Review

Origin of CD8$^+$ Effector and Memory T Cell Subsets

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It is well accepted that CD8$^+$ T cells play a pivotal role in providing protection against infection with intracellular pathogens and some tumors. In many cases protective immunity is maintained for long periods of time (immunological memory). Over the past years, it has become evident that in order to fulfill these multiple tasks, distinct subsets of effector and memory T cells have to be generated. Until today, however, little is known about the underlying mechanisms of subset differentiation and the timing of lineage fate decisions. In this context, it is of special importance to determine at which level of clonal expansion functional and phenotypical heterogeneity is achieved. Different models for T cell subset diversification have been proposed; these differ mainly in the time point during priming and clonal expansion (prior, during, or beyond the first cell division) when differentiation programs are induced. Recently developed single-cell adoptive transfer technology has allowed us to demonstrate that individual precursor cell still bears the full plasticity to develop into a plethora different T cell subsets. This observation targets the shaping of T cell subset differentiation towards factors that are still operative beyond the first cell division. These findings have important implications for vaccine development, as the modulation of differentiation patterns towards distinct subsets could become a powerful strategy to enhance the efficacy and quality of vaccines. *Cellular & Molecular Immunology*. 2007;4(6):399-405.

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CD8$^+$ memory T cells

Naïve CD8$^+$ T cells constitutively migrate through lymphoid tissues, where they eventually encounter their cognate antigen (e.g., a processed pathogen-derived peptide) together with appropriate co-stimulation, both delivered by activated specialized antigen-presenting cells (APCs). This crucial event of T cell priming not only initiates clonal expansion, through which the overall population size of antigen-reactive T cells gets enormously enhanced during the effector phase, it also results in the generation of T cells with distinct phenotypical characteristics that correlate with functionally discrete T cell subsets (1). Shortly after expanded antigen-reactive T cells reach a maximal frequency during the effector phase, which quite constantly occurs about 7-10 days after initial antigen challenge (2), the overall population collapses, with frequencies decreasing dramatically. This contraction phase is mediated by events inducing programmed cell death and is not directly linked to the clearance of antigen (3). If an infection is entirely cleared during the primary effector phase, a small fraction of long-lived memory cells can persist in the absence of further antigen encounter for long periods of time (4). Memory T cells have generally been described as a population of specialized T cells that - in contrast to naïve T cells - can (re-)activate specific effector functions extremely quickly (eventually within minutes) and from which an enlarged recall effector cell population can rapidly be expanded. For long-term maintenance of memory T cells in the absence of antigen, weak homeostatic proliferation seems to keep the overall frequency of memory T cells with defined specificities remarkably constant (5-7). Maintenance of antigen-reactive T cell populations is very different when antigen persists beyond the initial effector phase, e.g., during chronic viral infection. Although antigen-reactive CD8$^+$ T cells exhibit similar kinetics of expansion and contraction during the effector phase, the subsequent fate of this population is mainly dependent on the consequences of frequent antigen re-encounter (8). T cell populations maintained long-term during chronic viral infections are characterized by very high turnover rates, and their phenotypical and functional characteristics are strongly affected by frequent antigen-mediated activation (8); these

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cell populations are very distinct from "true" memory T cells, which are maintained in the absence of antigen restimulation.

The concept of "job sharing" by multiple effector and memory T cell subsets

It is well accepted that effector and memory T cells display diversity with respect to their effector functions, homing potential, and proliferative capacity (9, 10). Short-living effector T cells (T_{EC}) dominate the expansion phase, migrate to peripheral organs, and display immediate effector function. Some T_{EC} respond with a very restricted functional repertoire, while others wield multiple effector functions in parallel, such as production of IFN-γ, TNF-α, and/or IL-2. The latter T_{EC} have been termed "polyfunctional" or "pluripotent" T cell subpopulations and might be of special importance for T cell-mediated protection against a variety of infectious diseases, including HIV (9). Long-living memory cells can largely be assigned to two classes: effector memory T cells (T_{EM}) preferentially home to peripheral tissues and respond to antigen encounter with immediate effector functions but poor numeric expansion (11, 12), while central memory T cells (T_{CM}) home to lymphoid organs and can vigorously expand upon antigen reencounter. Most studies have characterized T_{CM} as cell populations producing large quantities of IL-2 (11, 12). Acquisition of full effector functions by T_{EM} and T_{CM} requires further differentiation signals (11, 13).

The functional diversity of $CD8^+$ T cells complicates their analysis, and much effort has been made to correlate functional properties with phenotypical appearance. Several markers have been identified, including the lymph node homing receptor CD62L, the α chain of the IL-7 receptor (CD127), and CD27, a member of the tumor necrosis factor (TNF) receptor superfamily. Combination of CD127/CD27 and CD62L allows discrimination between $CD8^+$ subsets that correlate with the functional properties reported for T_{CM} ($CD127/CD27^{high}CD62L^{high}$), T_{EM} ($CD127/CD27^{high}CD62L^{low}$), and T_{EC} ($CD127/CD27^{low}CD62L^{low}$) cells in mice as well as humans (14, 15).

In line with their distinct phenotypical and functional characteristics, the three major subsets (T_{EC}, T_{EM}, and T_{CM}) also display differences in their ability to respond to infection and provide protection. T_{EC} are the principal mediators of protection early during primary infection, whereas T_{CM} exhibit significant proliferative potential upon re-infection and are therefore seen by several investigators as the crucial population conferring long-lasting protective immunity against infection (12). However, proliferative capacity does not necessarily correlate with protection: for example, the specific induction of $CD62L^{high}$ T_{CM}-like cells (after immunization with heat-killed *Listeria monocytogenes*) results in vigorous proliferation and expansion of the cells after challenge with live *Listeria*, but they are unable to protect against infection as determined by clearance of the bacteria (16). Obviously, for rapidly replicating intracellular bacteria such as *Listeria*, T_{CM} are not capable of conferring effective protection. Interestingly, a recent study demonstrated that antigen-specific T_{CM} can confer protection against infection with lymphocytic choriomeningitis virus (LCMV) but not against vaccinia virus; T_{EM}, on the other hand, protected against both types of viruses. It is noteworthy that LCMV is a pathogen that replicates rather slowly in lymphoid organs, a behavior that most likely gives T_{CM} the time and a suitable environment to develop into effector cells. In contrast, vaccinia virus replicates rapidly in peripheral organs (ovaries); in this situation, protection relies mostly on immediate effector functions, and the functional transition from T_{CM} into protective T_{EM} or T_{EC} cannot occur quickly enough or is not possible at all outside lymphatic tissues. Thus, immediate protection against rapidly replicating peripheral pathogens seems to critically depend on the presence of sufficient numbers of long-living effector cells, at best residing at the specific entry site of the pathogen (12, 13, 17); protection against slowly replicating pathogens, however, can also be fulfilled by T_{CM}.

Differentiation pathways from naïve to effector and memory T cells

Over the past years, much effort has been applied to understand the differentiation pathway from naïve to memory $CD8^+$ T cells (1). The identification of the time point of memory induction is especially important for the identification of memory-determining conditions or factors, which could become interesting targets for vaccination strategies. Activation and expansion of $CD8^+$ T cells can be initiated by brief exposure to antigen: *in vitro*, the presence of antigen for as little as 2 hours is already sufficient to induce proliferation, and 24 hours of antigen exposure *in vivo* is sufficient to induce development of effector functions and memory characteristics on the bulk level of $CD8^+$ T cells (18-20). The initial antigen encounter leading to T cell priming, which is most probably represented by the physical interaction of a naïve antigen-reactive T cell with a mature antigen-presenting dendritic cell, obviously represents a crucial event for subsequent T cell differentiation.

However, a fundamental question remains: when during or after the priming phase is the lineage fate decision that distinguishes short-living effector T cells (and their subsets) and long-living memory T cells (and their subsets) really made? Two contradictory models have been proposed. According to the "linear differentiation model", memory T cells develop out of naïve cells in a continuum from T_{EC} over T_{EM} to T_{CM} (21), which would place the time point of memory T cell commitment most likely at the contraction phase. According to the "progressive differentiation model", differentiation depends on applied signal strength early in the immune response; during the priming period, naïve antigen-reactive $CD8^+$ T cells do not receive stimuli of identical "strength", a term subsuming a composition of TCR-MHC/peptide affinity, antigen concentration, access to co-stimulatory molecules, and cytokines. As a consequence, the cells reach different activation stages, defining their

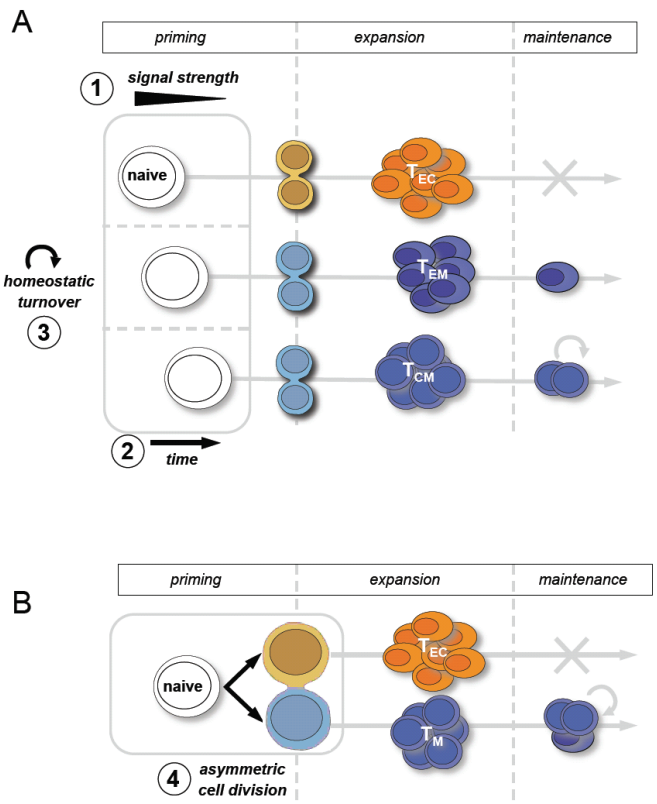

Figure 1. Models of T cell subset diversification. (A) Naïve antigen-specific T cells take a unidirectional lineage fate decision before the first antigen-driven cell division (grey box indicates time frame for lineage fate decision). Daughter cells are terminally tied to this decision yielding a homogenous phenotype of the ensuing progeny. ① "Signal strength model": Depending on the combined intensity of antigen-dependent, co-stimulatory and cytokine-driven signals, acting on a naïve T cell, a specific effector phenotype is imprinted onto this cell's progeny – with strong signals turning differentiation towards short-living effector T cells (T_{EC}) and weak signals imprinting effector memory T cell (T_{EM}) or central memory T cell (T_{CM}) fates (cyclic arrow indicates potential of homeostatic proliferation). ② "Latecomer model": During the course of an immune response the availability of stimulatory signals rises, peaks and declines. Naïve T cells joining late into an ongoing response are exposed to mainly weak signals and thus tend towards acquisition of T_{CM} memory fate. ③ "Homeostatic proliferation model": Lineage fate decisions might take place stochastically during homeostatic proliferation of naïve T cells gradually creating phenotypic diversity within a clonotypic T cell population. Antigen and inflammatory signals subsequently select individual predetermined T cells for expansion. (B) Naïve antigen-specific T cells take a bi-directional lineage fate decision during the first cell division. ④ "Asymmetric cell division model": The first antigen-induced cell division of naïve T cells leads to an uneven distribution of cellular components onto the first two daughter cells. The daughter proximal to the APC-T cell interphase becomes precursor for effector cells, whereas the distal daughter gives rise to memory T cells.

phenotype and fate (22, 23). This model would postulate that "true" memory T cells might already be detectable early during an immune response. First evidence for the early presence of memory cells during the initial priming period came from an activation-dependent transgenic reporter gene expression model in which a small number of reporter-positive T cells were present at the peak of the immune response and persisted over time as memory cells (24). In accordance with these findings, stable numbers of memory cells were detected starting on day 4 after infection with *Listeria monocytogenes* or LCMV when using CD127 as a memory marker (14, 15). Functional evidence for the early presence of memory cells also came first from experiments in the *Listeria* infection model: re-infection as early as 5 days after primary infection resulted in typical memory expansion of antigen-specific T cells. These cells were as efficient in providing protection against subsequent challenge with *Listeria* as memory cells generated late after primary infection (25, 26). Although these data clearly indicate that T cell subsets with typical characteristics of memory T cells are present early during the priming phase, one can debate - a matter of terminology - whether a recently activated T cell should be called a memory T cell. As a solution to this "problem", the term memory precursor cells (MPCs), as opposed to short-lived effector cells (SLECs), has been proposed for cell subpopulations at early stages of an immune response that are maintained long-term (27, 28).

Several groups have provided evidence that all memory $CD8^+$ T cells first transit through a stage during which they acquire at least some effector functions (such as cytokine production or cytotoxicity) before they further subdivide into T_{CM} and T_{EM}; this was also expressed by changing the terminology from MPCs to MPECs (memory precursor effector cells) (27-30). At first glance, the concept of MPECs challenges the "progressive differentiation model", which postulates that T_{CM} originate from weakly differentiated T cells. However, it has been documented by several groups that a substantial proportion of memory T cells expressing markers associated with the T_{CM} subset can exert some immediate effector functions (such as production of IFN-γ) (31), which may indicate that there is still a lack of appropriate markers to distinguish "true" T_{CM} from T cells that have acquired some effector functions or that the pool of T_{CM} itself contains more heterogeneity than commonly believed. Despite the difficulties in identifying proper lineage markers, it could recently be demonstrated using extensive adoptive transfer studies that MPCs with a T_{EM}-associated phenotype ($CD127^{high}/CD62L^{low}$) cannot (re-)convert into T cells with phenotypical or functional characteristics of T_{CM} *in vivo* (13). In contrast, MPCs with a T_{CM}-associated phenotype ($CD127^{high}/CD62L^{high}$) were capable of efficiently differentiating into T_{EM} upon antigen restimulation (13). These data indicate that it is very unlikely that after acquisition of effector functions, T cells can revert into cell populations with high proliferative capacity upon antigen re-challenge (a hallmark of the T_{CM} subset) and the potential for self-renewal by homeostatic proliferation.

Origin of T cell subset heterogeneity

In order to shed light on the mechanisms of T cell subset

diversification and the time points of true lineage fate decisions, it is necessary to understand the clonal level at which subset heterogeneity is achieved (32, 33). According to the progressive differentiation model (11) and the autopilot theory (34), the signal strength during the initial priming of naïve T cells, before the first cell division, induces a differentiation program determining the extent of differentiation of subsequent daughter cells (23). A "strong" integrated priming signal, which could substantially differ between individual naïve T cells (e.g., affected by differences in TCR-MHC/peptide affinity), would lead to a differentiation pattern shifted towards effector cells, while a weaker signal would result in less differentiated daughter cells, such as T_{CM} (Figure 1A-①). According to this model, the differentiation fate of a single naïve T cell results from genetic imprinting of a developmental program during the priming phase, which guarantees the transfer of similar characteristics to subsequent daughter cells; whether all daughter cells derived from a single naïve precursor T cell differentiate exactly to the same extent ("one cell - one subset model") or the offspring are characterized by a limited range of T cell subsets is unknown. 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 naïve precursor cells (35). 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 naïve T cells bearing the same TCR (36). However, none of these observations can discriminate between origin from a single precursor cell or a small pool of clonotypic naïve T cells (33), making it very difficult to prove or disprove the proposed models.

Related to the progressive differentiation model, it has been proposed that T_{CM} preferentially develop from naïve precursor cells recruited at later stages into the immune response ("latecomers", Figure 1A-②) (37, 38). Such latecomer cells might receive a qualitatively weaker priming signal compared to naïve T cells recruited substantially earlier into the immune response, resulting in a differentiation shift of daughter cells towards T_{CM} subsets. Some groups might even favor a scenario in which the entire T_{CM} compartment is derived from the last naïve T cells participating in an immune response (38). Unfortunately, the studies supporting the "latecomer" origin of T_{CM} subsets have been performed by adoptive transfer using high numbers of naïve TCR-transgenic T cells (1×10^4-1×10^6). Physiological precursor frequencies of naïve epitope-specific CD8$^+$ T cells have been estimated to be in the range of 50-200 cells per individual for different epitope specificities, and recent studies have demonstrated that increasing the precursor frequency to "unphysiological numbers" by adoptive transfer (a number as low as 500 cells already has to be regarded as unphysiological (39)) results in altered differentiation patterns, with a substantial shift towards T_{CM} phenotypes (39, 40).

As an alternative to the models describing T cell subset differentiation at the level of the priming phase, differentiation capabilities could already be predetermined to some extent within the repertoire of naïve precursor cells (Figure 1A-③). Together with numeric differences of naïve precursor cells, some degree of phenotypical and functional diversification could pre-form within clonotypic (bearing the same T cell receptor) precursor populations, as has been described for homeostatic cell proliferation of naïve T cells (41-43). In fact, homeostatic proliferation can lead, in a completely antigen-independent manner, to subsequent daughter cell populations with phenotypical and functional characteristics indistinguishable from conventional effector and memory T cells. Therefore, it is difficult to exclude that the subset heterogeneity upon clonal expansion is - at least in part - a reflection of diversity already present at the precursor level.

A very recent study indicated that segregation into the two major T cell subsets, short-living effector cells and long-living memory T cells, might occur with the first cell division (44) (Figure 1B-④). This interpretation is based on the finding that the first antigen-induced cell division of naïve T cells is asymmetric, leading to an uneven distribution of cellular components in the first two daughter cells. Although not conclusively proven, it was suggested that daughter cells located proximal to the APC-T cell interface become exclusively precursors for short-living effector cell subsets, whereas distal daughter cells give rise to memory T cell subsets. Diversification into different effector and memory subtypes has not been implemented into this model; while it is unlikely that the full spectrum of diversity can be determined on the level of a single asymmetric cell division, segregation into more diverse T cell subsets could follow the initial priming phase, promoted for example by variable additional antigen encounters or exposure to distinct environmental factors.

In vivo analysis of the fate of single naïve T cell precursors

In order to address the question of whether a single T cell can give rise to only a limited or to a broad spectrum of effector and memory T cell subsets upon clonal expansion, an experimental system allowing the fate of a single naïve precursor T cell to be tracked during an immune response had to be developed. Supported by the observation that primary infection with intracellular pathogens can lead to very strong expansion of epitope-specific CD8$^+$ T cell populations, which are believed to be derived from a relatively low number of naïve precursor cells, we postulated that the progeny of a single cell added to the naïve precursor pool (by adoptive transfer) should be detectable within the expanded population. Indeed, aided by the development of novel flow cytometry acquisition software (Summit V4.2, Dako), which allows acquisition of all lymphocytes from an entire organ (such as the spleen) within a single file, our group recently succeeded in developing a single-cell adoptive transfer model (45). We used genetically labeled (CD45.1) TCR-transgenic T cells specific for Ovalbumin (OT-I) and

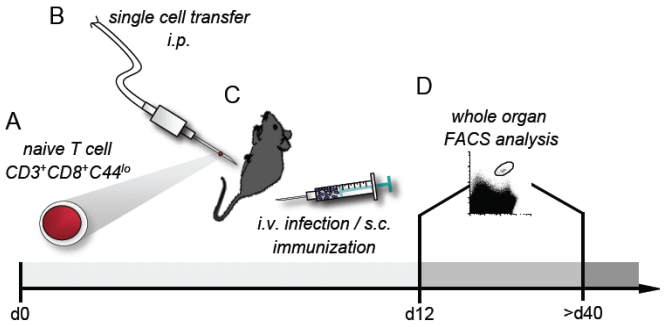

Figure 2. Principles of the single cell microinjection method. (A) Enrichment of naïve ($CD3^+CD8^+CD44^{lo}PI^{neg}$) OT-I TCR transgenic splenocytes by FACS. (B) Subsequently, the enriched cells were further diluted and applied onto a glass slide. Single cells were then picked under microscopic control by aspiration into a glass microinjection needle, and immediately transferred into the peritoneal cavity of a recipient mouse. (C) Shortly after T cell transfer, recipient mice were either infected with *L.m.*-Ova or immunized with cognate protein and adjuvant respectively. (D) FACS analysis of whole organs from single cell injected mice was performed either during the late effector or in the memory phase of infection/immunization.

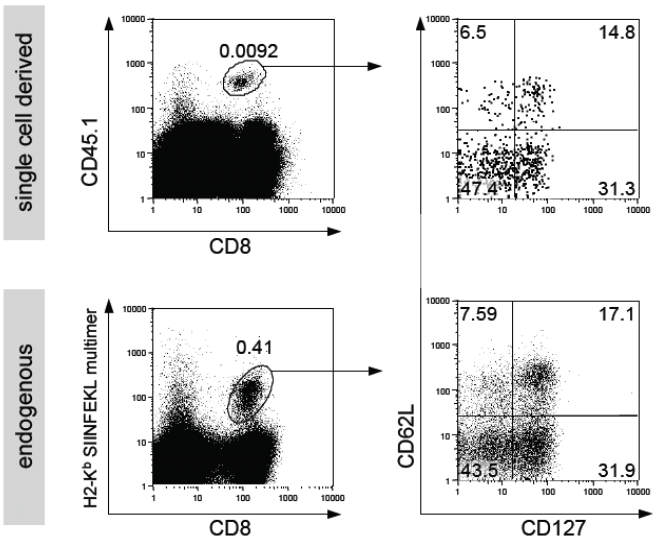

Figure 3. Phenotypical diversity of daughter cells from a single naïve T cell precursor. A C57BL/6 mouse ($CD45.2^+$) received a single naïve $CD45.1^+/CD8^+$ OT-I T cell by intraperitoneal microinjection and was infected with *L.m.*-Ova immediately after transfer. Twelve days later, the whole spleen was analyzed by flow-cytometry for the presence of the single-cell derived progeny population (stained for $CD45.1^+CD8^+$, upper row) or the endogenous SIINFEKL-specific endogenous population identified by MHC multimer staining (MHC Streptamers; lower row) in the same mouse. The dot plots on the right show the CD62L and CD127 expression patterns of the single cell derived and the endogenous population (parental gate is shown).

sorted them for truly naïve (CD44-negative) T cells (Figure 2). In order to ensure that only a single T cell was adoptively transferred, individual cells were aspirated under microscopic control into a sharp glass needle and subsequently flushed into the peritoneum of CD45.2-positive C57BL/6 recipient mice. Kinetic studies indicated that naïve T cells applied *via* the intraperitoneal route migrate within a few hours into lymphoid organs, especially into the spleen. Immediately after adoptive transfer of single naïve T cells, recipient mice were infected with *Listeria monocytogenes* expressing Ovalbumin (*L.m.*-Ova), and clonally expanded SIINFEKL-specific T cell populations were analyzed as early as 1 week after primary infection using H2-K^b/SIINFEKL multimers (MHC streptamers (46)) or intracellular cytokine staining. Co-staining for CD45.1 surface expression allowed discrimination between single cell-derived (CD45.1-positive) and endogenous (CD45.1-negative) SIINFEKL-specific T cell populations (Figure 3A).

Stem cell-like plasticity of naïve precursor cells and subset shaping during expansion

As shown in Figure 3A, using this novel single-cell transfer model, we were able to recover SIINFEKL-specific T cell populations derived from single naïve precursor cells following antigen challenge. This allowed us to determine for the first time the differentiation capabilities of a single naïve T cell into effector and memory T cell subsets, and the results were surprisingly clear-cut (45). In all cases in which we were able to recover single cell-derived cells (~25% of recipient mice), the cell populations contained a broad spectrum of effector T cell subsets with variable effector functions (Figure 3A). In addition, we could demonstrate that together with a diverse pool of short-living effector cells, daughter cells derived from the same naïve precursor cell contained both major subsets of memory T cells, T_{CM} and T_{EM}. These findings conclusively demonstrate that the "one cell - multiple subsets" model is sufficient to explain the origin of subset diversification, at least for $CD8^+$ T cells, and demonstrate the enormous, stem cell-like plasticity of naïve T cells. These findings also exclude the possibility that factors operative before the initial priming phase, such as homeostatic proliferation, have major relevance in determining subset differentiation patterns. Surprisingly, by comparing single cell-derived T cell populations with endogenous (polyclonal) T cell populations recognizing the same epitope in the same individual mice, we discovered that the functional and phenotypical differentiation patterns of these two populations were virtually identical (45). This "synchrony" of differentiation was unexpected, since it is unlikely that the initial priming conditions for the single adoptively transferred T cell are identical to the priming conditions for each naïve precursor from the polyclonal endogenous population. In fact, this synchrony of T cell differentiation patterns is not in line with a major importance of (imprinting) factors prior to the first cell division for subsequent subset diversification (Figure 1A). Even when "latecomer cells" were generated by adoptive transfer of

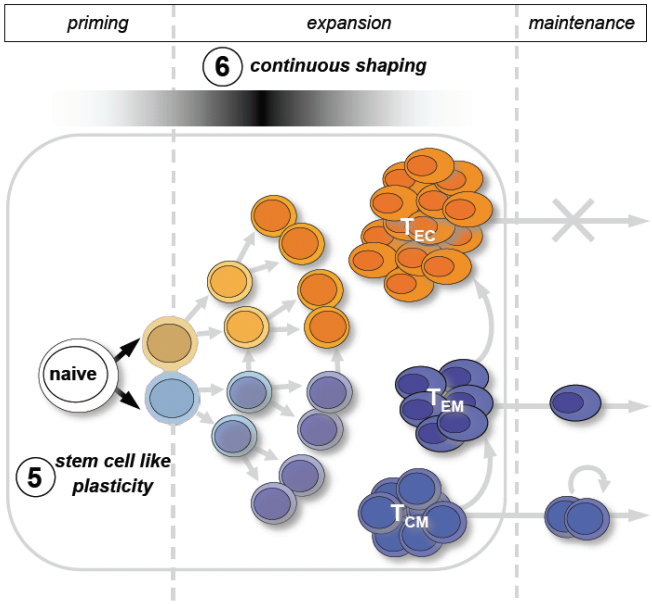

Figure 4. Memory differentiation of a single naïve CD8 T cell. After antigen-specific stimulation *in vivo*, a single naïve CD8 T cell develops into all major T cell subsets (T_E, T_{EM} and T_{CM}) and finally survives as long-living memory T cells (⑤ stem cell like plasticity). Furthermore, the single cell-derived progeny is found to be synchronized with the endogenous antigen-specific polyclonal T cell response. This may be explained by an ongoing differentiation capacity of proliferating T cells beyond the first cell division (grey box) sequentially influenced by the immunization-dependent environment (e.g., infection or protein/adjuvant immunization). Later influences during the expansion phase reduce potential intrinsic stochastical variabilities, explaining the distinct and conserved patterns of $CD8^+$ memory T cells (⑥ continuous shaping).

single naïve T cells (48 hours) after infection, perfect synchrony with endogenous T cell populations was maintained (45). It could also be demonstrated that the synchrony of clonal T cell differentiation patterns is not limited to the *Listeria* infection model, as similar results were obtained following subcutaneous immunization with soluble antigen mixed with adjuvant (45).

Taken together, these recent findings favor a model in which 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 as a result of "active" differentiation factors that 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. A first asymmetric cell division (44) could initiate the process of diversification from a single precursor cell, but it certainly requires several additional branching steps to explain the formation of the complexity of effector and memory T cell subset diversity found for many immune responses. Whether the first cell division is decisive for the dichotomy into long-living and short-living subsets still requires further analysis, as the persistence of T cells derived from the first cell division has not been specifically addressed.

Clinical relevance for the improvement of immunotherapies

The identification of major factors determining the patterns of subset diversification during immune responses will be an important task for future research, since this knowledge is likely to provide novel strategies for the improvement of T cell-based vaccines or for redirecting non-protective T cell responses *in vivo* (12). Recent findings (as summarized above) are likely to switch the current focus on the priming phase before the first cell division to events that are still operative at later stages during immune responses. Such mechanisms might be more accessible therapeutically, for example by the application of appropriate cytokine cocktails or additional antigen preparations during the expansion phase. The enormous stem cell-like plasticity of T cell subset diversification from a single T cell might also have relevance for adoptive T cell therapies. Perhaps it is not always necessary to generate large numbers of *in vitro*-expanded effector T cells, as is currently common for adoptive immunotherapy (47). If less differentiated antigen-specific T cell subpopulations can be isolated directly from appropriate donors (e.g., by improved MHC multimer-guided sorting strategies (46)), it might be possible to expand highly potent effector and memory T cell populations from very low numbers of adoptively transferred T cells. Indeed, adoptive transfer of donor lymphocyte infusions containing low numbers of EBV- or CMV-reactive T cells has been highly effective in the treatment of virus-associated complications (48), although the side effects caused by alloreactive T cells are often severe. Purification of the protective cell populations from leukocyte samples is a promising approach to decrease the negative side effects of this therapeutic approach and to make the therapy applicable to a broader range of patients and diseases.

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