Concurrent Generation of Effector and Central Memory CD8 T Cells during Vaccinia Virus Infection

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Abstract

It is generally thought that during the contraction phase of an acute anti-viral T cell response, the effector T cells that escape activation-induced cell death eventually differentiate into central memory T cells over the next several weeks. Here we report that antigen-specific CD8 T cells with the phenotype and function of central memory cells develop concomitantly with effector T cells during vaccinia virus (vv) infection. As soon as 5 days after an intraperitoneal infection with vv, we could identify a subset of CD44hi and CD62L+ vv-specific CD8 T cells in the peritoneal exudate lymphocytes. This population constituted approximately 10% of all antigen-specific T cells and like central memory T cells, they also expressed high levels of CCR7 and IL-7R but expressed little granzyme B. Importantly, upon adoptive transfer into naive congeneric hosts, CD62L+, but not CD62L− CD8 T cells were able to expand and mediate a rapid recall response to a new vv challenge initiated 6 weeks after transfer, confirming that the CD62L+ vv-specific CD8 T cells are bona fide memory cells. Our results are thus consistent with the branched differentiation model, where effector and memory cells develop simultaneously. These results are likely to have implications in the context of vaccine design, particularly those based on vaccinia virus recombinants.

Introduction

After a viral infection, naïve T cells expand enormously and differentiate into effector cells that control the infection [1,2]. After clearance of antigen, most of the effector T cells are eliminated by activation-induced cell death (AICD). However during this process, a few cells develop into memory T cells that survive for extended periods of time and mediate a rapid and robust recall response following a subsequent infection by the same pathogen (reviewed in [3–5]). Based on the homing characteristics and effector functions, considerable heterogeneity seems to exist within the memory CD8 T cell population and at least two subsets have been widely described [6–8]. One is designated as effector-memory T cells (CD8EM) that do not express the lymph node homing molecules CD62L and CCR7. This cell subpopulation is highly cytolytic, express high levels of molecules required for cell killing such as granzymes, but express little IL-7R and persist after antigen clearance predominately in nonlymphoid tissues [7]. The other is called the central memory cells (CD8CM), which do not express the CD62L and CCR7, but mediate a rapid recall response following a subsequent infection by the same pathogen. The presence of CCR7 and IL-7R but expressed little granzyme B. Importantly, upon adoptive transfer into naive congeneric hosts, CD62L+, but not CD62L− CD8 T cells were able to expand and mediate a rapid recall response to a new vv challenge initiated 6 weeks after transfer, confirming that the CD62L+ vv-specific CD8 T cells are bona fide memory cells. Our results are thus consistent with the branched differentiation model, where effector and memory cells develop simultaneously. These results are likely to have implications in the context of vaccine design, particularly those based on vaccinia virus recombinants.

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this study, we address this issue in the context of vaccinia virus. We were able to identify antigen-specific CD8 T cells with typical features of memory T cells, concomitantly with effector T cells at early time points after infection.

Results and Discussion

Identification of antigen-specific CD8 T cells with a memory phenotype during an acute listeria monocytogenes infection in cell transfer settings

We initially tested if memory-like cells can be identified at early time points during Lm infection. We reasoned that if effector and memory T cells are simultaneously generated from the asymmetric division of a T cell after it responds to a microbial challenge, a subpopulation with central memory phenotype should be identified concurrently with the effector subset early after infection. To test this, we used an adoptive transfer model using CD8 T cells from P14 (specific for LCMV gp33–41) mice crossed to T-GFP mice. The advantage of this system is that it allows the detection of antigen specific cells by MHC-peptide tetramer staining and simultaneously distinguishes the naive from activated T cells because, GFP expressed by naive T cells is turned off once the cells start dividing after activation [14]. C57 mice were transferred with CD8 T cells isolated from naive P14 xT-GFP mice (Fig. 1a left panel) and 3 days later, infected ip with recombinant Lm expressing gp33 epitope (rLmgp33, 10^5 CFU). Eight days after infection, their peritoneal exudate lymphocytes (PEL) were tested for the presence and phenotype of the transferred donor cells. We chose to monitor the expression of CD62L because it is a key marker that segregates the effector and transferred donor cells. We examined the expression of CD62L because it is a key marker that segregates the effector and memory T cell subsets in combination with CD44, which is expressed at high levels in all effector and memory but not in naive T cells [14,19]. This analysis revealed the presence of two major subsets. A majority of gp33 tetramer+ CD8 T cells (85.3±6.7%) were CD44hi CD62Lhi, a profile that is reminiscent of the effector phenotype. However a significant proportion of cells (14.7±5.9%) was CD44hi CD62L− a phenotype that generally typifies central memory cells [6,14] (Fig. 1, right panel and 1 b). Moreover they also had lost GFP expression (Fig. 1c), indicating that they were not input naive cells, but were activated and had undergone cell division. Thus, our data suggest that even at the acute phase of a primary response, a population of post-mitotic CD44hi CD62Lhi CD8 T cells can be detected.

Antigen-specific CD8+ T cells with a central memory phenotype can also be detected at early time points after a natural infection with vaccinia virus

One limitation of the above experiments is that the ‘unnatural’ numbers of antigen-specific T cell precursors present in the adoptive transfer system may not accurately reflect the situation that occurs during a natural infection. For instance, because of excessive precursor numbers, the kinetics T cell differentiation may be faster than that occurring in a natural infection. Thus, to determine whether the observed CD8+ T cell subset with a memory phenotype (CD44hi CD62Lhi) also occurs under physiological conditions, we studied the antigen-specific CD8 T cell differentiation during vaccinia virus infection. First, we determined the kinetics of vaccinia-specific T cell response in different organs following an ip infection with vv using the recently described immunodominant B8R20–27 peptide (TSYPFSV) [20] / MHC pentamers, VV-specific CD8 T cell response peaked on day 8 and the maximal response was seen in the PEL, where the B8R20–27 pentamer+ cells constituted 10–20% of CD8 T cells, followed by spleen, but few antigen-specific cells were detectable in the other lymphoid organs (Fig. 2a, b). Because the maximal response was seen in the PEL compartment, we used PEL CD8 T cells for further studies.

We examined the B8R20–27 pentamer+ CD8 T cells for expression of CD62L and CD44 at different times after infection. Strikingly, ~10–20% of B8R20–27 pentamer+ cells were CD44hi CD62Lhi and these cells could be detected as soon as any pentamer positive cells could be detected, starting on day 5 after infection (Fig. 2c). Moreover, they were continually present throughout the 30-day observation period (Fig. 2c, d). CD62Lhi cells peaked on day 8 (constituting ~20%) and remained at approximately 10% of B8R20–27 pentamer+ cells throughout the study period (Fig. 2c, d). Thus, antigen-specific T cells with CD62Lhi CD44hi phenotype can also be detected at early periods during a normal immune response to a viral challenge. To our knowledge, this is the first report of detection of CD62Lhi pentamer+ antigen specific CD8 T cells during an acute infection. We further characterized this subset using markers that differentiate naive, effector and memory T cells in a four-color flow cytometric analysis. On day 7 post-infection (at the peak of response), the CD62L negative and CD62L positive B8R20–27 pentamer+ CD8 gated cells were examined for the expression of CCR7, IL-7R, IL-15R, IL-18R (by external staining) and granzyme B (by internal staining). As Fig. 3a shows, a majority of CD62L− B8R20–27 pentamer+ cells did not express CCR7 or IL-7R, but expressed IL-15R, IL-18R and granzyme B, typical of effector cells [8,21]. In contrast, a majority of CD62Lhi B8R20–27 pentamer+ cells expressed CCR7 and IL-7R but little granzyme B. However they also expressed IL-15R and IL-18R. We also tested the phenotypic profile of CD62L+ and CD62L− B8R20–27...

Figure 1. Identification of CD44hi CD62Lhi CD8 T cell population during an acute infection with listeria monocytogenes. C57 mice were adoptively transferred with CD8 T cells isolated from uninfected naive P14XT-GFP mice (a, input, left panel) and infected with rLmgp33. Eight days later, PELs from infected mice were examined for the presence and phenotype of transferred cells. A representative dot plot (a, right panel) and cumulative data from 6 mice (b) of CD44 and CD62L expression by CD8 and gp33Db tetramer-gated cells is shown. The bar graph in (c) shows the mean fluorescence intensity (MFI) of GFP expression by the transferred input donor cells and CD62L+ and CD62L gp33Db tetramer+ cells in recipient mice after infection. Data are presented as mean±s.d. from two independent experiments with 3 mice per experiment. doi:10.1371/journal.pone.0004089.g001
pentamer+ cells on day 5 post-infection, which is the earliest time at which any B8R20–27 pentamer+ CD8+ T cells could be discerned (Fig. 2c). Even here, the CD62L+ antigen-specific cells expressed CCR7 and IL-7R while the majority of CD62L- cells were CCR7-, IL-7R- (Fig. 3b). Thus, antigen-specific CD8+ T cells exhibiting a central memory phenotype can be detected as soon as 5 days after a vaccinia infection. Although naive T cells also express CCR7 and IL-7R and do not express granzyme B, the CD62L+ B8R20–27 pentamer+ cells are unlikely to be naive cells because they expressed high levels of CD44, IL-15R and IL-18R (Fig. 3a, b) and NKG2A (data not shown). Moreover, because naive T cells specific for a given TCR specificity are thought be extremely few (estimated as 10–100 cells/animal in mice) [2,22], no study has reported the presence of naive antigen-specific T cells during an infection. Further, naive T cells circulate from blood to lymphoid organs and are thought not to traffic to tissue sites of infection such as PEL [23]. Additionally, it is highly unlikely that naive cells are being recruited into the peritoneal cavity even after the clearance of the antigen (typically vaccinia infection is resolved completely in 10–14 days) [24]. Taken together, our results

Figure 2. Kinetics and phenotype of antigen-specific CD8+ T response during an acute infection with vaccinia virus. C57BL/6 mice were i.p. infected with vv and at different time points after infection, the presence of B8R20–27 pentamer+ CD8+ T cells in different tissues was assessed by flow cytometry. Representative dot plot of results on day 8 (a) and cumulative data from 12 mice at different time points (b) on the presence of B8R20–27 pentamer+ CD8+ T cells are shown (SPL, spleen; BM, bone marrow; PLN, peripheral (inguinal and axillaries) lymph nodes; MLN, mesenteric lymph node; Para-A-LN, Para aortic lymph node). (c) Mice were infected with vv as in (a) and at indicated times post-infection, their PELs were tested for the presence and phenotype of B8R20–27 CD6+ T cells. Representative dot plots in the left panel show the percent of B8R20–27 pentamer+ CD8+ T cells and the right panel shows CD62L and CD44 expression by the B8R20–27 pentamer+ CD8+ gated cells (n = 4). (d) shows the cumulative data on B8R20–27 pentamer+ T cells as a percent of CD8 T cells in PEL (upper panel) and CD62L+ B8R20–27 pentamer+ T-cells as a percent of total B8R20–27 T-cells (lower panel).

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strongly suggest that the CD62L⁺ B8R20–27 pentamer⁺ cells are not naïve, but have already been activated with antigen and express the phenotypic features of central memory T cells and that this cell population can be found concomitantly with effector T cells during a primary immune response.

CD62L⁺ B8R20–27 pentamer⁺ cells mount long-term memory response to rechallenge with vv

A hallmark of memory CD8 T cells is long-term survival and ability to mediate a rapid recall response following rechallenge with the pathogen. Because the CD62L⁺ B8R20–27 pentamer⁺ cells exhibited the phenotypic characteristics of central memory cells, we tested if they are also capable of memory functions. Since the presence of neutralizing antibodies may complicate the analysis of CD8 T cell memory function during a second infection in the same mouse [24], we performed adoptive transfer experiments for these studies using congenic mice (Fig. 4a). We first tested the potential for in vivo survival and function of CD62L⁺ and CD62L⁻ CD8 T cell populations after transfer to naïve uninfected recipient mice. C57 (Thy1.2) mice were infected with vv and 15 days later, their PEL CD8 T cells were negatively selected using the R&D kit. The CD62L⁻ and CD62L⁺ B8R20–27 pentamer positive cells constituted ~10 and ~0.9%, respectively, of PEL CD8 T cells. The CD8 T cells were further immunomagnetically sorted and equivalent numbers (10⁶ donor cells/mouse) of CD62L⁻ and CD62L⁺ CD8 T cells (C57, Thy1.2) were i.v. injected into naive congenic mice (C57, Thy1.1). Because TCR cross linking might restimulate the cells, we did not use pentamer staining for sorting the cells. Since the CD62L⁺ B8R20–27 pentamer⁺ cells constituted 10% of total B8R20–27 pentamer⁺ CD8 T cells in the PEL and we transferred equal numbers of CD62L⁻ and CD62L⁺ CD8 T cells, we actually transferred ~10 times more CD62L⁻ than CD62L⁺ B8R20–27 pentamer⁺ cells/
recipient mouse (equivalent to \(10^7\) CD62L- and \(10^6\) CD62L+ pentamer+ cells). To test the survival and proliferative capacity of transferred donor cells, the recipient mice were challenged 8 days after transfer with vv i.p. and their PELs were examined for Thy1.2+ donor-derived pentamer+ cells 3 days after viral challenge. Despite the fact that \(~10\) times more CD62L- than CD62L+ cells had been transferred, pentamer+ CD8 T cells of donor origin (Thy1.2) could only be detected in the CD62L+ cell transferred mice (Fig. 4b, left panel), suggesting that the CD62L+ cells could survive in a naive host. The CD62L- cells isolated during the acute phase of infection failed to survive in antigen-naı¨ve hosts after transfer probably because they represent fully differentiated effector cells that are destined to die upon sudden withdrawal from antigen and/or the cytokine milieu associated with infection (a situation different from the endogenous CD62L+ cells seen at late periods after infection shown in Fig. 2c, which probably represent “effector memory” cells). In contrast, the CD62L+ cells were capable of survival probably because they represent central memory type of cells that are capable of survival in the absence of antigen. Moreover, a substantial portion of CD62L+ donor cells were also capable of interferon-γ production in response to ex-vivo stimulation with B8R20–27 peptide (Fig. 3b, right panel). In addition, the viral burden in the CD62L+ CD8 T cell transferred mice was substantially lower compared to CD62L- CD8 T cell transferred mice (Fig. 4c).
A significant number of donor-derived CD8 T cells and showed significant expansion (Fig. 4d). Under these conditions, it is only the CD62L+ recipient mice for 6 weeks before challenging with vv infection. Even repeated the adoptive transfer experiments and this time rested the memory cells should be capable of long-term survival. To test this, we generated early after vaccinia virus infection survive in vivo for at least 8 days and mount an effector response to rechallenge, true memory cells should be capable of long-term survival. To test this, we repeated the adoptive transfer experiments this time rested the recipient mice for 6 weeks before challenging with vv infection. Even under these conditions, it is only the CD62L+ transferred cells that showed significant expansion (Fig. 4d). The donor derived CD62L+ cells detected appear to be vaccinia-specific because when we transferred similar numbers of CD62L+ CD8 T cells from naive (vv uninfected) mice, we could hardly detect any donor cells in the PEL of vv infected recipients (data not shown). Moreover, the recipient mice that received the CD62L+ cells (from vv-infected donors) showed significantly reduced viral burden compared to CD62L- cell transferred mice (Fig. 4c). Thus, the CD62L+ CD8 T cells generated after 15 days of infection appear to be memory T cells.

It has recently been suggested that memory precursors can be identified within the effector cell population at early time points after infection and that the conversion of effector to central memory CD8 T cells occurs gradually starting from the first week of stimulation in vivo [9,25]. Thus a possibility remained that by day 15 after vv infection, memory cells could have arisen from differentiated effector T cells. To test this possibility, we repeated the adoptive transfer experiments using effector and memory phenotype cells isolated from mice 7 days after infection (the limited number of antigen-specific T cells produced precluded us from using the earliest time point of post-infection day 5). CD62L+ and CD62L- CD8 T cells from the PELs of 7 day vv-infected Thy1.2 mice was isolated by FCAS-based cell sorting and transferred to naive Thy1.1 mice. The recipient mice were rested for 6 weeks, infected with vv and 3 days later their PEL examined for the presence of donor-derived Thy1.2+ CD8 T cells. Even here, a significant number of donor-derived CD8 T cells and interferon γ production could be detected in the CD62L+ cell transferred mice but not in the CD62L- cell transferred mice (Fig. 4f, g). Moreover, viral titers were also lower in the CD62L+ cell transferred mice compared to CD62L- cell transferred mice (Fig. 4h). Thus, as early as 7 days after an acute vaccinia infection, central memory-like CD8 T cells, with capacity to survive in the absence of antigen for long periods of time and to expand and protect against a reinfection can be detected.

Taken together our results suggest that central memory CD8 T cells are generated simultaneously with effector T cells during vaccinia infection. These results are in agreement with the studies from the Reiner’s group which found that memory CD8 T cells arise by asymmetric division of antigen-stimulated cells, implying that both cell types can arise from a common progenitor very early in the response to infection [17]. However it should be cautioned that from our studies, we can not definitively conclude that the same antigen-specific CD8 T cell precursor is giving rise to both effector and memory cells.

Unlike our results with vaccinia infection, cells with the phenotypic and functional characteristic of central memory cells are not seen in the acute phase of LCMV infection and based on those results, it has been concluded that memory cells are descendants of effector cells and develop gradually after the clearance of antigen [25]. Why is the situation different in vaccinia infection is not clear. However, numerous variables including antigen density and persistence, costimulation, cytokines and CD4+ help are known to determine the quantity, quality and location of memory cells [26]. Although both vv and LCMV are acute infections, the antigen load and persistence is more in LCMV than vv. Moreover, the induction of costimulatory molecules as well as the response to blockade of costimulatory interaction also differs between LCMV and vv [27]. Furthermore, we have previously shown that cytokines IL-2 and IL-15 can differentially regulate the fate of antigen activated CD8 T cells into becoming either effector or memory cells [14]. Thus, it is possible that differences in the antigen density/costimulatory signals or cytokine response between LCMV and vaccinia infection could account for the differences between our results and those reported with LCMV in the literature. A study by Bachman et al also reported that the balance between effector and memory T cells is governed by the degree of antigen load and time [28]. However, using gp33-specific TCR Tg CD8 T cells as surrogate antigen-specific cells, they found that although CD62L+ memory cells proliferate in vivo upon infection with both LCMV and vaccinia, they clear LCMV but not vaccinia efficiently. This is not consistent with our results because we found both proliferation and protection to be mediated by central memory rather than effector cells following vaccinia rechallenge. Differences in the experimental systems might account for these differences. For e.g. Bachman et al used LCMV gp33-specific TCR Tg memory CD8 T cells generated during an LCMV infection as surrogate vaccinia-specific cells to detect responses against a recombinant vaccinia expressing gp33 and thus are looking at one surrogate epitope-responder T cells, we used memory CD8T cells during a native vaccinia infection (containing all naive vaccinia-responder cells) to measure protection following rechallenge. Similarly, Richards et al also used a influenza NP68 epitope-specific T cells as surrogate vaccinia specific T cells to measure responses to a recombinant vv expressing NP68 epitope and found that proteolytic cleavage of CD62L is required for efficient clearance of vaccinia infection [29]. This is however, not inconsistent with our results since the wild type vaccinia-specific T cells (in this study) are not defective for CD62L cleavage.

Finally, it is also noteworthy that most studies on LCMV have focused on events in the spleen after an iv infection, while we characterized the cell populations after vaccinia infection in the peritoneal cavity after an ip infection. Thus, another possibility is that the activated CD8 T cell fate may also vary with different routes of infection and/or at different tissue sites of immune response. Further studies are needed to test these hypotheses, which may have implications particularly in improving the design of vaccinia-based vaccines.

Materials and Methods

**Mice**

C57BL/6 (C57; H-2Db, Thy1.2+), C57BL/6 (C57; H-2Db, Thy1.1+) were purchased from Jackson Laboratory. P14 X T-GFP mice have been described [14]. All mice were maintained in specific pathogen free conditions and used when they were 6–10 weeks of age. All animal experiments had been approved by the
Adoptive transfer and listeria infection

These were done as described earlier [30]. Briefly, naive CD8\(^+\) T cells were purified from the splenocytes of P14×T-GFP mice [14] by negative selection using the murine CD8 subset isolation kit (R&D Systems). Negatively selected CD8 T cells were then stained with succinated purified CD8 T cells using the murine CD8 subset isolation kit (R&D Systems). Positively and negatively selected CD8 T cells were subsequently isolated using a Miltenyi miniMACS system. In some experiments (to test memory function after 7 days of infection), the CD62L\(^+\) and CD62L\(^-\) CD8 T cells were FACSorted using FACSaria cell sorter (Becton Dickinson, San Jose, CA). Age and sex-matched recipient C57 (H-2Db, Thy1.1+) mice were i.v. injected with (1×10\(^6\) cells/mouse) purified CD62L\(^+\) or CD62L\(^-\) cells. At day 8 or 6 weeks post-transfer, recipient mice were i.p. challenged with vaccinia virus (10\(^6\) PFU/mouse in 200 µl PBS) and 3 days later, their peritoneal exudates lymphocytes (PEls) and ovaries were harvested. When examining the recipient mice PELs for the presence of donor derived T cells, the cells were also stained with Thy1.2 Ab to distinguish them from the Thy1.1 host CD8 T cells. For testing IFN-\(\gamma\) response, syngeneic bone marrow-derived DC were pulsed with B0R20-27 peptide (TSY-FESV; 5 µg/ml; synthesized at ProImmune), washed and used to stimulate PELs of secondary recipient mice. Non peptide-pulsed BMDC served as controls. After 18 hours of co-cultures, the intracellular IFN-\(\gamma\) production was measured as described previously [30].

Virus titration

Viral titration

Serial dilutions of homogenates of ovaries harvested from recipient mice were inoculated on CV-1 cells and after 2 days, stained with neutral red/formalin and the plaques were counted manually.

Statistical analysis

Differences in values between experimental groups were examined for significance with Graph Pad Prism software using Student \(t\) test. We considered probability (\(P\)) values \(< 0.05 as significant. Values are presented as means±SEM.

Author Contributions

Conceived and designed the experiments: NM. Performed the experiments: AL MM VH. Analyzed the data: NM. Wrote the paper: AL NM.

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