Trends in Cancer

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Opinion

Immunogenomics of Colorectal Tumors: Facts and Hypotheses on an Evolving Saga

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Immunotherapy with immune checkpoint inhibitors is an approved treatment option for a subpopulation of patients with colorectal cancers that display microsatellite instability. However, not all individuals within this subgroup respond to immunotherapy, and molecular biomarkers for effective patient stratification are still lacking. In this opinion article, we provide an overview of the different biological parameters that contribute to rendering colorectal cancers with microsatellite instability potentially sensitive to immunotherapy. We critically discuss the reasons why such parameters have limited predictive value and the implications therein. We also consider that a more informed knowledge of response determinants in this tumor subtype could help understand the mechanisms of immunotherapy resistance in microsatellite stable tumors. We conclude that the dynamic nature of the interactions between cancer and immune cells complicates conventional biomarker development and argue that a new generation of adaptive metrics, borrowed from evolutionary genetics, may improve the effectiveness and reliability of clinical decision making.

Highlights

Different mutational burden only partially explains the different response of MSI and MSS CRCs to immunotherapy.

Neoantigen load, as measured using available prediction algorithms, is not sufficiently accurate for implementation into clinical decision making.

Abundant immune infiltration in the tumor tissue is likely to have high prognostic value, but not an equally high predictive value in terms of response to immunotherapy.

The intrinsic characteristics of MSI and MSS CRCs determine differences in their evolutionary paths, which inevitably influence the way the immune system sculpts tumor clonal and subclonal dynamics.

Immunotherapy in Colorectal Cancer (CRC): Appearances Can Be Deceiving

Cancer cells produce mutated antigens, **neoantigens** (see Glossary), that are captured by and presented at the surface of dendritic cells, in association with major histocompatibility complex (**MHC**) molecules. Dendritic cells deploy their cargo of tumor neoantigens to prime **CD4**$^+$ T helper cells and to trigger the activation of **CD8**$^+$ cytotoxic T cells, which travel to the tumor. Upon infiltration within the tumor microenvironment, activated cytotoxic T cells bind to the cancer cells and destroy them by inducing apoptosis. Cancer cells can forestall this immune attack by expressing PD-L1, a membrane-bound ligand that interacts with the PD-1 receptor exposed on effector T cells and, by doing so, prompts T cell inactivation. Antibodies against PD-L1 or PD-1 intercept this inhibitory immune checkpoint and re-engage immune-mediated cancer cell killing [1].

On the above premises, response to PD-L1 or PD-1 targeted agents is expected to occur in tumors that: (i) contain many neoantigens, (ii) display an abundance of immune infiltrates, and (iii) express high levels of PD-L1. This line of thinking offers a straightforward explanation to why immunotherapy is specifically active in CRC with microsatellite instability (MSI); the DNA mismatch repair deficiency that typifies such tumors makes cancer cells unable to correct errors that occur at microsatellite regions during physiological DNA replication, thus favoring the accumulation of DNA mutations that are likely to give rise to a great amount of immunogenic neoantigens [2–4] (Figure 1). Of note, MSI tumors carry a prevalence of frame-shift mutations, which normally lead to a variably long string of newly introduced amino acids before the DNA sequence runs into a stop codon. As such, frame-shift mutations are predicted to be more antigenic than single nucleotide variations, which generate single (hence, more neutral) amino acid substitutions [5]. Notwithstanding their immunostimulatory makeup, only approximately 50% of MSI CRC tumors regress on treatment with immune checkpoint inhibitors (ICIs) [3]. From an opposite and complementary perspective, microsatellite stable (MSS) CRCs are almost invariably refractory to immunotherapy. Still, they often exhibit large immune infiltrates [6,7], may be composed of cancer cells with high PD-L1 expression [8], and may have a mutational burden that, albeit less considerable than that borne by MSI cases, is not overtly different from that harbored by other cancer types that respond better to ICIs [9]. Collectively, these observations underscore the complexity of tumor susceptibility to immunotherapy and suggest that mechanisms of immune evasion occur even when T cell stimulation is, in principle, resurrected by checkpoint blockade. Here, we discuss how different CRC genomic features can shape the tumor immune contexture and, in turn, how the immune microenvironment can influence the genetic composition of cancer cells.

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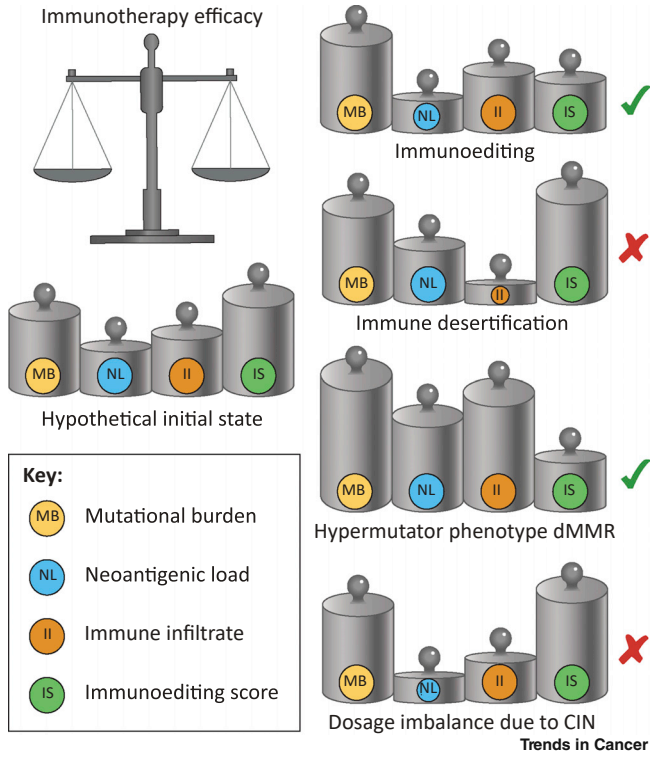

Figure 1. The Interplay between Cancer Cells and Immune Cells Evolves over Time in Different Contexts and Impacts the Efficacy of Immunotherapy.

A tumor in a hypothetical initial state characterized by a given mutational burden, neoantigen load, immune infiltrate, and immunoediting score, can follow different paths. (i) The selective pressure applied by the active immune infiltrate reduces the ratio between the observed neoepitopes and the expected ones (immunoediting). The presence of an active infiltrate is assumed to be a permissive ground for response to immunotherapy but timing should be accurate because immunoediting, at its limit, could theoretically abrogate immunogenicity. (ii) Immune desertification reduces the amount of immune infiltrate and thus does not affect the representation of neoantigens with respect to the total mutational burden. In this situation, immunotherapy could hardly be beneficial. (iii) Tumors with a hypermutator phenotype due to deficient mismatch repair (dMMR) produce more neoantigens and have more abundant immune infiltrates. This should sensitize them to immunotherapy. (iv) An antigen dosage imbalance due to chromosomal instability (CIN) can result in loss of genomic segments carrying immunogenic clonal mutations and/or in preferential HLA binding of self-peptides over neoepitopes (Figure 2). This reduces the relative number of expressed neoantigens, even in the presence of a high mutational burden, leading to less infiltrate. The immunoediting score formally does not account for the expression of neoantigens, so in this case it will not change. Given the reduction in neoantigenic load, immunotherapy is likely to be ineffective. In reality, all these mechanisms can coexist and coevolve along time, making it difficult to find a single, static, predictive biomarker for immunotherapy response.

Our considerations provide a perspective on both the opportunities and the hurdles afforded by coupling genomic analyses with the assessment of immune cell representation and function in CRC.

Tumor Mutational Burden and Neoantigen Load: The Importance of Being Exposed

Preclinical studies have demonstrated that PD-1/PD-L1 blockade cannot unleash antitumor T cell responses in the absence of fully primed and committed antigen-specific T cells [10,11]. But where are these antigens from? Each tumor mutation, if not synonymous, can lead to the production of an altered protein product, which, as such, is entitled to potentially act as a neoantigen. By this reasoning, the mutational burden of a tumor could be considered as a surrogate of its antigenicity,

Glossary

Bayesian inference: statistical inference method based on the Bayes' theorem, which defines the probability of an event using prior knowledge on the probability of related events.
CD3: a T cell co-receptor involved in the activation of naïve $CD8^+$ cytotoxic T cells and naïve $CD4^+$ T helper cells. It is a common immunohistochemical marker for all T cells.
CD4: a co-receptor for the T cell receptor that binds the invariant part of class II MHC proteins on antigen-presenting cells. It is expressed by T helper cells.
CD8: a co-receptor for the T cell receptor that binds the invariant part of class I MHC protein on cells. It is expressed by cytotoxic T lymphocytes.
Greedy algorithms: heuristic algorithms that search for the global optimal solution with a stepwise exploration of the solution spaces and, at each step, make the local best choice.
Haplotype: a set of alleles clustered on the same chromosome and inherited together.
HLA: human leukocyte antigen system, the gene complex encoding the major histocompatibility complex (MHC) proteins in humans.
IFNγ: an immunoregulatory, proinflammatory cytokine produced predominantly by natural killer cells and macrophages as part of the innate immune response, and by $CD4^+$ Th1 and $CD8^+$ cytotoxic effector T cells. IFNγ activates macrophages and stimulates the surface expression of class I and class II MHC molecules.
Integer programming: an optimization problem where variables are restricted to integers to find a solution that minimizes or maximizes a given expression and respects some mathematical constraints.
MHC: major histocompatibility complex, the genetic region encoding molecules involved in antigen presentation to T cells. Class I MHC molecules, expressed by essentially all nucleated cells, bind and present eight to ten amino acid peptides to $CD8^+$ cells. Class II MHC proteins, expressed primarily by professional

based on the so-called 'antigenic roulette' theory whereby the probability of developing 'winning neoantigens' increases proportionally to the extent of mutational burden [12]. Assessing the mutational burden could thus give a measure of how aggressively a tumor would be attacked by an active immune system, once suppressive brakes such as checkpoints molecules are pharmacologically neutralized; in other words, tumor mutational burden could be interpreted as a biomarker of immunotherapy sensitivity.

The correlation between tumor mutational burden and response to checkpoint inhibitors has been observed in different tumor settings [13,14] and has also received causal validation experimentally; for example, artificially enhancing the mutational burden in a CRC cell line through clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9)-based knockout of *MLH1*, a mismatch repair gene involved in correcting DNA replication errors, heavily sensitized to immunotherapy in mouse models [15]. However, the clinical applicability of this association cannot be generalized. In a recent meta-analysis of 27 different tumor types, mutational burden has been found to justify only half of the differences observed in the objective response rate across various cancers [9]; many tumor subtypes show a response to immunotherapy that is better than would be predicted by mutational burden and others have a response that is lower than would be predicted. The latter scenario paradigmatically applies to MSS CRC, in which the median number of coding somatic mutations per megabase (a classical definition of tumor mutational burden) is similar to that of renal-cell carcinoma and higher than that of mesothelioma and sarcoma; nonetheless, the response of MSS CRC to ICIs hovers around 0.5%, whilst it ranges between 10% and 25% in the other settings [9]. This discrepancy suggests that MSS CRC tumors have a proclivity for conveying immune suppressive cues that are not sufficiently antagonized by immunotherapy (Box 1). In general, the limited correlation between tumor mutational burden and immunotherapy sensitivity translates into a practical difficulty in defining thresholds that enable univocal patient stratification, with heterogeneous cutoffs proposed in different studies [16,17].

Tumor mutational burden may be inadequate in predicting immunotherapy sensitivity because it only partially contributes to tumor neoantigen load. For a somatic DNA variant to turn into a neoantigen, it must be transcribed, translated, processed to be loaded onto MHC molecules, and eventually recognized by a T cell receptor. This process is heavily influenced by how much the protein product differs from its wild type counterpart and by the **HLA haplotype** of the individual patients, which determines if and with what affinity the presentation of the antigen will occur (Box 2). Copious HLA-binding prediction tools, mainly focused on class I MHC binders, are currently available. Because these packages have been developed using supervised learning approaches trained on mass spectrometry experiments or protein structure and docking predictors [18], their output is typically constituted by a number of candidates that vastly exceeds the representation of 'real' neoantigens capable of triggering functional antitumor responses in patients [19–24]. The suboptimal performance of such algorithms hampers their implementation as **neoepitope** prediction tools for routine treatment decisions.

Other parameters should also be taken into account for the evaluation of the quality of a neoantigen. There is increasing evidence that neoantigens derived from clonal mutations or from tumors with low levels of heterogeneity (thus present in a substantial fraction of cells) may elicit more effective immune responses than neoantigens derived from subclonal mutations [25,26]. This notion may have profound implications in the context of MSI CRC. On the one hand, the genetic instability inherent in this tumor subtype makes it prone to continuously produce potential neoantigens [27]; on the other hand, dynamic neoantigen generation inevitably introduces a certain extent of subclonal diversity, which could penalize immune stimulation. This assumption may account for the poor response to immunotherapy displayed by a fraction of MSI CRCs. Tumors can also fail to present potentially immunogenic neoepitopes, commonly due to the loss of β2-microglobulin (a subunit of class I MHC) or the disruption of HLA alleles [28]. The fact that not all DNA mutations translate into neoantigens; the observation that neoantigen frequency influences immunogenicity; and finally, the possibility for cancer cells to disrupt the antigen presentation machinery, all attest to the shortcomings of using conventional genomic profiling for immunotherapy response prediction.

antigen-presenting cells such as dendritic cells and macrophages, bind and present 13–25 amino acid peptides to $CD4^+$ T cells.
Neoantigen: a newly expressed tumor antigen that arises from genetic alterations in tumor cells and therefore is not present in normal cells.
Neoepitope: the part of a neoantigen that is specifically recognized by an antigen receptor.
Optimization problem: the problem of finding the best solution from the set of possible solutions that satisfies all constraints.
Th1 cells: a differentiation state of $CD4^+$ T effector cells characterized by production of IFNγ (and other cytokines) upon T cell activation and clonal expansion.

Box 1. Different Routes for Immune Desertification

Tumors can proactively contrast immune cell infiltration [55]. In some cases, T cells are observed at the tumor margins but do not penetrate the tumor tissue. Therefore, a potentially effective T cell-mediated immune response is aborted by physical exclusion. In other cases, the tumor mass displays only a low degree of immune infiltration, likely due to the presence of an immune-suppressive microenvironment that prevents further recruitment and expansion of immune cells (Figure 1). With the exception of those with a high Immunoscore, MSS CRCs are a prominent example of these so-called immune desert tumors; quite strikingly, MSS CRC tumors may show even lower levels of cytotoxic T cell activation than the normal colonic mucosa [8].

One frequent cause of absent or sparse intratumoral T cell content is impaired immune cell extravasation through changes in the cellular and molecular composition of the tumor-associated vasculature [56]. Another route for manifestation of the T cell exclusion phenotype is activation of the β-catenin pathway, which results in transcriptional repression of immunostimulatory chemokines. The limited availability of such chemokines lessens the recruitment of dendritic cells and, in turn, reduces the attraction and activation of $CD8^+$ cytotoxic T cells [57]. This mechanism is of particular relevance in the CRC setting, in which β-catenin is frequently activated on a genetic basis. In particular, biallelic loss of *APC* (a gene that inhibits β-catenin activity) has been detected in 62% of MSS cases and 20% of MSI cases, where it is typically associated with downregulation of T cell infiltration [28,58]. The lower incidence of *APC* mutations in MSI tumors may account, among other factors, for their higher susceptibility to immunotherapy.

Tumors can also oppose immune cell deployment through the production of soluble factors that thwart antitumor immune responses [55]. In mouse models of MSS CRC, stromal fibroblasts have been found to secrete copious amounts of TGFβ, which blunts the recruitment and/or activation of $CD3^+$ and $CD4^+$ cells and downregulates the expression of IFNγ effector molecules [46,47]. High TGFβ levels correlate with transcriptional traits of abrogated T cell differentiation in MSS CRC clinical gene expression datasets [46], suggesting that TGFβ-mediated immune suppression also occurs in humans. Interestingly, MSS CRC tumors that become resistant to the EGFR antibody cetuximab (a standard-of-care therapy in patients with metastatic disease) feature a higher content of stromal TGFβ but also, somehow paradoxically, a more abundant infiltration of dendritic cells and lymphocytes compared with their cetuximab-naïve counterparts [49]. Whether these contrasting phenotypes represent a synchronous balanced reaction to EGFR inhibition, or are part of a sequential adaptation program in which the immunostimulatory switch precedes and is counteracted by immune suppressive stromal remodeling, remains to be determined [59].

Immune Infiltrate in CRC: Enough Is Not Enough

A correlation between neoantigen load and immune infiltrate has been repeatedly documented [29,30]. On this basis, it is not coincidental that MSI CRCs, with their high mutational burden and antigenic load, show an active immune microenvironment [6,31,32]. Both gene expression analyses and histopathological immunophenotyping have demonstrated that MSI CRCs exhibit higher densities of **Th1 cells** with massive **IFNγ** production, effector-memory T cells, and *in situ* proliferating T cells than their MSS counterparts [6,33]. Moreover, MSI CRCs are frequently infiltrated with mutation-specific cytotoxic T cells, confirming the presence of active neoantigens. Efforts have been made to develop a universal standardized scoring system, named Immunoscore, for the categorization of the immune infiltrate [7]. This method, which is based on the quantification of two lymphocyte populations (**CD3** and CD8) both at the tumor center and at the invasive margins, has proven to explain some biological features of colorectal tumors. MSI CRCs usually have a more favorable prognosis compared with MSS tumors and display a high Immunoscore in the majority of cases. A subgroup of MSS CRCs with good prognosis also have a high Immunoscore. Hence, Immunoscore shows an advantage over MSI status as a biomarker of recurrence and survival, given that it also predicts outcome in MSS CRC patients [6].

The higher prognostic significance of Immunoscore compared with MSI status does not reflect into a higher predictive value for stratifying patients likely to respond to immunotherapy. The range of MSS CRCs with high Immunoscore varies from 21% to 50% [6,7], but a comparable response rate to ICIs has never been registered in individuals with such tumors. Thus, a high Immunoscore appears to be indicative of an immune contexture that has sufficient strength to mitigate tumor progression, but not enough competence to unleash the potential of immune checkpoint blockade. It should be also

Box 2. HLA Typing Algorithms

Determining HLA alleles is necessary to identify which products of missense somatic mutations can be efficiently presented by MHC molecules, a prerequisite for mutant peptides to become potential neoantigens. Pioneer methodologies were based on restriction fragment length polymorphism (i.e., on the use of restriction enzymes recognizing polymorphic restriction sites in linkage disequilibrium with different HLA alleles) and on deconvolution of the individual alleles using lengths of the digested fragments [60]. Now, next-generation sequencing (NGS) enables identification of somatic mutations and HLA alleles with a single assay. Given the clinical relevance of accurate HLA typing, not only for cancer immunotherapy but also in other clinical settings, such as transplantation medicine, several efforts have been devoted to obtaining an NGS-tailored protocol that reaches the same performance as sequence-based typing, the clinical gold standard. This assay, which is based on DNA PCR amplification and Sanger sequencing, is afflicted with high costs, labor-intensiveness, and a certain degree of inaccuracy [61].

NGS algorithms are divided into two main categories: those that align reads on a reference made of all the known variable alleles of MHC loci, obtained from the IMGT/HLA database [62]; and those that perform a *de novo* assembly of the reads before comparing them with the known alleles. All algorithms are meant to find the alleles with the highest probability of having generated the sequenced reads, employing **Bayesian inference**, **greedy algorithms**, or **integer programming** to solve this **optimization problem**. Finding the right solution is, however, a challenge, owing to similarities of the loci sequences, our still incomplete knowledge of all the existing HLA alleles, and HLA codominance in heterozygous individuals. A recent study has benchmarked the performance of several HLA typing methodologies [61]; the median success rate, defined as the number of alleles with correct determination divided by the number of all determined alleles, is 61% (range, 12%–99%). The best performing tools are OptiType, an alignment-based approach that includes information from intron sequences (at present, it is only available for class I MHC) [63]; and PHLAT, again an alignment-based method that considers the distribution of HLA alleles in the human population when evaluating those that most likely have originated a given set of reads [64]. An extensive evaluation of all these approaches in the context of cancer is still missing, so it remains unclear whether complex scenarios, for example, somatic mutations and chromosomal rearrangements that involve HLA loci, will be properly addressed with the existing packages.

considered that solid tumors may display intralesional spatial heterogeneity for markers of active immune infiltration, as reported in non-small cell lung cancer [25]. If also present in CRC, this characteristic might explain the poor predictive power of Immunoscore, which is inevitably influenced by the selection bias of tissue sampling.

Immune Evasion: Showing Off Does Not Pay Off

The fact that not all tumors with an abundant and active immune infiltrate respond to ICIs highlights how preponderant the mechanisms of immune evasion are in cancer. This is not surprising, considering the high grade of plasticity that enables cancer cells to adapt to and survive adverse microenvironmental conditions. The interaction between the immune system and cancer cells has its inception in the early stages on neoplastic transformation and develops along the tumor natural history through a continuous, dynamic, and evolving course of events. This interaction is classically divided into three subsequent phases [34]: (i) elimination, when premalignant lesions are culled by effector immune cells that recognize tumor neoantigens; (ii) equilibrium, a variably lasting condition in which the growth of incipient tumors is counterbalanced by an ongoing anticancer immune response that is steadily becoming ineffective; (iii) escape, the moment when neoplastic cells elude immune surveillance to establish progressive lesions. Hence, by definition, a cancer that becomes clinically manifest in an immunocompetent subject must have implemented some mechanisms to evade immune pressure.

The incessant sculpting of cancer cells by the immune system is defined as genetic immunoediting, a Darwinian evolutionary process whereby cell clones expressing antigenic neoepitopes are dramatically depleted while poorly immunogenic, hardly recognizable cancer cell subpopulations become positively selected (Figure 1). The tumor cell population adjustment caused by genetic immunoediting can be measured analytically as a reduction in the ratio between observed and expected

neoantigen rates ('immunoediting score') [8]. The weight of genetic immunoediting in shaping tumor evolution can be captured by the recently described correlation between class I/II MHC genotypes and oncogenic mutational patterns [35,36]. Mutations that are poorly presented by MHC molecules have been shown to occur at a higher frequency in tumors, most probably because they have less immunological relevance. Similarly, disruption of HLA alleles is associated with an enrichment of predicted antigens binding the HLA lost allele, likely because peptides that are no longer presented evade negative selective pressure [37]. Thanks to the availability of scores that measure the likelihood and efficacy of peptide presentation, built on *in silico* tools that predict peptide binding affinities, the assessment of interindividual variation in HLA genotypes can provide information on the prevalence of mutations in cancer at the single-patient level [38].

In the case of CRC, the longitudinal analysis of multiple metastases in two patients has revealed that immunoedited tumor clones tend to be eliminated over time [39]. Albeit counterintuitive, this finding could be explained by considering that immunoedited cancer cell subpopulations are remnants of the persisting activity of a 'hot' immune microenvironment, which can still target weakly immunogenic cell variants as soon as they accrue novel neoantigens as a consequence of DNA replication. Besides being molded by time, the dynamic interplay between CRC cells and the immune system also experiences spatial diversification; indeed, independent metastatic lesions in the same patient were found to display a heterogeneous repertoire of neoantigenic subclones and different immunoediting scores [39].

Genetic immunoediting appears to be a relatively common hallmark of CRC, but it is less prevalent in MSS tumors [40]. One possibility is that the low absolute number of accumulated mutations in MSS tumors makes it difficult to accurately calculate the immunoediting score. Another interpretation stems from the notion, discussed above, that heterogeneous tumors with multiple subclonal neoantigens are less immunogenic than tumors with a dominance of clonal neoantigens [25,26]. Chromosomal instability (CIN), which typifies MSS CRC, is characterized by widespread somatic copy number alterations (SCNAs) that may contribute to mutational/neoantigen heterogeneity, thus rendering these tumors more resilient to the immune attack (Figures 1 and 2A). In CIN cases of lung cancer, for example, more than 13% of subclonal mutations (range, 0%–56%) are due to SCNA loss of genomic segments carrying clonal mutations [41]. This result has been confirmed in a recent study demonstrating that 50% of lung cancers show evidence of at least one historically clonal neoantigen being subclonally lost owing to subclonal copy number events [42]. In addition, high levels of arm and chromosome SCNAs could cause a general dosage imbalance against potential neoantigens, or neoantigen competitive disadvantage for loading onto MHC compared with the great amount of self-peptides without *de novo* mutations (Figures 1 and 2B). All these mechanisms afford cancer cells with the ability to survive immune attack, even in the presence of an abundant immune infiltrate. Other routes that tumors can take to evade immune surveillance rely on the physical or functional exclusion of immune cells from the tumor tissue, either through production of immune suppressive cues by stromal and inflammatory cells or because of architectural abnormalities in the vasculature (Box 1).

Therapeutic Implications: Some Like It Hot

All the above considerations highlight the pervasiveness of resistance to immunotherapy, but they also offer some hints on potential mechanisms to improve therapeutic response. One possibility to turn immune 'cold' tumors into 'hot' tumors is to enhance stromal inflammation. For instance, experiments in a number of preclinical models have shown that antiangiogenic agents can sensitize to PD-1 checkpoint blockade by promoting tumor necrosis (with the ensuing potentiation of antigen presentation by intratumoral phagocytes) and by facilitating the extravasation of effector T cells as a consequence of therapy-induced blood vessel normalization [43]. However, this approach is likely to be unproductive in CRC, as CRC liver metastases typically become vascularized by the nonangiogenic mechanism of vessel cooption (a process whereby tumors incorporate pre-existing vessels from the surrounding tissues rather than stimulating new vessel sprouting) [44]. Depletion of protumorigenic macrophages [45] and inhibition of TGFβ [46,47] have been also demonstrated to boost immune cell activation in CRC xenografts or genetically modified mice. In a complementary perspective, weakly antigenic CRC cell lines can become more immunogenic when treated with DNA

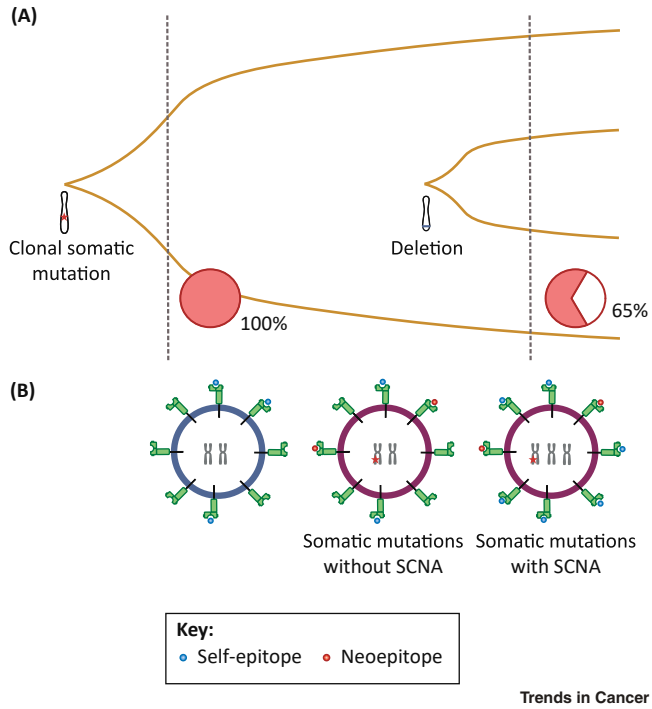

Figure 2. Somatic Copy Number Alterations (SCNAs) Affect Clonality and Dosage Imbalance of Neoepitopes versus Self-Peptides.

(A) A focal deletion in a DNA locus harboring a previously clonal, and possibly antigenic, somatic mutation reduces the fraction of cells with that mutation. Subclonal mutations are less immunogenic than clonal mutations. (B) Arm or whole chromosome amplifications can create an imbalance in the expression of germline versus mutated alleles (encoding self-epitopes and neoepitopes, respectively), lowering the relative number of neoepitopes presented to the immune system.

mutagenic agents, such as temozolomide [15], or with drugs that trigger immunogenic cell death (a modality of cancer cell death that renders dying cells visible to immune cells). In this context, the EGFR antibody cetuximab is known to induce immunogenic cell death in CRC cells [48]; accordingly, cetuximab-treated CRC tumors in metastatic patients have more abundant lymphocytic infiltration than the treatment-naïve counterparts [49] (Box 1).

Tumors that initially respond to ICIs may become insensitive to therapy after a certain amount of time (a condition known as secondary, or acquired, resistance). Intriguingly, some of the mechanisms responsible for intrinsic refractoriness may be also involved in the onset of secondary resistance, including loss of expression of PD-1/PD-L1, class I MHC, or IFNγ [50]. Protein downregulation can be due to promoter methylation [51,52], a notion that has spurred the design of several clinical trials with ICIs and epigenetic drugs [52]. While the majority of such trials are being conducted in melanoma patients, some are recruiting subjects with various types of advanced solid tumors. It will be interesting to see whether these combinations will be active in CRC.

Concluding Remarks

The criteria proposed so far for the enrichment of patients potentially responsive to immunotherapy are based on static parameters, extrapolated from a freeze-frame of the tumor along its natural history: the mutational load of a biopsy, the quality and quantity of the immune infiltrate, or the expression of immune checkpoints in an archival surgical sample. The information obtained from such an approach, although easily amenable to clinical implementation, does not grasp the dynamic nature of the interactions between

the tumor ecosystem and organismal immunity and limits the biological understanding of how these two entities coevolve from cancer onset to full-blown progression. A time-resolved picture of the landscape of mutations and neoantigens, coupled with an appraisal of the specificity and strength of the immune response as it impacts upon the tumor, would be key to address the many open issues that complicate our interpretation of sensitivity and resistance to immunotherapy. This knowledge would be particularly crucial for CRC, a tumor setting where the different evolutionary paths of MSI and MSS subtypes inevitably lead to divergent dynamic interactions with the immune system over time (see Outstanding Questions). Methodologically, an endeavor of this kind implies the development of both experimental and computational tools. Cocultures of primary tumor epithelia *en bloc* with endogenous, syngeneic tumor-infiltrating lymphocytes are feasible and, at least in principle, upgradable into even more holistic models by introducing additional immune components from lymph nodes or blood [53]. The availability of such cocultures is expected to streamline future investigations by providing a manipulatable platform to explore heterotypic crosstalks basally and under drug pressure. Analytical metrics to study neoantigen subclonal dynamics are also emerging and can be further improved by capitalizing on the wealth of algorithms that are routinely employed for delineating the clonal and mutational evolution spectra of cancer cells. At the clinical level, longitudinal monitoring of circulating tumor DNA (ctDNA) may provide important information on how tumor mutational burden and neoepitope prevalence change along time spontaneously and over the course of immunotherapy [54]; moreover, the current availability of technologies to measure ctDNA methylation patterns will prove useful to detect and study epigenetic mechanisms of resistance [54]. Ultimately, experimental, analytical, and clinical models that incorporate the evolutionary constraints imposed by the immune microenvironment in time and space will catalyze future efforts to disentangle the links between tumor genetic instability and heterogeneity, dynamic neoantigen production, and immunoediting. This knowledge will be a prelude to actualizing the promise of precision immunotherapy.

Outstanding Questions

Is the identification of static and generalizable biomarkers of response to immunotherapy feasible?

How can we bridge the gap between predicted neoantigens and those that are actually functional?

Why do some MSI CRCs not respond to immunotherapy and to what extent can this resistance be related to their high heterogeneity?

Why is the response of MSS CRCs to immunotherapy worse than any biologically sound parameter would predict and to what extent can this resistance be attributed to their SCNAs?

Is it possible to experimentally and computationally model the coevolution of cancer cells and the immune system in order to obtain a time- and space-resolved illustration of tumor progression from a global immunogenomic perspective?

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