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***Pan-cancer vaccines: breaking
tolerance by targeting stress-
induced post-translational
modifications in tumours.***

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the degree of Doctor of Philosophy

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Abstract

Cancer immunotherapy aims to induce potent pro-inflammatory immune responses that can lead to elimination of tumours. The challenge of this approach is to induce cancer-specific immune responses that do not cross react with healthy tissues and are not subject to the anti-inflammatory suppressive tumour environment. One approach is to use a vaccine therapy that targets cancer-associated antigens.

The objective of this study was to evaluate the immunogenicity of citrullinated and homocitrullinated epitopes in cancer patients. Here it is shown that cancer patients have a repertoire to these stress induced post-translational modification (siPTM) peptides which can be measured when their PBMCs (peripheral blood mononuclear cells) are cultured and stimulated with the modified peptides. The number of lung cancer patients with a CD4 proliferative response to citrullinated enolase 241-260 (E241) ($p=0.0021$) and vimentin 28-49 (V28) ($p=0.0230$) peptides or ovarian cancer patients with a CD4 proliferative response to citrullinated E241 ($p=0.0078$) and citrullinated binding immunoglobulin protein 189-208 (BIP) ($p=0.0286$) peptides was significantly reduced compared to healthy donors. In addition, the proliferating CD4 T-cells in cancer patients produced less of the cytokine IFN γ (Interferon- γ) compared to healthy donors ($p<0.0001$). A population of activated antigen specific cells that do not proliferate was identified in cancer patients. This population was defined as CD4+ T-cells expressing the transferrin receptor CD71 which is expressed on CD4+ T-cells following activation by an antigen. In lung cancer patients, this population (CD4+CD71+) was higher in the expression of Latency Associated Peptide (LAP, a peptide that associates with the inhibitory transforming growth factor- β (TGF- β)) than in healthy donors ($p=0.0424$) and was inversely correlated with proliferation ($p=0.0003$). In a suppression assay, CD4+T-cells expressing LAP were isolated from PBMCs of lung cancer patients and found to suppress proliferation ($p=0.0156$). These results suggest that upon stimulation with a modified antigen, cells either become proliferative Th1 (T-helper 1) cells producing IFN γ or convert into a suppressive Th3 (T-helper 3) cells expressing LAP.

In a lung tumour microarray (TMA) the expression of vimentin (a cytoskeletal protein expressed during epithelial mesenchymal transition), citrullinated vimentin, peptidyl arginine deiminase enzymes (PADI2 and PADI4 involved in the process of citrullination), CD4 T-cell infiltration, MHC-II, programmed cell death ligand-1 (PDL-1), autophagy markers and LAP was measured. Vimentin expression was significantly ($p=0.025$) associated with a bad prognosis, whereas citrullinated vimentin ($p=0.016$), PADI2 ($p=0.048$) and Ambra1 ($p=0.009$) were associated with a good prognosis. There was a positive correlation with expression of citrullinated vimentin and patients' age ($p=0.003$) but not with gender, TMN stage or tumour size. There was also a positive correlation between expression of PADI2 ($p<0.0001$) and PADI4 ($p=0.001$) enzymes, MHC-II expression ($p=0.008$), p62 ($p=0.001$) and PD-L1 expression ($p=0.012$) with citrullinated vimentin expression. There was a negative correlation between the expression of LAP on tumour cells and citrullination of vimentin ($p=0.018$) suggesting LAP maybe inhibiting the expression of citrullinated epitopes. The co-expression of LAP in tumour infiltrating lymphocytes (TILs) and citrullinated vimentin was a prognostic indicator ($p=0.018$) with patients whose tumours expressed citrullinated vimentin and had low LAP in TILs surviving 78 months while in contrast those whose tumours did not express citrullinated vimentin and had high LAP in TILs survived 33 months.

In murine experiments with C57Bl/6 mice bearing Py8119 breast cancer, immunisation with citrullinated peptides resulted in 50% improved survival ($p=0.0270$) but no synergy was seen with an anti-LAP antibody. Upon culling the mice when their tumours reached a size of $\geq 10\text{mm}$, flow cytometry was used to examine the expression of LAP and LAG3 (lymphocyte activation gene 3) on CD4⁺ T-cells isolated from the murine tumours. Th3 cells were described as CD4 highly suppressive cells expressing LAP and LAG3. In the group vaccinated with citrullinated peptides, the levels of LAP+LAG3⁺ on CD4⁺ TILs were lower compared to the controls ($p=0.0400$) suggesting that the vaccination was able to polarize responses from Th3 to Th1. Vaccination of the same model with citrullinated peptides in combination with anti-LAG3 resulted in improved survival

($p=0.0056$) and lower levels of Th3 cells identified by expression of CD4, LAP and LAG3 ($p=0.0435$). The Py8119 tumour model was identified as a model that expressed high levels of both LAP+ and LAG3+ CD4+TILs. However, in a model that expressed lower levels of LAP+ CD4 TILs, the B16F1 melanoma model, vaccination with citrullinated peptides lowered tumour volume on day 15 and 18 ($p=0.0057$ and 0.0269 respectively) but following this time point tumours exponentially outgrew the immune control and overall survival was not improved although was statistically close to significance ($p=0.0637$). In the latter model, vaccination with citrullinated peptides lowered the levels of CD4+LAP+ TILs ($p=0.0087$) suggesting that this tumour model escapes immune control through regulatory mechanisms other than Th3 cells.

These results suggest that modified antigens may be a good target for anti-tumour immunity but that some cancer patients may have developed an antigen specific LAP+ Th3 regulatory population which would either need to be polarized to Th1 with vaccination or inhibited with an antibody targeting either TGF- β /LAP or LAG3. The ModiFy trial, is the first in human trial which will reveal whether vaccination with citrullinated peptides alone or in combination with a checkpoint inhibitor such as agents targeting Programmed cell death receptor-1 (PD-1) can control tumour growth and polarize responses to Th1.

1 Chapter 1: Introduction

Cancer immunotherapy aims to induce an immune response that can clear the tumour. This field has evolved from the theory of immune editing to the highly successful checkpoint inhibitor antibodies that are currently approved for clinical use in a number of cancer settings. The challenge for all cancer immunotherapies is to induce an immune response that is tumour specific, that does not recognise healthy tissues, is not removed by thymic deletion and is not suppressed by anti-inflammatory tumour microenvironment. The advantage of immunotherapy is that it can induce responses that are long lasting, patient specific and that can control metastasis as well as primary tumour. However, cancer immunotherapies still require refinement in order to provide the best holistic outcomes for patients, this may include administering combination therapies to improve the efficacy of individual treatments.

1.1 Immune editing

The description of tumour growth repression by the immune system was first envisaged in the early 1900s. In the third quarter of the 20th century, the theory that cancer cells possess specific antigens which could provoke immune elimination started to develop and the term immunosurveillance emerged [1-3]. In more recent years, descriptions of tumours with reduced immunogenicity which can escape destruction by the immune system started to surface. This led to the hypothesis of immune editing to encompass the spectrum of interaction between the tumour and the host immune system [4, 5]. Three phases of immune editing were described; the elimination or surveillance phase where the immune system can remove the tumour, the equilibrium phase where incomplete elimination of the tumour occurs and the escape phase where the tumour wins the battle against the immune system and grows [6]. Figure 1-1 taken from Dunn et al 2004 [6] beautifully illustrates the concept with its' phases.

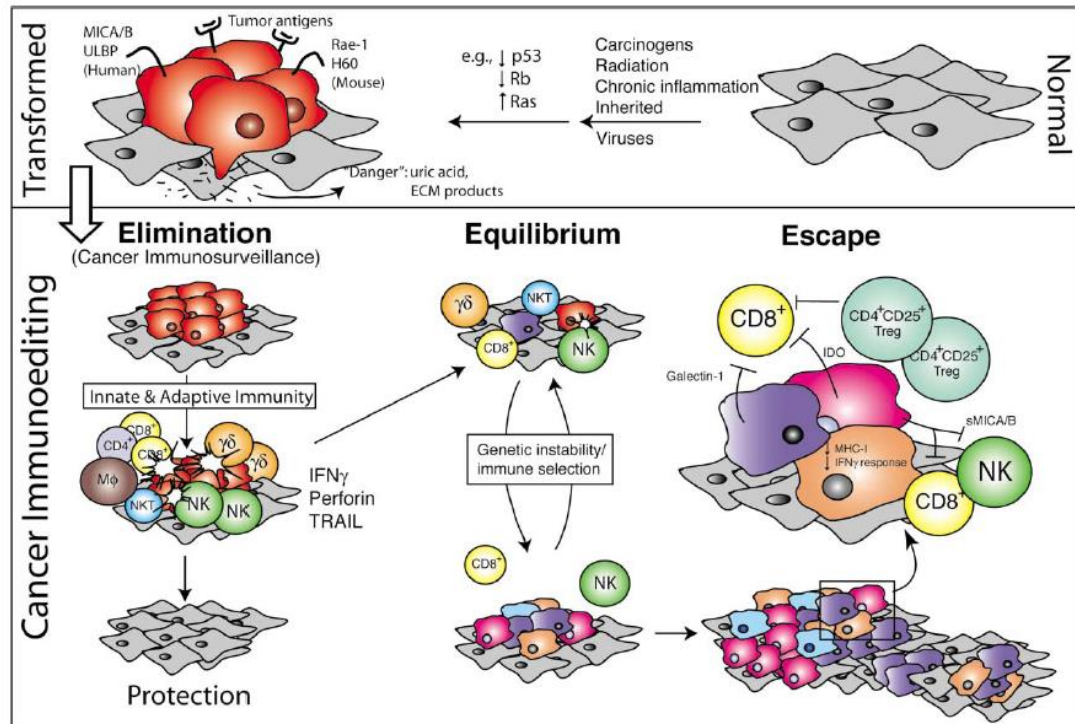


Figure 1-1: The three phases of cancer immunoediting process; elimination, equilibrium and escape [6].

In the context of the elimination phase, the immune system can recognise tumour antigens [7, 8]. Immune cells infiltrating the tumour microenvironment, both innate [9-11] and adaptive [12-14], are involved in the immune responses and surveillance against cancer [8]. Innate immune responses are mediated by cells such as natural killer (NK) cells, neutrophils, mast cells, macrophages, monocytes, and dendritic cells either by independent direct killing or through activating cells of the adaptive immune system [10, 11]. Adaptive immune responses are implemented through the actions of B-lymphocytes (humoral responses) or cell mediated killing by T-lymphocytes [12-14]. Clonal tumour specific T-cell expansion in patients with regressing melanomas provided strong evidence of T-cell mediated antitumour immunity [15]. Furthermore, production of IFN γ and perforin were described to be two key effectors of cytokine mediated anti-tumour immunity [5, 16]. The relevance of IFN γ in immune surveillance and anti-tumour immunity was first illustrated when IFN γ insensitive fibrosarcoma cell lines exhibited heightened tumorigenicity in Balb/c mice compared to control tumour cells [17]. IFN γ is a central coordinator of immune responses and is produced by CD4 $^{+}$ T helper-1 (Th1) cells, CD8 $^{+}$ T-cells [18-21], B-cells [22], NK cells [23, 24] and antigen presenting cells (APCs) [25]. It stimulates proliferation of cytotoxic T-cells [26, 27] and upregulates expression of major histocompatibility complex (MHC) class II on APCs resulting in antigen presentation and activation of cytotoxic CD4 T-cells [20, 28, 29] therefore initiating fast recognition of antigenic material.

Effective immune responses can eradicate cancer cells and lead to tumour rejection, however, cancer cells can adopt resistance mechanisms to abrogate the immune responses and escape surveillance leading to tumour progression [8]. Such mechanisms of resistance and immune evasion include recruitment of immunosuppressive cell types, increased activity of inhibitory pathways and defective tumour antigen presentation [30-32]. Tumours can employ suppressive CD4 $^{+}$ CD25 $^{+}$ T-regulatory cells (T-regs) to inhibit both naturally occurring or even therapeutically induced immune responses [33, 34]. Indeed, the degree of T-reg infiltration in tumours correlates with clinical outcome [35, 36] and their depletion has been reported to result in tumour

rejection [33, 34]. Another example of suppressive cell recruitment by tumour was reported by Terabe et al [37], in this case IL13 producing CD4⁺ NK T-cells where inhibition of IL13 or loss of NKT cells in mice resulted in resistance of tumour recurrence. Further to this, tumours can produce a variety of suppressive cytokines such as IL10 and TGF- β to dampen and negatively influence immune cell activation and function [38]. Moreover, it is known that the expression of inhibitory receptors (IRs) on T-cells play an essential role in controlling excessive T-cell activation and protection against autoimmunity. Tumours hijack these IRs to impede anti-cancer immune killing by creating an immunosuppressive tumour microenvironment. Functionally, they reduce T-cell proliferation and production of cytokines [39, 40]. Examples of inhibitory pathways seized in favour of tumours are illustrated in Figure 1-2 [41] and include Programmed cell death receptor-1 (PD-1) on T-cells, programmed cell death ligand-1 (PDL-1) expressed abnormally on tumour cells, cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) on T-cells, lymphocyte activation gene 3 (LAG3) on T-cells and T-cell immunoglobulin and mucin- containing protein 3 (TIM3) on T-cells [41]. Moreover, tumours have been classified according to their immune cell infiltration into being immunologically “cold” with constrained immune cell infiltration, “hot” with high quantity of immune cell infiltration, “excluded” where immune cells exist only in the tumour border and “desert” which are tumours completely devoid of immune cells [42, 43]. The level of immune cell infiltration has been positively associated with prognosis and can predict responses to immunomodulating therapies [44, 45].

Underscoring the immune system’s natural ability to eradicate tumour cells has paved the way for the development of immunological therapies to enhance and support immune cells in their battle against cancer.

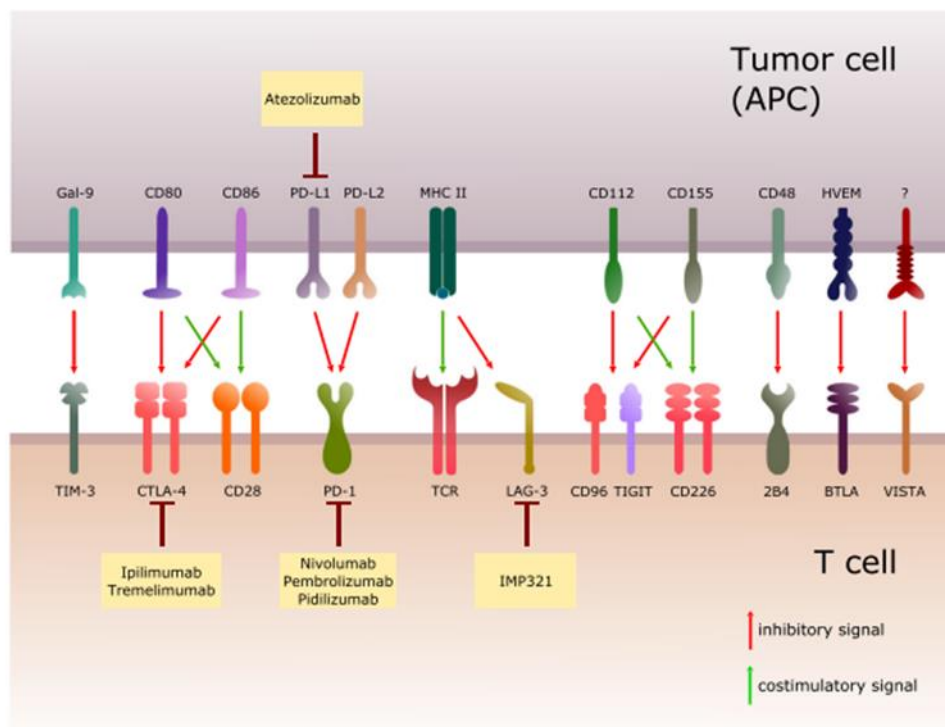


Figure 1-2: Illustration of expression of inhibitory receptors (IRs) and costimulatory receptors on T-cells with their ligands on tumour cells or APCs taken from Catakovic et al [41]. Additionally, antibodies blocking the IRs or their ligands are portrayed aiming to reverse exhaustion of T-cells.

1.2 Checkpoint inhibitors

Monoclonal antibodies (mAbs) targeting T-cell IRs and their ligands on tumour cells have seen the most progression in the world of immunomodulatory drugs against cancer. Releasing the breaks from these inhibitory pathways, the so called “off switches” has pioneered the development of checkpoint inhibitors. Checkpoint blockade agents such as those targeting PD-1/PDL-1 pathway and CTLA-4/CD80/86 pathway block these inhibitory mechanisms either by blocking receptors on T-cells or their corresponding ligands on tumour cells in order to re-invigorate anti-tumour immune responses. Ipilimumab, a fully human IgG1 mAb that blocks CTLA-4 a coinhibitory receptor expressed on T-cells was able to activate T-cells, induce durable responses and so was the first to gain access into clinic [46, 47]. PD-1 is a negative regulator expressed on T-cells with its ligand PDL-1 expressed on normal tissue to induce immune tolerance and abnormally on tumour cells to evade immune surveillance [48-50]. PDL-1 on tumour tissue is expressed as a mechanism of “adaptive resistance” which can be induced specifically in response to IFN γ production by the immune system [51]. Pembrolizumab and Nivolumab (PD-1 inhibitors) were amongst the first to gain approval and have shown tumour regressions, improvements in long-term clinical responses specifically in solid tumours in the face of somewhat manageable toxicity profile [51-53]. Shortly following the approval of PD-1 inhibitors, a range of drugs targeting PDL-1 were approved including atezolizumab, avelumab and durvalumab. A list of all approved checkpoint inhibitors is shown in Table 1-1. These checkpoint blocking agents have made a breakthrough in the treatment of different cancer types [54, 55] and are now broadly used; however, the objective tumour responses are limited to a cohort of patients [47, 56] and some tumour types remain challenging. Immunologically “cold” tumours believed to have little tumour infiltrating immune cells are thought to be resistant to checkpoint inhibitors [57, 58] and therefore their effectiveness is based on unleashing pre-existing immune recognition [57]. Additionally, their use is limited by acquired resistance and undesirable side effects. For this reason, attention has turned to the next generation of targets; LAG3, TIGIT and TIM3 [59-61] as either

monotherapies or in combination with already established immunotherapeutics, chemotherapy, radiation therapy or cancer vaccines.

Table 1-1: List of approved checkpoint monoclonal antibodies (last updated 16/11/2022)

Monoclonal antibody drug	Target	Food and Drug administration (FDA) indications	National Institute for Health and Care Excellence (NICE) indications
Ipilimumab	CTLA-4	Metastatic Melanoma Renal Cell Carcinoma Microsatellite instability high (MSI-H) or mismatch repair deficient (dMMR) Colorectal Cancer Hepatocellular Carcinoma (HCC) Non-Small Cell Lung Cancer (NSCLC) Malignant Pleural Mesothelioma	Melanoma Renal cell carcinoma NSCLC Malignant pleural mesothelioma Colorectal cancer
Tremelimumab	CTLA-4	HCC NSCLC	-
Pembrolizumab	PD-1	Metastatic melanoma NSCLC Head and neck squamous cell carcinoma Classical Hodgkin lymphoma Primary mediastinal large B-cell lymphoma Urothelial carcinoma MSI-H or dMMR colorectal cancer Gastric cancer Oesophageal cancer Cervical cancer	Melanoma NSCLC Urothelial carcinoma Classical Hodgkin lymphoma Head and neck squamous cell carcinoma Renal cell carcinoma Colorectal cancer Oesophageal carcinoma Breast cancer Endometrial carcinoma Cervical cancer

		HCC Merkel cell carcinoma Renal cell carcinoma Endometrial carcinoma Tumour mutational burden-high (TMB-H) cancer Cutaneous squamous cell carcinoma Triple-negative breast cancer	Gastric cancer Small intestine cancer Biliary cancer
Nivolumab	PD-1	Metastatic Melanoma NSCLC Renal Cell Carcinoma Hodgkin's Lymphoma Squamous cell Head and Neck Cancer Urothelial Carcinoma MSI-H or dMMR Colorectal Cancer HCC Oesophageal Carcinoma Malignant Pleural Mesothelioma Gastric Cancer	Melanoma Renal cell carcinoma NSCLC Malignant pleural mesothelioma Urothelial carcinoma Squamous cell cancer of the head and neck Classical Hodgkin lymphoma Gastrointestinal cancer
Cemiplimab	PD-1	Metastatic or locally advanced cutaneous squamous cell carcinoma Basal cell carcinoma NSCLC	Cutaneous squamous cell carcinoma
Dostarlimab	PD-1	dMMR endometrial cancer dMMR solid tumours	Endometrial cancer
Atezolizumab	PDL-1	Metastatic melanoma NSCLC Small cell lung cancer Urothelial cancer HCC	Urothelial carcinoma NSCLC Small cell lung cancer Breast cancer HCC

		Triple negative breast cancer	
Avelumab	PDL-1	Merkel cell carcinoma Renal cell cancer Urothelial cancer	Merkel cell carcinoma Renal cell carcinoma Urothelial carcinoma
Durvalumab	PDL-1	NSCLC Small cell lung cancer Biliary tract cancer Urothelial cancer	NSCLC
Relatlimab	LAG-3	Metastatic melanoma	-

1.3 Other immunomodulatory therapies

Historically, the first cases of harnessing the immune system to eradicate tumour were described in the context of using viruses to elicit immune responses against virally induced tumours. With advances in science, oncolytic viral therapies for established tumours were genetically engineered and one such therapy is T-Vec (talimogene laherparepvec). T-Vec is a genetically modified herpes simplex virus which is engineered to selectively replicate in tumour cells but not normal tissue leading to cancer cell lysis and production of granulocyte macrophage colony-stimulating factor (GM-CSF) which in turn induces influx of dendritic cells and activation of tumour specific effector T-cells [62]. T-Vec has shown clinical response rates in patients with melanoma and was the first viral therapy to be approved for clinical use [62, 63].

Another therapy that utilizes the immune system to work against cancer cells is cytokine therapy. Cytokines are messengers that mediate communication between immune cells, they are produced in response to an infective or tumour antigen and result in stimulation or enhancement of cytotoxic effector cells [64]. The two approved cytokine therapies are IL2 in renal cancer and melanoma [65-67] and IFN α in melanoma and haematological malignancies [68, 69]. However, their use as monotherapies is severely limited by the associated degree of toxicities seen with the required high doses. Cytokine therapy has been used in combination with Adoptive cell transfer therapy (ACT) to facilitate an optimal growth and functional environment for the transferred anti-tumour T-cells [64, 70]. ACT is the infusion of autologous T-cells isolated and expanded *ex-vivo* to respond to tumour antigens. Tumour infiltrating lymphocytes (TILs) were later used and expanded using IL2 to enhance this therapeutic approach which has given durable clinical responses [71-73]. Further development, led to the genetic engineering of 2 types of ACTs; T-cell receptor (TCR) engineered T-cells and Chimeric antigen receptor T-cells (CAR). The former allows for recognition of tumour specific antigen sequence, but toxicity can be limiting related to excessive cytokine release and mimicry of tumour antigens to self-antigens expressed on normal tissue [74, 75]. ACT with T-cells transduced

with TCR against NY-ESO-1 cancer/testis antigen has seen success in synovial sarcoma and melanoma with clinical response and limited toxicity [76]. CAR T-cells on the other hand includes an antibody fragment aimed against an antigen which is highly expressed on tumour cells. CAR T-cells have seen more success in haematological malignancies [77, 78] but less so in solid tumours [79] and have had similar struggles balancing toxicity and clinical efficacy [80].

1.4 Cancer vaccines

Prophylactic vaccines against virally induced cancers such as Human Papilloma virus (HPV) in cervical cancer and Hepatitis B virus in hepatocellular carcinoma have been very effective in preventing the development of these cancers [81, 82]. In contrast, therapeutic cancer vaccines need to elicit immune responses against established cancer to induce regression and reclaim control over tumour growth. As a general concept, cancer vaccine therapy involves the administration of a tumour associated antigen (TAA) to activate APCs and induce a T-cell response or the administration of APCs themselves (Dendritic cells (DCs)) loaded with TAA. TAAs can be divided into categories of self-antigens and mutated neoantigens. Self-antigens include cancer testis antigens, differentiation antigens and overexpressed self-antigens. Cancer testis antigens were a very attractive target due to their expression on germ cells and tumour cells only and examples of these antigens which have been targeted by vaccination are BAGE and NY-ESO1 [83-85]. Vaccination of head and neck cancer patients with a combination of three cancer testis antigens resulted in TAA-specific antitumour immunity and improvement of overall survival [86, 87]. On the other hand, differentiation antigens such as Melan A, gp100, tyrosinase and prostate specific antigen (PSA) [85] are expressed on normal cells and tumour cells from the same tissue type and so have the potential of causing high toxicity. Overexpressed self-antigens are found in low levels in normal tissue but as the name implies, they have higher expression on tumour cells. Examples of those are HER2, p53 and Survivin [85]. Tumour neoantigens (neoepitopes) arise as a consequence of mutational genetic alterations in the tumour, these have avoided thymic regulation and are

recognised by the immune system as non-self [43, 88]. Tumours with high mutation rate are usually those associated with an environmental carcinogen as an initiator such as lung, melanoma, and bladder cancers [89, 90]. Not all neoepitopes are immunogenic but the higher the tumour mutational burden the more likely a neoepitope can elicit an immune response [43]. Examples of neoepitopes are those arising from mutational frameshifts [91] or post-translational modifications such as citrullination [92], homocitrullination [93] phosphorylation [94] and glycosylation [95] (Figure 1-3 [96]).

Three major categories of cancer vaccine therapy have evolved: nucleic acid vaccines (DNA and RNA vaccines), protein/peptide vaccines and cell vaccines (tumour or dendritic cell vaccines). Nucleic acid vaccines deliver a concentrated source of TAA but require the additional steps of transcription and translation in case of DNA vaccines or just translation in case of RNA vaccines before presentation on APCs and induction of T-cell responses [97, 98]. DNA/RNA vaccines have the advantage of being comparatively low in cost, easy to design and stable at room temperature but their disadvantage is their poor cellular uptake and so the delivery method is key to their ability to present antigen [99, 100]. VGX3100, a DNA vaccine which targets HPV-16 and 18 E6 and E7 proteins delivered to cervical cancer patients via electroporation has displayed promise in a phase 2b placebo control trial [101]. An RNA based individualised poly neoepitope vaccine injected into the nodes of melanoma patients has shown vaccine specific anti-tumour immunity and reduced the number of metastatic episodes with sustained progression free survival [102]. Another RNA vaccine showing promise is a liposomal RNA vaccine FixVac (BNT111) targeting melanoma self-antigens (NY-ESO-1, MAGE-A3, tyrosinase and transmembrane phosphatase with tensin homology (TPTE)) given intravenously has shown clinical responses and T-cell responses specific to the antigens in melanoma patients who have previously received anti-PD1 therapy [103]. Viral vectors such as poxvirus, adenovirus and Herpes simplex virus-1 (HSV-1) have been used in engineering nucleic acid vaccines to carry and encode for TAA [104-106]. PROSTVAC for example is a cancer vaccine that consists of two poxvirus vectors containing transgenes encoding for self-antigen Prostate specific

antigen (PSA) in combination with costimulatory molecules. Preclinical data for PROSTVAC was promising, however, phase II trials showed limited responses [104, 107, 108] but it is now being considered in combination with checkpoint inhibitors (NCT02933255, NCT03532217). One limitation of using viral vectors is that immune responses may be diverted away from the TAA and induced against the virus [88].

Vaccination with peptides/proteins expressed by cancer cells has also been heavily explored and involve taking up of the peptide/protein by APCs, presentation on MHC molecules to induce T-cell immunity. Initially, short peptides (SPs, <15 amino acids) were used, and these consisted of peptides encoding CD8 epitopes and restricted to MHC class I which meant they can bind to any nucleated cell expressing MHC class I [85, 88, 109]. Nucleated cells unlike professional APCs (DCs) lack costimulatory molecules needed for proper T-cell activation [85]. In this situation, SPs induced anergy and led to accumulation of T-cells at the vaccination site and T-cell death because of lack of costimulatory signals [110, 111]. Synthetic long peptides (SLPs, >20 amino acids) on the other hand are only processed and presented by professional APCs on MHC class I and II therefore capable of activating both CD4 and CD8 cells without causing anergy [111-114]. Vaccination with SLPs is starting to show promise in clinical studies [115, 116], for example melanoma patients treated with long NY-ESO-1 peptide gave CD4 and CD8 T-cell responses in a phase I trial [115]. Further, conjugating SLPs with the appropriate adjuvants leads to enhanced pro-inflammatory responses [117]. Improved efficacy of SLPs has been seen when linked to Toll-like receptor (TLR) ligands which enhance immune responses through providing costimulatory signals and inducing DC maturation [117-119].

The third category of therapeutic cancer vaccines are cell vaccines either in the form of tumour cell or DC vaccines. Tumour cells have been isolated from patients' own tumours (autologous) or non-patient specific (allogeneic) and irradiated *ex-vivo* then administered to induce T-cell responses to any of the antigens expressed by the tumour cells. Cells are mostly genetically modified to produce cytokines [120, 121] or express costimulatory molecules [122]. A drawback of using tumour cells as a vaccine

is obtaining enough cells for therapy [123-125]. GVAX is a tumour cell vaccine modified to produce granulocyte-macrophage colony-stimulating factor (GM-CSF) that has been trialled in NSCLC [126] and prostate cancer [127] but did not seem elicit clinical tumour responses and so combination with checkpoint blockade agents is being considered [128]. Further to tumour cells, the potential of DCs as cell vaccine therapy to induce anti-tumour immunity has been recognised and immensely studied [129, 130]. They represent the complexity of cancer vaccines on how they can be loaded with various TAA in the form of tumour derived peptides or DNA/RNA to present to and induce T-cell responses. A prime representative model of DC based vaccine pulsed with a prostate tumour specific antigen is Sipuleucel-T (Provenge). IMPACT was the pivotal phase III trial for Sipuleucel-T that showed 4.1 months improvement in overall survival for patients with advanced prostate cancer, but no difference was observed in progression free survival or time to progression [131].

Many aspects need to be carefully considered for the success and for an optimal cancer vaccine design including the choice of antigen target and how to overcome the immunosuppressive tumour microenvironment. One must bear in mind that not all TAA are immunogenic and capable of inducing a durable immune response, therefore identification of immunogenic epitopes is key to success of using them as therapy. This largely depends on the epitope avoiding thymic regulation and inducing high affinity T-cell responses. High affinity T-cells to self-antigens are normally deleted during thymic regulation, while low affinity ones are not positively selected leaving moderate affinity T-cells which require high levels of the antigen for recognition and activation [88]. Tumours rarely express high levels of self-antigens which could be a reason for failure of self-antigen based cancer vaccines to induce impressive tumour regression [88]. As for neoepitopes, most are specific to each tumour and personalised vaccines would therefore be both expensive and time consuming. Further to this, variability within the same tumour can exist and escape of tumour cells that do not express the mutation can occur [132]. Post-translational modifications in cancer proteins are an attractive target for cancer vaccines as they form epitopes that have

averted thymic deletion and are generic to a range of tumours [43, 88]. Combination of cancer vaccines with checkpoint blockade agents is one approach that is being considered to improve efficacy and response rates to cancer vaccines by overcoming tumour immunosuppression and lifting T-cell inhibition [85].

1.5 Citrullination and homocitrullination (stress induced post-translational modifications siPTMs)

Autophagy is a process of degradation and recycling which is constitutively active in APCs but in other cells it is induced by stress [133, 134]. In the tumour microenvironment, stressful conditions such as nutrient starvation, hypoxia and DNA damage induce autophagy to remove unwanted cell organelles and digest proteins to preserve and provide additional energy [133, 134]. Autophagy is upregulated in rapidly proliferating tumour cells to promote cell survival [133, 135]. The process of autophagy enables post translational modification of proteins giving rise to modified self-epitopes in tumour cells [134] which are presented on MHC class II in APCs and epithelial cells [136]. Citrullination is one common stressed induced post-translational modification (siPTM) which involves the conversion of positively charged arginine to uncharged citrulline and is dependent on autophagy [134] (Figure 1-3 [96], Figure 1-4 [92]). The reaction is catalysed by a family of peptidyl arginine deiminase (PADI) enzymes activated by high levels of calcium ions [134, 136]. In viable cells high levels of calcium can only occur in double membrane organelles such as nuclei and autophagosomes. PADI2 and PADI4 are the most commonly expressed enzymes. PADI4 is mainly expressed in the nuclei and citrullinates regulatory proteins to allow gene regulation [137]. In contrast, PADI2 is mainly expressed in the cytoplasm and is activated within autophagosomes to aid protein digestion, release of energy and presentation to CD4 T-cells on MHC class II [134]. Ireland et al [134], eloquently show that the presentation of citrullinated epitopes on APCs to T-cells is dependent on both autophagy and PADI enzymes. Citrullination have been implicated in the pathogenesis of autoimmune diseases including rheumatoid arthritis (RA), type I diabetes, multiple sclerosis, sarcoidosis, psoriasis, and celiac disease [138-142]. Indeed, antibodies to citrullinated

proteins are used as part of the diagnostic process of RA. Furthermore, citrullination induced by autophagy has been reported in the joint synovium of RA patients [143]. However, it has been shown that CD4 T-cell responses generated against citrullinated epitopes by means of vaccination induce tumour rejection without causing autoimmune toxicities [144-146]. Peptide vaccination does not stimulate antibody responses which is essential for the pathogenesis of autoimmune diseases [92]. This is suggestive that T-cells alone cannot induce autoimmunity or RA in the absence of joint/tissue damage and antibody responses to provoke pathogenic consequences but can aid in inducing antibody responses. Nonetheless, it is probably wise to exclude patients with autoimmune diseases from trials of vaccination with citrullinated peptides to avoid untoward exacerbation and to monitor patients carefully for any autoimmunity.

In cancer, citrullination is involved in rapid cell turnover, and it interferes with intermediate filament assembly [147-149]. Vimentin, an intermediate filament cytoskeletal protein found in mesenchymal cells is of particular interest because it is commonly citrullinated [145, 150, 151]. During epithelial-mesenchymal transition (EMT); vimentin is essential for the acquisition of fibroblast-like characteristics [152]. EMT has been a recognised component of tumour progression and invasiveness in carcinomas [153]. Increased expression of vimentin has been reported in different tumour cell lines including prostate cancer, breast, gastro-intestinal, hepatic, melanoma and lung cancer [154]. Immunisation of HLA-DR4 transgenic mice with two citrullinated vimentin epitopes resulted in a survival benefit and 80% survival was seen even when immunisation was delayed to day 10 after tumour implantation [145]. This study demonstrated that the anti-tumour responses were mediated by citrullinated peptide specific cytotoxic CD4 T-cells, IFN γ dependent and CD8 independent. Cytotoxicity of citrullinated epitope specific CD4 T-cells was proved by Granzyme B production. Furthermore, they showed that inhibition of autophagy and PAD1 reduced T-cell recognition of the citrullinated epitopes suggesting dependency of citrullination on autophagy and PAD1 enzymes.

Another protein degraded during autophagy and often citrullinated is α -enolase (ENO-1) [155, 156]. ENO-1 is a plasminogen receptor and catalytic enzyme involved during glucose metabolism (glycolysis) [157]. During tumour cell growth, increased glycolysis to generate energy is key and so it is not surprising that the expression of ENO-1 has been reported in a range of tumours where it plays a vital role in invasion and metastasis [158-161]. Furthermore, antibodies against ENO-1 have been correlated with better survival [159]. Vaccination with citrullinated ENO-1 epitopes induced citrullination specific IFN γ Th1 (T-helper 1) responses and tumour rejection in HLA transgenic mice implanted with B16 melanoma, LLC/2 lung cancer and Pan02 tumour cell lines [146]. The later study also showed tumour rejection in C57/Bl6 mice implanted with parenteral melanoma B16F1 suggesting that the citrullination specific responses are not restricted to HLA-DR4 allele. Moreover, they demonstrated a CD4 proliferative repertoire to citrullinated ENO-1 in healthy donors assessed by thymidine proliferation assay and showed that responders were not all HLA-DR4 positive. Further to this, Brentville et al [144], showed that immunisation of mice with the combination of two citrullinated vimentin epitopes and a citrullinated ENO-1 epitope referred to as Modi-1 induced delay in tumour growth which was mediated by cytotoxic CD4 T-cells and when surviving mice were rechallenged with the same tumour, 71% survived without the need for another immunisation dose. They proved that CD8 T-cell depletion had no impact on the tumour rejection and that the immune responses were dependent on tumour expression of MHC class II and mediated by CD4 cells. Moreover, the post immunisation anti-tumour responses were present on HLA DR4 and DP4 mice implying no HLA restriction. These studies suggest that citrullinated vimentin and ENO-1 proteins are an excellent target for a cancer vaccine inducing high frequency cytotoxic CD4 T cells and that responses are not HLA restricted meaning they can be given to a broader population [144-146, 162, 163]. Modi-1 vaccine has now been initiated in a phase I/II human ModiFy trial.

Other peptide targets for citrullination include nucleophosmin (NPM), binding immunoglobulin protein (BIP) and cytokeratin-8 (Cyto8). NPM is a nuclear protein which is essential for cellular metabolism and plays a role in

cellular growth and proliferation [164, 165]. It is another protein reported to be overexpressed in a variety of human tumours promoting tumorigenesis [166-172]. Hyperexpression of has been associated with disease recurrence and progression in bladder cancer [173] and associated with clinicopathological findings in hepatocellular carcinoma [166]. It is also citrullinated by PADI enzymes, presented on MHC class II and reported to be a good target for inducing CD4 anti-tumour responses [165]. BIP (also known as glucose regulated protein 78 (GRP78)) is expressed in the endoplasmic reticulum (ER) where it functions as a master regulator and a chaperone protein during ER stress [174]. During rapid cell turnover and proliferation, an increased need for protein synthesis is required which results in increased ER activity and stress. BIP plays an essential role in degradation of misfolded proteins and is also required during stress induced autophagy [175, 176]. ER stress and hyperexpression of BIP have been linked with tumour progression, metastasis, and resistance to therapy [177-179]. Furthermore, T-cell responses against citrullinated BIP have been detected in autoimmune diseases such as rheumatoid arthritis and diabetes [141, 180, 181]. Cyto8 is an intermediate filament protein essential for the cytoskeleton of epithelial cells [182]. It has been identified as a self-antigen in several autoimmune disease [183-185], coronary artery disease [182] and implicated in the escape mechanisms of cancer cells [186]. Cyto8 expression was also reported to be a poor prognostic indicator in lung and hepatic cancer [187, 188]. This makes citrullinated BIP and Cyto8 other potential targets for a cancer vaccine to generate anti-tumour immune responses.

Another siPTM is homocitrullination (Figure 1-3 [96], Figure 1-5 [189]); the result of carbamylation which happens when cyanate or its active form isocyanic acid interacts with the amine group of lysine residues and leads to a change in charge providing self-altered antigens for the recognition by T-cells [190-193]. Under inflammatory conditions the reaction is mediated by myeloperoxidase (MPO) produced by immune cells [194] which in the presence of hydrogen peroxide (H_2O_2) from cancer cells [195] converts thiocyanate to cyanate and isocyanic acid [196, 197]. H_2O_2 is described to be high in malignant tumour cells and is associated with antioxidant imbalances

that occur during oncogenic transformation [195]. When isocyanic acid interacts with the amine group of lysine residues, carbamylation happens producing homocitrulline (hCit) which has antigenic properties that are recognised by T-cells [192, 193]. Similar to citrullinated peptides, hCit peptides can be processed during autophagy and in the presence of IFN γ they are presented on upregulated MHC class II [198]. Non-APC cells such as tumour cells do not express MHC class II unless induced by inflammation and IFN γ production [199]. Cook et al [198], demonstrated that vaccination with hCit peptides gave a 50-90% survival in mice implanted with B16 melanoma model which constitutively express MHC class II but no benefit was seen in mice bearing MHC class II negative tumours suggesting the hCit peptides are presented by MHC class II. They then confirmed this by engineering a B16 melanoma model to express MHC class II under the influence of IFN γ and showed that mice regained anti-tumour benefit from vaccination with hCit peptides and that tumour regression correlated with MHC class II expression. The responses were mediated by CD4 Th1 cells since depletion with anti-CD4 antibody abrogated the effects of the vaccine and responses did not generate IL17 or IL10 but were IFN γ induced. They also showed that upon inhibition of MPO the benefits of hCit vaccination were revoked confirming that MPO mediated homocitrullination. This suggests that homocitrullination can also be a good target for cancer vaccine.

In summary, modified proteins (citrullinated and homocitrullinated peptides) are degraded and presented by MHC-II for CD4 recognition. In murine tumour models, immunisation with citrullinated or homocitrullinated epitopes resulted in strong anti-tumour immunity and long-term survival mediated by cytotoxic CD4 Th1 cells with no reported toxicities or autoimmunity [93, 145, 146, 162, 163, 165, 190-193]. The hypothesis of the study was that tumours expressing siPTMs would attenuate the T-cell response to these antigens. Production of IFN γ from the cytotoxic CD4 T-cells responding to siPTMs will create a pro-inflammatory environment which tumours lack and result in up-regulation of MHC class II on tumour cells, facilitating recognition and direct cancer cell killing without the need for CD8 cells [145].

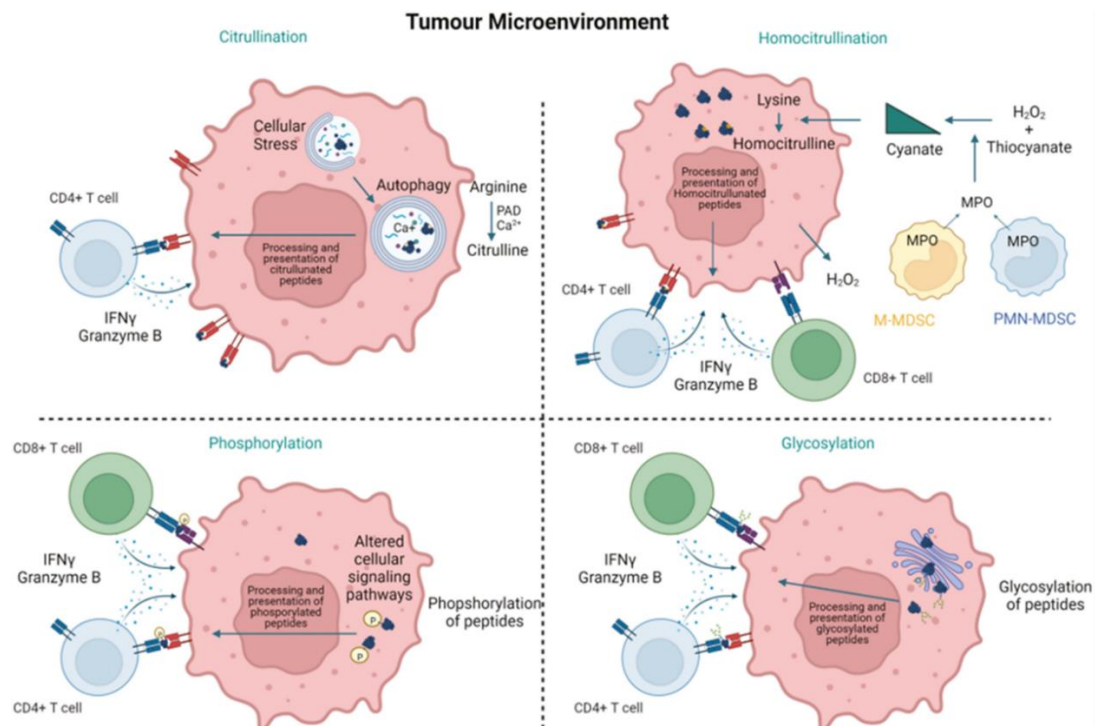


Figure 1-3: post-translational modifications in cancer proteins pose an attractive target for cancer vaccines by generating neoepitopes. This illustration extracted from Shah et al [96] depicts the processes that generate post-translational modifications in tumours; citrullination, homocitrullination, phosphorylation and glycosylation.

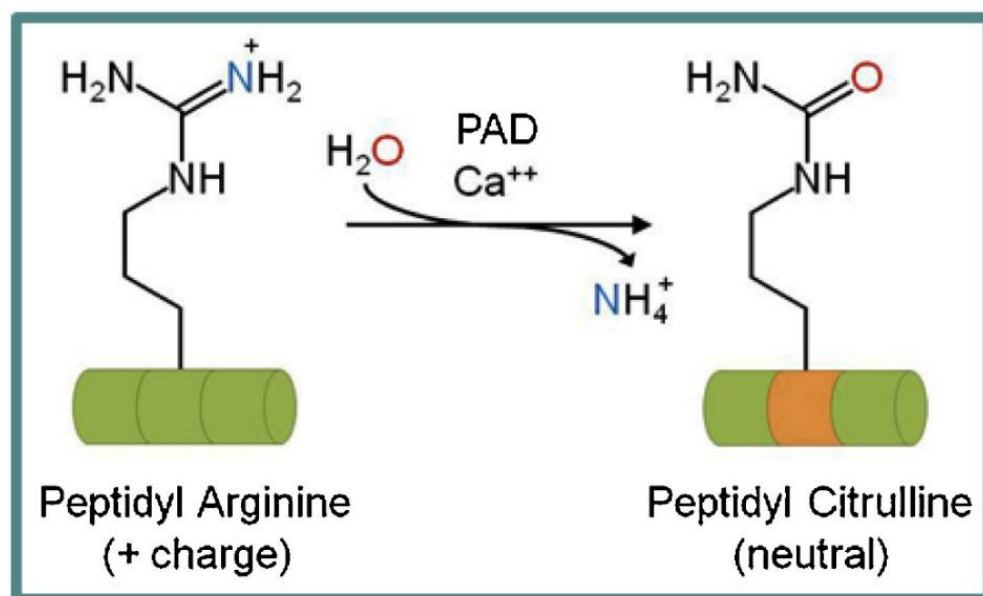
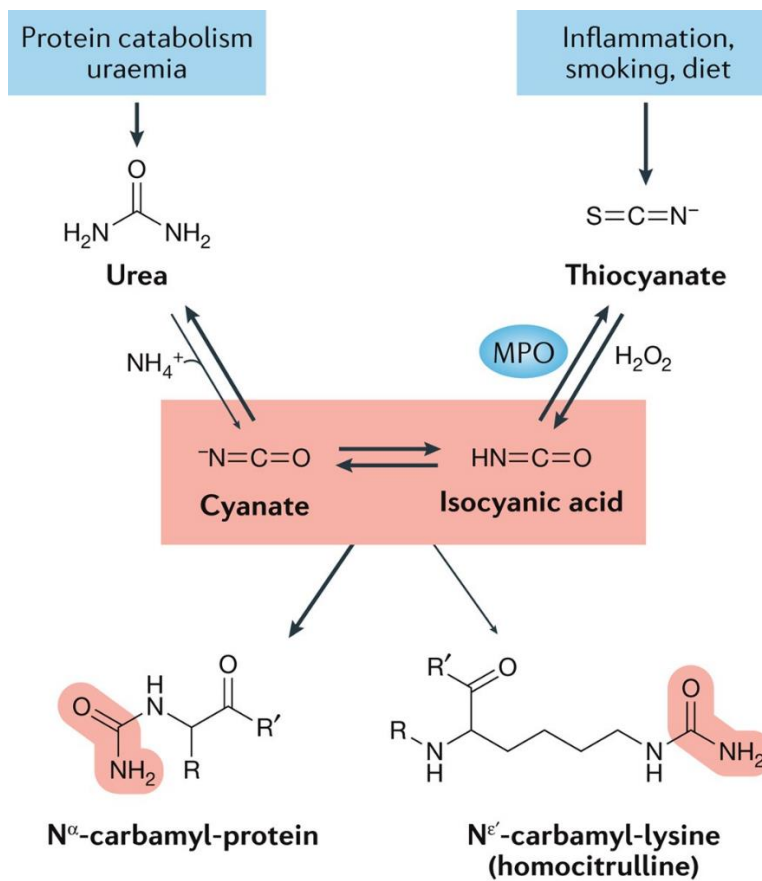


Figure 1-4: Schematic illustration taken from Brentville et al [92] of the post-translation modification process where the positively charged arginine is converted to neutral citrulline through an enzymatic reaction catalysed by PADI (peptidylarginine deiminase) enzymes which are activated by of high levels of calcium ions (Ca^{++}). A process known as citrullination which is enabled by autophagy.



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Figure 1-5: Schematic representation of homocitrullination taken from Delanghe et al [189] where Myeloperoxidase (MPO), produced by immune cells during inflammation, in the presence of hydrogen peroxide (H_2O_2), from cancer cells, catalyses the conversion of thiocyanate to cyanate and isocyanic acid. Isocyanic acid interacts with the amine group of lysine residues leading to carbamylation and producing homocitrulline.

1.6 CD4 T-cell differentiation

CD4 T-cells represent part of the adaptive immune system and function to modulate both adaptive and innate immune cells through the release of various cytokines. Naïve CD4 T-cells are activated following antigenic encounter through the interaction between T-cell receptor (TCR) and antigens complexed with MHC class II and presented on APCs [200, 201]. This interaction is augmented by costimulatory signals which together initiate downstream signalling pathways that eventually result in proliferation and differentiation into effector CD4 T-cells [200, 201]. The main costimulatory receptor expressed on naïve T-cells is CD28 and its' ligands are CD80 (B7-1) and CD86 (B7-2) which are expressed on dendritic cells [200]. Other costimulatory receptors/ligands include CD28 homolog inducible co-stimulator (ICOS)/ICOS-L, CD27/CD70, 4-1BB/4-1BBL and OX40/OX40L [200, 202, 203]. Various differentiated effector CD4 T-cells have been described including Th1, Th2, follicular helper (Tfh) T-cells, Th17 and Tregs (regulatory CD4 T-cells) with other subtypes constantly researched and plasticity between the types being a very well recognised phenomenon [200]. The type of effector cell the naïve CD4 cell differentiates to is dictated by cytokines in the cell environment, type of APC, burden of antigen and costimulatory molecules [204, 205]. An illustration of CD4 differentiation with the main cytokines driving the differentiation and main functions of each subtype is shown in Figure 1-6 [206]. CD4 inhibitory receptors such as PD1, CTLA4, LAG3, TIM3 are then implemented to dampen the immune response [41].

The most crucial cytokines required for differentiation into Th1 cells are IL12 and IFN γ [200, 207] with T-box transcription factor being the master regulator of Th1 differentiation which also suppresses Th2 and Th17 cell differentiation [208-210]. Th1 cells are implicated in rejection of intracellular pathogens, tumour rejection and in autoimmunity [145, 163, 211]. They mainly function through release of IFN γ and IL2 [211]. CD4 Th1 cells can induce direct cellular apoptosis through release of cytokines, they can also upregulate MHC class II through secretion of IFN γ which enhances recognition and killing, and they finally attract other immune cells such as

CD8 T-cells to promote killing of antigen expressing cells [88]. IL4 and IL2 on the other hand are critical for differentiation into Th2 cells with the main transcription factor driving Th2 differentiation being IL4 induced STAT6 which upregulates GATA3 that in turn suppresses Th1 differentiation [212-215]. IL6 from APCs and other non-immune cells is also required for Th2 differentiation and it inhibits Th1 differentiation [216, 217]. Th2 cells function through secreting IL4, IL5, IL9, IL13, IL10, IL25 to eliminate extracellular parasites and are involved in allergic diseases including asthma [200, 211, 218]. Tfh cells are another subtype that typically expresses CXC motif receptor 5 (CXCR-5), and their differentiation is driven by IL6 and IL21 as the main cytokines [219, 220]. Tfh cells partake in antigen specific B-cell humoral immunity [219, 221, 222]. Meanwhile, Th17 cell differentiation happens over 3 stages; differentiation stage governed by IL6 and TGF- β [223-225], amplification stage where IL21 is the main influencer, and stabilization phase mediated by IL23 [200]. ROR γ t (retinoic acid receptor-related orphan receptor gamma-T) is the master regulator for Th17 differentiation [200, 226]. Th17 cells implement their function through secretion of IL17A, IL17F, IL21 and IL22 [200]. They participate in elimination of extracellular bacteria and fungi, and they are also involved in autoimmune disease generation [226-228]. Although TGF- β is essential for Th17 cell differentiation, in higher concentrations it induces Forkhead transcription factor FOXP3 expression and differentiation into inducible Tregs (iTregs) [229, 230]. Naturally occurring Tregs have FOXP3 already expressed when released from the thymus while iTregs differ in that FOXP3 expression happens in the periphery after antigenic priming and these cells are typically CD4⁺CD25⁺FOXP3⁺ (Figure 1-7) [230, 231]. TGF- β is the main cytokine leading to Treg differentiation [230, 232, 233]. Smad2 and Smad3 (small mother against decapentaplegic 2 & 3) are other transcription factors activated through TGF- β pathways and they also induce Treg differentiation [234-236]. Tregs function through secreting IL10, TGF- β and IL35 [200], they have a negative regulating effect on immune responses and play a vital role in immunologic tolerance [237, 238]. The two most well-defined FOXP3 negative subsets of CD4⁺ iTregs are type 1 regulatory T-cells (Tr1) and T-helper 3 cells (Th3) (Figure 1-7 [231]) [239]. Tr1 cells produce higher levels of IL10 through which they exert their

suppressive function [240]. While Th3 cells typically produce higher levels TGF- β and are CD4+ cells expressing the inhibitory latency associated peptide (LAP) [241]. Th3 cells also produce IL8, IL9, IL10 and small amount of IFN γ but exert their suppressive function mainly through TGF- β [242, 243]. These Th3 cells have been identified in healthy human blood and were defined as CD4+LAP+Foxp3-CD69+ [242]. However, they were found to be much higher in cancer patient blood and even higher in the tumour microenvironment of colorectal cancer patients [244]. In the tumours of colorectal cancer patients, they were identified as CD4+LAP+Foxp3- co-expressing LAG3 and CD25 and exhibited 50-fold more suppression than the conventional FOXP3 expressing Tregs [244, 245].

CD4 Th1 cells and Tregs are the main subtypes involved in anti-tumour immunity and tumour promoting immune suppression respectively. The role of the other subtypes in the tumour microenvironment is yet to be clearly defined [246]. Emerging evidence has showed that CD4 T-cells are capable of cytotoxicity directly as well as indirectly and independent of CD8 T-cells [145, 163]. Aside from delivering immunologic help and cytokine centred effector functions, CD4 T-cells are capable of direct cancer cell killing effects through release of lytic granules containing granzyme B and expression of perforin [247-249]. However, one of the “Hallmarks of Cancer” is the escape from immune annihilation [250]. As discussed previously, tumour destruction by the immune cells may be hindered by the adoption of immunosuppressive environment in the escape phase of immune editing [251]. One example is through the release of the inhibitory cytokine TGF- β or the recruitment of suppressive regulatory Th3 subset of cells expressing of the inhibitory LAP [251].

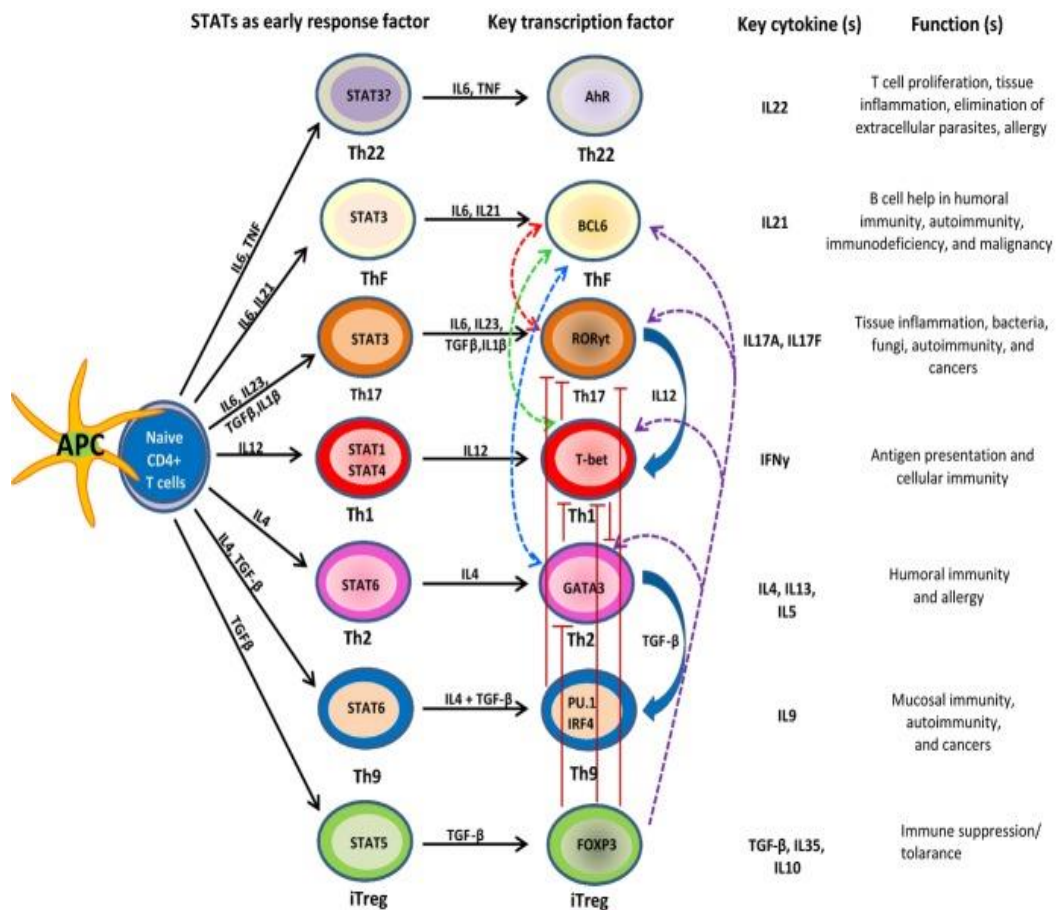


Figure 1-6: Illustration of CD4 T-cell differentiation and plasticity into the main subsets Th1, Th2, Th17, Th22, Th9, follicular helper (ThF) T-cells and inducible T-regulatory cells (iTregs) showing the main cytokines influencing the differentiation and those secreted by the differentiated cell type. The figure is taken from Subhash K Tripathi et al [206].

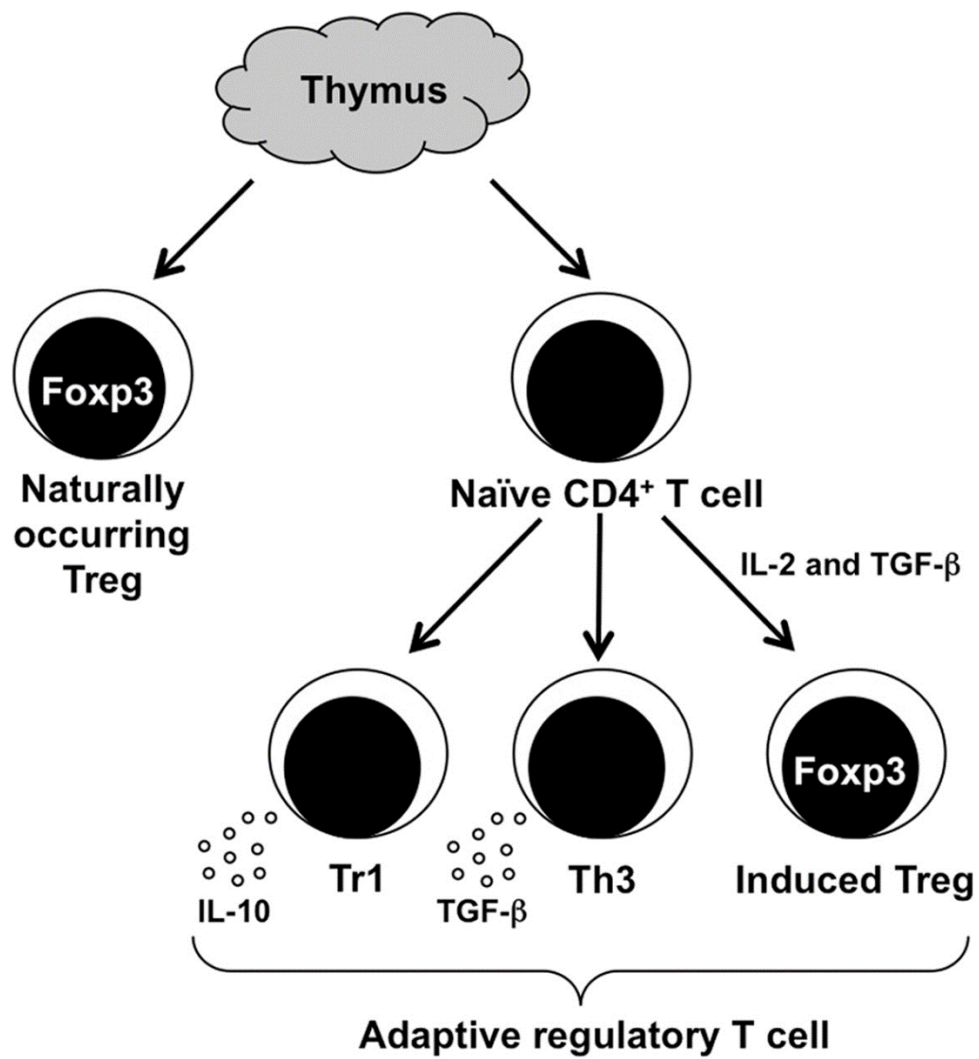


Figure 1-7: Illustration of the subsets of CD4⁺ regulatory T-cells (Tregs) taken from Tetsuya Nishimoto et al [231]. Where Foxp3 expression happens in the thymus for naturally occurring Tregs while it is induced in the periphery for the induced Tregs. Also showing the Foxp3 negative subsets of Tregs Tr1 (IL10 producing) and TH3 (TGF-β producing).

1.7 TGF- β /LAP/GARP pathway

TGF- β synthesis, activation and function is an area of ongoing research. TGF- β initially exists as a homodimeric propeptide (proTGF- β) and complexes with latency associated peptide (LAP). Mature TGF- β and LAP are cleaved by furin enzymes in the Golgi but remain non-covalently associated, this is called latent TGF- β or small latent complex (SLC) [252, 253]. Latent TGF- β localizes to the extracellular membrane (ECM) through a number of mechanisms. It can form disulphide bonds with latent TGF- β binding protein (LTBP) to form the large latent complex (LLC) which then localizes to the ECM through cysteine residues of the LTBP [254-256]. TGF- β in this form is inactive and is in the centre of a ring like structure within the complex [257]. Another mechanism by which TGF- β anchors to the cell surface is through association with Glycoprotein A repetitions predominant (GARP) [258, 259]. GARP is expressed predominantly on the surface of T-regulatory cells (Tregs) where it enhances their suppressive effect on other cells [260] through the secretion of TGF- β and IL10 [261, 262]. Furthermore, GARP is upregulated upon T-cell receptor stimulation [263]. GARP also exists on the surface of other non-Treg cells such as hepatic satellite cells, platelets, mesenchymal stromal cells and cancer cells [259, 264-268]. GARP is associated with latent TGF- β through non-covalent and disulphide linkage in a similar fashion to LTBP [269]. Activation of TGF- β is through multiple mechanisms including integrin, proteases and thrombospondin which are seen during inflammation and angiogenesis [253, 270]. Active TGF- β is involved in the regulation of a variety of cell types; immune and non-immune. Activated and secreted TGF- β , inhibits proliferation of effector cells, cytokine production and the differentiation of T-effector cells into Th1 cells (T-helper 1) [232, 271]. Additionally, it is also described in chronic feline immunodeficiency virus infection that latent TGF- β bound to membrane expressed GARP can convert Th cells into antigen induced Tregs [272, 273]. TGF- β is also involved in tissue homeostasis and regeneration through controlling epithelial cell growth, proliferation, apoptosis, production of ECM proteins and cytoskeletal organization [274, 275]. Abnormalities in TGF- β pathway are well documented in cardiovascular diseases such as valvular

heart disease, aneurysms, arteriovascular malformations and cardiac fibrosis [276, 277]. TGF- β is also implicated in fibrotic diseases, cancer and autoimmune diseases [275, 278, 279]. In the context of cancer, the roles of TGF- β have been termed the “TGF- β paradox” because of its opposing tumour suppressor and promotor roles [280] in extension to its’ role in immune surveillance. In early cancer, it acts as a tumour suppressor and cytostatic regulator through inducing cell cycle arrest and apoptosis therefore preventing unrestrained proliferation of epithelial cells and restraining immune cells proliferation [280, 281]. In established advanced cancers, mutations in TGF- β signalling suppressor pathway have been reported in several human cancers where the result is an increase EMT, invasion and metastasis [282-285].

Preclinical and clinical studies both in humans and animal models have explored different agents to inhibit the TGF- β /LAP/GARP signalling pathway. Human clinical trials have evolved mainly in agents targeting TGF- β , but no trials were found specifically targeting LAP upon researching clinicaltrials.gov. Even those targeting TGF- β pathway have moved into clinic with caution due to the functional variety of the pathway in controlling different cell types. For instance, vascular and inflammatory effects namely haemorrhagic valvular defects of the heart and aortic aneurysms were seen in animal studies when the pathway was silenced with long term and continuous treatment [286-289]. In a phase I trial of a TGF- β receptor I kinase inhibitor (Galunisertib); 1 out of 79 patients showed a mild degree of cardiac pathology measured by Doppler ultrasound with continuous high dosing of the drug [290]. Furthermore, 235 patients treated with Galunisertib did not show cardiac toxicity [291]. Following this, TGF- β inhibitors were thought to be safe and trials progressed with intermittent dosing schedules to avoid the toxicities seen with continuous dosing. Other human phase I trials with monoclonal antibodies targeting TGF- β (Fresolimumab) and animal studies (with LY2109761) reported skin toxicities such as epithelial hyperplasia, skin rashes and non-malignant keratoacanthomas similar to other approved targeted therapies such as sorafenib and vemurafenib [289, 292]. In NSCLC, a genetically modified tumour vaccine of human NSCLC cell

lines transfected with genes encoding for RNA antisense of TGF- β (Lucanix or Belagenpumatucel-L) was well tolerated in phase I and II which also suggested some dose related survival benefit, 15% partial response in late stage disease and immune responses amongst responders [293, 294]. However, a phase III trial using it as maintenance following primary treatment did not reach its end point of improved overall survival (OS), although marginal survival advantage was noted when starting treatment within 12 weeks of completing chemotherapy or following radiotherapy [295]. In metastatic melanoma, Morris et al [292] reported that 6 out of 29 patients who received the monoclonal antibody Fresolimumab showed a response on MRI in a phase I trial. Notably, this is similar to early trials with Ipilimumab in melanoma where 5 out of 46 patients showed a clinical response [296], only for later trials to show long-term benefit. Additionally, TGF- β inhibition is thought to promote osteoblastic activity [297, 298] and so it upholds a promising effect for trophic metastatic bone disease.

Combination treatment of agents inhibiting the TGF- β pathway with either chemotherapy or radiotherapy or immunotherapeutic drugs have also commenced to test for synergistic effects. Rodon et al and Brandes et al [299, 300], reported that the combination of Lomustine (an alkylating chemotherapy agent) with Galunisertib in glioblastoma multiforme patients did not improve efficacy or OS but when Galunisertib was used alone there was a trend toward better outcome and better tolerability compared to standard treatment single agent Lomustine. In the arm treated with Galunisertib monotherapy, it was noted that 9 patients with high pre-treatment levels of TGF- β showed a median OS of 11 months while the remaining 30 patients had a median OS of 4.7 months and low levels of TGF- β although this did not reach significance [300]. In the pre-clinical setting, the combination of TGF- β inhibition with either Doxorubicin or Fluorouracil reduced cancer growth in breast cancer models and gastric cancer respectively [301, 302]. In a phase II trial in breast cancer the combination of radiotherapy and Fresolimumab was reported to have better systemic immune responses (abscopal effect) and longer OS when the higher dose of Fresolimumab was used (10mg/kg as opposed to 1mg/kg) [303]. Radiation is

an inducer of TGF- β and TGF- β is thought to be implicated in the resistance to radiation and immunotherapy [304]. Completed phase Ib/II trials have tested the synergy between Galunisertib and Nivolumab in NSCLC and hepatocellular carcinoma (NCT02423343) as well as the combination of Galunisertib and Durvalumab in refractory pancreatic cancers (NCT02734160) but results are yet to be published.

TGF- β can anchor to the cell surface through the association with Glycoprotein A repetitions predominant (GARP) [258, 259]. GARP is expressed on the surface of Tregs where it enhances their suppressive effect on other cells [260] through the secretion of TGF- β and IL10 and inhibits proliferation of effector T-cells [261, 262]. Grégoire de Stree et al [305], showed anti-tumour immune responses and tumour regression of tumours resistant to anti-PD1 therapy with the use of a monoclonal antibody targeting GARP-TGF β 1 which prevented the release of TGF- β 1 from T-reg in mice without the global inhibition of other TGF- β 2 and TGF- β 3. They also confirmed that human melanomas consisted of T-reg expressing GARP. NCT03821935 is an early phase I clinical trial currently recruiting patients to test a similar monoclonal antibody (ABBV-151) in combination with a PD1 blocking agent.

LAP+ CD4 T-cells (Th3 cells) have been described as a highly suppressive subset of Tregs and associated with poorer prognosis in cancer patients [244, 245, 306]. In animal models these Th3 cells have been targeted through anti-LAP antibodies. Gabriely et al [307], explore the effects of anti-LAP antibody treatment in models of melanoma, colorectal cancer, and glioblastoma. They report that treatment of mice with an anti-LAP antibody reduces tumour growth, improves anti-tumour immunity, and boosts immune memory by decreasing LAP+ Tregs and TGF- β secretion as well as enhancing CD8 T-cell activation and CD8 tumour infiltration. They also show enhanced anti-tumour effects of their dendritic cell vaccination when combined with anti-LAP antibody. However, LAP inhibition could target many immune cells as its expressed on CD4+, CD8+, NK cells, dendritic cells, and B cells [308-311]. Anti-LAP antibodies blocking the actions of Th3 cells may improve the efficacy of vaccines including those that target siPTMs in cancer

by polarising responses to Th1. However, this has not previously been tested and will be explored as part of this work.

2 Chapter 2 Methods and materials

2.1 Human blood samples

Written informed consent was obtained from all subjects participating in this study. Peripheral blood was obtained from patients attending Nottingham City Hospital (Nottingham BioBank APC170 or REC ref. 13-EM-0218 or IRAS project ID 238448). Blood from healthy donors was taken following review and ethical approval by Nottingham University (Ethics ref: 161-1711).

Clinical details of 28 lung cancer (LG & OP) and 19 ovarian cancer (OV) patients are given in Table 2-1. Blood samples from LG36, LG38, LG42 and LG43 were used to establish the frequency of CD4+LAP+ cells and to assess the ability of this population to suppress the proliferation of target cells (Section 2.1.2). All other patient cells were used to determine proliferation in response to modified peptides (Section 2.1.1).

Table 2-1: clinical details of lung cancer patients (LG and OP) and ovarian cancer patients (OV) who donated blood to the assays.

<u>Donor code</u>	<u>Disease</u>	<u>Treatment</u>
LG1	Metastatic adenocarcinoma of lung (EGFR -ve, PD-L1 staining of tumour cells -ve)	Carboplatin and pemetrexed
LG2	Metastatic small cell lung cancer	Finished chemoradiotherapy 18 months prior to experiment. Currently on observation.
LG3	Metastatic adenocarcinoma of lung (EGFR +ve, T790 mutation +ve)	Osimertinib
LG4	Metastatic adenocarcinoma of lung (EGFR -ve)	Treatment naive
LG9	Metastatic adenocarcinoma of lung (PD-L1 staining of <1% of tumour cells, EGFR -ve)	Carboplatin and pemetrexed
LG10	T4N2M1a adenocarcinoma of lung and previously resected renal cell carcinoma 3years prior to experiment.	Pembrolizumab
LG11	T2bN2M1b adenocarcinoma of lung	Pembrolizumab
LG12	Metastatic small cell lung cancer	Carboplatin and etoposide
LG13	T4N3M0 poorly differentiated non-small cell lung cancer (NSCLC)	Pembrolizumab

LG17	T2N3M1a adenocarcinoma (PD-L1 staining of 100% of tumour cells)	Pembrolizumab. Previously had carboplatin and pemetrexed
LG18	T4N2M0 (stage 3B) adenocarcinoma of lung (PD-L1 staining of 50% of tumour cells, EGFR/ALK -ve)	Treatment naïve
LG19	Stage 4 adenocarcinoma of lung (PD-L1 staining of >50% of tumour cells)	Pembrolizumab
LG20	T3N1M1a adenocarcinoma of lung (PD-L1 staining of 70% of tumour cells)	Treatment naïve
LG22	T1aN0M1c (liver & brain metastasis) lung adenocarcinoma (PD-L1 staining of 70% of tumour cells).	Pembrolizumab. Previous brain metastasis craniotomy
LG23	Squamous cell carcinoma of the lung TxN2M1b (solitary bone metastasis) (PD-L1 staining of 75% of the tumour cells)	Treatment naïve. Had previous pneumonectomy 12 years prior to experiment.
LG24	Adenocarcinoma of the lung T4N3M1c (PD-L1 staining of 90% of the tumour cells)	Pembrolizumab
LG25	Adenocarcinoma of the lung T4N2M0 (stage 3B) (PD-L1 staining of 80-90% of the tumour cells)	Pembrolizumab
LG26	Squamous cell carcinoma T2N2M1a (adrenal metastasis) (PD-L1 staining of 70-80% of the tumour cells)	Pembrolizumab
LG27	Squamous cell carcinoma T4N3M1a (PD-L1 expression in >50% of tumour cells)	Pembrolizumab
LG28	Adenocarcinoma of lung T4N1M1c (liver, spleen, pleural and adrenal metastasis) (PD-L1 staining of 20-30% of the tumour cells)	Stopped maintenance pemetrexed 4 weeks prior to experiment after first line cisplatin and pemetrexed due to progression of disease.
LG29	T1cN2/3M1c adenocarcinoma of the lung with 20-30% of tumour cells staining positive for PD-L1	Second line pembrolizumab. First line was carboplatin and pemetrexed 7 months prior to experiment.
LG30	Metastatic adenocarcinoma of the lung with 60% of tumour cells staining for PD-L1	Combination carboplatin, pemetrexed and pembrolizumab
LG36	Metastatic adenocarcinoma with contralateral lung metastasis. EGFR/ALK/ROS1 were wild type.	On maintenance pembrolizumab and pemetrexed after finishing 4 cycles of carboplatin, pemetrexed and pembrolizumab.
LG38	T3N3M1b adenocarcinoma with 80% PDL1 expression. EGFR/ALK/ROS1 were wild type.	On pembrolizumab

LG42	T1bN0M1a adenocarcinoma with PD-L1 expression of <1%. ALK/EGFR/BRAF/ROS1 negative.	Carboplatin, pemetrexed and pembrolizumab.
LG43	Metastatic carcinosarcoma of the lung (multifocal adenocarcinoma with 50% sarcomatoid component) with PD-L1 expression 95%. ALK/EGFR/BRAF/ROS1 negative.	On maintenance pemetrexed and pembrolizumab. Finished 4 cycles of carboplatin, pemetrexed and pembrolizumab.
OP1	T1cN0M0 (2.4cm) squamous cell carcinoma of lung	Blood was taken preoperatively
OP3	T2bN0M0 (4.2cm) adenocarcinoma of lung	Blood was taken preoperatively
OV1	Serous ovarian/primary peritoneal Cancer	Treatment naïve
OV3	Stage 4 ovarian/ primary peritoneal	Not on treatment at time when blood was taken. Last chemotherapy was gemcitabine and carboplatin 6 months prior to experiment.
OV5	Stage 4 ovarian cancer	Treatment naïve
OV7	Stage 3C high grade ovarian adenocarcinoma	Treatment naïve
OV8	Stage 4 ovarian papillary serous carcinoma	Finished carboplatin and paclitaxel 3 months prior to experiment
OV9	BRCA +ve stage 4 high grade serous ovarian carcinoma	On maintenance bevacizumab but finished carboplatin and paclitaxel 3 months prior to experiment
OV10	Stage 4 serous ovarian carcinoma	Tamoxifen. No previous systemic treatment
OV11	Stage 1 high grade serous ovarian Ca (R ovarian mass only)	Treatment naïve. Refused surgery.
OV12	Stage 3C high grade ovarian serous carcinoma	Combination chemotherapy with carboplatin, paclitaxel and bevacizumab
OV13	Stage 4 high grade papillary serous ovarian carcinoma	Treatment naïve
OV14	Stage 4 BRCA +ve ovarian carcinoma	Bled on suspicion of progression. Previous carboplatin, paclitaxel and maintenance bevacizumab
OV15	Stage 3C high grade papillary serous ovarian ca, BRCA -ve. Also, Rheumatoid arthritis.	On Niraparib. Previous carboplatin and paclitaxel. For arthritis patient is on prednisolone, sulfasalazine, hydroxychloroquine and leflunomide

OV16	Stage 3B high grade serous ovarian adenocarcinoma	Treatment naive
OV17	Stage 3C serous adenocarcinoma of likely ovarian origin	Treatment naive
OV18	Stage 3C ovarian endometrioid adenocarcinoma	Off treatment. Previous carboplatin and gemcitabine 6months prior to experiment
OV19	Stage 3C low grade ovarian serous adenocarcinoma	Ultra-radical surgery 1 months prior to experiment. Bleed prior to commencing adjuvant chemotherapy
OV20	Stage 3C high grade serous adenocarcinoma of primary peritoneal origin	Bleed on suspicion of progression but not yet on treatment. Previous letrozole.
OV21	Stage 3C high grade papillary serous ovarian carcinoma, BRCA -ve	Finished carboplatin and caelyx 6weeks prior to experiment
OV22	Stage 4 primary peritoneal adenocarcinoma	Treatment naive

2.1.1 Proliferation assay on human peripheral bloods mononuclear cells (PBMCs)

2.1.1.1 Isolation of PBMCs

Approximately 20-94ml of peripheral blood from cancer patients and healthy donors was drawn into lithium heparin tubes (Becton Dickinson, 12356939). Samples were maintained at room temperature and processed within 2 hours of collection. PBMCs were isolated by density gradient centrifugation over Histopaque-1077 (Sigma, 10771). Briefly, whole blood was diluted with an equal volume of RPMI (Sigma, R8758) supplemented with HEPES (20mM final concentration, Sigma, HO887), L-glutamine (2mmol/L, Sigma, G7513) and penicillin streptomycin solution (0.1mg/ml streptomycin and 100units/ml penicillin, Sigma, P4333). Diluted blood was gently layered over histopaque at an approximate ratio of 1:2 (histopaque:diluted blood) and centrifuged at 400xg for 30minutes with gentle acceleration and no brake at room temperature. Autologous plasma (top layer) was removed and used to make complete media (RPMI supplemented with HEPES (20mM final concentration), L-glutamine (2mmol/L), penicillin streptomycin solution (0.1mg/ml streptomycin and 100units/ml penicillin) and

10%v/v autologous plasma. PBMCs were transferred from the interface into two 50ml conical tubes, washed with 50mls of Phosphate Buffered Saline (PBS, Sigma, D8537) and centrifuged at 300xg for 10mins at room temperature. Pellets were combined, resuspended in 50ml of PBS and centrifuged at 300xg for 5mins at room temperature. The washed cell pellet was resuspended in 10-20ml of complete media. Cell number was determined on an automated cell counter (Countess, Invitrogen) using trypan blue to determine viability (Sigma, T8154 - 50µl of PBMCs mixed with 50µl of trypan blue). Approximately 1×10^6 cells were removed and stained for expression of CD4⁺ and CD25. In selected donors the expression of LAP was also determined (see section 1.1.1.4 for staining methodology).

2.1.1.2 CD25 depletion

PBMCs from 7 patients with cancer were divided into two equal aliquots. One aliquot from each donor was depleted of CD25 cells whilst the other aliquot was left un-depleted. PBMCs from all other patients were CD25 depleted. CD25 depletion was performed on Miltenyi LS columns (Miltenyi Biotec, 130-042-401) using an anti-CD25 isolation kit (Miltenyi CD25 Microbeads II, 130-092-983) following the manufactures instructions. Briefly, isolated PBMCs were centrifuged at 300xg for 5minutes at room temperature and the supernatant aspirated. The remaining cell pellet was completely resuspended in 80µL of MACS buffer per 10^7 cells (MACS buffer: 500ml of PBS with 5ml of heat inactivated foetal calf serum (HI-FCS, Sigma, F9665) and 2ml of filtered 0.5M Ethylenediaminetetraacetic acid (EDTA, Gibco 15575-038)) before the addition of 20µL of anti-CD25 microbeads per 10^7 cells. Cells and microbeads were mixed gently, incubated for 15minutes at 4°C, washed with 2ml of MACS buffer per 10^7 cells, centrifuged at 300xg for 5minutes, re-suspended in 500µL of MACS buffer and applied to a pre-activated LS column. The LS column was activated by the addition of 3.0ml of MACS buffer. CD25 negative cells were eluted from the column by the addition of 3 x 3.0ml of MACS buffer and collected in a 50ml conical tube. The cell volume was made to 50ml with MACS buffer and approximately 5×10^5 - 10×10^5 cell aliquot was removed and direct immunofluorescence staining was carried out to analyse expression of CD4 and CD25. In selected

donors LAP expression was also quantified (see section 1.1.1.4 for staining methodology).

2.1.1.3 CFSE loading

Freshly isolated, CD25 depleted and non-depleted PBMCs were loaded with carboxyfluorescein succinimide ester (CFSE, ThermoFisher, C34554) to determine proliferation responses following culture with modified peptides. Briefly, a 5mM stock solution of CFSE was prepared by dissolving 1 vial of Cell Trace into 18 μ l of DMSO (supplied with the CSFE). Stock CFSE was further diluted 1:100 into warm (37°C) sterile PBS 1:100 to give a working concentration of 50 μ M CFSE. PBMCs were resuspended in 2-5ml of CFSE loading buffer (PBS supplemented with 5%v/v HI-FCS) at a concentration range of 0.5-10 $\times 10^6$ cells/ml. Cells were transferred into the bottom of a clean 50ml conical and the tube held at an angle. 110 μ l of working CFSE solution was added per ml of PBMCs to the inner wall of the tube so that the cells and working solution did not come into contact. The tube was capped securely, the contents mixed rapidly and incubated for 5minutes at room temperature in the dark. Cells were washed in 50mls of CFSE loading buffer centrifuged at 300xg for 10mins, resuspended in 10ml complete media, counted and the volume adjusted to give 1-3 $\times 10^6$ cells per ml. CFSE-loaded cells were plated in 24 well plates at 1-3 $\times 10^6$ cells per well in a total volume of 2ml per well. Cultures were stimulated with either vehicle, modified peptides (at 10 μ g/ml final concentration). For positive controls cultures were stimulated with PHA (Sigma, L8754) to achieve polyclonal stimulation (10 μ g/ml final concentration) or CPI (5 μ g/ml final concentration, Cellular Technology Ltd, CTL-CPI-005) to determine CD4 specific proliferation. Peptides were synthesized at >90% purity by Genscript (USA) with arginine (R) replaced by citrulline (cit) or lysine (K) replaced by homocitrulline (hcrit) and stored lyophilised at -80°C. Prior to usage peptides were reconstituted to 1mg/mL in phosphate buffered saline (PBS). Peptide sequences are listed in Table 2-2.

Table 2-2: Peptide sequences where arginine (R) replaced by citrulline (cit) or lysine (K) replaced by homocitrulline (hcrit).

Peptide aa coordinates	Abbreviation	Sequence
α-Enolase 241-260^{cit}	E241	VIGMDVAASEFF-cit-SGKYDLD
Vimentin 28-49^{cit}	V28	cit-SYVTTST-cit-TYSLGSAL-cit-PSTS
Vimentin 415-433^{cit}	V415	LPNFSSLNL-cit-ETNLDLPL
BIP 189-208^{cit}	BIP	TIAGLNVN-cit-IINEPTAAAIA
NPM 266-285^{cit}	NPM	AKFINVVKNC-cit-MTDQEAIQ
Cytokeratin-8 101-120^{cit}	Cyto8	KFASFIDKV-cit-FLEQQNKMLE
Aldolase 74-93^{hcrit}	A74	IGGVILFHETLYQ-hcrit-ADDGRP
Aldolase 140-157^{hcrit}	A140	hcrit-DGADFA-hcrit-WRCVL-hcrit-IGEH
Vimentin 116-135^{hcrit}	V116	NYID-hcrit-VRFLEQQN-hcrit-ILLAEI

2.1.1.4 Surface marker and intracellular staining

On the day blood samples were taken (day 0), aliquots of isolated PBMCs taken pre and post CD25 depletion were stained for CD4 (anti CD4 eFluo450, eBiosciences, clone RPA-T4, 1µl/test) and CD25 (anti CD25 PE, Miltenyi, clone REA570, 2µl/test). In addition, 9 lung cancer donors PBMCs (LG22 to LG30) were also stained for LAP expression (anti-LAP APC, Miltenyi, clone CH6-17E5.1, 5µl/test). On day 4, 7 and 10, a 250µl aliquot from each culture condition were harvested and stained with one of the panels in Table 2-3. Briefly, Brefeldin A, an intracellular cytokine transport inhibitor, (eBioscience, 00-4506-51) was added to each aliquot of cultured cells at a final dilution of 1:1000 to enhance the detection of intracellular IFNγ. Samples were incubated for 3 hours at 37°C, centrifuged at 350xg for 3 minutes, and the supernatant discarded. Antibody panels were added as master mixes (25uL per sample) to minimise staining variation across cultures. Samples were incubated at 4°C for 30 minutes, washed in 600µl of PBS, re-centrifuged at 350xg for 3 minutes and the cell pellet fixed in 50µl of fixation buffer (eBioscience, 00-8222-49) at 4°C for 30 minutes. Samples

were washed in 1ml of permeabilization buffer (eBioscience, 00-8333-56 - diluted 1:10 in distilled water) and re-centrifuged at 470xg for 3 minutes. Intracellular antibodies, prepared in permeabilization buffer, were also applied in a master-mix and allowed to bind at room temperature in the dark for 25minutes. Samples were washed with 600µl of PBS, re-centrifuged at 470xg for 3 minutes and the cells resuspended in 200µl of fixation buffer before analysis on a MACsQuant10 flow cytometer and MACsQuant software version 2.11. To determine the compensation matrix and gating strategy initial samples were stained with all but one of the antibodies in the panel. For example, in panel A to aid with where to place the CD4 quadrant gate, a sample was stained with all the antibodies in panel A except anti-CD4. This was repeated for samples to aid with gating for all antibodies. A gating strategy example is shown in Figure 2-1.

Table 2-3: Samples from human PBMCs were stained using direct immunofluorescence for the expression of extracellular and intracellular cytokines with one of the following panels of antibodies.

Panel A				
Antibody	Conjugate	Clone	Supplier	Concentration/ 25µl test
Anti-CD4	eFluor450	RPA-T4	eBioscience	1µl
Anti- CD8	VioGreen	REA734	Miltenyi	1µl
Anti-CD71	PE	REA902	Miltenyi	0.5µl
Anti-LAP (TGF-β)	APC	CH6- 17E5.1	Miltenyi	2.5µl
Anti-CD25	PE-vio770	REA570	Miltenyi	0.5µl
Anti-IFNγ	APC- efluor780	4S.B3	Invitrogen	1.25µl
Panel B				
Antibody	Conjugate	Clone	Supplier	Concentration/ 25µl test
Anti-CD4	eFluor450	RPA-T4	eBioscience	1µl
Anti- CD8	VioGreen	REA734	Miltenyi	1µl
Anti-CD71	PE	REA902	Miltenyi	0.5µl
Anti-CD134	PEvio770	REA621	Miltenyi	1.25µl
Anti-CD25	PE-vio770	REA570	Miltenyi	0.5µl

Anti-IFNγ	APC- eFluor780	4S.B3	Invitrogen	1.25 μ l
Anti- Granzyme B	APC	REA226	Miltenyi	2.5 μ l
Panel C				
Antibody	Conjugate	Clone	Supplier	Concentration/ 25μl test
Anti-CD4	eFluor450	RPA-T4	eBioscience	1 μ l
Anti- CD8	VioGreen	REA734	Miltenyi	1 μ l
Anti-CD71	PE	REA902	Miltenyi	0.5 μ l
Anti-LAP (TGF-β)	APC	CH6- 17E5.1	Miltenyi	2.5 μ l
Anti-IL17A	APCviolet770	REA1063	Miltenyi	0.5 μ l
Anti- IL10	PE Cy-7	JES3-9D7	eBioscience	1.25 μ l
Panel D (all ovarian patients)				
Antibody	Conjugate	Clone	Supplier	Concentration/ 25μl test
Anti-CD4	eFluor450	RPA-T4	eBioscience	1 μ l
Anti- CD8	VioGreen	REA734	Miltenyi	1 μ l
Anti-CD134	PEviolet770	REA621	Miltenyi	1.25 μ l
Anti-IFNγ	APC- eFluor780	4S.B3	Invitrogen	1.25 μ l
Anti- Granzyme B	APC	REA226	Miltenyi	2.5 μ l

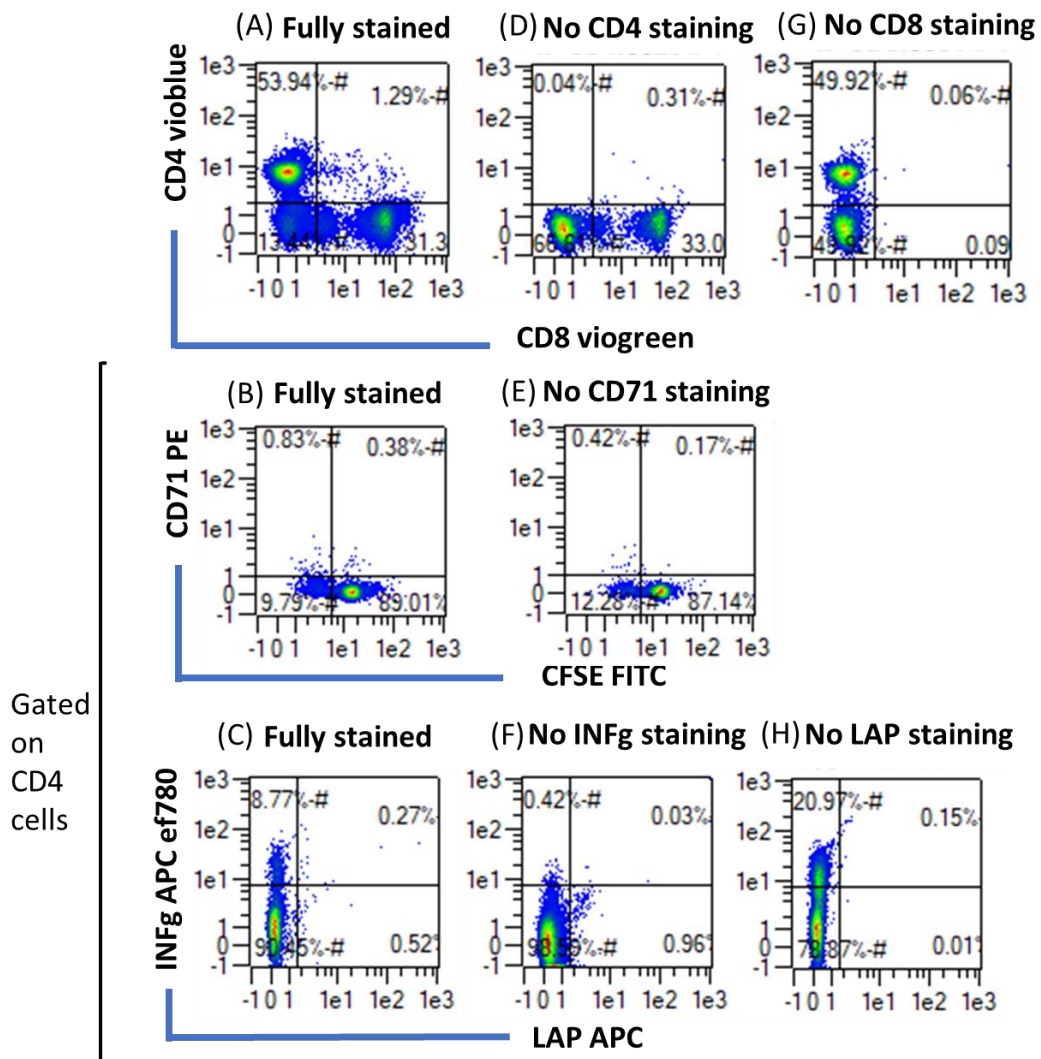


Figure 2-1: Gating strategy for flow cytometry analysis. (A), (B) and (C) are direct immunofluorescence flow cytometry plots of samples fully stained with a panel containing anti-CD4, anti-CD8, anti-CD71, anti-LAP(TGF- β) and anti-IFN γ antibodies while (D) is a sample stained with all but the anti-CD4, (E) is a sample stained with all but the anti-CD71, (F) is a sample stained with all but the anti-IFN γ , (G) is a sample stained with all but the anti-CD8 and (H) is a sample stained with all but the anti-LAP (TGF- β) antibody.

2.1.2 CD4+LAP+ isolation and suppression assays on cancer patient PBMCs

The ability of CD4+LAP+ PBMCs to suppress T cell proliferation was accessed in 4 lung cancer patients (LG36,38,42 and 43, Table 2-1). PBMCs were isolated as described in section 1.1.1.1. A median of 87.5ml (range 85-100ml) of peripheral blood was taken from each patient. The median PBMC count was 1.098×10^8 (range 0.32×10^8 to 2.66×10^8). However, a maximum of

1×10^8 PBMCs were used to isolate CD4+LAP+ cells. Briefly, following isolation, PBMCs were centrifuged at 350xg for 5 minutes and the supernatant discarded. The PBMC pellet was fully re-suspended in 50 μ l of PBS per 1×10^6 PBMCs before the addition of 2 μ l/ 1×10^6 cells of anti-LAP APC (Miltenyi Biotec, 130-125-197, clone CH6-17E5.1) and 5 μ l/ 1×10^6 cells of anti-CD4 eFluor-450 (Invitrogen, 48-0049-42, clone RPA-T4). PBMCs were incubated at 4°C for 30 minutes, washed in 50ml of PBS, re-centrifuged at 350xg for 5 minutes, and resuspended in 3ml of sorting buffer (PBS supplemented with 1%v/v HI-FCS and 1mM EDTA). Cells were sorted using a Sony Cell Sorter (model SH800S) into CD4+LAP+ and CD4+LAP- populations and collected into 5ml of Optimizer™ (Gibco, A10221-01) supplemented with 5%v/v heat inactivated Human AB Filtered -Pooled Serum (PHS) (Tetubio, 088HSER-ABP). The median number of CD4+LAP+ cells collected was 241,724 (range 214,128 to 314,904) and the median number of CD4+LAP- cells collected was 1,736,584 (range 810,000 to 4,492,255). The CD4+LAP- cells were loaded with CFSE as described in section 1.1.1.3. The median cell count post loading was 600,000 (range 4,000,00 to 2,200,000).

100 μ l of each of CD4+LAP+ and CFSE loaded CD4+LAP- sorted populations were added to a U-bottom 96 well plate at a 1:1 ratio (cell counts ranging between 0.4×10^5 and 1×10^5 for each cell type) to give a final volume of 200 μ l. CFSE loaded CD4+LAP- and non-loaded CD4+LAP- cells served as a negative control. Human T-Activator CD3/CD28 Dynabeads (ThermoFisher, 11131D) were used as a T-cell receptor (TCR) stimulus and added to the appropriate wells as per manufacturer's instructions (2 μ L beads per 8×10^4 total cells). Prior to their addition, Dynabeads were washed in 1ml of bead washing medium (PBS supplemented with 2mM EDTA and 0.1%v/v HI-FCS) and resuspended in Optimizer™ supplemented with 5%v/v PHS. IL-2 (Peprotech, 200-02) was added to all wells to a final concentration of 30 Units/ml. Cultures were incubated for 4 days at 37°C. On day 4, all 200 μ l volume of each well was harvested, centrifuged at 350xg for 3 minutes and the supernatant discarded. The cell pellets were resuspended in 200 μ l of fixation buffer and analysed on a MACSQuant16 flow cytometer. The

percentage of proliferating CD4+LAP- cells was measured and the percentage of suppression was calculated using the following formula:

“% suppression = (% proliferation of CD4+LAP- in the absence CD4+LAP+) minus (% proliferation of CD4+LAP- in the presence of CD4+LAP+) divided by (% proliferation of CD4+ LAP- in the absence CD4+LAP+)”.

2.2 Immunohistochemistry (IHC)

The Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria were followed throughout the study.

2.2.1 Patient cohort in tumour microarray (TMA)

The study cohort was comprised of patients subjected to therapeutic resection of histologically identified primary NSCLC (non-small cell lung carcinoma) between the 1st of January 1996 and 31st of July 2006 at the City Hospital, Nottingham, UK and was conducted under the ethical approval of the Biobank (study number ACP000030). All patients treated in the given time period were included in the study, except in the cases where the biological material was unavailable and from 3 patients who received neo-adjuvant therapy.

Samples from 220 patients of whom 106 (48%) were male and 114 (52%) were female were analysed. There were 29 cases (13%) of well differentiated, 91 cases (41%) of moderately differentiated and 60 cases (27%) of poorly differentiated NSCLC adenocarcinomas. TNM stage was assigned according to the 7th edition TNM classification. The cohort included 32 patients (15%) with stage IA, 52 patients (24%) with stage IB, 46 patients (21%) with stage IIA, 27 patients (12%) with IIB, 41 patients (19%) with stage IIIA and 1 patient with stage IV disease. The mean age at the diagnosis was 79.5 years (range of 36 – 91 years). Follow up was calculated from the time of the resection to the 1st of May 2013 giving a median follow up of 36 months for all patients (range 0–204 months), 25.5 months for the deceased patients (range 0 – 198 months) and 123.5 months for the surviving patients (range 82 – 204 months). At the time of analysis, 173 patients (79%) were deceased, and 43 patients (20%) were alive.

None of the patients included in this study were documented to have received radiotherapy, chemotherapy or biological therapy prior to the surgical resection. Following surgery, 11 patients received radiotherapy and 9 patients received at least 1 cycle of adjuvant chemotherapy (22 unknown) with only 2 patients receiving both types of treatment. Adjuvant chemotherapy was in the form of combination treatment of cisplatin, vinorelbine, carboplatin, gemcitabine, docetaxel, and etoposide, which could have affected overall survival.

All tumours received following resection in the histopathology lab were incised, fixed immediately in formaldehyde and processed through to embedding in paraffin wax, ensuring optimal tissue fixation and preservation for histological examination. Tissue microarrays were built as previously described [312].

2.2.2 IHC staining

IHC was performed using Novolink Polymer Detection systems Novocastra (Leica Biosystems RE7150K) following the manufacturer's protocol. Briefly, 4µm tissue sections were placed in a hot plate set at 60°C for 10 minutes, dewaxed in xylene, and rehydrated using Leica Autostainer XL. Antigen retrieval was achieved using a rotary microwave oven; slides were immersed in 500ml of citrate buffer (pH 6.0) or in the case of anti-human PD-L1 antibody immersed in EDTA (pH 9.0) and placed in the centre of the oven for 20 minutes at 800W. Slides were cooled down immediately for 10 minutes with tap water, then placed in a humidity chamber and covered with TBS (Tris Buffered Saline). Endogenous peroxidase activity was neutralized using 100µl of Dako peroxidase block solution for 5 minutes, followed by application of protein block solution to reduce non-specific binding of primary antibodies and polymer. Monoclonal mouse anti-human CD4 antibody (4B12, Dako, 1:3000 dilution), monoclonal mouse anti-vimentin (3B4, Dako, 1:250 dilution), anti-citrullinated vimentin anti-serum antibody (GB17002, Eurogentec, 1:100 dilution), monoclonal mouse anti-human PD-1 antibody (EH33, Cell Signaling, 1:75 dilution), polyclonal rabbit anti-Ambra1 antibody (Abcam, 1:20 dilution), monoclonal mouse anti-HLA-DP, DQ, DR antibody (CR3/43, Dako, 1:3000 dilution), rabbit polyclonal to PADI2/PAD2

(Abcam, 1:150 dilution) and mouse monoclonal to PADI4/PAD4 (OTI4H5, Abcam, 1:700 dilution) were incubated with the tumour sections for 1 hour at room temperature except for staining with monoclonal rabbit anti-human PD-L1 antibody (E1L3N, Cell Signalling, 1:25 dilution) and polyclonal goat anti-human LAP (TGF- β 1) (R&D, 1:20 dilution) where the incubation was overnight at 4°C. Finally, 3-3' Diaminobenzidine tetrahydrochloride (Novolink DAB substrate buffer plus) was freshly prepared and used as chromogen. Counter staining was performed using haematoxylin for 6 minutes. Negative controls were done by omitting the primary antibody. All antibodies were commercially validated using Western Blot. Citrullinated vimentin antibody was validated internally [313].

2.2.3 IHC scoring

TMA slides were captured digitally and stored as high-resolution image files and visualize using Aperio Image Scope 12.3.2 software. All markers were assessed independently by two assessors who were blinded to the clinicopathological data of the patients. All protein marker staining was evaluated by Histo-score system (H-score) which was calculated semi-quantitatively assessing the percentage of positivity (0-100%) and staining intensity (Negative: Score 0, Weak: Score 1, Moderate: Score 2, and Strong: Score 3). H-score is the sum of the percentage of positivity (0-100) and the intensity (0-3) giving a score ranging between 0-300. Marker expression was stratified into low or high staining using the X-tile software program [314]. Immune markers (CD4 and PD1) were assessed to 'number of TILs/mm²' using Aperio Image Scope for the analysis of the areas and stromal T cells were evaluated and classified in 3 categories 'Sparse/Moderate/Dense'.

2.3 Murine experiments

2.3.1 Murine tumour cell lines and anti-tumour studies

The murine melanoma B16F1 cell line (ATCC-CRL-6323), murine breast carcinoma Py8119 line (ATCC-CRL-3278), murine lung carcinoma LLC2 line (ATCC-CRL-1642), murine colon cancer CT26 line (ATCC-CRL-2638) and murine breast carcinoma 4T1 line (ATCC-CRL-2539) were obtained from the ATCC (Virginia, USA) and the murine colon

adenocarcinoma MC38 cell line (Kerafast ENH204-FP) was obtained from Kerafast (Boston, USA). B16F1, CT26 and 4T1 were cultured in RPMI1640 (Sigma R8758) supplemented with 10%v/v heat inactivated foetal bovine serum (HI-FCS, Sigma F9665). LLC2 and MC28 were cultured in Dulbecco's Modified Eagle's Medium (Gibco 41966-029) supplemented with 10%v/v HI-FCS. Py8119 cell line was cultured in Kaighn's modification of Ham's F12 (ATCC 30-2004) supplemented with 5%v/v HI-FCS. Cell lines were mycoplasma tested and used within 20 passages. For the tumour model in transgenic HHDII/DP4 mice, the B16F1 melanoma cell line was engineered to express interferon γ (IFN γ)-inducible human HLA-DP4 (iDP4) and HHDII (HLA-A2) and the mouse MHC (major histocompatibility) I and II were knocked out (B16F1 HHDII/iDP4) [163].

HLA-A2.1+/+ HLADP4+/+ hCD4+/+ (HHDII/DP4) transgenic mice (EM:02221, European Mouse Mutant Archive) express human CD4 in replacement of murine CD4 and are deficient in both murine MHC class I and II. Murine MHC are replaced with the chimeric HLA-A2 (HHDII) and human DP4 alleles. C57Bl/6 and Balb/c mice (from Charles River) were used when they were between 8-12 weeks of age under a Home Office approved project license and approval from the University of Nottingham and Nottingham Trent University ethics boards. Mice were challenged with 1×10^5 B16F1 iDP4 cells, $1-3 \times 10^4$ B16F1 cells, $5-10 \times 10^5$ Py8119 cells, $1-2 \times 10^6$ LLC2 cells, 5×10^3 MC38 cells, 1×10^5 CT26 cells or 2.5×10^3 4T1 cells subcutaneously on the right flank on day 1 of the studies. Tumours were measured twice a week and tumour volumes were calculated using the formula "volume = $(\pi / 6) \times (WX(L^2))$ " where W is width and L is length of tumour. Mice were humanly euthanized when measurements were ≥ 10 mm.

For anti-tumour studies, groups of 10 mice were randomly assigned to a therapy group or a control, untreated group and processed without investigator blinding. Mice were either implanted with Py8119 breast cancer tumour model in Corning Matrigel GFR Membrane Matrix (Fisher Scientific, 11553620) or the B16F1 melanoma cell line in PBS. Mice were implanted with tumour cells on day 1 followed by vaccination with citrullinated peptides on days 3, 7 and 10 for the Py8119 model or days 4, 11 and 18 for the

B16F1 model. Peptide vaccination comprised of a combination of 25µg dose of each of citrullinated alpha enolase 11-25 (sequence IFDS-Cit-GNPTVEVDLY where cit represents citrulline amino acid), citrullinated cytokeratin8 8-26 (sequence KSYKMSTSGP-Cit-AFSS-Cit-SFT) and citrullinated glutamate receptor 316-340 (sequence EPKSSCYNTHEK-cit-IYQSNMLN-cit-YLI) peptides delivered subcutaneously (s.c.). Peptides were stored as lyophilised powder at -80°C prior to reconstitution at 1mg/ml in PBS and emulsified with 5 µg/mouse of CpG ODN 1826 (InvivoGen, vac-1826-1) and monophosphoryl lipid A (MPLA) (InvivoGen, vac-mpla). Anti-LAP antibody (Ultra-LEAF™ purified anti-mouse/human LAP (TGF-β1), BioLegend, clone TW7-28G11, part number 146704) was administered via the intraperitoneal (i.p.) route in PBS at 200µg/dose/mouse on days 3, 7, 10, 14 & 17 for the Py8119 model and at 100µg/mouse on days 4, 8, 11, 15, 18 & 22 for the B16F1 model. The rationale behind using a different dose in the two tumour models was that B16F1 expressed lower levels of LAP compared to the Py8119 as shown in the results section and so it is expected that less antibody is needed. Anti-LAG3 antibody (*In-vivo* Mab Rat, anti-Mouse LAG-3, IgG1 kappa, 2BScientific, clone C9B7W, part number BE0174-25MG) was administered via the i.p. route in PBS 250µg/dose/mouse on days 7, 10 and 14. A diagram illustrating the designs adopted in the anti-tumour studies is shown in Figure 2-2.

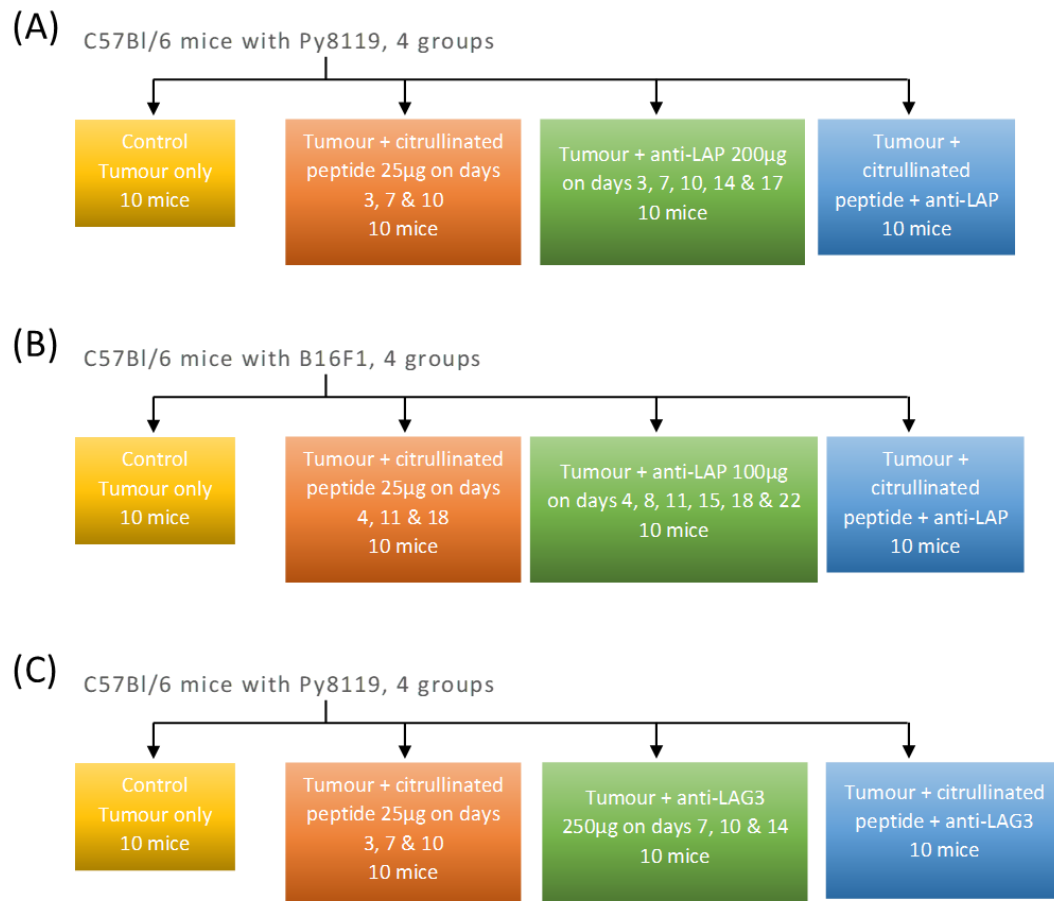


Figure 2-2: A diagram of 3 anti-tumour studies where (A) C57Bl/6 mice were implanted with Py8119 tumour model on day 1 and 4 groups of 10 mice each were randomly allocated to no treatment, treated with 3 citrullinated peptides s.c. on days 3, 7 and 10, treatment with anti-LAP antibody i.p. on days 3, 7, 10, 14 & 17 and a final group treated with the combination of the 3 citrullinated peptides with anti-LAP therapy using the same dosing regimen. (B) C57Bl/6 mice were implanted with B16F1 tumour model on day 1 and 4 groups of 10 mice each were randomly allocated to a no treatment group, a group treated with 3 citrullinated peptides s.c. on days 4, 11 and 18, a group treated with anti-LAP antibody i.p. on days 4, 8, 11, 15, 18 & 22 and a final group treated with the combination of the 3 citrullinated peptides with anti-LAP therapy using the same dosing regimen. (C) C57Bl/6 mice were implanted with Py8119 tumour model on day 1 and 4 groups of 10 mice each were randomly allocated to no treatment, treated with 3 citrullinated peptides s.c. on days 3, 7 and 10, treatment with anti-LAG3 antibody i.p. on days 7, 10 & 14 and a final group treated with the combination of the 3 citrullinated peptides with anti-LAG3 therapy using the same dosing regimen.

2.3.2 *Ex-vivo* staining of murine tumours and splenocytes.

Tumours and spleens from each mouse were dissected individually once they reached culling tumour size ($\geq 10\text{mm}$). Tumours were dissociated using scissors and forceps in petri dishes containing 10ml of RPMI1640 and processed separately. Spleens were disaggregated using forceps in petri dishes containing 10ml of RPMI1640. The 10ml of media containing cells from the tumours and spleens were transferred into individual 15ml falcon tubes and clumps were briefly allowed to settle. A 1ml aliquot from the dissociated tumours and a 200 μl aliquot from the dissociated spleens were taken into Eppendorfs. Samples were centrifuged at 350xg for 3 minutes, and the supernatant discarded. Tumour cells were resuspended in 50 μl of the staining antibody panel outlined in Table 2-4. Isolated splenocytes were initially treated with red cell lysis buffer (Roche, 11814389001) according to the manufacturer's recommendations. Briefly, splenocytes were resuspended in 100 μl of red cell lysis buffer, incubated for 2 minutes, washed with 1ml of PBS and centrifuged at 350xg for 3 minutes. The supernatant was discarded and the splenocytes resuspended in 50 μl of staining antibody panel outlined in Table 2-4. Stained samples from both spleens and tumours were incubated at 4°C for 30 minutes, washed in 600 μl of PBS, re-centrifuged at 350xg for 3 minutes and the cell pellet fixed in 200 μl of fixation buffer (eBioscience, 00-8222-49). Samples were analysed on the MACsQuant16 flow cytometer using MACsQuant software version 2.13.

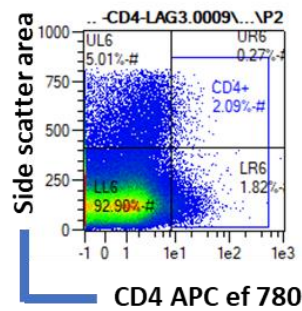
For some samples splenocytes and tumours were further stained for the intracellular expression of Foxp3. In this instance, cell pellets (stained for extracellular markers outlined in Table 2-4) were fixed in 50 μl of fixation buffer, incubated at 4°C for 30 minutes, washed in 1ml of permeabilization buffer (eBioscience, 00-8333-56 - diluted 1:10 in distilled water) and re-centrifuged at 470xg for 3 minutes. The supernatant was discarded, and the pellet resuspended in 50 μl of permeabilization buffer and incubated at 4°C for 20 minutes. Foxp3 antibody (Foxp3 vio-blue, clone FJK-16s, eBioscience 48-5773-82, 1 μl per 50 μl test), prepared in permeabilization buffer, was added (50 μl per sample, prepared as a master mix) and allowed to bind at room temperature in the dark for 25 minutes. Samples were washed with 600 μl of

PBS, re-centrifuged at 470xg for 3 minutes and the cells resuspended in 200µl of fixation buffer. Samples were analysed on the MACsQuant16 flow cytometer and MACsQuant software version 2.13. An example of gating strategy is shown in Figure 2-3.

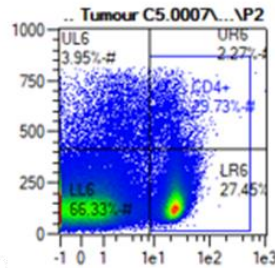
Table 2-4: Samples from murine splenocytes and tumours were stained for expression of extracellular cytokines using direct immunofluorescence with the following panel of antibodies.

Panel A				
Anti-mouse Antibody	Conjugate	Clone	Supplier	Concentration/ 50µl test
Anti-CD4	APCeF780	RM4-5	Invitrogen	1µl
Anti-LAP (TGF-β)	PECy7	TW7-16B4	Invitrogen	1µl
Anti-GARP	PE	YGIC86	Invitrogen	1.25µl
Anti-LAG3	vio-blue	C9B7W	Invitrogen	5µl

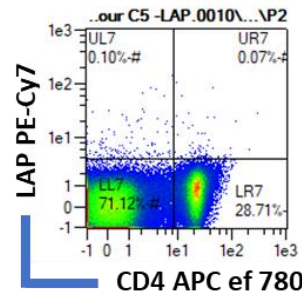
(A.i) No CD4 staining



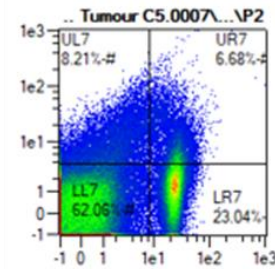
(A.ii) Fully stained



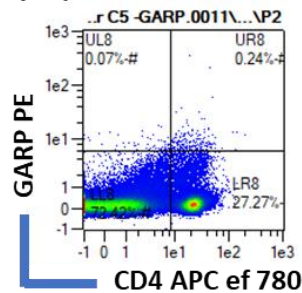
(B.i) No LAP staining



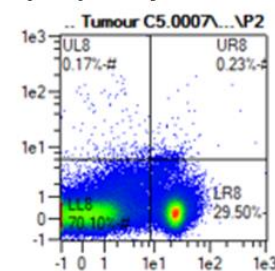
(B.ii) Fully stained



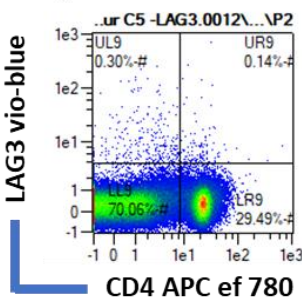
(C.i) No GARP staining



(C.ii) Fully stained



(D.i) No LAG3 staining



(D.ii) Fully stained

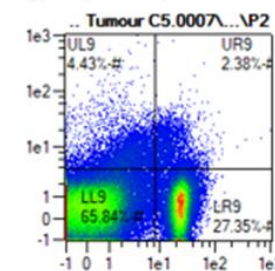


Figure 2-3: Gating strategy on a tumour sample. After dissociated of a Py8119 tumour from a C57Bl mouse, 4 samples were taken for direct fluorescence staining where one was fully stained with CD4 APC ef780, LAP PE-Cy7, GARP PE and LAG3 vio-blue and the others were stained with 3 out of the 4 antibodies. Samples were analysed in the MACSQuant16 flow cytometer. A.ii, B.ii, C.ii and D.ii are samples that were fully stained with all 4 antibodies. (A.i) a sample not stained with a CD4 antibody, (B.i) a sample not stained with a LAP antibody, (C.i) a sample not stained with a GARP antibody and (D.i) a sample not stained with a LAG3 antibody.

2.4 Statistical analysis

Results from proliferation assays were analysed using non-parametric statistical tests in Graphpad Prism version 8.3.0 for Windows. A significant proliferative response determined by CFSE dilution was defined as the population of CD4⁺CFSE^{low} cells seen in response to a peptide stimulation which is greater than 1% and at least double that of the vehicle control. Similarly, a significant CD71 response to a peptide was defined as a population of CD4⁺CD71⁺ cells greater than 1% and double that of the vehicle control. In all cases *p* values <0.05 were considered statistically significant.

All statistical analysis of IHC data was performed using the SPSS package version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Patients whose deaths resulted from non-lung cancer related causes were censored at the time of death. The cohort was categorised into groups by defining the optimal cut off points for marker expression utilising X-tile software (X-Tile Bioinformatics Software, Yale University, version 3.6.1) [314]. Survival analysis was performed using Kaplan-Meier curves and the statistical significance of differences in disease-specific survival between groups was estimated using the log-rank test. The χ^2 test was used to determine the association between antigen expression (low versus high) and traditional clinical variables. The Cox proportional-hazards model was used for multivariate analysis in order to determine the relative risk and independent significance of individual factors. In all cases two-sided *p* values of <0.05 were considered statistically significant.

Statistical analysis for murine data was performed using non-parametric tests in Graphpad Prism version 9.4.1 for Windows. Comparison of marker expression between tumour infiltrating lymphocytes and splenocytes was done using paired non-parametric Wilcoxon test. Comparison of marker expression between the different tumour models was done using unpaired non-parametric Mann-Whitney test. Elispot data was compared using paired *t* test with Geisser-Greenhouse correction. Overall survival and tumour free survival were compared between the therapy groups using log-rank (Mantel-Cox) test. Comparison of marker expression between

the different therapy groups in the anti-tumour studies was done using unpaired non-parametric Mann-Whitney test. Data was considered statistically significant at the level of $p < 0.05$ for all tests and p values are stated where relevant in the figures/data.

3 Chapter 3 Human repertoire to citrullinated peptides and stratification into Th1 or Th3 responders with characterisation of Th3 cells.

3.1 Introduction

Lung cancer is the leading cause of cancer related deaths with 1.7 million dying of lung cancer worldwide in 2018 [315]. In 2016, lung cancer was the second most common cancer in both men and women and remains the highest cause of cancer mortality in the UK [316]. Lung cancer survival is influenced by age, gender, socioeconomic class and disease stage. When diagnosed in the latest stage, 1 year survival is 19% while 5 year survival is only 3% [316]. Historically, therapeutic agents were the non-specific cytotoxic killing of chemotherapy drugs. The identification of mutations in cancer promoting genes, so called “oncogenic drivers”, has led to the discovery of more defined molecular targeting agents. Such agents which targeted the membrane bound receptor tyrosine kinase epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK) and receptor tyrosine kinase encoded by ROS1 gene (ROS1) are now established in clinical practice. These drugs have shown good therapeutic outcomes but are only suitable for a small cohort of patients expressing the relevant mutation and tumour progression typically occurs within one year of initiating therapy [317]. The most common mechanism of resistance described to EGFR inhibitors is the acquisition of T790M mutation in the EGFR kinase domain [318] which led to the development of further agents such as Osimertinib which target this mutation.

As Lung cancer is mainly induced by the chemical carcinogens it has a high mutational burden [319] which generates specific neo-epitopes that can serve as immune targets [89]. When the immune system fails to reject the tumour, cancer cells rapidly grow and establish an increasingly immunosuppressive tumour environment; a process termed “the escape phase of immune editing” [251] as described in more detail previously (Chapter 1, Section 1.1). One of the ways tumour cells evade the immune

system is through the expression of programmed death ligand 1 (PD-L1). The last decade has witnessed the emergence of immune checkpoint inhibitors (programmed cell death 1 (PD-1)/programmed death ligand 1(PD-L1)) which have changed the therapeutic spectrum in many cancer types including non-small cell lung cancer (NSCLC) [320-324]. These immunotherapeutic agents work through cancelling out the inhibitory signal created by tumour cells and allowing T-cell recognition and killing. With only a proportion of patients expressing PD-L1 and hence having therapeutic benefit; the development of new therapies to act synergistically in combination with checkpoint inhibitors is necessary.

Ovarian cancer has an incidence of around 295,000 cases and a mortality of 184,000 in 2018 worldwide [325]. It is the 6th most common cancer in females and the 6th cause of cancer related death in females in the UK [326]. Its incidence in the UK has remained stable since the 1990s but it is anticipated to rise by 15% between the years 2014 and 2035 [326]. 60% of cases are diagnosed in the late stage when 1-year survival is only 54%. 5-year survival drops from 68% for stage 2 disease, to 27% for stage 3 and 13% for stage 4 ovarian cancer [326]. Optimal debulking surgery remains the cornerstone of ovarian cancer treatment with complete cytoreduction of all macroscopic disease being the single most important independent prognostic factor [327, 328]. This is usually coupled with adjuvant, neoadjuvant or both therapies with platinum-based chemotherapy. 80% respond to such treatment but around 70% recur within 5 years [329, 330]. The Gynaecologic Oncology Group (GOG) trial 218 and the International Collaborative Ovarian Neoplasm (ICON-7) [331, 332] were the two trials that were pivotal in the approval of the first targeted agent in ovarian cancer; Bevacizumab a monoclonal antibody against vascular endothelial growth factor (VEGF) which inhibits angiogenesis. Recently, SOLO 1&2, Study-19 and NOVA trials demonstrated the benefit of poly ADP ribose polymerase (PARP) inhibitors best seen but not limited to patients with BRCA (BReast CAncer gene) mutations [333-336]. PARP inhibitors utilize the concept of synthetic lethality; that is, they inhibit repair of DNA breaks which leads to cell death when the homologous recombination repair (HRR) pathway is defected due to BRCA

mutations. However, several mechanisms of resistance have been described to PARP inhibitors [337]. Furthermore, the use of checkpoint inhibitors in ovarian cancers has been slow despite PD-L1 expression being associated with a poor prognosis as in other tumour types [338]. Hamanishi et al. [339], were the first to report benefit from checkpoint inhibitors in humans and more clinical trials are ongoing. However, other immunotherapies may need to be developed to improve outcomes for ovarian cancer patients. These may include the use of vaccines that target siPTMs.

Utilization of neoepitopes to develop a vaccine therapy which works to prime an immune response to tumour specific antigens may well be ideal for synergy with checkpoint inhibitors. However, as most neoepitopes are specific to each patient, personalised vaccines are both expensive and time consuming. Vaccination with neoepitopes generated during post-translational modification seems to be attractive as these are generic to various types of cancers [43, 88]. Citrullinated and homocitrullinated peptides are post-translational modifications that have shown to induce strong tumour rejection when used to immunize mice [93, 144-146, 165, 340]. These need to be explored in cancer patients as potential monotherapies or in combination with other immunotherapies.

3.2 Aims

In an attempt to explore new treatments options for such patients, we explore the T-cell repertoire to siPTMs in lung and ovarian cancer patients' blood and compare that to healthy donors. We also examine the phenotype of CD4⁺ T-cells responding to siPTMs. Furthermore, the expression of LAP on antigen stimulated CD4 cells isolated from the PBMCs of lung cancer patients and its affiliation with function and proliferation is investigated. CD4⁺LAP⁺ cells are also sorted from the PBMCs of lung cancer patients to examine their suppressive abilities.

3.3 Results

3.3.1 Is there a T-cell repertoire in cancer patients that recognises post translational modifications (siPTMs)?

Previous studies carried out by our research group have shown that HLA transgenic mice made a T-cell response to citrullinated vimentin, α -enolase, nucleophosmin, BIP and cytokeratin and to homocitrullinated aldolase and vimentin which resulted in strong tumour rejection *in-vivo* [93, 144-146, 165, 340]. These responses are mediated by CD4 T-cells and depend upon IFN γ stimulating MHC-II expression on the tumour. Previous studies have also shown that normal human donors make an *in-vitro* CD4 Th1 response to these antigens. The question remained could cancer patients make a similar T-cell response?

CFSE dilution proliferation assays were performed on 25 lung and 19 ovarian cancer patients and responses compared to 29 healthy donors. A proliferative response to stimulation with siPTM was defined as a CD4⁺ loss of CFSE greater than 1% and double that of the background vehicle proliferation and the donor was classified as a “responder”. Previous studies had shown that removal of CD25⁺ T-cells in healthy donors prior to stimulation with a peptide resulted in enhanced responses and so this was also tested in cancer patient samples. An example of proliferation results in a lung cancer patient (LG30) is shown in (Figure 3-1); where CD4⁺ proliferative response (percentage of CFSE^{low} cells from the total CD4⁺ cells) to citrullinated BIP on day 10 increases from 1.25% in a CD25 non-depleted sample to 27.8% in the CD25 depleted sample. Figure 3-2 shows in contrast to healthy donors, the difference between the number of responders in CD25 depleted samples and CD25 non-depleted samples in 6 lung cancer donors did not reach statistical significance when a Fisher’s exact test was done ($p=0.5881$). However, to make the results comparable with the healthy donors all subsequent samples were CD25 depleted.

25 lung cancer patients, 19 ovarian cancer patients and 29 healthy donors were tested for CD4⁺ proliferative response (CFSE dilution) to citrullinated and homocitrullinated peptides (Figure 3-3, Table 3-1 & Table

3-2) and compared using Fisher's exact test. 10.0% (n=2 out of 20, p=0.0021) of lung cancer patients and 15.8% (n=3 out of 19, p=0.0078) of ovarian cancer patients showed a CD4⁺ proliferative response to α -enolase 241-260 (E241) whereas 55.2% (n= 16 out of 29) of healthy donors responded suggesting that the cancer had somehow attenuated the response to this antigen. Similar reduced responses which were of statistical significance were observed in lung cancer patients to citrullinated vimentin 28-49 (V28) where 23 lung cancer patients and 28 healthy donors were tested for responses and 26.1% (n=6) and 60.7% (n=17) responded respectively (p=0.0230). This reduction in response to citrullinated V28 did not reach statistical significance for ovarian cancer patients; where 19 were tested and 52.6% (n=10, p=0.7645) showed a CD4⁺ proliferative response. Equally, responses to citrullinated BIP 189-208 (BIP) were attenuated in ovarian cancer patients but not lung cancer patients. PBMCs isolated from 14 ovarian cancer patients, 17 lung cancer patients and 19 healthy donors were cultured with BIP and a CD4⁺ proliferative response was seen in 35.7% (n=5, p=0.0286), 58.8% (n= 10, p=0.2814) and 78.9% (n=15) of donors respectively. In contrast, similar numbers of responders were observed to citrullinated vimentin 415-433 (V415), NPM 266-285 and cytokeratin-8 101-120 (cyto8) in cancer patients and healthy donors. Responses to V415 were tested in 23 lung cancer patients and seen in 13.0% (n=3, p=0.3054), 19 ovarian cancer patients and seen in 21.1% (n=4, p=0.7365) and 28 healthy donors and seen in 28.6% (n=8). CD4⁺ responses to citrullinated NPM were noted in 20.0% of lung cancer patients (n=3 out of 15 tested, p >0.9999), 8.3% of ovarian cancer patients (n=1 out of 12 tested, p=0.3704) and 25.0% of healthy donors (n=5 out of 20 tested). The final citrullinated peptide used in culture was cyto8. To which responses were seen in 40.0% of lung cancer patients (n=2 out of 5 tested, p >0.999), 41.7% of ovarian cancer patients (n=5 out of 12 donors tested, p >0.9999) and 41.2% of healthy individuals (n=7 out of 17). (Figure 3-3, Table 3-1 & Table 3-2)

PBMCs from these donors were also tested for CD4⁺ proliferative responses to 3 homocitrullinated (hcrit) peptides; aldolase 74-93hcrit (A74), aldolase 140-157hcrit (A140) and vimentin 116-135hcrit (V116); none of which

showed statistical significance in the number of responders when comparing the 2 cancer patient groups to the healthy donors (Figure 3-3, Table 3-1 & Table 3-2). In lung cancer patients, 10, 16 and 16 donors had their PBMCs cultured with A74, A140 and V116 respectively and 40.0% (n=4), 43.8% (n=7) and 56.3% (n=9) showed CD4⁺ proliferative responses to these peptides, respectively. In ovarian cancer patients, 33.3% of donors tested for A74, A140 and V116 separately showed a CD4⁺ proliferative response where 3 donor PMBCs were isolated and cultured with each of these peptides individually and 1 in each case showed a CD4⁺ response. 13, 13 and 12 healthy individuals were tested for responses to A74, A140 and V116 respectively. 46.2% (n=6), 46.2% (n=6) and 83.3% (n=10) of healthy donors showed a CD4⁺ proliferative response to homocitrullinated A74, A140 and V116 respectively. It is to note that the number of donors tested with each of the 3 homocitrullinated peptides in the ovarian cancer group and that with cyto8 in the lung cancer group were less than 10 donors and so to be read with caution.

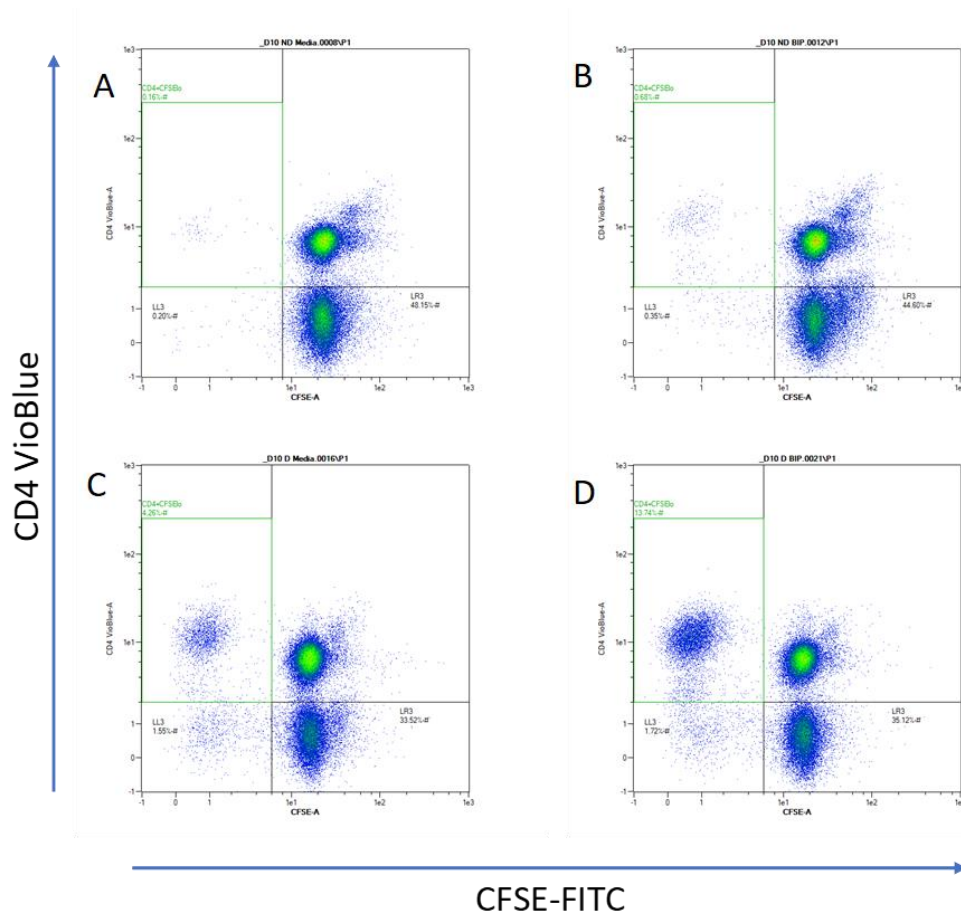


Figure 3-1: CD25 depletion of PBMCs increases proliferative responses identified as CD4+CFSElow population. PBMCs from lung cancer donor LG30 were isolated, CD25 depletion was done in (C) and (D) but not (A) and (B), on day 10 samples were analysed by direct fluorescence and flow cytometric analysis. A: vehicle well in CD25 non-depleted sample, B: BIP stimulated well in CD25 non-depleted sample, C: vehicle well in CD25 depleted sample and D BIP stimulated well in CD25 depleted sample.

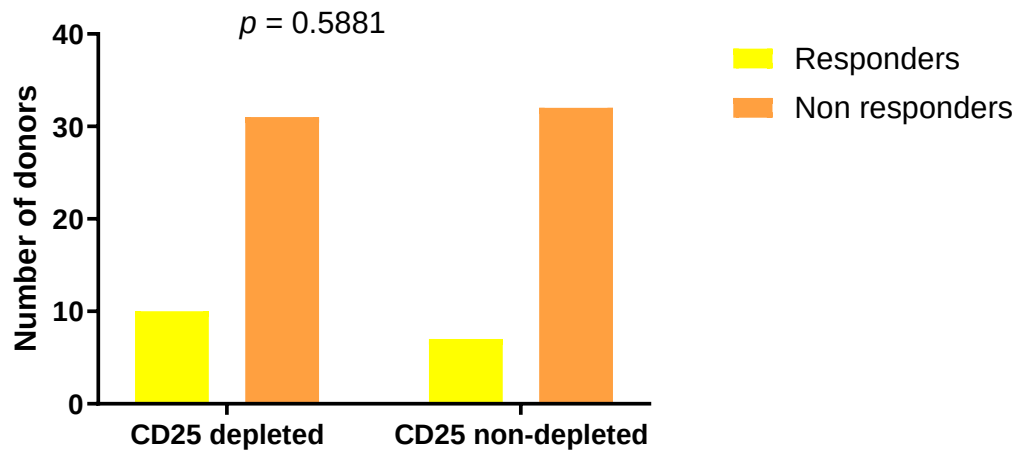


Figure 3-2: CD25 depletion of PBMCs from lung cancer patients did not result in better responses to culture with siPTMs ($p = 0.5881$ Fisher's exact test). A proliferative response to culture with a siPTM was defined as a CD4+ loss of CFSE greater than 1% and double that of the background vehicle proliferation. If this criterion was met, the donor was classified as a "responder" to the siPTM.

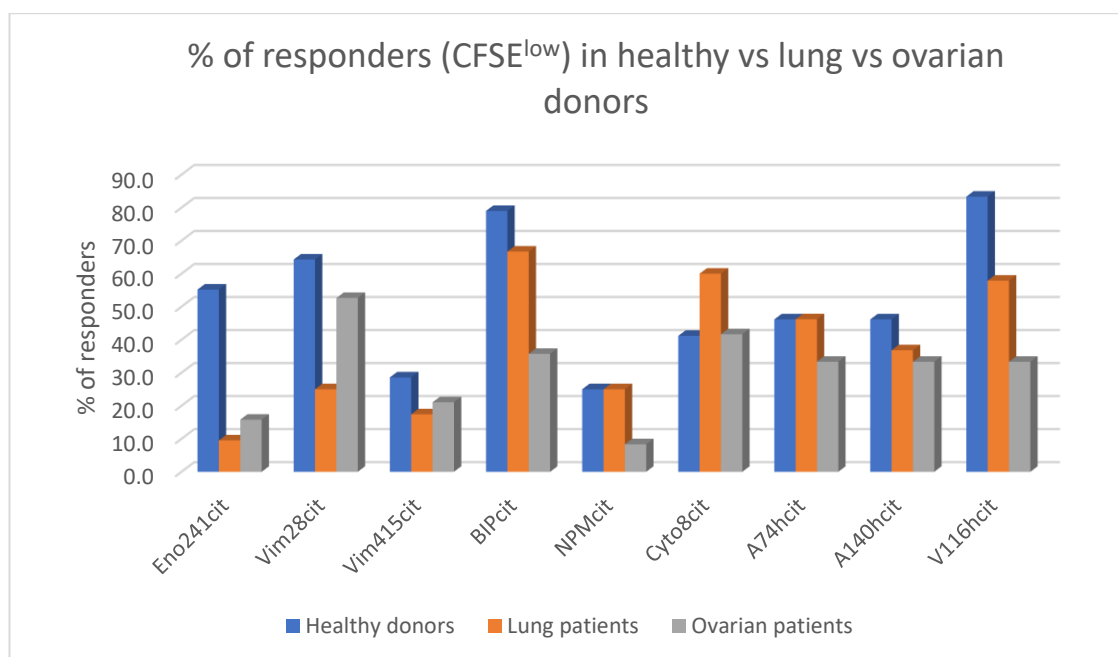


Figure 3-3: A histogram showing the percentage of donors who responded by CFSE dilution to culture with on of the 9 peptides in three donor groups; healthy individuals, lung cancer and ovarian cancer patients. Responder is defined as a donor which shows CD4+ loss of CFSE greater than 1% and double the negative control in response to culture with a siPTM. For number of donors tested and statistics refer to Table 3-1 and Table 3-2.

Table 3-1: Comparison between number of responders (a donor with CD4+CFSElow in siPTM stimulated well = double the background and >1%) to each peptide in lung cancer patients and healthy donors using Fisher's exact test.

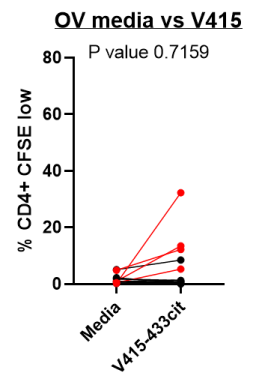
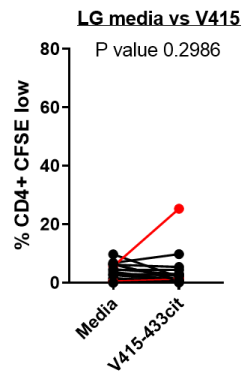
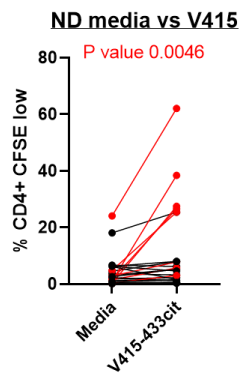
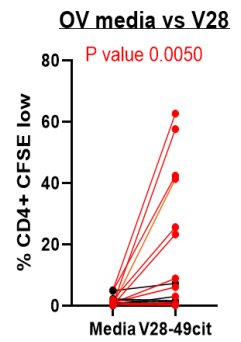
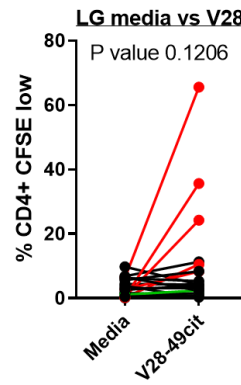
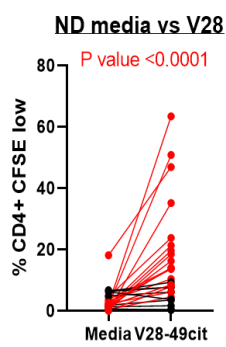
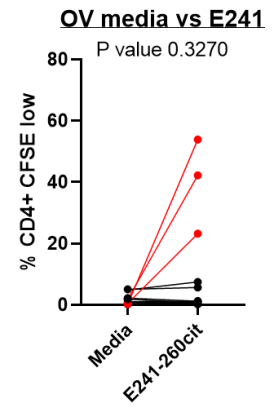
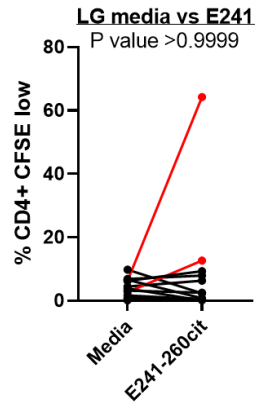
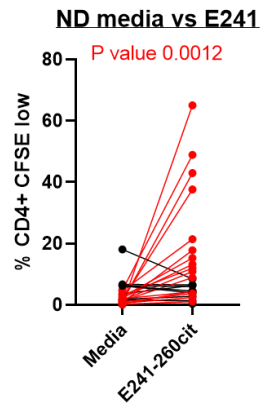
		Lung cancer patients # (%)	Healthy donors # (%)	P value (Fisher's exact test)
E241	Responders	2 (10.0%)	16 (55.2%)	0.0021
	Non-responders	18 (90.0%)	13 (44.8%)	
V28	Responders	6 (26.1%)	17 (60.7%)	0.0230
	Non-responders	17 (73.9%)	11 (39.3%)	
V415	Responders	3 (13.0%)	8 (28.6%)	0.3054
	Non-responders	20 (87.0%)	20 (71.4%)	
BIP	Responders	10 (58.8%)	15 (78.9%)	0.2814
	Non-responders	7 (41.2%)	4 (21.1%)	
NPM	Responders	3 (20.0%)	5 (25.0%)	>0.9999
	Non-responders	12 (80.0%)	15 (75.0%)	
Cyto8	Responders	2 (40.0%)	7 (41.2%)	>0.9999
	Non-responders	3 (60.0%)	10 (58.8%)	
A74	Responders	4 (40.0%)	6 (46.2%)	>0.9999
	Non-responders	6 (60.0%)	7 (53.9%)	
A140	Responders	7 (43.8%)	6 (46.2%)	>0.9999
	Non-responders	9 (56.3%)	7 (53.9%)	
V116	Responders	9 (56.3%)	10 (83.3%)	0.2232
	Non-responders	7 (43.8%)	2 (16.7%)	

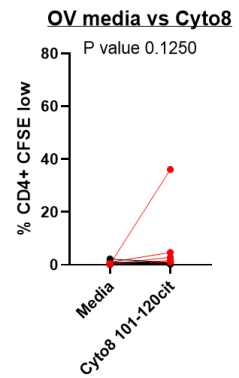
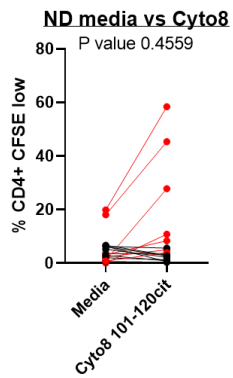
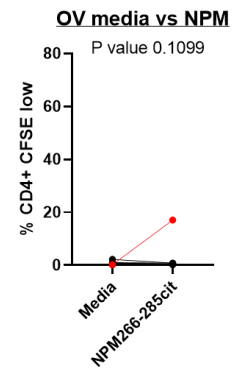
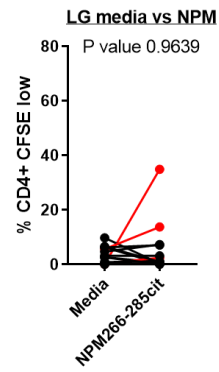
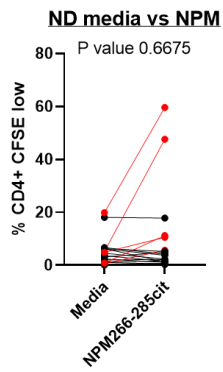
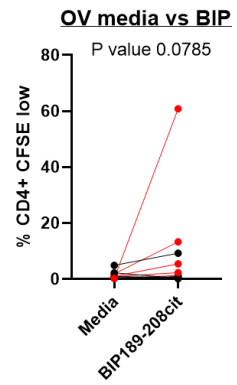
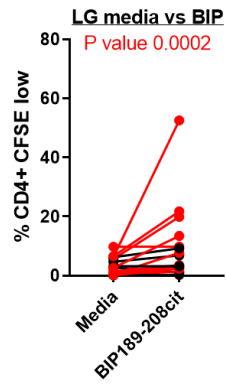
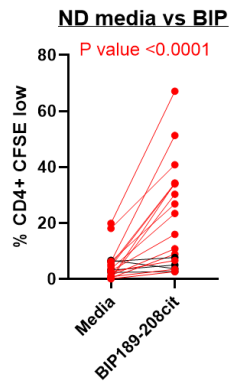
Table 3-2: Comparison between number of responders (a donor with CD4+CFSElow in siPTM stimulated well = double the background and >1%) to each peptide in ovarian cancer patients and healthy donors using Fisher's exact test.

		Ovarian cancer patients # (%)	Healthy donors # (%)	P value (Fisher's exact test)
E241	Responders	3 (15.8%)	16 (55.2%)	0.0078
	Non-responders	16 (84.2%)	13 (44.8%)	
V28	Responders	10 (52.6%)	17 (60.7%)	0.7645
	Non-responders	9 (47.4%)	11 (39.3%)	
V415	Responders	4 (21.1%)	8 (28.6%)	0.7365
	Non-responders	15 (78.9%)	20 (71.4%)	
BIP	Responders	5 (35.7%)	15 (78.9%)	0.0286
	Non-responders	9 (64.3%)	4 (21.1%)	
NPM	Responders	1 (8.3%)	5 (25.0%)	0.3704
	Non-responders	11 (91.7%)	15 (75.0%)	
Cyto8	Responders	5 (41.7%)	7 (41.2%)	>0.9999
	Non-responders	7 (58.3%)	10 (58.8%)	
A74	Responders	1 (33.3%)	6 (46.2%)	>0.9999
	Non-responders	2 (66.7%)	7 (53.9%)	
A140	Responders	1 (33.3%)	6 (46.2%)	>0.9999
	Non-responders	2 (66.7%)	7 (53.9%)	
V116	Responders	1 (33.3%)	10 (83.3%)	0.1538
	Non-responders	2 (66.7%)	2 (16.7%)	

3.3.2 Is there a preferential T-cell response in cancer patients to siPTMs of certain antigens?

To compare T-cell responses to siPTMs for specific antigens, the percentage of CD4⁺CFSE^{low} population in response to culture with each peptide was compared to that seen in the negative control well (media or vehicle) in each donor group separately (lung cancer, ovarian cancer patients and healthy donors) (Figure 3-4). A paired statistical test (Wilcoxon test) was used to determine the P value. Proliferating CD4⁺CFSE^{low} cells in response to culture with E241 and V415 were significantly higher than the CD4⁺CFSE^{low} population in the negative control for the healthy donor group (p=0.0012 and 0.0046 respectively) but this comparison was not significantly different in the lung and ovarian cancer patients. However, responses to V28 was significantly different than the media for ovarian cancer patients as well as healthy donors (p=0.0050 and <0.0001 respectively) but not lung cancer patients (p=0.1206). Responses to BIP, NPM and cyto8 were significantly different only to BIP in lung cancer patients (p=0.0002) and healthy donors (p<0.0001) but not ovarian cancer patients (p=0.0785). When comparing the proliferating CD4⁺CFSE^{low} cells in response to culture with each of the homocitrullinated peptides to the same population in the media well; it was noted that A140 and V116 were significantly different in both lung cancer patients (p=0.0050 and 0.0002 respectively) and healthy donors (p=0.0024 and 0.0005 respectively) but not ovarian cancer patients possibly due to the low number of ovarian cancer donors tested with the homocitrullinated peptides. Responses to A74, NPM and cyto8 were not significantly different in any of the donor groups. Graphical representation for the 3 homocitrullinated peptides in ovarian cancer patients and for cyto8 in lung cancer patients were excluded due to the low number of donors. (Figure 3-4)





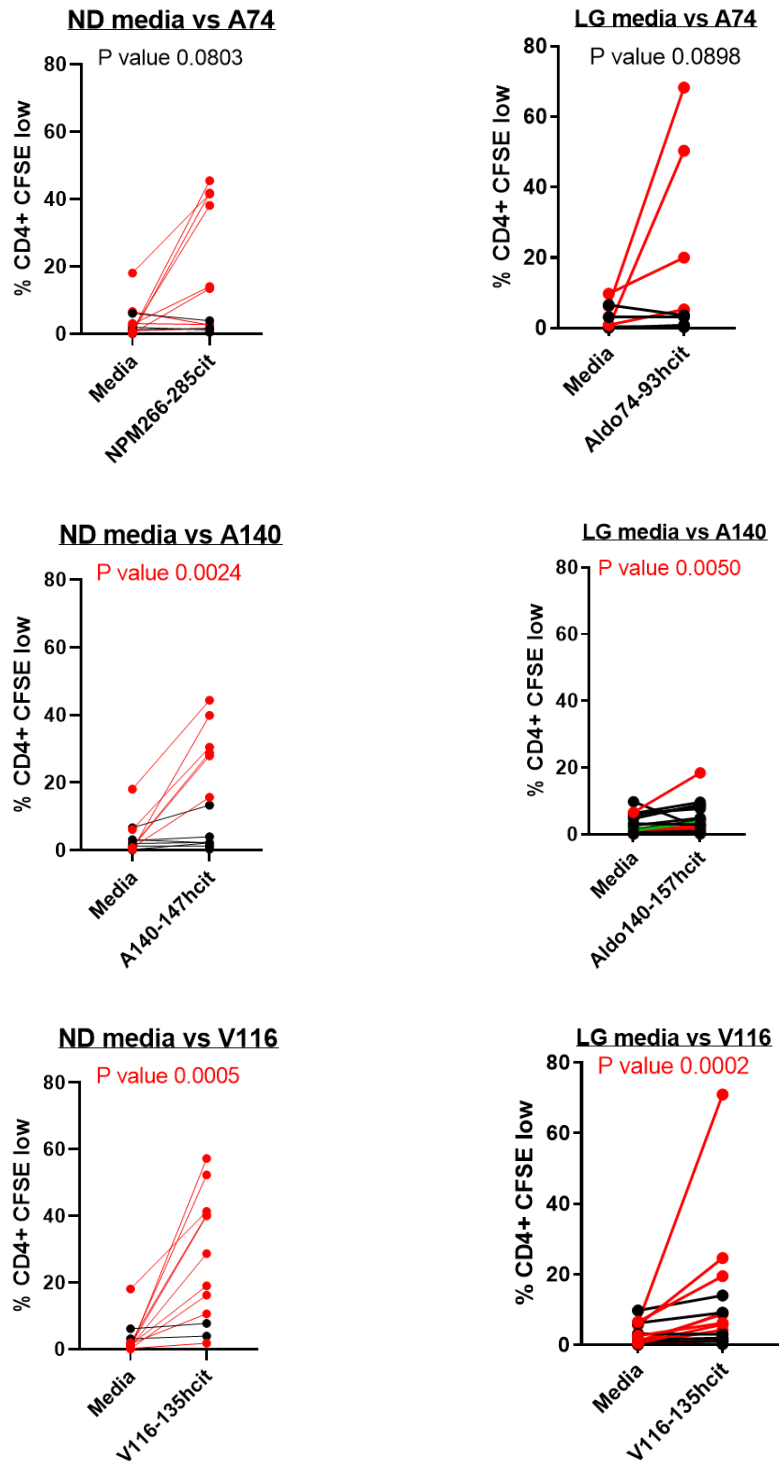


Figure 3-4: Comparison between the percentage of CD4+CFSElow population in response to each peptide with its corresponding CD4+CFSElow in the negative control using Wilcoxon statistical test. ND: normal healthy donor, LG: lung cancer patient and OV: ovarian cancer patient. PBMCs were isolated, CD25 depleted, CFSE labelled and cultured with or without siPTMs. On day 7-10 direct fluorescence and flow cytometry was done. Responders (in red) defined as CD4+ loss of CFSE greater than 1% and double that of the background vehicle proliferation.

3.3.3 What cytokines do the proliferating T-cells responding to siPTMs in cancer patients produce?

We showed that the siPTM peptides can induce proliferation, but this could be directed by a number of cell types. To determine whether the proliferating cells are pro-inflammatory we looked for the Th1 associated cytokine IFN γ . The percentage of proliferating cells (CD4⁺CFSE^{low}) producing IFN γ in donors responding to stimulation with siPTM was compared between healthy, lung cancer and ovarian cancer donors (Figure 3-5). An unpaired non-parametric Mann-Whitney test was used to determine the statistical significance. It was shown that proliferating CD4 T-cells producing IFN γ (CD4⁺CFSE^{low}IFN γ ⁺) in both lung and ovarian cancer patients' responding to a siPTM, were lower compared to healthy donors ($p=0.0090$ and <0.0001 respectively). Furthermore, CD4⁺CFSE^{low} cells producing IFN γ in response to a peptide in ovarian cancer patients were lower compared to lung cancer patients ($p=0.0043$). A Kruskal-Wallis test comparing all three groups collectively was also of statistical significance ($p < 0.0001$). This suggests that even the T-cells in cancer patients that do proliferate, less cells are producing IFN γ . The question then arose did these cells produce immunosuppressive cytokines? IL10 and LAP expression were only tested in lung cancer patients and healthy donors not ovarian patients. Our experiments showed that less than 4% of CD4⁺CFSE^{low} cells isolated from both healthy donors and lung cancer patients produced IL10 (data not shown). Also, there was no significant difference in the percentage of CD4⁺CFSE^{low} cells expressing LAP between healthy and lung cancer patients (Figure 3-6). Perhaps the cells producing the immune suppression in cancer patients were terminally differentiated and did not proliferate. The early activation marker CD71 was therefore used to determine if T-cells were activated by siPTMs but did not proliferate.

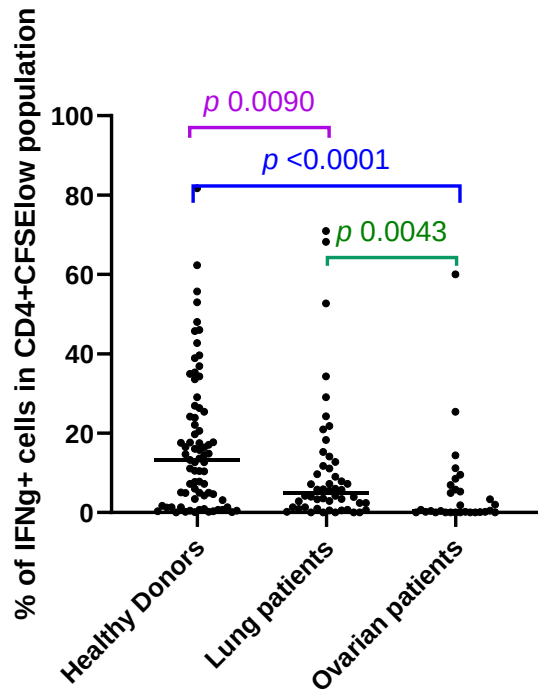


Figure 3-5: Lung cancer (LG) and ovarian cancer (OV) blood donors have lower percentage of IFN γ + cells in CD4+CFSElow cell population compared to healthy donors (HD). Comparison done using Mann-Whitney statistical test. PBMCs were isolated, CD25 depleted, CFSE labelled and cultured with or without a siPTMs. On day 7-10 direct fluorescence and flow cytometry was done on samples. The percentage of IFN γ + cells were measured in the CD4+CFSElow population (proliferating cells).

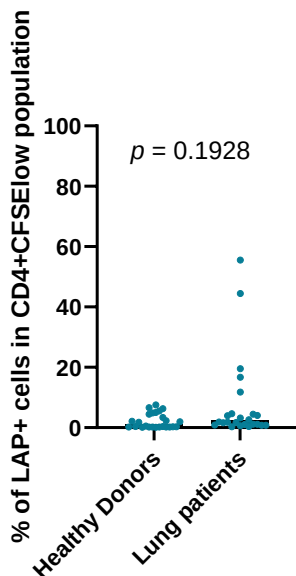


Figure 3-6: Comparison between healthy donors and lung cancer patients in percentage of LAP+ cells in CD4+CFSElow cell population using Mann-Whitney statistical test. PBMCs were isolated, CD25 depleted, CFSE labelled and cultured with or without a siPTMs. On day 7-10 direct fluorescence and flow cytometry was done on samples. Percentage of LAP+ cells were measured in the CD4+CFSElow population (proliferating cells).

3.3.4 Can activated T-cells responding to siPTMs in cancer patients be detected by CD71 expression?

To examine T-cell activation in response to siPTM peptide cultured PBMCs were stained for CD71 on days 4, 7 and 10 as per methodology. CD71, a transferrin receptor and an early activation marker was tested in 13 lung patients and 7 healthy donors in order to identify a population of CD4⁺ cells that was perhaps activated but were incapable of proliferation. A CD71 response to a peptide was defined as a population of CD4⁺CD71⁺ cells greater than 1% and double that of the negative control. There was no significant difference between the number of CD71 responders to culture with each of the siPTM in the lung cancer patients and healthy individual groups (Figure 3-7, Table 3-3). The percentage of CD71 responders to E241 and V28 approached significance and maybe increasing the number of healthy donors would uncover this observation and add statistical significance. It was noted that the response to these two peptides was lower, perhaps immune suppressed, in cancer patients compared to healthy donors. 11.1% (n=1 out of 9 tested) of lung patients showed CD71 expression in response to E241 compared to a higher percentage of 57.1% (n=4 out of 7 tested) in the healthy donor group (p=0.1058). Activated CD4⁺CD71⁺ cells in response to V28 were seen in 16.7% (n=2 out of 12 tested) in the lung cancer group compared to 57.1% (n=4 out of 7) in the healthy donor group (p=0.1287).

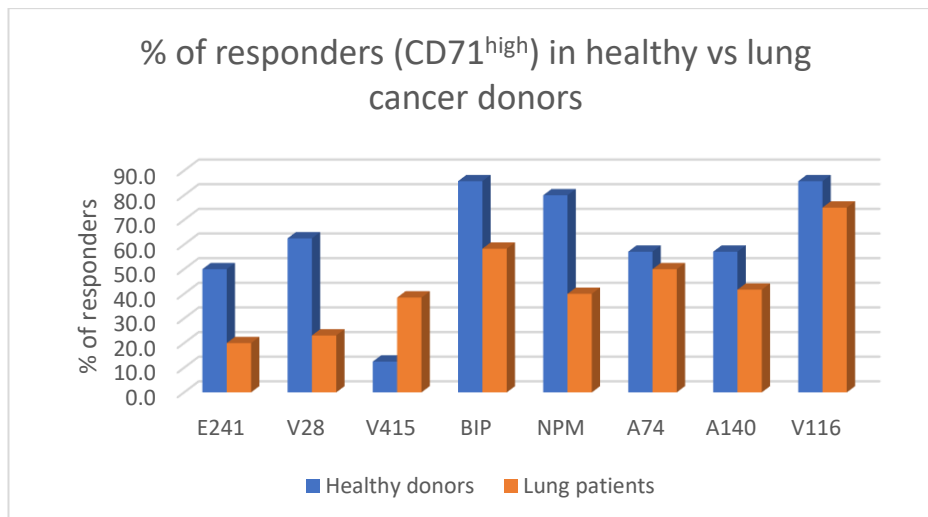


Figure 3-7: A histogram showing the percentage of donors who responded to culture with siPTM by CD71 expression in healthy individuals and lung cancer patients. CD71 responder is defined as a donor with CD4+CD71+ population greater than 1% and double the negative control in response to culture with a siPTM. For number of donors tested and statistics refer to Table 3-3.

Table 3-3: Comparison between number of responders (a donor with CD4+CD71^{high} = double the background and >1%) to each peptide in lung cancer patients and healthy donors using Fisher's exact test.

		Lung cancer patients # (%)	Healthy donors # (%)	P value (Fisher's exact test)
E241	Responders	1 (11.1%)	4 (57.1%)	0.1058
	Non-responders	8 (88.9%)	3 (42.9%)	
V28	Responders	2 (16.7%)	4 (57.1%)	0.1287
	Non-responders	10 (83.3%)	3 (42.9%)	
V415	Responders	4 (33.3%)	1 (14.3%)	0.6027
	Non-responders	8 (66.7%)	6 (85.7%)	
BIP	Responders	6 (54.6%)	6 (85.7%)	0.3156
	Non-responders	5 (45.5%)	1 (14.3%)	
NPM	Responders	3 (33.3%)	4 (80.0%)	0.2657
	Non-responders	6 (66.7%)	1 (20.0%)	
A74	Responders	2 (40.0%)	4 (57.1%)	>0.9999
	Non-responders	3 (60.0%)	3 (42.9%)	
A140	Responders	5 (45.5%)	4 (57.1%)	>0.9999
	Non-responders	6 (54.6%)	3 (42.9%)	
V116	Responders	8 (72.7%)	6 (85.7%)	>0.9999
	Non-responders	3 (27.3%)	1 (14.3%)	

3.3.5 What cytokines do the T-cells in cancer patients showing the early activation marker CD71 produce?

The profile of CD4⁺ cells expressing CD71 in lung cancer patients and healthy donors was examined by looking for the percentage of cells that were IFN γ positive and LAP positive (Figure 3-8, Figure 3-9). Comparison was done between the two groups of donors using an unpaired non-parametric statistical test (Mann-Whitney test) to determine the P value. In cancer patients, LAP⁺ expression by CD4⁺CD71⁺ cells was higher than in healthy donors ($p=0.0424$, Figure 3-8). Also, the IFN γ ⁺ cells in the CD4⁺CD71⁺ population was significantly lower in lung cancer patients compared to healthy donors ($p= 0.0445$, **Error! Reference source not found.**). This suggests that perhaps in cancer patients activated cells are prevented from exhibiting a proliferative response due to the suppressive effect of LAP which is more evident than in healthy donors. This led to examining the correlation between proliferation and the expression of LAP on CD4⁺CD71⁺ cells.

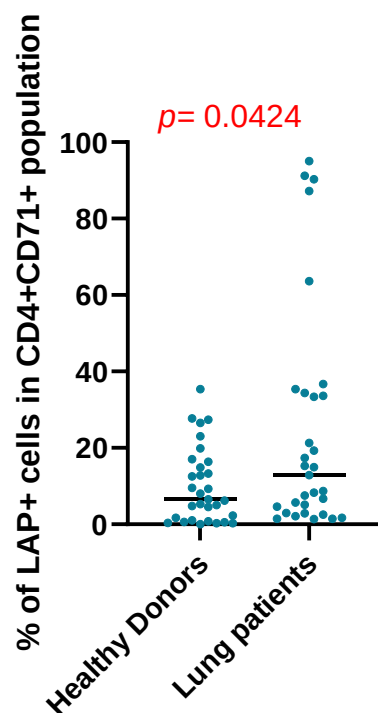


Figure 3-8: Comparison between healthy donors and lung cancer patients in percentage of LAP⁺ cells in CD4⁺CD71⁺ cell population using Mann-Whitney test. PBMCs were isolated, CD25 depleted, CFSE labelled and cultured with or without a siPTMs. On day 7-10 direct fluorescence and flow cytometry was done on samples. LAP⁺ cells were measured in the CD4⁺CD71⁺ population (Antigen specific activated cells).

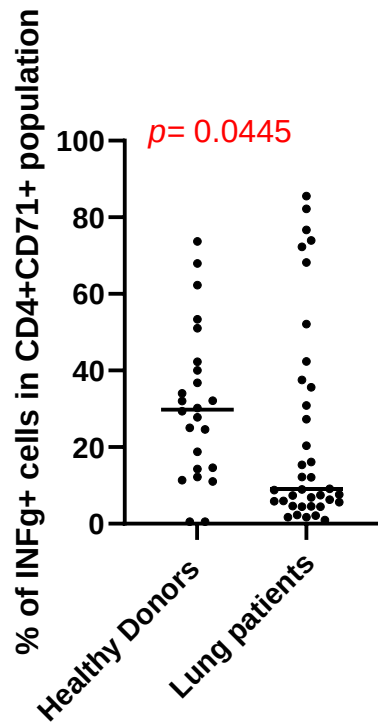


Figure 3-9: Comparison between healthy donors (HD) and lung cancer patients (LG) in percentage of IFN γ + cells in CD4+CD71+ cell population using Mann-Whitney test. PBMCs were isolated, CD25 depleted, CFSE labelled and cultured with or without a siPTMs. On day 7-10 direct fluorescence and flow cytometry was done on samples. IFN γ + cells were measured in the CD4+CD71+ population (Antigen specific activated cells).

3.3.6 Is there a correlation between T-cells responding to siPTMs in cancer patients and the LAP positive cells in the blood?

As described in the methodology the expression of LAP and CD25 by CD4⁺ cells were measured in 9 lung cancer patients at day zero prior to culture (Figure 3-10,). This was done both prior and post CD25 depletion. It was noted that there is a population of CD4⁺LAP⁺ cells which was removed by CD25 depletion. Figure 3-10 shows the reduction of CD4⁺LAP⁺ cells from 11.58% prior to CD25 depletion to 2.82% after CD25 depletion in LG25 donor as an example. However, the difference between the CD4⁺ cells expressing LAP prior to and post CD25 depletion in all the 9 lung cancer donors did not quite reach statistical significance when a Wilcoxon test was done ($p=0.0547$, Figure 3-11).

The Spearman correlation test was used to determine the correlation between proliferation index in the lung cancer patients and the expression of LAP in CD4⁺ cells (LAP+CD25-) (Figure 3-12). Proliferation index was used to normalise the data, this was calculated for each individual donor by dividing the percentage of CD4⁺CFSE^{low} cells responding to a peptide by the percentage of CD4⁺CFSE^{low} in the media. A negative correlation ($r -0.2579$, $p=0.0104$) was seen between proliferation and the percentage of CD4⁺LAP⁺ cells present at day zero (Figure 3-12A) this was significant for the CD25 depleted samples ($r -0.2731$, $p=0.0348$, Figure 3-12C) but not for the CD25 non-depleted samples ($r -0.2637$, $p=0.1097$, Figure 3-12B). This suggests that CD4⁺LAP⁺CD25- maybe the more suppressive population of cells dictating control of proliferative responses.

Moreover, the results showed a correlation between T-cells responding to siPTMs in cancer patients and the percentage of LAP expressed by siPTMs specific cells (Figure 3-13 & Figure 3-14). The level of LAP expressed by peptide specific CD71⁺ cells was strongly negatively correlated by Spearman correlation with the degree of proliferation seen in response to that same peptide (CD4⁺CFSE^{low} cells) ($r -0.4424$, $p=0.0003$, Figure 3-13). Figure 3-14 shows a different graphical representation where proliferation was more likely to happen if the expression of LAP in peptide

specific CD4⁺CD71⁺ cells was lower (p=0.0135 Mann-Whitney test). This suggests that antigen specific cells were being differentiated into Th3. However, the expression of LAP by CD71⁺ did not correlate significantly with the production of IFN γ by CFSE^{low} cells (Figure 3-15, p=0.4901) indicating that CD71⁺LAP⁺ cells prevent proliferation but not the production of IFN γ .

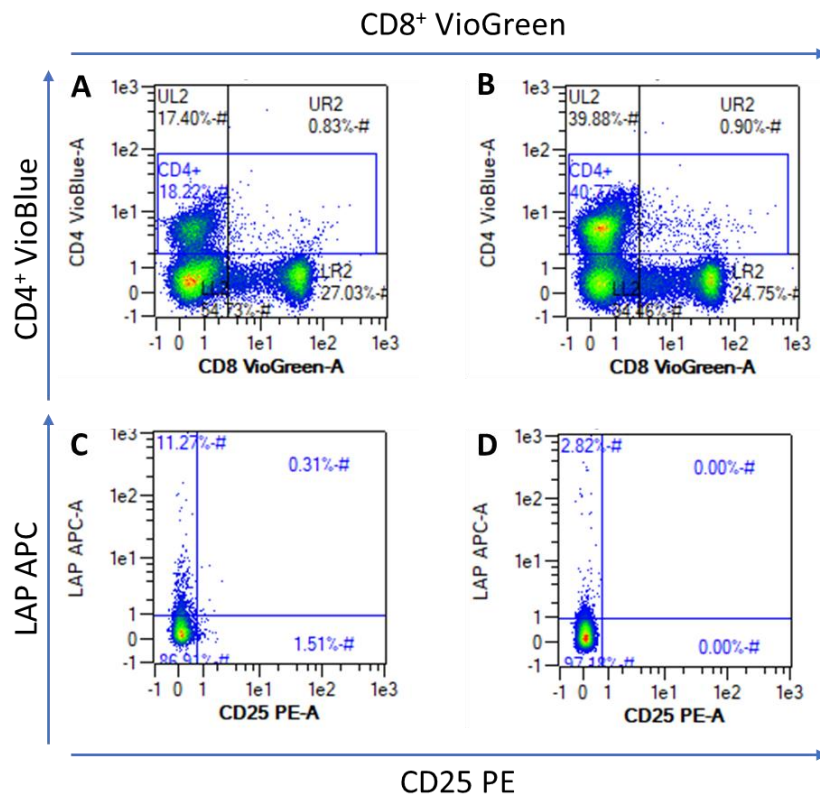


Figure 3-10: Direct fluorescence and flow cytometry of PBMCs isolated from a cancer patient showing day zero percentage of LAP⁺ and CD25⁺ cells in the CD4⁺ cell population prior to CD25 depletion and post CD25 depletion. (A) sample taken prior to CD25 depletion showing gating on CD4⁺ cells, (B) sample taken after CD25 depletion showing gating on CD4⁺ cells, (C) sample taken prior to CD25 depletion with gating set on CD4⁺ cells (as shown in (A)) showing expression of LAP and CD25 and (D) sample taken after CD25 depletion with gating set on CD4⁺ cells (as shown in (B)) showing expression of LAP and CD25.

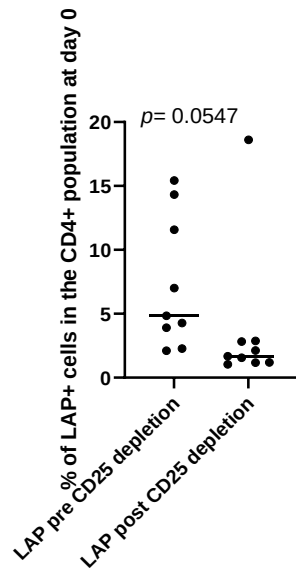


Figure 3-11: Percentage of LAP+ cells in CD4+ cell population at day zero prior to and post CD25 depletion in 9 lung cancer donors using Wilcoxon statistical test assess by direct fluorescence and flow cytometry on PBMCs isolated from cancer patients.

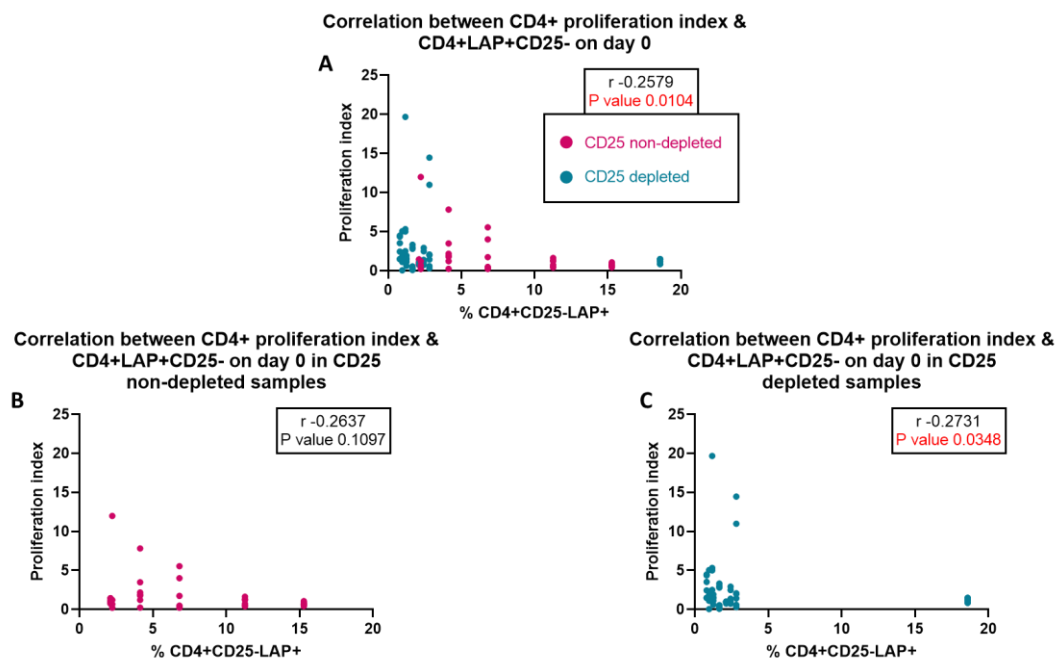


Figure 3-12: Correlation between proliferation index and CD4+LAP+CD25- cells assessed by direct fluorescence and flow cytometry on PBMCs isolated from cancer patients on day 0. Proliferation index calculated for each individual donor by dividing the percentage of CD4+CFSElow cells responding to a peptide by the percentage of CD4+CFSElow in the media (A) all samples included regardless of whether it was prior to CD25 depletion or afterwards, (B) CD25 non-depleted samples and (C) CD25 depleted samples. Statistical analysis done using Spearman correlation test.

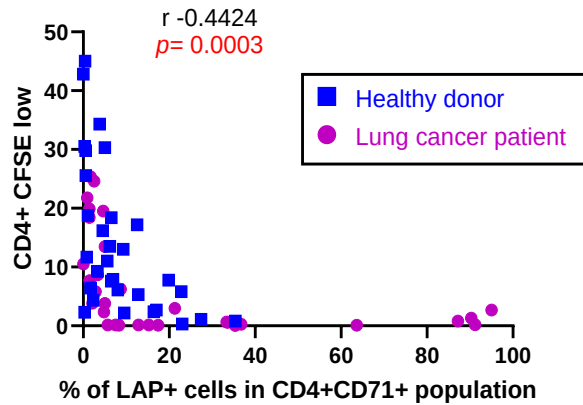


Figure 3-13: Correlation between the percentage of LAP+ cells in the antigen specific CD4+CD71+ population and the percentage of CD4+CFSElow cells responding to the same peptide. Spearman correlation test was used for statistical analysis. PBMCs were isolated, CD25 depleted, CFSE labelled and cultured with or without a siPTMs. On day 7-10 direct fluorescence and flow cytometry was done on samples to assess for expression of CD4, LAP, CD71 and loss of CFSE dye.

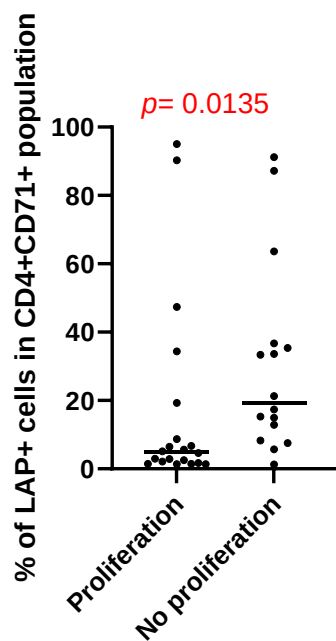


Figure 3-14: Relation between the presence or absence of proliferation and the percentage of LAP+ cells in the CD4+CD71+ population using Mann-Whitney test. PBMCs were isolated, CD25 depleted, CFSE labelled and cultured with or without a siPTMs. On day 7-10 direct fluorescence and flow cytometry was done on samples to assess for expression of CD4, LAP, CD71 and loss of CFSE dye.

Correlation between LAP in CD71⁺ and IFN γ in CFSE^{low} in lung cancer patients

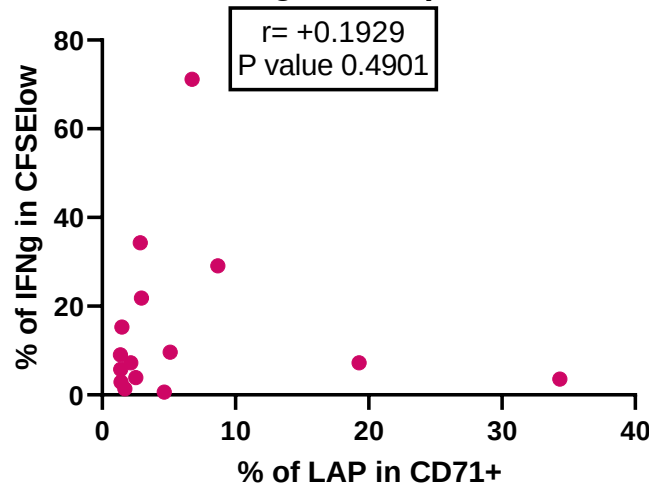


Figure 3-15: Correlation between peptide specific CD71⁺ LAP expression and the production of IFN γ by CFSE^{low} cells. PBMCs were isolated, CD25 depleted, CFSE labelled and cultured with or without a siPTMs. On day 7-10 direct fluorescence and flow cytometry was done on samples to assess for expression of CD4, LAP, CD71, IFN γ and loss of CFSE dye. Spearman correlation t test was used for statistical analysis.

3.3.7 Characterization of Th3 cells: Do LAP expressing cells truly have suppressive ability?

The results show that cancer patients have a repertoire to siPTMs and that they can mount an IFN γ producing CD4 T-cell response (Th1 response) but some of the responses to certain peptides are halted perhaps because cancer patients have higher levels of suppressive CD4 T-cells expressing LAP (Th3 response) in their blood compared to healthy donors. We show that antigen specific cells activated in response to culture with siPTMs (CD4+CD71+) and expressing LAP are inversely correlated to proliferation suggesting they are terminally differentiated with suppressive ability. Indeed, Scurr et al [244], report that LAP⁺ cells isolated from the tumour of colon cancer patients are ~50 fold more suppressive than the conventional Foxp3 expressing T-regs. Proving that antigen specific CD4+CD71+LAP⁺ cells isolated from the blood of cancer patients are suppressive would have been technically difficult. Isolation of CD4+CD71+LAP⁺ population would require 10 days of culture with siPTMs to obtain an antigen specific population and then sorting a minute population of LAP⁺ cells that would perhaps be unhealthy cells. Instead, we isolated CD4+LAP⁺ cells from the PBMCs of 4

lung cancer patients and cultured them with CD4+LAP- cells to assess the suppressive ability of the LAP+ cells and replicate what is documented in literature. First, PBMCs were isolated, stained with CD4 and LAP antibodies and sorted into CD4+LAP+ and CD4+LAP- populations. CD4+LAP- cells were labelled with CFSE dye and cultured with a TCR stimulus (CD3/CD28 beads). CD4+LAP+ cells were either added to culture or not and the proliferation of LAP- cells was assessed following 4 days of culture. Figure 3-16 shows an example of flow cytometry histogram from a lung cancer donor where proliferation of CD4+LAP- cells in the absence of CD4+LAP+ cells reduced from 37.11% to 26.63% upon the addition of CD4+LAP+ cells to culture. Figure 3-17A shows that the percentage of proliferating cells (CD4+LAP-) was consistently lower in the presence of CD4+LAP+ cells in all lung cancer donors ($p=0.0156$, Wilcoxon paired non-parametric test). The percentage of suppression was calculated using the formula = $[(\text{proliferation of LAP- in the absence LAP+} - \text{proliferation of LAP- in the presence of LAP+}) \div \text{proliferation of LAP- in the absence LAP+}] \times 100$. The median percentage of suppression of CD4+LAP+ cells was found to be 51.7% ($p=0.0156$, Wilcoxon paired non-parametric test, Figure 3-17B). This confirms that the LAP+ cells are able to suppress proliferative responses.

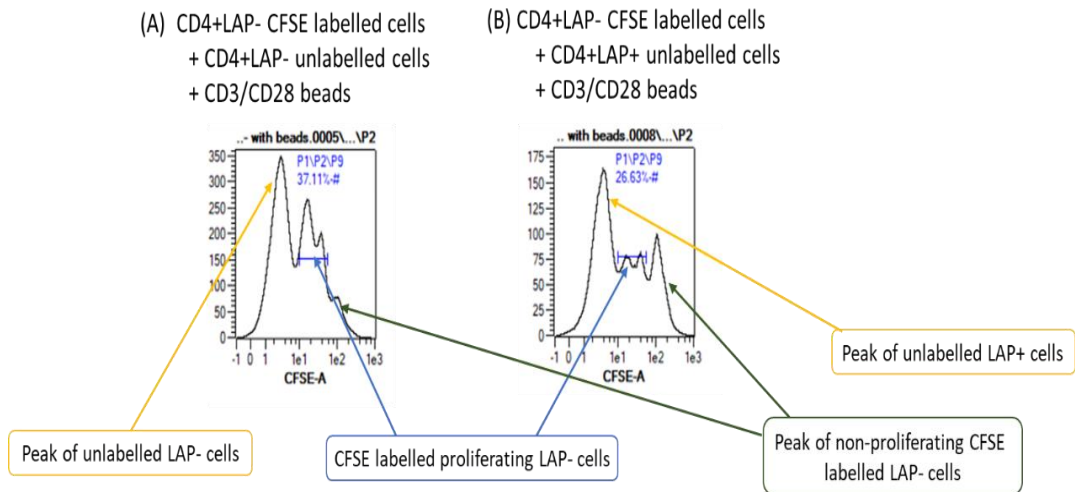


Figure 3-16: A histogram example showing the reduction of the percentage of proliferating CD4+LAP- cells upon the addition of CD4+LAP+ cells to culture in the presence of TCR stimulus CD3/CD28 beads. PBMCs from a lung cancer patient were isolated, stained with CD4 and LAP antibodies, sorted into CD4+LAP- and CD4+LAP+ populations. CD4+LAP- population was labelled with CFSE dye and assessment of proliferation was done by direct fluorescence and flow cytometry on day 4. (A) Histogram from well containing CD4+LAP- (CFSE labelled) and CD4+LAP- (not CFSE labelled) cultured at 1:1 ratio in the presence of CD3/CD28 beads. (B) Histogram from well containing CD4+LAP- (CFSE labelled) and CD4+LAP+ (not CFSE labelled) cultured at 1:1 ratio in the presence of CD3/CD28 beads. Proliferation reduced from 37.11% to 26.63%, percentage of suppression 28%.

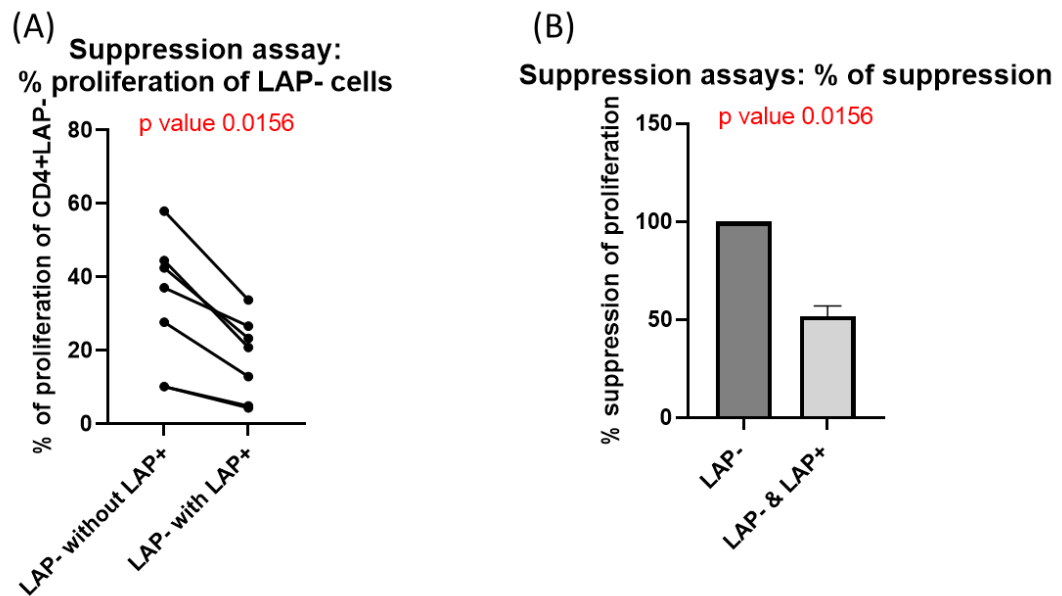


Figure 3-17: PBMCs from 4 lung cancer patients were isolated, stained with CD4 and LAP antibodies, sorted into CD4+LAP+ and CD4+LAP- population. CD4+LAP- population was loaded with CFSE dye and cultured with a TCR stimulus (CD3/CD28 beads) with or without CD4+LAP+ cells (2 donors had enough cells to duplicate culture conditions). On day 4, proliferation was assessed by direct fluorescence and flow cytometry. Paired, non-parametric analysis (Wilcoxon test) was used to show that proliferation was lower in the presence of CD4+LAP+ cells. (A) shows percentage of proliferating CD4+LAP-cells while (B) shows percentage of suppression calculated by $[(\text{proliferation of LAP- in the absence LAP+} - \text{proliferation of LAP- in the presence of LAP+}) \div \text{proliferation of LAP- in the absence LAP+}] \times 100$.

3.4 Discussion

It has been shown in murine models that CD4 T-cells can induce a potent anti-tumour immunity and promote long-term survival. This is mediated by CD4 T-cells recognising modified stress induced self-epitopes (citrullinated and homocitrullinated peptides), the release of IFN γ and upregulation of MHC class II which in turn stimulates further infiltration by cytotoxic T-cells and secretion of cytokines [145, 146, 162, 163]. It has previously been shown that healthy donors have a T-cell repertoire to siPTMs [163]. Here, it is shown that both lung and ovarian cancer patients also have a T-cell response to these siPTMs, but the responses are attenuated when compared to healthy donors. It was shown that this repertoire may not only be antigen and HLA type dependent but also dictated by the cancer type. In lung cancer there were fewer patients who showed a CD4 T-cell response to E241 and V28 than those seen in healthy donors, suggesting they have been influenced by the cancer. This attenuation is not evident for responses to other peptides including citrullinated V415, BIP and NPM as well as homocitrullinated A74, A140 and V116. In ovarian cancer, the tumour immune suppression of responses to E241 and BIP is apparent but not to the other peptides. Additionally, it is evident that certain cancer types may have preferential T-cell responses to certain antigens. This is obvious in our results for lung cancer favouring responses to citrullinated BIP, homocitrullinated A140 and V116. While ovarian cancers are as similarly responsive as healthy donors to citrullinated V28. This may help guide the selection of the peptide combination to be included in a vaccine therapy.

In healthy donors the phenotypic profile of the proliferating cells was that associated with IFN γ production suggesting a Th1 response. In cancer patients not only is attenuation seen to the number of patients showing a CD4 proliferative response to a specific epitope but in addition even those CD4 cells that proliferate seem to be less likely to produce IFN γ . In the context of immune surveillance, it is perhaps that cells initially make a good Th1 response to siPTMs but if the tumour mutates and adopts immunosuppressive traits, in the escape phase of immune editing, then these

stimulated cells can be converted to Th3 or have regulatory phenotype suppressing proliferation and cytokine production [251].

Th3 (T-helper 3) cells have been defined as a subset of Tregs which are induced upon antigen stimulation, suppress effector T-cells through TGF- β secretion and promote tumour progression and immune tolerance. This subset of cells is also capable of producing IFN γ but at much lower levels than Th1 [341, 342]. We now show a population of siPTM peptide specific CD4 cells which are terminally differentiated and incapable of proliferating. These were identified as being activated and expressing the early activation marker CD71. CD71, a transferrin receptor, has been reported to be expressed on CD4 T-cells following antigen activation along with other markers such as CD25, CD69 and CD154 (CD40 ligand) which have also been described as activation markers [343-345]. With a retrospective view, perhaps a marker such as CD154 which is reported to be robust and highly sensitive would have been better to use in this instance [346-348]. However, CD71 still provides a valid marker for T-cell activation. In our lung cancer patients these antigen specific T-effector type of CD4 cells expressing CD71 have high proportions of LAP expression compared to their counterpart in healthy donor blood. Furthermore, these antigen specific activated LAP expressing cells showed a strong negative correlation with proliferation. Thus, suggesting that these cells have differentiated into a Th3 type of immune suppressive cells. To this effect we conclude that antigen specific cells differentiate into either Th1 (low in LAP expression and high in IFN γ production) or Th3 cells (high LAP expression but low numbers of IFN γ expressing cells) which may well differ between the antigens as to which immune cells dominate. In the clinic, this differentiation can be helpful as a biomarker to direct the personalisation and selection of patients who are more likely to have a therapeutic response to this modified antigen approach as a vaccine therapy.

The presence of T-cells expressing the inhibitory surface marker LAP has been previously described in lung adenocarcinoma, colorectal and head and neck cancer patients' plasma and tumour infiltrating lymphocytes (TILs) [244, 245, 306, 307, 349]. These cells have been reported to be higher in

cancer patients' blood and TILs compared to blood from healthy individuals and normal tissue. Skurr et al. [244] and Mahalingam et al. [245] further described the effector function of the LAP expressing CD4 cells as being highly suppressive and perhaps more inhibitory than the conventional CD4⁺Foxp3⁺. When CD25 depleting our PBMCs it became evident that we removed some cells that were expressing LAP. Yet, it appeared that the CD4⁺LAP⁺CD25⁻ have a strong suppressive effect in the ability to proliferate as shown in the inverse correlation of the CD25 depleted sample more than the non-depleted sample. Further to this, CD4⁺LAP⁺ cells isolated from PBMCs of cancer patients showed suppression of CD4⁺LAP⁻ cell proliferation by an average 51.7% replicating the published data that these cells are highly suppressive in function [244].

Anti-LAP or TGF- β neutralising therapy has been shown to lessen tumour progression and to have anti-tumour immunity in melanoma, glioblastoma and colorectal tumour models [307]. Also, the combination of blocking TGF- β and PD-L1 in urothelial cancer in animal models has been shown to promote immune responses [350]. Moreover, early clinical trials with TGF- β inhibitors in different cancer types are merging to show promising results but perhaps will have a more enhanced effect in combination with other therapies [351, 352]. However, toxicity concerns represent a challenge for therapies targeting the TGF- β pathway. One particular concern with anti-TGF- β therapies is the valvular heart dysfunction seen in pre-clinical studies [287, 353], this was somewhat overcome by intermittent rather than continuous dosing [354]. Another limiting toxicity in early human trials was cytokine storm in the face of no clinical benefit [355]. It is yet to be revealed whether therapies targeting LAP/TGF- β will ever find the balance between meaningful clinical efficacy and toxicities. Our results propose that modified antigens are good targets for anti-tumour immunity, but cancer patients have a specific LAP⁺ regulatory population which would either need to be inhibited with a LAP/TGF- β neutralizing antibody or polarized to Th1 with vaccination. The next chapter dives into the expression of the citrullinated antigen Vimentin, markers involved in the citrullination pathway as well as expression

of LAP in a NSCLC tumour microarray and the implication of these markers on patient survival and prognosis.

4 Chapter 4: Tumour expression of siPTMs, citrullination process and LAP expression in a lung tumour microarray (TMA).

4.1 Introduction

In the tumour microenvironment, hypoxia and lack of nutrients result in additional energy requirements by stressed cells which in turn induce a process of degradation and recycling called autophagy resulting in citrullination of proteins. This siPTMs is a consequence of enzymatic deimination of the amino residue arginine to citrulline within proteins [134]. The reaction is catalysed by a family of peptidylarginine deiminase (PADI) enzymes in the presence of calcium ions. In viable cells high levels of calcium can only occur in double membrane organelles such as nuclei and autophagosomes. PADI2 and PADI4 are the most commonly expressed enzymes. PADI4 is mainly expressed in the nuclei and citrullinates proteins to allow gene regulation [137]. In contrast, PADI2 is mainly expressed in the cytoplasm and is activated within autophagosomes to aid protein digestion, release of energy and presentation to CD4 T-cells on MHC class II [134]. Modified proteins including citrullinated peptides are degraded and presented by MHC-II for CD4 recognition. Our group has shown that in the tumour, production of cytokines from the cytotoxic CD4 T-cells responding to siPTMs result in MHC class II-dependent tumour recognition and direct cancer cell killing without the need for CD8 cells [145]. Vimentin, an intermediate filament cytoskeletal protein involved in the epithelial-mesenchymal transition, has been reported to have increased expression in lung cancer and is associated with metastasis and poor outcome [154]. In murine tumour models, immunization with citrullinated vimentin epitopes resulted in strong anti-tumour immunity and long-term survival [144, 145]. However, we do not know how well this *in-vivo* data will translate to the clinic. Results from the previous chapter show that lung cancer patients have a CD4 T-cell repertoire to citrullinated peptides but responses to one citrullinated vimentin peptide were lower compared to healthy donors perhaps suggesting that responses can be attenuated by the cancer. Upon exploring the phenotype of the

responding CD4 T-cell population we discover that responses maybe hindered by a LAP+ Th3 population which is known to be increased in cancer patients as one mechanism of escaping immune surveillance. Next, it is important to determine whether patients' tumours express markers of siPTMs including citrullinated vimentin. These markers may have prognostic implications as well as improving our understanding of which cancer indications may respond well to Modi-1 vaccine therapy.

4.2 Aims

In a lung cancer microarray, we examine the expression of vimentin, citrullinated vimentin, PADI2, PADI4, autophagy markers, MHC-II, CD4 tumour infiltrating cells, PD-L1 and LAP and their relation to patient prognosis and survival.

4.3 Results

4.3.1 Patient cohort

Analysis was carried out on a historic cohort of NSCLC patients undergoing surgical resection with tumours microarrays prepared as described [312] . Patient data for this cohort shows that higher age, female gender, well differentiated tumours and tumour size less than 3cm, no node involvement and TNM stage 1 were all associated with longer survival (Figure 4-1, Table 4-1). Patients older than 76 years of age at the time of resection had mean survival of 101 months, patients more than 60 but less than 76 years of age had a mean survival of 60 months whereas patients less than 60 years of age had a mean survival of 40 months ($p < 0.0001$). Female patients had a mean survival of 82 months, while male patients had a mean survival of 53 months ($p = 0.003$). Ex-smokers and current smokers had mean survival of 71 and 68 months respectively whereas never smokers had a mean survival of 49 months, but this was not significant. Patients with well differentiated tumours had a mean survival of 86 months compared to 62 months for moderately differentiated and 56 months for poorly differentiated tumours ($p = 0.012$). Patients with a tumour size of less than 3cm had a mean survival of 82 months, patients with tumours larger than 3cm but smaller than 6cm had a mean survival of 72 months and patients with a tumour larger than

6cm or with tumours invading other organs, had a mean survival of 43 months ($p=0.002$). Patients with no node involvement (N0) had 91 months mean survival, patients with metastasis in ipsilateral lymphatic nodes (N1) had 49 months mean survival and patients with metastasis in other lymph nodes (N2) had 22 months mean survival ($p<0.0001$). Finally, patients had 104 months mean survival with TNM stage I, 55 months mean survival with TNM stage II and 30 months mean survival with TNM stage III and IV disease ($p<0.0001$). All significant univariant determinants of survival were assessed for independence in a cox regression multivariant analysis. Age ($p=0.005$), gender ($p=0.012$), and TMN ($p <0.0001$) were independent predictors of overall survival (Table 4-2) in line with documented studies [356].

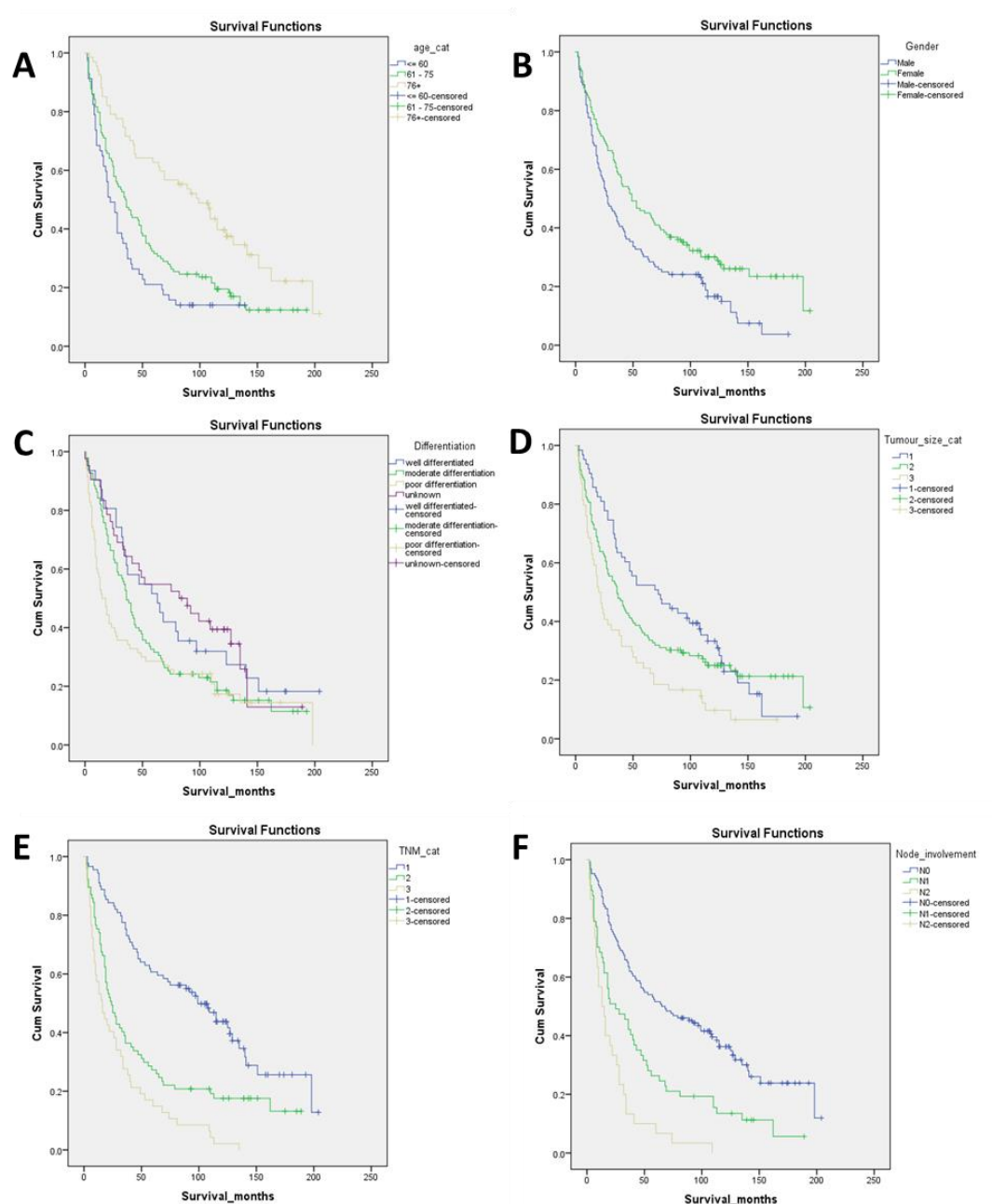


Figure 4-1: Kaplan-Meier plots showing correlation of categorised clinicopathological variables with patient survival. Significance was determined using log rank test. A) Age: ≤ 60 in blue, 61-75 in green and ≥ 76 in yellow ($p < 0.0001$), B) Gender: male in blue and female in green ($p = 0.003$), C) Differentiation: well differentiated in blue, moderately differentiated in green and poorly differentiated in yellow and unknown in purple ($p = 0.012$), D) Tumour size: T1 (<3cm) in blue, T2 (3-6cm) in green and T3 (>6cm) in yellow ($p = 0.002$), E) TNM stage: stage 1 in blue, stage 2 in green and stage 3 + 4 in yellow ($p < 0.0001$) and F) Nodal involvement: N0 (no nodes) is blue, N1 (ipsilateral nodal involvement) is green and N2 (distant nodal involvement) is yellow ($p < 0.0001$).

Table 4-1: Summary of univariate analysis of each independent variable and survival.

Variable	Category	% expression	Mean DSS (months)	95% CI (months)	p-value
Age	<=60	24	40	28.7-51.5	<0.0001
	61-75	48	60.2	48.7-71.7	
	76+	28	100.7	83.1-228.4	
Gender	Male	49	53	43.1-62.9	0.003
	Female	51	82.2	68.6-95.8	
Smoking history	Ex-smokers	48	71	58.7-83.9	0.289
	Current smokers	40	68	55.2-81.8	
	Never smoked	2	49	20.4-77.1	
Tumour differentiation	Well	16	86	61.2-111.4	0.012
	Moderate	49	62	49.9-75.2	
	Poor	35	56	39.2-73.5	
Tumour size	<3cm	27	82	66.6-97.2	0.002
	3-6 cm	50	72	58.2-85.4	
	>6cm	23	43	29.8-56.2	
Nodal involvement	No node	59	91	77.5-104.8	<0.0001
	Ipsilateral	27	49	34.6-63.6	
	Distant	14	22	13.5-30.4	
TNM	1	42	104	88.0-119.5	<0.0001

	2	36	55	40.4-69.1	
	3,4	22	30	20.4-39.6	

Table 4-2: Cox proportional hazards analysis of various clinicopathological variables for overall survival. All significant univariant determinants of survival were assessed for independence in a cox regression multivariant analysis.

Variable	Sig.	Exp(B)	95% CI for Exp(B)	
			Lower	Upper
Age	0.005	2.044	1.236	3.380
Gender	0.012	1.556	1.102	2.197
Differentiation	0.217	0.750	0.877	2.259
Tumour size	0.438	1.247	0.713	1.848
TNM	<0.0001	0.309	0.547	0.840

4.3.2 Do NSCLC tumours express vimentin and/or citrullinated vimentin and is this related to patients' prognosis?

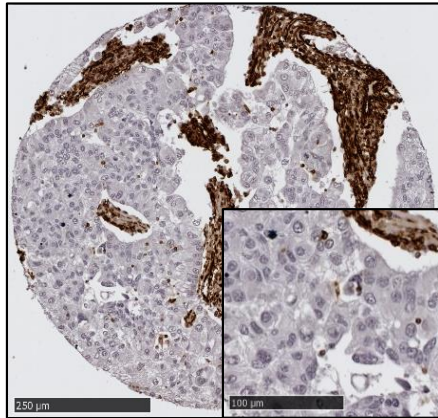
Vimentin can be citrullinated by PAD1 enzymes and may be part of the degradative process in EMT (epithelial-mesenchymal transition). IHC staining of 220 TMA samples was performed. Samples were stained using a monoclonal antibody which detects vimentin or an antibody which specifically detects citrullinated vimentin. Representative example images show that expression vimentin and citrullinated vimentin were predominantly cytoplasmic (Figure 4-2). However, the staining patterns shows a difference in expression of vimentin and citrullinated vimentin where the latter is in the autophagosome and the former in the cytoplasm.

Multivariate analysis was performed, and the analysis shows that vimentin expression is a poor prognostic indicator in our lung cancer TMA but is not independent of age, gender and TNM in predicting patient survival. Vimentin staining was predominately seen in the cytoplasm (Figure 4-2B). High or intermediate staining was defined by X-tiles program as a H score of greater than 30, while low staining was defined as less than or equal to a H score of 30 (Figure 4-2A,B). Patients whose tumours over express vimentin had a mean survival of 43 months whereas patients whose tumours express low levels of vimentin had a mean survival of 70 months ($p=0.025$; Figure 4-3A, Table 4-3). This is in line with our hypothesis that vimentin expression is a predictor of EMT leading to metastasis and poor prognosis.

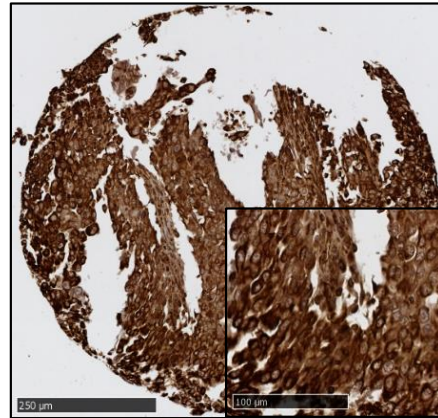
On the other hand, expression of citrullinated vimentin was a good prognostic indicator and is independent of TNM in predicting survival ($p=0.05$). Expression of citrullinated vimentin was predominately cytoplasmic where a H score cut off point of 75 was identified by X-tiles program as dividing the groups into high and low expression (Figure 4-2 C,D). High expression of citrullinated vimentin in patient's tumours was associated with a mean survival of 81 months, whereas patients whose tumours express low levels of citrullinated vimentin, had a mean survival of 47 months ($p=0.016$, Figure 4-3B, Table 4-3). As vimentin predicted poor survival and citrullinated vimentin is a predictor of good prognosis then this suggests that citrullinated

vimentin maybe a target for tumour immune responses limiting tumour growth at all stages.

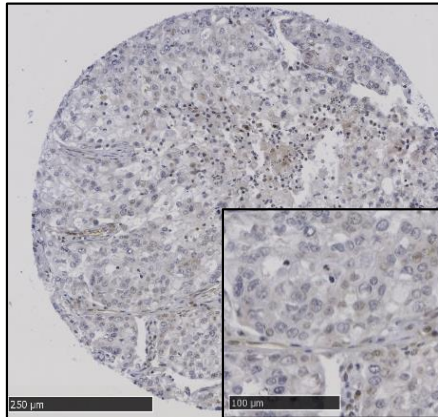
A: Low vimentin (H score 0)



B: High vimentin (H score 220)



C: Low citrullinated vimentin (H score 0)



D: High citrullinated vimentin (H score 190)

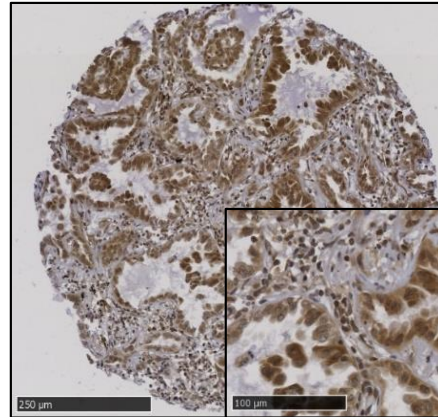


Figure 4-2: Representative example of IHC staining of lung tumour cells for vimentin and citrullinated vimentin which are both predominately expressed in the cytoplasm. A: low vimentin expressing tumour with H score of zero, B: is high vimentin expressing tumour with H score of 220, C: low citrullinated vimentin expressing tumour with H score zero and D: high citrullinated vimentin expressing tumour with H score 190.

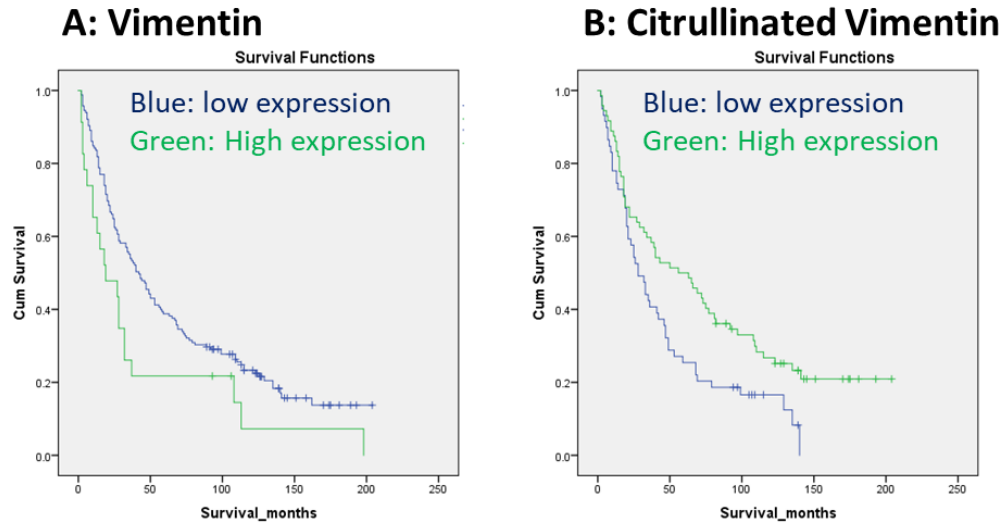


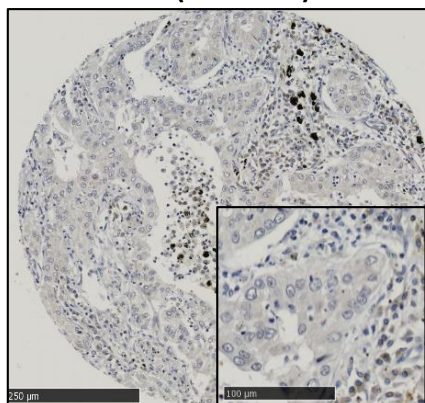
Figure 4-3: Kaplan-Meier plots for disease-specific survival for (A) cytoplasmic vimentin where blue is low staining and green is high or intermediate staining ($p=0.025$), (B) cytoplasmic citrullinated vimentin where blue is low staining and green is high staining ($p=0.016$). Statistical significance was determined using log-rank test.

4.3.3 Are PADI2 and PADI4 enzymes related to prognosis and/or the expression of citrullinated vimentin?

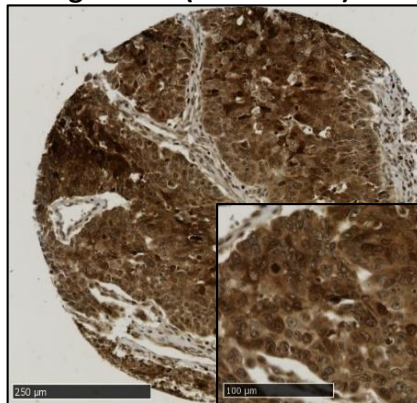
Citrullination is mediated by PADI enzymes with the two most widely expressed being PADI4 and PADI2. In this cohort, IHC analysis of PADI4 showed that staining was predominantly seen as nuclear staining. High staining was defined by X-tiles program as an H score of greater than 251 while low staining was defined as an H score of less than or equal to 251 (Figure 4-4 C,D). There was no correlation between PADI4 expression and survival ($p=0.314$, Figure 4-5B, Table 4-3). There was a positive correlation between PADI4 expression with p62 ($p=0.003$) and citrullinated vimentin expression ($p=0.001$, Table 4-4). These results suggest that PADI4 may be important in citrullination of vimentin that this may occur during autophagy.

PADI2 expression was predominantly seen as cytoplasmic staining (Figure 4-4B). High staining was defined by X-tiles program as an H score of greater than 41 (Figure 4-4B). Over expression of PADI2 was associated with a mean survival of 79 months, whereas patients whose tumours had a low expression of PADI2, had a mean survival of 58 months ($p=0.048$, Figure 4-5A, Table 4-3). These results suggest that PADI2 is a predictor of good survival but is not independent of age, gender and TNM. There was also a positive correlation between PADI2 expression with citrullinated vimentin ($p<0.0001$, Table 4-4) and p62 ($p=0.003$). These results suggest that PADI2 may also be important in citrullination of vimentin and that this may occur during autophagy.

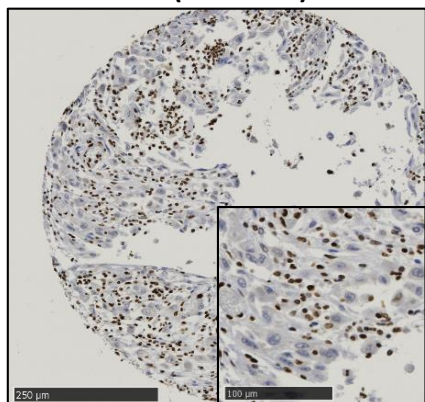
A: Low PAD2 (H score 0)



B: High PAD2 (H score 300)



C: Low PAD4 (H score 0)



D: High PAD4 (H score 300)

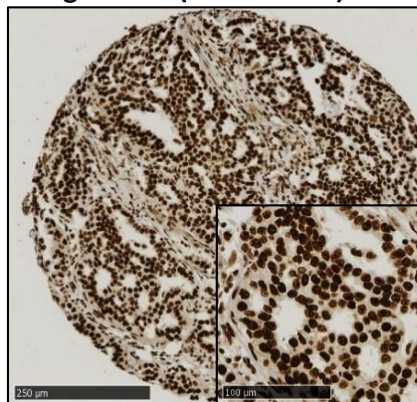
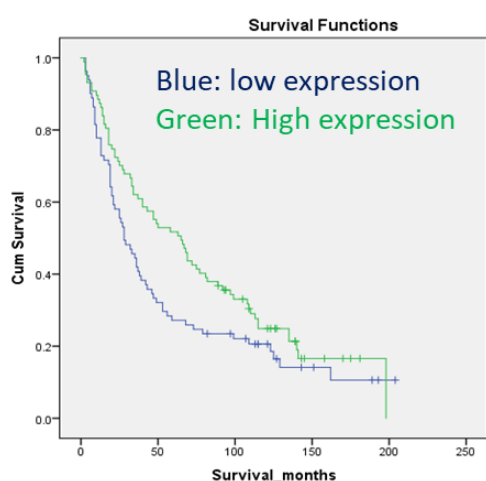


Figure 4-4: Representative example of IHC staining of lung tumour cells for PAD2 and PAD4 which are predominately expressed in the cytoplasm and nucleus respectively. A: low PAD2 expressing tumour with H score of zero, B: is high PAD2 expressing tumour with H score of 300, C: low PAD4 expressing tumour with H score zero and D: high PAD4 expressing tumour with H score 300.

A: Cytoplasmic PAD2



B: Nuclear PAD4

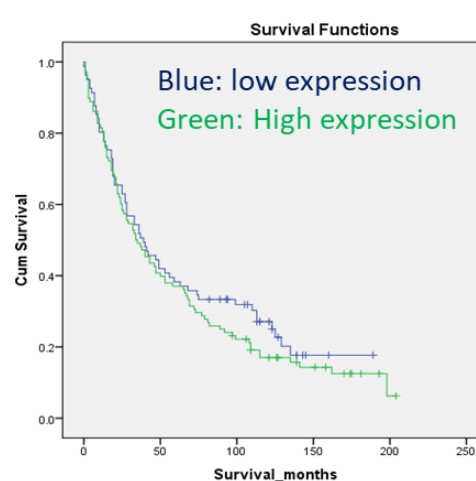
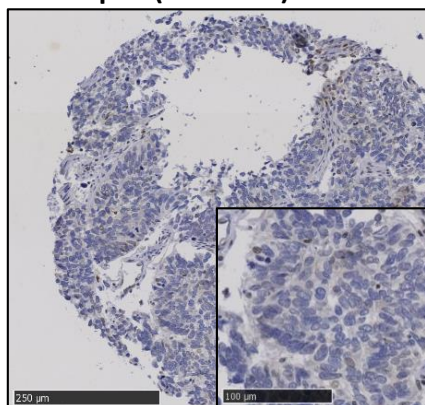


Figure 4-5: Kaplan-Meier plots for disease-specific survival for (A) cytoplasmic PAD2 where blue is low staining and green is high staining ($p=0.048$), (B) nuclear PAD4 where blue is low staining and green is high staining ($p=0.314$). Statistical significance was determined using log-rank test.

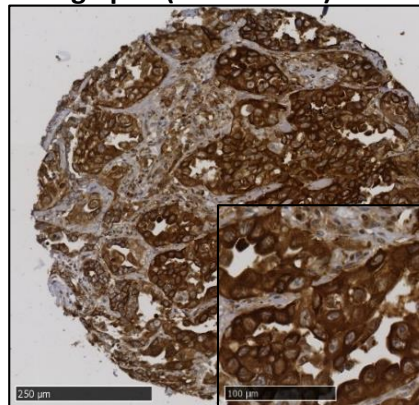
4.3.4 Is autophagy related to prognosis and/or citrullination?

PADI enzymes can only be activated by high levels of calcium. To achieve milli-molar concentrations of calcium within a viable cell, it needs a double membrane vesicle such as an autophagosomes. Autophagy can be assessed by immunohistochemical expression of Ambra1 and/or p62 (Figure 4-6) [357-360]. High staining for p62 was defined by X-tiles program as an H score of greater than 110 while low staining was defined as an H score of less than or equal to 110 (Figure 4-6 A,B). There was no correlation between p62 expression and survival ($p=0.360$; Figure 4-7A, Table 4-3). There was a positive correlation with p62 and expression of PADI2 ($p=0.003$), PADI4 ($p=0.003$), PDL1 ($p=0.01$) and citrullinated vimentin ($p=0.001$, Table 4-4). Ambra1 high staining was defined by X-tiles program as an H score of greater than 25 while low staining was defined as an H score of less than or equal 25 (Figure 4-6 C,D). Over expression of Ambra1 was associated with a mean survival of 82 months whereas patients whose tumours had low expression of Ambra1 had a mean survival of 51 months ($p=0.009$, Figure 4-7B, Table 4-3). These results suggest that Ambra1 is a predictor of good survival but is not independent of age, gender and TNM. There was a positive correlation between expression of Ambra1 and patient's age (0.016), TNM ($p=0.001$) and an inverse correlation with vimentin expression ($p=0.001$). There was no correlation between expression of p62 and Ambra1 suggesting that they measure different stages of autophagy. Ambra1 is involved in the initiation of autophagy and may be involved in the degradation of vimentin during EMT [357, 361]. In contrast p62 measures late autophagy when PADI enzymes have been activated and vimentin citrullinated.

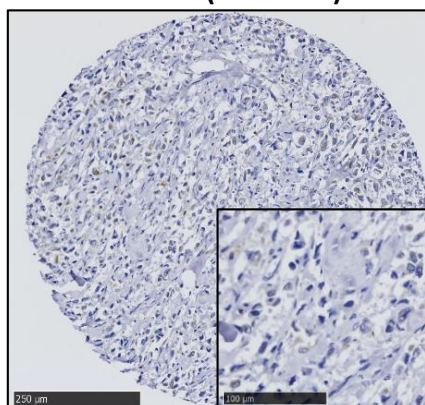
A: Low p62 (H score 0)



B: High p62 (H score 300)



C: Low Ambra1 (H score 0)



D: High Ambra1 (H score 300)

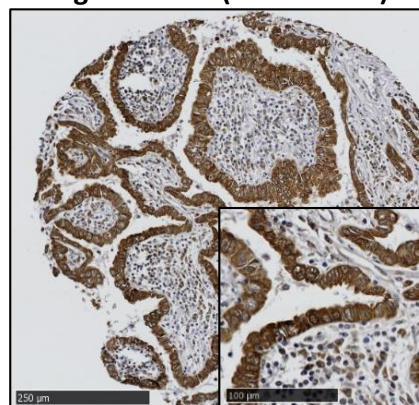
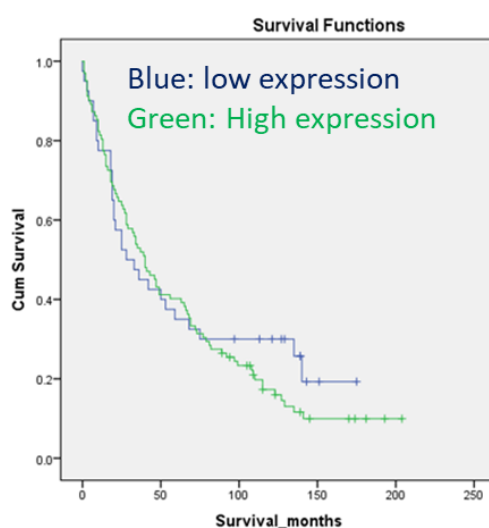


Figure 4-6: Representative example of IHC staining of lung tumour cells for p62 and Ambra-1. A: low p62 expressing tumour with H score of zero, B: is high p62 expressing tumour with H score of 300, C: low Ambra-1 expressing tumour with H score zero and D: high Ambra-1 expressing tumour with H score 300.

A: P62



B: Ambra-1

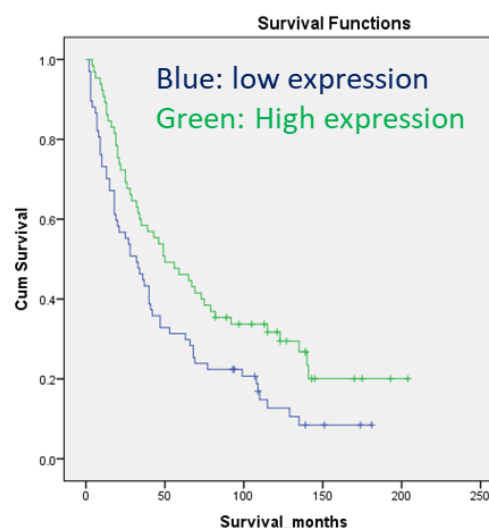


Figure 4-7: Kaplan-Meier plots for disease-specific survival for (A) p62 where blue is low staining and green is high staining ($p=0.360$), (B) Ambra-1 where blue is low staining and green is high staining ($p=0.009$). Statistical significance was determined using log-rank test.

4.3.5 Is the expression of MHC class II related to survival and/or citrullination?

We have shown in our tumour models that citrullinated vimentin can induce cytotoxic CD4 T cells that directly kill tumours expressing MHC-II. In the absence of inflammation most tumours do not express MHC-II, but it can be induced by IFN γ secreting T-cells. The TMA was therefore stained for expression of MHC-II on tumour cells. Analysis of MHC-II was seen predominantly as cell surface staining (Figure 4-8B). High staining was defined by X-tiles program as an H score of greater than 100. Expression of MHC-II was not associated with survival ($p=0.248$, Figure 4-9A, Table 4-3). There was a positive correlation between MHC-II expression with patient's age (0.001), CD4 infiltration ($p=0.027$) and citrullinated vimentin expression ($p=0.008$, Table 4-4). This is in line with our hypothesis that citrullinated vimentin is a target for CD4 T cells which limit tumour growth.

4.3.6 Is there a relation between CD4 T-cell infiltration and NSCLC prognosis?

Previous studies have shown that citrullinated vimentin can induce potent CD4 T cells responses in tumour models. CD4 T cell infiltration within the NSCLC was therefore assessed. High staining was defined by X-tiles program as an H score of greater than 163 (Figure 4-8D). There was no correlation between CD4 infiltration and survival ($p=0.232$, Figure 4-9B, Table 4-3). There was a positive correlation between CD4 infiltration and MHC-II expression ($p=0.032$) but not citrullinated vimentin. CD4 T cells can produce IFN γ which can up regulate PD-L1 to prevent toxicity a process known as adaptive resistance.

4.3.7 Is there a relation between PD-L1 expression, survival and/or citrullination?

PD-L1 expression within the NSCLC was therefore assessed (Figure 4-8 E,F). High staining was defined by X-tiles program as an H score of greater than 76 (Figure 4-8F). There was no correlation between PD-L1 and survival ($p=0.107$, Figure 4-9C, Table 4-3). There was a positive correlation between PD-L1 with patient's age ($p=0.003$), p62 ($p=0.001$) and citrullinated

vimentin expression ($p=0.012$, Table 4-4). This is in line with our hypothesis that citrullinated vimentin is a target for tumour immune responses but that these are limited by PD-L1 expression and may suggest a combination of checkpoint inhibition and vaccination against citrullinated vimentin may be a promising approach.

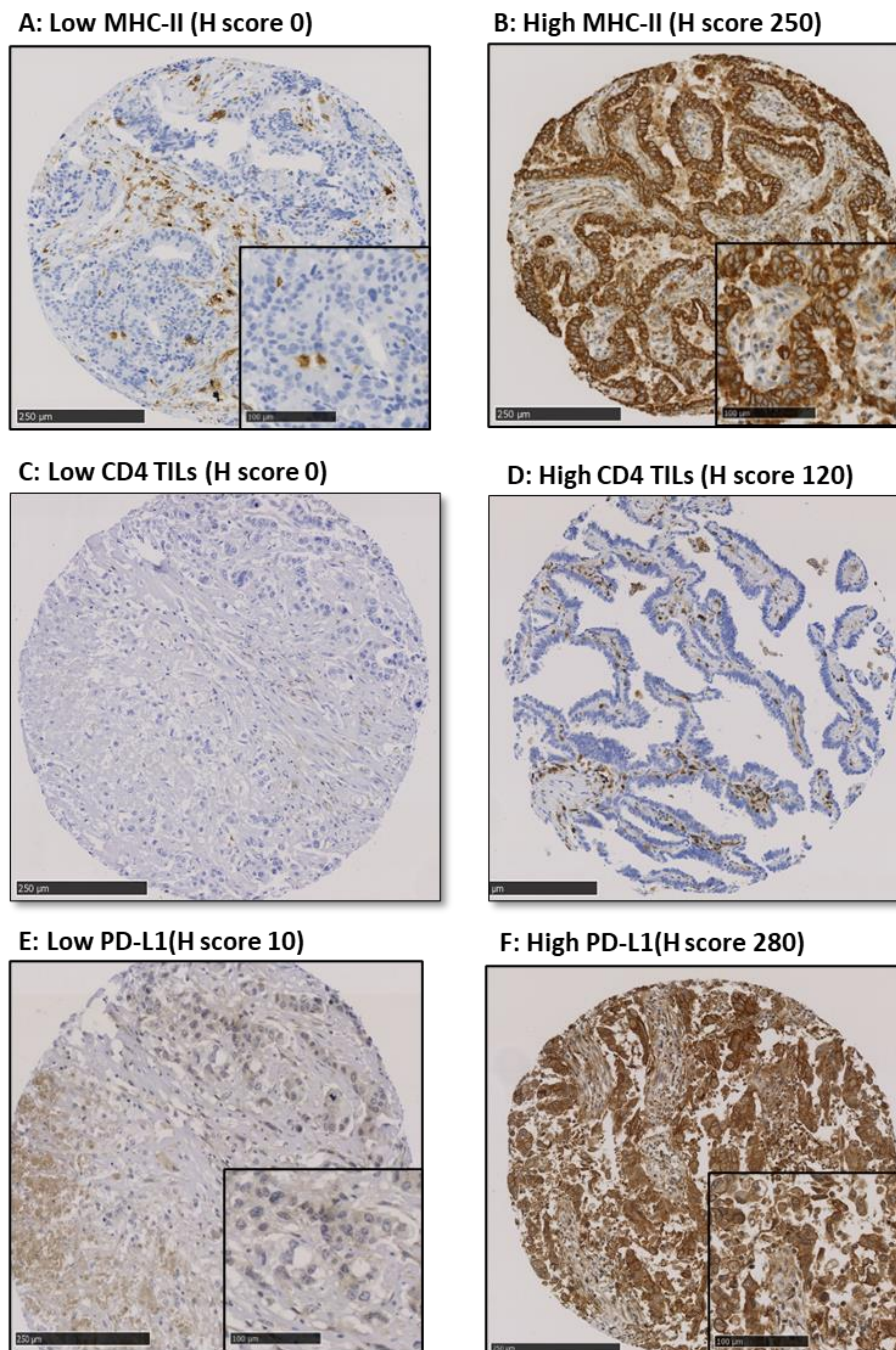
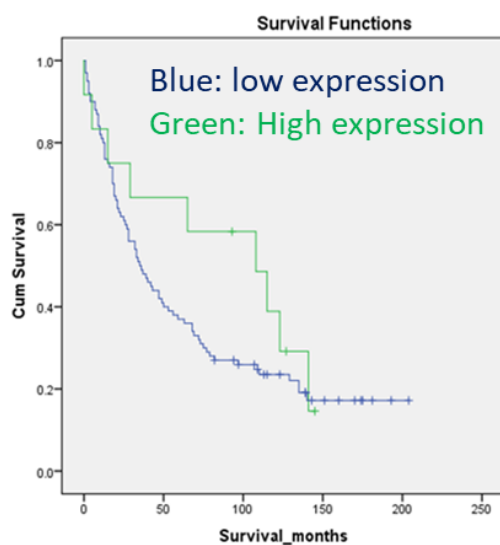
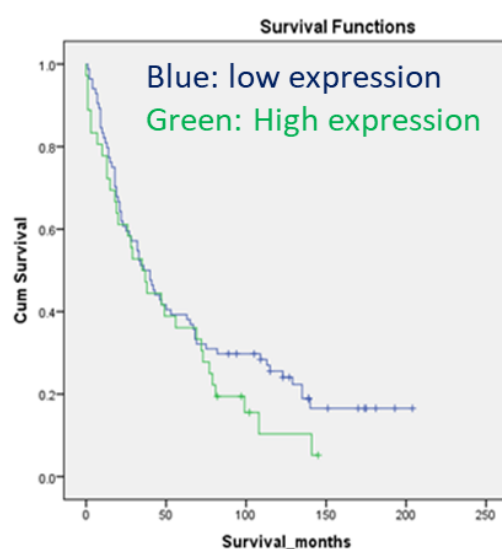


Figure 4-8: Representative example of IHC staining of lung tumour cells of MHC-II, infiltrating CD4 T-cell and PD-L1. A: low MHC-II expressing tumour with H score of zero, B: is high MHC-II expressing tumour with H score of 250, C: low CD4 TILs with score of zero, D: high CD4 TILs with score of 120, E: low PD-L1 expressing tumour with score of 10, F: high PD-L1 expressing tumour with score of 280.

A: MHC-II



B: CD4 TILs



C: PD-L1

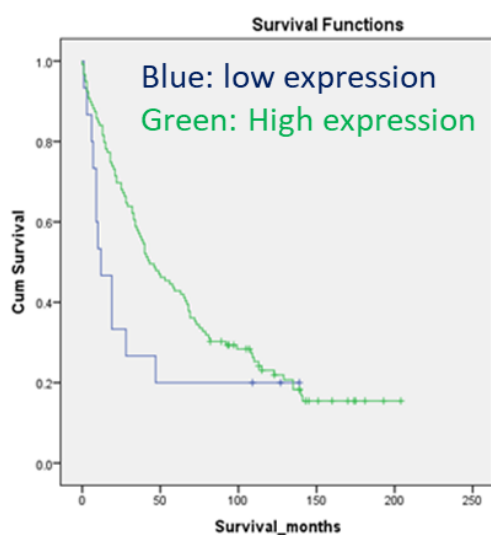


Figure 4-9: Kaplan-Meier plots for disease-specific survival for (A) MHC-II where blue is low staining and green is high staining ($p=0.248$), (B) CD4 TILs where blue is low staining and green is high staining ($p=0.232$) and (C) PD-L1 where blue is low staining and green is high staining ($p=0.107$). Statistical significance was determined using log-rank test.

4.3.8 Correlation of citrullinated vimentin expression with clinicopathology, expression of vimentin, PADI enzymes, PDL1 expression, MHC-II, Ambra1, p62 and CD4 infiltration

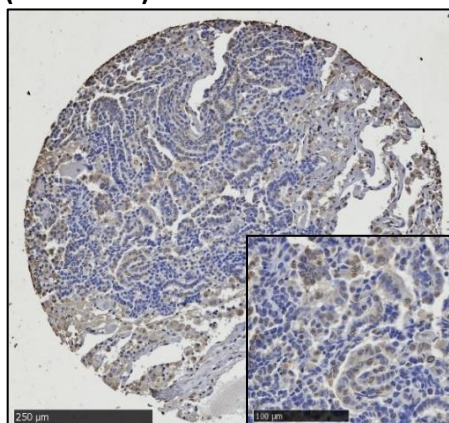
Expression of citrullinated vimentin was correlated with clinicopathology and co-expression of markers of citrullination using univariate analysis (Table 4-4). There was a positive correlation with expression of citrullinated vimentin and patients' age ($p=0.003$) but not with gender, TMN stage or tumour size. There was also a positive correlation between citrullinated vimentin expression and expression of PADI2 ($p<0.0001$), PADI4 ($p=0.001$), MHC-II ($p=0.008$), PD-L1 (0.012) and p62 ($p=0.001$). There was no correlation between citrullinated vimentin expression and vimentin, CD4 infiltration or Ambra1 expression.

4.3.9 Is the expression of LAP related to prognosis?

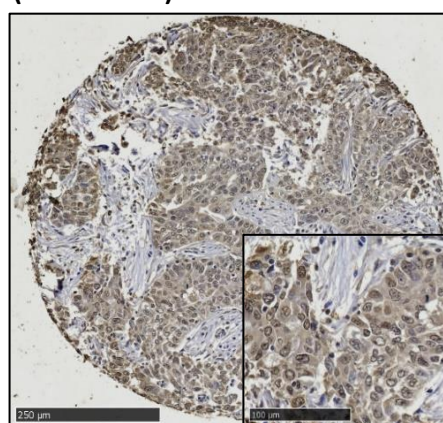
Results from chapter 3 shows that LAP expression is seen on some siPTM reactive T-cells in the peripheral blood of cancer patients. As LAP can be expressed both within tumours and on the surface of CD4 T cells, its expression on both tumour cells and tumour infiltrating lymphocytes (TILs) was measured (Figure 4-10). High staining for LAP-TILs was defined by X-tiles program as an H score of greater than 19 while low staining was defined as an H score of less than or equal to 19 (Figure 4-10 C,D). LAP-TILs staining was not associated with survival ($p=0.232$, Figure 4-11B, Table 4-3). There was an inverse correlation between LAP-TILs and metastases ($p=0.003$) suggesting that these cells may inhibit an immune response allowing tumour spread.

High staining for LAP-tumour was defined by X-tiles program as an H score of greater than 65 while low staining was defined as an H score of less than or equal to 65 (Figure 4-10 A,B). LAP-tumour staining was not associated with survival ($p=0.235$, Figure 4-11A, Table 4-3). There was an inverse correlation between LAP expression on tumour cells and citrullinated vimentin ($p=0.018$) and PADI4 ($p=0.009$) suggesting LAP within the tumour may be inhibiting citrullinated vimentin expression.

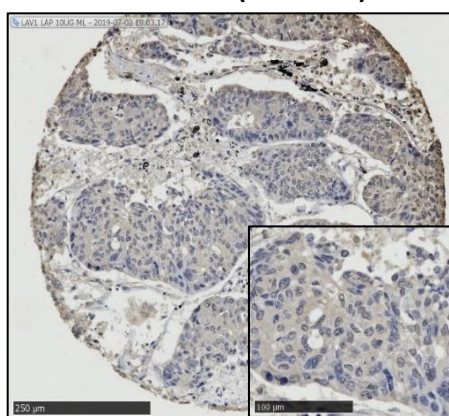
**A: Low LAP on tumour cells
(H score 15)**



**B: High LAP on tumour cells
(H score 150)**



C: Low LAP on TILs (H score 0)



D: High LAP on TILs (H score 20)

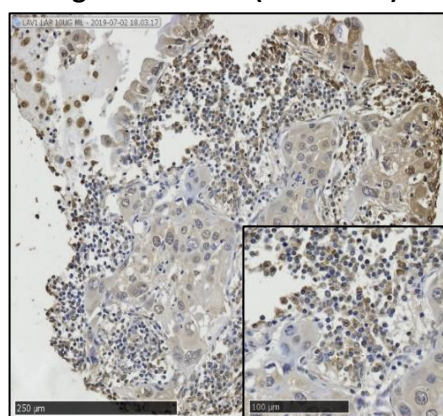
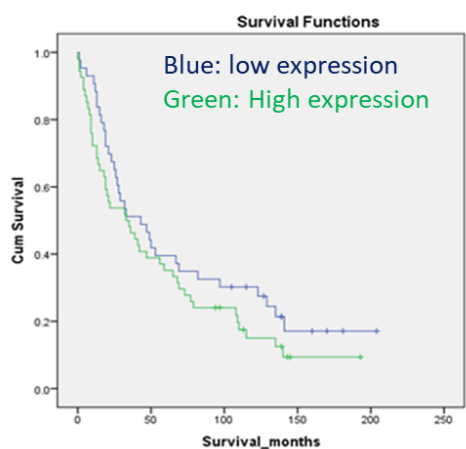


Figure 4-10: Representative example of IHC staining of lung tumour cells for LAP in cytoplasm and LAP in TILs. A: low LAP expressing tumour with H score of 15, B: is high LAP expressing tumour with H score of 150, C: low LAP-TILs staining with H score of zero, D: is high LAP-TILs staining with H score of 20.

A: LAP on tumour cells



B: LAP on TILs

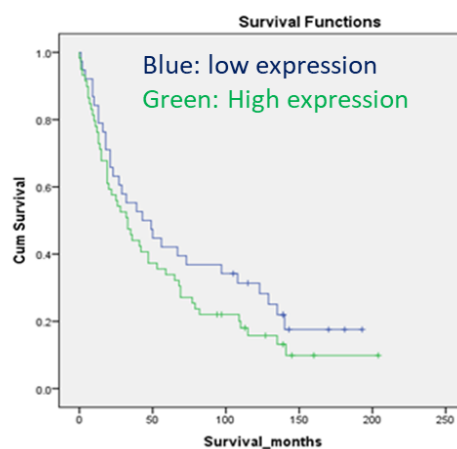


Figure 4-11: Kaplan-Meier plots for disease-specific survival for (A) LAP-tumour cells where blue is low staining and green is high staining ($p=0.235$), (B) LAP-TILs where blue is low staining and green is high staining ($p=0.232$). Statistical significance was determined using log-rank test.

4.3.10 Co-expression of citrullinated vimentin and LAP-TILs

As CD4 T-cells isolated from some lung cancer patients' blood responding to citrullinated vimentin epitopes expressed LAP; the co-expression of LAP-TILs and citrullinated vimentin was assessed on the TMA and there was a correlation with survival ($p=0.018$, Figure 4-12). Patients whose tumours expressed citrullinated vimentin but had few LAP positive TILs had a mean survival of 78 months with 22% of them surviving for more than 12 years. The worst prognostic group were patients whose tumours failed to express citrullinated vimentin but had high numbers of LAP positive TILs as they had a mean survival of 33 months with only 2% of them surviving for more than 8 years. This suggests that the LAP positive TILs were also inducing a suppressive environment which may have reduced citrullination of vimentin with tumours.

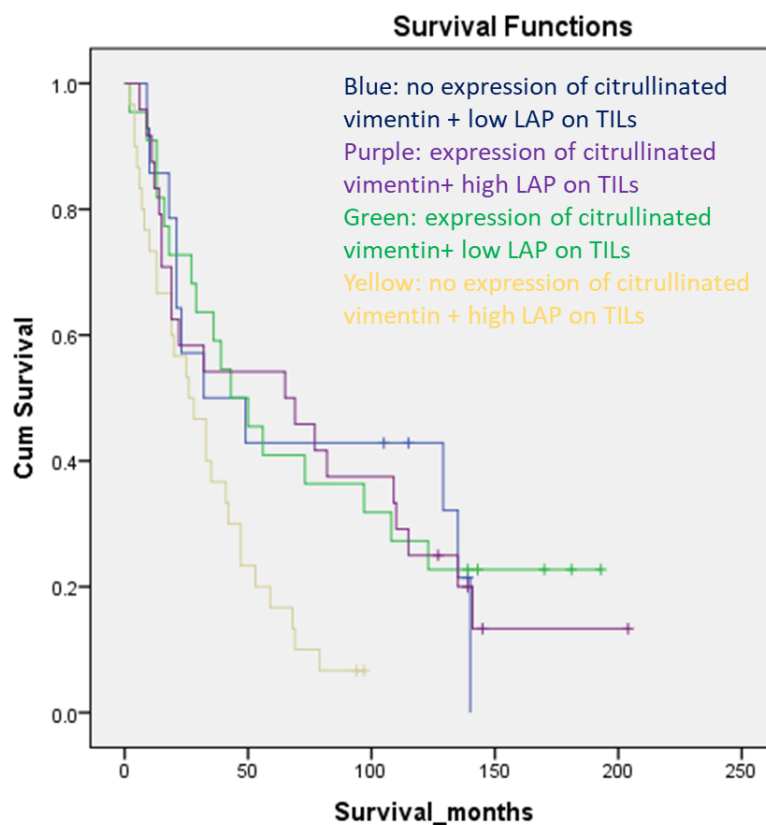


Figure 4-12: Kaplan-Meier plots for disease-specific survival for co-expression of LAP on TILs and citrullinated vimentin where blue are tumours with no expression of citrullinated vimentin and low LAP on TILs, purple are tumours with expression of citrullinated vimentin and had high LAP on TILs, green are tumours which expressed citrullinated vimentin and had low LAP on TILs and yellow are tumours which lacked expression of citrullinated vimentin and had high LAP on TILs ($p=0.018$). Statistical significance was determined using log-rank test.

Table 4-3: Univariant analysis of expression of vimentin, citrullinated vimentin, PADI2, PADI4, CD4 TILs, PD-L1, MHC-II, Ambra-1, P62 and LAP and their relation to survival. Statistical significance was determined using log-rank test (value greater than 0.05 was considered significant). Due to the small number of samples in the TMA, a cox regression analysis of independence for all these variables was not possible.

Variable	Category	% expression	Mean DSS (months)	95% CI (months)	p-value
Vimentin	weak	88	69.6	59.1-80.2	0.025
	strong	12	42.9	18.8-67.1	
Citrullinated Vimentin	weak	45	47.0	35.5-63.7	0.016
	strong	65	80.8	63.7-97.9	
PADI4	Negative	42	72.4	57.2-87.7	0.314
	Positive	58	64.4	52.0-76.8	
PADI2	Weak	48	57.9	43.6-72.3	0.048
	Strong	52	79.0	64.6-93.4	
CD4 TILs	Weak	70	70.2	54.9-85.6	0.232
	strong	30	49.8	35.0-64.7	
PDL1	Weak	11	39.1	13.3-65	0.107
	Strong	89	72.1	59.6-84.4	
MHC-II	Weak	90	70.6	56.2-84.9	0.248
	Strong	10	92.5	61.9-123.2	
Ambra	Weak	51	51.3	38.3-64.3	0.009
	Strong	49	82.4	64.3-100.5	
P62	Weak	28	69.4	48.3-90.6	0.360

	strong	72	64.4	52.2-76.6	
LAP in the cytoplasm of tumour	Weak	45	76.1	54.3-97.8	0.235
	strong	55	59.7	43.2-76.2	
LAP in TILs	weak	40	76.7	54.4-98.9	0.232
	strong	60	60.6	44.1-77.1	

Table 4-4: Univariate analysis showing correlation of citrullinated vimentin expression with vimentin, PADI2, PADI4, CD4, PD-L1, MHC-II, Ambra-1, p62 and LAP. Statistical significance was determined using log-rank test (value greater than 0.05 was considered significant).

		Citrullinated Vimentin		p-value
		Low	High	
Gender	Male	26.0%	26.7%	0.304
	Female	19.1%	47.3%	
Age	<=60	16%	23.7%	0.003
	61-75	20.6%	45.0%	
	76+	8.4%	31.3%	
TNM stage	I	15.1%	23.5%	0.303
	II	20.2%	18.5%	
	III and IV	12.6%	10.1%	
Tumour size	<3 cm	9.3%	15.5%	0.452
	3-6cm	22.5%	26.4%	
	>6cm	14%	12.4%	
Vimentin	weak	44.5%	47.1%	0.088
	strong	2.5%	5.9%	
PADI4	weak	26.2%	17.5%	0.001
	strong	17.5%	38.9%	
PADI2	weak	28.9%	16.5%	<0.0001
	strong	14.9%	39.7%	
CD4 TILs	weak	38	41	0.528
		(73.1%)	(64.1%)	

	strong	14 (26.9%)	23 (35.9%)	
PD-L1	weak	10 (18.5%)	3 (4.4%)	0.012
	strong	44 (81.5%)	65 (95.6%)	
MHC-II	weak	45.6%	43.7%	0.008
	strong	1.0%	9.7%	
Ambra	Weak	25.4%	21.3%	0.281
	strong	23.8%	29.5%	
P62	weak	20.2%	8.9%	0.001
	strong	25.8%	45.2%	
LAP in tumour	weak	15.6	28.9	0.018
	strong	33.3	22.2	

4.4 Discussion

To strengthen our hypothesis that siPTMs provide a good target for the development of novel anti-tumour therapy, we examined the process of citrullination and its' relation to survival in a tumour microarray of non-small cell lung cancer (NSCLC). In line with previous studies age, gender, tumour differentiation, tumour size and TNM stage were shown to be important predictors of survival in our TMA and age, gender and TNM remain independent predictors of survival in a multivariate analysis [356]. Vimentin is an important cytoskeletal protein which plays a vital role in cellular integrity[154]. During epithelial-mesenchymal transition (EMT); vimentin is essential for the acquisition of fibroblast-like characteristics[152]. EMT has been a recognised component of tumour progression and invasiveness in carcinomas[153]. Increased expression has been reported in different tumour cell lines including prostate cancer, breast, gastro-intestinal, hepatic, melanoma and lung cancer[154]. Our staining patterns shows a difference in expression of vimentin and citrullinated vimentin where the latter is in the autophagosome and the former in the cytoplasm. We hypothesis that citrullinated vimentin is the vimentin that is being degraded by autophagy and therefore not high in the cells that are actively using vimentin for cytoskeletal structure. Expression of vimentin was a predictor of poor survival but was not independent of age, gender or TNM stage. These results suggest that more aggressive tumours are those expressing high levels of vimentin facilitating their epithelial-mesenchymal transition and hence invasion and metastasis[362]. In complete contrast, expression of citrullinated vimentin was a predictor of good survival. In addition, we also saw a correlation between the expression of citrullinated vimentin and the PADI2/PADI4 enzymes which drive the citrullination process. In this cohort the expression of PADI2 but not PADI4 was associated with a good prognosis. Others have also shown that PADI2 was over expressed in lung cancer [363], but did not correlate expression with prognosis.

In viable cells citrullination occurs within autophagosomes and can result in citrullination of proteins such as vimentin. Subsequent presentation on MHC class II and recognition by CD4 T-cell will control tumour growth and

improve prognosis[145]. In this context citrullinated vimentin expression was correlated with p62 expression, whereas vimentin was associated with Ambra1. Ambra1 is expressed in the early stages of autophagy whereas p62 is degraded in the later stages, suggesting degradation of vimentin is an early event and presentation of citrullinated vimentin may be associated with later stages of autophagy. There was a correlation between citrullinated vimentin expression and MHC-II expression. As autophagy is triggered to promote cell survival by degrading intracellular components to generate energy; it is usually associated with a more aggressive tumour and poor survival[364, 365]. In this study, expression of Ambra1 was associated with improved survival suggesting it may have alternative roles. If autophagy results in citrullination, in the presence of inflammation and expression of MHC-II it may alert the immune system conferring an improved prognosis. In this context there was a correlation between CD4 infiltration and MHC-II expression. There was also a correlation between citrullinated vimentin expression and PD-L1 expression. Cells can express PD-L1 as a mechanism to prevent toxicity from IFN γ in a process known as adaptive resistance[366], this may imply that the citrullinated vimentin had stimulated an immune response which was then curtailed due to PD-L1 expression. This is in line with the immune responses to citrullinated vimentin that were detected in the peripheral blood of lung cancer patients. Thus, suggesting that boosting these responses by vaccination with citrullinated vimentin and preventing blockade with checkpoint inhibitors may be an ideal synergistic combination.

As LAP expression in peptide specific CD4 T-cells isolated from the blood of lung cancer patients inhibited their proliferation; the expression of LAP was also examined in the TILs and tumour cells in the lung TMA. Co-expression of citrullinated vimentin and LAP in the TILs was a strong prognostic indicator with patients whose tumours expressed citrullinated vimentin but had low numbers of LAP+TILs surviving 78 months and 22% of patients surviving more than five years. In contrast, patients whose tumours failed to express citrullinated vimentin and had high numbers of LAP+TILs only survived 33 months and only 2% survived 5 years. Similarly, a negative correlation was noted between the LAP in tumour cells and citrullination of

vimentin and PADI4. These results suggest that if the tumour microenvironment promotes the polarisation of antigen specific Th3 cells expressing LAP it confers a poor prognosis and progressive tumours perhaps through evading the immune system. This is in line with other studies showing the expression of immunosuppressive molecules namely LAP on tumours is associated with poor outcome[244, 245, 306, 307]. However, while tumour staining can suggest correlations between markers and prognosis it does not allow us to explore the causal links between these markers and therefore other methods are required to determine whether LAP expressing cells directly impact on prognosis. Results obtained from PBMCs of cancer patients show that patients do have a population of antigen specific Th3 cells that are terminally differentiated and can suppress immune responses to siPTMs. In the next chapter the anti-tumour immune responses and survival of murine models were examined. In particular the role of vaccination with citrullinated peptides and whether an additional therapy to neutralise Th3 cells is needed or if vaccination with the modified antigens is sufficient to polarize the response in favour of Th1.

5 Chapter 5 Screening of animal models for expression of LAP and Anti-tumour studies in murine tumours

5.1 Introduction

The previous chapters display that cancer patients have a repertoire to citrullinated peptides, but responses maybe attenuated by a terminally differentiated regulatory Th3 population identified by expression of LAP. It is also shown that patients with low levels of citrullinated epitopes and high levels of LAP in a NSCLC TMA represent the worst prognosis group. Here, in murine models we explore the effects of vaccination with citrullinated peptides on survival and explore potential combination therapies *in-vivo*. One obvious combination would be with anti-LAP targeting treatment but Th3 cells have been described to co-express LAG3 [244] and so anti-LAG3 therapy in combination with citrullination peptide vaccine is also studied.

5.1.1 LAG3 and therapies targeting LAG3

LAG3 (also known as CD223) gene is located in chromosome 12 and the protein is made up of 498 amino acids [367]. It is a type I transmembrane protein with four extracellular immunoglobulin superfamily (IgSF) domains (D1–D4) [367, 368] which is expressed on many immune cells including CD4, CD8 lymphocytes, regulatory T-cells (T-regs), natural killer cells and B-cells [368-372]. Its' ligands include MHC class II on APCs or tumour cells, galectin-3, α -synuclein, lymph node sinusoidal endothelial cell lectin, and fibrinogen-like protein 1 (FLP-1) on tumour cells [373-378]. Its binding to MHC class II is at a different site to that which CD4 binds and LAG3 binds with greater affinity compared to CD4 T-cells [369, 379, 380]. In normal physiological conditions LAG3 negatively regulates T-cell function and activation but the downstream signalling pathway is not well understood [40, 381]. Continual TCR stimulation in cases of chronic inflammation such as that in tumour microenvironment results in sustained co-expression of IRs including LAG3 [40]. In such non-physiological cases, when LAG3 binds to its ligands it exerts negative regulation and immune inhibition by impeding

proliferation of effector cells with reduction of cytokine production, inducing T-cell exhaustion [40, 382] and thus downregulating anti-tumour immunity. A negative feedback loop to control LAG3 expression and its inhibitory immune regulation is through its cleavage and shedding of the soluble form of LAG3, this is mediated by ADAM10 and ADAM17 (A Disintegrin And Metalloproteinase domain-containing protein) which increase following T-cell activation [383]. Indeed, high levels of soluble LAG3 tested at the time of diagnosis has been described as a good prognostic indicator with increased disease free and overall survival in a cohort of patients with breast cancer [384].

LAG3 and PD1 have been shown to be co-expressed in several tumour microenvironments of murine models including B16-F10 (melanoma), M38 (colon adenocarcinoma), CT26 (colon carcinoma) and Sa1N (fibrosarcoma) [385, 386]. Synergy in blocking both these IRs simultaneously has been shown to have a better therapeutic benefit in these tumour models compared to monotherapy with either of the agents [385-387]. LAG3 expression on tumour infiltrating lymphocytes (TILs) has also been described in many human tumours including melanoma [388], hepatocellular carcinoma [389], ovarian cancer [390], microsatellite instability colon tumours [391] and non-small cell lung cancer [392]. In patients with chronic lymphocytic leukaemia, high levels of expression of LAG3 on NK cells and T-cells correlated with clinical outcome and treatment of PBMCs from these patients with a LAG3 blocking antibody (Relatlimab) restored T-cell responses [393]. Further to this, a T-reg subset expressing LAG3 have been described to be expanded in the blood and TILs obtained from melanoma and colon cancer patients and functionally these cells release immunosuppressive cytokines IL-10 and TGF- β [372]. Induction of release of inhibitory cytokines by LAG3 expressing cells has also been described in other tumours such as muscle invasive bladder cancer [394]. A subset of Foxp3 negative TILs that expressed latency associated peptide (LAP), a peptide that complexes with TGF- β , have been described to be highly suppressive Th3 (T-helper 3) cells in patients with colon cancer and were found to majorly co-express LAG3 and exert their inhibitory effects through release of TGF- β [244]. Therefore, a

deduction could be made that inhibition of LAG3 could target a highly suppressive population of activated TILs.

Many clinical trials are underway for drugs targeting the LAG3 pathway. Eftilagimod Alpha (IMP321), a soluble LAG-3 fusion protein and MHC-II agonist that works through activation of APCs and priming cytotoxic T-cells, has been shown to be safe in dose escalating phase I trials in renal cell cancer, pancreatic cancer in combination with gemcitabine and breast cancer in combination with paclitaxel (NCT00351949, NCT00732082 and NCT00349934). The latter trial in breast cancer has also reported immunological effects in the form of increase in activated APCs and cytotoxic effector-memory CD8 T cells which correlated with tumour shrinkage and clinical benefit [395]. NCT02676869, another combination trial, has recently completed recruitment for a phase I study of IMP321 in combination with anti-PD1 pembrolizumab in unresectable melanoma and results are yet to be published. Relatlimab (BMS-986016) a monoclonal antibody is another drug mechanistically targeting LAG3 that has been tested in clinical trials in combination with anti-PD1 and recently been granted approval by FDA. RELATIVITY-047 (NCT03470922) was the pivotal phase 2/3 trial that tested the combination of Relatlimab with Nivolumab in patients with metastatic melanoma who received no prior treatment, they reported greater PFS with the combination treatment over monotherapy with Nivolumab and a frequency of grade 3 or 4 side effects in 18.9% of patients receiving the combination treatment [396]. Although the median PFS of Relatlimab with Nivolumab (10.1 months) was similar to that reported by CheckMate-067 for the combination of Ipilimumab and Nivolumab (11.5 months), the frequency of grade 3 or 4 adverse events in the latter was much higher (59%) [397] thus making the new combination clinically attractive and leading to its' clinical approved. Another monoclonal antibody targeting LAG3 developed by Novartis (LAG525) has entered clinical trials to test safety and efficacy in combination with anti-PD1 drugs (NCT02460224, NCT03499899, NCT03365791) and initial reports suggest the combination is as well tolerated as the single agent treatment with anti-PD1 with clinical benefit seen in patients with high expression of LAG3 in baseline tumour tissue

[398]. Capitalization on the synergy between agents targeting LAG3 and PD1 pathways has led to development of bispecific agents, one such drug is MGD013 which is undergoing clinical testing (NCT03219268). The development of bispecific agents is complex and faces scrutiny for its clinically efficacy, safety and justification over the use of dual monotherapies, however, one clinical attraction these agents pose is the potential ease of dosing and administration of a single agent over two [399].

Given that we have shown siPTM specific T-cells in cancer patients can express LAP and have a Th3 phenotype, the combination of siPTM vaccines and antibodies inhibiting LAP or LAG may improve clinical outcomes. This must first be tested in pre-clinical animal models before being moved to the clinic.

5.2 Aims

First, a murine tumour model which shows high expression of LAP and LAG3 is identified.

This model is then used to examine the impact of immunization with citrullinated peptides on survival and if immune boosting synergy of combination treatment with anti-LAP therapy is observed in anti-tumour murine studies.

Finally, with the perspective that a LAG3 targeting agent (Relatlimab) has already gained access to clinic, albeit in combination with Nivolumab, while agents pursuing the TGF- β /LAP/GARP pathway are struggling to find the balance between meaningful efficacy and toxicity with limited success thus far; this chapter will also scrutinise the effects of blocking LAG3 on survival and immune responses alone and in combination with treatment with citrullinated peptides in a murine anti-tumour study.

5.3 Results

5.3.1 Identifying a tumour model and mice strain with high LAP expression

HHDI/IDP4, C57Bl/6 and Balb/c mice were examined for the expression of LAP by CD4⁺ tumour infiltrating lymphocytes and splenocytes.

Tumour models studied for LAP expression on CD4⁺ splenocytes and CD4⁺TILs were melanoma B16F1 in HHDII/iDP4 mice, melanoma B16F1 in C57Bl/6 mice, breast Py8119 in C57Bl/6 mice, lung LLC2 in C57Bl/6 mice, colon MC38 in C57Bl/6 mice, colon CT26 in Balb/c mice and breast 4T1 in Balb/c mice. Mice were implanted subcutaneously (s.c.) with HLA matched tumour cells and tumour growth was monitored. When tumours reached a size of 10mm-15mm mice were culled, tumours and spleens were removed and stained using a flow cytometry antibody panel (Chapter 2, section 2.3.2, Figure 2-3 shows gating strategy). The median level of CD4⁺LAP⁺ cells in the splenocytes was 5.70% in HHDII/iDP4, 1.68% in C57Bl/6 and 3.15% in Balb/c mice. The highest expression of CD4⁺LAP⁺ TILs was seen in Py8119 and MC38 tumours in C57Bl/6 mice with a median of 69.57% and 78.75% of CD4⁺TILs expressing LAP respectively (Figure 5-1). B16F1 in HHDII/iDP4, B16F1 in C57Bl/6, LLC2 in C57Bl/6, CT26 in Balb/c and 4T1 in Balb/c mice showed much lower levels of LAP expression by CD4⁺TILs with a median of 12.30, 4.73, 9.67, 11.60, 9.30% respectively (Figure 5-1). C57Bl/6 mice bearing B16F1, Py8119, LLC2 tumours and Balb/c mice bearing CT26 tumour models showed a significantly higher expression of LAP by CD4⁺TILs compared to splenocytes (p 0.0156, <0.0001, 0.0313, 0.0078 respectively, Wilcoxon paired non-parametric test, Figure 5-3, Figure 5-4, Figure 5-5 & Figure 5-7). The exception was B16F1 HHDII/iDP4 (p 0.4609, Wilcoxon paired non-parametric test, Figure 5-2) and Balb/c 4T1 (p=0.500, Wilcoxon paired non-parametric test, Figure 5-8) where statistical significance was not reached, in the latter due to low number of splenocytes and TILs examined. No statistical analysis was done in C57Bl/6 MC38 tumour models as only 2 mice were studied (Figure 5-6). Given that the highest expression of LAP by CD4⁺TILs was seen in C57Bl/6 mice bearing Py8119, the tumour model was picked to study the effects of vaccination with citrullinated peptides and anti-LAP antibody therapy on tumour growth and levels of Th3 cells within tumours and if there is synergy between vaccination with citrullinated peptide and anti-LAP antibody therapy.

LAP in total CD4 TILs: Comparison of different tumour models

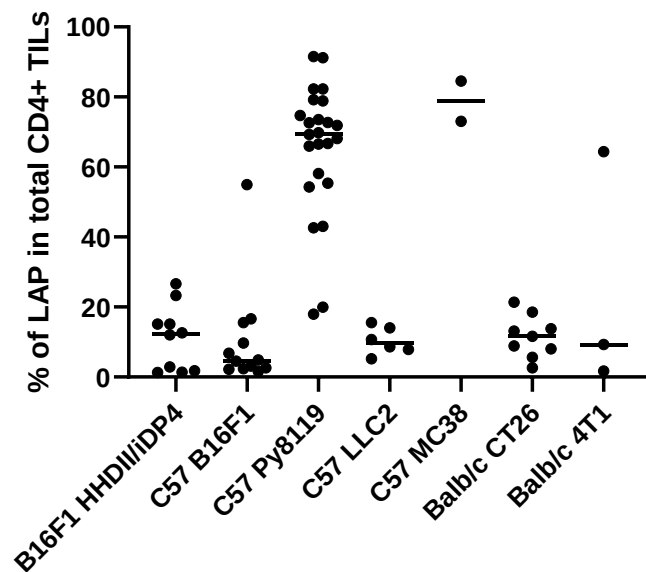


Figure 5-1: The levels of CD4+LAP+ on TILs were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated B16F1 HHDII/iDP4 tumours (n=10), B16F1 (n=12), PY8119 (n=24), LLC2 (n=6) and MC38 (n=2) tumours in C57Bl/6 mice and on CT26 (n=9) and 4T1 (n=3) tumours in Balb/c mice.

B16F1 HHDII/iDP4 TBM unimmunized: LAP in total CD4s

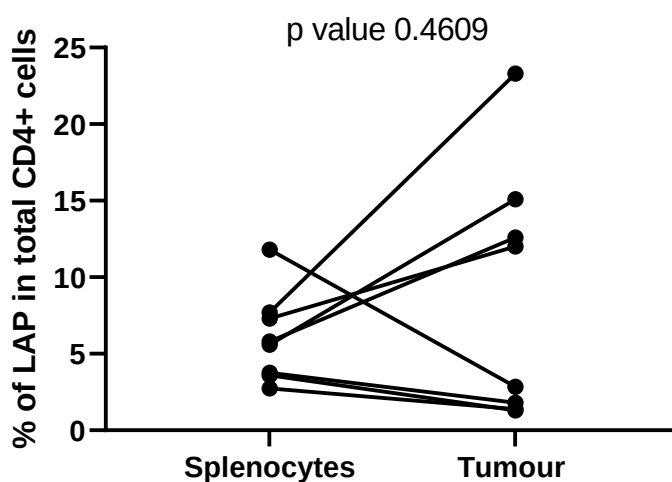


Figure 5-2: LAP expression by CD4+splenocytes and CD4+TILs compared using paired non-parametric Wilcoxon test (p 0.4609). Spleens and tumours from B16F1 in HHDII/iDP4 mice were disaggregated, stained by direct immunofluorescence and flow cytometric analysis was done. TBM: tumour bearing mice.

C57 B16F1 TBM unimmunised: LAP in total CD4s

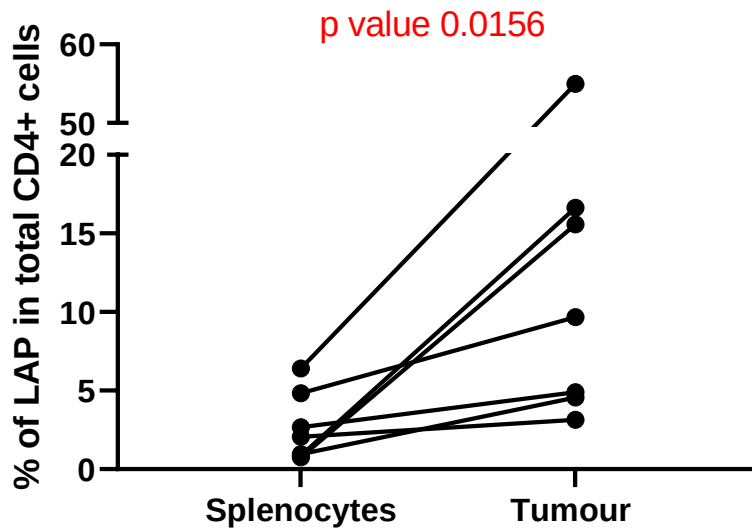


Figure 5-3: LAP expression by CD4+splenocytes and CD4+TILs compared using paired non-parametric Wilcoxon test (p 0.0156). Spleens and tumours from C57Bl/6 mice bearing B16F1 tumours were disaggregated, stained by direct immunofluorescence and flow cytometric analysis was done. TBM: tumour bearing mice.

C57 Py8119 TBM unimmunised: LAP in total CD4s

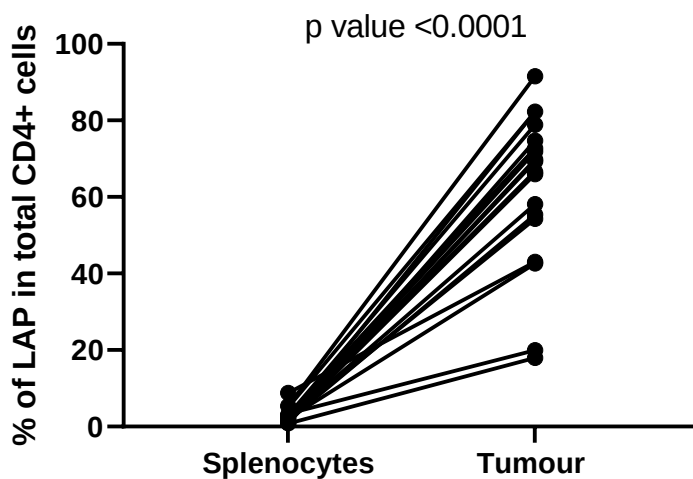


Figure 5-4: LAP expression by CD4+splenocytes and CD4+TILs compared using paired non-parametric Wilcoxon test (p <0.0001). Spleens and tumours from C57Bl/6 mice bearing Py8119 tumours were disaggregated, stained by direct immunofluorescence and flow cytometric analysis was done. TBM: tumour bearing mice.

C57 LLC2 TBM unimmunised: LAP in total CD4s

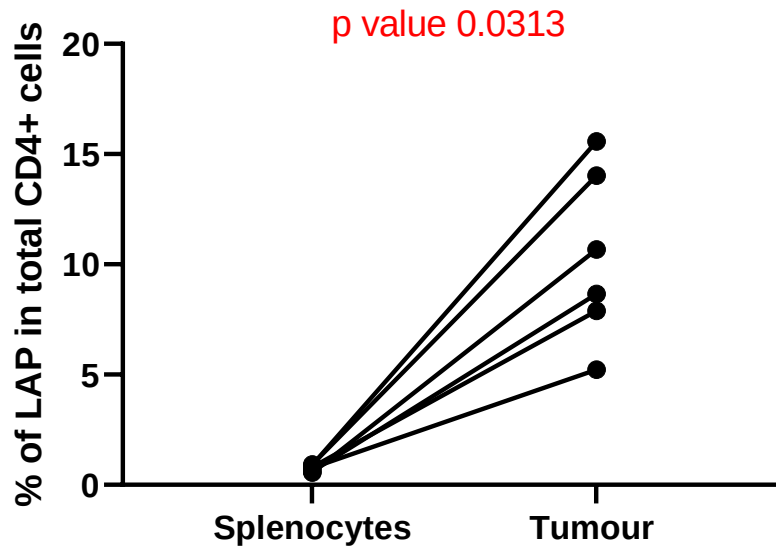


Figure 5-5: LAP expression by CD4+splenocytes and CD4+TILs compared using paired non-parametric Wilcoxon test (p 0.0313). Spleens and tumours from C57Bl/6 mice bearing LLC2 tumours were disaggregated, stained by direct immunofluorescence and flow cytometric analysis was done. TBM: tumour bearing mice.

C57 MC38 TBM unimmunised: LAP in total CD4s

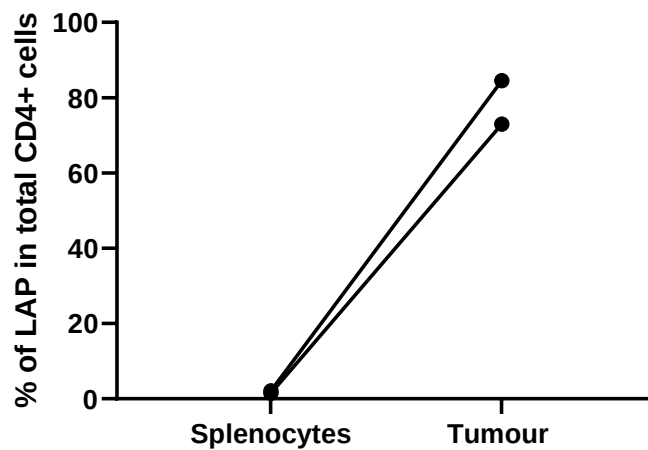


Figure 5-6: LAP expression by CD4+splenocytes and CD4+TILs from C57Bl/6 mice bearing MC38 tumours (no statistical analysis down as $n=2$). Spleens and tumours were disaggregated, stained by direct immunofluorescence and flow cytometric analysis was done. TBM: tumour bearing mice.

Balb/c CT26 TBM unimmunised: LAP in total CD4s

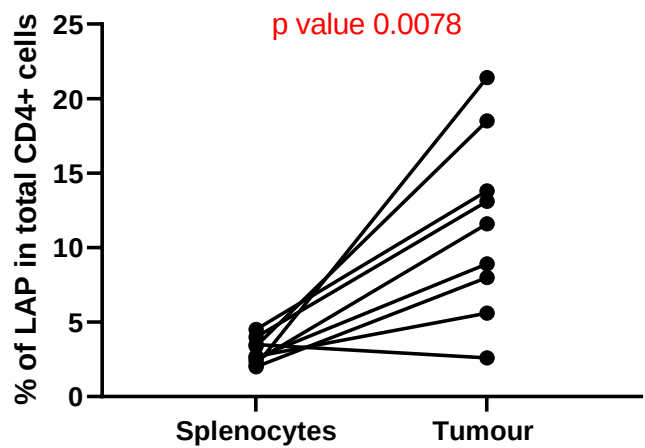


Figure 5-7: LAP expression by CD4+splenocytes and CD4+TILs compared using paired non-parametric Wilcoxon test (p 0.0078). Spleens and tumours from Balb/c mice bearing CT26 tumours were disaggregated, stained by direct immunofluorescence and flow cytometric analysis was done. TBM: tumour bearing mice.

Balb/c 4T1 TBM unimmunised: LAP in total CD4s

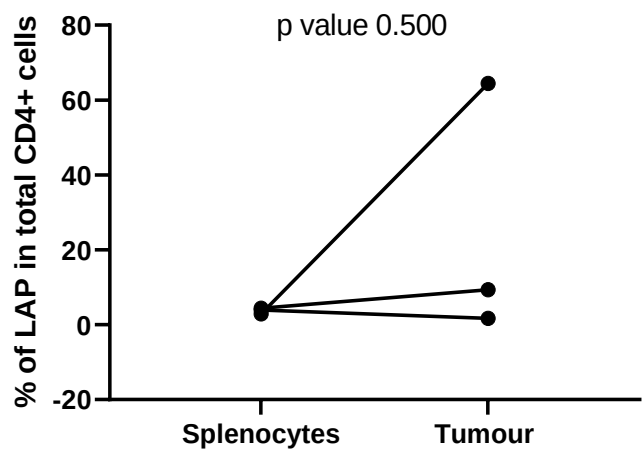


Figure 5-8: LAP expression by CD4+splenocytes and CD4+TILs compared using paired non-parametric Wilcoxon test (p 0.500). Spleens and tumours from Balb/c mice bearing 4T1 tumours were disaggregated, stained by direct immunofluorescence and flow cytometric analysis was done. TBM: tumour bearing mice.

5.3.2 Screening of citrullinated peptides for use in vaccination of C57Bl/6 mice

Ideally, a combination study would be performed using the peptides previously characterised as giving immune responses in cancer patients (chapter 3). However, previous data from the group showed that the citrullinated peptides used in the human assays did not mount an immune response in C57Bl/6 mice. These peptides showed CD4 T-cell mediated IFN γ immune responses and anti-tumour immunity in the HLA transgenic mouse models [144, 145, 163]. This perhaps reflects that these peptides are not presented by the specific MHC class II alleles of C57Bl/6 mice. Therefore, to use the Py8119 tumour model it was necessary to select alternative citrullinated peptide that give immune responses in C57Bl/6 mice. The work in this section selecting the citrullinated peptides to immunise C57Bl/6 mice was carried out by other members of the group.

Based on murine MHC class II predicted binding scores from the IEDB prediction algorithm (<https://www.iedb.org>) of native wild type peptides which had arginine amino acid within their binding core, the immunogenicity of citrullinated peptides were screened in C57Bl/6 mice. Three citrullinated peptides showed IFN γ responses individually and in combination as assessed by cytokine Enzyme linked immunosorbent spot (ELISpot) assay on splenocytes from immunised C57Bl/6 mice. Mice immunised with citrullinated α -Enolase 11-25 (sequence: IFDS-cit-GNPTVEVDLF where cit represents citrulline amino acid) on day 1, 8 and 15 showed a mean of 454 IFN γ spots per million splenocytes as compared to 34 IFN γ spots per million splenocytes in the negative control ($p < 0.0001$, ANOVA with Geisser-Greenhouse correction, Figure 5-9A). With the addition of CD4 or CD8 blocking antibodies, the responses reduced to a mean of 127 and 312 spots per million splenocytes respectively ($p = 0.0013$, 0.0124 respectively, ANOVA with Geisser-Greenhouse correction, Figure 5-9A) suggesting that the response was mainly mediated by CD4 cells. Immunization with citrullinated Cytokeratin8 8-26 (sequence: KSYKMSTSGP-Cit-AFSS-Cit-SFT) gave a mean of 367 IFN γ spots per million splenocytes compared to 46 IFN γ spots per million splenocytes to the negative control ($p < 0.0001$, ANOVA with

Geisser-Greenhouse correction, Figure 5-9B). The addition of blocking antibodies resulted in reduction of the mean to 26 spots per million splenocytes with CD4 antibody but no significant reduction upon the addition of CD8 antibody (mean of 338 spots per million splenocytes) ($p=0.0002$, 0.2719 respectively, ANOVA with Geisser-Greenhouse correction, Figure 5-9B) suggesting that the response to citrullinated Cytokeratin8 8-26 is CD4 mediated. Immunization with the third identified citrullinated peptide Glutamate receptor (Glurecep) 316-340 (sequence: EPKSSCYNTHEK-cit-IYQSNMLN-cit-YLI) gave a mean of 885 IFN γ spots per million splenocytes when compared to 41 IFN γ spots per million splenocytes to the negative control ($p=0.0009$, ANOVA with Geisser-Greenhouse correction, Figure 5-9C). The response to citrullinated Glurecep316-340 was CD4 specific since the addition of CD4 antibody resulted in reduced IFN γ responses (mean of 325 spots per million splenocytes) which was not seen upon the addition of CD8 blocking antibody (mean of 921 spots per million splenocytes) ($p=0.0096$, 0.9919 respectively, ANOVA with Geisser-Greenhouse correction, Figure 5-9C). Vaccination of mice with the combination of all three peptides also showed IFN γ immune responses as assessed by *ex-vivo* ELISpot assays performed using murine IFN γ capture to each of the individual peptide which reached statistical significance for citrullinated Cytokeratin8 8-26 and citrullinated Glurecep316-340 but not to citrullinated α -Enolase 11-25 ($p= 0.0129$, 0.0122 and 0.1033 respectively, ANOVA with Geisser-Greenhouse correction, Figure 5-9D).

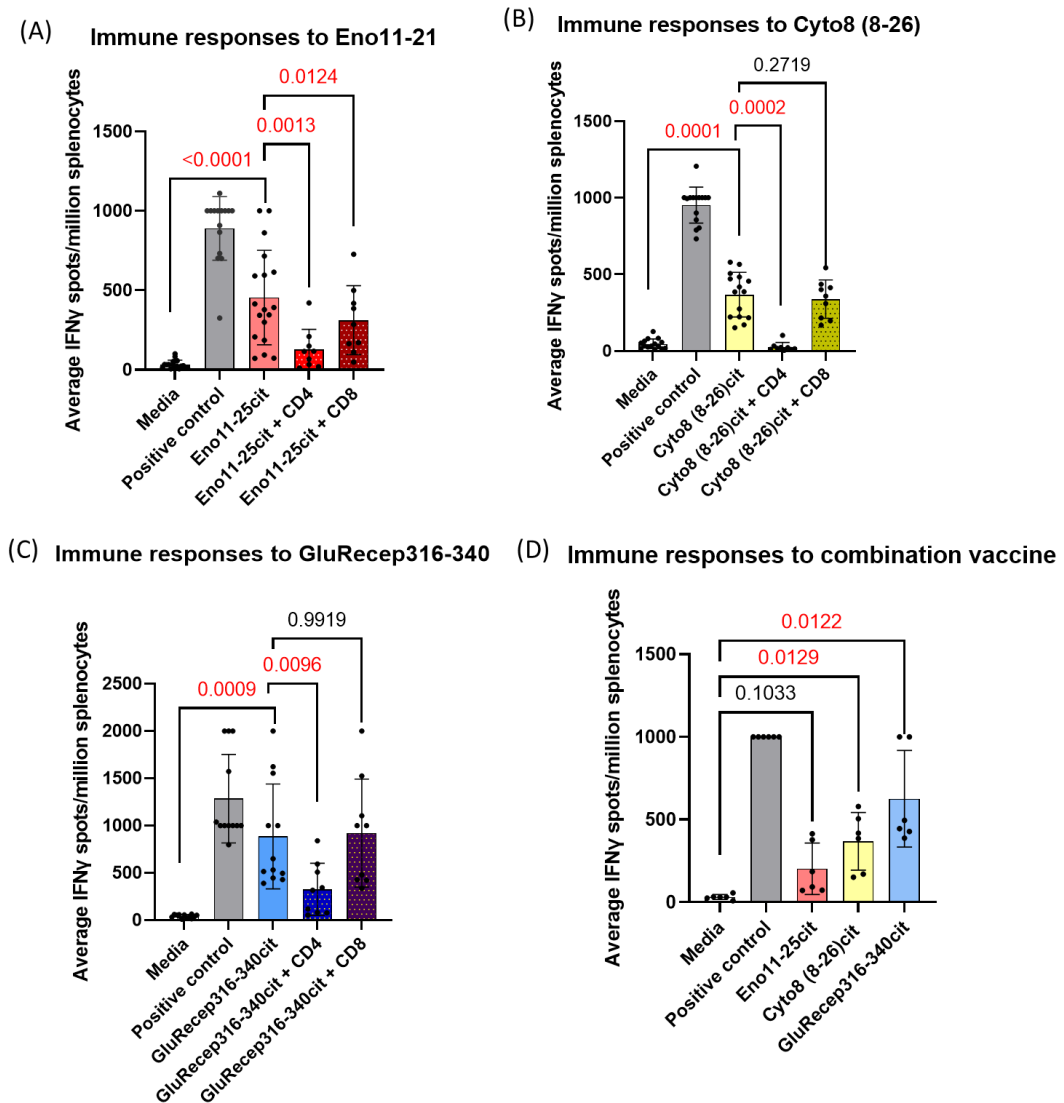


Figure 5-9: Immunisation of C57Bl/6 mice with (A) Citrullinated α -Enolase 11-25, (B) Citrullinated Cytokeratin8 8-26, (C) Citrullinated GluRecep316-340 and (D) Combination of the 3 peptides in A, B & C showed IFN γ responses to the citrullinated peptides as assessed by ex-vivo ELISpot assays performed using murine IFN γ capture on splenocytes. Most of the responses were CD4 mediated and not CD8 as proven by the reduction of responses upon addition of CD4 blocking antibody but not CD8 blocking antibody. Statistical analysis done using ANOVA with Geisser-Greenhouse correction.

These three peptides were next assessed in anti-tumour studies as monotherapies and in combination to examine their effect on overall survival of C57Bl/6 mice implanted with two different tumour models, melanoma B16F1 and melanoma B16F1 transduced with interferon-alfa (IFN α). The former model is reliant on the vaccine upregulating MHC class II while the latter model is engineered where IFN α leads to upregulation of MHC class II so that it is constitutively expressed by tumour cells. In the melanoma B16F1

model, 10 mice were used as control untreated, 10 mice were immunized with citrullinated Cytokeratin8 8-26 alone and 10 mice were immunized with a combination of citrullinated α -Enolase 11-25 + citrullinated Cytokeratin8 8-26 on days 3, 10 and 17. Mice immunised with citrullinated Cytokeratin8 8-26 showed a significant survival advantage compared to unimmunised mice with 40% of mice surviving 28 days post tumour implantation ($p=0.0002$, Log-rank (Mantel-Cox) test, Figure 5-10A) and 20% being completely tumour free ($p=0.0111$, Log-rank (Mantel-Cox) test, Figure 5-10B). Citrullinated Cytokeratin8 8-26 and citrullinated α -Enolase 11-25 in combination showed 70% overall survival and 40% of mice were tumour free at termination of study ($p<0.0001$ and <0.0001 respectively, Log-rank (Mantel-Cox) test, Figure 5-10C & D). 10 C57Bl/6 mice bearing melanoma B16F1 INF α model immunized with all three peptides on days 4, 11 and 18 showed a survival advantage and tumour free survival benefit over the 10 unimmunized mice ($p= 0.0044$ and 0.0355 respectively, Log-rank (Mantel-Cox) test, Figure 5-11). In the immunized group overall survival was 90% and tumour free survival was 60% at termination of the study on day 52 compared to unimmunized mice where overall survival and tumour survival were both 20%. It has also been reported that immunization of C57Bl/6 mice with citrullinated α -Enolase 11-25 alone after implantation with B16F1 tumour model gave a 50% survival advantage ($p= 0.0026$) [146].

Based on the immune responses observed by these three peptides and the survival advantage they gave in the tested tumour models in C57Bl/6 mice; I adopted this citrullinated peptide combination as a vaccine to use in C57Bl/6 mice to study its effects on Th3 cells and if there is synergy with anti-LAP antibody therapy.

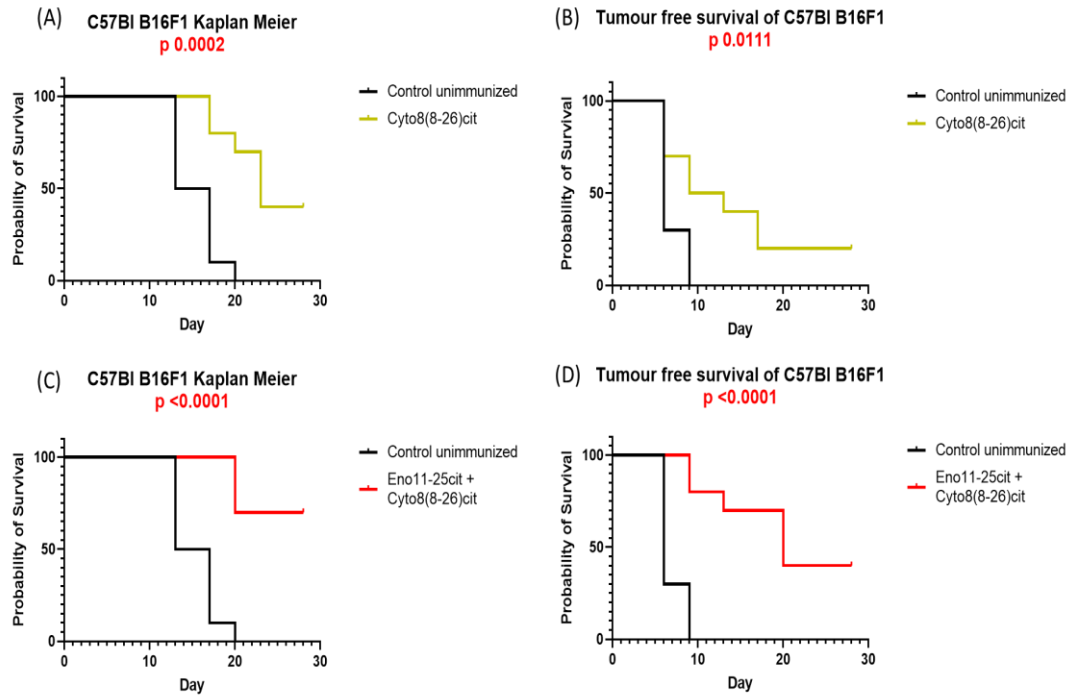


Figure 5-10: Kaplan Meier survival curves showing overall survival of C57Bl/6 mice bearing melanoma B16F1 tumour model when immunized with citrullinated Cytokeratin8 8-26 alone (A) and when immunized with a combination of citrullinated Cytokeratin8 8-26 + citrullinated α -Enolase 11-25 (C) on days 3, 10 and 17 compared to the unimmunized group (Log-rank (Mantel-Cox) test). (B) and (D) show Kaplan Meier plots of C57Bl/6 mice surviving without measurable tumour (termed tumour free survival) after injection with melanoma B16F1 tumour model and immunization with citrullinated Cytokeratin8 8-26 alone (B) and immunization with a combination of citrullinated Cytokeratin8 8-26 + citrullinated α -Enolase 11-25 (D) on days 3, 10 and 17 compared to the unimmunized group (Log-rank (Mantel-Cox) test).

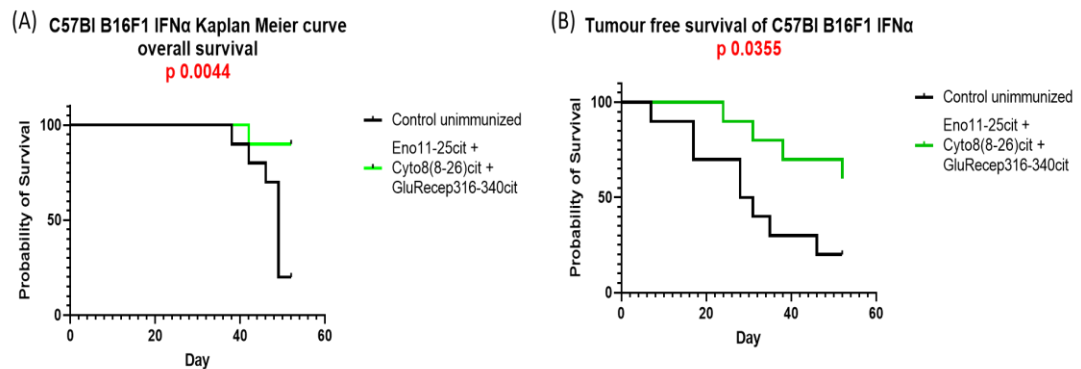


Figure 5-11: (A) Kaplan Meier survival curves showing overall survival of C57Bl/6 mice bearing melanoma B16F1 IFN α tumour model when immunized with a combination of citrullinated α -Enolase 11-25, citrullinated Cytokeratin8 8-26 and citrullinated Glurecep 316-340 on days 4, 11 and 18 compared to the unimmunized group (Log-rank (Mantel-Cox) test). (B) Kaplan Meier plots of C57Bl/6 mice surviving without tumour (termed tumour free survival) after injection with melanoma B16F1 IFN α tumour model and immunization with a combination of citrullinated α -Enolase 11-25, citrullinated Cytokeratin8 8-26 and citrullinated Glurecep 316-340 on days 4, 11 and 18 compared to the unimmunized group (Log-rank (Mantel-Cox) test).

5.3.3 Does vaccination with citrullinated peptides or anti-LAP antibody therapy reduce Th3 levels within tumours and tumour growth

C57Bl/6 mice bearing Py8119 tumour model were used to study CD4+LAP+LAG3 (Th3) levels and tumour growth survival relating to therapy with citrullinated peptides with or without anti-LAP treatment. A diagram illustrating the experiment design is shown in Figure 5-12. Tumours were measured every 3-4 days (representation of tumour volumes growth curves for each group shown in Figure 5-13). The first sign of tumour was observed on day 3 for all groups, while only one of the 10 mice in each of control untreated, citrullinated peptide vaccine treated and citrullinated peptide + anti-LAP combination treated groups showed signs of tumour; 7 out of 10 mice in anti-LAP treated group showed tumour growth. By day 7, all mice in the control untreated and anti-LAP treated group showed a measurable tumour and by day 24 all mice in these 2 groups were culled due to tumour size. In contrast, in the group treated with citrullinated peptides and the group treated with combination therapy there were 5 mice in each group which were still tumour free upon termination of the study (day 42). Tumour regression was observed in mouse C4 treated with citrullinated peptide vaccine where its tumour volume reduced from 33mm³ on day 17 to 0mm³ on day 21 and continued to have unmeasurable tumour on date of termination of study. Similarly, mouse D2 treated with combination of citrullinated peptide + anti-LAP regressed from 33mm³ on day 17 to 0mm³ on day 21. D6 mouse treated with combination of citrullinated peptide + anti-LAP starting to show minimal signs of regression where its' tumour shrunk from 339mm³ on day 17 to 234mm³ on day 21.

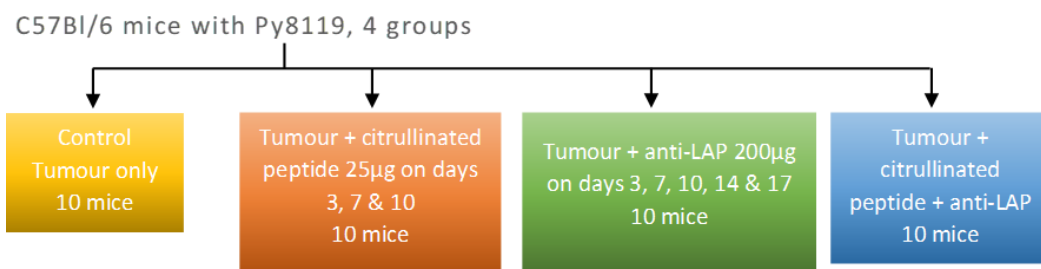


Figure 5-12: C57Bl/6 mice were implanted with Py8119 tumour model on day 1 and 4 groups of 10 mice each were randomly allocated to no treatment, treated with 3 citrullinated peptides s.c. on days 3, 7 and 10, treatment with anti-LAP antibody i.p. on days 3, 7, 10, 14 & 17 and a final group treated with the combination of the 3 citrullinated peptides with anti-LAP therapy using the same dosing regimen.

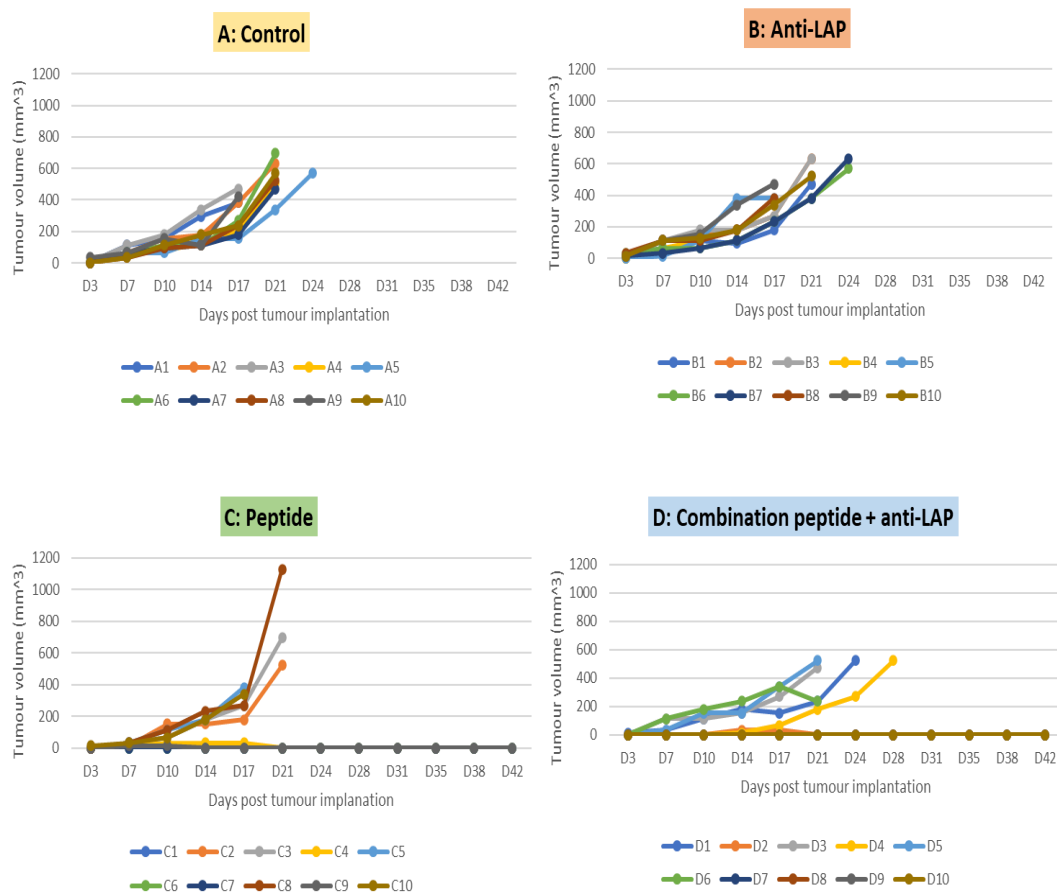


Figure 5-13: Representation of tumour growth in C57Bl/6 mice bearing Py8119 tumour model where (A) control untreated, (B) anti-LAP treated group, (C) citrullinated peptides treated group and (D) combination of citrullinated peptides and anti-LAP treated group. Tumour growth in mice was analysed by measuring the tumour size with callipers (length and width). Volume was estimated by the formula: $\text{Volume} = (\pi/6) \times (\text{width} \times \text{length}^2)$.

50% overall survival was seen in each of the group treated with citrullinated peptide (p 0.0270, Log-rank (Mantel-Cox) test, Figure 5-14B) and the group treated with combination of citrullinated peptides + anti-LAP (p 0.0011, Log-rank (Mantel-Cox) test, Figure 5-14C) when compared to untreated group, these mice were completely tumour free. Anti-LAP treatment on its own did not show any survival advantage compared to the untreated group (p 0.9693, Log-rank (Mantel-Cox) test, Figure 5-14A). When comparing survival between the citrullinated peptide treated group and the group treated with combination therapy, no significant difference was seen and so it can be concluded that the survival benefit in the combination group was achieved due to vaccination with the citrullinated peptides and that the addition of anti-LAP antibody did not add any further benefit in this model (Figure 5-14E).

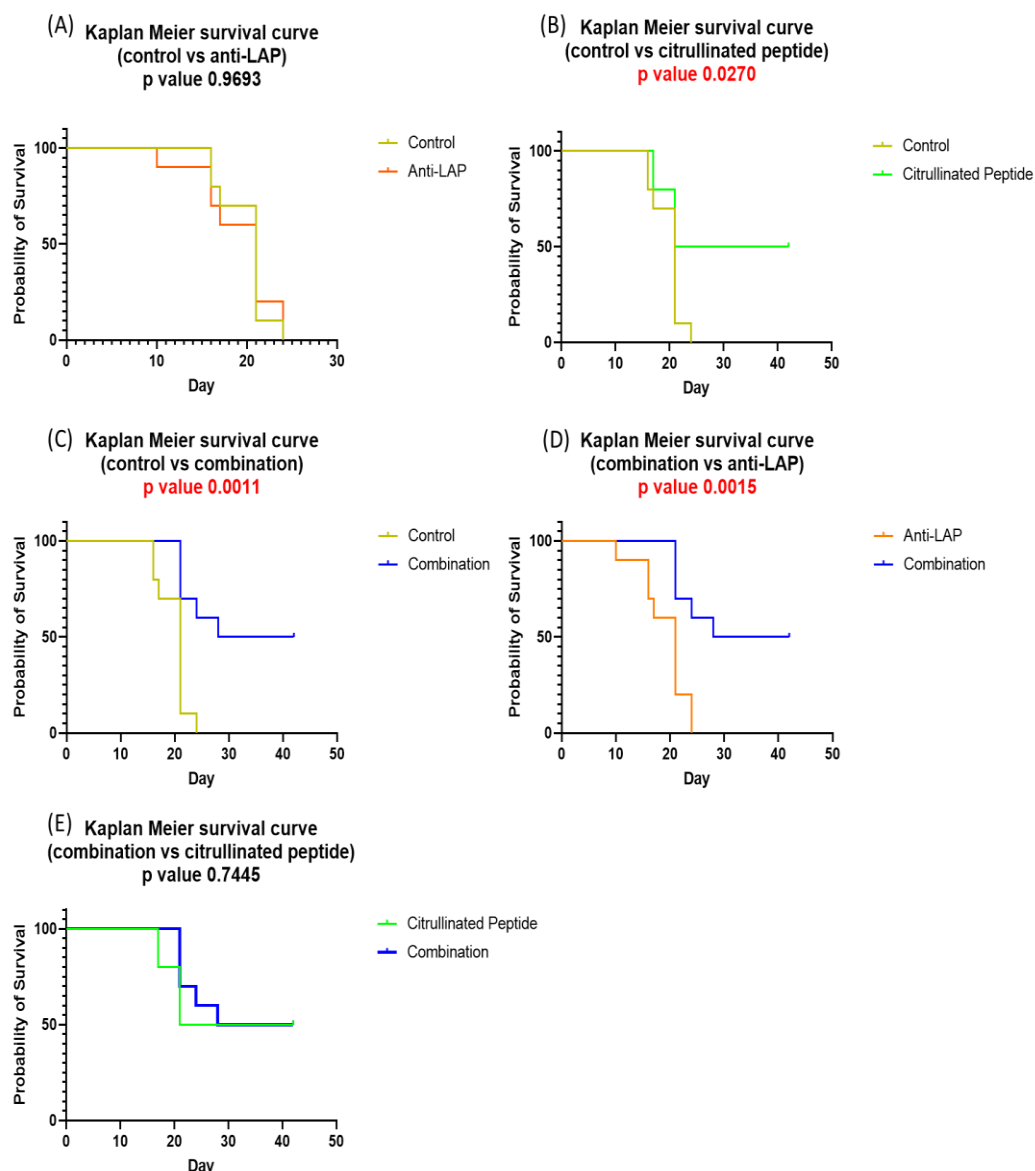


Figure 5-14: Comparison of Kaplan Meier survival curve between (A) control untreated vs anti-LAP treated groups ($p=0.9693$), (B) control untreated vs citrullinated peptides treated groups ($p=0.0270$), (C) control untreated vs combination therapy with citrullinated peptides + anti-LAP treated groups ($p=0.0011$), (D) Anti-LAP alone vs combination therapy with citrullinated peptide + anti-LAP treated groups ($p=0.0015$) and (E) citrullinated peptides alone vs combination therapy with citrullinated peptides + anti-LAP treated groups ($p=0.7445$) of C57Bl/6 mice bearing Py8119 tumour model (Log-rank (Mantel-Cox) test).

At the point of termination, tumours and spleens from each group were tested for expression of CD4, LAP, GARP and LAG3 (Table 5-1, Figure 5-15, Figure 5-16 and Figure 5-17). GARP is one mechanism by which the LAP-TGF- β complex anchors to the cell surface and is expressed mainly on T-regs [258-260]. It is measured here as a secondary indirect mechanism to look at the effects of immunization on LAP expressing cells. LAG3 on the

other hand is co-expressed with LAP on Th3 cells [244]. The focus and main purpose of this staining was to study the differences in CD4+LAP+ population between the groups. The CD4+LAP+ population was significantly lower in the group treated with citrullinated peptides compared to control group ($p=0.0380$, Mann-Whitney test, Figure 5-15) but not in the group treated with anti-LAP antibody or the combination therapy ($p > 0.999$, 0.3531 respectively, Mann-Whitney test). A possible explanation would be that treatment with citrullinated peptides has resulted in a Th1 response and shifted the Th1/Th3 balance in the tumour microenvironment by converting Th3 cells into Th1 cells. Anti-LAP antibody on its own was not able to significantly reduce the CD4+LAP+ levels possibly owing to insufficient blockade of LAP and low affinity of anti-LAP IgG2b antibody used *in-vivo*. There were only three tumours examined for their expression of CD4+LAP+ markers in the group treated with combination therapy which could explain the lack of statistically significant reduction in the expression of LAP by CD4+TILs. Similarly, the group treated with citrullinated peptides showed lower levels of CD4+GARP in the tumour ($p=0.0400$, Mann-Whitney test, Figure 5-16) and possibly a trend towards lower CD4+LAG3 TILs which did not quite reach statistical significance compared to the untreated group ($p=0.0553$, Mann-Whitney test, Figure 5-17). CD4+ TIL infiltration was not different between the groups ($p=0.2237$, Kruskal-Wallis test, Figure 5-18). Figure 5-19 shows that most of the inhibitory population lowered by treatment with citrullinated peptides was double positive for LAP and LAG3 expression suggesting that it is an activated Th3 population which is suppressed by the citrullinated peptide treatment, and this could perhaps be the most suppressive population stratified in this instance ($p=0.0502$, Kruskal-Wallis test). Testing for IFN γ production by CD4+ TILs and establishing the ratio between IFN γ and LAP may solidify the hypothesis that citrullinated peptides mount a Th1 response compared to a Th3.

Table 5-1: Tumours and splenocytes were disaggregated upon culling of C57Bl/6 mice bearing Py8119 tumour model where group (A) was control untreated, group (B) treated with anti-LAP, group (C) treated with citrullinated peptide vaccine and group (D) treated with a combination of anti-LAP and citrullinated peptide vaccine. Cells were stained by direct immunofluorescence for CD4, LAP, GARP & LAG3 expression and analysed by flow cytometry. All % shown in the total are of the total CD4+ cells. S denotes CD4+ splenocytes and T denotes CD4+TILs.

Date terminated	Mouse Gp#	Total CD4+	LAP+		GARP+		LAG3+		LAP+GARP+		LAP+LAG3+		GARP+LAG3+	
		TILs	S	T	S	T	S	T	S	T	S	T	S	T
D16	A1	33.13	5.41	91.53	1.76	3.39	1.48	77.4	1.71	3.38	1.01	77.15	0.61	3.37
D16	A3	20.11	5.01	82.25	1.71	5.61	1.58	66.77	1.63	5.59	1.12	66.35	0.73	5.57
D17	A9	22.04	3.36	19.93	0.74	0.77	1.28	5.57	0.7	0.74	0.77	5.3	0.37	0.69
D21	A2	3.82	1.04	69.31	0.42	23.54	0.91	56.35	0.39	23.28	0.45	54.76	0.28	23.54
D21	A4	5.69	1.11	74.74	0.33	15.7	0.89	65.52	0.3	15.5	0.41	63.81	0.22	15.65
D21	A6	3.1	1.68	66.74	0.76	16.74	2.12	54.51	0.7	16.74	1.08	52.15	0.66	16.74
D21	A7	5.25	1.61	82.25	0.58	28.98	1.24	74.86	0.52	28.98	0.59	73.58	0.43	28.98
D21	A8	3.95	2.06	72.63	0.94	5.73	2.19	56.64	0.88	5.53	1.17	54.73	0.82	5.63
D21	A10	3.53	1.42	71.89	0.38	34.83	0.72	64.92	0.33	34.83	0.31	63.68	0.24	34.83
D24	A5	9.89	8.81	43.06	3.13	31.05	1.69	25.74	3.07	30.3	1.49	24.79	1.08	21.6
D16	B5	17.91	5.46	81.55	1.12	3.05	1.32	68.74	1.06	3.03	1	68.48	0.38	3.03
D16	B9	44.28	5.18	90.28	1.18	3.84	1.24	76.87	1.12	3.84	0.81	76.62	0.43	3.81
D17	B8	16.28	2.94	75.64	0.69	9.94	5.19	56.54	0.57	9.92	1.01	55.73	0.41	9.7
D21	B1	4.17	2.06	60.58	0.54	7.31	0.92	39.88	0.48	7.31	0.6	38.36	0.44	7.31
D21	B2	5.27	1.32	80.56	0.43	6.62	1.22	62.4	0.4	6.41	0.58	60.9	0.37	6.2
D21	B3	5.85	4.11	70.39	2.11	5.84	3.13	50.7	1.95	5.84	2.37	49.43	2.02	5.84
D21	B10	4.21	1.74	67.83	0.7	9.59	1.44	53.96	0.66	9.5	0.89	51.61	0.6	9.59
D24	B6	17.49	12.02	71.01	4.11	57.88	5.45	62.52	4.1	56.53	4.43	61.04	2.68	54.41
D24	B7	9.05	3.84	65.25	1.17	39.87	0.5	38.59	1.12	39.4	0.33	38.24	0.23	32.88
D17	C5	29.73	3.99	22.48	1.05	0.76	1.92	8	0.99	0.74	1.02	7.53	0.53	0.72
D17	C10	28.92	4.25	14.81	0.65	0.71	2.12	6.12	0.62	0.7	1.09	5.52	0.36	0.67
D21	C2	4.81	1.15	70.79	0.4	9.61	1.27	56.47	0.34	9.61	0.52	54.31	0.32	9.61
D21	C3	5.01	1.26	63.87	0.36	14.71	1.15	49.16	0.33	14.71	0.5	47.11	0.26	14.71
D21	C8	16.17	0.99	26.66	0.36	0.55	1.03	8.3	0.31	0.49	0.33	7.57	0.23	0.5
D21	D3	3.08	0.95	35.12	0.29	2.39	0.71	14.8	0.26	2.31	0.32	13.97	0.21	2.15
D21	D5	3.4	1.38	73.5	0.53	2.3	1.06	49.12	0.47	2.12	0.48	47.88	0.32	2.12
D24	D1	6.91	4.2	46.95	1.38	31.62	2.14	36.23	1.26	30.21	1.43	35.07	0.92	28.88

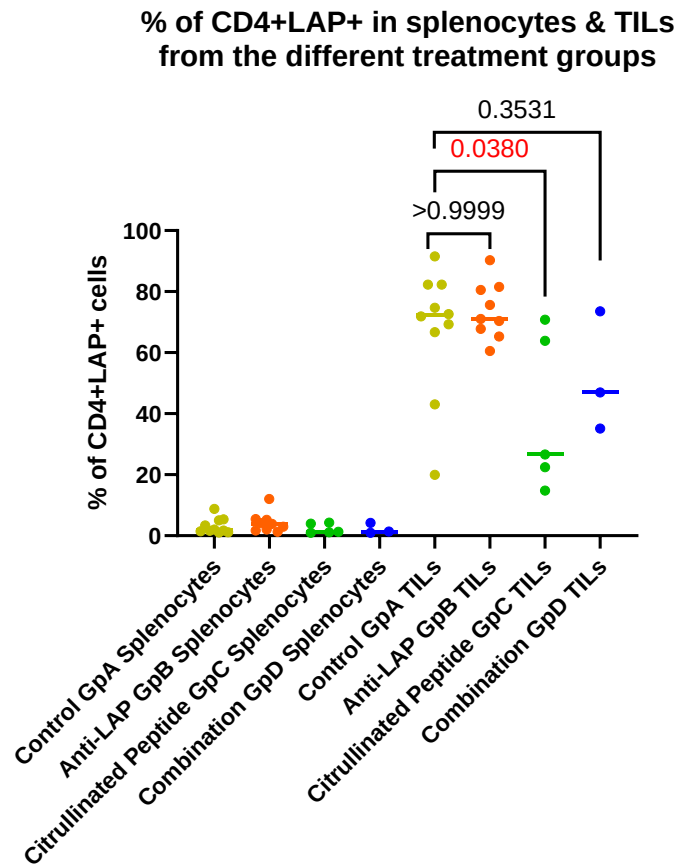


Figure 5-15: The levels of CD4+LAP+ were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours and spleens from C57Bl/6 mice bearing Py8119 tumour model and the different treatment groups compared. Statistical analysis on TILs was done and p values calculated using Mann-Whitney test. When corrections for multiple comparisons was done the statistical significance disappears likely secondary to the small numbers test.

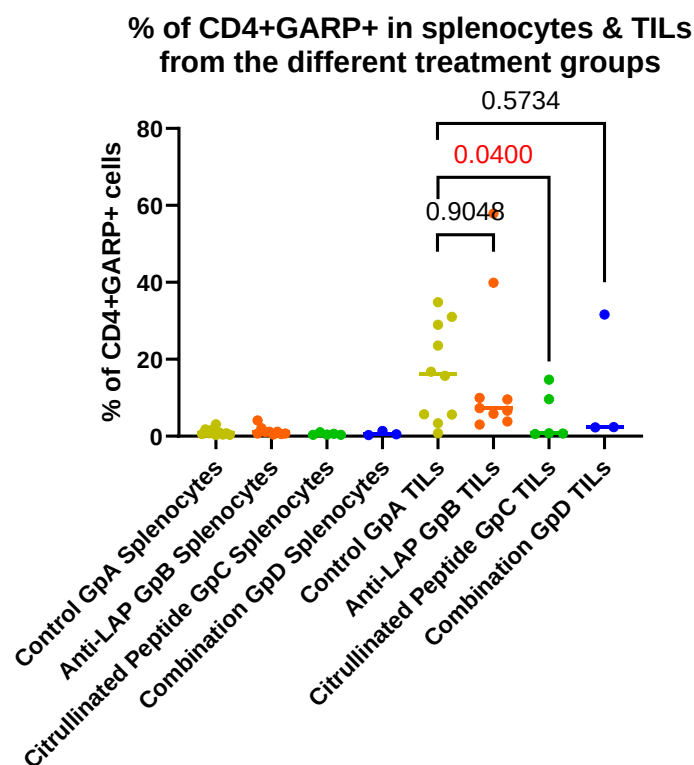


Figure 5-16: The levels of CD4+GARP+ were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours and spleens from C57Bl/6 mice bearing Py8119 tumour model and the different treatment groups compared. Statistical analysis on TILs was done and p values calculated using Mann-Whitney test. When corrections for multiple comparisons was done the statistical significance disappears likely secondary to the small numbers test.

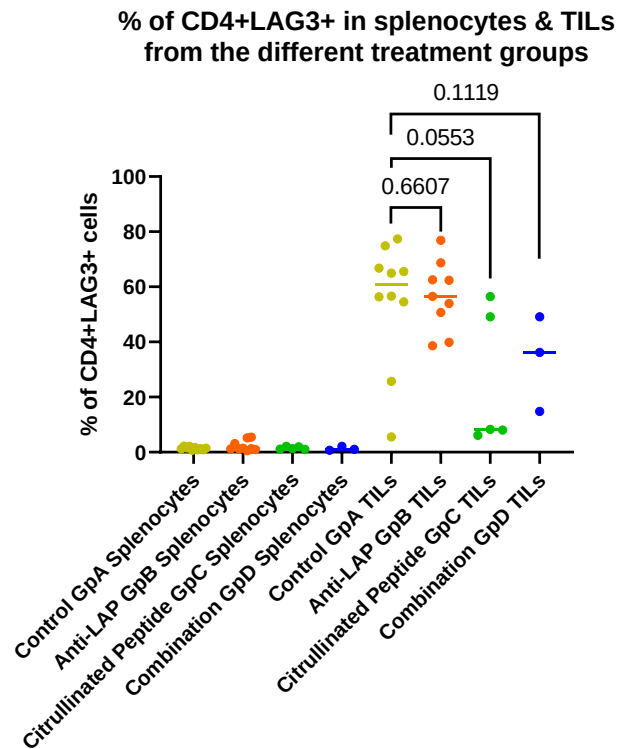


Figure 5-17: The levels of CD4+LAG3+ were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours and spleens from C57Bl/6 mice bearing Py8119 tumour model and the different treatment groups compared. Statistical analysis on TILs was done and p values calculated using Mann-Whitney test.

% of CD4+ TILs in the different treatment groups

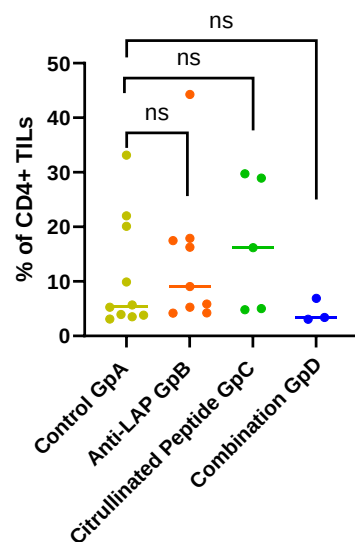


Figure 5-18: The levels of CD4+ were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours from C57Bl/6 mice bearing Py8119 tumour model and the different treatment groups compared. Statistical analysis on TILs was done and p values calculated using non-parametric Anova with corrections for multiple comparisons (Kruskal-Wallis test).

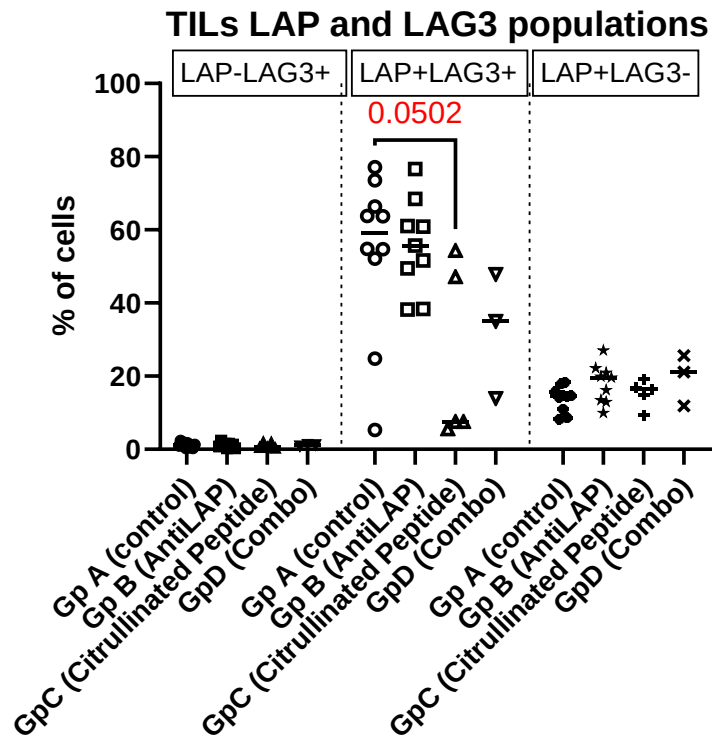


Figure 5-19: The levels of CD4+LAP+ and CD4+LAG3+ were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours from C57Bl/6 mice bearing Py8119 tumour model and the different groups of mice compared. Statistical analysis on TILs and p values calculated using non-parametric Anova with corrections for multiple comparisons (Kruskal-Wallis test). The difference between the treatment groups in the expression of LAP-LAG3+, LAP+LAG3+ and LAP+LAG3- populations by total CD4+ TILs is shown. P value is only shown if statistical significance is reached.

5.3.4 Does therapy with citrullinated peptide vaccination in combination with or without anti-LAP antibody still reduce Th3 levels and give a survival benefit in a tumour model that has lower levels of Th3 TILs?

Treatment with citrullinated peptides in C57Bl/6 mice bearing PY8119 tumours showed a survival benefit and lowered Th3 levels in tumour infiltrating lymphocytes. Gabriely et al [307], described anti-tumour responses and reduction in tumour growth in murine tumour models including B16F1 melanoma with anti-LAP therapy. In an attempt to replicate results and to examine the effects of treatment with citrullinated peptides in a model with lower Th3 levels we adopted a similar study design as 5.3.3 in C57Bl/6 mice bearing B16F1 tumour model. A diagram illustrating the experiment design is shown in Figure 5-20. The first sign of tumour was observed on day 4 for the control untreated group, while tumours started appearing on day 8 for the other three groups treated with anti-LAP alone, citrullinated peptide vaccine alone and both therapies in combination. By day 25 all mice in untreated group were culled due to tumour size. This happened on day 29 for the group treated with anti-LAP while for the 2 groups treated with citrullinated peptide vaccine alone and that treated with combination therapy there was one mouse in each group which was still tumour free on termination of study (day 36). Tumour regression was not observed in any of the treatment groups. Figure 5-21 is a representation of tumour volumes measured in every 3-4 days in each of the groups.

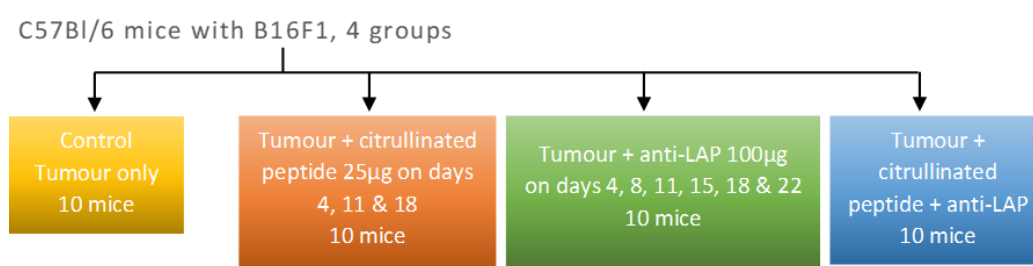


Figure 5-20 C57Bl/6 mice were implanted with B16F1 tumour model on day 1 and 4 groups of 10 mice each were randomly allocated to a no treatment group, a group treated with 3 citrullinated peptides s.c on days 4, 11 and 18, a group treated with anti-LAP antibody i.p. on days 4, 8, 11, 15, 18 & 22 and a final group treated with the

combination of the 3 citrullinated peptides with anti-LAP therapy using the same dosing regimen as the signal treatment arms.

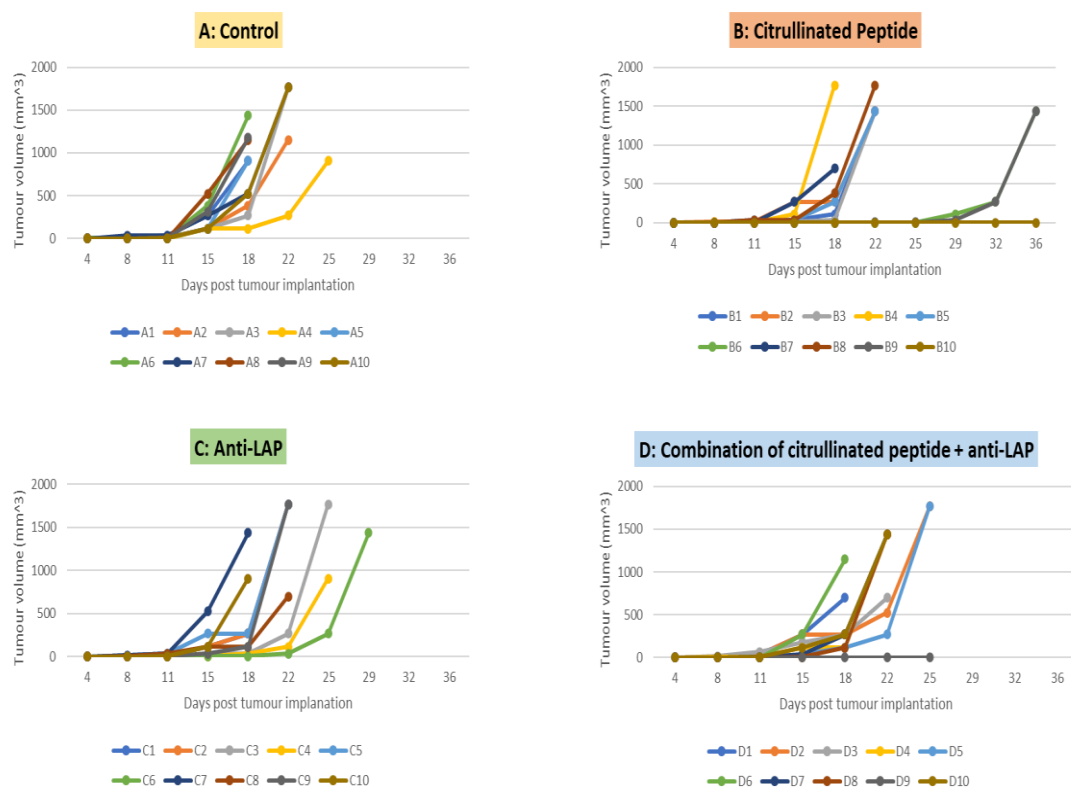


Figure 5-21: Representation of tumour growth in C57Bl/6 mice bearing B16F1 tumour model where (A) control untreated, (B) citrullinated peptide treated group, (C) anti-LAP treated group and (D) combination of citrullinated peptide and anti-LAP treated group. Tumour growth in mice was analysed by measuring the tumour size with callipers (length and width). Volume was estimated by the formula: $\text{Volume} = (\pi/6) \times (\text{width} \times \text{length}^2)$.

On day 15, it was noted that tumour volumes in the group treated with citrullinated peptides were lower than those seen in the untreated group ($p=0.0057$, Mann-Whitney test, Figure 5-22A). The tumour volumes in the group treated with anti-LAP alone and the group treated with combination therapy were not significantly lower than the control on day 15 but they were close to significance ($p=0.0549$ and 0.0916 respectively, Mann-Whitney test). On day 18 all treatment groups showed lower tumour volumes compared to the control group ($p=0.0269$, 0.0269 and 0.0208 respectively, Mann-Whitney test, Figure 5-22B). Following this exponential tumour growth was seen in all group (Figure 5-21). Furthermore, although not statistically significant the group treated with citrullinated peptide vaccine seem to have a trend of better

tumour free survival ($p=0.0637$, Log-rank (Mantel-Cox) test, Figure 5-23A) compared to the untreated group. This trend of improvement in tumour free survival was not observed in the group treated with anti-LAP alone ($p=0.3914$, Log-rank (Mantel-Cox) test, Figure 5-23B) or the combination therapy ($p=0.1957$, Log-rank (Mantel-Cox) test Figure 5-23C) compared to the untreated group. No survival benefit was seen to any of treatment regimens compared to the untreated group ($p=0.1577$, Log rank (Mantel-Cox) test, Figure 5-24A) although when comparing the survival of the untreated to the group treated with citrullinated peptide vaccine it was statistically close to significance ($p=0.0540$, Figure 5-24B, Log rank (Mantel-Cox) test). This may suggest that reducing Th3 has allowed the immune response to control growth of small tumours but eventually the tumours outgrew the immune response.

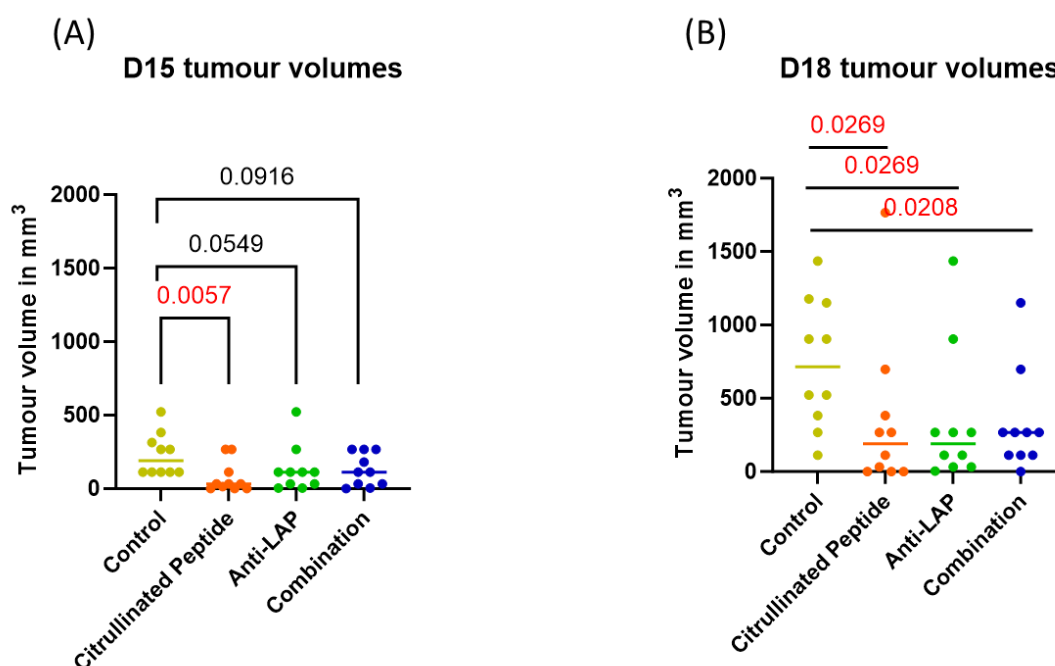


Figure 5-22: Comparison between the tumour volumes on (A) day 15 and (B) day 18 for each of the 4 groups of C57Bl/6 mice bearing B16F1 tumour model. Tumour growth in mice was analysed by measuring the tumour size with callipers (length and width). Volume was estimated by the formula: $\text{Volume} = (\pi/6) \times (\text{width} \times \text{length}^2)$. Statistical analysis done using Mann-Whitney test. When applying corrections for multiple comparisons (Kruskal-Wallis test) only the difference between control group and citrullinated peptide treated group remains statistically significant likely due to the small numbers examined.

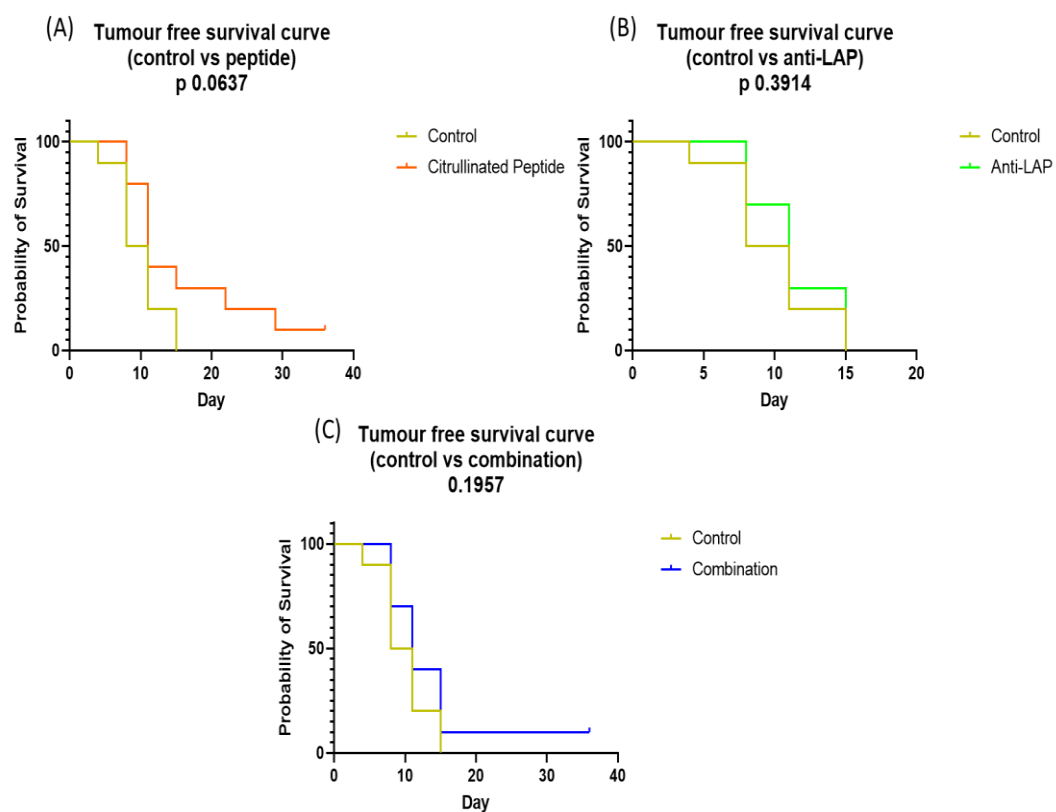


Figure 5-23: Comparison of tumour free survival of C57Bl/6 mice bearing B16F1 tumour model where (A) group treated with citrullinated peptide is compared to untreated group (p 0.0637), (B) group treated with anti-LAP is compared to untreated group (p 0.3914) & (C) group treated with combination therapy of citrullinated peptide + anti-LAP is compared to untreated group (p 0.1957). Statistical comparison done by Log rank (Mantel-Cox) test.

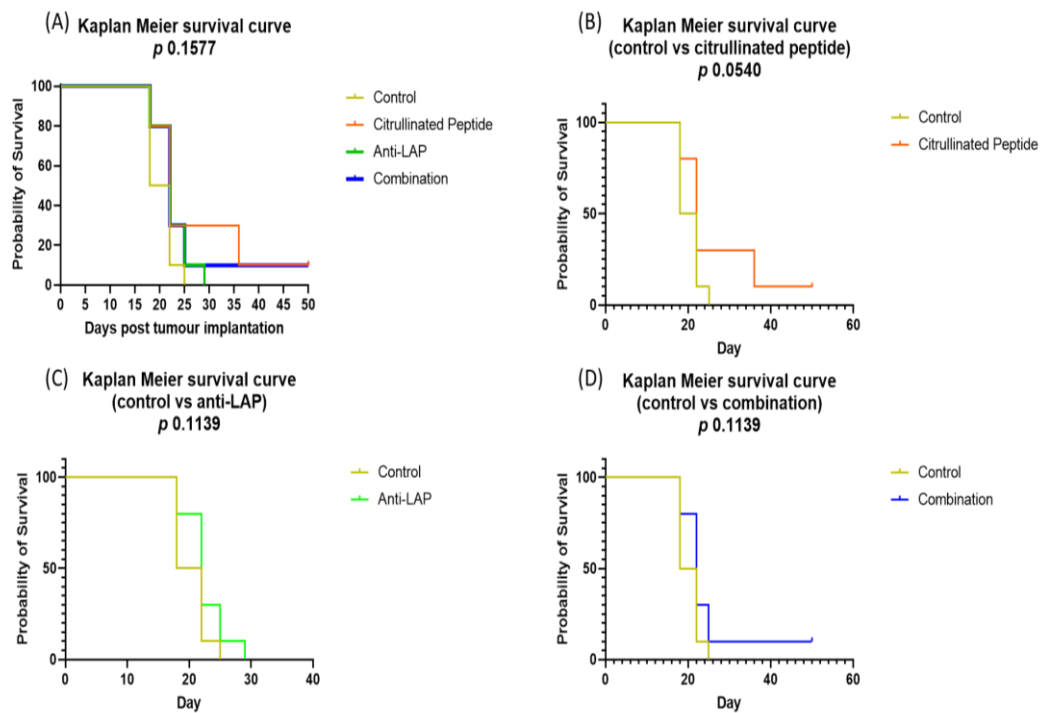


Figure 5-24: Comparison of Kaplan Meier survival curve between (A) control untreated, citrullinated peptides treated group, anti-LAP and combination of citrullinated peptides + anti-LAP therapy ($p=0.1577$), (B) control untreated vs citrullinated peptides treated groups ($p=0.0540$), (C) control untreated vs anti-LAP ($p=0.1139$), (D) control untreated vs combination therapy with citrullinated peptides + anti-LAP treated groups ($p=0.1139$) of C57Bl/6 mice bearing B16F1 tumour model (Log-rank (Mantel-Cox) test).

At the point of termination, 5-6 tumours from each group were tested for expression of CD4, LAP, GARP and Foxp3 (Table 5-2). The focus and main purpose of this staining was to study the differences in CD4+LAP+ population between the groups. The CD4+LAP+ population was significantly lower in the group treated with citrullinated peptides and those treated with anti-LAP antibody ($p= 0.0427$ and 0.0076 respectively, Kruskal-Wallis test, Figure 5-25) this is surprising as these tumours grew so large the mice had to be culled. However, the results suggest that the anti-LAP antibody is inducing an immunological effect in the tumour even though this does not translate to improved survival. This implies that despite low numbers of CD4+LAP+ cells the immune response was not strong enough to control aggressive tumour growth and suggest other immunosuppressive mechanisms may have been involved. No significant reduction in CD4+LAP+ was seen in the group

treated with the combination therapy ($p= 0.4525$, Kruskal-Wallis test) possibly due to the small number of tumours analysed.

Table 5-2: Tumours were disaggregated upon culling of C57Bl/6 mice bearing B16F1 tumour model where group (A) was control untreated, group (B) treated with citrullinated peptide, group (C) treated with anti-LAP and group (D) treated with a combination of anti-LAP and citrullinated peptide vaccine. Cells were stained by direct immunofluorescence for CD4, LAP, GARP & Foxp3 expression and analysed by flow cytometry. All % shown in the total are of the total CD4+TILs.

Date terminated	Mouse Gp#	LAP+	GARP+	Foxp3+	LAP+GARP+	LAP+FOXP3+	GARP+FOXP3+
D18	A1	2.61	1.7	1.23	0.41	0.15	0.07
D18	A5	6.76	9.5	2.05	1.11	0.77	0.6
D18	A6	1.61	0.73	3.05	0.24	0.13	0.14
D18	A7	2.22	1.05	0.38	0.4	0.02	0.02
D18	A8	2.4	0.85	2.03	0.34	0.15	0.15
D18	B7	1.92	1.3	5.16	0.33	0.35	0.75
D22	B1	0.51	0.22	0.55	0.1	0	0
D22	B2	0.81	0.31	0.79	0.15	0.05	0.01
D22	B3	0.73	0.4	0.37	0.18	0	0
D22	B5	1.52	0.7	0.49	0.19	0.01	0.01
D22	B8	1.15	0.41	0.6	0.14	0.01	0
D18	C7	1.1	0.77	7.41	0.18	0.18	0.42
D18	C10	0.93	0.48	1.59	0.14	0.1	0.07
D22	C1	1.02	0.42	0.65	0.14	0.01	0
D22	C2	0.53	0.16	1.25	0.06	0.01	0
D22	C5	0.91	0.59	0.5	0.14	0.01	0.01
D22	C9	0.47	0.13	0.29	0.06	0	0
D18	D1	2.85	1.69	2.41	0.38	0.41	0.6
D18	D6	1.55	0.87	1.91	0.15	0.13	0.39
D22	D3	2.57	1.63	0.13	0.37	0.03	0
D22	D8	0.71	0.27	1.68	0.1	0.02	0

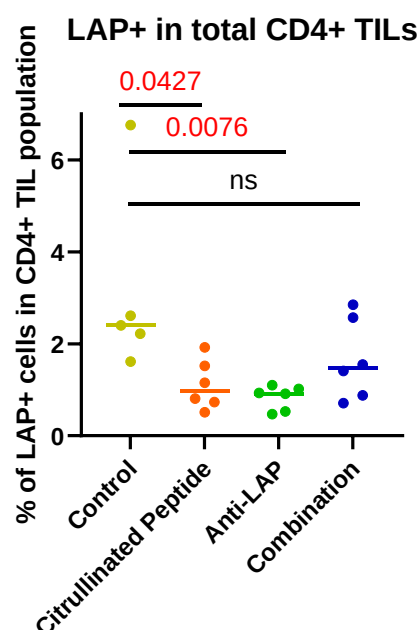


Figure 5-25: The percentage of LAP+ cells in the CD4+ TIL population were analysed by direct immunofluorescence and flow cytometric analysis done on disaggregated tumours from C57Bl/6 mice bearing B16F1 tumour model. Statistical analysis on TILs was done and p values calculated using non-parametric Anova with corrections for multiple comparisons (Kruskal-Wallis test) to compare each treatment group to the control untreated group.

5.3.5 Does therapy with citrullinated peptides impact LAG3 cell levels and is there a survival benefit and synergy with anti-LAG3 treatment

While the anti-LAP antibody did not appear to synergise with vaccination, it is still possible that another checkpoint antibody would be more successful. At a time when therapies with neutralising antibodies against inhibitory receptors is constantly evolving; we wanted to examine synergy between citrullinated peptide vaccination and an antibody targeting the inhibitory and exhaustion marker LAG3. First the levels of LAG3 expression by CD4+ TILs were examined in HHDII/iDP4, C57Bl/6 and Balb/c mice. The expression of CD4+LAG3+ cells in splenocytes was low with a median of 0.955% in C57Bl/6 mice, 2.75% in HHDII/iDP4 and 0.3% in Balb/c mice. The highest expression of CD4+LAG3+ TILs was seen in C57Bl/6 bearing Py8119 tumour cell line with a median percentage of CD4+ TILs expressing LAG3 being 56.64% followed by Balb/c mice bearing CT26 tumour cell line where the median was 26.9% (Figure 5-26). B16F1 in

HHDI/IDP4, B16F1 in C57Bl/6 mice, MC38 in C57Bl/6 mice and 4T1 in Balb/c mice showed much lower levels of LAG3 expression by CD4+TILs with a median of 12.65, 3.15, 8.45 and 1.10% respectively (Figure 5-26).

CD4+LAG3+ TILs: comparison between murine tumour models

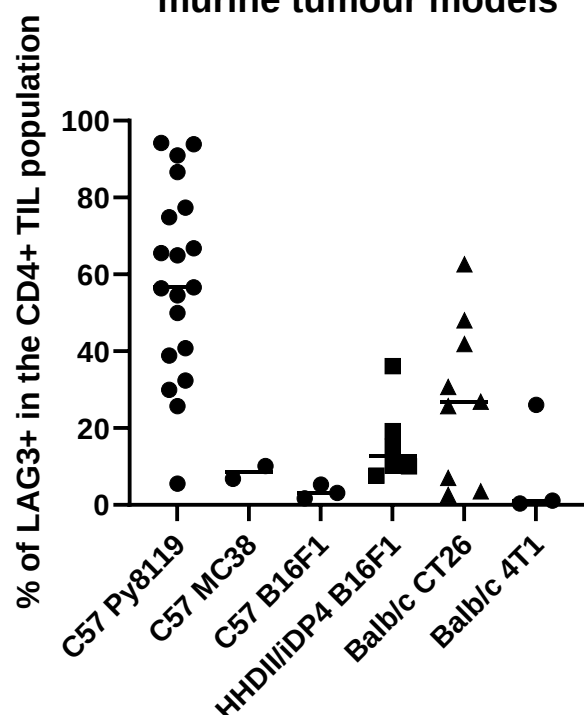


Figure 5-26: The levels of CD4+LAG3+ on TILs were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated PY8119 (n=19), MC38 (n=2) and B16F1 (n=3) tumours in C57Bl/6 mice, B16F1 tumour in HHDI/IDP4 mice (n=6) and on CT26 (n=9) and 4T1 (n=3) tumours in Balb/c mice.

Based on this the C57Bl/6 mice bearing Py8119 tumour model was picked to examine synergy between citrullinated peptide vaccination and an anti-LAG3 antibody. 10 mice in each group were either untreated or received treatment with citrullinated peptides or with anti-LAG3 antibody or were treated with a combination of citrullinated peptides and anti-LAG3. A diagram illustrating the experiment design is shown in Figure 5-27. First sign of tumour was observed on day 3 for all groups. By day 7 all mice in the 4 groups but one mouse treated with citrullinated peptides (mouse B2) showed tumour growth. By day 24 all mice in control untreated group were culled due to tumour size. All mice in the group treated with citrullinated peptides and the group treated with anti-LAG3 were culled by day 28. It is important to

note that mice in these 2 groups (citrullinated peptide group and anti-LAG3 group) were mostly culled by day 24 similar to the control group with 2 mice in each of the 2 groups making it to day 28. 9 out of 10 mice in the combination therapy group were culled by day 28 and the last mouse was culled on day 31. Tumour regression was observed in three mice in the group treated with combination therapy. One mouse (D4) had a tumour volume of 94mm^3 on day 10 which regressed to 33mm^3 on day 14 and remained the same size on day 17 but then grew to 507mm^3 on day 21 when it was culled. Another mouse (D5) showed a tumour volume of 65mm^3 on day 10 which then regressed to 33mm^3 on day 14, 14mm^3 on day 17 and remained the same size on day 21 but then grew to 113mm^3 on day 24 and was culled on day 31. The tumour of the third mouse (D2) grew to a volume of 113mm^3 on day 14 then regressed to 65mm^3 on day 17 but started growing again on day 21 and was culled on day 28. Figure 5-28 is a representation of tumour volumes measured every 3-4 days in each of the groups.

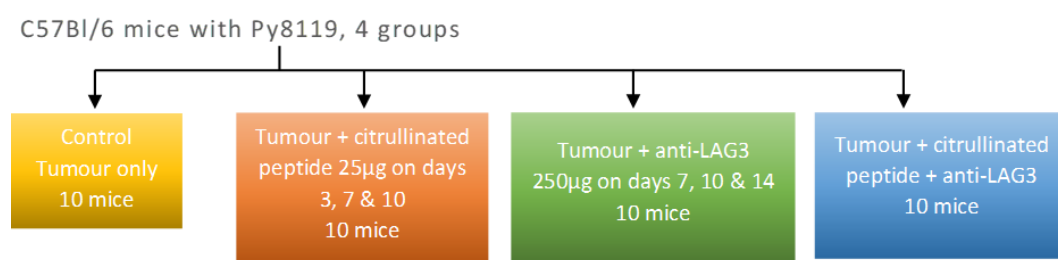


Figure 5-27: C57Bl/6 mice were implanted with Py8119 tumour model on day 1 and 4 groups of 10 mice each were randomly allocated to no treatment, treated with three citrullinated peptides s.c. on days 3, 7 and 10, treatment with anti-LAG3 antibody i.p on days ,7, 10 & 14 and a final group treated with the combination of the three citrullinated peptides with anti-LAG3 therapy using the same dosing regimen as the signal treatment arms.

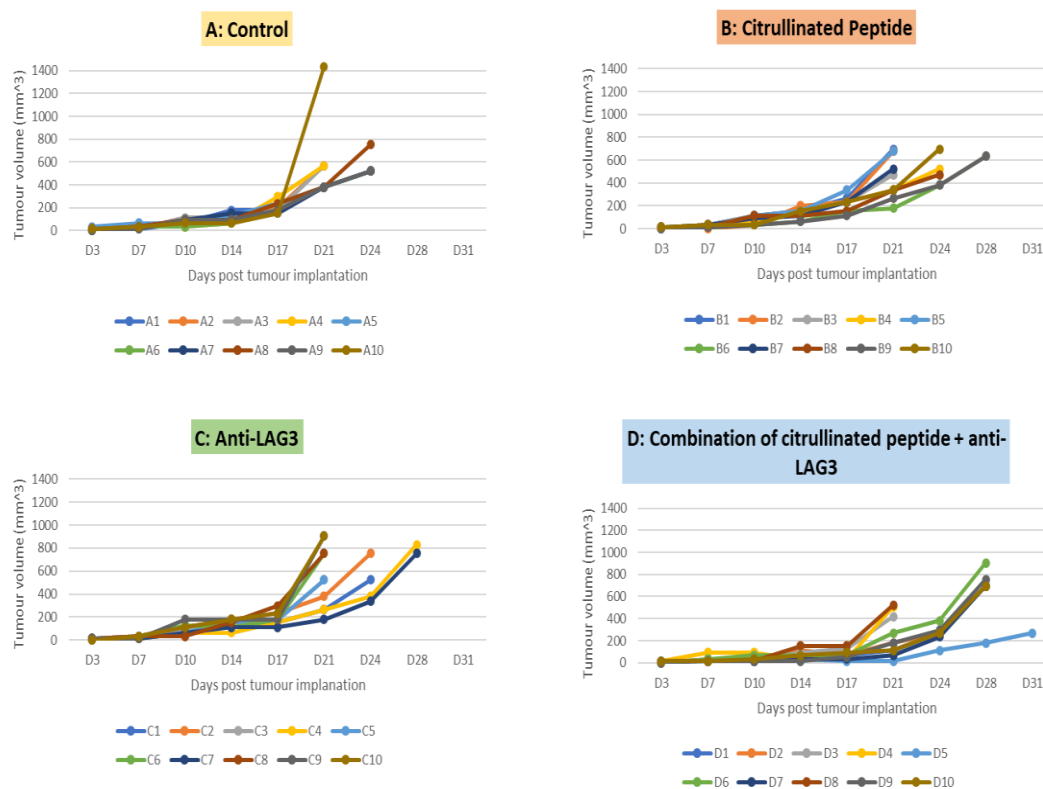


Figure 5-28: Representation of tumour growth in C57Bl/6 mice bearing Py8119 tumour model where (A) control untreated, (B) citrullinated peptide treated group, (C) anti-LAG3 treated group and (D) combination of citrullinated peptide and anti-LAG3 treated group. Tumour growth in mice was analysed by measuring the tumour size with callipers (length and width). Volume was estimated by the formula: $\text{Volume} = (\pi/6) \times (\text{width} \times \text{length}^2)$.

Unlike the results shown in section 5.3.3 where 50% overall survival was seen in the group vaccinated with citrullinated peptide ($p=0.0270$, Log-rank (Mantel-Cox) test, Figure 5-14B); this study did not show any survival advantage in the group treated with citrullinated peptide compared to the control untreated group ($p=0.3747$, Log-rank (Mantel-Cox) test, Figure 5-29A). It is worth pointing out that both studies shared the same murine tumour model and immunization schedule and that tumour growth rates were similar in both studies as well (Figure 5-13 & Figure 5-28). Therapy with anti-LAG3 alone did not confer a survival advantage either ($p=0.6730$, Log-rank (Mantel-Cox) test, Figure 5-29B). However, the combination therapy resulted in an overall survival benefit compared to the control untreated group ($p=0.0056$, Log-rank (Mantel-Cox) test, Figure 5-29C). This advantage was also statistically significant when comparing the combination therapy group to the group treated with citrullinated peptide alone ($p=0.0371$, Log-rank

(Mantel-Cox) test, Figure 5-29D) and to the group treated with anti-LAG3 alone ($p=0.0255$, Log-rank (Mantel-Cox) test, Figure 5-29E).

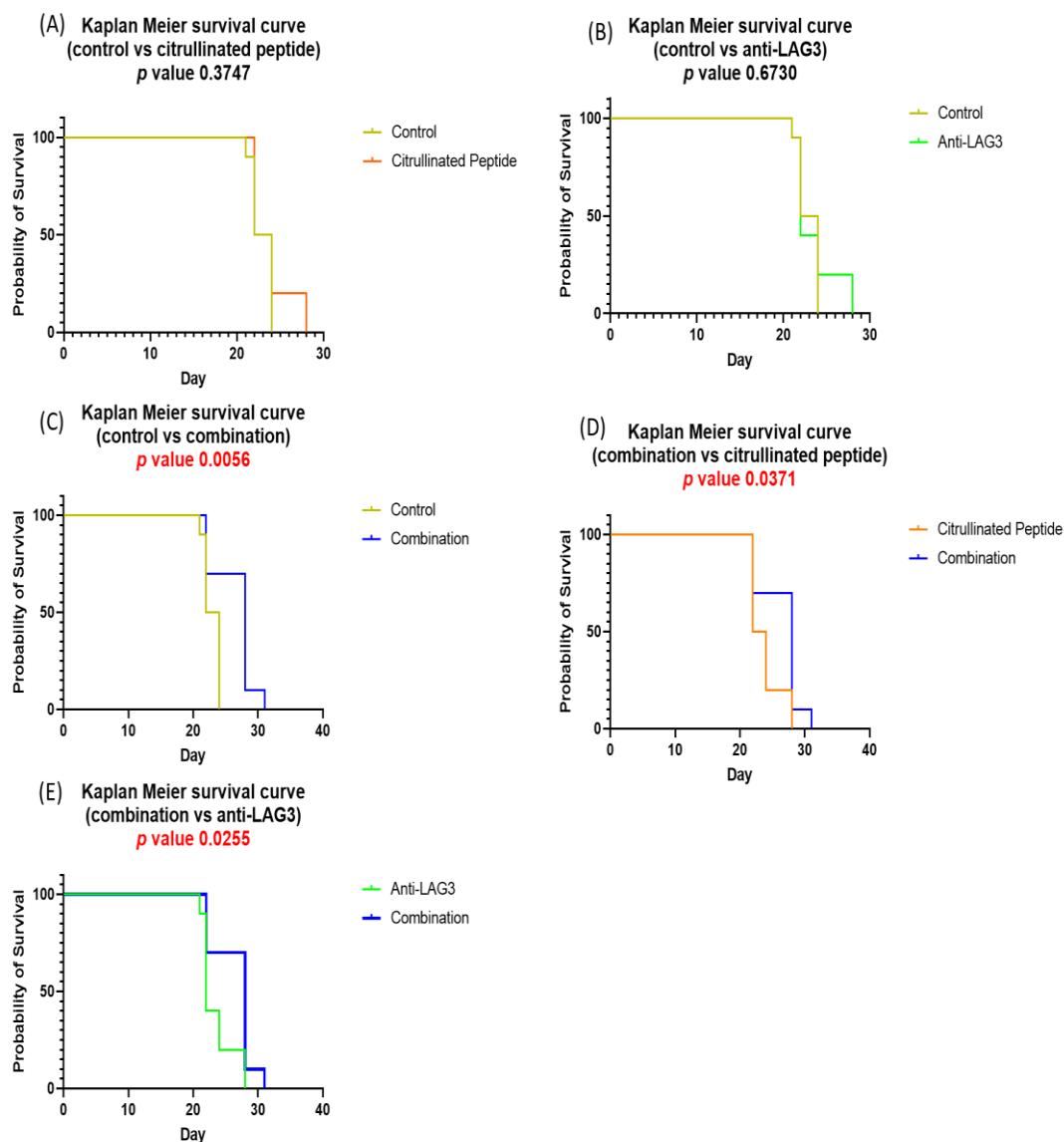


Figure 5-29: Comparison of Kaplan Meier survival curve between (A) control untreated vs citrullinated peptide treated groups ($p=0.3747$), (B) control untreated vs anti-LAG3 treated groups ($p=0.6730$), (C) control untreated vs combination therapy with citrullinated peptide + anti-LAG3 treated groups ($p=0.0056$), (D) citrullinated peptide alone vs combination therapy with citrullinated peptide + anti-LAG3 treated groups ($p=0.0371$) and (E) anti-LAG3 alone vs combination therapy with citrullinated peptide + anti-LAG3 treated groups ($p=0.0255$) of C57Bl/6 mice bearing Py8119 tumour model (Log-rank (Mantel-Cox) test).

At the point of termination, tumours and spleens from each group were tested for expression of CD4, LAP, GARP and LAG3 (Table 5-3, Figure 5-30, Figure 5-33, Figure 5-32 & Figure 5-31). The CD4+ TIL infiltration was not significantly different between the groups (Kruskal-Wallis test, Figure 5-30). The focus and main purpose of this staining was to study the differences in CD4+LAG3+ and CD4+LAP+ populations between the groups. None of the treatment groups resulted in lower levels of LAG3 or GARP (Figure 5-32 & Figure 5-31). A possible explanation to the former could be that the anti-LAG3 antibody is one that blocks function rather than depletes or kills LAG3 expressing cells. The CD4+LAP+ population was significantly lower in the group treated with combination therapy compared to control group ($p=0.0202$, Kruskal-Wallis test, Figure 5-33) but not in the group treated with citrullinated peptide or anti-LAG3 antibody further implying that the combination therapy induced a Th1 response and lowered the Th3 levels which resulted in better survival. Neither of the citrullinated peptide alone or anti-LAG3 antibody on its own were able to significantly reduce CD4+LAP+ population.

Figure 5-34 shows the levels of LAP-LAG3+, LAP+LAG3+ and LAP+LAG3- populations in the different treatment groups. It demonstrates that the combination therapy resulted in a higher expression of the LAP-LAG3+ population ($p=0.0021$, Mann-Whitney test), a lower expression of the double positive population LAP+LAG3+ ($p=0.0435$, Mann-Whitney test) and a lower expression of the LAP+LAG3- population ($p=0.0057$, Mann-Whitney test) compared to the untreated group. LAG3 is known to be an activation marker which is expressed to dampen immune responses [40, 381]. Therefore, one can conclude that the increase in LAP-LAG3+ population is suggesting an increase in activated Th1 population which could be proven by designing an experiment to show that this population produces IFN γ . Furthermore, the combination therapy resulted in reduction of the LAP+LAG3+ population which could be hypothesized as the most inhibitory population being activated Th3 cells. The last population of purely LAP+ cells which are LAG3- are an inactive inhibitory population which in this study was also reduced as a result of combination therapy. This pattern of expression

could suggest an increase in Th1 response to the combination therapy with reduction in the suppressive Th3 population.

Table 5-3: Tumours and splenocytes were disaggregated upon culling of C57Bl/6 mice bearing Py8119 tumour model where group (A) was control untreated, group (B) treated with citrullinated peptides, group (C) treated with anti-LAG3 and group (D) treated with a combination of citrullinated peptides and anti-LAG3. Cells were stained by direct immunofluorescence for CD4, LAP, GARP & LAG3 expression and analysed by flow cytometry. All % shown in the total are of the total CD4+ cells. S denotes CD4+ splenocytes and T denotes CD4+TILs.

Date terminated	Mouse Gp#	Total CD4+	LAP+		GARP+		LAG3+		LAP+GARP+		LAP+LAG3+		LAP-LAG3+		LAP+LAG3-		GARP+LAG3+	
		TILs	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T
Day 22	A1	6.16	-	79.19	-	92.73	-	93.84	-	78.64	-	78.41	-	15.43	-	0.79	-	90.92
Day 22	A2	6.54	-	73.58	-	93	-	94.21	-	73.18	-	73	-	21.21	-	0.58	-	90.9
Day 22	A3	4.73	-	68.09	-	79.92	-	86.62	-	63.94	-	65.33	-	21.29	-	2.77	-	77.38
Day 22	A4	14.53	-	91.18	-	72.8	-	90.95	-	71.46	-	88.03	-	2.92	-	3.15	-	72.15
Day 24	A5	2.97	2.11	54.27	0.55	24.26	0.72	38.86	0.43	22.42	0.3	34.78	0.42	4.08	1.91	19.49	0.3	22.42
Day 24	A6	2.33	1.75	65.94	0.68	43.79	0.7	49.96	0.59	42.97	0.38	48.4	0.32	1.56	1.42	17.55	0.37	42.16
Day 24	A7	4.29	1.58	55.37	0.59	18.63	0.64	32.41	0.45	18.17	0.29	30.68	0.35	1.73	1.33	24.68	0.34	16.96
Day 24	A8	3.2	2.01	58.11	0.66	25.8	0.86	40.78	0.52	24.61	0.41	37.37	0.45	3.14	1.64	20.75	0.4	23.47
Day 24	A9	1.89	2.1	42.6	0.68	19.36	1	29.94	0.57	17.47	0.48	26.01	0.52	3.93	1.71	16.59	0.35	17.61
Day 22	B1	2.1	-	54.95	-	42.68	-	59.4	-	37.52	-	51.29	-	8.11	-	3.66	-	40.53
Day 22	B2	5.37	-	79.46	-	77.8	-	86.17	-	70.24	-	76.56	-	9.61	-	2.91	-	76.4
Day 22	B3	11.16	-	86.5	-	46.63	-	83.42	-	45.26	-	79.96	-	3.46	-	6.54	-	45.14
Day 22	B5	5.29	-	74.97	-	21.76	-	63.47	-	20.43	-	59.56	-	3.91	-	15.41	-	20.32
Day 22	B7	1.28	-	52.11	-	82.57	-	85.11	-	50.42	-	50.79	-	34.35	-	1.35	-	78.34
Day 24	B4	5.5	2.5	84.08	0.96	60.37	0.7	69.04	0.82	60.03	0.38	67.68	0.32	1.36	2.24	16.4	0.36	58.06
Day 24	B8	2.71	2.48	55.95	1.15	20.93	1.07	32.49	0.95	20.18	0.54	30.76	0.53	1.73	2.01	25.19	0.55	19.21
Day 24	B10	3.69	2.48	64.34	1	29.13	0.95	44.21	0.82	27.4	0.5	41.56	0.45	2.65	2.08	22.78	0.45	27.27
Day 28	B6	2.84	3.46	26.19	4	68.52	1.03	93.82	2.51	26.16	0.64	26.14	0.39	67.68	2.82	0.05	0.78	67.96
Day 28	B9	6.91	2.78	29.59	2.52	81.85	0.38	96.73	1.77	29.49	0.27	29.49	0.11	67.24	2.5	0.1	0.3	81.51
Day 22	C3	2.68	-	68.95	-	58.15	-	76.85	-	49.84	-	64.8	-	12.05	-	4.15	-	56.49
Day 22	C5	5.9	-	73.87	-	23.92	-	69.58	-	22.7	-	63.8	-	5.78	-	10.07	-	23.08
Day 22	C6	6.88	-	22.84	-	20.39	-	38.56	-	11.2	-	13.77	-	24.79	-	9.07	-	17.61
Day 22	C8	5.22	-	68.63	-	93.69	-	95.52	-	68.38	-	68.22	-	27.3	-	0.41	-	92.28
Day 22	C10	5.27	-	71.74	-	75.65	-	82.31	-	62.59	-	64.66	-	17.65	-	4.09	-	73.08
Day 24	C1	5.27	3.5	68.23	1.13	32.91	0.86	44.75	1.01	32.44	0.57	43.8	0.29	0.95	3.06	24.43	0.43	30.89
Day 24	C2	5.22	3.72	67.65	1.14	37.86	1.32	49.72	0.95	37.36	0.9	47.94	0.42	1.78	2.94	19.71	0.63	36.75
Day 28	C4	5.99	3.83	35.94	3.36	82.46	0.56	95.71	2.15	35.88	0.34	35.9	0.22	59.81	3.49	0.05	0.47	82.01
Day 28	C7	1.65	3.78	21.27	5.25	62.43	1.32	92.85	2.92	21.22	0.54	21.22	0.78	71.63	3.25	0.05	0.92	61.33
Day 22	D3	5.26	-	43.72	-	83.26	-	90.03	-	43.06	-	42.69	-	47.34	-	1.04	-	80.86
Day 22	D4	22.19	-	90.54	-	40.24	-	82.88	-	39.84	-	82.08	-	0.8	-	8.46	-	38.44
Day 22	D8	26.05	-	31.91	-	53.2	-	91.28	-	29.06	-	31.11	-	60.17	-	0.8	-	51.66
Day 28	D1	4.76	3.94	17.04	6.31	44.66	1.48	89.07	3.49	16.99	0.73	17.01	0.75	72.06	3.22	0.03	1.09	43.72
Day 28	D2	2.86	5.67	16.4	6.78	54.37	0.77	92.04	4.33	16.38	0.38	16.4	0.39	75.64	5.29	0	0.59	53.76
Day 28	D6	4.39	3.8	7.49	3.33	33.49	0.66	95.42	2.08	7.46	0.36	7.48	0.3	87.94	3.45	0.01	0.44	33.34
Day 28	D7	8.42	4.46	23.13	4.74	43.41	0.79	85.82	2.95	22.5	0.45	22.9	0.34	62.92	4.02	0.23	0.62	42.88
Day 28	D9	7.91	4.5	47.15	2.98	64.29	0.67	89.62	2.19	40.56	0.4	46.87	0.27	42.75	4.1	0.28	0.48	64.12
Day 28	D10	7.86	3.74	18.92	4.26	47.52	0.67	94.78	2.67	18.76	0.39	18.88	0.28	75.9	3.35	0.05	0.55	47.45
Day 31	D5	14.93	2	39.67	1.88	58.54	0.52	75.75	1.29	32.06	0.41	34.7	0.11	41.05	1.6	4.97	0.45	54.85

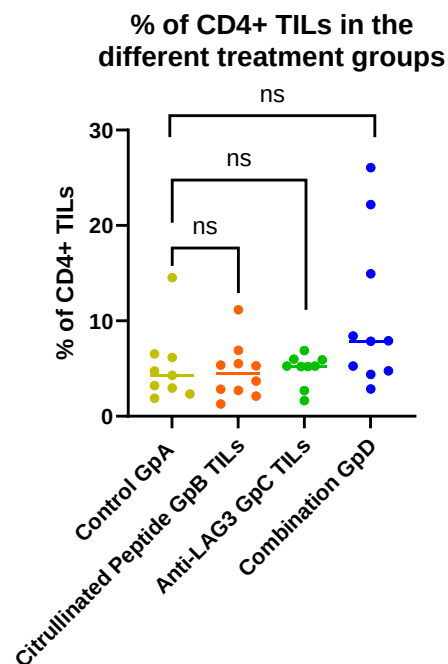


Figure 5-30: The levels of CD4+ TILs were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours from C57Bl/6 mice bearing Py8119 tumour model and the different treatment groups compared. Statistical analysis and p values calculated using non-parametric Anova with corrections for multiple comparisons (Kruskal-Wallis test).

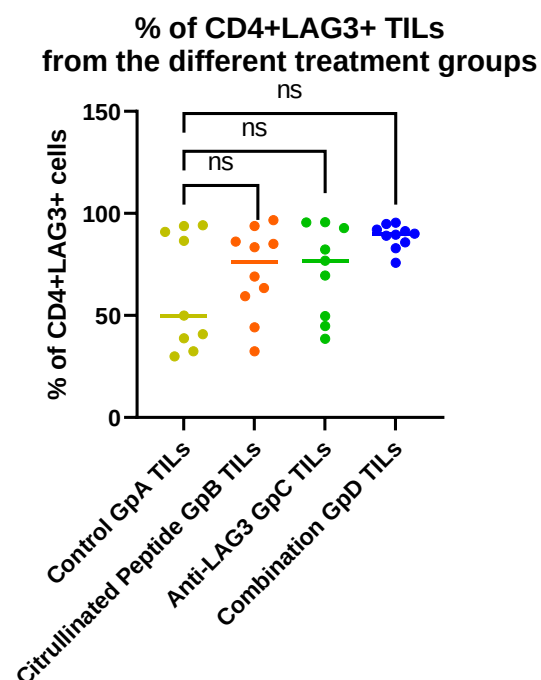


Figure 5-31: The levels of CD4+LAG3+ TILs were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours from C57Bl/6 mice bearing Py8119 tumour model and the different treatment groups compared. Statistical analysis and p values calculated using non-parametric Anova with corrections for multiple comparisons (Kruskal-Wallis test).

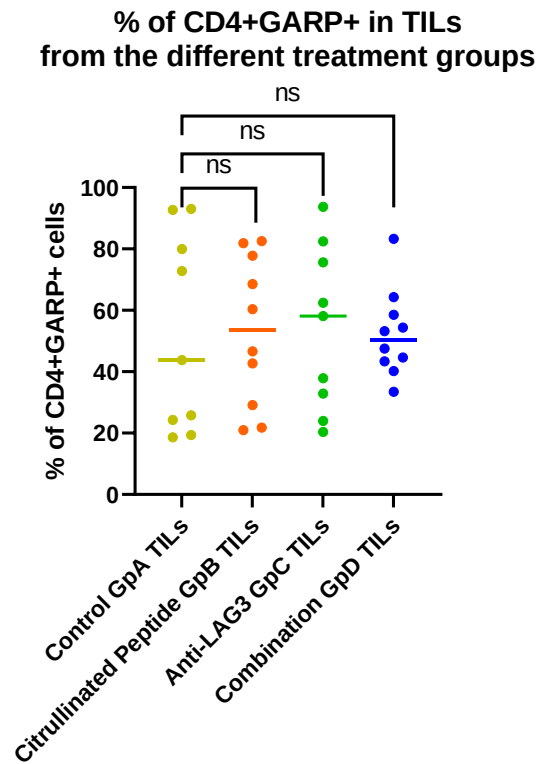


Figure 5-32: The levels of CD4+GARP+ TILs were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours from C57Bl/6 mice bearing Py8119 tumour model and the different treatment groups compared. Statistical analysis and *p* values calculated using non-parametric Anova with corrections for multiple comparisons (Kruskal-Wallis test).

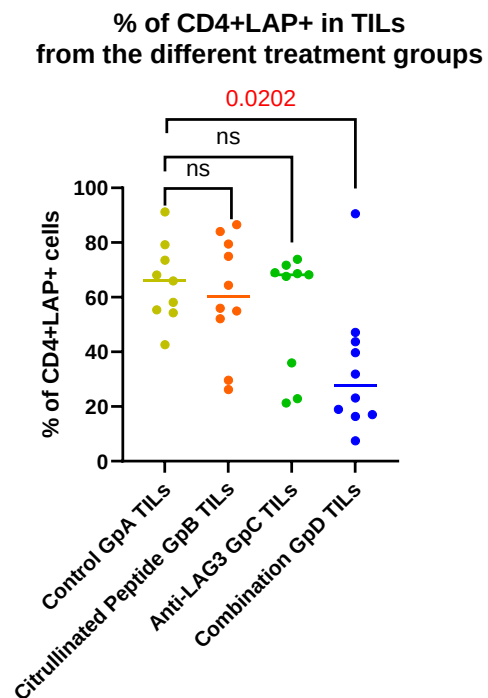


Figure 5-33: The levels of CD4+LAP+ TILs were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours from C57Bl/6 mice bearing Py8119 tumour model and the different treatment groups compared. Statistical analysis and p values calculated using non-parametric Anova with corrections for multiple comparisons (Kruskal-Wallis test).

TILs LAP and LAG3 populations

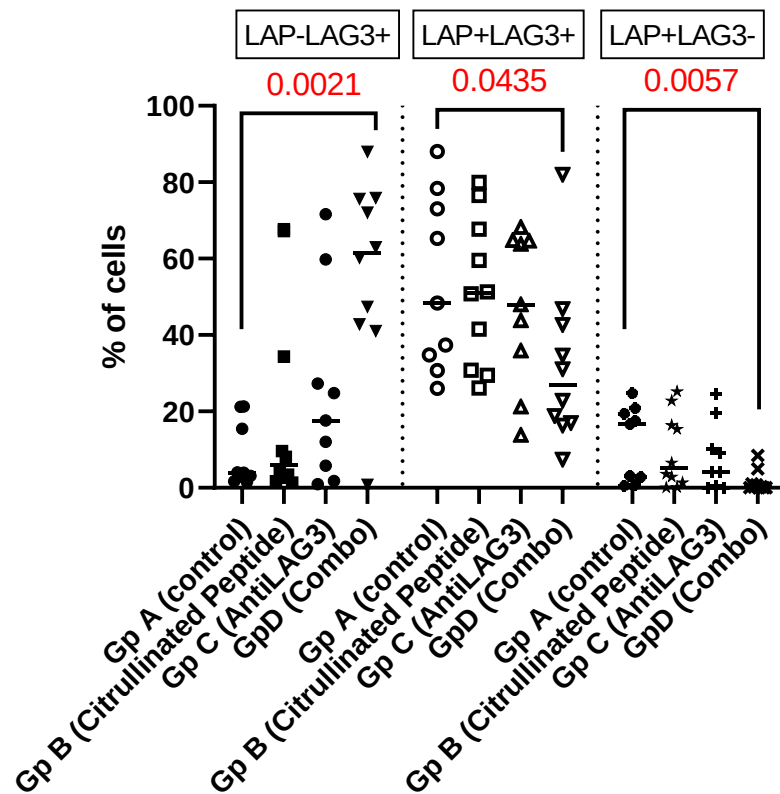


Figure 5-34: The levels of CD4+LAP+ and CD4+LAG3+ were analysed by direct immunofluorescence and flow cytometric analysis on disaggregated tumours from C57Bl/6 mice bearing Py8119 tumour model and the different groups of mice compared. Statistical analysis on TILs and p values calculated using Mann-Whitney test. The difference between the treatment groups in the expression of LAP-LAG3+, LAP+LAG3+ and LAP+LAG3- populations by total CD4+ TILs is shown. P value is only shown if statistical significance is reached, when corrected for multiple comparisons the difference between the control group and combination treated group in the percentage of LAP+LAG3+ on CD4+TILs became statistically insignificant.

5.4 Discussion

Data obtained from analysis of blood revealed that cancer patients have a repertoire to citrullinated peptides, but these responses were attenuated compared to healthy donors. Proliferating CD4⁺ cells responding to citrullinated peptides produced an IFN γ response (Th1 response), although the IFN γ levels were lower in cancer patients compared to healthy donors. However, cancer patients have a citrullinated peptide specific population of activated CD4⁺ cells expressing CD71 marker which co-expressed the inhibitory marker LAP. These cells inversely correlated with proliferation which suggests that cancer patients had an antigen specific terminally differentiated CD4⁺ population that could be the reason for attenuated responses to citrullinated peptides. Th3 cells identified by the expression of LAP are well documented in literature to be higher in cancer patients compared to healthy donors and these cells accumulated in the tumours but were also identified in lower levels in the peripheral blood of cancer patients [244, 245]. These cells were described to have highly suppressive function which I have also shown by isolating LAP expressing CD4⁺ cells from PBMCs of cancer patients and assessing their suppressive ability on proliferation of LAP⁻ cells. From this data, one can conclude that cancer patients may drive a Th1 response and acquire benefit from therapy with citrullinated peptides, but a cohort of patients may well have an overwhelming level of Th3 cells that would need an additional therapy to neutralise these cells. In this section, an attempt is made to study the effects of citrullinated peptides on survival and on immune infiltrating TILs in murine models and whether combination with an agent to neutralize Th3 cells provides additional benefit.

Evaluating different murine models, including melanoma B16F1 in HHDII/iDP4 mice, B16F1 in C57Bl/6 mice, breast Py8119 in C57Bl/6 mice, lung LLC2 in C57Bl/6 mice, colon MC38 in C57Bl/6 mice, colon CT26 in Balb/c mice and breast 4T1 in Balb/c mice, revealed that similar to human results the murine TILs expressed higher levels of LAP compared to peripheral splenocytes in most models with the exception of B16F1 in HHDII/iDP4 mice and 4T1 in Balb/c mice. Further to this, the levels of

CD4⁺LAP⁺ TILs differed between the tumour models with the highest expression seen in Py8119 in C57Bl/6 mice and MC38 in C57Bl/6 mice. It is important to note that the Py8119 model in C57Bl/6 also showed the highest expression of LAG3 which suggests it is a highly suppressive and regulated tumour. For this reason, the Py8119 in C57Bl/6 was picked to study the anti-tumour effects of vaccination with citrullinated peptides on survival as well as on Th3 cell levels and to examine synergy with anti-LAP therapy. The study which terminated on day 42 showed that three doses of citrullinated peptides given after tumour implantation resulted in 50% overall survival and tumour free survival. Although the combination therapy of citrullinated peptides and anti-LAP gave a 50% overall survival benefit compared to the untreated control group; this benefit was clearly incurred by therapy with the citrullinated peptides and no additional benefit from the combination with anti-LAP antibody. This conclusion was drawn since no difference in survival was seen when comparing the combination therapy to the citrullinated peptide therapy alone and also therapy with anti-LAP alone did not give any survival advantage. Upon humane termination of mice when their tumour size reached $\geq 10\text{mm}$; TILs were analysed for expression of CD4, LAP, GARP and LAG3. GARP is one mechanism by which the LAP-TGF- β complex anchors to the cell surface and is expressed mainly on T-regs [258-260]. It is measured here to solidify the effects of immunization on LAP expressing cells. LAG3 is co-expressed with LAP on Th3 cells [244]. The group treated with citrullinated peptides showed significantly lower LAP and GARP expression on CD4⁺TILs and gave a trend towards lower LAG3 expression on CD4⁺TILs when compared to the control untreated group. This suggests that treatment with citrullinated peptides switched Th3 to Th1 cells as there was no difference in CD4 levels indicating that this is not a result of infiltration of additional Th1 cells. Moreover, it transpired that the LAP⁺ population that was lowered by citrullinated peptide therapy co-expressed LAG3. LAG3 is known to be an activation marker which is expressed by immune cells as a negative regulator to control extreme T-cell activation [381, 400]. Scur *et al* [244], describe the co-expression of LAP with other markers including LAG3 on CD4⁺ Th3 TILs of patients with colorectal cancer and describe this population to be highly activated and suppressive in function. Therefore, the

presence of better survival and this pattern of marker expression on TILs of mice treated with citrullinated peptides suggests an induction of Th1 response and reduction of Th3 cells. Measuring IFN γ and TGF- β production by the CD4+TILs in this group would solidify this hypothesis. On the other hand, the anti-LAP antibody used to immunize the mice in this study not only gave no survival benefit; it also did not lower the levels of CD4+LAP+ cells. One can deduce that the anti-LAP antibody failed to deplete LAP expressing cells as its isotype as a mouse IgG2b antibody cannot mediate antibody dependent cellular cytotoxicity (ADCC) or activate complement. However, an argument could be made that the anti-LAP antibody used for immunization is a blocking rather than a depleting one and so the levels of LAP expression would not be decreased in this instance. In contrast to these results, Gabriely *et al* [307], show the same anti-LAP antibody treatment of C57Bl/6 mice bearing B16F1 reduced the levels of CD4+LAP+ TILs.

To reproduce a similar study to Gabriely *et al* [307], the tumour model B16F1 in C57Bl/6 mice was chosen to further assess the anti-LAP antibody and if synergy is seen with the citrullinated peptides. It must be noted that the melanoma B16F1 cell line in C57Bl/6 mice expressed much lower levels of CD4+LAP+ cells compared to the breast Py8119 in C57Bl/6. In the former model, the CD4+LAP+ cell levels were indeed reduced by treatment with citrullinated peptides alone and anti-LAP therapy alone. This supports the theory that the anti-LAP antibody is a low affinity one which in the context of the B16F1 was able to saturate the low number of the Th3 cells but was overwhelmed with the abundance of Th3 cells in the Py8119 model. The results in the B16F1 model were also supportive of the findings shown with the Py8119 model demonstrating that immunisation with citrullinated peptides on their own are capable of shifting the balance away from a Th3 response and in favour of a Th1 response. In replication to the Gabriely *et al* [307] results, the tumour volumes on day 18 of the study with B16F1 model were lower in the group treated with anti-LAP compared to the control untreated group and this was also true in the group treated with citrullinated peptides alone and the combination therapy of citrullinated peptides with anti-LAP. This reduction in tumour volumes was noted earlier (day 15) in the group

treated with citrullinated peptides. However, none of the treated groups of mice showed improvement in overall survival or tumour free survival although this was very close to statistical significance in the group treated with citrullinated peptides. A possible explanation to why the citrullinated peptides treatment ensued a better survival in the Py8119 model but not the B16F1 model is that the Py8119 tumour is a regulated tumour containing high T-cell infiltration with both Th1 and Th3 cells in the tumour microenvironment allowing citrullinated peptides to alter the balance in favour of Th1 cells and improve survival. Whereas the melanoma B16F1 tumour is a cold tumour protected from immune infiltration where Th3 cells in this instance play a smaller role in the tumour microenvironment with some other regulatory method impacting its' aggressive nature. It imperative to also remember the limitations of animal models and their comparability to human tumours where perhaps in the latter there is more time to allow for development of a regulatory phenotype. Based on the results from the two anti-tumour studies, one could reason that citrullinated peptides on their own are sufficient to drive a Th1 response and overcome the suppressive Th3 cells without the need for a LAP neutralizing agent in mouse xenograft models. It is important to note that Gabriely et al [307], showed improvement of survival with anti-LAP antibody only in orthotopic glioblastoma model and improvement in tumour free survival in B16 melanoma model expressing ovalbumin when prophylactic vaccination with tumour specific antigen was given. For that reason, no comparison was done with the results obtained from our models.

Following the outcomes from RELATIVITY-047 (NCT03470922) [396] which showed that Relatlimab (BMS-98621) an anti-LAG3 in combination with anti-PD1 nivolumab improved progression free survival in metastatic melanoma compared to nivolumab alone; FDA approved the use of the first anti-LAG3 treatment in humans. LAG3 binds to MHC class II with higher affinity than CD4 T-cells; thus, preventing CD4 T-cell activation and effector function [381, 400]. Additionally, LAG3 is expressed in many T-cell subsets where it induces immune inhibition and T-cell exhaustion [369, 381, 400, 401]. Consequently, it made sense to test synergy of citrullinated peptides with an anti-LAG3 therapy in a murine model since it has been reported that

immune responses to citrullinated peptides are predominantly CD4 mediated [144, 145]. The Py8119 tumour model is one which also expressed high levels of LAG3 in CD4+ TILs and so anti-tumour study was designed in this model. Treatment with the citrullinated peptides alone did not result in a similar survival advantage as seen in the previous study. Moreover, the *ex-vivo* staining did not show an increase in Th1 response and reduction in CD4+LAP+ cells in the group treated with citrullinated peptides as concluded earlier. This is difficult to explain when the same murine tumour model and the same dosing schedule of citrullinated peptides was used, however, it implies that the citrullinated peptide vaccination does not give a strong anti-tumour response in this model that could be replicated in every instance. Similarly, therapy with anti-LAG3 antibody alone did not result in improvement of overall survival. However, combination therapy resulted in a lower CD4+LAP+ cell level in the tumour microenvironment which translated to improved survival. A population of CD4+TILs co-expressing LAP and LAG3 were lower in the combination therapy group compared to the untreated group suggesting a double positive population that is perhaps activated Th3 cells. Further to this, there was an increase in the LAP-LAG3+ CD4+TIL population which could translate into an activated population implying a Th1 response. Likewise, testing for IFN γ and other pro-inflammatory or anti-inflammatory cytokines production by CD4+ TILs would solidify the hypothesis that this response mounted by combination therapy is indeed a Th1 response. Perhaps sorting the activated TIL population and performing an IFN γ Elispot is a method to try to further examine this hypothesis. This experiment proposes that immune responses and survival benefit may be enhanced when vaccination with citrullinated peptides is combined with therapy to neutralise LAG3.

6 Chapter 6: Discussion

Cancer vaccines are faced with the challenge of generating immune responses that can overcome tumour immunosuppression and have little activity in normal tissue to avoid significant autoimmune toxicities. Vaccines targeting self-antigens have seen limited success as high affinity T-cells recognising self-antigens are deleted during thymic regulation. In contrast, high affinity T-cells recognising tumour mutations or neoepitopes avoid thymic regulation and pose an attractive target for vaccine therapy. However, success in generation of vaccines against neoepitopes are only successful in tumours with a high mutational burden and are patient specific making them only useful for development of personalised vaccines which is both expensive and time consuming [402-404]. On the other hand, citrullination, a post-translational modification, is one process undertaken by a variety of cancer types producing neoepitopes that can be used in vaccine therapy and is not personalised. Citrullination occurs during stressful conditions such as tumour cell hypoxia and nutrient deprivation. It is dependent on autophagy and PAD1 enzyme activity which are ways of alerting the immune system to abnormal stressful conditions. These stressful conditions are not a characteristic of normal cells which grow and differentiate under strict control. In contrast, tumour frequently outgrow their niche and have high energy requirements triggering high levels of autophagy. Yet, citrullinated epitope generation and induction of high affinity CD4 T-cells on their own are not sufficient for anti-tumour immunity. Inflammation, IFN γ production and subsequent upregulation of MHC class II which presents the peptides produced within the autophagosome are required to allow for immune recognition and tumour rejection. When a citrullinated vaccine is given, the antigens are processed by APCs, presented on MHC class II to activate citrulline specific CD4 T-cells. CD4 T-cells can act indirectly producing chemokines to attract other cells as well as produce pro-inflammatory and cytotoxic cytokines [247, 405]. Indirectly, Th1 CD4 T-cells create the necessary pro-inflammatory environment through production of IFN γ which in turn induces expression of MHC class II on tumour cells that do not constitutively express MHC class II and lead to further immune infiltration and

tumour cell death [406, 407]. Indeed, it has been shown that vaccination with citrullinated epitopes in murine models induced Th1 CD4 responses which resulted in impressive tumour rejection of a variety of tumours and improved survival [144-146, 165]. Furthermore, similar antitumour responses were seen in murine models where the tumour only upregulates MHC class II under the influence of IFN γ rather than constitutively suggesting sufficient secretion of IFN γ mediated by CD4 T-cells to upregulate MHC class II expression [145, 146]. Results from NSCLC TMA strengthens this by showing a positive correlation between citrullinated vimentin and MHC class II expression on tumour cells and between CD4 T-cell infiltration and MHC class II expression suggesting that when citrullinated epitopes activate CD4 T-cells there is resultant upregulation of MHC class II expression. Additionally, in the TMA the expression of citrullinated vimentin correlated with better survival. This alongside the observed CD4 T-cell repertoire in the PBMCs of lung and ovarian cancer patients to citrullinated epitopes suggests that patients like the murine models would be candidates to incur benefit from vaccination. Alternatively, CD4 T cells can differentiate into cytotoxic CD4 T cells and directly kill tumours. The requirement for MHC-II on tumours, rather than APCs within the tumour environment, to see anti-tumour immunity in our animal models suggests that immunisation with citrullinated peptides stimulate cytotoxic CD4 T-cells.

TCR mediated detection of tumour antigens and subsequent activation of high affinity T-cells is one aspect of cancer vaccine therapy, but a second hurdle is the immune suppression imposed by the tumour microenvironment. One process by which the tumour escapes immune surveillance is through the employment of regulatory T-cells that release suppressive cytokines such as TGF- β and IL10 to inhibit anti-tumour immune responses and favour tumour progression [408]. There are reports of Tregs specifically activated in response to tumour antigens [409-413]. Th3 cells are a subset of regulatory type of CD4 T-cells that have been identified in human tumours and recently gained attention due their heightened suppressive ability compared to the conventional FOXP3 expressing Tregs [244, 245]. CD4 Th3 cells have been defined by their expression of LAP but are negative for FOXP3 and they co-

express CD69 and LAG3 [242, 244, 245]. Here, the results from PBMCs suggest that attenuation of the CD4 T-cell repertoire to citrullinated peptides in cancer patients could be owed to a Th3 population which were terminally differentiated non-proliferating antigen specific CD4 population that is defined by their expression of activation marker CD71 and inhibitory molecule LAP. Retrospectively, perhaps LAG3 and/or CD69 expression alongside LAP would have been better markers to identify this Th3 population. Moreover, the NSCLC TMA results suggest that the worst prognosis in patients is seen when there are low levels of citrullinated vimentin together with high expression of LAP by the tumour. To this effect, examining synergy between citrullinated peptide vaccine and a LAP neutralizing therapy was considered. In a murine model harbouring the breast tumour model Py8119 which expresses high levels of LAP and LAG3, vaccination with citrullinated peptides was able to improve survival and lower levels of Th3 cells defined by co-expression of LAP and LAG3 on CD4 T-cells whereas the combination with an anti-LAP antibody did not add benefit. The slow progression and perhaps lack of success of therapies targeting LAP/TGF- β in human trials coupled with their expected toxicities owing to its promiscuous expression in healthy cells suggests that extensive work is still needed in the development and design of such therapies. Perhaps an alternative way to target Th3 cells such as therapies against LAG3 would be better for investigating synergy with citrullinated vaccines.

Further to regulatory T-cells there is a wealth of inhibitory pathways that the tumour hijacks to negatively impact T-cell function and differentiation including PD1, CTLA4, LAG3 and TIM3. In an era when checkpoint inhibitors are used in abundance clinically, a combination of checkpoint inhibitor to synergise with citrullinated peptide vaccination maybe the natural course into clinic. An anti-LAG3 antibody was a tempting approach given its a recent access into approved clinical use (Relatlimab) combined with LAG3 expression on Th3 cells. In a murine model bearing Py8119, the combination treatment with citrullinated peptide vaccine and an anti-LAG3 antibody improved survival, increased CD4 tumour infiltration and significantly lowered Th3 levels. However, the benefit seen with citrullinated peptide vaccination

alone in the previous experiment (mentioned above with anti-LAP antibody) was not replicated suggesting that the vaccine does indeed need to be enhanced by other immune activating therapies. PDL1 expression is a negative regulatory mechanism adopted by tumours to escape immune control, a process named adoptive resistance [366], in response to inflammation and IFN γ releases by immune cells. The NSCLC TMA results highlight a positive correlation between PDL1 expression and citrullinated vimentin suggesting that as immune responses are triggered by citrullinated vimentin which is then tolerized by the tumours upregulating PDL1 expression to regain control. In fact, work by the group has shown that the combination of citrullinated peptide vaccine with anti-PDL1 gives synergistic benefits in murine models (data not published). Hence, a combination therapy with citrullinated peptide vaccine and blockade of PD1/PDL1 pathway is potentially the ultimate synergistic treatment.

A first in human phase 1/2 trial (ModiFY NCT05329532) has started recruiting in 2022 to investigate a so-called Modi-1 vaccine which consists of two citrullinated vimentins and a citrullinated α -enolase peptide. Target populations are patients with renal cell carcinoma (RCC), triple negative breast cancer (TNBC), advanced non-neoadjuvant HPV negative squamous cell head and neck cancer (SCCHN) and high grade serous ovarian carcinoma (HGSOC). One patient cohort will be in the neoadjuvant setting for SCCHN patients as monotherapy or in combination with checkpoint inhibitors. The selection of tumour types has been based on high tumour expression of vimentin and α -enolase. The primary end point is the assess safety, tolerability, and dosing levels of Modi-1 as monotherapy and in combination with checkpoint inhibitors when it is standard of care in RCC, TNBC and SCCHN. The secondary end point is to assess clinical overall response rates, duration of response, progression free survival and immune responses. One HGSOC patient has shown a dramatic 44% regression after 8 weeks of treatment suggesting that at least in this patient our proposed mechanism of action is correct. However, it will be good to identify biomarkers of responding patients. In this context, cellular immune responses will be assessed in pre and post vaccination PBMCs through IFN γ ELISpot

and CFSE proliferation assays. Marker expression (LAP and LAG3) alongside IFN γ responses are used to stratify patients into Th1 or Th3 responders. The aim of vaccination would be to induce a Th1 response as seen in the murine models but if a patient has high levels of baseline Th3 cells then the hope would be for the vaccine to flip that in favour of Th1. Pre-treatment biopsy samples and post-treatment surgical samples in the neoadjuvant SCCHN group will be assessed for expression of vimentin, α -enolase, PADI2/4, autophagy markers, MHC class II, T-cell infiltration, PD1/PDL1, CTLA4 expression as well as RNA sequencing and TCR repertoire analysis. The relationship between immune responses and Th1/Th3 stratification with the clinical outcomes is one interesting result to anticipate. It could be predicted that the group of patients with immunologically “hot” tumours, RCC and SCCHN in this instance, are likely to see better responses from this vaccine therapy and the combination with checkpoint inhibitors will boost these responses. The abundance of TILs within these immunologically regulated tumours makes the flip of balance from Th3 to Th1 more likely to have an impact. However, the combination of vaccine to induce proliferation, enlarge the pool of tumour specific T-cells and kick start tumour T-cell infiltration with reviving T-cells through checkpoint blockade has been reported in poorly immunogenic tumours [414, 415]. Once a small number of T-cells infiltrate an immunologically “cold” tumour they will be followed by a larger wave of activated cells [416].

However, the question of provoking autoimmunity such as RA like toxicity is one that imposes itself. Citrullination has been linked to autoimmune diseases including RA, type I diabetes, multiple sclerosis, sarcoidosis, psoriasis, and celiac disease [138-142] but it has been suggested that citrulline specific CD4 T-cells on their own do not result in autoimmunity but do aid antibody responses. Tissue damage and antibody responses to citrullinated proteins are essential to induce autoimmune diseases [92]. It is therefore anticipated that although peptide vaccination will not induce autoimmunity it may make it worse in patients with pre-existing conditions. In the murine models vaccinated with citrullinated epitopes, the antitumour immune responses mediated by CD4 T-cells were not

accompanied by autoimmune toxicities [144-146]. Even so, restraint should be exercised with human trials of citrullinated peptide vaccine, and an exclusion criterion ought to be history of autoimmune disease with such toxicities carefully monitored. The duration since receiving chemotherapeutic agents is another aspect that should be considered cautiously and a wash out period allowed before the administration of citrullinated vaccine. Chemotherapy induces non-specific cellular stress and so in this instance one can assume that autophagy induced citrullination is perhaps a likely occurrence in normal tissue increasing the chances of autoimmune toxicities from citrullinated vaccination.

This ModiFY trial of Modi-1 will determine if a citrullinated peptide vaccine either alone or in combination with anti-PD1 can control tumour growth without inducing toxicity. This will pave the way for further trials of this and other siPTM vaccines that are generic and not personalised and may be the future for cancer vaccines.

7 References

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