CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central–memory cells

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We explored the relationship between the time of naive CD4+ T cell exposure to antigen in the primary immune response and the quality of the memory cells produced. Naive CD4+ T cells that migrated into the skin-draining lymph nodes after subcutaneous antigen injection accounted for about half of the antigen-specific population present at the peak of clonal expansion. These late-arriving T cells divided less and more retained the central–memory marker CD62L than the T cells that resided in the draining lymph nodes at the time of antigen injection. The fewer cell divisions were related to competition with resident T cells that expanded earlier in the response and a reduction in the number of dendritic cells displaying peptide–major histocompatibility complex (MHC) II complexes at later times after antigen injection. The progeny of late-arriving T cells possessed the phenotype of central–memory cells, and proliferated more extensively during the secondary response than the progeny of the resident T cells. The results suggest that late arrival into lymph nodes and exposure to antigen-presenting cells displaying lower numbers of peptide–MHC II complexes in the presence of competing T cells ensures that some antigen-specific CD4+ T cells divide less in the primary response and become central–memory cells.

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Abbreviation used: HA, hemagglutinin.

Naive T lymphocytes are activated through their TCRs by peptide–MHC complexes displayed on dendritic cells in secondary lymphoid tissue (1). Upon activation, T cells undergo rapid proliferation, differentiating into effectors capable of migrating into sites of infection and producing antimicrobial lymphokines (2). A contraction phase then results in the elimination of the vast majority of T cells, leaving behind a stable population of memory cells (2). Two types of memory T cells have been defined based on expression of the cell surface homing receptors CD62L and CCR7 (3–6). Central–memory cells, which express both CD62L and CCR7, are located primarily in secondary lymphoid organs and produce IL-2 and proliferate well when exposed to antigen but are poor producers of effector lymphokines such as IL-4 or IFN-γ. Effector–memory cells do not express either CD62L or CCR7, reside in secondary lymphoid organs and nonlymphoid tissue, produce effector cytokines efficiently, and may not proliferate as well as central–memory cells (7), although this latter feature remains controversial (8). Effector–memory cells may benefit the host by eliminating microbes quickly within nonlymphoid tissue, whereas central–memory cells are thought to replenish the memory cell pool.

The conditions that favor the generation of central– and effector–memory cells during the primary immune response are unknown. Here we tested the possibility that asynchronous exposure to antigen as CD4+ T cells recirculate through antigen-containing lymphoid organs is one determining factor. Most infections and vaccinations result in deposition of foreign antigen in local tissues, which in turn leads to antigen presentation within a subset of the secondary lymphoid organs. For example, subcutaneous injection of antigen results in antigen presentation

exclusively in the lymph nodes that drain the injection site (9). Because naive T lymphocytes migrate randomly through all secondary lymphoid organs (10, 11), this creates a situation in which antigen-specific T cells that happen to reside in the antigen-draining lymph node at the time of antigen exposure will be activated immediately, whereas others that enter this lymph node from other parts of the body will be activated later. These "resident" and "late-arriving" T cells are likely to experience different levels of TCR and CD28 stimulation caused by changes in peptide–MHC and costimulatory ligand densities that occur over time after antigen enters the body. Differences in activation signal strength could then influence the numbers of effector and central–memory cells that are produced (12).

We addressed the role of late-arriving T cells on cell division and memory phenotype by preventing these cells from entering antigen-draining lymph nodes. The results demonstrate that the late arrivers account for a substantial proportion of the total antigen-specific CD4$^+$ T cell population that accumulates during the primary response. These late arrivers are enriched for cells with the central–memory cell phenotype and exhibit superior proliferation during the secondary immune response.

RESULTS
Kinetics of T lymphocyte entry and egress from skin–draining lymphoid tissue
Before attempting to measure the contribution of late-arriving CD4$^+$ T cells during a primary response to a subcutaneous antigen, it was first necessary to characterize the recirculation pattern of a defined population of naive CD4$^+$

T cells through skin-draining lymph nodes. Naive CD4$^+$ T cells from TEa TCR transgenic mice specific for a peptide from the I-Eα MHC II molecule (pEα) bound to I-A^b were injected intravenously into normal C57BL/6 (B6) recipient mice, which lack the relevant antigen. The rate at which the TEa cells appeared in the cervical lymph nodes was then measured by flow cytometry. The naive TEa T cells entered the lymph nodes of the recipient quickly, achieving a half-maximal level after only 2 h (Fig. 1 A). As expected, very few TEa T cells were found in the cervical lymph nodes of mice that received anti-CD62L antibody before the cell transfer (Fig. 1 B), because CD62L is required for naive T cell migration from the blood into lymph nodes via high endothelial venules (13). This interpretation was supported by the finding that anti-CD62L antibody-treated recipients had elevated numbers of TEa cells in their spleens (Fig. 1 B), as naive T cell migration into the spleen does not depend on CD62L (14). Thus, anti-CD62L antibody treatment was effective at preventing transferred naive CD4$^+$ T cells from homing to lymph nodes.

The effectiveness of anti-CD62L antibody at blocking naive T cell entry into lymph nodes was exploited to determine the time that naive T cells spend in lymph nodes. Naive TEa T cells were injected intravenously into normal mice and then given 24 h to reach the steady-state level of recirculation. Anti-CD62L antibody was then injected to prevent any TEa T cells that left a lymph node from entering any other lymph node. The number of TEa T cells present in the cervical lymph nodes declined sharply after injection of anti-CD62L antibody, whereas the number of TEa cells in untreated controls did not change over a 6-d period (Fig. 1 C). About 24 h
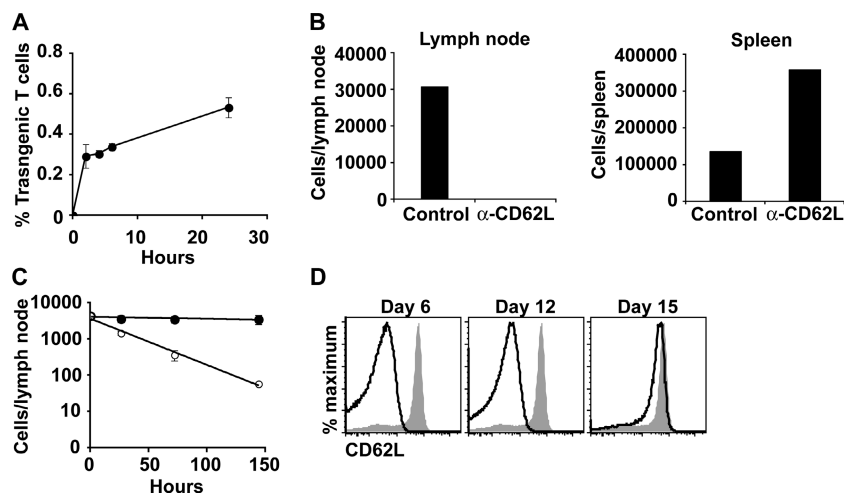

Figure 1. Naive CD4$^+$ T cell trafficking in skin–draining lymph nodes. (A) Naive TEa CD4$^+$ T cells in the cervical lymph nodes of recipient mice were monitored at different times after injection of 10^6 cells. CD4 and CD90.1 were used to identify TEa T cells, and percentages relative to endogenous CD4$^+$ T cells are shown. (B) Mice were given control Ig or anti-CD62L antibody, and shortly thereafter TEa T cells. The number of TEa cells in the cervical lymph nodes or spleen 5 d after transfer are shown. (C) Mice received TEa T cells, and the next day control Ig (●) or anti-CD62L antibody (○). The number of TEa cells in the cervical lymph nodes over time is shown. (D) CD62L expression on endogenous CD4$^+$ T cells at the indicated times after treatment with anti-CD62L antibody are shown (white histograms). Gray histograms show CD62L expression on CD4$^+$ T cells from untreated animals. Error bars in A and C represent standard deviation ($n = 2$). Data are representative of three independent experiments.

was required for half of the TEa cells that were initially present to exit the lymph nodes of anti-CD62L–treated mice. Collectively, these data suggest that naive lymphocytes enter lymph nodes rapidly from the blood, in a CD62L-dependent fashion, and spend about a day in this location before returning to the circulation. As shown by several previous studies (14–16), we found that a single injection of anti-CD62L antibody led to effective removal of CD62L from the T cell surface for ∼12–14 d (Fig. 1 D) and blocked the entry of naive CD4+ T cells into lymph nodes during this time frame (unpublished data). Thus, our subsequent analyses of the effects of CD62L blockade were limited to this time period.

Late-arriving T cells contribute to a localized primary immune response

Anti-CD62L antibody treatment was then used to assess the contribution of late-arriving T cells to a localized primary immune response. The idea was that administration of anti-CD62L antibody immediately before immunization of normal mice that had already received TCR transgenic T cells would not interfere with the response of the transferred T cells that resided in the draining lymph nodes at the time of immunization, but would prevent the participation of transferred T cells that would normally have entered these lymph nodes from other secondary lymphoid organs. To minimize deleterious effects of intraclonal competition on activation (17) and memory cell formation (18), only 10^4 TEa cells were

transferred, resulting in ∼10 cells in the deep cervical lymph nodes of each recipient under steady-state conditions (Fig. 2 A). The next day, recipient mice were injected intraperitoneally with anti-CD62L or control Ig, and intradermally in the ear with 30 ng of a recombinant antigen EαRFP containing the Eα peptide (9), plus LPS as an adjuvant (19). 30 ng was chosen because it produces peptide–MHC II complexes in the draining lymph nodes for ∼10 d after injection (unpublished data) as might be expected for many vaccines. TEa cells in the antigen-draining cervical lymph nodes of control Ig-treated mice increased 200-fold to a peak on day 6 and then declined (Fig. 2, A and B) as reported in other primary CD4+ T cell responses (20, 21). Anti-CD62L antibody reduced the accumulation of TEa cells in the antigen-draining lymph nodes to ∼50 and 10% of the control levels on days 6 and 10, respectively (Fig. 2 B). The local nature of this response was indicated by the finding that TEa cells increased only 10-fold in the nondraining mesenteric lymph nodes of control Ig-treated mice and not at all in the mesenteric lymph nodes of anti-CD62L–treated mice (Fig. 2 C). The complete lack of accumulation of TEa cells in the nondraining mesenteric lymph nodes of anti-CD62L–treated mice suggested that the 10-fold increase in control mice was caused by TEa cells that proliferated in the draining lymph nodes but retained CD62L and then migrated to the mesenteric lymph nodes. Together, these data show that late-arriving CD4+ T cells, either naive cells or CD62L+ cells that were activated in the
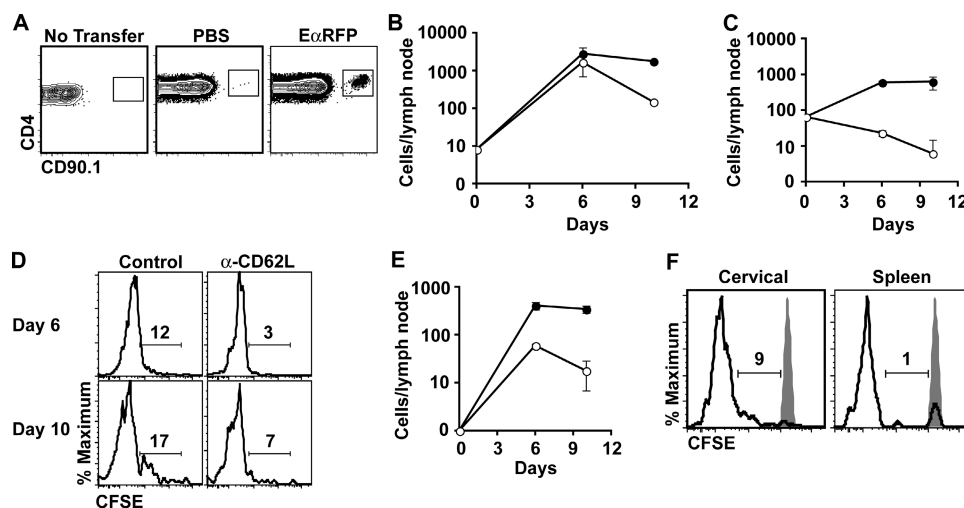

Figure 2. The contribution of late-arriving T cells to the local immune response in skin-draining lymph nodes. (A) 10,000 TEa T cells were injected into recipient mice, which were then injected with PBS (middle) or 30 ng of EαRFP plus LPS in the ear (right). 6 d after antigen injection, cervical lymph node cells from these mice, or mice that did not receive TEa cells (left), were identified as CD4+ and CD90.1+ cells. Contour plots of CD4 versus CD90.1 on CD11b−, B220−, and CD8α− cells are shown. (B) Recipients of 10^4 TEa cells were injected with control Ig (●) or anti-CD62L antibody (○) followed shortly thereafter by 30 ng of EαRFP plus LPS in the ear. Transgenic cells were detected as described in A in the cervical (B) or mesenteric lymph nodes (C) of the treated mice at

the indicated times. (D) CFSE histograms of TEa T cells in the cervical lymph nodes from control or anti-CD62L–treated mice shown in B. (E) Numbers of cells that had divided one to six times (using gates from D) from mice treated with control Ig (●) or anti-CD62L (○). The gate used to identify cells that had divided one to six times was based on the intensity of unstained endogenous CD4+ T cells, and the CFSE level of undivided TEa cells from unprimed animals. (F) CFSE histograms of TEa T cells from cervical lymph nodes and spleens of mice 6 d after immunization with EαRFP plus LPS are shown. Gray histograms represent naive TEa T cells from unprimed mice. All error bars represent SD ($n = 2$). Data are representative of three independent experiments.

draining lymph nodes and then left and returned, contribute substantial numbers of cells to the total pool of antigen-specific CD4^{+} T cells that expand in the lymph nodes draining a subcutaneous antigen injection site.

Cell division history

The CFSE dye dilution method was used in an attempt to identify the late-arriving T cells based on the assumption that these cells might be expected to divide fewer times than the resident T cells due to increased intraclonal competition or reduced levels of antigen. The TEa cells in the cervical lymph nodes of mice that received EαRFP and control Ig 6 d earlier were heterogeneous with respect to cell division history as indicated by the fact that ~12% had divided one to six times (Fig. 2 D). These less divided cells became a larger percentage of the total on day 10 (Fig. 2 D), perhaps as a result of the most divided cells migrating out of the lymph nodes (22, 23). The less divided population was greatly reduced in the cervical lymph nodes of mice that received EαRFP and anti-CD62L antibody (Fig. 2, D and E). The large reduction of the less divided cells by anti-CD62L antibody treatment is consistent with the idea that the less divided cells in the control Ig-treated mice were T cells that migrated into the draining lymph node after antigen injection and underwent their divisions in this location. However, it was possible that these cells had actually divided in some other location, perhaps in response to small amounts of antigen leaking from the injection site into the blood, and then entered the draining lymph nodes. This possibility was tested by examining the CFSE profiles of TEa cells in the spleen, which is the site of immune responses to blood-borne antigens. The small number of TEa cells in the spleen 6 d after antigen injection consisted of two populations—one that had not divided at all and thus consisted of naive cells, and one that had divided more than six times. Less than 1% of the TEa cells in the spleen at this time had the one to six cell division history characteristic of the less divided cells in the draining cervical lymph nodes (Fig. 2 F). The absence of these intermediates in the spleen ruled out the possibility that the TEa cells in the draining lymph nodes with the one to six cell division history underwent these divisions in the spleen and then migrated to the draining lymph nodes. It also suggests that most of the cells with the one to six cell division history in the draining lymph nodes are not leaving this location at a high rate during the first week of the response. Collectively, these results suggest that the less divided cells in the draining cervical lymph nodes, that were lost after anti-CD62L treatment, were cells that entered this location late after antigen injection and divided there. Conversely, the highly divided cells that appeared in the spleen most likely underwent their divisions in the draining lymph nodes and then migrated to the spleen.

If the less divided cells identified in Fig. 2 D were truly late arrivers, then TEa cells transferred into mice at late times after EαRFP injection should display the less divided pattern because all of the cells in the draining lymph nodes in this case would have entered after antigen injection. As shown in

Fig. 3 B, 43% of the TEa cells transferred into mice injected intradermally 3 d earlier with EαRFP, and assayed 6 d after transfer, had divided one to six times (Fig. 3 B; sum of middle gates) compared with 17% of the TEa cells that were in mice at the time of EαRFP injection (Fig. 3 C). Thus, forcing all of the TEa cells to enter the draining lymph nodes after antigen injection increased the fraction of the cells that divided one to six times, supporting the possibility that the less divided cells, that were eliminated by anti-CD62L antibody treatment, were late arrivers. Notably, most of the TEa cells that divided after transfer into mice that were injected with EαRFP 3 d earlier retained expression of CD62L, even those that divided more than six times. (Fig. 3 B). The late-arriving TEa cells with a one to six cell division history from the draining lymph nodes of mice that were injected with antigen after T cell transfer also retained CD62L expression, whereas many of the cells that divided more than six times lost CD62L (Fig. 3 C). Therefore, late-arriving CD4^{+} T cells were less likely to lose CD62L than resident CD4^{+} T cells that were in the lymph nodes at the time of antigen injection.

Mechanism underlying the fewer cell divisions experienced by late–arriving T cells

The basis for the fewer cell divisions experienced by late-arriving T cells was explored next. One possibility was that late-arriving T cells divided less because of competition with the much larger population of resident T cells that had already proliferated at an earlier time after antigen injection (17, 24–26). To test the effects of intraclonal competition on late-arriving T cells, we designed a system in which two different populations of DO11.10 TCR transgenic CD4^{+} T cells specific for ovalbumin peptide–I-A[d] complexes could be monitored simultaneously. Naive CD90.2^{+} DO11.10 cells were injected intravenously into normal BALB/c mice, which then received an intradermal injection of ovalbumin peptide plus LPS in the ear. Control mice received just the
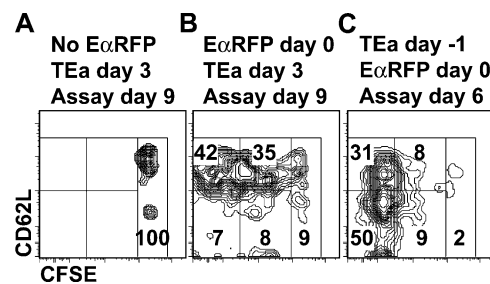

Figure 3.   Cell division history of late–arriving T cells. Mice were injected intradermally with PBS (A) or 30 ng EαRFP plus LPS (B) and given 10[4] CFSE-labeled TEa T cells 3 d later. TEa T cell division and CD62L expression in draining lymph nodes was analyzed 6 d after cell transfer. The contour plots display CFSE versus CD62L levels on CD4^{+}, CD90.1^{+}, CD11b^{−}, B220^{−}, CD8α^{−} lymphocytes. Percentages of cells that had undergone zero, one to six, or more than six divisions are shown according to their CD62L expression. (C) Other mice were given 10[4] CFSE-labeled TEa T cells first and then injected with EαRFP plus LPS and analyzed 6 d later. Data are representative of three independent experiments.

intradermal injection of ovalbumin peptide plus LPS. 6 d later, both sets of mice were injected intravenously with 10^4 CFSE-labeled naive CD90.1$^+$ DO11.10 cells, and the frequency of the CD90.1$^+$ cells was measured 6 d later. The first population of unlabeled CD90.2$^+$ cells served as a source of preexisting competitors that had already expanded to their maximal number in response to antigen, whereas the second population of CFSE-labeled CD90.1$^+$ cells served as a source of late arrivers. As shown in Fig. 4 A, the majority of CFSE-labeled CD90.1$^+$ DO11.10 cells that were transferred into antigen-primed recipients that did not receive competitors displayed the heterogeneous cell division history characteristic of late-arriving T cells. When transferred into antigen-primed mice that already contained an expanded population of CD90.2$^+$ DO11.10 cells, the CFSE-labeled CD90.1$^+$ DO11.10 cells divided on average one time less (Fig. 4 A) and accumulated about half as well as they did in the absence of intraclonal competition (Fig. 4 C).

To rule out any role for endogenous OVA-specific CD4$^+$ T cells in the competition, the experiment was also done using RAG-deficient hemagglutinin (HA) TCR transgenic recipients, in which all of the CD4$^+$ T cells are specific for a single influenza HA peptide. CFSE dye dillution was not informative in these recipients because of homeostatic proliferation by the DO11.10 cells in the absence of OVA injection (unpublished data). However, CD90.1$^+$ DO11.10 cells that were transferred into OVA-injected HA TCR transgenic recipients of competing CD90.2$^+$ DO11.10 cells expanded 2–3-fold less than CD90.1$^+$ DO11.10 cells that were transferred into OVA-injected HA TCR transgenic recipients that did not receive competitors (Fig. 4, B and C). Thus, intraclonal competition with early-arriving T cells probably accounts for some of the reduced cell division of late-arriving T cells.

We also tested the possibility that late-arriving T cells divided less than resident T cells simply because the number of peptide–MHC II complexes remaining in the lymph nodes was lower at later times after antigen injection. This possibility could be tested directly after injection of EαRFP using the Y-Ae monoclonal antibody, which is specific for the pEα–I-A^b complexes recognized by TEa T cells (27). Previous work using this approach showed that dendritic cells are by far the most abundant producers of pEα–I-A^b complexes in the cervical lymph nodes after intradermal injection of EαRFP in the ear (9). As shown in Fig. 5, the percentage of CD11c$^+$ dendritic cells that displayed pEα–I-A^b complexes peaked in the draining lymph nodes within 24 h after intradermal injection of EαRFP and then declined rapidly over the next 24 h. Thus, TEa cells that entered these lymph nodes after 48 h would be exposed to fewer pEα–I-A^b complexes than those residing in the lymph node at the time of antigen injection, providing a likely explanation for the reduced number of cell divisions experienced by the late-arriving population.

Relationship between clonal frequency and antigen dose

The reduced cell division history and retention of CD62L raised the possibility that late-arriving CD4$^+$ T cells give rise
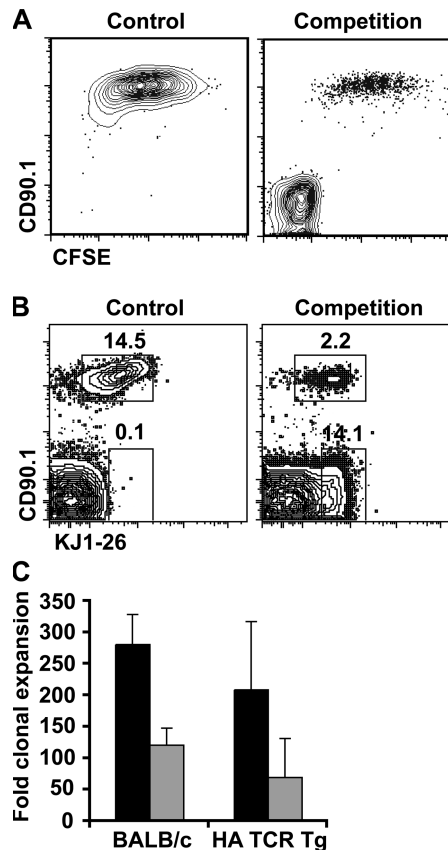

Figure 4. Late-arriving T cells and intraclonal competition. BALB/c mice (A) or RAG-deficient HA TCR transgenic mice (B) received 10^4 CD90.2$^+$ DO11.10 T cells (competitors) or no cells at all, followed by an intradermal injection of OVA plus LPS the next day. 6 d after antigen injection, all mice received 10^4 CFSE-labeled CD90.1$^+$ DO11.10 T cells (late arrivers). The antigen-draining cervical lymph nodes were analyzed 6 d after transfer. In BALB/c recipients, CD90.1 and CFSE levels were measured on CD4$^+$, KJ1-26$^+$, CD11b$^-$, B220$^-$ lymphocytes. CFSE-labeled CD90.1$^+$ DO11.10 T cells are visible in the top portion of both plots in A, whereas unlabeled CD90.2$^+$ DO11.10 T cells are visible in the lower left corner of the right plot in A. CFSE dye dilution was not informative in the HA TCR transgenic recipients because of homeostatic proliferation of the transferred cells in the absence of OVA. Therefore, in these recipients, the competitor DO11.10 cells were identified as CD4$^+$, CD90.1$^-$, KJ1-26$^+$, B220$^-$, CD11b$^-$ cells (visible in the bottom box in the right panel of B), and the late-arriving DO11.10 cells as CD4$^+$, CD90.1$^+$, KJ1-26$^+$, B220$^-$, CD11b$^-$ cells (visible in the top box in both panels in B). The mean fold expansion ($\pm$ SD, $n = 2$) for late arrivers in the absence (black bars) or presence (gray bars) of competitors was defined as the number of late arrivers present on day 6 divided by the number present in mice that did not receive antigen, and is shown in C. Data are representative of three independent experiments.

to central–memory cells. We wanted to test this possibility by purifying late arrivers via cell sorting based on their CFSE profile and then transferring these cells into new recipients. However, because so few TEa cells with the one to six cell division history were present in the draining lymph modes of mice that received 10^4 TEa cells and then EαRFP, it was
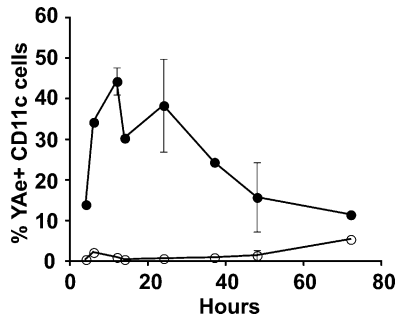
Figure 5. Display of pEα–MHC II complexes on dendritic cells in antigen–draining lymph nodes over time. 50 μg of EαRFP plus LPS (●) or LPS alone (○) was injected intradermally into the ears of mice, and the levels of pEα–MHC II complexes on CD11c$^+$ dendritic cells were analyzed by Y-Ae staining at different times thereafter. The mean percentage of Y-Ae$^+$ cells is shown. Data were pooled from multiple experiments and error bars represent SDs (n = 1–3).

technically impossible to sort enough TEa memory cells for retransfer. An obvious solution to this problem was to generate the late arrivers in mice transferred with a much higher number of naive TEa cells. However, this solution was potentially problematic because initial frequency is an important determinant of memory cell phenotype. For example, Marzo et al. showed that CD62L^{low} effector–memory CD8$^+$ T cells were not generated efficiently from an initially high number of naive cells (18). One explanation for this effect was that intraclonal competition for peptide–MHC complexes prevented the T cells from obtaining a strong enough TCR signal to become CD62L^{low} memory cells. If so, then it should be possible to produce efficient memory cell differentiation from a large number of naive cells by increasing the antigen dose used for immunization.

We explored this possibility by testing the relationship between initial T cell frequency and antigen dose. As shown in Fig. 6 A, the fraction of highly divided, CD62L^{low} TEa cells present in the draining cervical lymph node 6 d after intradermal injection of 30 ng of EαRFP increased from 12 to 43 to 64% as the number of transferred TEa cells decreased from 10^5 to 10^4 to 10^3. Similarly, the percentage of highly divided, CD62L^{low} TEa cells in mice that received 10^6 TEa cells increased from 33 to 42% as the EαRFP dose increased from 0.5 to 5 μg (Fig. 6 B). The fact that the cell division and CD62L profile achieved by the progeny of 10^4 cells exposed to 30 ng antigen closely resembled that achieved by 10^6 cells exposed to 5 μg was evidence that the inhibiting effect of high T cell frequency on memory cell differentiation could be overcome by increasing the antigen dose.

Late-arriving T cells and their impact on memory

The results in Fig. 6 showed that numerous CD62L^{low} cells could be produced from a large number of naive cells as long as a high dose of antigen was used for immunization. This finding set the stage for sorting late-arriving T cells and determining whether or not these cells were capable of extensive

proliferation during the secondary response as expected for central–memory cells. One million CFSE-labeled naive TEa T cells were transferred to naive mice, which were then immunized with a high dose of EαRFP (10 μg) in the ear. 17 d were then allowed to pass during which the TEa cells underwent the expansion and contraction phases of the primary response and stabilized numerically at the beginning of the memory phase (Fig. 7 A). As was observed at earlier times, the TEa cells recovered from the draining lymph nodes at this time were heterogenous with respect to CFSE dye dilution and CD62L and CCR7 expression (Fig. 7 B). The cells that divided one to six times expressed high levels of both molecules, which are phenotypic markers of central–memory cells (3), whereas the cells that divided 7 or more times contained substantial populations of CD62L^{low} and/or CCR7^{low} cells. The TEa cells were then sorted into two populations based upon their CFSE intensities: CFSE$^{1–4}$, which consisted of late-arriving cells that had undergone one to four cell divisions, and CFSE$^{>6}$, which consisted of the dimmest CFSE-labeled cells that had undergone at least seven divisions (Fig. 7 C). CFSE$^{1–4}$ cells were chosen instead of CFSE$^{1–6}$ cells to rigorously eliminate highly divided cells. 2,000 cells of each type were then injected into separate sets of naive B6 recipients, and a new sensitive magnetic bead enrichment technique described in Materials and methods was used to detect the transferred cells in spleen, lymph nodes, or lungs of the recipient mice.

A day after transfer, between 15 and 50 TEa cells were detected in the spleen and lymph nodes of recipients of CFSE$^{1–4}$ or CFSE$^{>6}$ cells (Fig. 7, D and E). Although, these were very small numbers, they were likely legitimate given that no such cells were detected in the spleen and lymph nodes from five mice that did not receive TEa cells (Fig. 7 D, top). The mice were then injected with EαRFP plus LPS intravenously to assess the antigen-driven proliferation of the transferred cells throughout the body. 4 d later, TEa cells were detected in recipients of either cell type at greater than the initial levels, indicating that both populations underwent antigen-driven proliferation (Fig. 7, D and E). However, ∼5–10 times more TEa cells were detected in the lymph nodes and spleens of recipients of CFSE$^{1–4}$ cells than the recipients of CFSE$^{>6}$ cells (Fig. 7 E). Very small populations of TEa cells were detected in the lungs of recipients of both CFSE$^{1–4}$ cells and CFSE$^{>6}$ cells. These results showed that the CFSE$^{1–4}$ cells, which divided fewer times in the primary response and had the phenotype of central–memory cells, were capable of greater proliferation during a secondary response than the progeny of the more highly divided resident T cells.

DISCUSSION

Our results show that naive CD4$^+$ T cells that enter the skin-draining lymph nodes after antigen drains into this location contribute significantly to the total population of T cells that respond to a nonreplicating soluble antigen. This situation is a consequence of several features of lymphocyte recirculation. Naive T cells migrate randomly from one secondary
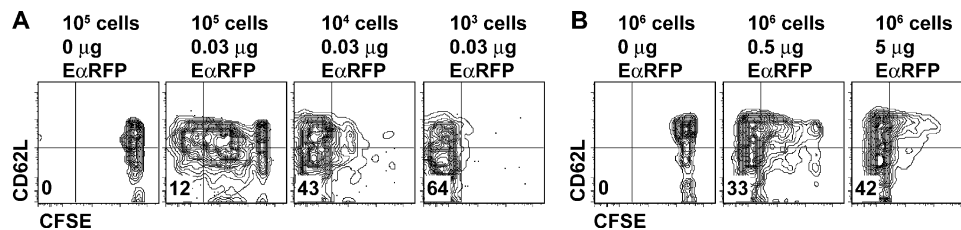
Figure 6. The relationship between clonal frequency and antigen dose. (A) Naive B6 recipient mice were injected with 10³, 10⁴, or 10⁵ CFSE-labeled TEa T cells and then immunized intradermally in each ear with 0 or 30 ng of EαRFP plus LPS, or (B) 10⁶ CFSE-labeled TEa T cells and then immunized with 0, 0.5, or 5 μg of EαRFP plus LPS. 4 (B) or 6 d (A) later,

the TEa cells were identified as CD4⁺, CD90.1⁺, B220⁻, CD11b⁻, CD8α⁻ lymphocytes and analyzed for CFSE dye dilution and CD62L expression. The percentage of CD62L^low cells with greater than six cell divisions is indicated on each contour plot. Data are representative of three independent experiments.

lymphoid tissue to another via the blood and lymphatic vasculature (11). Our results confirm earlier work showing that naive T cells enter lymphoid organs quickly from the blood (28) and then spend about a day in each lymphoid organ that they enter (10). It then follows that the population of naive T cells specific for any given antigen will be randomly distributed among the spleen, several hundred lymph nodes, and mucosal lymphoid organs. Even if the rate of entry of naive T cells into inflamed lymphoid organs increases several fold as suggested by Soderberg et al. (29), it would still take weeks for all T cells in this population to pass through any one of these organs. Therefore, when a foreign antigen is deposited in a local site, as would be the case in most infections or vaccinations, some antigen-specific T cells will happen to be in the relevant secondary lymphoid organs at the time that antigen is first presented, whereas others will continue to arrive in this location over time. Our results show that these late-arriving T cells account for at least half of the total population of antigen-specific CD4⁺ T cells that accumulate in the draining lymph nodes in the first several weeks after intradermal injection of antigen.

Competition with the large population of T cells that expanded early in the response contributed to the reduced cell division of late-arriving T cells. Previous work indicated that competition between naive T cells expressing the same TCR is related to competition for peptide–MHC complexes on APC (17). Notably, however, competition with T cells that proliferated earlier in the response only caused a 2–3-fold reduction in the expansion potential of the late arrivers. Thus, although competition was a restraining force on the activation of the late arrivers, it was not an absolute barrier. Competition may, therefore, favor the production of less-differentiated memory cells by limiting, but not preventing the activation of late arrivers.

The declining number of dendritic cells that displayed relevant peptide–MHC II complexes in these lymph nodes over time was likely another explanation for the reduced cell division of late-arriving T cells. Notably, the late-arriving T cells with the one to six cell division history were easily detected in the draining lymph nodes even 1 wk after the relevant peptide–MHC II complexes disappeared. This finding suggests that many of the late-arriving CD4⁺ T cells failed to execute

the full proliferation program after antigen presentation ceased, consistent with the recent finding that CD4⁺ T cells stopped dividing as soon as antigen presentation ceased (30).

Our results also showed that the late-arriving T cells from the primary response were much better at proliferating during a secondary response than resident T cells. Jelley-Gibbs et al. (31) recently showed that naive virus antigen-specific CD4⁺ T cells became more persistent memory cells after transfer into animals 1 wk after viral clearance than those transferred into animals at the time of infection. Since the cells transferred 1 wk into the infection would all be late arrivers, these results provide further support for the possibility that long-lived memory cells can be preferentially generated from late-arriving T cells. Our results extend those of Jelley-Gibbs et al. (31) by showing that late-arriving T cells are produced naturally by the combination of a locally presented antigen and the asynchronous circulation of naive T cells through the antigen-containing secondary lymphoid organs.

Our finding that lowering the initial frequency of naive TEa cells increased the efficiency of their activation supports the conclusion of Marzo et al. (18) that the clonal frequency of naive T cells is an important determinant of memory cell fate. Our findings extend that work by showing that the inefficient activation of large numbers of TCR transgenic T cells could be overcome by increasing the antigen dose. In addition, we found that the fraction of CD62L^low cells generated from a large number of naive cells after immunization with a high dose of antigen was maintained into the memory phase of response. Therefore, we conclude that adoptive transfer of large numbers of naive cells can be used to model the natural situation of low frequency as long as sufficient antigen is used for immunization.

Our approach identified two populations of cells in the draining lymph node after antigen injection: a late-arriving population with a one to six cell division history and a CD62L^high CCR7^high phenotype that was blocked by anti–CD62L treatment, and a resident population that was resistant to CD62L blockade, experienced more than six cell divisions and contained a significant number of cells that had lost CD62L and/or CCR7. The fact that the progeny of late-arriving T cells retained CD62L and CCR7 and the potential for extensive proliferation during the secondary response fits
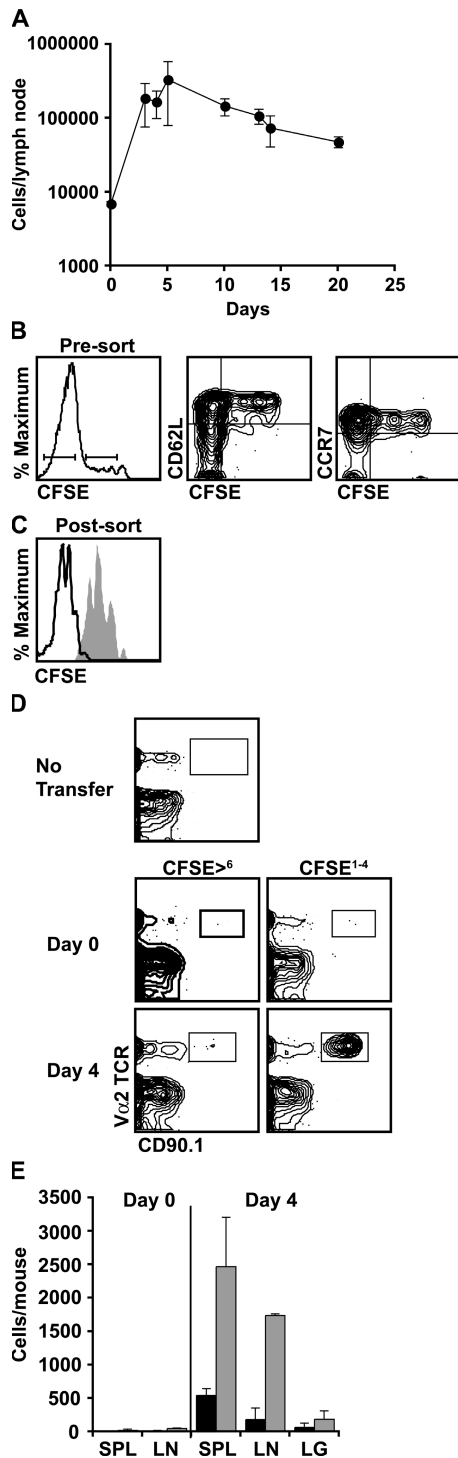
Figure 7. **The relationship between cell division history and memory cell potential.** Naive B6 recipients injected with 10^6 CFSE-labeled CD90.1+ TEa T cells were given 10 μg EαRFP plus LPS intradermally in each ear. The mean number (± SD) of TEa T cells in the draining lymph nodes at the indicated times after antigen injection is shown in A. (B) 17 d after antigen injection, CD4+, CD90.1+ T cells from the draining lymph nodes were enriched by negative selection with magnetic beads and sorted into two populations: cells that had undergone one to four divisions (CFSE$^{1-4}$; right gate) and cells that had undergone more than

with the possibility that these cells were central–memory cells (3). In contrast, the resident cells with CFSE cell division histories greater than six had properties of effector–memory cells, including a significant population of CD62L^{low} cells. The fact that these cells were not found in large numbers in the lungs after secondary transfer and priming of recipient mice argues against this possibility. However, it is possible that effector–memory cells do not undergo division in the secondary response (7) and, therefore, may be difficult to detect, even in nonlymphoid tissue. This fraction also contained a population of CD62L^{high} cells and thus may have been contaminated with central–memory cells.

Finally, our results offer a simple, anatomical explanation for how memory cells are generated from naive precursors. Effector–memory cells may be generated from naive T cells that reside in the lymph nodes at the time that antigen first enters this location. These resident cells proliferate many times in response to a high density of peptide–MHC II and costimulatory ligands on dendritic cells, and in the process lose lymph node homing molecules and gain molecules required for migration to inflamed tissues. At later times after antigen enters the body, other antigen-specific T cells enter the draining lymph nodes and proliferate less because of competition with previously activated resident T cells and exposure to a lower density of peptide–MHC II and costimulatory ligands. The less intense activation of these cells allows retention of lymph node homing capacity and strong proliferative potential during secondary immune responses. Thus, it is possible that reduced cell division in the primary response caused by late arrival into secondary lymphoid organs constitutes a natural mechanism for generating central–memory T cells.

MATERIALS AND METHODS

Animals. 6–8-wk-old male B6 and BALB/c recipient mice were purchased from The Jackson Laboratory. DO11.10 BALB/c RAG-deficient (32) and TEa TCR transgenic mice (33) expressing the CD90.1 allele were bred on site. HA TCR transgenic (34) RAG-deficient BALB/c mice and DO11.10 BALB/c RAG-deficient mice expressing the CD90.1 allele were obtained from Dr. Alex Khoruts (University of Minnesota). All mice were housed in a specific pathogen-free facility at the University of Minnesota, and experiments were conducted according to federal and institutional guidelines and with

six divisions (CFSE$^{>6}$; left gate). CD62L and CCR7 levels on presorted cells are shown. (C) Post-sort analysis showed clear separation of the CFSE$^{>6}$ (white histogram) and CFSE$^{1-4}$ (gray histogram) cells. 2,000 cells of each type were then transferred into new naive B6 recipients. (D) 1 d after transfer (middle), or 1 d after transfer and 4 d after intravenous injection of EαRFP plus LPS (bottom), spleen, lymph nodes, and lungs were analyzed for the presence of TEa cells using the anti-CD90.1 magnetic bead enrichment technique (as described in Materials and methods). The contour plots display CD90.1 versus TCR Vα2 expression on CD4+, B220−, CD11b−, CD8α− lymphocytes, with TEa cells shown in the box. A contour plot from a mouse that did not receive TEa T cells is shown in the top panel of D as a negative control. (E) The mean number of TEa T cells in spleen (SPL) and lymph nodes (LN) on day 0 and spleen, lymph nodes, and lungs (LG) on day 4 after antigen challenge are shown. Error bars repre-sent SD (n = 2). Data are representative of at least three independent experiments.

the approval of the University of Minnesota Institutional Animal Care and Use Committee.

Antigens and antibodies. OVA peptide 323–329 was purchased from Research Genetics. The EαRFP protein was purified from bacterial lysates using a Nickel resin His-Bind column (Novagen) as previously described (9). Contaminating endotoxin was removed using TritonX-114 phase separation (35). Anti–mouse CD62L was purified from ascites of the Mel-14 hybridoma (American Type Culture Collection) using protein G–Sepharose. Control rat IgG was purchased from Sigma–Aldrich. Antibodies were injected intraperitoneally in a single dose of 250 μg per mouse.

Adoptive transfer. Pooled spleen and lymph node cells from naive TCR transgenic donor mice were used for adoptive transfer. In some cases, these cells were labeled before transfer with 5 μM CFSE (Molecular Probes) as previously described (36, 37).

TEa TCR transgenic cells from donor mice were identified by flow cytometry after staining of a small sample with FITC-labeled anti-CD4 (eBioscience), PE-labeled anti-TCR Vα2, and PerCP-labeled anti-CD90.1 (BD Biosciences) antibodies. The fraction of TEa cells identified in this sample was then used to calculate the desired number of TEa cells from an unlabeled sample to be transferred by intravenous injection into naive CD90.2$^+$ B6 mice. Recipient mice were injected intradermally in the ear with 30 ng, 0.5, 5, or 10 μg of EαRFP. 1 μg LPS (List Biological) was used as an adjuvant for each ear injection. In some cases, mice were given an intraperitoneal injection of control or anti-CD62L antibody 15 min before antigen administration.

TEa T cells were detected in lymph node or spleen cell suspensions from recipient mice by staining with PE-labeled anti-CD4 and PerCP-labeled anti-CD90.1 (BD Biosciences) antibodies as previously described (9). CD62L was detected with an APC-labeled anti-CD62L antibody (eBioscience). Detection of CCR7 was accomplished using an ELC-Fc fusion protein (obtained from Jason Cyster, University of California-San Francisco, San Francisco, CA), followed by biotin-labeled anti–human Fc antibody (Jackson ImmunoResearch), and streptavidin-labeled APC. The number of transferred T cells was calculated by multiplying the frequency of CD90.1$^+$, CD4$^+$ cells by the number of live lymphocytes.

In some experiments two congenic DO11.10 CD4$^+$ T cell populations, CD90.1$^+$ and CD90.2$^+$, were used to study intraclonal competition. 10,000 naive CD90.2$^+$ DO11.10, identified as described by Kearney et al. (38), were injected into naive BALB/c or HA TCR transgenic RAG-deficient BALB/c mice, some of which were then injected intradermally with 5 μg of OVA peptide plus 1 μg of LPS. 6 d later, a second population of 10^4 CFSE-labeled CD90.1$^+$ DO11.10 cells was injected intravenously into these mice. 6 d after this transfer, cervical lymph nodes were stained with APC-labeled anti-B220, anti-CD8α, and anti-CD11b (BD Biosciences) (to exclude non–T cells), PE-labeled anti–KJ1-26 (Caltag), and PerCP-labeled anti-CD90.1 antibodies to distinguish the two populations of DO11.10 cells.

In some cases, TEa memory cells were generated in one set of recipients and then transferred to new naive recipients. For this purpose B6 mice that received 10^6 naive TEa cells were injected intradermally in the ear with 10 μg of EαRFP and LPS. 17 d later, cells from cervical lymph nodes of these mice were stained with biotin-labeled anti-CD90.2 antibody and a biotin-labeled cocktail of antibodies specific for cells other than CD4$^+$ T cells (provided with the Mouse CD4 T cell Isolation kit [Miltenyi]). Anti-biotin antibody-labeled magnetic beads were then added and the cells were passed over a magnetized LS column. The cells that passed through the column were highly enriched for CD90.1$^+$ TEa cells. Cells with a one to four or greater than six cell division history were then sorted from this enriched population. The fraction of "untouched" TEa cells in these populations were then determined from a small sample as described in the preceding paragraph. 2,000 CFSE$^{1–4}$ or CFSE$^{>6}$ TEa cells were then injected intravenously into naive B6 recipients, some of which were injected 1 d later with 100 μg of EαRFP plus LPS intravenously.

The small number of sorted memory cells transferred required that an enrichment method be used to detect them in recipient mice. 1 d after transfer, or 4 d after antigen injection, spleen and mesenteric, inguinal, cervical, axillary, and brachial lymph node cells from each recipient mouse were stained with biotin-labeled anti-CD90.1 antibody and then streptavidin-coated magnetic beads (Miltenyi). The cells were then passed over magnetized LS columns (Miltenyi), and bound cells were collected. A small portion of each sample was removed for a viable cell count. The remaining cells in each sample were stained with biotin-labeled anti-CD90.1, PerCP-labeled streptavidin (BD Bioscience), PE-Cy7–labeled anti-CD4, APC-labeled anti-CD62L (eBioscience), PE-labeled anti–TCR Vα2, and Pacific blue–labeled anti-B220 (Caltag), anti-CD11b, and anti-CD8α antibodies (to exclude cells other than CD4$^+$ T cells). Because calibration experiments (unpublished data) showed that the magnetic bead enrichment method captures all of the cells in the original sample, it was possible to calculate the total number of TEa cells in that sample by multiplying the percentage of CD4$^+$, B220$^-$, CD11b$^-$, CD8α$^-$, CD90.1$^+$, TCR Vα2$^+$ cells by the total number of all cells as determined by the viable count.

Flow cytometry was performed on a FACSCalibur or LSR II device (Beckson Dickinson) and analyzed using FlowJo software (Tree Star, Inc.). Sorting of CFSE-labeled CD4 T cells was performed on a FACSDiva or FACSAria (Beckson Dickinson) at the University of Minnesota Cancer Center Flow Core.

Detection of peptide–I-A^b complexes. 50 μg of EαRFP was injected intradermally into the ears of mice. At various times after injection, draining cervical lymph nodes were collected and treated with collagenase and EDTA as previously described (9, 39). Dendritic cells were then enriched from cell suspensions using anti-CD11c microbeads and LS columns (Miltenyi). Cells were stained with PE-Cy5.5–labeled anti-CD11c (Caltag), Pacific blue–labeled B220 (BD Bioscience), biotin-labeled YAe antibody (27), and streptavidin-conjugated PE-Cy7 (eBioscience) and analyzed by flow cytometry.

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