The function and dysfunction of memory CD8$^+$ T cells in tumor immunity

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Abstract: The generation and maintenance of $CD8^+$ T cell memory is crucial to long-term host survival, yet the basic tenets of $CD8^+$ T cell immunity are still being established. Recent work has led to the discovery of tissue-resident memory cells and refined our understanding of the transcriptional and epigenetic basis of $CD8^+$ T cell differentiation and dysregulation. In parallel, the unprecedented clinical success of immunotherapy has galvanized an intense, global research effort to decipher and de-repress the anti-tumor response. However, the progress of immunotherapy is at a critical juncture, since the efficacy of immuno-oncology agents remains confined to a fraction of patients and often fails to provide durable benefit. Unlocking the potential of immunotherapy requires the design of strategies that both induce a potent effector response and reliably forge stable, functional memory T cell pools capable of protecting from recurrence or relapse. It is therefore essential that basic and emerging concepts of memory T cell biology are rapidly and faithfully transposed to advance therapeutic development in cancer immunotherapy. This review highlights seminal and recent reports in $CD8^+$ T cell memory and tumor immunology, and evaluates recent data from solid cancer specimens in the context of the key paradigms from pre-clinical models. We elucidate the potential significance of circulating effector cells poised downstream of neoantigen recognition and upstream of T cell dysfunction and propose that cells in this immunological ‘sweet spot’ may prolong survival and serve as the substrate for checkpoint blockade.

Naïve T cell activation

$CD8^+$ T cell responses are initiated in secondary lymphoid organs (SLOs) when naïve $CD8^+$ T cells (Tn) are activated by migratory dendritic cells (DC) presenting antigen-derived peptides loaded on major histocompatibility complex (MHC) class I molecules. Tn cells carrying epitope-specific T cell receptors (TCR) may undergo activation, dysfunction, survival or deletion, contingent upon the following interdependent variables: i) the cytokine/chemokine/metabolite milieu, ii) status of the dendritic cell (DC) (e.g. activation, co-stimulatory/adhesion molecule profile, tissue of origin), iii) TCR affinity for presented peptide, iv) epitope antigenicity (amino acid sequence, MHC binding affinity, concentration) v) presence/quality of $CD4^+$ T cell help and vi) duration and frequency of contact at the immunological synapse [1-5]. During acute viral infection, Tn recognize antigenic peptides presented by migratory DC that have sensed pathogen- or danger-associated molecular patterns (e.g. dsRNA via TLR3) and subsequently expressed co-stimulatory molecules (e.g. CD80/CD86, CD40L, OX40L, 41BBL, CD70). After receiving sufficient signal 1 (TCR signaling), signal 2 (co-stimulation e.g. CD80/CD86) and signal 3 (inflammatory cytokine e.g. IFNα/β, IFNγ, IL-2, IL-12, IL-21, IL-33, TNFα), $CD8^+$ T cells clonally expand and give rise to vast numbers of effector $CD8^+$ T cells (Teff). Teff subsequently migrate to the infected tissue through the bloodstream via chemokine receptor (e.g. CCR5) and adhesion molecule (e.g. LFA-1) interactions where they recognize their cognate peptide:MHC-I complex on target cells and exert cytolytic functions (secretion of perforin, GZMb, TNFα, IL-2, IFNγ) to lyse infected cells. Following the effector phase, 90-95% of Teff cells undergo apoptosis whilst a pool of clonally expanded, antigen-experienced cells persist to provide durable immunological memory [6]. Memory T cells are present at 10-100 times their precursor frequency, and bear a distinctive migratory, molecular,

epigenetic, metabolic, phenotypic and functional profile relative to Tn and Teff cells [6-8]. These properties enable memory T cells to traffic throughout the blood, SLOs and tissues in a quiescent state yet hyper-proliferate and elicit augmented effector responses during antigen re-encounter; thereby coordinating rapid pathogen elimination.

$CD8^+$ memory T cell generation

Several studies have suggested that T cells are programmed to become memory during the early stages of the priming phase [9]. In vaccinated humans, memory $CD8^+$ T cells arise from a rapidly dividing effector pool formed in the first 14 days post challenge, subsequent to re-engagement of naïve like chromatin landscapes [10]. Similarly, in the lymphocytic choriomeningitis virus (LCMV) model, long-lived memory $CD8^+$ T cells emerge from de-differentiation of fate-permissive Teff cells [11]. These findings concur with single cell RNAseq (scRNAseq) analysis of early $CD8^+$ T cell specification during adoptive transfer in the LCMV model, in which Teff and memory differentiation emerge from an early burst of transcriptional activity followed by epigenetic refinement [12]. Work in the *Listeria monocytogenes* and LCMV models have previously classified subsets of Teff cells based upon their ability to give rise to memory $CD8^+$ T cells. These precursor subsets are defined by differential expression of the IL-7 receptor (CD127) and the killer cell lectin-like receptor G1 (KLRG1). Memory precursor effector cells (MPEC; $CD127^{hi}KLRG1^{neg}$) are characterized by BCL2 expression, a longer lifespan and proliferative potential in response to homeostatic cytokines (IL-7/IL-15) or antigenic re-challenge, whilst short-lived effector cells (SLEC; $CD127^{lo}KLRG1^{hi}$) have a shorter lifespan and reduced homeostatic proliferative capacity [13-15]. The recent finding that effector differentiation precedes memory formation is complicit with this ‘separate precursor’ model, and the long-held

knowledge that memory potential is non-equivalent amongst Teff cells, since certain effectors may preferentially re-engage naïve like programs that specify memory fate. Although not necessarily contradictory, it is also noteworthy that production of memory $CD8^+$ T cells has also been reported to occur in the absence of an overt effector response [16].

Data from several infection models have shown that SLEC differentiation is favored by increased signal 1 (prolonged antigen exposure, affinity/avidity/concentration low intraclonal competition) and signal 3 (elevated inflammatory cytokine burden, IFNγ, IL-12 directly or via CXCR3-mediated trafficking to the infected site), whilst brief TCR stimulation, truncated infection periods (e.g. via administration of antibiotics), defects in inflammatory cytokine signaling, enhanced anti-inflammatory cytokine availability (e.g. TGFb, IL-10) or the presence of regulatory T cells promotes MPEC development or derivation of less differentiated memory subsets [15]. Costimulation via CD28-CD80/CD86 is also required during priming to prevent anergy and adaptive tolerance, whilst ligation of TNF super family receptors (TNFSRs) on $CD8^+$ T cells (CD27, OX40, 41BB, CD30) promotes proliferation, survival and enhances the quality of the recall response [17-20]. Similarly, ligation of HVEM receptor on $CD8^+$ T cells by BTLA (on CD8α DC) is required for Teff cell survival and development of protective immune memory in response to bacterial and viral infection, in part via promoting MPEC persistence [21]. Another key factor in the generation of memory $CD8^+$ T cells is $CD4^+$ T cell help. $CD8^+$ T cells primed in the absence of $CD4^+$ T cells have impaired long-term survival and display defective ability to respond against secondary challenge [22]. The mechanisms behind the requirement of $CD4^+$ T cells are not completely understood, however the interaction between CD40 on $CD8^+$ T cells with CD40L on $CD4^+$ T cells and the secretion of IL-15 from these cells have shown to be relevant in the generation Teff

cells with enhanced ability to become memory [23,24]. More recently, CTLA-4 on $CD4^+$ T regulatory (Treg) cells has been shown to force memory T cell quiescence, suggesting that helper and regulatory $CD4^+$ T cell subsets may be required for optimal memory $CD8^+$ T cell generation and homeostasis, respectively [25].

Circulating memory $CD8^+$ T cell subsets

Memory $CD8^+$ T cells are heterogeneous, and can be defined as one of four major subsets according to their surface markers, effector potential, stemness and ability to home lymphoid organs and non-lymphoid tissues (Figure 1). Circulating memory $CD8^+$ T cells can be classified as stem central memory (Tscm), central memory (Tcm) and effector memory (Tem), whereas memory $CD8^+$ T cells that become established within the infected/challenged tissue and do not re-circulate are termed tissue resident memory (Trm). Tscm cells are present in mouse, human and non-human primates and are endowed with the greatest stem potential of all memory subsets, allowing them to give rise to Tcm and Tem cell populations upon antigen stimulation[26]. Tscm cells have a naïve-like phenotype with low expression of CD44 (mouse), high levels of CD62L and co-express antigen-experienced $CD8^+$ T cells molecules such as CD122, the Stem Cell Antigen 1 (SCA-1), B cell lymphoma 2 (BCL-2), CXC-chemokine receptor 3 (CXCR3), and CD95 [26]. Tcm and Tem cells were originally described in mouse and human based on the expression of CD44, CCR7 and CD62L, and CD45RO and CCR7 respectively [27]. Tcm cells display reduced effector function and have a stem-cell-like phenotype given their ability to generate new Tem cells after antigen recognition [28]. In mice, Tcm cells are $CD44^+CD62L^+CCR7^+$ while in human these cells are $CD45RO^+CCR7^+$ (and $CD62L^+$). Expression of CCR7 and CD62L facilitate migration through the high endothelial venules (HEV) into secondary lymphoid organs, where

Tcm cells preferentially accumulate [28]. Tcm/Tscm cells show common transcriptomic, epigenetic and proteomic features (e.g. high basal STAT5) that cluster them separately from Tem cells [29]. In comparison, Tem cells are more differentiated, display a molecular fingerprint associated with Teff cell function (cytolytic Teff genes) and exhibit immediate effector function upon antigen re-encounter [30]. Mouse Tem cells have a $CD44^{+}CD62L^{-}$ phenotype, whilst human Tem cells are defined by $CD45RO^{+}CCR7^{-}$, with KLRG1 expression being common to Tem in both species [31]. In humans, the markers CD27 and CD28 can be used to further define circulating memory $CD8^{+}$ T cells. Both markers are expressed by naïve, Tscm and Tcm cells, whereas Tem cells can be divided into Tem 1 ($CD28^{+}CD27^{+}$), Tem 2 ($CD28^{+}CD27^{-}$ or $CD28^{-}CD27^{+}$), or Tem 3 ($CD28^{-}CD27^{-}$) that exhibit progressively enhanced effector potential *ex vivo* [32]. Terminal differentiation of human $CD8^{+}$ T cells is demarcated by re-expression of CD45RA within the Tem cell pool, giving rise to Temra cells (Terminally differentiated effector memory cells re-expressing CD45RA; $CCR7^{-}CD28^{-}CD27^{-}CD45RA^{+}$) [33]. Temra cells exhibit potent effector function, poor proliferative capacity, low IL-2 production and are enriched for phenotypic and functional (defective telomerase activity) traits of senescence [33]. One marker associated with Temra cells is CD57, which correlates with a history of extensive cell division, short telomeres, replicative senescence, ageing, cytomegalovirus (CMV) status, decreased *ex vivo* IFNγ but enhanced cytotoxic function (i.e. GZMb and perforin expression) [33]. Temra cells may also (re)express KLRG1, which is enriched in populations specific for viruses with latency periods [34]. Interestingly, $CD57^{+}KLRG1^{-}$ and $CD57^{+}KLRG1^{+}$ $CD8^{+}$ T cells retain effector function but the latter subset fail to proliferate and have diminished expression of CD27, CD28 and CD127, indicating more terminal differentiation [34].

Signals 1-3 form complex molecular circuitries, which enact key transcription factors (T-bet, Eomes, Blimp-1, Bcl-6, Tcf7, Foxo1) to determine precursor fate and memory $CD8^+$ T cells subset differentiation. These findings have been expertly reviewed elsewhere [6]. An oversimplified consolidation of this data is that strong TCR signals, IL-2 (inducing Tbet and BLIMP-1) and IL-12 (upregulating Tbet) favor Tem cell (and SLEC) differentiation, whilst abrupt signal 1, IL-21, IL-10, TCF7, FOXO1, EOMES and Bcl-6 support Tcm cell (and MPEC) specification, as summarized in [35]. Tcm cells express higher levels of the latter two transcription factors, require Bcl-6 and sustain Eomes expression via the Tcf-1-Wnt axis [36]. Together with augmented IL-7/IL-15-driven Stat5 phosphorylation and induction of Bcl-2 this forms a module which confers enhanced survival and self-renewal to Tcm/Tscm cells relative to Tem cells [37]. Transcriptional networks downstream of increased inflammation and TCR signaling (which favor Teff cell development during priming) in contrast drive Tem/Temra cell differentiation [15]. However, whether subset commitment depends on the malleability of a single naïve $CD8^+$ T cell population via alterations in TCR stimulation (signal strength model) or successive rounds of antigen exposure (decreasing potential model) has been contended [6,38,39]. It is noteworthy that a model in which repetitive antigen exposure drives stepwise Tscm>Tcm>Tem>Temra cell differentiation is supported by recent functional, transcriptomic and proteomic data and the redistribution of these subsets following chronic immune stimulation [8,40-42]. In accordance with this, $CD8^+$ T cells in healthy human blood are predominantly of a naïve phenotype (40%), Tem and Temra cells are present at approximately equal proportions (20-25%) and a minority are of a Tcm cell phenotype [43,44]. However, this is highly variable between donors and changes with age or antigen experience such that Temra cells (but also to an extent Tem and Tcm cells) gradually increase at the

expense of naïve pools [44]. This phenomenon of 'immunosenescence' is exemplified in chronic infection (e.g HIV), auto-inflammatory disease (e.g. Rheumatoid arthritis) and cancer, and can be tracked within antigen specific $CD8^+$ T cells (e.g. HIV, CMV, EBV), where progressive differentiation may result in clonal deletion [33]. It should be noted that, despite the discrete properties and phenotypes of memory $CD8^+$ T cell subsets observed in a variety of experimental and clinical settings, the concept of linear differentiation remains a framework imposed upon a likely fluid spectrum of cell fates; consequently, exceptions and regular revisions to this model are common and necessary. An additional layer of complexity is that phenotypes used to describe memory $CD8^+$ T cell subsets derive largely from analysis of resting cells in the circulation. Since activation *in vitro* and *in vivo* drastically affects expression of the majority of markers used to define classical subsets, application of this nomenclature in the context of an ongoing or experimental immune responses can be challenging [45].

Tissue resident memory $CD8^+$ T cells

Tem cells within tissues were historically considered to be circulatory, however tissue-reident memory $CD8^+$ T (Trm) cells were formally described in 2009 [46]. Trm cells have been shown to stably reside in the skin, lung, intestine, brain, female reproductive tract, salivary glands and others, where they provide rapid and potent protective immunity against re-infecting pathogens [46-52]. Trm cells are long-lived, mediate immediate protective immunity and are the most abundant T cell lineage in organisms with natural infection experience [53,54]. Phenotypically, Trm cells constitutively express CD69, integrin αE(CD103)β7 (commonly referred to as CD103) and are devoid of CD62L and CCR7 [55]. Given that CD103 is the ligand for E-cadherin, which is

expressed in epithelial cells, it has been proposed that CD103 is responsible for residency in epithelial tissues [56]. CD103 is also induced by TGFβ (which is key to Trm development) and competes for E-cadherin binding with KLRG1 creating a circuit in which TGFβ favors Trm cell abundance via induction of CD103 and interception of the KLRG1-E-cadherin axis [55,57]. CD69 upregulation abrogates tissue egress by degrading sphingosine 1-phosphate (S1P) receptor 1 (S1P1R), disabling $CD8^+$ T cells to respond to S1P gradients, which is highly abundant in blood and lymph [58]. Interestingly, Trm cells from unrelated tissues share a core transcriptional program that is different from Tem, Tcm and Tn cells, but may also diverge on the basis of auxiliary, tissue-specific gene expression characteristics reflective of the site of origin [55,59-61].

Several transcription factors are involved in the generation and maintenance of Trm cells. Downregulation of Eomes during Trm cell development is necessary for CD103 induction, whereas low levels of T-bet are necessary for the expression of IL-15 receptor (a key signal for the maintenance of these cells in the tissues) [55,62]. Furthermore, the Trm cell differentiation program is controlled by the expression of Blimp-1 and the homolog of Blimp-1 in T cells (Hobit) transcription factors together with downregulation of the transcription factor Krüppel-like factor 2 (Klf2), which represses the expression of S1PR1 (receptor for S1P) thereby inhibiting tissue egress [63,64]. RUNX3 was also recently described as a transcription factor required for the establishment of Trm cells in different tissues and solid tumors, operating via induction of tissue-residency genes and the suppression of loci related to tissue egress [65]. Trm cell commitment appears to be two stage process (Bcl-2 and CD69 induction followed by CD103 expression), and Tem as well as Tcm cells can give rise Trm cells in different tissues [55,66,67]. It should be noted that, in a similar manner to their impact on

Tem cells, $CD4^+$ T cell help has been shown to guide Trm cell formation [68].

Functionally, the positioning of Trm cells at sites of previous antigen encounter provides host organisms with a means of rapid response to reinfection and protection from reactivated latent viruses [69]. Upon antigen recognition, Trm cells likely mediate both immediate lytic activity via high constitutive production of GZMb and orchestrate an alarm state at the local tissue site, recruiting and activating NK cells, DC and other lymphocytes via secretion of IFNγ, IL-2 and TNFα [51,70,71]. Interestingly, Trm cells recruit Tem cells into the tissues in an IFNγ-dependent manner, potentially inducing bystander activation since recruited populations are $GZMb^+$ [51,72]. Two recent studies have extended these findings to show that Trm cell reactivation promotes their *in situ* local proliferation and the recruitment of new Trm cells into the tissues without displacement of pre-existing populations [72,73]. It is of relevance to tumor immunity and vaccination strategies that Trm cell induction is, intuitively, site specific. For example, cutaneous HSV infection establishes a virus-specific Trm cell pool at the challenge site, but not the contralateral flank, providing protection upon challenge in the former but not the latter [46]. Similarly heterosubtypic (cross strain) protection from influenza virus can be achieved by influenza-specific lung Trm cells generated through intranasal live attenuated influenza but not systemic administration of injectable inactivated or live attenuated influenza [74]. Intriguingly, although Trm cells in various barriers sites are maintained by IL-15, their turnover and persistence also appears to be tissue and/or context-specific [55]. Trm cells in multiple target tissues have been reported to exhibit extended life spans [59,75]. However, unlike the skin, lung Trm cells undergo rapid turnover, with attrition after infection being partly counterbalanced by ongoing recruitment from the circulation [66].

In humans, pioneering work to produce a spatial map of T cells in tissues using brain dead organ donors has illustrated that blood and lymph nodes have a diffuse distribution of naïve (most abundant)>Temra/Tem>Tcm (least abundant) subsets, whereas the spleen and lungs contain mainly Tem and Temra cells and in the Jejunum, Ileum and Colon are predominantly of a Temra cell phentoype (approx. 80%) [76]. Interestingly, CD103 expression was preferentially localized to the $CD45RO^+$ fraction of $CD8^+$ T cells (in the Jejunum, Ileum, colon and lung), whilst Temra cells were largely, but not entirely $CD103^-$ [76]. Only a small fraction of Trm cells produce IFNγ or IL-2 following stimulation with PMA and Ionomycin (PMA/Io), thus the full scope and magnitude of effector function in human Trm cells is likely under appreciated [43,76]. Subsequent work by the same group demonstrated that a shift towards more differentiated phenotypes (Tem cells > Temra cells, increased $\%CD57^+$ cells) occurred as a function of viral specificity, age and /or CMV status in both Trm cell and circulatory compartments [76,77]. Of relevance, work in clinical samples unveiled that lung-derived Trm cells but not skin or circulating $CD8^+$ T cells elicit polyfunctional responses to influenza challenge, confirming tissue-specific immunity of Trm cells seen *in vivo* is common to humans [78,79] .

Memory $CD8^+$ T cells and immune homeostasis

Genetic, pharmacological or pathogen-derived memory $CD8^+$ T cell deficiency or dysfunction renders the host susceptible to potentially fatal opportunistic infection and tumor development, whilst de-restricted Teff cell responses precipitate lethal autoimmunity, allergy or inflammatory tissue destruction. There is therefore strong evolutionary pressure to develop tightly regulated, multilateral mechanisms of immune homeostasis. In the memory $CD8^+$ T cell pool, immune homeostasis is orchestrated

291 in several layers. The overall size of the CD8$^+$ memory T cell pool is maintained by
292 balancing attrition with compensatory homeostatic proliferation driven by IL-7 and IL-
293 15 [80,81]. These cytokines reconstitute lymphopenic hosts by peripheral expansion
294 which simultaneously converts naïve and Tcm cells to a Tem-like 'memory phenotype'
295 with augmented effector potential [82,83]. Memory CD8$^+$ T cells are also restrained by a
296 myriad of T cell intrinsic and extrinsic regulators of effector function including Treg
297 cells, intracellular quiescence factors [25,84-87], cell surface proteins involved in ATP
298 hydrolysis (CD38, CD39, CD73) [88], antigen presenting cell-derived IFNγ-inducible
299 catabolic enzymes (i.e. IDO) [89], nitric oxide [90], arginase 1 [91], prostaglandin E2 and
300 anti-inflammatory cytokines (TGFβ, IL-10, VEGF, IL-35) [91,92] and T cell inhibitory
301 receptors (TCIR), many of which are currently targeted or under investigation in
302 immune oncology. The latter include well characterized receptors who's cognate
303 ligands are expressed on various cells in the tumor microenvironment (TME) and
304 lymph nodes, such as PD-1 (PD-L-1/PDL-2; antigen presenting cells (APC), tumor
305 cells or epithelial cells), CTLA-4 (CD80/86 on professional APC), Tim-3 (galectin-9 on
306 APC and tumor cells), and LAG-3 (MHC-II on APC) [93]. The abundance of TCIRs is
307 restricted to activated CD8$^+$ T cells, and terminally differentiated Tem/Temra cells,
308 whilst their ligands are found on activated APC or epithelial cells, illustrating spatial
309 and temporal restriction to balance immunity and tolerance.

310 **Memory CD8$^+$ T cell dysregulation**

311 Perturbation of signals 1, 2 or 3 can dysregulate memory CD8$^+$ T cell responses. This
312 includes the onset of self-tolerance and anergy; two differentiation programs that
313 share an overlapping molecular basis which manifests in hypo-responsiveness to self-
314 peptide [94]. The deletion of autoreactive T cell clones during central tolerance is

incomplete. Therefore, peripheral self-tolerance is a necessary evolutionary strategy that prevents autoimmunity via inhibition of effector responses to cognate antigen following sub-optimal co-stimulation (i.e. in the absence of DAMP/PAMP signalling on APC). Context and system-dependent differences (including cytokine environment and TCR avidity) may bring about variable degrees of hyporesponsiveness, altering the requirement for antigen persistence, as well as the magnitude or co-occurrence of defects seen in cytokine production/proliferation, in some instances leading to T cell deletion [94-99]. Self-tolerance may also result from induction of TCIRs, via suppression from immune regulatory cell populations (e.g. Treg cells) or the action of anti-inflammatory cytokines/cc (e.g. IL-10) [94,98]. *In vivo*, tolerance can be rescued by IL-2, IL-7 or lymphopenia, but this occurs transiently with resumption of tolerance occurring in the absence of antigen, suggesting commitment to an epigenetically programmed tolerogenic cell fate [98]. Similar to self-tolerance, stimulation of T cell clones with antigen or anti-CD3 in the absence of costimulation *in vitro* results in proliferative inhibition via a process termed anergy [100], which is rescuable via addition of exogenous cytokines [101,102]. However, it has been suggested that anergy and tolerance can be discriminated on the basis of functional and molecular characteristics, despite overlapping features [94]. Self-tolerance is engaged through a $CD8^+$ T cell intrinsic gene expression profile distinct to naïve or memory $CD8^+$ T cells. Relative to memory $CD8^+$ T cells, tolerant $CD8^+$ T cells exhibit enhanced expression of TCIRs (e.g. LAG-3), transcriptional repressors (EGR1/2, DUSP2), loss of key transcription factors (EOMES, T-BET, GATA-3), diminished expression of cytokine receptors and chemokine receptors (e.g. IL12RB1, CXCR3, CCR5) and crucially, lack of effector genes induction (e.g. IFNγ, PRF1) [94]. *In vitro* anergy induces NFAT in the absence of AP-1, leading to NFAT homodimers that induce Egr2, Ikaros, E2F

transcription factors and the E3 ubiquitin ligase family which inhibit IL-2, TNFα, IFNγ and other effector genes [103]. Models of *in vivo* anergy are associated with defective calcium signaling and nuclear translocation of NFAT2 in the absence of NFAT1 leading to anergy-associated gene expression [104].

$CD8^+$ T cells experience persistent antigen exposure in a range of pathologies and microenvironments resulting in the onset of an unconventional cell fate often described as T cell exhaustion [105]. During acute viral infection, host $CD8^+$ T cell responses clear pathogen during the effector phase, contract and form functional memory $CD8^+$ T cells. A failure to rapidly eliminate pathogen results in chronic infection, associated with unremitting antigen load and high levels of inflammation that drives exhaustion. Seminal studies using the LCMV clone 13 mouse model of chronic viral infection led to the prototypic description of exhaustion as a state of T cell hyporesponsiveness [106-108]. Despite common misconceptions, exhausted T (Tex) cells are not entirely devoid of effector function, since they contribute to viral control [109]. Rather, Tex cells exhibit a broad spectrum of dysfunctional states, characterised by stepwise loss of i) IL-2 production ii) *in vitro* cytotoxicity iii) IFNγ/TNFα production, iv) degranulation and in some instances ultimately v) physical deletion [94,108,110,111]. Progression to a terminal Tex cell fate coincides with altered metabolism and broad expression of TCIRs including, PD-1, CTLA-4, LAG-3, CD160, BTLA and Tim-3 [112-114]. The severity of exhaustion has been further defined by altered transcription factor expression. In the LCMV clone 13 infection model, a circulating progenitor pool of TNFα, and IFNγ-producing $EOMES^{lo}PD\text{-}1^{int}$ Tex cells gave rise to a tissue homing, poorly proliferative, but cytotoxic $EOMES^{hi}Tbet^{lo}PD\text{-}1^{hi}$ Tex cell progeny upon antigen restimulation [115]. Given that T-bet represses PD-1, LAG-3 and other TCIR in Teff cells, loss of this transcription factor marks transition into severe exhaustion that facilitates increased

365 negative signaling [115]. Conversely, NFAT signaling enhances the expression of PD-1
366 and Tim-3 [116]; thus, a balance between T-bet and NFAT may be crucial determinants
367 of the TCIR profile of Tex cells. Interestingly BLIMP-1 and BATF also appear to display
368 a distinct-context-dependent role in Tex cells; the former is correlated with TCIR
369 expression but is necessary for GZMb expression, whilst the latter is induced by PD-
370 1 signaling to suppress effector function [117,118]. Thus, in chronic viral infections there
371 is a progenitor subset of Tex cells whose function is supported by T-bet which may
372 stall severe exhaustion, whilst in progressively exhausted $CD8^+$ T cells BLIMP-1 and
373 EOMES provide residual cytotoxic function whilst BATF and NFAT limit effector
374 potential [119]. It is also of note that the NFAT-EGR2 axis appears central in anergy, and
375 thus may be a master regulator of T cell hyporesponsiveness [116]. Targeting TCIRs
376 with blocking antibodies has been suggested to reverse exhaustion in chronic infection
377 and tumors, however this appears to be stage and to an extent system-dependent. In
378 LCMV chronic infection, targeting Tim-3 and PD-1 synergistically restores effector
379 function of $CD8^+$ T cells [120]. It has also been suggested that there is a differential
380 sensitivity amongst $Tbet^{hi}PD\text{-}1^{int}EOMES^{lo}$ (reversible Tex cell phenotype) and
381 $Tbet^{neg}PD\text{-}1^{hi}EOMES^{hi}$ (irreversible Tex cell phenotype) subsets to PD-L1 blockade in
382 LCMV chronic infection [115,121]. Similar to what has been proposed in tolerance, chronic
383 infection appears to impose epigenetic re-programming associated with T cell
384 exhaustion[122,123]. In this module transcription factors, cytokine and TCR signaling loci
385 appear in closed chromatin conformations at later stages of infection coincident with
386 increased accessibility of the PD-1 locus [124,125]. It has been suggested that this
387 epigenetically fixed state of $CD8^+$ T cell dysfunction is accountable for checkpoint
388 blockade activity[126]. In agreement with this, two recent reports showed that i) PD-L1
389 blockade in the LCMV infection model only transiently engaged effector transcriptional

circuitry but did not alter the epigenetic landscape of Tex cells or induce functional memory T cells and ii) determined a specific epigenetic basis of Tex cells in murine and human chronic viral infections [122,123]. Indeed, Tex cells have been widely described in chronic viral infection of higher primates, including humans with Hepatits C virus (HCV) infection, Hepatitis B virus (HBV) induced-hepatitis and both simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) infection [127]. $CD8^+$ T cells in chronic SIV and HIV exhibit cardinal phenotypic (TCIR expression), functional and molecular features of exhaustion described above. HIV-specific resting and activated $CD8^+$ T cells showed a $Tbet^{int}EOMES^{hi}$ population marked with multiple TCIRs, corresponding to the severely exhausted T cells found in LCMV chronic viral infection models [128], whilst CMV-specific $CD8^+$ T cells showed balanced EOMES and Tbet expression. HIV-specific $Tbet^{int}EOMES^{hi}$ $CD8^+$ T cells exhibited a Tem1 cell phenotype with poor effector function, and persisted long after anti-retroviral therapy initiation suggesting that exhaustion was not reversed and that these cells may remain long after removal of high antigen load in humans [128]. Another report classified $CD8^+$ T cells of HIV patients based on EOMES and CD57 expression showing that $EOMES^{int}CD57^+(Tbet^{hi}GZMb^+PRF^+)$ cells retained functionality and correlated with HIV control, whereas $EOMES^{hi}CD57^+(Tbet^{int}GZMb^{int}PRF^{lo})$ cells were dysfunctional [129]. A subsequent study showed that the frequency of activated/Tex cells ($CD38^+PD\text{-}1^+$) correlated with viral load in plasma and rapid clinical progression in HIV infection [130]. However, in line with findings in the LCMV model, it seems that Tex cells in chronic SIV and HIV infection may exert residual cytolytic function to contribute to viral control, since their depletion leads to virus rebound/disease progression in SIV [131,132]. $CD8^+$ T cell exhaustion therefore appears to result from the convergence of chronic antigen stimulation and inflammation, leading to augmented TCIR signalling, *de novo* gene

415 expression, dysregulation of $CD8^+$ T cell transcription factor networks and epigenetic
416 reprogramming.

417 As mentioned above, over-differentiation or immunosenescence of the $CD8^+$ T cell
418 memory compartment is observed in ageing, chronic infection and cancer; resulting in
419 elevated apoptosis in addition to increased frequencies of terminally differentiated
420 memory $CD8^+$ T cells (e.g. Temra cells) at the expense of progenitor pools (naïve
421 $CD8^+$ T cells, Tscm cells and Tcm cells) that manifests in defective immune memory
422 [33]. This accelerated ageing of the immune system has been shown to accompany T
423 cell senescence, a triphasic process of cell cycle arrest that occurs following DNA
424 damage (either via insult such as irradiation or through exposure of DNA via telomere
425 erosion) (Phase 1) and involves a DNA damage response (Phase 2) and growth arrest
426 (Phase 3) [133]. In senescent $CD8^+$ T (Ts) cells this process involves signaling via p53,
427 MAPK, p38, and CDK inhibitors, and is linked to progressive differentiation as marked
428 by CD57, KLRG1, loss of CD27 and CD28 expression and re-expression of CD45RA
429 [133]. However, distinguishing highly polyfunctional, pre-senescent $CD8^+$ T cells
430 (including $CD57^+$ cytotoxic $CD8^+$ T cells and CD45RA re-expressing Temra cells)
431 appears challenging and relies on KLRG1 and CD57 dual expression as a minimum
432 [34]. Loss of telomerase, BCL-2 and phosphorylation of AKT^{Ser473} may also mark truly
433 senescent $CD8^+$ T cell populations [134]. Senescent $CD8^+$ T cells are sustained by IL-
434 15 to persist *in vivo* and home to inflamed tissues, through interaction with ICAM-1,
435 the extracellular matrix and Fractalkine (CCR7-,CD62L-,CD11a+CD18+, CD49e+,
436 CX3CR1+). Most crucially, when stimulated with appropriate APC/co-stimulatory
437 signals (41BBL) these cells down regulate CD45RA, become activated, proliferate and
438 mediate potent cytotoxic effector function including IFNγ, TNFα and a reduced amount
439 of IL-2 [135].

Similar to Tex cells, Ts cells share a loss of proliferation and IL-2 production accompanied by high TCIR expression but these programs differ in many features [133]. Ts differentiation is associated with CD45RA re-expression, expression of CD57 and KLRG1, the acquisition of enhanced IFNγ, TNFα, cytotoxicity, shortened telomeres and reduced telomerase activity (many of which are also linked to pre-senescence) [133]. In contrast**,** Tex cells have been described as $CD57^-$, $KLRG1^-$ exhibiting progressive loss of effector function given by their low expression of IFNγ and TNFα. Finally, the epigenetic status of Tex and Temra cells is divergent, with the IFNγ locus being hyper and hypo-methylated, respectively[33,105,133,136,137].

The inception and inhibition of anti-tumor immunity

The unprecedented survival rates achieved with checkpoint blockade have fueled renewed optimism in cancer immunotherapy. However, only a minority of patients are sensitive to treatment and few experience durable clinical benefit [138]. Key correlates of a therapeutic response to checkpoint inhibition include high tumor mutational burden (TMB) and T cell infiltration; implying that mutation-encoded neoepitopes serve as a substrate for tumor specific Teff cells and that these neoantigen reactive T (NART) cells are actively suppressed by the targeted TCIRs [139-142]. However, the increasingly appreciated transience of Teff cell reinvigoration and prevalence of relapse collectively signify a defect in durable immune memory post checkpoint blockade. As a field, we have therefore failed to design immunotherapeutic strategies that reliably forge stable, functional memory T cell pools capable of protecting from recurrence, indicating a lack of essential knowledge in the ontogeny and dysregulation of anti-tumor T cell responses. Pioneering studies have shown that cross-presentation by tumor resident DC and direct presentation on tumor cells can prime $CD8^+$ T cell cells at the tumor site, eliciting an efficient anti-tumor immune response in the absence

of lymph nodes [143]. More recently however, it has been proposed that tumor antigens are most often presented in the tumor-draining lymph-nodes by migratory DC derived from the tumor site [144,145]. Following priming, formation of functional immune memory in the presence of chronic viral and tumor antigen is impaired, the basis of which remains only partly understood[146]. T cell extrinsic barriers of Teff and memory $CD8^+$ T cell function in anti-tumor immunity likely include i) inefficient priming (insufficient antigen load via low mutation rate, a high sub-clonal neoantigen burden and/or poorly expressed tumor antigens, similarity of tumor epitopes to self-peptides, inability of epitopes to bind HLA, lack of danger signals low levels of co-stimulation or inflammatory cytokines/chemokines, checkpoint ligand expression or tolerogenic function(s) of APCs and lack of $CD4^+$ T cell help) ii) local regulatory cell suppression (Tregs, myeloid-derived suppressor cells, tumor associated macrophages, cancer associated fibroblasts), iii) soluble inhibitory factors in the TME (e.g. TGFβ, IL-10, reactive oxygen species) iv) tumor-intrinsic resistance (expression of anti-apoptotic molecules, mutations in tumor antigen processing/presentation machinery and IFNγ signaling, down-regulation or loss of heterozygosity of MHC alleles), loss of neoantigens, inhibitors of cytolytic compounds, expression of FASL and checkpoint ligands) v) physical exclusion of T cells from the tumor vi) Metabolic and hypoxic constitution of the TME [147-150]. T cell intrinsic hurdles to generate a functional tumor specific memory T cell pool include: i) the deletion of tumor-associated self-antigens and potentially NART cells by central tolerance ii) low avidity TCR-peptide:MHC interactions iii) increased sensitivity of T cells to apoptosis, iv) inability to migrate to the tumor site, v) TCIR expression and vi) T cell dysfunction (anergy, tolerance, exhaustion) [94]. The anti-tumor immune response therefore shares common features with those discussed above for chronic viral infection. This includes prolonged antigen

stimulation, a predominance of T cell inhibitory networks, and regulatory cell expansion[94]. Some key differences in anti-tumor immunity include priming conditions, where lower antigenicity of self- or modestly altered non-self peptides, the absence of danger signals to activate APC and the initial lack of inflammation, collectively there are reduced signals 1, 2 and 3 in cancers compared to viral infection. Thus, the context of tumor-specific T cell priming in early disease is similar to conditions conducive to tolerance, yet consequent antigen chronicity and increased inflammation thereafter recapitulate cardinal aspects of exhaustion[94,98,151,152]. It is therefore unsurprising that T cells exhibiting TCIR expression, defective cytokine production, altered cytokine production, modulated TCR signalling and epigenetic reprogramming have been widely observed in experimental and clinical settings of cancer[105,153].

Memory CD8$^+$ T cells in tumor immunity: Pre-clinical models

CD8$^+$ T cells in pre-clinical cancer models exhibit profound TCIR expression and are typically unable to reject even highly immunogenic tumors. However, experimental interventions such as checkpoint blockade, adoptive cell therapy, vaccination or induced lymphopenia can lead to tumor regression via inhibition of suppressive signals, delivering agonistic co-stimulation or cytokine signals, depletion of Tregs or provision of Teff cells devoid of inhibitory receptors [154,155]. Given phenotypic, functional and transcriptomic similarities, it has been proposed that an overlapping program of T cell dysfunction occurs in tumors similar to T cell exhaustion seen in chronic viral infection. For example, the co-expression of PD-1 and Tim-3 has been used to define dysfunctional tumor infiltrating CD8$^+$ T cells in colon and mammary mouse tumors, where the blockade of these two molecules restores the functionality of CD8$^+$ T cells [156]. Another study has shown that co-blockade of TIGIT and PDL-1 can resurrect functionality of intratumoral CD8$^+$ T cells [157]. However, targeting of different

515 checkpoints clearly elicits tumor regression via divergent mechanisms that may not
516 always reflect reversal of dysfunction. Indeed, whilst α-PD-1 treatment specifically
517 induces the expansion of PD-1^{hi}TIM-3^{+}CD8^{+} T cells inside the tumor (which may point
518 to either transient rewiring of effector machinery or disengagement of CD8 T cell
519 dysfunction), α-CTLA-4 treatment induces the proliferation of peripheral ICOS^{+} Th1
520 CD4^{+} and CD8^{+} T cells and depletes Tregs, suggesting that these agents i) have vastly
521 different mechanisms of action beyond antagonism ii) de-repress CD8^{+} T cells via
522 disrupting CD8^{+} T cell intrinsic (PD-1) and/or CD8^{+} T cell extrinsic (Treg cell depletion)
523 regulation iii) mobilize independent memory or Teff cell subsets and thus iv) may
524 exhibit a differential impact on CD4^{+} and CD8^{+} T cell memory induction [155]. Loss of
525 effective CD8^{+} T cell responses in tumors also appears to involve transcription factor
526 dysregulation. Rescue of T-bet and EOMES phosphorylation was seen concomitant
527 with tumor clearance following α-PD-1 combined with α-CTLA-4 in a CT26 GVAX
528 tumor model [158]. Ablation of the key anergy gene *Ikaros* induced tumor rejection in a
529 melanoma model and loss of the transcription factor MAF augmented anti-tumor
530 responses in established melanoma [159,160]. More recently, the transcription factor Egr2
531 (implicated in T cell anergy) together with LAG-3 and 4-1BB expression was used to
532 define dysfunctional CD8^{+} T cells in the tumor microenvironment that can be
533 reactivated using blocking antibodies against these two molecules [127]. Furthermore,
534 these dysfunctional LAG-3^{+}4-1BB^{+}CD8^{+} T cells expressed a wide range of inhibitory
535 and stimulatory receptors including 2B4, TIGIT, CD160, CTLA-4, OX-40, GITR, NRP1
536 and ICOS and downregulated the IL-7 receptor which is essential for memory CD8^{+} T
537 cell survival [13]. The ATPase CD39 has been recently used to define exhausted CD8^{+}
538 T cells in a mouse model as well as in melanoma and breast cancer patients. Tumor-
539 infiltrating CD39^{hi}CD8^{+} T cells produce less TNF-α, IL-2 and express more PD-1, Tim-

3, LAG-3, TIGIT and 2B4 compared with the $CD39^{int}$ and $CD39^{neg}$ tumor infiltrating $CD8^+$ T cells [161]. In addition to TCIRs, several immunosuppressive and pro-tumorogenic factors, including adenosine, indoleamine 2,3 dyoxigenase (IDO), vascular endothelial growth factor (VEGF), type I interferons, glucose, Treg cells and myeloid-derived suppressor cells (MDSC) have been implicated in $CD8^+$ T cell exhaustion or dysfunction [153]. However, direct evidence for the role of these factors in mouse tumor models is scarce. VEGF has been shown to induce an exhausted phenotype in tumor infiltrating $CD8^+$ T cells characterized by the expression of several inhibitory receptors including PD-1, CTLA-4 and TIM-3. Interestingly, VEGF blocking antibodies synergize with α-PD-1 antibodies promoting $CD8^+$ T cell reinvigoration and slowing tumor growth [162]. Treg cells induce a dysfunctional state of tumor-infiltrating DC, promoting the induction of $PD\text{-}1^+TIM\text{-}3^+$ exhausted $CD8^+$ T cells that produced lower amounts of IFN-γ and TNF-α inside the tumor [163]. Thus, TCIR expression appears to identify tumor reactive T cells that experience negative TCIR signaling, transcription factor dysregulation, loss of cytokine-mediated homeostasis and extrinsic regulation, with checkpoint inhibition (CPI) eliciting anti-tumor responses by inducing heterogeneous effector T cell pools via interception of several pathways. However, this evidence does not elucidate the inception of tumor specific T cell dysfunction.

Several recent studies have used inducible experimental neoantigen expression in tissues to model the physiology of tumorigenesis. Elegant work demonstrated that chronic neoantigen stimulation induced biphasic tumor-specific T cell dysfunction that is initiated in early tumorigensis. Using an inducible SV40 T antigen, it was shown that neoepitope exposure resulted in first a plastic (Day 8) then irreversibly fixed (day 35) state which could not be rescued *in vitro* via IL-2 or anti-PD-1 [152]. More specifically, *in*

565 *vivo*, at day 35 post neoantigen induction, neoantigen-specific T cells showed
566 enhanced T-BET and Ki67 following anti-PD-1/PDL-1 but no reinvigoration of IFNγ or
567 TNFα production [152]. Importantly, immunization of mice with epitopes for two TCR-
568 transgenic $CD8^+$ T cell clones elicited comparable effector responses and migration to
569 the TME for corresponding adoptively co-transferred cells. However, cells specific for
570 the persistently (but not transiently) expressed neoantigen selectively developed
571 dysfunction; demonstrating that chronic neoantigen exposure rather than elements of
572 the TME were the key drivers of dysfunction [152]. Molecular analysis of these cells
573 illustrated that an overlapping but not identical transcriptional profile existed for chronic
574 viral infection and tumor-specific dysfunctional cells. However, importantly, context-
575 specific differences were evident and tumor-specific $CD8^+$ T cells also shared common
576 gene signatures with tolerised $CD8^+$ T cells. The molecular basis of the aberrant
577 response showed that, relative to Teff cells, late dysfunctional cells exhibited
578 diminished key effector and memory transcription factors (*Eomes*, *Tbet*), with
579 progressive loss of genes involved in memory differentiation (*Tcf7*, *Foxo1*) and
580 attenuated expression of regulators of Trm cell fate commitment (*Klf2*, *S1pr1*) whilst
581 at day 8 Teff cells up-regulated anergy or hypofunction related loci (*Egr2*, *E2f1*, *E2f2*)
582 [152,164]. At Day 34 memory $CD8^+$ T cells upregulated multiple genes that were also
583 enriched in late stage patient melanoma samples. These included transcription factors
584 (e.g. *Blimp-1*, *Batf*, *Dusp1*), TCIR (*Ctla4*, *Lag3*, *CD137*) and down regulation of
585 memory and quiescence factors (*Tcf7*, *Foxo1*, *Bach2*) [152]. Another difference to the
586 LCMV chronic infection model was the progressive loss of both *Tbet* and *Eomes*
587 expression in the tumor-specific dysfunction setting, which differed from the switch of
588 $Tbet^{hi}PD\text{-}1^{int}$ into $Tbet^{lo}$ $EOMES^{hi}PD\text{-}1^{hi}$ discussed in previous sections of this review
589 [115,152]. Given that loss of EOMES and T-bet are necessary for CD103 and IL-15R

590 expression in Trm development (see **Tissue resident memory CD8$^+$ T cells** section
591 above), this observation may reflect activity of the Trm program in solid tumors.

592 Since CD8$^+$ T cells in inducible neoantigen cancer models exhibit a truncated effector
593 phase, it remains possible that memory CD8$^+$ T cell generation and CD8$^+$ T cell
594 dysfunction occur in the absence of canonical fate commitment, and that memory cells
595 are formed without complete effector de-differentiation [11]. Results from these models
596 suggest that tumor-specific CD8$^+$ T cell dysfunction represents a unique program of
597 differentiation, distinguishable from acute/chronic infection, or tolerance that is caused
598 by chronic neoantigen exposure in the TME. How this molecular program of
599 dysfunction is altered in models testing neoepitopes derived from mutated self-
600 proteins (that may have a broad range of affinities) remains to be seen. Work from
601 Schietinger's group has subsequently shown that the irreversible dysfunction in this
602 model is linked with epigenetic reprogramming and a fixed chromatin state [151]. In this
603 report, changes in epigenetic landscape occur during the first 14 days (plastic state)
604 and not thereafter (fixed state), whilst PD-1 expression steadily increases. The fixed
605 state was consistent with inaccessible enhancer regions at the *Ifng* and *Tcf* family loci
606 whilst accessibility to the *Pdcd1* locus and predicted NFATC1- binding sites of anergy-
607 inducing (*Egr1/2*) or TCIR-encoding loci was increased [151]. A crucial finding in this
608 report was that adoptively transferred memory CD8$^+$ T cells also underwent rapid
609 dysfunction upon neoantigen exposure, implying that even the development of
610 functional memory may not overcome tumor-specific CD8$^+$ T cell dysfunction. The
611 fixed chromatin state was also seen in human tumor infiltrating lymphocytes (TIL) from
612 melanoma and NSCLC (a common feature between species being altered *Tcf7*
613 accessibility) and correlated with the presence of surface markers, including co-
614 expression CD38 and CD101, which marked cells unable to respond to stimulation,

615 although a minor subset in these cultures were still able to produce cytokine (i.e. the
616 CD38-CD101- cells). Treatment of dysfunctional $CD8^+$ T cells with IL-15 *in vitro* did
617 not reverse dysfunction, however it has been shown that IL-15 only epigenetically
618 altered specific loci (*Tcf7)* in $CD8^+$ T cells that convert from a Tscm/Tcm to Tem cell
619 phenotype during homeostatic proliferation [30], and thus intuitively would be insufficient
620 to completely reverse dysfunction. In line with both of these findings, IL-15 has been
621 shown to sustain rather than reverse exhausted $CD8^+$ T cell pools at the tumor site
622 [165].

623 A combined inference of work in mouse models of cancer is therefore that chronic
624 antigen stimulation and negative co-inhibitory signaling appear to produce a positive
625 feedback loop reinforcing tumor specific $CD8^+$ T cell dysfunction to a stabilized
626 epigenetic state of CPI non-responsiveness. Moreover, where effective, the
627 antagonism of single or multiple negative signaling cascades (e.g. the PD-1:BATF
628 module) may not re-shape Tex cells *per se* but offer transient reprieve from one of the
629 central orchestrators of the dysfunctional program, without altering remodeled
630 chromatin, as demonstrated in the mouse model of LCMV [122]. This theme may be
631 imperative to improving long-term memory T cell responses. It is perhaps of crucial
632 relevance that murine dysfunctional NART cells had gene expression profiles that
633 showed considerable overlap with MART-1 specific $CD8^+$ T cells isolated from late
634 stage human cancers [152]. This speaks to a vast amount of data attesting that tumor
635 specific $CD8^+$ T cell dysfunction is also a major feature and therapeutically critical facet
636 of T cells in human cancer.

637 **Memory T cell subsets in tumor immunity: Studies in clinical samples**

TILs isolated from colon, renal, lung, ovarian, bladder and melanoma tumors have been phenotyped using various combinations of markers to define activation status (e.g. HLA-DR, CD38, Ki67), cytotoxicity (PRF, GZMb), transcription factor profile (EOMES, Tbet), tissue residency (CD69, CD103) and linear differentiation (CD45RA, CCR7, CD27, CD28) [166,167]. The majority of tumor infiltrating $CD8^+$ T cells exhibit dysfunction-associated phenotypes, including broad and intense TCIR expression (e.g.PD-1, LAG-3, TIM-3 and TIGIT) [105]. For example, in clear cell renal cell carcinoma TILs exhibited increased markers of residency (CD69), activation (CD38) and TCIR (ICOS, LAG-3, PD-1, TIM-3) relative to T cells in normal tissue [167]. In non-small cell lung cancer (NSCLC) the frequency of $GZMb^+CD8^+$ T cells in early lung adenocarcinoma was decreased relative to adjacent lung, whilst $CD8^+PD\text{-}1^+$ T cell frequency was increased [166]. A separate study in early stage NSCLC revealed that relative to adjacent tissue, tumor lesions contained increased activated ($HLA\text{-}DR^+$), Tem cells that co-expressed PD-1, Tim-3, CTLA-4, LAG-3 and TIGIT that were largely $KLRG1^-CD127^-$, with $PD\text{-}1^+$ cells specifically enriched for activation markers and TCIRs (TGIT, TIM-3, CD137, CD38 and Ki67), but displaying lower EOMES expression [168]. In this investigation, increased activation of $CD8^+$ T cells was observed relative to normal tissue, and to a greater extent in current or ex-smokers compared to never smokers. Despite TCIR expression, $CD8^+$ T cells appeared capable of synthesizing IFNγ and IL-2 in response to synthetic stimuli (PMA/Io) and IL-2 in response to autologous tumor antigens, suggesting that $CD8^+$ T cells may be preferentially activated in response to mutagens but that functional competence is retained or can be recovered at the tumor site by at least a subset of cells [168]. In terms of linear differentiation Kargl et al. showed that $CD8^+$ TIL in NSCLC were predominantly Tem, with a smaller population of Temra cells and that lung

adenocarcinoma (LUSC) had a higher Temra to Tem cell ratio compared to LUSC [169]. $CD8^+$ T cells from NSCLC in a second report were shown to be of a Tem or Temra cell phenotype and able to produce IFNγ and IL-2 upon PMA/Ionomycin stimulation following IL-2 pre-treatment [170]. In melanoma, two reports showed that $CD8^+$ T cells were largely $CD45RO^+CCR7^-CD27^+CD28^+$ (Tem1) though a smaller Temra cell population were present [171]. Moreover, in patients with advanced melanoma, NY-ESO-1-specific memory $CD8^+$ T cells displayed a dysfunctional phenotype ($CD45RO^+CCR7^-TIM\text{-}3^+PD\text{-}1^+$) and lower *in vitro* production of IFN-γ, TNF-α and lL-2 compared to $TIM\text{-}3^-PD\text{-}1^+$ and $TIM\text{-}3^-PD\text{-}1^-$ $CD8^+$ T cells [172,173]. In clinical specimens, activation markers, TCIRs expression and a Tem cell phenotype therefore appear to be associated with exposure to, or specificity for tumor antigens.

Intriguingly, a report by Baitsch et al, showed that virus-specific and vaccine-induced $CD8^+$ T cells specific to Melan-A/MART-1 melanoma antigens in the periphery exhibited small but significant differences (higher expression of TIM3 and CTLA4 but lower XCL1 in the latter) though both were noted to be late differentiated effector cells. Tumor-specific $CD8^+$ T cells in the tumor infiltrated lymph nodes however, showed preferential overlap with LCMV-derived Tex cells, suggesting that tumour specific T cell exhaustion or dysfunction is localised to the tissue site, but not a feature of cells within the circulation [174]. Two articles form Rosenberg's lab identified that $PD\text{-}1^+$ $CD8^+$ T cells contained tumor-specific pools in melanoma. Firstly, it was discovered that Melan-A/MART1 specific $CD8^+$ T cells were predominantly (though not exclusively) $PD\text{-}1^+$. In this report, PD-1 expression tracked with signs of ongoing activation (HLA-DR, CTLA-4, Ki67) and *ex vivo* dysfunction (lower IFNγ and IL-2 production) [174]. In the second, report $PD\text{-}1^+CD45RO^+CD8^+$ Tem cells in the blood were found to contain circulating tumor-reactive $CD8^+$ T cells that recognize neoantigens in the tumor [175]. In

agreement with this we have also identified NART cells in the tumor of NSCLC patients, and found these cells to be largely PD-1$^+$, with heterogenous expression of LAG-3, GZMb and CTLA-4 [147]. These findings therefore confirm that PD-1 expression coincides with tumor reactivity in humans and extends this to include NART cells.

In NSCLC it was shown that the level of co-expression of TCIRs on memory CD8$^+$ T cells associates with stage of disease and loss of functional competence, but that CD8$^+$ T cells expressing intermediate levels of TCIRs may retain function [176]. Merad's group showed that CD8$^+$PD-1$^+$ T cells correlated with TCR clonality, whilst Kargl et al demonstrated that tumor-specific (private) clonal expansion was correlated with *in vitro* reactivity to autologous tumor cells [166,169]. Collectively, these findings imply that activation and/or exhaustion correlates with clonal expansion to tumor antigens [169]. Recently, scRNAseq analysis was used to deconvolute the multicellular ecosystem of the TME in melanoma in a report by Garraway's group. In this study, TCR expansion was associated with enrichment of an exhaustion molecular signature, further underlining that clonal expansion may predispose commitment to a dysfunctional state [177]. An in-depth scRNAseq and TCRseq profile from PBMC, adjacent tissue and TILs of six patients with hepatocellular carcinoma has supported this model where expanded clonotypes enrich for exhaustion and suggested a cell fate trajectory from naïve > Tem > Tex cells may occur in liver cancer [178]. Interestingly, this report paid attention to two subsets of CD8$^+$ T cells that have been ill defined in human tumors; mucosal associated invariant T (MAIT) cells and an intermediate subset of *GZMK*-expressing cells positioned between the effector and Tex state[178]. The study of MAIT cells in tumors remains in its infancy, but has been recently reviewed elsewhere[179]. Clearly much work is required to reconcile these potential pathways of differentiation with programs of gradual dysfunction observed in pre-clinical data. Two important

conclusions can be drawn from the data on memory $CD8^+$ T cells in human samples; i) Tumor reactivity is linked to a Tem cells expressing TCIRs (but not TEMRA cells, which have lower TCIR expression) ii) Clonal expansion or disease progression predicts T cell dysfunction. However, this does not explain the multitude of other dynamic states that memory $CD8^+$ T cells appear to adopt within human TILs, especially those unveiled by recent scRNAseq studies. Indeed, the co-existence of phenotypically diverse, antigen experienced $CD8^+$ T cells is a common observation in human TILs. Whilst a consistent finding is the presence of dysfunctional $CD8^+$ T cells, less attention has been paid to tumor-specific $CD8^+$ T cells within the TME and blood that retain *ex vivo* cytotoxicity consistent with functional Teff or Tem cells. Hallmarks of these cells are i) an ability to circulate in the periphery ii) more primitive states linear of differentiation (e.g. $CD27^+$ or $CD28^+$), ii) lower degrees of dysfunction, as shown by decreased TCIRs expression, iii) the presence of activation markers and iv) *ex vivo* cytolytic or Teff cell function [167,168,171,176,180]. These studies and others, therefore suggest that, like viral infections, tumor-specific memory $CD8^+$ T cells may be present in solid cancers at multiple stages of differentiation and that an earlier stage of differentiation may predict function. This paradigm is consistent with mouse models of checkpoint blockade discussed above, where less differentiated (plastic) cells, comprised the subset responsive to anti-PD-1 therapy compared to stably dysfunctional cells [121,152].

The enhanced activity of less antigen-experienced T cells can be extrapolated to the setting of adoptive transfer. Results of pre-clinical experiments using Tcm and Tem cell subsets (generated by IL-15 or IL-2 in vitro, respectively) showed that less differentiated (lymph node-homing Tcm cells) had superior anti-tumor activity, suggesting that expansion of a progenitor population is required to supply the anti-

tumor response (possibly by retaining non-exhausted pools) [181]. Consistent with this, infusions of human Teff cells bearing ectopic TCRs were inferior to Tcm cells of the same specificity *in vivo*, with the latter giving rise to Teff and memory $CD8^+$ T cells [182]. Furthermore, in a T cell competent patient-derived xenograft (PDX) mouse model, adoptively transferred Tcm and Tem cells derived from breast cancer infiltrate and rejected tumors [183]. *In vitro* generated Tscm cells transferred into lymphodepleted mice have also showed enhanced capacity to mediate rejection of melanoma tumors compared to Tcm and Tem cells [26]. In this report, the authors suggest that, given their lower TCR signaling upon antigen recognition, Tscm cells survive better in environments with persistent antigen stimulation such as tumors, potentially resisting entry into a dysfunctional state. In the clinic, TIL therapy of metastatic melanoma showed that infusions of polyclonal TIL with superior T cell persistence correlated with better clinical outcome [184], and that TIL retaining a 'young' ($CD27^+CD28^+$ expression, longer telomeres) phenotype can mediate regression in melanoma [185]. On aggregate, these data indicate that less differentiated, circulating memory $CD8^+$ T cell subsets of humans and mice exhibit favorable anti-tumor activity *in vivo*.

Remarkably, it has also been shown that peripheral activation of effectors may be integral for the success of immunotherapy. Recent data from Nolan and Engleman's laboratories demonstrated that sustained systemic immunity across different tissues is required for tumor rejection in a range of immunotherapy models [186]. These pre-clinical data, and the transient rewiring of Tex cells described by Pauken et al. may explain the temporary clinical response observed in an anti-PD-1 treated NSCLC patients, decline of which coincided with the contraction and dysfunction of NART cells in the blood [141]. Intratumoral expansion of Tem cells also was seen to associate with response to anti-PD-1 therapy in clinical samples, however it is not evident whether

these cells were dysfunctional prior to therapy or emerged from increased migration of newly primed cells into the tumor [187]. Of note, a high frequency of $CD27^+CD28^+$ Tem cells in the blood of late stage Ipilimumab-treated patients also correlated with response rate and overall survival whilst Temra cells frequency negatively associated with overall survival [188]. Furthermore, in clear cell RCC $CD8^+$ T cells with lower levels of activation markers and TCIR (termed immune silent or activated) in the tumor were linked to disease-free survival, whilst cells exhibiting co-expression of multiple TCIR (immune regulated) were associated with worse outcome [167]. Work from Wherry's group additionally suggested that activation or reinvigoration of circulating cells is associated with clinical response to anti-PD-1 [167]. Several correlative *in silico* studies further support that intratumoral Tem cells or activated Teff cells may offer protection in primary disease as well as following CPI treatment. Charaentong et al. have made *in silico* predictions that suggest activated $CD8^+$ T cells could be major substrates for immune checkpoint inhibition (CPI) in solid tumors, whilst Tem cells could be important for control of primary disease [189]. This is in accord with previous work highlighting a correlation of Tbet expression and Tem cell signatures with clinical outcome in solid tumors [190,191]. These data therefore suggest that i) Tscm and Tcm cells capable of differentiating into Teff cells are the most potent memory T cell subsets for tumor rejection in adoptive cell therapy (due to their enhanced persistence, expansion, lymph-node homing and resistance to dysfunction). ii) T cell subsets associated with survival in primary disease and CPI are Teff, Tem and activated $CD8^+$ T cells. iii) Dysfunctional $CD8^+$ T cells and Temra cells may appear later in disease or negatively associate with outcome.

The current body of T cell profiling data from solid cancer specimens raises several central questions, especially when considered in the context of basic T cell immunology and murine tumor models. Firstly, what are the cellular, molecular, clinical and tumor-associated factors which determine T cell differentiation in the anti-tumor response in humans? The current data suggest that clinical stage, clonal T cell expansion, metastasis, stromal architecture (e.g. presence of tertiary lymphoid structures) histological subtype or mutagen exposure may influence the level of dysfunction or activation, but beyond that there is little evidence [166,169,171,176]. A second question is whether T cell differentiation links with immune editing? Clonal expansion in melanoma and liver cancer TIL was linked to an 'exhausted' molecular profile by scRNAseq or high PD-1 expression, whilst loss of heterozygosity at HLA alleles in non small cell lung cancer (NSCLC) associated with an increased cytolytic score, suggesting that tumor antigen recognition exerts selection pressure to alter the tumor genomic landscape and synchronously shapes co-evolution of memory $CD8^+$ T cell differentiation [149,177,178]. Moreover, therapeutic NART cell infusion clearly causes loss of neoantigen presentation by tumors, demonstrating that selection pressure can drive evolutionary tumor escape [149]. Thus, existing evidence supports that clonal expansion and immune editing likely co-evolve, associated with increased PD-1 expression. Related to this it is worth considering that $CD8^+$ T cells specific for edited or lost neoantigens may persist in the TME. Indeed, although Tex cells in viral infections are maintained after antigen withdrawal, the turnover of tumor reactive cells in humans is uncertain, and tissue resident populations such as those in the lung in fact experience rapid attrition [66]. This becomes particularly cogent when considering the impact of surgery on immune memory and in the context of clinical decisions to offer adjuvant or neoadjuvant CPI, i.e. will removal of the main source of antigen impede formation

812 of memory following treatment and/or will Tex cells recover? Longitudinal studies will
813 likely determine this conundrum. Thirdly does the nature of antigen shape the T cell
814 response? T cells in cancer may recognise tumor-associated, tumor-specific or viral-
815 derived and mutation-encoded neoantigens. However, whether T cells recognising
816 these antigens adopt phenotypes consistent with divergence of tolerance induction
817 (i.e. due to degrees of self-similarity, or resemblance to viral epitopes) or
818 chronicity/level/dosage of exposure (i.e. ubiquitous truncal neoantigens present in
819 every tumor cells and thus appearing early in tumor development compared to branch,
820 sub clonal antigens that may appear later in tumor evolution) remains to be seen
821 [147,150,192]. In this regard it is interesting that the burden of clonal neoantigens and high
822 affinity frameshift insertion and deletion encoded neoantigens associate with response
823 to checkpoint blockade, yet how the pool of cells fostered by these favourable genomic
824 landscapes differs from low mutational burden patients is largely unknown [147,193].
825 Fourthly, what is the differentiation program, ontogeny and fate of tumor reactive
826 memory $CD8^+$ T cells in humans? The limited data on phenotypes of NART cells and
827 MART-1/Melan-A in humans suggest high TCIR expression but also heterogeneity,
828 provoking the idea that specific tumor reactive clonotypes may differentiate from
829 functional and dysfunctional states [147,174]. In acute viral infection and vaccination, we
830 have discussed that memory $CD8^+$ T cells emerge from effector de-differentiation [11].
831 *In vivo*, NART cells appear to become activated then rapidly and progressively adopt
832 dysfunctional states [152]. In human TILs we find Tem, Temra and Teff cells and
833 phenotypically dysfunctional cells some of which may be connected by clonotype.
834 Thus, there appears to be a cell fate trajectory in tumor specific cells that is vastly
835 different cells differentiating in optimal conditions of immune memory (acute viral
836 infection) that leads to a spectrum of differentiation whereby less antigen experienced

cells (e.g. those recently migrated to the TME) are functional and those with prolonged antigen exposure gradually acquire high TCIRs expression. The multitude of phenotypes emerging from high dimensional flow cytometry and scRNAseq analysis may also arise from different priming environments (tumor or APC in situ vs lymph node) have different specificities (for tumor versus common pathogens) and/or be interconvertible. Regardless of the pathway of differentiation, the ultimate cell fate of tumor specific T cells in humans requires better definition. Tumor driven T cell dysfunction appears to be distinguishable from classical T cell exhaustion in viral infections, consequently the level of assumed dysfunction in these cells requires full clarification. Fifth, connected to this is whether there such thing as a tumor-reactive T cell phenotype? There is certainly a predisposition of pathogens to evoke responses dominated by specific T cell subsets. For example, in humans, the majority of respiratory syncytial virus (RSV), influenza (Flu) and Epstein bar virus (EBV) specific $CD8^+$ T cells show a Tem1 ($CD45RA^-CCR7^-CD28^+CD27^+$) profile, whilst HIV-specific $CD8^+$ T cells tend to be Tem 2-3 and CMV-specific $CD8^+$ T cells split between Tem1 or Temra [194]. Furthermore, clonal dominance may influence this hierarchy, since CMV-specific $CD8^+$ T cells show a different phenotype in healthy vs HIV infected individuals (increased Temra cells in the latter) [42]. For tumor specific T cells this is less clear, multimer technologies are an immensely powerful tool, being implemented widely and expertly in the study of NART cells and other tumor specific T cell populations, but the current data does not provide a consensus on a tumor reactive phenotype [195]. Despite PD-1 and TCIR co-expression proving useful to enrich for tumor specificity, these markers are also expressed on activated cells [175]. Co-expression of multiple TCIR, the presence of CD38 and CD101 or high levels of PD-1 expression perhaps may be the most accurate predictors of tumor reactivity, since these are a feature of chronic

stimulation, not likely to be shared by bystander cells [196]. However, these phenotypes likely only enrich for a subset of tumor reactive cells which are dysfunctional. Recently primed or functional anti-tumor Teff cells and circulating Tem cells present in the TME, blood, adjacent tissue or LN may be more challenging to distinguish given that such phenotypes are common to viral specific T cell populations. Indeed, although pathways of differentiation may be gleamed from coupled TCRseq and scRNAseq analysis of $CD8^+$ T cells in human TILs, an integral missing component to these data is antigen specificity. Finally, which T cell subset elicits therapeutic responses to CPI? This will clearly depend on the TCIR targeted and the context. For anti-PD-1/PD-L1 evidence suggests that intratumoral T cells with high TCIR expression expand in the TME [187]. On the other hand the most exhausted $PD\text{-}1^{hi}$ cells in tumor and chronic infection settings appear to be refractory to rescue and responses in the clinic and *in vivo* rather associate with peripheral effector cell expansion [140,185,121,151,152]. It remains possible therefore that several subsets are mobilised in response to anti-PD-1, but that de-repression of a key non-exhausted effector pool facilitates durable clinical benefit.

Whilst functionally relevant TCRs may be recovered from cells expressing high TCIR levels, the intrinsic dysfunction of such populations may limit their utility in adoptive cell transfer. This limitation would be evident both when preventing *in vitro* expansion and by an inability to induce a response *in vivo* via compromised persistence or inability to recirculate to the LN to serve as progeny. Efforts to reverse exhaustion may assist in generation of functional TIL products from such populations, and may include cytokines (e.g. IL-21), agonistic antibodies or epigenetic modifiers [126,197,198]. Although potentially not as efficient, reversal of exhaustion *in vitro* may be possible with current methods of rapid expansion, given the ability of several groups to detect neo-antigen

reactivity in expanded products, though it is uncertain if these cells were Tex or functional *ex vivo* [199]. Engineering of exhaustion-resistant TILs (i.e. use of CRISPR technology to remove TCIRs) for adoptive cell therapy, or combining adoptive cell therapy with checkpoint blockade may avoid recrudescence of T cell dysfunction and improve the efficacy of cellular cancer therapeutics [200]. Whilst these findings underline the crucial contribution of circulating $CD8^+$ T cell in anti-tumor immunity, emerging evidence also points towards a key role for Trm cells in some cancers.

Tissue resident memory cells in anti-tumor immunity

The role of Trm cells in tumor protection is yet to be fully discerned. Two reports in mouse models of melanoma indicate these cells may have anti-tumor activity. Trm cells driven by autoimmune vitiligo were shown to protect from melanoma in a CD103-dependent manner [201] and OVA-encoding vaccinia virus was shown to generate Trm cells that delayed growth of OVA-expressing melanoma [67]. The prevalence, if not relevance of Trm cells in clinical specimens however, is clear. Tumor samples from patients with ovarian, endometrial, breast and lung cancers exhibit infiltration of $CD8^+CD103^+$ TIL, the abundance of which correlates with prolonged survival and better prognosis [202-206]. Counterintuitively, in ovarian and lung tumors, the $CD103^+CD8^+$ TIL subset express the highest levels of inhibitory immune checkpoints such as PD-1, TIM-3, CTLA-4 and LAG-3, indicating that Trm cells may preferentially adopt a dysfunctional phenotype, likely due to chronic antigen stimulation [207]. However, it is not certain whether a subset of Trm cells in tumors retain functionality [204,206]. Although a $CD103^+CD8^+$ T cell signature associated with prolonged survival in NSCLC, and total $CD8^+$ T cells from $CD103^{hi}$ TIL produced increased GZMb, no difference was observed between $CD103^+$ and $CD103^-$ $CD8^+$ T cells in the production of GZMb, IFNγ or CD107, and PFN expression was lower in $CD103^+$ cells [206]. This

implies that $CD103^+CD8^+$ T cell accumulation may, like intense PD-1 expression, reflect a history of cells with previous effector function that have converted to Trm/ Tex cells, or that Trm cells confer a survival advantage through indirect mechanisms. Interestingly, a major function of Trm cells is recruitment of cells from the circulation [51,72,73]. Given the significant role of circulating Teff or Tem cells in anti-tumor immunity, it remains possible that Trm cells confer protection via recruitment of bystander circulating, tumor-specific T cells. Indeed, this mechanism may contribute to heterosubtypic immunity in influenza models and may facilitate the immigration of recently primed effectors from the tumor draining lymph nodes [74].

In melanoma tumors, nearly 60% of all $CD8^+$ T cells have a $CD45RO^+CD69^+CCR7^-$ phenotype with nearly 50% being $CD103^+$ [207]. However, the presence of Trm cells in melanoma tumors has not been correlated with enhanced survival or better prognosis, suggesting an unknown mechanism by which a Trm cell phenotype is associated with good prognosis and survival in some types of tumors while not in others [207]. This could be accounted for by a difference in subsets of Trm cells and their relative ability for cytotoxicity, dysfunction retention/ turnover and recruitment at different sites. Both mouse Trm cells in the lung and Trm cells of NSCLC samples were shown to have increased sensitivity to apoptosis, a feature of lung Trm cell biology linked to maintenance of antigen diversity and prevention of autoinflammatory tissue damage at this sensitive host site [66,208]. Direct, *ex vivo* analysis of lung Trm cells has shown that IL-2 can selectively induce cytotoxic features in $CD103^+$ Trm cells, and that blocking CD103 reduced *in vitro* lysis of autologous targets in the context of PD-1/PD-L-1 blockade, suggesting that in the appropriate cytokine environment or following CPI, Trm cells become potent anti-tumor effectors [208].

A recent report examining the TCR diversity between metastatic lesions of melanoma patients suggests that Trm in tumors are less competent (e.g. lower IFNγ, IL-2) than circulating populations, and that TCR diversity in Trm cells among lesions exceeds that expected by changes in genomic landscape (although this prediction may be challenging) [207]. It was suggested by the authors of the article that the interlesional diversity in TCR sequences may explain differential responses to checkpoint inhibitors and therefore that Trm cells are a major target of these therapies. Whether Trm cells contribute directly or indirectly to tumor destruction during checkpoint blockade in the clinic is currently unknown. The data above propounds that Trm cells may indirectly or directly promote anti-tumor immunity yet may be selectively prone to TCIRs linked dysfunction, and that the relative contributions of these features may be context and possibly tissue specific.

A potential disadvantage of a stable pool of Trm cells in the TME is the retention of cells with a reduced capacity for anti-tumor function, competing for trophic factors with *de novo* primed or functional circulating Tem/Teff cells pools. This may occur due to either cumulative antigen-driven dysfunction, or through maintenance of $CD8^+$ T cells with specificity for epitopes that have been edited, down-regulated or lost. A recent report in a breast cancer model suggests that recent arrivals in the tumor exhibit functionality, but that Trm cells established previously are dysfunctional [165]. Tumor-reactive Trm cells in this model persisted at the tumor site independent of antigen and were sustained by TAM-derived IL-15, where Trm cells act as a ‘sink’ for cytokine. This is in keeping with the persistence of Tex cells in the absence of antigen in viral infection *in vivo* and in the clinic. Furthermore, in line with this it is likely that induction of a Trm cell profile in the tumor may both allow recruitment of functional circulating cells, and facilitate direct anti-tumor responses but as these cells become

dysfunctional or relevant epitopes are eliminated in later phases, Trm cells may exert a negative impact by occupying the niche and preventing accommodation of more functional or relevant cells (Figure 2). Indeed, it is possible that enforcing the Trm program, which incurs loss of T-bet and EOMES transcription factors and ostensibly reduced Teff potential may be a mechanism of tumor immune evasion[47,62,76,77]. Two reports this year have shown that existing Trm cells proliferate and give rise to secondary Trm cells upon re-challenge, and that initial seeding populations are not replaced upon recruitment of antigen-specific and bystander populations- implying once more that irrelevant cell specificities may be accrued in the tumor, harnessing cytokine resources at the expense of functional recent arrivals [72,73]. Furthermore, the second generation of Trm cells in the TME would presumably inherit the inhibitory chromatin landscape of their progenitors, further expanding the dysfunctional pool. Interestingly, a major molecular mechanism of anti-PD-1 is the increase of cell motility, it is thus a possibility that mobilization of Trm cells may favor enhanced intratumoral responses by permitting entry to the niche [209]. However, the turnover and attrition of Trm cells is site-specific and this may have crucial implications of local anti-tumor responses in different malignancies. Evidence from pulse-chase experiments in the influenza model shows that Trm cells in the lung have a short half-life [66,210], and that the gradually waning numbers after infection reflect the net effect of this loss partly counterbalanced by continual reseeding from circulating memory $CD8^+$ T cells pools [61]. Whether this is also true in lung cancer is unknown.

Similar to circulating tumor specific-cells, the origin of tumor reactive Trm cells is undefined, though this subset is likely to emerge from the Tem or Tcm cell pool. Whether Trm cells acquire dysfunctional characteristics or whether tumor reactive cells acquire dysfunction and a Trm cell gene expression signature synchronously is

also not clear. It is possible that the high concentration of TGFβ inside the tumor induces CD103 on cells that do not bear transcriptional hallmarks of Trm cells, or equally that TGFβ driven *bona fide* Trm cell formation is directly accountable for increased frequency of Trm cells in solid tumors relative to adjacent tissue [55,206,208,211]. Further research to converge the nascent fields of Trm cell biology and T cell dysfunction is required to better define this process and the role of Trm cells in tumor immunity. In keeping with the 'streetlight hypothesis', our current attention may be guided to analysis of effector functions common to circulating $CD8^+$ T cells, whilst thus far under-appreciated facets of Trm cell biology may be more significant in the anti-tumor response.

Conclusion

The generation and maintenance of $CD8^+$ T cell memory subsets is crucial to host survival. Dysregulation of the central orchestrators in these networks leads to defective immune memory and host pathology. Recent work has made evident the complexity of memory T cell ontogeny, epigenetic reprogramming and the fundamental role that Trm cells play in immediate protection at portals of pathogen entry. Transposing our evolving knowledge of anti-tumor immunity onto this framework is a demanding but essential challenge, given the promise of immunotherapy and clinical need to broaden and optimize its application. The recent pre-clinical data suggest that following immune checkpoint inhibitors in the clinic i) the inability to revert the dysfunctional state and ii) the onset of fixed dysfunction in existing or *de novo* memory cells may both contribute to a lack of durable immune memory [122,152]. However, TIL therapy can lead to durable and complete responses and a minority of

patients receiving immune checkpoint inhibitors experience long-term clinical benefit, suggesting either or both of these limitations may be overcome. It will be imperative to monitor memory T cell function in this sub group of patients to decipher the requirements for generation of functional tumor specific memory.

The reversal of T cell dysfunction and the availability of neoepitopes represent two recently defined hurdles for tumor reactive memory $CD8^+$ T cell maintenance and generation, respectively. Together with an inhibitory TME and lack of infiltration we now have four major hurdles to overcome for the development of effective immunotherapy in solid cancers. Therefore, combinatorial treatments providing i) a stimulatory priming environment (e.g. TLR agonists) ii) source of antigen (personalised neoepitope or tumor specific/associated antigen vaccines) or antigen-specific T cells (e.g. targeting neoepitopes) iii) enhanced infiltration (e.g. anti-TGFb, or anti-VEGF) and iv) a means to prevent exhaustion and/or regulation (e.g. CPI) may ultimately be fruitful if proven economically and clinically feasible[212-214]. Correspondingly, a high frequency of endogenous NART effector cells in an immunological ‘sweet-spot’ that are effectively primed, but non-exhausted may provide these benefits to prolong survival during primary disease or enhance responses to CPI. This is consistent with the mounting evidence which supports a major contribution of systemic activation and circulating Tem cells in effective anti-tumor responses. Unveiling the mechanisms which can form and maintain functional, tumor specific effector and memory cells remains key to the success of next generation immunotherapeutic strategies in solid cancers.

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Figure legends

Figure 1. Linear differentiation refreshed.

A composite of seminal work that has defined the lineage relationships of human CD8 memory T cells. The conversion of naïve cells to Teff and consequent de-differentiation gives rise to diverse memory cells subsets with specific migratory potential. Re-stimulation of T subsets gives rise to progeny later in the scheme. See main text for a detailed description.

Figure 2. A putative model of co-evolution of T cell dysfunction and tumor genomics within the TME: Phase I, Circulating tumor-specific Teff or Tem migrate to the tumor alongside bystander cells via chemotactic and inflammatory signaling**.** Teff/Tem convert to Trm and elicit cytotoxic effector function whilst experiencing chronic antigen stimulation. Selection pressure from T cell responses drives tumor evolution, including loss of class I presentation. Phase II, Tumor-specific cells undergo clonal expansion, consume IL-15 and experience progressive dysfunction. IL-15 resources for incoming circulating Tem/Teff are depleted as dysfunctional T cells with specificity to lost antigen dominate the niche, facilitating tumor escape and disease progression.

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