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Phenotypic and spatial heterogeneity of CD8$^+$ tumour infiltrating lymphocytes

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Abstract

CD8$^+$ T cells are the workhorses executing adaptive anti-tumour response, and targets of various cancer immunotherapies. Latest advances have unearthed the sheer heterogeneity of CD8$^+$ tumour infiltrating lymphocytes, and made it increasingly clear that the bulk of the endogenous and therapeutically induced tumour-suppressive momentum hinges on a particular selection of CD8$^+$ T cells with advantageous attributes, namely the memory and stem-like exhausted subsets. A scrutiny of the contemporary perception of CD8$^+$ T cells in cancer and the subgroups of interest along with the factors arbitrating their infiltration contextures, presented herein, may serve as the groundwork for future endeavours to probe further into the regulatory networks underlying their differentiation and migration, and optimise T cell-based immunotherapies accordingly.

Keywords CD8$^+$ T cells, Tumour-infiltrating lymphocytes, T cell nomenclature, T cell exclusion, Caner immunotypes, Cancer immunotherapy.

Introduction

Tumour infiltrating lymphocytes (TILs) are the frontliner immune cells that populate the tumour microenvironment (TME), the very interface of cancer-immunity confrontation, in solid tumours. CD8$^+$ T lymphocytes are considered one of if not the most important constituents of TILs with quasi-universal prognostic benefits owing to their capability to unleash direct cytotoxicity upon encounter with tumour cells expressing the cognate antigen [1, 2]. In reality, however, the presence or even abundance of CD8$^+$ TILs per se does not consistently translate into tumour control or regression, favourable survival outcomes, or response to T-cell-based immunotherapy to the same effect size [3], suggesting failure to divulge relevant modifying factors through undifferentiated evaluation of all CD8$^+$ T cells as a congruent whole. Recent advances in high-throughput, high-definition cellular characterisation modalities such as single-cell RNA sequencing (scRNA-seq), T cell receptor

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sequencing (TCR-seq), and Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) have uncovered an unprecedented level of $CD8^+$ TIL heterogeneity [4], while spatial transcriptomic (ST) technologies have enabled faithful reconstructions of the positional details inevitably lost during the tissue dissociation process required for scRNA-seq, permitting in-depth analysis of the contact-mediated and paracrine actions between cell populations that are intrinsically location-dependent [5]. Accumulating data on the immensely complex multi-omic landscape of $CD8^+$ TILs suggest that only subpopulations with certain functional and differentiation statuses are empowered by the pre-requisite molecular machinery and stand at the immunological vantage points to act as the principal mediators of anti-tumour immunity and response to immunotherapy [6].

The blowout of high-dimensional data has nonetheless stirred up a taxonomical kerfuffle as it inundates the conventional T-cell classification systems. Before advancing the discussion, it is therefore crucial to reiterate the inherent limitations of the current T cell nomenclature and the caveats when making inferences based on it. Any cell clustering or annotation attempts are more or less arbitrary in nature and at risk of misinterpreting or under-representing the true biological significance of cells. Functional overlaps between pre-defined cell populations frequently exist, and misclassification is common. Besides, the transition from one distinguishable cell phenotype to another routinely involves a gradual, uninterrupted spectrum, but overly refined discretisation to meticulously mirror the continuum may produce unjustified sophistication and compromise generalisability, thus calling for an optimal resolution to meaningfully group cells.

A reference cell tree brilliantly named 'consensus ontology' has been envisaged, where the collection of all given cells in both physiological and pathological states over the lifespan of an organism can be moulded into a three-dimensional tree-like architecture with lineage, differentiation, and elapsed time being the localising axial parameters, decorated with the key transcriptional programmes, molecular features, signalling pathways, and, where relevant, spatial information of the cells [7]. Such an epochal idea has provided much inspiration for this review, where we aim to present a lineage-orientated, function-centred panorama of the $CD8^+$ TILs based on prevailing immunological knowledge, zoom in on the subpopulations of interest with regard to anti-cancer immunity and therapeutic benefits, and complement the discussion with pivotal cell-extrinsic and spatial determinants of their successful tumour infiltration.

CD8+ T cell evolution in cancer

Early T cell activation

Naïve $CD8^+$ T cells (T_N) are the positively and negatively selected, heterodimeric αβTCR-bearing, and lineage-committed single positive ($CD4^-CD8^+$) T cells that have not encountered the cognate antigen. Cytometric definition of T_N entails exclusive expression of the CD45RA isoform of leukocyte common antigen ($CD45RA^+CD45RO^-$) along with auxiliary naïve T cell markers, many of which are homing receptors such as C-C motif chemokine receptor 7 (CCR7) and CD62L (also known as L-selectin) that allows homing to lymphoid organs and tissues via blood circulation and then manoeuvring through the white pulp of the spleen or the T cell zone of lymph nodes to survey antigens. They are the last common precursors that beget all $CD8^+$ T cell subtypes found in the secondary lymphoid organs (SLOs) and tumours [1, 8]. There exist numerous obstacles that act as physiological deterrents to autoimmunity and need to be surmounted before T_N can be activated to effectors (T_{EFF}) with the full tumoricidal potential. In nascent tumorigenesis, immunologic ignorance can occur when pathological expression of tumour-associated self-antigens is below threshold, not displaying a recognisable differential pattern, or restricted to the immune-privileged sites; or when transformed cells expressing tumour-specific neoantigens are shielded by surrounding tissues from detection and translocation to tumour-draining lymph nodes (TDLNs) for T cell priming. On the other hand, antigen recognition in the absence of a co-stimulus or signal transduction and augmentation by dedicated antigen-presenting cells (APCs) in an appropriately conditioned inflammatory milieu results in a hyporesponsive 'anergic' state of $CD8^+$ T cells with limited proliferative and effector capacities [9]. Meanwhile, over-exposure to self-antigens can induce a 'tolerised' state that is deficient in cytotoxicity and prone to apoptosis, a phenomenon known as 'peripheral deletion' [10, 11].

In the meantime, the effector-memory equilibrium is known to be under tight regulation of orchestrated, potentially antigen-independent, and frequently counterbalancing determinants in relation to cytokines (e.g., effector-predisposing interleukin-12 [IL-12], and IFN-γ versus memory-favouring IL-7, IL-15, IL-21, and transforming growth factor [TGF]-β), TCR signal strength and antigen affinity, antagonising transcription factor duos (e.g., pro-effector T-bet versus pro-memory Eomesodermin, BLIMP-1 versus BCL-6, ID2 versus ID3, and STAT4 versus STAT3), epigenetic remodelling, and metabolic machineries [12]. Resolution of an acute infection customarily leads to a clear-cut inclination towards memory differentiation, survival and self-renewal under the composite force of the regulatory network [13]. In contrast, with an unremitting threat such as a developing tumour,

differing chronology of antigen exposure and stimulation precludes fate unambiguity and accentuates the relevance of the variant, intermediate, and hybrid states of memory, effector, and exhausted $CD8^+$ T cells. An entr'acte from constant antigen stimulation, most commonly in the refuge of TDLNs [14, 15] and the blood circulation, is of utmost importance in initiating the memory-associated programmes while postponing the path to exhaustion, a fate ultimately manifested in most tumour-reactive $CD8^+$ T cells [4, 10, 14, 16–19].

The transmuted memory lane in cancer

Immunological memory denotes the ability to stage a more expeditious and pronounced response upon re-encounter with an earlier insult. Stemness and migrating capacity are the two pillars that uphold the depth (that is, the stretches of the possible effector progenies) and breadth (the tissue versatility and specificity) of the immune protection memory $CD8^+$ T cells (T_{MEM}) are capable of evoking. A substantial proportion of T_{MEM} continually traverse the blood and/or the lymphatics to maximise the antigen screening range. The circulating T_{MEM} were initially divided into central memory cells (T_{CM}) and effector memory cells (T_{EM}) based on expression of CCR7 and CD62L (thus having access to lymphoid tissue), or the lack thereof, respectively [20]. Subsequent investigation has revealed the extraordinary longevity and tremendous developmental and proliferative potentials of T_{CM} [21–25]. Additionally, stem cell memory T cells (T_{SCM}) have been identified as a body of $CD45RA^+IL\text{-}7R\alpha^+$ cells sharing overlapping cardinal memory and recirculation features with T_{CM} but are even less differentiated and demonstrate enhanced self-renewal, plasticity, and multipotency [26–30], though Galletti et al. have demonstrated that the apparent gene expression profile differences could very well be accounted for by differential inclusion of $PD\text{-}1^+TIGIT^+$ progenitors committed to exhausted-like progenies [31]. T_{EM}, in comparison, are characterised by non-lymphoid tissue (NLT) tropism, more immediate effector functions upon restimulation, but less secondary expansion capacity [25].

Although T_{CM} and T_{SCM} are abundant in the peripheral blood of cancer patients [17] and theoretically constitute a systemic reserve of effector $CD8^+$ TILs, such untainted memory phenotypes are difficult to maintain with the systemic perturbations and local TME of tumour-bearing individuals. Tumour-specific bona fide memory cells have been identified in TDLNs [14], but a plethora of single-cell and TCR-profiling studies on melanoma [18], hepatocellular carcinoma [32], colorectal carcinoma [33], and pan-cancer types [4] have unanimously indicated that infiltrating $CD8^+$ T cells that are tumour-reactive almost exclusively reside in an exhausted state of various degrees, and that the heavily expanded, cancer antigen-recognising intra-tumoural TCR clonotypes are rarely detected in the memory compartment of the peripheral circulation [18]. This could suggest inadequate development of a circulating memory repertoire specifically targeting tumours across different cancer types, and even if spawned, these memory cells may be rapidly depleted after mobilisation. Therefore, the precise size of the circulating tumour-reactive memory compartment and magnitude of contribution from these cells to naturally occurring anti-cancer immunity prior to acquiring exhaustion features are to be further assessed in human cancers.

That being said, the superior proliferative potential of T_{CM} and T_{SCM} can be leveraged in various forms of cancer immunotherapies. T_{SCM} can be generated ex vivo from naïve precursors using clinical-grade culture protocols utilising IL7 and IL15 [27], or a combination of IL-7, IL-21 and the WNT agonist TWS119 [34]. Recently, MEK inhibition has also been shown to be capable of inducing T_{SCM} and functionally superior T_{CM} [30]. Compared to chimeric antigen receptor (CAR)-T cell therapy derived from unselected T cells, adoptive transfer of CAR-T_{SCM} and T_{CM} have engendered expanded, persistent, and intensified anti-tumour responses against haematologic malignancy xenografts [34–36] with reduced risk of cytokine release syndrome [35], and, in combination with PD-1 blockade, achieved tumour control in OVCAR-3-inoculated mice [37]. Likewise, engineered expression of IL-7 along with CCL19 on CAR-T cells boosted memory responses to solid tumours and prolonged survival in mice [38].

Tissue-resident memory

The observation of ample long-lived, T_{EM}-like $CD8^+$ T cells in NLT heralded the revelation of tissue-resident memory T cells (T_{RM}) [39], which have been formally recognised with skin [40] and intestinal [41] transplantation in murine models and subsequently found prevalent in SLOs and both barrier and non-barrier tissues in mice and humans [42, 43]. The non-migratory nature of T_{RM} stems from the expression of integrins CD49a and CD103, the latter of which is the integrin subunit αE (ITGAE) that serves as a canonical T_{RM} marker, and CD69, an early activation marker and down-regulator of relocation-promoting sphingosine 1-phosphate receptor-1 (S1PR1), in addition to a concomitant lack of tissue egress mediators [42, 43]. An array of universal (e.g., CXCR6 and VIM) and site-specific (e.g., CCR4, CCR10, and CXCR4 for skin versus CCR9 for small intestine predilection) cell matrix and adhesion molecules also reliably delineate T_{RM} [44, 45]. Additionally, upregulation of transcription factors (TFs) such as BLIMP-1, HOBIT, RUNX3 and NOTCH is pivotal to T_{RM} formation and has

further instituted T_{RM} as separate entities to the circulating memory T cells in mouse models [46–48]. Suppression of KLF2 is another T_{RM} hallmark that provides an additional binding force for T_{RM} to the surrounding tissue via resultant down-regulation of S1PR1 [49]. Many of these T_{RM}-enriched TFs, including Runx3, Notch, and Hif1a, are known harbingers of effector T cell functions, which underlie the phenotypic preparedness of T_{RM} in repelling invading pathogens and rejecting transformed cells [47, 48, 50]. However, it remains uncertain the extent to which the core transcriptional signature is shared with human T_{RM}. Notwithstanding the discrepancies in Hobit expression pattern [48], recent studies have corroborated perpetuation of some of these T_{RM} TFs in humans, and identified other ones of interest such as AHR, KLF4, and Hic1 [44, 51, 52].

Mounting evidence has brought T_{RM} to the centre of attention in quelling numerous cancers, particularly those of epithelial origins. It has been recognised that T_{RM} cells are central to immunosurveillance, especially in tissue compartments not readily accessible by circulating memory, as well as curtailment and, in an ideal situation, prompt elimination of transformed cells and subclinical tumours, analogous to their presumed contribution in local containment of latent viruses [53–56]. Park et al. have notably shown in their seminal work that tumour-specific T_{RM} unfailingly prevented melanoma development upon rechallenge independent of circulating memory depletion, and upon T_{RM} knockdown, tumour outgrowth manifested in approximately 20% of mice with occult melanomas [54]. This is echoed by T_{RM}-mediated protection against secondary breast tumour rechallenge in the ipsi- but not contralateral mammary fat pad [55]. T_{RM} can also derive from intratumoural T_{MEM}/T_{EM} [57] and reside in the lymph nodes [58], offering protection against metastases at distant sites. Furthermore, tumour-associated clones of T_{RM} generated post-immune checkpoint blockade (ICB) were able to persist for up to 9 years [59], illustrating the long-lasting protection that T_{RM} confer. The consequent selective pressure from T_{RM} immunosurveillance, however, may paradoxically favour the clonal growth of cancer cells that are adept at immune evasive and suppressive manoeuvres, such as the cases in lung cancers where tumour cells escape T_{RM} hammering by down-regulation of MHC-I molecules [60] and engaging epithelial-to-mesenchymal transition [61], which epitomises the phenomenon of immunoediting that signifies the complex dynamic of the cancer-immunity equilibrium [62].

In established solid tumours of many types, enrichment of T_{RM} transcriptomic signatures in tumours is also found invariably correlated with more intense anti-tumour activity with respect to cytolytic molecule expression and pro-inflammatory cytokine release, and better prognoses, irrespective of the overall amount of $CD8^+$ T cell infiltrates [59, 63–71]. As known expressors of immune checkpoint molecules [63, 66, 71, 72], infiltrating T_{RM} represent one of the prime salvageable targets of ICB therapy [55, 67, 71, 73, 74], where reinvigoration and expansion of T_{RM} have been consistently observed following ICB, and pre-treatment T_{RM} signature prognosticated response to ICB-based regimens [71, 73]. However, accurate measurement of the anti-tumour momentum and therapeutic efficacy attributable to conventional T_{RM} and their progenies is challenging due to the entangled $CD8^+$ T cell fates within the TME, as TILs with T_{RM}-like phenotypes could very well develop from activated T cells of distinct lineages secondarily acquiring T_{RM} features [55, 70, 75, 76]. Gavil et al. have demonstrated that, in the context of a tumour, the common T_{RM} markers (CD69 and CD103) failed to reflect tumour residence, nor did CD62L exclude the same [75]. Instead, chronic antigen stimulation endowed incoming tumour-specific $CD8^+$ T cells with a distinct resident programme that matured in concert with exhaustion [75]. TME factors such as TGF-β, which is known to induce CD103 [52, 61, 68, 77], could also be contributing. Furthermore, recently conducted in-depth TIL characterisation studies have resolved T_{RM} subpopulations of extended significance. In tumours and vitiligo-affected skins of metastatic melanoma patients who responded well to immunotherapy, subcluster of T_{RM} distinguished by abundant expression of certain pro-inflammatory cytokines and chemokines predicted better patient overall survival among other clinical variables, whereas the T_{RM} cluster defined by TCR signalling transcripts did not [59]. In ovarian cancers, a group of progenitor T_{RM} descended from a recently activated $CD103^-CD69^+$ re-circulating population was pinpointed as the reservoir of tumour-reactive effectors and associated with improved outcomes [70]. Therefore, additional investigations are warranted for more rigorous ways to distinguish bona fide T_{RM} from T_{RM}-like cells in the TME and resolve the subsets with the most tumoricidal capacity and the manipulable therapy targets.

The transcriptomic and epigenetic stigmata of exhaustion

Exhaustion entails the compromise in anti-tumour immunity due to prolonged stimulation of immune cells, which ubiquitously occurs in tumour-reactive $CD8^+$ TILs and leads to dwindling overall magnitude of T cell anti-tumour response in the long term [78]. TCR activation is naturally an antecedent to and propagator of T cell exhaustion. Low-affinity interactions usually lead to functional inertness or failure to instigate a self-sustaining tumour-reactive T cell population [79], whereas exceedingly strong TCR signals may predispose tumour-reactive $CD8^+$ T cells to accelerated exhaustion [80–82]. A minimum length of time is also required for initiation

towards exhaustion differentiation [15, 83, 84], as evidenced by conserved lineage flexibility of virus-specific $CD8^+$ T cells when removed from chronic infection in the first weeks [85], and yet the time point at which $CD8^+$ T cells commit to the exhaustion fate in cancer remains a subject of active investigation. Recently, an interrogation of chromatin accessibility and transcriptomic alterations of early activated $CD8^+$ T cells in tumour-bearing mice has revealed that acquisition of exhaustion hallmarks commenced as early as within 6 h of tumour antigen exposure, well in advance of the previously thought timeframe of days to short weeks [86]. Also, elements of exhaustion (loss of effector function, upregulation of immune checkpoint molecules, and contracted proliferative capacity) are regulated independently and ripen asynchronously [86–88], where, for instance, IFN-γ and TNF loss preceded that of proliferative capacity by a large margin [86]. These findings suggest that extrapolation of $CD8^+$ T cell exhaustion state from isolated parameters is an error-prone and misleading practice, and instead an all-inclusive, weighted scoring fabric may be necessitated.

Liberal usage of the term 'exhaustion' frequently constitutes a misnomer in that cells commonly designated as exhausted $CD8^+$ T cells (T_{EX}) encompass a wide spectrum that spans across near-complete preservation of function to absolute indolence [4, 89], and that the degree of reprogrammability by ICB is not indicated by the somewhat deceptive terminology [90]. Despite these variations, once lineage committed, all T_{EX} are, without exception, constrained by the transcriptional rewiring and epigenetic scarring that persisted in progenies and precluded complete reversal to the pristine naïve or memory-level of functional reserve even after antigen withdrawal [31, 91, 92]. TCR signalling-mediated upregulation of nuclear factor of activated T-cells (NFAT) family of TFs lays the foundation for the extensive and incompletely understood regulatory network of interdependent TFs that perpetuate the exhaustion programme [84]. NFAT holds critical roles in both T cell activation/effector function and tolerance/exhaustion, the former of which typically requires partnering of NFAT with AP-1 proteins [93]. With relative scarcity of AP-1 secondary to, for instance, insufficient MAPK signalling from inadequate co-stimulation, uncomplexed NFAT is diverted to monomeric binding motifs unaccompanied by an adjacent AP-1 site, inducing transcription of exhaustion-related genes encoding inhibitory molecules (e.g., *Pdcd1* [encoding PD-1], *Lag3*, *Havcr2* [TIM3], *Tigit*, *Ctla4*) and repressive TFs (e.g., *Tox*, *Egr1*, *Egr2*, and *Nr4a*) [86–88, 93–97]. In conformation with the dynamics of the down-stream transcripts, NFAT motifs are enriched in the differentially accessible chromatin regions of T_{EX} [84, 86, 97], and the predicted NFAT binding sites in the exhaustion-related genes also increased in accessibility [87, 88]. The partner-dependent functional bivalency of NFAT provides an opening for manipulating the exhaustion-versus-effector differentiation, where overexpressing c-Jun, a canonical AP-1 factor, granted the CAR T cells exhaustion resistance [98]. Akin to NFAT, BATF has bipartite roles after TCR signalling induction, and overexpressing BATF promoted BATF-IRF4 cooperation, thereby skewing CAR TILs towards an effector phenotype [99].

Among other NFAT-induced TFs, TOX has gained recognition in recent years as an essential potentiator of exhaustion [87, 92, 100–102], without which differentiation into T_{EX} is averted altogether [100, 103]. Although acute infections saw temporary presence of TOX at low levels, sustained high-intensity TOX expression in chronic infections and tumours upregulates inhibitory receptors, further modulates the TF network, and eventually engraves modification of the corresponding exhaustion-specific chromatin landscape in an NFAT-independent, self-propagating manner [84, 87, 100], thereby inflicting the indelible epigenetic scars on T_{EX} that forbid them from full recovery of memory/effector functions after antigen withdrawal [92] or ICB rescue [104]. With that said, TOX-knockout tumour-specific T cells failed to persevere in tumours and did not exhibit superior effector functions compared to the wild-type counterparts [87], which shows that TOX is necessary for T_{EX} survival [102] and exemplifies the diverging regulatory networks underlying different exhaustion modules.

Stemness sustains response in the face of exhaustion

Founding and upkeep of tissues and cellular cohorts are routinely overseen by precursors with regenerative and stem-like attributes, and T_{EX} are no exception. Despite the distinct exhaustion-associated epigenetic scars, an extensive and still expanding body of studies has firmly established a distinguishable subgroup of T_{EX} with reserves for longevity, self-renewal, and proliferation transcending those of the rest, which is correspondingly named 'precursor exhausted' or 'progenitor exhausted' T cells (T_{PEX}) but also sometimes referred to as 'stem-like' and 'memory-like' cells [16, 76, 89, 103, 105–110]. The relevance of T_{PEX} in cancer and immunotherapy was presaged by cornerstone works in chronic viral infections [105, 111, 112], and notably a $PD\text{-}1^+CXCR5^+CD8^+$ T cell population possessed a gene signature resembling memory precursor cells and haematopoietic stem cell progenitors, and proliferative bursts after PD-1 blockade while differentiating into more terminally differentiated effector-like progenies [105], although T_{PEX} are typically lacking in cytotoxicity themselves [17]. The existence and behaviour of such cells have been then substantiated in lung cancer [110] and melanomas [76, 90], and ablation of these cells dampened efficacy of checkpoint blockade.

Further studies have validated significant post-treatment intra-tumoural accumulation of T_{PEX} exclusively in ICB responders, where lineage tracking analyses credited the cell population increase to the composite effect of local expansion of pre-existing tumour-specific clonotypes and likely an influx of extratumoural T cells, in light of the novel TCR clonotypes not present within the tumour prior to therapy [74, 107, 113].

Unsurprisingly, T_{PEX} may co-express exhaustion (e.g., TOX and PD-1), memory (e.g., EOMES, SLAMF6 [Ly108 in mice], BCL6, ID3, and IL7R[CD127]), and naïve-like cell markers (e.g., CCR7 and CD62L) [84, 105, 114]. T_{PEX} differentiation is governed by an expansive set of TFs such as FOXO1 [115, 116], BCL6 [114], MYB [117], BACH2 [118], and HMGB2 [119]. Notably, T cell factor 1 (TCF1, encoded by *Tcf7*) has emerged as a key TF that dependably defines T_{PEX} [76, 84, 108, 109]. TCF1 is a well-known enactor of Wnt-β-catenin signals and assumes a myriad of roles in T cell development and maturation pending the precise circumstance [120, 121]. At the post-antigen encounter bifurcating point of $CD8^+$ T cell fate, TCF1 conspicuously steer differentiation of effector-phase lymphocytes towards the stem-like endpoints as opposed to short-lived effectors, giving rise to T_{CM} in acute infection [22, 122] and T_{PEX} in chronic infection and tumour models [29, 92, 114, 123–125], suggesting the transcriptional schemes that instil stemness in these two cell populations are, at the very least, partially overlapping [14, 31, 113, 126]. On the contrary, as TCF-1 suppresses TGF-β-induced CD103 expression [77], T_{RM} are known to be $TCF1^-$ [70, 76, 77], despite displaying a stem-like phenotype comparable to that observed in T_{CM} and T_{PEX}. Moreover, involvement of TCF1 in T_{PEX} generation is likely redundant and relies on coordinated changes in a set of additional factors [125], where *Tcf7* knockout did not significantly alter the abundance of T_{PEX} TIL [76]. On the other hand, TCF1 has been demonstrated to be indispensable for the maintenance of the T_{PEX} phenotype and arbitrates tumour control and response to ICB as well as cancer vaccination, with clear superiority of the $TCF1^+CD8^+$ T cell population over the $TCF1^-$ counterparts in these regards [76, 90, 109, 127]. In fact, a recent study has shown that TCF1 is required for optimal priming of tumour-specific $CD8^+$ T cells and ICB efficacy in the context of poorly but not highly immunogenic tumours [124], resembling the critical role of TCF1 in 'pre-programming' the chromatin landscape of T_{CM} for proper upregulation of the necessary cell cycle, DNA replication and glycolysis-related genes during a recall response [122].

Nevertheless, closer examination of T_{PEX} has shown that TCF1 alone is insufficient to demarcate the subsets of biological significance contained within the heterogenous cell population [103]. A recent study conducted in the murine LCMV infection model unveiled that $CD62L^+$ T_{PEX}, whose development relies on MYB, are transcriptionally distinct and functionally superior to the CD62L ones, and bring forth the proliferative burst following PD-1 blockade[117]. Cells with a comparable transcriptomic profile have been identified in $CD8^+$ CAR-T cells responding to B-cell acute lymphoblastic leukaemia, with FOXP1 being a newly identified hub TF that orchestrates stemness and anti-tumour response, although the equivalent to this subpopulation fortified with stemness was not readily evident in the cells infiltrating solid tumours in the same study[149]. Similarly, $TCF1^+$ T_{PEX} with circulating capacities and greater proliferative potentials are found to be associated with an $SLAMF6^+$ as opposed to $SLAMF6^-$phenotype in a murine lung adenocarcinoma model[163].

En route to terminal exhaustion

Tumour-specific T_{PEX} on the ground, along with other stem-like populations, eventually embark on the forced march in the direction of terminal exhaustion (Fig. 1A), at which point irrecoverable loss of intrinsic and therapeutically inducible anti-tumour activity manifests. Although terminally exhausted $CD8^+$ T cells (T_{TEX}) exuberantly express *IFNG*, *PRF1* and *GZMB* transcripts [4, 18], diminished polyfunctionality, survival, and expanding capacities fundamentally restrict the cumulative anti-tumoural potential of individual T_{TEX}, which are not amenable to ICB reinvigoration despite plentiful expression of checkpoint molecules such as PD-1 and TIM-3 [88, 109, 113]. Markers for T_{TEX} also notably include CD39 (ENTPD1) [4, 90, 113, 126], an ectonucleotidase that, in conjunction with CD73, potently generates extracellular adenosine, which is known to inhibit T cell signalling cascades [128–130]. Accordingly, Vignali et al. have recently shown that not only do T_{TEX} take on a powerless stance against tumour cells, but they also exert immunosuppressive effects comparable to those of $Foxp3^+$ regulatory T cells (T_{REG}) isolated from the same tumour environment, primarily through hypoxia-driven enhancement of CD39 density and activity [131], in accordance with the benefits observed with exploratory usage of CD39/CD73 monoclonal antibodies [132] and dual inhibition of CD39 and TIM3 [90] in mouse tumour models. Contrarily, the authors did not observe significant alterations in tumour growths or T_{TEX}-mediated immune suppression with germline deletion of or neutralising antibody administration against IL-10 [131], a pleiotropic cytokine with well-recognised anti-inflammatory and immunomodulatory functions whose involvement in cancers is obscured by the discordant findings in available studies [133]. Interestingly, an engineered IL-10-Fc fusion protein actualised T_{PEX}-independent re-expansion and cytotoxic function revitalisation of T_{TEX}

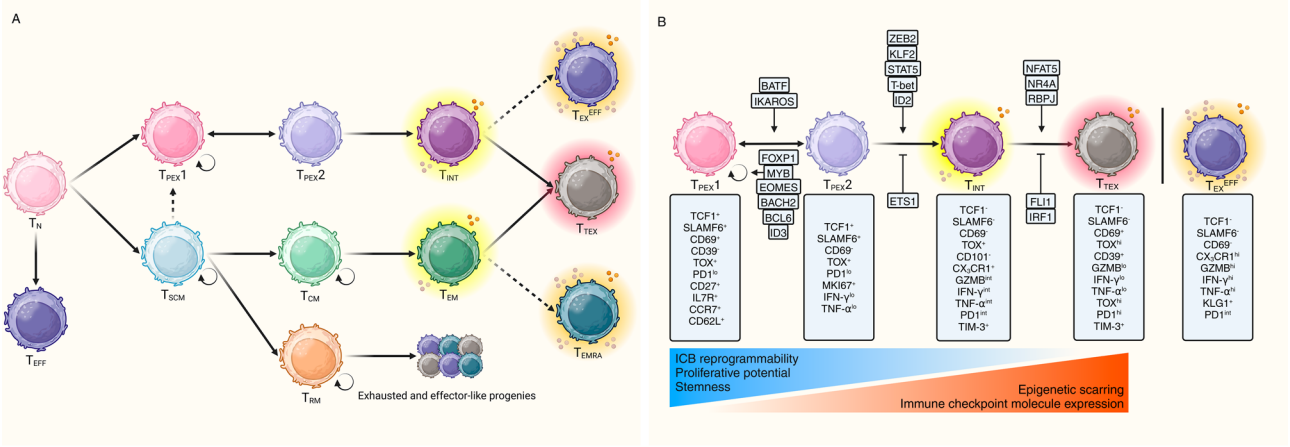

Fig. 1 Outlining CD8$^+$ T cell evolution in cancer. **(A)** On the way to terminally differentiated states, naïve CD8$^+$ T cells in cancer move through a meshwork of transcriptomically distinct phenotypes. The cells may stroll down the exhausted (top) or memory (bottom) thoroughfare, though memory cells can give rise to exhausted cells after acquiring the epigenetic imprints and the T_{RM} branch has clear transcriptional idiosyncrasies. In general terms, progression through either lane sees the waning of stem-like features and reprogrammability by immunotherapy and eventual fate convergence at the T_{TEX} terminus. T_{EX}^{EFF} and T_{EMRA} are lineage-wise juxtaposed with T_{PEX} but infused with heightened effector capacities. **(B)** Subsets of T_{EX} are identifiable by the expression of an assortment of TFs and surface proteins. T_{PEX} are vital to maintaining the exhausted T cell populations due to their stem-like features and capabilities to self-renew, as indicated by TCF1 expression, whereas TOX marks the inability of exhausted cells to fully reconstitute the memory or effector functions. The quiescent $T_{PEX}1$ subset is distinguished from the mobilised ones by SLAMF6 and CD69 expression profiles. The activated state of $T_{PEX}2$ is illustrated by the acquisition of the proliferative marker MKI67 and effector molecules IFN-γ and TNF-α, whose expression plateaus in T_{INT} before returning to low levels in T_{TEX}. In addition to escalation of immune checkpoint molecules (e.g., PD-1), T_{TEX} also express terminal exhaustion makers (e.g., CD39 and TIM-3). T_{EX}^{EFF} may form in place of T_{TEX} only under certain circumstances such as T_H-derived IL-21 induction. T_{EX} differentiation is under the oversight of an extensive, tightly regulated TF network, as shown in the figure. Notably, many of the TFs maintaining the T_{PEX} population are implicated in memory formation, and those propelling their differentiation support the effector cells during acute infection.

through fostering mitochondrial pyruvate carrier-dependent oxidative phosphorylation [134], echoing other preclinical [135] and clinical [136, 137] studies. However, the following phase II trials combining PEGylated IL-10 and anti-PD-1 antibodies led to doubling of treatment discontinuation rate due to adverse events while bringing no added survival benefits in metastatic non-small cell lung cancer patients [138]. Taken together, these findings highlight the fact that, in addition to being functionally subpar, T_{TEX} can potentially exert suppressive actions owing to susceptibility to the various TME elements, and that therapeutic strategies alternative to ICB, especially those pertaining to reprogramming T cell metabolism, could potentially exploit the remaining, albeit very limited, plasticity of T_{TEX}, though how they may best synergize with other modalities of immunotherapy and accurately target the intended cells is to be explored.

Meanwhile, progression from T_{PEX} to T_{TEX} entails a sophisticated succession of events subject to an array of cell-intrinsic and extrinsic modifiers and thus markedly variable in pace, but generally aligns with the alterations in expression levels of the master regulators, namely a hierarchical loss of TCF1-instituted stemness and culmination of TOX-associated epigenetic imprinting, which foreshadow the downstream transcriptional and functional changes [88, 102]. Though direct transitioning from T_{PEX} to T_{TEX} has been observed [139], T_{PEX} differentiation into a metabolically rewired and actively proliferating intermediate-exhausted phenotype (T_{INT}), marked by a Tim3$^+$CD101$^-$ phenotype or CX_3CR1 expression in chronic infections [17, 139, 140], proves to be a pre-requisite step to liberate the full anti-tumour potential and ICB responsiveness of T_{PEX} [103, 140–143], since T_{PEX} are mostly quiescent by default, as attested by low expression of cell cycling genes such as *MKI67* [17]. This is validated by the latest advances, where toning down *Ikzf1* (encoding IKAROS, which suppresses TCF1) has successfully retarded T_{PEX}-to-T_{INT} differentiation, but failed to evoke added antitumour effects [142]. Admittedly, the transitory T_{EX} populations were evident in earlier TME profiling studies [19, 32, 89, 90], but their structured categorization was pioneered by Beltra et al., where, using Ly108 (a proxy marker for TCF1) and CD69, they have distinguished the interconverting quiescent ($T_{PEX}1$, Ly108$^+$CD69$^+$) and proliferating ($T_{PEX}2$, Ly108$^+$CD69$^-$) subsets of T_{PEX}, and established the developmental trajectory from $T_{PEX}2$ to Ly108$^-$CD69$^-$ T_{INT} and ultimately Ly108$^-$CD69$^+$ T_{TEX} [103], thus completing the $T_{PEX}1 \rightarrow T_{PEX}2 \rightarrow T_{INT} \rightarrow T_{TEX}$ paradigm that was consolidated by succeeding studies [142, 144–146] (Fig. 1B). Increasing evidence suggests that the $T_{PEX}2$-to-T_{INT} junction harbours a critical epigenetic, transcriptional and metabolic checkpoint, whose regulation incorporates inputs from activation of the mTOR

pathway [142], which serves as a control panel integrating cellular metabolism sensor signals [147], and a range of TFs, including T-bet [103, 142, 148], BATF [148], KLF2 [149], ETS1 [142], and STAT5 [150]. In fact, augmentation of Stat5 activity by employing an orthogonal IL-2:IL2Rβ pair system in established T_{EX} cells not only stimulated T_{INT} formation, especially when combined with PD-L1 blockade, but also improved their durability and effector functions in the face of persistent antigen stimulation, and suppressed differentiation to T_{TEX} [150].

Indeed, T_{INT} may still bear therapeutically accessible developmental pliability and effector potential. A growing body of studies has shed light onto an alternative differentiation path from the T_{INT} waypoint to an effector-like exhausted (T_{EX}^{EFF}) population that is inducible by IL-21-producing $CD4^+$ helper T cells (T_H) [139], TF ZEB2 [144], and lower TCR avidity [81, 146], and lineage-wise juxtaposed with T_{TEX} but possesses strong effector or NK cell characteristics [81, 139, 146]. Multiple lines of evidence derived from murine chronic infection models are in support of this bifurcating model placing T_{INT} at the branch point, including the in silico predictions based on pseudotime and RNA velocity trajectory analyses, retained phenotypic plasticity in T_{INT} but not T_{EX}^{EFF} or T_{TEX} upon adoptive transfer, and shared open chromatin regions between T_{INT} with either T_{EX}^{EFF} or T_{TEX} and the paucity thereof between T_{EX}^{EFF} and T_{TEX} [81, 139, 146]. This novel model is of great therapeutic relevance, as it provides the theoretical foundation for a window of opportunity, perhaps the last one before the end-stage epigenetic ossification takes place, to expand the aggregate effector activity by diverting T_{INT} towards the T_{EX}^{EFF} fate in lieu of T_{TEX} [151].

To complicate matters even further, concurrent 'non-T_{PEX}-origin' trajectories to T_{TEX} progressing through memory subsets have been described [4, 70, 72, 74, 144, 152], which are generally organised into two interlaced paths marked by the resident-versus-circulating duality of memory (Fig. 1A). In a pan-cancer TIL atlas constructed from scRNA-seq data from 316 patients across 21 cancer types, Zheng et al. projected that $CD8^+$ T_N could ultimately develop into either T_{EX} or T_{EMRA} [4], concordant with recent identification of a non-T_{EX} $ZNF683^{high}$ T_{EFF}/T_{EM} endpoint that was accentuated in marrow-infiltrating $CD8^+$ T cells in Richter syndrome responding to PD-1 blockade [152]. Along the path to T_{EX}, T_N first progressed to $IL7R^+$ T_{MEM} (T_{CM}/T_{SCM}) and then split into the $GZMK^+$ T_{EM} and $ZNF683^+CXCR6^+$ T_{RM} branches before uniting at the $GZMK^+$ T_{EX} and, finally, T_{TEX} fate [4]. A similar scheme was outlined in an NSCLC dataset, involving progression to T_{TEX} (CD8-LAYN) through a transitional population (CD8-GZMH) from circulating (CD8-KFL2/CD8-GZMK) and tissue-resident (CD8-XCL1) precursors, where the T_{RM} population is deemed the main source of the more differentiated tumour antigen-specific T cells based on TCR sharing [72] – a clonal predilection that was also seen in ovarian [70] and neoadjuvant ICB-treated oral cancers [74]. Taken together, suffice it to say, notwithstanding the concrete accomplishments in the nuanced dissection of the phenotypes, current models may still merit refinement and reconciliation for an unequivocal representation of $CD8^+$ TIL development and evolution, which is made particularly challenging by the confounding qualities unique to chronic antigen stimulation and TME, the logistical and technical constraints, and the taxonomical dilemmas for the betwixt and between cell populations.

Navigating the haemolymphatics and TME: a spatial odyssey

The relevance of tumour-specific $CD8^+$ T cell phenotypes is nullified if detached from the position coordinates, given the contact-dependent nature of their tumoricidal weaponries. Indeed, the quantity and spatial distribution of $CD8^+$ TILs demonstrated drastic variations across the cancer types, anatomical sites, and lesion classifications (i.e., primary, recurrent, or metastatic) [2, 4]. This observation has prompted development of Immunoscore, a histological type-agnostic grading system quantifying $CD3^+$ and $CD8^+$ lymphocyte densities in the tumour core and the invasive margin that has shown prognostic values in colorectal and other cancers [2, 153–156], effectively categorising tumours into immune infiltration-based 'immunotypes', namely the heavily infiltrated 'hot', the infiltration-devoid 'cold', and, between these two extremes, the uniformly poorly infiltrated 'immunosuppressed', and the 'excluded' tumours where infiltrates are limited to the invasive margin [155]. Importantly, the immunotypes cannot be explicated by tumour cell antigenicity alone and are in fact telltale snapshots that mark the culmination of the long march of $CD8^+$ T cells as they migrate through different circulatory compartments, penetrate multiple barriers, and persevere in spite of suppressive environments, to complete the larger cancer-immunity cycle [157]. Along the journey, $CD8^+$ T cells adjust the balance of tissue anchoring and egress factors to facilitate or restrain translocation, perform tissue-specific adaptive manoeuvres, and pass through several key anatomical and microenvironmental locales that profoundly shape their dynamics (Fig. 2), leading to reverberations in the intratumoural $CD8^+$ T cell landscape.

Tumour-specific CD8+ T cells gear up in TDLNs

The conventional school of oncology often view TDLNs as receptables forming the catchment for metastatic seeding, which is predisposed by the architectural and functional deviations from the non-draining nodes

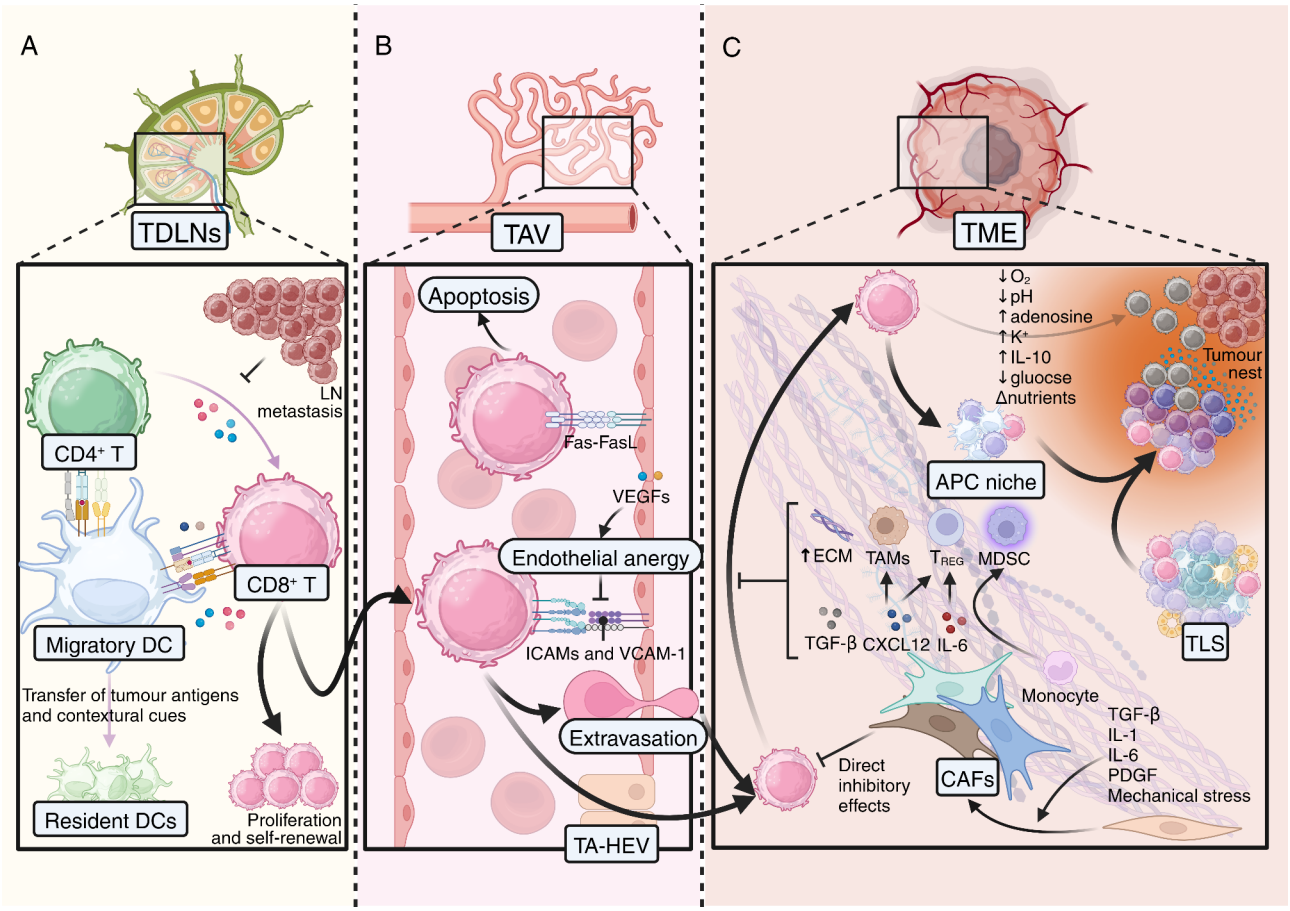

Fig. 2 Critical anatomical and microenvironmental waypoints dictating success of $CD8^+$ T cell infiltration. **(A)** TDLNs are the ideal venue for tumour antigen-bearing migratory DCs from the TME to condition the T_H and $CD8^+$ T cells. The migratory DCs also pass on the antigens and TME cues to their resident counterparts. With provision of the primary, costimulatory, and cytokine signals by the DCs and T_H sans the inhibitory TME elements, $CD8^+$ T cells have the privilege of remaining in the stem-like states and serving as a reservoir of tumour-repressive cells. Establishment of metastases in the LNs demolishes the supportive context for these population-sustaining $CD8^+$ T cells. **(B)** Tumour-associated vascular endothelium is a major barrier against T cell infiltration. Endothelial expression of FasL can precipitate direct $CD8^+$ T cell death. Aberrant adhesion molecule expression secondary to VEGF-induced endothelial cell anergy renders it impregnable to $CD8^+$ T cells. Tumour-associated high endothelial venules are important sites for $CD8^+$ T cell extravasation and aggregation. **(C)** CAFs orchestrate the tumour-associated stroma and mediate T cell exclusion in a multitude of ways, as illustrated. The APC niches and TLSs are on-site powerhouses that harbour stem-like $CD8^+$ T cells and, by safeguarding their survival, greatly amplifies anti-tumour immunity. Conversion to the more terminally differentiated phenotypes ensues the longer the dwell time and the closer the distance to the tumour cells, which are known to foment an immunosuppressive aura.

provoked by tumour-derived lymph-borne factors [158]. Removal of uninvolved TDLNs, however, failed to improve outcomes in preclinical models or cancer patients [159–161]. In this day and age, the significance of TDLNs as a nexus between the cancer and immune cell ends of the tug-of-war has been brought back to attention and reinforced, and their role in facilitating generation of $CD8^+$ TILs reaffirmed, for the key window for antigen presentation and T cell priming arises as tumour antigens, T lymphocytes, and dendritic cells (DCs) converge in the lymphatic endothelial cell (LEC)-lined and macrophage-interspersed subscapular sinuses (SCS) of TDLNs [158, 162]. Current evidence suggests that CCR7-expressing type I conventional DCs (cDC1s) migrated from the TME are the main DC subset that internalise and transport tumour antigens in large quantities and potently prime $CD8^+$ T cells through MHC-I-mediated cross-presentation or cross-dressing (direct transfer of peptide-MHC-I complex) [162–168] and $CD4^+$ T cells through MHC-II-mediated direct presentation, which reciprocally augment the immunostimulatory proficiency of DCs [169]. Meanwhile, the TME IFN-I-induced ISG^+ cDC2s also demonstrated capability to activate $CD8^+$ T cells by cross-dressing in the absence of cDC1s [170]. Once arrived at the TDLN, the migratory DCs may in turn transfer tumour antigen to their resident counterparts to prepare the inbuilt DC terrain for $CD8^+$ T priming [168, 171], where the DC status across the tissue origin and the LN may also be synchronised with co-transfer of contextual cues [172].

Given their nurturing multilayered cellular infrastructure and strategic anatomical locations, TDLNs are naturally the reservoirs of tumour-specific $CD8^+$ TILs. An abundance of studies have provided evidence of $CD8^+$ T cell transmigration from TDLNs to the tumour [14, 15, 141, 159, 163, 173–176] and vice versa [15, 177], as well as enrichment of tumour-reactive $CD8^+$ clonotypes in TDLNs [175, 176, 178]. Unencumbered by the stress from persistent antigen stimulation and TME factors, activated tumour-specific $CD8^+$ T cells in TDLNs are typically found in the minimally exhausted T_{PEX} or T_{PEX}-like states [79, 117, 141, 163, 173, 174, 178, 179], whereas those TILs clonally related to them predominantly display the T_{TEX} phenotypes [141, 173, 178]. In fact, expansion of pre-existing or de novo LN-dwelling stem-like populations is vital to ICB response [14, 105, 175, 176, 180] (Fig. 3A) and potentially accountable for a greater share of efficacy than those circulating or residing in the TME, as surgical removal of TDLNs [14, 181], co-administration of FTY720, an inhibitor of T cell egress from lymphoid organs [175, 176, 181], was sufficient to negate the antitumour effects of ICB, and targeted delivery of ICB monoclonal antibodies to the TDLNs eclipsed systemic therapy in terms of efficacy and permitted dose sparing [179]. Furthermore, studies have consistently shown that the responses of TDLNs to ICB and the integrity of TLDNs as an immune reservoir and line of defence in general can be profoundly undermined by presence of metastases [141, 182–185]. In both melanoma murine models and human patients, LN metastases precipitated an altered immune contexture involving IFN-II-associated accumulation of regulatory T cells (T_{REG}s) and PD-$L1^+$ DCs that suppressed $CD8^+$ T cells [183, 184]. These tolerogenic cells were also observed in LNs with HNSCC metastasis and, along with naïve/quiescent ($CD45RO^{lo}PD\text{-}1^{lo}TCF\text{-}1^{hi}$) $CD4^+$ T cells, formed the immunosuppressive niches encircling T_{PEX} and T_{INT} post-ICB [141]. Overall, these evidences offered an additional explanation to the prognostic values of TDLN statuses from the perspective of $CD8^+$ T cell anti-tumour immunity, revealed the underappreciated role of TDLN statuses in predicting the response to ICB, and conceptualised the combination of ICB with agents targeting LN metastases, the latter of which, however, are yet to be developed.

Overcoming the vascular endothelial barrier

Following potentiation of tumour-specific $CD8^+$ T cells in the TDLNs, an endurance run through systemic circulation, tissue environment, and microvasculature awaits before they are able to reach the designated destination in the centre of tumour. Execution of such an extended process that requires extreme precision relies on coordination and fine-tuning of various homeostatic control systems. After priming, exit from the LNs is classically conducted with upregulation of S1PR1 in the context of high concentration of S1P in efferent lymphatics and synchronous curtailment of CCR7 and CD69 [6, 186], whereas sympathetic neural inputs typically promote retention of T cells in the LNs [187, 188]. Intact chemotactic systems are then a prerequisite for tumour homing, which include the CCR5-CCL5, CXCR3-CXCL9/

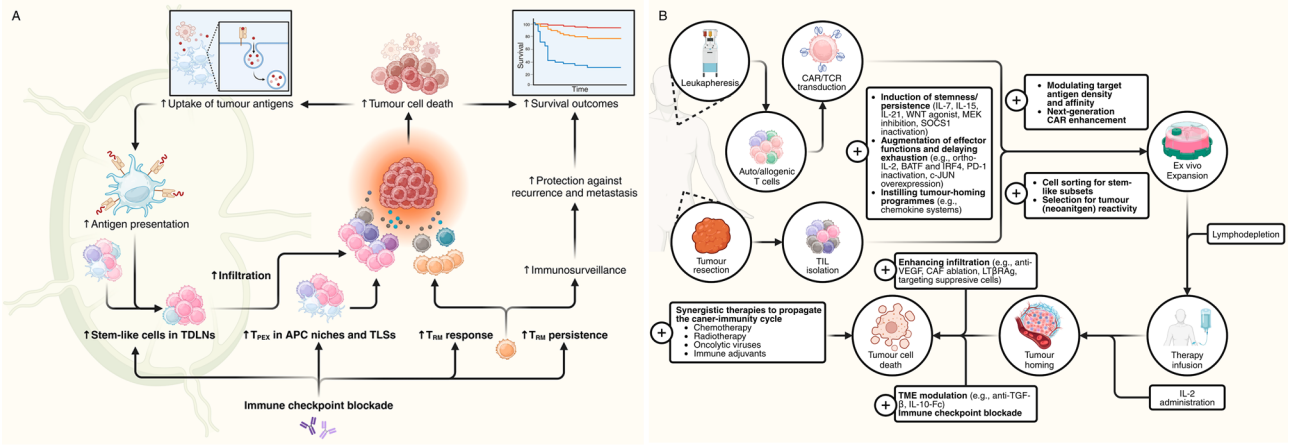

Fig. 3 Modifying $CD8^+$ T response in cancer. **(A)** Immune checkpoint blockade radically changes the $CD8^+$ T landscape via mobilisation of the stem-like populations, which primarily occurs in the company of supportive cells in the TDLNs and microenvironmental niches. Cancer cell death as a result of amplified response fosters a self-amplifying cycle owing to tumour antigen release and subsequent increased uptake and priming by APCs. ICBs can simultaneously stimulate T_{RM}, which contributes to increased tumour killing and long-lasting immunosurveillance that deters recurrence and metastasis. **(B)** In addition to the conventional steps implicated in adoptive cell therapies (lymphocyte procurement, isolation, activation and expansion, pre-infusion lymphodepletion and post-infusion IL-2 administration), there have been multiple strategies in the phases of ex vivo manipulation and in vivo augmentation (marked with plus signs) to maximise the utility of transferred cells. A deeper understanding of the transcriptional, epigenetic, and metabolic controls of $CD8^+$ T phenotypes will shed light on novel ways to engineer cells for the desirable attributes.

CXCL10/CXCL11, CXCR5-CXCL13, CX_3CR1-CX_3CL1, and CXCR6-CXCL16 axes for $CD8^+$ T cells [105, 109, 139, 189–194], where the transition from T_{PEX} to T_{INT}/T_{TEX} may be accompanied by specific changes in expression of chemokine receptors [139, 177, 191]. Conversely, the elaborate interactions can be sabotaged or manipulated by solid tumours to diminish the driving force for cytotoxic T cell migration while fostering preferential recruitment of suppressive immune cells including T_{REG}s, myeloid-derived suppressor cells (MDSCs), and tumour-associated macrophages (TAMs) and neutrophils (TANs) [189, 192]. The directionality of chemoattractant concentration gradient, which steers the $CD8^+$ T cell traffic, also bears obvious significance as, for instance, the interplay between $CXCR4^+$ T_{EFF} and tumour-associated lymphatic vessel-derived CXCL12 resulted in marginalisation and egress of TILs, which undermines tumour control and therapeutic response [177].

Before $CD8^+$ T cells could set foot on the core of the tumour, they can be repulsed by multiple chemical, physical, and cellular ramparts, and the tumour vasculature represents the first tumour-orchestrated line of defence that must be surmounted. In acute inflammation, leukocyte extravasation typically follows a multi-step progression, where friction for arresting cell locomotion is jointly supplied by selectins and adhesion molecules such as ICAMs and VCAM-1. Tumours resort to formation of blood vessels (termed 'neo-angiogenesis'), which is stimulated by mechanical strain, hypoxia, and angiogenic molecules, with the VEGF family (VEGFA - F) being the most prominent, to fulfill the metabolic demands for neoplastic growths [195–197]. VEGF has the ability to directly influence immune cells, promoting development of T_{REG}s, M2-macrophags, and MDSCs, impairing DC maturation and T cell development, and inducing $CD8^+$ TIL exhaustion [195, 198–201]. When faced with relentless malignancy-driven bombardment by VEGF and other angiogenic factors, endothelial cells lining the tumour-associated blood vessels are prevented from activation by pro-inflammatory cytokines and conspicuously deviate from those in inflammation in terms of surface protein expression. Differentially expressed by neoangiogenic endothelium are immune checkpoint molecules [202, 203], FasL that selectively mediate $CD8^+$ T cell demise [204], and, importantly, fewer adhesion molecules [205, 206], referred to as 'endothelial cell anergy', thereby veiling the portal of entry for tumour-specific $CD8^+$ T cells. These observations serve as the mechanistic underpinning for combining anti-angiogenic agents with ICBs [194, 199, 201, 204, 207], where clinical trials have verified additive if not synergistic effects across several cancer types [208–213], which may be seen regardless of the intra-tumoural immune checkpoint molecule expression status [208, 211]. Notably, recent advances have demonstrated endothelial cells comprising the post-capillary venule (PCV)-derived MECA-79^+ tumour-associated high endothelial venules (TA-HEV) as major breach sites for $CD8^+$ T cells extravasation at baseline and in anti-CTLA-4 (+/- anti-PD-1) therapy, and increased number and maturation of these endothelial cells ushered a higher percentage of stem-like ($SMALF6^+$) $CD8^+$ TILs [214]. In preclinical models, anti-VEGFR2, anti-PD-L1 and LTβRAg triple therapy were sufficient to induce differentiation of PCVs into TA-HEVs that indeed facilitated lymphocyte infiltration and T_{PEX} accumulation [215, 216].

Cancer-associated fibroblasts at the crux of CD8+ T-excluding and suppressing stroma

The tumour-associated stroma, apart from requitting the cancer-derived factors with direct facilitative and prohibitive influences on neoplastic cell-led tumour progression and metastatic transition, and thus holding cardinal non-immune-related biological and prognostic implications [196, 217–219], lies in between the extravasated $CD8^+$ T cells and the nest of tumour cells, and contains various cellular and inanimate components with shielding effects. Being one of the most abundant and functionally significant constituents of tumour stroma, cancer-associated fibroblasts (CAFs) are evidently discernible from normal quiescent fibroblasts [218, 220, 221], commonly arise from co-option of resident fibroblasts by mechanical stress and cancer cell-secrete chemokines and growth factors (e.g., TGF-β, IL-1, IL-6, and PDGF), but are otherwise of remarkable plasticity (i.e., reactivity to external cues) and heterogeneity in origins and phenotypes and across tumour/tissue types, for which a unanimous classification system is lacking despite tentative, crude categorisation into myofibroblastic (myoCAFs, typically dependent on TGF-β for differentiation and ECM-associated genes/αSMA^{high}), inflammatory (iCAFs, IL-1-dependent and IL-6/$CXCL12^{high}$), and antigen-presenting CAFs (apCAFs, HLA-DR/MHC-II^{high}) [219–226].

Transcriptomic analyses have established the inverse correlations between myoCAF-associated signatures and abundance of $CD8^+$ T cells as well as immunotherapy efficacy [219, 227–229]. Integration of spatial information allowed pinpointing of several myoCAF subsets in particular that mediate T cell repression and exclusion predominantly by deposition of extracellular matrix (ECM, a known trigger of $CD8^+$ T cell exhaustion in cancer [230] and impediment to lymphocyte locomotion when densely packed or mal-aligned [231, 232]), as well as TGF-β secretion and interacting with other suppressive cell populations [221, 222, 227, 228, 233–236]. Costa et al. are among the first groups to portray myoCAFs at higher resolutions, where, using six fibroblast markers, they ascertained one particular myoCAF

subset that tends to cluster near epithelial tumour cells in TNBCs and attracted, maintained, and differentiation into $CD25^{high}FOXP3^{high}$ T_{REG}s with augmented suppressive effects on $CD8^+$ T cells [234]. Further deciphering endeavours followed thereafter. For example, in KPR mouse pancreatic adenocarcinomas, TGF-β-signalling-dependent $LRRC15^+$ myoCAFs are shown to be in close proximity of and capable of suppressing $CD8^+$ T cells [222]. In early- and advanced-stage lung cancers, $MYH11^+\alpha SMA^+$ and $FAP^+\alpha SMA^+$ myoCAFs have been respectively identified to obstruct T cell contact with tumour cells, albeit in different ways, where the former form a monolayer of elongated cells enveloping the tumour parenchyma and deposit type IV collagen fibres, and the latter are organised into patches or multiple layers and collagen XI and XII-producing instead [221].

In contrast, CAFs exhibiting an inflammatory or immune modulatory phenotype sculpt the immune cell landscape via extensive ligand-receptor interactions [224, 237]. CAF-emanated IL-6 recruits $FOXP3^+$ TILs [235], invokes CD73 expression in $\gamma\delta T_{REG}$s [130], and induces differentiation of TME monocytes into MDSCs through STAT3 signalling [238]. Even though CAF production of CXCL12 can attract $CD8^+$ T cells in infiltrated tumours [193], it oftentimes culminates in T cell exclusion [239, 240]. This could be the combinatory result of $CD8^+$ T cells being misdirected towards the tumour margin [239–241], the paradoxical repelling effects of CXCL12 at high concentrations [242], and the CXCR4-CXCL12 apparatus-orchestrated CAF crosstalk with T_{REG}s [234], $SPP1^+$ TAMs [243], and other myeloid populations [244]. Moreover, Ma et al. have computationally derived from harmonised scRNA and ST data a multitude of ligand-receptor interactions through which iCAF can directly corrupt $CD8^+$ T cell robustness [224]. Notably, the LGALS1-PTPRC interaction is among the most frequently seen on ST slices, which triggers NFAT signalling and thus $CD8^+$ T cell exhaustion [224]. On the other hand, apCAFs may render anergic or convert naïve $CD4^+$ T cells to T_{REG}s with antigen-specific MHC-II-TCR crosslinking void of backing from co-stimulatory molecules [226, 245], and direct antigen-dependent interaction with CAFs can precipitate PD-L2 and FasL-mediated lethality to the antigen-specific $CD8^+$ T cells [246]. That being said, in lung cancers, apCAFs are found to activate and protect $CD4^+$ T cells from apoptosis and consequently tumour-suppressive rather than permissive [247]. Additionally, non-canonical markers have also proven to be useful for setting apart non-ternary CAF subsets with immunomodulatory effects. A distinct cluster of CAFs has been discovered in HNSCC that sequester T_{PEX} ($TCF1^+GZMK^+$) by enhanced MHC-I and CXCLs expressions and impede their effector transformation through leveraging galectin-9 [240]. In pancreatic ductal adenomas (PDACs), CD105 effectively partitioned CAFs into two fractions displaying opposite correlations with immune cell subpopulations, with the $CD105^-$ subsets being positively associated with proliferation of the $CD8^+$ subsets, whilst the $CD105^+$ counterparts had a negative association [248]. Specifically, the $CD105^-MHCII^+CD74^+$ CAFs were the sole mesenchymal subset securing a positive correlation with antigen-experienced but non-terminally exhausted $CD8^+$ TILs [248].

Considering the myriad of ways $CD8^+$ T cells are obstructed and subjugated, therapies targeting CAFs have been extensively investigated, and combination with therapeutic strategies carrying direct empowering effects on $CD8^+$ T cells (e.g., ICBs and CAR-T therapy) attempted as a natural course of action for the prospect of "infiltrate and eliminate" [233, 239, 242]. As an embodiment of this approach, antecedent treatment with CAR-T targeting FAP^+ CAFs in a recent study on murine PDAC models dismantled the desmoplastic stroma and dispersed the associated immunosuppressive environment, allowing full engagement of ICB-reprogrammable endogenous $CD8^+$ T cells and CAR-T cells targeting tumour-associated antigen (Meso-CAR-T) with tumour cells [249]. Nonetheless, given the phenotypic multiplicity of CAFs and their restraining effects on tumours [226, 247, 250, 251], indiscriminate ablation of CAFs may accelerate cancer progression even with increased $CD8^+$ T cell infiltration [250], hence the imperative to selectively deplete the reprehensible CAF subsets for clear-cut clinical benefits.

Seeking sanctuary in the immune-permissive microenvironmental enclaves

Once advanced to the immediate vicinity of tumour cells, $CD8^+$ TILs plunge into the hypoxic, acidified, adenosine and potassium ion-imbued, and nutrient-deficient or perturbed quagmire, which overwhelmingly favours $CD8^+$ T dysfunction and sealing of the exhaustion fate [252, 253]. In accordance with this, intra- or peri-tumoural T_{PEX} are rarely found flanked by tumour cells unbuffered, but tend to congregate in spatial niches conducive for population maintenance and effector differentiation [76, 106, 143, 174, 190, 191, 216, 254–256], and in some cancer types, the density and quality of these structures may set patients with favourable outcomes apart from those with poor prognosis [106, 257], and ICB responders from non-responders in the scenario of intact T cell priming in TDLNs and unhindered infiltration [143]. Cells with both antigen-presenting and immunomodulatory capabilities, especially DCs, are almost invariably implicated in the organisation of these $CD8^+$ T-accommodating alcoves [174, 190, 191, 257–259]. DCs close the distance with the inbound $CD8^+$ T cells through specific interactions

between chemokines and their receptors (e.g., CXCL16-CXCR6 [191] and CXCL9/CXCL10-CXCR3 [190, 194, 257]), granting the primary and co-stimulatory licences [169, 174, 255, 260], and unleash cytokines crucial for survival and induction of effector functions, such as IL-12 [261] and IL-15 [143, 191]. Prostaglandin E_2 [262] and immune checkpoint molecules like TIM-3 [258] and PD-L1 [259] are notable for interfering with the DC-$CD8^+$ TIL communication, thus explaining at least part of the benefits seen in respective therapies. Spatial profiling studies have also substantiated that DCs can complex with the $CD4^+$ T_H subsets they primed to form dyadic powerhouses that exert strengthened chemoattractant and invigorating influences on $CD8^+$ T cells [143, 169, 263].

Tertiary lymphoid structures (TLSs) are the most sophisticated form of ectopic lymphoid tissues that can be found in tumours. Representing the pinnacle of immune cell reciprocity within the TME, TLSs are structurally less organised (i.e., unencapsulated and variable in T and B cell compartmentalisation) but cellular composition-wise reminiscent of SLOs, where each type of cells undertake corresponding roles that ultimately contribute to a brisk and self-replenishing local adaptive response [264], as tumours with TLSs had denser and less dysfunctional intra-tumoural T cell infiltrates [254, 265–267]. Like in SLOs, presence of HEVs [216, 268] and a stromal network primarily consisting of follicular reticular cells (FRCs) [264] in TLSs secures the microanatomical space for lymphocyte passage and residence. The TLS-defining $CD20^+$ B cells can be intermingled with T cells or gather in follicles with or without germinal centres, and nurtured by the $CD4^+$ follicular helper or $CD21^+$ follicular dendritic cells [264]. In addition to the canonical role of producing antibodies, TLS B cell subsets have been reported to be capable of energizing T lymphocytes by antigen presentation and cytokine secretion [266, 269–271]. In the meantime, the $CD4^+$ T_H and DC duo have been found enriched in TLSs, predominantly in the T cell zones [254, 263], and so have tumour-reactive and stem-like $CD8^+$ T cells [76, 106, 256, 265, 272], which integrate the inputs from the neighbouring immune cells and proceed to tackle the tumour cells.

TLSs are generally associated with improved survival outcomes and immunotherapy responses [265, 266, 269, 270], and yet, on scrutinisation, the localisation and maturity level of TLSs wield pivotal influences [267, 273], where extra-tumoural positioning and immaturity may underpin the negative prognostic impacts of TLSs in clear cell renal cell carcinoma [273, 274]. TLS formation beholds a choreography of lymphocytes, myeloid cells and mesenchymal cells under the guidance of molecular factors including lymphoid chemokines (e.g., CXCL13, CCL19, and CCL21), LTs, IFN-Is, TNFs, IL-17, many of which have been appropriated to experimentally induce TLSs in murine models [264]. $CXCL13^+CD8^+$ T cells have been described, were determined to be tumour-reactive and predictive of ICB response [275], and indeed correlated with TLS presence [272, 276], though it was reported that their dysfunctional states and contribution to an immunosuppressive milieu might ultimately lead to unfavourable outcomes [276].

Perspectives

The notion that not all $CD8^+$ TILs are created equal stands truer than ever. An outpouring of multi-dimensional and high-resolution data over the past few years spotlighted the stem-like exhausted and memory cells as the subsets of the highest tumour-suppressive yield at baseline and upon therapeutic intervention. Their transcriptomic and epigenetic properties are being characterised and phylogenetics elucidated, to a level of certainty, precision, and scale previously unfathomable, revealing invaluable insights into the promising biomarkers for prognostication, subpopulations for successful adoptive transfer, and strategies for engineering CAR-T cells (Fig. 3B).

Innovative and painstaking iterative processes with state-of-the-art technologies paved the way for these ground-breaking discoveries. Recent implementation of in vivo sc-CRISPR screening to TFs concerning $CD8^+$ T cell differentiation and exhaustion in cancer may have just constructed the most comprehensive TF directory for T cell fate to date [142]. Synergising available omics modalities represents an alternative tactic for new discoveries. Mapping and deconvolution algorithms are routinely applied to scRNA-seq and ST data to confer spatial coordinates to individual cells and deduce the cellular composition of each ST spot [5, 224, 236, 240]. Building on the previously known markers for tumour specificity, which are frequently also tokens of exhaustion (e.g., CD39, PD-1, TIM-3 and TOX) [4, 18, 68, 277], coupling of scRNA-seq, sc-TCR-seq, and TCR specificity testing data helped to fish out the tumour-reactive stem-like $CD8^+$ T cells from the swarm of bystanders, which are otherwise difficult to tell apart due to transcriptomic similarity [18, 56, 127, 275, 278]. Disciplined application of these investigative tools to the matched longitudinal samples enabled a chronicle of the tumour-reactive clonotype dynamics in naturally occurring anti-tumour immunity and immunotherapies [18, 56, 275], laying down the ground truth against which computational inferences can be compared, and the methods refined. Furthermore, the recent invention of Zman-seq, which combines scRNA-seq with time-stamping by sequential injections of fluorescent anti-CD45 antibodies that selectively tag circulating immune cells but not those already infiltrated the tumour, allows cataloguing

of transcriptomic states with different durations of TME residence and consequently deciphering of the temporal dynamics [279]. Much to the excitement of the academia and the medical community, the methodological revolution seems to have provided the necessary means to materialise the envisioned all-encompassing cell tree for $CD8^+$ T cells in health and disease [7]. The much-anticipated nomenclatural reform, however, has lagged behind, and work still needs to be done to mend the gap between the newly acquired high-dimensional information and legacy observations from the pre-omics era. Besides, caution should be exercised when interpreting data from the viral models, due to the overlapping but nuanced phenotypes [56, 81, 84] and asynchronous pace of exhaustion [86] between the exhausted $CD8^+$ T subsets in chronic viral infections and those in tumours.

That key steps of $CD8^+$ T cell priming and maturation take place across anatomical locations outside of the tumour mandates a cross-the-body relay in parallel with the phenotypic evolution of tumour-specific $CD8^+$ T cells, which is an extremely error-prone process. Any misstep could end up with flawed immunotypes that render tumour cells inaccessible to the tumouricidal arsenal of $CD8^+$ T cells, which operate exclusively in close quarters. Conversely, drawing on the continually expanding mechanistic insights, each nucleating point of $CD8^+$ T anti-tumour immunity, be it the TDLNs, APC-rich niches, or TLSs, may represent an opportunity for therapeutic amplification, and the vascular and stromal barriers for ablation, especially in the 'excluded' immunotype. Similarly, the phenotypic pliability of $CD8^+$ T cells, whilst hijacked by TME factors for exhaustion induction, is a double-edged sword malleable to therapeutic honing in recognised and less obvious ways. In broader terms, $CD8^+$ T cells are inscribed with marks of systemic immunological wellbeing, which is dictated by age, sex, hormone levels, antigen exposure history, and comorbidities of individuals, but also newly established determinants such as commensal microbiota. An elegant testament bolstering the linkage is the recent discovery of PD-1 blockade efficacy enhancement by gut *L. johnsonii* and *C. sporogenes*, which collaborate to convert tryptophan to IPA, a metabolite that promotes H3K27 acetylation of the super-enhancer region of *Tcf7*, thereby diverting $CD8^+$ T cells towards differentiation into T_{PEX} [280]. The impacts of systemic immune health factors on $CD8^+$ T-mediated anti-tumour immunity are therefore also worthy of further investigation, from which individualised and easy-to-implement immune-boosting diet and lifestyle interventions can be devised.

In conclusion, $CD8^+$ T cells are indispensable for executing adaptive antineoplastic response, as they are positioned at the converging point downstream of miscellaneous immunomodulatory pathways to administer the coup de grâce to tumorous cells by unleashing direct cytotoxicity. The vast phenotypic and spatial heterogeneity of $CD8^+$ TILs are being unveiled as we venture forth into the age of omics. Expanding on the preceding efforts to activate, reprogramme, and facilitate the infiltration of tumour-specific $CD8^+$ T cells, the first-of-its-kind FDA approval for TIL infusion therapy in melanoma has officially advanced the forefront of immeasurable possibilities into clinical practice [281]. Working towards a consensus ontogeny to synthesise the multimodal data and delving into the mechanistic underpinnings will help to maximise the yield of $CD8^+$ TIL-based cancer therapies, ultimately translating into better outcomes for patients.

Abbreviations

αSMA	alpha smooth muscle actin
ACAT-seq	Assay for Transposase Accessible Chromatin with high-throughput sequencing
AHR	aryl hydrocarbon receptor
AP-1	activator protein 1
APCs	antigen-presenting cells
BACH2	BTB and CNC homologue 2
BATF	Basic Leucine Zipper ATF-Like Transcription Factor
BCL-6	B-Cell Lymphoma 6
BLIMP-1	B-lymphocyte-induced maturation protein 1
CAFs	cancer-associated fibroblasts (myoCAFs – myofibroblastic CAFs; iCAFs – inflammatory CAFs; apCAFs – antigen-presenting CAFs)
CAR	chimeric antigen receptor
CCL/R	C-C motif chemokine ligand/receptor
cDC1/2s	type I/II conventional dendritic cells
CRISPR	clustered regularly interspaced short palindromic repeats
C. sporogenes	Clostridium sporogenes
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
CX_3CL/R	C-X3-C motif chemokine ligand/receptor
CXCL/R	C-X-C motif ligand/receptor
DCs	dendritic cells
ECM	extracellular matrix
Egr1/2	early growth response 1/2
ENTPD1	ectonucleoside triphosphate diphosphohydrolase-1
EOMES	Eomesodermin
FAP	fibroblast activation protein
FasL	Fas ligand
FOXO1	forkhead box protein O1
FOXP1/3	forkhead box protein P1/3
FRCs	follicular reticular cells
GZMB/H/K	granzyme B/H/K
H3K27	histone H3 lysine 27
Havcr2	Hepatitis A Virus Cellular Receptor 2
Hic1	hypermethylated in cancer 1
Hif1a	hypoxia inducible factor 1 subunit alpha
HLA-DR	human leukocyte antigen, DR isotype
HMGB2	high mobility group box 2
HNSCC	head and neck squamous cell carcinoma
HOBIT	homolog of Blimp-1 in T cells
ICAM	intracellular adhesion molecule 1
ICB	immune checkpoint blockade
ID2/3	inhibitor of DNA binding 2/3
IFN-I/II	type I/II interferons
IFN-γ/IFNG	interferon gamma
Ikzf1	IKAROS family zinc finger 1
IL	interleukin
IL7R	interleukin 7 receptor
IRF4	interferon regulatory factor 4
IPA	indole-3-propionic acid
ISG	interferon-stimulated genes

ITGAE	integrin subunit alpha E
KLF	Kruppel-like factors
Lag3	lymphocyte-activation gene 3
LAYN	Layilin
LEC	lymphatic endothelial cell
LGALS1	galectin 1
L. johnsonii	Lactobacillus johnsonii
LRRC15	leucine rich repeat containing 15
LTβRAg	lymphotoxin beta receptor agonist
LTs	leukotrienes
MAPK	mitogen-Activated Protein Kinase 1
MDSCs	myeloid-derived suppressor cells
MEK	also known as MAPKK (mitogen-activated protein kinase kinase)
MHC-I/II	major histocompatibility complex I/II
MKI67	marker of proliferation Ki-67
mTOR	mammalian target of rapamycin kinase
MYH11	myosin heavy chain 11
NFAT	nuclear factor of activated T-cells
NK	natural killer (cells)
NLT	non-lymphoid tissue
NOTCH	neurogenic locus notch homolog protein 1
Nr4a	nuclear receptor subfamily 4 A
OVCAR-3	(NIH) ovarian carcinoma cell line 3
PCV	post-capillary venule
PD-1	programmed death-1
PDACs	pancreatic ductal adenomas
Pdcd1	programmed cell death 1
PDGF	platelet-derived growth factor
PD-L1/2	programmed death-ligand 1
PRF1	perforin 1
PTPRC	protein tyrosine phosphatase receptor type C
RUNX3	runt-related transcription factor 3
S1PR1	sphingosine 1-phosphate receptor-1
scRNA-seq	single-cell RNA sequencing
SCS	subscapular sinuses
SLAMF6	SLAM family member 6
SLOs	secondary lymphoid organs
SPP1	secreted phosphoprotein 1
ST	spatial transcriptomic
STAT	signal transducer and activator of transcription
TA-HEV	tumour-associated high endothelial venules
TAM/Ns	tumour-associated macrophages/neutrophils
TCF1	transcription factor 1
T_{CM}	central memory cells
TCR-seq	T cell receptor sequencing
TDLNs	tumour-draining lymph nodes
T_{EFF}	effector $CD8^+$ T cells
T_{EM}	effector memory cells
T_{EMRA}	effector memory re-expressing CD45RA (terminally differentiated effector memory) $CD8^+$ T cells
T_{EX}	exhausted $CD8^+$ T cells
T_{EX}^{EFF}	effector-like exhausted
TFs	transcription factors
TGF-β	transforming growth factor beta
T_H	$CD4^+$ helper T cells
TIGIT	T cell immunoreceptor with Ig snd ITIM domains
TILs	tumour infiltrating lymphocytes
TIM3	T cell immunoglobulin mucin 3
T_{INT}	intermediate-exhausted phenotype
TLSs	tertiary lymphoid structures
TME	tumour microenvironment
T_{MEM}	memory $CD8^+$ T cells
T_N	naïve $CD8^+$ T cells
TNF-α	tumour necrosis factor alpha
TOX	thymocyte selection-associated HMG box
T_{PEX}	progenitor exhausted T cells
T_{REG}	($CD4^+$) regulatory T cells
T_{RM}	tissue-resident memory T cells
T_{SCM}	stem cell memory T cells
T_{TEX}	terminally exhausted $CD8^+$ T cells
WNT	wingless-related integration site (signalling pathway)
VCAM	vascular cell adhesion molecule
VEGF(R)	vascular endothelial growth factor (receptor)
VIM	vimentin
XCL1	X-C motif chemokine ligand 1
ZEB2	zinc finger E-box binding homeobox 2
ZNF683	zinc finger protein 683

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethical approval
This study did not involve the participation of human or animal subjects, and, therefore, was exempt from formal assessment by the ethics committee for clinical research of our centre.

Competing interests
The authors declare no competing interests.

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