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Characterization of a novel subset of T cells from human spleen that lacks L-selectin

D. L. WALLACE & P. C. L. BEVERLEY *ICRF Human Tumour Immunology Group, The Courtauld Institute of Biochemistry, London*

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SUMMARY

Human L-selectin (LAM-1, Leu-8, TQ1, DREG 56) is a member of the 'selectin' family of adhesion molecules. Antibodies to L-selectin have been shown to block the binding of T cells to peripheral lymph node high endothelial venules (HEV). Most unstimulated peripheral blood T cells express high levels of L-selectin whilst it is only weakly expressed on the majority of T cells from secondary lymphoid organs. We show here (a) that T cells from tonsil and lymph node up-regulate L-selectin when released from their microenvironment, (b) that in contrast, spleen contains a stable L-selectin negative subset, (c) that this subset remains surface L-selectin negative after stimulation even though the T cells can respond by proliferation, (d) that this subset expresses minimal levels of LAM-1 mRNA and (e) that mucosal lymphocyte antigen (MLA) positive and T-cell receptor (TcR)$\gamma\delta$ positive T cells found within the L-selectin negative population are similar to subsets of T cells found amongst lamina propria (LP) and intraepithelial lymphocytes (IEL) of the gut.

INTRODUCTION

Lymphocytes continuously recirculate via blood and lymph through organized lymphoid tissues, such as lymph node, spleen, gut-associated lymphoid tissues, other exocrine mucosal sites and peripheral tissues.[1] This allows antigen-reactive effect and memory cells to migrate to and localize at sites of potential antigen presentation. *In vivo* studies have shown that lymphocytes from blood adhere to and migrate through characteristic endothelial cells lining the post-capillary high endothelial venules (HEV) of lymph nodes and mucosa-associated tissues.[2] This process is highly organ specific and *in vitro* experiments using the Stamper and Woodruff frozen section assay have shown that it appears to be mediated by complementary receptors on lymphocytes and tissue-specific HEV.[3] At least four separate recognition systems have been been described which mediate lymphocyte homing to peripheral lymph nodes, skin, gut or mucosal-associated tissues and inflamed synovium.[4-6]

Molecules from a variety of adhesion families such as integrins, selectins and the cartilage link protein-related CD44 are thought to be involved in selective migration of lymphocytes into secondary lymphoid organs. The murine cell surface molecule LPAM-1 (lymphocyte Peyer's patch adhesion HEV molecule-1), which has an α-chain analogous to the α-chain of VLA4 (CD49d, a member of the integrin family of adhesion receptors) has been shown to mediate the homing of lymphocytes to Peyer's patches but not to lymph nodes.[7] Specific lymphocyte–HEV adherence has also been shown in humans. Hermes-3, a monoclonal antibody (mAb) against human H-CAM (CD44), specifically blocks binding to HEV from mucosal tissue but not peripheral lymph node HEV, as does mAb to VLAα_4.[8] In contrast mAb to L-selectin (Mel-14 in the mouse and antibodies including LAM-1, TQ1, Leu-8 and Dreg 56 in humans) can block binding of murine and human lymphocytes to peripheral lymph node but not Peyer's patch HEV.[9-11]

Little is known about receptors involved in the homing of lymphocytes to the gut. It has been shown, however, that the mucosal lymphocyte antigen (MLA) is expressed on 95% of intraepithelial lymphocyte (IEL) T cells and 40% of LPL T cells,[12] and that the MLA heterodimer consists of an α-chain distinct from α_4 which is associated with a β-chain of the β_7 integrin type.[13]

Expression of L-selectin on human T lymphocytes varies according to the microenvironment in which cells are found, and to their state of activation/differentiation. T cells that express low LFA3 (CD58) levels have a uniform high level of expression of L-selectin whilst T cells that express high LFA3 show a bimodal pattern of expression.[14] In the mouse it has been shown that long-lived antigen-specific CD4$^+$ T cells are enriched for CD45RB$^-$ and Mel-14$^-$ T cells.[15] CD4$^+$ T cells from the lamina propria[16] and from germinal centres in lymph nodes and tonsil[17] express little or no L-selectin. When cells are activated *in vitro* with mitogen down-regulation is induced but loss of expression

Abbreviations: HEV, high endothelial venules; IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes; mAb, monoclonal antibody; MLA, mucosal lymphocyte antigen; PBL, peripheral blood lymphocytes; VLA, very late antigen.

Correspondence: Dr D. Wallace, ICRF Human Tumour Immunology Group, The Courtauld Institute of Biochemistry, 91 Riding House Street, London W1P 8BT, U.K.

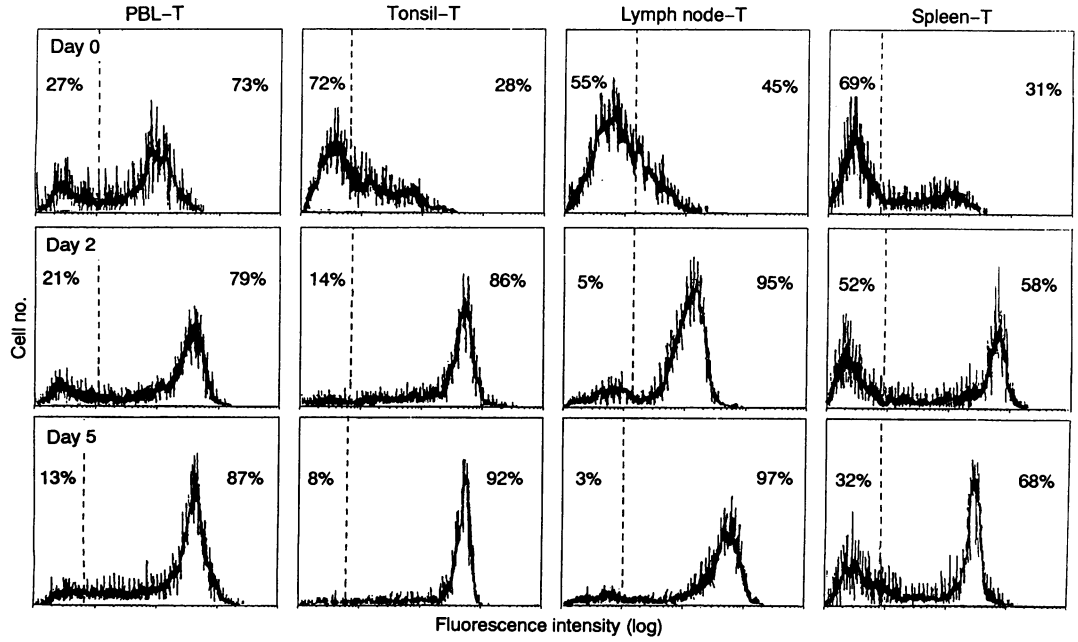

Figure 1. Alterations in expression of Leu-8 on purified peripheral blood, tonsil, lymph node and spleen T cells over a period of 5 days. T cells were incubated at 37° at 4×10^6/ml in RPMI-1640 containing 10% FCS. Cells were stained on days 0, 2 and 5 using PE-conjugated Leu-8 mAb. The negative control is represented by the dotted line. The percentage of Leu-8$^-$ and Leu-8$^+$ cells is shown. Results were compiled from several experiments.

is reversed when the stimulus is removed.[18] It has also been shown that freshly isolated human spleen cells which express low levels of L-selectin (23%) show increased levels (40–50%) when cultured for several days in medium alone.[19]

We have investigated the expression and regulation of L-selectin on T cells from a variety of secondary lymphoid organs. A minority of freshly isolated T cells from tonsil and lymph node express L-selectin at a low level, but after 48 hr in culture the majority of cells have up-regulated L-selectin to the same high level as that found on peripheral blood T cells. In contrast spleen contains two subsets of T cells. One expresses high levels of L-selectin whilst in the other surface expression is minimal. In this study the phenotype of the L-selectin negative subset was investigated, attempts made to induce expression of L-selectin, and the presence or absence of mRNA for L-selectin determined.

MATERIALS AND METHODS

Cell preparation

Spleens were obtained from two patients with idiopathic thrombocytopenic purpura (ITP), one with a gastric ulcer, three with oesophageal cancer, two with pancreatic cancer and one with sickle cell anaemia. Spleens came from male and female patients with ages ranging from 13 to 80. Tonsil, lymph node and spleen cells were isolated by teasing through a metal sieve, and mononuclear cells were obtained from these and from peripheral blood by centrifugation over Ficoll–Paque (Pharmacia, Uppsala, Sweden). T cells were then obtained by rosetting with AET-treated sheep red blood cells (SRBC). Residual non-T cells were removed by incubating with a mixture of monoclonal antibodies (mAb) against B cells, natural killer (NK) cells and monocytes/macrophages, washing, then incubating with sheep anti-mouse immunoglobulin-coated dynabeads, dynabead-coated cells were removed with a Dynal magnetic particle concentrator (Dynal AS, Oslo, Norway). The resulting T-cell populations were >95% CD3$^+$. For separation of Leu-8 (L-selectin) negative spleen T cells, fresh or frozen E-rosetted spleen T cells were incubated at 37° for 5–14 days in RPMI-1640 containing 10% foetal calf serum (FCS) without any other additives. Dead cells were removed by fractionation on Ficoll–Paque. Leu-8$^+$ T cells were removed using the magnetic particle concentrator after incubation with Leu-8 mAb at a saturating concentration followed by sheep anti-MIg-coated dynabeads. The resulting Leu-8$^-$ populations were <3% positive for Leu-8.

Antibodies

The following mAb were used; OKM1 (CD11b), HB55 (HLA-DR) from ATCC (Rockville, MD); Leu-8/Leu-8-phycoerythrin (PE), Leu-11 (CD16), interleukin-2 receptor (IL-2R) (CD25), Leu-16 (CD20), T-cell receptor (TcR)$\alpha\beta$–biotin and irrelevant isotype control antibodies from Becton Dickinson (Mountain View, CA); 4B4 (CD29) from Coulter Clone (Hialeah, FL); SAM-1 (VLAα_5) and GOH3 (VLAα_6) from Serotec (Kidlington, U.K.); HML-1 (MLA) from Immunotech (Marseilles, France); BerACT8 (MLA) from Dako (High Wycombe, U.K.); 9.3 (CD28) from Oncogen (Seattle, WA); TcRδ1 ($\gamma\delta$) from T Cell Sciences (Cambridge, MA). The following were kind gifts—PD7 (CD45RB) from D. Mason, SN130 (CD45RA) from G. Janossy, Fl0-44-2 (CD44) from R. Dalchau, H10-84 (CD54) from P. Mannoni, Hermes 3 (CD44) from S. Jalkanen, B-5G10 (VLAα_4) from M. Hemler, 134-2C2 (CD26) from J. Vives, QS4120 (CD4) from Q. Sattentau, TS2/9 (CD58) from T. Springer, BU12 (CD19) from N. Ling. UCHT1 (CD3), UCHT4 (CD8), UCHL1 (CD45RO) from ICRF. Anti-subclass-specific antibodies were obtained from Southern Biotechnology Associates Inc (Birmingham, AL).

Immunofluorescence

2×10^5 purified T cells were stained in round-bottomed flexible microtitre plates (Dynatech, Billingshurst, U.K.) with saturating amounts of ascites or hybridoma supernatant followed by

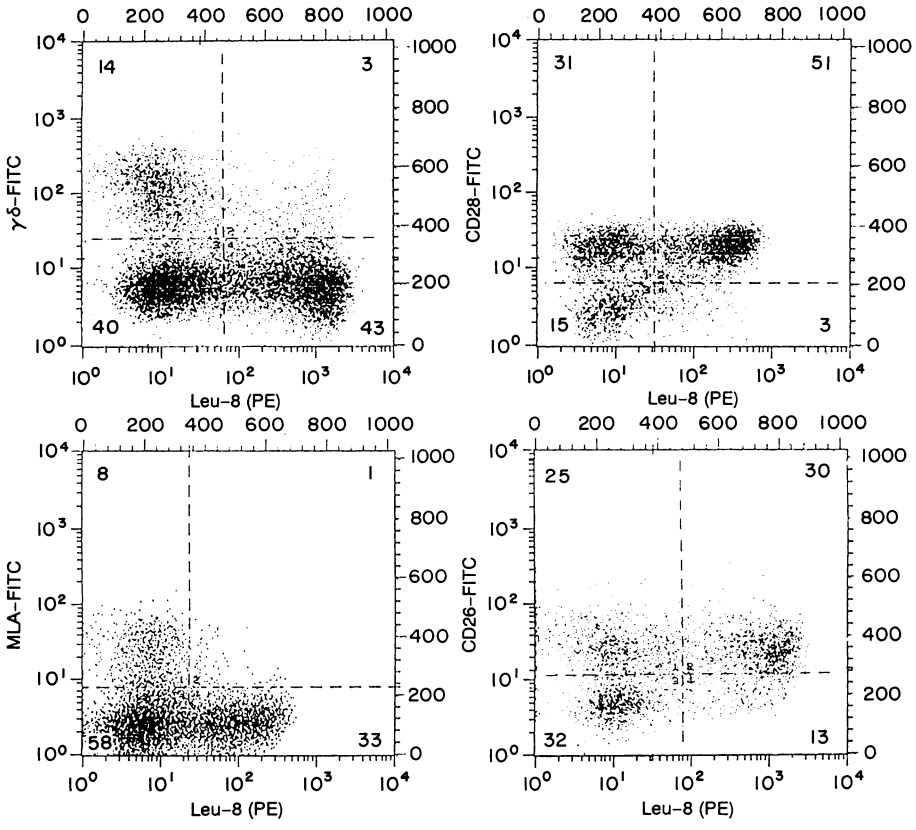

Figure 2. Two-colour immunofluorescence analysis of the expression of HML-1, γδ, CD26 and CD28 on splenic T cells after 5 days culture in medium alone. Goat anti-mouse IgG subclass-specific FITC conjugates were used to detect mAb against surface antigens (*y*-axis) while PE-conjugated Leu-8 mAb was used to detect L-selectin (*x*-axis). The cut-off point for positive and negative cells as determined by reference to the isotype-matched irrelevant negative control mAb is shown by the dotted line. Results taken from three experiments.

fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse subclass-specific mAb, with biotinylated mAb together with Phycoprobe avidin-PE (Biogenesis, Bournemouth, U.K.), or with mAb directly conjugated with fluorescein or PE. For dual-colour immunofluorescence cells were first incubated with unconjugated antibody followed by anti-subclass FITC then with 20% normal mouse serum and finally with an optimal concentration of biotinylated/directly PE-conjugated mAb. Thirteen thousand cells/sample were analysed on a FACScan (Becton Dickinson).

Proliferation assays

2×10^5 purified T cells together with 1×10^4 mitomycin C-treated autologous antigen-presenting cells were incubated in triplicate in flat-bottomed microtitre plates with PHA-P (Wellcome Research Laboratories, Beckenham, U.K.) 1 μg/ml, with or without phorbol myristate acetate (PMA) (Sigma Chemical Co., Poole, U.K.) 3 ng/ml in RPMI-1640 containing 10% FCS. Cultures were incubated for 72 hr and pulsed for the last 6 hr with 1 μCi/well of [^{3}H]TdR. The contents were harvested onto a filter using an automatic harvester (Skatron, Lier, Norway) and radioactivity measured in a Betaplate reader (LKB Wallac, Turku, Finland).

Northern blotting

After incubation in medium alone for 5–14 days, spleen T cells were split into an unseparated fraction and a negatively selected Leu-8$^-$ fraction. The T cells were lysed with guanidinium thiocyanate and ultracentrifuged on a cesium chloride gradient to obtain RNA. 15 μg/track of total RNA was electrophoresed on a formaldehyde gel and blotted onto gene screen nylon filters. A LAM-1cDNA probe (a kind gift from T. Tedder)[20] was labelled with ^{32}P using the random primer technique. Hybridization was performed at 65° for 16 hr. The membrane was then washed twice in 0·5 × SSC + 0·1% SDS at 50° and autoradiographs exposed with intensifying screens for 4–10 days at −70°.

RESULTS

Up-regulation of L-selectin on T cells

When separated T cells from fresh peripheral blood, tonsil, lymph node and spleen were incubated in medium alone then stained with Leu-8 mAb, there was little change in the percentage of Leu-8$^+$ peripheral blood T cells, yet there was dramatic up-regulation both in the number of Leu-8$^+$ cells and level of expression of Leu-8 on both tonsil and lymph node T cells (Fig. 1). This was not due to selective loss of Leu-8$^+$ T cells as control studies showed that viability was around 95% (data not shown). In addition although initial numbers of Leu-8$^+$ cells varied between 5 and 50% (low level expression only) the same levels of up-regulation were always detected. These levels of up-regulation were also detected when unseparated suspensions of cells were used and double stained with CD3 and Leu-8 mAb

Table 1. Expression of surface molecules defining subpopulations of human T cells on L-selectin positive, L-selectin negative and total human T cells*

Antigen	Total spleen T cells	Leu-8$^-$ T cells	Leu-8$^+$ T cells
CD4	51·9±7	40·8±9	67·0±5
CD8	30·8±8	26·8±10	33·1±9
$\alpha\beta$	86·2±5	84·7±7	89·7±6
$\gamma\delta$	6·7±5	8·7±6	2·6±1
CD45RA	34·4±2	30·9±12	39·2±10
CD45RB	94·5±4	95·3±3	93·0±7
CD45RO	63·9±8	56·1±9	74·7±7
CD29(VLAβ_1)	85·7±11	84·1±11	85·3±18
CD49d(VLAα_4)	60·0±11	63·7±8	52·2±24
CD44(Hermes-3)	96·5±5	96·5±6	97·7±3
CD26	32·1±10	27·2±8	37·8±11
CD28	60·9±14	48·2±13	77·8±15
MLA	7·4±2	10·1±3	4·8±3

* Spleen T cells were incubated in medium alone for 5–10 days, stained using a panel of mAb against surface molecules and anti-subclass-specific FITC-conjugated mAb and then double stained with Leu-8–PE. The percentage of positives in the total T-cell population, the L-selectin negative subset (Leu-8$^-$) and L-selectin positive subset (Leu-8$^+$) was calculated. Means±standard deviation were calculated from three to nine separate staining experiments using a minimum of three different spleens.

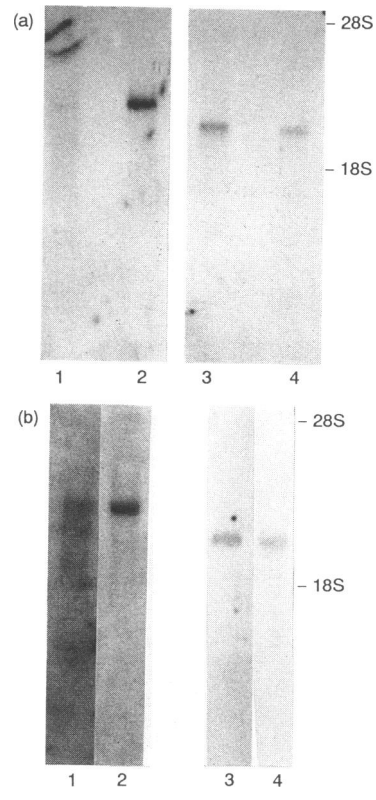

Figure 3. (a) Northern blot analysis of Leu-8$^-$ spleen T cells (lane 1) and unseparated spleen T cells (lane 2) cultured for 7 days before isolation and then probed with labelled LAM-1 cDNA probe. The same blot was stripped and reprobed with β-actin (lane 3 Leu-8$^-$, lane 4 unseparated spleen T cells). (b) Northern blot analysis using the LAM-1 cDNA probe of Leu-8$^-$ spleen T cells (lane 1) and unseparated spleen T cells (lane 2) after culture for 7 days, isolation and subsequent stimulation with PHA+PMA for 14 hr. Lanes 3 and 4 show the same samples stripped and reblotted with β-actin.

(data not shown). This up-regulation could not be prevented by the addition of cytokines such as interleukin-2 (IL-2), IL-3, IL-4, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), by mixtures of different accessory cell fractions or by varying concentrations of human serum (data not shown). In contrast staining of spleen (Fig. 1) showed that by day 5 there was a residual population of Leu-8$^-$ T cells (30–50%). Even when spleen T cells were further cultured for 25 days this negative population did not disappear.

Phenotypic analysis of L-selectin negative spleen T cells

In order to determine the phenotype of this population of L-selectin negative cells, spleen T cells were cultured in medium alone, stained with a panel of mAb against cell surface antigens and then double stained with Leu-8 mAb. Table 1 summarizes the expression of a number of molecules associated with recognition and adherence by T lymphocytes during recirculation as well as with different functional subsets such as CD4 (helper/inducer), CD8 (cytotoxic/suppressor), CD45RO (memory) and CD45RA (naive). The phenotype of the total spleen T-cell population as well as T cells gated on Leu-8$^-$ and Leu-8$^+$ cells was examined. No significant differences were found in the expression of most adhesion molecules, CD4/CD8 or CD45RA/CD45RO on either the Leu-8$^+$ and Leu-8$^-$ populations when compared to the total population. Hermes-3 (CD44) and VLAα_4 (CD49d) mAb which selectively block binding to HEV of mucosal origin were both equally expressed on the majority of Leu-8$^+$ and Leu-8$^-$ populations. Both subpopulations expressed activation molecules equally (HLA-DR 31% and CD25 14%) at levels somewhat higher than that found among peripheral blood T cells (data not shown). Figure 2 shows individual FACScan profiles demonstrating some of the differences between L-selectin negative and L-selectin positive spleen T cells. Data obtained from a total of six spleens indicated that 75%±SD 15 of TcR$\gamma\delta$ positive and 68%±SD 12 of MLA positive cells were found within the L-selectin negative population. Although the L-selectin negative subset contained only 9% of TcR$\gamma\delta$ positive and 10% of MLA positive cells, this is higher than the total percentage found in peripheral blood (around 2% for both). In addition the L-selectin positive sub-population was enriched for both CD26$^+$ and CD28$^+$ cells (Fig. 2) indicating that there is complex heterogeneity of T-cell subsets within the spleen.

Northern blot analysis of L-selectin negative spleen T cells

Since the majority of L-selectin negative spleen T cells did not express high levels of activation markers such as CD25 and did not have a 'blast'-like appearance they were unlikely to be activated cells which had down-regulated L-selectin.[18] In order to confirm this Northern blots of total cellular RNA from spleen T cells that had been cultured in medium alone were hybridized with the pLAM-1 cDNA probe. Figure 3a (lanes 1 and 2) shows that the unseparated fraction hybridized with a major band of

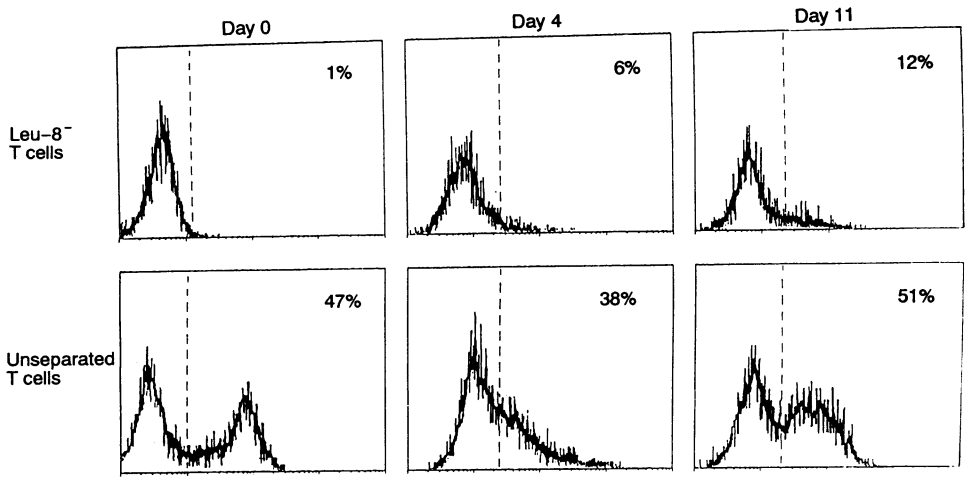

Figure 4. Splenic E-rosetted T cells were incubated in medium alone for 5 days. Some L-selectin negative T cells were negatively selected using Leu-8 mAb, these and unseparated cells were then stimulated with PHA + PMA for 12 hr at 37°. The cells were then washed five times and put back into culture. Immunofluorescence using PE-conjugated Leu-8 mAb was carried out on the day of separation (day 0) and on days 4 and 11.

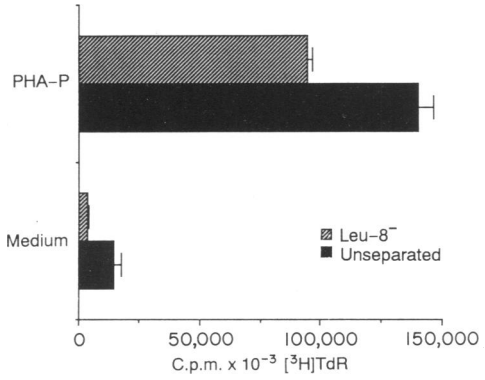

Figure 5. Splenic E-rosette positive T cells were incubated in medium alone for 7 days and a Leu-8⁻ fraction then isolated by negative selection. This fraction along with an unseparated spleen T-cell fraction and (supplemented with 10% autologous mitocycin C-treated T-depleted E negative spleen) was stimulated with a mitogenic dose of PHA-P at 37° for 72 hr and pulsed with [^{3}H]TdR for the last 6 hr.

2·3 kilobase (kb) whilst the surface negative fraction shows only a faint band. Reprobing with an actin probe (Fig. 3a), lanes 3 and 4) showed that this was not due to lack of total RNA. It has previously been shown that although stimulation with PHA + PMA for 14 hr decreases surface expression of L-selectin, RNA transcripts are increased.[21] When L-selectin negative spleen T cells are incubated for 14 hr with PHA + PMA there may be a small increase in mRNA level although it remains much lower than the levels detected in the total T-cell population (Fig. 3b). The level of L-selectin mRNA is therefore quantitatively different in the two subsets of spleen T cells. In similar experiments on intestinal lamina propria cells of non-human primates no mRNA for L-selectin was detected.[22]

Effects of mitogenic stimulation on L-selectin negative spleen T cells

Following stimulation of peripheral blood T cells with PHA + PMA there is down-regulation of surface expression of L-selectin which is restored after 6 days. As shown in Fig. 4 PHA + PMA stimulation of L-selectin negative spleen T cells induced only low levels of L-selectin, although these cells were able to respond by proliferation to PHA-P (Fig. 5). In contrast stimulation of unseparated spleen T cells led to down-regulation and subsequent up-regulation of L-selectin as has been reported for peripheral blood T cells.[18]

DISCUSSION

The results shown in Fig. 1 demonstrating that lymph node and tonsil L-selectin negative T cells are able to up-regulate their expression of L-selectin, suggest that the microenvironment may play a role in maintaining down-regulation of this molecule. In contrast, the spleen contains an L-selectin positive T-cell population as well as an L-selectin negative population. This L-selectin negative subset consists of cells the majority of which do not up-regulate L-selectin when cultured *in vitro* and which appear to express minimal levels of mRNA. Phenotypic analysis showed that both populations contain a mixture of $CD4^+$/$CD8^+$ and $CD45RA^+$/$CD45RO^+$ cells, although there were differences in the distribution of CD26, CD28, MLA and TcR$\gamma\delta$ between the two populations.

The TcR$\gamma\delta$ positive, L-selectin negative subset found in the spleen has some similarities to a TcR$\gamma\delta$ positive, L-selectin negative subset found amongst murine IEL T cells (Schmitz et al, 1988). Similarly the mucosal lymphocyte associated antigen is found on 95% of IEL and 40% of LP T cells, and the majority of intraepithelial lymphocytes (IEL) and lamina propria (LP) T cells are L-selectin negative.[12] The MLA positive, L-selectin negative spleen T-cell subpopulation therefore shows phenotypic similarities to populations found in the lamina propria of the gut, and like LP T cells of non-human primates expresses minimal levels of L-selectin mRNA.[22] This subset is also similar to a small subset found in peripheral blood,[14] suggesting that the MLA positive subset within the spleen may represent a population having a specialized pathway of migration to the gut. This is supported by evidence that the MLA(HML-1)α-chain is found in association with a β_7 chain belonging to the integrin familiy of adhesion molecules.[23] Similarly β_7 is associated with a VLAα_4 suggesting that there may be a β_7 integrin

family that plays a role in the localization of T cells in the gut. In addition there is a structural relationship between human $\alpha_4\beta_7$ and the murine Peyer's patch homing molecule LPAM-1 which consists of an α_4-chain in association with a β_p integrin subunit. Likewise, M290 a surface molecule found predominantly on murine IEL, consists of a β_7-chain associated with a cleaved α-chain.[24] Furthermore, in sheep, it has been shown that L-selectin negative memory T cells from the gut migrate preferentially back to the gut.[25]

It has been shown that mature single CD4$^+$ or CD8$^+$ human thymocytes express L-selectin at a level similar to that found on peripheral blood T cells[14] and this raises the question of whether differentiation of L-selectin negative spleen T cells could occur extrathymically. Studies using germ-free[26] or thymectomized[27] mice, which show extrathymic differentiation and maturation can occur especially amongst IEL TcR$\gamma\delta$ positive T cells, might support this possibility.

Although extensive recirculation occurs through the spleen very little is known about the mechanisms controlling entry into and localization within it. Entry does not appear to involve adhesion to HEV as in lymph node, and the possibility remains that the L-selectin negative population could contain a population which localizes in the spleen by another as yet uncharacterized adhesion molecule.

Our results demonstrate the presence of several subsets of T cells within the spleen that bear distinct arrays of surface molecules in particular L-selectin, TcR$\gamma\delta$ and MLA. Such heterogeneity suggests the presence of several separate recirculation pathways associated with the spleen.

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