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The Effects of Clonal Heterogeneity on Cancer Immunosurveillance

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Keywords

intratumor heterogeneity, cancer immunosurveillance, immunotherapy, immune checkpoint inhibition

Abstract

Intratumor heterogeneity (ITH) is associated with tumor progression in several clinical and experimental settings and contributes to therapeutic resistance. Its relation to cancer immunosurveillance is complex. Clonally heterogeneous tumors are associated with decreased immunosurveillance and are less responsive to immune checkpoint inhibition, but the mechanistic basis underlying these observations remains unclear. One possibility is that tumors that are under active immunosurveillance are relatively homogeneous because immunosurveillance prevents the outgrowth of immunogenic subclones. Alternatively, high ITH might directly impair immunosurveillance due to lower dosages of subclonal antigens, competition between antigens and immunodominance, the induction of detrimental T cell differentiation programs, or negative feedback loops. Here we review the evidence for these scenarios and outline hypotheses that could underlie the negative association between clonal heterogeneity and cancer immunosurveillance.

INTRODUCTION

The cancer immunosurveillance paradigm posits that cancer growth is controlled, to some extent, by the immune system (reviewed in Dunn et al. 2002). Indeed, immune checkpoint inhibitors (CPIs) have revolutionized the treatment of many advanced cancers and can offer durable remissions, albeit in a minority of patients (Bellmunt et al. 2017, Hellmann et al. 2018, Larkin et al. 2015, Powles et al. 2014, Reck et al. 2016, Schmid et al. 2018, Socinski et al. 2018).

Genetic intratumor heterogeneity (ITH) can enable therapeutic resistance through selection of resistant subclones (Dagogo-Jack & Shaw 2018, Marusyk et al. 2020). In the current era of precision medicine and widespread use of immunotherapy as standard of care, ITH and its potential interplay with the immune response have been intensively researched.

Understanding the potential impact of ITH on immunosurveillance may hold the key to developing effective immunotherapies for the majority of patients who do not experience clinical benefit. The directionality of the association between ITH and cancer immunosurveillance remains unclear, engendering the question: Does the tumor shape the immune response, or does the immune response shape the tumor? Here we review clinical data on the association between ITH and cancer immunosurveillance and the experimental evidence and mechanistic studies supporting different (but not mutually exclusive) answers to the above question. Finally, we highlight unanswered questions and future directions.

INTRATUMOR HETEROGENEITY AS A PROGNOSTIC BIOMARKER

There is increasing evidence that ITH has implications for prognosis after surgical resection. In the TRACERx [Tracking Non-Small-Cell Lung Cancer (NSCLC) Evolution through Therapy] study, the degree of subclonal copy number alterations (CNAs) was associated with an increased risk of relapse in a cohort of over 90 patients with early-stage NSCLC after surgical resection (Jamal-Hanjani et al. 2017). Likewise, NSCLC tumors that harbored a greater proportion of clonal mutations (Zhang et al. 2014) or clonal neoantigens (McGranahan et al. 2016) were associated with more durable remission and improved overall survival, respectively. Smaller studies have also found associations of higher subclonal mutational burdens with relapse after surgery in other cancers (Masoodi et al. 2019). However, some patients included in these studies received adjuvant therapy following surgery, making it difficult to tease apart the prognostic and predictive impacts of ITH. Nonetheless, a more recent study in over 80 patients with stage II surgically resected colorectal cancer who did not receive adjuvant therapy also demonstrated an increased risk of relapse associated with subclonal CNAs (Lahoz et al. 2021). Bearing in mind the caveats of adjuvant therapy, these observations imply that heterogeneous tumors may be inherently more aggressive.

CORRELATION OF INTRATUMOR HETEROGENEITY WITH SIGNATURES OF IMMUNE ACTIVITY

The impact of ITH on prognosis could be driven by its effects on cancer immunosurveillance or by non-immune-mediated mechanisms. In support of the former, ITH is associated with several biomarkers of antitumor immunity. For example, several studies have revealed a negative association between ITH and the degree of immune cell infiltration or the cytolytic score (Fernández et al. 2020, Karn et al. 2017, Li et al. 2020, Lin et al. 2020, McDonald et al. 2019, Morris et al. 2016, Oshi et al. 2021). Although statistically significant, the effect size in many of these studies was small. Moreover, these studies were based on single-region tumor sampling, which may limit accurate estimates of ITH (McGranahan & Swanton 2017). Likewise, many of these studies relied on bioinformatic tools to infer the degree of ITH based on the variant allele

frequency (VAF) distribution of mutations (Mroz et al. 2015). While useful as an estimate of ITH, VAF distribution is also influenced by the degree of somatic CNAs (SCNAs) and tumor purity (Noorbakhsh et al. 2018). Given that aneuploidy itself may be associated with reduced immune infiltration (Davoli et al. 2017), robust conclusions from these data require careful controls.

Several multiregion studies have also analyzed the association of ITH with immune cell infiltration. The Shah lab sampled multiple tumor regions from patients with ovarian cancer having undergone debulking surgery (Zhang et al. 2018). Tumors highly infiltrated with immune cells were overall more homogeneous, with a lower heterogeneity index and lower numbers of subclonal SCNAs. Consistent with this, work from our group demonstrated that in lung adenocarcinomas, tumors with a higher degree of $CD8^+$ T cell infiltration were less heterogeneous (Rosenthal et al. 2019). Interestingly, this association was not seen for lung squamous cell carcinomas. A pancancer analysis also demonstrated a negative correlation between ITH and immune cell infiltration for most cancer types, except for lung squamous cell carcinomas and head and neck squamous cell carcinomas (Morris et al. 2016), making it tempting to speculate that the association of ITH and immunosurveillance might differ between squamous cell carcinomas and adenocarcinomas.

In summary, despite their caveats, multiple single-region studies, each using different tools to estimate ITH and thus subject to different biases, converged on the conclusion that ITH is negatively associated with immunosurveillance, a conclusion also supported by observations from multiregion studies.

INTRATUMOR HETEROGENEITY AS A PREDICTIVE BIOMARKER FOR RESPONSE TO IMMUNE CHECKPOINT INHIBITORS

While the association of ITH with prognosis may be influenced by adjuvant therapy and nonimmune selective pressures, a causal link to tumor immunosurveillance may be inferred from the utility of ITH as a predictive biomarker of response to CPI therapy.

As CPIs work in part through derepressing (neo)antigen-reactive αβ T cells, it is not surprising that tumor mutational burden (TMB), a surrogate for neoantigen burden, has emerged as a clinically relevant biomarker across many solid cancers (Huang et al. 2021). Indeed, the FDA (US Food and Drug Administration) has recently approved the use of pembrolizumab for patients with advanced, TMB-high (≥10 mutations/megabase) solid tumors. However, a high-TMB/high-neoantigen burden is not always predictive of CPI response. In cancers where $CD8^+$ αβ T cell levels were not correlated with neoantigen load, a low TMB actually predicted response to CPI therapy (McGrail et al. 2021), suggesting cells other than αβ T cells may play a role (Crowe et al. 2002; Cui et al. 1997; Gentles et al. 2015; Girardi et al. 2001; Mikulak et al. 2019; Wu et al. 2019, 2022).

In other cancers, it is likely that TMB/neoantigen burden is simply too crude as a biomarker and that ITH has a dominant impact on its predictive utility. The notion that incorporating clonality into measurements of neoantigen burden strengthens its association with prognosis and response to CPI therapy was first introduced by McGranahan et al. (2016). Several subsequent studies have confirmed an association between a high proportion of clonal mutations and CPI response (Bortolomeazzi et al. 2021, Litchfield et al. 2021, McGranahan et al. 2016, Riaz et al. 2017). Likewise, others have reported an association between a high proportion of subclonal mutations and progression on CPI therapy (Miao et al. 2018). In addition to relative proportion, it appears a greater absolute clonal TMB also predicts response to CPI therapy. A recent meta-analysis from our group of more than 1,000 CPI-treated patients across multiple cancer types demonstrated that the strongest predictor of response was clonal TMB, in contrast to subclonal TMB, which was not predictive (Litchfield et al. 2021).

Another meta-analysis of several cohorts of CPI-treated melanoma patients showed that tumors with greater clonal diversity had worse outcomes compared to more clonal tumors, independent of TMB (Wolf et al. 2019). Similarly, in a cohort of NSCLC patients, high-ITH tumors responded worse to anti-PD(L)1 therapy (Fang et al. 2021).

HYPOTHESES

The available data indicate that ITH is negatively associated with biomarkers of cancer immunosurveillance and response to CPI therapy. However, it is challenging to determine the directionality in this relationship. Does cancer immunosurveillance shape tumor evolution, such that ITH reflects a lack of immune pruning? Or does ITH actively impair cancer immunosurveillance?

INTRATUMOR HETEROGENEITY AS A RESULT OF IMPAIRED IMMUNOSURVEILLANCE

Tumors can exist in a state of equilibrium with the immune system (Dunn et al. 2002). Active immunosurveillance could result in pruning of immunogenic subclones, thus leaving behind a relatively homogeneous tumor (**Figure 1*a***). On the other hand, heterogeneous tumors may be the result of a lack of immunosurveillance—for example, in so-called cold tumors, which lack

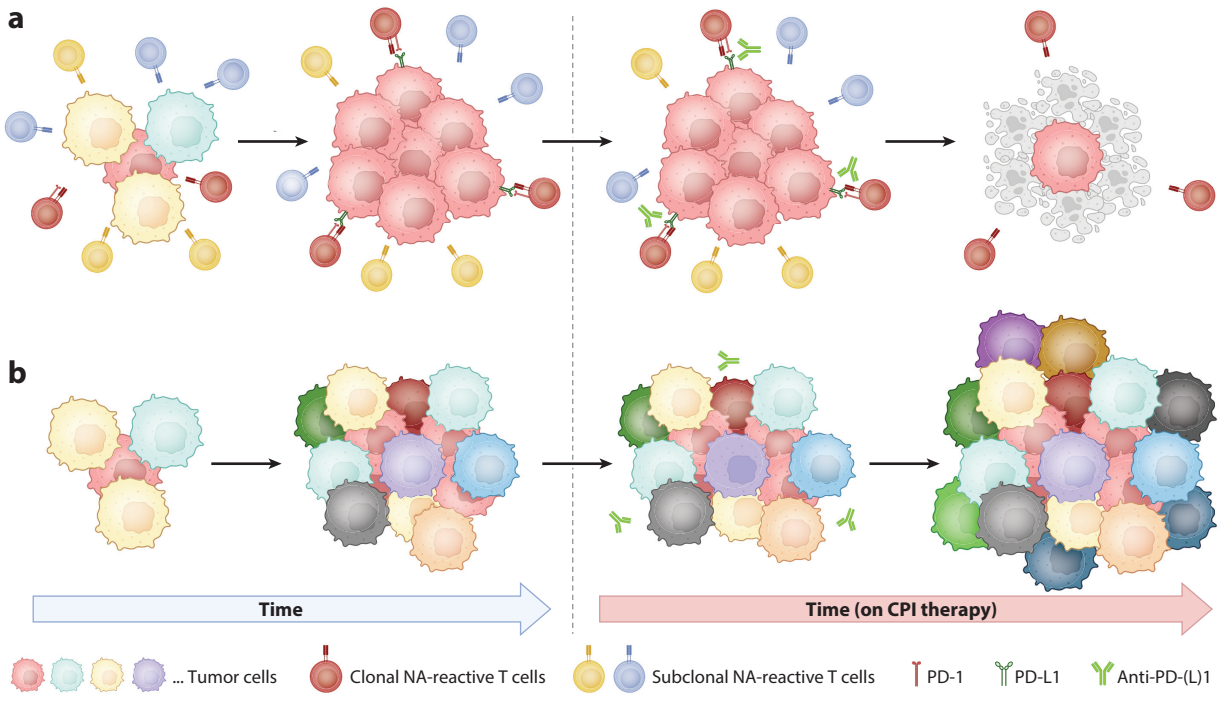

Figure 1

Immunosurveillance restricts ITH through pruning of subclonal neoantigens. (*a*) Clinically apparent homogeneous tumors (low ITH) may be a legacy of effective immune pruning of subclonal neoantigens. By definition, resultant homogeneous tumors have escaped immunosurveillance, often by induction of immune inhibitory feedback mechanisms such as PD-1/PD-L1 signaling. This would be consistent with the observations that tumors with low ITH are more likely to respond to CPI therapy. (*b*) Conversely, heterogeneous tumors may reflect a lack of immune pruning, for example, in so-called cold tumors. In such cases, CPI therapy may be less effective given the lack of effector immune cells within the TME. Abbreviations: CPI, checkpoint inhibitor; ITH, intratumor heterogeneity; NA, neoantigen; TME, tumor microenvironment. Figure adapted from images created with BioRender.com.

intratumoral immune infiltrates (**Figure 1*b***). Two key observations would support this hypothesis. First, one would expect to find evidence of elimination of immunogenic subclones in patients. Second, the immune system should restrict ITH in experimental (mouse) models.

Elimination of Immunogenic Subclones

With regard to the former, an observational study in clinical ovarian cancer found that tumors with the highest ITH harbored the fewest $CD8^+$ tumor-infiltrating lymphocytes (TILs) (Zhang et al. 2018). Fewer than expected subclonal (but not clonal) neoantigens were found in tumors harboring the highest number of $CD8^+$ TILs. This suggested that the acquisition of immunogenic subclones was held back by infiltrating $CD8^+$ T cells. Similarly, a higher-than-expected number of subclonal neoantigens was observed in immune cold (but not hot) tumors (Rosenthal et al. 2019), possibly indicating that neoantigens were better preserved in tumors with decreased immunosurveillance. Interestingly, in cold tumors, subclonal copy number loss of truncal neoantigens was observed as a mechanism of immunoediting, perhaps reflecting a legacy of immunosurveillance in an ancestral hot tumor that subsequently turned cold.

In addition to these observational clinical studies, cancer immunoediting has also been observed in experimental mouse models (Dunn et al. 2002, DuPage et al. 2012). Moreover, studies of patients on CPI therapy also indicate that tumors may lose target antigens after treatment (Verdegaal et al. 2016). Anagnostou et al. (2017) analyzed tumors from patients with NSCLC prior to and after acquired resistance to CPI therapy. They found that neoantigens lost posttreatment were immunogenic and that many of these were subclonal. A recent study also suggests that subclonal neoantigens may not be inherently poor immune targets (Lussier et al. 2021). The authors induced subclonal mutations in a nonimmunogenic, low-mutational-burden sarcoma cell line using a nonlethal dose of irradiation in vitro. Transplantation of these irradiated tumor cells into mice resulted in heightened susceptibility to CPI therapy compared to the parental cell line, in an antigen-dependent manner. The authors speculated that subclonal neoantigens may drive further immune rejection through epitope spreading. In addition, screens for neoantigen-reactive T cells in patients with metastatic urothelial carcinoma treated with anti-PD-L1 therapy found no enrichment for recognition of clonal versus subclonal neoantigens, although the latter made up only a small proportion of all antigens screened (Holm et al. 2022). Riaz et al. (2017) analyzed pre- and on-treatment samples from CPI-treated melanoma patients. Responders showed depletion of neoantigens in on-treatment biopsies and this was not observed in nonresponders. Together these findings indicate that immunological pruning of subclones can occur in patients.

The Immune System Restricts Intratumor Heterogeneity

Experimental mouse studies indicate that the immune system can restrict tumor heterogeneity. In two recent studies, a multicolor barcoding strategy revealed that injected tumor cells formed considerably more heterogeneous tumors in immunodeficient mice compared to immunocompetent mice (Maire et al. 2020, Milo et al. 2018). In apparent contrast, in a genetically engineered mouse model with mismatch repair deficiency, tumors in T cell–depleted mice were more heterogeneous compared to those formed in immunocompetent mice (Westcott et al. 2021a), as shown by a shift of the VAF distribution toward more subclonal mutations, possibly because of selective targeting of clonal neoantigens by T cells (McGranahan et al. 2016). While this study also indicates that the immune system can sculpt the tumor, in this case this resulted in more rather than less heterogeneity. The discrepancy between these studies might be explained by the difference between spontaneous versus transplanted tumor models. In the latter, multiple highly immunogenic subclones can proliferate unimpaired in vitro but may then be rapidly eliminated after transplantation into immunocompetent hosts, a major evolutionary bottleneck expected to strongly

restrict heterogeneity. Nonetheless, this may be analogous to the treatment setting in which CPI therapies result in drastic disruptions to tumor homeostasis. As has been observed in the clinic, this may result in the elimination of immunogenic subclones or neoantigens (Anagnostou et al. 2017, Verdegaal et al. 2016) and cause a bottleneck that would be expected to reduce tumor heterogeneity (Riaz et al. 2017). In genetically engineered mouse models, tumors develop much more gradually, which may be more reflective of tumor evolution in the absence of therapy. In this setting, bottlenecks are weaker, creating an environment in which subclonal diversity can develop.

Intratumor Heterogeneity as a Result of Impaired Immunosurveillance: Concluding Thoughts

The available evidence suggests that elimination of immunogenic subclones can occur, both in mice and in patients. This is most apparent in settings where the cancer-immune equilibrium is disturbed drastically and suddenly, such as in transplantable murine tumor models and in the clinical setting, following CPI therapy. Nonetheless, signals of immunoediting under more homeostatic conditions have been observed in some studies, such as selection against neoantigens in expressed genes (Rosenthal et al. 2019) or shaping of oncogenic mutations by their ability to be presented on MHC (major histocompatibility complex) class I molecules (Marty et al. 2017).

If tumors remain relatively homogeneous when in a state of immune equilibrium but become heterogeneous only after they escape immunosurveillance, one would expect so-called immunological scars early during tumor evolution, but not in more recent subclones. Indeed, subclonal neoantigens have been predicted to be more immunogenic than clonal neoantigens (Jiménez-Sánchez et al. 2017). Moreover, decreased immunoediting has been observed for subclonal but not clonal mutations in NSCLC (Rosenthal et al. 2019). Interestingly, as described above, immunoediting of subclonal mutations was observed in highly infiltrated ovarian cancers (Zhang et al. 2018), possibly indicating continued immunosurveillance in hot tumors.

Several challenges remain when determining whether immunoediting in untreated patients restricts ITH. First, given the imperfection in neoantigen prediction algorithms, subtle signals of immunoediting remain difficult to detect in relatively noisy data. Second, it is likely that only a minority of the presented neoantigen space is effectively targeted by T cells, due, for example, to competition between antigens (as discussed below). This implies that loss of a particularly relevant antigen may be overlooked in analyses focused on the total neoantigen burden per tumor. Third, longitudinal studies, including those initiated in early stages of tumor development, are needed to truly determine whether immunosurveillance restricts ITH. A recent study compared primary tumors and recurrences in short- and long-term survivors of pancreatic cancer (STSs and LTSs, respectively) (Łuksza et al. 2022). Primary tumors of LTSs showed more immune infiltration, suggestive of increased immunosurveillance (Balachandran et al. 2017). Compared to STSs, recurrent samples in LTSs acquired fewer new neoantigens, and newly acquired clones had lower immune fitness, which is indicative of immunoediting. However, while recurrences of LTSs were more homogeneous compared to those of STSs, this was also the case for primary tumors, making it challenging to interpret whether ongoing immunosurveillance restricted heterogeneity at recurrence.

IMPAIRED IMMUNOSURVEILLANCE AS A RESULT OF INTRATUMOR HETEROGENEITY

An alternative, although not mutually exclusive, explanation for the association between ITH and decreased immunosurveillance is that ITH directly enables immune evasion. Several experimental studies provide support for this hypothesis. Wolf et al. (2019) found that after grafting a mouse melanoma cell line into immunocompetent mice, tumor growth was accelerated when the cell line

was previously UVB irradiated. This was hypothesized to be due to increased ITH resulting from novel UVB-induced subclonal mutations. Single-cell clones generated from the UVB-irradiated cell line were readily rejected. Furthermore, a series of mixing experiments showed that tumor rejection was progressively impaired when increasing the number of clones that were injected simultaneously. These clones were mapped back to the cell line's phylogenetic tree, and injecting clones from different branches resulted in tumor growth, whereas injecting the same number of clones within a single branch led to tumor rejection. These data provide direct evidence that more heterogeneous tumors impair cancer immunosurveillance. In support of this, others have also observed that tumors formed from single-cell clones were more easily rejected than were those from their parental polyclonal tumor cell lines (DiMarco et al. 2021, Germano et al. 2017, Westcott et al. 2021a). What might explain such a phenomenon?

Intratumor Heterogeneity Enables Immune Evasion by Limiting Antigen Dosage

In a tumor where subclones compete for space, increased ITH may limit the expansion of individual subclones, diluting the abundance of subclonal antigens. Experimental data suggest that minor subclones can be selectively pruned by T cells but fail to be eliminated if they make up too small a fraction of the tumor (Gejman et al. 2018). This is consistent with the observation that lowly expressed (clonal) antigens fail to induce a protective immune response, but instead promote T cell dysfunction through failed priming (**Figure 2**) (Westcott et al. 2021b). Conceivably, the overall abundance of antigen that is presented to T cells determines whether a productive immune response is mounted. Failure to present antigens at sufficiently high levels could be impaired either because expression level is low (in all tumor cells) or because of low cancer cell fraction (subclonality). This is consistent with the observation that hot (but not cold) NSCLC samples frequently show transcriptional repression of neoantigens as a possible immune evasion mechanism (Rosenthal et al. 2019). Sequencing studies have revealed that many nonmalignant tissues form a patchwork of small clonal expansions of untransformed cells that contain somatic

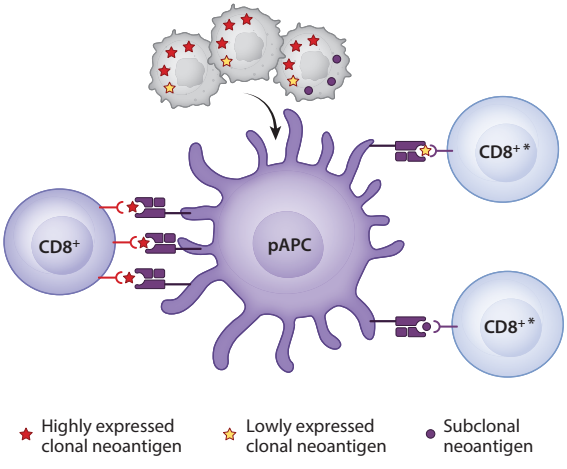

Figure 2

Limiting antigen dosage impairs immune surveillance. Low antigen dosage may impair efficient priming of T cells. Both subclonal neoantigens and lowly expressed clonal neoantigens may result in low antigen dosage ($CD8^{+*}$; the asterisk indicates inefficiently primed). Abbreviation: pAPC, professional antigen-presenting cell. Figure adapted from images created with BioRender.com.

mutations (Lee-Six et al. 2019, Yizhak et al. 2019, Yoshida et al. 2020). It is likely that at least some of these mutations represent neoantigens. While the absence of damage signals likely is one important reason, their low abundance may also explain why these clones evade elimination by T cells, which is consistent with a recent observation that more immunogenic *TP53* mutations are relatively well tolerated in non-neoplastic lesions compared to tumors (Hoyos et al. 2022).

Intratumor Heterogeneity Represses Immunosurveillance Through Competition and Immunodomination

Heterogeneous tumors contain multiple evolutionary branches, each harboring potentially immunogenic antigens. The lack of recent clonal sweeps may increase the total antigenic diversity of tumors and reduce the relative abundance of each individual (subclonal) antigen. This large subclonal antigen burden could compete with itself, but also with clonal antigens, for MHC binding (Boulanger et al. 2018). This relative reduction in antigen dosage may then impair efficient priming of neoantigen reactive T cells (Westcott et al. 2021b) and tumor rejection (Gejman et al. 2018). Furthermore, subclonal neoantigen reactive T cells may consume limited growth factors such as interleukin-2 (IL-2). Within the tumor microenvironment (TME), this may reduce the ability of clonal neoantigen reactive T cells to survive (Busse et al. 2010). Within secondary lymphoid organs, this may also impair the efficient priming and generation of long-lived functional clonal neoantigen reactive T cells (Williams et al. 2006). Furthermore, T cells may persist in the TME in the absence of cognate T cell receptor (TCR) stimulation through IL-15-mediated homeostatic turnover, outcompeting newly primed T cell clones (Boldajipour et al. 2016). This could drown out responses against clonal neoantigens and prevent any single response from reaching sufficient magnitude to elicit meaningful tumor control. This could be particularly relevant in tumors with high ITH but low TMB, which is associated with reduced survival in melanoma patients (Wolf et al. 2019).

ITH might also lead to impaired immunosurveillance through the establishment of antigen dominance hierarchies based on the magnitude of the immune response they elicit (immunodominance). Immunodomination refers to the situation in which an immune response against one antigen suppresses the response against a second antigen (Schreiber et al. 2002, Yewdell 2006). Immunodominance hierarchies may be established against different antigens expressed on the same tumor cells (clonal antigens), which may be the case in tumors with high mutational burden but low ITH. In this case, the impact on immunosurveillance would be expected to be minor, as the dominant and subdominant response are directed against the same target cell. However, in heterogeneous tumors, immunodomination might impair cancer immunosurveillance in two ways.

First, a dominant response against a subclonal antigen may effectively target that subclone, but at the same time suppress the response against other subclones that express different antigens. Conceivably, once that subclone is eliminated and the dominant response contracts, previously subdominant responses could resurface, resulting in stepwise pruning of immunogenic subclones. A continued restructuring of epitope dominance hierarchies likely occurs through tumor evolution and has yet to be fully elucidated. In longitudinal studies of HIV-1 infection, the early T cell response is directed toward a typically narrow range of epitopes, with subsequent pulses of expansion and contraction to novel epitopes observed later in infection, consistent with sequential shifts in immunodominance hierarchies over time (Turnbull et al. 2009). However, there may be situations in which the immune system is locked into a response against one subclone without being able to eliminate it. This could, for example, arise in the case of immunoregulation, T cell exhaustion, or loss of specific HLA (human leukocyte antigen) class I alleles. A large proportion of cancers show loss of heterozygosity of HLA molecules, which is often subclonal (McGranahan et al. 2017). Dominant antigens presented on the lost HLA allele may still be cross-presented by

nonmalignant professional antigen-presenting cells (pAPCs), keeping the immune system focused on an antigen that cannot be eliminated (Schreiber et al. 2002).

Second, among other factors (Yewdell 2006), the order in which immune responses are established may determine the place of the antigen in the dominance hierarchy. This suggests that a response against an early (clonal) antigen may limit the development of responses against subclonal antigens arising later in tumor evolution (Schreiber et al. 2002). Evidence for this hypothesis comes from mouse studies in which antigens are introduced at different time points. To what extent this would also be relevant in the context of a naturally evolving tumor is still unclear.

Subdominant T cell responses appear to be associated with higher expression levels of PD-1 and in two mouse studies, anti-PD1 therapy specifically boosted subdominant responses (Friedman et al. 2020, Memarnejadian et al. 2017). However, other studies have not found a preferential targeting of subdominant responses by CPIs (Burger et al. 2021, Chen et al. 2018). The phenotype of the subdominant response, and thereby its capacity to be reinvigorated by CPI therapy, may depend on the antigens and tumor models used. However, there is consistency between studies that subdominant responses can be boosted by vaccination (Burger et al. 2021, Chen et al. 2018). This may present a viable therapeutic strategy to broaden the immune response and prevent escape of subclones expressing only subdominant antigens.

In conclusion, from a theoretical perspective, ITH could impair cancer immunosurveillance if a dominant subclonal antigen leads to suppression of responses against other subclonal or clonal antigens. Experimental studies in mice suggest that this may indeed play a role in cancer immune escape. However, most studies have coexpressed multiple antigens in the same tumor cell. The extent to which dominance hierarchies and immune escape are shaped by subclonality still remains largely unclear. Importantly, to the best of our knowledge, there is currently no evidence that immunodominance plays a role in shaping the immune response against cancer in humans. The presence, phenotype, and reversibility of subdominant responses in humans therefore remains to be unequivocally demonstrated.

Intratumor Heterogeneity Engages a Detrimental Immune Response

Finally, an effective immune response against a subclonal antigen might hinder further immunosurveillance against other subclonal and clonal antigens. So-called hot inflamed tumors that engage an immune response are generally viewed as favorable. Indeed, T helper 1 cell (Th1)-skewed and cytolytic immune responses have most often been associated with cancer protection (Ayers et al. 2017, Gao et al. 2016, Rooney et al. 2015, Shankaran et al. 2001), while some data also implicate Th2 responses (Dalessandri et al. 2016, Hemelrijck et al. 2010). Nonetheless inflammation can be tumor promoting, and chronic tissue inflammation is strongly linked to the development of cancers within these tissues (Coussens & Werb 2002). Beyond clinical correlation, murine models unequivocally demonstrate a tumor-promoting role for some facets of the immune response, supporting tumor cell fitness either directly or through remodeling the TME. For example, deletion of tumor necrosis factor (*Tnf*) protects mice from de novo cutaneous carcinogenesis (Moore et al. 1999). Moreover, NF-κB and IKKβ, critical molecular links for TNF-α signaling, were both found to reduce tumor development when selectively deleted in either tumor cells or inflammatory cells (Greten et al. 2004, Pikarsky et al. 2004). Indeed, chronic blockade of TNF-α in the clinical setting is not associated with increased risk of carcinogenesis, and early-phase studies of anti-TNF to treat cancer, both as monotherapy and in combination with CPIs, hint at some efficacy (Madhusudan et al. 2004, 2005; Montfort et al. 2021). Even interferon gamma (IFN-γ), a cytokine consistently linked with protection against cancer in both murine models and clinical disease (Ayers et al. 2017, Gao et al. 2016, Shankaran et al. 2001), has been linked to tumor

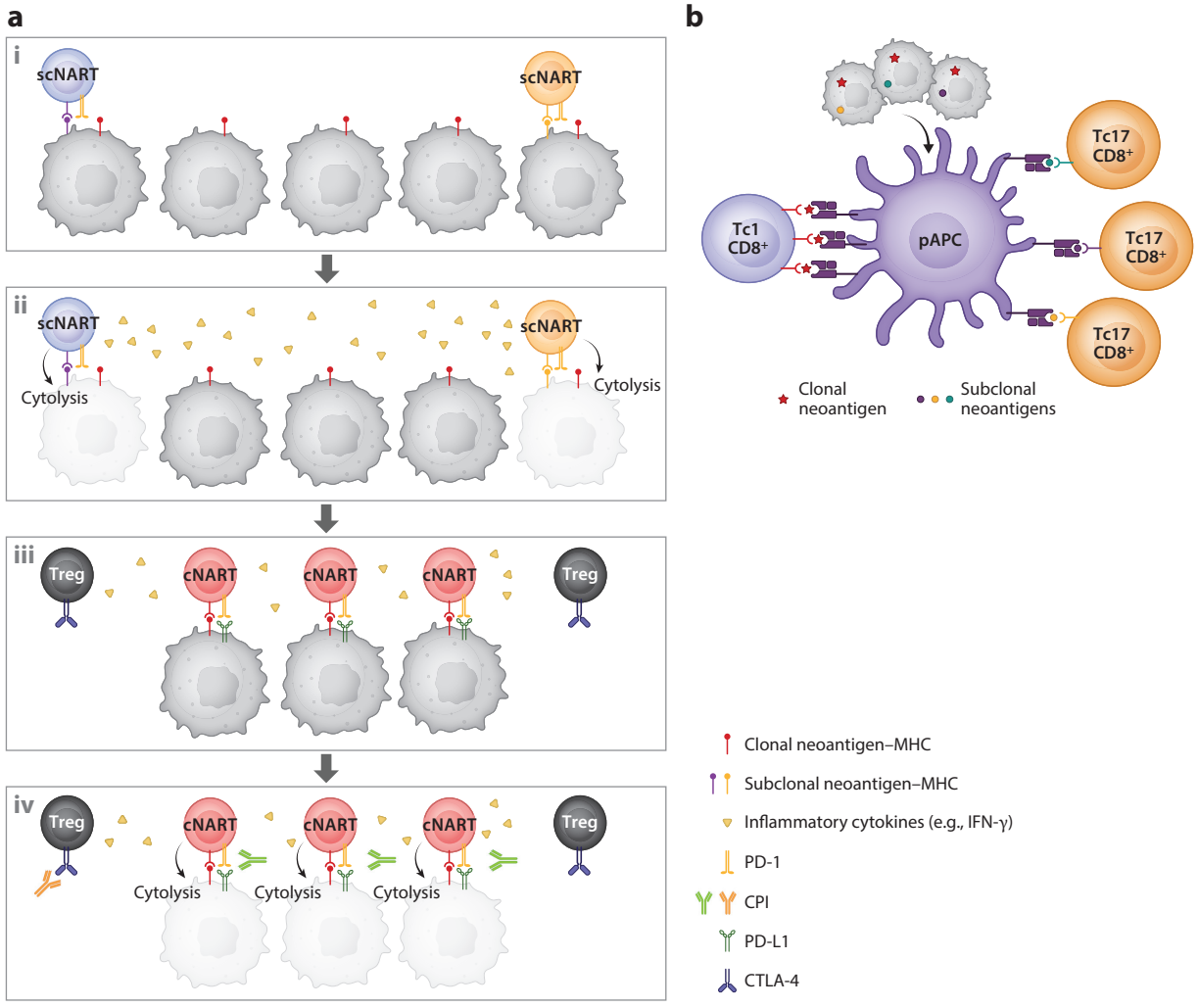

Figure 3

ITH engages a detrimental immune response. (*a*) scNARTs do not confer sterilizing immunity as they only recognize (subpanel *i*) and kill a proportion of cognate tumor cells (*ii*). The associated release of proinflammatory cytokines driven by subclonal neoantigen recognition induces a tolerance program within the TME, which hinders effective immunosurveillance by other T cells including cNARTs (*iii*), which may be reversed by CPI therapy (*iv*). (*b*) Subclonal neoantigen–MHC complexes may be present at lower concentrations on pAPCs, resulting in lower-avidity TCR engagement, thus favoring tumor-promoting IL-17 production from antigen-specific T cells. Abbreviations: cNART, clonal neoantigen reactive T cell; CPI, checkpoint inhibitor; ITH, intratumor heterogeneity; MHC, major histocompatibility complex; pAPC, professional antigen-presenting cell; scNART, subclonal neoantigen reactive T cell; Tc1, type 1 cytolytic $CD8^+$ T cell; Tc17, type 17 $CD8^+$ T cell; TCR, T cell receptor; TME, tumor microenvironment; Treg, regulatory T cell.

promotion in settings of chronic exposure (Benci et al. 2019). In this context, one can appreciate how ITH may induce a chronic but not necessarily sterilizing immune response. In this case, subclonal neoantigen reactive T cells directly kill only a proportion of cognate tumor cells while at the same time producing proinflammatory cytokines such as TNF-α or IFN-γ, which in turn drive expression of an immune tolerance program in the TME (Benci et al. 2019, Williams et al. 2020) (**Figure 3*a***).

A recent study by Burger et al. (2021) demonstrated that neoantigen quality may drive disparate $CD8^+$ effector T cell functions. Using a murine tumor model with enforced expression of model tumor antigens of differential MHC binding affinity, they demonstrated that the high-affinity peptide induced an immunodominant response enriched for effector and exhausted signatures. The weaker-binding antigen led to a subdominant response enriched for a less differentiated $TCF1^+$ progenitor state with impaired cytotoxic function. Interestingly, this was accompanied by differentiation of subdominant $CD8^+$ T cells toward a potentially tumor-promoting type 17 $CD8^+$ T cell (Tc17) lineage. The Th17 response has been linked to tumor promotion, likely through the recruitment of myeloid-derived suppressor cells (Coffelt et al. 2015, Daley et al. 2016, Jin et al. 2019). Interestingly, the observations by Burger et al. (2021) were dependent on an antigen dominance hierarchy such that weak MHC binders could induce tumor-rejecting type 1 cytolytic $CD8^+$ T cell (Tc1) responses when paired with even weaker MHC binders. These findings suggest a role for antigen-TCR avidity in determining effector responses. In the context of ITH, subclonal neoantigens may mimic these low-avidity interactions, as they are likely to be present at lower concentrations in the TME and thus also have lower surface peptide-MHC representation on pAPCs. Higher ITH and a higher proportion of subclonal neoantigens could therefore actively promote tumors through sustaining IL-17 production in the TME (**Figure 3*b***).

Impaired Immunosurveillance as a Result of Intratumor Heterogeneity: Concluding Thoughts

Because of the complex life histories of clinically observed tumors, there is a need for experimental model systems to determine whether increased ITH directly undermines cancer immunosurveillance. To the best of our knowledge, the transplantation study from Wolf et al. (2019) of melanoma cell lines irradiated by ultraviolet B light is currently the only study that directly addresses this question in a controlled setting. There is an urgent need for other models in which the extent of heterogeneity can be controlled. This will be easiest to achieve in transplantation studies. Similar approaches will need to be developed in genetically engineered mouse models (with more gradual tumor evolution), for example, by temporal control of specific antigens expressed stochastically in the tumor, by early versus late induction of APOBEC (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide) enzymes, or by knockout of mismatch repair genes.

Therapeutic intervention may cause marked changes in ITH in patients. Therapeutic selection pressures may create strong bottlenecks that decrease ITH. Conversely, treatments such as chemotherapy or radiotherapy may increase ITH by generating new subclonal mutations (Lussier et al. 2021, Wolf et al. 2019). Assessing tumor clonality before and after such therapies combined with an analysis of the TME could help clarify to what extent changes in ITH impact subsequent immunosurveillance. Moreover, it will be interesting to determine whether timing of CPI therapy at a point of maximal reduction in ITH (e.g., treatment initiation at the point of maximal response to targeted therapy or chemotherapy, rather than after treatment progression) will be beneficial.

We have reviewed the available evidence supporting a direct detrimental effect of ITH on immunosurveillance. ITH can of course also promote immune escape more indirectly by generating genetic diversity, which provides a substrate for the positive selection of resistant subclones. How ITH promotes therapeutic resistance through Darwinian and non-Darwinian mechanisms has been recently reviewed elsewhere (Vendramin et al. 2021). Here we propose several mechanisms that could explain a direct adverse impact of ITH on cancer immunosurveillance: antigen dosage, competition, immunodomination, and the induction of detrimental responses as collateral damage. While preclinical experimental evidence exists for these phenomena, whether they play a relevant role in humans in general, and in the context of tumor heterogeneity in particular, is still an open question.

Specifically, future work could be directed toward determining how important antigen dosage is in determining the magnitude of a T cell response, and whether clonality and expression level can compensate for each other. Of particular interest is the question whether competition between antigens can limit the overall magnitude of the anticancer immune response. The concept of immunodominance is intriguing, but its direct relevance in human cancer needs to be unequivocally demonstrated, as well as how immunodominance hierarchies are shaped by clonality. Finally, it will be of interest to determine whether successful immune responses against one subclone can induce collateral damage by inducing detrimental responses against other subclones.

FUTURE ISSUES

1. Are tumors more heterogeneous in immunodeficient or in immunocompetent spontaneous tumor models in mice?
2. Do tumors in cancer patients become more heterogeneous over time if immunosurveillance is limited?
3. Are tumors more heterogeneous in immunosuppressed patients (due to, e.g., transplantation, AIDS, immunosuppressive drugs, or high age)?
4. Is direct impairment of immunosurveillance observed in experimental models—particularly in gradually evolving cancer models?
5. Can clonality and expression level compensate for each other? In other words, is antigen dosage the ultimate factor determining the strength of the T cell response?
6. To what extent are immunodominance hierarchies observed in cancer patients (beyond mouse models)? What is the phenotype of dominant and subdominant responses? Are responses against subclonal antigens more often subdominant compared to those against clonal antigens?
7. Is differentiation of subdominant responses toward potentially tumor-promoting phenotypes observed more commonly (in other mouse models or in patients)?
8. Does T cell targeting of a subclone induce negative feedback loops in neighboring tumor cells representing another subclone? Does that increase the threshold for targeting those subclones?

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