Alveolar macrophages that suppress T-cell responses may be crucial to the pathogenetic outcome of pulmonary sarcoidosis

M.A. Spiteri*, S.W. Clarke*, L.W. Poulter**

ABSTRACT: The alveolar macrophage (AM) population is widely recognized to be heterogeneous; distinct subpopulations can be identified by the use of macrophage-specific monoclonal antibody (MoAb) probes.

We have isolated a macrophage subset that appears to react with both MoAbs that have previously discriminated between dendritic cells and classic macrophages. In the bronchoalveolar lavage (BAL) of patients with active sarcoidosis the proportion of this specific AM subpopulation increases dramatically (30.4±4.01% compared to 6.14±1.56% in normal BAL). This AM subset not only increases in direct proportion to the lavage lymphocytosis, but also exhibits sarcoid-related differences in surface receptor expression, physiology and induction of T-cell responses. An increased number of these AM expressed a separate antigen RFD9 (which identified epithelioid cells), and had raised fibronectin content, increased phagocytosis, and high lysosomal enzyme activity.

Of functional significance, we found that while in normal volunteers this specific AM subset was capable of down-regulating by as much as 40% the induction of T-cell responses set up by other stimulator macrophages, in sarcoid patients this suppressor activity was enhanced, such that T-cell responses were completely abolished. In some studies this action was masked by the reduced enhancing capacity of sarcoid Inducer AM.

We postulate that the presence of an increased proportion of these suppressor AM (together with their sarcoid-specific features) in active sarcoidosis is of crucial significance in determining the fate of granulomata in the lungs of these patients.


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It is now recognized that macrophages are necessary to initiate and maintain the T-cell mediated immune reactions that lead to granuloma formation within the lung interstitium of patients with sarcoidosis. However, the macrophage population is heterogeneous, and distinct subpopulations have been identified in both normal and sarcoid bronchoalveolar lavage (BAL) [1, 2]. Differences in the proportion of these alveolar macrophage (AM) subsets exist between the two groups. In particular, the proportion of a specific AM subpopulation (unique in expressing phenotypic markers of both dendritic cells and phagocytes) increases dramatically with the progression of sarcoid inflammation in the lung [3].

Macrophages within this subset react with both macrophage-specific monoclonal antibody (MoAb) probes, RFD1 and RFD7, that have previously been shown to identify dendritic cells and classic macrophages, respectively, [4, 5]. When isolated from normal BAL, these double phenotype macrophages (RFD1+D7+) exhibit physiological and functional features that are distinct from those in other AM subpopulations [6]. These RFD1+D7+ macrophages adhere to glass, have increased phagocytic capacity, express Fe and C3b surface receptors, and also contain fibronectin. Of greater significance, this AM subset is capable of down-regulating the induction of T-cell responses set up by other stimulator macrophages present in normal BAL. In support of these observations, Holt et al. [7] in animal experiments, elegantly showed that the in vitro antigen-presenting capacity of non-adherent Fe receptor negative mononuclear cells could be inhibited by the presence of endogenous adherent Fe receptor positive macrophages.

As previous reports have shown the proportion of these suppressor macrophages in sarcoid BAL to be directly related to clinical activity [8], and to be modulated by therapeutic regimes [9], the question arises as to the critical influential role this suppressor AM subpopulation may play in determining the fate of
the granulomata within the sarcoid lungs, and hence the clinical course of the disease.

The present study was, therefore, set up to obtain a relatively homogeneous population of putative suppressor AM from the BAL of patients with active pulmonary sarcoidosis, using the previously well-established combined techniques of specific macrophage probes and density gradients [6]. This allowed a detailed characterization of any sarcoid-related differences in surface receptor expression, physiology and function of the cells within this AM subpopulation, which could potentially be of importance to the clinical outcome of pulmonary sarcoidosis.

Material and methods

Subjects

A total of 25 patients with clinically active sarcoidosis were recruited: all nonsmokers, 20 males, 5 females; mean ± standard error of mean (SEM) age 35 ± 4.7 yrs. None of the patients had received any form of treatment prior to the study. All had bilateral interstitial shadowing on their chest radiographs (stage III classification), and restrictive ventilatory defect on physiological function (forced expiratory volume in one second 67 ± 0.22% of predicted value; forced vital capacity 78.3 ± 0.76% total lung capacity; diffusion capacity for carbon monoxide 69.3 ± 0.81%). The control population consisted of 25 healthy controls: all nonsmokers, 19 males, 6 females; age 35 ± 4.7 yrs. All had normal chest radiographs and physiological function (forced expiratory volume in one second 67 ± 0.22% of predicted value; forced vital capacity 78.3 ± 0.76% total lung capacity; diffusion capacity for carbon monoxide 69.3 ± 0.81%). The control population consisted of 25 healthy controls: all nonsmokers, 19 males, 6 females; mean ± SEM age 23 ± 1.4 yrs, with normal chest radiographs and pulmonary function. None had a past history of lung disease or any viral illness in the two weeks preceding the study. All subjects were recruited following formal written consent; the study had received prior approval by the Local Ethics Committee.

Bronchoalveolar lavage

BAL was performed using a 6 mm fibreoptic flexible bronchoscope (Olympus model BT-IT20D) following intravenous midazolam (Hypnovel) (relative dose depending on individual characteristics of age, weight and clinical assessment) 15 min prior to the procedure. The right middle lobe was anaesthetized with 2% lignocaine and lavaged with successive 20 ml aliquots of sterile buffered 0.9% isotonic saline, to a total of 180 ml. The lavage fluid was gently aspirated after each aliquot and collected into a sterile siliconized glass bottle maintained at 4°C.

Processing of samples

The lavage fluid was filtered through a single layer of coarse gauze and centrifuged at 480 x g, at 4°C for 5 min. The cell pellet was then washed twice in RPMI 1640 medium, after which the cells were counted in a modified Neubauer haemocytometer and viability assessed by cellular exclusion of trypan blue. The final cell concentration in each sample was adjusted to 1 x 10⁶ cells·ml⁻¹ using supplemented RPMI 1640, containing 1.25% 200 mM L-glutamine, 10% heat inactivated fetal calf serum (FCS) 100 μg·ml⁻¹ streptomycin and 100 IU·ml⁻¹ penicillin.

Separation of macrophage subpopulations

The above cell suspension was plated onto sterile plastic 85 mm diameter tissue culture grade petri dishes (Nunc, Denmark), with no more than a total of 6.0 x 10⁶ cells on each and a medium suspension depth of 3 mm. These were incubated for 2 h at 37°C in an atmosphere of 5% humidified CO₂. The supernatant containing the nonadherent cell population was then collected and the plate washed three times with medium to remove any further nonadherent cells. The adherent cells were gently scraped off the plates using a sterile rubber policeman. The adherent and nonadherent cell populations were centrifuged at 480 x g, 4°C for 5 min, resuspended in medium at 1 x 10⁶ cells·ml⁻¹ and 2 x 10⁶ cells·ml⁻¹, respectively, and kept on ice until required.

Isolation of suppressor RFD1+D7+ macrophages

The adherent cell suspension (1 x 10⁶ cells·ml⁻¹) was layered onto a 2 ml metrizamide (Nyegaard) gradient, and spun at 650 x g for 10 min at room temperature. The light density fraction was then removed, washed twice in medium, and resuspended in supplemented RPMI. This was kept on ice at 4°C. Subsequent staining with MoAbs RFD1 and RFD7 showed that over 90% of the cells obtained exhibited the phenotype RFD1+D7+ (table 1).

Table 1. – Homogeneity of isolated AM subsets

<table>
<thead>
<tr>
<th>Subset</th>
<th>RFD1+D7⁻</th>
<th>RFD1+D7+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>97.0±2.41</td>
<td>97.4±1.60</td>
</tr>
<tr>
<td>Sarcoid</td>
<td>96.4±1.40</td>
<td>98.2±1.41</td>
</tr>
</tbody>
</table>

The homogeneity is expressed as the mean ± SEM percentage of macrophage-like cells (within each isolated sample) that express positivity to the MoAb probe/s that identify the appropriate AM subpopulation. AM: alveolar macrophage; MoAb: monoclonal antibody.

Isolation of inducer RFD1+D7⁻ macrophages

Lymphocytes were removed from the nonadherent population by rosetting with neuraminidase-treated sheep red blood cells, followed by separation on a ficoll-Hypaque gradient (Nycomed, Norway). The resulting cell interface was harvested, washed twice and resuspended in supplemented RPMI 1640 medium. Subsequent analysis with the MoAbs RFD1 and RFD7
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showed that over 90% of the cells obtained were RFD1+D7- (the phenotype of interdigitating cells, [5]) (table 1).

Cytospin preparation

Cytospins were prepared on a Shandon Cytospin 2 using 100 μl aliquots of each of the above cell suspensions (2 x 10^5 cells·ml^{-1}). One cytospin from each sample was stained for morphology, while the remainder were air-dried for one hour at room temperature, fixed in 1:1 mixture of chloroform-acetone for 10 min, wrapped in plastic film and stored at -20°C until use. Cell morphology was determined by using a Diff-Quick (Dade Diagnostics) differential white cell stain.

Proportion of RFD1+D7+ AM to lymphocytosis

The proportion of RFD1+D7+ macrophages was related to the number of morphologically identifiable lymphocytes in each of the sarcoid and normal unfractionated BAL samples. The RFD1+D7+ AM were identified by double immunofluorescence techniques [10], and lymphocytes by Diff-Quick staining of lavage cytospins.

Table 2. — Panel of monoclonal antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>M.W kD</th>
<th>Specificity in normal tissue</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFDR1</td>
<td>28/33</td>
<td>Identifies a framework epitope on the HLA-DR molecule</td>
<td>RFHSM</td>
<td>[4]</td>
</tr>
<tr>
<td>RFD1</td>
<td>28/33</td>
<td>Identifies interdigitating cells and a small proportion of B-cells</td>
<td>RFHSM</td>
<td>[5]</td>
</tr>
<tr>
<td>RFD7</td>
<td>77</td>
<td>Identified mature phagocytic macrophages</td>
<td>RFHSM</td>
<td>[5]</td>
</tr>
<tr>
<td>RFD9</td>
<td>-</td>
<td>Identifies epithelioid cells and tingible body macrophages</td>
<td>RFHSM</td>
<td>[11]</td>
</tr>
<tr>
<td>EMB11 (CD68)</td>
<td>110</td>
<td>Identifies all cells of the monocyte - macrophage lineage</td>
<td>Dakopatts, Denmark</td>
<td></td>
</tr>
<tr>
<td>UCHM1 (CD14)</td>
<td>52</td>
<td>Identifies antigen present on the majority of blood monocytes</td>
<td>P.C.L. Beverley University College London</td>
<td>[13]</td>
</tr>
<tr>
<td>Anti C3b receptor</td>
<td>205</td>
<td>Reacts with the receptor for the third component of human complement</td>
<td>Dakopatts, Denmark</td>
<td>[14]</td>
</tr>
<tr>
<td>Anti fibronectin</td>
<td>-</td>
<td>Reacts with fibronectin in human cells</td>
<td>Dakopatts, Denmark</td>
<td>[15]</td>
</tr>
<tr>
<td>MoAb 10.1</td>
<td>71</td>
<td>Reacts with Fc receptors FcR1 on human mononuclear cells</td>
<td>N. Hogg Imperial Cancer Research Laboratories London</td>
<td>[16]</td>
</tr>
</tbody>
</table>

RFHSM: Royal Free Hospital School of Medicine; CD: cluster designation; MW: molecular weight; MoAb: monoclonal antibody.

Immunocytological analysis

Immunocytological analysis of the RFD1+D7+ AM subpopulation, isolated from 10 of the sarcoid and 10 of the normal BAL samples, was performed with a panel of MoAb probes (table 2) [4, 5, 11-16] using standard immunoperoxidase [17], and double immunofluorescence [10] staining methods. Background staining was identified by comparison with negative control cytospins on which the MoAb was omitted; positive specificity controls were always prepared using sections of human palatine tonsil. In the case of the fluorescent studies, a Zeiss microscope equipped with epi-illumination and barrier filters appropriate for fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) was used for recording the presence of RFD1+D7+ cells. Background fluorescence was noted using controls as described above. At least 150 cells were counted in each prepared cytospin, and the percentage of positive cells to the MoAb/s used recorded.

Lysosomal enzyme activity

Lysosomal enzyme activity was investigated using a standard histochemical reaction for acid phosphatase [18] on unfixed cytospins of isolated RFD1+D7+
abstract

Macrophages, obtained from lavage samples of 10 sarcoid patients and 10 normal volunteers. All preparations were counterstained with haematoxylin. The percentage of RFD1+D7+ macrophages that gave a positive result in each test sample was read using an Olympus light microscope with high magnification (x600).

Phagocytosis

Macrophages from each separated RFD1+D7+ subpopulation (1 x 10^6 cells), obtained from five sarcoid patients and five normal subjects, were incubated with fluorescein-coated latex beads of 1.0 μm diameter (Polysciences, Northampton, UK) in a concentration of 100 beads·cell⁻¹ for 2 h at 37°C in an atmosphere of 5% humidified CO₂. Control plates were set up in which phagocytosis was blocked by the inclusion of cytochalasin B (Imperial Chemical Industries Ltd, UK) in a concentration of 2.0 x 10⁻⁶ M, prepared fresh in a solution of 0.2% dimethylsulphoxide [19]. Following incubation, each cell suspension was washed twice to remove excess beads. Cytospin preparations were made and latex beads within the cells were identified by the presence of fluorescein on their surface using a Zeiss standard microscope fitted with epi-illumination and appropriate filters for FITC. A minimum of 100 cells was counted on each cytospin preparation and the presence of five or more latex beads in a cell was considered to constitute phagocytosis.

Allogeneic mixed lymphocyte cultures (MLC)

Aliquots of 1 x 10⁴ unfractionated BAL cells and similar aliquots of each RFD1+D7+ and RFD1+D7- macrophage subpopulations (isolated from five sarcoid patients and four normal volunteers) were, respectively, co-cultured with a standard population of 1 x 10⁵ allogeneic normal peripheral blood mononuclear cells (PBMC) from a constant donor. In all experiments, each culture was set up in triplicate and incubated for five days at 37°C in 5% humidified CO₂. Tritiated thymidine (HT) was then added to each MLC, which was incubated for a further 18 h and harvested using a semi-automatic cell harvester (Titerek-Flow, Laboratory Inc., McLean Va). The amount of incorporated ³HT was measured in a liquid scintillation counter and expressed as average counts per minute (cpm) of triplicate cultures.

Statistics

Quantitative data were expressed as the mean ± standard error of mean (SEM). Wherever relevant, appropriate significance between results was determined using the Student’s t-test. Significance was taken at p<0.05.

Results

Bronchoscopic findings

No evidence of any bronchial infection or inflammation was found in any normal subject. Signs of endobronchial mucosal inflammation were observed in 6 out of 25 active sarcoid patients. In this study, the mean±SEM percentage return of total lavage fluid instilled was 70±9.5% in the sarcoid group, compared to 72±11% in the normal group.

Differential cell counts

The mean±SEM total BAL cell yield in the normal volunteers was 9±1.4 x 10⁶ cells, of which the absolute number of AM was 8.3±1 x 10⁶ cells. In contrast, the total BAL cell yield in sarcoid patients was 16.5±2.5 x 10⁶, of which the absolute number of AM was 11.2±0.9 x 10⁶. The mean proportion of lymphocytes and polymorphonuclear cells in sarcoid BAL was 35% and 5%, respectively; whilst that in normal lavage was 9% and 2%, respectively.

The viability of unfractionated macrophage-like cells and separated AM subsets in both normal and sarcoid samples was >90% by trypan blue exclusion. All cells in the separated populations were morphologically identified as macrophages.

Immunocytochemical analysis

In unfractionated active sarcoid BAL, 50.4±8.62% of macrophage-like cells expressed positivity for RFD1 compared to 13.2±3.51% in the normal BAL (p<0.001). Much of this increase in sarcoidosis was due to the increased proportion of doubly labelled macrophages (30.4±4.01%), in contrast to only 6.14±1.56% in the normal group (p<0.001) (fig. 1). Analysis of the phenotypic features of the RFD1+D7+ AM subpopulation isolated from normal and sarcoid groups is summarized in table 3 a and b. Sarcoid-related differences were notable between the two groups. In particular, an increased proportion of RFD1+D7+ AM in active sarcoid BAL showed expression of a separate antigen RFD9 and contained higher fibronectin content than equivalent normal AM. Furthermore, the intensity of fibronectin staining was much stronger in RFD1+D7+ AM from active sarcoid patients.
Fig. 1. - The proportion of the different AM fractions identified by the macrophage-specific monoclonal antibody probes RFD1 and RFD7 in normal and sarcoid lavage. Values are significantly different between the two groups (see text). AM: alveolar macrophage.

Alveolar macrophage subsets

<table>
<thead>
<tr>
<th>AM subset*</th>
<th>RFDR1</th>
<th>RFD9</th>
<th>EBM11</th>
<th>UCHM1</th>
<th>Anti-C3b</th>
<th>Anti-FcR1</th>
<th>Anti-fnnectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 Mean</td>
<td>98.3</td>
<td>&lt;1</td>
<td>95.4</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>20.3</td>
<td>30.3</td>
</tr>
<tr>
<td>SEM</td>
<td>1.21</td>
<td></td>
<td>0.61</td>
<td></td>
<td></td>
<td>1.21</td>
<td>0.21</td>
</tr>
<tr>
<td>D1D7 Mean</td>
<td>95.4</td>
<td>5.44</td>
<td>96.3</td>
<td>&lt;1</td>
<td>35.7</td>
<td>85.3</td>
<td>95.3</td>
</tr>
<tr>
<td>SEM</td>
<td>3.51</td>
<td>1.12</td>
<td>0.21</td>
<td>&lt;1</td>
<td>0.45</td>
<td>1.24</td>
<td>3.41</td>
</tr>
</tbody>
</table>

b. Sarcoid patients

<table>
<thead>
<tr>
<th>AM subset*</th>
<th>RFDR1</th>
<th>RFD9</th>
<th>EMB11</th>
<th>UCHM1</th>
<th>Anti-C3b</th>
<th>Anti-FcR1</th>
<th>Anti-fnnectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 Mean</td>
<td>100</td>
<td>&lt;1</td>
<td>95.5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>25.7</td>
<td>50.3</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td></td>
<td>0.52</td>
<td></td>
<td></td>
<td>0.91</td>
<td>1.51</td>
</tr>
<tr>
<td>D1D7 Mean</td>
<td>95.6</td>
<td>45.5</td>
<td>96.2</td>
<td>&lt;1</td>
<td>36.4</td>
<td>92.2</td>
<td>100</td>
</tr>
<tr>
<td>SEM</td>
<td>2.41</td>
<td>3.12</td>
<td>0.41</td>
<td>&lt;1</td>
<td>0.17</td>
<td>1.45</td>
<td></td>
</tr>
</tbody>
</table>

The above values represent the mean and standard error of mean (SEM) of total morphologically identifiable macrophages within each separated AM subset, that express positivity with the monoclonal antibodies RFDR1, RFD9, EBM11, UCHM1, anti-C3b & anti-Fc receptor (FcR1), and anti-fibronectin. *: refers to the degree of intensity of the staining; #: for clarity, D1=RFD1+D7- macrophages, D1D7=RFD1+D7+ macrophages; AM: alveolar macrophage; fnnectin: fibronectin.

Table 3. - Phenotypic features of macrophage subsets isolated from normal volunteers

Correlation of BAL lymphocytosis with RFD1+D7+ AM concentration

Sarcoid patients had significantly higher proportions of lymphocytes in their BAL (35.3±9.11%) than normal subjects (9.4±3.10%) (p<0.001). Similarly, greater numbers of RFD1+D7+ cells were isolated from sarcoid lavage than normal (p<0.001). In both groups, the concentration of RFD1+D7+ AM in the samples was noted to be directly proportional to the lymphocytosis (fig. 2 a and b). The correlation coefficient was calculated to be 0.936 (p<0.001) in the normal group and 0.947 (p<0.001) in the sarcoid group.

Acid phosphatase reaction

In normal BAL, only 70.5±1.11% of RFD1+D7+ cells were acid phosphatase (ACP) positive, while in active sarcoid BAL 90.6±4.13% of RFD1+D7+ macrophages were ACP positive (p<0.001).

Phagocytosis

Similarly, phagocytosis was observed to be significantly increased in RFD1+D7+ AM from sarcoid BAL (84.2±1.51%), when compared to normal RFD1+D7+ cells (73.4±8.11%) (p<0.001). Changes in cell morphology were accompanied by total inhibition of phagocytosis in all cultures containing cytochalasin B.

Effect of AM subpopulations on allogeneic PBM proliferation

 Cultures were set up using 1 x 10^4 aliquots of unfractioned macrophages and each of the separated RFD1+D7+ and RFD1+D7- subsets with a standard responder population of 1 x 10^5 allogeneic normal PBM in four normal volunteers (table 4a) and five sarcoid patients (table 4b).
SUPPRESSOR ALVEOLAR MACROPHAGES IN SARCOIDOSIS

Fig. 2. — The relation between the proportion of morphologically identifiable lymphocytes and RFD1+D7+ macrophages in the lavage of: a) normal subjects; and b) patients with pulmonary sarcoidosis.

Table 4. — Allogeneic mixed lymphocyte reactions in normal subjects

<table>
<thead>
<tr>
<th></th>
<th>a. Normal subjects</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td>110±4.1</td>
<td>87±15.9</td>
<td>196±6.1</td>
<td>80±28.3</td>
<td></td>
</tr>
<tr>
<td>BALM</td>
<td>153±34.7</td>
<td>86±29.6</td>
<td>237±89.0</td>
<td>41±4.5</td>
<td></td>
</tr>
<tr>
<td>PBM</td>
<td>84529±2655</td>
<td>8604±1451</td>
<td>1132±71.0</td>
<td>4464±555</td>
<td></td>
</tr>
<tr>
<td>PBMM</td>
<td>147±13.7</td>
<td>157±79.8</td>
<td>108±20.6</td>
<td>30±4.0</td>
<td></td>
</tr>
<tr>
<td>BAL + PBM</td>
<td>4100±1121</td>
<td>7958±368</td>
<td>1309±210</td>
<td>4735±376</td>
<td></td>
</tr>
<tr>
<td>D1D7</td>
<td>84±13.2</td>
<td>87±7.1</td>
<td>140±44.1</td>
<td>182±20.0</td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>121±19.8</td>
<td>81±8.7</td>
<td>94±31.5</td>
<td>96±28.8</td>
<td></td>
</tr>
<tr>
<td>D1D7 + PBM</td>
<td>131188±642</td>
<td>9825±1124</td>
<td>198±8.0</td>
<td>9357±589</td>
<td></td>
</tr>
<tr>
<td>D1 + PBM</td>
<td>40520±3156</td>
<td>20404±775</td>
<td>4181±1685</td>
<td>14360±1644</td>
<td></td>
</tr>
<tr>
<td>Background</td>
<td>9±3.2</td>
<td>4±0.3</td>
<td>15±6.7</td>
<td>5±0.9</td>
<td></td>
</tr>
</tbody>
</table>

The background values represent the amount of radioactivity in wells containing culture medium alone. BAL: unfractionated bronchoalveolar lavage cells; BALM: mitomycin treated unfractionated bronchoalveolar lavage cells; PBM: peripheral blood mononuclear cells; PBMM: mitomycin treated peripheral blood mononuclear cells; BAL + PBM: mitomycin treated bronchoalveolar lavage cells admixed with PBM, D1 and D1D7 represent the isolated alveolar macrophage subsets. D1 + PBM, D1D7 + PBM: the isolated subsets admixed with PBM. For details of these reactions, see text.

While RFD1+D7- macrophages from both normal and sarcoid groups clearly support allogeneic PBM proliferation, this response is suppressed by RFD1+D7+ cells from the same subjects. Furthermore, when compared to the normal group, preliminary observations suggest that RFD1+D7+ AM from sarcoid patients seem to have an increased suppressive influence to the extent that allogeneic PBM responses are abolished in three out of five patients. It also appears that RFD1+D7- AM from the same sarcoid patients appear to have reduced T-cell enhancing capacity when compared to equivalent normal cells.
The effect on RFD1+D7- cell inducing activity by RFD1+D7+ macrophages

In separate MLC experiments, varying concentrations of both AM subsets from five healthy (fig. 3a) and five active sarcoid patients (fig. 3b) were added to a fixed concentration (1 x 10⁶) of allogeneic PBM; the proportion of each AM subset was adjusted so as to maintain a constant total number of macrophages (1 x 10⁸) within each MLC. Analysing the results of these AM subsets in the normal group (fig. 3a) RFD1+D7- AM alone consistently failed to stimulate allogeneic PBM. In contrast RFD1+D7- AM alone (in concentrations of 4 x 10⁵ to 1 x 10⁶ per well) caused three to fourfold increase in PBM proliferation. Lower concentrations of RFD1+D7- AM caused a comparatively reduced PBM response, but this will still significantly greater than PBM autologous reactivity (even when 1 x 10³ RFD1+D7- cells were used). In co-culture studies using both AM subsets, it was observed that as the decreasing proportion of RFD1+D7- cells within the MLC was substituted by a corresponding proportional increase in RFD1+D7+ cells, PBM proliferation became progressively suppressed and eventually abolished at high concentrations of RFD1+D7+ macrophages.

Data obtained from equivalent experiments using AM subsets from sarcoid patients (fig. 3b) show that not only are RFD1+D7+ macrophages in sarcoid BAL more suppressive of allogeneic PBM proliferation than normal equivalents, but that the enhancing capacity of sarcoid RFD1+D7- cells (seen best at their higher concentrations) is subdued at lower concentrations.

When this happens, the over suppressive influence of RFD1+D7+ AM on allogeneic MLC is masked by the reduced enhancing capacity of RFD1+D7- AM.

In all MLC experiments, blank wells containing only culture medium but no cells consistently gave readings of less than 20 cpm.

Discussion

This study has shown that the presence of sarcoid inflammation in the lung induces specific changes within the local heterogeneous AM population. In particular, a specific macrophage subset emerges in the BAL of patients with active sarcoidosis. Cells within this subset react with both MoAb probes previously described to discriminate between dendritic cells and classic macrophages in tissue. Our observations show that the dramatic increase in proportion of this AM subpopulation is accompanied by sarcoid-related differences in surface receptor expression, physiology and T-cell inducing capability. Functional studies with these doubly-labelled macrophages show that their suppression of T-cell proliferation is enhanced in sarcoidosis. Furthermore, an increased proportion of cells within this suppressor AM subpopulation also express a separate antigen, RFD9 (which identifies epithelioid cells in tissue) and have enhanced fibronectin content.

It could be argued that, as the homogeneity in the isolated AM subsets was at best 97%, the observed functional as well as phenotypic and physiological results were possibly influenced by contaminating cells. However, all separated cell subpopulations were
counter-staining immunofluorescence methods after isolation and before use in experiments. While a minimal degree of contamination with other AM subtypes occurred (<2%), a proportion of morphologically identifiable macrophages (5.0±1.4%), that were EBM11+ but UCHM1-, was found repeatedly in the isolated samples. It is assumed that these macrophages have not yet undergone full differentiation. We believe that the influence that this small proportion of cells could have on the overall results is negligible.

In support, the functional reconstitution experiments showed that even at the lowest concentration of 1 × 10^3, RFD1+D7+ AM still suppressed the allogeneic MLR to the same extent as the higher concentration (fig. 3a and b).

Many workers have previously described sarcoid-related changes in surface phenotype, specific receptor expression, phagocytosis and T-cell inductive capabilities of AM. However, unlike the current study, none of these observations were carried out on isolated phenotypically and functionally distinct AM subpopulations. More relevant to our data, animal studies have shown a similar increase in the number of cells, with a macrophage-like morphology, capable of down-regulating T-cell responses in the presence of disease [7, 20]. The increased presence of such suppressor AM in active sarcoid BAL could explain why unfractonated sarcoid AM consistently reduced T-cell proliferation in previous experiments [3, 21]. At the time, such a finding had appeared contradictory in the light of previous reports of sarcoid AM showing increased DR expression [22, 23] and enhanced antigen presenting capacity [24]. However, it would appear that under normal circumstances in man, the AM population represents a dynamic system made up of a delicate balance of phenotypically distinct immune inducer and suppressor macrophages [6]. Our data suggest that with the presence of sarcoid inflammation, this balance has shifted towards the emergence of a specific AM subtype that not only increases in direct proportion to the degree of local lymphocytosis but is also capable of suppressing T-cell responses. This raises the intriguing question: What promotes the increase in numbers of these AM in sarcoidosis?

One could postulate that in a susceptible patient, under an appropriate as yet unidentified stimulus, a macrophage-lymphocyte interaction sparks off the development of a localized cellular immune response responsible for the initial alveolitis described in early sarcoidosis [25, 26]. Consequent on this reaction, biologically active mediators are released by activated T-cells into the local milieu. One such substance is interferon-gamma (IFN-γ), increased levels of which have been shown in active sarcoid lavage [27]. IFN-γ is a potent activator of macrophages: indeed it has been observed in vitro to increase HLA-DR expression on sarcoid AM [22]. Other in vitro experiments have observed that IFN-γ can increase the proportion of RFD1+ cells developing, while suppressing D7-antigen expression [28]. It is therefore possible that within the sarcoid microenvironment, one of the roles of the secreted IFN-γ is to induce the emergence of D1-antigen on macrophages that already express the D7-phenotype. By suppressing the further development of the D7-phenotype (the phenotype of classic macrophages), IFN-γ can induce a switch in the resident AM population towards an increasing proportion of suppressor RFD1+D7+ macrophages. If such events were reflected in vivo, they would certainly explain the shifts observed towards particular AM subpopulations in the BAL of patients with active sarcoidosis.

What influence could these suppressor RFD1+D7+ macrophages have on the outcome of the epithelioid cell granuloma? It is recognized that sarcoid granulomata are usually benign and resolve spontaneously. This would imply that the immune response set up has effectively removed the inciting agent, or has markedly decreased the antigen load (without leaving any secondary sequelae). For this to happen in the face of an increasing spiral of cell activation, it would not be wrong to envisage that controlling influences must be in operation. Current studies have shown that the increase in suppressor RFD1+D7+ macrophages in BAL, is directly proportional to the lymphocytosis present. In addition, the suppressor function of these cells seems to be enhanced in sarcoid patients, when compared to normal equivalents. It is thus tempting to postulate that the marked rise in suppressor macrophages in active sarcoidosis is aimed at controlling T-lymphocyte responses from potentially escalating and resulting in disruption of the local integral alveolar-capillary units. In support, Ainslie et al. [8] noticed reduced levels of CD7 (a marker of blast formation in T-cells), and HLA-DR expression of CD4+ T-lymphocytes, at the same time as proportions of suppressor RFD1+D7+ macrophages were increasing in sarcoid BAL. It would follow that such macrophages could arise as part of a secondary response to stimuli in the immediate milieu, in order to contain the events arising from the initial macrophage-T-cell interaction. Could this also explain why the enhancing effect of inducer RFD1+D7- macrophages in sarcoid patients is diminished when compared to equivalent macrophages in the normal? Indeed, macrophages from active sarcoid patients have been observed to release increased amount of prostaglandin E_2 (PGE_2) tumour necrosis factor (TNF) and IFN-γ [27, 29, 30]. These cytokines possess inhibitory activity, with direct down-regulation of T-cell responses [31]. PGE_2 can also inhibit the expression of major histocompatibility complex (MHC) Class II molecule [32]. The potential effect of IFN-γ in inducing a switch in AM phenotype has already been discussed.

With such a sophisticated, inbuilt, macrophage-mediated regulation of local T-cell responses, it would be expected that the reaction would be self-limiting. Indeed this is the case in the majority of patients with pulmonary sarcoidosis [33]. What, therefore, goes wrong with the controlling mechanisms in the small proportion of sarcoid patients, in whom a more progressive disease develops, and results in cellular and matrix disorganization of the alveolar structures with
Phenotypic change their phenotype (and perhaps their function) and cells? In support, IFN-γ has been observed under local stimuli, weakened RFD1+D7+ macrophages is still unknown [39]. Could it be that yet again nonspecific production [38], the physiological role of the epithelioid function these (RFD1+D7+D9+) cells have. Apart from observation provokes the intriguing question of what important structural units of the sarcoid granuloma, this low). be seen whether an exaggerated presence of this sub­

of RFD1+D7+ suppressor macrophages increases with growth factor, in addition to enhanced expression of increased levels of fibronectin and AM- derived membrane fibronectin receptors on macrophages the immediate milieu! After all, the controlled release normal repair process? In fact, in advanced stages of this situation, cell-to-cell interaction would escalate, and as a result an overproduction of biologically active substances could be released into the immediate microenvironment. Data in this study show that not only were the majority of RFD1+D7+ suppressor macrophages positive for fibronectin, but that in sarcoidosis the intensity of this staining on the individual RFD1+D7+ macrophages was markedly increased. Could therefore a sustained heightened immune reaction result consequent on persistent antigen stimulation and, in a last attempt to combat this, the RFD1+D7+ suppressor macrophages release fibronectin (and possibly other similar substances) into the immediate milieu! After all, the controlled release of such substances would appear to be required for the normal repair process? In fact, in advanced stages of the disease, patients have been shown to have increased levels of fibronectin and AM- derived growth factor, in addition to enhanced expression of membrane fibronectin receptors on macrophages [35-37]. As it has been observed that the proportion of RFD1+D7+ suppressor macrophages increases with progressive radiographic changes [8], it remains to be seen whether an exaggerated presence of this subset is present at site of injury in Stage IV sar­

oid patients (at a time when lavage lymphocyte numbers are low).

These RFD1+D7+ macrophages also show an increased expression of a separate antigen D9 (which identified epithelioid cells). As epithelioid cells are important structural units of the sarcoïd granuloma, this observation provokes the intriguing question of what function these (RFD1+D7+D9+) cells have. Apart from nonspecific angiotensin converting enzyme (ACE) production [38], the physiological role of the epithelioid cell is still unknown [39]. Could it be that yet again under local stimuli, weakened RFD1+D7+ macrophages change their phenotype (and perhaps their function) and undergo morphological differentiation into epithelioid cells? In support, IFN-γ has been observed in vitro to promote epithelioid cell and giant cell formation when added to cultured macrophages [40].

In conclusion, supportive evidence has been given for the emergence of a specific AM subset in pulmonary sarcoidosis, capable of suppressing T-cell responses and possessing sarcoid-related features. These cells could play a critical role in determining the fate of granulomata (their resolution or progression to fibrosis) in the lungs of sarcoïd patients. Further evaluation of this specific subset could provide criteria for staging disease activity, as well as guiding therapy in these patients. In support, proportions of these AM in sarcoïd BAL correlated well with clinical status [8] and are modified by therapeutic regimes [9].

References

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