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Perspective

The thin red line between the immune system and cancer evolution

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ABSTRACT

The cancer immunoediting theory describes the dual ability of endogenous antitumor immunity to inhibit or promote progressing cancers. Tumor-specific neoantigens arising from somatic mutations serve as targets for the endogenous T-cell-mediated antitumor immunity and therefore possess a crucial role for tumor development. Additionally, targeting these molecules is conceptually appealing because neoantigens are not expressed in healthy tissue and therefore confer less toxicity and greater specificity when used in therapeutic interventions. Moreover, intratumor neo-antigenic heterogeneity is believed to play a pivotal role in the activation of adaptive immunity and in the efficacy of immunotherapies that are based on immune checkpoint inhibition. In this respect, mutual interactions between tumor cells and immune lymphocytes regulate the levels of antitumor immunity, but also shape tumor heterogeneity through the selective outgrowth of tumor subclones. Therefore, the exploration of the mechanistic pathways and the identification of the genomic aberrations underlying the clonal evolution of tumors is considered mandatory for improving the clinical outcomes of therapies, as it will assist in the selection of the appropriate therapeutic decisions so as to delay, avoid, or overcome resistance through the identification of the most effective therapeutic strategies.

Introduction

During tumor clonal evolution, clones with distinct molecular features emerge as the result of resistance to intrinsic selective pressures, which are mediated by continuous and dynamic interactions between the tumor and elements of the immune system that constitute the endogenous antitumor immunity [1]. Tumor resistant phenotypes may also emerge during immunotherapies and in the course of cytotoxic and targeted therapies, which may additionally affect tumor immune reactivity, at least to a certain extent [2]. Clonal expansion of resistant tumors includes multiple molecular mechanisms that emerge during the continuous dynamic interactions between immune lymphocytes and tumor cells; during this process the former eliminate tumor immunogenic clones and, in this way, convey antitumor protective immunity, but also select for specific tumor clonal variants. The remaining, unselected tumor clones escape immune attack via the accumulation of genetic alterations, a process that is known as immunoediting and which may variously hamper antitumor immunity [3]. Although immunoediting explores the cell interactions taking place between the endogenous antitumor armamentarium and the tumor, accumulating evidence suggests that this process may also occur during the course of immunotherapies or other types of therapy that aim at reinvigorating the exhausted tumor reactive T-cell-mediated immunity [3]. In this article, we provide our perspective on the biological processes underlying the interconnected relationship between endogenous antitumor immunity, tumor heterogeneity and immune resistance, on the grounds of neo-antigenic heterogeneity. We also discuss the importance of investigating the dynamic and continuous interplay between endogenous antitumor immunity and tumor cells in order to gain valuable insight into the biological pathways regulating the anti-tumor response during immunotherapies.

Tumor heterogeneity and immune resistance

Genetic heterogeneity among the malignant cell clones dominating a particular tumor poses a serious obstacle for effective disease management. Such clones are genetically diverse and potentially confer a survival advantage to the growing tumor by ultimately promoting the evolution of genetically diverse tumor subclones in low frequencies [2]. This implies that not all subclones are equally susceptible to a certain therapeutic modality and that some will survive and progress, a property that could cause therapeutic imbalance and give rise to resistant tumor

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phenotypes [2]. This observation is becoming more apparent in the context of immunotherapies where, despite the durable clinical responses seen in a subset of patients, most patients do not respond to treatment or relapse after an initial response. Disease progression in this case may be due to acquired immune resistance or to the selection of resistant tumor clones that were present before treatment, albeit at low frequencies [4]. However, in either case, tumor progression seems to manifest in an environment of a constantly developing antitumor response that is either preexisting, shaped by both genetic and environmental factors, or activated during immune checkpoint blockade or, as mentioned above, in the course of other immunomodulatory treatments such as radiotherapy, chemotherapy and several targeted therapies [2]. It can therefore be easily understood that the interplay between endogenous antitumor immunity and tumor cells can result in tumor heterogeneity, even before the tumor becomes clinically apparent, and this may play a critical role in establishing resistance mechanisms before the administration of immunotherapy or any other form of anti-cancer therapy [1]. Thus, it becomes important to understand the spatial distribution of genetically diverse tumor clones within the tumor microenvironment (TME) prior to or after therapy in order to be able to estimate the extent to which tumor heterogeneity influences immune resistance. To this end, it is reasonable to consider that while the tumor mutational burden (TMB) is found throughout the tumor mass, mutations can be differently distributed among the tumor regions, generating a spatial separation of tumor subclonal populations within the heterogenic tumor microenvironment; this can in turn cause an underestimation of the status of mutations and their clonal distribution. It also implies that the tumor is not overpopulated by a single dominant clone but rather by several genetically distinct subclones that are found at distinct tumor sites. In this context, it has been shown that even in cases where a dominant clone harboring a primary mutant driver gene overpopulates the tumor, subclones at low frequencies that carry additional mutations may provide nonredundant functions that cooperatively promote therapeutic resistance and minimize clinical outcomes [5]. Therefore, the genetic basis for cancer progression comprises multiple tumor-promoting signaling pathways that are activated by different mutations; the latter must be identified and targeted therapeutically so as to achieve precision oncology – based therapies that can be applied to several types of cancer. A necessary prerequisite for this will be a thorough molecular diagnosis and reasonable combinatorial treatments to target not only any primary driver mutation arising in dominant tumor clones, but also mutations occurring in low-frequency subclones, so as to avoid progression to therapeutic resistance.

Neo-antigenic heterogeneity and antitumor immunity

Genomic instability leading to intratumoral mutational diversity with high TMB and neoantigen expression in individual tumors may have a significant impact on antitumor immunity and therefore it is critical to gain information regarding the effect of T-cell-derived immunity on clonal vs subclonal tumor populations. According to the immunoediting theory the selective pressure of the antitumor immune response towards neoantigens markedly decreases tumor heterogeneity via eradication of immunogenic tumor clones [6]. Hence, tumors heavily infiltrated with effector programmed cell death 1+ (PD-1+) CD8+ T cells have a favorable prognostic outcome in various types of cancer, further emphasizing the need for targeting this subset of lymphocytes during immunotherapies, especially in the form of immune checkpoint inhibition (ICI). To this end, monoclonal antibodies targeting PD-1 or PD-1 ligand (PD-L1) provide unprecedented prolongation of cancer patients' survival. However, only a restricted number of patients experience benefits from ICI and even though high TMB has been associated with durable responses during ICI, clinical responses to ICI may also be observed in patients with low TMB [7,8]. Thus, TMB alone may not adequately predict favorable clinical responses to ICI. On the other hand, PD-L1 has been disputed as a predictive biomarker because of robust clinical responses in patients with low PD-L1 expression [8,9].

Such inconsistencies in the clinical outcomes of patients with low TMB and low PD-L1 levels could be explained by intratumoral heterogeneity (ITH) that occurs due to subclonal neoantigen expression. Tumor-specific neoantigens are generated through somatic mutations and constitute attractive targets for therapeutic intervention since they constitute "foreign" molecules recognized by neoepitope-specific CD4+ and CD8+ T cells not affected by central tolerance, thus conferring higher antitumor reactivity and less off-target side effects [6]. In the study by McGranahan and colleagues [5], patients with early-stage non-small cell lung cancer therapeutically treated with anti-PD1 were examined for correlations between neoantigen heterogeneity in their tumor samples and clinical responses. There was a significant prolongation of progression-free survival (PFS) in patients with clonal tumors characterized by high clonal neoantigen burden and low neoantigen heterogeneity (neoantigen ITH $\leq$ 1%) as compared to PFS in patients with heterogeneous or low clonal neoantigen burden tumors (i.e., subclonal tumors having high neoantigen ITH and low neoantigen burden). Moreover, clonal tumors were infiltrated by PD-1+ CD8+ T cells specific for clonal neoantigens and also exhibited high expression of genes associated with antitumor responses and predicting responses to immunotherapy including those coding for (i) CD8A and CD8B (expressed by cytolytic effector cell populations); (ii) TAP-1, TAP-2, and STAT-1 (associated with antigen presentation pathways); (iii) CXCL10 and CXCL9 (involved in T-cell migration pathways); and (iv) IFN-γ and granzymes (associated with effector T-cell function). Similar results were obtained with melanoma patients treated with anti-CTLA4 [5], proposing that clonal tumors, based on neoantigen expression, are more immunogenic as compared subclonal tumors (demonstrating high levels of neoantigen heterogeneity) and that clonally expressed neoantigens may act as predictive biomarkers for selecting patients most likely to respond to ICI. Thus, it seems plausible that T-cell-mediated immune selection during immunoediting contributes to the emergence of clonal tumor. By contrast, lack of immune selection due to no or low T-cell infiltration in the tumor, would result in heterogenous tumors (Fig. 1).

Accordingly, the T-cell receptor (TCR) profile has been associated with responses to ICI and overall survival [10], further supporting the notion that the neoantigen composition of the tumor greatly influences the outcome of T-cell targeting immunotherapies. However, there is also substantial evidence to support the presence of neoantigen heterogeneity within individual tumors via the accumulation of mutations during tumor evolution [8]. Accordingly, increased neoantigen heterogeneity dictates that single neoepitopes will be expressed by distinct tumor subclones at lower frequencies, thereby reducing the possibility to generate robust antitumor immunity; at the same time this diminishes the potential of T cells to recognize and target all tumor cells, and therefore restrains control of tumor subclones providing them with the opportunity to repopulate the tumor later on. On the other hand, limited neoantigen ITH implies that neoantigens are clonally expressed in the majority of tumor cells, thus constituting a significant percentage of the neoantigen burden, and may therefore trigger a more pronounced antitumor T-cell response leading to their eradication. Importantly, therapeutic targeting of neoantigens that derive from mutations in driver oncogenes has emerged as an appealing strategy, especially in tumors with low TMB, given that such driver mutations would not only be tumor-specific, but would also be expected to reside in all tumor clones, because they are essential drivers of disease progression [8].

The clonal, as opposed to the subclonal expression of neoantigens, may be mandatory for eliciting robust antitumor responses, as evidenced by the relatively high levels of their cognate T-cell clone frequencies. Accordingly, high levels of intratumoral neoantigen heterogeneity have been associated with relapses following ICI, whereas sensitivity to this particular type of treatment appears to increase in tumors with clonal expression of neoantigens [5]. At this point it is worth mentioning that clonal expression of tumor neoantigens does not necessarily presuppose their equal distribution in the TME; on the contrary, they may be

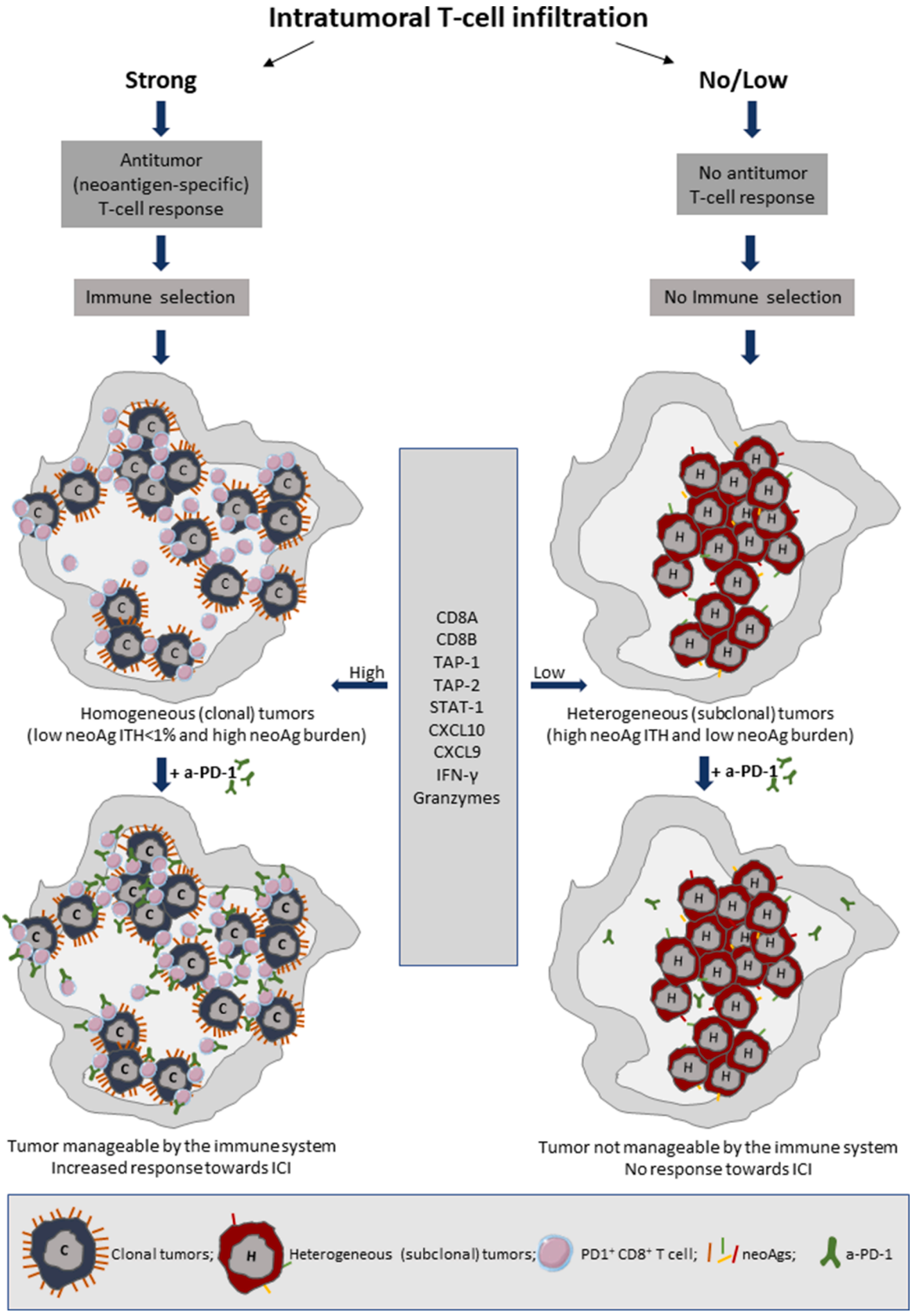

Fig. 1. During immunoediting, tumor-(neoantigen)specific T-cell infiltrates by developing antitumor neoantigen-specific responses select for clonal tumors predictive for a response to ICI. Instead, by no or low T-cell infiltrates there is lack of immune selection and the tumor remains heterogenous with lack of response to ICI. Immune-related genes are upregulated in clonal tumors. ITH: intratumoral heterogeneity; neoAgs: neoantigens.

spatially restricted, generating topographical differences in the immunogenicity and capacity of different tumor compartments to generate tumor-specific T-cell responses [11]. Such responses are mediated by either CD8+ or CD4+ T-cell subsets, depending on the nature of neoantigens and MHC expression of the tumor subclones [11]. TCR sequencing performed in various regions of tumors from lung cancer patients have revealed considerable differences in TCR clonality and expansion; such TCR heterogeneity has in turn been shown to correlate with neoantigen heterogeneity, suggesting a prominent role for neoantigens in inducing spatial variations in the tumor-specific T-cell repertoire [12].

Endogenous antitumor immunity, tumor clonality and response to ICI

The intratumoral immunity targeting tumor neoantigens mostly constitutes the endogenous antitumor immunity which, according to the immunoediting theory, determines tumor evolution [3]. Our knowledge of the regulation of endogenous antitumor immunity has been greatly enhanced through the discovery of immune checkpoint inhibitors (e.g., CTLA-4, PD-1), followed by the generation of monoclonal antibodies targeting such inhibitory receptors and their corresponding ligands. Immunotherapy based on ICI has uncovered the indispensable role of endogenous T-cell immunity in controlling tumor growth [7]. There is also enough clinical evidence to suggest that in order for anticancer treatments to be effective, they must induce *de novo* antitumor immunity or reinvigorate the endogenous antitumor response [13]; in this way dynamic alterations in the intratumor immune landscape generate a transition from a pre-existing antitumor immune response to a treatment-regulated immune response. Hence it can be concluded that the infiltration of inflamed tumors by immune (CD8+) T cells is a prerequisite for effective endogenous antitumor immunity. In contrast, non-inflamed tumors lack immune lymphocyte infiltrates and as such remain unresponsive to ICI (Fig. 1). Nonetheless, we should also bear in mind that such non-inflamed tumors may still express neoantigens that can be recognized by T cells [12] and that there are still strategies which can render such tumors susceptible to immune attack [9]. For example, the observations that CTLA-4 blockade induces infiltration of tumor-reactive T cells intratumorally and PD-1 inhibition activates intratumoral T cells, support the combination of these two antibodies as the most efficient strategy for generating antitumor immunity in non-T-cell-inflamed tumors. T-cell exclusion from the tumor could also be due to the absence of T-cell recruiting chemokines. For example, constitutive activation of the β-catenin pathway has been found to downregulate STING activation, followed by deficient production of type I interferons and defective recruitment of mature dendritic cells, which in turn results in low levels of CXCL9 and CXCL10 and insufficient T-cell infiltration [14]. Thus, inhibition of β-catenin signaling could lead to recruitment of T cells into the tumors, and as such provide a platform for combination treatment with ICI. Therapeutic cancer vaccines that are based on neoantigen targeting could also enable tumor infiltration by eliciting the response of vaccine-specific and neoantigen-targeting T cells; this represents an alternative therapeutic approach which aims at generating a *de novo* T-cell-inflamed tumor and at combining vaccines with ICI. Based on these observations, the intratumoral landscape and the endogenous antitumor immunity may have leading roles in regulating the outcome of therapeutic responses to ICI. Therefore, it is critical to examine the differences between clonal versus subclonal expression of neoantigens in tumors with high TMB and their respective clinical responses to ICI, so as to be able to assess the question regarding the extent to which the neo-antigenic landscape impacts intratumoral antitumor immunity. To this end, it is of particular importance to refer to clinical studies in which genetic alterations were increased during chemotherapies with creation of new tumor subclones suggesting that chemotherapy can increase TMB and intratumor heterogeneity resulting in decreased clinical response [8].

As also mentioned above, even though clonal expression of neoantigens is associated with clinical efficacy following ICI, tumor cells can still employ mechanisms of acquired immune resistance which may hamper the generation of antitumor immunity, as for example those that affect the tumor antigen-presentation machinery [4]. Such types of escape tumors may develop during immunoediting under the selective immune pressure of T cells directed against immunogenic neoantigens. Subsequently, it is reasonable to consider that immune selection impacts neoantigen heterogeneity by eliminating the majority of tumor cells and resulting into more clonal tumors. This implies that T-cell infiltration of tumors is a prerequisite for tumor clonality and subsequently for clinical efficacy during ICI therapies. By contrast, the absence of intratumoral T cells (i.e., tumors without endogenous antitumor immunity) could result in subclonal neo-antigenic heterogeneity, thereby creating tumor heterogeneity that is associated with clinical failures during ICI. One could also argue that high levels of tumor neo-antigenic heterogeneity dampen antitumor immune reactivity even in T-cell-infiltrated tumors, thus raising the question of whether the heterogeneity of the tumor is shaped by the magnitude of the immune pressure or whether the extent of tumor heterogeneity determines the robustness of the antitumor immune response. These possibilities could result in the differential intratumoral expression of neoantigens and therefore in the emergence of intratumoral TCR heterogeneity, which in turn reflects the differences in the potential of different tumor compartments to generate antitumor immunity. In this context, further unraveling of the process of tumor clonal evolution in response to selective immune pressure will be of paramount importance for the integration of neo-antigenic heterogeneity that will confer positive clinical outcomes following ICI and for enabling the design of more effective treatment strategies.

Conclusions

With the advancement of new technological platforms that enable detailed investigations of intratumoral heterogeneity, our knowledge of the evolutionary processes of tumors can be applied to improve the design of clinical trials, so as to gain better therapeutic efficacy and prevent or delay the development of therapeutic resistance. Surely, further investigations on intratumoral heterogeneity are warranted in order to understand the complex and continuous interactions between tumor and immune cells and the role of tumor heterogeneity in the response of patients to immunotherapies. Exploration of the mechanisms underlying intratumoral heterogeneity, will help to gain mechanistic insight in the induction of more effective antitumor immunity. The identification of genes functionally involved in the therapeutic response, along with high throughput whole exome sequencing, will enable the identification of genomic predictive biomarkers of clinical efficacy that will represent novel therapeutic targets. If one considers that intratumoral heterogeneity is driven by both immune and tumor cell-derived factors, it is only logical to assume that the pipeline for novel cancer therapeutics will be based on combinatorial treatments that aim at tumor cell destruction with concomitant generation of diverse and persistent antitumor immune responses. We anticipate that appreciating the importance of intratumor heterogeneity in shaping the immune response, combined with the evolving technologies that allow the discovery of mechanistic pathways implicated in the development of such heterogeneity, will improve our knowledge on tumor evolution and facilitate the design of novel, more effective therapeutic modalities.

Authors' contributions

CNB: Conceptualization, Writing – original draft, Visualization; **MG**: Writing – review & editing; **MA**: Writing – review & editing; **SPF**: Visualization, Writing – review & editing. All authors provided critical feedback, helped shape the final version of the manuscript and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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