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REVIEW

immunology
Insights into immunity in health and disease

Immunotherapeutic targets in non-small cell lung cancer

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Abstract

Non-small cell lung cancer (NSCLC) is one of the most common types of cancer in the world and has a 5-year survival rate of ~20%. Immunotherapies have shown promising results leading to durable responses, however, they are only effective for a subset of patients. To determine the best therapeutic approach, a thorough and in-depth profiling of the tumour microenvironment (TME) is required. The TME is a complex network of cell types that form an interconnected network, promoting tumour cell initiation, growth and dissemination. The stroma, immune cells and endothelial cells that comprise the TME generate a plethora of cytotoxic or cytoprotective signalling pathways. In this review, we discuss immunotherapeutic targets in NSCLC tumours and how the TME may influence patients' response to immunotherapy.

KEYWORDS
immunotherapy, non-small cell lung cancer, NSCLC, TME, tumour microenvironment

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INTRODUCTION

Lung cancers (LCs) are classified into two types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). LCs are the most common malignant disease globally, accounting for 18% of cancer-related deaths [1]. There are several treatment approaches for LC, which include radiation therapy, surgery, systemic treatments like chemotherapy, molecularly targeted therapy, hormonal-based therapy, and immunotherapy. In NSCLC, it has been reported that approximately 56% of patients with early stage (I and II) disease undergo surgery. However, the majority of patients with stage III NSCLC (62%) receive chemotherapy or radiotherapy [2]. Significant advances and efforts have been made over the last few decades to improve the treatment of LC and to improve which therapies work in which patients, which is likely to lead to better outcomes [3].

The emergence of immune checkpoint inhibitor (ICI) therapy has revolutionized cancer management, particularly in patients with LC [4, 5]. There are still significant limitations to using ICI therapies in LC as only a subset of patients is responsive [6]. As a result, improving ICI effectiveness is one of the top priorities in LC treatment, which can be accomplished by the development of better predictive biomarkers [7, 8]. The tumour microenvironment (TME) and mutational status of the tumour can be used to assess ICI treatment response in NSCLC patients. Numerous studies have recently been conducted to identify and characterize the TME, with a focus on PD-L1 expression, immune cell infiltration and signalling pathways [9–11]. The classification of tumour types into 'hot' (T cell—inflamed) and 'cold' (low T cell infiltration) has shown predictive and prognostic values [12, 13].

IMMUNOTHERAPY

Immunotherapies involve releasing the brakes off the immune system to decloak the tumour and recognize tumour-associated neoantigens, which induce an immune defence and result in tumour suppression [14]. This enables the host immune system to act with any tumour histology or driver mutation [15]. Different approaches to cancer immunotherapies have been developed, such as enhancing effector mechanisms as well as counteracting inhibitory and suppressive mechanisms. One of the approaches for neutralizing immunosuppressive mechanisms is to employ antibodies against immune checkpoint proteins [16]. Tumours commonly use immune checkpoint activation to evade the immune system; thus, ICIs are employed, as treatment approaches, to reinvigorate the immune responses against tumour cells in the TME, which is made up of immune cell types, as well as extracellular matrix (ECM), all of which are closely associated with tumour cells [17]. The presence of tumour-infiltrating lymphocytes (TILs) in the TME can be characterized as an immune-inflamed (infiltrated or also known as 'immunologically hot'), immune-desert (non-infiltrated or also known as 'immunologically cold') and immune-excluded (peripheral immune infiltration around tumour cells) [18]. The immune-inflamed tumours are able to express immune checkpoint proteins, which, in turn, contribute to the stimulation and activation of immune system responses and, as a result, have better outcomes to ICI therapies [19]. Pembrolizumab and Nivolumab, both programmed death 1 (PD-1) inhibitors, and Atezolizumab, a PD-L1 inhibitor, have shown improved responses compared to standard of care chemotherapy, earning them approval as the second-line of treatment for patients with metastatic NSCLC [20–22]. However, Pembrolizumab is the only single-agent checkpoint inhibitor approved in the first-line setting, and only in patients who have a high PD-L1 expression [23]. Dual ICI therapies, combining two types of checkpoint blockade, have also been studied. Ipilimumab (the CTLA-4 inhibitor) in combination with Nivolumab has shown promising results, with improved antitumor activity and progression-free survival (PFS) [24] (Figure 1). Recently, the U.S. Food and Drug Administration (FDA) approved Nivolumab (Opdivo, Bristol-Myers Squibb Company) in combination with platinum-doublet chemotherapy for adult patients with resectable NSCLC, regardless of PD-L1 status, in the neoadjuvant setting based on CHECKMATE-816 (NCT02998528) trial [25], the first FDA-approved neoadjuvant therapy for early stage NSCLC [26].

CTLA4

The first therapeutic target identified for ICI therapy was cytotoxic T lymphocyte-associated protein 4 (CTLA4), a $CD4^+$ lymphocyte surface protein that blocks the immune response output of this type of cell when activated [27]. In 2011, the first monoclonal anti-CTLA4 antibody, Ipilimumab, was approved for the treatment of melanoma [28]. Ipilimumab therapy was first studied in NSCLC as a monotherapy in phase II studies as an add-on to standard treatments in a sequential regimen, and it was found to improve survival [29]. According to the 'Efficacy by Histology' analysis, Ipilimumab appeared to be more effective in patients with squamous histology, which is consistent with the higher T cell infiltration seen in this histological subtype. Ipilimumab was then approved by the FDA for use in the first-line setting in

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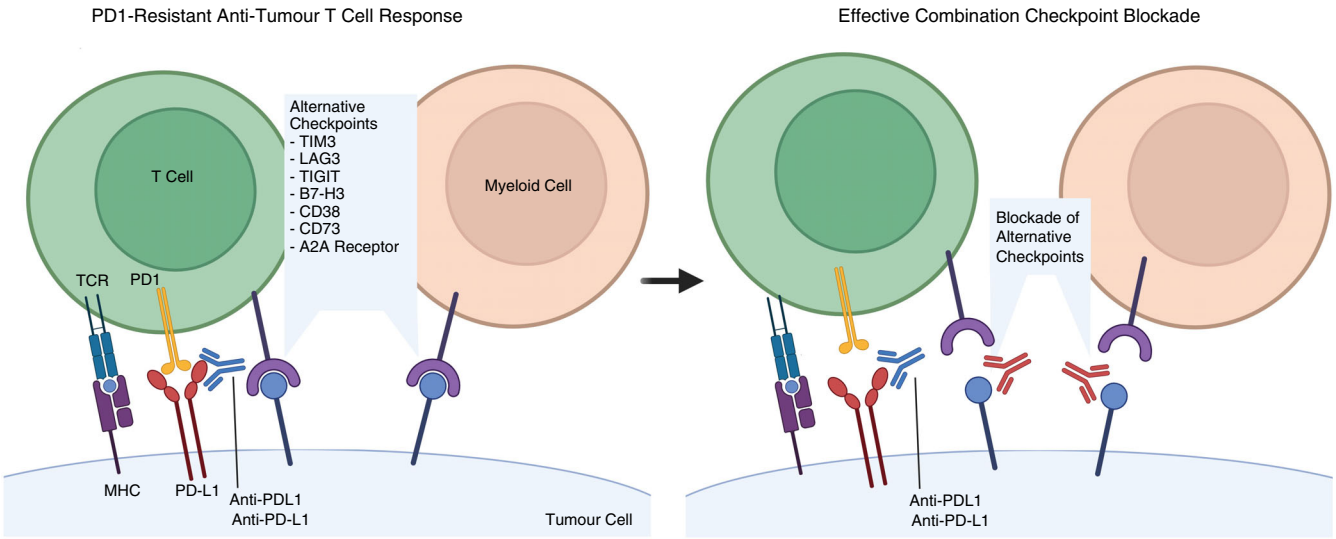


FIGURE 1 Immune checkpoint proteins. Adapted from Ref. [211]. Created with BioRender.com.

combination with anti-PD-1/PD-L1 antibodies for metastatic NSCLC patients, who had PDL-1 expression of ≥1% and no ALK or EGFR gene aberrations [30].

PD-1/PD-L1

The interaction of the PD-1 checkpoint receptor on the surface of activated T cells with its ligands (PD-L1 and PD-L2) carried by tumour cells serves an immunosuppressive function [31–33]. Pre-treatment tumour samples with high PD-L1 expression have shown to have a more robust anti-tumour adaptive immune response [34, 35]. Antibodies against PD-1/PD-L1 are used to prevent PD-1/PD-L1 interaction and, as a result, promote the anti-tumour immune response [36, 37]. Immunohistochemistry (IHC) measurement of PD-L1 expression is widely utilized and well-established in clinical settings [21, 23, 38]. However, PD-L1 expression is still not an ideal biomarker because of its spatial and temporal heterogeneity [39]. In a recent study in NSCLC, PD-L1 expression was reported to be substantially linked with the biopsy site [40]. The highest levels of PD-L1 expression were found in adrenal, liver, and lymph node metastases; however, bone and brain metastases had lower levels of PD-L1 expression. Moreover, there was a link between clinical outcomes and PD-L1 expression in lung and distant metastasis but not when found in the lymph nodes. These findings suggest that the PD-L1 from different sites may have distinct expression patterns, and therefore predictive values. The Blueprint Study showed that the abundance of PD-L1-positive tumour cells using 22C3, 28–8 and SP263 clones were comparable, while the SP142 assay showed a fewer number of stained tumour cells [41, 42]. When high discordance of PD-L1 expression was found on immune cells, interpreting the results became challenging [42]. Studies have shown that the prevalence of PD-L1 expression in squamous NSCLC is higher than in adenocarcinoma NSCLC, which may explain why ICIs work better in squamous NSCLC tumours [43–46]. It was also shown that squamous NSCLC samples had 2.5 times higher PD-L1 expression than adenocarcinoma specimens [47]. Persistent PD-1 expression on TILs was detected in tumour samples, indicating impaired T cell function and immune responses [48]. The immunosuppressive environment created by surrounding tumour cells, as well as prolonged exposure of immune cells to tumour antigens, may be the causes of the high and constitutive PD-1 expression, which eventually contributes to a defective immune response and the expression of other inhibitory checkpoint proteins [49, 50]. Studies have shown that as tumour cells progress, PD-1^+ $CD4^+$ and PD-1^+ $CD8^+$ T cell effector function deteriorates, implying an inhibitory role of PD-1 expression in antitumor immune response as well as the mechanism of immune evasion generated by tumour cells via PD-1/PD-L1 expression [51–54]. A study by Kumagai et al. conducted on $CD8^+$PD-1^+ subpopulations from different tumour types, including NSCLC, found that higher PD-1 expression on $CD8^+$ TILs is reflective of interactions with tumour antigens and may predict response to anti-PD-1/PD-L1 blockade [55]. Aside from $CD4^+$ and $CD8^+$ T cells, the expression of PD-1 has been reported in other immune cell types, like NK cells [56]. Patients with NSCLC were found to have defective tumour-infiltrating NK cells expressing PD-1, and NK cell dysfunction was associated with higher levels of cell surface PD-1 expression [56].

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LAG3

Various immune cells express the lymphocyte activation gene 3 (LAG-3 or CD223) [57]. LAG-3$^+$ T lymphocytes bind to cancer cell-released ligands, such as fibrinogen like 1 (FGL1) [58], inhibiting cytokine secretion and activation by disrupting TCR signalling [59]. Studies have found that LAG-3 and PD-1 are simultaneously expressed on TILs [60, 61]. PD-1 can mark a wide range of T cell exhaustion phenotypes, from mild to anergic, whereas LAG-3 exhibits heavily exhausted PD-1$^+$ CD8$^+$ T cells [60, 61]. LAG-3 synergizes with other checkpoint inhibitors, thus, dual immune checkpoint blockade with antibody against LAG3, such as IMP321 and Relatlimab, in addition to antibodies against PD-1/PD-L1 has provided encouraging preclinical outcomes in a variety of tumour types, resulting in several clinical phase I/II trials being conducted [59]. Tumour- or peripheral blood-derived regulatory T cells (Tregs) have been found to express LAG-3 in patients with NSCLC, melanoma and colorectal cancer (CRC) [62–64]. The expression level of LAG3, as well as the infiltration of LAG3$^+$ cells, have been linked to tumour progression, poor prognosis, and less favourable clinical outcomes in various tumour types, including NSCLC [60], CRC [65] and follicular lymphoma [66].

TIM-3

T cell immunoglobulin mucin-3 (TIM-3) is a T cell inhibitory immune checkpoint that has been shown to negatively regulate autoimmunity [67, 68]. TIM-3 was first discovered on T helper (Th)-1 cells as well as CD8$^+$ T cells. TIM-3 ligand Galectin-9 has been shown to cause cell death and tolerance in activated T cells [69, 70]. TIM-3 was identified as a novel ICI candidate due to its frequent co-expression with PD-1 on the surface of T cells in tumour samples [71]. TIM-3 is also expressed by myeloid cell subsets, and myeloid TIM-3 has been found to have multiple functions in innate immunity. First, TIM-4, a TIM protein family member, sends an 'engulfment signal' to macrophages by recognizing phosphatidylserine expressed on apoptotic cells [72]. Similarly, TIM-3 can recognize phosphatidylserine on apoptotic cells, resulting in cross-presentation by CD8$^+$ dendritic cells (DCs) [73]. Co-blocking TIM-4 and TIM-3 with mAbs reduces apoptotic cell clearance, which contributes to the synthesis of anti-double-stranded DNA [73]. TIM-3 and TIM-4 work together to maintain immunological homeostasis by clearing cells that are supposed to die. TIM-3 on tumour-associated DCs, on the other hand, appears to have a negative effect on antitumor immunity. Galectin-9 has been found to be overexpressed, and the galectin-9-TIM-3 pathway has been linked to immunosuppression in numerous types of cancer [74]. Evidence suggests that, in addition to CD8$^+$ TILs, CD4$^+$ Tregs also express TIM-3 [75–77]. In LC, studies have found a link between TIM-3$^+$ Tregs and poor clinical outcomes in NSCLC patients [78]. It was found that nearly 60% of Foxp3$^+$ TILs express TIM-3, and nearly 70% of TIM-3$^+$ CD4$^+$ TILs were Foxp3$^+$. As a result, there was a link between the abundance of TIM-3$^+$ Tregs and nodal metastasis in NSCLC patients [78]. TIM-3 expression was also found in natural killer (NK) cells and was linked to a shorter overall survival (OS) in patients with lung adenocarcinoma, implying that TIM-3+ NK cells may play a prognostic role as a biomarker [79]. Galectin-9 expression, on the other hand, has been linked to poor survival in cancer patients [77, 80]. Schulkens et al. showed that galectin-9 was associated with a worse clinical outcome in patients with NSCLC [80].

VISTA

V-domain immunoglobulin suppressor of T cell activation (VISTA) has been found to be highly expressed on Foxp3$^+$ Tregs and naïve CD4$^+$ T cells [81]. VISTA is a type 1 transmembrane protein with a single N-terminal immunoglobulin (Ig) V-domain that has high homology with the PD-L1 protein [82]. The role of VISTA in the immune response is not fully understood [83]. In addition to its role as a ligand expressed on antigen-presenting cells (APCs), VISTA may act as a receptor on T cells [83]. Evidence suggests that because VISTA has an immune-suppressive function, VISTA deficiency and anti-VISTA treatment may reactivate immune responses against tumours [84]. In NSCLC, VISTA has been found to play an immunomodulatory role. A link between VISTA expression on lymphocytes and the numbers of Tregs has been discovered in both lung squamous and adenocarcinomas histologies [85]. Tissue samples with VISTA expression ≥10% had more Tregs. Furthermore, increased TILs and specific genetic variations, as well as PD-1 axis markers, have all been linked to VISTA expression in human NSCLC tumours [86]. The expression of VISTA was significantly linked to PD-1/PD-L1 expression and EGFR mutation in NSCLC patients [86]. It was discovered that the pattern of VISTA expression on immune cells varies across tumour types [87]. VISTA expression, for example, is higher in CD3$^+$ T cells than in macrophages in NSCLC, in contrast to most other cancers [86]. This distinct pattern could be explained by the fact that TMEs in NSCLC are typically lymphocyte enriched, whereas TMEs in other cancers, such as CRC, are myeloid infiltrated [87, 88].

TIGIT

T-cell immunoreceptor with Ig and ITIM domains (TIGIT) is an Ig superfamily receptor that suppresses

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innate and adaptive immune responses [89–92]. TIGIT functions in a complex regulatory system consisting of one competing costimulatory receptor (DNAM-1/CD226), and multiple inhibitory receptors, like CD112R/PVRIG and CD96/TACTILE, as well as multiple ligands, such as CD155 (PVR/NECL-5) and CD112 (Nectin-2/PVRL2) [92–96]. There is some resemblance with the pathway involving CD28/CTLA-4/CD80/CD86, in which both costimulatory and inhibitory receptors compete for similar ligands to bind [97]. It has been shown that human NK cells, Tregs, follicular Th cells and activated $CD4^+$ and $CD8^+$ T cells express TIGIT [91, 92, 98, 99]. In cancer, human $CD8^+$ TILs and tumour antigen-specific $CD8^+$ T cells were found to co-express TIGIT and PD-1 [100, 101]. Moreover, exhausted $CD8^+$ T cell subsets were found to express TIGIT along with other immune inhibitory receptors, such as LAG-3 and TIM-3, as well [100, 101]. TIGIT could be overexpressed on Tregs and also upregulated in the TME of cancer patients [102, 103]. TIGIT was found to suppress immune responses through a number of pathways. The pathways include inhibiting T cell function through binding to CD155 on DCs [93], preventing CD155-mediated CD226 activation [104, 105], mediating the effector function of NK and T cells, and promoting Treg stability and immunosuppressive role [102]. It is worth mentioning that CD226 is a costimulatory receptor typically expressed by monocytes, NK cells and T cells [105]. Co-inhibiting TIGIT and PD-1 may improve the function of tumour antigen-specific $CD8^+$ T cells [100, 106]. Given LC, it was found that co-blocking TIGIT and PD-L1 (Tiragolumab/Atezolizumab) had a better clinical outcome than blocking PD-L1 alone as the first-line setting for patients with PD-L1 positive NSCLC and no EGFR/ALK tumour aberrations [107]. As a result, the FDA granted a Breakthrough Therapy Designation (BTD) in January 2021 for the combination of Tiragolumab and Atezolizumab as a first-line setting for metastatic NSCLC patients with PD-L1 expression [107]. Lung adenocarcinoma tumours, on the other hand, were found to overexpress CD155 [108]. CD155 not only promotes tumour cell progression and invasion [109], but its overexpression is associated with poor patient outcomes [110].

TUMOUR MICROENVIRONMENT

The TME is an essential indicator of the initiation and progression of tumours [111]. The immune response taking place in the respiratory system involves prompt phagocytosis of inhaled material, which includes pathogens and foreign particles, by the most common leukocytes in the lower airways (i.e., alveolar macrophages) through pattern recognition receptors [112, 113]. Respiratory epithelial cells contribute to the immune reaction by applying a mechanical function as well as regulating different molecular factors, such as secondary cytokines and chemokines, cell adhesion molecules, reactive oxygen species (ROS) and other lung-related factors like β-defensins and surfactant proteins [114]. DCs, $CD8^+$ and $CD4^+$ T lymphocytes (primarily the Th1 subtype) are other immune cells found in lung tissue [115–117]. NSCLC has distinct cellular and molecular properties, as well as unique mutational heterogeneity [9]. Such a heterogeneity extends to both tumours and their surrounding TME. Immune cell infiltration into the NSCLC TME has been found to be tumour stage dependent, implying that the TME plays a role in carcinogenesis as well as treatment response/resistance [9]. TME states in IO sensitive and resistant disease are shown in (Figure 2).

Cell types within the TME

$CD4^+$ T cells

A subset of $CD4^+$ T cells that express the transcription factor Foxp3 ($CD4^+$ $CD25^{hi}$ $Foxp3^+$ cells) and the interleukin 2 alpha (IL-2α) receptor is known as regulatory T cells (Tregs) [118]. Tregs suppress the inflammatory process; however, their overabundance in the TME is associated with a weakened anti-tumour immune response and a poor prognosis [119]. A higher effector/Treg ratio has been linked to better clinical outcomes in a variety of solid cancers, according to clinical studies [120, 121]. Tregs suppress effector T cells by inhibiting their activation, migration, functionality and survival [122, 123]. Furthermore, Tregs initiate an immunosuppressive process in the TME to limit the enrichment of activated antigen specific $CD8^+$ T cells [124]. Notably, $Foxp3^+$ Tregs boost cytokine production, which promotes effector $CD4^+$ and $CD8^+$ TIL dysfunction as well as checkpoint inhibition [125]. Systemic Treg depletion has been shown to make mice more prone to autoimmunity [126], indicating that a specific target to reduce intratumoral $Foxp3^+$ Tregs may be beneficial for maintaining therapeutic safety [127]. Furthermore, Treg accumulation in the TME was found to correlate with activation and differentiation of cancer-associated fibroblasts (CAFs) and myeloid-derived suppressor cells (MDSCs), implying that Tregs are regulating a complex cellular/molecular suppressive network in the TME [128]. In patients with NSCLC, increased enrichment of tumour infiltrating Tregs has been linked to a poorer outcome and survival [129, 130].

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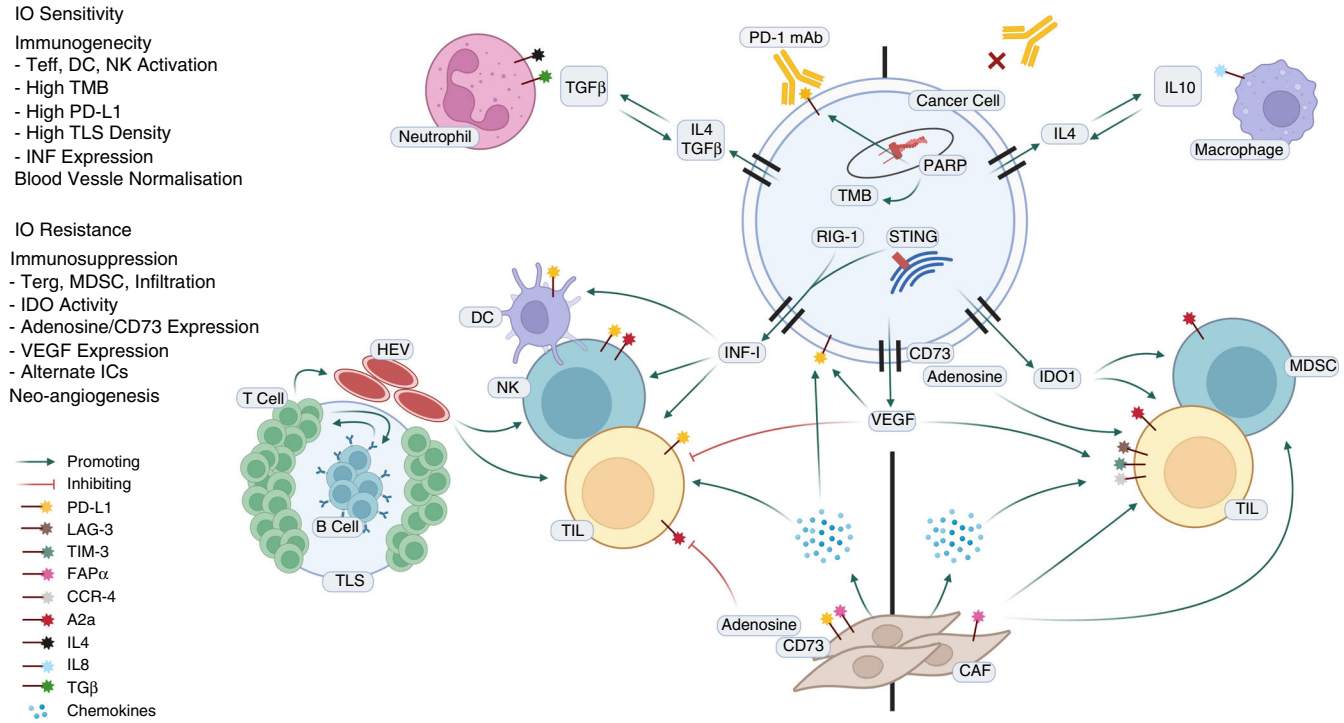


FIGURE 2 Cell types and interactions within the tumour microenvironment (TME). Adapted from Ref. [57]. Created with BioRender.com. The TME of NSCLCs involves multiple cell types and cell signalling pathways, which may affect response or resistance to immunotherapy (IO). NSCLC, non-small cell lung cancer.

Neutrophils

Neutrophils are the most frequent circulating leukocytes in patients with cancer [131]. Although neutrophils have a well-established role in the inflammatory response, their function in tumorigenesis requires further investigation [132]. Neutrophils have been shown to mediate tumour progression by releasing cytokines (such as IFN), and growth factors (such as VEGF) into the microenvironment surrounding tumour cells [133]. VEGF accelerates tumour growth by increasing angiogenesis, whereas IFN has been found to inhibit tumour promotion by recruiting and activating innate and adaptive immune cells [133]. Moreover, studies have indicated that when neutrophils are recruited to tumours, they release matrix metalloproteinases (MMPs) and VEGF, inducing ECM reorganization and angiogenesis, respectively [132]. Neutrophil diversity has been observed in various cancers, including NSCLCs, and a high neutrophil-to-lymphocyte ratio has been linked to a worse prognosis in NSCLC patients [134, 135].

Myeloid-derived suppressor cells

MDSCs proliferate during chronic infection and cancer and have been shown to inhibit NK cell and T cell activity [136, 137]. MDSCs have been shown to have a negative predictive role for ICI outcome in melanoma cancer [138, 139]. These cells suppress the immune system by producing immunosuppressive cytokines, enhancing arginase (ARG) and prostaglandin E2 (PGE2) levels and releasing ROS [140]. PGE2 is a proinflammatory factor that promotes tumour development and an inhibitor of host anti-tumour immunity [141]. ARG1 is involved in the impairment of T cell functions through lowering the expression level of T-cell receptor (TCR)-associated CD3ζ and ε chains [142]. Tumour cells, on the other hand, produce a great deal of a tryptophan-metabolizing enzyme known as indoleamine 2,3dioxygenase 1 (IDO1), which hastens the first step in the kynurenine pathway (KP), inducing an immunosuppressive TME through the recruitment of MDSCs [143]. MDSCs and Tregs were found to be the most abundant immune cells in LC patient specimens [144]. Also, the most common subsets of MDSCs in LC are granulocytic-like $CD33^{+}CD11b^{+}CD14^{-}$ MDSCs and monocytic $CD33^{+}CD11b^{+}CD14^{+}$ MDSCs [145]. According to studies, NSCLC patients with a low abundance of G-MDSCs and a high abundance of Tregs may respond better to Nivolumab therapy, demonstrating the importance of the Tregs to MDSC ratio in immunotherapy [145, 146].

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Cancer-associated fibroblasts

Because of their immunosuppressive activity, CAFs play a crucial part in tumour progression and metastasis, TME architecture and therapeutic resistance [147]. CAF activation is caused by the secretion of PDGF, FGF, TGF-β, EGF and CTGF by tumour cells, as well as the secretion of damage-associated molecular patterns (DAMPs) released by necrotic tumour cells or damaged tissues [148]. Clinical studies have shown that CAF markers correlate with ineffective NK and $CD8^+$ T cells by releasing multiple cytokines and chemokines (primarily IL-6 secretion) [148, 149]. As a result, targeting CAF-dependent pathways and CAF depletion may have therapeutic implications in patients with cancer [148]. In LC, it was found that CAFs could develop chemoresistance in A549 cells by producing IGF-2, which acts as an inducer of the ABC transporter P-GP [150]. CAFs from cisplatin-treated lung adenocarcinoma were also shown to confer chemoresistance by regulating IL-11 and activating the STAT3 anti-apoptotic pathway [151].

Tumour-associated macrophages

Macrophages are the most common type of innate immune cells in the TME, accounting for up to 50% of tumour mass [152]. Understanding macrophage phenotype polarization is critical for determining their role in tumorigenesis. Macrophage activation in tumour tissue could be induced with two distinct phenotypes: M1 macrophages (tumoricidal) and M2 macrophages (tumorigenic) [153]. These phenotype expressions are influenced by signals from their microenvironment, such as cytokine secretion [154]. In normal tissue, macrophages may exist in a state of equilibrium between the M1 and M2 phenotypes. However, in regressing tumours, macrophage polarization of the M1 phenotype has been usually observed, whereas in progressive cancers, the phenotype is shifted towards the M2 phenotype and away from the M1 phenotype [155]. Surface receptors of M2 macrophage are involved in the secretion of growth factors, such as VEGF and TNF-α, as well as pro-inflammatory cytokines [153]. In the case of LC, the presence of tumour-associated macrophages (TAMs) in the TME has been linked to cancer proliferation, epithelial–mesenchymal transition and metastasis, as well as a poor prognosis [156, 157]. Furthermore, it was found that LC cells promote TAM activation in the TME, forming a positive loop between tumour cells and TAMs that develops further tumour progression [152, 158, 159].

Dendritic cells

Tumour antigens within the TME are effectively engulfed and processed by immature DCs [160]. DAMP promotes the activation and maturation of DCs, enhancing their ability to present antigens to lymphocytes. Matured DCs move to lymph nodes where they stimulate $CD8^+$ and $CD4^+$ T cell activation [160]. Maturated DCs also produce cytokines and co-stimulatory molecules such as B7 and TNF family members to complete T cell activation. Furthermore, by releasing IL-12, these cells activate NK cells [132]. Conventional DCs have recently been discovered to infiltrate the TME and release IFN-III to promote antitumor immune responses [161]. IFN-III induces the production of IL-12p70, which results in the differentiation and activation of Th1 cells and effector $CD8^+$ T-cells, ultimately resulting in better clinical outcomes in cancer patients [161]. Conventional DCs are classified into two types: conventional type 1 DCs (cDC1s) and conventional type 2 DCs (cDC2s) [162]. cDC1s serve as APCs for $CD8^+$ T cells, whereas cDC2c primes $CD4^+$ T cell responses [163]. Studies on NSCLC tumours have shown that multiple specific gene signatures and the expression of genes TLR3 and TOP2A have been linked to the infiltration of DCs in NSCLC tumours [164–166]. Accordingly, the presence of DCs in the TME of NSCLC patients has been associated with a higher survival rate [167].

$CD8^+$ T cells

Cytotoxic T lymphocyte (CTL) infiltration into TME is stimulated by specific chemokines, including CXCL9, CXCL10, CXCL11 and CXCL16, as well as CCL3, CCL4, CCL5 and CCL20 [168]. The release of CCL5 by tumour cells and the release of CXCL9 by APCs in response to IFN-γ appear to be the keys, as $CCL5^{hi}$ $CXCL9^{hi}$ tumours were found to have a high level of TIL infiltration and to respond well to ICIs [169]. CTLs have been shown to target and kill tumour cells via TCR interaction with MHC class I and apoptosis induction, respectively. Apoptosis can be induced by granzyme B (GZMB) and perforin secretion as well as death receptor ligation, TRAIL and FasL [160]. However, tumour cells suppress MHC-I expression and endure mutations that impair antigen processing and presentation in order to avoid CTL-mediated death. Tumour cells also overexpress anti-apoptotic molecules, like BCL-2, while downregulating death receptors Fas [160]. The presence and abundance of TILs in the TME may have prognostic value, especially in the early stages of LC [170]. TILs from patients with LC were found to have a downregulation of perforin and granzyme, implying a dysfunctional state of $CD8^+$ T cells [171, 172]. Nonetheless, patients with fewer exhausted T cells, as evidenced by a low PD-1 to

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CD8 ratio, may have a more favourable tumour immune microenvironment and benefit more from immunotherapy in advanced NSCLCs [11, 171].

NK cells

NK cells are characterized as $CD3^{-}/CD56^{+}$ cells that destroy any potentially dangerous cell [173]. These cells account for approximately 15% of all human circulating lymphocytes [174]. NK cells function against tumour cells by producing GZMB and perforin or by stimulating TNF-related apoptosis inducing ligand (TRAIL)- and FasL-mediated apoptosis [173]. NK cells also regulate T cell proliferation by destroying activated T cells and stimulating Th1 polarization through the release of IFN-γ [174]. Through the release of chemotactic cytokines, like XCL1 and CCL5, NK cells attract myeloid cells and effector lymphocytes to inflamed tissues [175]. In cancer, NK cells are known to have a crucial function in promoting an antitumor immunity and to be associated with better clinical outcomes in cancer patients [176]. These cells were also found to secrete pro-inflammatory cytokines and chemokines such as TNF-a, IFN and GM-CSF in order to activate T cells, macrophages, DCs and neutrophils, thereby stimulating antitumor immunity [173]. The success of ICIs in NSCLC patients was found to be related to the activation status of peripheral NK cells as well as NK cell infiltration into the TME [177–179]. It was demonstrated that patients with NSCLC who had higher NK cell gene expression responded better to ICI and had a longer survival [177].

Molecular characteristics of the TME

Tumour mutation burden

The efficacy of ICIs has been linked to the tumour mutation burden (TMB) [180–182]. TMB-high tumours, Range, 5–15 mutations/Megabase (mut/Mb), such as SCLC [183, 184] and NSCLC [180], are highly immunogenic and are linked to better objective response rate (ORR), PFS and/or long-term clinical outcomes in patients with ICI treatment. SCLC tumours show fewer mutations per megabyte than NSCLC, which may be related to the less effective immunomodulatory treatment options observed in this subtype of LC [185]. Thus, through the infiltration and development of antigen-specific effector T-cells, high immunogenicity could result in the production of more neoantigens and, as a result, increased sensitivity to treatment response [17]. This increased number of mutations may be due to MSI-H and DNA damage repair deficiency in the tumours from patients who had long-term responses to PD-1 therapy, which accounts for 21% of patients who had a complete response and 53% who had radiographic responses [19, 186]. Moreover, C to A transversions and the deletion of particular genes may also be associated with the clinical response [187]. A high-transversion mutational profile, so-called the 'molecular smoking signature' produced by tobacco smoke carcinogens [180], may cause an increase in TMB and, therefore, be responsible for the effectiveness of ICI therapy, particularly in smokers compared to never-smokers among LC cases [185]. The response to ICIs is also influenced by the tumour efficiency in presenting neoantigens on MHC-I to T-cells, as the CTL-mediated anti-tumour immunity depends on it. The antigen presentation efficiency can be reduced markedly via the homozygosity of the HLA-A, HLA-B and HLA-C genes or reduction/lack of MHC-I expression, thereby leading to lower affinities of neoantigens [187]. Due to immune selective pressure, several independent immune-evasion strategies have been distinguished in early stage untreated NSCLCs. Specifically, it has been revealed that clonal neoantigens could be subjected to copy number loss via HLA loss of heterozygosity, or reduced transcription through promoter hypermethylation, which occurs in ~23% of neoantigens, or other mechanisms [188]. Moreover, the lower HLA-I and B2M gene expression in squamous cell carcinoma (SCC) subtype than adenocarcinoma and normal tissue may define the lack of correlation between OS and neoantigen burden discovered in SCC tumours [189]. The genomic and clinical data of cancer patients suffering from advanced disease who were treated with ICIs, as well as patients who were not treated with ICIs and whose tumour samples underwent NGS (Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets [MSK- IMPACT]), were analysed in the landmark study by Rizvi et al. [190] They found an association between higher somatic TMB, which constituted the top 20% in each histology group, and improved clinical outcomes, like OS, across all patients. The TMB cutoffs varied markedly between cancer types; for instance, a high TMB cutoff for NSCLC was 13.8 mut/Mb [190]. the FDA approved Pembrolizumab use in patients who suffer from advanced solid cancers with high TMB (≥10 mutations per mega base evaluable with the MSK-IMPACT or the FoundationOne CDx analyses), based on the rates of overall response from a pre-planned retrospective study from the KEYNOTE-158 trial taking place in June 2020 [191, 192].

Driver mutations

Mutations and rearrangements in anaplastic lymphoma kinase (ALK) and epidermal growth factor receptor

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(EGFR) genes have been discovered in NSCLC patients as tumour driver gene mutations. Tyrosine kinase inhibitors (TKIs) showed impressive efficacy in cancer patients; however, it has been found that tumour cells with ALK or EGFR mutations are resistant to these treatments; thus, ICIs appear to be a potential alternative for these patients [193]. It should be noted that TKIs may cause side effects ranging from mild to severe in NSCLC patients [194]. Skin rash or acne, diarrhoea, cardiac effects (e.g., QT interval alterations) and fetal side effects, such as pneumonia, myocardial infarction and cerebral infarction, could be some examples [194]. Studies were conducted to assess the effectiveness of PD-1/PD-L1 blockers according to molecular genotypes, with a focus on patients with ALK and EGFR genomic variations. Interestingly, ORR or PFS was significantly lower in ALK-positive or EGFR-mutant patients who received PD-1 inhibitors than individuals who did not have any of those mutations [195]. These results were compatible with the prospective data released by the KEYNOTE-010 and Checkmate-057 trials [20, 21]. A cohort study on ALK-positive or EGFR-mutant NSCLC patients aimed at identifying potential molecular mechanisms found that most NSCLC patients with ALK or EGFR rearrangements did not have PD-L1 positive or CTL-infiltrated tumours, which are assumed to be the main effective factors for ICI treatment [195]. The lack of an inflammatory microenvironment might explain why immune checkpoint blockers are ineffective in these populations [195]. However, in the IMpower150 trial (a randomized, open label, phase III study), NSCLC patients with sensitizing EGFR mutations who received Atezolizumab + Bevacizumab + Carboplatin + Paclitaxel (ABCP) had an improved survival compared to those given Bevacizumab + Carboplatin + Paclitaxel (BCP), indicating promising results for the combination of Atezolizumab plus Bevacizumab and chemotherapy in patients with EGFR mutations [196].

Hypoxia

The uncontrolled and rapid proliferation of cancerous cells can result in hypoxia, a common feature of the microenvironment in almost all solid tumours, as well as insufficient blood supply [197]. The median oxygen level in most malignant tumours is around 10 mmHg; however, the oxygen pressure in normal tissues is estimated to be around 40–60 mmHg [198]. The degree of hypoxia differs by tumour types, and oxygen levels in hypoxic areas of tumours, which typically have an average oxygen level of less than 2%, are lower than in normal tissues [197]. The concentration of oxygen in healthy human lung tissue is almost 5.6% O_2. In comparison, in NSCLC, it is reduced to 1.9%–2.2% [199], indicating a relative dependence of oxygen levels on the source tissue of origin [198]. Hypoxia can produce intratumoral oxygen gradients, which lead to tumour cell heterogeneity and plasticity. Hypoxia can also cause genomic and proteomic alterations within cancerous cells, promoting TME transformation. These alterations may provoke cell cycle arrest, differentiation, cellular apoptosis and necrosis [200]. In contrast, various changes may provoke tumour development, invasion and metastasis. They may also initiate and develop anaerobic metabolism and angiogenesis, which aid tumour cells in their survival or evasion of their oxygen-depleted environment [201]. Molecularly, a transcription factor known as hypoxia-inducing factor (HIF) accumulates in response to a decrease in oxygen levels, regulating tumour cell compatibility with hypoxia. HIF-1, HIF-2 and HIF-3, members of the human HIF family, have been found to interact with consensus hypoxia-response element binding sites and stimulate various transcription factors important in cellular oxygen homoeostasis and hypoxic signalling pathways [200, 202–204].

Extracellular matrix

The cancer cell-ECM not only acts as a scaffold in the cells and lymphatic vascular system, but it also helps to maintain the inflammatory environment required for tumour progression and metastasis [205, 206]. The effects of the ECM, which serves as a storage depot for cytokines, growth factors, and other molecules, are mediated by the integrins that connect the actin cytoskeleton to the ECM [155]. In terms of interactions with ECM proteins, laminine-5 and collagen expressions would change in NSCLC, with the former associating with EGFR-AKT signalling overexpression [207]. Hypoxia-induced Lysyl-oxidase (LOX) upregulation may promote tumour invasion by controlling the focal adhesion kinase (FAK) signalling pathway and forming cross-links across collagen fibres [208]. Furthermore, a complicated interaction between ECM and cancer cells is suggested, in which cancer cells directly regulate the structure and function of the ECM by stimulating the production of some MMPs, such as MMP-1, -2 and -9, the polymorphisms of which were found to affect the risk and survival of NSCLC [117, 209, 210].

CONCLUSION

Given the importance of immunotherapy in LC treatment, better approaches to remodelling and reinvigorating immune responses against tumours may result from

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characterizing the TME. The TME is a dynamic and heterogenous network that plays a significant role in tumour growth and progression. The interaction of various cell types and signalling pathways within the TME, including proinflammatory and immunoregulatory pathways, results in an immunosuppressive environment required for the survival and invasion of the surrounding tumour. Determining the best treatment strategies to deal with such an immunosuppressive environment needs an understanding of how to re-educate the immune response against tumour cells. Moreover, appreciation of the tumour-stroma interface in the premalignant stage may provide critical cues of key players involved in tumour-immune escape and tumour dissemination. Despite significant therapeutic advances over the last decade, the lack of validated predictive and prognostic biomarkers of response to these therapies remains a challenge. This could be addressed by performing a comprehensive analysis of NSCLC tumours with multiomic spatial profiling technologies to gain a deeper understanding of the TME.

AUTHOR CONTRIBUTIONS

Concept and idea: Arutha Kulasinghe and Ken O'Byrne. *Original – draft writing*: all authors. *Original – editing and review*: all authors.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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