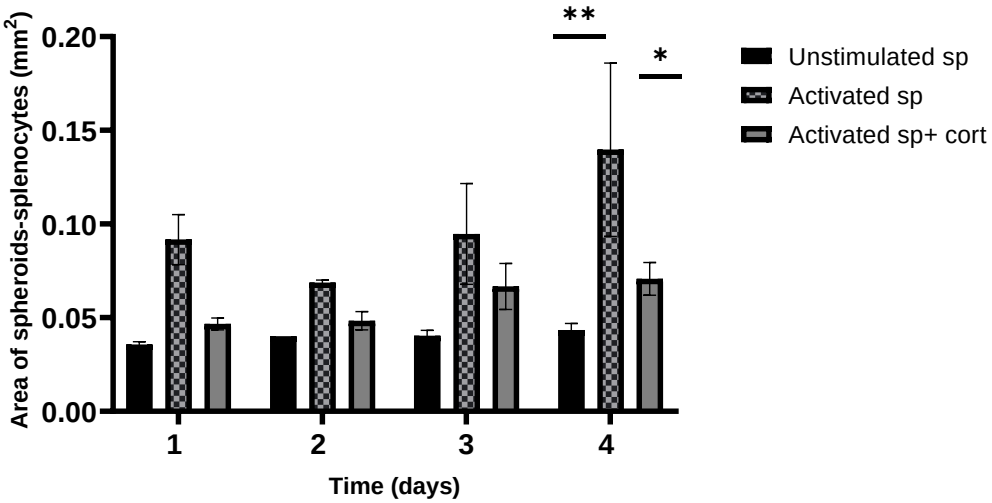


Figure 22: Larger areas of Sph-Sp were observed in PMA & ionomycin activated splenocytes co-cultures and treatment with cortisol decreased the areas of these co-cultures
C57BL/6J derived ID8 cells spheroids were previously prepared and fluorescently stained green over 7 days. Splenocytes were extracted from C57BL/6J female mice, fluorescently stained red then either stimulated with PMA & ionomycin and incubated at 37˚C & 5% CO_2 for 3h or left untreated. Spheroids and SP/ PMA & ionomycin activated SP were co-cultured at a ratio of 1:3 respectively. Activated splenocytes were either treated with pharmacological concentration of cortisol (5muM) or left without treatment. The treatment with cortisol continued for 4 days. Spheroids with no treatment and no additions were used as a control. Data are expressed as mean ± SEM. 2-way analysis of variance followed by Tukey's multiple comparisons test was used for the statistical analysis. *p<0.05, **p<0.01. *ASP-Activated splenocytes, USP-Unstimulated splenocytes, Sph-Sp- Spheroid-splenocytes.*

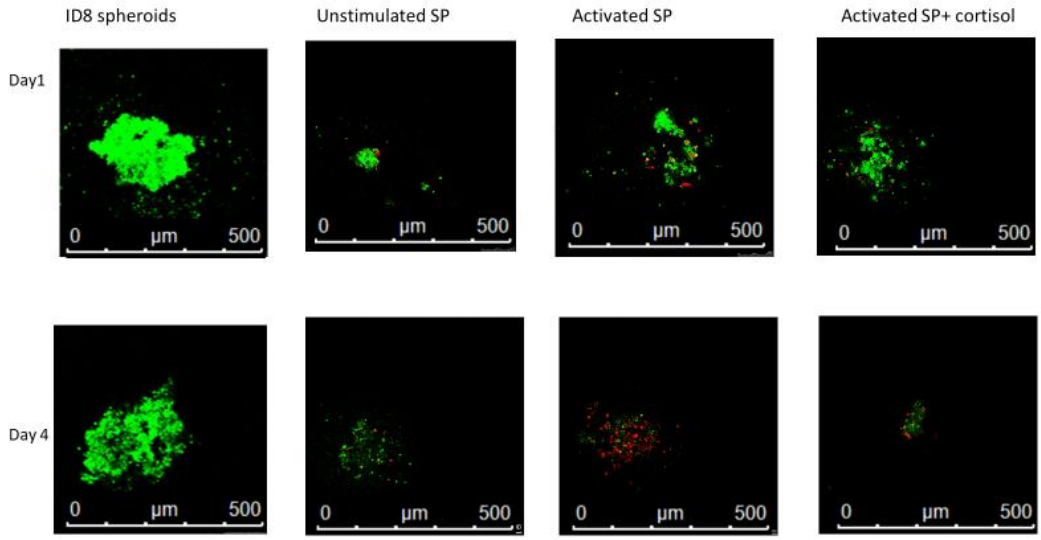


Figure 23: Activated splenocytes dispersed ID8 spheroid effectively and cortisol reverted this effect

Representative confocal images of Sph-Sp co-cultures of spheroid with unstimulated SP or PMA/ionomycin activated splenocytes in the presence and absence of cortisol. Magnification X100. *Green- ID8 spheroids cells, red-splenocytes, orange/yellow-spheroids infiltrated with splenocytes.*

2.8.4.2 Cortisol decreased the area of Sph-Sp in co-cultures of activated spleens and the gold standard GR antagonist (RU486) partially antagonised the effect of cortisol

It was shown from the previous findings that when PMA and ionomycin were used to activate splenocytes, the splenocytes could then disrupt spheroid integrity (figure 21&22) and this effect was inhibited by cortisol. The hypothesis is that the effect from cortisol (maintaining the spheroids integrity) was due to the activation of the cortisol receptor. Thus, it was hypothesised that the cortisol antagonist mifepristone (RU486) would reverse this effect.

A co-culture of SP or PMA/ ionomycin activated SP were treated with RU486, 30 minutes before cortisol. Light microscope images were captured daily for 4 days and assessed. Representative images are presented in (figure 24). RU486 compromised the integrity of the Sph-Sp by breaking the spheroids which facilitated the effect of activated SP. The areas of the Sph-Sp were measured and illustrated in (figure 25). A pattern decrease in Sph-Sp area was observed in the presence of cortisol and RU-486. RU-486 did not significantly affect the area of Sph-Sp. This is possibly because RU-486 is known to be a non-selective antagonist, and is specifically a progesterone receptor antagonist (267).

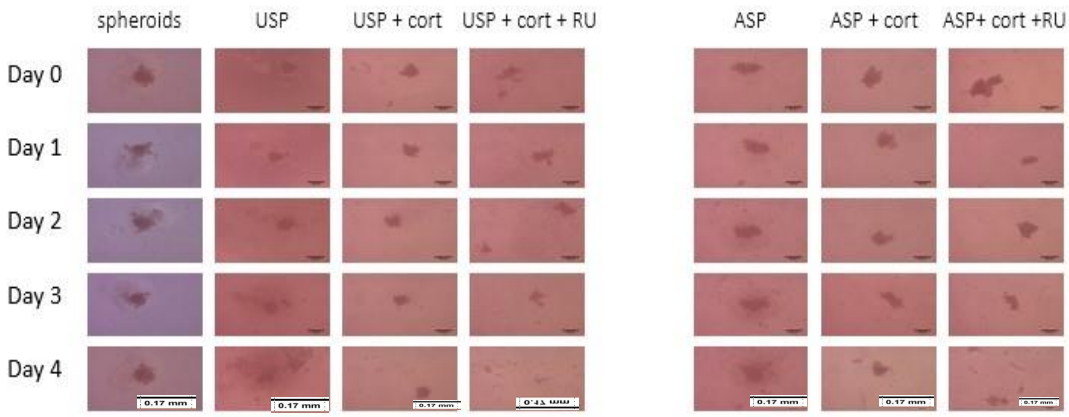


Figure 23: Cortisol formed smaller, more compact Sph-Sp and RU486 the GR antagonist partially antagonised this effect by breaking the spheroids

ID8 spheroids were previously prepared and fluorescently stained green and co-cultured with splenocytes or PMA/ionomycin activated SP extracted from spleens of C57BL/6J female mice and fluorescently stained red at a ratio of 1:3. Cells were either treated with 5 muM cortisol or 5 muM RU-486 30 mins before adding cortisol. Spheroids, USP and ASP were used as controls. Treatment continued daily for 4 consecutive days and light microscope images were captured daily over the time course. Representative light microscopy images are presented. Magnification X100. *USP- unstimulated splenocytes, ASP- Activated splenocytes with PMA (Phorbol 12-myristate 13-acetate) & ionomycin, Sph-Sp-spheroi-splenocytes, cort-cortisol & RU- RU486 (mifepristone).*

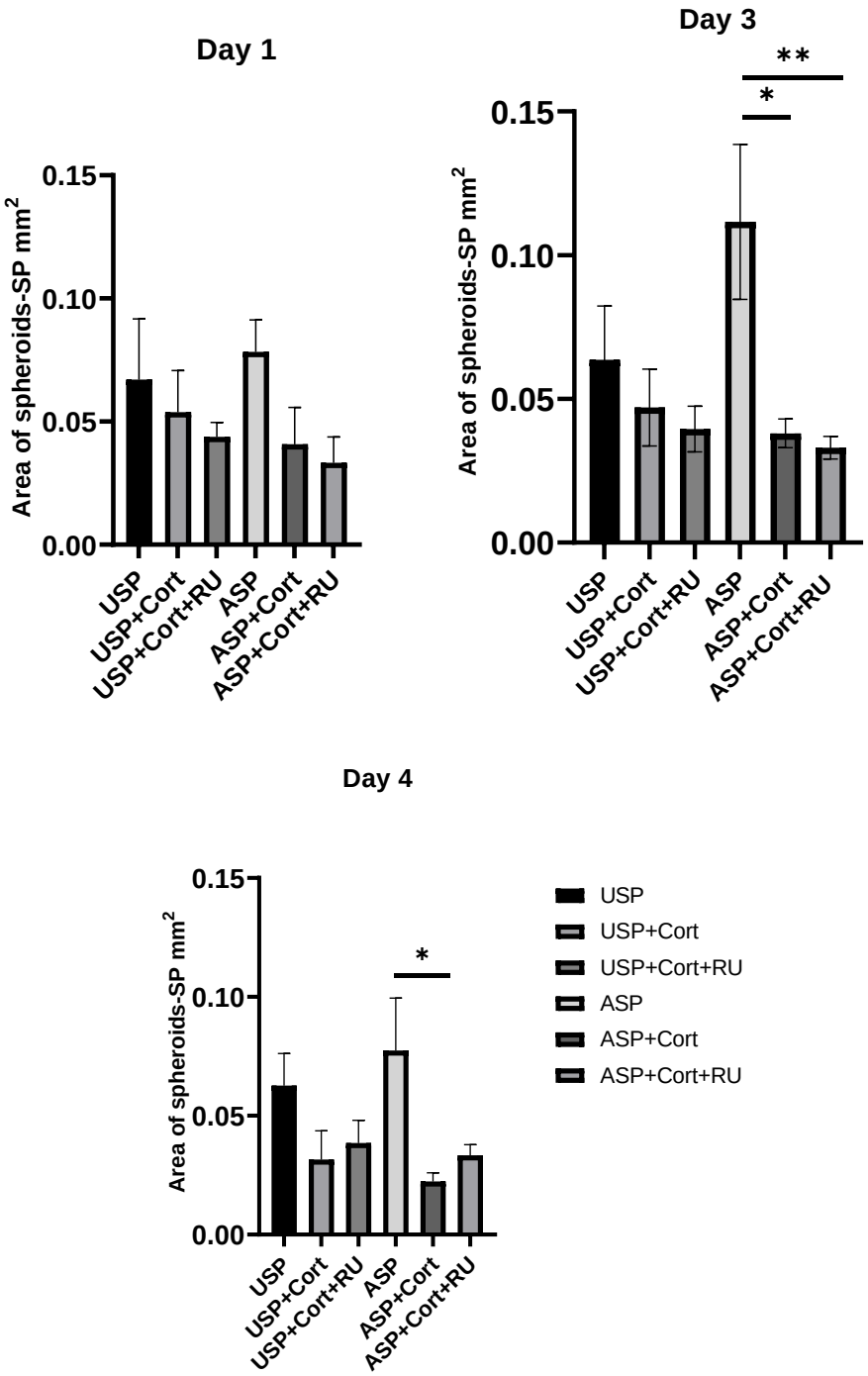


Figure 24: Cortisol decreases the area of Sph-Sp and the GR antagonist RU-486 appears to revert this by dispersing the spheroids

Splenocytes were extracted from *ex vivo* spleens isolated from female mice (C57BL/6J), fluorescently stained with lipophilic stain, and divided into 2 groups. First was an activated group which was treated with PMA/ionomycin, and the second group was unstimulated. Both groups were co-cultured with previously prepared and fluorescently stained ID8 spheroids at a ratio of 3:1 respectively. Cells were either treated with 5muM cortisol or 5muM RU-486, 30 mins before adding cortisol. Spheroids, USP and ASP were used as controls. Data is presented as mean ± SEM of 2 technical replicates and 3 biological replicates. ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis. *p<0.05, **p<0.01. *USP- unstimulated splenocytes, ASP- Activated splenocytes, Cort- cortisol, GR- Glucocorticoid receptor antagonist, RU- RU486 (mifepristone), PMA- Phorbol 12-myristate 13-acetate and Sph-Sp=Spheroid-Splenocytes.*

2.8.4.3 The effect of stress on spheroid-PMA/ionomycin activated splenocyte co-cultures was successfully reverted by novel GR antagonists

Because the RU486 is a non-selective antagonist, novel GR antagonists were used. Relacorilant and CORT125281 are currently under clinical trials in breast and ovarian cancer. In this work they facilitated the anti-tumour action of PMA/ionomycin activated splenocytes and dissociated the cancer cells spheroids as illustrated in figure 26. The areas of the spheroid-splenocyte co-cultures were measured and illustrated in figure 27. The dissociation of Sph-Sp by activated splenocytes was partially reverted by the stress hormone cortisol (5muM) as smaller, more compact co-cultures were observed but not to a significant level. All GR antagonists facilitated the effect of ASP by disintegrating the spheroids (figure 26&27). To confirm if the change in the areas of Sph-Sp co-cultures were associated with splenocytes infiltration and dissociation of the spheroids, confocal images were captured on day 1,3 and 4 of the co-culture protocol as illustrated in figure 28. This may suggest that the observed stress-splenic T lymphocytes activation inhibition is not likely to be reverted by GRs antagonists.

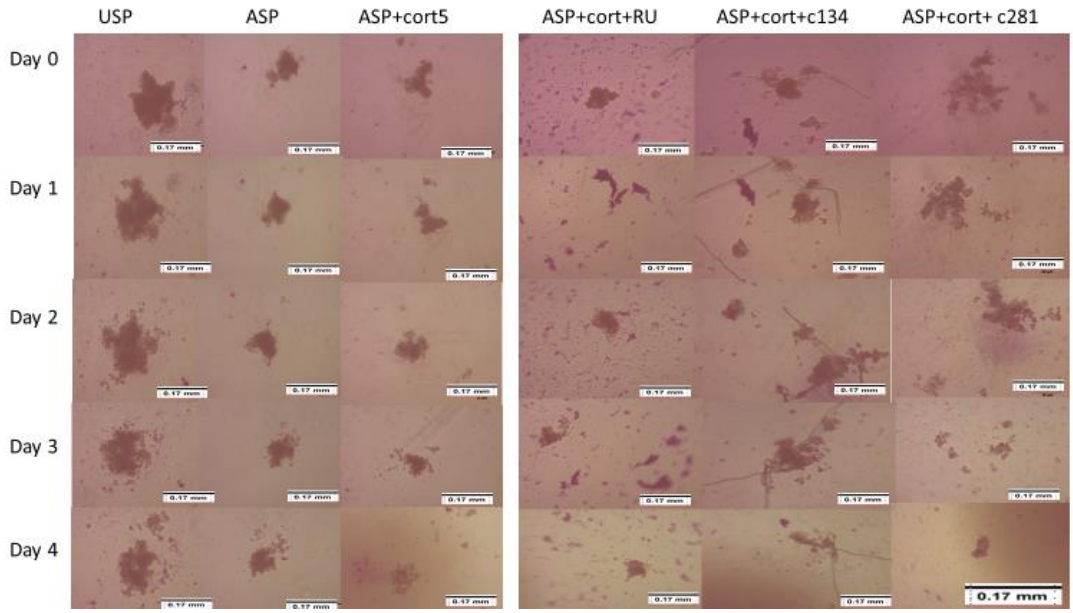


Figure 25: More specific GR antagonists potentiated the anti-tumour immunity of PMA/ionomycin activated splenocytes

Splenocytes were extracted from spleens isolated from female mice (C57BL/6J), fluorescently stained red and divided into 2 groups, first is activated with PMA/ionomycin and second is unstimulated then co-cultured with ID8 spheroids. Cells were either treated with 5 µM cortisol or 5 µM RU486 (mifepristone), Relacorilant (c134) or CORT125281 (c281), 30 mins before adding cortisol. USP and ASP were used as controls. Treatment continued daily for 4 consecutive days and light microscope images were captured daily. Representative light microscope images are presented. Magnification X100. *USP- Unstimulated splenocytes, ASP- Activated splenocytes with PMA (Phorbol 12-myristate 13-acetate) & ionomycin, Cort- Cortisol & RU - RU486 and Sph-Sp-Spheroid-Splenocytes, c134-relacorilant, c281-cort125281.*

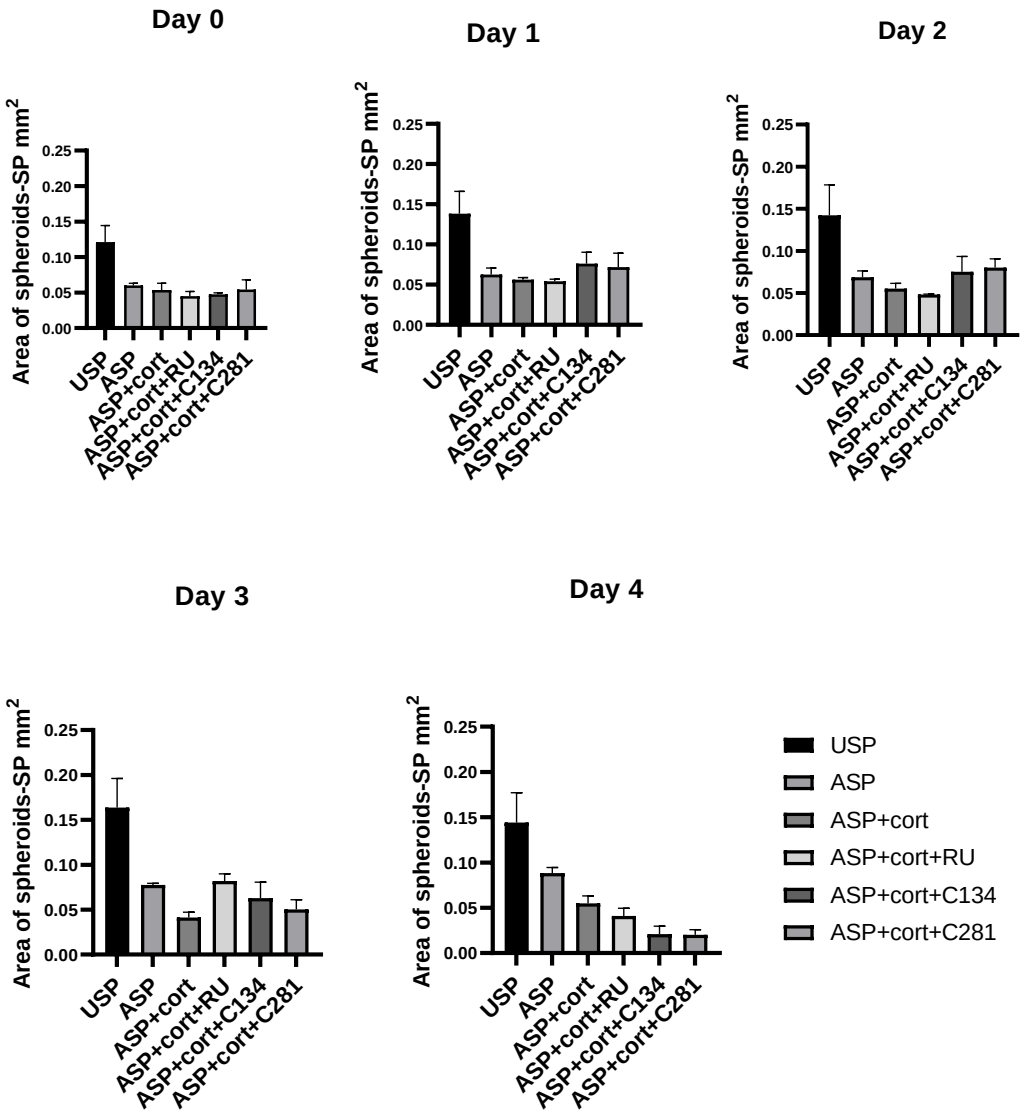


Figure 26: GRs antagonists facilitated the cytotoxic activity of PMA/ionomycin activated splenocytes

Areas of Sph-Sp co-cultures were measured by ImageJ. Data is presented as mean ± SEM of 3 technical replicates. ANOVA followed by Kruskal-Walli's test for multiple comparisons was used**.** No significant effects were observed from cortisol and GRs antagonists on Sph-Sp areas. *USP- unstimulated splenocytes, ASP- Activated splenocytes with PMA (Phorbol 12-myristate 13-acetate) & ionomycin, Cort-cortisol & RU- RU486 (mifepristone), c134-Relacorilant, c281-CORT125281 and Sph-Sp-Spheroid-Splenocytes.*

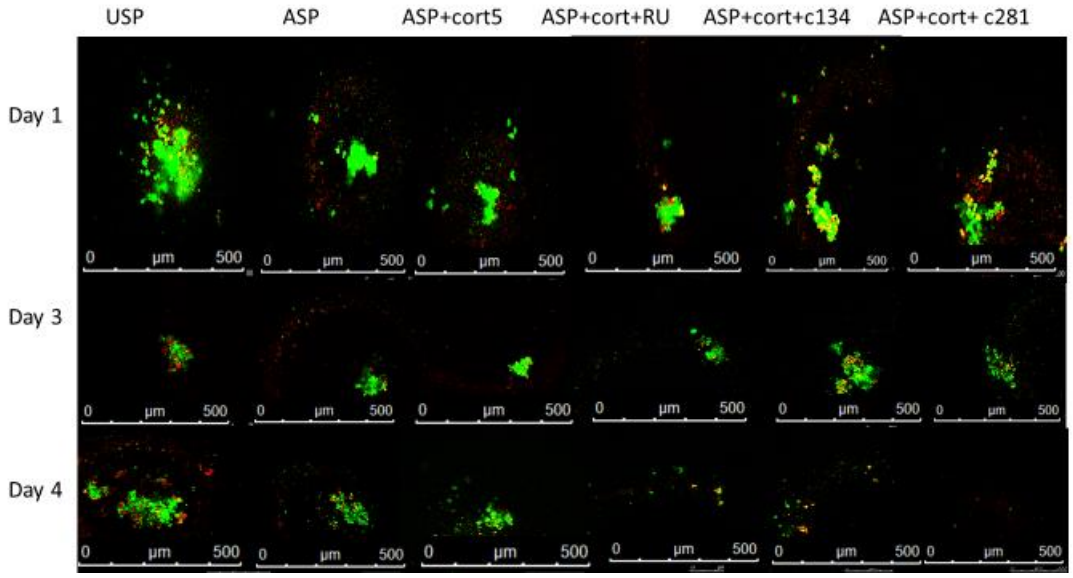


Figure 27: Representative confocal images of Sph-Sp co-cultures taken over a course of 3 days. Magnification X100

USP- unstimulated splenocytes, ASP- Activated splenocytes with PMA (Phorbol 12-myristate 13-acetate) & ionomycin, cort-cortisol & RU-RU486 (mifepristone), c134-(Relacorilant), c281-(CORT125281 and Sph-Sp-Spheroid-Splenocytes. Green- ID8 spheroids cells, red-splenocytes, orange/yellow=spheroids infiltrated with splenocytes.

2.8.5 Restraint stress and DNA damage in immune cells

2.8.5.1 Chronic restraint stress (RS) induces DNA damage in the immune cells splenocytes

Restraint stress reduced T cell activation and thus their ability to infiltrate ID8 spheroids as observed from the previous experiments (figures 17,17,20,23 and 28). There are many mechanisms through which stress can affect the cells directly. One possible mechanism is DNA double strand break (DSB). Based on evidence from the literature showing that stress hormones can increase double strand DNA damage and cause apoptosis in pre-cancerous and cancerous cells (103, 107). It was therefore hypothesised that the suppression in T cells activation could be in part due to DNA damage. Thus, the gamma histone γ-H2AX assay was used to quantify the extent of DNA DSBs in splenocytes from stressed and non-stressed female mice.

Immunofluorescence analysis of splenocytes using confocal microscopy illustrated significantly larger DNA damage in splenocytes subjected to chronic restraint stress when compared to control (no stress), $p<0.01$. This can be seen in figure 29, where there is larger increase in cells with 4 or more foci when compared with the no stress

control splenocytes. Overlay images were used to assess and quantify the number of FITC green foci within cells, cells fluorescing as turquoise were regarded as having too many foci to count and scored as damaged (figure 30). Restraint stress significantly increased the number of γ-H2AX positive splenocytes. γ-H2AX is released in response to DNA double strand breaks which cause the highest form of damage to DNA (270).

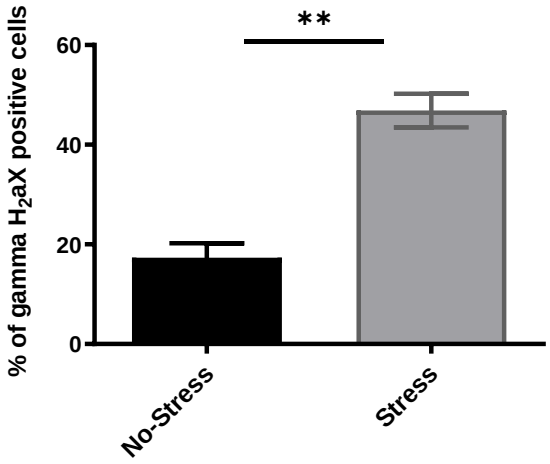


Figure 28: Chronic restraint stress increases DNA damage in splenocytes
The two groups of female C57BL/6J mice (n=3), were subjected to restraint stress for 2h over 6 weeks stress (S) compared to control (NS). Splenocytes were immunofluorescently labelled FITC green to detect and quantify gamma H2AX positive cells. Cells with 4 or more foci were scored positive and expressed as a percentage of the total cells. Data is presented as mean ± SEM of 3 technical replicates. T test was used for the statistical analysis. *p<0.05, **p<0.01.

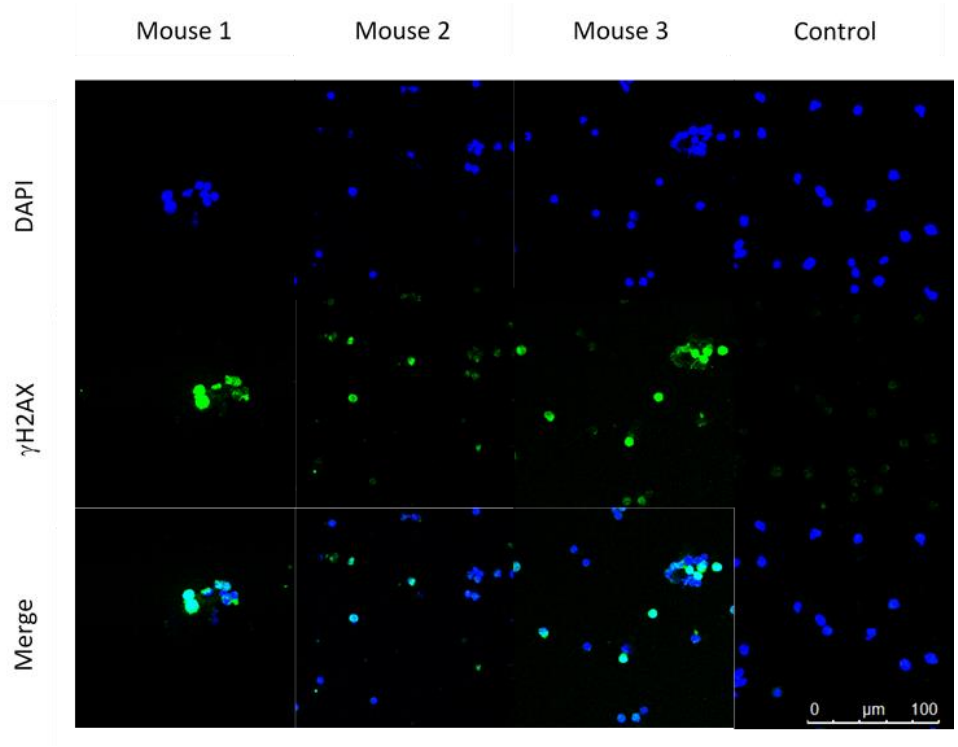


Figure 29: Representative confocal images of splenocytes fluorescently stained to detect γ- H2AX positive cells. Magnification x40 oil. γ-H2AX is a marker for double strand DNA damage
6 C57BL/6J female mice were stratified into 2 groups (n=3) and named according to procedures: stress and no stress (control). Mice from the stress group were subjected to restraint stress for 2 h over 6 weeks. Splenocytes were then isolated from spleens of stressed and non-stressed mice and fluorescently stained to detect γ-H2AX positive cells using primary and secondary antibodies. The secondary antibody: anti-mouse /rabbit IgG fluorescein isothiocyanate (FITC) were used to detect gamma γ- H2AX (green colour) and 4',6-diamidino -2-phenylindole (DAPI) the blue stain was used to detect cells nuclei. A total of 50 cells were counted from each sample (due to the low adherence of splenocytes to the cover slips) and those with 4 or more green foci were scored positive and expressed as a percentage of the total cells, the cells coloured turquoise were counted positive as they were considered to have too many foci.

2.8.5.2 Cortisol increases DNA damage in T lymphocytes

To support the previous finding that chronic stress induced SP' DNA damage (figures 29 & 30) and mimic this effect *in vitro*, it was investigated whether a high acute exposure of splenocytes to cortisol can cause DNA damage in T lymphocytes. T lymphocytes were extracted from spleens isolated from C57BL/6J female mice and divided into 2 groups: control (no cortisol) and cortisol (cort). Samples from the cortisol group were exposed to high concentration of cortisol (10muM) for 30 mins to mimic the stress performed in the experiments displayed in figure 29, then immunofluorescently stained to detect and quantify gamma γ-H2AX as an indicator for DNA DSBs. Immunofluorescence analysis of $CD3^+$ T lymphocytes using confocal microscopy illustrated greater DNA damage in cells exposed to 10 µM cortisol when

compared with control (no cortisol). This can be seen in figure 31. Overlay images of γ-H2AX foci (green) on DAPI blue stained cells is illustrated in figure 32 where more γ-H2AX foci in (b) treatment than in (a) control can be observed. It should be noted that T lymphocytes proved harder to analyse under the confocal microscope, as there was reduced cell viability. Acute exposure to high concentration of cortisol significantly increased the number of γ-H2AX positive T lymphocytes <0.05, suggesting that DNA damage can alter the function of effector T lymphocytes.

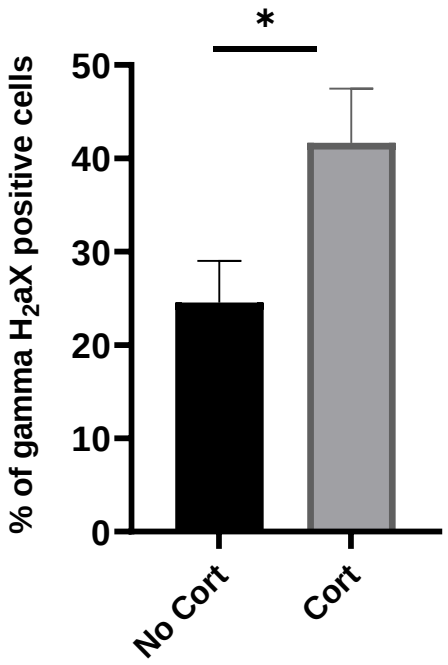


Figure 30: High concentration of cortisol induced DNA damage in T lymphocytes on acute exposure T lymphocytes were extracted from the spleen of C57BL/6J female mice and tested positive for CD3. They were divided into 2 groups: control (no cortisol) and cortisol (cort). Samples from the cortisol group were exposed to high dose of cortisol (10muM) for 30 mins to mimic the stress in figure 19 then immunofluorescently stained to detect and quantify γ-H2AX to assess for DNA DSBs. Each reading represents the mean ± SEM of 3 technical replicates of 1 mouse, and t test was used for the statistical analysis. *p<0.05, **p<0.01. Cort-Cortisol.

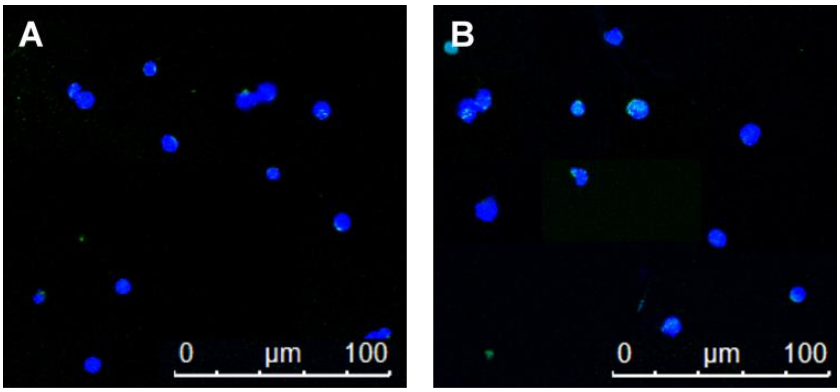


Figure 31: Representative confocal microscopy overlay images of CD3$^+$T lymphocytes (x40 oil)
(A) control (No cortisol) and (B) treatment (10µM cortisol). To quantify DNA damage, cells with 4 or more green foci were scored positive and expressed as a percentage of the total cells. The cells coloured turquoise were counted positive as they were considered to have too many foci. Hydrogen peroxide (H_2O_2) was used as a positive control and negative control samples without a primary antibody were prepared to exclude any background interferences. *DSBs-Double strand breaks.*

2.8.5.3 Physiological concentration of cortisol did not induce DNA damage

In the previous experiment a pharmacologically high concentration of cortisol was used, whereas in this experiment, the effect of acute exposure to physiologically relevant concentrations of cortisol on DNA damage in splenocytes was examined to determine if the cortisol induced DNA damage is mediated by GRs. Splenocytes were treated *in vitro* with either 1muM cortisol or 1muM cortisol / 1muM RU-486 for 30 mins. Cells were then immunofluorescently stained to detect and quantify γ-H2AX as an indicator for DNA damage. Cortisol had no significant effect on DNA damage in splenocytes and no effect was observed from RU486 (figure 33).

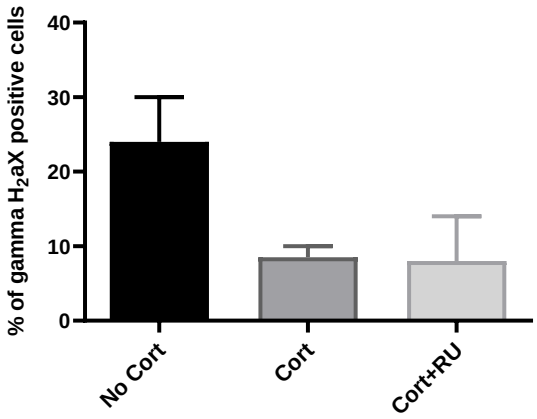


Figure 32: Splenocytes treated with physiologically relevant concentration of 1 µM cortisol for 30 mins showed less DNA damage and RU-486 did not reverse this effect
Splenocytes were isolated from a spleen of C57BL/6J female mice and treated *in vitro* with either 1µM cortisol or 1muM cortisol / 1muM RU-486 for 30 mins. Following exposure, cells were immunofluorescently stained to detect and quantify gamma-H2AX positive cells as an indicator for DNA DSBs. Hydrogen peroxide (H_2O_2) was used as a positive control, splenocytes only were used as negative control and splenocytes with no primary antibody were used as a negative control to eliminate any background interferences. Each reading represents the mean ± SEM of 2 technical replicates, for the statistical analysis, one way analysis of variance (post hoc Tukey multiple comparisons) was used, *p<0.05, **p<0.01. 2 biological replicates were used. There was no statistical significance between the groups. *DSBs-Double strand breaks, Cort-Cortisol, RU-RU486 (mifepristone).*

To further explore the impact of stress on the anti-tumour activity in EOC, the impact of stress on DCs maturation and activation was investigated. DC is a key cell bridging the innate and adaptive immune responses because it plays a pivotal role in antigen presentation and the initiation of the adaptive anti-tumour immune response (271).

2.8.6 The impact of stress on the antigen presenting activity of dendritic cells

2.8.6.1 Restraint stress for 2 weeks impairs the expression of the activation markers in splenic DCs

Previous experiments have concentrated on the T cell population of the spleen. It was hypothesised that other cell types such as dendritic cells (DCs), the sentinel antigen presenting cells, could be affected by stress at an early stage of the cancer (2 and 4 weeks). Thus, the effect of stress on DCs antigen presenting activity was investigated by flow cytometry measuring the expression of the activation marker MHCII and the co-stimulatory factor CD40 in splenic and lymph nodes (LN) DCs. Gating strategy based on $CD11c^+$ DCs is illustrated in figure 34. Splenic DCs were activated by challenging mice with intraperitoneal injections of ID8 cells. The

percentage of activated splenic DCs ($CD11c^+$ $MHCII^+$ & $MHCII^+$, $CD11c^+$ $CD40^+$ & $CD40^+$) after 2 weeks of RS is demonstrated in figure 35 and quantified as illustrated in figure 36. Stress significantly reduced the expression of the activation markers MHCII, $p<0.01$ and CD40, $p<0.01$ in splenic DCs. The study was repeated once, and the results were successfully reproduced. The results from the 2 independent biologic repeats were combined and illustrated in figure 36.

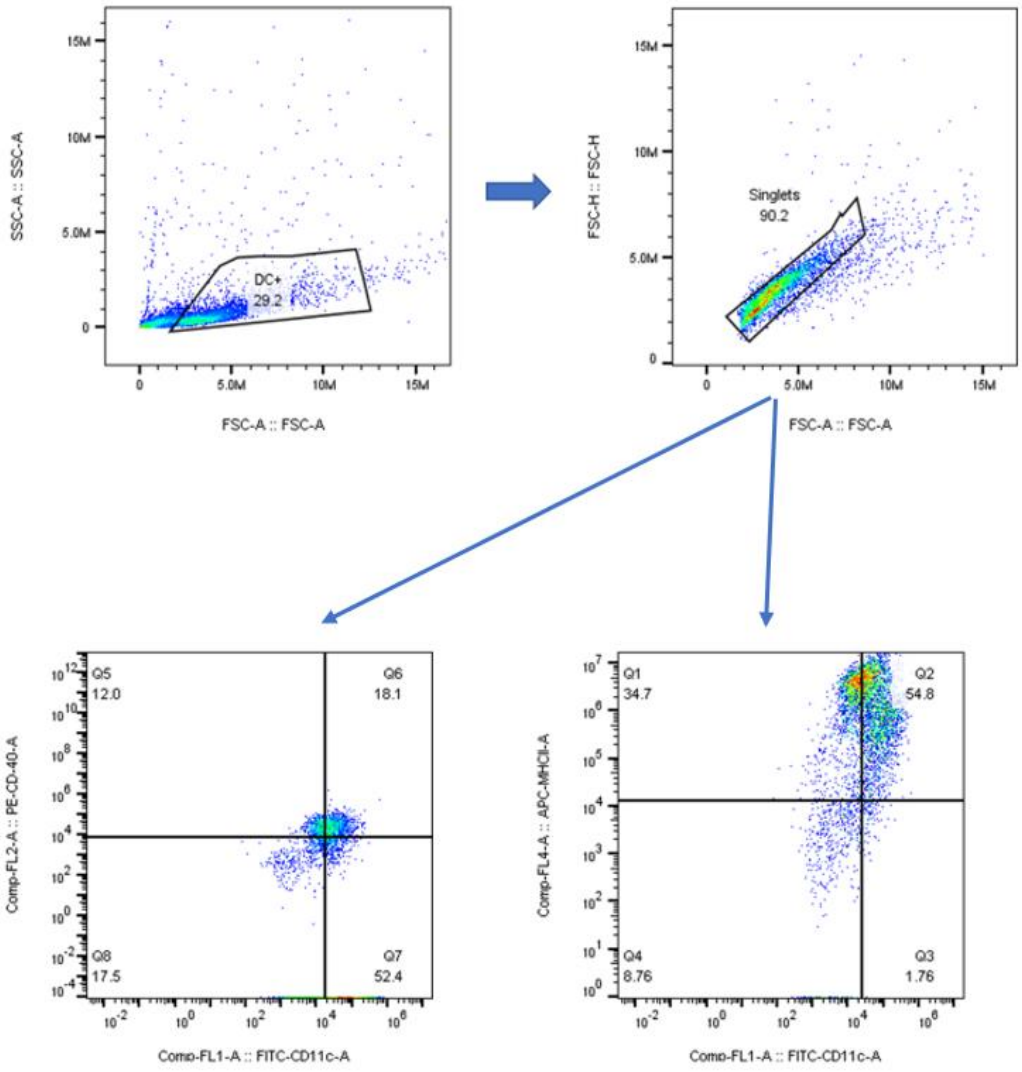


Figure 33 Figure 34: Gating strategy used to identify $CD11c^+$dendritic cells with activation markers MHCII and CD40 generated from co-culture of *ex vivo* bone marrows (BM) and splenocytes (SP) and named BMSP

BMSP were gated by their characteristics forward scatter (FSC) & side scatter (SSC). Gated on BMSP including dead granulocytes and debris, positive populations were identified as possible DCs (A). Gated on single cells of the expected DCs (B), DCs were identified as positive for CD11c. Stimulated DCs were defined as $CD11c^+$ and $MHC11^+$ (C) and $CD11c^+$ and $CD40^+$ (D). Single stain samples were used on immature DCs for compensation to minimise fluorescence overlapping. The gate was applied to all samples.

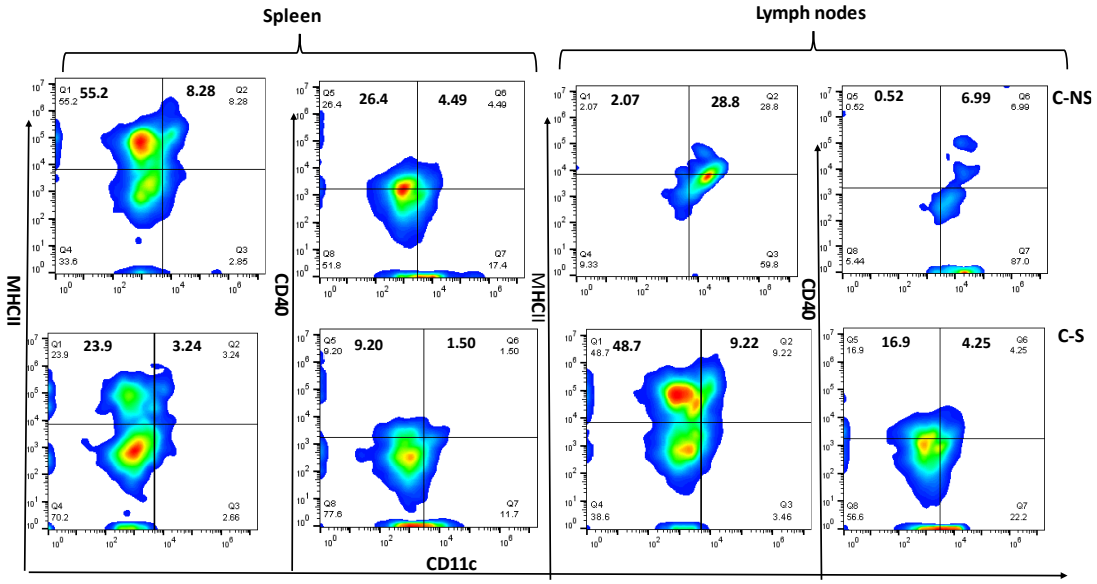


Figure 34: Representative flow cytometry images analysed by Flowjo illustrating the percentage of activation markers expressed by splenic DCs

Representative flow cytometry images analysed by BD Flowjo software illustrating the percentage of activation markers expressed by splenic DCs. The expression of the activation markers MHCII and CD40 by splenic DCs of stressed and non-stressed mice were identified by antibodies conjugated with fluorescent stains: CD11c by Green fluorescence-FITC (FL1), CD40 by orange fluorescence PE (FL2)) and MHC II by red fluorescence APC (FL4). BD Accuri C6 flow cytometry was used for sample process and generating data. Flowjo software was used for data analysis. Unexpectedly, the positive populations that are observed for $CD11c^{+}$ DCs are much less than expected. This is likely to be due to a technical problem with the staining antibody.

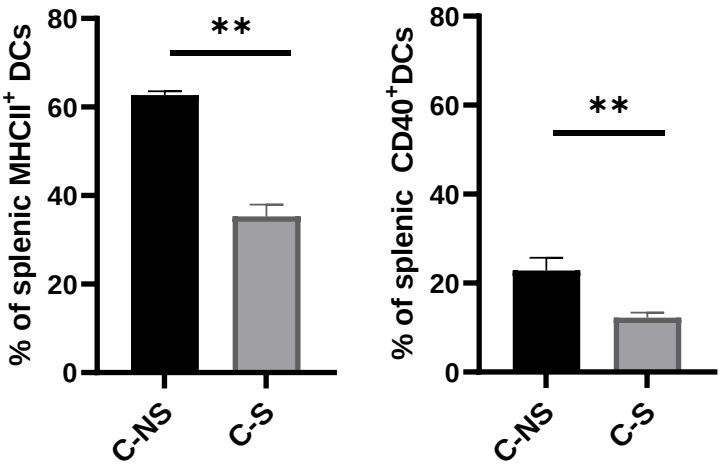


Figure 35: Stress for 2 consecutive weeks following IP injections of ID8 cancer cells impaired the expression of MHCII/CD40 in splenic dendritic cells

Stress significantly reduced the % of expression of the activation markers MHCII & CD40 in splenic DCs. The study was repeated once more, and the same results were observed from samples of the repeat experiment (n=5). Samples from both experiments were pooled (n=10). 10, eight-week-old female C57BL/6J mice were injected with $7x10^6$ ID8 (EOC) cells in 200 µl PBS IP, then stratified into 2 groups of n=5 and named according to the procedure to be undertaken: Cancer-Stress (C-S) and Cancer-No stress (C-NS). Mice from the stress group were subjected to restraint stress (RS) daily for 2 h by placing them in a 50 ml perforated falcon tubes. The mice from the NS group were left in their cages. RS started 1 week before the IP injections and continued for 2 weeks. The mice underwent gross necropsy to harvest spleens from which dendritic (DCs) were extracted by PAN DCs kit and stained for flow cytometry analysis. The percentage of activation and maturation markers was identified by antibodies conjugated with the fluorescent stains: CD11c by Green fluorescence-FITC (FL1), CD40 by orange fluorescence PE (FL2)) and MHC II by red fluorescence APC (FL4). BD Accuri C6 flow cytometry was used for sample processing and generating data. Flowjo software was used for data analysis. Data is presented as mean ± SEM of 2 technical replicates and combined from 2 independent biologic repeats. T test was used for statistical analysis, *p<0.05, **p<0.01. *IP - Intraperitoneal, C-S- Cancer-Stress, C-NS-Cancer-No stress, RS- Restraint stress.*

2.8.6.2 Restraint stress for 2 weeks does not affect the percentage of expression of MHCII and CD40 in LN DCs

Because LN are the secondary lymphoid organ for antigen presentation by DCs to T lymphocytes and the initiation of adaptive anti-tumour immune response, LN DCs were also analysed for activation markers following the ID8 cells challenge and 2 weeks restraint stress. The percentage of activated LN DCs ($CD11c^+MHCII^+$ and $MHCII^+$, $CD11c^+CD40^+$ and $CD40^+$) after 2 weeks of RS is illustrated in figure 35 and quantified in figure 37. Stress did not significantly affect the percentage of LN DCs expressing the activation markers MHCII and CD40 in the presence of ovarian cancer cells (ID8). This may suggest that stress at an early stage of EOC does not impair the

ability of LN DCs to express the activation markers MHCII/CD40 which could be because LNs are small, therefore isolating DCs from them was technically challenging.

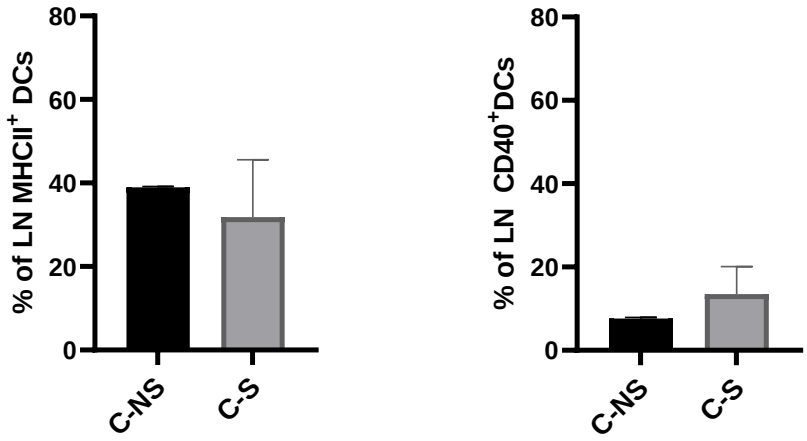


Figure 36: Stress for 2 consecutive weeks following IP injections of ID8 cancer cells did not supress the expression of the activation markers MHC II and CD40
10, eight weeks old female C57BL/6J mice were injected with $7x10^6$ ID8 (EOC) cells in 200 µl PBS IP, then stratified into 2 groups of n=5 and named according to the procedure to be undertaken: Cancer-Stress (C-S) and Cancer-No stress (C-NS). Mice from the stress group were subjected to restraint stress (RS) daily for 2 h by placing them in a 50 ml perforated falcon tubes. The mice from the NS group were left in their cages. RS started 1 week before the IP injections and continued for 2 weeks. Fresh lymph nodes from both S and NS groups were harvested to isolate DCs by PAN DCs kit. DCs were then stained with fluorescently conjugated antibodies to identify the activation markers MHCII and CD40 by flow cytometry analysis. Data is presented as mean ± SEM of 2 technical replicates, and t test was used. No significant effects were observed. *IP-Intraperitoneal, C-S- Cancer-Stress, C-NS-Cancer-No stress, RS-Restrain stress.*

2.8.6.3 ID8 spheroids-splenocytes co-cultures from 2 weeks stressed mice show larger areas over a course of 4 days when compared with the non-stressed mice

It was demonstrated previously (figures 17 and 20) that RS can impair the splenocytes ability to infiltrate ID8 spheroids and damage them in the absence of ovarian tumour antigens. The impact of 2 weeks of RS following the exposure to ovarian tumour antigens and the SP ability to infiltrate ID8 spheroids was next investigated. We hypothesised that T lymphocytes within splenocytes exposed *in vivo* to ID8 cells would develop tumour antigens specific immunity and recognise these cells on further exposures. Previously prepared and fully formed C57BL/6J ID8 spheroids were co-cultured with SP extracted from spleens of NS and S groups following ID8 injections and 2 weeks RS. Confocal microscope images were captured over a course

of 3 days and representative samples are illustrated in figure 38. SP from unstressed mice demonstrated an ability to compromise the integrity of ID8 spheroids. However, SPs from stressed mice were not able to disintegrate ID8 spheroids as larger Sph-Sp areas were observed in stressed mice compared to NS ones. This coincides with the effects observed from stress on ID8 cells spheroids in the absence of cancer (figures 17 and 20) and suggests that stress has the potential to impair SP ability to infiltrate ID8 spheroids despite previous exposure to these antigens (ID8 cells) where T lymphocytes within splenocytes are expected to recognise the tumour antigens and mount a tumour antigen specific attack. This may also suggest that the observed impairment in the DCs activation markers is not driven by the ovarian tumour.

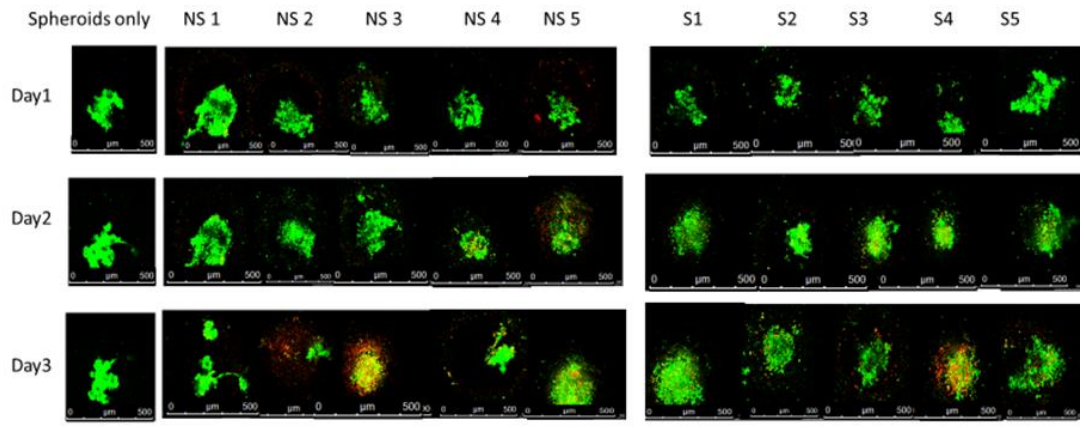


Figure 37: Larger spheroids-Splenocytes areas were observed in co-cultures with splenocytes from stressed mice compared to non-stressed ones
SP were isolated from spleens of S &NS mice (n=5) after they were subjected to 2 weeks of RS and challenged with $7x10^6$ ID8 cells/200ul PBS IP injections. SP were fluorescently stained red then co-cultured with previously prepared and fluorescently stained green C57BL/6J ID8 spheroids at a ratio of 3:1, respectively. Confocal images were captured over a course of 3 days. Magnification X100. Scale 0-500 µm. *S- Stress NS-No Stress RS- Restraint Stress SP-Splenocytes IP- Intraperitoneal injection Yellow-Spheroids infiltrated with splenocytes.*

2.8.6.4 Restraint stress for 4 weeks does not affect the expression of MHCII and CD40 in lymph node and splenic DCs

The next experiment performed was to investigate the impact of prolonged restraint stress on the expression of the activation markers MHCII and CD40 in splenic and LN DCs at a later stage of OC (progression) when the tumour is likely to be more resistant to the immune response. Splenic and LN DCs were activated by challenging mice with

intraperitoneal injections of ID8 cells followed by 4 weeks of RS. The expression of activation markers in splenic and LN DCs ($CD11c^+MHCII^+$ & $MHCII^+$ DCs, $CD11c^+CD40^+$ & $CD40^+$ DCs) after 4 weeks exposure to RS is shown in figure 39 and quantified as illustrated in figures 40a & b. Although a decreasing trend in the percentage of expression of the activation markers was observed from stress in both splenic and LN DCs, the difference was not statistically significant. This may suggest that splenic and LN DCs are more sensitive to the effect of stress on shorter exposures (2 weeks) than on 4 weeks as a significant change in the expression of activation markers was observed in splenic DCs at 2 weeks but not at 4. alternatively, the immune response to tumour antigens may have dampened because of tumour progression.

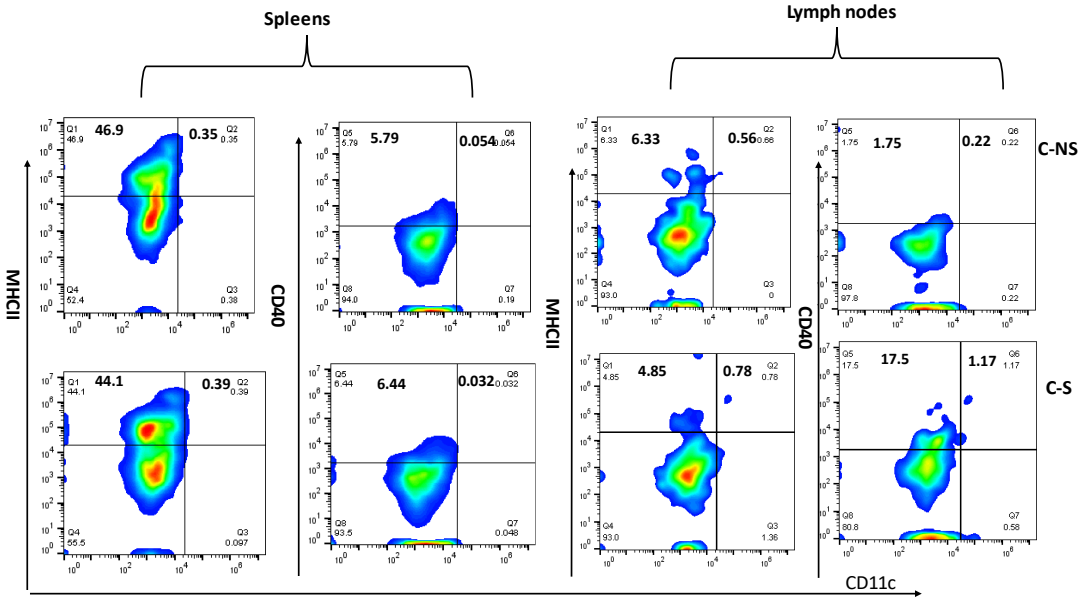
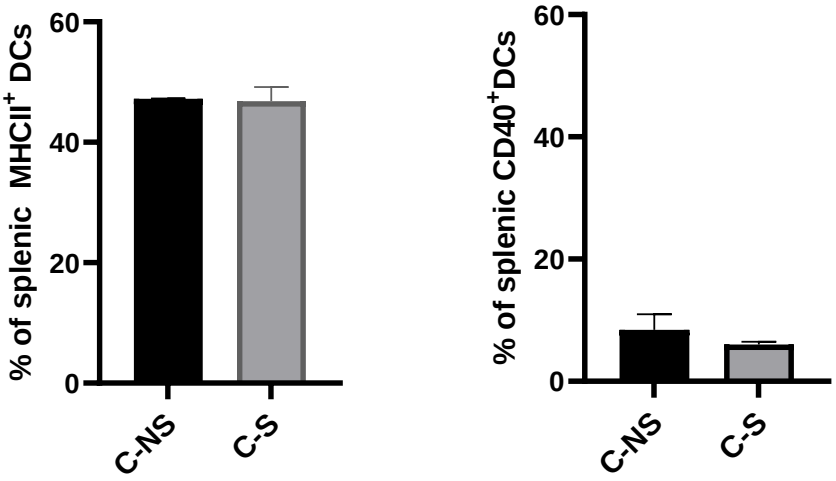


Figure 38: Representative flow cytometry images illustrating the percentage of activation markers expressed by splenic & LN DCs in mice challenged with ID8 cells followed by 4 weeks of RS
The activation markers MHCII & CD40 were identified by antibodies conjugated with fluorescent stains: CD11c by Green fluorescence-FITC (FL1), CD40 by orange fluorescence PE (FL2)) and MHC II by red fluorescence APC (FL4). BD Accuri C6 flow cytometry was used for sample process and generating data. BD Flowjo software was used for data analysis. Unexpected CD11c positive populations were observed which is likely to be due to a technical problem with the antibody used to stain CD11c.

A



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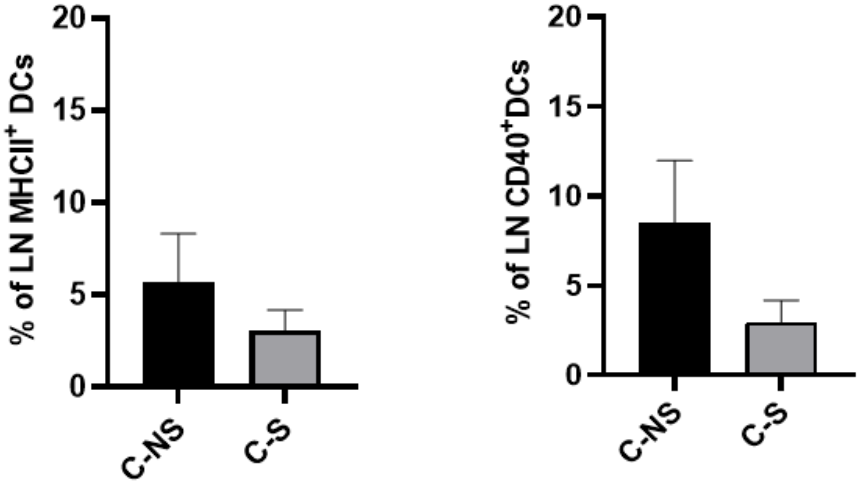


Figure 39: Stress for 4 consecutive weeks did not impair the antigen presenting activity of splenic and lymph node DCs as measured by % of DCs expressing the markers

10, eight weeks, old female C57BL/6J mice were injected with 7x10 ^{6} ID8 cells in 200 ul PBS IP, then stratified into 2 groups of (n=5) and named according to the procedure to be undertaken: Cancer-Stress (C-S) and Cancer-No stress (C-NS). Mice from the stress group were subjected to restraint stress (RS) daily for 2 h by placing them in a 50 ml perforated falcon tubes. The mice from the NS group were left in their cages. RS started 1 week before the IP injections and continued for 4 weeks. The mice underwent gross necropsy to harvest spleens and lymph nodes from which dendritic (DCs) were extracted for flow cytometry analysis. The percentage of activation and maturation markers were identified by antibodies conjugated with fluorescent stains: CD11c by Green fluorescence-FITC (FL1), CD40 by orange fluorescence PE (FL2)) and MHC II by red fluorescence APC (FL4). BD Accuri C6 flow cytometry was used for sample process and generating data. Flowjo software was used for data analysis. (A) is the percentage of expression of MHCII and CD40 in splenic DCs, (B) is the percentage of expression of MHCII and CD40 in LN DCs. Data is presented as mean ± SEM of 2 technical replicates, and t test was used for statistical analysis, *p<0.05, **p<0.01. No significant differences were observed. *IP-Intraperitoneal, C-S- Cancer-Stress, C-NS-Cancer-No stress, RS-Restrain stress.*

2.8.6.5. Prolonged chronic stress (4 weeks) has a more severe effect on the T cells ability to infiltrate the spheroids

The impact of prolonged 4 weeks restraint stress on SP ability to infiltrate ID8 spheroids after ID8 cells injections was next investigated. Previously prepared and fully formed C57BL/6J ID8 spheroids were co-cultured with SP extracted from spleens of C-NS and C-S groups following 4 weeks RS. The extracted SP were first activated with PMA/Ionomycin then co-cultured with ID8 spheroids. Confocal microscope images were captured over a course of 4 days and representative samples are illustrated in figure 41. SP from stressed mice were less likely to infiltrate the ID8 spheroids than SP from non-stressed mice despite activation with PMA/Ionomycin and previous exposure to tumour antigens.

This suggests that T lymphocytes of the SP are less able to recognise ID8 cells antigens after a prolonged 4-week period of restraint stress which may reflect the role of stress and cortisol in modulating SP and lymphocytes by increasing DNA damage in these cells (figures 29 & 31), and this has been further investigated in sections 2.8.5.1-3. Also, SP from both S and NS mice were unable to disintegrate ID8 spheroids, suggesting reduced ability of SP to compromise the integrity of ID8 spheroids at a later stage of OC, possibly due to other tumour related factors that are likely to modulate the immune response at a later stage (4 weeks) during cancer progression.

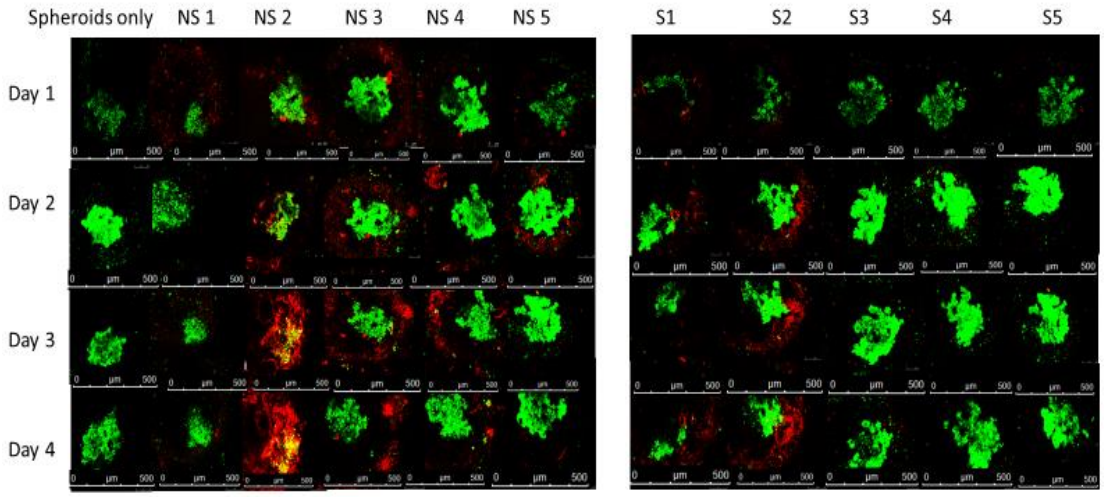


Figure 40: Prolonged 4-weeks RS reduced the ability of SP T lymphocytes to infiltrate ID8 spheroids
Ex vivo SP were isolated from S and NS mice (n=5) after they were subjected to 4 weeks of RS and challenged with IP injections of $7x10^6$ ID8 cells/200ul PBS. They were fluorescently stained red then activated with PMA & ionomycin and co-cultured with previously prepared and fluorescently stained green ID8 spheroids at a ratio of 3:1 respectively. Confocal images were captured over a course of 4 days. Magnification X100. Scale 0-500 µm. *NS- No Stress, S-Stress, RS- Restraint Stress, SP-Splenocytes, yellow-Spheroids infiltrated with splenocytes, IP-Intraperitoneal.*

2.8.6.6 Less weight gain was observed in mice subjected to 4 weeks restraint stress following ID8 ovarian cancer cells injections

To investigate the tumour burden from the 2 and 4-weeks cancer stress study, different parameters were assessed. Mice were weighed weekly (figure 42a) and ovarian tumours were collected and weighed following necropsy (figure 42b). The tumours' progress to secondary organs and angiogenesis were observed (figures 42c & d). At the end of 2-weeks stress, there was no observed difference in weight between stressed and non-stressed mice. However, at the end of 4-weeks stress, significantly less weight gain was observed in the stressed mice compared to their control non-stressed ones. Significantly more angiogenesis, $p<0.0001$ was observed at necropsy in stressed mice at 2 and 4 weeks of stress compared to their control non-stressed ones. Finally, no significant differences in tumour weight and progression were observed between stressed and non-stressed mice both at 2 and 4-weeks stress. This suggests that prolonged exposure to restraint stress (4 weeks) has more severe impact on mice weight compared to shorter exposure (2 weeks) but there is no difference between the two duration of stress exposure on tumour

burden. However, tumour burden is increasing at a later stage of OC (4 weeks) which is expected because of tumour progression.

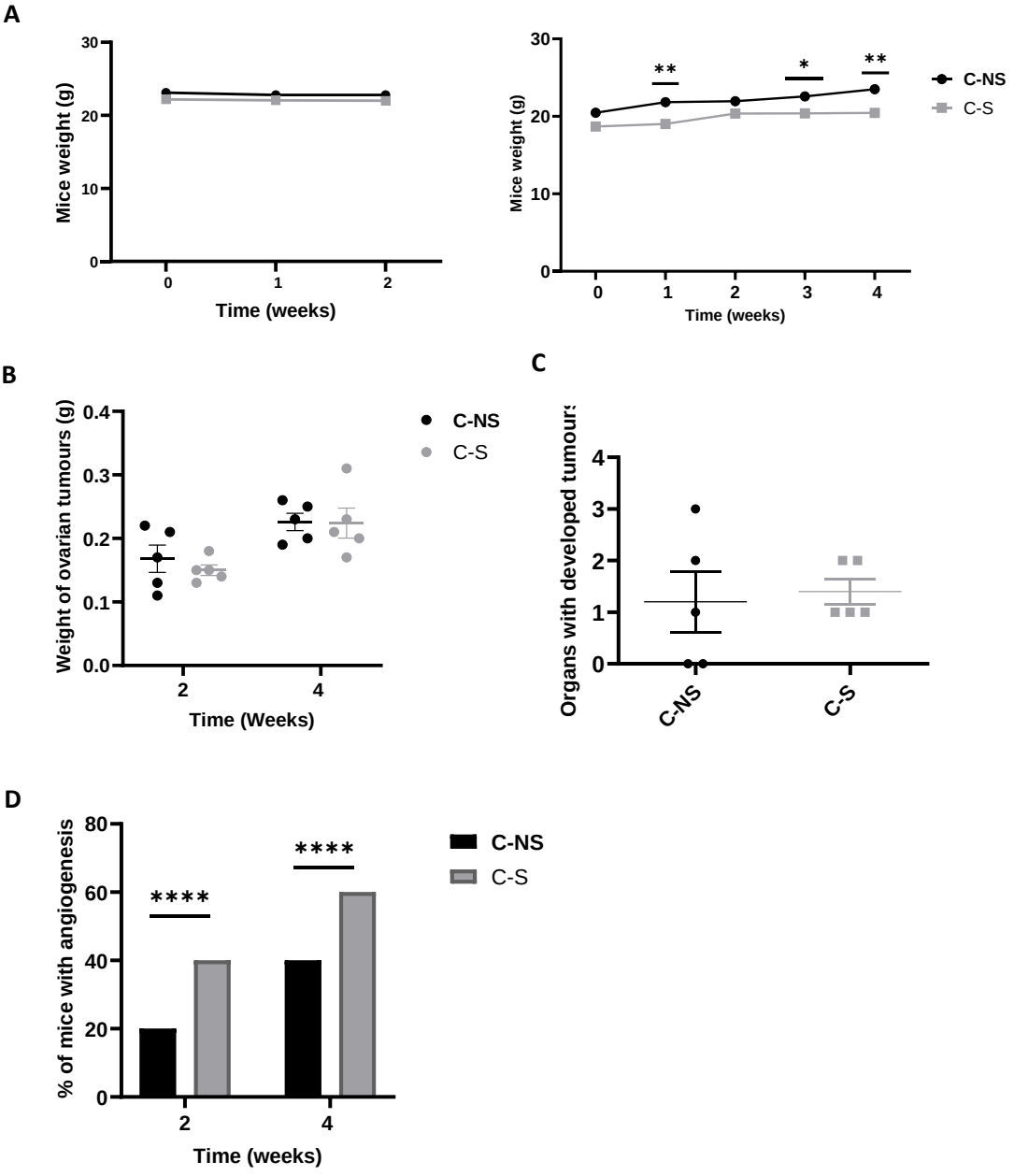


Figure 41: Stress increases angiogenesis and Prolonged stress decreases mice weight
20, eight weeks old female C57BL/6J mice were injected with $7x10^6$ ID8 cells in 200 ul PBS IP, then stratified into 4 groups of (n=5) and named according to the procedure to be undertaken: Cancer-Stress(C-S) (stress for either 2 or 4 weeks) and Cancer-No stress (C-NS). Mice from the stress groups both 2 and 4 weeks were subjected to restraint stress (RS) daily for 2 h by placing them in a 50 ml perforated falcon tubes. Mice from the NS groups were left in their cages. RS started 1 week before the IP injections and continued for either 2 or 4weeks. Weekly weight was taken (A). All mice underwent gross necropsy at the end of their stress periods (2 or 4 weeks) and were assessed for tumour's weight (B), tumour's progression (C) and angiogenesis (D). Stress significantly reduced mice weight at 4 weeks and increased angiogenesis following 2 and 4 weeks of stress. Data is presented as means ± SEM. Statistical tests 2-way ANOVA and t test were used, *p<0.05, **p<0.01, ***p<0.001. *IP-Intraperitonially, RS-Restraint stress, SP-Splenocytes.*

2.8.7 Activation of *in vitro* generated DCs with ovarian tumour lysate

2.8.7.1 Ovarian tumour lysate does not significantly activate DCs generated from splenocytes-bone marrow (BMSP) co-culture

Because restraint stress reduced splenic DCs activation, the observed reduced antigen presenting activity of DCs from RS was recapitulated *in vitro* to probe a mechanism of action. To do this, fresh frozen ovarian tumour lysates were prepared and used as an immunogenic source of tumour specific antigens to pulse DCs generated from *ex vivo* bone marrows (BM) and SP. The stress hormone cortisol and RU486 were used. The percentage of activated splenic DCs ($CD11c^+MHCII^+$, $CD11c^+CD40^+$) is shown in figure 43 then presented as fold increase from DCs unstimulated (figures 44a & b). To investigate the level of expression of the activation markers from the pulsed DCs, mean fluorescence intensity (MFI) was analysed (figure 43), and presented as fold increase from unstimulated DCs (figures 44c & d. Ovarian tumour lysate did not activate the DCs to a statistically significant level despite the observed potential for this effect.

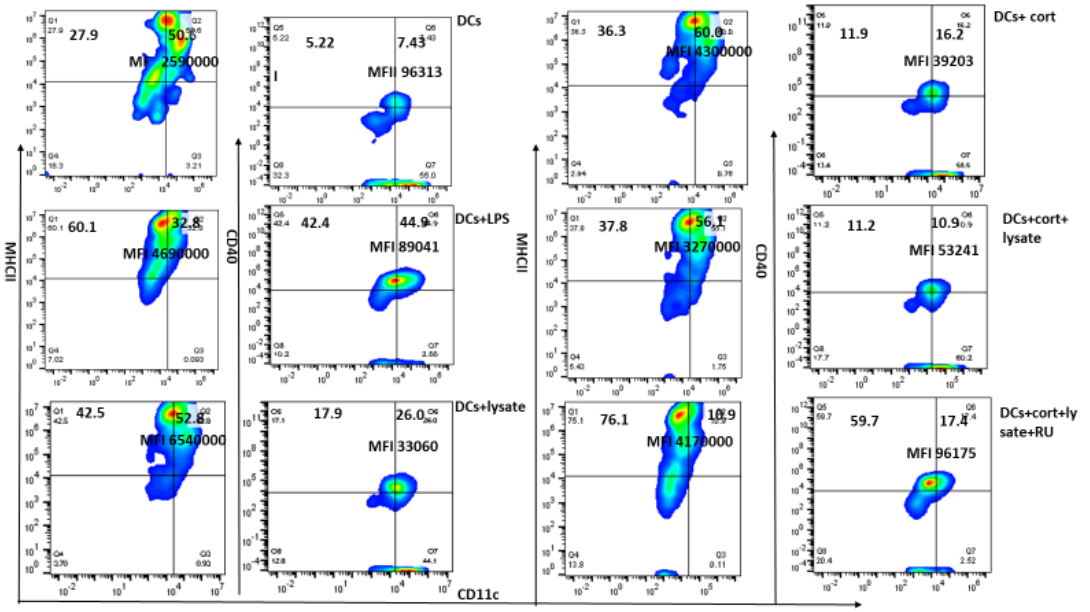


Figure 42: Representative flow cytometry images illustrating the percentage of expression and mean fluorescence intensity for the activation markers: MHCII and CD40 on DCs

The activation markers were identified by antibodies conjugated with fluorescent stains: CD11c by Green fluorescence-FITC (FL1), CD40 by orange fluorescence PE (FL2)) and MHC II by red fluorescence APC (FL4). BD Accuri C6 flow cytometry was used for sample process and generating data. Flowjo software was used for data analysis. 20,000 events were acquired and gated on CD11c expression and side scatter properties. Cells without any stains and individually labelled DCs were used for colour compensation. 2 technical replicates were prepared for each sample. 3 biological replicates were prepared. LPS is the ligand for TLR4, used as positive control. Different CD11c Ab was used.

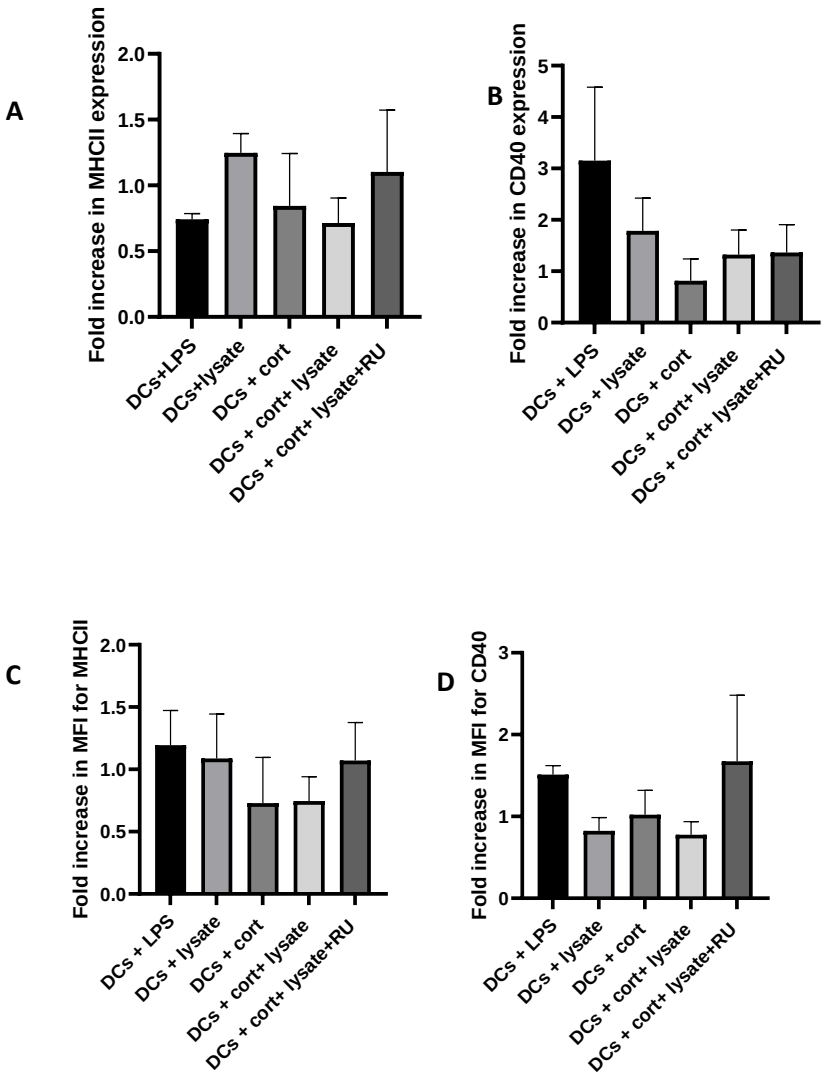


Figure 43: Ovarian tumour lysate did not activate DCs generated *in vitro* from *ex vivo* tissues
DCs were generated from co-culture of *ex vivo* C57BL/6J bone marrows and splenocytes at a ratio of 1:7 respectively over a period of 9 days in a growth media supplemented with GM-CSF. Media with non-adherent cells were transferred to clean petri dishes and fresh media was added every 3 days. On day 9, the cells were harvested, co-cultured with ovarian tumour lysate prepared by mechanical disaggregation of freshly frozen primary ovarian tumour tissues at a ratio of 1:5 (DCs: tumour lysate) respectively and treated with either 5 µM cortisol or 5µM cortisol and 5µM of the GR antagonist RU486, 30 min before the cortisol then incubated for 24h. Subsequently, cells were fluorescently stained with conjugated anti-mouse antibodies for flow cytometry analysis to detect the following DCs surface markers: CD11c by Green fluorescence -FITC (FL1), CD40 by orange fluorescence PE (FL2)) and MHCII by red fluorescence APC (FL4). BD Accuri C6 flow cytometry was used for sample process and generating data. Flowjo software was used for data analysis. (A) Is the fold increase of MHCII expression in $CD11c^{+}$DCs relative to unstimulated DCs and (B) is the fold increase of CD40 expression in $CD11C^{+}$DCs relative to unstimulated DCs. (C) is the fold increase in MFI for MHCII expression in $CD11c^{+}$DCs relative to MFI for unstimulated DCs and (D) is the fold increase in MFI for CD40 expression in $CD11c^{+}$DCs relative to MFI for unstimulated DCs. LPS; The (TLR4) agonist was used as a positive control and DCs alone were used as a negative control. 2 technical replicates were prepared for each point and 3 biological repeats were done. Data were combined from 3 independent experiments. Data are presented as mean ± SEM. One-way ANOVA was used for statistical analysis, *p<0.05, **p<0.01. No significant differences were observed between the groups.

2.8.7.2 DCs pulsed with ovarian tumour lysate showed upregulation of the expression of 2 genes mediating type 1 interferons production and cortisol suppressed them

To investigate the impact of RS on DCs activation mechanistically, DNA damage in splenic and LN DCs was investigated by detecting and quantifying gamma H2AX immunofluorescently. Unfortunately, it was very challenging to have sufficient adherent DCs to the cover slips to conduct the assay. Therefore, the results were not compelling to draw a valid conclusion.

It was previously demonstrated (figure 44) that when DCs were pulsed with fresh frozen ovarian tumours, they showed increased expression of the activation markers MHCII & CD40. Cortisol dampened this activation and RU486 reverted the inhibition caused by cortisol and facilitated the lysate activation of DCs. It was postulated that this activation of DCs is mediated through stimulation of interferons genes (STING) in response to cytosolic sensing of double strand DNA from ovarian tumour cells- c GAS-STING pathway resulting in the production of type 1 interferons (IFNs) (figure 12). Type 1 IFNs are potent inducer of the adaptive anti-tumour immunity. Therefore, an experiment was designed to investigate the expression of some genes mediating the c GAS-STING pathway using one biological replicate. The expression of Tank Binding Kinase1 (TBK1) and TMEM173 (gene encodes the protein STING) were assessed by qRT-PCR, and the amplification of these genes is illustrated in figure 45. TBK1 and TMEM173 were amplified by the ovarian tumour lysate but downregulated by cortisol. RU486 reverted the effect of cortisol. No statistically significant effects were observed. Only one biologic replicate was used. Initial results are promising but need to be replicated in at least 2 more biological replicates and the expression of 2 more genes including interferon alpha or beta would allow a robust conclusion.

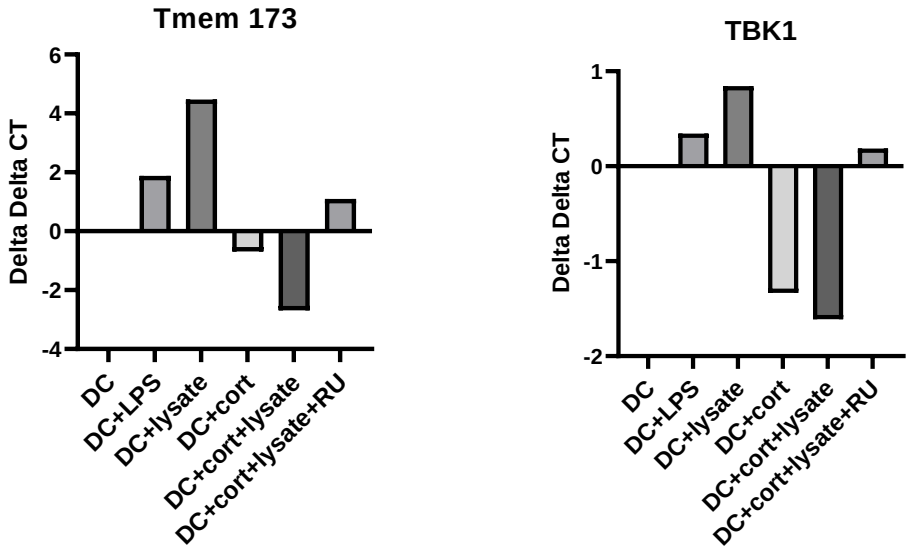


Figure 44: Cortisol downregulated the expression of two genes mediating type 1 IFNs production in ovarian tumour pulsed DCs and RU486 upregulated their expression

DCs were generated from co-culture of *ex vivo* C57BL/6J bone marrows and splenocytes at a ratio of 1:7 respectively over a period of 9 days in a growth media supplemented with GM-CSF. Media with non-adherent cells were transferred to clean petri dishes and fresh media was added every 3 days On day 9, the cells were harvested, co-cultured with ovarian tumour lysate prepared by mechanical disaggregation (3 cycles of freeze and thaw) of freshly frozen primary ovarian tumour tissues at a ratio of 1:5 (DCs: tumour lysate) respectively and treated with either cortisol 5µM or cortisol 5µM and GR inhibitor (RU486 5µM) 30 min before the cortisol, subsequently incubated for 24h. RNAs were extracted and transcribed to produce c DNA by thermocycler PCR following Qiagen protocols (42). Quantitative real time PCR was done to assess the expression of genes of interest. Data is presented as mean ± SEM of 2 technical replicates, one way ANOVA was used for statistical analysis, *$p<0.05$, **$p<0.01$.

2.9 Discussion

RS for 3 consecutive weeks significantly impairs the early activation of splenic T lymphocytes and dampens their ability to disintegrate ID8 ovarian spheroids. This impaired anti-tumour immunity was observed in splenic T lymphocytes from stressed naïve and primed mice. Further, the negative stress effect on SP was not fully reversed by the gold standard GR antagonist RU-486 or the novel more selective GR antagonists: Relacorilant and CORT125281 and found to be mediated by DNA damage predisposing to apoptosis. Furthermore, a significant reduction in the expression of the activation markers MHCII and CD40 in splenic DCs was observed at an early stage of EOC (2 but not 4 weeks of exposure) may suggest a reduced ability of these cells to present antigens and an increased sensitivity to the stress effect at an early stage of OC. The impaired expression of MHCII/CD40 in splenic DCs was thought to be mediated through suppression in the c GAS-STING pathway which when activated in cells such as DCs results in the production of type 1 interferons subsequently activating DCs to induce an immune response (272). Preliminary work has been started to examine the expression of genes mediating type 1 IFNs production. However, due to time constraints, further work is needed to unravel this pathway.

The observed impaired ability of splenic T lymphocytes from stressed naïve and primed mice to infiltrate and disintegrate ID8 spheroids suggests that the damage in SP is likely to be driven by stress and not by ID8 cells. Therefore, RU-486 was first used to reverse the stress effect on SP and restore the anti-tumour immunity. But this did not happen, possibly because RU-486 has an affinity to both GR and progesterone receptors (266). Two novel more selective GR antagonists (Relacorilant and CORT125281) which are currently under clinical trials in breast and ovarian cancers (273) were then used. Although a loss in the integrity of ovarian tumour spheroids was observed which facilitates the anti-tumour effect of splenic T lymphocytes, the GR antagonistic effect was not statistically significant. This may imply that the stress-induced inhibition of T lymphocytes is either not mediated by the GR or that the SP are unable to recover and restore their function.

To explore the mechanism underlying the observed impaired anti-tumour immune response in SP, they were examined for DNA damage. Evidence from the literature demonstrated in precancerous (103) and cancerous cells (107) that stress hormones can affect these cells directly resulting in a long-lasting effect such as DNA damage, genomic instability, alteration in DNA repair and apoptosis. One mechanism through which DNA damage occurs is double strand break (DSB) which causes the highest damage to the DNA when induced by physical, chemical, or biological factors (14). Therefore, SP from stressed naïve mice were assayed for DNA DSB.

Interestingly, a significant increase in DNA damage was observed implying that SP are undergoing apoptosis, which could, at least in part, explain the impaired activation of splenic T lymphocytes and the subsequent reduced antitumour activity observed from stress in the absence of IP tumour injections and at the early stages of cancer (2 weeks). This is consistent with the literature which correlates impaired T lymphocytes infiltration into ovarian tumours with poor prognosis and their enhanced infiltration is strongly correlated with favourable prognosis. Thus, ovarian tumours are known as immunogenic (146, 274).

To support the observed stress-induced DNA damage in splenic T lymphocytes, a significant increase in DNA damage was observed in *ex vivo* T lymphocytes when treated with cortisol. However, this effect is to be confirmed through more biological replicates and with GR antagonist. Further, when SP were treated with relevant physiological doses of cortisol, no increase in DNA damage was observed. This could be due to the short acute exposure to cortisol as opposed to the detrimental long-term effect observed from prolonged stress.

DNA repair mechanisms are usually activated in response to DNA damage. Therefore, assessing for DNA repair in splenocytes such the homologous and non-homologous pathways would strengthen the support to our results and increase their validity. The stress induced DNA damage in immune cells is a new and exciting finding which can contribute effectively to cancer treatment. It highlights the importance of incorporating stress management strategies with the treatment regimens of OC patients starting at an early stage.

Moreover, splenic T lymphocytes from primed and 4-weeks stressed mice lost their ability to recognise ovarian tumour antigens. Which may suggest that splenic T lymphocytes are less able to recognise ID8 ovarian tumours antigens after a prolonged period of restraint stress. This may reflect our finding on the role of cortisol in modulating T lymphocytes by inducing DNA damage. Or it could be due to tumour related factors that are likely to affect the immune system and modulate its function during cancer progression.

Restraint stress for two weeks following ovarian cancer cells injections impaired the expression of maturation and activation markers MHCII & CD40 in in splenic DCs but not at 4 weeks exposure. This could be due to the observed damage in SP from which the DCs were isolated. The increased severe effect of stress on the splenocytes following 4 weeks exposure suggest more detrimental damage from stress on SP making them less sensitive to stress which is consistent with the literature (275).
Studies in rats exposed to restraint stress (1 h) followed by a gap of 4 h with forced swimming exercise (15 min) daily for 2, 4 and 8 weeks, showed that the severity of stress influenced lymphoid organs (e.g. decreased spleen, thymus and axillary lymph nodes weights, splenic lymphocyte numbers and apoptosis) was duration dependent and the sensitivity of these organs is higher at a shorter exposure to stress. Following a stress recovery period, the effect of stress on lymphoid organs parameters exposed to shorter stress was reversed but not on those exposed to longer duration (275).

Surprisingly, stress did not have a significant effect on the ability of LN DCs from primed mice to express activation markers at 2- and 4-weeks exposures. LN are the secondary lymphoid organ where the antigen presentation takes place to prime effector T cells and induce tumour antigen specific immunity. This could be due to the very small number of DCs that were collected. The size of LNs is small which made their identification, isolation and DCs extraction challenging (42). A study in adults' primates (monkeys) showed a psychological stress-induced alteration in LN neural structure that was associated with long term regulatory change in the initiation, maintenance, and resolution of the immune response.

In a healthy immune system, transformed cells are identified and destroyed as part of the homeostatic immune surveillance. The early impairment in the antitumour response is likely to promote tumour growth and may contribute to the shift in the immune surveillance from the elimination to the escape state and thus promoting the invasive potential of the EOC cells (93, 152).

The restraint-stress impaired antigen presenting activity at an early stage of cancer is important finding. It highlights another possible contributary factor to the immunosuppressive nature of OC and suggest that patients should receive support with stress management immediately following diagnosis with OC.

2.10 Conclusion

This study shows that restraint stress has the potential to impair the antitumour immunity at an early stage of EOC by suppressing the antigen presenting activity of DCs and impairing the cytotoxic effect of splenocytes at the tumour microenvironment. The latter effect is also observed in the absence of cancer and is likely to be mediated through DNA damage in the splenic T lymphocytes. This may-in part demonstrates a possible mechanism of action underlying the inhibitory effect of the stress hormone cortisol on the cytotoxic activity of T cells, which provides possible targets for pharmacological and/or biobehavioural interventions that is likely to promote good stress effects and minimise chronic harmful effects. Modulating GRs with gold standard or novel antagonists is a possible way to partially reverse the negative effect of stress and restore the natural anti-tumour activity of effector immune cells.

Alternatively, biotherapeutics with probiotic that has a potential anti-tumour effect would provide a safe, non-invasive complementary therapy for ovarian cancer patients. *Escherichia coli Nussle 1917 (EcN)* is a well characterised and clinically valued probiotic (276). An ant-metastatic effect for *EcN* in EOC metastasis was observed previously (unpublished data).

In this chapter, the impact of stress on the immune response was investigated at an early stage of EOC to propose possible underlying mechanism. In the next chapters, the effect of *EcN* on EOC metastasis will be reinvestigated for reproducibility and

mechanistically explore this effect. Then the impact of stress on EOC metastasis will be explored to elucidate a potential role for *EcN* in protecting against stress-mediated effects.

Therefore, it was hypothesized that *EcN* has a protective anti-tumour effect against stress in EOC metastasis and this effect is likely to reverse any negative effects from stress on the immune response.

3. Chapter 3-The immunomodulatory impact of the probiotic *Escherichia Nissle 1917 (EcN)* on ovarian cancer metastasis

3.1 Introduction

OC is the deadliest gynaecologic cancer (277-279). This is mainly due to its asymptomatic nature during the early stages of development and the lack of specific and sensitive early diagnostic tests resulting in late diagnosis at an advanced stage when it is metastatic (280). Despite advances in treatments over the last few decades, platinum-chemotherapy with paclitaxel and cytoreductive surgeries are the current gold standard treatments (281). This is in addition to targeted therapies against vascular endothelial growth factor (VEGF) (282, 283) and poly (ADP-ribose) polymerase (PARP) inhibitors for platinum-sensitive recurrent ovarian cancer (284, 285). Relapse and chemoresistance often occur in 70-80% of advanced-stage patients and the 10 years survival does not exceed 15% (286) for advanced stages. Current treatments for OC are cytotoxic and associated with life threatening side effects. Therefore, there is a need for novel anti-tumour therapies that are non-toxic or that exert less toxicity to give patients a better quality of life.

3.1.1 Probiotics and cancer

Our enhanced understanding of the gut microbiome complex composition and its essential role in human health and disease is mainly due to advances in technologies and the use of second-generation sequences (187, 272). Such advancements have led to a rise of growing interest in developing new approaches to manipulate the composition, distribution, and function of the gut microbiome to treat, prevent or diagnose many pathologies including cancer. This has hence led to the increased interest in studying microbial therapies such as probiotics.

Biotherapeutics have been the focus of recent research as an alternative protective treatment in cancer (287, 288). Probiotics which are used to restore the microbiota ecosystem, are defined as live microorganisms that can confer health benefits when consumed in adequate amounts (289). Probiotics have been studied and are increasingly valued for their various benefits, particularly their promising role in the diagnosis, prevention, and treatment of cancer (290). The underlying mechanism for their anti-tumour effects was initially believed to be exerted through activation of

the host immune system, changing transit time and motility of the colon, suppressing pro-carcinogens and carcinogens, inhibiting bacteria that are involved in the transformation of pro-carcinogens to carcinogens, and reducing intestinal pH (291). However recent evidence has shown that the probiotics' anti-tumour effects are also exerted through modification of the biological processes that are involved in cancer such as apoptosis, oxidative stress, proliferation, inflammation, and metastasis (292-295). Additionally, probiotics have been used as a supplementary product during and after chemotherapy to prevent or treat chemotherapy associated side effects such as infectious complications and diarrhoea and have resulted in improved patients' quality of life (296).

3.1.2 Probiotic and cancer metastasis

Oncobiosis (i.e. dysbiosis in the neoplastic diseases) can be driven by lifestyle choices such as smoking, diet, obesity, changes in diurnal rhythm, underlying diseases, and medications (297). Oncobiosis is thought to play a prominent role in cancer pathogenesis through interactions between the microbiome and cancer cells such as immune modulations and oxidative stress. Such interactions may modulate gene expression leading to a cascade of changes in cell functions and metabolism. These primary changes can further modulate other events including, modulation of anti-tumour immunity resulting in tumour promoting inflammation, epithelial-to-mesenchymal transition (EMT), dissemination, invasion, angiogenesis and metastasis (298).

Metastasis is a hallmark of malignant cells and involves interruption of the basement membrane and local invasion to stromal cells, passing through the circulation alive (intravasation) and extravasation to secondary organs to manifest a clinically evident metastasis (298). The role of probiotics mainly Lactic Acid Bacteria (LAB) in cancer metastasis has been demonstrated in cell lines, animal models and human studies. probiotics are found to interfere with the process of invasion and metastasis at different stages. Firstly in cell to cell adhesion, cell-free supernatants (CFS) from *L.*

casei and *L. rhamnosus GG* have been shown *in vitro* to prevent colon cancer cell invasion (299).

Secondly, in epithelial mesenchymal transition (EMT), live *L. casei* was found to induce anti proliferative effect and apoptotic cell death in murine and human cell lines as well as experimental tumour models of colon carcinoma (300). Thirdly, in the modulation of tumour microenvironment, different animal studies have demonstrated the anti-metastatic effect for the probiotic lactobacilli mediated through modulation of the tumour microenvironment. For instance, intralesional injections of *L. casei* YIT9018 in C57BL/6j mice bearing highly metastatic melanoma resulted in suppression of the tumour and enhanced survival of the mice. Meanwhile, IV injections of the same strain into mice which were injected with melanoma cells, protected them against pulmonary metastasis (301). And finally, with cancer stem cell (CSC) maintenance, it was demonstrated that administration of 9 combined Gram-positive bacteria activated NK cells which enhanced the differentiation of cancer stem cells and consequently supressed the tumour growth (302).

3.1.3 Probiotics and ovarian cancer

Although it is still not clear how OC starts, chronic inflammation has been linked to OC carcinogenesis. A prospective study showed an association between high circulatory level of C reactive protein (CRP), a biomarker of inflammation, and increased risk of invasive OC (303). Also, OC has been characterised by oncobiosis which involves disruption in the microbiome composition and distribution observed in different compartments, namely the tumour tissues, ovaries, peritoneum, intestine, plasma, upper and lower genital tract. These compartments were found to be colonised by gram negative bacteria with a potential for an increased inflammation and reduced microbiome diversity. Peritoneal microbial colonies are thought to be associated with the formation of metastasis in OC (304-306). Besides oncobiotic transformation, genital bacterial, viral, or fungal pathogens (e.g., *Chlamydia Trachomatis* and Human papillomavirus (HPV) are likely to drive inflammation and increase the risk of ovarian oncogenesis and progression (307-

309). Such inflammation can be manifested via different pathways, either through increasing oxidative stress causing DNA damage and accumulation of mutations or through stimulation of Pattern Recognition receptors (PRRs) TLR2, 4 and 5, which are the ligands for bacterial lipopolysaccharides (LPS) (310) and flagellin (311) respectively. LPS is a component of the outer membrane of Gram-negative bacteria which protects the bacteria against external toxins, antibiotics, and bile acids. LPS is highly immunogenic (312).

3.1.4 The probiotic *Escherichia Coli Nissle* 1917 (*EcN*)

Since microbes, mainly Gram negative, are a major driver for OC oncogenesis, progression, invasion, and metastasis, the manipulation of microbiota with probiotic may benefit OC patients. *EcN* is one of the most-studied probiotic strains. Although it is gram negative bacteria, it has characteristics which makes it a suitable candidate as probiotic such as the lack of virulence, its competition with pathogens at the epithelial site, its ability to strengthen tight epithelial junctions. Most importantly, it expresses LPS which enables it to exert immune-modulatory effect without immunotoxicity when used at an appropriate dose (211), (figure 46). *EcN* offers benefits against gastrointestinal tract (GIT) related pathologies such as ulcerative colitis and has been well evaluated and valued for many years (313). However, its anti-tumour effect and the possible signalling pathway for this effect has not been elucidated to date, especially in cancers distant from the GIT such as OC.

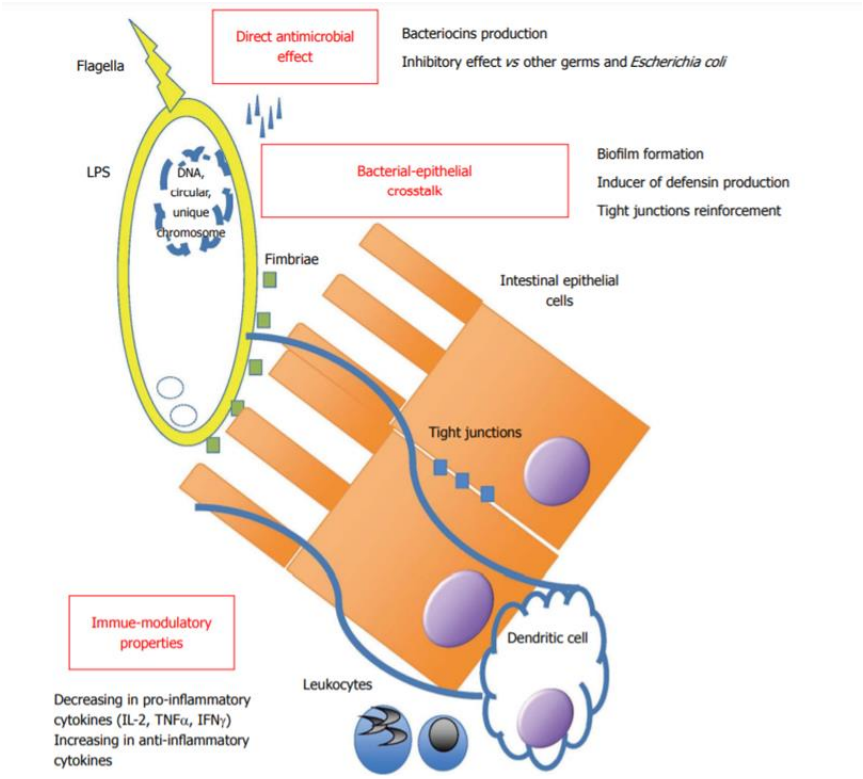


Figure 45: Structure and mechanisms of action of *Escherichia coli Nissle* 1917
EcN exerts its action via three mechanisms. Firstly, it has direct antimicrobial effect by producing bacteriocin or directly suppressing pathogens. Secondly, it can undertake bacterial epithelial cross talk through its Fimbriae and biofilm formation, reinforcement of tight epithelial junctions and the production of defensin. Finally, it can modulate the immune system by increasing anti-inflammatory cytokines and decreasing pro-inflammatory cytokines such as IL-2, TNFα, IFN-γ. LPS= Lipopolysaccharide, IL-2= Interleukin-2, TNFα= Tumour necrosis factor alpha and IFN-γ: Interferon gamma. Adapted from (211).

3.1.5 Toll like Receptors (TLRs) stimulation by PRR activates the innate and adaptive immune response

The gut microbiome and immune system cross talk at the epithelial mucosa. At steady state the microbiota shapes the function and development of the immune system while the immune system response by developing tolerance to commensals and reserving immune response to pathogens. Through compartmentalization, the immune system minimises the direct contact of microbiota with the mucosa by producing mucous layers and developing tight junctions to restrict trans-epithelial permeability (176, 185, 187). Disruption in the microbiota homeostasis may result in immune-mediated disorders such as auto immunity allergy (44).

TLRs are type of Pattern Recognition Receptors (PRR) which are expressed mainly on immune cells such as macrophages and dendritic cells, though can be expressed on

other cells such as the epithelial, myofibroblasts and even on the tumour cells (314, 315). Thus, they play a crucial role in the rapid innate immune response. They are primarily triggered by pathogen-associated molecular pattern (PAMP) and damage associated molecular pattern (DAMP). Each TLR subtype recognises specific molecular pattern. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the surface of the cells and triggered by external microbial components of both commensals and pathogens such as lipopeptides, flagellins, and LPS. Whilst TLR3, 7, 8 and 9 are expressed inside the cells and are triggered by viral components (316). On the other hand, stimulation of TLRs on cancer cells result in tumour suppression or promotion.

Stimulation of TLR 2,4 and 7/8 on a tumour cell result in tumour progression mediated by proliferation, resistance to apoptosis and production of immune suppressant cytokines. Whereas stimulation of TLR 2, 3, 4, 5, 7/8, and 9 when combined with chemo or immune therapy induces anti-tumour immunity and tumour suppression. Further, stimulation of TLRs on APCs, NK cells in the tumour microenvironment, activates CTLs subsequently, inducing tumour suppression (317) (figure 47).

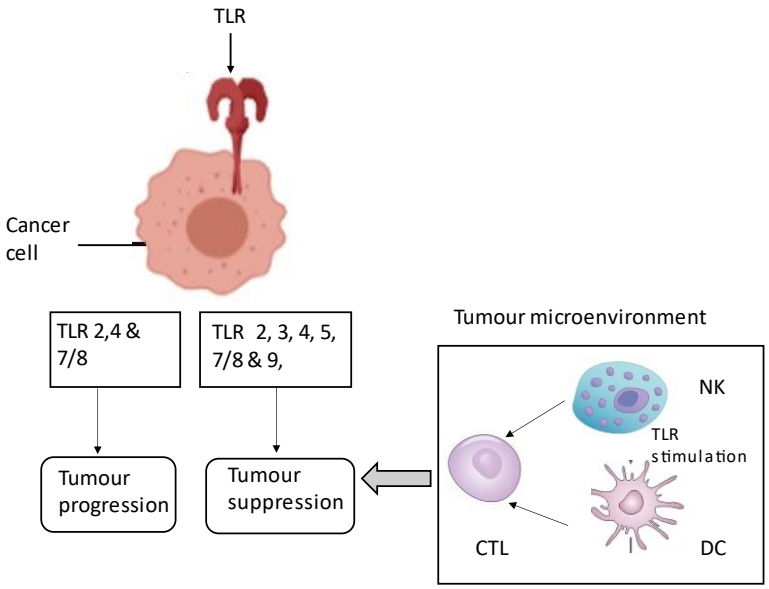


Figure 46: TLRs stimulations in cancer
Stimulation of TLR 2,4 and 7/8 result in tumour progression whereas stimulation of TLR 2, 3, 4, 5, 7/8, and 9 when combined with chemo or immune therapy induces anti-tumour immunity and tumour suppression. Activation of DCs and NK cells in the tumour microenvironment activates CTLs leading to tumour inhibition. *TLR-Toll like receptors, DCs-Dendritic cells, NK-Natural killing, and CTL-Cytotoxic T lymphocyte*. Adapted from (317).

Amongst all the TLRs that have been studied, TLR4 is the most extensively investigated (318). TLR4 activation stimulates 2 different signalling pathways involving different adaptor proteins and transcription factors, these are: Myeloid Differentiation primary response gene 88 (Myd-88) dependent pathway which activates nuclear factor-kappa B (NF-κB) and stimulates the production of pro-inflammatory cytokines, while the other is (Myd-88) independent and activates Interferon-Regulatory Factors (IRFs) resulting in expression of genes encoding for type 1 interferons and production of inflammatory type 1 IFNs cytokines(272, 319). It has been shown that activation of TLR4 in immune cells mediates the innate immune response to pathogens (304, 320). However, the expression of TLR4 and Myd-88 on OC cells is associated with poor prognosis (318). Indeed, the expression of TLR4 on OC cells is found to mediate paclitaxel resistance through activating NF-kB (321). Therefore, TLR4 is a potential therapeutic target to sensitise OC cells to chemotherapy and reduce their migration and invasion.

Triggering of TLRs at steady state by commensals does not stimulate inflammation, instead, it enables them to control epithelial homeostasis and gut integrity as well as providing a protection against microbial infections. This could be due to their sequestration by the epithelial layer, so they are not directly contacting the gut mucosa (322), as well as their ability to induce the release of regulatory molecules. These molecules include retinoic acid (RA), Transforming Growth Factor β (TGF-β) and Interleukin 10 (IL-10), produced by dendritic cells (DC). RA and TGF β both support the differentiation of T helper cells into $Foxp3^+$ regulatory T (T Reg) cells and the inhibition of the inflammatory Th17 in lamina propria. (323) IL-10 activates regulatory $Foxp3^+$ Treg cells. Tregs suppress the activation, proliferation, and the effector function of the immune cells in the gut to maintain tolerance and prevent autoimmunity (323). Commensal bacteria thus play a critical role, stimulating the adaptive immune system to maintain homeostasis.

including OC and are mostly associated with immune suppression (324). Coukos and his team demonstrated in 104 individuals with ovarian carcinoma that $CD4^+CD25^+FOXP3^+$ Treg cells supress the tumour specific T cell immunity, increase tumour growth and reduces survival (325).

Recently, it was found that innate lymphoid cell group 3 (RORγt+ ILC3) which reside in the lamina propria can stimulate the release of IL 22, which is a member of the IL-10 family and upregulates the gene expression for antimicrobial protein REGIIIγ to maintain the gut integrity and homeostasis. On the other hand, IL 23 is a member of the IL-12 family and is primarily released from activated macrophages and dendritic cells (326). It is found to bridge the innate and adaptive immune response to defend the host against infections and tumour progress. This effect is likely to be achieved through the activation of the inflammatory cell, T helper 17 (Th17). Additionally, IL-23 promotes the antigen presenting activity of DCs and production of proinflammatory cytokines (326-328).

An enhanced understanding of the relationship between the bacterial ecosystem of the gut and the progression of ovarian cancer would open venues for better treatments where microbial interventions could be used as complementary therapies to improve patients outcomes (329). In this chapter, the anti-tumour effect of the probiotic *EcN* on OC metastasis, the leading cause of death in cancer, will be evaluated to probe a mechanism of action for this role.

3.2 Hypothesis

We hypothesize that the probiotic *Escherichia coli Nissle 1917 (EcN)* has a protective anti-tumour effect in OC metastasis and this effect is exerted through modulation of the innate and adaptive immune response mediating inflammatory pathways.

3.3 Aims

We aim to investigate the impact of the probiotic *EcN* on EOC metastasis and identify the signalling pathway for this effect.

3.4 Objectives

- Evaluate the impact of the probiotic *EcN* on EOC metastasis. We aim to confirm our previous finding (unpublished data) regarding the antitumour effect of *EcN* to demonstrate reproducibility and thus, robustness and reliability.
- Probe the signalling pathway for *EcN*. We aim to evaluate the immune modulatory effects of *EcN* using a syngeneic mouse model for EOC. We propose that *EcN* modulates the adaptive immune response through $CD8^+$ cytotoxic T cells and $Foxp3^+$ regulatory T cells (Tregs). Also, we suggest that *EcN* modulates the innate immune response through Toll Like Receptors 4 and 5 (TLR4 & TLR5) and the cytokines IL23 & IL22.

3.5 Material and method

3.5.1 Culture and manipulation of bacterial strains

The Probiotic *Escherichia coli* strain *Nissle 1917 (EcN)* and other control *E. coli* strains shown in *Table 4* below, were routinely grown either in liquid medium, Luria-Bertani (LB) broth, composed of tryptone (pancreatic digest of casein) 10 g/L, yeast extract 5 g/L and Nacl 5 g/L (330) (*Fisher, Loughborough, UK)* with shaking at 150 rpm overnight 37°C, or on solid LB agar media (LB broth supplemented with 1.5 % (w/v) agar) technical grade agar (*Fisher , UK*) at 37°C. Isolation of single colonies of bacterial cultures was achieved by streaking cultures on LB agar which was then used to inoculate LB broths.

Prior to use in mice feeding experiments, *EcN* strain was rendered resistant to rifampicin to allow its recovery and characterisation from faeces. The *EcN* rifampicin strain was created using the serial passage method (331). This method involved sub culturing *EcN* cells in LB broth supplemented by rifampicin (*Sigma-Aldrich,*

Gillingham, UK) up to 100 µg/ml. Its purity was confirmed by growing on LB agar and comparing colonies morphology with those from stock, none-treated strain. Further, its growth on LB agar was ensured by growing the rifampicin adapted strain in four different concentrations of Rifampicin and compared against a negative control with no rifampicin.

Bacterial strains	Use	Source
E coli Nissle 1917	A proved probiotic strain mainly used in yogurt drinks as Mutaflor® suspension.	Ardeypharm GmbH, Herdecke, Germany
E coli K-12 MG1655	Used as the representative of commensal non-pathogenic wild-type E. coli of human intestine. It was used in this work as a control in PCR identification of EcN recovered from mice faecal pellets.	Blattner (332)
E coli EPI300 ™	Laboratory strain of E. coli used in cloning of plasmid vectors. It was used in this work as a control in PCR identification of EcN recovered from mice faecal pellets.	Epicentre Technologies Corporation, Madison, USA

Table 4: Bacteria strains

EcN viability was tested in the water used to supply probiotics to mice statically at room temperature and at 37° for 24 hours. Viability was evaluated by plating on LB agar solid media. Total viable count in 24 hours was established as colony-forming unit per one ml of media (cfu/ml) to reflect the uncertainty of how many cells formed the colony (58) and compared with initial count (time 0, table 5). The experiment was repeated twice.

Time (hours)	Viability colony-forming unit (cfu/ml)	
	Room temperature	37 °C°
0	$6.13x10^8$	$8.5\ x10^7$
24	$7.1x10^7$	$4.1\ x10^7$

Table 5: Viability of *EcN* at room temperature and 37 C measured as colony-forming units

3.5.2 Preparation of probiotic feed

Pure cultures of *Escherichia coli Nissle* rifampicin resistant (*EcN*-Rif$^+$) were grown in LB broth inside sterile glass bottles for 12 hours, with shaking (150 rpm) at 37°C to achieve culture density of 1x 10^9 CFU/ml (mid- logarithmic phase of growth, normalised to optical density of 1). The cultures were transferred to 50 mL sterile centrifuge tubes and centrifuged in Heraeus Labofuge® 400 R model centrifuge (*Thermo Scientific*) at 4500 rpm for 10 min at room temperature. The supernatant was removed, and bacterial pellet was then resuspended in same volume of water and transferred to mice water bottles (200 ml each). The probiotic suspensions (1x 10^9 CFU/mL) were supplied to the cancer-probiotic group (treatment) and water only to the cancer-no probiotic group (control) over three consecutive days (Tuesday, Wednesday, and Thursday) for 10 weeks, preparing fresh feed every 24 hours (at 9:00 am). Probiotic feeds and water control feeds were replaced by normal water for the rest of the week.

3.5.3 Cell Line

On the first week of the study, the murine epithelial ovarian cancer cells (ID8) (kindly donated by Dr Premal Thaker, Washington University, St Louis, USA) were used to induce ovarian tumour growth. Cells were cultured in DMEM media consisting of 4%

FBS (Foetus Bovine serum) and 1% Pen/Strep and ID8 supplement (5µg/ml insulin, 5µg/ml transferrin, 5µg/ml sodium selenite)**.** Cells were then kept at 25-50% confluency and passaged until 90% confluency was reached. They were then detached with trypsin protein and resuspended to get four million cells/200µl of PBS.

3.5.4 Mice and diet

Thirty, 8 weeks old female C57BL/6j mice weighing 18±2g were obtained from ENVIGO laboratories (Oxfordshire), and accustomed and handled for two weeks to allow them to acclimatise to researchers. To develop ovarian cancer mouse model, the mice were then injected with 200µl of ID8 cells ($4x10^6$cell/ml) intraperitoneally (IP), and then stratified and divided into two groups (n=15) according to feed supplement: cancer-no probiotic (CC) (control) fed with water only (200 ml) and cancer-probiotic (CP) (treatment) fed with *EcN* ($1x10^9$ CFU/ml). This was chosen as the optimal concentration of *EcN* from a concentration time log graph and incorporated in water (200 ml). A power analysis was performed from a study using a similar mouse model, and thus it was known that with a type I error rate of 0.05, a sample size of n = 10 provides approximately 80% power to detect at least a 20% difference. A sample size 15 was thus chosen to allow for tissue freezing and paraffin-embedding to perform immune analyses.

The supplementary diet was prepared freshly each day for three days each week and administered immediately after the IP injections every week until the end point of the experiment which was the first sight of ascites (swelling of the abdomen indicative of the end stage of ovarian cancer). The experiment was ended at 10 weeks. All animals received normal diet, RM No. three SDS (Special Diet Services) Expanded Diet *(LBS*, *Surrey)* 5g/mouse daily in addition to fresh tap water on the non-treatment days and were maintained in a room with a 12-hour light/dark cycle at 19°c-21°c and 40%-60% humidity (controlled remotely). Cage temperature was set at 37°C (controlled by providing appropriate nesting material). All procedures were approved by the animal welfare at the University of Brighton, personal licence number is 181347B.

3.5.5 Procedures

3.5.5.1 Consumption of probiotic and control

The bottles containing the probiotic in water or water only, were weighed before feeding the mice and twenty-four hours after, to measure the amount consumed each day by each group. Cumulative sum of consumption over the three days each week was then calculated for each group to ensure that the mice were drinking the supplementary diet.

3.5.5.2 Weight of animals

The mice were also weighed every week throughout the experiment and the average weekly weight for each group was calculated to further monitor the uptake of the probiotic.

3.5.5.3 Faecal pellet assay

For the functional measure of the uptake of probiotic, faecal pellets studies were carried out at the beginning and at an interval of three weeks. The two cancer groups were assessed on week one, four and seven. For each group, the animals were placed individually in empty cages for thirty minutes to settle and then the faecal output which was generated within the next hour was collected and monitored. The pellets were counted to assess the probiotics' colonic activity (frequency of transition).

3.5.5.4 Recovery, enumeration, and identification of probiotic from mice faecal pellets

To ensure that the probiotic was effective, it was first proved that *EcN* could survive the pressure in the mice gut and was recoverable from faeces. After 7 weeks of probiotic treatment, fresh faecal pellets were collected from mice following 4 days without probiotic feed and used to isolate *EcN*. The pellets were collected from 14 mice, 7 random mice from each group: CC and CP. The pellets were transferred to sterile plastic tubes, weighed then suspended in sterile PBS (1mL) and mixed by vortexing. Pellet suspensions were further diluted (serially); three dilutions for each sample, in sterile PBS were then streaked on LB agar supplemented with Rifampicin

75 µg/mL and incubated at 37 °C overnight. A total viable count was calculated as total viable count collected per 1 g faecal pellet (Table 3). Control LB plates (without selective rifampicin (rif)) were included at each dilution to check efficiency of rifampicin based on presence of background but bacterial species. Pure cultures of *EcN* wild-type and *EcN* rif+ were also assessed for growth on LB (with and without rif) and for colony morphology on agar. After viable colony count, representative samples of colonies were recovered from CC & CP. *EcN* cultures samples were kept in fridge for further analysis to confirm *EcN* identity using an *E coli Nissle* PCR specific method.

3.5.5.5 Identification of probiotic from mice faecal pellets using PCR

Strain identity of *EcN* recovered from faecal pellets was confirmed by *EcN* strain specific PCR method developed by Blum-Oehler (333). Faecal pellet-recovered colonies (Rif resistant strains), positive controls (*EcN* wild type and *EcN* Rif+ stock cultures) and negative control *E. coli* strains (MG1655 and EPI300) were used. Total DNA was isolated from fresh colonies of these strains by colony boil method (334). Briefly, a single bacterial colony was suspended in 50 µL sterile $T_{50}E_{50}$ buffer (50mM Tris-Cl pH8, 50mM EDTA) inside a sterile 1.5 Eppendorf tube, then boiled at 95°C for 20 minutes. The boiled colony was then centrifuged at 17,000 g for 1 minute and the supernatant containing DNA template was recovered and used in PCR. The PCR employed Forward primer pMuta5: 5'- AACTGTGAAGCGATGAACCC-3' and reverse primer pMuta6: 5'- GGACTGTTCAGA GAGCTATC-3' (333). Primers were purchased from *Eurofins MW/Operon London, UK*.

The PCR were performed using Taq DNA Polymerase (Qiagen) kit, and consisted of 25- µL reactions, each contained 13.3 µl nuclease free H_2O, 5 µl of 5x Q solution, 2.5 µl of 10 x buffer (with15 mM MgCl2, giving 1.5 mM $MgCl_2$ per reaction), 1.5 µl of primer mix (10 [pmol] of forward primer: 10 [pmol] of reverse primer), 0.5 µl dNTPs (1 mM, giving 0.5 mM of dNTPs), 0.2 µl *Taq* (5 U/µl, giving 0.04U per reaction) and 2 µl of DNA template. No template PCR control (2 µl of water added instead of template was added to the experiment).

Reactions were mixed and transferred in 0.2 mL PCR tubes (VWR) and DNA amplification was carried out in thermocycler MJ Research PTC-200 Bio-Rad using the following thermocycler conditions:

Stage 1: Initial denaturation at 95°C for 5 min (1 cycle)

Stage 2: Amplification

Annealing at 52 °C for 45 sec

Extension at 72°C for 1 min (30 cycles)

Denaturation at 94°C for 30 sec

Stage 3: Final extension at 72°C for 5 min (1 cycle)

Stage 4: Final hold at 4°C overnight.

3.5.5.6 Necropsy

The experiment was ended at the first sight of ascites on week 10, mice were euthanised and a gross necropsy was performed to look for the presence of metastasis and ascites. The tumour tissues were harvested and weighed. Eight tissues from each group were frozen in liquid nitrogen and seven were fixed then embedded in paraffin for staining by immunohistochemistry (IHC) or immunofluorescence to study tumour infiltration by immune subsets ($CD8^+$ & $Foxp3^+$ (Tregs)). The spleens were put in a fresh media; six millilitres of DMEM (Dulbecco's Modified Eagle Medium), mashed and frozen to isolate white blood cells for further analysis.

3.5.5.7 Real time quantitative PCR (RT q PCR)

Total RNA was isolated from frozen tumour tissues (ovaries) following a protocol from commercially available kit (*RNeasy mini kit, part 1*, Qiagen) for extracting RNA from animal tissues. This was performed using less than 20 mg tissue lysate buffer (RLT) to homogenise the lysate, as well as ethanol, RW1 and RPE buffers to purify the

RNA and collect it on a spin column membrane which is then washed with RNase free water and centrifuged to elute RNA. RNA was then reverse transcribed to make complimentary cDNA as per protocol from Qiagen (QuantiTect Reverse Transcription Kit, Qiagen Group) RT kit. Primers for Real-time PCR (Foxp3, CD8, IL22, IL23, TLR4, TLR5) were purchased ready from Qiagen (QuantiTect Primer Assay, Qiagen, GmbH) for SYBR Green based real-time RT-PCR. Reaction mixtures were prepared using Qiagen Syber green kit (Qiagen) for 25 µl RT- q PCR reaction to be used in PCR machine two steps. The first step (the hold) is denaturation at 95 °C for 5 minutes then the second step is cycling which involves annealing at 95°C for 5 seconds in the first cycle followed by elongation at 60 °C for 10 seconds and ramping between both cycles for forty cycles with a temperature rise of 1 °C each cycle acquiring Syber green at cycle 2. Each reaction runs for 60 minutes. Beta actin was the housekeeping gene used as endogenous control and the comparative cycle threshold (Ct) was used to calculate Delta CT and thus calculate the relative amount of transcript.

3.6 Statistical analysis

Data was analysed statistically using Graph Pad prism software version 9 to conduct analysis of variance ANOVA. For continuous data assuming normal variance 2-way ANOVA or one way ANOVA followed by Tukey's post hoc test for multiple comparisons between the groups. For discrete data and column analysis, t-test is used to compare means, or one column statistic followed by Wilcoxon rank test. P value of less than 0.05 was significant.

3.7 Results

A series of experiments were designed to ensure the uptake of the supplementary feed (the probiotic in water and the water only). The first experiment was to measure the amount of consumption every day (figure 48), while the second was to take the weekly weight of the mice (figure 49). Finally, to further ensure the uptake of the feed and the survival of the probiotic throughout its passage, viable colonies assay was designed to retrieve and characterise viable faecal colonies (table 500). PCR using a primer unique for *EcN* was performed to confirm the identity of the bacteria

(figure 4). For the functional measure of the uptake and transit, faecal pellet assay at 3 weeks interval was designed to assess gut motility by measuring the number and weight of faecal pellets excreted over the time (figure 51a & b). Both groups were consuming the supplementary feed, suggesting that the supplementary feed is palatable. No difference was observed between the groups for the amount that was consumed

3.7.1 Consumption of the probiotic (*ECN*) and water is observed from both groups.

To ensure that the probiotic was palatable, and the mice were drinking it. The amount consumed by both groups each day was monitored by weighing the bottles prior to feeding and 24h later. The difference in the weight represents the amount that has been consumed. The sum of these amount over 3 days represents the cumulative amount, with no significant differences between the groups for a total period of 10 weeks (figure 48).

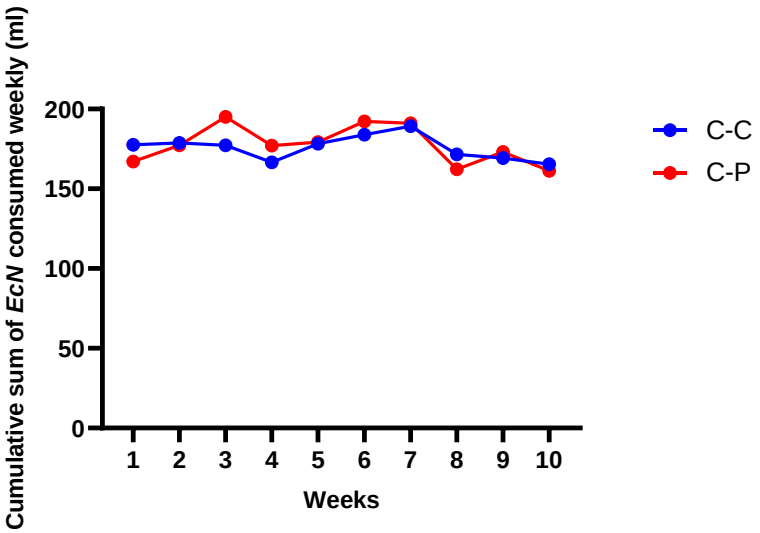


Figure 47: Cumulative sum of probiotic consumption in mice fed probiotics vs water
30 C57bl/6j female mice were injected with $4x10^6$ ID8 ovarian cancer cells IP then stratified into 2 groups (n=15): cancer-control (C-C). and cancer-probiotic (C-P). The probiotic group received $1x10^9$ cfu/ml of rifampicin resistant *EcN* in 200ml of water. Meanwhile, the control group received 200 ml of water only. The supplementary feed was freshly prepared daily and administered for 3 consecutive days each week until the end point was reached which is the development of ascites. The daily consumption was measured each week for 10 weeks. The difference in the weight of the feeding bottles 24h post the feed and before the start of the feed was measured each day of the administration of the feed (3 days a week). The cumulative sum of the amount consumed each week was calculated for each group. *EcN-E coli Nissle*, *IP-intraperitonially.*

3.7.2 Weekly weight of mice

To further ensure the uptake of the supplementary feed by mice from both groups, mice weights were measured. Both groups of mice gained weight. No significant difference was observed in the average weekly weight of the control vs probiotic groups (figure 49).

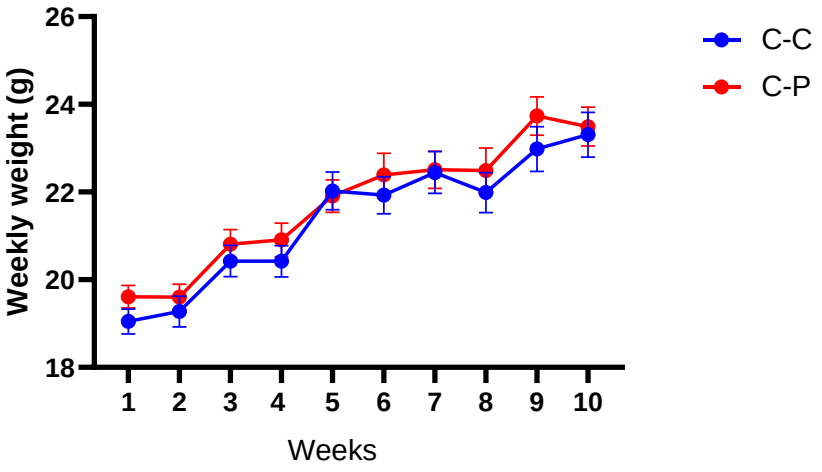


Figure 48: The average weekly weight of mice fed probiotics vs water
30 C57bl/6j female mice were injected with $4x10^6$ ID8 ovarian cancer cells IP then stratified into 2 groups n=15 and named according to the supplementary feed they received: cancer-control (C-C) and cancer-probiotic (C-P). The probiotic group received $1x10^9$ cfu/ml of rifampicin resistant *EcN* in 200ml of water. Meanwhile, the control group received 200 ml of water only. The supplementary feed was freshly prepared daily and administered for 3 consecutive days each week until the end point was reached which is the development of ascites. Mice from both groups: probiotic-fed and water only fed) were weighed each week for 10 weeks (the first sign of ascites). The average weekly weight for both groups was measured. *EcN-Escherichia coli Nissle 1917*, *IP-intraperitonially*, *C-C-Cancer-Control, C-P-Cancer-Probiotic*.

3.7.3 The probiotic *EcN* has the potential to reduce the frequency of transition along the gut as observed from the faecal pellets number and weight collected at 3 weekly intervals

To assess the functional effect of the supplementary feed. Faecal pellets were collected and weighed at three-week intervals: weeks (1,4&7) to assess the effect of the probiotic on transition along the gut. Both groups showed continuous increase in the average pellets count and weight with time, (figures 50a & b) respectively.

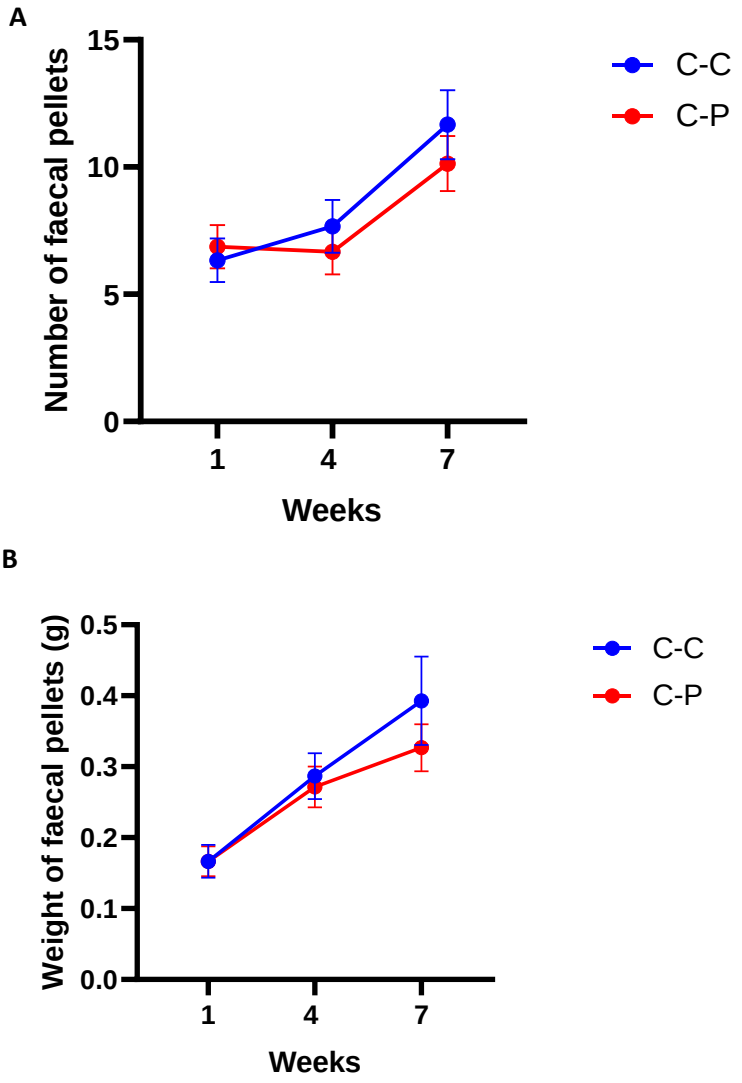


Figure 49: A reduction in the gut transition was observed in the probiotic-fed group

Faecal pellets were collected from both groups: cancer-control (C-C) and cancer-probiotic (C-P) (n=15) and counted every 3 weeks. On week 1, 4 &7, mice were put in an individual compartment for half an hour to acclimate then left for further one hour. The faecal pellets were collected, counted, and weighed. Tow way ANOVA was applied. No significant difference in the number of faecal pellets collected (A) nor in the weight of these pellets (B) was observed between the groups. *C-C-Cancer-Control, C-P-Cancer-Probiotic.*

3.7.4 Recovery of viable *EcN* colonies from mice faecal pellets at 4 days following the consumption of the probiotic

To ensure the uptake of *EcN* by the mice and its ability to survive the competition throughout its passage along the gut, a viable colony assay was designed to recover *EcN* colonies from faecal pellets excreted 4 days post the consumption of the probiotic. The recovered colonies were seeded on agar cultures treated with the broad-spectrum antibiotic (rifampicin). Only viable colonies of the rif+ *EcN* strain were observed from the probiotic-fed group as illustrated in (table 6). No viable *EcN* colonies were detected from the control samples (water only fed). This may suggest that the probiotic can survive the competition in the gut.

Samples	Total viable per 1g pellet cfu/g		Total viable per whole pellet cfu/pellet	
	Mean	SD	Mean	SD
CP1	$3.15x10^3$	$4.58x10^2$	$1.01x10^3$	$1.42x10^2$
CP2	$1.95x10^4$	$1.0x10^4$	$4.10x10^3$	$2.12x10^3$
CP3	$6.78x10^5$	$1.58x10^5$	$2.03x10^5$	$4.74x10^4$
CP4	$2.60x10^3$	$6.38x10^2$	$6.23x10^2$	$1.53x10^2$
CP5	$3.38x10^5$	$2.37x10^5$	$2.37x10^4$	$1.66x10^4$
CP6	$8.81x10^2$	$3.32x10^2$	$4.93x10^2$	$1.86x10^2$
CP7	$4.53x10^5$	$3.08x10^5$	$5.43x10^4$	$3.69x10^4$
CC1	0	0	0	0
CC2	0	0	0	0
CC3	0	0	0	0
CC4	0	0	0	0
CC5	0	0	0	0
CC6	0	0	0	0
CC7	0	0	0	0

C-C- Cancer control, C-P- cancer-probiotic

Table 6: Recovery of viable colonies of E. *coli Nissle 1917* (rifampicin resistant) from mice faecal pellets 4 days post the consumption of the probiotic

Prior to the use of the probiotic in the experiments, *EcN* strain was rendered resistant to the broad-spectrum antibiotic (rifampicin) to allow its recovery and characterisation from faeces. A random sample of 7 mice from 15 were picked up from each group: cancer-control and cancer-probiotic, 4-days post the last probiotic feed. The mice were put in individual cages for 1h and the faecal pellets were collected, weighed, and resuspended in PBS. 3 serial dilutions were prepared for each sample and 3 repeated agar plates with rifampicin were prepared for each dilution. The table shows the average number and standard deviation of viable *EcN* colonies from three repeated agar plates for each sample calculated as colony forming unit per whole collected pellets and per 1g of faecal pellets.

3.7.5 Identification of probiotic from mice faecal pellets using PCR method

To confirm the identity of the recovered viable colonies from faecal pellets, PCR was run using a primer unique for *EcN* against positive and negative controls (figure 51). The picture confirms the identity of *EcN.*

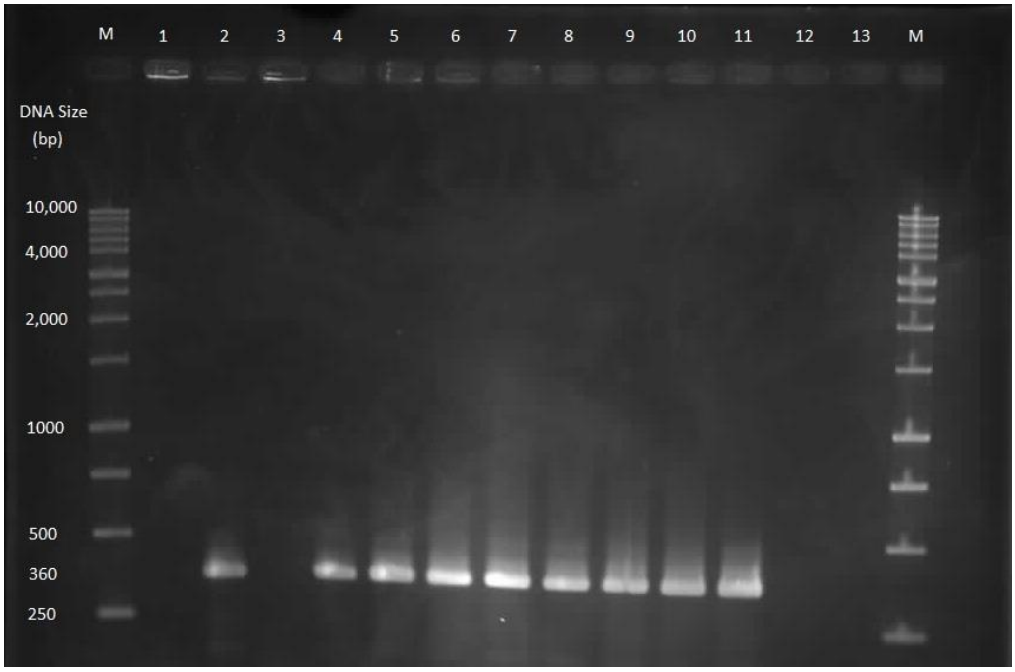


Figure 50: Amplified picture of segments of DNA from PCR method for *EcN*
EcN positive controls (*EcN* 1917 wild type and *EcN* (Rif$^+$) stock cultures), *EcN* Rif$^+$ recovered from faecal pellets from cancer probiotic (C-P) groups' samples and negative control *EcN* (MG1655 and EP1300). M- 1kb Promega DNA ladder; 1- (K12 MG1655), 2- *EcN* wild type, 3 - EPI300, 4= *EcN* Rif$^+$ stock, 5-11 *EcN* Rif+ recovered from faecal pellets, 12=-sterile water. PCR products were purified and separated on 1.0 % agarose gel.

3.7.6 Tumour burden

A series of assessments were conducted to investigate the impact of the probiotic *EcN* on ovarian tumour burden.

3.7.6.1 The probiotic EcN successfully reduced the weight of tumours collected from different organs

To assess the impact of the probiotic *EcN* on tumour burden, tumours collected from each group were weighed and compared. *EcN* significantly reduced the weight of tumours $p<0.05$, suggesting a potential protective role for *EcN* against ovarian tumour burden (figure 52). The number of tumours that were developed by each

mouse were counted as another measure for tumour burden. *EcN* had no effect on the number of tumours.

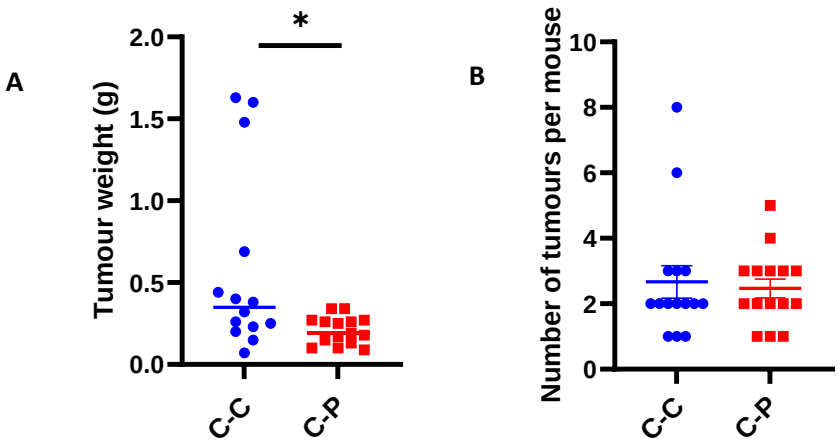


Figure 51: The probiotic *EcN* successfully reduced the weight of tumours collected from different organs

Cancer-control (C-C) and cancer-probiotic (C-P) underwent gross necropsy at the first sight of ascites-accumulation of fluid in the abdomen-which is the sign of metastasis in ovarian cancer. Tumours were harvested from each mouse and weighed. *EcN* significantly reduced the weight of tumours collected from each group, p= 0.0166, (A). But has no effect on the number of tumours observed from each mouse (B). T test for discrete data to compare the mean of the groups was applied. *EcN-Escherichia coli Nissle 1917*, *C-C-Cancer-Control, C-P-Cancer-Probiotic.*

3.7.6.2 EcN does not affect metastasis and ascites

To assess the impact of *EcN* on ascites production and metastasis, following gross necropsy, mice were observed for ascites and tumour development. There were no significant differences in the percent of mice presenting with ascites or metastasis (figure 53a), or mice with metastasis to the IP cavity and diaphragm (figure 53b) or the amount of ascites produced (figure 53c) between mice given probiotics vs control.

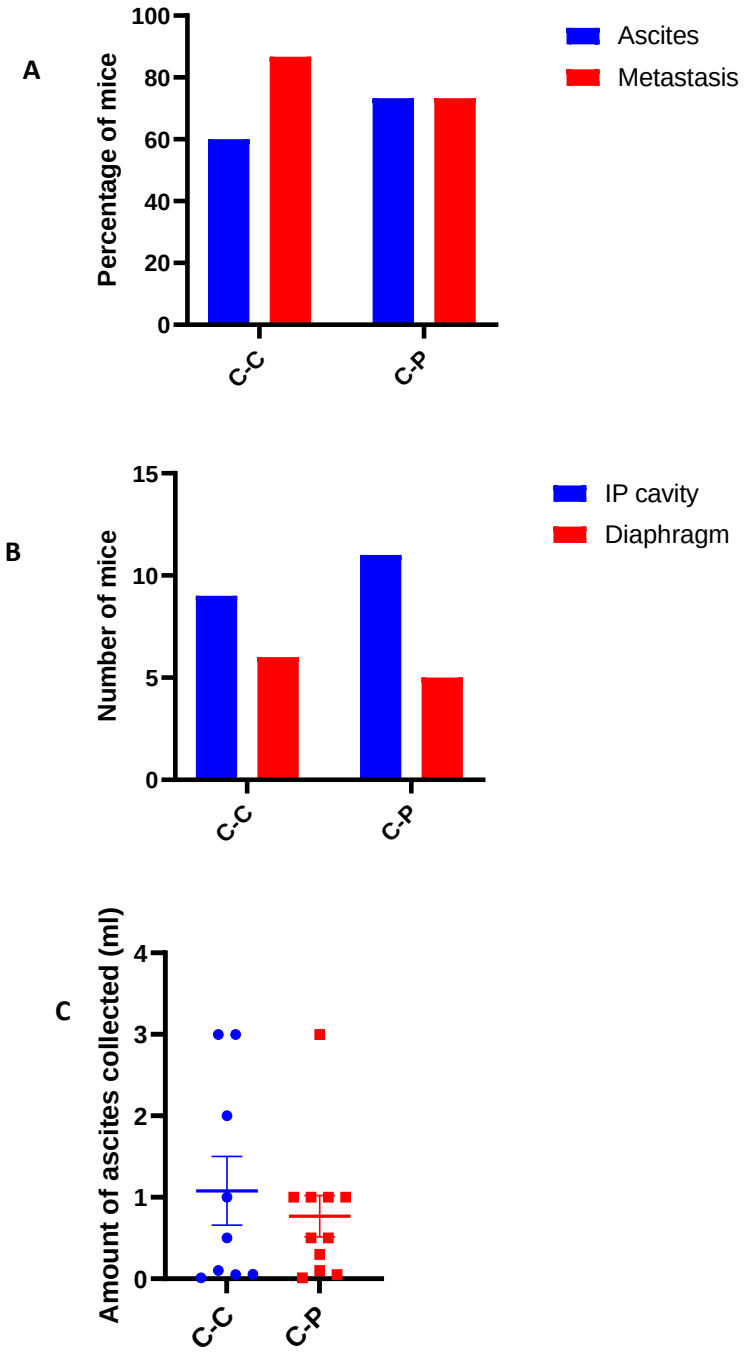


Figure 52: A potential protective effect was observed from the probiotic against metastasis and ascites

At the end point of the study comparing cancer-control (C-C) against cancer-probiotic-(C-P) group (n=15), which is the first visual sight of ascites, both groups underwent gross necropsy to look for tumour metastasis and ascites. Row statistics test for group analysis and t test for discrete data were applied. No significant difference was observed for: Metastasis and ascites (A), tumour progress to the IP cavity and diaphragm (B), and for the amount of ascites that was collected (C). *IP-Intraperitoneal cavity, C-C-Cancer-Control, C-P-Cancer-Probiotic.*

3.7.7 *EcN* downregulates the expression of TLR4 and upregulates the expression of 1L-23 in the ovarian tumours

A possible underlying mechanism for the anti-tumour effect of *EcN* is to manipulate the microbiome to reverse the inflammation induced by oncobiosis/dysbiosis in OC metastasis. This is likely to be mediated through modulation of the immune response. To investigate the immune-modulatory effect of the *EcN* on the innate immune response in tumour-bearing mice, TLR4, TLR5 and IL-23 were measured. *EcN* significantly down regulated the expression of TLR4 through LPS mediated stimulations in the ovarian tumours $p<0.01$, (figure 54), suggesting a potential anti-inflammatory role for *EcN*. However, the expression of TLR-5 which is triggered through stimulation mediated by the flagella of *EcN* (335), was observed in the ovarian tumour, but it was not affected by *EcN* (figure 55).

To further explore the signalling pathway, cytokines IL22 and IL23, which mediate TLR4 signalling pathway were investigated. *EcN* significantly upregulated the expression of IL23, $p<0.05$ (figure 56), demonstrating the presence of this cytokine in the ovarian tumour and a possible role for IL23 in the signalling pathway. The expression of interleukin 22 (IL-22) was investigated for its key role at maintaining tissues integrity (336). IL-22 was not found to be expressed in mouse tumours.

To explore the role of the adaptive immune response in the signalling pathway mediated by *EcN*, markers for key cells of the adaptive immune response were investigated. CD8 is a marker for effector cytotoxic T lymphocytes and Foxp3 is a marker for regulatory T cells (T regs). Expression of both markers were observed in the ovarian tumours (figures 57 & 58), suggesting that these cells are present in the tumour but neither CD8 or FOXP3 were affected by *EcN.* Taken together, the ant-tumour effect of *EcN* is likely to be through modulation of the innate immune response mediated by TLR4 and IL-23.

3.7.7.1 EcN suppressed TLR-4 in the ovarian tumours as illustrated in figure 54

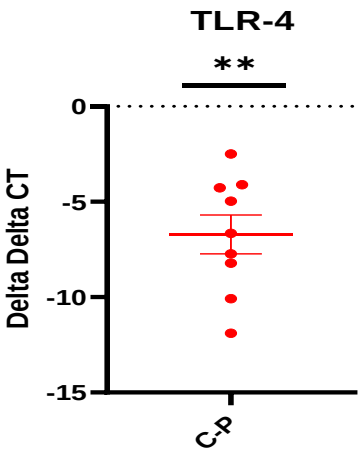


Figure 53: *EcN* suppressed TLR-4 in the ovarian tumours of the probiotic-fed group
At the first sight of ascites, both groups (probiotic-fed and water fed) underwent gross necropsy and tissues were harvested. Tissues from eight mice from each group were frozen for RTq PCR and 7 were fixed for immunohistochemistry analysis. Total RNA was isolated from frozen tumour tissues (ovaries) then reverse transcribed to make complimentary c DNA. Primer for TLR4 for SYBR Green based real-time RT-PCR was used. Beta actin was the housekeeping gene used as endogenous control and the comparative cycle threshold (Ct) was used to calculate Delta Delta CT and thus calculate the relative amount of transcript. Delta Delta CT from duplicate experiments showed a significant difference between the groups **p. One sample t test was applied.

3.7.7.2 EcN has no effect on the expression of TLR-5 in the ovarian tumour as illustrated in figure 55

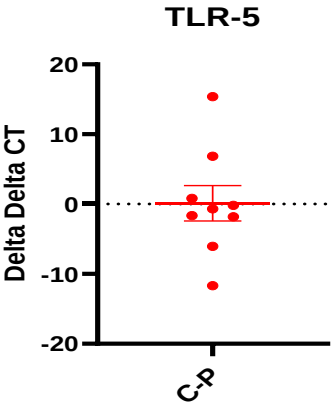


Figure 54: *EcN* has no effect on the expression of Toll Like Receptors 5 (TLR-5)
Expression of TLR-5 was observed in both groups: water-fed group (control) and probiotic-fed (treatment). At the first sight of ascites, both groups underwent gross necropsy and tissues were harvested. Tissues from eight mice from each group were frozen for PCR analysis and 7 were fixed for immunohistochemistry analysis. Total RNA was isolated from frozen tumour tissues (ovaries) then reverse transcribed to make complimentary c DNA. Primer for TLR-5 for SYBR Green based real-time RT-PCR was used. Beta actin was the housekeeping gene used as endogenous control and the comparative cycle threshold (Ct) was used to calculate Delta Delta CT and thus calculate the relative amount of transcript. Delta Delta CT from duplicate experiments showed no significant difference between the groups, one sample t-test was applied.

3.7.7.3 EcN upregulated the cytokine IL-23 in the ovarian tumour as shown in figure 56

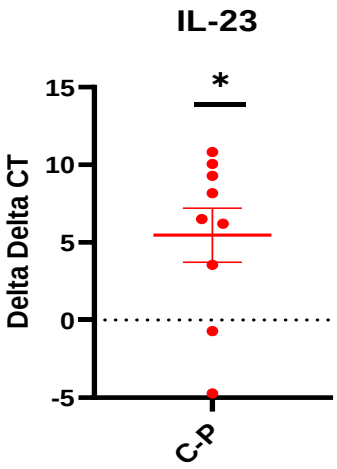


Figure 55: *EcN* upregulates the expression of cytokine IL-23 in the ovarian tumours
At the first sight of ascites, both groups: Probiotic-fed and water-fed groups underwent gross necropsy, tissues were harvested. Tissues from eight mice from each group were frozen for PCR and 7 were fixed for immunohistochemistry analysis. Total RNA was isolated from frozen tumour tissues (ovaries) then reverse transcribed to make complimentary c DNA. Primer for IL-23 for SYBR Green based real-time RT-PCR was used. Beta actin was the housekeeping gene used as endogenous control and the comparative cycle threshold (Ct) was used to calculate Delta Delta CT and thus calculate the relative amount of expression. Delta Delta CT from duplicate experiments showed a significant difference between the groups *p. One sample t test for discrete data was applied.

3.7.7.4 EcN has no effect on the expression of the cytokine IL-22

Interleukin 22 (IL-22) was not detectable in the tumour of either the water-fed group (control) or probiotic-fed (treatment), suggesting the absence of this cytokine from the tumour.

3.7.7.5 *EcN* has no significant effect on effector cytotoxic CD8+ T lymphocyte in the ovarian tumour as shown in figure 57.

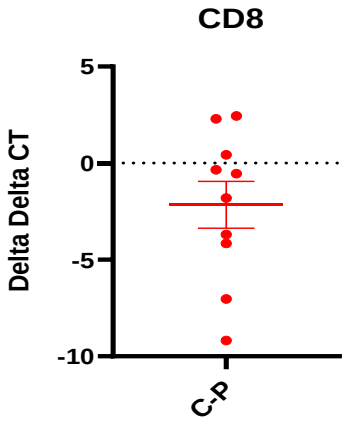


Figure 56: *EcN* has no effect on CD8+ cytotoxic T cells in the ovarian tumours
Less expression of CD8+ T cells was observed in the probiotic-fed group. At the first sight of ascites, both groups underwent gross necropsy and tissues were harvested. Tissues from eight mice from each group were frozen for PCR and 7 were fixed for immunohistochemistry analysis. Total RNA was isolated from frozen tumour tissues (ovaries) then reverse transcribed to make complimentary c DNA. Primer for CD8 for SYBR Green based real-time RT-PCR was used. Beta actin was the housekeeping gene used as endogenous control and the comparative cycle threshold (Ct) was used to calculate Delta Delta CT and thus calculate the relative amount of transcript. Delta Delta CT from duplicate experiments showed no significant difference between the groups. One sample t test was applied.

3.7.7.5 EcN has no significant effect on FOXP3+ regulatory T lymphocyte (T regs) in the ovarian tumours as illustrated in figure 58

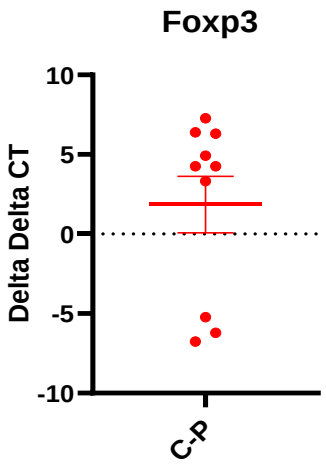


Figure 57: *EcN* has no significant effect on T regs cells in the ovarian tumour
Similar expression of Foxp3 the marker for regulatory T cells (T Reg) was observed from both groups: water-fed group (control) and probiotic-fed (treatment). At the first sight of ascites, both groups underwent gross necropsy and tissues were harvested. Tissues from eight mice from each group were frozen for PCR and 7 were fixed for immunohistochemistry analysis. Total RNA was isolated from frozen tumour tissues (ovaries) then reverse transcribed to make complimentary c DNA. Primer for Foxp3 for SYBR Green based real-time RT-PCR was used. Beta actin was the housekeeping gene used as endogenous control and the comparative cycle threshold (Ct) was used to calculate Delta Delta CT and thus calculate the relative amount of transcript. Delta Delta CT from duplicate experiments showed no significant difference between the groups. One sample t test was applied.

3.8 Discussion

The probiotic *Escherichia Nissle 1917 (EcN*) was able to successfully reduce tumour's weight in the probiotic-fed mice. This potential anti-tumour effect is likely to be mediated through modulation of the innate immune response at the tumour microenvironment via inflammatory pathways. Because a statistically significant downregulation in the expression of TLR4 and upregulation in the expression of IL-23 were observed in the ovarian tumour in the presence of the probiotic and both genes mediate inflammatory pathways. A protective anti-tumour effect was previously observed from *EcN* incorporated in saccharin water on OC metastasis (unpublished data). To verify our results for robustness and reliability in addition to probe a possible signalling pathway, the study was repeated.

It was found that the supplementary feed is palatable from the amount they consumed every day as well as the weight they gained every week. The uptake of the probiotic was further ensured by recovering viable colonies of *EcN* from faecal pellets excreted four days post probiotic feed and the identity of *EcN 1917* was confirmed by PCR. The ability to recover viable colonies of *EcN* from faecal pellets of probiotic-fed mice 4 days after they consumed the probiotic proved that *EcN* was able to survive the pressure from other pathogens, gastric acid, and bile salts in addition to the immune response throughout their passage in the gut. A functional measure for the uptake and transit of the probiotic was undertaken by monitoring the faecal output, where a continuous rise in the number of faecal pellets collected every three weeks was observed. The probiotic group showed less transition which indicates a possible direct effect of *EcN* on the gut motility and the probiotic is likely to reduce the gut motility. This effect is possibly mediated by the neurotransmitter serotonin (5HT) as explained in a study conducted by J Nzakizwanayo and his colleagues which demonstrated that *EcN* is able to enhance the bioavailability of serotonin (5-HT) in gut tissues through modulation of synthesis and clearance (337) along with evidence from previous studies highlighting the critical role of GI microbiota in regulating 5-HT in the host system (338). 5-HT is a transmitter predominantly produced in the gut

and interact with serotonergic receptors locally in the gut to regulate many gut functions like gut motility (339, 340).

Interestingly, *EcN* successfully reduced tumour weight and showed an overall reduction in tumour burden even though its effect on metastasis was not significant, which was different to what we had previously observed with the saccharin model where *EcN* significantly reduced metastasis (unpublished data). The effect of *EcN* previously observed on metastasis could be attributed to additional metabolic effects triggered by the sugar and have contributed to its anti-tumour role. Our finding suggests that the probiotic *EcN* has the potential for anti-tumour effect in OC and it is a promising complementary therapy in OC metastasis. In this study, the impact of *EcN* at the end stage of OC was investigated, it would be useful to look at early-stage disease and see if the onset of metastasis was delayed.

To probe the signalling pathway for *EcN* potential antitumor effect, it was decided to investigate its possible role in modulating the innate immune response. First, the expression of Toll Like Receptors (TLRs) in the ovarian tumour was examined. TLR4 is usually triggered by the bacterial component Lipopolysaccharide (LPS) and results in a cascade of signals mediated by adaptor proteins most commonly myeloid differentiation primary response gene 88 (*Myd88*). Subsequently leads to activation of transcription factors such as NF-kB and the release of proinflammatory cytokines from cells. Alternatively, activation of TLR4 by LPS can activate the transcription factor interferon regulatory factor IRF3 through different adaptor proteins resulting in expression of genes encoding type 1 interferons (272). These signalling pathways in turn direct the function of the adaptive immune system (341).

Evidence from the literature suggests that Toll-like receptor 4 expression on solid tumour cells is correlated with immune tolerance, tumour progression and metastasis as illustrated in a review by LI and colleagues (329). Moreover, (TLR-4) signalling pathways appears to be a key player in the initiation and progression of EOC (342). The ability of *EcN* to stimulate the downregulation of TLR4 suggests that

EcN has anti-inflammatory role which is in line with the postulated anti-tumour traits of this and other probiotics when it takes place at the intestine (229). In contrast, *EcN*-mediated downregulation of TLR4 was observed in the ovarian tumour which is expected to further contribute to the ovarian immune suppressive microenvironment, but based on the evidence from the literature, expression of TLR4 in tumours including ovarian tumour is correlated with disease progress, metastasis and poor prognosis as mentioned above (342). This implies that *EcN* has the potential for a promising role against the ovarian tumour metastasis.

EcN did not show an effect on TLR5 despite the observed expression in the ovarian tumour and the presence of flagellin (component of *EcN*), the ligand for TLR5. This suggests that this receptor is not likely to be involved in the *EcN* signalling pathway.

EcN significantly increased the expression of IL-23 in the ovarian tumour. IL-23 is released in response to stimulation of TLRs expressed on myeloid cells, and consequently it stimulates the differentiation of T cells into Th17 (an effecter T cell) (figure 59). Also, IL-23 is found to promote the antigen presenting activity of DCs and production of proinflammatory cytokines (37-39). It has been shown in few human studies (343, 344) that Th17 has the potential to exert antitumor immunity in established epithelial cancers such as epithelial ovarian cancer. This effect is found to be facilitated by recruiting other effector T cells subsets (345). It has also been shown that both intratumorally myeloid DCs and macrophages are potent inducers of Th17. However, such induction is found to be dependent on the expression of IL-23 and IL-1β as blockage of either of these expressions showed a significant reduction in Th17 and IL17. The reported role for IL-23 in activating Th17, DCs and macrophages demonstrates a potential anti-tumour role for IL-23 mediated by activation of DCs and macrophages as well as activation of Th17. Our results demonstrate amplification of IL-23 in the ovarian tumour in the presence of *EcN*, thus, a role for IL-23 in the anti-tumour signalling pathway of *EcN* is illustrated. Furthermore, Wolf and his colleagues concluded in their studies (346, 347) that high intra-tumoral expression of IL23 p19 m RNA is associated with superior clinical outcome in ovarian

cancer which further support the potential protective effect for *EcN* in EOC and the role for IL-23 in the mechanism of *EcN* action.

Moreover, the induction of Th17 is found to be suppressed by Tregs in the same ovarian microenvironment (345). However, our results do not show a significant increase in the ovarian tumour's Tregs in the presence of *EcN*. Therefore, it is unlikely for *EcN* to dampen the anti-tumour activity of Th17 in the ovarian tumour. It could be argued that *EcN* suppresses the expression of TLR4 on the ovarian tumours which could mean less production of Th17. However, Th17 is likely to be released in response to IL-23 from activated macrophages and dendritic cells in the tumour. Nonetheless, assessing the expression of IL-17, the effector cytokine that is released by Th17, in the ovarian tumour would better enlighten our understanding of the *EcN* signalling pathway especially that Th17 appears to play a key anti-tumour role in EOC. In summary, the increased expression in IL-23 by *EcN* suggests a role for this cytokine in the anti-tumour activity of *EcN*.

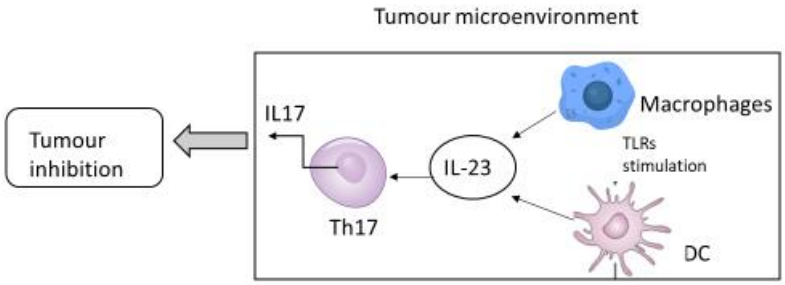


Figure 58: Illustration of the signalling pathway for TLRs activation in myeloid cells at the tumour microenvironment
Stimulation of TLRs on myeloid cells such as dendritic cells or macrophages triggers the release of the cytokine IL-23 which induces T helper 17 (Th17) cells to release the inflammatory cytokine IL-17. Adapted from(127).

The absence of IL-22 from the ovarian tumour could possibly be because IL-22 is a cytokine which has a regulatory role associated with maintaining the integrity of barriers and protecting from invading pathogens, therefore its function is relevant to body barriers such as the skin, respiratory tract, intestine, liver, pancreas (348).

Both cytotoxic tumours infiltrating $CD8^+$ T cells and $Foxp3^+$ regulatory T cells (Tregs) (components of key importance in the adaptive immune system) were present in the ovarian tumours but were possibly not affected by *EcN* because no significant difference was observed between the groups for these cells. This suggests that the anti-tumour effect of *EcN* is not likely to be mediated through these adaptive immune cells. Further work is required to explore the role of *EcN* in modulating the adaptive immune response.

3.9 Conclusion

The probiotic *EcN* significantly reduces tumour weight in a syngeneic mouse model of epithelial ovarian cancer metastasis. This anti-tumour effect is likely to be mediated by TLR-4 and IL-23 in the ovarian tumour, (figure 60). The probiotic could have the potential for benefits in patients with cancer. Our results may suggest that when *EcN* is administered to patients with OC metastasis along with their current treatment it reduces their tumour burden and give them a better quality of life. It is worth repeating this experiment using saccharin as well as looking for Th17 marker to enhance our understanding of its signalling pathway at a distant organ such as the ovaries. Our intriguing finding unravel a novel role for *EcN* in cancer.

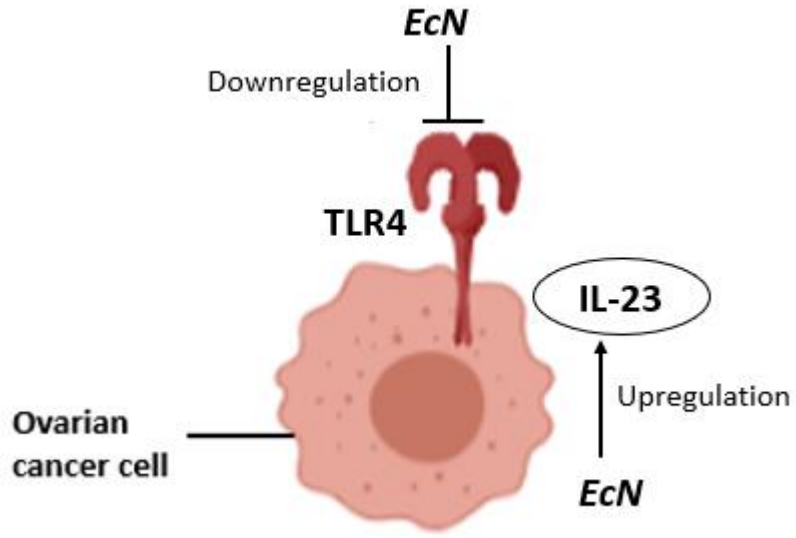


Figure 59: Postulated signalling mechanism for the anti-tumour effect of *EcN* in ovarian cancer metastasis

EcN through its immunomodulatory role, downregulates the expression of TLR4 and upregulates the expression of IL23 in the ovarian tumour which are likely to enhance its anti-tumour potential. In EOC TLR4-Toll like receptor 4, IL23-Interleukin 23, *EcN-Escherichia coli Nissle*.

Based on the interesting potential for *EcN* to stimulate the downregulation of TLR4 in the ovarian tumour and the upregulation of IL-23, it was decided to investigate the impact of this probiotic on stress in advanced stages of EOC (metastasis) with the intension for use as a complementary therapy. We propose that *EcN* can combat the effect of stress by enhancing the anti-tumour immune response in the ovarian tumour.

4. Chapter 4-The impact of probiotics, psychological stress in epithelial ovarian cancer

4.1 Introduction

4.1.1 Gut microbiota and stress

Stress is one of the essential survival mechanisms in nature. It is defined as an acute threat to the homeostasis of an organism that is caused by a physical or psychological stressors and results in adaptive responses to enable survival (349). Based on its duration and effect on an individual's health, stress can be divided into acute (minutes to hours) and chronic (days and weeks), manifesting beneficial or detrimental effects, respectively. Stress stimulates neuroendocrine response through the HPA axis and SNS resulting in the production of the stress hormones glucocorticoids and catecholamines. Persistent release of these hormones increases their level in the blood and within central and peripheral tissues, consequently causing detrimental physiological and psychological morbidities such as cardiovascular, digestive, and immune diseases as well as reduced happiness and unhealthy lifestyle (41, 72, 350). In the UK, at least one third of work-related illness is caused by stress, resulting in a big loss of working time (351). Moreover, clinical, and epidemiological studies over the last few years have shown a role for stress, chronic depression, and lack of social support in increasing the risk of cancer progression (61, 352). Indeed stress-related psychosocial factors were linked with poor survival in patients diagnosed with cancer and increased cancer mortality (60).

The gut microbiota has a symbiotic relationship with its host and plays a key role in regulating the host's physiology and immunity in the gut (353). It has long been recognised that the brain regulates the gut activities such as motility, permeability, and secretion. However, it has recently been found that the gut microbiota can also affect the host's brain development and functions in the adulthood. Microbiota is a key regulator of processes that influence stress regulation and behaviours such as neurogenesis and neuroinflammation (340, 354).The cross talk between the brain and the gut takes place through the brain-gut microbiota axis (355-357). This bidirectional interaction is regulated by hormonal, neural and immune pathways and it is now the proposed regulatory mechanism for the maintenance of gut function and homeostasis in health and disease (357-359). Dysregulation of the brain-gut

microbiota homeostasis (i.e. dysbiosis) induced by infection by pathogens or other factors such as antibiotics, diet and stress results in alteration in central processes (including neurogenesis, neuroinflammation and neurotransmission), subsequently leading to stress-related disorders such as altered GI motility, secretion, permeability and behaviour (355, 358, 360). The use of animals raised in a germ free environment (GF) has enabled a thorough investigation of the microbiota-HPA relationship by comparing them against their conventional counterpart (358).

Evidence from animal models demonstrates a role for different types of psychological stress (such as restraint conditions and maternal separation) in disturbing the integrity of the gut epithelia, causing translocation of the gut microbiota and activation of the mucosal immune response. The disruption of the gut epithelia was found to affect the postnatal development of the HPA stress response because of the disturbed composition and distribution of the resident microbiota at an early stage of life. These changes in the gut microbiota and the subsequent changes in the HPA stress response were shown to be associated with a change in behaviours including anxiety and depression (356, 361-363).

Both animal and human studies show that manipulation of the gut microbiota with probiotics to adjust their ecological balance in the gut can result in reduced anxiety and depression as well as an alleviation of stress symptoms. It was demonstrated in GF mice that exposure to mild RS caused an excessive increase in corticosterone and adrenocorticotrophin hormones (ACTH) when compared with specific pathogen free (SPF) controls. The stress response was completely reverted when GF mice were mono colonised with *Bifidobacterium infantis* over time (361). This highlights the importance of the microbiota composition in the development of the appropriate stress response. Moreover, mice deficient in adaptive immune cells (B and T cell) and subjected to psychological stress displayed a change in their baseline memory and anxiety response along with hyperactivated HPA axis. These stress-induced effects were reversed by probiotic, suggesting a role for the microbiota in regulating the stress response. This is likely to be achieved through maintaining gut homeostasis.

The gut microbiota and immune system crosstalk at the epithelial mucosa from early stages of life, whereby the host tolerates many antigens colonising its gut and only mount an immune response against pathogenic antigens, thus resulting in diverse and abundant microbiota to maintain homeostasis (364). While the microbiota shapes the development, maturation and function of the immune system by modulating the activation and differentiation of different lymphocytes, balance the production of immunoglobulin A and promoting the development of lymphoid structures (365). Disruption of this homeostasis results in systemic translocation, inflammation and release of proinflammatory cytokines which on turn activates the HPA axis (figure 62) (358).

Further, administration of the probiotic *L. helveticus NS8* to rats subjected to chronic restraint stress depression, improved their behaviour (anxiety and depression) and cognition function. This effect was the same or better than what was seen in control groups treated with the selective serotonin reuptake inhibitor anti-depressant (citalopram) (12). Furthermore, daily consumption of the probiotic *Lactobacillus casei* strain *Shirota* significantly reduced exam stress associated abdominal dysfunction in healthy medical students when compared against placebo in randomised double blind-placebo controlled study. This effect is thought to be mediated by restoration of the microbiota diversity by the probiotic (14).

It is postulated that probiotics modulate the stress response by modifying neurotransmitters synthesis and release. The probiotic *B. infantis 35624* showed a potential anti-depressant activity in rats following administration for 14 days relative to controls with no probiotic, the anti-depressant effect was observed through an increase in the plasma level of tryptophan (a precursor of serotonin (5-HT) which is a key neurotransmitter in the brain-gut axis) (366). Alternatively, probiotics regulate the immune response through cytokines release. It was demonstrated in a human study that administration of the probiotic *B. infantis* to patients with irritable bowel syndrome normalised the HPA response. This effect is thought to be mediated through modulation of the pro to anti-inflammatory cytokines (367). It is important

to note that multiple mechanisms are likely to be involved at the same time (figure 61).

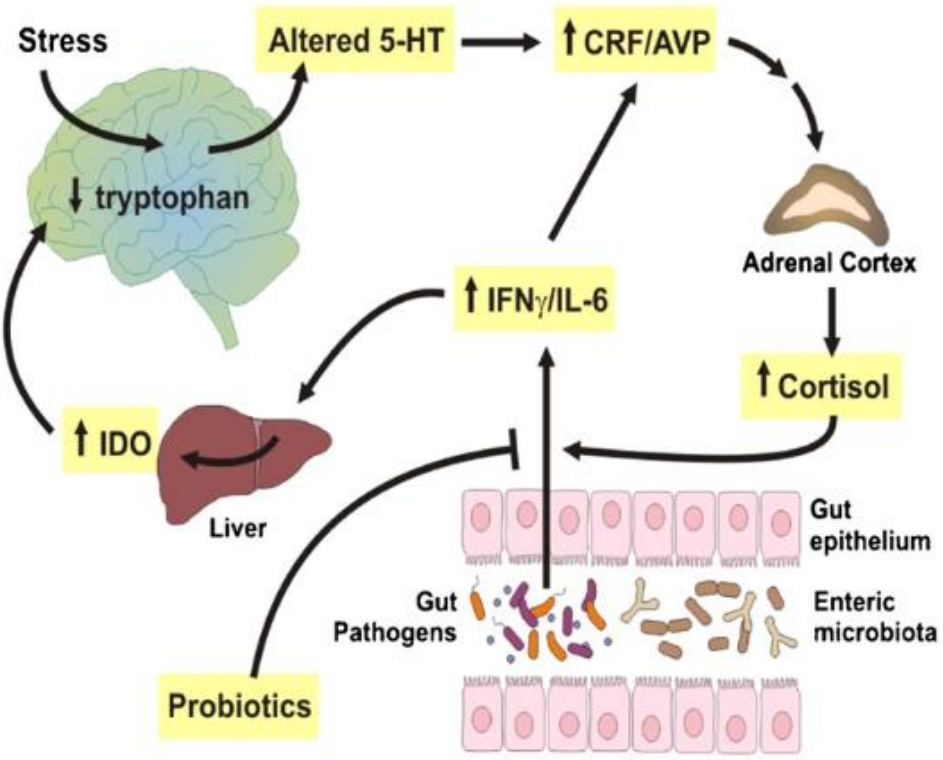


Figure 60: Illustrates disruption in the bidirectional brain-gut-microbiota
Stress can alter barrier function in the gut increasing gut 'leakiness' and leading to an increase in pro-inflammatory cytokines which in turn can alter indoleamine 2,3-dioxygenase (IDO) activity. This leads to altered tryptophan availability. Pro-inflammatory cytokines such as IFN γ, IL-6 together with 5-HT influence the release of CRF and AVP from the paraventricular nucleus of the hypothalamus. Certain probiotic bacteria can alter gut barrier function and via the vagus nerve may impact on key central neurotransmitter systems. *CRF-Corticotrophin-releasing factor, AVP-Arginine Vasopressin, IL-6-Interleukin 6 and IFN γ-Interferon gamma*. Taken from (358).

4.1.2 Metastasis, the immune response and Psychologic stress

Metastasis is resistant to conventional treatment; therefore, it is the major cause of death in cancer (368). It is a complex process which involves a cascade of steps including angiogenesis, proliferation, invasion, embolization, and evasion of the immune surveillance; all these steps must be achieved for metastasis to manifest clinically (51, 369). The outcome of metastasis depends on multiple factors including metastatic potential of cancer cells, host homeostatic mechanisms (immune surveillance) and the specific organ's microenvironment which determines the extent of cancer cells proliferation, angiogenesis, invasion, and survival (234).

Therefore, treating metastasis should extend beyond cancer cells to include the host factors which contribute to the progress and survival of metastatic cancer cells (368, 370) for instance, the immune response. Dhabhar et al demonstrated possible mechanisms underlying the negative impact of chronic stress on the immune response in cancer patients. They showed that chronic stress can affect immune cells trafficking and function leading to modulated functional end effect of the immune response which may speed up cancer progression and metastasis. The underlying mechanisms for stress-immune modulations are likely to be through suppression of the protective immunity, or Induction/exacerbation of the inflammatory immune response or enhancement of the regulatory/immune suppressive immunity (72, 232, 233).

Evidence from preclinical and clinical studies has identified a role for the stress response in promoting the key elements of cancer metastasis. For example, in ovarian cancer cell line, the stress hormone noradrenaline has been shown to increase the expression of VEGF (a direct angiogenic molecule which play a key role in angiogenesis and neovascularisation of the malignancies) (371). The observed increase in VEGF was abrogated by propranolol (non-specific β-blocker. Similarly, clinical studies in OC patients showed a decrease in tumour and sera VEGF in patients who received high social support (249, 372). Further, Zhao and colleagues observed growth in prostate cancer cell line by cortisone (the metabolite of cortisol) in an androgen independent way. This was found to be mediated by the development of glucocorticoid-responsive androgen receptors mutants (373). Furthermore, stress was found to disrupt neuroendocrine circadian rhythm causing an enhanced tumour growth and metastasis as observed in mice engrafted with breast cancer cells and subjected to disturbed light/dark shifts (374).

4.1.3 Probiotic as a possible intervention in OC metastasis

Management of stress by psychosocial interventions such as mindfulness and social support may influence the stress-related behaviour processes and clinical health outcome in cancer patients (72, 375, 376). However, more research is required to

examine how soon should the intervention start post diagnosis with cancer, the format for delivering the intervention and which subgroups of patients is likely to clinically benefit the most from these interventions (72).

A relationship between gut microbiota dysbiosis and cancer initiation, progression and treatment has been observed particularly in colorectal cancer (377). The immune- inflammation modulatory properties of probiotics have made them the prime attention of many researchers to prevent cancer onset and progression, improve the clinical efficacy and attenuate the adverse effects of anti-cancer therapies (378, 379).

4.1.4 c GAS-c GAMP-STING pathway in the ovarian tumour

It was demonstrated earlier in chapter 3 that *EcN* has the potential for a protective anti-tumour effect mediated by modulation of the innate immune response in the ovarian tumour. Therefore, it was decided to investigate the impact of probiotic and RS on the inflammatory pathway c GAS-c GAMP-STING in the ovarian tumour. The STING pathway is activated in response to cytosolic double strand DNA from exogenous source such as bacteria or viruses or from endogenous source such as tumour cells. This pathway is found to bridge innate with adaptive immune responses by stimulating the expression of genes mediating the production of type 1 interferons (IFNs). Triggering of TLR4 with LPS can also result in Type 1 IFNs production. Type 1 IFNs are found to induce T cells mediated anti-tumour immunity (380). (The pathway is illustrated in chapter 2, section 2.1.4.

4.2 Hypothesis

We hypothesize that the probiotic *EcN* can combat the negative effects of stress in ovarian tumour by restoring the protective immune response.

4.3 Objectives

- To Identify the impact of RS on OC metastasis, the major cause of death in cancer
- To investigate the role of the probiotic *EcN* in protecting against the negative effects of stress in OC metastasis.

4.4 Material and methods

4.4.1 Culture and manipulation of bacterial strains

EcN strain was rendered resistant to rifampicin to allow its recovery and characterisation from faeces. *EcN* rifampicin resistant strain was created using the serial passage method (331) as described previously in chapter 3 section 3.5.1.

4.4.2 Preparation of probiotic feed

Pure cultures of *Escherichia coli Nissle* rifampicin resistant (*EcN-Rif+)* were grown in LB broth inside sterile glass bottles for 12 hours, with shaking (150 rpm) at 37°C to achieve culture density of 1x 10^9 CFU/ml (mid-logarithmic phase of growth from a concentration time log graph). The cultures were transferred to 50 mL sterile centrifuge tubes and centrifuged in Heraeus Labofuge® 400 R model centrifuge (Thermo Scientific) at 4500 rpm for 10 min at room temperature. The supernatant was removed, and bacterial pellet was then resuspended in the same volume of saccharin water (Sigma) (660mg saccharin in 1L of sterile water) and transferred to mice water bottles (200 ml each). Saccharine is a polysaccharide which is not used by *EcN* as a source of energy but sweet enough to make probiotic feed palatable to mice. The probiotic suspensions (1x 10^9 CFU/mL) in saccharin water were supplied to the 4 groups (n=5): cancer-stress-probiotic (C-S-P), cancer-no stress-probiotic (C-P), stress-probiotic (S-P) and no stress-probiotic (P) over three consecutive days every week (Tuesday, Wednesday, and Thursday) at 12 pm for 10 weeks, freshly prepared every

24 hours. Probiotic feeds and saccharin water control feeds were replaced by normal water for the rest of the week.

4.4.3 Cell line

On week (0) of the study (figure 2), the 2 cancer groups; C-S-P and C -P were injected with $4x10^6$ cells of murine epithelial ovarian cancer cells (ID8) in 200ul of PBS intraperitonially. ID8 cells were kindly donated by Dr Premal Thaker, Washington University, St Louis, USA were used to induce ovarian tumour growth. The cells were cultured as described previously in chapter 3 section 3.5.3.

4.4.4 Mice and diet

Twenty (20), 6 weeks old female C57BL/6j mice weighing 18 ± 2 g were bought from Charles River (C.R) laboratories (Margate, Kent, United Kingdom). The mice were handled for 1 week to acclimatise to researchers (myself) then stratified into four groups (n=5) and named: cancer stress-probiotic (C-S-P), cancer no stress-probiotic (C-P), stress-probiotic (S-P) and no stress-probiotic (P). The mice and diet used in the study with a timeline are illustrated in figure 62.

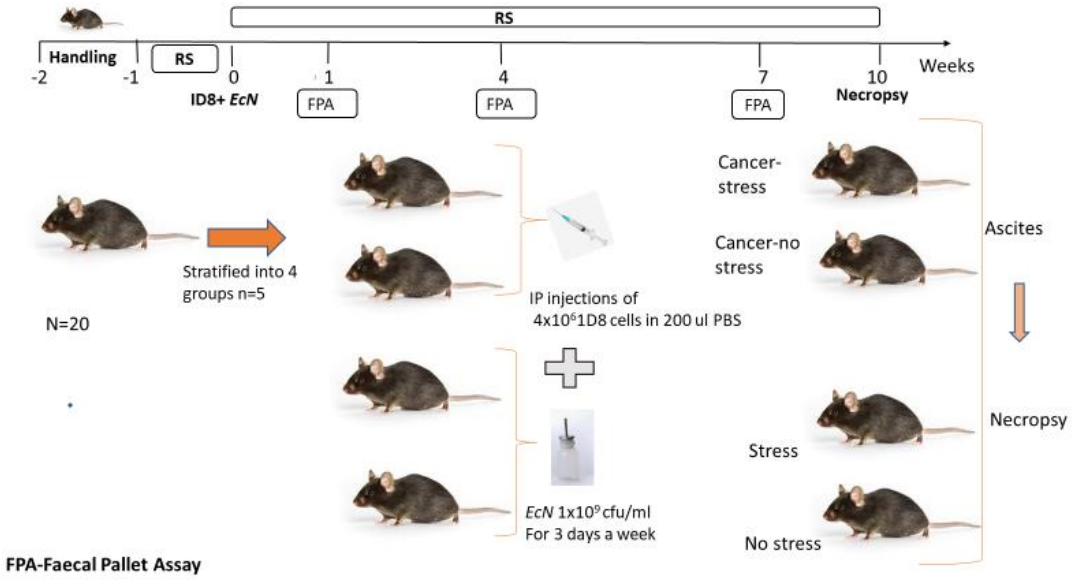


Figure 61: Illustration of the mice, diet and essays that were used in the study with timeline
Twenty (20), 6 weeks old female C57BL/6j mice weighing 18±2g were bought from Charles River (C.R) laboratories (Margate, Kent, United Kingdom). The mice were handled for 1 week (-2 to -1) to acclimatise to researchers then stratified into four groups (n=5) and named: cancer stress-probiotic (C-S-P), cancer no stress-probiotic (C-P), stress- probiotic (S-P) and no stress-probiotic (P). The stress groups were subjected to RS daily for one week (-1 to 0) while the No stress groups were kept in their cages. At week 0, all 4 groups were fed with 200 ml of the supplementary diet *(EcN* $1x10^9$ cfu/ml incorporated in saccharin water). The feeds were freshly prepared each day for three consecutive days each week and started on the second week of the study immediately after the IP cancer injections. They were fed every week on Tuesdays, Wednesdays, and Thursdays until the end point of the study which was the first visual sight of ascites. For the rest of the week, all animals received normal diet; RM No. three SDS (Special Diet Services) Expanded Diet (LBS, Surrey) 5g/mouse daily in addition to fresh tap water and were maintained in a room with a 12-hour light/dark cycle at 19°c-21°c and 40%-60% humidity (controlled remotely). Cage temperature 37c° (controlled by providing appropriate nesting material). The RS continued until the end point of the study (week 10). Faecal pellet assay (FPA) was performed at weeks (1,4&7) to test the functional uptake of the probiotic by assessing its activity on the gut motility. All procedures were approved by the animal welfare at the University of Brighton (personal licence number is I81347B). At week 10, all mice underwent necropsy and tissues were harvested for further analysis. *RS-Restraint stress, FPA-Faecal Pellet Assay.*

4.4.5 Study power and sample size

A power analysis was performed from a study using similar mouse model, it was known that with a type I error rate of 0.05, a sample size of n = 10 provides approximately 80% power to detect at least a 20% difference. Originally, 40 mice were bought so that each group would have 10 (n=10). However, due to the unprecedented problem with COVID and the limited budget that was available. The mice were aged and were replaced with only 20 (n=5).

4.4.6 Procedures

4.4.6.1 Restraint Stress (RS)

Mice from the stress groups: C-S-P and S-P (n=5) underwent RS for 2h daily from 10-12 am in 50ml perforated falcon tubes as described earlier in chapter 2 section 2.6.3. RS was started at (-1 week), one week prior to the administration of IP injections of ID8 (0 week) to the cancer groups and continued daily until the first visual sight of ascites (figure 2).

4.4.6.2 Consumption of probiotic

The bottles containing the probiotic in saccharin or saccharin only, were weighed before feeding the mice and twenty-four hours after, to measure the amount consumed each day by each group. Cumulative sum of consumption over the three days each week was then calculated to ensure that the mice were drinking the supplementary diet

4.4.6.3 Weight of animals

Mice were weighed before the initiation of RS, following the IP injections of ID8 cells and weekly afterwards to further monitor the uptake of the supplementary feeds.

4.4.6.4 Faecal pellet assay

For the functional measure of the uptake of the probiotic, faecal pellets assay was carried out at the beginning and at an interval of three weeks to measure the effect of the probiotic on the gut motility. All groups were assessed on week one, four and seven (figure 2) as previously described in chapter 3 section 3.5.5.3. Briefly, mice were placed in separate cages for 1h and their faecal pellets were collected. The pellets were counted and weighed to assess the colonic activity of the probiotic (frequency of transition).

4.4.6.5 Recovery, enumeration of viable colonies of the probiotic EcN from mice faecal pellets

To ensure that the probiotic is being effective, it should be first proved that *EcN* could survive the pressure in the mice gut, and it was recoverable from faeces. After 7 weeks of probiotic treatment, fresh faecal pellets were collected from mice following 4 days without probiotic feed as described in chapter 3 section 3.5.5.4. Three mice were picked up randomly from each group and used to grow viable colonies on LB agar media supplemented with rifampicin 75 µg/mL, then incubated at 37 °C overnight.

4.4.7 Necropsy

The experiment was ended at the first visual sight of ascites which was week 10. Mice were euthanised and underwent a gross necropsy to look for the presence of metastasis and ascites in the cancer groups. Tumour tissues were then harvested, weighed and frozen for further immune analysis by RT qPCR to assess for gene amplifications of markers on cytotoxic T lymphocytes (CD8+), markers for T helper 17 (IL17+) and some STING pathway genes mediating the release of type 1 interferons (IFNs); STING (Tmem 173) and Tank Binding kinase 1(TBK1). Ascites was collected and measured to assess tumour burden, blood was collected, and plasma was isolated and frozen for cytokines analysis.

4.4.8 Real time quantitative Polymerase Chain Reaction (RT q PCR)

To look for gene amplifications of immune cells markers and inflammatory genes such as type 1 INFs, total RNAs were isolated from frozen ovarian tumours, then reverse transcribed to make complimentary c DNA following Qiagen, UK protocols as described previously in chapter 3 section 2.7. Primers for Real-time PCR (CD8, IL17, Tmem173, TBK1) were purchased ready from Qiagen (QuantiTect Primer Assay, Qiagen, GmbH) for SYBR Green based real-time RT-PCR. Reaction mixtures were prepared using Qiagen SYBR green kit (Qiagen) for 25 µl RT- q PCR reaction to be used in PCR machine.

4.5 Statistical analysis

Data was analysed statistically using Graph Pad prism software version 9 to conduct analysis of variance ANOVA. For continuous data assuming normal variance 2-way ANOVA or one way ANOVA followed by Tukey's post hoc test for multiple comparisons between the groups. For discrete data and column analysis, t-test is used to compare means, or one column statistic followed by Wilcoxon rank test. P value of less than 0.05 was significant.

4.6 Results

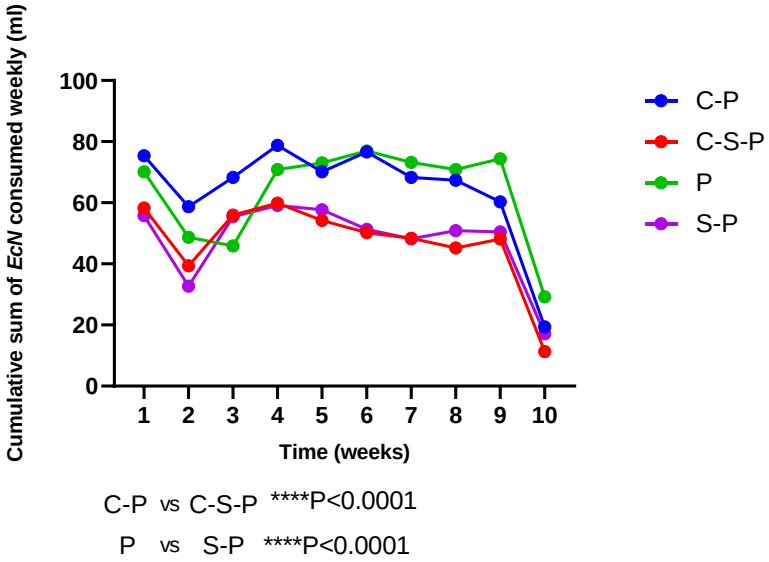
A series of procedures were designed to ensure the supplementary feeds (probiotic in saccharin water/saccharin water only) were palatable and the mice were taking the probiotic.

4.6.1 Restraint stress reduced the amount of probiotic consumed by mice

To ensure that the supplementary feeds (probiotic in saccharin water/saccharin water) were palatable, and the mice were drinking them, the amount consumed by both groups was monitored each day by weighing the bottles prior to feeding and 24h later. The difference in the weight represents the amount that has been consumed. The sum of these amounts over 3 days represents the cumulative amount. All groups were drinking the supplementary feeds. RS significantly reduced the amount of probiotic consumed by mice from both cancer and no cancer groups $P<0.0001$ (figure 63a). This may suggest that the probiotic is palatable, but the amount consumed is affected by the stress. To further ensure the uptake of feeds, the mice were weighed before the start of RS (week -1), following the ID8 injections (week 0) and weekly afterwards for 10 weeks until the first visual sight of ascites. All groups gained weight over the 10 weeks. Daily restraint stress over 10 weeks significantly reduced the weekly weight that was gained by mice with and without cancer when compared with the no stress groups, $p<0.0001$, (figure 63b). This confirms our previous finding in chapter 2, section 2.8.6.6, where stress reduced the weight gained by mice challenged with ID8 injections and stressed for 4 weeks. The results indicate that the supplementary feeds are palatable because the mice were

gaining weight, but the stress is affecting their consumption. Thus, the probiotic is not showing any protection against the stress effect on weight.

A



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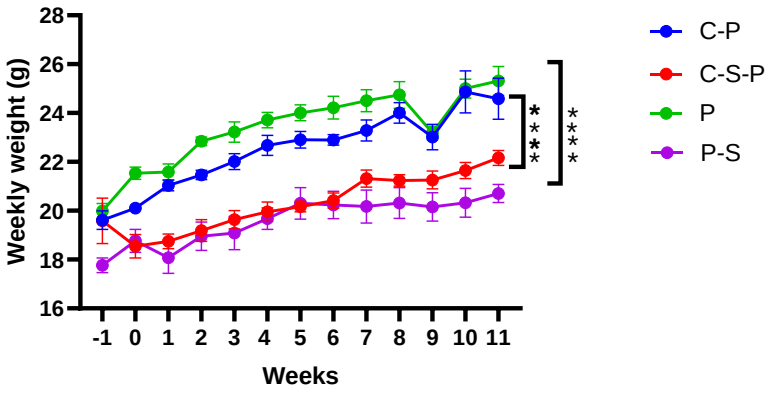

Figure 62: Restraint stress significantly reduce the amount of probiotic consumed by mice in the presence and absence of cancer

Twenty, 6 weeks old female mice C57bl/6j were stratified into 4 groups (n=5) and named cancer stress-probiotic (C-S-P), cancer no stress-probiotic (C-P), stress probiotic (S-P) and no stress probiotic (P). The stress groups underwent restraint stress by placing them in perforated 50 ml falcon tubes for 2 h a day while the other two groups were left in their cages. One week later, the cancer groups were injected with $4x10^6$ ID8 cells (EOC cells) IP. All groups were then fed with $1x10^9$ cfu/ml of the probiotic *EcN* incorporated in saccharin water for 3 consecutive days each week, until ascites was observed at week 10 (the end point of the study). The difference in the weight of the feeding bottles 24h post the feed and before the start of the feed was measured each day of the administration of the feed (3 days a week). Cumulative sum of consumption per week was calculated for each group and plotted against time. Unfortunately, one mouse from each stress groups: C-S-P and S-P deceased during the first week of RS prior to cancer injections. Therefore, the RS continued with n=4 in each stress group. The stress groups with and without cancer consumed significantly less probiotic p<0.0001 (A). The weight of mice from stress groups was significantly less than that of the no stress groups. p< 0.0001 (B). (2-way ANOVA followed by Tukey's multiple comparisons between the column means). *IP-Intraperitonially, cfu-colony forming units, EOC-Epithelial Ovarian Cancer, C-P-cancer-probiotic, C-S-P-cancer-stress-probiotic, P-probiotic, S-P-stress-probiotic.*

4.6.2 Restraint stress reduces the gut motility as observed from the number and weight of the faecal pellets

To ensure that the probiotic is functional, its effect on the gut motility was assessed by measuring the frequency of transition from the number and weight of faecal pellets excreted from each mouse over 1h. Restraint stress significantly reduced the number of pellets that were excreted from the cancer group, $p < 0.01$ and no cancer group $p < 0.01$, (figure 64a). Also, RS significantly reduced the weight of the excreted pellets in the cancer group $p < 0.05$ and no cancer group $p < 0.001$, (figure 64b). The data suggest that stress is likely to reduce the frequency of transition along the gut (there was more constipation) in both cancer and no cancer groups. The effect of the probiotic that is observed on the gut's transition (reduced motility) in the presence of stress coincides with the effect that was observed previously in the absence of stress (chapter 3, section **3.7.3**). However, the stress significantly decreased the gut motility which may suggest that the stress is enhancing the effect of the probiotic on the gut.

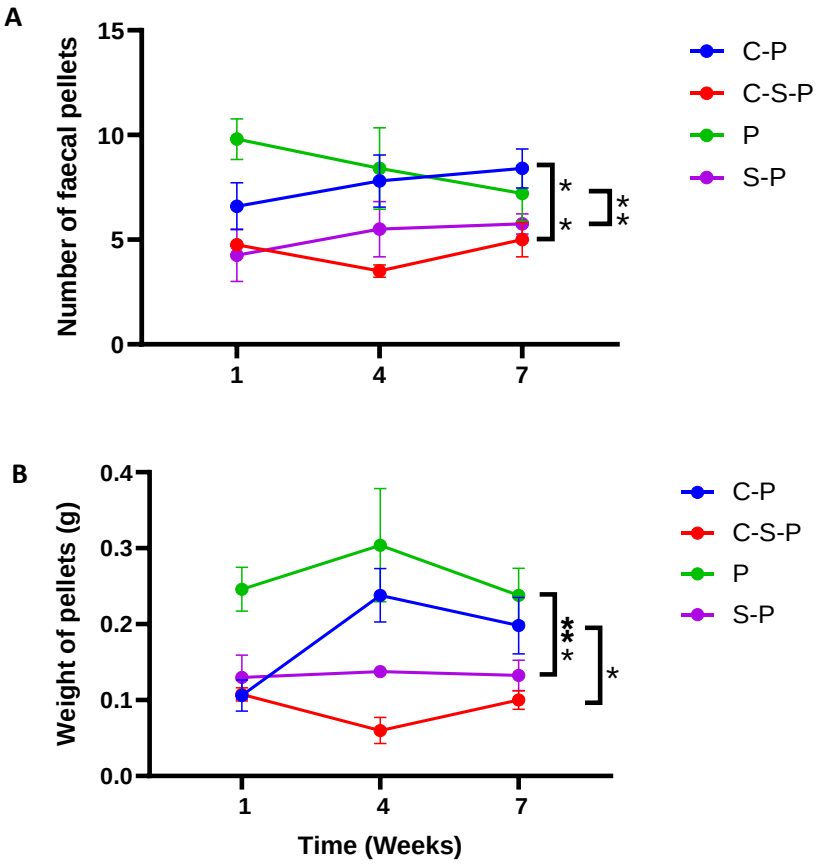

Figure 63: Restraint stress reduces the frequency of transition along the gut
Mice from each group (n=5): C-P, C-S-P, P & S-P underwent faecal pellet assay at week 1, 4 and 7 to assess the colonic activity of the probiotic. Mice were placed individually in empty cages for thirty minutes to settle and then the faecal output which was generated within the next an hour was collected, counted, and weighed. Restraint stress significantly decreased the colonic transition across the gut. Restraint stress significantly reduced the number of pellets that were excreted from cancer and no cancer groups **p (A). Also, RS significantly reduced the weight of the excreted pellets in the cancer groups *p and no cancer groups ***p (B) (2-way ANOVA followed by Tukey's multiple comparisons between the column means). *C-P-cancer-probiotic, C-S-P-cancer-stress-probiotic, P-probiotic, S-P-stress-probiotic*.

4.6.3 No *EcN-rif*^{r} viable colonies were enumerated on rifampicin treated agar plates

To ensure the uptake of *EcN* by the mice and its ability to survive the competition throughout its passage along the gut, a viable colony assay was designed to recover *EcN* colonies from faecal pellets excreted 4 days post the consumption of the probiotic. A random sample of 3 mice from 5 were picked up from each group (C-P, C-S-P, P and P-S) 4-days post the last probiotic feed. The mice were put in individual cages for 1h and the faecal pellets were collected, weighed, and resuspended in PBS.

3 serial dilutions were prepared for each sample and 3 repeated agar plates with rifampicin were prepared for each dilution

The recovered colonies were seeded on agar cultures treated with the broad-spectrum antibiotic (rifampicin) and incubated overnight. No *EcN* viable colonies were observed on LB agar plates supplemented with rifampicin 75ul/ml from the cancer groups in both the stress and no stress groups. However, inconsistent growth was observed from samples taken from the no cancer groups in both the stress, and no stress groups. The results were collated from 2 technical repeats. This could either be due to inability of *EcN* to survive the competition from acidic pH, other pathogens or the immune system even though it was demonstrated in chapter 3 that it is able to maintain viability following excretion. Alternatively, it could be due to errors during faecal dilutions before enumeration on agar plates.

4.6.4 Restraint stress significantly increased metastasis

Throughout the study, mice were observed until the development of ascites at week 10. The mice then underwent gross necropsy to observe for ascites and tumours development. Significantly more metastasis $p<0.05$ was observed in the stressed mice compared to the no stressed group, but no significant differences were observed in the percent of mice presenting with ascites (figure 65a) or in the amount of ascites produced (figure 65b). Finally, significantly more metastasis to secondary organs was observed in the stressed mice $p< 0.05$ (figure 65c). These results indicate a role for RS in promoting cancer metastasis and the inability of the probiotic *EcN* to provide protections against metastasis in the presence of stress.

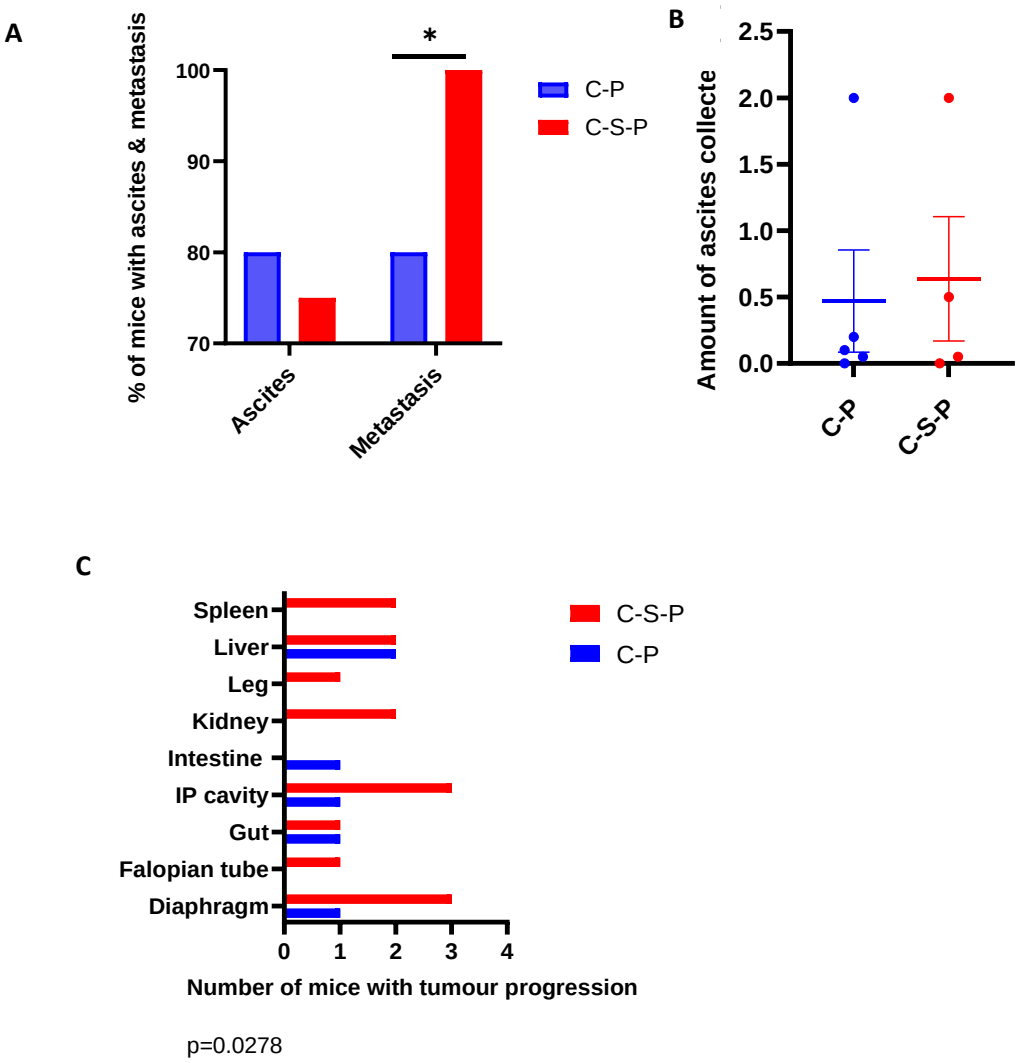

Figure 64: Restraint stress significantly increased metastasis

Two cancer groups (n=5); C-P and C-S-P were challenged with 4x10^6 ID8 cell/200ul PBS, IP, then fed with 1x10^9 cfu/ml of the probiotic *EcN* incorporated in saccharin water for 3 consecutive days each week until ascites was observed at week 10. The stress group (C-S-P) was subjected to 2-hours daily restraint stress in a 50 ml perforated falcon tubes for one week prior to the cancer injections and continued afterwards until the first visual sight of ascites. Meanwhile mice from the control group (C-NS-P) were left in their cages. At the first visual sight of ascites, all mice were euthanised and underwent gross necropsy to assess for ascites and metastasis. Significantly more metastasis was observed in the stressed mice *p (A). No significant difference was observed between the groups in the amount of ascites that was developed, t test was applied, (B). A significant increase in tumour progression to secondary organs was observed in the stressed mice, *p(C). Two-way ANOVA and Tukey's multiple comparison test. *C-P- Cancer- No stress and probiotic, C-S-P-Cancer-stress, and probiotic. IP-Intraperitonially, cfu-colony forming unit, ID8 cells- Murine epithelial ovarian cancer cells to induce ovarian tumour growth.*

4.6.5 No effect from the stress and probiotic was observed on the gene expression of 3 markers from the adaptive and innate immune response

To explore the impact of stress and probiotic on the adaptive immune response in the ovarian tumour, gene amplification for CD8 (a marker for cytotoxic tumour infiltrating T lymphocytes (CTL) was measured. Restraint stress decreased the amplification of the gene for CD8 but not to a significant level (figure 66), suggesting that this key component cell of the adaptive immune response is unlikely to be affected by the stress and probiotic.

To investigate the impact of *EcN* on the innate immune response in the presence of RS, the expression of two genes mediating the inflammatory pathway c GAS--STING was assessed in the ovarian tumour: Tmem173 (gene encodes for the protein STING) and Tank Binding Kinase 1 (TBK1) gene. An upregulation in the expression of TBK1 and downregulation in the expression of Tmem173 was observed in the presence of stress and probiotic. However, the level of the expression did not reach significance (figure 67). This may imply that this pathway is not affected by the stress and *EcN.*

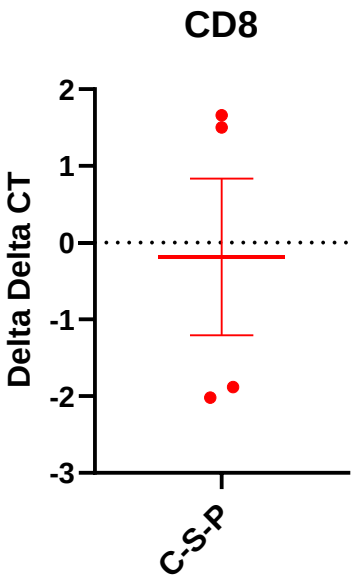

Figure 65: Restraint stress has the potential to decreases the amplification of CD8; a marker for effector cytotoxic T lymphocytes (CTL)

Two cancer groups C-P (n=5) and C-S-P (n=4) were challenged with $4x10^6$ ID8 cell/200ul PBS IP, then fed with $1x10^9$ cfu/ml of the probiotic *EcN* incorporated in saccharin water for 3 consecutive days each week until ascites was observed at week 10. The stress group (C-S-P) was subjected to 2-hours daily restraint stress in a 50 ml perforated falcon tubes for one week prior to the cancer injections and continued afterwards until the first visual sight of ascites, meanwhile mice from the control group (C-P) were left in their cages. At the first visual sight of ascites, all mice underwent gross necropsy to assess for ascites and metastasis and harvest tissues to freeze at -80° for immune-analysis. Total RNAs were isolated from frozen tumour tissues (ovaries) then reverse transcribed to make complimentary c DNA. Primer for CD8 for SYBR Green based real-time RT-PCR was used. Beta actin is the housekeeping gene used as endogenous control and the comparative cycle threshold (Ct) was used to calculate Delta Delta CT and thus calculate the relative amount of transcript. Delta Delta CT from duplicate experiments showed no significant difference between the groups. One sample t test was applied. *C-P - Cancer- No stress and probiotic, C-S-P-Cancer-stress and probiotic.*

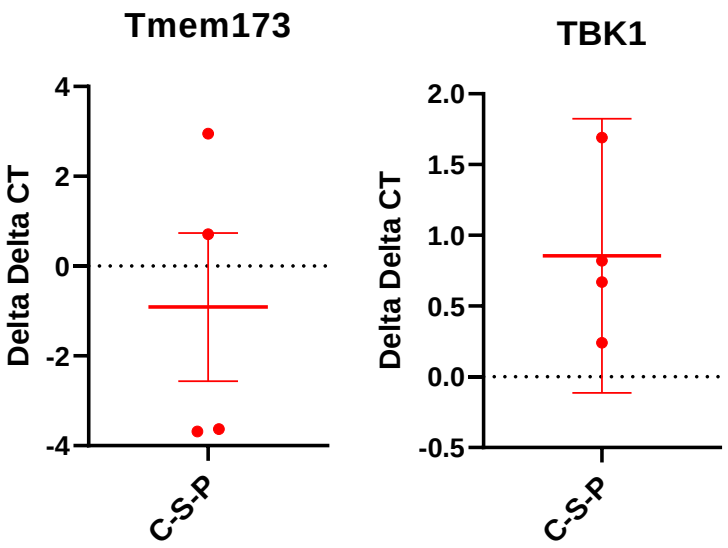

Figure 66: Restraint stress has no significant effect on the expression of Tmem173 (STING) and TBK1 Two cancer groups (n=5); C-P and C-S-P were challenged with $4*10^6$ ID8 cell/ml IP, then fed with $1x10^9$ cfu/ml of the probiotic *EcN* incorporated in saccharin water for 3 consecutive days each week until ascites was observed at week 10. The stress group (C-S-P) was subjected to 2-hours daily restraint stress in a 50 ml perforated falcon tubes for one week prior to the cancer injections and continued afterwards until the first visual sight of ascites, meanwhile mice from the control group (C-P) were left in their cages. At the first visual sight of ascites, all mice underwent gross necropsy to assess for ascites and metastasis and harvest tissues to freeze at -80° for immune-analysis. Total RNAs were isolated from frozen tumour tissues (ovaries) then reverse transcribed to make complimentary c DNA. Primers for Tmem173 and TBK1 for SYBR Green based real-time RT-PCR were used. Beta actin is the housekeeping gene used as endogenous control and the comparative cycle threshold (Ct) was used to calculate Delta Delta CT and thus calculate the relative amount of transcript. Delta Delta CT from duplicate experiments showed no significant difference between the groups for the expression of TBK1 and Tmem173. One sample t test was applied. *C-P- Cancer- No stress and probiotic, C-S-P-Cancer-stress, and probiotic. Tmem173- STING; stimulating interferon gene. TBK1- Tank binding kinase 1 and c GAS- complementary Cyclic GMP-AMP (cGAMP) synthase*.

4.7 Discussion

Our data suggests that stress overrides the potential reduction in tumour burden and health promoting effects of the probiotic *EcN* by increasing metastasis and tumour progression to secondary organs, reducing weight gain, and suppressing appetite. Some of these negative effects from stress were observed in our previous syngeneic cancer-stress mouse model which was used to investigate the impact of stress on DCs and T lymphocytes activations in addition to tumour burden at an early stage of OC. No added benefits were observed from the probiotic against tumour burden or stress negative effects.

The observed pathophysiology of the gut such as altered gut motility, suppressed appetite, and reduced weight gain were all observed previously in our lab in the presence of stress without a probiotic. These modulated GIT physical functions are believed to be mediated through dysregulation of the bidirectional brain-gut-microbiota axis as demonstrated in different studies (381, 382). Therefore, it is expected that administration of probiotic would restore the microbiota balance and consequently reinstate the gut homeostasis. Conversely, *EcN* did not reverse the negative effects of the stress on the gut. This could be due to the stress-mediated dysbiosis and subsequent translocation of pathobiont (commensals with pathogenic potential, where they can express pathogenic components) to the systemic circulation, activation of the systemic immune response and LPS-mediated inflammation which may promote cancer development and progression (127). Also, stress is likely to alter the release of serotonin which in turn further activates the HPA-axis (stress response) due to the compromised negative feedback which regulates the stress hormones release, resulting in more systemic inflammation (figure 62). Therefore, *EcN* is facing more pressure to compete against throughout its passage along the gut which may have dampened its probiotic functions. This may explain the inconsistency in the number of viable colonies retrieved from faecal pellets of the no cancer groups and the absence of any viable colonies from the cancer groups where cancer further contributes to the disruption of the gut

homeostasis. Perhaps, administration of more than one strain of probiotics is needed to restore the microbiota balance and abrogate the stress effect on the gut.

In this study, no protection was observed from the probiotic *EcN* against the effect of stress on metastasis. This could be due to synergistic perturbation effect from cancer and stress on the gut microbiota composition, diversity, and function, in addition to the disruption to the gut integrity and increased permeability. Subsequently, systemic translocation occurs which is likely to trigger the mucosal immune response resulting in chronic inflammation and neuroendocrine activation (383). Activation of the immune response mainly through PRR with microbial PAMP drives the release of proinflammatory cytokines such as interferon gamma (IFN γ) and interleukin 6 (IL 6). These cytokines have the potential to activate the HPA-axis by acting directly on the hypothalamus or indirectly altering the availability of tryptophan and serotonin (5-HT) through the enzyme IDO leading to an increased release of cortisol and disturbed gut microbiota (figure 1) (355, 356, 384). The presence of IDO in the ovarian tumour microenvironment has been associated with suppressive immunity and poor prognosis (385). *EcN* is expected to restore the gut homeostasis by combatting the perturbation. This is likely to be achieved through its immunomodulatory property mediated by suppression of proinflammatory cytokines and enhancement of anti-inflammatory ones. In addition to its ability to reinforce the epithelial tight junction and supress the growth of pathogens (211). However, these effects seem to be overridden by the impact of stress.

It was shown in human studies (343, 344) that Th17 is an effector T cell which has the potential to exert antitumor immunity in established epithelial cancers such as ovarian cancer (345). It was found that Th17 cells recruit other effector T cells subsets to the ovarian tumour making it inflamed/hot tumour and thus susceptible for immune destruction (149). Testing for the presence of Th17 cells in the ovarian tumour would have enhanced our understanding of the signalling pathway underlying *EcN* anti-tumour activity in OC. The induction of Th17 is found to be dependent on the expression of IL-23 and IL-1β as blockage of either of these

expressions showed a significant reduction in Th17 (345). And it was demonstrated in chapter 3 that upregulation of IL-23 mediates the potential antitumour effect of *EcN*. Therefore, it was decided to assess the expression of IL-17 (a marker for Th-17) in the ovarian tumour to explore the activity of *EcN* in the presence of stress. However, due to a technical problem with the primer and time constrains, it was not possible to investigate the expression of this cytokine.

Moreover, it was observed earlier in chapter 2 that RS has the potential to decrease the activation of DCs at an early stage of cancer (2 weeks). Therefore, it was speculated that administering *EcN* to the stressed mice would reduce the tumour burden by abrogating the effect of stress on DCs activation, consequently enhancing the antigen presenting activity of DCs and the anti-tumour immunity. However, this did not happen possibly because of the oncobiosis/dysbiosis caused by the cancer and RS, resulting in dysregulation of the brain-gut-microbiota bidirectional homeostasis and disruption of the probiotic activity.

Finally, it has been observed throughout this study that *EcN* potential anti-tumour activity is mediated through modulation of the innate immune response. Therefore, it was decided to investigate the impact of the probiotic *EcN* in the presence of stress on type 1 interferons response and innate immune sensing of cancer. cGAS-STING pathway (a vital component of the innate immunity and likely to play a key role in anti-cancer immunity (illustrated in chapter 2)) was partially investigated (114). However, to draw a conclusion on the impact of *EcN* on type 1 IFNs production in the presence of RS, further exploration of this pathway in the ovarian tumour is required by looking at all the genes that are involved in the pathway particularly the expression of type 1 interferons (272) .

4.8 Conclusion

The negative impact of stress supersedes the potential anti-tumour effect that was previously observed from *EcN*. Nonetheless, the anti-tumour effect of *EcN* in the absence of stress has been reproduced in EOC (chapter 3). Therefore, *EcN* is a valued probiotic with promising potential for anti-tumour effect at distant areas from the gut but its role against stress may require further investigation.

Administering of *EcN* to patients with ovarian cancer metastasis as a complementary therapy along with other anti-cancer therapies and social support for an early stress management is likely to improve clinical outcomes.

5. Chapter 5- General discussion and conclusions

5.1 Discussion

In this thesis, it was shown that RS can damage SP in the presence and absence of tumour antigens and impairs the anti-tumour immunity of T lymphocytes and dendritic cells. The impairment in the anti-tumour activity of splenic T cells was first observed in co-cultures of 3D ovarian spheroids models and *ex vivo* SP either from ID8 antigens-naïve mice or ID8-primed ones by IP injections. Splenocytes from stressed mice were less able to infiltrate and disintegrate the ovarian spheroids compared to SP from non-stressed mice suggesting a reduction in cytotoxic activity. Further, prolonged stress (4-weeks exposure) showed more severe effect on SP as they were not able to recognise ID8 antigens despite previous priming, being isolated from mice with ID8 ovarian tumours. Furthermore, a decreased expression of the activation markers MHCII/CD40 was observed in splenic DCs subjected to 2 but not 4 weeks of RS following ID8 cells injection suggesting a reduced ability to present antigens.

It is possible that the stress-induced SP damage is duration dependent and is consistent with findings in the literature (275, 386). Studies in rats exposed to restraint stress (1 h) followed by a gap of 4 h with forced swimming exercise (15 min) daily for 2, 4 and 8 weeks, showed that the severity of stress influenced lymphoid organs (e.g., decreased spleen, thymus and axillary lymph nodes weights, splenic lymphocyte numbers and apoptosis). Furthermore, stress was found to be duration dependent, and the sensitivity of these organs was higher at a shorter exposure to stress. Following a stress recovery period (1 month), the effect of stress on lymphoid organs parameters of rats exposed to shorter stress was reversed but not on those exposed to longer duration (275). These findings could explain the observed impaired ability of DCs to express activation markers at 2 but not 4 weeks stress exposures and the reduced ability of SP from 4-weeks stressed mice to recognise ovarian tumour antigens despite previous exposure. Additionally, a study in adults primates (monkeys) showed a psychological stress-induced alteration in LN neural structure that was associated with long term regulatory change in the initiation, maintenance and resolution of the immune response (386).

Although the literature suggests similar stress-induced damage is expected to be seen in LN DCs, in this study no significant change was observed in the expression of the activation markers MHCII/CD40 in LN DCs isolated from the inguinal LN of stressed mice compared to non-stressed ones. Knowing that LN is the first site for tumour antigens specific priming of T cells, it was expected to observe a similar effect from stress on LN DCs to that observed in splenic DCs. This could be due to the smaller number of DCs isolated from each LN compared to splenic DCs. LNs are very small, therefore isolating DCs from them was technically challenging. Also, the calculated expression of markers in splenic DCs was the average of pooled data from 2 biological repeats whereas for LN DCs, the average expression in individual LNs from one biological experiment was calculated; this may explain the larger variability of results. For more consolidated conclusions on the impact of stress on the LN DCs activation markers' expression, the experiment should be repeated with pooled LNs.

To begin to understand the mechanism underlying stress-induced SP damage, DNA damage was investigated. A significant increase in DNA double strand breaks (a severe damage in the structure of DNA) in SP from stressed mice was observed and further confirmed in splenic T lymphocytes treated with cortisol *in vitro*. The data suggest that SP are undergoing apoptosis which is likely to explain the impaired anti-tumour activity of splenic T lymphocytes and the inability of GR antagonists to fully recover their function. Further work is needed to investigate the ability of these cells to repair DNA damage and restore their cytotoxic activity.
Stress-induced DNA damage in SP is a novel finding which has not been demonstrated in immune cells previously.

Moreover, it was also demonstrated in this study that RS has the potential to supress the anti-tumour immune response at an early stage of OC (2 weeks) implying that the impact of stress at an early stage is crucial for an early intervention. The immunosuppressant effect of stress is well recognised in the literature (58, 236, 392). However, our finding shows that the immunosuppression in OC starts at an early stage, impairing the antigen presenting activity of DCs, consequently compromising

the priming of cytotoxic T lymphocytes and thus the activation of the adaptive immune response. Such a finding is promising and may suggest the importance of early complementary interventions following diagnosis with OC including social support, promoting healthy lifestyle and mental wellbeing as a possible strategy for reducing the impact of stress and restoring the anti-tumour activity of DCs and splenic T lymphocytes.

Interestingly, the immunomodulatory trait of a well characterised (genotype and phenotype) and clinically valued probiotic *Escherichia coli Nissle 1917* (*EcN)* (387) inspired the investigation of its anti-tumour activity using an immunocompetent syngeneic mouse model. The study identified a potential anti-tumour role for *EcN* in OC metastasis which is likely to be mediated through immune modulation of the innate immune response involving (TLR-4) signalling pathways and IL-23. *EcN* showed downregulation of TLR-4 in ovarian tumours which is consistent with other evidence from the literature (342, 388). For example, it was demonstrated in a human OC cell line (CAOV-4) that treatment with the probiotic L. lactis down regulated the expression of TLR-4 which correlated with the observed apoptosis (342). Other evidence suggests a key role for activation of (TLR-4) signalling pathways and subsequent inflammation in the initiation and progression of EOC (388). Upregulation in IL-23 by *EcN* was also observed. IL-23 is associated with activation of the effector subset of T cells (Th17). The presence of Th17 in epithelial cancers such as ovarian cancer was found to promote protective anti-tumour immunity (343, 344). Our finding suggests that the probiotic *EcN* has a potential anti-tumour effect at tissues distant from the gastrointestinal tract which could benefit ovarian cancer patients by reducing tumour burden when it is administered as a complementary therapy in metastasis.

Finally, the well characterised clinical values for *EcN* at preventing and treating diseases at the epithelial and extraepithelial level which are attributed to its immunomodulatory role (387), led to the investigation of the role of *EcN* in protecting against the impaired anti-tumour immunity induced by RS. It was hypothesised that *EcN* has the potential to enhance the anti-tumour immunity and

reduce tumour burden in the ovarian tumour and thus combats the negative impact of stress. But this was not observed, instead a significant increase in metastasis and tumour progression was observed from the stress. This is possibly due to the enhanced dysbiosis mediated by stress and cancer leading to chronic inflammation. Nonetheless, *EcN* is a valued probiotic with promising potential for anti-tumour effect at distant areas from the gut, consumption of this probiotic as a complementary therapy in OC metastasis is likely to confer benefits. However, its role against stress may require further investigations.

5.2 Conclusions

An enhanced understanding of the molecular mechanisms underlying the effect of stress on the immune response highlights targets for novel intervention to restore the natural anti-tumour immune response.

This research showed that restraint stress has the potential to impair the antitumour immunity at an early stage of EOC by suppressing the antigen presenting activity of DCs and impairing the cytotoxic effect of splenocytes at the tumour microenvironment. The latter effect is also observed in the absence of cancer and is likely to be mediated through DNA damage and subsequently apoptosis of splenic T lymphocytes. This may-in part demonstrates a possible mechanism of action underlying the inhibitory effect of the stress hormone cortisol on the cytotoxic activity of T cells, which provides possible targets for pharmacological and/or biobehavioural interventions that is likely to promote good stress effects and minimise chronic harmful effects. Modulating GRs with gold standard or novel antagonists is a possible way to partially reverse the negative effect of stress and restore the natural anti-tumour activity of effector immune cells.

Alternatively, biotherapeutics with probiotic that has a potential anti-tumour effect would provide a safe, non-invasive complementary therapy for ovarian cancer patients. It was demonstrated in chapter 3 of this thesis that the probiotic *EcN* significantly reduced tumour weight in a syngeneic mouse model for epithelial ovarian cancer metastasis. This potential anti-tumour effect was found to be

mediated by modulation of innate immune response components namely: TLR4 and IL23 in the ovarian tumour (figure 60).

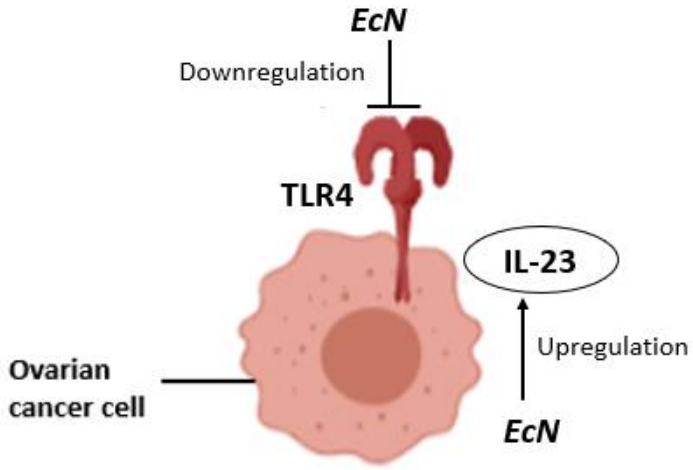

Figure 60: Postulated signalling mechanism for the anti-tumour effect of *EcN* in ovarian cancer metastasis. *EcN* through its immunomodulatory role, downregulates the expression of TLR-4 and upregulates the expression of IL-23 in the ovarian tumour which are likely to enhance its anti-tumour potential in EOC. *TLR4-Toll like receptor 4, IL-23-Interleukin 23, EcN-Escherichia coli Nissle, EOC-epithelial ovarian cancer.*

The negative impact of stress superseded the potential anti-tumour effect that was previously observed from *EcN*. Nonetheless, *EcN* is a valued probiotic with promising potential for anti-tumour effect at distant areas from the gut but its role against stress may require further investigation.

Our results may suggest that when ovarian cancer patients consuming *EcN* regularly as complementary therapy along with their other anti-cancer therapies in addition to receiving social support and stress management strategies starting immediately following diagnosis. It would give them a future hope to have a better quality of life and clinical outcomes.

5.3 Limitations of study

5.3.1 Limitations of 3D spheroid model

3D spheroids model was used to better mimic the natural biological tumour including the architecture, cell-cell and cell-extracellular environment interactions, which are essential for cellular differentiation, transformation, proliferation, expression of

genes, proteins and response to stimuli. Also, spheroids can recapitulate the variation in access to nutrients, metabolites, and oxygen between the interior and exterior of the spheroid presenting hypoxic core, quiescence cells in the middle and proliferative exterior cells similar to the natural tumour (389). Though, the lack of stromal cells including fibroblasts, endothelial cells and immune cells and their signalling effects in the tumour microenvironment limits the ability of spheroid cultures to appropriately replicate the natural biological tumour (390, 391). Additionally, the cell response to hypoxia involves characteristics gene expression profile resulting in the activation of different signalling pathways that regulate processes such metabolism, angiogenesis, invasion and metastasis (392). Compromised diffusion of nutrients and metabolite may also affect the viability of cells in the spheroids core. These effects were found to be size dependent (389).

ID8 is a late passaged C57BL/6 murine ovarian surface epithelial cell (MOSEC) which is transformed by subsequent serial passage *in vitro* (392, 393). It is a well characterised and a highly published cell line (394). ID8 was used in preference to freshly isolated primary tumours because the cells survive longer and allow for reproducible clones, which minimise the variations between experiments (389). Repeated passage of ID8 cells *in vitro* contribute to the loss of the cells morphology and growth inhibition attributing to the high invasiveness nature, consequently, ID8 cells have poor ability to form tight spheroids because they grow as multi-layered cluster of cells (389, 392). Although the methodology that was applied enabled successful formation of ID8 spheroids that are reproducible, they were irregular in shape, small and compact which impeded successful analysis of these spheroids for further experimental analysis such as RNA extractions, IHC sectioning and assessment of infiltration using pipelines. The problem with variation in access to nutrients, oxygen across the spheroids were overcome by limiting the size of the spheroids.

The presented data indicate the need for a pre-selection of tumour spheroids of homogeneous volume and shape (morphology) to minimise data variability before

the use of these spheroids in a cytotoxicity test. Further, visual analysis of confocal images is subjective thus, it is likely to introduce bias and jeopardise the data accuracy. Although a pipeline was built up in our lab to quantify confocal images of ovarian spheroids-splenocytes co-cultures, the irregular shape of the co-cultures hindered the successful application of this pipeline. Therefore, applying a quantification method to assess the spheroids-Splenocytes co-cultures for T cells infiltration into ID8 ovarian cancer spheroids would enable a robust and valid conclusion on the role of stress on T cells tumour infiltration.

5.3.2 Limitations of in vivo model

ID8 syngeneic mouse model of EOC is the most widely used mouse model in the field of EOC preclinical research (393). It has an intact immune system and in response to IP injections of ID8 cells, it develops numerous intraperitoneal tumours resemblant to those observed in EOC patients at stage III-IV (394). Therefore, it is well suited to study ant-tumour immunity and tumour microenvironment. The ID8 syngeneic mouse model has been genetically manipulated to include the most frequently mutated/deleted genes in EOC. For example, ID8 cells clones were used with deleted genes *Trp53*, *Brca1*, and *Brca2* to generate mutations. Recently, more ID8 clones (CRISPR-generated models) have been developed with multiple gene deletion or overexpression to better mimic the heterogeneity in EOC so that our understanding of disease would be enhanced, and more effective therapies would be developed (393, 395). Despite the many advantages for this model it has a limitation which is the interspecies variation in the immune system of different mouse models which requires the validation of any finding using another EOC mouse model (395). Newer genetically engineered mouse models (GEMM) of HGSC have been developed which extended our understanding of the pathobiology of this disease. However, many mice are required due to viability problems, also tumours take very long time (months) to develop which limit the use of these models (393, 395). Therefore, there is no such superior mouse model, the choice depends mainly on the research question, the use of different mouse models increases the confidence and allow for more accurate therapeutic translation (393).

Loss of valuable laboratory time and resources mainly animals were due to the unprecedent situation with COVID-19. A total of 40 C57BL/6J female mice were bought for the probiotic and stress study. Due to 12 weeks of lock down, the mice age exceeded the maximum age needed to undertake the experiment. Therefore, mice were replaced by a smaller cohort because of the limited budget that was available, consequently less power attained.

Although rodents are physiologically like human in their organs and systems that are involved in stress response, there are some variations which may have a major impact on the study outcomes such as: Light/dark cycles; the circadian rhythmic changes of the stress hormones release throughout the day massively affect the response to stress and their basal metabolic activity (mice have 30 times faster metabolic rate than human). Subsequently, much higher doses of drugs should be used to relief the effect of stress (47). This problem was overcome by housing the mice under fixed 12h light/dark cycles. Additionally, pharmacological doses of cortisol were used.

5.4 Future studies

DNA damage (likely to cause apoptosis) was found to mediate stress-induced impaired anti-tumour activity of splenic T lymphocytes and may mean that stress causes DNA damage and apoptosis in DCs. Therefore, DNA damage was assessed in splenic and LNs DCs at 2 and 4 weeks from ID8 injections as a possible underlying mechanism for its stress-mediated impaired anti-tumour activity. However, technical issues experienced (including low adherence of DCs to glass/plastic surfaces for IF analysis) resulted in loss of many cells throughout the washing steps. Also, only few cells were available for the assay because DCs were needed for other immune assay such as flow cytometry analysis. Therefore, the results were not compelling. Assessing for DNA damage in DCs with large number of DCs may result in more compelling results. Also, assessing for T cells subsets would enlighten and deepen

our understanding of the specific impaired T cells mediating the compromised anti-tumour immunity mediated by the stress.

Further, investigation of genes involved in the production of the inflammatory type 1 interferons (e.g., cGAS- STING pathway in DCs and ovarian tumour) as a possible underlying mechanism for the stress-mediated impaired activation of DC, would provide a potential target for restoring the anti-tumour immune response in EOC. This may lead to an enhanced inflammation in the cold ovarian tumour (increasing the heat) and enhancing the activation of DCs and thus help to translate our findings into clinical benefits.

Furthermore, DCs pulsed with whole ovarian tumour lysate provides a source of known and unknown source of antigens which could be used as a source of activating T cells to undertake long lasting tumour specific immunity.

Although *EcN* is shown to have a promising anti-tumour activity in ovarian cancer, all probiotics that have been used individually in cancer patients showed limited prophylactic and therapeutic efficacy (396). The use of cocktail of probiotics is likely to provide synergistic effect as opposed to single strain use and could be a promising complementary therapy in EOC metastasis whereby enhancing the efficacy of immune/chemotherapy and reducing their toxicity.

Novel contribution to knowledge

The overarching hypothesis for this study is that stress has a negative impact on the anti-tumour immunity at an early stage of OC and at metastasis mediated by two key immune cells: DCs (the professional APCs) and splenic T lymphocytes (effector T cells). Also, the probiotic *EcN* has a health promoting characters and potential for anti-tumour role which can override the negative impact of stress on the anti-tumour immunity in ovarian cancer metastasis.

Data presented in this research demonstrate evidence which help to further understand the stress-induced pathophysiology mediating immune-cancer interaction and their impact on the anti-tumour immune response to ovarian cancer and an underlying molecular pathway was postulated. Also, evidence is presented

for the immune modulatory role of the probiotic *EcN* in ovarian cancer metastasis (a cancer which is distant from the intestinal epithelial). The research focus was on the molecular mechanisms mediating the immune response to stress in OC and those that are mediating the role of *EcN* in OC metastasis to identify potentials for novel complementary therapies to restore the anti-tumour immunity in OC metastasis. Sufficient evidence was produced in this research to conclude the following novel contributions.

- Stress negatively impacts the anti-tumour immune response to ovarian tumour starting at an early stage of OC by impairing the activation and maturation of splenic DCs.
- Stress impairs activation of splenic T lymphocytes and their anti-tumour immunity against OC specific antigens at the tumour microenvironment. This was demonstrated in a novel 3D ovarian culture (spheroids) which mimic the cellular structure, tumour architecture and the oxygen/nutrient gradients that are observed in the biological tumour and impact cancer immunity.

- Double strand breaks in DNA which are likely to induce apoptosis in splenic T lymphocytes were found to mediate the compromised anti-tumour immunity observed in these cells under the effect of prolonged stress. This evidence was supported *in vitro* in T lymphocytes treated with cortisol. This data was published as an abstract at the ESMO conference 2019 (397).

- A role for the probiotic *EcN* at reducing tumour burden in OC metastasis was demonstrated in an *in vivo* mouse model for EOC metastasis and a molecular mechanism was postulated for its immune modulatory mediated anti-tumour role. This is likely to add values to this probiotic and thus could attribute to the clinical management of ovarian cancer when administered as complementary therapy in EOC metastasis.

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