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Intratumor Heterogeneity and Antitumor Immunity Shape One Another Bidirectionally

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ABSTRACT

Over the last decade, it has become clear that the genomic landscapes of tumors profoundly impact their immunogenicity and how tumor cells interact with immune cells. Whereas past discoveries mainly focused on the interplay between tumor immunogenicity and tumor mutational burden (TMB), under the assumption that a higher mutation load would give rise to a better patient response to immune checkpoint blockade therapies, we and others have underlined intratumor heterogeneity (ITH) as an important determinant of the magnitude of the antitumor response and the nature of the tumor microenvironment. In this review, we define TMB versus ITH and how the two factors are being inferred from data, examine key findings in the cancer immunogenomics literature deciphering the complex cross-talk between TMB, ITH, and antitumor immunity in human cancers and *in vivo* models, and discuss the mutual influence of ITH and immunity—how the antitumor response can give rise to tumors with higher ITH, and how higher ITH can put shackles on the antitumor response.

Introduction

The last 20 years have seen a major shift in our understanding of cancer biology, driven by two major advancements: (i) the deciphering of thousands of cancer genomes and their complex landscapes, spearheaded by The Cancer Genome Atlas (1) and (ii) the harnessing of the immune system to counteract cancer by immune checkpoint blockade (ICB) therapy (anti-CTLA4 or PD-1/PD-L1 antibodies) or cell therapy (adoptive transfer, chimeric antigen receptor technology, etc.; refs. 2, 3). As the current ICB therapy given in the clinic has a durable response in only a limited set of patients, with 36% of patients with metastatic melanoma undergoing 5-year progression-free survival at best (4), accompanied by decline in response rates for adoptive cell therapy compared with its success in the pre-ICB era (5), the need for better patient matching using genetic, transcriptomic, epigenetic, metabolic, and proteomic biomarkers that can predict the optimal clinical outcome is needed.

Observations of an initial association between responsiveness to ICB and tumor mutational burden (TMB), especially in tumors with a high TMB—i.e., in melanoma (6, 7), non–small cell lung cancer (NSCLC; ref. 8), and the microsatellite instability–high (MSI-H) form of colorectal cancer (9)—established a common assumption that high TMB confers a better response to ICB therapy. However, this assumption has been challenged by other observations, as we will further discuss herein, which called for the identification of other genetic components and refinement of the concept of TMB in the context of clinical response. One such component is intratumor heterogeneity (ITH), which is now considered a key obstacle to the success of immunotherapy. The interrelations between TMB, ITH, and the tumor microenvironment (TME), with an emphasis on the immune cell compartment, are of prime interest and are the subject of this review.

How Is ITH Defined and What Is Its Evolutionary Role in Tumor Development?

Genetic aberrations, ranging from point mutations to chromosomal rearrangements, gains and losses, are the driving force behind cancer cells' acquisition of additional genetic alterations and adaptation to new microenvironments (10). Some of these genetic alterations may lead to a fitness advantage that results in the generation of genetically distinct cancer subclones. In the context of this review, ITH refers to the variations in the genomic-driven subclonal structure of a cancer. The degree of ITH reflects the number of distinct clones composing the tumor and the degree of their genetic diversity, the combination of which influences tumor aggressiveness. ITH is further defined by its uneven distribution, spatially or temporally, of its genomic diversification in an individual tumor, fostered by accumulated genetic mutations (11, 12). It should be noted that the very definition of the terms 'clone' and 'subclone' are somewhat vague, ambiguous, and fluid (13); in principle, since tumors are thought to derive from a single tumor cell, clones and subclones are clusters of cells which have a mutational landscape which has evolved away from the original 'founder' single tumor cell.

ITH can be inferred and calculated from genomic data in numerous ways. First, mutation calling can be performed using sequencing data by a variety of bioinformatic tools such as MuTect (14) and be annotated by algorithms such as Oncotator (15). A minimal sequence depth of 100× can be used. Variants supported by 5 reads or more are accepted, and germline variants appearing in public databases (such as dbSNP) are removed. Furthermore, variants identified in sequenced matching normal samples are filtered out as well.

A few highly useful computational tools were developed to measure clonal and subclonal mutational composition and evolution and thus

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calculate ITH from genomic and exonic data, such as EXPANDS (16), PyClone (17), SciClone (18), and CHAT (19). These methods use somatic mutation and copy-number variants to infer cellular abundance, and clonal population architecture. Only mutations identified in two or more clones are accepted, and clustering of mutations is set between 10 and 15 clusters. ClonEvol (20) can be used for inferring and visualizing clonal evolution trees. Using CHAT, combining the estimates derived from clustering of cellular abundance of somatic mutations and copy-number variants enables further stratification to tumors with low versus high TMB/ITH (below and above the median within the cohort of patients; ref. 21). ITH and clone numbers can also be evaluated by the variant allele frequency (VAF) of exonic mutations, as it was assessed that mutation with VAF >0.25 are likely to be clonal mutations (21, 22), or by calculating the Shannon diversity index of tumors (21, 23). When multiregion sequencing is performed, clonal mutations are considered as such if they are present within all tumor region sequenced, whereas nonubiquitous mutation present in only a fraction of the regions are considered subclonal (24). In addition, ITH can also be assessed histologically using the different morphology of tumor clones (25). Finally, and importantly, ITH of tumors can be imaged in the clinic using CT, PET, and MRI, which could facilitate the identification of tumor with high or low ITH in a nonintrusive manner (26).

Although beyond the scope of this review, it should nevertheless be emphasized that ITH can be achieved not only through genomic events (mutations, indels, copy-number aberrations) but also by epigenetic events and metabolic states within the tumor (12). It is also sculpted by the heterogeneity of the TME, such as the composition of immune cells within various tumor regions, which also exert significant evolutionary pressures that may cause particular cancer clones to continuously proliferate due to immune-cell evasion (27, 28), ultimately sculpting a tumor that is composed of multiple clones and subclones.

The degree of ITH varies considerably between cancer types. Thyroid cancers are typically composed of 1 to 3 main clones, whereas the number of main clones in melanoma can exceed 10 (25). Notably, a close relationship can be drawn between the number of clones and TMB, such as in melanoma and lung cancer (29). However, the two features are not interchangeable, and counting TMB without considering the degree of ITH might be misleading, especially when immunogenicity is being considered (discussed further below).

It is also of vast importance to discriminate between clonal mutations, which are shared between all tumor clones and are thus indicative of low ITH, and subclonal mutations, which are unique to each tumor clone and are thus indicative of high ITH. One of the reasons the distinction between clonal and subclone mutations is highly important is that some of the nonsynonymous mutations can eventually give rise to immunogenic tumor antigens unique to the specific tumor of a specific patient, known as neoantigens. Thus, the distinction can also be expanded to clonal versus subclonal neoantigens (24). When this distinction is taken into account, the interactions between ITH and TMB become more complex. Melanoma and lung cancer, for instance, are characterized by a high TMB, but the vast majority of the nonsilent mutations are, in fact, clonal, whereas other tumors, such as low-grade glioblastoma and prostate cancer, accumulate a lower mutational burden in total, but a much higher proportion of this burden is subclonal (11).

Indeed, highly heterogeneous tumors, which have been shown to be more aggressive than clonal tumors, may have several advantages over lowly heterogeneous tumors. Highly heterogeneous tumors could include dominant clones with strong immune-escape mechanisms, such as genomic and epigenomic neoantigen silencing (30), loss of HLA genes (31), or impaired IFNγ-sensing pathways (32), and the abundance of such resistant clones may interfere with immune elimination of the tumor. Moreover, clones with subclonal antigens may undergo "dilution" within vastly heterogeneous tumors, allowing them to effectively evade an immune attack (33). Importantly, different clones can cooperate with one another, with some reports, such as in glioblastoma and breast cancer, showing how minor subclones can enhance the growth of the entire tumor in a non–cell-autonomous manner (34–36). Finally, increased ITH can enhance the generation of metastatic subclones, which are enriched within the tumor core due to accelerated tumor evolution and a microenvironment that drives the formation of these subclones (37).

TMB, ITH, and Patient Survival

An ongoing debate in the field is whether TMB, ITH, or their combination are associated with and can predict patient survival and responsiveness to ICB (key findings, highlighting the discourse in the field, are summarized in **Table 1**). An intuitive assumption is that excessive TMB translates into a high neoantigen load, which should increase the potential to benefit from ICB (38). Early reports have shown that tumors with increased TMB due to environmental carcinogens (UVB, tobacco smoke) or genomic instability [apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) mutagenesis, mismatch repair deficits] are associated with better checkpoint blockade sensitivity (6–9, 39–43). In uveal melanoma, which have substantially lower TMB compared with cutaneous melanoma, durable responses following ICB are much more rare, and tumor-infiltrating lymphocytes (TIL) extracted from patients have drastically decreased reactivity towards autologous tumor cells (44). It was later suggested that the correlation between TMB and patient survival could, in fact, be generalized across most solid tumors (45). These observations, however, were later refined and other factors were suggested as interacting biomarkers that cooperate with TMB to improve ICB responsiveness, such as a T-cell–inflamed gene expression profile (GEP; ref. 46) and the number of TILs (47). Mechanistically, it was suggested that TMB is positively correlated with a T-cell differentiation switch from a naïve-like PD-1$^-$ to an antigen-experienced PD-1$^+$ state (which include both effector and dysfunctional TILs), in both CD8$^+$ and, most prominently, CD4$^+$ T cells, as was demonstrated for NSCLC (48), accompanied by superior cytotoxic ability (6) as measured by calculating the geometric mean expression of granzyme A and perforin (CYT score; ref. 49).

However, there are some key shortcomings to the assumption that TMB translates into a high neoantigen load. Unlike the vast majority of human data, which show a retrospective association of TMB and ICB benefit posttreatment, the mutational count *prior* to the treatment has limited predictive power, as was shown for patients with metastatic melanoma treated with anti–PD-1 antibodies in which only a small, nonsignificant elevation in nonsynonymous mutations was observed in patients responding to anti–PD-1 compared with nonresponders, and some patients with a low TMB do respond to anti–PD-1 while some patients with a high TMB do not (50). Moreover, some cancers with a low mutational burden, such as clear-cell renal cell carcinoma (ccRCC), also respond to ICB, without any association to TMB (51). It was further found in a melanoma mouse model that single-cell–derived clones (SCC) with an even degree of heterogeneity but with very different TMBs still harbored similar immunogenicities (21).

Table 1. Summary of key findings of TMB/ITH interactions with patient survival and ICB response.

Ref. no.	Cancer type	ICB type	Findings
(6)	Cutaneous melanoma	Anti-CTLA4	Association between high TMB and neoantigen load with clinical benefit
(7)	Cutaneous melanoma	Anti-CTLA4	Association between high TMB and neoantigen load with clinical benefit
(8)	NSCLC	Anti–PD-1	Association between high TMB and clinical benefit
(9)	MSI-H type colorectal cancer	Anti–PD-1	Efficient treatment as first line therapy, unlike MSS type colorectal cancer
(39)	Colorectal cancer (mice models)	Anti-CTLA + anti–PD-1	Genetic deletion of DNA mismatch repair genes *in vivo* results in higher neoantigen load and better immune clearance
(40)	Multiple MSI-H tumors (including MSI-H type colorectal cancer)	Anti–PD-1	∼20% of patients of 12 different MSI-H cancers undergo complete response
(41)	SCLC	Anti-CTLA+ anti–PD-1	Association between high TMB and clinical benefit
(42)	NSCLC	Anti-CTLA + anti–PD-1	Association between high TMB and clinical benefit
(43)	NSCLC	Anti–PD-1	Association between high APOBEC mutation count and clinical benefit
(44)	Uveal and cutaneous melanoma	n/a	Uveal melanoma, known to be ICB refractory, is characterized by much lower TMB compared with ICB responsive cutaneous melanoma
(46)	Meta-analysis of multiple solid tumors	Anti–PD-1	Modest association of high TMB and clinical benefit, better association when TMB is interacting with T-cell–inflamed GEP
(47)	Colorectal cancer (all types)	n/a	Association with high neoantigen load in both MSI-H and MSS subtypes
(48)	NSCLC	n/a	High TMB correlated with antigen-experienced CD8 and CD4 TIL phenotype, but also with dysfunctional TILs
(50)	Cutaneous melanoma	Anti–PD-1	High TMB cannot predict clinical benefit from ICB
(24)	NSCLC and melanoma	Anti–PD-1 for NSCLC, anti-CTLA4 for melanoma	Tumors with low subclonal neoantigen burden have better clinical benefit, especially if interacting with high TMB; tumors with high subclonal neoantigen burden have poor response
(51)	ccRCC	Anti–PD-1 and anti–PD-L1	No association between high TMB and clinical benefit, even when TMB is divided to clonal and subclonal TMB
(52)	Cutaneous melanoma	n/a	No correlation between TIL infiltration and neoantigen count
(21)	Cutaneous (human and mice models)	Anti–PD-1, anti-CTLA4	Mouse tumors with increased ITH are less immunogenic and do not respond to anti–PD-1; mouse tumors with low ITH are highly immunogenic regardless of their degree of TMB; high ITH tumors are characterized with lower CD8 T infiltration and higher Treg numbers; human melanoma patient cohorts with low ITH have better overall survival without ICB and better response to ICB
(53)	Acral, mucosal, and cutaneous melanoma	n/a	No correlation between TIL infiltration and TMB
(55)	Meta-analysis of multiple solid tumors	n/a	Weak association between high TMB with clinical benefit across cancer types
(56)	Meta-analysis of multiple solid tumors	n/a	Weak association between high TMB with clinical benefit across cancer types
(25)	Meta-analysis of multiple solid tumors	n/a	Tumors with low ITH have better overall survival
(58)	Breast cancer	n/a	Tumors with high ITH are associated with lower survival, lower CD8 and CD4 infiltration, and higher Treg numbers
(60)	RCC	Anti–PD-1	Low ITH is associated with better clinical benefit
(63)	Meta-analysis of multiple MSS tumors	Anti-CTLA4, anti–PD-1, anti–PD-L1, and their combinations	Complex association between high clonal TMB and low subclonal TMB and clinical benefit
(64)	Meta-analysis of multiple solid tumors	Anti-CTLA4, anti–PD-1, anti–PD-L1	High clonal, but not subclonal, TMB predicted better clinical benefit

More importantly, the seemingly intuitive association between the abundance of tumor antigens and a strong immune response is, in fact, disputable, as no correlation between the abundance of antigens, TMB load, or structural chromosomal aberrations and T-cell infiltration or density has been found (52, 53) and no correlation between TMB and neoantigen detection has ever been proven (54). Thus, with more reports demonstrating the low predictive power of TMB screening in patients prior to ICB therapy, it has been suggested that the association between TMB and ICB response should be revisited, refined, and reconsidered (55, 56).

At the same time, ITH is becoming a highly acceptable genomic metric that needs to be considered alongside TMB, with significant consequences for antitumor immunity (**Fig. 1**). A pan-cancer analysis, for example, showed better survival and ICB response in patients with low heterogeneity (25), as was demonstrated also for lung cancer (24), melanoma (21, 57), breast cancer (58, 59), ccRCC (60), and ovarian cancer (61). These observations emphasize the importance of employing ITH and the degree of mutation clonality as an additional genomic biomarker for assessing immunotherapy success.

Interestingly, patients with lung cancer treated with ICB had a better outcome when their neoantigen landscape was enriched with clonal neoantigens and had a worse outcome when it was enriched with subclonal neoantigens (24). A cohort of human melanoma

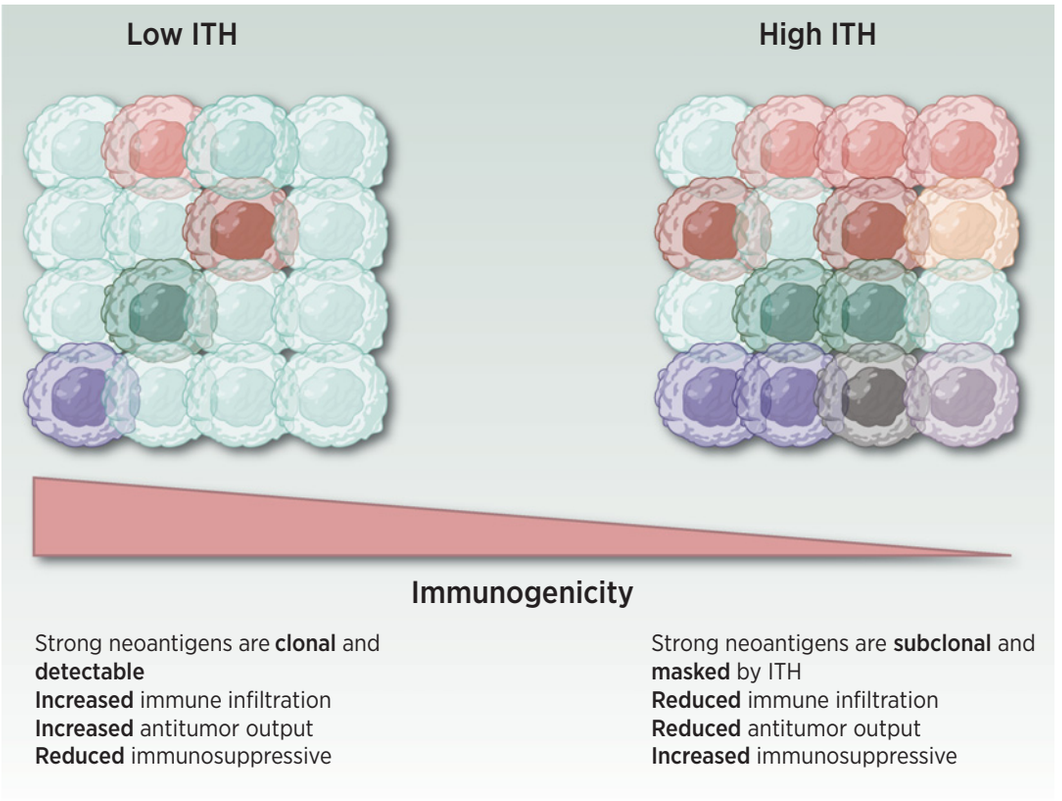

Figure 1.
Immune characteristics of tumor with different ITH. Comparison between immunologic features of low versus high ITH. Adapted from an image created with BioRender.com.

patients treated with anti–PD-1 also showed better survival with higher clonal TMB (62). These observations were recapitulated in a broader context of a pan-cancer analysis of microsatellite stable (MSS) tumors (63).

In a study correlating genomic factors of seven tumor types (melanoma, head and neck, urothelial, renal, lung, breast, colorectal cancer), it was found that clonal, but not subclonal TMB also predicted positive ICB outcome (64). This finding supports the concept of neoantigen "dilution," in which strong antigens exert immunogenicity when they are clonally abundant, but when they are subclonal, their ability to induce an effective antitumor response is significantly hampered. Strong evidence for such neoantigen "dilution" was demonstrated in subclonal immunogenic neoantigens detected using HLA peptidomics (65) in SCCs generated from parental melanoma cell lines, which were completely undetected in the parental cell line (21). Moreover, when the clonal fraction of immunogenic subclones in a tumor is "diluted" by mixing them in decreasing numbers with non-immunogenic subclones, the ability to reject the tumor is significantly hampered (33).

Mutual Impact of ITH and Antitumor Response

In ITH research, it is accepted to study the development of ITH in an evolutionary biology perspective (11). Accordingly, ITH and antitumor immunity shape each other in multiple mechanisms of coevolution. Due to selective pressure from the immune system that prunes and eliminates tumor clones with strong neoantigens, the tumor evolves to present novel subclones that, on top of other "traditional" growth advantages, such as excessive proliferative and metastatic capabilities, lower the immunogenicity of the entire tumor, either by the "dilution" of strong clonal neoantigens or by the immune-escape mechanisms of the individual subclones themselves (**Fig. 2**). This principle is demonstrated in comparisons of the genomic alterations in human melanoma tumors before and after anti–PD-1 treatment, in which ICB nonresponder tumors were found to accumulate novel subclones after treatment, while the responder tumors lost subclones detected before treatment (62). Increased ITH also shapes the immune TME, manifested in the reduced presence and altered phenotypes of immune cells in the tumor. The immune system, on its part, effectively restricts tumor clonality to avoid excessive ITH (66). We believe that understanding the interplay between these concepts is instrumental for the development of future cancer therapies.

A useful readout for tumor immunogenicity is the distinction between immune "hot" and immune "cold" tumors, denoting the high or rare abundance of immune infiltrates, respectively. In a mouse model developed in the Samuels lab that uncouples TMB and heterogeneity by generating highly heterogeneous and highly homogenous tumor cell lines with varying TMB and injecting them into immunocompetent mice, we showed that tumors with low ITH are more immunogenic and "hot" regardless of their TMB, manifested in increased numbers of $CD8^+$ TILs *in situ* with better effector function, accompanied by reduced $CD4^+FOXP3^+$ regulatory T cell (Treg) numbers (21). In accordance, in patients with lung adenocarcinoma,

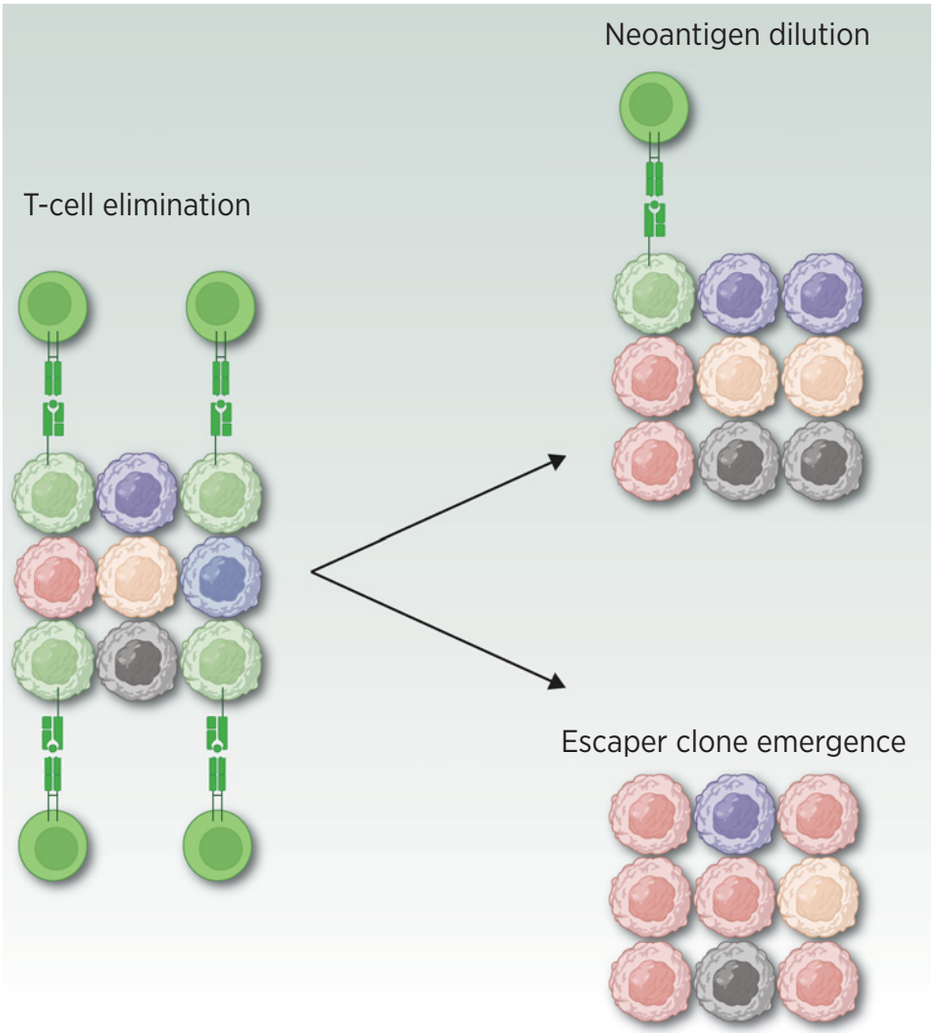

Figure 2.
Main mechanisms of immune/ITH coevolution. Tumors with low ITH are constantly being pressured by the immune system in the form of clonal neoantigen specific $CD8^+$ T cells, which can detect peptide–MHC-I complexes (in green) and eliminate the cells carrying those neoantigens (also in green), This gives rise to two parallel processes which decrease the immunogenicity of the tumor as a result of elevated ITH: the decrease in the fraction of cells carrying clonal neoantigens (neoantigen dilution; top) and the emergence of clones which have mechanisms to evade the immune system (brown, red, and blue cells, bottom). Adapted from an image created with BioRender.com.

tumor regions with low clonal TMB tend to be "colder" with lower levels of infiltrates, with patients with multiple "cold" regions having a poorer survival rate (67). Beyond the mere presence of TILs in a tumor, their spatial distribution in distinct components of the tumor is also important. In ovarian cancer, tumors with abundant levels of epithelial, rather than stromal, $CD8^+$ T cells were characterized with lower ITH and depletion of subclonal, but not clonal, neoantigens, suggesting the immunoediting and selective elimination of subclones with subclonal neoantigens (68).

The association between tumor "coldness" and ITH could stem from the "dilution" of potent antigens. Indeed, it was demonstrated *in vivo* that such dilution can dampen the antitumor response (33). Alternatively, the association could be due to immune escape, which might be more widespread as the prevalence of minor subclones increases. Clones that escape immune elimination by immune escape, such as immunoediting (the process by which the selective pressure by the immune system reduces the abundance of neoantigens by genomic processes), HLA loss, or impaired antigen presentation, are expected to have a significant evolutionary advantage (3, 68). In accordance, in lung adenocarcinoma, immunoediting continuously takes place in previously "hot" tumor regions that have become "cold," accompanied by a constant increase in ITH (30). Interestingly, TMB has a nonlinear association with loss of HLA genes, as HLA loss is abundant in tumors with intermediate, but not high, TMB. It is postulated that in these intermediate TMB tumors, other immune-escape mechanisms take place. An intriguing line of research would be to investigate HLA loss in tumors enriched for clonal versus subclonal mutations (69).

When considering the tumor ITH and the immune response, one also must address the heterogeneity within the immune infiltrates, in particular, T-cell clonality. Accordingly, T-cell receptor (TCR) sequencing of $CD8^+$ T cells in patients with NSCLC revealed that TCR diversity is positively correlated with nonsynonymous TMB (70). Similarly, the TCR repertoire significantly correlated with clonal composition in ovarian tumors with high epithelial $CD8^+$ T-cell infiltration (68). Moreover, minor subclones may alter the TME by secreting chemokines and cytokines that affect the T-cell repertoire within the TME, as was demonstrated in pancreatic cancer SCCs that express high levels of chemokine CXCL1 and block T-cell infiltration and ICB responsiveness in a murine model of pancreatic cancer (71). Minor subclones can also impact tumor-infiltrating immune cells other than T cells. In breast cancer, $IL11^+$

clones stimulated IL11R$^+$ mesenchymal stromal cells to recruit neutrophils that can support metastasis outgrowths (35). Interestingly, tumor-infiltrating neutrophils positively correlated with increased ITH in lung cancer (72).

Future Directions—from Basic Research to Clinical Intervention

High ITH is now widely considered an obstacle to cancer immunotherapy (3, 73). Given its importance, it should be factored into patient "tailoring" to immunotherapy. Indeed, it can be harnessed to improve immunotherapy. First, it could serve as a genomic readout alongside TMB, as it was shown that clonal TMB should be segregated from subclonal TMB, as the latter has better predictive power (64). Second, the notion that clonal, but not subclonal, neoantigen burden better correlates with response to ICB highlights the importance of focusing on clonal neoantigens as therapeutic targets (24). Third, understanding how minor subclones can decrease the immunogenicity of the entire tumor in specific cancer types may lead to new therapeutic agents, such as IL11$^+$ sucblones in breast cancer, which may be suppressed by future anti-IL11 therapy (35).

Establishing novel mouse models to further distill the role of clonality in tumor aggressiveness as well as deepen our understanding of the relation between ITH and T-cell immunity would be highly beneficial. Such models could be used, for example, to experimentally validate the contribution of tumors with decreased ITH to the mediation of an antitumor immune response, by elevating the clonal fraction of certain clones in the tumor in a controlled fashion. Such models could also be used to control the relative percentage of each SCC in a cell mixture. Namely, they could aid in the investigation of the hypothesis raised in this review that elevation of the relative representation of particular SCCs will expose them to higher cytotoxic activity and neoantigen-mediated elimination by T cells, while lowly represented SCCs will reduce their exposure and, thus, elimination by T cells.

Importantly, these highly controlled mouse models will greatly aid in assessing the effect of the tumor composition on and its response to cancer synthetic peptide vaccines containing neoantigens presented on the tumor cells, in the presence and absence of checkpoint inhibitors. Of particular interest will be a comparison of vaccinations based on neo-peptides that cover a large spectrum of the neoantigen repertoire (all evolutionary tumor branches) versus ones with only a fraction of the neoantigen repertoire (few branches). This has implications for the clinical use of tumor vaccination, as it may be difficult to identify peptide candidates from all subclones of a tumor or from all subclones of potential metastases. Indeed, such data could define strategies for a potent, rationally designed cancer vaccination therapy that accounts for tumor ITH.

To summarize, increased ITH and the emergence of subclonal TMB with reduced clonal TMB give rise to weaker antitumor immunity, reduced efficacy of ICB, and poorer patient survival. Thus, using total TMB as a sole genomic metric to assess and predict a patient's benefit from ICB is far from an optimal approach, and ITH must be considered as well. Moreover, ITH and antitumor immunity follow each other in an evolutionary arms race. Finally, a better understanding of the interrelations between ITH and antitumor immunity may shed light on new therapeutic opportunities.

Authors' Disclosures

Y. Wolf reports a patent for Vaccination with cancer neoantigens issued to Yeda Research and Development Co. Ltd. Y. Samuels reports a patent for Vaccination with cancer neoantigens issued to Yeda Research and Development Co. Ltd.

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