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Adenosine Regulates CD8+ T Cell Activation by Disrupting the ARP2/3 and Cofilin Pathway could Prevent Immunological Synapse Formation and Motility

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A B S T R A C T

Immunosurveillance is a powerful defines mechanism by which both innate and adaptive arms of the immune system mediate to protect from tumour development. The tumour microenvironment (TME) plays a crucial role in regulation of CD8+ cytotoxic T lymphocytes (CTLs) and suppresses their function. When these CTLs migrate from tumour-draining lymph nodes (TDLN) into the tumour to form tumour-infiltrating lymphocytes (TIL) they quickly lose their CTL function. Tumour growth is associated with the frequent production of immunosuppressive molecules within the tumour microenvironment that inhibit anti-tumour CTL effector function resulting in the secretion of tumour progression molecules. Immunotherapies that block immune inhibitory receptors have developed the treatment strategy for advanced-stage tumours. Here, we review this study to improve the effector function of CD8+ T cells following adoptive transfer to eliminate tumour cells by validating a selected set of proteins that are significantly changed in their levels of expression through proteomic analysis obtained from a tandem mass tagging (TMT) comparison of the whole proteome of purified populations of CL4 CD8+ T cells isolated from TDLNs with those that form TILs in RencaHA tumour-bearing mice. To conduct these experiments *in vitro* and *vivo*, a highly characterized murine Renal carcinoma (Renca) model was used. Renca cells were transfected with a plasmid construct containing the HA protein from influenza virus A/PR/8 H1N1 as a cell surface neo-tumour-specific antigen and Thy1.1+, KdHA-specific, CL4 TcR transgenic CD8.

As a result, we demonstrated that *in vivo* ARP2/3 was significantly decreased (>10 fold) in TILs compared to TDLNs. Moreover, we also showed that when *in vitro* activated CL4 cells were exposed to adenosine, which plays a crucial role in modulating both the ARP2/3 Complex and Cofilin proteins, ARP2/3 levels decreased (>1.5 fold) compared to untreated CL4 cells, resulting in possible prevention of, for example, the formation of the immunological synapse. Adenosine treatment, however, led to increased levels of Cofilin.

Keywords: *Populations of CL4 CD8 T cells isolated from the TDLN, Populations of TIL in tumour-bearing mice, Immunological synapse*

الأدينوزين ينظم تنشيط الخلايا التائية +CD8 عن طريق تعطيل مسار ARP2/3-Cofilin والذي يمنع تكوين التشابك المناعي والحركة

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الملخص

المراقبة المناعية هي آلية قوية تحدد من خلالها الجهاز المناعي بنوعيه الفطري والمكتسب والتواسط في الحماية من تطور الورم. تلعب البيئة المحيطة بالورم دورًا حاسمًا في تنظيم الخلايا التائية السامة **CD8+** وكبح وظيفتها. يرتبط نمو الورم بالإنتاج المتكرر للجزيئات المثبطة للمناعة داخل البيئة المحيطة بالورم والتي تمنع وظيفة الخلايا التائية السامة المضادة للورم مما يؤدي إلى إفراز جزيئات التي تطور الورم. هذه الدراسة تهدف إلى تحسين وظيفة الخلايا التائية **CD8+** بعد النقل المتبني للخلايا، للقضاء على الخلايا السرطانية من خلال التحقق من صحة مجموعة مختارة من البروتينات التي تغيرت بشكل كبير في مستويات افرازها من خلال التحليل البروتيني الذي تم الحصول عليه من مقارنة وسم الكتلة المترادفة (**TMT**) للبروتينات الكاملة لمجموعات نقية من الخلايا التائية **CD8+ CL4** المعزولة من **TDLNs** مع تلك التي شكلت **TILs** في الفئران الحاملة للورم **RencaHA**. لإجراء هذه التجارب في المختبر وفي الجسم الحي، تم استخدام نموذج سرطان الكلى الفأري (**Renca**) عالي التوصيف. تم تحويل خلايا **Renca** باستخدام بنية بلازميد تحتوي على بروتين التجلط **HA**. ونتيجة لذلك، أظهرت النتيجة في الجسم الحي أن مستويات **ARP2/3** انخفضت بشكل ملحوظ (< 10 مرات) في **TILs** مقارنة بـ **TDLNs**. وايضا، عرضنا في النتائج أنه عندما تعرضت خلايا **CL4** المنشطة في المختبر للأدينوزين، وهو يلعب دورًا رئيسيًا في تنظيم كل من بروتينات المعقدة **ARP2/3 Complex** و**Cofilin**، انخفضت مستويات **ARP2/3** < (< **1.5** مرة) مقارنة بخلايا **CL4** غير المعرضة للادينوزين، مما أدى إلى احتمالية منع تكوين التشابك المناعي. ايضا، أدى تعرض الخلايا الأدينوزين إلى زيادة مستويات **Cofilin**.

الكلمات المفتاحية: *مجموعات الخلايا التائية CL4 CD8 المعزولة من TDLN، مجموعات الخلايا التائية TIL في الفئران الحاملة للورم، التشابك المناعي*

1. Introduction

1.1 Tumour Immunosurveillance

Cancer is still one of the main causes of death worldwide, around 325,000 people were diagnosed with cancer in 2010 in the UK. Every two minutes someone in the UK is diagnosed with cancer and more than 1 in 3 people in the UK will develop some form of cancer during their lifetime. The majority of cancer treatments currently in use involve the surgical removal of tumour tissue; chemotherapy, which works by targeting DNA synthesis and cell division; and radiotherapy, which aims to kill tumour cells using ionising agents. However, there is a necessity to find new ways to target tumours due to difficulties in surgery, and the localised toxicity and lack of specificity of conventional chemo- and radio-therapeutic approaches [1,2,3]. Studies in the past two decades have been focused on the development of therapeutic vaccines, in order to combat cancer cells. Thus, for many years research has been focused on trying to identify proteins which play a crucial role in the growth and survival of cancer cells which may serve as targets for intervention.

1.1.1 Tumour growth

The process of tumour development also occurs as a result of factors, including genetic mutation, physiological transformation of tumour cells and the microenvironment within tumour sites. Moreover, the resulting in oncogenesis, tumour suppressor genes are inactivated by the genetic instabilities or stimulation of oncogenes. Growth of tumour and metastatic spread are caused by a number of intrinsic factors as well as the suppression of the immune system, through the production of other factors such as IL6, IL10 and IL4 [4,5]. The function of IL-6 prevent inflammation, but IL-10 and IL-4 can turn Th1 response into Th2 response. Transforming growth factor beta (TGF-β) also contributes to insensitivity to antigrowth signals. Tumour cells proliferate is often prevented by (TGF-β). However, the lack of insensitivity of TGF-β resulting in further development of tumour cells results from mutation in the TGF-β receptors, SMAD4 or retinoblastoma [6]. TGF-β can also control of anti-tumour immune responses [7]. Another factor that also promotes tumour development is the resistance to apoptosis. This is not so critical during the initiation step but in the later stages, it becomes a crucial part of the resistance of tumour cells caspase activity which is often a target for both chemotherapy and anti-tumour immune response [8].

1.2 CD8+ T cell activation

Naïve CD8+ T lymphocytes within peripheral lymphoid organs recognize tumour-derived peptide and other ligands on antigen presenting cells and undergo proliferation and expansion of anti-tumour specific lymphocytes which of these differentiate into effector and memory lymphocytes [9]. This priming results from at least three signals. Naïve CD8+ T cell priming is regulated by the interaction of the TcR with MHC class I/ peptide complexes

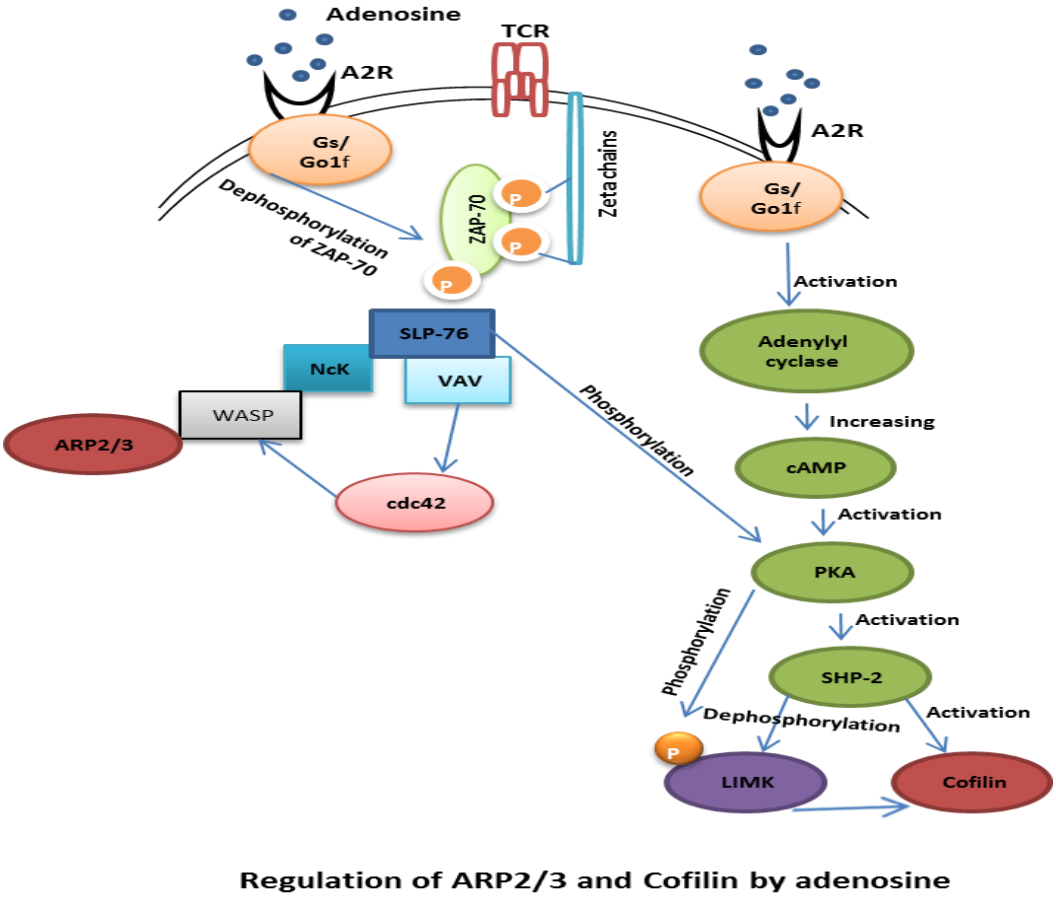

Figure 1: Illustration of the signalling pathways of cofilin and ARP2/3 by adenosine.

which lead to the induction of signal one. The subsequent formation of an immunological synapse that is generated triggers TcR movement to lipid rafts on the cell membrane, thereby at the centre (cSMAC) generate a TcR and co-stimulatory molecules, in contrast on the side (pSMAC) presents adhesion molecules [10,11]. Furthermore, central supramolecular activation clusters (cSMAC) contain high structural associations of several signalling clusters which provide the effective activation of T cell; thereby (cSMAC) is recognized by the PKCθ protein kines. The peripheral (pSMAC) its region that is surrounding the (cSMAC) in which location of lymphocyte function-associted antigen-1 (LFA-1) and the cytoskeletal protein are clustered [12]. TcR/CD3 complex consist of several molecules such as α and β chains and cytoplasmic signalling CD3, γ, δ, ε, and ζ.

1.3 Regulation of actin cytoskeleton dynamics in T cells by adenosine

1.3.1 The cofilin and ARP2/3 pathway

The regulation of actin filaments at the leading edge in T cells is integral in the process of cell motility; in both, actin-depolymerizing and actin-polymerizing. The ARP2/3 complex nucleates and elongates the actin cytoskeleton to generate the branched actin filaments at the leading edge resulting in the driving of the cell front forward and the formation of protrusions [13]. Whereas, the depolymerizing aspects induced by proteins such as cofilin to disassemble the barbed-end actin filaments result in a recycling of actin monomers to form further rounds of polymerization at the leading edge [14].

In addition, activation of adenosine receptors A2a and A2b are associated with G_s α subunit of the heterotrimeric G-protein stimulates adenylyl cyclase activity which promotes the concentration of intracellular cAMP [15]. The presence of high cAMP concentration activates LIM kinase can

induce through the action of PKA, which phosphorylates an inhibitory residue on cofilin Figure 1 whereas activated SHP-2 by PKA dephosphorylate LIMK the cofilin is activated [16]. TCR activation causes, Src kinase proteins, Lck and Fyn to phosphorylate and activate ZAP-70. However, in the presence of adenosine signalling, ZAP-70 is de-phosphorylated such that SLP-76 is not activated. Thus, Nck and Vav will inhibit WASp and Arp2/3 is not recruited, resulting in a decrease in actin polymerization. ZAP70 also regulates the activation of cofilin which causes actin de-polymerization via SLP-76. Activation of A2A receptors by extracellular adenosine leads to an increase in levels of intracellular cAMP and subsequent PKA activated which then activates SHP-2 dephosphorylating LIMK, which subsequently dephosphorylates cofilin causing it to binding to actin filaments, which are then severed and thus depolymerized.

Studies have previously shown that several transduction signalling including Ca2+, cAMP and PtdIns 3-kinase can regulate and recruit cofilin-induced dephosphorylation. Accordingly, dephosphorylation of the cofilin pathway was associated with SSH [17]. Remarkably, evidence has shown that the activity of SSH can regulate cofilin via the dephosphorylation and inactivation of LIMK which plays a role in regulating cofilin. Furthermore, phosphorylated LIMK phosphorylates and inactivates cofilin [18].

Phosphorylation of Zap-70 is reduced by adenosine Figure 1. which suppresses the transduction signalling pathways linked to T cell proliferation and differentiation [19]. Zap-70 regulates proteins associated with the motility of T cells and the formation of immunological synapses, such as ARP2/3 and cofilin which are essential for the remodelling of actin filaments [20]. Phosphorylated SLP-76 activates Vav and Nck-Nck activates WASp whereas Vav binds to Cdc42. WASp can bind Arp2/3 thus Arp2/3 can bind pre-existing actin filaments for initiating a new actin filament polymerization. Moreover, the inhibition of cofilin is induced by phosphorylated LIMK; however, adenosine inhibits Zap-70 and consequently SLP-76, resulting in cofilin remaining in an active state. However, the formation of protrusions does not happen due to the dephosphorylated state which inactivates Arp2/3-mediated polymerizing by the inhibiting of Vav and Nck which are not recruited. As a result, WASp is not bound and in turn Arp2/3 is not activated [20].

Several cytokine transcriptions including IL-2 and effector molecules such as granzyme B and perforin associated with lymphocyte growth and expansion were inhibited by adenosine through increasing the dephosphorylation of the STAT5 transcription factor [21].

1.4 Does adenosine act as an immunosuppressive metabolite?

Adenosine has been recognised to inhibit the proliferation of pro-inflammatory cytokine production which is released from DCs, favouring the loss of their capacity to trigger T cells [22]. Similar T cells could be impeded and result in the decrease of their cytotoxic functions and IFN-γ production due to extracellular adenosine [23].

The existing adenosine level is typically low in concentration in body fluids [24]. Pro-inflammatory ATP which could produce adenosine is divided into a two-step process: CD39 degrades ATP or ADP to AMP and in turn, CD73 hydrolyses AMP into yielding adenosine. Strikingly, AMP and ADP are phosphorylated by other extracellular enzymes such as adenylate kinase and nucleoside diphosphate kinase, resulting in antagonising CD39 activity [25].

2. Research objectives

This study was based on data obtained from a Tandem Mass Tagging (TMT) comparison between the entire proteome of purified populations of CL4 CD8+ T cells isolated from the TDLN with those that form TIL in RencaHA tumour-bearing mice. Although, similar proteomic analyses have been performed in vitro with Jurkat cells and cultured lymphoblasts [26,27], to our knowledge this is the first comprehensive data set from populations of tumour-specific CD8+ T cells activated in vivo in tumour-bearing hosts. The data allowed us to identify particular protein that have candidates significantly changed in their levels of expression between FACS-sorted CL4 CTL from the TDLN and TIL of (Renal carcinoma heamagglutinin) RencaHA tumour-bearing BALB/c mice.

Within this list of proteins that were significantly decreased (>1.5 fold) in the TIL compared with the TDLN were two key proteins that orchestrate motility of the actin cytoskeleton in the generation of the Immunological Synapse. ARP2/3, and Cofilin.

The immunological synapse (IS) plays a critical role in stimulation of naïve T cells, as well as in target cell recognition and lysis by CTL. During this process cell surface interactions and topological re-arrangement of proteins result in intracellular signalling and the formation of a central supramolecular activation complex (cSMAC) [28].

Actin filaments within CTL have two important mechanisms in antigen recognition. The first role is a formation of an immunological synapse, and in cSMAC activation clustering and molecular movement first on a micrometre-scale on the surface of T cells [29,30]; resulting in large-scale molecular–rearrangement [31]. The next role appears to include signalling complexes which rely upon the formation of scaffold of actin filaments [32,33]. The actin cytoskeleton of the CTL also has a key role in the morphological alterations within the CTL; coordinating the polarization of the cSMAC

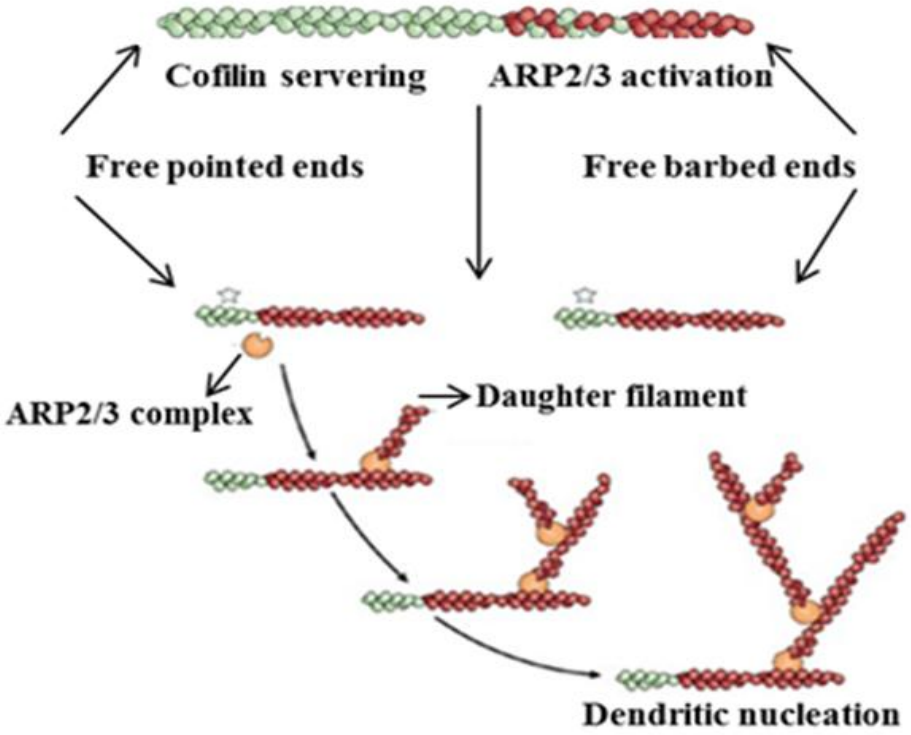

Figure 2: Cofilin severing promotes an increase in barbed and pointed ends, of actin filaments derived from the mother filament. Activated APR2/3 facilitates dendritic nucleation [1].

and receptors to the end of the cell proximal to the target. In addition to directing the CTL killing the cytoskeleton, also facilitates the transportation of secretory molecules including the cytotoxic proteins, derived from secretory lysosomes, to the interface between the two cells at the immunological synapse [34]. This, any disorder of actin filaments results in the blockade of efficient CTL function [20]. Exposure of T lymphocytes to certain chemokines before they encounter APC results in the rapid actin polymerization, which induces the formation of lamella podium; (a 1-5µm extensive cytoplasmic protrusion at a front end of the cell that includes a dendritic nucleation of actin filament), a distal anchoring uropod, the re-organisation of the intracellular organelles and movement of the front end of the cell. [35,36].

The ARP2/3 complex promotes actin polymerization, and so plays an important role in cell movement and formation of IS. Initially, the barbed end of the actin filament is linked to ATP, which is then hydrolysed to ADP and inorganic phosphate. When this occurs the ARP2/3 complex becomes dissociated. Disassociation of the inorganic phosphate promotes Cofilin activity, leading to filament fragmentation [20]. The majority of Cofilin is important in turnover of actin monomeric subunits, where it severs mother filaments (older pre-existing filaments) to generate new barbed ends resulting in the newly polymerized actin filament to become elongated, which favours dendritic nucleation [37].

There are several mechanisms by which Cofilin can induce actin depolymerisation: by destabilizing the actin filament or altering the actin structure to make it twisted as well as destroying the bonds between actin subunits [38]. Thus, disrupting the ARP2/3 and Cofilin pathway could influence CTL activity by preventing IS formation and motility.

3. Materials and Methods

3.1 Media.

3.1.1 Complete medium.

RPMI (1640) tissue culture medium without L-glutamine (Sigma-Aldrich, Poole, UK), was supplemented with 10% vol./vol. FCS (Foetal Calf Serum) or FBS (Foetal Bovine Serum) which was heat inactivated at 56ºC for 1hr prior to use (Invitrogen, Paisley, UK), 2 mM L-glutamine (Invitrogen), 50U/ml penicillin/streptomycin (Invitrogen) and 5×10-5 M 2-mercapto-ethanol (Invitrogen).

3.1.2 Tumuor cell medium.

This was prepared in a similar manner to the complete medium but with the addition of 100µg/ml geneticin (Invitrogen).

3.1.3 MACS buffer.
The buffer used for magnetic cell sorting consisted of 1×PBS (University of Bristol), 0.5% wt./vol. albumin from bovine serum (BSA) (Sigma-Aldrich) and 2mM of 0.5M EDTA (Sigma-Aldrich). FACS staining buffer was prepared in a similar manner to the MACS buffer but without the addition of EDTA.

3.2 Cell counting.
Cells were counted and their viability was assessed using a standard method. Briefly, equal volumes of cells and trypan blue (Sigma-Aldrich) were mixed and were then transferred to a haemocytometer counting chamber (Sigma-Aldrich). The haemocytometer counting chamber was viewed under a light microscope. Cell viability was assessed via trypan blue exclusion, where live cells appear bright under the microscope.

3.3 Mice strains.
6- to 8- wk old conventional Thy1.2+ BALB/c, BALB/c Thy1.1+ CL4+/- [39], were bred and housed at the University of Bristol Animal Service Unit, and maintained under specific pathogen-free conditions with guidelines set by the U.K Home Office.

3.4 Renca HA tumour cells injection of mice.
6- to 8-wk old conventional Thy1.2+ BALB/c mice were injected with tumour cells subcutaneously (s.c.) on the scruff of the neck on Day 0. The mice were first anaesthetised using isoflurane (Merial Animal Health LTD, Essex, UK) and the injected with 1x106 cells in 100µl PBS.

3.5 Culturing of Renca tumour cell lines.
RencaNT cells were cultured in a complete medium. RencaHA tumour cells were cultured in a complete medium Supplement with 100µg/ml geneticin (Invitrogen). 1×106 tumour cells (RencaNT or RencaAH) were cultured in a T75 flask (Corning, NY, U.S.A) under sterile conditions at 37 ºC in a humidified incubator with 5% vol./vol. CO2. Once confluent, the cells were trypsinized and split into new flasks.

3.6 Cell isolation.

3.6.1 Isolation of lymphocytes from peripheral lymphoid tissue.
Mice were culled by cervical dislocation and then completely spayed all over with 70% vol./vol. ethanol. Lymph nodes (LN) and spleens were collected using sterile forceps and placed in complete RPMI on ice. Spleens were cut into small pieces to allow efficient release of splenocytes. Single-cell suspensions were generated by disrupting the tissue in 2ml of complete medium. Cells were forced through 40µm cell strainers (BD Falcon) into 35mm Petri dishes (Corning Inc.). Using sterile syringe plungers. The cells were transferred into a 15ml conical tube (Greiner Bio-one, Germany), and the petri dish was rinsed with 10ml complete medium. At this point, red blood cells (RBC) generated from spleen disruption were lysed by the addition of 900µl distilled water for 5 sec. Lysis was stopped by adding 100µl of 10 x PBS followed by 10ml complete medium. Cell debris was removed by passing the suspension through a 40µm sterile cell strainer (BD Falcon).

3.6.2 Naïve CL4 CD8+ Purification.
Lymphocytes from lymph nodes and spleens of transgenic CL4 mice underwent positive selection for CD8+ T cells using the Miltenyi CD8+ T cell isolation kit accordance with the manufacturer's instructions (Miltenyi Biotec Ltd., Bisley, UK). Briefly, lymphocytes were re-suspended at 1×107 cells per 90µl of cold MACS buffer and incubated with 10µl of anti-CD8 magnetic MACS beads (Miltenyi Biotec Ltd.). Before being vortexed and incubated at 4ºC for 20 mins. They were then washed with 10ml of cold MACS buffer and centrifuged at 400g for 5 mins at 4ºC. Ls MACS separation columns, used for positive selection (Miltenyi Biotec Ltd.), were placed in MACS midi magnets (Miltenyi Biotec Ltd.) and washed with 6ml of cold MACS buffer. Pelleted cells were resuspended with 1ml of cold MACS buffer before being transferred onto the LS column through a 40µl cell strainer (BD Falcon) to prevent the column from clotting, and they were then washed with another 1ml of cold MACS buffer. The eluent was collected and passed over the MACS column one more time to prevent the loss of CD8+ T cells. At this point, a fraction of the negative sample was taken for purity. The column was washed again with 9ml of cold MACS buffer and then removed from the magnets. CD8+ T cells were collected by forcing 5ml of MACS buffer through the column using a syringe plunger. This protocol routinely yields more 95% purify of the purified CD8+ T cells.

3.6.3 Isolation of peripheral lymphocytes from tumour-bearing mice.
Non-tumour-draining lymph nodes (non-TDNL) consisting of inguinal and popliteal lymph nodes and TDNL which consist of cervical and auxiliary lymph nodes, and spleens were collected from conventional mice or tumour-bearing Thy1.2+ BALB/c mice. 14-

18 days following tumour cell injection Non-TDLN, TDLN and spleens were collected from euthanized mice and placed in complete medium on ice. A single-cell suspension of lymphoid cells was generated as described above.

3.6.4 Isolation of tumour infiltrating immune cells.
Tumour cells were removed from mice 14-18 days following tumour cell injection and placed in serum free medium on ice. They were transferred into a 35mm Petri dish (Corning Inc.) in a 5ml serum-free medium. Tumours were cut into small pieces using sterile forceps to generate an efficient cell suspension and then transferred into 40µm cell strainer (BD Falcon) and placed in clean 35mm Petri dishes (Corning Inc). A single-cell suspension was generated using a sterile syringe plunger. Cells were washed with serum-free medium and transferred into a 50ml conical tube (Greiner bio-one). This last step was repeated several times including a final wash to the 35mm Petri dish. Lymphocytes and myeloid cells separation from tumour cells were performed using Ficoll gradient separation. 15ml of Histopaque 1077(Sigma-Aldrich) was transferred into a 50ml conical tube (Greiner bio-one). Cells were slowly and carefully layered over the Histopague 1077 (Greiner bio-one) and centrifuged at 450g for 25 mins at room temperature with the brake off. The interface layer, which is comprised of the lymphocytes and myeloid cells, was transferred into a 15ml conical tube (Greiner bio-one) using a Pasteur pipette. Excess Histopaque 1077 was removed by washing and centrifuging the cells with 10ml of serum-free medium at 300g for 5 mins.

3.7 Proliferation of naïve CL4 CD8+ T cells.
In order to generate activated CL4 CD8+ T cells, either 24 or 96 well plates were coated with α-CD3 (10µg/ml) for 24 hours. Following this the MACS purified CL4+ CD8 T cells (see section 2.8) were incubated for 2 days with either α-CD28 (5µg/ml) for 48 hours (37◦C, 5% CO2). Naive CL4 CD8 T cells were obtained following MACS isolation and did not undergo incubation with any stimulatory molecules.

3.8 Protein Extraction.
The CL4 CD8+ T cells were harvested from the well plate using a Pasteur pipette, and wells were washed with complete media before counting. Cells were then pelleted and washed twice in 10mls of PBS. The cells were re-suspended in 1ml PBS before being pelleted the re-suspended in PBS. Whole cell protein was obtained by sonicating at 20% amplitude in 5

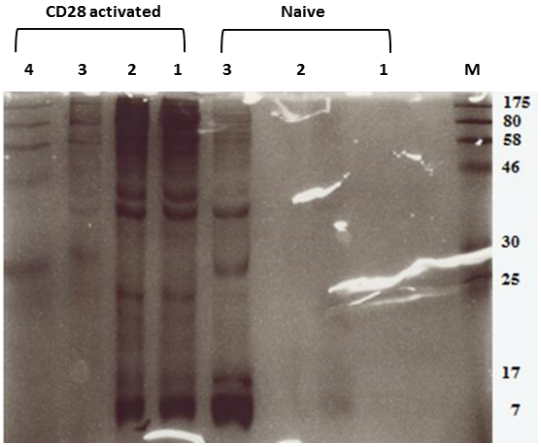

Figure 3: Coomassie blue staining. Samples were loaded into 12% SDS-PAGE gel of Coomassie blue staining. Wells were from left to right first loaded by pre-stained protein, 1 and 2 then 3, were 6 and 10 million Naïve CL4+ cells respectively. CD28 activated cell wells were loaded: in lane 1 are 10 million, 2 and 3 are 14 and 11 million respectively. Finally, the last well was loaded with 6 million α-CD3/ α-CD28 activated CL4 CD8+ T cells.

second intervals 5 times with 10 second breaks in between each pulse. Following this, the cell lysates were boiled at 95◦C for 5 minutes and stored at 20◦C until use. Cells were kept on ice following harvesting to limit the action of protease enzymes that could decrease the protein concentrations.
The decision was made to use a cell lysis buffer to limit the action of the cell proteases. This process was identical until the final re-suspension, where they left on ice for 10 minutes following re-suspension in hypotonic buffer (1M HEPES, 1M MgCL, 2.5M KCL, 1M DTT, dH2O and proteases inhibitors) which caused the cells to swell, and RIPA buffer (1m Tris, 5M NaCL, 10% deoxycholate and protease inhibitors) (Sigma-Aldrich, Boechringer-Mannheim, BDH and Fisher Chemical). The cells were then sonicated and frozen at -20◦C.

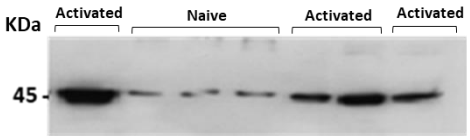

Figure 4: Western blot probe of naïve and activated CL4+ CD8+ whole cell protein for β-actin

3.9 Protein Assay.
Quantitative protein analysis was obtained via colorimetric analysis. BSA standards and entire cell lysate dilutions were plated out into a 96 flat bottom

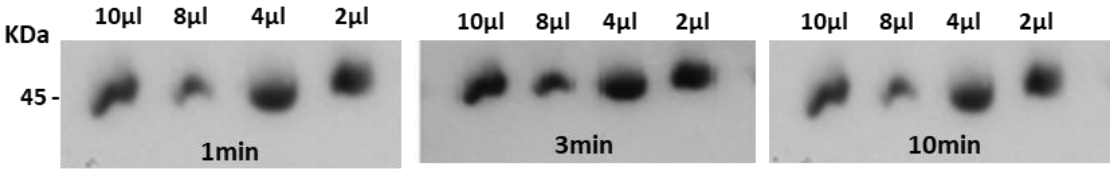

Figure 5: Titration of proteins derived from Naïve CL4 CD8+ T cell lysates. Naïve cell samples were probed for β-actin. 1 X SDS-sample buffer was added to give a 15 µl sample which was loaded into gel Sample protein levels contained within the 15µl varied from 10 - 2 µl, as shown. The exposure time was set for 1, 2 and 10mins.

well plate before 40µl Bio-Rad Protein Assay Dye Reagent (Bio-Rad, California, USA) was added. The plate was incubated for 5 minutes before being read using a Spectra MAX 190 plate microplate reader.

3.10 SDS-PAGE.

Initial analysis via SDS-Page was undertaken on a 12% SDS gel with Pre-stained Protein Marke (BioLabs, Massachusetts, USA) in a vertical gel apparatus. Protein samples were mixed with samples loading buffer (0.5M Tris HCL PH 6.8, Glycerol, 10% SDS, 2-mecraptoethanol, 10% w/v, Bromophenol Blue, dH2O) loaded and run at 150V for 10 minutes or until samples had reached resolving gel then run at 200V for 40 minutes. Coomassie 940% methanol, 10% acetic acid Coomassie Brilliant Blue staining (50% methanol, 10% acetic acid, 0.2% Brilliant blue) for protein visualization was undertaken for 1 hour on a gentle rocker before de-staining (45% methanol, 10% acetic acid). Imaging was obtained using UVP UV-trans illuminator.

3.11 Western Blotting.

Following whole cell protein extraction, standard western blotting protocols were followed using antibodies to detect β-actin, ARP2/3. In order for the proteins to be electrophoretically transferred, the arrangement of the western blot apparatus was as follows; three cut to measure Amersham Hybond blotting paper (GE Healthcare, Buckinghamshire, UK) were soaked in semi-dry western blotting buffer (5.82g Tris, 2.93g glycine, 200MeOH). A cut piece of Amersham Hybond-P nitrocellulose membrane (GE Healthcare), was placed on top following a brief methanol and transfer buffer wash. The gel was pre-equilibrated for 2 minutes in transfer buffer, placed on top of the membrane before adding three cuts, transfer buffer-soaked pieces of Amersham Hybond Blotting paper. The transfer was run at 15V for 1 hour before being placed in blocking solution (5% w/v non-fat milk powder, 1x TBS, 0.1% Tween-20) for 1 hour. Following blocking, the membrane was washed three times for 5 minutes each in a 1x TBS/T solution, then incubated with the primary antibodies, rabbit α-Cofilin (1:1000 dilution) (Cell Signalling Technology #3312, Massachusetts, USA) rabbit α-β actin (1:1000 dilution) (Cell Signalling Technology #4967, Massachusetts, USA), or mouse α-ARP3 (1:14000 dilution) (Cell Signalling Technology A5979, Missouri, USA), at 4◦C overnight. These were detected using α-mouse (1:2000 dilution) (Cell Signalling Technology #7076) or α-rabbit (1:2000 dilution) (Cell Signalling Technologies #7074) HRP-linked antibodies, incubated at room temperature for 1 hour. Membranes were washed with 1 x TBS/T solution before the addition of Amersham ECL Western Blotting Detection reagents (GE Healthcare). Imaging of bands was obtained using Amersham Hyperfilm ECL (GE Healthcare).

3.12 KdHA peptide pulsing of Renca cells in vitro.

Renca cells are harvested as described in 2.6 and counted and adjusted to 2 x 106ml in complete

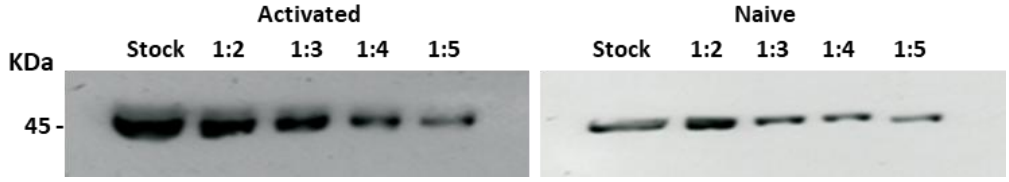

Figure 6: Naïve and activated CL4 CD8+ T cell lysates identified by Coomassie blue staining

Table 1: The protein concentration of a set of cell lysates were gained from CL4 CD8+ T cells.

Samples	Set of purified cells	Protein Concentration (µg)	Protein Concentration (µg/µl)	Protein Concentration (µg/cell)
Naive1	8×10^6	518.22	2.16	6.47×10^{-5}
Niave2	10×10^6	636.44	2.65	6.36×10^{-5}
Naive3	10×10^6	625.37	2.61	6.25×10^{-5}
Naive4	5.5×10^6	453.33	1.88	8.24×10^{-5}
Activated	7.9×10^6	7730.28	32.1	9.76×10^{-4}
TDLN	2×10^6	131.9	1.1	6.55×10^{-4}
TIL	2×10^5	13.19	0.2638	6.55×10^{-4}

medium. Kd-HA peptide-pulsing of the Renca cells is carried out in 1ml aliquots in a 15 ml conical tube with 4µg/ml of KdHA peptide [IYSTVASSL], 90 mins at 37oC. 5mls of calcium magnesium buffer was added after 90 mins incubation then the cells are pelleted by centrifugation at 400G for 5 mins. The pulsed Renca cells are then re-suspended accordingly at the desired concentration in an appropriate buffer or medium.

4. Results

Previous studies in our laboratory showed that KdHA–specific CL4 CTL that are generated in the TDLN of RencaHA tumour-bearing BALB/c mice lose their CTL activity following when the form TIL. Following a TMT analysis between the entire proteome of CL4 CD8+T cells from the TDLN cells and TIL compartment of RencaHA tumour-bearing mice a Pathway Analysis revealed that several proteins were significantly altered in their expression between these two cell types. Amongst the proteins that were identified ARP2/3 and Cofilin are key molecules involved in motility of the actin cytoskeleton during the formation of the Immunological Synapse, which is necessary for both priming of naive CD8+ T cells, and for CTL lysis of target cells. Both these proteins are shown to decrease by approximately 2-fold when CL4 CTL from the TDLN of RencaHA tumour bearing form TIL.

The experiments outlined in this report are aimed at the validating the TMT analyses and to determine how specific immunosuppressive molecule, (such as Adenosine), that are generated within the tumour micro-environment can modulate the expression and function of Cofilin and ARP2/3 during actin cytoskeleton regulation and IS formation.

4.1 Protein assay.

It orders to compare the expression of Cofilin and ARP2/3 between naïve and activated CL4 CTL it was important to quantify the levels of entire cell protein in the samples before Western blots analyses was performed on the cell samples. However, before Western blots are performed on the cell samples it was necessary to quantify the levels of protein in the extracts of each sample. This was done using Bio-Rad Bradford protein assay. In this particular experiment we obtained 11.6 and 10.2 µg/ml of protein from the two naïve population samples, and 31.8µg/ml from anti-CD3/anti-CD28-activated CD8 T cells. These data highlighted the fact that in activated cells there is much more protein than in naïve cells. However, the naïve sample reading was above the standard blanks and the bands on the SDS-PAGE were very faint in several cases of the Coomassie blue staining Figure.3.

4.2 Analysing the quantitative data

The analysis of the ARP2/3 and Cofilin in naive and activated CL4 CD8+ T cells relied on obtaining a set of results that could be compared accurately. The hypotonic buffer was applied as described previously, and also using a similar protocol to Bradford protein assay (Bio-Rad) for protein extracts. The cell samples used: 4 naive samples were obtained from the purification of CL4 CD8+

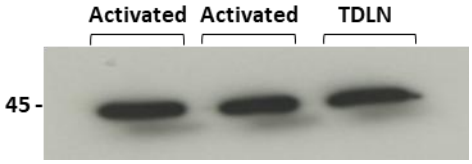

Figure 7: Western blotting analyses of naïve activated and TDLN-derived CL4 CD8+ T cells. 20µg of each sample was loaded onto the gel by using different concentrations: from left to right (7.7µl of naïve, 3µl of activated and 7µl of TDLN) These samples were probed for using β- actin as primary antibody followed by α-rabbit IgG as a secondary antibody.

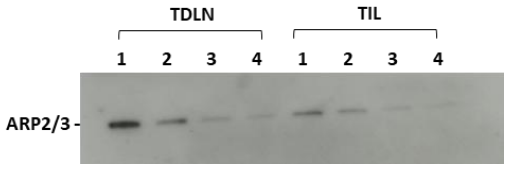

Figure 8: Western analyses of TDLN-derived and TIL CL4 CD8+ T cell protein. Protein samples were probed for ARP2/3. All samples were loaded onto the gel in four different concentrations: 1.9µg, 0.95µg, 0.48µg and 0.24µg and a 2µl of 5 x SDS-sample buffer was added to all except for sample number four to which was added 4µl.

cells, resulting in a high number, (on average between 20-30 million cells in Table 1). It was possible to collect both naive samples and a reasonable number of the α-CD3/α-CD28 activated cells.

4.3 SDS-PAGE Protein Analysis

There was an initial challenge at the start of this research project, namely whether or not it was possible to isolate and measure the amount of T cell-derived protein. This step was previously carried out by isolation, expansion and extraction of protein from the aforementioned cell types. The wells of a 12% resolving gel and 3% staking gel were loaded with 15µl amounts of the cell sample, and then they underwent Coomassie blue staining for 1 hour and were washed three times for 20 mins during the de-staining. Firstly, analyses of the protein samples showed that the levels of protein amongst the naïve the CL4+ CD8+ samples are significantly less than the activated CD8 T cells Figur.3.

4.4 Western Blot Analysis

4.4.1 β- actin control

Firstly, Western blot analyses of β-actin expression was performed to determine if there is enough protein for subsequent successful imaging of Cofilin and ARP2/3.

4.4.2 Cofilin and ARP2/3 staining

The successful identification of β-actin Figure.4, provided evidence that there was protein in these samples. Therefore, to perform Western blotting analyses for detecting Cofilin it was important to use the same protocol used for β-actin but with the anti-Cofilin mAb as the primary antibody.

The Western blot analyses were repeated initially the same way as before using the same primary antibody dilution was added in accordance with the manufacturer's instructions (1:1000). All the steps were the same; the samples were loaded in the same way to introduce any other variables into the experiment.

It was revealed that the noticeable bands from naïve T cells protein in Figure 5-7 were not as clear as the bands we saw in the protein quantification experiment. Moreover, the intensity of the band did not directly correlate with the concentration of protein in the sample Figure 5-7. This may be related to the exposure time exposure for the Hyperfilm or whether the samples have maintained a high enough concentration of protein concentration. Thus, reducing the concentration and exposure time would be important in the next step for all prior Western blot and Coomassie blue staining experiments which were carried out, it was

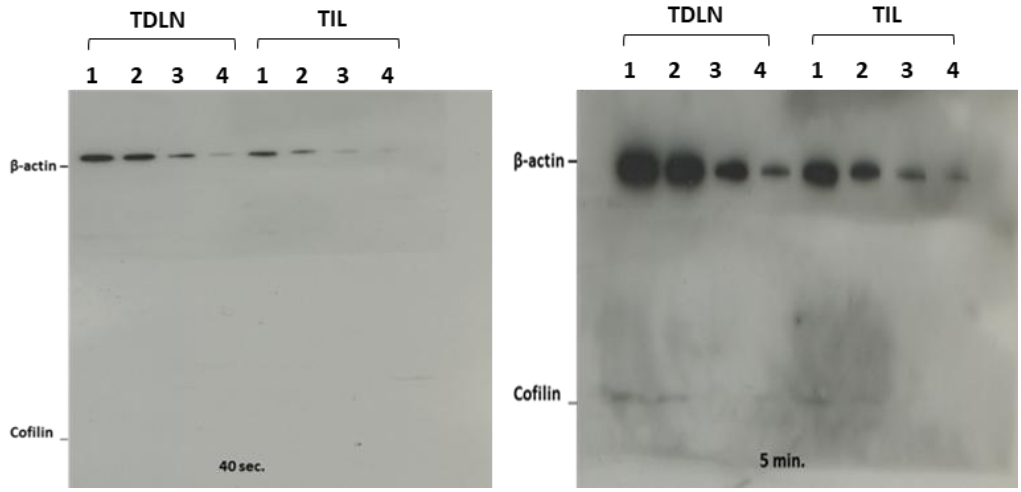

Figure 9: Western blotting configuration. TDLN and TIL protein samples were probed with β-actin and Cofilin. All samples were loaded onto the gel in four different concentrations: 1.9µg, 0.95µg, 0.48µg and 0.24µg and a 2µl of 5 x SDS-sample buffer was added to all except for sample number four to which was added 4µl.

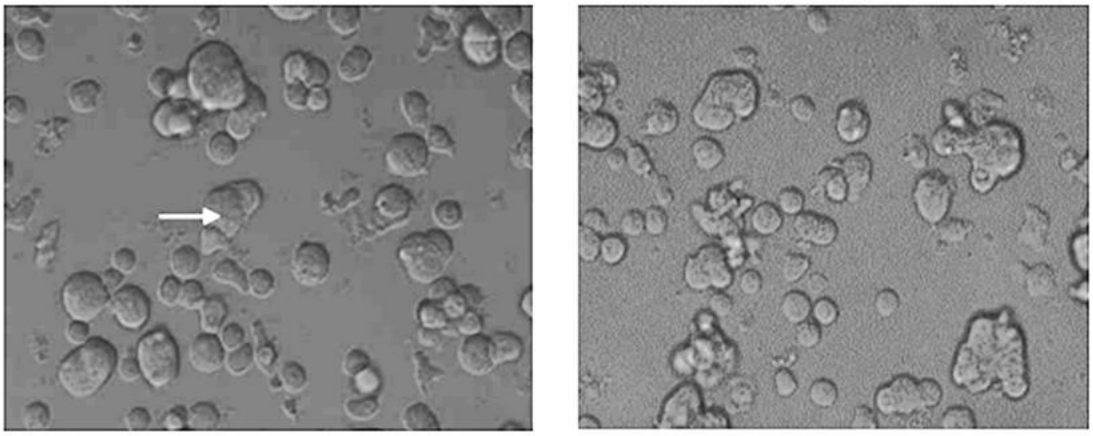

Figure 10: Imaging of activated CL4 CD8+ T cells to RencaHA with Kd peptide, RenecaWT left to right coupling in vitro.

recommended to try repeating the protocol, using a lower concentration of protein in samples as well as decreasing the exposure time. These repeated blots were conducted by probing for β-actin.

The blots shown in Figure 6-10 were carried out again with various dilutions of the original protein lysate at the concentrations shown at the top of each panel. 1x SDS sample buffer was added before loading the samples onto the gels. The data shows that there are identical dark bands in both the naïve and activated sample at higher dilutions in The Coomassie blue stained gel, and there are bands of decreasing thickness in both the naïve and the activated samples when probed for β-actin Figures.6-10. It was revealed that the whole cell lysate protein and also β-actin were more concentrated when compared to the naive cells. The activated CD8+ cells comprise a high level of protein and also are as big as the naive sample which was derived from 1.4 x 10^7 cells whereas the activated cell sample was derived from more than 4 x 10^6 cells. However, due to the fact that the protein was obtained in a buffer containing β-mercaptoethanol and Bromophenol Blue salts, both of which are incompatible at low levels in protein assay kits, we were not able to quantitate the amount of protein in these samples.

In this next stage, it was decided to combine a hypotonic solution with a RIPA buffer instead of the protein sample containing β-mercaptoethanol and Bromophenol Blue. This would lead to greater efficiency of cell lysis and the better yield of soluble protein and the fact that protease inhibitors were included resulted less protein degradation. However, the aforementioned process of compatible concentrations has been calculated as the final concentration of components which prevent an interference with the protein assay. The breaking of disulphide molecules and protein degradation was achieved by adding 5 X SDS-sample buffer and boiling before samples were loaded on the gel.

Based on previous Western blotting, we decided to decrease the protein concentration and/or exposure time for all protein samples.

It was crucial to find the optimal dilution of protein which could give clear and neat uniform bands. The samples were serially diluted to achieve a number of different amounts and 5 x SDS- sample buffer was also added.

The next Coomassie stain and Western blotting carried out used protein samples from naive, activated and TDLN-derived CL4 CD8+ T cells probed for β-actin Figures 7-10. The samples were loaded onto the wells as in the previous experiment and the bands and background appeared both clear and even. However, there are in fact faint duplicated bands caused by the opening of the cassette.

In order to ensure that samples were loaded into the well gel again the same procedure was carried out as before. Four different concentrations, of the TDLN protein sample were used and the levels Cofilin were probed. Figure 8 shows that there is a difference in concentration of the bands between the Western blotting was probed with ARP2/3 and this was incubated with Cofilin. Again, it can be observed that the bands are clear and even, including a high background for 5 minutes of exposure time.

Western blotting for β-actin and Cofilin was also carried out. The bands reveal although there is β-actin when exposed for 40 seconds, Cofilin gave no

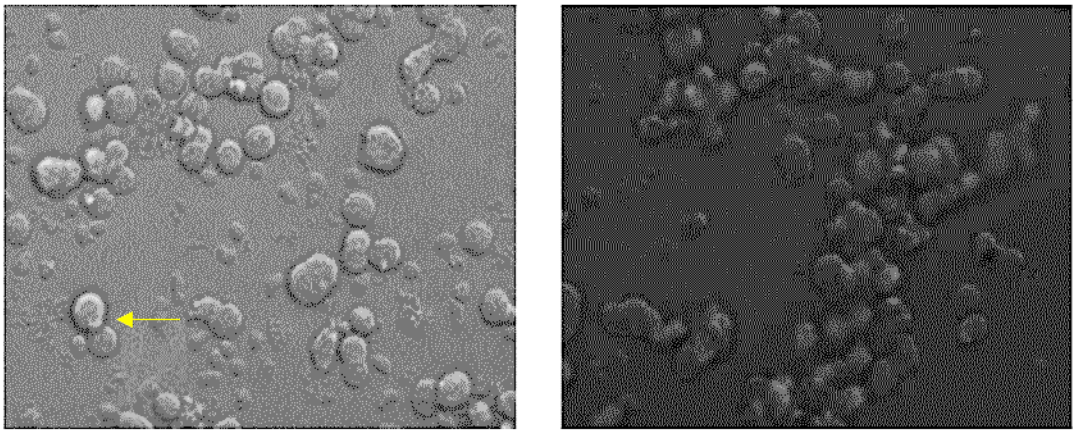

Figure 11: Image of TDLN and TIL CL4 CD8+ T cells left to right coupling with RencaHA with Kd peptide

signals. As can be seen, the β-actin bands were much stronger after 5 mins whereas the Cofilin bands appeared very faint and barely detectable Figure 9.

5 Bio-imaging of coupling between Renca cells and CL4 CD8+ T cells.

The goal of bio-imaging is to determine and observe the formation of immunological synapses between CL4 CD8+ from the TDLN and TIL (as previously mentioned) and how, the formation of a synapse or not relates to the levels of ARP2/3 and Cofilin, and the role that these molecules play in rapidly mobilizing several surface proteins to the region of cell-cell contact. Once the TCR recognizes the antigen presented by the APC, selected receptors on the T cell and their exact ligands form the immunological synapse or the supermolecular activation cluster (SMAC), which divide the molecules in the central compartment of the synapse (cSMAC) and those in the periphery of the synapse form (pSMAC). As can be seen in Figure 10. The binding of RencaHA with K^d peptide and activated CD8+ T cells in vitro appeared clearly as a strong coupling indicated by the pink arrow on the left image Figure 10. They were obtained from transgenic mice using the protocol described in Section 3.6. In contrast to the RencaWT cells: CD8+ T cells interaction is just attached and stacked. All cells migrate around having the protrusion lamellipodia. Confocal microscopy was used to imagine the TDLN and TIL CL4 CD8+ T cells obtained from immunised tumour Reneca HA-bearing mice, which were sorted and stained before imaging. The results in observing the TDLN were very active, in contrast to the TIL which was very inactive and failed to produce coupling, leading to either a reduction in the levels of Cofilin and ARP2/3 proteins or the concentration of tumour antigens influencing anti-tumour CD8+ T cell functions Figure 11.

5. Conclusion

This investigation has hallmarked a major number of protein variances in genetic expression between the TDLN-derived lymphocytes and TILs of tumour-bearing mice. It was carried out to analyse the concentration of two types of protein, which acted in the actin cytoskeleton within both naïve and activated CL4 CD8+ T cells: these are ARP2/3 and Cofilin, the result of a slight difference between naïve and activated T cells as in Figure 7. Moreover, there is a slight difference between TDLN and TILs CL4 CD8+ T cells as in Figure 9 which were probed with ARP2/3 antibody and β-actin antibody as control. In contrast, Cofilin can be seen as barely detectable bands and this may be due to the protein going down during electrophoresis at the bottom in the gel.

MACS isolation was used to harvest the initial CL4 CD8+ T cells which were derived from mouse spleen and lymph nodes. Following the activation with α-CD3 and α-CD28 mAbs and protein isolation by sonicating, β- mercaptoethanol and Bromophenol Blue buffer were added to dilute the initial samples, but this protocol was not suitable for using in the protein assay as the colour interferes with the Bradford assay.

Hence, using the alternative buffer containing a hypotonic and RIPA was essential to lyse the cells to ensure the quantification of the whole cell protein concentration through a Bradford assay.

The whole cell protein concentration was compared

directly by the loading of an SDS-PAGE gel followed by Western blotting using specific antibodies for probing ARP2/3 and Cofilin. The resulting ARP2/3 level showed a little higher in naïve T cells compared to the activated T cells Figure 7. Initial Coomassie blue and Western blotting emphasized significant differences: for instance, the bands were not running directly into the lower part of the gel; also, the protein standard was smeared and not formed of straight, uniform bands Figure 5. The Tris buffer component of the gel was altered to resolve these issues, as well as confirming that the inner buffer chamber keeps the SDS-PAGE running buffer fully by filling the outer chamber practically to the top with running buffer. The uneven bands may be generated by slightly decreasing the inner chamber volume due to minor leakage of the SDS-PAGE running buffer. The alteration of the anode covers of the apparatus also aided in the running of the gel and a more efficient separation of the bands; providing less smearing and giving the protein standard a clearer and shaper appearance Figure 7.

The expression of ARP2/3 in naïve T cells Figure 7 is expected. Because it binds to the sides of actin filament to allow dendrite nucleation and promote a new daughter filament of actin polymerization [36]. There are many microvilli on the leading edge of naïve T cells, where the numerous adhesion molecules are located, and their function includes promoting specific adhesion to target molecules during the migration through the endothelium of the blood vessels. In the presence of stimulatory cytokine this leads to a response result in microvilli breakdown in a process to aid the penetration of the T cell from end to end of the endothelium cell wall [40].

As a result of this situation, the naïve T cells contain high amounts of ARP2/3 resulting in a large promotion in net actin filament in order to recognise targets and co-stimulatory molecules leading to support the protruding microvilli from the cell surface. This is termed dendritic nucleation. Cofilin plays a critical role in severing the actin filament on both barbed and pointed ends, but after severing it does not continue binding to the barbed ends of actin filaments and depolymerization of F-actin into monomeric G-actin Figure 7. The initial Western blotting detected and investigated the protein concentrations after 48-hour initial proliferation of cells were lysed. It is sometimes believed that the levels of Cofilin naive are similar to the levels of Cofilin in activated cells. Thus, activated cells have probably undergone the proliferation and stimulation phase during the TcR: stimulatory molecules of CD-3 and CD28 interaction. It is becoming clear at that point that Cofilin is most likely promoted in comparison to naïve T cells, which may aid in the breaking down of the microvilli, the flattening of the cell surface induced severance of the protruding F-action by Cofilin. The formation of immunological synapse is best described in Figure 10 between activated CL4 CD8+ T cells and RencaHA tumour cells, whereas TDLN and TIL CL4 CD8+ T cells coupling with RencaHA is merely attaching and moving around Figure 11.

6. Further work

1- Purification and activation of CL4 CD8+ T cell derived from lymph nodes and spleens of BALB/c mice *in vitro*.
2- Using specific immunosuppressive Adenosine to modulate CL4 CD8+ T cell activation.
3- Use Western blotting to detect whether or not there are any significant alterations of APR2/3 and Cofilin in naïve activated, TDLN and TIL-derived CL4 $CD8^+$ T cells.
4- Use PhosTag Western blotting or gel shift (electrophoretic mobility shift assay) to probe for phosphorylated or dephosphorylated Cofilin in TDLN and TIL-derived CL4 $CD8^+$ T cells as well as in CL4 $CD8^+$ T cells exposed to immunosuppressive adenosine.
5- Compare the ability of TDLN and TIL-derived CL4 $CD8^+$ T cells to couple with RencaHA tumour cells *in vitro*.
6- Determine the ability of adenosine-treated CL4 $CD8^+$ T cells to couple with RencaHA cells.

References

[1] Wang, W., R. Eddy, and J. Condeelis, *The cofilin pathway in breast cancer invasion and metastasis.* Nat Rev Cancer, 2007. **7**(6): p. 429-40.
[2] Pages, F., et al., *Effector memory T cells, early metastasis, and survival in colorectal cancer.* New England Journal of Medicine, 2005. **353**(25): p. 2654-2666.
[3] Honoson HL, e.a., *Eradication of established tumor by CD8+ T cells adoptive immunotherapy.* Immunity, 2000. **13**(2): p. 265-44.
[4] Stassi, G., et al *Thyriod cancer resistance to chemotherapeutic durgs via autocrine production of interleukin-4 and interleukin-1o.* Cancer Res, 2003. **63**(20): p. 6784-90.

[5] Wang, T., et al., *Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells*. nAT mED, 2004. **10**(1): p. 48-54.
[6] Zitvogel, L., A. Tesniere, and G. Kroemer, *Cancer despite immunosurveillance: immunoselection and immunosubversion*. Nature Reviews Immunology, 2006. **6**(10): p. 715-727.
[7] Gorelik, L.a.F., R. A., *Transforming growth factor-beta in T-cell biology*. Nat Rev Immunol, 2002. **2**(1): p. 46-53.
[8] Kroemer, G. and S.J. Martin, *Caspase-independent cell death*. Nature Medicine, 2005. **11**(7): p. 725-730.
[9] Acuto, O. and D. Cantrell, *T cell activation and the cytoskeleton*. Annu Rev Immunol, 2000. **18**: p. 165-84.
[10] Montixi, C., et al., *Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains*. EMBO J, 1998. **17**(18): p. 5334-48.
[11] Nel, A.E. and N. Slaughter, *T-cell activation through the antigen receptor. Part 2: role of signaling cascades in T-cell differentiation, anergy, immune senescence, and development of immunotherapy*. J Allergy Clin Immunol, 2002. **109**(6): p. 901-15.
[12] Potter, T.A., et al., *Formation of supramolecular activation clusters on fresh ex vivo CD8+ T cells after engagement of the T cell antigen receptor and CD8 by antigen-presenting cells*. Proc Natl Acad Sci U S A, 2001. **98**(22): p. 12624-9.
[13] Pollard, T.D., *Cellular motility powered by actin filament assembly and disassembly*. Harvey Lect, 2002. **98**: p. 1-17.
[14] Ichetovkin I, G.W., Condeelis J, Cofilin produces newly polymerized actin filaments that are preferred for dendritic nucleation by the Arp2/3 complex. Curr Biol, 2002. 12: p. 79-84.
[15] Gessi, S., et al., *Adenosine and lymphocyte regulation*. Purinergic Signalling, 2007. **3**(1-2): p. 109-116.
[16] Edwards, D.C., et al., *Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics*. Nature Cell Biology, 1999. **1**(5): p. 253-259.
[17] Wang, Y., F. Shibasaki, and K. Mizuno, *Calcium signal-induced cofilin dephosphorylation is mediated by slingshot via calcineurin*. Journal of Biological Chemistry, 2005. **280**(13): p. 12683-12689.
[18] Soosairajah, J., et al., *Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin*. Embo Journal, 2005. **24**(3): p. 473-486.
[19] Linnemann, C., et al., *Adenosine regulates CD8 T-cell priming by inhibition of membrane-proximal T-cell receptor signalling*. Immunology, 2009. **128**(1): p. e728-e737.
[20] Dustin, M.L. and J.A. Cooper, *The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling*. Nat Immunol, 2000. **1**(1): p. 23-9.
[21] Hoskin, D.W., et al., *Inhibition of T cell and natural killer cell function by adenosine and its contribution to immune evasion by tumor cells (Review)*. International Journal of Oncology, 2008. **32**(3): p. 527-535.
[22] Ryzhov, S., et al., *Adenosine A(2B) receptors in regulation of dendritic cell differentiation and function*. Faseb Journal, 2008. **22**.
[23] Stagg, J. and M.J. Smyth, *Extracellular adenosine triphosphate and adenosine in cancer*. Oncogene, 2010. **29**(39): p. 5346-5358.
[24] Sitkovsky, M.V., et al., *Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors*. Annu Rev Immunol, 2004. **22**: p. 657-82.
[25] Beavis, P.A., et al., *CD73: a potent suppressor of antitumor immune responses*. Trends Immunol, 2012. **33**(5): p. 231-7.
[26] Schmidt, H., et al., *Effector granules in human T lymphocytes: the luminal proteome of secretory lysosomes from human T cells*. Cell Commun Signal, 2011. **9**(1): p. 4.
[27] Bonzon-Kulichenko, E., et al., *Quantitative in-depth analysis of the dynamic secretome of activated Jurkat T-cells*. J Proteomics, 2011. **75**(2): p. 561-71.
[28] Stinchcombe, J.C., et al., *The immunological synapse of CTL contains a secretory domain and membrane bridges*. Immunity, 2001. **15**(5): p. 751-61.
[29] Wulfing, C. and M.M. Davis, *A receptor/cytoskeletal movement triggered by costimulation during T cell activation*. Science, 1998. **282**(5397): p. 2266-2269.
[30] Grakoui, A., et al., *The immunological synapse: A molecular machine controlling T cell activation*. Science, 1999. **285**(5425): p. 221-227.
[31] Monks, C.R., et al., *Three-dimensional segregation of supramolecular activation clusters in T cells*. Nature, 1998. **395**(6697): p. 82-6.
[32] Valitutti, S., et al., *Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton*. J Exp Med, 1995. **181**(2): p. 577-84.
[33] Villalba, M., et al., *A novel functional interaction between Vav and PKCtheta is required for TCR-induced T cell activation*. Immunity, 2000. **12**(2): p. 151-60.
[34] Stinchcombe, J.C. and G.M. Griffiths, *The role of the secretory immunological synapse in*

killing by CD8(+) CTL. Seminars in Immunology, 2003. **15**(6): p. 301-305.
[35] Sanchez-Madrid, F. and M.A. del Pozo, *Leukocyte polarization in cell migration and immune interactions.* EMBO J, 1999. **18**(3): p. 501-11.
[36] Machesky, L.M. and K.L. Gould, *The Arp2/3 complex: a multifunctional actin organizer.* Curr Opin Cell Biol, 1999. **11**(1): p. 117-21.
[37] Wang, W.G., R. Eddy, and J. Condeelis, *The cofilin pathway in breast cancer invasion and metastasis.* Nature Reviews Cancer, 2007. **7**(6): p. 429-440.
[38] Galkin, V.E., et al., *Actin depolymerizing factor stabilizes an existing state of F-actin and can change the tilt of F-actin subunits.* J Cell Biol, 2001. **153**(1): p. 75-86.
[39] Morgan, D.J., et al., *CD8(+) T cell-mediated spontaneous diabetes in neonatal mice.* Journal of Immunology, 1996. **157**(3): p. 978-983.
[40] Burkhardt, J.K., E. Carrizosa, and M.H. Shaffer, *The actin cytoskeleton in T cell activation.* Annu Rev Immunol, 2008. **26**: p. 233-59.