NSCLC's TIME

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Contributor: Alexander Chi

Immune checkpoint blockade (ICB) has become a major treatment for lung cancer. Better understanding of the tumor immune micro-environment (TIME) in non-small cell lung cancer (NSCLC) is urgently needed to better treat it with this type of therapy. In this review, we describe and explore how NSCLC's TIME relates to response to ICB, as well as how to treat those with unresponsive types of TIME, which will significantly impact future research in lung cancer immunotherapy.

Keywords: NSCLC ; LUAD ; LUSC

1. Introduction

The systemic therapeutic options for advanced-stage non-small cell lung cancer (NSCLC) have expanded greatly in recent years to include not only chemotherapy and targeted therapies but also immune checkpoint inhibitors (ICI) [1]. Clinical outcome in patients with PD-L1 expressing treatment-naïve stage IV or previously-treated NSCLC has significantly improved with the emergence of anti-PD-1 and anti-PD-L1 ICIs [2][3][4][5]. In the first-line setting, significant survival advantage over standard chemotherapy with anti-PD-1/anti-PD-L1(anti-PD-(L)1) monotherapy has been consistently observed in EGFR and ALK wild-type stage IV patients with tumor cell PD-L1 expression ≥ 50% [4][5]. For those with PD-L1 expression < 50%, combining an anti-PD-1 antibody with standard chemotherapy has become the first-line treatment of choice on the basis of both superior progression-free survival (PFS) and overall survival (OS) observed over standard chemotherapy in randomized controlled phase 3 trials [6][7]. In previously-treated EGFR and ALK wild-type patients with any PD-L1 expression, a survival advantage over chemotherapy from anti-PD-(L)1 monotherapy was also consistently found [8][9][10]. This advantage over chemotherapy appears to be largest in patients with high PD-L1-expressing tumors (tumor cells: ≥50%, or tumor infiltrating immune cells: ≥10%). In addition, durable response significantly longer than that of chemotherapy was observed in responders to anti-PD-(L)1 antibodies [9]. Overall, the majority of current clinical evidence demonstrated that EGFR and ALK wild-type advanced-stage NSCLC patients with high PD-L1-expressing tumors benefited the most from anti-PD-(L)1 ICIs, despite quantitative variations between the currently available PD-L1 immunohistochemistry (IHC) assays [5][11]. However, PD-L1 expression level alone does not always predict for response to anti-PD-(L)1 ICIs [12]. Independent from PD-L1, a high tumor mutational burden (TMB), which correlates with tumor neoantigen load and effector T cell interferon (IFN)-γ gene signatures, was also shown to correlate with therapeutic benefit from ICIs [5][12][13][14][15][16]. PD-L1 expression level, TMB, or effector T cell IFN-γ gene signatures may each correlate with certain characteristics of a tumor immune micro-environment (TIME) that will be optimal for PD-(L)1 immune checkpoint blockade (ICB). However, none of them alone can be used to reliably select for all responders to anti-PD-(L)1 ICIs. More thorough understanding of NSCLC's TIME is required in order to select NSCLC patients more reliably for ICIs. In this review, the classification of different types of TIME that may exist in NSCLC and their characteristics are discussed in the context of NSCLC's response to ICB. Furthermore, strategies to augment ICI's therapeutic efficacy in NSCLC patients who respond poorly are explored.

2. TIME Classification Applicable to NSCLC and Its Correlation with Response to ICB

One of the major immune-inhibitory mechanisms in the tumor micro-environment is the upregulation of PD-1 expression in tumor-infiltrating lymphocytes (TILs), leading to $CD8^+$ T cell suppression and regulatory T (T_{reg}) cell proliferation upon interaction with its ligands (PD1 ligands 1 and 2: PD-L1 and PD-L2, respectively), which are upregulated on tumor cells through constitutive oncogenic signaling, or an adaptive response to interferon signaling-triggered antitumor immunity [17]. Because of this underlying mechanism, antitumor activity of the TILs can be restored through PD-(L)1 immune checkpoint blockade, and this has led to durable response in a subset of patients with different solid tumors [18][19]. In NSCLC's tumor microenvironment (TME), PD-L1 can be expressed in tumor and/or immune cells. Interestingly, response to anti-PD-L1 antibody has been correlated with PD-L1 expression in tumor-infiltrating immune cells, but not in tumor cells [19]. This is

likely related to the removal of myeloid cell-mediated immune suppression, leading to increased T cell activation resulting from enhanced antigen presentation upon PD-(L)1 blockade [20][21][22][23]. The TIME of poor responders to anti-PD(L)1 therapy has initially been characterized into the following types on the basis of histological observations before and after treatment with an anti-PD-L1 antibody: little or no tumor-infiltrating immune cells (immunological ignorance), intra-tumoral immune cell infiltration with minimal or no PD-L1 expression (a non-functional immune response), and an excluded immune infiltrate around the outer edge of the tumor cell cluster [19]. These types of TIME have no evidence of functional effector T cells (Table 1).

Table 1. General classification of the tumor immune micro-environment.

References	Method	Criteria	TIME Classification	Major Features	Additional Features
Herbst et al. [19]	**IHC**	**PD-L1 expression**	**Responsive**	**Before Rx**	**Before Rx**
		(TC and IC)		**Increased PD-L1 expression**	**Increased expression of another checkpoint (NSCLC):**
		CD8$^+$ T cell infiltration		**(TC, IC)**	**B7-H3, CTLA-4, TIM3, LAG3, IDO1, PD-L2**
					Decreased CX3CL1; increased CTLA-4
					Increased IFN-γ and IFN-γ-inducible genes (e.g., IDO1 and CXCL9)
				After Rx	**After Rx**
				Increased PD-L1 expression	**Increased tumor IFN-γ expression**
				(TC, IC)	**Gene expression pattern of immune activation:**
				CD8 and Th1 T cell activation	**granzyme-A, B; Perforin, EOMES, IFN-γ, TNF**
					CXCL10, CD8A, CTLA 4
			Non-Responsive	**Pre-Rx and After Rx**	**After Rx**
			Immunological ignorance	**Little or no TILs**	**No overexpression of genes associated with immune activation**
			Non-functional immune response	**TIL without PD-L1 expression**	**No overexpression of genes associated with immune activation**
					(with pre-treatment CD 8 T cell infiltrate)
			Excluded infiltrate	**Immune infiltrate at tumor margin**	**Same as the two types above, except with increased CTLA-4 expression**
					Proliferation and PD-L1 expression in immune cells at tumor margin
Teng et al. [24]	**IHC**	**PD-L1 expression (TC)**	**Type I (adaptive immune resistance)**	**PD-L1 (+), TIL (+)**	**Immunogenic mutations associated with**
		TILs			**increased TILs of higher PD-1, CTLA-4 expression**
			Type II (immune ignorance)	**PD-L1 (-), TIL (-)**	**No pre-existing T cell infiltration**
			Type III (intrinsic induction)	**PD-L1 (+), TIL (-)**	**More common in oncogenic mutation-driven NSCLC**
					LUAD: PD-L1 expression-associated EGFR mutations

References	Method	Criteria	TIME Classification	Major Features	Additional Features
			Type IV (tolerance)	PD-L1 (-), TIL (+)	Increased myeloid cells Activation of other immune checkpoints and suppressive pathways

Another TIME classification system that also applies to NSCLC has been proposed [24]. In this system, the TIME is classified by the level of tumor PD-L1 expression and TILs: type I, PD-L1+ and TILs+; type II, PD-L1- and TILs-; type III, PD-L1+ and TILs-; type IV, PD-L1- and TILs+ (Table 1). Type I TIME is consistent with a state of adaptive immune resistance with T cell exhaustion mediated by the PD-1–PD-L1 inhibitory immune axis, which has been effectively targeted with anti-PD-(L)-1 blockade. Here, PD-(L)1 expression in the tumor-infiltrating $CD8^+$ T cells has been essential to PD-(L)1 ICI therapeutic efficacy [19][25][26]. Type II TIME, which represents a state of immunological ignorance, has been associated with a lack of response to ICB [19][24]. Type III TIME represents a state of constitutive PD-L1 expression on tumor cells resulting from oncogenic signaling pathway activation, which is more prevalent in oncogenic mutation-driven cancers, such as adenocarcinoma of the lung (LUAD). Increased PD-L1 expression has been observed on NSCLC cells with activating gene alterations in KRAS, EGFR, and ALK, which has been associated with upregulated MAPK, PI3K–AKT–mTOR signaling, and JAK–STAT3 activation [27][28][29][30][31][32][33]. However, such expression is not due to the presence of functional TILs [34]. Subsequently, response to anti-PD-(L)1 ICIs alone is poor, despite PD-L1 expression in tumor cells. This has been reported in NSCLC patients with EGFR mutations and ALK rearrangements, which are also associated with low tumor neoantigen load [35][36]. Type IV TIME describes a state of ineffective IFN-γ signaling that fails to induce any PD-L1 expression [37], or an environment of immune exhaustion through additional immune checkpoints. For NSCLC, alternative immune checkpoints, such as B7x and HHLA2, were found to be expressed in the majority of PD-L1-negative cases, which inhibited T cell receptor (TCR)-mediated $CD4^+$, $CD8^+$ T cell proliferation, and T cell cytokine production [38].

The four-type classification system captures the main features of a TIME responsive to PD-(L)1 immune check point blockade, a state of adaptive immune resistance or T cell exhaustion that relies heavily on the PD-(L)1 immune checkpoint: increased PD-(L)1 expression on tumor and immune cells, and prominent tumor infiltration by functional TILs. This type of TIME is also described as an “inflamed” TIME. On the other hand, the main feature of an unresponsive or “cold” TIME is a lack of functional TILs in the TIME, which can be characterized with a lack of TILs (type II: immunological ignorance, excluded infiltrate, or type III: intrinsic induction), or the presence of non-functional TILs (type IV: tolerance; non-functional immune response). These types of TIMEs are associated with or without PD-L1 expression, which further demonstrates the limitations of using PD-L1 expression alone to select patients for anti-PD-(L)1 ICIs and a need for treatment strategies to augment tumor response to ICIs in cancers with an unresponsive TIME. Overall, different TIME subtypes represent variations in different aspects or steps of antitumor immunity generation and maintenance, involving a variety of factors that are intrinsic to tumor cells and extrinsically present in the TME. They will all need to be further understood in order to better characterize the TIME and effectively target tumors with unresponsive types of TIME [39][40].

3. TIME Subtype Classification Based on Analysis of Immunogenomic Data from the Cancer Genome Atlas (TCGA)

To further understand the cancer immune landscape, researchers used various immunogenomic methods to classify the TIME across 33 cancers into the wound-healing, IFN-γ-dominant, inflammatory, lymphocyte-depleted, immunologically quiet, and TGF-β-dominant subtypes on the basis of the distinct distribution of five immune-oncologic gene signatures (macrophages/monocytes, lymphocyte infiltrate, TGF-β response, IFN-γ response, and wound healing) [41]. Their characteristics are summarized in Table 2.

Table 2. Characteristics the TCGA TIME subtype classification.

TIME Subtypes	Wound Healing ǂ	IFN-γ Dominant	Inflammatory	Lymphocyte Depleted	Immunologically Quiet	TGF-β Dominant
Leukocyte fraction *	Intermed.	High	Intermed.	Low	Low	Highest
Lymphocyte fraction (25–55%)	High	Highest	High	Intermed. low	Lowest	Intermed.
TIL (H and E)	High	Highest	Intermed. low	Low	Lowest	Intermed.

TIME Subtypes	Wound Healing ‡	IFN-γ Dominant	Inflammatory	Lymphocyte Depleted	Immunologically Quiet	TGF-β Dominant
Immune cell composition						
T cells						
CD8 T cells (<15%)	Intermed. high	Highest	High	Intermed. low	Lowest	Intermed.
CD4 T cells (<35%)						
Th1	Lowest		Elevated		Elevated	Elevated
Th2	Highest	Highest	Lowest	Intermed.	Low	Intermed. high
Tfh (<10%)	High	Highest	Intermed.	Low	Lowest	Intermed. low
Tregs (<5%)	High	Highest	Intermed. high	Low	Lowest	High
Macrophages (38–60%)				Elevated	Most elevated	Elevated
M0 (<15%)	Highest	High	Intermed. low	Intermed.	Lowest	High
M1 (<10%)	Intermed.	Highest	Intermed.	Intermed. low	Lowest	Intermed.
M2 (>20%)	Intermed. low	Lowest	Intermed.	High	Highest	High
Tumor proliferation rate	Highest	Highest	Low	High	Lowest	High
Survival						
OS	Intermediate	Intermediate	Best	Worst	Worse	Worst
PFI	Intermediate	Intermediate	Best	Worst	Worse	Worst
NSCLC subtype	Predom. in LUSC; third common in LUAD **	Second most common in LUAD and LUSC	Predom. In LUAD ***	LUSC **		
Factors of immunogenecity						
DNA damage						
Tumor neoantigen load						
SNVs	Highest	Second highest			Lowest	
Indels	Highest	Second highest			Lowest	
ITH	Elevated	Elevated	Lowest			
Enriched oncogenic driver mutations	APC, JAK1, PIK3CA, FGFR3	PIK3CA, FGFR3	CDH1, PIK3CA, FGFR3	EGFR		
TCR diversity	Intermediate	Highest	Intermediate	Low	Lowest	Highest
Immunomodulators						
Expression						
CXCL10		Highest			Lowest	Second Highest
EDNRB	Low	Lowest			Highest	
BTLA				High	High	

TIME Subtypes	Wound Healing ‡	IFN-γ Dominant	Inflammatory	Lymphocyte Depleted	Immunologically Quiet	TGF-β Dominant
Networks modulating the immune response						
Predominant immune cells		**CD8 T cells**	**CD8 T cells, CD4 T cells**	**CD4 T cells**		**CD4 T cells**
Intracellular regulatory networks						
TGF-β (somatic mut+)		**↓Leuk Fract.**	**↑Leuk Fract.**			**↓Leuk Fract.**
	↑[r] DC, M0, M1, M2, [r] NK, plasma cells	**↑E, [a] Mast, M0/2, [a] DC, [r] NK, TγΔ**	**↑M1, M2, N, CD4, Treg**	**↑M0,M1, [a] DC**	**↑M0, Treg, [mr] CD4**	**↑[r] DC**
	↓[a] NK, Treg, Tfh, CD8	**↓CD8, Treg, Tfh, [a] NK**	**↓DC, M0, Tfh, [m] B cells, plamsa cells**	**↓monocytes**	**↓[n] CD4, CD8**	
Extracellular comm. networks						
		IFN-γ (+)	**IFN-γ (+)**			
		TGF-β (+)	**TGF-β, TGF-βR(+)**			**TGF-β, TGF-βR(+)**
T cell and macrophage-related signaling	**CD80-CTLA4**	**LAG-3, CD27/28**	**CD27, PD-1**	**TLR4, VEGFB**	**TLR4**	**TLR4**
	CD70-CD27	**TIGIT, ICOS, CTLA, PD-1**	**CCR4, 5; CXCR3 DARC**		**EDN3-EDNRB, CX3CL1-CX3CR1**	**ITGB2**
	IL1A/1B-IL1R2	**CXCR3, CCR1,4,5**				**CD276**
	CXCL9-CXCR3	**BTLA**				

The wound healing, IFN-γ-dominant, and inflammatory subtypes are associated with relatively higher lymphocyte fractions (LF), which is the highest in the IFN-γ-dominant TIME. Type II helper T cells (Th2) and regulatory T cells (Tregs) were also elevated in the wound healing and IFN-γ-dominant TIME subtypes, as observed in a TGF-β-dominant TIME. The lymphocyte-depleted, immunologically quiet, and TGF-β-dominant TIME subtypes are associated with noticeably higher fractions of M2 macrophages and lower fractions of M1 macrophages. The highest and lowest M1/M2 ratios were observed in the IFN-γ-dominant and the immunologically quiet subtypes, respectively. Overall, the inflammatory subtype was associated with the best overall survival (OS). Only increased LF in the wound healing and IFN-γ dominant TIMEs significantly correlated with increased OS. This was likely related to the lower tumor proliferation rate associated with the inflammatory TIME. The lymphocyte-depleted, immunologically quiet, and TGF-β-dominant subtypes were associated with lower LF, worse survival, and higher incidence of progression. Factors associated with increased immune activation, such as lymphocyte infiltration, TCR richness, and increased fractions of Th17 and Th1 cells are associated with improved survival, while features of immune suppression, such as the wound healing (high angiogenic gene expression), macrophage regulation, and TGF-β signatures are associated with shortened survival [41].

The proportions of different TIME subtypes vary substantially among different cancers. The inflammatory, IFN-γ-dominant, and wound-healing subtypes are most common in lung adenocarcinoma (LUAD), while wound-healing and IFN-γ-dominant subtypes predominate in lung squamous cell carcinoma (LUSC). The immunologically quiet TIME is absent in both LUAD and LUSC. Consistent with their predominant TIME subtypes, LUAD and LUSC have the highest leukocyte fractions among all solid tumors analyzed, which partially explains their response to ICIs [9][41][42][43]. Increases in lymphocyte and macrophage signatures are associated with increased OS for LUAD and prolonged progression-free interval (PFI) for both LUAD and LUSC. This is most likely related to the increased fractions of $CD8^+$ T cells and M1 macrophages in their predominant TIME subtypes. When broken down to specific immune cells, monocytes, mast cells (resting), dendritic cells (DCs), and memory B cells are prominently associated with prolonged OS for LUAD, whereas Tfh cells, γδ T cells, $CD8^+$ T cells, activated NK cells, and M1 macrophages are associated with prolonged OS for LUSC. Tregs, $CD8^+$ T cells, CD4 T cells, resting mast cells, M1 macrophages, DCs (resting), and memory B cells are associated

with prolonged PFI for both LUAD and LUSC, thus suggesting the importance of an overall active immune infiltrate for achieving a durable response and prolonged survival after ICB in lung cancer patients.

The tumor neo-antigen load is highest in the wound healing and IFN-γ dominant TIMEs and lowest in the immunologically quiet TIME. Higher tumor neo-antigen loads in the first two types of TIMEs are associated with increased PFI, but the opposite has been observed in the inflammatory, lymphocyte-depleted, and immunologically quiet TIME subtypes [41]. This finding may relate to the presence of a normal adaptive antitumor immune response to increased tumor neo-antigens in the first two TIME subtypes but the presence of immune tolerance and immunological ignorance/exclusion in the latter three TIME subtypes. The way in which the level of tumor neoantigens associates with the level of TILs in each TIME subtype remains to be further investigated. Among all factors of immunogenicity, elevated SNV neoantigen load, non-silent mutations, and intra-tumoral heterogeneity (ITH) generally correlate with increased leukocyte fraction within the TIME. This usually represents elevated $CD8^+$ T cells, M1 macrophages, and $CD4^+$ memory T cells, and decreased Treg, mast, DC, and memory B cells. These correlations are strongest for in an inflammatory TIME, with weaker correlations observed in the wound healing, IFN-γ dominant, and the lymphocyte depleted TIMEs.

Different levels of driver mutation enrichment are found in different TIME subtypes, with most of them identified in the wound healing and IFN-γ dominant TIMEs, which are also predominant TIME subtypes in LUSC and LUAD. These alterations are associated with different levels of tumor neoantigens and/or the expression of various immunomodulators (IMs) (Table 3).

Table 3. Mutations associated with the most common neoantigens and enriched in different TIME subtypes based on TCGA data.

TIME Subtype	Neoantigen-Related Driver Mutations	Enrichment
Wound healing	**KRAS, KRAS G12, PIKC3A, TP53**	**APC (OM), JAK1 (OM), TP53 *, FAT1, PPP2R1A, BRCA1, RB1, PIK3CA (OM), PTPRD, SPTA1, *CTNNB1* *, *FGFR3* * (OM), SMARCA4, KRAS G12, DACH1, PTEN *, SMARCA1, JAK1, *KRAS* *, MSH3**
IFN-γ-dominant	**PIKC3A, TP53**	**CASP8, HLA-A, HLA-B, ZNF750, TP53 *, MLH1, NF1 *, FAT1, PPP2R1A, BRCA1, RB1 *, PIK3CA(OM), PTPRD, SPTA1, DACH1**
Inflammatory	**BRAF**	**BRAF, CDH1 (OM), PBRM1 ***
Lymphocyte-depleted	**IDH1**	**EGFR (OM), *CTNNB1* ***
Immunologically quiet	**TP53, IDH1**	***IDH1 R132H*, ATRX, *CIC* *, TP53 ***
TGF-β-dominant	**KRAS G12**	**KRAS G12**

Some are associated with increased leukocyte fraction (TP53, HLA-B, BRAF, PTEN, NF1, APC, and CASP8), while others are associated with decreased leukocyte fraction (IDH1 R132H, GATA3, KRAS, NRAS, CTNNB1, and NOTCH1). Their association with tumor neoantigen generation, IM expression, and ultimately leukocyte fraction provides further evidence for tumor intrinsic gene alterations' role in the sculpting of the TIME, which warrants further exploration to guide the treatment of NSCLC and other solid tumors [41].

The pattern of IM expression varies in different TIME subtypes. Stimulatory modulator CXCL10 is most highly expressed in the IFN-γ-dominant TIME, while inhibitory modulators, such as EDNRB and BTLA, are most highly expressed in the more immune-suppressive TIME subtypes. A balance between T cell activation and suppression is found in more immune-stimulatory TIME subtypes, which is evidenced by the expression of both stimulatory and inhibitory IM genes, such as SLAMF7, TNFSF4 (OX40L), IL10, CD40, and IDO1. On the contrary, modulators associated with immune infiltration are more frequently deleted in the immunologically quiet TIME (e.g., TGFB1, KIR2DL1, KIR2DL3), which is consistent with a lack of TILs in this TIME subtype. Overall, TIME subtypes with increased $CD8^+$ T cell infiltration have been associated with the expression of stimulatory IMs, while those with increased infiltration by CD4 T cells and macrophages were associated with increased TGF-β signaling (Table 2). This pattern of IM expression reflects the predominance of different extracellular signaling networks associated with the fraction of different immune cells in the TIME [41].

Intrinsic tumor mutations interact with external signaling networks in a particular TIME with different driver mutations modulating IM expression in a TIME subtype-specific manner through common transcription factors (TFs). For example, ATM mutations and co-occurring STK11 and SMARCA4 mutations may drive wound healing TIME-specific gene

expression through STAT5A in LUAD, while KEAP1 mutations, which often co-occur with STK11 and SMARCA4 mutations, drive the expression of genes specific to the immunologically quiet and TGF-β-dominant TIMEs through IRF8 in LUAD [41][44]. In LUSC, NFE2L2 mutation may drive the expression of wound healing and IFN-γ-dominant TIME-specific genes through IRF4, as well as the TGF-β dominant TIME specific gene expression through NFKB2 [41]. TIME characterization may be further enhanced with identifying T cell associated receptors and ligands that are uniquely present or absent in particular TIME subtypes, such as the absence of CTLA, LAG-3, TIM-3, TIGIT, ICOS, and IL2A in the inflammatory TIME, or the presence of IL1B and VEGFB in the TGF-β dominant TIME [41].

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