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Recirculatory and Sessile CD4\(^+\) T Lymphocytes Differ on CD45RC Expression\(^1\)

Francisco Ramírez\(^2,3\) and Don Mason

CD4\(^+\) T cell subsets are unequally distributed in rat secondary lymphoid organs. Those with the memory phenotype CD45RC\(^{low}\) are present at a higher frequency in Peyer’s patches (PP) than in lymph nodes and spleen, and increase in numbers with age in all three tissues, particularly in the PP. Homing experiments revealed that CD4\(^+\) T cells that recirculate through secondary lymphoid organs are mainly CD45RC\(^{high}\). It was also apparent that the ability of recirculating cells to enter different lymphoid organs varies; less cells enter PP than the spleen or lymph nodes. Our results also reveal the existence of a nonrecirculating population of CD4\(^+\) T cells in secondary lymphoid organs, which are predominantly, if not exclusively, CD45RC\(^{low}\). Our results show that secondary lymphoid organs differ in their CD4\(^+\) T cell subset composition as a consequence of having different ratios of recirculatory:nonrecirculatory CD4\(^+\) T cells, and these cells display a different CD45RC phenotype. The Journal of Immunology, 2000, 165: 1816–1823.

The primary lymphoid organs, thymus and bone marrow, are responsible for the generation of mature virgin lymphocytes. These naive lymphocytes enter the pool of recirculatory lymphoid cells that migrate between the secondary lymphoid organs. The most accepted route of lymphocyte recirculation postulates that naive cells extravasate from the blood into secondary lymphoid organs and return to the blood via the efferent lymphatics. Naive lymphocytes follow this pathway until they die or they encounter their specific Ag and are activated (1). Lymphocyte activation takes place in the secondary lymphoid organs, where Ags are trapped in appropriate microenvironments to initiate immune responses. Activated lymphocytes proliferate and differentiate into effector and memory cells. It has been proposed that these cells, in contrast to naive cells, migrate to the body tissues where they can meet Ag and eliminate it. If memory/effector cells are not stimulated in the tissues they re-enter the lymph nodes (LN)\(^4\) through the afferent lymphatics, where they mix with naive cells en route to the efferent lymphatics and blood (1, 2). Although several studies analyzing the recirculation of sheep lymphocytes support this view (2), some studies, employing rats as a model, do not find considerable differences between the pattern of recirculation of CD4\(^+\) T cells with naive and memory phenotypes (3–5).

CD4\(^+\) T cells can be divided into populations reflecting different stages of maturation and activation on the basis of the expression of several membrane molecules. The following three markers are particularly important in rat studies: Thy-1, RT6, and CD45RC (6). In rats, Thy-1 is expressed on thymocytes and recent thymic emigrants (RTE) and is down-regulated a few days after cells leave the thymus (7, 8). RT6 and CD45RC are absent on thymocytes, and their expression is first detected in the periphery on RTE. Most T cells express RT6 but are heterogeneous in the expression of CD45RC (7–9). Activated T lymphocytes down-regulate the expression of both molecules (10, 11). CD45RC expression defines two subpopulations of functionally distinct, mature CD4\(^+\) T cells. The CD45RC\(^{high}\) population contains naive cells that produce more IFN-\(\gamma\) and IL-2 on activation than CD45RC\(^{low}\) cells; they provide help to B cells in primary responses, are reactive in graft-vs-host assays, can cause autoimmunity, and are the precursors of the CD45RC\(^{low}\) population (9, 12–17). In contrast, the CD45RC\(^{low}\) subpopulation contains the majority of T helper cells for secondary humoral responses, produces more IL-4 than the CD45RC\(^{high}\) subpopulation, proliferates to recall Ags, and contains cells that suppress some autoimmune manifestations (6, 9, 12–14, 16–18). The CD45RC phenotype of mature CD4\(^+\) T cells in the rat is not stable (19, 20). It has been proposed recently that naive rat CD4 T cells are contained in the CD45RC\(^{high}\) pool while the memory CD4 population contains CD45RC\(^{high}\) and CD45RC\(^{low}\) cells which differ in the requirement for Ag. Memory CD45RC\(^{low}\) cells regain expression of the CD45RC epitope in the absence of Ag (21), but both CD45RC\(^{high}\) and CD45RC\(^{low}\) memory cells can coexist in the same animal (9).

Differences in cytokine production between secondary lymphoid organs have been described (22–24), suggesting that different secondary lymphoid organs are specialized to generate different effector functions depending on their anatomical localization. Some of these studies suggest that leukocytes from mucosal-associated lymphoid tissues produce, on activation, Th2 responses. Given that the expression of CD45RC on CD4\(^+\) T cells defines cell subsets with different cytokine production, we decided to analyze the distribution of CD4\(^+\) T cell subsets in rat secondary lymphoid organs. A marked difference in the expression of CD45RC, and other maturation markers, was observed between lymphoid organs. Peyer’s patches (PP) contain the highest proportion of CD4\(^+\) CD45RC\(^{low}\) Thy-1\(^-\) L-selectin\(^-\) T cells. This is the phenotype of Ag-experienced, mature CD4\(^+\) T cells. In the present work, we explore the
mechanism underlying this difference in CD4+ T cell subset composition in rat secondary lymphoid organs.

Materials and Methods

**Animals**

PVG.RT1b, PVG.RT7b, PVG.RT1, AO, and Lewis rats were from the specific pathogen-free unit of the Medical Research Council (MRC) Cellular Immunology Unit (Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.). Rats were removed from the specific pathogen-free environment at the start of the experiments.

**Antibodies**

Anti-rat mAbs used in this work were as follows: OX6 (anti-MHC class II) (25), OX7 (anti-Thy-1) (26), OX8 (anti-CD8a) (27), OX12 (anti-Ig κ-chain) (28), OX22 (anti-CD45RC) (9), OX32 (anti-CD45RC noncompetitive with OX22) (9), OX39 (anti-IL-2Rα) (10), and OX40 (recognizes a CD4+ T cell activation molecule) (10). OX21 (anti-human C3b inactivator) was used as a control mAb (29). These mAbs were from the MRC Cellular Immunology Unit. The rat mAb P4/16 (anti-rat RT6.1) (30) was donated by Dr. J. Kamioka (Department of Histology, University of Groningen, Groningen, The Netherlands). PE-conjugated streptavidin, FITC-conjugated W3/25 (anti-rat CD4) (32), and FITC-conjugated rabbit anti-mouse (RAM) Ig F(ab')2 (2) were from Serotec (Kidlington, U.K.). PE-conjugated donkey anti-mouse (DAM) Ig was from Chemicon International (Temecula, CA). Quantum Red-conjugated streptavidin (St. Louis, MO), FITC-conjugated RAM Fab and affinity-purified RAM IgG were prepared by Steve Simmonds (Sir William Dunn School of Pathology). FITC-conjugated OX14 and OX16 (anti-rat IgG2b) mAbs were a generous gift from Dr. S. Hunt (Sir William Dunn School of Pathology). Biotinylated and FITC-conjugated mAbs were prepared as previously described (33).

**Preparation of cell suspensions**

Spleen (SPL), mesenteric LN (MLN), cervical LN (CLN), and PP were removed from rats, and cells were isolated by pressing fragments of the tissue through a sieve into ice cold PBS containing 0.2% BSA. Rat thoracic duct lymphocytes (TDL) were obtained by cannulation of the duct (34). Cells were collected at 4°C overnight into flasks containing PBS and 20 U/ml heparin. PBL were obtained by cardiac puncture, collection of the blood in the presence of heparin, and centrifugation on metrizoate-Ficoll.

**Isolation of subsets of lymphocytes**

CD4+ T lymphocytes were isolated from TDL by negative selection using RAM Ig-coated sheep RBC (33). TDL were depleted of MHC class II+CD8α+IL-2Rα+, and B cells by use of the mAbs OX6, OX8, OX39, and OX12. Cell purities were assessed by labeling pre and postdepletion samples and analyzing them on the FACScan flow cytometer (Becton Dickinson, San Jose, CA). Purities were always 97–99%.

**Immunofluorescence staining and analysis**

For single-color staining, cells were incubated with tissue culture supernatant of the appropriate mAb, washed, and then incubated with FITC-conjugated RAM F(ab')2 in the presence of normal rat serum. For two-color staining, cells were first incubated with mouse mAb, washed, incubated with FITC-conjugated RAM Fab or PE-conjugated DAM Ig in the presence of normal rat serum, washed, and then incubated with biotinylated mAb followed by PE-conjugated streptavidin or by FITC-conjugated W3/25, respectively. Samples stained with anti-rat RT6 mAb P4/16 were labeled with FITC-conjugated OX14 and OX16. Three-color staining was done using mouse mAb, PE-conjugated DAM Ig, FITC-conjugated W3/25, biotinylated mAbs, and Quantum Red-conjugated streptavidin sequentially. Cells were analyzed by FACScan using Lysis or CellQuest software (Becton Dickinson).

**Migration experiments**

CD4+ T cells from PVG.RT7b rats were purified from TDL and injected into PVG.RT7b rats. The number of cells injected varied between 18 and 135 million cells per rat. After a different time ranging from 3 h to 12 wk, animals were sacrificed and samples from the main secondary lymphoid organs were analyzed by flow cytometry; in many experiments blood samples were also taken. Injected cells were identified by the expression of the CD45.7 epitope using the mAb H5S41. The extent of the entry of injected cells into individual organs was determined by the percentage of CD4+ cells that were H5S41−. To make comparisons between animals injected with different number of cells, the data were transformed in a standard value defined by the ratio between the %CD4+ H5S41+ cells in a particular organ and the %CD4+ H5S41+ cells in the MLN, which showed consistently the highest level of entry. The CD45RC expression of the injected cells was characterized by gating on the H5S41+ population.

**Statistical analysis**

The Student's t test was employed in all analyses.

**Results**

**PP contain a higher proportion of CD4+ T cells with a memory phenotype than LN and SPL**

The expression of CD45RC on CD4+ T cells from the main secondary lymphoid organs (MLN, CLN, SPL, and PP) is shown in Fig. 1. CD4+ cells from MLN, CLN, and SPL showed similar CD45RC profiles, equivalent to that of CD4+ T cells from the thoracic duct (12). In contrast, PP contained a higher percentage of CD4+ CD45RClow T cells. These observations were also true for rats of different age, strain, and sex (Table I). Sex and strain of the animal did not seem to affect the pattern of CD45RC expression. However, CD45RC expression was found to change with age. MLN and PP cells from PVG rats of 7, 27, and 93 wk of age were simultaneously analyzed (Fig. 2). Young animals contained a high proportion of CD4+ T cells with intermediate CD45RC expression (Fig. 2, A and D). There is evidence that CD45RClow cells have recently left the thymus (Ref. 35 and see below). This population decreases with age, although the percentage of CD45RC+ cells remained roughly constant in the first months of life (Fig. 2, B and E). Table I contains data from animals from 3 to 43 wk. In older animals there is a pronounced increase in the CD45RClow population, particularly in the PP (Fig. 2F). The accumulation of lymphocytes with a memory phenotype in older animals seems to be a general phenomenon, as it has been reported in other species (20, 36, 37).

Peripheral CD4+ CD45RClow T cells are heterogeneous and include RTE, activated cells, and mature cells with a memory function (6). To characterize the CD45RClow population present in the secondary lymphoid organs, and to define which of these subpopulations are increased in the PP, analyses of RT6 and Thy-1

**FIGURE 1.** CD45RC and RT6 expression on CD4+ T cells is lower in PP than in MLN, CLN, and SPL. MLN, CLN, SPL, and PP were removed from a 12-wk-old male PVG rat. Cell suspensions were prepared, labeled with anti-CD4 and anti-CD45RC or anti-RT6 or OX21 mAbs, and analyzed by FACScan. Histograms represent the expression of CD45RC and RT6 on gated CD4+ cells. The broken line histogram indicates the staining with the control mAb OX21. The broken vertical line marks the position of the gate used to distinguish RT6-negative and -positive populations defined by OX21 staining, and CD45RC-low and -high populations. The number in each histogram indicates the percentage of RT6+ or CD45RChigh cells. This experiment is representative of a series of experiments summarized in Table I.
expression were performed. Fig. 1 shows the expression of RT6 on CD4\(^+\) cells from the secondary lymphoid organs of a single animal, and Table I summarizes the information obtained from rats of different sex, age, and strain. The results showed that PP contain slightly more CD4\(^+\) RT6\(^-\) cells than the other organs and these differences are statistically significant, but less pronounced than the difference seen for CD45RC expression. Strain, sex, and age did not affect the pattern of RT6 expression, at least in the range of 3–43 wk of age (Table I). In contrast, and in agreement with previous reports (7, 8), it was found that Thy-1 expression on peripheral CD4\(^+\) T cells strongly depends on the age of the animal. Fig. 3 shows that Thy-1 expression on CD4\(^+\) T cells decays dramatically with age and this decrease is roughly equivalent in all the lymphoid organs analyzed (results from SPL are not shown). These results suggest that SPL, CLN, MLN, and PP contain a similar percentage of RTE. However, in animals younger than 10 wk, PP and CLN contain slightly fewer CD4\(^+\) Thy-1\(^+\) cells than does MLN (Fig. 3).

It has been shown that CD4\(^+\) T cells activated in vitro and in vivo lose the expression of CD45RC (10, 13) and that activated CD4\(^+\) T cells do not express RT6 (11). To determine whether activation could account for the higher percentage of CD45RC\(^{low}\) and RT6\(^-\) cells in the PP, the expression of two activation markers, IL-2Ra and OX40, was analyzed in the different lymphoid organs (Table I). SPL, CLN, MLN, and PP all contained a small proportion of activated CD4\(^+\) T cells. This percentage was higher in SPL and PP. The higher level of activated CD4\(^+\) T cells in PP could account for the lower content of CD4\(^+\) RT6\(^-\) cells in these organs, but it was not sufficient to account for the differences in CD45RC expression as the PP contained, on average, 10% more activated CD4\(^+\) T cells than LN, but contained 20% more CD4\(^+\) CD45RC\(^{low}\) cells.

Three-color analyses showed that MLN and PP contain a population of CD4\(^+\) T cells with the characteristic phenotype of memory cells CD45RC\(^{low}\) OX7\(^-\) L-selectin\(^-\), and this population was larger in the PP (Fig. 4). CD4\(^+\) cells from SPL and CLN showed a similar phenotype than MLN cells (data not shown). These observations show that the increased representation of CD45RC\(^{low}\) cells in PP is not due to an increased representation of RTE, confirming the results from Fig. 3. Furthermore, L-selectin is involved in lymphocyte trafficking through the high endothelial venules (38). The results also suggest that a high proportion of CD4\(^+\) CD45RC\(^{low}\) cells from PP do not access these lymphoid organs from blood.

Table I. CD45RC, RT-6, IL-2Ra, and OX40 expression by CD4\(^+\) T cells from secondary lymphoid organs

<table>
<thead>
<tr>
<th>Organ</th>
<th>CD45RC</th>
<th>RT6</th>
<th>IL-2Ra</th>
<th>OX40</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLN</td>
<td>72 (9)</td>
<td>89 (7)</td>
<td>11 (4)</td>
<td>6 (4)</td>
</tr>
<tr>
<td>CLN</td>
<td>64 (9)</td>
<td>82 (7)</td>
<td>9 (2)</td>
<td>10 (5)</td>
</tr>
<tr>
<td>SPL</td>
<td>63 (9)</td>
<td>80 (7)</td>
<td>17 (7)</td>
<td>10 (7)</td>
</tr>
<tr>
<td>PP</td>
<td>46 (10)</td>
<td>75 (6)</td>
<td>16 (7)</td>
<td>19 (8)</td>
</tr>
</tbody>
</table>

\(^{a}\) MLN, CLN, SPL, and PP were removed from male and female rats of different strains (Lewis, AO, DA, PVG.RT1\(^{d}\), PVG.RT1\(^{u}\), and PVG.RT7\(^{b}\)) and ages (3–43 wk). Cell suspensions were prepared, labeled with anti-CD4, anti-CD45RC, anti-RT6, anti-IL-2Ra, or anti-OX40 mAbs, and analyzed by FACSscan. After gating on CD4\(^+\) cells, the percentage of cells stained for the different markers was recorded; values on the table are the mean (SD) from n = 29 for CD45RC, n = 18 for RT6, n = 7 for IL-2Ra, and n = 10 for OX40. The differences in CD45RC expression are highly statistically significant between all the organs except CLN vs SPL. The differences in RT6 expression are highly statistically significant between all the organs except PP vs SPL, which is significant, and CLN vs SPL, which is not significant. The differences in IL-2Ra expression are statistically significant in CLN vs SPL and CLN vs PP, but other differences are not significant. The differences in OX40 expression are highly significant between PP vs CLN and MLN, and significant between CLN vs MLN and PP vs SPL. The other comparisons are not significant.

FIGURE 2. CD45RC expression on CD4\(^+\) T cells changes with age. MLN and PP were removed from three male PVE rats at 7, 27, and 93 wk of age. Cell suspensions were prepared, labeled with anti-CD4 and anti-CD45RC mAbs, and analyzed by FACSscan. Histograms represent the expression of CD45RC on gated CD4\(^+\) cells. The broken vertical line marks the position of the gate used to distinguish cells labeled with low and high intensity for CD45RC. The number in each histogram indicates the percentage of CD45RC\(^{low}\) cells. Similar findings were obtained with CLN (data not shown). This experiment is representative of several experiments, some of which are summarized in Table I.

FIGURE 3. Thy-1 expression on CD4\(^+\) T cells from secondary lymphoid organs varies with age. MLN, CLN, and PP were removed from rats of different strains (Lewis, AO, DA, PVG.RT1\(^{d}\), PVG.RT1\(^{u}\) and PVG.RT7\(^{b}\)), ages (5–93 wk), and both sexes. Cell suspensions were prepared, labeled with anti-CD4 and anti-Thy-1 mAbs, and analyzed by FACSscan. After gating on CD4\(^+\) cells the percentage of Thy-1\(^+\) cells was recorded. The symbols represent values from individual animals or the mean from two or three animals (n = 18).

FIGURE 4. CD45RC, Thy-1 and L-selectin expression on CD4\(^+\) T cells from MLN and PP. Lymphoid organs were removed from a 9-wk-old male PVE rat. Cell suspensions were prepared, labeled for three-color analysis with anti-CD4, anti-CD45RC, and anti-Thy-1 or anti-selectin mAbs, and analyzed by FACSscan. Dot plots represent the expression of CD45RC and Thy-1 (upper) and CD45RC and L-selectin (lower) on gated CD4\(^+\) cells from MLN (left) and PP (right). The numbers represent percentage of cells contained in the respective quadrants.
were conducted to identify the cause. CD4+ T cells were also analyzed for the expression of CD44, and homogeneous staining was obtained in all lymphoid organs (data not shown). Therefore, the level of CD44 expression in the rat strains analyzed does not distinguish memory and naive CD4+ T cells as reported in some mice strains (39). CD4+ T cells from the thoracic duct of PVG.RT7b rats were purified and injected i.v. CD4+ T cells enter lymphoid organs to a greater extent than the CD45RClow population does.

The accumulation of lymphocytes in a particular lymphoid organ depends on the rates of entry and exit, as well as maturation, proliferation, and cell death within it. As CD4+ T cell subsets were unequally distributed in secondary lymphoid organs, experiments were conducted to identify the cause. CD4+ T cells from the thoracic duct of PVG.RT7b animals were purified and injected i.v. into PVG.RT7b animals. This experimental setting allowed the analysis of CD45RC expression of injected cells that accumulated in different lymphoid organs over time. Shortly after cell injection, most of the injected cells that entered secondary lymphoid organs were CD45RChigh (Fig. 5). The percentage of cells expressing CD45RC was higher in the donor population than in the CD4+ T cell subsets. This was true in all organs analyzed but was more obvious in PP. Fig. 5A shows results from an analysis performed 46 h after the injection of cells; the same pattern was seen from 3 h to 16 days after injection. These experiments are summarized in Fig. 5B, where the data from 10 animals are depicted. Also included, for comparison, is the percentage of injected CD4+ T cells that express CD45RC (CD45RC+ injected). These results show the absence of a correlation between the phenotype of the CD4+ T cells contained in the organs and the phenotype of the injected cells that accumulate after a short time in the organs. More CD4+ CD45RClow T cells accumulated in the PP than in the other organs, but this small difference in homing, although statistically significant, cannot account for the representation of at least one lymphoid organ cannot be explained by the differential homing of CD4+ T cell subsets to the secondary lymphoid organs.

The CD4+ CD45RClow T cell population increases with time in secondary lymphoid organs.

To investigate how the expression of CD45RC on injected cells changed with time, animals were injected with CD4+ T cells from PVG.RT7b rats and analyzed at different times after inoculation. Based on forward and side scatter profiles on the FACScan, the existence of new blastlike cells in the donor population of animals injected 2 wk earlier was apparent. This population appears first in the PP (data not shown). At 2 wk the CD45RC profile was similar to analyses performed at shorter times, when ~90% of the injected cells in all organs expressed CD45RC, as shown in Fig. 5A. After 3 wk, the percentage of donor HIS41+ CD45RClow cells increased and became larger proportionally with time. Fig. 6 shows the CD45RC expression on host and donor CD4+ T cells in secondary
lymphoid organs of an animal sacrificed 9 wk after injection. The increase in the percentage of HIS41\(^+\) CD45R\(_{\text{low}}\) cells in all organs analyzed was evident compared with animals analyzed 48 h after injection (Fig. 5A vs. Fig. 6). Again PP showed a distinctive pattern, with the most striking change in CD45R expression. These experiments do not address whether the increase in HIS41\(^+\) CD45R\(_{\text{low}}\) cells results from the expansion of CD45R\(_{\text{low}}\) cells, from the phenotypic change in the CD45R\(_{\text{high}}\) cells, or from the loss of CD45R\(_{\text{high}}\) cells. Note that the CD45R profile of the injected cells was clearly divided into two populations. The CD45R\(_{\text{int}}\) population is absent from the donor cells 9 wk after their injection, but not from the host CD4\(^+\) population (Fig. 6). As we showed above, this population is very conspicuous in young animals (Fig. 2), expresses Thy-1 (Fig. 4), and also decreases dramatically after thymectomy (R. Dyke, unpublished observations). Taken together, these data strongly suggest that CD45R\(_{\text{int}}\) cells have left the thymus recently. After injection into animals these cells must follow their normal development, probably progressively gaining CD45R expression (8).

**CD4\(^+\) T cells do not migrate equally to different lymphoid organs**

The level of CD4\(^+\) T cell recirculation into the different lymphoid organs was analyzed. Animals were injected with CD4\(^+\) RT7\(_b\) cells, sacrificed at different times after injection, and the percentage of CD4\(^+\) T cells that were of donor origin in the different lymphoid compartments was determined. This value is an estimate of the mass of CD4\(^+\) T cells belonging to the recirculatory pool in every lymphoid organ. The number of injected cells correlated linearly with the percentage of injected cells detected 48 h after injection (Fig. 7A). The strong correlation between these two magnitudes allows quantitative comparisons between different organs. Organs were analyzed at 48 h because in a shorter time period many injected cells accumulated in the SPL in agreement with previous observations (41); between 24 and 48 h after injection, the distribution of injected cells in the different organs reached the equilibrium (data not shown). Fig. 7B summarizes the information from 10 experiments. To allow comparison between different experiments, we present the data as the ratio between the percentage of donor CD4\(^+\) T cells in each lymphoid compartment and the percentage of donor CD4\(^+\) T cells in MLN for that experiment. MLN generally showed the highest level of cell entry. Similar in value to the entry in blood. Consistently, less CD4\(^+\) T cells of donor origin were detected in the PP than in the other lymphoid organs analyzed (Fig. 7B). The values represented in Fig. 7B were further corrected considering that SPL and PBL contain a population of non-T CD4\(^+\) cells. This population, by mixing with the CD4\(^+\) T cells in the analysis, decreases the detection of the injected population in these compartments. It was determined by labeling with anti-CD5 or anti-TCR that T cells comprise, on average, 86 and 93% of the CD4\(^+\) cells in SPL and PBL, respectively, and 97–100% in MLN, CLN, and PP (data not shown). After correction by these factors, the relative values of CD4\(^+\) T cell recirculation in each organ (considering PBL as reference) were 1.00 for PBL, 0.99 for SPL, 0.95 for MLN, 0.88 for CLN, and 0.70 for PP. So, the minimal size of the nonrecirculatory CD4\(^+\) T population is 1% in SPL, 5% in MLN, 12% in CLN, and 30% in PP. These values are probably an underestimate as we are assuming that all the injected CD4\(^+\) T cells in the blood recirculate. Animals analyzed after longer times following injection showed the same qualitative differences between organs, with a reduction in the percentage of injected cells detected.

**Secondary lymphoid organs contain a nonrecirculating CD4\(^+\) T cell population enriched with CD45R\(_{\text{low}}\) cells**

The data from previous sections suggest that the size of the recirculatory CD4\(^+\) population varies between secondary lymphoid organs, and inversely correlates with the percentage of CD4\(^+\) CD45R\(_{\text{low}}\) cells, implying that secondary lymphoid organs contain a nonrecirculating CD4\(^+\) population that is predominantly CD45R\(_{\text{low}}\). To test this hypothesis, animals were cannulated in the thoracic duct for 48 h and thus became depleted of lymphocytes; secondary lymphoid organs were then removed to analyze the CD45R expression in the CD4\(^+\) T cells remaining in the organs. Fig. 8A shows a representative experiment and Fig. 8B summarizes the data from four experiments. The results show that after 48 h of thoracic duct cannulation the proportion of the CD45R\(_{\text{low}}\) population increased in all organs analyzed relative to the sham-operated control, consistent with predomination of CD45R\(_{\text{low}}\) cells in the nonrecirculatory pool and the CD45R\(_{\text{high}}\) cells in the recirculatory pool. The increase in CD45R\(_{\text{low}}\) cells...
are depicted. Bars represent mean ± SD (n = 4 for MLN, CLN, and PP, and n = 2 for SPL). Statistical analyses were performed using Student’s t test; *, significant (p < 0.05); **, highly significant (p < 0.01).

The proportion of CD4+ CD45RClow cells increases in secondary lymphoid organs after 48-h cannulation of the thoracic duct. PVG rats were cannulated in the thoracic duct and were depleted of lymphocytes for 48 h. Sham-cannulated animals from the same litter were used as controls. Rats were kept in Bollman cages during lymph collection. After 48 h, animals were sacrificed, their MLN, CLN, SPL, and PP were removed, and cell suspensions were prepared and labeled with anti-CD4 and anti-CD45RC mAbs. CD45RC expression was analyzed by FACScan after gating on the CD4+ population. A, CD45RC expression histograms from a single representative experiment are shown. The broken vertical line marks the position of the gate used to distinguish cells labeled with low and high intensity for CD45RC. The number in each histogram indicates the percentage of CD45RClohigh cells. B, Data from four different experiments are depicted. Bars represent mean ± SD (n = 4 for MLN, CLN, and PP, and n = 2 for SPL). Statistical analyses were performed using Student’s t test: *, significant (p < 0.05); **, highly significant (p < 0.01).

Discussion
The data described in this paper give new insights into the recirculation and maturation of CD4+ T cells. First, differences in the memory pool size between different secondary lymphoid organs are demonstrated, and PP were the organ containing the largest population of cells with memory phenotype. The second implication resulting from our data is that CD4+ T cells that migrate to the secondary lymphoid organs are predominantly, but not exclusively, CD45RClohigh. The third result is the existence of a nonrecirculating pool of CD4+ T cells which are predominantly CD45RClow, with PP being the organ containing the largest nonrecirculating pool. The fact that PP contain the largest memory, activated, and nonrecirculatory population may be due to the high level of exposure to Ags from the gut.

Our observations may be integrated with results published by others to suggest the following model of CD4+ T cell dynamics. Naive CD45RClohigh cells recirculate through the secondary lymphoid organs until they encounter specific Ags and are activated. Activated cells and their effector and memory progeny are CD45RClow. This population recirculates throughout lymphoid and nonlymphoid tissues but also accumulates in the lymphoid organs and increases in frequency with age. The Ag-experienced CD45RClow cells can reexpress the CD45RC isoform in the absence of its specific Ag, and in the presence of Ag the memory cells maintain the CD45RClow phenotype (21). These observations suggest that a fraction of the CD45RClow population, which accumulates in secondary lymphoid organs, consists of cells that are in the presence of their specific Ag. Interestingly, the increase in the number of cells with memory phenotype in older mice does not occur in the absence of Ag (43), in agreement with this view. An important point which requires further investigation is the localization of memory cells that reexpress CD45RC. We have characterized the majority of recirculatory CD4+ cells as CD45RClohigh, and it is possible that the reverting memory cells are contained in this recirculatory population. Similarly, we found enrichment for CD45RClow cells in secondary lymphoid organs after prolonged cannulation, but our approach does not exclude the possibility that a few sessile CD4 cells express CD45RC. Memory CD45RClohigh CD4+ T cells do not have a distinctive phenotype and more information is required to characterize their dynamics.

Analyses of cell proliferation in vivo have shown that a high proportion of mouse CD45RBlohigh CD4+ T cells, in many aspects the analogous population to rat CD45RClohigh CD4+ T cells (44, 45), are cycling in secondary lymphoid organs (35). These cells may be the sessile CD4+ T cells with memory phenotype in secondary lymphoid organs. Their progeny is probably responsible for spreading the memory response throughout the body. It is known that CD45RClohigh cells collected from the thoracic duct can transfer memory humoral responses (9), and we detected a few CD45RClow recirculatory cells (Fig. 5). It has been suggested that activated T cells randomly enter LN and PP but they only survive in the tissue of origin where they divide (46). Similarly, it is possible that some recirculatory CD45RClohigh cells only survive in the presence of an appropriate environment that provides signals to avoid apoptosis, such as Ag, APC, extracellular matrix, and others. This would explain the high rate of CD45RClow cell loss that has been observed in transfer experiments (47).

Studies performed in mice have provided similar results to some of our findings. Mouse PP also contain a larger population of CD4+ T cells with memory phenotype than SPL and LN (23). It has also been shown that most of the CD4+ T cells that migrate to the LN are CD45RBlohigh CD44low and L-selectin−, suggestive of a naive phenotype (48).

The experimental procedure used to study the accumulation of CD4+ T cells in lymphoid organs was intended to be as physiological as possible. TDL were used in all experiments as they are the cells that normally recirculate and enter the blood. It was necessary to maintain the cells at 4°C during collection and purification procedures, and this is known to affect lymphocyte recirculation during the first hours after injection. However, our analyses were performed after 24 h, when this effect had ended (49). Ethical guidelines do not allow us to keep cannulated animals for more...
than 48 h. This is a limitation in experiments where the composition of the sessile population is to be analyzed. It has been estimated that the number of cells released after 24 h of cannulation comprises about one third of the recirculatory pool (41). Classical experiments by Gowans (42) showed that the release of lymphocytes from the thoracic duct decreases steadily until the fourth day, when a stable equilibrium is reached and 30% of the initial output is released daily. We predict that our results (Fig. 8) would have been more significant if the analysis could have been performed on day 4. The majority of lymphocytes collected from the thoracic duct are small lymphocytes, and a small percentage of TDL are blast cells (42). It is interesting to point out that at day 4 of cannulation, the output of small lymphocytes decays dramatically, but when a stable equilibrium is reached and 30% of the initial output is released daily, it is not cautious to derive quantitative information about the size of the recirculatory population to be analyzed. It has been estimated that some of the recirculatory CD45RClow cells are activated cells (6). According to the hypothesis outlined above, we propose that after losing the recirculatory pool of resting naïve cells by cannulation, consisting mainly of CD45RChigh the cells that enter the lymph are predominantly the progeny of sessile memory CD45RClow cells. However, it has not been determined in long-term cannulation experiments like the one shown in Fig. 8, whether there is lymphocyte proliferation in response to the displacement of homeostasis provoked by lymphocyte loss (52). So, until this point is analyzed it is not cautious to derive quantitative information about the size of the recirculatory and sessile populations from the long-term cannulation experiments.

The data presented in this paper are consistent with a model where most recirculatory CD4+ T cells are CD45RChigh; the smaller representation of CD45RChigh cells in lymphoid organs compared with PBL or TDL is due to the existence of a nonrecirculatory population predominantly CD45RClow, the size of which varies between lymphoid organs.

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References


