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Antigen-driven shedding of L-selectin from human γδ T cells

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SUMMARY

Activation of lymphocytes leads to the modulation of a number of surface molecules. We have investigated the expression of one such molecule, L-selectin, following activation of γδ T cells with *Mycobacterium tuberculosis*. L-selectin is modulated during lymphocyte entry into lymph nodes; this modulation reflects the recirculation and homing potential of lymphocytes. We find that stimulation of γδ T cells by *M. tuberculosis* antigens results in shedding of L-selectin from γδ T cells. Re-expression of L-selectin occurs on removal of antigen suggesting that the regulation of expression is controlled by the presence or absence of antigen. The γδ T-cell receptor (TCR)-positive, L-selectin negative population of peripheral blood lymphocytes appears to be resting cells, as assessed by forward- and light-scatter analysis. We further find that γδ T cells isolated from a site of infection, the pleural fluid of a tuberculosis patient, are L-selectin negative, and that L-selectin is re-expressed following culture of the pleural fluid γδ T cells in the absence of antigen. These results demonstrate that, in addition to stimulation with polyclonal mitogens, antigen stimulation can also promote the surface shedding of L-selectin and that γδ T cells have the potential to home to sites of infection supporting their role in the immunological defence against infectious micro-organisms.

INTRODUCTION

T cells expressing the γδ T-cell receptor (TCR) represent approximately 5% of the human peripheral blood T-cell population of which some 70% are γ9δ2. γδ T cells are known to be selectively stimulated by a range of micro-organisms, including mycobacteria,[1–3] although their function is not yet clear. It has been suggested that they are involved as a first line of defence against micro-organisms, possibly through recognition of common antigen(s).[4] Direct evidence of a protective role for γδ T cells has recently been obtained for *Listeria monocytogenes* infections in mice.[5] More recently, γδ T cells have been found to be involved in establishing primary immune responses in mice infected with *L. monocytogenes* and *Nippostrongylus brasiliensis*.[6]

T cells expressing the γδ TCR accumulate at the site of antigenic challenge in both mouse[7] and man;[8] this accumulation suggests that γδ T cells are able to home in to sites of inflammation. In this study we have investigated the homing and recirculation potential of γδ T cells by investigating the regulation of L-selectin expression following antigenic stimulation.

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L-selectin directs the migration of lymphocytes into peripheral lymph nodes and belongs to a family of adhesion molecules that are involved in the earliest events of the acute inflammatory response leading to the initial adhesive interactions between leucocytes and endothelial cells.[9,10] Expression of L-selectin is uniquely regulated on the surface of leucocytes; within minutes of stimulation with chemotactic factors or other activating agents, L-selectin is shed from the surface of neutrophils and monocytes.[11] L-selectin is also shed from lymphocytes following activation with phorbol esters, mitogenic lectins and anti-CD3 antibodies.[12,13] Shedding also occurs in a Vβ selective fashion following *in vivo* stimulation with bacterial superantigens.[14]

In this study we have investigated the homing and recirculating potential of γδ T cells by monitoring L-selectin expression following antigenic stimulation with a preparation derived from *M. tuberculosis*. The results indicate how the migration of γδ T cells to sites of infection is regulated.

MATERIALS AND METHODS

Preparation of low molecular weight molecules of M. tuberculosis *M. tuberculosis* $H_{37}Rv$ was grown in Dubos medium for 2 weeks and the bacteria killed by γ-irradiation (2.5 MRad from a cobalt source). After centrifugation at 4000 ***g***, the bacterial pellet was washed twice and resuspended in phosphate-buffered

saline (PBS). The bacteria were lysed by sonication (100 W). Insoluble bacterial components were removed by centrifugation (20 000 *g*), and the cell-free extract treated with 1% (v/v) trifluoroacetic acid (TFA; Romil, Leicestershire, UK) to precipitate proteins and peptides larger than 5000 MW, which were removed by centrifugation at 13 000 r.p.m.. The supernatant was neutralized with Tris pH 11 and then applied to a Biogel P-2 column (Pharmacia, Uppsala, Sweden) for desalting using high-performance liquid chromatography (HPLC) water to elute the sample. This rendered a crude protein-free preparation; low molecular weight molecules were further isolated by Sephadex G-50 (Pharmacia Biotech, Uppsala, Sweden) chromatography.

Separation of peripheral blood mononuclear cells (PBMC) and cell sorting

Healthy, purified protein derivative (PPD) positive volunteers were used as a source of peripheral blood mononuclear cells

Table 1. Expression of L-selectin by γδ T cells from human peripheral blood

Donor	γ9 T cells (% of total T-cell population)	% L-selectin negative (% γ9 T cells)	L-selectin positive (% γ9 T cells)
1	1·6	0·7 (43·8)	0·9 (56·3)
2	3·8	1·6 (42·1)	2·2 (57·9)
3	0·8	0·3 (37·5)	0·5 (62·5)
4	5·6	2·9 (51·8)	2·7 (48·2)
5	2·9	1·4 (48·3)	1·5 (51·7)
6	3·4	0·8 (23·5)	2·6 (76·5)
7	3·0	2·2 (73·3)	0·8 (26·7)

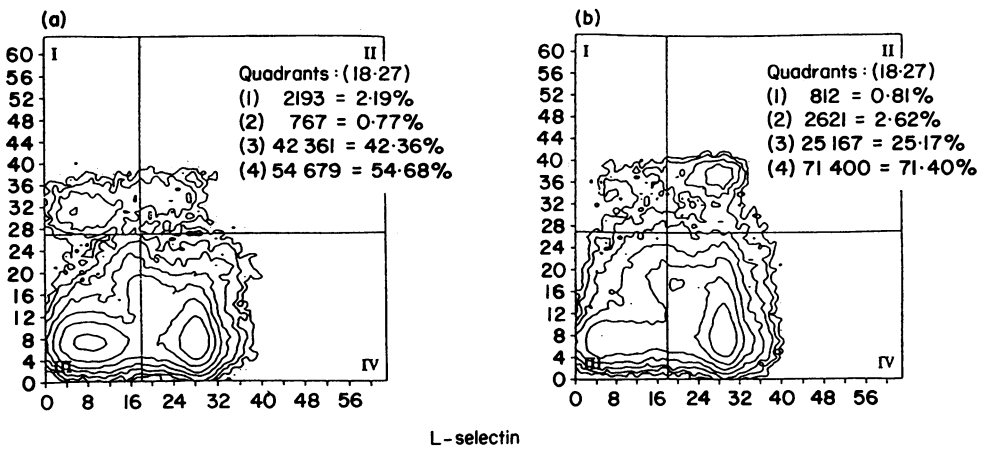

Figure 1. The expression of L-selectin by peripheral blood γδ T cells of healthy donors. Panel (a) shows a donor in which the γ9 TCR, L-selectin-negative population was in excess. Panel (b) shows a donor in which the γ9 TCR, L-selectin-positive population was in excess.

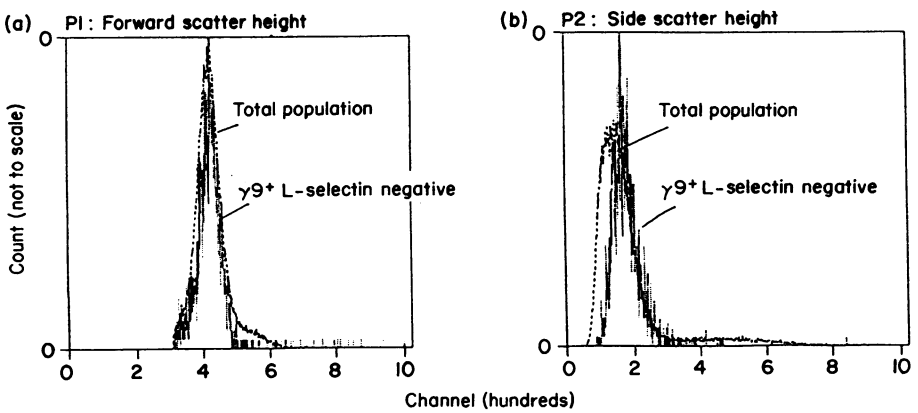

Figure 2. The activation state of peripheral blood γδ T cells. Forward-scatter (a) and side-scatter (b) analysis of freshly isolated lymphocytes.

(PBMC). PBMC were isolated by Ficoll–Hypaque (Pharmacia) density gradient centrifugation. Accessory cell-depleted T cells were prepared by passing PBMC through a nylon wool column. Non-adherent cells were washed three times in RPMI-1640 (Flow, Buckinghamshire, UK) and resuspended in the same medium supplemented with 10% human type AB serum (Flow), 2×10^{-5} M 2-mercaptoethanol, 50 μg/ml ampicillin, 100 U/ml streptomycin and 2 mM L-glutamine. Nylon wool adherent cells were recovered by passing ice-cold saline (0.85% NaCl w/v) through the column and gently sequeezing the nylon wool. This adherent cell popu-lation was used as a source of APC after irradiation (3000 Rad).

T cells were sorted on the basis of L-selectin expression using a miniMACS system (Multenyi Biotec, Bergisch Gladbach, Germany), following the manufacturers instructions. Briefly, 1×10^7 cells were labelled with fluorescein isothyocynate (FITC)-conjugated anti-L-selectin monoclonal antibody (mAb) and incubated with anti-mouse immunoglobulin microbeads. After washing, the cell suspension was passed through the MiniMACS column. Positive (L-selectin positive)

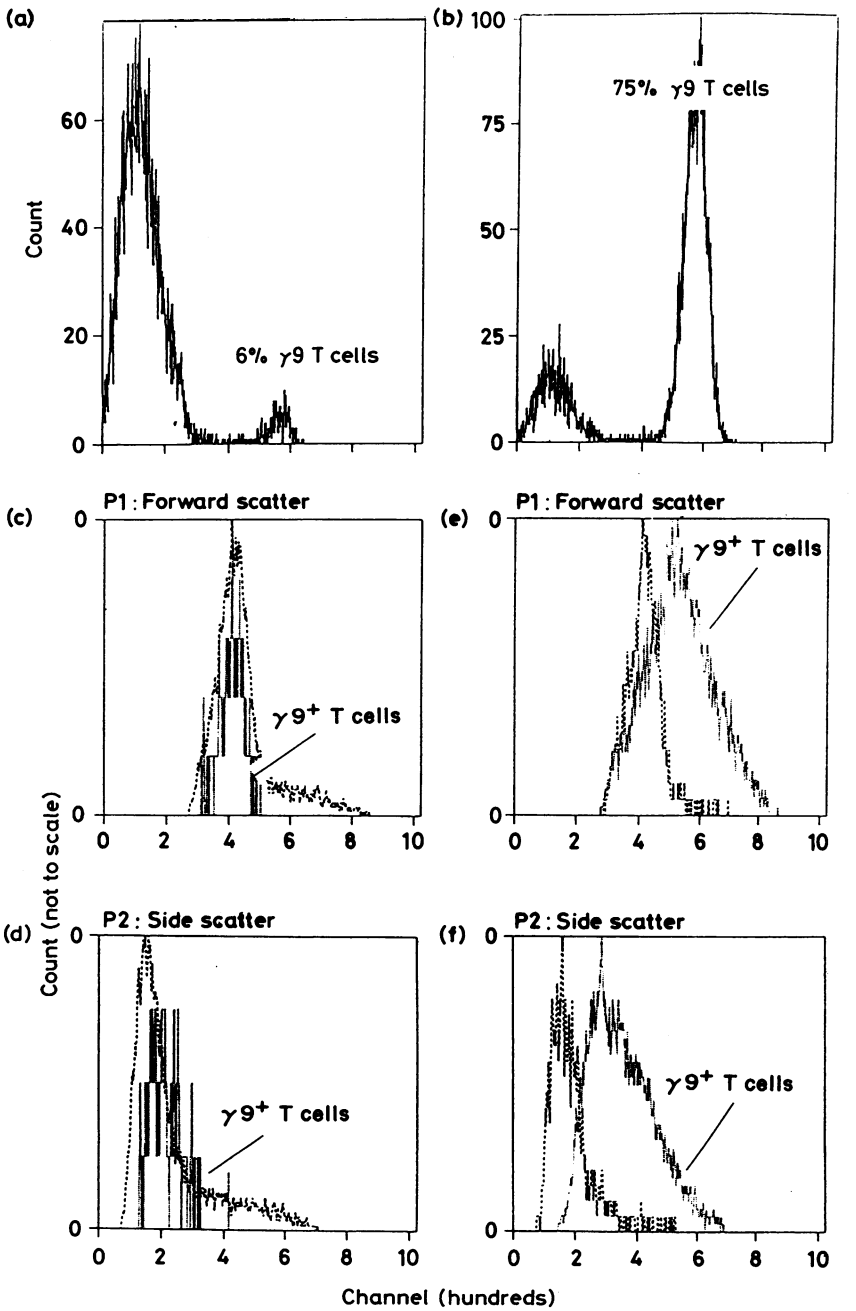

Figure 3. Proliferation of γδ T cells in response to stimulation with low molecular weight *M. tuberculosis* antigens. Panels (a) and (b) show FACS analysis in the absence and presence of antigen, respectively. Panels (c) and (d) show forward- and side-scatter analysis in the absence of antigen. Panels (e) and (f) show forward- and side-scatter analysis in the presence of antigen.

and negative (L-selectin negative) selected cells were collected separately.

Proliferation assays

Proliferative responses against the *M. tuberculosis* low molecular weight preparation were measured in triplicate cultures (200 μl/well) in 96-well round-bottomed plates containing 3×10^5 T cells and 3×10^4 irradiated antigen-presenting cells (APC) in culture medium. Cells were incubated at 37° in an atmosphere of 5% CO_2. After 5 days 10 U/ml of recombinant interleukin-2 (rIL-2) (Boehringer, East Sussex, UK) was added to each well and the cells cultured for a further 3 days. 18 hr prior to harvesting, 0.5 μCi of [^{3}H]thymidine (Amersham International, Amersham, UK) was added. Cells were harvested and thymidine incorporation measured. Results were expressed as mean c.p.m. of the triplicate samples.

Immunofluorescence analysis

Exact replicates of the proliferation assay cultures were used for immunofluoresence analysis. Cells from triplicates were pooled, washed twice with cold PBS, resuspended in PBS containing 1% BSA and 0.01% sodium azide, and stained with murine mAb anti-TCR γ9 (diversi-TγV2, T Cell Sciences, Cambridge, MA) for 30 min at 4° followed by incubation with goat–anti-mouse phycoerythrin (PE) (Sigma, St Louis, MO) and FITC-conjugated anti-L-selectin (Leu8, Beckton-Dickinson, Mountain View, CA). Samples were washed twice with PBS after each incubation with antibody and finally resuspended in fixing solution (PBS plus 1% BSA and 1% formaldehyde). Analysis was performed using a FACScan (Becton-Dickinson).

Direct analysis of γδ T cells from peripheral blood and pleural effusion

Because of the small percentage of γδ T cells in peripheral blood, 100 000 freshly isolated lymphocytes were analysed for expression of L-selectin and γδ TCR. The pleural effusion of a single tuberculosis patient was also examined for L-selectin and γδ TCR expression. The analysis by immunofluorescence was carried out as described above for cultured T cells.

Detection of soluble L-selectin

In order to detect the presence of L-selectin that had been shed into the medium, 10–15 μl of supernatant from cultured cells was dot-blotted onto a nitrocellulose membrane. The membrane was incubated for 1 hr in blocking solution (100 mM NaCl, 50 mM Tris pH 7.4, 0.1% Tween 20, 3% BSA). The membrane was then incubated with anti-L-selectin mAb (Leu8, Becton-Dickinson) for 1 hr, followed by biotinylated anti-mouse antibody (Bioguex, San Ramon, CA) and peroxidase-conjugated ExtrAvidin (Sigma). Incubation was at room temperature for 1 hr followed by thorough washing of the membrane. After the final wash the membrane was incubated with ECL western blotting peroxidase substrate (Amersham) for chemiluminescent detection following the manufacturer's instructions. After exposure, Hyperpaper ECL Western (Amersham) was developed in an X-ray developer and scanned using a Scanmaster 3 + (Howlek, Inc., Hudson, NH). The integrated density of the dots was calculated using the 'whole-band analyser' software (Millipore Corporation Imaging System, Ann Arbor, MI). The negative control consisted of supernatant from cells incubated in RPMI-1640 in the absence of *M. tuberculosis* antigens.

RESULTS

L-selectin expression defines two subpopulations of peripheral blood γδ T cells

The expression of L-selectin by peripheral blood γδ T cells of healthy donors was analysed by flow cytometry. In each individual two populations of γδ T cells could be distinguished, one of which was L-selectin positive and the other L-selectin negative (Table 1). In most cases the proportion of positive and negative cells were approximately equal, although individual variation was observed. Figure 1 shows two extreme cases in which the percentage of L-selectin negative cells (Fig. 1a) or positive cells (Fig. 1b) was in excess. It has been proposed that L-selectin is shed during cell activation[15] and results from this study show this to be the case for γδ T cells stimulated *in vitro* with antigen. To check whether the L-selectin negative population of γδ T cells represents a subpopulation of cells that had been activated *in vivo*, possibly as a result of exposure to environmental antigens, we analysed the size and granularity of the cells by forward and side scatter

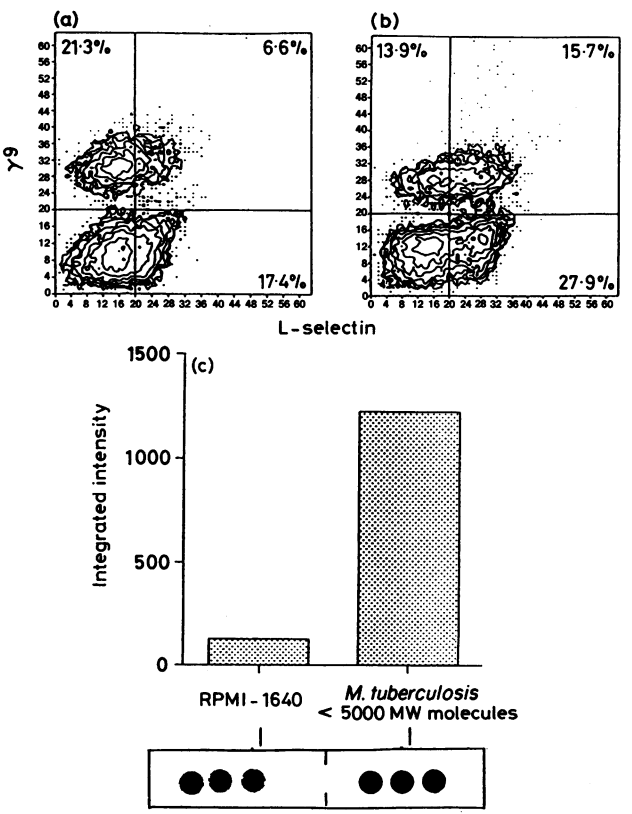

Figure 4. Re-expression of L-selectin following removal of antigen. Panel (a) shows low levels of L-selectin expression on γδ T cells stimulated by *M. tuberculosis* antigens. Panel (b) shows an increase in L-selectin-positive γ9 TCR expressing T cells following removal of antigen and incubation for a further 24 hr in RPMI-1640 medium alone. Panel (c) shows the presence of soluble L-selectin in the supernatant of *M. tuberculosis*-stimulated cells.

(Fig. 2). The results indicate that in spite of their lack of L-selectin expression, these cells were in fact resting cells, based on their size and granularity. This does not, however, exclude the possibility that they are memory cells that have already reacted to antigen.

Shedding of L-selectin from γδ T cells following antigenic stimulation and re-expression on removal of antigen

Peripheral blood T cells were stimulated with a non-proteinaceous preparation of *M. tuberculosis* low molecular weight molecules. This material is known to selectively stimulate γδ T cells.[16,17] Cell proliferation was assessed by flow cytometry analysis which revealed an increase in the percentage of $\gamma 9^+$ T cells in response to mycobacterial antigens when compared with cultures containing RPMI-1640 alone (Figs 3a and b). Forward-scatter and side-scatter analysis on the $\gamma 9^+$ T-cell population confirm that the cells are larger and more granular, consistent with cells in proliferation (Figs 3c, d, e and f).

Double staining for γδ TCR and L-selectin revealed that most of the antigen-primed cells were L-selectin negative (Fig. 4a). Following 8 days of culture with *M. tuberculosis*, the cells were washed twice, resuspended in RPMI-1640 and cultured for 24 hr. Figure 4(b) shows there was re-expression of L-selectin.

To rule out the possibility of selective proliferation of the L-selectin-negative population, L-selectin-positive cells were sorted, stimulated with mycobacterial antigens, and analysed for L-selectin expression at the end of the culture period, and 24 hr after removal of antigen. The results (Fig. 5), show a loss of L-selectin expression during culture with antigen, which is regained after removal of antigen. The interpretation that L-selectin was shed during stimulation is further supported by the finding of soluble L-selectin in the supernatant of cultures stimulated with mycobacterial antigens, but not in the supernatant of cells cultured in RPMI-1640 alone (Fig. 4c).

Modulation of L-selectin on cells in pleural fluid

Cells from the pleural fluid of a patient with tuberculosis were stained for γδ TCR and for L-selectin. Most of the cells, along with those stained positive for the γδ TCR, were L-selectin negative (Fig. 6a). However, after incubation overnight in

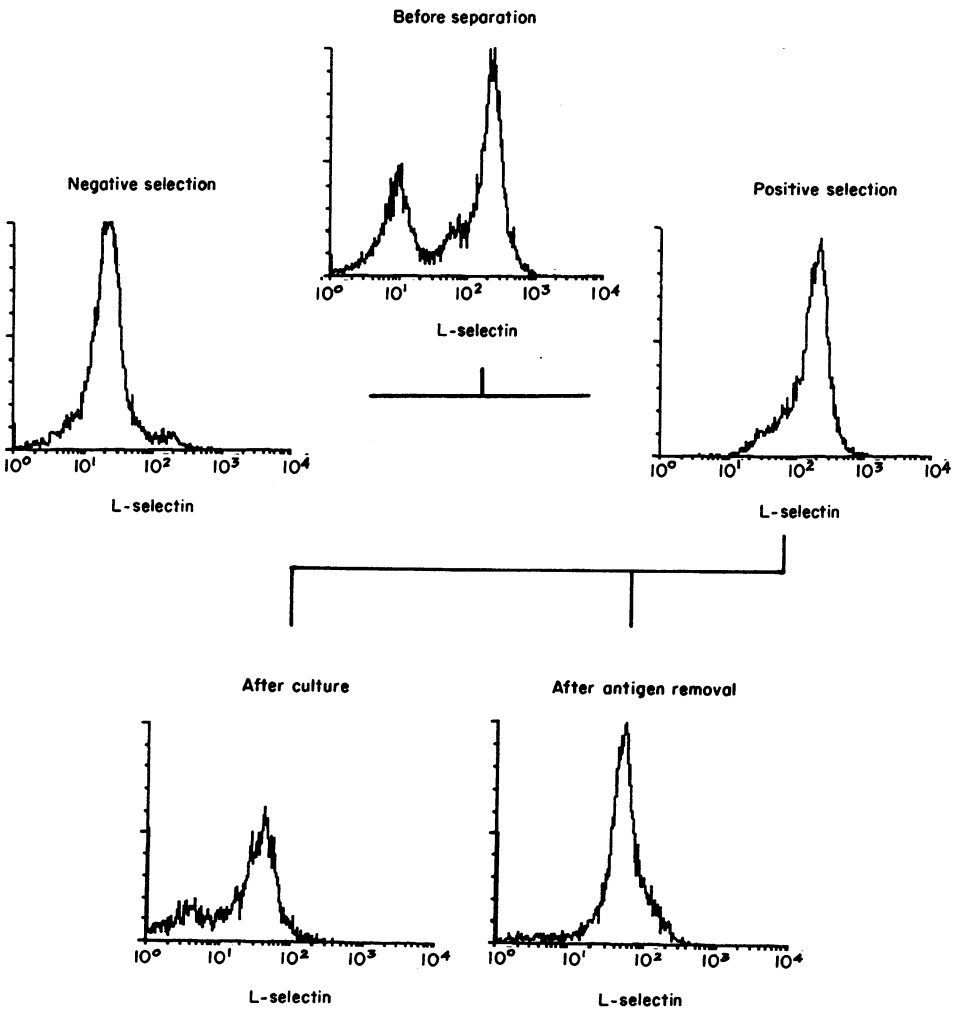

Figure 5. Shedding and re-expression of L-selectin from L-selectin-positive selected cells. L-selectin-positive cells were magnetically sorted and stimulated with mycobacterial antigens for 8 days. The figure shows a decrease in L-selectin expression immediately after culture, with a return to prestimulation levels of expression after removal of antigen.

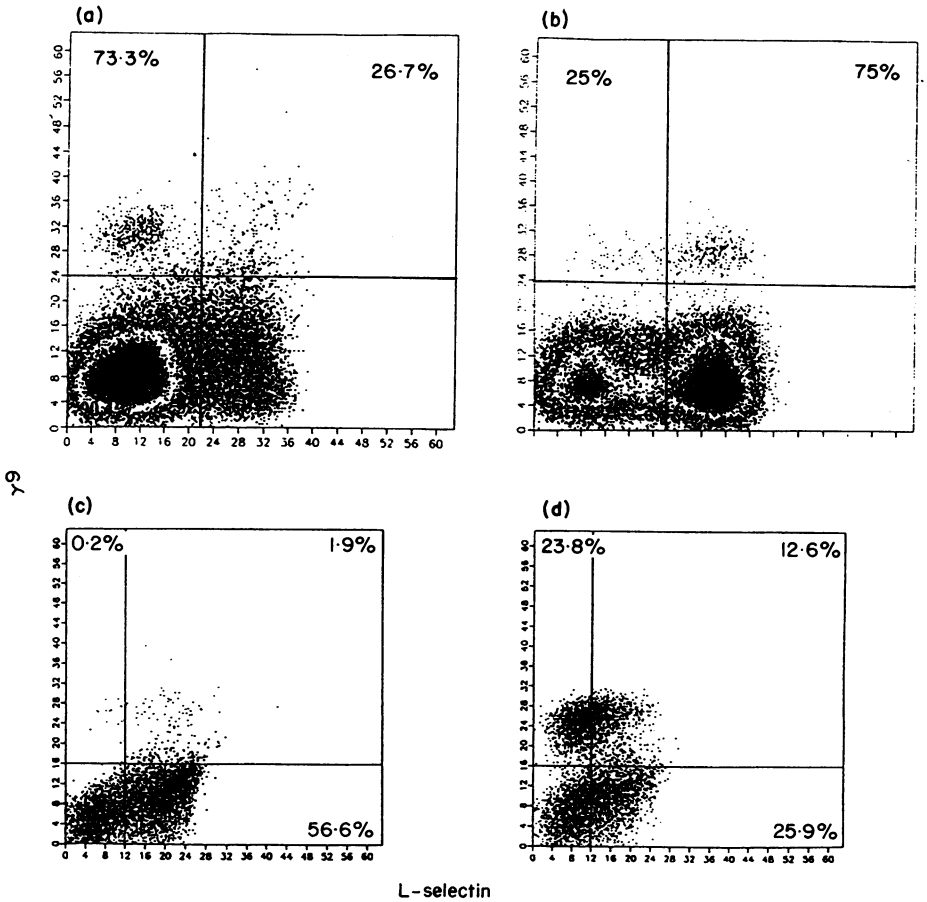

Figure 6. Low levels of L-selectin expression on T cells from the pleural fluid of a tuberculosis patient. The majority of cells, including TCR γ9-expressing cells, were L-selectin negative (a). After washing and incubation overnight in RPMI-1640, the majority of cells became L-selectin positive (b). Panels (c) and (d) show cells from the pleural fluid cultured for 8 days in RPMI-1640 alone or in the presence of *M. tuberculosis* antigens, respectively.

RPMI-1640 medium, the majority of cells, including γδ T cells, had re-acquired L-selectin expression (Fig. 6b). For analysis purposes the percentage of L-selectin-positive and -negative cells takes into account only the $\gamma 9^+$ T cells. Cells from the pleural fluid were also cultured in the absence (Fig. 6c) or in the presence (Fig. 6d) of *M. tuberculosis* antigens. An increase in the percentage of $\gamma 9^+$ T cells following antigen stimulation was observed; as in the case of the PBMC from healthy individuals most of the γδ T cells shed L-selectin.

DISCUSSION

In this study we show that expression of L-selectin on the surface of γδ T cells from freshly isolated peripheral blood defines two subpopulations, one L-selectin negative and the other L-selectin positive, and that this molecule is shed *in vitro* following antigenic stimulation. The antigen used was a preparation of low molecular weight molecules from *M. tuberculosis* that has been shown to selectively stimulate the proliferation of human γδ T cells.[16,17] As it has been shown that down-regulation of L-selectin follows cell activation[15] and that $\gamma 9^+$ T cells are expanded extrathymically as a result of antigen stimulation by environmental mico-organisms[18,19] it seemed likely that the L-selectin-negative subpopulation of γδ T cells were in a state of activation as a result of continuous contact with such environmental micro-organisms. However, forward-scatter and side-scatter analysis performed on the L-selectin negative subpopulation from fresh peripheral blood suggested that they were in a resting state. It is possible that these are memory cells that have already reacted to antigen. Alternatively an activation-independent mechanism might be responsible for the lack of L-selectin in some γδ cells; such a mechanism, involving cross-linking of L-selectin, has been proposed.[20] Interestingly bovine γδ T cells express high levels of L-selectin but few are capable of homing to the lymph nodes presumably because of inefficient shedding of L-selectin;[21] γδ T cells accumulate along the vascular wall of venules that support lymphocyte extravasation into peripheral lymph nodes but they do not extravasate from the blood into the parenchyma of the lymph nodes.

Although the regulation of L-selectin expression following activation has been extensively studied, few such studies have investigated the antigenic-driven regulation of this molecule. The demonstration in the present work that surface L-selectin is shed from γδ T cells following stimulation with antigen *in vitro* is compatible with the finding that L-selectin shedding follows cell activation[15,22] and that there is a differential regulation on T cells during the virgin-to-memory cell transition; most virgin

T cells (CD45RA high/RO low) are L-selectin positive, while among memory T cells (CD45RA low/RO high) there are subpopulations of positive and negative cells.[23] This is further supported by preliminary results from our laboratory which suggest that bacillus Calmette–Guérin (BCG) vaccination leads to shedding of L-selectin from the surface of γδ T cells as well as from other lymphocytes. It should be noted however, that not all γδ T cells became L-selectin negative on antigen stimulation.

Cells in different lymphoid compartments differ in their ability to regulate L-selectin expression. Wallace *et al.* have found that spleens contain an L-selectin-negative population which does not up-regulate L-selectin expression,[24] whereas human lymph node and tonsil L-selectin-negative cells re-express L-selectin when cultured *in vitro,* suggesting that the micro-environment may play a role in maintaining the down-regulation. Picker *et al.* have found that L-selectin is preferentially down-regulated in mucosal lymphoid tissue and retained in peripheral lymph nodes,[23] supporting the view that the local micro-environment influences L-selectin expression and contributes to the formation of memory cells with tissue-selective homing behaviour. Galea *et al.* have shown that among CD4-negative T cells, γδ T cells exhibit the greatest capacity to migrate through endothelial cells.[25] The demonstration that the pleural effusion of a tuberculosis patient contains L-selectin-negative γδ T cells that reaquire expression of L-selectin when suspended overnight in medium also supports the view that the presence of antigen can contribute to the local environment which controls L-selectin expression. Thus the results presented here demonstrate that the homing and recirculation potential of γδ T cells, as assessed by expression of L-selectin, can be regulated by activation-independent mechanisms, by antigenic stimulation, and by factors present in their micro-environment. If γδ T cells play a role in controlling infections they need to be present at the sites of infection; these results suggest how this migration and homing could be regulated.

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