Analysis of T cell subsets and β chemokines in patients with pulmonary sarcoidosis

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Abstract

Background – Sarcoidosis is a systemic granulomatous disorder of unknown origin characterised by accumulation of T lymphocytes and macrophages in multiple organs. Several cytokines and adhesion molecules may contribute to the accumulation of T lymphocytes in pulmonary sarcoidosis. The distribution of T lymphocyte subsets, T cell bearing CD11a and β chemokines such as regulated on activation normal T expressed and secreted (RANTES), macrophage inflammatory peptide 1α (MIP-1α), and macrophage chemoattractant protein 1 (MCP-1) in bronchoalveolar lavage (BAL) fluid and peripheral blood were compared in untreated patients with sarcoidosis and normal subjects.

Methods – Flow cytometric analysis with monoclonal antibodies to cell surface antigens was used to identify T lymphocyte subsets in the BAL fluid of untreated patients with sarcoidosis (n = 40) – either without (group A, n = 12) or with (group B, n = 28) radiological evidence of pulmonary involvement – and in 22 normal subjects. The level of different β chemokines was estimated by enzyme linked immunosorbent assay (ELISA).

Results – A high percentage of CD3+ cells, CD4+ cells expressing HLA-DR antigen, and a high CD4/CD8 ratio were detected in the BAL fluid of patients compared with normal subjects. In particular, CD4+ CD29+ memory T cells were significantly increased in patients with sarcoidosis. Furthermore, these cells were higher in those in group B than group A. The level of RANTES in the BAL fluid of patients was significantly higher than in normal subjects and correlated well with the percentage, number, and expression of CD29 on CD4 cells. The expression of CD11a (α chain of lymphocyte function associated antigen-1, LFA-1) on CD3+ cells in the BAL fluid of patients with sarcoidosis was not different from that of normal subjects. However, the expression of CD11a on CD3+ cells in the BAL fluid of patients in group A was significantly lower than that of patients in group B and normal subjects.

Conclusions – These results suggest a possible interaction between activated memory T cells bearing CD11a and RANTES which may contribute to the pulmonary involvement in patients with sarcoidosis.

Keywords: adhesion molecules, β chemokines, pulmonary sarcoidosis.

Sarcoidosis is a chronic systemic disorder characterised by the presence of non-caseating granulomas and accumulation of T lymphocytes and macrophages in multiple organs. T lymphocytes, alveolar macrophages, and several cytokines are thought to play an important role in sarcoidosis, although the aetiology of the disease is still unknown. Recent studies using monoclonal antibodies to cell surface antigens have demonstrated the presence of subsets of T cells and expression of cell adhesion molecules on peripheral blood lymphocytes such as CD11a/CD18 (lymphocyte function associated antigen 1, LFA-1) in sarcoidosis. Furthermore, analysis of bronchoalveolar lavage (BAL) fluid in patients with pulmonary sarcoidosis has also shown the presence of activated CD4+ and CD8+ T lymphocytes expressing HLA-DR antigen, increased alveolar macrophages, and predominance of CD29+ memory T cells.

Leucocyte adhesion molecules such as LFA-1 of the β2 family are involved in leucocyte-endothelial adherence in the lung, and the mechanism of T cell migration into the lung is also initially dependent on the presence of adhesion molecules of both endothelial cells and leucocytes. The β2 family shares a common β chain (CD18) which is combined with three different α chains (LFA-1, CD11a; Mac-1, CD11b; p150/95, CD11c), and intercellular adhesion molecule 1 (ICAM-1) has been identified as a ligand for these molecules. Several studies have shown that the expressions of adhesion molecules and their ligand are increased in chronic inflammatory diseases. In addition, β chemokines such as macrophage chemoattractant protein 1 (MCP-1), regulated on activation normal T expressed and secreted (RANTES), and macrophage inflammatory peptide 1α (MIP-1α) are closely related to the expression of adhesion molecules and the migration of inflammatory cells into the lung. MCP-1 is chemoattractant to monocytes and the activating factor produced and secreted by fibroblasts, type 2 epithelial cells, and macrophages. RANTES is a selective chemotactic factor for memory T cells and recent reports...
have described the role of MIP-1α in attracting T cells.16

In the present study we investigated the different types of T lymphocytes and the expression of adhesion molecules in peripheral blood and BAL fluid in patients with sarcoidosis. The presence of chemokines suggested that RANTES may contribute to the accumulation of T lymphocytes in the lungs of these patients.

Methods

STUDY POPULATION

T cell subsets were studied from 40 untreated patients with sarcoidosis (26 women) of mean (SD) age 43 (17) years and 22 normal subjects (five women) of mean (SD) age 27 (11) years. At the time of the study six patients and five volunteers were smokers. The normal subjects had no history of pulmonary diseases. The diagnosis of pulmonary sarcoidosis was based on histological examination of biopsy samples from the lungs. We also examined tissue samples from lymph nodes, skin, or liver. The presence of non-caseating epithelioid cell granulomas and typical clinical, radiological, and biochemical findings were used to evaluate the activity of the disease process. Patients were divided according to the chest radiological findings into two groups. Group A consisted of 12 patients who had normal chest radiographs (stage 0), 12 uveitis and one skin. Group B consisted of 28 patients of which 19 were stage I with bilateral hilar lymphadenopathy (BHL) but clear lung fields, seven were stage II with BHL and parenchymal infiltration, and two were stage III with parenchymal infiltration without BHL. The results of pulmonary function tests in patients in groups A and B were as follows: mean (SD) vital capacity 106.8 (5.2)% predicted and 113.2 (17.8)% predicted, forced expiratory volume in one second/forced vital capacity 89.4 (7.5)% predicted and 80.8 (8.3)% predicted, respectively, but there were no significant differences between the two groups. Patients in group A had no symptoms and three patients in group B had cough. Human T-lymphotrophic virus type 1 (HTLV-1) seropositive patients were excluded from the study since HTLV-1 carriers are thought to have raised T cell activation markers.17 All patients gave their informed consent.

BRONCHOALVEOLAR LAVAGE AND CELL PREPARATION

Bronchoalveolar lavage was performed using a standard technique. The patients were premedicated intramuscularly with atropine (0.5 mg). After local anaesthesia with 4% lignocaine a flexible fiberoptic bronchoscope (BF-P20, Olympus, Tokyo) was wedged into a subsegment of the right middle lobe for lavage. An aliquot of 50 ml sterile physiological saline solution at body temperature was instilled through the bronchoscope and the fluid was immediately retrieved by gentle suction using a sterile syringe. Saline instillation was performed four times. The lavage fluid was passed through two sheets of gauze and centrifuged at 40 g for 10 minutes at 4°C and the supernatant was stored at −80°C until used. After washing twice with phosphate buffered saline solution (PBS) cells were suspended with 10% heat inactivated fetal calf serum (FCS) and counted using a haemocytometer. An aliquot was then adjusted to 2 × 10⁵ cells/ml and a 0.2 ml sample of each cell suspension was spun down onto a glass slide at 160 g for two minutes using a cytocentrifuge (Cytospin 2, Shandon Instruments, Pennsylvania, USA). The slides were later dried, fixed, and then stained using the May-Giemsa method. Two hundred cells were identified under the photomicroscope. The remaining cells were resuspended in PBS, supplemented with 10% fetal calf serum, and incubated in plastic flasks for 90 minutes at 37°C in humidified 5% carbon dioxide/air for depletion of alveolar macrophages. The cells were then centrifuged at 500 g for five minutes at 4°C, the supernatant was discarded, and the cells were resuspended in PBS. The cells were washed twice in PBS and passed through a 100 µm nylon mesh, and finally adjusted to a concentration of 1 × 10⁶/ml. Viable cells constituted more than 90% of non-adherent cells which were collected for flow cytometric analysis using the trypsin blue exclusion test.

MONOCLONAL ANTIBODIES

The monoclonal antibodies shown in table 1 were used. Fluorescein isothiocyanate (FITC) conjugated CD4, CD8, anti-human lymphocyte antigen-DR (HLA-DR), CD11a antibodies, and phycoerythrin (PE) conjugated anti-CD3, CD4 and CD8 antibodies were purchased from Becton Dickinson (Mountain View, California, USA) and FITC conjugated anti-CD45RA and CD29 antibodies were purchased from Coulter Immunology (Hialeah, Florida, USA). Mouse IgG1 conjugated with FITC or PE was purchased from Becton Dickinson and used to determine the borderline between stained and unstained cells in flow cytometric analysis.

TWO-COLOUR DIRECT IMMUNOFLUORESCENCE STAINING

One hundred µl of whole blood taken from the 40 patients with sarcoidosis and the 22 controls by venipuncture with ethylenediaminetetraacetic acid (EDTA) was placed into a 12 × 5 mm polystyrene tube (Falcon Plastics, Oxnard, California, USA) and 5 µl of each

| Table 1 Monoclonal antibodies used in direct immunofluorescence analysis |
|-----------------------------|--------------------------|--------------------------|
| Antigen cluster | Monoclonal antibody | Expression on lymphocytes |
| CD3 | Leu4 | T cells |
| CD4 | Leu3a | Helper T cells |
| CD8 | Leu2a | Suppressor/cytotoxic T cells |
| CD11a | LFA-1α | α chain of the LFA-1 complex |
| CD29 | B4 | β chain of β integrin |
| CD45RA | 2H4 | Naive T cells |
| CD29 | HLA-DR | Memory T cells |

LFA-1 = leucocyte function associated antigen 1.
monoclonal antibody was added. The tubes were incubated for 15 minutes at room temperature in darkness and 2 ml of 1 × FACS lysing solution (Becton Dickinson) was added to destroy red blood cells. The cells were mixed vigorously, incubated for 10 minutes at room temperature, and then washed once in cold PBS containing 0.1% sodium azide. The cells were finally resuspended in cold PBS containing 0.5% paraformaldehyde.

The concentration of cells in the BAL fluid was adjusted to 1 × 10^6/ml. A total of 5 µl of each monoclonal antibody was placed into a polystyrene tube and 100 µl of the cell suspension (1 × 10^6 cells) was added. The cells were incubated for 30 minutes on ice in the dark, washed once in cold PBS containing 0.1% sodium azide, and then resuspended in cold PBS containing 0.5% paraformaldehyde. The fixed cells were kept in darkness at 4°C until analysis.

TWO-COLOUR FLOW CYTOMETRY

Stained cells were analysed on a flow cytometer equipped with an argon ion laser turned to 488 nm (FACScan, Becton Dickinson, FACS Division), and a computer system ( Consort 30, Becton Dickinson) was used for data acquisition and analysis. A minimum of 10 000 events was collected for each sample. A cell gate containing lymphocytes was established on the basis of forward and side light scatter. Flow cytometry is not an ideal technique because of measurements based on assumptions. However, the purity of the lymphocytes was confirmed as CD3+ cells more than 90% using CD3 antibodies. To determine the borderline between stained and unstained cells, the cells were also stained with mouse IgG1 conjugated FITC or PE. The FITC and PE fluorochromes were excited at the same wavelength (488 nm) with an argon laser beam. The FITC and PE fluorescence was collected using, respectively, 530 and 573 nm bandpass interference filters and dichroic mirrors. Electronic compensation was used to prevent contamination by FITC fluorescence in the PE channel. The percentages were calculated based on the number of lymphocytes found in each quadrant. The intensities of CD11a and CD29 were expressed as the mean fluorescence intensity (MFI) in an arbitrary number of mean fluorescence. Interassay reproducibility was checked using beads (CalibRITE, Becton Dickinson) and a software program (AutoCOMP, Becton Dickinson).

MEASUREMENT OF MCP-1, RANTES AND MIP-1α

Sufficient quantity of BAL fluid was available in 12 patients and nine normal subjects for estimating the level of β chemokines after concentrating the supernatant with Centriprep-3 (Amicon a GRACE Company, Beverly, Massachusetts, USA) which is used to concentrate low molecular weight components with a cut off molecular weight of 3000 daltons. In this concentration procedure the recovery of each β family chemokine was greater than 90%, and the magnification of concentration was calculated by the ratio of protein consistency in unconcentrated to concentrated BAL fluid supernatant, which was measured using the DC protein assay kit (BIO RAD), and the original level of the chemokines was corrected by this ratio. The level of MCP-1, RANTES and MIP-1α was quantified using enzyme linked immunosorbent assay kits (Quantikine, R&D Systems, Minneapolis, Minnesota, USA). As bronchoalveolar lavage has a dilutional effect on the recovery of cytokines, measurements are occasionally standardised to albumin or urea. There was a good correlation between the non-standardised and standardised values by albumin concentration. The detection limits were 5.0, 2.5 and 2.0 pg/ml for MCP-1, RANTES and MIP-1α, respectively. Cross reactions with other cytokines were not observed.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Volume recovered (%)</th>
<th>Total cells (× 10^6/ml)</th>
<th>Macrophages (%)</th>
<th>Lymphocytes (%)</th>
<th>No of lymphocytes (× 10^6/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects (n=22)</td>
<td>63.7 (45.3–76.7)</td>
<td>1.3 (0.4–4.3)</td>
<td>88.0 (69.0–95.8)</td>
<td>8.6 (2.6–30.0)</td>
<td>0.1 (0.02–0.4)</td>
</tr>
<tr>
<td>Sarcoidosis (n=40)</td>
<td>58.7 (24.3–76.7)</td>
<td>2.3 (0.7–9.2)†</td>
<td>63.1 (23.3–94.3)†</td>
<td>34.7 (5.5–75.8)†</td>
<td>0.7 (0.1–5.9)†</td>
</tr>
</tbody>
</table>

†p<0.001 compared with normal subjects (Mann-Whitney U test); differences remained significant after Bonferroni correction.

STATISTICAL ANALYSIS

All values were expressed as median (range). The Mann-Whitney U test was used to compare differences between unpaired samples and the Wilcoxon signed rank test was used to compare differences between paired samples. Where necessary, the results of the analysis were further corrected using Bonferroni’s method, and Spearman’s rank correlation was used to examine the relationship between various parameters. Statistical analysis was performed using the Statview-J 4.02 software package. p values of <0.05 were considered significant.

Results

DIFFERENTIAL CELL COUNT OF BAL FLUID

As shown in table 2, the volume of BAL fluid did not differ between patients with sarcoidosis and normal subjects. However, the patients had a significantly higher number of cells, percentage and number of lymphocytes, and a significantly lower percentage of alveolar macrophages than normal subjects. The absolute number of alveolar macrophages in patients with sarcoidosis was not different from the normal subjects (data not shown). In ad-
The number of lymphocytes was significantly higher in patients in group B than in those in group A (group A: 0.3 (0.1–2.3), group B: 1.0 (0.3–5.9), p<0.01). There were no differences in percentages of neutrophils, eosinophils, and basophils between the patients and normal subjects (patients: 1.3%, 0.2%, and 0%; normal subjects: 2.1%, 0.8% and 0%, respectively).

LYMHCY SUBSETS IN PERIPHERAL BLOOD AND BAL FLUID

The percentages of CD3+, CD3+HLA-DR+, CD4+, CD4+CD45RA+, CD4+CD29+, CD8+, CD8+HLA-DR+, CD4/CD8 ratio in peripheral blood were similar in patients and normal subjects (data not shown). Only the percentage of CD4+HLA-DR+ cells in peripheral blood was significantly higher in patients than in normal subjects (patients: 5.9 (0.5–26.4), normal subjects: 2.5 (1.3–3.5), p<0.05). In BAL fluid the percentages of CD3+HLA-DR+, CD4+, and CD4+HLA-DR+ cells and the CD4/CD8 ratio were significantly higher in patients than in normal subjects (table 3). In patients with sarcoidosis the percentages of CD3+, CD3+HLA-DR+, CD4+, and CD4+HLA-DR+ cells were significantly higher in BAL fluid than in peripheral blood (fig 1). Phenotypic analysis of CD4+ cells in the BAL fluid of patients showed a significantly higher percentage of CD4+CD29+ cells as memory T cells than in normal subjects, while the percentage of CD4+CD45RA+ cells as naive T cells was similar in patients and normal subjects (table 3). In addition, the percentage of CD4+CD29+ cells was significantly higher in patients in group B than in those in group A (group A: 45.2 (26.7–64.2); group B: 67.5 (41.9–79.2), p<0.05). A higher percentage of CD4+CD29+ cells was present in BAL fluid (50.7 (26.7–79.2)) than in peripheral fluid (22.0 (9.6–45.9), p<0.0001) of patients with sarcoidosis.

Table 3 Proportion of T cell subsets in bronchoalveolar lavage fluid. Values are median (range)

<table>
<thead>
<tr>
<th></th>
<th>CD3+ (%)</th>
<th>CD3+HLA-DR+ (%)</th>
<th>CD4+ (%)</th>
<th>CD4+HLA-DR+ (%)</th>
<th>CD4+CD45RA+ (%)</th>
<th>CD4+CD29+ (%)</th>
<th>CD8+ (%)</th>
<th>CD8+HLA-DR+ (%)</th>
<th>CD4/CD8 ratio</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>82 (56.0–90.5)</td>
<td>35.9 (6.7–48.3)</td>
<td>35.1 (17.4–58.8)</td>
<td>18.8 (3.8–33.9)</td>
<td>1.3 (0.3–2.8)</td>
<td>33.7 (17.9–48.8)</td>
<td>32.8 (19.2–74.4)</td>
<td>13.5 (1.2–30.3)</td>
<td>1.0 (0.2–2.2)</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>88.7 (58.5–97.2)</td>
<td>63.2 (21.8–86.7)</td>
<td>60.4 (30.0–95.3)</td>
<td>44.9 (5.4–76.6)</td>
<td>2.6 (0.2–13.6)</td>
<td>50.7 (26.7–79.2)</td>
<td>23.2 (4.9–49.1)</td>
<td>11.9 (3.1–28.7)</td>
<td>2.5 (0.7–19.4)</td>
</tr>
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</table>

* p<0.001 compared with normal subjects (Mann-Whitney U test); differences remained significant after Bonferroni correction.

** BAL FLUID LEVELS OF MCP-1, RANTES AND MIP-1α**

Figure 2 shows the levels of β chemokines in BAL fluid obtained from patients with sarcoidosis and normal subjects. The level of RANTES in patients with sarcoidosis (5.9 (0.6–55.1) pg/ml) was significantly higher than in normal subjects (1.2 (0.7–7.4) pg/ml, p<0.01), while the level of MIP-1α in the patients (2.1 (0.2–4.4) pg/ml) did not differ from that in normal subjects (1.9 (0.2–5.8) pg/ml). Although the level of MCP-1 was also higher in patients with sarcoidosis (28.9 (1.3–57.5) pg/ml) than in normal subjects (15.7 (2.2–7.4) pg/ml), the difference was not statistically significant.
T cell subsets and β chemokines in sarcoidosis

Discussion

This study has confirmed our earlier findings and those of other investigators that the percentages of lymphocytes, CD3+HLA-DR+ and CD4+HLA-DR+ cells, representing activated T cells, and CD4/CD8 ratio in the BAL fluid of patients with sarcoidosis are significantly higher than in normal subjects. The results also show that these activated T cells are higher in BAL fluid than in the peripheral blood of these patients. In addition, a high percentage of peripheral CD4+HLA-DR+ cells was detected in patients, indicating that CD4+ cells are also activated even in the circulation, although migration of a proportion of lung CD4+HLA-DR+ cells into the circulation may explain this finding.

CD4+ cells are classified into two major phenotypically and functionally distinct subsets – CD4+CD45RA+ naive cells and CD4+CD29+ memory cells. A higher than normal percentage of CD4+CD29+ cells was seen in the BAL fluid of patients with sarcoidosis which was also higher than in the peripheral blood of these patients. In addition, more memory T cells were detected in the BAL fluid of patients with radiological evidence of pulmonary involvement than in those without.

In contrast, sarcoidosis did not influence the number of CD4+CD45RA+ cells in the BAL fluid and these naive T cells represented only a small proportion of the CD4+ cells in the BAL fluid. These results are in agreement with those reported previously by others and indicate that accumulation of activated CD4+CD29+ memory T cells at the site of inflammation may be primarily involved in the pathogenesis of pulmonary sarcoidosis.

The attachment of T cells to endothelial cells is one of the first critical events in lymphocyte migration to inflammatory sites. CD11a/CD18 (α/β chain of LFA-1) is expressed on human T and B lymphocytes, NK cells, monocytes, and macrophages, and plays an important role in the firm adhesion of these cells to ICAM-1 on endothelial cells followed by migration into the affected tissue. Shakoor and Hambelin reported that the expression of CD11a on peripheral lymphocytes was similar in Caucasian patients with sarcoidosis and normal individuals of the same race, while a significant difference in the cells of both compartments was noted in Afro-Caribbeans. Furthermore, analysis of CD18 expression on peripheral lymphocytes showed a significant difference between patients with sarcoidosis and normal subjects of both races. However, these workers did not perform BAL fluid analysis. Our study has shown that, although there was no significant difference in CD11a expression on lymphocytes in the BAL fluid of patients with sarcoidosis and normal subjects, patients without radiological evidence of thoracic involvement had less expression of CD11a in BAL fluid than those with radiological involvement and healthy subjects. This finding may be explained by the low level of lymphocytes, CD4+HLA-DR+ and CD4+CD29+ cells, as detected in the BAL fluid of the former patients, since it has been reported...
that conversion of T cells from naive to memory cells increases the level of expression of LFA-1. It is also possible that lymphocytes, especially activated memory T cells bearing highly expressed CD11a adhesion molecules, migrate into organs other than the lung in patients with sarcoidosis, resulting in diminished levels of adhesion molecules in patients without thoracic involvement.

Cytokines released locally from inflamed tissue into the blood stream may upregulate adhesion molecules, and consequently, the migration of leucocytes into the inflammatory region. In this context, increased plasma or serum levels of tumor necrosis factor α (TNF-α) or interleukin 1β (IL-1β) released by alveolar macrophages has been reported in patients with sarcoidosis. MCP-1, produced or secreted by fibroblasts, type II epithelial cells, and macrophages, is a chemoattractant for monocytes and macrophages and regulates a number of adhesion molecules and the expression of cytokines. Car et al described high levels of MCP-1 in the BAL fluid of patients with pulmonary sarcoidosis. However, our results showed that the level of MCP-1 in patients with sarcoidosis was not different from that in normal subjects, which suggests that MCP-1 is probably not involved directly in the pathogenesis of pulmonary sarcoidosis.

On the other hand, MIP-1α exhibits a wide variety of biological activities such as activation of monocytes or basophils and chemotaxis of macrophages or neutrophils, and recent studies by Denis described it as a chemoattractant to CD8+ T cells. Increased BAL fluid levels of MIP-1α and its expression on alveolar macrophages within the airspace and interstitium have been reported in patients with sarcoidosis. However, the levels of MIP-1α in the BAL fluid of our patients were not raised. This difference may be attributed to different patient populations since the patients in our study were all Japanese and were at a stage early in the course of the disease. Furthermore, the prognosis of sarcoidosis appears to be clinically more favourable in Japanese patients than in other populations. Our study has provided the first evidence that the level of another β chemokine, RANTES, is increased in the BAL fluid of patients with sarcoidosis. In addition, a significant correlation was found between the level of RANTES and the percentage or number of CD4+CD29+ cells. RANTES is primarily a T cell product and selectively attracts memory T cells in vitro. Gilat et al recently reported that RANTES induced CD4+ T cell adhesion to extracellular matrix and that such adhesion was partially inhibited by a monoclonal antibody specific for CD29, the common β1 chain of VLA receptors. In this context, our study has also shown that the concentration of RANTES correlated well with the expression of CD29 antigen on CD4+ cells, and the mean concentration of RANTES was similar to that reported to induce T cell binding to endothelial cells. Considered together, our results suggest that RANTES, rather than MCP-1 and MIP-1α, may play an important role in thoracic involvement in patients with sarcoidosis through the recruitment or activation of memory T cells.

We demonstrated high levels of activated memory T cells bearing CD11a and high RANTES concentrations in the lungs of patients with pulmonary sarcoidosis. These results suggest that the interaction between these inflammatory cells and RANTES may contribute to thoracic involvement in patients with sarcoidosis. Since we could not define the direct effect of RANTES on T cells in the BAL fluid of these patients, further studies are required to define the role of memory T cells and RANTES in sarcoidosis.


22 Sanders ME, Makgoba MW, Shallow SO, Stephany D, Springer TA, Young HA, et al. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UHCL1, CDw29, and gp-1) and have enhanced IFN-γ production. J Immunol 1988;140:1401–7.


