



Does ageing modulate interactions between mesothelioma cells, macrophages, and tumour endothelial cells?

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Abstract

It is becoming increasingly clear that the tumour microenvironment (TME) adopts a changing and increasingly complex landscape as tumours evolve. Central to the TME, and alongside malignant cells, are tissue resident and recruited macrophages, other immune cells, and endothelial cells, with the latter critical for angiogenesis and tumour development. Tumour vessels provide oxygen and nutrients and are portals for immune cells. Tumour cells, immune cells and endothelial cells engage in multi-directional crosstalk that ultimately influence tumour progression and treatment responses. Adding to complexity, the TME often consists of oxygenated, and oxygen deprived or hypoxic regions, with the latter significantly contributing to disease progression and treatment resistance. However, the function of immune cells and endothelial cells change with ageing, and this underexplored area likely influences the aged TME and disease outcomes in the elderly. Solid cancers such as mesothelioma with known carcinogen exposure (asbestos) take decades to reach a diagnosable size, often emerging in people aged 60 years or more. Here, we discuss the influence of ageing on the function of tumour-associated immune cells, focussing on macrophages, and their possible interactions with endothelial cells, and how this might impact the evolving mesothelioma TME in elderly people.

Keywords: Mesothelioma, macrophages, endothelial cells, tumour microenvironment

1. The Carcinogenic Process in Mesothelioma

Many cancers do not yet have identifiable carcinogens that can be used to track initial exposure and long-term disease aetiology. In contrast, the carcinogen (asbestos fibres) that causes mesothelioma, confirmed in the 1960s by Wagner^[1], is trackable. It is now well established that there is a lengthy latency period (over 30 years) between known asbestos exposure and detectable mesothelioma^[2], meaning this disease mostly emerges in people over 60 years old. This is confirmed by age-response relationship studies that report a slow increase in mesothelioma diagnosis until people reach 50 years old, after which a sharp increase is seen^[3-5]. This lengthy timeframe provides ample opportunity for multiple genetic, molecular, and cellular changes, including changes associated with ageing.

The underlying genetic mechanisms leading to carcinogenesis in mesothelioma are not yet fully understood, but there is evidence that it starts with mesothelial cells phagocytosing asbestos fibres^[6], summarized in Figure 1. Asbestos fibres induce production of reactive oxygen metabolites that could be responsible for causing DNA point mutations, as well as strand and chromosomal breaks, in combination with mitotic damage ADDIN ENRfu ADDIN ENRfu and increased cell division, all resulting in cell survival and neoplastic transformation^[7,8]. Genetic changes of mesothelial cells as they differentiate into malignancy include upregulating transforming growth factor-beta (TGF- β) and platelet-derived growth factor (PDGF)-A transcripts^[9,10]. Whilst these events may happen relatively quickly *in-vitro* (between one to six weeks depending on the cell line tested^[11,12]), it is not known how long genetic and molecular changes take in asbestos-exposed humans, with type of asbestos fibre, dose, and duration of exposure affecting disease outcomes. However, there is also evidence that local acute and chronic inflammatory responses contribute to the carcinogenic process and, in this regard, it is possible that age-associated changes, such as low level inflammation, or inflammaging^[13,14] in the local environment contribute to the ultimate emergence of immune-resistant mesothelioma.

The potential role of the ageing process in mesothelioma aetiology is further highlighted by the fact that universal driver mutations



have not been found, and that mesothelioma is generally regarded as having a low tumour mutation burden with unusual genetic aberrations^[15]. Deletion of the cyclin D dependent kinase inhibitor 2A (CDKN2A) gene and the co-located methylthioadenosine phosphorylase (MTAP) gene on chromosome 9 are the most common mutations, both affect cell cycle. Other common mutations are seen in the BRCA1-associated protein-1 (BAP1), neurofibromin2 (NF2), and tumour protein (TP)53 genes^[16-18]. Early events occurring after asbestos exposure, including an increasingly inflammatory environment, likely drive aberrant activation of intracellular pathways and transcriptional processes that induce malignant transformation^[19], with the distinct possibility that some of these processes occur with, and perhaps because of, age-related changes.

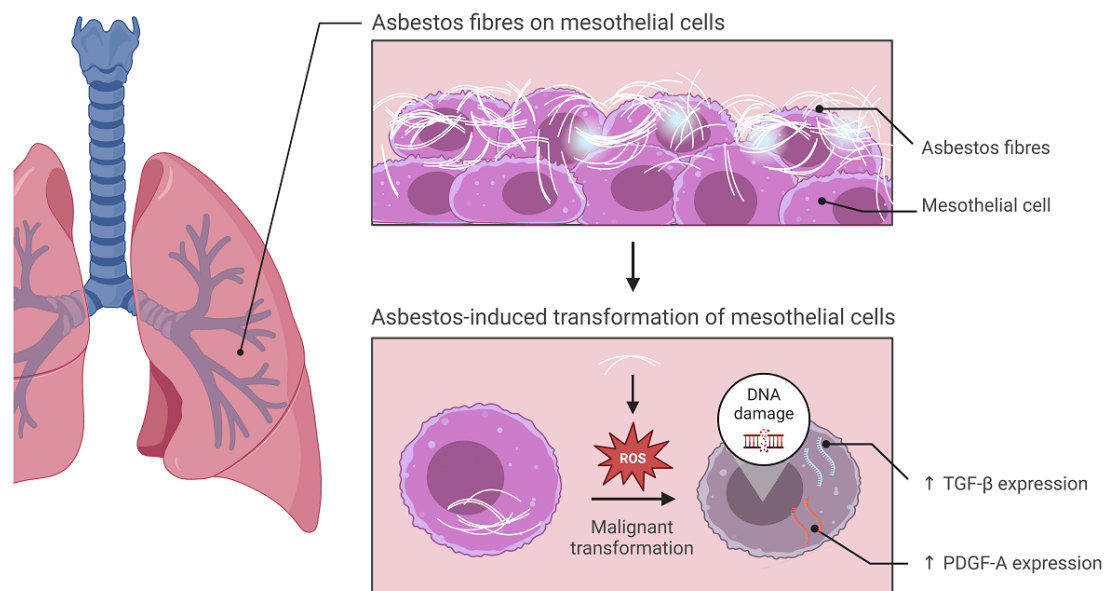


Figure 1. The effect of asbestos fibres on mesothelial cells. Inhaled asbestos fibres induce production of ROS and initiate malignant transformation of mesothelial cells. Malignant mesothelial cells upregulate TGF- β and PDGF-A. Created in [BioRender.com](https://www.biorender.com). ROS: reactive oxygen species; TGF- β : transforming growth factor beta; PDGF: platelet-derived growth factor; $\uparrow$: increased.

2. Macrophages and Mesothelioma Carcinogenesis

Macrophages may be crucial players in mesothelioma development. Our *in-vivo* studies in mice show that macrophages are required for mesothelioma growth, as co-inoculation of macrophages with mesothelioma tumour cells leads to faster tumour growth, whilst depleting macrophages using the anti-F4/80 antibody induces tumour regression in young and elderly mice^[20].

Early events induced by inhaled asbestos fibres lodged in the lungs include haemorrhage, destruction of the elastic membrane under visceral pleura, and inflammation^[21]. The early inflammatory response is characterised by a polymorphonuclear infiltrate^[22,23] that cannot clear lodged asbestos fibres. Whilst most asbestos fibres in the lungs are phagocytosed by alveolar macrophages, long asbestos fibres cannot be readily phagocytosed, resulting in persistent abnormal macrophage/fibre clumps. Macrophages unable to eliminate asbestos fibres, have been termed ‘frustrated’^[24]. Asbestos fibres activate macrophages *in-vitro*^[25,26], and increased numbers of macrophages have been recovered from bronchoalveolar lavages (BAL) of asbestos-exposed and asbestosis patients^[27,28], as well as from the BAL of experimental animals after asbestos injury^[29]. These activated frustrated macrophages display increased expression of IgG Fc receptors, surface ruffles, and filopodia^[30]. Up to six months later, fibre clusters partially covered by mesothelium are surrounded by macrophages, and regenerating mesothelial cells are visible^[31]. Frustrated macrophages produce nicotinamide adenine dinucleotide phosphate (NADPH), reactive oxygen species (ROS)^[32] and pro-inflammatory factors including interleukin-1 (IL-1), interleukin-8 (IL-8), interleukin-6 (IL-6), and tumour necrosis factor- α (TNF- α) that activate survival-associated signalling pathways in tumour cells^[33,34], summarised in [Figure 2](#).

High-mobility group protein box 1 (HMGB1), an endogenous damage-associated molecular pattern (DAMP) ‘danger’ molecule that binds DNA and possesses pro-inflammatory properties^[35], is released by mesothelial cells upon asbestos exposure^[36]. HMGB1 can activate macrophages via multiple pattern recognition receptors including Toll Like Receptor (TLR)4^[35]. When HMGB1 binds TLR4 on macrophages the NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome is activated, resulting in secretion of IL-1, IL-18, and HMGB1, thereby establishing a chronic inflammatory loop^[37]. HMGB1 activation further impairs macrophage phagocytosis and induces TNF- α secretion that protects mesothelial cells from death signals, concomitantly sustaining chronic inflammation^[38,39]. Transforming mesothelioma cells proliferate^[38] and express CD47, a “don’t eat me” signal, helping them avoid macrophage phagocytosis^[40].

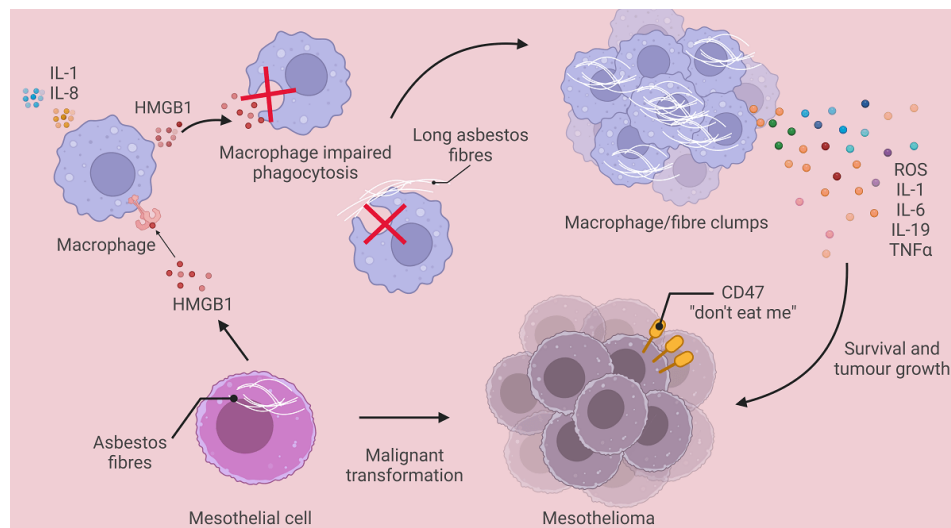


Figure 2. The effect of asbestos fibres on macrophages. This figure summarises the cross talk between mesothelial cells and macrophages injured by asbestos fibres. Activated macrophages may clump due to an inability to breakdown asbestos fibres and release inflammatory factors such as ROS and IL-6 that induce upregulation of CD47 on transforming mesothelial cells, thereby creating immune-resistant mesothelioma. Created in [BioRender.com](https://www.biorender.com). ROS: reactive oxygen species; IL: interleukin; TNF- α : transforming growth factor alpha; HMGB1: high-mobility group protein box 1.

3. Mesothelioma-Associated Macrophages

Mesothelioma cells produce high levels of the monocyte chemoattractant protein (MCP-1; also known as C-C motif chemokine ligand 2, CCL2), to recruit monocytes^[41] and induce tumour-promoting macrophages (that have also been referred to as alternatively activated, M2 or M2-like macrophages) by secreting IL-10, TGF- β and macrophage colony stimulating factor (M-CSF), these factors are seen in patient pleural effusions (PE)^[42]. Increased numbers of tumour-associated macrophages (TAMs) that express CD68 (a scavenger receptor for the hemoglobin-haptoglobin complex) and display an immunosuppressive, tumour-promoting phenotype, that includes the high affinity scavenger receptor (CD163), the mannose receptor (CD206) and the IL-4R receptor (IL-4R), have been reported in human mesothelioma^[41,43]. The presence of these tumour-promoting TAMs that release IL-6, IL-10 and IL-34 has been linked to increased tumour cell proliferation and chemotherapy resistance^[41]. TAMs, in turn, induce a cancer stem cell-like phenotype in tumour cells via IL-1/IL-1R activation^[44].

Human and murine mesotheliomas are heavily infiltrated by macrophages, which can comprise greater than 20% of total cellularity^[20,43,45], and their high prevalence is associated with a poor prognosis^[42]. We have shown that TAMs change as mesothelioma progresses. Starting with a more pro-inflammatory, anti-tumour phenotype (also referred to as classically activated or M1-like) and shifting as tumours develop to adopt a more mixed anti-inflammatory and pro-tumourigenic phenotype^[20]. We described this mixed phenotype as M3 macrophages, however, it is now recognised that functionally versatile macrophages are rapid responders to local signals and adopt an array of molecular and functional phenotypes in response to changing local environments. The mixed phenotype macrophages proliferated in-situ^[20] and proliferative TAMs have been postulated to be a hallmark of human solid tumours, as well as a prognostic marker of malignancy^[46]. Moreover, the mixed phenotype macrophages were not uniformly dispersed throughout tumours, instead they were in distinct micro-niches^[20] that might be associated with hypoxic regions. Others, using a different murine mesothelioma model, found that monocyte-derived, small peritoneal/pleural macrophages (SPM) rapidly increased in mesothelioma, and preferentially activated kirsten rat sarcoma viral oncogene homolog (KRAS) and TNF- α /nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) signaling pathways, contributing to tumour-promoting TAMs^[47]. Furthermore, depletion of SPM enabled tumour rejection. This was translatable to humans, as an SPM gene signature was identified in PEs and tumours from patients with untreated mesothelioma. Colin *et al.*, using an orthotopic xenograft model consisting of human mesothelioma cells injected into immunodeficient athymic mice, also reported an expansion of CD206⁺ tumour-promoting TAMs during mesothelioma progression^[48].

Mesothelioma TAMs play a major role in tumour development by providing factors that promote matrix components, tumour cell proliferation and angiogenesis. TAMs also suppress the anti-tumour immune response via multiple mechanisms including production of TGF- β , nitric oxide (NO) and hydrogen peroxide (H₂O₂)^[49]. PE-derived macrophages, used as surrogate mesothelioma TAMs, were shown to display an immune suppressive phenotype that released high levels of prostaglandin E₂ (PGE₂), and suppressed CD4⁺ and CD8⁺ T cell proliferation^[50]. The authors further showed that PE-macrophages secreted soluble factors associated with tumour invasion, angiogenesis, and immune suppression, such as IL-6, TGF- β , VEGF and IL-12^[50], highlighting the functional diversity demonstrated by macrophages in response to their environment, summarised in [Figure 3](#).

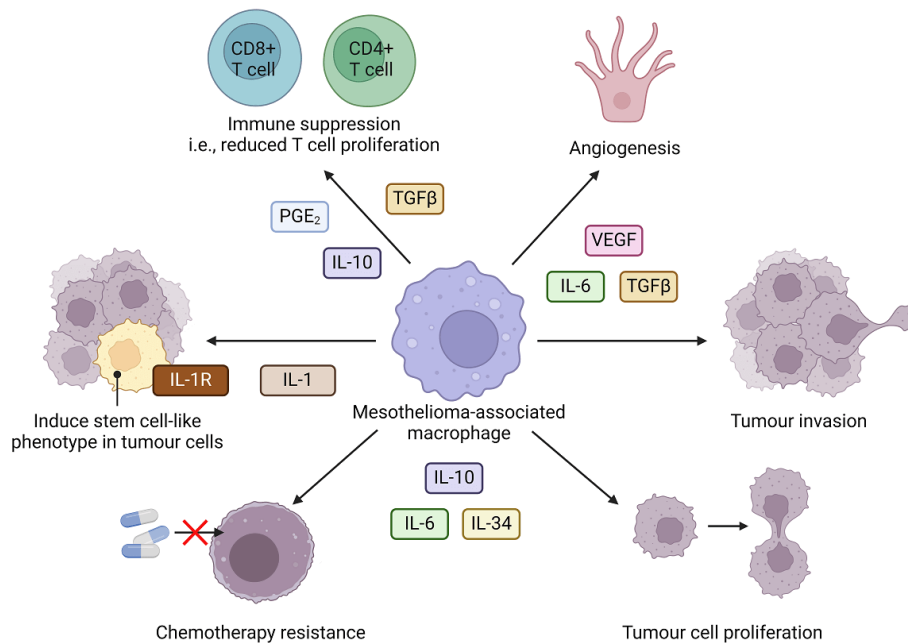


Figure 3. The role of mesothelioma-associated macrophages in tumor progression. Mesothelioma-associated macrophages support disease progression by secreting factors that drive angiogenesis, tumour cell proliferation, tumour invasion, contribute to treatment resistance and induce stem-like cells and immune suppression. Created in BioRender.com. TGF- β : transforming growth factor beta; PGE₂: prostaglandin E₂; IL-1R: interleukin 1 receptor; IL: interleukin; VEGF: vascular endothelial growth factor.

4. The Mesothelioma Tumour Microenvironment

The human mesothelioma TME also contains regulatory T cells (Tregs) that suppress the function of effector T cells, such as CD8⁺ cytotoxic T lymphocytes (CTLs)^[51,52]. High levels of programmed death protein ligand-1 (PD-L1, or CD274) are expressed in the TME^[46] by immune cells, particularly macrophages, as well as by mesothelioma cells, although the latter is contestable^[34]. Macrophages may be responsible for reprogramming T helper (Th)-1 cells into Tregs via TGF- β and IL-10. Dysfunctional CTLs and tissue-resident memory (Trm) cells have been described in mesothelioma, their dysfunction likely due to pre-existing Tregs and expression of the Eomes transcriptional factor, a regulator of CD8⁺ T cell function^[53]. Patient PE also contain activated CTLs and Th cells that express exhaustion (checkpoint) molecules, such as programmed death protein-1 (PD-1) or CD279. PD-1 on CD8⁺ T cells binds its ligands PD-L1 and PD-L2 which suppress effector T cell function (i.e., their tumour killing capacity). Other checkpoint molecules found in PEs include T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3, or CD366) and lymphocyte activation gene-3 (LAG-3, CD223 or FDC protein) that suppress T cell activity^[54]. Natural killer (NK) cells with an immunosuppressive profile and lower cytotoxic function are also present in mesothelioma samples^[55]. However, it should be noted that the mesothelioma TME differs between patients and histological subtypes^[56]. An association between the aged environment and different mesothelioma TEMs has yet to be identified.

Murine mesothelioma models display similar infiltrating M2-like cells^[20,48] and Treg^[57], as well as low numbers of dendritic cells (DCs), T cells, B cells and NK cells^[58]. DCs are the most likely antigen presenting cell (APC) to take up mesothelioma antigens as they traverse the tumour site and move to draining lymph nodes to cross-present tumour antigen. We have shown, using transfection/transgenic models, that mesothelioma growth in young and elderly mice is associated with constitutive tumour antigen cross-presentation and CTL induction, meaning that DCs retain their APC function in the aged and cancerous setting, however, these CTLs fail to prevent disease progression suggesting a severe defect in DCs and/or T cells^[59,60].

We have reported defects in human DCs with healthy ageing^[61], and in humans with mesothelioma^[62]. For example, we found that circulating elderly blood myeloid (m)DC1s, mDC2s, and plasmacytoid (p)DCs numbers diminished with ageing. We also found that whilst lipopolysaccharide (LPS)/IFN γ or CD40Ligand(L) stimulation induced elderly blood mDC1s, mDC2s, and plasmacytoid DCs (pDCs) to up-regulate comparable levels of T cell co-stimulatory molecules (CD40, CD80 and/or CD86), pro-inflammatory cytokines (IFN γ , TNF- α , IL-6 and/or IL-12), and induce CD8⁺ and CD4⁺ T cell proliferation, they maintained their antigen processing ability, implying incomplete maturation. Moreover, elderly LPS/IFN γ -activated mDC1s and pDCs adopted a regulatory phenotype, due to greater expression of PD-L1 and TGF- β than their younger counterparts, that may impact the efficacy of immune responses in the elderly^[63].

In mesothelioma-bearing mice, we found that elderly tumour antigen-specific CTLs lost their ability to lyse target cells, whereas young CTLs did not^[59]. Whilst this might explain why tumours in elderly hosts continued to progress, it does not explain why tumours

in young hosts also continued to progress. Nonetheless, it does help explain why chemotherapy is not as effective in elderly hosts, as even though there is likely elevated tumour antigen presentation, on account of increased tumour cell death, CTL functionality remained compromised in elderly mice^[59]. In contrast, chemotherapy enhanced CTL function in young mice; CD8⁺ T cell depletion studies confirmed that these CTLs were responsible for chemotherapy-induced mesothelioma regression^[59,64].

Cancer-associated fibroblasts (CAFs) are recruited into tumours, likely by frustrated macrophages. CAFs further enhance the migratory and invasive abilities of mesothelioma tumour cells, recruit immune and vascular cells, and remodel the extra cellular matrix (ECM)^[65].

5. Mesothelioma, Macrophages and Ageing

We have data suggesting that macrophages in older hosts function differently to those from younger hosts. Our intra-tumoural IL-2/anti-CD40 antibody immunotherapy is curative for at least 80% of young adult mice with mesothelioma^[66], whereas the cure rate reduces to approximately 30% in elderly mice^[64]. Depleting F4/80⁺ macrophages yields contrasting age-related results, as the IL-2/anti-CD40 immunotherapy cure rate almost doubled in elderly mice, whilst outcomes for macrophage-depleted young mice worsened. Moreover, macrophage depletion increased *in-vivo* anti-tumour CTL cell activity in elderly mice, but decreased CTL activity in young mice^[64].

Our data show that mesothelioma tumours grow faster in elderly compared with young mice, and this that corresponds with an increase in TAMs^[64]. We also reported that macrophages increase in bone marrow (BM) and spleens during healthy ageing, suggesting these sites have an increased potential to supply cancer-promoting macrophages^[64]. Furthermore, we found that all tumour-bearing elderly, but not young, mice demonstrated signs of cachexia such as decreased body weight, which was exacerbated by immunotherapy. Interestingly, macrophage depletion prevented therapy-induced cachexia^[64], suggesting macrophages play a key role in driving cancer cachexia in the elderly, particularly during therapy, and likely sabotage elderly anti-tumour immune responses.

Age-associated macrophage dysregulation has been shown in *in-vitro* studies using splenic macrophages from Balb/c mice; elderly macrophages produced less TNF- α and IL-1 β than their younger counterparts in response to LPS^[67]. Yet, BM-derived macrophages from elderly C57BL/6J mice produced more TNF- α and IL-6 than those from younger mice in response to LPS^[68]. In contrast, elderly-derived peritoneal macrophages from Balb/c and C57BL/6J mice displayed elevated production of anti-inflammatory IL-10 and TGF- β in response to IL-4^[69,70]. Macrophages from the eye, muscles, lymph nodes, spleen and BM have also been shown to release increased IL-10 with ageing^[69-71]. However, liver and adipose tissue macrophages from elderly C57BL/6J mice produced more IL-6 and TNF- α ^[72,73]. We proposed that each tissue microenvironment might exert its own influence on macrophage function during ageing^[64]. The impact of ageing on TAMs has yet to be fully elucidated. It is possible that interactions with aged endothelial cells (ECs) as macrophages access tumours modulate their function and phenotype. It is possible role that we are describing senescent macrophages likely induced by surrounding aged cells and cancer cells. The importance of senescent macrophages is described by others^[74,75].

6. Endothelial Cells, Immune Cells, Cancer and Ageing

The endothelium is made up of a single cell layer of ECs that line vascular and lymphatic systems. ECs play essential physiological roles including vascular stabilization, hemostasis, modulating vascular permeability, and regulating the movement of cells into and out of the circulatory system^[12]. Under normal healthy physiological conditions, ECs are non-proliferating quiescent cells that allow diffusion of solutes to underlying cells, and do not interact with immune cells. ECs only engage with immune cells after activation by pro-angiogenic signals, hypoxia and inflammation. Activated ECs up-regulate adhesion molecules, chemokines and integrins that interact with, and activate, immune cells so that they up-regulate relevant ligands enabling the multi-step process that follows. ECs sequentially express selectins. The first is E-selectin, that ligates sialofucosylated glycan determinants on protein and lipid scaffolds of immune cells, enabling their tethering and slow rolling on the EC surface. Immune cells are then exposed to chemokines immobilized by glycosaminoglycans on ECs leading to engagement of G-protein-coupled chemokine receptors on immune cells. In response, immune cells up-regulate integrins, particularly very late activation protein 4 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1), that bind vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on ECs, leading to firm adhesion^[76]. Transendothelial migration (or diapedesis) is facilitated via transient dismantling of EC junctions (paracellular migration) or migration through individual EC (transcellular migration).

It is becoming widely accepted that ECs are key regulators of the immune response^[14,35,77-79]. Due to their front-line location ECs are early detectors of foreign pathogens and endogenous danger signals in blood. Bacterial endotoxin (such as LPS) as well as pro-inflammatory cytokines, such as TNF- α , activate ECs, inducing pro-inflammatory cytokine and chemokine production that amplify an immune response^[79]. There is evidence that ECs are selective regarding the type of immune cell they attract. For example, ECs expressing chemokine (C-X-C motif) ligand 10 (CXCL10) and E-selectin, favour Th1 cell recruitment^[14,80]. ECs may secrete growth-regulated oncogene alpha (GRO α) or MCP-1 to attract neutrophils or monocytes, respectively^[81,82]. Moreover, ECs may act as APCs via MHC class I and II molecule expression, and selectively regulate the influx of antigens-specific T cells^[83-85]. Further, ECs can modulate

immune cell function, for example, ECs may impact DC maturation^[86] as well as macrophage and T cell polarization, via cytokine secretion^[87,88]. ECs have been shown to influence the aged bone marrow microenvironment, in which they function as APCs and induce T cells to acquire a more pro-inflammatory phenotype than young ECs^[89]. Moreover, younger ECs were associated with lower levels of ROS and better migratory and tube-forming abilities than elderly ECs.

7. Endothelial Cells and Vascular Ageing

ECs change with the ageing process, referred to as vascular ageing. For example, EC proliferation decreases with age, meaning ECs adopt a senescent profile^[36,90]. Accumulating senescent ECs in vessels, such as the aortic wall of aged mammals, induces EC dysfunction, and increased permeability. Senescent ECs lose their ability to produce NO, leading to decreased vasodilation during ageing. Furthermore, senescent ECs exhibit increased expression and secretion of various cytokines, known as the Senescence-associated secretory phenotype (SASP)^[90-92]. SASP cytokines cause low-grade chronic inflammation, cellular fibrosis and apoptosis, and stimulate macrophage, T and B cell infiltration which together exacerbate vascular ageing.

Vascular ageing appears to be the result of interactions with multiple cell types and their secreted products over time leading to endothelial injury. More recently, the potential role of mitochondria in multicellular interactions and vascular ageing has been revealed^[93-95]. Mitochondria are the main source of intracellular ROS, but are also targets of ROS damage. An imbalance between mitochondrial ROS ((mt)ROS) leads to overproduction and/or decreased antioxidant enzymes in the mitochondria and cytosol, causing oxidative stress, which is closely associated with cell senescence, vascular inflammation, arterial stiffness and vascular ageing.

MtDNA contain unmethylated cytosine-phosphate-guanine motifs, similar to bacterial DNA, this may be because mitochondria evolved from ancient eubacteria. This means that mtDNA can be identified as foreign once outside cells, and function as a DAMP resulting in immune activation and inflammation^[96]. Increased levels of circulating mtDNA with ageing^[97] has been correlated with elevated inflammatory factors, such as TNF- α , IL-6, regulated upon activation, normal T cell expressed and secreted (RANTES), also known as CCL5, and the IL-1 receptor antagonist (IL-1Ra) to promote vascular ageing. Excessive or prolonged increases in vessel permeability, may contribute to the chronic inflammation and cancer seen in the elderly.

8. Immune Cells, Fibroblasts and Vascular Ageing

Macrophages play a key role in vascular ageing on account of their ability to release pro- and anti-inflammatory factors, as well as their role in lipid metabolism. Mitochondria in macrophages are reported to be crucial effectors in vascular ageing^[49,98,99]. Mitochondria represent the energy powerhouse for macrophages under inflammatory conditions such as hypoxia and hyperglycemia via glycolysis or fatty acid oxidation. The latter are seen in ageing due to vascular and metabolic dysfunction. Vascular ageing has been best characterised in atherogenesis and starts when accumulating lipids or lipoproteins are modified in the vascular sub-endothelium, resulting in macrophage recruitment and activation. Macrophages then take lipids up via scavenger receptors such as CD36, and excess lipid that cannot be degraded leads to the formation of foam cells^[100,101]. In early atherogenesis development, mitochondria in macrophages from coronary artery disease plaques consume more oxygen and produce more energy (ATP) than their healthy counterparts^[102]. Moreover, these M2-like macrophages demonstrate higher mitochondrial content, including increased levels of mtDNA and have a higher oxygen consumption rate than their pro-inflammatory M1-like counterparts^[103]. Interestingly, enhanced mitochondrial respiration improves lipid metabolism in M2-like macrophages^[104] that may provide a protective effect on vascular ageing^[95,105]. However, with disease progression lipid-loaded foamy macrophages reduce oxygen consumption leading to apoptosis. Overexpression of mtROS in macrophages can aggravate atherosclerosis by stimulating NF- κ B pathway activation and recruiting monocytes that exacerbate the vascular inflammatory cascade^[106].

Neutrophils, plasma cells, and mast cells are elevated in aged arteries, along with elevated IL-1 β , IL-6, and IL-10 in plasma^[106-109]. These cells, as well as fibroblasts, secrete factors associated with vascular ageing. Fibroblasts in the aortic adventitia layer demonstrate excessive proliferation and differentiation in aged vessels which exacerbate vascular sclerosis and fibrosis^[110]. Moreover, activated fibroblasts secrete cytokines and chemokines to recruit immune cells and modulate ECs, driving endothelial dysfunction, and aggravating vascular ageing.

9. Endothelial Cells, Angiogenesis and Ageing

Angiogenesis, the process of vessel formation from pre-existing vascular beds, supports tissues during high metabolic demand such as growth, physiological stress, and tissue injury^[111]. Angiogenesis is guided by proliferating ECs. Vascular endothelial growth factor (VEGF) A binding VEGF receptor 2 (VEGFR2) on ECs triggers the development of navigating tip ECs and proliferating stalk ECs that form new vascular sprouts by following the VEGFA gradient^[112,113].

Aged individuals appear to have impaired angiogenesis and to be at higher risk of pathological vessel formation^[114-116]. Age-related changes in angiogenesis likely result from vascular ageing and/or endothelial cell senescence^[117,118]. This is supported by data showing that endothelial cells from aged mice show decreased proliferation and migration^[46,119] contributing to delayed wound

healing. Moreover, elderly patients are reported to have reduced capillary density^[116,120-122] and elderly mice show reduced angiogenesis in response to ischemia^[114,123].

The ageing process is associated with accumulating exposure to harmful stimuli. Oxidative stress is increased in ageing and is associated with increases in oxidatively damaged proteins, lipids, and DNA that affect blood vessel growth. Multiple mechanisms contribute to age-related increases in oxidative stress, including increased production in endogenous antioxidant/oxidant pathways^[37,124,125]. Mitochondrial production of ROS is increased in ageing animals. Mitochondria-derived H₂O₂ aggravates NFKB activation in aged ECs, elevating low-grade vascular inflammation and further promoting oxidative stress by activating NADPH oxidases, matrix metalloproteinase, and TGF- β expression. As a result, elastin fragments and collagen content increases in aged arteries^[126-130], contributing to arterial stiffness and cardiovascular disease (CVD).

EC senescence can be induced by stimuli associated with ageing^[131], such as hypoxia, disturbed flow and oxidative stress, including ROS, high glucose, β -amyloid peptides and inflammation^[132-134]. Senescent EC accumulate in ageing tissues and contribute to tissue dysfunction^[90]. These senescent ECs adopt a pro-inflammatory SASP phenotype^[91,92]. Structural and functional changes in senescent ECs include vascular leak, thrombosis and immune dysregulation. Senescent ECs stop proliferating and undergo other functional changes. Superoxide, a ROS produced by immune cells and generated by mitochondria inhibits angiogenesis by acting as a NO scavenger that inhibits endothelial nitric oxide synthase (eNOS) activity. Thus, increased superoxide in aged endothelium impairs vasodilation and vessel formation. However, somewhat confusingly, Coleman *et al.* showed that age-related stress can also induce non-activated, anti-inflammatory senescent ECs^[135] mediated by NFKB inhibition^[136,137]. It is possible that the proportion of pro-inflammatory and anti-inflammatory senescent ECs in aged hosts determines inflammatory responses, and when dysregulated, drives pathological outcomes such as cancer. Age-related changes in monocytes/macrophages, endothelial cells and T cells are summarised in Figure 4.

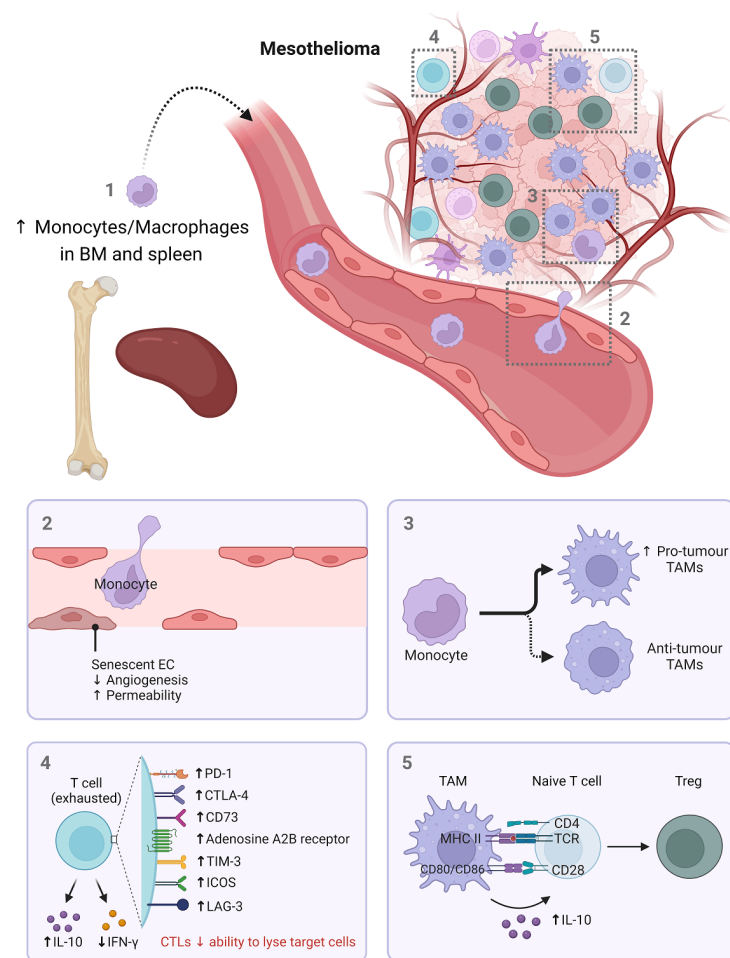


Figure 4. Age-related changes in monocytes/macrophages, endothelial cells and T cells. Ageing is associated with an increase in monocytes and macrophages in the bone marrow and spleen which can be a source of TAMs (1). The migration of these cells to the tumour site may be impacted by aged vessels, as senescent endothelial cells exhibiting decreased angiogenesis and increased permeability (2). In the tumour, the ageing environment drives the development of pro-tumor TAMs (3) and exhausted T

cells (4). Furthermore, the presence of pro-tumour TAMs promotes Treg differentiation. Created in BioRender.com. BM: bone marrow; EC: endothelial cell; PD-1: programmed death ligand-1; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; TIM-3: T cell immunoglobulin and mucin domain-containing protein 3; ICOS: inducible T-cell co-stimulator; LAG-3: lymphocyte activation gene-3; CTL: cytotoxic T lymphocyte; MHC: major histocompatibility complex, TAMs: tumour-associated macrophages; TCR: T cell receptor; Treg: regulatory T cell; IL: interleukin; IFN- γ : interferon gamma; ↓ : decreased; ↑ : increased.

10. Angiogenesis and Cancer

Angiogenesis plays a major role in tumor growth, and the elderly are at increased risk of most forms of cancer. Relevant to cancer and ageing, one of the pathways central to angiogenesis is hypoxia. Hypoxia increases expression of transcription factors or co-activators such as hypoxia-inducible factor-1 (HIF-1) and PPAR γ coactivator (PGC)-1 α that induce production of angiogenic factors, such as VEGF and eNOS. A common factor for most angiogenic pathways is that they intersect with ageing-related pathways, particularly regulators of cell senescence, such as telomere length, sirtuins, and the cyclin-dependent kinase inhibitors, p16 (Ink4a), p19 (Arf). Senescent ECs and slowed angiogenesis may account for the observation that in some cancers, disease progression is slower in elderly compared with younger patients. For example, studies in aged mice showed that B16 melanoma growth was slower^[138] and associated with decreased tumour vasculature relative to younger mice^[138,139,140,141]. Moreover, young mice with B16 pulmonary metastases had a poorer prognosis than 12 month old mice^[141]. The Lewis lung carcinoma model has been shown to grow more slowly in older mice^[142]. 4T1 breast tumours also grew slower in elderly mice^[135,136] and reduced tumour microvessel counts may also account for slower disease progression and lower grade tumours in elderly breast cancer patients^[143,144]. Similarly, colorectal cancer is more aggressive in younger people, who nonetheless have a higher survival rates than their older counterparts^[145,146]; this is replicated in murine CT26 and MC38 colon cancer with tumours progressing faster in younger mice^[136]. However, others have reported the opposite, with CT26 colon tumours growing slower in younger mice^[147].

We found that mesothelioma grows faster in older mice^[61]. This appears similar to people with mesothelioma, as younger pleural mesothelioma patients at 40 years old live between 4–9 years, whilst life expectancy diminishes to 1–3 years at 80 years old, suggesting older people experience a more aggressive, immune-evasive, treatment-resistant cancer^[148]. Similarly, prostate cancer in older men is more aggressive than in younger men^[149]. The differences in cancer progression in the elderly might be accounted for by the type and location of tumours. For example, cancers with a higher hypoxic load might progress faster than those with higher oxygenated environments, noting that the elderly are already susceptible to the development of hypoxic regions due to vascular ageing.

11. Tumour Endothelial Cells, Hypoxia and Cancer

The TME represents a complex and changing network of cancer and stromal cell types that interact with, and modulate, each other over time. At the early stages of development, tumour cells rely on diffusion of oxygen and nutrients from surrounding tissue. However, as tumour cells proliferate this becomes inadequate with the TME becoming hypoxic. Cancer cells respond to this hypoxic environment by expressing angiogenic factors such as HIF, VEGFA, PDGF and/or angiopoietin 2 (ANGPT2), as well as pro-angiogenic chemokines and receptors to initiate neoangiogenesis in a process termed the “angiogenic switch”^[111,150].

Tumour vessels develop differently to normal vessels and are excessively branched, disorganized and leaky^[85,151]. Unlike normal ECs (NECs), tumour ECs (TECs) are characterized by an irregular multi-layered endothelial lining, a discontinuous basement membrane and an inconsistent smooth muscle and pericyte sheath^[152,12]. Unstable vessel walls promote leakiness leading to higher interstitial pressure, poor perfusion and inconsistent blood flow, causing hypoxic regions^[153] that foster neo-angiogenesis^[85,154].

Single cell RNA-Seq and bioinformatic analyses have shown that TECs are genetically, phenotypically, functionally and metabolically different to NECs^[155,156]. TECs demonstrate chromosomal instability and abnormality, including aneuploid karyotypes, deletions, translocations or supernumerary centrosomes, all likely due to hypoxia, redox alterations, and epigenetic modulation (e.g., demethylations)^[157,158]. Unlike NECs, TECs show increased potential of self-renewal and are highly proliferative. This is because TECs are highly transcriptionally active (with up to four-fold higher RNA content), are hyper-glycolytic and mostly use aerobic glycolysis to meet their energy demands^[154,159]. TEC show high gene expression heterogeneity. Single cell RNA-Seq data reveal numerous TEC subtypes, such as tip and stalk ECs that are involved in neo-angiogenesis, or postcapillary venous and activated postcapillary ECs that display immunoregulatory capacity^[154,155,160]. TEC phenotypes may be determined by the tumour milieu, hypoxia-induced ROS and cellular stress, as well as by vascular ageing in the elderly.

12. TECs and Their Immunoregulatory Properties

TECs represent the first-line encounter for immune cells and tumour cells^[154] and can activate, inhibit or selectively barricade effector immune cells^[155,161,162]. Tumour cells up-regulate VEGF, fibroblast growth factor 2 (FGF2), EGF-like domain-containing protein 7 (EGFL7) and NO that regulate blood flow^[163] and prevent rolling and adhesion of immune cells by inhibiting up-regulation of adhesion molecules on TECs, even under inflammatory stimulation^[19,164–168]. The vasoconstrictive peptide Endothelin 1 (ET1) directly affects angiogenesis via VEGF and HIF-1^[169], and is associated with reduced ICAM-1 expression and decreased tumour-infiltrating

lymphocytes^[159]. Moreover, secretion of soluble adhesion molecules (MCAM/sCD146 and Endoglin) by tumour cells indirectly inhibit T cell recruitment by competing with receptors on TECs^[170,171]. Therefore, TECs become less responsive to pro-inflammatory stimulation and down-regulate expression of adhesion molecules and/or chemokines^[154,172]. However, TECs can also up-regulate expression of specific adhesion molecules, with the common lymphatic endothelial and vascular endothelial receptor (CLEVER-1) shown to preferentially select Tregs and suppressive macrophages^[173,174]. Therefore, TECs regulate immune cell trafficking and may select suppressive cells, whilst inhibiting effector immune cells^[175]; changes associated with ageing have yet to be elucidated.

NECs and TECs can express MHC class I and II molecules and select and activate antigen-specific T cells. However, they do not express the T cell costimulatory molecules, CD80 and CD86^[161], meaning they can only present processed antigens to antigen experienced memory T cells. Moreover, TECs acting as APCs have been shown to control the formation of tertiary lymphoid structures (TLS) within tumours, which are associated with positive responses to checkpoint antibody therapy^[176]. However, TECs can down-regulate MHC I/II molecules in response to the local milieu and lose their antigen presenting function^[155].

ECs can upregulate checkpoint molecules to inhibit T cell activation^[177]. For example, ECs in response to pro-inflammatory cytokines, including IFN γ and TNF- α , can elevate PD-L1 and PD-L2 expression^[178,179]. Moreover, Fas ligand (FasL) expression can be induced on TECs by VEGF-A, IL-10 and PGE2. TECs expressing FasL have been shown to selectively reduce effector CD8⁺ T cell, but not Treg, tumour infiltration^[180]. The enzyme indoleamine-2,3-dioxygenase (IDO) prevents T cell proliferation, induces T cell apoptosis and promotes Treg activation via tryptophan metabolism^[181]. TECs up-regulate IDO in response to IFN stimulation, to promote an immunosuppressive microenvironment^[182]. The effect of ageing on TEC expression of checkpoint molecules in mesothelioma has yet to be fully elucidated, however we have shown that lymph node CD8⁺ and CD4⁺ T cells in healthy elderly mice express higher levels of several checkpoint molecules than their younger counterparts including CD73, the adenosine A2B receptor, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4 or CD152), PD-1, inducible T-cell co-stimulator (ICOS, or CD278), LAG-3, and IL-10, compared to young mice; the presence of mesothelioma did not change their expression levels^[182]. Therefore, T cells expressing high levels of checkpoint molecules interacting with TECs expressing their ligands are likely to experience loss of function in mesothelioma tumours in elderly people. Indeed, we found decreased IFN- γ by elderly CD8⁺ and CD4⁺ T cells in mesothelioma, compared to their younger counterparts, implying loss of function^[182].

13. Mesothelioma, Angiogenesis and Ageing

Angiogenesis plays a key role in mesothelioma progression with blood and PE samples from patients demonstrating up to three-fold higher serum VEGF levels compared to other malignancies or healthy volunteers, and high serum VEGF levels are a negative prognostic factor^[183-185]. Harada *et al.* showed that IL-6 secreted by mesothelioma cells promoted increased VEGF expression in mesothelioma cell lines via the signal transducer and activator of transcription (STAT)3 pathway^[186]. Others have shown that mesothelioma CAFs and TAMs also release VEGF to amplify VEGF production by mesothelioma cells and further promote EC recruitment and angiogenesis^[187,188]. Recent papers using single cell RNA-Seq confirmed the presence of a large proportion of endothelial cells and angiogenic molecules in human mesothelioma samples; age was not addressed^[189,190]. The influence of age on VEGF release and its downstream effects has not yet been addressed in mesothelioma.

Human mesothelioma tumours consist of significant areas of hypoxia, particularly in dominant tumour masses, these hypoxic regions positively correlated with intensity of metabolic activity^[191]; the data were not stratified for age. An *in-vitro* study showed that culturing human mesothelioma cell lines under hypoxic conditions upregulated HIF-1 α /2 α as well as the glucose transporter (Glut)-1 target relative to normoxic controls^[192]. Therefore, the hypoxic regions likely further induced VEGF release by tumour cells and ECs thereby promoting tumour growth and augmenting aggressive behaviour by mesothelioma cells, because their clonogenicity, mobility and invasive characteristics were significantly elevated, as was resistance to cisplatin^[193]. Furthermore, a higher microvessel density (MVD) has been reported in mesothelioma biopsies compared to other malignancies^[194] which was independently related to poor survival, even when adjusted for other known prognostic factors, including age. These data prompted testing of several antiangiogenic drugs with or without chemotherapy in mesothelioma patients, but the effects were unremarkable and sometimes led to significant toxicity^[157,64,195-197]. Perhaps age played a role in this poor response. For example, an unintended consequence of antiangiogenic drugs is the induction of hypoxia in tumours^[197] which drives angiogenesis and tumour growth, in part through the recruitment and conversion of TAMs towards pro-tumourigenic and pro-angiogenic functions, noting that M2-like macrophages increase in BM and spleen with healthy ageing^[61]. Moreover, we have reported increased pro-tumourigenic CD206⁺ TAMs in elderly mesothelioma tumours^[20]. This means the pro-tumourigenic process is amplified. Furthermore, tumour cells with lower drug sensitivity are more aggressive and are selected for, leading to tumour outgrowth and resistance to anti-angiogenic therapy in the elderly. Age may also contribute to elevated toxicity mediated by pro-tumourigenic macrophages, as we have shown in old (but not young) mice with mesothelioma and treatment-related cachexia^[59,61].

14. Summary and Conclusions

The vascular and the immune systems are intimately linked, both share metabolic and growth factor stimuli and are responsive to their local milieu, particularly hypoxia. Both systems are altered during healthy ageing, and more so in response to factors released

by cancer cells, yet there are few studies examining the links between ageing, cancer, the immune and vascular systems. Here, we argue that mesothelioma is an especially useful model for understanding the impact of ageing and cancer on TECs and immune cells. This is because there is a lengthy latency period of over 30 years between known asbestos exposure and detectable mesothelioma, meaning this disease mostly emerges in elderly people. Like other cancers with less well-defined carcinogens and unknown latency periods, mesothelioma sabotages normal endothelial and immune cell physiological functions by releasing factors such as VEGF, and by recruiting and modulating suppressive cell types including TAMs, and Tregs that amplify EC recruitment and modulate their function (Figure 5). There is a clear gap in knowledge, as we do not yet understand the role ageing plays in mesothelioma aetiology and disease progression, this is true in many cancers.

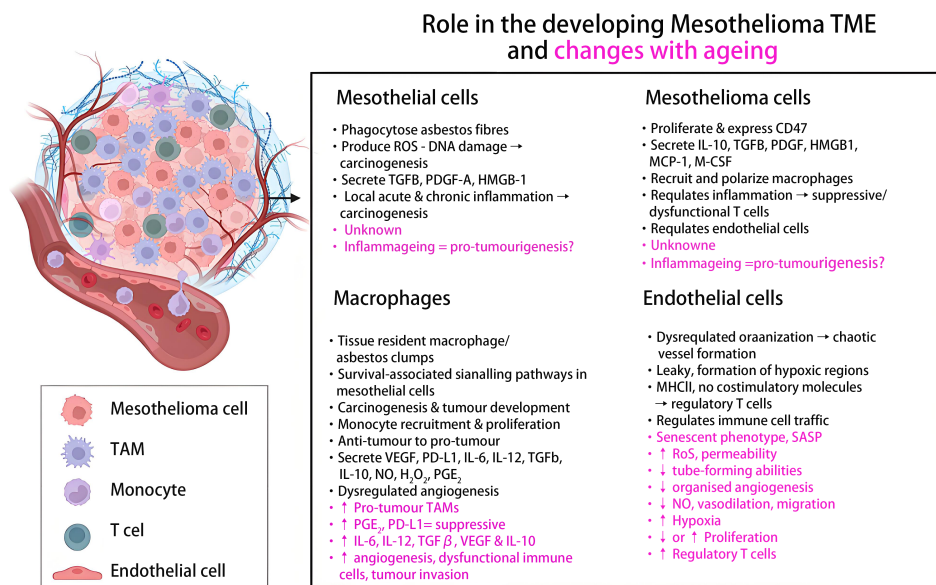


Figure 5. The influence of ageing on interactions between mesothelial cells, mesothelioma cells, macrophages and endothelial cells in the developing tumor microenvironment. Created in [Biorender.com](https://www.biorender.com). TAM: tumour-associated macrophage; TME: tumour microenvironment; ROS: reactive oxygen species; TGF-β: transforming growth factor beta; PDGF-A: platelet-derived growth factor-A; HMGB-1: high-mobility group protein box 1; VEGF: vascular endothelial growth factor; PGE₂: prostaglandin E₂; PD-L1: programmed death-ligand 1; IL: interleukin; NO: nitric oxide; MCP-1: monocyte chemoattractant protein-1; M-CSF: macrophage-colony stimulating factor; MHC: major histocompatibility complex; SASP: senescence-activated secretory phenotype; ↓ : decreased; ↑ : increased.

Declarations

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Authors contribution

All authors contributed equally to this work.

Conflicts of interest

Nelson DJ is a research academic at Curtin University and is involved in a number of research projects. One project is funded by an immunotherapy start-up company, Selvax. Selvax played no role in the preparation, or generation of data described in this manuscript. Nelson DJ is an Editorial Board member of *Ageing and Cancer Research & Treatment*. Other authors have no conflicts of interest to declare.

Ethical approval

Not applicable.

Consent to participate

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