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Technology meets TILs: deciphering T cell function in the -omics era

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Summary

T cells are at the centerstage of cancer immunology due to their ability to recognize mutations within tumor cells and directly mediate cancer cell killing. Immunotherapies to rejuvenate exhausted T cell responses have transformed the clinical management of several malignancies. In parallel, the development of novel multidimensional analysis platforms such as single-cell RNA-sequencing and high-dimensional flow cytometry has yielded unprecedented insights into immune cell biology. This convergence has revealed substantial heterogeneity of tumor-infiltrating immune cells, both within single tumors, across tumor types, and among cancer patients. Here, we discuss the opportunities and challenges of studying the complex tumor microenvironment with -omics technologies that generate vast amounts of data, highlighting the opportunities and limitations of these technologies with a particular focus on interpreting high-dimensional studies of $CD8^+$ T cells in the tumor microenvironment.

The promise of immunotherapy

Cancer immunotherapy – the augmentation of anti-tumor immunity – has revolutionized the treatment of many cancers. Dozens of immunotherapies have received FDA approval, and thousands of immuno-oncology agents are under development (Upadhaya et al., 2020). Current types of cancer immunotherapy include cytokines, depleting antibodies, adoptive cell therapy, oncolytic viruses, cancer vaccines, and immune checkpoint inhibitors (ICIs) (Esfahani et al., 2020). As key mediators of anti-tumor immunity, T cells are the target of

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most of these therapeutic strategies. ICIs, in particular, are the most widely used cancer immunotherapy and modulate signaling pathways of exhausted $CD8^+$ T cells in order to enhance the anti-tumor response (Robert, 2020). The unprecedented success of ICIs in the clinical management of cancer combined with the transience or complete lack of responses in most patients has spurred significant efforts over the past decade to gain a better understanding of $CD8^+$ T cell differentiation and dysfunction in cancer to ultimately improve immunotherapy response rates.

Studies of T cell differentiation were revolutionized by the use of peptide-MHC (pMHC) I multimers, allowing the tracking of antigen-specific T cells in a manner independent of their functionality (Altman et al., 1996). Much early work on antigen-specific T cell differentiation was performed in mouse models, especially the lymphocytic choriomeningitis virus (LCMV) system that enables the investigation of T cells specific for identical epitopes under acute and chronic antigen stimulation settings (Wherry et al., 2007; Zajac et al., 1998). Until recently, studies of antigen-specific T cell differentiation in humans had focused almost exclusively on viral infections, including yellow fever virus (YFV) (Akondy et al., 2017; Akondy et al., 2009; Miller et al., 2008), Epstein-Barr Virus (EBV) (Amyes et al., 2003; Long et al., 2019; Murray et al., 1992), Cytomegalovirus (CMV) (Khan et al., 2002a; Klenerman and Oxenius, 2016; Pourgheysari et al., 2007), and influenza (Gotch et al., 1987; Hufford et al., 2014; Koutsakos et al., 2019).

These studies showed that, following antigen exposure, naïve $CD8^+$ T cells differentiate into effector cells and express high amounts of cytotoxic proteins, cytokines, and markers of division (Fig. 1). Antigen clearance results in de-differentiation of a subset (Kaech et al., 2003) of effector cells into memory cells, which lack effector molecules but are primed for rapid recall of cytotoxic function (Youngblood et al., 2017). In cases of antigen persistence, such as cancer, $CD8^+$ T cells exist in several differentiation states (Eberhardt et al., 2021; Hudson et al., 2019a; Im et al., 2016; Philip and Schietinger, 2019). One such subset, PD-1^+ stem-like cells, has been of immense interest in the field of cancer immunology as this subset of $CD8^+$ T cells serves as a reservoir maintaining a relatively stable pool of antigen-specific T cells during antigen persistence (Siddiqui et al., 2019). Most importantly, these stem-like cells mediate the proliferative burst of antigen-specific $CD8^+$ T cells observed after PD-1 pathway blockade, a finding first described in the murine LCMV model (Im *et al.*, 2016).

The heterogeneity of the tumor microenvironment

In contrast to the early focus on viral infections, hundreds of studies have been performed in recent years to characterize T cell responses to cancer. This increased interest in anti-tumor T cell responses has coincided with a revolution in biological techniques that has advanced tumor immunology. In particular, next-generation sequencing has provided the ability to measure the expression of thousands of genes in pools of cells, *in situ* within tissue sections, or in single cells. Other technologies, including flow cytometry and gene editing, have greatly increased their capabilities and adoption by researchers in the cancer immunotherapy era.

These advances in methodology have revealed many important features of tumor immunology; most notably, the diversity of tumor-infiltrating immune cells. This diversity includes differences in anti-tumor immunity between cancer patients as well as the variety of immune cell types within a single tumor microenvironment (TME). Further, not only are many cell types present within tumors, but each of these cell types can also be present in multiple states within the same TME, a feature true not only of T cells, but also of B cells (Wei et al., 2021), myeloid cells (Veglia et al., 2021), fibroblasts (Helms et al., 2020), as well as tumor cells themselves (Patel et al., 2014). In this Perspective, we will discuss the opportunities and challenges that have arisen as modern immunology techniques have encountered the complexity of the TME, with a particular focus on anti-tumor $CD8^+$ T cell immunity.

Specificity is key: bystanders within the TME

Heterogeneity in clinical responses was an early feature of ICI use in cancer (Brahmer et al., 2010; Topalian et al., 2012), prompting the search for clinical correlates of immunotherapy response. One of the first variables tested for correlation with clinical response to PD-1 pathway blockade was pre-existing infiltration of tumors by $CD8^+$ T cells (Herbst et al., 2014; Tumeh et al., 2014). In many cases, a higher level of $CD8^+$ T cell infiltration predicts response to ICIs (Galon and Bruni, 2019; Tumeh *et al.*, 2014), but this correlation has not been found in all studies (Braun et al., 2020; Herbst *et al.*, 2014). Studies in patients with various cancers have shown that a substantial proportion - in some cases even the majority - of intratumoral $CD8^+$ T cells are bystander cells; that is, they are not specific for tumor antigens and are either predominantly reactive to common human pathogens such as EBV, CMV, and influenza (Cheng et al., 2021; Oliveira et al., 2021; Rosato et al., 2019; Scheper et al., 2018; Simoni et al., 2018; Sudmeier et al., 2022) or have unknown antigen specificity.

Thus, the composition of the intratumoral $CD8^+$ T cell compartment is not only the result of available and recognized tumor antigens but also of the immunological history of the patient. Without knowledge of their antigen specificity, this infiltration by microbe-specific $CD8^+$ T cells has the potential to seriously confound studies assessing the number, phenotype, and differentiation of tumor-specific $CD8^+$ TILs. This presence of bystander cells is not only restricted to $CD8^+$ TILs, as CMV-specific $CD4^+$ T cells have also been reported with in the TME of various cancers (Li et al., 2022; Oliveira et al., 2022). However, less is known about bystander infiltration of $CD4^+$ T cells due to inherent difficulties of their detection with pMHC multimers when compared to $CD8^+$ T cells (Christophersen, 2020).

In some tumors, even the most abundant $CD8^+$ clones may not be tumor-specific. Elderly patients – in which cancer incidence is high - can accumulate oligoclonal $CD8^+$ T cell repertoires in a process termed memory inflation (Klenerman, 2018). Many of these clones are CMV-specific (Khan et al., 2002b; Wieland et al., 2018). In a nonagenarian melanoma patient we recently found that the ten most abundant $CD8^+$ T cell clones in circulation accounted for nearly 60% of the entire blood $CD8^+$ T cell repertoire, with the most dominant clone comprising 30% (Wieland *et al.*, 2018). Importantly, while these peripherally expanded clones were less prevalent in tumor and did not expand in response to anti-PD-1 therapy (thus likely representing virus-specific bystanders), they still ranked

among the top tumor-infiltrating $CD8^+$ T cell clones due to their vast abundance in the periphery.

While these and other data suggest that a negative enrichment of $CD8^+$ T cell clonotype frequency in tumor versus blood can identify bystander cells, bystander cells can also be found at similar or even slightly higher frequencies than in blood (Ning et al., 2022; Rosato *et al.*, 2019; Scheper *et al.*, 2018). This is likely due to the relative exclusion of naïve T cells from tumors (Egelston et al., 2018; Sudmeier *et al.,* 2022) and highlights the impact of the respective denominator for such comparisons. While techniques allowing for the direct determination of antigen specificity such as tetramer staining, MANAFEST and TCR sequencing of cells with confirmed antigen/tumor specificity are tedious (Caushi et al., 2021; Danilova et al., 2018; Oliveira *et al.,* 2022; Oliveira *et al.,* 2021; Rosato *et al.,* 2019; Simoni *et al.*, 2018), they are necessary to accurately distinguish bystanders from tumor-specific T cells.

This presence of bystander $CD8^+$ T cells in the absence of their cognate antigen has also been recapitulated in mouse tumor models, demonstrating that pre-existing virus-specific $CD8^+$ T cells can infiltrate tumors and acquire phenotypic traits such as PD-1 expression (Erkes et al., 2017; Rosato *et al.*, 2019; Sudmeier *et al.*, 2022) which are often erroneously associated with T cell dysfunction or even equated with tumor specificity (Danahy et al., 2020). Importantly, bystander recruitment seems to be mostly restricted to antigen-experienced cells (Erkes *et al.,* 2017; Ning *et al.,* 2022; Rosato *et al.,* 2019; Sudmeier *et al.*, 2022) and thus likely results in substantially higher frequencies of tumor-specific $CD8^+$ T cells in standard mouse models due to the relative absence of microbe-specific memory $CD8^+$ T cells in mice kept under standard pathogen-free conditions.

Bystander infiltration into tumors has two major implications for the study of tumor-infiltrating $CD8^+$ T cells. First, since tumor-specific $CD8^+$ T cells are required for efficient tumor killing, the presence of bystanders may complicate the correlation of $CD8^+$ T cell infiltration with response to ICIs. A potential contributor to this phenomenon might be the variable fraction of bystanders compared to *bona fide* tumor-specific cells in different tumors and among individuals. Another contributor may be the potentially beneficial, antigen-independent role of bystander cells for the maintenance of a permissive local immune milieu by secretion of various cytokines and chemokines. This too may be variable: cells that have experienced multiple sequential stimulations by their cognate antigen exhibit increased antigen-independent effector functions in the TME (Danahy *et al.*, 2020). These data suggest that functional bystander cells are not equal from an anti-tumor perspective and that their antigen specificity and thus antigenic history (i.e. time since last antigen encounter and number of antigenic encounters throughout their lifetime) will significantly impact their anti-tumor functions. Experiments in mice exposed to various pathogens in a controlled manner prior to tumor inoculation (Danahy *et al.*, 2020; Erkes *et al.*, 2017; Ning *et al.*, 2022; Rosato *et al.*, 2019) or the use of “dirty” mice (Beura et al., 2016) will continue to improve our understanding of the role of bystander $CD8^+$ T cells and their potential to be specifically harnessed to promote anti-tumor immune responses.

Looks can be deceiving

A second implication of bystander cell infiltration is their potential to confound the study of tumor-specific T cells within the TME. As discussed above, tumor specificity of T cells cannot be inferred from their simple presence within the TME. As a corollary, the phenotype and number of tumor-infiltrating cells should not be confused with the phenotype and number of tumor-specific cells.

The marked heterogeneity of antigen specificities within the tumor raises the important question to what degree the observed phenotypic heterogeneity of $CD8^+$ TILs can be attributed to divergent antigen specificities and thus differential histories of antigenic stimulation versus differentiation of the tumor-specific cells themselves. As noted, $CD8^+$ T cell differentiation has been extensively studied in the context of both acute and chronic antigen exposure in both preclinical mouse models as well as humans (Akondy *et al.*, 2017; Kaech *et al.*, 2003; Miller *et al.*, 2008). While acute antigen exposure results in the ultimate development of polyfunctional memory cells, chronic antigen stimulation leads to the alternate T cell differentiation state of exhaustion. Although the ultimate products of acute and chronic antigen exposure (i.e., memory and exhausted $CD8^+$ T cells) differ in their functional capacity, they still share a substantial number of expressed molecules at various stages of their differentiation trajectories, while simultaneously exhibiting significant heterogeneity along each trajectory (Figure 1).

Hence, multiple markers are required to accurately describe the differentiation state of a given $CD8^+$ T cell. In settings in which the number of employed markers is small, such as in low-dimensional flow cytometry or immunohistochemistry, determining distinct differentiation states may mislead data interpretation. One misleading marker is PD-1 itself, whose presence on $CD8^+$ T cells is commonly equated to an exhausted phenotype; however, PD-1 is more accurately described as an activation marker reflecting TCR stimulation. Indeed, PD-1 expression is not limited to exhausted T cells but also observed on antigen-specific cells early after antigenic stimulation (Ahn et al., 2018), with memory precursors - fated to become long-lived memory $CD8^+$ T cells - expressing higher levels of PD-1 than terminal effector cells (Hudson et al., 2019b).

The above observations were made in a mouse model of acute viral infection, which provides detailed information about the antigen specificity of the analyzed $CD8^+$ T cells and when a cell first/last encountered its cognate antigen. With some notable exceptions of experimental studies in viral infections (Akondy *et al.*, 2017; Akondy *et al.*, 2009; Fuertes Marraco et al., 2015; Miller *et al.*, 2008), temporal information is typically not available to study human $CD8^+$ T cell differentiation. This is particularly true in tumor studies, where a snapshot of the TME is generated at the time of resection or biopsy.

Pitfalls of trajectory analysis

The resulting difficulty in determining distinct lineage relationships between complex human T cell populations *in vivo* has spurred the use of differentiation trajectory algorithms to develop a biological framework for tumor-infiltrating lymphocyte differentiation. Key

methods of this kind include trajectory analysis using pseudotime and RNA velocity to infer differentiation pathways among cells analyzed by scRNA-seq (La Manno et al., 2018; Trapnell et al., 2014). However, while these methods can be useful for hypothesis generation, they are not substitutes for experimental determination of lymphocyte differentiation trajectories and may lead to erroneous results even in relatively simple experiments.

As an example, to determine the differentiation pathways of virus-specific $CD8^+$ T cells, Kurd et al. (Kurd et al., 2020) transferred P14 cells – TCR-transgenic $CD8^+$ T cells specific for a LCMV epitope – to wild type mice. After infection of recipient mice with the acute strain of LCMV Armstrong, the authors isolated donor P14 cells at various time points and performed scRNA-sequencing (Fig. 2A–B). Since the stage of differentiation and time after antigen exposure are known for every cell in this experiment, this is an excellent dataset to compare the ground truth (real time) with inferred pseudotime. We re-analyzed their data set, performing dimensionality reduction of sequenced cells with Uniform Manifold Approximation and Projection (UMAP) and trajectory inference with Slingshot (McInnes et al., 2018; Street et al., 2018). The actual, real-time trajectory of $CD8^+$ T cell differentiation followed a clockwise pattern in UMAP space (Fig. 2C) whereas trajectory inference predicted the opposite differentiation pathway as determined by biological experimentation: naïve cells were predicted to differentiate into memory cells first, followed by subsequent transition into late and early effectors (Fig. 2D).

Even in this exceptionally “clean” example, where $CD8^+$ T cells with an identical antigen receptor of known specificity are transferred to a congenically distinct host and subsequently isolated, trajectory analysis from scRNA-seq data infers a wildly incorrect differentiation pathway compared to the known ground truth. In human tumors, such analyses are often performed on scRNA-seq data from heterogenous pools of tumor-infiltrating T cells comprised of cells with multiple TCR clonotypes, multiple antigen specificities and thus distinct differentiation histories, often from multiple patients, each isolated at a single time point. Any trajectory inference from such a heterogeneous population of cells should thus be treated with skepticism. In many cases, it appears that bystander and tumor-specific T cells are found in mutually exclusive differentiation states (Oliveira *et al.*, 2021; Sudmeier *et al.*, 2022). Given the inability of exhausted $CD8^+$ T cells to form central memory cells after antigen removal (Abdel-Hakeem et al., 2021; Hensel et al., 2021; Tonnerre et al., 2021; Yates et al., 2021), the poor response of memory $CD8^+$ T cells to sustained antigenic load (West et al., 2011), and the epigenetically-enforced irreversibility of the exhausted T cell state (Pauken et al., 2016), two distinct tumor-infiltrating T cell populations may not share a common differentiation pathway and any attempt to infer trajectories between such states would be incorrect. An extreme example is a recent study (Wilk et al., 2020) that used RNA velocity analysis from scRNA-seq to propose a “differentiation bridge from plasmablasts to developing neutrophils”, a conclusion seemingly at odds with basic immunological principles (Alquicira-Hernandez et al., 2021).

In our example (Fig. 2), the discrepancy between real-time and pseudotime trajectories is due to a conflict between inherent biological properties of $CD8^+$ T cell differentiation and the design of differentiation trajectory algorithms. Upon activation, naïve T cells undergo

extensive proliferation and acquire an effector gene expression pattern including high levels of *MKI67*, *GZMB*, *PRF1*, *IL2RA* (encoding CD25), and *PDCD1* (encoding PD-1) (Fig. 2B,E). This is accompanied by downregulation of memory/stemness-associated genes such as *SELL* (encoding CD62L), *IL7R*, *TCF7*, and *CCR7* (Fig. 2B,E). After antigen clearance, a subset of effector cells will ultimately de-differentiate into memory $CD8^+$ T cells that re-acquire a more naïve and quiescent-like state but simultaneously maintain epigenetic marks of an effector phase such as demethylation of loci encoding for effector molecules such as granzyme B (Akondy *et al.*, 2017; Youngblood *et al.*, 2017). Similar reversals of gene expression are also observed in the differentiation of exhausted $CD8^+$ T cells (with *CX3CR1* and *CD69*, for example (Beltra et al., 2020; Hudson *et al.*, 2019a)). Unfortunately, this gene expression pattern – inherent to T cell biology - violates "a key assumption that enables pseudotemporal ordering," namely "that genes do not change direction very often, and thus samples with similar transcriptional profiles should be close in order" (Bacher and Kendziorski, 2016). The result in our example is that naïve and memory cells, which are the most transcriptionally similar yet temporally distant, are projected to be adjacent in differentiation state (Fig. 2F). Similar issues likely exist with $CD4^+$ T cells, and inference of their differentiation trajectories may be further complicated by their potential to differentiate into distinct helper T cell subsets, compared to the (relatively) simplistic lifestyle of cytotoxic $CD8^+$ T cells. Thus, although it is tempting to use trajectory inference techniques to infer differentiation relationships between cell subsets, experimental validation is required before making firm conclusions about immune cell differentiation pathways.

Another method commonly used to infer immune cell phenotype or differentiation state is gene set enrichment analysis (GSEA) (Subramanian et al., 2005). GSEA determines whether differences in expression levels of a pre-defined set of genes are statistically significant between two populations. When using GSEA or gene ontology data for analysis, it is critical to properly frame the comparison between the gene set(s) and populations being analyzed and to also consider confounding biological processes that may be present between two otherwise-different cell types. For example, despite their distinct differentiation states, both effector and exhausted $CD8^+$ T cells will express high levels of cytotoxicity-related genes compared to naïve or memory cells. In our analysis of $CD8^+$ T cells following acute LCMV infection, expression of an exhaustion-associated gene set is high in effector cells isolated 5–7 days following infection (Fig. 3A,B). However, no exhausted cells are present in this dataset; the $CD8^+$ T cells expressing these genes are not exhausted but are effectors that share an overlapping gene expression program with exhausted T cells. It is also worth noting that any errors or omissions in annotation or construction of gene sets will be carried forward in the analysis. For example, the gene set in our example was constructed from microarray data (Doering et al., 2012), likely omitting more recently discovered or annotated genes associated with T cell exhaustion. However, careful use of gene sets does have several advantages, including the availability of thousands of gene sets for analysis (Liberzon et al., 2011) and less sensitivity to scRNA-seq dropout.

One key advantage to studying lymphocytes with scRNA-seq is the ability to determine antigen receptor sequences (B cell receptors/BCRs and T cell receptors/TCRs) (Morgan and Tergaonkar, 2022; Pai and Satpathy, 2021; Pauken et al., 2022). At a broad level, measuring TCR diversity yields important information on the clonality and breadth of T cell responses.

On the single-cell level, since lymphocytes with the same antigen receptor sequence are clonally related and share antigen specificity, BCR/TCR sequencing can deliver profound biological insight into lymphocyte differentiation and is a necessary addition for scRNA-seq studies of lymphocytes. When sequenced to an appropriate depth, analysis of cells with the same BCR/TCR clonotype will reveal differentiation states available to lymphocytes with a given antigen receptor sequence within a particular tissue or tumor. This insight is strongest when paired with experimental determination of antigen specificity; several recent, elegant studies have used peptide-MHC I multimers and/or *in vitro* stimulation assays to determine bystander and/or tumor-specific TCRs which are then linked with scRNA-seq gene expression data (Caushi *et al.*, 2021; Eberhardt *et al.*, 2021; Oliveira *et al.*, 2022; Oliveira *et al.*, 2021; Sudmeier *et al.*, 2022). Even without experimental determination of antigen specificity, careful use of databases (Bagaev et al., 2020) containing TCR sequences with known specificity can assist in the identification of bystander, self-reactive, or tumor-associated antigen-specific cells (Park et al., 2020a; Sudmeier *et al.*, 2022). Although determination of their antigen specificity is more difficult due to the recognition of both linear and conformational epitopes, studies of tumor-specific B cells are also possible (Mazor et al., 2022; Wieland et al., 2020).

Markers of tumor-specific T cells

Unfortunately, the cost of scRNA-seq makes it prohibitive for many sample sets, and in all cases the number of cells analyzed is fewer than in other methods, particularly flow cytometry. Thus, protein markers capable of distinguishing tumor-specific from bystander T cells have become a topic of particular interest. Memory $CD8^+$ T cells to common human pathogens exist in a continuum of differentiation states that likely are imparted by differences in their antigenic history and strength of antigenic stimulation. Human memory T cells can exhibit a wide range of phenotypes with YFV-specific cells ultimately acquiring a $CCR7^+CD45RA^+PD\text{-}1^{neg}TCF\text{-}1^+$ T stem cell memory (T_{SCM}) phenotype (Gattinoni et al., 2011) and cells specific for other common human pathogens such as Influenza (Flu), EBV, and CMV expressing a mix of the aforementioned markers (Fig. 1) (Debes et al., 2004; Sekine et al., 2020).

However, both YFV- and Influenza-specific memory cells lack expression of TOX, a transcription factor heavily associated with $CD8^+$ T cell exhaustion and tumor-specificity (Alfei et al., 2019; Khan et al., 2019; Oliveira *et al.*, 2021; Scott et al., 2019). Unfortunately, TOX expression can also be induced in mouse and human memory T cells by cytokine signaling, independent of TCR stimulation (Maurice et al., 2021). $CD8^+$ T cells reactive to EBV and CMV, two common human herpesviruses that periodically reactivate and can thus repeatedly stimulate cognate $CD8^+$ T cells, show a marked heterogeneity in terms of the aforementioned memory markers and can express significant levels of PD-1 as well as TOX (Sekine *et al.*, 2020). Despite the expression of these “exhaustion” markers, these cells are highly cytolytic and polyfunctional (Sekine *et al.*, 2020), highlighting that the exclusive reliance on these markers for differentiation state determination can falsely label cells as tumor-specific or exhausted. Of note, EBV- and CMV-specific $CD8^+$ T cells can co-express TCF-1 and TOX, making them particularly resemble the antigen-specific stem-like $CD8^+$

T cells found in chronic viral infection and some tumors (Eberhardt *et al.*, 2021; Im *et al.*, 2016; Siddiqui *et al.*, 2019).

Stem-like $CD8^+$ T cells are characterized by co-expression of the transcription factors TOX and TCF-1 and possess substantial proliferative capacity and the potential to give rise to more differentiated cytotoxic progeny (Im *et al.*, 2016; Leong et al., 2016; Utzschneider et al., 2016). The proliferative burst observed after immune checkpoint blockade depends on the presence of these cells, which makes them an intense topic of study (Im *et al.*, 2016). In the steady state, stem-like $CD8^+$ T cells serve as a resource to maintain antigen-specific T cell responses in the face of chronic antigen stimulation through slow self-renewal and differentiation (Im *et al.*, 2016; Leong *et al.*, 2016; Utzschneider *et al.*, 2016). These cells are also referred to as progenitor exhausted cells and have been found in various settings of persistent antigen such as chronic viral infections, cancer and most recently in autoimmune settings such as diabetes (Gearty et al., 2021). Note that despite potentially confusing similarities in nomenclature, human stem-like $CD8^+$ T cells are highly distinct from T memory stem cells (T_{SCM}) that have been described based on the expression of the CD45RA isoform and form a distinct and quite rare subset of memory T cells (Gattinoni *et al.*, 2011) emerging slowly after antigen clearance (Akondy *et al.*, 2017; Fuertes Marraco *et al.*, 2015). In contrast to T_{SCM}, human stem-like $CD8^+$ T cells express the CD45RO isoform and high levels of PD-1 (Eberhardt *et al.*, 2021) (Fig. 1).

In contrast to the somewhat promiscuous expression of PD-1 and TOX on exhausted and polyfunctional cells, CD39 has been identified and confirmed in several studies to faithfully demarcate exhausted tumor-specific $CD8^+$ T cells (Duhen et al., 2018; Hanada et al., 2022; Simoni *et al.*, 2018). CD39 is also expressed on tumor-specific conventional $CD4^+$ (Kortekaas et al., 2020; Oliveira *et al.*, 2022) TILs, although its constitutive expression on regulatory T cells precludes its use to capture tumor-specific Tregs (Borsellino et al., 2007). Furthermore, *CXCL13* transcript expression has been reported to be enriched among exhausted tumor-specific $CD4^+$ and $CD8^+$ T cells (Hanada *et al.*, 2022; Thommen et al., 2018; Veatch et al., 2022; Zheng et al., 2022). While CD39 appears to be a specific marker to enrich for tumor-specific $CD8^+$ TILs without requiring cognate tumor antigen identification, CD39 expression alone does not necessarily capture all tumor-specific $CD8^+$ T cells in the TME, as tumor-specific stem-like cells do not express CD39 (Eberhardt *et al.*, 2021; Oliveira *et al.*, 2021). Rather, CD39 marks an exhausted, dysfunctional differentiation state that is available primarily to tumor-specific $CD8^+$ T cells within the TME.

Studying T cell differentiation states

In most studies, the majority of intratumoral tumor-specific $CD8^+$ T cells have been found in the $CD39^+$ terminally exhausted state (Caushi *et al.*, 2021; Li et al., 2019; Oliveira *et al.*, 2021; Philip et al., 2017; Simoni *et al.*, 2018), while stem-like cells are present at higher frequencies in tumor-draining lymph nodes (Buchwald et al., 2020; Connolly et al., 2021). Combined with the high frequency of $TCF\text{-}1^+$ bystanders, it has remained unclear if intratumoral $TCF\text{-}1^+$ cells are truly tumor-reactive (Philip and Schietinger, 2019). The answer likely depends on the tumor type and nature of the surrounding tissue. Using tetramer staining for tumor-associated viral antigens, we recently identified intratumoral

TCF-1$^+$ tumor-specific cells in human papilloma virus (HPV)$^+$ head and neck cancer (HNC) (Eberhardt *et al.*, 2021), which arises mostly in lymphoid tissues such as tonsils. Other studies examining tumors in non-lymphoid tissues have found very few TCF-1$^+$ tumor-specific cells (Caushi *et al.*, 2021; Li *et al.*, 2019; Oliveira *et al.*, 2021; Philip *et al.*, 2017). Importantly, while HPV-specific T cells were readily detectable in the HPV$^+$ HNC tumors, these cells were undetectable in the peripheral blood directly ex vivo (Eberhardt *et al.*, 2021), a finding consistent with other studies (Caushi *et al.*, 2021; Oliveira *et al.*, 2021). Overall, these data demonstrate a differential distribution of tumor-specific CD8$^+$ T cells and subsets not only macroscopically between blood and tissue but also between lymphoid and non-lymphoid tissues. This suggests that tumor-specific stem-like cells mostly reside in lymphoid tissues, with the possible exception of the cells residing in tertiary lymphoid structures. This distribution aligns nicely with data from the murine LCMV model, where viral antigen is present in all tissues, but stem-like cells are resident in lymphoid organs (Im et al., 2020).

This divergent localization presents challenges to the faithful detection of different CD8$^+$ T cell subsets and their associated markers by flow cytometry. Without cells available from tumor and lymphoid tissues (or blood), accurate quantification of cell- or tissue-restricted proteins can be difficult. One such example is TIM-3, a key marker and co-inhibitory molecule co-expressed with CD39 on differentiated tumor-infiltrating CD8$^+$ T cells (Sade-Feldman et al., 2018). Since TIM-3$^+$ CD39$^+$ CD8$^+$ T cells are rarely found in circulation (Eberhardt *et al.*, 2021; Hudson *et al.*, 2019a; Im *et al.*, 2020; Oliveira *et al.*, 2021; Sudmeier *et al.*, 2022) (Fig. 4A), it is difficult to assess the quality of TIM-3 staining and true fraction of TIM-3$^+$ cells using circulating T cells alone. However, simultaneous staining of PBMCs and TILs (the latter acting as a positive control) facilitates assessment of the TIM-3$^+$ population (Fig. 4B–C). Unfortunately, such samples are not readily available in all studies, and measurements of the TIM-3$^+$ fraction in circulating T cells of healthy adults has thus varied wildly, from <1% (Bachmann et al., 2012; Kawashima et al., 2020; Wu et al., 2013) to more than 40% (Bonifacius et al., 2021) and various points in between (Cai et al., 2015; Liu et al., 2010; Sada-Ovalle et al., 2015; Wu et al., 2011; Xu et al., 2015) (Fig. 4D). Such variability highlights the importance of sample diversity, biological controls, and careful downstream analysis of studies using cytometry-based approaches.

Another challenge for cytometry-based approaches is the analysis, visualization, and reporting of high dimensional data. This challenge dates to the introduction of mass cytometry (or CyTOF, cytometry by time-of-flight), which at its introduction greatly increased the number of measured parameters per cell (Bandura et al., 2009). CyTOF measures the cellular abundance of isotope-labeled antibodies, theoretically enabling collection of up to 120 parameters, although this number is limited in practice due to current conjugation chemistries and the availability of sufficiently purified heavy metal isotopes to currently about 50 parameters (Bandura *et al.*, 2009; Gadalla et al., 2019; Iyer et al., 2022).

Recent years have seen the proliferation of advanced fluorescence flow cytometers with five or more lasers and equipped with 64 or more detectors, allowing the full-spectrum assessment of each fluorophore rather than limiting its detection to a narrow bandwidth, a technique known as spectral flow cytometry. Coupled with advances in fluorophore

chemistry, it is now possible to analyze expression of 40 or more proteins on single cells using fluorescence flow cytometry (Park et al., 2020b), thus nearly closing the gap with mass cytometry in terms of measurable parameters per cell. Compared to CyTOF or scRNA-seq, modern fluorescence-based flow cytometry offers an outstanding balance between the number of parameters acquired per cell, data acquisition rates, reagent and instrumentation cost, widespread availability of required equipment, and ease of downstream analysis (Bonilla et al., 2021).

Unfortunately, these properties of fluorescence flow cytometry create a tradeoff: while a higher number of measured parameters increases the potential biological insight gathered from an experiment, the complexity of data acquisition, analysis, and presentation increase in parallel (Fig. 5A). Compensation – correction for spectral overlap in fluorescence flow cytometry experiments – is both more crucial and complicated in high-parameter experiments. Several excellent articles have been written on the technical aspects of high-parameter flow cytometry data collection and analysis (Ferrer-Font et al., 2020; Fox et al., 2020; Liechti et al., 2021), but we will provide an example demonstrating how small inaccuracies collection or analysis can lead to erroneous conclusions from cytometry data.

We recently collected 22-color flow cytometry data of brain metastasis-infiltrating $CD8^+$ T cells on a four-laser spectral flow cytometer (Sudmeier *et al.*, 2022). All antibodies in the staining panel were expected to bind to at least some proportion of T cells; some cells had 16 or more bound fluorophores. Thus, both the difficulty of compensation and sensitivity to compensation error in this experiment were high. Given the high number of parameters, we used UMAP to reduce data collected from $CD8^+$ T cells to two dimensions for visualization (McInnes *et al.*, 2018). This analysis showed an exhausted $CD8^+$ T cell population with high expression of immune checkpoint molecules PD-1 and CTLA-4 (Fig. 5B–C).

As expected, nearly all $CD8^+$ T cells were negative for expression of FOXP3, a transcription factor expressed in regulatory $CD4^+$ T cells (Rudensky, 2011) (Fig. 5D). However, a false positive population of $FOXP3^+$ $CD8^+$ T cells can be generated through errors in analysis – or even choices in data visualization. Compressing the scale of FOXP3 fluorescence intensity produces the appearance of a small population of $FOXP3^{hi}$ $CD8^+$ T cells, even when data are properly compensated (Fig. 5E). When compounded by an error in compensation, the result is a striking - and false - $FOXP3^+$ population that represents 12% of $CD8^+$ T cells (Fig. 5F).

The experimental details needed to decipher such an erroneous result are often not reported alongside the data; unlike scRNA-seq experiments, deposition of raw cytometry data is not yet (Kozlov, 2022) required for most publications. While dimensionality reduction methods such as UMAP (McInnes *et al.*, 2018) or tSNE (Van der Maaten and Hinton, 2008) are frequently used to visualize and analyze high-dimensional data (Keyes et al., 2020), their use often results in limited inspection and presentation of direct fluorescence measurements. In our example, “old-fashioned” examination of the individual parameter channels in low-dimensional space (Fig. 5G–H) is crucial to revealing these issues. Further, the inclusion of scales is critical to assess antibody staining, particularly when using color scales in dimensionality reduction projections. Finally, when in doubt, positive control samples or

populations should be used to validate expression of key markers. In this example, FOXP3 expression on $CD8^+$ T cells can be compared to a known positive population ($CD4^+$ T_{regs}) to quantify the true level of $FOXP3^+$ cells in this sample and to guide gating strategies (Fig. 5H). However, as described above, these controls may not always be available if the expression of the marker in question is cell-type or spatially restricted.

Induced sources of heterogeneity

Experimental uncertainty is also introduced into flow cytometry and scRNA-seq experiments through artifacts from sample processing. Batch effects result from the separate sample collection, processing, and/or data acquisition of different samples, and can introduce artificial heterogeneity. When data are collected from different patients, cell types, or tissues at different times, technical variation may also obscure true biological differences (Hicks et al., 2018). This is a particular problem in samples like tumors where significant biological heterogeneity is already present. Preclinical studies often allow the simultaneous processing of samples from different treatment groups or separate animals, but batch effects remain a particular challenge in human studies, where patient samples are often collected over many weeks or months. In such cases, batch effects can be quantified and controlled by including a reference cell population in each technical sequencing run (Stuart et al., 2019). For single-cell studies, an additional strategy is to prepare samples and acquire data from different individuals and/or experimental conditions simultaneously. In flow cytometry experiments, this can be done by barcoding individual samples with unique fluorophores (Krutzik and Nolan, 2006), but this technique is more relevant in single-cell sequencing studies where cells from each analyzed sample can be barcoded with a unique, DNA-labeled sequencing antibody specific for a ubiquitously-expressed antigen (Stoeckius et al., 2018). This technique, Cellular Indexing of Transcriptomes and Epitopes by Sequencing (Stoeckius et al., 2017), or CITE-seq, is also a powerful method for detecting hundreds of proteins simultaneously on single cells. Panels of over 250 antibodies have been used to characterize immune tumor infiltrate with this method (Pombo Antunes et al., 2021), and large CITE-seq antibody cocktails are now available commercially, providing unprecedented insights into the heterogeneity at the protein level. As fluorescence spillover is not an issue in CITE-seq experiments, it is an excellent complementary and confirmatory technique to flow cytometry.

In human studies, T cell phenotype may also be affected by the current and previous medical and pharmacological history of the patient cohort. Importantly, immunotherapies may directly change T cell phenotype, abundance, and clonality within the tumor, and this likely varies by treatment type. PD-1 pathway blockade does not markedly change exhausted T cell phenotype, which is limited by stability of a dysfunctional epigenetic state (Pauken *et al.*, 2016). Instead, in both preclinical mouse models and human tumors, PD-1 pathway blockade results in a higher abundance of classically-exhausted $CD8^+$ T cells (Bassez et al., 2021; Hudson *et al.*, 2019a; Zhang et al., 2021) caused by division and differentiation of the $TCF\text{-}1^+$ stem-like population (Im *et al.*, 2016). Most – but not all – human tumor studies show responding cells originate from pre-existing clones found in the pre-treatment tumor (Bassez *et al.*, 2021; Chang et al., 2020; Liu et al., 2021; Yost et al., 2019). As with differentiation trajectory analysis, the interplay of methodology with T cell biology in such studies is critical to interpretation of these results. In the chronic LCMV infection model, the

stem-like population is spatially restricted to lymphoid tissues and contains a more diverse TCR repertoire (Chang *et al.*, 2020; Im *et al.*, 2020). In human cancer, the TCR repertoire varies spatially throughout the tumor microenvironment (Joshi et al., 2019; Sudmeier *et al.*, 2022). Thus, appropriate sequencing depth and spatial sampling are critical to accurately define changes in T cell phenotype and TCR repertoire in response to immunotherapy.

In contrast to PD-1 pathway blockade monotherapy, its combination with IL-2 administration results in drastic changes in antigen-specific $CD8^+$ T cell phenotype (Hashimoto et al., In press; West et al., 2013). Immunologic effects of other medications should also be considered when assessing TIL phenotype and function in patients. Glucocorticoids, for example, are used both to directly treat various hematological malignancies and also to manage sequelae of other cancers (Keith, 2008) but may promote T cell dysfunction in the tumor microenvironment (Acharya et al., 2020). Systemic chemotherapy agents may also induce changes in T cell phenotype within the tumor microenvironment (Zhang *et al.*, 2021).

Tissue disruption and cell isolation is an additional processing step with the potential to confound interpretation of cell states. Enzymatic digestion with various proteases is commonly used to generate viable single-cell suspensions from tumors and other tissues (Carney and Malmgren, 1967; Corgnac et al., 2021; Reichard and Asosingh, 2018; Rodriguez de la Fuente et al., 2021). While this method is employed to obtain the highest possible number of viable cells, these digestion steps can cleave or otherwise alter extracellular proteins, creating false negatives in flow cytometry or CITE-sequencing experiments (Autengruber et al., 2012; Hines et al., 2014). A more insidious effect of enzymatic digestion is its effects on cellular transcriptomes. Incubation of immune cells at room temperature for as little as two hours can impact gene expression (Massoni-Badosa et al., 2020). Collagenase digestion at 37 °C causes large changes in cellular transcriptomes, including upregulation of immune-associated genes such *FOS*, *JUN*, and *NR4A1* (O'Flanagan et al., 2019). Even though these changes may only be present in a subset of digested cells, the magnitude of these changes can be high, requiring careful inspection of clusters in single-cell experiments in addition to careful analysis of stressrelated genes in bulk RNA-sequencing experiments (van den Brink et al., 2017). As a potential remedy, some groups and commercial suppliers have investigated fixation to "lock-in" cell states before subsequent downstream processing (Alles et al., 2017). While this method may introduce transcript length and GC content-dependent bias (Wang et al., 2021), it also enables cell isolation based on fluorescence sorting with intracellular markers (Thomsen et al., 2015).

Location, location, location

Generation of single-cell suspensions from tumors or other tissue may result in the underestimation of target cell abundance (Stark et al., 2018; Steinert et al., 2015) and causes the loss of spatial information. Recent studies have shown that spatial restriction of $CD8^+$ T cell subsets is not limited to differences among tissues, but T cell phenotype is also closely linked to position within the TME. For example, TCR repertoires vary across space in tumors (Joshi *et al.*, 2019), and $TCF\text{-}1^+$ cells are more likely to be situated in

tertiary lymphoid structures or stromal antigen-presenting “niches” (Jansen et al., 2019). Spatial biology has advanced significantly in recent years, allowing the detection of many proteins and gene expression signatures *in situ* in tissue sections. While low-parameter immunohistochemistry has been a mainstay of cancer pathology for decades, new methods have been developed to measure multiple proteins by microscopy, with multiplexing ranging from a handful to 40 or more detected markers (Tan et al., 2020). When combined with the ability to image whole tumor sections, the spatial architecture of cell types within the TME can be defined and even correlated with clinical outcomes (Berry et al., 2021; Jansen *et al.*, 2019).

Interrogating the spatial architecture of the TME has already provided significant insights, such as demonstrating the importance of immune cell co-localization - spanning from small lymphoid aggregates to fully-fledged tertiary lymphoid structures (TLS) - for anti-tumor immunity and response to ICIs (Berry *et al.*, 2021; Helmink et al., 2020; Jansen *et al.*, 2019; Schumacher and Thommen, 2022). As mentioned, tumor-specific T cells express the B cell chemoattractant CXCL13 and are thus equipped to potentially remodel their microenvironment, with $CXCL13^+$ cells being found in close proximity to tumor-infiltrating B cells and within TLS (Gu-Trantien et al., 2017; Ukita et al., 2022). TLS and smaller immune structures have been suggested to provide a protective niche for the local maintenance of stem-like $CD8^+$ T cells (Jansen *et al.*, 2019; Pagliarulo et al., 2022). However, most protein-based multiplex imaging approaches do not provide antigen-specificity information of interrogated cells, hindering discrimination of bystander and tumor-specific cells. One notable exception is *in situ* tetramer staining, which has shown the presence of EBV- and influenza-specific $CD8^+$ T cells in glioblastoma (Ning *et al.*, 2022).

A potential solution to this problem lies in spatial transcriptomics, a new technique that permits the measurement of RNA levels – often on a whole-transcriptome basis – *in situ* within tissue sections (Rao et al., 2021). While spatial transcriptomics currently provides neither the resolution nor the sequencing depth of scRNA-seq, integration of the two modalities can elucidate significant biological insight (Longo et al., 2021). Spatially-resolved methods that can capture complete transcriptomes may be a particular boon for tumor immunology. Targeted amplification with these techniques can recover lymphocyte antigen receptor sequences; we recently used targeted TCR amplification combined with spatial transcriptomics to show that clones with a $CD39^+$ phenotype were preferentially enriched within tumor beds, whereas clones expressing TCF-1 were found in the surrounding stroma (Hudson and Sudmeier, 2022; Sudmeier *et al.*, 2022). Similar techniques have also been used to identify B cell receptor sequences in tumors (Meylan et al., 2022). Long-read sequencing could also be used to detect mutated mRNAs and copy number variations in cancer, aiding in localization of neoantigen-expressing tumor cells (Erickson et al., 2022; Lebrigand et al., 2022). The combination of these modalities will allow extremely detailed studies of the TME, such as determining how the distribution and gene expression patterns of neo-antigen-specific T cell clones are influenced by expression of their cognate antigens.

Putting it all together

The emergence of new multidimensional technologies such as scRNA-seq, high-parameter flow cytometry, and spatial transcriptomics have transformed our view of anti-tumor immune responses. In the future, as these technologies expand in capability and are adopted more widely, they will provide deep insights into immune cell behavior within the TME. Such knowledge has the potential to form the foundation of rationally-designed and more effective immunotherapies. To achieve this goal, however, the sheer volume of data generated by these technologies must be matched by focused experimental questions, accurate analysis, clear interpretation, and supportive mechanistic studies.

Recent years have witnessed a slowdown in FDA approval of ICIs, and the vast majority of approvals have targeted the PD-1/PD-L1 inhibitory pathway (de Miguel and Calvo, 2020). To accurately identify targets for next-generation ICIs, determination of antigen specificity within the TME must be a crucial component of future immuno-oncology studies. Studies identifying tumor-specific T cells (Eberhardt *et al.*, 2021; Oliveira *et al.*, 2022; Oliveira *et al.*, 2021; Simoni *et al.*, 2018) have already provided important insights into their differentiation states; studying the signaling pathways within and in spatial proximity of tumor-specific cells is an important task to identify effective and specific ICI targets. Understanding tumor-specific T cells will also inform strategies for engineering improved function of CAR and TCR transgenic T cells, which have been widely used for hematologic malignancies and are now seeing use in other tumor types (Leidner et al., 2022; Majzner et al., 2022; Singh and McGuirk, 2020). While infiltration of bystander T cells may complicate the study of tumor-specific cells, they also represent an important therapeutic target, particularly to increase infiltration of “cold” tumors or promote inflammation and antigen processing in patients with poor pre-existing anti-tumor T cell responses (Lapteva et al., 2014; Rosato *et al.*, 2019; Wang et al., 2020). Thus, the study of antiviral immune responses in the infectious disease and basic immunology fields also directly benefits immuno-oncology research efforts.

Generating therapies and therapeutic strategies – or even working biological hypotheses – from this deluge of -omics data may represent the greatest current challenge in immunology and most other fields of biology (Anderson, 2008; Denecker and Lelandais, 2022; Krassowski et al., 2020). As we have discussed here, it is crucial to be aware of the intrinsic limitations and potential sources of error in modern immunology techniques. As ever more raw data are collected, processed data attract more of our attention, often obscuring experimental error and true biological complexity in the process (Chari et al., 2021; Gorin et al., 2022). To combat this, the use of complementary techniques can test and control for both biological, experimental, and analytical sources of error. For example, CITE-seq and scRNA-seq respectively measure protein and transcript levels in single cells, providing two independent measurements of gene and gene product expression. CITE-seq and flow cytometry both measure single-cell protein expression but have different cell throughput and sources of experimental error. However, while these complementary and multidimensional techniques provide unprecedented insights into human anti-tumor immune responses, they are intrinsically descriptive, should be mostly used for hypothesis generation and ultimately require experimental validation for establishing causal relationships.

While recent advances, such as an *ex vivo* tumor platform to assess patient response to ICIs (Voabil et al., 2021), have permitted more mechanistic study of human tumors, preclinical tumor immunology models remain an irreplaceable cornerstone to evaluate findings in the - omics era. These models allow the normalization of many variables across treatment groups, most notably antigen specificity, tumor burden, age, and genetic background. Much work has been performed to make these models better recapitulate human cancer, including varied processes for tumor initiation and the use of different tumor antigens (Connolly et al., 2022). In these models, the use of technologies such as scRNA-seq, high-parameter flow cytometry, and spatial transcriptomics can deliver unparalleled phenotypic data that can be directly attributed to an experimental treatment or condition. Such studies combining hypothesis-driven science with emerging immunological techniques have the potential to create accurate models of immune function, provide invaluable insights into anti-tumor immune responses, and ultimately to improve patient outcomes.

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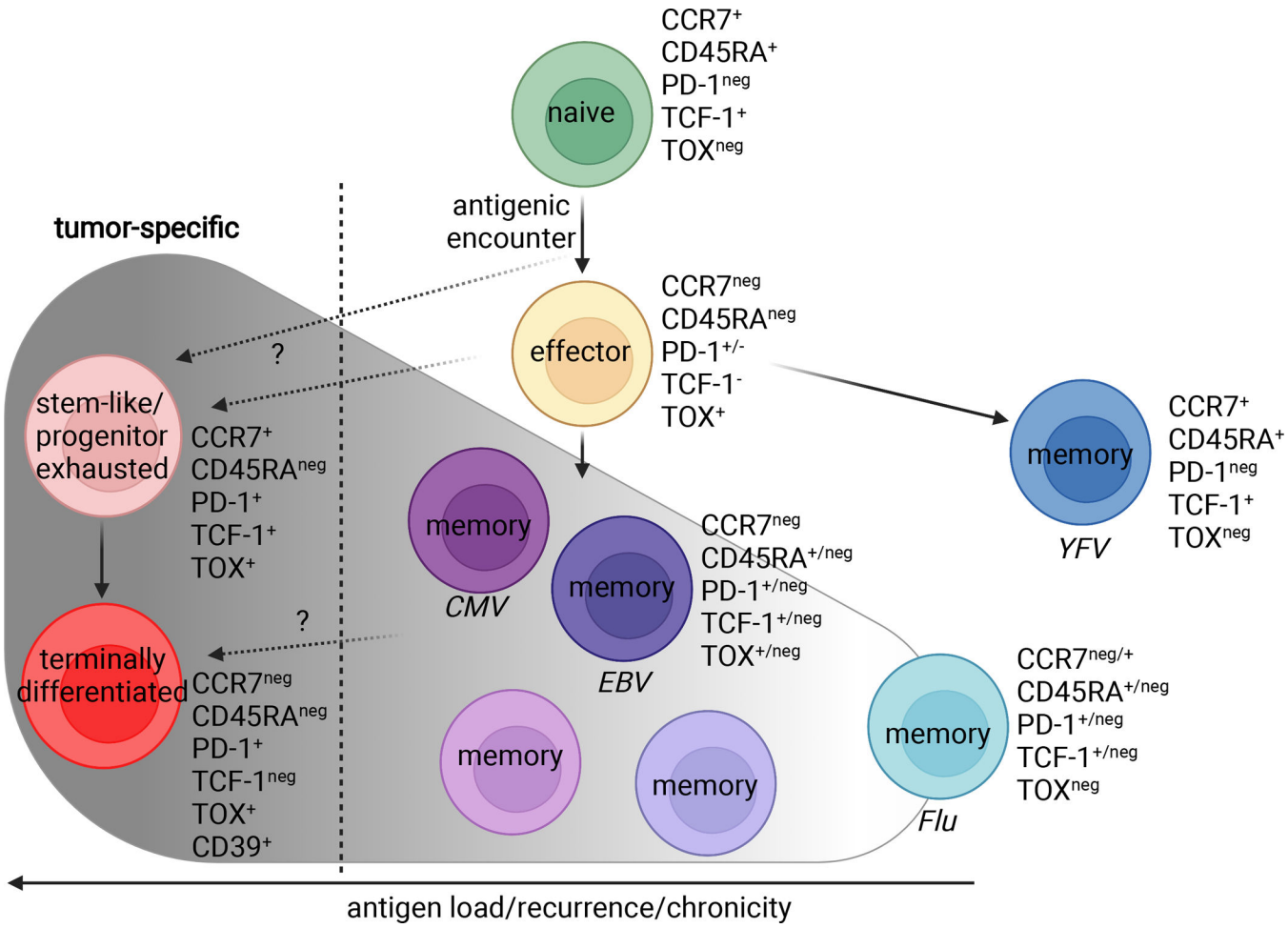

Figure 1:

Human CD8$^+$ T cell differentiation and associated phenotypic markers. Tumor-specific phenotypes are shown in red. Upon antigenic encounter, naïve CD8$^+$ T cells differentiate into effector cells and further differentiate into different states depending on antigen clearance and chronic/recurrent antigen loads. Memory T cells specific for yellow fever virus (YFV) and the common human pathogens Influenza virus (Flu), Epstein-Barr virus (EBV) and cytomegalovirus (CMV), as well as tumor-specific cells (stem-like/progenitor exhausted and terminally differentiated) are shown along a schematic continuum of antigen load/recurrence. Of note, CMV- and EBV-specific memory CD8$^+$ T cells can adopt various phenotypes and even resemble the phenotype of tumor-specific stem-like CD8$^+$ T cells. Figure created with BioRender.

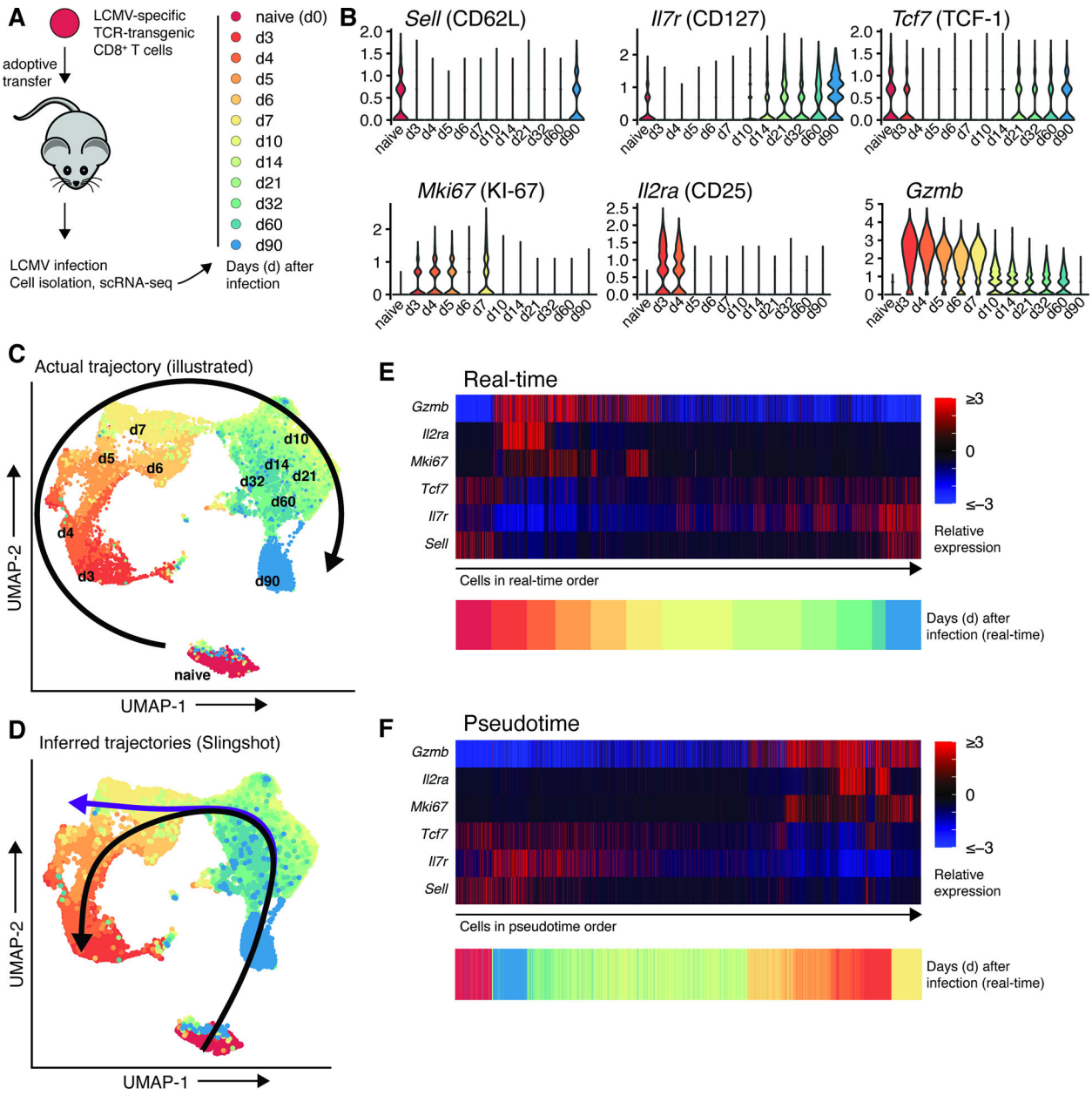

Figure 2:

Inference of a reverse differentiation trajectory from scRNA-seq data. A: TCR-transgenic CD8$^+$ T cells were transferred to mice which were subsequently infected with LCMV Armstrong. At illustrated timepoints, cells were sorted and subjected to scRNA-seq. B: Expression of T cell differentiation markers at indicated time points. C: UMAP projection of sequenced cells, with an illustrated arrow showing actual cell differentiation trajectory. D: Identical UMAP projection, with inferred trajectories. E: Heatmap of T cell differentiation marker expression, with cells in real-time order. F: Heatmap of T cell differentiation marker expression, with cells in pseudotime order. Raw data are from (Kurd *et al.*, 2020). Code and data to reproduce the scRNA-seq analysis are available on Mendeley Data, doi: 10.17632/3dvt79c7yt.1.

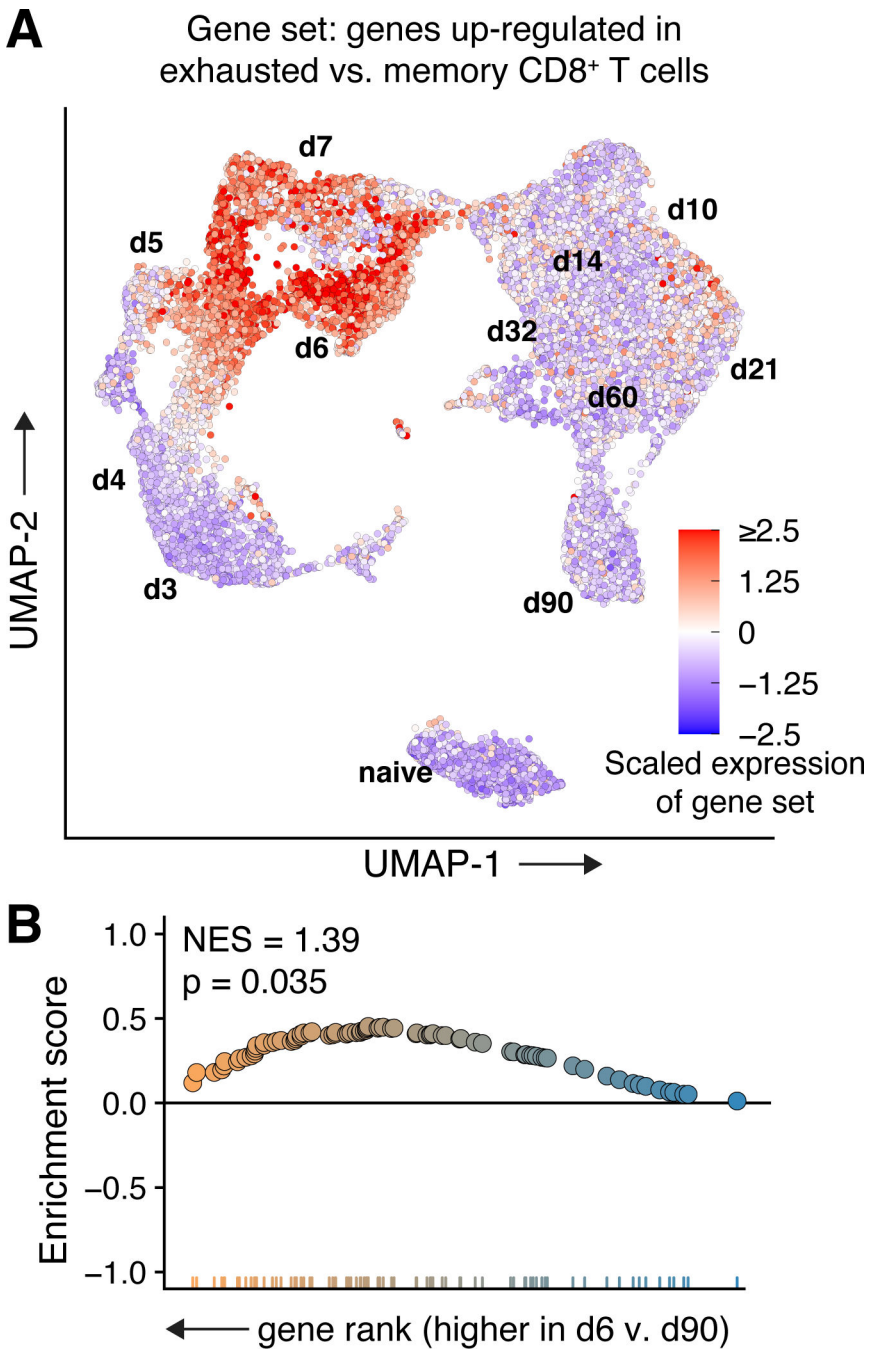

Figure 3:

Expression of an exhaustion-associated gene set in non-exhausted CD8$^+$ T cells. A: Relative expression of the GSE41867_MEMORY_VS_EXHAUSTED_CD8_TCELL_DAY30_LCMV_DN gene set (Godec et al., 2016) in CD8$^+$ T cells following acute infection. B: Exhaustion-associated genes are significantly enriched in day 6 vs day 90 cells, despite neither subset being biologically exhausted.

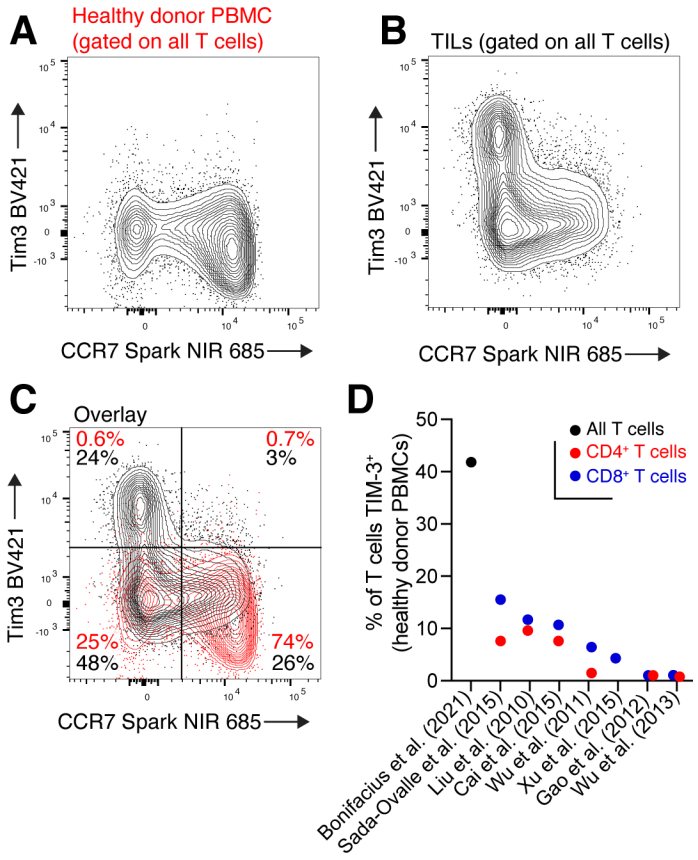

Figure 4:

Quantification of TIM-3 on human T cells. A: CCR7 and TIM-3 staining on circulating T cells of a healthy donor. With this tissue only, it is unclear if TIM-3 staining was successful, and if so, where TIM-3^{+} cells should be gated. B: CCR7 and TIM-3 expression on tumor-infiltrating T cells, stained at the same time as PBMCs in (A). C: Overlay of CCR7 and TIM-3 staining in healthy donor PBMCs (red) and TILs (black). D: Estimates of TIM-3 expression on circulating T cells of healthy donors from various studies. Flow data in panels A-C were previously reported (Sudmeier *et al.*, 2022).

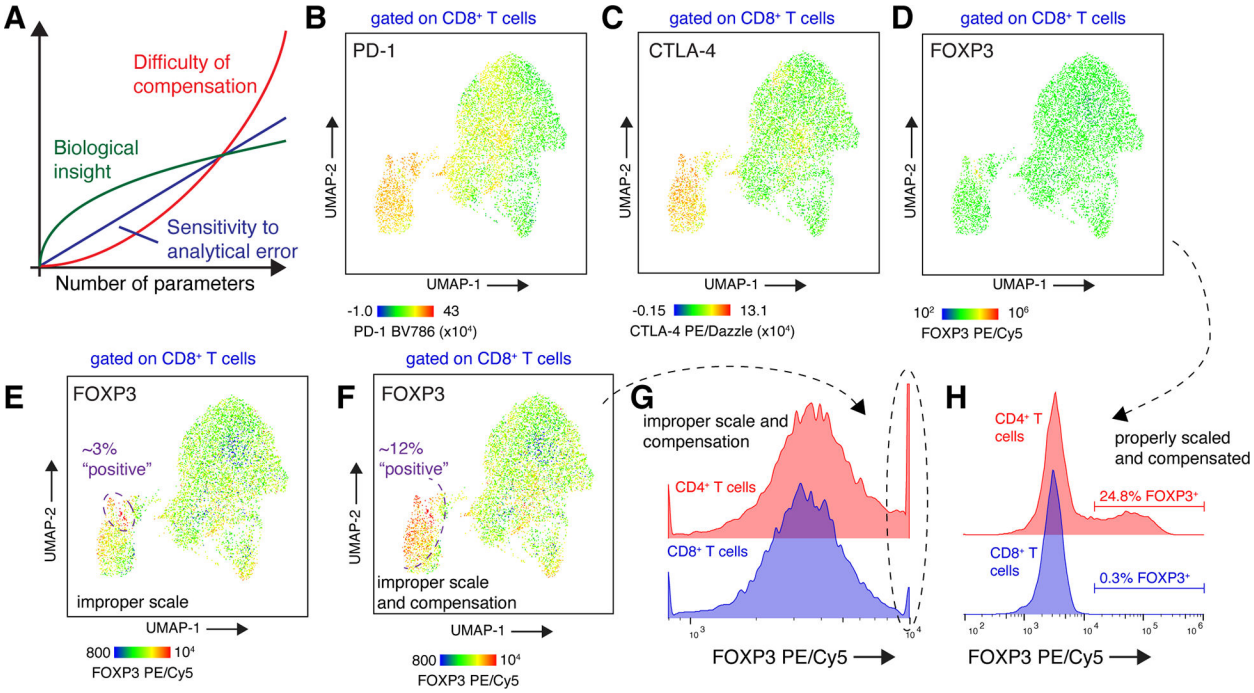

Figure 5:

Perils of data visualization and analysis in high-parameter flow cytometry. A: As more parameters are collected in a flow cytometry experiment, the potential biological insight increases. Unfortunately, both the difficulty of analysis and sensitivity of the experiment to such error also increase. An example is shown in panels B-H, where tumor-infiltrating lymphocytes from a brain metastasis patient were analyzed with a T cell-focused, 22-color flow cytometry panel. B-D: UMAP projection of $CD8^+$ T cells, with cells colored by expression of PD-1 (B), CTLA-4 (C), and FOXP3 (D). Data shown are properly compensated and scaled. E: UMAP projection of $CD8^+$ T cells, with cells colored by expression of FOXP3. The color intensity scale has been modified compared to panel (D); data are properly compensated. F: UMAP projection of $CD8^+$ T cells, with cells colored by expression of FOXP3 (same scale as panel E). Fluorescence spillover of CTLA-4 PE/Dazzle into FOXP3 PE/Cy5 has been undercompensated by 10%. (G) An alternate display method, such as a histogram, using an internal positive control for FOXP3 expression would reveal improper scaling of FOXP3 fluorescence intensities (circled population). H: The true percentage of $FOXP3^+$ $CD8^+$ T cells in this sample is <0.5%. Data to reproduce the flow cytometry analysis are available on Mendeley Data, doi: 10.17632/3dvt79c7yt.1.