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Qualitative Differences Between Naive and Memory T Cells Make a Major Contribution to the More Rapid and Efficient Memory CD8+ T Cell Response

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CD8+ T cells are present at a higher frequency following a primary response, and these memory cells exhibit qualitative differences from naive cells. The importance of these differences vs increased precursor frequency in making a memory response more rapid and efficient has been unclear. Adoptive transfer of 2C TCR transgenic CD8+ T cells into normal recipients, followed by i.p. challenge with allogeneic P815 tumor, results in a long-lived memory population that includes both endogenous host CD8+ T cells and 2C cells. The 2C cells can be identified, using 1B2 mAb specific for the TCR, and thus used as an indicator of the properties of the memory cells. The memory cells have a heterogeneous surface phenotype, and their distribution in lymphoid organs, blood, and peripheral sites is distinct from that of naive cells. Upon rechallenge with Ag, memory cells access the peritoneal cavity much more rapidly than do naive cells (12 h vs 5 days). This appears to result from a requirement for naive cells to interact with Ag before they can efficiently migrate to inflammatory sites, while this is not required for memory cells. In addition, memory cells exhibit some cytolytic activity before rechallenge with Ag, and potent cytolytic activity is present in the peritoneal cavity within 12 h of rechallenge. Comparison of primary and memory responses in mice having similar frequencies of Ag-specific precursors demonstrated that the more rapid migration and the immediate effector function of at least some memory cells contribute very substantially to making a memory response at a peripheral site more rapid and efficient. *The Journal of Immunology*, 1998, 161: 674–683.

Naive T cells clonally expand in response to an initial exposure to Ag and persist in higher numbers following the primary response (1–3). Upon reexposure to Ag, these memory cells make a more rapid and vigorous response. The increased frequency of Ag-specific cells is a contributing factor in the more vigorous memory response in comparison with a primary response. Memory T cells also differ qualitatively from naive T cells, however, in that they express higher levels of several adhesion and activation molecules (4). This may, at least in part, contribute to their ability to be more readily activated by lower doses of Ag (5, 6) and their lesser dependence on costimulatory receptor-ligand interactions (7–9).

Several of the surface proteins that have altered expression on memory cells are also likely to contribute to the distinct recirculation pathways of the two cell types, with memory cells selectively trafficking from blood to peripheral tissues while naive cells selectively traffic from blood to lymph nodes (10–12). This broader immune surveillance by memory cells could contribute to more rapid responses, particularly to Ag challenge at peripheral sites. Finally, recent reports have indicated that at least some memory CD8+ T cells either retain active effector function or very rapidly reacquire it upon exposure to Ag (13, 14), in contrast to the 2 to 3 days of differentiation required for naive cells to gain these functions. Whether the greater speed and efficiency of a memory response is simply due to increased precursor frequency, or is influenced by the qualitative differences between naive and memory cells, has remained controversial. An evaluation of the extent to which these factors contribute to enhanced memory responses requires in vivo examination of the Ag-specific cells during the response.

Adoptive transfer of TCR transgenic T cells into normal recipients, coupled with use of an anti-TCR mAb to identify the transferred cells, provides a powerful means of studying Ag-specific cells in vivo (5). The locations, numbers, and surface phenotype of the cells can be readily determined by flow cytometry during the course of a response, while the relatively small number of transgenic cells in the recipient does not significantly skew the normal immune response. Using this approach to study the CD8+ T cell memory response to lymphocytic choriomeningitis virus (LCMV) infection, Zimmerman et al. (13) have characterized the surface phenotype of the memory cells and demonstrated that at least some of the cells are directly cytolytic when isolated from the recipients. A substantial fraction of the memory cells were found to be undergoing cell division, as assessed by BrdU labeling; whether persistent viral Ag was involved in stimulating this was not examined. Because systemic infection with lymphocytic choriomeningitis virus results in widely disseminated Ag, and Ag is presented to CD8+ T cells by host APC, issues of T cell trafficking would be difficult to study in this model.

Adoptive transfer of CD8+ T cells transgenic for the 2C TCR (15), specific for Ld allotype, allows the in vivo response to challenge with allogeneic tumor in the peritoneal cavity to be visualized using the 1B2+ mAb specific for the 2C TCR (16). Since

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the Ag recognized by the 2C receptor is native H-2L<sup>d</sup> complexed with the p2CA peptide (17), re-presentation to the 2C cells by host APC cannot occur. Thus, the location of stimulatory Ag is limited and can be determined by flow cytometry, allowing study of the trafficking of the Ag-specific cells and their site(s) of activation. When adoptive transfer recipients are challenged by i.p. injection of P815 allogeneic tumor, a primary response occurs that involves both endogenous host alloantigen-specific CD8<sup>+</sup> T cells and the transferred 2C cells, with the ability to identify the 2C cells making it possible to track Ag-specific cells during the response. The response is characterized by initial clonal expansion of the 1B2<sup>+</sup> 2C cells in the peritoneal cavity beginning on days 5 and 6 and peaking on day 8 (18). As expansion occurs in the peritoneal cavity; 1B2<sup>+</sup> cells that have been activated but are no longer proliferating migrate into the peripheral lymphoid organs and blood. The total number of 1B2<sup>+</sup> cells then decreases as tumor is eliminated and reaches a steady state level by about day 20 at a higher number than was present before antigenic challenge.

These previously challenged adoptive transfer recipients have a stable population of Ag-specific memory cells that can be identified using the 1B2 mAb and have made it possible to examine the CD8<sup>+</sup> T cell memory response with respect to memory phenotype, trafficking, sites of activation, and development of effector function. The results demonstrate that, while a higher frequency of Ag-specific cells contributes to a more efficient memory response, qualitative differences in trafficking to the site of Ag and development of effector function make major contributions to memory cells being more rapid and effective than naive cells in mounting a response.

Materials and Methods

**Mice**

Transgenic mice expressing the 2C TCR (15), a gift from Dr. Dennis Loh (Hoffman-LaRoche, Nutley, NJ), were bred to wild-type C57BL/6 mice to generate mice heterozygous for the 2C TCR transgene, and these were used as the source of TCR transgenic CD8<sup>+</sup> T cells in all of the experiments. C57BL/6/NxSnJ mice from Charles River Laboratories (Wilmington, MA) were used as recipients for the adoptive transfer at 4 to 6 wk of age. Mice were housed in a specific pathogen-free environment at all times.

**Antibodies**

The 1B2 mAb specific for the 2C TCR (16) (a gift from Dr. Herman Eisen, Massachusetts Institute of Technology, Boston, MA) was purified from culture supernatant of the 1B2 hybridoma using a protein A-Sepharose column with elution by citric acid at pH 3.5. The purified mAb in PBS was biotinylated by incubation for 4 h at room temperature with 0.15 mg/ml biotin and 1 mg/ml mAb. Other Abs used were Mel14, Mel14-FITC, anti-CD44-FITC (Pgp1), anti-CD25-FITC, anti-CD8<sup>α</sup>-phycoerythrin (PE), anti-CD8<sup>α</sup>-cytochrome, goat-anti-rat-FITC, streptavidin-phycoerythrin (SA-PE), streptavidin-APC (SA-APC), SA-perCP, anti-H-2K<sup>b</sup>-biotin, anti-H-2K<sup>b</sup>-FITC, and rat- and mouse-FITC isotype controls. All conjugated Abs were purchased from PharMingen (San Diego, CA), with the exceptions of the goat-anti-rat-FITC and isotype controls that were purchased from Jackson ImmunoResearch (West Grove, PA). SA-PerCP was purchased from Becton Dickinson (San Jose, CA).

**Cell lines**

The mastocytoma P815 (H-2<sup>d</sup>), lymphoma RDM4 (H-2<sup>d</sup>), the thymoma EL4 (H-2<sup>b</sup>), and H-2<sup>L</sup>-transfected EL4 cells (EL4-L<sup>d</sup>) were grown in RPMI 1640 media (Cellgro, Herndon, VA) with 10% FCS, 0.2% glutamine, 0.1% pen/strep, 0.1% HEPES, 0.1% nonessential amino acids, 0.01% sodium pyruvate, and 0.05% β-mercaptoethanol. Cultures were always 80 to 90% viable (by trypan blue exclusion), and cells were washed with PBS before use for i.p. injections or in vitro chromium release assays.

Adaptive transfer of TCR transgenic cells and Ag priming

Lymph node cells (axillary, brachial, cervical, inguinal, periaortic, mediastinal, and mesenteric) from heterozygous 2C transgenic mice were re-mobilized, homogenized, and washed. Three times with 10<sup>6</sup> CD8<sup>+</sup> cells (2.3 × 10<sup>5</sup>) were transferred into sex-matched naive C57BL/6 mice by tail vein injection.Recipient mice were rested for a period of 1 to 5 days before priming by i.p. injection of 5 × 10<sup>6</sup> P815 tumor cells. Where noted, 1B2<sup>+</sup> cells and P815 tumor were injected simultaneously i.p. The primary response was analyzed over 2 to 20 days following this initial priming. Memory responses were analyzed in mice that had been primed with P815 tumor 40 to 60 days previously and were then rechallenged by i.p. injection of 5 × 10<sup>6</sup> P815 or RDM4 tumor cells, and the response was analyzed from 12 h to 30 days after rechallenge.

Flow cytometric analysis of primary and memory responses

Mice were sacrificed at varying times after priming or rechallenge, and the LN and spleen were removed, homogenized, and ammonium chloride treated to lyse red blood cells. Brachial, axillary, and cervical LN were pooled as distant peripheral nodes, and periaortic, mediastinal, and mesenteric nodes as draining LN. Peripheral blood was drawn from the heart using a heparin-loaded syringe, and the red blood cells were lysed by ammonium chloride treatment. The peritoneal cavity was washed with 50 ml of PBS, and the resulting peritoneal exudate lymphocytes (PEL) were adherence depleted for 90 min in complete media at 37°C. Each cell population was counted for total cell number using trypan blue to exclude dead cells.

Cells (1–2 × 10<sup>6</sup>) isolated from each site were stained with 1B2-biotin mAb, PE-conjugated anti-mouse CD8<sup>α</sup>, and one of the following FITC-conjugated mAb: CD25, CD44, a4 (VLA-4), or Mel14. After washing, SA-PerCP or SA-APC were added for detection of 1B2-biotin. Stained cells were fixed with 1% formaldehyde and analyzed by three- or four-color flow cytometry using the CellQuest software package (Becton Dickinson). Cytometer settings were identical for all time points within a given experiment. For determination of the total number of 1B2<sup>+</sup> CD8<sup>+</sup> cells, 50 to 100 × 10<sup>3</sup> events were analyzed (see Fig. 2A), and the percent of 1B2<sup>+</sup> CD8<sup>+</sup> cells was multiplied by the total number of cells recovered from the site. This reliably detects the 2C cells. Staining and detecting in this way shows a background of less than 0.01% 1B2<sup>+</sup> CD8<sup>+</sup> cells in LN from normal C57BL/6 mice that have not received 2C cells (Ref. 18; data not shown), while LN from mice that have received 3 × 10<sup>6</sup> transferred cells have about 0.23% 1B2<sup>+</sup> CD8<sup>+</sup> cells in the LN. In contrast, background in this quadrant is minimal in the transferred mice when the 1B2 mAb is replaced with an isotype control (data not shown).

Phenotypes of the 2C cells from the various sites were determined by gating on the 1B2<sup>+</sup> CD8<sup>+</sup> cells and collecting 200 to 500 events examining the FITC fluorescence of the phenotype marker Abs. Gates denoting low and high expression of each surface marker were set based on the phenotype of naive T cells, as shown in Figure 2B. Replacing the specific Abs with isotype control Abs resulted in almost no events falling into the high gate for each marker (data not shown). Cells from all sites were separately stained for the presence of P815 tumor using anti-H-2K<sup>b</sup>-biotin, with a second step SA-Cy or SA-APC, and anti-H-2K<sup>b</sup>-FITC. This protocol stains all endogenous cells (H-2K<sup>b</sup>) with cytochrome (FL3) or APC (FL4) and any P815 tumor present (H-2K<sup>c</sup>) with FITC (FL1). By excluding all FL3<sup>+</sup> events, any tumor present can be easily detected down to a frequency as low as 0.01% of total cells (data not shown). The total number of tumor cells at the various sites was determined by multiplying the percent of tumor cells in the population by the total number of cells recovered from each site.

Chromium release assay and calculation of lytic units

PEL were assayed for lytic activity following depletion of adherent cells using a standard 4-h<sup>3</sup>Cr release assay with EL4-L<sup>d</sup> target cells. LU at 50% target cell lysis (LU<sub>50</sub>) were calculated, with 1 LU<sub>50</sub> defined as the number of effector cells required to lyse 50% of the target cells. For some experiments, lytic activity is expressed based on the number of 1B2<sup>+</sup> cells in the population (as determined by flow cytometry), rather than on total cells. EL4 cells were used as the syngeneic control in all lytic assays. Up to day 5 of the primary response, some lysis of EL4 targets by PEL could be detected, consistent with published results by Nishi et al. (19). By day 6 of the primary response, lysis of EL4 targets was always less than 5%. On days where release from control targets was higher than 5%, the value was subtracted from the percent lysis of the specific target to achieve a more appropriate estimate of specific target lysis.

<sup>1</sup>Abbreviations used in this paper: PE, phycoerythrin; SA, streptavidin; LN, lymph node; PEL, peritoneal exudate lymphocytes; VLA, very late Ag.
detected in the peritoneal cavity on day 6 (18). The number of i.v. injected 2C TCR transgenic mice were in-
troduced into naive C57BL/6 recipients using 2 to 3
lymph node (LN) cells from 2C TCR transgenic mice were in-
using a third Ab and gating on the CD8
in the recipients by staining with anti-CD8 mAb and the 1B2 mAb
C57BL/6 mice received 10^6 1B2
transferred recipients challenged by i.p. injection of P815 tumor cells. The tumor cell line, bearing the L_d/p2Ca for which the 2C TCR is
Mice were sacrificed at various times after priming, and the total number
above that of recipients that were not primed with Ag (Fig. 1), and
mains constant for at least 2 to 3 mo at a frequency 5- to 10-fold
the number decreases at all sites through day 20 and then re-
memory cells were done using cells from mice that had not been activated in the primary response.
1B2
the ability of memory cells to recirculate through LN (22). Al-
though 1B2
cells could not be detected in unimmunized recipients by sixty days posttransfer (Fig. 1), some 1B2
cells with a naive phenotype (CD44lo, VLA-4lo) could be seen in the primed mice (Fig. 2B), suggesting either a reversion of some memory cells to a naive phenotype or an enhanced survival of naive cells that had not been activated in the primary response.

The number of 1B2
cells in the peritoneal cavity increases about 10-fold within 12 h of rechallenge with P815 tumor i.p. (Fig. 3A) and continues to increase through day 3 (Fig. 3B). As this is occurring, the 1B2
cells are decreasing in number in the spleen and blood through the first 1 to 2 days of the response (Fig. 3B). The rapid increase in the peritoneal cavity occurs too quickly to be accounted for by proliferation of the small number of 1B2
cells present at this site at the time Ag is injected. Rather, it appears that the majority of these are cells migrating into the peritoneal cavity from the spleen and blood. A similar pattern of decreasing numbers of Ag-specific cells in the spleen and blood as cells appear in the peritoneal cavity is seen in the primary response of 2C cells in adoptive transfer recipients challenged i.p. with P815 (18). The primary and memory responses differ dramatically, however, with respect to the timing of these events. 1B2
cells increase in number in the peritoneal cavity within 12 h in a memory response (Fig. 3, A and B), but not until day 5 after challenge in a primary re-
response (18). After day 2 to 3, the number of 1B2
cells in the peritoneal cavity begins to decrease. During this time, however, there is a large increase in the numbers of 1B2
cells in the spleen and blood (Fig. 3B). At longer times, 1B2
cells persist, primarily in the spleen and blood, at higher numbers than were present in the memory population before rechallenge (data not shown).

In a primary response to P815 in adoptive transfer recipients, tumor is not eliminated from the peritoneal cavity until days 8 to 10, and from the spleen shortly thereafter (18). In contrast, tumor was very rapidly eliminated in the secondary response, becoming undetectable in both the peritoneal cavity and all lymphoid organs by 12 to 36 h after challenge (see below). This suggested that Ag-specific CD8
T cells, both endogenous and transferred 2C cells, were able to initiate a much more rapid effector response than naive cells. To determine the presence of lytic function at various stages in the response, L_d-specific lytic activity was mea-
sured using an EL4 cell line expressing H-2L^d as the target (EL4-L_d) in standard 4-h chromium release assays. Use of EL4-L^d as the target measures L_d-specific killing mediated by both the

Results

Location and phenotype of memory cells
To directly examine the activity of memory CD8
T cells in vivo, lymph node (LN) cells from 2C TCR transgenic mice were in-
jected i.v. into naive C57BL/6 recipients using 2 to 3 × 10^6
1B2
CD8
cells/mouse. Cells bearing the 2C TCR were detected in the recipients by staining with anti-CD8 mAb and the 1B2 mAb specific for the 2C TCR (16), and phenotype was characterized using a third Ab and gating on the CD8
1B2
1B2
cells maintain a naive phenotype in the recipients, and the numbers in the spleen, lymph nodes, and blood remain relatively constant for greater than 2 wk, after which there is a gradual decline in the number of 1B2
cells at all sites (Ref. 18; Fig. 1).

When recipient mice are primed by i.p. injection of the P815 tumor cell line, bearing the L^d/p2Ca for which the 2C TCR is specific (17), 1B2
cells having an activated phenotype are first detected in the peritoneal cavity on day 6 (18). The number of 1B2
cells then increases in the peritoneal cavity, draining LN, spleen, and blood to reach a maximum at day 8 (Fig. 1). After day 8, the number decreases at all sites through day 20 and then re-
mains constant for at least 2 to 3 mo at a frequency 5- to 10-fold above that of recipients that were not primed with Ag (Fig. 1), and 1B2
cells remain easily detectable in primed mice for at least 6 mo (data not shown). These long term memory cells remain func-
tional and respond rapidly to a secondary challenge with Ag as evidenced by clonal expansion beginning as early as 2 days after rechallenge (Fig. 1). All of the following experiments examining memory cells were done using cells from mice that had been primed at least 40 days previously by i.p. injection of P815 tumor.

In comparison with naive recipients, 1B2
cells in primed mice account for a greater percentage of the total cells in the LN, spleen, peritoneal cavity, and blood (Fig. 2A). This is particularly the case for blood and for the peritoneal cavity, where 1B2
cells are un-
detectable in naive recipients while a small but easily detectable population is present in the primed mice. The majority of 1B2
cells at all sites expressed high levels of CD44 and VLA-4 (Fig. 2B), a well-documented memory phenotype (20, 21). Naive 1B2
cells lose L-selectin from the surface during the initial phase of a primary in vivo response, but about half of the cells reacquire an L-selectin^{high} phenotype within a few days, while retaining high levels of CD44 and VLA-4 (18). Consistent with this, about half of the cells present in the spleen and peritoneal cavity of the primed mice express high levels of L-selectin (Fig. 2B). The fraction ex-
pressing high L-selectin is greater in LN, consistent with earlier work suggesting that L-selectin reexpression would contribute to the ability of memory cells to recirculate through LN (22).

Traffic, clonal expansion, and acquisition of lytic activity in response to rechallenge with Ag

The number of 1B2
cells in the peritoneal cavity increases about 10-fold within 12 h of rechallenge with P815 tumor i.p. (Fig. 3A) and continues to increase through day 3 (Fig. 3B). As this is oc-
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cells are decreasing in number in the spleen and blood through the first 1 to 2 days of the response (Fig. 3B). The rapid increase in the peritoneal cavity occurs too quickly to be accounted for by proliferation of the small number of 1B2
cells present at this site at the time Ag is injected. Rather, it appears that the majority of these are cells migrating into the peritoneal cavity from the spleen and blood. A similar pattern of decreasing numbers of Ag-specific cells in the spleen and blood as cells appear in the peritoneal cavity is seen in the primary response of 2C cells in adoptive transfer recipients challenged i.p. with P815 (18). The primary and memory responses differ dramatically, however, with respect to the timing of these events. 1B2
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In a primary response to P815 in adoptive transfer recipients, tumor is not eliminated from the peritoneal cavity until days 8 to 10, and from the spleen shortly thereafter (18). In contrast, tumor was very rapidly eliminated in the secondary response, becoming undetectable in both the peritoneal cavity and all lymphoid organs by 12 to 36 h after challenge (see below). This suggested that Ag-specific CD8
T cells, both endogenous and transferred 2C cells, were able to initiate a much more rapid effector response than naive cells. To determine the presence of lytic function at various stages in the response, L_d-specific lytic activity was mea-
sured using an EL4 cell line expressing H-2L^d as the target (EL4-L_d) in standard 4-h chromium release assays. Use of EL4-L^d as the target measures L_d-specific killing mediated by both the
endogenous and 2C CD8\(^+\) T cells. We estimate that about half of the lytic activity measured in these experiments is attributable to the 2C CTL based on studies using the 1B2 mAb to block killing by the 2C cells. It must be emphasized, however, that the purpose of these studies was not to establish the lytic activity of the 2C cells specifically, but of the entire Ag-specific effector CD8\(^+\) T cell pool that includes both 2C cells and endogenous host cells. The transferred 2C cells serve as a marker for the Ag-specific CD8\(^+\) population.

A low level of Ld\(^+\)-specific lytic activity can be detected for cells from the peritoneal cavity and spleen of previously primed adoptive transfer recipients before rechallenge with Ag (Fig. 4). By 12 h after rechallenge, Ld\(^+\)-specific lytic activity has increased dramatically in the peritoneal cavity. Thus, it appears that the memory population includes some cells that may have a low level of lytic activity in the absence of Ag stimulation and that lytic activity can increase very rapidly upon stimulation. Although tumor was no longer detectable by 12 to 36 h, lytic activity persisted in the peritoneal cavity and even increased in some experiments, through days 4–5, before returning to background levels. In the experiment shown in Figure 4B, there was a transient decrease in lytic activity at 36 h, and this was seen to varying degrees in three independent experiments (e.g., see Fig. 7C). This may result from loss of the rapidly activated subset of effectors from the peritoneal cavity (through death or migration from the site), followed by differentiation to effector status of a different subset of memory cells that behave more like naive cells in that they require 2 to 3 days to develop lytic function.

In comparison with the very rapid acquisition of lytic activity in the peritoneal cavity in the memory response, lytic activity does not develop until about day 6 in the primary response (Fig. 4B). Although the speed of acquisition of lytic function, and rate of tumor clearance, are dramatically increased in the secondary response, the maximal lytic activity that develops is significantly reduced in comparison to the primary response (Fig. 4B). However, equivalent levels of lytic activity are found when the lytic activities of primary and memory effector populations are compared on the basis of the number of 1B2\(^+\) cells (Fig. 4C) (the assumption being that the relative numbers of 2C cells are proportional to the total number of Ag-specific CTL), i.e., the primary and memory CTL have comparable lytic activities at the level of the individual cells. Thus, it appears likely that the greater activity generated in the primary response can be accounted for simply by the more extensive clonal expansion that occurs, probably as a

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**FIGURE 2.** Numbers and phenotype of 1B2\(^+\) memory cells in adoptive transfer recipients after priming with P815 tumor cells. C57BL/6 mice received 3 × 10\(^6\) 1B2\(^+\)CD8\(^-\) cells by i.v. injection and were challenged 1 day later by i.p. injection of 5 × 10\(^6\) P815 cells. Cells from these mice were analyzed 60 days later (Memory). In parallel, cells were analyzed from mice that had received the same number of 1B2\(^+\)CD8\(^-\) cells 3 days before the analysis (Naive). Draining LN, spleen, blood, and peritoneal cavity cells were isolated and analyzed by flow cytometry as described in Materials and Methods. A. Percent of 1B2\(^+\) cells at the various sites. B. Phenotype of 1B2\(^+\) cells at the various sites. 1B2\(^+\) cells were analyzed by gating on 1B2\(^+\)CD8\(^-\) events.
result of the tumor Ag reaching a much higher level by the time naive cells gain entry into the peritoneal cavity and begin expanding.

Sites of clonal expansion of memory cells

During a primary response in adoptive transfer recipients, the only location where large numbers of 1B2+ cells that are CD25+ and blasts can be detected is within the peritoneal cavity during days 5 to 7 (Fig. 5A; Ref. 18), suggesting that the majority of clonal expansion occurs there as opposed to within the secondary lymphoid tissue. In contrast, during days 2 to 3 of the memory response a significant percentage of 1B2+ cells at almost all sites are blasts, reaching as high as 50% of the 1B2+ cells in the draining LN, spleen, and peritoneal cavity (Fig. 5A). This suggests that some clonal expansion probably occurs at these sites, but the possibility of migration of blasts from the peritoneal cavity to these sites cannot be ruled out. Despite this increase in activated cells in the draining LN and spleen, there is little increase in lytic function of the cells at either of these sites at this time or any time thereafter (Fig. 5B; data not shown). This is again in contrast to the primary response where, by day 6 and later, 1B2+ cells are increasing in the spleen as they exit the peritoneal cavity after having undergone activation there. These cells are no longer blasts but retain potent lytic activity (Fig. 5B). Thus, following the initial response in the peritoneal cavity, expanded numbers of nonblasting but lytically active 1B2+ cells are present in the spleen in the primary response, while expanded numbers of blasting 1B2+ cells with little lytic activity are present in the spleen in the memory response.

Blast formation at multiple sites during the memory response is unlikely to be due to recognition of disseminated Ag at these sites, since tumor cannot be detected at any site by 12 h after rechallenge (data not shown, and see below). Thus, the blast transformation

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**FIGURE 3.** Trafficking and clonal expansion of 1B2+ memory cells in response to Ag challenge. Adoptive transfer recipients that had been primed with P815 tumor 60 days previously were rechallenged by i.p. injection of $5 \times 10^7$ P815 cells. Mice were sacrificed at the times indicated (day 0 being the day of rechallenge), and cells from spleen, peritoneal cavity, and blood were analyzed by flow cytometry as described in Materials and Methods. A, Percentage of 1B2+ cells in the peritoneal cavity before rechallenge (0 h) and 12 h after rechallenge. B, Number of 1B2+ cells in the spleen and peritoneal cavity (left axis) and percentage of 1B2+ cells in the blood (right axis) following rechallenge with P815 tumor. Two mice were examined at each time point. The results shown are the average and range of the values found.

**FIGURE 4.** Development of lytic activity during the memory response to rechallenge with P815 tumor cells. Lytic activity of cells isolated from adoptive transfer recipients at varying times after challenge with P815 was determined in a 4-h $^{51}$Cr release assay using EL4-L d targets. A, Cells were removed from the peritoneal cavity and spleen of previously primed (62 days) recipients before rechallenge (0 h) or 12 h after rechallenge by i.p. injection of P815 tumor cells. Cells from two mice at 12 h were examined separately. B, Time course for lytic activity during the primary and memory response. For the primary response, mice were adoptively transferred with 2C cells on day 21 and challenged by i.p. injection of P815 tumor on day 0. For the memory response, adoptive transfer recipients were primed by i.p. injection of P815 and rechallenged 62 days later (day 0 on graph) by i.p. injection of P815. Cells were harvested from the peritoneal cavity at the indicated times, and cytolytic activity was determined. Cells from two mice were examined at each time point. Values shown are the average, and bars indicate the range. C, Lytic activity of peritoneal exudate lymphocyte effectors at the peak of the primary response (day 8) and memory response (60 h) following i.p. challenge with P815 cells. The effector:target ratio was calculated based on the number of 1B2+ cells in the populations.
and expansion of $1B2^+$ cells in the memory response occurs 2 days after detectable Ag has been eliminated, suggesting that at least some of this expansion may not be caused directly by Ag but by inflammatory factors.

**Specificity of the memory response: trafficking and activation**

Memory $1B2^+$ cells have access to the peritoneal cavity in the absence of any inflammation or Ag (Fig. 2A) and rapidly traffic to that site when rechallenged with Ag (Fig. 3B). We were interested in determining whether the rapid trafficking of memory $1B2^+$ cells required Ag or whether it might occur in response to just an inflammatory stimulus. Adoptively transferred recipients that had been previously primed with P815 were therefore rechallenged with a third-party allogeneic tumor, the RDM4 lymphoma of AKR ($H-2K^k$) origin that is not cross-reactively recognized by the $2C$ receptor (data not shown). Challenge by i.p. injection of $5 \times 10^6$ P815 or RDM4 ($H-2^k$) tumor cells resulted in a substantial influx of $1B2^+$ cells into the peritoneal cavity by day 3 (Fig. 6A). Concomitant with this increase in $1B2^+$ cells in the peritoneal cavity, the number of $1B2^+$ cells decreased by 3 to $5 \times 10^4$ in the spleen, and there was a decrease in the percent $1B2^+$ cells in the blood (data not shown). While significant, the increase in number of $1B2^+$ cells was about 10-fold less than when the mice were challenged with P815 tumor.

The $1B2^+$ cells in the peritoneal cavity of mice rechallenged with RDM4 were about 30% blasts and about 20% CD25$^+$ (data not shown); i.e., they had a surface phenotype comparable to that of cells in mice that had not been rechallenged (Fig. 2B). This suggested that the $1B2^+$ cells were able to migrate to the peritoneal cavity in response to the nonspecific inflammatory stimulus provided by the RDM4 tumor but were not becoming further activated in the absence of specific Ag. Consistent with this, although $1B2^+$ cells were present in significant numbers, there was very little Ld$^+$ specific lytic activity in comparison to that in mice rechallenged with the specific Ag (Fig. 6B).

**The contribution of precursor frequency to the memory response**

Determination of the extent to which qualitative differences between naive and memory cells contributes to the more rapid and
efficient memory response would require that differences in frequencies of the specific cells be eliminated as a factor. Simply varying the number of 2C cells transferred into the recipients cannot accomplish this, since the majority of the CD8 response to the tumor is by endogenous host T cells. Attempting to adjust the size of the memory population by varying the number of tumor cells injected for the primary challenge would also be difficult, since the tumor grows very rapidly for several days before being rejected. We found, however, that injection of naive recipient mice with P815 tumor and 2C CD8+ T cells simultaneously into the peritoneal cavity results in a lower frequency of memory 1B2+ cells at the conclusion of the response than does i.v. injection of 2C cells and i.p. tumor challenge. This usually results in a frequency of memory cells in the spleen, LN, and blood that is comparable to the frequency of naive 1B2+ cells in mice receiving the transfer by i.v. injection (Fig. 7A). The lower frequency probably results from less clonal expansion occurring because the tumor Ag is more rapidly eliminated when 2C cells are placed in the peritoneal cavity simultaneously with tumor (data not shown). It would be expected that the endogenous host memory population would be correspondingly reduced in frequency for the same reason. This provided a means of comparing primary vs memory responses where the precursor frequencies are comparable.

In the experiment shown in Figure 7, three sets of mice were examined. These included mice that received 2C cells 3 days previous to challenge (“Primary”), mice 60 days after 2C transfer by i.v. injection and priming by i.p. injection of P815 (“Hi Memory”), and mice 40 days after simultaneous i.p. injection of 2C and P815 cells (“Low Memory”). The number of 1B2+ cells in the spleens of these same mice was also determined by flow cytometry (Fig. 7B). Here too, the increase in the number of cells in the spleens before rechallenge with P815 tumor. The numbers indicate 1B2+ CD8+ cells as a percent of the total lymphocytes. B-E, Mice were rechallenged by i.p. injection of 5 x 10^6 P815 tumor, and the number of tumor cells present in the peritoneal cavity at the indicated times was determined by flow cytometry (B). The peritoneal cavity exudate cells were adherence depleted and assessed for lytic activity in a 4-h ^51Cr release assay using EL4-Ld targets (C), and the number of 1B2+ CD8+ cells was determined by flow cytometry (D). The number of 1B2+ CD8+ cells in the spleens of these same mice was also determined by flow cytometry (E). For B-E, two mice were examined at each time point, and values shown are averages and ranges.

FIGURE 7. Kinetics of the primary response in comparison with memory responses at differing precursor frequencies. Mice examined in the experiment included: Primary, mice that received 3 x 10^6 1B2+ cells by i.v. injection (3 days previously) and had not been challenged with tumor; Hi Memory, mice that received 3 x 10^6 1B2+ cells by i.v. injection and were challenged 60 days previously by i.p. injection of 5 x 10^6 P815 cells; Low Memory, mice that received 1 x 10^6 1B2+ cells and 5 x 10^6 P815 cells injected simultaneously into the peritoneal cavity. A. The frequency of 1B2+ CD8+ cells in the spleens before rechallenge with P815 tumor. The numbers indicate 1B2+ CD8+ cells as a percent of the total lymphocytes. B-E, Mice were rechallenged by i.p. injection of 5 x 10^6 P815 tumor, and the number of tumor cells present in the peritoneal cavity at the indicated times was determined by flow cytometry (B). The peritoneal cavity exudate cells were adherence depleted and assessed for lytic activity in a 4-h ^51Cr release assay using EL4-Ld targets (C), and the number of 1B2+ CD8+ cells was determined by flow cytometry (D). The number of 1B2+ CD8+ cells in the spleens of these same mice was also determined by flow cytometry (E). For B-E, two mice were examined at each time point, and values shown are averages and ranges.
relationship between the level of Ag, the timing of its elimination, and the extent of clonal expansion that occurs is clearly complex and almost certainly influenced by factors other than just the amount of Ag available for the T cells to interact with. It is clear from these results, however, that memory CD8 T cells can respond much more rapidly and clear Ag sooner than naive cells are able to, even when comparable numbers of precursors are present at the time of challenge with Ag.

**Discussion**

Mice that have received CD8+ T cells from animals transgenic for the 2C TCR develop a stable pool of 1B2+ CD8+ 2C memory cells following a primary i.p. challenge with P815 tumor that expresses the L7/p2Ca Ag recognized by the 2C receptor (Fig. 1). These memory cells are long-lived, remaining present for at least 6 mo following priming, and provide identifiable Ag-specific cells within the memory population that includes endogenous alloantigen-specific CD8+ T cells. Persistent Ag is very unlikely to be involved in maintenance of the memory pool. Only the P815 tumor can present the native Ld alloantigen recognized by the 2C receptor, so a long-lived pool of Ag associated with host APC would not be present, and tumor becomes undetectable by flow cytometry within 8 to 10 days of the primary challenge (18). Although we have not used more sensitive detection methods, it is very unlikely that any tumor cells remain present long term, given the very aggressive growth of the P815 tumor. While it appears that Ag persistence may be important for maintenance of memory CD4+ T cells (24), its importance in maintaining CD8+ T memory cells has been more controversial (25–29). Our results support the hypothesis that Ag persistence may not be critical for CD8+ T cell memory. Recent experiments of Tanchot et al. (30) have suggested that maintenance of memory CD8+ T cells requires TCR-MHC class I interactions but that a nonspecific class I is sufficient for this and Ag is not required.

Despite the absence of persistent Ag, a substantial fraction of the 1B2+ memory cells express CD25 and are in cycle, as indicated by high forward light scatter (Fig. 2). This is consistent with previous studies that have used BrdU labeling and have shown that a significant fraction of memory cells are dividing (10, 13, 23). The potential role of persistent Ag could not be addressed in those experiments, however, either because the Ag stimulus was viral infection (10, 13, 23), which could allow low level persistence in the host, or because the activating Ag was not known (10, 23). The presence of CD25high 1B2+ blasts at long times after priming (Fig. 2) indicates that Ag persistence is not necessary to drive the continuing division of at least some of the memory cells. This conclusion was also reached in experiments where HY-specific memory CD8+ T cells were transferred into female recipients (30). The memory cells continued to divide despite the lack of HY Ag in the recipients. Continuing division of memory cells in the absence of specific Ag may be driven by the interaction with nonspecific class I that appears necessary for their survival (30), by environmental growth factors (31, 32), or both.

The majority of 1B2+ cells in previously primed recipients have a surface phenotype characteristic of CD8+ memory cells; they express high levels of CD44 and VLA-4. In contrast, a smaller fraction express low L-selectin levels, and this varies depending on the site examined (Fig. 2B). Although activated CD8+ T cells express low L-selectin, it is again up-regulated on a significant fraction of cells following the response (22, 33, 34). During the primary response to P815 in the peritoneal cavity, virtually all of the responding 1B2+ cells (>90%) convert to an L-selectinlow phenotype at the peak of the response on day 8. By day 11, however, about half have converted back to L-selectinhighb while remaining CD44high and VLA-4high (18). A small fraction of the 1B2+ cells in previously primed recipients have a phenotype characteristic of naive cells, expressing low levels of CD44 and VLA-4 and high L-selectin (Fig. 2B). These could potentially be naive cells that did not respond to the initial challenge. This appears unlikely, however, since naive cells do not remain present at detectable levels beyond about day 30 in recipients that have not been primed with Ag (18). Thus, it appears more likely that these are cells that responded during the initial challenge but retain a naive phenotype, consistent with evidence from Tough and Sprent (23) indicating that some memory T cells, and particularly CD8+ cells, retain a naive phenotype.

Heterogeneous expression of surface receptors involved in migration and homing is likely to promote more effective immune surveillance by the memory population, allowing recirculation through both the lymph nodes and peripheral tissue. Consistent with this possibility is the finding that a greater number of 1B2+ cells express high L-selectin and low VLA-4 and CD44 levels in the lymph nodes than at other sites (Fig. 2). Furthermore, the distribution of the memory 1B2+ cells is very different from that of naive cells (Fig. 2A). Memory cells are present in much higher proportion in the blood, which would allow for rapid migration into peripheral sites of inflammation. Unlike naive cells, they are also found in small numbers in the peritoneal cavity, consistent with the ability of memory cells to traffic through peripheral tissue in the absence of any apparent inflammation (10, 11, 20).

Memory 1B2+ cells respond to Ag challenge much more rapidly than do naive cells with respect to both the speed of migration to the site of challenge and the acquisition of effector function. In a primary response, 1B2+ cells do not become detectable in the peritoneal cavity until days 5 to 6 after challenge. P815 tumor is detectable in spleen and draining LN by days 2 to 4, and the fraction of 1B2+ cells expressing low L-selectin and high VLA-4 increases at these sites during this time, although CD25 up-regulation and blast transformation are not occurring. During days 4 to 6, the number of 1B2+ cells is declining in the spleen, and they are becoming detectable in the peritoneal cavity where they express high CD25 and are blasting. These observations suggest that efficient migration of naive cells to the peripheral site of Ag may require interaction with Ag in the spleen and draining LN to promote altered expression of homing receptors and that migration does not occur until Ag has reached these sites. This model is consistent with earlier studies showing that VLA-4 is up-regulated on CD8+ T cells in response to viral infection or contact Ags and implicating this integrin in being involved in migration to peripheral sites (35–37).

In contrast to naive cells, the decrease in memory 1B2+ cells in the spleen and LN and entry into the peritoneal cavity begin within 12 h of rechallenge (Fig. 3B). Although a small number of 1B2+ cells are present in the peritoneal cavity before rechallenge, it is unlikely that proliferation of these cells could account for the substantial increase in the number of 1B2+ cells at this site by 12 h. The memory 1B2+ cells enter the peritoneal cavity well before tumor cells become detectable in the spleen or LN. In fact, tumor has become almost undetectable by 12 h in the peritoneal cavity and is never found in the spleen or LN during a secondary response. This suggests that rapid migration of memory cells to the site of Ag does not depend on Ag recognition in lymphoid organs.
This conclusion is supported by the fact that a substantial number of 1B2+ memory cells migrate into the peritoneal cavity at early times in response to challenge with RDM4 tumor (Fig. 6A), although they fail to develop lytic activity in the absence of Ag that can be recognized by the 2C receptor. In contrast, naive 1B2+ cells cannot be found in the peritoneal cavity at these times when challenge is with the RDM4 tumor (18). Thus, these results strongly support the conclusion that memory cells rapidly traffic to an inflammatory site in an Ag-independent manner, while trafficking of naive cells is much slower (by several days) and is likely to require interaction with Ag in lymphoid organs before migration.

Rapid entry alone appears unlikely to account for the increased speed of the memory response. When naive 1B2+ cells are injected directly into the peritoneal cavity of a naive mouse along with P815 tumor, so that naive 1B2+ cells are present at the site of Ag in greater numbers than in the memory response, tumor is not cleared until day 4 or 5 (data not shown). In addition to much more rapid migration into the peritoneal cavity, the memory populations present in the spleen and peritoneal cavity exhibit a low level of cytolytic activity before rechallenge, and potent cytolytic activity develops in the peritoneal cavity within 12 h of rechallenge (Fig. 4A). Virus-specific memory populations have recently been demonstrated to have immediate effector function without a requirement for Ag-dependent differentiation, as measured by target cell lysis (13, 38) and IFN-γ production (14), and a population of human effector CD8+ T cells that have cytolytic activity without in vitro stimulation has been identified (39). Given the likelihood that P815 tumor is not present in the adoptive transfer recipients at long times after a primary challenge, it appears that Ag persistence is not necessary to maintain this cytolytic effector function. Our results also suggest that the memory population may include a subset of cells with immediate effector function, accounting for the lytic activity detected at 12 h in the peritoneal cavity, as well as a subset that must undergo Ag-dependent differentiation to acquire effector function, accounting for the second peak of lytic activity seen on day 3 after rechallenge (Figs. 4 and 7). If so, this second population also migrates very rapidly to the peritoneal cavity since the lytic activity peaks on day 3, in contrast to the primary response where lytic activity peaks on day 8, i.e., 3 days after the naive cells enter the peritoneal cavity (18).

Rapid trafficking of memory cells to the site of Ag, together with immediate effector effector function of at least a subset of the memory cells, might be expected to contribute substantially to the greater speed and efficiency of a memory response in comparison with a primary response by naive cells. This was directly demonstrated by experiments comparing the primary response of 1B2+ cells to the memory response when the number of 1B2+ cells present at the time of challenge with P815 was comparable (Fig. 7). Despite similar numbers of precursors, entry of 1B2+ cells into the peritoneal cavity, development of cytolytic effector function, and elimination of tumor all occurred about 4 days sooner in the previously primed mice than in naive adoptive transfer recipients, and the P815 tumor never reached as high a number in the primed mice as in the naive mice. Precursor frequency clearly does make some contribution, however, since mice with a higher memory cell frequency developed detectable cytolytic activity in the peritoneal cavity and cleared tumor even more rapidly (Fig. 7). Thus, increased precursor frequency, more rapid trafficking to peripheral sites, and the immediate effector function of at least some of the cells all contribute substantially to making a memory response to antigenic challenge at a peripheral site more rapid and efficient than a primary response.

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References