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Evaluating the effect of immune cells on the outcome of patients with mesothelioma

By

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ABSTRACT

FACULTY OF MEDICINE

Cancer Immunology

Thesis for the degree of Doctor of Philosophy

EVALUATING THE EFFECT OF IMMUNE CELLS ON THE OUTCOME OF PATIENTS WITH MESOTHELIOMA

Serena Jamie Tzu Wen Chee

Mesothelioma is a rare cancer usually affecting the pleura. It is characteristically associated with inhalation of asbestos fibres and accounts for 1% of cancers in the United Kingdom. Median survival remains poor at 4-18 months despite treatment.

Immunotherapy has established itself as an important treatment option in many solid tumours where survival benefit has been shown to be associated with CD8 infiltration. In mesothelioma, there are 3 small studies that suggest that CD8 infiltration may confer survival benefit.

Here, a systematic assessment was undertaken of the prognostic and predictive value of infiltrating adaptive and innate immune cells in a large cohort of patients with advanced mesothelioma. A tissue microarray from 302 samples was constructed. Markers of adaptive immune response CD4+ T helper and CD8+ Cytotoxic T cells, FOXP3+Tregs, CD45RO+Memory T cells and B-cells (CD20+), and innate immune response; macrophages (CD68+), natural killer cells (CD56+) and neutrophils (NP57+) were evaluated.

Surprisingly, CD8+ tumour infiltrating lymphocytes (TILs) did not predict for outcome. On multivariate analysis a high CD4+, high CD20+ and low NP57+ count were linked to better outcome in the epithelioid tumours. A low FOXP3+ predicted for good outcome in both epithelioid and non-epithelioid tumours.

Next, multiplex immunohistochemistry was utilised to further evaluate CD4+ and CD8+ T cell subtypes. This established the presence of MHC class II expression on epithelioid mesothelioma tumour cells and confirmed that some CD4+ T cell subsets (Tissue resident memory cells and T follicular helper cells), were associated with better outcome in epithelioid mesothelioma. The intriguing question of why CD4 + T cells function as the outcome determining immune effectors in mesothelioma, remains to be determined.

Mesothelioma-associated pleural fluid was evaluated to determine its utility as a surrogate for immune events in the solid tumour by transcriptomic analysis. T cells in the pleural fluid exhibited a phenotype characteristic of quiescent/naive cells.

In summary, the work presented in this thesis demonstrates that CD4+ and not CD8+ TILs correlate with clinical outcome in epithelioid mesothelioma. Future work will focus on characterising why and how the CD4+ TILs contribute to this effect.

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Academic Thesis: Declaration Of Authorship

I, Serena Jamie Tzu Wen Chee

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

EVALUATING THE EFFECT OF IMMUNE CELLS ON THE OUTCOME OF PATIENTS WITH MESOTHELIOMA .....

.....

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
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7. Parts of this work have been published as: Chee SJ, Lopez M, Mellows T, Gankande S, Moutasim KA, Harris S, et al. Evaluating the effect of immune cells on the outcome of patients with mesothelioma. British journal of cancer. 2017;117(9):1341-8.

Signed: .....

Date: 19th July 2019.....

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Definitions and Abbreviations

ADCC	**Antibody dependent cellular cytotoxicity**
AEC	**3-amino-9-ethylcarbazole**
ANG2	**Angiopoietin 2**
ANOVA	**Analysis of variance**
APC	**Antigen presenting cell**
Apo2L	**Apoptosis 2 ligand**
Arg1	**Arginase 1**
ATP	**Adenosine Triphosphate**
B7-H1	**B7 homolog 1**
B7-H3	**B7 homolog 3**
B7-H4	**B7 homolog**
BAP1	**BRCA associated protein 1**
BATF	**Basic leucine zipper transcription factor ATF-like**
Bc1	**B cell 1**
Bc2	**B cell 2**
BCL2	**B cell lymphoma 2**
Bcl6	**B cell lymphoma 6**
BCR	**B cell receptor**
Blimp	**B-Lymphocyte-Induced Maturation Protein**
Breg	**B regulatory cell**
CAR	**Chimeric antigen receptor**
CCL	**Chemokine ligand**
CCR	**Chemokine receptor**

CD	**Cluster of differentiation**
CDKN2A/ARF	**Cyclin-dependent kinase inhibitor 2A/alternative reading frame**
CI	**Confidence interval**
CM	**Central memory**
CPA	**Clinical pathology accreditation**
CRTAM	**Cytotoxic and regulatory T cell molecule**
CRUK	**Cancer Research United Kingdom**
CSD	**Cancer Sciences Division**
CSF	**Colony stimulating factor**
CTL	**Cytotoxic T lymphocyte**
CTLA-4	**Cytotoxic T lymphocyte associated protein 4**
CXCL	**Chemokine (CXC motif) ligand**
CXCR	**Chemokine (CXC motif) receptor**
cyTOF	**Cytometry Time of Flight (Mass cytometry)**
DC	**Dendritic cell**
DEG	**Differentially expressed gene**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
ECM	**Extracellular matrix**
EGF	**Epidermal growth factor**
EGFR	**Epidermal growth factor receptor**
ELF4	**E74 Like ETS Transcription Factor 4**
ELISA	**Enzyme-linked immunosorbent assay**
ELISPOT	**Enzyme-linked ImmunoSpot**

EMT	**Epithelial-mesenchymal transition**
EPP	**Extra-Pleural Pneumonectomy**
eTreg	**Effector T regulatory cell**
FAS	**Apoptosis stimulating fragment**
FasL	**Apoptosis stimulating fragment ligand**
FAK	**Focal adhesion kinase**
Fc	**Fragment crystallises**
FcR	**Fragment crystallises Receptor**
FFPE	**Formalin fixed paraffin embedded**
FoxP3	**Forkhead box Protein 3**
GARP	**Glycoprotein A repetitions predominant**
GATA-3	**GATA binding protein 3**
GC	**Germinal centre**
GITR	**Glucocorticoid induced TNF receptor related protein**
GM-CSF	**Granulocyte macrophage colony stimulating Factor**
GNLY	**Granulysin**
GZB	**Granzyme B**
HAVCR2	**Hepatitis A Virus Cellular Receptor 2**
H&E	**Haematoxylin and Eosin**
HECT	**Homologous to the E6-AP Carboxyl Terminus**
Hi	**High**
HIV	**Human Immunodeficiency Virus**
HLA	**Human leucocyte antigen**

HLA-E	**Human leucocyte antigen alpha chain E**
HOBIT	**Homolog Of Blimp-1 In T Cells**
HPF	**High Powered Field**
HR	**Hazard ratio**
HRP	**Horseradish peroxidase**
HTA	**Human tissue authority**
ICAM1	**Intercellular adhesion molecule 1**
ICI	**Immune checkpoint inhibitor**
ICOS	**Inducible T cell costimulator**
ICOSL	**Inducible T cell costimulator ligand**
IDO	**Indoleamine 2,3-dioxygenase**
IFN	**Interferon**
IFNγ	**Interferon gamma**
IHC	**Immunohistochemistry**
IL	**Interleukin**
IL R	**Interleukin Receptor**
iNOS	**inducible Nitric oxide synthase**
IRF4	**Interferon regulatory factor**
iTreg	**Induced T regulatory cell**
KIR	**Killer cell immunoglobulin like receptors**
KLF	**Krüppel-like factor**
KLRG1	**Killer cell lectin like receptor subfamily G member 1**
KLRK1	**Killer Cell Lectin Like Receptor K1**
Lag3	**Lymphocyte activation gene 3**
LAP	**Leucocyte Alkaline Phosphatase**

LN2	**Liquid nitrogen**
Lo	**Low**
LREC	**Local Research Ethics Committees**
Ly6C	**Lymphocyte antigen 6 complex**
M1	**Type 1 Macrophage**
M2	**Type 2 Macrophage**
mAbs	**Monoclonal Antibody**
MARF	**Mesothelioma Applied Research Foundation**
MDSC	**Myeloid derived suppressor cell**
MHC	**Major Histocompatibility Complex**
MICA	**MHC class 1 related gene A**
MICB	**MHC class 1 related gene B**
MMP	**Matrix metalloprotinase**
mRNA	**Messenger RNA**
N1	**Type 1 Neutrophil**
N2	**Type 2 Neutrophil**
NCR1	**Natural Cytotoxicity Triggering Receptor 1**
NDFIP1	**Nedd4 Family Interacting Protein 1**
NEDD4	**Neural Precursor Cell Expressed, Developmentally Down-Regulated 4**
NEQAS	**National external quality assessment services**
NF2	**Neurofibromatosis type 2**
NHS	**National Health Service**
NK	**Natural Killer**
NKT	**Natural Killer T cell**

NO	**Nitric oxide**
NRES	**National Research Ethics Service**
NSCLC	**Non small cell lung cancer**
nTreg	**Natural T regulatory cell**
OS	**Overall survival**
padj	**Benjamini-Hochberg–adjusted *P* value**
PBMC	**Peripheral blood monocyte cell**
PCR	**Polymerase chain reaction**
PD-1	**Programmed death 1**
PDCD1	**Programmed cell death 1**
PD-L1	**Programmed death ligand 1**
PD-L2	**Programmed death ligand 2**
PDGF	**Platelet derived growth factor**
PGE_2	**Prostaglandin E2**
PTGER4	**Prostaglandin E Receptor 4**
PPARα	**Peroxisome proliferator activated enzyme receptor Alpha**
PTPN22	**Protein tyrosine phosphatase nonreceptor 22**
pTreg	**Peripheral T regulatory cell**
RB1	**Retinoblastoma 1**
R&D	**Research and Development**
RAET1E	**Retinoic acid early transcript 1E**
RARA	**Retinoic acid receptor alpha**
RASGRP1	**Ras-guanyl nucleotide exchange factor**
REC	**Research Ethics Committee**

RNA	**Ribonucleic acid**
RNAseq	**RNA based next generation sequencing**
RORA	**Related Orphan Receptor A**
Rorc	**Retinoic acid receptor related orphan receptor C**
RORγ	**Retinoic acid receptor related orphan receptor gamma**
ROS	**Reactive oxygen species**
RPMI	**Roswell Park Memorial Institute medium**
RTU	**Ready to use**
RUNX2	**Runt-related transcription factor 2**
SELL	**Selectin L**
SLAM	**Self-ligand receptor of the signaling lymphocytic activation molecule**
SMAD	**Similar to Mothers against Decapentaplegic**
SOP	**Standard Operating Procedure**
SPN	**Sialophorin**
Stat	**Signal transducer and activator of transcription**
T-B	**T cell zone-B cell follicle interface**
T-bet	**T box transcription factor**
TAA	**Tumour associated antigen**
TAM	**Tumour associated macrophage**
TAN	**Tumour associated neutrophil**
Tbx21	**T box transcription gene 21**
Tc	**T cytotoxic cell**
TCF	**Transcription factor**

T_{CM}	**Central memory T cell**
TCR	**T cell receptor**
T_{EFF}	**Effector T cell**
T_{EM}	**Effector memory T cell**
TEMRA	**Effector memory RA T cell**
Tfh	**Follicular helper T cell**
T_{FR}	**T follicular regulatory cell**
TGF-β	**Transforming growth factor**
Th	**Helper T cell**
Tie2	**Tyrosine kinase with immunoglobulin like and EGF l like domains 1**
TIGIT	**T cell immunoreceptor with Ig and ITIM domains**
TIL	**Tumour infiltrating lymphocyte**
TIM-3	**T cell immunoglobulin and mucin domain containing 3**
TLR	**Toll like receptor**
TMA	**Tissue microarray**
TME	**Tumour microenvironment**
TNF	**Tumour necrosis factor**
TNFSF	**Tumour necrosis factor superfamily**
Tr1	**Type 1 regulatory T cell**
TRAF	**TNF Receptor Associated Factor**
TRAIL	**TNF related apoptosis inducing ligand**
Treg	**Regulatory T cell**
T_{RM}	**Resident memory T cell**

TSA	**Tumour specific antigen**
T_{SCM}	**Stem memory T cell**
T_{SEN}	**Senescent T cell**
tTregs	**Thymus T regulatory cell**
UHS	**University Hospital Southampton**
UK	**United Kingdom**
UNC13D	**Unc-13 Homolog D**
UoS	**University of Southampton**
VATS	**Video Assisted Thoracic Surgery**
VEGF	**Vascular Endothelial Growth Factor**
WT	**Wild Type**
WT1	**Wilms Tumour 1**
XCL1	**X-C Motif Chemokine Ligand 1**
γδ	**Gamma delta**
ZBTB7B	**Zinc Finger And BTB Domain containing 7B**
ZEB2	**Zinc Finger E-Box Binding Homeobox 2**
ZNF	**Zinc Finger Protein**

Chapter 1 Introduction

1.1 Mesothelioma

Mesothelioma is a malignancy of mesothelial cells, which form the superficial surface of serosal membranes such as the pleura, peritoneum, tunica vaginalis and pericardium. Pleural mesothelioma is the most common subtype, accounting for 90% of cases. It is mainly associated with asbestos exposure with a lag time from exposure to diagnosis of up to 30-40 years (Scherpereel et al., 2010). There is also a genetic component, with family clusters of patients who possess BAP1 mutations having an increased likelihood of developing mesothelioma and uveal melanoma (Testa et al., 2011).

Mesothelioma usually presents late, with diagnosis typically made by radiological findings in combination with pleural biopsies/pleural fluid cytology. The median survival following a diagnosis of mesothelioma is 4-18 months despite treatments like chemotherapy, radiotherapy and surgery.

Three histological subtypes of mesothelioma are recognised. Epithelioid mesothelioma carries the best prognosis, sarcomatoid mesothelioma is the most aggressive and tumours with mixed morphology have intermediate outcomes. Mesothelioma is a rare cancer that accounts for less than 1% of cancers diagnosed in the United Kingdom. 83% of cases occur in males, reflecting occupational exposure to asbestos. The UK has the highest incidence of mesothelioma worldwide and the incidence is predicted to peak in the UK in 2020. Worldwide however, the incidence will continue to rise as asbestos is still mined and used in industry in many countries outside of Europe and Australia. It is not yet clear if inhalation of carbon nanotubes, increasingly used in the fields of electronics and medicine, causes similar sequalae as inhalation of asbestos fibres (Jaurand et al., 2009).

1.1.1 Aetiology

Asbestos is the name of a group of naturally occurring minerals that have been used by man for thousands of years. In the 20th century, asbestos was extensively used in many industries, as it is inexpensive, fire-resistant and durable. Asbestos exposure is associated with 80% of cases of mesothelioma.

Types of asbestos include those with serpentine fibres such as chrysotile (white asbestos), which accounts for 90% of asbestos used and amphibole fibres such as crocidolite (blue asbestos) and

amosite (brown asbestos) (Mossman et al., 2013). The UK was once the highest importer of amosite asbestos in the world. Recognition of the harmful effects of asbestos exposure occurred as early as 1918 by the US government. Despite research evidence and mainstream acceptance that asbestos was carcinogenic, bans on the use of asbestos lagged behind by several decades. In the UK, amphibole asbestos was banned in1985 and chrysotile asbestos was banned in 1999. Although asbestos is banned in over 50 countries, a worldwide ban has not been passed and asbestos is still used extensively in China, Russia, Canada, the United States and India.

All types of asbestos can cause mesothelioma, however, amphibole fibres have been associated with a higher incidence of mesothelioma (Gibbs and Berry, 2008). Particular properties of the asbestos fibres affect tumourigenicity: Long thin, amphibole asbestos fibres are the most tumorigenic (Stanton et al., 1981) with long-term persistence in tissue, once inhaled. Persistence of asbestos fibres generates on-going inflammation that contributes to a tumorigenic environment (Coussens and Werb, 2001).

Although the majority of cases of mesothelioma are associated with asbestos exposure, there is also a subset of patients (20-25%) with no known asbestos exposure (Jasani and Gibbs, 2012). Recognised causes of mesothelioma include exposure to non-asbestos mineral fibres such as erionite, radiation and genetic mutations, all of which in one way or another lead to transformation of mesothelial cells.

1.1.2 Pleural space

Physiologically, a small volume of pleural fluid exists between the visceral and parietal pleura. This acts as a lubricant allowing frictionless movement of the lungs during respiration. Many diseases including primary and secondary malignancies are associated with a pleural effusion, which is an accumulation of pleural fluid in the pleural space.

Pleural fluid accumulates by a combination of increased fluid production and impaired lymphatic outflow (Stathopoulos and Kalomenidis, 2012). A suggested pathway of increased pleural fluid production is an interaction between tumour cells and the host vasculature and immune cells resulting in enhanced plasma extravasation into the pleural space (Stathopoulos and Kalomenidis, 2012). The presence of a pleural effusion has been shown at autopsy to be best predicted by blockage of the mediastinal lymph nodes rather than the extent of pleural involvement (Meyer, 1966).

94% of pleural malignancies have an associated pleural effusion (Woolhouse et al., 2018). Mesothelioma is the most common primary malignancy of the pleura and associated with a pleural effusion in 90% of cases.

70% of patients with mesothelioma develop pleural effusions containing malignant cells (Sneddon et al., 2018).

Pleural fluid is easily accessible and pleural fluid cytology is accepted as a basic investigation for patients with a pleural effusion. The mean sensitivity of cytology in a malignant pleural effusion is 60% but this is decreased in mesothelioma (Roberts et al., 2010). The reliability of diagnosis of mesothelioma on cytology is variable, with a mean sensitivity of 16-73% (Segal et al., 2013, Woolhouse et al., 2018). A biopsy is advocated for diagnosis but sometimes cytology is adequate.

1.1.3 Mesothelial cells

Mesothelial cells form the single cell surface of the mesothelium and regulate serosal response to injury and infection (Mutsaers, 2002). The mesothelium is a complex organ, which functions as a protective barrier and a low-friction interface to allow organs to move freely (Mutsaers, 2002). Mesothelial cells play an active role in inflammation and secrete pro and anti-inflammatory mediators such as nitric oxide, reactive oxygen species, cytokines, growth factors, chemokines and prostaglandins with a view to maintaining or restoring tissue homeostasis (Mutsaers, 2002, Antony et al., 1995, Choe et al., 1998). Mesothelial cells also play a role in fluid and cell transport and tissue repair (Mutsaers, 2002).

Inflammation, activation of resident cells, a chemotactic gradient and upregulation of adhesion molecules are required for leucocytes to migrate to the serosal space (Topley et al., 1996). A specific immune response requires presentation of foreign antigen by MHC class II molecules, usually present on professional antigen presenting cells (APCs), to T helper (Th) cells. Non-professional APC such as endothelial cells, keratinocytes and mesothelial cells have also been shown to be able to express MHC class II and present antigen following cytokine stimulation (Sprent, 1995, Nickoloff and Turka, 1994). Mesothelial cells can act as the sole antigen presenting cells and, in the presence of IFNγ, can induce the proliferation of previously activated memory/effector CD4+ Th cells (Valle M et al., 1995). High levels of ICAM-1 (intercellular adhesion molecule 1) are present on the surface of non-professional APCs such as mesothelial cells which acts as an accessory molecule for antigen presentation to memory T cells instead of CD80 and CD86 (Sprent, 1995, Nickoloff and Turka, 1994).

1.1.4 Pathogenesis of malignant transformation

Inhaled asbestos fibres migrate from the lung to the pleural space (Holt 1983) and accumulate in the visceral and parietal pleura (Boutin et al., 1996, Jaurand and Fleury-Feith, 2005). Here, the fibres are taken up by phagocytic cells and mesothelial cells, which subsequently die due to the physical damage caused by the fibres, leading to a new iteration of phagocytosis and cell death. The net effect is repetitive tissue damage and repair, and chronic inflammation (Sekido, 2013).

The exact mechanism of carcinogenesis of asbestos is not known but there are several hypotheses. These can be divided into factors that initiate and those that promote a neoplastic process.

Possible mechanisms for initiation of carcinogenesis include:

1) - Reactive oxygen species (ROS) generated on the surface of asbestos fibres lead to DNA damage such as base hydroxylation and strand breaks. ROS are produced by macrophages when unable to digest the phagocytosed asbestos fibres (Sekido, 2013, Liu et al., 2000, Wang et al., 1987).

2) - Mesothelial cells phagocytose asbestos fibres (Jaurand et al., 1979). Asbestos fibres inside the mesothelial cells physically impinge on mitotic spindles and disrupt the cell cycle with chromosome mis-segregation (Sekido, 2013, Yegles et al., 1993). Interference with chromosomes or mitotic spindles may result in chromosomal structural abnormalities and aneuploidy of mesothelial cells (Sekido, 2013). Asbestos fibres have also been shown to cause chromosome damage (Wang et al., 1987), most likely again by physically interfering with DNA repair complexes. Unlike macrophages, mesothelial cells are less likely to die following phagocytosis of asbestos fibres allowing persistence of these chromosomal abnormalities.

3) - Asbestos fibres absorb a variety of proteins and chemicals to their surface, which may result in accumulation of additional carcinogens such as benzo(a)pyrene from cigarette smoke (Sekido, 2013, Mossman et al., 1984). Asbestos fibres also bind important cellular proteins such as chromatin binding proteins, RNA binding proteins, cytoprotective proteins amongst many others and the deficiency of such proteins may be detrimental to the function of mesothelial cells (Sekido, 2013, Nagai et al., 2011).

A number of factors both initiate and promote a neoplastic process:

4) – Asbestos-exposed mesothelial cells and macrophages release a variety of cytokines and growth factors that induce inflammation in response to injury by asbestos fibres. Inhalation studies of asbestos fibres have found they induce an acute inflammatory response, followed by mesothelial cell proliferation (Mossman et al., 2011). As the asbestos fibres are unable to be eliminated by phagocytosis, a continuous cycle of chronic inflammation ensues. Persistently activated macrophages release TNFα, which inhibits mesothelial cell death and as a consequence, increases the risk of malignant transformation (Yang et al., 2006). Asbestos activates the MAP kinase pathway leading to a cascade of phosphorylation and expression of specific genes involved in the production of inflammatory factors that may play a role in cancer progression (Zanella et al., 1996, Jaurand and Fleury-Feith, 2005). Additionally, when mesothelial cells undergo necrotic cell death following asbestos exposure, they release high mobility group box 1 protein, which also promotes an inflammatory response (Yang et al., 2010).

It is likely that a combination of factors that initiate and promote the carcinogenic process over many years lead to malignant transformation of mesothelial cells to mesothelioma and formation of the tumour promoting microenvironment.

Features common to most cancer development, so called Hallmarks of Cancer, also contribute to mesothelioma development (Hanahan and Weinberg, 2011). This includes self-sufficiency in growth signals such as PDGFA and TGFβ in mesothelioma cells, insensitivity to growth inhibitory signals by inactivation of molecules such as p14 and p16 that are important in the Rb and p53 tumour suppressor pathway so allow unchecked tumour growth, evasion of apoptosis by elevated levels of anti-apoptotic molecules in mesothelioma (Broaddus et al., 2005), immortalisation of the cell by expression of telomerase in 90% of mesothelioma cells (Dhaene et al., 2000) , sustained angiogenesis by production of VEGF by mesothelioma cells and tissue invasion (Hanahan and Weinberg, 2011, Robinson and Lake, 2005).

1.1.5 Genetic factors contributing to the incidence of mesothelioma

Less than 5% of workers with significant exposure to asbestos go on to develop mesothelioma (Below et al., 2011, Burdorf et al., 2003, Sekido, 2013, Sluis-Cremer et al., 1992) leading to the hypothesis that mesothelioma occurs in genetically pre-disposed individuals exposed to a causative agent.

1.1.6 Genomics and epigenetics of mesothelioma

Mesothelioma is mainly caused by chronic inflammation and reactive oxygen species that promote DNA damage (strand breaks, crosslinks and double strand breaks) leading to genomic instability and epigenetic alterations (Sage et al., 2018). The impact of reactive oxygen species can be amplified by germline mutations affecting the ability of the cell to repair DNA damage (Sage et al., 2018). In mesothelioma, chromosome 22 is frequently lost along with rearrangements in the 1p, 3p, 9p and 6q regions (Robinson and Lake, 2005). Somatic mutations in CDKN2A and NF2 and copy number alterations are caused by genomic instability in mesothelioma (Sage et al., 2018).

Epigenetic differences have been found between mesothelioma and normal pleura such as over-expression of key epigenetic regulators such as EZH2 and SUZ1q (Christensen et al., 2009, McLoughlin et al., 2017). These are associated with poor survival in mesothelioma (Christensen et al., 2009, McLoughlin et al., 2017).

BAP1 (BRACA1 associated protein-1), CDKN2A/ARF (cyclin-dependent kinase inhibitor 2A/alternative reading frame) and NF2 (neurofibromatosis type 2) are the most frequently mutated tumour suppressor genes in mesothelioma (Sekido, 2013). A specific mutation in a single common driver gene has not been found and it is likely that a combination of mutations accumulated over many years leads to the development of mesothelioma (Hylebos et al., 2016).

1.1.6.1 BAP1

BAP1 is localised to chromosome 3p21.1 and has been shown to be an important tumour suppressor gene in mesothelioma. BAP1 has been implicated in chromatin dynamics, DNA damage response and regulation of cell cycle and growth (Eletr and Wilkinson, 2011).

Mutations can be inherited or acquired. Inherited mutations within BAP1 are rare (Melaiu et al., 2018)however 58% of histology samples and 59% of cytology samples from patients with mesothelioma were found to have a BAP1 mutation (Pulford et al., 2017).

BAP1 is localised at the endoplasmic reticulum and modulates calcium release into the cytosol and mitochondria, which promotes apoptosis of the cell. Reduced levels of BAP1 cause reduction in calcium flux preventing apoptosis in damaged cells (Bononi et al., 2017). As a result, more asbestos-damaged cells survive, leading to a higher rate of neoplastic transformation.

BAP1 germline mutant carriers have a high frequency of cancers, including mesothelioma, lung cancer and uveal melanoma. However, on average, these patients survive longer than patients in the BAP-WT equivalent cohorts (Baumann et al., 2015).

BAP1 inactivation may be also be important as an epigenetic regulator (Yap et al., 2017).

1.1.6.2 CDKN2A

The CDKN2A gene on chromosome 9 encodes proteins that regulate cell cycle regulatory pathways, p53 and RB1. CDKN2A is frequently deleted or mutated in many cancer cells and has been found in 70% of mesotheliomas. In 2 of 6 families with a predisposition to mesothelioma and melanoma, a BAP1 mutation or CDKN2A germline mutation was found suggesting CDKN2A mutations might also contribute to rare familial cancer syndromes (Betti et al., 2016). CDKN2A encodes p16 and p14. Loss of p14 in mesothelioma contributes to p53 repression (Yap et al., 2017, Urso et al., 2016, Walter et al., 2015), loss of p16 prevents apoptosis of mesothelioma cells as p16 inhibits CDK4 and CDK6 that are required for phosphorylation and activation of the Rb protein that promotes progression from G1 to S in the cell cycle (Yap et al., 2017).

1.1.6.3 NF2

The NF2 gene is located in the chromosomal region 22q12 and encodes Merlin, a tumour suppressor gene inactivated in 40% of mesotheliomas (Sato and Sekido, 2018). Merlin inhibits the Hippo pathway and negatively regulates focal adhesion kinase (FAK), which is a protein kinase that controls cell adhesion, invasion and migration (Sato and Sekido, 2018). FAK is overexpressed in cancer and recruits immunosuppressive cells into the TMA. Merlin inhibits FAK phosphorylation at Try397, which disrupts the interaction of FAK with p85 on PI3K and SRC (Yap et al., 2017, Poulikakos et al., 2006). Inactivation of Merlin may sensitise mesothelioma cells to FAK inhibition as weak cell-cell adhesions increase dependence on cell-extracellular matrix induced FAK signalling (Yap et al., 2017).

The rate of NF2 mutations is highest in Sarcomatoid mesothelioma and lowest in epithelioid tumours (Bueno et al., 2016). This suggests that NF2 mutations may be involved in the epithelial-mesenchymal transition (Sato and Sekido, 2018). It has been shown that the restoration of NF2 expression in NF2 deficient mesothelioma cells significantly inhibited their growth (Sato and Sekido, 2018).

1.1.7 Surrogate markers

It has been established that pleural effusions contains immune cells and cytokines that vary between diseases and tumour types.

More recently, it has been shown that the same principle mutations that are found in mesothelioma solid tumours, such as BAP1, CDKN2A and NF2 alterations, were also found in the

short-term tumour cell cultures from pleural effusions associated with mesothelioma (Sneddon et al., 2018).

Pleural fluid is an attractive surrogate option for monitoring the tumour environment in mesothelioma. It is accessible and can be sampled repeatedly to allow assessment of tumour progression and response to treatment (Sneddon et al., 2018).

There is increasing evidence that surrogates such as circulating tumour DNA in blood might be usable to monitor disease response in many cancers. The proximity of pleural fluid to the tumour and presence of tumour cells in a large number of mesothelioma-associated pleural effusions arguably makes the pleural fluid a more representative surrogate than blood. However, to date it is not clear how closely the associated pleural effusion reflect the solid mesothelioma tumour.

1.1.8 Treatment

1.1.8.1 Chemotherapy

Chemotherapy with pemetrexed and cisplatin is recommended by NICE for patients with previously untreated mesothelioma. Median overall survival time with this combination first line treatment was 12.7 months (Ceresoli et al., 2006). Where licensed, bevacizumab (anti-VEGF) may be added to this regime (Woolhouse et al., 2018). There are no licensed drugs for patients with previously treated mesothelioma and patients are usually given a combination of single or dual agent regimens.

1.1.8.2 Radiotherapy

There is a role for palliative radiotherapy for symptom control for localised pain in mesothelioma. Previously, prophylactic radiotherapy was given to the chest wall of patients with mesothelioma at the site of any tract from thoracoscopic surgery to prevent tumour growth through the tract; however, the PIT trial demonstrated no role for this treatment. There is little evidence for hemithorax radiotherapy (Woolhouse et al., 2018).

1.1.8.3 Surgery

Extra-Pleural Pneumonectomy (EPP) was previously conducted for a select group of patients with early stage mesothelioma and good performance. However, recent meta-analysis has shown there is no survival benefit and recent guidelines do not recommend EPP (Woolhouse et al., 2018). An extended pleurectomy decortication procedure is not recommended outside of a clinical trial; the MARS2 trial is currently underway, comparing extended pleurectomy decortication following chemotherapy vs chemotherapy alone.

1.1.8.4 Immunotherapy

The large phase IIb DETERMINE study of tremelimumab, an anti-CTLA4 antibody, demonstrated no improvement in overall survival in mesothelioma (Maio et al., 2017).

PDL1 is expressed in 40% of mesotheliomas. PDL1 is associated with non-epithelioid histology and worse survival (Thapa 2017). A phase 1b clinical trial of Pembrolizumab (anti-PD1 antibody) reported a 20% response rate and median overall survival of 18 months (Alley et al., 2017).

Currently, in the UK, there are several clinical trials of immunotherapy for mesothelioma underway. The Confirm study is a phase IIb/III trial recruiting patients 2:1 to nivolumab, (anti-PD1 antibody) vs placebo (Fennell et al., 2018) A trial of an oral FAK inhibitor was discontinued in 2015 but the FAK-PD1 study, a phase I/IIa study of a combination of FAK (Defactinib) and PD1 (Pembrolizumab) inhibitors and is open to patients with Mesothelioma, NSCLC and pancreatic cancer (NCT02758587).

1.2 Cancer Immunology

Tumours are not simply collections of malignant cells, but comprise a complex mix of cancer cells that co-locate and interact with cells that make up the tumour microenvironment (TME). These include innate and adaptive immune cells, endothelial cells and fibroblasts (Balkwill et al., 2012). Numerically, cancer cells may be far outnumbered by other cells in the TME (Hadrup et al., 2013).

In many solid cancers, there is abundant evidence of the key role that immune responses against the tumour play in determining the progress and outcome of the cancer. During the generation of cancerous change, new antigens are frequently generated, either derived from oncogenic viruses such as human papilloma viruses or as a result of the mutations occurring during the malignant transformation and progression. When confronted by such new antigens, the immune system is likely to generate an adaptive anti-tumour response that may comprise a range of effector mechanisms including cytotoxic T lymphocytes and antibodies. In addition to the adaptive immune response, the components of the innate immune response can also make important contributions to the anti-cancer defences. Thus, macrophages can adopt various phenotypes that may either have anti-cancer effects (so-called M1 macrophages) or they may exert tumour-supporting effects (so-called M2 macrophages). Another important cellular element in anti-tumour defences are the Natural Killer (NK) cells. These cells can kill tumour cells not through recognition of tumour-associated neo-antigens, but through interaction with a variety of other membrane-associated receptors and molecules. The overall effectiveness of the anti-tumour immune response is the resultant between the effectiveness of the anti-tumour

cytotoxic immune mechanisms and the tumour's wide range of protective escape mechanisms aimed at avoiding detection by the immune system or aimed at actively suppressing any immune effector mechanisms.

1.2.1 Generation of a new/primary immune response

Protein antigens must be taken up by specialized, so-called "professional" antigen presenting cells (APC). These cells are members of the dendritic cell family. They have highly developed properties by which they process potentially antigenic proteins into forms that can be presented to naïve CD4+ helper T cells – the first components of the complex inter-cellular interaction that generates the full immune response. Potentially antigenic proteins are endocytosed into vesicles to which lysosomal enzymes are added (phagolysosomes). The proteins are digested by proteases to form a "soup" of peptides; newly synthesized MHC molecules from the endoplasmic reticulum are contained in endosomes that fuse with the phagolysosomes. Following addition of the newly synthesized MHC molecules to the peptide mixture, appropriately structured peptides are taken into the groove that is a central part of the MHC molecular structure. The peptide-loaded MHC molecules are then cycled out to the surface of the APC where they are available for presentation to naïve T cells.

It is classically accepted that this is the route by which antigens/proteins that originate outside the cell are processed and the MHC molecules into which the peptides are loaded are the MHC Class II. When antigens/proteins arise within the cell, such as from viruses or cancer cells, they are processed slightly differently, being broken down by the proteolytic mechanisms of the proteasomes and the resulting peptides are loaded into MHC Class I molecules. It is now also recognized that antigenic peptides that arise within the cell can get loaded onto MHC Class II – a process called "cross-presentation".

During the processing of the antigen-containing proteins, the APC becomes activated and it migrates to the regional draining lymph nodes. On the journey, while "processing" the antigenic protein/polypeptide, it undergoes a "maturation" which involves up-regulation of the expression of a set of key molecules on the cell surface. These include antigen-presenting MHC molecules and so-called co-stimulatory molecules (CD80, CD86, CD40 and others). These molecules and receptors are a vital part of the essential and powerful signalling system that the APC needs to give to activate naïve T cells. Upon entering the regional lymph node, the activated APCs enter the paracortical area of the node where they traffic among the naïve T cells "presenting" their load of MHC-associated antigens for "inspection" and recognition by the T cell antigen receptor (TCR). A critical aspect of antigen-presentation is that peptides loaded into MHC Class II

molecules are recognized only by T cells that express the CD4 molecule in association with their TCR – CD4+ T cells; peptides associated with MHC Class I are only recognized by T cells that express the CD8 molecule in association with their TCR – CD8+ T cells.

Recognition and binding of the MHC-bound peptide by the naïve T cell gives it the first of 3 major activation signals that it requires to be awakened from its "slumbering or catatonic state". The second signal is delivered via the co-stimulatory molecules, which interact with their ligands expressed by the T cell. Thus, CD80 and 86 on the APC interact with CD28 on the T cell; CD40 on the APC interacts with CD40L on the T cell, and so on for the many activating co-stimulatory molecules. The precise combination of interactions and the affinities with which the receptors interact, initiates various possible differentiation pathways in the awakening naïve T cell.

The third signal is delivered in the form of various possible cytokines. For example, IL12, IFNγ, IL4, TGFβ may be produced by the APC, and sometimes by adjacent T cells. The exact composition of the cytokines present as signal three, is crucially influential in directing the differentiation pathway down which the awakening CD4+ T cell will progress.

1.2.2 T cell differentiation pathways

Naïve CD4+ T cells are referred to as Th0 cells because they are precursors that have yet to follow any differentiation pathway. The first function of CD4+ T cells, after activation by interaction with antigen presented by APCs, is that of helper cells. They can "help" other T cells to mature, such as CD8+ cells, and they can also help B cells to produce antibodies of the various classes. In addition, however, CD4+ T cells can follow a number of differentiation pathways, depending on the combinations of co-stimulatory and cytokine signals to which they are exposed.

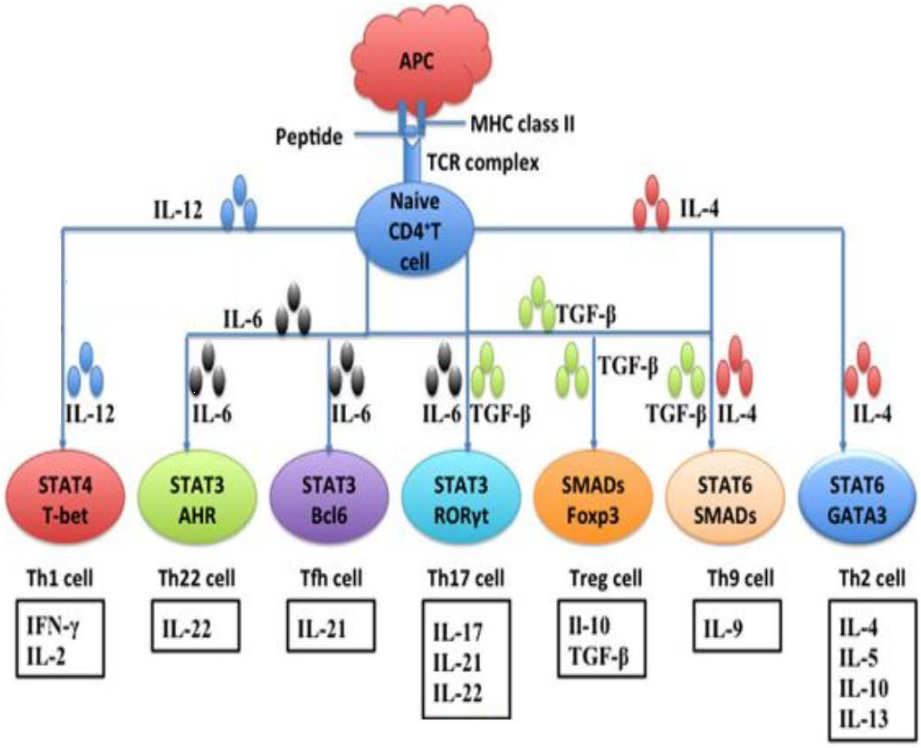


Figure 1: T-cell activation and differentiation. Adapted from (Ren et al., 2017).

The subpopulations of CD4+ T helper cells (Th1, Th2, Th9, Th17, Th22, Tfh), T regulatory cells, memory T cells and cytotoxic CD4+ T cells will be discussed.

1.2.3 CD4+ T cells

1.2.3.1 Th1

Th1 cells, characterised by strong expression of IFNγ, are pro-inflammatory and important in protection against intracellular pathogens like viruses. Excess Th1 cell activity can cause tissue damage, autoimmune and inflammatory diseases (Wan and Flavell, 2009).

Th1 cells are classically recognised as anti-tumourigenic due to their role in activation of cytotoxic CD8+ T cells, macrophages and NK cells (Wan and Flavell, 2009, Vella, 1994, Vegran et al., 2015).

Naïve Th0 cells differentiate towards a Th1 phenotype through contact with IFNγ, which is produced by activated NK cells, and IL12, which is secreted primarily by dendritic cells (Ivanova and Orekhov, 2015). Together, IFNγ and IL12 induce T-bet (T-box 21) expression, the master transcription factor of Th1 cells (Ivanova and Orekhov, 2015, Muranski and Restifo, 2013, Wan

and Flavell, 2009, Nurieva and Chung, 2010). The Th1 phenotype is relatively stable as T-bet can induce its own expression either directly or indirectly via a positive feedback loop(Ivanova and Orekhov, 2015). T-Bet can suppress GATA-3, the alternative transcription factor responsible for Th2 differentiation (Ivanova and Orekhov, 2015).

Th1 cells are characterised by secretion of IFNγ and IL2, along with pro-inflammatory cytokines such as TNFα and TNFβ (Wan and Flavell, 2009). IFNγ is essential for the activation of macrophages, CD8+ T cells and NK cells (Wan and Flavell, 2009, Vella, 1994, Vegran et al., 2015). IFNγ also induces MHC class II expression on cells that do not usually do so, such as epithelial cells, thus enabling them to act as APCs (Vella, 1994). IL2 supports the primary antigen response by promoting proliferation of both the Th1 cells and cytotoxic CD8+ T cells, and also the secondary immune response by promoting development of memory CD8+ cells. In addition, IL-2 supports the differentiation of naïve T cells to express FoxP3 thus promoting the formation of immunosuppressive Tregs to regulate the Th1 pathway (Luckheeram et al., 2012, Li et al., 2017).

Together, the dendritic cells, Th1 and cytotoxic cells create a tumour suppressive environment leading to control or elimination of the tumour (Disis, 2010).

The predominant Th phenotype in mesothelioma is not yet known.

Reduction in Th1-related IFNγ production and chemokine receptor CXCR3 expression has been observed in cell lines chronically exposed to asbestos (Maeda et al., 2011). This suggests that chronic asbestos exposure may reduce anti-tumour Th1 activity (Maeda et al., 2011) and contribute to tumour escape in mesothelioma.

1.2.3.2 Th2

Th2 cells are characterised by the production of IL4, IL5 and IL13 and are mainly involved in humoral responses to extracellular organisms or parasites and development of atopy and allergic reactions. Th2 cells are also important for lung mucosal immunity and they "help" B cells to make antibodies, IgE class being particularly involved in anti-parasite immunity and allergic responses (Wan and Flavell, 2009).

Th2 cells are mainly pro-tumourigenic through production of cytokines that promote tumour growth (Vegran et al., 2015) as well as not producing IFNγ, so are unable to activate innate and CD8+ T cells to mount an anti-tumour response (Ruffell et al., 2010).

Naïve Th0 cells differentiate towards a Th2 phenotype in the presence of IL4. The cell that initially produces IL4 has not yet been identified. GATA-3 is the master transcription factor for Th2 differentiation and is up regulated by IL4 (Ivanova and Orekhov, 2015, Muranski and Restifo, 2013, Wan and Flavell, 2009). Similar to T-bet in Th1 cells, GATA-3 forms its own positive feedback loop (Wan and Flavell, 2009, Ivanova and Orekhov, 2015).

IL4 plays a major role in allergic inflammation as well as inducing other pro-inflammatory mediators like IL6 and GM-CSF (Luckheeram et al., 2012). Initially, IL4 may be able to improve anti-tumour immunity by promotion of acute inflammation and leucocyte recruitment before the development of a pro-tumourigenic chronically inflamed tumour microenvironment (Kemp and Ronchese, 2001, Ruffell et al., 2010). IL4 may also contribute to tumour promotion by direct immunosuppressive effects on CD8+ T cells as seen in a murine model (Ruffell et al., 2010, Kemp and Ronchese, 2001).

IL4 and IL5 are required for the activation and proliferation of B cells respectively.

Both IL4 and IL13 induce M2 TAMs, causing immunosuppression by up regulating immunosuppressive cytokines and reducing inflammatory cytokines, driving tumour immune tolerance and neo-angiogenesis (Ruffell et al., 2010, Vegran et al., 2015, Dong, 2017). IL4 induces cathepsin activity in TAMs promoting angiogenesis, growth and invasion of tumour cells (Dong, 2017, Gocheva et al., 2010). IL4 induced M2 TAMs may also potentiate metastasis by activation of EGFR signalling in malignant epithelial cells (Dong, 2017, DeNardo et al., 2009).

The type II IL4 receptor binds both IL4 and IL13 and is expressed by many non-haemopoetic cells such as epithelial cells and so may affect tumour growth independent of the immune system (Ruffell et al., 2010, Murata et al., 1999).

The TAMs and B cells in the Th2 environment promote infiltration of Tregs to the TME. Tregs secrete TGFβ and inhibit the generation of CTLs thus contributing to the pro-tumourigenic TME (Disis, 2010).

The presence of Th2 cells in the TME has been associated with increased metastasis and worse survival in some cancers (Chang et al., 2015).

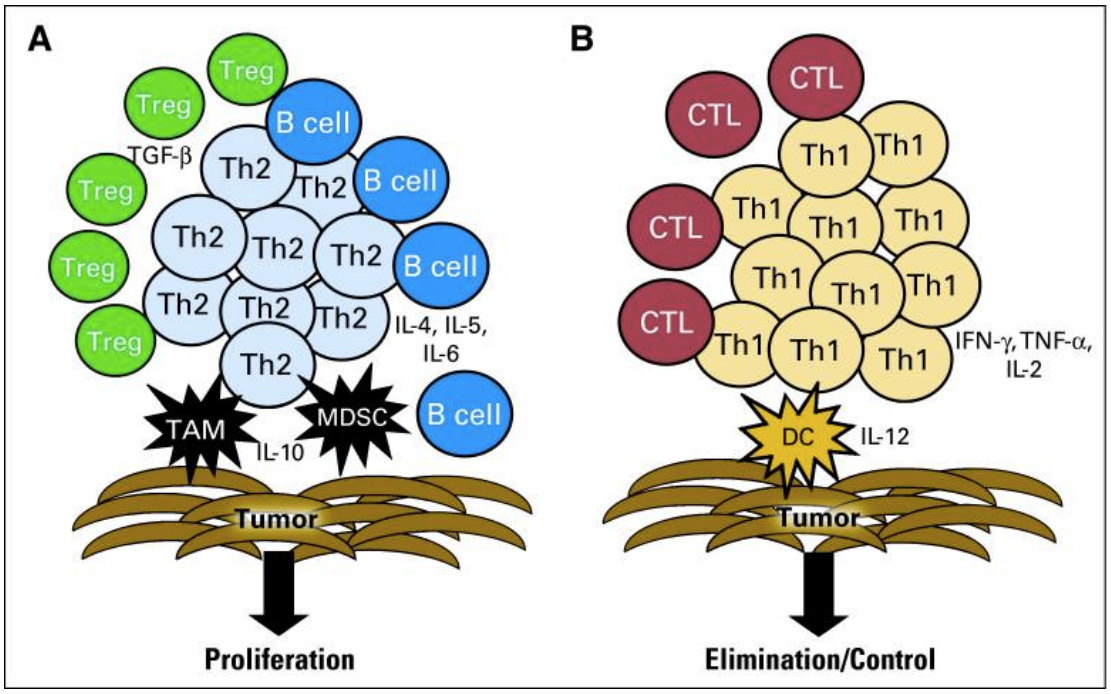


Figure 2: Th2 and Th1 tumour specific immunity. Figure from (Disis, 2010).

1.2.3.3 Th9

Th9 cells confer protection against helminth infections. They are associated with allergy, inflammatory and autoimmune diseases.

Th9 cells are anti-tumourigenic via the production of granzyme B and cytokines in solid tumours (Kaplan 2015).

Th9 cells differentiate via stimulation of naïve T cells with IL4 and TGF-β, or by stimulation of Th2 cells with TGF-β (Muranski and Restifo, 2013, Ivanova and Orekhov, 2015, Purwar et al., 2012, Vegran et al., 2015). Th9 cells were initially thought to be a subtype of Th2 cells but have been shown to be a distinct subset of cells. Th9 differentiation and IL9 production is inhibited by IFNγ and indirectly through the induction of IL27 from dendritic cells (Murugaiyan et al., 2012).

Th9 cells are characterised by IL9 and IL21 production (Vegran et al., 2015, You et al., 2017). During Th9 differentiation, these cells express IL9 and cease to express IL4, IL5 and IL13, the cytokines associated with a Th2 phenotype (Vegran et al., 2015, Purwar et al., 2012). Th9 cells are not the only cells that secrete IL9, which can also be secreted by Th2, Th17 and Treg cells (Vegran et al., 2015).

The master transcription factor for Th9 cells has not yet been identified.

The role of Th9 cells in cancer immunology is not yet established. The anti-tumour effect of Th9 cells might be explained by the release of cytotoxic granzyme B; IL9 dependent effects such as inducing mast cell cytotoxicity, promoting DC survival and activating epithelial cells to produce CCL20 which attracts DCs and CD8+ T cells into the tumour bed; and IL21 dependent effects such as inducing IFNγ production by NK cells and CD8+ T cells, promoting CD8+ T cell proliferation and increasing NK cell cytolytic activity (Vegran et al., 2015).

1.2.3.4 Th17

Th17 cells are important in acute and chronic tissue inflammation, and protect against a variety of predominantly extracellular bacterial and fungal pathogens in the epithelial and mucosal barriers (Dong, 2017, Ivanova and Orekhov, 2015, Muranski and Restifo, 2013). They have been implicated in autoimmune disease.

Differentiation towards a Th17 subset allows these cells to acquire properties of the stem memory T cells (T_{SCM}) which can efficiently form memory, generate more differentiated progeny, self-renew and are long-lived (Muranski and Restifo, 2013). Th17 cells are plastic and can initially provide an inflammatory response by recruiting myeloid cells then switch to a cytotoxic Th1-like phenotype (Muranski and Restifo, 2013). They are also able to switch to other phenotypes including Th2, Treg and Tfh (Guery and Hugues, 2015).

The role of Th17 in cancer is controversial (Muranski and Restifo, 2013). Both pro and anti-tumour properties have been described. Th17 cells infiltrate tumours and their concentrations are highly elevated in comparison with surrounding tissues, implying a specific role in tumours (Ivanova and Orekhov, 2015).

Th17 cells are polarized from naïve Th0 cells by IL6, IL21, IL23 and TGF-β (Hirahara et al., 2010, Ivanova and Orekhov, 2015, Wan and Flavell, 2009). The master transcription factor of Th17 cells is retinoic acid-related orphan receptor γ (RORγ) (Muranski and Restifo, 2013, Wan and Flavell, 2009, Ivanova and Orekhov, 2015).

Th17 cells produce IL17A, IL17F, IL21, IL22. IL17 induces pro-inflammatory cytokines such as IL6, IL1 and TNFα and pro-inflammatory chemokines. IL17R is widely expressed by immune and non-immune cells and IL17 has effects beyond immune mediated responses (Muranski and Restifo, 2013).

IL21 is a self-amplifying cytokine for Th17 differentiation and also activates T cells and NK cells, and induces B cell differentiation into plasmocytes and memory cells (Luckheeram et al., 2012).

Th17 cells are regulated by innate system-derived pro-inflammatory cytokines such as IL23, IL6, and IL1 (Dong, 2017). Th17 cell development and function is highly regulated by IL23, which is important for Th17 survival, inflammatory potential and is highly expressed in many tumours (Ivanova and Orekhov, 2015, Langowski et al., 2006, Dong, 2017).

Th17 cells are commonly found in precancerous and cancerous lesions (Muranski and Restifo, 2013). Th17 cells may cause tissue damage by engagement with the antigen-specific target, or from recruitment of macrophages and neutrophils into the TME (Muranski and Restifo, 2013).

Th17 cells can promote a pro-tumourigenic TME in chronically inflamed tissue by IL17 induced myeloid derived suppressor cell (MDSC) recruitment and angiogenesis (Chang et al., 2014, Dong, 2017). Th17 cells demonstrate plasticity to a Treg phenotype, thus contributing to an immunosuppressive and pro-tumour TME (Guery and Hugues, 2015).

Conversely, Th17 cells can contribute to an anti-tumour TME by recruitment of NK cells, CD8+ T cells, Th1 cells and DCs into the inflammatory TME and activation of CD8+ T cells (Guery and Hugues, 2015). Th17 cells also demonstrate plasticity to a Th1 phenotype, which promotes anti-tumour IFNγ and TNFα mediated killing (Guery and Hugues, 2015).

Th17 cells have been reported in many types of cancers with varying impact on survival (Dong, 2017). In colon and pancreatic cancer, increased Th17 cells were associated with poor prognosis (Bailey et al., 2014). In patients with early stage ovarian cancer and malignant pleural effusions increased Th17 cells were associated with better survival (Dong, 2017, Kryczek et al., 2009).

Mesothelial cells express IL17 receptors and work on mesothelioma cell lines has shown that mesothelioma cells create an environment that strongly polarizes macrophages and CD4+ T cells to produce IL17. Here, IL17 exerts a strongly proliferative effect on the mesothelioma cell, favouring its survival (Izzi et al., 2013).

1.2.3.5 Th22

Th22 cells have been identified as a distinct lineage and are present in inflammatory conditions such as psoriasis, allergic airway disease and autoimmune diseases. Th22 cells have also been identified in cancer.

Th22 cells differentiate from naïve Th0 cells influenced by TNFα and IL6 (Tamasauskiene and Sitkauskiene, 2017). The master transcription factor for Th22 cells is not known.

Th22 cells are mainly characterised by their production of IL22. Other cells such as Th1, Th2, Th17, CD8+ T cells and NK cells also produce IL22 (Ivanova and Orekhov, 2015). Th22 cells do not produce IFNγ, IL4 or IL17A thus differentiating them from Th1, Th2 and Th17 cells respectively (Muranski and Restifo, 2013, Eyerich et al., 2009). Th22 cells also express CCR10, CCR4 and CCR6 (Muranski and Restifo, 2013, Duhen et al., 2009).

IL22 is a member of the IL10 family; it mediates host defence against pathogens and influences multiple cell types like epithelial cells and fibroblasts (Ivanova and Orekhov, 2015). IL22 primarily affects epithelial cells, fibroblasts and stromal cells that possess an IL22 receptor.

In tuberculous pleural effusions, Th22 cells were stimulated possibly due to pro-inflammatory cytokines and chemokines produced by pleural mesothelial cells (Ye et al., 2012). Pleural mesothelial cells were able to act as antigen presenting cells to stimulate CD4+ T cell proliferation and Th22 differentiation (Ye et al., 2012).

Increased Th22 cells and IL22 have been shown to be associated with various tumours such as lung and colorectal, and to be associated with tumour cell proliferation (Ivanova and Orekhov, 2015, Kobold et al., 2013, Huang et al., 2015).

The role of Th22 cells in mesothelioma is not yet known but they may play a role due to the ability of IL22 to promote regeneration of epithelial cells following injury but may also contribute to carcinogenesis by inducing proliferation and inhibiting apoptosis of epithelial cells (Dudakov et al., 2015).

1.2.3.6 T follicular helper cells

Tfh cells play an important role in mediating humoral immunity through interacting with B cells. Within germinal centres, Tfh cells help B cells proliferate and somatically mutate to produce long-lived, high-affinity plasma cells for antibody production and memory B cells (Crotty, 2014, Ivanova and Orekhov, 2015, Qi, 2016).

Tfh cells differentiate from naïve Th0 cells in the presence of IL6 and IL21. IL6 is produced by DCs and induces IL21 production by naïve Th0 cells in an autocrine manner (Dienz et al., 2009). IL6 and IL21 are required to upregulate Bcl6, the master transcription factor of Tfh cells. Bcl6 suppresses the transcription factors required for Th1, Th2 and Th17 differentiation.

Tfh cells are characterized by the expression of CXCR5, ICOS, PD1, IL21 and IL4 (Ma et al., 2012, Vijayanand et al., 2012, Wan and Flavell, 2009, Nurieva and Chung, 2010, Jankovic et al., 2010, Nurieva et al., 2008). Bcl6 is required for expression of CXCL5 (Nurieva and Chung, 2010, Crotty, 2014). CXCR5 expression is required for the early Tfh cells to home to the border of the B cell follicle, attracted by the expression of its ligand, CXCL13 from follicular DCs or stromal cells in lymphoid organs (Ohmatsu et al., 2007, Nurieva and Chung, 2010, Crotty, 2014).

Here, the early Tfh cells interact with activated B cells presenting antigen, and deliver help to the B cells resulting in their differentiation into short lived extra-follicular plasmablasts or their migration in the follicles to form GCs (Ma et al., 2012).

Tfh cells are critical for GC formation and function, they regulate GC size, restrict low-affinity B cell entry into the GC, support high affinity B cell occupancy of the GC and select high affinity B cells during affinity maturation (Crotty, 2014, Nurieva and Chung, 2010). Within the GC, Tfh continue to provide help to the B cells, supporting the generation of long-lived plasma cells and memory B cells (Ma et al., 2012). This requires formation of stable conjugates between CD28, ICOS, PD1 and CD40L on the Tfh cells with CD86, ICOS-L, PD-L1 and CD40 on the B cells respectively (Ma et al., 2012). IL4, IL21 and CD40L produced by the Tfh help to maintain GC B cells and induce proliferation (Crotty, 2014). IL4 and IL21 also induce class switching in B cells (Crotty, 2014).

Tfh cells have a high capacity to form long-term transferrable memory (Crotty, 2014, Muranski and Restifo, 2013). On leaving the GC, Tfh cells can become resting memory Tfh cells by upregulating IL7Rα and down regulating Bcl6 (Crotty, 2014, Choi et al., 2013, Kitano et al., 2011, Shulman et al., 2013). 20-25% of central memory (CM) CD4+ T cells are CXCR5+ memory Tfh cells (Crotty, 2014, Chevalier et al., 2011). Memory Tfh cells recall their previous lineage and preferentially reacquire their lineage-specific effector function upon reactivation (Crotty, 2014, Hale et al., 2013). Activated memory B cells induce memory Tfh cells to rapidly re-express Bcl6, demonstrating the close link between Tfh cells and B cells (Crotty, 2014, Ise et al., 2014).

Tfh positively influence the influx of other anti-tumourigenic cells of the adaptive immune system into the TME by the production of CXCL13 (Bindea et al., 2013). Infiltration of tumours by Tfh cells increases with tumour progression and is associated with formation of intratumoural follicular structures and survival in colorectal and breast cancer (Bindea et al., 2013, Vegran et al., 2015, Gu-Trantien et al., 2013).

CXCR5 and CXCL13 expression associated with Tfh cells, were found to be up regulated in T cells from cell lines continually exposed to asbestos which may indicate that Tfh cells play a role in the TME of mesothelioma (Kumagai-Takei et al., 2018).

1.2.3.7 T regulatory cells

Tregs are CD4+ T cells specialized in immune suppression that regulate the potentially harmful effects of Th cells (Sakaguchi et al., 2008, Corthay, 2009). Tregs are important in maintaining immune homeostasis and self-tolerance, controlling autoimmunity, infection, graft-versus-host disease, inflammation and tumour immunity (Chaudhary and Elkord, 2016, Takeuchi and Nishikawa, 2016, Wan and Flavell, 2009).

Different types of Tregs can develop: naturally occurring Treg; nTreg otherwise known as tTregs develop in the thymus with high affinity TCR specificity for self-antigens. Induced Treg; iTreg otherwise known as peripheral Treg (pTregs) are differentiated in the periphery from naïve T cells following TCR stimulation by sub-optimal antigen presentation in the presence of TGFβ and IL2 (Chaudhary and Elkord, 2016, Takeuchi and Nishikawa, 2016, Wan and Flavell, 2009, Lee et al., 2011). Unlike the majority of naïve T cells produced in the thymus, tTregs are mature and "antigen primed" on leaving the thymus (Sakaguchi et al., 2008). The role of tTregs is to maintain tolerance to self and prevent auto-immunity, while pTregs maintain peripheral tolerance at sites of inflammation (Chaudhary and Elkord, 2016, Whiteside, 2015, Sakaguchi et al., 2008, Yadav et al., 2013).

Treg subsets are defined by expression of the FOXP3 transcription factor, their master regulator and the IL2α receptor chain (CD25) (Chaudhary and Elkord, 2016, Wan and Flavell, 2009, Sakaguchi et al., 2008).

Tregs exert their suppressive activity via suppressive cytokines (TGFβ, IL10, IL35), immune checkpoints and inhibitory receptors (PD-1, CTLA-4, TIM-3, LAG-3, ICOS), direct cytotoxicity, IL2 depletion, degradation of ATP and induction of tolerogenic DCs (Chaudhary and Elkord, 2016, Takeuchi and Nishikawa, 2016, Shevach, 2009).

Inhibitory immune checkpoint molecules have a physiological role to prevent excessive T cell activation (Chaudhary and Elkord, 2016). CTLA-4 binds with CD80/86 preferentially over CD28, preventing early T cell activation by blocking maturation of the APCs (Chaudhary and Elkord, 2016, Buchbinder and Desai, 2016) LAG-3, TIM-3 and PD-1 negatively regulate T_{EFF} and CTLs, and promote the proliferation and activity of Tregs (Chaudhary and Elkord, 2016, Nirschl and Drake, 2013).

The role of Tregs in cancer is not resolved, with a potential dual role (Whiteside, 2015). Tregs control inflammation and self-tolerance, they may be protective prior to tumour formation acting

to limit inflammation associated tumour development (Chaudhary and Elkord, 2016, Whiteside, 2015, Erdman et al., 2010).

Tumours activate Tregs and promote an immunosuppressive TME by the presentation of neo-epitopes (Chaudhary and Elkord, 2016, Savage et al., 2014, Lu and Robbins, 2016). Tregs are recruited to the TME or draining LN; here, they are chronically exposed and activated by TAAs resulting in increased Tregs compared to T_{EFF} and CTLs (Chaudhary and Elkord, 2016, Gobert et al., 2009). Tregs can exert their immunosuppressive function by downregulating immune cells with anti-tumorigenic functions. Thus they can inhibit the effects of NK, DC, T_{EFF} and CTLs, they can induce apoptosis of CD8+ T cells by Fas/FasL pathway, they can suppress the activity of CD4+ and γδ+ T cells by production of inhibitory cytokines IL10 and TGFβ and upregulating CTLA-4 and ICOS (Chaudhary and Elkord, 2016, Whiteside, 2015).

In the TME of many human cancers, highly suppressive effector Tregs (eTregs) expressing suppression molecules like TIGIT and CTLA-4, infiltrate heavily and account for a significant proportion of CD4+ T cells (20-50%) compared to healthy blood (5-10%) (Takeuchi and Nishikawa, 2016, Sakaguchi et al., 2010). A possible mechanism for this is the abundance of self-antigens from proliferating and dying tumour cells that are recognized by and activate Tregs which have been stimulated by intratumoural DC release of TGFβ (Takeuchi and Nishikawa, 2016, Nishikawa et al., 2005).

Up regulation of inhibitory receptors such as PD-1, CTLA-4, TIM-3 and LAG-3 on tumour infiltrating Tregs may be a mechanism for regulating the immunosuppressive Tregs and maintain homeostasis in the TME (Whiteside, 2015, Butt and Mills, 2014).

In many cancers, a high infiltration of intratumoural Tregs is associated with a poor prognosis due to suppression of antitumour immunity (Takeuchi and Nishikawa, 2016, Whiteside, 2015, Fridman et al., 2012). Tregs have been demonstrated in mesothelioma tumours and a high infiltration in the TME is associated with a shorter survival (Anraku et al., 2008).

However, in some cancers, there are reports suggesting Treg infiltration might be associated with a better prognosis (Whiteside, 2015, Badoual et al., 2006b). FOXP3+ Tregs have been associated with improved survival and locoregional tumour control in a diverse range of cancers, such as in colorectal and breast cancer (Chaudhary and Elkord, 2016, Whiteside, 2015, Rech et al., 2010, Salama et al., 2009). Tregs may be protective in these cancers by controlling inflammation associated with tumour development (Chaudhary and Elkord, 2016).

1.2.3.8 CD4+ Memory T cells and peripheral immunity

Long-term memory is required to mount an effective response on subsequent/secondary antigen exposure and is a crucial feature of adaptive immunity (Muranski and Restifo, 2013).

The multiple phenotypes and plasticity of Th cells complicates the formation of CD4+ memory T cells which may be less robust than their CD8+ counterparts. (Muranski and Restifo, 2013, MacLeod et al., 2009).

It is unclear if CD4+ memory T cells are long-lived and there has been speculation that once CD8+ T cells and long-lived protective antibody have been generated there might no longer be a requirement for CD4+ memory T cells (MacLeod et al., 2009).

Naïve T cells express CD45RA and after antigen experience, memory T cells instead express CD45RO (MacLeod et al., 2009, Ahmed and Gray, 1996). CCR7 is expressed by all naïve T cells and $CD45RA^{-}CCR7^{+}$ central memory T cells (T_{CM}) which home these cells to lymphoid tissue, whereas $CD45RA^{-}CCR7^{-}$ effector memory T cells (T_{EM}) migrate to non-lymphoid tissue (Farber et al., 2014, Golubovskaya and Wu, 2016). Stem cell memory T cells (T_{SCM}) express Fas and CD122 (memory marker), they are highly proliferative, self-renewing, can differentiate into other effector and memory subsets and mediate a superior anti-tumour response (Farber et al., 2014, Gattinoni et al., 2011). This has important therapeutic implications for cancer immunotherapy (Gattinoni et al., 2011).

In contrast to CD8+ memory T cells that rely on IL15 for homeostasis, IL17 for survival, and do not require MHC or antigens, CD4+ memory T cells depend on TCR signalling and/or MHC class II molecule contact for survival and function (Farber et al., 2014, Kassiotis et al., 2002, Bushar et al., 2010).

1.2.3.8.1 CD4+ Tissue Resident Memory cells (T_{RMS})

In humans, both CD4+ and CD8+ tissue resident memory cells, CD4+ T_{RM} ($CCR7^{-}CD69^{+}$) and $CD8^{+}CD103^{+}$ T_{RM}, are predominant in multiple organs (Farber et al., 2014, Sathaliyawala et al., 2013, Mueller et al., 2013). Human T_{RM} cells have tissue-specific properties suggesting local environmental influence. (Farber et al., 2014) Less is known about CD4+ T_{RMS} than CD8+ T_{RMS}.

Physiologically, T_{RMS} reside in epithelial barriers, can respond rapidly and independently to pathogens and are important for long-term immunity (Jiang et al., 2012, Clark, 2015, Park and Kupper, 2015, Wilk et al., 2017). T_{RMS} are specific for the barrier tissue in which they reside. When

T_{RMS} gather in sterile tissues such as the pleura, they may drive inflammatory disease (Park and Kupper, 2015).

CD103 has been identified as a marker of tissue-resident memory in CD8+ T cells. However it has been shown that CD103 alone is not a reliable marker for tissue-resident memory in CD4+ T cells (Bromley et al., 2013). CD4+CD103+ cells have been found as tissue-resident cells as well as in draining lymph nodes (Bromley et al., 2013). A better marker for tissue residency in CD4+ T cells appears to be CD69 (Jiang et al., 2012, Park and Kupper, 2015).

CD4+ T_{RMS} have been shown in infection and inflammatory disease. They are defined by being retained locally in tissue and are able to quickly produce IFNγ and IL17 in response to recognised pathogens (Turner and Farber, 2014).

CD4+ T_{RMS} have been demonstrated in the lung, gut and female genital tract in response to viral, bacterial and mycobacterial infections (Turner and Farber, 2014).

They may be implicated in chronic inflammatory diseases such as inflammatory bowel disease, psoriasis and asthma. The mechanisms of differentiation of CD4+ T_{RMS} are not well understood.

Further work is required to stratify CD4+ tissue resident memory cells in general as well as in cancer.

1.2.3.9 CD4+ effector cells

In addition to helper functions, some of the CD4+ T cell sub-types have effector functions. Cytotoxic CD4+ T cells have long been recognized as important in anti-infection immune responses and have also been observed to possess anti-tumour properties.

1.2.3.9.1 CD4+ CTLs

CD4+ CTLs have been demonstrated in viral infections, autoimmune conditions and cancers. They demonstrate cytotoxic properties similar to those of CD8+ T cells and NK cells. CD4+ CTLs have two methods of effecting cytotoxicity; i) induction of Fas-induced target cell apoptosis by expression of the Fas ligand and ii) secretion of granzyme B and perforin to kill in an MHC class II restricted manner (Malyshkina et al., 2017).

CD4+ CTLs originate from various cells including Th0, Th1, Th2, Th17 and Tregs (Takeuchi and Saito, 2017). However the majority of CD4+ CTLs are thought to originate from Th1 cells with

expression of IFNγ, IL2 and TNFα (Takeuchi and Saito, 2017). Transcription factors T-bet and Eomes have been associated with differentiation of CD4+ CTL (Takeuchi and Saito, 2017).

Markers for CD4+ CTLs have not been clearly identified but some markers that are associated with cytotoxicity in CD8+ T cells and NK cells can be used such as NKG2A and NKG2D, which indicate that these cells share similar functions (Takeuchi and Saito, 2017). CRTAM (class I restricted T cell molecule) has also been identified as a marker of CD4+ CTLs. In a cell line experiment, only cells expressing CRTAM developed into cytotoxic CD4+ T cells after incubation with IL2 (Takeuchi and Saito, 2017).

The role of CD8+ cytotoxic T cells in infection and cancer has been well characterized. Tumours can evade detection by the immune system if they do not express tumour-associated antigens that are recognized as foreign (Mutti et al., 1998, Ioannides and Whiteside, 1993). To escape detection by CD8+ T cells, virally infected cells or tumour cells may downregulate MHC class I expression on their cell surface. The peptides may instead be expressed on MHC class II (Takeuchi and Saito, 2017).

CD4+ CTLs can target APCs expressing MHC class II and exert a direct cytotoxic effect via release of perforin and granzymes. Additionally, IFNγ release by activated CD4+ T cells may lead to up-regulation of MHC class II molecules on cells that do not usually express this surface marker, like mesothelial and tumour cells, making them targets for CD4+CTLs (Quezada et al., 2010, Zanetti, 2015). It is not clear how often class II restricted killing of the target cell occurs in vivo (Takeuchi and Saito, 2017).

There is little data in the literature about the expression of MHC class II on malignant mesothelioma cells. Four primary mesothelioma cell cultures from pleural effusions were established and examined for the presence of accessory molecules required for antigen presentation, ICAM-1, MHC class I and II, CD80, CD86 (Mutti et al., 1998). Tumour cells from these primary mesothelioma cell cultures were found to express MHC class I and II, ICAM-1 and CD86, which are necessary for antigen presentation (Mutti et al., 1998). The presence of MHC class II on mesothelioma cells in solid tumours has not yet been established. However, given these tumour cells arise from malignant transformation of mesothelial cells which are known to express MHC class II molecules on their cell surface, it is possible that these mesothelioma cells will also express MHC class II.

1.2.4 CD8+ T cells

Naïve CD8+ T cells differentiate in the lymph node similarly to CD4+ T cells with TCR engagement by peptide-MHC complex, ligation of co-stimulatory receptors, and specific cytokine signals (Condotta and Richer, 2017, Mittrucker et al., 2014). However, they recognise antigen presented on MHC class I rather than MHC class II molecules.

Following activation, the antigen-specific CD8+ T cells undergo clonal expansion and develop into effector T cells (Condotta and Richer, 2017, Mittrucker et al., 2014, Kaech et al., 2002b). These CD8+ T_{EFF} cells migrate into the periphery and are able to fulfil distinct functions on re-encounter with antigen (Mittrucker et al., 2014).

After the primary response, 90-95% of pathogen-specific effector CD8+ T cells die by apoptosis (Mittrucker et al., 2014, Kaech et al., 2003). A small proportion persist as long-lived T memory cells that proliferate and convert rapidly into effector cells on re-exposure to specific antigen (Condotta and Richer, 2017, Mittrucker et al., 2014, Kaech and Cui, 2012).

1.2.4.1 CD8+ CTLs

Similar to Th cells, different subtypes of CD8+ T cells have been described - Tc1, Tc2, Tc9 and Tc17 (Mittrucker et al., 2014).

1.2.4.1.1 Tc1

Cytotoxic T lymphocytes (Tc1) are the best described population and play an important role in the host defence against intracellular pathogens and cancer (Mittrucker et al., 2014).

Naïve Tc cells differentiate to a Tc1 phenotype by exposure to inflammatory cytokines, IL2 and IL12. This is associated with up-regulation of surface markers including CD69, CD44, CD25 and KLRG1 (killer cell lectin like receptor) and downregulation of CD62L, CD27 and CD127 (Mittrucker et al., 2014, Pipkin et al., 2010, Joshi et al., 2007). Effector Tc1 cells are influenced by transcription factors T-bet, Blimp-1, and interferon regulatory factor (IRF4) (Mittrucker et al., 2014).

Tc1 cells have the ability to kill target cells by secreting cytokines like IFNγ and TNFα, releasing cytotoxic granules containing perforin and granzymes resulting in apoptosis of the target cell and they can cause target cell destruction by Fas/FasL interactions (Mittrucker et al., 2014).

CD8+ cytotoxic T cells and NK cells both produce IFNγ and directly kill target cells so play a vital role in the anti-tumour response (Dunn et al., 2004, Ruffell et al., 2010). CD8+ TILs also mediate tumour rejection through recognition of tumour antigens (Reiser and Banerjee, 2016).

Two types of tumour antigens have been identified that can be recognised by T cells: highly/aberrantly expressed normal proteins called tumour-associated antigens (TAAs) or abnormal mutated proteins (neoantigens)/oncogenic viral proteins called tumour specific antigens (TSAs) (Ruffell et al., 2010, Takeuchi and Nishikawa, 2016).

CD8+ T cell priming with tumour antigen occurs mainly in draining lymph nodes but can also occur in the tumour itself by tumour cells or cross presenting APCs (Reiser and Banerjee, 2016, Marzo et al., 1999, Thompson et al., 2010).

CD8+ T cells can be chronically stimulated by tumours leading to changes in their phenotype and function (Reiser and Banerjee, 2016). Chronically stimulated CD8+ T cells are functionally impaired and this may enable tumour progression (Reiser and Banerjee, 2016, Klebanoff et al., 2006).

1.2.4.1.2 Tc2

Tc2 cells play a role in allergy and inflammation. They are polarized by IL4 expression. Their transcription is regulated by GATA3. They produce IL4, IL5 and IL13 similar to Th2 cells, and some granzymes and perforin. In airway inflammation, Tc2 cells demonstrate low levels of cytotoxicity and enhance inflammation (Mittrucker et al., 2014, Tang et al., 2012).

1.2.4.1.3 Tc9

Tc9 cells play a role in inflammatory bowel disease and allergy. They are polarised by TGFβ and IL4. Their lineage determining transcription factor is not clear. They produce IL9 and IL10. Tc9 cells expressing IL9 have been shown to be anti-tumourigenic in a murine model (Mittrucker et al., 2014, Lu et al., 2014).

1.2.4.1.4 Tc17

Tc17 cells are polarized by TGFβ, IL6 and IL21. They are regulated by RORγt, similar to Th17 cells. They express IL17 and IL21. Tc17 cells contribute to autoimmunity, defence against viral infections and possess anti-tumour properties. Tc17 cells display impaired cytotoxicity as they express less IFNγ, granzyme B and perforin than Tc1 cells (Mittrucker et al., 2014, Huber et al., 2009). Tc17 cells have been shown to be protective in viral infections such as hepatitis C (Mittrucker et al., 2014, Billerbeck et al., 2010). The evidence for Tc17 activity in cancer is contradictory.

1.2.4.2 CD8+ Memory cells

CD8+ memory cells are important for the induction of a rapid secondary immune response. There are various models that have been suggested for the generation of memory T cells.

CD8+ memory T cells can be divided into CD8+ central memory T_{CM} ($CD62L^{+}CCR7^{+}$), CD8+ effector memory T_{EM} ($CD62L^{-/low}CCR7^{-}$) and CD8+ tissue resident T_{RM} (CD69+CD103+) cells.

During the activation of CD8+ T cells, most T_{EFF} cells express high levels of KLRG1 and low levels of CD127, which marks the cell as terminally differentiated and these are short lived effector cells (Samji and Khanna, 2017). A small proportion of T_{EFF} retains CD127 expression, do not express KLRG1 and survive to become memory cells (Samji and Khanna, 2017).

In infection, the T_{EFF} cells clear antigen then undergo rapid, apoptosis-induced contraction, leaving a small subset of CD8+ memory T cells (Kaech et al., 2002a, Reiser and Banerjee, 2016). Memory CD8+ T cells are maintained through IL15 and IL17 which promote memory CD8+ T cell survival and self-renewal and do not require the presence of antigen (Kaech and Cui, 2012). Inflammatory IL15 promotes antigen-experienced memory $CD8^{+}$ T cell trafficking to inflamed tissues (Nolz and Harty, 2014, Condotta and Richer, 2017).

1.2.4.2.1 CD8+ T_{CM}

T_{CM} are mainly $CD62L^{hi}$ $CCR7^{hi}$ cells and home to secondary lymphoid organs and bone marrow. T_{CM} are less differentiated and slower to demonstrate effector function but are more able to proliferate, self-renew and produce IL2 compared to T_{EM} cells (Reiser and Banerjee, 2016, Bachmann et al., 2005). Similar to T_{EM}, they are able to circulate through the blood stream.

1.2.4.2.2 CD8+ T_{EM}

T_{EM} are mainly $CD62L^{lo}CCR7^{lo}$ and are most commonly found in non-lymphoid tissue such as the spleen, blood and liver. They have increased effector function and cytotoxicity compared to T_{CM}, but reduced proliferative potential and ability to produce IL2 (Kaech and Cui, 2012).

1.2.4.2.3 CD8+ T_{RM}

T_{RM} are important for providing protective immunity in their tissue of residence (Condotta and Richer, 2017, Djenidi et al., 2015). They have increased effector function and cytotoxicity compared to T_{CM} but decreased proliferative potential and ability to produce IL2 (Kaech and Cui, 2012).

T_{RM} have been defined on the basis of surface expression of CD8, CD103, CD69, PD1 and TIM3 (Condotta and Richer, 2017, Djenidi et al., 2015). CD8+CD103+ cells do not necessarily express GZB but can up-regulate these cytotoxic mediators quickly on contact with their specific antigen or when exposed to a type I IFN milieu (Piet et al., 2011). Down regulation of GZB may be to prevent cytotoxic damage to the single cell epithelial layer when not required (Piet et al., 2011).

On activation, T_{RM} produce IFNγ that recruits other immune cells into the tissue. (Condotta and Richer, 2017, Shin and Iwasaki, 2013a).

In the TME there is not an absence of antigen so it may be more appropriate to describe these cells as "persistent" CD8+ T cells rather than memory T cells (Reiser and Banerjee, 2016).

Our own data has shown that T_{RM} linked with a robust anti-tumour response in non-small cell lung cancer (Ganesan et al., 2017). $CD8^{+}CD103^{hi}$ TILs demonstrated features of enhanced proliferation and cytotoxicity, suggesting a more robust anti-tumour response and a higher density of these cells was associated with survival (Ganesan et al., 2017).

It is likely that a combination of these different memory cells is required for an optimal secondary immune response, the T_{EM} and T_{RM} confer immediate effector function and first line of defence while the T_{CM} generate a large number of secondary effector cells (Kaech and Cui, 2012). Subsets of tumour specific T_{EM} and T_{CM} have been identified in breast and colorectal cancer patients (Reiser and Banerjee, 2016).

Chronic inflammation has been found to adversely affect the number of CD8+ T cells, CD8+ memory formation, secondary expansion and the protective capacity of CD8+ memory T cells (Condotta and Richer, 2017, Stelekati et al., 2014). These mechanisms may influence the TME in many cancers including mesothelioma, which is characterized by chronic inflammation.

1.2.5 B Lymphocytes

B cells are responsible for humoural immunity and play an essential role in the adaptive immune response. B cells interact with T cells to participate in antigen presentation, produce antibodies, produce cytokines that regulate functions of T cells, DCs and APCs, regulate wound healing and influence tumour immunity (Sarvaria et al., 2017, LeBien and Tedder, 2008, Tsou et al., 2016).

Peripheral B cells are divided into subsets that include transitional B cells, which mature within the spleen into naïve follicular B cells, germinal centre B cells, memory B cells and antibody secreting plasmablasts and plasma cells (Sarvaria et al., 2017).

1.2.5.1 B cell maturation

Immature B cells originate in the bone marrow. Here, they undergo positive selection to ensure the heavy and light chain of the B cell receptor (BCR) fit together followed by negative selection to prevent auto-reactivity. Immature B cells then migrate to the spleen where they are called transitional type (T1) B cells. Here they are exposed to red blood cells and any T1 B cells that respond to self-antigen become anergic (T3). If a T1 B cell survives, it migrates to the follicle and becomes a T2 B cell. At this point, these naïve follicular B cells are responsive to antigen and considered to be mature. The mature B cells will circulate through lymphoid tissue being exposed to antigens. If they encounter an antigen that stimulates a response they migrate to the T-B border to interact with stimulated T cells.

1.2.5.2 B cell activation and differentiation

B cell activation requires two signals. The first signal is recognition of antigen by the BCR. These cells will then start to divide.

The next step can be T cell dependent or T cell independent. In the T cell dependent setting, upon binding with the BCR, the antigen is internalised, digested and forms a complex with MHC class II molecules on the B cell surface. The MHC class II molecule loaded with peptide will form a complex with the TCR from the Th cell. Co-stimulatory molecule binding is also required, CD40 on APCs and CD40L on Th cell. Once this binding has occurred, CD4 will strengthen the interaction. Once the interaction is strong enough, the T cell will release IL4, which will interact with ILR (interleukin receptor) on the surface of the B cell. The Th cell will then secrete IL5, which is a B cell expansion factor. With both IL4 and IL5, the B cell is activated and will start to proliferate. A germinal centre will be generated where B cells undergo affinity maturation and class switching. From the germinal centre, B cells will be generated that can produce antibodies with increased specificity for the antigen and also memory B cells which will be able to respond more quickly on subsequent exposure to the same antigen. The terminal differentiation of a B cell is to become an antibody producing plasma cell. This process takes 7-10 days. Plasma cells in the spleen or lymph node are short lived but plasma cells that migrate to the bone marrow are long lived.

The T cell-independent setting allows the B cell to become activated by a wider range of molecules. T cells can only recognise peptide presented on an MHC class II molecule but antibodies on the B cell surface are able to recognise a wide range of molecules including

peptides, carbohydrates, lipids and DNA molecules. The B cell response is rapid but the antibodies produced are of low affinity. Additional co-stimulatory signals such as TLR or extensive BCR crosslinking are required for activation. This reaction does not generate a germinal centre or memory B cells.

1.2.5.3 Effector function

B cells produce antibodies and cytokines that exert effector function (Spaner and Bahlo, 2011, Lund, 2008). B effector 1 (Bc1) and B effector 2 (Bc2) cells are formed by interactions with Th1/Tc1 type T cells and Th2/Tc2 type T cells respectively and secrete characteristic cytokines Bc1 (IFNγ, IL12 and TNFα), and Bc2 (IL2, IL4, IL6, IL13 and TNFα) (Spaner and Bahlo, 2011).

Plasma cells produce antibodies against the specific antigen that was initially recognised by the naïve B cell. Antibody binding to cell surface antigens coats the target cell, referred to as "opsonisation". Although binding of antibody to cell surface antigens does not normally kill the target cell directly, it recruits potential cytotoxic effector mechanisms. These may either be in the form of complement, the formation of antibody complement complexes leads to the formation of "membrane attack complexes" which create holes in the target cell membrane and the pathogen or target cell is killed. Immunoglobulins of IgG1 and IgGIII classes are able to fix complement. Alternatively, the antibodies coating (opsonizing) a target cell can interact with innate immune cells including macrophages, NK cells and granulocytes. The Fc tail-piece of the antibody interacts with Fc-receptors such as CD16, to activate cytotoxic mechanisms in the associated innate immune cell – a process referred to as Antibody-Dependent Cellular Cytotoxicity (ADCC).

B cells can act as APCs stimulating memory and effector cells in the local environment. They can also produce GZB to function directly as cytotoxic B cells (Hagn et al., 2012).

1.2.5.4 Bregs

Regulatory B cells (Bregs) are immunosuppressive and play a role in the immune regulation of cancer and inflammation (Sarvaria et al., 2017, Yuen et al., 2016).

Bregs are thought to develop from marginal zone or CD5+ transitional/naïve B cells (Spaner and Bahlo, 2011, Walker and Goldstein, 2007). Differentiation into a Breg phenotype can occur in the TME by interactions with tumour-produced metabolites of 5-lipoxygenase that activate PPARα in B cells (Wejksza et al., 2013, Yuen et al., 2016).

Bregs mainly secrete IL10 but can also secrete TGFβ and IL35 (Yuen et al., 2016, Shen and Fillatreau, 2015). Bregs may exert their inhibitory effects via both immunosuppressive cytokine secretion of IL10 and TGFβ, and direct lysis of T cells (Spaner and Bahlo, 2011, Tsou et al., 2016, Mauri and Ehrenstein, 2008).

Regulatory B cells can directly and indirectly suppress Th1 and CD8+ cytotoxic cell responses, through anti-inflammatory mediators like IL10 or promoting the differentiation of naïve CD4+ cells into Tregs or inducing CD4+ T cell death through the expression of FasL (Sarvaria et al., 2017, Yuen et al., 2016, Balkwill et al., 2012, Tian et al., 2001).

In autoimmunity, B cell suppression of Th1 type responses during chronic inflammation may be beneficial, but in cancer, this effect prevents detection and elimination of tumour cells, promoting tumourigenesis (Yuen et al., 2016).

1.2.6 Innate immune response

The innate immune response is the first line of defence against pathogens. It is activated by physico-chemical perturbation of the tissue microenvironment – usually by "sensing" microbial products such as lipopolysaccharides, DNA, RNA or other polysaccharides and proteins. Families of receptors such as Toll-like receptors (TLRs), Lectin-binding receptors and many others, recognize general molecular patterns but are much less specific than receptors of the adaptive immune system that recognise antigens. While many cell types, particularly in barrier tissues such as epithelia, are able to be activated via these "danger" and pattern recognition receptors, particular cell types including macrophages, dendritic cells and NK cells are the major effectors of innate immune responses. The sensing of danger or noxious perturbation of the cell microenvironment must be responded to rapidly – within minutes and hours. The responses involve rapid up-regulation of various defences including cytotoxic proteins, cytokines and anti-oxidant defences. The innate immune responses usually generate an inflammatory response.

Tumour-associated inflammation is recognized as a key hallmark of cancer (Grivennikov et al., 2010, Hanahan and Weinberg, 2011). Initially this inflammation may be useful in suppressing tumour growth but later may be co-opted by the tumours to promote growth and metastasis.

1.2.6.1 Macrophages

The main roles of tissue-resident macrophages are firstly to maintain homeostatic equilibrium, removing debris from apoptotic cells and remodelling stroma and secondly, to provide the first line of defence against pathogens. Tissue-resident macrophages arise from myeloid progenitor cells and monocytes migrating into tissue (Sica and Mantovani, 2012, Lievense et al., 2013).

A wide spectrum of functional states of Tumour associated macrophages (TAMs) have been described and M1 and M2 macrophages represent extreme ends of this spectrum (Sica and Mantovani, 2012). The division into these two phenotypes is an oversimplification and many of the macrophages may be able to exhibit both M1 and M2 characteristics (Noy and Pollard, 2014, Qian and Pollard, 2010). In early tumour development, TAMs have been reported to skew towards a M1 phenotype and in the later stages a M2 phenotype (Colegio et al., 2014, Franklin et al., 2014, Shalapour and Karin, 2015).

M1 macrophages are activated upon encounter with IFNγ or in association with cytokines such as TNFα and GM-CSF. They are characterized by IL12 and IL23 production and activation of the polarized Th1 cell response (Lievense et al., 2013, Mantovani, 2014). They possess good capabilities as antigen presenting cells and produce effector molecules and pro-inflammatory cytokines such as TNFα and IL6, which contribute to tumour progression (Lievense et al., 2013, Mantovani, 2014, Shalapour and Karin, 2015). They can also act as potent anti-tumour effectors, demonstrating cytotoxic activity against neoplastic cells and are controlled by CD4+ Treg cells that favour tumour progression (Mantovani, 2014).

In the transition from a non-cancerous to a cancerous growth, the cytokines and growth factors create a Th2 type microenvironment (Noy and Pollard, 2014). This is characterized by TGF-β1, arginase-1 and an increase in IL4 producing CD4+ Th2 cells (Noy and Pollard, 2014, DeNardo et al., 2009). Tumour-associated factors can polarize macrophages into the M2 phenotype when they are exposed to signals such as growth factors produced by tumour cells like CSF1 and GM-CSF, or IL4 from activated CD4+ Th2 cells (Gocheva et al., 2010, Mantovani, 2014, Noy and Pollard, 2014).

M2 macrophages promote wound healing, angiogenesis and tissue remodelling (Lievense et al., 2013). They produce anti-inflammatory cytokines like IL10, chemokines like CCL17 (Katakura et al., 2004), CCL22 and CCL24 (Owen and Mohamadzadeh, 2013), produce MMPs, express the mannose (CD206), and scavenger (CD163) receptors and are poor at antigen presentation (Lievense et al., 2013). Hypoxia in the TME induces M2 macrophages to produce pro-angiogenic factors like VEGF-A (Lievense et al., 2013, White et al., 2004). M2 TAMs contribute to the dampening of inflammation by downregulating M1 mediated functions and promote wound

healing by their association with anti-inflammatory molecules such as IL10, TGF-β and arginase-1 (Shalapour and Karin, 2015, Mantovani, 2014, Lievense et al., 2013). M2 TAMs also promote cancer cell migration and intravasation, which occur in the later stage of tumour development (Shalapour and Karin, 2015).

1.2.6.1.1 TAMs

Tumour-associated macrophages (TAMs) have a major and complex role in the tumour microenvironment accounting for up to 60% of the tumour stroma (Lievense et al., 2013). In multiple cancers TAM infiltration predicts for poor prognosis (Mantovani, 2014).

Macrophages can act in a pro or anti-inflammatory manner and are able to switch phenotype in response to different stimuli (Allavena et al., 2008). Macrophages are polarized to a pro-tumour phenotype in established tumours but may have anti-tumour effects in pre-malignant or the early stages of tumour development (Noy and Pollard, 2014).

Macrophages can gain anti-tumour properties by the release of lysis mediators, phagocytosis and T cell activation (Jackaman and Nelson, 2014).

Hypoxia and cell death are characteristic of cancer and steer macrophages towards an attempt to restore homeostasis (Ruffell et al., 2012). This mechanism has been co-opted by tumours to promote growth by angiogenesis, lymphangiogenesis, tissue remodelling, invasion, metastases and development of immunosuppressive microenvironments that inhibit anti-tumoural immune surveillance (Ruffell et al., 2012).

Solid tumours are immunosuppressive environments where macrophages recruit and induce regulatory T cells and suppress the action of CD4+, CD8+ and NK cells to protect the tumour (Noy and Pollard, 2014, Ruffell et al., 2012). TAMs have immunosuppressive potential through factors like TGFβ, inducible nitric oxide synthase (iNOS), arginase-1 (Arg1) and IL10 (Mantovani, 2014, Sica et al., 2000, Noy and Pollard, 2014).

TAM suppression of CD8+ T cells might alternatively rely on B7 molecules - B7-H1 (PD-L1) (Kuang et al., 2009), B7-H4 (Kryczek et al., 2006) and B7-H3 (Chen et al., 2013) in hepatocellular, ovarian and lung cancer respectively (Mantovani, 2014, Ruffell et al., 2012, Noy and Pollard, 2014). TAMs can induce these molecules on the cancer cell surface via IL10 production, thus allowing immune escape (Mantovani, 2014, Chen et al., 2013).

Macrophages express PD-1 and CTLA-4 ligands, which are usually present on activated effector cells to control the immune response (Noy and Pollard, 2014).

1.2.6.1.2 TAMs in mesothelioma

Macrophages are recruited to the lungs in response to inhaled asbestos fibres, which they are unsuccessful in removing (Lievense et al., 2013). In response, macrophages produce molecules like IL6, TNFα and IFNγ, which are responsible for the activation and maintenance of a chronic state of inflammation (Noy and Pollard, 2014, Mantovani, 2014).

In mesothelioma, a worse outcome was associated with increased numbers of circulating monocytes as well as higher densities of TAMs, which demonstrated an immunosuppressive M2 phenotype in non-epithelioid disease (Burt et al., 2011).

Macrophages co-cultivated in vitro with mesothelioma cells skew to an immunosuppressive M2 phenotype and release anti-inflammatory prostaglandin E2 (PGE_2) (Lievense et al., 2013, Izzi et al., 2009). PGE_2 induces FoxP3 and the development of immunosuppressive Tregs (Izzi et al., 2009, Lievense et al., 2013, Sharma et al., 2005).

Mesothelioma tends to occur in older people with a long lag time between exposure and diagnosis. It is unclear whether there is a M1 or M2 predominance in aging. Aging has been associated with an inability to respond to M1 stimuli (Mahbub et al., 2012) and elderly mice showed a decrease in M1 macrophages and increase in IL10 secretion suggesting M2 macrophages (Jackaman and Nelson, 2014). This suggests that tissue macrophages will more readily support tumour growth in aging hosts (Jackaman and Nelson, 2014) and raises the possibility that a shift to an M1 phenotype may improve T cell immunity in aging hosts (Allavena et al., 2008). However, mesothelioma does occur in younger patients and this may be due to increased genetic predisposition or early exposure to asbestos.

1.2.6.2 Natural Killer cells

Natural killer (NK) cells are innate cytotoxic lymphocytes that play an important role in controlling viral infections and tumour progression. In animal models and humans, deficiency or impairment of NK cells has been associated with an increased incidence of various cancer types (Orange, 2013, Morvan and Lanier, 2016).

NK cells are activated by stress molecules like interferons, or cytokines like IL15, IL18 and IL2 from DCs and macrophages (Vivier et al., 2011). Cytokine receptors such as IL15R, IL21R and IL2R are involved in maturation and effector function of NK cells (Vivier et al., 2011). IL15 is essential for NK cell maturation and survival (Vivier et al., 2011, Caligiuri, 2008).

Activated NK cells secrete IFNγ and TNFα. IFNγ activates macrophages and TNFα promotes direct NK killing. They also secrete other cytokines like IL10, growth factors like GM-CSF and chemokines like CCL3, CCL4, CCL5 (Vivier et al., 2011, Morvan and Lanier, 2016).

NK cells express receptors that can either stimulate or dampen NK cell reactivity (Vivier et al., 2011, Vivier et al., 2004, Bryceson et al., 2006). Activating receptors like NKG2D and NKp46 recognize stress-induced ligands on the surface of the target cell and play a role in cytotoxicity induction (Guillerey et al., 2016, Nishimura et al., 2015). Suppressive receptors include KIRs and heterodimers CD94-NKG2A or NKG2B (Nishimura et al., 2015, Guillerey et al., 2016).

The main method of NK cell-mediated killing is by the binding of ligands between the NK and target cells. This induces release of cytotoxic granules containing perforin and granzymes, leading to perforation and apoptosis of the target cell(s) (Lieberman, 2003, Voskoboinik et al., 2006, Nishimura et al., 2015, Morvan and Lanier, 2016, Waldhauer and Steinle, 2008). This process of secretory lysosome exocytosis is very tightly controlled (Topham and Hewitt, 2009). NK cells are inhibited by MHC class I molecules that are recognised by NKG2A or KIR family NK receptors on their cell surface (Bessoles et al., 2014). NK cell activating receptor (NKG2D) can recognise other signals of aberrant cells such as MHC class I chain related molecules (MICA and MICB) and the resultant ligand binding can signal target cell killing (Topham and Hewitt, 2009). Other activating receptors include natural cytotoxicity receptors like NKp46 (Topham and Hewitt, 2009). Healthy cells that express self-MHC class I molecules and low amounts of stress-induced self molecules are spared (Morvan and Lanier, 2016, Vivier et al., 2011, Raulet and Guerra, 2009).

A second pathway that can be used by NK cells involves molecules of the TNF family as a death receptor pathway to kill tumour cells in a perforin-independent manner (Wallin et al., 2003, Morvan and Lanier, 2016, Guillerey et al., 2016). Soluble and membrane-bound TNF can be expressed by NK cells which, when activated, induce ligands such as the TNF related apoptosis ligand (TRAIL) and FAS ligand at their cell surface which interact with the corresponding receptors on tumour cells contributing to NK cytotoxicity (Morvan and Lanier, 2016, Wallin et al., 2003, Waldhauer and Steinle, 2008). Caspase-dependent apoptosis occurs when these ligands bind to their death receptors on target cells (Smyth et al., 2005, Morvan and Lanier, 2016).

NK cells can also kill by antibody-dependent cell-mediated cytotoxicity (ADCC). Here, infected or mutated cells are opsonized with antibody, the Fc (tailpiece fragment) of which can be recognised by CD16 on NK cells. This activates NK cells leading to release of cytotoxic granules inducing target cell apoptosis (Topham and Hewitt, 2009).

NK mediated cytotoxicity of target cells can accelerate cytotoxic T cell responses and induce antigen-specific CD4+ T cells that prime CD8+ T cells and B cells thus activating an adaptive immune response (Vivier et al., 2011, Robbins et al., 2007, Krebs et al., 2009).

1.2.6.2.1 NK cells in cancer

Abnormal cells like tumour cells can initiate NK effector function including cytokine production and proliferation, and cytotoxicity by up-regulating the activating receptor ligands to overcome inhibitory signals or by loss of self-identifying molecules like MHC class I that bind to NK cell inhibitory receptors (Morvan and Lanier, 2016).

NK cells may have an early protective role but once a cancer is established are likely to be anergic due to tumour-derived suppressive factors like TGF-β (Fridman et al., 2012). Tumours can downregulate expression of the IL15 receptor, which blocks maturation of NK cells (Morvan and Lanier, 2016). NK cells may still contribute to the anti-tumour response by secreting cytokines or chemokines that attract or activate other immune cells in the TME (Morvan and Lanier, 2016).

There is evidence for NK cell immune editing of tumours in addition to the more major role played by the adaptive immune system (Schreiber et al., 2011). An immunoregulatory role occurs via their impact on other cells of the TME such as neutrophils, DCs, macrophages and downstream T and B cell activity (Vivier et al., 2011, Guillerey et al., 2016). NK cells also contribute to immunosurveillance by elimination of senescent cells, which secrete cytokines and chemokines that attract NK cells (Guillerey et al., 2016, Iannello et al., 2013).

In the TME, NK cells usually reside in the tumour stroma away from the tumour cells (Albertsson et al., 2003, Fridman et al., 2012). There are typically few NK cells in established tumours possibly due to inefficient homing and are not likely to significantly affect tumour cell elimination (Albertsson et al., 2003, Waldhauer and Steinle, 2008). Decreased NK activity has been detected in many studies of cancer patients (Morvan and Lanier, 2016, Pross and Lotzova, 1993).

There is a relative abundance of circulating NK cells compared to the TME and NK cells have been shown to resist the formation of metastasis in a murine model, suggesting this may be their predominant role (Gorelik et al., 1982, Morvan and Lanier, 2016).

In cell lines, exposure to asbestos has the potential to suppress the function of NK cells (Nishimura et al., 2015), which may influence their effect in mesothelioma.

1.3 Immune responses and Cancer

As any cell/tissue in the body undergoes malignant transformation and starts to form a cancer, some of the mutations that occur result in the generation of mutated proteins which may be recognizable by the immune system as neo-antigens. It is probable that for very many cancers, the immune surveillance and recognition of the "foreignness" of the tumour cells generates an effective immune response that eliminates the cancer before it ever reaches the point of being detectable clinically. The anti-cancer immune response generates antigen-specific effector mechanisms including CD8+ cytotoxic effectors of different types, CD4+ memory and effectors of different types, B-cell derived antibodies and also inhibitory mechanisms in the form of Regulatory T cells (Tregs). The cancer generates defensive strategies to avoid detection by the immune system or to inhibit the efficacy of the effector mechanisms. The ultimate outcome of the cancer including the response to therapy is the resultant of the balance between the immune system and the cancer's self-protective mechanisms.

The role of tumour infiltrating lymphocytes (TILs) in solid tumours is well established. In many solid tumours, including colorectal, lung, breast and oropharyngeal cancer, TILs confer a survival benefit (Galon et al., 2006, Al-Shibli et al., 2008, Garcia-Martinez et al., 2014, Ward et al., 2014, Gooden et al., 2011). A meta-analysis of 23 studies of solid tumours demonstrated the presence of CD8+ TILs conferred a prognostic advantage and a similar analysis of 6 studies of CD4+ TILs showed a significant effect on overall survival (Gooden et al., 2011). A more recent meta-analysis of nearly 18,000 tumours in 39 cancer types found higher levels of T-cell fractions to be generally associated with better survival (Gentles et al., 2015).

It has become clear that the effect of tumour-infiltrating lymphocytes varies between tumour types and that survival benefit is dependent on a complex interplay of cell types in the tumour microenvironment.

1.3.1 CD8+ CTLs

CD8+ TILs have been the most extensively studied cell in cancer immunology. The evidence from many tumour types is that CD8+ TILs are associated with a good outcome. To date, the majority of

data have come from single stained CD8+ IHC samples. The positive effect of CD8+ TILs is likely to be an oversimplification as there is evidence of different phenotypes of CD8+ T cells including CD8+ Tregs and CD8+ T_{RMS}. Similarly; the presence of a CD8+ T cell in the TME does not indicate its activation status or cytotoxic ability.

CD8+ CTLs have the potential to eliminate tumours by direct and indirect effects of IFNγ or by antigen-specific recognition of tumour antigens expressed in MHC class I molecules and cytotoxicity mediated by perforin/granzyme or receptor (Fas/TNF) mechanisms (Harimoto et al., 2013).

IFNγ is produced by many cells; including NK cells, CD8+ CTLS and Th1 cells. It has been shown to contribute directly to the anti-tumour response by preventing tumour cell growth and proliferation, and promoting apoptosis (Wall et al., 2003, Ivashkiv, 2018). Indirect effects include up-regulation of MHC class I and II expression, that promotes the function of Th1, CD8+ CTLs and macrophages and also suppression of Treg function (Ivashkiv, 2018).

Tumour-specific CD8+ CTLs are induced by professional APCs that present the TAA together with other factors that are required for successful antigen presentation such co-stimulatory molecules, their ligands and adhesion molecules (Harimoto et al., 2013, Mutti et al., 1998). There may be tumour-based factors that decrease the expression of co-stimulatory molecules on the DCs, making these cells tolerogenic and unable to stimulate CD8+ CTLs (Harimoto et al., 2013). It has been demonstrated in a mouse model that DCs were able to expand tumour specific CD8+ CTLs in the early stage of tumour development but their function was impaired in later stage tumours (Harimoto et al., 2013).

The functional properties of CD8+ TILs are likely to influence the outcome of the tumour, for example if they are activated or able to express cytotoxic effector molecules such as GZB (Harimoto et al., 2013). It has been shown in a murine model injected with 2 related tumour cell lines, that CD8+ cells from mice injected with the faster growing cell line were less activated (expressed less CD69), expressed less IFNγ and expressed no GZB, even on stimulation with IL2 (Harimoto et al., 2013).

Most *in vitro* CD8+ CTL work has demonstrated their ability to kill rapidly and efficiently (Halle et al., 2016). This may not translate directly to the *in vivo* environment where there are many other factors at play. *In vivo* murine work has demonstrated that in virally infected cells, the CD8+ CTLs killed very few cells per day and that MHC class I down-regulation strongly reduced the CTL-mediated, antigen-specific contact-dependent killing (Halle et al., 2016).

Tumour cells evade immunosurveillance by CD8+ CTLs in a variety of ways. They have poor antigen presenting properties as they are able to down regulate their surface expression of MHC class I molecules and often lack surface expression of adhesion and co-stimulatory molecules (Guinan et al., 1994, Maher and Davies, 2004) and are poorly immunogenic as they are closely related to self (Maher and Davies, 2004). Tumour cells may also kill CD8+ CTLs by expression of death receptor ligands like Fas (Maher and Davies, 2004).

1.3.2 T_{RMs}

T_{RMS} have been found to confer a survival benefit in solid tumours. T_{RMS} are a long-lived population of cells that are localised in tissue and able to quickly exert effector function on activation (Turner and Farber, 2014).

CD8+ T_{RMS} have been recognized for nearly 20 years but more recently the role of CD4+ T_{RMS} has been explored.

CD69 is expressed on most CD4+ and CD8+ T_{RMS} while CD103 is only expressed on a subset (Kumar et al., 2017). CD69 is associated with early activation or in response to pro-inflammatory cytokines. T_{RM} cells express elevated levels of CD69 compared to memory cells in the lymph node and spleen, which may be the result of continued stimulation with persistent antigen at tissue sites (Turner and Farber, 2014).

T_{RMS} have the ability to reside in peripheral tissue independently of other populations of memory cells (Turner and Farber, 2014). In a murine model, they have been shown to develop and survive independent of local antigen recognition in the absence of MHC class I expression (Davies et al., 2017). This may be important in cancer especially in view of eventual down-regulation of MHC class I by tumour cells. T_{RM} also express high levels of IFN-induced transmembrane protein 3 (IFITM3), an IFN-stimulated gene that promotes survival (Shin and Iwasaki, 2013b, Shin and Iwasaki, 2013a).

Compared to circulating T_{EM}, human T_{RMS} express more adhesion and inhibitory molecules, pro-inflammatory and regulatory cytokines (Kumar et al., 2017). T_{RMS} express high levels of GZB (Shin and Iwasaki, 2013b, Shin and Iwasaki, 2013a) In an infection model of Herpes Simplex Virus (HSV), the T_{RM} expressed lower levels of perforin and GZB in the skin where the infection was cleared but retained high expression of GZB and perforin in the ganglia, perhaps due to engagement of the TCR by latently infected or reactivating neurons (Shin and Iwasaki, 2013b, Shin and Iwasaki, 2013a). In cancer, persistence of the antigen may lead to increased expression of cytotoxic

granules by the TRMs to try and contain the tumour cells. CD8+ T_{RMS} in the ganglia can undergo repeated proliferative responses on HSV reactivation despite being subjected to chronic stimulation (Carbone, 2015). This may be an important contributory factor to the benefit of T_{RMS} in cancer where there is chronic inflammation and persistent antigen in the TME.

TRMs are able to recruit other immune cells to the site by release of IFNγ. In addition the TRMs promote NK and DC activation (Carbone, 2015). T_{RMS} also secrete TNFα that leads to APC maturity, however it is unclear if antigen presentation is required to maintain this population (Schenkel et al., 2013, Schenkel and Masopust, 2014).

1.3.3 B cells

While the focus in cancer immunology has been on CD8+ T cells, T cells do not work in isolation (Spaner and Bahlo, 2011, Yuen et al., 2016). B cells account for up to 25-40% of all cells in some tumours (Yuen et al., 2016).

There is conflicting evidence for whether B cells exert anti or pro-tumour responses and this is likely to be influenced by many factors including the heterogeneity of B cell types and interactions with other cells in the TME.

B cells are most commonly found in the draining lymph nodes but can be found at the invasive margin of tumours (Balkwill et al., 2012). Inflammation produces chemo-attractants that cause B cells to enter the TME (Spaner and Bahlo, 2011, Colotta et al., 2009).

In the TME, B cells can demonstrate anti-tumour activity by antigen-driven clonal expansion, class switching and affinity maturation leading to the production of tumour-specific antibodies (Sarvaria et al., 2017, Yuen et al., 2016).

Tumour cells undergo cell death by apoptosis and necrosis which produce antigens that are recognised by tumour-reactive antibodies and B cells (Spaner and Bahlo, 2011, Zeh and Lotze, 2005, Schafer and Werner, 2008). However, tumours are weakly immunogenic and express self-antigens that may not produce high affinity antibodies that would be associated with an effective anti-tumour response (Spaner and Bahlo, 2011). A weak response may conversely contribute to the pro-tumourigenic inflammatory process (Spaner and Bahlo, 2011, Houghton and Guevara-Patino, 2004).

Antibodies play a role in the opsonisation of tumour cells, activation of the complement cascade and in antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC is effected via the Fc portion of the antibody binding to and activating NK cells which are then able both to lyse target cells without priming and also to secrete cytokines like IFNγ to recruit adaptive immune cells (Yuen et al., 2016).

B cells exert effector function beyond antibody production and produce a range of anti-inflammatory cytokines like IL35, IL10 and TGFβ or pro inflammatory cytokines like IL12, IL6, IL15 and GMCSF that have complex and paradoxical effects in the TME (Li, 2016). It is not clear whether intra-tumoural B cells are Bc1 cells, Bc2 cells or Bregs (Spaner and Bahlo, 2011).

Tumour infiltrating B cells can function as APCs to sustain activity of memory and effector T cell (Tsou et al., 2016).

B cells may also exert anti-tumour effects directly by cytotoxicity associated with GZB secretion or indirectly by activation of Th1, CD8+ CTLs and macrophages with IFNγ secretion. (Sarvaria et al., 2017)

Tumour progression occurs despite the anti-tumour effect of B cells. This may be due to B cells promoting a pro-inflammatory angiogenic environment and recruiting immunosuppressive cells and supressing T cell activity (Sarvaria et al., 2017, Tsou et al., 2016).

1.3.4 Tregs

Tregs have traditionally been associated with suppression of effector T cells and are thought to contribute to an immunosuppressive TME and poor clinical outcome in many cancers. However, there have been some studies that have demonstrated a good outcome associated with Treg infiltration. Most studies have used one or two markers like FoxP3 and CD25 to define the Treg population. However, it should be noted that these markers are not restricted to Tregs so the reliability of those studies is uncertain.

Similar to other T cell populations, the Tregs have been shown to be heterogeneous. Thymus-derived nTregs exert their suppressive function on effector cells via GZB/perforin and Fas/FasL mechanisms (Frydrychowicz et al., 2017). The peripherally induced iTregs exert their function via secretion of TGFβ, IL10 and IL35 (Frydrychowicz et al., 2017).

A variety of mechanisms have been suggested for Treg suppression of effector T cells in the tumour. These include direct suppression of effector T cells by immunoregulatory cytokines, competition with effector T cells for access to antigen-presenting dendritic cells, interaction with DCs to affect their function, competition with effector T cells for IL2, inhibition by cytolysis of effector cells, hampering T cell activation thus inducing apoptosis of these cells (Frydrychowicz et al., 2017, Jang et al., 2017, Ward-Hartstonge and Kemp, 2017, Arce-Sillas et al., 2016).

Tregs are able to suppress a range of other cells in the TME including B cells, macrophages and DCs. Tregs promote B cell anergy and hinder the production of antibodies (Frydrychowicz et al., 2017). Inhibition of co-stimulatory and antigen-presenting molecules and inflammatory cytokines in APCs inhibits their ability to stimulate T cell responses (Frydrychowicz et al., 2017).

More recently, it has been shown in pancreatic cancer, that Tregs suppress the expression of co-stimulatory ligands on tumour-associated DCs that are necessary for activation of CD8+ T cells (Jang et al., 2017).

Tregs up-regulate inhibitory molecules on their cell surface like CTLA4, ICOS and LAG3 that prevent the APCs from activating effector T cells (Frydrychowicz et al., 2017, Maher and Davies, 2004). Tregs in the TME often express higher levels of inhibitory markers and are more suppressive than circulating Tregs (Ward-Hartstonge and Kemp, 2017).

Tregs may also exert protective effects for the host, such as suppression of pro-inflammatory Th17 cells and bacteria-driven inflammation which have both been associated with carcinogenesis (Frydrychowicz et al., 2017).

1.4 Checkpoint molecules – T cell exhaustion

1.4.1 Exhausted CD8+ T cells

When T cells are exposed to long-term persistent contact with their cognate antigens, there is the risk that uncontrolled activation and tissue damage may occur as collateral effects. Therefore, in many situations involving chronic exposure to antigen – such as various infections or in cancers, a supposedly protective self-inhibitory change takes place in the T cells which renders them inactive – a so-called state of “exhaustion”.

T cell exhaustion results in diminished effector function and cytokine production, resistance to reactivation and sustained expression of inhibitory receptors (Reiser and Banerjee, 2016, Crespo

et al., 2013, Legat et al., 2013, Wherry, 2011). CD8+ T cell exhaustion is reversible, which has therapeutic implications (Reiser and Banerjee, 2016).

When T cells become “exhausted”, they up-regulate a number of receptors with inhibitory functions including PD1, CTLA4, TIM3 and LAG3 (Reiser and Banerjee, 2016, Wherry, 2011).

CD8+ TILs in tumours display a higher proportion of an exhausted phenotype compared to normal tissue or blood (Ahmadzadeh et al., 2009, Hadrup et al., 2013, Wang et al., 2010). In melanoma, CD8+ TILs co-expressed more PD1 and CTLA4 with impaired cytokine production, corresponding to an exhausted phenotype and impaired anti-tumour responses (Ahmadzadeh et al., 2009, Hadrup et al., 2013).

PD-1 activation by PD-L1 and PD-L2 and CTLA-4 activation by B7-1 (CD80) and B7-2 (CD86) directly inhibits TCR and BCR signalling (Noy and Pollard, 2014). CTLA-4 competes with co-stimulatory CD28 binding, so inhibiting the activation and function of cytotoxic T cells (Noy and Pollard, 2014).

Dual blockade of CTLA4 and PD1 reversed T cell exhaustion, increased cytokine release and proliferation of effector cells and inhibited Tregs (Duraiswamy et al., 2013).

While expression of inhibitory receptors is often associated with exhaustion, (Legat et al., 2013) it has been shown that PD1 expression in healthy donors does not represent exhaustion but rather an effector memory phenotype (Legat et al., 2013, Duraiswamy et al., 2013, Baitsch et al., 2011).

The expression of eight inhibitory receptors by CD8+ T cells in healthy humans was examined (Legat et al., 2013). Their data indicate that expression of inhibitory receptors does not necessarily identify dysfunctional cells (Legat et al., 2013). It may be that these are markers more of differentiation and maturity than exhaustion.

1.4.2 Immune checkpoint molecules

Immune checkpoint molecules help to tightly control immune responses by activating or suppressing T cell activation. There are a range of stimulatory checkpoint molecules such as ICOS, CD137 and OX40. There are also many inhibitory molecules including CTLA4, PD1, TIM3 and LAG3. These have been extensively studied and form the basis of immunotherapy with checkpoint inhibitors.

Each of the immune checkpoint inhibitors has a different target and mechanism of action. For example an anti-CTLA-4 agent would help with T cell activation whereas an anti-PD1 agent will

prevent PD1 from binding with its ligand so allowing the activated T cells to continue with its effector function.

CTLA4 is mainly expressed on naïve CD4+ and CD8+ T cells and Tregs. It binds to CD80 and CD86 preferentially over CD28, thus inhibiting T cell activation. It can also remove CD80 and CD86 from the surface of the APC. It limits the signalling potential of the TCR through phosphorylation upon ligand binding.

PD-1 is a transmembrane inhibitory immunoreceptor expressed by activated T cells that negatively regulates immune responses by interaction with its ligands PD-L1 and PD-L2 (Ceresoli et al., 2016). PD1 is mainly expressed on activated T cells, B cells, APCs and NK cells. It acts as an "off switch" and engages with ligands to inhibit T cell proliferation, reduce cytokine production and decrease expression of survival proteins. Blockade of PD-1 or PD-L1 allows the activated T-cells to exert effector function on tumour cells.

TIM3 is a type I cell surface glycoprotein. It is mainly expressed on activated T cells (CD4+ and CD8+ T cells, Tregs), innate immune cells and on certain tissue. It binds to its ligand galectin-9 leading to decreased production of IFNγ and reduced T cell proliferation. There is evidence for positive and negative effects of TIM3 on the TCR signalling and it may depend on the cellular context (positive for naïve, effector, memory T cells, negative for exhausted T cells) or the ligand it engages with (Du et al., 2017). TIM3 has been noted to be upregulated on Th1 cells leading to apoptosis, is a marker for exhausted CD8+ T cells in chronic infection and is upregulated on antigen-specific CD8+ T cells and CD8+ TILs (Du et al., 2017). The mechanism of action of TIM3 is currently not clear.

LAG3 is mainly expressed on activated T cells and NK cells. It binds to MHC class II molecules. The mechanism of action is not clear. LAG3 exerts a negative regulatory effect on proliferation and activation of T cells (Marcq et al., 2017a)

In mesothelioma, PD1, CTLA4 and TIM3 have been demonstrated on immune cells in the TME but there was no expression of LAG3 (Marcq et al., 2017a). TIM3 expression in mesothelioma samples was found to be an independent prognostic factor (Marcq et al., 2017a).

TIM3, LAG3 and PD1+ T cells have been demonstrated to varying amounts the pleural effusions of patients with mesothelioma (Marcq et al., 2017b).

There has been great interest in understanding the role of checkpoint molecules as a means of modulating the immune system with immunotherapy.

1.5 Cancer Immunotherapy

Cancer treatments have traditionally involved surgery, chemotherapy and radiotherapy with no new treatments entering the arena for the last few decades and little improvement in survival for most cancers.

Immunotherapy covers a broad range of treatments that aim to therapeutically modulate the immune system to treat disease. This has been used in a range of diseases including cancer. Types of cancer immunotherapy include the use of checkpoint inhibitors, monoclonal antibodies, vaccines, cytokines and adoptive cell transfer.

Using the immune system to target cancer was attempted in the 19th century but was stopped in favour of chemotherapy and radiotherapy. Further advances were made in the 1970s with the use of a Bacille Camette-Guerin (BCG) vaccine for preventing bladder cancer recurrence. In the 1990s, the first monoclonal antibody was approved for therapeutic use in non-Hodgkin's lymphoma. In 2010, the first dendritic cell cancer vaccine for prostate cancer was approved.

In 2011, the first checkpoint inhibitor, Ipilimumab, a fully human monoclonal antibody that blocks cytotoxic T lymphocyte 4 (CTLA-4) was approved for use in metastatic melanoma (Hodi et al., 2010). The median survival for these patients with stage III and IV metastatic melanoma without immunotherapy was 11 months. In this study, 20% of patients treated were alive at 3 years and in some cases treatment has led to long-term disease control and survival at 10 years (Hodi et al., 2010, Robert et al., 2015, Schadendorf et al., 2015). However, there is no way of predicting in advance which patients will benefit from Ipilimumab.

Immune checkpoint inhibitors (ICIs) block immune checkpoint molecules or their ligands that are overexpressed in TILs and tumour cells to re-establish anti-tumour immunity (Buchbinder and Desai, 2016, Chaudhary and Elkord, 2016).

ICIs were initially designed to enhance T_{EFF} and CTL activity but may also play a role in Treg depletion or impairment (Chaudhary and Elkord, 2016).

The effectiveness of cancer immunotherapy is blocked by Tregs (Ivanova and Orekhov, 2015). Tregs however could be therapeutically harnessed in a variety of ways including local depletion and checkpoint inhibitors (Sakaguchi et al., 2008).

Treg depletion can provoke and enhance tumour immunity in animal models but in itself may not establish sufficiently robust anti-tumour activity (Takeuchi and Nishikawa, 2016, Sakaguchi et al., 2008). It has been shown that self-reactive CD8+ T cells become irreversibly anergic after Treg suppression which is a mechanism for self–tolerance (Takeuchi and Nishikawa, 2016, Maeda et

al., 2014). Therefore, for immunotherapy to be successful, a combination of Treg depletion and re-priming of T_{EFF} from naïve T cells would be required (Takeuchi and Nishikawa, 2016).

A number of immune checkpoint inhibitors have been licensed and an increasing number have been the subject of clinical trials in many different cancers types.

1.5.1 CTLA-4 (cytotoxic T lymphocyte 4)

Blockade of CTLA-4, allows T cell activation and leads to immune-mediated tumour cell death when used by itself or in combination with other agents (Grosso et al, 2013). This has led to the development of fully humanized monoclonal antibodies, Ipilimumab (Bristol-Myers Squibb, Princeton, NJ) and Tremelimumab (Pfizer, New York, NY). Ipilimumab has also been studied in conjunction with chemotherapy and other immunotherapies in a variety of solid tumours.

In mesothelioma, there were 2 promising small phase II studies of Tremelimumab, which led to a double blind, randomised placebo control study, DETERMINE (Calabro et al., 2013, Calabro et al., 2015). However, this failed to meet its primary endpoint of overall survival in Tremelimumab versus placebo in unresectable pleural or peritoneal mesothelioma in the second and third line setting. (Astrazeneca 2015) Tremelimumab in combination with durvalumumab (an anti PD-L1 antibody) is being studied in a phase II trial (Calabro et al., 2018).

1.5.2 PD-1/PD-L1

PD1 is expressed on the surface of activated T cells. Blockade of PD1 significantly increases cytolytic activity of T cells and tumour cell lysis. Antigen recognition by T cells produces inflammatory cytokines that up-regulate PD-L1 expression in the tissue/tumours. PD1/PD-L1 ligation leads to immune tolerance (Alsaab et al., 2017).

The expression of PD-L1 has been reported in up to 45% of mesothelioma samples in different series, with a higher rate in sarcomatoid mesothelioma, and has been associated to poor prognosis (Marcq et al, 2015).

Anti-PD1 therapies such as Nivolumab and Pembrolizumab have been licensed for use as single agents and in combination in several solid tumours including melanoma and non-small cell lung cancer. A trial of Nivolumab (Fennell et al., 2018) is currently underway in mesothelioma.

An anti-PDL1 therapy, Avelumab (human anti-PD-L1 IgG1 monoclonal antibody) is licenced in the United States for use in metastatic merkle cell carcinoma.

PD-L1 blockade has also demonstrated promising efficacy in patients with mesothelioma. In a phase IB study, 20 patients with unresectable pleural or peritoneal mesothelioma, progressing after prior platinum/pemetrexed containing chemotherapy, were treated with avelumab, an anti-PD-L1 antibody (Hassan et al., 2016).

The association between PD-L1 expression and efficacy remains controversial, mainly because of the use of different companion tests and different cutoffs of positivity in the development of the various drugs (Ceresoli et al, 2016) In the avelumab study, mesothelioma patients were not selected based on PD-L1 positivity (Hassan et al., 2016).

1.5.3 TIM-3

T cell immunoglobulin mucin (TIM) 3 is up regulated in tumour antigen-specific CD8+ TILs (Sakaguchi et al., 2010). Anti-TIM3 antibodies have been shown to increase proliferation and cytokine production of these cells (Sakaguchi et al., 2010). The use of anti-TIM3 antibodies alone has not shown efficacy in pre-clinical models but have shown dramatic reductions in tumour growth when used in combination therapy (Sakaguchi et al., 2010).

A phase I-Ib/II open label trial is ongoing to assess safety and efficacy of anti-TIM3 mAbs as a single agent and in combination with PD1 mAbs in adults with advanced malignancies (NCT02608268). Another phase I trial is also on-going with a different anti-TIM3 antibody (NCT 02817633).

There is increasing interest in harnessing the potential of combination immunotherapy. This is already licenced for use in melanoma. However, the benefits of combination therapy must be balanced with the additional risk of toxicity.

Currently around 20% of patients derive significant benefit from immunotherapy. It is difficult to predict which patients will benefit and which treatment will offer the most benefit.

There has been increasing recognition that factors like the TME and tumour heterogeneity contribute significantly to clinical outcomes of patients with cancer. It is clear that one size does not fit all. It is likely that going forward a successful treatment strategy for cancer will involve understanding the immune and genetic profile of the individual patient's tumour in order to tailor a personalised treatment plan. With the increasing understanding of tumour signatures and

development of new therapies this is already in place for a select group of patients in the research setting. The ultimate goal is for stratified and personalised cancer treatment to become standard practice.

The work in this thesis aims to contribute to understanding the immune events in mesothelioma that influence the clinical outcome.

1.6 Investigative approaches for analysis of the anti-tumour immune response

A number of investigative approaches will be undertaken to further the understanding of the role immune cells in mesothelioma**.** The approaches are aimed at first characterizing and quantifying the phenotypes of infiltrating immune cells within the tumour and comparing them with cells in surrogate tissues (Blood and/or pleural fluid). Then attempts will be made to define the functional activities and intercellular interactions by more detailed study of cells extracted from the tumour environment. The methodological approaches include:

Immunohistochemistry (IHC)

Extraction of cells and analysis by Flow Cytometry (FACS)

Bulk RNA-seq/Single cell-RNAseq

Surrogate tissues: pleural fluid

1.7 Aims and Objectives of this thesis

1) The over-arching hypothesis to be tested in this thesis is that the immune response against mesothelioma is the critical determinant of the clinical outcome.
2) The secondary hypotheses are:
    a. The main effector cells contributing to the defence against mesothelioma are CD8+ CTL and T_{RM}
    b. The main effector cells contributing to the defence against mesothelioma are CD4+ CTL and T_{RM}
    c. The main effector cells contributing to the defence against mesothelioma are B cells and antibodies

3) Less differentiated mesotheliomas create a microenvironment that is anti-immune/pro-tumour

The hypotheses will be addressed as follows:

1) Define the gross nature of the immune response in mesothelioma – quantification of numbers of infiltrating lymphocytes and their phenotypes
2) Look for evidence that immune response correlates with clinical outcome
3) Define the phenotypes of infiltrating immune cells by IHC on biopsies and tumours
4) More detailed analysis of cells and their interactions – Bulk RNA-seq
    a. Seek confirmatory evidence in pleural fluid

Chapter 2 Materials and Methods

2.1 Study design

About 50 new cases of mesothelioma are diagnosed at University Hospital Southampton (UHS) each year.

A review identified that in the last 10 years, in the order of 500 cases have been evaluated and pathological tissue stored. Initially, formalin fixed, paraffin embedded tissue from patients with a known diagnosis of mesothelioma was retrieved from the pathology archive at UHS and where sufficient material was available, it was sampled for construction of a tissue microarray (TMA). This allowed up to 60 individual tumours to be analysed simultaneously, making multiple staining and comparison feasible.

The series of TMA was probed for markers for immune effector, including CD3, CD4, CD8, CD45RO (memory), FoxP3 (Treg), CD20 (B cells), CD56 (Natural Killer cells), neutrophils (NP57) macrophages (CD68) and also for WT1, which is used as a diagnostic marker for mesothelioma.

During the period of the study, patients with a clinically or radiologically suspected mesothelioma for which a pleural fluid aspiration or Video Assisted Thoracic Surgery (VATS)/Surgical Thoracoscopy was planned were approached.

A further group of patients with pleural effusions but not a suspected diagnosis of mesothelioma who were undergoing a planned diagnostic/therapeutic pleural aspiration were approached to participate in this study as a control group to enable comparisons to be made between pleural fluid samples taken from the two groups.

In those who were willing to participate, pleural fluid and where possible pleural biopsies were taken in the context of their planned procedure and stored fresh frozen. Samples of pleural fluid and pleural biopsies were taken in the context of clinical treatment so conferred minimal additional risk or burden.

Pleural fluid would usually be drained from the patient for diagnostic and therapeutic purposes during thoracic surgery and surplus pleural fluid that would otherwise be discarded was collected for study purposes. If the patient was having a diagnostic/therapeutic pleural fluid aspiration, an additional 200ml-2000ml of fluid, depending on the planned procedure being undertaken, was taken for research purposes.

At the time of the pre-operative/planned blood sampling wherever possible, or at a later date, 50 ml of additional blood was drawn for analysis of immune markers.

A review of patient notes including electronic case notes has enable correlation of immunohistochemical evaluation with clinical outcome in patients with mesothelioma.

2.1.1 Patient identification and recruitment

For the study of retrospective formalin fixed paraffin embedded tissue, records were identified from the histological database and review of patient records. Both male and female patients over the age of 18 with a diagnosis of mesothelioma were included. It was envisioned that up to 500 stored tissue samples would be available for analysis by TMA. Not all samples had sufficient material for analysis.

For the retrospective study of formalin fixed paraffin embedded tissue from the pathology archives, NHS consent for use of samples in research is part of the standard University Hospital Southampton (UHS) consent form.

No formal statistical calculations were performed as this was an exploratory study to understand the biology of the disease. From past experience with tissue microarray analysis the size of the effect (for example the effect of T cell density on outcome) cannot be predicted accurately and therefore as large a cohort of historical samples as possible was collected to build the tissue microarray.

For the prospective collection of blood, pleural fluid and where possible pleural biopsies; it was estimated there are 50 new cases of mesothelioma diagnosed at University Hospital Southampton per year. The aim was to recruit 50-75 patients with a suspected diagnosis of mesothelioma, and 50-75 patients with a pleural effusion but without a suspected diagnosis of mesothelioma, to the study. It was estimated that 50% of the total number of patients with a suspected diagnosis of mesothelioma would agree to participate in the study and aimed to recruit a similar number to the group with a pleural effusion without a suspected diagnosis of mesothelioma.

For prospective collection of samples, eligible patients with a potential clinical or radiological diagnosis of mesothelioma, or with a pleural effusion if part of the control group, were approached in the medical/surgical ward or outpatients department. Patient information sheets were provided to eligible patients inviting them to consider participation. Patients were undergoing planned pleural procedures/thoracic surgical intervention and consent for participation in the study was taken at the same time as consent for the planned procedure. In the inpatient or outpatient setting, consent is usually taken just before a pleural procedure and in

order to collect additional pleural fluid samples in the context of the planned procedure, these patients had less than 24 hours to consider taking part in the study. Specific REC approval was sought to allow this. Where possible however, patients were given 24 hours or longer to consider entry to the study. Having received prior written and verbal explanation of the study, patients who wished to participate were asked to provide written informed consent.

Consent included permission for prospective sampling of pleural fluid and blood and where possible an additional pleural biopsy to be taken and storage of samples for analysis relating to this study and for the use of FFPE histopathology material, where adequate surplus material existed following completion of full diagnostic work.

2.1.2 Study plan

2.1.2.1 Blood samples

A blood sample was taken at the pre-admission/speciality clinic or on the ward together with routine blood tests where possible. If this was not possible, the blood sample was taken on admission for surgery.

Each blood sample consisted of 40ml of anticogulated blood and 10ml of clotted blood from 1 venesection, 50ml of blood in total. These blood samples were necessary to identify circulating immune cells for correlation with immune cells found in the pleural fluid and pleural biopsies.

2.1.2.2 Pleural fluid and pleural biopsies

In the context of a planned diagnostic/therapeutic pleural fluid aspiration or chest drain insertion, additional pleural fluid that was surplus to diagnostic requirement was collected for the purposes of the study, the exact amount depended on the procedure being undertaken. In the context of a diagnostic pleural aspiration up to an additional 200-500ml might be collected. In the context of a therapeutic pleural aspiration or chest drain insertion, up to 2000ml might be collected.

During a VATS/Surgical Thoracoscopy all pleural fluid in the pleural cavity is removed for therapeutic purposes. A small sample of this pleural fluid is usually sent for diagnostic purposes but the rest is discarded. From this pleural fluid surplus to diagnostic requirements and would normally be discarded, up to 2000ml was taken for study purposes.

In addition to pleural biopsies taken for diagnostic purposes, one addition biopsy was taken for study purposes. In the event that the biopsies taken for diagnostic purposes were non-diagnostic, the pleural biopsy taken for study purposes was surrendered to the hospital pathologist for standard clinical procession rather than research.

Simple histopathological details were recorded and access to formalin-fixed paraffin-embedded tissue (FFPE) surplus to diagnostic requirement was requested for immunohistochemistry.

All samples were anonymised upon receipt in the laboratory and identified only by a unique patient identification number according to the standard operating procedure in place for this procedure (CSD/SOP/003). The sample number and date of receipt was also recorded. On conclusion of the study any remaining samples or tissue will be donated to the University of Southampton Research Human Tissue Authority (HTA) Tissue Bank (Human Tissue Act License 12009, Southampton and South West Hampshire Research Ethics Committee: 280/99).

2.1.3 Inclusion and Exclusion criteria

2.1.3.1 Inclusion criteria

For the study of retrospective paraffin embedded tissue:

Known diagnosis of mesothelioma

Patient aged 18 or over

For the prospective collection of pleural fluid/pleural biopsies and blood:

Suspected diagnosis of mesothelioma

Presence of pleural effusion in control group

Patient aged 18 or over

Patients with the ability to understand the study requirements and provide written informed consent.

2.1.3.2 Exclusion criteria

None identified (apart from the absence of consent)

2.1.4 End of Study Participation

Active participation in the study ceased following collection of blood, pleural fluid and where possible a pleural biopsy. Recording of histopathological details occurred once the final diagnostic histopathological report was available and correlation made with clinical outcome as part of the formation of a database.

End of study will be defined by acceptance of publication reporting on the data

2.1.5 Clinical and histopathological data collection

Where available, clinical and histopathological information was extracted and recorded onto study specific case report forms. Data points recorded included:

- Age at diagnosis
- Histological tumour type
- Immunohistochemical markers
- Clinical biomarkers such as haemoglobin, platelet count, albumin levels and performance status
- Treatment such as chemotherapy or radiotherapy
- Surgical interventions/Procedures such as VATS/Thoracoscopy/Thoracotomy/Chest drain insertion/Talc pleurodesis
- Length of time of survival from diagnosis (if applicable)

Data was extracted from patients clinical records (generally from computer based records of histopathology reports, blood results and patient records) held by Southampton University Hospitals NHS Trust by clinically trained research personnel with authorisation to access such details and stored within a secure facility within the Southampton CR-UK Clinical Centre. Data was anonymised at the point of entry onto the case report forms to allow it to be linked to the relevant sample, but with all identifying information removed. The Chief Investigators have retained overall responsibility for the recording and quality of the data. The link code and all personal data has been stored in a secure, access controlled location in the clinical trials office according to standard operating procedures of UHS/Cancer Sciences division and the University of Southampton Research Human Tissue Authority (HTA) licensed tissue bank.

2.1.6 Statistical analysis

This study aimed to conduct an exploratory analysis of immune events in mesothelioma. No formal statistical calculations were undertaken to arrive at a sample size.

Retrospective samples of tissue from patients with an established diagnosis of mesothelioma over the last 10 years were accessed for analysis. We expected there to be approximately 500 samples, but we expected that not all of these would have sufficient material for analysis. The analysis has studied markers of immune function, correlated with mesothelioma sub-type and clinical outcome.

Prospective samples of blood, pleural fluid and where possible pleural biopsies were collected from patients with a suspected diagnosis of mesothelioma and patients with a pleural effusion without suspected mesothelioma. Markers of immune cell type and function from the samples from patients in the 2 groups will be compared as a full set using statistical tests like the independent samples t-test if the results are normally distributed or the Mann-Whitney test if the results are not normally distributed. .

The statistical packages PRISM and SPSS have been utilised for statistical analysis.

2.1.7 Sample processing and storage

Historical samples: Analysis of paraffin embedded tissue samples of known mesothelioma from the pathology archives were undertaken in a linked anonymised fashion.

Prospective sampling: Informed consent was sought. Samples were anonymised and stored in the Southampton CRUK centre. All analyses were undertaken on samples that are identified only by a unique laboratory sample number.

Blood samples were taken by the clinical team in the context of planned/pre-assessment blood tests where possible and processed on the same day. Peripheral blood mononuclear cells (PBMCs) were isolated from anti-coagulated blood by centrifugation over Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) and either used immediately or cryopreserved in 50% decomplemented human antibody serum, 40% complete RPMI 1640 medium and 10% DMSO. Cells were transferred to liquid nitrogen after 24 hours. Serum was isolated from clotted blood samples by centrifugation and stored at -80°C.

Pleural fluid samples were taken directly to the laboratory on the day of collection, 30mls of pleural fluid was snap frozen in liquid nitrogen. The remaining pleural fluid was centrifuged and 30ml of supernatant was fresh frozen at -80 C. Red cell lysis was undertaken on the cell pellet. A cell count was undertaken with Tryptan Blue and Improved Neubauer Haemocytometer according the standard operating procedure CSD/SOP/306. The cell pellet was cryopreserved in 50% decomplemented human antibody serum, 40% complete RPMI 1640 medium and 10% DMSO. Cells were transferred to liquid nitrogen after 24 hours.

Pleural biopsies were placed in ice and flash frozen in liquid nitrogen to provide for specimen integrity and a wide array of options for tissue analysis. The specimens were placed into a sterile 2 ml cryovial, which was then tightly capped and submerged in liquid nitrogen for "flash freezing". The vials were transferred from the temporary LN2 transport container to a liquid nitrogen storage tank for long-term storage.

Patient material collected during this study has been stored within a secure facility in the University of Southampton Research facility. Material will be held as linked anonymised samples and labelled with a study specific number. Patients were asked to provide signed consent for the indefinite storage of samples for use by the investigators for analyses relating to the objectives of this study, or in the event of samples or tissue remaining on conclusion of the study, for the samples to remain in the University of Southampton Human Tissue Authority tissue bank for use in future ethically approved research.

2.1.8 Data Storage

All essential documents, including source documents, will be retained for a minimum period of 5 years following the end of the study until data evaluation is complete. Analytical data from this study will be stored electronically on password protected data files on workstations within the Southampton CR-UK Clinical Centre by the investigators in a secure, access controlled environment following the standard operating procedure of the UHS/UoS and the UoS HTA licensed tissue bank until the final report of the project has been submitted and accepted for publication.

Patient confidentiality was and continues to be maintained by removing patient identifiable labels other than an assigned study specific number to create linked anonymised samples.

Data will be collected and retained in accordance with the Data Protection Act 1998.

2.1.9 Compliance with regulations

This study was performed subject to REC and R&D approval.

The study was conducted in accordance with the Data Protection Act 1998, Human Tissue Act 2004, International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines; and the Research Governance Framework for Health and Social Care.

2.1.10 Funding

This study was funded through the Mesothelioma Applied Research Foundation: The Lance S. Ruble, Janelle Bedel and Ferraro Law Firm Grant and the Wessex CRN.

2.2 Tissue Microarray

2.2.1 Case selection

Ethical approval for this study (NRES Southampton & South West Hampshire LREC 10/H0504/32) was in place at our institution.

Patients included in the cohort had a pathological diagnosis of mesothelioma dated at least 2 years prior to analysis. 302 consecutive formalin fixed paraffin embedded (FFPE) tissue blocks archived between 2004 and 2012 were assessed by haematoxylin-eosin (H&E) staining to ensure they contained sufficient tissue for further immunohistochemical evaluation. A tissue microarray (TMA) was generated from this cohort. Some cases could not be evaluated as there was tissue loss in the formation of the TMA. A subcohort of ~170 cases was evaluated for CD3+ T-cells and CD56+ natural killer cells. All samples were taken at the time of diagnostic biopsy or surgical intervention when no systemic treatment had been given.

Clinical data was collected from patient records. Where survival data was not available locally, the date and cause of death was retrieved from the national cancer registry (Public Health England) following a formal data-access application.

2.2.2 Histopathological analysis-Tissue microarray

Tumour histology was re-reviewed by pathologists GJT, KAM and SG. H&E stained slides from all available cases were assessed by a consultant pathologist and three representative areas of tumour were marked for TMA construction.

Triplicate 1mm cores were taken from the corresponding formalin fixed paraffin embedded tissue block using a semi-automated system (Aphelys Minicore 2, Mitogen, Harpenden, UK) to generate a TMA as previously (Ward et al., 2014). 4μm TMA sections were used for haematoxylin-eosin and immunohistochemical staining. The formation of the TMA and subsequent IHC staining, was undertaken by the Research Pathology team at UHS.

Immunohistochemistry was performed using an automated platform (Dako Autostainer) in a CPA-accredited clinical cellular pathology department using antibodies optimized to national diagnostic standards (NEQAS). The antibodies used were anti-human CD3 1:200 (clone F7.2.38; Dako, Carpinteria, California, USA), anti-human CD4 1:50 (clone 4B, Dako), CD8 RTU (Clone C8/14 4B, Dako), CD20 1:250 (Clone L26, Dako), CD45RO 1:2500 (Clone UCHL-1, Dako), FoxP3 1:100 (Clone 236A/E7, AbCam, Cambridge, UK), CD68 RTU (Clone PG-M1, Dako, NP57 1:100 (Dako), CD56 RTU (Clone 123C3, Dako), Wilms Tumour 1 RTU (Clone 6F-H2, Dako).

Triplicate random high-power fields (x400) were counted manually per core across three cores on the Olympus dotSlide (SC). The average of 3-9 counts per patient was calculated to allow for intra-tumoural heterogeneity. The variability in the number of counts per patient relates to the number of viable cores present on the TMA.

The mean of each antibody count was taken as the cutoff point between high and low counts.

2.2.3 Statistical analysis

Statistical analyses were performed using SPSS Version 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Survival time was measured from the time of diagnosis to the time of death. Patients for whom the date of death was unknown were censored from the survival analyses. Kaplan-Meier plots with log-rank tests, univariate and multivariate Cox proportional hazard models were used to analyse the survival data. A p-value of <0.05 was considered significant.

The cohort was divided into epithelioid and non-epithelioid (biphasic and sarcomatoid) mesothelioma sub-types for the purposes of analysis as there were only 41 cases of sarcomatoid mesothelioma.

2.3 Multiplex immunohistochemistry of epithelioid mesothelioma

2.3.1 Case selection

Patients included in this cohort had a known diagnosis of epithelioid mesothelioma. The time from diagnosis to death for these patients was known. In total, 44 patients were selected for this study, 24 patients with good survival and 20 with poor survival. Good survival was taken as survival of greater than 800 days (26 months) from the date of diagnosis and poor survival was taken as survival of greater than 30 days but less than 150 days (5 months). These figures were chosen, as the average survival for patients with a diagnosis of mesothelioma is 4-18 months.

2.3.2 Histopathological analysis

A multiplexed immunohistochemistry (IHC) method was utilised for repeated staining of a single paraffin-embedded tissue slide. The slide was first stained with a standard primary antibody followed by a biotin-linked secondary antibody and horseradish peroxidase (HRP)-conjugated streptavidin to amplify the signal. Peroxidase-labelled compounds were revealed using 3-amino-9-

ethylcarbazole (AEC), an aqueous substrate that results in red staining, and counter stained using hematoxylin (blue). The slide was scanned at high resolution. The coloured reaction product was then removed using an organic solvent-based de-staining buffer. The process was repeated for each of the antibodies on the same slide.

The antibodies used were CD4 RTU (Clone 4B12 Dako), CD8 RTU (Clone IR2361-2 Dako), CD103 1:500 (Clone EPR4155(2) AbCam), Granzyme B 1:50 (Clone GrB-7 Dako), CD69 1:200 (Clone Ag1094 Proteintech), FOXP3 1:100 (Clone 236A/E7 AbCam), BCL6 1:30 (Clone PG-B6p Dako), CXCL13 1:100 (Polyclonal, product code PA5-47035 Invitrogen), TIM3 1:50 (Clone D5D5R Cell Signalling), WT1 RTU (Clone 6F-H2 Dako), anti HLA Class 1 1:500 (Clone EMR8-5 AbCam), anti HLA-DPB1 1:2500 (Clone ERP11226 AbCam).

Bright field images were inverted in imaging processing software (Image J) and red green blue channels were separated to create pseudo-colour images.

Multiplex IHC is an umbrella term for a variety of techniques that enable the same piece of tissue to be stained with multiple antibodies and evaluated. This can be achieved by the simultaneous use of multiple fluorescent dyes, using different colours. This approach uses proprietary platforms such as the Vectra system and is not currently available in our centre. An alternative approach is to use a single or small number of traditional chromogens, and to remove and/or quench the antibody-dye complex after capturing the image of staining by each antibody. The 'stripping' allows a 2nd staining, followed by repeated image acquisition. By repeating this process multiple times, many features (at the moment in our laboratory up to 7 stains) can be evaluated on one slide. Digital manipulation is then used to create 'pseudo colour' to represent each stain and thus allows visual and digital evaluation of the location and quantity of particular features in tissue.

There are several benefits of multiplex IHC over single stained IHC. With multiplex IHC, compound images can be constructed showing multiple cell types simultaneously. Hence, the spatial relationship between cell types within the tumour microenvironment can be appreciated and co-expressed markers can be evaluated. This allows detailed phenotypic differentiation of the cells of interest. Multiplex IHC conserves tissue, which is important as the tissue sample may be required for an increasing number of diagnostic and research tests to allow the patient access to treatment options. The same tissue slice can be stained for multiple markers sequentially and can even be stained with different coloured antibodies at the same time, thus further increasing the number of potentially identifiable features such as cell types or intracellular features.

The downside to multiplex staining is that it needs to be performed on full-face sections of tissue, which is costly. Full-face tissue is required as smaller tissue sections as used in TMAs, are likely to degrade in the process of multiple staining and elution of antibodies. The process is also time consuming as each stain/image acquisition/strip cycle takes roughly a working day; thus 5 'colours' take a week to complete. A greater number of antibody stains on the tissue can present challenges for interpretation of the multiplex IHC and the complex image evaluation is time consuming in its own right.

2.3.3 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0c for Mac OS X GraphPad Software, La Jolla California USA. Survival time was measured from the time of diagnosis to the time of death. Mann-Whitney U and Spearman R tests were used to analyse the data. A p-value of <0.05 was considered significant.

2.4 FACS and RNAseq Mesothelioma associated pleural fluid

2.4.1 Case selection

Ethical approval for this study, NRES Committee South West Cornwall and Plymouth 13/SW/0128 was in place at our institution.

Patients included in the cohort had a suspected diagnosis of mesothelioma and were undergoing either a therapeutic pleural aspiration or VATS surgical drainage of their pleural effusion between 2013 and 2015. Pleural fluid samples were processed as described in section 2.1.7. The cell pellet from the pleural fluid was cryopreserved and stored in liquid nitrogen. Cryopreserved cell pellets from 23 patients with a confirmed diagnosis of mesothelioma were utilised for bulk sorting by flow cytometry and RNAseq analysis.

Clinical data was collected from patient records.

2.4.2 Histopathological analysis

Tumour histology was reviewed as part of routine clinical care.

Immunohistochemistry was performed on whole face sections using an automated platform (Dako Autostainer) in a CPA-accredited clinical cellular pathology department using antibodies optimized to national diagnostic standards (NEQAS). The antibodies used were anti-human CD4 1:50 (clone 4B, Dako), CD8 RTU (Clone C8/14 4B, Dako).

Nine randomly selected high-powered fields (x400) were counted manually across each section. The average of 9 counts per patient was calculated to allow for intra-tumoural heterogeneity. The cutoff point between high and low counts was taken as per the mean values of CD4+ and CD8+ T cells from the mesothelioma TMA counts described in Chapter 3. This value was 15.1 for CD4+ T cells and 20.8 for CD8+ T cells.

2.4.3 FACS sorting

Cryopreserved cells from pleural fluid were defrosted and re-suspended in MACS buffer (phosphate-buffered saline containing 2mM EDTA and 0.5% bovine serum albumin) for flow sorting. The cells were incubated with FcR block (Miltenyi Biotec) then stained with anti-CD45-FITC (HI30; Biolegend), anti-CD4-PE (RPA-T4; BD Biosciences), anti-CD3-PE-Cy7 (SK7; Biolegend), anti-CD8a-PerCP-Cy5.5 (cSK1; BD Biosciences), anti-HLA-DR-APC (L243; BD Biosciences), anti-CD14-APC-H7 (MϕP9; BD Biosciences), anti-CD19-PerCP-Cy5.5 (clone HIB19; Biolegend) and anti-CD20- PerCP-Cy5.5 (clone 2H7; Biolegend) antibodies. Dead cells were excluded using DAPI (D9542; Sigma Aldrich). Stained samples were analyzed using BD FACSAriaTM (BD Biosciences) and FlowJo software (Treestar), and CD4+ and CD8+ T cells were sorted into ice-cold TRIzol LS reagent (Ambion).

2.4.4 RNA sequencing

Total RNA was purified using a miRNAeasy micro kit (Qiagen, USA). Purified total RNA (5 ng) was amplified following the smart-seq2 protocol. cDNA was purified using AMPure XP beads (1:1.1 ratio, Beckman Coulter). From this step, 1 ng of cDNA was used to prepare a standard Nextera XT sequencing library (Nextera XT DNA sample preparation kit and index kit, Illumina). Samples were sequenced using HiSeq2500 (Illumina) to obtain 50-bp single-end reads. Quality control steps were included to determine total RNA quality and quantity, optimal number of PCR pre-amplification cycles, and cDNA fragment size. Samples that failed quality control were eliminated from further downstream steps.

2.4.5 RNAseq analysis

RNA-Seq data was mapped against the hg19 reference using TopHat (v1.4.1., --library- type fr-secondstrand -C) and the RefSeq gene annotation downloaded from the UCSC Genome Bioinformatics site. Sequencing read coverage per gene was counted using HTSeq-count (-m union -s yes -t exon -i gene_id, http://www-huber.embl.de/users/anders/HTSeq/). To identify genes differentially expressed between patient groups, we performed negative binomial tests for

paired and unpaired comparisons by employing the Bioconductor package DESeq2 disabling the default options for independent filtering and Cooks cutoff. Genes were considered differentially expressed between any pairwise comparison when the DESeq2 analysis resulted in a Benjamini-Hochberg–adjusted *P* value < 0.05. The Qlucore Omics Explorer 3.2 software package was used for visualization and representation (heat maps, principal component analysis) of RNA-Seq data.

2.4.6 Knowledge-based network generation and pathway analysis

The biological relevance of differentially expressed genes identified by DESeq2 analysis (significantly impacted pathways, biological processes, molecular interactions) was further investigated using the Adviabio iPathwayGuide (https://www.advaitabio.com/ipathwayguide) and the ToppGene Suite (http://toppgene.cchmc.org).

2.4.7 Statistical analysis

For the DESeq2 analysis, a Benjamini-Hochberg–adjusted *P* value of <0.05 was considered significant.

Chapter 3 Tissue Microarray

3.1 Introduction

The role of the immune system in cancer is well established and is critically involved in immunoediting and surveillance; tumour progression results from immune escape (Dunn et al., 2004, Schreiber et al., 2011). In many solid tumours, tumour infiltrating lymphocytes (TILs) confer a survival benefit (Galon et al., 2006, Al-Shibli et al., 2008, Garcia-Martinez et al., 2014, Ward et al., 2014, Gooden et al., 2011).

In mesothelioma, the impact of TIL density is less clear. Previous small surgical studies have suggested that tumour CD8+ T-cell infiltration is associated with better survival (Yamada et al., 2010, Anraku et al., 2008). More recently, a larger series demonstrated tumour CD4+ T-cell infiltration confers a survival advantage in epithelioid mesothelioma (Ujiie et al., 2015). These three cohorts consisted mainly of patients who were fit enough to undergo surgical resection.

The link between T-cell density in the tumour and outcome for advanced disease is not resolved. The work presented sought to characterise the link between morphological density of immune cells in mesothelioma and outcome in a predominately treatment naïve cohort. An assessment of adaptive and innate immune cells in a cohort of 302 patients from a large UK centre was undertaken, using immunohistochemical evaluation of immune cells.

The mesothelioma tissue microarray (TMA) was evaluated for T cells (CD3+ cells), followed by an assessment of helper T cells (CD4+ cells), cytotoxic T cells (CD8+ cells), memory T cells (CD45RO+ cells) and regulatory T cells (FoxP3+ cells). The other adaptive immune cells evaluated were B cells (CD20+ cells), along with cells of the innate immune system; Macrophages (CD68+ cells), Neutrophils (NP57+ cells) and NK cells (CD56+ cells). The tumours were also evaluated for Wilms tumour 1 (WT1) expression.

The WT1 gene is believed to contribute to growth and differentiation of the mesothelium. It is a positive marker for mesothelioma with a sensitivity of 72-91% and specificity of 88-100% (Woolhouse et al., 2018). It is used as part of a panel to differentiate between mesothelioma and adenocarcinoma. WT1 has been evaluated as a prognostic marker for mesothelioma in a smaller series (Cedres et al., 2014).

3.2 Results

Clinical and demographic characteristics of our patient cohort are shown in Table 1. The median age was 72 (41-90) years, 81% of the patients were men. Of the 302 tumours, 172 were classified as epithelioid (57%), 82 as biphasic (27%) and 41 as sarcomatoid (14%) subtypes. Seven cases (2%) had an undefined morphology. The median follow-up was 278 days (9.3 months) and the minimum follow-up time was 30 days. There were 293 deaths from mesothelioma over the study period. Survival analysis by histological subtype was consistent with published data. The median overall survival for epithelioid mesothelioma in our cohort was 342 days (11.4 months) (95% CI 277-406 days) and for non-epithelioid mesothelioma was 205 days (6.8 months) (95% CI 146-260 days), with 216 days (7.2 months) (95% CI 152-279 days) for biphasic mesothelioma and 187 days 6.2 months) (95% CI 112-261 days) for sarcomatoid mesothelioma (Table 1).

Of the 302 patients, 259 (86%) underwent palliative symptom management with thoracoscopy and pleural biopsy followed by talc pleurodesis; and 7 (2%) underwent image guided pleural biopsy. 36 (12%) underwent a radical surgical approach including decortication/pleurectomy/ pleuro-pneumonectomy (Table 1).

Post diagnosis treatment data was available from 166 patients. Of these 61 (37%) underwent chemotherapy and 105 (63%) did not. Treatment data was not available for 136 patients who had been referred to our centre for diagnostic procedures but were then managed at other sites (Table 1).

Table 1 Demographics of patient cohort

Variable		**N (%)**	**Median OS days (months)**	**95% CI**	**p-value**
Overall group		302(100%)	278 (9.2)	247 to 308	
Age	<65	63 (21%)	349 (11.6)	257 to 440	0.157
	≥65	239 (79%)	263 (8.7)	229 to 296	
Sex	Male	243(81%)	275 (9.2)	243 to 306	0.682
	Female	59 (19%)	291 (9.7)	194 to 387	
Subtype	***Epithelioid***	172 (57%)	342 (11.4)	277 to 406	**<0.0001**
	Non-epithelioid	130 (43%)	205 (6.8)	146 to 260	
	Biphasic	82 (27%)	216 (7.2)	152 to279	
	Sarcomatoid	41 (14%)	187 (6.2)	112 to261	
	Undefined	7 (2%)			
Intervention					
Chemotherapy status known	Overall	166	280 (9.3)	239 to 320	**<0.0001**
	Yes	61 (37%)	460 (15.3)	360 to 559	
	No	105 (63%)	205 (6.8)	159 to 250	
Chemotherapy status unknown		136			
Surgery	Overall	295	279 (9.3)	247 to 310	0.735
	Radical	36 (12%)	330 (11.0)	185 to 474	
	Palliative	259 (86%)	274 (9.1)	242 to 305	
No Surgery		7(2%)			

OS Overall survival, CI Confidence interval, significant log rank p value<0.05

We examined expression of WT1 in this unselected cohort of patients with mesothelioma. A representative image of low and high expression of WT1 is shown in Figure 1. There is heterogeneity in expression of WT1 ranging from weak to strong, but this was not associated with differential survival in either morphology in our dataset.

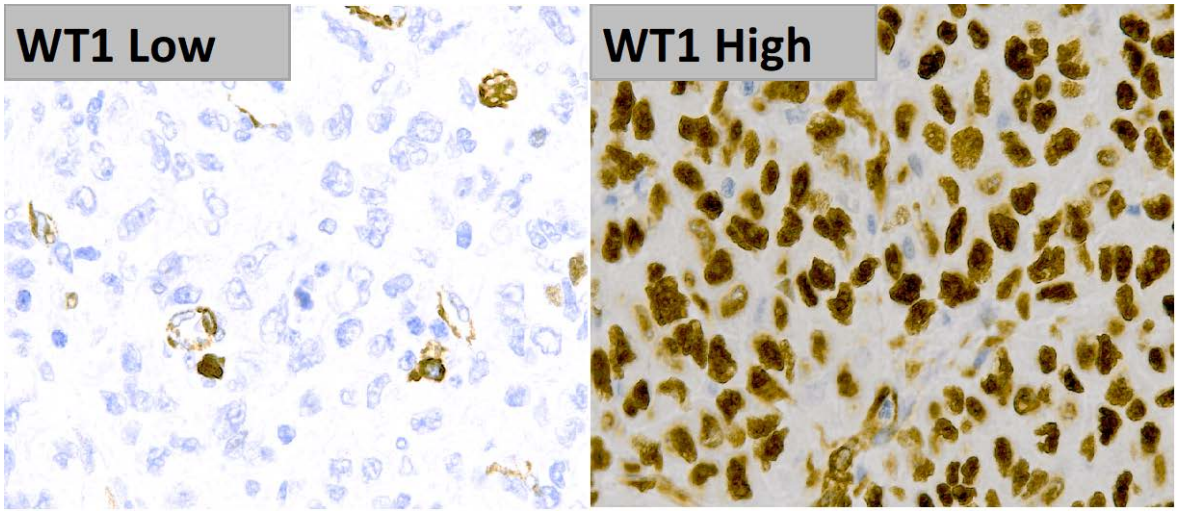


Figure 1 Representative low and high WT1 tumour cell densities (x400) magnification. Chromogenic IHC of WT1 showing nuclear staining (Brown) with weak background staining of FFPE human mesothelioma cells. Nuclear counterstaining with haematoxylin (Blue).

The adaptive immune response, particularly involving cytotoxic T-cells, has been shown to influence the prognosis of many tumour types. We examined expression of markers for T-cells (CD3+), helper T-cells (CD4+), cytotoxic T-cells (CD8+), memory T-cells (CD45RO+), regulatory T-cells (FOXP3+), and B-cells (CD20+) in the epithelioid and non-epithelioid subtypes. Representative images of CD4+ and CD8+ T cells are shown in Figure 2.

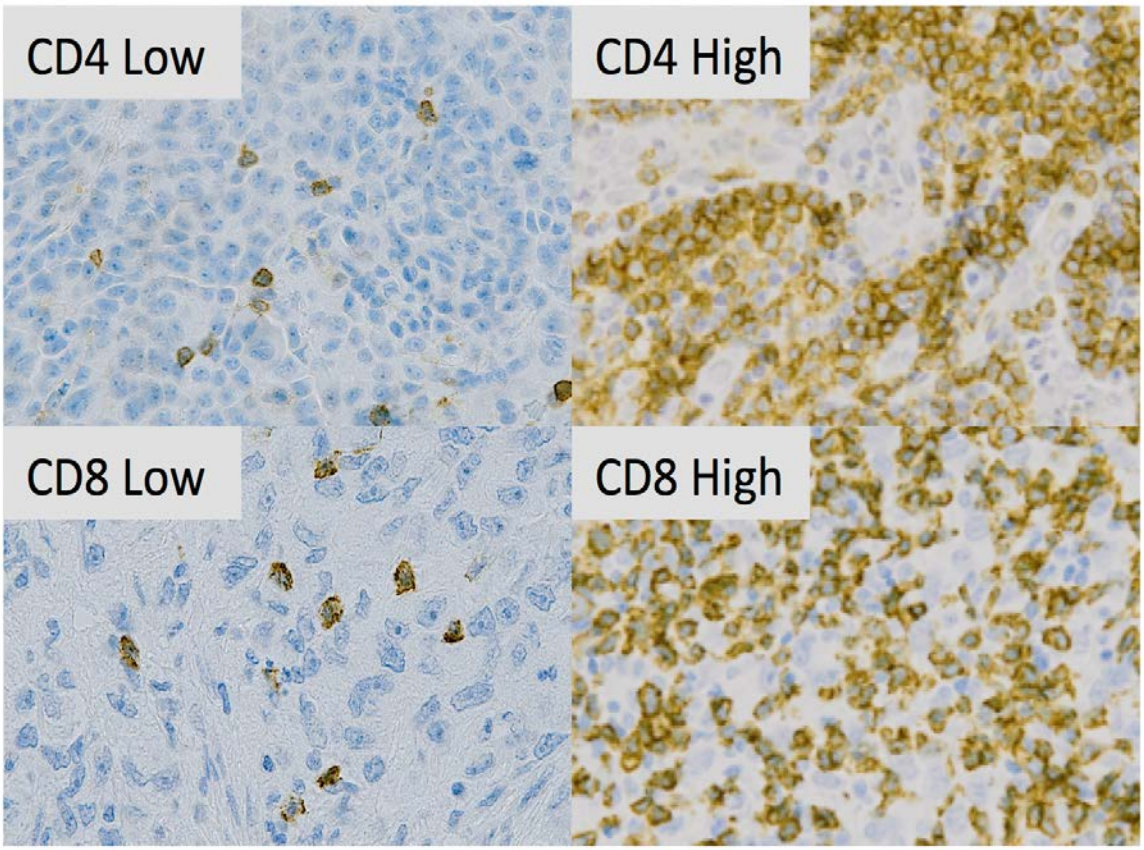


Figure 2 Representative low and high T cell densities (x400) magnification. Chromogenic IHC of CD4 showing membranous staining (Brown) and CD8 showing brown membranous and cytoplasmic staining (Brown). Nuclear counterstaining with haematoxylin (Blue).

The tumour microenvironment also contains innate immune cells and evaluation of the expression of markers of macrophages (CD68+), neutrophils (NP57+) and natural killer cells (CD56+) was undertaken.

The mean count/high power field was used to define the cut point between high and low values per marker evaluated. Median overall survival with 95% confidence interval and p-values for the epithelioid and non-epithelioid groups are shown in Table 2.

Table 2 Univariate analysis of overall survival and immune parameters

Marker		Mean counts /high powered field	N (%)	Median OS days (months)	95% CI	p-value
Adaptive						
CD3	Epithelioid		84	410 (13.7)	348 to 471	0.387
		Low <34	61 (73%)	**373** (12.4)	287 to 458	
		High >34	23 (27%)	**490** (16.3)	305 to 674	
	Non-epithelioid		86	170 (5.7)	124 to 215	0.123
		Low <34	48 (56%)	170 (5.7)	102 to 237	
		High >34	38 (44%)	169 (5.6)	126 to 211	
CD4	Epithelioid		158	319 (10.6)	250 to 387	**0.005**
		Low <15.1	107 (68%)	**275** (9.2)	240 to 309	
		High >15.1	51 (32%)	**490** (16.3)	350 to 629	
	Non epithelioid		126	205 (6.8)	150 to 259	0.383
		Low <15.1	87 (69%)	212 (7.1)	145 to 278	
		High >15.1	39 (31%)	185 (6.2)	117 to 252	
CD8	Epithelioid		172	342 (11.4)	277 to 406	0.983
		Low <20.1	123 (72%)	342 (11.4)	264 to 419	
		High >20.1	49 (28%)	373 (12.4)	186 to 559	
	Non epithelioid		130	205 (6.8)	149 to 260	**0.019**
		Low <20.1	80 (62%)	**278** (9.3)	228 to 327	
		High >20.1	50 (38%)	**152** (5.1)	130 to 173	
CD45RO	Epithelioid		159	342 (11.4)	276 to 407	0.339
		Low <22	121 (76%)	342 (11.4)	275 to 408	
		High >22	38 (24%)	296 (9.9)	81 to 510	
	Non-epithelioid		128	203 (6.8)	147 to 258	0.105
		Low <22	77 (60%)	252 (8.4)	176 to 327	
		High >22	51 (40%)	164 (5.5)	129 to 198	
FOX P3	Epithelioid		158	369 (12.3)	285 to 452	**0.024**
		Low <4	112 (71%)	**374** (12.5)	314 to 433	
		High >4	46 (29%)	**272** (9.1)	214 to 329	
	Non-epithelioid		127	205 (6.8)	146 to 263	**0.012**
		Low <4	74 (58%)	261 (8.8)	173 to 348	

		High >4	53 (42%)	141 (4.7)	116 to 165	
Marker		**Mean counts /hpf**	**N (%)**	**Median OS days (months)**	**95% CI**	**p-value**
CD20	Epithelioid		155	313 (10.4)	245 to 380	**0.008**
		Low <15	104 (67%)	**279** (9.3)	220 to 337	
		High >15	51 (33%)	**488** (16.2)	358 to 617	
	Non-epithelioid		125	197 (6.6)	147 to 246	0.227
		Low <15	104 (83%)	205 (6.8)	141 to 268	
		High >15	21 (17%)	185 (6.2)	117 to 252	
Innate						
CD68	Epithelioid		164	341 (11.4)	275 to 406	**0.026**
		Low <27	111 (68%)	**373** (12.4)	277 to 468	
		High >27	53 (32%)	**284** (9.5)	200 to 367	
	Non-epithelioid		126	205 (6.8)	149 to 260	0.927
		Low <27	62 (49%)	247 (8.2)	175 to 318	
		High >27	64 (51%)	164 (5.5)	98 to 229	
NP57	Epithelioid		158	313 (10.4)	242 to 383	**0.006**
		Low <1.6	129 (82%)	**356** (11.9)	264 to 447	
		High >1.6	29 (18%)	**272** (9.1)	202 to 341	
	Non-epithelioid		120	197 (6.6)	150 to 243	0.291
		Low <1.6	104 (87%)	203 (6.8)	145 to 260	
		High >1.6	16 (13%)	164 (5.5)	105 to 222	
CD56	Epithelioid		89	426 (14.2)	364 to 487	0.786
		Low <3	69 (78%)	410 (13.7)	342 to 477	
		High >3	20 (22%)	438 (14.6)	96 to 779	
	Non-epithelioid		83	177 (5.9)	132 to 221	0.239
		Low <3	61 (73%)	169 (5.6)	138 to 199	
		High >3	22 (27%)	247 (8.2)	130 to 363	
Other						
WT1	Epithelioid		160	319 (10.6)	246 to 391	0.802
		Low <42	68 (42%)	319 (10.6)	232 to 405	
		High >42	92 (58%)	312 (10.4)	203 to 420	
	Non-		126	197 (6.6)	143 to 250	0.875

	epithelioid	Low <42	102 (81%)	185 (6.2)	135 to 234	
		High >42	24 (19%)	212 (7.1)	151 to 272	

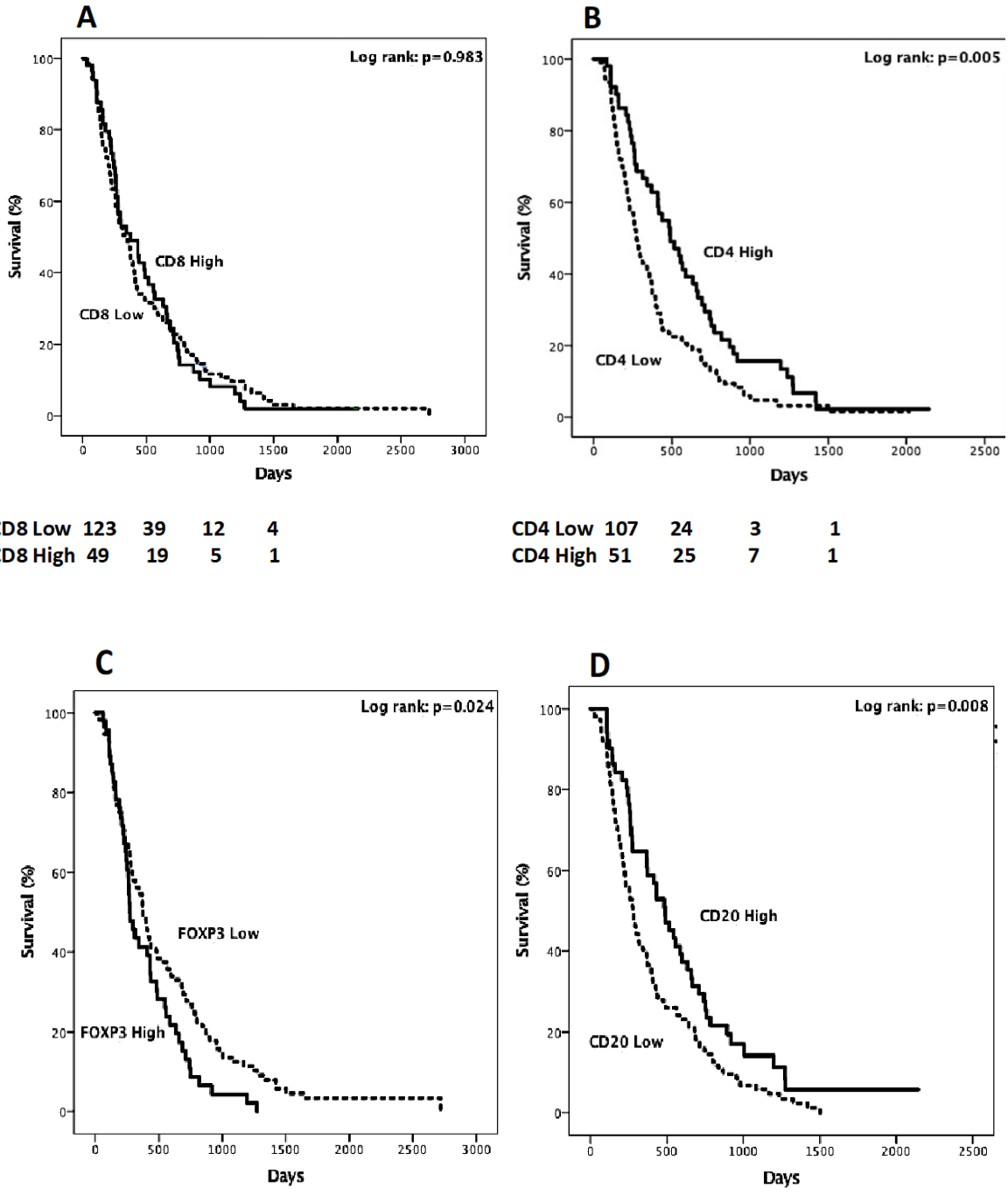
OS Overall survival, CI Confidence interval, significant log rank p value <0.05

The mean value of CD4+ and CD8+ T cell counts in this mesothelioma cohort was compared to CD4+ and CD8+ T cell counts from a non-small cell lung cancer cohort (Table 3). The mean values for Mesothelioma and Lung cancer were similar

Table 3 Mean values of CD4+ and CD8+ counts in Mesothelioma and Non-small cell lung cancer

	Number	Range	Mean	Standard deviation
Epithelioid Mesothelioma CD4+ cells	155	0.00 to 142.33	16.57	22.04
Non small cell lung cancer CD4 + cells	54	1.67 to 38.11	16.32	9.68
Epithelioid Mesothelioma CD8+ cells	167	0.11 to 57.00	16.11	12.74
Non small cell lung cancer CD8+ cells	116	0.30 to 93.30	15.56	14.07

In the epithelioid group, CD8+ T-cell counts were not associated with survival (p=0.983) (Figure 3A). However, high CD4+ (p=0.005); low FOXP3+ (p=0.024) and high CD20+ counts (p=0.008) were associated with a better outcome in epithelioid mesothelioma. The relevant Kaplan-Meier curves are shown in Figures 3B, 3C and 3D. These markers were associated with a longer overall survival of 7, 3 and 7 months respectively compared to the CD4+ low, FOXP3+ high and CD20+ low groups.



CD8 Low	123	39	12	4
CD8 High	49	19	5	1

CD4 Low	107	24	3	1
CD4 High	51	25	7	1

FOXP3 Low	112	43	13	4
FOXP3 High	46	13	2	0

CD20 Low	104	27	7	0
CD20 High	51	24	5	2

Figure 3

Kaplan-Meier survival curves of adaptive immune markers associated with survival in epithelioid mesothelioma (A) Kaplan-Meier curves for epithelioid mesothelioma survival

according to CD8+ T cell counts (log-rank test, P=0.983) (B) Kaplan-Meier curves for epithelioid mesothelioma survival according to CD4+ T-cell counts (log-rank test, P= 0.005) (C) Kaplan-Meier curves for epithelioid mesothelioma survival according to FOXP3+ T-regulatory cell counts (log-rank test, P=0.024) (D) Kaplan-Meier curves for epithelioid mesothelioma survival according to CD20+ B-cell counts (log-rank test, P=0.008). The numbers under the Kaplan Meier curves show the number of patients alive at Time point 0, 500, 1000 and 1500 days respectively for low and high counts for each marker analysed.

In the non-epithelioid group, positive prognostic markers were low CD8+ (p=0.019) and low FOXP3+ (p=0.012) T-cell counts. Kaplan-Meier curves are shown in Figure 4A and 4B. Both markers were associated with a 4 month longer overall survival in the non-epithelioid group compared to the CD8+ high and FOXP3+ high groups.

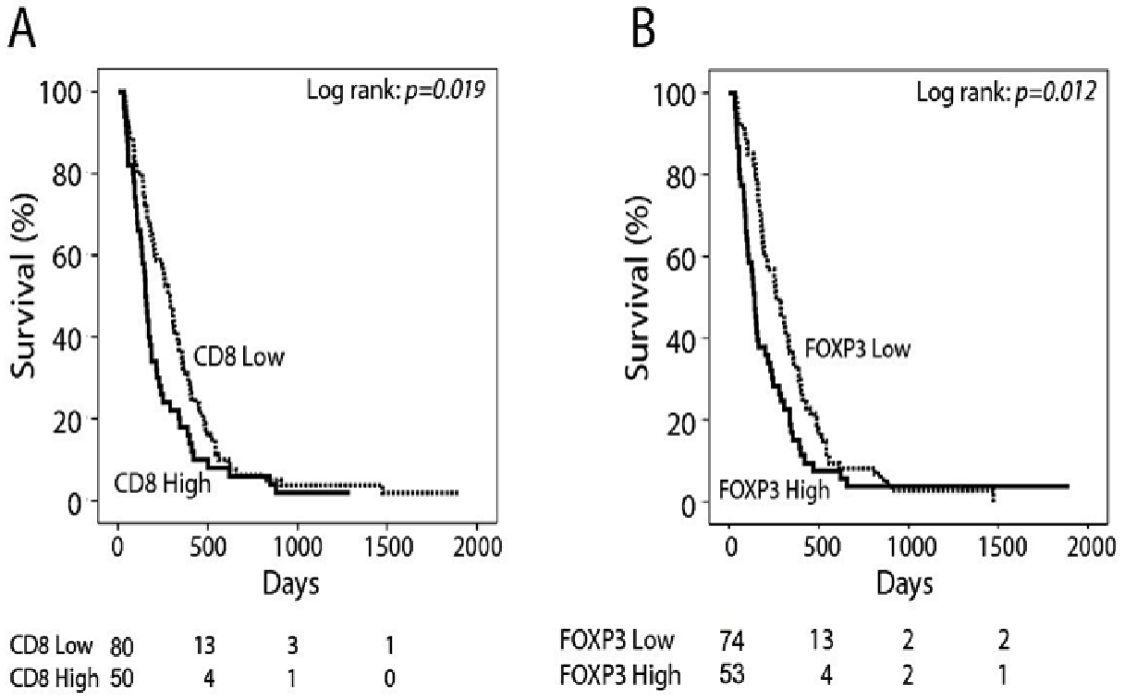


Figure 4

Kaplan-Meier survival curves of adaptive immune markers associated with survival in non-epithelioid mesothelioma (A) Kaplan-Meier curves for non-epithelioid mesothelioma survival according to CD8+ T-cell counts (log-rank test, P=0.019) (B) Kaplan-Meier curves for non-epithelioid mesothelioma survival according to FOXP3+ T-regulatory cell counts (log-rank test, P=0.012). The numbers under the Kaplan Meier curves show the number of patients alive at Time point 0, 500, 1000 and 1500 days respectively for low and high counts for each marker analysed.

In the epithelioid group, it was found the positive predictors of outcome from innate immune cells were low CD68+ (p=0.026) and low NP57+ (p=0.006) counts. Kaplan-Meier curves are shown in Figures 5A and 5B. These markers were both associated with a 3 month longer overall survival in the epithelioid group compared to the CD68+ high and NP57+ high groups. In the non-epithelioid group, none of the markers of innate immunity tested were significantly associated with survival.

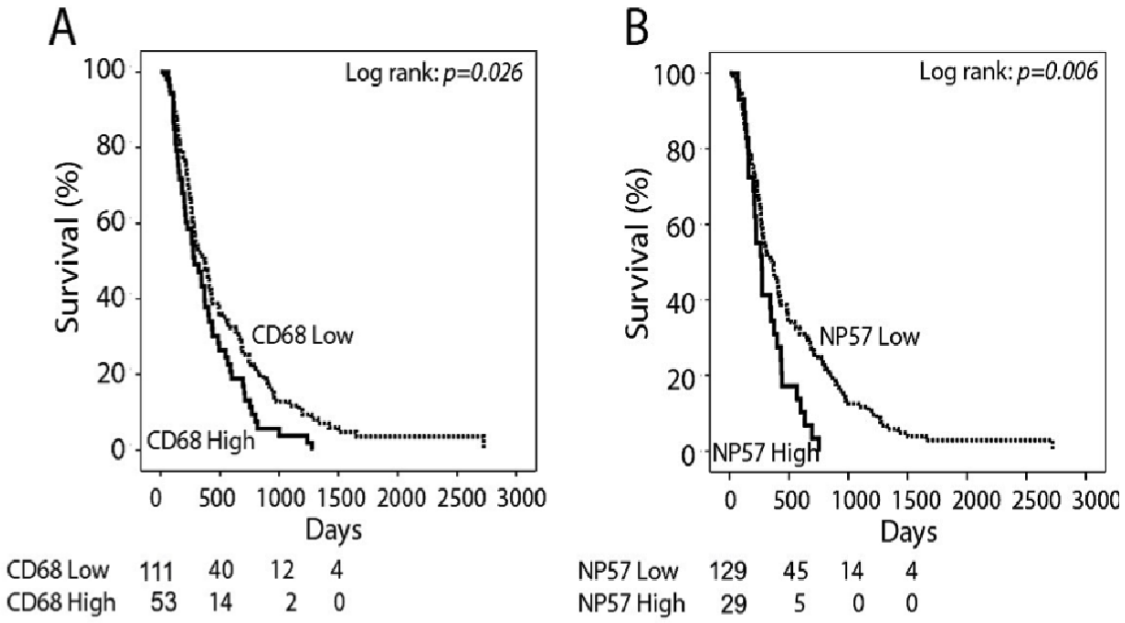


Figure 5

Kaplan-Meier survival curves of innate immune markers associated with survival in epithelioid mesothelioma (A) Kaplan-Meier curves for epithelioid mesothelioma survival according to CD68+ macrophage counts (log-rank test, P=0.026) (B) Kaplan-Meier curves for epithelioid mesothelioma survival according to NP57+ neutrophil counts (log-rank test, P=0.006). The numbers under the Kaplan Meier curves show the number of patients alive at Time point 0, 500, 1000 and 1500 days respectively for low and high counts for each marker analysed.

Multivariate analysis was performed next based on the parameters that were significant on univariate analysis. In the epithelioid group, a high CD4+ (p=0.003); high CD20+ (p=0.010); low FOXP3+ (p=0.000414) and low NP57+ (p=0.038) counts remained significantly associated with survival. In the non-epithelioid group, a low number of FOXP3+ cells associated with survival, p=0.043. The data for the multivariate analysis of both groups is shown in Table 4.

Table 4 Multivariate analysis of overall survival in whole cohort

	Marker	HR	95% CI	p-value
Epithelioid n= 131				
T helper cells	CD4	0.499	0.313 to 0.795	**0.003**
Cytotoxic T cells	CD8	0.939	0.579 to 1.522	0.797
T regulatory cells	FOX P3	2.399	1.476 to 3.900	**<0.001**
B cells	CD20	0.560	0.361 to 0.869	**0.010**
Macrophages	CD68	1.325	0.909 to 1.930	0.143
Neutrophils	NP 57	1.595	1.026 to 2.481	**0.038**
Non-epithelioid n=111				
T helper cells	CD4	0.800	0.465 to 1.375	0.420
Cytotoxic T cells	CD8	1.470	0.939 to 2.302	0.092
T regulatory cells	FOX P3	1.554	1.015 to 2.381	**0.043**
B cells	CD20	1.122	0.619 to 2.034	0.704
Macrophages	CD68	0.961	0.646 to 1.429	0.843
Neutrophils	NP 57	1.338	0.769 to 2.325	0.303

HR Hazard Ratio, CI Confidence interval, Cox proportional hazards regression model, significant p value <0.05.

The ratio of CD4+/CD8+ with a cut point of 1 between low and high counts was analysed. A ratio of >1 was associated with longer survival only in the epithelioid group, 99 low, 59 high (158 total) p=0.047. Kaplan-Meier curves are shown in Figures 6A and 6B.

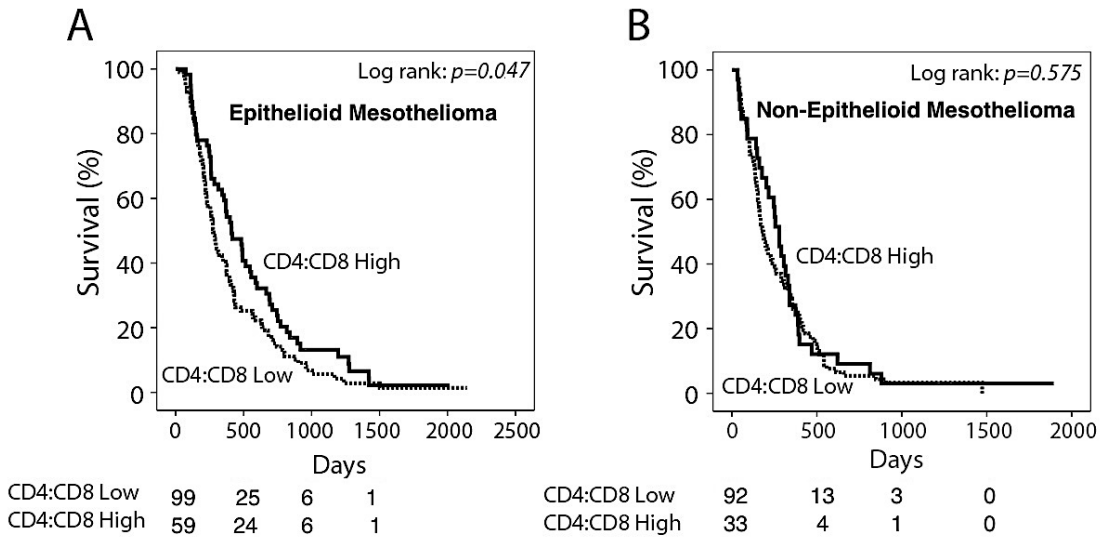


Figure 6

Kaplan-Meier survival curves of CD4:CD8 ratio with a cut point of 1 in overall, epithelioid and non-epithelioid groups (A) Kaplan-Meier curves for epithelioid mesothelioma survival according to CD4:CD8 ratio (log-rank test, P=0.047) (B) Kaplan-Meier curves for non-epithelioid mesothelioma survival according to CD4:CD8 ratio (log-rank test, P=0.575). The numbers under the Kaplan Meier curves show the number of patients alive at Time point 0, 500, 1000 and 1500 days respectively for low and high counts for each marker analysed.

Of 166 patients managed in our own centre, 61 had received chemotherapy. These 61 chemotherapy treated patients had a significantly better survival than those who were not given chemotherapy ($p < 0.0001$), likely reflecting both patient selection and effect of treatment. In the 61 patients treated with chemotherapy, a high CD4+ T cell count identified patients who lived longer ($p=0.034$); no effect was seen for CD8+ T-cells.

Of 94 patients with epithelioid histology, 42 were treated with chemotherapy. Chemotherapy administration but also high CD4+, high CD20+, low FOXP3+ counts were independent prognostic factors ($p=0.001$, $p=0.005$, $p=0.015$, $p=0.046$) in multivariate analysis. In the group of 72 non-epithelioid cases, 19 patients had been treated with chemotherapy; only chemotherapy administration was independently associated with survival ($p=0.029$) (Table 5).

Table 5 Multivariate analysis of overall survival in cohort receiving chemotherapy

	Marker	HR	95% CI	p-value
Epithelioid n= 42				
T helper cells	CD4	0.429	0.237-0.778	**0.005**
Cytotoxic T cells	CD8	0.920	0.456-1.857	0.816
T regulatory cells	FOX P3	2.023	1.014-4.036	**0.046**
B cells	CD20	0.504	0.290-0.876	**0.015**
Macrophages	CD68	0.986	0.564-1.726	0.961
Neutrophils	NP 57	1.157	0.602-2.224	0.661
Chemotherapy		2.546	1.500-4.323	**0.001**
Non-epithelioid n=19				
T helper cells	CD4	0.777	0.310-1.945	0.590
Cytotoxic T cells	CD8	1.492	0.735-3.030	0.268
T regulatory cells	FOX P3	0.858	0.379-1.939	0.712
B cells	CD20	2.347	0.967-5.697	0.059
Macrophages	CD68	0.947	0.518-1.731	0.859
Neutrophils	NP 57	1.123	0.551-2.290	0.749

Chemotherapy		2.253	1.084-4.681	**0.029**

HR Hazard Ratio, CI Confidence interval, Cox proportional hazards regression model, significant p value <0.05.

Next, CD4+ T cells in the epithelioid mesothelioma tissue microarray were correlated with FOXP3+ cells, CD8+ T cells and B cells.

The correlation between the number of CD4+ T cells and FOXP3+ cells in Good and Poor survival are shown (Figure 7A). The Pearson correlation is 0.535 and p=0.000006, the Spearman's rho correlation is 0.401 and p=0.001. However, when represented graphically, the CD4+ T cells and FOXP3+ cells from the good and poor survival groups appear to be evenly distributed with a R2 linear value of 0.118. A log10 scale has been chosen to better represent the data as it is more normalised.

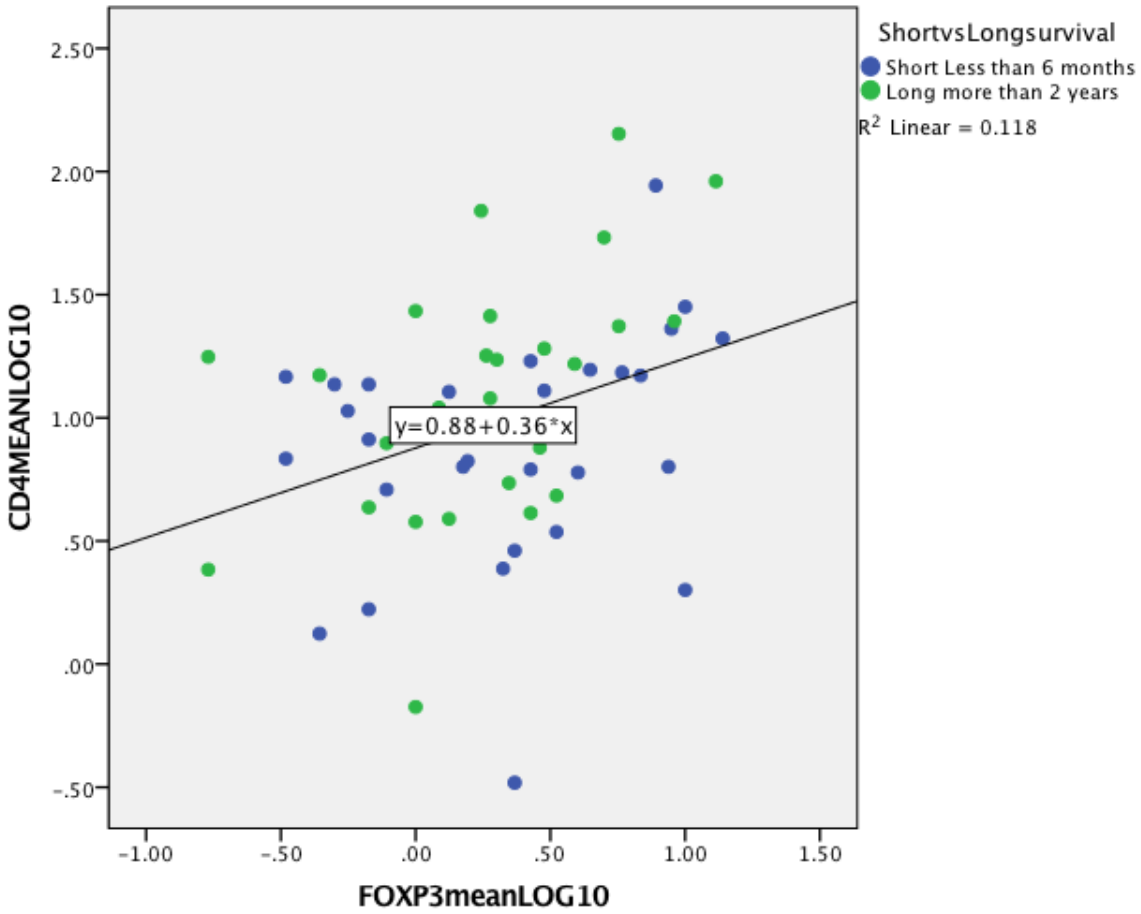


Figure 7A

Correlation of CD4+ T cells and FoxP3+ cells in epithelioid mesothelioma

The top and bottom quartiles for CD4+ T cell counts were correlated with FOXP3+ cell counts (Figure 7b). The patients with good survival tended to have a high CD4+ T cell infiltrate and the patients with a poor survival tended to have a high FOXP3+ cell infiltrate.

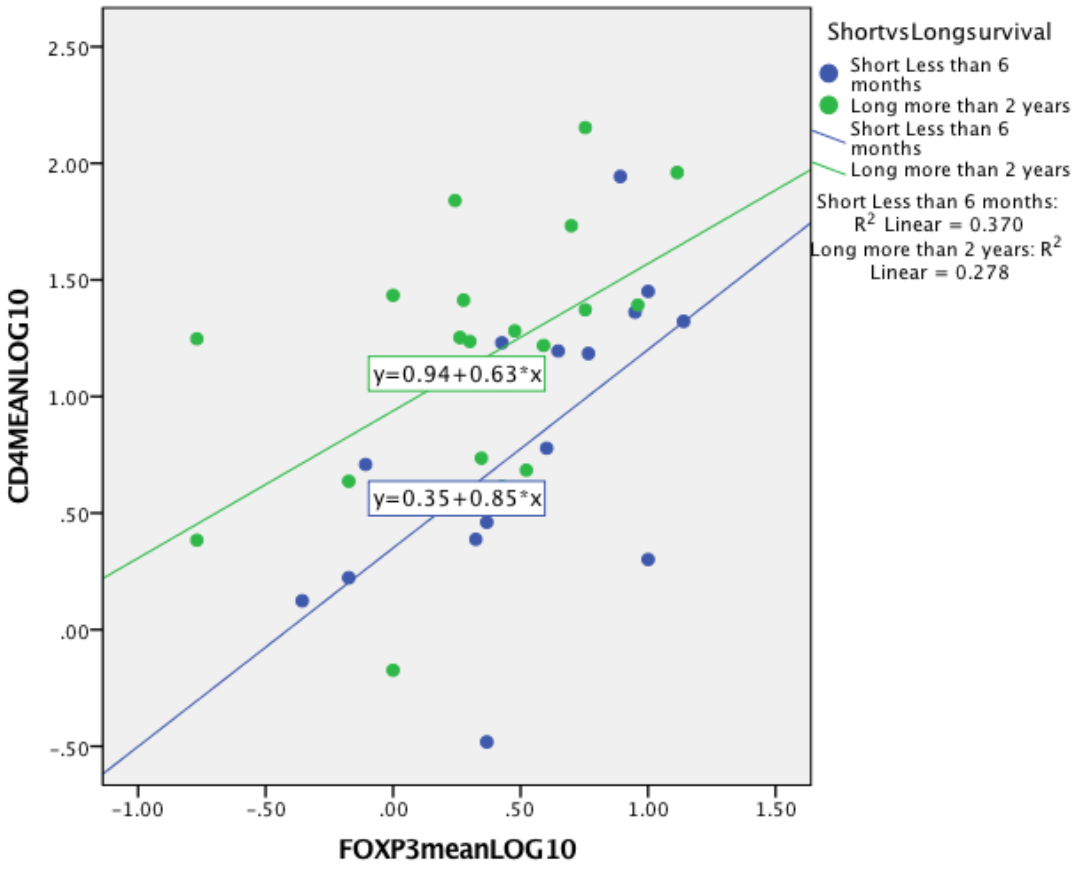


Figure 7B

Correlation of top and bottom quartiles of CD4+ T cells with FOXP3+ cells in epithelioid mesothelioma

The correlation between the number of CD4+ and CD8+ T cells in Good and Poor survival groups are shown (Figure 8A). The Pearson correlation is 0.623 and p=<0.001 the Spearman's rho correlation is 0.623 and p=<0.001. When represented graphically, the CD4+ and CD8+ T cells in the good and poor survival groups appear to be evenly distributed with a R2 linear value of 0.364.

The top and bottom quartiles of CD4+ T cell counts were correlated with the CD8+ T cell counts (Figure 8b). This shows a R2 linear value of 0.496 in the poor survival group and 0.350 in the good survival group.

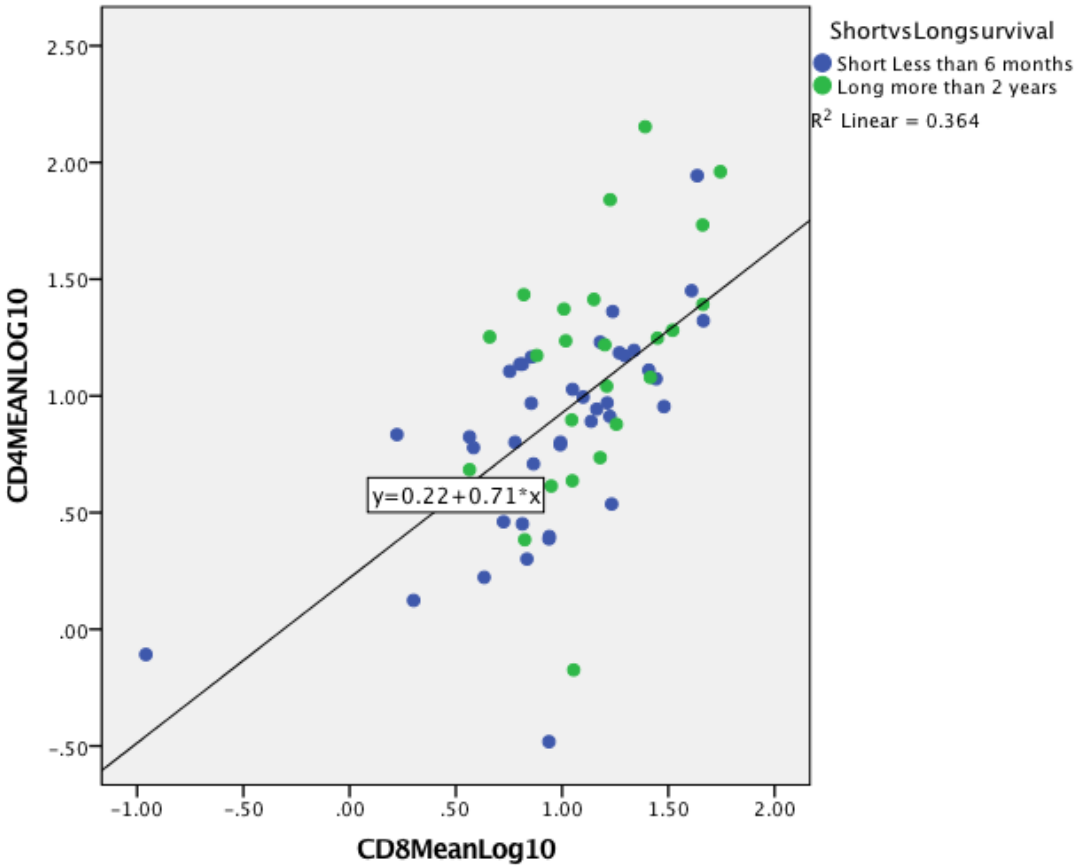


Figure 8A

Correlation of CD4+ T cells with CD8+ T cells in epithelioid mesothelioma

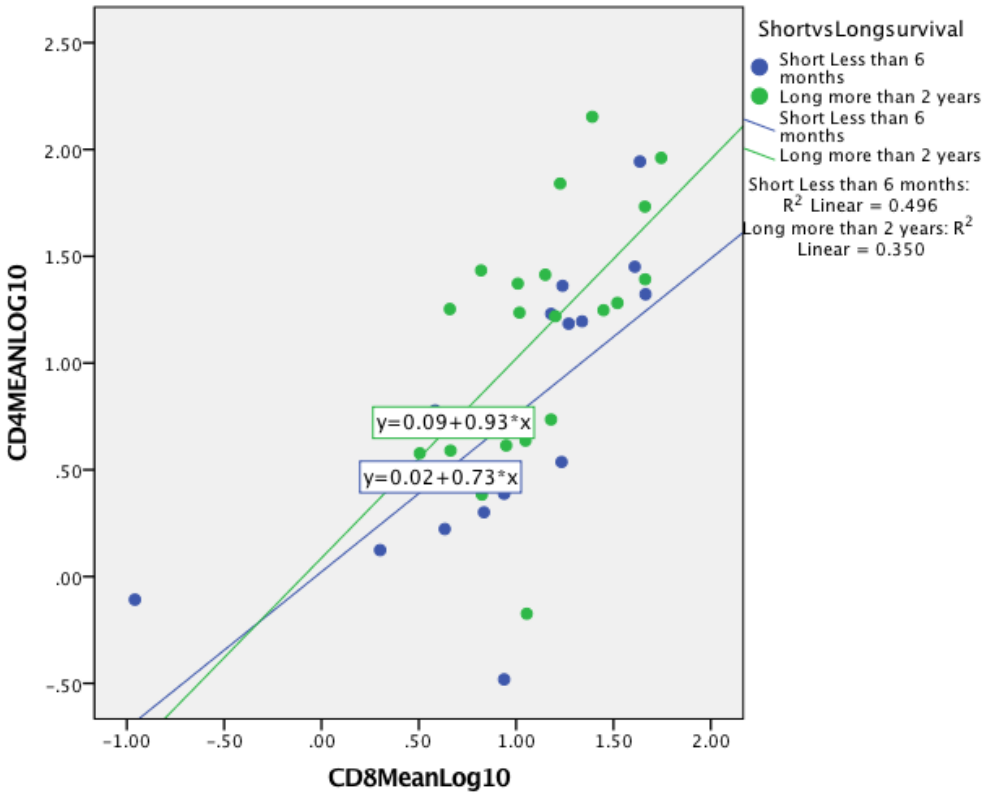


Figure 8B

Correlation of top and bottom quartile of CD4+ T cells with CD8+ T cells in epithelioid mesothelioma

The correlation between the number of CD4+ T cells and CD20+ B cells in Good and Poor survival are shown (Figure 9A). The Pearson correlation is 0.540 and p=<0.001 the Spearman's rho correlation is 0.471 and p=<0.001. When represented graphically, the cells appear to be evenly distributed in the good and poor survival groups.

The upper and lower quartiles for CD4+ T cell counts were correlated with CD20+ B cell counts (Figure 9B). This shows a strong correlation between CD4+ and CD20+ cell counts in the poor survival group with a R2 linear value of 0.605 but a R2 linear value of 0.129 in the good survival group.

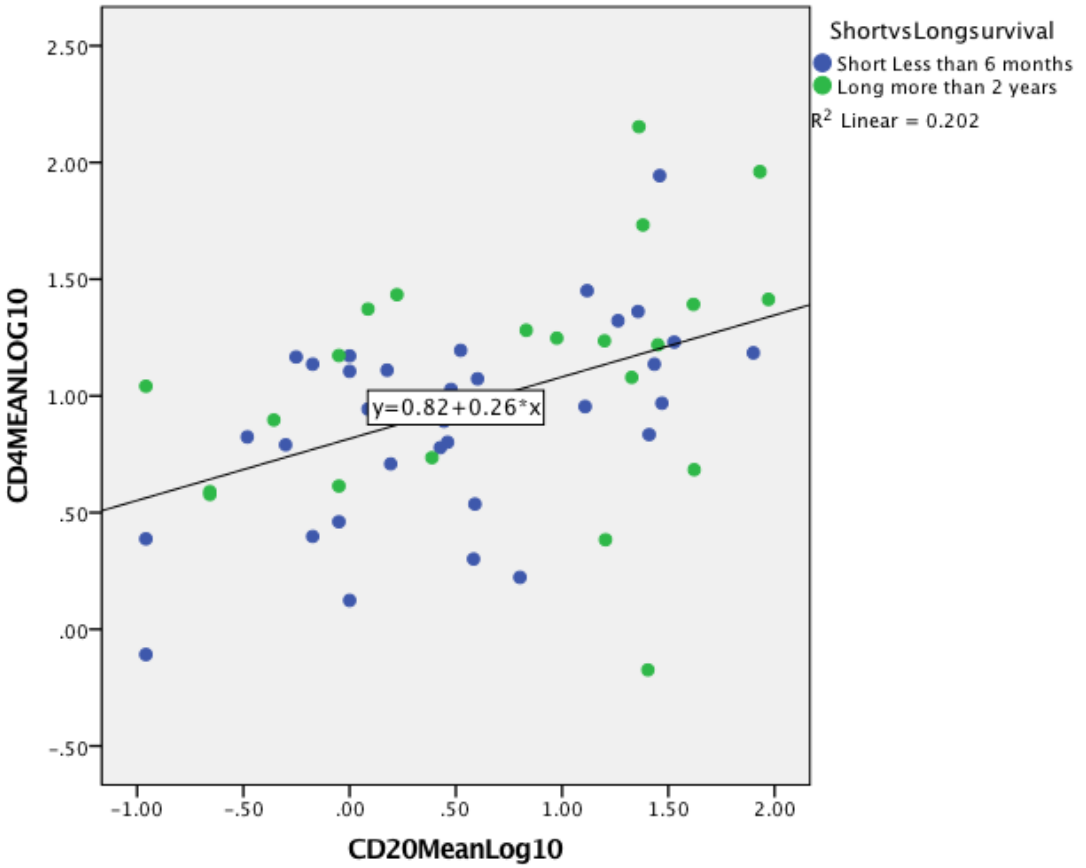


Figure 9A

Correlation of CD4+ T cells with CD20+ B cells in epithelioid mesothelioma

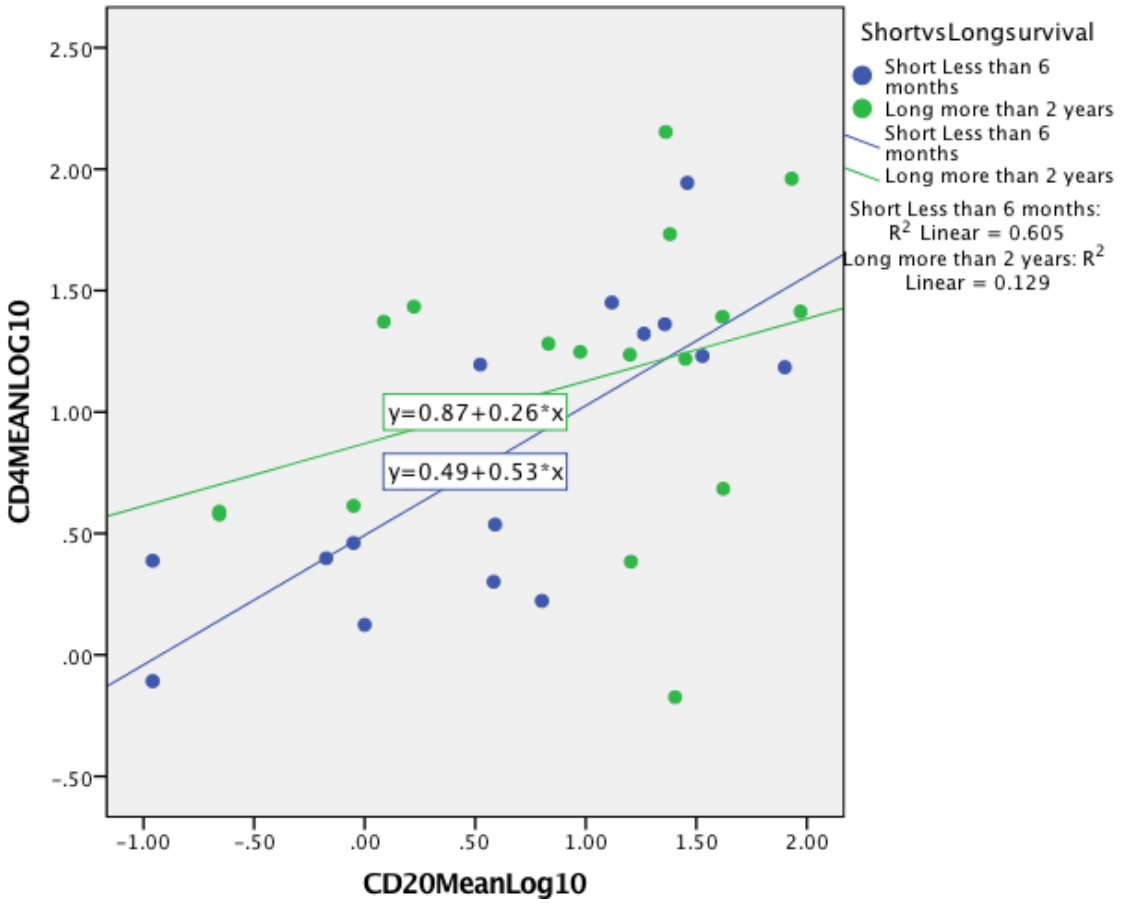


Figure 9B

Correlation of top and bottom quartile of CD4+ T cells with CD20+ B cells in epithelioid mesothelioma

3.3 Discussion

A large unselected cohort of patients was evaluated for evidence of whether immune attack occurs in mesothelioma and how this might affect survival. To our knowledge, this is the largest cohort of mesothelioma containing all subtypes analysed to date. The data represent a patient population in whom treatment was given with palliative intent from the outset (88%). Only 37% of the cohort received palliative chemotherapy and the cohort is clinically distinct from published datasets that have evaluated TIL density in mesothelioma to date and where the focus has been on operable disease (Yamada et al., 2010, Ujiie et al., 2015, Anraku et al., 2008).

Markers of adaptive (CD3+, CD4+, CD8+, T regulatory, T memory and B-cells) and innate immunity (macrophages, natural killer cells and neutrophils) were studied. Epithelioid and non-epithelioid

mesothelioma are clinically distinct diseases, and the data demonstrate they are immunologically different also; adaptive immune cell infiltrates differentially link to outcome between subtypes.

Unexpectedly, high CD8+ density appears not to be beneficial for survival in mesothelioma patients. In the epithelioid group the CD8+ T-cell density was indifferent for outcome; in the non-epithelioid group, a low number of CD8+ T-cells linked with a survival advantage, although this observation was not maintained in the multivariate analysis. This is in contrast to observations in many other solid cancers where a high density of CD8+ TILs has been shown to confer a survival advantage (Al-Shibli et al., 2008, Galon et al., 2006, Garcia-Martinez et al., 2014, Ward et al., 2014, Gooden et al., 2011). Exceptions to this general trend are patients with uveal melanoma (Bronkhorst and Jager, 2012) and renal cell carcinoma (Nakano et al., 2001).

A high density of CD4+ TILs conferred a survival advantage in the epithelioid group, both on univariate and multivariate analysis. CD4+ T cells are important for activating a range of tumour-reactive immune cells including CD8+ T-cells and B-cells (Ding et al., 2010). Recent work has demonstrated that the immunogenic mutations (between 70-95%) are recognised by CD4+ T-cells in 3 independent mouse models (Kreiter et al., 2015). If the mutanome is recognised by CD4+ T-cells in mesothelioma also, this might explain the dominance of CD4+ T-cells and favourable outcome in our series. Recent data however do not suggest that there is a significant difference in mutational load between epithelioid and sarcomatoid mesothelioma (Bueno et al., 2016), suggesting that number of mutations alone is not the explanation for the difference in the presence of CD4+ T-cells. However distinct mutational patterns emerged between the histological subgroups and it will be intriguing to test if the might be a differential effect on immune recognition, for example by activation of pathways that drive EMT (Bueno et al., 2016). Further work to establish the targets of CD4+ T-cell recognition is needed to better understand the balance of effector and regulatory CD4+ T cells in mesothelioma. This would then also allow a linkage to mutational status and immune attack and targeting of such mutations, for example by vaccination.

A key CD4+ T-cell population that limits the function of effector and helper T-cells are $FOXP3^{+}CD4^{+}$ regulatory T-cells. Consistent with the current understanding of their role, we observe that a high number of FOXP3+ T-cells was significantly associated with poorer survival in both morphological sub groups of mesothelioma. The effect of FOXP3+ T-reg likely has disease specific features. While in many solid tumour types a lower T-reg density is good for the patient (Shang et al., 2015), there are some exceptions, such as head and neck squamous cell carcinoma, where higher FOXP3+ T-cell density is linked to better locoregional control (Badoual et al., 2006b). It may simply be though that this apparent contradiction reflects a parallel influx of protective and suppressive

immune cells, as in head and neck cancer where globally the number of immune cells is tightly linked to survival (Ward et al., 2014, Wood et al., 2016).

There is correlation between the different immune cell types infiltrating the tumour. This demonstrates that the subsets of tumour infiltrating lymphocytes are interlinked. Some tumours with a high CD4+ T cell infiltrate have similarly high CD8+ T cell counts. This may reflect the gross number of cells in a TIL high tumour. FOXP3+ cells are a subset of CD4+ T cells and while there is a correlation between these cells, a high CD4+ T cell count does not necessarily equate to a high FOXP3+ infiltrate. There is a correlation between the number of CD4+ T cells and CD20+ B cells particularly in the poor survival group. This may be due to an interaction between the two cell types with one influencing the other to infiltrate the tumour microenvironment.

Evaluation of whether the sensitivity of quantification of the immune cell infiltrate could be improved by assessing the relative density of CD4+ and CD8+ T-cells was carried out. An easy way of expressing this is by calculating the ratio of CD4+/CD8+ T-cells, a measure that then becomes independent of the absolute abundance of the T-cells in the tissue. A high CD4+/CD8+ ratio has been previously evaluated in different types of cancer and was found to be associated with a good outcome in cervical squamous cell carcinoma and a poor outcome in colorectal cancer (Shah et al., 2011, Diederichsen et al., 2003). In this cohort, a CD4+/CD8+ ratio of greater than 1 was associated with better survival in the overall and epithelioid groups and this parameter warrants further analysis in other cancers to determine if it might be a useful prognostic marker for survival.

There is increasing recognition of the important role B-cells play in adaptive immune attack in the tumour microenvironment. Tumour associated B-cells induce and regulate T-cell immune responses through antigen presentation and CD4+ T-cell activation, contributing to the differentiation of CD4+ T-cells and polarization of Th1 and Th2 subsets (Baumgarth, 2011, Lund and Randall, 2010, Bao et al., 2014). In this cohort, a high density of CD20+ cells in the epithelioid group was associated with better survival. This remained significant on multivariate analysis and the data are consistent with Ujiie *et al.* (Ujiie et al., 2015) Similar to T-cells, there is variation in the prognostic effect of B-cell infiltration between tumour types. In colorectal cancer, an increased B-cell gene expression was significantly associated with survival (Bindea et al., 2013). Data from our group has demonstrated a high B-cell infiltrate to be associated with better outcome in head and neck squamous cell carcinoma (Wood et al., 2016). It is unclear however what the B-cells might contribute immunologically, and whether are simply drawn to the tumour in response to cytokine stimulation. In contrast to the morphological observations in humans, in a HPV 16 regulated murine squamous cell carcinoma model, administration of a B cell depleting monoclonal antibody

significantly improved response to chemotherapy (Affara et al., 2014), and raises the possibility that B-cells might also exert tumour-promoting effects.

Tumour associated inflammation is recognised as a key hallmark of cancer (Grivennikov et al., 2010, Hanahan and Weinberg, 2011) and innate immune cells such as macrophages and neutrophils form an important and complex part of the tumour microenvironment. Tumour associated macrophages can adopt a pro-tumourigenic role by promotion of angiogenesis and metastases and preventing T-cell attack on tumour cells and once the tumour is established, the macrophages are polarized to a pro-tumour phenotype (Noy and Pollard, 2014). In this cohort, a low CD68+ count was significant in the epithelioid group, similar to findings by Ujiie *et al* (Ujiie et al., 2015), but not in the non-epithelioid group. The association of a low infiltration of tumour associated macrophages and survival is consistent with the theory of chronic asbestos induced inflammation driving the development of mesothelioma in a process of "frustrated phagocytosis" (Bograd et al., 2011).

Tumour associated neutrophils similarly can adopt a pro-tumourigenic role by involvement in angiogenesis and creating a pro-invasive and pro-metastatic environment and may also play an anti-tumourigenic role (Hanahan and Coussens, 2012). Consistent with this, in this cohort, a low number of NP57+ cells was associated with better survival in the epithelioid group. It is known that a high circulating Neutrophil to Lymphocyte ratio in mesothelioma is adversely associated with survival in patients undergoing systemic therapy (Kao et al., 2010). The biological role of tumour associated neutrophils is not clear (Fridlender and Albelda, 2012). To our knowledge, our group is the first to detect the prognostic value of a low neutrophil count on survival in epithelioid mesothelioma.

Beyond histological typing into epithelioid, sarcomatoid and mixed types, markers that predict response to treatment in mesothelioma are lacking. In other solid tumours such as breast, rectal and oesophageal cancer immunological features that predict for pathological complete response and survival following chemotherapy have been examined. In a breast cancer cohort, a pre-treatment high CD4+/CD8+ ratio was an independent predictor of pathological complete response after neo-adjuvant chemotherapy and was associated with better prognosis (Garcia-Martinez et al., 2014) as was the presence of CD20+ B-cells (Brown et al., 2014). The question arose of whether TIL density would link to treatment response in mesothelioma. This was indeed the case: in 61 patients who had received chemotherapy CD4+ T-cell high cases identified patients who had a better outcome. This remained true also if only the 42 patients with epithelioid histology and who received chemotherapy were considered and suggest that CD4+ T cells may also have a predictive value in mesothelioma.

In summary, these data demonstrate for the first time an association of survival with high CD4+, low FOXP3+, high CD20+, low NP57+ and low CD68+ counts in epithelioid mesothelioma, treated with palliative intent. Low CD8+ and low FOXP3+ T-cell densities emerge as prognostic in non-epithelioid mesothelioma. Given that epithelioid and non-epithelioid mesothelioma behave very differently clinically, the underpinning differences in potential immunological drivers of the different subtypes are intriguing and warrant further study and will benefit from mapping onto the emerging stratifiers using genomic analyses (Bueno et al., 2016). Emerging data suggesting that sarcomatoid mesothelioma may be more responsive to immunotherapy than the epithelioid subtype (Cedres et al., 2015, Mansfield et al., 2014), but more work is needed to understand the reason behind this difference. These data illustrate that the morphological differences, linked to outcome also find their reflection in the adaptive and innate immune events that are present in the cancer. Their functional understanding will open the door towards rational targeting of immune pathways to improve the outcomes of patients with this devastating disease.

Chapter 4 Multiplex immunohistochemistry

4.1 Introduction

In most solid tumours, the presence of CD8+ cytotoxic T cells has been associated with better survival. However, recent work has demonstrated the importance of tissue resident memory cells (T_{RMs}) with particular emphasis on CD8+ T_{RMs}. Tissue resident memory cells are a specialized subset of cells that reside in tissue and reactivate quickly on encounter with their target antigen. In lung cancer, our group has recently shown that density of CD8+ T_{RMs} in tumour tissue is associated with better survival outcomes, independent of $CD8^+$ TIL density (Ganesan et al., 2017).

However, data from the mesothelioma TMA presented in the previous chapter (chapter 3) demonstrated that while the number of infiltrating CD8+ T cell was variable across tumours, there was no positive relationship between survival and CD8+ TILs, thus appearing to negate the role of these cells for anti-tumour immunity in mesothelioma. Therefore, in this chapter we wished to explore two possible explanations: first that some important aspect of CD8+ CTL -related biology had been missed and second that, if not CD8+ T cells, then possibly CD4+ T cells might have an important role.

In order to ensure we had not missed some important phenotypic or functional characteristics of the CD8+ infiltrating cells, we therefore also sought to define both the phenotypes of the CD8+ T cells in greater detail and to seek evidence of mechanisms employed by the tumour to avoid detection and killing by CD8+ T cells. Tumour cells may downregulate cell surface expression of MHC class I – required by CD8+ T cells for recognition of antigen and performance of their effector functions. Also, they can present self-antigens, which may result in chronically stimulated CD8+ CTLs becoming exhausted or senescent.

In contrast to the lack of relationship between outcome and CD8+ T cells in epithelioid mesothelioma, CD4+ TILs were positively associated with survival. This led us to hypothesize that, rather than CD8+ T cells in mesothelioma, CD4+ TILs are somehow functioning as effector cells to drive a protective anti-tumour immune response. It is unclear how these CD4+ TILs are functioning as effector cells independent of CD8+ T cells. Traditionally, cytotoxicity – the key anti-tumour effector function, has been associated with cells like NK cells and CD8+ T cells. However, recently, there has been renewed interest in a subset of cytotoxic CD4+ T cells that express markers associated with cytotoxicity such as Granzyme A, Granzyme B and Perforin (Takeuchi and Saito, 2017). The aim of the work in this chapter was to test the hypothesis that CD4+

effector/cytotoxic T cells are present in the mesothelioma tumour tissue and their presence might correlate with clinical outcome.

In order to pursue this line of investigation, it was necessary to stain tumour tissue with multiple antibodies in order to characterize and define the phenotypes of infiltrating T cells. A major impediment to the analysis of multiple cell types within a tumour was, up to now, the difficulty of staining multiple markers simultaneously. However, in recent years, it has become possible to evaluate these relationships with multiplex immunohistochemistry (IHC) and this method has been established in our laboratory in the last year. I have applied this approach to the evaluation of T cells in my tissue samples to evaluate the protective role of CD4+ T cells in mesothelioma.

In order for a CD4+ effector T-cell to have cytotoxic function, presentation of antigen in MHC II molecules on the target cells is required. It has been demonstrated previously that mesothelioma tumour cell lines express MHC class II on their surface (Mutti et al., 1998). MHC class II expression has also been demonstrated on mesothelioma cells by cyTOF (Lee et al., 2018). We hypothesised that the expression of MHC class II on the surface of tumour cells in mesothelioma may allow interaction of these cells with CD4+ T cells. MHC class II expression is induced on the surface of epithelial cells by exposure to interferon gamma (IFN-γ). Many cells including NK cells, NKT cells, macrophages, activated CD4+ Th1 cells and CD8+ T cells produce IFN-γ.

4.2 Multiplex immunohistochemistry staining in epithelioid mesothelioma

The aim of using multiplex staining in epithelioid mesotheliomas was to evaluate the presence of various cell types within the intra-tumoural immune cell infiltrate. In particular, even though my previous work indicated there was no correlation between CD8+ T cells and survival, I wished to determine the presence or absence of CD8+ T_{RM} in tumour tissue. This will allow a comparison with the number and features of CD4+ T cells expressing markers characteristic of either tissue residency (CD69 or CD103) and/or cytotoxicity (GZB). The CD4+ T cells will additionally be characterized for the presence of other CD4+ T cell subsets including tissue resident, regulatory and follicular helper cells.

The tumour tissue sections from patients in this cohort were stained with three antibody panels, selected to focus on particular phenotypic markers. The first panel was aimed at defining CD4+ T cell subsets and consisted of anti-CD4, Granzyme B, CD69, CD103, FOXP3, BCL6 and CXCL13 antibodies. The second panel focused on MHC class I and II expression on tumour cells and

consisted of anti-WT1, anti-HLA-class I and HLA-DPB1 antibodies. The third panel aimed at further refinement of CD8+ T cell phenotypes and consisted of anti-CD8, CD103, TIM3 and Granzyme B antibodies.

Granzyme B was chosen as a marker of cytotoxicity as in our hands this produces good IHC staining. CD69 is an early activation marker and has also shown to remain expressed in tissue-resident CD4+ T cells; CD103 has been used as a marker of tissue residency in T cells. FOXP3 is the key transcription factor in T regulatory cells. BCL6 is expressed by T follicular helper cells. CXCL13 has been shown to be expressed by CD4+ T follicular helper cells.

Wilm's tumour antigen (WT1) is expressed by 72-91% of mesothelioma tumour cells. All tumours analyzed in this cohort had been reported as WT1 positive by diagnostic immunohistochemistry.

TIM3 is expressed by activated T cells. TIM3 has been associated with an exhausted state but more recently our group has shown that TIM3 expressing T cells are highly activated and functional (Clarke et al, J Exp Med 2019 in press)

The number of cells per 520 X 300 μm (0.16mm^2) was evaluated in each tumour tissue section. In the CD4+ multiplex immunohistochemistry panel, the number of single stained CD4+, CD103+, FOXP3, BCL6+ and Granzyme B (GZB+) cells was determined. The tissue was then evaluated for the presence of dual positive and triple positive cells. The CD4+ cell phenotypes represented by the co-expression of these markers is shown in table 1.

Variable	Cell Type
CD4+CD103+ cells CD4+CD69+ cells	CD4+ Tissue Resident Memory cells (T_{RM})
CD4+GZB+ cells	Cytotoxic CD4+ T cells
CD4+CD103+GZB+ cells CD4+CD69+GZB+ cells	Cytotoxic CD4+ T_{RM} cells
CD4+ FOXP3+ cells	T regulatory cells
CD4+BCL6+FOXP3- cells CD4+CXCL13+ cells CD4+BCL6+CXCL13+ cells	T follicular helper cells
CD4+BCL6+FOXP3+ cells	T follicular regulatory cells

Table 1 CD4+ T cell phenotypes in Multiplex immunohistochemical analysis of epithelioid mesothelioma

The second multiplex was evaluated for the number of single stained WT1+ tumour cells, followed by evaluation of the number of WT1+ HLA Class 1+ and WT1+ HLA-Class II (HLADPB1)+ cells. In addition, the staining intensity of HLA Class 1 and HLADPB1 was assessed on the WT1+ HLA Class 1+ and WT1+HLADPB1+ tumour cells respectively.

Finally, the number of single stained CD8+ cells was enumerated, followed by evaluation of CD8+ cells dual and triple stained with a combination of CD103, GZB and TIM3 antibodies (Table 2).

Variable	Cell type
CD8+CD103+ cells	CD8+ Tissue resident memory cells (T_{RM})
CD8+GZB+ cells	Cytotoxic CD8+ T cell
CD8+CD103+GZB+ cells	Cytotoxic CD8+ T_{RM}
CD8+CD103+TIM3+ cells	Highly functional CD8+ T_{RM}
CD8+CD103+TIM3+GZB+ cells	Cytotoxic Highly functional CD8+ T_{RM}

Table 2 CD8+ T cell phenotypes in Multiplex immunohistochemical analysis of epithelioid mesothelioma

4.3 Results

All tumours analysed were epithelioid mesotheliomas.

First, tissue from 24 patients with good survival and 20 patients with poor survival was examined for expression of CD4+, markers of tissue residency (CD69+ and CD103+), markers of cytotoxicity (GZB+), markers of T regulatory cells (CD4+ FOXP3+), markers of T follicular helper (BCL6, CXCL13) and T follicular regulatory cells (CD4+BCL6+FOXP3+). Disintegration of the tissue during the multiplex process meant not all cases underwent complete evaluation.

Representative pseudo-colour images from a patient with good survival are shown for CD4+ cells in Blue (Figure 1), CD69+ cells in Red (Figure 2) and combined in Figure 3.

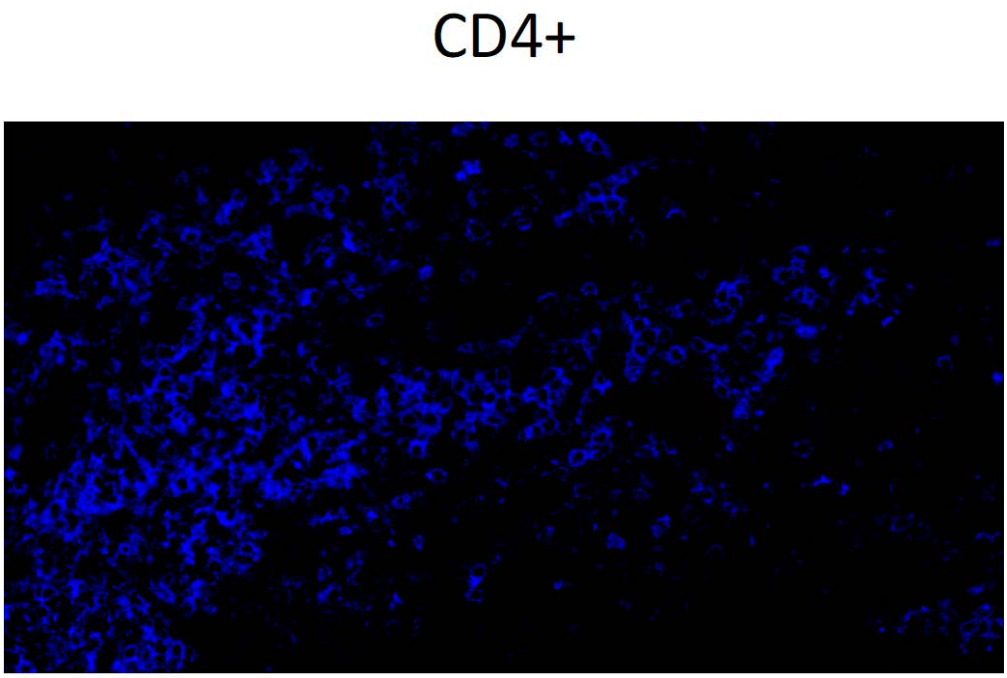


Figure 1 CD4+ cells (Blue pseudo-colour) in Epithelioid tumour from patient with good survival

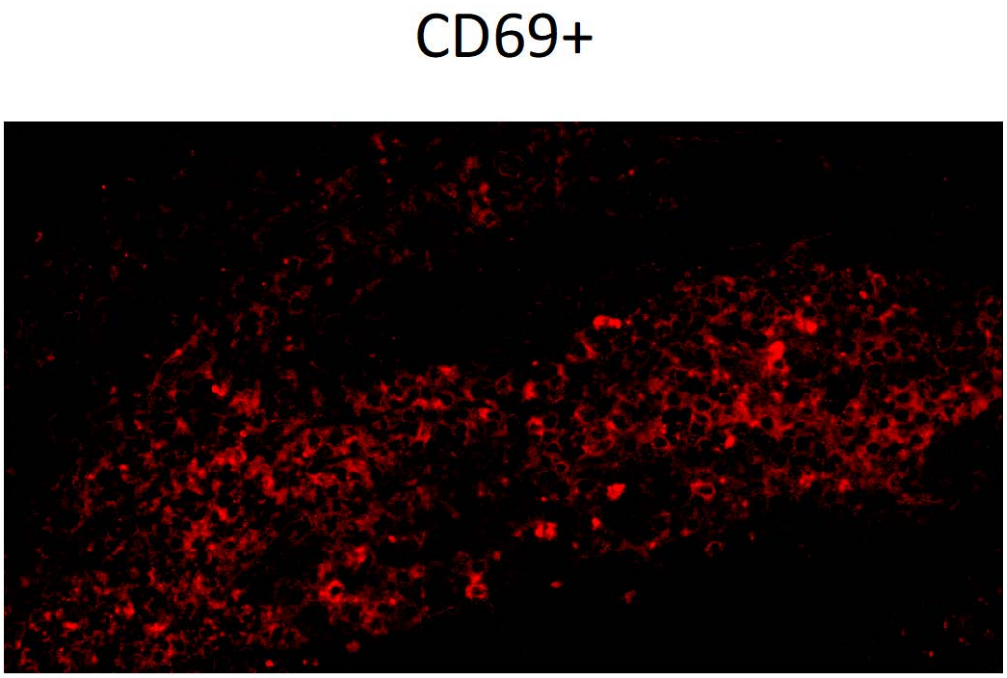
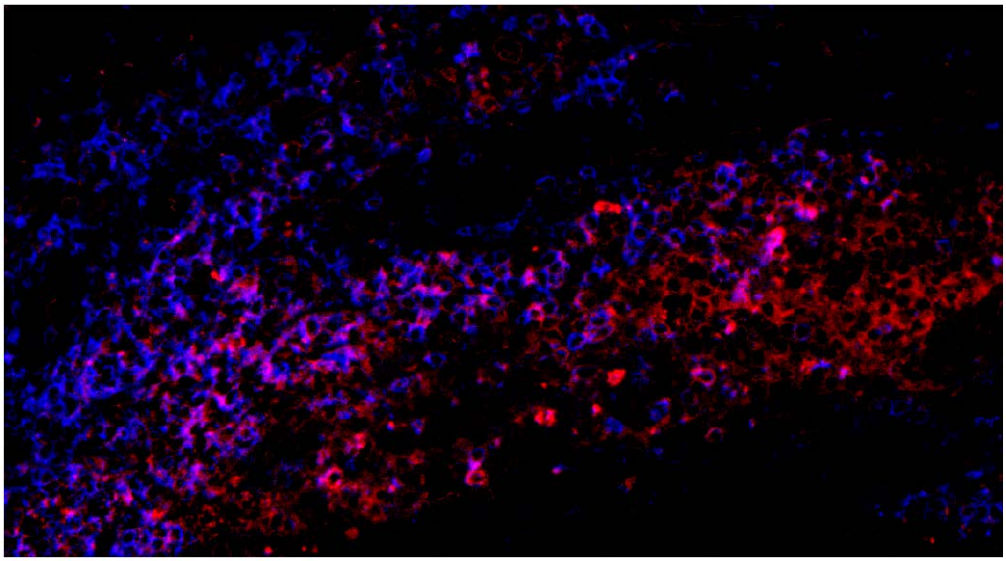


Figure 2 CD69+ cells (Red pseudo-colour) in Epithelioid tumour from patient with good survival

CD4+CD69+



CD4+CD69+ magnified image

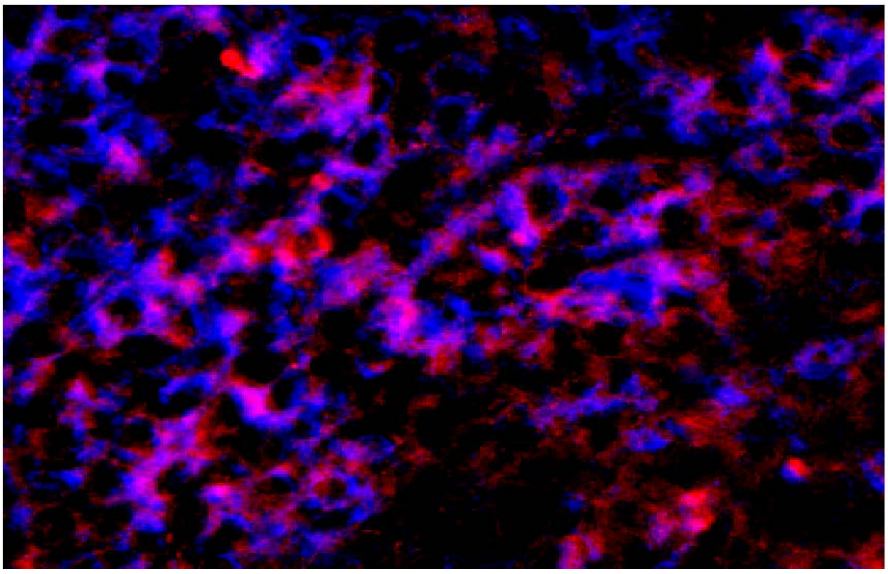


Figure 3 CD4+CD69+ cells (Blue and Red pseudo-colour respectively) in Epithelioid tumour from patient with good survival including magnified image. Double positive cells represented in Magenta

The median value for CD4+ cells is higher in the good survival group compared to the poor survival group (p=0.0009, Mann-Whitney test). The Mann-Whitney test was chosen as the CD4 data from the larger mesothelioma TMA cohort does not follow a normal (Gaussian) distribution. Within the CD4+ T cell subsets, the median value for CD4+CD69+ (activated CD4+/T_{RM}) cells, as well as CD4+BCL6+CXCL13+ and CD4+CXCL13+ (T_{FH}) cells was higher in the good survival group compared to the poor survival group (p=0.0199, p= 0.0008 and p=0.0117 respectively). Distribution of these cell types is shown in Figure 4.

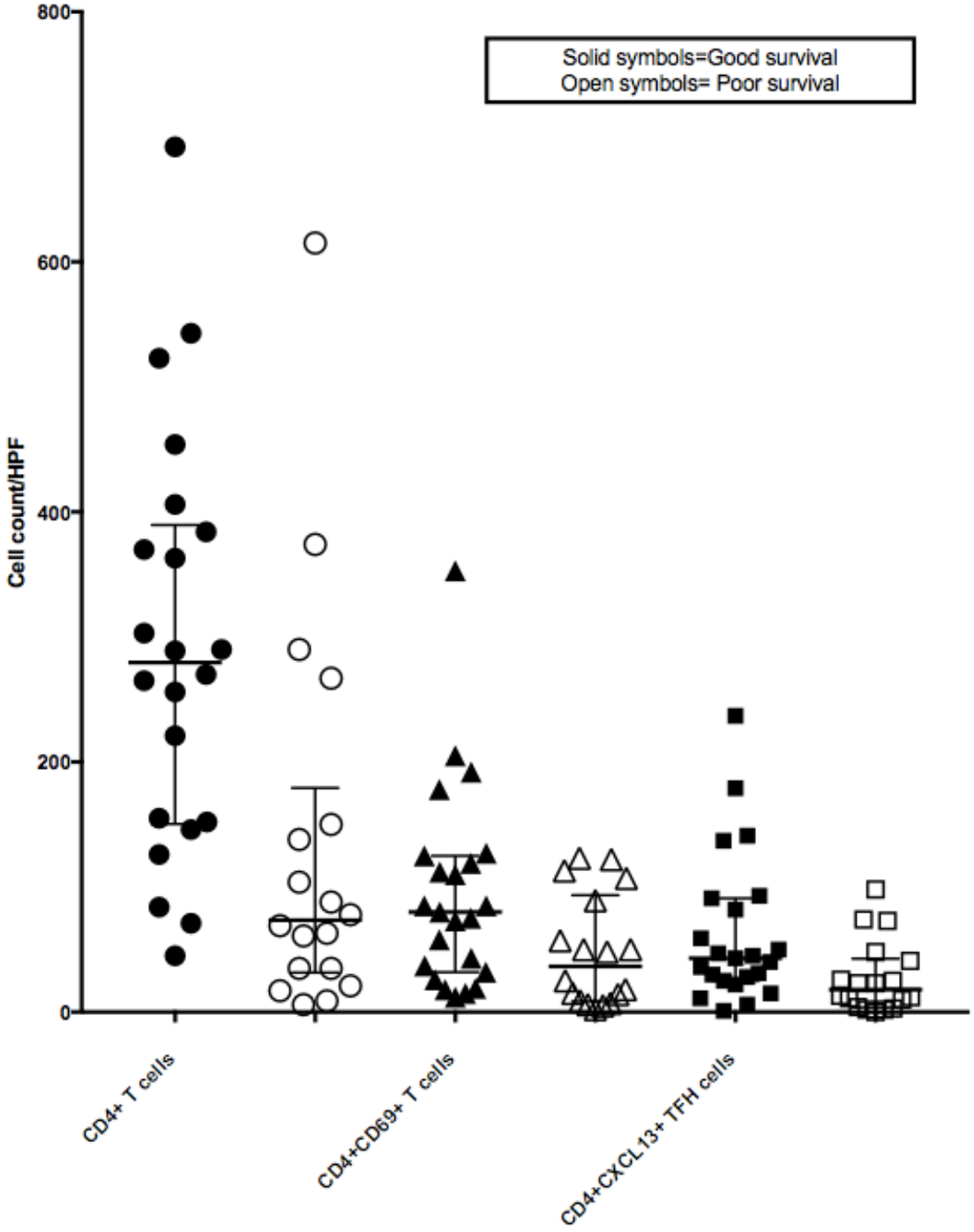


Figure 4 Distribution of CD4+ T cells and CD4+ T cell subsets CD4+CD69+ (Tissue Resident CD4+ T cells) and CD4+CXCL13+ TFH cells for Good and Poor survival groups (Median and interquartile range)

The median value for CD4+GZB+ (CD4+ CTLs) trended toward but did not reach statistical significance (p=0.0617) between the good and poor survival groups. Other cell types such as CD4+CD103+ (T_{RMS}), CD4+CD103+GZB+ (cytotoxic T_{RMS}), CD4+FOXP3+ (Tregs), CD4+FOXP3+BCL6+ (T_{FRS}) and CD4+BCL6+FOXP3-(T_{FH}) were indifferent for survival between the good and poor survival groups. A summary of these data for CD4+ T cells and CD4+ T cell subsets are shown in Table 3.

Variable	**Cell type**	**Median of cells/HPF Good survival group n=22**	**Median of cells/HPF Poor survival group n=18**	**P value Mann Whitney test**
CD4+	T helper cell	280	74	**0.0009**
CD4+GZB+	Cytotoxic CD4+ T cell	12.5	4	0.0617
CD4+CD69+	Tissue resident CD4+ T cell	80	36.5	**0.0199**
CD4+CD69+GZB+	Cytotoxic Tissue resident CD4+ T cell	5	5	0.7002
CD4+CD103+	Tissue resident CD4+ T cell	4	3	0.1452
CD4+CD103+GZB+	Cytotoxic Tissue resident CD4+ T cell	0	1	0.6522
CD4+ FOXP3+	T regulatory cell	42	17	0.1688
CD4+FOXP3+BCL6+	T follicular regulatory cell	13	9	0.4849
CD4+BCL6+FOXP3-	T follicular helper cell	10	2	0.1228
CD4+CXCL13+	T follicular helper cell	43	18	**0.0117**
CD4+BCL6+CXCL13+	T follicular helper cell	5	1	**0.0008**

Table 3 Comparison of median values of CD4+ T cells and CD4+ T cells subtypes between good and poor survival groups, significant Mann Whitney test p value <0.05

The TMA data presented in Chapter 3 demonstrate that WT1 expression is not associated with effects on survival. WT1 is expressed on 72-91% of mesothelioma tumour cells and indeed our own data has demonstrated the presence of WT1 antigen specific T cells in mesothelioma associated pleural fluid (unpublished data). Given the association of CD4+ T cell infiltration with survival in epithelioid mesothelioma, we sought to understand the possible interaction between CD4+ T cells and mesothelioma tumour cells.

The next multiplex immunohistochemistry examined the expression of MHC class II expression on WT1+ tumour cells in epithelioid mesothelioma. The same cohort was stained for WT1 and HLA-DPB1. WT1 expression alone did not differentiate between patients in the good and poor survival groups ($p=0.0978$). However, some of the WT1+ tumour cells expressed MHC class II on their cell surface and the difference between the number of WT1+HLADPB1+ tumour cells in the good and poor survival groups was highly significant ($p=<0.0001$). The difference between the ratio of WT1+ HLADPB1+ cells /Total number of WT1+ cells in the good and poor survival groups was also highly significant ($p=<0.0001$). (Table 4)

Variable	**Cell type**	**Median of cells/High powered field (HPF)** **Good survival group n=24**	**Median of cells/High powered field (HPF)** **Poor survival group n=19**	**P value**
WT1+	Tumour cells	197	165	0.0978
WT1+HLADPB1+	MHC class II expressing Tumour cells	88.5	8	**<0.0001**
		Median of ratio of WT1+HLADPB1+/ Total WT1+ cells	**Median of ratio of WT1+HLADPB1+/Total WT1+cells**	**P value**
Ratio of WT1+HLADPB1+ /Total WT1+ cells		0.405	0.05	**<0.0001**

Table 4 Comparison of median values of WT1+ tumour cells and WT1+HLADPB1+ tumour cells/high power field and ratio of WT1+HLA-DPB1+/Total WT1+ cells between good and poor survival groups, significant Mann Whitney test p value <0.05

Figure 5 and 6 show WT1+HLADPB1+ expression in the good and poor outcome groups respectively. The double-positive tumour cells show as pink rings around a blue nucleus. The bright red single stain is HLA-DPB1 expression by non-tumour cells. The pink rings that are not around a blue nucleus represent non-specific WT1 staining.

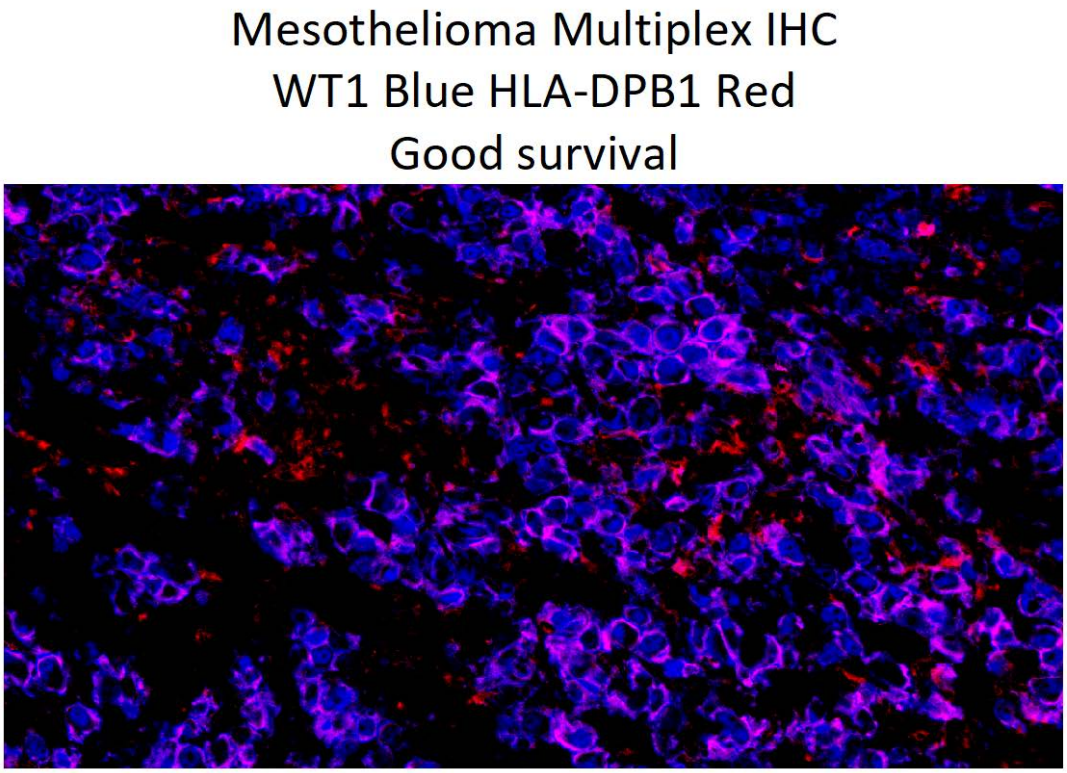


Figure 5 WT1+HLA DPB1+(Blue and Red pseudo-colours respectively) in Epithelioid mesothelioma tumour from patient with good survival

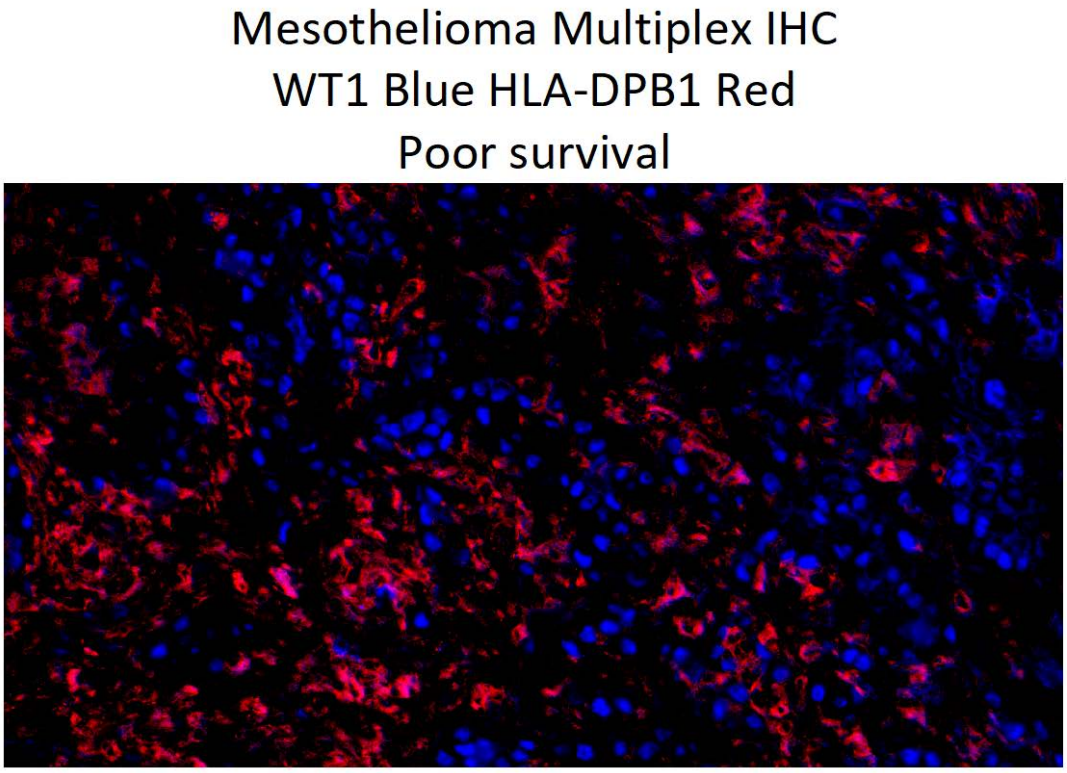


Figure 6 WT1+HLA DPB1+(Blue and Red pseudo-colours respectively) in Epithelioid mesothelioma tumour from patient with poor survival

Next, a correlation between CD4+ T cell numbers and WT1+HLADPB1+ tumour cells was examined. The Spearman R value was 0.506, p=0.0012. The other significant CD4+ subsets (CD4+CD69+, CD4+CXCL13+) also demonstrated correlation to WT1+HLADPB1+ expression on tumour cells. The Spearman R value were 0.408, p= 0.0089 for the correlation between CD4+CD69+ T cells and WT1+HLADPB1+ tumour cells, and 0.4041, p=0.0097 for the CD4+CXCL13+ T cells and WT1+HLADPB1+ tumour cells respectively.

The intensity of HLADPB1+ staining on WT1+ tumour cells varied between samples. A semi-quantitative scoring of the intensity of HLADPB1+ staining in WT1+ tumour cells (1+ =weak, 2+ =moderate, 3+ =strong) and the proportion WT1+ tumour cells that were also HLADPB1+ (1 <10%, 2 >10% and <50%, 3 >50%) was undertaken. The median of the combined score was 5 in the good survival group and 3 in the poor survival group (p=<0.0001).

Next, MHC class I expression on WT1+ tumour cells in epithelioid mesothelioma was evaluated. The difference between the number of WT1+HLA Class 1+ cells was significant between the good and poor survival groups (p=0.0222) (Table 5).

Variable	**Cell type**	**Median of cells/High powered field (HPF)** **Good survival group n=24**	**Median of cells/High powered field (HPF)** **Poor survival group n=19**	**P value**
WT1+HLA Class 1+	MHC class I expressing Tumour cells	182	102	**0.0222**

Table 5 Comparison of median values of WT1+HLA Class 1+ tumour cells/high power field between good and poor survival groups, significant Mann Whitney test p value <0.05

The intensity of HLA Class 1 staining on WT1+ tumour cells also varied between samples. A semi-quantitative scoring of the intensity of HLADPB1+ staining in WT1+ tumour cells (1+ =weak, 2+ =moderate, 3+ =strong). Here, only the intensity of staining was evaluated. The median of the intensity score was greater in the in the good survival group at 2.5 and was 2 in the poor survival group (p=0.001).

Lastly, tumour infiltration of CD8+ T cells and subsets of CD8+CD103+, CD8+GZB+, CD8+CD103+GZB+, CD8+CD103+TIM3+ and CD8+CD103+TIM3+ GZB+ was evaluated. Neither CD8+ T cells nor any of the CD8+ subtypes was significantly different between the good and poor survival groups (Table 6).

Variable	**Cell type**	**Median of cells/HPF Good survival group n=20**	**Median of cells/HPF Poor survival group n=15**	**P value**
CD8+	CD8+ T cell	106.5	84	0.9934
CD8+CD103+	CD8+ Tissue Resident Memory Cell (T_{RM})	4.5	6	0.5242
CD8+GZB+	Cytotoxic CD8+ T cell	8	12	0.3094
CD8+CD103+TIM3+	Highly functional CD8+ T_{RM}	0	1	0.1027
CD8+CD103+GZB+	Cytotoxic CD8+ T_{RM}	2	1.5	0.2319
CD8+CD103+TIM3+GZB+	Cytotoxic Highly	0	1	0.0556

	functional CD8+ T_{RM}			

Table 6 Comparison of median values of CD8+ T cells and CD8+ T cells subtypes between good and poor survival groups, significant Mann Whitney test p value <0.05

4.4 Discussion

The results presented in chapter 3 indicated clearly but surprisingly, that in epithelioid mesothelioma, CD8+ TILs were not increased in numbers in subjects with good survival outcome. By contrast, there was a significant difference in the numbers of CD4+ TIL between the two groups. The aim of the work in the present chapter was therefore, to characterize the subsets of CD4+ and CD8+T cells to see whether clear evidence could be obtained to understand this observation.

A number of observations emerged. Similar to the results presented in the previous chapter, there were greater numbers of CD4+ T cells in tumours with good prognosis than those with poor prognosis. The median number of CD4+ T cells (per hpf) was ca. 4-fold higher, 280 in the good prognosis versus 74 in the poor prognosis group (p=0.0009). On further phenotyping, the tissue resident CD4+CD69+ cells and some subsets of T follicular helper cells were also significantly greater in number in the good prognosis group. Cytotoxic CD4+ T cells were present in both the tumours with good and poor outcome and with increased numbers in the good survival group that trended towards but did not reach statistical significance. So if these cells are playing a role, it is not simply their numbers but more likely, their functional capabilities that are the key factor.

CD4+CD69+ cells are tissue resident and between the good and poor prognosis groups, their numbers differed significantly. However, while CD4+CD69+GZB+ cells were present in both groups, expression of this marker of cytotoxicity did not differentiate between the groups.

In the larger cohort of patients presented in the previous chapter, a low number of FOXP3+ cells was associated with a better outcome. Both T regulatory cells and T follicular helper cells express FOXP3. In this smaller patient sample, neither were significantly associated with survival. However, CD4+CXCL13+ cells, a subtype of T follicular helper cells were present in significantly

greater numbers in the good survival group compared with the poor survival group. T follicular helper cells are usually characterised by CXCR5+ expression. There are some populations of Tfh cells that do not express CXCR5 but these have been shown to have a similar gene profile to CXCR5+ Tfh cells (Rao et al., 2017). T follicular helper cells play an essential role in the formation of the germinal centre and tertiary lymphoid structures to support B cell proliferation and differentiation. High intra-tumoural levels of Tfh cells have been associated with better survival in breast cancer and melanoma (Gu-Trantien et al., 2013, Nurieva et al., 2019). IL21 and CXCL13 produced by Tfh cells may confer a protective effect by attracting activated and memory T cells to the tumour microenvironment (Qin et al., 2018). The possible mechanisms by which these cells may contribute to the anti-tumour immune response needs to be investigated using a more extensive range of approaches and techniques.

This study has demonstrated for the first time that MHC class II expression on epithelioid mesothelioma cells (WT1+HLADPB1+) is associated with a better outcome. It is not clear which cells are producing the interferon gamma that is inducing MHC class II expression on the surface of tumour cells but this is potentially important as this may represent a target to be manipulated therapeutically. It may be this is induced by the presence of Th1 CD4+ cells which requires further investigation. It is also known that pulmonary epithelial cells are a source of interferon gamma in infection and it may also be possible that the pleural epithelial cells are the source of interferon gamma. The other cells that produce interferon gamma such as CD8 + T cells, NK cells, and macrophages were not shown to be significantly associated with survival in epithelioid mesothelioma in the previous chapter. Interferon gamma has been previously trialled in the treatment of mesothelioma with limited anti-tumour activity (Monnet et al., 2002). It is likely that the protective effect is less to do with the presence of interferon gamma per se, but more the complex interplay of factors within the tumour microenvironment that is associated with elevated levels of interferon gamma.

The main accepted function of CD8+ T cells in anti-tumour immunity is of cytotoxicity. CD8+GZB+ cells represent actively cytotoxic CD8+ T cells and CD8+CD103+GZB+ cells are cytotoxic CD8+ T_{RM} cells. CD8+ T cell numbers in epithelioid mesothelioma are not associated with outcome in this cohort and further phenotyping of CD8+ T cells subpopulations such as CD8+ tissue resident and cytotoxic cells failed to reveal in other phenotypic differences that might have been relevant to survival.

The lack of differences between tumours with good and poor prognoses raises questions about the phenotypes and functional characteristics of the intra-tumoural CD8+ T cells. Exactly what CD8+CD103+GZB- cells are would require either further stains for functionally relevant markers,

or alternative methodological approaches such as transcriptomic analysis, as they are likely to represent tissue resident memory CD8+ T cells that may be exhausted.

Hence, it would be important to know whether the cells without a cytotoxic phenotype (GZMB negative) may be functionally impaired, for example through being in a state of “exhaustion”.

Because of the lack of correlation of CD8+ T cells of any type with outcome, the question had arisen of whether expression of MHC-Class I might be a factor. Perhaps surprisingly, this study has demonstrated a significantly increased expression of HLA Class 1 in better prognosis tumours. Thus, both the numbers of WT1+ tumour cells expressing HLA Class 1+ and the intensity of staining of HLA Class 1 were significantly reduced in poor compared to good survival groups in epithelioid mesothelioma. Together, these data suggest possible downregulation of MHC class I in tumour cells in epithelioid mesothelioma to evade surveillance by immune cells, particularly in the poor survival group. This does leave open the possibility that CD8+ CTLs are making a contribution to tumour cell killing in the good prognosis group despite the numbers of CD8+ T cells being similar across the groups.

To understand the potential protective role of CD4+ T cells will require the use of a broader range of techniques. Although staining with a wider array of antibodies may increase our awareness of the functional capacities of these cells, more sophisticated techniques such as RNAseq will allow an in-depth analysis of their transcriptomic programmes to definitively assess the nature and phenotype for cytotoxic CD4+ and CD4+T_{RM} cells.

Chapter 5 Mesothelioma associated Pleural fluid FACS and RNAseq

5.1 Introduction

Pleural fluid is easily accessible making it attractive as a possible surrogate for analysis of biological activity in the pleural space in both infectious and malignant diseases. Pleural fluid is used for diagnostic purposes in routine clinical care and cancer cells are found in around 60% of mesothelioma associated pleural effusions (Roberts et al., 2010). In both lung cancer and mesothelioma, the cancer cells in pleural fluid have been shown to contain the same mutations as those in the solid tumour (Sneddon et al., 2018, Carter et al., 2017).

The immune infiltrate has been shown to have a significant impact on clinical outcome in most solid tumours. The data presented in the previous chapter shows this to also be the case in mesothelioma.

Previous studies have analysed the immune infiltrate in pleural fluid from cancer-associated effusions as well as from effusions associated with infection and inflammation. Pleural effusions associated with cancer have been shown by flow cytometry analysis to contain more naïve and central memory T cells than their benign counterparts (Scherpereel et al., 2013).

The work presented in this chapter sought to understand if immune cells in the pleural fluid reflected those in the solid tumour, making this compartment potentially useful as a surrogate target for investigation. The immune infiltrate of T cells and macrophages in pleural effusions and their solid tumour components was shown not to correlate in 4 of 5 patients in a small study (Lievense et al., 2017).

Significant numbers of immune cells are present in the pleural fluid and we sought to gain a better understanding of the role of these cells, specifically focusing on the role of CD4+ and CD8+ T cells. Immune cells cryopreserved from pleural fluid were stained with antibodies to evaluate and sort CD4+ and CD8+ T cells, B cells and macrophages by flow cytometry. The CD4+ and CD8+ T cells were further evaluated by RNA-seq for differential gene expression.

5.2 Results

The clinical demographics of the 23 patients evaluated in this cohort are shown in Table 1. The median age was 74 (range 61-83) years, 90% were men. 16 (70%) were classified as epithelioid and 7 (30%) were non-epithelioid mesothelioma. This is comparable to the larger cohort of patients presented in Chapter 3.

Variable		**N (%)**
Age	<65	1 (4%)
	>65	22 (96%)
Sex	Male	20 (90%)
	Female	3 (10%)
Histology	Epithelioid	16 (70%)
	Non-epithelioid	7 (30%)

Table 1: **Patient demographics for sorted pleural fluid samples**

The number of CD4+ and CD8+ T cells in the mesothelioma tumour samples was evaluated by immunohistochemistry. The mean count/high power field was used similar to the TMA evaluation presented in Chapter 3.

Flow cytometric analysis and bulk sorting of CD4+ and CD8+ T cells was undertaken for 23 samples of mesothelioma-associated pleural fluid. The gating strategy is shown in Figure 1.

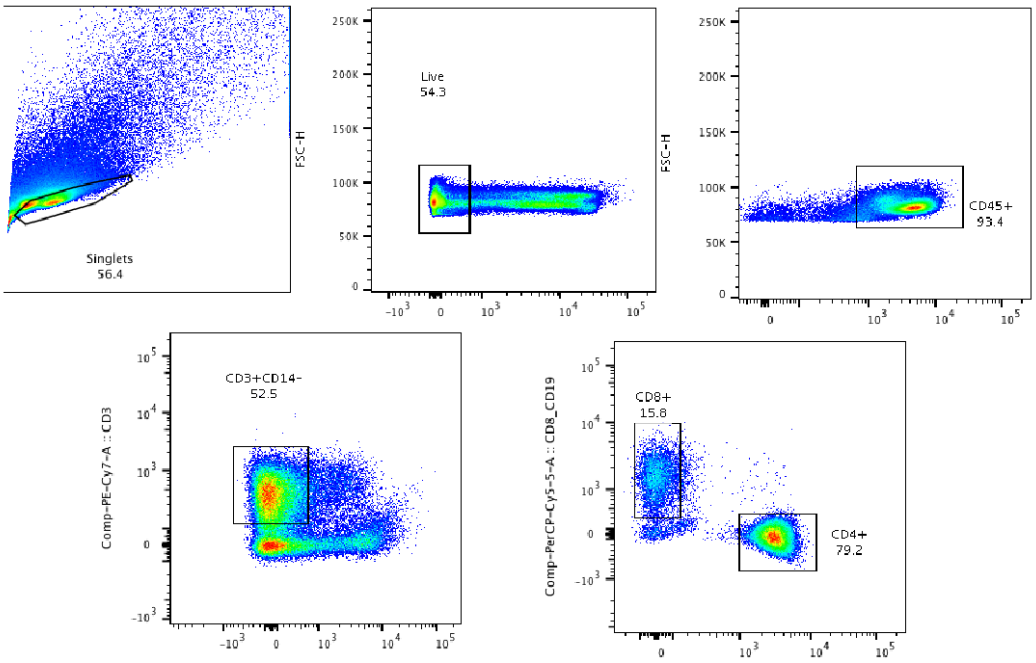


Figure 1: Gating strategy for sorting CD4+ and CD8+ T cells

The distribution of T cell subtypes in the sorted populations is shown as the mean with standard deviation in Figure 2. The CD4+ T cell proportion outnumbers the CD8+ T cell proportion in all cases.

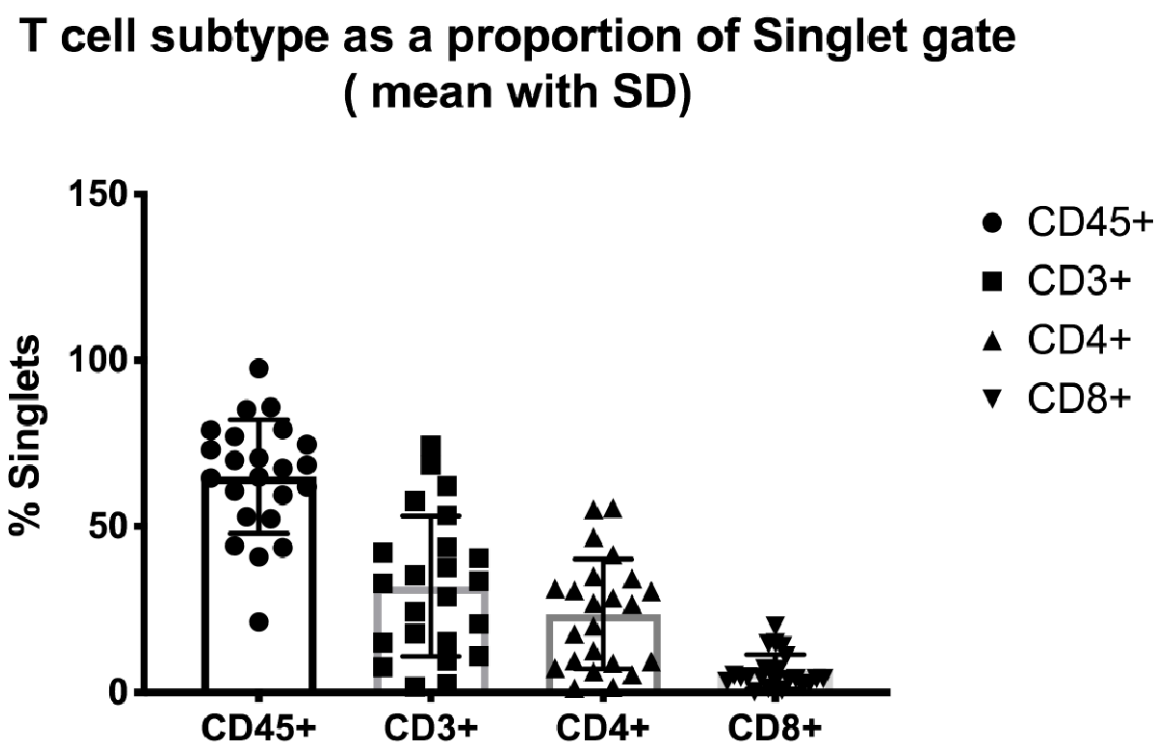


Figure 2: T cell subtype as a proportion of Singlet gate in pleural fluid samples sorted by flow cytometry (Mean with SD)

To identify the core transcriptional signature of the CD4+ and CD8+ T cells present in pleural fluid associated with mesothelioma, RNA-seq was performed on samples from 23 patients with pleural effusions associated with mesothelioma, categorised as epithelioid and non-epithelioid types. This was compared to transcriptional profiles from non-matched normal lung tissue and NSCLC (non-small cell lung cancer) generated from 36 patients previously evaluated in our group.

2572 genes were differentially expressed by CD4+ T cells in mesothelioma-associated pleural fluid vs normal lung and 2652 genes were differentially expressed by CD4+ T cells in mesothelioma-associated pleural fluid vs NSCLC, Benjamini-Hochberg–adjusted *P* value of <0.05 and fold change of 1.5 (Figure 3).

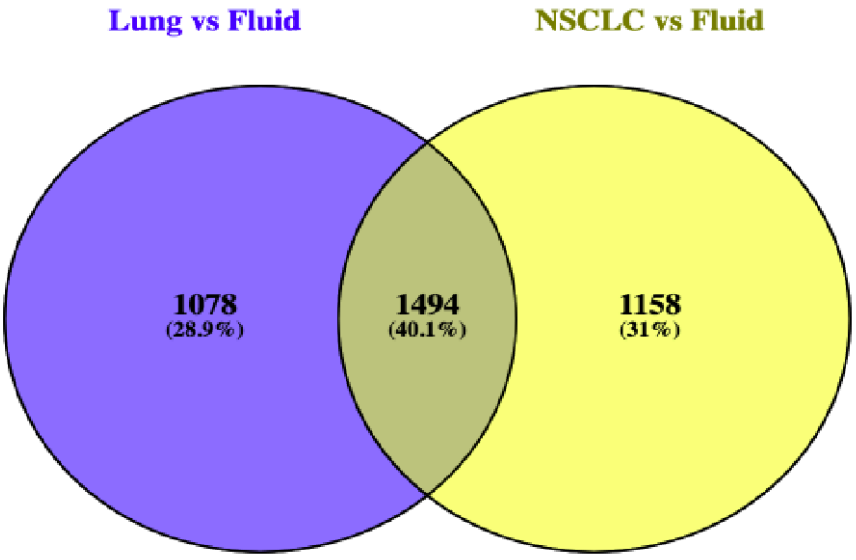


Figure 3: Venn diagram of differentially expressed genes – CD4+ T cells in normal lung vs mesothelioma-associated pleural fluid and CD4+ T cells in NSCLC vs mesothelioma-associated pleural fluid, padj<0.05, fold change 1.5

4379 genes were differentially expressed by CD8+ T cells in mesothelioma-associated pleural fluid vs normal lung and 5159 genes were differentially expressed by CD8+ T cells in mesothelioma-associated pleural fluid vs NSCLC (Benjamini-Hochberg–adjusted *P* value of <0.05, fold change 1.5) (Figure 4).

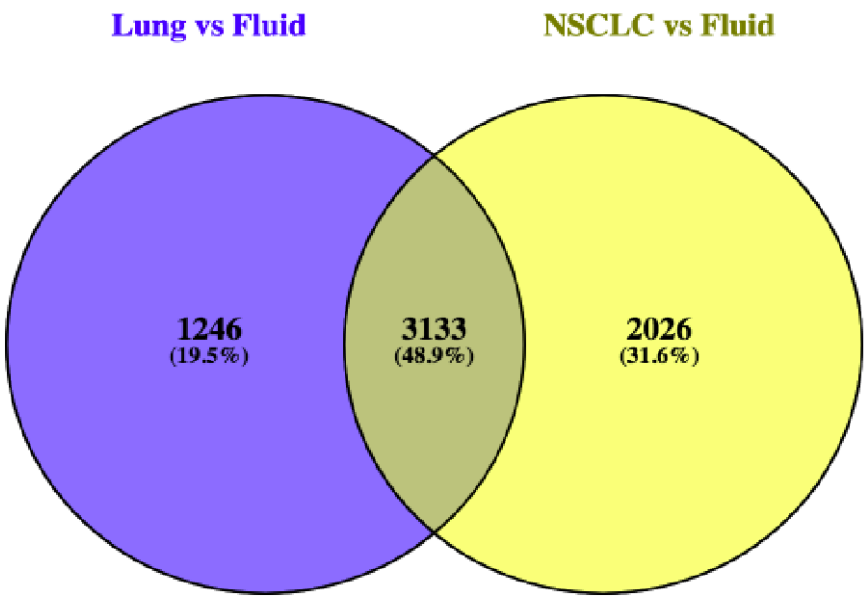


Figure 4: Venn diagram of differentially expressed genes – CD8+ T cells in normal lung vs mesothelioma-associated pleural fluid and CD8+ T cells in NSCLC vs mesothelioma-associated pleural fluid, padj<0.05, fold change 1.5

The expression of transcripts did not differ in the mesothelioma-associated pleural fluid between high and low intra-tumoural CD4+ T cells or between epithelioid and non-epithelioid subtypes.

Principle component analysis (figure 5 and 6) and hierarchical clustering (not shown) showed that CD4+ and CD8+ T cells from mesothelioma-associated pleural fluid clustered together compared to both normal lung and NSCLC.

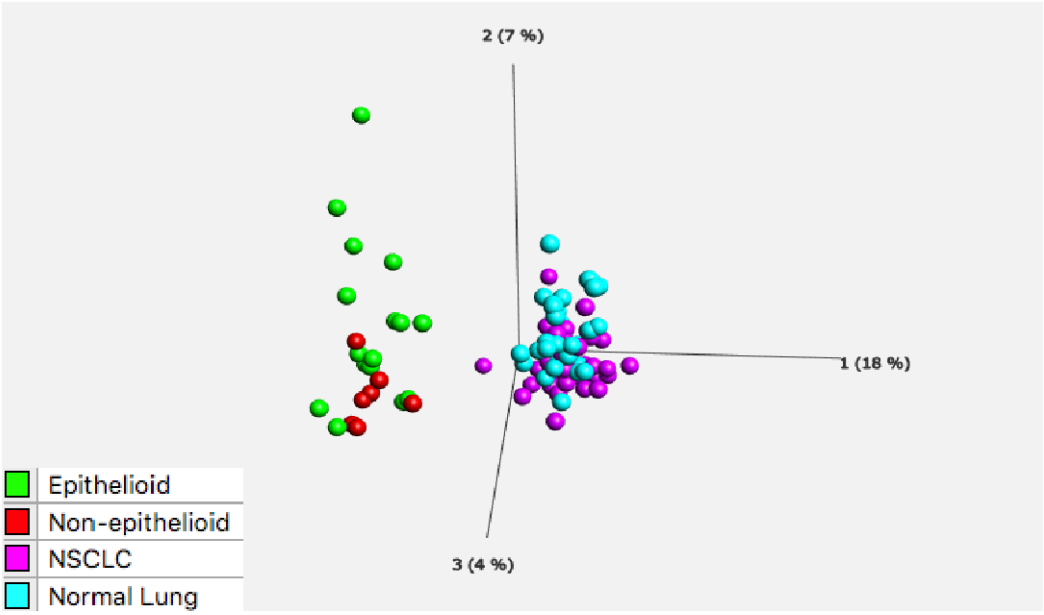


Figure 5: CD4+ T cells in mesothelioma-associated pleural fluid vs normal lung and NSCLC (padj<0.05, fold change 1.5)

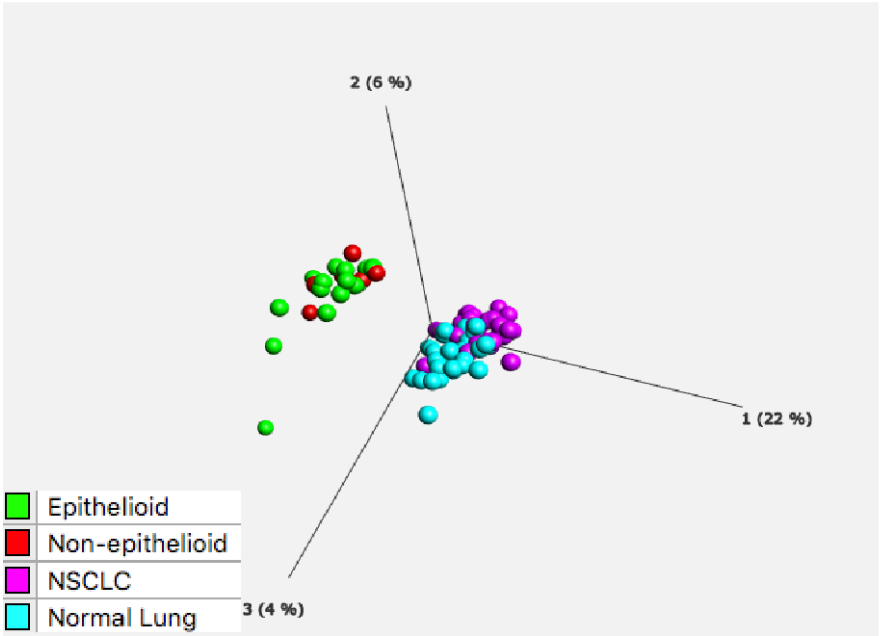


Figure 6: CD8+ T cells in mesothelioma-associated pleural fluid vs normal lung and NSCLC (padj<0.05, fold change 1.5)

CD4+ T cells

CD4+ T cells are sub-divided into the following categories based on their functionality. CD4+ helper cells: Th1, Th2, Th9, Th17 and Th22; CD4+ T regulatory cells, CD4+ T follicular helper cells,

cytotoxic CD4+ T cells and memory CD4+ T cells. CD4+ T cells are found abundantly in pleural fluid associated with mesothelioma and this analysis sought to understand their phenotype and function.

Differentially expressed genes for CD4+ T cell differentiation and activation, CD4+ T cell memory and CD4+ T cell cytotoxicity were analysed in mesothelioma-associated pleural fluid compared with normal lung and mesothelioma-associated pleural fluid compared with NSCLC.

CD4+ T cell differentiation and activation

A comparison of differentially expressed genes associated with CD4+ T cell differentiation was evaluated in mesothelioma-associated pleural fluid vs normal lung and mesothelioma-associated pleural fluid vs NSCLC. The fold change and Benjamini-Hochberg–adjusted *P* values (q values) are shown in supplementary table 1a, Appendix A.

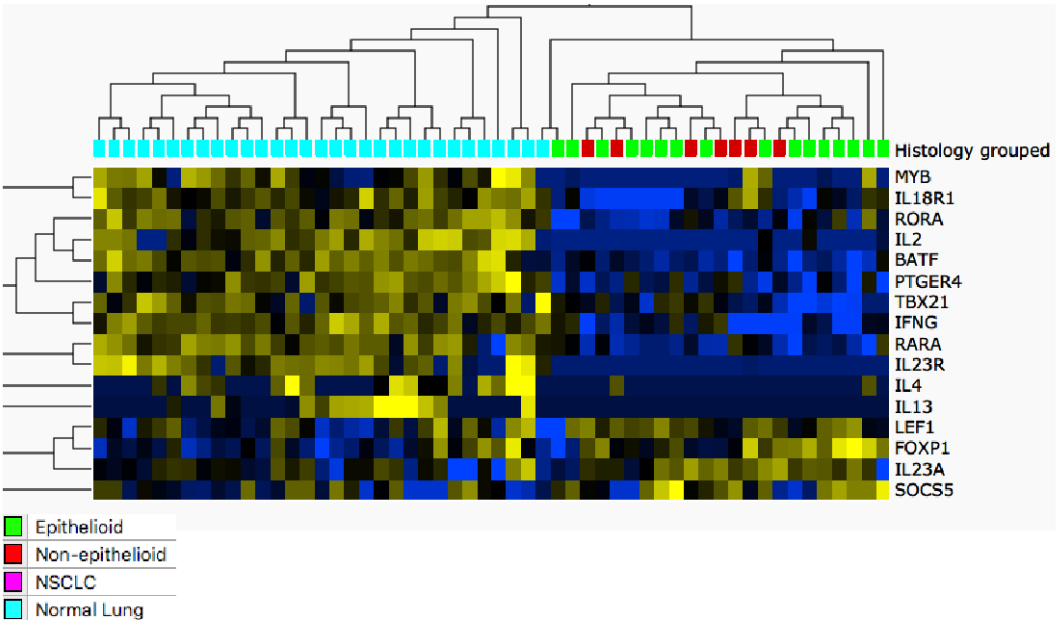


Figure 7: Heat map of differentially expressed CD4+ T cell differentiation and activation genes between normal lung and mesothelioma-associated pleural fluid, padj<0.05, fold change 1.5

The genes that are upregulated in mesothelioma-associated pleural fluid are also expressed in normal lung tissue CD4+ T cells (figure 7)

Of interest are the downregulated genes in mesothelioma-associated pleural fluid CD4+ T cells compared to those from normal lung, shown in the heat map (figure 7). Genes responsible for key

drivers of the CD4+ T cell differentiation pathway are notably downregulated in the mesothelioma associated pleural fluid.

TBX21 encodes for T-bet, the key lineage defining Th1-specific transcription factor that controls the expression of INFG, the signature Th1 cytokine. TBX21 also inhibits development of Th2 and Th17 cells. T-bet is not expressed in naïve T cells (Lazarevic et al., 2013). There is some expression of TBX21 in the mesothelioma associated pleural fluid but overall it is downregulated in comparison to the expression in normal lung tissue.

IFNG encodes for interferon gamma, an important cytokine expressed by many cells including activated CD4+ Th1, CD8+ CTLs and NK cells. Interferon gamma plays a role in enhancing antigen presentation, macrophage activation, as well as forming a positive feedback loop to enhance Th1 differentiation and suppress Th2 differentiation. Importantly, this is downregulated in most of the mesothelioma-associated pleural fluid samples.

IL2 is produced by activated Th1 CD4+ cells and can also be produced by CD8+ T cells, NK cells and dendritic cells. IL2 promotes CD4+ T cell proliferation and differentiation into Th1 and Th2 cells and inhibits Th17 differentiation (Paul and Zhu, 2010, Szabo et al., 2003, Littman and Rudensky, 2010, Jiang et al., 2016). IL2 acts on CD4+ T cells in an autocrine manner in a positive feedback loop, driving proliferation through its cognate receptor IL-25. IL2 is downregulated across all samples of mesothelioma-associated pleural fluid. This indicates that the CD4+ T cells in mesothelioma-associated pleural fluid are less activated and proliferating less than their counterparts in normal lung.

IL4 is a key cytokine produced by Th2 cells. IL4 acts in an autocrine manner on CD4+ T cells to induce Th2 differentiation and proliferation. IL4 is downregulated in most of the samples of mesothelioma-associated pleural fluid along with IL13. IL13 is another key Th2 cytokine that has a similar biological function to IL4 apart from not playing a role in T cell activation due to the lack of IL13R on the surface of Th0 cells (Mak and Saunders 2006).

MYB together with GATA3 are key transcription factors essential for Th2 differentiation and maturation (Naito and Taniuchi, 2010) (Nakata et al., 2010). MYB is downregulated in most samples of mesothelioma associated pleural fluid.

RORA (Related Orphan Receptor A) is involved in the lineage specification of CD4+ T cells into Th17 (Yang et al., 2008). This is downregulated in the most of the mesothelioma associated pleural fluid samples.

IL18R1 is present widely on Th17 cells, mostly on Th1 cells and on a proportion of induced Tregs but not on naïve CD4+ T cells or Th2 cells (Yu et al., 2008, Harrison et al., 2015). IL18R1 is associated with the ability of CD4+ and CD8+ memory T cells to respond to cytokines such as IL18 in combination with IL12 or IL21 to produce interferon gamma in the absence of antigen exposure (Ingram et al., 2011). In infectious disease, this allows memory T cells to respond to inflammation- inducing infections independent of antigen presentation and the T cell receptor (Ingram et al., 2011). IL18R1 is preferentially expressed on TEMRA and effector memory cells as compared to expression on naïve and central memory cells (Bofill et al., 2004). In CD8+ T cells, IL18Ralpha downregulation is associated with exhaustion (Ingram et al., 2011). IL18R is mostly downregulated on CD4+ T cells in mesothelioma associated pleural fluid.

BATF (basic leucine zipper transcription factor ATF-like) together with IRF4 is important for Th9 and Th17 differentiation and also controls Tfh differentiation by regulating the BCL6 and c-MAF genes (Ise et al., 2011, Jabeen et al., 2013, Schraml et al., 2009, Sahoo et al., 2016). This gene is downregulated globally in the mesothelioma associated pleural fluid samples.

PTGER4 is a prostaglandin receptor for prostaglandin E2. PTGER4 as part of COX2-PGE2-EP2/EP4 signalling promotes induction of IL23R, leading to proliferation of Th17 cells (Lee et al., 2019). PTGER4 is mostly downregulated in the mesothelioma associated pleural fluid.

IL23R is expressed on Th0, Th1, Th17 CD4+ T cells as well as on memory T cells. Naïve T cells express small amounts of IL23R. IL23 is the main inducer of Th0 to Th17 differentiation (Li et al., 2016). IL23R is uniformly downregulated in the mesothelioma-associated pleural fluid.

RARA (Retinoic acid receptor alpha) is required for the induction of regulatory T cells and required for CD4+ effector cell activation (Hill et al., 2008, Hall et al., 2011). Naïve CD4+ T cells have also been shown to have high expression of RARA (Hall et al., 2011). In a murine knockout experiment, T cells lacking RARA proliferated less efficiently in response to T cell stimulation and displayed early TCR activation defects (Hall et al., 2011). Expression of RARA is mostly downregulated in mesothelioma associated pleural fluid.

Taken together, there is global downregulation of IL2 that is associated with differentiation and proliferation of Th1 and Th2, IL23R associated with Th17 differentiation and BATF that is associated with Th9 and Th17 differentiation.

Key genes responsible for Th cell lineage such as TBX21 which is responsible for Th1 cell differentiation and RORA which is involved in Th17 differentiation and MYC which is involved in Th2 differentiation are downregulated in most samples of mesothelioma-associated pleural fluid.

The majority of mesothelioma-associated pleural fluid samples demonstrated downregulation of IFNG an important marker of Th1 activation. Other markers of activation of effector cells and T regulatory cell induction such as RARA were also similarly mainly downregulated.

This would indicate that there is a downregulation of genes associated with Th1, Th2, Th9, Th17, T regs and T effector cells, as well as genes associated with activation and proliferation of the CD4+ T cells in mesothelioma-associated pleural fluid compared to normal lung tissue.

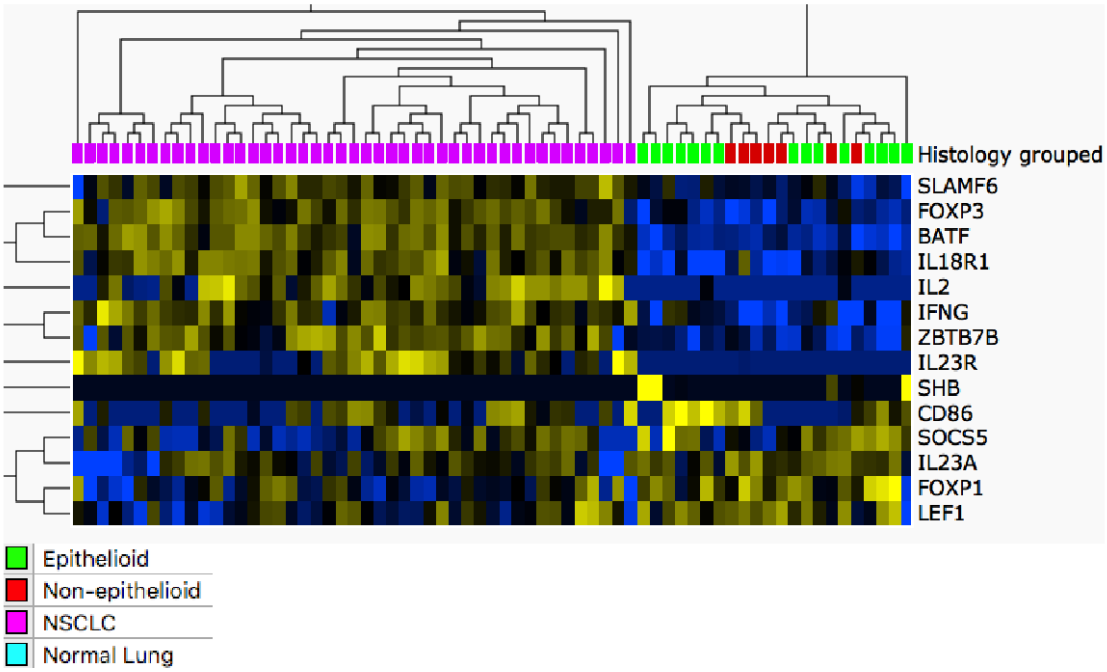


Figure 8: Heat map of differentially expressed CD4+ T cell differentiation and activation genes between NSCLC and mesothelioma-associated pleural fluid, padj<0.05, fold change 1.5

A similar picture is seen when the comparison is made between differentially expressed genes in NSCLC and mesothelioma-associated pleural fluid CD4+ T cells where the genes upregulated in the pleural fluid are also expressed in the NSCLC samples (figure 8). The fold change and Benjamini-Hochberg–adjusted *P* values (q values) are shown in supplementary table 1b, Appendix A.

Here, the expression of IL2 which is related to T cell proliferation and Th1 differentiation, BATF which is responsible for IL4 expression in Tfh, Th17 and Th9 cells, and IL23R which is mainly

expressed on activated/memory T cells are globally downregulated in mesothelioma associated pleural fluid similar to the previous comparison with normal lung.

In addition, the gene expression of FOXP3 and ZBTB7B is also downregulated across all samples of pleural fluid. FOXP3 is the master regulator for T regulatory cells and ZBTB7B is a key regulator of lineage commitment of CD4+ cells.

IL18R1 and IFNG expression is also downregulated in most samples of mesothelioma-associated pleural fluid when compared to NSCLC similar to the previous comparison with normal lung.

SLAMF6 promotes differentiation into Th17 cells and is downregulated in most of the sample of mesothelioma associated pleural fluid.

This comparison indicates a downregulation of key genes associated with Th1, Th17, T reg differentiation and Th1 cell proliferation in mesothelioma-associated pleural fluid when compared with NSCLC.

CD8+ T cells

CD8+ T cell differentiation produces cytotoxic and memory phenotypes. Cytotoxic Tc1 cells, as well as Tc2, Tc9 and Tc17 cells have been described. CD8+ memory cells are divided into central memory, effector memory, resident memory populations.

CD8+T cell infiltration has been shown in the TMA data presented in chapter 3 to be not associated with survival in mesothelioma, unlike in many other solid tumours. In this analysis, we sought to understand the phenotype and function of the CD8+ T cells in the mesothelioma-associated pleural fluid for insights into this behaviour.

Differentially expressed genes for CD8+ T cell differentiation and activation, CD8+ T cell memory and CD8+ T cell cytotoxicity were analysed for mesothelioma-associated pleural fluid compared with normal lung and mesothelioma-associated pleural fluid compared with NSCLC.

CD8+ T cell differentiation and activation

A comparison of differentially expressed genes associated with CD8+ T cell differentiation was evaluated in mesothelioma-associated pleural fluid vs normal lung and mesothelioma-associated pleural fluid vs NSCLC. The heat map for the comparison of mesothelioma-associated pleural fluid

and normal lung is shown in figure 9. The fold change and Benjamini-Hochberg–adjusted *P* values (q values) are shown in supplementary table 2a, Appendix A.

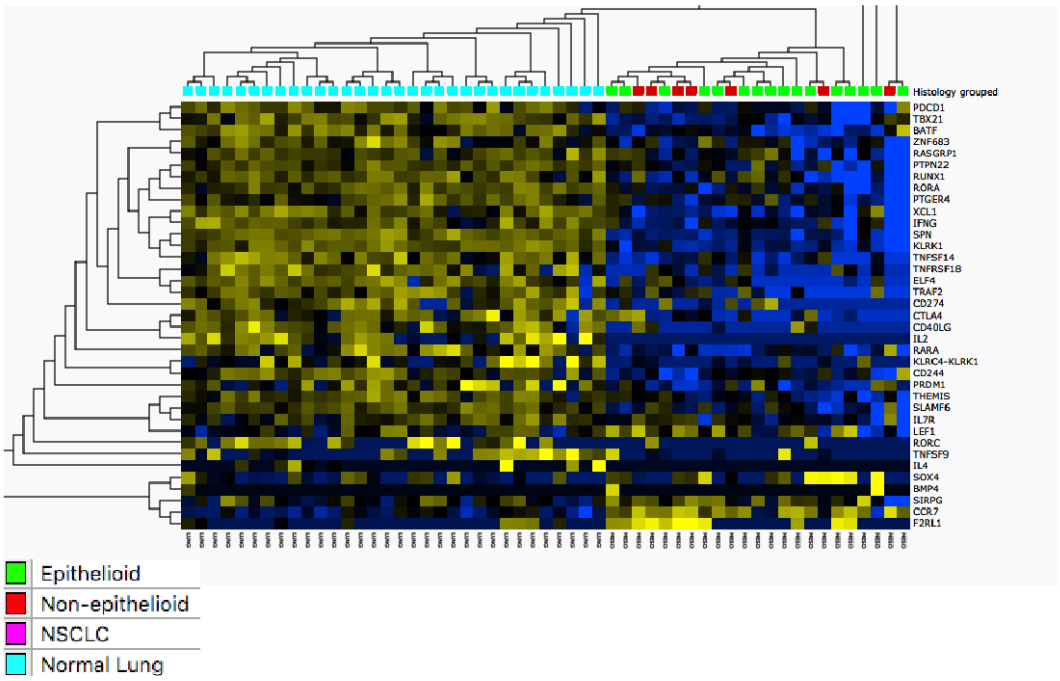


Figure 9: Heat map of differentially expressed CD8+ T cell differentiation and activation genes between normal lung and mesothelioma-associated pleural fluid, padj<0.05, fold change 1.5

PDCD1 (programmed cell death 1) is the central inhibitory receptor regulating CD8+ T cell exhaustion during chronic infections and cancer. It is mostly downregulated in mesothelioma-associated pleural fluid compared to normal lung.

TBX21 encodes Tbet, a critical driver of CD4+ Th1 differentiation but also of effector CD8+ T cell differentiation. T bet operates in a graded manner in CTLs, with a moderate amount allowing for memory cell fates but larger amounts promoting terminal differentiation (Joshi et al., 2007, Dominguez et al., 2015). TBX21 is mostly downregulated in mesothelioma-associated pleural fluid.

BATF is important in the differentiation of many lymphocytes. In CD8+ T cells, BATF is a central regulator of early effector differentiation after antigen exposure and BATF deficient cells show defects in differentiation and proliferation (Kurachi et al., 2014). BATF is mostly downregulated in mesothelioma-associated pleural fluid, which may explain the lack of effector function in these cells.

ZNF683 (Hobit) is expressed in effector T cells, particularly in quiescent and long-lived effector CD8+ T cells but not in naïve or memory CD8+ T cells or CD4+ T cells (Post et al., 2017, Vieira Braga

et al., 2015). Expression of Hobit strongly correlated with T-bet and IFNG expression in CD8+ T cells (Vieira Braga et al., 2015). ZNF683 is mostly downregulated in mesothelioma-associated pleural fluid compared to normal lung.

RASGRP1 (Ras-guanyl nucleotide exchange factor) has been shown to play a selective role in T cell signaling in CD8+ T cell maturation (Priatel et al., 2010). RASGRP1 deficient CD8+ T cells display an anergic phenotype that is partially reversible on addition of IL2 (Priatel et al., 2010). RASGR1 is mostly downregulated in mesothelioma-associated pleural fluid.

PTPN22 (protein tyrosine phosphatase nonreceptor 22) is a negative regulator of T cell signaling (Sood et al., 2016). In a murine model, PTPN22-/- mice show increased TCR signaling particularly in effector cells (Sood et al., 2016). PTPN22 may play a dual role as lack of PTPN22 expression promotes CD8+ T cell activation and cytokine production in a viral murine model but inhibits proliferation ((Jofra et al., 2016). PTPN22 is mostly downregulated in mesothelioma-associated pleural fluid indicating inhibition of CD8+ T cell activation.

RORA and RORC are required for effector function and differentiation of CD8+ CTLs (Miao et al., 2017, Kaech and Cui, 2012, Dominguez et al., 2015, Omilusik et al., 2015). Both are downregulated in mesothelioma-associated pleural fluid.

PTGER4 is a PGE2 receptor and is overexpressed in chronic viral infections by more terminally exhausted CD8+ CTLs compared to naïve CD8+ T cells (Kabashima et al., 2002, Chen et al., 2015). These were mostly downregulated in the mesothelioma-associated pleural fluid.

XCL1 is produced by activated CD8+ T cells and NK cells to control the movement of CD4+ and CD8+ T cells and also controls IFNG production by CD4+ T cells (Ordway et al., 2007). XCL1 expression is mostly downregulated in mesothelioma-associated pleural fluid.

IFNG is produced by a wide variety of cells including NK and NKT cells in the innate immune system and CD4+ Th1 cells and CD8+ CTLs in the adaptive immune response. IFNG is mostly downregulated in the mesothelioma-associated pleural fluid compared with normal lung.

SPN encodes a glycoprotein involved in antigen specific activation of T cells. It is found on many cells including T lymphocytes. It is globally downregulated in CD8+ T cells in mesothelioma-associated pleural fluid indicating these cells are not activated.

KLRK1 is important in NK cells and acts as a co-stimulatory molecule to the TCR in CD8+ T cells by amplifying T cell activation (Refseq 2010). KLRK1 is globally downregulated in mesothelioma-associated pleural fluid.

TNFSF14 is expressed on many cells including monocytes, NK cells and CD8+ T cells. In CD8+ T cells it may function as a co-stimulatory factor for activation and stimulate CD8+ T cell proliferation. It is mostly downregulated in mesothelioma-associated pleural fluid.

TNFRSF18 encodes for GITR (Glucocorticoid-induced TNFR-related protein), which is expressed in all T cells subsets but particularly in Tregs. GITR is a co-stimulatory molecule which enhances T cell proliferation, survival and cytokine production (Ronchetti et al., 2012). In CD8+ T cells, GITR is expressed on T cell activation and its absence prevents proliferation. GITR has been found to be enhances in tumour specific CD8+ T cells (Ronchetti et al., 2012). TNFRSF18 is mostly downregulated in mesothelioma-associated pleural fluid.

ELF4 has been shown to directly activate KLF4 to induce cell cycle arrest in naïve CD8+ T cells (Yamada et al., 2009). ELF4 negatively regulates the proliferation of naïve CD8+ T cells and restricts the expansion of CD8+ T cells with a memory phenotype (Yamada et al., 2009). Loss of ELF4 resulted in deregulated T cell population expansion during homeostasis (Yamada et al., 2009). By inducing the expression of KLF4, ELF4 maintains the expression of CD62L and CCR7 on memory cells (Yamada et al., 2009). ELF4 is mainly downregulated in mesothelioma-associated pleural fluid.

TRAF2 belongs to a family of intracellular signaling molecules. In a murine model, TRAF2 negative mice show impairment in T cell memory responses with reduction in effector cytotoxic CD8+ T cells to re-stimulation (Cannons et al., 2002). TRAF2 is downregulated in most samples of mesothelioma-associated pleural fluid.

CD274 encodes for PDL1, which is an inhibitory receptor ligand widely expressed on immune cells and tumour cells. Naïve CD8+ T cells do not express PDL1 and only express high levels of PDL1 on activation by IFN-γ and TNF-α (Gibbons Johnson and Dong, 2017). PDL1 also plays a role in survival in the contraction phase of an immune response (Pulko et al., 2011). CD274 is mainly downregulated in mesothelioma-associated pleural fluid.

CTLA4 is a costimulatory molecule that negatively regulates early CD8+ T cell activation and proliferation. In normal T cell subsets, CTLA4 is more highly expressed on CD4+ T cells than CD8+ T cells (Chan et al., 2014). CTLA4 expression is mostly downregulated in mesothelioma-associated pleural fluid.

CD40LG codes for CD40Lwhich is expressed on CD4+ and CD8+ T cells and mediates a wide range of immune functions. It has been found to allow CD8+ T cells to promote their own and bystander differentiation and expansion via dendritic cells (Tay et al., 2017). Lack of CD40L results in lower

number of memory CD8+ T cells (Wherry et al., 2004). It is mostly downregulated in mesothelioma-associated pleural fluid.

IL2 can be produced by CD8+ T cells. In newly activated naïve CD8+ T cells, cell that do not express IL2 preferentially attain effector features while autocrine IL2 production was associated with the formation of a memory phenotype (Kahan et al., 2015). IL2 is globally downregulated in mesothelioma-associated pleural fluid possibly alluding to a lack of memory formation.

RARA (retinoic acid receptor alpha) has been shown to upregulate tissue homing receptor and control CD8+ T cell survival after activation (Guo et al., 2014). RARA was mainly downregulated in mesothelioma-associated pleural fluid.

KLRC4-KLRK1 encodes the KLRK1 (NKG2D) protein that is expressed on the surface of NK cells and CD8+ T cells. In CD8+ T cells it recognises a stressed target to enhance TCR signalling to activate a cytotoxic response (Prajapati et al., 2018). This is mostly downregulated in mesothelioma-associated pleural fluid.

CD244 is expressed by memory phenotype CD8+ T cells and NK cells. CD244 is required for optimal activation of CD8+ T cells (McNerney et al., 2005). CD244 is mostly downregulated in mesothelioma-associated pleural fluid and may indicate a lack of activation of the cells in these samples.

PRDM1 encodes for Blimp1 that promotes CD8+ T cells into short-lived effector cells rather than central memory cells (Welsh, 2009). Blimp1 is induced in T cells by IL2 but expression represses IL2 gene transcription (Martins et al., 2008). Blimp1 mRNA is high in the short-lived effector cells but low in memory cells (Rutishauser et al., 2009). Blimp1 expression was also elevated in effector memory cells but low in central memory cells (Rutishauser et al., 2009). PRDM1 is mostly downregulated in mesothelioma-associated pleural fluid, which may be in keeping with a predominately central memory phenotype in these samples.

TNFSF9 encodes a cytokine of the TNF ligand family that acts as a ligand for 41BB. This is present in activated CD8+ T cells and involved in antigen presentation, the generation of cytotoxic T cells survival and proliferation (refseq 2008). It is mainly downregulated in mesothelioma-associated pleural fluid, potentially indicating the cells are less activated in this compartment.

F2RL1 encodes PAR2 (protease-activated receptor 2). Overexpression of F2RL1 has been linked to many epithelial cancers including breast, lung and colon cancer (Wu et al., 2019). PAR2 stimulates VEGF for angiogenesis and is linked to cancer cell migration (Versteeg et al., 2008). Selective PAR2 blockade has been investigated as a potential drug target in inflammatory diseases and cancer.

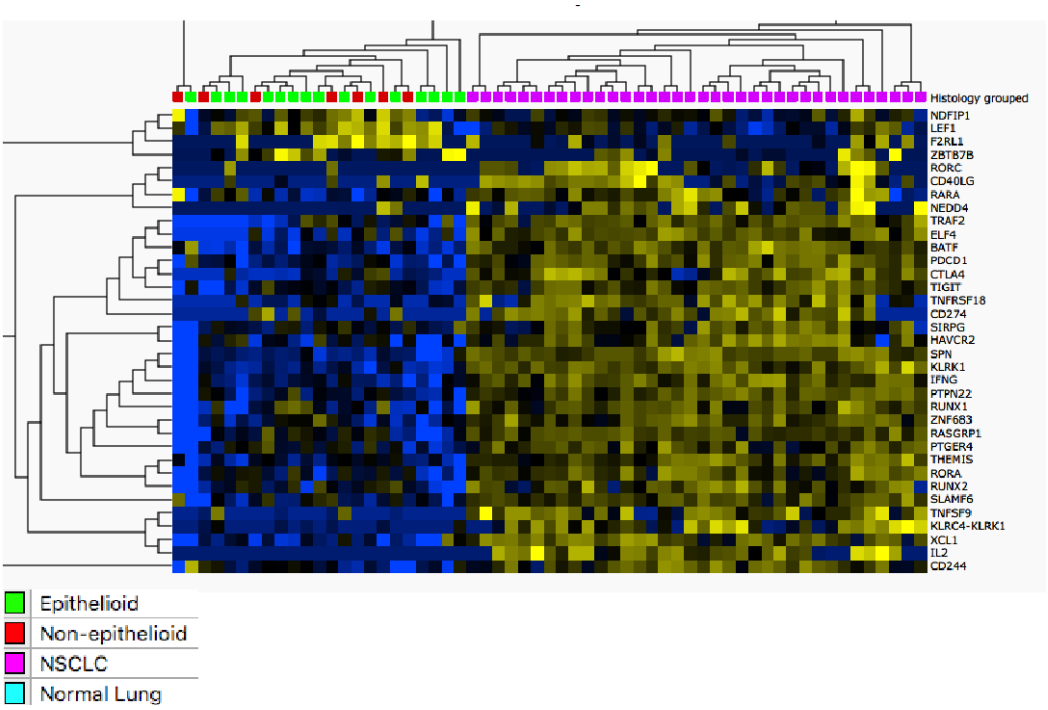


Figure 10: Heat map of differentially expressed CD8+ T cell differentiation and activation genes between mesothelioma-associated pleural fluid and NSCLC, padj<0.05, fold change 1.5

Next, a comparison of differentially expressed genes associated with CD8+ T cell activation and differentiation were evaluated in mesothelioma-associated pleural fluid compared with NSCLC (figure 10). The fold change and Benjamini-Hochberg–adjusted *P* values (q values) are shown in supplementary table 2b, Appendix A.

Many of the differentially expressed genes are similar to those seen in the comparison between CD8+ T cells in mesothelioma-associated pleural fluid and normal lung tissue.

There were 6 additional genes of interest that were differentially expressed in the comparison between CD8+ T cells in mesothelioma-associated pleural fluid and NSCLC.

ZBTB7B (ThPOK) is implicated in commitment of CD4+ T cell linage. It also plays a role in CD8+ T cells in promoting clonal expansion and strengthening the secondary CD8+ T cell response (Setoguchi et al., 2009). Memory CD8+ T cells retain expression of ThPOK more than effector CD8+ T cells (Setoguchi et al., 2009). In peripheral CD8+ T cells, forced expression of ThPOK causes gain of CD4+ T cell characteristics including increased IL2 production (Jenkinson et al., 2007).

ZBTB7B is upregulated in mesothelioma-associated pleural fluid which may be in keeping with a less cytotoxic phenotype with increased memory features.

NDFIP1 is a HECT-type ubiquitin ligase activator that has been shown to enforce a cell-intrinsic CD8+ T cell checkpoint that desensitises TCR signalling during exposure to high antigen levels (Wagle et al., 2018). NDFIP1 is upregulated in mesothelioma-associated pleural fluid compared with NSCLC and this may indicate tolerance and anergy in these CD8+ T cells.

RUNX2 is a runt related transcription factor that has been shown to be important for long term memory CD8+ T cell persistence in a murine viral model (Olesin et al., 2018). RUNX2 is downregulated in mesothelioma–associated pleural fluid.

TIGIT (T-cell immunoglobulin and ITIM domains) is a checkpoint inhibitor that is expressed on activated CD8+ T cells and negatively regulates T cell function. TIGIT expression on CD8+ T cells in myeloma showed a dysfunctional phenotype characterized by decreased proliferation and inability to produce cytokines (Guillerey et al., 2018). TIGIT is highly expressed in tumour infiltrating lymphocytes and exhausted CD8+ T cells (Johnston et al., 2014). TIGIT is mostly downregulated in the mesothelioma-associated pleural fluid which may be in keeping with this compartment not being similar to the tumour microenvironment or that the CD8+ T cells in the pleural fluid are less activated.

HAVCR2 (TIM3) is widely expressed on T cells, NK cells and monocytes (Anderson et al., 2016). It is expressed on activated CD8+ T cells and is associated with T cell exhaustion. More recently it has been associated with highly functional cells. HAVCR2 is downregulated in mesothelioma-associated pleural fluid, which may reflect a non-activated cell population.

NEDD4 is a E3 ubiquitin ligase which has been shown in infectious diseases to promote autophagy (Pei et al., 2017). NEDD4 is downregulated in mesothelioma associated pleural fluid.

Taken together, this is likely to reflect quiescent, non-activated and possibly tolerant and anergic CD8+ T cells in the mesothelioma-associated pleural fluid compared to both normal lung and NSCLC. TIGIT and CTLA4 are both induced on early activation of the cell and are both downregulated in mesothelioma-associated pleural fluid. ZBTB7B which is associated with memory and less cytotoxicity and NDFIP1 which is associated with TCR signaling and may represent tolerance and anergy in these cells are both upregulated in mesothelioma-associated pleural fluid. TIM3 is a marker that is usually associated with exhaustion but our group has shown recently that this may be associated with highly activated cells, this is downregulated in mesothelioma-associated pleural fluid which may reflect a less activated population of cells.

T cell naïve/central memory/effector memory

Figure 11: Heat map of differentially expressed CD4+ T cell Naïve/Memory/Effector genes between mesothelioma-associated pleural fluid vs normal lung and NSCLC, padj<0.05 ,fold change 1.5

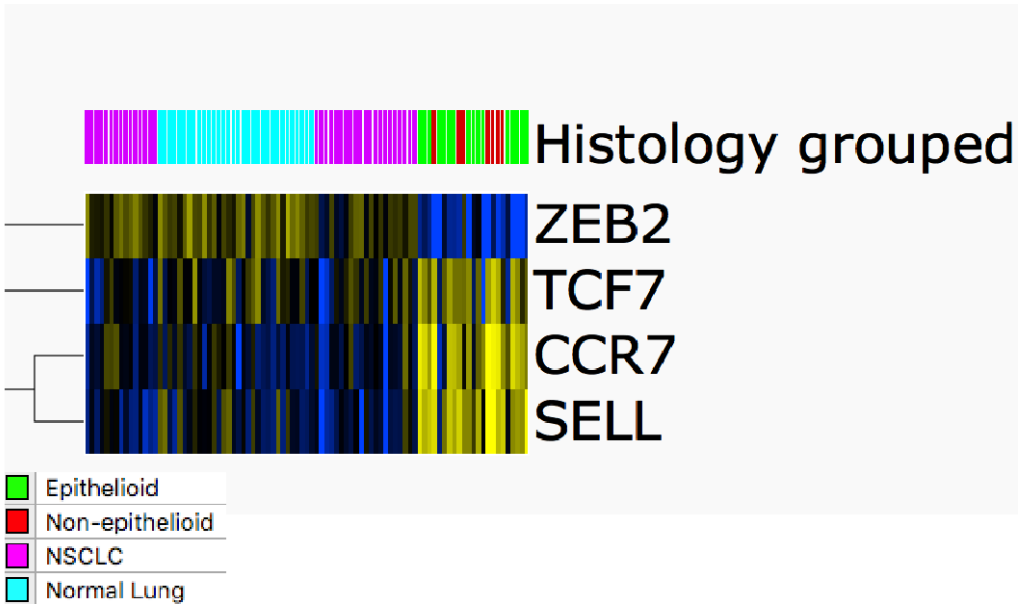


Figure 12: Heat map of differentially expressed CD8+ T cell Naïve/Memory/Effector genes between mesothelioma-associated pleural fluid vs normal lung and NSCLC, padj<0.05 ,fold change 1.5

Previous studies have shown that naïve and central memory cells form the predominant proportion of CD4+ T cells in malignant pleural effusions including mesothelioma (Scherpereel et al., 2013, Atanackovic et al., 2004). In both the comparisons between mesothelioma-associated pleural fluid and normal lung or NSCLC in the sorted CD4+ and CD8+ T cells, gene expression of SELL which codes for CD62L and CCR7 were upregulated in mesothelioma-associated pleural effusions, reflecting what has been previously shown with surface marker expression by flow cytometry. (Figures 11 and 12 above). TCF7 encodes for the transcription factor Tcf1 and this is also upregulated in mesothelioma-associated pleural fluid. Tcf1 is expressed in high levels in naïve cells, is downregulated in effector cells and is required for the formation of central memory cells (Arsenio et al., 2014, Danilo et al., 2018).

ZEB2 is a transcription factor expressed in effector and effector memory T cells (Arsenio et al., 2014, Best et al., 2013). The downregulation of ZEB2 in mesothelioma-associated pleural fluid indicate these cells are not of an effector phenotype.

The upregulation of SELL, CCR7 and TCF7; and downregulation of ZEB2 in mesothelioma-associated pleural fluid is in keeping with a mainly naïve or central memory phenotype as demonstrated in the previous studies.

The fold change and Benjamini-Hochberg–adjusted *P* values (q values) are shown in supplementary table 3a and 3b respectively, Appendix A.

T cell Cytotoxicity

Genes associated with cytotoxicity are downregulated in mesothelioma pleural fluid when compared to normal lung and NSCLC in both CD4+ (figure 13) and CD8+ (figure 14) cohorts. The fold change and Benjamini-Hochberg–adjusted *P* values (q values) are shown in supplementary table 4a, Appendix A.

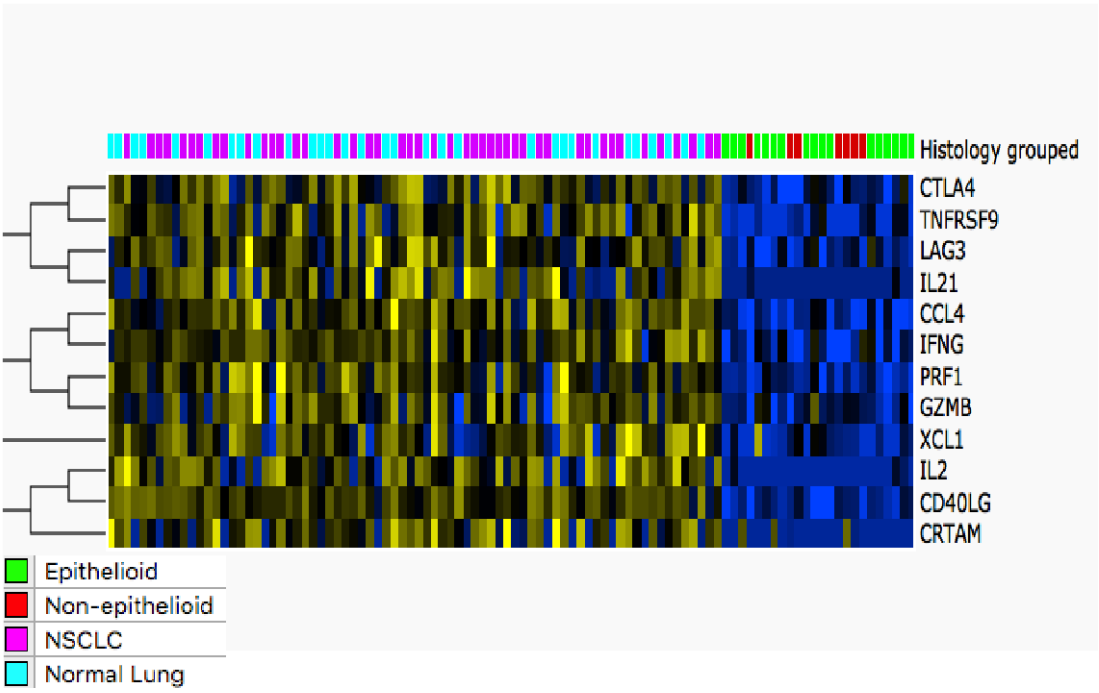


Figure 13: Heat map of differentially expressed cytotoxicity genes in CD4+ T cells between mesothelioma-associated pleural fluid vs normal lung and NSCLC, padj<0.05, fold change 1.5

In CD4+ T cells, genes associated with cytotoxic activity such as PRF1(perforin), are globally downregulated in mesothelioma-associated pleural fluid compared with normal lung and NSCLC. Other major genes associated with cytotoxicity such as GZMB (granzyme B) that is secreted by T cells to induce target cell apoptosis and CRTAM which is a marker for CD4+ CTLs are downregulated in most samples of mesothelioma associated pleural fluid. GNLY which is contained in the cytotoxic granules of T cells and released on antigen stimulation is only differentially expressed with a fold change of 1.5 in the comparison between mesothelioma-associated pleural fluid and normal lung but not when compared with NSCLC (data not shown).

Genes associated with early TCR-mediated activation such as TNFRSF9 (4-1BB), which is a co-stimulatory molecule able to induce activation of CTLs and large amounts of interferon gamma, is globally downregulated in mesothelioma-associated pleural fluid. CTLA4 and LAG3 encode for the respective proteins that are expressed on T regulatory cells and activated T cells. These are both mostly downregulated in mesothelioma associated pleural fluid.

IL21 is expressed by activated CD4+ T cells and is essential for Th proliferation, Th17 cell differentiation, has been shown to upregulate NK and CD8+ T cell cytotoxicity, and helps with differentiation of B cells into plasma cells (Spolski and Leonard, 2008). It is globally downregulated in mesothelioma-associated pleural fluid.

CD40LG is responsible for expression of CD40L on activated T cells (Lederman et al., 1992). It acts as a co-stimulatory molecule in macrophage and B cell activation. It is globally downregulated in mesothelioma-associated pleural fluid.

CCL4 codes for a chemokine that attracts CD8+ CTLs. It is globally downregulated in mesothelioma-associated pleural fluid.

XCL1 is a chemokine produced by CD4+ and CD8+ T cells, NK cells and NKT cells. It is important for dendritic cell cytotoxic responses (Lei and Takahama, 2012). It is mostly downregulated in mesothelioma-associated pleural effusions.

IL2 is globally downregulated and IFGN is mostly downregulated in mesothelioma associated pleural fluid as presented in the section on T cell differentiation.

Taken together, the CD4+ T cells in mesothelioma-associated pleural fluid are relatively quiescent and inert compared to those in normal lung and NSCLC.

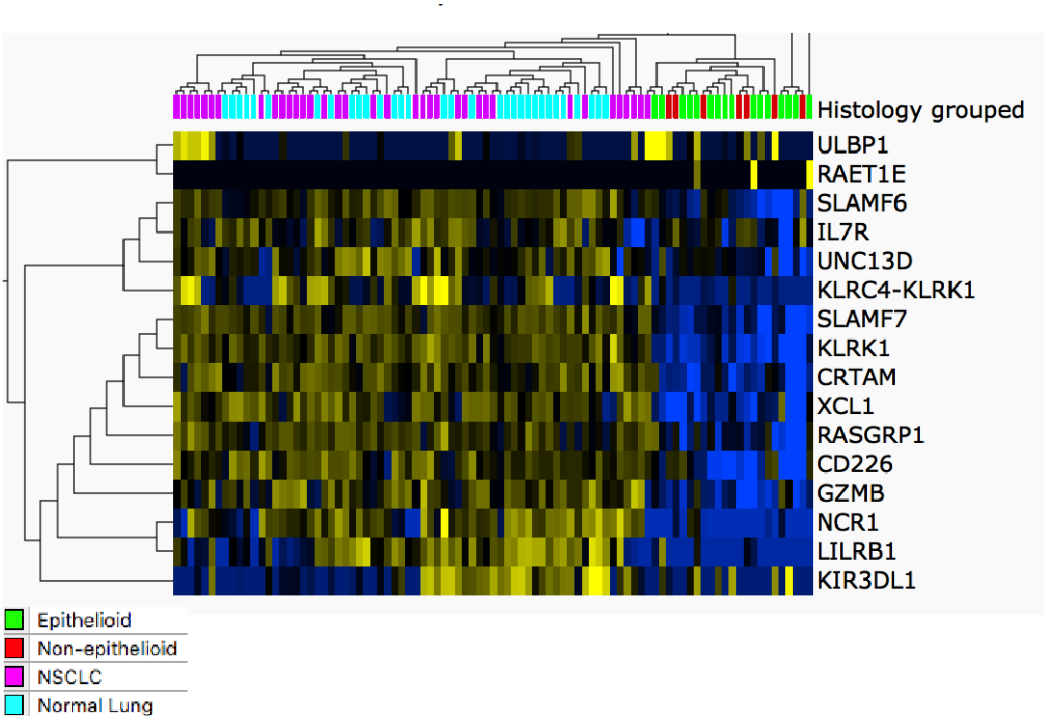


Figure 14: Heat map of differentially expressed cytotoxicity genes in CD8+ T cells between mesothelioma-associated pleural fluid vs normal lung and NSCLC, padj<0.05, fold change 1.5

Comparison of differentially expressed genes in CD8+ T cells between mesothelioma-associated pleural fluid vs normal lung and NSCLC reveals some expected genes associated with cytotoxicity that have been discussed earlier. The genes associated with cytotoxicity are once again either

mostly or wholly downregulated in the mesothelioma-associated pleural fluid. These include KLRC4-KLRK1, KLRK1, CRTAM, XCL1 and GZMB. The fold change and Benjamini-Hochberg–adjusted *P* values (q values) are shown in supplementary table 4b, Appendix A.

An additional 5 genes are differentially expressed in the comparison of CD8+ T cell cytotoxicity gene expression between NSCLC and mesothelioma-associated pleural fluid.

SLAMF6 is expressed on NK cells, CD8+ T cells and B cells. SLAMF6 reduces activation-induced cell death, enables cells to secrete more interferon gamma and enhance their cytolytic capabilities (Eisenberg et al., 2018). SLAMF6 is mostly downregulated in mesothelioma-associated pleural fluid.

UNC13D helps to transport cytolytic granules from CD8+ T cells and NK cells to the membrane of the target cell to trigger apoptosis. UNC13D is downregulated in mesothelioma-associated pleural fluid.

SLAMF7 has been shown to be associated with cytotoxicity in CD8+ T cells (Comte et al., 2017). SLAMF7 is globally downregulated in mesothelioma associated pleural fluid.

CD226 is a costimulatory and adhesion molecule and mediates activation signals for cytotoxicity by binding to CD155 and/or CD122 on target cells. It contributes to the immunosurveillance of tumours (Ayano et al., 2015). CD226 is mostly downregulated in mesothelioma-associated pleural fluid.

NCR1 (natural cytotoxic receptor 1) is a cytotoxicity activating receptor on NK cells and T cells. It is mostly downregulated in mesothelioma-associated pleural fluid.

This again demonstrates the lack of cytotoxicity in the CD8+ T cells in mesothelioma-associated pleural fluid compared with NSCLC.

Gene ontology and pathway analysis

Gene ontology for biological processes and pathway analysis has been conducted via the ToppGene Suite (http://toppgene.cchmc.org) to gain insight into the function relevance of these differentially expressed genes. Gene ontology for biological processes and pathway analysis supports the DEG analysis findings, showing that pathways from CD4+ cells from mesothelioma-associated pleural fluid are depleted in biological processes and pathways such as regulation of T cell activation, interferon gamma production, T cell receptor signalling pathway compared to normal lung and non small cell lung cancer. Similarly, pathways from CD8+ cells from mesothelioma-associated pleural fluid also support the DEG analysis findings, showing depletion

of biological processes and pathways in T cell activation and T cell receptor signalling pathways compared to normal lung and non small cell lung cancer. The top 15 biological processes and pathways for each parameter are shown in supplementary tables 5-8, Appendix A.

5.3 Discussion

The data presented in this chapter reveal that the transcriptomic signature of CD4+ T cells in the pleural fluid in patients with mesothelioma is mainly downregulated for key markers that determine lineage to Th1, Th2, Th17 and Tregs. Markers associated with proliferation such as IL2 and activation such as IFGN, CTLA4, LAG3 are also downregulated in most samples of mesothelioma-associated pleural fluid.

Previous studies have found naïve (CD45RA+CD62L+CCR7+) and central memory T cells (CD45RO+CD62L+CCR7+) represent a higher proportion of CD4+ T cells in the pleural fluid in both mesothelioma and other cancers compared to benign effusions (Scherpereel et al., 2013). The present data are in keeping with this observation, where SELL and CCR7 gene expression are upregulated in mesothelioma associated pleural fluid, and ZEB2 gene expression is downregulated, possibly indicating a predominance of naïve and central memory cells and lack of effector and effector memory cells.

These data show that the transcriptome of the CD4+ T cells in mesothelioma- associated pleural fluid is quiescent and in keeping with a naïve/central memory phenotype when compared to normal background lung tissue and NSCLC.

The question remains as to why the immune cells in the pleural fluid, which are physically in contact with the mesothelioma tumour mass and that also contains malignant cells in 60% of cases, are not activated. One possibility is the cells in the pleural fluid are in a resting state or the effector cells are exhausted/senescent and undergo apoptosis at a faster rate. Another is that the effector cells are not able to enter the inflamed pleural space. Naïve and central memory cells express CD62L and are trafficked to inflammation via lymphatic drainage. Effector cells need to extravasate into the site of inflammation like the pleural space by expressing P and E selectin ligands (Kim and Harty, 2014). It has been shown that effector cells express functional P and E selectin ligands but not most memory cells (Nolz et al., 2011).

Recent studies have reported on the expression of immune checkpoint targets in the effusions of patient with mesothelioma such as TIM3, LAG3, PD1 and PD-L1 with a view of using these cells as a surrogate for the immune events in the tumour (Marcq et al., 2017b). Another small study has

shown the nature of the immune infiltrate in malignant pleural effusions fluctuates over time and is not reflective of the tumour (Lievense et al., 2017).

Whether mesothelioma associated pleural fluid can be utilised as a surrogate for immune events in the tumour remains unresolved. Future work includes comparison of mesothelioma associated pleural fluid to PBMCs which may be more sensitive in identifying differentially expressed gene expression of immune cells in the pleural fluid compartment.

Chapter 6 Conclusion and Future work

Mesothelioma is a rare cancer mainly affecting the pleura. It is usually associated with inhalation of asbestos fibres. It accounts for 1% of cancer in the United Kingdom. Median survival remains poor despite treatments such as chemotherapy.

Mesothelioma is driven by chronic inflammation following from the inability of the immune cells to deal with a physical "antigen", the inhaled asbestos fibres. In many solid tumours, CD8+ TILs have been shown to confer a survival advantage. In mesothelioma, there are 3 small surgical studies that suggested this might be the case. Immunotherapy has been established as an important treatment option in many solid tumours where survival benefit has been shown to be associated with CD8+ T cell infiltration.

This raised a number of hypotheses to be addressed by the work presented in this thesis.

The work presented here started by obtaining an overview of the immune cells present in the tumour microenvironment in a real world cohort of patients with treatment-naïve mesothelioma in Chapter 3. The analysis of the immune infiltrate was then looked at in relation to the clinical outcomes. Here, adaptive and innate immune cells were evaluated, including T cells (CD4+, CD8+, CD45RO+ memory cells, FOXP3+ T regulatory cells), B cells, NK cells, Macrophages, Neutrophils. Expression of WT1, a mesothelioma tumour-associated antigen, was also evaluated.

Surprisingly, CD8+ T cell counts were not associated with survival in epithelioid mesothelioma. However, a high CD4+ T cell infiltrate was significantly linked to survival. The other adaptive immune cells significantly associated with survival in epithelioid mesothelioma were a high B cell infiltrate and low FOXP3+ (T regulatory cell) infiltrate. A low neutrophil count was also significantly associated with survival in epithelioid mesothelioma. In the non-epithelioid group, all immune cell markers apart from a low FOXP3+ count were indifferent for survival.

These findings were published in the British Journal of Cancer (Chee et al., 2017). Based on these findings, the work in the next chapter (Chapter 4) sought to understand the reasons why CD8+ T cells did not confer a survival advantage and to define the phenotype of CD8+ T cells and the presence of MHC class I on tumour cells. This was followed by characterisation of the phenotype of CD4+ T cells that conferred a survival advantage in epithelioid mesothelioma. The hypothesis to be tested here was that CD4+ effector cells/cytotoxic CD4+ T cells are present in epithelioid mesothelioma and their numbers are correlated with clinical outcome.

Here, 24 cases of epithelioid mesothelioma with good survival (>800 days) and 20 cases with poor survival (>30 days and <150 days) were evaluated with multiplex immunohistochemistry: 3 panels of markers were evaluated. A CD8+ T cell panel with CD8, CD103, TIM3 and Granzyme B to look at CD8+ Tissue resident memory cells, cytotoxic T cells and T_{RMS}, highly functional and cytotoxic CD8+ T_{RMS}. In the second panel we evaluated WT1, MHC class I and II expression. In the third, we characterised CD4+ T-cells with antibodies to identify CD4, Granzyme B, CD69, CD103, FOXP3, BCL6 and CXCL13. These would identify CD4+ tissue resident memory cells, cytotoxic CD4+ T cells and TRMs, T regulatory cells, T follicular helper cells and T follicular regulatory cells in the various marker combinations (Chapter 4, Table 1, page 92).

The CD8+ T cells and the CD8+ T cell subsets showed no significant differences between the good and poor survival groups. However, in the good survival group, both the number and staining intensity of MHC class I on tumour cells was greater than that in the poor survival group. This does leave open the possibility that the lack of correlation of CD8+ T cell numbers with outcome is not a function of their numbers or functional phenotypes but in some cases, of their incapacity to recognize and interact with tumour cells through the lack of tumour cell expression of critical MHC- class 1 molecules.

Next, an exploration of the phenotype of CD4+ T cells that conferred a survival advantage in epithelioid mesothelioma was undertaken. Here, it was demonstrated that the median number of CD4+ T cells was 4 times greater in the good survival group compared to the poor survival group and the tissue resident and some subsets of T follicular helper cells were significantly higher in the good survival group compared to the poor survival group. Cytotoxic CD4+ T cells were present in both groups and trended towards, but did not quite reach statistical significance with an increase in the cases with better outlook ($p=0.0617$). If CD4+ cytotoxic cells were to be able to attack the tumour, it would be expected that the tumour cells should express MHC class II molecules.

We were able to confirm this: using WT1 as a tumour marker we observed that WT1+ tumour cells expressed MHC class II on their cell surface. This was significantly higher in the good survival group compared with the poor survival group ($p<0.0001$). The ratio of WT1+HLADBP1+ cells to WT1 cells was also significantly higher in the good survival group (0.405) compared with the poor survival group (0.05) ($p <0.0001$).

Semi quantitative scoring of the intensity of MHC II staining using an antibody for HLADPB1+ staining and the proportion of WT1+ cells that were also HLADPB1+, were combined. The median of the combined score was 5 in the good survival group and 3 in the poor survival group. So it was interesting to find that there was indeed expression of MHC-Class-II by the tumour cells and there was a positive correlation with the numbers of infiltrating CD4+ T cells and the outcome.

In the next chapter (Chapter 5), I presented results of analysis of the phenotypes and transcriptomic signatures of various cell fractions isolated from mesothelioma-associated pleural fluid. Pleural fluid is easily accessible and used as a diagnostic tool. It is established that tumour cells are present in pleural effusions. It was not clear if this mesothelioma- associated pleural fluid could be used as a surrogate for immune events in the tumour. I compared the CD4+ and CD8+ T cells from pleural fluid with CD4+ and CD8+ T cells from normal lung and also NSCLC, which have been characterized in detail in our group. This comparison was made as disaggregation of mesothelioma tumour was technically challenging. The analysis revealed that the immune cells in the pleural fluid had a quiescent, naïve and memory phenotype compared to those in normal lung and NSCLC. A comparison of immune cells in the mesothelioma- associated pleural fluid compartment with PBMCs from healthy donors might reveal important signals from the mesothelioma associated pleural fluid. This will be considered in future work.

Below, I have outlined the original plan of the thesis and how these hypotheses have been addressed.

1) ***The over-arching hypothesis to be tested in this thesis is that the immune response against mesothelioma is the critical determinant of the clinical outcome.***

The primary hypothesis that the immune response is critical in mesothelioma arose from observations that tumour prognosis is associated with lymphocyte infiltration, that vaccine approaches have had some success and that treatment with immunotherapy has shown benefit in some patients. This primary hypothesis has been partially addressed in Chapter 3. From analysis of tumour samples from 302 patients with mesothelioma, there is clear evidence that the immune events in epithelioid mesothelioma play an important role in outcome. The role of immune cells in non-epithelioid tumours is less clear.

2) ***The secondary hypotheses were:***
    a. ***The main effector cells contributing to the defence against mesothelioma are CD8+ CTL and T_{RM}***

It was surprising to find that CD8+ CTLs and T_{RMS} showed no correlation with the outcomes of patients with mesothelioma. This is in contrast to their effect in other solid tumours. This secondary hypothesis was developed and then addressed in Chapter 4.

    b. ***The main effector cells contributing to the defence against mesothelioma are CD4+ CTL and T_{RM}***

Work so far shown in Chapter 3 and extended in chapter 4 has demonstrated that CD4+ T cells are significantly associated with better survival in patients with epithelioid mesothelioma. Further analysis revealed that, while CD4+ CTLs are present in epithelioid mesothelioma in greater numbers in the good survival group than the poor survival group, the numbers trended towards but do not reach statistical significance. I demonstrated that a higher number of CD4+CD69+ T_{RMS} is associated with better survival in epithelioid mesothelioma. Multiplex analysis also revealed the presence of subsets of T follicular helper cells that are associated with a good outcome. However, it is clear that further detailed analysis of the phenotypic and functional nature of these cells is required to understand the mechanisms that confer this survival advantage. Furthermore, it is important to determine the presence, phenotypes and functional roles of other T cell subsets such as Th1, Th2, Th17 helper and Treg cells.

Analysis by flow cytometry and RNAseq of cells present in mesothelioma-associated pleural fluid has indicated that the characteristics of the T cells in pleural fluid are very different from those within the tumour. Hence, this compartment is likely not useful as a surrogate for immune events in the tumour and further work is required to either compare this compartment to PBMCs or to overcome the technical challenges of performing RNAseq on the mesothelioma tumour tissue itself.

c. The main effector cells contributing to the defence against mesothelioma are B cells and antibodies

B cells in the tumour microenvironment of epithelioid mesothelioma have been shown to be associated with a better survival in the data from Chapter 3 (Chee et al., 2017). More detailed analysis of the phenotype and functions that confer this survival advantage is required. This will be addressed in future work.

3) Less differentiated mesotheliomas create a microenvironment that is anti-immune/pro-tumour

The observations in Chapter 3 have shown that in less differentiated (non-epithelioid) mesotheliomas, there were virtually no correlations of the immune responses with survival. This suggests that these less well-differentiated tumours have developed escape mechanisms, which may be immunosuppressive, inhibiting immune effector functions or rendering the tumours invisible to the immune cells. Analysis of these possibilities is required and will be addressed in future work.

Plan for future work:

1) Consolidate the observations in Chapter 4: Analysis of phenotypes of CD4+ cells infiltrating tumours in relation to survival: Extend antibody range to explore phenotypes of CD4+ cells (Th1, Th2, Th17). Examine CD8+ cells both as control and in pursuit of understanding the lack of correlation with outcome. Explore presence of naïve and memory phenotype of CD8+ T cells (CD45RA and CD45RO).
2) Explore expression of checkpoint molecules by CD8+ T cells to determine whether they are non-functional as a result of being "exhausted".
3) Explore the phenotypes of B cells.
4) Explore the phenotypes of macrophages in terms of M1 and M2 functions, and to explore possible correlations with T cell numbers and types.
5) Explore the presence of characteristics of NK cells within the TME. The down-regulation of MHC Class-1 in the poor-prognosis tumours might have been expected to render them as targets for the cytotoxic effects of NK cells.

The work presented in this thesis has demonstrated that CD4+ T cells play a central role in the outcome of patients with epithelioid mesothelioma. Future work will aim to understand this role and the relationship with B cells and further characterise the phenotype of CD8+ T cells in mesothelioma. Limitations of this study are that RNAseq was not carried out on immune cells from mesothelioma tumour due to challenges in disaggregating immune cells from this compartment. As a result, the immune cells from mesothelioma-associated pleural fluid were compared to immune cells from normal lung and lung cancer. This comparison with active immune cells in normal lung and NSCLC tissue may have caused the signals from the mesothelioma associated pleural fluid to be "drowned out". This will be addressed in future work where we will revisit the technical issues of disaggregation of mesothelioma tumour and also compare this compartment to PBMCs.

CD8+ T cells have been shown to be associated with outcome in many solid tumours. Surprisingly this has not been shown in this cohort of patients with mesothelioma. The CD8+ T cells here do have cytotoxic and tissue-resident memory features but numerically do not differentiate between the good and poor survival groups. However, the expression of MHC class I on tumour cells in the poor survival group was significantly reduced compared to the good survival group and this may indicate that CD8+ T cells do play a role in the TME of patients with mesothelioma.

The available data on the role of CD4+ T cells in cancer has been less straightforward than is the case for CD8+ T cells. This may be due to the plasticity of CD4+ cells (Hadrup et al., 2013). In a meta-analysis of 6 studies, CD4+ TILs were shown to be significantly associated with overall survival (Gooden et al., 2011). In head and neck cancer, multivariate analysis showed that CD4+CD69+ cells were associated with overall survival (Badoual et al., 2006a). The CD4+ T cells may be exerting an anti-tumour effect via direct cytotoxicity, the release of cytokines, recruitment of other cells or providing help to B cells in the form of T_{FH} cells. It was shown in work presented in Chapter 3 that B cell infiltrate was associated with good outcome tumours in epithelioid mesothelioma.

It may be that T_{FH} cells are recruiting and activating higher affinity B cells in TLS or germinal centres. In breast cancer, CXCL13+T_{FH} cells are associated with organized TLS containing reactive GCs that are associated with good clinical outcomes (Gu-Trantien et al., 2013).

Key questions remain about the function and phenotype of CD4+ T cells in the TME, if they are providing help for other effector cells, their relationship with B cells and how they might be helping with B cell function. This will lead on to characterization of B cells in future work

It is clear that immune cells do play a significant role in mesothelioma. Mesothelioma develops from exposure from a known causative agent, the asbestos fibre, with a long lag time and it is clear that chronic inflammation is a key factor in promoting the malignant progression. This may account for the differences in the immune infiltrate between mesothelioma and other solid tumours. CD8+ T cells are key prognostic and predictive markers in most solid tumours, but do not appear to play a central role in late stage mesothelioma. It may be that these cells are less functional due to chronic stimulation leading to exhaustion. Mesothelioma remains a disease with a poor outcome and with limited treatment options available. Going forward, it will be important to understand the role of CD4+ T cells and B cells in mesothelioma with a view to exploiting this understanding for therapeutic gain.

Appendix A

	Gene symbol	FoldChange (log2)	q-value
Upregulated in mesothelioma-associated pleural fluid	FOXP1	-0.759703987	1.45E-06
	LEF1	-0.834038605	0.000838359
	IL23A	-1.152291029	0.005081695
	SOCS5	-1.167784425	0.015661097
Downregulated in mesothelioma-associated pleural fluid	IL23R	4.572599689	1.69E-30
	BATF	1.627021605	2.85E-14
	IL2	3.728872308	1.03E-13
	PTGER4	1.080217378	9.11E-07
	IFNG	2.248319341	9.22E-07
	RARA	1.259154224	1.19E-06
	RORA	0.907758499	2.21E-06
	IL13	3.057006407	3.21E-06
	TBX21	1.141948	0.003666134
	IL4	1.880177467	0.008234866
	MYB	1.568926851	0.012660538
	IL18R1	1.134531903	0.02076543

Supplementary table 1a: CD4+ T cell differentiation and activation normal lung vs mesothelioma-associated pleural fluid. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5 (log2 fold change >0.75 or <-0.75)

	Gene symbol	FoldChange (log2)	q-value
Upregulated in mesothelioma-associated pleural fluid	FOXP1	-1.032975049	2.82E-12
	LEF1	-0.858721449	4.64E-07
	SOCS5	-1.27054381	0.006471701
	IL23A	-1.036867561	0.017739439
	CD86	-1.508541775	0.017944863
	SHB	-1.640827137	0.023462955
Downregulated in Mesothelioma pleural fluid	BATF	2.224109972	6.00E-45
	IL23R	4.57166462	2.01E-30
	FOXP3	2.23472715	6.76E-17
	IL2	3.997930229	8.42E-16
	ZBTB7B	1.526076702	1.19E-07
	IL18R1	1.925447951	2.52E-07
	IFNG	2.047145623	1.00E-06
	SLAMF6	0.793555066	0.008307019

Supplementary table 1b: CD4+ T cell differentiation and activation NSCLC vs mesothelioma-associated pleural fluid. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5 (log2 fold change >0.75 or <-0.75)

	Gene symbol	FoldChange (log2)	q-value
Upregulated in mesothelioma-associated pleural fluid	CCR7	-3.026400255	9.22E-20
	F2RL1	-2.796527333	0.000196793
	SOX4	-1.81215828	0.000602968
	LEF1	-0.861901476	0.008536421
	BMP4	-2.613618989	0.008567386
	SIRPG	-0.780650681	0.025050423
Downregulated in mesothelioma-associated pleural fluid	SPN	3.551417054	7.31E-40
	IL2	6.732260924	2.42E-25
	KLRK1	1.846574864	5.39E-20
	TBX21	2.335361408	1.28E-09
	BATF	1.738831533	1.06E-08
	TNFSF14	2.412416834	1.65E-08
	RARA	1.887139203	1.26E-07
	RORA	1.354789876	2.02E-06
	IFNG	1.982643992	3.64E-06
	XCL1	1.992372925	1.05E-05
	THEMIS	1.239542614	2.61E-05
	SLAMF6	1.387987534	2.82E-05
	CD244	1.416342037	8.86E-05
	PTPN22	1.057126918	0.000275026
	PDCD1	1.375041717	0.000281467
	ELF4	1.689270696	0.000345051
	TNFRSF18	1.811325491	0.000559517
	KLRC4-KLRK1	1.56926906	0.00076134
	PTGER4	0.895347226	0.00077304
	CD40LG	2.253755073	0.000904497
	ZNF683	1.362536721	0.002118151
	RUNX1	1.008463221	0.003218796
	TRAF2	1.382552143	0.007016051
	IL7R	0.870613047	0.007968262
	PRDM1	0.78258914	0.008992663
	CTLA4	1.447060526	0.018722678
	TNFSF9	1.448742715	0.032894297
	IL4	2.162323836	0.034807556
	RORC	1.857174031	0.038571503
	CD274	1.378830734	0.047036492
	RASGRP1	0.813368209	0.047567803

Supplementary table 2a: CD8+ T cell differentiation and activation mesothelioma-associated pleural fluid vs normal lung. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5 (log2 fold change >0.75 or <-0.75)

	Gene symbol	FoldChange (log2)	q-value
Upregulated in mesothelioma-associated pleural fluid	F2RL1	-2.182611435	0.006999989
	LEF1	-2.018176701	1.47E-08
	ZBTB7B	-1.472323452	0.012915055
	NDFIP1	-0.797862948	3.08E-05
Downregulated in mesothelioma-associated pleural fluid	IL2	7.755137716	4.21E-28
	SPN	2.703501481	2.89E-25
	KLRK1	1.937982176	4.21E-22
	IFNG	3.203761397	1.17E-16
	BATF	2.107754634	3.20E-13
	PDCD1	2.402703921	4.12E-11
	PTPN22	1.758701068	2.28E-10
	CTLA4	3.298790333	1.70E-08
	XCL1	2.361998277	8.00E-08
	THEMIS	1.59570363	9.06E-08
	TNFRSF18	2.82819553	1.17E-07
	TNFSF9	2.581757877	1.15E-06
	KLRC4-KLRK1	2.155342764	1.73E-06
	ZNF683	1.749754293	2.60E-06
	HAVCR2	2.128686887	2.68E-06
	TIGIT	1.619486835	5.95E-06
	RORA	1.249927205	3.03E-05
	ELF4	1.854214489	5.72E-05
	SLAMF6	1.266005046	8.67E-05
	CD244	1.268902592	0.000177075
	PTGER4	0.967692895	0.000382255
	RASGRP1	1.176514483	0.000693737
	TRAF2	1.525077834	0.000826852
	NEDD4	2.4105514	0.001282052
	RUNX2	1.142328628	0.002890738
	RUNX1	0.990347258	0.004218469
	RARA	0.946376914	0.004584497
	RORC	2.606455531	0.004634949
	CD274	1.688018091	0.017422817
	SIRPG	0.75374242	0.025678384
	CD40LG	1.761204274	0.03783719

Supplementary table 2b: CD8+ T cell differentiation and activation mesothelioma-associated pleural fluid vs NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5 (log2 fold change >0.75 or <-0.75)

	Gene symbol	Fold Change (log2)	q-value
Upregulated in mesothelioma-associated pleural fluid			
CD4+ normal lung vs mesothelioma-associated pleural fluid	ZEB2	2.027549147	3.14E-06
CD4+ NSCLC vs mesothelioma-associated pleural fluid	ZEB2	1.001831406	0.040235786
Downregulated in mesothelioma-associated pleura fluid			
CD4+ normal lung vs mesothelioma-associated pleural fluid	SELL	-2.12323786	1.03E-18
	CCR7	-1.624723495	1.75E-10
	TCF7	-1.210505023	2.42E-06
CD4+ NSCLC vs mesothelioma-associated pleural fluid	SELL	-2.128997688	2.99E-30
	CCR7	-1.434119263	4.08E-11
	TCF7	-1.307665733	2.78E-08

Supplementary table 3a: CD4+ T cell Naïve/Memory/Effector mesothelioma-associated pleural fluid vs normal lung and NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5 (log2 fold change >0.75 or <-0.75)

	Gene symbol	Fold Change (log2)	q-value
Upregulated in mesothelioma –associated pleural fluid			
CD8+ normal lung vs mesothelioma-associated pleural fluid	ZEB2	2.572448682	2.08E-11
CD8+ NSCLC vs mesothelioma-associated pleural fluid	ZEB2	2.119298351	1.01E-08
Downregulated in mesothelioma-associated pleura fluid			
CD8+ normal lung vs mesothelioma-associated pleural fluid	CCR7	-3.026400255	9.22E-20
	SELL	-2.358945411	1.79E-17
	TCF7	-0.993290794	5.33E-05
CD8+ NSCLC vs mesothelioma-associated pleural fluid	CCR7	-3.938896718	6.79E-26
	SELL	-3.70845968	4.28E-34
	TCF7	-1.777074966	2.73E-08

<u>Supplementary table 3b:</u> CD8+ T cell Naïve/Memory/Effector mesothelioma-associated pleural fluid vs normal lung and NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5 (log2 fold change >0.75 or <-0.75)

	Gene symbol	Fold Change (log2)	q-value
Upregulated in mesothelioma-associated pleural fluid			
CD4+ normal lung vs mesothelioma-associated pleural fluid	N/A	N/A	N/A
CD4+ NSCLC vs mesothelioma-associated pleural fluid	N/A	N/A	N/A
Downregulated in mesothelioma-associated pleural fluid			
CD4+ normal lung vs mesothelioma-associated pleural fluid	CCL4	3.257041452	3.22E-19
	CD40LG	2.487664797	9.94E-17
	IL2	3.728872308	1.03E-13
	PRF1	2.075116499	1.85E-12
	GZMB	2.481133204	1.75E-09
	IL21	3.08272663	4.25E-07
	IFNG	2.248319341	9.22E-07
	XCL1	2.359217823	2.05E-06
	CRTAM	2.220411536	3.09E-06
	TNFRSF9	1.88189916	1.22E-05
	CTLA4	1.49756078	0.000124187
	LAG3	1.090827172	0.001991259
CD4+ NSCLC vs mesothelioma associated pleural fluid	TNFRSF9	3.099456051	1.06E-19
	IL2	3.997930229	8.42E-16
	IL21	4.160066885	1.45E-15
	CCL4	2.362256175	1.85E-14
	CTLA4	2.431385661	4.19E-14
	CD40LG	1.971074538	4.41E-14
	LAG3	2.156610911	1.95E-12
	PRF1	1.389303507	6.67E-10
	IFNG	2.047145623	1.00E-06
	XCL1	1.991281824	4.52E-06
	CRTAM	2.001270281	3.48E-05
	GZMB	1.721070279	4.87E-05

Supplementary table 4a: CD4+ T cell cytotoxicity Mesothelioma associated pleural fluid vs normal lung and NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5 (log2 fold change >0.75 or <-0.75)

	Gene symbol	Fold Change (log2)	q-value
Upregulated in mesothelioma-associated pleural fluid			
CD8+ normal lung vs mesothelioma-associated pleural fluid	RAET1E	-3.700090029	5.18E-11
	ULBP1	-1.668082103	0.009459866
CD8+ NSCLC vs mesothelioma-associated pleural fluid	N/A	N/A	N/A
Downregulated in mesothelioma-associated pleura fluid			
CD8+ normal lung vs mesothelioma-associated pleural fluid	KLRK1	1.846574864	5.39E-20
	SLAMF7	2.243302977	7.51E-12
	NCR1	3.517915048	3.88E-09
	GZMB	1.63472092	9.42E-07
	LILRB1	2.873873169	9.88E-07
	CRTAM	1.567977468	7.86E-06
	XCL1	1.992372925	1.05E-05
	SLAMF6	1.387987534	2.82E-05
	KLRC4-KLRK1	1.56926906	0.00076134
	KIR3DL1	2.163914627	0.002208344
	UNC13D	0.826104482	0.00434219
	IL7R	0.870613047	0.007968262
	CD226	1.315073128	0.008932107
	RASGRP1	0.813368209	0.047567803
CD8+ NSCLC vs mesothelioma-associated pleural fluid	KLRK1	1.937982176	4.21E-22
	SLAMF7	2.308399318	1.14E-13
	CRTAM	2.335291075	2.39E-12
	NCR1	3.657790548	6.00E-10
	XCL1	2.361998277	8.00E-08
	GZMB	1.879461513	1.74E-07
	KLRC4-KLRK1	2.155342764	1.73E-06
	SLAMF6	1.266005046	8.67E-05
	RASGRP1	1.176514483	0.000693737
	CD226	1.468105464	0.00292088
	LILRB1	1.845299967	0.003822901

Supplementary table 4b: CD8+ T cell cytotoxicity mesothelioma-associated pleural fluid vs normal lung and NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5 (log2 fold change >0.75 or <-0.75)

Gene Ontology ID	Gene Ontology term	Set Size	Candidates	q-value
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	75	105	2.89E-72
GO:0006613	cotranslational protein targeting to membrane	76	109	2.94E-72
GO:0045047	protein targeting to ER	78	120	1.17E-70
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	77	120	3.40E-69
GO:0072599	establishment of protein localization to endoplasmic reticulum	78	124	3.40E-69
GO:0006413	translational initiation	92	198	8.52E-66
GO:0070972	protein localization to endoplasmic reticulum	79	149	5.43E-62
GO:0019080	viral gene expression	89	211	4.08E-59
GO:0019083	viral transcription	84	197	3.67E-56
GO:0000956	nuclear-transcribed mRNA catabolic process	83	212	6.83E-52
GO:0006612	protein targeting to membrane	82	212	8.82E-51
GO:0090150	establishment of protein localization to membrane	92	355	2.78E-40
GO:0006414	translational elongation	133	764	1.87E-38
GO:0006412	translation	133	764	1.87E-38
GO:0006402	mRNA catabolic process	91	377	3.56E-37

Supplementary table 5a: Genes expressed to a greater extent in CD4+ T cells in mesothelioma associated pleural fluid compared to normal lung. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Gene Ontology ID	Gene Ontology term	Set Size	Candidates	q-value
GO:0001775	cell activation	189	1559	1.76E-18
GO:0045321	leukocyte activation	172	1386	1.96E-21
GO:0002252	immune effector process	163	1332	1.07E-19
GO:0006954	inflammatory response	113	806	4.26E-18
GO:0006952	defense response	198	1850	2.80E-17
GO:0009607	response to biotic stimulus	179	1633	1.25E-16
GO:0002682	regulation of immune system process	190	1776	1.48E-16
GO:0034097	response to cytokine	149	1287	6.84E-16
GO:0002443	leukocyte mediated immunity	117	918	1.47E-15
GO:0051707	response to other organism	173	1604	2.06E-15
GO:0002521	leukocyte differentiation	87	591	2.07E-15
GO:0043207	response to external biotic stimulus	173	1606	2.31E-15
GO:0071345	cellular response to cytokine stimulus	139	1188	3.13E-15
GO:0050900	leukocyte migration	81	534	3.66E-15
GO:0002274	myeloid leukocyte activation	95	684	4.24E-15

Supplementary table 5b: Genes expressed to a lesser extent in CD4+ T cells in mesothelioma associated pleural fluid compared to normal lung. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Pathway ID	Pathway	Source	Set Size	Candidates	q-value
1268690	Eukaryotic Translation Elongation	BioSystems: REACTOME	81	98	6.90E-84
M189	Ribosome	MSigDB C2 BIOCARTA (v7.1)	77	88	6.90E-84
1268691	Peptide chain elongation	BioSystems: REACTOME	78	93	3.47E-82
1268681	Formation of a pool of free 40S subunits	BioSystems: REACTOME	83	107	3.47E-82
1268688	L13a-mediated translational silencing of Ceruloplasmin expression	BioSystems: REACTOME	86	119	1.03E-80
1269120	Viral mRNA Translation	BioSystems: REACTOME	77	93	2.15E-80
1268686	GTP hydrolysis and joining of the 60S ribosomal subunit	BioSystems: REACTOME	85	119	3.72E-79
1339156	Selenocysteine synthesis	BioSystems: REACTOME	77	96	2.05E-78
1268692	Eukaryotic Translation Termination	BioSystems: REACTOME	77	97	8.43E-78
1269718	Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	BioSystems: REACTOME	78	100	8.91E-78
1268680	Cap-dependent Translation Initiation	BioSystems: REACTOME	86	127	4.08E-77
1268679	Eukaryotic Translation Initiation	BioSystems: REACTOME	86	127	4.08E-77
1339149	Selenoamino acid metabolism	BioSystems: REACTOME	84	123	8.86E-76
1268689	SRP-dependent cotranslational protein targeting to membrane	BioSystems: REACTOME	81	116	2.89E-74
1268678	Translation	BioSystems: REACTOME	94	165	3.05E-74

Supplementary table 5c: Pathways expressed to a greater extent in CD4+ T cells in mesothelioma associated pleural fluid compared to normal lung. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Pathway ID	Pathway	Source	Set Size	Candidates	q-value
M9809	Cytokine-cytokine receptor interaction	MSigDB C2 BIOCARTA (v7.1)	53	265	1.07E-09
83051	Cytokine-cytokine receptor interaction	BioSystems: KEGG	52	270	4.16E-09
1269547	Chemokine receptors bind chemokines	BioSystems: REACTOME	20	48	7.07E-09
M4844	Chemokine signaling pathway	MSigDB C2 BIOCARTA (v7.1)	36	189	6.63E-06
M60	Calcineurin-regulated NFAT-dependent transcription in lymphocytes	MSigDB C2 BIOCARTA (v7.1)	16	45	8.35E-06
137993	Calcineurin-regulated NFAT-dependent transcription in lymphocytes	BioSystems: Pathway Interaction Database	16	48	2.01E-05
99051	Chemokine signaling pathway	BioSystems: KEGG	33	182	5.35E-05
M4047	Selective expression of chemokine receptors during T-cell polarization	MSigDB C2 BIOCARTA (v7.1)	11	26	1.23E-04
1470924	Interleukin-10 signaling	BioSystems: REACTOME	15	49	1.26E-04
1457780	Neutrophil degranulation	BioSystems: REACTOME	61	492	5.68E-04
1474301	IL-17 signaling pathway	BioSystems: KEGG	20	93	6.83E-04
842771	Inflammatory bowel disease (IBD)	BioSystems: KEGG	16	65	9.79E-04
M235	Calcium signaling in the CD4+ TCR pathway	MSigDB C2 BIOCARTA (v7.1)	10	28	1.51E-03
137941	Calcium signaling in the CD4+ TCR pathway	BioSystems: Pathway Interaction Database	10	28	1.51E-03
P00031	Inflammation mediated by chemokine and cytokine signaling pathway	PantherDB	30	191	1.96E-03

Supplementary table 5d: Pathways expressed to a lesser extent in CD4+ T cells in mesothelioma associated pleural fluid compared to normal lung. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Gene Ontology ID	Gene Ontology term	Set Size	Candidates	q-value
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	74	105	5.28E-68
GO:0006613	cotranslational protein targeting to membrane	75	109	5.33E-68
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	77	120	1.98E-66
GO:0045047	protein targeting to ER	76	120	5.28E-65
GO:0072599	establishment of protein localization to endoplasmic reticulum	76	124	1.68E-63
GO:0006413	translational initiation	91	198	1.74E-61
GO:0070972	protein localization to endoplasmic reticulum	78	149	5.47E-58
GO:0019080	viral gene expression	87	211	7.40E-54
GO:0019083	viral transcription	82	197	4.64E-51
GO:0000956	nuclear-transcribed mRNA catabolic process	83	212	3.38E-49
GO:0006612	protein targeting to membrane	81	212	5.09E-47
GO:0016032	viral process	149	853	1.79E-39
GO:0090150	establishment of protein localization to membrane	94	355	3.01E-39
GO:0044419	interspecies interaction between organisms	157	960	3.62E-38
GO:0044403	symbiotic process	152	911	6.17E-38

Supplementary table 6a: Genes expressed to a greater extent in CD4+ T cells in mesothelioma associated pleural fluid compared to NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Gene Ontology ID	Gene Ontology term	Set Size	Candidates	q-value
GO:0001816	cytokine production	114	875	1.69E-12
GO:0050863	regulation of T cell activation	64	354	1.69E-12
GO:0046649	lymphocyte activation	108	818	2.22E-12
GO:0001775	cell activation	167	1559	9.43E-12
GO:0045321	leukocyte activation	152	1386	2.20E-11
GO:0032609	interferon-gamma production	35	133	3.11E-11
GO:0051249	regulation of lymphocyte activation	79	538	3.11E-11
GO:0034097	response to cytokine	143	1287	3.30E-11
GO:0002694	regulation of leukocyte activation	88	640	3.93E-11
GO:0042110	T cell activation	78	537	5.76E-11
GO:1903037	regulation of leukocyte cell-cell adhesion	58	336	6.08E-11
GO:0002682	regulation of immune system process	179	1776	8.37E-11
GO:0001817	regulation of cytokine production	100	789	8.73E-11
GO:0050865	regulation of cell activation	91	690	1.07E-10
GO:0007159	leukocyte cell-cell adhesion	61	372	1.07E-10

Supplementary table 6b: Genes expressed to a lesser extent in CD4+ T cells in mesothelioma associated pleural fluid compared to NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Pathway ID	Pathway	Source	Set Size	Candidates	q-value
1268690	Eukaryotic Translation Elongation	BioSystems: REACTOME	80	98	3.27E-78
M189	Ribosome	MSigDB C2 BIOCARTA (v7.1)	76	88	3.27E-78
1268691	Peptide chain elongation	BioSystems: REACTOME	77	93	1.76E-76
1268681	Formation of a pool of free 40S subunits	BioSystems: REACTOME	81	107	8.36E-75
1269120	Viral mRNA Translation	BioSystems: REACTOME	76	93	8.36E-75
1268692	Eukaryotic Translation Termination	BioSystems: REACTOME	77	97	5.04E-74
1269718	Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	BioSystems: REACTOME	78	100	5.63E-74
1268688	L13a-mediated translational silencing of Ceruloplasmin expression	BioSystems: REACTOME	84	119	1.30E-73
1339156	Selenocysteine synthesis	BioSystems: REACTOME	76	96	4.94E-73
1268686	GTP hydrolysis and joining of the 60S ribosomal subunit	BioSystems: REACTOME	83	119	4.28E-72
1268680	Cap-dependent Translation Initiation	BioSystems: REACTOME	84	127	5.17E-70
1268679	Eukaryotic Translation Initiation	BioSystems: REACTOME	84	127	5.17E-70
1268678	Translation	BioSystems: REACTOME	91	165	8.83E-66
1268689	SRP-dependent cotranslational protein targeting to membrane	BioSystems: REACTOME	78	116	9.17E-66
1339149	Selenoamino acid metabolism	BioSystems: REACTOME	80	123	9.28E-66

Supplementary table 6c: Pathways expressed to a greater extent in CD4+ T cells in mesothelioma associated pleural fluid compared to NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Appendix A

Pathway ID	Pathway	Source	Set Size	Candidates	q-value
M9809	Cytokine-cytokine receptor interaction	MSigDB C2 BIOCARTA (v7.1)	50	265	1.34E-08
83051	Cytokine-cytokine receptor interaction	BioSystems: KEGG	50	270	1.38E-08
M60	Calcineurin-regulated NFAT-dependent transcription in lymphocytes	MSigDB C2 BIOCARTA (v7.1)	17	45	9.70E-07
137993	Calcineurin-regulated NFAT-dependent transcription in lymphocytes	BioSystems: Pathway Interaction Database	17	48	2.33E-06
1269332	TNFs bind their physiological receptors	BioSystems: REACTOME	13	30	7.69E-06
P00031	Inflammation mediated by chemokine and cytokine signaling pathway	PantherDB	32	191	2.81E-04
137922	IL12-mediated signaling events	BioSystems: Pathway Interaction Database	16	61	4.27E-04
M9904	T cell receptor signaling pathway	MSigDB C2 BIOCARTA (v7.1)	22	108	4.42E-04
1470924	Interleukin-10 signaling	BioSystems: REACTOME	14	49	4.77E-04
M54	IL12-mediated signaling events	MSigDB C2 BIOCARTA (v7.1)	16	63	4.77E-04
83080	T cell receptor signaling pathway	BioSystems: KEGG	21	103	5.50E-04
M4047	Selective expression of chemokine receptors during T-cell polarization	MSigDB C2 BIOCARTA (v7.1)	10	26	5.89E-04
M2064	The 4-1BB-dependent immune response	MSigDB C2 BIOCARTA (v7.1)	8	18	1.58E-03
1269310	Cytokine Signaling in Immune system	BioSystems: REACTOME	80	763	2.02E-03
138018	Downstream signaling in naive CD8+ T cells	BioSystems: Pathway Interaction Database	13	50	2.25E-03

Supplementary table 6d: Pathways expressed to a lesser extent in CD4+ T cells in mesothelioma associated pleural fluid compared to NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Gene Ontology ID	Gene Ontology term	Set Size	Candidates	q-value
GO:0009141	nucleoside triphosphate metabolic process	59	241	5.82E-15
GO:0006119	oxidative phosphorylation	46	153	5.82E-15
GO:0009144	purine nucleoside triphosphate metabolic process	55	216	5.82E-15
GO:0015986	ATP synthesis coupled proton transport	46	156	5.82E-15
GO:0015985	energy coupled proton transport, down electrochemical gradient	46	156	5.82E-15
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	51	193	1.32E-14
GO:0009145	purine nucleoside triphosphate biosynthetic process	51	194	1.44E-14
GO:0006754	ATP biosynthetic process	49	181	1.48E-14
GO:0009142	nucleoside triphosphate biosynthetic process	53	210	1.78E-14
GO:0009201	ribonucleoside triphosphate biosynthetic process	51	199	3.24E-14
GO:0009205	purine ribonucleoside triphosphate metabolic process	51	206	1.41E-13
GO:0044403	symbiotic process	128	911	5.00E-13
GO:0009199	ribonucleoside triphosphate metabolic process	51	213	5.26E-13
GO:0044419	interspecies interaction between organisms	131	960	1.94E-12
GO:0016032	viral process	119	853	7.63E-12

Supplementary table 7a: Genes expressed to a greater extent in CD8+ T cells in mesothelioma associated pleural fluid compared to normal lung. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Gene Ontology ID	Gene Ontology term	Set Size	Candidates	q-value
GO:0001775	cell activation	42	133	2.21E-12
GO:0045321	leukocyte activation	40	126	4.44E-12
GO:0042110	T cell activation	46	165	6.15E-12
GO:0002682	regulation of immune system process	40	131	9.74E-12
GO:0030155	regulation of cell adhesion	44	171	3.20E-10
GO:0007264	small GTPase mediated signal transduction	43	171	1.14E-09
GO:0002252	immune effector process	36	127	1.15E-09
GO:0046649	lymphocyte activation	36	127	1.15E-09
GO:0002684	positive regulation of immune system process	32	103	1.15E-09
GO:0002274	myeloid leukocyte activation	40	154	1.26E-09
GO:0050863	regulation of T cell activation	34	116	1.26E-09
GO:0140352	export from cell	29	88	1.73E-09
GO:0030098	lymphocyte differentiation	34	119	2.36E-09
GO:0002263	cell activation involved in immune response	41	165	2.41E-09
GO:0032940	secretion by cell	30	97	3.74E-09

Supplementary table 7b: Genes expressed to a lesser extent in CD8+ T cells in mesothelioma associated pleural fluid compared to normal lung. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Pathway ID	Pathway	Source	Set Size	Candidates	q-value
82942	Oxidative phosphorylation	BioSystems: KEGG	42	133	2.21E-12
1270127	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	BioSystems: REACTOME	40	126	4.44E-12
1268678	Translation	BioSystems: REACTOME	46	165	6.15E-12
M19540	Oxidative phosphorylation	MSigDB C2 BIOCARTA (v7.1)	40	131	9.74E-12
1270121	The citric acid (TCA) cycle and respiratory electron transport	BioSystems: REACTOME	44	171	3.20E-10
83097	Alzheimer's disease	BioSystems: KEGG	43	171	1.14E-09
1268680	Cap-dependent Translation Initiation	BioSystems: REACTOME	36	127	1.15E-09
1268679	Eukaryotic Translation Initiation	BioSystems: REACTOME	36	127	1.15E-09
1270128	Respiratory electron transport	BioSystems: REACTOME	32	103	1.15E-09
83036	Ribosome	BioSystems: KEGG	40	154	1.26E-09
1268689	SRP-dependent cotranslational protein targeting to membrane	BioSystems: REACTOME	34	116	1.26E-09
M189	Ribosome	MSigDB C2 BIOCARTA (v7.1)	29	88	1.73E-09
1268688	L13a-mediated translational silencing of Ceruloplasmin expression	BioSystems: REACTOME	34	119	2.36E-09
M16024	Alzheimer's disease	MSigDB C2 BIOCARTA (v7.1)	41	165	2.41E-09
1268692	Eukaryotic Translation Termination	BioSystems: REACTOME	30	97	3.74E-09

Supplementary table 7c: Pathways expressed to a greater extent in CD8+ T cells in mesothelioma associated pleural fluid compared to normal lung. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Pathway ID	Pathway	Source	Set Size	Candidates	q-value
M13519	Graft-versus-host disease	MSigDB C2 BIOCARTA (v7.1)	18	41	6.83E-04
83124	Graft-versus-host disease	BioSystems: KEGG	18	41	6.83E-04
1269314	Interferon gamma signaling	BioSystems: REACTOME	29	94	1.30E-03
83060	Apoptosis	BioSystems: KEGG	37	138	1.30E-03
M18615	Allograft rejection	MSigDB C2 BIOCARTA (v7.1)	16	37	1.30E-03
M4844	Chemokine signaling pathway	MSigDB C2 BIOCARTA (v7.1)	46	189	1.30E-03
1269508	Rho GTPase cycle	BioSystems: REACTOME	38	145	1.30E-03
M9904	T cell receptor signaling pathway	MSigDB C2 BIOCARTA (v7.1)	31	108	1.30E-03
83123	Allograft rejection	BioSystems: KEGG	16	38	1.31E-03
1269363	Effects of PIP2 hydrolysis	BioSystems: REACTOME	13	27	1.52E-03
M5669	Natural killer cell mediated cytotoxicity	MSigDB C2 BIOCARTA (v7.1)	36	137	1.52E-03
1269311	Interferon Signaling	BioSystems: REACTOME	47	202	2.13E-03
M60	Calcineurin-regulated NFAT-dependent transcription in lymphocytes	MSigDB C2 BIOCARTA (v7.1)	17	45	2.63E-03
99051	Chemokine signaling pathway	BioSystems: KEGG	43	182	2.93E-03
1457780	Neutrophil degranulation	BioSystems: REACTOME	92	492	3.32E-03

Supplementary table 7d: Pathways expressed to a lesser extent in CD8+ T cells in mesothelioma associated pleural fluid compared to normal lung. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Gene Ontology ID	Gene Ontology term	Set Size	Candidates	q-value
GO:0044419	interspecies interaction between organisms	157	960	7.17E-18
GO:0044403	symbiotic process	151	911	7.17E-18
GO:0016032	viral process	142	853	4.99E-17
GO:0006413	translational initiation	58	198	4.99E-17
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	54	193	7.58E-15
GO:0009145	purine nucleoside triphosphate biosynthetic process	54	194	7.58E-15
GO:0006414	translational elongation	126	764	7.58E-15
GO:0006412	translation	126	764	7.58E-15
GO:0009142	nucleoside triphosphate biosynthetic process	56	210	1.19E-14
GO:0009201	ribonucleoside triphosphate biosynthetic process	54	199	1.70E-14
GO:0006518	peptide metabolic process	144	940	1.70E-14
GO:0009057	macromolecule catabolic process	196	1453	2.45E-14
GO:0009144	purine nucleoside triphosphate metabolic process	56	216	3.30E-14
GO:0044265	cellular macromolecule catabolic process	170	1201	3.66E-14
GO:0006119	oxidative phosphorylation	46	153	3.71E-14

Supplementary table 8a: Genes expressed to a greater extent in CD8+ T cells in mesothelioma associated pleural fluid compared to NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Gene Ontology ID	Gene Ontology term	Set Size	Candidates	q-value
GO:0007049	cell cycle	380	1971	6.79E-10
GO:0022402	cell cycle process	317	1632	2.25E-08
GO:0000280	nuclear division	254	1256	3.86E-08
GO:0000278	mitotic cell cycle	222	1083	1.17E-07
GO:0140014	mitotic nuclear division	222	1083	1.17E-07
GO:1903047	mitotic cell cycle process	222	1083	1.17E-07
GO:0048285	organelle fission	257	1301	1.43E-07
GO:0001816	cytokine production	186	875	1.43E-07
GO:0042110	T cell activation	127	537	1.43E-07
GO:0006259	DNA metabolic process	220	1080	1.57E-07
GO:0050863	regulation of T cell activation	91	354	6.24E-07
GO:0051726	regulation of cell cycle	252	1295	6.30E-07
GO:0030155	regulation of cell adhesion	166	777	6.93E-07
GO:0046649	lymphocyte activation	171	818	1.96E-06
GO:0007264	small GTPase mediated signal transduction	132	594	2.70E-06

Supplementary table 8b: Genes expressed to a lesser extent in CD8+ T cells in mesothelioma associated pleural fluid compared to NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Pathway ID	Pathway	Source	Set Size	Candidates	q-value
1268678	Translation	BioSystems: REACTOME	59	165	3.84E-19
1268680	Cap-dependent Translation Initiation	BioSystems: REACTOME	49	127	1.23E-17
1268679	Eukaryotic Translation Initiation	BioSystems: REACTOME	49	127	1.23E-17
83036	Ribosome	BioSystems: KEGG	53	154	1.04E-16
1268688	L13a-mediated translational silencing of Ceruloplasmin expression	BioSystems: REACTOME	46	119	1.04E-16
1268690	Eukaryotic Translation Elongation	BioSystems: REACTOME	41	98	2.63E-16
1268686	GTP hydrolysis and joining of the 60S ribosomal subunit	BioSystems: REACTOME	45	119	5.12E-16
1268692	Eukaryotic Translation Termination	BioSystems: REACTOME	40	97	9.30E-16
M189	Ribosome	MSigDB C2 BIOCARTA (v7.1)	38	88	9.30E-16
1268681	Formation of a pool of free 40S subunits	BioSystems: REACTOME	42	107	9.50E-16
1269120	Viral mRNA Translation	BioSystems: REACTOME	39	93	9.50E-16
1269718	Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	BioSystems: REACTOME	40	100	2.44E-15
1268689	SRP-dependent cotranslational protein targeting to membrane	BioSystems: REACTOME	43	116	3.91E-15
1268691	Peptide chain elongation	BioSystems: REACTOME	38	93	5.86E-15
1339156	Selenocysteine synthesis	BioSystems: REACTOME	38	96	1.97E-14

Supplementary table 8c: Pathways expressed to a greater extent in CD8+ T cells in mesothelioma associated pleural fluid compared to NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Pathway ID	Pathway	Source	Set Size	Candidates	q-value
1269650	Generic Transcription Pathway	BioSystems: REACTOME	200	879	2.62E-08
1269741	Cell Cycle	BioSystems: REACTOME	141	624	3.27E-05
M1	Fanconi anemia pathway	MSigDB C2 BIOCARTA (v7.1)	21	47	7.04E-04
1269763	Cell Cycle, Mitotic	BioSystems: REACTOME	114	517	1.42E-03
1269508	Rho GTPase cycle	BioSystems: REACTOME	42	145	2.84E-03
M4844	Chemokine signaling pathway	MSigDB C2 BIOCARTA (v7.1)	51	189	2.84E-03
1309102	HDR through Homologous Recombination (HRR)	BioSystems: REACTOME	25	69	2.91E-03
P00031	Inflammation mediated by chemokine and cytokine signaling pathway	PantherDB	51	191	2.95E-03
377262	Fanconi anemia pathway	BioSystems: KEGG	21	55	4.89E-03
1269507	Signaling by Rho GTPases	BioSystems: REACTOME	93	430	1.12E-02
1339113	SUMOylation of DNA replication proteins	BioSystems: REACTOME	18	46	1.12E-02
99051	Chemokine signaling pathway	BioSystems: KEGG	47	182	1.12E-02
1268743	SUMO E3 ligases SUMOylate target proteins	BioSystems: REACTOME	31	104	1.20E-02
1309103	Homologous DNA Pairing and Strand Exchange	BioSystems: REACTOME	17	43	1.24E-02
1269121	Export of Viral Ribonucleoproteins from Nucleus	BioSystems: REACTOME	14	32	1.24E-02

Supplementary table 8d: Pathways expressed to a lesser extent in CD8+ T cells in mesothelioma associated pleural fluid compared to NSCLC. DEGs identified with a FDG adjusted p-value of <0.05 (ie, q value <0.05) and fold change of >1.5 or <-1.5

Appendix B

Glossary of Terms

List of References

AFFARA, N. I., RUFFELL, B., MEDLER, T. R., GUNDERSON, A. J., JOHANSSON, M., BORNSTEIN, S., BERGSLAND, E., STEINHOFF, M., LI, Y., GONG, Q., MA, Y., WIESEN, J. F., WONG, M. H., KULESZ-MARTIN, M., IRVING, B. & COUSSENS, L. M. 2014. B cells regulate macrophage phenotype and response to chemotherapy in squamous carcinomas. *Cancer Cell,* 25**,** 809-821.

AHMADZADEH, M., JOHNSON, L. A., HEEMSKERK, B., WUNDERLICH, J. R., DUDLEY, M. E., WHITE, D. E. & ROSENBERG, S. A. 2009. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood,* 114**,** 1537-44.

AHMED, R. & GRAY, D. 1996. Immunological memory and protective immunity: understanding their relation. *Science,* 272**,** 54-60.

AL-SHIBLI, K. I., DONNEM, T., AL-SAAD, S., PERSSON, M., BREMNES, R. M. & BUSUND, L. T. 2008. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clin Cancer Res,* 14**,** 5220-7.

ALBERTSSON, P. A., BASSE, P. H., HOKLAND, M., GOLDFARB, R. H., NAGELKERKE, J. F., NANNMARK, U. & KUPPEN, P. J. 2003. NK cells and the tumour microenvironment: implications for NK-cell function and anti-tumour activity. *Trends Immunol,* 24**,** 603-9.

ALLAVENA, P., SICA, A., GARLANDA, C. & MANTOVANI, A. 2008. The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. *Immunol Rev,* 222**,** 155-61.

ALLEY, E. W., LOPEZ, J., SANTORO, A., MOROSKY, A., SARAF, S., PIPERDI, B. & VAN BRUMMELEN, E. 2017. Clinical safety and activity of pembrolizumab in patients with malignant pleural mesothelioma (KEYNOTE-028): preliminary results from a non-randomised, open-label, phase 1b trial. *Lancet Oncol,* 18**,** 623-630.

ALSAAB, H. O., SAU, S., ALZHRANI, R., TATIPARTI, K., BHISE, K., KASHAW, S. K. & IYER, A. K. 2017. PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome. *Frontiers in Pharmacology,* 8**,** 561.

ANDERSON, A. C., JOLLER, N. & KUCHROO, V. K. 2016. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. *Immunity,* 44**,** 989-1004.

ANRAKU, M., CUNNINGHAM, K. S., YUN, Z., TSAO, M. S., ZHANG, L., KESHAVJEE, S., JOHNSTON, M. R. & DE PERROT, M. 2008. Impact of tumor-infiltrating T cells on survival in patients with malignant pleural mesothelioma. *J Thorac Cardiovasc Surg,* 135**,** 823-9.

ANTONY, V. B., HOTT, J. W., KUNKEL, S. L., GODBEY, S. W., BURDICK, M. D. & STRIETER, R. M. 1995. Pleural mesothelial cell expression of C-C (monocyte chemotactic peptide) and C-X-C (interleukin 8) chemokines. *Am J Respir Cell Mol Biol,* 12**,** 581-8.

ARCE-SILLAS, A., ÁLVAREZ-LUQUÍN, D. D., TAMAYA-DOMÍNGUEZ, B., GOMEZ-FUENTES, S., TREJO-GARCÍA, A., MELO-SALAS, M., CÁRDENAS, G., RODRÍGUEZ-RAMÍREZ, J. & ADALID-PERALTA, L. 2016. Regulatory T Cells: Molecular Actions on Effector Cells in Immune Regulation. *Journal of Immunology Research,* 2016**,** 1720827.

ARSENIO, J., KAKARADOV, B., METZ, P. J., KIM, S. H., YEO, G. W. & CHANG, J. T. 2014. Early specification of CD8+ T lymphocyte fates during adaptive immunity revealed by single-cell gene-expression analyses. *Nat Immunol,* 15**,** 365-372.

ATANACKOVIC, D., BLOCK, A., DE WEERTH, A., FALTZ, C., HOSSFELD, D. K. & HEGEWISCH-BECKER, S. 2004. Characterization of effusion-infiltrating T cells: benign versus malignant effusions. *Clin Cancer Res,* 10**,** 2600-8.

AYANO, M., TSUKAMOTO, H., KOHNO, K., UEDA, N., TANAKA, A., MITOMA, H., AKAHOSHI, M., ARINOBU, Y., NIIRO, H., HORIUCHI, T. & AKASHI, K. 2015. Increased CD226 Expression on CD8+ T Cells Is Associated with Upregulated Cytokine Production and Endothelial Cell Injury in Patients with Systemic Sclerosis. *J Immunol,* 195**,** 892-900.

BACHMANN, M. F., WOLINT, P., SCHWARZ, K., JAGER, P. & OXENIUS, A. 2005. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol,* 175**,** 4686-96.

BADOUAL, C., HANS, S., RODRIGUEZ, J., PEYRARD, S., KLEIN, C., AGUEZNAY, N. E. H., MOSSERI, V., LACCOURREYE, O., BRUNEVAL, P., FRIDMAN, W. H., BRASNU, D. F. & TARTOUR, E. 2006a. Prognostic Value of Tumor-Infiltrating CD4⁺ T-Cell Subpopulations in Head and Neck Cancers. *Clinical Cancer Research,* 12**,** 465.

BADOUAL, C., HANS, S., RODRIGUEZ, J., PEYRARD, S., KLEIN, C., AGUEZNAY NEL, H., MOSSERI, V., LACCOURREYE, O., BRUNEVAL, P., FRIDMAN, W. H., BRASNU, D. F. & TARTOUR, E. 2006b. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res,* 12**,** 465-72.

BAILEY, S. R., NELSON, M. H., HIMES, R. A., LI, Z., MEHROTRA, S. & PAULOS, C. M. 2014. Th17 Cells in Cancer: The Ultimate Identity Crisis. *Frontiers in Immunology,* 5**,** 276.

BAITSCH, L., BAUMGAERTNER, P., DEVÊVRE, E., RAGHAV, S. K., LEGAT, A., BARBA, L., WIECKOWSKI, S., BOUZOURENE, H., DEPLANCKE, B., ROMERO, P., RUFER, N. & SPEISER, D. E. 2011. Exhaustion of tumor-specific CD8+ T cells in metastases from melanoma patients. *The Journal of Clinical Investigation,* 121**,** 2350-2360.

BALKWILL, F. R., CAPASSO, M. & HAGEMANN, T. 2012. The tumor microenvironment at a glance. *J Cell Sci,* 125**,** 5591-6.

BAO, Y., LIU, X., HAN, C., XU, S., XIE, B., ZHANG, Q., GU, Y., HOU, J., QIAN, L., QIAN, C., HAN, H. & CAO, X. 2014. Identification of IFN-gamma-producing innate B cells. *Cell Res,* 24**,** 161-76.

BAUMANN, F., FLORES, E., NAPOLITANO, A., KANODIA, S., TAIOLI, E., PASS, H., YANG, H. & CARBONE, M. 2015. Mesothelioma patients with germline BAP1 mutations have 7-fold improved long-term survival. *Carcinogenesis,* 36**,** 76-81.

BAUMGARTH, N. 2011. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol,* 11**,** 34-46.

BELOW, J. E., COX, N. J., FUKAGAWA, N. K., HIRVONEN, A. & TESTA, J. R. 2011. Factors that impact susceptibility to fiber-induced health effects. *J Toxicol Environ Health B Crit Rev,* 14**,** 246-66.

BESSOLES, S., GRANDCLEMENT, C., ALARI-PAHISSA, E., GEHRIG, J., JEEVAN-RAJ, B. & HELD, W. 2014. Adaptations of Natural Killer Cells to Self-MHC Class I. *Front Immunol,* 5**,** 349.

BEST, J. A., BLAIR, D. A., KNELL, J., YANG, E., MAYYA, V., DOEDENS, A., DUSTIN, M. L., GOLDRATH, A. W. & IMMUNOLOGICAL GENOME PROJECT, C. 2013. Transcriptional insights into the CD8(+) T cell response to infection and memory T cell formation. *Nat Immunol,* 14**,** 404-12.

BETTI, M., ASPESI, A., BIASI, A., CASALONE, E., FERRANTE, D., OGLIARA, P., GIRONI, L. C., GIORGIONE, R., FARINELLI, P., GROSSO, F., LIBENER, R., ROSATO, S., TURCHETTI, D., MAFFE, A., CASADIO, C., ASCOLI, V., DIANZANI, C., COLOMBO, E., PICCOLINI, E., PAVESI, M., MICCOLI, S., MIRABELLI, D., BRACCO, C., RIGHI, L., BOLDORINI, R., PAPOTTI, M., MATULLO, G., MAGNANI, C., PASINI, B. & DIANZANI, I. 2016. CDKN2A and BAP1 germline mutations predispose to melanoma and mesothelioma. *Cancer Lett,* 378**,** 120-30.

BILLERBECK, E., KANG, Y. H., WALKER, L., LOCKSTONE, H., GRAFMUELLER, S., FLEMING, V., FLINT, J., WILLBERG, C. B., BENGSCH, B., SEIGEL, B., RAMAMURTHY, N., ZITZMANN, N., BARNES, E. J., THEVANAYAGAM, J., BHAGWANANI, A., LESLIE, A., OO, Y. H., KOLLNBERGER, S., BOWNESS, P., DROGNITZ, O., ADAMS, D. H., BLUM, H. E., THIMME, R. & KLENERMAN, P. 2010. Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. *Proc Natl Acad Sci U S A,* 107**,** 3006-11.

BINDEA, G., MLECNIK, B., TOSOLINI, M., KIRILOVSKY, A., WALDNER, M., OBENAUF, A. C., ANGELL, H., FREDRIKSEN, T., LAFONTAINE, L., BERGER, A., BRUNEVAL, P., FRIDMAN, W. H., BECKER, C., PAGES, F., SPEICHER, M. R., TRAJANOSKI, Z. & GALON, J. 2013. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity,* 39**,** 782-95.

BOFILL, M., ALMIRALL, E., MCQUAID, A., PEÑA, R., RUIZ-HERNANDEZ, R., NARANJO, M., RUIZ, L., CLOTET, B. & BORRÀS, F. E. 2004. Differential expression of the cytokine receptors for human interleukin (IL)-12 and IL-18 on lymphocytes of both CD45RA and CD45RO phenotype from tonsils, cord and adult peripheral blood. *Clinical and experimental immunology,* 138**,** 460-465.

BOGRAD, A. J., SUZUKI, K., VERTES, E., COLOVOS, C., MORALES, E. A., SADELAIN, M. & ADUSUMILLI, P. S. 2011. Immune responses and immunotherapeutic interventions in malignant pleural mesothelioma. *Cancer Immunol Immunother,* 60**,** 1509-27.

BONONI, A., GIORGI, C., PATERGNANI, S., LARSON, D., VERBRUGGEN, K., TANJI, M., PELLEGRINI, L., SIGNORATO, V., OLIVETTO, F., PASTORINO, S., NASU, M., NAPOLITANO, A., GAUDINO, G., MORRIS, P., SAKAMOTO, G., FERRIS, L. K., DANESE, A., RAIMONDI, A., TACCHETTI, C., KUCHAY, S., PASS, H. I., AFFAR, E. B., YANG, H., PINTON, P. & CARBONE, M. 2017. BAP1 regulates IP3R3-mediated Ca(2+) flux to mitochondria suppressing cell transformation. *Nature,* 546**,** 549-553.

BOUTIN, C., DUMORTIER, P., REY, F., VIALLAT, J. R. & DE VUYST, P. 1996. Black spots concentrate oncogenic asbestos fibers in the parietal pleura. Thoracoscopic and mineralogic study. *Am J Respir Crit Care Med,* 153**,** 444-9.

BROADDUS, V. C., DANSEN, T. B., ABAYASIRIWARDANA, K. S., WILSON, S. M., FINCH, A. J., SWIGART, L. B., HUNT, A. E. & EVAN, G. I. 2005. Bid mediates apoptotic synergy between tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and DNA damage. *J Biol Chem,* 280**,** 12486-93.

BROMLEY, S. K., YAN, S., TOMURA, M., KANAGAWA, O. & LUSTER, A. D. 2013. Recirculating Memory T cells are a Unique Subset of CD4(+) T Cells with a Distinct Phenotype and Migratory Pattern. *Journal of immunology (Baltimore, Md. : 1950),* 190**,** 970-976.

BRONKHORST, I. H. & JAGER, M. J. 2012. Uveal melanoma: the inflammatory microenvironment. *J Innate Immun,* 4**,** 454-62.

BROWN, J. R., WIMBERLY, H., LANNIN, D. R., NIXON, C., RIMM, D. L. & BOSSUYT, V. 2014. Multiplexed quantitative analysis of CD3, CD8, and CD20 predicts response to neoadjuvant chemotherapy in breast cancer. *Clin Cancer Res,* 20**,** 5995-6005.

BRYCESON, Y. T., MARCH, M. E., LJUNGGREN, H. G. & LONG, E. O. 2006. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev,* 214**,** 73-91.

BUCHBINDER, E. I. & DESAI, A. 2016. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol,* 39**,** 98-106.

BUENO, R., STAWISKI, E. W., GOLDSTEIN, L. D., DURINCK, S., DE RIENZO, A., MODRUSAN, Z., GNAD, F., NGUYEN, T. T., JAISWAL, B. S., CHIRIEAC, L. R., SCIARANGHELLA, D., DAO, N., GUSTAFSON, C. E., MUNIR, K. J., HACKNEY, J. A., CHAUDHURI, A., GUPTA, R., GUILLORY, J., TOY, K., HA, C., CHEN, Y. J., STINSON, J., CHAUDHURI, S., ZHANG, N., WU, T. D., SUGARBAKER, D. J., DE SAUVAGE, F. J., RICHARDS, W. G. & SESHAGIRI, S. 2016. Comprehensive genomic analysis of malignant pleural mesothelioma identifies recurrent mutations, gene fusions and splicing alterations. *Nat Genet,* 48**,** 407-16.

BURDORF, A., DAHHAN, M. & SWUSTE, P. 2003. Occupational characteristics of cases with asbestos-related diseases in The Netherlands. *Ann Occup Hyg,* 47**,** 485-92.

BURT, B. M., RODIG, S. J., TILLEMAN, T. R., ELBARDISSI, A. W., BUENO, R. & SUGARBAKER, D. J. 2011. Circulating and tumor-infiltrating myeloid cells predict survival in human pleural mesothelioma. *Cancer,* 117**,** 5234-44.

BUSHAR, N. D., CORBO, E., SCHMIDT, M., MALTZMAN, J. S. & FARBER, D. L. 2010. Ablation of SLP-76 signaling after T cell priming generates memory CD4 T cells impaired in steady-state and cytokine-driven homeostasis. *Proceedings of the National Academy of Sciences,* 107**,** 827-831.

BUTT, A. Q. & MILLS, K. H. 2014. Immunosuppressive networks and checkpoints controlling antitumor immunity and their blockade in the development of cancer immunotherapeutics and vaccines. *Oncogene,* 33**,** 4623-31.

CALABRO, L., MORRA, A., FONSATTI, E., CUTAIA, O., AMATO, G., GIANNARELLI, D., DI GIACOMO, A. M., DANIELLI, R., ALTOMONTE, M., MUTTI, L. & MAIO, M. 2013. Tremelimumab for patients with chemotherapy-resistant advanced malignant mesothelioma: an open-label, single-arm, phase 2 trial. *Lancet Oncol,* 14**,** 1104-11.

CALABRO, L., MORRA, A., FONSATTI, E., CUTAIA, O., FAZIO, C., ANNESI, D., LENOCI, M., AMATO, G., DANIELLI, R., ALTOMONTE, M., GIANNARELLI, D., DI GIACOMO, A. M. & MAIO, M. 2015. Efficacy and safety of an intensified schedule of tremelimumab for chemotherapy-resistant malignant mesothelioma: an open-label, single-arm, phase 2 study. *Lancet Respir Med,* 3**,** 301-9.

CALABRO, L., MORRA, A., GIANNARELLI, D., AMATO, G., D'INCECCO, A., COVRE, A., LEWIS, A., REBELATTO, M. C., DANIELLI, R., ALTOMONTE, M., DI GIACOMO, A. M. & MAIO, M. 2018. Tremelimumab combined with durvalumab in patients with mesothelioma (NIBIT-MESO-1): an open-label, non-randomised, phase 2 study. *Lancet Respir Med,* 6**,** 451-460.

CALIGIURI, M. A. 2008. Human natural killer cells. *Blood,* 112**,** 461-9.

CANNONS, J. L., BERTRAM, E. M. & WATTS, T. H. 2002. Cutting Edge: Profound Defect in T Cell Responses in TNF Receptor-Associated Factor 2 Dominant Negative Mice. *The Journal of Immunology,* 169**,** 2828.

CARBONE, F. R. 2015. Tissue-Resident Memory T Cells and Fixed Immune Surveillance in Nonlymphoid Organs. *J Immunol,* 195**,** 17-22.

CARTER, J., MILLER, J. A., FELLER-KOPMAN, D., ETTINGER, D., SIDRANSKY, D. & MALEKI, Z. 2017. Molecular Profiling of Malignant Pleural Effusion in Metastatic Non-Small-Cell Lung Carcinoma. The Effect of Preanalytical Factors. *Ann Am Thorac Soc,* 14**,** 1169-1176.

CEDRES, S., MONTERO, M. A., ZAMORA, E., MARTINEZ, A., MARTINEZ, P., FARINAS, L., NAVARRO, A., TORREJON, D., GABALDON, A., RAMON, Y. C. S. & FELIP, E. 2014. Expression of Wilms' tumor gene (WT1) is associated with survival in malignant pleural mesothelioma. *Clin Transl Oncol,* 16**,** 776-82.

CEDRES, S., PONCE-AIX, S., ZUGAZAGOITIA, J., SANSANO, I., ENGUITA, A., NAVARRO-MENDIVIL, A., MARTINEZ-MARTI, A., MARTINEZ, P. & FELIP, E. 2015. Analysis of expression of programmed cell death 1 ligand 1 (PD-L1) in malignant pleural mesothelioma (MPM). *PLoS One,* 10**,** e0121071.

CERESOLI, G. L., BONOMI, M. & SAUTA, M. G. 2016. Immune checkpoint inhibitors in malignant pleural mesothelioma: promises and challenges. *Expert Rev Anticancer Ther,* 16**,** 673-5.

CERESOLI, G. L., ZUCALI, P. A., FAVARETTO, A. G., GROSSI, F., BIDOLI, P., DEL CONTE, G., CERIBELLI, A., BEARZ, A., MORENGHI, E., CAVINA, R., MARANGOLO, M., PARRA, H. J. & SANTORO, A. 2006. Phase II study of pemetrexed plus carboplatin in malignant pleural mesothelioma. *J Clin Oncol,* 24**,** 1443-8.

CHAN, D. V., GIBSON, H. M., AUFIERO, B. M., WILSON, A. J., HAFNER, M. S., MI, Q. S. & WONG, H. K. 2014. Differential CTLA-4 expression in human CD4+ versus CD8+ T cells is associated with increased NFAT1 and inhibition of CD4+ proliferation. *Genes and immunity,* 15**,** 25-32.

CHANG, S. H., MIRABOLFATHINEJAD, S. G., KATTA, H., CUMPIAN, A. M., GONG, L., CAETANO, M. S., MOGHADDAM, S. J. & DONG, C. 2014. T helper 17 cells play a critical pathogenic role in lung cancer. *Proc Natl Acad Sci U S A,* 111**,** 5664-9.

CHANG, Y., XU, L., AN, H., FU, Q., CHEN, L., LIN, Z. & XU, J. 2015. Expression of IL-4 and IL-13 predicts recurrence and survival in localized clear-cell renal cell carcinoma. *International Journal of Clinical and Experimental Pathology,* 8**,** 1594-1603.

CHAUDHARY, B. & ELKORD, E. 2016. Regulatory T Cells in the Tumor Microenvironment and Cancer Progression: Role and Therapeutic Targeting. *Vaccines (Basel),* 4.

CHEE, S. J., LOPEZ, M., MELLOWS, T., GANKANDE, S., MOUTASIM, K. A., HARRIS, S., CLARKE, J., VIJAYANAND, P., THOMAS, G. J. & OTTENSMEIER, C. H. 2017. Evaluating the effect of immune cells on the outcome of patients with mesothelioma. *Br J Cancer,* 117**,** 1341-1348.

CHEN, C., SHEN, Y., QU, Q. X., CHEN, X. Q., ZHANG, X. G. & HUANG, J. A. 2013. Induced expression of B7-H3 on the lung cancer cells and macrophages suppresses T-cell mediating anti-tumor immune response. *Exp Cell Res,* 319**,** 96-102.

CHEN, J. H., PERRY, C. J., TSUI, Y.-C., STARON, M. M., PARISH, I. A., DOMINGUEZ, C. X., ROSENBERG, D. W. & KAECH, S. M. 2015. Prostaglandin E2 and programmed cell death 1 signaling coordinately impair CTL function and survival during chronic viral infection. *Nature medicine,* 21**,** 327-334.

CHEVALIER, N., JARROSSAY, D., HO, E., AVERY, D. T., MA, C. S., YU, D., SALLUSTO, F., TANGYE, S. G. & MACKAY, C. R. 2011. CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol,* 186**,** 5556-68.

CHOE, N., TANAKA, S. & KAGAN, E. 1998. Asbestos fibers and interleukin-1 upregulate the formation of reactive nitrogen species in rat pleural mesothelial cells. *Am J Respir Cell Mol Biol,* 19**,** 226-36.

CHOI, Y. S., YANG, J. A., YUSUF, I., JOHNSTON, R. J., GREENBAUM, J., PETERS, B. & CROTTY, S. 2013. Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. *J Immunol,* 190**,** 4014-26.

CHRISTENSEN, B. C., HOUSEMAN, E. A., GODLESKI, J. J., MARSIT, C. J., LONGACKER, J. L., ROELOFS, C. R., KARAGAS, M. R., WRENSCH, M. R., YEH, R. F., NELSON, H. H., WIEMELS, J. L., ZHENG, S., WIENCKE, J. K., BUENO, R., SUGARBAKER, D. J. &

KELSEY, K. T. 2009. Epigenetic profiles distinguish pleural mesothelioma from normal pleura and predict lung asbestos burden and clinical outcome. *Cancer Res,* 69**,** 227-34.

CLARK, R. A. 2015. Resident memory T cells in human health and disease. *Sci Transl Med,* 7**,** 269rv1.

COLEGIO, O. R., CHU, N. Q., SZABO, A. L., CHU, T., RHEBERGEN, A. M., JAIRAM, V., CYRUS, N., BROKOWSKI, C. E., EISENBARTH, S. C., PHILLIPS, G. M., CLINE, G. W., PHILLIPS, A. J. & MEDZHITOV, R. 2014. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature,* 513**,** 559-63.

COLOTTA, F., ALLAVENA, P., SICA, A., GARLANDA, C. & MANTOVANI, A. 2009. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis,* 30**,** 1073-81.

COMTE, D., KARAMPETSOU, M. P., YOSHIDA, N., KIS-TOTH, K., KYTTARIS, V. C. & TSOKOS, G. C. 2017. Signaling Lymphocytic Activation Molecule Family Member 7 Engagement Restores Defective Effector CD8+ T Cell Function in Systemic Lupus Erythematosus. *Arthritis Rheumatol,* 69**,** 1035-1044.

CONDOTTA, S. A. & RICHER, M. J. 2017. The immune battlefield: The impact of inflammatory cytokines on CD8+ T-cell immunity. *PLoS Pathog,* 13**,** e1006618.

CORTHAY, A. 2009. How do regulatory T cells work? *Scand J Immunol,* 70**,** 326-36.

COUSSENS, L. M. & WERB, Z. 2001. Inflammatory cells and cancer: think different! *J Exp Med,* 193**,** F23-6.

CRESPO, J., SUN, H., WELLING, T. H., TIAN, Z. & ZOU, W. 2013. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. *Curr Opin Immunol,* 25**,** 214-21.

CROTTY, S. 2014. T follicular helper cell differentiation, function, and roles in disease. *Immunity,* 41**,** 529-542.

DANILO, M., CHENNUPATI, V., SILVA, J. G., SIEGERT, S. & HELD, W. 2018. Suppression of Tcf1 by Inflammatory Cytokines Facilitates Effector CD8 T Cell Differentiation. *Cell Rep,* 22**,** 2107-2117.

DAVIES, B., PRIER, J. E., JONES, C. M., GEBHARDT, T., CARBONE, F. R. & MACKAY, L. K. 2017. Cutting Edge: Tissue-Resident Memory T Cells Generated by Multiple Immunizations or Localized Deposition Provide Enhanced Immunity. *J Immunol,* 198**,** 2233-2237.

DENARDO, D. G., BARRETO, J. B., ANDREU, P., VASQUEZ, L., TAWFIK, D., KOLHATKAR, N. & COUSSENS, L. M. 2009. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell,* 16**,** 91-102.

DHAENE, K., WAUTERS, J., WEYN, B., TIMMERMANS, J. P. & VAN MARCK, E. 2000. Expression profile of telomerase subunits in human pleural mesothelioma. *J Pathol,* 190**,** 80-5.

DIEDERICHSEN, A. C., HJELMBORG, J., CHRISTENSEN, P. B., ZEUTHEN, J. & FENGER, C. 2003. Prognostic value of the CD4+/CD8+ ratio of tumour infiltrating lymphocytes in colorectal cancer and HLA-DR expression on tumour cells. *Cancer Immunol Immunother,* 52**,** 423-8.

DIENZ, O., EATON, S. M., BOND, J. P., NEVEU, W., MOQUIN, D., NOUBADE, R., BRISO, E. M., CHARLAND, C., LEONARD, W. J., CILIBERTO, G., TEUSCHER, C., HAYNES, L. & RINCON, M. 2009. The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4⁺ T cells. *The Journal of Experimental Medicine,* 206**,** 69.

DING, Z. C., BLAZAR, B. R., MELLOR, A. L., MUNN, D. H. & ZHOU, G. 2010. Chemotherapy rescues tumor-driven aberrant CD4+ T-cell differentiation and restores an activated polyfunctional helper phenotype. *Blood,* 115**,** 2397-406.

DISIS, M. L. 2010. Immune regulation of cancer. *J Clin Oncol,* 28**,** 4531-8.

DJENIDI, F., ADAM, J., GOUBAR, A., DURGEAU, A., MEURICE, G., DE MONTPREVILLE, V., VALIDIRE, P., BESSE, B. & MAMI-CHOUAIB, F. 2015. CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. *J Immunol,* 194**,** 3475-86.

DOMINGUEZ, C., AMEZQUITA, R., GUAN, T., MARSHALL, H., JOSHI, N., KLEINSTEIN, S. & M KAECH, S. 2015. *The transcription factors ZEB2 and T-bet cooperate to program cytotoxic T cell terminal differentiation in response to LCMV viral infection*.

DONG, C. 2017. Helper T Cells and Cancer-Associated Inflammation: A New Direction for Immunotherapy? *J Interferon Cytokine Res,* 37**,** 383-385.

DU, W., YANG, M., TURNER, A., XU, C., FERRIS, R. L., HUANG, J., KANE, L. P. & LU, B. 2017. TIM-3 as a Target for Cancer Immunotherapy and Mechanisms of Action. *Int J Mol Sci,* 18.

DUDAKOV, J. A., HANASH, A. M. & VAN DEN BRINK, M. R. M. 2015. Interleukin-22: immunobiology and pathology. *Annual review of immunology,* 33**,** 747-785.

DUHEN, T., GEIGER, R., JARROSSAY, D., LANZAVECCHIA, A. & SALLUSTO, F. 2009. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol,* 10**,** 857-63.

DUNN, G. P., OLD, L. J. & SCHREIBER, R. D. 2004. The three Es of cancer immunoediting. *Annu Rev Immunol,* 22**,** 329-60.

DURAISWAMY, J., KALUZA, K. M., FREEMAN, G. J. & COUKOS, G. 2013. Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. *Cancer Res,* 73**,** 3591-603.

EISENBERG, G., ENGELSTEIN, R., GEIGER-MAOR, A., HAJAJ, E., MERIMS, S., FRANKENBURG, S., UZANA, R., RUTENBERG, A., MACHLENKIN, A., FREI, G., PERETZ, T. & LOTEM, M. 2018. Soluble SLAMF6 Receptor Induces Strong CD8(+) T-cell Effector Function and Improves Anti-Melanoma Activity In Vivo. *Cancer Immunol Res,* 6**,** 127-138.

ELETR, Z. M. & WILKINSON, K. D. 2011. An emerging model for BAP1's role in regulating cell cycle progression. *Cell Biochem Biophys,* 60**,** 3-11.

ERDMAN, S. E., RAO, V. P., OLIPITZ, W., TAYLOR, C. L., JACKSON, E. A., LEVKOVICH, T., LEE, C. W., HORWITZ, B. H., FOX, J. G., GE, Z. & POUTAHIDIS, T. 2010. Unifying roles for regulatory T cells and inflammation in cancer. *Int J Cancer,* 126**,** 1651-65.

EYERICH, S., EYERICH, K., PENNINO, D., CARBONE, T., NASORRI, F., PALLOTTA, S., CIANFARANI, F., ODORISIO, T., TRAIDL-HOFFMANN, C., BEHRENDT, H., DURHAM, S. R., SCHMIDT-WEBER, C. B. & CAVANI, A. 2009. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest,* 119**,** 3573-85.

FARBER, D. L., YUDANIN, N. A. & RESTIFO, N. P. 2014. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol,* 14**,** 24-35.

FENNELL, D. A., KIRKPATRICK, E., COZENS, K., NYE, M., LESTER, J., HANNA, G., STEELE, N., SZLOSAREK, P., DANSON, S., LORD, J., OTTENSMEIER, C., BARNES, D., HILL, S., KALEVRAS, M., MAISHMAN, T. & GRIFFITHS, G. 2018. CONFIRM: a double-blind, placebo-controlled phase III clinical trial investigating the effect of nivolumab in patients with relapsed mesothelioma: study protocol for a randomised controlled trial. *Trials,* 19**,** 233.

FRANKLIN, R. A., LIAO, W., SARKAR, A., KIM, M. V., BIVONA, M. R., LIU, K., PAMER, E. G. & LI, M. O. 2014. The cellular and molecular origin of tumor-associated macrophages. *Science,* 344**,** 921-5.

FRIDLENDER, Z. G. & ALBELDA, S. M. 2012. Tumor-associated neutrophils: friend or foe? *Carcinogenesis,* 33**,** 949-55.

FRIDMAN, W. H., PAGES, F., SAUTES-FRIDMAN, C. & GALON, J. 2012. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer,* 12**,** 298-306.

FRYDRYCHOWICZ, M., BORUCZKOWSKI, M., KOLECKA-BEDNARCZYK, A. & DWORACKI, G. 2017. The Dual Role of Treg in Cancer. *Scandinavian Journal of Immunology,* 86**,** 436-443.

GALON, J., COSTES, A., SANCHEZ-CABO, F., KIRILOVSKY, A., MLECNIK, B., LAGORCE-PAGES, C., TOSOLINI, M., CAMUS, M., BERGER, A., WIND, P., ZINZINDOHOUE, F., BRUNEVAL, P., CUGNENC, P. H., TRAJANOSKI, Z., FRIDMAN, W. H. & PAGES, F. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science,* 313**,** 1960-4.

GANESAN, A. P., CLARKE, J., WOOD, O., GARRIDO-MARTIN, E. M., CHEE, S. J., MELLOWS, T., SAMANIEGO-CASTRUITA, D., SINGH, D., SEUMOIS, G., ALZETANI, A., WOO, E., FRIEDMANN, P. S., KING, E. V., THOMAS, G. J., SANCHEZ-ELSNER, T., VIJAYANAND, P. & OTTENSMEIER, C. H. 2017. Tissue-resident memory features are linked to the magnitude of cytotoxic T cell responses in human lung cancer. *Nat Immunol,* 18**,** 940-950.

GARCIA-MARTINEZ, E., GIL, G. L., BENITO, A. C., GONZALEZ-BILLALABEITIA, E., CONESA, M. A., GARCIA GARCIA, T., GARCIA-GARRE, E., VICENTE, V. & AYALA DE LA PENA, F. 2014. Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. *Breast Cancer Res,* 16**,** 488.

GATTINONI, L., LUGLI, E., JI, Y., POS, Z., PAULOS, C. M., QUIGLEY, M. F., ALMEIDA, J. R., GOSTICK, E., YU, Z., CARPENITO, C., WANG, E., DOUEK, D. C., PRICE, D. A., JUNE, C. H., MARINCOLA, F. M., ROEDERER, M. & RESTIFO, N. P. 2011. A human memory T cell subset with stem cell-like properties. *Nat Med,* 17**,** 1290-7.

GENTLES, A. J., NEWMAN, A. M., LIU, C. L., BRATMAN, S. V., FENG, W., KIM, D., NAIR, V. S., XU, Y., KHUONG, A., HOANG, C. D., DIEHN, M., WEST, R. B., PLEVRITIS, S. K. & ALIZADEH, A. A. 2015. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med,* 21**,** 938-945.

GIBBONS JOHNSON, R. M. & DONG, H. 2017. Functional Expression of Programmed Death-Ligand 1 (B7-H1) by Immune Cells and Tumor Cells. *Frontiers in immunology,* 8**,** 961-961.

GIBBS, G. W. & BERRY, G. 2008. Mesothelioma and asbestos. *Regul Toxicol Pharmacol,* 52**,** S223-31.

GOBERT, M., TREILLEUX, I., BENDRISS-VERMARE, N., BACHELOT, T., GODDARD-LEON, S., ARFI, V., BIOTA, C., DOFFIN, A. C., DURAND, I., OLIVE, D., PEREZ, S., PASQUAL, N., FAURE, C., RAY-COQUARD, I., PUISIEUX, A., CAUX, C., BLAY, J. Y. & MENETRIER-CAUX, C. 2009. Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. *Cancer Res,* 69**,** 2000-9.

GOCHEVA, V., WANG, H. W., GADEA, B. B., SHREE, T., HUNTER, K. E., GARFALL, A. L., BERMAN, T. & JOYCE, J. A. 2010. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev,* 24**,** 241-55.

GOLUBOVSKAYA, V. & WU, L. 2016. Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy. *Cancers (Basel),* 8.

GOODEN, M. J., DE BOCK, G. H., LEFFERS, N., DAEMEN, T. & NIJMAN, H. W. 2011. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *Br J Cancer,* 105**,** 93-103.

GORELIK, E., WILTROUT, R. H., OKUMURA, K., HABU, S. & HERBERMAN, R. B. 1982. Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. *Int J Cancer,* 30**,** 107-12.

GRIVENNIKOV, S. I., GRETEN, F. R. & KARIN, M. 2010. Immunity, inflammation, and cancer. *Cell,* 140**,** 883-99.

GU-TRANTIEN, C., LOI, S., GARAUD, S., EQUETER, C., LIBIN, M., DE WIND, A., RAVOET, M., LE BUANEC, H., SIBILLE, C., MANFOUO-FOUTSOP, G., VEYS, I., HAIBE-KAINS, B., SINGHAL, S. K., MICHIELS, S., ROTHE, F., SALGADO, R., DUVILLIER, H., IGNATIADIS, M., DESMEDT, C., BRON, D., LARSIMONT, D., PICCART, M.,

SOTIRIOU, C. & WILLARD-GALLO, K. 2013. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest,* 123**,** 2873-92.

GUERY, L. & HUGUES, S. 2015. Th17 Cell Plasticity and Functions in Cancer Immunity. *Biomed Res Int,* 2015**,** 314620.

GUILLEREY, C., HARJUNPÄÄ, H., CARRIÉ, N., KASSEM, S., TEO, T., MILES, K., KRUMEICH, S., WEULERSSE, M., CUISINIER, M., STANNARD, K., YU, Y., MINNIE, S. A., HILL, G. R., DOUGALL, W. C., AVET-LOISEAU, H., TENG, M. W. L., NAKAMURA, K., MARTINET, L. & SMYTH, M. J. 2018. TIGIT immune checkpoint blockade restores CD8⁺ T-cell immunity against multiple myeloma. *Blood,* 132**,** 1689.

GUILLEREY, C., HUNTINGTON, N. D. & SMYTH, M. J. 2016. Targeting natural killer cells in cancer immunotherapy. *Nat Immunol,* 17**,** 1025-36.

GUINAN, E. C., GRIBBEN, J. G., BOUSSIOTIS, V. A., FREEMAN, G. J. & NADLER, L. M. 1994. Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. *Blood,* 84**,** 3261-82.

GUO, Y., LEE, Y.-C., BROWN, C., ZHANG, W., USHERWOOD, E. & NOELLE, R. J. 2014. Dissecting the role of retinoic acid receptor isoforms in the CD8 response to infection. *Journal of immunology (Baltimore, Md. : 1950),* 192**,** 3336-3344.

HADRUP, S., DONIA, M. & THOR STRATEN, P. 2013. Effector CD4 and CD8 T cells and their role in the tumor microenvironment. *Cancer Microenviron,* 6**,** 123-33.

HAGN, M., SONTHEIMER, K., DAHLKE, K., BRUEGGEMANN, S., KALTENMEIER, C., BEYER, T., HOFMANN, S., LUNOV, O., BARTH, T. F., FABRICIUS, D., TRON, K., NIENHAUS, G. U., SIMMET, T., SCHREZENMEIER, H. & JAHRSDORFER, B. 2012. Human B cells differentiate into granzyme B-secreting cytotoxic B lymphocytes upon incomplete T-cell help. *Immunol Cell Biol,* 90**,** 457-67.

HALE, J. S., YOUNGBLOOD, B., LATNER, D. R., MOHAMMED, A. U., YE, L., AKONDY, R. S., WU, T., IYER, S. S. & AHMED, R. 2013. Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. *Immunity,* 38**,** 805-17.

HALL, J. A., CANNONS, J. L., GRAINGER, J. R., DOS SANTOS, L. M., HAND, T. W., NAIK, S., WOHLFERT, E. A., CHOU, D. B., OLDENHOVE, G., ROBINSON, M., GRIGG, M. E., KASTENMAYER, R., SCHWARTZBERG, P. L. & BELKAID, Y. 2011. Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. *Immunity,* 34**,** 435-447.

HALLE, S., KEYSER, K. A., STAHL, F. R., BUSCHE, A., MARQUARDT, A., ZHENG, X., GALLA, M., HEISSMEYER, V., HELLER, K., BOELTER, J., WAGNER, K., BISCHOFF, Y., MARTENS, R., BRAUN, A., WERTH, K., UVAROVSKII, A., KEMPF, H., MEYER-HERMANN, M., ARENS, R., KREMER, M., SUTTER, G., MESSERLE, M. & FORSTER, R. 2016. In Vivo Killing Capacity of Cytotoxic T Cells Is Limited and Involves Dynamic Interactions and T Cell Cooperativity. *Immunity,* 44**,** 233-45.

HANAHAN, D. & COUSSENS, L. M. 2012. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell,* 21**,** 309-22.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

HARIMOTO, H., SHIMIZU, M., NAKAGAWA, Y., NAKATSUKA, K., WAKABAYASHI, A., SAKAMOTO, C. & TAKAHASHI, H. 2013. Inactivation of tumor-specific CD8(+) CTLs by tumor-infiltrating tolerogenic dendritic cells. *Immunol Cell Biol,* 91**,** 545-55.

HARRISON, O. J., SRINIVASAN, N., POTT, J., SCHIERING, C., KRAUSGRUBER, T., ILOTT, N. E. & MALOY, K. J. 2015. Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3^{+} Treg cell function in the intestine. *Mucosal immunology,* 8**,** 1226-1236.

HASSAN, R., THOMAS, A., PATEL, M. R., NEMUNAITIS, J. J., BENNOUNA, J., POWDERLY, J. D., TAYLOR, M. H., DOWLATI, A., CHEN, F., LEACH, J., VAISHAMPAYAN, U. N., VERSCHRAEGEN, C. F., DELORD, J.-P., GROTE, H. J., VON HEYDEBRECK, A., CUILLEROT, J.-M. & GULLEY, J. L. 2016. Avelumab (MSB0010718C; anti-PD-L1) in patients with advanced unresectable mesothelioma from the JAVELIN solid tumor phase Ib trial: Safety, clinical activity, and PD-L1 expression. *Journal of Clinical Oncology,* 34**,** 8503-8503.

HILL, J. A., HALL, J. A., SUN, C.-M., CAI, Q., GHYSELINCK, N., CHAMBON, P., BELKAID, Y., MATHIS, D. & BENOIST, C. 2008. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. *Immunity,* 29**,** 758-770.

HIRAHARA, K., GHORESCHI, K., LAURENCE, A., YANG, X. P., KANNO, Y. & O'SHEA, J. J. 2010. Signal transduction pathways and transcriptional regulation in Th17 cell differentiation. *Cytokine Growth Factor Rev,* 21**,** 425-34.

HODI, F. S., O'DAY, S. J., MCDERMOTT, D. F., WEBER, R. W., SOSMAN, J. A., HAANEN, J. B., GONZALEZ, R., ROBERT, C., SCHADENDORF, D., HASSEL, J. C., AKERLEY, W., VAN DEN EERTWEGH, A. J., LUTZKY, J., LORIGAN, P., VAUBEL, J. M., LINETTE, G. P., HOGG, D., OTTENSMEIER, C. H., LEBBE, C., PESCHEL, C., QUIRT, I., CLARK, J. I., WOLCHOK, J. D., WEBER, J. S., TIAN, J., YELLIN, M. J., NICHOL, G. M., HOOS, A. & URBA, W. J. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med,* 363**,** 711-23.

HOUGHTON, A. N. & GUEVARA-PATINO, J. A. 2004. Immune recognition of self in immunity against cancer. *J Clin Invest,* 114**,** 468-71.

HUANG, Y. H., CAO, Y. F., JIANG, Z. Y., ZHANG, S. & GAO, F. 2015. Th22 cell accumulation is associated with colorectal cancer development. *World J Gastroenterol,* 21**,** 4216-24.

HUBER, M., HEINK, S., GROTHE, H., GURALNIK, A., REINHARD, K., ELFLEIN, K., HUNIG, T., MITTRUCKER, H. W., BRUSTLE, A., KAMRADT, T. & LOHOFF, M. 2009. A Th17-like developmental process leads to CD8(+) Tc17 cells with reduced cytotoxic activity. *Eur J Immunol,* 39**,** 1716-25.

HYLEBOS, M., VAN CAMP, G., VAN MEERBEECK, J. P. & OP DE BEECK, K. 2016. The Genetic Landscape of Malignant Pleural Mesothelioma: Results from Massively Parallel Sequencing. *J Thorac Oncol,* 11**,** 1615-26.

IANNELLO, A., THOMPSON, T. W., ARDOLINO, M., LOWE, S. W. & RAULET, D. H. 2013. p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells. *J Exp Med,* 210**,** 2057-69.

INGRAM, J. T., YI, J. S. & ZAJAC, A. J. 2011. Exhausted CD8 T cells downregulate the IL-18 receptor and become unresponsive to inflammatory cytokines and bacterial co-infections. *PLoS pathogens,* 7**,** e1002273-e1002273.

IOANNIDES, C. G. & WHITESIDE, T. L. 1993. T cell recognition of human tumors: implications for molecular immunotherapy of cancer. *Clin Immunol Immunopathol,* 66**,** 91-106.

ISE, W., INOUE, T., MCLACHLAN, J. B., KOMETANI, K., KUBO, M., OKADA, T. & KUROSAKI, T. 2014. Memory B cells contribute to rapid Bcl6 expression by memory follicular helper T cells. *Proc Natl Acad Sci U S A,* 111**,** 11792-7.

ISE, W., KOHYAMA, M., SCHRAML, B. U., ZHANG, T., SCHWER, B., BASU, U., ALT, F. W., TANG, J., OLTZ, E. M., MURPHY, T. L. & MURPHY, K. M. 2011. The transcription factor BATF controls the global regulators of class-switch recombination in both B cells and T cells. *Nature immunology,* 12**,** 536-543.

IVANOVA, E. A. & OREKHOV, A. N. 2015. T Helper Lymphocyte Subsets and Plasticity in Autoimmunity and Cancer: An Overview. *Biomed Res Int,* 2015**,** 327470.

IVASHKIV, L. B. 2018. IFNgamma: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. *Nat Rev Immunol*.

IZZI, V., CHIURCHIU, V., D'AQUILIO, F., PALUMBO, C., TRESOLDI, I., MODESTI, A. & BALDINI, P. M. 2009. Differential effects of malignant mesothelioma cells on THP-1 monocytes and macrophages. *Int J Oncol,* 34**,** 543-50.

IZZI, V., CHIURCHIÙ, V., DOLDO, E., PALUMBO, C., TRESOLDI, I., BEI, R., ALBONICI, L. & MODESTI, A. 2013. Interleukin-17 Produced by Malignant Mesothelioma-Polarized Immune Cells Promotes Tumor Growth and Invasiveness. *European Journal of Inflammation,* 11**,** 203-214.

JABEEN, R., GOSWAMI, R., AWE, O., KULKARNI, A., NGUYEN, E. T., ATTENASIO, A., WALSH, D., OLSON, M. R., KIM, M. H., TEPPER, R. S., SUN, J., KIM, C. H., TAPAROWSKY, E. J., ZHOU, B. & KAPLAN, M. H. 2013. Th9 cell development requires a BATF-regulated transcriptional network. *The Journal of clinical investigation,* 123**,** 4641-4653.

JACKAMAN, C. & NELSON, D. J. 2014. Are macrophages, myeloid derived suppressor cells and neutrophils mediators of local suppression in healthy and cancerous tissues in aging hosts? *Exp Gerontol,* 54**,** 53-7.

JANG, J.-E., HAJDU, C. H., LIOT, C., MILLER, G., DUSTIN, M. L. & BAR-SAGI, D. 2017. Crosstalk between Regulatory T Cells and Tumor-Associated Dendritic Cells Negates Anti-tumor Immunity in Pancreatic Cancer. *Cell Reports,* 20**,** 558-571.

JANKOVIC, D., KUGLER, D. G. & SHER, A. 2010. IL-10 production by CD4+ effector T cells: a mechanism for self-regulation. *Mucosal Immunol,* 3**,** 239-46.

JASANI, B. & GIBBS, A. 2012. Mesothelioma not associated with asbestos exposure. *Arch Pathol Lab Med,* 136**,** 262-7.

JAURAND, M. C. & FLEURY-FEITH, J. 2005. Pathogenesis of malignant pleural mesothelioma. *Respirology,* 10**,** 2-8.

JAURAND, M. C., KAPLAN, H., THIOLLET, J., PINCHON, M. C., BERNAUDIN, J. F. & BIGNON, J. 1979. Phagocytosis of chrysotile fibers by pleural mesothelial cells in culture. *Am J Pathol,* 94**,** 529-38.

JAURAND, M. C., RENIER, A. & DAUBRIAC, J. 2009. Mesothelioma: Do asbestos and carbon nanotubes pose the same health risk? *Part Fibre Toxicol,* 6**,** 16.

JENKINSON, S. R., INTLEKOFER, A. M., SUN, G., FEIGENBAUM, L., REINER, S. L. & BOSSELUT, R. 2007. Expression of the transcription factor cKrox in peripheral CD8 T cells reveals substantial postthymic plasticity in CD4-CD8 lineage differentiation. *The Journal of experimental medicine,* 204**,** 267-272.

JIANG, T., ZHOU, C. & REN, S. 2016. Role of IL-2 in cancer immunotherapy. *Oncoimmunology,* 5**,** e1163462.

JIANG, X., CLARK, R. A., LIU, L., WAGERS, A. J., FUHLBRIGGE, R. C. & KUPPER, T. S. 2012. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature,* 483**,** 227-31.

JOFRA, T., DI FONTE, R., EDGAR HUTCHINSON, T., DASTMALCHI, F., GALVANI, G., BATTAGLIA, M., SALEK-ARDAKANI, S. & FOUSTERI, G. 2016. *Protein tyrosine phosphatase PTPN22 has dual roles in promoting pathogen versus homeostatic-driven CD8 T-cell responses*.

JOHNSTON, ROBERT J., COMPS-AGRAR, L., HACKNEY, J., YU, X., HUSENI, M., YANG, Y., PARK, S., JAVINAL, V., CHIU, H., IRVING, B., EATON, DAN L. & GROGAN, JANE L. 2014. The Immunoreceptor TIGIT Regulates Antitumor and Antiviral CD8+ T Cell Effector Function. *Cancer Cell,* 26**,** 923-937.

JOSHI, N. S., CUI, W., CHANDELE, A., LEE, H. K., URSO, D. R., HAGMAN, J., GAPIN, L. & KAECH, S. M. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity,* 27**,** 281-95.

KABASHIMA, K., SAJI, T., MURATA, T., NAGAMACHI, M., MATSUOKA, T., SEGI, E., TSUBOI, K., SUGIMOTO, Y., KOBAYASHI, T., MIYACHI, Y., ICHIKAWA, A. & NARUMIYA, S. 2002. The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *The Journal of clinical investigation,* 109**,** 883-893.

KAECH, S. M. & CUI, W. 2012. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat Rev Immunol,* 12**,** 749-61.

KAECH, S. M., HEMBY, S., KERSH, E. & AHMED, R. 2002a. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell,* 111**,** 837-51.

KAECH, S. M., TAN, J. T., WHERRY, E. J., KONIECZNY, B. T., SURH, C. D. & AHMED, R. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol,* 4**,** 1191-8.

KAECH, S. M., WHERRY, E. J. & AHMED, R. 2002b. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol,* 2**,** 251-62.

KAHAN, S., BAKSHI, R., LUTHER, R., HARRINGTON, L., WEAVER, C. & ZAJAC, A. 2015. IL-2 production by activated naïve and effector CD8 T cells forecasts memory formation. (LYM5P.700). *The Journal of Immunology,* 194**,** 134.5.

KAO, S. C., PAVLAKIS, N., HARVIE, R., VARDY, J. L., BOYER, M. J., VAN ZANDWIJK, N. & CLARKE, S. J. 2010. High blood neutrophil-to-lymphocyte ratio is an indicator of poor prognosis in malignant mesothelioma patients undergoing systemic therapy. *Clin Cancer Res,* 16**,** 5805-13.

KASSIOTIS, G., GARCIA, S., SIMPSON, E. & STOCKINGER, B. 2002. Impairment of immunological memory in the absence of MHC despite survival of memory T cells. *Nat Immunol,* 3**,** 244-50.

KATAKURA, T., MIYAZAKI, M., KOBAYASHI, M., HERNDON, D. N. & SUZUKI, F. 2004. CCL17 and IL-10 as Effectors That Enable Alternatively Activated Macrophages to Inhibit the Generation of Classically Activated Macrophages. *The Journal of Immunology,* 172**,** 1407.

KEMP, R. A. & RONCHESE, F. 2001. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. *J Immunol,* 167**,** 6497-502.

KIM, M. T. & HARTY, J. T. 2014. Impact of Inflammatory Cytokines on Effector and Memory CD8+ T Cells. *Front Immunol,* 5**,** 295.

KITANO, M., MORIYAMA, S., ANDO, Y., HIKIDA, M., MORI, Y., KUROSAKI, T. & OKADA, T. 2011. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity,* 34**,** 961-72.

KLEBANOFF, C. A., GATTINONI, L. & RESTIFO, N. P. 2006. CD8+ T-cell memory in tumor immunology and immunotherapy. *Immunol Rev,* 211**,** 214-24.

KOBOLD, S., VOLK, S., CLAUDITZ, T., KUPPER, N. J., MINNER, S., TUFMAN, A., DUWELL, P., LINDNER, M., KOCH, I., HEIDEGGER, S., ROTHENFUER, S., SCHNURR, M., HUBER, R. M., WILCZAK, W. & ENDRES, S. 2013. Interleukin-22 is frequently expressed in small- and large-cell lung cancer and promotes growth in chemotherapy-resistant cancer cells. *J Thorac Oncol,* 8**,** 1032-42.

KREBS, P., BARNES, M. J., LAMPE, K., WHITLEY, K., BAHJAT, K. S., BEUTLER, B., JANSSEN, E. & HOEBE, K. 2009. NK-cell-mediated killing of target cells triggers robust antigen-specific T-cell-mediated and humoral responses. *Blood,* 113**,** 6593-602.

KREITER, S., VORMEHR, M., VAN DE ROEMER, N., DIKEN, M., LOWER, M., DIEKMANN, J., BOEGEL, S., SCHRORS, B., VASCOTTO, F., CASTLE, J. C., TADMOR, A. D., SCHOENBERGER, S. P., HUBER, C., TURECI, O. & SAHIN, U. 2015. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature,* 520**,** 692-6.

KRYCZEK, I., BANERJEE, M., CHENG, P., VATAN, L., SZELIGA, W., WEI, S., HUANG, E., FINLAYSON, E., SIMEONE, D., WELLING, T. H., CHANG, A., COUKOS, G., LIU, R. &

ZOU, W. 2009. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood,* 114**,** 1141-9.

KRYCZEK, I., ZOU, L., RODRIGUEZ, P., ZHU, G., WEI, S., MOTTRAM, P., BRUMLIK, M., CHENG, P., CURIEL, T., MYERS, L., LACKNER, A., ALVAREZ, X., OCHOA, A., CHEN, L. & ZOU, W. 2006. B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. *J Exp Med,* 203**,** 871-81.

KUANG, D. M., ZHAO, Q., PENG, C., XU, J., ZHANG, J. P., WU, C. & ZHENG, L. 2009. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med,* 206**,** 1327-37.

KUMAGAI-TAKEI, N., YAMAMOTO, S., LEE, S., MAEDA, M., MASUZZAKI, H., SADA, N., YU, M., YOSHITOME, K., NISHIMURA, Y. & OTSUKI, T. 2018. Inflammatory Alteration of Human T Cells Exposed Continuously to Asbestos. *International Journal of Molecular Sciences,* 19**,** 504.

KUMAR, B. V., MA, W., MIRON, M., GRANOT, T., GUYER, R. S., CARPENTER, D. J., SENDA, T., SUN, X., HO, S.-H., LERNER, H., FRIEDMAN, A. L., SHEN, Y. & FARBER, D. L. 2017. Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. *Cell Reports,* 20**,** 2921-2934.

KURACHI, M., BARNITZ, R. A., YOSEF, N., ODORIZZI, P. M., DIIORIO, M. A., LEMIEUX, M. E., YATES, K., GODEC, J., KLATT, M. G., REGEV, A., WHERRY, E. J. & HAINING, W. N. 2014. The transcription factor BATF operates as an essential differentiation checkpoint in early effector CD8+ T cells. *Nature immunology,* 15**,** 373-383.

LANGOWSKI, J. L., ZHANG, X., WU, L., MATTSON, J. D., CHEN, T., SMITH, K., BASHAM, B., MCCLANAHAN, T., KASTELEIN, R. A. & OFT, M. 2006. IL-23 promotes tumour incidence and growth. *Nature,* 442**,** 461-5.

LAZAREVIC, V., GLIMCHER, L. H. & LORD, G. M. 2013. T-bet: a bridge between innate and adaptive immunity. *Nat Rev Immunol,* 13**,** 777-89.

LEBIEN, T. W. & TEDDER, T. F. 2008. B lymphocytes: how they develop and function. *Blood,* 112**,** 1570-80.

LEDERMAN, S., YELLIN, M. J., INGHIRAMI, G., LEE, J. J., KNOWLES, D. M. & CHESS, L. 1992. Molecular interactions mediating T-B lymphocyte collaboration in human lymphoid follicles. Roles of T cell-B-cell-activating molecule (5c8 antigen) and CD40 in contact-dependent help. *J Immunol,* 149**,** 3817-26.

LEE, H. M., BAUTISTA, J. L. & HSIEH, C. S. 2011. Thymic and peripheral differentiation of regulatory T cells. *Adv Immunol,* 112**,** 25-71.

LEE, J., AOKI, T., THUMKEO, D., SIRIWACH, R., YAO, C. & NARUMIYA, S. 2019. T cell-intrinsic prostaglandin E(2)-EP2/EP4 signaling is critical in pathogenic T(H)17 cell-driven inflammation. *The Journal of allergy and clinical immunology,* 143**,** 631-643.

LEGAT, A., SPEISER, D. E., PIRCHER, H., ZEHN, D. & FUERTES MARRACO, S. A. 2013. Inhibitory Receptor Expression Depends More Dominantly on Differentiation and Activation than "Exhaustion" of Human CD8 T Cells. *Front Immunol,* 4**,** 455.

LEI, Y. & TAKAHAMA, Y. 2012. XCL1 and XCR1 in the immune system. *Microbes Infect,* 14**,** 262-7.

LI, Y., STRICK-MARCHAND, H., LIM, A. I., REN, J., MASSE-RANSON, G., DAN, L., JOUVION, G., ROGGE, L., LUCAS, S., BIN, L. & DI SANTO, J. P. 2017. Regulatory T cells control toxicity in a humanized model of IL-2 therapy. *Nat Commun,* 8**,** 1762.

LI, Y., WANG, H., LU, H. & HUA, S. 2016. *Regulation of Memory T Cells by Interleukin-23*.

LIEBERMAN, J. 2003. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol,* 3**,** 361-70.

LIEVENSE, L. A., BEZEMER, K., AERTS, J. G. & HEGMANS, J. P. 2013. Tumor-associated macrophages in thoracic malignancies. *Lung Cancer,* 80**,** 256-62.

LIEVENSE, L. A., BEZEMER, K., CORNELISSEN, R., KAIJEN-LAMBERS, M. E., HEGMANS, J. P. & AERTS, J. G. 2017. Precision immunotherapy; dynamics in the cellular profile of pleural effusions in malignant mesothelioma patients. *Lung Cancer,* 107**,** 36-40.

LITTMAN, D. R. & RUDENSKY, A. Y. 2010. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell,* 140**,** 845-58.

LIU, W., ERNST, J. D. & BROADDUS, V. C. 2000. Phagocytosis of crocidolite asbestos induces oxidative stress, DNA damage, and apoptosis in mesothelial cells. *Am J Respir Cell Mol Biol,* 23**,** 371-8.

LU, Y., HONG, B., LI, H., ZHENG, Y., ZHANG, M., WANG, S., QIAN, J. & YI, Q. 2014. Tumor-specific IL-9-producing CD8+ Tc9 cells are superior effector than type-I cytotoxic Tc1 cells for adoptive immunotherapy of cancers. *Proc Natl Acad Sci U S A,* 111**,** 2265-70.

LU, Y. C. & ROBBINS, P. F. 2016. Cancer immunotherapy targeting neoantigens. *Semin Immunol,* 28**,** 22-7.

LUCKHEERAM, R. V., ZHOU, R., VERMA, A. D. & XIA, B. 2012. CD4(+)T cells: differentiation and functions. *Clin Dev Immunol,* 2012**,** 925135.

LUND, F. E. 2008. Cytokine-producing B lymphocytes-key regulators of immunity. *Curr Opin Immunol,* 20**,** 332-8.

LUND, F. E. & RANDALL, T. D. 2010. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat Rev Immunol,* 10**,** 236-47.

MA, C. S., DEENICK, E. K., BATTEN, M. & TANGYE, S. G. 2012. The origins, function, and regulation of T follicular helper cells. *The Journal of Experimental Medicine,* 209**,** 1241-1253.

MACLEOD, M. K., CLAMBEY, E. T., KAPPLER, J. W. & MARRACK, P. 2009. CD4 memory T cells: what are they and what can they do? *Semin Immunol,* 21**,** 53-61.

MAEDA, M., NISHIMURA, Y., HAYASHI, H., KUMAGAI, N., CHEN, Y., MURAKAMI, S., MIURA, Y., HIRATSUKA, J., KISHIMOTO, T. & OTSUKI, T. 2011. Reduction of CXC chemokine receptor 3 in an in vitro model of continuous exposure to asbestos in a human T-cell line, MT-2. *Am J Respir Cell Mol Biol,* 45**,** 470-9.

MAEDA, Y., NISHIKAWA, H., SUGIYAMA, D., HA, D., HAMAGUCHI, M., SAITO, T., NISHIOKA, M., WING, J. B., ADEEGBE, D., KATAYAMA, I. & SAKAGUCHI, S. 2014. Detection of self-reactive CD8(+) T cells with an anergic phenotype in healthy individuals. *Science,* 346**,** 1536-40.

MAHBUB, S., DEBURGHGRAEVE, C. R. & KOVACS, E. J. 2012. Advanced age impairs macrophage polarization. *J Interferon Cytokine Res,* 32**,** 18-26.

MAHER, J. & DAVIES, E. T. 2004. Targeting cytotoxic T lymphocytes for cancer immunotherapy. *British Journal of Cancer,* 91**,** 817-821.

MAIO, M., SCHERPEREEL, A., CALABRO, L., AERTS, J., CEDRES PEREZ, S., BEARZ, A., NACKAERTS, K., FENNELL, D. A., KOWALSKI, D., TSAO, A. S., TAYLOR, P., GROSSO, F., ANTONIA, S. J., NOWAK, A. K., TABOADA, M., PUGLISI, M., STOCKMAN, P. K. & KINDLER, H. L. 2017. Tremelimumab as second-line or third-line treatment in relapsed malignant mesothelioma (DETERMINE): a multicentre, international, randomised, double-blind, placebo-controlled phase 2b trial. *Lancet Oncol,* 18**,** 1261-1273.

MALYSHKINA, A., LITTWITZ-SALOMON, E., SUTTER, K., ZELINSKYY, G., WINDMANN, S., SCHIMMER, S., PASCHEN, A., STREECK, H., HASENKRUG, K. J. & DITTMER, U. 2017. Fas Ligand-mediated cytotoxicity of CD4+ T cells during chronic retrovirus infection. *Scientific Reports,* 7**,** 7785.

MANSFIELD, A. S., RODEN, A. C., PEIKERT, T., SHEININ, Y. M., HARRINGTON, S. M., KRCO, C. J., DONG, H. & KWON, E. D. 2014. B7-H1 expression in malignant pleural mesothelioma is associated with sarcomatoid histology and poor prognosis. *J Thorac Oncol,* 9**,** 1036-40.

MANTOVANI, A. 2014. Macrophages, Neutrophils, and Cancer: A Double Edged Sword. *New Journal of Science,* 2014**,** 14.

MARCQ, E., SIOZOPOULOU, V., DE WAELE, J., VAN AUDENAERDE, J., ZWAENEPOEL, K., SANTERMANS, E., HENS, N., PAUWELS, P., VAN MEERBEECK, J. P. & SMITS, E. L. J. 2017a. Prognostic and predictive aspects of the tumor immune microenvironment and immune checkpoints in malignant pleural mesothelioma. *Oncoimmunology,* 6**,** e1261241.

MARCQ, E., WAELE, J., AUDENAERDE, J. V., LION, E., SANTERMANS, E., HENS, N., PAUWELS, P., VAN MEERBEECK, J. P. & SMITS, E. L. J. 2017b. Abundant expression of TIM-3, LAG-3, PD-1 and PD-L1 as immunotherapy checkpoint targets in effusions of mesothelioma patients. *Oncotarget,* 8**,** 89722-89735.

MARTINS, G. A., CIMMINO, L., LIAO, J., MAGNUSDOTTIR, E. & CALAME, K. 2008. Blimp-1 directly represses Il2 and the Il2 activator Fos, attenuating T cell proliferation and survival. *The Journal of experimental medicine,* 205**,** 1959-1965.

MARZO, A. L., LAKE, R. A., LO, D., SHERMAN, L., MCWILLIAM, A., NELSON, D., ROBINSON, B. W. & SCOTT, B. 1999. Tumor antigens are constitutively presented in the draining lymph nodes. *J Immunol,* 162**,** 5838-45.

MAURI, C. & EHRENSTEIN, M. R. 2008. The 'short' history of regulatory B cells. *Trends Immunol,* 29**,** 34-40.

MCLOUGHLIN, K. C., KAUFMAN, A. S. & SCHRUMP, D. S. 2017. Targeting the epigenome in malignant pleural mesothelioma. *Transl Lung Cancer Res,* 6**,** 350-365.

MCNERNEY, M. E., GUZIOR, D. & KUMAR, V. 2005. 2B4 (CD244)-CD48 interactions provide a novel MHC class I-independent system for NK-cell self-tolerance in mice. *Blood,* 106**,** 1337-1340.

MELAIU, O., GEMIGNANI, F. & LANDI, S. 2018. The genetic susceptibility in the development of malignant pleural mesothelioma. *J Thorac Dis,* 10**,** S246-s252.

MEYER, P. C. 1966. Metastatic carcinoma of the pleura. *Thorax,* 21**,** 437-43.

MIAO, T., SYMONDS, A. L. J., SINGH, R., SYMONDS, J. D., OGBE, A., OMODHO, B., ZHU, B., LI, S. & WANG, P. 2017. Egr2 and 3 control adaptive immune responses by temporally uncoupling expansion from T cell differentiation. *The Journal of Experimental Medicine,* 214**,** 1787.

MITTRUCKER, H. W., VISEKRUNA, A. & HUBER, M. 2014. Heterogeneity in the differentiation and function of CD8(+) T cells. *Arch Immunol Ther Exp (Warsz),* 62**,** 449-58.

MONNET, I., BREAU, J. L., MORO, D., LENA, H., EYMARD, J. C., MENARD, O., VUILLEZ, J. P., CHOKRI, M., ROMET-LEMONNE, J. L. & LOPEZ, M. 2002. Intrapleural infusion of activated macrophages and gamma-interferon in malignant pleural mesothelioma: a phase II study. *Chest,* 121**,** 1921-7.

MORVAN, M. G. & LANIER, L. L. 2016. NK cells and cancer: you can teach innate cells new tricks. *Nat Rev Cancer,* 16**,** 7-19.

MOSSMAN, B. T., EASTMAN, A. & BRESNICK, E. 1984. Asbestos and benzo[a]pyrene act synergistically to induce squamous metaplasia and incorporation of [3H]thymidine in hamster tracheal epithelium. *Carcinogenesis,* 5**,** 1401-4.

MOSSMAN, B. T., LIPPMANN, M., HESTERBERG, T. W., KELSEY, K. T., BARCHOWSKY, A. & BONNER, J. C. 2011. Pulmonary endpoints (lung carcinomas and asbestosis) following inhalation exposure to asbestos. *J Toxicol Environ Health B Crit Rev,* 14**,** 76-121.

MOSSMAN, B. T., SHUKLA, A., HEINTZ, N. H., VERSCHRAEGEN, C. F., THOMAS, A. & HASSAN, R. 2013. New insights into understanding the mechanisms, pathogenesis, and management of malignant mesotheliomas. *Am J Pathol,* 182**,** 1065-77.

MUELLER, S. N., GEBHARDT, T., CARBONE, F. R. & HEATH, W. R. 2013. Memory T cell subsets, migration patterns, and tissue residence. *Annu Rev Immunol,* 31**,** 137-61.

MURANSKI, P. & RESTIFO, N. P. 2013. Essentials of Th17 cell commitment and plasticity. *Blood,* 121**,** 2402-14.

MURATA, T., TAGUCHI, J., PURI, R. K. & MOHRI, H. 1999. Sharing of receptor subunits and signal transduction pathway between the IL-4 and IL-13 receptor system. *Int J Hematol,* 69**,** 13-20.

MURUGAIYAN, G., BEYNON, V., PIRES DA CUNHA, A., JOLLER, N. & WEINER, H. L. 2012. IFN-γ Limits Th9-Mediated Autoimmune Inflammation through Dendritic Cell Modulation of IL-27. *The Journal of Immunology,* 189**,** 5277.

MUTSAERS, S. E. 2002. Mesothelial cells: their structure, function and role in serosal repair. *Respirology,* 7**,** 171-91.

MUTTI, L., VALLE, M. T., BALBI, B., ORENGO, A. M., LAZZARO, A., ALCIATO, P., GATTI, E., BETTA, P. G. & POZZI, E. 1998. Primary human mesothelioma cells express class II MHC, ICAM-1 and B7-2 and can present recall antigens to autologous blood lymphocytes. *Int J Cancer,* 78**,** 740-9.

NAGAI, H., ISHIHARA, T., LEE, W. H., OHARA, H., OKAZAKI, Y., OKAWA, K. & TOYOKUNI, S. 2011. Asbestos surface provides a niche for oxidative modification. *Cancer Sci,* 102**,** 2118-25.

NAITO, T. & TANIUCHI, I. 2010. The network of transcription factors that underlie the CD4 versus CD8 lineage decision. *Int Immunol,* 22**,** 791-6.

NAKANO, O., SATO, M., NAITO, Y., SUZUKI, K., ORIKASA, S., AIZAWA, M., SUZUKI, Y., SHINTAKU, I., NAGURA, H. & OHTANI, H. 2001. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res,* 61**,** 5132-6.

NAKATA, Y., BRIGNIER, A. C., JIN, S., SHEN, Y., RUDNICK, S. I., SUGITA, M. & GEWIRTZ, A. M. 2010. c-Myb, Menin, GATA-3, and MLL form a dynamic transcription complex that plays a pivotal role in human T helper type 2 cell development. *Blood,* 116**,** 1280-1290.

NICKOLOFF, B. J. & TURKA, L. A. 1994. Immunological functions of non-professional antigen-presenting cells: new insights from studies of T-cell interactions with keratinocytes. *Immunol Today,* 15**,** 464-9.

NIRSCHL, C. J. & DRAKE, C. G. 2013. Molecular pathways: coexpression of immune checkpoint molecules: signaling pathways and implications for cancer immunotherapy. *Clin Cancer Res,* 19**,** 4917-24.

NISHIKAWA, H., KATO, T., TAWARA, I., SAITO, K., IKEDA, H., KURIBAYASHI, K., ALLEN, P. M., SCHREIBER, R. D., SAKAGUCHI, S., OLD, L. J. & SHIKU, H. 2005. Definition of target antigens for naturally occurring CD4(+) CD25(+) regulatory T cells. *J Exp Med,* 201**,** 681-6.

NISHIMURA, Y., KUMAGAI-TAKEI, N., MATSUZAKI, H., LEE, S., MAEDA, M., KISHIMOTO, T., FUKUOKA, K., NAKANO, T. & OTSUKI, T. 2015. Functional Alteration of Natural Killer Cells and Cytotoxic T Lymphocytes upon Asbestos Exposure and in Malignant Mesothelioma Patients. *Biomed Res Int,* 2015**,** 238431.

NOLZ, J. C. & HARTY, J. T. 2014. IL-15 regulates memory CD8+ T cell O-glycan synthesis and affects trafficking. *J Clin Invest,* 124**,** 1013-26.

NOLZ, J. C., STARBECK-MILLER, G. R. & HARTY, J. T. 2011. Naive, effector and memory CD8 T-cell trafficking: parallels and distinctions. *Immunotherapy,* 3**,** 1223-33.

NOY, R. & POLLARD, J. W. 2014. Tumor-associated macrophages: from mechanisms to therapy. *Immunity,* 41**,** 49-61.

NURIEVA, O., HUBACEK, J. A., URBAN, P., HLUSICKA, J., DIBLIK, P., KUTHAN, P., SKLENKA, P., MELISKA, M., BYDZOVSKY, J., HEISSIGEROVA, J., KOTIKOVA, K., NAVRATIL, T., KOMARC, M., SEIDL, Z., VANECKOVA, M., VOJTOVA, L. & ZAKHAROV, S. 2019. Clinical and genetic determinants of chronic visual pathway changes after methanol - induced optic neuropathy: four-year follow-up study. *Clin Toxicol (Phila),* 57**,** 387-397.

NURIEVA, R. I. & CHUNG, Y. 2010. Understanding the development and function of T follicular helper cells. *Cell Mol Immunol,* 7**,** 190-7.

NURIEVA, R. I., CHUNG, Y., HWANG, D., YANG, X. O., KANG, H. S., MA, L., WANG, Y. H., WATOWICH, S. S., JETTEN, A. M., TIAN, Q. & DONG, C. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity,* 29**,** 138-49.

OHMATSU, H., SUGAYA, M., KADONO, T. & TAMAKI, K. 2007. CXCL13 and CCL21 are expressed in ectopic lymphoid follicles in cutaneous lymphoproliferative disorders. *J Invest Dermatol,* 127**,** 2466-8.

OLESIN, E., NAYAR, R., SAIKUMAR-LAKSHMI, P. & BERG, L. J. 2018. The Transcription Factor Runx2 Is Required for Long-Term Persistence of Antiviral CD8(+) Memory T Cells. *ImmunoHorizons,* 2**,** 251-261.

OMILUSIK, K. D., BEST, J. A., YU, B., GOOSSENS, S., WEIDEMANN, A., NGUYEN, J. V., SEUNTJENS, E., STRYJEWSKA, A., ZWEIER, C., ROYCHOUDHURI, R., GATTINONI, L., BIRD, L. M., HIGASHI, Y., KONDOH, H., HUYLEBROECK, D., HAIGH, J. & GOLDRATH, A. W. 2015. Transcriptional repressor ZEB2 promotes terminal differentiation of CD8⁺ effector and memory T cell populations during infection. *The Journal of Experimental Medicine,* 212**,** 2027.

ORANGE, J. S. 2013. Natural killer cell deficiency. *J Allergy Clin Immunol,* 132**,** 515-25; quiz 526.

ORDWAY, D., HIGGINS, D. M., SANCHEZ-CAMPILLO, J., SPENCER, J. S., HENAO-TAMAYO, M., HARTON, M., ORME, I. M. & GONZALEZ JUARRERO, M. 2007. XCL1 (lymphotactin) chemokine produced by activated CD8 T cells during the chronic stage of infection with Mycobacterium tuberculosis negatively affects production of IFN-γ by CD4 T cells and participates in granuloma stability. *Journal of Leukocyte Biology,* 82**,** 1221-1229.

OWEN, J. L. & MOHAMADZADEH, M. 2013. Macrophages and chemokines as mediators of angiogenesis. *Front Physiol,* 4**,** 159.

PARK, C. & KUPPER, T. S. 2015. THE EMERGING ROLE OF RESIDENT MEMORY T CELLS IN PROTECTIVE IMMUNITY AND INFLAMMATORY DISEASE. *Nature medicine,* 21**,** 688-697.

PAUL, W. E. & ZHU, J. 2010. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol,* 10**,** 225-35.

PEI, G., BUIJZE, H., LIU, H., MOURA-ALVES, P., GOOSMANN, C., BRINKMANN, V., KAWABE, H., DORHOI, A. & KAUFMANN, S. H. E. 2017. The E3 ubiquitin ligase NEDD4 enhances killing of membrane-perturbing intracellular bacteria by promoting autophagy. *Autophagy,* 13**,** 2041-2055.

PIET, B., DE BREE, G. J., SMIDS-DIERDORP, B. S., VAN DER LOOS, C. M., REMMERSWAAL, E. B. M., VON DER THÜSEN, J. H., VAN HAARST, J. M. W., EERENBERG, J. P., TEN BRINKE, A., VAN DER BIJ, W., TIMENS, W., VAN LIER, R. A. W. & JONKERS, R. E. 2011. CD8(+) T cells with an intraepithelial phenotype upregulate cytotoxic function upon influenza infection in human lung. *The Journal of Clinical Investigation,* 121**,** 2254-2263.

PIPKIN, M. E., SACKS, J. A., CRUZ-GUILLOTY, F., LICHTENHELD, M. G., BEVAN, M. J. & RAO, A. 2010. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity,* 32**,** 79-90.

POST, M., CUAPIO, A., OSL, M., LEHMANN, D., RESCH, U., DAVIES, D. M., BILBAN, M., SCHLECHTA, B., EPPEL, W., NATHWANI, A., STOIBER, D., SPANHOLTZ, J., CASANOVA, E. & HOFER, E. 2017. The Transcription Factor ZNF683/HOBIT Regulates Human NK-Cell Development. *Frontiers in Immunology,* 8.

POULIKAKOS, P. I., XIAO, G. H., GALLAGHER, R., JABLONSKI, S., JHANWAR, S. C. & TESTA, J. R. 2006. Re-expression of the tumor suppressor NF2/merlin inhibits invasiveness in mesothelioma cells and negatively regulates FAK. *Oncogene,* 25**,** 5960-8.

PRAJAPATI, K., PEREZ, C., ROJAS, L. B. P., BURKE, B. & GUEVARA-PATINO, J. A. 2018. Functions of NKG2D in CD8(+) T cells: an opportunity for immunotherapy. *Cellular & molecular immunology,* 15**,** 470-479.

PRIATEL, J. J., CHEN, X., HUANG, Y.-H., CHOW, M. T., ZENEWICZ, L. A., COUGHLIN, J. J., SHEN, H., STONE, J. C., TAN, R. & TEH, H. S. 2010. RasGRP1 Regulates Antigen-Induced Developmental Programming by Naive CD8 T Cells. *The Journal of Immunology,* 184**,** 666.

PROSS, H. F. & LOTZOVA, E. 1993. Role of natural killer cells in cancer. *Nat Immun,* 12**,** 279-92.

PULFORD, E., HUILGOL, K., MOFFAT, D., HENDERSON, D. W. & KLEBE, S. 2017. Malignant Mesothelioma, BAP1 Immunohistochemistry, and VEGFA: Does BAP1 Have Potential for Early Diagnosis and Assessment of Prognosis? *Dis Markers,* 2017**,** 1310478.

PULKO, V., HARRIS, K. J., LIU, X., GIBBONS, R. M., HARRINGTON, S. M., KRCO, C. J., KWON, E. D. & DONG, H. 2011. B7-h1 expressed by activated CD8 T cells is essential for their survival. *Journal of immunology (Baltimore, Md. : 1950),* 187**,** 5606-5614.

PURWAR, R., SCHLAPBACH, C., XIAO, S., KANG, H. S., ELYAMAN, W., JIANG, X., JETTEN, A. M., KHOURY, S. J., FUHLBRIGGE, R. C., KUCHROO, V. K., CLARK, R. A. & KUPPER, T. S. 2012. Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. *Nat Med,* 18**,** 1248-53.

QI, H. 2016. T follicular helper cells in space-time. *Nat Rev Immunol,* 16**,** 612-25.

QIAN, B. Z. & POLLARD, J. W. 2010. Macrophage diversity enhances tumor progression and metastasis. *Cell,* 141**,** 39-51.

QIN, L., WASEEM, T. C., SAHOO, A., BIEERKEHAZHI, S., ZHOU, H., GALKINA, E. V. & NURIEVA, R. 2018. Insights Into the Molecular Mechanisms of T Follicular Helper-Mediated Immunity and Pathology. *Front Immunol,* 9**,** 1884.

QUEZADA, S. A., SIMPSON, T. R., PEGGS, K. S., MERGHOUB, T., VIDER, J., FAN, X., BLASBERG, R., YAGITA, H., MURANSKI, P., ANTONY, P. A., RESTIFO, N. P. & ALLISON, J. P. 2010. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med,* 207**,** 637-50.

RAO, D. A., GURISH, M. F., MARSHALL, J. L., SLOWIKOWSKI, K., FONSEKA, C. Y., LIU, Y., DONLIN, L. T., HENDERSON, L. A., WEI, K., MIZOGUCHI, F., TESLOVICH, N. C., WEINBLATT, M. E., MASSAROTTI, E. M., COBLYN, J. S., HELFGOTT, S. M., LEE, Y. C., TODD, D. J., BYKERK, V. P., GOODMAN, S. M., PERNIS, A. B., IVASHKIV, L. B., KARLSON, E. W., NIGROVIC, P. A., FILER, A., BUCKLEY, C. D., LEDERER, J. A., RAYCHAUDHURI, S. & BRENNER, M. B. 2017. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature,* 542**,** 110-114.

RAULET, D. H. & GUERRA, N. 2009. Oncogenic stress sensed by the immune system: role of natural killer cell receptors. *Nat Rev Immunol,* 9**,** 568-80.

RECH, A. J., MICK, R., KAPLAN, D. E., CHANG, K. M., DOMCHEK, S. M. & VONDERHEIDE, R. H. 2010. Homeostasis of peripheral FoxP3(+) CD4 (+) regulatory T cells in patients with early and late stage breast cancer. *Cancer Immunol Immunother,* 59**,** 599-607.

REISER, J. & BANERJEE, A. 2016. Effector, Memory, and Dysfunctional CD8+ T Cell Fates in the Antitumor Immune Response. *Journal of Immunology Research,* 2016**,** 14.

REN, W., LIU, G., YIN, J., TAN, B., WU, G., BAZER, F. W., PENG, Y. & YIN, Y. 2017. Amino-acid transporters in T-cell activation and differentiation. *Cell Death Dis,* 8**,** e2655.

ROBBINS, S. H., BESSOU, G., CORNILLON, A., ZUCCHINI, N., RUPP, B., RUZSICS, Z., SACHER, T., TOMASELLO, E., VIVIER, E., KOSZINOWSKI, U. H. & DALOD, M. 2007. Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog,* 3**,** e123.

ROBERT, C., SCHACHTER, J., LONG, G. V., ARANCE, A., GROB, J. J., MORTIER, L., DAUD, A., CARLINO, M. S., MCNEIL, C., LOTEM, M., LARKIN, J., LORIGAN, P., NEYNS, B., BLANK, C. U., HAMID, O., MATEUS, C., SHAPIRA-FROMMER, R., KOSH, M., ZHOU, H., IBRAHIM, N., EBBINGHAUS, S. & RIBAS, A. 2015. Pembrolizumab versus Ipilimumab in Advanced Melanoma. *N Engl J Med,* 372**,** 2521-32.

ROBERTS, M. E., NEVILLE, E., BERRISFORD, R. G., ANTUNES, G. & ALI, N. J. 2010. Management of a malignant pleural effusion: British Thoracic Society Pleural Disease Guideline 2010. *Thorax,* 65 Suppl 2**,** ii32-40.

ROBINSON, B. W. & LAKE, R. A. 2005. Advances in malignant mesothelioma. *N Engl J Med,* 353**,** 1591-603.

RONCHETTI, S., NOCENTINI, G., PETRILLO, M. G. & RICCARDI, C. 2012. CD8+ T cells: GITR matters. *TheScientificWorldJournal,* 2012**,** 308265-308265.

RUFFELL, B., AFFARA, N. I. & COUSSENS, L. M. 2012. Differential macrophage programming in the tumor microenvironment. *Trends Immunol,* 33**,** 119-26.

RUFFELL, B., DENARDO, D. G., AFFARA, N. I. & COUSSENS, L. M. 2010. Lymphocytes in cancer development: polarization towards pro-tumor immunity. *Cytokine Growth Factor Rev,* 21**,** 3-10.

RUTISHAUSER, R. L., MARTINS, G. A., KALACHIKOV, S., CHANDELE, A., PARISH, I. A., MEFFRE, E., JACOB, J., CALAME, K. & KAECH, S. M. 2009. Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity,* 31**,** 296-308.

SAGE, A. P., MARTINEZ, V. D., MINATEL, B. C., PEWARCHUK, M. E., MARSHALL, E. A., MACAULAY, G. M., HUBAUX, R., PEARSON, D. D., GOODARZI, A. A., DELLAIRE, G. & LAM, W. L. 2018. Genomics and Epigenetics of Malignant Mesothelioma. *High Throughput,* 7.

SAHOO, A., WALI, S. & NURIEVA, R. 2016. T helper 2 and T follicular helper cells: Regulation and function of interleukin-4. *Cytokine & growth factor reviews,* 30**,** 29-37.

SAKAGUCHI, S., MIYARA, M., COSTANTINO, C. M. & HAFLER, D. A. 2010. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol,* 10**,** 490-500.

SAKAGUCHI, S., YAMAGUCHI, T., NOMURA, T. & ONO, M. 2008. Regulatory T cells and immune tolerance. *Cell,* 133**,** 775-87.

SALAMA, P., PHILLIPS, M., GRIEU, F., MORRIS, M., ZEPS, N., JOSEPH, D., PLATELL, C. & IACOPETTA, B. 2009. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol,* 27**,** 186-92.

SAMJI, T. & KHANNA, K. M. 2017. Understanding memory CD8(+) T cells. *Immunol Lett,* 185**,** 32-39.

SARVARIA, A., MADRIGAL, J. A. & SAUDEMONT, A. 2017. B cell regulation in cancer and anti-tumor immunity. *Cell Mol Immunol,* 14**,** 662-674.

SATHALIYAWALA, T., KUBOTA, M., YUDANIN, N., TURNER, D., CAMP, P., THOME, J. J., BICKHAM, K. L., LERNER, H., GOLDSTEIN, M., SYKES, M., KATO, T. & FARBER, D. L. 2013. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity,* 38**,** 187-97.

SATO, T. & SEKIDO, Y. 2018. NF2/Merlin Inactivation and Potential Therapeutic Targets in Mesothelioma. *Int J Mol Sci,* 19.

SAVAGE, P. A., LEVENTHAL, D. S. & MALCHOW, S. 2014. Shaping the repertoire of tumor-infiltrating effector and regulatory T cells. *Immunol Rev,* 259**,** 245-58.

SCHADENDORF, D., HODI, F. S., ROBERT, C., WEBER, J. S., MARGOLIN, K., HAMID, O., PATT, D., CHEN, T. T., BERMAN, D. M. & WOLCHOK, J. D. 2015. Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J Clin Oncol,* 33**,** 1889-94.

SCHAFER, M. & WERNER, S. 2008. Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Biol,* 9**,** 628-38.

SCHENKEL, J. M., FRASER, K. A., VEZYS, V. & MASOPUST, D. 2013. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol,* 14**,** 509-13.

SCHENKEL, JASON M. & MASOPUST, D. 2014. Tissue-Resident Memory T Cells. *Immunity,* 41**,** 886-897.

SCHERPEREEL, A., ASTOUL, P., BAAS, P., BERGHMANS, T., CLAYSON, H., DE VUYST, P., DIENEMANN, H., GALATEAU-SALLE, F., HENNEQUIN, C., HILLERDAL, G., LE PECHOUX, C., MUTTI, L., PAIRON, J. C., STAHEL, R., VAN HOUTTE, P., VAN MEERBEECK, J., WALLER, D. & WEDER, W. 2010. Guidelines of the European Respiratory Society and the European Society of Thoracic Surgeons for the management of malignant pleural mesothelioma. *Eur Respir J,* 35**,** 479-95.

SCHERPEREEL, A., GRIGORIU, B. D., NOPPEN, M., GEY, T., CHAHINE, B., BALDACCI, S., TRAUET, J., COPIN, M. C., DESSAINT, J. P., PORTE, H. & LABALETTE, M. 2013. Defect in recruiting effector memory CD8+ T-cells in malignant pleural effusions compared to normal pleural fluid. *BMC Cancer,* 13**,** 324.

SCHRAML, B. U., HILDNER, K., ISE, W., LEE, W.-L., SMITH, W. A. E., SOLOMON, B., SAHOTA, G., SIM, J., MUKASA, R., CEMERSKI, S., HATTON, R. D., STORMO, G. D., WEAVER, C. T., RUSSELL, J. H., MURPHY, T. L. & MURPHY, K. M. 2009. The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature,* 460**,** 405-409.

SCHREIBER, R. D., OLD, L. J. & SMYTH, M. J. 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science,* 331**,** 1565-70.

SEGAL, A., STERRETT, G. F., FROST, F. A., SHILKIN, K. B., OLSEN, N. J., MUSK, A. W., NOWAK, A. K., ROBINSON, B. W. & CREANEY, J. 2013. A diagnosis of malignant pleural mesothelioma can be made by effusion cytology: results of a 20 year audit. *Pathology,* 45**,** 44-8.

SEKIDO, Y. 2013. Molecular pathogenesis of malignant mesothelioma. *Carcinogenesis,* 34**,** 1413-9.

SETOGUCHI, R., TANIUCHI, I. & BEVAN, M. J. 2009. ThPOK derepression is required for robust CD8 T cell responses to viral infection. *Journal of immunology (Baltimore, Md. : 1950),* 183**,** 4467-4474.

SHAH, W., YAN, X., JING, L., ZHOU, Y., CHEN, H. & WANG, Y. 2011. A reversed CD4/CD8 ratio of tumor-infiltrating lymphocytes and a high percentage of CD4(+)FOXP3(+) regulatory T cells are significantly associated with clinical outcome in squamous cell carcinoma of the cervix. *Cell Mol Immunol,* 8**,** 59-66.

SHALAPOUR, S. & KARIN, M. 2015. Immunity, inflammation, and cancer: an eternal fight between good and evil. *J Clin Invest,* 125**,** 3347-55.

SHANG, B., LIU, Y., JIANG, S. J. & LIU, Y. 2015. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep,* 5**,** 15179.

SHARMA, S., YANG, S. C., ZHU, L., RECKAMP, K., GARDNER, B., BARATELLI, F., HUANG, M., BATRA, R. K. & DUBINETT, S. M. 2005. Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer. *Cancer Res,* 65**,** 5211-20.

SHEN, P. & FILLATREAU, S. 2015. Antibody-independent functions of B cells: a focus on cytokines. *Nat Rev Immunol,* 15**,** 441-51.

SHEVACH, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity,* 30**,** 636-45.

SHIN, H. & IWASAKI, A. 2013a. Tissue-resident memory T cells. *Immunol Rev,* 255**,** 165-81.

SHIN, H. & IWASAKI, A. 2013b. Tissue-resident memory T cells. *Immunological reviews,* 255**,** 165-181.

SHULMAN, Z., GITLIN, A. D., TARG, S., JANKOVIC, M., PASQUAL, G., NUSSENZWEIG, M. C. & VICTORA, G. D. 2013. T follicular helper cell dynamics in germinal centers. *Science,* 341**,** 673-7.

SICA, A. & MANTOVANI, A. 2012. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest,* 122**,** 787-95.

SICA, A., SACCANI, A., BOTTAZZI, B., POLENTARUTTI, N., VECCHI, A., VAN DAMME, J. & MANTOVANI, A. 2000. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. *J Immunol,* 164**,** 762-7.

SLUIS-CREMER, G. K., LIDDELL, F. D., LOGAN, W. P. & BEZUIDENHOUT, B. N. 1992. The mortality of amphibole miners in South Africa, 1946-80. *Br J Ind Med,* 49**,** 566-75.

SMYTH, M. J., CRETNEY, E., KELLY, J. M., WESTWOOD, J. A., STREET, S. E., YAGITA, H., TAKEDA, K., VAN DOMMELEN, S. L., DEGLI-ESPOSTI, M. A. & HAYAKAWA, Y. 2005. Activation of NK cell cytotoxicity. *Mol Immunol,* 42**,** 501-10.

SNEDDON, S., DICK, I., LEE, Y. C. G., MUSK, A. W. B., PATCH, A. M., PEARSON, J. V., WADDELL, N., ALLCOCK, R. J. N., HOLT, R. A., ROBINSON, B. W. S. & CREANEY, J. 2018. Malignant cells from pleural fluids in malignant mesothelioma patients reveal novel mutations. *Lung Cancer,* 119**,** 64-70.

SOOD, S., BROWNLIE, R. J., GARCIA, C., COWAN, G., SALMOND, R. J., SAKAGUCHI, S. & ZAMOYSKA, R. 2016. Loss of the Protein Tyrosine Phosphatase PTPN22 Reduces Mannan-Induced Autoimmune Arthritis in SKG Mice. *The Journal of Immunology***,** 1502656.

SPANER, D. & BAHLO, A. 2011. B Lymphocytes in Cancer Immunology. *In:* MEDIN, J. & FOWLER, D. (eds.) *Experimental and Applied Immunotherapy.* Totowa, NJ: Humana Press.

SPOLSKI, R. & LEONARD, W. J. 2008. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol,* 26**,** 57-79.

SPRENT, J. 1995. Antigen-presenting cells. Professionals and amateurs. *Curr Biol,* 5**,** 1095-7.

STANTON, M. F., LAYARD, M., TEGERIS, A., MILLER, E., MAY, M., MORGAN, E. & SMITH, A. 1981. Relation of particle dimension to carcinogenicity in amphibole asbestoses and other fibrous minerals. *J Natl Cancer Inst,* 67**,** 965-75.

STATHOPOULOS, G. T. & KALOMENIDIS, I. 2012. Malignant pleural effusion: tumor-host interactions unleashed. *Am J Respir Crit Care Med,* 186**,** 487-92.

STELEKATI, E., SHIN, H., DOERING, T. A., DOLFI, D. V., ZIEGLER, C. G., BEITING, D. P., DAWSON, L., LIBOON, J., WOLSKI, D., ALI, M. A., KATSIKIS, P. D., SHEN, H., ROOS, D. S., HAINING, W. N., LAUER, G. M. & WHERRY, E. J. 2014. Bystander chronic infection negatively impacts development of CD8(+) T cell memory. *Immunity,* 40**,** 801-13.

SZABO, S. J., SULLIVAN, B. M., PENG, S. L. & GLIMCHER, L. H. 2003. Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol,* 21**,** 713-58.

TAKEUCHI, A. & SAITO, T. 2017. CD4 CTL, a Cytotoxic Subset of CD4(+) T Cells, Their Differentiation and Function. *Frontiers in Immunology,* 8**,** 194.

TAKEUCHI, Y. & NISHIKAWA, H. 2016. Roles of regulatory T cells in cancer immunity. *Int Immunol,* 28**,** 401-9.

TAMASAUSKIENE, L. & SITKAUSKIENE, B. 2017. Role of Th22 and IL-22 in pathogenesis of allergic airway diseases: Pro-inflammatory or anti-inflammatory effect? *Pediatr Neonatol*.

TANG, Y., GUAN, S. P., CHUA, B. Y., ZHOU, Q., HO, A. W., WONG, K. H., WONG, K. L., WONG, W. S. & KEMENY, D. M. 2012. Antigen-specific effector CD8 T cells regulate allergic responses via IFN-gamma and dendritic cell function. *J Allergy Clin Immunol,* 129**,** 1611-20.e4.

TAY, N. Q., LEE, D. C. P., CHUA, Y. L., PRABHU, N., GASCOIGNE, N. R. J. & KEMENY, D. M. 2017. CD40L Expression Allows CD8(+) T Cells to Promote Their Own Expansion and Differentiation through Dendritic Cells. *Frontiers in immunology,* 8**,** 1484-1484.

TESTA, J. R., CHEUNG, M., PEI, J., BELOW, J. E., TAN, Y., SEMENTINO, E., COX, N. J., DOGAN, A. U., PASS, H. I., TRUSA, S., HESDORFFER, M., NASU, M., POWERS, A., RIVERA, Z., COMERTPAY, S., TANJI, M., GAUDINO, G., YANG, H. & CARBONE, M. 2011. Germline BAP1 mutations predispose to malignant mesothelioma. *Nat Genet,* 43**,** 1022-5.

THOMPSON, E. D., ENRIQUEZ, H. L., FU, Y.-X. & ENGELHARD, V. H. 2010. Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. *The Journal of Experimental Medicine,* 207**,** 1791.

TIAN, J., ZEKZER, D., HANSSEN, L., LU, Y., OLCOTT, A. & KAUFMAN, D. L. 2001. Lipopolysaccharide-activated B cells down-regulate Th1 immunity and

prevent autoimmune diabetes in nonobese diabetic mice. *J Immunol,* 167**,** 1081-9.

TOPHAM, N. J. & HEWITT, E. W. 2009. Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology,* 128**,** 7-15.

TOPLEY, N., LIBEREK, T., DAVENPORT, A., LI, F. K., FEAR, H. & WILLIAMS, J. D. 1996. Activation of inflammation and leukocyte recruitment into the peritoneal cavity. *Kidney Int Suppl,* 56**,** S17-21.

TSOU, P., KATAYAMA, H., OSTRIN, E. J. & HANASH, S. M. 2016. The Emerging Role of B Cells in Tumor Immunity. *Cancer Res,* 76**,** 5597-5601.

TURNER, D. L. & FARBER, D. L. 2014. Mucosal resident memory CD4 T cells in protection and immunopathology. *Front Immunol,* 5**,** 331.

UJIIE, H., KADOTA, K., NITADORI, J. I., AERTS, J. G., WOO, K. M., SIMA, C. S., TRAVIS, W. D., JONES, D. R., KRUG, L. M. & ADUSUMILLI, P. S. 2015. The tumoral and stromal immune microenvironment in malignant pleural mesothelioma: A comprehensive analysis reveals prognostic immune markers. *Oncoimmunology,* 4**,** e1009285.

URSO, L., CALABRESE, F., FAVARETTO, A., CONTE, P. & PASELLO, G. 2016. Critical review about MDM2 in cancer: Possible role in malignant mesothelioma and implications for treatment. *Crit Rev Oncol Hematol,* 97**,** 220-30.

VALLE M, T., DEGL'INNOCENTI M, L., BERTELLI, R., FACCHETTI, P., PERFUMO, F., FENOGLIO, D., KUNKL, A., GUSMANO, R. & MANCA, F. 1995. Antigen-presenting function of human peritoneum mesothelial cells. *Clinical & Experimental Immunology,* 101**,** 172-176.

VEGRAN, F., APETOH, L. & GHIRINGHELLI, F. 2015. Th9 cells: a novel CD4 T-cell subset in the immune war against cancer. *Cancer Res,* 75**,** 475-9.

VELLA, F. 1994. Molecular biology of the cell (third edition): By B Alberts, D Bray, J Lewis, M Raff, K Roberts and J D Watson. pp 1361. Garland Publishing, New York and London. 1994. *Biochemical Education,* 22**,** 164-164.

VERSTEEG, H. H., SCHAFFNER, F., KERVER, M., ELLIES, L. G., ANDRADE-GORDON, P., MUELLER, B. M. & RUF, W. 2008. Protease-activated receptor (PAR) 2, but not PAR1, signaling promotes the development of mammary adenocarcinoma in polyoma middle T mice. *Cancer Res,* 68**,** 7219-27.

VIEIRA BRAGA, F. A., HERTOGHS, K., KRAGTEN, N., DOODY, G., A BARNES, N., REMMERSWAAL, E., HSIAO, C.-C., D MOERLAND, P., WOUTERS, D., DERKS, I., VAN STIJN, A., DEMKES, M., HAMANN, J., ELDERING, E., NOLTE, M., TOOZE, R., J.M. TEN BERGE, I., VAN GISBERGEN, K. & VAN LIER, R. 2015. *The Blimp-1 homologue Hobit identifies effector-type lymphocytes in humans*.

VIJAYANAND, P., SEUMOIS, G., SIMPSON, L. J., ABDUL-WAJID, S., BAUMJOHANN, D., PANDURO, M., HUANG, X., INTERLANDI, J., DJURETIC, I. M., BROWN, D. R., SHARPE, A. H., RAO, A. & ANSEL, K. M. 2012. Interleukin-4 production by Follicular Helper T cells requires the conserved Il4 enhancer HS V. *Immunity,* 36**,** 175-187.

VIVIER, E., NUNES, J. A. & VELY, F. 2004. Natural killer cell signaling pathways. *Science,* 306**,** 1517-9.

VIVIER, E., RAULET, D. H., MORETTA, A., CALIGIURI, M. A., ZITVOGEL, L., LANIER, L. L., YOKOYAMA, W. M. & UGOLINI, S. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science,* 331**,** 44-9.

VOSKOBOINIK, I., SMYTH, M. J. & TRAPANI, J. A. 2006. Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol,* 6**,** 940-52.

WAGLE, M. V., MARCHINGO, J. M., HOWITT, J., TAN, S.-S., GOODNOW, C. C. & PARISH, I. A. 2018. The Ubiquitin Ligase Adaptor NDFIP1 Selectively Enforces a CD8(+) T Cell Tolerance Checkpoint to High-Dose Antigen. *Cell reports,* 24**,** 577-584.

WALDHAUER, I. & STEINLE, A. 2008. NK cells and cancer immunosurveillance. *Oncogene,* 27**,** 5932-43.

WALKER, W. E. & GOLDSTEIN, D. R. 2007. Neonatal B cells suppress innate toll-like receptor immune responses and modulate alloimmunity. *J Immunol,* 179**,** 1700-10.

WALL, L., BURKE, F., BARTON, C., SMYTH, J. & BALKWILL, F. 2003. IFN-gamma induces apoptosis in ovarian cancer cells in vivo and in vitro. *Clin Cancer Res,* 9**,** 2487-96.

WALLIN, R. P., SCREPANTI, V., MICHAELSSON, J., GRANDIEN, A. & LJUNGGREN, H. G. 2003. Regulation of perforin-independent NK cell-mediated cytotoxicity. *Eur J Immunol,* 33**,** 2727-35.

WALTER, R. F., MAIRINGER, F. D., TING, S., VOLLBRECHT, C., MAIRINGER, T., THEEGARTEN, D., CHRISTOPH, D. C., SCHMID, K. W. & WOHLSCHLAEGER, J. 2015. MDM2 is an important prognostic and predictive factor for platin-pemetrexed therapy in malignant pleural mesotheliomas and deregulation of P14/ARF (encoded by CDKN2A) seems to contribute to an MDM2-driven inactivation of P53. *Br J Cancer,* 112**,** 883-90.

WAN, Y. Y. & FLAVELL, R. A. 2009. How diverse--CD4 effector T cells and their functions. *J Mol Cell Biol,* 1**,** 20-36.

WANG, L. X., LI, Y., YANG, G., PANG, P. Y., HALEY, D., WALKER, E. B., URBA, W. J. & HU, H. M. 2010. CD122+CD8+ Treg suppress vaccine-induced antitumor immune responses in lymphodepleted mice. *Eur J Immunol,* 40**,** 1375-85.

WANG, N. S., JAURAND, M. C., MAGNE, L., KHEUANG, L., PINCHON, M. C. & BIGNON, J. 1987. The interactions between asbestos fibers and metaphase chromosomes of rat pleural mesothelial cells in culture. A scanning and transmission electron microscopic study. *Am J Pathol,* 126**,** 343-9.

WARD, M. J., THIRDBOROUGH, S. M., MELLOWS, T., RILEY, C., HARRIS, S., SUCHAK, K., WEBB, A., HAMPTON, C., PATEL, N. N., RANDALL, C. J., COX, H. J., JOGAI, S., PRIMROSE, J., PIPER, K., OTTENSMEIER, C. H., KING, E. V. & THOMAS, G. J. 2014. Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer. *Br J Cancer,* 110**,** 489-500.

WARD-HARTSTONGE, K. A. & KEMP, R. A. 2017. Regulatory T-cell heterogeneity and the cancer immune response. *Clinical & Translational Immunology,* 6**,** e154.

WEJKSZA, K., LEE-CHANG, C., BODOGAI, M., BONZO, J., GONZALEZ, F. J., LEHRMANN, E., BECKER, K. & BIRAGYN, A. 2013. Cancer-produced metabolites of 5-lipoxygenase induce tumor-evoked regulatory B cells via peroxisome proliferator-activated receptor alpha. *J Immunol,* 190**,** 2575-84.

WELSH, R. M. 2009. Blimp hovers over T cell immunity. *Immunity,* 31**,** 178-180.

WHERRY, E. J. 2011. T cell exhaustion. *Nat Immunol,* 12**,** 492-9.

WHERRY, E. J., BARBER, D. L., KAECH, S. M., BLATTMAN, J. N. & AHMED, R. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 16004-16009.

WHITE, J. R., HARRIS, R. A., LEE, S. R., CRAIGON, M. H., BINLEY, K., PRICE, T., BEARD, G. L., MUNDY, C. R. & NAYLOR, S. 2004. Genetic amplification of the transcriptional response to hypoxia as a novel means of identifying regulators of angiogenesis. *Genomics,* 83**,** 1-8.

WHITESIDE, T. L. 2015. The role of regulatory T cells in cancer immunology. *Immunotargets Ther,* 4**,** 159-71.

WILK, M. M., MISIAK, A., MCMANUS, R. M., ALLEN, A. C., LYNCH, M. A. & MILLS, K. H. G. 2017. Lung CD4 Tissue-Resident Memory T Cells Mediate Adaptive Immunity Induced by Previous Infection of Mice with Bordetella pertussis. *J Immunol,* 199**,** 233-243.

WOOD, O., WOO, J., SEUMOIS, G., SAVELYEVA, N., MCCANN, K. J., SINGH, D., JONES, T., PEEL, L., BREEN, M. S., WARD, M., GARRIDO MARTIN, E., SANCHEZ-ELSNER, T., THOMAS, G., VIJAYANAND, P., WOELK, C. H., KING, E. & OTTENSMEIER, C. 2016. Gene expression analysis of TIL rich HPV-driven head and neck tumors reveals a distinct B-cell signature when compared to HPV independent tumors. *Oncotarget,* 7**,** 56781-56797.

WOOLHOUSE, I., BISHOP, L., DARLISON, L., DE FONSEKA, D., EDEY, A., EDWARDS, J., FAIVRE-FINN, C., FENNELL, D. A., HOLMES, S., KERR, K. M., NAKAS, A., PEEL, T., RAHMAN, N. M., SLADE, M., STEELE, J., TSIM, S. & MASKELL, N. A. 2018. BTS guideline for the investigation and management of malignant pleural mesothelioma. *BMJ Open Respir Res,* 5**,** e000266.

WU, V., YEERNA, H., NOHATA, N., CHIOU, J., HARISMENDY, O., RAIMONDI, F., INOUE, A., RUSSELL, R. B., TAMAYO, P. & GUTKIND, J. S. 2019. Illuminating the Onco-GPCRome: Novel G protein-coupled receptor-driven oncocrine networks and targets for cancer immunotherapy. *J Biol Chem,* 294**,** 11062-11086.

YADAV, M., STEPHAN, S. & BLUESTONE, J. A. 2013. Peripherally induced tregs - role in immune homeostasis and autoimmunity. *Front Immunol,* 4**,** 232.

YAMADA, N., OIZUMI, S., KIKUCHI, E., SHINAGAWA, N., KONISHI-SAKAKIBARA, J., ISHIMINE, A., AOE, K., GEMBA, K., KISHIMOTO, T., TORIGOE, T. & NISHIMURA, M. 2010. CD8+ tumor-infiltrating lymphocytes predict favorable prognosis in

malignant pleural mesothelioma after resection. *Cancer Immunol Immunother,* 59**,** 1543-9.

YAMADA, T., PARK, C. S., MAMONKIN, M. & LACORAZZA, H. D. 2009. Transcription factor ELF4 controls the proliferation and homing of CD8+ T cells via the Krüppel-like factors KLF4 and KLF2. *Nature immunology,* 10**,** 618-626.

YANG, H., BOCCHETTA, M., KROCZYNSKA, B., ELMISHAD, A. G., CHEN, Y., LIU, Z., BUBICI, C., MOSSMAN, B. T., PASS, H. I., TESTA, J. R., FRANZOSO, G. & CARBONE, M. 2006. TNF-alpha inhibits asbestos-induced cytotoxicity via a NF-kappaB-dependent pathway, a possible mechanism for asbestos-induced oncogenesis. *Proc Natl Acad Sci U S A,* 103**,** 10397-10402.

YANG, H., RIVERA, Z., JUBE, S., NASU, M., BERTINO, P., GOPARAJU, C., FRANZOSO, G., LOTZE, M. T., KRAUSZ, T., PASS, H. I., BIANCHI, M. E. & CARBONE, M. 2010. Programmed necrosis induced by asbestos in human mesothelial cells causes high-mobility group box 1 protein release and resultant inflammation. *Proc Natl Acad Sci U S A,* 107**,** 12611-6.

YANG, X. O., PAPPU, B. P., NURIEVA, R., AKIMZHANOV, A., KANG, H. S., CHUNG, Y., MA, L., SHAH, B., PANOPOULOS, A. D., SCHLUNS, K. S., WATOWICH, S. S., TIAN, Q., JETTEN, A. M. & DONG, C. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity,* 28**,** 29-39.

YAP, T. A., AERTS, J. G., POPAT, S. & FENNELL, D. A. 2017. Novel insights into mesothelioma biology and implications for therapy. *Nat Rev Cancer,* 17**,** 475-488.

YE, Z. J., ZHOU, Q., YUAN, M. L., DU, R. H., YANG, W. B., XIONG, X. Z., HUANG, B. & SHI, H. Z. 2012. Differentiation and recruitment of IL-22-producing helper T cells stimulated by pleural mesothelial cells in tuberculous pleurisy. *Am J Respir Crit Care Med,* 185**,** 660-9.

YEGLES, M., SAINT-ETIENNE, L., RENIER, A., JANSON, X. & JAURAND, M. C. 1993. Induction of metaphase and anaphase/telophase abnormalities by asbestos fibers in rat pleural mesothelial cells in vitro. *Am J Respir Cell Mol Biol,* 9**,** 186-91.

YOU, F. P., ZHANG, J., CUI, T., ZHU, R., LV, C. Q., TANG, H. T. & SUN, D. W. 2017. Th9 cells promote antitumor immunity via IL-9 and IL-21 and demonstrate atypical cytokine expression in breast cancer. *Int Immunopharmacol,* 52**,** 163-167.

YU, Q., CHANG, H.-C., AHYI, A.-N. N. & KAPLAN, M. H. 2008. Transcription factor-dependent chromatin remodeling of Il18r1 during Th1 and Th2 differentiation. *Journal of immunology (Baltimore, Md. : 1950),* 181**,** 3346-3352.

YUEN, G. J., DEMISSIE, E. & PILLAI, S. 2016. B lymphocytes and cancer: a love-hate relationship. *Trends Cancer,* 2**,** 747-757.

ZANELLA, C. L., POSADA, J., TRITTON, T. R. & MOSSMAN, B. T. 1996. Asbestos causes stimulation of the extracellular signal-regulated kinase 1 mitogen-activated

protein kinase cascade after phosphorylation of the epidermal growth factor receptor. *Cancer Res,* 56**,** 5334-8.

ZANETTI, M. 2015. Tapping CD4 T Cells for Cancer Immunotherapy: The Choice of Personalized Genomics. *The Journal of Immunology,* 194**,** 2049.

ZEH, H. J., 3RD & LOTZE, M. T. 2005. Addicted to death: invasive cancer and the immune response to unscheduled cell death. *J Immunother,* 28**,** 1-9.

Bibliography