Focus Article:

Intratumoral CD8$^+$ T cells with stem cell-like properties: Implications for cancer immunotherapy

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Short Title:

Stem cell-like PD-1$^+$ Tcf1$^+$ CD8$^+$ T cells in tumors

Abstract:

Preclinical studies identify an intratumoral PD-1$^+$ TCF1$^+$ CD8$^+$ T cell subset with stem cell-like properties that mediates cellular expansion and tumor control in response immunotherapy.

Patients with cancer frequently harbor tumor-specific cytotoxic $CD8^+$ T cells. Although these cells usually fail to control tumor progression, a substantial fraction of patients experience durable benefit from T cell-targeted immunotherapy. This has been hard to understand since tumor-specific $CD8^+$ T cells, which are characterized by the expression of co-inhibitory receptors such as Programmed Death-1 (PD-1), are hypofunctional and considered terminally and irreversibly differentiated or "exhausted". Nevertheless, when signaling via the inhibitory PD-1 receptor is prevented (as in immune checkpoint blockade therapy), $PD\text{-}1^+$ $CD8^+$ T cells expand at the population level and this often correlates with clinical benefit. Recent studies demonstrate that this proliferative response derives from a PD-1-expressing, yet relatively undifferentiated, subset of intratumoral $CD8^+$ T cells (*1*) (*2*). Besides improving the basic understanding of the tumor immune response, these findings have important implications for translational immunotherapy research and consequently clinical cancer care.

IDENTIFICATION OF TUMOR-INFILTRATING $CD8^+$ T CELLS WITH STEM CELL-LIKE PROPERTIES

Single cell RNA sequencing and high dimensional flow and mass cytometry analyses have uncovered considerable diversity among $CD8^+$ T cells infiltrating murine and human tumors (*3*) (*4*) (*5*). However, it often remained unclear which of these T cells were tumor-specific, whether T cell subtypes were clonally related and, if so, whether the observed diversity was generated systemically or in the tumor. Most importantly, the functional relevance of intratumoral $CD8^+$ T cell subsets remained unknown.

As with other seminal discoveries in tumor immunity, initial insights into the cellular basis for immunotherapy success came from studies of chronic viral infections in mice. These studies identified a subset of virus-specific $CD8^+$ T cells that sustained the immune response to chronic viral infection and that was needed for cellular expansion in response to PD-1 blockade (*6*) (*7*). These findings raised the question whether similar mechanisms support tumor immune responses and whether these mechanisms play a role in immunotherapy.

Indeed, several recent papers identified intratumoral PD-1$^+$ CD8$^+$ T cells that behave similarly to the ones found in chronic infection (*1*) (*2*) (*4*) (*8*)**.** One subset of tumor-infiltrating PD-1$^+$ CD8$^+$ T lymphocytes (TIL) consists of terminally differentiated cells with limited expansion capacity, which combine effector (e.g. Granzyme B) and exhaustion gene expression programs whereby multiple co-inhibitory receptors are co-expressed (PD-1, LAG3, TIM3, 2B4). A second subset of PD-1$^+$ TIL lacks an effector gene program but combines memory (e.g. T cell factor 1 (TCF1) (encoded by *TCF7*)) and exhaustion features, with more limited inhibitory receptor expression (PD-1, LAG3 but not TIM3 or 2B4) (**Fig. 1**). These latter cells, which further express SLAMF6 (Ly108) and CXCR5, display stem cell-like properties as they can yield terminally differentiated cells and reproduce themselves during cell division (self-renewal) within the tumor microenvironment (TME) (*1*). Based on their memory properties and peristence despite the presence of antigen PD-1$^+$ TCF1$^+$ progenitor cells are also referred to as “memory-like”, while “exhausted” is used to describe differentiated PD-1$^+$ TCF1$^-$ progeny (*1*). Alternative terms used to denote these subsets are “progenitor exhausted” and “terminally exhausted” (*2*).

Multiplex stainings confirm the presence of PD-1$^+$ TCF1$^+$ cells in human melanoma biopsies and show that such cells are rare (*1*) (*2*), in agreement with a small fraction of lung cancer TIL detected by flow cytometry based on CXCR5 expression (*4*). The PD-1$^+$ TCF1$^+$ or CXCR5$^+$ progenitor TIL are less frequent than a TIL subset identified based on intermediate levels of PD-1 (*8*) indicating that not all PD-1^{int} cells are progenitors. These differences underscore a need to harmonize the use of markers to identify and functionally characterize progenitors among TIL. The PD-1$^+$ TCF1$^+$ TIL are associated with the stroma of human tumors, in proximity to vessels in murine tumors, but do not localize to the tumor area (*1*) (*8*). They are also observed in tertiary lymphoid structures in melanomas (*1*) and lung cancers (*8*) but can also persist outside and independent of tertiary lymphoid structures, perhaps in specialized vascular niches.

Single cell analyses identify transcriptomically distinct PD-1$^+$ TIL subsets corresponding to PD-1$^+$ TCF1$^+$ progenitor and PD-1$^+$ TCF1$^-$ differentiated cells in murine (*2*) and human tumors (*1*) (*4*) (*3*). PD-1$^+$ TCF1$^+$ TIL show molecular and functional similarities to the corresponding cells

derived from the spleen of chronically infected mice (*1*) (*2*) (*6*), a remarkable observation given the distinct biological contexts and tissue origins of these cells. It seems conceivable that PD-1$^+$ TCF1$^+$ progenitor cells arise in additional situations of antigen persistence, such as graft rejection and autoimmunity, two disease categories with great needs for research breakthroughs and improved therapies. Further, the transcriptome of PD-1$^+$ TCF1$^+$ CD8$^+$ TIL considerably overlaps with that of follicular helper T cells but not with that of tissue resident memory cells (T_{RM}), both of which play important roles in tumor immunity and immunosurveillance. Interestingly, however, differentiated PD-1$^+$ TCF1$^-$ TIL display similarity with T_{RM} cells, suggesting that T_{RM} cell characteristics are acquired during the differentiation of memory-like TIL in the TME.

Functionally, the properties of human TIL were established using *in vitro* re-stimulation assays, which reveal increased expansion and differentiation potential of CXCR5$^+$ or PD-1^{int} TIL subsets compared to CXCR5$^-$ or PD-1^{hi} TIL (*4*) (*8*). The importance of PD-1$^+$ TCF1$^+$ TIL in immunotherapy-driven tumor control was formally established in preclinical mouse models using adoptive cell transfers (*2*) and experiments with conditional and selective ablation of such cells in the TME whereby the influx of new T cells into the tumor was prevented (*1*).

CONTRIBUTIONS OF SYSTEMIC VERSUS LOCAL IMMUNITY TO TUMOR CONTROL

According to the prevalent view, naïve tumor-specific T cells are primed in the tumor draining lymph node, which leads to upregulation of the effector/memory marker CD44 (in mice). Priming yields memory and effector precursor cells whereby the latter are commonly thought to infiltrate non-hematopoietic tissues. Based on prolonged antigen exposure, likely together with additional tumor-specific signals, effector T cells upregulate co-inhibitory receptors. The new data imply that also primed, but relatively undifferentiated (TCF1$^+$), tumor-reactive CD8$^+$ T cells infiltrate the TME where cells acquire exhaustion-associated features (PD-1) and persist locally (**Fig. 1**). These tumor-resident PD-1$^+$ TCF1$^+$ cells form the cellular reservoir of tumor-specific CD8$^+$ T cells that are able to expand and give rise to an enlarged pool of differentiated cells in response to immunotherapy. There is no evidence that immunotherapy induces the dedifferentiation of terminally differentiated cells (**Fig. 1**). In murine immunotherapy experiments,

intratumoral T cells can be sufficient for tumor control whereby PD-1$^+$ TCF1$^+$ TIL are necessary for the effect (*1*). Thus, the intratumoral immune response seems to play a more substantial role for tumor control than previously thought. The sustained production of differentiated cells from PD-1$^+$ TCF1$^+$ progenitors in the TME can be considered as a supplementary immune axis that eventually operates independently of the classical cancer immune cycle, which involves the draining lymph nodes. The relative contribution of local and systemic effects to tumor control likely varies and remains to be determined.

RENEWAL, EXPANSION, AND DIFFERENTIATION OF PD-1$^+$ TCF1$^+$ TIL

Since PD-1$^+$ TCF1$^+$ TIL are important for tumor control (*1*) (*2*), it is critical to understand how these cells arise and how their maintenance and turnover is regulated. Mouse models show that TIL expansion and the self-renewal of PD-1$^+$ TCF1$^+$ TIL during immunotherapy depends on TCF1 (*1*). Although this was not always addressed directly, additional factors can be implicated in the regulation of progenitor TIL function. For example, TOX sustains the production of differentiated PD-1$^+$ TCF1$^-$ TIL (*9*). Furthermore, the presence of conventional dendritic cells 1 in tumors, CD28-dependent co-stimulation, and the cytokine IL-12 are needed for TIL expansion in response to immune checkpoint blockade. It seems likely that all these factors act at least in part on PD-1$^+$ TCF1$^+$ TIL. Interestingly, IL-12 has been shown to silence TCF1 expression in CD8$^+$ T cells during dendritic cell vaccination and this facilitates effector differentiation, suggesting that IL-12 promotes the differentiation of PD-1$^+$ TCF1$^+$ TIL by suppressing TCF1.

The generation of terminally differentiated or exhausted T cells is promoted by persistent TCR stimulation, likely in conjunction with additional TME-derived signals such as the cytokine IL-27, which stimulates the acquisition of the full set of co-inhibitory receptors. Inhibitory receptor acquisition and effector differentiation further involve the transcription factors c-MAF, PRDM1, NR4A1, and TOX (*9*). Remarkably, TOX deficiency reveals that TIL dysfunction can be uncoupled from inhibitory receptor expression (*9*). Although these studies provide important insights, the precise impact of these factors on PD1$^+$ TCF1$^+$ progenitors versus differentiated PD1$^+$ TCF1$^+$ TIL should be assessed directly.

IMPROVING CLINICAL IMMUNOTHERAPY AND OUTCOME PREDICTION

Even though antibodies blocking PD-1 and PD-L1 show success in an increasing number of different solid tumors, the response rates remain relatively low. Current biomarkers to predict successful immunotherapy against solid tumors are the tumor mutational burden, the expression of PD-L1 by tumor cells, and the abundance of TIL. Unfortunately, these markers are usually not sufficient to predict treatment outcome in individual patients.

The importance of PD-1$^+$ TCF1$^+$ TIL in preclinical mouse models raises the obvious question whether the presence of these cells predicts the response to immunotherapy. Consistent with this possibility, the expression of TCF1 by TIL found in human melanoma correlates with clinical benefit of immune checkpoint blockade (*5*). A caveat is that TCF1 is not only expressed by PD-1$^+$ TIL, which are enriched for tumor antigen specificity, but also by bystander TIL (naïve or memory cells) that are likely less relevant for tumor immunity. Multiplex analysis of PD1$^+$ TCF1$^+$ or PD-1^{int} TIL in pretreatment biopsies from patients with melanoma or lung cancer reveals the presence of progenitor TIL in non-responder patients at frequencies comparable to that observed in responders (*2*) (*8*). While it seems likely that the absence of progenitor TIL will be associated with treatment failure, the data indicate that the mere presence of these cells does not guarantee treatment response. Unexpectedly, the presence of a lower than median frequency of PD1$^+$ TCF1$^-$ (differentiated) relative to PD1$^+$ TCF1$^+$ (progenitor) TIL prior to immune checkpoint inhibition correlates with prolonged progression free survival (*2*). This observation raises the possibility that immune checkpoint blockade preferentially benefits patients in which the pretreatment generation of differentiated TIL is inefficient despite the presence of progenitor TIL. The ratio of progenitor to differentiated TIL prior to treatment may thus represent a biomarker for therapy outcome prediction. Post-treatment analysis of melanoma TIL suggests that the PD-1$^+$ TCF1$^+$ TIL expand and/or persist preferentially in response to immune checkpoint blockade (*1*). Although these observations require rigorous confirmation and extension, they suggest that a thorough understanding of the generation, maintenance and differentiation of PD-1$^+$ TCF1$^+$ TIL will be essential to predict treatment response and to fully

exploit the therapeutic potential of these cells.

The new insights will likely have a direct translational application also for further improving adoptive T cell therapy (ACT). Here, TIL are isolated, expanded *in vitro* and reinfused into lymphodepleted cancer patients. Although this procedure shows increasing efficacy, the clinical response rates are relatively low and TIL amplification *in vitro* may even fail. As the expansion potential of TIL resides in memory-like cells, it will be essential to fully characterize progenitor cells in TIL isolates and to determine how the *in vitro* culture conditions impacts their phenotype and functionality. For example, *in vitro* expansion of human TIL in the presence of IL-2 leads to the differentiation of $CXCR5^+$ progenitor TIL into $CXCR5^-$ cells, whereas the $CXCR5^+$ phenotype is maintained when TIL are expanded in IL-15 (*4*). Culture conditions may be optimized further to more consistently obtain TIL products with preserved and possibly enhanced stem cell-like properties in order to improve and sustain the therapeutic efficacy of ACT. Indeed, mouse models show that activation of naive $CD8^+$ T cells in the presence of high potassium levels followed by adoptive cell transfer into tumor bearing mice improves the generation of $CXCR5^+$ progenitor TIL and tumor control (*10*).

CONCLUDING REMARKS

Recent work identifies a stem cell-like TIL subset that plays a crucial role in limiting tumor progression in response to immunotherapy. Unfortunately, despite the importance of these cells, untreated tumors almost always progress and immunotherapy often fails to mediate durable tumor control. Although this is likely based on a multitude of progenitor TIL-intrinsic as well as TME-associated negative regulatory factors, current immunotherapy approaches may not be adequate to unleash the full functional potential of this stem cell-like TIL subset. By elucidating the regulatory circuits that control the generation, maintenance, and function of these cells it should be possible to fully exploit the therapeutic potential of tumor-specific $PD\text{-}1^+$ $TCF1^+$ progenitor TIL to further improve immunotherapy against cancer.

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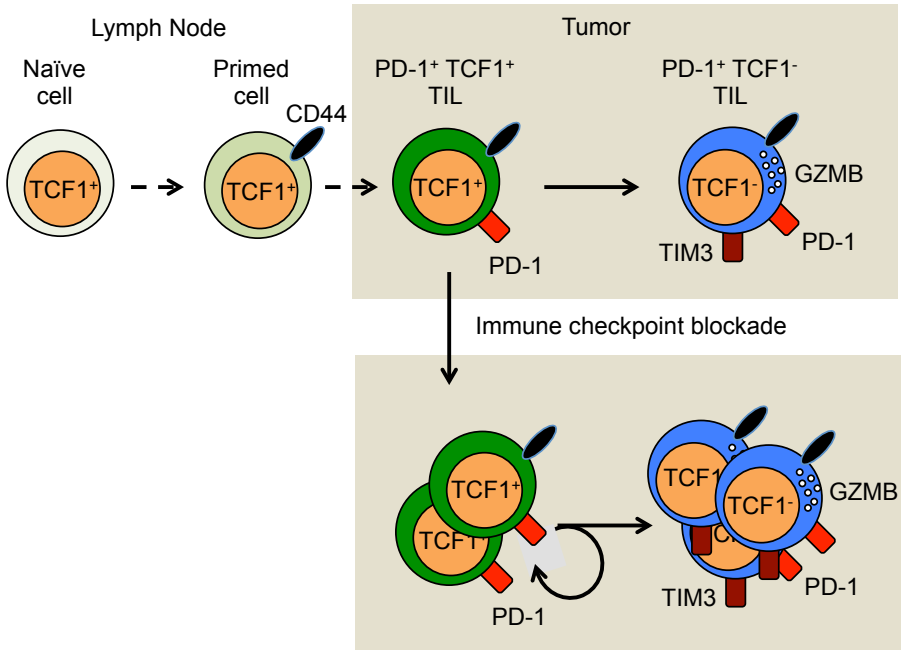

Fig. 1: T cell expansion in response to Immunotherapy depends on intratumoral PD-1$^+$ TCF1$^+$ CD8$^+$ T cells

Priming of naive tumor-specific CD8$^+$ T cells (TCF1$^+$) in the tumor draining lymph node upregulates the effector/memory marker CD44 (in mice) and allows relatively undifferentiated (TCF1$^+$) cells to infiltrate the tumor microenvironment, where they acquire exhaustion features (PD-1), persist and can give rise to differentiated PD-1$^+$ TCF1$^-$ cells that exhibit additional exhaustion features (TIM3) and have cytolytic potential (GZMB). In response to immune checkpoint blockade, tumor-resident PD-1$^+$ TCF1$^+$ TIL can expand, self-renew and yield an enlarged pool of differentiated PD-1$^+$ TCF1$^-$ TIL that have cytolytic potential. There is no evidence that differentiated PD-1$^+$ TCF1$^-$ TIL dedifferentiate.