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## **Assessing T cell differentiation at the single-cell level**

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## ONE NAÏVE T CELL - MULTIPLE FATES IN CD8<sup>+</sup> T CELL DIFFERENTIATION

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The mechanism by which the immune system produces effector and memory T cells is largely unclear. To allow a large-scale assessment of the development of single naive T cells into different subsets, we have developed a technology that introduces unique genetic tags (barcodes) into naive T cells. By comparing the barcodes present in antigen-specific effector and memory T cell populations in systemic and local infection models, at different anatomical sites, and for TCR-pMHC interactions of different avidities, we demonstrate that under all conditions tested, individual naive T cells yield both effector and memory CD8<sup>+</sup> T cell progeny. This indicates that effector and memory fate decisions are not determined by the nature of the priming antigen-presenting cell or the time of T cell priming. Instead, for both low and high avidity T cells, individual naive T cells have multiple fates and can differentiate into effector and memory T cell subsets.

## INTRODUCTION

Activation of naïve antigen-specific T cells is characterized by a vigorous proliferative burst, resulting in the formation of a large pool of effector T cells. Following pathogen clearance, ~95% of activated T cells die, leaving behind a stable pool of long-lived memory cells <sup>1</sup>. Two fundamentally different mechanisms could give rise to the production of effector and memory T cells during an immune response. First, single naïve T cells may be destined to produce either effector T cells or memory T cells, but not both (one naïve cell - one fate). As an alternative, effector and memory T cells could derive from the same clonal precursors within the naïve T cell pool (one naïve cell - multiple fates). As the fate decisions that control T cell differentiation could either be taken during initial T cell priming (i.e. before the first cell division) or at later stages, at least four conceptually different models describing effector and memory T cell differentiation can be formulated (Fig. S1).

A first model predicts a separate origin of effector and memory T cells as a result of differential T cell priming by APCs. In this scenario, fate decisions would be taken before the first cell division and even though cells destined to become memory cells may transiently display traits associated with effector T cells (e.g. expression of Granzyme B or IFN- $\gamma$ ; see below), their ability for long-term survival would be predetermined. In line with this model, several studies have provided evidence that the fate of CD8<sup>+</sup> T cells may, to some extent, be programmed during initial activation <sup>1-5</sup>.

A second model, which relies on recent data from Chang *et al.* <sup>6</sup>, likewise suggests that the priming APC plays the crucial role in determining effector or memory T cell fate, but by a strikingly different mechanism and with an opposite prediction concerning the lineage relationship of effector and memory T cells. Specifically, analysis of T cell – APC conjugates has shown that the first division of activated T cells can be asymmetric, with the daughter T cell that is formed proximal to the APC being more likely to contribute to the effector T cell subset and the distal daughter T cell being more likely to generate memory T cells <sup>6</sup>. Assuming that all primary daughter cells survive and yield further progeny, these data would predict that single naïve T cells contribute to both the effector and the memory subset.

In contrast to these two models that are based on a determining role of the priming APC, two other models predict that T cell fate is determined by the cumulative effect of signals that not only naïve T cells but also their descendants receive. The first of these models, termed the 'decreasing potential model', argues that T cell progeny that receive additional stimulation after priming undergo terminal differentiation towards the effector subset, whereas descendants that do not encounter these signals may transiently display certain effector functions, but will ultimately become memory T cells <sup>7</sup>. In support of this model, it has been demonstrated that continued inflammatory signals <sup>8,9</sup> and prolonged antigenic stimulation <sup>10</sup> can lead descendant CD8<sup>+</sup> T cells to preferentially develop into effector cells.

If the descendants of all individual naïve T cell have an equal chance of receiving signals for terminal differentiation, the standard decreasing potential model predicts that memory and effector T cells will be derived from the same population of naïve T cells. There is however evidence that the environmental factors that promote either terminal differentiation or memory T cell development may alter over the course of infection <sup>10</sup>. A fourth model therefore argues that the progeny of T cells that are activated early or late during infection will receive distinct signals, and hence assume (partially) different fates <sup>11-14</sup>.

A large number of studies in which cell differentiation was analyzed at the population level have been informative in revealing which effector properties can be displayed by T cells that subsequently differentiate into memory T cells (reviewed in <sup>15</sup>). In particular, two recent studies using IFN- $\gamma$  or granzyme B reporter mice have shown that memory T cells can arise from cells that have previously transcribed IFN- $\gamma$  or granzyme B genes <sup>5,16</sup>. It is however important to realize that these studies reveal little with regard to the developmental potential of individual naïve T cells. Specifically, the fact that T cells that have a particular effector capacity can become memory T cells does not indicate that all naïve T cells yield such effector cells, nor does it indicate that all memory T cells have gone through an effector phase.

To determine the developmental potential of naïve T cells, it is essential to develop technologies in which T cell responses can be analyzed at the single naïve T cell level. In early work that aimed to follow T cell responses at the clonal level, TCR repertoire analysis has been used to assess the kinship of T cell populations <sup>17,18</sup>. However, as several naïve T cell clones can share the same TCR, it has been argued that such analyses do not necessarily monitor T cell fate at the single T cell level <sup>19,20</sup>. Recently, Stemberger *et al.* have reported on a more elegant approach to address naïve T cell potency <sup>14</sup>. Using the transfer of single naïve CD8<sup>+</sup> T cells into mice, this study provides direct evidence that a single naïve CD8<sup>+</sup> T cell can form both effector and memory cell subsets. However, the statistical power of single cell transfer studies obviously has limitations. In addition, if homeostatic proliferation would occur prior to antigen-driven proliferation in this system, this would limit the conclusions that can be drawn with regard to the pluripotency of a single naïve T cell.

In this study, we have developed a technology that allows the generation of naïve T cells that carry unique genetic tags (barcodes), and we describe how this technology can be utilized for the large-scale assessment of the developmental potential of single naïve T cells. Using physiological frequencies of barcode-labeled naïve CD8<sup>+</sup> T cells of different functional avidities, we demonstrate that in both systemic and local infection models, effector and memory CD8<sup>+</sup> T cell subsets share the same precursors in the naïve T cell pool. These data demonstrate that under all conditions analyzed, single naïve T cells do not selectively yield effector or memory T cells. Rather, T cell differentiation into effector and memory T cell subsets occurs by a one naïve cell - multiple fates principle.

## RESULTS

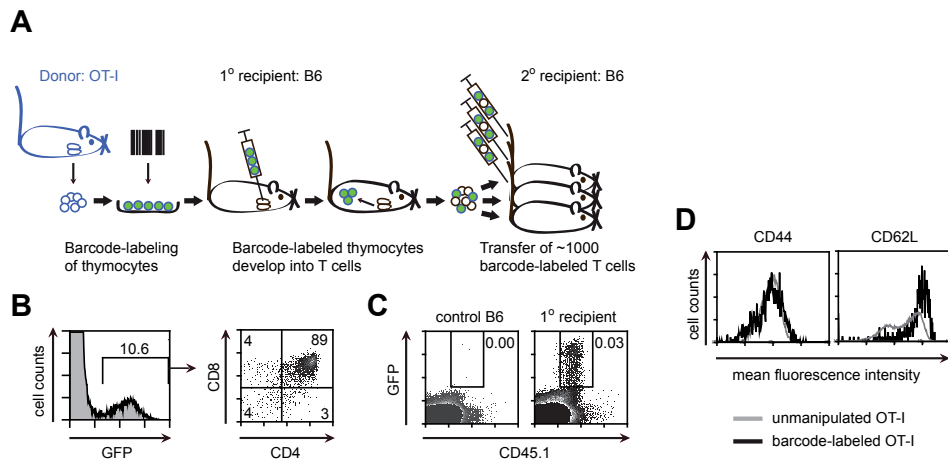
### Generation of a barcode-labeled naïve T cell pool

To study the lineage relationship between effector and memory CD8<sup>+</sup> T cells, we aimed to follow the progeny of individual naïve CD8<sup>+</sup> T cells during the course of infection. Recently, we have developed a genetic tagging technology that allows lineage analysis in a high-throughput fashion<sup>21</sup>. In this technology, unique DNA barcodes are introduced into cell populations of interest. As these barcodes are transmitted to all progeny, microarray-based analysis of barcodes present in different cell populations can be used to reveal their common or separate ancestry.

A potential concern in using this technology for fate mapping within the T cell lineage is that the retroviral transduction procedure that is used to introduce barcode sequences requires T cell activation, which could influence subsequent T cell fate (note that lentiviral transduction strategies to modify quiescent murine T cells have not been developed). To permit T cell fate mapping without a confounding effect of the barcoding procedure itself, we set out to develop a technology that allows the generation of 'untouched' naïve barcode-labeled T cells (Fig. 1A). To this purpose, total thymocytes from young OT-I TCR-transgenic mice were transduced using a retroviral library containing approximately 3,500 unique genetic tags plus a GFP marker gene (the barcode-library). One day after transduction, approximately 10% of thymocytes showed GFP expression (Fig. 1B), and the large majority (89%) of GFP-positive thymocytes was CD4<sup>+</sup>CD8<sup>+</sup> (double-positive; DP) (Fig. 1B). Based on the kinetics of thymocyte maturation, it is likely that both proliferating double-negative (DN) and DP thymocytes contributed to the generation of these barcode-labeled cells<sup>22</sup>, "unpublished data").

To investigate whether barcode-labeled thymocytes could be utilized to generate barcode-labeled naïve T cells, GFP-expressing OT-I thymocytes were sorted and injected intra-thymically into unmanipulated primary recipient mice. Three weeks after intra-thymic injection, primary recipients contained a clear population of peripheral CD8<sup>+</sup> T cells that were GFP-positive (barcode-labeled) and donor-derived (CD45.1<sup>+</sup>) (Fig. 1C). Importantly, these barcode-labeled CD8<sup>+</sup> T cells showed a naïve phenotype, as evidenced by the expression profiles of CD62L and CD44 (Fig. 1D).

To test whether barcode-labeled OT-I cells behave similarly to unmanipulated OT-I cells upon activation, both were co-transferred into secondary recipients. Recipient mice were then infected with a *Listeria monocytogenes* strain expressing ovalbumin (LM-OVA). Absolute peak heights of T cell responses cannot be compared because of the difficulty in determining the exact number of transferred barcode-labeled cells (see Materials and Methods). However, the kinetics of expansion, contraction and survival into the memory phase were comparable between barcode-labeled and unmanipulated OT-I cells (Fig. 2A). Furthermore, both types of OT-I cells displayed a similar activated phenotype (CD44<sup>hi</sup>, CD62L<sup>lo</sup>, CD43<sup>hi</sup>, CD127<sup>lo</sup>, mostly KLRG-1<sup>hi</sup>) (Fig. 2B + Fig. S2).



**Figure 1. Genetic tagging of naïve T cells.** (A) Experimental set-up for the generation and use of naïve, barcode-labeled T cells. Donor thymocytes are transduced with a barcode library. Transduced cells are sorted and injected intra-thymically into primary (1°) recipient mice. 3 weeks later, mature T cells are isolated from 1° recipients, pooled and transferred into 2° recipients. (B) Total CD45.1<sup>+</sup> OT-I thymocytes were transduced with the retroviral barcode library. One day after transduction, cells were stained and analyzed by flow cytometry. CD4 and CD8 expression is shown for the GFP<sup>+</sup> (barcode-labeled) population. Numbers indicate percentages. Data are representative of two independent experiments. (C and D) Sorted GFP<sup>+</sup> CD45.1<sup>+</sup> OT-I thymocytes were injected into the thymi of CD45.2<sup>+</sup> B6 1° recipient mice (n=3). 3 weeks later, spleen and LN cells were isolated, pooled, enriched for CD8<sup>+</sup> cells, stained for the indicated surface markers and analyzed by flow cytometry. Data are representative of two independent experiments. (C) Expression of CD45.1 and GFP on gated CD8<sup>+</sup> cells from unmanipulated (control) B6 or 1° recipient mice. Numbers indicate percentages. (D) Expression of CD44 and CD62L on unmanipulated (CD45.1<sup>+</sup>) and barcode-labeled (GFP<sup>+</sup> CD45.1<sup>+</sup>) CD8<sup>+</sup> T cells.

Having established a strategy to generate naïve barcode-labeled T cells, we investigated the potential of these cells for lineage tracking. Any effective lineage tracking strategy must meet two criteria. First, it must be able to reveal a high degree of relatedness between two cell populations known to be derived from a common progenitor. Second, it must demonstrate the absence of relatedness when assessing two populations known to be derived from separate progenitors. To create an experimental setting in which related and unrelated cell populations co-exist within a single mouse, secondary recipient mice received naïve barcode-labeled OT-I cells and were challenged with both an OVA<sub>(257-264)</sub>-expressing influenza strain (WSN-OVA) and OVA<sub>(257-264)</sub>-expressing tumor cells (EL-4-OVA). In this setting, priming of naïve T cells occurs independently and simultaneously in the lymph nodes that drain the two separate sites of antigenic challenge<sup>21</sup>. Five days after infection, when activated T cells are still largely confined to the site of priming, lymph node cells were isolated and divided into 2 pools (sample A and B) per lymph node. These lymph node

half-samples were independently expanded *in vitro* to boost T cell numbers prior to barcode analysis. As expected, the same barcodes were found in both half-samples of the same lymph node (Fig. 2C, upper left and middle plot). This indicates that cellular barcoding using naïve barcode-labeled T cells is able to establish that these two T cell populations are progeny of the same naïve T cell pool. In contrast, T cells isolated from two separate lymph nodes of the same mouse harbored a different set of barcodes (Fig. 2C, upper right plot), indicating that the technology can also correctly identify two T cell populations as being unrelated (i.e. derived from two different naïve T cell pools). As a further control, barcode content was compared between T cell populations recovered from different mice. These ‘between-mice’ comparisons are of use to reveal the background overlap that could, for instance, arise in case of clonal dominance of a small number of thymocytes during reconstitution of primary recipients. Notably, the overlap in barcode content between such evidently unrelated samples was low (Fig. 2C, lower plot).

To quantify the degree of relatedness between different samples, the correlation between barcode signals was calculated (see Materials and Methods). Analysis of lymph node samples from 3 mice revealed a consistently high positive correlation between barcode signals from two cell populations known to be related (sample A and B of the same LN) (Fig. 2D). In contrast, negative correlation values were obtained in all cases where two cell populations known to be unrelated were compared (Fig. 2D). In conclusion, these data show that the introduction of genetic tags in thymocytes can be used to generate naïve T cell populations suited for lineage tracking.

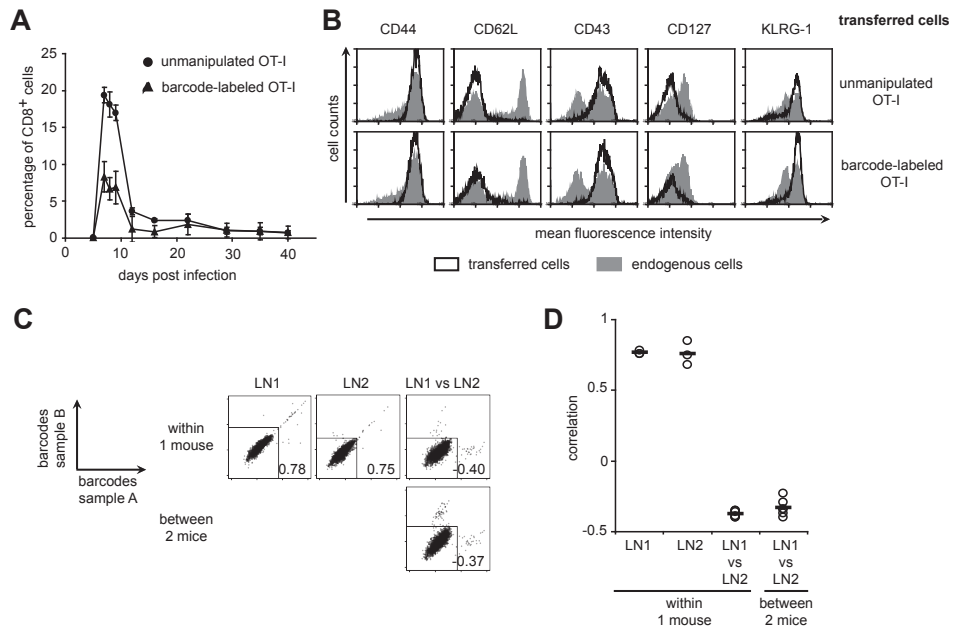
In all subsequent experiments intra-population comparisons were included as a barcode ‘sampling control’ to determine the reliability of barcode analysis for a given population, and ‘between-mice’ comparisons were included to reveal the background overlap seen in a particular experiment. These two control comparisons of cells that are by definition related or unrelated set the boundaries for all biological analyses.

#### **CD8<sup>+</sup> T cells that are present during effector and resting memory phase are derived from the same naïve T cells**

Traditionally, memory T cells are defined on the basis of their long-term persistence after antigen clearance. More recently, it has been suggested that precursors of memory cells can already be identified around the peak of the response, by the expression of high CD127 and low KLRG-1 levels<sup>9,23</sup>. As a first test of the lineage relationship of effector and memory T cells, we therefore performed a preliminary experiment to examine whether CD127<sup>hi</sup>KLRG-1<sup>lo</sup> and CD127<sup>lo</sup>KLRG-1<sup>hi</sup> CD8<sup>+</sup> T cells are derived from the same or distinct naïve T cells.

To this end, naïve barcode-labeled OT-I T cells were injected into secondary recipient mice that were subsequently infected with LM-OVA. Ten days after infection, CD127<sup>hi</sup>KLRG-1<sup>lo</sup> and CD127<sup>lo</sup>KLRG-1<sup>hi</sup> CD8<sup>+</sup> T cells were sorted from spleen and lymph nodes and each subset was divided into two half-samples (sample A and B) (Fig. S3A). As pilot experiments had shown that barcode copy numbers were too





**Figure 2. Barcode-labeled T cells can be utilized for lineage relationship analysis.**

(A) CD45.1<sup>+</sup> B6 2<sup>o</sup> recipient mice (n=5) received unmanipulated CD8<sup>+</sup>CD45.2<sup>+</sup> OT-I cells plus naïve barcode-labeled CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells and subsequent i.v. LM-OVA infection. Average responses of transferred OT-I cells are depicted; error bars indicate standard deviations. (B) Naïve, barcode-labeled or unmanipulated CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells were injected i.v. into CD45.2<sup>+</sup> B6 2<sup>o</sup> recipient mice (n=7 and n=2, respectively). Mice were subsequently infected i.v. with LM-OVA. At day 7 post-infection, blood cells were stained for the indicated surface markers and analyzed by flow cytometry. Filled gray histograms represent total endogenous CD45.1<sup>+</sup>CD8<sup>+</sup> T cells; black lines represent transferred CD45.1<sup>+</sup> OT-I cells. One representative mouse per group is shown. Data are representative of 2 independent experiments. (C and D) Naïve, barcode-labeled CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells were injected into CD45.2<sup>+</sup> B6 2<sup>o</sup> recipient mice. Mice were subsequently challenged with WSN-OVA (intra-nasal) and EL-4 OVA tumor cells (s.c.) the next day. At day 5 post-infection, lung-draining and tumor-draining lymph node cells were isolated, and each lymph node sample was split into two half-samples (sample A and B). Each half-sample was cultured separately for 3-4 days *in vitro* in the presence of 10µg/ml IL-7 and 20U/ml IL-2. Barcode analysis was performed independently on sample A and B of both lymph nodes (LN1 and LN2). Barcodes with a P-value of <0.001 were considered to be present above background. Rectangular dividers indicate which barcodes are present above background. Note that rather than fixing the absolute position of dividers in terms of intensity, it is the statistical probability of barcode presence that is kept constant for all samples in a given experiment. Data are representative of two independent experiments. (C) Representative 2-D plots of barcode analyses. Numbers indicate the correlation between signals from sample A and B. (D) Correlation analysis of barcodes present in T cells from the same or from distinct LN. Data are shown for 3 mice from 2 independent experiments. Mice of which barcodes in one of the 2 draining lymph nodes could not be sampled reliably were excluded from further analysis.

low for representative recovery from CD127<sup>hi</sup>KLRG-1<sup>lo</sup> cells, both half-samples of this subset were expanded separately *in vitro* to boost cell numbers prior to barcode analysis.

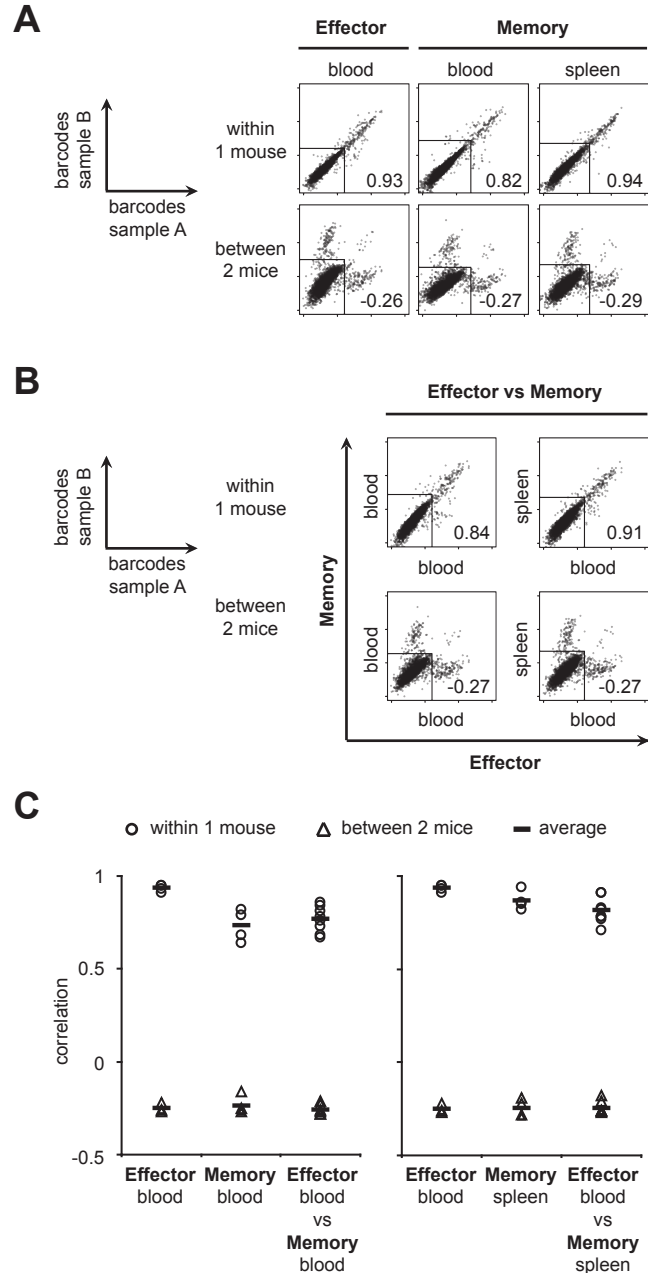
For all mice analyzed, barcodes were recovered representatively from the CD127<sup>lo</sup>KLRG-1<sup>hi</sup> subset (Fig. S3B, upper left plot + Fig. S3C), as indicated by the high correlation between barcode signals from sample A and B. Barcode signals from CD127<sup>hi</sup>KLRG-1<sup>lo</sup> half-samples were also well correlated, although some barcodes were only recovered from one of the two half-samples (Fig. S3B, upper middle plot + Fig. S3C). Furthermore, all 'between-mice' comparisons showed the expected inverse correlation (Fig. S3B, lower plots + Fig. S3C triangles).

Importantly, comparison of barcodes detected within the CD127<sup>lo</sup>KLRG-1<sup>hi</sup> and the CD127<sup>hi</sup>KLRG-1<sup>lo</sup> subset showed a strong correlation of barcode signals (Fig. S3B, upper right plot + Fig. S3C), demonstrating a high relatedness of both subsets. A small fraction of barcodes was only detected in one of the two subsets. However, this fraction of outliers was similar to that detected within intra-CD127<sup>hi</sup>KLRG-1<sup>lo</sup> comparisons. In line with this, the correlation of CD127<sup>lo</sup>KLRG-1<sup>hi</sup> versus CD127<sup>hi</sup>KLRG-1<sup>lo</sup> barcode comparisons was similar to that of intra-CD127<sup>hi</sup>KLRG-1<sup>lo</sup> comparisons (Fig. S3C). Note that when two related populations are compared of which one is sampled sub-optimally, the inter-sample correlation approximates the lowest of the two intra-sample correlations (Fig. S4A and B). To further test the potential significance of the observed difference between CD127<sup>hi</sup>KLRG-1<sup>lo</sup> and CD127<sup>lo</sup>KLRG-1<sup>hi</sup> subsets, data were reanalyzed after exclusion of outliers present within the intra-subset comparison, a strategy that can be utilized to facilitate the detection of small differences (Fig. S4C and D). Also after this filtering, no clear population of either CD127<sup>lo</sup>KLRG-1<sup>hi</sup> or CD127<sup>hi</sup>KLRG-1<sup>lo</sup>-specific barcodes was observed (Fig. S4E). Together, these preliminary data indicate that CD127<sup>lo</sup>KLRG-1<sup>hi</sup> and CD127<sup>hi</sup>KLRG-1<sup>lo</sup> by and large share the same set of progenitors.

To address the kinship of effector and memory phase CD8<sup>+</sup> T cells in a direct manner, we analyzed the barcode content of antigen-specific T cells that are present within the same mouse at different time points following infection. To this purpose, secondary recipient mice were infected with LM-OVA and blood was drawn for barcode analysis at the peak of the effector response. Animals were then kept until the resting memory phase, when both blood and spleen samples were obtained for barcode analysis. Analysis of sampling controls indicated that barcodes could be identified reliably during the memory phase and also in a small sample of peripheral blood obtained during the effector phase (Fig. 3A, upper plots). 'Between-mice' comparisons demonstrated that these unrelated cell populations were also correctly identified as such (Fig. 3A and B, lower plots + Fig. 3C, triangles).

Importantly, when the barcode repertoire present in effector and memory phase samples was compared, it was apparent that the two were highly correlated, independent of whether the memory sample was drawn from blood (Fig. 3B, upper left plot + Fig. 3C) or spleen (Fig. 3B, upper right plot + Fig. 3C). Furthermore, few

**Figure 3. CD8<sup>+</sup> T cells that are present during effector and resting memory phase are derived from the same naïve T cells.** CD45.2<sup>+</sup> B6 2<sup>o</sup> recipient mice (n=4) were injected with naïve, barcode-labeled CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells and subsequently infected i.v. with LM-OVA. Barcode analysis was performed on a 250-300µl blood sample drawn at day 8 post-infection (effector phase), and on a blood sample as well as on spleen isolated at day 28 post-infection (memory phase) from the same mice. Blood and spleen samples were divided into two halves (sample A and B) that were independently analyzed for barcode content. Barcodes with a P-value <0.005 were considered to be present above background. On average, 150 barcodes were detected per mouse. Data are representative of two independent experiments. **(A and B)** Representative 2-D plots of barcode comparisons. Numbers indicate the correlation between signals from sample A and B. **(C)** Correlation analysis of barcodes present in sample A and B from effector phase blood and memory phase blood (left plot) or effector phase blood and memory phase spleen (right plot). Data from 4 mice are depicted.



if any barcodes were identified that were selectively present in either the effector phase or the memory phase T cell population. These data demonstrate that CD8<sup>+</sup> T cells participating in the effector response and those present during memory phase in blood and spleen are derived from the same naïve CD8<sup>+</sup> T cells.

#### **CD8<sup>+</sup> T cells that are present in different lymphoid organs are derived from the same naïve T cells**

Prior work has suggested that the bone marrow can serve as a site of T cell priming<sup>24</sup>. In addition, there is evidence to suggest that the bone marrow may contain a specialized memory T cell compartment<sup>25</sup>. Based on these data it could be postulated that while effector and memory phase T cells in blood and spleen are derived from the same naïve T cells, bone-marrow resident T cells may form a separate pool.

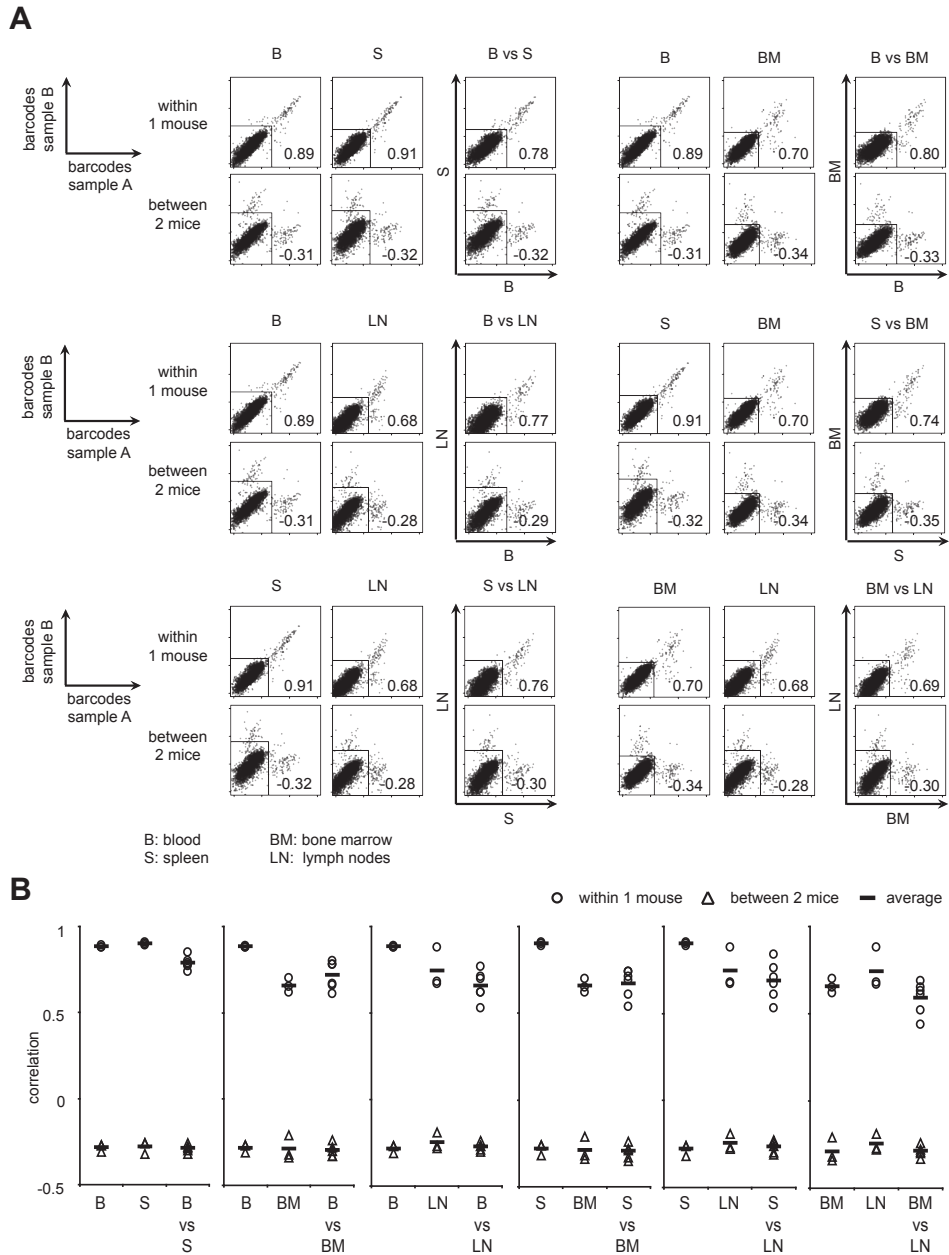
To investigate the lineage relationship of CD8<sup>+</sup> T cells that are found in different organs during the effector and memory phase, naïve barcode-labeled OT-I T cells were transferred into secondary recipient mice. Mice were then infected with LM-OVA and barcode analysis was performed on cells isolated from blood, spleen, bone marrow and lymph nodes at the peak of the effector response or during the memory phase. Barcodes were recovered efficiently from all four organs during the effector phase, albeit with a somewhat lower efficiency for lymph nodes and bone marrow (Fig. 4A and Fig. 4B, circles), consistent with the lower number of barcode-labeled T cells isolated from these organs.

To assess whether T cells present at these distinct sites share common progenitors within the naïve T cell pool, barcode content of the different T cell populations was compared. In all these comparisons, barcode content of T cells from the different anatomical sites was highly correlated and similar to the poorest sampled intra-organ comparison (Fig. 4A and Fig. 4B, circles). This demonstrates that the progeny of naïve T cells activated within an antigen-specific T cell response seeds all secondary lymphoid organs, including the bone marrow. Note that this analysis also validates the use of blood samples for lineage analysis during the effector phase (Fig. 3), as barcodes found in a 300µl blood sample are representative of barcodes found in all other lymphoid organs at the peak of the effector response.

Barcode analysis was subsequently performed on cells isolated from blood, spleen, lymph node and bone marrow samples at a late time point post-infection (day 28) (Fig. S5A and B). Efficiency of barcode recovery was again high for spleen and blood, but lower for bone marrow and lymph nodes, consistent with the lower number of GFP<sup>+</sup> cells in these organs. Nevertheless, in all possible comparisons the inter-organ correlation was similar to the correlation of the least efficiently sampled intra-organ comparison. Together, these data indicate that CD8<sup>+</sup> T cells that are present in different lymphoid organs are largely, if not fully derived from the same set of antigen-specific precursors within the naïve T cell repertoire.

#### **Shared T cell families in resting memory and secondary T cell responses**

To investigate whether all naïve T cell clones of which the progeny persisted into



**Figure 4. Effector phase CD8<sup>+</sup> T cells present in blood, spleen, bone marrow and lymph nodes are derived from the same naïve T cells.** CD45.2<sup>+</sup> B6 2<sup>o</sup> recipient mice (n=3) were injected with naïve, barcode-labeled CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells and subsequently infected i.v. with LM-OVA. Barcode analysis was performed at day 8 post-infection on cells recovered from blood (B), spleen (S), bone-marrow (BM) and lymph nodes (LN). All samples were divided into two halves (sample A and B) that were

resting memory phase also had descendants that were able to respond to secondary infection, naïve barcode-labeled OT-I T cells were transferred into secondary recipients that were subsequently infected with LM-OVA. At the resting memory phase, cells from blood, spleen, lymph nodes and bone marrow were isolated and pooled. Two parts were then used for independent barcode analysis and a third part was transferred into a tertiary recipient mouse that was thereafter infected with LM-OVA. At the peak of the secondary response, spleen samples were isolated for barcode analysis.

Analysis of the resting memory and secondary expansion sampling controls showed that barcodes could be reliably identified at both time points (Fig 5A, upper left and middle plot + Fig 5B). As expected, 'between-mice' comparisons were poorly correlated, although higher than in previous experiments due to the larger number of participating barcodes in this experiment (Fig 5A, lower plots + Fig 5B, triangles).

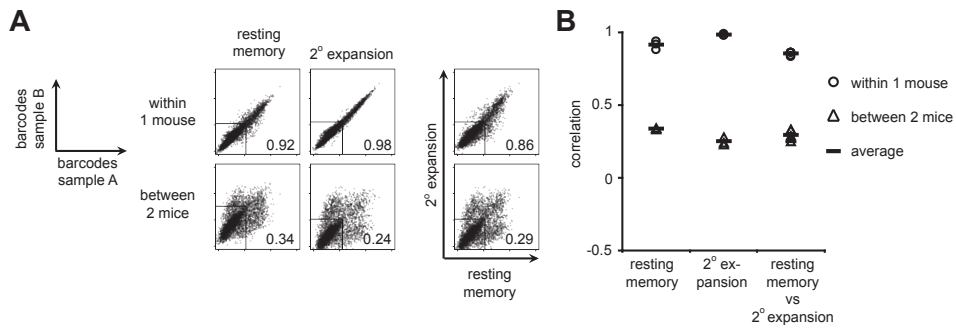
Subsequent comparison of the barcode repertoire present in resting memory and secondary expansion samples showed that at both time points, essentially the same barcode pool was present (Fig 5A, upper right plot + Fig 5B). Thus, these data show that all naïve T cell clones that contribute to the resting memory population also have progeny that respond to secondary antigen encounter. This indicates that the kinship of effector and memory T cells applies not only to resting memory T cells, but also to memory T cells as defined by their ability to respond to secondary antigen encounter.

#### Kinship of effector and memory phase CD8<sup>+</sup> T cells upon local infection

To establish whether the observed kinship of effector and memory subsets is restricted to systemic infection models, we determined the lineage relationship of these subsets in a local influenza A infection model. As pilot experiments had shown that the number of barcode-labeled cells in a blood sample drawn at the peak of the influenza-specific T cell response was too low for reliable barcode sampling, barcodes were read from a spleen sample obtained by partial splenectomy at the peak of the effector response. Mice were then kept until the resting memory phase, when the remainder of the spleen was analyzed for barcode content.

Sampling control comparisons demonstrated that both during effector and memory phase, barcodes could be read reliably (Fig 6A, upper left and middle plot + Fig 6B). 'Between-mice' controls showed the expected low correlation, although the number of shared barcodes was relatively high, due to the higher amount of barcodes participating in this experiment (Fig 6A, lower plots + Fig 6B, triangles). Importantly,

- ▷ independently analyzed for barcode content. Barcodes with a P-value <0.02 were considered to be present above background. On average, 150 barcodes were detected per mouse. S vs. B comparisons are representative of two independent experiments, LN and BM barcodes were analyzed in one experiment. **(A)** Representative 2-D plots of barcode comparisons. Numbers indicate the correlation between signals from sample A and B. **(B)** Correlation analysis of barcodes present in sample A and B. Data from 3 mice are depicted.



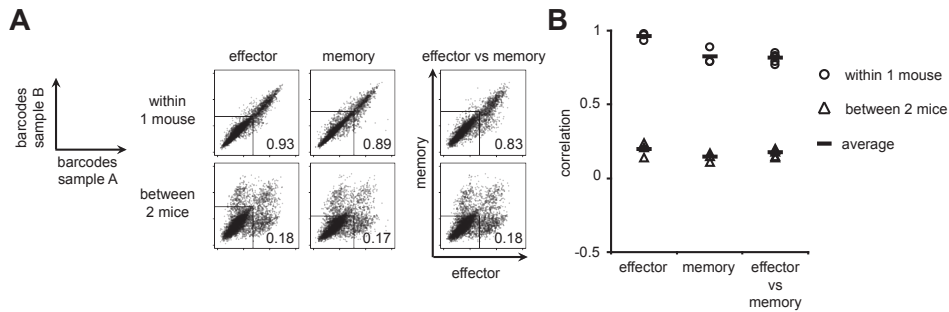
**Figure 5. Naïve T cell clones that contribute to the resting memory population, also have progeny that respond to secondary antigen encounter.** B6 2° recipient mice ( $n=3$ ) were injected with naïve, barcode-labeled CD8<sup>+</sup> OT-I cells and subsequently infected i.v. with LM-OVA. 28 days post-infection, blood, spleen, LN and BM cells were isolated, pooled per mouse and divided into 3 parts. 2 parts were used for barcode analysis (sample A and B, resting memory phase) and the third was transferred into a B6 3° recipient mouse that was then infected with LM-OVA. 5 days later, barcodes were analyzed from spleen half-samples (2° expansion). Barcodes with a P-value <0.01 were considered to be present above background. On average, 1080 barcodes were detected per mouse. **(A and B)** Representative 2-D plots of barcode comparisons. Numbers indicate the correlation between signals from sample A and B. **(C)** Correlation analysis of barcodes present in sample A and B. Data are derived from 3 2° recipient and 3 3° recipient mice analyzed within one experiment. These data confirm results obtained in an independent pilot experiment in which 1° expansion (effector phase) and 2° expansion memory populations could be compared for one mouse.

when barcode signals were compared between effector and memory phase samples, it was apparent that also during WSN-OVA infection, barcode content was highly correlated at both time points (Fig 6A, upper right plot + Fig 6B). This demonstrates that also in a local infection model, effector and memory phase T cells are progeny of the same naïve T cells.

#### Relatedness of effector and memory T cells is independent of TCR avidity

As the naïve T cell repertoire consists of a variety of clones that recognize the same antigen but with different avidities, we performed two experiments to address whether the lineage relationship of effector and memory subsets is dependent on the strength of the TCR - pMHC interaction.

To assess the lineage relationship of effector and memory CD8<sup>+</sup> T cells in an oligoclonal antigen-specific T cell population we made use of 'Limited' (Ltd) mice that express the OT-I TCR $\beta$  chain together with a V $\alpha$ 2 TCR $\alpha$  chain minilocus, which can recombine to form a 'limited' repertoire of TCRs<sup>26</sup>. This focused repertoire of TCRs expressed by Ltd T cells recognizes the OVA epitope with a range of avidities, as measured by K<sup>b</sup>-OVA tetramer binding<sup>27</sup>. Presumably due to the fact that only a single V segment is available for recombination, thymic selection of Ltd thymocytes is inefficient. Because of this low efficiency, it was technically not feasible to generate



**Figure 6. Kinship of effector and memory phase CD8<sup>+</sup> T cells upon local infection.** B6 2<sup>o</sup> recipient mice (n=4) were injected with naïve, barcode-labeled CD8<sup>+</sup> OT-I cells and subsequently infected i.n. with WSN-OVA. Barcode analysis was performed on a ¼ spleen sample obtained by partial splenectomy at day 9 post-infection (effector phase), and on a spleen sample isolated at day 25 post-infection (memory phase) from the same mice. Effector and memory phase samples were divided into two halves (sample A and B) that were independently analyzed for barcode content. Barcodes with a P-value <0.001 were considered to be present above background. On average, 800 barcodes were detected per mouse. **(A and B)** Representative 2-D plots of barcode comparisons. Numbers indicate the correlation between signals from sample A and B. **(C)** Correlation analysis of barcodes present in sample A and B. Data from 4 mice are depicted. These data confirm results obtained in an independent pilot experiment in which effector and memory phase populations could be compared for one mouse.

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naïve barcode-labeled Ltd cells by intra-thymic injection of transduced thymocytes. Therefore, barcode labeling was performed on *in vitro* activated peripheral Ltd T cells. Following transfer into recipient mice and LM-OVA infection, barcode content of effector and memory T cells was compared. Barcode sampling of the effector phase was less efficient in this preliminary experiment, possibly due to a reduced clonal burst size of *in vitro* activated T cells. Nevertheless, comparisons of effector and memory samples showed that barcodes in effector phase and secondary expansion phase T cells were largely shared (Fig. S6).

To study the kinship of effector and memory T cells in relation to T cell avidity in a system that does allow the use of naïve barcode-labeled T cells, we made use of a recombinant Listeria strain (LM-Q4-OVA) that expresses a variant of the OVA<sub>257-264</sub> epitope. Functional recognition of this Q4 variant by OT-I T cells requires approximately 18-fold more ligand than recognition of the parental epitope<sup>28</sup>. In line with the notion that Q4 forms a low avidity ligand, OT-I responses induced by LM-Q4-OVA have a reduced magnitude and earlier contraction<sup>28</sup>.

Barcode-labeled naïve OT-I T cells were transferred into recipient mice that were challenged with LM-Q4-OVA at day 0 and re-challenged at day 27. At the peak of the effector response, barcodes were read from a spleen sample obtained by partial splenectomy and memory barcodes were read from the remainder of the spleen at the peak of the secondary response. Control comparisons showed that both during the effector and the secondary expansion phase, barcodes could be identified reliably

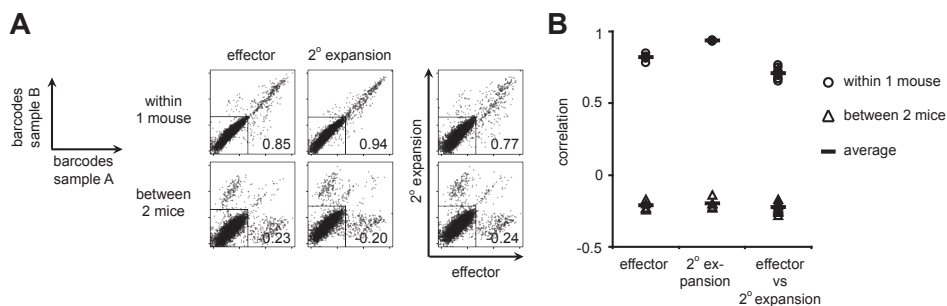


(Fig 7A, upper left and middle plot + Fig 7B), and that barcodes present in different mice were largely distinct (Fig 7A, lower plots + Fig 7B, triangles). In contrast, the barcode content of T cells present during effector and secondary expansion phase was highly correlated (Fig 7A, upper right plot + Fig 7B). This demonstrates that also for a lower avidity T cell response, effector and memory cells are derived from the same naïve T cells.

## DISCUSSION

To date, our understanding of the process by which activation of naïve T cells leads to formation of effector and memory T cells remains incomplete with some studies favoring a principle of 'one naïve cell - one fate' and others of 'one naïve cell - multiple fates'<sup>29</sup>. The aim of this study was to determine which of these two principles applies to *in vivo* CD8<sup>+</sup> T cell differentiation.

Previous fate mapping studies were, with the exception of a study by Stemberger et al.<sup>14</sup>, performed on the T cell population level, which precludes conclusions on the developmental potential of single naïve T cells. To allow large-scale fate mapping of naïve T cells at the single cell level, we have developed a technology in which naturally cycling thymocytes are provided with unique genetic tags (barcodes) by



**Figure 7. Relatedness of effector and memory T cells is independent of TCR avidity.** B6 2° recipient mice (n=5) were injected with naïve, barcode-labeled CD8<sup>+</sup> OT-I cells and subsequently infected i.v. with LM-Q4-OVA. 27 days later, mice were re-challenged with LM-Q4-OVA. Barcode analysis was performed on a ¼ spleen sample obtained by partial splenectomy at day 7 post-infection (effector phase), and on a spleen sample isolated at day 5 after re-challenge (2° expansion) from the same mice. Effector and 2° expansion samples were divided into two halves (sample A and B) that were independently analyzed for barcode content. Barcodes with a P-value <0.005 were considered to be present above background. On average, 250 barcodes were detected per mouse. **(A and B)** Representative 2-D plots of barcode comparisons. Numbers indicate the correlation between signals from sample A and B. **(C)** Correlation analysis of barcodes present in sample A and B. Data from 5 mice analyzed within one experiment are depicted. Data were confirmed in a second experiment (5 mice) performed with LM-A2-OVA, a *Listeria* strain containing another lower functional avidity variant of the OVA epitope (SAINFEKL).

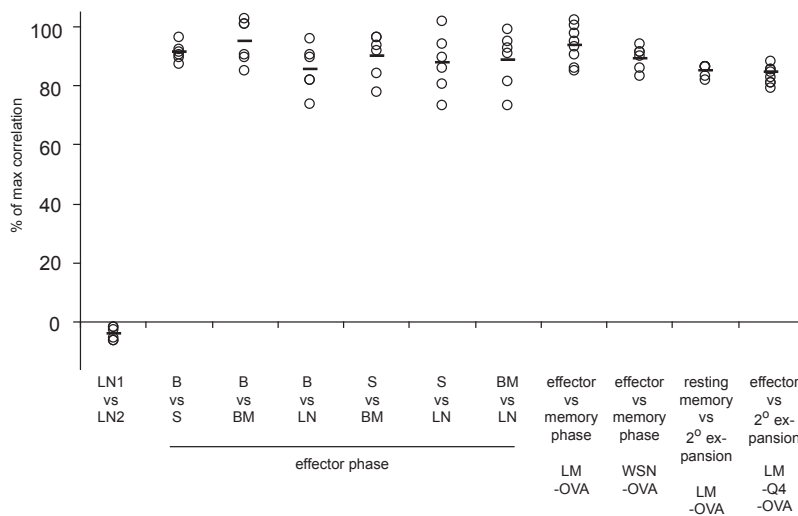
retroviral transduction. Intra-thymic injection of these genetically labeled thymocytes into unmanipulated recipient mice is then used to create a pool of naïve barcode-labeled T cells. This strategy allows the use of genetic tagging for the analysis of T cell fate without any potential confounding effect of the *in vitro* T cell activation that is used for standard T cell gene modification. By following the progeny of physiological numbers of these naïve barcode-labeled OT-I T cells during systemic or local infection, the lineage relationship of different T cell subsets could be assessed at the single cell level.

The current large-scale analysis of naïve CD8<sup>+</sup> T cell fate complements and extends the finding of Stemberger *et al.*, who utilized single cell transfer to assess the formation of CD8<sup>+</sup> T cell subsets<sup>14</sup>. While the Stemberger study stands out as the first example of *in vivo* analysis of T cell fate at the single naïve T cell level, a number of issues remained. First, in single cell transfer studies it is difficult to exclude homeostatic proliferation as a confounding factor. Second, the possibility remained that selective differentiation into either effector or memory subsets would not occur during systemic T cell responses -in which inflammatory signals are omnipresent- but would take place upon local infection. Most importantly, the fact that a single naïve T cell can form both effector and memory cells does not necessarily imply that all antigen-specific naïve T cells, including T cells activated by a low avidity stimulus, follow this pathway of differentiation.

Here we provide strong evidence that effector and memory T cells present under different conditions of infection and at different anatomical sites are in all cases derived from the same single naïve T cells. Furthermore, this shared ancestry of effector and memory cells applies both to T cells that are activated by a low avidity and by a high avidity stimulus.

An important technical advance of the current approach for fate mapping is that it allows one to demonstrate that in case two unrelated T cell populations are present within an animal, such populations can readily be distinguished, something that is impossible in single cell transfer systems. Specifically, the lack of kinship of T cells present in two different lymph nodes early after priming (Fig. 8, LN1 vs LN2) forms a crucial control to remove any concerns on homeostatic proliferation of T cells prior to antigen-driven activation, something that would obviously limit the ability to visualize a difference in ancestry of different T cell subsets.

The difference in ancestry of T cell populations residing in different lymph nodes early after infection forms a very strong contrast to the shared ancestry of all other T cell populations examined (Fig. 8). Specifically, 1) Effector and memory T cells are progeny of the same naïve T cells, both when memory is defined by long-term persistence and when defined by capacity for secondary expansion; 2) Effector and memory T cells are progeny of the same naïve T cells, both in systemic and local infection models, and independent of the anatomical site in which they reside and 3)



**Figure 8. Relatedness of different T cell subsets.** Results from barcoding experiments in Fig. 2-7 depicted as the percentage of maximal attainable correlation. 100% reflects the mean correlation of all intra-sample correlations for each individual experiment. 0% reflects the mean correlation of all ‘between mice’ comparisons for each individual experiment. (Note that the average correlation of two sampling controls forms a reasonable estimate and possibly a slight overestimate of the maximal attainable correlation in case the two cell populations would be fully related, Figure S4B). From the effector vs memory phase comparison during LM-OVA infection, only the effector blood vs memory blood comparison is depicted. Circles represent correlations within individual mice, bars indicate group averages. B=blood; S=spleen; BM=bone marrow; LN=lymph nodes.

Effector and memory T cells are progeny of the same naïve T cells in T cell responses of different functional avidities (Fig. 8).

These data argue against a model in which effector or memory fate is imprinted by distinct APC-derived signals delivered during initial priming. Likewise, a model assuming disparate fates for naïve T cells that are primed early or late during infection is not supported by the data. Instead, the current data indicate that under a variety of conditions the dominant pathway of CD8<sup>+</sup> T cell differentiation is ‘one naïve cell - multiple fates’.

## MATERIALS AND METHODS

**Mice.** CD45.1<sup>+</sup> and CD45.2<sup>+</sup> C57BL/6 (B6), as well as CD45.1<sup>+</sup> and CD45.2<sup>+</sup> TCR-transgenic OT-I and Limited (Ltd) mice were bred and housed in the animal department of the Netherlands Cancer Institute (NKI). All animal experiments were approved by the Experimental Animal Committee of the NKI.

**Retroviral transductions.** Total thymocytes isolated from 4-8 weeks old CD45.1<sup>+</sup> OT-I donor mice were transduced by spin-infection (90min. 2000rpm) in the presence

of 10ng/ml recombinant murine IL-7 (Peprotech) and retroviral supernatant that had been incubated with 10µg/ml DOTAP (Roche). Retroviral supernatants containing the barcode-library<sup>21</sup> were generated as described<sup>30</sup> and diluted prior to use to reach a transduction efficiency of approximately 10%. 4h after transduction, thymocytes were washed and cultured o/n in culture medium (IMDM/ 8%FCS/ 100U/ml penicillin/ 100µg/ml streptomycin/  $5 \times 10^{-6}$  M 2-ME) supplemented with 10ng/ml IL-7. Ltd cells were isolated from spleen and lymph nodes, activated for 2 days in culture medium containing 2 µg/ml Concanavalin A (Calbiochem) and 1 ng/ml IL-7, and subsequently transduced by spin infection in the presence of 10ng/ml recombinant murine IL-7 and retroviral supernatant. Transduced Ltd cells were sorted on the basis of GFP expression and absence of staining with anti-B220 and anti-CD4 mAb.

**Intra-thymic injection of transduced thymocytes.** One day after transduction, thymocytes were purified using Lympholite (Cedarlane) and GFP<sup>+</sup> cells were sorted by FACS. Sorted cells were injected intra-thymically into 4-6 weeks old primary (1°) recipient B6 mice. 1° recipients were sedated i.p. with azepromazine (1µg/g body weight, Ceva Sante Animale) and anesthetized i.p. with a mixture of midazolam (7.5µg/g body weight; Roche), fentanyl citrate (0.47µg/g body weight; VetaPharma) and fluanisone (15µg/g body weight; VetaPharma). For pain relief, carprofen (5µg/g body weight; Pfizer) was given s.c. The fur overlying the thymus was disinfected with iodine and a small (max 1cm) longitudinal incision was made in the skin along the sternum. The salivary gland was pushed aside and each thymic lobe was injected with 25µl cell suspension. Thereafter, the skin was closed with 2-3 stitches. Each mouse received 0.5-1x10<sup>6</sup> sorted, GFP<sup>+</sup> thymocytes.

**Isolation, purification and transfer of naïve OT-I T cells.** Barcode-labeled OT-I cells were isolated ~3 weeks after intra-thymic injections from spleen and LNs (cervical, axillary, brachial, mesenteric, inguinal, lumbar) of 1° recipient mice. CD8<sup>+</sup> cells were enriched by negative selection (Mouse CD8 T Lymphocyte Enrichment Set; BD Biosciences). Enriched CD8<sup>+</sup> cells were pooled from several 1° recipients, and then analyzed by flow cytometry and/or transferred i.v. into several 8-10 weeks old B6 2° recipients (~1000 barcode-labeled cells / 2° recipient). This pooling and distribution approach guarantees that each 2° recipient receives an equal number of barcode-labeled T cells. In addition, it serves as an important control to ensure that the technology allows the detection of differences in kinship (see: 'between mice' controls). In all experiments, the number of transferred barcode-labeled cells as indicated in the legends is based on the analysis of very low percentages (~0.01%) of GFP<sup>+</sup> T cells and therefore an approximation. On average, ~2x10<sup>3</sup> barcode-labeled CD8<sup>+</sup> T cells were isolated per 1° recipient. Non-barcode-labeled OT-I cells were isolated from spleen and LNs of OT-I mice.

**Listeria and influenza A infections and tumor challenge.** 2° recipient mice were infected i.v. with 2.5x10<sup>4</sup> (1° infection) or 2.5x10<sup>5</sup> CFU (2° infection) of a *Listeria monocytogenes* strain expressing ovalbumin (LM-OVA)<sup>31</sup>. Infection with

LM-Q4-OVA, a *Listeria monocytogenes* strain expressing the SIIQFEKL sequence<sup>28</sup> occurred at  $2.5 \times 10^3$  (1° infection) or  $2.5 \times 10^5$  CFU (2° infection) i.v. Alternatively, mice were anesthetized with ether and infected intra-nasally (i.n.) with  $10^3$  PFU of the recombinant influenza A/WSN/33 strain (WSN-OVA) that expresses the H-2K<sup>b</sup>-restricted OVA<sub>257-264</sub> epitope<sup>32</sup>, followed by s.c. injection of  $10^7$  EL4-OVA thymoma cells the next day<sup>30</sup>.

**Recovery and *in vitro* expansion of transferred cells.** Transferred OT-I or Ltd cells were recovered from spleen, BM (femur, tibia, iliac crest), LN (cervical, axillary, brachial, mesenteric, inguinal, lumbar) and blood. Blood was either drawn from the tail vein, cheek (~300µl; in experiments in which mice were kept for longitudinal analysis), or heart (~800µl; after sacrificing the mice). To recover barcode-labeled spleen cells from living mice, partial splenectomy was performed. Recovered cells were either used for flow cytometry or for barcode analysis. To allow for efficient barcode recovery, spleen, LN and BM samples were enriched for Vα2<sup>+</sup> cells by MACS (Miltenyi Biotec; anti-PE Microbeads). Where indicated, cells were expanded *in vitro* for 3-4 days prior to Vα2 enrichment. All samples were divided into two equal parts (sample A and B) from which barcodes were recovered independently. This division was performed after Vα2 enrichment or, in case *in vitro* expansion was performed, prior to *in vitro* expansion.

**Partial splenectomy.** Mice were anesthetized with isoflurane and the skin overlying the spleen was shaved and disinfected. A <1cm incision was made in the skin and peritoneum. ¼ of the spleen was resected and the wound on the spleen was closed with Histoacryl® Topical Skin Adhesive (TissueSeal). Thereafter, peritoneum and skin were closed with ~3 stitches and buprenorphine (0.1µg/g body weight; Schering-Plough) was given as pain relief.

**Flow cytometry.** Cell surface stainings were performed with the following reagents: Vα2-PE (B20.1), CD62L-PE (MEL-14), CD45.1-PE (A20), CD43-PE (1B11), SA-PE-Cy7, CD4-APC (L3T4), CD8α-APC-Cy7 (53-6.7), KLRG-1-biotin (2F1), CD4-APC (RM4-5), B220-APC (RA3-6B2) (BD Biosciences), CD45.1-APC (A20; Southern Biotech), CD127-PE (A7R34), CD44-PE-Cy7 (IM7), CD127-APC (A7R34) and CD45.2-APC (104) (eBiosciences). Dead cells were excluded by DAPI staining. Analyses were performed on a CyAn<sub>ADP</sub> (Summit v4.3; Beckman Coulter). Cell sorting was carried out on a FACSAria Cell Sorter (Diva 5.0.1; BD Biosciences) or MoFlo Highspeed Sorter (Summit v3.1; Beckman Coulter).

**Barcode recovery, microarray hybridizations and data analysis.** Genomic DNA was isolated and barcode sequences were amplified by nested PCR as described<sup>21</sup>. PCR products were purified, labeled with Cyanine-3 or Cyanine-5 and co-hybridized to the barcode microarray. Fluorescence signals were quantified and normalized, and duplicates were averaged. Barcodes present above background were defined for each sample, based on the probability a signal differed from a calculated background distribution<sup>21</sup>. The rectangular divider in 2-D barcode plots indicates which barcodes are present above background, based on the indicated probability (see legends). Note that rather than fixing the absolute position of dividers in terms of intensity, it is

the statistical probability of barcode presence that is kept constant for all samples in a given experiment. The Pearson correlation between fluorescent signals of barcodes present within two samples was calculated. Calculations included a randomly drawn fraction of barcodes that were not present in a given sample (reference population), as described previously<sup>21</sup>. Note that the use of such a reference population within the calculations restricts correlation values to a range of 1 (full kinship) to approximately -0.5 (lack of kinship).

**Online supplemental material.** Fig. S1 depicts different models for the formation of effector and memory T cell subsets. Fig. S2 shows that activated barcode-labeled and unmanipulated OT-I T cells have a similar phenotype. Fig. S3 depicts the relatedness of CD127<sup>lo</sup>, KLRG-1<sup>hi</sup> and CD127<sup>hi</sup>, KLRG-1<sup>lo</sup> CD8<sup>+</sup> T cells. Fig. S4 shows how barcode analysis is interpreted when barcode sampling is suboptimal. Fig. S5 indicates that resting memory T cells present in different lymphoid organs are largely derived from the same naïve T cells. Fig. S6 shows that effector and memory T cells in an oligoclonal antigen-specific T cell response are related.

**Abbreviations used.** 2-D, two dimensional; Ltd, Limited.

## ACKNOWLEDGMENTS

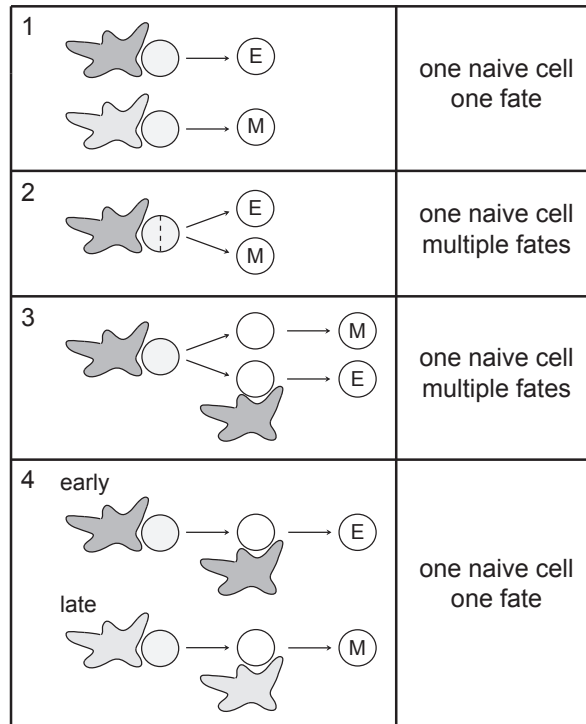
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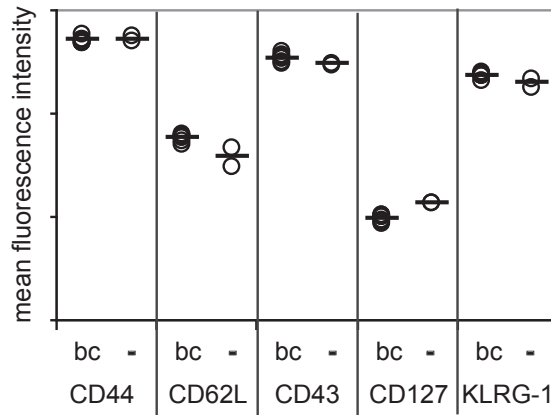
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## SUPPLEMENTARY FIGURES

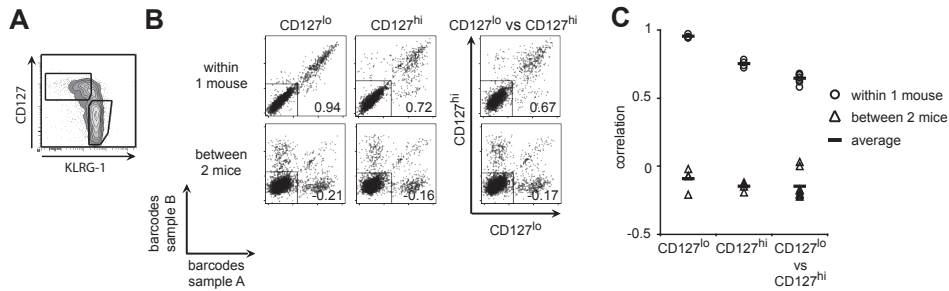


**Figure S1. Models predicting effector and memory T cell lineage relationship.** Schematic representation of four of the proposed models describing the lineage relationship of effector (E) and memory (M) T cells. Dendritic cells with different stimulating capacities are depicted in light and dark blue. Naïve T cells are drawn in pink. Note that other (variations on these) models have been proposed, but the fundamental distinguishing feature of the different models for the current study is whether they predict that single naïve T cells produce single or multiple subsets.

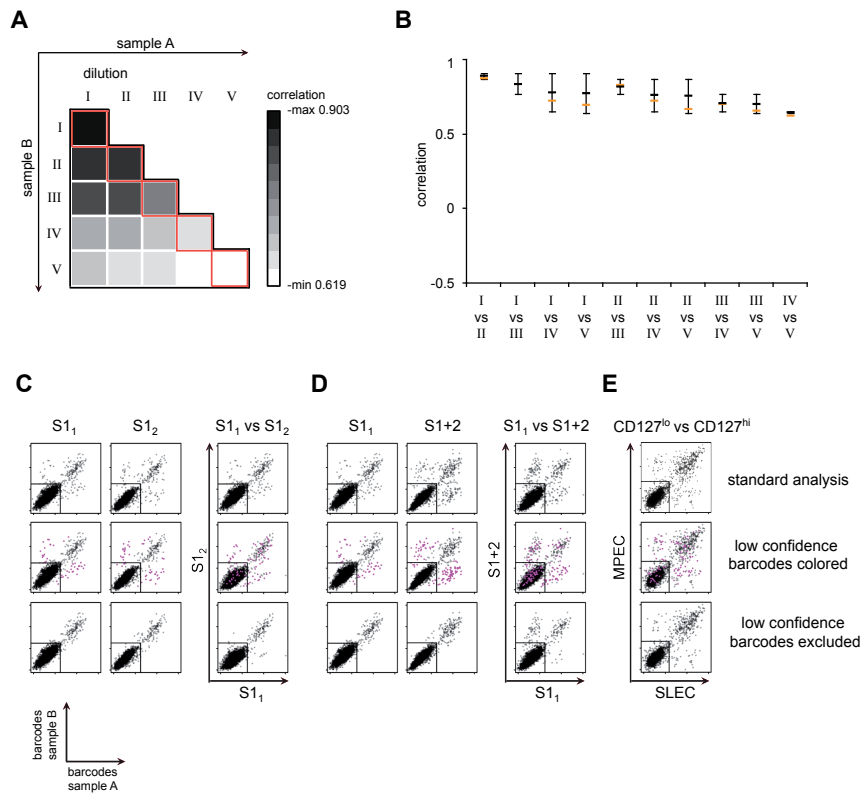




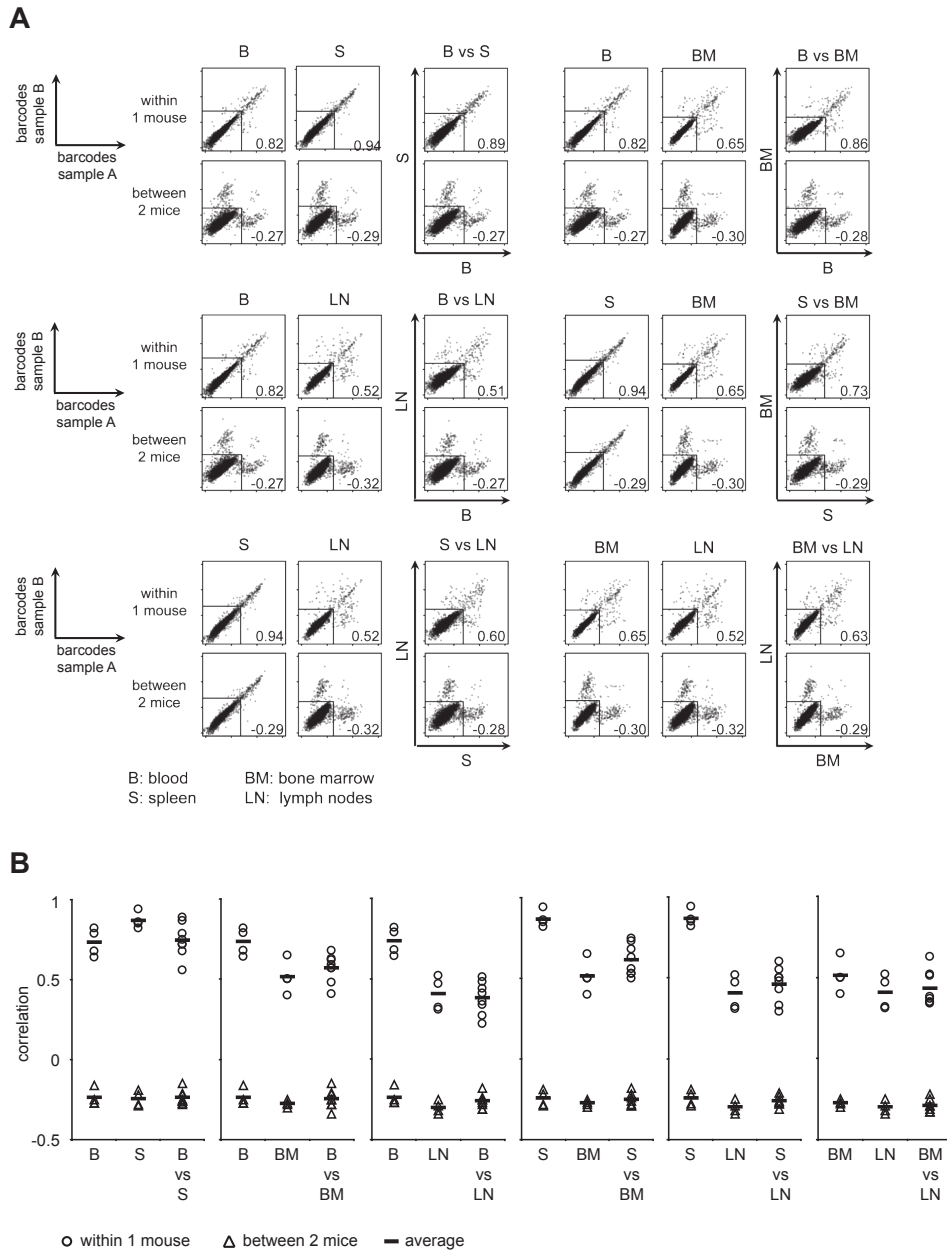
**Figure S2. Phenotypic characterization of barcode-labeled and unlabeled OT-I T cells after LM-OVA infection.** CD45.2<sup>+</sup> B6 2<sup>o</sup> recipient mice were injected with naïve, barcode-labeled (bc) CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells or unmanipulated (-) CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells (n=7 and n=2 respectively). Mice were subsequently infected i.v. with LM-OVA. At day 7 post-infection, blood cells were stained for the indicated surface markers and analyzed by flow cytometry. Circles represent individual mice, bars indicate group averages. Data are representative of two independent experiments.



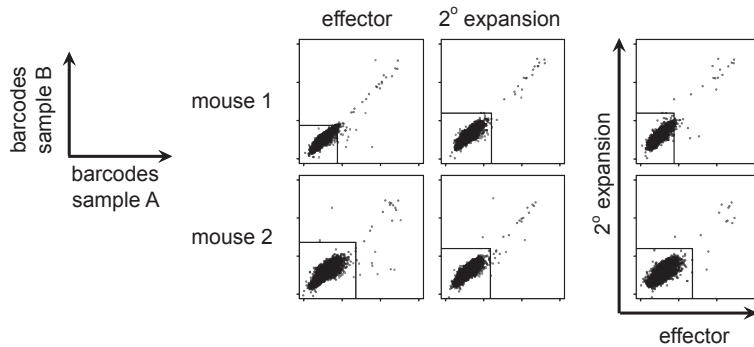
**Figure S3. CD127<sup>lo</sup>KLRG-1<sup>hi</sup> and CD127<sup>hi</sup>KLRG-1<sup>lo</sup> are related.** CD45.2<sup>+</sup> B6 2<sup>o</sup> recipient mice (n=4) were injected with naïve, barcode-labeled CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells and subsequently infected i.v. with LM-OVA. 10 days after infection, spleen and lymph node cells were isolated, enriched for CD8<sup>+</sup> cells and sorted into CD8<sup>+</sup>, CD45.1<sup>+</sup>, CD127<sup>lo</sup>KLRG-1<sup>hi</sup> and CD8<sup>+</sup>, CD45.1<sup>+</sup>, CD127<sup>hi</sup>KLRG-1<sup>lo</sup> cells. Data are derived from 4 mice, analyzed within one experiment. **(A)** Flow cytometric sorting plot, gated on CD8<sup>+</sup>CD45.1<sup>+</sup>GFP<sup>+</sup> cells; sorting gates are indicated. **(B and C)** Both sorted populations, abbreviated as CD127<sup>lo</sup> and CD127<sup>hi</sup> were divided into two halves (sample A and B) that were independently analyzed for barcode content. CD127<sup>hi</sup> half-samples were separately expanded for 3 days *in vitro* in the presence of OVA<sub>257-264</sub>-loaded splenic DCs at a T:DC ratio of 10:1 prior to barcode analysis. Barcodes with a P-value <0.0001 were considered to be present above background. On average, 430 different barcodes were detected per mouse. **(B)** Representative 2-D plots of barcode comparisons. Numbers indicate the correlation between signals from sample A and B. **(C)** Correlation analysis of barcodes present in sample A and B. Data from 4 mice analyzed within one experiment are depicted.



**Figure S4. Barcode data analysis in case of suboptimal sampling.** CD45.2<sup>+</sup> B6 2<sup>o</sup> recipient mice were injected with naïve, barcode-labeled CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells and subsequently received an i.v. infection with LM-OVA at day 0 and day 30. Five days later, genomic DNA was isolated from the spleen of two 2<sup>o</sup> recipient mice. **(A and B)** To determine how suboptimal sampling would affect correlation analyses on cell populations of which one is sampled less efficiently than the other, 1.5% of the spleen 1 DNA was diluted in 4 steps of 2-fold dilutions (dilutions I-V with I being the highest and V the lowest concentration). For each dilution, 2 samples were independently analyzed for barcode content. Barcodes with a P-value of <0.0001 were considered to be present above background. **(A)** The correlation of intra-dilution (red squares) and inter-dilution comparisons is depicted in a heat-map. **(B)** The correlation of different inter-dilution comparisons (orange horizontal bars) is depicted in relation to the correlation of the two corresponding intra-dilution comparisons and their mean correlation (highest, lowest and middle black horizontal bar respectively). Note that when correlation analyses are performed for two samples that have substantially different intra-sample correlation values (e.g. I vs IV, I vs V, II vs V), this value approximates that of the poorest intra-sample comparison. **(C and D)** To test whether the exclusion of barcodes detected with low confidence would facilitate the detection of barcodes that are differentially present, part of DNA of spleen 1 was divided in two samples (S<sub>1</sub><sub>1</sub> and S<sub>1</sub><sub>2</sub>) and part of spleen 1 DNA was mixed with part of spleen 2 DNA (S<sub>1</sub>+2). All three samples were diluted and split in 2 halves (sample A + B) for independent barcode analysis. Standard barcode analysis is shown in the first row. To statistically determine which barcodes are likely to be outliers (i.e. detected with low confidence), a linear model was fitted through the barcode signals from the intra-sample comparisons and the studentized residuals were calculated per barcode. Barcodes with residuals ≥3 (97.5% chance of being an outlier) in the intra-sample comparisons were either colored (second row) or excluded (third row). Barcodes with a P-value of <0.0005 were considered to be present above background. **(C)** Barcode comparison between two related samples (S<sub>1</sub><sub>1</sub> vs S<sub>1</sub><sub>2</sub>) in relation to the corresponding intra-sample comparisons. **(D)** Barcode comparison between two partially unrelated samples (S<sub>1</sub><sub>1</sub> vs S<sub>1</sub>+2) in relation to the corresponding intra-sample comparisons. **(E)** Barcode comparison between CD127<sup>lo</sup>KLRG-1<sup>hi</sup> (CD127<sup>lo</sup>) and CD127<sup>hi</sup>KLRG-1<sup>lo</sup> (CD127<sup>hi</sup>) from the experiment shown in Fig. S3. Standard barcode analysis is shown in the first row. Barcodes with residuals ≥3 in the intra-sample comparisons were either colored (second row) or excluded (third row) as described for C and D.



**Figure S5. Memory CD8<sup>+</sup> T cells present in different organs harbor the same set of barcodes.** CD45.2<sup>+</sup> B6 2<sup>o</sup> recipient mice (n=4) were injected with naïve, barcode-labeled CD8<sup>+</sup>CD45.1<sup>+</sup> OT-I cells and subsequently received an i.v. infection with LM-OVA. Barcode analysis was performed on blood, spleen, BM and LN cells isolated at day 28 post-infection. All samples were divided into two halves (sample A and B) that were independently analyzed for barcode content. Barcode comparisons are made between sample A and B taken from the same mouse ('within 1 mouse') or ▶



**Figure S6. Relatedness of effector and memory T cells in an oligoclonal antigen-specific T cell response.** B6 2° recipient mice were injected with barcode-labeled Ltd cells and subsequently infected i.v. with LM-OVA. 27 days later, mice were re-challenged with LM-OVA. Barcode analysis was performed on a 250-300µl blood sample drawn at day 8 post-infection (effector phase), and on spleen isolated at day 5 after re-challenge (2° expansion) from the same mice. Blood and spleen samples were divided into two halves (sample A and B) that were independently analyzed for barcode content. Barcodes with a P-value  $<1 \times 10^{-8}$  were considered to be present above background. On average, 25 barcodes were detected per mouse. 2-D plots of barcode comparisons are shown for 2 mice within one experiment. All barcodes present in more than 1 mouse were excluded. To limit analysis to barcodes detected with high reliability, barcodes with residuals  $\geq 3$  (97.5% chance of being an outlier) in the intra-sample comparisons (depicted in purple in the intra-sample comparisons) were excluded in the inter-sample comparisons, as described for Fig. S3 C and D. Note that this filtering procedure precludes the calculation of correlation values. Data from one experiment are depicted.

- ▷ from two different mice ('between 2 mice'). Barcodes with a P-value  $<0.005$  were considered to be present above background. **(A)** Representative 2-D plots of barcode comparisons. Numbers indicate the correlation between signals from sample A and B. **(B)** Correlation analysis of barcodes present in sample A and B isolated from blood (B), spleen (S), bone marrow (BM) and lymph nodes (LN). Data from 4 mice analyzed within one experiment are depicted.

