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Serial Transfer of Single-Cell-Derived Immunocompetence Reveals Stemness of $CD8^+$ Central Memory T Cells

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SUMMARY

Maintenance of immunological memory has been proposed to rely on stem-cell-like lymphocytes. However, data supporting this hypothesis are focused on the developmental potential of lymphocyte populations and are thus insufficient to establish the functional hallmarks of stemness. Here, we investigated self-renewal capacity and multipotency of individual memory lymphocytes by in vivo fate mapping of $CD8^+$ T cells and their descendants across three generations of serial single-cell adoptive transfer and infection-driven re-expansion. We found that immune responses derived from single naive T (Tn) cells, single primary, and single secondary central memory T (Tcm) cells reached similar size and phenotypic diversity, were subjected to comparable stochastic variation, and could ultimately reconstitute immunocompetence against an otherwise lethal infection with the bacterial pathogen *Listeria monocytogenes*. These observations establish that adult tissue stem cells reside within the $CD62L^+$ Tcm cell compartment and highlight the promising therapeutic potential of this immune cell subset.

INTRODUCTION

The hematopoietic system is subject to constant loss of many of its cellular constituents. This loss, however, is counterbalanced by continuous replenishment of all differentiated blood cell lineages from a common source of hematopoietic stem cells (HSCs) (Morrison et al., 1995; Spangrude et al., 1988). Although T and B lymphocytes are ultimately derived from HSCs, random somatic recombination of T and B cell receptor loci during lymphocyte development creates a highly diverse antigen receptor repertoire and precludes the directed marrow-borne replenishment of defined antigen-specific lymphocyte populations (Wakim and Bevan, 2010). Cessation of de novo T cell development after thymic involution further detaches T cell homeostasis from the influence of HSCs (Buchholz et al., 2011; Hale et al., 2006). Despite these constraints, protective T cell immunity—requiring intermittent or even constant generation of antigen-specific effector T cells—can be maintained over extensive periods of time (Hammarlund et al., 2003; Snyder et al., 2008; Williams and Bevan, 2007). This apparent stability of T cell memory, despite high cellular turnover and negligible replenishment from the bone marrow, has spurred the notion that T cell immunity could be maintained via stem-cell-like antigen-specific memory T cells (Fearon et al., 2001; Gattinoni et al., 2012; Neuenhahn and Busch, 2009; Stemberger et al., 2009). The crucial feature of stem cells is their capability to generate a variety of more differentiated daughters (multipotency) and of daughters that retain the undifferentiated state and developmental potential of their ancestors (self-renewal) (Till et al., 1964). As an integral part of this operational stem cell definition, both qualities—multipotency and self-renewal—must potentially emerge from a single "colony-forming" cell (Becker et al., 1963; Siminovitch et al., 1963; Wu et al., 1968). Thus, to identify whether a tissue is maintained via stem-cell-based mechanisms and, if so, which subcompartment of the tissue harbors stem cell potential, it is essential to determine multipotency and self-renewal capacity of individual cells (Morrison et al., 1997; Weissman, 2000). Such a "clonogenic" approach, based on the retrospective evaluation of single-cell-derived developmental potential, was first realized for the HSC (Osawa et al., 1996; Smith et al., 1991) and remains an indispensable predecessor to the phenotypic delineation of stemness in any tissue context (Schroeder, 2008).

Various phenotypic signatures have been proposed to identify stem-cell-like memory T cells (Ciocca et al., 2012; Gattinoni et al., 2009; Luckey et al., 2006; Muranski et al., 2011; Turtle et al., 2009; Zhang et al., 2005). However, the clonogenic potential of individual memory T cells, to self-renew and provide a diverse offspring of effector T cells that reconstitute immunocompetence, has not been investigated and cannot be inferred from previous studies focusing on population analyses (Buchholz et al., 2013b; Schumacher et al., 2010). Such limitations are underscored by recent studies showing substantial variability of immune responses derived from single T cells in vivo—a finding that had not been predicted by population analyses (Buchholz et al., 2013a; Gerlach et al., 2013; Plumlee et al., 2013; Tubo et al., 2013). Therefore, despite the apparent longevity of immunological memory, it remains unknown whether antigen-experienced T cell populations are maintained via stem-cell-based mechanisms and, if so, which phenotypic subcompartment contains the putative stem cell of T cell memory.

Here, we investigated multipotency, self-renewal, and immune reconstitution capacity of individual memory T cells. We analyzed the phenotypic composition of epitope-specific T cell populations at early and late time points after infection with the bacterial pathogen *Listeria monocytogenes* (Lm) and could confirm the presence of CD62L$^-$ Tem cells and CD62L$^+$ Tcm cells—as well as the latter's superior proliferation and differentiation capacity (Sallusto et al., 2004). In order to then investigate stemness of cells belonging to the Tcm cell compartment, we performed an extensive series of in vivo clonogenic assays. We adoptively transferred single-epitope-specific T cells into immunocompetent or immunocompromised hosts that were subsequently infected with Lm. In these assays, progeny size and diversity, originating from single Tn and single Tcm cells, were found to be remarkably similar. During serial single-cell adoptive transfers, single Tn, single primary Tcm, and single secondary Tcm cells were equally efficient in generating epitope-specific T cell populations. In addition, progeny derived from single primary Tcm cells contained secondary Tcm cells, which were again capable of generating a diverse offspring when individually transferred to new hosts and exposed to infection. Finally, progeny derived from single secondary Tcm cells sufficed to reconstitute antigen-specific immunocompetence against otherwise lethal *Listeria* challenge of immunocompromised hosts. Through these observations, our work establishes multipotency, self-renewal, and functional "tissue" reconstitution as traits of individual Tcm cells. It thereby provides experimental demonstration of this phenotypic subset's clonogenic potential and thus of its stemness.

RESULTS

Long-Lived Memory after Acute Infection Consists of CD8$^+$CD44^{hi} Central Memory and Effector Memory T Cells

In search of a memory T cell subset with stem-cell-like features, we first monitored the phenotypic composition of long-term persistent, epitope-specific CD8$^+$ T cell populations generated in response to acute infection with *Listeria monocytogenes*-expressing chicken ovalbumin (Lm-OVA). After infection of immunocompetent hosts with Lm-OVA, live bacteria are cleared within a few days and bacterial antigen is fully removed during the first week of infection (Mercado et al., 2000). Because proliferation of short-lived effector T cells is chiefly dependent on antigen exposure (Sarkar et al., 2008), we expected, after resolution of acute infection, a relative enrichment of long-lived memory T cells over time. We further argued that if self-renewing stem-cell-like memory T cells existed, they must be contained within one or more of the phenotypic subsets present during late memory phases. By day 500 postinfection (p.i.), populations derived from 500 adoptively transferred naive OT-I T cell receptor (TCR)-transgenic T cells specific for peptide OVA$_{257\text{-}264}$ (SIINFEKL), as well as populations derived from polyclonal endogenous CD8$^+$ T cells specific for the same epitope, consisted mainly of CD44^{hi}CD62L$^+$ Tcm cells and lower numbers of CD44^{hi}CD62L$^-$ Tem cells (Figure 1A). These memory T cells also expressed surface markers CXC motif chemokine receptor 3 (CXCR3) and CD122 (Figure 1B). Cells of both subsets were also found to uniformly lack the marker of short-lived effector T cells killer cell lectin-like receptor subfamily G member 1 (KLRG1). Although CD62L$^-$ Tem cells displayed some heterogeneity concerning expression of CD27, CD127, T-box transcription factor expressed in T cells (T-bet), and transcription factor Eomesodermin (Eomes), CD62L$^+$ Tcm cells were homogeneously positive for these markers (Figures S1A and S1B available online). Recent work has ascribed stem-cell-like qualities to a murine CD8$^+$ CD62L$^+$ T cell subset displaying phenotypic characteristics of both naive (CD44^{lo}) and antigen-experienced (CXCR3$^+$CD122$^+$) T cells. This subset was described in a setting of graft-versus-host disease (GVHD) (Zhang et al., 2005) and after pharmacological induction of Wnt signaling (Gattinoni et al., 2009). However, in our infection model, which lacks chronic antigen exposure and additional stimulation of the Wnt pathway, CD44^{lo}CD62L$^+$ T cells could not be detected within the antigen-experienced CXCR3$^+$CD122$^+$ compartment (Figures 1C and S1C–S1F). Thus, although Tcm cells were enriched in long-term memory, we could not find evidence for a similar accumulation of the putative CD44^{lo}CD62L$^+$ stem-cell-like subset of CD8$^+$ T cells.

High Expansion and Differentiation Potential of CD8$^+$ Central Memory T Cells

Functional evaluation of classical memory subsets sorted via flow cytometry at least 100 days after primary infection (Figure S2A) showed a superior capacity of adoptively transferred Tcm cells to re-expand and reconstitute memory and effector T cell subsets—including the Tcm cell subset itself (Figures 2A, 2B, and S2B). Further on Tcm cells derived from bone marrow, lymph node, or spleen showed similar proliferative expansion (Figure 2C). This argues against preferential maintenance of CD8$^+$ memory T cells in specialized niches of the bone marrow—as had previously been suggested for CD4$^+$ T cells (Tokoyoda et al., 2009). Importantly, Tcm-cell-derived progeny, rested for 700 days after infection, was again devoid of CD44^{lo} cells (Figures S2C–S2E). Although showing high developmental capacity of Tcm cell populations derived from diverse anatomical sites, our population-based experiments—like those previously performed by others—could not assign stemness to the Tcm cell compartment. To overcome this limitation, we reasoned it essential to investigate the clonogenic potential of individual T cells.

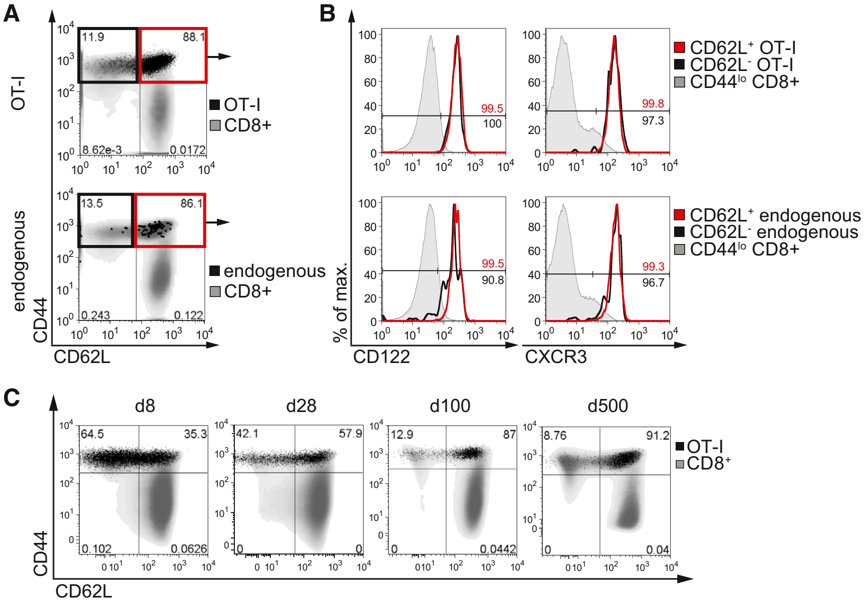

Figure 1. Long-Lived Memory after Acute Infection Consists of CD8$^+$CD44^{hi} Central Memory and Effector Memory T Cells

Progenies derived from 500 adoptively transferred naive OT-I CD45.1$^+$ T cells or endogenous H2-K^b/SIINFEKL-Streptamer$^+$ populations detected in spleen of C57BL/6 recipients at day 8 (d8), d28, d100, and d500 postinfection (p.i.) with Lm-OVA (5 × 10^3 cfu) and analyzed for CD44 and CD62L expression.

(A) Progenies derived from OT-I T cells (pregated on CD45.1$^+$CD8$^+$CD19$^-$ cells, upper dot plot) or from polyclonal SIINFEKL-specific endogenous CD8$^+$ T cells (pregated on H2-K^b/SIINFEKL-Streptamer$^+$ CD45.1$^-$CD8$^+$CD19$^-$ cells, lower dot plot) recovered at day 500 p.i. and analyzed for CD44 and CD62L expression (two independent experiments, n = 2, gray density plots indicate CD45.1$^-$CD8$^+$ cells).

(B) The CD44^{hi}CD62L$^+$ Tcm and CD44^{hi}CD62L$^-$ Tem cell subsets from (A) were further analyzed for CD122 and CXCR3 expression (red histogram: upper panel CD44^{hi}CD62L$^+$ OT-I, lower panel CD44^{hi}CD62L$^+$ endogenous Streptamer$^+$ population; black histogram: upper panel CD44^{hi}CD62L$^-$ OT-I, lower panel CD44^{hi}CD62L$^-$ endogenous Streptamer$^+$ population; gray histogram: CD44^{lo}CD45.1$^-$CD8$^+$ cells). Fraction of marker-positive cells within respective subsets is indicated.

(C) CD44 and CD62L expression of OT-I progenies detected at d8, d28, d100, and d500 p.i. Black dot plots indicate OT-I CD45.1$^+$ T cells (pregated on CD122$^+$CXCR3$^+$ cells), gray density plots indicate CD45.1$^-$CD8$^+$ cells (two independent experiments, n = 2).

Similar Developmental Capacity of Single Naive and Central Memory T Cells

As an initial step, we compared immune responses originating from single Tcm cells with those generated by their naive precursors. Memory T cells were derived from populations of 500 naive OT-I T cells that had been transferred into primary recipient mice and exposed to Lm-OVA infection. At least 100 days after primary infection, single CD62L$^+$ Tcm cells were sorted via flow cytometry, retransferred into naive secondary recipients, and rechallenged by subsequent Lm-OVA infection. In parallel, we transferred single naive OT-I T cells into a primary infection setting. We used eight distinct OT-I TCR-transgenic mouse strains, each identified by a unique signature of congenic markers CD45.1 or CD45.2 and CD90.1 or CD90.2 (OT-I congenic matrix) (Buchholz et al., 2013a), as a source for both naive and memory T cells (Figure S3). Through up to 8-fold single-cell cotransfer into one host, this allowed efficient single-cell fate mapping during primary and secondary responses. We found no significant difference in between the median proliferative output of single Tcm and that of single naive T cells 8 days p.i. (Figure 3A). In addition, the strong variability of progeny sizes described for monoclonal immune responses derived from single naive CD8$^+$ T cells (Buchholz et al., 2013a; Gerlach et al., 2013) was equally present in responses derived from single Tcm cells (Figures 3A and 3B). Like single Tn cells (Buchholz et al., 2013a; Gerlach et al., 2010, 2013; Stemberger et al., 2007), single Tcm cells could produce a phenotypically diverse offspring consisting of CD27$^-$CD62L$^-$ effector T (Tef) cells, CD27$^+$CD62L$^-$ Tem and CD27$^+$CD62L$^+$ Tcm cells, or an offspring biased toward one of these subsets (Figure 3B). Furthermore, naive- and central-memory-derived population expansion was accompanied by a relative decrease of T cells expressing memory surface markers CD27 and CD62L (Figures 3B and 3C). This inverse correlation of expansion and memory precursor phenotype proved to be virtually identical in both settings (Figure 3D). Thus, single Tcm cells generated immune responses of equal size and stochastic variation and of similar phenotypic patterning as did their naive precursors. These observations show that key developmental traits are preserved during the transition to memory and that—like Tn cells—single monoclonal Tcm cells are multipotent to produce a phenotypically diverse progeny.

Serial Adoptive Transfer of Single Cells Demonstrates Self-Renewal Capacity of Individual Central Memory T Cells

A prerequisite to conclusively assign stemness to a phenotypically defined cellular subset is that at least one cell from this subset must be capable of self-renewal, i.e., of generating a phenotypically diverse progeny that contains at least one cell capable of generating a secondary progeny with similar characteristics as the primary one (Morrison et al., 1997; Siminovitch et al., 1963; Weissman, 2000). Such a genealogical line cannot be identified when analyzing populations of memory T cells. In order to provide stringent conclusions concerning the mode of memory maintenance, we investigated the self-renewal capacity of Tcm cells in a setting comprising multiple generations of single-cell adoptive transfers and infection-driven re-expansions. In these serial clonogenic assays, the origin of all T cells observed could be traced along a genealogical line encompassing up to three defined ancestors—a single Tn cell, a single primary Tcm cell, and a single secondary Tcm cell (Figure 4A). In detail, we first transferred a single naive OT-I CD45.1$^+$ T cell per recipient and immunized with Lm-OVA. One week after this first generation single-cell transfer (1st SCT), recipients were assayed in blood for progeny recovery. At least 2 months

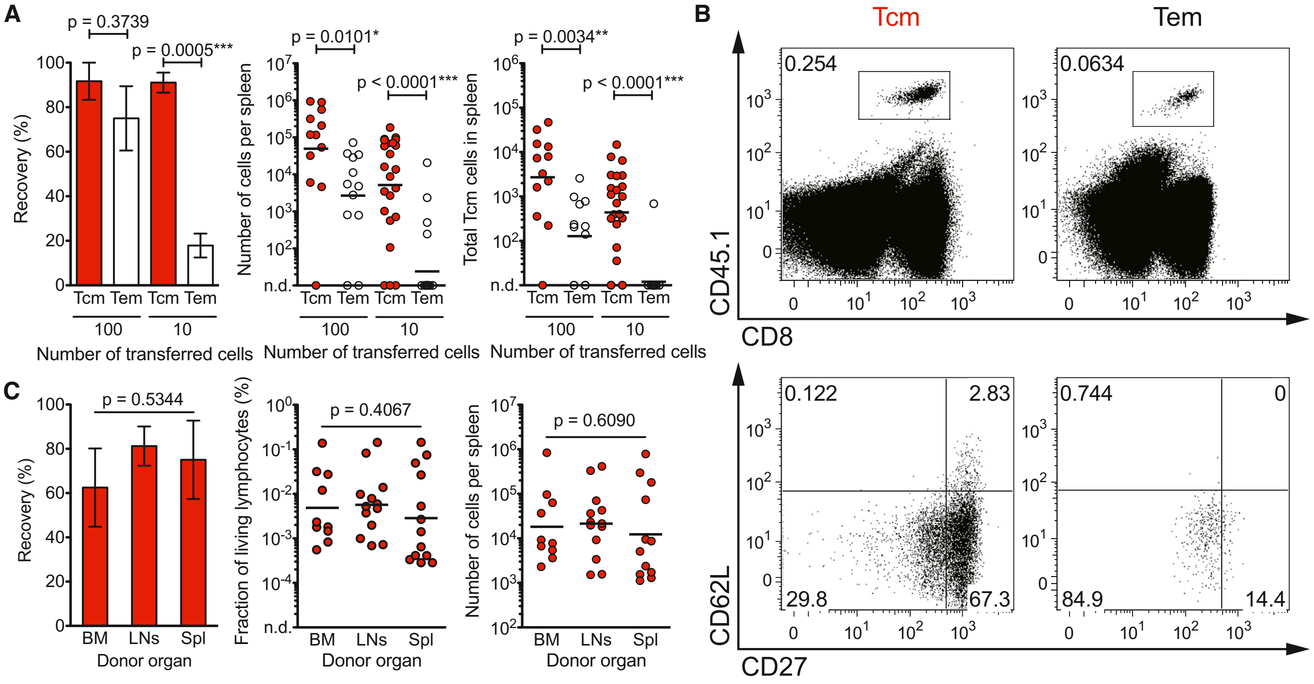

Figure 2. High Expansion and Differentiation Potential of CD8$^+$ Central Memory T Cells

CD8$^+$ memory T cells were derived from progenies of 500 adoptively transferred naive OT-I CD45.1$^+$ T cells at least 100 days p.i. with Lm-OVA (5 × 10^3 cfu).

(A) Adoptive retransfer of 100 or 10 CD62L$^+$ (Tcm) or CD62L$^-$ (Tem) OT-I CD45.1$^+$ memory T cells analyzed in spleen 8 days p.i. with Lm-OVA (three independent experiments, $n_{100} = 12$, $n_{10} = 24$). Comparison of recovery rates (unpaired, two-tailed t test, bars indicate mean ± SD), total cell numbers, and total number of Tcm cells generated after transfer of Tcm and Tem cells, respectively (two-tailed Mann Whitney test, median indicated). Recovery rates are defined as fraction of all recipients (n) in which progeny was detectable.

(B) Flow cytometry analysis of peripheral blood from representative recipients in (A) that had received 100 Tcm or Tem OT-I CD45.1$^+$ cells. CD45.1$^+$CD8$^+$ cells were analyzed for expression of CD62L and CD27.

(C) Adoptive retransfer of 10 OT-I CD45.1$^+$ Tcm cells purified from bone marrow (BM), lymph nodes (LNs), or spleen (Spl). Comparison of recovery rates (1-way ANOVA, bars indicate mean ± SD) and relative and absolute sizes of progenies (Kruskal-Wallis test, median indicated) in spleen 8 days after Lm-OVA infection (two independent experiments, n = 16).

p.i., single CD45.1$^+$CD62L$^+$ cells were sorted via flow cytometry. These single primary Tcm cells were subsequently transferred into naive secondary recipients (2nd SCT). After infection with Lm-OVA, the procedure was repeated again at more than 2 months p.i. by sorting single secondary Tcm cells and transferring them into naive tertiary recipients (3rd SCT) that were then infected with Lm-OVA (Figure 4A). To our knowledge, this is the first report of a trigenerational serial adoptive single-cell transfer of any mammalian cell type. Importantly, even upon transfer of 100 cells, serial transplantation was not possible for Tem cells (Figure S4). As described previously (Buchholz et al., 2013a; Stemberger et al., 2007), progeny from single naive T cells could be detected in approximately 20% of recipients. This recovery rate remained constant for successive transfers of individual primary and secondary Tcm cells (Figure 4B) and is comparable to the reconstitution efficiency of single HSCs (Osawa et al., 1996). During the acute response phase, variability of peak expansion and its correlation to memory precursor phenotype appeared similar, irrespective of whether offspring had originated from single Tn, primary Tcm, or secondary Tcm cells (Figure 4C). During the memory phase, long-term maintenance of progenies detected at peak expansion and numbers of single-cell-derived Tcm cells present within these progenies were found not to differ significantly between each generation of transfer (Figures 4D–4F). Importantly, the degree of expansion that had originated from a single ancestor did not predetermine the stochastic expansion behavior of its Tcm daughter cells (Figures 5A and 5B). Tcm cells derived from both large and small primary expansions could themselves generate large and small secondary responses (Figure 5C). This observation argues in favor of a persistently broad developmental potential of single Tcm cells and against accumulation of inherited restrictions. Hence, in a stringent setting of repetitive single-cell adoptive transfer and infection-driven re-expansion, Tn as well as primary and secondary Tcm cells appear to harbor and maintain broad proliferation and differentiation potential—demonstrating for the first time true self-renewal capacity of individual Tcm cells.

Single Central Memory T Cells Remain Multipotent throughout Serial Adoptive Transfers

The putative stem cell status of Tcm cells hinges not only on their self-renewal capabilities but also on their sustained multipotency to generate a diverse and protective offspring—equivalent to tissue reconstitution provided by other types of adult stem cells. Investigating this reconstitution capacity, we found that by the time of peak expansion, single secondary Tcm cells had

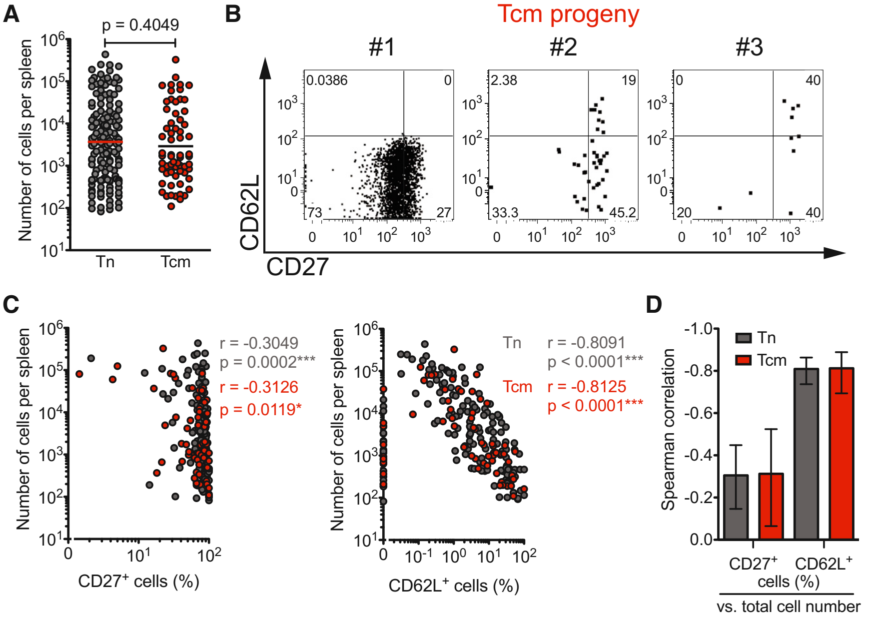

Figure 3. Similar Developmental Capacity of Single Naive and Central Memory T Cells

Progenies recovered from spleen 8 days after adoptive transfer of single Tn or Tcm OT-I matrix cells and infection with Lm-OVA (5×10^{3} cfu).

(A) Absolute number of descendants derived from single Tn (n = 148) and single Tcm (n = 64) cells (six and three independent experiments, respectively, two-tailed Mann-Whitney test, bars indicate median).

(B) Flow cytometry analysis indicates the size as well as the CD27 and CD62L phenotype of three expanded progenies (#1–3) recovered from the same recipient after adoptive transfer of single Tcm OT-I matrix cells.

(C) Scatter plots depict correlation of size and percentage of CD27 or CD62L expression in progenies derived from single Tn (gray) or single Tcm (red) cells (indicated are Spearman correlation coefficients r and respective p values).

(D) Comparison of correlation coefficients (Spearman coefficient and 95% confidence interval) from (C).

generated a tertiary progeny encompassing Tef, Tem, and tertiary Tcm cells (Figure 6A). Offspring showed diverse migratory behavior to lymphoid and nonlymphoid tissues (Figure 6A) and expressed transcription factors Eomes and T-bet (Figure 6B). Diversity was also evident for production of cytokines interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor-necrosis-factor-α (TNF-α) (Figure 6C). In line with our previous findings (Figures 3A–3C and 4C), tertiary progeny composition derived from one secondary Tcm cell could differ substantially to that derived from another (Figures 6 and S5).

Progeny Derived from Single Central Memory T Cells Can Restore Immunocompetence

Curative bone marrow reconstitution to lethally irradiated hosts is the major therapeutic hallmark provided by transplantation of HSCs. To test immune-reconstitution capacity of individual Tcm cells and compare it to that provided by naive T cells, we attempted to protect severely immunocompromised recombinase-activating-gene-2 and common-γ-chain-deficient ($Rag2^{-/-}Il2rg^{-/-}$) animals—lacking B, T, and NK cells—against an otherwise lethal bacterial infection. $Rag2^{-/-}Il2rg^{-/-}$ animals received no T cells, ten Tn cells, or ten tertiary Tcm cells derived from a genealogical line of three single cell ancestors—a Tn, a primary Tcm, and a secondary Tcm cell. 12 days after subsequent prime-boost vaccination with replication-deficient modified vaccinia virus Ankara-expressing OVA (MVA-OVA), $Rag2^{-/-}Il2rg^{-/-}$ animals that either had or had not received adoptive T cell transfer were challenged with Lm-OVA at a dose lethal to naive wild-type animals. Whereas $Rag2^{-/-}Il2rg^{-/-}$ animals that had not received adoptive T cell transfer showed high bacterial titers, recipients of Tn or tertiary Tcm cells both displayed sterile protection at 3 days p.i. (Figures 7A and 7B). At this time after infection, both Tn and Tcm cells had generated comparable numbers of offspring (Figure S6). Similar results were obtained when ten tertiary Tcm cells were transferred to P14^{tg} $Rag1^{-/-}$ recipients (Figure 7C). Because the P14 TCR is specific for a major histocompatibility complex I-restricted epitope of lymphocytic choriomeningitis virus, P14^{tg} $Rag1^{-/-}$ mice cannot provide adaptive immune responses against Lm-OVA but contain nearly physiological numbers of CD8^{+} T cells. In consequence, the successful transfer of immunocompetence to these animals cannot be critically dependent on lymphopenia-driven homeostatic mechanisms. Thus, a minute number of tertiary Tcm cells, whose ancestors had passed through three generations of single-T-cell-derived proliferation and diversification, were readily able to reconstitute full immunocompetence to severely immunocompromised hosts.

DISCUSSION

More than a decade ago, Fearon et al. (2001) put forward the hypothesis that akin to maintenance of other tissues, persistence of immune memory could be dependent on stem-cell-based mechanisms. This hypothesis was mainly sparked by the observations that certain B cells express a transcriptional repressor, which prevents their development into terminally differentiated plasma cells (Reljic et al., 2000). They are instead retained in an undifferentiated state out of which they can reenter germinal center reactions and produce new plasma cell daughters. These findings were interpreted as evidence for a stem-cell-like capacity of memory lymphocytes to self-renew and maintain a differentiated offspring.

Subsets of memory T cells were proposed to possess a similar developmental potential (Lanzavecchia and Sallusto, 2002). In support of this idea, studies relying on genomic signatures (Holmes et al., 2005), multidimensional phenotyping (Newell et al., 2012), and computational inference from single-cell fate mapping (Buchholz et al. 2013a) have positioned CD8^{+} memory T cells and specifically the Tcm cell subset in an intermediate position between naive and effector T cells. Further on, core molecular (Cui et al., 2011; Gattinoni et al., 2011; Muranski et al., 2011; Rao et al., 2010, 2012), genomic (Luckey et al.,

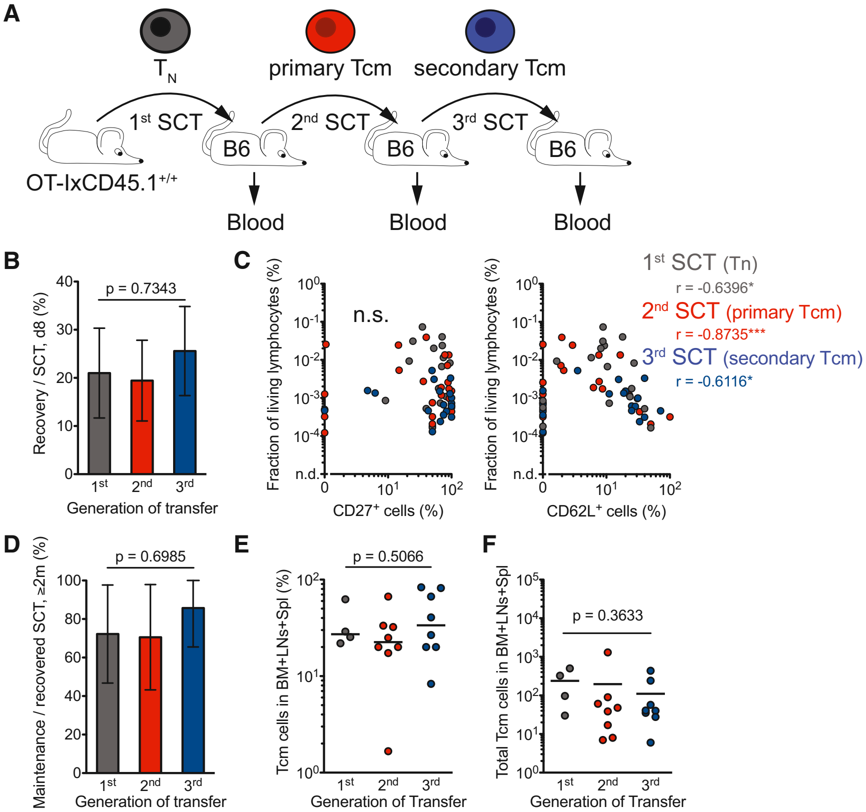

Figure 4. Serial Adoptive Transfer of Single Cells Demonstrates Self-Renewal Capacity of Individual Central Memory T Cells

(A) Experimental strategy to assess self-renewal capacity of individual $CD8^+$ Tcm cells by successive first-, second-, and third-generation single-cell transfers (SCT) of OT-I $CD45.1^+$ Tn, derived primary Tcm, and derived secondary Tcm cells into C57BL/6 recipients (B6), followed by Lm-OVA infection and blood sampling at d8 postinfection.

(B) Comparison of recovery rates (1-way ANOVA, bars indicate mean ± SD) during successive SCT generations of Tn (first generation) and derived primary Tcm (second generation) and secondary Tcm (third generation) cells (three independent experiments, $n_1 = 90$, $n_2 = 111$, $n_3 = 100$). Recovery rates are defined as fraction of all recipients (n) in which progeny was detectable at peak expansion.

(C) Scatter plots depict correlation in between single-cell-derived progeny size and percentage of CD27- or CD62L-expressing cells at peak expansion. First, second, and third generation SCTs are depicted in gray, red, and blue, respectively (indicated are Spearman correlation coefficients r and significance, n.s. $p > 0.05$; $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$).

(D) Maintenance rates of single-cell-derived progenies (1-way ANOVA, bars indicate mean ± SD). Maintenance rates are defined as the fraction of all single-cell-derived progenies detected at peak expansion, which could also be recovered at ≥2 months p.i.

(E and F) Scatter plots depict percentage (E) and absolute number (F) of Tcm cells in single-cell-derived progenies, harvested from total bone marrow (BM), lymph nodes (LNs), and spleen (Spl) of C57BL/6 recipients, at ≥2 months after first-, second-, or third-generation SCT (Kruskal-Wallis test, median indicated).

2006), and cell morphological (Chang et al., 2007; Ciocca et al., 2012) signatures, previously associated with tissue stem cells, could be identified within the memory T cell pool. Importantly, such correlative studies rely on the assumption that a phenotypic signature, previously identified among the stem cells of one specific tissue, will be indicative of tissue stem cells in general. Although such an assumption can be useful to guide phenotypic delineation of a putative stem-cell-containing subset within a complex tissue, it cannot replace the direct interrogation of this subset's developmental potential.

Postulated "T memory stem cells" would have to be able to persistently maintain an "epitope-specific tissue" (Buchholz et al., 2013b)—i.e., an epitope-specific population of T cells—by renewing themselves upon division while also generating a diverse offspring of effector T cells that ultimately protect the host in case of reinfection. These operational stem cell features (self-renewal, multipotency, and functional tissue reconstitution) would in addition have to be of "clonogenic" nature—meaning they would have to potentially emerge from a single stem cell.

Modeled on prototypic studies performed in the hematopoietic field (Dykstra et al., 2007; Osawa et al., 1996; Smith et al., 1991), we have performed here a set of in vivo clonogenic assays that allow for the first time a stringent evaluation of stemness in the $CD8^+$ memory T cell compartment. These assays—based on mapping the developmental history of individual T cells across multiple generations of single-cell adoptive transfer and infection-driven re-expansion—grant unprecedented insight into the genealogical relations within a complex immune response. They establish for the first time that individual $CD62L^+$ Tcm cells indeed meet the stringent operational criteria that define adult tissue stem cells. Beyond self-renewal and the multipotent generation of a diverse offspring, we identify single Tcm cells as a source for full reconstitution of immunocompetence. Recent work has suggested that a $CD44^{lo}CD62L^+$ naive-like subset of murine $CD8^+$ memory T cells harbors stem-cell-like capacity (Gattinoni et al., 2009; Zhang et al., 2005). However, a stringent functional analysis of this subset's stemness, like the one presented here for Tcm cells, has not been performed. In our physiological infection model, naive-like memory T cells could not be detected. It is possible that the frequency of a minute $CD44^{lo}$ memory T cell subset was below the sensitivity of our assays. A major role of such a subset in maintaining $CD8^+$ T cell memory is, however, irreconcilable with the observed capacity of at least 20% of individually transferred Tcm cells to self-renew and generate a diverse offspring. Thus, stem cell potential is not restricted to a rare memory T cell subset, but rather is present within a substantial fraction of $CD8^+$ Tcm cells. Our data do not formally exclude the existence of even less differentiated T cells than Tcm cells within the memory T cell compartment. But because single naive T cells and single Tcm cells are virtually indistinguishable concerning their capacity for proliferation, differentiation, and protective reconstitution of immunity, intermediates are unlikely to do better. It is important to note that these observations do not equate to a general functional identity of

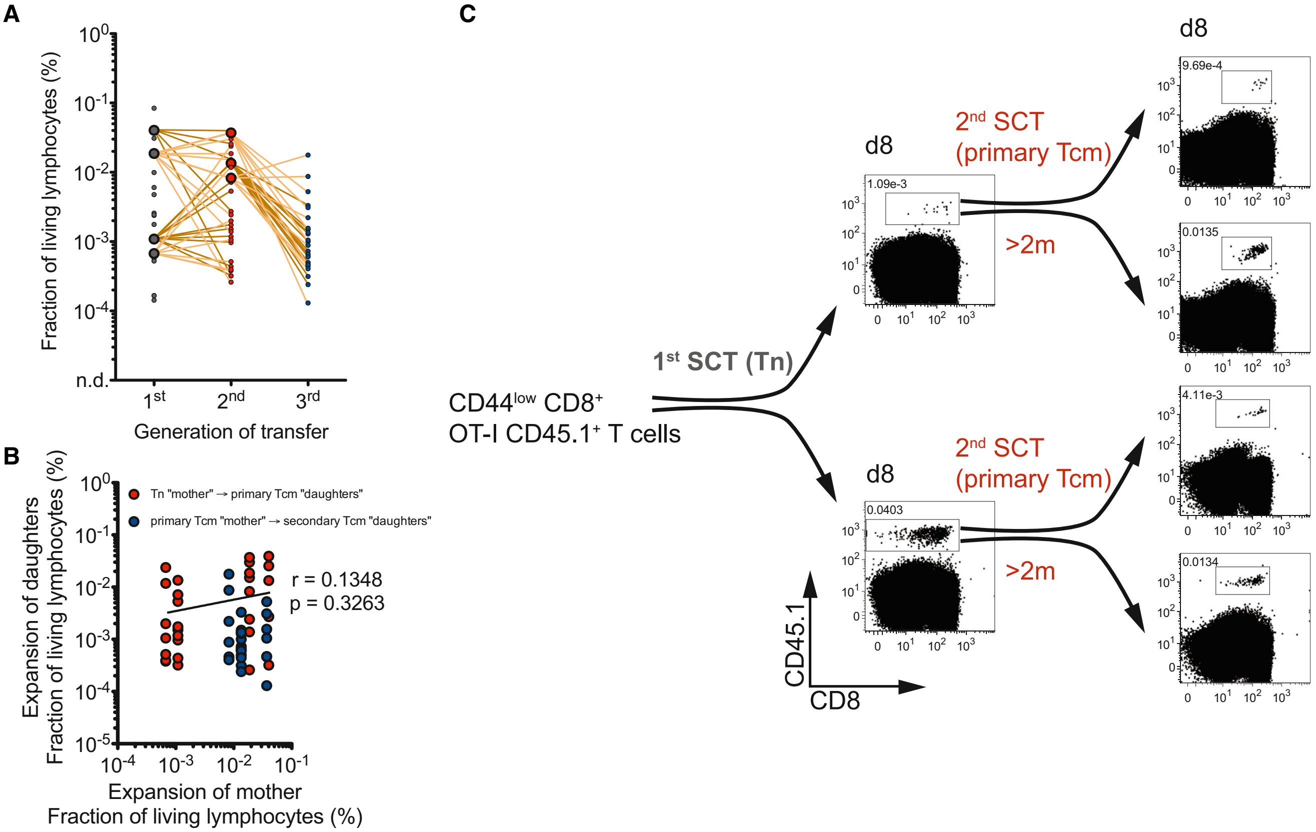

Figure 5. Degree of Ancestral Expansion Does Not Predetermine Proliferative Behavior of Single Central Memory T Cells

(A) Aligned scatter plots depict the size of progenies derived from individual Tn (large and small gray dots), individual primary Tcm (large and small red dots), or individual secondary Tcm (blue dots) cells, measured in blood 8 days after single-cell transfer (SCT) and Lm-OVA infection. All lines originating from a large dot connect a "mother" population to all of its "daughter" populations. Each daughter population is derived from an individual "maternal" Tcm cell by single cell transfer and infection-driven re-expansion. Serial single cell transfers were always performed at ≥2 months p.i. (three independent experiments).

(B) The correlation plot depicts peak expansion size of mother populations (x axis) versus that of derived daughter populations (y axis) (indicated is spearman correlation coefficient r and respective p value).

(C) Plots depict size of single-cell-derived OT-I CD45.1$^+$ T cells detected in peripheral blood at 8 days p.i. with Lm-OVA. Left: A large and a small "mother" population each derived from an individual Tn cell. Right: Two pairs of large and small daughter populations, each generated by single-cell transfer of single primary Tcm cells derived from large and small mother populations.

Tcm and Tn cells. In vivo imaging has suggested that the cell-autonomous advantage of Tcm versus Tn cells could largely be based on the capacity of Tcm cells to migrate beyond lymph node T cell areas and provide early encounter with pathogens trapped in the subcapsular sinus (Sung et al., 2012). In our experimental setting, based on Lm-OVA infection, antigen encounter occurs mainly in the T cell areas of the spleen via CD8$^+$ dendritic cells (DCs) (Neuenhahn et al., 2006; Verschoor et al., 2011)—probably equalizing deficits in migration of Tn cells. In this setting, we find equal expansion and differentiation kinetics for individual Tn and Tcm cells.

Naive-like memory T cells have also been identified in humans and nonhuman primates (Gattinoni et al., 2011; Lugli et al., 2013). Here as well, they have been proposed to be of major importance for maintaining T cell immunity and suggested to possess stem-cell-like features. In the light of our current study, these assumptions warrant reinvestigation in a setting allowing for stringent clonogenic assays, e.g., by implementing humanized mouse models and single-cell transplantation as recently done for human HSCs (Notta et al., 2011). Currently, it cannot be excluded that human T cell memory is structured in a different manner as its murine counterpart. The idea, however, that minute populations of epitope-specific T cells are the sole repository of stemness in immune memory appears problematic in the face of substantial stochastic variation, intrinsic to immune responses derived from single CD8$^+$ Tn cells (Buchholz et al., 2013a; Gerlach et al., 2013; Plumlee et al., 2013) as well as single Tcm cells. Both T-cell-intrinsic and -extrinsic cues can be envisioned as drivers of this variability. Independent of the question of how variability arises, a certain size of the T stem cell pool is likely to be essential for balancing it. In fact, we found that 10%–20% of recovered progenies (derived from individual Tn and Tcm cells) did not contain detectable numbers of CD62L$^+$ T cells at peak expansion. In addition, approximately 20% of single-cell-derived progenies were detectable at peak expansion but not during the memory phase (despite analysis of complete bone marrow, spleen, and lymph nodes). These observations could hint toward a stochastic loss of stemness during antigen-driven expansion

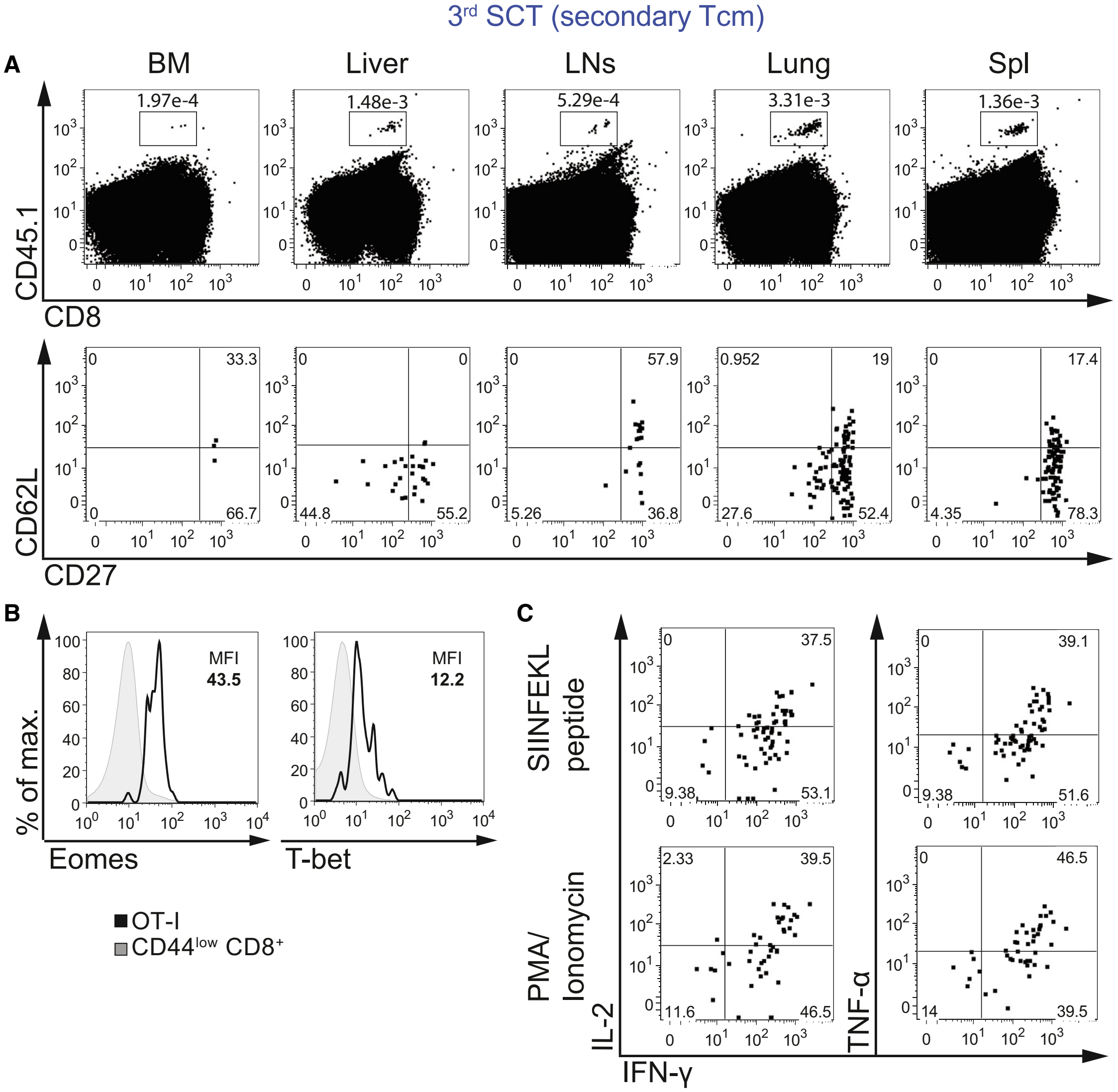

Figure 6. Single Central Memory T Cells Remain Multipotent throughout Serial Adoptive Transfers

One exemplary single-cell-derived progeny recovered after three successive SCTs of OT-I CD45.1$^+$ T cells. Analysis of the tertiary recipient was performed 12 days after third-generation SCT and infection with Lm-OVA (5 × 10^3 cfu).

(A) Flow cytometry analysis of single-cell-derived CD45.1$^+$CD8$^+$ progeny size (top) and the respective CD27 and CD62L phenotype (bottom) in bone marrow (BM), liver, lymph nodes (LNs), lung, and spleen (Spl).

(B) Intracellular staining for expression of Eomes and T-bet (black histogram, OT-I; gray histogram, CD44^{lo}CD45.1$^-$CD8$^+$ cells). Median fluorescence intensity (MFI) of OT-I T cells (CD45.1$^+$CD8$^+$ cells) is indicated.

(C) Flow cytometry analysis of IFN-γ, IL-2, and TNF-α expression of CD45.1$^+$CD8$^+$ cells after antigen-specific (SIINFEKL peptide) or antigen-independent (PMA/ionomycin) restimulation of tertiary recipient splenocytes.

and differentiation—reminiscent of "neutral drift" dynamics described for certain types of tissue stem cells (Snippert et al., 2010)—or to a further subdivision of the CD44^{hi}CD62L$^+$CD8$^+$ T stem cell compartment, with less than 80% of cells possessing true stemness. Importantly, when CD62L$^+$ progeny could be recovered at memory time points and single Tcm cells were adoptively retransferred, the response patterns generated showed again stochastic variation and appeared independent of inherited developmental histories. This emphasizes the capacity of Tcm cells to self-renew into a multipotent developmental state. Antigen-driven clonal expansion generates out of a few Tn cells a substantially enlarged pool of Tcm cells. This could be a crucial phenomenon guaranteeing robust memory T cell maintenance, despite stochastic expansion and differentiation processes. Similar features of population-based robustness have been proposed for homeostasis provided through other tissue stem cells (Mascré et al., 2012; Simons and Clevers, 2011; Snippert et al., 2010).

Importantly, with the high prevalence of antigen-specific T memory stem cells reported here, the classical Tcm cell compartment appears as an ideal source of T cells for immunotherapy against chronic infection and malignancy. Recent clinical trials

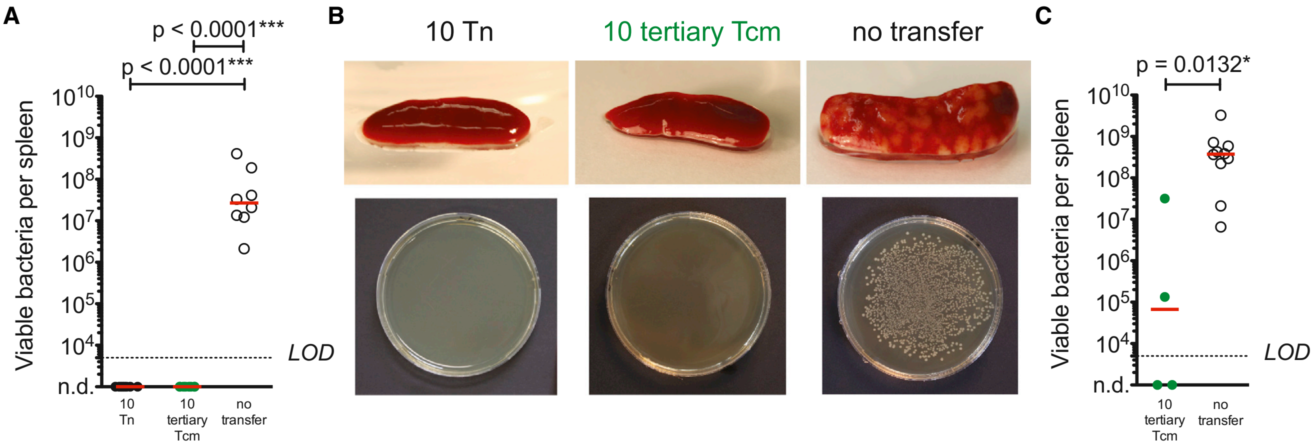

Figure 7. Progeny Derived from Single Central Memory T Cells Can Restore Immunocompetence

(A) Ten Tn or ten tertiary Tcm cells (derived from a single secondary Tcm) were transferred into immunodeficient lymphopenic *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients and prime-boost vaccinated with MVA-OVA (1–2 × 10^8 pfu i.v.) directly and 12 days after transfer. At 24 days after transfer, mice were challenged with 2 × 10^5 cfu Lm-OVA i.v. and bacterial burden was determined in spleen 3 days p.i. (three independent experiments, n = 8, two-tailed Mann-Whitney test, median indicated).

(B) Exemplary macroscopic aspect and bacterial culture from spleens of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice from (A) with or without prior transfer of ten Tn or ten tertiary Tcm cells.

(C) Ten tertiary Tcm cells (derived from a single secondary Tcm) were transferred into immunodeficient nonlymphopenic P14^{tg} *Rag1*$^{-/-}$ mice and prime-boost vaccinated with MVA-OVA (1–2 × 10^8 pfu i.v.) directly and 12 days after transfer. At 24 days after transfer, mice were challenged with 2 × 10^6 cfu Lm-OVA i.v. and bacterial burden was determined in spleen 3 days p.i. (two independent experiments, $n_{Tcm} = 4$, $n_{no\ transfer} = 10$, two-tailed Mann Whitney test, median indicated).

using donor-derived virus-specific memory T cells for the treatment of life-threatening infections in patients undergoing allogeneic stem cell transplantation have shown substantial success (Cobbold et al., 2005; Walter et al., 1995). Clinical efficacy of such interventions is still difficult to predict. Interestingly, however, it has been reported that even very low numbers of memory T cells can be sufficient to transfer protective immunity (Schmitt et al., 2011). Immunotherapy of cancers has provided some of the first spectacular clinical cases showing that adoptive transfer of T cells expressing recombinant tumor-reactive receptors can cure otherwise treatment-resistant malignancies (Brentjens et al., 2013; Grupp et al., 2013; Porter et al., 2011). For success of these therapies, long-term persistence of adoptively transferred T cells is of major importance (Berger et al., 2008; Jensen and Riddell, 2014; Maus et al., 2014). This indicates dependency on the regenerative quality rather than the sheer quantity of transplanted T cells. The identification of adult tissue stem cells within the Tcm cell compartment provides an intriguing explanation for these observations and should help to further refine current therapeutic approaches.

EXPERIMENTAL PROCEDURES

Mice and Infections

C57BL/6 mice were obtained from Harlan (Winkelmann, Borchen). Female OT-I matrix donor mice (Buchholz et al., 2013a), *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, and P14^{tg} *Rag1*$^{-/-}$ mice were derived from in-house breeding under specific-pathogen-free conditions and entered experiments at an age of 8–16 weeks. For primary infection of C57BL/6 mice, 5,000 cfu Lm-OVA (Pope et al., 2001) were injected i.v. For primary and boost immunization of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ and P14^{tg} *Rag1*$^{-/-}$ mice, 1–2 × 10^8 pfu MVA-OVA were injected i.v. (Staib et al., 2004). Bacterial challenge of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ or P14^{tg} *Rag1*$^{-/-}$ mice was performed by i.v. injection of 2 × 10^5 or 2 × 10^6 cfu Lm-OVA, respectively. All animal experiments were performed in accordance with national guidelines and were approved by local authorities.

Flow Cytometry and Intracellular Cytokine Staining

Leukocytes were isolated from blood, spleen, lymph nodes (axillary, inguinal, brachial, cervical, lumbar, mesenteric), bone marrow, liver, and lung as described previously (Schiemann et al., 2003). For flow cytometric analysis, samples were stained with fluorochrome-labeled antibodies specific to CD8 (5H10), CD16/32 (Fc-block 2.4G2 [unlabeled, BD Biosciences]), CD19 (1D3), CD27 (LG.7F9), CD44 (IM7 [BD Biosciences]), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD90.1 (OX7 [BD Biosciences]), CD90.1 (HIS51), CD90.2 (53-2.1), CD122 (TMβ1), CD127 (A7R34), CXCR3 (CXCR3-173), KLRG-1 (2F1) for 30 min at 4°C. Where indicated, fluorochrome-labeled H2-K^b/OVA$_{257\text{-}264}$ Streptamers (IBA) were used. Live/dead discrimination was performed with either ethidium monazide (EMA) or propidium iodide (PI). For intracellular cytokine staining, splenocytes were restimulated for 5 hr in vitro in medium containing 10^{-6} M SIINFEKL or 25 ng/ml PMA and 1 μg/ml Ionomycin, in the presence of Brefeldin A (BD Biosciences) at 2 μg/ml. Fixation and permeabilization of cells were performed according to the manufacturer's suggestions (BD PharMingen). Intracellular staining with fluorochrome-labeled antibodies specific for IL-2 (JES6-5H4), TNF-α (MP6-XT22, BD Biosciences), IFN-γ (XMG1.2), Eomes (Dan11mag), and T-bet (4B10) was done at 4°C for 30 min. Unless noted otherwise, antibodies and reagents were purchased from eBioscience. Data were collected on a CyAn ADP Lx 9 color cytometer (Beckman Coulter). Number of acquired events ranged between 0.5–3 × 10^6 for blood samples and 10^7–10^8 for all other organ systems. Analysis was performed with FlowJo software (TreeStar).

Cell Sorting and Adoptive Transfer of T Cells

Single naive CD44^{lo}CD8$^+$ cells were sorted from peripheral blood preparations of OT-I matrix donor mice as previously described (Buchholz et al., 2013a). Primary, secondary, or tertiary memory OT-I T cells were isolated from preparations of bone marrow, lymph nodes, and spleen of C57BL/6 mice that had at least 2 months before received 1–500 naive, primary memory, or secondary memory OT-I T cells and had subsequently been infected with Lm-OVA. Samples were stained with antibodies specific to the suitable congenic marker as well as CD62L, CD4, and CD19. 1–500 congenic marker positive and CD62L$^+$CD4$^-$CD19$^-$ or CD62L$^-$CD4$^-$CD19$^-$ cells were sorted. Successive single-cell sorts for assembly of multiple single OT-I matrix cells and subsequent i.p. transfer were performed as previously described (Buchholz et al., 2013a). Cell sorting was performed on a MoFlo XDP (Beckman Coulter).

Determination of Bacterial Burden

In brief, spleens were isolated 3 days after high-dose bacterial challenge with 2×10^5 or 2×10^6 cfu Lm-OVA for infection of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ or P14^{tg} *Rag1*$^{-/-}$ mice, respectively, homogenized through 100 μm cell strainers, and resuspended in 5 ml sterile PBS. 100 μl of the cell suspensions were diluted 1:10, 1:100, and 1:1,000 in 0.1% Triton X-100/PBS to release intracellular Lm-OVA from the cells. Aliquots of 10 μl per respective dilution were plated in triplicates on BHI plates and incubated overnight at 37°C. Colony-forming units were counted on the following day, and the amounts of Lm-OVA per organ were calculated according to the respective dilutions.

Statistical Analysis

p values were calculated with Student's t test, Mann-Whitney test, 1-way ANOVA, or Kruskal-Wallis test (GraphPad Prism), as specified in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.05.018.

AUTHOR CONTRIBUTIONS

P.G. performed and designed experiments, analyzed data, and wrote the paper; V.R.B. designed experiments, analyzed data, and wrote the paper; C.S. analyzed data; M.F. performed statistical analysis; L.H. performed cell sorting; M.S. performed and supervised cell sorting; I.D. provided MVA-OVA; T.H. supervised statistical analysis; S.R.R. designed experiments; and D.H.B. designed experiments, analyzed data, supervised the study, and wrote the paper.

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