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A Single Naive CD8$^+$ T Cell Precursor Can Develop into Diverse Effector and Memory Subsets

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SUMMARY

Upon first antigen encounter, naive CD8$^+$ T cells get activated, clonally expand, and can develop into very distinct subsets, such as short-living effector cells or different memory subpopulations. The origin of subset diversification is currently unknown, but qualitative and quantitative differences in early signals received by individual precursor cells have been suggested as a major determinant. We show that transfer of a single antigen-specific naive T cell into a normal recipient mouse allowed recovery of clonally expanded daughter cells upon immunization. With this experimental approach, we conclusively demonstrated that a wide range of diversity could develop out of a single precursor cell, including different types of effector and memory T cells. Interestingly, single-cell-derived subset diversification resembled that of polyclonal T cell responses in the same individual mouse, although differentiation patterns differed between immunization strategies. These data implicate that subset diversification is both shaped and synchronized during the expansion phase.

INTRODUCTION

Upon initial priming, naive CD8$^+$ T cells can acquire a variety of effector functions, including cytotoxicity and cytokine production (Williams and Bevan, 2006). Some of these effector cells (T_E) respond with only a restricted functional repertoire, whereas others can exert multiple effector functions in parallel (such as production of interferon gamma [IFN-γ], tumor necrosis factor alpha [TNF-α], or interleukin 2 [IL-2]). Only a few primed cells are maintained long-term as memory T cells, a heterogeneous population (Sallusto et al., 1999, 2004) comprising at least two distinct subtypes: central memory (T_{CM}) and effector memory (T_{EM}) T cells. In the mouse, short-living T_E express low amounts of the IL-7 receptor alpha chain (CD127) and downregulate L-selectin (CD62L) (Huster et al., 2004). Long-living T cells are characterized by their constitutive expression of CD127. CD62L expression is used to further discriminate T_{CM} (CD62L^{high}) from T_{EM} (CD62L^{low}). T_E preferentially migrate to nonlymphoid tissues, where some survive as T_{EM} for long periods of time (Masopust et al., 2001; Reinhardt et al., 2001). T_{EM} are capable of rapidly activating effector functions but proliferate poorly in response to antigen. In contrast, T_{CM} mainly home to lymphoid organs and vigorously re-expand upon antigen re-encounter.

Several models have been proposed to explain the generation of subset diversity. The "progressive differentiation" model (Sallusto et al., 2004) defines the generation of subsets via a gradual differentiation process from weakly to terminally differentiated cells, where variability in "signal strength" (a multifactorial parameter comprising TCR-MHC-peptide affinity, antigen concentration, and access to costimulatory receptors and cytokines) during the priming phase defines how far individual T cell clones differentiate; this could be achieved by genetic imprinting of a developmental program, which would guarantee the transfer of similar characteristics to subsequent daughter cells. Related to this model, it has been proposed that T_{CM} preferentially develop from naive precursor cells, which are recruited at later stages into the immune response ("latecomers") (Catron et al., 2006; D'Souza and Hedrick, 2006). Alternatively, differentiation capabilities could be predetermined within the repertoire of naive precursor cells. In line with this hypothesis, it has recently been shown that quantitative differences in precursor frequencies can "dictate" differentiation patterns in effector and memory T cells upon immunization. Perhaps together with numeric differences, some degree of phenotypical and functional diversification could already preform within clonotypic (bearing the same T cell receptor) precursor populations, as it has been described for homeostatic cell proliferation of naive T cells (Goldrath et al., 2000; Hamilton et al., 2006; Murali-Krishna and Ahmed, 2000). Segregation into T cell subsets could also follow the initial priming phase, promoted by variable additional antigen encounters or exposure to distinct environmental factors. However, the finding that a brief priming phase (less than 24 hr) is enough to induce the complete diversity of T cell subsets (Kaech and Ahmed, 2001; Mercado et al., 2000;

van Stipdonk et al., 2001), including memory T cells, without further need for antigen encounter has focused most attention on the initial priming phase.

Many groups have tackled the question of T cell subset origin experimentally. The finding that the T_{EM} and T_{CM} compartments have substantially different T cell receptor (TCR) repertoires has been interpreted as support for recruitment from distinct naive precursor cells (Baron et al., 2003). On the other hand, the finding that individual clonotypes of epitope-specific TCRs can be found in different subsets supports a model in which variable subsets can arise from naive T cells bearing the same TCR (Bouneaud et al., 2005); however, this observation cannot discriminate between origin from a single precursor cell or a small pool of clonotypic naive T cells (Reiner et al., 2007). In addition, all of these studies have the intrinsic problem that their readouts are indirect parameters. The recent finding that the first cell divisions of naive T cells can be asymmetric (Chang et al., 2007), perhaps caused by uneven distribution of cellular components between two daughter cells, indicates that diversification might indeed occur from individual cells. However, so far it has only been possible to study the phenotype of daughter cells derived from the first cell division, which limits the interpretations that can be made regarding T cell subsets found later during an immune response. A definitive answer to this fundamental question can be obtained only with an experimental system that offers the possibility to monitor the differentiation of a single naive T cell in vivo.

In this report, we describe the development of such a single-cell adoptive transfer model, and we demonstrate unequivocally that highly diverse subsets could arise from a single antigen-specific precursor cell. Surprisingly, the patterns of different effector and memory subsets derived from single T cells mirrored the diversity found for endogenous T cell responses specific for the same epitope in the same mouse.

RESULTS

Development of a Single-Cell Adoptive Transfer Model

Primary T cell responses to infectious agents can induce expansion into large antigen-reactive T cell populations, although the number of precursor cells is thought to be very low (50–200 cells) (Williams and Bevan, 2006). Therefore, we hypothesized that supplementation of the precursor repertoire with only a few naive T cells (here FACS-enriched CD45.1$^+$ CD44^{low} OT-I TCR-transgenic cells) should be sufficient to subsequently detect expanded daughter cells during the effector phase. In a first step, titration experiments were performed in which approximately 10,000, 1,000, 100, or 10 OT-I cells were adoptively transferred by intravenous (i.v.) injection, and subsequently recipient mice were infected with Ovalbumin-expressing *Listeria monocytogenes* (Pope et al., 2001) *(L.m.-Ova).* Seven days after transfer and infection, in all cases expanded CD45.1$^+$ OT-I T cells were readily detectable, even in the 10 cell group (see Figure S1A available online). Interestingly, intraperitoneal (i.p.) application of naive T cells showed very similar transfer efficiency. This observation was further supported by the finding that upon i.p. injection without infection, OT-I cells migrated very efficiently to the spleen within the first 24 hr after transfer without changing their naive phenotype (Figure S7A). In addition, we found no evidence for homeostatic cell proliferation during the first 48 hr upon transfer (Figure S7B). These data motivated us to do similar experiments transferring a single cell. We developed an injection system in which a single naive OT-I T cell is aspirated under microscopic control into a glass injection needle, from which it is subsequently flushed into the peritoneal cavity (Figure S1B). In about 25% of recipient mice, we could recover an expanded CD45.1$^+$ OT-I population on day 7 (Figure S2) or day 12 (Figure 1A) upon immunization; each event in the shown dot plots represents a daughter cell derived from the same single precursor cell. In summary, these data demonstrate that it is possible to experimentally track the fate of a single naive precursor cell upon clonal expansion in vivo.

Diverse Effector T Cell Populations Arise from Single Naive Precursor Cells

As shown in Figure 1A, the single-cell-expanded populations upon primary *Listeria* infection demonstrate a broad spectrum of heterogeneity, which is indicated by scattered distribution of surface markers as well as migration to different organs (spleen, lymph nodes). Staining for CD62L and CD127 surface expression, which begins to become most reliable around 10–12 days after *L.m.* infection (Huster et al., 2004, 2006a), demonstrates segregation into distinct phenotypical subsets (Figure 1A; for day 7 after infection analyses, see also Figure S2). In fact, the degree of diversity was basically identical to that generated in mice that received higher numbers of naive precursor cells (Figure 1B) or among natural polyclonal SIINFEKL-reactive CD8$^+$ T cell populations, which can be studied in the same individual mice with MHC multimers such as Streptamers (Figure 1C; Knabel et al., 2002). The similarities between endogenous and single-cell-derived cell populations indicate that naive OT-I cells reflect the full spectrum of differentiation capabilities characteristic of wild-type naive T cells. Comparably, intracellular cytokine stainings demonstrated the presence of subsets with different functional properties (Figure 2) derived from single precursor cells, based on IFN-γ and IL-2 coexpression or production of TNF-α (Figure 2B). Just as for phenotypical surface markers, also patterns in effector function of single-cell-derived populations were almost identical when compared to endogenous epitope-reactive T cells (Figure 2B) or when increasing the precursor frequency to $\sim$100 cells (Figure 2C). Further insights into the repertoire of effector functions of single-cell-derived populations were obtained by degranulation assays (Figure 2E; Betts et al., 2003), which confirmed the presence of heterogenic subpopulations. In summary, these data demonstrate subset diversification from a single naive precursor cell during the effector phase,

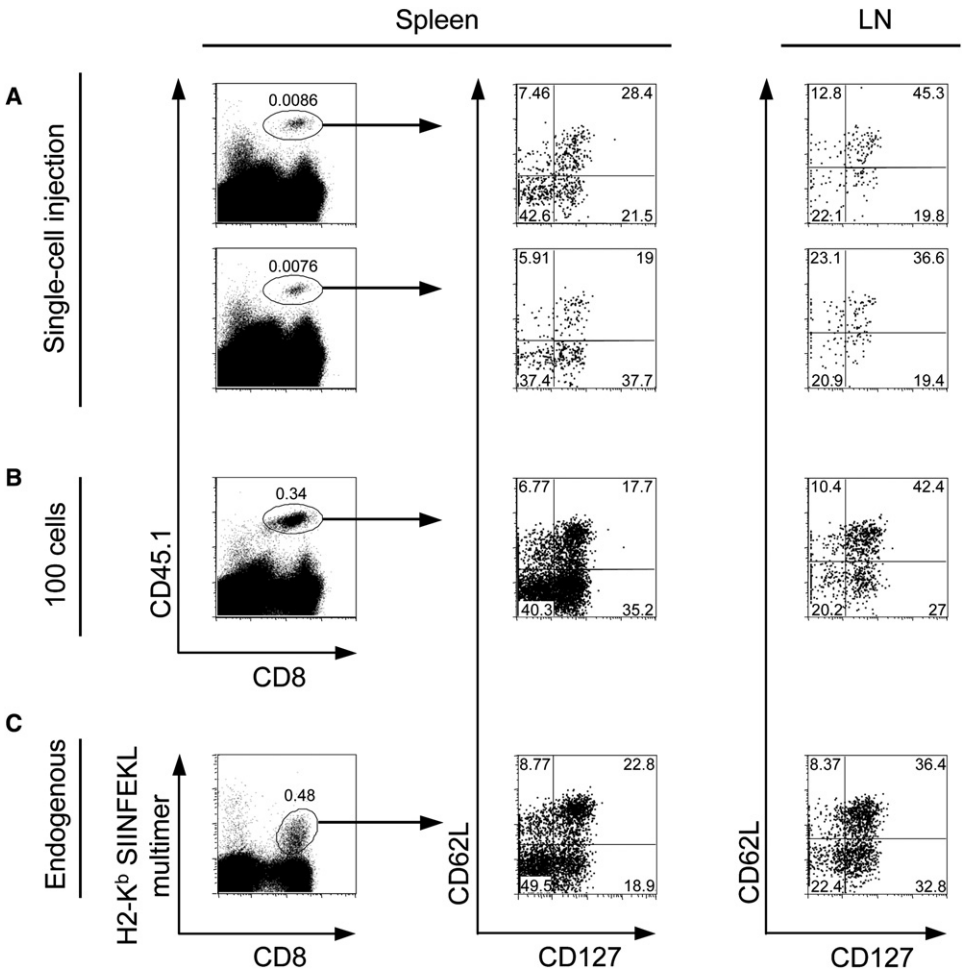


Figure 1. Phenotypical Diversity of T Cells Expanded from a Single Naive Precursor Cell during the Effector Phase

(A and B) C57BL/6 mice (CD45.2$^+$) received (A) a single naive CD45.1$^+$ CD8$^+$ OT-I T cell (two representative mice, upper two rows) by intraperitoneal microinjection or (B) 100 naive OT-I cells. Immediately after transfer, recipient mice were infected with *L.m.-Ova* (0.1 × LD_{50}), and 12 days later, T cells were analyzed in the spleen and lymph nodes (LN) by flow cytometry. Each row summarizes data from an individual mouse. Dot plots to the left show the identification of transferred OT-I cells in the spleen by staining for CD45.1 (y axis) and CD8 (x axis); percentages among total splenocytes are indicated for the shown gates. Dot plots in the middle and right show CD62L (y axis) and CD127 (x axis) expression patterns of in vivo expanded CD45.1$^+$ OT-I cells in the spleen and LN, respectively (parental gate is shown).

(C) Analyses of endogenous SIINFEKL-specific T cells in spleen and LN identified by MHC multimer (Streptamer) staining 12 days after infection with *L.m.-Ova* (0.1 × LD_{50}); identification of the cell populations is indicated in the left dot plot (gated on CD45.1$^-$ cells). Within each quadrant, percentages for defined subpopulations referring to the indicated parental gates are shown.

which is almost identical compared to endogenous epitope-specific T cell populations.

Memory Responses Derived from Single Naive Precursor Cells

The staining patterns for CD62L and CD127 on day 12 after immunization (Figure 1) indicate the presence of memory T cell precursors and suggest that both types of memory subsets, T_{EM} and T_{CM}, might arise from a single precursor cell. In order to demonstrate the presence of memory T cells more directly, a cohort of mice that had received a single naive OT-I cell prior to primary infection was rechallenged 5 weeks later with *L.m.-Ova*. Similar to the primary effector phase, we could detect a CD45.1$^+$ OT-I population in about 25% of recipient mice upon reinfection (Figure 3). This finding shows that cells with characteristics of long-living memory T cells can evolve from a single naive precursor cell. Compared to the primary response, recall population sizes were about 8 times larger, which corresponded to an almost identical increase in the number of endogenous SIINFEKL-specific T cells in the same cohort of mice (Figure S3), indicating that the two populations were synchronized rather than competitive in their in vivo kinetics. Re-expanded single-cell-derived populations demonstrated highly heterogeneous phenotypical (Figure 3A) and functional (data not shown) patterns, again very similar to endogenous T cell populations (Figure 3B; Huster et al., 2004, 2006b). These data

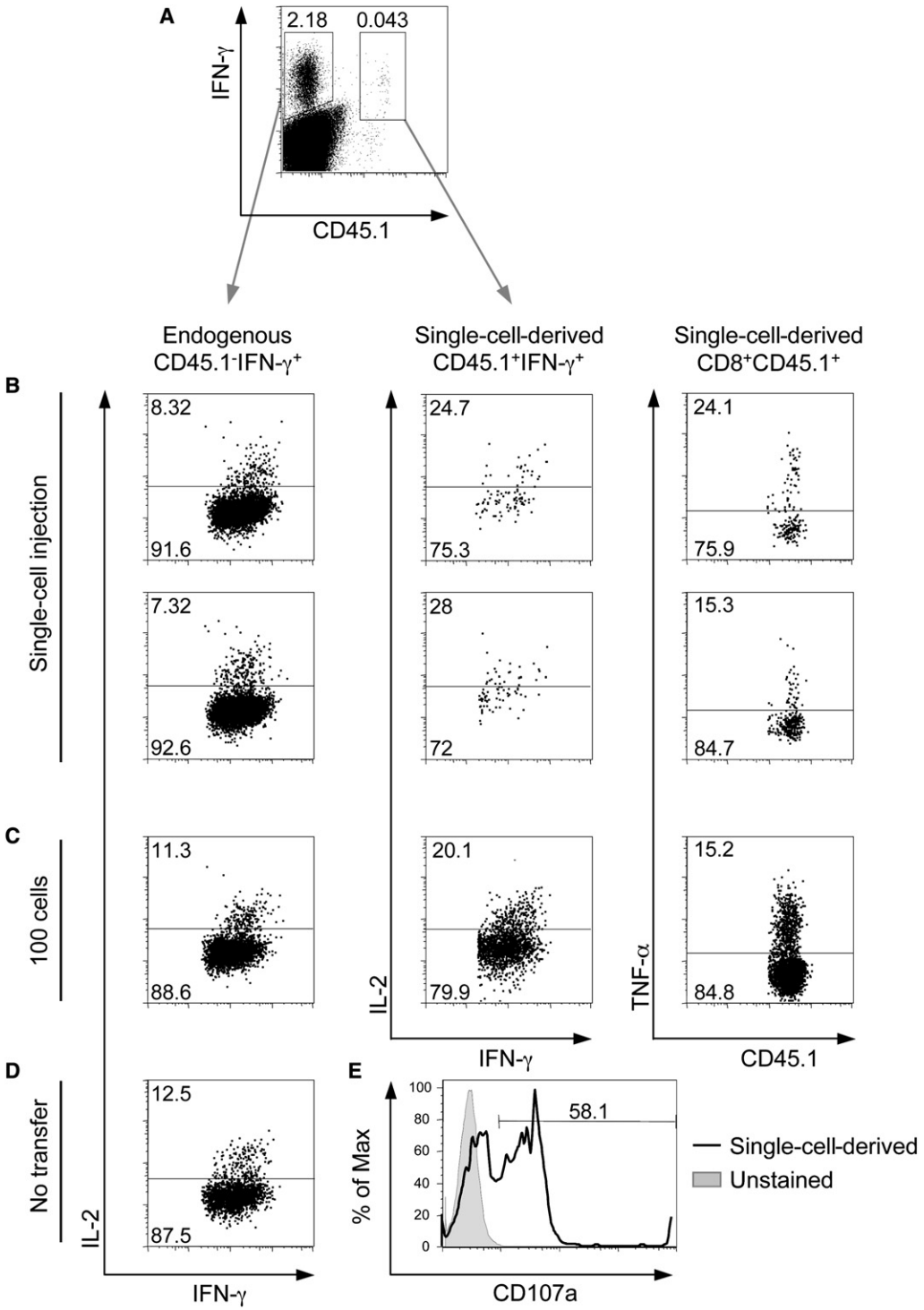


Figure 2. Functional Diversity of T Cells Expanded from a Single Naive Precursor Cell during the Effector Phase

(A) Representative dot plot (pregated on $CD8^+$ T cells) indicating the gating strategy for identification of endogenous ($CD45.1^-$) and single-cell-derived ($CD45.1^+$) IFN-γ-producing effector populations. Cells were pregated on live $CD8^+$ cells, and staining for IFN-γ (y axis) versus CD45.1 (x axis) is displayed.

(B–D) Analysis of subpopulations defined as described in (A) for IFN-γ (x axis) versus IL-2 (y axis) are shown in the left two dot plots. The dot plots to the far right show TNF-α production (y axis) by $CD45.1^+$ (x axis) single-cell-derived cells. Gates segregating subpopulations are indicated, and percentages for each area are shown.

(B and C) C57BL/6 mice ($CD45.2^+$) received (B) a single naive $CD45.1^+$ $CD8^+$ OT-I T cell (two representative mice) by intraperitoneal microinjection or (C) 100 naive OT-I cells intravenously.

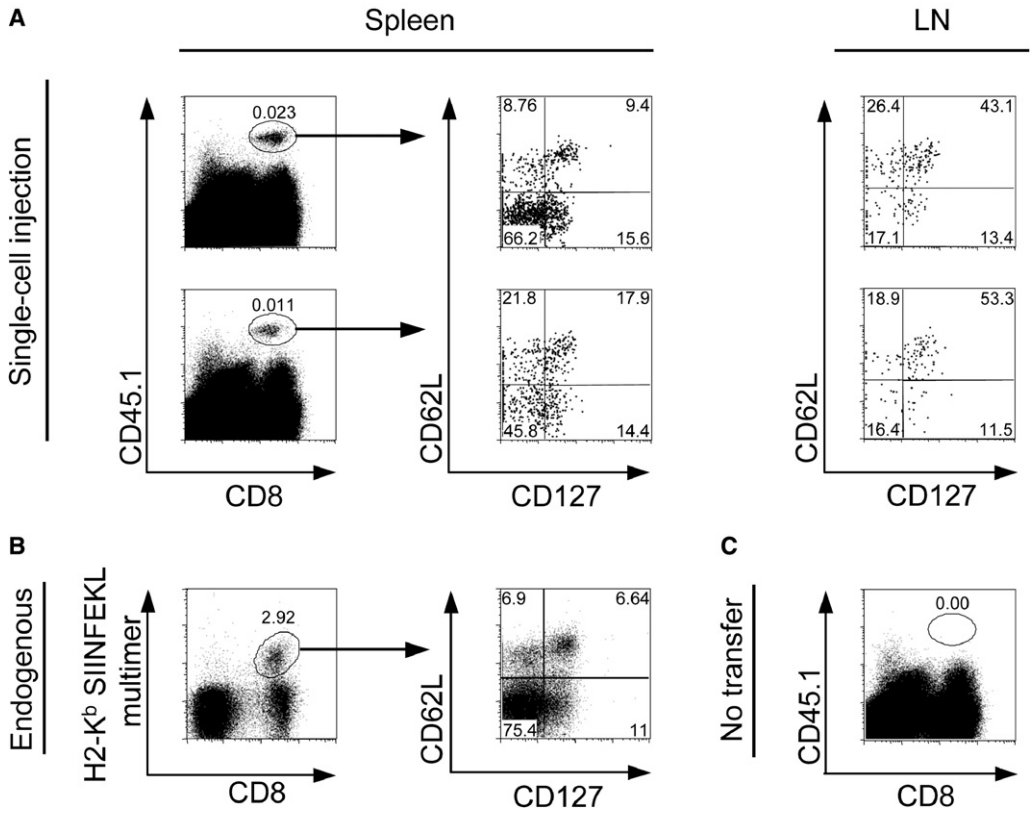


Figure 3. Phenotypical Diversity of T Cells Expanded from a Single Naive Precursor Cell upon Recall Expansion

C57BL/6 mice (CD45.2$^+$) received a single naive CD45.1$^+$ CD8$^+$ OT-I T cell by intraperitoneal microinjection. Immediately after transfer, recipient mice were infected with *L.m.-Ova* (0.1 × LD_{50}); mice received a second injection with *L.m.-Ova* (5 × LD_{50}) 5 weeks later, and T cells were analyzed on day 5 after infection in the spleen and lymph nodes (LN) by flow cytometry.

(A) Each row summarizes data from an individual mouse. Dot plots to the left show the identification of transferred OT-I cells by staining for CD45.1 (y axis) and CD8 (x axis); percentages among all splenocytes are indicated for the shown gates. Dot plots in the middle show CD62L (y axis) and CD127 (x axis) expression patterns of in vivo expanded CD45.1$^+$ OT-I cells in the spleen (parental gate is shown). Dot plots to the right display corresponding stainings in LN.

(B) Similar analyses as described in (A) were performed for endogenous SIINFEKL-specific T cells identified by MHC multimer (Streptamer) staining; identification of the cell populations is indicated in the left dot plot (gated on CD45.1$^-$ cells); the displayed data belong to the same mouse shown in the upper row in (A).

(C) Example of a control mouse that did not receive any CD45.1$^+$ CD8$^+$ OT-I T cells.

implicate that a single naive precursor cell can give rise to memory T cells, which become the origin of an enlarged and heterogeneous effector cell population upon antigen rechallenge.

Different Memory Subsets Derived from Single Naive Precursor Cells

The recall expansion clearly demonstrated that the presence of memory T cells developed from a single naive precursor cell. However, diversity patterns of subsequently re-expanded cell populations do not necessarily reflect the presence of both major subsets of memory T cells, because mainly T_{CM} are capable of proliferating in response to antigen re-encounter. In order to determine whether both T_{CM} and T_{EM} subsets are present in recipients of a single adoptively transferred cell, we attempted to visualize single-cell-derived memory T cells 5 weeks after primary immunization (without reimmunization). Because of the low frequencies of memory T cells, these experiments were possible only with improved flow cytometry acquisition software (Summit V.4.3.1, Dako) that allows acquisition of up to 7 × 10^7 events per sample. As summarized in Figure 4 for an individual mouse, we were able to visualize memory T cell populations derived from a single naive precursor cell in several different organs (spleen, lymph node, and lung). In the spleen (Figure 4A), CD45.1$^+$ OT-I

(D) Control mice did not receive any adoptively transferred cells but were otherwise treated identically. Immediately after transfer, recipient mice were infected with *L.m.-Ova* (0.1 × LD_{50}), and 12 days later, T cells from the spleen were analyzed by intracellular cytokine staining upon in vitro restimulation in the presence of OT-I peptide (SIINFEKL, 10^{-6} M). Data for the same individual mice as in Figure 1 are shown.

(E) Degranulation upon peptide restimulation in the presence of Brefeldin A was determined by anti-CD107a staining. Histogram shows CD45.1$^+$ cells from a single-cell-injection experiment; unstained control is shown in the overlap mode (gray).

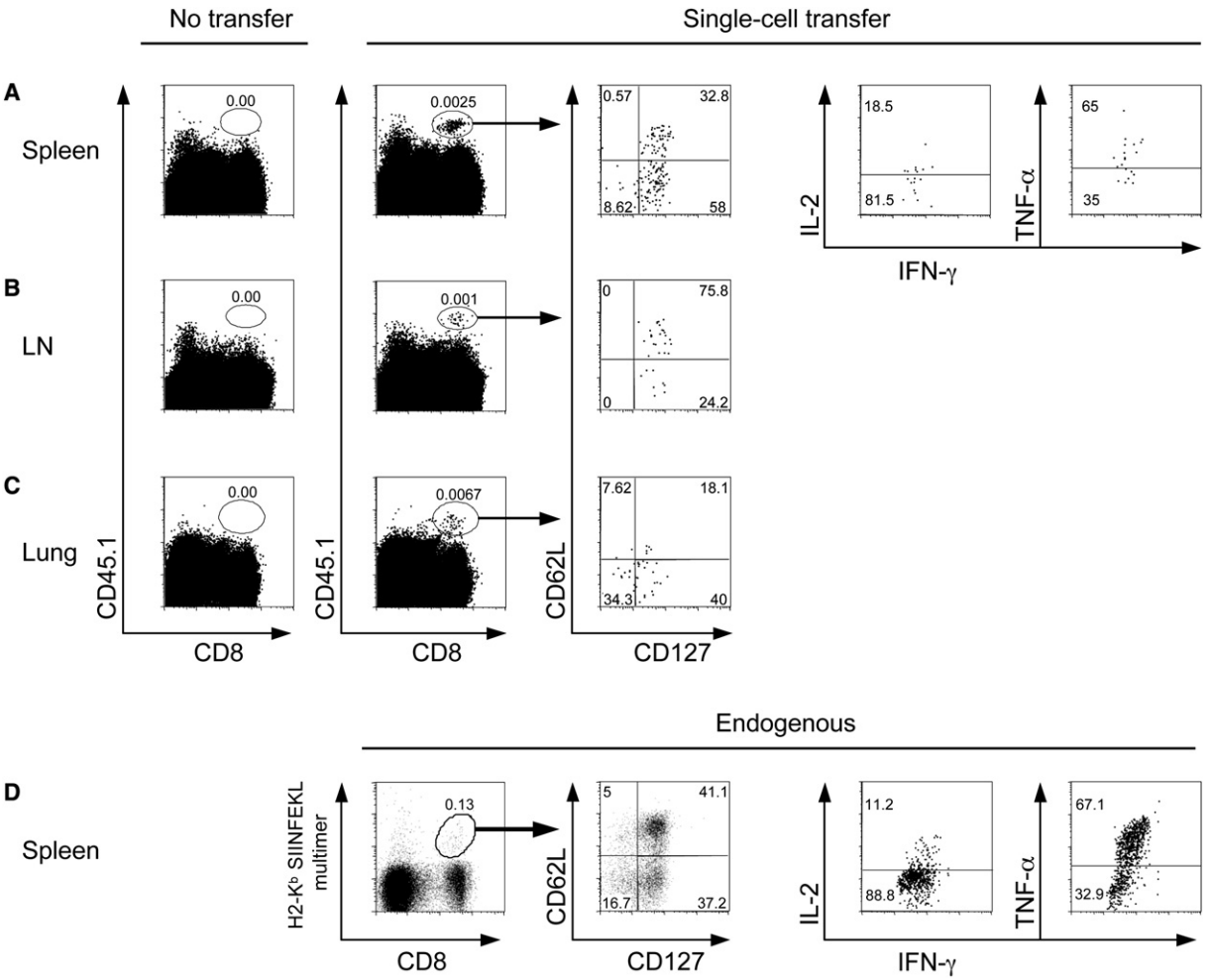


Figure 4. Phenotypical and Functional Diversity of Memory T Cells Generated from a Single Naive Precursor Cell

C57BL/6 mice (CD45.2$^+$) received a single naive CD45.1$^+$ CD8$^+$ OT-I T cell by intraperitoneal microinjection. Immediately after transfer, recipient mice were infected with *L.m.-Ova* (0.1 × LD_{50}), and 5 weeks later, T cells were analyzed by flow cytometry in different organs as indicated (all displayed data belong to the same individual mouse).

(A–C) Analysis of single-cell-derived populations in different organs. Left dot plots show stainings for a control mouse (no T cell transfer); all other dot plots show memory T cells from a mouse after single-cell transfer. OT-I cells were identified by staining for CD45.1 (y axis) and CD8 (x axis); percentages among viable cells are indicated for the shown gates. CD45.1$^+$ CD8$^+$ T cells were further analyzed for CD62L (y axis) and CD127 (x axis) expression. The two dot plots to the right show IFN-γ (x axis) and IL-2 (y axis) or TNF-α (y axis) expression patterns of IFN-γ$^+$ CD45.1$^+$ cells.

(D) Identification of endogenous SIINFEKL-specific T cells in the spleen by MHC multimer (Streptamer) staining; the relevant cell population is indicated in the left dot plot (gated on CD45.1$^-$ cells); gated antigen-specific cells were further analyzed for CD62L (y axis) and CD127 (x axis) expression. The two dot plots to the right show expression of IFN-γ (x axis) versus IL-2 (y axis) or TNF-α (y axis) on endogenous SIINFEKL-reactive IFN-γ$^+$ cells as described in Figure 2.

populations were almost exclusively positive for CD127, a characteristic feature of memory T cells (Huster et al., 2004; Kaech et al., 2003). Additionally, costaining for CD62L, a marker commonly used to segregate T_{EM} from T_{CM}, clearly distinguished two subpopulations (CD62L^{high} and CD62L^{low}). The presence of both memory subsets at this time point after infection is typical for the spleen (Busch et al., 1998). Again, the phenotype of endogenous SIINFEKL-specific T cells detected by MHC multimer staining was similar (Figure 4D). Intracellular cytokine staining in the spleen further confirmed the presence of different memory T cell subsets (Figure 4A), and the complexity found within the endogenous SIINFEKL-specific T cell population was comparable (Figure 4D). Frequencies of memory T cells in other organs were too low to allow for functional analysis. T_{CM} preferentially migrate to lymphoid organs, and, accordingly, primarily CD62L^{high} CD45.1$^+$ memory T cells were found in lymph nodes of single-cell recipients (Figure 4B). The opposite has been described for T_{EM}, which are CD62L^{low} and migrate to nonlymphoid organs such as the lung. We observed the same for single-cell-derived T_{EM} subpopulations (Figure 4C), similar to endogenous SIINFEKL-specific T cells in the same individual mouse (not shown). In summary, these data demonstrate

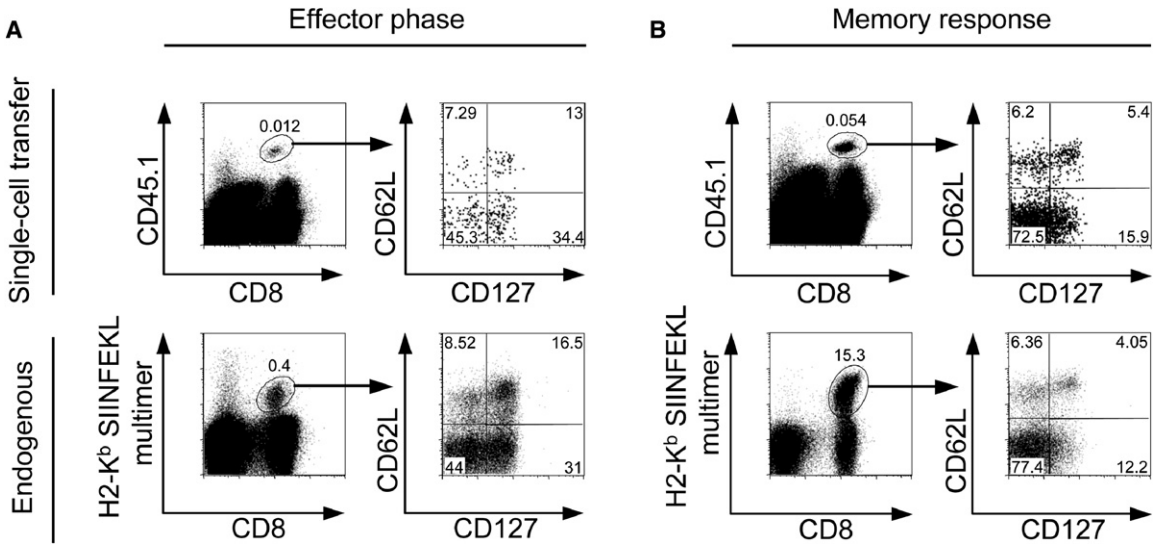


Figure 5. Phenotypical Characteristics of Single-Cell-Derived Memory T Cells after Secondary Adoptive Transfer and Recall Infection

C57BL/6 mice (CD45.2$^+$) received a single naive CD45.1$^+$ CD8$^+$ OT-I T cell by intraperitoneal microinjection. Immediately after transfer, mice were infected with *L.m.-Ova* (0.1 × LD_{50}). After 12 days, cells from spleen and LN were pooled and either analyzed by flow cytometry ([A], effector phase) or transferred into a second naive CD45.2$^+$C57BL/6 mouse. After more than 5 weeks after primary transfer, recipient mice were infected with 5 × LD_{50} *L.m.*-Ova, and after 6 days, splenocytes were analyzed by flow cytometry ([B], memory response). (A) and (B) show representative data for a corresponding donor-recipient pair. Dot plots to the left show the identification of transferred OT-I cells by staining for CD45.1 and CD8 or SIINFEKL-specific CD45.1$^-$ T cells identified by MHC multimer (Streptamer) staining; percentages among all splenocytes are indicated for the shown gates. Dot plots to the right show CD62L and CD127 expression patterns of in vivo expanded SIINFEKL-specific (transferred or endogenous) cells in the spleen (parental gate is shown) after primary and secondary infection.

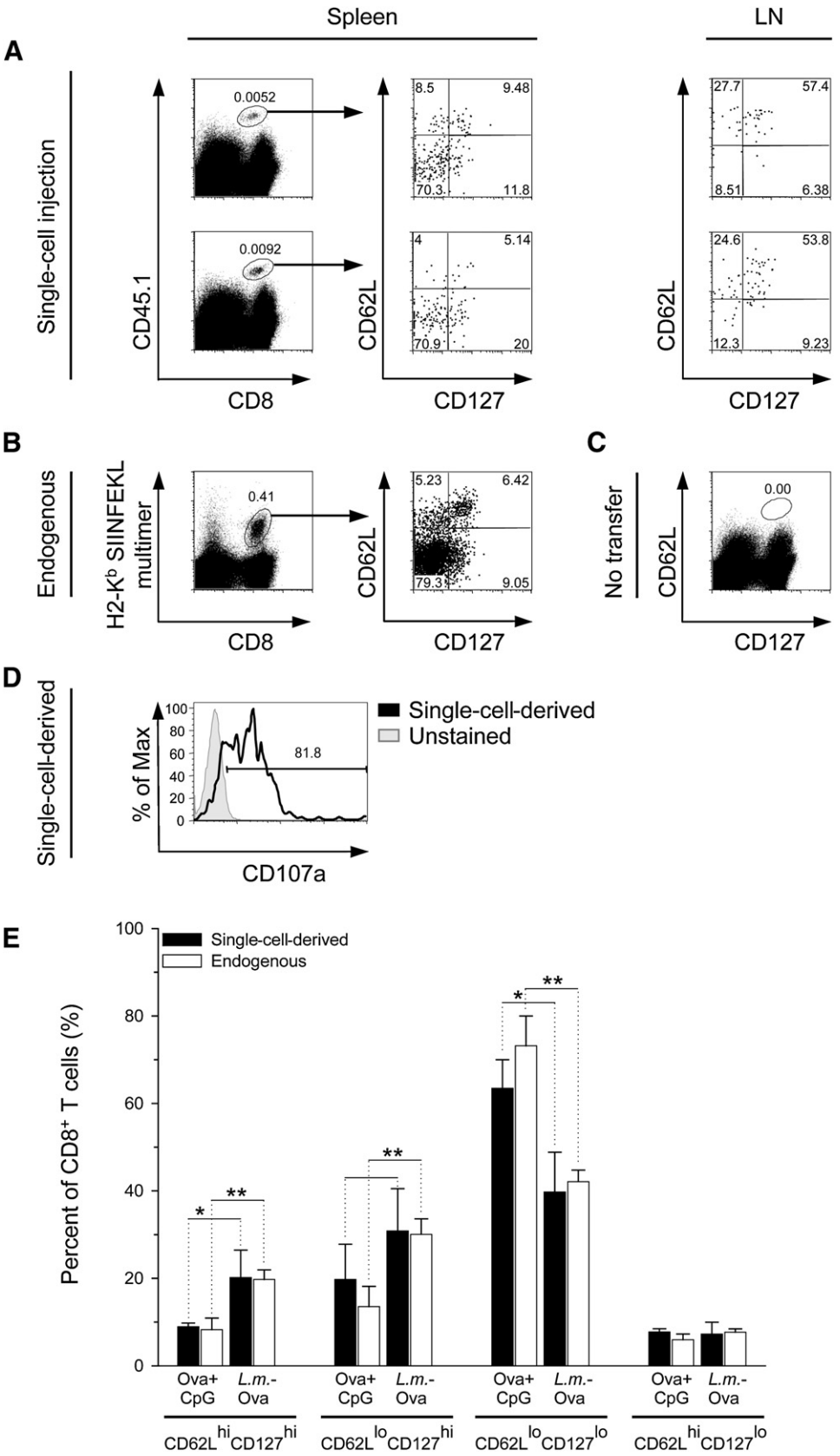
that phenotypically and functionally distinct memory T cell subsets can arise from the same naive precursor T cell.

Effector and Memory Subsets Originate from the Same Single Naive Precursor Cells

Single-cell-derived memory T cells were detectable in *Listeria*-infected recipient mice with a similar recovery rate as compared to analyses during the effector phase, which indicates that both observations are directly linked to each other. However, these data cannot prove that daughters from the same precursor cell that gave rise to diversified subtypes during the effector phase also became memory T cells. In order to demonstrate this directly, we performed repetitive adoptive transfer experiments that allowed following the fate of single-cell-derived progeny at all stages of the immune response (Figure S4 and Figure 5). Twelve days after single-cell transfer and primary *Listeria* challenge, splenocytes and lymph node cells from individual mice were pooled; 1/3 of the cells was then used to demonstrate the presence of diversified single-cell-derived progeny during the effector phase (Figure 5A). The remaining cells were adoptively transferred into a naive recipient mouse, which was challenged 5 weeks later with *L.m-Ova* and subsequently analyzed for T cell responses 6 days later. As shown in Figure 5B, recipient mice demonstrated a typical SIINFEKL-specific memory response, which contained single-cell-derived and endogenous T cell populations. These data unequivocally demonstrate that short-living effector cells as well as memory T cells can arise from the same precursor cell. In addition, also after adoptive transfer, single-cell-derived populations showed almost identical phenotypical differentiation patterns as compared to endogenous cell populations (Figure 5B).

Synchrony of Immunization-Dependent Signatures of Subset Diversification

So far, we have shown that a broad spectrum of effector and memory T cells can arise from a single precursor cell. Perhaps even more surprising was the finding that the patterns of diversification for single-cell-derived cells mirrored the subset heterogeneity found for endogenous T cell populations with the same epitope specificity. Because different vaccination strategies have been described to mediate distinct differentiation patterns (Bachmann et al., 2006; Badovinac et al., 2005; Huster et al., 2006a), we decided to analyze whether a similar correlation could be found in a different immunization model. For this purpose, mice received a subcutaneous vaccination of 100 μg ovalbumin together with 10 nmol CpG as adjuvant immediately after adoptive transfer of a single naive OT-I cell. Twelve days later, lymphocytes were analyzed in different organs for the presence of single-cell-derived daughter cells. As shown in Figure 6A for splenocytes from two representative mice, we were again able to detect single-cell-derived T cell populations with a similar recovery rate as described before for *Listeria* infection experiments (~25%). However, in contrast to the previous experiments (Figure 1), the phenotypical differentiation


A
Spleen
LN
Single-cell injection
0.0052
0.0092
8.5
9.48
70.3
11.8
4
5.14
70.9
20
27.7
57.4
8.51
6.38
24.6
53.8
12.3
9.23
CD45.1
CD8
CD62L
CD127
B
Endogenous
H2-Kb SIINFEKL multimer
0.41
5.23
6.42
79.3
9.05
C
No transfer
0.00
D
Single-cell-derived
% of Max
81.8
Single-cell-derived
Unstained
CD107a
E
Single-cell-derived
Endogenous
Percent of CD8+ T cells (%)
Ova+ CpG
L.m.-Ova
CD62Lhi CD127hi
CD62Llo CD127hi
CD62Llo CD127lo
CD62Lhi CD127lo

patterns differed substantially, especially characterized by an enlarged subpopulation of CD62L^{low} CD127^{low} cells and diminished CD127^{high} subsets (Figure 6A), which correlated functionally with increased numbers of degranulating (CD107a-positive) cells (Figure 6D). But again, when comparing single-cell-derived T cells with endogenous SIINFEKL-specific cell populations (Figure 6B), there was no obvious difference in their differentiation patterns. This became even more clear and backed up by statistical analyses when comparing the percentages of subset prevalence in larger groups of mice that underwent single-cell transfer and different immunization procedures (Figure 6E). Whereas both immunizations resulted in substantially distinct subset distributions, the diversification patterns of single-cell-derived and endogenous T cells within each group were remarkably similar.

Also Single Naive Latecomer Cells Develop into Diverse T Cell Subsets

It has recently been suggested for CD4$^+$ and CD8$^+$ T lymphocytes that central memory T cells might evolve preferentially from naive T cells that are recruited at later time points (e.g., day 2–3) into an immune response. These latecomer cells might encounter suboptimal priming conditions, which are not sufficient to drive their full effector cell differentiation. Such a correlation has, for example, been demonstrated by adoptive transfer of naive CD8$^+$ TCRtg T cells upon infection with VSV or *Listeria monocytogenes* (D'Souza and Hedrick, 2006); a large proportion of these latecomer cells developed into a phenotype characteristic for T_{CM}, which was already detectable by an increased CD62L^{high} subset about a week after infection and which was different from endogenous T cells identified by MHC multimers in the same individual mice. However, it needs to be mentioned that the number of transferred T cells in these reports were relatively high (10^4–10^6), which has recently been shown to lead to "unphysiological" T cell differentiation with a preference to a T_{CM} phenotype (Badovinac et al., 2007; Marzo et al., 2005).

Based on these previously published data, we decided to analyze the fate of single naive latecomer T cells adoptively transferred 2 days (48 hr) after *L.m.-Ova* infection (Figure S5). As controls, some mice received 100 naive T cells and the differentiation patterns were compared to endogenous SIINFEKL-specific CD8$^+$ T cell populations. We found that although the overall size of single-cell-derived daughter cell populations from latecomers was substantially lower compared to adoptive transfer directly together with infection (Figures 1 and 7), in all cases the phenotypical and functional differentiation patterns were highly diverse. Furthermore, also single-cell-derived latecomer T cell populations demonstrated a differentiation pattern of daughter cells, which closely resembled that of endogenous polyclonal T cells (Figure 7). In addition, transfer experiments with 100 naive T cells 2 days (48 hr) after infection demonstrated exactly the same picture, and no trend toward larger frequencies of CD62L^{high} subsets, which have been associated with T_{CM}-like characteristics, could be observed (Figure S6). These data strongly support the interpretation that subset diversification is both shaped and synchronized continuously during the expansion phase.

DISCUSSION

In this study, we demonstrate that it is possible to follow the fate of a single naive CD8$^+$ T cell transferred into a polyclonal environment. Upon immunization with the intracellular bacterium *Listeria monocytogenes* or vaccination with Ovalbumin and CpG, distinct phenotypes expressing varying combinations of CD62L, CD127, IFN-γ, TNF-α, IL-2, and/or degranulation could be recovered. In addition, daughter cells derived from a single precursor cell developed into both major subsets of memory T cells, central memory (T_{CM}), and effector memory (T_{EM}) T cells. These data directly demonstrate that subset heterogeneity can arise from a single naive precursor cell. In addition, we uncovered in all cases after single-cell inoculation that subset diversifications mirrored the subset heterogeneity found for endogenous T cell responses. This latter finding is particularly important, because it might reorient the current discussion concerning the origin of subset diversification to differences in the range of signals provided to clonal progeny during the proliferation phase.

Because of the small number of naive precursor T cells specific for a given antigen and the relatively robust differentiation patterns found for many infection and vaccination models, some degree of intraclonal diversification upon

Figure 6. Subset Differentiation of Single-Cell-Derived and Endogenous Antigen-Reactive T Cells upon Immunization with Ovalbumin and CpG

(A and B) C57BL/6 mice (CD45.2$^+$) received a single naive CD45.1$^+$ CD8$^+$ OT-I T cell by intraperitoneal microinjection (two representative mice are shown). Immediately after transfer, recipient mice were immunized subcutaneously with Ovalbumin and CpG, and 12 days later, T cells were analyzed in the spleen (A). Dot plots to the left show the identification of transferred OT-I cells by staining for CD45.1 and CD8 or (B) of endogenous (CD45.1$^-$) cells by MHC multimer (Streptamer) staining (displayed data correspond to the same mouse shown in the upper row of [A]); percentages among total splenocytes are indicated for the shown gates. Dot plots to the right show CD62L and CD127 expression patterns of in vivo expanded transferred or endogenous SIINFEKL-reactive cells in the different organs (parental gates are shown).

(C) Example of a control mouse that did not receive any CD45.1$^+$CD8$^+$ OT-I T cells.

(D) Surface expression of CD107a from single-cell-derived CD45.1$^+$ OT-I cells. Splenocytes from one representative mouse are shown.

(E) Comparison of CD62L and CD127 expression patterns of single-cell-derived progeny upon subcutaneous immunization with Ovalbumin + CpG or upon challenge with *L.m.-Ova* (0.5 × LD_{50}). After 12 days, different CD62L- and CD127-expressing subsets (CD62L^{hi}CD127^{hi}, CD62L^{lo}CD127^{hi}, CD62L^{lo}CD127^{lo}, and CD62L^{hi}CD127^{lo}) were analyzed by flow cytometry and plotted as percentage of CD8$^+$ cells. Black bars represent single-cell-derived cells, white bars cells from endogenous antigen-reactive cells (*$p < 0.02$; **$p < 0.003$). Standard deviations are indicated by error bars; n = 6–10 mice per group.

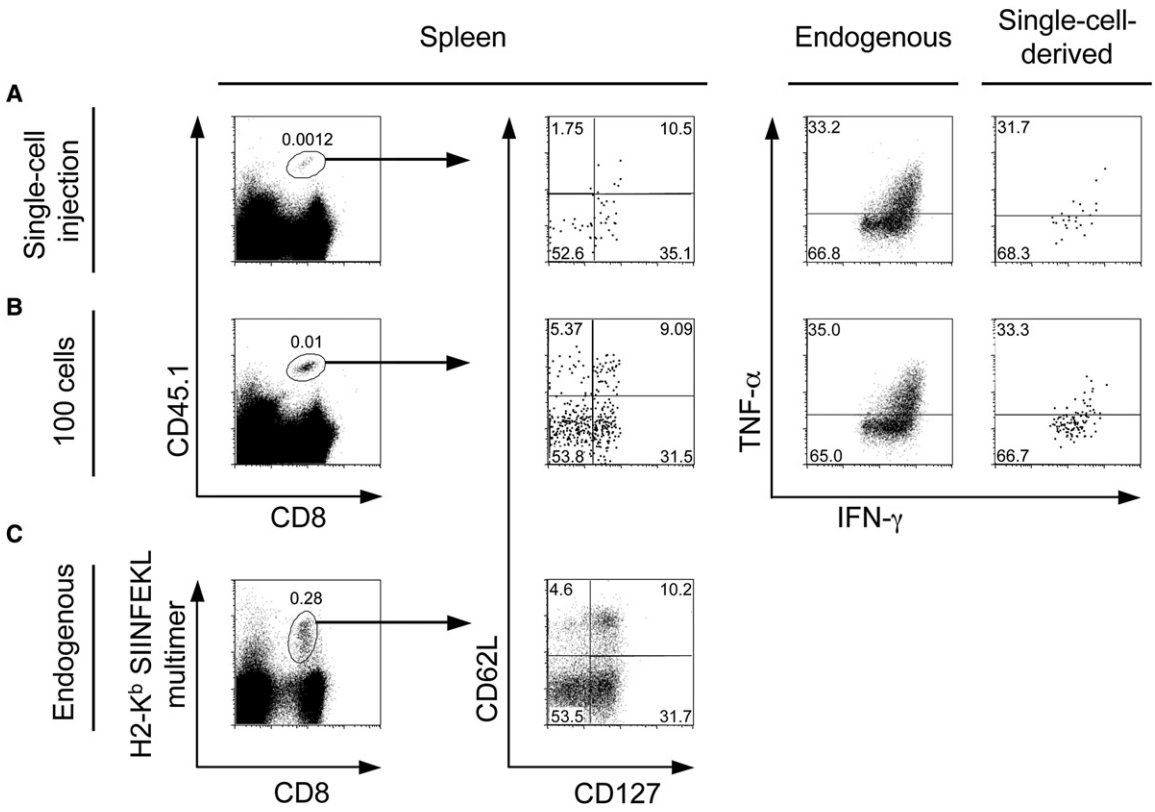


Figure 7. Functional Diversity of Daughter Cells from Latecomer Precursor Cells

C57BL/6 mice (CD45.2$^+$) were infected with *L.m.-Ova* (0.1 × LD_{50}) and received naive CD45.1$^+$ CD8$^+$ OT-I T cells 48 hr after infection. At day 12 after infection, single-cell-derived and endogenous Ova-reactive CD8$^+$ T cells were analyzed by flow cytometry.

(A and B) Transfer of a single naive CD45.1$^+$ CD8$^+$ OT-I T cell (A) or 100 naive OT-I cells (B) by intraperitoneal microinjection. OT-I cells were identified by staining for CD45.1 (y axis) and CD8 (x axis); percentages among viable cells are indicated for the shown gates. CD45.1$^+$ CD8$^+$ T cells were further analyzed for CD62L (y axis) and CD127 (x axis) expression. The two dot plots to the right show IFN-γ (x axis) versus TNF-α (y axis) expression patterns of IFN-γ$^+$ CD45.1$^+$ OT-I cells (right) compared to IFN-γ$^+$ CD45.1$^-$ endogenous SIINFEKL-responsive cells (left).

(C) Analyses of endogenous SIINFEKL-specific T cells in the spleen (shown data correspond to the same individual mouse as in [A]) identified by MHC multimer (Streptamer) staining 12 days after infection with *L.m.-Ova* (0.1 × LD_{50}); identification of the cell populations is indicated in the left dot plot (gated on CD45.1$^-$ cells).

priming of individual T cells might have been expected, but it was demonstrated only recently that identical TCR sequences are indeed present within different subset compartments, such as T_{CM} and T_{EM} (Bouneaud et al., 2005). However, the demonstration of intraclonal diversity among T cell subsets does not answer the question of when during an immune response (before or during priming, later in the expansion phase) this diversification is really achieved. During thymic selection, cell proliferation might already increase the number of cells derived from individual clones bearing the same TCR, and this number can be further amplified by homeostatic proliferation (Goldrath et al., 2000). Homeostatic proliferation has been studied in detail, and it has been shown that some degree of diversification, including the acquisition of effector functions and the development of phenotypes characteristic of memory T cells, can occur in the absence of antigen encounter (Goldrath et al., 2000; Hamilton et al., 2006; Murali-Krishna and Ahmed, 2000). These findings indicate that the "naive" T cell pool can already be diversified intraclonally, and perhaps this predetermined diversity has dictating consequences for the differentiation patterns observed during T cell responses. Also, numeric differences of clonotypic precursor frequencies have been suggested to dictate T cell subset diversification (Badovinac et al., 2007; Marzo et al., 2005). Therefore, we decided to develop an experimental system in which (multicell) diversity before the immune response is excluded; this was achieved by adoptive transfer of single naive T cells and subsequent (immediate) challenge with cognate antigen. The resulting data clearly demonstrate that all major subsets, from effector cells to different types of memory T cells, can develop from a single precursor T cell, making a major impact of predetermined diversity for T cell subset generation highly unlikely. In particular, concerns regarding potential shaping of the transferred single cell by homeostatic proliferation prior to priming—although difficult to fully exclude experimentally—are unlikely for the following reasons. First, single T cells were transferred into normal recipient mice, a situation where homeostatic proliferation has reported to be very slow

upon adoptive transfer (Hataye et al., 2006). We even looked experimentally for potential homeostatic cell division upon i.p. injection of naive OT-I cells by CSFE labeling. Whereas OT-I cells migrated very efficiently to the spleen within the first 24 hr upon transfer without changing their naive phenotype, we found no evidence for homeostatic cell proliferation during the first 48 hr upon transfer. Obviously, homeostatic proliferation is (if at all) an extremely rare event during the critical phase (before priming) in our experimental model. Second, immunization was performed together with the cell transfer in order to minimize the time gap between transfer and T cell priming (in the *Listeria* infection model, compelling data demonstrate that most of the priming occurs during the first 24 hr upon infection) (Mercado et al., 2000; Prlic et al., 2006). Third, if homeostatic proliferation of the single transferred T cell prior to priming would have played a crucial role for T cell subset diversification, diversification should have been observed only in a few cases or at least with a high variability. We found exactly the opposite. Finally, perhaps the strongest argument against an involvement of homeostatic proliferation prior to priming for diversification is the fact that single-cell-derived daughter cells diversify almost identical to endogenous T cells. Naive precursor T cells should have had much more time to get potentially shaped by homeostatic proliferation, but this still doesn't result in detectable differences (or increased variability) as compared to single-cell-derived T cells.

If pre-existing factors within the naive repertoire can be excluded as a major contributor to subset diversification, what about the initial priming phase (before the first cell division). It has been discussed extensively that it is unlikely that individual naive T cells will receive identical initial activation signals during in vivo T cell responses. This might be due to differences in the TCR affinity for different clonotypes within a polyclonal repertoire of naive epitope-specific T cells, to variability in the quality and/or kinetics of antigen-presentation or expression of costimulatory molecules by antigen-presenting cells, or to primary antigen encounter of individual naive T cells in different lymphoid organs or subcompartments. Also, the time point of recruitment into the immune response could be an important factor, as indicated by the fact that it was found that latecomers might more likely differentiate toward a central memory T cell phenotype. In summary, the physiological variability in the initial signal strength during priming of individual naive T cells has been suggested to be a major determinant for subset diversification (Gett et al., 2003). However, the precise characterization of single-cell-derived diversification patterns in comparison to endogenous (polyclonal) T cell responses presented in this report might challenge this model, as indicated by the fact that we find that phenotypical as well as functional diversification was very similar for single-cell-derived and endogenous populations. In all cases where we could recover progeny from single cells in mice later during the immune response, the patterns of subset diversification were almost identical (provided the mice were immunized in a similar fashion), although it is unlikely that in all cases single cells had exactly the same priming conditions. Furthermore, the differentiation patterns of endogenous polyclonal epitope-specific T cell populations mirrored the results found for single-cell-derived progeny, indicating that the general process of diversification of naive T cells recruited into an immune response is synchronized between cells, even between cells that might have been primed under different initial conditions (prior to the first cell division). This interpretation is further supported by the finding that slightly increasing the number of transferred T cells to ~100 cells did not change the differentiation pattern compared to single-cell-derived T cells or endogenous polyclonal populations. At first glance, this observation contradicts a recent report demonstrating that changes in precursor frequencies can strongly affect differentiation patterns (Marzo et al., 2005); however, a major difference in this particular study was the use of much higher numbers of T cells (up to 1×10^7) for adoptive transfer, generating an unphysiologically large repertoire of naive antigen-reactive T cells. A recent study analyzed this question in more detail and came to the conclusion that only with very low numbers of adoptively transferred T cells (10 to 50 cells), comparable T cell responses to endogenous antigen-reactive T cells can be achieved (Badovinac et al., 2007). Even when single naive T cells were adoptively transferred slightly later (48 hr) after the onset of infection, this did not obviously affect the general phenotypical and functional differentiation pattern of daughter cell populations. A recent report has suggested a different outcome when adoptively transferring $CD8^+$ TCRtg T cells 2 days after VSV infection (D'Souza and Hedrick, 2006). Here, latecomer T cells were characterized by a larger subset of $CD62L^{high}$ cells on day 6–9 after infection, which was interpreted as a differentiation shift toward central memory T cells. We performed similar experiments in the *Listeria* infection model transferring a single cell 48 hr after infection, and no evidence for changes on the amount of CD62L surface expression as compared to day 0 transferred cells could be detected. Perhaps even more important, under latecomer conditions, the differentiation patterns (e.g., CD62L versus CD127) were again found to be very similar to endogenous Ova-specific $CD8^+$ T cells in the same individual mice, supporting the interpretation that exposure to environmental factors during the expansion phase shape (and therefore synchronize) the subset heterogeneity. Although our data might not be entirely comparable to the study of D'Souza and Hedrick (2006), who used a different infection model and adoptive transfer strategy (1×10^6 TCRtg cells/mouse), it is more likely that the previously reported shift of latecomer cells toward a T_{CM}-like phenotype was linked to the use of unphysiologically high numbers of adoptively transferred TCRtg T cells, as has been demonstrated recently by other groups (Marzo et al., 2005; Badovinac et al., 2007).

Synchrony of the kinetics of antigen-specific T cell responses with different specificities in individual mice has been reported previously (Busch et al., 1998), and this phenomenon has been linked to the finding that a short initial "priming" phase (<24 hr) is already sufficient to initiate

a subsequently antigen-independent differentiation program, comprising all features of clonal expansion and contraction as well as memory T cell generation. Although these data clearly demonstrate that major features of T cell kinetics and their differentiation are initiated during the early priming phase and are independent from further antigen encounter, the synchronized differentiation patterns observed for progeny from "single-cell-," "multi-cell-," and endogenous polyclonal epitope-specific T cells suggest that additional factors during the expansion phase (beyond the first cell division) shape the profile of T cell subsets. Because we confirm previous reports (now on a single-cell resolution) that subset diversification for T cells with the same epitope specificity can be qualitatively different depending on the immunization procedure (Bachmann et al., 2006; Badovinac et al., 2005; Huster et al., 2006a), it seems unlikely that subset diversification is solely due to initial programming and an intrinsic plasticity inherent within clonal precursors. Our data favor a model where exposure to external environmental factors during the expansion phase shape (and therefore synchronize) the subset heterogeneity developing during immune responses. This could be envisioned to occur by "active" differentiation factors, which promote the progression of individual daughter cells to acquire distinct functional or phenotypical properties, or by "passive" factors that primarily affect the survival of (intrinsically?) generated subsets. The identification of the main factors determining the overall differentiation patterns during the expansion phase will be an important task for future research, because they are likely to provide novel strategies for the improvement of T cell-based vaccines or to redirect nonprotective T cell responses in vivo.

EXPERIMENTAL PROCEDURES

Mice and Infection

C57BL/6 (CD45.2$^+$) mice were obtained from H. Winkelmann. $OVA_{257–264}$ peptide-specific T cell receptor transgenic C57BL/6 OT-I (CD45.1$^+$) mice were derived from in-house breeding. All mice were bred under specific pathogen-free conditions at the mouse facility at the Technical University Munich, and sex-matched mice were used at 6–8 weeks for transfer and infection experiments.

Infection experiments were performed by intravenous (i.v.) injection of recombinant ovalbumin-expressing *Listeria monocytogenes* (*L.m.-Ova,* kindly provided by H. Shen, Philadelphia, PA) (Pope et al., 2001). For primary infection, mice were infected with 0.1 × LD_{50} (5000 bacteria). Rechallenge experiments were performed after 5 weeks with a second injection of *L.m.-Ova* (5 × LD_{50}).

Subcutaneous vaccination was performed by injecting 100 μl physiological sodium chloride solution containing 100 μg chicken ovalbumin (OVA, Sigma Aldrich) and 10 nM phosphorothioate-modified CpG-DNA oligonucleotide (1826, 5′-TCCATGACGTTCCTGACGTT-3′, Coley).

Animal experiments were approved by the local authorities (209.1/211-2531-100/04).

Cell Sorting and Adoptive Transfer of T Cells

For adoptive transfer experiments, naive CD8$^+$ cells (CD44^{low}) from the spleen of CD45.1$^+$OT-I mice were highly enriched by FACS sorting (MoFlo XDP, Dako). For FACS enrichment, splenocytes were stained with anti-CD3-APC (145-2C11, BD Bioscience), anti-CD8-PE (5H10; Caltag), and anti-CD44-FITC (IM7, BD Bioscience), and CD3$^+$CD8$^+$ CD44^{low} cells were sorted into pure FCS. Live/dead discrimination was performed with propidium iodide (Molecular Probes). The purity of the sorted cells was controlled on a CyAn ADP 9 color (Dako) and found to be >99%.

For titration experiments with adoptive transfer of enriched T cells, C57BL/6 mice (CD45.2$^+$) received varying numbers (10–10,000) of naive CD45.1$^+$ OT-I CD8$^+$ T cells by either i.v. or i.p. application. The given cell numbers were adjusted to 200 μl injection volume, and the true cell count was confirmed by recounting aliquots of injection solutions (accuracy ±10%). Immediately after transfer, recipient mice were infected with *L.m.-Ova*.

For the transfer of single cells, enriched naive CD45.1$^+$ OT-I CD8$^+$ T cells were diluted to an approximate concentration of 20 cells per 20 μl, and a 20 μl drop was applied onto a glass slide. Under microscopic control (Axiovert S100, Zeiss), a single cell was aspirated into the tip of a glass microinjection needle, and the presence of only a single cell was further confirmed by extensive microscopy of the needle content. With the same needle, the cell was immediately transferred into the peritoneal cavity of a recipient mouse by flushing the needle with 200 μl of a physiological sodium chloride solution. Transferred cells were analyzed after either 7 or 12 days or 5 weeks after primary infection or 5 days after recall infection.

FACS and Intracellular Cytokine Staining

After either 7 or 12 days or 5 weeks after primary infection or 5 days after secondary infection, lymphocytes were isolated from the spleen, lymph nodes, lung, and liver as described previously (Schiemann et al., 2003). For FACS analysis, cells were incubated with ethidium monazide (EMA) and anti-CD16/32 (Fc-block 2.4G2, BD Biosciences) followed by staining with a combination of anti-CD8 (5H10; Caltag), anti-CD45.1 (A20, BD Biosciences), anti-CD62L (MEL-14, BD Biosciences), anti-CD127 (A7R34, eBioscience), and H2-K^b/SIINFEKL multimers (Streptamers; IBA, Germany) (Knabel et al., 2002; Schiemann et al., 2003) for 45 min at 4°C. Data were collected by flow cytometry on a CyAn ADP 9 color (Dako) by up to 70 Mega event acquisition (via Summit V.4.3.1, Dako) and analyzed with FlowJo software (TreeStar).

For intracellular cytokine staining, splenocytes were restimulated for 6 hr in vitro in the presence of 10^{-6} M $OVA_{257–264}$ or with DMSO (Sigma) as a control in the presence of Brefeldin A (Golgi Plug, BD Biosciences). Cells were then incubated with EMA and Fc-block, followed by surface staining (anti-CD8 and anti-CD45.1), fixation, and permeabilization as recommended by the manufacturer (BD Biosciences). Intracellular staining with anti-IFN-γ (XMG1.2, eBioscience), anti-IL-2 (JES6-5H4, eBioscience), and anti-TNF-α (MP6-XT22, BD Biosciences) was done at 4°C for 30 min.

Degranulation analysis (with anti-CD107a added at the beginning of restimulation; clone 1D4B, BD Biosciences) was performed as described for the intracellular cytokine staining with additional blocking of endosome acidification with Monensin (Golgi-Stop, BD Biosciences). Data were acquired and analyzed as mentioned above.

Supplemental Data

Seven figures are available at http://www.immunity.com/cgi/content/full/27/6/985/DC1/.

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