Real-time tracking of cell cycle progression during CD8\(^+\) effector and memory T-cell differentiation

Ichiko Kinjyo\(^1,\dagger\), Jim Qin\(^1\), Sioh-Yang Tan\(^1\), Cameron J. Wellard\(^2,3\), Paulus Mrass\(^1,\dagger\), William Ritchie\(^1\), Atsushi Doi\(^4\), Lois L. Cavanagh\(^1\), Michio Tomura\(^5,\dagger\), Asako Sakaue-Sawano\(^6\), Osami Kanagawa\(^5,\dagger\), Atsushi Miyawaki\(^6\), Philip D. Hodgkin\(^2,3\) & Wolfgang Weninger\(^1,7,8\)

The precise pathways of memory T-cell differentiation are incompletely understood. Here we exploit transgenic mice expressing fluorescent cell cycle indicators to longitudinally track the division dynamics of individual CD8\(^+\) T cells. During influenza virus infection in vivo, naive T cells enter a CD62L\(^\text{int}\) state of fast proliferation, which continues for at least nine generations. At the peak of the anti-viral immune response, a subpopulation of these cells markedly reduces their cycling speed and acquires a CD62L\(^\text{hi}\) central memory cell phenotype. Construction of T-cell family division trees in vitro reveals two patterns of proliferation dynamics. While cells initially divide rapidly with moderate stochastic variations of cycling times after each generation, a slow-cycling subpopulation displaying a CD62L\(^\text{hi}\) memory phenotype appears after eight divisions. Phenotype and cell cycle duration are inherited by the progeny of slow cyclers. We propose that memory precursors cell-intrinsically modulate their proliferative activity to diversify differentiation pathways.
**Results**

**Fucci mice facilitate tracking of the cell cycle in T cells.** In Fucci mice, cells in G0/G1 and S/G2/M cell cycle phases express mKusabira-Orange 2 (mKO2) and mAzami-Green (mAG), respectively26 (Fig. 1a). The schematic in Fig. 1a represents the originally designed Fucci probe pattern for cell cycle progression, where Fucci probes are matched to cell cycle phases26. To dissect proliferative kinetics of CD8+ T cells after antigen activation, Fucci (Fucci-G1/Fucci-SG2-M-double transgenic) mice were crossed with T-cell receptor transgenic OT-I mice27, in which CD8+ T cells recognize OVA257–264 (SIINFEKL) peptide presented in the context of the major histocompatibility complex-I. As expected from the previous observation that Fucci cells in quiescent G0 phase were mKO2++ (ref. 28), naïve CD8+ T cells isolated from naïve Fucci-OT-1 mice were predominantly mKO2++ mAG− (mKO2++; Supplementary Fig. 1a), reflecting their resting state. Following stimulation with SIINFEKL peptide *in vitro*, we found that mKO2++ naïve cells became mKO2+ mAG+ (double positive), mKO2− mAG+ (mAG+), mKO2− mAG− (double negative (DN)) after entry into the first cell cycle (Supplementary Fig. 1a; 20 h). Cell cycle profiling using DNA stains confirmed that mAG+ cells exhibited higher DNA content than 2 N corresponding to S/G2/M phases, and mKO2 positivity was matched to the 2 N state (Supplementary Fig. 1b). At time points when CD8+ T cells are known to proliferate vigorously, cycling cells were found as mAG+ or DN cells with very low mAG or mKO2 intensity (Supplementary Fig. 1a; 50 h). The DN state arises from the gap between the degradation of mAG-hGeminin(1/110) and accumulation of mKO2-hCdt1(30/120) at the early G1 phase26. Thus, when cells cycle with a short G1 phase, a substantial fraction of cells may be found as DN (Supplementary Note 1). In addition, we cannot formally exclude a certain degree of heterogeneity in Fucci transgene expression, which could also contribute to the DN state in the current transgenic mice. Therefore, in this study we decided to take advantage of the Fucci system by mainly focusing on mAG− or mKO2-positive cells to track the real-time proliferative dynamics *in vivo* during an immune response, which could not be addressed with classical methods.

**Slow-cycling memory precursors appear in influenza infection.** To examine the cell cycle kinetics of individual virus-specific CD8+ T cells in the course of infection, we adoptively transferred Fucci/OT-I cells to recipient mice that were subsequently infected with influenza A virus PR8 engineered to express ovalbumin (PR8-OVA)29. As early as 2 days post infection (p.i.), Fucci/OT-I cells in the mediastinal lymph nodes (MLN) entered the cell cycle at a time when memory T-cell precursors first appear *in vivo*, very little information is available on the proliferative signature of T cells. In addition, dynamic studies that determine the correlation between proliferative behaviour and cell fate plasticity of individual T cells within populations over prolonged time periods have not been performed.

To gain insight into these outstanding questions, we utilized transgenic mice that express the fluorescent ubiquitination-based cell cycle indicator ("Fucci"), in which cells become reversibly fluorescent depending on their cell cycle state26. This enabled us to investigate the dynamics of cell cycle progression at the single-cell level during immune responses *in vivo* and *in vitro*. Correlation of the differentiation state of T cells and proliferative activity revealed that naïve T cells initially undergo vigorous proliferation after the encounter of antigens. After nine or more division cycles, a subpopulation of T cells separated from the fast cyclers characterized by slowing down of proliferation speed and paralleled by acquisition of a central memory precursor phenotype. Slow cycling and phenotype were an inherited feature of these cells. Thus, our findings suggest that activated T cells can reset their cell cycle machinery to initiate memory cell differentiation programmes separately from the fast-cycling effector pool.
became mKO2\(^+\) mAG\(^-\), indicating that they became quiescent (Supplementary Fig. 3).

Taking advantage of the Fucci system, we dissected the cell cycle status of highly divided cells after complete dye dilution. Consistent with previous reports\(^3^1\), a population of CD44\(^+\)CD62L\(^hi\) Tcm-like cells appeared on day 7 p.i. in the spleens and MLNs, but not in the lungs (Fig. 2a). When gating on CD62L\(^hi\) cells, approximately half of them were mKO2\(^-\) mAG\(^-\), indicating slowing of their cell cycle as compared with CD62L\(^lo\) effector T cells, which remained mAG\(^+\) (Fig. 2b). Conversely, mKO2\(^+\) cells in the MLN and spleen contained a sizeable population of CD62L\(^hi\) cells, while mAG\(^+\) cells were largely CD62L\(^lo\) (Fig. 2a).

To confirm that the mKO2\(^+\) T cells that reappeared on day 7 p.i. were indeed slower cyclers than their mAG\(^+\) counterparts, mice received a single intraperitoneal injection of BrdU on that day (Fig. 2c). After 3 h, while about 60% of mAG\(^+\) cells incorporated BrdU, mKO2\(^+\) cells mostly stayed BrdU\(^-\). The proportion of BrdU\(^-\) mKO2\(^+\) cells increased over the next 5 h, but never reached the level of mAG\(^+\) cells. As only cells in the S phase can incorporate BrdU, BrdU\(^-\) mKO2\(^+\) cells must be the cells that have newly entered the G\(_1\) phase after recent

**Figure 1** Dynamic cell cycle progression of virus-specific CD8\(^+\) T cells shown by Fucci probes. (a) Fucci fluorescent signals in cell cycle dynamics. The originally designed Fucci system, in which mutually expressed Fucci probes correspond to each cell cycle phase. The cells in G\(_{0/1}\) show high intensity of mKO2-hCdt1(30/120), and S/G2/M cells represent the accumulation of mAG-hGem(1/110). (b) Magnetic-activated cell sorting-purified Fucci/OT-I (CD45.2\(^+\)) cells were labelled with Cell Trace Violet (CTV) dye and transferred into CD45.1\(^-\) CD44\(^hi\) CD62L\(^hi\) Tcm-like cells appeared on day 7 p.i. in the spleens and MLNs, but not in the lungs (Fig. 2a). When gating on CD62L\(^hi\) cells, approximately half of them were mKO2\(^-\) mAG\(^-\), indicating slowing of their cell cycle as compared with CD62L\(^lo\) effector T cells, which remained mAG\(^+\) (Fig. 2b). Conversely, mKO2\(^+\) cells in the MLN and spleen contained a sizeable population of CD62L\(^hi\) cells, while mAG\(^+\) cells were largely CD62L\(^lo\) (Fig. 2a).

To confirm that the mKO2\(^+\) T cells that reappeared on day 7 p.i. were indeed slower cyclers than their mAG\(^+\) counterparts, mice received a single intraperitoneal injection of BrdU on that day (Fig. 2c). After 3 h, while about 60% of mAG\(^+\) cells incorporated BrdU, mKO2\(^+\) cells mostly stayed BrdU\(^-\). The proportion of BrdU\(^-\) mKO2\(^+\) cells increased over the next 5 h, but never reached the level of mAG\(^+\) cells. As only cells in the S phase can incorporate BrdU, BrdU\(^-\) mKO2\(^+\) cells must be the cells that have newly entered the G\(_1\) phase after recent

**Figure 1** Dynamic cell cycle progression of virus-specific CD8\(^+\) T cells shown by Fucci probes. (a) Fucci fluorescent signals in cell cycle dynamics. The originally designed Fucci system, in which mutually expressed Fucci probes correspond to each cell cycle phase. The cells in G\(_{0/1}\) show high intensity of mKO2-hCdt1(30/120), and S/G2/M cells represent the accumulation of mAG-hGem(1/110). (b) Magnetic-activated cell sorting-purified Fucci/OT-I (CD45.2\(^+\)) cells were labelled with Cell Trace Violet (CTV) dye and transferred into CD45.1\(^-\) CD44\(^hi\) CD62L\(^hi\) Tcm-like cells appeared on day 7 p.i. in the spleens and MLNs, but not in the lungs (Fig. 2a). When gating on CD62L\(^hi\) cells, approximately half of them were mKO2\(^-\) mAG\(^-\), indicating slowing of their cell cycle as compared with CD62L\(^lo\) effector T cells, which remained mAG\(^+\) (Fig. 2b). Conversely, mKO2\(^+\) cells in the MLN and spleen contained a sizeable population of CD62L\(^hi\) cells, while mAG\(^+\) cells were largely CD62L\(^lo\) (Fig. 2a).

To confirm that the mKO2\(^+\) T cells that reappeared on day 7 p.i. were indeed slower cyclers than their mAG\(^+\) counterparts, mice received a single intraperitoneal injection of BrdU on that day (Fig. 2c). After 3 h, while about 60% of mAG\(^+\) cells incorporated BrdU, mKO2\(^+\) cells mostly stayed BrdU\(^-\). The proportion of BrdU\(^-\) mKO2\(^+\) cells increased over the next 5 h, but never reached the level of mAG\(^+\) cells. As only cells in the S phase can incorporate BrdU, BrdU\(^-\) mKO2\(^+\) cells must be the cells that have newly entered the G\(_1\) phase after recent

**Figure 1** Dynamic cell cycle progression of virus-specific CD8\(^+\) T cells shown by Fucci probes. (a) Fucci fluorescent signals in cell cycle dynamics. The originally designed Fucci system, in which mutually expressed Fucci probes correspond to each cell cycle phase. The cells in G\(_{0/1}\) show high intensity of mKO2-hCdt1(30/120), and S/G2/M cells represent the accumulation of mAG-hGem(1/110). (b) Magnetic-activated cell sorting-purified Fucci/OT-I (CD45.2\(^+\)) cells were labelled with Cell Trace Violet (CTV) dye and transferred into CD45.1\(^-\) CD44\(^hi\) CD62L\(^hi\) Tcm-like cells appeared on day 7 p.i. in the spleens and MLNs, but not in the lungs (Fig. 2a). When gating on CD62L\(^hi\) cells, approximately half of them were mKO2\(^-\) mAG\(^-\), indicating slowing of their cell cycle as compared with CD62L\(^lo\) effector T cells, which remained mAG\(^+\) (Fig. 2b). Conversely, mKO2\(^+\) cells in the MLN and spleen contained a sizeable population of CD62L\(^hi\) cells, while mAG\(^+\) cells were largely CD62L\(^lo\) (Fig. 2a).

To confirm that the mKO2\(^+\) T cells that reappeared on day 7 p.i. were indeed slower cyclers than their mAG\(^+\) counterparts, mice received a single intraperitoneal injection of BrdU on that day (Fig. 2c). After 3 h, while about 60% of mAG\(^+\) cells incorporated BrdU, mKO2\(^+\) cells mostly stayed BrdU\(^-\). The proportion of BrdU\(^-\) mKO2\(^+\) cells increased over the next 5 h, but never reached the level of mAG\(^+\) cells. As only cells in the S phase can incorporate BrdU, BrdU\(^-\) mKO2\(^+\) cells must be the cells that have newly entered the G\(_1\) phase after recent
Figure 2 | The mKO2-positive cells reappearing on day 7 p.i. are slower cycling cells. Magnetic-activated cell sorting-purified Fucci/OT-I (CD45.2+) cells were labelled with CTV dye and transferred into CD45.1+ recipients prior to intranasal (i.n.) PR8-OVA influenza A virus infection. (a) Representative flow plots of mediastinal lymph nodes (MLNs), lungs and spleens on day 7 p.i. The histograms show CD44 and CD62L levels for mAG+ (blue), mKO2+ (orange) and DN (black dot) cells from infected mice against naive cells in uninfected mice (grey solid). Data are representative of three independent experiments with three to five mice each. (b) Backgating analysis for expression profiles of mAG versus mKO2 in CD62Lhi and lo subsets from the spleens of mice on day 7 p.i. CD62Lhi (red) and lo (blue) gates were determined as shown in the histogram and compared for mAG versus mKO2 in dot plots. Data are representative of three independent experiments with three to five mice each. (c) BrdU incorporation in virus-specific CD8+ T cells on day 7 p.i. The percentages of BrdU+ cells in mAG+, mKO2+ or DN subpopulations in spleens were assessed at 3, 5 and 8 h after BrdU intraperitoneal administration on day 7 p.i. Data are representative of three independent experiments with four mice each. Bars show mean with s.d. (n = 12, *P < 0.001, **P < 0.01; two-way analysis of variance (ANOVA)). (d) mAG+ CD62Llo, DN CD62Llo, mKO2+ CD62Llo and mKO2+ CD62Lhi subsets within the CD8+ CD45.2+ cell population were sorted on day 7 p.i. as single cells and cultured in rIL-2 (10 ng ml−1) containing medium. The wells with a single cell at the time 0 were observed by time-lapse imaging to count the cell number at 48 h. Data were summarized from three independent experiments. Bars show mean with s.d. (n = 35, *P < 0.001 versus KO+ CD62Llo; NS, not significant. ANOVA). (e) Memory-specific cell surface markers on virus-specific CD8+ T cells on day 7 p.i. Top panel: histograms of each marker are shown for mAG+ (blue line) versus mKO2+ (orange line) compared with naive cells in uninfected mice (grey solid). Bottom panel: histograms of each marker are shown for CD62Lhi (red line) versus CD62Llo (blue solid). Data are representative of three independent experiments with three mice each.
cytokinesis. The BrdU− mKO2+ cells remaining after 8 h likely contained not only the cells that have just moved from the G2 to M phase but also those that stayed in the G1 phase for 8 h, indicating that the mKO2+ population contained the slower cycling cells.

Furthermore, culture of sorted single cells isolated on day 7 after PR8-OVA infection showed significantly less expansion of mKO2+ cells compared with mAG+ CD62L10 cells ex vivo (Fig. 2d). Thus, activated virus-specific Fucci transgenic CD8+ T cells were found as mAG+ or DN cells during the initial vigorous expansion phase, and some of them slowed down their cell cycle speed paralleled by the accumulation of mKO2-hCdt1(30/120) on day 7 p.i.

Cell surface marker profiling revealed that both mAG+ and mKO2+ cells on day 7 p.i. displayed an activated phenotype with elevated expression of CD44, CD27, Ly6C and CXCR3 (Fig. 2e). The mAG+ population showed higher expression of CD71 (transferrin receptor protein 1), indicating their highly proliferative status32,33, and slightly higher expression of KLRG-1. Consistent with a memory precursor phenotype, mKO2+ cells expressed higher levels of IL-7Rα. In addition, messenger RNA expression levels of transcription factors ELF4 and KLF2, which are known to bind and activate CD62L and S1PR1 expression levels of transcription factors ELF4 and KLF2, were upregulated in mKO2+ cells similarly to CD8+ memory control (Supplementary Fig. 4). In contrast, the expression levels of interferon (IFN)-γ and IL-2 were higher in mAG+ cells than mKO2+ cells (Supplementary Fig. 4). These data suggest that slow-cycling mKO2+ cells exhibit a Tcm precursor phenotype.

Memory precursors arise from highly proliferative T cells. The fact that mKO2+ CD62L10 cells had diluted cell tracker dye indicated that they may have arisen from the effector cell pool. Alternatively, they may have developed as an independent population with a constantly high CD62L level that initially went through a similar expansion as effector T cells. Following activation, naïve T cells proliferated and also downregulated CD62L expression37 (Fig. 3a). By day 4 p.i., most of the virus-activated cells showed intermediate level of CD62L, as a relatively homogenous population, compared with the distinct CD62Lhi and CD62Llo subsets present on day 7 p.i. To determine whether both CD62Lhi and CD62Llo populations developed from the same activated CD62Lint T-cell pool, we sorted CD44hiCD62Lint T cells within the 4–8th division on day 4 p.i., and transferred them to secondary recipient mice that were concurrently infected with PR8-OVA. After 10 days, we found that the transferred T cells continued to proliferate, and that they gave rise to both CD62Lhi and CD62Llo populations (Fig. 3b). Therefore, virus-activated T cells retain their potency to become effector or memory cells during the early expansion phase, and at some later time point, split into either effector or memory differentiation pathways after multiple division cycles.

Real-time tracking of CD8+ T-cell divisions in vitro. Recent evidence suggests that during primary immune responses, individual naïve T cells give rise to separate families with distinct differentiation profiles, namely highly proliferative effector and less-expanding Tcm families11,12,38. These observations are somewhat at odds with our data that suggest that Tcm precursors undergo initial vigorous proliferation prior to switching to a slow-cycling mode. If indeed distinct proliferative families would be generated, one would expect to find heterogeneity in cell cycle times, and a high correlation (inheritance) of times between generations within individual T-cell families. To better understand the cycling characteristics of T-cell families, we measured actual cell cycle duration by time-lapse imaging in vitro. We sorted in vitro activated Fucci CD8+ T cells from early- (first and third) and late- (eighth) division generations, and performed single-cell time-lapse imaging of cells placed in a microgrid array for up to 90 h (Fig. 4a; Supplementary Movie 1). Next, we manually tracked each cytokinesis by identifying individual cells, and measured their cell cycle duration. Since it was not possible to determine the exact time to the first cytokinesis in the acquired movies, we started measurements after the observed first cytokinesis for two subsequent division rounds (Fig. 4a; Supplementary Fig. 5). To distinguish each cell arising from the same progenitor, we named the cells from the 1st divisions as ‘mothers (M1 and M2)’ and the cells from the 2nd divisions as ‘daughters (D1, D2 or D3, D4)’. For further analysis, daughters’ groups are distinguished as ‘siblings (D1 versus D2, D3 versus D4)’ from the same mother or ‘cousins (D1/D2 versus D3/D4)’ originating from different mothers from the same initial progenitor (C1). By collecting cell cycle time data, we found cells from the 1st and 3rd generations showed similar and fast cell cycle times with a mean of 13.4 ± 5.4 and 14.3 ± 4.8 h, respectively (Fig. 4b). In generation-8 cells, in addition to fast-cycling cells (early dividers) with similar proliferation and inheritance features to the cells from earlier generations, we also observed the occurrence of a subset of slow- and non-cycling cells, some of which had division times of more than 24 h (Fig. 4b; Supplementary Fig. 6a). This was evidenced by the fact that their first cytokinesis was not observed until the 2nd imaging day (late dividers) and their daughters did not divide again before the end of the observation period (Fig. 4b, data below). Consistent with their long division times, the slow-dividing cells had markedly increased periods of mKO2 positivity (Fig. 4c; Supplementary Fig. 6b). These differences in division patterns between the 1st/3rd generation and 8th generation were highly significant (Supplementary Fig. 6c).

Furthermore, we found that the cells with longer mKO2+ phases were smaller in cell size than fast-proliferating mAG+ cells, and were mostly CD62Lhi (Fig. 5a,b) (Fig. 5c). When we cultured sorted small mKO2+ CD62Lhi and S1PR1 (sphingosine-1-phosphate receptor) promoters34-36 were upregulated in mKO2+ cells similarly to CD8+ memory control (Supplementary Fig. 4). In contrast, the expression levels of interferon (IFN)-γ and IL-2 were higher in mAG+ cells than mKO2+ cells (Supplementary Fig. 4). These data suggest that slow-cycling mKO2+ cells exhibit a Tcm precursor phenotype.

Correlation of cell cycle times between T-cell relatives. To examine whether there was evidence for inheritance of cell cycle times within division trees arising from individual T cells, we analysed the correlation of horizontally (that is, siblings and cousins) and vertically (that is, mother versus daughter) related cell cycle times. The correlation between cousins was higher than that of mothers and daughters. Nevertheless, cell cycle times were not strictly identical between generations, but rather showed variations with s.d. of more than 4 h.

The fact that siblings showed high correlation of cycling times could be cell intrinsic, due to equal partitioning of the cycling machinery in the two daughters after cytokinesis, or due to environmental factors within the microgrid. To distinguish between these possibilities, we performed time-lapse imaging of separately stimulated CD8+ T-cell populations expressing either cytoplasmic green fluorescent protein (GFP)39 or membrane-targeted tdTomato40 seeded in the same wells (Fig. 6b). Analysis
Figure 3 | Proliferating virus-specific CD8\(^+\) CD62L\(^{int}\) cells segregate into CD62L\(^{hi}\) and CD62L\(^{lo}\) subsets. Magnetic-activated cell sorting-purified Fucci/OT-I (CD45.2\(^+\)) cells were labelled with CTV dye and transferred to CD45.1\(^+\) recipients prior to PR8-OVA influenza A virus infection. (a) Surface expression of CD62L against CTV dilution on virus-specific CD8\(^+\) T cells in MLNs and spleens on days 2, 3, 4 and 7 p.i. Data are representative of three independent experiments with three to five mice each. (b) CD62L\(^{int}\) Fucci/OT-I cells in the 4th–8th divisions were sorted from splenocytes on day 4 p.i. and transferred into the concurrently infected 2nd recipients. Ten days after transfer, 2nd recipients were analysed for the change of CD62L level. The percentage of CD62L mean fluorescence intensities against naive CD8\(^+\) T cells is shown to compensate differences in fluorescence between sorted and analyzer instrument. Symbols show mean with s.d. (n = 7). Data are representative of three independent experiments with three mice each.

showed that same-colour siblings divided synchronously (Fig. 6c), but non-relatives from differentially-coloured progenitors divided without correlation (Fig. 6d), indicating that cell intrinsic, rather than environmental, factors determine cycling times of activated T cells.

Inheritance of cell cycle times in sequential divisions. Thus far, our data have shown that within a cycling population of activated T cells two basic cycling patterns with respective early and late dividers can be identified in the 8th generation (Fig. 7a). To determine whether these patterns were an inherited feature, we...
arbitrarily categorized cycling times of in vitro stimulated Fucci CD8+ T cells into fast (<600 min), medium (600–800 min), slow (>1,000 min) and undivided cells. This analysis revealed that 8th-generation cells gave rise to a much higher proportion of slow dividers and undivided daughters as compared with earlier generations (Fig. 7b). Inheritance was then tested by grouping mothers into fast, medium and slow dividers, and comparing them with the division category of daughters (Fig. 7c). In the 1st and 3rd generation, both fast- and slow-cycling mothers gave rise to a similar distribution of cycling times of progeny, which is consistent with stochastic resetting of cycling times between generations as described above. In contrast, in the 8th-generation slow-dividing mothers gave rise to a much higher proportion of slow- or non-dividing daughters than faster dividing mothers (Fig. 7c). We concluded that at stage slow division was an intrinsic, inherited feature of Tcm-like cells arising in vitro.

Transcriptome analysis of small slow-cycling CD8+ T cells. To gain further insight into the characteristics of slow-cycling smaller cells that segregated from the activated T-cell pool, we performed a genome-wide microarray expression analysis. Larger and smaller cell-sized CD8+ OT-I cells were sorted on day 7 after in vitro stimulation or influenza virus infection in vivo. In heatmap and clustering analyses, the large cells clustered with effector T-cell control samples, while the smaller cells clustered with memory control samples (Fig. 8a), which indicates that large cells have a closer gene expression pattern with effector cells, but smaller cells are more similar to memory cells. Analysis of selected genes showed that the larger sized cells expressed more cytotoxic molecules and cell cycle regulators associated with their effector phenotype and active proliferation (Figs 5c and 8b). In contrast, smaller sized cells had acquired homing receptors such as CCR7 and CXCR3, and expressed transcription factors such as ELF4 and KLF2, reported to be critical for memory cells (Fig. 8b). Gene set enrichment analysis further demonstrated that smaller sized cells were enriched for genes previously shown to be upregulated in memory cells31 (Fig. 8c), suggesting they are on their way to differentiate into memory cells rather than being effector cells. The larger cells were enriched for genes related to the cell division process, DNA replication, cell cycle regulation, microtubule cytoskeleton and the DNA repair process compatible with their proliferative phenotype (Fig. 8d). Taken together, the slower cell cycle times and higher CD62L expression level of small sized T cells, arising during the peak of the influenza response in vivo and after eight divisions in vitro, match the gene expression profile of bona fide memory T cells.

Discussion
Proliferation critically determines the quality of immune responses by regulating the number of available effector and memory T cells. In addition, proliferative dynamics are linked to the differentiation state of T cells. Characterization of T-cell cycling dynamics during a time when memory T-cell precursors appear during immune responses has been challenging, due to technical limitations of conventional proliferation assays. Using the Fucci cell cycle reporter system, we have longitudinally dissected the proliferative behaviour of CD8+ T cells in the course of immune responses in vivo and in vitro. We show that memory T-cell precursors initially undergo fast proliferation indistinguishable from effector T cells, and then switch to a heritable slow-cycling mode paralleled by their acquisition of a memory cell phenotype. Our data suggest a memory T-cell differentiation pathway, whereby the fast-cycling T-cell pool maintains a flexible programme that enables direct differentiation into Tcm precursors and effector cells during a phase of anti-viral immune responses when the T-cell population is still expanding (Fig. 9). This plasticity of memory cells seems to be guided by cell intrinsic modulation of cell cycle progression, which potentially protects these cells from exhaustion due to slow-cycling characteristics and small cell size.
The rapid expansion of antigen-specific T-cell clones following encounter of cognate antigen is a cardinal feature of the adaptive immune response, and this process has been examined extensively in a variety of infection and immunization models. While it is well established that cell extrinsic and environmental factors, such as major histocompatibility complex/antigen–T cell receptor (TCR) interactions, co-stimulation and cytokines, as well as specific anatomical locations are critical for the proliferation and differentiation of naive CD8+ T cells, it is less clear whether lymphocytes have any intrinsic mechanism to regulate their cell cycle duration. Our time-lapse imaging data of cycling T cell in vitro demonstrate that T-cell siblings derived from the same mother cell displayed synchronous cell cycle progression during the initial expansion phase (until the 9th generation). Considering that the cell cycle is composed of consecutive G1–S–G2–M phases, which are regulated by distinct cell cycle checkpoints, it is likely that siblings inherit the founders’ cell cycle machinery components during cytokinesis when T cells clonally expand. Previous reports have revealed that asymmetric division during the first division may act as a mechanism for CD8+ T cells fate determination. This, prior to the first division, asymmetric distribution of signalling molecules is established during interactions with antigen-presenting cells, and cells receiving less of such molecules proceed towards central memory cells. While our data do not contradict these results, they suggest that during subsequent divisions, which are more IL-2 dependent and can occur without further antigen stimulation, asymmetry may not play a role, resulting in daughter cells that more closely resemble each other. Nevertheless, division times between unrelated fast-cycling cells do show variations, with cycling times apparently reset after each generation. This is reminiscent of B cells, in which the cycling machinery is inherited in each individual generation, but that some, as yet unknown, stochastic process, randomizes division times equally for the two daughters after each division.
Summary from three independent experiments from Fig. 4. (a) Correlation analysis of cell cycle times between 'siblings' (top), 'mother–daughter' (middle) and 'cousins' (bottom) from sorted 1st, 3rd and 8th generations. Data are cumulative of three independent experiments from Fig. 4. Significance of correlation was determined by Spearman’s rank correlation coefficient ($\rho$), significance (P) and number of events (N). (b) Scheme shows the strategy for sorting and tracking of non-relative GFP+ or membrane-tdTomato+ (mTomato+) cells from their respective 8th generations. (c) Left: representative snapshots with synchronous divisions of two mTomato+ or two GFP+ cells (top) or with a mTomato+ and a GFP+ cell (bottom) (scale bar, 10.0 $\mu$m). Right: frequency of observed division types. Data are cumulative from three independent experiments. Bars represent mean ± s.d. (N = 45). (d) Correlation analysis of cell cycle times between GFP+ and mTomato+ cells. Data are cumulative from three independent experiments. Significance of correlation was determined by Spearman’s rank correlation coefficient ($\rho$), significance (P) and number of events (N).

Previous studies have shown that a single naive CD8+ T cell can achieve both effector and memory subset differentiation27–28, supporting the idea that distinct T-cell subsets may develop by intraclonal diversification during immune responses. Additional evidence using barcoded or congenically labelled cell-transfer methods suggested that naive T cells have the potential to respond with heterogeneity to the initial antigen stimulation, resulting in the generation of diverse families with different capacity for expansion, as well as development into effector or central memory cells11,12. Nevertheless, it is conceivable that certain T-cell families are heterogeneous and may change their proliferative behaviour over time. Indeed, even in proliferative families, which were considered to be composed of fast-cycling cells of effector phenotype, CD62L+ were present11. Whether such intra-familial phenotypic heterogeneity is also reflected by varied cell cycle times would require the longitudinal tracking of individual dividing cells over several generations. In our experiments, we measured the cell cycle times of individual CD8+ T cells during sequential cytokinesis using time-lapse imaging and examined the relationship between families or
T cells within a given family. Consistent with the mentioned studies, 1, 12, 49, T-cell cycle times between progenies from the same founder showed high correlation while they varied between families. Importantly, however, a slower cycling subpopulation of central memory-like cells appeared after many divisions from the fast-cycling T-cell pool, suggesting that cycling capacity is not fixed within T-cell families. Additional support for this hypothesis comes from our adoptive transfer experiments using CD62L^{hi}CD44^{hi}CD8^{+} T cells in the 4–8th division generations, which excluded weakly activated T cells and/or ‘late comers’ to the antigen-presenting site, as those cells are known to preferentially obtain a memory phenotype. Upon transfer these cells maintained the capability to differentiate into both CD62L^{hi} and CD62L^{lo} cells in the presence of cognate antigen. Although

Figure 8 | Microarray analysis of larger and smaller sized T cells from influenza-infected mice. RNA samples were prepared from sorted populations of larger or smaller sized cells from spleens of influenza virus PR8-OVA-infected mice on day 7 p.i. or from in vitro 7 days culture after stimulation with plate-bound anti-CD3ε (1.0 μg ml^{-1}) and anti-CD28 mAb (0.5 μg ml^{-1}). Effector T-cell control samples were prepared from SIINFEKL (100 ng ml^{-1}) stimulated OT-I cells after 4 days of in vitro culture and sorted as CD8^{+}CD44^{hi}CD62L^{lo}. Control bona fide effector memory and central memory T cells were sorted from the spleens of PR8-OVA-infected mice on day 42 p.i. Naive cells were sorted as CD8^{+}CD44^{hi}CD62L^{lo} cells from uninfected C57BL/6 mice. Duplicate samples were prepared from independent experiments. (a) Clustering analysis and heatmap of gene expression values to depict the similarity of gene profiles between samples for the 934 significant genes (P < 0.01). The colour key shown on the top illustrates the relative expression level across all samples: blue represents expression above the mean and yellow represents expression lower than the mean. The in vivo samples of interest are labelled with a double underline and in vitro samples are labelled with a single underline. (b) The effector or memory phenotype-associated genes were compared between duplicate samples of larger and smaller sized cells sorted from the spleens of infected mice on day 7 p.i. The log fold change of the expression value (the larger cells/the smaller cells) is shown as black bars. The means with s.d. are shown. (c) Gene set enrichment analysis (GSEA) plot shows that the duplicated samples of sorted smaller cells from spleens on day 7 p.i. are enriched for previously reported gene sets for memory CD8^{+} cells (P < 0.001, false discovery rate (FDR) < 0.25). (d) GSEA plots showing the enrichment of cell cycle checkpoint or DNA repair gene sets in the duplicate samples of sorted larger cells from spleens on day 7 p.i. (P < 0.001, FDR < 0.25).
to segregate into the smaller CD62Lhi mKO2 (around days 4–6). On day 7 p.i., the proliferative CD8+ T cells have the plasticity to enter the central memory differentiation pathway to escape the contraction phase adds new perspectives for memory development in vaccination and immunotherapeutic strategies.

**Methods**

**Mice.** C57BL/6 (wild type) and B6.SJL/Ptprc<sup>+</sup> (CD45.1) mice were purchased from the Animal Research Centre (Perth, Australia). mKO2-CdGdl1/30/120 (Fucci-G1; #639) and mAG-hGem(1/110) (Fucci/Gc/M-#474) transgenic mice were described in a previous report<sup>26,28,53</sup>. Fucci-double transgenic mice (#639/#474) were backcrossed onto the C57BL/6 background, and further crossed with OT-1 TCR transgenic<sup>27</sup> (CD45.2) and B6.SJL/Ptprc<sup>+</sup> (CD45.1) mouse strains. DPE-gfp transgenic mice that express GFP under the control of the murine CD4 promoter<sup>59</sup>, and mt/mG transgenic mice that express membrane-targeted Tomato fluorescent protein under the chicken β-actin promoter<sup>40</sup> were described previously. All mice were maintained in specific pathogen-free conditions at the Centenary Institute animal facility. All experiments were performed in accordance with protocols approved by the Animal Ethics Committee at the University of Sydney and the Sydney Local Health District Animal Welfare Committee.

**Adoptive transfers and influenza A virus infection.** CD8+ T cells from spleen and LN’s of Fucci/OT-1 mice were purified using anti-CD8<sup>+</sup>-conjugated microbeads (Miltenyi Biotec) and labelled with 5 μM Cell Trace Violet (Ctv, Invitrogen) in pre-warmed PBS at 10<sup>5</sup> ml<sup>−1</sup>. Labelling was performed by incubating in a 37°C water bath for 20 min and stopped by adding 100% fetal calf serum (FCS) and followed by incubation in RPMI with 10% FCS. Cells were washed twice using PBS and counted. Labelled Fucci/OT-1 cells (10<sup>⁶</sup>) were adoptively transferred into B6.SJL/Ptprc<sup>+</sup> or Fucci/B6.SJL/Ptprc<sup>+</sup> mice. On the next day, the mice were anaesthetised by intraperitoneal injection of Ketamine/Xylazine (80/10 mg kg<sup>−1</sup>) and infected intranasally with the haemagglutinin units of OVA<sub>257–264</sub> peptides expressing influenza virus A/Puerto Rico/8/34 (PR8-OVA<sub>257–264</sub>) kindly provided by Dr. S. Turner, University of Melbourne) in 30 μl PBS.

**Flow cytometry.** Isolation of cells from lungs, MLNs and spleens, and surface staining was performed as described previously<sup>54</sup>. Cell suspensions from lungs were obtained by digestion with 2 mg ml<sup>−1</sup> collagenase IV (Sigma-Aldrich) for 20 min in an air incubator at 37°C. To obtain single-cell suspensions, tissue was passed through a metal cell strainer (80 μm; Seer filters), Cells were washed with fluorescence-activated cell sorting (FACS) buffer (2% FCS, 2 mM EDTA and 0.02% sodium azide/1 × PBS) and incubated with anti-CD61/32 (2.4.G2; BD Biosciences) for blocking Fc receptors. Cells were stained with biotin or fluorochrome-conjugated primary antibodies for 30 min on ice. Antibodies used for flow cytometry were purchased from BD Biosciences (CD3: 53-6.7, CD27: LG.7F9, CD44: IM7, CD45.2: 104), Biolegend (CXCR3: CXCR3-173, Ly6C: HK1.4), eBioscience (CD45.1: A20, CD261: MEL-14, CD71: R17217, KLRG-1; 2F1, IL-7Rα: P8, IL-15Rα: DNT15Ra) or Invitrogen (SA-Alexa Fluor 594, SA-Alexa Fluor 647) and used at a dilution of 1/400–1/12,000. After staining, cell suspensions were resuspended in 0.25 μg ml<sup>−1</sup> propidium iodide (PI; Molecular probes) or 0.5 μg ml<sup>−1</sup> 4′,6-diamidino-2-phenylindole (DAPI; Molecular probes) containing FACS buffer for exclusion of dead cells. Data were collected on an LSRII or Fortessa (BD Biosciences) and analysed with FlowJo software (Tree Star). Cell sorting was performed using a BD Aria II (BD Biosciences).

**BrdU labelling in vivo.** On day 7 p.i., influenza virus-infected mice were intraperitoneally injected with 100 μl of 1 mg ml<sup>−1</sup> BrdU (BD Biosciences) at 3, 5 and 8 h prior to tissue harvest. Cell suspensions from spleens of infected mice were stained for surface markers, and BrdU<sup>+</sup> cells were identified using the BrdU Flow Kit according to the manufacturer’s specifications (BD Biosciences).

**Whole-mount confocal microscopy.** On day 7 after infection, lungs were harvested and fixed in 10% sucrose, 4% formaldehyde solution at 4°C overnight. The lungs were embedded in 4% agarose (Sigma-Aldrich) prepared in triple-distilled water and cut as 200-μm sections with a vibratome (Vibratome 1000 Classic; SDR). Staining was performed by incubating sections with purified anti-mouse CD45.2 antibody (eBioscience) and anti-lamini antibody (L9393; Sigma-Aldrich) overnight at 4°C. The section was washed with 10% of FCS and incubated with Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) and Alexa Fluor 647 goat anti-rat IgG (Invitrogen) at 4°C for 4 h. After washing, the sections were mounted on slides using Mounting medium (DAKO) and imaged with a SP5 confocal microscope (Leica Microsystems) with × 63 oil immersion objective (HCX PL APO 63 × 1.3).
In vitro CD8+ T-cell stimulation and live-cell imaging. CD8+ T cells from spleen of naive Fucci/OT-I mice were purified and labelled with CTV as described above. Cells were stimulated with plate-bound anti-mouse CD3e mAb (1.0 μg ml−1; 145-2C11; BD Biosciences) and anti-CD28 mAb (0.5 μg ml−1; 37.51; BD Biosciences) in T-cell medium (TCM) consisting of RPMI 1640, 10% of FCS, 1 mM sodium pyruvate, 10 mM HEPES, 100 U ml−1 penicillin, 100 μg ml−1 streptomycin and 50 μM 2-mercaptoethanol (Gibco). On the next day, cells were washed and cultured in TCM with 10 ng ml−1 streptomycin and 50 μM 2-mercaptoethanol (Gibco). On the next day, cells were washed and cultured in TCM with 10 ng ml−1 streptomycin and 50 μM 2-mercaptoethanol (Gibco). On the next day, cells were washed and cultured in TCM with 10 ng ml−1 streptomycin and 50 μM 2-mercaptoethanol (Gibco). On the next day, cells were washed and cultured in TCM with 10 ng ml−1 streptomycin and 50 μM 2-mercaptoethanol (Gibco). On the next day, cells were washed and cultured in TCM with 10 ng ml−1 streptomycin and 50 μM 2-mercaptoethanol (Gibco). On the next day, cells were washed and cultured in TCM with 10 ng ml−1 streptomycin and 50 μM 2-mercaptoethanol (Gibco). On the next day, cells were washed and cultured in TCM with 10 ng ml−1 streptomycin and 50 μM 2-mercaptoethanol (Gibco).

Single-cell tracking and analysis of time-lapse imaging. Time-lapse image files were converted into movie format using Velocity software (PerkinElmer) to track cells. Only wells containing single cells at the beginning of imaging were selected and tracked manually for sequential cell division until the end of imaging. If cells exited from the well or if other cells entered the same well, or if the cells died during imaging, wells were excluded from further analysis. Cell death was determined by cell morphology in bright-field images. The cell cycle times of tracked cells were measured and subjected to frequency distribution and correlation analysis using Spearman’s rank correlation coefficient. For the permutation test, the raw data of cell cycle time from tracking cells in generation 8 were randomized and subjected to correlation analysis.

In vitro stimulation with OVA peptide. Total splenocytes from Fucci/OT-I mice were incubated with K+ restricted ovalbumin-derived SIINFEKL peptide (100 ng ml−1, Auspep) in TCM for indicated times before FACs analysis.

Quantitative real-time RT-PCR. Sorted populations from influenza virus-infected mice spleens were applied for quantitative PCR. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription (RT)-PCR was carried out with the Maloney MLV reverse transcriptase with Oligo (dT)15 Primer (Promega). Quantitative real-time PCR was performed using a Mx3000P qPCR system with MxPro qPCR software v2.0 (www.broadinstitute.org/gsea) was performed to determine whether the predefined PL-4. Obar, J. J. & Lefranc, M. 20. Bird, J. J. et al. Differentiation, and tissue pathology. Immunity 37, 709–720 (2012).

References

1. Kaech, M. S., Wherry, J. E. & Ahmed, R. Effector and memory T-cell differentiation: implications for vaccine development. Nat. Rev. Immunol. 2, 251–262 (2002).
2. Williams, M. A. & Bevan, M. J. Effector and memory CTL differentiation. Annu. Rev. Immunol. 25, 171–192 (2007).
3. Harty, J. T. & Badovinac, V. P. Shaping and reshaping CD8+ T-cell memory. Nat. Rev. Immunol. 8, 107–119 (2008).
4. Obar, J. J. & Lefrançois, L. Memory CD8+ T cell differentiation. Annu. N. Y. Acad. Sci. 1183, 251–266 (2010).
5. Jacob, J. & Baltimore, D. Modelling T-cell memory by genetic marking of memory T cells in vivo. Nature 399, 593–597 (1999).
6. Opferman, T. J., Ober, T. B. & Ashton-Rickardt, G. P. Linear differentiation of cytotoxic effectors into memory T lymphocytes. Science 283, 1745–1748 (1999).
7. Dwyer, W. N. & Hedrick, S. M. Cutting edge: latecomer CD8+ T cells are imprinted with a unique differentiation program. J. Immunol. 177, 777–781 (2006).
8. Teixeiro, E. et al. Different T-cell receptor signals determine CD8+ memory versus effector development. Science 323, 502–505 (2009).
9. Chang, J. T. et al. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. Science 315, 1687–1691 (2007).
10. King, C. G. et al. T-cell affinity regulates asymmetric division, effector cell differentiation, and tissue pathology. Immunity 37, 709–720 (2012).
11. Gerlach, C. et al. Heterogeneous differentiation patterns of individual CD8+ T cells. Science 340, 635–639 (2013).
12. Buchholz, V. R. et al. Disparate individual fates compose robust CD8+ T cell immunity. Science 340, 630–635 (2013).
13. Jameson, S. C. Maintaining the norm: T-cell homeostasis. Nat. Rev. Immunol. 2, 547–556 (2002).
14. Suri, C. D. & Sprent, J. Homeostasis of naive and memory T cells. Immunology 29, 848–862 (2002).
15. Van Snapdonk, M. J., Lemmens, E. E. & Schoonberg, S. P. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. Nat. Immunol. 2, 423–429 (2001).
16. Malek, T. R. The biology of interleukin-2. Annu. Rev. Immunol. 26, 453–479 (2008).
17. Veiga-Fernandes, H. & Rocha, B. High expression of active CDK6 in the cytolytic program of CD8+ memory cells favors rapid division. Nat. Immunol. 5, 31–37 (2004).
18. Yoon, H., Kim, T. S. & Braciale, T. J. The cell cycle time of CD8+ T cells responding in vivo is controlled by the type of antigenic stimulus. PLoS ONE 5, e15423 (2010).
19. Harty, J. T. & Badovinac, P. V. Shaping and reshaping CD8+ T-cell memory. Annu. Rev. Immunol. 27, 107–119 (2009).
20. Bird, J. J. et al. Helper T-cell differentiation is controlled by the cell cycle. Immunol. 99, 229–237 (1998).
21. Oehen, S. & Brudsha-Riem, K. Differentiation of naive CD8+ T cells. Science 340, 635–639 (2013).
22. Bett, V. A. & Hodgkin, D. P. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. Proc. Natl. Acad. Sci. USA 95, 9488–9493 (1998).
23. Jenkins, M. R. et al. Cell cycle-related acquisition of cytotoxic mediators defines the progressive differentiation to effector status for virus-specific CD8+ T cells. J. Immunol. 181, 3818–3822 (2008).
24. Lyons, A. B. & Parish, R. C. Determination of lymphocyte division by flow cytometry. J. Immunol. Methods 171, 131–137 (1994).
25. Bett, V. A. & Hodgkin, D. P. A cellular calculus for signal integration by T cells. Nat. Immunol. 1, 239–244 (2000).
26. Sakaue-Sawano, A. et al. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. Cell 132, 487–498 (2008).
27. Hoggquist, K. A. et al. T cell receptor antagonist peptides induce positive selection. Cell 76, 17–27 (1994).
28. Tomura, M. et al. Contrasting quiescent G0 phase with mitotic cell cycling in the mouse immune system. PLoS ONE 8, e73801 (2013).
29. Taddeo, R. M., Webby, R. J. & Turner, J. S. Addition of a prominent epitope affects influenza A virus-specific CD8+ T cell immunodominance hierarchies when antigen is limiting. J. Immunol. 177, 2917–2925 (2006).
30. Lawrence, W. C., Ream, M. R. & Braciale, J. T. Frequency, specificity, and sites of expansion of CD8+ T cells during primary pulmonary influenza virus infection. J. Immunol. 170, 5332–5340 (2003).

31. Yamada, T., Park, C. S., Mamonkin, M. & Lacorazza, H. D. Transcription factor ELF4 controls the proliferation and homing of CD8+ T cells via the Krüppel-like factors KLF4 and KLF2. Nat. Immunol. 10, 618–626 (2009).

32. Chao, C. C., Jensen, R. & Dailey, M. O. Mechanisms of L-selection regulation by activated T cells. J. Immunol. 159, 1686–1694 (1997).

33. Schlub, T. E. et al. Division-linked differentiation can account for CD8+ T-cell phenotype in vivo. Eur. J. Immunol. 39, 67–77 (2009).

34. Manjunath, N. et al. A transgenic mouse model to analyze CD8(+) effector T cell differentiation in vivo. Proc. Natl Acad. Sci. USA 96, 13932–13937 (1999).

35. Carlson, C. M. et al. Kruppel-like factor 2 regulates thymocyte and T-cell migration. Nature 442, 299–302 (2006).

36. Yo, M., Sakaue-Sawano, A., Noda, S., Miyawaki, A. & Miyoshi, H. Fucci-guided stochastic competition. Science 335, 1578–1581 (2012).

37. Chao, C. C., Jensen, R. & Dailey, M. O. Mechanisms of L-selection regulation by activated T cells. J. Immunol. 159, 1686–1694 (1997).

38. Schlub, T. E. et al. Division-linked differentiation can account for CD8+ T-cell phenotype in vivo. Eur. J. Immunol. 39, 67–77 (2009).

39. Manjunath, N. et al. A transgenic mouse model to analyze CD8(+) effector T cell differentiation in vivo. Proc. Natl Acad. Sci. USA 96, 13932–13937 (1999).

40. Muzumdar, D. M., Tasic, B., Miyamiichi, K., Li, L. & Luo, L. A global survey of T-cell development. Nat. Rev. Immunol. 8, 108–112 (2008).

41. Hawkins, E. D., Markham, J. F., McGuinness, L. P. & Hodgkin, P. D. A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. Immunity 27, 985–997 (2007).

42. Gerlach, C. et al. One naive T cell, multiple fates in CD8+ T cell differentiation. J. Exp. Med. 207, 1235–1246 (2010).

43. Lemaître, F., Moreau, H. D., Vede, L. & Bousso, P. Phenotypic CD8+ T cell diversification occurs before, during, and after the first T cell division. J. Immunol. 191, 1578–1585 (2013).

44. Hawkins, E. D., Markham, J. F., McGuinness, L. P. & Hodgkin, P. D. A single naive CD8+ T cell can develop into diverse effector and memory subsets. Immunity 27, 985–997 (2007).

45. Lawrance, W. C., Ream, M. R. & Braciale, J. T. Frequency, specificity, and sites of expansion of CD8+ T cells during primary pulmonary influenza virus infection. J. Immunol. 170, 5332–5340 (2003).

46. Dufy, K. R. et al. Activation-induced B cell fates are selected by intracellular stochastic competition. Science 335, 338–341 (2012).

47. Lemaitre, F., Moreau, H. D., Vede, L. & Bousso, P. Phenotypic CD8+ T cell diversification occurs before, during, and after the first T cell division. J. Immunol. 191, 1578–1585 (2013).

48. Gerlach, C. et al. One naive T cell, multiple fates in CD8+ T cell differentiation. J. Exp. Med. 207, 1235–1246 (2010).

49. Lemaître, F., Moreau, H. D., Vede, L. & Bousso, P. Phenotypic CD8+ T cell diversification occurs before, during, and after the first T cell division. J. Immunol. 191, 1578–1585 (2013).

50. Marchingo, J. M. et al. Antigen affinity, costimulation, and cytokine inputs sum linearly to amplify T cell expansion. Science 346, 1123–1127 (2014).

51. Jones, R. G. & Thompson, C. B. Reviving the enigma: signal transduction fuels T cell activations. Immunity 27, 173–178 (2007).

52. Araki, K. et al. mTOR regulates memory CD8 T-cell differentiation. Nature 460, 108–112 (2009).

53. Yo, M., Sakaue-Sawano, A., Noda, S., Miyawaki, A. & Miyoshi, H. Fucci-guided purification of hematopoietic stem cells with high repopulating activity. Biochem. Biophys. Res. Commun. 457, 7–11 (2014).

54. Tan, S. Y. et al. Phenotype and functions of conventional dendritic cells are not compromised in aged mice. Immunol. Cell Biol. 90, 722–732 (2012).

55. Day, D. et al. A method for prolonged imaging of motile lymphocytes. Immunol. Cell Biol. 87, 154–158 (2009).

56. Yamada, T., Park, C. S., Mamonkin, M. & Lacorazza, H. D. Transcription factor ELF4 controls the proliferation and homing of CD8+ T cells via the Krüppel-like factors KLF4 and KLF2. Nat. Immunol. 10, 618–626 (2009).

57. Tanaka, K. et al. Kruppel-like factor 2 is required for trafficking but not quiescence in postactivated T cells. J. Immunol. 186, 775–783 (2011).

58. Smyth, K. G. in Bioinformatics and Computational Biology Solutions using R and Bioconductor (eds Gentleman, R. C., Carey, V. J., Dudoit, S. R., Irizarry, R. & Huber, W.) 397–420 (Springer, 2005).

Acknowledgements

We thank members of the Weninger and Hodgkin laboratories for discussions, the Centenary Institute Flow Cytometry/Imaging Facility for cell sorting and microscopy. Animal Facility for mouse housing. Dr S. Turner (University of Melbourne) for providing PR8-OVA influenza virus, Dr D. Day (Swinburne University of Technology) and Microsurfaces Pty. Ltd. for providing the microgrids and the Ramaciotti Center at UNSW for microarray analysis. We acknowledge Ms R. Barugahare, M. Rizk and A. Cooray for maintaining mouse strains. This work was supported by a grant from the National Health and Medical Research Council (APP1030145 to W.W.) and a contract from the National Institutes of Health (BAA-NIAID-DAIT-HHSN2120110018C to W.W.).

Author contributions

I.K. and W.W. conceived the study. I.K., J.Q. and S.-Y.T. performed experiments. C.J.W. provided PR8-OVA influenza virus, Dr D. Day (Swinburne University of Technology) and Microsurfaces Pty. Ltd. for providing the microgrids and the Ramaciotti Center at UNSW for microarray analysis. We acknowledge Ms R. Barugahare, M. Rizk and A. Cooray for maintaining mouse strains. This work was supported by a grant from the National Health and Medical Research Council (APP1030145 to W.W.) and a contract from the National Institutes of Health (BAA-NIAID-DAIT-HHSN2120110018C to W.W.).

Additional information

Accession codes: Microarray expression data have been deposited to NCBI’s Gene Expression Omnibus with accession number GSE48219.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Kenjiyo, I. et al. Real-time tracking of cell cycle progression during CD8+ effector and memory T-cell differentiation. Nat. Commun. 6:6301 doi: 10.1038/ncomms7301 (2015).
Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:
Kinjyo, I; Qin, J; Tan, S-Y; Wellard, CJ; Mrass, P; Ritchie, W; Doi, A; Cavanagh, LL; Tomura, M; Sakaue-Sawano, A; Kanagawa, O; Miyawaki, A; Hodgkin, PD; Weninger, W

Title:
Real-time tracking of cell cycle progression during CD8(+) effector and memory T-cell differentiation

Date:
2015-02-01

Citation:
Kinjyo, I., Qin, J., Tan, S.-Y., Wellard, C. J., Mrass, P., Ritchie, W., Doi, A., Cavanagh, L. L., Tomura, M., Sakaue-Sawano, A., Kanagawa, O., Miyawaki, A., Hodgkin, P. D. & Weninger, W. (2015). Real-time tracking of cell cycle progression during CD8(+) effector and memory T-cell differentiation. NATURE COMMUNICATIONS, 6 (1), https://doi.org/10.1038/ncomms7301.

Persistent Link:
http://hdl.handle.net/11343/255789

License:
CC BY