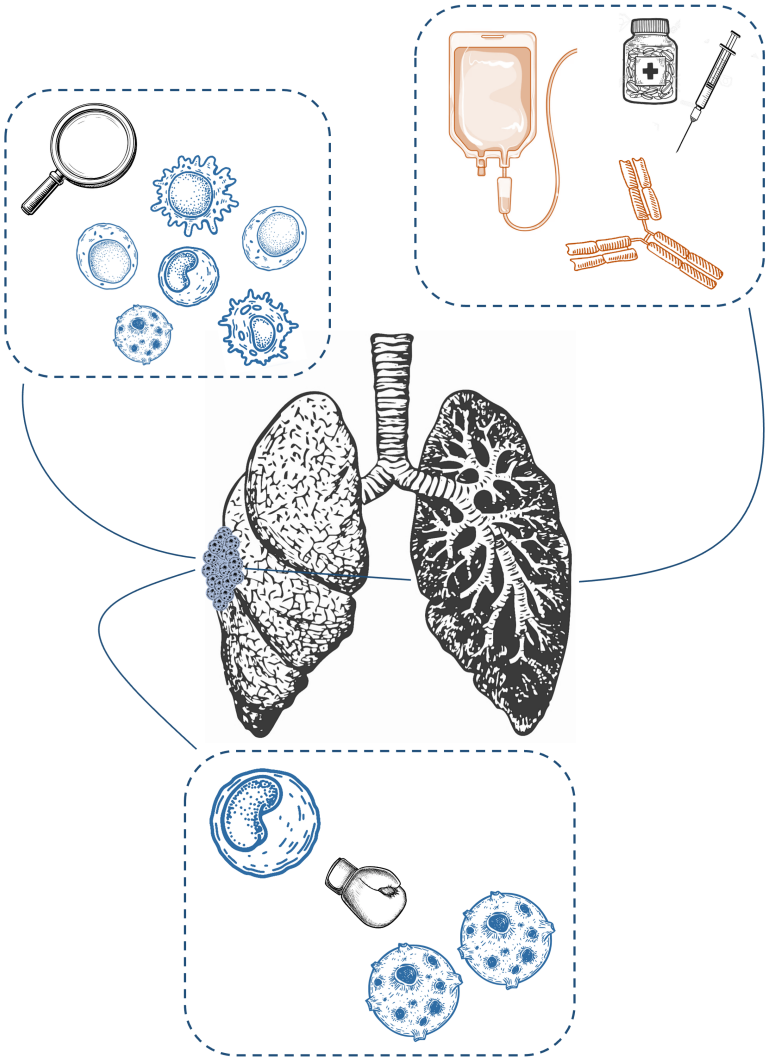
LIÈGE université
Gembloux
Agro-Bio Tech

Tumor microenvironment heterogeneity and novel therapeutic approaches in pleural mesothelioma



Alexis Fontaine

COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Tumor microenvironment heterogeneity and novel therapeutic approaches in pleural mesothelioma

Alexis FONTAINE

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteur: Luc Willems
Année civile: 2024

Abstract

Pleural mesothelioma (PM) is a rare and aggressive cancer with a poor prognosis, primarily caused by asbestos exposure and originating from the mesothelial cells of the pleura. For almost two decades, the standard first-line treatment for unresectable PM has involved a combination of cisplatin or carboplatin with pemetrexed. More recently, immunotherapy with immune checkpoint inhibitors (ICIs) has emerged as a frontline option, especially for the sarcomatoid subtype. However, despite these advancements, the benefits remain limited, with only a subset of patients responding to ICIs and a modest median overall survival improvement. Consequently, a new treatment paradigm combining chemotherapy with ICIs is being explored in various clinical trials, although results have been disappointing so far. The underlying biological mechanisms driving this general lack of response remain poorly understood, with emerging evidence nevertheless pointing to the crucial influence of the tumor microenvironment.

In this context, this thesis project aimed to deepen our understanding of the immune landscape in PM, with a particular focus on tumor-associated macrophages (TAMs), using single-nucleus RNA sequencing of PM biopsies. Simultaneously, the project sought to develop innovative therapeutic strategies by exploring two approaches: reprogramming monocytes with epigenetic modulators and evaluating the synergistic potential of metronomic chemotherapy (mCT) combined with doublet immunotherapy in preclinical models.

Through the snRNA-seq analysis of PM patient biopsies, the main immune cell types previously described in PM were identified, and the characterization of PM TAMs was significantly expanded. Notably, an association between hypoxic TAMs and poor survival in PM patients was identified. TGF-β3 emerged as a potential driver of the hypoxic TAM phenotype, suggesting novel therapeutic approaches. Moreover, comparative analysis with non-tumoral macrophages revealed a global angiogenic signature among TAMs, with CD93 and SPP1 identified as potential therapeutic targets.

In a complementary study, it was demonstrated that blood-derived monocytes exhibited inherent cytotoxic activity against PM cells, at a low level. Treatment with valproic acid (VPA), a histone deacetylase inhibitor, significantly enhanced this cytotoxicity as well as monocyte migration and their aggregation with tumor cells. Additionally, VPA down-regulated key receptors associated with an M2-like phenotype, such as CD163, CD206, and CD209, suggesting VPA as an interesting compound to improve PM treatment outcomes, through modulation of monocyte cytotoxicity.

Lastly, a preclinical study demonstrated that various chemotherapeutic agents administered on a metronomic schedule effectively reduced tumor growth in syngeneic preclinical PM models. Surprisingly, mice treated with a combination of ICIs and metronomic cisplatin and pemetrexed demonstrated heterogeneous responses, with stabilization of tumor growth observed in some cases. Notably, this variability in re-

sponse did not correlate with differences in immune cell infiltration at the end of treatment, underscoring the necessity for further investigation into the underlying mechanisms driving this response variability.

Résumé

Le mésothéliome pleural (MP) est un cancer rare et agressif, au pronostic défavorable, principalement causé par l'exposition à l'amiante et se développant à partir des cellules mésothéliales de la plèvre. Pendant près de deux décennies, le traitement de première ligne pour le MP non résécable a reposé sur une combinaison de cisplatine ou de carboplatine et de pemetrexed. Plus récemment, l'immunothérapie par inhibiteurs de points de contrôle immuns (ICI) s'est imposée comme une option de première ligne, notamment pour le sous-type sarcomatoïde. Cependant, malgré cette avancée majeure, les bénéfices demeurent limités : seule une fraction des patients répond aux ICIs, et l'amélioration de la survie globale reste modeste. En conséquence, un nouveau paradigme thérapeutique combinant chimiothérapie et ICIs est actuellement exploré dans différents essais cliniques, bien que les résultats obtenus jusqu'à présent aient été relativement décevants. Les mécanismes biologiques sous-jacents à cette absence générale de réponse restent mal compris, bien que de plus en plus d'études émergentes soulignent l'influence cruciale du microenvironnement tumoral.

Dans ce contexte, ce projet de thèse visait à approfondir notre compréhension du microenvironnement immunitaire au sein du MP, en focalisant essentiellement sur les macrophages associés aux tumeurs, via un séquençage ARN de noyaux uniques de biopsies de patients atteints de MP. Parallèlement, ce projet visait à développer de stratégies thérapeutiques innovantes en explorant deux approches : reprogrammer les monocytes avec des modulateurs épigénétiques et évaluer le potentiel synergique de la chimiothérapie métronomique en combinaison avec l'immunothérapie basée sur les ICIs dans des modèles précliniques de mésothéliome.

L'analyse de biopsies de patients a non seulement confirmé la présence des principaux types de cellules immunitaires précédemment décrits dans le MP, mais a également considérablement étendu notre compréhension des macrophages associés aux tumeurs. Une association entre les macrophages hypoxiques et une faible survie chez les patients atteints de MP a été identifiée. Le TGF-β3 a émergé comme un facteur potentiellement impliqué dans l'induction de ce phénotype hypoxique, suggérant de nouvelles approches thérapeutiques. De plus, une analyse comparative avec des macrophages non-tumoraux a révélé une signature pro-angiogénique globale parmi les macrophages tumoraux, avec les protéines CD93 et SPP1 identifiées comme cibles thérapeutiques potentielles.

Dans une étude complémentaire, il a été constaté que les monocytes dérivés du sang présentaient une activité cytotoxique intrinsèque contre les cellules de MP, bien que faible. Cependant, le traitement par l'acide valproïque (VPA), un inhibiteur de lysine désacétylases, a significativement amélioré la cytotoxicité, ainsi que la migration des monocytes et leur agrégation avec les cellules tumorales. En outre, le VPA a réduit l'expression des récepteurs clés associés à un phénotype de type M2, tels que CD163, CD206 et CD209, suggérant le VPA comme un candidat intéressant pour améliorer le

traitement du MP via la modulation de la cytotoxicité des monocytes.

Enfin, une étude préclinique a démontré que divers agents chimiothérapeutiques administrés de manière métronomique réduisaient efficacement la croissance tumorale dans des modèles précliniques syngéniques de MP. De manière suprenante, les individus traités avec une combinaison d'ICIs et de cisplatine et pemetrexed métronomique ont présenté une hétérogénéité de réponse, avec une stabilisation de la croissance tumorale observée dans certains cas. Cette variabilité de réponse n'a cependant pas été corrélée à des différences d'infiltration immunitaire à la fin du traitement, soulignant la nécessité d'études complémentaires pour identifier les mécanismes sous-jacents.

Acknowledgments

Bien que cette thèse soit le fruit d'un travail personnel, elle a néanmoins été grandement enrichie par les échanges, l'aide précieuse et les discussions avec de nombreuses personnes, qui ont ainsi largement contribué à la réussite de ce projet. Je tiens ainsi à remercier chaleureusement toutes ces personnes.

Premièrement, j'aimerais remercier mon promoteur de thèse, **Luc Willems**, de m'avoir permis d'effectuer ma thèse au sein de son laboratoire. Luc, merci pour ton encadrement, ta confiance, ta disponibilité ainsi que pour les nombreuses discussions enrichissantes qui m'ont permis d'avancer tout au long de ma thèse.

Je remercie également les membres de mon comité de thèse, constitué du Dr. **Arnaud Scherpereel**, du Dr. **Christophe Blanquard**, du Dr. **Malik Hamaidia** ainsi que du Prof. **Michaël Herfs** pour leur temps et les conseils qui ont contribué à la progression de ce projet. Un grand merci également au Dr. **Didier Jean** et au Prof. **Aurore Richel** pour leur contribution à l'évaluation finale de ce travail.

Je tiens également à remercier tous mes collègues du laboratoire d'Epigénétique et, particulièrement, les autres doctorants actuels et anciens avec qui j'ai pu travaillé de près ou de loin: Clotilde, Mélanie, Hélène, Mégane, Louise, Roxane, Aurélie, Xavier, Léa, Thomas 1 et Thomas 2. Merci pour la super ambiance, les échanges et l'entre-aide très présente qui m'ont permis de mener à bien pas mal d'expériences (et particulièrement les manips souris), et pour avoir rendu ces années de thèse si enrichissantes ! Merci pour les chouettes moments et soirées que l'on a passé ensemble.

Clotilde, merci pour ton encadrement pendant mon TFE, ainsi que pour les discussions et encouragements qui ont suivi pendant ma thèse. Tu es vraiment une personne géniale avec qui je suis très content d'avoir pu travailler ! Promis je ferai attention au pipetus à l'avenir. **Hélène**, j'ai été très content de partager un petit bout de sujet avec toi. Tu es une très belle rencontre, avec qui j'espère bien garder contact à l'avenir. **Mélanie**, bien que tu maltraites continuellement tes encadrants, merci pour toute l'aide que tu m'as apporté sur le projet ainsi que pour ton implication et les discussions et réflexions qui ont souvent été bien productives. Merci aussi pour ta bonne humeur ainsi que tes nombreuses blagues qui m'ont (presque) toujours fait rire. **Mégane**, merci pour toute l'aide apporté pendant les manips souris ainsi que de manière générale au labo ou pendant les (nombreuses) grèves. Tu es toujours prête à rendre service et à aider ! **Louise**, ce fut un plaisir d'être ton voisin de bureau (hum). Merci d'avoir pris ton rôle de remplaçante si à coeur et d'amener de la bonne humeur au sein du labo. Nos discussions de remise en question de l'univers me manqueront (un peu). **Roxane**, merci beaucoup pour ton aide sur le projet, tant au niveau de la manip souris que pour toutes les autres expériences. Je te souhaite bon courage pour la suite de ton projet. **Léa**, merci pour nos échanges bio-informatiques et l'aide que tu es toujours prête à donner! Bon courage pour le reste de ta thèse. **Aurélie**, **Xavier**, **Thomas**, et **Thomas**, bien que nous n'ayons pas eu l'occasion de collaborer beaucoup ensemble,

j'ai été très heureux de vous avoir comme collègues. J'ai apprécié nos échanges sur vos différents projets, même si je dois avouer que je n'en comprends (toujours) pas tous les détails ! Je remercie également **Jean-Rock**. Merci pour ton aide pour l'immunohisto, la gestion des commandes ainsi que pour l'organisation des team-buildings ! Un grand merci à **Christophe** et **Soline** pour leur aide, qui m'a permis de faire progresser le projet plus rapidement.

Merci également aux post-docs du laboratoire : **Malik** pour ton aide et les connaissances transmises, et **Mhothash** pour ta bonne humeur et les discussions enrichissantes.

Merci à la plateforme d'imagerie et en particulier à **Alex** pour toute l'aide apportée à l'analyse de mes expériences d'imagerie. C'était toujours un plaisir de pouvoir partager et discuter avec toi, aussi bien scientifiquement que sur d'autres sujets.

Je tiens également à remercier toute l'équipe de la plateforme bio-informatique et génomique : **Arnaud**, **David**, **Alice** et **Olivier** pour avoir répondu à mes nombreuses questions et m'avoir aidé à résoudre les quelques problèmes auxquels j'ai pu faire face en période d'urgence. Un merci tout particulier à **Latifa** pour ta disponibilité et ton aide pour le design de ma manip, ainsi qu'à **Manon** et **Emilie** pour m'avoir aidé dans la recherche de solutions.

Je souhaite également adresser un tout grand merci à **Sophie** pour toute l'aide, le temps, les explications et les conseils liés aux expériences souris, qui m'ont permis de mener à bien cette partie importante de ma thèse.

Merci également au Pr. **Yves Brostaux** pour m'avoir bien aidé sur les approches statistiques à utiliser dans bon nombre d'expériences.

Enfin sur un volet plus personnel, je tiens également à adresser un petit mot à toutes les personnes qui, dans ma vie privée, m'ont apporté leur soutien tout au long de cette thèse.

Premièrement, merci à mes **parents**, mon **frère** et mes **grand-parents** pour leur encouragements pendant mes études ainsi que pendant ma thèse. Vous avez fortement contribué à ma réussite.

J'aimerais aussi remercier **Jean-Louis**, **Bérengère**, **Robin** et **Marjorie** pour leur soutien tout au long de ma thèse, ainsi que pour les bons moments et les chouettes soirées partagés ensemble.

Merci également aux GB résiduels, **Victor**, **Rox**, **Vyckie**, **Claire**, **Naomi**, ainsi qu'à tous mes autres amis de notre super groupes de potes : **Malibubu**, **Denis**, **Pingy**, **Pouchou**, **Loli**, **Guillaume**, **Elboudumonde**, **Lallie**, **Tanguy** et **Maxence**. Toutes nos soirées, journées, week-ends et vacances étaient une source d'amusement, de décontraction et de décompression inestimable pendant cette thèse !

Enfin, la meilleure pour la fin, merci à **Florine** pour m'avoir supporté et encouragé tout au long de cette thèse. Merci pour ton soutien dans les moments plus stressants,

ton écoute quotidienne ainsi que pour tout le reste, qui rend mon quotidien si épanouissant.

Financements

Je souhaite également exprimer ma gratitude envers le Télévie, le FNRS ainsi que la Fondation Léon-Frédéricq pour leur soutien financier inestimable sans lequel ce travail n'aurait pu voir le jour.

Contents

List of Figures

List of Tables

Acronyms

18F-FDG-PET	fluorodeoxyglucose positron emission tomography
3meA	N3-methyladenine
5-Fu	5-fluorouracil
7meG	N7-methylguanine
8-OHdG	8-hydroxy-2'-deoxyguanosine
ABC	ATP-binding cassette
ACTA	actine alpha
ADCC	antibody-dependent cell cytotoxicity
ADCP	antibody-dependent cell phagocytosis
ADM	adrenomedullin
AICARFT	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase
ANG	angiopoietin
ANPEP	alanyl aminopeptidase
APC	antigen presenting cell
APP	antigen processing and presentation
Arg1	arginase 1
ASR	age-standardized rate
ATP	adenosine triphosphate
AUPR	area under the precision-recall curve
B2M	β_2 microglobulin
BAP-1	BRCA1-Associated Protein–1
BCL	binary base call
Bcl-2	B-cell lymphoma 2
BER	base excision repair
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CAF	cancer-associated fibroblast
CAR	chimeric antigenic receptor

CCL	chemokine ligand
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
cDC	conventional DC
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CEACAM	CEA-related cell adhesion molecule
CFSE	5,6-Carboxyfluorescein Diacetate, Succinimidyl Ester
CI	confidence interval
CK	cytokeratin
CLEC	human C-type lectin domain
CNV	copy-number variation
COL	collagen
COX	cyclooxygenase
CpG	cytosine–phosphate–guanine
CSF1	colony stimulating factor 1
CSF3R	colony stimulating factor 3 receptor
CT	computed tomography
CTA	cancer testis antigen
CTLA-4	cytotoxic T-lymphocyte 4
CTS	cathepsin
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DAPI	4',6-diamidino-2-phénylindole
DC	dendritic cell
DCK	deoxycytidine kinase
DCR	disease control rate
DDIT4	DNA damage inducible transcript 4
DDR	DNA damage repair
DEG	differentially expressed gene
dFdCDP	gemcitabine diphosphate
dFdCTP	gemcitabine triphosphate
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSB	double-strand breaks
dsDNA	double-stranded DNA

e.g.	exempli gratia
ECM	extracellular matrix
EGFL7	EGF-like domain-containing protein 7
EMA	European Medicines Agency
EMT	epithelial–mesenchymal transition
ENG	endoglin
ENO	enolase
EPD	extended pleurectomy/decortication
EPP	extrapleural pneumonectomy
FASL	FAS ligand
FBS	fetal bovine serum
FC	fold change
FDA	Food and Drug Administration
FELASA	federation of laboratory animal science association
FOXP	forkhead box P
G-CSF	granulocyte colony-stimulating factor
GARFT	glycinamide ribonucleotide formyltransferase
GM-CSF	granulocyte-macrophage colony-stimulating factor
GNLY	granulysin
GPNMB	glycoprotein NMB
GZMB	granzyme B
h	hour
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HDGF	heparin binding growth factor
HEV	high endothelial venule
HIF	hypoxia-inducible factor
HLA	human leukocyte antigen
HMGB1	high–mobility group box 1
HNSCC	head and neck squamous cell carcinoma
HoR	homologous recombination
HR	hazard ratio
IARC	International Agency for Research on Cancer
ICI	immune checkpoint inhibitor
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IGFBP7	insulin-like growth factor binding protein 7
IGHG	immunoglobulin heavy constant gamma
IGHM	immunoglobulin heavy constant mu

IGKC	immunoglobulin kappa constant
IGLC	immunoglobulin lambda constant
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible NO-synthase
irAE	immune-related adverse event
ITGB3	integrin subunit beta 3
JAK	janus kinase
Keap1	kelch-like ECH-associated protein 1
LA	lipid-associated
LAF	long asbestos fiber
LAG-3	lymphocyte-activation gene 3
LAMP	lysosome-associated membrane glycoprotein
LATS	large tumor suppressor kinase
LILRA4	leukocyte immunoglobulin like receptor A4
LMA	lympho-myeloid aggregate
LPM	large peritoneal/pleural macrophages
LPS	lipopolysaccharide
LYZ	lysozyme
M-MDSC	monocytic MDSC
MAGE	melanoma-associated antigen
MAPK	mitogen-activated protein kinase
MAPS	Mesothelioma Avastin Cisplatin Pemetrexed Study
mCT	metronomic chemotherapy
MDR1	multidrug resistance 1
MDSC	myeloid-derived suppressor cell
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minutes
MMP	matrix metalloproteinase
MMR	mismatch repair
MMRN	multimerin
MOC-31	anti-epithelial related antigen
moDC	monocyte-derived DC
mOS	median overall survival
mPFS	median progression-free survival
mRNA	messenger RNA
MS4A1	membrane spanning 4-domains A1
MSLN	mesothelin
MTA	microtubule-targeting agent

MTAP	methylthioadenosine phosphorylase
MTD	maximal tolerated dose
MYL	myosin light chain
NAD	nicotinamide adenine dinucleotide
NF-κB	nuclear factor-kappa B
NHEJ	non-homologous end-joining
NK	natural killer
NLRP3	NLR family pyrin domain containing 3
NMUR1	neuromedin U Receptor 1
NO	nitric oxide
NR	nuclear receptor
Nrf2	nuclear factor erythroid 2-related factor 2
NRP	neuropilin
NSCLC	non-small cell lung cancer
NY-ESO	New York esophageal squamous cell carcinoma
O6 Cl-ethylG	O6-chloroethylguanine
O6meG	O6-methylguanine
OATP1B3	organic anion transporting polypeptide 1B3
ORR	objective response rate
OSR	overall survival rate
PAX5	paired box 5
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCA	principal component analysis
PD-1	programmed cell death 1
PD-L1	programmed cell death ligand 1
pDC	plasmacytoid DC
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PECAM1	platelet endothelial cell adhesion molecule
PFA	paraformaldehyde
PFS	progression-free survival
PGE2	prostaglandin E_2
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PM	pleural mesothelioma
PMA	phorbol 12-myristate 13-acetate
PMN-MDSC	granulocytic/polymorphonuclear MDSC
PTEN	phosphatase and tensin homolog
PTPRC	protein tyrosine phosphatase receptor type C
RAGE	receptor for advanced glycation end products

RGS	regulator of G-protein signaling
RNA	ribonucleic acid
RNR	ribonucleotide reductase
ROI	region of interest
ROS	reactive oxygen species
RT	room temperature
S100	S100 calcium-binding protein
SAF	short asbestos fiber
SASP	senescence-associated secretory phenotype
scRNA-seq	single-cell RNA sequencing
SDF	stromal cell-derived factor
SELENOP	selenoprotein P
SEMA	semaphorin
SIRP	signal regulatory protein
SLO	secondary lymphoid organ
snRNA-seq	single-nucleus RNA sequencing
SNV	single nucleotide variant
SPM	small peritoneal/pleural macrophages
SPP1	secreted phosphoprotein 1
SSB	single-strand break
STAT	signal transducer and activator of transcription
T_{cm}	central memory T-cell
T_{em}	effector memory T-cell
T_h	helper T-cell
T_{reg}	regulatory T-cell
T_{scm}	stem cell memory T-cell
TAM	tumor-associated macrophage
TCR	T-cell receptor
TGF-β	transforming growth factor-beta
TIGIT	T-cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif domains
TIL	tumor-infiltrating lymphocyte
TIL-B	tumor-infiltrating B-cell
TIL-PC	tumor-infiltrating plasma-cell
TIM	tumor-infiltrating monocyte
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TIS	treatment-induced senescence
TLS	tertiary lymphoid structure
TMB	tumor mutational burden
TME	tumor microenvironment

TNF-α	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TNM	tumor-node-metastasis
TOX	thymocyte selection-associated high mobility group box protein
TRAC	T-cell receptor alpha constant
TRAIL	TNF-related apoptosis-inducing ligand
TS	thymidylate synthase
TSA	trichostatin A
TSP-1	thrombospondin-1
U	unit
UMAP	uniform manifold approximation and projection
UMI	unique molecular identifier
v/v	volume/volume
VATS	video-assisted thoracoscopic surgery
VCAN	versican
VEGF	vascular endothelial growth factor
VISTA	V-domain Ig suppressor of T-cell activation
VPA	valproate
VPF	vascular permeability factor
WBC	white blood cell
WHO	World Health Organization
WT1	Wilm's-tumor 1

1

General Introduction

1. Pleural mesothelioma

Mesothelioma is a highly aggressive and rare neoplasm that originates from mesothelial cells of the serous membranes encompassing the pleura, the peritoneum, the pericardium, and the tunica vaginalis testis. Pleural mesothelioma (PM) accounts for 70% to 90% of all mesothelioma cases, with the pleura therefore being the primary location for mesothelioma development **(Figure 1.1)** [1, 2]. PM mainly develops following asbestos exposure and has been officially recognized as an occupational disease since 1982 in Belgium [3].

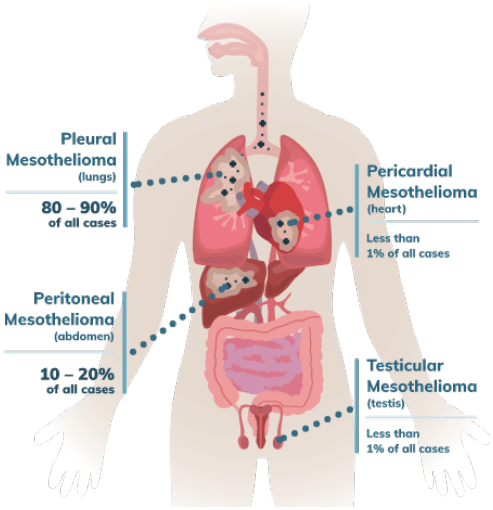


Figure 1.1: Mesothelioma localization and frequency. Mesothelioma originates from the mesothelial lining of the pleural cavity, the peritoneum, and more rarely the pericardium and the tunica vaginalis [4]

1.1. Pleura anatomy and histology

The pleura is a serous membrane structured as a double mono-layer of mesothelial cells lying on an underlying thin basal lamina and connective tissue [5, 6, 7]. One layer, referred to as the visceral pleura, directly covers the surface of the lungs, while the other one, the parietal pleural, lines the thoracic wall, the diaphragm and the mediastinum **(Figure 1.2)** [8]. Together, they delineate the pleural space containing the pleural fluid produced by parietal sub-pleural capillaries and supplemented by glycosaminoglycans secreted by mesothelial cells [6]. Although parietal and visceral pleura are similar, they also exhibit notable structural and functional differences such as the vascularization level or the lymphatic drainage [9].

The pleura provides a frictionless surface allowing intracoelomic movement and a physical barrier against pathogens. Besides these roles, mesothelial cells are also involved in antigen presentation, tissue repair or fluid transport [7].

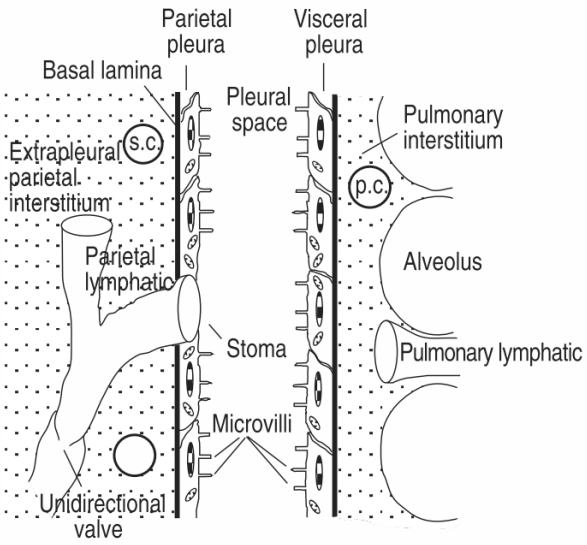


Figure 1.2: Pleura histology. Pleura is formed by two layers, the parietal and visceral pleura, delineating the pleural cavity. Both layers are constituted by a single layer of mesothelial cells disposed on a basal lamina and a connective tissue [10].

1.2. Etiology

Asbestos exposure stands as the foremost etiological factor in the development of PM (above 80%). Nevertheless, other proven factors including different mineral fibers, radiations, or genetic predispositions can also contribute to the onset of PM [11]. Additionally, preclinical evidence, although not supported by epidemiological studies, suggests that carbon nanotubes could also be responsible for PM development [12].

1.2.1. Asbestos

The term "asbestos" is a generic term that encompasses six distinct forms of natural silicate fibers that have been used in commercial applications [13]. Asbestos possesses an array of highly attractive properties for industrial applications including resistance to heat and corrosion, flexibility, effective electric and acoustic insulation characteristics, and high tensile strength, all at a cost-effective price [11, 14, 15]. For those reasons, asbestos has been widely used in different applications including insulation in shipbuilding and railway rolling stock, production of textile products, and thermal and acoustic insulation in domestic houses, particularly during and after World War II [11].

Based on their structural and chemical properties, asbestos fibers can be classified into two distinct groups: serpentines (short and curled fibers) and amphiboles (straight and long fibers) [14]. Serpentines are exclusively composed of chrysotile (white as-

bestos), the most widely used form worldwide while the amphiboles include crocidolite (blue asbestos), amosite (brown asbestos), actinolite, tremolite, and anthophyllites [13, 14]. Besides occupational exposure, cases of PM due to environmental (residential proximity to industrial asbestos sources), para-occupational exposure, and exposure to commercial asbestos-containing products have also been documented [16].

The connection between occupational exposure to asbestos and PM was first established through an epidemiological study of 33 proven PM cases in 1960 in South Africa [17]. Since 1987, all asbestos forms have been classified as a Group 1 carcinogen by both the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC), leading to its progressive ban in Europe. Some studies indicate variations in carcinogenic potential between different types of asbestos, with amphiboles showing a higher carcinogenic potency than serpentines. Indeed, a meta-analysis demonstrated that the likelihood of developing PM following non-occupational exposure to asbestos was significantly higher among individuals exposed to amphibole fibers than chrysotile [18]. Of interest, asbestos exposure does not always result in PM onset as only 10% of highly exposed people develop the tumor [19]. Besides PM, asbestos exposure has also been associated with various other cancers, including lung, ovarian, laryngeal, and gastrointestinal cancers based on epidemiological and histological evidence [20].

1.2.2. Genetic predispositions and *BAP-1* predisposition syndrome

Owing to the facts that only a minority of individuals (5-10%) exposed to asbestos develops PM and that familial clusters with an unusually high incidence of PM have been identified, despite small exposure to asbestos, the potential role of genetic predispositions in the etiology of PM has been considered [21, 22, 23]. Through desoxyribonucleic acid (DNA) analysis and preclinical experiments, germinal mutations in *BRCA1-Associated Protein–1 (BAP-1)* have been associated with PM development [23, 24].

BAP-1 is a tumor suppressor gene that codes for an enzyme playing major roles in DNA replication, cell cycle, and DNA damage repair (DDR) [25]. The influence of *BAP-1* alteration on PM development was identified through an analysis conducted on two families with abnormally high incidence of PM. The analysis revealed a common *BAP-1* heterozygous mutation among individuals who developed PM [21]. Furthermore, preclinical evidence demonstrated that heterozygous *BAP-1*-mutant mice exhibit a more rapid onset of mesothelioma when exposed to asbestos compared to wild-type mice [24, 26]. These studies therefore suggested that germline *BAP-1* mutation predisposes individuals to the tumorigenic effects of asbestos. However, it is noteworthy that germline *BAP-1* cancer syndrome cases contribute to only a small fraction of PM (1-7% of PM cases) [22]. Interestingly, individuals with germline BAP-1 mutations who develop PM exhibit less aggressive disease progression [27]. The underlying reasons for this phenomenon remain largely unknown; however, a significant reduction

in hypoxia-inducible factor (HIF)-1α activity in PM cells with homozygous *BAP-1* mutations has been recently proposed as a potential explanation for the reduced aggressiveness [28].

1.2.3. Other mineral fibers

In addition to asbestos, fluoro-edenite and erionite have also been recognized as inducer of PM. Erionite is a zeolite morphologically similar to asbestos and found in volcanic regions of Cappadocia, Italy, and the United States [22]. The carcinogenicity of erionite was suspected following the identification of remarkably high percentages of PM in three asbestos-free villages in Cappadocia [29]. Its carcinogenic nature was experimentally confirmed through intra-pleural injection into rats, leading to PM development [29, 30].

1.2.4. Radiations

Radiation is well known to be carcinogenic. Consistently with other cancers, PM could develop following ionizing radiotherapy for childhood primary tumors. Furthermore, individuals with occupational radiation exposure also face an increased risk of PM development [9, 22].

1.2.5. Carbon nanotubes

Engineered carbon nanotubes are nanomaterials composed of graphene sheets arranged into cylindrical fibers, sharing a morphological resemblance to asbestos fibers [31]. This similarity has raised concerns regarding their potential carcinogenicity and the possibility of inducing asbestos-like pathology [6]. Indeed, experimental studies showed that intrascrotal injection of carbon nanotubes in rats results in 87% of mortality due to peritoneal mesothelioma [32]. Similarly, a preclinical study demonstrated that 16% of rats developed peritoneal mesothelioma following trans-tracheal injection of carbon nanotubes [33]. However, despite these findings, epidemiological studies have not yet established a clear correlation between carbon nanotube exposure and mesothelioma development.

1.3. Epidemiology and incidence

PM exhibits a widely heterogeneous distribution worldwide similar to the global mesothelioma distribution as illustrated in **Figure 1.3**. In 2022, an estimate of 30,633 cases of mesothelioma and an age-standardized rate (ASR) of 0.28 per 100,000 inhabitants were reported globally, with the highest ASR observed in Northern Europe and Australia [34, 35]. In particular, Luxembourg, the United Kingdom, The Netherlands, and Belgium present the highest rate in Europe [34, 35]. This trend aligns with historical data, reflecting the widespread use of asbestos during the second part of the 20^{th} century [36]. While countries with high human development indexes have now seen a stabilization or decline in PM cases since the asbestos ban in the 1980s, countries such as Russia, Kazakhstan, China, and Brazil, along with other developing countries, are still producing and using asbestos [34]. PM will therefore continue to represent a health hazard for people living in those countries [36, 37].

Due to occupational exposure, mesothelioma mainly affects men compared to women, with 21,410 cases recorded in men compared to 9,223 in women worldwide in 2022 [34]. Moreover, owing to the latency period between exposure and tumor development, the median age of patients at diagnosis is 75 years [11].

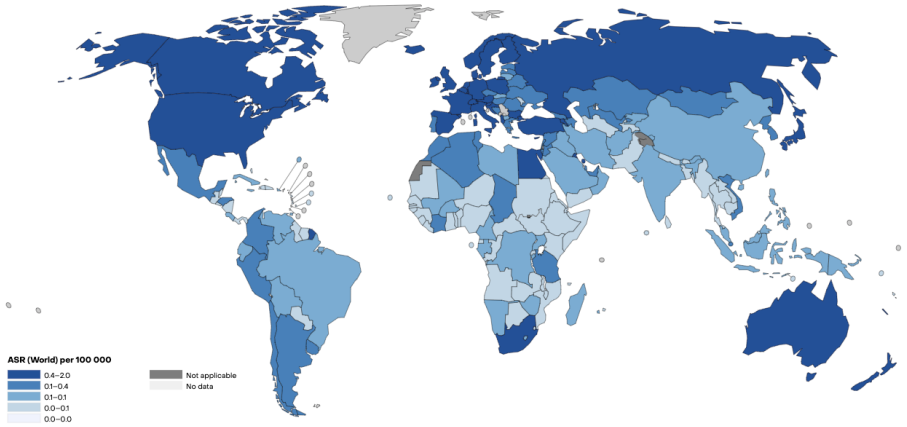


Figure 1.3: Incidence, expressed in ASR, of mesothelioma in 2022. Distribution of mesothelioma incidence worldwide, irrespective of gender [35]. ASR, age-standardized rate.

1.4. Classification

1.4.1. Histopathological classification

In its historical classification, PM has been categorized into three primary histopathological subtypes: epithelioid, sarcomatoid, and biphasic (or mixed), with the latter exhibiting characteristics from both the epithelioid and sarcomatoid subtypes **(Figure 1.4)** [38]. This discrete classification currently remains of clinical importance as it still drives the clinical management decision and shows prognosis significance. Indeed, patients diagnosed with the epithelioid subtype, representing 50-70% of cases, show a median overall survival (mOS) ranging from 13 to 17 months. In contrast, those with the sarcomatoid form, representing 10% of cases, have a significantly shorter survival period of 4 to 7 months, while patients with the biphasic form, 10-30% of cases, present an intermediate mOS of 8 to 11 months [39, 40]. It is noteworthy that the mOS of patients is also closely linked to the performance status at diagnosis [39]. Of interest, epithelioid PM can be further divided into different architectural patterns including tubulopapillary, trabecular, adenomatoid, solid, and micropapillary patterns, also demonstrating prognostic value [38].

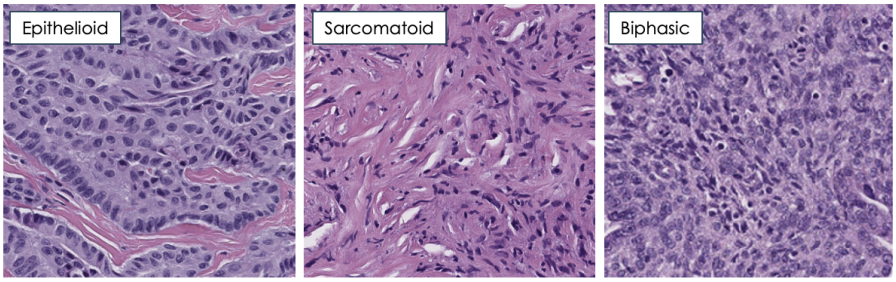


Figure 1.4: PM histopathological subtypes. The epithelioid subtype is composed of flat and cuboidal cells while the sarcomatoid subtype is characterized by spindle cells with abundant stroma. The biphasic subtype consists of a morphological mix of the epithelioid and the sarcomatoid subtypes [41].

1.4.2. Molecular classification

While the WHO histopathological classification has widely served as a key tool for patient stratification, variability in patient outcomes persists even within histopathological subtypes, particularly in the epithelioid one. Recent extensive efforts have therefore focused on enhancing the understanding of inter- and intra-patient variability at a molecular level and identifying more precise groups with prognostic values **(Figure 1.5)** [42].

Transcriptomic data were first harnessed to unveil molecular clusters through unsupervised consensus clustering. A pioneering classification leveraging transcriptomic data from primary cell lines emerged in 2014, delineating PM into two distinct groups,

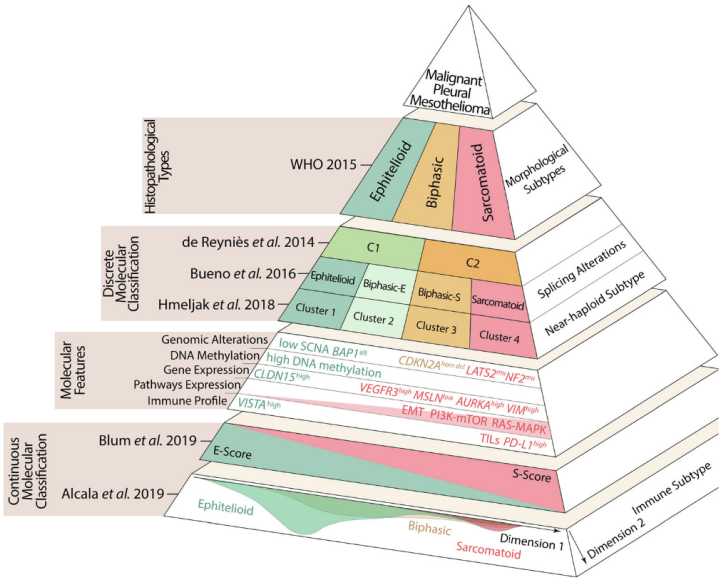


Figure 1.5: Schematic view of the different proposed histopathological and molecular classification models for PM. Evolution of the different classifications from discrete subtypes to continuous classification systems for PM through pivotal studies [42].

partly linked with histological subtypes and characterized with distinct prognostic outcomes [43]. In the large-scale study conducted by Bueno *et al.*, four discrete categories: sarcomatoid, epithelioid, biphasic-epithelioid, and biphasic-sarcomatoid were described [44]. It is worth noting that, although gene expression-based, this improved classification remains closely intertwined with histopathological classification. Similarly, through integrative omics analysis, another research group revealed the presence of four distinct molecular prognostic subsets, which were also found to be correlated with histology [45].

The major limitation of the aforementioned cluster-based approaches lies in the assumption of discrete clusters to define tumors and recapitulate inter-patient diversity. In contrast, Blum *et al.* introduced a continuous classification based on a deconvolution approach, suggesting that each tumor can be dissected into a mix of epithelioid-like and sarcomatoid-like components, with their proportions being strongly associated with prognosis [46]. Similarly, another group has further characterized PM heterogeneity as a continuum, emphasizing the involvement of vascular and immune components [47].

However, whether categorized as discrete or continuous, all these classification models predominantly focus on characteristics associated with the epithelioid and sarcomatoid components and lack a deep integration of genomic and epigenetic data. Based on the observation that the histopathological model only explains up to 10% of inter-

patient variability, a recent integrative omics analysis was performed. Three new non-interdependent sources of molecular variations were uncovered: ploidy factor, adaptive immune response factor, and cytosine–phosphate–guanine (CpG) island methylation phenotype factor **(Figure 1.6)** [48]. The integration of these three molecular factors along with the histological factor has significantly increased the explained inter-patient variance to an average of 33%. This study therefore opens new ways for novel patient stratification strategies in future clinical trials [48].

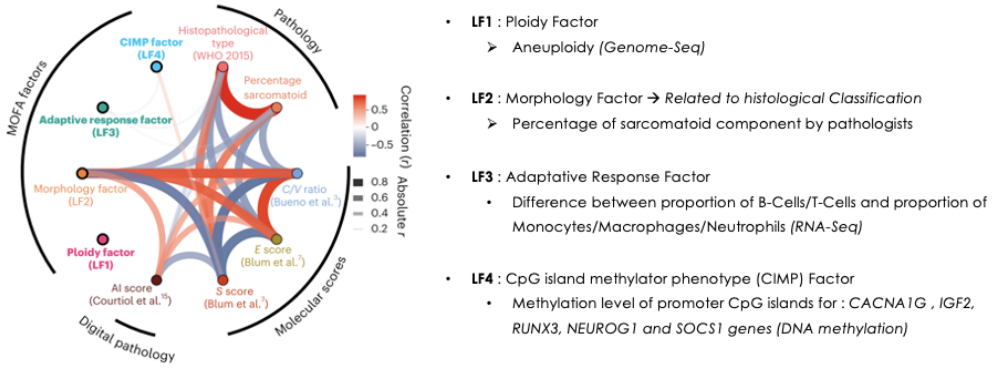


Figure 1.6: Sources of molecular variations in PM through multi-omics analysis. Network of the correlations between newly identified factors of heterogeneity and previously published molecular scores. Adapted from [48]. LF, latent factor; CIMP, CpG islands methylator phenotype; MOFA, multi-omics factor analysis.

1.5. Tumorigenesis

PM tumorigenesis can be divided into two different parts: asbestos translocation to pleura and malignant transformation of mesothelial cells into mesothelioma cells.

1.5.1. Translocation of asbestos

Asbestos fibers are inhaled and pass through the upper respiratory tract before ultimately reaching the alveolar cavity. The exact mechanism by which these fibers subsequently gain access to the pleural space remains largely unknown. The current model suggests that, driven by pressure and osmotic gradients, fibers translocate from alveoli to the pulmonary interstitial space through paracellular pathways. This translocation induces an inflammatory state that reverses lymph flow and trans-pleural pressure, facilitating the migration of fibers through the visceral pleura and into the pleural cavity [6, 49]. This process is thought to occur on several years, providing a reasonable explanation for the long latency period between asbestos exposure and PM development [50]. Interestingly, there is evidence suggesting that the latency period is inversely correlated with the duration and/or intensity of asbestos exposure [51].

Within the pleural cavity, short asbestos fibers (SAFs) (length < 5 μm) are drained by

the pleural fluid and subsequently eliminated through the stomata located at the surface of the parietal pleura. In contrast, long asbestos fibers (LAFs) (length > 5μm) tend to accumulate at these openings, thereby initiating events leading to PM development. Of interest, this model could elucidate the nearly systematic occurrence of PM at the parietal pleura even though there is no consensus on this point [6, 9].

1.5.2. Mechanisms of tumorigenesis

The pathogenesis of PM is a complex interplay of various processes primarily involving mesothelial cells and pleural macrophages. Globally, asbestos triggers oxidative stress and cytokine release, initiating a cascade of events that culminates in chronic inflammation and, ultimately, in the tumor development **(Figure 1.7)**.

Mesothelial cells internalize asbestos fibers through endocytosis, facilitated by their binding to Annexin A2 and integrins, the latter one requiring the presence of vitronectin [52]. It has been suggested that within mesothelial cells, these fibers can disrupt the mitotic spindle during cell division [53]. In addition, through their chemical composition, endocytosed asbestos fibers also trigger genomic damage by promoting the intracellular production of reactive oxygen species (ROS). Most asbestos varieties contain iron, which acts as a catalyst in generating hydroxyl radicals through the Fenton reaction [54]. The consequences of this process encompass DNA single-strand breaks, chromotrypsis events, and modifications to DNA bases, such as the occurrence of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a common base modification in PM [55]. Protein degradation, including DNA repair proteins, also occurs as a consequence of the intracellular oxidative stress, thereby preventing DDR.

Nonetheless, mesothelial cells exhibit a high susceptibility to asbestos-induced cytotoxicity, raising the question of how these damaged cells survive and proliferate, ultimately contributing to tumor formation [56]. An explanation comes from studies that have shown that, upon contact with asbestos fibers, mesothelial cells also secrete tumor necrosis factor alpha (TNF-α), while concomitantly expressing the tumor necrosis factor receptor (TNFR)1 [56, 57]. TNF-α activates nuclear factor-kappa B (NF-κB) in an autocrine manner, a pathway that induces transcription of genes promoting cell survival and proliferation [57]. These findings offered a mechanistic explanation for the observation that asbestos lacks pathogenicity in transgenic mice deficient in TNFR1 [58]. Additionally, upon asbestos contact, mesothelial cells also secrete inflammatory cytokines (interleukin (IL)-1β, IL-13, granulocyte colony-stimulating factor (G-CSF)) and growth factors (vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF)), that contribute to promote the proliferation of newly formed tumor cells [59]. Furthermore, a significant proportion of mesothelial cells undergo programmed necrosis, releasing high–mobility group box 1 (HMGB1) into the extracellular space.

In parallel, these cytokines, together with HMGB1 and chemokine ligand (CCL)2 secreted by adipocytes, foster the recruitment of monocytes and macrophages into the

pleural cavity. Pleural macrophages and newly recruited macrophages attempt to engulf asbestos fibers. However, macrophages will fail at engulfing LAFs, leading to a phenomenon known as "frustrated phagocytosis". During this process, macrophages will release substantial amounts of ROS in the extracellular medium, intensifying the oxidative stress. Furthermore, inflammasome activation driven by the binding of HMGB1 to receptor for advanced glycation end products (RAGE) induces the release of IL-1β, IL-18, IL-1α, and HMGB1, further amplifying the chronic inflammation process [53, 60].

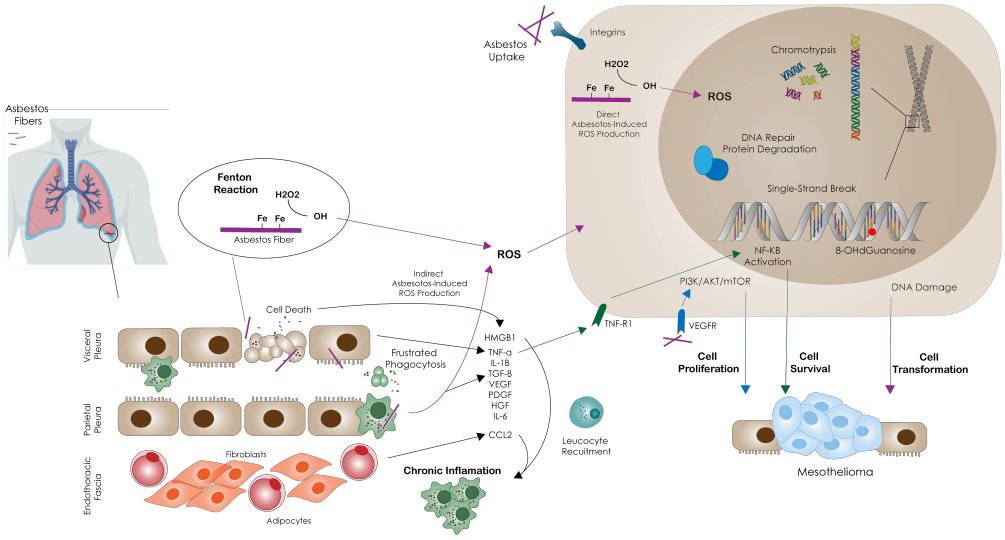


Figure 1.7: Pathogenesis of PM. When asbestos fibers reach the pleural space and come into contact with mesothelial cells, they produce inflammatory cytokines and growth factors, which attract monocytes that differentiate into macrophages. These macrophages attempt to engulf the fibers but are unable to do so effectively. This inability leads to a phenomenon known as "frustrated phagocytosis" where macrophages produce ROS, inflammatory cytokines, and free radicals. The combined effect of these inflammatory mediators from both mesothelial cells and macrophages creates a chronic inflammatory environment, which can eventually lead to the development of PM. ROS, reactive oxygen species; mTOR, mammalian target of rapamycin; Fe, fer; HMGB-1, high–mobility group box 1; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; HGF, hepatocyte growth factor; CCL2, chemokine ligand 2; IL, interleukin.

1.6. Diagnosis

The symptoms associated with PM are typically nonspecific and most often include weight loss, chest pain, cough, and dyspnea generally resulting from pleural effusions. The diagnostic approach outlined in current guidelines encompasses occupational asbestos exposure history, imaging techniques, thoracoscopy, and subsequent immunohistochemistry (IHC) [61].

Among the different imaging techniques used in cancer diagnosis, computed tomography (CT) with contrast enhancement has been a primary tool for PM detection [41, 61, 62]. This method facilitates the identification of pleural effusion, diffuse pleural thickening, and the presence of pleural nodules, indicative of the potential presence of PM **(Figure 1.8)**. However, CT exhibits some limitations, particularly in determining tumor dissemination to adjacent or distant tissues [62]. Consequently, in addition to CT, chest X-rays and fluorodeoxyglucose positron emission tomography (18F-FDG-PET) are also valuable diagnostic tools. Although not routinely employed, 18F-FDG-PET plays a role in evaluating metastatic spread, encompassing intrathoracic and extrathoracic lymph nodes as well as distant anatomical sites **(Figure 1.8)**. Collectively, these techniques are used for tumor identification and aid in determining the optimal biopsy site [61, 63]. Indeed, all those techniques lack sensitivity and specificity, and more confirmatory exams must always be undertaken in case of PM suspicion [61]. Additionally, imaging is also used for tumor staging through tumor-node-metastasis (TNM) classification [61].

Pleural biopsies are key elements to confirm PM diagnosis. Thoracoscopy serves a dual purpose, allowing diagnosis through biopsy sampling and managing pain by draining pleural effusions. The intricate diversity of histological characteristics due to the different subtypes, coupled with the frequent occurrence of metastatic disease within the pleura, makes the use of IHC staining essential. Indeed, IHC staining is crucial for distinguishing PM from mesothelial hyperplasia, fibrous pleuritis, and other tumors, particularly lung adenocarcinoma, and for identifying specific histopathological subtypes **(Figure 1.8)**. Assessment of at least two mesothelioma-associated markers (exempli gratia (e.g.). calretinin, Wilm's-tumor 1 (WT1) or cytokeratin (CK) 5/6) and two adenocarcinoma-associated markers (e.g. carcinoembryonic antigen (CEA), anti-epithelial related antigen (MOC-31) or Ber-EP4) is currently recommended [61]. CK5/6 staining is particularly useful for sarcomatoid PM identification. Additionally, diagnosis refinement can be achieved by assessing BAP-1 loss (more prevalent in the epithelioid subtype) and *cyclin-dependent kinase inhibitor 2A (CDKN2A)/ methylthioadenosine phosphorylase (MTAP)* deletions (more prevalent in the sarcomatoid subtype) [63].

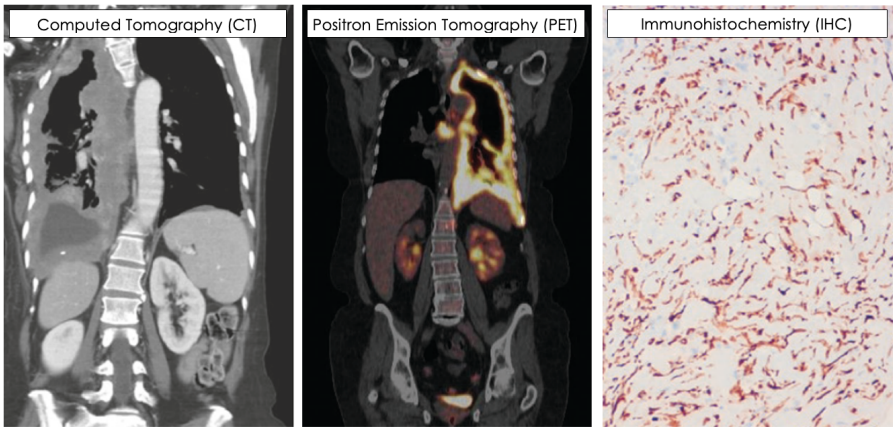


Figure 1.8: Diagnosis of PM. The diagnosis of PM typically involves initial screening using contrast-enhanced CT scans, sometimes complemented with 18F-FDG-PET imaging, and further confirmation through IHC analysis [64]. CT, computed tomography; 18F-FDG-PET, fluorodeoxyglucose positron emission tomography.

1.7. Treatments

Until now, PM has remained an incurable tumor with a dismal prognosis. Patients typically experience a 5-year overall survival rate (OSR) ranging from 5% to 10% and a mOS of around 12 months. First-line treatment for PM patients is based on two different approaches: (i) multimodal treatment combining surgery with (neo)adjuvant chemotherapy and/or perioperative radiotherapy and (ii) systemic therapies based on chemotherapy or immunotherapy [63, 38]. Besides those approved therapies, numerous other treatments are currently evaluated in clinical trials.

1.7.1. Standard multimodal treatment

The multimodal approach involves a combination of surgery, chemotherapy, and radiotherapy. Two main surgical procedures have been used over time. These include the extrapleural pneumonectomy (EPP), which entails the removal of the lung, pleura, pericardium, and diaphragm, and extended pleurectomy/decortication (EPD), defined as the removal of the pleura along with resection of portions of pericardium and diaphragm if macroscopically invaded by the tumor [65]. Due to lower perioperative mortality and morbidity, EPD is now the recommended surgical method, although EPP could still be used. Surgery is nevertheless used for a minority of early-stage patients, generally enrolled in clinical trials [38, 63].

In combination with surgery, intensity-modulated radiation therapy can be used. However, current guidelines state that it should only be considered within the context of clinical trials thereby precluding its use in routine due to the lack of phase III clinical trials in combination with EPD supporting its efficacy and suitability for

integration into standard clinical protocols [38, 63].

1.7.2. Standard systemic treatments

However, most PM patients are not eligible for multimodal therapy and their treatment options are primarily palliative. For over two decades, the combination of an alkylating agent (cisplatin/carboplatin) and an antifolate (pemetrexed/ralitrexed) has been the standard of care for unresectable PM. Indeed, a pivotal randomized phase III clinical trial showed an improvement in mOS with the pemetrexed/raltitrexed leading to mOS of 12.1 months, compared to 9.3 months with cisplatin alone [66].

More recently, immunotherapy based on immune checkpoint inhibitors (ICIs) has nevertheless supplanted chemotherapy, especially for the treatment of sarcomatoid subtypes of PM. In the phase III CheckMate 743 clinical trial (NCT0289929), the combination of anti-programmed cell death 1 (PD-1) and anti-cytotoxic T-lymphocyte 4 (CTLA-4) antibodies (nivolumab and ipilimumab, respectively) increased the 2-year OSR by 50% compared to platinum plus pemetrexed chemotherapy. Moreover, the mOS was extended from 14.1 months with standard chemotherapy to 18.1 months with immunotherapy [67, 68].

Of interest, there is no standardized second-line for PM patients [38, 63]. The predominant treatment options often encompass single-agent chemotherapy with vinorelbine or gemcitabine, or re-treatment with pemetrexed.

1.7.3. Other therapeutic approaches and ongoing clinical trials

Beyond the approved treatments previously mentioned, several additional therapeutic approaches are currently being explored for first-line or second-line management of unresectable PM.

Anti-angiogenesis

A phase III clinical trial (Mesothelioma Avastin Cisplatin Pemetrexed Study (MAPS); NCT00651456) demonstrated the efficacy of bevacizumab, an anti-angiogenesis antibody targeting VEGF in the frontline, when used in combination with standard chemotherapy, resulting in a mOS increase of 2.7 months in patients with PM compared to chemotherapy alone [69]. Although approved neither by the Food and Drug Administration (FDA) nor by the European Medicines Agency (EMA), this treatment is nonetheless included in guidelines in certain countries [70]. However, patients treated with bevacizumab experienced a statistically significant higher incidence of toxicity, particularly grade 3 or 4 hypertension, elevated creatinine levels, and arterial or venous thromboembolic events although quality of life remained comparable between the two treatment arms [69]. Besides bevacizumab, the combination of ramucirumab, an anti-VEGFR2 antibody, with gemcitabine (RAMES trial) improved mOS (7.5 to 13.8 months) in the second-line setting compared to gemcitabine alone [71], although the interpretation of the trial results is contentious owing to the potential for selection

bias existing between both study arms [72].

Chemoimmunotherapy

Chemoimmunotherapy stands as the new paradigm in cancer treatment. In recent years, several phase II trials have been conducted in PM **(Table 1.1)**. Based on the promising results of these trials, three pivotal phase III clinical trials, namely ETOP-BEAT Meso, DREAM3R, and IND227, are currently ongoing or have recently reached completion **(Table 1.2)**. Those trials evaluate the combination of the front-line chemotherapy along with atezolizumab (programmed cell death ligand 1 (PD-L1) inhibitor), durvalumab (PD-L1 inhibitor) and pembrolizumab (PD-1 inhibitor), respectively. Of interest, the BEAT-Meso trial also includes bevacizumab in both arms.

Results from the IND227 trial demonstrated a significant, albeit moderate, increase in mOS from 16.1 months in the control arm to 17.3 months in the chemoimmunotherapy arm, with particularly pronounced benefits observed in the non-epithelioid subgroup (8.2 vs. 12.3 months) [73]. Additionally, the study highlighted a considerable enhancement in the overall response rate in the combination arm, rising from 38% to 61%. However, an increase in adverse events was observed, with 28% of patients experiencing grade 3-4 adverse events in the combination arm compared to 16% in the chemotherapy-alone group. However, and importantly, this increase in adverse events did not correlate with a decrease in quality of life [73]. The early results of the ETOP-BEAT Meso trial seem to demonstrate the same trend, with a moderate and non-significant increase in mOS when considering no histological stratification and a significant increase when only non-epithelioid patients are considered (10 vs. 17.9 months) [74]. The ongoing DREAM3R trial explores the inclusion of durvalumab alongside platinum-pemetrexed chemotherapy, with either platinum-pemetrexed or nivolumab–ipilimumab as the control group (**Table 1.2**).

Table 1.1: Phase II chemoimmunotherapy clinical trials in PM. C = cisplatin, P = pemetrexed.

Name	ID	Cohort	Experimental arm	Completion Date
PrE0505	NCT02899195	55	C + P + Durvalumab	2023
DREAM	ACTRN12616001170415	54	C + P + Durvalumab	2020
JME-001	UMIN000030892	18	C + P + Nivolumab	2021

Table 1.2: Phase III chemoimmunotherapy clinical trials in PM. C = cisplatin, Ca = carboplatin, P = pemetrexed.

Name	ID	Cohort	Experimental arm	Control arm
DREAM3R	NCT04334759	214	C/Ca + P + Durvalumab	C/Ca + P Ipilimumab + Nivolumab
ETOP-BEAT Meso	NCT03762018	401	Ca + P + Bevacizumab + Atezolizumab	Ca + P + Bevacizumab
IND.227	NCT02784171	520	C/Ca + P + Pembrolizumab	C/Ca + P

Novel immunotherapies

Other immunotherapeutic approaches are currently under investigation, primarily in second-line or maintenance settings **(Figure 1.9)**. Besides nivolumab and ipilimumab, various other **ICIs**, alone or in combination, have been considered. Notably, a recent phase I/II clinical trial (NCT02460224) exploring the combination of ieramilimab (anti-lymphocyte-activation gene 3 (LAG-3)) and spartalizumab (anti-PD-1) has shown promising results in progressive PM patients previously treated with antineoplastic therapy [75]. Additionally, a phase I study (NCT03652077) evaluating INCAGN02390, an anti-T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) antibody, is currently underway in PM patients [76]. Results are also awaited from a phase I trial (NCT02812875) of CA-170, an novel oral ICIs targeting both PD-L1/PD-L2 and V-domain Ig suppressor of T-cell activation (VISTA), which has shown promising outcomes in preclinical cancer models [77]. Another promising immunotherapeutic approach involves **dendritic cell (DC) vaccines**. A small-scale clinical trial (NCT01241682) has already demonstrated their safety and feasibility [78]. However, a subsequent randomized phase II/III trial (NCT03610360) evaluating DC immunotherapy as a maintenance treatment following standard first-line chemotherapy yielded disappointing results, with no significant increase in mOS compared to best supportive care alone. **Peptide vaccines** targeting WT1 and mesothelin (MSLN), highly expressed in PM, have also been explored. A phase II trial (NCT01265433) demonstrated the safety of the WT1 peptide vaccine galinpepimut-S in previously treated PM patients, demonstrating median progression-free survival (PFS) and OSRs 36% and 25% longer, respectively, than those treated with placebo. Also based on tumor antigens, another emerging immunotherapeutic strategy involves **chimeric antigenic receptor (CAR) T-cells**. Promising results have emerged from phase I/II clinical trials utilizing anti-MSLN CAR T-cells for PM treatment, demonstrating favorable anti-tumor activity and safety outcomes [79]. Additionally, this therapy, in combination with anti-PD-1, was evaluated in a phase I trial, demonstrating a mOS of 23.9 months [80]. These promising results served as the basis for the phase II, which is currently ongoing. Finally, another alternative approach, termed **in situ immunogene therapy**, involves the intrapleural injection of a non-replicating adenoviral vec-

tor expressing interferon (IFN)α in addition to celecoxib (cyclooxygenase (COX)-2 inhibitor) and gemcitabine [81]. Following promising results from a phase II trial, a phase III trial (INFINITE; NCT03710876) is currently ongoing.

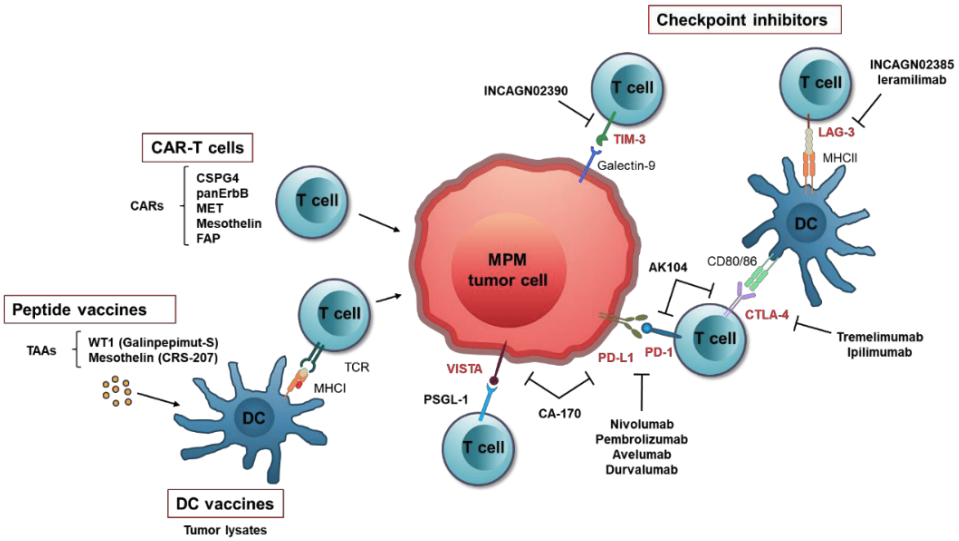


Figure 1.9: Currently evaluated PM therapeutic strategies. Current treatments under investigation include DC and peptide vaccines, CAR T-cells, and next-generation ICIs. *Modified from* [76].

2. Tumor microenvironment and pleural mesothelioma

Tumor microenvironment (TME) is defined as a complex and dynamic mixture of tumor, stromal, endothelial, and immune cells embedded in an extracellular matrix (ECM) [82]. Besides spatial intratumoral heterogeneity, TME composition is influenced by factors such as the patient's characteristics, histological subtypes, and treatment modalities. PM TME is defined as immunologically "altered" and characterized by varying degrees of T-cell infiltration. The altered TME can be further categorized as "immunosuppressed", characterized by intermediate infiltration of exhausted cluster of differentiation (CD)8^+ T cells and immunosuppressive cells within the tumor nest, or "excluded" where $CD8^+$ T cells are primarily restricted to the invasive margin. In PM, the TME is typically altered or excluded [83].

2.1. Spatial organization of the tumor microenvironment

Tumors could be divided into three distinct compartments: the tumor core, also referred to as the tumor nest, housing most tumor cells, the tumor stroma, enriched with stromal components like cancer-associated fibroblasts (CAFs) surrounding the tumor core and the invasive margin, delineating the transition zone between these two regions **(Figure 1.10)**. Importantly, there is notable heterogeneity in the spatial distribution of immune cells across these compartments, likely attributable to variations in tumor tissue origin and the high mobility of immune cells [84].

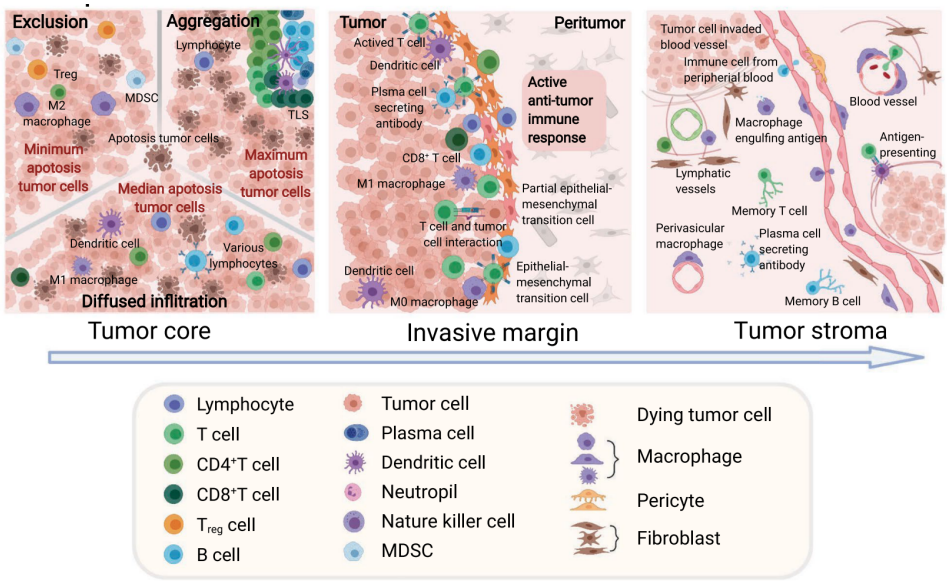


Figure 1.10: Spatial architecture of the TME. Tumors are generally divided into the tumor core, tumor stroma, and invasion margin located between the tumor core and the stroma [84].

Within those different compartments, patterned structures of immune cells have been identified. Tertiary lymphoid structures (TLSs) are organized cellular aggregates resembling secondary lymphoid organs (SLOs) that are formed under pathological conditions in non-lymphoid organs [85, 86]. These structures can be found within the three compartments of the tumor [87]. TLSs are delineated by a T-cell rich zone containing conventional DCs and high endothelial venules (HEVs), within which B-cells are organized in follicles containing follicular DCs ($CD21^+$), and plasma cells **(Figure 1.11 A)**. [87, 85]. Emerging evidence indicates that adaptive immune responses can be initiated within TLSs, recapitulating SLOs functions locally [85]. Recent studies demonstrate that the presence of TLSs before treatment correlates with favorable responses to PD-1 or combined PD-1 and CTLA-4 blockade in melanoma or soft tissue sarcoma [88, 89]. Therapeutic induction of TLSs could therefore be a promising therapeutic approach and has been shown to be feasible in some human cancers [90, 91]. Interestingly, TLSs have been identified in epithelioid PM and correlated to prolonged survival [92].

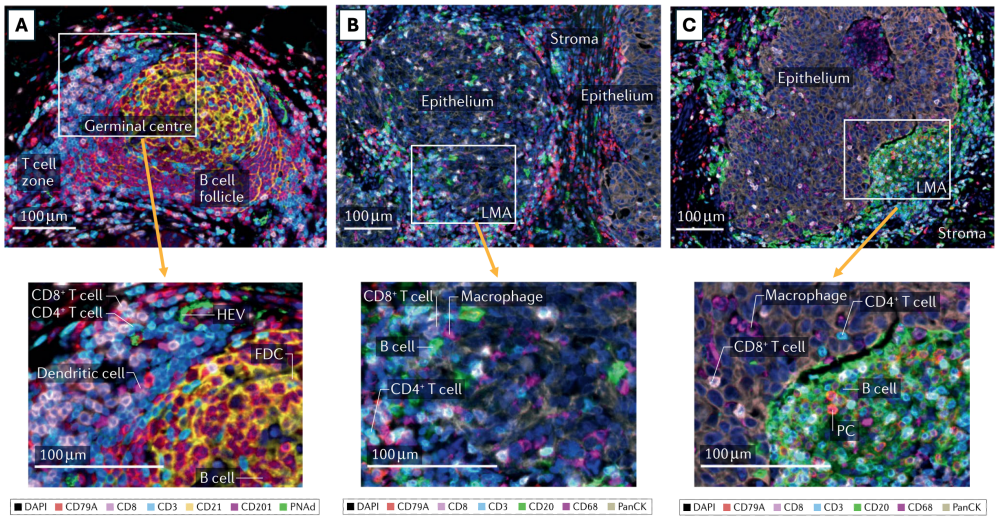


Figure 1.11: Structure of TLS and LMA: Immunofluorescence staining of untreated human high-grade serous ovarian cancer. A. Secondary follicular TLS. **B.** LMA with infiltration of the epithelial region by $CD4^+$ and $CD8^+$ T-cells and B-cells **C.** LMA with a dense stromal infiltrate of B-cells with adjacent infiltration of T-cells and TAMs [86]. TLS, tertiary lymphoid structure; LMA, lympho-myeloid aggregate.

Another structure, less organized than TLSs, is termed lympho-myeloid aggregate (LMA) which comprises non-follicular aggregates of B-cells, T-cells, and myeloid cells **(Figure 1.11 B-C)** [86]. Additional structures within the TME include the perivascular niche, the antigen presenting cell (APC) niche, the stem cell niche, the pre-metastatic niche, and the metastatic niche [84].

2.2. Infiltrating immune cells

2.2.1. Tumor-associated macrophages

Tumor-associated macrophages (TAMs) represent a substantial cell population within the TME, typically linked to tumor development, outgrowth, invasion, and poor therapeutic response in most tumors [93, 94]. However, in colorectal cancer, TAMs assessed through CD68 expression have also been associated with favorable prognoses [93, 95]. In PM, TAMss are the predominant immune cell population, but their role as a prognostic factor remains unclear due to conflicting findings regarding the predictive value of CD68 expression, which may be influenced by variations in therapies across the cohorts [96, 97, 98]. In the largest cohort (n=220), neither $CD68^+$ nor $CD163^+$ cells emerged as predictive of mOS. However, the tumoral ratio CD163/CD8 was identified as an independent prognostic factor for mOS, wherein a higher ratio correlated with significantly lower mOS in epithelioid PM [98].

It was initially postulated that TAMs solely arose from bone marrow-derived monocytes recruited via chemotaxis into the TME through chemokines such as CCL2, colony stimulating factor 1 (CSF1), VEGFA or vascular permeability factor (VPF) [99, 100]. However, subsequent evidence has indicated that tissue-specific embryonic-derived resident macrophages also infiltrate tumor tissues, although to a lesser extent than monocyte-derived TAMs [99]. In human PM, the relative contribution of both origins to the TAM population remains unknown, although this could be of particular importance. Intriguingly, depending on their origin, TAMs may exert distinct effects on tumor growth in PM. In a murine model of PM, a recent investigation demonstrated that monocyte-derived TAMs, defined as small peritoneal/pleural macrophagess (SPMs), adopted an M2-like phenotype and promoted tumor growth, as their selective depletion led to tumor regression, whereas tissue-resident TAMs, defined as large peritoneal/pleural macrophages (LPM), contributed to the anti-tumor response [101].

TAMs exhibit remarkable plasticity and perform diverse functions, making their classification into distinct subtypes challenging. Initially, TAMs were dichotomized into 'classically' activated M1 (inflammatory) or 'alternatively' activated M2 (immunosuppressive) macrophages, referring to the helper T-cell (T_h) polarization model. However, the advent of single-cell RNA sequencing (scRNA-seq) analyses has led to the emergence of a novel classification system [102, 100]. Seven primary subtypes, shared across various cancer types, have been delineated: interferon-primed TAMs, immune regulatory TAMs, inflammatory cytokine-enriched TAMs, lipid-associated TAMs, pro-angiogenic TAMs, RTM-like TAMs, and proliferating TAMs **(Figure 1.12)** [102].

Anti-tumoral functions include antibody-dependent cell cytotoxicity (ADCC), T-cell activation, phagocytosis, and pro-inflammatory cytokine production. However, evidence suggests that these functions are limited to the early phases of tumor develop-

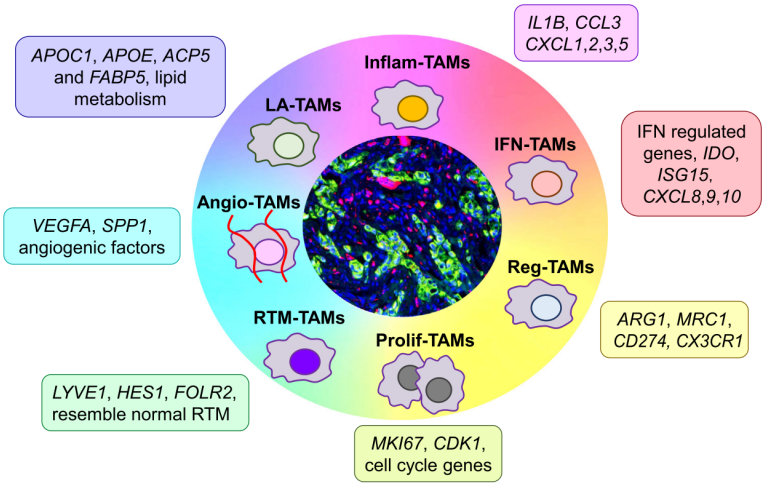


Figure 1.12: Main TAM subsets across tumors. Advanced single-cell technologies have led to the identification of seven distinct subsets of TAMs. These subsets exhibit unique molecular profiles and are consistently observed across various human cancer types [102]. TAM, tumor-associated macrophage; RTM, resident-tissue macrophages; LA, lipid-associated; Reg, regulatory; Prolif, proliferating; Angio, angiogenic; Inflam, inflammatory; IFN, interferon.

ment, as illustrated by a study showing that T-cell activation by TAMs is predominantly observed in early-stage tumors in human lung cancer [103]. Conversely, in established tumors and during late-stage tumor development, TAMs actively contribute to tumor progression and dissemination. They release nitric oxide (NO) and ROS, inducing DNA damage and fostering genetic instability [104]. Additionally, TAMs contribute to metastatic dissemination by releasing IL-1 and transforming growth factor-beta (TGF-β), which, alongside proteases, facilitate ECM remodeling and pathological fibrosis [105]. TAMs also promote neo-vascularization through the secretion of pro-angiogenic factors (e.g. VEGF). Moreover, within the TME, they induce immunosuppression by secreting anti-inflammatory cytokines (e.g., IL-10, TGF-β, indoleamine 2,3-dioxygenase (IDO)), notably fostering the expansion of regulatory T-cells and resulting in T-cell metabolic starvation [99].

In PM, TAMs have therefore emerged as an interesting target for therapeutic intervention. Preclinical investigations have highlighted that targeting the CSF1/ CSF1R axis, pivotal for TAM recruitment within the TME, in conjunction with either DC vaccination or ICIs, results in reduced tumor growth through enhanced functionality of $CD8^+$ T-cells [106, 107]. Another approach involves TAMs reprogramming to suppress their pro-tumor functions. An interesting strategy relies on galectin-9 targeting, which induces TAM reprogramming towards anti-tumor phenotype while concomitantly stimulating apoptosis in PM cells [108].

2.2.2. T-cells

T-cells constitute the second most abundant immune cell population in PM (10-20%) and play a pivotal role in immunotherapy based on ICIs. Conventional T-cell subsets include CD4$^+$ (T_h and regulatory T-cell (T_{reg}) and CD8$^+$ T-cells, each playing distinct roles within the TME. As for most cancers, CD8$^+$ T-cell infiltration has been associated with improved mOS after surgery, chemotherapy, or in the absence of treatment in epithelioid PM [98, 109, 110].

CD8$^+$ T-cells

CD8$^+$ T-cells have traditionally been depicted as cytotoxic cells characterized by their secretion of cytolytic enzymes. While this population, often referred to as "Tc1," dominates the CD8$^+$ landscape in various cancers, recent research has uncovered other CD8$^+$ subsets resembling their CD4$^+$ counterparts, and also infiltrating the TME **(Figure 1.13)** [111].

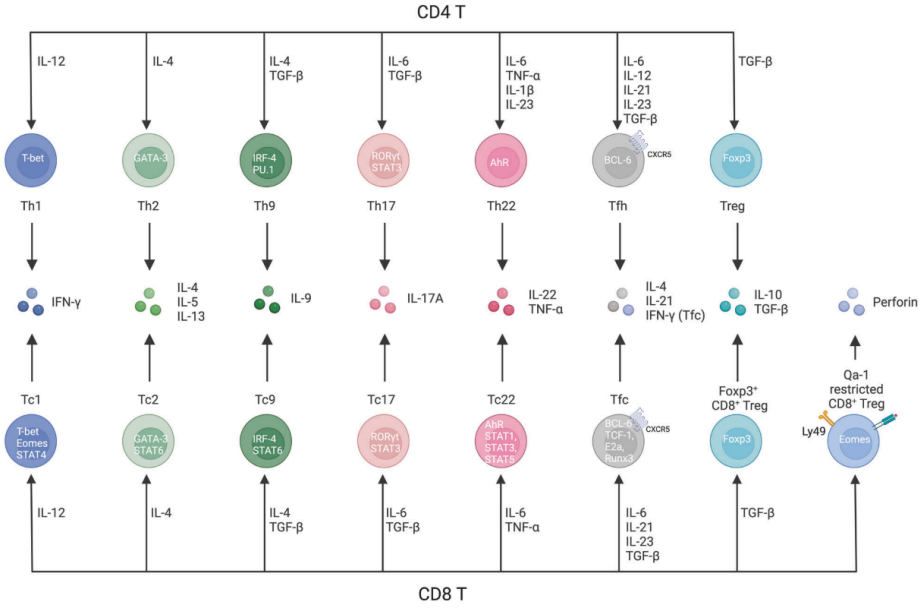


Figure 1.13: Historical and new CD8$^+$ and CD4$^+$ T-cells subsets identified in tumor tissues. New CD8$^+$ T-cells have recently been described based on their resemblance with CD4$^+$ T_h. They share common lineage-determining transcription factors and effector cytokine profiles [111].

Following activation, effector Tc1 can eliminate tumor cells through mechanisms such as engagement of death ligand/receptor complexes (e.g. FAS/FAS ligand (FASL)) or release of death-inducing granules (i.e. perforin, granzymes, cathepsin C and gran-

ulysin) inducing apoptosis or pyroptosis, the latter one being promoted by IFNγ [111, 112]. Upon antigen clearance, effector cells give rise to memory Tc1 cells (encompassing stem cell memory T-cell (T_{scm}), central memory T-cell (T_{cm}) and effector memory T-cell (T_{em})) that maintain cytotoxic traits and rapidly produce IFNγ upon reactivation. These cells have been proposed as potential predictive markers for responses to ICIs, as demonstrated in lung cancer or melanoma [113, 114]. Consistently, in a retrospective analysis of PM patients from the INITIATE clinical trial (NCT03048474) receiving nivolumab and ipilimumab, responder patients exhibited higher frequencies of blood CD45RA$^+$/C-C chemokine receptor type 7 (CCR7)$^-$ effector memory CD8$^+$ T-cells cells re-expressing CD45RA CD8$^+$ before treatment, suggesting it could also apply to PM [115]. Another critical phenotype observed in the TME is exhausted Tc1 [116]. Exhaustion arises from prolonged exposure to antigens and is defined by a gradual loss of effector functions [117]. These cells typically exhibit heightened expression levels of multiple co-inhibitory receptors, such as PD-1, LAG-3, TIM-3, CTLA-4, and T-cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT) alongside an epigenetic program driven by thymocyte selection-associated high mobility group box protein (TOX) [83, 116, 118]. Notably, cells expressing these exhaustion markers display varying degrees of impairment in cytotoxic function. PD-1, LAG-3, and TIM-3 were up-regulated in CD8$^+$ T-cells from PM samples compared to pleuritis [119].

Besides Tc1, other subsets of CD8$^+$ T-cells have been identified including Tc2, Tc9, Tc17, Tc22 and CD8$^+$ T_{reg} [111]. While certain subsets like Tc9 and Tc22 have demonstrated the ability to induce tumor regression into tumor-bearing mice [120], there remains a significant lack of investigation regarding the presence and functions of these cells in PM. A preclinical study however suggests that Tc17 could play a role in the response to anti-CTLA-4 antibodies in PM [121].

CD4$^+$ T-cells

Although less frequent than Tc1, CD4$^+$ T-cells, including T_{reg} are important components of the PM TME. Different subtypes of CD4$^+$ T_h have been described. Specifically, the T_h1 subset manifests anti-tumorigenic properties by enhancing the activity of anti-tumor cytotoxic CD8$^+$ cells, natural killer (NK)-cells and B-cells [122]. Additionally, they engage in the direct elimination of cancer cells through the secretion of IFNγ and TNF-α [123]. At the opposite, the T_h2 subset secretes anti-inflammatory molecules that paradoxically support tumor growth [123]. Emerging evidence suggests that CD4$^+$ T-cells may significantly influence the effectiveness of ICIs. In addition to T_h cells, cytolytic tumor-specific CD4$^+$ T-cells have also been identified in some cancers through scRNA-seq analysis and *ex vivo* confirmation [124].

T_{reg} (CD4$^+$forkhead box P (FOXP)3$^+$CD25^{hi}CD127^{low}) are another essential lymphoid population. These cells are highly immunosuppressive populations and maintain

immune tolerance through IL-10 and TGF-β secretion. Consistently, T_{reg} depletion has been associated with suppressed tumor growth in murine PM models and heightened cytotoxic T-cell activity [125, 126]. However, an alternative study reports that T_{reg} may not play a significant role in suppressing anti-tumoral responses in murine PM [127]. Interestingly, due to a skewed T-cell receptor (TCR) repertoire favoring self-recognition, T_{reg} tend to infiltrate tumors earlier than effector T-cells and exhibit greater responsiveness to tumor-derived self-antigens [128].

2.2.3. Monocytes

Monocytes are often overlooked as distinct cell populations within the TME due to challenges in distinguishing them from monocyte-derived TAMs and DCs. However, given their role as precursors to TAMs and DCs within the TME, monocytes hold significant importance in understanding tumor progression and therapeutic modulation. In PM patients, a high level of circulating monocytes has been correlated with a poor prognosis [96].

Blood monocytes have been categorized into three main subtypes: "classical" ($CD14^+$ $CD16^-$), "intermediate" ($CD14^+CD16^+$), and "non-classical" ($CD14^-CD16^+$) monocytes [129]. However, *in vivo* experiments have demonstrated their belonging to a phenotypic continuum where non-classical monocytes derive from classical ones, with intermediate being a transitional state [130].

Recent pan-cancer scRNA-seq analyses have identified the presence of two subtypes ($CD14^+$ and $CD16^+$) corresponding to both classical and intermediate/non-classical monocytes within the TME (referred to as tumor-infiltrating monocytes (TIMs)) [131, 132]. Upon extravasation into the TME, both subsets of TIMs exhibit up-regulation of tissue-resident markers such as *nuclear receptor (NR)4A1* (mainly for the Mono $CD16^+$), *NR4A2*, and *NLR family pyrin domain containing 3 (NLRP3)*, alongside inflammatory cytokines and chemokines including *IL-1*β, *CCL4*, *C-X-C motif chemokine ligand (CXCL)2*, and *C-X-C motif chemokine receptor (CXCR)4*. Conversely, they down-regulate the expression of a set of neutrophil-associated genes (*S100 calcium-binding protein (S100)A8*, *S100A9*, and *colony stimulating factor 3 receptor (CSF3R)* compared to their blood counterparts [131]. Furthermore, TIMs up-regulate macrophage-related genes (*CD163* and *CD68*), suggesting differentiation into TAMs within the TME [131]. Nevertheless, the signals governing the fate determination of monocytes, whether they persist as monocytes, undergo further differentiation, or undergo programmed cell death, are still incompletely understood [133].

Monocytes demonstrate several functionalities, including phagocytosis, antigen presentation, or ADCC. Similarly to macrophages, monocytes are capable of phagocytosis albeit to a lesser extent. *In vitro* studies have demonstrated that all three blood-derived monocyte subtypes can engulf fluorescent polystyrene beads [134]. Nevertheless, the significance of this phagocytic function *in vivo* in anti-tumor responses necessitates further investigation, particularly given their elevated expression levels of

signal regulatory protein (SIRP)α that negatively regulates phagocytosis upon binding with CD47, expressed at the tumor cell surface [129].

Monocytes have also been considered as APCs, contributing to T-cell activation. Tissue-resident monocytes expressing CCR7 use afferent lymphatics, while blood monocytes expressing CD62L traverse HEVs to reach lymph nodes [135]. Within secondary lymphoid organs, monocytes present antigenic peptides via major histocompatibility complex (MHC)-II. Intermediate and non-classical monocytes, with heightened MHC-II expression, are considered as APCs. However, debate persists on their antigen presentation capacity [129]. A recent study investigating exogenously administered monocyte antigen presentation abilities, demonstrated their reliance on endogenous DCs for antigen presentation indicating their deficiency in direct presentation to $CD8^+$ T-cells. This study nevertheless underscores a potential role in enhancing the anti-tumor response of CD8+ T-cells through antigen transfer to endogenous DCs [136].

Furthermore, monocytes exhibit the capacity to induce tumor cell death through cytokine-mediated pathways. Upon stimulation with IFNγ, they up-regulate TNF-related apoptosis-inducing ligand (TRAIL), enabling the activation of TRAIL/TRAIL receptor signaling in sensitive cells. Additionally, the $CD16^+$ population exhibits the ability to perform ADCC through the engagement of its CD16 receptor, triggering TNF-α secretion and up-regulation of TNFR on the target cell [137]. However, those cytotoxic functions have only been established *in vitro*, and the contribution of monocyte-mediated killing to *in vivo* anti-tumoral responses remains elusive [129]. Besides, investigations have revealed that monocytes isolated from cancer patients manifest diminished cytotoxic activity *in vitro* [138], suggestive of tumor-induced reprogramming.

In PM, cells within the TME produce various chemotactic factors that attract monocytes into the tumor. These factors notably include CCL2 that acts as the principal chemokine orchestrating monocyte influx [139]. Elevated levels of CCL2 have been identified in PM patients and correlated with tumor stage progression [140].

2.2.4. Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are immunosuppressive cells arising from a pathological (e.g. prolonged presence of myeloid growth factors and inflammatory signals) state of activation, leading to an immature phenotype with relatively weak phagocytic activity, increased background levels of ROS production, and high expression of anti-inflammatory cytokines [141, 142]. MDSCs are divided into two major subtypes, namely granulocytic/polymorphonuclear MDSCs (PMN-MDSCs) ($CD11b^+$ $CD14^-$ $CD15^+CD66b^+LOX\text{-}1^+$) and monocytic MDSCs (M-MDSCs) ($CD11b^+$ $CD14^+$ $CD15^-$ human leukocyte antigen (HLA)-DR^- $CD84^+$), along with a third small group referred to as early-immature MDSCs ($Lin-CD11b^+$ $CD34^+$ $CD33^+$ $CD117^+$ HLA-DR^-) **(Figure 1.14)** [133, 143, 142]. Besides cell surface markers, transcriptomic signatures of PMN-MDSCs and neutrophils as well as M-MDSCs and monocytes from the same patients are significantly different [144, 145]. Although PMN-MDSCs is generally predominant, in PM TME, PMN-MDSCs and M-MDSCs are equivalently abundant and represent approximately 10% of the immune infiltrate and have been both associated with worse mOS and PFS [119].

The main characteristic that defines MDSCs is their ability to inhibit immune responses, including those mediated by T-cells, B-cells, and NK cells, with M-MDSCs exhibiting a higher immunosuppressive capacity in cancer patients [133, 143]. These effects arise from underlying mechanisms such as the depletion of arginine and tryptophan, facilitated by the expression of effector enzymes including arginase 1 (Arg1), inducible NO-synthase (iNOS), IDO, alongside the production of ROS and prostaglandin E_2 (PGE2) [143, 142].

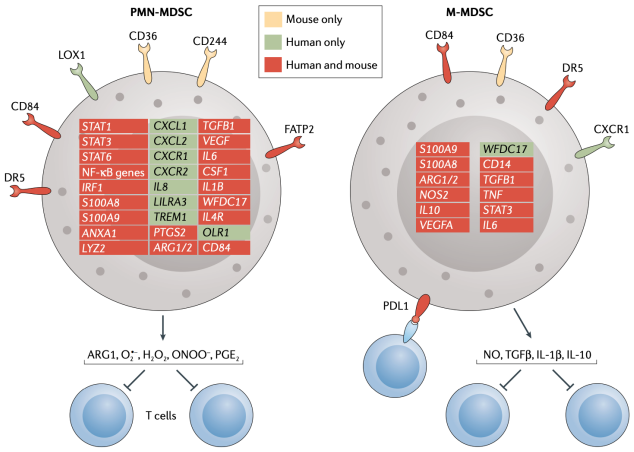


Figure 1.14: Phenotypic characterization of M-MDSCs and PMN-MDSCs [142]. PMN-MDSC, polymorphonuclear myeloid-derived suppressor cell; M-MDSC, monocytic myeloid-derived suppressor cell.

2.2.5. NK cells

NK cells ($CD3^{-}CD56^{+}$) are present within the TME of most PM cases but in small numbers, accounting for 0-5% of total immune cells and have not been correlated with prognosis in PM [146, 147].

In humans, peripheral blood NK cells are categorized into two main subsets: $CD56^{dim}$ $CD16^{hi}$ (NK1) and $CD56^{hi}CD16^{low}$ (NK2), each with distinct functions [148]. The NK1 subset is primarily involved in cytotoxic activity, whereas the NK2 subset is responsible for immuno-regulation through enhanced cytokine production [149, 148]. The fundamental function of $CD56^{dim}CD16^{hi}$ cells is therefore the elimination of cells that have lost or under-expressed HLA-I. In PM, recurrent deletion of the β_2 *microglobulin (B2M)* gene has been identified [48].

Both subsets of NK cells infiltrate the TME and undergo transcriptional modifications upon recruitment into the tumor. Through pan-cancer analysis, *regulator of G-protein signaling (RGS)1* has been identified as a specific transcriptomic marker for tumor-infiltrating NK cells. A subset of dysfunctional NK cells, originating from the NK1 subset, has also been described. It is characterized by a reduced cytotoxicity and an increased expression of the inhibitory receptors *CD158a* and *CD158e* but not *PD-1* and *CTLA4*. This subset has also been identified as a driver of T-cell dysfunction [150].

In a preclinical PM model, the combination of cisplatin and α-galactocysylceramide led to increased NK-cell numbers in both tumor and peripheral blood, along with elevated levels of cytolytic enzymes and cytokines, indicating the potential for enhancing the immune response through heightened NK-cell activity [151].

2.2.6. Dendritic cells

DCs play a crucial role as APCs, facilitating T-cell activation and differentiation. DCs are generally divided into conventional DCs (cDCs), which include cDC1s and cDC2s (that can be further divided into DC2 and DC3), plasmacytoid DCs (pDCs) and monocyte-derived DCs (moDCs), based on their ontogeny [152].

Studies have shown that circulating DCs (moDCs, cDCs, and pDCs) are significantly reduced in patients with PM compared to an age- and gender-matched control group of healthy individuals [153]. Consistently, IHC analyses demonstrated that DCs were rarely detected or found in small numbers in TME of PM patients [146]. Furthermore, *in vitro* experiments revealed that moDCs in PM exhibit functional deficiencies, as evidenced by reduced expression of co-stimulatory molecules (CD40, CD80, and CD86) and MHC molecules compared to healthy controls [153].

2.2.7. B-cells/Plasma cells

Tumor-infiltrating B-cells (TIL-Bs) and tumor-infiltrating plasma-cells (TIL-PCs) are integral components of the adaptive immune response [154]. Like T-cells, they

are typically linked to a positive prognostic outcome (in 50%) or neutral (41%) effect across various cancer types [155]. In epithelioid PM, higher counts of $CD20^+$ TIL-Bs have been associated with enhanced mOS, independently of the treatment modality used [98].

Recent scRNA-seqs studies revealed the presence of naive, activated and memory B-cells, germinal center B-cells, and plasma cells in TME with great diversity according to the tumor [86]. Furthermore, exhausted ($CD69^+$ $CD27^-CD21^-$) and regulatory ($IL\text{-}10^+IL\text{-}35^+TGF\text{-}\beta^+$granzyme B $(GZMB)^+$) TIL-B subsets have been described in lung cancer [156].

TIL-Bs exhibit several functions. They serve as mediators in the recruitment of various immune cell types, including T-cells, TAMs, and NK cells, achieved through the secretion of immunostimulatory cytokines such as CCL3, CCL4, CCL5, IL-2, IL-6, IFNγ, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [157, 86]. In addition, notable features of TIL-Bs lie in their ability to present antigens and to expand clonally, allowing them to shape the immune response towards particular antigens [86]. This capability extends to antigen spreading, where an initial $CD4^+$ T-cell response to a mutated neoantigen can trigger subsequent B-cell and T-cell responses directed towards wild-type flanking epitopes. Such mechanisms hold promise in mitigating neoantigen loss due to immune editing, thereby enhancing the efficacy of cancer immunotherapy approaches [86]. In addition to cell-based functions and upon differentiation into TIL-PCs, they generate antigen-specific antibodies capable of fostering ADCC and antibody-dependent cell phagocytosis (ADCP) [157, 86]. Tumor antigen-specific antibodies are of particular interest in PM as the amount of antigen required to trigger a T-cell response is markedly reduced when internalized through an immune complex compared to its native form, which represents a significant advantage, especially in low-immunogenic tumors [158].

In PM, TIL-Bs and TIL-PCs nevertheless constitute only a minor fraction of immune infiltration, sometimes undetectable and their precise roles and contributions within TME remain incompletely understood, although evidence suggests a potential central role in responses to immunotherapies in PM preclinical models, independently of antibody production [159, 157].

3. Cancer treatments

Since the 1950s, chemotherapy has been the cornerstone of anti-cancer treatments, either in monotherapy or in combination with radiotherapy or between multiple chemotherapeutic agents [160, 161]. In recent years, a paradigm shift has occurred with the advent of immunotherapy based on ICIs, supplanting chemotherapy in specific tumor types, including melanoma and renal cell carcinoma [162]. Despite the remarkable efficacy of ICIs as a monotherapy for certain tumors, a significant number of cancers exhibit resistance to this treatment approach. Consequently, ongoing efforts are directed towards exploring novel strategies to improve immunotherapy response, including its combination with chemotherapy or with histone deacetylase inhibitors (HDACis) [160, 161, 163]. These strategies are currently being evaluated in PM.

3.1. Chemotherapy

Cancer chemotherapy could be broadly defined as the administration of cytotoxic or cytostatic chemical agents to trigger cell death or inhibit the proliferation of rapidly dividing cancer cells [164]. Conventional chemotherapy, also known as maximal tolerated dose (MTD) chemotherapy, involves the use of high doses of non-specific compounds, often leading to substantial off-target effects. In contrast, a more recent treatment approach, known as metronomic chemotherapy (mCT), involves the frequent, repeated administration of low-dose drugs, aiming to minimize toxicity [165, 166].

3.1.1. Chemotherapeutic agents

Conventional chemotherapeutic agents are commonly divided into five main groups based on their principal mechanism of action: alkylating agents, antimetabolites, topoisomerase antagonists, microtubule-targeting agents (MTAs), and anti-tumor antibiotics **(Figure 1.15)** [167].

Alkylating agents

Alkylating agents constitute the predominant and historically the more ancient class of anti-neoplastic chemotherapy [169]. Alkylating agents are broadly defined as electrophilic compounds that, upon biological activation, trigger the transfer of an alkyl group on DNA, ribonucleic acid (RNA) or proteins. Their cytotoxic effect is mainly attributed to the alkylation of DNA bases inducing structural alterations that impede replication and transcription processes ultimately leading to cell death [169, 170, 171]. Typically, the alkylation reaction predominantly targets the N7 and the O6 positions of the guanine, the N1 and N3 positions of the adenine, and the N3 position of cytosine, although other locations and DNA bases may undergo alkylation but to a lesser extent [171]. Alkylating agents can be divided into two main groups: mono- and bifunctional agents, based on the number of reactive sites. Mono-functional alkylating agents contain one active group, resulting in covalent adducts with the target, while

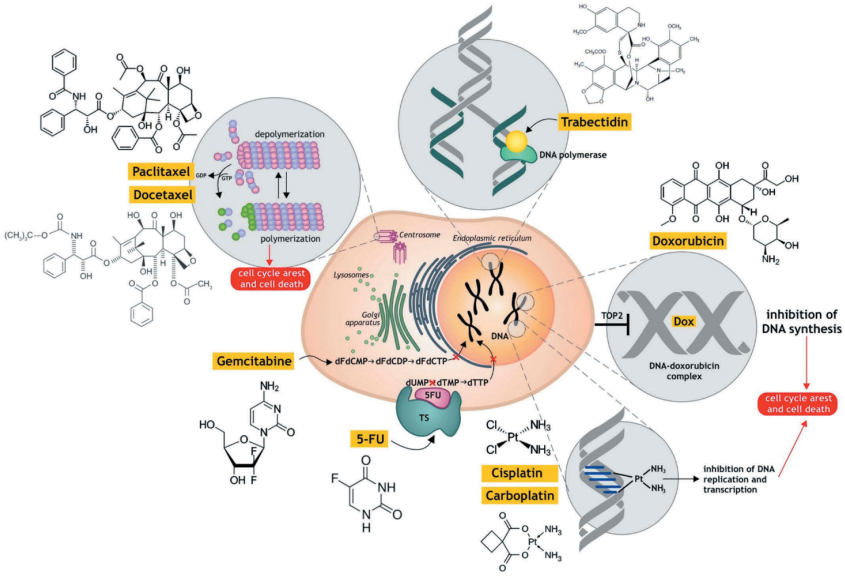


Figure 1.15: Main chemotherapeutic compounds and associated mechanisms of actions *[168].*

bifunctional alkylating agents contain two reactive groups allowing the formation of intra- or inter-strand crosslinks within DNA or between DNA and proteins [170, 171].

Mono-functional agents can be subdivided into methylating and chloroethylating agents based on the alkyl group they introduce. The first group includes hydrazine (e.g., procarbazine), triazenes (e.g., dacarbazine and temozolomide), as well as streptozotocin. These agents primarily induce methylation at the N7-position of guanine, leading to the formation of N7-methylguanine (7meG) and, to a lesser extent, at the N3-methyladenine (3meA) within double-stranded DNA (dsDNA) [172]. The highly unstable 7meG adduct typically results in an abasic site or the formation of 5-alkyl formamidopyrimidine [172, 171]. A third lesion is O6-methylguanine (O6meG), less frequent, but responsible for most cytotoxic and genotoxic effects associated with monofunctional alkylating agents. Chloroethylating agents constitute the second group of mono-functional agents, encompassing most nitrosoureas compounds. Notably, these molecules induce O6-chloroethylguanine (O6 Cl-ethylG) adducts, capable of forming cytotoxic inter-strand crosslinks with cytosine upon chemical rearrangement [170].

Bi-functional agents share similarities with mono-functional agents, but owing to the presence of two reactive active sites, they generate intra- or inter-strand crosslinks in addition to mono-adducts [170]. For example, nitrogen mustard compounds (melphalan, mechlorethamine, cyclophosphamide, ifosfamide, and chlorambucil) initially form N-monoadducts on N7-guanine, N3-adenine, and N7-adenine, subsequently progressing to guanine–guanine and guanine–adenine inter-strand crosslinks [171]. These

crosslinks can undergo conversion into DNA double-strand breaks that can be repaired through homologous recombination (HoR) or non-homologous end-joining (NHEJ). Notable bifunctional agents include nitrogen mustards, aziridine compounds (e.g., altretamine, mitomycin C, and thioTEPA), and busulfan.

In addition to these two categories, platinum-based compounds (e.g., cisplatin, carboplatin, and oxaliplatin) represent a distinct group, known as alkylating-like agents. These compounds do not directly alkylate DNA but form covalent adducts by binding to N7 of the guanine base, resulting in intra-strand (95%), mainly guanine-guanine or inter-strand (5%) crosslinks, thereby inhibiting DNA replication [169, 171, 173].

Antimetabolites

Antimetabolites are compounds that mimic naturally occurring bases/nucleosides or folic acid. They exert their anti-cancer effects by either inhibiting deoxyribonucleotide triphosphate (dNTP)-producing pathways, which typically rely on folic acid, or by direct incorporation into DNA during replication, thereby disrupting cellular replication and division. They can be categorized into two subgroups: purine and pyrimidine analogs, and antifolates [167, 169, 174].

Widely used antifolates include methotrexate, pemetrexed, raltitrexed, and pralatrexate [169]. These compounds inhibit dihydrofolate reductase (DHFR), thymidylate synthase (TS), glycinamide ribonucleotide formyltransferase (GARFT) and to a lesser extent 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT) albeit with different efficacy [174]. Both TS and DHFR are essential for pyrimidine synthesis, while GARFT and AICARFT are involved in purine biosynthesis [175]. Inhibition of nucleotide synthesis induces defects in DNA repair and replication leading to cell death.

Nucleoside analogs closely resemble endogenous nucleosides but exhibit alterations in either the deoxyribose or base structure. Following sequential phosphorylations, nucleotide analogs compete with endogenous dNTPs for incorporation into the nascent DNA strand by DNA polymerases [176]. The modification of the sugar component often disrupts the extension step from the mis-incorporated analog. Consequently, these compounds are commonly referred to as "chain terminators," although some compounds may still permit elongation. The stalling of replication forks upon chain termination can lead to induction of single-strand breaks (SSBs), DSBs, and other DNA lesions [177]. Interestingly, the non-terminal position of those analogs in the DNA chain impedes their detection and DNA repair by exonucleases [178]. Currently, base excision repair (BER) and mismatch repair (MMR) are believed to be essential repair pathways to remove nucleoside analogs into DNA [179]. In addition to impeding DNA replication, nucleoside analogs also inhibit key enzymes involved in DNA metabolism. Specifically, gemcitabine diphosphate (dFdCDP) and metabolites of clofarabine, fludarabine, and cladribine serve as substrates for ribonucleotide reductases (RNRs), ul-

timately causing its inhibition [176]. The inhibition of RNR results in a reduction of competing endogenous dNTP pools, thereby enhancing the integration of analogs in DNA [180]. Furthermore, gemcitabine triphosphate (dFdCTP) contributes to the inhibition of CTP-synthase and deoxycytidylate dCMP deaminase [176, 178]. Additionally, fluoropyrimides such as 5-fluorouracil (5-Fu) irreversibly impede TS [15]. Besides, 5-Fu also incorporates into RNA thereby altering messenger RNA (mRNA) expression and inhibiting mRNA splicing and pre-ribosomal RNA processing [181].

Topoisomerase antagonists

DNA topoisomerases are enzymes that play a pivotal role in maintaining the integrity of DNA structure by orchestrating topological adjustments through transient cleavage of DNA strands [182]. Topoisomerases are classified into two major classes: topoisomerase I, which induces transient SSB in DNA, and topoisomerase II, which causes transient DNA double-strand breaks (DSB). Following this distinction, topoisomerase inhibitors are classified as type I or type II depending on whether they inhibit topoisomerase I or topoisomerase II, respectively. Topotecan, irinotecan, belotecan, and camptothecin are type I topoisomerase inhibitors while etoposide, teniposide, doxorubicin, and mitoxantrone are type II [183].

Beyond this simplistic classification, topoisomerase inhibitors can be further categorized into groups based on their mechanism of inhibiting the enzyme's catalytic activity [184]. "Topoisomerase poisons", consists of clinically used inhibitors that specifically hinder the enzyme's catalytic activity by stabilizing the covalent DNA and topoisomerase complex, forming a ternary complex. This stabilization prevents the religation step of the catalytic reaction, resulting in the accumulation of DNA damage [184]. This group encompasses camptothecin and its derivatives as well as etoposide and teniposide [183, 185]. Doxorubicin, and mitoxantrone, an anthracycline and a synthetic drug, respectively, share similar mechanisms of action, although their interaction occurs through DNA intercalation, disrupting topoisomerase-II-mediated DNA repair [183, 184].

A second group comprises compounds that impair topoisomerase functions without interacting with the DNA-enzyme complex, referred to as "topoisomerase suppressors" and notably include merbaron, which compete with topoisomerase II for DNA binding [184].

Microtubule-targeting agents

MTAs are a class of chemotherapeutic agents that act as modifiers of microtubule dynamics. Microtubules are components of the cytoskeleton playing a key role in intracellular transport, regulation of cell morphogenesis and polarity, cell signaling, and chromosome separation during mitosis [186]. Microtubule inhibitors have been historically categorized into two groups: stabilizing and destabilizing agents according

to their effects on microtubule polymer mass at high concentrations and the binding sites on microtubules [187, 188].

Microtubule-stabilizing agents notably encompass taxanes (e.g. paclitaxel, docetaxel, and cabazitaxel) and epothilones (e.g. ixabepilone). These drugs exert their effects by binding to the β-tubulin in the lumen of microtubules, leading to microtubule stabilization, enhanced polymerization, and suppression of microtubule dynamics [187]. Consequently, this induces cell cycle arrest in the G2/M phase between metaphase and anaphase and eventually leads to apoptosis through activation of intrinsic mitochondrial apoptotic pathway [188, 189]. Indeed, and in addition to its binding to β-tubulin, a study showed that paclitaxel could bind to B-cell lymphoma 2 (Bcl-2), leading to the release of pro-apoptotic factors [189]. Destabilizing agents notably include vinca alkaloids (e.g. vinorelbine, vincristine, vinblastine, and vindesine), colchicine and analogs, steganacins, cryptophycins, and dolastatins. Their mechanism of action relies on the inhibition of microtubule polymerization [188].

However, these definitions were established based on *in vitro* and preclinical models utilizing cell lines with extended replicative capacity [190]. At clinically relevant concentrations, both classes achieve their effects without significantly altering the overall microtubule-polymer mass although they still induce reduced microtubule dynamics [191, 192]. Furthermore, it has been suggested that in human cancer, this mechanism accounts for only a small portion of the effects of MTAs [190]. Indeed, MTAs also exhibit mitosis-independent toxicity by inducing cell death in non-dividing cells through the modulation of cell signaling, vesicular trafficking, migration pathways, or vascular disruption [190].

Anti-tumor antibiotics

Some chemotherapeutic agents have been discovered through screening of microbial products and especially from *Streptomyces*. Those agents include the anthracyclines, bleomycin, and mitomycin [169].

Bleomycin contains a DNA-binding region and an iron-binding domain located at opposite ends. When it reacts with copper or iron, it forms a complex known as bleomycin-Fe(II)/Cu(II). This complex undergoes oxidation and binds to DNA, leading to apurinic/apyrimidinic sites or DNA DSBs and subsequent chromosomal aberrations through the formation of free radicals within DNA [193]. Research conducted on yeast indicates that the mutagenic effects of bleomycin are attributed to the resolution of apurinic/apyrimidinic sites through error-prone translesion synthesis [194].

In addition to serving as topoisomerase inhibitors, anthracyclines (doxorubicin, epirubicin, and daunorubicin), demonstrate additional functions. Their high-affinity binding to DNA via intercalation inhibits the synthesis of both DNA and RNA. Additionally, studies have shown that anthracyclines induce ROS production, thereby increasing their anticancer effects [169].

3.1.2. Conventional/cytotoxic chemotherapy

For over five decades, the predominant paradigm in chemotherapy was characterized by the MTD dogma, also referred to as "conventional chemotherapy". This methodology relies on administrating chemotherapy drugs at the highest doses tolerable by patients without causing life-threatening levels of toxicity [165]. Essentially, it involves short bursts of high-dose drug administration followed by prolonged drug-free intervals of two to three weeks to allow recovery of normal cells. Indeed, this methodology is associated with significant acute and cumulative toxic side effects, including acute myelosuppression, lymphopenia, nephrotoxicity, cardiac and neurological toxicity, and nausea leading to poor quality of life during treatment [165]. Those side effects therefore limit treatment duration [167]. In addition to these considerations, this treatment approach often contributes to tumor relapse by exerting extensive selective pressure, which allows chemoresistant clones to survive and further proliferate [195].

It is noteworthy that the influence of MTD chemotherapy on the immune system presents a paradox. On one hand, its inherent immunosuppressive activity enhances patient response by depleting immunosuppressive cells, while also potentially impairing its function. Indeed, and notwithstanding their direct effect on tumor cells, the therapeutic efficacy of some chemotherapeutic agents (e.g. anthracyclines, taxanes, oxaliplatin, and cyclophosphamide) partially hinges on the immune system, as part of the response is due to their capacity to heighten the immunogenicity of cancer cells, notably through immunogenic cell death induction, thereby re-activating the immune response [167]. Nevertheless, studies have demonstrated that even in the absence of immune suppression, MTD chemotherapy significantly compromises various immune functions. These include impairment of antigen presentation processes, reduced cell mobilization, and down-regulation of the expression of cell surface markers such as CD80 and CD86 [196]. A promising approach to leverage these benefits while avoiding toxicity leading to treatment discontinuation or the development of chemoresistance is through the implementation of mCT [195].

3.1.3. Metronomic chemotherapy

mCT involves the frequent and regular administration of low, less-toxic doses of chemotherapeutic drugs over extended periods, without prolonged drug-free intervals [164]. Initially developed to counteract the resurgence of vascular endothelial cells during breaks in conventional chemotherapy, the mCT schedule has shown promising results [165]. Early studies demonstrated that frequent administration of low doses of cyclophosphamide could exert **anti-angiogenic effects** in preclinical models of chemosensitive and chemoresistant lung cancer, breast cancer, and melanoma [197]. Moreover, beyond its potential direct cytotoxic effects on endothelial cells and their precursors, subsequent preclinical research revealed that mCT stimulates the secretion of the anti-angiogenic protein thrombospondin-1 (TSP-1) which can induce apopto-

sis in an autocrine manner by binding to CD36, and therefore acts as a endothelial-specific inhibitor of angiogenesis [198, 199]. Consistently, an increase in plasma TSP-1 has been observed in patients undergoing mCT with various chemotherapeutic agents [200, 201]. Additionally, multiple clinical trials have demonstrated that mCT leads to the down-regulation of pro-angiogenic factors including VEGF, HIF-1α and basic fibroblast growth factor (bFGF) [202].

Moreover, mCT exhibits a multitude of **immunomodulatory properties**. Preclinical models have revealed that low doses of cyclophosphamide or metronomic temozolomide effectively reduce the population and inhibit the functions of T_{reg}s [203, 204]. Accordingly, a decrease in blood T_{reg} has been observed in patients undergoing mCT with cyclophosphamide [205, 206, 207], although this reduction seems to be transient [206]. However, even transient reduction of T_{reg} allowed the restoration of anti-tumor T-cell response [206]. Additionally, mCT treatment can suppress MDSCs or prompt their differentiation into DCs, facilitate the maturation of DCs, improve the antigen presentation process and enhance the activation of cytotoxic NK and CD8+ T-cells [202, 208, 209]. Crucially, immune-modulatory responses frequently exhibit dose and schedule dependency, highlighting the significance of multiple schedule testing in preclinical models [210]. Currently, the most commonly tested drugs for mCT include cyclophosphamide, methotrexate, capecitabine, and vinorelbine [211, 209].

3.1.4. Chemoresistance

Chemoresistance is due to several intertwined mechanisms including inhibition of influx, enhanced efflux, lack of intracellular activation or enhanced inactivation, and interaction between cells within the TME [212].

Inhibition of influx

Cellular uptake of chemotherapy agents involves multiple mechanisms, including passive diffusion, transporter-mediated transport, and endocytosis (**Figure 1.16**) [213, 212]. Changes in the structure and composition of the cell membrane can impede both passive diffusion and endocytosis. Notably, studies have demonstrated that resistant tumor cells often exhibit unique membrane lipid profiles compared to their sensitive counterparts affecting the diffusion of doxorubicin and cisplatin [214, 215, 216]. Additionally, drug uptake is facilitated by transporter proteins, including members of the solute carrier superfamily that either facilitate passive diffusion along concentration gradients or actively transport substrates against concentration gradients [217]. While increased expression of these transporters generally enhances drug uptake, their subcellular localization is equally critical. In a recent investigation, it was found that a specific isoform of organic anion transporting polypeptide 1B3 (OATP1B3), by localizing to the lysosomal membrane, facilitates the transport of drugs into lysosomes therefore leading to their inactivation [217].

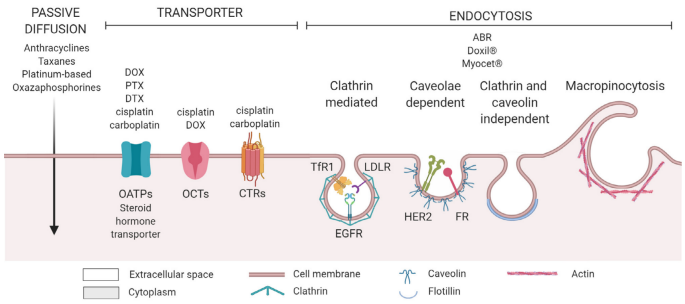


Figure 1.16: Pathways of chemotherapeutic drug uptake. Chemotherapy drugs can enter cells through various pathways, including passive diffusion, transport via plasma membrane transporters, and endocytosis. Remarkably, a single drug may use different mechanisms to be internalized by the cell [213]. OATP, organic anion transporters polypeptide; OCT, organic cation transporter; CTR, copper transporter.

Increased drug efflux

Drug efflux primarily occurs via ATP-binding cassette (ABC) transporters, among which multidrug resistance 1 (MDR1)) is one of the most extensively studied. MDR1 is widely expressed in cancers and has been associated with resistance to numerous therapies [218]. Other commonly investigated ABC transporters include ABCG2, ABCC1, and ABCC10 [212]. In addition to ABC transporters, other efflux transporters also contribute to chemoresistance. Preclinical studies demonstrated that a significant mechanism of cisplatin resistance involves its expulsion from the cell through copper-exporting P-type adenosine triphosphate (ATP)ases 1 and 2 (ATP7A and ATP7B) [219].

Lack of activation and intracellular inactivation

Two additional mechanisms contributing to chemoresistance are the detoxification of drugs and the failure to convert them into their active metabolites [212]. For example, cytarabine and gemcitabine depend on deoxycytidine kinases (DCKs) to phosphorylate them into their cytotoxic forms. Consistently, genetic alterations or decreased expression of DCKs have been identified in gemcitabine-resistant cancer cell lines [220]. However, once activated, drugs can also be inactivated by specific isoforms of aldehyde dehydrogenases (associated with cyclophosphamide resistance), glutathione-S-transferases (linked to platinum drug and doxorubicin resistance), cytochrome P450 (involved in gemcitabine and paclitaxel resistance), and uridine diphosphoglucuronosyltransferase [212].

Regulation from TME

Chemoresistance is also regulated by components of the TME. Specifically, CAFs contribute to resistance induction by secreting hyaluronan and matrix metalloproteinases (MMPs) [221]. Interaction with glycoproteins of the ECM indeed drives the activation of some metabolic pathways thereby inducing resistance in tumor cells [212]. A major axis is the stromal cell-derived factor (SDF)1/CXCR4 axis that, besides its role in tumor angiogenesis and metastasis, induces resistance to chemotherapeutic drugs in pancreatic or acute myeloid leukemia cells [222, 223].

3.2. Immune checkpoint inhibitors

Immune checkpoints are receptors expressed by immune cells that orchestrate dynamic regulation of functional outcomes during cellular interactions, particularly relevant to T-cell functionality and avoid inappropriate adverse effects impacting healthy cells [224, 225]. By utilizing monoclonal antibodies to target these checkpoints, it has become feasible to disrupt interactions that foster tumor growth. Presently, the FDA and the EMA have approved eleven ICIs targeting three four receptors, namely CTLA-4 (ipilimumab and tremelimumab), PD-1 (nivolumab, pembrolizumab, cemiplimab, dostarlimab, toripalimab), PD-L1 (atezolizumab, durvalumab and avelumab), and LAG-3 (relatlimab).

3.2.1. First generation of immune checkpoint inhibitors and their targets

The first monoclonal FDA-approved ICI, ipilimumab, targets **CTLA-4**, a crucial immune checkpoint that maintains immune tolerance and prevents autoimmune diseases [226]. In resting T-cells, CTLA-4 primarily resides intracellularly, but upon TCR engagement and co-stimulation via CD28 and CD80/86 binding, it translocates to the cell surface [227]. Once on the surface, CTLA-4 competes with CD28 for binding to CD80/86 for which it has a much higher affinity, thereby inhibiting further T-cell activation and proliferation (**Figure 1.17**) [225, 226]. This negative co-stimulation by CTLA-4 predominantly occurs at T-cell priming sites such as SLOs or TLSs. Blocking CTLA-4 therefore enhances T-cell priming and clonal diversity, mainly acting on $CD4^+$ T-cells [226, 228]. Based on preclinical evidence, another proposed mechanism of CTLA-4 inhibitors involves their potential to facilitate the mechanistic depletion of T_{reg}, which constitutively express high levels of CTLA-4, through ADCC [229, 230]. However, this notion has been more recently contradicted by analysis of tumors from patients treated with ipilimumab or tremelimumab, revealing no discernible effect of the treatment on the proportion of infiltrating T_{reg}, while showing increased CD4+ and CD8+ T-cell infiltration [231]. Hence, research currently focuses on finding new human antibodies that could induce T_{reg} ADCC in patients [231]. Anti-CTLA-4 monotherapy exhibits rather limited efficacy, primarily confined to melanoma, whereas

anti-PD-1 and PD-L1 have demonstrated effectiveness across a broad spectrum of tumors [225]. Blocking CTLA-4 increases the expression of PD-L1 in non-small cell lung cancer (NSCLC) cells *in vitro*, thereby facilitating immune evasion and justifying its combination with PD-1/PD-L1 blockade strategies.

PD-1 is a co-inhibitory receptor expressed upon initial activation through the TCR at the surface of T-cells. It is also expressed on B-cells, NK-cells, macrophages, DCs and monocytes as well as on some tumor cells [222]. In contrast to PD-1, **PD-L1** exhibits a broad expression across hematopoietic cells (e.g. T-cells, B-cells, DC, and macrophages) and non-hematopoietic cells (e.g. endothelial cells, keratinocytes, tumor cells) mainly upon exposure to inflammatory signals [226]. By binding to its receptors PD-L1 and PD-L2, PD-1 induces a negative feedback loop to dampen T-cell response, maintaining peripheral tolerance and preventing autoimmune adverse effects [222]. PD-1/PD-L1 binding triggers the recruitment of tyrosine phosphatases to the PD-1 tail, which dephosphorylates molecules downstream of the TCR thereby attenuating the stimulatory signals initiated by TCR interaction with the peptide-MHC-I complex and by co-stimulatory signals (CD28 and CD80/86) (**Figure 1.17**) [222, 225, 232]. Of interest, while PD-1 expression is reduced upon antigen clearance in infectious conditions, this is generally not the case in the TME, where sustained expression is observed [232]. This results in decreased T-cell activation, proliferation, and cytokine production through the exhaustion phenomenon. Hence, blocking the PD-1/PD-L1 signaling pathway predominantly affects exhausted $CD8^+$ T-cells. It prevents PD-1-mediated suppression of proximal TCR signaling, thereby reinstating the functionality of exhausted $CD8^+$ effectors [222, 225].

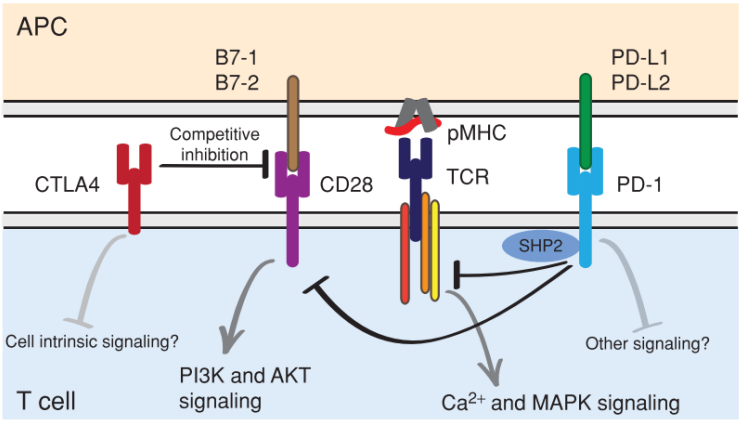


Figure 1.17: Mechanisms of CTLA-4 and PD-1 attenuation of T-cell activation. CTLA-4 prevents the co-stimulation by competing with CD28 for CD80/86. Upon binding with its ligand, PD-1 attenuates the downstream signaling of the TCR and CD28 [222]. APC, antigen-presenting cell.

Despite the promising responses observed with monotherapies, they are limited to a subset of patients. Consequently, multiple clinical trials demonstrated that patients treated with combined anti-CTLA-4 and anti-PD-1/PD-L1, had higher mOS compared to patients in the monotherapy group across various cancer types, notably including melanoma [233], NSCLC [234], and PM (not statistically confirmed) [235]. However, these combinations frequently result in increased adverse side effects and require thorough investigation alongside monotherapies, as they do not consistently translate into significant clinical benefits. This highlights the critical need to balance therapeutic efficacy with the risk of heightened toxicity [236].

Indeed, an important consideration regarding anti-CTLA-4 and anti-PD-1/PD-L1 is their potential for toxicity. Among the most prevalent adverse events are dermatitis, thyroiditis/hypothyroid, colitis, inflammatory hepatitis, and pneumonitis [225, 237]. However, these effects can often be mitigated by concurrent administration of corticosteroids without compromising anti-tumor efficacy. Interestingly, anti-PD-1/PD-L1 and anti-CTLA-4 therapies exhibit differential toxicities, with more severe immune-related adverse events (irAEs) associated with anti–CTLA-4 blockade occurring earlier and more frequently than those associated with anti–PD-1/PD-L1 monotherapy. Additionally, anti-CTLA-4 antibodies are linked to dose-dependent toxicities, whereas PD-1/PD-L1 blockade exhibits consistent toxicity rates across various agents and dose ranges [225]. While acute irAEs have been extensively documented, the observation that those acute events can develop into chronic irAEs has only recently gained interest. Retrospective data from melanoma patients treated with anti-PD-1/PD-L1 indeed suggest that chronic irAEs, defined as those persisting for more than 12 weeks after treatment discontinuation, are more prevalent than previously acknowledged, occurring in 43.2% of patients [238]. Similarly, in the CheckMate 743 trial, pneumonitis, rash, and renal dysfunction collectively accounted for over 20% of patients experiencing symptoms lasting more than a year [68]. Various strategies are currently under investigation to mitigate ICI-related adverse events. One approach involves probodies, which are antibodies with a peptide linked by a protease-cleavable linker, masking the epitope-binding site [239]. They are designed to be activated within the TME through the action of tumor-associated proteases. Another strategy involves the use of bi-specific antibodies. For example, cadonilimab is a tetrabody bispecific antibody, designed to target both PD-1 and CTLA-4, that reduces toxicity due to widespread co-expression of CTLA-4 and PD-1 on tumor-infiltrating lymphocytes but not on peripheral T-cells and overcome challenges associated with drug-drug interactions [240, 241].

Beyond toxicity, another critical consideration when using ICIs is the phenomenon of hyperprogression. This is broadly defined as an accelerated growth or a marked increase in the rate of tumor progression during or after ICI therapy, significantly deviating from baseline [242]. Hyperprogression is estimated to occur in approximately 5–30% of treated patients [243]. In the MAPS2 trial, 4% to 10% of second-line PM patients were identified as hyperprogressors when treated with nivolumab and ipili-

mumab or nivolumab alone, respectively [235]. Understanding the mechanisms underlying this phenomenon is crucial for improving further patient stratification and treatment strategies.

3.2.2. Next generation of ICIs and their targets

Besides anti-CTLA-4 and anti-PD-1/PD-L1, antibodies targeting other co-stimulatory or inhibitory receptors **(Figure 1.18)** have been extensively investigated and are currently being evaluated in multiple clinical trials [244].

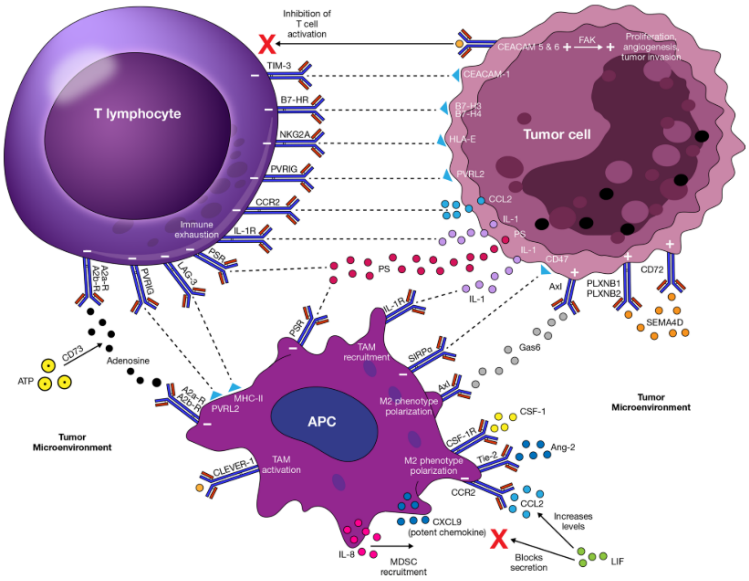


Figure 1.18: Targets for next-generation of ICIs. Summary of inhibitory immune checkpoint currently under investigation as targets for new generation of ICIs, inducing different effects on immune cell functions and tumorigenesis [244].

Among the co-inhibitory receptors under investigation, LAG-3, TIM-3, TIGIT and VISTA can be mentioned.

Recently, an anti-**LAG-3** antibody (relatlimab) has received approval for endometrial carcinoma treatment [245]. LAG-3 is a co-inhibitory receptor expressed on activated T-cells, NK-cells, B-cells and DCs. Due to its similarity to the CD4 receptor, LAG-3 binds to MHC class II on the surface of APCs with greater affinity than CD4 [246]. This interaction causes down-regulation of T-cell expansion and cytokine production and favors T_{reg} phenotype adoption to prevent tissue damage and autoimmunity [244].

TIM-3 fosters immune tolerance by binding to its principal ligands: galectin-9, phosphatidylserine, and CEA-related cell adhesion molecule (CEACAM)-1 [247]. This interaction inhibits IFNγ production and promotes T-cell exhaustion. Furthermore, it has been shown that specific binding to galectin-9 induces the expansion of MD-

SCs within the TME in preclinical models of mammary adenocarcinoma, thereby contributing to tumor growth [248]. TIM-3/galectin-9 pathway also trigger T-cell death [248]. Of interest, in contrary to LAG-3 and TIGIT, TIM-3 is also expressed on DCs, monocytes, and macrophages. Therapeutic blockade of TIM-3 will therefore affect multiple targets including T-cells but also NK cells, DCs and MDSCs [247].

TIGIT, expressed by activated NK cells and T-cells, exerts both direct and indirect immunosuppressive effects. It inhibits T-cell and NK cell functions by delivering a negative signal via binding to CD155 expressed on DCs, or by interfering with DNAM-1/CD155 binding, which otherwise delivers an activating signal and enhances cytotoxicity [249]. Furthermore, this binding also acts on DCs and induces IL-10 expression, inhibiting T-cell proliferation, while decreasing the expression of pro-inflammatory cytokines in DCs [250]. In addition to its interaction with DCs, TIGIT can also bind to receptors CD112 and CD113, along with CD155, expressed on tumor cells, that also deliver inhibitory signals to NK- and T-cells [249]. While TIGIT in monotherapy seems to have rather limited efficacy in some solid tumors (e.g. colon, rectum, breast, NSCLC, head and neck squamous cell carcinoma (HNSCC), and ovarian cancer), its combination with other ICIs seems promising [251]. Two anti-TIGIT antibodies, tiragolumab and ociperlimab are currently tested in combination with atezolizumab and tislelizumab, respectively, in phase III trials [252]. The combination with other ICIs seems promising, particularly given the low toxic profile of anti-TIGIT antibodies, with no grade 3–4 adverse events reported in monotherapy [252].

Similarly to TIGIT, **VISTA** is another immune checkpoint that is more specifically expressed by tumor-infiltrating T-cells, rendering it more tumor-specific and less toxic. VISTA exhibits both immunosuppressive and immunostimulatory functions. It serves as a stimulatory ligand for APCs, promoting immune activation. However, it also acts as a negative regulator for T-cells, suppressing their activation, proliferation, and cytokine production [253]. While its expression level is highly variable across different cancers, it appears to be highly expressed in epithelioid PM tumors, both in immune and tumor cells [45, 254].

It is noteworthy that co-stimulatory receptors such as **OX40**, **ICOS**, **CD137**, and **CD40** are also the focus of immune blockade and are currently under investigation [244].

3.2.3. Predictive factors of response to ICIs

One main limitation of ICIs is the overall low response rate. One of the pivotal challenges has therefore been the identification of biomarkers that can reliably predict treatment efficacy and facilitate patient stratification [224]. While numerous predictive biomarkers have been investigated and identified (**Figure 1.19**), tumor mutational burden (TMB), PD-L1 expression, and T-cell infiltration emerge as the primary ones.

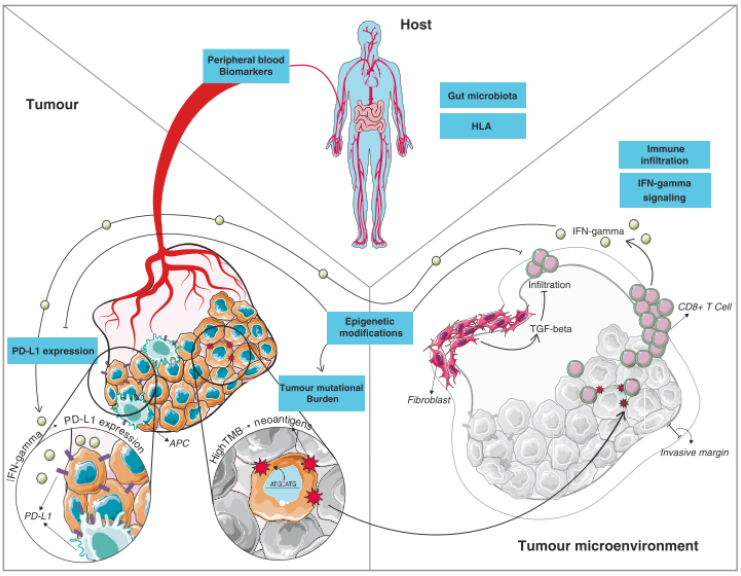


Figure 1.19: Potential predictive biomarkers to ICI response. Numerous factors encompassing TMB, PD-L1 expression, infiltration of CD8 T-cells, epigenetic modifications and intestinal microbiota have been investigated for their predictive nature to response to ICIs [224].

Tumor mutational burden

Tumor mutational burden (TMB) refers to the number of somatic coding mutations derived from single nucleotide variants (SNVs), and short insertions or deletions (INDELs), identified in tumor cells per megabase of the targeted genome region analyzed [255]. Its biomarker status is linked to the fact that a high number of non-synonymous mutations in exonic regions would result in increased neoantigens, some of which could be immunogenic and recognized by T-cells, thereby fostering improved anti-tumor immune responses [256]. Since 2020, TMB has been an FDA-approved predictive biomarker for the treatment of adult and pediatric unresectable solid tumors with pembrolizumab [257].

High TMB is positively correlated with responses to ICIs in tumors such as melanoma, lung, colorectal, head and neck, and bladder cancers, where high TMB is defined as the top 20th percentile within each cancer [258]. This association is attributed to the positive correlation between $CD8^+$ T-cell levels and neoantigen presence in these cancers. However, in tumors where neoantigen load does not correlate with $CD8^+$ T-cell levels, high TMB fails to predict response to ICIs [259]. Furthermore, the predictive value of TMB may be limited by technical challenges in sequencing. While whole-exome sequencing remains difficult to implement in clinical practice, targeted sequencing panels (e.g., FoundationOne CDx assay, MSK-IMPACT) covering 300 to 500 cancer-

related genes are often used as surrogates in clinical practice [224].

In addition, the current definition of TMB does not capture the genomic complexity of tumors with low levels of small-scale mutations (SNVs and INDELs) and high levels of chromosomal rearrangements, such as PM [260]. Patients with PM demonstrate higher-than-expected response rates to ICIs despite low TMB, potentially due to neoantigens arising from complex chromosomal rearrangements, which are not detected in conventional TMB assessment [260, 261]. Consistently, findings from the CheckMate743 clinical trial reveal that TMB alone does not predict the response to frontline nivolumab and ipilimumab treatment in PM patients [68].

PD-L1 expression

The significance of PD-L1 expression as a biomarker lies in two primary aspects. First, as a target of PD-1/PD-L1 antibodies, PD-L1 expression serves as a logical predictive biomarker for the response to anti-PD-1/PD-L1 therapy. It is currently the most extensively investigated and clinically utilized marker [257]. Secondly, PD-L1 expression correlates with parameters associated with immune activation within the tumor, such as activated $CD8^+$ T-cell responses and antigen presentation. This correlation suggests a higher likelihood of response to ICIs [262].

However, several limitations exist for the use of this marker. Thresholds to define positivity and negativity are not standardized and exhibit significant variability across different cancer types. Moreover, although the predictive role of PD-L1 expression would be expected to be consistent across various ICIs targeting PD-1 due to their shared pathway target, a recent meta-analysis in NSCLC demonstrated this was not the case [263]. Finally, while a correlation between PD-L1 expression on tumor cells and clinical response is observed in NSCLC, substantial responses can also occur in patients with PD-L1–negative tumors in other cases [228].

In PM, PD-L1 appears to lack predictive value for the response to ICIs. This observation is consistent across various trials, including the CheckMate743 trial, which evaluated nivolumab and ipilimumab as frontline therapy in a phase III study, and the NIBIT-MESO 1 trial, which investigated tremelimumab + durvalumab in frontline and second-line settings in a phase II study [264, 68]. Similarly, phase III trials such as CONFIRM (evaluating nivolumab) and PROMISE-MESO (evaluating pembrolizumab), both testing single ICI, also reported no significant predictive value of PD-L1 status [265, 266].

T-cell infiltration and scores

The TME plays a central role in determining responses to ICIs, with tumor-infiltrating T-cells emerging as key players [257]. The overall immune status, as reflected by infiltrating T-cells, can serve as a predictive marker. CD45RO+ T-cells have been recognized as independent predictive factors associated with positive outcomes across

various cancers [267]. To assess T-cell infiltration, the Immunoscore, a standardized scoring system, relying on the densities of two T-cell populations (CD3/CD45RO, CD3/CD8, or CD8/CD45RO) infiltrating both the tumor core and the invasive margin, has been developed [268].

Closely linked with T-cell infiltration but at the gene level, extensive efforts have been devoted to developing scores capable of embracing TME characteristics by assessing the expression levels of a select few genes. Several gene expression-based signatures have been established for predicting responses to ICIs notably including the T-cell-inflamed gene expression profile. This score is a clinically validated biomarker score consisting of a panel of 18 genes initially designed to predict the efficacy of pembrolizumab treatment across diverse solid tumor types, spanning bladder, gastric, HNSCC, anal canal, biliary, colorectal, esophageal, ovarian, and triple-negative breast cancers [269]. This signature is defined by the expression of genes associated with T-cell activation and effector functions, a hallmark of a T-cell-inflamed TME. Correspondingly, responders exhibit a high score in this signature, while non-responders typically display a low score [269].

Another promising signature with clinical relevance is derived from the transcriptomic expression levels of four inflammatory genes: *CD8A*, *signal transducer and activator of transcription (STAT)1*, *LAG3*, and *PD-L1*. This score has demonstrated prognostic value in melanoma, hepatocellular carcinoma, and gastric cancer [270, 271, 272]. Notably, it has been consistently associated with the response to nivolumab and ipilimumab in PM patients **(Figure 1.20)** [68].

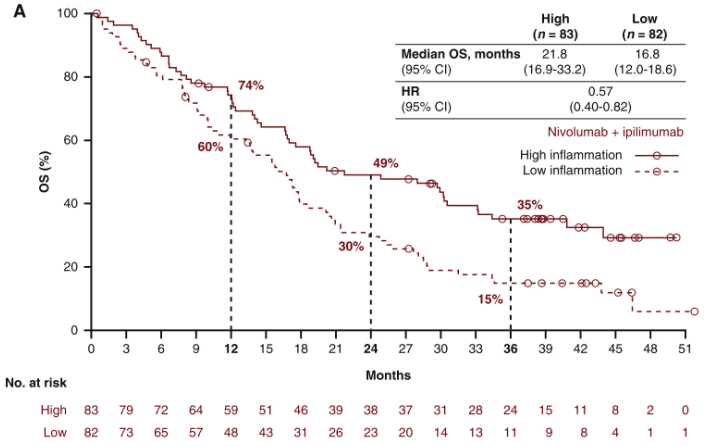


Figure 1.20: OS curve by a four-gene inflammatory signature score in patients from CheckMate 743 [68].

3.2.4. Immunoresistance to ICIs

Resistance to ICIs arises from various mechanisms that may overlap. These mechanisms can be broadly categorized into three groups: (i) inadequate generation of anti-tumor T-cells, (ii) dysfunction of tumor-specific T-cells, and (iii) impaired formation of T-cell memory (**Figure 1.21**) [273]. Additionally, resistance mechanisms can be classified as primary or acquired. Primary resistance refers to cases in which patients did not initially respond to ICI therapy, while acquired resistance encompasses cases in which patients initially responded but later relapsed [262]. However, nearly all mechanisms involved in primary resistance are also involved in acquired resistance, as these pathways are essential for both the development and maintenance of an effective anti-tumor response [262].

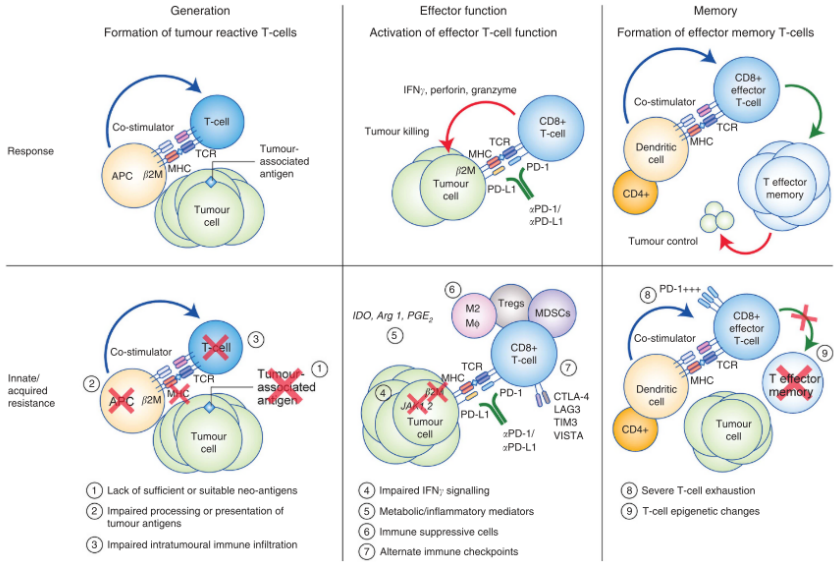


Figure 1.21: Mechanisms of immunoresistance. Resistance to ICIs can result from a variety of overlapping mechanisms that encompass insufficient generation of anti-tumor T-cells, malfunction of tumor-specific T-cells, and compromised development of T-cell memory [273].

Inadequate generation of anti-tumor T-cells

The most straightforward and intuitive mechanism of resistance to ICIs is linked to the absence of T-cells within the TME notably due to the low immunogenicity of tumor cells. It arises from various factors, including the lack of tumor-specific antigens, impaired presentation of these antigens, and the low clonal status of the tumor [262, 274].

Tumor-intrinsic factors notably encompass low genomic alterations and genetic and epigenetic alterations that influence both antigen formation and the antigen-presenting

machinery. This includes modifications in proteasome sub-units or transporters associated with antigen processing, and changes in ***B2M*** or ***MHC class I*** genes. Mutations and DNA epigenetic silencing through promoter methylation of *B2M* and *HLA* genes have been observed in multiple tumors [275, 274]. Studies have also revealed that a more **clonal signature** is linked to a response to ICIs, whereas excessive antigenic heterogeneity diminishes ICI efficiency [276].

Additionally, other intrinsic mechanisms contributing to T-cell exclusion have been described in melanoma. For example, signaling through the **mitogen-activated protein kinase (MAPK) pathway** induces VEGF and IL-8 production, thereby impeding T-cell recruitment [277]. Up-regulation of **Wnt/β-catenin** signaling pathway in tumor cells, leading to down-regulation of CCL4 production, a DC recruitment cytokine, has also been linked to reduced recruitment of tumor-infiltrating T-cells in patients [278, 279]. Furthermore, the loss of ***phosphatase and tensin homolog (PTEN)*** is correlated with enhanced **phosphoinositide 3-kinase (PI3K)** pathway and elevated levels of CCL2 and VEGF, reduced T-cell infiltration, and resistance to PD-1 blockade in preclinical models and patients [280].

Inadequate anti-tumor T-cell effector function

Upon successful neoantigen (cross)-presentation and T-cell activation, proper T-cell function may be impeded by the immunosuppressive TME or mutations in proteins involved in immune effector signaling pathways.

First, mutations in proteins involved immune signaling pathways may induce the neutralization of tumor-specific T-cell functions. The expression level of HLA at the tumor cell surface is closely linked with the **IFNγ signaling pathway** [281]. Mutations in ***janus kinase (JAK)1/2***, critical components of interferon signaling, have been associated with resistance to ICIs, through disruption of the tumor's response to IFNγ, affecting key processes like antigen presentation up-regulation or DC recruitment [282, 274]. Alternatively, considering that PD-L1 expression is augmented via IFNγ signaling, alterations in *JAK1/2* imply that the tumor may have developed alternative immune-evasive strategies beyond PD-L1 up-regulation and may decrease the efficacy of PD-L1/PD-1 axis blockade [281]. However, without therapies targeting this latter axis, IFNγ could lead to resistance through increased PD-L1 expression. Furthermore, prolonged exposure to IFNγ leads to increased expression of other co-inhibitory receptors, which could hinder the efficacy of ICIs, highlighting the complex and opposing role of this signaling pathway [283].

Additionally, T-cell effector functions can be impaired by **immunosuppressive cells**. Various immune suppressive cell types, such as T_{reg}s, MDSCs, and T_h cells secrete cytokines and other soluble factors that reduce the efficacy of ICIs. For example, IDO that is secreted by immunosuppressive TAMs or MDSCs, catalyzes the degradation of tryptophan. Both the reduction in local tryptophan concentration along the production

of immunosuppressive tryptophan metabolites contribute to the suppression of T-cell functions and proliferation [284]. Moreover, TAMs can directly affect PD-1 blockade efficacy by eliminating anti-PD-1 antibodies from PD-$1^{+}$$CD8^{+}$ T-cells [285].

Furthermore, the expression of **alternative co-inhibitory immune checkpoints**, such as CTLA-4, TIM-3, LAG-3, and VISTA, has been linked to resistance against PD-1 blockade [273].

Inadequate formation of T-cell memory

The formation of T_{em} cells is essential for achieving long-term, durable clinical benefits following ICI therapy. However, TME-induced epigenetic changes in T-cells may hinder the differentiation of effector T-cells into T_{em}, although the mechanisms driving T-cell expansion after ICI treatment remain largely unknown [273].

3.3. Epigenetic therapy: histone deacetylase inhibitors

In addition to chemotherapy and immunotherapy, another extensively investigated therapeutic approach in cancer involves epigenetic modulators. Epigenetic modifications encompass DNA methylation, histone modification, chromatin remodeling, and the influence of non-coding RNA. Among these, modulation of histone acetylation, especially through HDACis, has been extensively explored [286].

3.3.1. Histone deacetylases in cancer

Histone deacetylases (HDACs) are enzymes essential for regulating gene access to transcription factors through post-translational modifications. Specifically, HDACs remove acetyl groups from the ϵ-amino lysine residues on histones leading to chromatin compaction and transcriptional repression [287, 288]. Besides histones, HDACs also remove acetyl groups from non-histone proteins. Based on their primary homology to yeast HDACs and subcellular location, the eighteen human HDACs have been divided into four main classes [289, 290]. HDACs belonging to classes I (HDACs 1, 2, 3, 8), II (HDACs 4, 5, 6, 7, 9, 10), and IV (HDAC 11), also referred to as "classical HDACs", are zinc-dependent whereas members of class III (sirtuins 1, 2, 3, 4, 5, 6, 7) called "non-classical HDACS", are nicotinamide adenine dinucleotide (NAD)-dependent [291].

Cancer is frequently linked with epigenetic changes which include alterations in histone acetylation. A widespread decrease in monoacetylation at lysine 16 of histone 4 (H4-Lys16) is a notable feature in many human cancers [292]. Aberrant expression of classical HDACs has been reported in several cancers, including lung, breast, and colorectal cancers [293, 287]. This suggests a potential mechanism whereby the over-expression of HDACs act on the promoter regions of tumor-suppressor genes

resulting in their transcriptional repression, potentially fostering tumor initiation and progression [294]. Regardless of expression levels, functional alterations may also underlie the involvement of HDAC in tumor development [287]. Studies have suggested that genetic inactivation of HDACs could have tumorigenic effects and correlate with poorer survival outcomes, highlighting the paradoxical roles of these enzymes [287, 295]. In addition to histone modifications, HDACs play a critical role in acetylating non-histone proteins like p53 or NF-κB therefore regulating cell cycle, apoptosis, metastasis, angiogenesis, autophagy, and other cellular processes [287].

3.3.2. Histone deacetylase inhibitors

Given the evidence implicating HDAC activity in promoting tumor cell proliferation, metastasis, and angiogenesis, HDACis have thus emerged as promising therapeutic agents [293]. They function by binding to the catalytic site of HDACs, preventing their binding with their targets. HDACis are classified into four groups based on their structural similarity: hydroxamates, electrophilic ketones and cyclic peptides, short-chain fatty acids, and benzamides (**Table 1.3**) [296]. Currently, five HDACis (i.e vorinostat, belinostat, romidepsin, tucidinostat, and panobinostat) have been FDA-approved for cancer treatment [296].

Table 1.3: Classification and examples of HDACis.

Class of HDACis	Examples	Targeted HDACs
Hydroxamates	Trichostatin A (TSA)	Class I, II
	Panobinostat	Class I, II, IV
	Belinostat	Class I, II
	Vorinostat	Class I, II, IV
Short-chain fatty acids	Valproic acid	Class I, IIa
	Butyric acid	Class I, IIa
Benzamides	Entinostat	Class I
	Tucidinostat	Class I, IIb
	Tacedinaline	Class I, IIa
Cyclic peptides	Romidepsin	Class I

HDACis impact both tumor cells and healthy cells, including immune cells, which notably rely on HDAC activity for their differentiation and/or functions [297, 298]. Within tumor cells, HDACis primarily induce apoptosis and cell cycle arrest, inhibit angiogenesis, and suppress tumor cell proliferation and metastasis. Furthermore, they modulate the immunogenicity of tumor cells, potentially enhancing immune system response. Notably, in vivo studies involving T-cell depletion in mice have demonstrated that an intact immune system is necessary for HDACis to fully manifest their anti-tumor effects, providing valuable proof of concept. In PM cell lines, several studies have highlighted the down-regulation of the anti-apoptotic protein bcl-XL and subsequent induction of apoptosis upon exposure to sodium butyrate or suberoylanilide

hydroxamic acid. Moreover, in addition to their direct apoptotic effects, HDACis have demonstrated the ability to sensitize PM cells to the combination of cisplatin either in combination with pemetrexed or not [299, 300]. Additionally, synergistic effects have been observed when HDACis were combined with DNA hypomethylating agents, leading to enhanced lethality in PM cells and increased expression of cancer/testis antigens (i.e melanoma-associated antigen (MAGE)-A1, MAGE-A3, New York esophageal squamous cell carcinoma (NY-ESO)-1) in surviving tumor cells [301, 302]. However, despite encouraging preclinical findings, clinical trials have yielded disappointing outcomes in PM. In the VANTAGE-014 trial, vorinostat failed to improve mOS compared to placebo [303].

While HDACis are primarily recognized for their influence on tumor cells, emerging evidence also highlights their direct impact on immune cells [304]. However, the literature reflects a heterogeneous landscape, with conflicting findings regarding the impact of HDACis on different immune cell populations (**Figure 1.22**) [304]. Nevertheless, a point of consensus is the observed increase in CD8 T-cell infiltration into tumors, along with a corresponding rise in the CD8:T_{reg}s ratio following HDACi treatment [304].

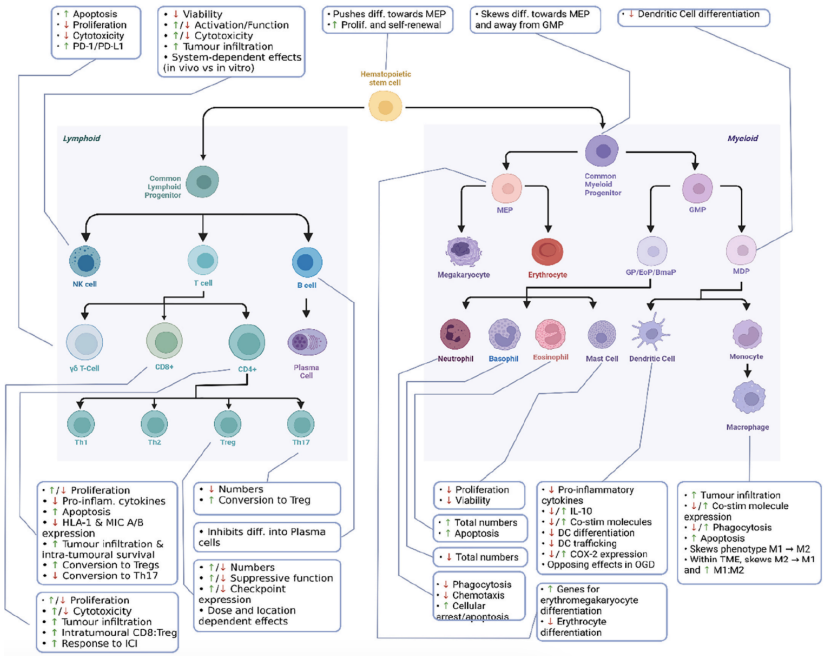


Figure 1.22: Effects of HDAC inhibition on immune cells. HDAC inhibition induce diverse effects across diverse experimental contexts, influenced by the cell type under investigation, the specific HDACi variant employed, and the targeted HDAC receptors. Additionally, nuances in experimental parameters, such as timing, route of administration, and type of study (*in vivo* and *ex vivo*), play pivotal roles [304].

2

Thesis Objectives

1. Thesis objectives

PM is a poor prognosis tumor, closely linked to asbestos exposure and affecting mesothelial cells of the pleura [61]. For over two decades, the standard treatment for unresectable PM has been a combination of cisplatin/carboplatin with pemetrexed, yielding a modest increase in mOS of approximately three months compared with cisplatin alone [66]. More recently, immunotherapy based on ICIs has emerged as a second frontline treatment, especially for the sarcomatoid subtype. ICIs have demonstrated a significant enhancement in the 2-year OSR by 50% and a mOS extension from 14.1 months to 18.1 months [67]. Nevertheless, this improvement remains limited, with only a subset of PM patients showing responsiveness to ICIs [67]. A new paradigm based on the combination of chemotherapy and ICIs is therefore currently being evaluated in several clinical trials. Recent results from the phase III clinical trials IND.227 and BEAT-Meso have nonetheless been disappointing. Without stratification by subtype, the IND.227 trial demonstrated a modest improvement in mOS by just over one month in the combination arm compared to chemotherapy alone, while the BEAT-Meso trial showed no significant improvement [73, 74]. Notably, although there was a significant improvement in the non-epithelioid subtype with chemoimmunotherapy, mOS are similar to doublet immunotherapy alone.

One of the major obstacles hindering the progress of these novel approaches is the lack of a thorough understanding of the optimal dosing and scheduling, crucial for achieving an additive or synergistic effect. Additionally, ongoing trials employ standard-of-care chemotherapy in both arms without a strong rationale regarding potential interactions with tested ICIs or their impact on the TME. Indeed, the crucial role of the TME in mediating therapeutic responses in PM has garnered increasing recognition but is still poorly characterized in PM compared to other solid tumors. The TME exhibits remarkable heterogeneity in its cellular composition, including endothelial, stromal, and immune cell populations that remain insufficiently characterized [305]. Notably, in PM, it comprises immunosuppressive cells such as TAMs and MDSCs, that could further prevent therapeutic effects [154]. Circulating monocytes, serving as TAM precursors, are associated with lower mOS among PM patients [96] and monocyte modulation towards anti-tumor function could therefore be of great interest.

In this context, my thesis project seeks to enhance our understanding of the immune microenvironment in PM, with a particular emphasis on monocytes and macrophages, using single-nucleus RNA sequencing (snRNA-seq). Concurrently, the project aims to develop novel therapeutic strategies. One approach involves the reprogramming of monocytes with epigenetic regulators, while the other explores the combination of mCT with doublet ICIs, proposing a new rationale for this treatment approach.

Owing to these different elements, this manuscript will be divided into three main studies (**Figure 2.1**):

- Deciphering the intra-tumor heterogeneity of monocyte/macrophages in PM by snRNA-seq.
- Characterization and epigenetic modulation of monocyte cytotoxicity against PM tumor cells.
- *In vitro* and preclinical rationale for metronomic chemotherapy and ICI combination in mesothelioma.

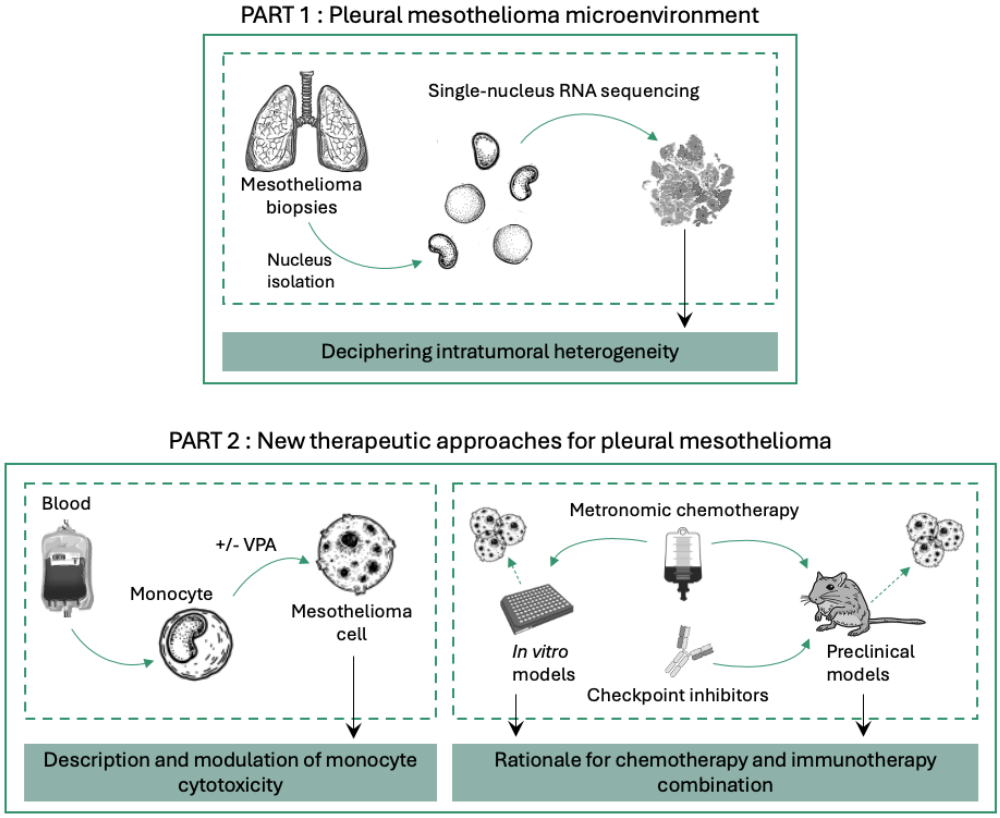


Figure 2.1: Graphical summary of thesis objectives.