CD8 single-cell gene coexpression reveals three different effector types present at distinct phases of the immune response

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To study in vivo CD8 T cell differentiation, we quantified the coexpression of multiple genes in single cells throughout immune responses. After in vitro activation, CD8 T cells rapidly express effector molecules and cease their expression when the antigen is removed. Gene behavior after in vivo activation, in contrast, was quite heterogeneous. Different mRNAs were induced at very different time points of the response, were transcribed during different time periods, and could decline or persist independently of the antigen load. Consequently, distinct gene coexpression patterns/different cell types were generated at the various phases of the immune responses. During primary stimulation, inflammatory molecules were induced and down-regulated shortly after activation, generating early cells that only mediated inflammation. Cytotoxic T cells were generated at the peak of the primary response, when individual cells simultaneously expressed multiple killer molecules, whereas memory cells lost killer capacity because they no longer coexpressed killer genes. Surprisingly, during secondary responses gene transcription became permanent. Secondary cells recovered after antigen elimination were more efficient killers than cytotoxic T cells present at the peak of the primary response. Thus, primary responses produced two transient effector types. However, after boosting, CD8 T cells differentiate into long-lived killer cells that persist in vivo in the absence of antigen.

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Abbreviations used: LCMV, lymphocytic choriomeningitis virus; PE, primary early; PL, primary plateau; PM, primary memory; PP, primary peak; SCD8, secondary response CD8; SM, secondary memory; Tc, T-cytotoxic; Tg, transgenic.

During immune responses, naive CD8 T cells are called on to develop multiple activities required to control antigen load, as well as to generate memory cells able to respond efficiently to rechallenge. All of these events are initiated by TCR triggering and occur within a very limited time frame. Addressing how these differentiation programs are established is of great interest to understand the establishment of successful immunity.

Most studies evaluating gene expression after T cell activation used in vitro–activated CD4 or CD8 T cells (1, 2) and studied cytokine expression directly or through the use of reporter genes. In both circumstances, IL-2 was induced before any division, and the Th1 or Th2 differentiation patterns were imprinted through successive divisions. Although these and other studies contributed significantly to delineate Th1–Th2 differentiation pathways (3), they appeared not to mimic in vivo CD8 differentiation because the induction of killer genes was not addressed and CD8 T cells were never reported to develop a Th2 cytokine profile during in vivo immune responses. Concerns were also raised on other possible differences between in vitro and in vivo environments. It was shown that the normal organ three-dimensional structure could significantly modify CD8 responses (4). It was also shown that in vitro restimulation could alter in vivo readouts; IFN-γ expression frequencies of 10% evaluated ex vivo were shown to increase to 90% (5), and TNF-α expression frequencies changed from <1% to 100% after a 4-h peptide stimulation in vitro (6). Also, inefficient or abortive immune reactions leading to deletion or anergy scored similarly to efficient memory cells in certain conditions of in vitro

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T cell stimulation (7, 8). These differences between in vitro and in vivo data raise the possibility that during an in vivo response, CD8 T cells may never meet the peptide or cytokine concentrations used to differentiate them in vitro. Conversely, the in vivo context may provide multiple additional environmental clues that may not be mimicked in in vitro cultures. Therefore, we aimed to directly study the induction of gene expression and gene association in CD8 T cells throughout immune responses in vivo, and considered the best approach to reach such an aim.

Differentiation programs involve the modification of the expression of multiple genes, which concur to define new cell properties. Because the induction of each gene expression requires multiple modifications to occur in the same cell (the induction/activation of signaling components, transcription factors, etc.), initiation of gene transcription is governed by probability laws, i.e., it is stochastic (for review see reference 9). New genes may be induced differently in each individual cell in such a way that each cell may express different amounts of the same gene and/or different gene combinations. To study such cell–cell variation, one should be able to quantify each mRNA type in each individual cell. Because the direct quantification of gene expression at a single cell level was thought impossible (10), studies of the induction of gene expression used reporter transgenes to quantify indirectly gene expression levels in single cells. These studies confirmed the stochasticity of gene induction (for review see reference 9). However, because knock-in technology does not yet allow multiple gene labeling, reporter genes could not be used to evaluate the establishment of differentiation programs that involve the coexpression of several genes by the same cell. It is possible that coexpression of different genes by the same cell is also stochastic. However, in the absence of available data, an elegant hypothesis was put forward (11). Because all CD8 effector molecules share several regulatory elements, it was proposed that once an individual cell would acquire some of these components any de novo expressed gene would be expressed preferentially in that cell (11). However, this hypothesis does not take into account that many of the regulatory components known to be involved in CD8 differentiation (Eomes, NFAT, NF-κB, Ikaros, etc.) (11) are not really CD8 specific, but are shared by several alternative differentiation pathways.

In this context, we thought the best way to evaluate putative cell heterogeneity during in vivo CD8 differentiation was to study individual cells throughout immune responses, and to quantify the expression of several genes simultaneously in each individual cell. We developed a methodology overcoming the multiple limitations preventing the direct quantification of multiple gene expression in single cells, and showed that we may now study 20 different genes simultaneously in each cell and accurately quantify 2–10^9 mRNA copies of each gene (10). In this study, we characterized two putatively different immune reactions that originate efficient CD8 memory: the response of anti-HY TCR-transgenic (Tg) cells to the male antigen (5, 12, 13) and of OT-1 naive TCR-Tg cells to *Listeria monocytogenes* OVA (14). We isolated individual cells at the different points of each immune reaction. In each cell, we simultaneously evaluated the expression of 14 T-cell effector genes, as well as several receptors for cytokines and chemokines reported to influence CD8 responses. This extensive single-cell study showed that both responses were similar, but that gene expression patterns were quite complex. Different effector genes were induced at different time points of the response, transcribed during different time periods, and could decline or persist independently of antigen. This heterogeneous behavior revealed CD8 types with different gene coexpression patterns and different in vivo behavior that were present at different phases of the response.

Globally, these data show how multiple genes important to CD8 function are induced and associate through the immune response and redefine different functional properties of CD8 T cells at the different phases of the immune reaction. We also show that, like B cells, CD8 T cells eventually differentiate into long-lived effectors after boosting that persists in vivo in the absence of antigen. In this context, this data also has a particular relevance to the discussion of what an effector or a memory T cell is.

RESULTS

Experimental approach

We isolated individual naive or primed TCR-Tg cells at different points of the immune response to male cells or to *L. monocytogenes* OVA stimulation. To ensure that all cells we studied had been stimulated by antigen, they were labeled with CFSE before "in vivo" transfer. In the first 24 h after "in vivo" activation, we selected $CD69^+$ cells that had not diluted CFSE because antigen-specific cells do not divide (5). At later time points, CD69 is down-regulated, but we ensured that all cells studied had diluted CFSE labeling, i.e., were stimulated and divided in response to antigen. The following points were selected: the early expansion phase, before exponential T cell growth (primary early expansion CD8s [PE-CD8s]); the peak of the exponential T cell growth (primary peak expansion CD8s [PP-CD8s]); the plateau of the response (primary plateau CD8s [PL-CD8s]); and at different time points of the memory stage (primary memory CD8 [PM-CD8]). Each individual cell was screened for the coexpression of 14 effector genes (Supplemental text, available at http://www.jem.org/cgi/content/full/jem.20062349/DC1). Th2/ T-cytotoxic 2 (Tc2) genes were never expressed. In contrast to in vitro CD8 activation, the expression of *Il2* and *Il10* was so rare that expression frequencies could not be estimated at the single-cell level. Eight remaining effector genes were expressed frequently. Because gene expression was similar in both responses, we describe first the anti-HY response in detail and show the analogies of the anti-OVA response after.

Individual effector genes have different kinetics of induction/down-regulation/coexpression

Naive anti-HY cells do not express effector molecules, with the exception of occasional cells expressing *Tgfb1* (5). After in

vivo activation, each effector gene had a different behavior. "Inflammatory" mediators were induced early upon activation. Surprisingly, their expression was transient, being down-regulated while CD8 T cells were still expanding vigorously. The *Tgfb1* was up-regulated at 7 h (unpublished data) and maximal frequency (70%; Fig. 1) and mRNA copies/cell (Figs. 1 and 2) were found in early expansion PE-CD8s, both declining thereafter. The *Tnf* was also induced at 7 h, but its expression down-regulated even earlier, before the exponential expansion phase (unpublished data). In contrast, the classical CD8 effector molecules were poorly expressed in PE-CD8s. Although PE-CD8s already have diluted CFSE labeling, demonstrating that they divided extensively (5), they rarely expressed *Ifng* or *Fasl*. Individual PE-CD8s could express *Prf*, *Gzma*, or *Gzmb*, but these molecules were rarely coexpressed by the same cell. These results suggested that PE-CD8s were not cytotoxic because target cell elimination through the perforin pathway requires the coexpression of perforin and granzymes by the same cell (for review see reference 15). Moreover, only few expressed *Fasl*, and killing mediated by FasL alone is not efficient (15, 16). Thus, early CD8 differentiation appeared to favor the expression of inflammatory molecules rather than the classic CD8 functions.

We next aimed to define TGF-β function in this response. TGF-β may be proinflammatory (promoting APC differentiation and being a powerful chemoattractant for neutrophils, monocytes, and macrophages) or antiinflammatory, inhibiting CD8 division (for review see reference 17). This latter effect requires the coexpression of two receptor types, RI (required for signal transduction) and RII (required for ligand capture) (18). However, our single-cell analysis showed individual CD8s did not coexpress Tgfbr1 and Tgfbr2 (Fig. 1). Thus, expanding CD8 cells may present this cytokine in trans, but may have developed mechanisms to escape TGF-β antiproliferation effect, probably because CD8s coexpressing RI and RII receptor types were eliminated from the cohort of dividing cells. Overall, these gene coexpression patterns suggest that CD8 T cells may develop effector functions early in the immune response, but these effector functions may be proinflammatory rather than cytotoxic. As described previously in the antilymphocytic choriomeningitis virus (LCMV) response (19), anti-HY CD8 T cells also down-regulated Ifngr2 expression, and thus could not respond to IFN-γ (Fig. 1).

PP-CD8s recovered by the peak of the exponential growth were very different from PE-CD8s (Fig. 1). Classical effector

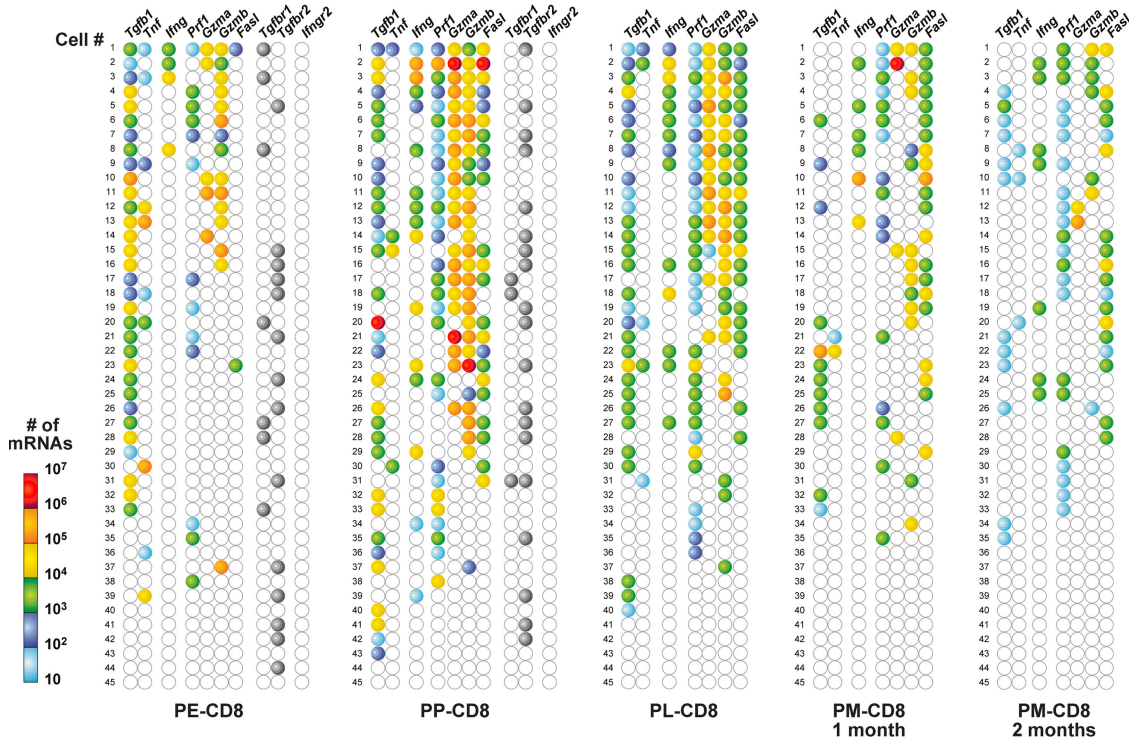

Figure 1. Coexpression of "effector" genes in male-specific CD8 single cells during the primary immune response. Anti-HY CD8 Tg single cells were sorted at different points of the response corresponding to the following: PE, early expansion phase; PP, peak of exponential growth; PL, plateau; and different time points after the end of the contraction phase (PM-CD8s). Each row shows the same individual cell that is numbered. Each column shows a different gene, representing the number of mRNA molecules/cell according to a color log scale. Empty symbols represent cells negative for that particular mRNA (<2 mRNA molecules). Gray symbols correspond to positive cells where mRNA levels were not quantified. For better visualization of coexpression patterns, individual cells were ordered by the degree of gene coexpression. The same expression patterns were obtained in two independent experiments.

CD8 mRNAs were now frequently expressed. Importantly, these mRNAs were coexpressed by the same cell, which is necessary to mediate effective killer functions (15, 16). Thus, ∼36% of PP-CD8s were *Ifng* mRNA$^+$. For the first time, a substantial fraction of CD8s also expressed *Fasl*. Considering gene coexpression, ∼45% of PP-CD8s coexpressed both *Prf1* and *Gzma* or/and *Gzmb* (Granzymes-*Gzms)*. Coexpression of these molecules together with *Ifng* and *Fasl* revealed that ∼30% of PP-CD8s might have been able to kill targets using either the perforin or the FasL pathway, and a further 30% might had the potential to use both pathways simultaneously. 29% of these T cells could potentially associate IFN-γ killing to cytotoxicity mediated by other killer molecules. Thus, PP-CD8 populations harbor a large cohort of potential killer effectors, in contrast to PE-CD8s.

These results allow us, for the first time, to investigate how different genes belonging to the same differentiation pathway become coexpressed by the same cell. Statistic analysis of gene association revealed that the gene coexpression was generally random, with only *Gzms* and *Fasl* associating preferentially (Fisher's exact test: FET $P < 0.01$). These results have particular relevance to the current theories of gene association during CD8 differentiation (11), as discussed later.

From the peak of the response onwards anti-HY CD8 T cells accumulate slowly forming a plateau up to day 15, when the contraction phase begins, CD8 T cells reaching steady-state numbers 1–2 wk (5). This plateau correlated to a drop in effector mRNAs copies in PL-CD8s (Figs. 1 and 2). Surprisingly, different effector genes also behaved very differently during the transition to memory. The expression frequencies of *Prf* and *Fasl* were relatively maintained, whereas other genes down-regulated. However, PM-CD8s lost the coexpression of effector killer genes (Fig. 1) reported to be required for efficient killer functions.

In contrast to anti-HY cells that are all CD44$^-$ and do not express effector molecules (5), "naïve" OT-1 cells are very cross-reactive and contain CD44^{int}, as well as a few CD44^{high} cells (20), and ∼35% of the cells already expressed *Tgfb1*. (Fig. 1 and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20062349/DC1). The kinetics of the OT-1 response (Fig. 3 a) was also more rapid than that of anti-HY cells (5). This rapid expansion was peculiar to the OT-1 clone because the kinetics of P14 cells response to *L. monocytogenes* GP33 was similar to the anti-HY response (unpublished data). However, when similar stages of the response were compared, the gene expression patterns found after *L. monocytogenes* stimulation (Fig. 3, b and c) were very similar to the HY response (Figs. 1 and 2). The *Tgfb1* was up-regulated, and *Tnf* was induced before any T cell division (Fig. 3 c) and peaked in PE-CD8s. In contrast, PE-CD8s showed poor coexpression of the cytotoxic mRNAs *Prf1* and *Gzms*. *Fasl* expression was also very rare (Fig. 3, b and c). PP-CD8s were also characterized by *Prf1* and *Gzms* coexpression by the same cell, and *Fasl* expression in a large fraction of CD8 T cells. During the transition to memory, these different genes also showed the same heterogeneous behavior we found in the anti-HY response. The expression frequencies of both *Prf1* and *Fasl* were relatively maintained, whereas *Ifng* and *Gzms* were down-regulated. The OT-1 PM-CD8s lost coexpression of killer genes in a manner similar to anti-HY PM-CD8s. Moreover, we observed this behavior in all individual mice (Fig. 3 c). However, we found that *Tgfb1* was less down-regulated (Fig. 3) than in anti-HY memory cells (Fig. 1). We are presently investigating if this higher expression of *Tgfb1* is a characteristic of this cross-reactive clone that expresses some *Tgfb1* at the naive stage (Fig. S1) or a different inflammatory imprinting of the *L. monocytogenes* response.

We thus conclude that effector genes expression patterns in different T clones (anti-HY or anti-OVA) and in different immune responses (response to male cells or response to *L. monocytogenes*) follow similar rules: the heterogeneous behavior of individual effector genes, the early expression/down-regulation of proinflammatory molecules, the coexpression of killer mRNAs in the same cell at the peak of the immune reaction, the maintenance of the expression of certain effector genes in the PM phase, but the loss of coexpression of killer molecules in the effector–memory transition. It must

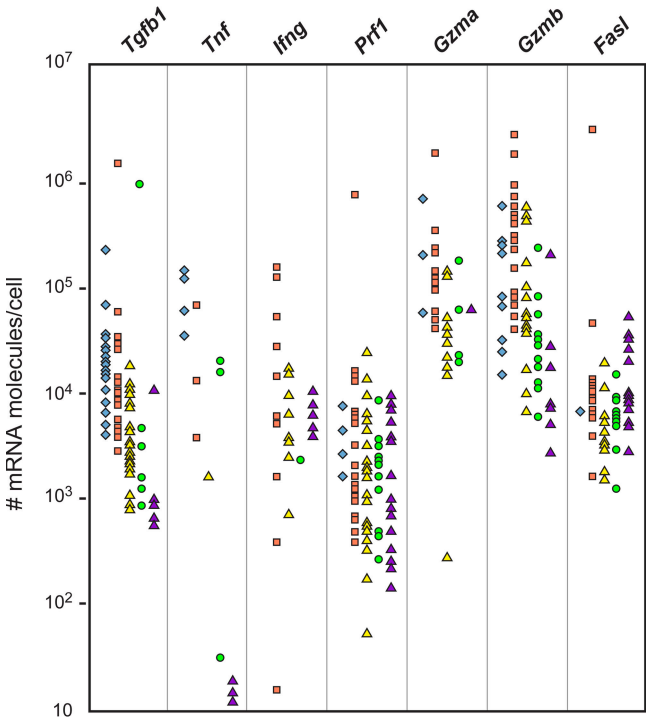

Figure 2. Variation of each gene expression level at different time points of the primary immune response. Individual CD8 Tg spleen lymphocytes specific to the male antigen were recovered at PE (blue diamond), PP (orange square), PL (yellow triangle), and at the memory phase 1 mo (green circle) and 2 mo (purple triangle) after immunization; 30 individual cells were studied at each time point. Negative cells are not figured. Results compare the expression levels of each gene in individual cells throughout the response, showing the absolute number of mRNAs/cell plotted in a log scale. They correspond to one of the two independent kinetic experiments we performed.

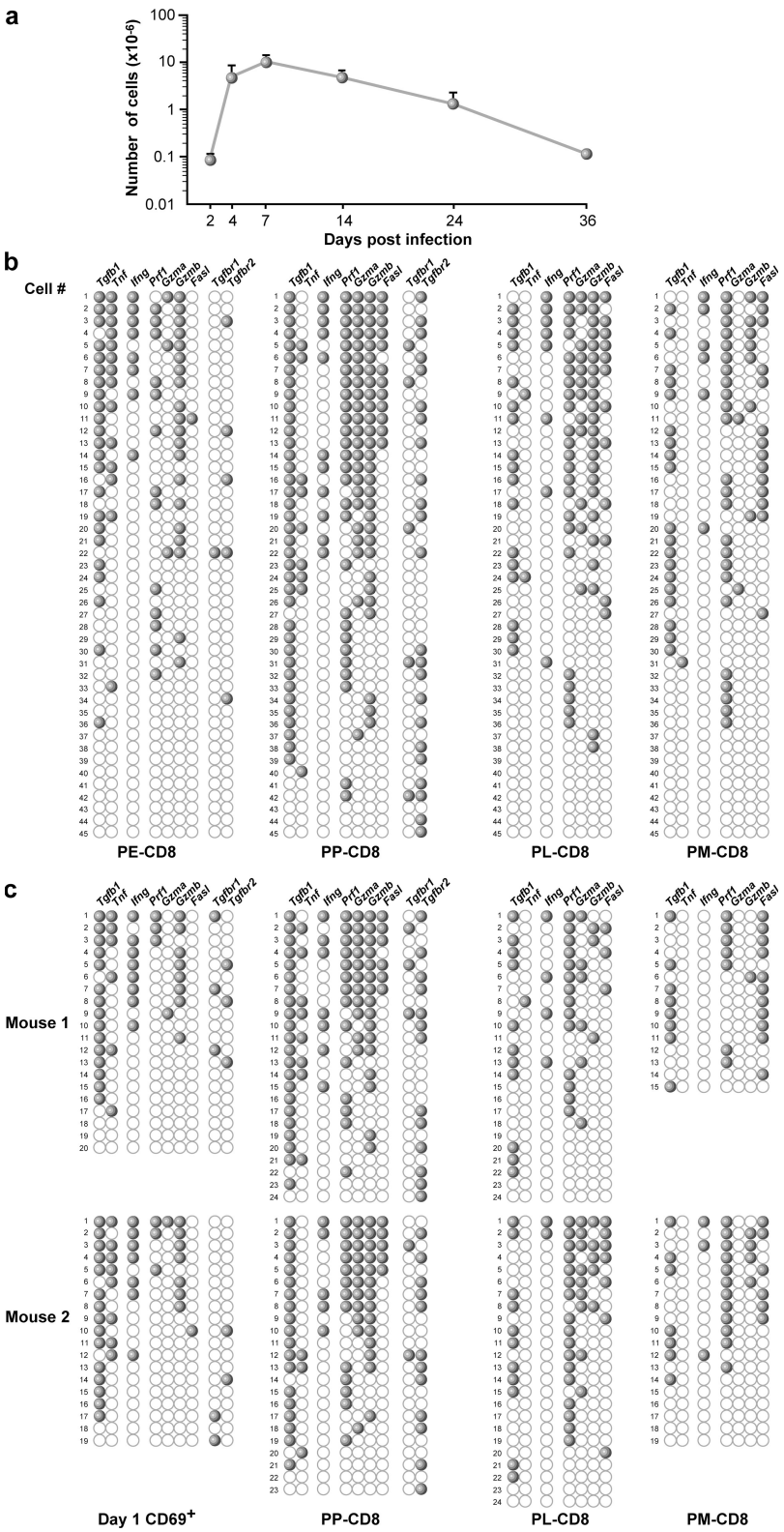

Figure 3. The primary response to *L. monocytogenes* immunization. OT-1 CD8 Tg cells specific of the OVA antigen were transferred to B6 mice, which were immunized with *L. monocytogenes* OVA. (a) Kinetics of the response. Results show the number of OT-1 cells recovered/mouse at different points after immunization and are the mean ± the SEM of three mice/time point. (b and c) Coexpression of effector genes at different points of the response. OT-1 cells were single-cell sorted, and their gene expression was depicted as described in Fig. 1. (c) OT-1 cells were recovered from two different individual mice in each time point. $CD69^+$ cells were activated cells that had not yet divided. Gene expression patterns are as described in Fig. 1.

be noted that throughout the primary response we found cells that responded to the Ag "in vivo," but did not express or coexpress effector molecules. Early in the response, these could correspond to cells that did not yet differentiate into effector cells. However, the presence of "functionless" cells at the peak of the response suggests that not all T cells dividing extensively in vivo necessarily differentiate into effector functions. Alternatively, transition to memory may not occur simultaneously; these "functionless cells" could correspond to CD8s that had already lost effector functions and differentiated into memory cells.

Early T cell differentiation generates "inflammatory effectors"

The different gene coexpression patterns of CD8 T cells at the beginning or at the end of the exponential expansion phase suggested that they could meditate different functions, the former being proinflammatory the latter being cytotoxic. Inflammation is characterized by local blood flow modifications and nonspecific trapping, when all types of circulating cells initially accumulate in the organ where the inflammation takes place (21). We used the anti-HY system to verify if T cells recovered early in the expansion phase could mediate such an inflammatory process. Indeed, only the anti-HY system allows attributing inflammatory properties to the T cells themselves. When adoptively transferring T cells to test for the induction of inflammation, we do not risk cotransferring either bacteria or bacterial products that are present at the beginning of the *L. monocytogenes* response, and that may also be able to induce inflammatory reactions by themselves.

To study inflammatory trapping we isolated anti-HY cells at different points of the immune response and injected them directly into the spleen of naive hosts. These hosts were injected i.v. with female target cells loaded or not with the HY peptide, and labeled with different intensities of CFSE. In the absence of "effector" CD8 T cells, the target cells reached the lymph nodes. When PE-CD8s were injected, target cells were prevented from reaching the lymph nodes (Fig. 4, a and b) and accumulated in the spleen (Fig. 4 b). Both antigen-loaded and nonloaded cells were trapped similarly, as it is characteristic of the inflammatory component of the trapping reaction (21). PE-CD8s also induced the accumulation of host monocytes/granulocytes in the spleen (unpublished data). In contrast, these cells were unable to control antigen

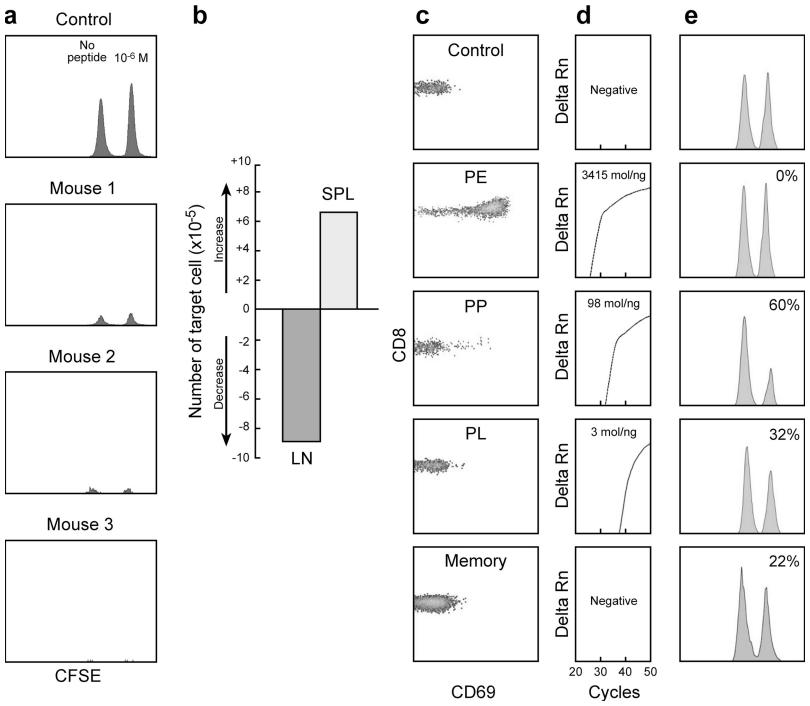

Figure 4. CD8 effector functions. (a and b) Trapping. Naive or PE-CD8s anti-HY–specific cells were injected in the spleen of *Cd3ε*$^{-/-}$ female mice. These mice were simultaneously injected i.v. with female CFSE^{+} target cells (a mixture of CFSE^{low} and CFSE^{high} targets, the latter loaded with the HY-peptide). 1 d later, we quantified the target cell recovery in the spleen and lymph nodes. (a) Gated CFSE^{+} cells in half of the lymph node (control) or total lymph node cells from individual mice injected with PE-CD8s. (b) Absolute number of targets in the lymph nodes and spleen. (c and d) Antigen loads. (c) Thy1.1^{+} naive "sensor" cells were injected into mice undergoing the immune reaction at different points of the responses. Results show CD69 expression in "sensor" cells 1 d after injection. (d) Quantification of Zfy-1 DNA in the spleen at different days after immunization. Similar results were obtained in the bone marrow. (e) In vivo killing. Targets were as described in the graphs in (a). CD8 cells were naive (control) or recovered at points of the response. Targets and CD8s were coinjected into the spleen of *Cd3ε*$^{-/-}$ female mice. The percentage of specific killing was evaluated as compared with the control performed in the same day. CD8 T cells and nonpulsed targets remained in similar numbers in the spleen during the 6 h required for optimal killing. All results are from one of three experiments.

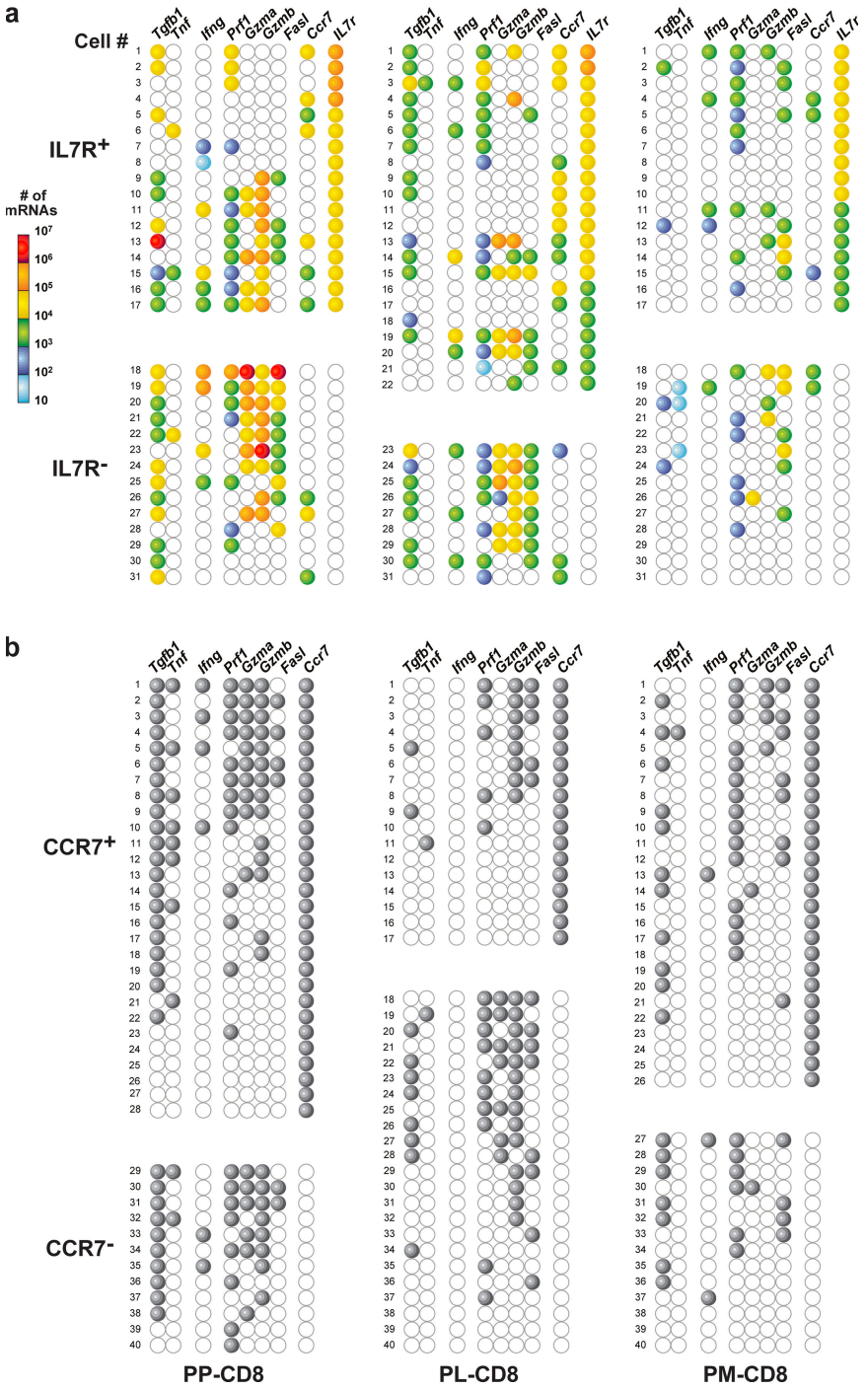

Figure 5. Correlation between *Il7r* and *Ccr7* expression and the coexpression of effector molecules. Each horizontal line shows the same individual cell. Columns show different genes and, when colored, represent the number of mRNA molecules/cell according to a color log scale. Empty symbols represent cells negative for that particular mRNA (<2 mRNA molecules). Gray symbols represent positive cells where mRNA levels were not quantified. (a) *Il7r*-expressing cells were ordered by decreasing amounts of *Il7r* copies/cell. *Il7r*-negative cells were ordered by the degree of effector gene coexpression. (b) *Ccr7*$^+$ and *Ccr7*$^-$ cells were ordered according to the degree of effector gene coexpression. Please note that individual *Ccr7*$^+$ cells did not show major differences in *Ccr7* expression levels.

load (see next section). These results show that effector cells are generated very early in the immune response, but they are inflammatory rather than cytotoxic. Moreover, it is usually believed that initiation of inflammatory reactions requires the presence of "danger" signals provided by pathogens (22). Our data demonstrate that T cells themselves can initiate at least

some type of inflammatory reactions because in the anti-HY system, such danger signals are absent.

"Killer effectors" coexpress killer genes

We next characterized antigen elimination in these mice. Antigen loads usually decline by the peak of the immune reaction, a finding we observed in both these responses. We confirmed that *L. monocytogenes* is eliminated at the peak of the exponential OT-1 cell growth (14). The CD69 expression of a new cohort of naive Thy1$^+$ cells injected into mice undergoing the anti-HY response indicates that male antigen loads in the anti-HY response are also highest at the beginning of the response, declining by the peak of exponential T cell growth (Fig. 4 c). In the *L. monocytogenes* system, we cannot exclude the presence of cross-presented peptides, whereas the anti-HY response relies on direct antigen presentation (23). Therefore, we can more accurately quantify antigen persistence by the direct quantification of the male-specific Zfy1 gene (zinc finger protein Y-linked) that detects male cells even when present at a 10^{-6} frequency (12). By this direct quantification, antigen loads were also highest at the beginning of the expansion phase and declined by the peak of the exponential T cell growth (Fig. 4 d).

The presence of high antigen doses early on, and its elimination at the peak of the response could be caused by the presence of two different effector cell types with different properties: an early "inflammatory effector" mediating inflammation, but unable to eliminate antigen; and a "killer effector" present only at the peak of response that can eliminate antigen, but cannot mediate inflammation. Alternatively, the antigen elimination at the peak of the response could be caused by the presence of a much higher number of effector cells. To address these alternatives, we aimed to compare the killer capacity of anti-HY PE-, PP-, PL-, and PM-CD8s on a per cell basis by injecting the same number of CD8s together with target cells directly in the spleen. We reasoned that this strategy should guarantee each recipient would receive the same number of effectors and that both effectors and targets would be present in the same location during the in vivo killer assay. Indeed, it is well known that CD8s change their homing capacities throughout the immune response. If injected i.v., CD8s recovered at different time points would likely migrate differently, not meeting their targets in the same way. This assay showed that PE-CD8s were unable to kill target cells. Maximal killing was carried out by PP-CD8s. PM-CD8s still maintained some killer activity, likely because of their *Fasl* expression (Fig. 4 d).

Therefore, the effector arm of this immune reaction harbors two distinct effector subtypes; a first inflammatory effector, which is generated before exponential expansion, and the classic killer effector, which is only present at the peak of the immune reaction.

Correlation of IL7R and CCR7 expression and the expression of effector molecules

The kinetics of IL-7R expression in these responses (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20062349/DC1) was as described in the LCMV response (24). The IL-7R expression was fully down-regulated in PE-CD8s and progressively up-regulated later on, with some IL-7R$^-$ cells persisting into the PM phase (24). Because of this progressive up-regulation, IL-7R expression levels distributed as a continuum from negative to IL-7R^{high}–expressing cells in PP and PL CD8s (24) (Fig. S2). Because IL-7R receptor expression was claimed to identify memory precursors (24), we correlated *Il7r* expression levels to effector gene coexpression (Fig. 5 a). When individual cells were ordered according to their *Il7r* levels, PP-CD8s and PL-CD8s (FET $<$ 0.01) expressing the highest *Il7r* levels showed the lowest coexpression of effector molecules. These results suggest that IL-7R^{high} cells that were identified as memory precursors (24) may be like memory cells, i.e., characterized by the lack of killer gene coexpression, and that they may already coexist with effectors well before the contraction phase. PP-CD8 IL-7R$^+$ and IL-7R$^-$ cells were believed to have similar effector functions (24), but this is likely caused by a less powerful discriminatory capacity of previous tests. Indeed, cell sorting does not allow separating cells with discrete variations of IL-7R expression levels. In contrast, we can identify discrete variations of *Il7r* expression levels, which may allow a better separation of putative memory precursors and effector subtypes. However, the *Il7r*$^-$ cells persisting into the memory phase also lost effector gene coexpression, and persisted for long time periods in the absence of antigen (Fig. 5 a).

The expression of CCR7 subdivides human CD8 memory cells into CCR7$^+$ central (T_{CM}) and CCR7$^-$ effector (T_{EM}) subtypes with very different properties, but in the mouse, the relative role of these cell types, as well as their lineage relationships, are very controversial (for review see reference 25). In the anti-HY response, we found no differences in gene expression/coexpression patterns between *Ccr7*$^+$ and *Ccr7*$^-$ PM-CD8s, but this response generated few *Ccr7*$^+$ memory cells in the spleen (Fig. 5 a). However, the response to *L. monocytogenes* generates abundant CCR7$^+$ PM-CD8, and we also found no differences between *Ccr7*$^+$ and *Ccr7*$^-$ cell effector gene coexpression patterns (Fig. 5 b). These results indicate that mouse CCR7$^+$ and CCR7$^-$ types in primary responses are not equivalent to human T_{CM} and T_{EM} subtypes because these human populations have very different effector gene coexpression (26).

Modification of gene expression patterns in the secondary immune reaction

We next investigated effector genes expression in secondary response CD8s (SCD8s). The anti-HY–primed CD8s respond vigorously in secondary reactions. Maximal cell numbers are recovered by day 7, and the contraction phase finishes by 2 wk after boosting (5). We expected all effector genes to be rapidly up-regulated during this vigorous response. Surprisingly, *Fasl* and *Prf1* expression frequencies (already high in PM-CD8s) did not increase by day 4 and were not much higher in secondary memory cells (SM-CD8s) than in PM-CD8s (Fig. 6 a). In contrast, *Tgfb1*, *Ifng*, *Gzma*, and *Gzmb*

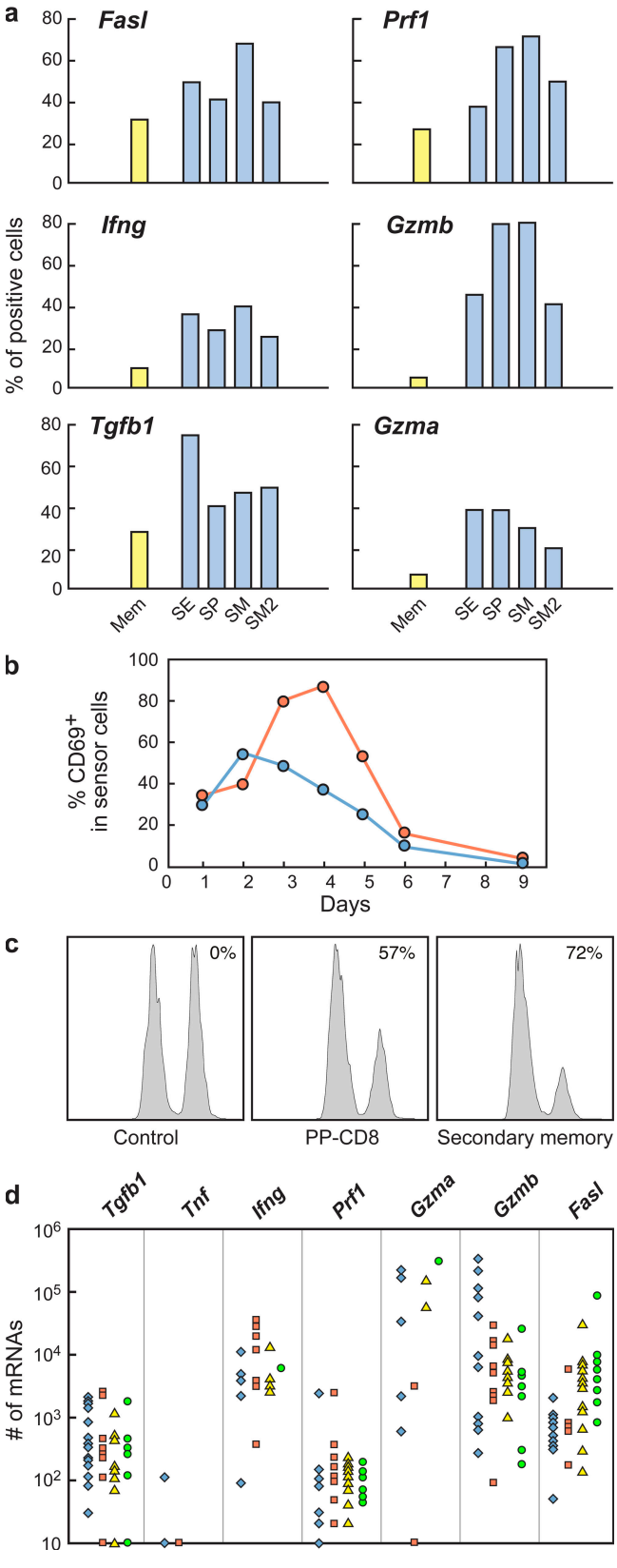

Figure 6. Secondary responses. PM-CD8s were boosted with male cells. (a) Individual cells were sorted at different time points after boosting. Results show gene expression frequencies in the PM-CD8 donor cells we used in this experiment (yellow bars) and in the secondary response at different time points after boosting (blue bars). Results correspond to >47 cells per time point. (b) Comparison of Ag loads in the primary (orange circle) and secondary (blue circle) responses. Primary and secondary hosts were studied simultaneously. Results show CD69 expression in "sensor" cells at different time points, as described in Fig. 4 c. The same results were obtained in three independent experiments. (c) Comparison of cytotoxic capacity of PP-effector cells and SM cells recovered 3 mo after antigen elimination. Killer tests were performed as described in Fig. 4 e. Both CD8 populations were studied in the same day, with results corresponding to one of two experiments. (d) Quantification of mRNA expression levels in secondary single cells recovered at days 4 (blue diamond), 7 (orange square), 15 (yellow triangle), and 33 (green circle) after boosting. Results show the number of mRNA copies/cell plotted in a log scale.

expression (which is rare in PM-CD8s) was rapidly reinduced in most cells (Fig. 6 a). SCD8s coexpressed multiple killer genes shortly after boosting, and controlled the accumulation of male cells even before the secondary expansion phase. The antigen concentration never reached the levels found in primary responses (Fig. 6 b). However, the most surprising aspect of this secondary response was the very different behavior of effector genes in the effector–memory transition. In the primary reaction, antigen elimination was followed by a drop in effector gene expression and a loss in gene coexpression (Figs. 1 and 2). In contrast, in the secondary reaction, the expression of all effector genes was maintained in a substantial proportion of cells well after antigen elimination (Fig. 6 a). As a consequence, many SM-CD8s coexpressed several killer genes simultaneously, i.e., they had a gene expression profile similar to PP-effectors (Supplemental text, available at http://www.jem.org/cgi/content/full/jem.20062349/DC1).

It was recently reported that SM cells were more efficient killers than PM cells (27, 28). This data was generated in the anti-LCMV response and interpreted as suggesting that successive boosting generated more effector–memory cells than the primary boosting. However, most of the anti-HY PM-CD8s were already CCR7$^-$ (Fig. 6), and they did not have the gene coexpression we found in SM-CD8s. SM-CD8 gene coexpression patterns rather resembled the killer cells recovered at the peak of the primary response. Thus, we wondered if SM-CD8s could, in reality, be effectors while persisting in vivo in the absence of Ag. To evaluate this possibility, we compared SM-CD8s to PP-CD8s killing on a per cell basis. Surprisingly, SM-CD8s persisting in vivo for 2–3 mo after antigen elimination were even more efficient killers than PP-effectors (Fig. 6 c). These results show that the outcome of primary and secondary reactions is totally different. Primary immune responses generate transitory effector cells and quiescent memory cells, whereas secondary responses generate permanent effector cells that persist in vivo in the absence of antigen.

CD8 T cells modify their transcriptional behavior in secondary immune responses

It is not yet known how the "noise" induced by stochastic gene expression is eventually controlled at single-cell level

(9, 11, 29). We observed that transcription levels in SM T cells (Fig. 6 d) were very different from that found in primary cells (Fig. 2). Cell–cell variation was much reduced. Paradoxically, the number of "effector" mRNAs/cell was approximately one to two logs lower than found in the primary response. Thus, the permanent expression of effector genes is associated with "stabilization" of mRNA expression in individual cells.

DISCUSSION

We describe how multiple effector genes are expressed throughout CD8 differentiation in in vivo immune responses. The results obtained were surprising. They do not support assumptions issuing from limited in vitro studies of CD8 responses. They are also not compatible with the single theory proposed to explain gene association during CD8 differentiation.

Previous studies of effector gene expression in Th1/Tc1 differentiation concentrated on cytokine expression by T cells activated in vitro with peptide or anti-CD3, correlating cytokine expression to cell division (1, 2). They reported the rapid induction of IL-2 before any division in all T cells. We also found that these Tg cells expressed abundant IL-2 after in vitro activation (7, 23), but we found very little *Il2* expression in vivo. This in vitro/in vivo difference was also found in CD4 responses that express abundant IL-2 after in vitro stimulation, but little *Il2* in in vivo responses (30).

CD8 effector genes are also believed to be rapidly induced after a few cell divisions (1, 2). However, we found that individual effector molecules could be induced at very different time points during the primary response. Inflammatory genes were expressed before any division. In contrast, CD8 cells had diluted CFSE, and underwent major expansion before the majority expressed *Ifng Grzms* or *Fasl*. It is also assumed that effector genes decline when the antigen is eliminated, but not all genes behaved this way. Inflammatory genes declined when the antigen was abundant, and T cells expanded vigorously. *Prf1* and *Fasl* expression were relatively maintained for a long time after antigen elimination. Therefore, during this primary immune response, individual effector genes could be induced at different time points, were expressed during different lengths of time, down-regulated at different time points, and, in some cases, not down-regulated at all. Importantly, such heterogeneous gene expression is not peculiar to TCR-Tg systems, but also occurs in polyclonal cells from normal immunized mice (unpublished data).

Several modifications of the transcriptional behavior in secondary responses were also unexpected. Memory T cells are believed to rapidly reexpress all effector genes after reactivation (31). However, boosting did not modify the transcription of *Prf1* and *Fasl*. All other effector genes were rapidly reinduced after boosting, but then they became permanently transcribed in a large fraction of CD8 T cells even after antigen elimination. These results show that during CD8 differentiation, the expression of all effector genes eventually evolves into an "antigen-independent" permanent transcription status, which was unsuspected. This sustained transcription was associated to other major differences in SCD8's transcription behavior. Cell–cell variation was reduced. Paradoxically, the number of "effector" mRNAs/cell was approximately one to two logs lower than that found early in the primary response. It is tempting to speculate that such differences are caused by the signal transduction modifications we described in memory cells (7). Because these effector genes are regulated by several Ca^{2+}-dependent transcription factors (11), and memory cells increase the frequency and reduce the amplitude of Ca^{2+} transients (7), transcription oscillations (32) likely follow the same trend. Increased frequency/decreased amplitude of Ca^{2+} transients and transcription oscillations would simultaneously justify SCD8's reduced mRNA levels, diminished cell–cell variation, and sustained transcription.

Our study also allows us to determine, for the first time, how different effector genes are coexpressed by the same effector cell. As CD8 effector genes share some regulatory elements, it was postulated that this sharing would determine preferential effector gene coexpression (11). In contrast, we found that most of them were coexpressed stochastically, even when transcription became permanent in SM cells. Although our study does not address gene regulation, the present results indicate that we need to look for different clues to understand how these genes are induced and maintained. Because individual effector genes behaved differently, gene-specific regulatory elements (rather than shared regulatory elements) should have a dominant role in conditioning their expression. The random association of effector molecules in the same cell further supports that "shared regulatory elements" are not sufficient to establish preferential gene coexpression. This is perhaps not too surprising because many of the regulatory factors described as being shared by CD8 effector genes (Eomes, NFAT, NF-κB, Ikaros, etc.) are not specific to the CD8 differentiation pathway. Other "gene-specific" and "pathway-specific" combinations must be involved to engage these regulatory factors in the very different pathways of differentiation they are involved.

This data also refines our previous classification of the different phases of the immune reaction. Classically, the immune response is divided into four sequential successive phases, each associated to a peculiar functional behavior: the expansion phase, where cells accumulate, but do not have effector functions; the effector phase, where cells express their effector functions and eliminate the antigen; and the contraction phase, which is initiated after antigen elimination when effectors die and memory precursors are selected to become quiescent memory cells in the memory phase. Our data shows that during the primary immune response, CD8 T cells actually go through two successive effector phases, inflammatory and cytotoxic. Inflammatory effectors are generated shortly after antigen stimulation, and they mobilize other cells to the place where the immune reaction takes place. "Cytotoxic effectors" are present at the peak of the exponential T cell growth, and they coexpress killer molecules and control antigen loads. PM cells lose killer capacity because they lose killer

genes coexpression. The behavior of CD8 T cells in secondary immune reactions diverges even further from the classic subdivision of CD8 responses. Thus, SCD8s differentiate into killer cells and control antigen loads before the beginning of the secondary expansion phase. Moreover, after the antigen was fully eliminated in secondary responses CD8s maintain the coexpression of effector genes and effector functions permanently; they are more efficient than the classical killer cells that are present at the peak of the primary immune reaction.

It has recently been described that anti-LCMV GP33-specific memory cells change after boosting, SM cells further down-regulating CCR7 expression, killing target cells better than PM cells, and increasing their tropism for nonlymphoid tissues (27, 28). This data was interpreted as suggesting that boosting induces the conversion of central memory T cells to effector memory T cells (27, 28). However, both effectors and effector–memory cells are $CCR7^-$, and our data suggests that cells present at the end of secondary responses are actually efficient effectors. Indeed, in both the anti-HY and the anti–*L. monocytogenes* systems, the majority of PM-CD8s were $CCR7^-$, and thus should be considered effector–memory T cells. However, these $CCR7^-$ PM-CD8s did not coexpress effector genes nor killed targets efficiently, i.e., were clearly different from PP-killer cells. Instead of comparing primary and SM as was done previously (27, 28), we compared SM-CD8s to the PP-effector cells. We found both cell types shared similar effector gene coexpression and that the SM-CD8s killer capacity on a *per cell* basis was superior to that of PP primary effectors. Moreover, the anti-HY system has some advantages in establishing that such effector cells can persist in the absence of nominal antigen. Indeed, the anti-HY clone is not cross-reactive (20). In contrast, anti-LCMV GP-33 cells recognize self-epitopes from the dopamine β-monooxygenase responsible for the conversion of dopamine to noradrenalin in the suprarenal glands, LCMV infection leading to suprarenal infiltration, and a drop in dopamine levels (33, 34). Thus, the LCMV GP-33 system does not allow excluding chronic self-stimulation as a mechanism for maintenance of memory or effector cell types. Globally, our data thus supports the notion that primary responses predominantly induce short-lived effector functions, whereas secondary responses generate a cohort of long-lived effector cells that persist in vivo in the absence of antigen.

Finally, it might be useful to compare the information obtained by this single-cell method to that of more global approaches, as the quantitative analysis of gene expression at population level in gene expression arrays (35). Arrays are relatively easy to perform, and allow screening for virtually the entire mouse genome, whereas the present methodology is laborious and only allows screening the expression of ~20 known genes each time. However, our results show that single-cell assays give important information that cannot be obtained by array studies. We can determine the frequency of expression of each gene. In an array's data, it is impossible to determine if a signal is caused by a minority of cells expressing high mRNA levels, or to a majority population expressing a gene at lower levels. Indeed, we show that individual genes are transcribed at very different levels. Transcription can range from $>10^7$ mRNAs/cell (*Gzmb* and *Gzma*) to 10^3 mRNAs/cell (*Tgfb1* and *Prf1*). Consequently, a single cell expressing *Gzmb* at 10^6 mRNAs/cell present at 1/1,000 frequency may give the same signal as 100% of the cells expressing *Tgfb1* at 10^3 mRNAs/cell, i.e., a rare nonrepresentative event at 10^{-3} frequency and a major property shared by all T cells may score similarly in population readouts. This major bias was evident when we quantified mRNA expression of the same PE-CD8s studied as a population or as single cells (10). In the first case, *Gzmb* was the most abundant gene expressed by the PE population, but our single-cell analysis revealed that such a signal was caused by rare cells expressing *Gzmb* at $>10^6$ copies/cell. In contrast, PE-CD8 population *Tgfb1* signal was much weaker than that of *Gzmb*, but our single-cell analysis revealed that this gene was expressed by >70% of PE-CD8s at $\sim10^3$ copies/cell. The other major limitation of gene expression arrays is their inability to evaluate if different genes are expressed by the same cell or by different individual cells. Our present data shows that coexpression studies are particularly relevant for the understanding of T cell behavior. Thus, we determined that proliferating T cells couldn't respond to the TGF-β they produce, because individual cells did not coexpress the *Tgfbr1* and *Tgfbr2*. This finding led us to envisage a proinflammatory role of early effectors that we confirmed by in vivo functional studies. In contrast, because individual cells expressing either *Tgfbr1* or *Tgfbr2* are present within this population, a study performed at a population level would score positive for both genes. Killer genes coexpression studies gave important clues on killer potential of different populations. Thus, PE-CD8s (that at a population level score positive for both *Prf1* and *Gzmb*) were shown not to coexpress these molecules at single-cell level, suggesting that they were not cytotoxic, which we did confirm by in vivo functional tests. Similarly, individual PM-CD8 did not coexpress *Prf1* and *Gzmb*, explaining why PM cells are quiescent, although they may score positive for these cytotoxic mRNAs in genetic arrays (35). It must be noted that, in many circumstances, single-cell coexpression of different genes cannot be evaluated at protein level because, as in the case of perforin, Abs recognizing native proteins in the mouse are not available. Thus, genetic arrays and single-cell analysis appear to have different complementary scopes. Genetic arrays are fundamental to identify potentially important genes that are differentially expressed in two different cell sets. Single-cell analysis, by evaluating different gene expression frequencies and their coexpression by the same cell, gives important information on cell heterogeneity and indicates potentially different T cell properties.

MATERIALS AND METHODS

Naive and memory Tg cells. Naive CD8 cells were obtained from C57BL/6 *Rag2*-deficient female mice expressing Tg-TCR specific to the male antigen HY(5, 12) or to the OVA peptide (OT-1 cells). PM Tg cells

were obtained from immunized mice 2–6 mo after priming. SM cells were obtained 2–3 months after the secondary immune response. In the anti-HY response, Tg CD8 T cells were stimulated in the same conditions in primary and secondary responses. In brief, 0.5×10^6 Tg lymph node cells and 10^6 $CD4^+$ T cells were injected i.v. into *Rag2*-deficient female mice, and immunized with 0.5×10^6 male bone marrow cells from *Cd3ε*-deficient mice and 5×10^6 female bone marrow cells. In the response to OVA, 10^6 OT-1 Tg cells were transferred i.v. into normal B6 mice and immunized i.v. with 10^3 *L. monocytogenes* OVA, which was a gift from L. Lefrançois (University of Connecticut Healthcare Center, Farmington, CT). The animal studies performed were approved by the Bureau de l'Expérimentation Animale du Ministère d'Éducation National, Enseignement Supérieur et Recherche.

Antibodies and immunofluorescence. Antibodies used were as follows: PE-labeled anti-CD69, biotin-labeled T3.70, anti-Ly-5.2 (104–2.1), CyChrome–labeled anti-CD8, and FITC-labeled anti–Thy-1.2. Biotinylated antibodies were revealed with streptavidin-allophycocyanin, and labeling was evaluated in a FACSCalibur (Becton Dickinson).

Evaluation of antigen load. To determine *L. monocytogenes* loads, livers were aseptically removed and separately homogenized in distilled water. Bacterial counts in liver homogenates were determined at various intervals on BHI agar supplemented with 5 μg/ml erythromycin. We determined male antigen loads remaining in immunized mice by two independent methods that gave overlapping results. We studied their capacity to activate naive Tg cells by injecting 0.5×10^6 naive $Thy1.1^+$ $CD69^{neg}$ Tg cells ("sensor cells") i.v. into mice undergoing immune responses at different time points after immunization and evaluating sensor cell CD69 expression 1 d later (13). Because this response relies on direct antigen presentation (23), and not cross-presentation, we also quantified male DNA directly. For that purpose, spleen and bone marrow cells were isolated during the immune response and samples containing 20 ng of DNA were real-time PCR amplified for the Zfy-1 gene (Zinc finger protein Y-linked) (12).

Trapping. Targets were $Ly5.2^+$ female splenocytes labeled with two concentrations of CFSE, 0.5 μM $CFSE^{low}$, and 5 μM $CFSE^{high}$. The $CFSE^{high}$ cells were pulsed with 10^{-6} M HY peptide (36). Anti-HY CD8 Tg cells were obtained from naive or immunized mice at different times after antigen stimulation by magnetic sorting. 0.5×10^6 purified Tg (>98%) naive or primed CD8 Tg cells were injected in the spleen of $Ly5.1^+$ *Cd3e*-deficient mice and a mixture of 5×10^6 $CFSE^{low}$ and 5×10^6 $CFSE^{high}$ targets were injected i.v. Target cell migration to the lymphnodes was evaluated 1 d later.

In vivo cytotoxicity assays. CD8 Tg cells and target cells were as described in the previous section. 0.5×10^6 Tg cells and a mixture of 10^6 $CFSE^{low}$ and 10^6 $CFSE^{high}$ targets were injected into the spleen of $Ly5.1^+$ *Cd3e*-deficient mice. First, we quantified $CFSE^{low}$ and $CFSE^{high}$ cells in the spleen and lymph nodes of mice injected with target cells alone or with targets and naive CD8 Tg cells. As expected, naive Tg cells did not modify target cell recovery. Thus, specific cytotoxicity was determined by evaluating $CFSE^{low}$ and $CFSE^{high}$ relative recovery in mice injected either with naive Tg cells or different sets of primed Tg cells, as described previously (37). We evaluated the kinetics of $CFSE^{high}$ target cells elimination and found maximal killing was by 6 h after injection.

Single-cell multiple parametric quantitative RT-PCR. This method has previously been described in detail (10). To ensure that each well contained a T cell *Cd3e*, mRNA was amplified simultaneously with the other genes. To ensure that amplifications performed in different days could be directly compared, we included two internal standards for quantitative evaluations. The first was an in vitro–synthesized RNA containing serial dilutions of a known number of molecules, ranging across all expression levels and undergoing the same RT and PCR amplifications, which allowed evaluation of both RT and PCR efficiencies. The second was a pooled cDNA prepared from activated T cells, which contained RNAs from all the genes we studied in previously determined known amounts. This second standard controlled individual gene amplification efficiencies.

Nomenclature. Throughout this study, we used the genetic nomenclature according to the guidelines from the International Committee on Standardized Genetic Nomenclature for Mice for genes and mRNA (http://www.informatics.jax.org). In this nomenclature, genes and mRNAs have the same abbreviation. The mRNAs studied were Perforin (*Prf1*), Granzyme A (*Gzma*), Granzyme B (*Gzmb*), FasL (*Fasl*), IFNγ (*Ifng*), TGFβ1 (*Tgfb1*), TNFα (*Tnf*), TNFβ (*Lta*), IL2 (*Il2*), IL4 (*Il4*), IL5 (*Il5*), IL10 (*Il10*), IL13 (*Il13*), IL15 (*Il15*), IL21 (*Il21*), IFNγRII (*Ifngr2*), TGFβRI (*Tgfbr1*), TGFβRII (*Tgfbr2*), IL7R (*Il7r*), CCR7 (*Ccr7*), and CD3ε (*Cd3e*).

Statistical analysis. Frequency estimates were determined according to the Poisson equations. All differences mentioned in the text were significant. Potential associations or dissociations in the expression of different genes were studied using the two-tailed Fisher's exact test. This test allows discrimination between random association and preferential gene coexpression, and is adequate to evaluate relative small samples. A P value of <0.05 was considered statistically significant.

Online supplemental material. Fig. S1 shows effector genes expression in naive OT-1 T cells. Fig. S2 shows expression of IL-7R in anti-HY–specific cells. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20062349/DC1.

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REFERENCES

1. Croft, M., L. Carter, S.L. Swain, and R.W. Dutton. 1994. Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J. Exp. Med.* 180:1715–1728.
2. Gett, A.V., and P.D. Hodgkin. 1998. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proc. Natl. Acad. Sci. USA.* 95:9488–9493.
3. Lee, G.R., S.T. Kim, C.G. Spilianakis, P.E. Fields, and R.A. Flavell. 2006. T helper cell differentiation: regulation by cis elements and epigenetics. *Immunity.* 24:369–379.
4. Surh, C.D., O. Boyman, J.F. Purton, and J. Sprent. 2006. Homeostasis of memory T cells. *Immunol. Rev.* 211:154–163.
5. Veiga-Fernandes, H., U. Walter, C. Bourgeois, A. McLean, and B. Rocha. 2000. Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nat. Immunol.* 1:47–53.
6. Panus, J.F., L.J. McHeyzer-Williams, and M.G. McHeyzer-Williams. 2000. Antigen-specific T helper cell function: differential cytokine expression in primary and memory responses. *J. Exp. Med.* 192:1301–1316.
7. Tanchot, C., S. Guillaume, J. Delon, C. Bourgeois, A. Franzke, A. Sarukhan, A. Trautmann, and B. Rocha. 1998. Modifications of CD8+ T cell function during in vivo memory or tolerance induction. *Immunity.* 8:581–590.
8. Kassiotis, G., S. Garcia, E. Simpson, and B. Stockinger. 2002. Impairment of immunological memory in the absence of MHC despite survival of memory T cells. *Nat. Immunol.* 3:244–250.
9. Raser, J.M., and E.K. O'Shea. 2005. Noise in gene expression: origins, consequences, and control. *Science.* 309:2010–2013.

10. Peixoto, A., M. Monteiro, B. Rocha, and H. Veiga-Fernandes. 2004. Quantification of multiple gene expression in individual cells. *Genome Res.* 14:1938–1947.
11. Glimcher, L.H., M.J. Townsend, B.M. Sullivan, and G.M. Lord. 2004. Recent developments in the transcriptional regulation of cytolytic effector cells. *Nat. Rev. Immunol.* 4:900–911.
12. Rocha, B., A. Grandien, and A.A. Freitas. 1995. Anergy and exhaustion are independent mechanisms of peripheral T cell tolerance. *J. Exp. Med.* 181:993–1003.
13. Tanchot, C., F.A. Lemonnier, B. Perarnau, A.A. Freitas, and B. Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science.* 276:2057–2062.
14. Liu, Z., and L. Lefrancois. 2004. Intestinal epithelial antigen induces mucosal CD8 T cell tolerance, activation, and inflammatory response. *J. Immunol.* 173:4324–4330.
15. Russell, J.H., and T.J. Ley. 2002. Lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* 20:323–370.
16. Corazza, N., S. Muller, T. Brunner, D. Kagi, and C. Mueller. 2000. Differential contribution of Fas- and perforin-mediated mechanisms to the cell-mediated cytotoxic activity of naive and in vivo-primed intestinal intraepithelial lymphocytes. *J. Immunol.* 164:398–403.
17. Luethviksson, B.R., and B. Gunnlaugsdottir. 2003. Transforming growth factor-beta as a regulator of site-specific T-cell inflammatory response. *Scand. J. Immunol.* 58:129–138.
18. Massague, J. 1998. TGF-beta signal transduction. *Annu. Rev. Biochem.* 67:753–791.
19. Haring, J.S., G.A. Corbin, and J.T. Harty. 2005. Dynamic regulation of IFN-gamma signaling in antigen-specific CD8+ T cells responding to infection. *J. Immunol.* 174:6791–6802.
20. Hao, Y., N. Legrand, and A.A. Freitas. 2006. The clone size of peripheral CD8 T cells is regulated by TCR promiscuity. *J. Exp. Med.* 203:1643–1649.
21. Zatz, M.M., and E.M. Lance. 1970. The distribution of chromium 51-labelled lymphoid cells in the mouse. A survey of anatomical compartments. *Cell. Immunol.* 1:3–17.
22. Matzinger, P. 1998. An innate sense of danger. *Semin. Immunol.* 10:399–415.
23. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science.* 297:2060–2063.
24. Kaech, S.M., J.T. Tan, E.J. Wherry, B.T. Konieczny, C.D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4:1191–1198.
25. Rocha, B., and C. Tanchot. 2006. The Tower of Babel of CD8+ T-cell memory: known facts, deserted roads, muddy waters, and possible dead ends. *Immunol. Rev.* 211:182–196.
26. Monteiro, M., C. Evaristo, A. Legrand, A. Nicoletti, and B. Rocha. 2006. Cartography of gene expression in CD8 single cells: novel CCR7- subsets suggest differentiation independent of CD45RA expression. *Blood.* DOI: 10.1182/blood-2006-06-027060.
27. Jabbari, A., and J.T. Harty. 2006. Secondary memory CD8+ T cells are more protective but slower to acquire a central–memory phenotype. *J. Exp. Med.* 203:919–932.
28. Masopust, D., S.J. Ha, V. Vezys, and R. Ahmed. 2006. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. *J. Immunol.* 177:831–839.
29. Raser, J.M., and E.K. O'Shea. 2004. Control of stochasticity in eukaryotic gene expression. *Science.* 304:1811–1814.
30. Garcia, S., J. DiSanto, and B. Stockinger. 1999. Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity.* 11:163–171.
31. Fitzpatrick, D.R., K.M. Shirley, and A. Kelso. 1999. Cutting edge: stable epigenetic inheritance of regional IFN-gamma promoter demethylation in CD44highCD8+ T lymphocytes. *J. Immunol.* 162:5053–5057.
32. Golding, I., J. Paulsson, S.M. Zawilski, and E.C. Cox. 2005. Real-time kinetics of gene activity in individual bacteria. *Cell.* 123:1025–1036.
33. Ohteki, T., A. Hessel, M.F. Bachmann, A. Zakarian, E. Sebzda, M.S. Tsao, K. McKall-Faienza, B. Odermatt, and P.S. Ohashi. 1999. Identification of a cross-reactive self ligand in virus-mediated autoimmunity. *Eur. J. Immunol.* 29:2886–2896.
34. Sandalova, T., J. Michaelsson, R.A. Harris, J. Odeberg, G. Schneider, K. Karre, and A. Achour. 2005. A structural basis for CD8+ T cell-dependent recognition of non-homologous peptide ligands: implications for molecular mimicry in autoreactivity. *J. Biol. Chem.* 280: 27069–27075.
35. Kaech, S.M., S. Hemby, E. Kersh, and R. Ahmed. 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell.* 111:837–851.
36. Opferman, J.T., B.T. Ober, and P.G. Ashton-Rickardt. 1999. Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science.* 283:1745–1748.
37. Wong, P., M. Lara-Tejero, A. Ploss, I. Leiner, and E.G.P. Am. 2004. Rapid development of T cell memory. *J. Immunol.* 172:7239–7245.

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SUPPLEMENTAL TEXT

Cell screening

We initially screened CD8 T cells recovered at different points of the immune reaction for the expression of 14 effector genes, and we found that the T_{H2} cytokine genes (*Il4*, *Il5*, and *Il13*), *Lta*, and *Il15* were never expressed, and that *Il10* expression was extremely rare. At some time points, we could find a single cell scoring positive for this cytokine, but, more frequently, all cells were negative. *Il2* mRNA expression was relatively more abundant because two to three cells per time point were usually scored. The expression frequency of these rare genes is too low to allow accurate evaluation at a single-cell level and is not described further.

Cell coexpression

We enumerated the secondary memory cells coexpressing *Prf1 and Gzmb* (and thus potentially able to use the efficient perforin killer pathway), as well as those coexpressing *Fasl*, which thus have the potential to use both perforin and Fas-L killer pathways simultaneously. We found 15% of the former and an additional 13% of the latter cell types in SM-CD8s. Thus, SM-CD8s had ~28% of cells potentially able to mediate effective killing. In contrast, in PM-CD8s only 3/45 cells coexpressed *Prf1* and *Gzmb*, and 1/45 PM-CD8 cells coexpressed *Prf1 Gzmb* and *Fasl* (Fisher's exact test, $P < 0.01$).

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