Germline BAP1 mutation alter the tumor immune microenvironment and impact the progression of Malignant Mesothelioma (MM)

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Abstract

Asbestos causes malignant transformation of primary human mesothelial cells (HM), leading to mesothelioma. The primary inherited risk factor linked to mesothelioma is the dysfunction of BRCA1 Associated Protein 1 (BAP1). People with inherited BAP1 mutations face an increased risk of developing MM. However, to date, we found that none of the mesothelioma patients carrying germline BAP1 mutations were professionally exposed to asbestos. Using a Bap1$^{+/-}$ mouse model, we found that, compared with their wild-type littermates, Bap1$^{+/-}$ mice exposed to low-dose asbestos fibres showed significant alterations of the peritoneal inflammatory response, and lower levels of several chemokines and cytokines. Consistent with these data, Bap1$^{+/-}$ mice had a significantly higher incidence of mesothelioma after exposure to very low doses of asbestos doses that rarely induced mesothelioma in wild-type mice. Additionally, our findings showed that tumors in Bap1$^{+/-}$ mice have more M2 type macrophages as compared to tumors in Bap1^{WT} mice. This puzzled us even more since M2 macrophages promote immunosuppressive microenvironment and should have adverse effects on therapies, but we see the opposite response in BAP1 mutant patients. We speculated if asbestos had an effect for the observed results. We created a mouse model from mesothelioma derived luciferase integrated cancer cell line (AN1) from Bap1$^{+/-}$ mice which was biallelic inactivated for BAP1 as found in 60% of BAP1 mutant MM patient tumors, to understand how BAP1 loss in mesothelioma impact tumour microenvironment without asbestos exposure. Here, I injected Bap1^{WT} and Bap1$^{+/-}$ with AN1 (Bap1$^{-/-}$) biallelic inactivated tumor cell line. Tumors were digested and tumor immune cells were collected. Harvested cells were immunoprofiled by flow cytometry. I did not find any significant differences in the immune cell population amongst the two genotypes i.e. Bap1$^{+/-}$ and Bap1$^{WT.}$ Additionally, I did not find any differences in the T cell population (CD8+, CD4+, their activation states and Tregs) and IFNY levels by flow cytometry. But I found that tumor associated macrophages (TAMs) in Bap1$^{+/-}$ showed reduced levels of MHCII expression as compared to Bap1^{WT}. I did not find the similar findings in other antigen presenting cells i.e. dendritic cells, B cells and macrophages outside of the TME. Additionally, I found that tumor promoting markers i.e. Trem2, CD9 and Ly6C were significantly reduced on TAM's in Bap1$^{+/-}$ as compared to Bap1^{WT} mice. These findings imply that while alterations in BAP1 status i.e. partial or complete loss are associated with MM development, the relationship between BAP1 status and survival outcomes is more complex and possibly influenced by other factors or molecular pathways which need to be explored further.

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List of Abbreviations

APC- Antigen-presenting cells

BAP1-TPDS - BAP1 tumor predisposition syndrome

BIMT - BAP1-inactivated melanocytic tumors

BAP1- BRCA1 Associated Protein 1

CLIP- class II-associated invariant chain peptide

DECM- decellularized tumor matrix

DALYs- disability-adjusted life years

ER- Endoplasmic reticulum

HMGB1- high mobility group box 1

HM- human mesothelial cells

ICI - immune checkpoint inhibitor

IRF-1- interferon regulatory factor-1

JAK-1- Janus Activated Kinases

LAG-3- Lymphocyte Activation Gene 3

MHC- Major histocompatibility complex

MM- Malignant mesotheliomas

MPeM- Malignant peritoneal mesothelioma

MPM- malignant pleural mesothelioma

NLS- nuclear localization sequences

SDI- socio-demographic index

TCR- T cell receptors (TCR)

TAMs- Tumor associated macrophages

TME- tumor microenvironment

TNFa- tumor necrosis factor alpha

TILs- tumor-infiltrating lymphocytes (TILs)

YY1-Yin Yang 1

Chapter 1: Introduction and Background

1.1 Cancer

Cancer is a complex spectrum of diseases characterized by the uncontrolled proliferation of abnormal or mutated cells within the body. These cells possess the capability to multiply rapidly and uncontrollably, leading to the formation of tumors (B Kalyanaraman.,2017). This condition represents a significant global health challenge, contributing to a substantial portion of worldwide mortality across nations of varying economic statuses (Torre et al.,2015). According to the World Health Organization (WHO), cancer imposes a heavy burden globally, accounting for 244.6 million Disability-Adjusted Life Years (DALYs) lost, affecting both men (137.4 million) and women (107.1 million).

Although the burden appears slightly higher in men than in women (9.6% vs 8.6%; $p = 0.219$), the impact is profound across both genders. Recent WHO updates indicate that cancer stands as the second leading cause of death globally, claiming 8.97 million lives annually. Its ranking among leading causes of mortality varies across different regions, with some areas experiencing a more significant burden than others. It remains first in the Western Pacific and America Regions, second in South-East Asia, Eastern Mediterranean Regions and European, but only fourth in the African Region (Mattiuzzi and Lippi.,2019). Despite efforts to combat cancer, it remains a formidable foe, necessitating continued research and interventions to mitigate its impact.

1.2 Mesothelioma

Malignant mesotheliomas (MM) are uncommon cancers (Hajj et al., 2021) arising predominantly from the pleura of mesothelial cells (80-90%), with occurrences in the peritoneum (10-15%) and rarely in the pericardium (<5%) (Baumann et al., 2015). Asbestos exposure is the primary cause of MM. However, due to a latency period of approximately 30 years and diagnostic challenges, many cases remain undetected until they reach advanced stages (Hajj et al., 2021). Asbestos is a collective term encompassing six types of mineral fibres: crocidolite, chrysotile, amosite, anthophyllite, tremolite, and actinolite (Xue et al., 2020).

In 2019, an estimated 34,511 new cases of mesothelioma, resulting in 29,251 deaths and 668,104 disability-adjusted life years (DALYs) globally. Han et al found that the burden rate of mesothelioma attributed to asbestos exposure correlated positively with the socio-demographic index (SDI) on a national scale (Han et al.,2022).

Malignant peritoneal mesothelioma (MPeM) arises from prolonged inflammation, with 70-90% of cases attributed to asbestos exposure (Wadowski et al., 2021). Research reveals that asbestos exposure prompts an inflammatory response characterized by mononuclear phagocytes transforming into macrophages. These macrophages ingest asbestos, generating reactive oxygen species and numerous mutagenic cytokines (Yang et al., 2006; Cersosimo et al., 2021).

Our investigations have shown that erionite, crocidolite, and chrysotile asbestos induce programmed cell necrosis in human mesothelial cells (HM). Necrotic cells release high mobility group box 1 (HMGB1) protein into the extracellular environment, initiating inflammation and activating the inflammasome pathway. This sustained inflammatory process results in the release of tumor necrosis factor alpha (TNFα) and other mutagenic cytokines, leading to DNA damage and potentially malignant transformation (Yang et al.,2010, Jube et al.,2012, Xue et al., 2020).

1.2.1 Epidemiology

Certain regions, particularly in Europe (Belgium, Malta, The Netherlands) and Oceania (New Zealand and Australia), have reported heightened incidences of Malignant Mesothelioma (MM). However, only 16% of the inhabitants contribute to the world population. The second group includes a significant part of Europe and the USA. Although asbestos bans have been implemented in numerous countries (Bianchi et al.,2014). MM incidence rates remain uncertain due to insufficient data from regions where asbestos use persists (Bianchi et al.,2014).

The incidence and mortality rates of global mesothelioma are uncertain because there is no data available from developed countries which continue to use large quantities of asbestos. Diagnostic accuracy has improved with the advent of novel molecular and immunohistochemical markers, yet misdiagnoses persist, particularly in developing nations. However, still, 14 per cent (high-resource countries) to 50 per cent (developing countries) of diagnoses are incorrect, leading to insufficient diagnosis (Carbone et al., 2019).

Asbestos exposure, particularly in males (mean patient age is > 60 years) remains a significant risk factor, although other mineral fibers and therapeutic radiation also contribute to MM development.

The most significant risk factor is asbestos exposure, which persists in 54-90% of all affected patients (Tischoff and Tannapfel.,2017). In environmentally exposed subjects, the male to female ratio reports about 1.2: 1.4 (Marinaccio et al.,2018). In 9 countries (United Kingdom, Sweden, France, Germany, Netherlands, Canada, United States, Australia, and New Zealand), MM mortality has decreased dramatically among males. It was significantly rising in 5 countries (Poland, Spain, China-Hong Kong, Japan, and the Republic of Korea) over the last ten years. Among females and in the last ten years MM mortality has declined significantly in only one country (Italy); while in 3 countries (Poland, Argentina, and the Republic of Korea), it has increased substantially underscoring the global variability in burden and mortality patterns. Despite asbestos bans in many nations, MM continues to pose a substantial threat to public health (Rahman. ,2018).

1.2.2 Etiology

Airborne asbestos exposure predominantly correlates with MM development (Porpodis et al., 2013) with various types of asbestos implicated in different geographic locations. Asbestos belongs to silicate family minerals which are further divided into two major groups: The serpentine form and the amphibole form. Asbestos is known to cause various diseases like laryngeal cancer, plaques/fibrosis and majorly Mesothelioma (Michele et al.,2012). Most pleural mesotheliomas are due to asbestos (70% to 90%) in men in Europe and North America. However, this proportion is lesser for peritoneal mesothelioma. In North America, asbestos exposure is lesser in women at any location, but in Europe, the ratio is higher and differs significantly by locale. Other types of mineral fibers (erionite, fluoro-edenite, and possibly balangeroite) can induce MM at specific geographic locations.While asbestos exposure remains a primary risk factor, a small percentage of MM cases result from germline deletions of BRCA1-associated protein 1 (BAP1). These alternative etiologies contribute to a fraction of MM cases, emphasizing the multifactorial nature of the disease since all mesotheliomas are not caused by asbestos exposure (Attanoos et al.,2017).

1.2.3 Types and Features

Mesothelioma, arising from mesothelial surfaces, exhibits anatomical and pathological diversity. Pleural Mesothelioma predominates (65%-70%), among MM cases, with Peritoneal Mesothelioma (30%) being less common. Other primary cardiac and pericardial mesotheliomas, as well as those of the tunica vaginalis, are rare entities (1%-2%). Pathological classification categorizes MM into epithelioid, fibrous sarcomatoid, biphasic/mixed, and other rare histologies (Ahmed et al.,2013). Recent classification revisions aim to refine distinctions between epithelioid and sarcomatoid tumors, reflecting their prognostic implications as follows:

1. Pleural Malignant Mesothelioma: It is the most common and accounts for the predominant subtype-80% of all cases. Pleural mesothelioma has an unusually dismal prognosis; its 5-year survival rate is only 10%, making it the most fatal among rare cancers (Tolani et al.,2018).

2. Peritoneal Malignant Mesothelioma -Peritoneal Malignant Mesothelioma is a rare tumour, less common than its pleural equivalent. It emerges from the peritoneum overlying mesothelial cells, it occurs preferentially in males with an average age ranging from 47 to 60.5 years, clinical symptoms are not specific, and the imagery remains little or non-contributive (Mery et al.,2014).

3. Primary cardiac and pericardial tumours - These are rare entities with an autopsy frequency of 0.001-0.03%. Cardiac and pericardial metastases are much more common than primary tumours. Pericardial malignant mesotheliomas account for up to 50% of primary pericardial tumours (Barroso et al.,2017).

4. Mesothelioma of the tunica vaginalis - It arises from the serosal membranes of the tunica vaginalis testis (TVT) (Butnor et al.,2019). It represents 0.3 to 0.5% of all malignant mesotheliomas. Asbestos exposure is frequently accompanied by sickness. It is often mistakenly diagnosed during surgery for other causes, due to its low occurrence and non-specific clinical presentation, and the prognosis is typically low (Trenti et al.,2019).

1.2.4 Prognosis

MM prognosis remains poor, with median survival ranging from 8 to 14 months. Prognostic markers encompass various clinical, serological, imaging, and molecular factors, aiding in risk stratification. Epithelioid histology typically associates with a better prognosis, while sarcomatoid variants exhibit poorer outcomes. Inflammatory markers like WBC, neutrophil to lymphocyte ratio (NLR) and C-reactive protein in various MM, prognosis studies have also found to be significantly elevated (Zandwijk et al.,2013).

Multiple organisations have analysed quite a large number of MM patients who enrolled in the treatment trials and identified the following poor prognostic factors: WBC count - 8.3×10^9/L or higher, older age, i.e. more than 75, Male, chest pain, non-epithelioid histology, Platelets greater than 400,000 L, poor performance status, LDH greater than 500 IU/L (Alastair J Moore et al., 2008). Decreased levels of haemoglobin, thrombocytosis, and elevated serum lactate dehydrogenase (LDH) also contributed to the poor prognosis (Zandwijk et al.,2013). Epithelioid is the most common amongst the subtypes and is associated with the best prognosis. The worst prognosis is of sarcomatoid variants consisting of spindle morphology (Frank et al., 2012). Despite advancements in prognostic assessment, MM continues to present therapeutic challenges, necessitating ongoing research for improved management strategies.

1.2.5 Symptoms

Usually, MM is diagnosed between 40 and 65 years of age and can show ambiguous, non-specific symptoms which may be quite variable depending on the degree and distribution of the disease in the peritoneum. Plaques are very common with mesothelioma patients. Patients most often complain of increased abdominal distension and abdominal pain (Enomoto et al., 2019). Typical patients with malignant pleural mesothelioma may have an unproductive cough, chest wall pain, dyspnea, pleural effusions mainly on the right side (60 per cent of cases)). The common symptoms are shortness of breath and pain (90%), while others include fatigue (36%), worry (29%), cough (22%), sweating (22%), and constipation (22%) (Porpodis et al.,2013). MM presents with nonspecific symptoms, often leading to delayed diagnosis. Clinical manifestations vary, with pleural and peritoneal involvement eliciting distinct symptoms. Diagnostic modalities, including imaging and thoracoscopy, facilitate accurate diagnosis, although challenges persist due to symptom heterogeneity.

1.2.6 Staging of MM

MM staging primarily relies on TNM ((T) tumours, (N) nodes, and (M) metastases)) classification, incorporating surgical, pathological, and radiographic criteria. Challenges in staging arise from tumor morphology and heterogeneity, necessitating multidimensional assessments mainly due to the impracticality of measuring tumours with irregular and highly variable morphology, unlike TNM staging of most solid tumours, MM classification does not require consideration of tumour size. The lung cancer diagram, which suggests that tumours invade pulmonary lymphatics, follows the N classification of MM. M classification of MM is binary. M1 indicates documented blood-borne metastasis. Distant metastases to the brain, bone, kidney and adrenal glands have reported. Still, they are rarely diagnosed, likely due to the comparatively rapid and fatal progression of local T4 disease involving vital intrathoracic organs (Richard et al.,2017). Tertemiz et al. found extrapleural dissemination in 87.7% of cases during postmortem studies. Tumour dissemination in extrathoracic sites seen in the liver (31.9%), spleen (10.8%), thyroid (6.9%), and the brain (3.0%) (Tertemiz et al.,2014).

Pathological staging classifies cases from surgical resection based on gross and microscopic analyses of pathological specimens (Richard et al.,2017). Review of the database of MM by the IASLC indicated that therapeutic (cTNM) staging minimally stratified survival and was inconsistent with pathological (pTNM) staging. Alternative staging models based on quantitative parameters explored, to improve the prognostic classification of MM. The rarity of this disorder, the difficulty of its diagnosis and the general ineffectiveness of available treatment have impeded attempts to improve MM staging (Gill et al.,2018).

Beyond TNM, histology remains the most significant prognostic determinant (Bonomi et al., 2017). The peritoneal carcinomatosis index (PCI) is the most frequently used and accepted classification to measure the peritoneal disease burden. AJCC (American Joint Committee on Cancer) eighth editions for staging, has a staging system for pleural mesothelioma. Still, it has no staging system for Malignant Peritoneal Mesothelioma (MPM). A new TNM staging method, proposed by Yan and colleagues, addresses this issue. T was allocated under this method based on the magnitude of the disease burden quantified by intraoperative PCI and classified into four subgroups: T1 (PCI 1-10), T2 (PCI 11-20), T3 (PCI 21-30), and T4 (PCI 31-39). Node status (N) allocated for histopathology of surgical specimens based on the presence (N1) or absence (N0) of positive lymph nodes. Extra-abdominal metastasis on the pre-operative imaging was assigned M1. Stage I disease included T1N0M0, stage II included T2-3N0M0, and stage III included T4N0M0 and N1 or M1 disease.5-year survival for stage I, II, and III using this staging system was 87%, 53%, and 29%, (Enomoto et al.,2019). Efforts are underway to further refine criteria and to validate 3-dimensional quantitative estimates of tumour size to potentially augment T classification in future editions of the staging system (Richard et al.,2017). Novel staging models aim to enhance prognostic accuracy, reflecting the evolving understanding of MM pathophysiology.

1.2.7 Pathogenesis

Asbestos exposure triggers molecular changes within mesothelial cells, culminating in malignant transformation. Reactive oxygen and nitrogen species generated by asbestos induce DNA damage, contributing to tumorigenesis (Quinn et al.,2015). The underlying pathogenesis of the disease is that the inhaled asbestos fibres enter the visceral pleura and the pleural space and then to the parietal pleura through the alveoli or retrograde through the lymphatic vessels (Roe and Stella.,2015). Chromosomal losses and dysregulated signalling pathways further drive MM progression, highlighting the intricate interplay of genetic and environmental factors. The typical molecular landscape of MM consists of multiple chromosomal losses leading to loss or inactivation of several tumour suppressor genes. Majorly, chromosomes 3p, 9p and 22q with tumour suppressor genes, are involved in causing MM. This leads to the loss of CDKN2A located at 9p21, NF2 at 22q12 and BRCA1-associated protein 1 (BAP-1) at 3p21.31-p21.2 (Hjerpe, Own and Dobra.,2020).

Several MM have very complex karyotypes with rearrangements of several chromosomes and extensive aneuploidy. Moreover, dysregulation in signal transduction pathways, related to cell survival and proliferation, has also been demonstrated (Boneli et al.,2017). The fibres themselves can also cause aberrant separation of chromosomes during mitosis and direct activation of tyrosine kinase receptors, in the absence of driver mutations (Abbott et al.,2020).

1.2.8 Diagnosis

Diagnosing MM remains challenging due to nonspecific clinical presentations. Imaging modalities, including chest X-ray and computed tomography, aid in detection, with thoracoscopy providing definitive tissue confirmation. Chest X-ray (CXR) typically starts with imaging and then goes on to computed tomography (CT). A novel approach may help differentiate malignant from benign lesions, such as quantitative textural and form analysis. Ultrasound is especially important to analyse pleural effusion. Thoracoscopy is used in the diagnostic phase as a standard procedure that allows good visualisation of the endothoracic anatomy with the direct evaluation of the locoregional tumour extension and to practice, where necessary, effective chemical pleurodesis (Abbott et al.,2020). It is a reliable diagnostic tool since it can give additional tissue confirmation. Computed tomography (CT) may show pleural-based nodularity. PET (Positron emission tomography) is very beneficial as mesothelioma has hypermetabolic characteristics. Thus, this technique cannot only be used for staging, but post-treatment follows up as well (Frank et al., 2012).

1.2.9 Biomarkers

Currently, there is no routine test used for testing prognostic or predictive biomarkers for MM. (Brcic and Kern.,2020). A combination of two positive (mesothelial) and two negatives (cancer-related) markers are considered the gold standard for immunohistochemical diagnosis of epithelioid and biphasic MM, while the rare sarcomatoid type has no specific markers (Lacerenza et al.,2020).

Existing biomarkers, such as soluble mesothelin-related peptide (SMRP) and claudin-4, offer insights into MM pathophysiology but exhibit limitations in diagnostic accuracy (Schulte and Husain.,2020). MM stains were positive for cytokeratin 5/6 (CK 5/6), calretinin, vimentin, epithelial membrane antigen, Wilms tumour 1 (WT-1) (Enomoto et al.,2019). Until today the only tumour biomarker to receive the approval of US Food and Drug Administration for clinical use is mesothelin. It has a very confined expression on non-cancerous healthy tissue; thus, it is a desirable therapeutic target for mesothelin-positive tumours. In a few situations, mesothelin can be shed from the surface of the cells by the action of tumour necrosis factor-a-converting enzyme and identified in the blood (Creaney et al.,2017). Ongoing efforts aim to identify novel biomarkers for early detection and prognostic assessment, enhancing MM management.

1.2.10 Treatment

Unfortunately, few treatment options exist for MM (Abbott et al.,2020). Current treatments for MM are limited to surgery, radiation, and chemotherapy (Tolani et al.,2018). Chemotherapy regimens, such as cisplatin and pemetrexed combination therapy, represent standard first-line treatments, albeit with modest efficacy. Resistance to conventional therapies underscores the need for novel treatment approaches and personalized interventions in MM management.

<u>Surgery</u>

In terms of pleurectomy, surgical therapy is only for patients who can handle surgical risks and have a low tumour stage (Abbott et al.,2020). Different surgical procedures are surgical pleurodesis by video-assisted thoracoscopic surgery (VATS), extrapleural pneumonectomy

(EPP) and debulking surgery (Alastair J Moore et al., 2008). Several non-randomised series reported long-term survivors following extra-pleural pneumonectomy (EPP), which involves removal of the diseased pleura in addition to the lung, pericardium and hemidiaphragm, usually as part of a multi-modality regime including neoadjuvant chemotherapy and adjuvant Hemi-thoracic radiotherapy (Blyth et al.,2018). In MM surgery, microscopic radical (R0) resection is not possible because of the anatomy, and the target of MM surgery is full macroscopic resection (R1). Surgery alone is not effective, it is generally undertaken with chemotherapy and/or radiation therapy and reserved for a subset of patients with early tumour, epithelioid histology, and good results (Cantini et al., 2020)

Radiotherapy

Radiation therapy (RT) may have a role in MM, as it can be used in combination with chemotherapy and can provide local tumour control if the patient has a good performance status (Abbott et al. ,2020). Combinations of RT with relevant agents, e.g. DNA-damage repair inhibitors and/or immunotherapies may deliver new treatment options (Blyth et al.,2018). Intensity-modulated radiotherapy (IMRT) is a sophisticated modality using small radiation beams at different angles in a 3-D conformal pattern, which allows more intense radiation at the target with high precision (Frank et al., 2012). However, the toxicity may be important and, thus, RT unless it helps to alleviate symptoms such as chest pain or bronchial/oesophagal obstruction, it alone has no benefit (Abbott et al. ,2020).

Chemotherapy

The combination of Cisplatin and Pemetrexed is followed as a standard first line therapy for MM. In 2003, a phase III randomised trial compared Cisplatin alone versus Cisplatin plus Pemetrexed in untreated malignant pleural mesothelioma with the combination, and the response rate was 41.3% compared to 16.7% for Cisplatin alone, Median time to progression was 5.7 vs 3.9 months. Median overall survival was 12.1 vs 9.3 months, both in favour of the combination arm. Therefore, it has also become a standard recommendation in the adjuvant combined modality approach to resectable disease (Frank et al., 2012). The National Comprehensive Cancer Network's recommendations for 2018 identify that the first-line systemic therapy for MM is Pemetrexed combined with Cisplatin and probably Bevacizumab (Abbott et al. ,2020).

Cisplatin

Cisplatin is a small and surprisingly simple molecule made up of one atom of Platinum bound to two amides and two chlorides. Cisplatin covalently binds in its reactive form to the DNA bases, forming adducts of DNA. In a coordinated effort by the cells to remove the lesions, these DNA adducts block transcription and DNA synthesis, which in turn activates a complex intracellular signal transduction cascade and cell cycle is arrested for DNA repair and in case of excessive damage the cells undergo apoptosis (Rocha et al.,2018).

Platinum is highly responsive but several patients ultimately relapse. Drug resistance has been observed in many patients who relapsed from interactions between a cell, and its environmental components which promote these internal mechanisms and subsequent Cisplatin resistance (Chen and Chang.,2019). To overcome resistance, Cisplatin is commonly used in combination with some other drugs in treating various cancers, including mesothelioma (Dasari et al.,2014).

Pemetrexed

Pemetrexed belongs to the class of antifolic agents, but it is distinguished from the other antifolates for its novel structure, possessing a unique 6-5 fused pyrrolo-[2,3-d] pyrimidine nucleus instead of the classical 6-6 core structure (pteridine or quinazoline) (Facchetti et al.,2017). Pemetrexed is an antagonist against folates. Because folate donate one-carbon units to the biosynthesis of purines, thymidine, and hence DNA, the interruption of folate metabolism by Pemetrexed contributes to ineffective DNA synthesis and failure of growth in tumour cells (Liang et al.,2019). The primary adverse effect of Pemetrexed is the dose-limiting toxicity of the drug and is associated with elevated pre-treatment levels of plasma homocysteine which predicts severe myelosuppression (Xin et al.,2014).

Resistance of MM to Cisplatin and Pemetrexed

A widely held misconception contends that all elderly patients, because of the toxicity of some chemotherapeutic agents, even with good performance status (performance status [PS] 0-1), are unable to tolerate aggressive chemotherapy (Xin et al.,2014). The effect of this therapy iscan be reversed, as testing showed that exogenous hypoxanthine and thymidine were required to prevent growth inhibition by Pemetrexed (Curtin and Hughes 2001). The cells resistant to

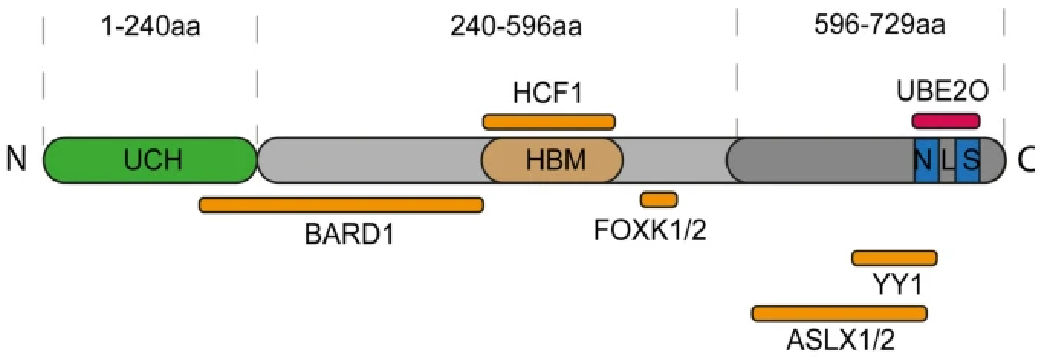
Pemetrexed showed overexpression of TYMS (Thymidylate Synthase) which is one of the major factors leading to resistance (Obata et al.,2013).

Although Cisplatin is the most frequently used chemotherapeutic agent used, in association with Pemetrexed, usually the results are disappointing. Since the score of residual peritoneal seeding after cytoreductive peritonectomy is the principal prognostic indicator; selected patients can support it. Hyperthermic intraoperative intraperitoneal chemotherapy (HIPEC), followed by early postoperative intraperitoneal chemotherapy, is a good strategy for a much longer survival (Murinello et al.,2010). Therefore, despite the improvement shown with the combination of Cisplatin and Pemetrexed, nearly two-thirds of patients still fail to show a response to this regimen (Frank et al., 2012).

MM treatment is not guided by histological or molecular features of the tumour. It mainly depends on the clinical stage and patient characteristics. Moreover, there are no other approved regimens for relapsed or refractory MM as Platinum-based chemotherapies and have also failed to show improvements in survival benefits and (Porpodis et al., 2013). There is no alternative when MM patients fail this treatment option as the combination confers a median progression-free survival (PFS) of 5.7 months (Porpodis et al., 2013). Moreover, an overall Response rate of combination chemotherapy is approximately 40%; almost half of all patients are primary resistant and develop resistance ultimately (Kim et al.,2018).

2.1 BRCA 1 associated protein (BAP1)

The primary genetic risk factor associated with mesothelioma involves the mutation of the BRCA1 Associated Protein 1 (BAP1), located on chromosome 3p21.1. BAP1 acts as a tumor suppressor gene (Hiltbrunner et al., 2021). Found in the nucleus, BAP1 functions as a deubiquitinating enzyme, regulating chromatin remodelling and participating in the DNA damage response. Recent research indicates that BAP1 also contributes to the modulation of calcium-induced apoptosis. Mutations in BAP1 may lead to the accumulation of DNA-damaged cells, increasing susceptibility to cancer development (Pulford et al., 2017).

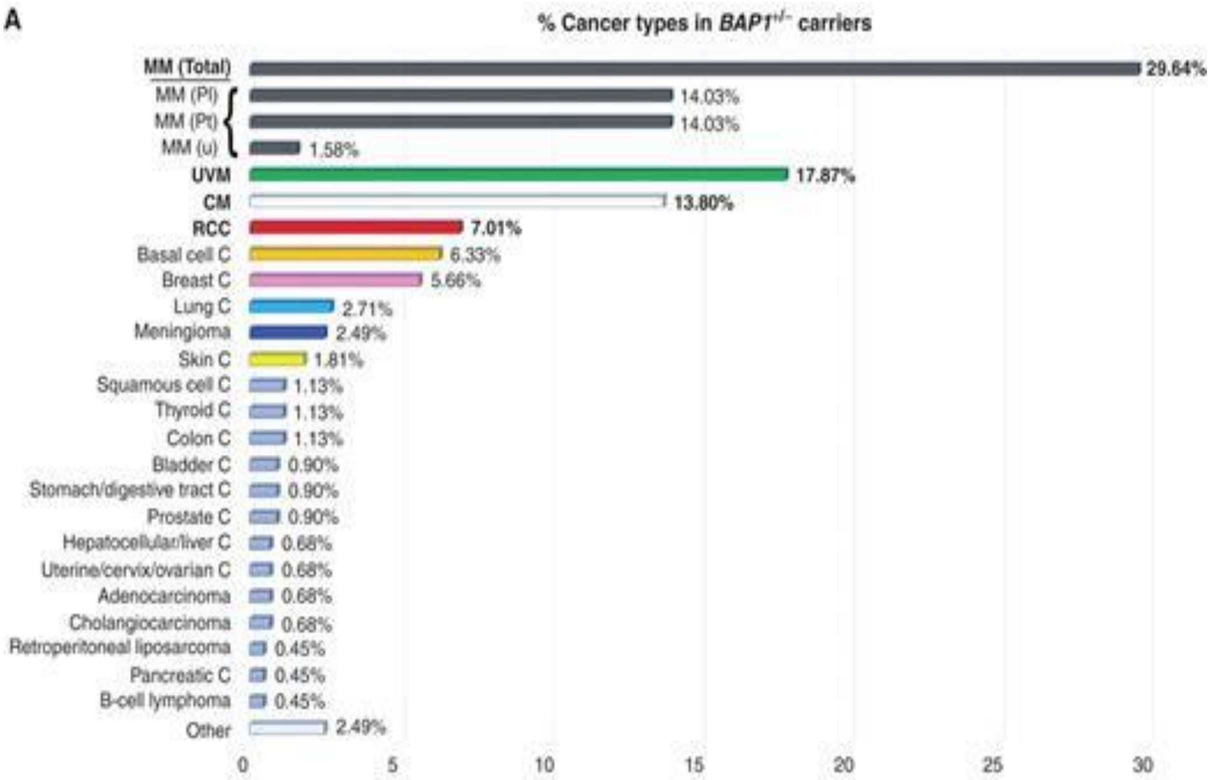


Schematic representation of BAP1 structure and interacting partners (Modified from Caporali et al.,2022)

The BAP1 protein comprises three distinct regions: an N-terminal section housing the catalytic triad responsible for deubiquitylation; a central region known to interact with BARD1, HCF1 via the HCF-1 binding motif (HBM), and FOXK1 and 2; and a C-terminal region containing two nuclear localization sequences (NLS). UBE2O can target these NLS, leading to cytoplasmic retention of BAP1. This section has been shown to engage with YY1 (Yin Yang 1) and ASLX1 and 2 (Caporali et al.,2022).

In 2011, our research team uncovered that the individuals with a heterozygous germline mutation in BAP1 i.e. BAP1$^{+/-}$, had an increased susceptibility to developing mesothelioma and uveal melanoma (Testa et al., 2011). Data encompassing 350 carriers of the BAP1$^{+/-}$ mutation, sourced from 45 articles published until September 30, 2019, revealed that 295 of them developed cancer, resulting in a total of 442 distinct cancer cases (as some individuals developed multiple cancers). Specifically, only carriers of germline BAP1 mutations with a familial history of BAP1-core cancers were considered (Carbone et al., 2020). These findings have been validated by multiple research groups (Carbone et al., 2020; Carbone et al., 2019; Kobrinski et al., 2020).

This condition, known as BAP1 cancer syndrome, increases the likelihood of BAP1- melanocytic tumors (MBAITs) as well as several other types of cancers. The most commonly seen cancers in individuals with BAP1 cancer syndrome, listed in decreasing frequency, are uveal melanoma, malignant mesothelioma, cutaneous melanoma, renal cell carcinoma, and basal cell carcinoma (as several of them developed more than one cancer) (Pilarski et al., 2022).



Cancer types present in *BAP1*$^{+/-}$ carriers (%age) (Adapted from Carbone et al.,2020)

Furthermore, the presence of heterozygous germline BAP1 mutations (BAP1$^{+/-}$) significantly heightens susceptibility to asbestos-induced mesothelioma in mice (Napolitano et al.,2016) illustrating the interplay between genetic factors and environmental exposure (G×E). Diminished BAP1 levels lead to compromised DNA repair mechanisms and trigger metabolic shifts, collectively fostering cancer initiation and progression (Carbone et al., 2020). Research has indicated that malignant mesothelioma (MM) emerging within the context of germline BAP1 mutations often correlates with prolonged survival and less aggressive clinical behaviour (Buamann et al., 2015; Frazin et al., 2015). Additionally, patients with BAP1 mutations exhibit enhanced responsiveness to first-line chemotherapy agents such as Pemetrexed and Cisplatin (Louw et al., 2022; Hassan et al., 2019).

BAP1 deleted tumors were found to have strong cytokine signalling and upregulation of the innate immune response based on gene set enrichment analysis. In contrast, tumors displaying intact BAP1 had upregulation of adaptive immunity and MHC-I/II antigen presentation (Shrestha et al.,2019). Furthermore, tumors with deleted BAP1 showed a lower proportion of plasma cells, natural killer (NK) cells, and B cells but higher mast cell and T cell infiltration, as well as higher expression of genes with a known role in immune checkpoint modulation (PD1, PD-L1 CD80, CTLA4, LAG3, and ICOS), compared with tumor with wild-type BAP1 (Wadowski et al.,2021).

Germline mutations in BAP1 have also been associated with heightened activation of the local innate immune response in peritoneal mesothelioma (Wadowski et al., 2021). Moreover, Kaler et al. illustrated that the loss of BAP1 leads to increased PROS1 expression in uveal melanocytes and melanoma cells. This upregulation subsequently triggers the phosphorylation and activation of the receptor tyrosine kinase MERTK on neighbouring macrophages, compelling them toward a suppressive M2-polarized state (Kaler et al., 2022). Hence, existing literature indicates that the deficiency of BAP1 function could serve as a pivotal factor in the dysregulation of tumor immunity.

1.2 Tumor associated macrophages and Mesothelioma.

The association between inflammation and cancer can be categorized into two pathways: the intrinsic pathway, which is driven by genetic events such as proto-oncogene activation through mutation, tumor-suppressor gene inactivation, chromosomal amplification, and deletion; and the extrinsic pathway, which is triggered by inflammatory conditions in specific anatomical sites. The convergence of these pathways leads to the activation of NF-KB transcription factor, HIF-1α, and STAT3 in tumor cells. These transcription factors stimulate the production of inflammatory cytokines, chemokines, and prostaglandins. Consequently, these mediators recruit leukocytes, predominantly monocytes, thus fostering the development of a cancer-related inflammatory microenvironment (Malekghasemi et al., 2020).

MPM is thought to develop within the framework of persistent inflammation. Approximately 70-90% of instances are linked to exposure to asbestos. Over extended periods, asbestos or similar mineral fibres can induce both direct harm to cells and DNA as well as produce free radicals, leading to ongoing inflammation by disrupting cytokine balance. Consequently, immune responses are triggered, sustaining the inflammatory milieu, and fostering epigenetic and genetic modifications within mesothelial cells, ultimately paving the way for malignant changes (Wadowski et al., 2021).

In the human MM tumor microenvironment, TAMs represent the majority of tumor-infiltrating cells, accounting for approximately 25-40% of total immune infiltrates. MM typically exhibits significant macrophage infiltration, without a direct correlation with tumor stage but notably impacting survival in the non-epithelioid subgroup. This phenomenon may be attributed to the presence of immunosuppressive cytokines that support the pro-tumoral role of infiltrated macrophages (Hiltbrunner et al., 2021). Miselis et al. demonstrated that targeting M2-like TAMs reduced mesothelioma growth and metastases (Cersosimo et al., 2021).

Throughout the transition from benign to invasive cancer cells, the tumor microenvironment (TME) becomes enriched with cytokines and growth factors, leading to the prevalence of Th2-like immunosuppression rather than a Th1-like pro-inflammatory milieu. This transition prompts M1-like TAMs to polarize into M2-like TAMs, resulting in the preferential accumulation of M2-like TAMs within the TME (Zhou et al., 2020). TAMs often co-express genes characteristic of both M1 and M2 phenotypes within individual cells, underscoring the existence of an intermediate TAM state (Yang et al., 2020). Although TAMs do not strictly adhere to M1 or M2 subtypes, they generally exhibit M2-like characteristics and facilitate tumor growth by fostering immune suppression (Mehla et al., 2019).

Recent investigations have revealed the presence of "M2" polarized macrophages in pleural effusions from mesothelioma (Rehrauer et al., 2018). Once tumors develop, macrophages undergo education to adopt an M2-like TAM phenotype, thereby promoting tumor growth and exerting immunosuppressive effects (Wang et al., 2021). TAMs constitute a significant portion of the immune cell infiltration within the tumor microenvironment of mesothelioma patients, with "M2"-polarized macrophages correlating with poorer outcomes (Rehrauer et al., 2018). Increased macrophage infiltration leads to reduced survival in non-epithelioid malignant pleural mesothelioma (MPM), although not in epithelioid MPM (Wadowski et al., 2021).

As per Linton et al., inflammation within mesothelioma may be seen as either a "benefactor" or an "adversary," as persistent inflammation may foster a conducive environment for cell survival while also potentially dampening anti-cancer responses. Hence, a thorough examination of the inflammatory milieu within the mesothelioma tumor microenvironment (TME) is imperative to rejuvenate immune responses and devise more effective therapeutic approaches, as suggested by Cersosimo et al. (2021).

1.3 Role of MHCII in cancer

Major histocompatibility complex (MHC) class II molecules are primarily expressed by professional antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages, and B cells (Liu et al., 2010). Upon expression mediated by CIITA, the MHC-II alpha and beta chains form complexes with the MHC class II-associated invariant chain (li) in the endoplasmic reticulum (ER). Ii occupies the peptide-binding groove of MHC-II, preventing peptide loading within the ER. Within acidic vesicles, Ii undergoes degradation, resulting in the formation of a short fragment known as the class II-associated invariant chain peptide (CLIP). The release of CLIP and the binding of peptide antigens are facilitated by the co-chaperone HLA-DM. HLA-DM also promotes the release of weakly bound peptides, ensuring that only strongly bound peptide complexes are presented on the cell surface (Axelrod, 2019).

Huang et al. demonstrated the pivotal role of MHC-II in bolstering immune surveillance against cancer, thereby fostering the infiltration of T cells into tumors, and augmenting anti-cancer immune responses. Their findings collectively identified three inducers of MHC-II and elucidated that the depletion of MHC-II, attributed to hyperactivated fatty acid synthesis, represents a potentially widespread mechanism contributing to cancer development (Huang et al., 2023). Additionally, for T helper (TH) cells to achieve full activation, they must recognize antigens, including those from tumors, via their T cell receptors (TCR), which engage with antigens only when presented within the context of MHC-II molecules on the surface of professional antigen-presenting cells (APCs), predominantly dendritic cells (DCs) and macrophages. Importantly, the ability to reject tumors or significantly impede their growth is directly correlated with the level of CIITA-driven expression of MHC class II molecules on the surface of cancer cells (Accolla et al., 2019).

Macrophages constitute the predominant immune cell population within the tumor microenvironment and exert a pivotal role in tumor progression. Elevated levels of macrophage infiltration in tumor tissues are linked to unfavourable clinical outcomes (Wang et al., 2017). Coletta et al. demonstrated that monocytes, when co-cultured with tumor cells or exposed to the decellularized tumor matrix (DECM), undergo differentiation towards a pro-tumoral macrophage phenotype characterized by low expression of MHC-II and CD86, along with heightened expression of CD206. These macrophages secrete pro-tumoral cytokines and chemokines and, significantly, exhibit diminished capacity to activate T lymphocytes (Coletta et al., 2021). Yang et al. revealed that TAMs exhibit distinct phenotypes delineated by the markers Ly-6C and MHC-II.

Notably, the most hypoxic TAMs demonstrate suppressed expression of FoxO1, suggesting that within the tumor microenvironment, hypoxia may function as a suppressor of FoxO1 expression in TAMs, consequently driving their transition towards a pro-tumoral state (Yang et al., 2017).

Shi et al. demonstrated that natural killer (NK) cells and macrophages possess the capability to eliminate tumor cells through direct cell-cell contact and/or indirectly via paracrine mechanisms mediated by cytokines. NK cells can also trigger the activation of the caspase cascade in tumor cells, leading to apoptosis (Shi et al., 2005). Furthermore, Li et al. revealed that the absence of MHC class II molecules enhances the activation and functionality of NK cells in mice. Their study indicates that while the development and maturation of NK cells remain unchanged, their functions such as IFN-γ production and cytotoxicity are markedly enhanced in the absence of MHC class II molecules (Li et al., 2020).

Evidence indicates that cancer cells have the capacity to express MHC-II, particularly under the influence of inflammatory cytokines such as IFN-γ (Zeng et al., 2022). Upon IFN-γ binding to its receptor on the cell surface, Janus Activated Kinases (JAK)-1 and JAK2 are activated, serving as intracellular tyrosine kinases that phosphorylate the transcription factor Signal Transducer and Activator of Transcription-1 (STAT1). Phosphorylated STAT1 forms dimers and translocate to the nucleus, where it binds to cis-acting GAS elements within the promoter regions of IFN-responsive genes, including CIITA. Notably, upregulation of CIITA following IFN-γ stimulation does not occur in cells deficient in JAK1 or STAT1, indicating the indispensability of JAK/STAT signalling for IFNγ-mediated MHC-II induction. Once bound to the GAS element, STAT1 is stabilized through interaction with the ubiquitous transcription factor USF-1, which binds to nearby E-box elements in the CIITA promoter (Coletta et al., 2021).

Kerdedani et al. demonstrate that fibroblasts expressing MHCII are commonly found in lung non-small cell carcinomas and are associated with areas of high CD4 T cell density rather than low. In murine models of lung cancer, targeted ablation of MHCII in fibroblasts specifically impaired local immunity and expedited tumor growth. While MHCII fibroblasts lacked expression of classical costimulatory molecules, they retained the ability to activate the TCR/CD28 pathway in effector T cells and enhance T cell metabolism. Additionally, they found that IFN-γ drives MHCII expression in epithelial cells and that the tumor microenvironment regulates MHCII expression in fibroblasts via IFN-γ (Kerdedani et al., 2022).

While tumor cells typically do not express MHC-II constitutively, the presence of IFNγ within the tumor microenvironment can induce MHC-II expression in tumor cells (referred to as tsMHC-II). Increasing evidence underscores the crucial role of tsMHC-II in activating CD4+ T cells. Hypermethylation of HLA-DR and HLA-DQ genes, along with the absence of tsMHC-II expression, has been linked to poorer survival outcomes in patients with oesophageal squamous cell carcinoma. In B cell lymphomas, inactivating mutations in the histone acetyltransferase CREBBP have led to reduced MHC-II expression, highlighting the significance of histone acetylation in MHC-II expression. Furthermore, loss of interferon signalling can also diminish MHC-II transcription (Balasubramanian et al., 2022).

Additionally, it has been observed that depletion of dendritic cells (DCs) or macrophages did not affect the ability of mice to reject Ciita+ tumor cells in some experiments, raising the possibility that MHC-II+ tumor cells may serve as antigen-presenting cells (APCs) to prime antitumor immunity. In a small cohort of unselected melanoma patients, tsMHC-II was not associated with survival, but it has been linked to an improved response to anti-PD-1/anti-PD-L1 therapy. This suggests that in some MHC-II+ murine models, inflammation may induce rapid T cell suppression via the PD-1/PD-L1 axis, and immune checkpoint inhibitor (ICI) therapy may be necessary to unveil the pro-immunogenic effect of tsMHC-II. An intriguing yet underexplored hypothesis is that induction of tsMHC-II may prompt selective pressure to upregulate immunoinhibitory molecules on tumor-infiltrating lymphocytes (TILs), such as Lymphocyte Activation Gene 3 (LAG-3), an immunoinhibitory receptor that binds MHC-II, among others. Murine models suggest a potential causative relationship between tsMHC-II expression and increased immunogenicity (Axelrod, 2019).

Recently, ubiquitination has emerged as a significant regulator of MHC-II expression in antigen-presenting cells (APCs). Cho et al. demonstrate that internalized pMHC-II undergoes efficient recycling in both mature DCs and activated B cells but not in immature DCs and resting B cells. They utilized mice with ubiquitination-defective MHC-II, revealing that the inability of pMHC-II to recycle in immature DCs or resting B cells stems from its ubiquitination by March-I. These findings illustrate that the lack of pMHC-II recycling in resting APCs does not result from an inherent deficiency in MHC-II's recycling ability in these cells but rather reflects a redirection from an efficient recycling pathway to lysosomal degradation (Cho et al., 2015).

In contrast, numerous tumors exhibit aberrant expression of MHC class II molecules. Myeloma cells, representing the malignant counterparts of plasma cells, often reactivate MHC class II genes due to the constitutive activation of transcription from pIV. MHC class II-positive myelomas may evade immune surveillance in vivo through various mechanisms, such as inducing T-cell anergy via the absence of co-stimulatory molecule expression. The presence of these highly malignant MHC class II-positive tumors suggests that the loss of MHC class II expression is not always necessary for immune evasion (Reith et al., 2005).

Interestingly, some tumors that lose expression of MHC-I may still maintain expression of MHC-II, although the functional significance of this phenomenon remains unclear. Similarly, certain melanoma cell lines, despite not expressing MHC-II in response to IFN-γ, exhibit high levels of MHC-I. These findings suggest independent regulation of MHC-I and MHC-II in cancer, with potential separate implications for cancer immunotherapy. A lingering question is the effect, if any, of tumor cell-expressed MHC-II (tsMHC-II) on CD4+ regulatory T cells (Tregs), which are often abundant and strongly immunosuppressive in tumors. Tregs rely on T cell receptor (TCR) stimulation by MHC-II for their suppressive activity. However, the favourable association of tsMHC-II with improved immune-mediated outcomes suggests that tsMHC-II may fail to activate Tregs, although solid evidence for this is lacking (Axelrod, 2019).

Expression of MHC-II on tumor cells fosters antitumor immunity and facilitates the infiltration of CD4+ T cells, leading to increased expression of CXCR3-binding cytokines involved in T cell recruitment. However, as tumors adapt to this microenvironment, either during cancer progression or in response to immunotherapies targeting the PD-1/L1 axis, they acquire immunosuppressive signals through alternative checkpoints that counteract MHC-II expression, such as LAG-3. The presence of MHC-II expression in tumor cells initially promotes an antitumor immune milieu characterized by the recruitment of CD4+ T cells, but ultimately leads to the engagement of PD-1 and LAG-3, which suppress antitumor immunity (Johnson et al., 2018).

Expression of tsMHC-II has been linked to improved progression-free (PFS) and overall survival (OS) in melanoma and classic Hodgkin lymphoma patients treated with anti-PD-1/anti-PD-L1 therapy, but not with anti-CTLA-4 therapy. This suggests that increased expression of MHC-II or related pathway components by tumor cells or in the bulk tumor population is associated with better prognosis and enhanced antitumor immunity. Consequently, there is a hypothesis that strategies to increase tsMHC-II may be therapeutic, especially when combined with immunotherapies. Studies in mouse models have explored tsMHC-II by multiple groups, with most demonstrating that transduction of tumor cells with ectopic MHC-II or CIITA increases immune-mediated tumor rejection. However, some conflicting reports suggest that MHC-II or CIITA has no effect or, in certain cases, accelerates tumor growth (Axelrod, 2019).

The concept of enhancing immunogenicity in tumor cells through MHC class II expression has raised hopes for bolstering antitumor immune responses by utilizing tumor cells rendered MHC class II positive via CIITA expression vectors. While this approach did not yield success in a model of lung carcinoma, it enhanced tumor immunogenicity, prompted rejection, and triggered tumor-antigen-specific immune memory in a mouse model of mammary adenocarcinoma. Encouraging outcomes were also observed with the combination of CIITA overexpression and downregulation of Ii expression. These MHC class II-positive, Ii-negative tumor cells demonstrated both prophylactic and therapeutic potential in primary and metastatic cancer studies in mice. Additionally, a promising anticancer strategy involves therapeutic monoclonal antibodies targeting MHC class II molecules. However, the effectiveness of this novel treatment may benefit from a deeper understanding of the regulation of MHC class II expression in cancer cells (Reith et al., 2005).

Utilizing CCLE data, Zeng et al. demonstrated that skin cancer exhibits the highest expression of MHC-II, although MHC-II expression is also observed in other cancer types. Cancer cell-intrinsic MHC-II expression emerges as a crucial determinant for the antitumor immune response, suggesting future endeavours should aim at targeting cancer cells through enhancing their intrinsic MHC-II expression (Zeng et al., 2022).

Aims and objectives of thesis.

OBJECTIVE: The objective of this proposal is to study how tumor immunosuppressive microenvironment promotes longer survival in $BAP1^{+/-}$ patients as we hypothesize that immune cells in $Bap1^{wt}$ and $Bap1^{+/-}$ have differences in the immune cells on functional levels.

AIM 1. To. Characterize the immune cell populations at the basal level and in the tumor microenvironment of $Bap1^{wt}$ and $Bap1^{+/-}$ mice.

AIM 2. To determine if these differences explain the prolonged survival of $Bap1^{+/-}$ individuals.

Chapter 2: Material and Methods

2.1 Material and Methods

This section describes the methods used within this specific study and any observed changes as compared to the general detailed methods explained in chapter 2.

2.1.1

In vivo models

We adhered to the guidelines set forth by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (http://www.aaalac.org), and obtained approval from the University of Hawaii Institutional Animal Care and Use Committee (Protocol #) for all animal care and experiments. Bap1$^{+/-}$ constitutive mice (C57BL/6 background) were utilized and bred from mice containing *loxP* sites flanking *BAP1* exons 4 and 5, and mice expressing a constitutive general Cre delete. While homozygous Bap1 deficiency in mice leads to embryonic lethality, Bap1$^{+/-}$ mice are viable and in good health. Bap1$^{+/-}$ mice exhibited approximately half the Bap1 protein expression in relevant tissues compared to their wild type littermates. Throughout the experiments, all mice were housed under a 12-hour light-dark diurnal cycle and provided with standard rodent chow, water and libitum.

Isolation of immune cells

Mice received an intraperitoneal injection of 1 million AN1 luciferase positive Bap1 null tumor cells. Tumor growth was assessed by administering luciferin and imaging with IVIS on day 15. On day 20, mice were euthanized using carbon dioxide, and tumors were collected. Tumor immune cells were isolated using the TAM isolation protocol. Tumors were weighed and digested with collagenase IV in serum-free medium for 45 minutes. The tumors were minced and filtered through a 70-micron filter. Cells were harvested, and red blood cells (RBCs) were lysed. Immune cells were isolated using the Ficoll density centrifugation method.

Flow Cytometry

Tumor-derived cells were placed into individual wells of a 96-well plate and treated with a viability dye (LIVE/DEAD zombie dye, Biolegend) for 20 minutes. Following staining, the cells underwent a wash with FBS, followed by blocking of Fc receptors using TruStain fcX (anti-mouse CD16/32) antibody (Biolegend) in 5% FBS for 10 minutes on ice. Phenotyping of immune cells was conducted by centrifuging and resuspending 1×10^6 tumor immune cells with antibodies for 30 minutes on ice in the absence of light. Post-staining, cells were washed twice with 1x PBS, and their fluorescence was measured using an Attune Nxt Flow Cytometer (Invitrogen). Analysis of results was carried out using FlowJo 11 (Becton Dickinson). Antibody concentrations for flow cytometry experiments were determined through titration experiments, and BD Comp Beads (BD Biosciences) were employed for compensation. Positivity gates were established using the fluorescence-minus-one (FMO) technique.

qRT-PCR analysis

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, #74134) and assessed through spectrophotometric analysis for quantification. Subsequently, complementary DNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen, catalogue number 4368814) as per the manufacturer's guidelines. Quantitative PCR (qPCR) was conducted in triplicate using TaqMan Universal Master Mix II (Invitrogen, catalogue number 4440040) along with TaqMan Probes (Invitrogen) on a Step One Plus system (Applied Biosystems). The samples were subjected to triplicate analysis, and mRNA levels were determined using the comparative Ct method (ΔΔCt method) after normalization with the geometric mean of two reference genes (GAPDH and ACTB).

Western blot analysis

Western blot analysis was employed to assess protein expression levels. Cell lysates were prepared using M-PER reagent (Thermo Fisher Scientific, 78501), supplemented with protease and phosphatase inhibitors (2 mM Na3VO4, 2 mM NaF, 1 mM PMSF, and a protease inhibitor cocktail, along with 1 mM DTT), and quantified via the Bradford assay (Bio Rad Laboratories). The protein lysates were separated by SDS-PAGE electrophoresis, transferred onto PVDF membranes, and then subjected to incubation with primary antibodies. Following this, peroxidase-conjugated secondary antibodies were applied, and the signals were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA) following the manufacturer's instructions, utilizing an Amersham Imager 680 (GE).

Positive selection of F4/80 positive tumor immune cells

Tumor immune cells (Lymphocytes and monocytes) were collected using Ficoll density centrifugation method. Tumor associated macrophages were isolated using Octomacs kit (130-042-108 Miltenyi). The isolated cells were resuspended in the degassed macs buffer and incubated with anti F4/80 microbeads (130-110-443 Miltenyi) for fifteen minutes followed by 10 minutes centrifugation at 500g. The cells were resuspended in 500 uL buffer and passed through prewashed MACS columns.

Unlabelled cells were passed through the filter coloumn, and positively labelled F4/80 cells were collected from the filters using a plunger.

Image analysis

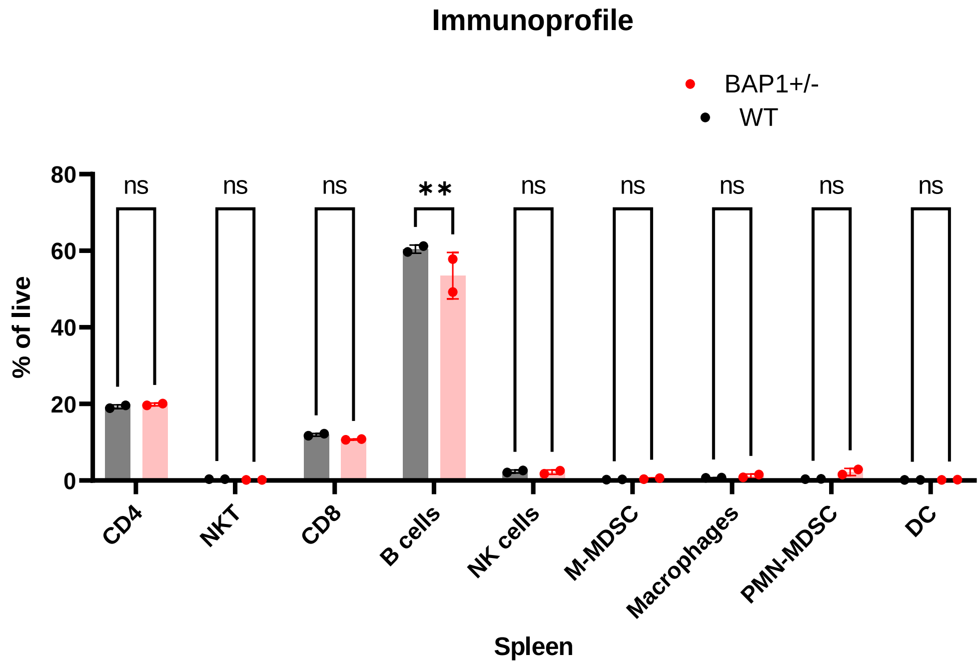
For histological examination, tumors were first treated with 10% buffered formalin, then dehydrated in ethanol, embedded in paraffin, and finally stained with haematoxylin and eosin. Immunohistochemistry (IHC) utilized antibodies targeting MHCII (Thermofischer), FOXP3 (Thermofischer), and F4/80 (14-4801-81 eBioscience). Images were captured using Leica LAS X microscope software and averaged across 25 distinct regions per tumor section.

Chapter 3. Results

3.1 No differences were observed in the immune cell population between Bap1^{wt} and Bap1$^{+/-}$ mice on the basal levels as seen in spleen, peritoneum, and bone marrow.

Cells were collected from spleen, bone marrow and peritoneum (lavage) from Bap1^{wt} and Bap1$^{+/-}$ mice and readout outs were done by flow cytometry for Immunoprofiling Bap1$^{+/-}$ and Bap1^{wt} mice. We speculated differences in the basal immune cell population between Bap1^{wt} and Bap1$^{+/-}$ mice.

(a)



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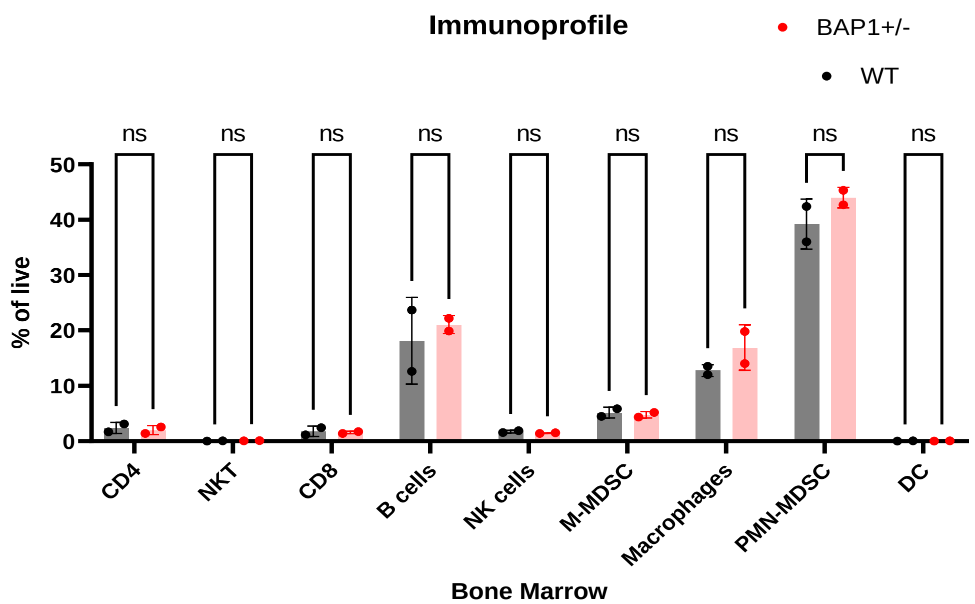

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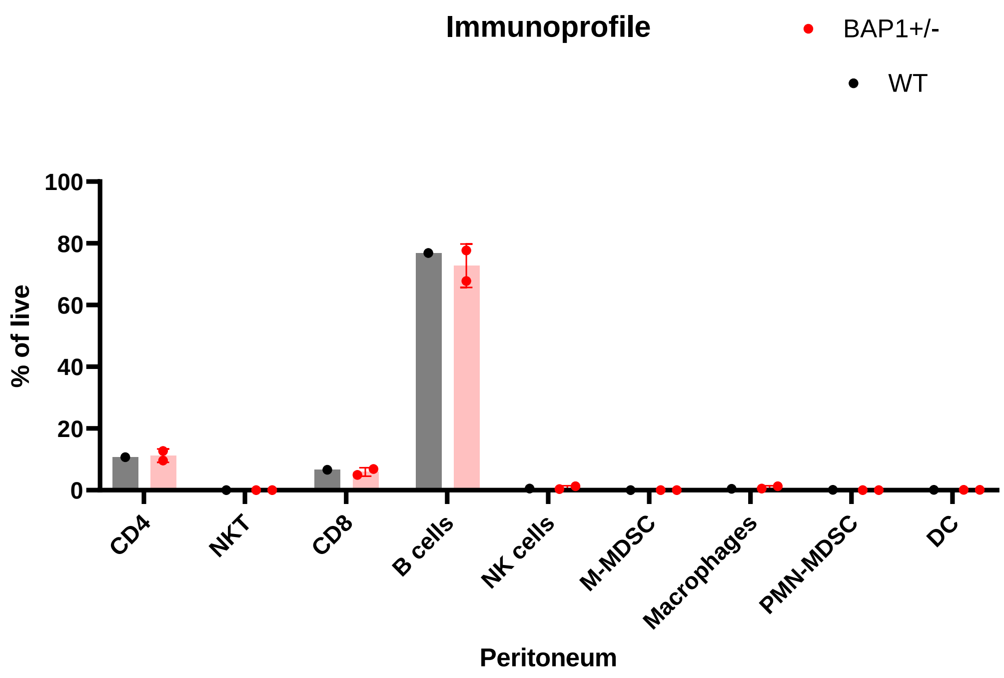


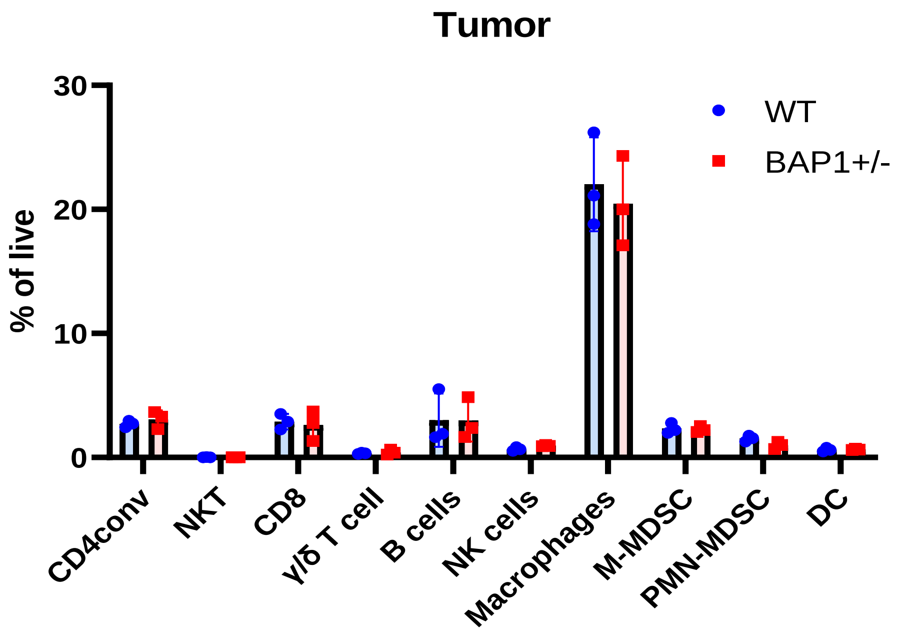
Figure 1: (a), (b), (c) comparison of different immune cell populations in the spleen, bone marrow and peritoneum from Bap1$^{+/-}$ and Bap1^{wt} mice.

Conclusion: No difference were observed in the immune cell population between Bap1^{wt} and Bap1$^{+/-}$ in the spleen, bone marrow and peritoneum.

3.2 No differences were observed in the tumor immune cell population in Bap1^{wt} and Bap1$^{+/-}$ mice.

Six-month-old female mice (n=3 Bap1^{wt}, n=3 Bap1$^{+/-}$) were injected 1 million of AN1 Bap1 null tumor cell line (Bap1$^{-/-}$) biallelic inactivated for Bap1. IVIS was performed on Day15 with a positive peritoneal signal. Mice were sacrificed and tissues were harvested on day 25. Spleen was used as a control. Tumors were minced and immune cells were extracted using Ficoll density gradient centrifugation method. Readout was done by Flow cytometry for Immunoprofiling. We speculated differences in the tumor immune cell population amongst the two genotypes.

(a)



(b)

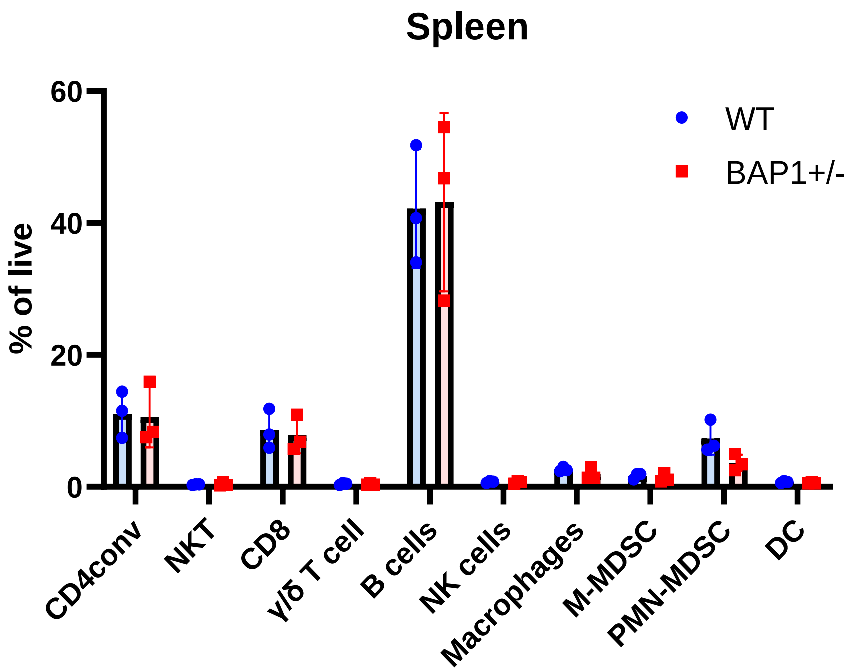


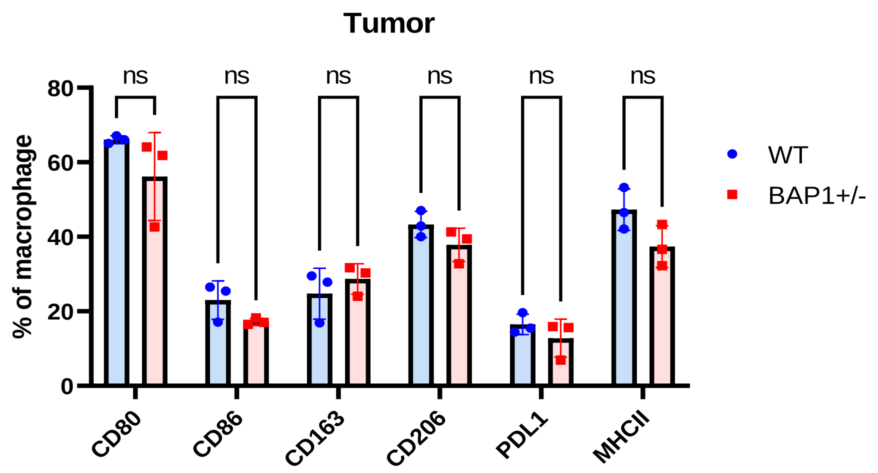
Figure 2: (a), (b) comparison of different immune cell populations in Tumor and spleen samples in Bap1^{wt} and Bap1$^{+/-}$ mice.

Conclusion: No differences were observed in the tumor immune cell population in Bap1^{wt} and Bap1$^{+/-}$ mice.

3.3 No difference was observed in the M1 macrophage (CD86, CD80) and M2 macrophage (CD163, CD206) cell population in Bap1^{wt} and Bap1 $^{+/-}$ tumor and spleen samples. Reduction of MHCII expression was observed on macrophage MFI values in Bap1$^{+/-}$ tumor samples as compared to Bap1^{wt}.

Six-month-old female mice (n=3 Bap1^{wt}, n=3 Bap1$^{+/-}$) were injected 1 million of AN1 Bap1 null tumor cell line (Bap1$^{-/-}$) biallelic inactivated for Bap1. IVIS was performed on Day15 with a positive peritoneal signal. Mice were sacrificed and tissues were harvested on day 20. Spleen was used as a control. Tumors were minced and immune cells were extracted using Ficoll density gradient centrifugation method. Readout was done by Flow cytometry for M1 and M2 macrophage markers, PDL-1 and MHCII expression.

(a)



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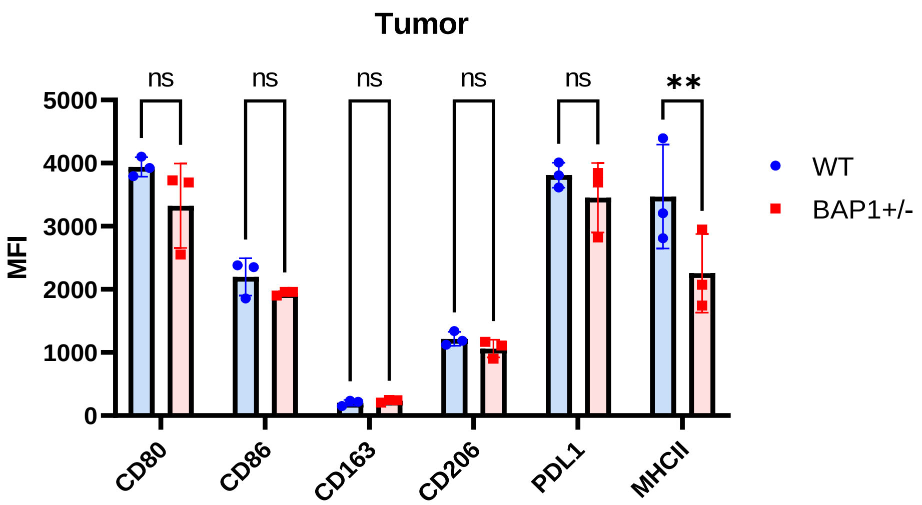

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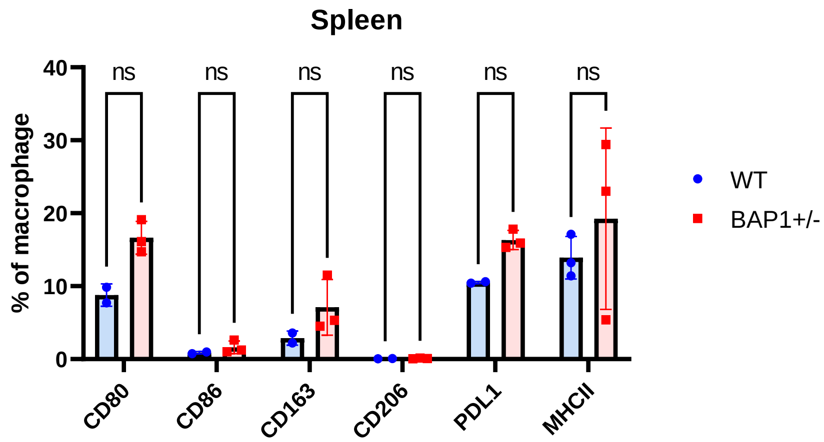


(d)

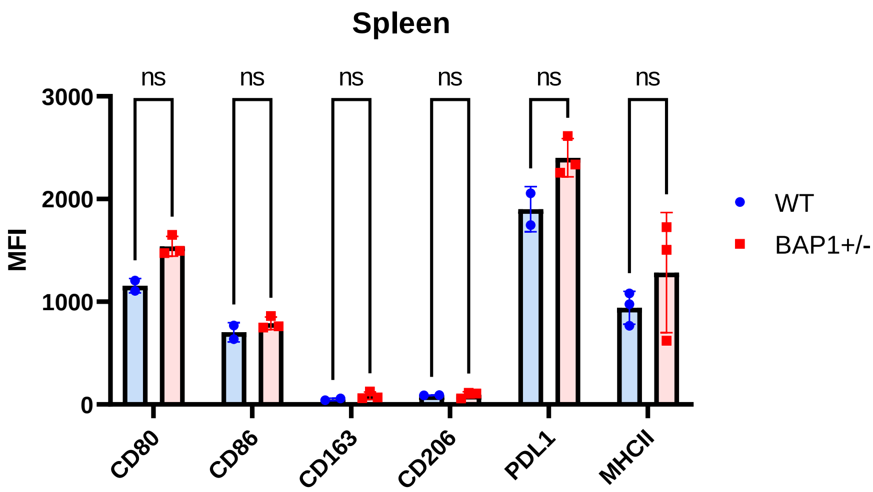
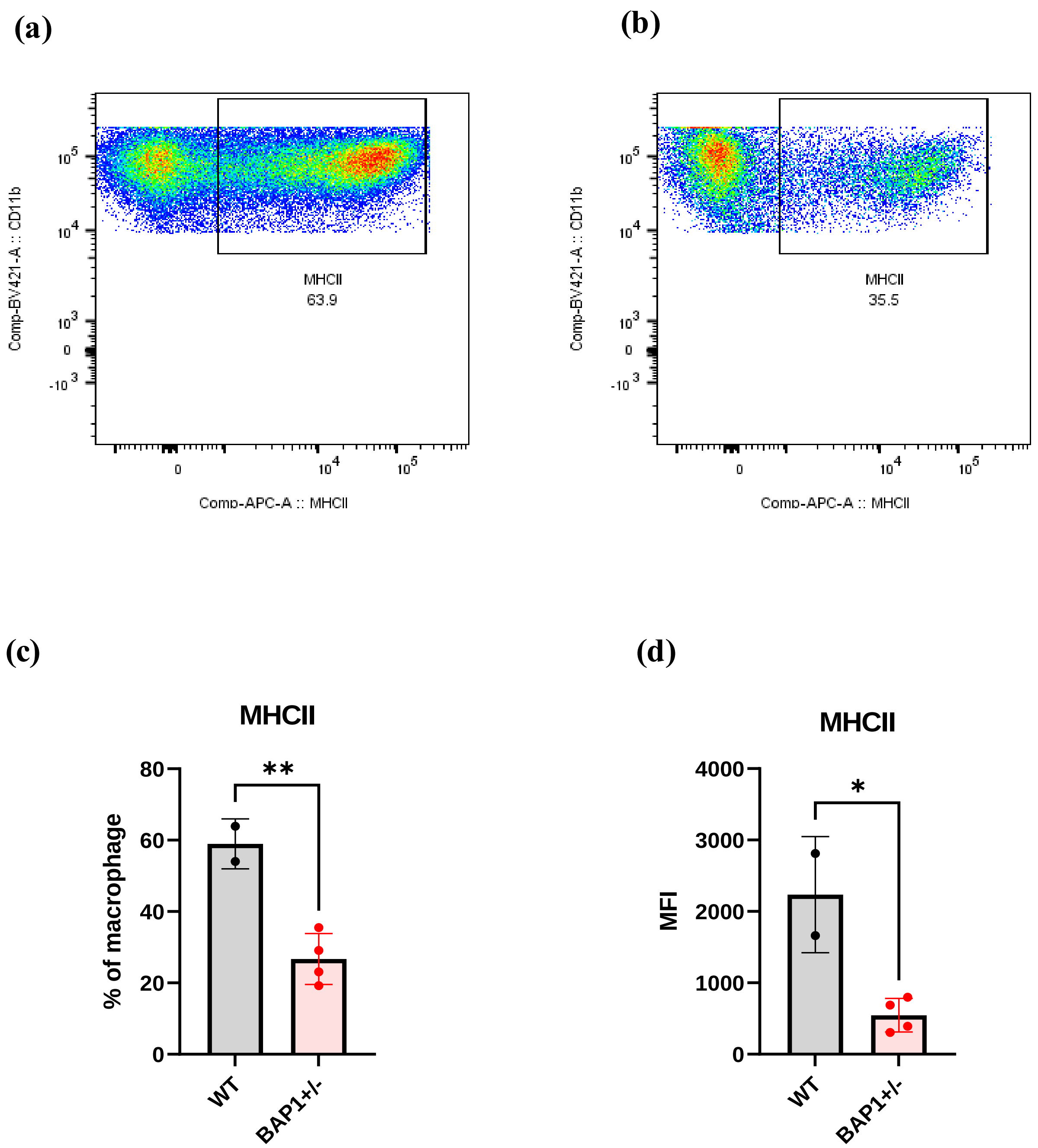


Figure 3: (a), (b) Percentages and Mean Fluorescence Intensity values for macrophage population in tumor. (c), (d) Percentage and MFI value of macrophages in spleen in Bap1 ^{wt} and Bap1 $^{+/-}$ mice.

Conclusion: MFI values show reduced expression of MHCII in the tumor associated macrophages in Bap1 $^{+/-}$ mice as compared to Bap1 ^{wt} mice.

3.4 MHCII reduction in Bap1$^{+/-}$ mice does not depend on mice age and found to be true for eight weeks old younger mice as observed previously in six months old mice. B cells and DC's do not show reduction of MHCII in Bap1$^{+/-}$ mice.

Eight weeks-old female mice (n=3 Bap1^{wt}, n=3 Bap1$^{+/-}$) were injected with 1 million of AN1 Bap1 null tumor cell line (Bap1$^{-/-}$) biallelic inactivated for Bap1. IVIS was performed on Day15 with a positive peritoneal signal. Mice were sacrificed and tissues were harvested on day 20. Spleen was used as a control. Tumors were minced and immune cells were extracted using Ficoll density gradient centrifugation method. Readout was done by Flow cytometry for MHCII expression.



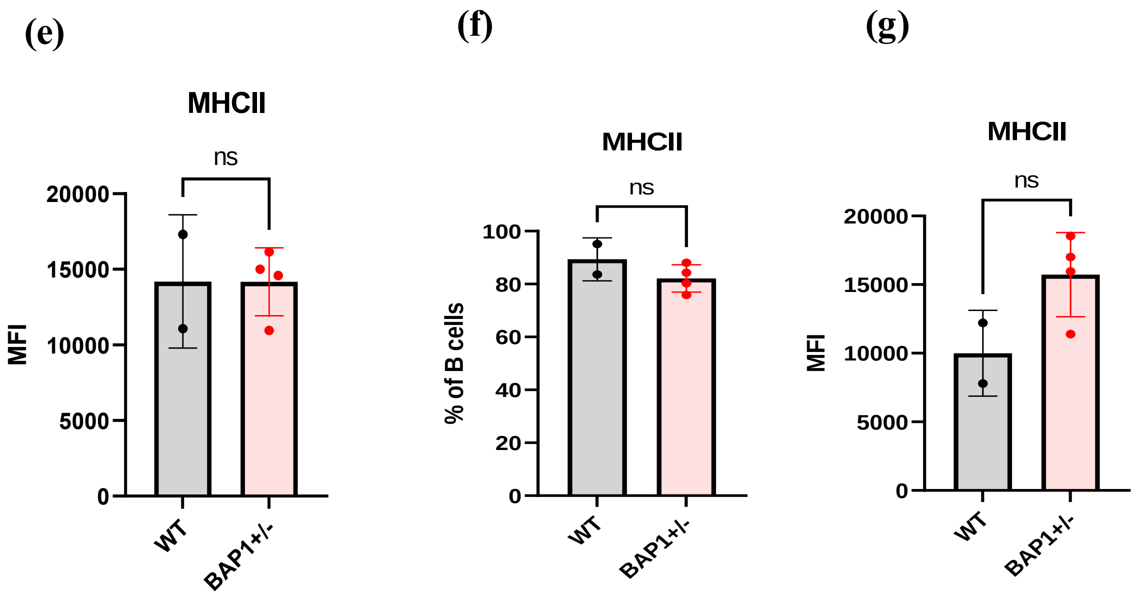
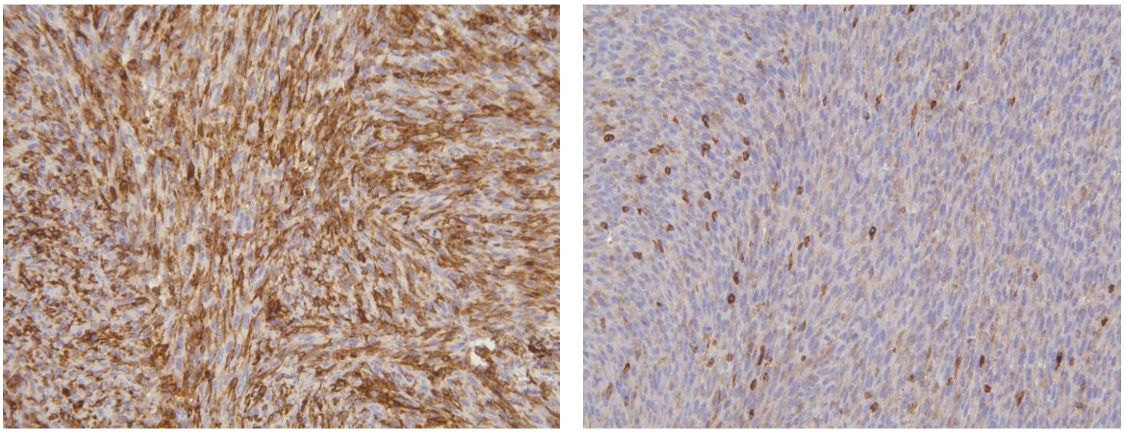


Figure 4: (a) and (b) raw values of MHCII expression in Tumor associated macrophages. (c) and (d) graphical representation of MHCII with percentage of tumor associated macrophages and MFI values. (e) and (f) percentage and MFI values of live B cells in tumors. (g) MFI values of live DC in tumors which are positive for MHCII expression in $Bap1^{wt}$ and $Bap1^{+/-}$ mice.

Conclusion: Tumor associated macrophages shows reduced expression of MHC II in TAMs in $Bap1^{+/-}$ mice as compared to $Bap1^{wt}$ mice. No difference was observed in the MHC II expression in tumor associated B cell and Dendritic cell population in $Bap1^{+/-}$ and $Bap1^{wt}$ mice.

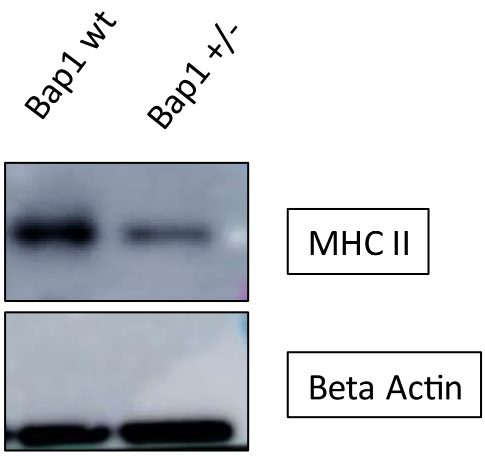
3.5 Immunohistochemistry and Immunoblot show reduced expression of MHCII in MM tumors obtained from Bap1$^{+/-}$ mice in comparison to Bap1^{wt} mice.

(a)



Bap1 ^{wt} Bap1 $^{+/-}$

(b)



<u>Figure 5</u>: (a) Reduced expression of MHCII positive tumor cells by immunohistochemistry (b) Reduced expression of MHCII in Bap1$^{+/-}$ as compared to Bap1^{wt} by western blot analysis in tumor immune cell population.

<u>Conclusion:</u> Bap1$^{+/-}$ tumor immune cells have reduced MHC II expression to Bap1^{wt}.

3.6 No differences were observed in Bap1^{WT} and Bap1$^{+/-}$ T regulatory cell populations and IFNY expression levels.

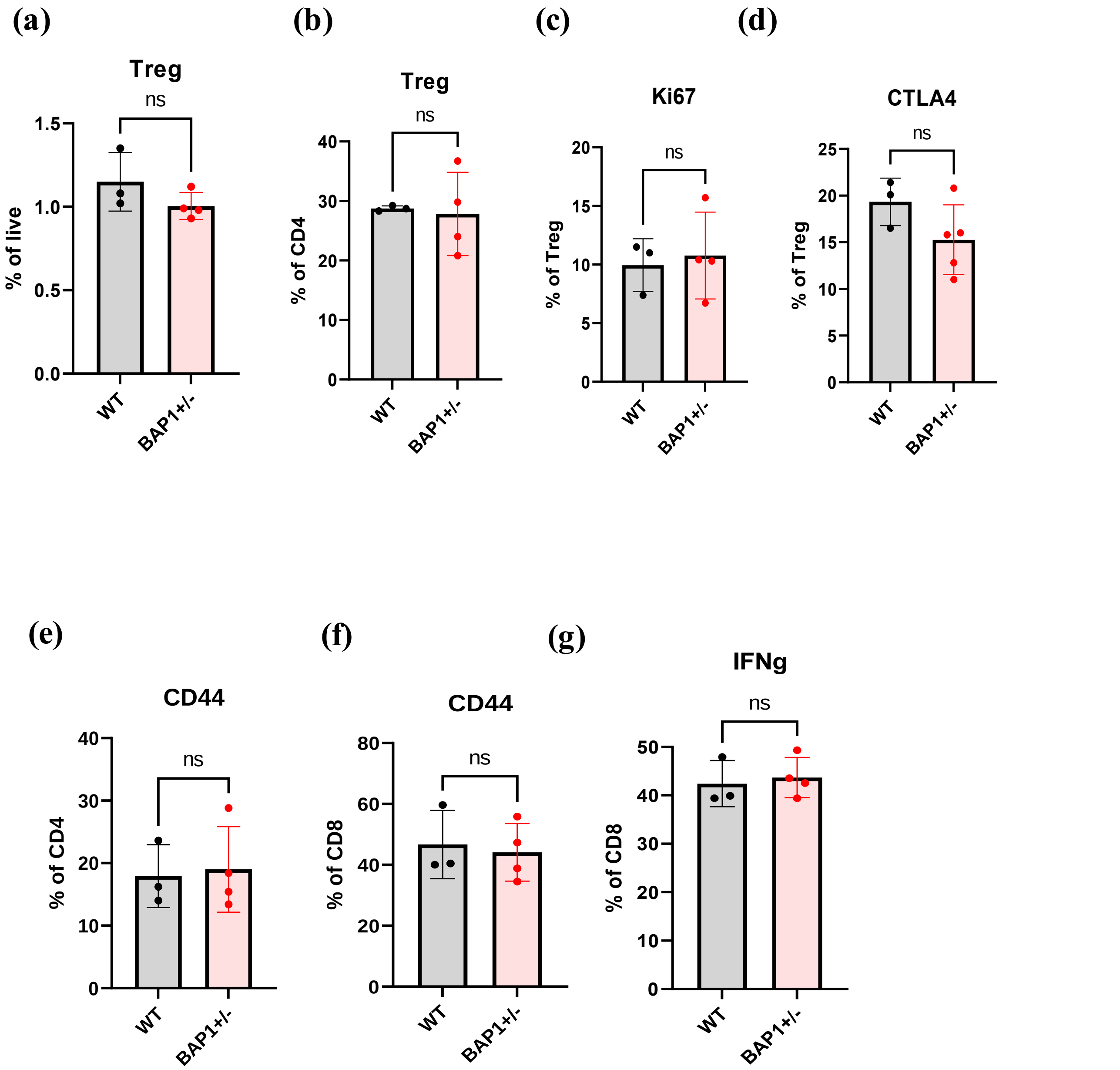


Figure 6: (a), (b), (c), (d) Percentage of live cells, percentage of CD4 positive cells, proliferation marker Ki67 and exhaustion marker CTLA-4 for T regulatory cells. (e), (f) CD4 positive and CD8 positive for CD44 activation marker in tumor cell population (g) Comparison of IFNγ expression in Bap1 $^{+/-}$ and Bap1 ^{wt} mice.

Conclusion: No differences were observed in the T regulatory cells population and IFNγ expression in Bap1$^{+/-}$ and Bap1^{wt} mice.

3.7 Bap1$^{+/-}$ show reduce expression of Ly6C, TREM2 and CD9 as compared to Bap1^{wt}

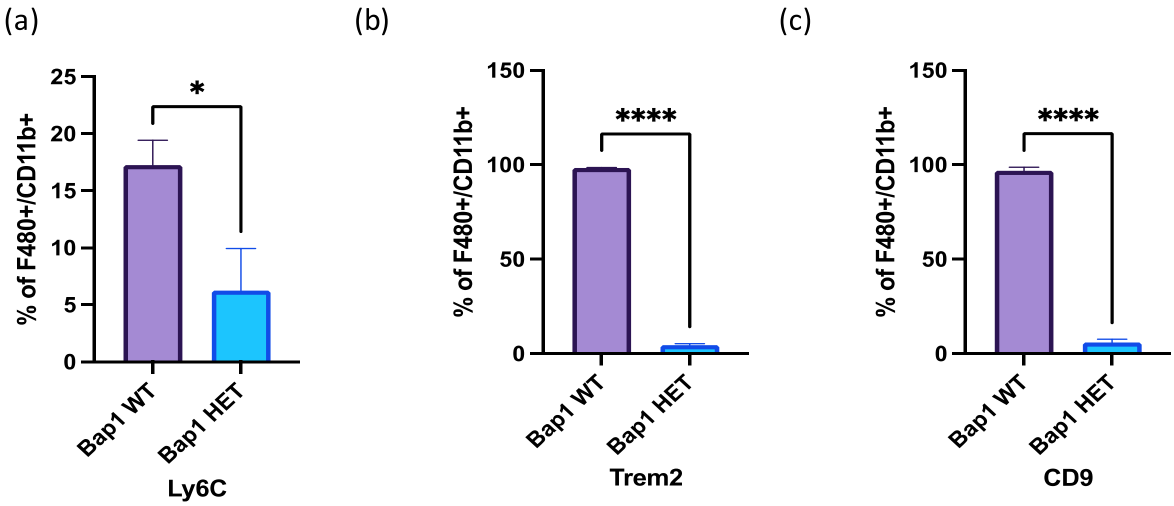


Figure 7: Comparison of tumor facilitating macrophage markers (a) LY6c (b) Trem2 (c) CD9

Conclusion: Bap1$^{+/-}$ tumor immune cells have reduced Ly6c, Trem2 and CD9 expression as compared to to Bap1^{wt}

Chapter 4: Discussion and conclusion

For years, scientists have grappled with understanding how asbestos causes cancer (Carbone et al.,2019, Carbone et al.,2004, Mossman et al.,1990). Research has revealed that exposure to asbestos leads to an inflammatory response, predominantly characterized by the differentiation of mononuclear phagocytes into macrophages. These macrophages ingest asbestos fibres, subsequently releasing reactive oxygen species and various cytokines, which have been identified as mutagenic agents (Yang et al., 2006; Cersosimo et al., 2021). Our lab and others have shown that erionite, crocidolite, and chrysotile asbestos can induce programmed cell necrosis in human mesothelial cells (HM), resulting in the release of high mobility group box 1 (HMGB1) protein into the extracellular environment. HMGB1 serves as a proinflammatory mediator, initiating inflammation, activating the inflammasome pathway, and perpetuating chronic inflammatory processes. These processes culminate in the release of tumor necrosis factor alpha (TNFα) and other mutagenic cytokines, ultimately causing DNA damage that may lead to malignant transformation (Xue et al., 2020 June et al.,2012., Yang et al.,2010., Qi et al.,2013).

The primary inherited risk factor linked to mesothelioma is the dysfunction of BRCA1 Associated Protein 1 (BAP1), a deubiquitinating enzyme located on chromosome 3p21.1, acting as a tumor suppressor gene (Hiltbrunner et al., 2021). Our laboratory discovered in 2011 that carriers with heterozygous germline BAP1 mutation (BAP1$^{+/-}$) developed mesothelioma and uveal melanoma. Subsequent research by multiple teams confirmed and expanded upon these findings (Carbone et al., 2020; Carbone et al., 2019; Kobrinski et al., 2020).

People with inherited BAP1 mutations face an increased risk of developing MM. However, they appear to have significantly better survival rates of 5-10 years, show improved responses to chemotherapy, and have less aggressive and invasive tumors compared to those without the mutation (Carbone et al.,2022; Pastorino et al., 2018; Baumann et al., 2015; Hassan et al., 2019). Furthermore, the extended survival observed in individuals correlate with complete loss of BAP1. This is because 60-70% of MM cases develop somatic BAP1 mutations, and these patients also experience modest survival improvement (Yuce et al.,2024; Farzin et al.,2015; Ghafoor et al.,2022; Louw et al.,2022)

Additionally, MM is generally associated with professional exposure to asbestos. However, to date, we found that none of the mesothelioma patients carrying germline BAP1 mutations were professionally exposed to asbestos. Therefore, we hypothesized that germline BAP1 mutations might influence the asbestos-induced inflammatory response that is linked to asbestos carcinogenesis, thereby increasing the risk of developing mesothelioma after minimal exposure. Using a Bap1$^{+/-}$ mouse model, our laboratory found that, compared with their wild-type littermates, Bap1$^{+/-}$ mice exposed to low-dose asbestos fibres showed significant alterations of the peritoneal inflammatory response, and lower levels of several chemokines and cytokines. Consistent with these data, Bap1$^{+/-}$ mice had a significantly higher incidence of mesothelioma after exposure to very low doses of asbestos doses that rarely induced mesothelioma in wild-type mice (Napolitano et al.,2016) Additionally, Napolitano et al showed that Bap1$^{+/-}$ mice have more M2 type macrophages as compared to Bap1^{WT} mice. This puzzled us even more since M2 macrophages promote immunosuppressive microenvironment (Wang et al., 2021) and should have adverse effects on therapies but we see the opposite response in BAP1 mutant patients (Buamann et al.,2015; Frazin et al., 2015; Pastorino et al., 2018; Baumann et al., 2015; Hassan et al., 2019).

We speculated if asbestos had an effect for the observed results. We created a mouse model from mesothelioma derived luciferase integrated cancer cell line (AN1) from Bap1$^{+/-}$ mice which was biallelic inactivated for BAP1 as found in 60% of BAP1 mutant MM patient tumors to understand how BAP1 loss in tumour impact the tumour microenvironment in the absence of asbestos exposure. Studying the TME in the context of germline BAP1$^{+/-}$ and BAP1^{WT} mice seems a promising avenue and could provide a critical insight into how the microenvironment impacts tumor behaviour and patient outcomes.

Here, I injected Bap1^{WT} and Bap1$^{+/-}$ with AN1 (Bap1$^{-/-}$) biallelic inactivated mesothelioma cell line. Tumors were digested and tumor immune cells (including TAMs) were collected by using Ficoll density centrifugation method. Harvested cells were immunoprofiled by flow cytometry but I did not find any significant differences in the immune cell population amongst the two genotypes i.e. Bap1$^{+/-}$ and Bap1$^{WT.}$ Additionally, I did not find any differences in the T cell population (CD8+, CD4+, their activation states and Tregs) and IFNY levels by flow cytometry. But I found that tumor associated macrophages in Bap1$^{+/-}$ showed reduced levels of MHCII expression as compared to Bap1^{WT} and not in other antigen presenting cells i.e. dendritic cells, B cells and macrophages outside of the TME. Additionally, I found that markers related to aggressive tumor progression i.e. Trem2, CD9 and Ly6C were significantly reduced on TAM's in Bap1$^{+/-}$ as compared to Bap1^{WT} mice. This suggests that factors beyond the biallelic inactivation of BAP1 might contribute to survival outcomes. These findings imply that while alterations in BAP1 status i.e. partial or complete loss are associated with MM development, the relationship between BAP1 status and survival outcomes is more complex and possibly influenced by other factors or molecular pathways. In conclusion my research lead to the following findings:

Mice carrying heterozygous BAP1 mutations ($BAP1^{+/-}$) and injected with the BAP1 null mouse mesothelioma cell line AN1, contained TAM population with reduced expression of MHC II., in addition:

1. No differences in MHC class II were observed among these two different strains of mice in their macrophages located outside of the TME.
2. No differences were observed in the T cell population in the $Bap1^{+/-}$ and $Bap1^{wt}$ mice.
3. $Bap1^{wt}$ mice have increased levels of markers related to aggressive tumor progression (Ly6C, CD9 and TREM-2) on TAMs as compared to $Bap1^{+/-}$ mice.

Chapter 5. Future Perspectives

My findings have shed light on a novel avenue for comprehending the differences in the tumor microenvironment between Bap1^{wt} and Bap1$^{+/-}$ mice. In the future, it would be advantageous to explore whether there is a decrease in MHCII in germline human Bap1$^{+/-}$ tumors similar to what has been observed in mice. Furthermore, we aim to elucidate the mechanisms underlying the reduction of MHCII in Bap1$^{+/-}$ mice and its potential impact on treatment response and survival. I hypothesize that Bap1 interacts within the nucleus to deubiquitylate the class II major histocompatibility complex transactivator (CIITA), which governs MHCII expression in antigen-presenting cells. Consequently, diminished Bap1 levels leads to increased degradation of *Ciita* within the nucleus, resulting in reduced MHCII expression in Bap1$^{+/-}$ mice. Previous research by Buxade et al demonstrated that the transcription factor NFAT5 is essential for *Ciita* and MHCII expression in macrophages, with NFAT5-deficient macrophages exhibiting impaired activation of MHCII-dependent responses in CD4^{+} T lymphocytes, but not in dendritic cells and other antigen-presenting cells (Buxade et al.,2018).

Research indicates that secreted metabolites like lactate, create gradients that convey spatial information about tumor metabolism, influencing the polarization of macrophages. These gradients also trigger varying activation levels in signalling pathways, notably the Kras/MAPK pathway, within macrophages associated with tumors. Consequently, lactate levels within tumor environments govern signalling activities and the polarization of M2 macrophages (Mehla et al., 2019). Lactate promotes M2 macrophage polarization through the activation of the ERK/STAT3 signalling pathway (Mu et al., 2018). Persistent inflammation in the TME can lead to macrophage accumulation within the tumor. Higher concentrations of lactic acid tend to attract fewer macrophages to the tumor site. Indeed, elevated lactic acid levels impede monocyte migration in vitro. While macrophages struggle to infiltrate tumors that produce substantial lactic acid, they can still adopt an M2-like phenotype within such environments (Ohashi et al., 2017). Our findings have revealed significant differences in glycolysis and TCA cycle metabolites between BAP1^{wt} and BAP1$^{+/-}$ fibroblast cell cultures. BAP1$^{+/-}$ cells exhibit heightened glucose consumption and lactate release in the culture medium, indicating a faster glycolytic rate compared to BAP1^{wt} cells (Bononi et al.,2017). To further investigate this phenomenon within the Bap1 tumor microenvironment, I propose conducting lactate assays on tumor-derived macrophages from both Bap1^{wt} and Bap1$^{+/-}$ tumors in mice.

I would further like to elucidate how the reduction of MHCII and the germline Bap1 status contribute to improved survival and treatment outcomes for mesothelioma patients with Bap1 mutations. To investigate this, I propose to conduct experiments on mice employing either of the two strategies. One approach involves injecting $Bap1^{wt}$ and $Bap1^{+/-}$ mice with tumor cells with AN1 cell line ($Bap1^{-/-}$) and subsequently treating both the groups ($Bap1^{WT}$ and $Bap1^{+/-}$) with a CD-40 monoclonal antibody, known to elicit a potent immune response in mice mimicking immunotherapy in humans.

In summary, my work sheds light on a novel direction for understanding the differences in the tumor microenvironment between $Bap1^{wt}$ and $Bap1^{+/-}$ mice, offering new insights for Bap1 mutants predisposed to early mesothelioma development.

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