Intratumoral heterogeneity in cancer progression and response to immunotherapy

Intratumoral heterogeneity in cancer progression and response to treatment

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Running Title: Spatiotemporal diversity of the tumor microenvironment as an oncogenic driver.

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Abstract

Most (if not all) tumors emerge and progress under a strong evolutionary pressure imposed by trophic, metabolic, immunological and therapeutic factors. The relative impact of these factors on tumor evolution changes over space and time, ultimately favoring the establishment of a neoplastic microenvironment that exhibits considerable genetic, phenotypic and behavioral heterogeneity in all its components. Here, we discuss the main sources of intratumoral heterogeneity and its impact on the natural history of the disease, including sensitivity to treatment, as we delineate potential strategies to target such a detrimental feature of aggressive malignancies.

Introduction

A large body of work performed over the past decade has enabled the reconstruction of the cellular architecture, functional orientation and evolutionary trajectory of multiple cancer types. It is now clear that most tumors are complex ecosystems that emerge and evolve under a robust selective pressure from their microenvironment, which involves trophic, metabolic, immunological and therapeutic components. Such a pressure promotes the diversification of both malignant and non-malignant (i.e., endothelial, stromal and immune) compartments of the tumor microenvironment (TME), culminating with a degree of intratumoral heterogeneity (ITH) that enables aggressive disease progression and resistance to treatment{Vitale, 2019, 30765144}.

Multiregional genome sequencing studies have revealed considerable variations in the genetic makeup of malignant cells not only across distinct anatomical locations and disease stages (e.g., primary *vs.* metastatic lesions), but also across distinct regions of the same tumor (spatial ITH). Along similar lines, longitudinal studies have demonstrated that genetic features of the same lesion can vary quite significantly over time (temporal ITH){Wang, 2016, 27270107}. Importantly, ITH does not manifest exclusively at the genetic level, but encompasses epigenetic, transcriptional, phenotypic, metabolic, and secretory components{Teixeira, 2019, 30664780;Sharma, 2019, 31747591;van Galen, 2019, 30827681;Grosselin, 2019, 31152164}. Such components can vary independently from each other (*e.g.*, genetically stable tumors presenting high epigenetic variability) or in a tightly interconnected manner (*e.g.*, genetical and epigenetic alterations cooperating at defining transcriptomic and phenotypic profiles){Flavahan, 2017, 28729483}. Moreover, ITH also affects non-malignant compartments of the TME, including (but not limited to) cancer-associated fibroblasts (CAFs){Costa, 2018, 29455927}, tumor-infiltrating lymphocytes (TILs){Aoki, 2020, 31857391;Li, 2019, 30595452;Azizi, 2018, 29961579}, as well as various myeloid cell populations like tumor-associated macrophages (TAMs) and

dendritic cells (DCs){Chevrier, 2017, 28475899}. Thus, the abundance, localization and functional orientation of each cellular components of the TME evolves over space and time to dictate ITH.

Such a spatiotemporal evolution is determined by the dynamic nature of its sources, which include cancer cell-intrinsic processes such as genetic instability as well as the microenvironmental features such as the local availability of specific growth factors, nutrients and immune regulators{McGranahan, 2017, 28187284}. Mechanisms of clonal selection, cooperation and competition operate in the context of multidirectional interactions between malignant cells and the other cellular compartments of the TME. As an example, while various genetic and epigenetic defects in malignant cells impact the abundance and activity of TILs{McGranahan, 2017, 29107330}, the effector activity of the latter has a major influence on the proliferative potential and clonal configuration of the tumor{Zhang, 2018, 29754820;Rosenthal, 2019, 30894752}.

Two common principles govern the evolution of ITH in all compartments of the TME. First, each cell type appears to exist in a continuum of phenotypic and/or behavioral states associated with a degree of plasticity that enables rapid shifts in response to microenvironmental perturbations{Neftel, 2019, 31327527;Azizi, 2018, 29961579;Lambrechts, 2018, 29988129;Li, 2019, 30595452}. Second, ITH (and its sources) can be tolerated only to a specific threshold, beyond which not only cell survival is compromised but also the architecture of the compartment or the entire tumor is at risk{Andor, 2017, 28432052} (**Figure 1**). Importantly, ITH is paramount for tumors to emerge as they elude natural immunosurveillance, progress and resist (immuno)therapy{Dagogo-Jack, 2018, 29115304;McGranahan, 2017, 28187284}. Accordingly, high genetic and non-genetic ITH has been associated with poor patient outcome in a variety of oncological settings{Li, 2016, 27322744;Lin, 2017, 28302680;Jamal-Hanjani, 2017, 28445112}. However, the crucial features that define ITH and its spatiotemporal evolution are still largely uncharted. Here, we discuss the major sources of ITH, its impact on disease progression and

treatment sensitivity, as well as potential strategies to target such a particularly unfavorable feature of aggressive neoplasms.

Dynamic heterogeneity of cancer cells

Single or multi-sample sequencing studies on patient biopsies have delineated the tumor mutational landscape and its evolutionary trajectory, demonstrating widespread genetic ITH on both spatial and temporal dimensions{Dagogo-Jack, 2018, 29115304;McGranahan, 2017, 28187284} (**Figure 2**). ITH has been detected on somatic single-nucleotide variants, short insertions or deletions (indels), copy number variants as well as structural or numerical chromosomal aberrations{Jamal-Hanjani, 2017, 28445112;Raynaud, 2018, 30212491}. These genetic alterations can originate and accumulate in all cancer cells (*i.e.*, clonally) or a fraction thereof (*i.e.*, subclonally) in the course of tumor progression and response to therapy{Teixeira, 2019, 30664780;Gerstung, 2020, 32025013}. In this context, clonality (which is a measure of genetic ITH) depends not only on the type and severity of the underlying genetic insult, but also on (1) the genomic region involved, and (2) a balance between intrinsic (*e.g.*, genomic instability) and extrinsic (*e.g.*, clonal competition, immunosurveillance) factors that determine clonal fitness{Salmon, 2019, 30867580}.

The current view is that genetic ITH is crucial for cancer cell proliferation, invasion and resistance to therapy as it confers plasticity to evolving tumors, constituting a fertile soil for evolution{Dagogo-Jack, 2018, 29115304;McGranahan, 2017, 28187284}. Clonal diversity shapes genomic features, including tumor mutational burden (TMB) and karyotypic profile, as well as (in a mutually reciprocal manner) its oncogenic makeup and immunogenicity. Thus, progressing tumors tend to acquire driver mutations that favor at least some degree of genetic instability (*e.g.*, mutations in DNA repair genes), treatment resistance (*e.g.*, PTEN loss){Juric, 2015, 25409150} and immunoescape (*e.g.*, loss of MHC class I genes){Keenan, 2019, 30842677}. Such alterations often arise through convergent evolution, as exemplified by the convergent somatic loss of *PTEN* observed in patients with metastatic breast cancer resisting phosphatidylinositol-3-kinase (PI3K) inhibitors{Juric, 2015, 25409150}, as well as by the loss

of the MHC class I locus in patients with lung carcinomas and others tumors that escape immunosurveillance{Montesion, 2020, 33127846;McGranahan, 2017, 29107330}. As driver mutations generally confer a survival and/or growth advantage, they are positively selected and emerge clonally in most primary cancers{Hu, 2020, 32424352}, although a certain degree of subclonality may intervene along with tumor progression{Jamal-Hanjani, 2017, 28445112;Gerstung, 2020, 32025013;von Loga, 2020, 31949146}. On the contrary, passenger mutations (which do not offer selective advantages) evolve neutrally{Martincorena, 2017, 29056346}, and their branched evolution is considered the main source of clonal diversity in established tumors. In this context, genetic ITH has a major impact on the antigenic landscape of the TME. Non-synonymous mutations and indels in protein-coding genes represent indeed main sources of tumor neoantigens (TNAs), which are important targets for tumor-specific $CD8^+$ cytotoxic T lymphocytes (CTLs){Schumacher, 2019, 30550719}. Of note, in a majority of tumors not only the amount of genetic defects but also their type{Gerstung, 2020, 32025013} changes during disease progression, ultimately resulting in patterns of regional and temporal neoantigen subclonality{Rosenthal, 2019, 30894752}. Moreover, subclonal karyotypic alterations are recurrent in tumors with high levels of chromosomal instability (CIN){Watkins, 2020, 32879494}, exemplifying the relevance of somatic copy number variations (CNVs) for tumor evolution. All these processes have important therapeutic implications, as an elevated clonal diversity negatively affects disease outcome and sensitivity to treatment in numerous oncological settings{Rosenthal, 2019, 30894752}. Indeed, high but tolerable degrees of ITH with respect to somatic mutations and CNVs constitute a negative prognostic factor for patients affected by a variety of solid tumors{Jamal-Hanjani, 2017, 28445112;Morris, 2016, 26840267;Andor, 2016, 26618723}.

Accumulating evidence points to a relevant role for epigenetic processes in ITH. Thus, while normal development epigenetic pathways are key for the establishment and maintenance of cell fate decisions, cancer cells harness epigenetic aberrations to transition easily between cell states {Flavahan, 2017,

28729483}. Temporal shifts in DNA methylation patterns have been detected in progressing lung cancers and recurrent glioblastomas{Teixeira, 2019, 30664780}. Similarly, various epigenetic processes including histone acetylation, chromatin remodeling and gene regulation by non-coding RNAs have been shown to display ITH along the spatial and/or temporal dimension{Flavahan, 2017, 28729483}. Although generally reversible, these epigenetic changes can be inherited by the cell progeny, thus influencing the clonal landscape of the tumor and its evolution{Patten, 2018, 30038216}. Moreover, they can affect cancer proliferation and its immunogenicity by deregulating driver genes and/or genes potentially coding for antigenic entities including TNAs, (latent) viral proteins and tumor-associated antigens (TAAs, which derive from overexpression or ectopic expression of wild-type proteins for which central tolerance is leaky){Ishak, 2018, 30064665}. Consistently, epigenomic ITH in malignant cells has been associated with poor disease outcome in patients with various tumors including acute myeloid leukemia (AML){Li, 2016, 27322744}, Ewing sarcoma{Sheffield, 2017, 28134926} and hepatocellular carcinoma{Lin, 2017, 28302680}.

Ultimately, genetic and epigenetic processes delineate transcriptomic, proteomic and phenotypic patterns that – upon interaction with microenvironmental elements including nutritional and immunological cues – dictate behavioral and immunological ITH{Neftel, 2019, 31327527;Gaiti, 2019, 31092926}. The former influences rather 'canonical' processes involved in disease progression, such as proliferative attitude and invasiveness{Sharma, 2019, 31747591}, while the latter revolves around antigenicity (the feature of cells that can be recognized by the immune system), adjuvanticity (the feature of cells that can prime immune responses) and immunoevasion (the feature of cells that can suppress or evade immunity){Losic, 2020, 31941899}. Thus, genomic and transcriptomic studies in lung and liver carcinoma revealed that tumor regions with differential transcriptional regulation and proliferation rate often exhibited heterogeneous patterns of T-cell infiltration or activation{Sharma, 2019, 31747591;Losic, 2020, 31941899}. Moreover, multiregional single-cell RNA sequencing detected

considerably degree of transcriptional ITH for genes linked to immunity in lung cancer, on both the spatial{Ma, 2019, 30821712} and temporal{Suda, 2018, 29933065} dimensions. Interestingly, some components of ITH in malignant cells such as metabolic ITH appear to influence the interface between behavioral and immunological ITH. As an example, highly glycolytic cancer cells not only can redirect glucose to anabolic reactions in support of proliferation{Zhu, 2019, 30976106}, but also secrete increased amount of lactate, which exerts immunosuppressive effects{Brand, 2016, 27641098}. Metabolic flexibility actually stands out as a major feature of progressing tumors{Xiao, 2019, 31434891}, probably reflecting the multipronged selective advantages conferred by the ability to fine tune metabolism in response to environmental cues. Indeed, a heterogeneous pattern of glucose metabolism has been associated with the preferential usage of different carbon sources including lactate in non-small cell lung cancer (NSCLC){Hensley, 2016, 26853473}. Moreover, metabolic analyses have revealed the co-existence of highly and slowly proliferating cell subpopulations in glioblastoma, which displayed a glycolytic metabolism coupled to chemosensitivity or an oxidative metabolism coupled to chemoresistance, respectively{Hoang-Minh, 2018, 30322894}. Cancer cell subpopulations displaying elevated metabolic heterogeneity (including the ability of employ extracellular lactate as a carbon source) and highly metastatic potential have also been documented in melanoma{Tasdogan, 2020, 31853067}, suggesting that multiple neoplasms can harness metabolic ITH in support of progression. Interestingly, recent studies suggest that metabolic heterogeneity (as those driven by hypoxia in pediatric ependymoma) can drive large-scale epigenetic ITH {Michealraj, 2020, 32445698;Sulkowski, 2020, 32494005}.

Single-cell transcriptomic studies have also demonstrated the existence of hierarchically organized cancer cell subpopulations harnessing an elevated plasticity as a source of behavioral ITH. Thus, developmental analyses have revealed the coexistence of malignant cell subtypes differing for differentiation status and stemness potential within multiple tumors including AML{van Galen, 2019,

30827681}, glioma{Neftel, 2019, 31327527} and medulloblastoma{Ocasio, 2019, 31863004}. The relative abundance of these subpopulations varied remarkably, both across tumor types and cancers of the same type. Moreover, such cancer cell subsets display distinct immunogenic potentials{van Galen, 2019, 30827681} and, at least in glioma, are capable to switch between phenotypic states{Neftel, 2019, 31327527}. Intriguingly, cancer stem cells (CSCs) - the immunoprivileged cell subpopulation that is believed to drive initiation, progression and therapeutic resistance of most human neoplasms – display karyotypic and phenotypic ITH{Bhaduri, 2020, 31901251}, suggesting that at least some degree of ITH may be required for tumor evolution.

In conclusion, the malignant compartment of most tumors displays an elevated genetic, epigenetic, transcriptomic, phenotypic, and metabolic ITH that confers superior plasticity to the tumor system at both the behavioral and immunological level in support to disease progression and resistance to therapy.

Dynamic heterogeneity of immune-stromal patterns

Recently, numerous single cell RNA sequencing and mass cytometry studies have provided in-depth insights into the cellular architecture of various human tumors including Hodgkin's lymphoma{Aoki, 2020, 31857391}, glioma{Müller, 2017, 29262845}, melanoma{Li, 2019, 30595452}, as well as mammary{Azizi, 2018, 29961579;Bartoschek, 2018, 30514914;Costa, 2018, 29455927}, colorectal{Zhang, 2018, 30479382}, pulmonary{Lambrechts, 2018, 29988129}, and renal cell carcinoma{Chevrier, 2017, 28475899}, unveiling not only considerable interpatient variability, but also a high degree of ITH affecting malignant cells as well as TILs, other immune cells of the TME (i.e., DCs, TAMs) and CAFs.

These studies identified various subsets of T cells residing at precise anatomical locations of the TME, in adjacent normal tissues or in the peripheral blood{Zhang, 2018, 30479382;Li, 2019, 30595452}. The main populations of T cells infiltrating solid tumors encompass effector T (T_E) cells (which mediate effector functions against an existing antigenic challenge), resident memory T (T_M) cells (which act as sentinels and can rapidly respond to the re-emergence of a past antigenic challenge), regulatory T (T_{REG}) cells (which mediate immunosuppressive effects), and exhausted T cells (which are dysfunctional due to chronic antigen exposure){van der Leun, 2020, 32024970} (**Figure 3**). In the aforementioned studies, T cells subsets were generally characterized for differentiation status (*e.g.*, naïve *vs.* T_E or T_{REG} cells), proliferative aptitude (*e.g.*, quiescent *vs.* cycling), clonality (*i.e.*, the relative abundance of a specific T cell clone within a T cell subset), expression of effector molecules such as interferon gamma (IFNG) and metabolic profile (*e.g.*, glycolytic *vs.* oxidative). Altogether, these features are normally employed to define the functional properties of a specific T cell, and hence its ability to inhibit or support tumor growth. However, TILs (as well as other immune cells of the TME and CAFs) do not necessarily assume either of two opposed states but rather exist in a continuum of highly plastic functional phenotypes that

contribute to spatiotemporal ITH (**Figure 1**). Thus, it is now clear that the dichotomic distinction between pro-inflammatory M1-like and anti-inflammatory M2-like TAMs is an oversimplification that does not properly reflect the complexity of the TAM compartment{Azizi, 2018, 29961579;Lambrechts, 2018, 29988129}. Likewise, the expression of the co-inhibitory receptor programmed cell death 1 (PDCD1, best known as PD-1) by tumor-infiltrating $CD8^+$ T cells is insufficient to define exhaustion, as it appears to co-exists with a continuum of functional states, including states with (at least some) effector activity{Azizi, 2018, 29961579;Li, 2019, 30595452;Sade-Feldman, 2018, 30388456}. Moreover, even truly dysfunctional T cells have been shown to exhibit elevated degrees of ITH{Miller, 2019, 30778252}, which is relevant as dysfunctional T cells are the most abundant T cell subset in tumors such as melanoma, where they exhibit an unexpectedly high proliferation and clonal expansion rate{Li, 2019, 30595452}. Of note, the spectrum of states in which tumor-infiltrating immune cells exist appears to be relatively conserved, even across different neoplasms from different patients{Gerhard, 2020 #1199;Mantovani, 2017, 28117416}. Conversely, the relative proportion of each cell subset exhibits considerable degrees of heterogeneity{Gerhard, 2020 #1199;Mantovani, 2017, 28117416}, potentially pointing to the balance between different immune cell states as to a major factor in the regulation of anticancer immune responses.

The local distribution, relative abundance and functional orientation of the immune and stromal TME compartments are largely dictated by the evolutionary pressure imposed by microenvironmental features (*e.g.*, local oxygen levels) and malignant cell themselves, including surface proteins such as the PD-1 ligand CD274 (best known as PD-L1){Zou, 2016, 26936508} as well as secreted factors such as the immunosuppressive cytokine transforming growth factor beta 1 (TGFB1){Batlle, 2019, 30995507}. Importantly, all these features can vary considerably across different tumor regions, hence influencing disease progression and response to treatment in a spatially heterogeneous manner{Binnewies, 2018, 29686425}. Thus, while so-called “hot” tumors are generally sensitive to various treatments including

immunotherapy with immune checkpoint inhibitors (ICIs) (**Box 1**) as their TME is enriched in CTLs, their "cold" counterparts exhibit elevated resistance to therapy, reflecting a TME with limited immune infiltration. Similarly, "excluded" tumors are rather insensitive to therapy as CTLs are confined to the periphery of the TME by immunosuppressive signals or a dense stroma. This latter scenario exemplifies well the importance of spatial ITH for cancer progression and response to therapy beyond merely numerical considerations (excluded tumors are indeed confronted by high numbers of CTLs). That said, classifying the immune-stromal patterns of solid tumors based on the hot/cold/excluded triad only also stands out as an oversimplification. Indeed, a large number of immune cell subtypes have been identified, each of which exhibit a distinct functional and spatial landscape and is associated with specific clinical parameters{Thorsson, 2018, 29628290}. As an example, tertiary lymphoid structures (TLSs, which are highly organized, functionally competent aggregates of DCs, B cells and T lymphocytes) have recently emerged as key sites for the initiation of anticancer immunity with a prominent impact on disease outcome, at least in some tumors including sarcoma, melanoma, and renal cell carcinoma{Sautès-Fridman, 2019, 31092904}. Of note, T_{REG} cells also display (at least some degree) of functional heterogeneity linked to spatial ITH{van der Leun, 2020, 32024970;Aoki, 2020, 31857391}. In particular, while some T_{REG} subsets are spatially restricted to zones displaying deficient antigen presentation{Aoki, 2020, 31857391}, others appear to populate tumor regions enriched in CAFs{Costa, 2018, 29455927}.

Importantly, both the immune and stromal compartments of the TME are exquisitely sensitive to microenvironmental conditions, and hence dynamically evolve (naturally and upon treatment) to determine disease outcome{Maynard, 2020, 32822576;Binnewies, 2018, 29686425}. A recent single-cell RNA sequencing study revealed substantial variations in the cellular composition of the TME accompanying the progression from non-invasive dysplasia to invasive pancreatic ductal adenocarcinoma{Bernard, 2019, 30385653}. Such changes encompassed the depletion of CTLs and DCs coupled to the accumulation of immunosuppressive cells such as myeloid-derived suppressor cells

(MDSCs) and specific CAF subsets. Decreased effector functions, limited clonality and increased exhaustion of the T-cell compartment, frequently coupled to T_{REG} cell accumulation and an expanding CAF compartment have also been observed in the TME of various progressing tumors, including mammary{Bartoschek, 2018, 30514914} pulmonary{Mascaux, 2019, 31243362} and pancreatic{Öhlund, 2017, 28232471} carcinomas. Likewise, multiregional sequencing studies have demonstrated considerable spatial heterogeneity in the immune and stromal compartments{Zhang, 2018, 29754820}. For instance, distinct metastatic lesions from the same ovarian cancer patient have been shown to display 'cold' or 'hot' immunological profiles correlating with progression and regression, respectively{Jiménez-Sánchez, 2017, 28841418}. A similar spatial ITH in the immune compartment has been documented across distinct neoplastic lesions or regions from the same lesion in individual patients with hepatocellular carcinomas, high-grade serous ovarian and NSCLC{Losic, 2020, 31941899;Zhang, 2018, 29754820;Rosenthal, 2019, 30894752}. In this setting, the (sub)clonal configuration of specific tumor regions (and thus spatial ITH) appears to be robustly influenced by the functional orientation of the immune system{Zhang, 2018, 29754820}. Reinforcing the importance of spatiotemporal ITH for disease outcome, the number of cold regions or lesions in individual patients appears to inversely correlate with disease control and good prognosis{AbdulJabbar, 2020, 32461698}.

In most of the aforementioned settings, adaptive immunity was operational only in tumor regions displaying an evolving TNA landscape, pointing to clonal evolution dictated by immune cells (immunoediting). Accordingly, multiregional sequencing studies on biopsies from pulmonary{Joshi, 2019, 31591606} and esophageal tumors{Yan, 2019, 30975989} demonstrate that an elevated spatial ITH in both the TIL compartment and TCR repertoire correlates with the local amount of non-synonymous mutations or neoantigens. That said, most tumor-infiltrating $CD8^+$ CTLs display limited (although heterogeneous) reactivity against tumor-derived antigens, suggesting that their impact on tumor growth may originate from bystander effects{Scheper, 2019, 30510250}. Moreover, most of these

studies did not keep the migratory capacity of specific immune subsets{Zhang, 2018, 30479382} and the replenishment of tumor-infiltrating immune cells from the circulation or adjacent normal tissues{Wu, 2020, 32103181} under careful consideration, which is very important for the interpretation of immunological ITH along both the spatial and temporal dimensions.

Spatial ITH in the stromal compartment has also been identified as a factor that influences the impact of the tumor stroma on disease progression and response to treatment{Bartoschek, 2018, 30514914;Zhang, 2018, 29754820}. For instance, an elevated degree of ITH has been documented in tumor endothelial cells (TECs) from lung cancer{Goveia, 2020, 31935371}, delineating not only TEC clusters with "conventional" endothelial functions, but also TEC subpopulations with prominent immunomodulatory activity{Goveia, 2020, 31935371}. Although the temporal ITH of TECs remains fully unexplored, it is tempting to speculate that their first-line position with respect to systemic fluctuations calls for an elevated plasticity, and hence a pronounced capacity to evolve in response to nutritional, metabolic and therapeutic challenges.

Despite this and other unknowns, the immune, stromal (and endothelial) compartment of the TME exhibit a dynamic spatiotemporal heterogeneity in terms of relative abundance, distribution and functional orientation with major influence on disease progression and response to treatment.

Interaction between intrinsic and extrinsic determinants of ITH

ITH originates from multiple sources and is shaped by numerous factors, all of which can vary on the spatial and/or temporal dimension along with disease progression. A major developmental/histological component is operational in this setting, as both cell and tissue of origin ultimately dictate the propensity of a given tumor to diversify as it progresses and responds to therapy{Hoadley, 2018, 29625048}. Moreover, tissue-specific features are key determinants of the immune, stromal and endothelial compartments of the TME{Salmon, 2019, 30867580}. Operating on top of developmental/histological determinants, various genetic and epigenetic defects{Gaiti, 2019, 31092926} as well as environmental factors and therapeutic agents{Wolf, 2019, 31522890;Russo, 2019, 31699882} are major propellers of ITH in both malignant and non-malignant cells.

Genomic instability, an aberrant state of most (if not all) solid tumors (or regions thereof) that renders cancer cells particularly prone to accumulate genetic alterations, is a major source of ITH. Microsatellite instability (MSI) favors the expansion or contraction of short repeated DNA tracts downstream of defects in DNA mismatch repair (MMR){Hause, 2016, 27694933}. MSI has been associated with extreme ITH in multiple neoplasms including gastro-esophageal adenocarcinomas{von Loga, 2020, 31949146} and glioma{Touat, 2020, 32322066}, generally coupled with abundant TIL infiltration and improved prognosis{Hause, 2016, 27694933}. Indeed, MSI-high (MSI-H) tumors often present a hypermutator phenotype, which increases their likelihood of generating TNAs that can be recognized by the immune system{Le, 2015, 26028255}. In line with this notion, experimental induction of MSI in mammary, colorectal and pancreatic mouse cancer cells accrues mutational and neoantigenic load, culminating in limited tumor growth in immunocompetent hosts{Germano, 2017, 29186113}. Interestingly, MSI is directly connected to an epigenetic mark on histone 3 (*i.e.*, K36 trimethylation) that is required for the recruitment of the MMR heterodimeric complex mutS homolog 2 (MSH2)/MSH6 (Ref. {Fang, 2018,

30181289}). Thus, cancer bearing mutations in histone 3 residues that are required for K36 trimethylation (e.g., G34) or defects in SET domain containing 2, histone lysine methyltransferase (SETD2, which catalyzes trimethylation) generally display an elevated mutation frequency and MSI {Fang, 2018, 30181289}. Increased ITH coupled to changes in local immune composition has also been observed in neoplasms with mutations in other DNA repair genes (*e.g.*, *POLD1*, *BRCA2*){Rizvi, 2015, 25765070;Jenzer, 2019, 31549213}, deregulation of cytidine deaminases from the APOBEC family{Roper, 2019, 30840888}, and karyotypic aberrations emerging in the context of CIN{Davoli, 2017, 28104840} (**Box 2**).

Genetic and epigenetic events form the evolutionary substrate for the establishment of ITH, but numerous factors shape it over the spatial and temporal dimensions. Clonal selection is one of such determinants, acting prominently in the early phase of tumor development{Barthel, 2019, 31748746}. Indeed, malignant and non-malignant compartments of the TME constantly compete with each other for nutrients, oxygen and trophic factors, which may dwindle in progressing tumors or regions thereof{Kedia-Mehta, 2019, 31073180}. Localized changes or temporal alterations in other TME factors including vascularization, stiffness, and inflammation also shape ITH{Dagogo-Jack, 2018, 29115304}. Moreover, emerging cancer (sub)clones are subjected to a strong selective pressure from the adaptive immune system, which shapes clonal architecture (and thus genetic and epigenetic ITH) via immunoediting{Zhang, 2018, 29754820;Rosenthal, 2019, 30894752}. In this setting, cancer cells harness a variety of mechanisms to evade recognition and elimination by the immune system (all of which originate from existing ITH and enable disease progression in the context of additional ITH). Such mechanisms include limited antigenicity downstream of repressed antigen expression or defects in the molecular machinery for antigen presentation{Rosenthal, 2019, 30894752;McGranahan, 2017, 29107330} as well as alterations in cancer cell adjuvanticity{Senovilla, 2012, 23019653} and the establishment of a highly immunosuppressive TME{Binnewies, 2018, 29686425}. Of note, antigen loss

appears to occur in specific tumor regions{Zhang, 2018, 29754820} (spatial ITH) as well as in the course of disease progression{Angelova, 2018, 30318143} (temporal ITH), although the relevance of antigenic selection for the progression of untreated tumor remains debated{Van den Eynden, 2019, 31768072}.

Notably, immunoescape can be directly enabled by ITH-promoting genetic alterations in cancer cells. These include mutations in genes controlling (1) genomic stability, such as MYC proto-oncogene, bHLH transcription factor (*MYC*), KRAS proto-oncogene, GTPase (*KRAS*), and tumor protein p53 (*TP53*), whose alteration promotes the expression of co-inhibitory molecules like PD-L1, CTL exclusion and/or infiltration by immunosuppressive cells; (2) metabolic fitness, such as phosphatase and tensin homolog (*PTEN*) and serine/threonine kinase 11 (*STK11*), whose mutation impairs CTL recruitment, expansion and cytotoxicity; and (3) differentiation such as catenin beta 1 (*CTNNB1*), whose hyperactivation results in T-cell exclusion{Spranger, 2018, 29326431}. Likewise, deregulation in epigenetic controllers such as the zeste 2 polycomb repressive complex 2 subunit (*EZH2*) or DNA methyltransferase 1 (*DNMT1*) impairs cancer cell antigenicity (downstream of MHC Class I downregulation){Burr, 2019, 31564637} or adjuvanticity (downstream of inhibited secretion of pro-inflammatory cytokines){Kitajima, 2019, 30297358}.

Finally, ITH evolves over space and time in the context of a complex multidirectional interaction between all TME compartments. For instance, both malignant and myeloid cells can alter the distribution and functional orientation of the immunological TME by exposing or releasing various immunoregulatory factors encompassing danger signals, growth factors, cytokines, and cell surface proteins{Li, 2018, 29958801;Mariathasan, 2018, 29443960}. Similarly, specific CAF subsets can influence the immunological TME both directly, by secreting extracellular matrix components plus pro-fibrotic cytokines (*e.g.*, TGFB1) and hence favor CTL exclusion{Sahai, 2020, 31980749}, or immunosuppressive factors{Costa, 2018, 29455927}, and indirectly, by changing the proliferation

rate{Ligorio, 2019, 31155233}, stemness{Su, 2018, 29395328} and metabolism{Demircioglu, 2020, 32157087} of cancer cells. In this context, cancer cells can also favor CAF heterogeneity through multiple mechanisms, including a paracrine loop centered on TGFB1 (Ref. {Biffi, 2019, 30366930}). Importantly, various signals involved in the spatial and temporal reconfiguration of the TME originate from dying cells, which are frequent in the TME even in the absence of therapeutic challenges{Galluzzi, 2018, 30305710}. The type and abundance of such signals are largely influenced by cytoprotective responses activated by cells coping with microenvironmental stress, as well as by their ultimate outcome (*i.e.*, cell survival vs. cell death){Galluzzi, 2020, 32209603} (**Box 3**).

Taken together, these observations indicate that ITH emerges and evolves via a process involving the balance between multiple developmental, tumor-intrinsic and extrinsic forces, reciprocal influence between TME compartments, and selective pressures from a dynamic microenvironment.

Impact of intratumor heterogeneity on responses to immunotherapy

Ample evidence suggests that ITH increases the likelihood of malignant cells to survive conventional chemotherapy, radiation therapy (RT) and targeted anticancer agents (**Box 4**). Moreover, ITH has a major impact on the efficacy of various immunotherapies, in particular immune checkpoint inhibitors (ICIs){Kalbasi, 2020, 31570880}. MMR-deficient tumors are extraordinarily sensitive to ICIs{Le, 2017, 28596308}, largely reflecting their TNA load and elevated immune infiltration, an observation that supported the first-in-history approval of the PD-1 blocker pembrolizumab for the treatment of MSI-H tumors irrespective of histological derivation{Lemery, 2017, 29020592}. Nevertheless, not all MMR-deficient tumors respond to ICIs, and acquired resistance mechanisms can develop{Le, 2017, 28596308}. Indeed, both the degree of MSI and CTL infiltration appear to be heterogeneous in MMR-deficient tumors{Mandal, 2019, 31048490}, resulting in regions with limited antigenicity and scarce immune control that may support resistance. Alongside, the hypermutated phenotype of MMR-deficient tumors confers an elevated plasticity to the antigenic profile{von Loga, 2020, 31949146;Wolf, 2019, 31522890}, *de facto* increasing the probability that poorly antigenic cancer cell clones emerge despite active ICI-driven immunity{Miao, 2018, 30150660}. Other ITH-related variables influencing the response of MSI-H tumors to ICIs include their tendency to undergo robust immunoediting{Grasso, 2018, 29510987} and to switch to a glycolytic profile{Vasaikar, 2019, 31031003} during progression, which altogether favor immunoevasion. Moreover, recent data suggests that tumor-infiltrating CTLs may be difficult to reinvigorate by PD-1 blockers{Yost, 2019, 31359002}, and that superior sensitivity to ICIs may rely on a small set of novel T-cell clones that expand in the periphery{Yost, 2019, 31359002; Wu, 2020, 32103181}. Thus, an elevated ITH in the intratumoral CTL compartment may not necessarily support the clinical efficacy of ICIs.

Besides influencing ICI efficacy, ITH also confounds clinically used predictors of disease outcome. The TMB globally predicts the efficacy of ICI-based immunotherapy in multiple cancer types{Samstein, 2019, 30643254;Hellmann, 2018, 29657128}, but tumors with an elevated TMB display heterogeneous responsiveness, and some cancers with limited TMB are efficiently controlled by ICIs{Miao, 2018, 30150660}. Recently, an excessive boost in TMB (and hence genetic ITH) has been linked to decreased melanoma immunosurveillance and response to immunotherapy, at least partially owing to clonal divergence{Wolf, 2019, 31522890}. In this context, the predictive value of TNA load is influenced by ITH-related factors beyond TNA clonality, including the type, incidence and sources of TNA-generating mutations{Turajlic, 2017, 28694034;Grasso, 2018, 29510987} and TNA quality{Balachandran, 2017, 29132146}, which is also influenced by MHC haplotype and regional expression levels{Alspach, 2019, 31645760;Chowell, 2018, 29217585}. Of note, ICI-unresponsive tumors with high TMB often present defects in antigen presentation{Sade-Feldman, 2017, 29070816;Chowell, 2018, 29217585;McGranahan, 2017, 29107330}. Additional TMB-counteracting forces linked to ITH include elevated degrees of aneuploidy and consequent CTL exclusion{Davoli, 2017, 28104840}, epigenetic alterations and chromatin remodeling that control the expression immune genes{Miao, 2018, 29301960}, stemness-related immunoevasion{Miao, 2019, 31031009}, and metabolic profiles linked to poor antigen presentation and inhibited immune effector functions{Harel, 2019, 31495571}.

PD-L1 levels in diagnostic biopsies are also commonly employed to predict ICI sensitivity in patients with various tumors{Reck, 2016, 27718847}, but PD-L1 expression displays a considerable degree of spatial and temporal ITH{Yan, 2019, 30975989} caused by the heterogenous deregulation of IFNG signal transducers like Janus kinase 1 (JAK1) and JAK2 (Ref. {Zaretsky, 2016, 27433843}). Such an ITH may explain why a fraction of patients with PD-L1^{+} tumors fail to respond to ICIs and some individuals with PD-L1^{-} neoplasms robustly do so{Keenan, 2019, 30842677}. ITH also influences the prognostic value of TILs, as documented in a variety of tumors{Tumeh, 2014, 25428505;Van Allen,

2015, 26359337}. The TIL compartment is indeed intrinsically heterogeneous, varying in composition, distribution, clonality and functional orientation across tumor types, stages, and regions{AbdulJabbar, 2020, 32461698}. Thus, the clinical activity of ICIs may not only depend on abundant CTL infiltration{Cristescu, 2018, 30309915;Mariathasan, 2018, 29443960} and localization at the tumor-invasive margin{Tumeh, 2014, 25428505}, but also on the relative abundance of specific CTL population including (but not limited to) distinct memory-like{Siddiqui, 2019, 30635237;Sade-Feldman, 2018, 30388456} or exhausted{Miller, 2019, 30778252} T cells. Moreover, emerging data suggest that various TME compartments including $CD4^+$ T cells, B cells, TAMs, TLSs, DCs, CAFs and endothelial cells influence the clinical efficacy of ICIs{Oh, 2020, 32497499;Hollern, 2019, 31730857}. Thus, additional studies clarifying the precise contribution of these compartments and their ITH to the efficacy of immunotherapy in cancer patients are urgently awaited.

Importantly, ICI has a direct impact on temporal ITH, with specific changes predicting the extent and durability of antitumor responses. In particular, MSI-H tumors responding to ICIs often display signs of early immunoediting including a longitudinal decrease in TMB and TNA load{Mandal, 2019, 31048490;Riaz, 2017, 29033130}, which has been ascribed to TNA selection including loss of subclonal TNAs. Conversely, defects in antigen presentation and interferon signaling emerging in the course of ICI-based immunotherapy have been associated with poor disease outcome in patients with melanoma, as well as colorectal and lung cancer{Zaretsky, 2016, 27433843;Sade-Feldman, 2017, 29070816;Le, 2017, 28596308}. Moreover, ICI-responsive tumors display an extensive transcriptomic remodeling coupled to a functional rewiring of the TIL compartment toward cytotoxicity{Riaz, 2017, 29033130}, lending further support to the notion that the efficacy of ICIs involves the reinvigoration pre-existing $CD8^+$ T cell immunity{Ribas, 2018, 29567705}. However, the dynamics of effector responses driven by ICIs, their ITH, and their specific targets remain debated. In this context, the hypothesis that PD-1 blockers reactivate terminally-differentiated exhausted CTLs has been argued by evidence documenting

the expansion of tumor-resident exhausted T cell progenitors{Miller, 2019, 30778252} and stem-like memory $CD8^+$ T cells{Sade-Feldman, 2018, 30388456;Siddiqui, 2019, 30635237}, as well as a process of clonal replacement of specific T-cell subtypes{Wu, 2020, 32103181}. Finally, ICIs influence T_{REG} cells{Kamada, 2019, 31028147} and remodel the myeloid TME compartment as they reinvigorate DCs{Mayoux, 2020, 32161104} and recruit macrophages that acquire heterogeneous phenotypic and behavioral states{Gubin, 2018, 30343900}.

In conclusion, both spatial and temporal ITH have a major influence on disease outcome in cancer patients receiving (immuno)therapy, suggesting that strategies targeting ITH may constitute valid partners for the development of novel combinatorial regimens with improved efficacy.

Therapeutic strategies to target intratumoral heterogeneity

As discussed above, ITH offers a means to developing tumors for escaping immunosurveillance and resisting therapy, thus constituting a promising target for treatment (**Table 1**). One potential approach in this sense relies on targeting ITH at its sources, based on two opposite rationales: (1) an excessive boost in ITH may prove intolerable for cancer cell fitness, and (2) a decrease in ITH may (re)instate natural immunosurveillance as well as sensitivity to conventional treatments or immunotherapy{Marusyk, 2020, 32289271; Vitale, 2015,28741522}. The former approach may be preferable for tumors that are highly heterogenous at baseline (i.e., exhibit gross karyotypic alterations), as these have previously been shown to be intolerant to extra degrees of ITH{Andor, 2017, 28432052;Sansregret, 2018, 29297505}. Thus, various agents targeting components of the molecular machinery for mitosis, such as the kinase monopolar spindle 1 (MSP1){Jemaa, 2013, 23933817}, have been shown to promote cancer cell death as a consequence of grossly exacerbated CIN. Conversely, decreasing ITH may be preferable for tumors with limited ITH at baseline, and can involve either pharmacological inhibitors of ITH-promoting processes, such as epigenetic regulators{Jones, 2019, 30723290}, or the targeted depletion of ITH-promoting cell subpopulations including (but not limited to) tumor- or metastasis-initiating cells as well as cancer cell subsets with a glycolytic profile{Cascone, 2018, 29628419;Renner, 2019, 31577944}. Among other caveats, the specificity of these approaches is limited, which is particularly relevant for TILs, whose phenotypic profile, functional orientation and effector functions are highly dependent on clonal architecture, nutrient availability, genetic stability and epigenetic regulation{Andrejeva, 2017, 28683294}.

Several immunological approaches can be conceived to target ITH, ideally in combination with strategies for remodeling an exhausted, immunosuppressive and/or fibrotic TME. One such approaches relies on the relatively unselective cytolysis or oncolysis of cancer cells with pharmacological agents or viruses

that drive immunogenic cell death (ICD), a particularly immunostimulatory variant of cell death{Galluzzi, 2020, 32209603}. In this setting, dying cancer cells release abundant amounts of antigens, danger signals and cytokines within the TME, potentially resulting in the activation of potent, multi-epitope immune response that overcome (at least some degree of) ITH. Oncolytic viruses (OVs) may be particularly effective against tumors with high ITH because of their rather unspecific tropism for cancer cells, which relies on entities that normally develop limited ITH (*e.g.*, surface receptors for viral entry that are not under selective pressure){Bommareddy, 2018, 29743717}. Viral oncolysis culminates with robust waves of ICD coupled to the potential establishment of a TME permissive for (immuno)therapy{Gujar, 2018, 29275092}. However, the efficacy of OVs as standalone therapeutics is limited, and combinatorial regimens involving ICIs appear to be required for effective tumor eradication. Additional limitations of the oncoviral platform are related to the identification of precise schedules that (at least initially) enable robust viral replication (which involves some degree of immunosuppression) coupled to subsequent anticancer immunity{Bommareddy, 2018, 29743717}. A similar lytic strategy can be achieved with cationic ampholytic peptides that preferentially kill cancer cells based on poorly specific surface properties (*e.g.*, electrical charge){Kepp, 2020, 31595049}. In preclinical studies, these agents were shown to potently induce ICD initiating at the mitochondrial and reticular compartments{Zhou, 2016, 26962684}. Peptide-mediated oncolysis results in abundant antigen release, danger signaling and initiation of CTL-mediated anticancer immunity despite elevated ITH{Yamazaki, 2016, 27082453}. Likewise, RT can be harnessed to initiate anticancer immunity downstream of ICD activation{Yamazaki, 2020, 32747819}, especially in cancer with high ITH (which normally display defects in DNA repair){Burrell, 2013, 24048066}. However, RT can also exert a variety of immunosuppressive effects including latent TGFB1 activation, and not all TME compartments exhibit the same radiosensitivity, implying that radiation therapy may actually foster, rather than limit, ITH (especially in the non-malignant TME){Rodriguez-Ruiz, 2020, 31873291}. Of note, both oncolytic

peptides and radiotherapy are particularly effective when combined with ICIs (at least in preclinical tumor models), underscoring the fact that robust therapeutic responses generally involve the reconfiguration of the entire TME towards anticancer immunity.

At least theoretically, a second strategy to target ITH involves T cells specific for antigens that are invariably homogeneous, such as fully clonal TNAs shared by the entire malignant compartment{Schumacher, 2019, 30550719}. This approach involves an in-depth, multiregional profiling of the TNA landscape coupled to the engineering or selection of patient-derived T cells with TCRs specific for a clonal TNA{Yamamoto, 2019, 31591590}. Besides cost and feasibility (some tumors may not express a targetable clonal TNAs), one major limitation of this approach reflects temporal ITH coupled to TNA loss. This issue may be bypassed, at least in part, by targeting (1) TNAs encoded by hot-spot mutations in driver genes or oncogenic fusions (which generally cannot be lost as they underlie tumor survival or progression) or (2) TAAs homogeneously expressed by cancer cells (even though TAAs are generally poor initiators of anticancer immunity as compared to TNAs){Weber, 2020, 32243795;Yamamoto, 2019, 31591590}. Other potential obstacles include the emergence of T cell exhaustion, an immunosuppressive or fibrotic TME, as well as the intrinsic resistance of tumors with defects in cell death signaling or antigen presentation.

Antigen presentation defects can be circumvented with chimeric antigen receptors (CARs){June, 2018, 29972754} or bispecific T-cell engagers (BiTEs){Slaney, 2018, 30012854} specific for surface tumor antigens, both of which enable CTL activity in the absence of antigen presentation by MHC molecules. Despite the regulatory approval of CAR-expressing T cells specific for CD19 (a common B-cell antigen) for the therapy of some B-cell malignancies, the CAR T cell platform is still poorly effective against solid tumors, requires considerable antigen density, and causes toxicity linked to target expression by normal tissues, cytokine release syndrome, or immunological neurotoxicity{Majzner, 2019, 31501612}.

Finally, antigen loss represents a prominent cause of resistance to CAR T cells, and instances of exhaustion have also been described{O'Rourke, 2017, 28724573}. Interestingly, low-dose RT reportedly elicits antigen-independent killing by CAR T cells upon upregulation of death receptors on malignant cells{DeSelm, 2018, 30415658}, which stands out as a promising strategy to target ITH downstream of antigen loss.

An alternative MHC-unrestricted strategy to target ITH involves unconventional lymphoid cells that recognize non-peptide antigens (*e.g.*, lipids, small metabolites), presented by monomorphic antigen-presenting molecules such as CD1 (which is recognized by γδ T cells) and MR1 (which is recognized by mucosal-associated invariant T cells){Godfrey, 2015, 26482978}. Notably, various cancer cells present such molecules on their surface. Moreover, an MR1-restricted T cell population recognizing hitherto unidentified ligands homogeneously expressed by malignant cells but not their healthy counterparts has recently been documented{Crowther, 2020, 31959982}. In this setting, MR1-restricted T cells specifically killed cancer cells irrespective of tissue of origin and MHC haplotype{Crowther, 2020, 31959982}. However, MR1 resembles conventional MHC Class I molecules in requiring beta 2 microglobulin (B2M) for presentation{Yamaguchi, 2002, 11785959}. Thus, MR1-restricted T cells may not be active against neoplasms with loss-of-heterozygosity at the *B2M* locus, which is a common mechanism through which tumors with elevated ITH evade immunosurveillance{Sade-Feldman, 2017, 29070816}. Moreover, whether cancer cell killing by MR1-restricted T cells results in long-term immunological memory remains to be elucidated. Solving this and other unknowns about MR1-dependent presentation will shed light on the true potential of MR1-restricted T cells as a tool to target ITH. Finally, a potential strategy to target ITH relies on the redirection of genetically-modified T cells against multiple clonal and/or subclonal TNAs or TAAs{Weber, 2020, 32243795}, which is likely to minimize the ability of heterogenous tumors to evade recognition upon antigen loss. However, genetic defects compromising the sensitivity of cancer cells to immune effectors (*e.g.*, *CASP8* or *JAK2*

mutations), or enabling local immunosuppression (*e.g.*, PD-L1 upregulation) may still offset therapeutic efficacy.

Altogether, these observations support the potential of harnessing ITH as a target for the development of novel, effective anticancer (immuno)therapies.

Concluding remarks

Abundant evidence demonstrates that an elevated ITH negatively influences clinical responses to a variety of treatments including immunotherapy, placing ITH in a preferential position as a target for the development of novel combinatorial regimens. However, while targeting spatial ITH in the malignant compartment of the TME may be feasible (at least theoretically), temporal ITH may emerge (unless tumors are fully eradicated) and drive aggressive disease progression in the context of resistance to therapy. Moreover, most neoplasms generally develop and respond to therapy in a context of a multidirectional relationship among and within malignant and non-malignant TME compartments{McGranahan, 2017, 28187284}, which may offer unexpected mechanisms to preserve plasticity despite therapeutic regimens. Elucidating the sources of ITH in all TME compartments and the key spatiotemporal features of ITH dynamics is paramount for the development of effective treatments for neoplasms with high ITH.

Major progress in this context has been achieved by the characterization (and therapeutic exploitation) of (some) molecular mechanisms underlying the unique phenotypic and functional plasticity of the TME. These pathways include (but are not limited to) complex signaling networks governing the functional orientation of immune and stromal cells, which are generally hijacked by malignant cells to obtain nutritional support, elude natural immunosurveillance, and resist to (immune)therapy{Sahai, 2020, 31980749;DeNardo, 2019, 30718830}. Importantly, most of these processes are regulated by epigenetic mechanisms, and hence may potentially be reverted for therapeutic purposes{Albrengues, 2015, 26667266;Jones, 2019, 30723290}. Moreover, it seems that the malignant and non-malignant compartments of the TME differ with respect to metabolic ITH and plasticity, potentially opening up a window for therapeutic interventions{Xiao, 2019, 31434891;Renner, 2019, 31577944;Cascone, 2018, 29628419}.

It is now clear that all (immuno)therapies have a major impact on spatial and temporal ITH, which in turn influences the degree and durability of responses. Precisely understanding the patterns of spatial and temporal ITH in the microenvironment of neoplasms responding or resisting to treatment may enable not only the identification of predictive biomarkers, but also the characterization of novel therapeutic targets. Using ICI-based immunotherapy as an example, it will be important to elucidate the precise impact of ICIs on immune cell subsets other than CTLs (*e.g.*, unconventional T cells), as well as the relevance of hitherto poorly understood processes (*e.g.*, T cell rejuvenation, clonal replacement), or structures (*e.g.*, TLSs){Oh, 2020, 32497499}.

In view of all these considerations, we believe that efforts should be made to rethink cancer therapy to account not only for the composition but also for the heterogeneity and plasticity of the tumor ecosystem, as well as for the interactions that exist between and among TME compartments and how they evolve during cancer progression and response to therapy.

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Table 1. Therapeutic strategies to kill tumors with elevated intratumoral heterogeneity.

Strategy	Methods	Limitations	Notes	Ref
ITH level modulation	Boosting ITH to intolerable levels	• Restricted to neoplasms relying on ITH for progression or resistance • Restricted to neoplasms with constitutively high ITH • Limited by the presence of ITH in specific regions or stages • Potential development of acquired resistance due to plasticity of cancer cells with high ITH • Limited specificity	• Can be particularly effective against tumors with high genomic or chromosomal instability • Increased ITH may lead to immunoediting and evasion	{Andor, 2017, 28432052;Sansregret, 2018, 29297505}
	Inhibiting ITH-promoting processes or depleting ITH-promoting cell subpopulations	• Restricted to neoplasms relying on ITH for progression or resistance • Potentially ineffective on tumors with high ITH • Limited by the presence of ITH in specific tumor regions or stages • Potential development of acquired resistance due to plasticity of cancer cells with high ITH • Limited specificity	• Can be effective against tumors with epigenetic ITH using pharmacological inhibitors of epigenetic regulators • Potential application against tumor- or metastasis-initiating cells as well as cancer cell subsets with a highly glycolytic profile	{Jones, 2019, 30723290;Cascone, 2018, 29628419;Renner, 2019, 31577944}
Antigen spreading	ICD induction with oncolytic viruses	• Difficulties in identifying precise treatment schedules • Potential immunosuppression due to viral replication • Viral clearance • Presence of neutralizing antiviral antibodies • Limited antitumor activity as monotherapy	• Can be effective against tumors with high ITH due to rather unspecific tropism • Antigen spreading is linked to the establishment of a TME permissive for (immuno)therapy • High molecular flexibility of the viral platform • Some oncolytic viruses have already entered the clinical practice	{Bommareddy, 2018, 29743717;Gujar, 2018, 29275092}
	ICD induction with oncolytic peptides or radiation therapy	• Immunosuppressive effects (*e.g.*, latent TGFB1 activation) • Limited specificity • Distinct sensitivity of TME compartments • Potential increase in ITH in non-malignant TME compartments	• Recent technical progress has improved the precision by which ionizing radiation can be specifically delivered to tumor • Ideal for combination with immune checkpoint inhibitors	{Kepp, 2020, 31595049;Yamazaki, 2016, 27082453;Burrell, 2013, 24048066;Rodriguez-Ruiz, 2020, 31873291}

Homogeneous antigen targeting	TCR engineered T-cells against clonal TNAs or TAAs	• Elevated cost • Long experimental set-up and procedures • Fully clonal TNAs/TAAs are rare • Poor immunogenicity of TAAs coupled to toxicity linked to TAA expression by normal tissues • Potential TNA/TAA loss over time • Engineered T-cell exhaustion • Establishment of immunosuppressive or fibrotic TME • Restricted to tumors with proficient antigen presentation and cell death signaling	• Antigen loss can be limited by targeting TNAs encoded by hot-spot mutations in driver genes or oncogenic fusions	{Schumacher, 2019, 30550719;Yamamoto, 2019, 31591590;Weber, 2020, 32243795}
	CAR T cells and BiTEs	• Limited effectiveness against solid tumors • Requirement of high antigen density • Toxicity linked to target expression by normal tissues • Other immune-related toxic effect (e.g., cytokine release syndrome) • Potential target loss over time • Engineered T-cell exhaustion • Establishment of immunosuppressive or fibrotic TME	• High molecular flexibility allows for the improvement of potency, persistence and safety (e.g., "armored" CAR T cells) • Low-dose RT may be employed to limit resistance upon antigen escape	{DeSelm, 2018, 30415658;June, 2018, 29972754;Majzner, 2019, 31501612;O'Rourke, 2017, 28724573;Slaney, 2018, 30012854}
	Tumor-specific unconventional lymphoid cells	• Demonstrated only for MR1-restricted T cells • Ligands not identified • Ineffective against B2M-deficient tumors • No evidence of long-term immunological memory • Establishment of immunosuppressive or fibrotic TME	• Unconventional lymphoid cells usually recognize non-peptide antigens (e.g., lipids, small metabolites), presented by monomorphic antigen-presenting molecules	{Godfrey, 2015, 26482978;Crowther, 2020, 31959982;Yamaguchi, 2002, 11785959}
Multi-antigen targeting	T cells targeting multiple clonal and/or subclonal TNAs or TAAs	• Limited effectiveness against solid tumors • Requirement of high antigen density • Toxicity linked to target expression by normal tissues • Engineered T-cell exhaustion • Establishment of immunosuppressive or fibrotic TME	• Can be performed with TCR- or CAR-expressing T cells • Possible use of multiple monospecific T cell clones, or a single T cell clone encoding receptors for distinct antigens (e.g., dual- or multi-targeted CAR T cells)	{Weber, 2020, 32243795}

	• Restricted to tumors with proficient antigen presentation and cell death signaling	

Abbreviations. B2M, beta 2 microglobulin; CAR, chimeric antigen receptor; ITH, intratumor heterogeneity; MR1, major histocompatibility complex, class I-related; RT, radiation therapy; TAA, tumor-associated antigen; TCR, T cell receptor; TME, tumor microenvironment; TGFB1, transforming growth factor beta 1; TNA, tumor neoantigen.

Box 1. Immune checkpoint inhibitors.

The term immune checkpoint inhibitors (ICIs) refers to a group of modern, antibody-based immunotherapeutic agents that target co-inhibitory receptors expressed on lymphoid cells, including (but not limited to) $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+CD25^+FOXP3^+$ regulatory T (T_{REG}) cells. As of today, no less than six distinct ICIs have been approved by the US Food and Drug Administration (FDA) and equivalent agencies worldwide for use in dozens of oncological indications, encompassing agents specific for cytotoxic T lymphocyte-associated protein 4 (CTLA4), such as ipilimumab, programmed cell death 1 (PDCD1, best known as PD-1), such as nivolumab and pembrolizumab, and the main PD-1 ligand CD274 (best known as PD-L1), such as durvalumab, atezolizumab and avelumab. Moreover, these as well as other, hitherto experimental, ICIs are being investigated – as standalone immunotherapeutics or in combination with a variety of clinical regimens – in hundreds of clinical trials for efficacy in hitherto immunoresistant tumors. Although the precise mechanism of action of ICIs remains to be fully elucidated, it seems that PD-1- and PD-L1-targeting agents mostly enhance the effector phase of anticancer immunity, at least in part by preventing (at least some degree of) or reverting (at least to some degree) functional exhaustion in tumor-infiltrating CTLs. Conversely, CTLA4 blockers appear to mostly boost the priming phase of anticancer immunity, possibly via a compound effect that also involved the depletion of T_{REG} cells. That said, recent findings indicate that the clinical efficacy of ICIs may involve other immune cell populations, including not only type I helper (T_H1) $CD4^+$ T cells and natural killer (NK) cells, but also dendritic cells (DCs) and tumor-associated macrophages (TAMs). Moreover, it appears that combinatorial immunotherapeutic regimens involving more than one ICI elicit immunological effects that cannot be simply accounted for by an additive activity from each individual ICI. Despite these and other unknowns, ICIs have literally revolutionized the clinical management of patients with a variety of neoplasms, including (but not limited

to) melanoma, non-small cell lung carcinoma (NSCLC) and urothelial carcinoma. However, only a relatively limited (<20% in most cases) fraction of patients respond to ICI-based immunotherapy, and intratumoral heterogeneity (ITH) has been linked to ICI resistance in multiple oncological settings (as discussed in the main text). These observations highlight the potential of ITH not only as potential target for the development of combinatorial immunotherapeutic regimens with superior efficacy, but also as a predictive biomarker of response to ICIs (which is particularly relevant given the cost and side effects of the latter){Sharma, 2020, 31925406;Galluzzi, 2018, 30232229}.

Box 2. Chromosomal instability and intratumor heterogeneity.

Chromosomal instability (CIN) is a form of genomic instability that favors the accumulation of gross karyotypic alterations due to frequent errors in sister chromatid segregation or global mitosis regulation{Sansregret, 2018, 29297505}. CIN occurs over relatively extended periods of tumor progression in most cancer types{Gerstung, 2020, 32025013}, *de facto* acting as a major disease driver{Teixeira, 2019, 30664780}. CIN boosts both genomic and transcriptomic ITH as it frequently reshuffles the overall karyotypic configuration of malignant cells. However, a recent study has revealed that extreme genetic ITH does not correlate with CIN across a variety of cancers{Raynaud, 2018, 30212491}. At least in part, this observation can be explained by the fact that CIN can have extreme consequences for cellular homeostasis, and hence can be tolerated only up to a specific threshold. Thus, while tolerable degrees of CIN foster tumor progression by providing the malignant compartment with an elevated ITH as a substrate for evolution within the TME, excessive CIN is negatively selected by compromising cancer cell fitness. In line with this notion, tolerable CIN levels coupled to elevated sub-clonality in the malignant compartment have been associated with very poor disease outcome in patients with various neoplasms{Jamal-Hanjani, 2017, 28445112}. CIN also has a major impact on cancer cell immunogenicity. Indeed, aneuploidy (which is invariably generated by CIN) has been consistently associated with limited immune infiltration{Davoli, 2017, 28104840}. In this context, whole-genome duplication (WGD) events operate as key drivers of ITH via CIN, at least in part as they increase the ability of cancer cells to tolerate CIN{Sansregret, 2018, 29297505}. That said, the relevance of WGD for tumorigenesis and ITH vary depending on tumor type, as the inferred frequency of WGD across tumors range from 10% to 80%, in a context in which WGD can be either an early or a late event during disease progression{Gerstung, 2020, 32025013;Jamal-Hanjani, 2017, 28445112}.

Box 3. Links between cellular adaptation to stress and intratumoral heterogeneity.

All compartments of the tumor microenvironment (TME) are continuously exposed to microenvironmental perturbations that initiate cytoprotective mechanisms aimed at the restoration of cellular homeostasis, such as the DNA damage response, the unfolded protein response and autophagy. When microenvironmental perturbations are mild and/or restricted in time, these and other evolutionary conserved mechanisms endow cells with the capacity to cope with stress and ultimately resume their baseline activity. Conversely, in the presence of severe and/or prolonged stressful conditions, adaptative response fail, and cells undergo one of two terminal fates, *i.e.*, regulated cell death or cellular senescence. Of note, the adaptative capacity of each cell, and hence its likelihood to survive stress, is determined by a variety of cell-intrinsic (e.g., mutations, epigenetic alterations) and extrinsic (e.g., oxygen tension, glucose availability) factors, and hence varies across different tumors as well as different areas of the same neoplasm. Moreover, irrespective of whether adaptative responses to stress are successful (and hence support cell survival) or not (and hence culminate with regulated cell death or cellular senescence), virtually all cytoprotective mechanisms are tightly connected to the regulation of microenvironmental homeostasis. Indeed, cells mounting adaptative responses to stress expose or release a variety of bioactive factors that *de facto* operate as intratumoral heterogeneity (ITH) modifiers. For instance, cancer cells coping with DNA damage expose killer cell lectin like receptor K1 (KLRK1, best known as NKG2D) on their membrane, which facilitates their elimination by natural killer (NK) cells, and secrete abundant levels of type I interferon (IFN), which promotes multipronged immunostimulatory effects. Similarly, cancer cells unsuccessfully attempting to cope with some chemotherapeutic agents or radiation therapy through autophagy release elevated amounts of ATP, which *per se* mediates immunostimulatory and chemotactic effects, but can rapidly be converted into adenosine by extracellular nucleotidases, and hence promote local immunosuppression. Since these nucleotidases are often abundantly expressed by

malignant and myeloid components of the TME, the gradient of ATP release by cancer cells undergoing immunogenic cell death (ICD) is rather steep, resulting in highly localized effects that promote ITH. Similar considerations apply to a variety of signals emitted by malignant and non-malignant cells coping with microenvironmental perturbations, highlighting the intimate interconnection between cellular adaptation to stress and both spatial and temporal ITH{Galluzzi, 2018, 30305710}

Box 4. Impact of intratumoral heterogeneity on clinical responses to conventional anticancer therapy.

A large body of evidence indicates that an elevated degree of spatial and temporal intratumoral heterogeneity (ITH) supports the resistance of neoplastic cells to conventional chemotherapy, radiation therapy and targeted anticancer agents{Dagogo-Jack, 2018, 29115304}. Indeed, the (epi)genetic, phenotypic and functional components of ITH strongly promote clonal diversification, hence increasing the chances for the emergence (and selection) of a treatment-resistant clone. This applies (but is not limited) to intralesional ITH in (1) the mutational frequency or expression levels of (actionable) target genes, as exemplified by the limited response of tumors with subclonal mutations in KRAS proto-oncogene, GTPase *(KRAS)* or epidermal growth factor receptor (*EGFR*) or with heterogeneous erb-b2 receptor tyrosine kinase 2 (*ERBB2*, best known as HER2) copy number amplification to targeted therapies; (2) the functionality of cytoprotective mechanisms triggered or inhibited by conventional therapeutics, as exemplified by the inverse correlation between ITH in the DNA damage response and therapeutic efficacy of genotoxic agents (*e.g.*, ionizing radiation or temozolomide); and (3) global metabolic profile and proliferative potential, resulting in cancer cell subpopulations differing on vulnerability to a wide range of conventional therapeutics{Quintanal-Villalonga, 2020, 32152485;Dagogo-Jack, 2018, 29115304}. In these settings, the selective pressure imposed by treatment promotes the expansion of resistant subclones, ultimately leading to therapeutic failure and disease recurrence. These subclones can pre-exist in untreated tumors (ITH-driven intrinsic resistance) or appear during treatment (ITH-driven acquired resistance). In both settings, the elevated plasticity conferred to cancer cells by ITH stands out as a major determinant for resistance, favoring the genetic or epigenetic escape mechanisms including the target downregulation, secondary mutations and signaling pathway bypass, which have been linked with resistance to targeted anticancer agents including ALK receptor

tyrosine kinase (ALK), B-Raf proto-oncogene, serine/threonine kinase (BRAF), EGFR, PARP1 or phosphatidylinositol-3-kinase (PI3K) inhibitors. Plasticity is also linked to (1) global transcriptomic rewiring, dictating (as an example) the resistance of breast cancer patients to standard taxane plus anthracycline-based chemotherapy{Kim, 2018, 29681456}, (2) considerable cellular reprogramming culminating in the generation of treatment-resistant stem-like cells, as shown in basal cell carcinomas and melanomas surviving resisting to Sonic Hedgehog (SHH) and mitogen-activated protein kinase (MAPK) inhibition, respectively{Quintanal-Villalonga, 2020, 32152485}; and (3) increased genomic instability resulting in the acquisition of a hypermutator phenotype, as reported in gliomas and colorectal carcinomas resisting temozolomide and EGFR inhibitors{Touat, 2020, 32322066;Russo, 2019, 31699882}.

Legends to Figures

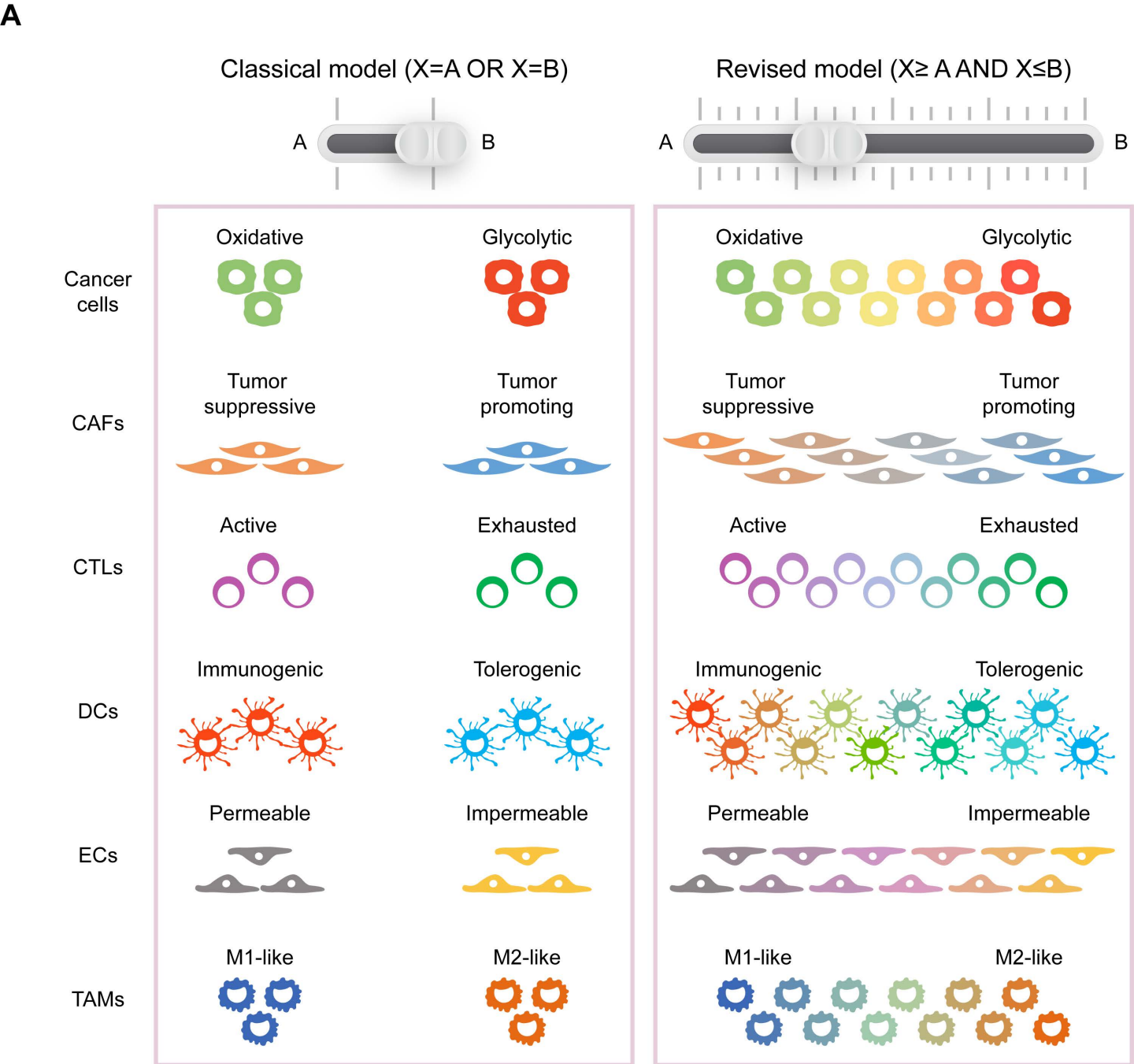
Figure 1. **Principles governing intratumoral heterogeneity.** Intratumoral heterogeneity (ITH) is governed by two main principles. **A**. At odds with previous models, each cellular compartment of the tumor microenvironment (TME) does not exist in two mutually exclusive states, but rather can assume a spectrum of phenotypic, metabolic and behavioral states spanning across two extremes. **B**. At least some degree of ITH is required for the spatial and temporal evolution of the TME as an ecosystem encompassing several, mutually interacting components and exposed to physical, nutritional, and therapeutic perturbations. However, ITH can only be tolerated below a specific threshold, as an excessive genetic, epigenetic, phenotypic or behavioral drift would compromise cell survival and/or the architecture of the entire tumor. CAF, cancer-associated fibroblast; CTL, cytotoxic T lymphocyte; DC, dendritic cell; EC, endothelial cell.

Figure 2. **Spatial and temporal intratumoral heterogeneity in malignant cells.** The relative abundance of specific cancer cell clones in distinct regions of the tumor microenvironment can exhibit considerable variability, delineating areas with relatively high or low intratumoral heterogeneity (ITH). In response to microenvironmental perturbations including therapeutic challenges, ITH further varies as per the evolutive advantage display by some cancer cell clones over others. In some settings, the selective pressure from treatment alters the relative abundance of specific cancer cell clones but overall does not influence the regional degree of ITH. In other scenarios, ITH can be greatly amplified (or reduced, not exemplified) by therapy. Micrographs of hematoxylin/eosin-stained biopsies from a thymic carcinoma before and after treatment were courtesy of Giorgio Inghirami (Weill Cornell Medical College, New York, NY, US)

Figure 3. **Intratumoral heterogeneity in the lymphoid compartment.** Single cell RNA sequencing (scRNAseq) analyses of endogenous mouse mammary carcinomas developing in five female C57BL/6

mice as described in Ref. {Buque, 2020, 32732875} exemplify the considerable degree of heterogeneity that characterizes the $CD45^{+}CD3^{+}$ lymphoid compartment of tumors of the same type evolving in different hosts. UMAPs were courtesy of Kenneth Gouin and Simon Knott (Cedars Sinai, Los Angeles, CA, US). T_H1, type 1 helper; T_{REG}, regulatory T.

References


A
Classical model (X=A OR X=B)
Revised model (X≥ A AND X≤B)
A
B
A
B
Cancer cells
CAFs
CTLs
DCs
ECs
TAMs
Oxidative
Glycolytic
Tumor suppressive
Tumor promoting
Active
Exhausted
Immunogenic
Tolerogenic
Permeable
Impermeable
M1-like
M2-like

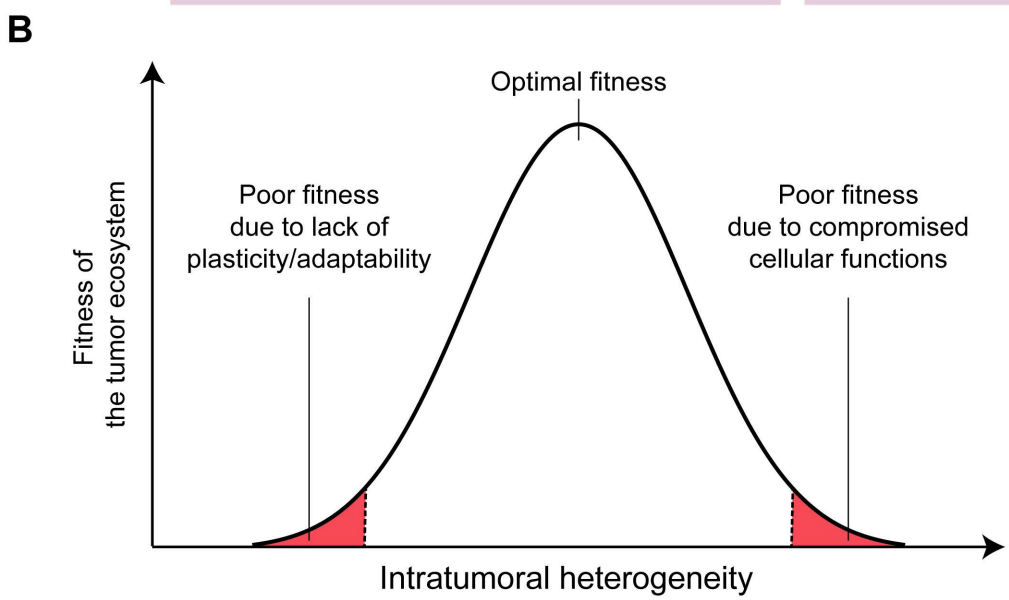

B
Optimal fitness
Poor fitness due to lack of plasticity/adaptability
Poor fitness due to compromised cellular functions
Fitness of the tumor ecosystem
Intratumoral heterogeneity

Figure 1

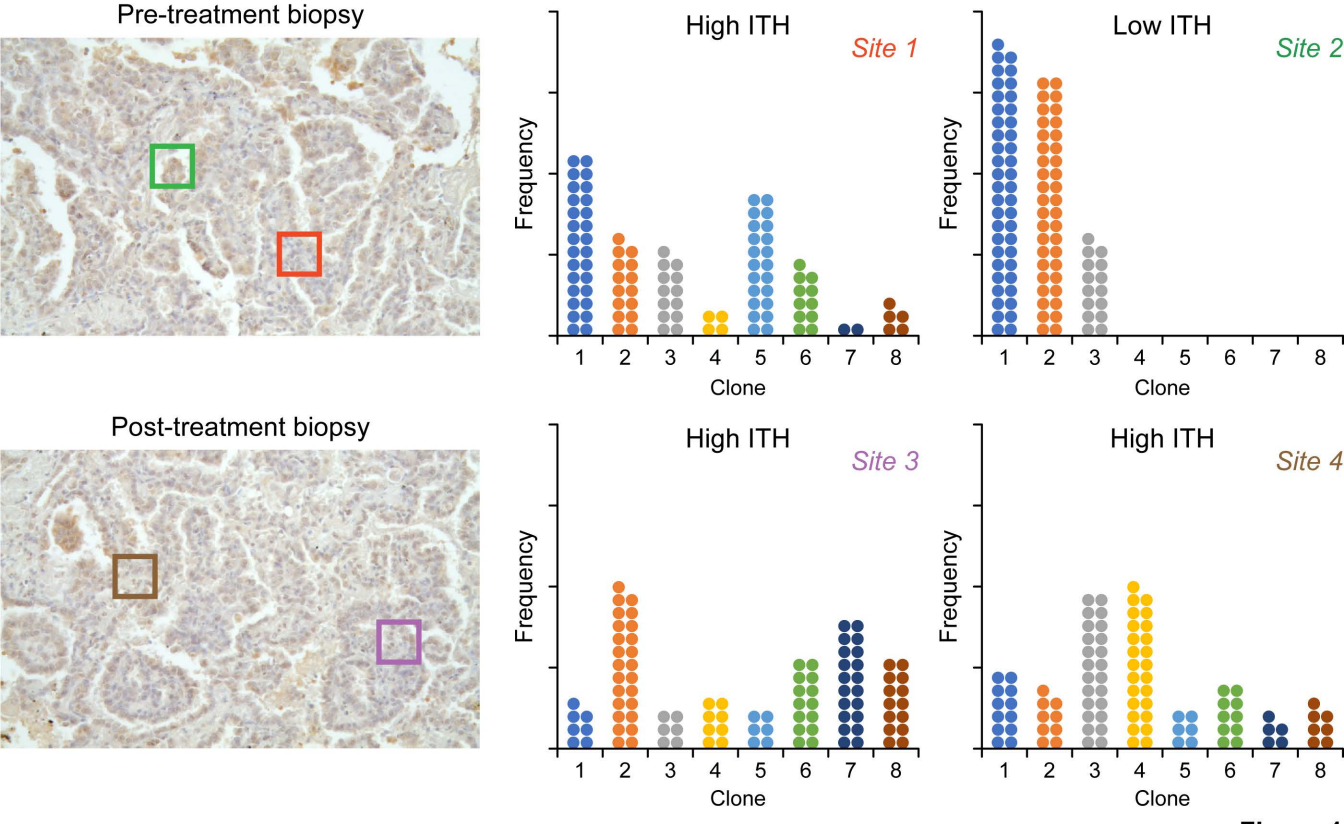

Pre-treatment biopsy
High ITH
Site 1
Low ITH
Site 2
Post-treatment biopsy
High ITH
Site 3
High ITH
Site 4
Frequency
Clone
1
2
3
4
5
6
7
8

Figure 1

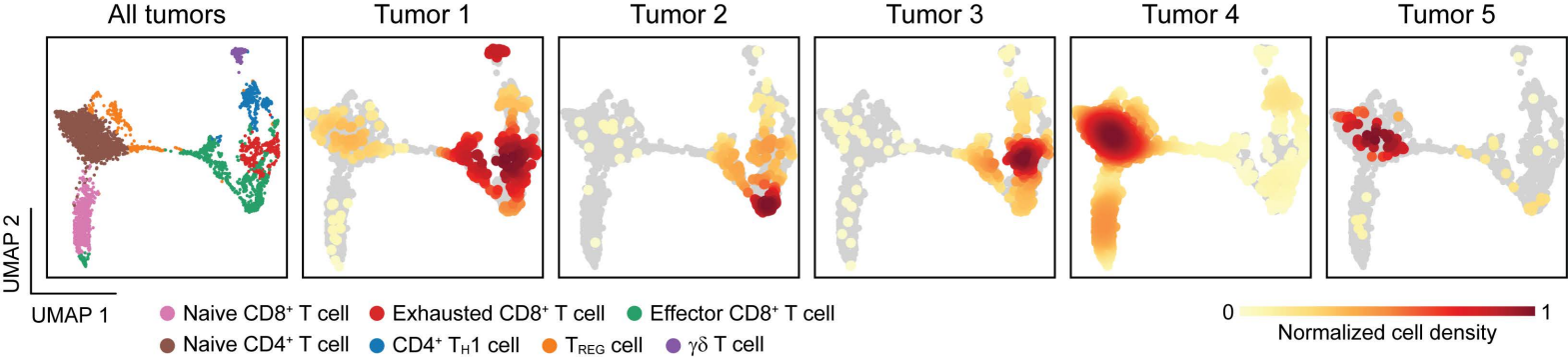

All tumors
Tumor 1
Tumor 2
Tumor 3
Tumor 4
Tumor 5
UMAP 2
UMAP 1
Naive CD8⁺ T cell
Exhausted CD8⁺ T cell
Effector CD8⁺ T cell
Naive CD4⁺ T cell
CD4⁺ T_H1 cell
T_{REG} cell
γδ T cell
0
1
Normalized cell density

Figure 3