HHS Public Access

Author manuscript

Nat Rev Cancer. Author manuscript; available in PMC 2024 January 08.

Published in final edited form as:

Nat Rev Cancer. 2023 May ; 23(5): 295–316. doi:10.1038/s41568-023-00560-y.

Dynamics and specificities of T cells in cancer immunotherapy

Giacomo Oliveira[1,2,3,#], **Catherine J. Wu**[1,2,3,4,#]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

[2]Harvard Medical School, Boston, MA, USA

[3]Broad Institute of MIT and Harvard, Cambridge, MA, USA

[4]Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA

Abstract

Recent advances in cancer immunotherapy — ranging from immune- checkpoint blockade therapy to adoptive cellular therapy and vaccines — have revolutionized cancer treatment paradigms, yet the variability in clinical responses to these agents has motivated intense interest in understanding how the T cell landscape evolves with respect to response to immune intervention. Over the past decade, the advent of multidimensional single-cell technologies has provided the unprecedented ability to dissect the constellation of cell states of lymphocytes within a tumour microenvironment. In particular, the rapidly expanding capacity to definitively link intratumoural phenotypes with the antigen specificity of T cells provided by T cell receptors (TCRs) has now made it possible to focus on investigating the properties of T cells with tumour-specific reactivity. Moreover, the assessment of TCR clonality has enabled a molecular approach to track the trajectories, clonal dynamics and phenotypic changes of antitumour T cells over the course of immunotherapeutic intervention. Here, we review the current knowledge on the cellular states and antigen specificities of antitumour T cells and examine how fine characterization of T cell dynamics in patients has provided meaningful insights into the mechanisms underlying effective cancer immunotherapy. We highlight those T cell subsets associated with productive T cell responses and discuss how diverse immunotherapies might leverage the pre-existing tumour-reactive T cell pool or instruct de novo generation of antitumour specificities. Future studies aimed at elucidating the factors associated with the elicitation of productive antitumour T cell immunity are anticipated to instruct the design of more efficacious treatment strategies.

Introduction

Immune cells are the cellular underpinnings of cancer immunotherapy[1,2]. For T cells, antitumour reactivity is defined by their unique T cell receptors (TCRs), capable of recognizing specific antigens presented in the context of human leukocyte antigen (HLA)

giacomo_oliveira@dfci.harvard.edu; cwu@partners.org.

Author contributions G.O. researched data for the article. Both authors contributed substantially to discussion of the content, wrote the article, and reviewed and/or edited the manuscript before submission.

Competing interests C.J.W. is an equity holder of BioNTech. G.O. declares no competing interests.

Peer review information *Nature Reviews Cancer* thanks Paul Robbins, who co-reviewed with Sri Krishna; Kellie N. Smith; and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

molecules[3]. Under physiological conditions, such as an encounter with a pathogen, antigen-triggered T cell stimulation via TCRs leads to robust immune responses, culminating in the generation of effector lymphocytes, able to traffic across tissues and eradicate target antigens, and of memory cells, which can persist and provide long-term protection in the event of antigen rechallenge[4].

In the native tumour setting, however, T cells typically fail to eradicate tumour cells despite their documented ability to infiltrate the tumour microenvironment (TME) and to recognize, to a variable extent, epitopes presented on cancer cells[5]. While lack of response could be ascribed, in part, to limited antigenicity on the tumour side[6–8], studies in mouse models of chronic antigen stimulation[9–11] and characterizations of tumour-infiltrating lymphocytes (TILs) in immunologically ‘hot’ human tumours[12–14] have highlighted that antitumour T cells can progressively acquire a dysfunctional state during cancer progression, enabling tumour immune evasion[15,16]. Indeed, restoring the functionality of the tumour-specific T cell pool within the TME has broadly been the focus of modern era immunotherapies that have transformed the clinical care of patients with cancer over the past decade[17]. Still, only a minority of patients achieve long-term remission[18–20], and a better understanding of the properties and dynamics of antitumour T cells will be key to improving our ability to deploy and manipulate T cell immunity against cancer.

Deconvolution of the antitumour T cell landscape has been remarkably challenging for several reasons: (1) T cells can acquire a broad spectrum of functional states[14,21] and, hence, pinpointing the exact subsets responsible for effective antitumour responses has not been straight-forward; (2) TILs display an extremely diverse range of specificities[22], and definitive identification of TCRs with confirmed tumour specificity at scale is currently technically demanding; and (3) T cell responses are dynamic across time and tissue compartments and, hence, correct inference of tumour-specific T cell trajectories necessitates meticulous and possibly longitudinal sample collections. Over the past decade, several critical technological advances have enabled the more comprehensive capture of the complex landscape of T cells in patients with cancer (Box 1). First, single-cell profiling technologies, such as transcriptome (single-cell RNA (scRNA)) sequencing and single-cell TCR sequencing (scTCR-seq), have provided the ability to unambiguously assign phenotypic characteristics to T cell clonotypes at the resolution of individual cells[23,24]. Second, given the countless possibilities generated by V(D)J recombination, TCRs can also be used as molecular barcodes to trace the dynamics of antigen-specific populations over time and across different compartments[25,26]. Finally, TCR reconstruction in reporter cell lines has given rise to the opportunity to interrogate the antigen specificity and biochemical properties of TCRs[27,28]. When used in combination, these capabilities provide the means to comprehensively investigate the properties of antitumour T cells within the native TME and upon immunotherapeutic intervention. Herein, we summarize our current understanding of the antitumour T cell repertoire within the human TME and how it is shaped by diverse immunotherapies through the lens of recent single-cell profiling studies.

TILs in the cancer immune landscape

T cell states within the native tumour microenvironment

The extent of T cell infiltration within the tumour site has long been recognized as an important prognostic factor for patients with cancer, even before immune-checkpoint blockade (ICB) became an integral part of the therapeutic arsenal for cancer treatment[29–31]. Indeed, with respect to the rather simplistic dichotomy of immunologically ‘cold’ versus ‘hot’ tumours, numerous studies have consistently demonstrated that the presence of T cells within tumours or at their margins (for example, quantitated by immunohistochemistry)[32] or the detection of cytotoxic activity within the tumour (estimated from bulk gene expression profiling)[33] are associated with improved clinical outcomes. More recently, single TIL characterization, at an ever-increasing level of granularity and across different cancer types, has provided evidence for how T cells within the TME can exist in a spectrum of different phenotypic cell states, each defined by diverse degrees of differentiation, cytotoxicity, and proliferative and stemness potential[14,34,35]. A broad array of TIL subsets has been described across studies; while many have been characterized in specific cancer types, disease stages (localized versus metastatic) or tissues (tumour versus lymph node versus peripheral blood), recurrent phenotypic and transcriptional patterns have been identified[12,13,21,36–42], although often designated with different nomenclatures[14]. As described below, we synthesize the wealth of marker and phenotypic information afforded by these recent single-cell studies and propose a streamlined lexicon of T cell subsets (Fig. 1).

Across tumour tissue types, ‘hot’ TMEs can be predominantly infiltrated by antigen-experienced $CD4^+$ and $CD8^+$ T cells, originating from naive precursors undergoing activation and differentiation upon encounter and recognition of their cognate antigens. Antigen-experienced cells captured within the TME can be broadly compartmentalized into memory T cells (T_{Mem} cells) and exhausted T cells (T_{Ex} cells)[43]. T_{Mem} cells comprise heterogeneous subpopulations of lymphocytes with a varying ability to persist and generate a progeny of effector cells[44,45]. T_{Mem} cells at different stages of differentiation — namely stem cell memory T cells (T_{SCM} cells), central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells) — can also be found at high frequencies in blood, secondary lymphoid organs and peripheral organs[46–48]. T_{Mem} cells are all characterized by the expression, albeit at different levels, of T cell-specific transcription factor 1 (TCF1, encoded by the gene *TCF7*), homeostatic cytokine receptors (such as IL-7 receptor (IL-7R)) and homing receptors for secondary lymphoid organs (for example, L-selectin (also known as CD62L) and CC-chemokine receptor 7 (CCR7)). T_{Mem} cells are commonly generated during physiological immune responses, when the inciting pathogen is cleared or under control (as in certain chronic infections), which serves to establish a persisting pool of antigen-specific T cells ready to mount a secondary immune response in the case of antigen rechallenge[49–51].

The second broad class of T cells in the TME are those characterized by the high expression of a group of proteins collectively associated with a state termed ‘exhaustion’[16,52–54], namely programmed cell death protein 1 (PD1), cytotoxic T lymphocyte-associated antigen 4 (CTLA4), lymphocyte activation gene 3 (LAG3), T cell immunoglobulin mucin receptor

3 (TIM3) and the CD39 antigen (which is encoded by *ENTPD1*). Expression of these markers typically characterizes cells at a late stage of differentiation with variable degrees of impairment of cytotoxic function consequent to chronic antigenic stimulation provided by persistent tumour antigens within the TME[55]. This is also accompanied by functional adaptation as an increased production of chemokines, such as CXC-chemokine ligand 13 (CXCL13), is commonly observed among T_{Ex} cells[56]. Importantly, upregulation of similar markers is also observed early after T cell activation, indicating that the 'exhausted' T cell compartment can potentially encompass T cells in different phases of response to antigen stimulation[55,57], corresponding to diverse functional roles. In contrast to T_{Mem} cells, TIL clones with an intratumoural T_{Ex} cell phenotype are rarely detected in circulation but instead are confined to the site where their cognate antigens are abundant as demonstrated by studies investigating the distribution of TIL clones in tumours and peripheral blood[58,59].

Within the TME, chronic antigen stimulation leads to a gradual loss of effector functions through a complex epigenetic and transcriptional programme orchestrated by the transcription factor thymocyte selection-associated high mobility group box protein (TOX)[60–62]. Studies in mouse models of chronic stimulation (either virus or tumour driven) have elegantly shown that *TOX* expression results in the generation of both terminally exhausted T cells (T_{TE} cells) and progenitor exhausted T cells (TPE cells)[60–62]. While T_{TE} cells show production of effector molecules at the transcriptional level (*PRF1* (encoding perforin 1) and the genes encoding granzymes), their terminal exhaustion severely hampers their cytotoxic potential, resulting in non-functional T cell responses that lack the ability to regenerate functional effectors and are prone to activation-induced cell death[15]. Even if terminally differentiated, T_{TE} cells can still proliferate in an antigen-dependent fashion, thereby explaining their numerical predominance within the TME[53], where they likely exhibit the first rapid (dys)functional control of proliferating tumour cells; the T_{TE} cell compartment can also be replenished by T_{PE} cells, which represents a reservoir of T_{Ex} cells still capable of self-renewal and differentiation due to preserved expression of TCF1 (refs. 11,63). While T_{PE} cells lack major cytotoxic properties, they contribute to the regeneration of a line of defence constituted by terminally differentiated effector T cells[9,10]. Of note, as addressed in mouse models and validated in humans, TILs with T_{PE} cell properties are often found in lymph nodes and intratumoural tertiary lymphoid structures (TLS)[9,64,65], suggesting that maintenance of T_{PE} cells requires nurturing interactions with other immune cells available at those sites.

Finally, a proportion of T_{Ex} cells shows transcriptional signatures of tissue-resident memory T (TRM) cells[66–68]. As TRM cell markers, such as αE integrin (also known as CD103 (ref. 69) and encoded by *ITGAE*) and the transcription factor homologue of BLIMP1 in T cell (HOBIT; encoded by *ZNF683*)[70,71], are usually co-expressed with PD1, CD39 and other exhaustion markers even in normal tissues[68,72], we favour the designation of this subset as T_{RM}-like T_{Ex} cells when found in the TME. While it is difficult to pinpoint if such cells represent T_{RM} cells after chronic antigenic stimulation or a variation of T_{TE} cell differentiation within the tumour, T_{TE} and T_{RM} cells clearly represent a continuum in the spectrum of TIL phenotypes as recently highlighted in a pan-cancer single-cell TIL study[21].

While these subsets have been described mostly for $CD8^+$ TILs, the same categorization applies to antigen-experienced $CD4^+$ cellular counterparts. As $CD4^+$ T cells can undertake more differentiation programmes than $CD8^+$ T cells, including follicular helper T (T_{FH}) cell and regulatory T (T_{reg}) cell fates[73,74], this enhanced complexity is also reflected in the $CD4^+$ T cell composition within the TME[75–77]. Indeed, T_{FH} TILs, identified by co-expression of CXC-chemokine receptor 5 (CXCR5), TCF1 and PD1 (ref. 78), exhibit a transcriptional profile that closely resembles that of T_{PE} cells[9]. In addition, the TME is populated by numerous $CD4^+$ T_{reg} cells expressing CD25 (also known as IL-2RA) and the transcription factor forkhead box protein P3 (FOXP3)[13,36,79,80]. The frequency of immunosuppressive $CD4^+$ T_{reg} cells is markedly increased at tumour sites compared with peripheral blood, as described in a recent pan-cancer study profiling the TME at single-cell resolution[21], where immunosuppressive $CD4^+$ T_{reg} cells were detected with different states of activation. Notably, in non-small-cell lung cancer (NSCLC), the extent of activated T_{reg} cells expressing the CD137 activation antigen inversely correlated with clinical outcomes and response to ICB[36,79].

T cell clonality

While $CD4^+$ and $CD8^+$ T cells can be present within the TME with diverse phenotypes, their exact contribution to tumour-directed immunity ultimately depends on the primary signals they receive via TCR engagement[81]. TCRs confer T cells their antigen specificity. Due to the almost infinite combinatorial permutations provided by V(D)J recombination during thymic development[82], TCR sequences also provide unique molecular barcodes that can enable the detection and tracking of T cell clonal dynamics over time or across distinct anatomic sites[25]. Given these informative characteristics, tools for the direct sequencing or inference of TCR structure from whole exomes or transcriptome data have been the subject of several experimental or computational efforts over the past decade (extensively reviewed in ref. 83).

Evidence of expansion of dominant clones within the TME has been conventionally used as indirect proof for local recognition of tumour antigens. When applied to cellular subsets of TILs, isolated based on discrete immunophenotypic markers, TCR sequencing of such bulk populations has offered the potential for a more detailed understanding of the extent to which particular T cell states are preferentially associated with clonal T cell expansion within the TME and the ability to infer T cell clonal dynamics and differentiation trees within the TME. For example, TCR sequencing of melanoma TILs sorted based on PD1 expression revealed the vast majority of expanded clonotypes to segregate within the exhausted compartment[84,85]. Notably, most TIL clones exhibited a non-overlapping distribution either in the $PD1^+$ or $PD1^-$ compartment, thus indicating that individual T cell clones tended to preferentially acquire either an exhausted or a non-exhausted phenotype. Analysis of TIL clonality based on sorting for the T_{RM}-like T_{Ex} cell markers (CD103 and CD39) has generated similar results[69].

In recent years, the availability of single-cell transcriptome profiling, coupled with scTCR-seq (in which paired TCR α-chain and β-chain information is detected), has provided unprecedented resolution for the characterization of clonotype dynamics within the TME.

We recently performed high-resolution profiling of melanoma TILs and found that the T_{Ex} cell compartment exhibited the least TCR diversity, consistent with high intratumoural clonal expansion[58]. TCR clones from T_{Ex} cells could also be traced in T_{PE} and T_{TE} cell clusters, suggesting that there is, at least in this tumour type, a continuum in the spectrum of differentiation across these cell states[13,58]. Notably, T_{Ex} cell oligoclonality was consistent among both $CD8^+$ and $CD4^+$ TIL compartments[86], albeit the size of dominant clonotypes was more prominent in the former[13,58]. Clonally expanded TCRs could also be observed within the T_{Mem} cell compartment, yet they were distinct and non-overlapping with those from the T_{Ex} cell clusters, indicating that antigen specificity plays a crucial role in shaping T cell phenotypic properties[58]. Other investigators have similarly shown that TIL clonotypes preferentially segregate within so-called ‘primary clusters’ (mostly consisting of $HOBIT^+$-expressing clusters, reminiscent of T_{RM} cell profiles) across a spectrum of cell states in the TME of NSCLC, renal cell carcinoma (RCC), endometrial cancer and colorectal carcinoma (CRC)[59]. While single-cell profiling of diverse tumour types (that is, basal cell carcinoma, squamous cell carcinoma, RCC and NSCLC) has shown that individual cells from the same TCR clonotype family can reside in distinct transcriptional clusters[41,42,79,87–89], most of these states could be traced back to either one of the two broad categories of T_{Mem} or T_{Ex} cells. Within this conceptual framework, the TCR repertoire of exhausted and non-exhausted TIL fractions overlapped only minimally, with clonally expanded $CD8^+$ TILs preferentially displaying an exhausted expression profile. Of note, the same studies have also demonstrated that, within the T_{Ex} cell compartment, a range of transcriptionally exhausted phenotypes can be observed not only among the different TCR clonotypes but also among TILs with identical TCRs (thus belonging to the same clonotype family). As investigation of the transcriptional states of individual TCR clonotypes is still in its infancy, it is preliminary to speculate on the factors underlying such phenotypic heterogeneity. Indeed, several parameters can potentially affect the transcriptional profile of TIL clonotypes, including their localization in different tumour or non-tumour areas, their interactions with the surrounding cells, and the levels of cognate antigens to which they are exposed.

In addition to the measurement of T cell clonality in the TME, other studies have attempted to infer antitumour specificity based on the paired analysis of distinct anatomic sites, with the assumption that clones expanded in the tumour and/or draining lymph nodes, rather than in peripheral blood and/or normal tissues adjacent to the tumour, are more likely enriched in tumour specificities. Indeed, a study of the RCC TME reported that the TCR diversity was lower (that is, with more clonally expanded TILs) than in normal tissue[89]. Thus, consistent with the notion of cellular expansion driven by recognition of tumour antigens, oligoclonality was associated with the acquisition of an exhausted phenotype and progressively increased with more advanced disease stages. In the separate setting of native melanoma, we likewise showed that $CD8^+$ TCR clonotypes expanded in T_{Ex} cell compartments within the TME were only rarely detected in peripheral blood and accounted, on average, for ~0.1% of the circulating T cell repertoire[58]. Of note, clonotypes identified in the intratumoural T_{Mem} cell compartment were easily detected in the periphery and represented up to 10–15% of circulating T cells[58].

Several studies in other diverse tumour settings have identified similar findings. One study includes data generated from a set of CRC tumours that were profiled at single-cell resolution[42]. Here, T cell clonotype analysis revealed that T_{Ex} cell clones expanded in the TME were barely detectable in peripheral blood, while T_{Mem} cell TCR clones were commonly shared among the TME, adjacent normal mucosa and systemic circulation. Preferential localization of T_{reg} cell clones characterized by high expression of CTLA4 within the TME was also identified. In another study, a similar pattern of T cell clonal distribution was observed in patients with hepatocellular carcinoma for which paired TCR profiling of blood, normal and tumour tissue was performed[41]. In a third study, in NSCLC, analysis of TCR clones within the $CD39^+$ and $CD39^-$ intratumoural compartments revealed that only dominant intratumoural $CD39^-$ (and not $CD39^+$) clones could be detected in peripheral blood, thus demonstrating the preferential localization within the tumour of T_{Ex} cell clones also when defined by CD39 expression[90]. Finally, in a pan-cancer analysis of TILs, integrated scRNA-seq and scTCR-seq analyses confirmed an increased expansion and proliferation of clonotypes from $CD8^+$ T_{Ex} cell subtypes across tumours, which were more enriched within the TME than in blood or normal tissues[21].

Overall, analyses of tissue distribution and expansion levels of T cell clones have pointed towards T_{Ex} TILs as the reservoir of T cells with antigen specificities directed against tumour antigens. This is not surprising, since chronic antigen stimulation can generate the observed progressive dysfunction, leading to the acquisition of a T_{Ex} cell phenotype and impairing the generation of a T_{Mem} cell pool able to recirculate throughout the body[13,58].

Defining those TILs with tumour-specific reactivity

Localization of T cell clonotypes within the TME has been considered in the past as indirect evidence of antitumour specificity. However, several studies have now documented that a major portion of TILs lacks any antitumour specificity. In a large cohort of patients with lung cancer and CRC, Simoni et al.[91] elegantly showed that $CD8^+$ TILs with a non-exhausted phenotype recognized a wide range of viral epitopes, deriving from endemic viruses such as Epstein–Barr virus, human cytomegalovirus and influenza. Due to a lack of activation or exhaustion markers as well as an inability to recognize tumour antigens (Box 2), such T_{Mem} cells present in the TME were classified as ‘bystanders’. The fact that viral-specific lymphocytes display a preferential memory phenotype is not surprising, since memory cells are commonly generated following acute infections when the antigen load is cleared[92]. Nonetheless, this study clearly demonstrated that a substantial proportion of TILs lack direct antitumour function and that the sole presence within the TME of T cells does not automatically equate to their antitumour activity.

Therefore, the characterization of ‘true’ antitumour TILs necessitates distinction from bystanders, namely the demonstration of T cell reactivity against tumour cells or tumour antigens in order to be classified as the former. Indeed, a major barrier to fully deconvoluting the tumour specificity of TILs is the requirement for definitive confirmation of the reactivity of the TCR of interest against autologous tumour cells. This can be achieved through in vitro challenge of the TCR-expressing cells against a source of such tumour cells — often not easily accomplished, since most tumour types propagate poorly in vitro[93]. As

a consequence, our knowledge of TIL antitumour reactivity comes predominantly from results generated in the melanoma setting, for which the establishment of patient-derived cell lines has been most feasible. Gros et al.[84] and Pasetto et al.[85] first demonstrated that $CD8^+$ TILs expressing inhibitory receptors, such as PD1, LAG3 and TIM3 (that is, T_{Ex} cells), in melanoma showed the highest levels of activation (that is, surface upregulation of CD137), secretion of effector cytokines (such as interferon-γ (IFNγ)) and target lysis when purified and challenged ex vivo against autologous tumour cells. Such results were then confirmed and extended to head and neck squamous cell carcinoma (HNSCC) specimens, where antitumour reactivity segregated with T_{RM}-like T_{Ex} cells[69]. Taken together, these studies pioneered the linking of discrete T cell phenotypes to antitumour recognition within the native TME, and showed that, despite an exhausted phenotype, such cells retained some level of functional competence which could be measured in vitro. Additional studies, while not directly assessing tumour reactivity, confirmed the exhausted phenotype of T cells specific for tumour antigens detected by peptide–major histocompatibility complex (MHC) multimer staining[91,94] (Table 1). In contrast, the non-exhausted fraction of TILs across these studies displayed low or negligible reactivity towards autologous tumours[69,84], thereby further underscoring how the T_{Mem} cell compartment likely does not represent a major reservoir of T cells with antitumour specificity.

The detection of such a substantial fraction of viral-specific bystander T cells in the TME raises the as-yet unresolved question of how and why they were recruited to the TME. We speculate that bystander recruitment might occur in an antigen-independent fashion, since: (1) cells with a T_{Mem} phenotype are able to recirculate among secondary lymphoid organs and peripheral tissues through the expression of distinct homing (CD62L and CCR7) and migratory receptors (intercellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1))[95]; (2) most tumours display both high vascularization and inflammation (albeit to different extents depending on tumour type), potentially leading to T cell attraction and colonization; and (3) viral-specific T cells are detected at high frequencies in the circulation[58], which increases their probability of recirculation within the TME. Irrespective of the mechanisms leading to viral-specific T cell homing into the TME, we cannot exclude that, once there, they may potentially participate in antitumour activity following indirect and TCR-independent activation triggered by inflammatory cues as recently suggested[96].

Probing the antitumour reactivity of TIL TCRs

scTCR-seq provides the unequivocal assignment of paired TCR α-chains and β-chains to each analysed T cell and, as such, this information has now prompted many investigators to clone and express TCRs identified in the TME into reporter cells and to then screen their reactivity in vitro against autologous tumours. This line of investigation was inaugurated by Scheper et al.[97], in which they reconstructed clonally expanded intratumoural TCRs from patients with solid cancers (including melanoma, CRC and ovarian cancer). In this initial study, up to 15 TCRs per patient were investigated, and only a few of the expanded TCR clonotypes from CRC or ovarian cancer showed substantial tumour recognition in vitro but tumour-reactive TCRs were instead more frequent in melanoma, in line with its increased tumour mutational burden. Hence, TIL clonal expansion (that is, TIL quantity)

does not automatically equate to antitumour reactivity (that is, TIL quality), which varies highly depending on the tumour type. In our recent in-depth analysis of the $CD8^+$ and $CD4^+$ T cell landscape of the human melanoma TME, we complemented single-cell transcriptome analysis of TILs with the deconvolution of antigen specificity of hundreds of clonally expanded TCRs[58]. Through implementation of a miniaturized system to clone and transduce dominant TCRs into primary T cells from healthy donors and subsequently testing hundreds of TIL TCR-redirected T cells for their antigen-specific activation upon co-culture with patient-derived melanoma cell lines, we found that more than 80% of TCRs expressed in T_{Ex} cells exhibited strong autologous tumour recognition and had a preferential skewing towards T_{TE} and T_{RM}-like T_{Ex} cell clusters[58]. Of note, almost all tumour-specific clonotypes also had a detectable fraction in the T_{PE} cell niche. Hence, in line with mouse models of tumour immunobiology[10,63], we demonstrated that antitumour T cells within the TME are present in a continuum of differentiation from T_{PE}, T_{TE} and T_{RM}-like expression states. In stark contrast, T_{Mem} TILs accounted for less than 2% of the tumour-specific compartment[58].

Further studies have integrated scTCR-seq and transcriptomic profiling together with in vitro screening for TCR specificity against a limited panel of tumour antigens (Box 2) to evaluate the breadth of antitumour specificities within the TME. Lowery et al.[98] demonstrated that, in metastatic breast cancer, melanoma and CRC, $CD8^+$ and $CD4^+$ TILs expressing TCRs with confirmed specificity for tumour private neoantigens mapped preferentially to dysfunctional and exhausted clones with both high or low levels of clonal expansion. Furthermore, the transcriptional profile of neoantigen-specific cells overlapped with that of T_{TE} cells. Indeed, using a gene signature enriched in T_{Ex} cells, the authors were able to predict TCR clonotypes with putative tumour reactivity, which was experimentally confirmed in vitro against tumour antigens or autologous tumour cells in ~50% of cases. Two studies in NSCLC, before or after neoadjuvant anti-PD1 treatment, converged on similar findings. Caushi et al.[88] showed that neoantigen-reactive $CD8^+$ TCRs isolated from circulating T cells could be traced back to the TME, where they displayed an exhausted profile. Through the screening of ex vivo, expanded NSCLC TILs with autologous antigen-presenting cells (APCs) expressing tandem minigenes, Hanada et al.[90] identified neoantigen-reactive T cells, sequenced their TCRs and confirmed their reactivity against mutated tumour proteins in vitro. Single-cell analysis of tumour samples showed that such specificities mapped exclusively to $CD8^+$ and $CD4^+$ exhausted cell clusters, identified by CD39 and CXCL13 expression. Following a reverse strategy, through reconstruction and screening of dominant TCRs from $CD39^+$ versus $CD39^-$ TILs, neoantigen-reactive TCRs were again only detected in the former compartment[90]. Similar studies investigating the transcriptional profile of neoantigen-reactive TILs in bile duct and pancreatic cancers documented the presence of rare but dominant $CD8^+$ or $CD4^+$ neoantigen-reactive T cell clones with a similar $CXCL13^+$ T_{Ex} cell profile[99]. In human papillomavirus (HPV)-positive HNSCC, scRNA sequencing (scRNA-seq) of peptide–MHC multimer-sorted $PD1^+$ TILs specific for oncoviral HPV proteins documented a differentiation state that could span from T_{PE} cells to T_{TE} cells[100]. In another cohort of patients with HNSCC, deconvolution of TCR specificity through the use of TScan technol- ogy (Table 1) revealed the presence of TILs specific for shared tumour-associated antigens (TAAs) among exhausted cells with a T_{RM}-like cell profile[101]. Most of

the aforementioned studies used viral antigens as negative controls to validate the specificity of screened TCRs: TILs expressing virus-specific TCRs were almost exclusively confined to the PD1^{-} and CD39^{-} compartments and expressed memory markers (TCF1, IL-7R, CD62L and CCR7), consistent with the transcriptional profile of T_{CM} or T_{EM} cells[58,88,90,97]. While bystander T cell clones could be detected as numerous and even expanded in some instances, this was not specific to the TME as the same clones could also be abundantly detected in normal tissues and/or peripheral blood. The intratumoural clonal expansion of T_{Ex} cells (sustained by recognition of tumour antigens) generally outcompetes one of the individual bystander T cells, which is likely sustained by perfusion of cells from blood, where such T_{Mem} cell clonotypes are highly expanded.

Overall, this series of in-depth studies have consistently demonstrated that tumour-specific T cells in solid tumours reside almost exclusively in the T_{Ex} cell compartment, sharing remarkably similar transcriptional and phenotypic properties across different tumour types. Additionally, since these studies evaluated tumour reactivity across different antigenic classes (that is, TAAs and neoantigens), it is possible to conclude that the specificity for tumour-expressed antigens, and not the antigen class per se, drives T cell exhaustion within the TME. The signatures of tumour-reactive TILs are now increasingly available for prediction of putative tumour-reactive TILs in tumour biopsies: the detection of surface expression of PD1 and CD39 among TILs can be integrated into clinical analyses to quantify T cells potentially specific for tumour antigens. In addition, the detection of gene signatures enriched in *CXCL13* expression from the transcriptome of TILs has gained considerable interest as this gene encodes a chemokine that mediates recruitment of B cells to tumours and is essential for the formation of TLS, where functional TILs can be preserved[102]. Indeed, assessment of the levels of putative tumour-reactive CXCL13^{+} T_{Ex} TILs has been increasingly used as a predictive measure in many solid tumours with high tumour mutational burden as it has been associated with improved outcome after immunotherapy[56,103–105].

Finally, a separate consideration should be made for CD4^{+} TILs. Relatively fewer studies have focused on helper T cells, but the wide-spread use of single-cell sequencing and reactivity screening of TILs has now provided evidence of their specificity for both private neo-antigens and TAAs among TILs[106,107] (Box 2) and has linked them to an unexpected richness of T cell states, including cytotoxic T_{Ex}, T_{FH} and T_{reg} cells[108–110]. The detection of tumour-specific CD4^{+} TIL clones has raised the question of whether these cells can directly recognize tumour cells, which usually lack constitutive expression of HLA class II. In vitro measurement of TCR reactivity against autologous tumours has revealed distinct patterns of interaction between CD4^{+} TILs and cancer[86]. We showed that melanoma-specific CD4^{+} T cells (T_{FH} and T_{reg} cells) could be indirectly activated in the TME by APCs, taking up tumour antigens from apoptotic cancer cells and presenting them in the context of HLA class II[86]. Surprisingly, CD4^{+} TILs can also directly recognize melanoma cells: such tumour-specific CD4^{+} lymphocytes mapped to PD1^{+} CXCL13^{+} T_{Ex} cell clusters in the TME and displayed cytotoxic properties (expressing IFNγ and granzyme A)[110]. This was apparent in the context of concurrent TME inflammation, leading to HLA class II upregulation in tumour cells. Alternatively, a fraction of cytotoxic CD4^{+} T_{Ex} TILs from melanoma could also engage peptide–HLA class I complexes on the surface of tumour cells in a CD4–CD8

co-receptor-independent fashion[86]. Finally, we reported the direct activation of $CD4^+$ T cells in a subset of melanomas with aberrantly constitutive HLA class II expression[86]. We found that positivity for HLA class II expression in melanomas (10–30% of all cases)[111] is strongly associated with an extremely high tumour mutational burden (>1,000 missense exonic mutations per sample)[86,112] and that the TME of HLA class II-positive melanomas is populated by large numbers of neoantigen-reactive $CD4^+$ or $CD8^+$ T_{Ex} TILs with cytotoxic capacity, as well as by numerous clonally expanded neoantigen-specific $CD4^+$ T_{reg} cells[86]. These data provide compelling evidence that HLA class II- positive melanomas can engage and possibly orchestrate the activity of T_{reg} cells to shape an immunosuppressive TME capable of escaping from cytotoxic antitumour responses[86]. As neoantigen-specific $CD4^+$ T cells can be detected not only in melanoma but also in tumours with a moderate or elevated mutational burden (such as breast cancer and CRC[98]), it becomes intriguing to speculate whether such TILs could potentially be stimulated by tumour cells to elicit immune suppression. Furthermore, while tumour-specific T_{reg} cell TCRs can be stimulated by cancer cells, it is currently unknown whether the tumour cells can induce $CD4^+$ TILs with immunosuppressive functions or, more likely, engage pre-existing T_{reg} cells through antigenic mimicry. Future studies will be needed to assess how tumour-specific T_{reg} cells differentiate within or outside the TME.

Manipulating T cells with immunotherapies

Cancer immunotherapy aims to empower T cells against tumours, but each immunotherapeutic approach harnesses the distinct properties of antitumour T cells in a different fashion (Fig. 2). First, immunotherapies can either directly or indirectly impact the functionality of tumour-reactive T cells that pre-exist within the TME prior to treatment, fostering their reinvigoration and thereby serving to amplify antitumour responses within the TME or in extratumoural sites (lymph nodes, peripheral blood or normal tissues). Alternatively, immunotherapies may elicit novel T cell responses through de novo generation of novel T cell specificities not yet exhausted by the chronic exposure to tumour antigens within the TME. The release of tumour antigens (termed epitope spreading) from cancer cells killed by T cells elicited during immunotherapy may serve to further amplify the magnitude and breadth of both pre-existing and de novo T cell responses. Overall, the distinct immunotherapies are divergent from one another in relation to the number of antigen specificities they can target and their ability to rewire T cell phenotype and overcome exhaustion.

Immune-checkpoint blockade

ICB represents the most widely used immunotherapeutic approach for solid tumour malignancies, with an ever-growing number of monoclonal antibodies targeting distinct inhibitory receptors entering the clinical arena[113]. Antibodies targeting PD1, CTLA4 and LAG3 have already been approved by the FDA for the treatment of several different solid tumour types[114,115]. While ICB has undoubtedly been shown to induce objective responses even in the challenging setting of metastatic disease, only a minority of patients (estimated to be <30%) achieve sustained and/or complete clinical responses[116–118]. Therefore, it remains imperative to understand the immunological correlates of productive antitumour immune responses following ICB treatment. Tracking of T cell clones in tumour biopsies

and peripheral blood before and during ICB treatment has enabled a better understanding of the effects of ICB on T cell composition and function, especially in patients treated with anti-PD1 therapy (Fig. 3a).

Intuitively, ICB is thought to act primarily on the exhausted antitumour T cells within the TME through a process of reinvigoration of their functionality, which can be termed 'cytotoxic revival'. For this to be effective, tumour-specific T cells are required to: (1) be already present within the TME prior to ICB initiation and (2) retain, despite exhaustion, a residual cytotoxic functionality that could be unlocked and unleashed through the removal of inhibitory signals. Direct demonstration of TIL reinvigoration would entail the real-time measurement of cytotoxic molecule release within the tumour during treatment, which is infeasible in human samples, but several studies have reported an association between the extent of pre-treatment TME T cell infiltration (in turn linked to the tumour mutational burden) and clinical response[29–31]. Additionally, a high frequency of T_{RM}-like T_{Ex} TILs has been observed in anti-PD1-responsive tumours (Fig. 3a), with one study in breast cancer demonstrating that in vitro exposure to ICB could restore the functionality of $CD39^+$ T_{RM}-like T_{Ex} TILs[119].

Notably, three recent single-cell-based studies have provided strong support for such functional revival of TILs in the context of neoadjuvant anti-PD1 treatment (Fig. 3b). Of note, in this specific setting, ICB administration shortly (2–4 weeks) before surgery allows for the direct evaluation of T cell dynamics early after ICB treatment in an intact immune ecosystem (encompassing the native TME and draining lymph nodes). In a cohort of patients with breast cancer, Bassez et al.[120]. showed that response to neoadjuvant PD1 blockade was accompanied by intratumoural expansion of pre-existing $PD1^+$ $CXCL13^+$ exhausted (that is, consistent with bona fide tumour-specific) $CD8^+$ clonotypes expressing high levels of the genes encoding cytotoxic effector molecules (*PRF1* and *GZMB*). In HNSCC, Luoma et al.[101]. demonstrated that response to neoadjuvant PD1 was associated with expansion of exhausted T_{RM}-like TILs with transcriptional evidence of cytotoxicity. Most clonotypes expanded after anti-PD1 therapy could be detected in pre-treatment biopsies, underscoring the requirement for clinical response of a pre-existing repertoire of antitumour T cells, which could then be reinvigorated through exposure to anti-PD1 treatment, and implicating the revival of cytotoxicity as a rapid mechanism of ICB response in tumours with a large number of TILs in baseline tumour biopsies. In another study of patients with HNSCC[121], we have preliminarily documented that lack of response to neoadjuvant ICB was indicated by a paucity of expanded T_{Ex} cell clones in the native TME, with the few expanded T_{Ex} cell clones present lacking T_{RM} cell features or cytotoxicity potential, and instead exhibiting extreme levels of exhaustion. This observation is in line with the hypothesis that ICB is unable to efficiently restore the functionality of TIL clones at a terminal stage of dysfunction[122–124]. Cytotoxic T_{RM}-like T_{Ex} cell clones were contracted in biopsies collected after 5 weeks of treatment in patients experiencing profound pathological response (that is, achieving almost complete cancer eradication)[121], supporting the notion that antigen availability determines the persistence of terminally differentiated T cells[52,53,55].

In addition to its local activity, ICB, most extensively characterized following anti-PD1 blockade, has also been found to act systemically through the mobilization and recruitment

of new TCR clonotypes to the tumour site from the periphery, in a phenomenon termed ‘clonal replacement’ (Fig. 3b). In non-melanoma skin cancers, longitudinal tracking of TCR clones revealed that ~70% of clonally expanded post-ICB TILs were undetectable within the TME prior to adjuvant therapy[87]. Newly detected clones had a preferential T_{Ex} cell state (and hence, bona fide tumour-specific). While the influx of novel T cell clones was detected at ~9 weeks after immunotherapy, evidence of clonal replacement could be found as early as 4 weeks after ICB in the neoadjuvant setting (especially following combined treatment with anti-PD1 and anti-CTLA4 agents)[101]. Of note, the occurrence of clonal replacement per se has not been associated with response to ICB but rather the extent of expansion of emergent clones was consistently higher in patients responding to anti-PD1 therapy[87]. Since T cells with newly detected specificities could be recruited from extratumoural sites, additional studies have focused on investigating the systemic effects of ICB. Comparison of the TCR repertoire across tumour and blood has shown that emergent clono- types could be traced in circulation early after ICB treatment (~2 weeks), where they exhibited transcriptional features consistent with proliferation and activation[101]. Similarly, patients with melanoma responding to anti-PD1 or anti-CTLA4 showed significantly more clonal expansion and T cell turnover following therapy compared with non-responders[125–128]. Productive antitumour responses were associated with early detection of circulating expanded clonotypes bearing signatures of activated cytotoxic T cells[126]. In patients with HNSCC and NSCLC treated with neoadjuvant anti-PD1 therapy, the clones that were peripherally expanded were found enriched within the post-treatment TME[101,129]. Therefore, activation and expansion outside of the TME of antitumour T cells, then recruited to the tumour site, seem to be a key mechanism of response to ICB. Consistent with this hypothesis, Wu et al.[59] have shown that response to anti-programmed cell death protein 1 ligand 1 (anti-PDL1) therapy correlated with expansion of clonotypes within the peripheral blood as well as with increased T cell infiltration in both normal tissues and tumour. Interestingly, additional studies have reported that TCR clones newly detected after ICB treatment originated from draining lymph nodes[130,131]. It is tempting to speculate that such novel antitumour T cell clones were able to persist with a less differentiated state at extratumoural sites as the absence of tumour-induced chronic stimulation could have reasonably prevented the acquisition of a terminally exhausted phenotype. Subsequently, upon recruit- ment within the TME, these newly emerging clones could interact with cancer cells in the presence of ICB, allowing cytotoxic responses to be uncoupled locally from the acquisition of terminal exhaustion.

Finally, in some patients with NSCLC and melanoma, TIL clonal composition did not change dramatically upon ICB treatment but rather, in responding patients, pre-existing clones reverted to a T_{PE}-like cell state, characterized by CXCL13 positivity and low expression of inhibitory receptors[79]. This mechanism, termed ‘clonal revival’, could be, in part, ascribed to local expansion and differentiation of pre-existing tumour-specific T_{PE} TILs within the TME, and partly to the recruitment from extratumoural niches (especially lymph nodes) (Fig. 3b) of novel non-exhausted precursors that, in the presence of the ICB antibodies, could differentiate into functional effectors due to the disruption of inhibitory tumour signals. In both scenarios, reinvigoration of antitumour responses is fostered by increased numbers of T_{PE} cell precursors able to regenerate functional cytotoxic

TILs. Evidence supporting this phenomenon includes the observation that an increased frequency of T_{PE}-like TILs correlates with improved outcomes in melanoma and lung and ovarian cancers[12,21,79,132]. Interestingly, so far, T_{PE}-like cell resurgence has not been detected early after therapy in the setting of neoadjuvant PD1 blockade[101,121] but only in studies investigating the response to ICB at later time points (that is, 2–3 months post-treatment)[12,21,79,132], thus suggesting that clonal revival might require more time to manifest.

It should be emphasized that the aforementioned mechanisms of response to ICB are not mutually exclusive but could represent complementary, or even synergistic, events occurring at different temporal phases following treatment (Fig. 3b). For all mechanisms, their efficacy likely depends on the quality of the native T cell compartment, either within or outside the TME. Within this framework, response to ICB could be conceptualized as a multistep process, in which cytotoxic revival reawakens an initial immediate wave of antitumour T cells, with this phenomenon more prominent in tumours with high tumour mutational burden and brisk infiltration of T_{RM}-like TILs. A second wave of antitumour immunity might then be elicited through the recruitment of novel T cell specificities from extratumoural sites. Clonal replacement is expected to contribute to ICB response, particularly in tumours with low-to-moderate T cell infiltration, and could be further boosted by epitope spreading. Finally, clonal revival would be the slowest to become evident as it would require the progressive accumulation and differentiation of T_{PE} cells. This is theorized to be crucial for the restoration of functional antitumour immunity in those tumours populated by severely exhausted T_{TE} TILs. Likewise, lack of response to ICB could be conceptualized as the failure to enact one or more of the aforementioned mechanisms of action due to quantitative and/or qualitative defects in the native antitumour immunity (Fig. 3a). Further studies in different cancer types and therapeutic settings will likely elucidate the exact requirements, timeline and contribution of these mechanisms of response to ICB.

Cancer vaccines

Cancer vaccines are designed to promote both the reinvigoration and elicitation of antitumour responses by selectively enriching for T cell reactivities towards predefined sets of tumour antigens. Indeed, through the increase of APC-mediated presentation of cancer antigens, vaccination can both boost pre-existing tumour responses or prime the de novo differentiation of T cell precursors, ‘steering’ the specificity of antitumour immune responses towards particular immunogenic antigens.

Several vaccine approaches have been developed through the modulation of its active components, including the type of targeted tumour antigens, formulation, immune adjuvant and delivery vehicles, as recently reviewed[133,134]. Several of these different vaccine formats have been tested in clinical trials (exhaustively reviewed in ref. 135) alone or in combination with conventional chemotherapy or ICB. Even though clinical testing has proved that vaccination against cancer is safe and feasible, complete responses are rare, and a significant benefit of vaccination in larger patient cohorts remains to be demonstrated. A major caveat to achieving clinical responses after vaccination is whether targeting immunogenic tumour antigens can elicit effective and persisting cytotoxic T cells. Novel vaccine approaches are

currently under development to incorporate tumour antigens predicted as immunogenic and to better stimulate antigen-specific T cells using specific formulations, immune adjuvants and delivery vehicles, alone or in combination with other immunotherapies[133,134].

Several studies have investigated how cancer vaccines impact the T cell repertoire by tracking the reactivity of circulating T cells towards the targeted antigens. Seminal studies on patients with melanoma, bladder cancer and NSCLC treated with either neoantigen-based or TAA-based vaccines have documented robust induction of both $CD4^+$ and $CD8^+$ T cell responses directed against the epitopes included in the vaccine formulation, which were not detected or present at low levels prior to therapy, thus formally demonstrating that cancer vaccines both elicit de novo responses and amplify pre-existing ones[136–140]. From these trials, up to 60–70% of predicted neoantigens were able to induce reactive T cells. Of interest, while vaccination protocols were aimed at eliciting $CD8^+$ T cells, responses mainly comprised polyclonal $CD4^+$ T cells, which were possibly favoured by the processing and presentation of long immunizing antigens on HLA class II molecules of APCs[136–138]. Through single-cell sequencing and multiparametric flow cytometric analysis, we recently completed a detailed characterization of neoantigen-specific $CD4^+$ T cells induced by a cancer vaccine in eight patients with melanoma[141]. Vaccine-reactive T cells expanded during the first months after vaccination and persisted for up to 4 years in treated patients. In the early phase after vaccination, $CD4^+$ T cells specific for neoantigens exhibited a cytotoxic profile with signatures of activation and exhaustion; such cells were able to traffic to within the TME, as evidenced by the detection of their TCRs in tumour lesions collected after treatment[141]. At later time points (up to 5 years post-vaccination), T cells harbouring the same clonotypes displayed a memory phenotype, at least in circulation[141]. A similar expansion of cytotoxic neoantigen-specific $CD4^+$ T cells was observed in 38 patients with NSCLC treated with a neoantigen peptide vaccination in combination with chemotherapy and PD1 blockade[139]. Neoantigen vaccines have been able to induce neoantigen-specific $CD4^+$ T cells possessing the ability to infiltrate immunologically cold tumours such as glioblastoma[142,143].

While T cell clonotypes specific for tumour antigens can be induced by vaccination, their direct antitumour capacity has been demonstrated only for a limited number of patients[136,137]. In this setting, the evaluation of T cell reactivity against tumour antigens that are not included within the vaccination platform offers the unique opportunity to indirectly measure the antitumour efficacy of the approach; indeed, non-vaccine-specific T cells can be expanded only following epitope spreading, when the tumour antigens become more available as a result of lysis of tumour cells. In melanoma, NSCLC and bladder cancer, neoantigen-based vaccination trials have documented epitope spreading following treatment, with emergence of specificities targeting a wide variety of neoantigens or TAAs not present in the vaccine formulation nor detectable prior to vaccination[138,139,141]. This phenomenon has been associated with improved progression-free survival, thus demonstrating that an increase in breadth of the tumour-specific T cell repertoire can contribute to disease control[138]. It is worth noting that the exact size and timing of epitope spreading and its derived immune responses within the TME remain unknown as most of the current studies have focused mainly on the analysis of antigen-specific cells residing in peripheral blood.

Adoptive T cell therapy

Adoptive T cell therapy (ACT) aims to quantitatively and qualitatively enhance antitumour immunity. This can be achieved in two ways: (1) by expanding ex vivo the pre-existing T cells possessing antitumour specificity provided by their endogenous TCR repertoire (TIL therapy) or (2) by redirecting the specificity of T cells ex vivo through gene manipulation with antitumour TCRs or chimeric antigen receptors (CARs), which can stimulate de novo antitumour responses (Fig. 2).

Trailblazing studies by the Rosenberg group first demonstrated how TILs isolated from surgically resected tumour fragments could be expanded in vitro, screened for recognition of autologous tumour cells and then reinfused into patients[144–147], and investigations of this adoptive TIL therapy approach initially achieved up to ~20% complete clinical responses[148]. A major advantage of TIL therapy is its polyclonality: analysis of infused TIL batches has consistently shown that TIL products contain a broad spectrum of T cell specificities, including TAAs, neoantigens and oncoviral proteins[149–151]. Rosenberg et al.[148] and Dudley et al.[152,153] subsequently fine-tuned the approach by adding a lymphodepleting preparative regimen (a concept since successfully adopted by CAR T cell studies) and post-infusion administration of IL-2, in an attempt to improve the in vivo persistence, and thus efficacy, of the transferred T cells. Indeed, numerous efforts since then have been dedicated to understanding the cellular and molecular basis of response to ACT, all converging on the persistence of transferred T cells as a main determinant[154,155]. Persistence of T cells has been, in turn, typically associated with a less differentiated phenotype as the acquisition of full effector functions in vivo, paradoxically, has been found to impair T cell fitness upon in vivo infusion[156–161]. More recently, by analysing TIL products at single-cell resolution using cytometry by time of flight (Box 1), Krishna et al.[162] documented that patients who responded to ACT had received larger numbers of TILs with stem cell-like properties as identified by high TCF1 expression and negativity for CD39 and CD69. Combined scRNA-seq and scTCR-seq analyses of TIL products from representative patients confirmed these findings: in the non-responder patient analysed, neoantigen-specific TCR clonotypes were less expanded and exhibited an exclusively T_{TE} cell profile. Consistently, such clonotypes showed poor persistence in peripheral blood upon infusion. In contrast, neoantigen-specific T cells from the infusion product of the responder patient expanded more and encompassed multiple T cell states, including not only T_{Ex} cells but also stem cell-like $TCF1^+$ T cells. This translated into improved T cell fitness in vivo as documented by clonotype detection in peripheral blood up to 75 months after infusion[162]. Overall, this analysis showed that ex vivo, expanded T cell products enriched in antitumour T cells with T_{Mem} features (for example, with regenerative potential) are associated with improved immunotherapy outcomes (Fig. 4). It is tempting to speculate that clinical response to TIL therapy might depend on the presence within the native TME of antitumour T cells with a T_{PE} cell phenotype with stem cell properties able to clonally expand ex vivo and provide tumour control upon re-infusion[163].

While TIL therapy represents a milestone in the development of ACT, it is limited by the requirement of pre-existing antitumour T cells within the tumour, whose numbers might not be sufficient for all patients, especially in the setting of tumour types with a lower tumour

mutational burden than, for example, melanoma. To overcome this limitation, many groups have focused on genetic manipulation to redirect T cell specificity against tumour antigens in the form of engineered TCRs or CARs, as extensively reviewed in refs. 2,164–166. The use of T cells that have been genetically engineered to express tumour-specific TCRs, first of all, requires the isolation, sequencing and validation of TCRs that are able to recognize tumour antigens when presented within the context of the HLAs of a given patient. As a consequence, this approach has been limited, in part, by the difficulty in finding high-affinity TCRs specific for public TAAs presented by HLA restrictions of high frequency (such as HLA-A*0201). Initial attempts using T cells transduced with two different TCRs recognizing the mela- noma differentiation antigen melanoma antigen recognized by T cells 1 (MART1) have documented limited efficacy against metastatic melano- mas, with responses that seemed to be dependent on the affinity of the isolated TCRs[167,168] and that were also able to recognize healthy tissues, thereby causing on-target off-tumour toxicity. Conversely, the use of T cells transduced with NY-ESO-1-specific TCRs has been shown to generate stable or complete responses in melanoma and myeloma, which were associated with elevated expression of this TAA and with expansion of TCR-modified T cells[169–172]. Of note, such effects were observed in the absence of severe toxicities. In contrast, the use of TCRs specific for melanoma-associated antigen (MAGE) was accompanied with severe on-target off-tumour recognition but also with lethal cross-recognition of cardiac proteins when affinity-enhanced TCRs were utilized[173,174], thus demonstrating the need for extensive preclinical testing when ACT approaches use TCRs specific for TAAs due to their possible low expression on healthy tissue. The identification of tumour-specific mutations through sequencing and the isolation of neoantigen-specific TCRs has provided a potential solution to overcoming this hurdle as such TCRs retain high specificity and avidity for the mutated peptides. Due to the patient-specific nature of tumour mutations, the implementation of neoantigen-specific TCR-based therapy requires personalized ACT approaches and should only be extended to multiple patients when neoantigens are generated by recurrent mutations that are presented on HLA alleles with high population frequency[175–177].

The advent of CARs has revolutionized the field of ACT as such constructs enable the targeting of surface tumour proteins in an HLA- independent fashion. CARs typically require thousands of target surface molecules to mediate an effective response: as a consequence, such an ACT approach is generally feasible only when surface proteins with high, broad and conserved expression on tumour cells are identified. Thus far, this has been challenging for solid tumours, owing also to their high intratumoural clonal heterogeneity and immunosuppressive potential; however, encouraging results have been obtained using a CAR specific for the disialoganglioside GD2 in neuroblastoma[178] and human epidermal growth factor receptor 2 (HER2) in sarcomas[179]. Conversely, CAR T cells have demonstrated remarkable success in treating haematological cancers, although mainly acute and chronic $CD19^+$ B cell leukaemias[180]. As CAR T cells have rapidly become an integral part of the therapeutic arsenal for relapsed and/or refractory $CD19^+$ haematological malignancies, most studies dissecting the molecular and cellular underpinnings of successful immune-gene therapy have been in this specific setting[181,182]. However, it is necessary to highlight a few differences in the immunobiology of CAR-redirected T cells compared to other adoptively transferred T cells: (1) CARs are synthetic molecules providing antigen

recognition at the same time as a non-physiological *in cis* costimulation via one or more costimulatory endodomains (which might vary depending on the specific CAR construct designs); and (2) for CD19 CARs, there is the possibility of constitutive tonic CAR signalling through recognition of CD19 on normal B cells even after clearance of tumour cells, clinically evident as prolonged B cell aplasia. Even with these caveats in mind, sustained clinical responses in this setting also depend on the level of persistence of gene-modified T cells[183]. In a study on 41 patients with chronic lymphocytic leukaemia and treated with CD19 CAR T cells, Fraietta et al.[181] showed that, in the peripheral blood of responder patients, there was a dramatic in vivo expansion of CAR T cells that coincided with B cell aplasia in the first 2 weeks after infusion, which was then followed by a decline; in contrast, non-responders displayed low or undetectable levels of circulating CAR T cells. By transcriptionally profiling CAR T cells, the investigators found that infused batches from patients achieving complete response were enriched in T_{Mem} cell-related genes, whereas T cells from non-responders upregulated programmes involved in effector differentiation, exhaustion and apoptosis, among others. Similarly, Deng et al.[182] profiled, through scRNA-seq, CD19 CAR T cell infusion products from 24 patients with aggressive B cell lymphomas. In line with the previous study, patients with complete responses received infusion products with threefold higher frequencies of $CD8^+$ T cells expressing memory signatures than those patients with partial responses or progressive disease.

Subsequent studies have started to shed light on the clonal dynamics of CAR T cells upon in vivo infusion by incorporating longitudinal sequencing of the endogenous TCR. Sheih et al.[184] described distinct patterns of clonal behaviour contributing to the circulating CAR T cell pool in the first 30 days after infusion in a cohort of 10 patients with $CD19^+$ B cell malignancies. CAR T cells undergoing expansion early after infusion showed a transcriptional profile of cytotoxic and proliferative T cells, while at later time points (that is, after clearance of the target antigen), CAR T cells reverted to a more quiescent cell state without acquiring exhaustion features. CAR T cells with non-expanding clonotypes were instead characterized by rapid acquisition of T_{Ex} cell phenotypes and soon became undetectable. However, it should be noted that the CAR T cell manufacturing protocol for this study introduced a bias, preventing generalization of the findings as it entailed the selection of $CD4^+$ T cells and T_{CM} $CD8^+$ T cells, which were then infused at a 1:1 ratio. Wilson et al.[185] performed a similar analysis on a cohort of patients with paediatric acute lymphoid leukaemia and, likewise, identified divergent differentiation trajectories of CAR T cells upon infusion, with a unique three-gene signature of CAR T cell effector precursors ($TIGIT^+$, $CD62L^{lo}$, $CD27^-$) that, when present in pre-infusion cell products, was consistently correlated with clinical response. Finally, Haradhvala et al.[186] recently reported longitudinal scRNA-seq and scTCR-seq analyses of aggressive B cell lymphomas from 32 patients treated with 2 different commercial CAR T cell products (axi-cel and tisa-cel). Substantial differences in the cellular dynamics of response were observed between the two products: responses to tisa-cel were associated with a striking expansion of rare $CD8^+$ T_{CM}-like cell populations from the infusion product, while treatment with axi-cel revealed that T cell lineages did not change as much between the infusion product and post-treatment and that, in responders, T cells often originated from more-differentiated populations. While all these studies focused on in vivo CAR T cell dynamics up to ~6 months post-infusion,

a landmark work by Melenhorst et al.[187] has provided the first evidence of long-term persistence of CAR T cells, up to 10 years post-infusion. Notably, the pool of long-lived CAR T cells was composed mainly of oligoclonal $CD4^+$ T cells, which still showed transcriptional signatures of active proliferation but not of exhaustion.

Altogether, these studies provide evidence that the ex vivo manufacturing protocols that enrich for antitumour T cells with T_{Mem} cell features are associated with T cell persistence and disease control in vivo[163], while those enriched for antitumour T_{Ex} cells have poor T cell fitness once infused in patients (Fig. 4).

Conclusions and future perspectives

TIL characterization has unequivocally proved that tumour-reactive T cells are present within the TME, yet exhaustion remains the major factor limiting their antitumour potential. However, not all T_{Ex} cells are the same, and the presence of pre-existing T_{RM}-like T_{Ex} cells and T_{PE} cells correlates with improved clinical outcomes upon immuno-therapeutic intervention[12,21,69], paving the way for their quantification as a predictive biomarker of response. Despite these findings, it remains unclear which events in the natural history of cancer, even before clinical diagnosis occurs, determine the presence or absence of an immunotherapy-actionable T_{RM}-like T_{Ex} or T_{PE} cell pool.

For instance, what role is played by the antigen specificities that are recognized? With present tools, we are unable to yet quantify the real size of the antitumour T cell repertoire nor the full spectrum of tumour antigens per cancer type. Intuitively, we can imagine that a broader set of tumour antigens, able to elicit a wider tumour-specific T cell repertoire, could generate a more varied, and hence more potent, anticancer response. In addition, antigen quality is also expected to play a central role: since chronic antigenic stimulation drives T cell exhaustion[16,17,52,55], it is reasonable to infer that the strength of the primary signal provided by the TCR (for example, TCR avidity) could directly affect the fate of antitumour T cells. Definitive answers to these questions have been elusive thus far as they would require simultaneous measurement of TCR antitumour reactivity and deconvolution of the target antigen. Several promising innovative technologies have the potential to close this knowledge gap, including the possibility to screen the reactivity and biochemical attributes of TCRs at scale against an increasing number of putative targets[188] (Table 1) and thus more definitively link the qualitative and quantitative attributes of antigen reactivity to the acquisition of defined dysfunctional T cell states. Large-scale TCR specificity analysis has already provided some remarkable insights: in melanoma, neoantigen-specific TCRs display higher avidities than TAA-specific TCRs, indicating that, in the case of neoantigens, epitope recognition is maximized by enhanced TCR avidity, which enables stronger and prolonged peptide–HLA–TCR interactions[58]. Conversely, for TAA-specific TCRs, the inferior avidity is counterbalanced by antigen abundance as TAAs are usually overexpressed in tumour cells. Furthermore, in NSCLC, neoantigen-specific TCRs have been shown to display higher avidities in responders to ICB than in non-responders[88]. A second major open question is in regard to the cellular interactions of antitumour T cells within the TME as ancillary signals (in addition to those emanating from the TCRs, such as costimulation or co-inhibition, or from the cytokine milieu) could

further shape T cell (dys)function[15,52,55,189]. This additional layer of complexity cannot be fully captured without technologies that are able to preserve the spatial architecture of the TME. This is particularly relevant in light of the increasing recognition of the importance of specific cellular niches within the TME. For instance, in many tumours, the presence of organ- ized aggregates of immune cells as TLS correlates with improved clinical outcomes[30,64,190,191], yet the exact contribution of these structures to intratumoural immune responses remains incompletely understood. The advent of spatially resolved profiling is now reshaping our ability to interrogate cellular interactions and architectural relationships between antitumour T cells and the other cell types present within the TME[23,35,192–195]. Multiple technologies have been developed to capture the full or partial transcriptome of cells within a tissue, for example, by incorporating protocols for the sequencing of barcodes associated with the spatial localization of cells or by imaging tissue specimens after specific in situ amplification of cell transcripts (reviewed in ref. 193). Furthermore, spatial proteomics allows for the characterization of expression of T cell-related proteins at a cellular and subcellular level[196]. When used in conjunction with scRNA-seq, the identified subtypes of TILs can be potentially localized within the different areas of the TME (centre of tumour areas, tumour margins, stromal areas and immune structures). Technological improvements enabling multidimensional spatial detection of TCRs and transcriptomes promise to decipher the full interactome of T cells with validated tumour reactivity[194]. By coupling in vitro validation of TCR specificities, single-cell profiling, and detection of TCR clonotypes and transcriptomes of TILs within the spatial architecture of the TME, future studies could potentially discern which interactions are assigned to ‘true’ antitumour T cells, how the levels of the cognate antigens presented by interacting cells affect their functionality, what the level of phenotypic heterogeneity between T cells is within different tumour areas, and how the differentiation fate of T cells is influenced by the surrounding cells. Furthermore, spatial localization of TIL clonotypes could shed light on the role of bystander T cells in indirectly sustaining productive antitumour immunity. Finally, how could all these discoveries translate into an advancement in cancer treatment? Undoubtedly, disentangling tumour reactivity from exhaustion will be key to successful immunotherapy, whichever type. A comprehensive mapping of the antitumour specificities and corresponding T cellular states within the TME and across the different tumour histologies will help to define the best target antigens for cancer vaccination and/or ACT. In this respect, different studies have already laid the foundations for this by defining signatures to isolate antitumour T cells[58,88,90,98], characterizing and either expanding or cloning their TCRs into non-exhausted T cells[163]. With such information, it is now feasible to predict, screen and identify putative tumour-reactive TCRs using clonality and transcriptional profiling of TILs. Indeed, the administration of antitumour TCR-transduced T cells produced from non-exhausted lymphocytes has already shown promising results[176,197]. Similar approaches could, in theory, be used to isolate potentially tumour-reactive T cells from the peripheral blood, enabling investigation of the transcriptional properties of the reservoir of antitumour T cells outside the TME and factors associated with their persistence. An in-depth characterization of antitumour TCR specificities will be instrumental, since it would likely provide information on the TCR attributes (type and number of targeted tumour antigens and avidity) that ensure the best performance of antitumour T cells. Ultimately, a more in-depth knowledge of the mechanisms of action of the different immunotherapy modalities

will surely inspire rational combinatorial approaches. Indeed, ACT with gene-modified T cells and cancer vaccines represent promising approaches to foster de novo T cell responses when the pre-existing ones are rare or severely exhausted, and combination with ICB could spare novel antitumour responses from TME-induced dysfunction. Conversely, the detection of pre-existing antitumour T cells with remnant cytotoxicity (T_{RM} -like T_{Ex} cells) and regenerative potential (T_{PE} cells) may point to a preference for the selection of ICB and/or TIL therapy. Altogether, analysis of the native TME can be expected to enable the tailoring of immune therapies with respect to the quantity and quality of the native antitumour T cell compartment. Overall, recent growth in the development of high-dimensional profiling techniques and their application to the characterization of the properties of the T cell repertoire directly from cancer biospecimens has allowed the dissection of cellular states of antitumour T cells in patients and preclinical models. By longitudinally tracking such T cell specificities in patients, we can gain a better understanding of the dynamics associated with the generation of productive antitumour responses. We now know that, in immunologically hot tumours, cancer outgrowth is accompanied by the induction of exhausted T cells with diverse antitumour potential and successful tumour control requires the reinvigoration and/or rewiring of such functionalities. With the continued advances in T cell profiling techniques and their application to human samples, we can better understand the biological events underlying successful immunotherapies and translate such findings into novel therapeutic options to foster antitumour T cell immunity in all patients with cancer.

Acknowledgements

G.O. acknowledges N. Cieri for scientific discussions. G.O. was supported by DF/HCC Kidney Cancer SPORE P50 CA101942. C.J.W. acknowledges support from NIH NCI R01CA155010.

Glossary

Antigenic mimicry:
Similarity between different antigens that results in their immune recognition by the same T cells

Central tolerance:
The clonal deletion of autoreactiveT cells in the thymus during ontogenesis to create a state in which immune cells are unresponsive to autoantigens

Cytometry by time of flight:
Technique that measures the abundance of metal isotope labels on antibodies and other tags (such as peptide–major histocompatibility complex (MHC) tetramers) on single cells using mass spectroscopy

Epitope spreading:
Also known as antigen spread. The broadening of the immune response from the initially targeted epitope to other epitopes on the same antigen or different antigens

HLA restrictions:

Requirement that T cells recognize antigens only when presented by germline-encoded HLA molecules that are polymorphic and specific for each subject

Major histocompatibility complex (MHC) multimer:
Oligomers of MHC molecules that are loaded with antigenic peptides, tagged with probes and assembled together so that they can provide a measurable signal on T cells once the complex binds to the antigen-specific T cell receptors (TCRs)

Secondary lymphoid organs:
Specialized tissues, such as the spleen and lymph nodes, where antigen-presenting cells instruct the activation of mature lymphocytes

Tandem minigenes:
Artificial gene constructs composed of consecutive gene fragments of T cell targets, which can be transfected into antigen-presenting cells to achieve the presentation of encoded epitopes

Tertiary lymphoid structures (TLS):
Organized aggregates of immune cells that form postnatally in non-lymphoid tissues

Tumour-associated neoantigens (TAAs):
Antigens encoded by unmutated genes in both normal and cancer cells but that exhibit a preferentially high expression in tumour cells

Tumour private neoantigens:
Antigens arising from mutation of the patient-specific tumour genome that causes tumour cells to express specific proteins that are not expressed on normal cells

V(D)J recombination:
Somatic rearrangement of pre-existing variable, diversity and joining gene segments of the TCR α-chain or β-chain genes, which results in the generation and expression of diverse TCRs in T cells

References

1. Sharma P & Allison JP Dissecting the mechanisms of immune checkpoint therapy. Nat. Rev. Immunol 20, 75–76 (2020). [PubMed: 31925406]
2. Restifo NP, Dudley ME & Rosenberg SA Adoptive immunotherapy for cancer: harnessing the T cell response. Nat. Rev. Immunol 12, 269–281 (2012). [PubMed: 22437939]
3. Alcover A, Alarcón B & Di Bartolo V Cell biology of T cell receptor expression and regulation. Annu. Rev. Immunol 36, 103–125 (2018). [PubMed: 29261409]
4. Kaech SM & Ahmed R Memory $CD8^+$ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. Nat. Immunol 2, 415–422 (2001). [PubMed: 11323695]
5. Boon T, Coulie PG, Van den Eynde BJ & van der Bruggen P Human T cell responses against melanoma. Annu. Rev. Immunol 24, 175–208 (2006). [PubMed: 16551247]
6. Galon J & Bruni D Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. Nat. Rev. Drug Discov 18, 197–218 (2019). [PubMed: 30610226]
7. Gerard CL et al. Turning tumors from cold to inflamed to improve immunotherapy response. Cancer Treat. Rev 101, 102227 (2021). [PubMed: 34656019]

8. Pai SI, Cesano A & Marincola FM The paradox of cancer immune exclusion: immune oncology next frontier. Cancer Treat. Res 180, 173–195 (2020). [PubMed: 32215870]
9. Im SJ et al. Defining $CD8^+$ T cells that provide the proliferative burst after PD-1 therapy. Nature 537, 417–421 (2016). [PubMed: 27501248]
10. Siddiqui I et al. Intratumoral Tcf1+PD-1+CD8+ T cells with stem-like properties promote tumor control in response to vaccination and checkpoint blockade immunotherapy. Immunity 50, 195–211.e10 (2019). [PubMed: 30635237]
11. Utzschneider DT et al. T cell factor 1-expressing memory-like CD8+ T cells sustain the immune response to chronic viral infections. Immunity 45, 415–427 (2016). [PubMed: 27533016]
12. Sade-Feldman M et al. Defining T cell states associated with response to checkpoint immunotherapy in melanoma. Cell 176, 404 (2019). [PubMed: 30633907] Single-cell transcriptome analysis in human melanoma identifies TCF1-expressing TILs associated with response to ICB.
13. Li H et al. Dysfunctional CD8 T cells form a proliferative, dynamically regulated compartment within human melanoma. Cell 176, 775–789.e18 (2019). [PubMed: 30595452] Single-cell transcriptome and TCR analysis of TILs in human melanomas, which describes the presence of dysfunctional and exhausted T cells with cytotoxic and proliferative potential.
14. van der Leun AM, Thommen DS & Schumacher TN CD8+ T cell states in human cancer: insights from single-cell analysis. Nat. Rev. Cancer 20, 218–232 (2020). [PubMed: 32024970]
15. Thommen DS & Schumacher TN T cell dysfunction in cancer. Cancer Cell 33, 547–562 (2018). [PubMed: 29634943]
16. Philip M & Schietinger A $CD8^+$ T cell differentiation and dysfunction in cancer. Nat. Rev. Immunol 22, 209–223 (2022). [PubMed: 34253904]
17. Pauken KE & Wherry EJ Overcoming T cell exhaustion in infection and cancer. Trends Immunol. 36, 265–276 (2015). [PubMed: 25797516]
18. Ottaviano M, De Placido S & Ascierto PA Recent success and limitations of immune checkpoint inhibitors for cancer: a lesson from melanoma. Virchows Arch. 474, 421–432 (2019). [PubMed: 30747264]
19. Keenan TE, Burke KP & Van Allen EM Genomic correlates of response to immune checkpoint blockade. Nat. Med 25, 389–402 (2019). [PubMed: 30842677]
20. Bagchi S, Yuan R & Engleman E Immune checkpoint inhibitors for the treatment of cancer: clinical impact and mechanisms of response and resistance. Annu. Rev. Pathol 16, 223–249 (2021). [PubMed: 33197221]
21. Zheng L et al. Pan-cancer single-cell landscape of tumor-infiltrating T cells. Science 374, abe6474 (2021). [PubMed: 34914499] Single-cell transcriptome atlas of TILs across different cancer types, highlighting the preferential exhaustion fate acquired by T cells within the TME. This paper also provides a re-analysis of data included in Sade-Feldman et al. (2019), identifying T_{PE} cells in melanoma samples and documenting that their level within the basal TME is associated with positive outcome after immunotherapy.
22. Schrama D, Ritter C & Becker JC T cell receptor repertoire usage in cancer as a surrogate marker for immune responses. Semin. Immunopathol 39, 255–268 (2017). [PubMed: 28074285]
23. Hudson WH & Wieland A Technology meets TILs: deciphering T cell function in the -omics era. Cancer Cell 41, 41–57 (2023). [PubMed: 36206755]
24. Newman AM & Alizadeh AA High-throughput genomic profiling of tumor-infiltrating leukocytes. Curr. Opin. Immunol 41, 77–84 (2016). [PubMed: 27372732]
25. Pauken KE et al. TCR-sequencing in cancer and autoimmunity: barcodes and beyond. Trends Immunol. 43, 180–194 (2022). [PubMed: 35090787]
26. Penter L, Gohil SH & Wu CJ Natural barcodes for longitudinal single cell tracking of leukemic and immune cell dynamics. Front. Immunol 12, 788891 (2021). [PubMed: 35046946]
27. Gros A et al. Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. Nat. Med 22, 433–438 (2016). [PubMed: 26901407]
28. Hu Z et al. A cloning and expression system to probe T-cell receptor specificity and assess functional avidity to neoantigens. Blood 132, 1911–1921 (2018). [PubMed: 30150207]

29. Galon J et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. J. Pathol 232, 199–209 (2014). [PubMed: 24122236]
30. Fridman WH, Pagès F, Sautès-Fridman C & Galon J The immune contexture in human tumours: impact on clinical outcome. Nat. Rev. Cancer 12, 298–306 (2012). [PubMed: 22419253]
31. Maibach F, Sadozai H, Seyed Jafari SM, Hunger RE & Schenk M Tumor-infiltrating lymphocytes and their prognostic value in cutaneous melanoma. Front. Immunol 11, 2105 (2020). [PubMed: 33013886]
32. Kather JN et al. Topography of cancer-associated immune cells in human solid tumors. eLife 7, e36967 (2018). [PubMed: 30179157]
33. Rooney MS, Shukla SA, Wu CJ, Getz G & Hacohen N Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell 160, 48–61 (2015). [PubMed: 25594174]
34. Paijens ST, Vledder A, de Bruyn M & Nijman HW Tumor-infiltrating lymphocytes in the immunotherapy era. Cell Mol. Immunol 18, 842–859 (2021). [PubMed: 33139907]
35. Gohil SH, Iorgulescu JB, Braun DA, Keskin DB & Livak KJ Applying high-dimensional single-cell technologies to the analysis of cancer immunotherapy. Nat. Rev. Clin. Oncol 18, 244–256 (2021). [PubMed: 33277626]
36. Guo X et al. Global characterization of T cells in non-small-cell lung cancer by single-cell sequencing. Nat. Med 24, 978–985 (2018). [PubMed: 29942094]
37. Clarke J et al. Single-cell transcriptomic analysis of tissue-resident memory T cells in human lung cancer. J. Exp. Med 216, 2128–2149 (2019). [PubMed: 31227543]
38. Savas P et al. Single-cell profiling of breast cancer T cells reveals a tissue-resident memory subset associated with improved prognosis. Nat. Med 24, 986–993 (2018). [PubMed: 29942092]
39. Tirosh I et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Science 352, 189–196 (2016). [PubMed: 27124452]
40. Jerby-Arnon L et al. A cancer cell program promotes T cell exclusion and resistance to checkpoint blockade. Cell 175, 984–997.e24 (2018). [PubMed: 30388455]
41. Zheng C et al. Landscape of infiltrating T cells in liver cancer revealed by single-cell sequencing. Cell 169, 1342–1356.e16 (2017). [PubMed: 28622514]
42. Zhang L et al. Lineage tracking reveals dynamic relationships of T cells in colorectal cancer. Nature 564, 268–272 (2018). [PubMed: 30479382]
43. Doering TA et al. Network analysis reveals centrally connected genes and pathways involved in $CD8^+$ T cell exhaustion versus memory. Immunity 37, 1130–1144 (2012). [PubMed: 23159438]
44. Kaech SM & Cui W Transcriptional control of effector and memory $CD8^+$ T cell differentiation. Nat. Rev. Immunol 12, 749–761 (2012). [PubMed: 23080391]
45. Cui W & Kaech SM Generation of effector $CD8^+$ T cells and their conversion to memory T cells. Immunol. Rev 236, 151–166 (2010). [PubMed: 20636815]
46. Sallusto F, Geginat J & Lanzavecchia A Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu. Rev. Immunol 22, 745–763 (2004). [PubMed: 15032595]
47. Gattinoni L et al. A human memory T cell subset with stem cell-like properties. Nat. Med 17, 1290–1297 (2011). [PubMed: 21926977]
48. Lanzavecchia A & Sallusto F Progressive differentiation and selection of the fittest in the immune response. Nat. Rev. Immunol 2, 982–987 (2002). [PubMed: 12461571]
49. Kaech SM & Wherry EJ Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity 27, 393–405 (2007). [PubMed: 17892848]
50. Hand TW & Kaech SM Intrinsic and extrinsic control of effector T cell survival and memory T cell development. Immunol. Res 45, 46–61 (2009). [PubMed: 18629449]
51. Reiner SL, Sallusto F & Lanzavecchia A Division of labor with a workforce of one: challenges in specifying effector and memory T cell fate. Science 317, 622–625 (2007). [PubMed: 17673652]
52. Wherry EJ T cell exhaustion. Nat. Immunol 12, 492–499 (2011). [PubMed: 21739672]
53. McLane LM, Abdel-Hakeem MS & Wherry EJ CD8 T cell exhaustion during chronic viral infection and cancer. Annu. Rev. Immunol 37, 457–495 (2019). [PubMed: 30676822]

54. Schietinger A & Greenberg PD Tolerance and exhaustion: defining mechanisms of T cell dysfunction. Trends Immunol. 35, 51–60 (2014). [PubMed: 24210163]
55. Wherry EJ & Kurachi M Molecular and cellular insights into T cell exhaustion. Nat. Rev. Immunol 15, 486–499 (2015). [PubMed: 26205583]
56. Liu B, Zhang Y, Wang D, Hu X & Zhang Z Single-cell meta-analyses reveal responses of tumor-reactive CXCL13+ T cells to immune-checkpoint blockade. Nat. Cancer 3, 1123–1136 (2022). [PubMed: 36138134]
57. Fooksman DR et al. Functional anatomy of T cell activation and synapse formation. Annu. Rev. Immunol 28, 79–105 (2010). [PubMed: 19968559]
58. Oliveira G et al. Phenotype, specificity and avidity of antitumour CD8+ T cells in melanoma. Nature 596, 119–125 (2021). [PubMed: 34290406] This work coupled single-cell profiling of CD8+ TILs with reconstruction and specificity screening of intratumoural TCRs and demonstrates that tumour-reactive TCRs able to recognize autologous melanomas are highly enriched among exhausted TILs, while viral specificities are prevalent among memory cells. A gene signature of tumour-specific TILs is also provided.
59. Wu TD et al. Peripheral T cell expansion predicts tumour infiltration and clinical response. Nature 579, 274–278 (2020). [PubMed: 32103181] By performing single-cell sequencing of transcriptomes and TCRs in patients with different types of cancer, the authors present evidence of clonal expansion of T cells in tumours, normal tissue and peripheral blood upon ICB. Analysis of lesions collected 2–3 months after immunotherapy documents that response is associated with the replenishment of intratumoural T cells from extratumoural sites.
60. Scott AC et al. TOX is a critical regulator of tumour-specific T cell differentiation. Nature 571, 270–274 (2019). [PubMed: 31207604]
61. Khan O et al. TOX transcriptionally and epigenetically programs CD8+ T cell exhaustion. Nature 571, 211–218 (2019). [PubMed: 31207603]
62. Alfei F et al. TOX reinforces the phenotype and longevity of exhausted T cells in chronic viral infection. Nature 571, 265–269 (2019). [PubMed: 31207605]
63. Miller BC et al. Subsets of exhausted CD8+ T cells differentially mediate tumor control and respond to checkpoint blockade. Nat. Immunol 20, 326–336 (2019). [PubMed: 30778252]
64. Schumacher TN & Thommen DS Tertiary lymphoid structures in cancer. Science 375, eabf9419 (2022). [PubMed: 34990248]
65. Connolly KA et al. A reservoir of stem-like CD8+ T cells in the tumor-draining lymph node preserves the ongoing antitumor immune response. Sci. Immunol 6, eabg7836 (2021). [PubMed: 34597124]
66. Okła K, Farber DL & Zou W Tissue-resident memory T cells in tumor immunity and immunotherapy. J. Exp. Med 218, e20201605 (2021). [PubMed: 33755718]
67. Mueller SN & Mackay LK Tissue-resident memory T cells: local specialists in immune defence. Nat. Rev. Immunol 16, 79–89 (2016). [PubMed: 26688350]
68. Gebhardt T, Palendira U, Tscharke DC & Bedoui S Tissue-resident memory T cells in tissue homeostasis, persistent infection, and cancer surveillance. Immunol. Rev 283, 54–76 (2018). [PubMed: 29664571]
69. Duhen T et al. Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. Nat. Commun 9, 2724 (2018). [PubMed: 30006565] Analysis of tumour reactivity of TILs against autologous tumours, which identifies CD103+ and CD39+ TILs as a reservoir of antitumour T cells in melanoma and HNSCC.
70. Parga-Vidal L et al. Hobit identifies tissue-resident memory T cell precursors that are regulated by Eomes. Sci. Immunol 6, eabg3533 (2021). [PubMed: 34417257]
71. Mackay LK et al. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. Science 352, 459–463 (2016). [PubMed: 27102484]
72. Park SL, Gebhardt T & Mackay LK Tissue-resident memory T cells in cancer immunosurveillance. Trends Immunol. 40, 735–747 (2019). [PubMed: 31255505]
73. Swain SL, McKinstry KK & Strutt TM Expanding roles for CD4+ T cells in immunity to viruses. Nat. Rev. Immunol 12, 136–148 (2012). [PubMed: 22266691]

74. Sallusto F & Lanzavecchia A Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. Eur. J. Immunol 39, 2076–2082 (2009). [PubMed: 19672903]

75. Oh DY & Fong L Cytotoxic CD4+ T cells in cancer: expanding the immune effector toolbox. Immunity 54, 2701–2711 (2021). [PubMed: 34910940]

76. Kim H-J & Cantor H CD4 T-cell subsets and tumor immunity: the helpful and the not-so-helpful. Cancer Immunol. Res 2, 91–98 (2014). [PubMed: 24778273]

77. Ahrends T & Borst J The opposing roles of CD4+ T cells in anti-tumour immunity. Immunology 154, 582–592 (2018). [PubMed: 29700809]

78. Qin L et al. Insights into the molecular mechanisms of T follicular helper-mediated immunity and pathology. Front. Immunol 9, 1884 (2018). [PubMed: 30158933]

79. Liu B et al. Temporal single-cell tracing reveals clonal revival and expansion of precursor exhausted T cells during anti-PD-1 therapy in lung cancer. Nat. Cancer 3, 108–121 (2022). [PubMed: 35121991] Using single-cell transcriptomics and TCR clonality, this work analyses the dynamics of T cell clones infiltrating lung cancer before and after immunotherapy. After ICB, the authors detect less exhausted TILs, derived from local expansion of pre-existing TILs and recruitment of their peripheral precursors, a phenomenon called ‘clonal revival’.

80. Oh DY et al. Intratumoral $CD4^+$ T cells mediate anti-tumor cytotoxicity in human bladder cancer. Cell 181, 1612–1625.e13 (2020). [PubMed: 32497499]

81. Smith-Garvin JE, Koretzky GA & Jordan MS T cell activation. Annu. Rev. Immunol 27, 591–619 (2009). [PubMed: 19132916]

82. Klein L, Kyewski B, Allen PM & Hogquist KA Positive and negative selection of the T cell repertoire: what thymocytes see (and don’t see). Nat. Rev. Immunol 14, 377–391 (2014). [PubMed: 24830344]

83. Pai JA & Satpathy AT High-throughput and single-cell T cell receptor sequencing technologies. Nat. Methods 18, 881–892 (2021). [PubMed: 34282327]

84. Gros A et al. PD-1 identifies the patient-specific $CD8^+$ tumor-reactive repertoire infiltrating human tumors. J. Clin. Invest 124, 2246–2259 (2014). [PubMed: 24667641] By co-culturing in vitro $PD1^+$ or $PD1^-$ TILs with autologous melanoma, this work is one of the first to demonstrate that $PD1^+$ TILs retain detectable antitumour potential.

85. Pasetto A et al. Tumor- and neoantigen-reactive T-cell receptors can be identified based on their frequency in fresh tumor. Cancer Immunol. Res 4, 734–743 (2016). [PubMed: 27354337]

86. Oliveira G et al. Landscape of helper and regulatory antitumour $CD4^+$ T cells in melanoma. Nature 605, 532–538 (2022). [PubMed: 35508657] The reactivity screening of TCRs sequenced among $CD4^+$ T cells infiltrating melanoma tumours demonstrates the presence of tumour-reactive helper T cells and T_{reg} cells specific for TAAs and private neoantigens. This study documents that neoantigen- reactive T_{reg} cells are abundant in highly mutated melanomas, where constitutive HLA-class II expression enables the engagement of potentially immunosuppressive neoantigen-specific T_{reg} cells.

87. Yost KE et al. Clonal replacement of tumor-specific T cells following PD-1 blockade. Nat. Med 25, 1251–1259 (2019). [PubMed: 31359002] Single-cell analysis of T cell clonal dynamics in basal and squamous cell carcinoma biopsies collected before and after anti-PD1 therapy shows that the expanding and responding T cell clones derive preferentially from novel clones that were not detected in pre-treatment specimens. This work documents the phenomenon of clonal replacement in samples collected >2 months after ICB.

88. Caushi JX et al. Transcriptional programs of neoantigen-specific TIL in anti-PD-1- treated lung cancers. Nature 596, 126–132 (2021). [PubMed: 34290408] TCRs specific for neoantigens isolated from blood are traced using scRNA-seq within lung cancers collected after neoadjuvant immunotherapy. The authors document that neoantigen-specific TILs are defined by a transcriptional programme characterized by expression of markers of exhausted and tissue-resident TILs and by an incompletely activated cytolytic programme.

89. Braun DA et al. Progressive immune dysfunction with advancing disease stage in renal cell carcinoma. Cancer Cell 39, 632–648.e8 (2021). [PubMed: 33711273]

90. Hanada K-I et al. A phenotypic signature that identifies neoantigen-reactive T cells in fresh human lung cancers. Cancer Cell 40, 479–493.e6 (2022). [PubMed: 35452604] Analysis of neoantigen-

specific TILs in lung cancers, which identifies CD39 and CXCL13 as important molecular markers for the isolation of antitumour specificities. The tracing of CD39+ T cell clones in peripheral blood demonstrates that dysfunctional antitumour specificities are not characterized by high persistence and systemic distribution.

91. Simoni Y et al. Bystander CD8$^+$ T cells are abundant and phenotypically distinct in human tumour infiltrates. Nature 557, 575–579 (2018). [PubMed: 29769722] Through the detection of peptide–HLA binding on T cells using cytometry by time of flight, the authors analyse the frequency and phenotype of TILs specific for viral antigens or tumour antigens in human lung cancer and CRC. They demonstrate that the TME is populated by abundant bystander TILs with specificity for viral antigens and lack expression of CD39, which can be used to distinguish bystander from neoantigen-reactive TILs.
92. Wherry EJ & Ahmed R Memory CD8 T-cell differentiation during viral infection. J. Virol 78, 5535–5545 (2004). [PubMed: 15140950]
93. Goodspeed A, Heiser LM, Gray JW & Costello JC Tumor-derived cell lines as molecular models of cancer pharmacogenomics. Mol. Cancer Res 14, 3–13 (2016). [PubMed: 26248648]
94. Newell EW, Klein LO, Yu W & Davis MM Simultaneous detection of many T-cell specificities using combinatorial tetramer staining. Nat. Methods 6, 497–499 (2009). [PubMed: 19543286]
95. Harjunpää H, Llort Asens M, Guenther C & Fagerholm SC Cell adhesion molecules and their roles and regulation in the immune and tumor microenvironment. Front. Immunol 10, 1078 (2019). [PubMed: 31231358]
96. Meier SL, Satpathy AT & Wells DK Bystander T cells in cancer immunology and therapy. Nat. Cancer 3, 143–155 (2022). [PubMed: 35228747]
97. Scheper W et al. Low and variable tumor reactivity of the intratumoral TCR repertoire in human cancers. Nat. Med 25, 89–94 (2019). [PubMed: 30510250] Reconstruction of TCRs from TILs and screening against autologous tumours demonstrates that, in human cancers, many specificities are not tumour-reactive, exhibit variable recognition of tumour antigens or can be stimulated by viral antigens.
98. Lowery FJ et al. Molecular signatures of antitumor neoantigen-reactive T cells from metastatic human cancers. Science 375, 877–884 (2022). [PubMed: 35113651] By mapping neoantigen-specific TCR clonotypes into T cell states identified through scRNA-seq of TILs in metastatic cancers, the authors identify a gene signature of CD8$^+$ or CD4$^+$ neoantigen-reactive TILs that is highly enriched in exhaustion genes.
99. Zheng C et al. Transcriptomic profiles of neoantigen-reactive T cells in human gastrointestinal cancers. Cancer Cell 40, 410–423.e7 (2022). [PubMed: 35413272]
100. Eberhardt CS et al. Functional HPV-specific PD-1+ stem-like CD8 T cells in head and neck cancer. Nature 597, 279–284 (2021). [PubMed: 34471285]
101. Luoma AM et al. Tissue-resident memory and circulating T cells are early responders to pre-surgical cancer immunotherapy. Cell 185, 2918–2935.e29 (2022). [PubMed: 35803260] This study analyses the dynamics of T cells early after neoadjuvant ICB administration in patients with HNSCCs and demonstrates that TILs undergoing expansion and reinvigoration are those exhibiting a tissue-resident memory-like phenotype and expressing exhaustion and cytotoxicity markers.
102. van de Pavert SA et al. Chemokine CXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation. Nat. Immunol 10, 1193–1199 (2009). [PubMed: 19783990]
103. Thommen DS et al. A transcriptionally and functionally distinct PD-1+ CD8+ T cell pool with predictive potential in non-small-cell lung cancer treated with PD-1 blockade. Nat. Med 24, 994–1004 (2018). [PubMed: 29892065]
104. Workel HH et al. A transcriptionally distinct CXCL13+ CD103+ CD8+ T-cell population is associated with B-cell recruitment and neoantigen load in human cancer. Cancer Immunol. Res 7, 784–796 (2019). [PubMed: 30872264]
105. Litchfield K et al. Meta-analysis of tumor- and T cell-intrinsic mechanisms of sensitization to checkpoint inhibition. Cell 184, 596–614.e14 (2021). [PubMed: 33508232]

106. Linnemann C et al. High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. Nat. Med 21, 81–85 (2015). [PubMed: 25531942]

107. Friedman KM et al. Tumor-specific CD4+ melanoma tumor-infiltrating lymphocytes. J. Immunother 35, 400–408 (2012). [PubMed: 22576345]

108. Cachot A et al. Tumor-specific cytolytic CD4 T cells mediate immunity against human cancer. Sci. Adv 7, eabe3348 (2021). [PubMed: 33637530]

109. Ahmadzadeh M et al. Tumor-infiltrating human CD4+ regulatory T cells display a distinct TCR repertoire and exhibit tumor and neoantigen reactivity. Sci. Immunol 4, eaao4310 (2019). [PubMed: 30635355]

110. Veatch JR et al. Neoantigen-specific CD4+ T cells in human melanoma have diverse differentiation states and correlate with CD8+ T cell, macrophage, and B cell function. Cancer Cell 40, 393–409.e9 (2022). [PubMed: 35413271]

111. Rodig SJ et al. MHC proteins confer differential sensitivity to CTLA-4 and PD-1 blockade in untreated metastatic melanoma. Sci. Transl Med 10, eaar3342 (2018). [PubMed: 30021886]

112. Shao XM et al. HLA class II immunogenic mutation burden predicts response to immune checkpoint blockade. Ann. Oncol 33, 728–738 (2022). [PubMed: 35339648]

113. Sharma P & Allison JP The future of immune checkpoint therapy. Science 348, 56–61 (2015). [PubMed: 25838373]

114. Vaddepally RK, Kharel P, Pandey R, Garje R & Chandra AB Review of indications of FDA-approved immune checkpoint inhibitors per NCCN guidelines with the level of evidence. Cancers 12, 738 (2020). [PubMed: 32245016]

115. No authors listed. FDA approves anti-LAG3 checkpoint. Nat. Biotechnol 40, 625 (2022). [PubMed: 35577946]

116. Bagchi S, Yuan R & Engleman EG Immune checkpoint inhibitors for the treatment of cancer: clinical impact and mechanisms of response and resistance. Annu. Rev. Pathol 16, 223–249 (2021). [PubMed: 33197221]

117. Morad G, Helmink BA, Sharma P & Wargo JA Hallmarks of response, resistance, and toxicity to immune checkpoint blockade. Cell 184, 5309–5337 (2021). [PubMed: 34624224]

118. de Miguel M & Calvo E Clinical challenges of immune checkpoint inhibitors. Cancer Cell 38, 326–333 (2020). [PubMed: 32750319]

119. Lee YJ et al. CD39+ tissue-resident memory CD8+ T cells with a clonal overlap across compartments mediate antitumor immunity in breast cancer. Sci. Immunol 7, eabn8390 (2022). [PubMed: 36026440]

120. Bassez A et al. A single-cell map of intratumoral changes during anti-PD1 treatment of patients with breast cancer. Nat. Med 27, 820–832 (2021). [PubMed: 33958794] This study analyses the dynamics of T cells after pre-surgical administration of ICB in patients with breast cancer and demonstrates that early expansion of TILs in responding patients mainly involves CD8+ T cells with pronounced expression of cytotoxic activity, immune-cell homing and exhaustion markers.

121. Afeyan A et al. Cytotoxic revival is implicated in response to neoadjuvant PD-1 blockade for head and neck squamous cell carcinoma. J. Immunother. Cancer 10, 503 (2022).

122. Yates KB et al. Epigenetic scars of CD8+ T cell exhaustion persist after cure of chronic infection in humans. Nat. Immunol 22, 1020–1029 (2021). [PubMed: 34312547]

123. Pauken KE et al. Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. Science 354, 1160–1165 (2016). [PubMed: 27789795]

124. Schietinger A et al. Tumor-specific T cell dysfunction is a dynamic antigen-driven differentiation program initiated early during tumorigenesis. Immunity 45, 389–401 (2016). [PubMed: 27521269]

125. Valpione S et al. Immune-awakening revealed by peripheral T cell dynamics after one cycle of immunotherapy. Nat. Cancer 1, 210–221 (2020). [PubMed: 32110781]

126. Fairfax BP et al. Peripheral CD8+ T cell characteristics associated with durable responses to immune checkpoint blockade in patients with metastatic melanoma. Nat. Med 26, 193–199 (2020). [PubMed: 32042196]

127. Huang AC et al. T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. Nature 545, 60–65 (2017). [PubMed: 28397821]
128. Kamphorst AO et al. Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. Proc. Natl Acad. Sci. USA 114, 4993–4998 (2017). [PubMed: 28446615]
129. Zhang J et al. Compartmental analysis of T-cell clonal dynamics as a function of pathologic response to neoadjuvant PD-1 blockade in resectable non-small cell lung cancer. Clin. Cancer Res 26, 1327–1337 (2020). [PubMed: 31754049]
130. Inamori K et al. Importance of lymph node immune responses in MSI-H/dMMR colorectal cancer. JCI Insight 6, 137365 (2021). [PubMed: 33755600]
131. Nagasaki J et al. PD-1 blockade therapy promotes infiltration of tumor-attacking exhausted T cell clonotypes. Cell Rep. 38, 110331 (2022). [PubMed: 35108529]
132. Anadon CM et al. Ovarian cancer immunogenicity is governed by a narrow subset of progenitor tissue-resident memory T cells. Cancer Cell 40, 545–557.e13 (2022). [PubMed: 35427494]
133. Hu Z, Ott PA & Wu CJ Towards personalized, tumour-specific, therapeutic vaccines for cancer. Nat. Rev. Immunol 18, 168–182 (2018). [PubMed: 29226910]
134. Sellars MC, Wu CJ & Fritsch EF Cancer vaccines: building a bridge over troubled waters. Cell 185, 2770–2788 (2022). [PubMed: 35835100]
135. Lin MJ et al. Cancer vaccines: the next immunotherapy frontier. Nat. Cancer 3, 911–926 (2022). [PubMed: 35999309]
136. Sahin U et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. Nature 547, 222–226 (2017). [PubMed: 28678784]
137. Ott PA et al. An immunogenic personal neoantigen vaccine for patients with melanoma. Nature 547, 217–221 (2017). [PubMed: 28678778]
138. Ott PA et al. A Phase Ib trial of personalized neoantigen therapy plus anti-PD-1 in patients with advanced melanoma, non-small cell lung cancer, or bladder cancer. Cell 183, 347–362.e24 (2020). [PubMed: 33064988]
139. Awad MM et al. Personalized neoantigen vaccine NEO-PV-01 with chemotherapy and anti-PD-1 as first-line treatment for non-squamous non-small cell lung cancer. Cancer Cell 40, 1010–1026.e11 (2022). [PubMed: 36027916]
140. Poran A et al. Combined TCR repertoire profiles and blood cell phenotypes predict melanoma patient response to personalized neoantigen therapy plus anti-PD-1. Cell Rep. Med 1, 100141 (2020). [PubMed: 33294862]
141. Hu Z et al. Personal neoantigen vaccines induce persistent memory T cell responses and epitope spreading in patients with melanoma. Nat. Med 27, 515–525 (2021). [PubMed: 33479501]
142. Keskin DB et al. Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. Nature 565, 234–239 (2019). [PubMed: 30568305]
143. Platten M et al. A vaccine targeting mutant IDH1 in newly diagnosed glioma. Nature 592, 463–468 (2021). [PubMed: 33762734]
144. Rosenberg SA, Spiess P & Lafreniere R A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. Science 233, 1318–1321 (1986). [PubMed: 3489291]
145. Rosenberg SA et al. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. J. Natl Cancer Inst 86, 1159–1166 (1994). [PubMed: 8028037]
146. Rosenberg SA et al. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N. Engl. J. Med 313, 1485–1492 (1985). [PubMed: 3903508]
147. Dudley ME, Wunderlich JR, Shelton TE, Even J & Rosenberg SA Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. J. Immunother 26, 332–342 (2003). [PubMed: 12843795]
148. Rosenberg SA et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin. Cancer Res 17, 4550–4557 (2011). [PubMed: 21498393]

149. Stevanoviç S et al. Landscape of immunogenic tumor antigens in successful immunotherapy of virally induced epithelial cancer. Science 356, 200–205 (2017). [PubMed: 28408606]

150. Tran E, Robbins PF & Rosenberg SA 'Final common pathway' of human cancer immunotherapy: targeting random somatic mutations. Nat. Immunol 18, 255–262 (2017). [PubMed: 28198830]

151. Parkhurst MR et al. Unique neoantigens arise from somatic mutations in patients with gastrointestinal cancers. Cancer Discov. 9, 1022–1035 (2019). [PubMed: 31164343]

152. Dudley ME et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J. Clin. Oncol 26, 5233–5239 (2008). [PubMed: 18809613]

153. Dudley ME et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J. Clin. Oncol 23, 2346–2357 (2005). [PubMed: 15800326]

154. Huang J et al. Survival, persistence, and progressive differentiation of adoptively transferred tumor-reactive T cells associated with tumor regression. J. Immunother 28, 258–267 (2005). [PubMed: 15838383]

155. Yee C et al. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. Proc. Natl Acad. Sci. USA 99, 16168–16173 (2002). [PubMed: 12427970]

156. Gattinoni L, Klebanoff CA & Restifo NP Paths to stemness: building the ultimate antitumour T cell. Nat. Rev. Cancer 12, 671–684 (2012). [PubMed: 22996603]

157. Gattinoni L et al. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J. Clin. Invest 115, 1616–1626 (2005). [PubMed: 15931392]

158. Hinrichs CS et al. Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. Proc. Natl Acad. Sci. USA 106, 17469–17474 (2009). [PubMed: 19805141]

159. Chen GM et al. Integrative bulk and single-cell profiling of premanufacture T-cell populations reveals factors mediating long-term persistence of CAR T-cell therapy. Cancer Discov. 11, 2186–2199 (2021). [PubMed: 33820778]

160. Busch DH, Fräßle SP, Sommermeyer D, Buchholz VR & Riddell SR Role of memory T cell subsets for adoptive immunotherapy. Semin. Immunol 28, 28–34 (2016). [PubMed: 26976826]

161. Chan JD et al. Cellular networks controlling T cell persistence in adoptive cell therapy. Nat. Rev. Immunol 21, 769–784 (2021). [PubMed: 33879873]

162. Krishna S et al. Stem-like CD8 T cells mediate response of adoptive cell immunotherapy against human cancer. Science 370, 1328–1334 (2020). [PubMed: 33303615] Using single-cell profiling and specificity screening of T cell products expanded from TILs for ACT, this study shows that neoantigen-specific clones with a stem-like memory phenotype are characterized by improved persistence upon infusion into patients with melanoma who responded to treatment. Conversely, neoantigen- specific T cells infused into non-responder patients displayed a signature of terminal exhaustion that correlates with poor in vivo fitness.

163. Kishton RJ, Vodnala SK, Vizcardo R & Restifo NP Next generation immunotherapy: enhancing stemness of polyclonal T cells to improve anti-tumor activity. Curr. Opin. Immunol 74, 39–45 (2022). [PubMed: 34710751]

164. Labanieh L, Majzner RG & Mackall CL Programming CAR-T cells to kill cancer. Nat. Biomed. Eng 2, 377–391 (2018). [PubMed: 31011197]

165. Leon E, Ranganathan R & Savoldo B Adoptive T cell therapy: boosting the immune system to fight cancer. Semin. Immunol 49, 101437 (2020). [PubMed: 33262066]

166. Newick K, O'Brien S, Moon E & Albelda SM CAR T cell therapy for solid tumors. Annu. Rev. Med 68, 139–152 (2017). [PubMed: 27860544]

167. Morgan RA et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314, 126–129 (2006). [PubMed: 16946036]

168. Johnson LA et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 114, 535–546 (2009). [PubMed: 19451549]

169. Robbins PF et al. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. Clin. Cancer Res 21, 1019–1027 (2015). [PubMed: 25538264]

170. Stadtmauer EA et al. Long-term safety and activity of NY-ESO-1 SPEAR T cells after autologous stem cell transplant for myeloma. Blood Adv. 3, 2022–2034 (2019). [PubMed: 31289029]

171. Rapoport AP et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. Nat. Med 21, 914–921 (2015). [PubMed: 26193344]

172. Robbins PF et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J. Clin. Oncol 29, 917–924 (2011). [PubMed: 21282551]

173. Morgan RA et al. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. J. Immunother 36, 133–151 (2013). [PubMed: 23377668]

174. Linette GP et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood 122, 863–871 (2013). [PubMed: 23770775]

175. Leidner R et al. Neoantigen T-cell receptor gene therapy in pancreatic cancer. N. Engl. J.Med 386,2112–2119(2022). [PubMed: 35648703]

176. Kim SP et al. Adoptive cellular therapy with autologous tumor-infiltrating lymphocytes and T-cell receptor-engineered T cells targeting common p53 neoantigens in human solid tumors. Cancer Immunol. Res 10, 932–946 (2022). [PubMed: 35749374]

177. Chandran SS et al. Immunogenicity and therapeutic targeting of a public neoantigen derived from mutated PIK3CA. Nat. Med 28, 946–957 (2022). [PubMed: 35484264]

178. Louis CU et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. Blood 118,6050–6056(2011). [PubMed: 21984804]

179. Ahmed N et al. Human epidermal growth factor receptor 2 (HER2) -specific chimeric antigen receptor-modified T cells for the immunotherapy of HER2-positive sarcoma. J. Clin. Oncol 33, 1688–1696 (2015). [PubMed: 25800760]

180. Haslauer T, Greil R, Zaborsky N & Geisberger R CAR T-cell therapy in hematological malignancies.Int.J.Mol.Sci 22,8996(2021). [PubMed: 34445701]

181. Fraietta JA et al. Determinants of response and resistance to CD19 chimeric antigen receptor(CAR)Tcelltherapyofchroniclymphocyticleukemia. Nat.Med 24,563–571 (2018). [PubMed: 29713085] In this study, the authors perform a comprehensive assessment of phenotypic and functional features of CD19 CAR T cells circulating in treated patients with chronic lymphocytic leukaemia. It shows that the presence of gene-modified cells with memory-like properties is associated with sustained remission, while in non- responder patients, the gene-modified cells differentiated towards an exhaustion programme.

182. Deng Q et al. Characteristics of anti-CD19 CAR T cell infusion products associated with efficacy and toxicity in patients with large B cell lymphomas. Nat. Med 26, 1878–1887 (2020). [PubMed: 33020644] Single-cell transcriptional profiling of CD19 CAR T cell infusion products is performed in this study to reveal the T cell features associated with response to ACT. Patients who experienced expansion of gene-modified T cells and achieved complete responses benefited from infusion of T cell products enriched in memory-like cells.

183. Shah NN & Fry TJ Mechanisms of resistance to CAR T cell therapy. Nat. Rev. Clin. Oncol 16, 372–385 (2019). [PubMed: 30837712]

184. Sheih A et al. Clonal kinetics and single-cell transcriptional profiling of CAR-T cells in patients undergoing CD19 CAR-T immunotherapy. Nat. Commun 11, 219 (2020). [PubMed: 31924795]

185. Wilson TL et al. Common trajectories of highly effective CD19-specific CAR T cells identified by endogenous T-cell receptor lineages. Cancer Discov.12,2098–2119(2022). [PubMed: 35792801]

186. Haradhvala NJ et al. Distinct cellular dynamics associated with response to CAR-T therapy for refractory B cell lymphoma. Nat. Med 28, 1848–1859 (2022). [PubMed: 36097221]

187. Melenhorst JJ et al. Decade-long leukaemia remissions with persistence of CD4+ CAR T cells. Nature 602, 503–509 (2022). [PubMed: 35110735]

188. Joglekar AV & Li G T cell antigen discovery. Nat. Methods 18, 873–880 (2021). [PubMed: 32632239]

189. Franco F, Jaccard A, Romero P, Yu Y-R & Ho P-C Metabolic and epigenetic regulation of T-cell exhaustion. Nat. Metab 2, 1001–1012 (2020). [PubMed: 32958939]
190. Sautès-Fridman C, Petitprez F, Calderaro J & Fridman WH Tertiary lymphoid structures in the era of cancer immunotherapy. Nat. Rev. Cancer 19, 307–325 (2019). [PubMed: 31092904]
191. Fridman WH et al. B cells and tertiary lymphoid structures as determinants of tumour immune contexture and clinical outcome. Nat. Rev. Clin. Oncol 19, 441–457 (2022). [PubMed: 35365796]
192. Longo SK, Guo MG, Ji AL & Khavari PA Integrating single-cell and spatial transcriptomics to elucidate intercellular tissue dynamics. Nat. Rev. Genet 22, 627–644 (2021). [PubMed: 34145435]
193. Rao A, Barkley D, França GS & Yanai I Exploring tissue architecture using spatial transcriptomics. Nature 596, 211–220 (2021). [PubMed: 34381231]
194. Liu S et al. Spatial maps of T cell receptors and transcriptomes reveal distinct immune niches and interactions in the adaptive immune response. Immunity 55, 1940–1952.e5 (2022). [PubMed: 36223726]
195. Hudson WH & Sudmeier LJ Localization of T cell clonotypes using the Visium spatial transcriptomics platform. STAR Protoc. 3, 101391 (2022). [PubMed: 35707680]
196. Lundberg E & Borner GHH Spatial proteomics: a powerful discovery tool for cell biology. Nat. Rev. Mol. Cell Biol 20, 285–302 (2019). [PubMed: 30659282]
197. Ag C et al. T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. Nat. Med 25, 1064–1072 (2019). [PubMed: 31235963]
198. Hadrup SR et al. Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. Nat. Methods 6, 520–526 (2009). [PubMed: 19543285]
199. Newell EW et al. Combinatorial tetramer staining and mass cytometry analysis facilitate T-cell epitope mapping and characterization. Nat. Biotechnol 31, 623–629 (2013). [PubMed: 23748502]
200. Bentzen AK et al. Large-scale detection of antigen-specific T cells using peptide-MHC-I multimers labeled with DNA barcodes. Nat. Biotechnol 34, 1037–1045 (2016). [PubMed: 27571370]
201. Zhang S-Q et al. High-throughput determination of the antigen specificities of T cell receptors in single cells. Nat. Biotechnol 36, 1156–1159 (2018).
202. Peng S et al. Sensitive detection and analysis of neoantigen-specific T cell populations from tumors and blood. Cell Rep. 28, 2728–2738.e7 (2019). [PubMed: 31484081]
203. Ng AHC et al. MATE-Seq: microfluidic antigen-TCR engagement sequencing. Lab Chip 19, 3011–3021 (2019). [PubMed: 31502632]
204. Wang Y et al. Using a baculovirus display library to identify MHC class I mimotopes. Proc. Natl Acad. Sci. USA 102, 2476–2481 (2005). [PubMed: 15699351]
205. Crawford F et al. Use of baculovirus MHC/peptide display libraries to characterize T-cell receptor ligands. Immunol. Rev 210, 156–170 (2006). [PubMed: 16623770]
206. Birnbaum ME et al. Deconstructing the peptide-MHC specificity of T cell recognition. Cell 157, 1073–1087 (2014). [PubMed: 24855945]
207. Gee MH et al. Antigen identification for orphan T cell receptors expressed on tumor-infiltrating lymphocytes. Cell 172, 549–563.e16 (2018). [PubMed: 29275860]
208. Huisman BD, Dai Z, Gifford DK & Birnbaum ME A high-throughput yeast display approach to profile pathogen proteomes for MHC-II binding. eLife 11, e78589 (2022). [PubMed: 35781135]
209. Dobson CS et al. Antigen identification and high-throughput interaction mapping by reprogramming viral entry. Nat. Methods 19, 449–460 (2022). [PubMed: 35396484]
210. Joglekar AV et al. T cell antigen discovery via signaling and antigen-presenting bifunctional receptors. Nat. Methods 16, 191–198 (2019). [PubMed: 30700902]
211. Kisielow J, Obermair F-J & Kopf M Deciphering $CD4^+$ T cell specificity using novel MHC-TCR chimeric receptors. Nat. Immunol 20, 652–662 (2019). [PubMed: 30858620]
212. Li G et al. T cell antigen discovery via trogocytosis. Nat. Methods 16, 183–190 (2019). [PubMed: 30700903]

213. Kula T et al. T-Scan: a genome-wide method for the systematic discovery of T cell epitopes. Cell 178, 1016–1028.e13 (2019). [PubMed: 31398327]

214. Sharma G,Rive CM& Holt RA Rapid selection and identification of functional $CD8^+$ T cell epitopes from large peptide-coding libraries. Nat. Commun 10, 4553 (2019). [PubMed: 31591401]

215. Banchereau R et al. Intratumoral $CD103^+$ $CD8^+$ T cells predict response to PD-L1 blockade. J. Immunother. Cancer 9, e002231 (2021). [PubMed: 33827905]

216. Spitzer MH & Nolan GP Mass cytometry: single cells, many features. Cell 165, 780–791 (2016). [PubMed: 27153492]

217. Svensson V, Vento-Tormo R & Teichmann SA Exponential scaling of single-cell RNA-seq in the past decade. Nat. Protoc 13, 599–604 (2018). [PubMed: 29494575]

218. Stoeckius M et al. Simultaneous epitope and transcriptome measurement in single cells. Nat. Methods 14, 865–868 (2017). [PubMed: 28759029]

219. Stuart T& Satija R Integrative single-cell analysis. Nat.Rev.Genet 20,257–272(2019). [PubMed: 30696980]

220. Bentzen AK & Hadrup SR Evolution of MHC-based technologies used for detection of antigen-responsive T cells. Cancer Immunol. Immunother 66, 657–666 (2017). [PubMed: 28314956]

221. Roudko V, Greenbaum B & Bhardwaj N Computational prediction and validation of tumor-associated neoantigens. Front. Immunol. 11, 27 (2020). [PubMed: 32117226]

222. Salmaninejad A et al. Cancer/testis antigens: expression, regulation, tumor invasion, and use in immunotherapy of cancers. Immunol. Invest 45, 619–640 (2016). [PubMed: 27603913]

223. Kawakami Y et al. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc. Natl Acad. Sci. USA 91, 6458–6462 (1994). [PubMed: 8022805]

224. Fisk B,Blevins TL,Wharton JT & Ioannides CG Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J. Exp. Med 181, 2109–2117 (1995). [PubMed: 7539040]

225. Parkhurst MR et al. Characterization of genetically modified T-cell receptors that recognize the CEA:691–699 peptide in the context of HLA-A2.1 on human colorectal cancer cells. Clin. Cancer Res 15, 169–180 (2009). [PubMed: 19118044]

226. Attermann AS, Bjerregaard A-M, Saini SK, Grønbæk K & Hadrup SR Human endogenous retroviruses and their implication for immunotherapeutics of cancer. Ann. Oncol 29, 2183–2191 (2018). [PubMed: 30239576]

227. Smith CC et al. Endogenous retroviral signatures predict immunotherapy response in clear cell renal cell carcinoma. J. Clin. Invest 128, 4804–4820 (2018). [PubMed: 30137025]

228. Gattoni-Celli S & Cole DJ Melanoma-associated tumor antigens and their clinical relevance to immunotherapy. Semin. Oncol 23, 754–758 (1996). [PubMed: 8970598]

229. Davis MMNot-so-negative selection. Immunity 43, 833–835(2015). [PubMed: 26588773]

230. Lang F, Schrörs B, Löwer M, Türeci Ö & Sahin U Identification of neoantigens for individualized therapeutic cancer vaccines. Nat. Rev. Drug Discov 21, 261–282 (2022). [PubMed: 35105974]

231. Schumacher TN,Scheper W&Kvistborg P Cancer neoantigens. Annu.Rev.Immunol 37, 173–200 (2019). [PubMed: 30550719]

232. Draper LM et al. Targeting of HPV-16+ epithelial cancer cells by TCR gene engineered T cells directed against E6. Clin. Cancer Res 21, 4431–4439 (2015). [PubMed: 26429982]

233. Iyer JG et al. Merkel cell polyomavirus-specific CD8+ and CD4+ T-cell responses identified in Merkel cell carcinomas and blood. Clin. Cancer Res 17, 6671–6680 (2011). [PubMed: 21908576]

234. Leko V & Rosenberg SA Identifying and targeting human tumor antigens for T cell-based immunotherapy of solid tumors. Cancer Cell 38, 454–472 (2020). [PubMed: 32822573]

235. Smith CC et al. Alternative tumour-specific antigens. Nat. Rev. Cancer 19, 465–478 (2019). [PubMed: 31278396]

Box 1

Human immunology toolkit for T cells

Several tools currently allow for a refined characterization of the intratumoural T cell compartment. First, enumeration of $CD8^+$ and $CD4^+$ T cells through immunohistochemistry can easily provide an accurate quantification of the intratumoural T cell compartment, and thereby classify tumours as immunologically 'hot' or 'cold' in diverse clinical settings[29–31]. By detecting the expression of a limited panel of surface markers, multiplexed immunohistochemistry and immunofluorescence can offer the possibility of capturing the states of selected immune populations while preserving quantitative and topographical information.

The possibility of dissociating tumour tissue into single-cell suspensions has enabled the collection of data from large fractions of cells, and this has been instrumental in the identification of rare subpopulations. Indeed, multiparametric flow cytometry using fluorochrome-conjugated antibodies has enabled the simultaneous examination of multiple parameters and classification of T cells based on the expression of a relatively small number of markers (<30). This capability can be utilized for the in vitro isolation of T cell subsets to measure their functional capabilities (such as proliferation and production of cytokines in response to stimulation). The use of purified metal isotopes as labels for >40 specific antibodies to stain individual cells and to detect labelled and ionized cells by the mass spectrometry-based tool cytometry by time of flight has further increased our ability to characterize the surface proteome of T cells[216]. The recent coupling of cell-isolating microfluidics with genome-wide deep sequencing has provided the ability to undertake unbiased investigations of thousands of T cells at single-cell resolution[217]. Single-cell RNA sequencing (scRNA-seq) has enabled the profiling of numerous analytes in single T cells, revealing a previously underestimated heterogeneity of transcriptional states[35]. The use of oligonucleotide barcoded antibodies in conjunction with protocols of scRNA-seq has allowed the comparison of signals derived from T cell transcriptomes and proteomes through cellular indexing of transcriptomes and epitopes by sequencing[218].

The interpretation of single-cell transcriptomes using computational pipelines has commonly relied on inference of the phenotypic relationships between T cell subsets, and to investigate preferential differentiation trajectories[35,219]. While such tools have been instrumental to the characterization of cell states of tumour-infiltrating lymphocytes (TILs), these are not fully sufficient to determine the specific killing capabilities of T cells, which are dependent on the sequence of their T cell receptor (TCR). Therefore, a more detailed description of the functional properties of TILs requires tools for the direct detection of TCR sequences and specificities. First, several protocols of TCR sequencing have been developed to amplify and detect the rearrangements of TCR α-chain and β-chain genes in T cells, both at the resolution of bulk populations or of single cells[23,24]. Given the high diversity of their sequences, TCRs have been used as clonal markers, thus providing the possibility for investigating the expansion, diversity and dynamics of antigen-specific T cell clones within the tumour microenvironment. Second, tools for antigen- specificity screening have now emerged that provide information regarding the

epitopes that can bind TCRs, thus stimulating the cytotoxic activity of T cells. Soluble recombinant peptide–major histocompatibility complex (MHC) multimeric complexes can faithfully reproduce TCR ligands and have been conjugated with different probes (fluorochromes, isotypes or DNA oligonucleotides)[220] and allow for the detection of antigen-specific T cells when coupled with other technologies (flow cytometry, cytometry by time of flight or scRNA-seq). The availability of computational pipelines for antigen immunogenicity prediction starting from tumour sequencing data and the ability to synthesize arrays of peptides now make the identification of candidate tumour antigens via screening assays feasible[221]. The reactivity of T cells in response to cognate antigens can be further assessed in vitro by measuring their level of activation (detection of CD137 by flow cytometry), degranulation (detection of CD107a (also known as LAMP1) and CD107b by flow cytometry) and cytokine release (detection of interferon-γ (IFNγ), tumour necrosis factor (TNF) or IL-2 by intracellular flow cytometry or enzymatic immune assays) upon stimulation with antigen-presenting cells pulsed with tumour antigens. Finally, knowledge of the TCR α-chain and β-chain pairs harboured by TILs has allowed the design of systems to clone and express candidate TCRs in T cell lines: in these controlled systems, the activity of the TCRs can be measured in response to a wide variety of stimulants, including tumour cells or antigen-presenting cells pulsed with peptides or modified through transduction or transfection to express candidate antigens.

All these tools can now be combined to simultaneously define the phenotype, specificity and spatial distribution of individual T cells within the tumour microenvironment.

Box 2

Classes of tumour antigens

Several classes of tumour antigens have been identified and described. The first class comprises those antigens encoded by unmutated genes that are shared between the genomes of cancer and normal cells. They are classified as tumour-associated antigens (TAAs), since their pattern of expression is aberrant in tumour cells. TAAs include the following:

- Cancer germ line antigens,which are expressed during fetal development and silenced in most adult normal tissues. They can be re-expressed in a variety of tumours due to dysregulated DNA methylation (for example, melanoma-associated antigen (MAGE) and NY-ESO-1)[222].
  - Tissue differentiation antigens, which are highly expressed in tumour cells but also in their normal tissue of origin (for example, melanoma antigen recognized by T cells 1 (MART1) and tyrosinase in melanoma)[223].
  - Overexpressed tumour antigens, which are expressed at low–intermediate levels in healthy tissue but are highly translated in certain cancer types (for example, human epidermal growth factor receptor 2 (HER2) in breast cancer and carcinoembryonic antigen (CEA) in epithelial cancer)[224,225].
  - Human endogenous retroviruses, which are proteins translated from remnants of ancient retroviral sequences located throughout the human genome[226]. As a result of DNA demethylation, they can be highly expressed in certain tumour types (such as renal cell carcinoma), resulting in the production of potentially immunogenic epitopes[33,227].

T cells specific for TAAs have been detected in several cancer types and have been shown to be enriched in melanoma tumours, where tissue differentiation antigens in particular are highly expressed[228]. Owing to expression of TAAs in normal cells and within the thymus, TAA-specific T cells are subjected to central tolerance, which negatively selects most of the high- avidity TAA-specific T cell receptors (TCRs), thus limiting their potential autoreactivity. As a consequence, TAA-specific T cells display moderate or low TCR avidities[58]. However, rare TAA- specific T cells with high avidity can escape thymic tolerance[229]. As a result of their non-specific tumour expression, TAAs can be shared among different cancer types, making them appealing targets for ‘off-the-shelf’ immunotherapeutic approaches. However, this comes with important limitations: elicitation of high-affinity TAA-specific T cells can induce the destructionof normal cells expressing even minuscule levels of the TAA, resulting in ‘on-target’ toxicity; in addition, responses against cross-reactive antigens can be observed, leading to ‘off-target’ toxicities.

A second class of tumour epitopes arises from those antigens genetically encoded only in tumour cells. Their expression is exclusive to tumour cells, and hence they qualify as tumour-specific antigens (TSAs). As a result, TSAs exhibit novel amino acid sequences that are not subjected to central tolerance (due to their lack of expression within the thymus) and that are rarely shared among different patients with cancer. TSAs comprise:

- Neoantigens, arising from tumour somatic mutations in protein- coding genomic portions[230,231]. These can derive from missense single nucleotide variants or from small DNA insertions and deletions in protein-coding genes. Alternatively, neoantigens can originate from large-scale genomic rearrangements (for example, chromosomal translocations, insertions and deletions) that generate the fusion of coding genes. Most of these mutations have no functional implications in cancer biology and are considered ‘passengers’, whilst a small amount of ‘drivers’ and often recurring mutations play a role in tumour evolution (for example, *TP53*, *NRAS* or *KRAS* mutations). Neoantigens may become immunogenic when the mutant amino acids confer to such epitopes an increased ability to bind to human leukocyte antigen (HLA) molecules.
- Viral oncoproteins, resulting from integration of viral genes into cancer cells. Indeed, in both solid and haematological cancers, cell transformation can be driven by infections with viruses (for example, human papillomavirus, Merkel cell polyomavirus and Epstein–Barr virus), which can result in the production of viral proteins by cancer cells[232,233].

Since TSAs are not subjected to thymic tolerance, they can be seen as ‘foreign’ antigens by the immune system, thus potentially stimulating high-affinity T cell responses. Indeed, T cells specific for TSAs have been detected across many tumour types. For these reasons, TSAs represent the ideal target antigens for immunotherapy, since they can stimulate potent antitumour responses in the absence of toxicity. The development of novel sequencing-based approaches has greatly facilitated the detection of neoantigens and the systematic evaluation of their immunogenicity; based on such efforts, many computational algorithms for the prediction of antigenic immunogenicity have been developed (reviewed in ref. 221). Still, neoantigens that are able to elicit antitumour T cell responses are very rare (~0.5–2% of predicted immunogenic neoantigens)[234]. Thus, gaining a better understating of the rules underlying their immunogenicity is expected to be instrumental to the generation of TSA-specific responses with immunotherapeutic approaches.

Finally, additional tumour antigens can arise from either protein-coding or non-coding regions undergoing aberrant transcription and splicing, aberrant translation, or post-translational modifications. Such tumour antigens, which are termed ‘unconventional’ (reviewed in ref. 235), possess a diverse range of tumour-specific expression. Due to the lack of tools for their systematic detection and prediction, this class of antigens remain largely unexplored in the analysis of antitumour T cell responses, and therefore novel technologies will be required to investigate their role in eliciting antitumour responses.

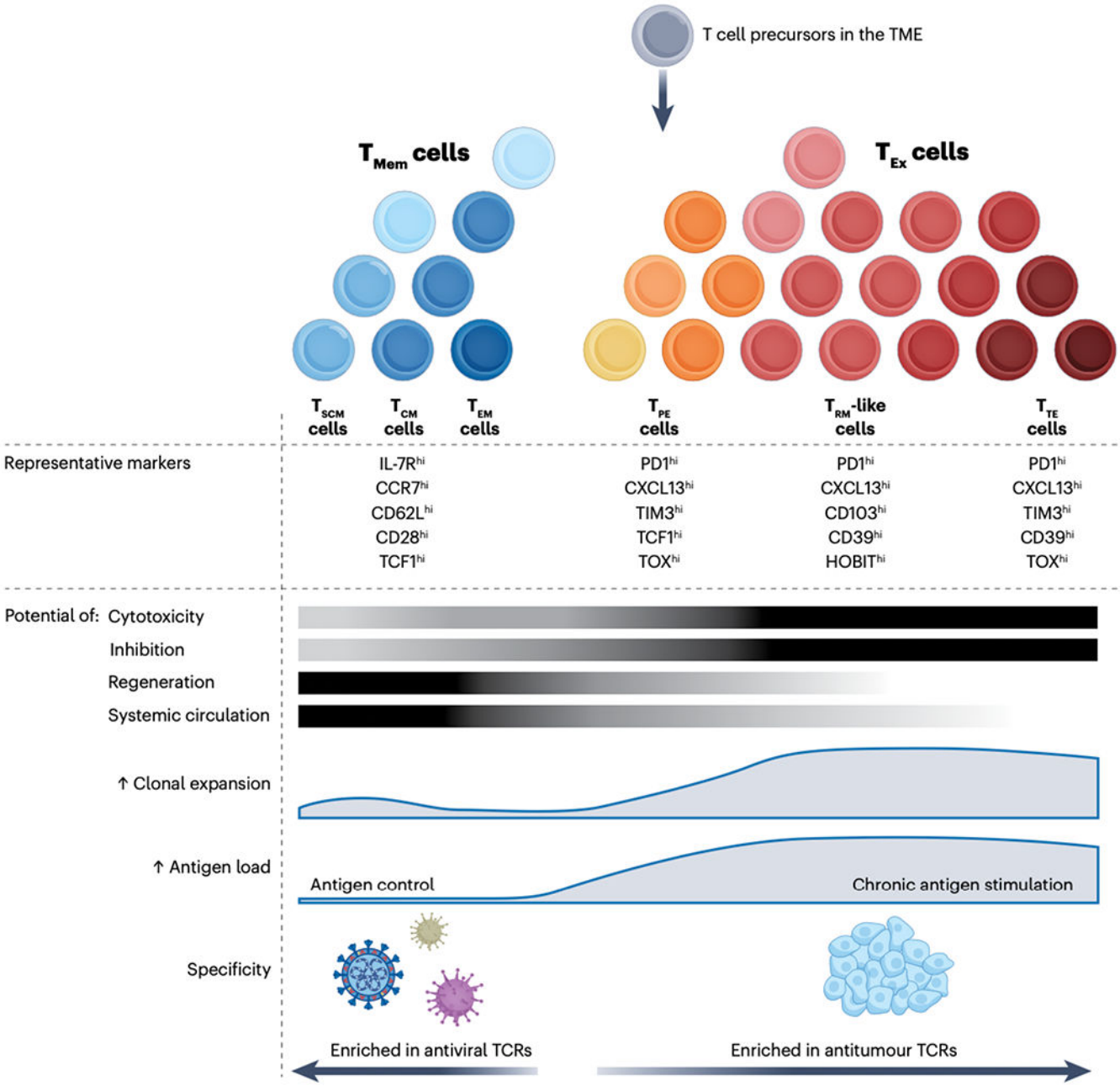

Fig. 1 |. Phenotypes of CD8$^+$ T cells within the native tumour microenvironment.

Upon activation and recognition of cognate antigens (denoted by the arrow at the top), naive T cells differentiate into antigen-experienced T cells that can be captured by single-cell RNA sequencing (scRNA-seq) as a spectrum of cellular states. As a synthesis of the current literature, we propose a streamlined division of the tumour-infiltrating lymphocyte (TIL) phenotypes into two major compartments: memory T cells (T_{Mem} cells; left, blue) and exhausted T cells (T_{Ex} cells; right, red, orange and yellow). Of note, such representation does not stringently reflect differentiation pathways and T cell hierarchies, which cannot be fully addressed in human tumour studies due to the lack of longitudinal tracing of T cell clones. T_{Mem} cells encompass subsets traditionally subdivided in peripheral blood as

stem cell memory T (T_{SCM}), central memory T (T_{CM}) or effector memory T (T_{EM}) cells that maintain signatures of lowly differentiated cells and retain the ability to regenerate a vast progeny of effector cells. In the native tumour microenvironment (TME), T_{Mem} cells do not exhibit tumour-specific localization, and this compartment has reduced overall clonal expansion within the TME. Screening of the T cell receptor (TCR) specificity of T_{Mem} cells has revealed them to be the preferential reservoir of T cells with antiviral reactivity and, therefore, consistent with bystander TILs, persisting in conditions where the cognate antigen levels are low and controlled. In contrast, T_{Ex} cells are highly enriched within the TME, where they demonstrate broad expression of signatures of activation and exhaustion. Their high expression of immune-checkpoint molecules renders them highly sensitive to cancer-induced inhibition. They have been found to consistently express common markers (for example, programmed cell death protein 1 (PD1) and CXC-chemokine ligand 13 (CXCL13)) across studies but display a phenotypic diversity, which translates into a range of capacities for cytotoxicity and extent of dysfunction. Progenitor exhausted T cells (T_{PE} cells) are early dysfunctional T cells that retain the capacity to regenerate a proportion of the T_{Ex} cell compartment but lack effector functions. In contrast, terminally exhausted T cells (T_{TE} cells), represent the last stage of differentiation: their cytotoxic capabilities are restrained by inhibitory signals from within the TME due to their high level of expression of immune-checkpoint molecules. Finally, a proportion of T_{Ex} cells exhibits expression of signatures related to the acquisition of a tissue-resident memory programme (T_{RM} cells), which confers concomitant expression of inhibitory molecules and high cytotoxic potential. We define this compartment as T_{RM}-like T_{Ex} cells because of their phenotypic connections with the T_{Ex} cell compartment, but we acknowledge that T_{RM} cells with a similar profile can differentiate in normal tissues upon acute infections to provide a first line of defence against pathogen recurrence. Tumour-specific T_{RM}-like T_{Ex} cells have been detected within the TME, thus demonstrating that such a phenotype can also be elicited upon chronic stimulation provided by tumour antigens. Across tumours, the T_{Ex} cell compartment is highly clonotypically expanded, consistent with likely recognition of tumour cells. Phenotypic analyses of TCR clonotype families have confirmed the clonal relatedness of diverse T_{Ex} cell subsets, possibly constituting different stages of differentiation driven by the recognition of tumour antigens. T_{Ex} and T_{Mem} TCR clonotypes have been found to only marginally overlap, indicating a distinct division of the specificities of the two compartments. Indeed, several studies of TIL specificity across different tumour types have now unambiguously demonstrated that the T_{Ex} cell compartment is almost exclusively enriched in antitumour TCRs. CCR7, CC-chemokine receptor 7; HOBIT, homologue of BLIMP1 in T cell; IL-7R, IL-7 receptor; TCF1, T cell-specific transcription factor 1; TIM3, T cell immunoglobulin mucin receptor 3; TOX, thymocyte selection-associated high mobility group box protein.

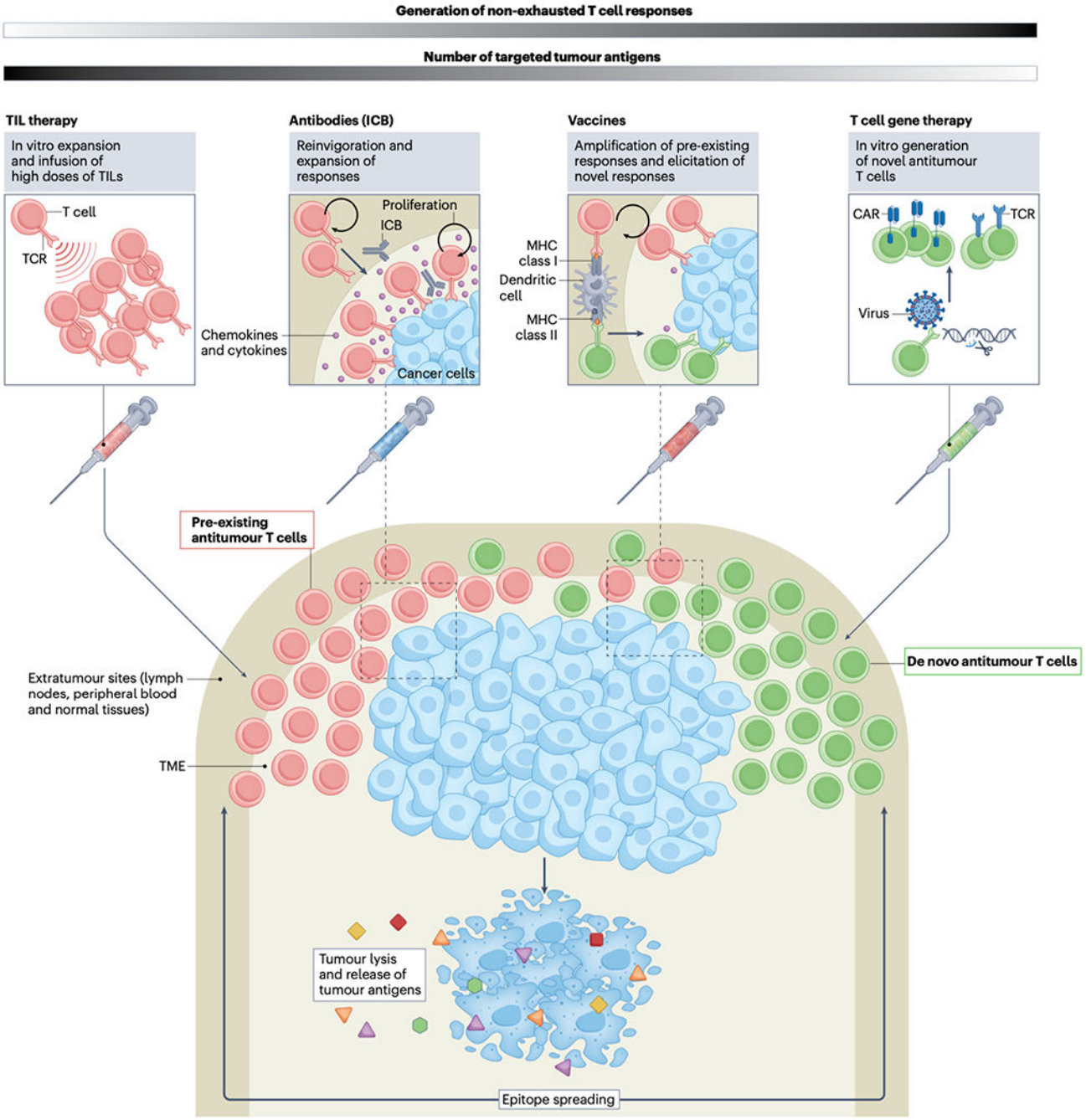

Fig. 2 |. Effect of immunotherapies on the T cell repertoire of patients.

Immunotherapies can rely on antitumour T cells already present within the native tumour microenvironment (TME) or in extratumoural sites (lymph nodes, peripheral blood and normal tissues). Such pre-existing T cell responses (red T cells) can be amplified (indicated by a circular arrow) in extratumoural sites or reinvigorated within the TME (as denoted by the release of cytokines and chemokines) in vivo, through the administration of cancer vaccines or immune-checkpoint blockade (ICB) therapies. Amplification of responses and release of cytokines within the tumour can favour the recruitment of T cells from

extratumoural sites. Pre-existing responses can be expanded ex vivo for the generation of T cell products for adoptive tumour-infiltrating lymphocyte (TIL) therapy. By targeting pre-existing responses, which are usually sustained by exhausted T cells specific for a wide array of tumour antigens, such immunotherapies are expected to stimulate polyclonal but dysfunctional T cells. Conversely, the elicitation of non-exhausted antitumour responses requires the de novo generation of antitumour T cells (green T cells). Immunotherapies can elicit de novo T cell responses directly in vivo, through the administration of cancer vaccines that can induce antigen-presenting cell-mediated priming of antigen-unexperienced T cells; alternatively, the specificity of functional T cells may be rewired in vitro by inserting natural T cell receptors (TCRs) or chimeric antigen receptors (CARs) using gene-manipulation technologies. Immunotherapies that induce de novo responses allow the steering of T cell responses towards the recognition of the targeted tumour antigens, optimally under conditions that minimize the induction of exhaustion. Finally, effective antitumour T cells induced by immunotherapies can lyse tumour cells, thus fostering the release of additional tumour antigens, which can be presented by antigen-presenting cells. This phenomenon, known as epitope spreading, can further contribute to the amplification and reinvigoration of pre-existing responses as well as to the induction of new T cell specificities. MHC, major histocompatibility complex.

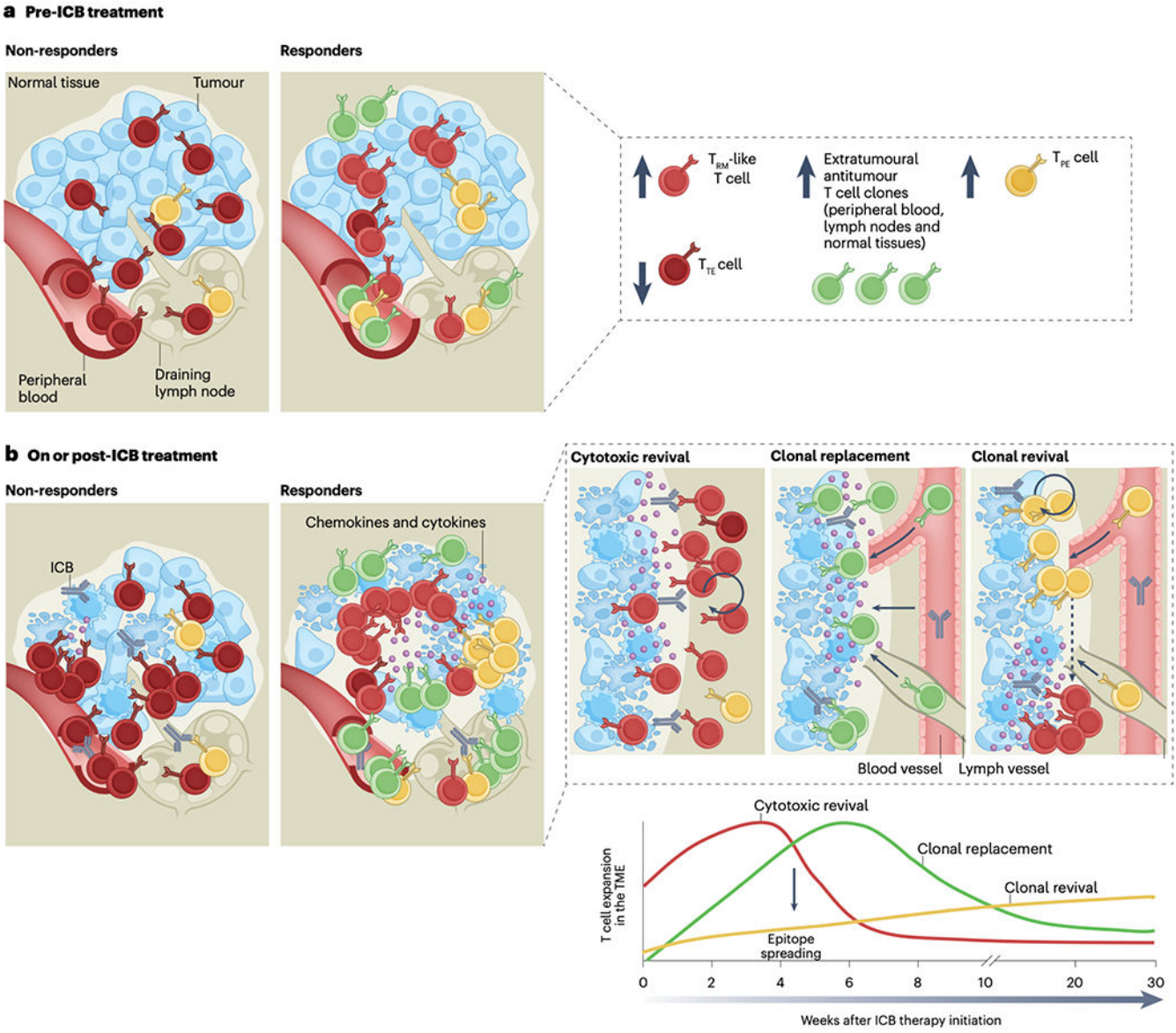

Fig. 3 |. Mechanisms of response to ICB and the T cell subsets involved.

a, Schema of the characteristics of the native T cell compartment associated with subsequent response (right) or non-response (left) to treatment with immune-checkpoint blockade (ICB). Complete or partial responses to ICB have been associated with a diverse T cell composition within the native tumour microenvironment (TME) as well as with a broader T cell repertoire in extratumoural sites (peripheral blood, normal tissues and draining lymph nodes). Depending on the tumour type, time of analysis and clinical setting, responders can be characterized by high frequencies of intratumoural tissue-resident memory T (T_{RM}) cell-like exhausted T cells with high cytotoxic capacity[38,69,121,215], increased fractions of progenitor exhausted T (T_{PE}) cells, which possess high regenerative capacity[9,12,63], or increased expansion and infiltration of putative tumour-reactive T cells in extratumoural sites[59]. These features are thought to underlie the mechanistic basis of responses to ICB.

In contrast, lack of such T cell populations and, consequently, high frequencies of terminally exhausted T (T_{TE}) cells that cannot be substantially reinvigorated predispose patients to ineffective immunotherapy responses. **b**, Mechanisms of action and dynamics of T cells upon response to ICB in patients. First, by disrupting the inhibitory axis, ICB antibodies can unleash the cytotoxic activity of antitumour T_{RM} cell-like exhausted T cells, which can further expand within the TME (denoted by the circular arrow). This mechanism, so-called 'cytotoxic revival', has been observed early after neoadjuvant ICB treatment (2–4 weeks) in head and neck squamous cell carcinoma and breast cancers[101,120,121]. Second, ICB can have a systemic effect, favouring the intratumoural infiltration of novel (or previously undetected) T cell specificities from extratumoural sites. Newly recruited T cell clones might mount an antitumour response in the presence of ICB antibodies, which could likely prevent the inhibition of cytotoxicity. Such 'clonal replacement' was first described as present at a median time of 9 weeks after treatment in patients with basal and squamous cell carcinoma[87]. These systemic dynamics of the T cell repertoire have been observed as predictive of response early after treatment (2–4 weeks) in several clinical settings and cancer types[125–128]. Finally, response could be sustained by progenitor or early dysfunctional tumour-specific T cells (T_{PE} cells) expanding and differentiating within the TME or recruited from lymph nodes and peripheral tissues. This has been observed in responding lung cancer and melanoma lesions, which were characterized by an accumulation of T_{PE} cells late after treatment (>2 months)[12,79]. This phenomenon has been termed 'clonal revival', since it is characterized by the accumulation of less dysfunctional cells that can potentially regenerate a progeny of effector cells (dashed arrow) capable of tumour cytotoxicity in the presence of the ICB-mediated disruption of inhibitory signals. We propose that such mechanisms might have different kinetics (as presented in the graph modelling the temporal dynamics of T cell responses) and might synergistically contribute to the response to ICB based on the qualitative and quantitative status of the antitumour T cell repertoire (part **a**).

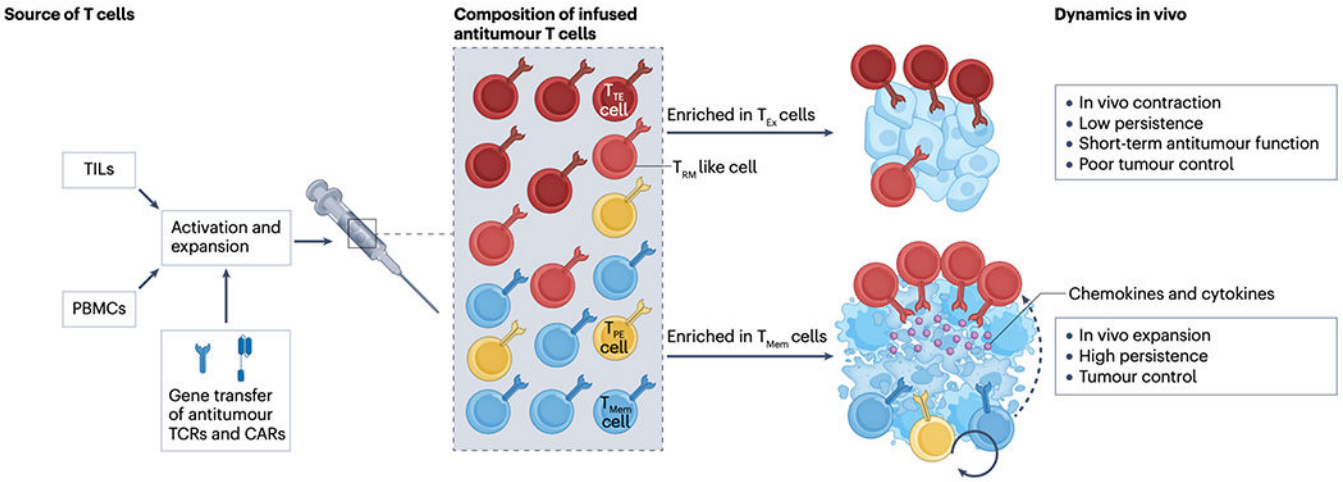

Fig. 4 |. The phenotypic composition of transferred T cells affects the outcome of adoptive T cell therapy.

The quality of infused T cells with antitumour potential is a major factor contributing to their in vivo persistence and functionality upon transfer to patients with cancer[162,181,182,184,185]. Antitumour T cells can be expanded from tumour-infiltrating lymphocytes (TILs) or peripheral blood mononuclear cells (PBMCs), and they can be further manipulated through the introduction of natural T cell receptors (TCRs) or chimeric antigen receptors (CARs) to redirect their specificity towards tumour antigens (left). Ex vivo activation of such T cells results in expansion of T cells with a diverse spectrum of phenotypes (middle), which can affect the dynamics and functionality of T cells once they are transferred into patients with cancer (right). Infusion products enriched in exhausted T (T_{Ex}) cell phenotypes with low regenerative potential (terminally exhausted T (T_{TE}) cells and tissue-resident T (T_{RM}) cell-like T_{Ex} cells, top right) can provide only short-term antitumour function and rapidly decline in vivo, thus providing a limited control of cancer cells. Indeed, infused T_{Ex} cell clones have been shown to contract early following infusion (2–4 weeks) in patients treated with adoptive T cell therapy (ACT) approaches, and the resulting low persistence of the antitumour T cells has been associated with disease progression or recurrence. Conversely, T cell products enriched with memory T (T_{Mem}) cells have been associated with high levels of persistence of transferred antitumour T cells and, in turn, with response to ACT (bottom right). The better fitness of infused T cells derives from the presence of cells with regenerative potential (T_{Mem} cells) or, to a limited extent, of progenitor exhausted T (T_{PE}) cells, which can expand in vivo, persist long term and differentiate to generate a large number of effectors that provides successful tumour control (dashed arrow). Therefore, ACT can benefit from approaches that favour the ex vivo generation and expansion of non-exhausted antitumour T cells.

Table 1 |

Novel tools for the identification of ligands of antitumour T cell receptors

Screening tool	Method	Number of screened TCRs	Number of peptide–HLA complexes screened	Refs.
Functional activation of TCR in T cells	T cells are transduced with a single TCR and their activation (upregulation of CD137, CD107a and CD107b, IFNγ, TNF and IL-2) is detected upon stimulation with antigen-presenting cells pulsed with candidate antigens	Individual TCR clones	~10–100	28,58
Dual fluorochrome-encoded peptide–HLA multimers	T cells are labelled with different combinations of fluorochrome-tagged peptide– HLA multimers detected by flow cytometry	Polyclonal T cell populations (TILs or PBMCs)	~10–60	94,198
Heavy metal ion-tagged peptide–MHC multimers	T cells are labelled with peptide–HLA multimers tagged with different heavy metal ions detected by CyTOF	Polyclonal T cell populations (TILs or PBMCs)	~100	199
DNA-barcoded peptide– MHC multimers	T cells are labelled with different peptide–HLA multimers tagged with DNA oligonucleotides; bulk or single-cell sequencing of DNA tags allows deconvolution of antigen specificity	Polyclonal T cell populations (TILs or PBMCs)	~1,000 (potentially higher)	200,201
Magnetic nanoparticle–peptide–HLA complexes barcoded with nucleic acids	T cells are labelled with peptide–HLA complexes that are linked to magnetic nanoparticles through DNA barcodes; magnetic separation and sequencing of barcodes allow deconvolution of antigen specificity; coupled with microfluidics, it enables the isolation of single TCR-expressing cells	Polyclonal T cell populations (TILs or PBMCs)	~1,000 (potentially higher)	202,203
Baculovirus display libraries	Sf9 insect cells are infected with a baculovirus encoding large libraries of peptide– HLA complexes, resulting in surface expression of peptide–HLA libraries; a single fluorescently labelled soluble TCR is used to stain peptide–HLA library-expressing Sf9 cells that can be enriched for TCR-binding targets upon serial rounds of TCR staining, flow cytometry sorting and expansion of positive cells; sequencing of enriched cells reveals TCR-binding peptides	Individual TCR clones	~10^5 peptides with a single HLA	204,205
Yeast display libraries	Yeast are modified to express single-chain peptide–HLA complexes with degenerate epitope libraries; these yeasts are then stained with soluble TCRs that can be labelled with fluorochromes or magnetic beads; serial rounds of selection of TCR-binding yeasts and amplification of the positive fractions enables the isolation of yeast expressing TCR-binding peptide–HLA complexes, that can be identified through sequencing	Individual TCR clones	~10^8 peptides with a single HLA	206–208
RAPTR	Gene engineering of a VSVG fusion protein allows expression of peptide–HLA complexes on viral particles; productive interactions between peptide–HLA- expressing viruses and TCRs can trigger viral infection, which genetically labels TCR-expressing T cells; single-cell sequencing of viral cassettes in T cells reveals the nature of the recognized peptide–HLA complexes	Polyclonal T cell populations (TILs or PBMCs)	~100	209
Bifunctional receptors	Libraries of extracellular single-chain peptide–HLA complexes are fused to an intracellular CD28–CD3ζ signalling domain or with a truncated membrane– intracellular TCR domain; the resulting bifunctional receptors are expressed in cell lines (typically Jurkat cells) with NFAT-GFP reporter genes; upon interaction with TCR-expressing effectors, bifunctional receptors are activated, leading to signal transduction and GFP expression, which allows the isolation of TCR-binding cells by flow cytometry; sequencing of gene cassettes encoding for bifunctional receptors on sorted populations reveals antigen specificity	Individual TCR clones	~10^4 peptides with a single HLA	210,211
Trogocytosis	HLA-negative cell lines (K562) are gene-modified to express libraries of single- chain peptide–HLA complexes; incubation of such cells with the TCR-expressing human T cell line (Jurkat) triggers the cell–cell transfer of specific TCR molecules from effector to target cells (trogocytosis); as a result, target K562 cells	Individual TCR clones	~104 peptides with a single HLA	212

Screening tool	Method	Number of screened TCRs	Number of peptide–HLA complexes screened	Refs.
	expressing cognate peptide–HLA complexes can be sorted based on TCR positivity and can be sequenced to identify the presented epitope			
Granzyme B reporter genes (TScan)	Target cell lines are engineered to express one or more HLAs, a library of epitope- encoding minigenes and a fluorescence protein that becomes activated by granzyme B cleavage; incubation with TCR-expressing T cells leads to activation of the reporter genes in target cells expressing specific peptide–HLA complexes and acquisition of a specific pattern of fluorescence; FACS of the fluorescent targets and sequencing of the minigene cassettes reveal the nature of the recognized epitope	Individual TCR clones or polyclonal T cells (TILs or PBMCs)	~10^4–10^5 peptides on multiple HLAs	213,214

CyTOF, cytometry by time of flight; FACS, fluorescence-activated cell sorting; HLA, human leukocyte antigen; IFNγ, interferon γ; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; RAPTR, receptor–antigen pairing by targeted retroviruses; TCR, T cell receptor; TIL, tumour-infiltrating lymphocyte; TNF, tumour necrosis factor; VSVG, vesicular stomatitis virus G.