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Unique Ability of Activated CD4$^+$ T Cells but Not Rested Effectors to Migrate to Non-lymphoid Sites in the Absence of Inflammation*

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Javed N. Agrewala, Deborah M. Brown, Nancy M. Lepak, Debra Duso, Gail Huston, and Susan L. Swain[1]

From the Trudeau Institute Inc., Saranac Lake, New York 12983

Recent studies suggest that effector T cells generated by immune responses migrate to multiple non-lymphoid sites, even those without apparent expression of antigen or inflammation. To investigate the ability of distinct CD4$^+$ T lymphocyte subsets to enter and persist in non-lymphoid, noninflamed compartments, we examined the migration and persistence of naïve, effector, and rested effector CD4$^+$ T cells generated *in vitro* following transfer to nonimmunized adoptive hosts. Th1 and Th2 effectors migrated to both lymphoid and non-lymphoid organs (peritoneum, fat pads, and lung). In contrast, rested effectors and naïve cells migrated only to lymphoid areas. Adhesion molecule expression, but not chemokine receptor expression, correlated with the ability to enter non-lymphoid sites. Donor cells persisted longer in lymphoid than in non-lymphoid sites. When hosts with naïve and memory donor cells were challenged with antigen, effectors developed *in situ*, which also migrated to non-lymphoid sites. Memory cells showed an accelerated shift to non-lymphoid migration, in keeping with memory effector formation. These results suggest that only recently activated effector T cells can disperse to non-lymphoid sites in the absence of antigen and inflammation, and as effectors return to rest, they lose this ability. These data also argue that memory cells in lymphoid sites are longer lived and not in equilibrium with those in non-lymphoid sites.

Naïve T cells are found concentrated in lymphoid organs such as lymph nodes and spleen, but they constantly recirculate, thus providing an effective surveillance system in the lymphoid organs. The naïve T cells enter the lymph nodes from the blood via the high endothelial venules and pass through the paracortex, a specialized T cell compartment. The high surface expression of CD62L and the chemokine receptor CCR7 on naïve T cells are associated with their migration into the lymph node by binding the peripheral lymph node addressin or Glycam-1 and by binding constitutively produced chemokines such as SLC (CCL21) or ELC (CCL19), respectively (1). When an immune response occurs, antigen-presenting cells (APC)[2] bearing specific peptides, especially activated dendritic cells (DC), are induced to migrate from the site of infection to the draining lymph node or spleen, where the naïve T cells first recognize antigen on DC and are activated to initiate their response (2).

Optimum activation of naïve cells in lymph nodes requires signals through the T cell receptor (TCR) via peptide bound to class II MHC as well as secondary signals from costimulatory ligands such as B7 and CD40 provided by activated DC. The combination of these signals leads to proliferation of naïve cells and begins the process of their differentiation to fully functional effectors capable of secreting high levels of cytokines and participating in helper and inflammatory reactions. The presence of polarizing cytokines such as IL-12 or IL-4 cause CD4$^+$ T cells to differentiate into Th1 or Th2 cells that produce distinct cytokine patterns and mediate different types of protective responses (3, 4). Effector CD4$^+$ T cells are also characterized phenotypically by a decrease in the expression of CD62L and a concomitant increase in CD44 expression.

Once effector cells are generated, their functional role requires that they relocate so they can interact with responding B cells and provide help (5) and/or to migrate to the sites of infection (6). Evidence indicates that these relocations are regulated by the acquisition or increase in expression of multiple adhesion molecules (7, 8) and a switch in the pattern of chemokine receptor expression (9) that causes the effectors to migrate to the B cell zones in lymphoid organs on one hand, or to exit the lymphoid organ and traffic to sites of infection and inflammation on the other hand. Lymphocytes also widely disseminate to sites with no apparent involvement in the response (10–14). Some recent evidence supports the concept that migration to these non-lymphoid sites is independent of antigen (10) but is increased by inflammation (8, 11).

In the infection models examined previously (13, 14), it is difficult to determine whether migration to tertiary, non-lymphoid, sites is an inherent property of effector cells because of their activation state and surface phenotype, or whether effec-

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[1] To whom correspondence should be addressed: Trudeau Institute, 154 Algonquin Ave., Saranac Lake, NY 12983. Tel.: 518-891-3080; Fax: 518-891-5126; E-mail: sswain@northnet.org.

[2] The abbreviations used are: APC, antigen-presenting cells; ATXBM, adult thymectomized, bone marrow reconstituted; DC, dendritic cells; LN, lymph node; MFI, mean fluorescence intensity; PEC, peritoneal exudate cells; PCCF, pigeon cytochrome C fragment; FACS, fluorescence-activated cell sorter; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; MHC, major histocompatibility complex; PE, phycoerythrin; IL, interleukin; GFP, green fluorescent protein; TCR, T cell receptor; VSV, vesicular stomatitis virus; PBL, peripheral blood; Tg, transgenic; Ag, antigen.

tor cells are called into these sites by inflammatory signals and/or small amounts of antigen. The published studies favor the idea that some recruitment may be independent of antigen and inflammation (11) but cannot rule out those factors. The wide dissemination of effectors could provide an important mechanism for the destruction of infected cells, which are in or have gone to non-lymphoid tissues.

Recently it has been demonstrated that during a vigorous response, highly activated CD8 and $CD4^+$ T effectors not only can migrate widely but they can also persist in these tertiary sites for months following infection (11, 13, 14). When effector responses to influenza are visualized *in vivo*, large numbers of effectors are recruited to the lung at the peak of effector generation, and then as soon as virus is cleared, the population rapidly contracts and the remaining cells acquire a more resting phenotype (6). $CD4^+$ memory T cell persistence in lymphoid sites does not require antigen (16). It has been claimed that memory cells in the site of likely infection are better able to provide protection. However, the role of antigen and inflammation in the persistence of primed cells in the non-lymphoid sites remains unclear.

Our laboratory has demonstrated previously that $CD4^+$ T cell effectors generated *in vitro* and transferred into recipient mice can persist as memory cells (15). We found that when *in vitro* generated $CD4^+$ T cell effectors are further cultured in the absence of antigenic stimulation, they revert to a resting state that phenotypically resembles memory cells (16, 17). When we adoptively transferred these "rested effector" cells, they migrated mainly to lymphoid sites and efficiently developed into long lived memory cells, which persist indefinitely without the need for any further division (17). This raises the possibility that recruitment to non-lymphoid sites is restricted to highly activated effectors. To better understand the mechanisms governing non-lymphoid migration of $CD4^+$ T cell subsets to non-inflamed sites, we compared the ability of naïve, Th1 and Th2 effectors, and rested effectors to migrate in an unimmunized adoptive host. CD4 cells were isolated from TCR transgenic mice, and effectors were generated *in vitro*.

Here we report that localization of $CD4^+$ T cell effectors to non-lymphoid organs can occur in the *absence* of antigen and inflammatory signals. We find that after transfer to uninfected hosts, activated, but not rested, effectors migrate in substantial numbers into tertiary sites. Furthermore, *in vivo* effectors, generated following *in situ* immunization with antigen in adjuvant, are also recruited into non-lymphoid organs. Resting memory cells became effectors more quickly following immunization and also are induced to migrate to tertiary sites. These results suggest that the ability to migrate to tertiary sites is an intrinsic but transient property of effectors. Of particular interest is that populations in the tertiary sites decay faster than those in secondary sites, arguing against the concept that memory cells continuously recirculate from tissues to lymphoid organs. We speculate that the ability of activated effectors to gain access to noninflamed organs or tissues may be a way to provide a mechanism to find and destroy disseminated infected cells and provide systemic protection from reinfection, but that such cells could also pose a danger by inducing autoimmunity in sites not normally exposed to immune reactions.

MATERIALS AND METHODS

Mice—AND.Thy 1.1 (H-$2^{b/k}$) and GFP.AND (H-$2^{k/k}$) TCR transgenic mice at 6–8 weeks of age were used to obtain donor cells and were transferred to recipient B10.BR × B6 F1(H-$2^{b/k}$) or B10.BR (H-$2^{k/k}$) mice, respectively. All mice were bred in the animal facility at Trudeau Institute. The animals were kept in animal isolators in an infection-free environment. Hosts were intact mice or adult thymectomized, bone marrow reconstituted mice (ATXBM) that were prepared by surgical thymectomy at 3–4 weeks of age, followed by irradiation with 950 rads, and bone marrow reconstitution at 1–3 weeks after surgery. Donor cells were transferred into ATXBM hosts at 1–3 weeks after bone marrow reconstitution. All animals were bred in the animal facility at Trudeau Institute. In all experiments, multiple animals were analyzed individually, and the mean percentage of total donor cells recovered ± S.D. was calculated.

Reagents—Medium used for all *in vitro* cultures was RPMI 1640 (Invitrogen) supplemented with 200 μg/ml penicillin, 200 μg/ml streptomycin, 4 mM L-glutamine, 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, and 7.5% fetal calf serum (Invitrogen).

Preparation of $CD4^+$ Naïve T Cells—Naïve $CD4^+$ T cells were enriched from spleen and lymph nodes as described previously (15). Briefly, pooled spleen and lymph node cells from TCR Tg mice were passed through a nylon wool column. The nonadherent cells were then treated with a panel of antibodies, including anti-CD8 (3.155), anti-heat-stable Ag (J11d), and anti-class II MHC (D3–137, CA4, and M5114) followed by complement. The resulting $CD4^+$ T cell population was then further purified into the small resting fraction by Percoll gradient separation. The purified cell populations were routinely >90% $CD4^+$ T cells, 95–98% of which had a naïve phenotype ($CD45RB^{hi}$, $CD62L^{hi}$, $CD44^{lo}$, and $CD25^{lo}$) and expressed the TCR transgene. Five 20×10^6 naïve cells were adoptively transferred by intravenous injection into syngeneic intact or ATXBM mice.

Generation of Effectors, Rested Effectors, and Memory Cells—Th1 and Th2 effectors and rested effectors were generated as described previously (15, 17). Highly purified naïve cells (1.5×10^5/ml) from AND TCR Tg mice were cultured with mitomycin C (100 μg/ml)-treated fibroblast APC expressing B7 and ICAM-1 (DECK.ICAM) (7.5×10^4/ml) and 5 μM pigeon cytochrome *c* fragment, amino acids 88–104 (PCCF). For the generation of Th1 effectors, cultures were incubated with IL-2 (11 ng/ml), IL-12 (2 ng/ml), and anti-IL-4 (clone 11B11; 10 μg/ml). Th2 effectors were cultured in the presence of IL-2 (50 units/ml), IL-4 (200 units/ml), and anti-interferon-γ (clone XMG1.2; 10 μg/ml). Effectors were harvested after 4 days of *in vitro* culture, whereas rested effectors were thoroughly washed and recultured in fresh medium for another 2–3 days. $1–10 \times 10^6$ effectors or rested effectors were adoptively transferred intravenously into intact or ATXBM mice.

After we had transferred the effectors, they returned to resting cells within a few days.[3] We refer to the donor cells remaining 3 weeks or more after effector transfer as memory cells (16, 17).

[3] J. Li, H. Hu, and S. L. Swain, unpublished data.

Immunofluorescent Staining and FACS Analysis—Single cell suspensions from spleen, lymph nodes, fat pads, PEC, and PBL were incubated with Cy-Chrome-labeled anti-CD4 (clone RM4-5; Caltag, Burlingame, CA) and biotin-labeled anti-Thy 1.1 (clone OX-70; Pharmingen) followed by streptavidin APC (Pharmingen) to detect donor cells. PE-labeled anti-CD44 (clone IM7; Pharmingen) and biotin-labeled anti-CD62L (clone Mel 14; Caltag) were used to detect surface expression of CD44 and CD62L. Anti-CD11a, anti-CD18, and anti-CD162 were also from Pharmingen. Control isotype-matched antibodies, including PE-labeled rat IgG2a, and fluorescein isothiocyanate- and PE-labeled rat IgG2b isotypes were also purchased from Pharmingen and used as controls with relevant staining reagents. Donor cells were gated as Thy1.1$^+$/CD4$^+$ or GFP$^+$/CD4$^+$ cells and were analyzed for the expression of CD44 and CD62L using FACSCalibur flow cytometers, and the data percent recovery in each organ was based on total donor cells (the % in FACS analysis × total cell recovery from the organ or site) as a fraction of total transferred cells. This also results in normalized data that can be directly compared from mouse to mouse and experiment to experiment. The raw data cannot be so compared, because the number of cells transferred varied in different experiments.

RNase Protection Assay—Total RNA was isolated from naïve, Th2 effectors, Th2 rested effectors, and Th2 *in vivo* memory cells using TRIzol (Invitrogen). mRNA levels were determined using the RiboQuant multipurpose ribonuclease protection assay system (Pharmingen) and the mCR-5 (CCR receptors) and mCR-6 (CXCR receptors) probe sets. The signal from bands was detected using the densitometric feature of Quantity One software (Bio-Rad) and normalized against the housekeeping gene L32.

Antigen Challenge of Adoptive Hosts with Naïve, Rested Effectors, and Effectors—In some experiments, adoptively transferred mice were injected intraperitoneally with 88 μg of PCCF peptide emulsified in complete Freund's adjuvant (CFA) (for recipients of naïve cells) or incomplete Freund's adjuvant (IFA) (for recipients of rested effectors and effectors). The animals were sacrificed on day 4 after immunization and analyzed for the presence of transferred cells in spleen, lymph nodes (paraaortic, inguinal, and mesenteric), PEC, and fat pads.

RESULTS

Th1 and Th2 Effector Cells Migrate to Both Lymphoid and Non-lymphoid Sites following Adoptive Transfer—Our laboratory (6) as well as others (7, 10, 11, 14, 18) have demonstrated that T cell effectors activated by infection or antigen in adjuvant can migrate to the site of inflammation where they secrete cytokines, display cytolytic activity, and participate in pathogen clearance. To analyze the regulation of CD4$^+$ T cell subset recruitment to non-lymphoid sites, we used a standard *in vitro* activation protocol to generate Th1 and Th2 effector cells that produced interferon-γ and IL-4, respectively (15, 17, 19). After 4 days, the resulting Th1 and Th2 populations were transferred intravenously into unimmunized intact or ATXBM. We transferred the CD4$^+$ T cell subsets to unimmunized mice where there would be no possibility of antigen or significant inflammation in non-lymphoid sites. Utilization of TCR Tg cells activated *in vitro* and transferred to mice without infection allows us to determine whether migration to non-lymphoid sites is an intrinsic property of activated effector cells or results from inflammation and/or antigen.

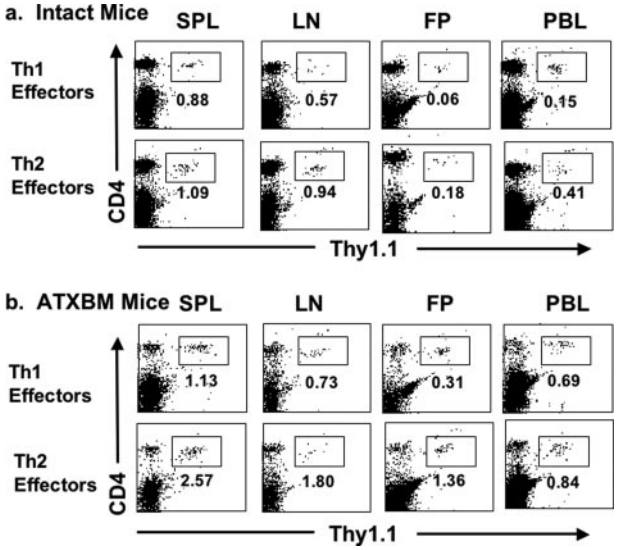

FIGURE 1. **Th1 and Th2 effector cells migrate to lymphoid and non-lymphoid sites following adoptive transfer.** Th1 and Th2 effectors were generated *in vitro* from AND.Thy1.1 TCR Tg naïve CD4$^+$ T cells. Effectors were then injected intravenously into naïve B10.Br. hosts (*a*) or ATXBM B10.BR hosts (*b*). After 4 days, mice were sacrificed; their organs were removed; and cells were isolated and stained with antibodies to CD4 and Thy1.1 for analysis by flow cytometry. The *rectangles* show how transferred cells were gated, and the *numbers* below the *boxes* represent the percentage of donor cells detected in spleen (*SP*), lymph nodes (*LN*), fat pads (*FP*), and PBL.

The donor Th1 and Th2 effectors were obtained from Thy1.1$^+$ TCR transgenic mice and were readily detected in the spleen and lymph nodes of intact mice 4 days after transfer (Fig. 1*a*). Strikingly, the transferred Th1 and Th2 effectors were also detectable in the fat pads of these mice. As expected, effector cells were also detected as circulating cells in the PBL. No significant differences were seen between the Th1 and Th2 polarized populations. Although effector cells were detectable in intact mice, the frequency of these cells was low, and thus particularly difficult to detect in the tertiary or non-lymphoid organs. In previous studies from our laboratory, we used ATXBM mice as recipients to visualize the transition from effector cell to memory cell (17, 20). To allow us to better track the low numbers of donor cells in non-lymphoid sites, we compared migration in intact *versus* ATXBM hosts, and we found it to show a similar pattern. As expected, the frequency of Thy1.1 cells is somewhat higher in ATXBM mice than in intact hosts (Fig. 1*b*). The paucity of host T cells clearly makes the detection of transferred cells more unambiguous. At later time points after transfer, effectors are more difficult to detect in intact hosts (data not shown). Because donor cells could be more extensively analyzed and memory generation and persistence followed in the T cell-deficient hosts, we show mostly data from such hosts. We have repeated many experiments in intact animals and have always seen the same patterns.

To more readily detect cells in low frequencies, we have also used TCR Tg mice that express green fluorescent protein (GFP). The cells obtained from the GFP$^+$ mice are bright green,

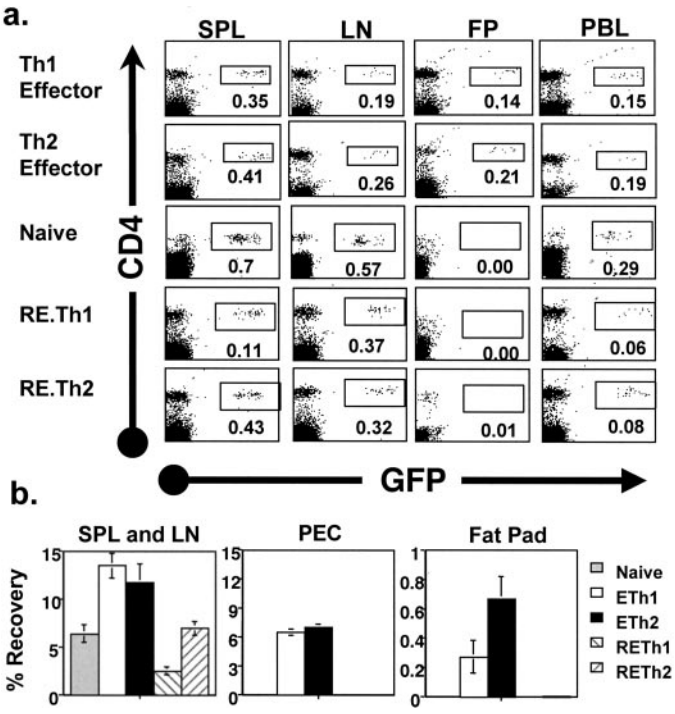

FIGURE 2. **Effectors but not naïve cells or rested effectors of CD4$^+$ T cells are found in tertiary sites.** CD4$^+$ naïve T cells, Th1 and Th2 effectors, and Th1 and Th2 rested effectors were generated *in vitro* from AND.GFP TCR Tg and transferred intravenously to ATXBM hosts as described in Fig. 1. After 4 days, peritoneal exudate cells were collected; the lymphoid organs (spleen (*SPL*) and LN) and fat pads were removed and analyzed by flow cytometry for percentage of donor cells. Donor cells were identified by gating on simultaneous expression of CD4 and GFP and are shown within the *rectangles*. The *numbers* below the *boxes* represent the percentage of donor cells detected in spleen (*SPL*) lymph nodes (*LN*) fat pads (*FP*) and peripheral blood (*PBL*) (*a*). Percent recovery was calculated as the absolute number of CD4$^+$/GFP$^+$ cells recovered per organ divided by the total number of cells transferred (*b*). The data show the mean ± S.D. of three mice per group.

which greatly facilitates their visualization. To evaluate the use of these mice, we transferred Th1 and Th2 effectors generated from AND.GFP naïve CD4$^+$ T cells in the wild type mice. Fig. 2*a* confirms that the migratory patterns of these Th1 and Th2 GFP-expressing effectors are similar, and we can readily find GFP$^+$ donor effector cells in the fat pads. We also find them in lung, liver, and peritoneal exudate as seen in later experiments. Preliminary studies indicated that the GFP-expressing effector cells persisted after transfer to ATXBM at levels equivalent to their wild type counterparts.[4] Fig. 2*b* shows the pattern of migration of T cell subsets in the lymphoid (combined spleen and lymph node) *versus* two non-lymphoid sites, the peritoneum and abdominal fat pads. Four days following transfer of effectors (Fig. 2*b, solid bars*), the peritoneum contained more than a third of the number of cells found in the combined lymphoid organs. Even the largely acellular fat pads contained a significant fraction of the total (up to 1 part in 20).

Taken together, these data demonstrate that activated CD4$^+$ T cell effectors cannot only traffic to the spleen and lymph node after transfer but that the activated cells can also migrate to non-lymphoid sites in the absence of both antigen and inflammation. No significant difference in pattern of distribution or frequency of Th1 *versus* Th2 effectors was seen in repeated experiments.

Rested Effectors Lose the Ability to Migrate to Non-lymphoid Sites—In vitro generated CD4$^+$ T cell effectors can revert to a resting state after further culture in the absence of antigenic stimulation (17). As they become rested, the cells come to express a more memory phenotype characterized by lower forward scatter, loss of CD25, lower levels of surface CD44, and higher levels of CD62L (17). Rested effectors no longer proliferate to γc cytokines such as IL-2, IL-4, IL-7, and IL-15,[4] and unlike effectors they do not divide upon transfer to adoptive hosts (17). Here rested effectors are generated by culturing Th1 or Th2 effectors for an additional 2–3 days in fresh media without additional antigen or cytokines. To determine whether migration to tertiary sites was a property dependent on the activated state of CD4$^+$ T cell effectors, naïve, Th1, and Th2 effectors and Th1 and Th2 rested effectors were transferred to adoptive hosts, and the percent recovery of transferred donor cells in various organs was determined based on the frequency of donor cells and total cell recovery from each organ (Fig. 2). This calculation makes comparison among experiments possible because the number of transferred cells often varied with each group, and because effectors were numerous, whereas rested effectors (especially Th1) were more rare. Earlier experiments established that recovered cells were directly proportional to transferred cells over a broad range (17).

By comparing recipients of these different subsets, it is clear that rested effectors, like naïve cells, do not appear in the PEC or fat pads 4 days after transfer. This result is representative of effector, naïve, and rested effector migration upon adoptive transfer seen in over four experiments. Naïve CD4$^+$ T cells were only detected in secondary lymphoid sites and not in fat pads or PEC. Rested effectors were like naïve cells and were found only in the spleen and lymph node (Fig. 2*b*, shown here as combined, but separate analysis shows rested effectors concentrated in the spleen as shown in Figs. 5 and 6). Both Th1 and Th2 effectors were uniquely able to enter non-lymphoid sites. Because rested effectors were not found in PEC or fat pads, we conclude that as effectors return to rest they quickly lose their ability to migrate to these non-lymphoid sites. The patterns of migration were the same irrespective of the number of cells transferred (not shown). For all subsets, the polarization of the cells to Th1 or Th2 profiles had little effect on their potential to migrate to different organs.

Effectors Display Enhanced Expression of Adhesion Molecules Relative to Rested Subsets of CD4$^+$ T Cells—The migration of different CD4 subsets to secondary *versus* tertiary sites is expected to be regulated by adhesion molecules, selectins, and chemokine receptors. Therefore, we examined the expression of cell surface receptors/ligands by the T cell subsets evaluated for migration to lymphoid *versus* non-lymphoid sites (Fig. 3 and Fig. 4). We evaluated the effect of transfer on expression of CD44 and CD62L molecules and examined expression by long term resting memory cells from spleen and lymph nodes derived from transfer of Th1 and Th2 effectors as described previously (17). Such memory cells are concentrated in lymphoid organs, particularly the spleen (Fig. 3). We hoped this analysis would point the way to candidate pathways regulating the

[4] G. Huston and S. L. Swain, unpublished data.

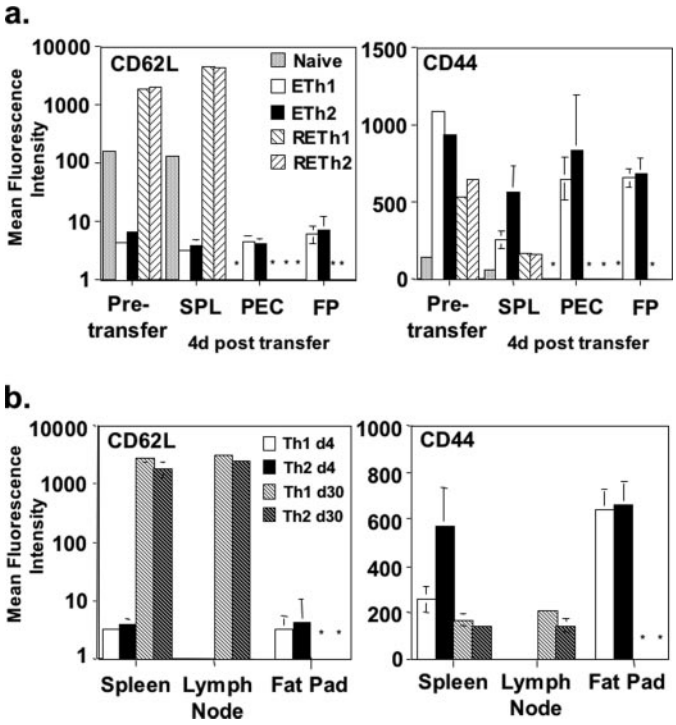

FIGURE 3. **Phenotype of CD4$^+$ T cell subsets before and after transfer.** Donor cells were isolated as described for Fig. 2, and their expression of CD62L and CD44 was determined. Subsets were transferred to ATXBM hosts, stained with fluorochrome-conjugated antibodies, and analyzed in various organs for 4 days and 30 days after transfer. Donor cells were identified by co-expression of CD4 and GFP, and expression of CD62L or CD44 was determined. The *graphs* show the mean fluorescence intensity of CD62L or CD44 expression on gated donor cells. The *bars* represent the mean ± S.D. of three mice per group for spleen (*SPL*), peritoneal exudate cells (*PEC*), lymph nodes (*LN*), and fat pads (*FP*). The level of expression of CD62L and CD44 on naïve, effectors, and rested effectors before transfer is shown as a comparison. *a,* expression of CD62L (*left*) and CD44 (*right*) on CD4$^+$ T cell subsets before and 4 days after transfer. Expression was determined by FACS and is expressed as mean fluorescence intensity. *Each bar* is the mean ± S.D. of three mice. The *missing bars* for naïve and RETh1 and RETh2 denote no cells recovered in that group and are shown by an *asterisk*. *b,* expression of CD62L and CD44 on transferred Th1 and Th2 effectors for 4 (*solid bars*) and 30 days after transfer. No cells were detected in the fat pads at 30 days and are indicated by an *asterisk*.

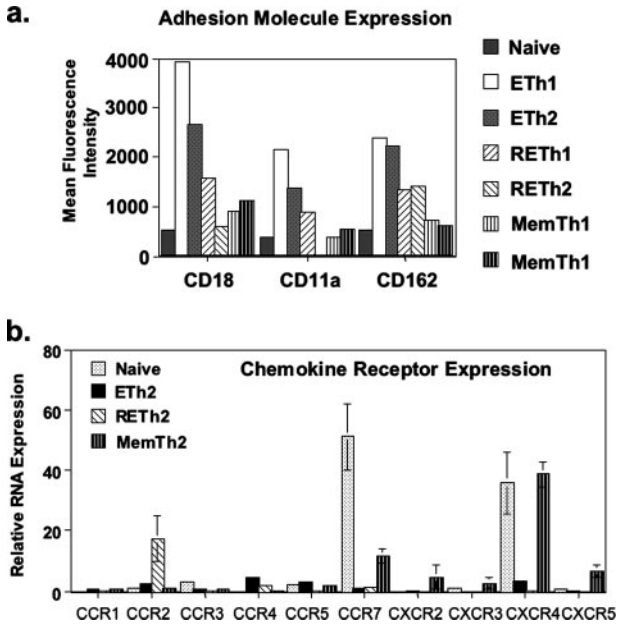

FIGURE 4. **Adhesion molecule expression but not chemokine receptor expression correlates with ability to migrate to tertiary sites.** *a,* expression of a panel of adhesion molecules was determined on CD4$^+$ T cell subsets, including naïve, effectors (*Eth*), and rested effectors (*REth*) and also memory (*Mem*) cells recovered 6 weeks after transfer of effectors to MHC class II knockout mice. Those that were highest on effectors, CD18, CD11a, and CD162, are shown as the mean fluorescence intensity. *b,* chemokine receptor RNA expression by CD4$^+$ T cells subsets was determined by RNase protection assays. Results represent the mean relative RNA expression determined in 3–4 separate experiments with S.E. The values for naïve, Th2 effectors, and Th2 rested effectors and Th2 memory are shown. Th1 counterparts were examined at the same time and were very similar, although Th1 rested effectors expressed less CCR2 than Th2, Th1 effectors expressed less CCR4 and more CCR5 than their Th2 counterparts, and Th1 memory expressed less CXCR5.

unique ability of effectors to migrate to non-lymphoid sites, which is dramatically reversed as cells become resting *in vitro*.

As expected from previous studies (17), Th1 and Th2 effectors were large activated cells with high forward *versus* side scatter and high expression of CD25, whereas naïve and rested effectors were smaller cells with no CD25 expression (not shown). We first examined CD62L and CD44 expression because these are known to be involved in migration and are standard markers used to identify T cell subsets (Fig. 3). We found that the mean fluorescence intensity and pattern of expression of transferred effectors (before transfer) was retained at 4 days post-transfer in the spleen, PEC, and fat pads, although only the effector populations were represented in the non-lymphoid organs. Naïve cells expressed high levels of CD62L and low levels of CD44 both before and after transfer. Highly activated CD4$^+$ T cell effector cells, expressed low levels of CD62L and high levels of CD44. Rested effectors shifted their pattern somewhat over the 4 days. They still expressed high CD62L but displayed intermediate CD44 levels, which were lower than those expressed before transfer, consistent with a continued progression to a more resting state.

We next analyzed the putative memory cell population retained 30 days after transfer for change in CD62L and CD44 expression. Fig. 3 compares expression on recovered donor cells after effector transfer at day 4 and day 30 post-transfer. The donor cells, derived from Th1 and Th2 effectors, recovered from spleen and lymph node, had reverted to a CD62L moderate to high CD44 moderate phenotype, which is characteristic of resting memory cells. At this time there were no donor cells recoverable in the fat pads as indicated by the *asterisks* in Fig. 3. These results were observed repeatedly. These results suggest that the activated effectors are unique in their very low expression of CD62L and in the highest expression of CD44, suggesting this pattern could contribute to the unique and transient migration of effectors to tertiary non-lymphoid sites.

We also analyzed expression of a number of other adhesion molecules by cell surface staining. Among those, the integrin chains CD18 and CD11a and the ligand for P-selectin, CD162, were high on effectors and lower on resting cells, including naïve, rested effectors, and memory cells as seen in Fig. 4*a*. Thus the high but transient expression on effectors of very high CD44 and high CD18, CD11a, and CD162 and loss of CD62L are correlated with the transient ability of effectors to migrate to PEC and fat pads.

We also evaluated shifts in the pattern of chemokine receptor expression by RNase protection to see if expression at the RNA level would correlate with the pattern of migration. Fig. 4*b* shows the compilation of data from 3 to 4 experiments per population for naïve and Th2 subsets. The only unique feature of effectors that migrate to tertiary sites *versus* the naïve and rested effector populations, which do not, was a slightly increased expression of RNA for CCR4 and CCR5. Naïve and memory cells expressed high levels of CCR7 and CXCR4, but this was not true of rested effectors. Rested effectors expressed the highest levels of CCR2. Th1 subsets showed a similar pattern for most receptors, and importantly, no unique expression of any of the receptors on Th1 effectors *versus* the resting populations was noted. Thus no particular chemokine receptor was correlated with the unique ability of effectors to migrate to the non-lymphoid sites. This is perhaps expected, because the transfers we did were into normal, unimmunized mice where there would be only basal constitutive chemokine production.

Donor Cells Remain in Lymphoid Organs as Memory Cells but Disappear from Tertiary Sites Over Time—We wanted to determine whether effectors in tertiary sites became resting and persisted like those that became memory cells in secondary lymphoid sites. The migration of GFP^+ effector cells to both lymphoid organs and tertiary sites after adoptive transfer allowed us to compare whether the memory cells that developed persisted equally in all organs. We followed the percent recovery of transferred Th2 effectors and Th2 rested effectors in the spleen, LN, and fat pads over 30 days (Fig. 5*a*). The data are represented as log % recovery.

Both effector and rested effectors persisted in secondary sites for 4 weeks with a slow decline that was somewhat more pronounced in the effector population. The rested effectors persisted with only minor decreases but were never found in the fat pads. The fat pad data are not shown for rested effectors because they were below the level of detection. In contrast, the effector cells persisted in the fat pads for about 3 weeks and then declined rapidly so that they were no longer detectable by 30 days. This suggests that the donor populations decay more rapidly in the tertiary site. In a separate experiment we also analyzed the relative recovery of different donor subsets at day 4 *versus* day 30 (Fig. 5*b*). Naïve and both Th1 and Th2 rested effectors went to the spleen and persisted there, but they were not found in fat pads or PEC at days 4 or 30. Th1 and Th2 effectors were highly represented in both the spleen and in both tertiary sites at day 4 (Fig. 5*b, dark bars*). Donor cells from effectors disappeared by day 30 from PEC and fat pads but were still found in the spleen. Because the donor cells are retained in secondary but not tertiary sites, it appears that the memory cells in the two different types of locations are not in equilibrium and suggests that memory cells do not circulate between lymphoid organs and tissues in noninflammatory conditions. It also appears that persistence of memory is regulated by the location of the cells.

Antigenic Challenge of Resting Donor Cells in Vivo Leads to Effectors, Which Migrate to Tertiary Sites—The data in Fig. 5 demonstrated that residual donor cells were not found in appreciable numbers in tertiary sites by 30 days after transfer.

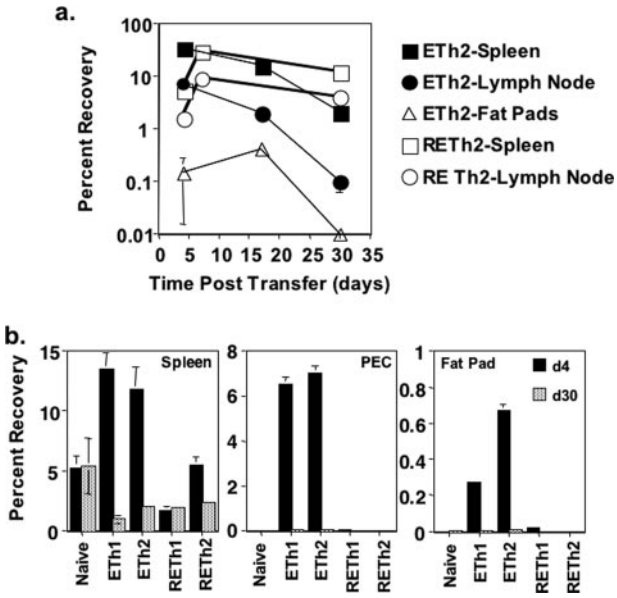

FIGURE 5. **Donor memory cells derived from effectors remain in lymphoid organs but disappear from tertiary sites over time.** *a,* Th2 effectors and rested effectors were generated as described for Fig. 2, transferred to ATXBM hosts, and their numbers followed by analyzing co-expression of CD4 and GFP in various organs on 4, 19, and 30 days after transfer. The positive and the percent recovery were calculated for each organ as described. The *bars* represent the mean ± S.D. of four mice assayed for each group. Data are displayed as log values. No donor cells were detectable in the fat pads (*FP*) of mice receiving rested effectors, and so those values are not shown. *b,* naïve, Th1 and Th2 effectors, and rested effectors were transferred to groups of ATXBM mice, and recovery was determined on days 4 (*dark bars*) and day 30 (*lighter bars*). The bars represent the mean ± S.D. of four mice assayed individually within each group.

We thus wanted to visualize whether *in situ* stimulation of resting donor cells in secondary sites would lead to their activation and the reacquisition of the capacity to migrate to tertiary sites. We reasoned that with optimal stimulation naïve cells would become primary effectors (like those we generate *in vitro*), as demonstrated previously (6, 21), and that donor cells remaining after effector transfer, which now were memory cells (17, 20), would become secondary effectors. In each case, once fully activated, they might be able migrate to PEC, fat pads, and other tertiary sites. Recipients of naïve cells and Th2 effector or Th2 rested effector cells, introduced 4 weeks previously, were stimulated with or without peptide antigen in adjuvant, and the percent recovery of donor cells was determined 4 days later (Fig. 6*a*). In all recipients, treatment with adjuvant without antigen (Fig. 6*a,* −*Ag* group) did not change the distribution of the cells from that before challenge (not shown but compare with Fig. 2). They were found only in spleen and LN and could not be detected in the PEC or fat pad. In contrast, immunization with antigen changed the migratory pattern of the naïve, rested effectors, and effectors (now rested effectors). In all these groups, the cells migrated to fat pads and PEC. However, in recipients of naïve cells, priming with Ag led to substantial expansion of donor cells and their appearance in PEC and to a lesser but significant extent (comparable with the % recovery in the earlier transfer of effector experiments) in the fat pads.

The memory cells generated by transfer of either effectors or rested effectors expanded dramatically in response to antigen in adjuvant (Fig. 6*a*). Within the secondary sites, total recovery

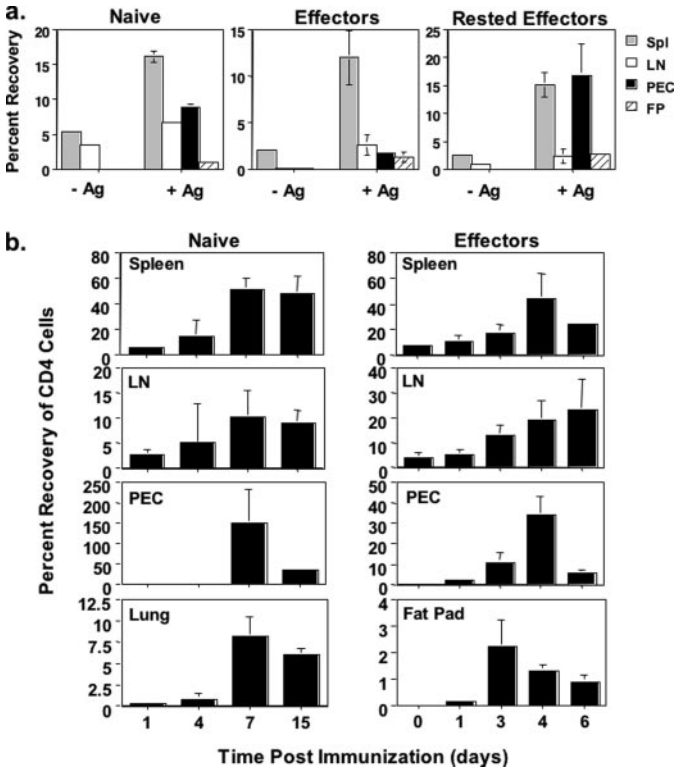

FIGURE 6. ***In vivo* generated effectors migrate to tertiary sites.** *a, in vivo* generated effectors go to tertiary sites. Naïve, Th2 effectors, and Th2 rested effectors derived from AND.GFP TCR Tg mice were transferred into ATXBM hosts and allowed to return to rest. After 30 days, recipients were injected intraperitoneally with 5 μM PCCF in IFA, whereas mice receiving naïve cells were injected with 5 μM PCCF in CFA (+*Ag*). Four days after immunization, when the Ag-specific cells have again differentiated into effectors (naïve) or secondary effectors, mice were sacrificed and their organs removed. Donor cells were enumerated in each sample by flow cytometry as described. The *graphs* show the mean ± S.D. of the percent recovery calculated as described for 3–4 mice per group. As a control, mice that had received naïve cells, effectors, or rested effectors 30 days previously were treated with adjuvant but no antigen (−*Ag*). *b,* kinetics of *in vivo* generation of migrating effectors. The peak of memory cell numbers in non-lymphoid sites occurs earlier than the influx of effector cells to those sites. AND.GFP TCR Tg naïve cells were transferred into ATXBM hosts and immunized with 88 μg of PCCF emulsified in CFA intraperitoneally (*left*). At 1, 4, 7, and 15 days after immunization, mice were sacrificed, and spleen, lymph node (*LN*), peritoneal exudate, and lung were removed and isolated cells analyzed for the presence of $CD4^+/GFP^+$ T cells by flow cytometry. The *graph* shows the mean ± S.D. percent recovery of $CD4^+/GFP^+$ T cells at different days after immunization. *Right,* Th2 effectors derived from AND.GFP TCR Tg mice were transferred into ATXBM hosts and rested *in vivo* for 30 days (day 0 on graph). Mice receiving effectors were then injected intraperitoneally with 88 μg of PCCF in IFA and sacrificed at various days post-immunization. The percent recovery of $CD4^+/GFP^+$ T cells is shown for each time point. The data represent the mean ± S.D. of 3–4 mice per group.

was highest in spleen as compared with lymph node. Following priming (4 days), the transferred effector cells (*in situ* memory cells) were also now well represented in fat pads and peritoneum. This suggests that the *in vivo* generated primary and memory effectors had similar migratory properties as the primary *in vitro* generated effectors analyzed in Fig. 2 and Fig. 5. Thus antigen challenge activated all $CD4^+$ T cells subsets *in vivo* and promoted their influx into tertiary sites by 4 days. Mice that received naïve cells or rested effectors showed the highest percentage of cells in the peritoneal cavity. It should be noted that in this case of *in vivo* priming, both antigen and inflammation are present. The recipient mice receiving rested effectors were challenged with peptide in IFA. Because the peptide/adjuvant is introduced intraperitoneally, the peritoneum is a potential site of inflammation.

The Kinetics of Development of Effectors with Ability to Migrate to Tertiary Sites—Previous studies have suggested the peak of primary effector cell generation *in vivo* is seen at 6–7 days based on number, cytokine production, and the ability to migrate to a site of infection and inflammation (6, 22). Secondary or memory effectors required only 3–4 days to develop (21). To determine and compare the kinetics of the generation of effectors with the capacity to migrate to tertiary sites from naïve and memory cells, we compared the recruitment of naïve cells with tertiary organs after a primary immunization with the recruitment of memory cells, resulting from the transfer of *in vitro* generated effectors, upon secondary challenge with antigen. For the primary response, we used the lung as a tertiary site, because peptide in the strong inflammatory adjuvant, CFA, was introduced intraperitoneally, and we wanted to look at an alternate non-lymphoid site least likely to contain antigen and to be inflamed. As shown in Fig. 6*b*, the percent recovery based on the original number of transferred cells in mice given naïve cells and injected with PCCF in CFA increased with time and peaked in the lymphoid organs (both spleen and lymph nodes) 7 days after injection. This correlates very well with our previous studies that indicated the kinetics of peak of effector generation as indicated by both donor cell recovery (6) and the cytokine secretion by *in vivo* effectors (6, 23). Because we immunized with PCCF peptide rather than whole protein, we reasoned that antigen would persist for only a short time. Indeed very large numbers of cells were found in the PEC, the most in any site, consistent with the possibility that inflammation enhanced effector cell recruitment. However, the lung also was the source of a substantial population of donor effectors at day 7. We did not look at fat pads because of their location relative to antigen/adjuvant injection.

By 15 days donor cells in the primed recipients of naïve $CD4^+$ T cells were beginning to decline in the PEC and lung. Recipients with memory cells derived from transferred effectors were primed with peptide in incomplete adjuvant that contains no heat-killed bacteria and is largely noninflammatory.[5] At day 30 after initial transfer, no cells were detected in tertiary sites (PEC and fat pads). The 1st day after challenge, a few cells could be detected in PEC, and the peak recovery was seen by day 3 or 4. In tertiary sites the cell numbers decay noticeably after their peak, suggesting that memory effectors as well as primary effectors decay in tertiary sites *in situ*.

DISCUSSION

The results presented here suggest that resting cells, including rested effector cells and memory cells, do not migrate or recirculate to tertiary non-lymphoid sites in the absence of inflammation and that the ability of $CD4^+$ T cells to enter such sites is restricted to very recently activated effectors (generated either *in vitro* or *in vivo*) and is an intrinsic, but transient, property of such effectors. Once in such tertiary sites, the transferred cells revert to a resting state and decline with time at a rate faster than the corresponding cells in secondary lymphoid

[5] L. Haynes, S. Eaton, and S. L. Swain, unpublished data.

organs, suggesting there is no recirculation of resting cells between the lymphoid and non-lymphoid sites in the absence of inflammation.

Effectors Migrate to Tertiary Sites under Noninflammatory Conditions—$CD4^+$ T cells in different activation states localize to distinct sites *in vivo*. We used a TCR transgenic model to generate homogeneous, well defined populations of naïve, Th1 and Th2 effector, and corresponding rested effector populations to pinpoint which subsets had the ability to migrate to non-lymphoid sites in the absence of either antigen or inflammation. Effectors are highly activated, and they migrate efficiently to non-lymphoid organs following adoptive transfer. We recovered donor cells in fat pads and the peritoneum. This suggests that migration to tertiary sites is inherent to the activation state of the cell, because effectors were detected in the peritoneal cavity and the fat pads in unimmunized recipients, which had no reason to have above background levels of inflammation. These data are in agreement with the results obtained by Masopust *et al.* (14) studying the response of $CD8^+$ T cells after systemic infection with vesicular stomatitis virus (VSV). It was demonstrated that VSV-specific $CD8^+$ T cell effectors, generated *in situ,* can migrate to various non-lymphoid sites such as the kidney, lung, and fat pad after viral infection (14). However, in the VSV study, the host is infected and so inflammation and traces of antigen in tertiary sites are hard to rule out. An earlier study had also seen a widespread appearance of responding cells in many sites during an influenza infection (13). Similarly, using an adoptive transfer system, $CD4^+$ T effector cells were found disseminated to the liver, lungs, and lamina propria after intravenous injection of specific peptide antigen emulsified in adjuvant (11). Our studies provide definitive evidence that neither antigen nor inflammation is needed for this widespread dissemination of effectors.

It is interesting to speculate on the possible positive and negative consequences of effectors entering non-lymphoid tissues. On the positive side, this would ensure that infected cells, wherever they might be, would be found by the widespread effectors and presumably destroyed. Also, because effectors persist for several weeks, they could provide a widespread protection against reinfection. On the negative side, entry of effectors to some tissues, where tissue-specific antigens are not normally exposed to the immune surveillance, might increase the chance of initiation of tissue-specific autoimmunity, especially if there were to be a cross-reaction between the pathogen inducing the effectors and a tissue-specific antigen. The transience of the ability of effectors to migrate to noninflamed tertiary sites may have been selected for because of such dangers of autoimmune induction and damage.

Our studies showed a strong correlation between a very high level of activation and expression of high levels of adhesion molecules (CD44, CD11a, and CD18) and the selectin ligand CD162 and low levels of CD62L with the ability of the effectors to disseminate. A few days of incubation of the effectors *in vitro* so they became rested effectors reversed these expressions to a large extent and also dramatically reversed the migration on transfer of the cells. This did not represent an impairment of cell function because the rested effectors persisted well (somewhat better than the effectors; see Fig. 5), and they responded well to priming with peptide 30 days later (Fig. 6). Chemokine receptor expression, at least as reflected in RNA levels, did not show any similar correlation. This is not surprising inasmuch as chemokine expression (except for the constitutively expressed homeostatic chemokines) is not expected to be high in the recipients, which have received no antigen or adjuvant.

Further studies will be needed to determine the contributions of individual adhesion molecules and selectins to this non-lymphoid migration. CD44, CD11a, CD18, and P-selectin-ligand interactions have been demonstrated previously to regulate recruitment to sites of inflammation (7, 8, 24), and loss of CD62L has also been functionally associated with such migration (25).

In earlier studies it was found that both the CD8 and $CD4^+$ T cell populations persisted for months in non-lymphoid organs after viral infection, suggesting that long lived memory cells can remain in tertiary sites ready for reexposure with antigen (11, 14, 26). In that case the tertiary site was the site of infection, viral replication, and much inflammation. In contrast, our results suggest that $CD4^+$ T effector cells that migrate to tertiary sites persist only for a limited time and then decline to undetectable levels by 4 weeks (Figs. 5 and 6*a*). We saw similar indications of preferential decay in tertiary sites, even following *in situ* stimulation with peptide antigen and adjuvant (Fig. 6*b*). Further studies will be needed to assess whether decay is delayed if inflammation and/or antigen is present. It may make sense in balancing the positive and negative aspects of having effectors and their rested counterparts widely disseminated that the population stays long enough to outlast an infection passing through the community, but not much longer.

Implications for Memory Cell Recirculation—After 30 days, $CD4^+$ memory T cells in this model were detected only in secondary lymphoid organs no matter what their origin (from naïve, effectors, or rested effectors). This seems to be incompatible with suggestions that memory cells recirculate through peripheral tissues as suggested previously by Mackay *et al.* (27). The memory cells in these studies displayed preponderance in spleen and LN, and the cells in those secondary organs did not seem to be in equilibrium with those in tertiary sites, where they were no longer detectable. This suggests that memory cells either recirculate differently and/or may not necessarily recirculate significantly. Rested effectors, which are on their way to being memory cells (17), migrate similarly to other resting cells. They could migrate to the spleen and LN (Fig. 2 and Fig. 6) and after they reverted to memory cells (by day 30), and they gave a greater response to antigen in IFA than naïve cells and a vigorous response to the peritoneal cavity by day 4, even though they were initially detected only in the spleen and lymph nodes.

The migratory properties of memory T cells have been difficult to elucidate, partly because of the difficulty in defining memory cell populations and an inability to track individual cells. For example, Lanzavecchia and co-workers (33) have suggested that two subsets of memory cells can be defined based on data collected from circulating antigen experienced T cells recovered from the blood. The $CCR7^+$ population is termed "central memory," and these are predicted to migrate to secondary lymphoid organs where SLC or ELC is well expressed. The $CCR7^-$ cells are termed "effector memory" and are also

defined by high levels of expression of adhesion molecules LFA-1 and VLA-4 (33). Effector memory is the one that is expected to be in non-lymphoid sites. However, our recent results in an influenza model suggest some $CD4^+$ T cells in the lung still retain CCR7 (6), so these definitions may not be firm. The effector memory cells could be analogous to those long lived cells found in non-lymphoid sites months after exposure to virus or antigen in adjuvant as has been described in several studies (11, 14, 26).

Long after an infection, most memory cells are found in the spleen. It has been suggested that the spleen acts as a reservoir for memory T cells because cells circulating through the blood are emptied directly into this lymphoid organ via the central arteriole (34). In the past, it was suggested that memory cells recirculate from the tissues back into lymphoid organs (27, 35), although others have considered the possibility that some memory cells remain at a low frequency within tertiary sites, without being part of a recirculating pool, and expand there upon re-exposure to antigen (36). Because the memory cells in the tertiary sites in our experiments decayed to undetectable levels more quickly than those in secondary sites, our results are most compatible with the latter viewpoint that suggests separate compartments exist in secondary *versus* tertiary sites with little recirculation between them.

Life Span of Effectors and Rested Effectors Is Influenced by Site—Is the decay of cells in different organs influenced by the environment? The shorter life span of the $CD4^+$ T cell population in the tertiary sites could be the result of different properties of the cells themselves or, alternatively, of the different milieu of the tertiary *versus* secondary component. We have recently found that the successful transition of $CD4^+$ effector T cells to long lived memory cells and their persistence over the 1st week is dependent on IL-7 (37), and this is consistent with other recent studies (29). The *in vivo* expression of IL-7 has not been established, but it is thought to be primarily produced constitutively by stromal cells in the thymus, bone marrow, and perhaps other lymphoid tissue. We are currently exploring whether differences in the availability of IL-7 could explain what appears to be differences in memory $CD4^+$ T cell survival in secondary *versus* tertiary sites.

In Vivo Generated Primary and Memory Effector Kinetics—The ability to migrate to tertiary sites was restricted to the peak of effector activity inasmuch as resting effectors for 3 days caused them to lose this ability. Memory cells present after 30 days were not found in tertiary sites, and based on earlier transfer experiments (34), it seems that memory cells also do not have the capacity to enter tertiary sites after transfer. After boosting, memory cells redeveloped the capacity to go to tertiary sites in 3–4 days, about the time they redevelop effector activity based on state of activation and cytokine production (21). This suggests that although memory cells can rapidly secrete cytokines after restimulation with antigen (38), they do not have the full spectrum of activities of effector cells. The fact that the restimulated memory cells are found first in lymphoid sites and would take several days to become effectors able to migrate to the sites of infection may explain the greater protective ability of effector cells over memory cells in a number of infectious disease models (39, 40).

Recent studies suggest that effectors that reach tertiary sites during an infection may remain in those sites in sufficient numbers and perhaps in a more activated form (6, 26) like the effector memory proposed by Lanzavecchia and co-workers (33). Some recent studies support the concept that $CD8^+$ T cells in tertiary sites, like the lung, can rapidly respond and provide superior protection (41), whereas others support an equal or greater general functionality of memory with the central memory phenotype (42, 43). Our studies suggest that $CD4^+$ effector T cells that reach noninflamed tertiary sites can revert to a resting phenotype, and because they decay more rapidly than those in secondary lymphoid sites, protective memory from $CD4^+$ T memory cells in such sites may be of limited duration. Further understanding of the functional potential of memory $CD4^+$ T and $CD8^+$ T cells in different locations is needed to determine whether there are differences between these cells in this regard and also to determine whether prolonged antigen stimulation in some tertiary sites can contribute to long term maintenance of cells in an activated site in some infections.

In summary, we have found that as $CD4^+$ T cell subsets differentiate, there is a distinct and transient effector state during which they can migrate to noninflamed tertiary sites. This dispersal at the peak of the response could result in a transient $CD4^+$ T cell response in the tertiary sites. This process is strictly regulated, because of the fact that as soon as effectors become resting they lose their ability to migrate to tertiary sites and because the cells that do become memory in those sites decay over several weeks.

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Unique Ability of Activated CD4$^+$ T Cells but Not Rested Effectors to Migrate to Non-lymphoid Sites in the Absence of Inflammation

Javed N. Agrewala, Deborah M. Brown, Nancy M. Lepak, Debra Duso, Gail Huston and Susan L. Swain

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