

INTERSTITIAL LUNG DISEASE

Involvement of dendritic cells in sarcoidosis

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Background: Sarcoidosis is a systemic granulomatous disease of unknown aetiology. It has been suggested that T helper type 1 (Th1) polarisation is associated with the pathophysiology of sarcoidosis, but the mechanism of skewing towards Th1 has not been elucidated. Dendritic cells (DCs) are known to regulate immune responses. This study was performed to determine whether DCs are involved in the aetiology of sarcoidosis.

Methods: The numbers of peripheral blood DCs in 24 patients with sarcoidosis were analysed and biopsy specimens from four patients were stained immunohistochemically using monoclonal antibodies.

Results: The numbers of both myeloid and lymphoid DC subsets were significantly decreased in the blood and mature DCs were found in the granulomas of patients with sarcoidosis. A number of interferon- γ (IFN- γ) producing T cells were also detected in the sarcoid granuloma, as well as many interleukin (IL)-4 producing T cells. Double staining of the biopsy specimen using anti-fascin and anti-CD3 antibodies showed an anatomical interaction between DCs and T cells.

Conclusions: These findings suggest that the blood DC subsets may migrate into the affected tissues, contributing to the formation of the granulomas in sarcoidosis. It is hypothesised that the migrating DCs may regulate the T cell response in sarcoidosis, at least in the granulomatous lesions.

Sarcoidosis is a systemic granulomatous disease, the aetiology of which remains unknown. In sarcoidosis the granulomas are organised structures composed of epithelioid cells, fibroblasts, and several immune cells such as T cells and histiocytes/macrophages. It has been shown that the granulomatous inflammation of sarcoidosis is characterised by the local T helper 1 (Th1) balance.¹ However, it is unclear how this dominant Th1 response is induced in the process of granuloma formation.

In the immune system, dendritic cells (DCs) function as specialised antigen-presenting cells (APCs) and play a central role in the initiation and regulation of T cell responses.^{2–3} Two major subsets of DC precursors have been identified in human peripheral blood⁴: the CD11c+ subset belongs to the myeloid lineage, while the CD11c– plasmacytoid subset (referred to as interferon (IFN)- α/β producing cells^{4–7}) is of lymphoid lineage. Both DC subsets may migrate to tissues of various organs including lymphoid tissue, and they are therefore regarded as a circulating pool for the tissue DCs.^{8–9}

Recent research on DC biology has suggested that the DC subsets regulate different Th responses. The CD11c+ myeloid subset has a primary capacity to polarise naive CD4+ T cells towards IFN- γ producing Th1 cells depending on interleukin (IL)-12 production, while the CD11c– subset preferentially induces IL-4 producing Th2 cells upon exposure to IL-3.^{10–11} Evidence is emerging to suggest that these DCs play an important role in various disease conditions. Indeed, we have recently reported that, in primary Sjögren's syndrome, the CD11c+ DC subset is decreased due to selective trafficking from peripheral blood to the salivary glands, which suggests that DCs make an essential contribution in the pathophysiology of Sjögren's syndrome.¹² In addition, recruitment of blood DCs to granulomatous lesions occurs in pulmonary tuberculosis, suggesting that DCs have a role in this condition.¹³ These findings have prompted us to examine whether DCs are also involved in sarcoidosis, another chronic inflammatory disease.

METHODS

Patients

Twenty four patients with sarcoidosis (eight men and 16 women) who presented to our hospital between May 2000 and February 2002 were enrolled in the study after providing informed consent. All patients were clinically and/or histologically diagnosed as having sarcoidosis. Their clinical stage was classified according to Wurm's radiological staging.^{14–15} Four patients were receiving treatment with corticosteroids at the time of the examination; the remaining 20 patients were not given corticosteroids during the study. The control group consisted of 24 age and sex matched volunteers. The characteristics of the patients and the control subjects are shown in table 1.

Media

The medium used in this study was RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin, and heat inactivated 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA, USA).

Enrichment and analyses of peripheral blood DCs

Peripheral blood DCs were enriched according to the protocol described previously.^{12–13} Briefly, the DC enriched population

Table 1 Characteristics of patients and controls

| Characteristics | Patients (n = 24) | Controls (n = 24) |
|----------------------------|-------------------|-------------------|
| M:F | 8:16 | 8:16 |
| Median (range) age (years) | | |
| Male | 50 (28–67) | 43 (21–74) |
| Female | 60 (35–81) | 42 (31–59) |
| Stage | | |
| 0 | 6 | |
| 1 | 10 | |
| 2 | 5 | |
| 3 | 3 | |

Table 2 Clinical and laboratory findings in patients with sarcoidosis

| Patient/age/sex (biopsied tissue) | Stage | Corticosteroid | WBC/ μ l | PBMC $\times 10^5$ /ml | Total DC/ml | CD11c+ cells/ml | CD11c- cells/ml |
|-----------------------------------|-------|----------------|--------------|------------------------|-------------|-----------------|-----------------|
| 1/50/M/skin (leg) | 1 | + | 8600 | 38.8 | 1549 | 849 | 700 |
| 2/60/F/skin (arm) | 1 | - | 4300 | 21.8 | 1288 | 996 | 292 |
| 3/55/F/lymph node (cervical) | 1 | - | 5600 | 17.9 | 1460 | 948 | 512 |
| 4/56/F/lymph node (cervical) | 2 | - | 4200 | 21.8 | 429 | 167 | 262 |
| 5/60/F | 2 | - | 5200 | 23.2 | 2312 | 1737 | 575 |
| 6/41/M | 3 | - | 4700 | 13.0 | 5135 | 3877 | 1258 |
| 7/67/M | 1 | - | 4000 | 20.4 | 5700 | 3757 | 1943 |
| 8/53/F | 0 | + | 9300 | 22.3 | 1052 | 345 | 707 |
| 9/35/F | 2 | + | 3900 | 22.5 | 701 | 411 | 290 |
| 10/66/M | 1 | + | 3400 | 66.7 | 1279 | 545 | 734 |
| 11/64/F | 0 | - | 5000 | 36.7 | 2256 | 1817 | 439 |
| 12/28/M | 0 | - | 5200 | 11.5 | 2701 | 1243 | 1458 |
| 13/57/F | 0 | - | 4400 | 17.0 | 5220 | 3696 | 1524 |
| 14/65/F | 1 | - | 3800 | 12.9 | 3491 | 1718 | 1773 |
| 15/64/F | 2 | - | 5300 | 28.7 | 5030 | 3869 | 1161 |
| 16/61/F | 1 | - | 6100 | 28.5 | 4460 | 3288 | 1172 |
| 17/32/M | 2 | - | 5200 | 17.4 | 6470 | 3358 | 3112 |
| 18/59/F | 3 | - | 4000 | 10.9 | 1041 | 826 | 215 |
| 19/31/M | 3 | - | 6600 | 4.6 | 706 | 75 | 631 |
| 20/54/F | 0 | - | 4800 | 8.0 | 4429 | 2436 | 1993 |
| 21/54/F | 1 | - | 5400 | 8.3 | 784 | 530 | 254 |
| 22/81/F | 1 | - | 6300 | 14.7 | 7360 | 5160 | 2200 |
| 23/68/F | 0 | - | 5200 | 49.9 | 2545 | 1057 | 1488 |
| 24/54/M | 1 | - | 5800 | 46.6 | 5432 | 2505 | 2927 |

WBC=white blood cells; PBMC=peripheral blood mononuclear cells; DC=dendritic cells.

was obtained from peripheral blood mononuclear cells (PBMCs) by negative immunoselection using anti-CD3 (HIT3a; PharMingen, USA), anti-CD14 (M5E2; PharMingen), and anti-CD19 (B43; PharMingen) monoclonal antibodies (mAbs). The DC enriched population (CD3-/CD14-/CD19- cells) was stained with phycoerythrin (PE) labelled anti-CD11c (Leu-5; Becton Dickinson, San Jose, CA, USA), a mixture of fluorescein isothiocyanate (FITC) labelled mAbs against lineage markers (lineage); CD3 (M2Ab; Exalpa, Boston, MA, USA), CD14 (UCHM1; Ancell, Bayport, MN, USA), CD15 (80H5; Immunotech, Marseilles, France), CD16 (3G8; PharMingen), CD19 (HIB19; PharMingen), CD56 (NCAM16.2; Becton Dickinson, France), and phycoerythrin cyanin 5.1 (PC5) labelled HLA-DR (IMMU357; Immunotech) and the cells were analysed by FACScan. The blood DC population was identified as the lineage-/HLA-DR+ fraction, which was further subdivided into a CD11c+ DC subset (CD11c+/lineage-/HLA-DR+ cells) and a CD11c- DC subset (CD11c-/lineage-/HLA-DR+ cells).

Absolute numbers (per ml) of peripheral blood DCs were calculated by multiplying the percentage of the lineage-/DR+ population in the DC enriched population by the count (per ml) of the DC enriched population. The absolute number (per ml) of each DC fraction was calculated by multiplying the percentage of each fraction within the lineage-/DR+ population by the total number of the lineage-/DR+ population.

Immunohistochemical staining

Biopsy specimens of lymph nodes from two patients with sarcoidosis (nos 6 and 18) and of skin (erythematous lesion) from two further patients (nos 1 and 7) were immediately fixed in 10% formalin solution, embedded in paraffin, and frozen. Histological examination was performed with haematoxylin and eosin staining and immunohistochemical staining using several mAbs. Anti-fascin mAb (55K-2; Dako), anti-HLA-DR mAb (LN-3; Nichirei, Tokyo, Japan), anti-IL-4 mAb (MAB304; RD, Minneapolis, MN, USA), anti-CD4 mAb (1F6; Nichirei), and anti-CD8 mAb (C8/144B; Nichirei) were used for paraffin treated samples, and anti-IFN- γ mAb (45.15; Ancell), anti-IFN- α mAb (Genzyme,

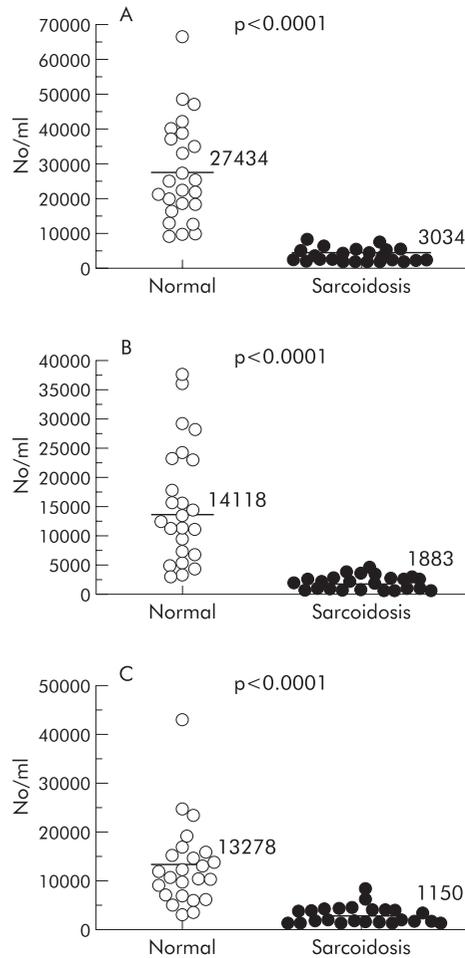


Figure 1 Mean number of (A) total blood dendritic cells (DCs), (B) CD11c+ cells and (C) CD11c- cells in patients with sarcoidosis. The numbers were significantly lower than in normal control subjects.

Cambridge, MA, USA), anti-DC-LAMP mAb (104.G4; Immunotech), and anti-IL-4 mAb were used for frozen samples. The avidin-biotin-peroxidase complex method using a labelled streptavidin-biotin kit (Dako, Carpinteria, CA, USA) with haematoxylin and diaminobenzidine was used for immunohistochemical staining.

In addition, immunohistochemical double staining was performed on the tissue sections using the alkaline phosphatase technique. Anti-fascin mAb and anti-CD3 mAb (08-0102; Zymed, San Francisco, CA, USA), anti-fascin mAb and anti-HLA-DR mAb, anti-DC-LAMP mAb and anti-fascin mAb, and anti-IL-4 mAb and anti-CD3 mAb were used as the primary and secondary antibodies, respectively.

Statistical analysis

The Mann-Whitney U test was used for statistical analysis with a StatView statistical program (Abacus Concepts, Berkley, CA). Differences were considered significant when *p* values were less than 0.05.

RESULTS

Numbers of blood DC subsets

The white blood cell (WBC) count was within the normal range (3000–8000 cells/ μ l) in 22 of the 24 patients (table 2). Two patients (nos 1 and 8) had a slightly increased WBC count. There was no significant difference in WBC count between the patients and the normal controls (data not shown). However, the total number of peripheral blood DCs was significantly decreased in patients with sarcoidosis compared with normal healthy volunteers (sarcoidosis: mean 3034 cells/ml (range 429–7360); controls: mean 27 434 cells/ml (range 9775–66 580); *p*<0.001, fig 1A). The numbers of CD11c+ and CD11c– DC subsets were also significantly

decreased in patients with sarcoidosis (CD11c+ DCs: sarcoidosis: mean 1883 cells/ml (range 75–5160); controls: mean 14 118 cells/ml (range 2426–36 366), *p*<0.001; CD11c– DCs: sarcoidosis: mean 1150 cells/ml (range 215–3112); controls: mean 13 278 cells/ml (range 3470–43 410); *p*<0.001, fig 1B, C). Although four of the 24 patients were being treated with corticosteroids at the time of the study, similar results were observed in the 20 patients who were not receiving corticosteroid treatment (total peripheral blood DCs: mean 3412 cells/ml; CD11c+ cells: mean 2148 cells/ml; CD11c– cells: mean 1259 cells/ml).

Immunohistochemical staining of sarcoid granuloma

Haematoxylin-eosin staining and immunohistochemical staining were performed on the biopsy samples of skin from two patients with sarcoidosis (nos 1 and 2) and of lymph node from two further patients (nos 3 and 4); similar results were obtained for all four specimens (figs 2, 3, and 4). Histological examination showed a pattern typical of sarcoid granuloma with an epithelioid core and a surrounding layer of infiltrated lymphocytes (fig 2A). A large number of CD4+ cells and a smaller but considerable number of CD8+ cells had infiltrated the lymphocyte layer (fig 2B and C). A number of fascin (mature DC specific marker) positive mononuclear cells were also found in this layer in sarcoid granulomas (fig 2D), as well as DC-LAMP (another DC specific marker) positive cells and HLA-DR positive cells (fig 2E and F). Double staining with anti-fascin mAb and anti-HLA-DR mAb showed that fascin positive cells were basically all HLA-DR positive (fig 3A and B). However, HLA-DR positive fascin negative cells were also detected. Furthermore, double staining showed that both fascin and DC-LAMP positive cells with prominent dendritic projections had infiltrated the lymphocyte layer (fig 3C). On the other

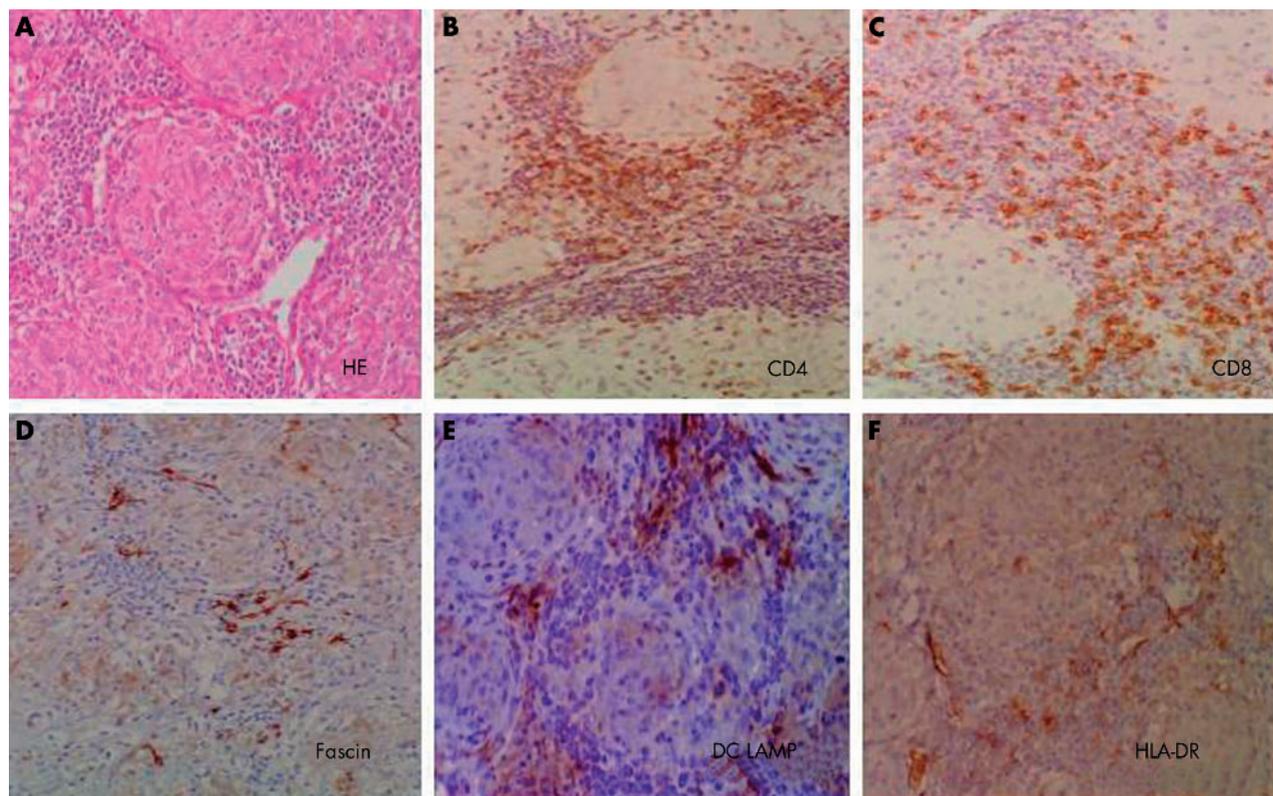


Figure 2 Immunohistochemical staining of lymph nodes of sarcoidosis. Representative data from one patient (no 3) are shown. (A) Haematoxylin and eosin; (B) anti-CD4– mAb; (C) CD8– mAb; (D) fascin– mAb; (E) DC-LAMP– mAb; (F) HLA-DR– mAb.

hand, double staining with anti-fascin mAb and anti-CD3 mAb showed that many CD3+ cells were in contact with fascin positive mononuclear cells in the lymphocyte layer (fig 3D and E), suggesting that DCs functionally interact with T cells in the granulomatous lesions of sarcoidosis.

We also found that many IFN- γ producing cells had infiltrated the lymphocyte layer (fig 4A). A substantial number of IL-4 producing cells were also detected in the lymphocyte layer and also inside the epithelioid core of the granuloma (fig 4B). Double staining of IL-4 and CD3 revealed the existence of three types of cells in the granuloma: IL-4+ CD3+ cells, IL-4+ CD3- cells, and IL-4- CD3+ cells (fig 4C and D). However, no IFN- α producing cells were seen in the biopsy samples. All of the histological findings described were observed in all four of the patients examined.

DISCUSSION

Accumulating evidence has shown that, under inflammatory conditions like infection, DCs infiltrate tissues in response to danger signals such as cytokines and chemokines.^{2,3} We therefore hypothesised that blood DCs migrate to sarcoid granulomatous lesions where they accumulate, causing a decrease in blood DCs. In support of this hypothesis, we found that the number of total blood DCs was significantly decreased in patients with sarcoidosis. This decrease was the result of a significant decrease in both CD11c+ myeloid and CD11c- plasmacytoid DC subsets. Histologically, a number of fascin+ HLA-DR+ cells and fascin+ DC-LAMP+ cells were found in the lymphocyte layer of the granuloma but not in the epithelioid core. Fascin is an actin bundling protein and is expressed in mature DCs,¹⁶ while DC-LAMP is a specific marker of lysosomes of mature DCs.¹⁷ HLA-DR, on the other

hand, is not a specific but an essential molecule of DCs. Based on these findings, we concluded that a number of mature DCs infiltrated exclusively into the lymphocyte layer of the sarcoid granuloma. The decrease in immature blood DCs and the accumulation of mature DCs in the granuloma suggest that DCs experience a maturation process during or after their movement from the blood to the granuloma. Previous studies have suggested the presence of DCs in sarcoid granulomatous lesions because of the detection of S-100 positive cells.¹⁸ However, S-100 protein is not established as a highly specific marker of DCs. This study shows unequivocally that DCs infiltrate into sarcoid granulomas in humans. We have recently suggested that the recruitment of blood DC subsets to inflammatory lesions might occur in patients with tuberculosis.¹³ However, the blood DC subtype that predominantly decreases and the extent of the decrease seem to differ between tuberculosis and sarcoidosis. In tuberculosis the numbers of myeloid DCs, but not lymphoid DCs, are significantly decreased in the blood, whereas in sarcoidosis both DC subsets are significantly lower, suggesting a different level of involvement of DCs in the pathophysiology of the two diseases.

In a murine model of sarcoidosis CD11c+ DC precursors increased in the peripheral blood and DC precursors were recruited from the circulation into *Propionibacterium acnes* induced granulomas in the liver.¹⁹ This apparently differs from the findings in our study of decreased DC subsets in the blood of patients with sarcoidosis. This discrepancy may reflect different study conditions; the murine study looked at a relatively acute phase of bacteria induced granuloma formation while we examined a steady state chronic phase of the disease.

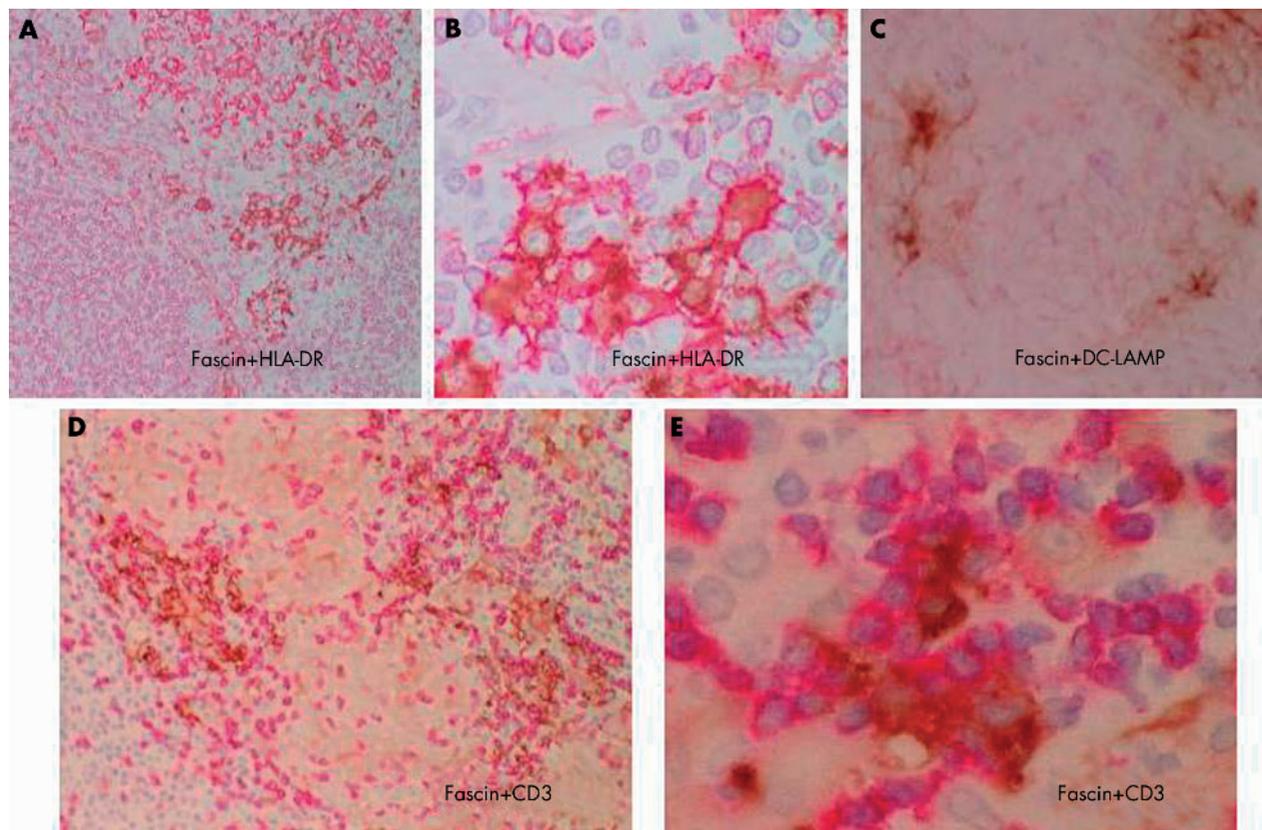


Figure 3 Immunohistochemical staining of lymph nodes of sarcoidosis. Representative data from two patients (no 2: A, B, C; no 3: D, E) are shown. (A, B) Double staining with anti-HLA-DR mAb (red) and anti-fascin mAb (brown). Original magnification $\times 100$ (A) and $\times 1000$ (B). (C) Double staining with anti-DC-LAMP mAb (red) and anti-fascin mAb (brown). Original magnification $\times 500$. (D, E) Double staining with anti-CD3 mAb (red) and anti-fascin mAb (brown). Original magnification $\times 200$ (D) and $\times 1000$ (E).

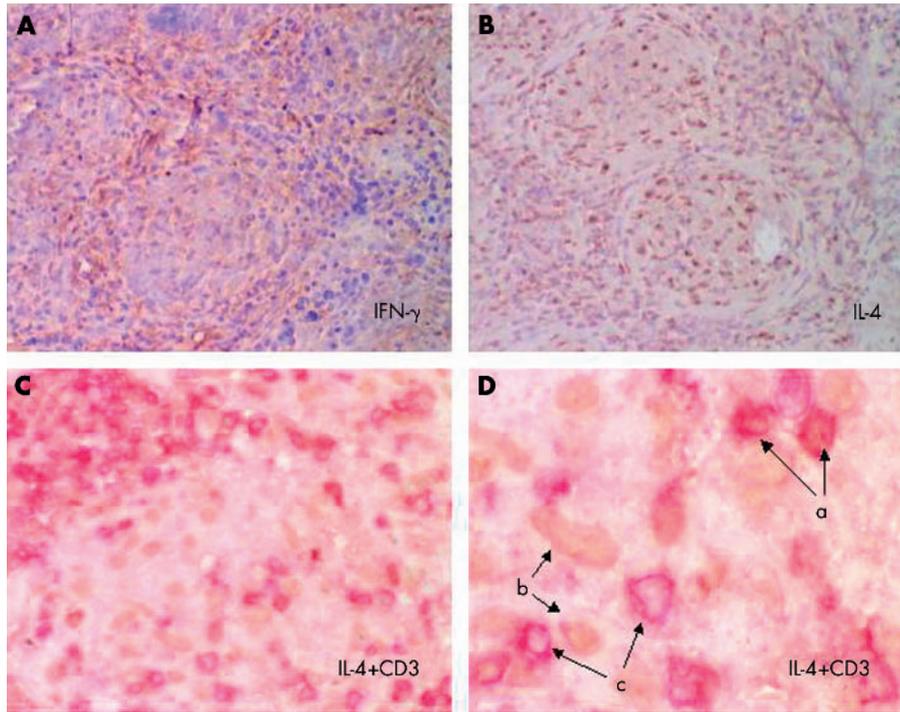


Figure 4 Immunohistochemical staining of lymph nodes and skin of patients with sarcoidosis. Representative data from two patients (no 3: A, B; no 1: C, D) are shown. (A, B) Staining with (A) anti-IFN- γ - mAb and (B) IL-4- mAb. Original magnification $\times 200$. (C, D) Double staining with anti-CD3 mAb (red) and anti-IL-4 mAb (brown). Original magnification $\times 500$ (C) and $\times 1000$ (D). (D) Staining of the epithelioid core of the granuloma showing three types of cells: (a) IL-4+ CD3+ cells, (b) IL-4+ CD3- cells, (c) IL-4- CD3+ cells.

We detected a number of IFN- γ producing T cells mostly in the lymphocyte layer of the granuloma. These might be CD4+ or CD8+ because many CD4+ and CD8+ T cells were found to be infiltrating the lymphocyte layer. Previous studies have shown that IFN- γ producing Th1 cells accumulate predominantly in sarcoid granulomatous lesions, emphasising the importance of Th1 rather than Th2 in sarcoidosis.^{1, 20-23} However, we also consistently found a significant number of IL-4 producing CD3+ T cells in the epithelioid core of the granuloma. This indicates the presence of Th2 cells or T cytotoxic type 2 (Tc2) cells, and suggests certain functional roles of these cells. One previous study suggested that Th2 cells were of relevance in pulmonary fibrosis in patients with sarcoidosis.²⁴ However, all patients who were histologically examined for affected tissues in this study demonstrated the presence of IL-4 producing T cells in the granuloma despite the absence of pulmonary fibrosis. One possibility is that the IL-4 producing T cells reflect a feedback mechanism that attenuates an overload of Th1 because IL-4 is known to counteract IFN- γ production of T cells.¹⁰ Interestingly, IL-4 producing CD3- cells were also detected in the granuloma, especially in the epithelioid core. These might represent mast cells which are known to be present in granulomatous tissues.²⁵

DCs play an essential role in the induction of antigen-specific T cell responses.^{2, 3} In the DC1/DC2 paradigm, DC1 (myeloid DCs) drive naive T cells towards Th1 cells, while DC2 (lymphoid DCs) promote the differentiation of naive T cells into Th2 cells.¹⁰ It is therefore possible that the IFN- γ producing T cells and the IL-4 producing T cells found in the sarcoid granuloma are generated by the interaction with DC1 and DC2, respectively. Indeed, double staining of fascin and CD3 showed an anatomical interaction between DCs and T cells in the lymphocyte layer of the sarcoid granuloma. Further studies of the expression profile of various cytokines and other molecules in these cells are needed to elucidate

more precisely the nature of the interaction between these cell types.

In conclusion, we found a significant decrease in both myeloid and lymphoid DC subsets in the blood and an accumulation of DCs in the lymphocyte layer of granulomas in patients with sarcoidosis, implying a recruitment of blood DCs to the granuloma. We also found IFN- γ producing T cells and IL-4 producing T cells in the sarcoid granuloma, which might be produced by an interaction with infiltrating DCs. These findings will help us to elucidate the pathophysiology of sarcoidosis and may open up new avenues of research into this enigmatic disease.

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LUNG ALERT

A rapid diagnostic test for ventilator associated pneumonia?

▲ Gibot S, Cravoisy A, Levy B, *et al*. Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia. *N Engl J Med* 2004;**350**:451–8

The diagnosis of ventilator associated pneumonia (VAP) is both contentious and frustrating for the intensivist. This study evaluates the effectiveness of using soluble triggering receptor expressed on myeloid cells (sTREM)-1, a member of the immunoglobulin superfamily expressed in response to bacterial or fungal infections, as a tool for the rapid diagnosis of VAP. A total of 148 patients requiring mechanical ventilation and with a clinical suspicion of pneumonia were prospectively enrolled in the study. Mini bronchoalveolar lavage was performed and the collected sample used for quantitative bacterial culture and assay of sTREM-1 and the inflammatory cytokines tumour necrosis factor (TNF)- α and interleukin (IL)-1 β . VAP was diagnosed on the basis of new persistent infiltrates on chest radiography, significant bacterial growth on culture ($\geq 10^3$ CFU/ml of lavage fluid), and either purulent tracheal secretions, pyrexia, leucocytosis, or leucopenia. The sTREM-1 assay reliably diagnosed pneumonia with a sensitivity of 98% and specificity of 90%. Using multiple logistic regression analysis among the predictors of pneumonia (clinical pulmonary infection score, sTREM-1, TNF- α and IL-1 β), sTREM-1 emerged as the strongest indicator (odds ratio 41.5).

The authors conclude that sTREM-1 assay may be a useful tool in both the rapid diagnosis of VAP and the reliable differentiation from non-infectious causes of fever and radiographic pulmonary infiltrates in ventilated patients.

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