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Phenotypic CD8+ T Cell Diversification Occurs before, during, and after the First T Cell Division

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Effector T cell responses rely on a phenotypically and functionally heterogeneous population of cells. Whether this diversity is programmed before clonal expansion or in later phases as a result of stochastic events or asymmetric cell division is not fully understood. In this study, we first took advantage of a sensitive in vitro assay to analyze the composition of single CD8+ T cell progenies. Heterogeneity was predominantly observed between progenies of distinct clones, but could also be detected within individual progenies. Furthermore, by physically isolating daughter cells of the first T cell division, we showed that differences in paired daughter cell progenies contributed to intraclonal diversification. Finally, we developed an in vivo limiting dilution assay to compare individual T cell progenies following immunization. We provided evidence for simultaneous intraclonal and interclonal diversification in vivo. Our results support the idea that T cell diversification is a continuous process, initiated before clonal expansion and amplified during the first and subsequent cell divisions. The Journal of Immunology, 2013, 191: 1578–1585.

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CD4+ and CD8+ T cell effector responses are generated from the progenies of individual Ag-specific T cells that proliferate and differentiate upon Ag recognition. Effector T cell pools are typically highly heterogeneous as revealed by multiparametric flow cytometry or single-cell PCR, and comprise cells with variable phenotypes and functional properties (1–5). The recent development of cytometry by time-of-flight (6) has provided an even more dramatic illustration of this complexity, revealing the existence of >200 distinct memory CD8+ T cell phenotypes (7). Effector and memory T cell diversity may promote the efficacy and flexibility of immune responses when dealing with a wide variety of infectious agents (8).

T cell heterogeneity could result from two complementary processes. First, interclonal diversification reflects the possibility that distinct Ag-specific T cell clones adopt different fates early on (e.g., prior to the first cell division) that will be imprinted in their progenies. By imaging the activity of a fluorescent IFN-γ reporter in T cells in vivo, we recently noted extensive heterogeneity in reporter activity before the first cell division (9). Second, intraclonal diversification permits generation of varied T cell phenotypes from a single T cell precursor. A striking example was provided by a study that followed the fate of single adoptively transferred CD8+ T cells (10) and revealed the generation of both effector and central memory T cells. Similar conclusions were reached in a study that used an elegant barcoding system to track the fate of individual T cell clones (11). TCR repertoire analyses also suggested that the progeny of a single T cell could contribute to phenotypically distinct T cell subsets (12, 13). Stochastic events occurring after the first T cell division, such as Ag re-encounter (14) or exposure to a distinct cytokine milieu, may account for intraclonal diversification. In addition, it has recently been proposed that the first T cell division occurs in an asymmetric fashion (15–17), a process that could involve asymmetric proteasome segregation (18) and have the potential to generate phenotypic and functional diversity within a T cell progeny.

Although evidence for both intraclonal as well as interclonal diversification exists, there is little information regarding their respective contribution during T cell activation. In addition, the possible contribution of divergent fate after the first T cell division remains to be precisely quantified. To address these issues, we used a short-term cloning strategy that allowed us to analyze the phenotypic and functional diversity in the progeny of single T cells activated by dendritic cells (DCs). To further assess whether the first T cell division contributes to intraclonal T cell diversity, we isolated paired daughter cells and compared the composition of their progenies. Finally, we developed an adoptive T cell transfer strategy under limiting conditions to track the fate of individual T cell progenies in vivo. Our results support the idea that T cell diversification occurs before, during, and after the initial T cell division.

Materials and Methods

Mice

CD4+CD8−CD44lowCD8+ T cells were purified from lymph nodes and spleens of Rag−/− OT-I and GFP- or cyan fluorescent protein (CFP)–expressing Rag−/− OT-I TCR transgenic mice were bred in our animal facility. All mice were housed in our animal facility under specific pathogen-free conditions. Animal experiments were performed in accordance with institutional guidelines for animal care and use.

Cell purification

CD4+CD8− CD44lowCD8+ T cells were purified from lymph nodes and spleens of Rag−/− OT-I mice by negative selection using the CD8+ T cell isolation kit (Miltenyi Biotec, Paris, France) and a biotinylated anti-mouse CD44 mAb (clone IM7; eBioscience, San Diego, CA). DCs were prepared from spleens of wild-type (WT) C57BL/6 mice [either untreated or injected intraperitoneally (i.p.) with 100 μg poly(I:C) 5 h before] by positive selection using CD11c+ microbeads (Miltenyi Biotec) after collagenase digestion. Unless stated otherwise, DCs were pulsed with 10−8 M OVA257–264 peptide (SIINFEKL; Polypeptide Group, Strasbourg, France) for 10 min at room temperature and washed extensively. When indicated, the lower-affinity Q4 OVA variant peptide was used (SIIQFEKL).

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Short-term progeny analysis

Purified naive OT-I cells (2 × 10^6 cells) were activated with OVA-pulsed DCs (2 × 10^6 cells) for 24 h at 37°C in 24-well culture plates. Activated OT-I cells were then cloned by limiting dilution at 0.5 cell/well into 60-well minitray culture plates (Nunc, Rochester, NY), in 10 μl complete RPMI 1640 media containing 25 U/ml human rIL-2 (Roche, Meylan, France). After cloning, each well was examined under the microscope, and only wells containing a single T cell after cloning were further analyzed. The absence of DCs after T cell cloning was systematically confirmed by flow cytometry. The number of cells in individual T cell progenies counted under the microscope is graphed against the number of live lymphocytes detected by flow cytometry. Growth curves of individual progenies were obtained by counting the content of each well under the microscope every day. Representative of three independent experiments.
Adoptive T cell transfer under limiting conditions

Naive CD8+ T cells were purified from Rag2−/− OT-I (CD45.2) and Rag2−/− OT-I GFP+ (CD45.2) mice and mixed at a 1:1 ratio. The indicated number of T cells was adoptively transferred into CD45.1 recipients that were injected in the footpad the next day with 2 × 10^7 DCs pulsed with 1 × 10^{-7} M OVA257–264 peptide. Four days later, the draining and nondraining lymph nodes were harvested. The totality of lymph node cells was stained and analyzed by flow cytometry.

Flow cytometry

R-PE-conjugated anti-CD25 (clone PC61), allophycocyanin-conjugated or allophycocyanin-eFluor 780-conjugated anti-CD62L (clone Mel-14), and PE-Cy7-conjugated anti-CD8 (clone 53-6.7) were purchased from eBioscience. Brilliant violet 421–conjugated anti-CD45.2 was purchased from BioLegend (San Diego, CA). PerCP-Cy5.5–conjugated anti-CD25 (clone XMG1.2), and PE-conjugated anti–IFN-γ (clone XMG1.2), and PE-conjugated anti-Vb5.1/5.2 (clone MR9-4) were purchased from BD Biosciences (Le Pont de Clai, France). Allophycocyanin-conjugated anti-human granzyme B (clone GB11) was purchased from Invitrogen (Carlsbad, CA). T cell progenies were transferred into FACS tube containing 50 µl diluted mAb and incubated at 4°C for 20 min, before direct analysis on a FACS-Canto II cytometer (BD Biosciences). Intracellular cytokine stainings were performed using the Cytofix/Cytoperm kit (BD Biosciences) following stimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (1 µg/ml). Given the small size of our samples, we controlled for the absence of cell contamination during FACS acquisition by including samples containing FACS buffer only.

Data processing and statistical analyses

For each progeny, the percentage of intraclonal variation was determined by dividing its SD by the SD of pooled progenies. Statistical significance was assessed by comparing these values with a target value of 100% using a one-sample t test (Prism version 5.0; GraphPad Software, La Jolla, CA). We created virtual progenies (whose size matched that of the experimental data) by randomizing fluorescence values from all progenies of an entire experiment using the “randbetween” function from Excel software (Microsoft, Issy-Les-Moulineaux, France). To compare paired daughter cell progenies, mean fluorescence intensity of each progeny was compared with an unpaired t test using Prism software. Normalized SDs were obtained by dividing the SD for one parameter by the corresponding geometric mean fluorescence.

Results

Analyzing single T cell progenies by flow cytometry

To dissect the origin of T cell diversity, we used a short-term in vitro assay aimed to analyze single T cell progenies (9). In this system, naive OT-I T cells are stimulated with peptide-pulsed DCs for 24 h and then cloned at 0.5 cell/well in 10 µl microwells (Fig. 1A).
After examination under the microscope, only wells containing a single T cell were further analyzed. After additional 3 d of culture, the early progenies of a single T cell would typically comprise 30–200 cells that could be analyzed by flow cytometry (Fig. 1A, 1B). To ensure cells recovered in individual wells originated from a single T cell, we cloned a mixture of WT and CFP-expressing OT-I T cells (Fig. 1C). Progenies recovered from wells that initially contained a single T cell were uniformly CFP⁺ or CFP⁻, confirming their clonal origin (Fig. 1D). This was not the case for wells in which two or more cells were initially detected, as these wells often contained a mixture of CFP⁺ and CFP⁻ cells, as expected (Fig. 1E). These results confirmed that our approach offers the ability to analyze single T cell progenies following a single round of activation, in the absence of restimulation or prolonged culture period. We observed a good correlation between the T cell numbers counted under the microscope and the number of events recorded by flow cytometry (Fig. 1F), with a typical recovery efficacy of 50%. Interestingly, substantial diversity was detected at the level of clone size and growth curves (Fig. 1G).

Measuring interclonal and intraclonal phenotypic diversity in T cells

To analyze the phenotypic diversity within and between clonal T cell progenies, we analyzed the expression of the following two important markers: CD25, which plays a crucial role in effector T cell homing properties (22, 23). When individual progenies were compared, we observed considerable clone-to-clone variations (Fig. 2A, 2B). Of note, levels of CD25 and CD62L staining appeared independent of the total progeny size (Supplemental Fig. 1). To exclude the possibility that these differences were attributable to the small size of the cell population analyzed, we randomized the values of CD25 (and CD62L) expression compiled in paired daughter cell progenies were randomized and assigned to two virtual paired progenies. Randomized progenies no longer exhibited clone-to-clone variation (Fig. 2C). The variability in experimental progenies appeared significantly \((p < 0.05)\) lower on average than the total diversity (all clones combined), but this was not the case for virtual progenies in 10 independent randomizations (Fig. 2D). These results confirmed that the observed interclonal diversity for CD25 and CD62L expression on T cells originates from the addition of clonal progenies that tend to be less diverse than the bulk of the population and differs from one another. These results support the view that phenotypic diversification is initiated before the first cell division.

Another striking observation was that the overall diversity for each of the markers largely varied from clone to clone. As shown in Fig. 2E, some T cell clones displayed homogenous expression of both markers, whereas others were more diverse for one or both markers. Thus, some T cell clones (80 of 135, 59%) displayed extensive phenotypic diversification as they expanded.

Similar results were obtained when the analysis of T cell progenies was performed at days 3 and 6, indicating that T cell diversity is detected at multiple time points (Supplemental Fig. 2). Altogether, our results support the idea that interclonal variations as well as intraclonal diversity in a subset of T cell clones contribute to the phenotypic heterogeneity of effector T cells.

Phenotypic T cell diversification arises in a variety of stimulation conditions

To test whether the large T cell heterogeneity observed was a peculiarity of our experimental system, we repeated the experiments using DCs pulsed with different peptide doses or with the Q4 OVA variant peptide that displayed a lower affinity for the OT-I TCR. As
shown in Supplemental Fig. 3, pronounced phenotypic diversification was observed in these conditions. We also assessed the role of DC maturation by analyzing the progenies of T cells stimulated by activated DCs that were isolated from poly(I:C)-treated mice. Again, a high level of interclonal and intraclonal diversity was seen in this setting (Supplemental Fig. 4A). Likewise, phenotypic diversification was evident when single naive T cells were cloned directly into microwells coated with recombinant PMHC and anti-CD28 (Supplemental Fig. 4B). Thus, both interclonal and intraclonal diversification appear to be a general hallmark of T cell activation, largely independent of the stimulation conditions used.

**Interclonal and intraclonal diversity in T cell production of IFN-γ and granzyme B content**

Next, we extended our analysis to functional T cell markers using intracellular staining. We found that only a fraction of the T cells within a given progeny produced IFN-γ in response to restimulation (Fig. 3A). Moreover, the frequency of IFN-γ-producing cells and the level of IFN-γ production varied between distinct progenies (Fig. 3A, 3B). Thus, heterogeneity in IFN-γ production was the result of both interclonal and intraclonal variations. Granzyme B content also varied from clone to clone. Variations in IFN-γ and granzyme B were significant, as demonstrated by the lack of differences in randomized progenies (Fig. 3B, 3C). Again, the diversity in IFN-γ and granzyme B levels in an individual progeny was, on average, significantly lower than that of the total population (Fig. 3D). Of note, a subset of clones (23 of 39, 59%) showed diversified expression of at least one of the molecules examined (Fig. 3E).

**Distinct contribution of paired daughter T cells can promote intraclonal diversity**

We next assessed the origin of intraclonal T cell diversity observed in some of the progenies. Specifically, we asked whether the first cell division contributed to this diversification by comparing the fate of paired daughter cells. Subcloning of daughter or granddaughters has been used previously to compare the fate of a given T cell clone cultured under distinct cytokine milieu (24, 25). Thus, we thought to physically separate daughter T cells immediately following the first cell division and monitor their respective fate using our short-term progeny assay (Fig. 4A). For approximately half of the T cell clones, CD25 and CD62L were

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**FIGURE 5.** Intraclonal and interclonal phenotypic diversification of CD8+ T cells both operate in vivo. (A) Experimental setup. Decreasing number of naive CD45.2 GFP+ and GFP- OT-I T cells (1:1 mixture) was transferred into CD45.1 recipients that were injected in the footpad with OVA-pulsed DCs. Four days later, the totality of cells from the draining lymph node was analyzed by flow cytometry. OT-I T cells were identified as CD45.2+ CD8+ Vß5+. (B) Immunization with DCs results in expansion and phenotypic diversification of GFP+ and GFP- OT-I T cells. FACS plots show CD25 and CD62L expression on gated OT-I T cells 4 d after transfer of 1 x 10^6 naive OT-I T cells and immunization with unpulsed or OVA-pulsed DCs. (C) Adoptive T cell transfer under limiting conditions. Mice were transferred with the indicated number of cells from GFP+GFP- OT-I T cell mixture and immunized with peptide-pulsed DCs. Pie charts depict the probability that an expanded OT-I GFP+ or GFP- T cell population could be detected in the draining lymph node in various conditions of T cell transfer. (D) Number of OT-I T cells detected 4 d postimmunization in non-draining and draining lymph nodes after transfer of 1 x 10^6 OT-I T cells. (E) Percentage of GFP+ cells among OT-I T cells (CD45.2+) detected in draining lymph nodes. (F) Representative FACS plots showing the composition of the expanded OT-I T cell population for the indicated condition of T cell transfer. (G) Representative FACS profiles showing CD25 and CD62L expression on GFP+ OT-I T cells in limiting conditions of adoptive transfer (1 x 10^5 OT-I T cells). (H) Overlaid FACS profiles of GFP+ and GFP- OT-I T cells detected in the same lymph node in limiting conditions of adoptive transfer (1 x 10^5 OT-I T cells). One example of divergent (left) and one example of similar (right) pattern are shown. (I) The distribution of CD25 expression was compared between GFP+ and GFP- T cell populations shown in (H). Representative of four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
expressed at similar levels in the progeny of each daughter cell. For the remaining clones, paired daughter cells generated progenies that significantly differed in their levels of CD25 and/or CD62L (Fig. 4B–D). Virtual daughter cell progenies (generated by data randomization) were not significantly different from one another, confirming that the differences detected are not the result of sampling limitations (Fig. 4C, 4D). Our results indicate that the first cell division most likely contributes to intraclonal diversity in a fraction of T cell clones.

**Monitoring intraclonal and interclonal T cell diversity in vivo**

To test the in vivo relevance of some of our findings, we developed an in vivo assay in which the fate of a very limited number of T cells could be tracked in immunized mice. To validate this approach, we first transferred a large cohort ($1 \times 10^5$ cells) of GFP$^+$ and GFP$^-$ OT-I T cells (both expressing CD45.2 and mixed at a 1:1 ratio) in CD45.1 recipients. Mice were then injected s.c. with OVA-pulsed DCs (Fig. 5A). On day 4, GFP$^+$ and GFP$^-$ had similarly expanded in the draining lymph node (and had not yet recirculated), and GFP$^+$ and GFP$^-$ effector T cells displayed a similarly diverse pattern of CD25 and CD62L expression (Fig. 5B). We reasoned that, by progressively decreasing the number of transferred T cells, we should reach a stage at which only one or very few GFP$^+$ and GFP$^-$ T cells would be present and stimulated in the draining lymph node. Whereas we could systematically detect T cell expansion in both GFP$^+$ and GFP$^-$ negative subsets when $1 \times 10^5$ and $1 \times 10^4$ T cells were transferred, this was no longer the case with the transfer of $1 \times 10^3$ T cells, suggesting that we had reached limiting conditions (Fig. 5C). In these conditions, GFP$^+$ and GFP$^-$ T cell expansion was detected in 72% of the lymph nodes analyzed, with an average of 71 ± 10 OT-I T cells being recovered when totality of the draining lymph node was analyzed by flow cytometry (Fig. 5D). No T cell expansion could be detected when $1 \times 10^2$ cells were transferred (Fig. 5C). Whereas the ratio of GFP$^+$:GFP$^-$ OT-I T cells was close to 1 in immunized mice transferred with $1 \times 10^5$ and $1 \times 10^4$ T cells, it was highly variable upon transfer of $1 \times 10^3$ cells (Fig. 5E, 5F), most likely reflecting the limited number of T cell clones recruited. Thus, very few (typically 0, 1, or 2) GFP$^+$ and GFP$^-$ T cells are present in the popliteal lymph node upon transfer of $1 \times 10^3$ cells, allowing us to detect the progenies of one or very few cells in the GFP$^+$ and GFP$^-$ subsets after immunization. Under limiting conditions, expanded T cells could still harbor a diverse profile of CD25 and CD62L expression, suggesting intraclonal diversification (Fig. 5G). In addition, we also noted variations between the T cell populations recovered from different mice, and, most importantly, we could also observe differences when the phenotype of expanded T cells in the GFP$^+$ and GFP$^-$ populations was compared in the same lymph node (Fig. 5H, 5I). These differences were significant, as they were abolished by randomization of pooled values. Thus, distinct T cell clones could generate different progenies of effectors in the same microenvironment, providing evidence for interclonal diversification in vivo.

**Discussion**

In the present work, we mapped the origin of phenotypic diversification during the course of T cell activation. Using a sensitive in vitro assay, we identified three checkpoints in the generation of a heterogeneous pool of effector T cells. First, large interclonal variability revealed that events occurring prior to the first cell division have a strong impact on the fate of T cell progenies. What

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**FIGURE 6.** A model for the generation of T cell heterogeneity during clonal expansion. (A–C) Previously proposed models for the diversification of T cells. (A) T cell fate is determined early on (prior to clonal expansion) and transmitted to the progeny. (B) T cells adopt distinct fates late during the expansion phase. (C) Asymmetric cell division during first division generates two possible fates within a progeny. (D) Continuous diversification generates a heterogeneous pool of T cells. Our data support a model in which T cell diversification is initiated prior to cell division and amplified during the first and subsequent divisions.
could be the origin of T cell interclonal diversification? These differences may originate from variation in TCR and cytokine signals (26) experienced by each T cell clone. Alternatively, differences could pre-exist in naive T cells. In this respect, it has been shown that even monoclonal T cells exhibit cell-to-cell variation in CD8 and soluble hematopoietic phosphatase 1 levels and that these differences accounted for the diversity in response to TCR stimulation (27). Second, we found that approximately half of the T cell clones analyzed exhibited significant diversity in phenotypic and functional markers, indicating that late events encompassing the successive divisions contribute to effector T cell heterogeneity. Although challenging, it will be of importance to clarify why some T cell progenies end up more homogenous than others. Third, through physical separation of paired daughter T cells, we provided evidence that T cell diversification can be initiated in the context of the first T cell division. Differences in paired daughter cells may originate from an asymmetric cell division, as recently proposed (15–18), or alternatively from random events occurring just after the cell division, such as variability in exposure to cytokines. We focused our analysis on two important markers, CD25 and CD62L, whose expression levels regulate T cell functional responses. The differences in CD25 expression observed in our experiments (up to 100-fold) are meaningful and expected to have substantial functional consequences, as predicted by previous studies (20). Of note, T cell phenotypic diversification appears to be a general phenomenon, observed with different peptide doses, affinities, or stimulating conditions. To study the composition of T cell progenies in vivo, we have also introduced an in vivo limiting dilution assay combined with the use of differently labeled T cell populations. In this approach, we decreased the number of transferred T cells to the point where, in most cases, a single T cell is activated in the popliteal lymph node draining the site of immunization. This methodology revealed that one T cell can give rise to a phenotypically diverse population of effectors (intraclonal diversification) in vivo, a finding consistent with a previous report in which single T cells were micromanipulated for adoptive transfer. Our approach is unique in that it allowed us to compare, in the same lymph node, two subsets (GFP+ and GFP−) originating from a limited number of clones. Divergent phenotypic profiles could be observed between GFP+ and GFP− subsets under limiting conditions (but not when large number of T cells was transfected), an observation that implies a significant level of interclonal differences in vivo and is consistent with two very recent studies that used barcoding and lineage tracing in vivo (28, 29). Thus, intraclonal and interclonal diversifications operate simultaneously in vivo.

Several models have been proposed to explain T cell heterogeneity, including early commitment, late T cell diversification, and asymmetric cell division (Fig. 6A–C). In the present study, our ability to analyze the composition of numerous T cell progenies and assess the fate of daughter cells enabled us to estimate simultaneously the contribution of these different mechanisms. Altogether, our results suggest that functional T cell diversification is a continuous process (Fig. 6D) largely initiated prior to clonal expansion, but later amplified during the first and subsequent cell divisions. Lastly, the in vitro and in vivo methodologies described in this work should provide new opportunities to track and dissect the development of a variety of T cell subsets during clonal expansion.

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Disclosures
The authors have no financial conflicts of interest.

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