TWO DISTINCT POOLS OF RECIRCULATING T LYMPHOCYTES: MIGRATORY CHARACTERISTICS OF NODAL AND INTESTINAL T LYMPHOCYTES

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Thymus-derived (T) lymphocytes display considerable functional heterogeneity, but although it has been suggested that certain cells involved in graft-vs.-host reactions might have different routes of migration (1), it is generally assumed that the recirculation of small lymphocytes is uniform.

Recently we have isolated small T-recirculating lymphocytes (TRL)[1] from different sources in the sheep and have used these TRL to examine the migrational routes of small T lymphocytes. The advantage of sheep, for such studies, is that the size of the sheep allows chronic drainage of peripheral lymph nodes (LN) and intestinal lymph to be followed simultaneously. An examination in autologous recipients of the distribution of intravenously (i.v.) infused ^{51}Cr-labeled TRL from the efferent lymph of peripheral LN, or alternatively from intestinal lymph, gave results that indicate the recirculating lymphocyte pool can be divided into two major subdivisions of small T lymphocytes.

Materials and Methods

Animals and Surgery. Sheep of either sex from commercial flocks of "white alpine" and "black face" 8-10 mo of age were used. They were kept in metabolism cages and given hay, water and salt lick ad libitum. The intestinal lymphatic duct was cannulated using the method described by Lascelles and Morris (2) and the lymphatic vessels efferent to the prescapular nodes by the method used previously (3). An i.v. infusion cannula was placed in the jugular vein and all lymph plasma collected was returned to the animal. The infusion was interrupted intermittently for taking blood samples.

Lymph Collection and Cell Counting. Lymph was collected in bottles containing sterile physiological saline with 500 U USP/ml of preservative-free heparin (Liquemin; F. Hoffmann-La Roche & Co. A. G., Basel, Switzerland) and 500 IU/ml each of penicillin and streptomycin (Flow Laboratories, Inc., Rockville, Md.). The collection times were kept constant at 6 h. Lymph collections used for obtaining TRL and labeling with radiochromium were never longer than 2 h for intestinal, and between 2 and 4 h for prescapular lymph. The viability of lymphocytes collected in this way was always 100% as assessed by trypan blue dye exclusion. Cell counts were made on lymph samples after appropriate dilution using a Coulter Counter model Fn with a 100-μm aperture (Coulter Electronics Inc., Hialeah, Fla.).

Isolation of TRL. The method described by Julius et al. (4) for the isolation of T lymphocytes in mice has been successfully applied by us to sheep recirculating lymphocytes. Thus, 35-ml plastic Brunswick syringe barrels (Opopharma A. G., Zürich, Switzerland) were packed with 3 g of

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[1] *Abbreviations used in this paper:* FCS, fetal calf serum; LN, lymph nodes; PBS, phosphate-buffered saline; RL, Recirculating lymphocytes; TDL, thoracic duct lymphocytes; TRITC, tetramethyl rhodamine-isothiocyanate; TRL, T-recirculating lymphocytes.

prewashed (4) nylon wool from Fenwal LP 1 Leuko-Pak Leukocyte filters (Fenwal Inc., Walter Kiddle & Co. Inc., Morton Grove, Ill.). The columns were pre-wet with 50–100 ml of Dulbecco's phosphate-buffered saline (PBS) containing 5% heat-inactivated fetal calf serum (FCS) (GIBCO, Biocult, Glasgow, Scotland) and incubated at 37°C for 1 h. The columns were then loaded with 5 × 10^8 lymphocytes in 10 ml of medium and the cells then washed into the nylon wool with 2 ml of warm (37°C) medium and left for 45 min at 37°C. After incubation the columns were washed slowly with medium at 37°C and the first 50 ml of effluent was collected. The cells were then pelleted at 300 g for 10 min at 4°C, washed once in Dulbecco's medium, and reconstituted to a concentration of 10^8 cells/ml in Eagle's minimal essential medium (MEM) containing 5% FCS ready for ^{51}Cr-labeling. After separation and ^{51}Cr-labeling the yield of TRL was always over 90%.

Antisera.   Rabbit anti-sheep Ig was absorbed on fetal lamb serum bound to Sepharose 4B and conjugated with tetramethyl rhodamine-isothiocyanate (TRITC) (Baltimore Biological Laboratories, Becton, Dickinson & Co., Cockeysville, Md.) as described by Cebra and Goldstein (5). The conjugated antisera were kept at 4°C in PBS containing 0.5% bovine serum albumin and 10 mM NaN$_3$.

Direct Immunofluorescence.   Direct labeling of surface Ig was done at 4°C. Samples of lymphocytes before separation and of TRL obtained after separation on nylon wool were washed three times in Eagle's MEM containing 10% heat-inactivated FCS and 10 mM NaN$_3$. The cells (3 × 10^6 lymphocytes in 0.1 ml of medium) were then incubated for 30 min with 0.1 ml of TRITC-conjugated rabbit anti-sheep Ig. The cells were then washed three times, smeared on slides, fixed in absolute ethanol, and mounted with phosphate-buffered glycerol. Fluorescence microscopy was done using a Leitz Orthoplan microscope with an Osram HBO-200 mercury lamp and an Opak-Fluor vertical illuminator (E. Leitz, Wetzlar, Federal Republic of Germany) was used. The percentage of Ig-bearing lymphocytes was determined by counting at least 1,000 cells. The number of Ig-bearing cells ranged between 20 and 45% before and 1–2% after separation.

Labeling of TRL with ^{51}Cr In Vitro.   The method described previously (3) was used. TRL were incubated for 30 min at 37°C with 30–50 µCi/ml Na$_2$ ^{51}CrO$_4$ (sp act 500–930 µCi of ^{51}Cr/mg Cr; Swiss Federal Institute for Reactor Research, 5303 Würenlingen, Switzerland) at a concentration of 10^8 TRL/ml in Eagle's MEM containing 10% heat-inactivated FCS. The labeled TRL were infused i.v. over 10 min via the jugular cannula. Before infusion two small aliquots were taken for cell counting and for subsequent measurement of radioactivity.

Counting of ^{51}Cr-Labeled TRL in Blood and Efferent Lymph.   Labeled TRL in venous blood were assayed as previously described (6) by counting 10 ml of heparinized blood in a Packard series 4000 gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Radioactivity in the plasma was corrected for by determining the hematocrit and counting 5 ml of plasma. The total white cell count of the sample was counted in a hemocytometer and the number of lymphocytes per milliliter was then determined from differential counts made on Leishman-stained smears. After taking a 50 µl aliquot of lymph for cell counting, lymph samples were counted in a gamma spectrometer as described previously (3).

Distribution of ^{51}-Cr-Labeled TRL in Different Organs.   At the end of each experiment the sheep were sacrificed with nembutal i.v. The lungs, liver, spleen, small intestine, and the prescapular, prefemoral, popliteal, posterior mediastinal, bronchial, hepatic, and mesenteric LN were removed. The entire lungs, liver, spleen, and small intestine were cut into small pieces. All samples were put into plastic counting vials, weighed, and then counted in the gamma spectrometer together with the lymph and the blood samples and the original aliquot of infused cells.

Results

The Recirculation of Intestinal Lymph TRL from Blood to Intestinal and Nodal Lymph.   The representative results of one of five experiments in which ^{51}Cr-labeled TRL obtained from intestinal lymph were infused i.v. back into the same animal and their reappearance in intestinal and prescapular lymph sequentially monitored for the next 72 h, are shown in Fig. 1. The unseparated recirculating lymphocytes used to obtain TRL for this experiment contained 34% Ig-bearing lymphocytes. After separation and ^{51}Cr labeling the yield of TRL was 91%, of which less than 2% were blast cells. The TRL were contaminated with
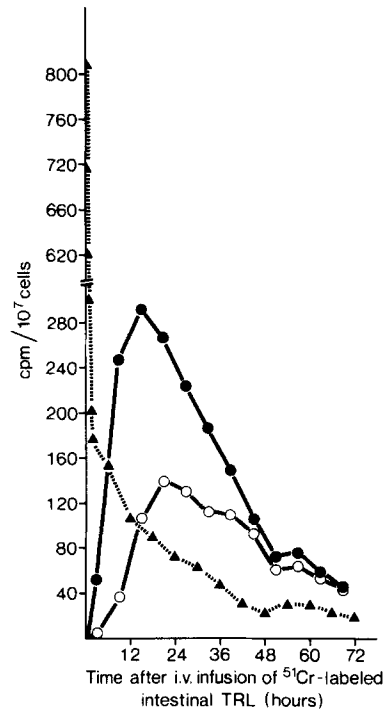
Fig. 1.   Changes in the concentration of autologous ⁵¹Cr-labeled intestinal TRL in venous blood (▲), intestinal lymph (●), and right prescapular lymph (○). 8.8 × 10⁸ intestinal TRL labeled with 4.8 × 10⁶ cpm ⁵¹Cr were infused i.v.

1.9% Ig-bearing cells. The concentration of labeled TRL in the blood (Fig. 1) fell by 80% in the first 3 h and then declined slowly so that it was about half that of the concentration in the lymph at 72 h. The appearance of labeled TRL in nodal and intestinal lymph showed a striking asymmetry. Their peak concentration in intestinal lymph was more than twice that in prescapular lymph. Not shown in this diagram is the left prescapular LN which gave an identical result to the right LN. The migration of intestinal lymph TRL from blood to lymph was therefore not random since higher relative recoveries were obtained from intestinal lymph than from peripheral LN. The animal was sacrificed at 72 h and 5.5% of the injected activity was recovered in the small intestine. The percentage of injected activity recovered per gram LN was the same (0.1%) for both the mesenteric LN and the peripheral LN.

The Recirculation of Nodal TRL from Blood to Intestinal and Nodal Lymph. The representative results of one of five experiments in which ⁵¹Cr-labeled nodal TRL were infused i.v. into autologous recipients are shown in Fig. 2. Nodal TRL were obtained from prescapular lymph which before separation contained 36% Ig-bearing lymphocytes. After separation and ⁵¹Cr labeling the yield of TRL was 90%, of which 1% was blast cells. The TRL were contaminated with 1.6% Ig-bearing cells. After their i.v. infusion the concentration of nodal TRL in the blood fell by around 70% in the first 3 h and then slowly declined over the next 3 days (Fig. 2) in a similar fashion to TRL from intestinal lymph (Fig. 1).
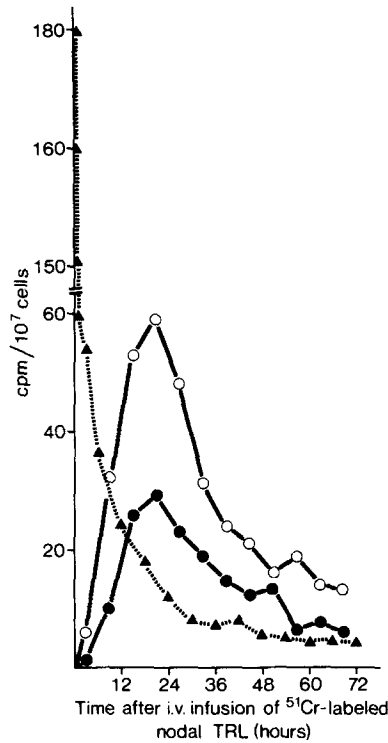
FIG. 2. Changes in the concentration of autologous ⁵¹Cr-labeled nodal TRL in venous blood (▲), right prescapular lymph (O), and intestinal lymph (●). 4.4 × 10⁸ nodal TRL labeled with 1.43 × 10⁶ cpm ⁵¹Cr were infused i.v.

The appearance of labeled TRL in intestinal and nodal lymph was asymmetric, but in this case the concentration of labeled TRL in intestinal lymph and nodal lymph were completely reversed (compare Fig. 2 with Fig. 1). This time the peak concentration of labeled TRL in prescapular lymph was twice that in intestinal lymph. Not shown in Fig. 2 is the contralateral prescapular LN which gave the same results.

The differences observed in the relative recoveries of labeled TRL from different sources in these two types of experiments were reflected in the absolute recoveries. When labeled TRL from intestinal lymph were infused i.v. their absolute recovery in intestinal lymph was 25% of the injected activity over the next 72 h. When labeled TRL from nodal lymph were infused i.v. their absolute recovery in intestinal lymph was in contrast only 12% of the injected activity. The reverse situation applied when the absolute recoveries of labeled TRL in the lymph draining a peripheral LN were computed. When labeled nodal TRL were infused i.v. their absolute recovery in lymph draining a prescapular LN was around 3% over the next 72 h but when labeled TRL from intestinal lymph were infused i.v. their absolute recovery in lymph draining a prescapular LN was about 1.5% of the injected activity. The recoveries from LN and small intestine after sacrificing the animal at 72 h showed further differences in the two types of experiments. Thus, after the infusion of nodal TRL, only 1.5% of the injected activity was recovered from the small intestine and the percentage of injected

activity recovered per gram LN was 0.13% for the peripheral LN, more than twice the 0.05% per gram recovered from the mesenteric LN.

It could be argued that factors present in intestinal or nodal lymph were passively absorbed onto the surface of TRL altering their surface membrane properties, and that this accounted for the different migratory characteristics we observed. This possibility was rendered unlikely by collecting nodal lymphocytes in 10 ml of fresh intestinal lymph and subsequently incubating them for 1 h at 37°C in a further fresh 10 ml of intestinal lymph before separation of TRL and ^{51}Cr labeling was performed. No differences occurred in the migration of nodal TRL, nor did the reverse experiment of collecting and incubating intestinal lymphocytes in nodal lymph alter the migration of TRL obtained from intestinal lymph.

The Site of Intravascular Selection of Intestinal TRL. Because intestinal lymph receives TRL from the blood by only two major sources, one via the high-endothelial post-capillary venules in the mesenteric LN and the other via afferent lymphatics draining the wall of the small intestine, selection of different TRL could only occur in the mesenteric LN or the small intestine. To decide between the two possibilities, ^{51}Cr-labeled TRL from nodal or intestinal lymph were infused i.v. into two groups of animals which were sacrificed 3 h later so that the distribution of labeled TRL in different organs could be determined. The results are given in Table I.

The most striking difference in the distribution of radioactivity was found in the small intestine. Up to 22.1% of the injected activity (mean 14.2%) was obtained in the small intestine after infusion of intestinal TRL compared with as little as 0.9% (mean 1.6%) after infusion of nodal TRL. The distribution of radioactivity in the lungs, liver, and spleen was the same in both groups of animals. The residual radioactivity in the blood (calculated from an assumed blood vol of 2.51) appeared to be higher with nodal TRL than TRL from intestinal lymph but the difference was not clear cut. Clearly a subpopulation of T lymphocytes which could recirculate from the blood through the lymphoid tissue in the wall of the small intestine was present in intestinal lymph but absent from nodal lymph.

Discussion

The experiments reported here demonstrate that a pronounced asymmetry exists in the recirculation from blood to lymph of small TRL. The interpretation of these experiments is not complicated by the presence of blast cells, because the cell populations which were used in these recirculation studies comprised over 98% resting small lymphocytes. When ^{51}Cr-labeled small TRL from intestinal lymph were infused i.v. their relative recovery in intestinal lymph was about twice that in nodal lymph. In contrast, the relative recovery in nodal lymph of ^{51}Cr-labeled nodal TRL infused i.v. was twice that in intestinal lymph. This suggests that intestinal lymph contains a mixture of around 50% intestinal TRL and 50% nodal TRL which, with the intestinal lymph providing about 80% of the thoracic duct output, would suggest a pool of recirculating small TRL consisting of about 40% intestinal TRL and 60% nodal TRL. Such an estimate is necessarily imprecise and the relative size of the two pools might well change during the lifetime of the animal.

TABLE I

Organ Distribution of ^{51}Cr-Labeled TRL from Intestinal or Nodal Lymph 3 h After i.v. Infusion

Number and specific activity of TRL	Lung	Liver	Spleen	Small intestine	Mesenteric LN	Peripheral LN	Blood*	Total % injected activity recovered
TRL from intestinal lymph								
9.4×10^8 TRL/3.9×10^6 cpm	519‡	589	93	746	61	34.5		
					0.07§	0.07		
	21.8‖	4.9	24	15.7	4.3	2.4	5.6	78.8
5.8×10^8 TRL/2.1×10^6 cpm	524	515	88	579	50.4	39.7		
					0.07	0.11		
	22.5	4.3	14.9	9.4	3.7	4.2	7.6	69
5.1×10^8 TRL/2.5×10^6 cpm	413	585	115	753	84.6	34.2		
					0.10	0.11		
	27.2	4.5	15.2	22.1	8.4	3.7	6.0	88
5.6×10^8 TRL/5.4×10^6 cpm	453	536	99	531	26.8	26.5		
					0.09	0.07		
	27.6	4.0	21.2	13.7	2.3	1.9	7.1	78
4.2×10^8 TRL/1.6×10^6 cpm	432	608	56	701	68.6	28.7		
					0.11	0.10		
	39	7.8	15.2	9.9	7.6	3.0	7.3	90
TRL from nodal lymph								
5.6×10^8 TRL/3.4×10^6 cpm	422‡	538	137	538	40.1	25.2		
					0.08§	0.14		
	35.8‖	3.4	32.6	1.9	3.4	3.5	11.3	92
2.7×10^8 TRL/2.5×10^6 cpm	393	612	120	644	44.1	29.4		
					0.06	0.10		
	17.8	2.1	14.1	1.4	2.8	2.8	15.6	57
7.2×10^7 TRL/897,600 cpm	678	580	88	664	51	40.6		
					0.06	0.13		
	27.5	3.2	17.5	1.8	3.3	5.2	6.8	65.3
6.1×10^8 TRL/5.5×10^6 cpm	458	465	90	726	46.9	38.9		
					0.06	0.07		
	31.3	3.7	21.4	2.0	2.8	2.6	12.6	76.4
5.8×10^8 TRL/3.9×10^6 cpm	546	725	104	546	26.1	37.1		
					0.04	0.06		
	33.2	4.3	21.5	0.9	1.1	2.1	6.7	70

* Calculated from an assumed blood vol of 2.5l.
‡ Weight in grams.
§ % Injected activity recovered/gram LN.
‖ % Injected activity recovered.

The organ distribution studies shown in Table I show unequivocally that intestinal TRL, but not nodal TRL, are in the small intestine in large numbers 3 h after i.v. infusion. This demonstrates that the intravascular selection of intestinal TRL occurs in the small intestine. The actual site where intestinal TRL recirculate through the wall of the small intestine was not examined microscopically. However, since high-endothelial PCV are present in the inter-follicular areas of Peyers' patches, these PCV are the most likely site of recirculation of intestinal TRL.

The specific activity of labeled TRL from intestinal lymph was the same in the mesenteric LN as in peripheral LN 3 h after their i.v. infusion. This is probably due to the fact that most of the labeled intestinal TRL are still in the small intestine at 3 h. The specific activity of labeled nodal TRL 3 h after i.v. infusion

was an average of 1.7 times higher in peripheral LN than in mesenteric LN, suggesting that a pool of nonlabeled intestinal TRL was diluting the labeled nodal TRL in the mesenteric LN. The recoveries of both labeled nodal and intestinal TRL after 3 h was the same in lungs, liver, and spleen and agreed with those previously found with unseparated nodal recirculating lymphocytes (RL) (6). The high accumulation of labeled RL in the lungs of sheep rather than the liver probably reflects a species difference and has been discussed previously (6).

Recently, using sheep Scollay et al. (7) have reported differences in the circulation of unseparated lymphocytes obtained from peripheral LN and intestinal lymph. They suggested that non-Ig-bearing T cells migrated randomly but that B-cell recirculation was nonrandom and was influenced by the class of their surface Ig. The studies reported here show that T-lymphocyte recirculation is not random and that a large scale recirculation of small T lymphocytes occurs in the small intestine; whether similar differences apply to B lymphocytes remains unresolved. Sprent has studied the organ distribution of ^{51}Cr-labeled TDL in mice sacrificed 4 h after i.v. injection (8). He observed comparable recoveries in the small intestine to those we obtained with labeled TRL from intestinal lymph. Sprent used TDL collected from the cysterna chyli (Fig. 3), which is the standard procedure for obtaining TDL from rodents. Therefore mouse TDL must contain a similar mixture of intestinal and nodal TRL to that in the intestinal duct of sheep. We had access to a pure population of nodal TRL, and therefore we were able to observe the differences in organ distribution of nodal and intestinal TRL. Furthermore, by establishing simultaneously chronic lymphatic fistulae in the intestinal duct and peripheral LN we were able to observe the differences in relative recoveries of intestinal and nodal TRL.

Fig. 3 shows a schematic representation of the recirculating lymphocyte pool which we propose for T lymphocytes. The pool of recirculating small T lymphocytes consists of two major subdivisions, an intestinal pool and a nodal pool. The nodal pool consists of small recirculating T lymphocytes which migrate from blood to lymph via high endothelial PCV in all LN. The intestinal pool consists of small recirculating T lymphocytes which cannot migrate from blood to lymph via PCV in LN but which do recirculate through the small intestine from which they pass via afferent lymphatics to the mesenteric LN which therefore (unlike peripheral LN) contains a mixture of intestinal and nodal TRL (see Fig. 3). Both intestinal and nodal TRL then emerge in the intestinal duct, pass into the cysterna chyli, and subsequently via the thoracic duct pass into the blood stream. Because we observed no differences in the recovery of either cell type in the spleen 3 h after their infusion i.v. (see Table I), we assume that both intestinal and nodal TRL have equal capacities to recirculate through the spleen.

Pearson et al. (9) recently reported an extensive recirculation of small thymus-derived lymphocytes in the fetus of sheep that are free from stimulation by extrinsic antigen and are agammaglobulinemic. They reported that when TDL were drained from fetuses, subsequently labeled with ^{3}H-cytidine, and then reinfused i.v., that larger numbers of labeled TDL could be found in the interfollicular area of the Peyers' patches and the mesenteric LN than in peripheral LN. Their findings suggest to us that the pool of intestinal TRL is
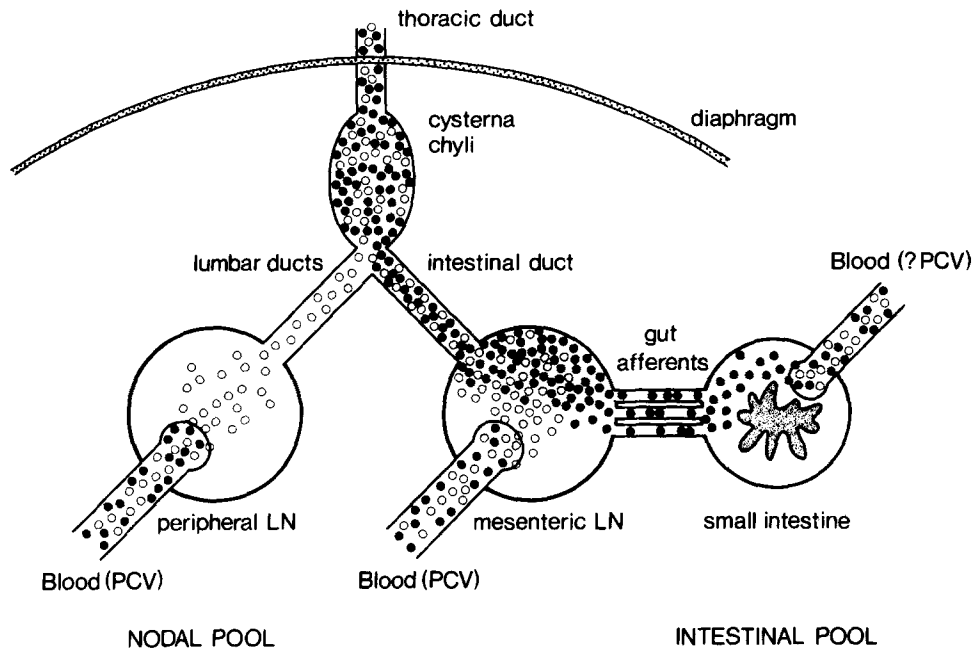
FIG. 3.   Diagram of the proposed two pools of recirculating small T lymphocytes. Intestinal TRL are depicted as closed circles and nodal TRL as open circles. For details see Discussion.

present in the fetus and that its initial development is independent of extrinsic antigen.

Summary

A pronounced asymmetry in the recirculation from blood to lymph of resting small recirculating T lymphocytes is described. When ^{51}Cr-labeled small T-recirculating lymphocytes (TRL) from intestinal lymph were infused intravenously their relative recovery in intestinal lymph was about twice that in nodal lymph. In contrast, the relative recovery in nodal lymph of ^{51}Cr-labeled nodal TRL was twice that in intestinal lymph. Intestinal TRL migrated in large numbers through the small intestine. Nodal TRL did not.

It is proposed that the pool of recirculating small T lymphocytes consists of two major subdivisions, an intestinal pool and a nodal pool. The nodal circulation comprises small TRL which traverse PCV in all lymph nodes (LN) but not the small intestine. The intestinal circulation comprises small TRL which do not traverse PCV in LN, but which do recirculate through the small intestine from which they pass via afferent lymphatics to the mesenteric LN and subsequently via the thoracic duct into the blood. It is suggested that the intestinal circulation is present in the fetus and that its initial development is independent of extrinsic antigen.

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