

## NAIVE AND MEMORY T CELLS SHOW DISTINCT PATHWAYS OF LYMPHOCYTE RECIRCULATION

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Immunologically experienced cells or their progeny are able to mediate a vigorous response upon antigenic restimulation. This "memory" response is thought to occur as a result of the clonal expansion of antigen-reactive cells and their persistence in the host (1, 2). However, other factors also contribute to the memory response. Memory T cells are functionally more potent than naive T cells, since they have an enhanced ability to produce IFN- $\gamma$  and IL-4 upon antigenic restimulation (3-5). Memory T cells also show increased expression of certain adhesion/activation molecules, which probably facilitates cell-cell interactions, antigen recognition, and triggering of the memory population (3-9). Adhesion molecules that show an increased expression on memory T cells include CD2 and its ligand, CD58, CD44, and CD11a. Memory T cells in humans also express the p180 isoform of CD45, termed CD45RO, whereas naive T cells express the p220/205 isoforms, CD45RA (10, 11). In some mice strains, memory T cells can be distinguished purely by the expression of Pgp-1 (12, 13), the mouse homologue of human CD44 (14, 15). The evidence that surface phenotype can distinguish naive from memory T cells is that: (a) lymphocytes from cord blood are entirely of naive phenotype (3); (b) the percentage of T cells of memory phenotype increases with age (9, 16); (c) proliferative responses to recall antigen resides purely within the memory phenotype (3, 13); and (d) naive T cells acquire the memory phenotype upon activation (3, 11). Interestingly, the altered expression of homing receptors such as MEL-14/Leu-8 and CD44 on activated/memory T cells (3, 12, 17-19) might result in altered patterns of recirculation for T cells upon transition to the memory state.

Memory T cells are thought to be long-lived cells that continuously recirculate between blood and lymphoid tissues (20-22). The extravasation of such cells to lymph nodes (LNs)<sup>1</sup> is mediated by "homing receptor" molecules on the lymphocyte cell surface that interact with "vascular addressins" expressed on high endothelial venules (HEV). Another recirculation route is the extravasation of certain lymphocytes to peripheral tissues such as skin, and their eventual drainage to a LN via the afferent lymph. Afferent lymphocytes are distinct both functionally and phenotypically from

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The Basel Institute for Immunology was founded and is supported by F. Hoffman-La Roche Ltd., Basel, Switzerland.

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<sup>1</sup> *Abbreviations used in this paper:* BrdU, bromo-deoxyuridine; HEV, high endothelial venule; LN, lymph node; PE phycoerythrin.

lymphocytes that enter LNs from the blood (23, 24), indicating that different criteria might operate for lymphocyte extravasation to lymphoid and nonlymphoid tissues (23).

In this report, we identified naive and memory T cells in sheep by their expression of a number of cell surface markers. Using the sheep model, we were able to address two important questions concerning immunological memory: do memory T cells continuously recirculate from blood to lymphoid tissue in the same manner as naive T cells, and are memory T cells long-lived?

### Materials and Methods

*Animals and Surgery.* White Alpine  $\times$  Black Jura ewes were obtained from Versuchsbetrieb Sennweid, Olsberg, Switzerland. Ewes (ranging in age between 10 and 18 mo) used for experiments requiring cannulation of lymph ducts were kept in metabolism cages and given food and water ad libitum.

The popliteal efferent lymph duct and one to two afferent lymph ducts draining the lower part of the right hind leg were cannulated as described (25). Lymph was collected from cannulated lymph ducts using sterile polyethylene collection bottles containing 1 ml of normal saline supplemented with 500 U heparin, 500 IU of penicillin, and 500  $\mu$ g of streptomycin. After surgery, collected cells were monitored by Giemsa staining of cytoposits, to ensure that any effects of trauma from surgery had subsided.

*mAbs.* Numerous mAbs have been produced against sheep leukocyte molecules. The details of these mAbs and the cell surface markers they recognize are summarized in Table I. Also used in this study was a previously uncharacterized mAb, termed F10-150, that recognizes the sheep CD11a (LFA-1) molecule (vide infra).

*Cell Suspensions.* Cells collected from the afferent or efferent lymph were washed two to three times in PBS/1% FCS. PBL were prepared using 60% Percoll. For the preparation of activated T cells, PBL were stimulated in the MLR for a period of 14 d, as described (26).

*Immunofluorescent Staining and Flow Cytometry.* For one-color immunofluorescence staining,  $10^6$  cells were reacted with 50  $\mu$ l of mAb supernatant for 10 min at 4°C. Cells were then washed once and resuspended in 50  $\mu$ l of FITC-goat anti-mouse Ig (Cappel Laboratories, Cochranville, PA). After a 10-min incubation at 4°C, cells were washed twice and analyzed using a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA). Propidium iodide was used to identify and exclude dead cells.

For two-color immunofluorescence, cells were stained for green fluorescence as described above, and were then stained for red fluorescence as follows. Unoccupied sites on the FITC-goat anti-mouse Ig were blocked using 20  $\mu$ l of 10% normal mouse serum, and then 50  $\mu$ l of biotinylated mAb was added to the cell suspension. After a 10-min incubation at 4°C, cells were washed once and incubated with 50  $\mu$ l of streptavidin-phycoerythrin (PE) (Southern Biotechnology, Birmingham, AL). After 1 min, cells were washed twice and analyzed on the FACScan.

Forward and 90° light scatter were used to gate on viable lymphocytes. For single-color fluorescence, 5,000 events were collected, and for two-color fluorescence, 10,000 events were collected. To analyze T cells, B cells, or T cell subsets for the expression of CD2, CD44, CD58, and CD11a, a two-color immunofluorescence staining was performed, and gates were set such that only positively stained cells in the red direction, i.e., CD5<sup>hi</sup>, CD4<sup>+</sup>, CD8<sup>hi</sup>,  $\gamma/\delta^+$ , or sIg<sup>+</sup> were analyzed for green fluorescence.

*BrdU Treatment.* 12-mo-old sheep were injected through an intravenous catheter with bromodeoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO) at a concentration of 15 mg/ml, once a day, at a dose rate of 15 mg/kg of body weight.

*BrdU Staining.* A two-color immunofluorescence method was used to analyze BrdU incorporation and cell surface phenotype. The entire procedure was performed in round-bottomed 96-well plates. Cells were stained first with biotinylated mAb to cell surface molecules followed by avidin-FITC. After washing in PBS, the cells were fixed by resuspending in 100  $\mu$ l of 70% ethanol. After a 20-min incubation at room temperature, cells were spun and resuspended in 100  $\mu$ l of 3 N HCl containing 0.5% Tween 20, and were incubated for

a further 20 min at room temperature. 100  $\mu$ l of PBS was added, and cells were then centrifuged and resuspended in 100  $\mu$ l of 0.1 M disodium tetraborate, pH 8.5, for 2–3 min. 100  $\mu$ l of PBS was added, the cells were spun, and then washed once in PBS 0.5% Tween 20. Cells were then stained with 20  $\mu$ l anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA). After a 20-min incubation, cells were washed with PBS/Tween and were incubated with PE-conjugated goat anti-mouse Ig (Southern Biotechnology). Cells were washed twice with PBS/Tween and were analyzed on the FACScan. Control staining was performed on unfixed cells to ensure that the fixation procedure did not influence the composition of the suspension or select for any particular cell type. All of the mAbs used in this study were still attached to the cell surface after fixation and HCl treatment, although the intensity of staining was always less than that of unfixed cells. Also, the second-step goat anti-mouse Ig-PE did not react with the primary mAb.

*In Vitro Proliferation of Sheep T Cells to Recall Antigen.* A 3.8-yr-old sheep that had been multiply injected with purified rabbit IgG was used as a blood donor to examine an in vitro proliferative response by T cells to a recall antigen. The last immunization of this sheep with rabbit Ig occurred 8 mo before the in vitro proliferation assays reported here. Naive and memory CD4<sup>+</sup> T cells from peripheral blood were separated on the basis of CD45R expression, using two-color immunofluorescence staining.

Cells were stained and sorted under sterile conditions using a FACS 440 (Becton Dickinson & Co.). Sorted cells were plated at 10<sup>5</sup>/well in sterile 96-well culture plates in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2-ME, nonessential amino acids, and antibiotics. Syngeneic PBL irradiated with 3,000 rad were used at 10<sup>5</sup>/well as feeder cells. Stimulation with antigen was performed for 5 d in a humidified incubator with 7% CO<sub>2</sub> at 37°C. Cultures were pulsed for 8 h with 1  $\mu$ C/well [<sup>3</sup>H]thymidine (Amersham International, Amersham, UK) and harvested onto glass filters. Thymidine incorporation was determined using a liquid scintillation counter (Packard Instrument Co. Inc., Downers Grove, IL).

*Immunoprecipitation and SDS-PAGE.* Procedures for immunoprecipitation and SDS-PAGE followed previously described methods (29).

## Results

*Expression of Adhesion Molecules by Sheep Lymphocytes.* The sheep has been used extensively for studies on lymphocyte recirculation, particularly with respect to the properties of lymphocytes entering and leaving single LNs via the afferent and efferent lymphatics (23, 25). The aim of this study was to identify naive and memory T cells in sheep, and examine their recirculation patterns and division rate.

TABLE I  
*mAbs Used and the Cell Surface Markers they Recognize*

Sheep leukocyte molecule	Other names	Size	mAb used	Expression in sheep	Reference
		<i>kD</i>			
CD2	T11	55	36F	$\alpha/\beta$ T cells	26
CD58	LFA-3/T11TS	42	L180-1	All leukocytes	27
CD11a/CD18	LFA-1	180,95	F10-150	All leukocytes	-
CD44	Pgp-1	94	25-32	Ubiquitous	14
CD45R	LCAp220	220	73B	B cells, some T	28
CD45	LCA	220,210,180	1-11-32	All leukocytes	28
CD4	T4	55	17D	~30–40% T cells	26
CD8	T8	36	38-65	~20–30% T cells	29
TCR- $\gamma/\delta$		44,36	86D	~30–60% T cells	30

Most of the molecules used to distinguish naive and memory T cells in humans have also been identified in sheep. These include CD2, CD58 (LFA-3), CD44, and CD11a (LFA-1), as well as the CD45R molecule. The expression of these molecules on sheep leukocytes is shown in Fig. 1. FACS analysis shows that CD2 subdivides sheep T cells into three populations: CD2<sup>-</sup>, CD2<sup>lo</sup>, and CD2<sup>hi</sup>. The CD2<sup>-</sup> subset is made up by the  $\gamma/\delta$  fraction of T cells, which can constitute up to 60% of PBL, depending upon the age of the animal (30). The relative proportions of the CD2<sup>lo</sup> and CD2<sup>hi</sup> subsets was found to be age dependent, and CD2<sup>hi</sup> cells were absent from neonatal blood (see below). Apart from humans, the sheep is the only species in which the ligand for CD2, CD58, or LFA-3/T11TS has been identified (27). Unlike

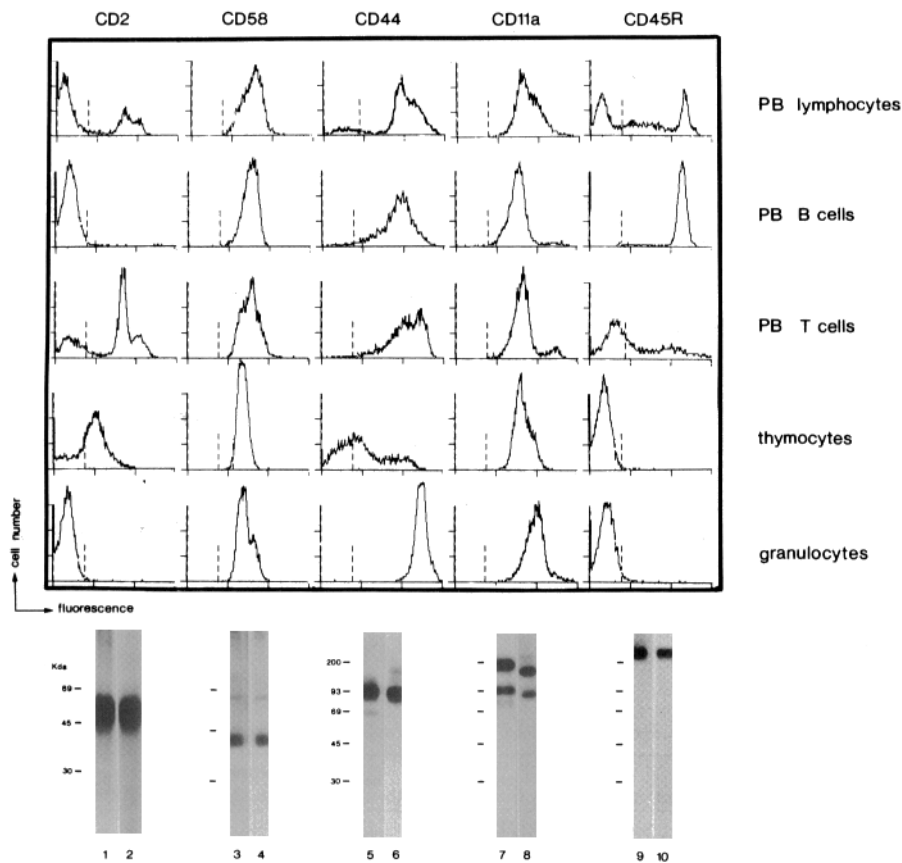


FIGURE 1. Expression and molecular characterization of adhesion molecules in sheep. Profiles show the staining of PBL, peripheral blood (PB) B cells, peripheral blood T cells, thymocytes, and granulocytes with anti-CD2, anti-CD58, anti-CD44, anti-CD11a, and anti-CD45R, as indicated. Peripheral blood T cells and B cells were identified and analyzed using two-color immunofluorescence, as described in Materials and Methods. The broken line in each plot signifies the point beyond which control antibodies showed no fluorescence staining. An SDS-PAGE analysis of the five molecules is shown on the bottom half of the figure. Lanes 1 and 2, CD2; lanes 3 and 4, CD58; lanes 5 and 6, CD44; lanes 7 and 8, CD11a/CD18; lanes 9 and 10, CD45R. Lanes 1, 3, 5, 7, and 9 were run under reducing conditions; lanes 2, 4, 6, 8, and 10 were run under nonreducing conditions.

humans, CD58 in sheep is expressed by all lymphocytes, whether they are naive or memory/activated. However, CD58 is expressed most strongly by dendritic cells (26) and B cells (Fig. 1). The CD44 adhesion molecule is one of the most widely distributed molecules in the body, and is expressed on virtually all leukocytes in sheep (14). Lymphocytes that are CD44<sup>-</sup> or CD44<sup>lo</sup> are those that are most sessile, such as cortical thymocytes and germinal centre cells. This expression is consistent with one proposed role for CD44 as a lymphocyte homing receptor (31). Another adhesion molecule, CD11a (LFA-1), recognized by a mAb F10-150, is expressed by all sheep leukocytes, including immature T cells and B cells (Fig. 1). F10-150 recognizes CD11a as judged by the characteristic molecular mass of this molecule (see below), the similar reactivity pattern of mAb F10-150 against sheep leukocytes compared with anti-CD11a mAbs in humans and mice (32, 33), and the ability of mAb F10-150 to block the sheep MLR and cytotoxic T cell killing against allogeneic targets (not shown). Lastly, a restricted epitope of CD45 (leukocyte common antigen) is expressed on all B cells (28), but also on NK-like cells (unpublished results), and some peripheral T cells, particularly during fetal and early post-natal life (see below). This molecule in sheep is identical to one of the restricted epitopes of human CD45, termed CD45RA, which has an  $M_r$  of 220 and 205 kD, and is expressed on B cells, NK cells, and naive T cells (34). In this report we will refer to the sheep molecule as CD45R.

The bottom half of Fig. 1 shows an SDS-PAGE analysis of sheep adhesion molecules. CD2 migrated as a 50–55-kD molecule, and its ligand, CD58, migrated at 42 kD, in accordance with previous reports (26, 27). The CD44 “lymphocyte homing receptor” migrated as a 94-kD molecule, whereas the CD11a/CD18 molecules (immunoprecipitated by the anti-CD11a mAb F10-150) migrated at 180 and 95 kD, respectively. Under nonreducing conditions, most molecules migrated slightly faster, although none contained interchain disulfide bonds. Sheep CD45R usually migrated as a single component of  $M_r$  220 kD, although on one occasion, a 205-kD component was evident.

*Phenotype of Naive and Activated T Cells in Sheep.* In our attempts towards identifying naive and memory T cells in sheep, we analyzed the expression of adhesion molecules and CD45R on T cells from the blood of fetal and neonatal lambs. In sheep, placentation is such that the fetus develops in a completely antigen-free environment and without maternally derived Ig. Fig. 2 shows that CD2, CD58, CD44, and CD11a were expressed at lower levels on neonatal T cells compared with the levels found on T cells from a 2-yr-old sheep. Also, CD45R was expressed by the majority of neonatal lamb T cells. T cells from fetuses at various stages of gestation showed a staining pattern similar to that obtained for T cells from newborn lambs (not shown).

In contrast to neonatal T cells, adult T cells were composed of two subpopulations: one identical in phenotype to naive T cells, and the other population expressing increased levels of CD2, CD58, CD44, and CD11a, and no CD45R (Fig. 2). This latter population appeared ~3–4 mo of age and usually comprised ~30–40% of T cells in sheep 1–2 yr of age. After a 14-d culture of neonatal T cells in the MLR, a FACS analysis showed that all adhesion molecules were markedly upregulated (Fig. 2). In addition, virtually all activated T cells expressed MHC class II molecules, had a large cell size (not shown), but no longer expressed CD45R.

*Response to Recall Antigen Resides within the Memory Phenotype.* We tested the proliferative response of the putative naive and memory T cell subsets in sheep to a recall

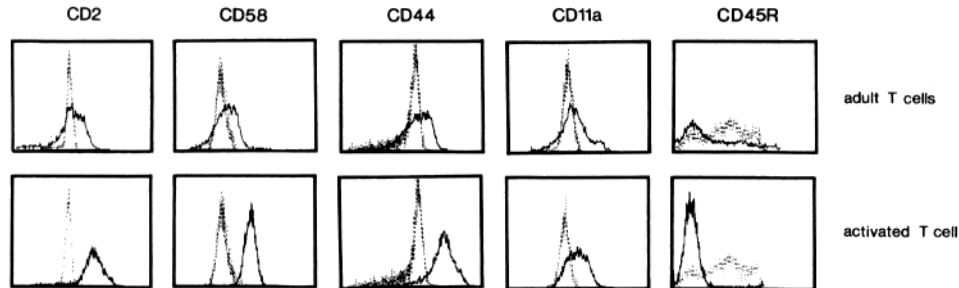


FIGURE 2. Phenotype of neonatal T cells and comparison to adult T cells and activated neonatal T cells. Neonatal blood was obtained from a 2-h-old lamb. Adult blood was from a 2-yr-old sheep. Lymphocytes were separated on 60% Percoll, stained in the green direction with mAb followed by FITC-goat anti-mouse Ig, and in the red direction with a mixture of anti-CD4-biotin and anti-CD8-biotin followed by avidin-PE. Lymphocytes were gated such that only CD4<sup>+</sup> and CD8<sup>hi</sup> T cells were analyzed. Broken lines are staining of neonatal T cells, whereas unbroken lines are staining of adult T cells (*top*) or activated neonatal T cells (*bottom*).

antigen. CD4<sup>+</sup> cells from a sheep immunized with rabbit Ig were sorted into CD45R<sup>+</sup> and CD45R<sup>-</sup> subsets (see Fig. 3 A). As in humans, increased expression of CD2, CD58, CD44, and CD11a correlated with the CD45R<sup>-</sup> phenotype (not shown). Fig. 3 B shows that, on a cell to cell basis, the proliferative response to the recall antigen used was ~15–20-fold greater within the putative memory fraction of sheep CD4<sup>+</sup> T cells (CD45R<sup>-</sup>) compared with the naive fraction (CD45R<sup>+</sup>). The proliferation by the memory fraction was specific for the recall antigen (rabbit Ig), since the same population showed a much smaller response to an unseen antigen, keyhole limpet hemocyanin (KLH). In addition, the memory fraction of an unimmunized sheep showed very little proliferation to rabbit Ig (data not shown).

*T Cells in Afferent Lymph Display a Memory Phenotype.* The above studies demon-

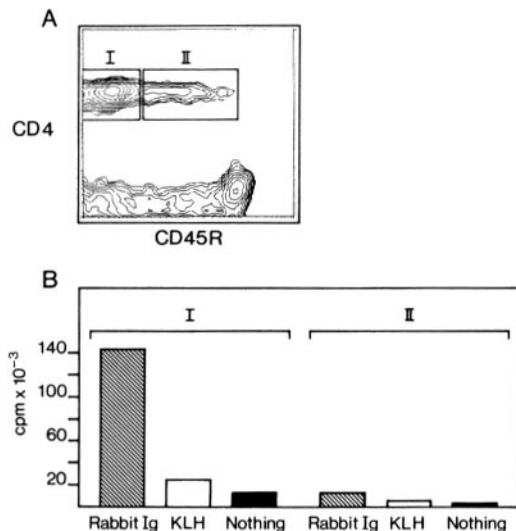


FIGURE 3. Response of CD4<sup>+</sup> T cells of naive and memory phenotypes to recall antigen. PBL were from a sheep multiply primed with rabbit IgG. Cells were isolated, dual stained with anti-CD45R-FITC and anti-CD4-PE, and sorted on the FACS into two populations, CD4<sup>+</sup>, CD45R<sup>-</sup> (designated population I) and CD4<sup>+</sup>, CD45R<sup>+</sup> (population II) (A). Sorted cells were stimulated in vitro for 5 d with an optimum concentration (20 μg/ml) of either rabbit IgG, an irrelevant antigen (KLH), or with nothing. B shows the response of the sorted cells to both antigens, as measured by [<sup>3</sup>H]thymidine incorporation (expressed as cpm, y axis).

strated that T cells in sheep, like those in man and mouse, can be divided into naive and memory phenotypes based on expression of adhesion molecules and CD45 isoforms. We examined the presence of naive and memory T cells in the three main compartments of recirculating cells: blood, afferent lymph, and efferent lymph. Fig. 4 shows a representative FACS analysis of T cells ( $CD4^+$  and  $CD8^+$ ) in afferent lymph, efferent lymph, and blood of a single animal. The expression of all adhesion molecules tested was markedly higher on T cells from the afferent lymph, and resembled the levels seen on the putative memory subset in blood. Moreover, CD45R, which is absent from activated/memory T cells, was also absent from T cells in afferent lymph. Afferent lymphocytes were only slightly larger than efferent lymphocytes, although they did express higher levels of MHC class I and class II molecules, as reported previously (23). The results indicated that all T cells within afferent lymph were purely of memory phenotype.

*Most T Cells in Efferent Lymph Display a Naive Phenotype.* In contrast to afferent lymph T cells, the majority of T cells in efferent lymph expressed CD45R, and showed low expression of adhesion molecules (Fig. 4), comparable with the phenotype of neonatal T cells, or the putative naive fraction in adult blood (size Fig. 2). However, not all cells in efferent lymph were of naive phenotype; usually a small proportion were of memory phenotype (0–10%), particularly in older sheep.

*Different Recirculation Properties of T Cell Subsets May Correlate with Memory Phenotype.* In a previous report, we noted that lymphocyte subsets possessed unequal capacity for extravasation into LNs and into peripheral tissue vascular beds (23). The observation here that naive and memory T cells preferentially accumulate in efferent and afferent lymph, respectively, indicated that the relative proportions of T cell subsets with memory or naive phenotypes may relate to the selective migration pattern of lymphocyte subsets. Fig. 5 shows the typical composition of T cell subsets and B

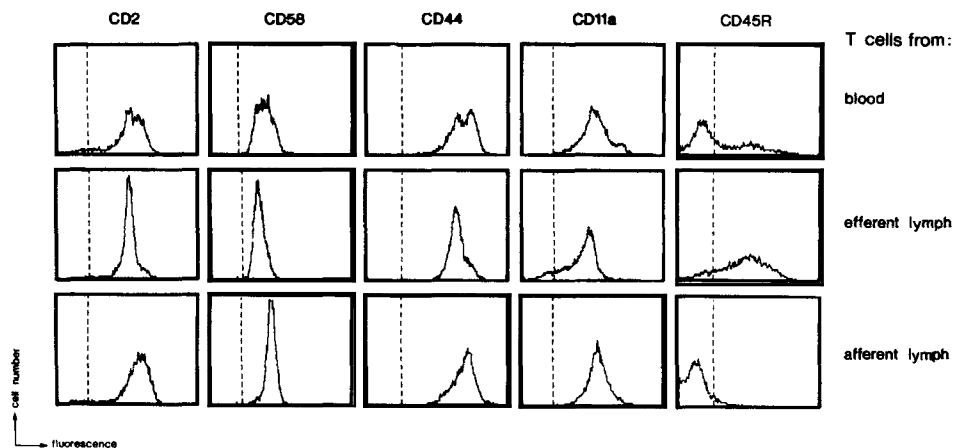


FIGURE 4. Phenotype of T cells within different compartments of the recirculating lymphocyte pool. Cells were stained in the red direction with anti-CD4 and anti-CD8 biotin followed by avidin-PE, and in the green direction with the indicated marker (see Materials and Methods). Broken lines indicate the level of fluorescence using negative control antibodies. Gates were set such that only  $CD4^+$  and  $CD8^+$  lymphoid cells from each cell preparation were analyzed for green fluorescence staining.

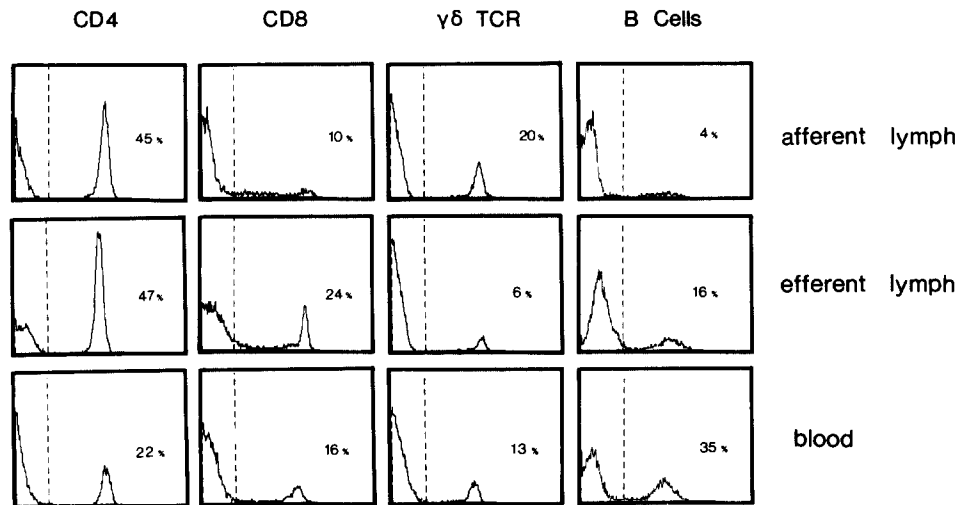


FIGURE 5. Differential accumulation of T cell subsets in various compartments of the recirculating pool. Lymphocytes in afferent lymph, efferent lymph, and blood were stained with mAbs specific for the T cell subsets CD4, CD8, or TCR- $\gamma/\delta$ , or with donkey anti-sheep Ig-FITC to identify B cells. The percentage of reactive cells for each marker is indicated for each staining.

cells in the different lymphoid compartments of a 12-mo-old sheep. The analysis of this and six other sheep demonstrated a selective enrichment for different subsets in various compartments. In all cases, CD4<sup>+</sup> cells were markedly enriched in both afferent and efferent lymph, compared with their relatively low levels in the blood (~22%). This indicated that CD4<sup>+</sup> cells had a strong propensity to bind to LN HEV and also to endothelial cells in peripheral tissues. However,  $\gamma/\delta$  T cells showed a completely different bias. They were enriched in afferent lymph, but were low in number in efferent lymph. Moreover, a previous report showed that  $\gamma/\delta$  T cells in sheep probably do not enter mesenteric LNs from the blood at all (30).  $\gamma/\delta$  T cells, which can comprise up to 60% of sheep PBL, comprised only 1% of mesenteric LN cells (30). By immunohistology, it was found that the majority, if not all, of these  $\gamma/\delta$  T cells had probably entered the node from the afferent lymph (23, 29). In any event,  $\gamma/\delta$  T cells were preferentially extracted by endothelium in peripheral tissues and not by HEV of LNs. The interpretation for CD8<sup>+</sup> cells is complicated by the fact that some NK cells and  $\gamma/\delta$  T cells express CD8, albeit at lower levels than for CD8<sup>+</sup>  $\alpha/\beta$  T cells. Certainly, CD8<sup>+</sup> cells were concentrated in efferent lymph compared with blood, and hence, a selective enrichment of this cell type probably also occurs at LN HEV.

The numbers of B cells, assessed by surface Ig staining, was very low in afferent lymph, consistent with previous observations (23, 35), and indicative that B cells pass from blood to peripheral tissues much more slowly than do T cells.

Fig. 6 shows that the three T cell subsets expressed different levels of adhesion molecules and CD45R. CD4<sup>+</sup> cells were enriched in the CD44<sup>hi</sup> subset, whereas  $\gamma/\delta$  T cells were enriched in the CD44<sup>lo</sup> subset. Also,  $\gamma/\delta$  T cells expressed lower levels of CD11a, and were negative for CD2. The CD11a<sup>hi</sup> subset of PBL, evidenced



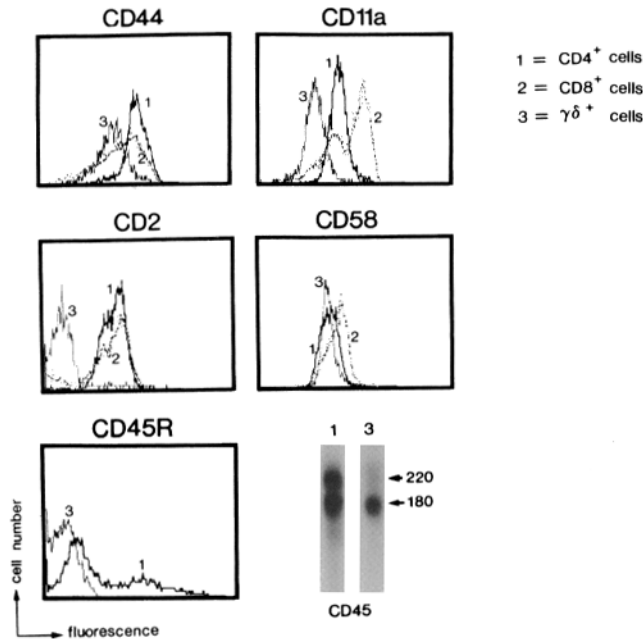


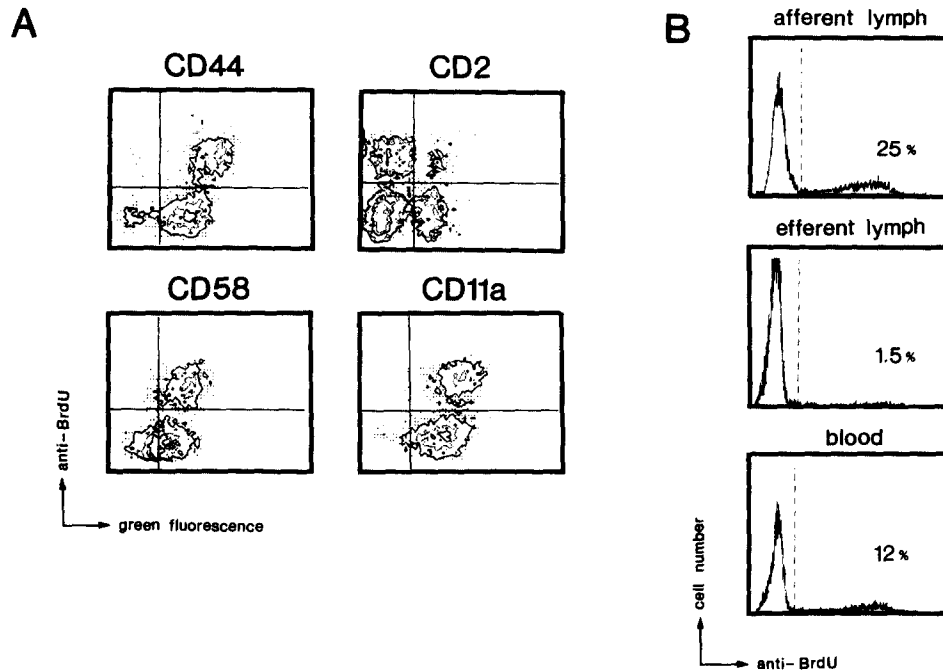
FIGURE 6. Expression of memory markers on  $CD4^+$ ,  $CD8^+$ , and  $\gamma/\delta^+$  T cell subsets in blood. The  $CD4^+$ ,  $CD8^+$ , or  $\gamma/\delta^+$  subsets in blood were identified using biotinylated mAbs followed by avidin-PE. Green fluorescence (anti-CD2, anti-CD58, anti-CD44, anti-CD11a, and anti-CD45R) was assessed on gated CD4, CD8, or  $\gamma/\delta$  T cell subsets. The key for the staining profile for each T cell subset is indicated on the top right hand side. The bottom right hand side of the figure shows an SDS-PAGE analysis of the CD45 molecule immunoprecipitated from (1) purified  $CD4^+$  cells and (3) purified  $\gamma/\delta^+$  cells. Each population was purified from blood by panning, and CD45 was immunoprecipitated with mAb 1-11-32 (28), which sees all isoforms of sheep CD45.

by the second peak in the FACS profile of Fig. 4, was composed entirely of  $CD8^+$  cells. These  $CD11a^{hi}$  cells were not evident in efferent lymph. PBL from six sheep were studied, and in all cases, the results obtained were similar to those shown in Fig. 5, indicating that the levels of adhesion molecules expressed on the three T cell subsets are tightly regulated.

The generally lower expression of adhesion molecules on  $\gamma/\delta$  T cells might, at first glance, indicate that these cells are mostly naive T cells. However, when  $\gamma/\delta$  T cells were analyzed for CD45R expression, virtually all were negative, both in blood (Fig. 6) and in afferent and efferent lymph (not shown). If expression of different isoforms of CD45 on  $\gamma/\delta$  T cells correlates with naive and memory function, as it does for  $CD4^+$  cells, this pattern of CD45 expression would indicate that the vast majority of circulating  $\gamma/\delta$  T cells are memory T cells, despite the fact that they express lower levels of adhesion molecules compared with  $CD4^+$  or  $CD8^+$  memory cells. To confirm that  $\gamma/\delta$  T cells did in fact express the p180 isoform of CD45,  $\gamma/\delta$  T cells were purified, from which CD45 was immunoprecipitated (Fig. 6).  $\gamma/\delta$  T cells from blood expressed almost exclusively the p180 form of CD45, whereas for  $CD4^+$  and  $CD8^+$  cells, both the p180- and p220-kD isoforms were immunoprecipitated.

**Cell Proliferation among Naive and Memory T Cells.** The anti-BrdU staining technique, which can be used to detect cells incorporating BrdU into their DNA during the S phase of the cell cycle (36), has the advantage over other techniques that measure cell proliferation in that it avoids the side effects caused by radioactive labels, and can be used in a two-color analysis with cell surface antigen-specific mAbs (37).

Fig. 7 shows a two-color immunofluorescence analysis of PBL from an 12-mo-old



**FIGURE 7.** Cell division within different populations of sheep lymphocytes and within different compartments of the recirculating pool. (A) Phenotypic analysis of dividing cells within blood of a 12-mo-old sheep. BrdU was administered intravenously for a period of 8 d. The phenotype of cells that had incorporated BrdU was assessed using two-color immunofluorescence (see Materials and Methods). After 8 d of labeling, the proportion of PBL that had incorporated BrdU was 34%. (B) BrdU incorporation by cells isolated from afferent lymph, efferent lymph, and blood. In this instance, BrdU labeling was for a period of 3 d, and BrdU incorporation was determined by a single-color immunofluorescence analysis. The proportion of cells that incorporated BrdU in each compartment is indicated accordingly.

sheep that had received BrdU for a period of 8 d. Approximately 35% of PBL had incorporated BrdU over this time period. The two-color analysis showed that all of the CD44<sup>hi</sup> cells had undergone at least one division over this time period, and that the staining intensity for CD44 was much greater for the BrdU<sup>+</sup> subset compared with the BrdU<sup>-</sup> subset. Similar results, albeit less obvious, were obtained with cells stained for CD2, CD58, and CD11a. Staining with anti-CD45R showed that the CD45R<sup>lo</sup> cells (naive T cells) were nondividing, whereas a proportion of the CD45R<sup>hi</sup> cells (B cells) were dividing (not shown). These results strongly suggest that the memory subset contained a high percentage of cells that had entered the S phase of the cell cycle over the time course studied.

If memory cells are a dividing population, then one would expect an accumulation of BrdU<sup>+</sup> lymphocytes within the afferent lymph. This was examined by cannulating the afferent and efferent lymph ducts of 12-mo-old sheep and analyzing BrdU incorporation. For technical reasons, the length of BrdU administration for these cannulated sheep was only 3 d. Fig. 7 B shows that afferent lymph did indeed contain many BrdU<sup>+</sup> cells (25%), in contrast to the efferent lymph, which contained

<2%. Blood contained a level of BrdU<sup>+</sup> cells that was in between these two values (12%). Two-color immunofluorescence analysis of PBL with T cell subset markers and anti-BrdU revealed that, over an 8-d labeling period, a comparable proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells had incorporated BrdU (~25%, compared with ~40% for the  $\gamma/\delta$  T cell subset). In afferent lymph, all three T cell subsets showed a comparable level of BrdU incorporation (~25% for the 3-d labeling period).

It should be noted that BrdU has a relatively short half-life in vivo, and since BrdU was administered only once per day, the values presented here may be an underestimation of the actual number of cells that entered the S phase of the cell cycle over the course of BrdU treatment. Despite this, the results demonstrate the much higher intrinsic proliferation of memory cells compared with naive cells, and of afferent lymphocytes compared with efferent lymphocytes. In these experiments, we could not distinguish between newly formed cells originating from primary lymphoid tissues, and cells that were dividing in the periphery; however, the sheep used for our experiments were 12 mo old, an age at which the thymus and ileal Peyer's patches have involuted.

### Discussion

The purpose of the present study was to compare the recirculation pathways and proliferation of naive and memory T cells. Such a study was contingent on the ability to phenotypically distinguish naive and memory T cells in sheep. Similar to man and mouse, memory T cells in sheep were CD45R<sup>-</sup> and expressed higher levels of adhesion molecules, whereas naive T cells were CD45R<sup>+</sup>, CD2<sup>lo</sup>, CD44<sup>lo</sup>, CD58<sup>lo</sup>, and CD11<sup>lo</sup>. Naive T cells were most numerous at birth, and decreased in number as the animal aged. After in vitro activation, naive T cells showed increased expression of CD2, CD44, CD58, and CD11a, and decreased expression of CD45R: phenotypic changes that also occur when naive cells undergo transition to memory cells in vivo. The observed higher expression of adhesion molecules on memory cells could not be attributed to increased cell size, since cells in the memory population of PBL were not blast cells and were only slightly larger than cells in the naive population (data not shown). Also, T cells in afferent lymph (predominantly memory cells) were only slightly larger than those in efferent lymph (predominantly naive cells) (23) or cells in neonatal blood (not shown). Finally, the response by sheep T cells to a recall antigen (Fig. 3) resided within the putative memory population and not in the naive population of cells.

This study shows that naive and memory T cells recirculate through the body by different pathways. Cells of the memory phenotype accumulated in the afferent lymph, indicative of a blood to tissue recirculation pathway, in contrast to the naive phenotype that accumulated in efferent lymph, indicative of a blood to LN recirculation pathway. The enrichment of the naive phenotype in efferent lymph indicates that these cells were preferentially extracted from the blood by HEV, since >90% of efferent lymphocytes are derived from cells entering the node at HEV and ~6% are derived from the afferent lymph (25). The low numbers of memory T cells in efferent lymph might be derived purely from the afferent lymph. The efficient recirculation of naive T cells through LNs is also evidenced by the extensive recirculation of lymphocytes between blood, LNs, and efferent lymph in sheep fetuses (38, 39). Recirculation is therefore a physiological property of naive lymphocytes. We

have not examined the cells in the afferent lymph of fetal sheep, which presumably are of naive phenotype; commencement of memory T cell recirculation through peripheral tissues must begin sometime after birth.

It is possible that the low numbers of memory T cells in efferent lymph might be due to retention and death of memory T cells in the LN, as is the case for T cell blasts injected into syngeneic recipients (40). The relatively rapid turnover for at least a proportion of the memory T cell population implies that a large number of these cells must also be dying. Naive and memory T cells may have an equal capacity to enter LNs via HEV, and the assumption that the composition of efferent lymph correlates with events occurring at LN HEV would be wrong. Recently we have produced a mAb to the sheep MEL-14/Leu-8 molecule and find that the molecule is expressed at high levels by all naive T cells, but is absent from a proportion (~30%) of memory T cells or afferent lymph T cells (Mackay, C. R., manuscript in preparation). However, whether lymphocyte binding to LN HEV is strictly related to MEL-14/Leu-8 expression is uncertain. In any event, it is mainly the naive phenotype that exits the node, and virtually all of these cells express high levels of MEL-14/Leu-8.

The recirculation pathways for naive and memory T cells described here may be of significance in immunological surveillance of the body for foreign antigens. In most instances, a primary immune response is initiated in a LN to which an antigen has drained, and, subsequent to this, memory lymphocytes leave via the efferent lymph and eventually drain into the blood through the thoracic duct. It seems appropriate that memory cells should migrate selectively through peripheral tissues such as skin, where antigens are first encountered. On the other hand, naive lymphocytes should recirculate rapidly and in large numbers through LNs, because the frequency of antigen-reactive cells within the naive population is so low; recirculating purely through LNs is the effective route for naive lymphocytes to make contact with a new antigen.

How naive and memory lymphocytes take different recirculation routes is unclear, but this presumably depends on differential expression of homing receptor molecules. Paradoxically, CD44 and CD11a, both of which have been reported to play a role in HEV recognition (31, 41, 42), are expressed at lower levels on those cells that have been extracted by the LN from the blood. This pattern of expression has also been noted in monkeys (43). Hence, LN homing may be mediated principally by another structure like MEL-14 in mice (44) and LAM-1/Leu-8 in humans (17, 45), and CD44 and CD11a might serve an accessory adhesion role. CD44 is a widely distributed molecule (14), and probably plays a more general role in cell adhesion for numerous cell types. In contrast to CD44, the MEL-14 homing receptor is down-regulated after T cell activation, and this causes alterations in the homing behavior of the activated cells (17–19). Also, rat T cell blasts generated *in vivo* migrate preferentially to the intestine, and not to LNs (40). These results are consistent with MEL-14/LAM-1 acting as LN homing receptors, whereas CD44 and CD11a probably act as general adhesion molecules and might be the principal molecules involved in lymphocyte extravasation in tissues such as skin. The memory/CD44<sup>hi</sup> subset in humans adheres more avidly to cultured endothelial cells, and is the cell type that accumulates in synovium and inflammatory infiltrates (46, 47; see also reference 43). Lymphocytes in afferent lymph of sheep might be preferentially extracted from blood because of their high levels of CD44. However, this preferential extraction has to

be reconciled with the homing behavior of T cell subsets. For instance,  $\gamma/\delta$  T cells accumulated in afferent lymph, and in this site they expressed high levels of CD44, CD58, and CD11a (but were CD2<sup>-</sup>; data not shown). Yet,  $\gamma/\delta$  T cells in blood expressed lower levels of CD44 and CD11a, indicating that a simple extravasation of these cells from blood to tissues based on expression of CD44/CD11a does not apply. However, the enrichment of  $\gamma/\delta$  T cells in afferent lymph might be expected, since the vast majority of  $\gamma/\delta$  T cells in blood expressed the p180 isoform of CD45, indicative of a memory phenotype. Based on the pattern established for CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells,  $\gamma/\delta$  T cells might also preferentially accumulate in afferent lymph by the same, as yet unknown mechanism.

It has generally been assumed that memory cells are a population of resting, long-lived cells. This paradigm may need to be reassessed, since recent experiments in rats indicate that B cell and T cell memory depends on the persistence of antigen (48, 49; and D. Gray, manuscript in preparation). These studies imply that maintenance of memory requires repeated antigenic stimulation and cell division by antigen-specific clones. The persistence of antigen in the host for long periods of time is quite plausible, since antigens in their native form have been detected on follicular dendritic cells 12 mo after immunization (50). Antigen retained on follicular dendritic cells might serve as a record of a host's previous immunological experiences, and therefore could act as a depot to provide for ongoing stimulation of either naive cells or cells that have previously been primed (49). The BrdU labeling experiments described here establish that a large proportion of cells displaying the memory phenotype had divided at least once during the 8-d labeling period. In addition, a sizeable proportion (25%) of afferent lymph cells, all of which possess the memory phenotype, had taken up BrdU after a much shorter labeling period (3 d), whereas very few efferent lymphocytes were labeled over the same period of BrdU treatment. This indicates that most peripheral T cells in sheep are long lived, since the ratio of T cells in the blood or afferent lymph compared with the total number of T cells in the recirculating pool is very small (51). The relatively rapid turnover for memory T cells and the slow turnover of naive T cells concords well with the results of Sprent and Basten (21), who showed an average lifespan for mouse thoracic duct T cells of 4–6 mo. Thoracic duct lymph is a composite of efferent lymph from all LNs in the body, and we show here that the efferent lymph of the popliteal node, which comprises predominately naive cells, does indeed contain very few cycling cells. In addition, experiments in rats also showed most peripheral T cells to be long lived, and that cycling cells accumulated with time more in blood than in LNs (52). However, the above findings differ somewhat from those of Freitas and colleagues (53, 54), who reported that the majority of peripheral B and T lymphocytes in the mouse have a lifespan of only 2–3 d. The basis for these differences is not yet known. In any event, a body of evidence now indicates that at least some components of T cell memory may not be contained within the long-lived T cell population. Rather, antigen-specific T cell clones might exist for extended periods of time by way of cell division through persistent antigenic stimulation.

### Summary

In this report, we have addressed two questions concerning immunological memory: the way in which naive and memory T cells recirculate through the body, and the intrinsic rate of division within the naive and memory populations. We identified

naive and memory T cells in sheep by their cell surface phenotype and their ability to respond to recall antigen. Memory T cells were CD2<sup>hi</sup>, CD58<sup>hi</sup>, CD44<sup>hi</sup>, CD11a<sup>hi</sup>, and CD45R<sup>-</sup>, as pertains in man. T cells that crossed from blood to the tissues of the hind leg and accumulated in the popliteal afferent lymph were all of memory phenotype. Conversely, T cells in efferent lymph, 90% of which entered the lymph node (LN) via high endothelial venules (HEV), were mostly of the naive phenotype (CD2<sup>lo</sup>, CD58<sup>lo</sup>, CD44<sup>lo</sup>, CD11a<sup>lo</sup>, and CD45R<sup>+</sup>). The marked enrichment of these two phenotypes in different recirculatory compartments indicated that memory T cells selectively traffic from blood to peripheral tissues to LN (via afferent lymph), whereas naive T cells selectively traffic from blood to LN (via HEV). We argue that the differential use of these two recirculation pathways probably optimizes lymphocyte interactions with antigen. The nonrandom distribution of T cell subsets in various recirculatory compartments may be related to the relative proportion of memory cells in each subset. In particular,  $\gamma/\delta$  T cells in blood were almost exclusively of memory phenotype, and accumulated preferentially in afferent, but not in efferent, lymph. Finally, using the bromo-deoxyuridine labeling technique, we found that at least a sizeable proportion of memory T cells, whether in blood or afferent lymph, were a dividing population of cells, whereas naive T cells were a nondividing population. This result supports an alternative model of lymphocyte memory that assumes that maintenance of memory requires persistent antigenic stimulation.

We thank Dr. B. Rocha for assistance with the BrdU staining technique, Dr. M.-F. Beya for producing the F10-150 cell line, Dr. T. Hünig for the anti-LFA-3 cell line, D. Thorpe for assistance with cell sorting, Drs. R. Palacios, A. de la Hera, D. Gray, and I. R. Mackay for reviewing the manuscript, Dr. A. Kelus for providing immunized sheep, and Nicole Schoepflin for help in preparing the manuscript.

*Received for publication 29 September 1989 and in revised form 5 December 1989.*

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