Replicative history marks transcriptional and functional disparity in the CD8⁺ T cell memory pool

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Article

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Abstract

Clonal expansion is a core aspect of T cell immunity. However, little is known with respect to the relationship between replicative history and the formation of distinct CD8⁺ memory T cell subgroups. To address this issue, we developed a genetic-tracing approach, termed the DivisionRecorder, that reports the extent of past proliferation of cell pools *in vivo*. Using this system to genetically ‘record’ the replicative history of different CD8⁺ T cell populations throughout a pathogen-specific immune response, we demonstrate that the central memory T cell (T_{CM}) pool is marked by a higher number of prior divisions than the effector memory T cell pool, due to the combination of strong proliferative activity during the acute immune response and selective proliferative activity after pathogen clearance. Furthermore, by combining DivisionRecorder analysis with single cell transcriptomics and functional experiments, we show that replicative history identifies distinct cell pools within the T_{CM} compartment. Specifically, we demonstrate that lowly divided T_{CM} display enriched expression of stem-cell-associated genes, and that such lowly divided cells are superior in eliciting a proliferative recall response. The latter data provide the first evidence that a stem cell like memory T cell pool that reconstitutes the CD8⁺ T cell effector pool upon reinfection is marked by prior quiescence.

Introduction

The CD8⁺ T cell compartment serves to provide protection against intracellular pathogens and also acts as a modifier of cancer growth. Upon antigen encounter, naïve T cells (T_N) undergo extensive gene-expression alterations, while entering a highly proliferative state, dividing every 4h to 6h^{1,2}. This phase of clonal expansion gives rise to a phenotypically and functionally diverse pool of effector T cells (T_{EFF}) that exceeds its precursor population size by > 10,000-fold^{3,4}. Unlike T_N cells, these T_{EFF} cells have the capacity to disseminate to peripheral tissues, and scan for and kill infected or transformed cells. Upon antigen clearance, around 95% of the T_{EFF} pool succumbs to apoptosis, leaving behind a small long-lived pool of memory T cells (T_M) that is equipped to provide long-term protection against recurring pathogens.

The central role of proliferation in the T cell response has inspired many to study the relationship between replication and T cell state. While earlier work hinted that memory precursor T cells have undergone limited clonal expansion^{5,6}, more recent work studying acute T cell responses in human subjects demonstrated that T_M, as a whole, are derived from precursor cells that have undergone an extensive number of divisions⁷. Furthermore, prior work has shown that cell cycle speed can differ substantially between phenotypic subsets at different time-points in the T cell response. Specifically, central memory T cells (T_{CM}), a subgroup of memory cells that are endowed with a high level of multipotency, have been documented to undergo homeostatic proliferation after pathogen clearance, while effector memory T cells (T_{EM}) have a low turnover rate^{8,9}. In contrast, during the effector phase, a T_{CM}-like state has been linked to lower division speed and reduced clonal burst size compared to their T_{EM}-like and terminally differentiated counterparts^{10–13}.

The phase-dependent association of proliferative activity with specific cell states, in combination with the reported phenotypic instability of certain T cell subsets^{14,15}, makes it difficult to deduce the replicative history (i.e. the cumulative number of divisions) of different memory T cell populations, and the possible relationship between division history and functional properties. Here, we develop a genetic-tracing approach—termed DivisionRecorder—that allows the measurement of prior division of cell pools over extensive rounds of division, and apply this approach to determine to what extent replicative history identifies distinct memory T cell states and memory T cell behaviors, focusing on three central issues: (1) What are the differences in replicative history between (precursor-)T_{CM} and T_{EM} in the effector and memory phase? (2) Is there heterogeneity in division history within the pool of central memory T cells? (3) If so, does this relate to their capacity to mount a secondary T cell response?

Results

Division-linked genetic labeling of cell pools using the DivisionRecorder platform

The genome contains a large number of hypervariable short tandem nucleotide repeats (STRs) that accumulate intra-allelic length mutations through DNA polymerase slippage during cell division. Such slippage mutations in endogenous STRs have been used to study cell lineage trees in various organisms and tissues^{16,17}. In addition, synthetic STRs have previously been employed in a probabilistic labeling approach to define stem cells in the intestinal epithelium and the mammary gland^{18,19}. To investigate the replicative history of memory T cells, we engineered a synthetic STR-reporter system to continuously ‘record’ proliferation in cell pools. This genetically encoded system, termed DivisionRecorder, utilizes a synthetic STR domain to achieve a cell division-linked low-probability acquisition of a fluorescent mark (Fig. 1A). The DivisionRecorder consists of two separate elements: (1) a retroviral-vector encoded module that contains a synthetic STR linked to an out of frame CRE recombinase gene; (2) A CRE-activity reporter module that irreversibly induces the expression of a red fluorescent protein (RFP). In its base configuration, all cells that contain the DivisionRecorder only express GFP (hereafter referred to as DR^{GFP} cells). As cells undergo successive divisions, slippage mutations that occur within the synthetic STR yield in-frame variants of the downstream CRE recombinase gene at a fixed, division-dependent, probability (p). The resulting CRE activity induces an irreversible activation of the RFP gene, giving rise to GFP⁺RFP⁺ cells (hereafter referred to as DR^{RFP} cells) that pass this genetically encoded label on to subsequent generations, resulting in a cumulative increase in the DR^{RFP} cell fraction within the DivisionRecorder⁺ (DR⁺, i.e., the sum of DR^{GFP} and DR^{RFP} cells) population as the cell pool expands (Fig. 1B). Importantly, when p is small (< 0.01) the DivisionRecorder yields a near-linear relationship between the DR^{RFP} cell fraction and the average number of divisions that the population has undergone through dozens of population doublings (Fig. 1C)²⁰, thereby allowing analysis of replicative history substantially beyond what can be achieved with classical cell labeling dyes²¹.

To test the utility of the DivisionRecorder, we established a reporter cell line carrying a lox-STOP-lox-RFP cassette. Following retroviral introduction of the GFP-STR-CRE module into this reporter line, a

progressive acquisition of DR^{RFP} cells was observed over time, whereas no label acquisition was observed when the STR was replaced with a stable DNA sequence (Fig. 1D, E). Moreover, the rate at which DR^{RFP} cells accumulated was dependent on the sequence stability of the STR^{22,23}, underpinning that p is linked to the likelihood of STR slippage (Fig. 1F). Also when immortalized embryonic fibroblasts from the Ai9 mouse strain that carries a lox-STOP-lox-RFP cassette in the *Rosa* locus²⁴ were modified with the DivisionRecorder, a low and predictable DR^{RFP} acquisition was observed, with a [G]33 STR conferring a p of 0.0052 ± 0.00074 (Fig. 1G), thereby enabling the measurement of replicative history over many cell divisions.

To test whether the DivisionRecorder can be used as a proxy for replicative history in the CD8⁺ T cell compartment *in vivo*, we generated Ai9;OT-I mice, in which all T cells recognize the OVA₂₅₇₋₂₆₄ epitope, thereby allowing examination of T cell pool in the context of equal TCR affinity. Ai9;OT-I T cells were isolated and retrovirally transduced to generate DivisionRecorder⁺ (DR⁺) OT-I T cells. Next, DR⁺ OT-I T cells were transferred into *Listeria monocytogenes*-OVA (*Lm*-OVA) infected mice, and the fraction DR^{RFP} cells was followed over time (Fig. 2A). At early time-points post cell transfer (d1-d4), a rapid increase in DR^{RFP} cells was observed (Fig. 2B, C), coinciding with the proliferative burst of the antigen-specific CD8⁺ T cell pool. To determine whether the observed accumulation of DR^{RFP} cells formed an accurate measure of prior cell division, DR⁺ OT-I T cells were labelled with CellTrace Violet (CTV) prior to cell transfer. Notably, analysis of the fraction DR^{RFP} cells for cell pools with different degrees of CTV dilution revealed a close correlation (Fig. 2D, E, $r_{\text{rm}} = 0.94$), providing direct evidence that *in vivo* DR^{RFP} acquisition reflects the extent of past division in the CD8⁺ T cell pool. In conclusion, we have established a genetically encoded tracing tool that allows the long-term measurement of division history in cell pools, that can be read-out by flow cytometry, and that is compatible with further down-stream methodologies such as single cell sequencing (see below).

CD8⁺memory T cells are derived from replicative 'mature' cells

Having validated the utility of the DivisionRecorder to measure T cell proliferation, we next set out to determine the replicative history of the total CD8⁺ T_M pool relative to that of the T_{EFF} pool. Analysis of the size of the DR⁺ OT-I T cell compartment in blood following *Lm*-OVA infection showed the characteristic rapid expansion phase, with T cell numbers peaking around day 6, and subsequent contraction into a stable memory pool (Fig. 3A). Notably, DR^{RFP} cells remained detectable following formation of T cell memory, thus allowing analysis of replicative history at late time points after infection (Fig. 3B).

In case memory T cells would primarily be derived from T cells that had undergone limited proliferation upon primary antigen encounter, the fraction of DR^{RFP} cells would be expected to decay during the contraction phase, due to the decline of the number of clonally expanded T_{EFF} (**Fig. S1**). However, analysis of DR^{RFP} frequencies in blood demonstrated that the fraction of DR^{RFP} cells did not decline, but instead continued to increase during contraction and memory phase (an increase of $2.07\% \pm 0.77\%$

between day 13 and 59, Fig. 3C). Furthermore, this increase in DR^{RFP} frequencies post pathogen clearance was not restricted to T cell responses induced by *Lm*-OVA infection, but was also observed upon infection with LCMV-OVA²⁵ (Fig. 3D). To assess whether the observed increase in the fraction DR^{RFP} cells observed in peripheral blood could be explained by anatomical redistribution of cells with distinct division histories, we analyzed DR^{RFP} labeling of T cells in spleen and liver, the primary sites of *Lm*-OVA infection. Strikingly, the fraction of DR^{RFP} cells increased throughout contraction and transition into the memory phase in all assessed anatomical compartments (Fig. 3E, F). Furthermore, the label increase observed after the peak of the T cell response was similar to that observed during the expansion phase (Fig. 3E, F), suggestive of major ongoing proliferation in the antigen specific T cell pool after pathogen clearance. Thus, in line with recent work⁷, our results support the notion of a replicative 'mature', rather than 'nascent', memory CD8⁺ T cell pool, and extends this observation beyond the peripheral blood compartment to secondary lymphoid organs.

It has been well documented that T_{CM} are able to maintain the memory pool through infrequent homeostatic cell division, and recent work has shown that precursor-T_{CM} slow down their replicative cycle early during the expansion phase¹⁰, suggesting limited clonal expansion of these cells during the early phase of the T cell response. However, it is difficult to translate cell cycle activity at a given time-point into cumulative proliferative history, and we therefore wished to directly test the relationship between cell state (e.g. T_{CM} or T_{EM}) and replicative history during different stages of the T cell response. To test for differences in division history between cell states in the memory phase (d86 post-infection), we calculated the fraction DR^{RFP} cells for memory phase T cells with varying expression levels of proteins associated with either multipotency or terminal differentiation. This analysis showed a positive correlation between replicative history and the expression of CD27 ($r_{\text{rm}} = 0.81$, $p = < 0.0005$) and CD62L ($r_{\text{rm}} = 0.62$, $p = < 0.0005$)—two proteins reporting a less differentiated, multipotent state^{15,26,27}. Furthermore, a negative relationship between replicative history and the expression of the terminal differentiation-associated proteins KLRG1 ($r_{\text{rm}} = -0.83$, $p = < 0.0005$) and CX₃CR1 ($r_{\text{rm}} = -0.75$, $p = < 0.0005$)^{14,15,28} was also observed (Fig. 4A). Likewise, defining distinct multipotent and terminally differentiated T cell subsets by joint expression or absence of CD62L and CD27 (Fig. 4B), and further partitioning by the expression of KLRG1 or CX₃CR1, revealed a positive association between division history and a less differentiated cell state (Fig. 4C). Thus, memory cells that exhibit the lowest expression of classical terminal differentiation marks, commonly defined as T_{CM}, have replicated more extensively, than terminally differentiated memory T cells, generally referred to as T_{EM}.

The above data show that the multipotent T_{CM} pool has undergone a large number of cumulative divisions, but do not inform whether this can primarily be attributed to selective homeostatic proliferation, or whether this can additionally be traced to substantial division during the active inflammation phase. To address whether multipotent (CD27^{HI}KLRG1^{LO}) and terminally differentiated (CD27^{LO}KLRG1^{HI}) T cells (see **Fig. S2A** for phenotypic marker expression) differ in proliferative history at the end of the acute phase of the T cell response, we compared the fraction of DR^{RFP} cells at day 5/6 after *Lm*-OVA infection.

Interestingly, at the peak of the effector T cell response, the fraction DR^{RFP} cells within the CD27^{HI}KLRG1^{LO} and CD27^{LO}KLRG1^{HI} populations was indistinguishable (Fig. 4D top, **4E**), showing that both phenotypic subsets had on average divided equally. Likewise, analysis of the fraction DR^{RFP} cells as a function of CD62L and CX3CR1 expression (Fig. 4E), and separate testing of DR^{RFP} frequencies within the CD62L^{HI} and CD62L^{LO} effector populations (**Fig. S2B**), showed that replicative history varied minimally across cell states at the peak T cell response. In line with the above, the division history of CD27^{HI}KLRG1^{LO} early-T_{CM} gradually increased during memory formation through selective proliferation within this subset, as confirmed by enriched Ki67 expression during contraction and in early-memory (Fig. 4F,G, **Fig S2C,D**). In contrast, the CD27^{LO}KLRG1^{HI} pool lost Ki67 expression during contraction and division history remained constant (Fig. 4F,G, **Fig S2C,D**), thereby additionally indicating that this terminally differentiated pool does not receive significant replenishment from the replicative active CD27^{HI}KLRG1^{LO} population (**Fig S2E**). In conclusion, the strong enrichment of highly divided cells in the T_{CM} pool is due to the combined effect of robust clonal expansion during the acute T cell response plus continued homeostatic proliferation of the T_{CM} pool during and following memory formation.

Quiescent memory T cells reside within the highly divided T_{CM} pool

Having established that the average T_{CM} has divided extensively, we next asked if replicative heterogeneity exists within this pool, and if such heterogeneity is coupled to particular transcriptomic features. To this end, we performed single-cell mRNA sequencing on DR^{GFP} and DR^{RFP} memory T cells (85 days post *Lm*-OVA infection, Fig. 5A). To retrospectively identify CD27^{HI}KLRG1^{LO} T_{CM} and CD27^{LO}KLRG1^{HI} T_{EM} cells were stained with barcode-labeled antibodies directed against CD27 and KLRG1. Using the MetaCell algorithm²⁹, 11,767 single cells (6,548 DR^{RFP} and 5,219 DR^{GFP}) were grouped into 22 transcriptionally distinct MetaCells (MCs, 141–1301 cells per MC, each constructed from cells derived from every biological replicate [*n* = 3], **Fig. S3A-C**). Notably, essentially all CD27^{LO}KLRG1^{HI} T_{EM} cells mapped to a single transcriptionally distinct MC (MC21), which was enriched for archetypical effector-associated genes including *Zeb2*, *Gzmb* and *Cx3cr1*, and displayed diminished expression of multipotency-associated genes *Sell* (CD62L), *Il7r* and *Cxcr3* (Fig. 5B-D, **Fig. S3D**). As revealed by the DR^{GFP} / DR^{RFP} ratio, and in concordance with the flow cytometric analyses (Fig. 4), cells in this MC showed limited prior proliferative activity (Fig. 5E).

In contrast to the transcriptional homogeneity of the T_{EM} pool, the CD27^{HI}KLRG1^{LO} T_{CM} population consisted of a transcriptionally highly diverse group of cells that mapped to 21 distinct MCs (Fig. 5B-D). Furthermore, a substantial depletion, or enrichment, of the fraction of DR^{RFP} cells was observed in a number of these MCs, indicative of differences in prior proliferative activity of these cell pools. (Fig. 5E). To investigate a putative association between transcriptional state and replicative history within the T_{CM} pool, we first evaluated the expression of genes associated with stem cell quiescence in various tissues³⁰. Notably, within MC13 and MC20—the T_{CM} MCs that showed the lowest and highest level of

DR^{RFP} labeling, respectively—an enrichment (MC13) and depletion (MC20) of quiescence-associated gene expression was observed (**Fig. S3E**). Moreover, quiescence-gene score was also predictive for replicative history across all MCs (Fig. 5F, $r = -0.66$, $p = 0.00078$), suggesting that the adoption of cellular quiescence drives heterogeneity in division history within the multipotent memory T cell pool.

To subsequently assess whether replicative history within the T_{CM} pool is also associated with the expression of genes that influence lymphocyte activity, we focused on the two T_{CM} MCs that showed either the lowest or highest level of DR^{RFP} acquisition. The minimally divided MC13 was defined by increased expression of genes linked to lymphocyte and hematopoietic stem cell survival, such as *Serpina3g*, *Traf1*^{31,32} and a series of *Gimap* family members (*Gimap3*, 4, 6, 7)^{33–35}, and lower expression of effector-associated genes (*Tbx21*, *Klf2*, *Ccr2*, *Lgals1*, *S100* family members; Fig. 5G). Furthermore, an inverse gene expression profile was observed in MC20, displaying elevated expression of effector-associated genes, such as *S100* family members, *Ccr2*, *Lgals1* and several killer lectin family members (Fig. 5G). Notably, although MC20 bears resemblance to T_{EM} cells in terms of effector-associated gene expression, the high expression of *Il7r* and *Bcl2*³⁶, together with the prominent CD27 and low KLRG1 surface protein expression (Fig. 5B-C), underscore that these highly proliferated cells are a subgroup of the T_{CM} pool. The polarized expression in pro-survival and effector-associated genes in highly and less divided T_{CM} clusters was also observed in a transcriptome-wide differential gene expression analysis (Fig. 5H). Moreover, comparison of the shared gene signatures of the 3 MCs with either the highest (MC17, MC19, MC20) or lowest (MC2, MC11, MC13) DR^{RFP}/DR^{GFP} ratio to a core T_{EFF} signature demonstrated that less divided T_{CM} expressed T_{EFF}-associated genes at a lower level (Fig. 5I). Taken together, these data describe a previously unappreciated transcriptional heterogeneity within the T_{CM} pool that is closely linked to the number of past divisions that these cells have undergone.

The secondary response is initiated from a pool of replicative quiescent memory T cells

Having observed a diversity in replicative history that distinguishes quiescent and effector-like cells within the T_{CM} pool, we next asked whether the replicative history of T_{CM} also predicts functional capacity. Analysis of antigen-induced *ex vivo* T cell activation demonstrated that T_{CM} cells that had undergone fewer divisions consistently produced more IL-2, and were less likely to degranulate shortly after antigen exposure than their highly divided T_{CM} counterparts (**Fig. S4A, B**).

A hallmark feature of T cell memory is its capacity to rapidly create a secondary T_{EFF} pool upon re-challenge. In various tissues, quiescent stem cells have been documented that break their dormancy upon tissue injury and exhibit profound re-population capacity^{37–40}. We hypothesized that, in a similar fashion, the formation of the secondary T_{EFF} pool may be supported by the proliferative capacity of a T_M pool that is quiescent during the memory phase. To test the relationship between replicative history and recall potential *in situ*, i.e without disruption of the T_M niche, we subjected recipient mice carrying DR⁺ memory OT-I T cells previously challenged with *Lm*-OVA to a secondary infection. If the capacity for renewed

expansion would be primarily restricted to stem cell-like memory T cells, the fraction of DR^{RFP} cells should show an initial decay upon reinfection—due to the increased preponderance of offspring derived from this previously quiescent population—followed by a gradual recovery throughout the contraction phase, as a result of novel division-dependent label acquisition. Notably, analysis of the fraction DR^{RFP} T cells in blood of reinfected recipient mice revealed a steep decline during the first days post-secondary infection that gradually recovered during secondary memory formation (Fig. 6A). Furthermore, the same transient reduction in fraction DR^{RFP} cells was observed upon secondary infection of mice challenged with LCMV-OVA (Fig. 6B), and this reduction was apparent in both the multipotent CD27^{HI}KRG1^{LO} and terminally differentiated CD27^{LO}KLRG1^{HI} populations (**Fig. S4C**), and was also observed in spleen and liver; Fig. 6C). Of note, DR^{RFP} cell accumulation during the secondary contraction phase occurred at a comparable rate as observed during the primary response, yielding a secondary memory T cell pool that—despite extensive renewed clonal expansion—had undergone a comparable number of divisions as the initial memory pool (**Fig. S4D, E**, median fold difference = 1.03). Thus, the replicative histories of the T_{EFF} and T_M pools of the secondary T cell response mimic those of the primary T cell response, supporting the notion that the secondary expansion wave is mounted by a group of previously quiescent T_{CM} cells. Fitting of the obtained data (as shown in Fig. 2 through Fig. 5) to a mathematical framework (see **Supplementary Information**) indicates that our observations are consistent with a model in which the primary wave of T_{EFF} cells gives rise to at least three distinct memory T cell behaviors: (1) T_{EM} cells that, consistent with prior data, permanently adopt a replicative inert state after the peak of the response; (2) continuously self-renewing T_{CM} cells that maintain the memory pool, but minimally contribute to a secondary expansion wave (**Fig. S4F**); and (3) replication competent quiescent T_{CM} cells that, upon renewed antigen encounter, expand to form a secondary pool of effector T cells (Fig. 6D, **Fig S4G**).

Discussion

Here, we report the development and application of a genetic tracing approach to dissect the replicative history of cell pools *in vivo*, and its link to cell state. Using this approach, we demonstrate that, as a whole, the multipotent CD8⁺ T_{CM} pool has undergone substantial proliferation at the peak of the expansion phase, and continues to proliferate following pathogen clearance, resulting in a replicative age of the T_{CM} pool that exceeds that of the T_{EFF} and T_{EM} pool. Previous work has shown that CD62L^{HI} T_{CM} precursor cells divide at a lower rate than terminally differentiated effector subsets¹⁰. In line with this, we observed a lower fraction of Ki67⁺ cells with the multipotent effector pool than the terminally differentiated pool early post infection. However, our data demonstrate that this difference does not result in a reduced total number of past divisions within the CD27^{HI}CD62L^{HI} effector T cell pool, and these findings may potentially be reconciled by the ability of highly proliferative CD62L^{LO} effector cells to phenotypically convert to a less differentiated CD62L^{HI} state^{14,15,28}.

Through the combination of the DivisionRecorder and single cell RNA sequencing, we also reveal a large heterogeneity in the composition of the T_{CM} pool, and observe an association between replicative history

and transcriptional state. First, next to the previously described population of T_{EM} , our data demonstrate the presence of T_{CM} that bear transcriptional similarities to T_{EFF} cells (**Fig. S5**) but, in contrast to T_{EM} , these T_{CM} remain highly proliferative in the absence of inflammation. (**Fig. S5**). Second, we identify a population of lowly divided T_{CM} that expresses reduced levels of T_{EFF} associated genes, and high levels of pro-survival genes and genes associated with quiescent stem cells³⁰. The low level of label acquisition by this group of T_{CM} may reflect an adoption of a semi-quiescent cell state early after the initiation of the T cell response that is maintained throughout the memory phase. Alternatively, this cell pool may enter a dormant state around the peak of the response, with no subsequent cell cycle activity until renewed antigen encounter (**Fig. S5**). Recent work has shown that a subgroup of T_{CM} precursor cells slow down their cell cycle progression early after antigen stimulation¹⁰. Furthermore, we observed a Ki67^{LO} T_{EFF} population marked by T_{CM} associated proteins at the peak of the T cell response, and jointly these observations provide some evidence that the precursors of lowly divided T_{CM} may adopt a dormant state early during the T cell response.

A hallmark of immunological memory is the ability to efficiently generate a new wave of T_{EFF} upon renewed infection. Our data demonstrate that this ability is predominantly confined to a subgroup of replicative nascent T_M cells. The combined observations of a less differentiated quiescent T_{CM} population, and the reconstitution of the secondary T_{EFF} pool by the output of these nascent progenitors, make a compelling argument for the presence of a bona fide stem cell population within the T_M pool. A growing body of work has examined a stem cell-like memory T cell (T_{SCM}) population^{41,42}, generally using cell phenotype to enrich and study these cells *ex vivo*. Using a function-driven, phenotype-agnostic, approach that does not require removal of cells from their niche, we observe a cell behavior that fits the profile of stem cell-like memory T cells *in situ*.

In high turnover tissues, such as the bone marrow^{38,43}, the intestinal epithelium^{39,44} and skin epidermis^{45,46}, two distinct behaviors of multipotent progenitor cells have been described: Actively dividing cells that promote normal tissue homeostasis, and quiescent cells that enter an cycling state upon niche-associated insults, such as tissue damage. We propose that the two T_{CM} behaviors we describe provide the T cell compartment with the same capacity for renewal. Thus, the T cell pool can be viewed as an autonomous tissue that abides by organizing principles akin to those of the hematopoietic system and solid organs.

Methods

DivisionRecorder vectorgeneration

Full sequences of the DNA oligos used are supplied in **Supplementary table 4**. In order to prevent expression of Cre recombinase during bacterial cloning, a synthetic intron—containing a splice donor, a branch site, a pyridine rich region, and a splice acceptor— was inserted into the Cre gene through three-fragment isothermal assembly. The synthetic intron was produced as two overlapping DNA fragments

(1st: CTGGTTATGCGGCGGAAGGTAAGTCAGTAGGCCATGTTAGAGGGTGGCCCAGGTATTGA, 2nd: TGGCCCAGGTATTGACCTATTTCCACCTTTCTTCTTCATCCTTAGATCCGAAAAGAAAAC, IDT).

Subsequently, a pCDH plasmid containing Cre recombinase was digested with BamHI to split the Cre gene (at basepair position 353), and the resultant linear plasmid (100 mM) was assembled together with the synthetic fragments. To prevent low level Cre translation occurring from alternative start sites, two ATG codons (position 78 and 84) were replaced by TGT codons. Finally, the Cre start codon was replaced by an EcoRI-spacer-XhoI site, to facilitate subsequent introduction of synthetic STRs. To generate the DivisionRecorder vector, two lox511 sites were introduced into the multiple cloning site of the pMX retroviral vector. Subsequently, an eGFP gene and the modified Cre recombinase gene were introduced directly upstream and downstream of the 5' lox511 site, respectively. Finally, a P2A element was inserted directly in between the eGFP gene and the 5' Lox511 site. Together, this resulted in a cassette comprising from 5' to 3': Kozak, an eGFP gene, a P2A site, a lox511 site, an EcoRI restriction site, spacer, an XhoI restriction site, a Cre recombinase gene, and a lox511 site. In its base configuration, Cre recombinase is out of frame. Synthetic STR domains were ordered as oligonucleotides (Invitrogen) and subsequently dimerized. STR dimers were inserted via the EcoRI and XhoI sites, sequences of STR oligonucleotides are supplied in Supplementary Table 4.

Cre-activity reporter vector generation

LoxP sites were introduced into the multiple cloning site of the pCDH-CMVp-MCS-PGK-BlastR vector through two sequential rounds of digestion/ligation. First, the 3' loxP site was introduced using EcoRI and BamHI restriction sites. The introduced region included a short spacer sequence and a XhoI site directly upstream of the LoxP. Second, the 5' loxP site was introduced using the XbaI and EcoRI restriction sites. This resulted in a modified MCS, comprising XbaI-loxP-EcoRI-spacer-XhoI-loxP-BamHI. Next, a scrambled non-sense open reading frame, containing multiple stop codons, was introduced into the EcoRI and XhoI sites. Finally, a katushka open reading frame was introduced via the BamHI site. This resulted in a vector containing from 5' to 3': The CMV promoter, a floxed scrambled open reading frame, a Katushka open reading frame, the PGK promoter, and a blasticidin resistance gene.

Establishment of cell lines

The Cre-activity reporter cell line (**Fig. S1**) was generated by retroviral transduction of HEK 293T cells with the Cre-activity reporter plasmid. Successfully transduced cells were selected using 2 µg/ml Blasticidin (InvivoGen). Transduced cells were subsequently seeded at 1% confluency, and resulting single cell-derived colonies were transferred to individual wells. Clones were then examined for efficiency of induction of Katushka expression upon transfection with Cre recombinase, and the best-performing clone was selected. Cre-activity reporter cells were cultured in IMDM (Gibco) supplemented with 8% fetal calf serum (FCS, Sigma), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 2 mM Glutamax (Gibco).

A mouse embryonic fibroblast (MEF) cell line from the Ai9 mouse strain was generated by modification of E14.5 embryonic fibroblasts with a retroviral vector encoding short-hairpin RNA directed against the p53

mRNA. Resultant cells were cultured in IMDM supplemented with 8% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM Glutamax.

Mice

C57BL/6J-Ly5.1, OT-I and Ai9 mice were obtained from Jackson Laboratories, and strains were maintained in the animal department of The Netherlands Cancer Institute (NKI). Ai9 and OT-I mice were crossed to obtain the Ai9;OT-I strain. All animal experiments were approved by the Animal Welfare Committee of the NKI, in accordance with national guidelines.

Generation of DivisionRecorder⁺ OT-I T cells

Platinum-E cells cultured in IMDM supplemented with 8% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM Glutamax were transfected with the DivisionRecorder vector using FuGeneTM6 (Roche). Retroviral supernatant was harvested 48h after transfection and stored at -80°C. Spleens from Ai9;OT-I mice were harvested and mashed through a 70 µm strainer (Falcon) into a single cell suspension and resulting splenocytes were subsequently treated with NH₄Cl to remove erythrocytes. Subsequently, splenocytes were cultured in T cell medium (RPMI (Gibco Life Technologies) with 8% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, Glutamax, 10mM HEPES, MEM Non-Essential Amino Acids (Gibco), 1mM Sodium pyruvate (Gibco), 50µM 2-mercaptoethanol), supplemented with 1ng/ml recombinant murine IL-7 (PeproTech) and 2 µg/mL ConcanavalinA (CalBiochem). After 48h, splenocytes were re-seeded on RetroNectin (Takara) coated plates in T cell medium supplemented with 60IU/mL human IL-2 and DivisionRecorder virus, and were centrifuged for 90min at 400g to allow spinfection. virus concentration was chosen such that a transduction efficiency of approximately 10-15% was achieved, in order to minimize the occurrence of multiple retroviral integrations. Cells were harvested 24h later and a small aliquot was stained with anti-CD8-PerCPy5.5, anti-Vb5-PeCy7, anti-CD45.2-AF700 and DAPI to determine the fraction viable OT-I T cells (DAPI⁺CD8⁺Vb5⁺CD45.2⁺) by flow cytometry (Fortessa, BD Bioscience), which generally was around ~80%. CD8⁺Vb5⁺CD45.2⁺ that expressed GFP were considered as DivisionRecorder⁺ OT-I cells. Within the initial population of DivisionRecorder⁺ OT-I cells the fraction of cells that already showed reporter activation (as inferred by tdTomato expression) 24h after transduction was consistently between 0.4 and 0.8%. Activated splenocytes were prepared for adoptive transfer (see below).

Infection, adoptive transfer and cell recovery

C57BL/6J-Ly5.1 mice were infected with 5,000-10,000 CFU of a recombinant *Listeria monocytogenes* strain that expresses ovalbumin or with 5,000 PFU artLCMV-OVA²⁵, kindly provided by Doron Merkler, University of Geneva. Approximately 24h later, infected mice received 5,000-40,000 DivisionRecorder⁺ OT-I T cells through intravenous tail vein injection. To analyze OT-I T cell responses in peripheral blood over time, 25-50 mL blood samples were obtained from the tail vein at the indicated time points, and were treated with NH₄Cl supplemented with 0.2 mg/ml grade-II DNaseI (Roche) to remove erythrocytes (see

Methods, Flow Cytometry). To obtain spleen and liver samples, mice were sacrificed, organs were harvested, and single cell suspensions were prepared by means of mashing through a 100µm or 70µm strainer (Falcon), respectively. Subsequently, erythrocytes were removed by treatment with NH₄Cl. To purify leukocytes from single cell suspensions of liver tissue, cell suspensions were separated over a 37.5% Percoll (Sigma) density gradient. Following Percoll density separation, the pelleted cells consisted predominantly of leukocytes, while hepatocytes precipitated at the interphase. Obtained blood, spleen and liver samples were further processed for flow cytometric analysis, scRNA-sequencing or functional in vitro assays, as indicated.

Validation of DivisionRecorder functionality

To assess the ability of the DivisionRecorder as represented in Fig. 1D to faithfully report on the replicative history of T cell populations, an alternative experimental set-up was employed. First, splenic CD8⁺ T cells were isolated using the Mouse CD8 T Lymphocyte Enrichment Set (BD Biosciences) and were subsequently stained with CellTraceä Violet (Thermofisher). Next, cells were activated for 16h in T cell medium supplemented with 0.05 µg/mL SIINFEKL peptide and 60 IU/mL IL-2. Following this activation step, cells were seeded onto RetroNectin® (Takara Bio) coated plates and were transduced with DivisionRecorder virus by spinfection for 4h in the presence of IL-2 and SIINFEKL peptide. Analysis of CellTraceä Violet signal by flow cytometry indicated that the cells had not undergone a full cell division post labeling. Subsequently, 6x10⁶ OT-I T cells were transferred into *Lm*-OVA infected recipients. Spleens were harvested 48h after adoptive transfer, processed into single cell suspensions and prepared for flow cytometric analysis. In order to reliably determine the fraction of DR^{RFP} cells per division during the initial stages of the proliferative burst, analysis of a large number of DivisionRecorder⁺ OT-I T cells events is required. For this reason, a transduction efficiency of ~60% was chosen in these experiments, instead of the 10-15% transduction efficiency used in other experiments. Note that a high transduction efficiency will result in the more frequent occurrence of cells that carry multiple retroviral integrations. The presence of cells with multiple integrations will result in a higher, yet stable, DR^{RFP} acquisition rate, as compared to the experimental set-up used in the remainder of the study.

Ex vivo analysis of degranulation and cytokine secretion potential of memory T cells

Spleens were harvested from recipient mice at >60 days post-infection, and CD8 T cells were isolated using the Mouse CD8 T Lymphocyte Enrichment Set (BD Biosciences). Following isolation, T cells were plated at 10⁶ cells per well in 96-well U bottom plates in T cell medium supplemented with 0.05 µg/mL SIINFEKL peptide to selectively activate OVA-specific T cells. Following a 4hr incubation, capacity of indicated T cell populations to either produce the indicated cytokines or to degranulate was assessed. To allow analysis of cytokine production, Brefeldin A (GolgiPlugä, BD Biosciences) was added 30 minutes after initiation of T cell stimulation. To allow analysis of degranulation, T cell medium was supplemented with anti-CD107a and anti-CD107b antibodies at the initiation of T cell stimulation, and Brefeldin A (GolgiPlugä, BD Biosciences) and Monensin (GolgiStopä, BD Biosciences) were added 30 minutes after

initiation of T cell stimulation. At the end of the T cell stimulation period, cells were stained for KLRG1 and CD27 and prepared for flow cytometric analysis (see below).

Flow cytometric analysis

Cells were taken up in PBS (Gibco) supplemented with 0.5% bovine serum albumin (BSA, Fisher Scientific), and stained with antibodies directed against the indicated cell surface proteins (1:200 dilution), for 30min on ice. To allow detection of intracellular cytokine production, cells were fixed and permeabilized with CytoFix/CytPermä (BD Biosciences) according to the manufacturer's protocol and subsequently stained using antibodies against IL-2, TNF α and IFN γ . To detect intranuclear Ki-67 expression, the Foxp3/Transcription factor Staining buffer set (eBioscience) was used. See Supplementary table 6 for specifics on all antibodies used. All samples were acquired on a BD LSR Fortessaä (BD Bioscience); DR^{GFP} and DR^{RFP} cells were identified as CD8⁺Vb5⁺CD45.2⁺GFP⁺tdTomato⁻ and CD8⁺Vb5⁺CD45.2⁺GFP⁺tdTomato⁺, respectively. Flow cytometry data analysis was performed using FlowJo V10 (See **Supplementary table 5**).

For the moving average analysis depicted in Fig3A and Fig S2, CD8⁺Vb5⁺CD45.2⁺GFP⁺ events were exported and further processed using the R package FlowCore⁴⁷. In brief, outlier events (i.e. antibody aggregates/cell doublets) were removed, fluorescence intensities of each of the cell surface proteins were normalized using an inverse hyperbolic sine transformation and subsequently scaled between 0 and 1. To obtain the depicted moving averages, the fraction of DR^{RFP} cells was calculated within windows that each contained 10% of total cells, starting with the 10% of cells with the lowest expression levels for the indicated marker, and with subsequent windows moving up by steps of 2.5%.

Single cell RNA sequencing and data analysis

Spleens of DivisionRecorder⁺ OT-I T cell recipient mice (n=3) were harvested >85 days post-infection. Splenocytes were stained with fluorochrome-conjugated antibodies directed against CD8, CD45.2 and Vb5 (See **Supplementary table 5**), to allow purification of DivisionRecorder⁺ cells by FACS. In addition, to infer surface protein abundance, splenocytes were stained with barcode-labeled TotalSeqä antibodies directed against CD27 (TotalSeq-A0191, Biolegend) and KLRG1 (TotalSeq-A0250, Biolegend). Following the isolation of DR^{GFP} and DR^{RFP} memory T cells by FACS (FACSria Fusion, BD Biosciences), obtained cell populations were barcode-labeled with distinct anti-mouse TotalSeqä Hashtag antibodies (TotalSeq-A0301, TotalSeq-A0302, TotalSeq-A0303, TotalSeq-A0304, TotalSeq-A0305, TotalSeq-A0306, Biolegend), and mixed 1:1, with an equal number of cells from each mouse to form the total pool of cells for scRNA-sequencing. Single-cell RNA isolation and library preparation was performed according to the manufacturer's protocol of the 10X Genomics Chromiumä Single Cell 3' kit, and the cDNA library was sequenced on two separate NextSeqTM runs. Accumulating data of the two individual runs, tallied to a total of ~7x10⁸ reads, and resulted in the detection of ~15,000 cells with a median of 2,143 detected genes per cell. Feature-barcode matrices were generated using the Cell Ranger software of the 10X Genomics Chromiumä pipeline. Cells that could be ascribed to multiple mice or to no mouse (inferred

from the detection of multiple or no Hastags), cells with a transcript (UMI) count lower than 2,000 and cells with a gene count higher than 3,000 genes, or a mitochondrial gene fraction higher than 0.1 were excluded from downstream analysis. Subsequent transcriptional profiling of the remaining 11,767 cells was performed using the Seurat⁴⁸ and MetaCell²⁹ algorithms.

UMI counts derived from the anti-CD27 and anti-KLRG1 TotalSeq antibodies were used to classify cells as either T_{CM} or T_{EM}. Thresholds used to assign cells were set manually, guided by the expression profile measured by flow cytometry. T_{CM} were defined as KLRG1^{LO}CD27^{HI}, T_{EM} were defined as KLRG1^{HI}CD27^{LO}. Remaining cells were unclassified.

To examine enrichment or depletion of DR^{RFP} cells within the different MetaCells, cell counts were first normalized across hashtags. Data obtained from the different mice were subsequently aggregated and used to calculate the ratio of DR^{RFP} versus DR^{GFP} cells in each MetaCell.

The immune signature gene list (Fig 4D and F) was composed of gene clusters involved, or proposed to be involved in T cell function. The full gene list is described in **Supplementary table 6**.

The core T_{EFF} signature was generated by selecting genes that were present in at least 2 out of 7 T_{EFF} gene signatures downloaded from the GSEA database (M9475, M5836, M5820, M3041, M3039, M3027 and M3013; resulting list in **Supplementary table 3**). To determine the shared upregulated genes of both highly and lowly divided cells, the top 1,000 enriched genes from the 3 highest and 3 lowest DR^{TOM}/DR^{GFP} MetaCells were selected. Shared upregulated genes were defined as genes that appeared in the top 1,000 of at least 2 out of 3 MetaCells. Shared downregulated genes were defined similarly, using the top 1,000 depleted genes of each MetaCell.

Statistical analysis

All statistical analyses were performed either with R (V3.6.1, 'Action of the Toes') or Graphpad (V8.4.1, Prism software). All data shown is representative of at least 2 independent experiments.

Declarations

Author contributions

The study was designed by K.B., L.K., F.A.S. and T.N.S., and supervised by T.N.S. and F.A.S.; K.B. and L.K. jointly performed, analyzed, and visualized all experimental work included in the manuscript; F.A.S and K.B designed and developed the retroviral DivisionRecorder construct. L.A.K. and L.J. performed optimization and validation experiments integral to the design of the DivisionRecorder; A.C.S and R.J.D.B. performed mathematical modelling, together with T.S.W, L.P and K.R.D; K.B. and L.K. wrote the manuscript with the input of co-authors; T.N.S. and F.A.S. critically reviewed and revised the manuscript.

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Competing interests

The authors declare no competing financial interests.

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Data availability

Data supporting the findings of this study are available from the corresponding author upon request.

Code availability

Codes were written in R and are available from the corresponding author upon request.

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Figures

Figure 1

DivisionRecorder activation is a proxy for replicative history. (A) Schematic overview of the DivisionRecorder system. (B) Cartoon depiction of the DivisionRecorder in a cell pool (C) Simulation of the minimal ODE model (See Supplementary Information 1 for detailed description and equations), depicting DRRFP acquisition as a function of population doublings for the indicated values of the DRRFP acquisition probability (p). (D, E) Cultures of reporter HEK 293T cells were transduced ($n=3$) with the DivisionRecorder containing either a stable nucleotide region (indicated as "no STR") or a repeat of 24 guanines (indicated as "with STR") directly upstream of the CRE recombinase gene. Representative plots (D) and summarizing line graphs (E) are shown. (F) Cultures of reporter HEK 293T cells were transduced with the DivisionRecorder containing either a low stability STR (repeat of [G]24 [$n=3$]) or a high stability STR ([CA]30 [$n=3$]) directly upstream of the CRE recombinase gene. (G, H) Immortalized DivisionRecorder+ (DR⁺) mouse embryonic fibroblasts were cultured, counted, and analyzed every 3-4

days. Representative FACS plots [G] or percentage of DRRFP cells within the DR⁺ population for three experimental replicates [H]. Best fits of the minimal ODE model (Supplementary Information 1) are depicted. 100 bootstraps were performed on each experimental replicate. Blue line represents the median of the bootstraps, grey lines represent individual fits. *p* indicates DRRFP acquisition probability with a 95% confidence interval of 0.0043 to 0.0063. All depicted data are representative of at least two independent experiments, lines and symbols indicate individual mice or samples. *P* values (F) were determined by ANCOVA test. ****P* < 0.001.

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Figure 2

The DivisionRecorder can be applied to study T cell division kinetics in vivo. (A) Overview of experimental setup. (B, C) DR⁺ OT-I T cells were transferred into recipient mice 24 hours post infection with Lm-OVA. Spleen samples were analyzed for the percentage of DRRFP cells at day 1-4 post transfer, using 7-8 mice per time-point. Representative pseudo-color density plots [B], and box plots in which the center lines signify the median, and whiskers represent min/max [C]. (D, E) CTV stained OT-I T cells were retrovirally transduced with the DivisionRecorder and transferred into recipient mice 24 hours post infection with Lm-OVA. 48 hours post-transfer, splenic DR⁺ OT-I T cells were analyzed for CTV dilution [D], and the percentage of DRRFP cells within each division peak was analyzed [E]. All depicted data are representative of at least two independent experiments, lines and symbols indicate individual mice or

samples. P values were determined by two-sided Kruskal-Wallis test, with Dunn's multiple comparisons test (C), or repeated measurement correlation test (E). ***P < 0.005

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Figure 3

The memory T cell pool consists of replicative 'mature' cells. (A) Response kinetics of DR⁺ OT-I T cells as measured in peripheral blood. Grey lines indicate individual mice (n=6), black line represents group mean. (B, C) Kinetics of the percentage of DRRFP cells within the DR⁺ OT-I T cell pool (n=6). Representative FACS plots of DRRFP and DRGFP frequencies at indicated time points [B], and summarizing line plots [C]. Percentages DRRFP in line plots are relative to the values detected at the first measurement (day 4 post transfer). (D) Paired strip-chart of DRRFP percentages within the DR⁺ OT-I T cell pool at indicated time points during a response against LCMV-OVA. (E) Representative plots depicting DRRFP frequencies in blood (Bl), spleen (Spl) and liver (Liv). (F) Box plots depicting the percentage of DRRFP cells detected in blood, spleen and liver samples of recipient mice at the indicated time points (n=6 per time point). Box plot center lines signify the median and whiskers represent min/max. P values were determined by two-sided Kruskal-Wallis test with Dunn's multiple comparisons test (F), or two-sided Wilcoxon's signed-rank test (D). ***P < 0.001; **P < 0.01; *P < 0.05; n.s., not significant.

Figure 4

Continuous division of early TCM drives disparity in replicative history within the memory T cell pool. Flow cytometric analysis of phenotypic markers on DR+ OT-I T cells at various time points post adoptive transfer. (A) Moving average of surface marker expression level (normalized fluorescence intensity) plotted against the percentage of DRRFP cells within each window during memory (day 86). Grey lines indicate individual spleen samples (n=6), means are shown in black. DRRFP percentages within each

window are corrected for the total percentage of DRRFP cells detected across all cells in that sample. (B) Gating strategy subdividing memory T cells (d86) in spleen samples, numbers indicate percentages. (C) Box plots depicting DRRFP fractions within specific subsets as identified in (B). Box plot center lines signify the median and whiskers represent min/max. (D) Comparison of the replicative history of CD27HIKLRG1LO and CD27LOKLRG1HI cells during effector (d6) and memory phase (d86). Lines connect populations from individual mice (n=6). (E) Moving average of surface marker expression of spleen samples during effector phase (day 6), depicted as in panel A. (F) Paired strip-chart of DRRFP fractions of the CD27HIKLRG1LO and CD27LOKLRG1HI phenotypes, comparing effector (day 5/6) and memory (day >60) phases. Data shown for Lm-OVA (top) and LCMV-OVA (bottom) infections. Lines connect cell populations from individual mice. (G) Ki67 expression by CD27HIKLRG1LO and CD27LOKLRG1HI OT-I cells in response to Lm-OVA. Representative flow cytometry plot of the fraction Ki67+ cells between the two subpopulations over time. P values were determined by two-sided Wilcoxon signed-rank test (C left, D and F) or by ANOVA and Tukey HSD test (C right). *P < 0.05; **P < 0.005; ***P < 0.001; n.s., not significant.

Figure 6

The secondary TEFF expansion wave is mounted from a group of quiescent precursors. (A) Kinetics of the percentage of DRRFP cells in peripheral blood upon secondary Lm-OVA infection. Values are relative to the percentage DRRFP cells detected at the first measurement (day 2 post-secondary infection). Grey lines indicate individual mice (n=6), black line represents group mean. (B) Paired strip-chart of DRRFP percentages detected in the blood at memory (day >60 post primary) and at the peak of the secondary response (occurring on either day 4 or 5 post-secondary infection). Memory pools were generated with LCMV-OVA, recall was performed with Lm-OVA. (C) Box plots depicting the percentage of DRRFP cells detected in blood, spleen and liver samples of recipient mice at indicated time-points (n=6 per time point) post-secondary infection. (D) Best fit of the phenotype model to the blood and spleen data (See Supplementary Information 3 for detailed description and equations) that describes the formation and behaviors of three distinct populations in T cell response. Cell numbers in the spleen (left) and DRRFP percentages (right) are shown for CD27LOKLRG1HI (red) and CD27HIKLRG1LO (blue) populations. Predicted numbers of quiescent TCM are shown in green and represent 4.7% of the total TCM memory population. Lines indicate best fits; bullets indicate observed data in the spleen (see Fig. S4G for how this fit describes the blood data). Dashed lines indicate the secondary infection time-point (d86 post cell transfer). Box plot center lines signify the median, whiskers represent min/max. P values were determined by two-sided Wilcoxon signed-rank test (B), or two-sided Kruskal-Wallis test with Dunn's multiple comparisons test (C). ***P < 0.001; **P < 0.01; *P < 0.05; n.s., not significant.

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