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REVIEW

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Tumor-intrinsic oncogene pathways mediating immune avoidance

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ABSTRACT

Immunotherapy is emerging as a major treatment for patients with cancer, predominantly via blocking immune inhibitory pathways and through adoptive T cell therapy. However, only a subset of patients shows clinical responses to these interventions. Emerging data indicates a correlation between clinical response and a pre-existing T cell-inflamed tumor microenvironment. Tumor-intrinsic β-catenin activation has been identified as mediating exclusion of T cells from the tumor microenvironment and other oncogene pathways are being explored similarly. Understanding the molecular mechanisms underlying immune avoidance should identify new therapeutic targets for expanding efficacy of immunotherapies.

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Introduction

Recent developments in immunotherapy approaches for cancer are having significant clinical impact. In particular, monoclonal antibodies (mAbs) targeting the inhibitory receptors CTLA-4 and PD-1 have shown marked efficacy and have been FDA-approved for the treatment of patients with metastatic melanoma, and for non-small cell lung cancer in the case of anti-PD-1[1-3]. Significant clinical activity of anti-PD-1 or anti-PD-L1 mAbs has also been observed in multiple additional cancer types, including triple-negative breast cancer, head and neck cancer, bladder cancer, renal cell carcinoma, and others[4-6]. However, despite these exciting advances, only a subset of patients experiences clinical benefit in each of these tumor types. As such, understanding molecular mechanisms of primary resistance to immunotherapies has become paramount.

Gene expression profiling of melanoma metastases has revealed that a subset of patients shows evidence of a T cell-inflamed tumor microenvironment at baseline[7]. This phenotype includes evidence for expression of T cell-specific transcripts, chemokines, and a type I IFN gene signature[3,7]. The T cell-inflamed phenotype, in addition to demonstrating presence of $CD8^+$ T cells, also shows the highest expression of immune-inhibitory pathways, including expression of PD-L1 and indoleamine-2,3-dioxygenase (IDO) as well as presence of $FoxP3^+$ regulatory T cells[8]. Thus, antitumor immune responses in these cases appear to be held in check by immune-intrinsic negative feedback mechanisms. In contrast, tumors lacking T cells within the tumor microenvironment (non-T cell-inflamed phenotype) lack these inhibitory factors but instead seems to escape immune destruction through exclusion of T cells from the tumor site (Fig. 1). Thus, the mechanisms of immune escape appear to be distinct in these two major subsets of tumors. Perhaps not surprisingly based on this biology, the majority of clinical responses to immunotherapies appear to be restricted to tumors displaying the T cell-inflamed tumor microenvironment. This is the case for therapeutic cancer vaccines[9], but also has been observed with the anti-CTLA-4 mAb ipilimumab and recently with anti-PD-1[10]. Interestingly, post-treatment biopsies in melanoma patients treated with anti-PD-1 have demonstrated a marked increase in $Ki67^+$ proliferating $CD8^+$ T cells penetrating deep into the tumor microenvironment in response to therapy[10]. These observations are consistent with preclinical data indicating that immunotherapies targeting immune-inhibitory pathways predominantly function through re-activation of $CD8^+$ T cells already present within the tumor microenvironment[11].

Viewed from the perspective of immunotherapy resistance, therefore, absence of a T cell-inflamed tumor microenvironment at baseline appears to be an important biomarker. As such, the problem of resistance can, at first approximation, be reduced to a question of identifying molecular mechanisms that explain why a subset of patients have metastatic lesions that disallow accumulation of T cells within the tumor microenvironment. A major source of inter-patient heterogeneity is likely derived from differences in somatic mutations between individual patients' cancers[12]. Collectively, these concepts have led to the hypothesis that differential activation of specific oncogene pathways might explain the phenomenon of immune exclusion in a subset of cancers. Successful identification of such pathways should lead to new therapeutic approaches that may enable T cell entry into non-inflamed tumors and expand the fraction of patients capable of responding to novel immunotherapies.

Immunological mechanisms leading to spontaneous T cell priming and tumor infiltration when it does occur

One approach toward understanding why some tumors lack a T cell infiltrate is to gain insights from the mechanisms required for a spontaneous antitumor T cell response and T

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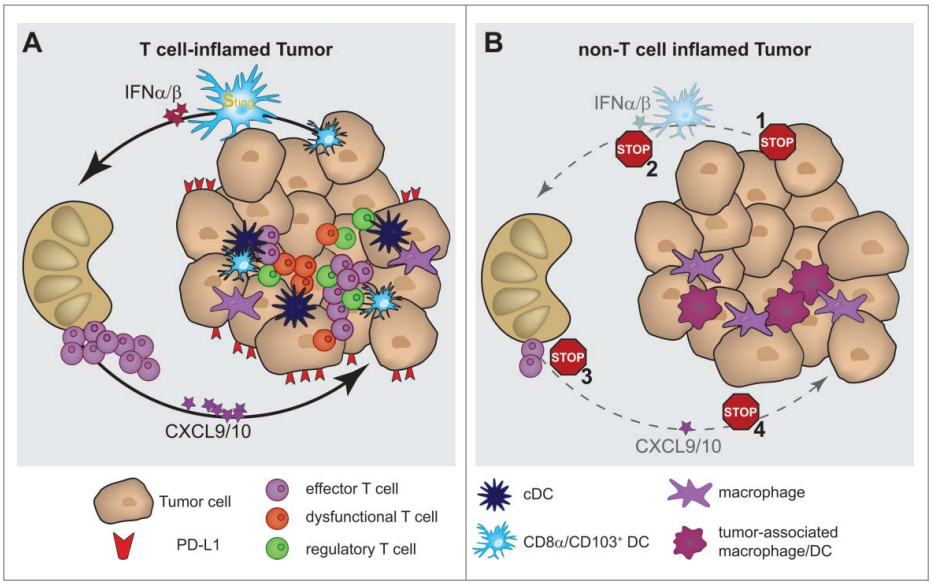

Figure 1. (T)cell-inflamed and non-(T)cell-inflamed phenotypes. (A) T cell-inflamed phenotype. CD8$\alpha^+$and/or CD103$^+$ dendritic cells (DCs) are sensing the tumor and upon stimulation through STING pathway activation, type I interferons are produced. These activated DCs migrate to lymph nodes to prime tumor antigen-specific T cells. Activated T cells are recruited back into the tumor microenvironment via CXCL9 and CXCL10 chemokines. Upon tumor infiltration and encounter with antigen, T cells produce IFNγ which leads to the upregulation of immune inhibitory mechanisms including PD-L1 and IDO, and also to produce chemokines that recruit regulatory T cells. (B) Non-T cell-inflamed phenotype. In this scenario the tumor microenvironment is lacking activated T cells, which could potentially be caused by 4 independent mechanisms (indicated by the indicated STOPs): (1) Lack of DC recruitment, (2) Lack of innate immune activation within DCs (3) Lack of adequate priming of antitumor T cells, or (4) Lack of trafficking of activated T cells into the tumor site. Despite lacking T cells, this phenotype still contains tumor-associated macrophages.

cell entry into the tumor microenvironment when it does occur. At minimum, tumors capable of priming a spontaneous antitumor T cell response must have antigens capable of being recognized by specific T cells. Although it has been suggested that non-T cell-infiltrated tumors might have far fewer antigens[13,14], preliminary data from our own laboratory has indicated that this is not likely the case. Analysis of the TCGA metastatic melanoma dataset has indicated comparable expression of differentiation antigens, cancer-germline antigens, as well as mutated self-proteins generating peptides presented by HLA-A*0201[15]. Thus, non-T cell-inflamed melanomas appear to have at least as many antigens as the T cell-inflamed tumors. However, the relevant innate immune pathways required to trigger a productive adaptive immune response against those antigens might not be engaged. Preclinical data have indicated that type I IFN signaling on host cells is necessary upstream from spontaneous T cell priming against tumor-associated antigens[3,16]. The mechanism of this effect is mediated via the Batf3-dependent subset of dendritic cells (DCs), which in the mouse express CD8α or CD103 and are superior at cross-presentation of antigens to CD8$^+$ T cells[3,17,18]. Interestingly, while the T cell-inflamed subset of human tumors shows evidence for a type I IFN gene signature, the non-T cell-inflamed tumors lack this type I IFN-driven gene expression profile[3,7,19]. Thus, the required innate immune pathways involved in this process might not be engaged. Recent work has indicated that the innate immune signals involved in triggering type I IFN production by host DCs in the tumor context occur predominantly via the STING pathway of cytosolic DNA sensing [16,20]. As such, interventions aimed at stimulating this pathway directly, for example using pharmacologic agonists of STING, are impressively therapeutic in preclinical tumor models[21] and attractive to consider for clinical development as a means to promote innate immune activation and an endogenous antitumor T cell response as a novel therapeutic. Some key steps in induction of antitumor immunity are depicted in Fig. 1A.

In the effector phase of the antitumor T cell response, expression of appropriate chemokines in the tumor microenvironment is critical for effector T cell trafficking into tumor sites. Gene expression profiling and confirmatory protein-based assays have confirmed that T cell-inflamed tumors show expression of a wide array of chemokines capable of recruiting CD8$^+$ effector T cells, whereas the non-T cell-inflamed subset lacks these chemokines[7,22,23]. Recent work has indicated that the mandatory chemokines for T cell entry into tumors are those that engage CXCR3, including CXCL9 and CXCL10[24]. Inasmuch as *in vitro* activation of the STING pathway in DCs triggers both production of type I IFNs and also the chemokines CXCL9 and CXCL10[16], it is plausible to consider that the minimal defect in non-T cell-inflamed tumors might be attributed to poor recruitment and/or activation of Batf3-lineage DCs into the tumor microenvironment. These concepts have formulated a reasonable working model as molecular explanations responsible for the non-T cell-inflamed tumor microenvironment phenotype are being pursued (illustrated in Fig. 1). Escape mechanisms from the immune system via exclusion could be at the level of recruitment of DCs and other innate immune cells (Stop 1, Fig. 1B), poor activation of DCs (Stop 2, Fig. 1B), inefficient priming of antigen-specific T cells (Stop 3, Fig. 1B), or failure to efficiently recruit primed T cells into the tumor microenvironment (Stop 4, Fig. 1B). All of these candidate arrests in the induction or execution of a tumor antigen-specific T cell response can be influenced by the tumor microenvironment, and could in principle be

impacted by specific oncogene pathways activated within the tumor cells.

Tumor-intrinsic Wnt/β-catenin pathway activation as a direct cause of T cell exclusion in melanoma

To begin to investigate the possibility that somatic differences at the level of the tumor cells themselves might explain the lack of a T cell-inflamed tumor microenvironment in a subset of cases, gene expression profiling of 266 individual melanoma metastases was analyzed in concert with exome sequencing of the same tumors[25]. Interestingly, exome sequence data revealed that seven tumors (14%) in the non-T cell-inflamed group showed gain of function mutations in the β-catenin gene. On closer examination, loss of function mutations in negative regulators of the β-catenin pathway (APC, Axin1, TCF1) were identified in an additional ten of the non-T cell-inflamed tumors (23%). Based on gene expression profiling of six defined β-catenin target genes, 48% of the non-T cell-inflamed tumors showed evidence for activation of the WNT/β-catenin pathway. The remainder of the tumors with evidence for β-catenin activation showed increased expression of either a Wnt-ligand family member (Wnt7b, 29.5%; 13 patients) or a receptor family member (Fzd3, 20.5%; 9 patients) or β-catenin itself (11%; 5 patients). Analysis of individual β-catenin target genes showed a negative correlation with CD8α expression in the tumor, whereas PD-L1 expression showed a positive correlation with CD8α as expected based on published results[8]. Immunohistochemistry confirmed high β-catenin protein expression predominantly in tumors that lacked $CD8^+$ T cells. These data therefore indicate a significant inverse correlation between β-catenin pathway activation and a T cell-inflamed tumor microenvironment[25].

To investigate the functional relevance of tumor-intrinsic β-catenin signaling in controlling the host immune response to melanoma, genetically engineered mice were constructed using a tamoxifen-regulated Cre driven by the tyrosinase promoter as developed by Marcus Bosenberg utilizing active Braf ($Braf^{V600E}$) and conditional PTEN deletion ($PTEN^{-/-}$), with or without a conditional active β-catenin mutant (CAT-STA)[26-28]. Tamoxifen-induced melanomas arising from $Braf^{V600E}/PTEN^{-/-}$ mice did have a modest T cell infiltrate as analyzed by flow cytometry and immunohistochemistry. However, melanomas induced by mutated Braf combined with active β-catenin ($Braf^{V600E}$/CT-STA) completely lacked a T cell infiltrate. Moreover, when PTEN deletion and stabilization of β-catenin ($Braf^{V600E}/PTEN^{-/-}$/CAT-STA) were combined, the melanomas that arose also lacked a T cell infiltrate. These results directly demonstrate that activation the β-catenin pathway within melanoma tumor cells can dominantly exclude immune cell activation and result in a non-T cell-inflamed tumor microenvironment.

Using this model system and based on fundamental knowledge of the mechanisms involved in spontaneous antitumor T cell responses, the mechanism by which tumor-intrinsic β-catenin activation antagonized antitumor immune responses was pursued. By combining the Cre-inducible expression of the model antigen SIY (SIYRYYGL)[29] with adoptive transfer of SIY-specific 2C TCR-transgenic T cells the extent of endogenous T cell activation could be determined. In fact, mice with SIY^+ tumors driven by $Braf^{V600E}$ mutation and PTEN deletion ($Braf^{V600E}/PTEN^{-/-}$) indeed showed spontaneous activation of 2C T cells as measured by CFSE dilution. However, no activation of 2C T cells was observed in mice bearing tumors driven by mutated Braf, PTEN deletion and active β-catenin ($Braf^{V600E}/PTEN^{-/-}$/CT-STA). These results indicated an early defect in immune priming, likely at the level of DC activation. Closer interrogation revealed that tumors expressing β-catenin showed a complete lack of recruitment of the Batf3-lineage DCs expressing the surface markers CD103 or CD8α. The mechanism of this defect was mapped to failed production of the critical chemokine CCL4 by the melanoma cells, which was downregulated by β-catenin via the transcriptional repressor ATF3 (see Fig. 2, zoom in). Importantly, this difference in baseline immune phenotype impacted on the ability of the mice to respond *in vivo* to immunotherapy. Whereas the combination

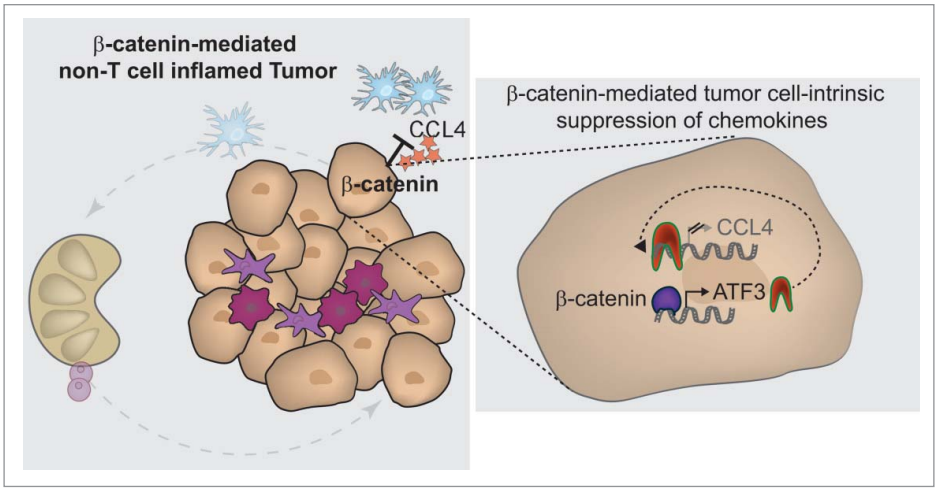

Figure 2. Molecular mechanism of β-catenin-driven immune escape. β-catenin-mediated immune avoidance occurs via inhibition of CCL4 production by the tumor cells themselves, as a result of induction of the transcriptional repressor ATF3, which blocks CCL4 gene transcription (see right zoom-in panel). This lack of CCL4-secretion results in failed recruitment of $CD103^+$ dendritic cells, thereby preventing cross-priming of antitumor T cells (indicated on left overview panel).

of anti-CTLA-4 + anti-PD-L1 mAbs slowed tumor growth in inducible $Braf^{V600E}$/$PTEN^{-/-}$ mice, there was no therapeutic effect in $Braf^{V600E}$/$PTEN^{-/-}$/CAT-STA mice. Thus, these data collectively have identified the Wnt/β-catenin pathway as the first defined tumor-intrinsic oncogene pathway that can abort the induction of antitumor T cell responses, prevent the T cell-inflamed tumor microenvironment, and generate resistance to checkpoint blockade therapy[25]. A diagram representing this mechanism in the context of a developing antitumor immune response is depicted in Fig. 2.

A model is emerging in which it is hypothesized that β-catenin can mediate direct immune evasion from an antitumor immune response through direct tumor immune avoidance. This process of immune avoidance can theoretically occur at any given time in tumor development, although data obtained to date have been generated with activation of β-catenin at the initial stage of tumorigenesis (Fig. 3 lower left). In concordance with the concept of immune evasion (3 E hypothesis, Elimination, Equilibrium and Escape, illustrated in Fig. 3 upper panel)[30], in which the tumor escapes from the immune system by elimination of tumor cells expressing immunogenic antigens followed by upregulation of immune inhibitory mechanisms that suppress the function of residual T cells of borderline avidity (leading to the T cell-inflamed phenotype, Fig. 3 upper right, Local immune suppression), we propose that tumor escape also can emerge through selection of tumor cells that possess activation of the Wnt/β-catenin pathway. This latter mechanism would lead to immune exclusion from the tumor microenvironment, corresponding to the second major phenotype observed clinically (non-T cell-inflamed, Fig. 3 lower right). A prediction of this model is that patients who develop secondary resistance to immunotherapies also may show acquisition of β-catenin pathway activation, a hypothesis that that is being investigated clinically.

Other candidate ancillary oncogene pathways active in subsets of cancers that could contribute to immune exclusion

Inasmuch as the Wnt/β-catenin pathway only explains around 48% of non-T cell-inflamed melanomas, it seems likely that additional molecular perturbations might function to limit host immunity in the remaining non-T cell-inflamed melanomas and in other cancer types as well. One potential candidate is activation of the STAT3 signaling pathway. Constitutively active STAT3 signaling in transplantable tumor cell lines has been reported to lead to decreased expression of proinflammatory mediators, while expression of a dominant negative STAT3 variant resulted in augmented expression of proinflammatory molecules[31,32]. These factors included the chemokines CCL5 and CXCL10, making them functionally relevant for immune cell recruitment. Additional evidence for this mechanism has been provided through more recent studies using a carcinogen-induced lung cancer model as well as a

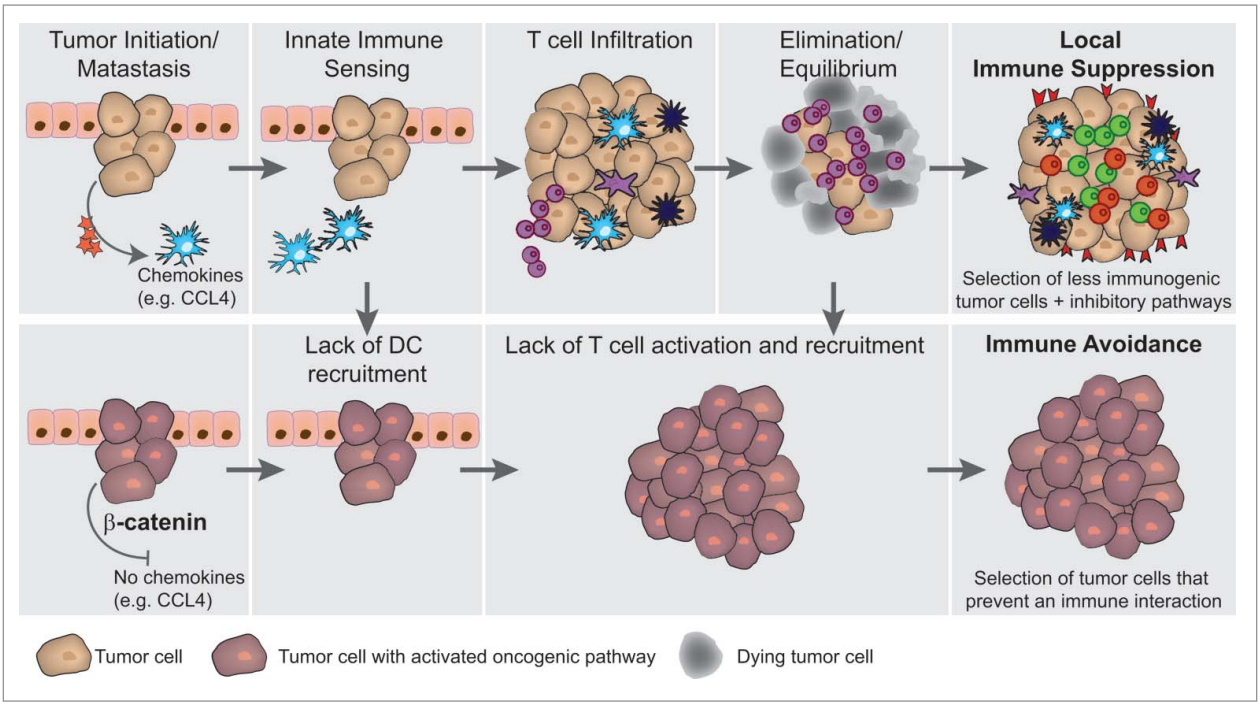

Figure 3. Oncogene-mediated immune avoidance in contrast to immunogenic escape through selection. Upper panel: Illustrated tumorigenesis of melanoma in the absence of β-catenin signaling. From left to right: tumor initiation is accompanied by recruitment of innate immune cells including $CD103^{+}$ dendritic cells via CCL4. Those dendritic cells become activated by danger signals, foremost through tumor-derived DNA sensing via the STING pathway. DC activation results in production of type I interferons and facilitates antigen-specific T cell activation (See Fig. 1 for details). Following T cell activation, tumor-specific T cells infiltrate the tumor and result in tumor cell killing, leading either to an equilibrium state in which the immune system can control the tumor growth, or to complete tumor elimination. If the tumor is not eliminated in its entirety, less-immunogenic tumor cells which survived the elimination grow out and an immune-suppressive tumor microenvironment becomes established (characterized by upregulated PD-L1, IDO, and Treg recruitment). Lower panel: Illustrated tumorigenesis in the presence of tumor-intrinsic β-catenin activation. If the initial tumor cells show upregulation of the Wnt/β-catenin pathway, an immediate block in recruitment of DCs cells results due to the lack of CCL4 secretion. It is conceivable that this block can also occur later in the tumor development if activating mutations triggering β-catenin activation are acquired over time, leading to aborted DC recruitment and blunted T cell activation and accumulation. Eventually in this scenario, T cell-negative tumors would grow out with the tumor cells "avoiding" T cell encounter.

genetically-induced prostate cancer model[33,34]. Using a conditional knockout model for STAT3, Ihara and colleagues showed an increased antitumor immune response in the absence of STAT3 signaling. This was associated with increased expression of CCL5 and CXCL10. This phenotype was associated with increased T cell accumulation and T cell function within the tumor microenvironment. Thus, STAT3 may represent another viable mechanistic pathway for diminishing immune cell recruitment into tumor sites and based on the currently available data this might interfere with recruitment of both DCs as well as T cells (Fig. 1B, Stop1 and 4).

Another molecular aberration in cancer cells that may be associated with immune modulatory effects is mutant p53. Intact p53 signaling has been associated with increased recruitment and activation of innate immune cells[35]. In a murine liver carcinoma model, reactivation of the p53 pathway resulted in tumor regression, which was associated with increased expression of proinflammatory chemokines. In a related study, tumor regression associated with re-expression of wildtype p53 was strongly dependent on the activation and recruitment of Natural Killer (NK) cells into the tumor microenvironment[36]. That recruitment was dependent on the p53-dependent production of the chemokine CCL2. Consistent with these data, a recent study analyzing triple negative breast cancer identified a correlation between wildtype p53 and the presence of T cells in the tumor microenvironment[37]. Cumulatively, these data suggest that steady-state p53 signaling could contribute to enhanced recruitment of innate immune cells as well as their activation (Fig. 1B, Stop1 and 2).

Another candidate oncogenic pathway that has potential impact on host immune responses is the NFκB signaling pathway. Activation of this pathway in cancer cells has been associated with tumor progression[38,39]. In a hepatocellular carcinoma model, increased immune-derived TNF signaling augmented NFκB signaling in liver cells and promoted tumor progression[40]. Constitutive activation of NFκB has also been shown to increase expression of tumor cell-derived chemokines, which could have positive immune effects[41]. Further, hyperactivation of NFκB within the tumor microenvironment has been shown to enhance the production of chemokines that recruit activated T cells [42]. Therefore, the impact of tumor-intrinsic NFκB activation on host immunity might depend on the cellular context, and whether tumor-promoting inflammatory cells are involved versus antitumor adaptive immunity. Additional cancer types-specific studies will be needed to determine if an NFκB driven tumor microenvironment is enhancing or dampening the antitumor immune response.

The PI3K/PTEN/AKT pathway is another interesting candidate to consider that could impact on host immune responses. Several studies focusing on inflammation-induced cancer progression have identified that active PI3K signaling, either through activating mutations in PIK3CA or loss of function mutations in PTEN, results in increased accumulation of tumor-associated macrophages, which in turn induce an immune suppressive microenvironment[43,44]. This phenomenon was associated with increased production of TNF, IL-6, CSF-1 VEGF-A and IL-8 by the tumor cells, which contributed to recruitment of macrophages and the induction of an M2 macrophage phenotype[45]. In contrast, recent reports in triple negative breast cancer have indicated that expression of PTEN was associated with the absence of T cells as well as low PD-L1 expression in the tumor microenvironment[46], arguing that loss of PTEN expression (and constitutive PI3K activation) is associated with presence of T cells in the tumor microenvironment. Similarly to intra-tumoral NFkB signaling, the impacts of active PI3K signaling need to be further studied in a tumor-specific context to draw definitive conclusions on its effect on T cell infiltration and those might be different between tumor types.

Host factors that may contribute to regulation of the T cell-inflamed phenotype

Besides differential activation of ancillary oncogene pathways within the tumor cells themselves, several host-derived factors could contribute to robustness of a spontaneous antitumor adaptive immune response. One consideration is the immunologic history of the patient and exposure to chronic viral infections. Life-long latent infection with cytomegalovirus (CMV) has been reported to consume the memory T cell pool and result in fewer T cell clones available to respond to new antigens[47]. Reduction in T cell repertoire diversity might have severe effects on the ability to recognize tumor-derived antigens in the context of cancer and in the face of immunotherapies. Additionally, emerging data have suggested that environmental factors, including the composition of the intestinal microbiota, might influence the magnitude of an antitumor immune response in the context of certain therapeutic interventions[48]. Thus, it is conceivable that the baseline composition of intestinal microbiota can influence the endogenous adaptive immune response toward the tumor. Another critical host factor is the constellation of germline polymorphisms in immune regulatory genes. Like predisposition toward autoimmune diseases, it is plausible that specific polymorphisms might favor the ability of the host to mount an adaptive immune response against their tumor[49-51].

Conclusions and future directions

The presence of a T cell-inflamed tumor microenvironment is indicative of an endogenous adaptive immune response against a given tumor, and is emerging as a useful predictive biomarker for response to immunotherapies. The molecular mechanisms that mediate the presence or absence of a T cell-infiltrated tumor are just beginning to be understood. Tumor-intrinsic β-catenin pathway activation has been identified as the first oncogene pathway mechanistically confirmed to mediate exclusion of immune cells from the melanoma tumor microenvironment. This pathway also may be relevant for other cancers beyond melanoma, and alternative oncogene pathways also could explain immune exclusion for some of the remaining melanoma cases and also for other cancer types. In addition, it is conceivable that activation of oncogenic pathways during the elimination phase can result in an alternative mechanism of immune escape through immune avoidance (Fig. 3, lower panel). Even more importantly, upregulation of these immune-inhibitory oncogene pathways could be predicted to result in secondary resistance after immunotherapies such as checkpoint inhibitors. Therefore, it will be of critical importance to analyze

such tumor-intrinsic pathways in patients with disease progression/recurrence after initial response. Host-derived elements are also being considered as contributory factors, including germline polymorphisms in immune regulatory genes, history of persistent viruses and a contracting T cell repertoire, as well as the specific composition of the intestinal microbiota. All of these factors are measurable, and should be analyzed prospectively from patients being treated with immunotherapeutic agents such as anti-PD-1 or anti-PD-L1 mAbs.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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