

# Changes to alveolar macrophage phenotype in HIV infected individuals with normal CD4 counts and no respiratory disease

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## Abstract

**Background** – It has previously been shown that HIV infected individuals with pneumonitis have identifiable abnormalities in alveolar macrophages obtained by bronchoalveolar lavage (BAL). In particular, alterations in the expression of alveolar macrophage surface antigens associated with macrophage function have been reported. To determine whether these changes reflect HIV infection or the respiratory episode itself, a population of HIV infected patients with no respiratory disease was studied.

**Methods** – Twenty two HIV antibody positive individuals with a peripheral blood CD4 count of >400/ $\mu$ l and 10 healthy volunteer controls underwent bronchoscopy and BAL. Cytospin preparations from the recovered cells were stained using immunoperoxidase and double immunofluorescence techniques with monoclonal antibodies RFD1, RFD7, EBM11/CD68 (mature macrophages), UCHM1/CD14 (monocyte marker), and HLA-DR (RFDR1). Differential cell counts were also performed.

**Results** – There was an increase in overall alveolar macrophage HLA-DR expression in the HIV population. This was not reflected in a change in the percentage of cells staining CD14 (monocytes) or CD68 (mature macrophages) positive. The relative proportions of cells staining RFD1+RFD7– (inducer cells), RFD1–RFD7+ (effector cells), and RFD1+RFD7+ (suppressive cells) were unchanged between HIV and control groups.

**Conclusions** – In a population of HIV infected individuals with normal CD4 counts and no respiratory disease there was an increase in overall alveolar macrophage HLA-DR expression which occurred independently of any alteration in the relative proportions of alveolar macrophage subpopulations.

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Keywords: macrophages, phenotypes, HIV, HLA-DR.

It has been shown by several groups that alveolar macrophages represent a heterogeneous population containing phenotypically definable subsets that exhibit distinct functional capacity in vitro.<sup>4-6</sup> Specifically, the use of monoclonal antibodies RFD1 and RFD7 (Royal Free Hospital School of Medicine, London, UK) allows antigen presenting cells, mature phagocytes, and suppressive macrophages to be discriminated.<sup>7</sup> Changes in the relative proportions of these alveolar macrophage subsets are associated with the immunopathology of several inflammatory lung diseases such as cryptogenic fibrosing alveolitis,<sup>8</sup> sarcoidosis,<sup>9</sup> and asthma.<sup>10</sup>

Similar aberrations have been observed in AIDS patients with respiratory disease – for example, we have found a reduction in expression of RFD1 and RFD7 and HLA-DR molecules on alveolar macrophages.<sup>2</sup> MHC class II is essential for antigen presentation. As accessory cell function is altered by HIV infection,<sup>11</sup> our observation suggests that a reduced capacity of lung macrophages to present antigen efficiently may be contributing to the immunocompromised state of the lung defences.

A question that remains, however, is whether such changes to the surface molecules of the lung macrophage pool precede or are an effect of the associated opportunistic infection in these symptomatic AIDS patients. Further observations that treatment with antiretroviral agents partially restores membrane antigen expression on these cells implies that the level of HIV infection itself may be contributing to these abnormalities.<sup>12</sup>

To begin to address these questions the current study sought to determine whether such alterations in the relative proportions of alveolar macrophage subsets occurred in HIV positive subjects with no respiratory disease and normal CD4 counts. The study further quantified the level of HLA-DR expression on these lung macrophage populations, thus testing the hypothesis that such changes may contribute to altered immunity before the emergence of respiratory disease.

## Methods

### SUBJECTS

Twenty two HIV antibody positive individuals with no history of acute or chronic respiratory disease determined by respiratory questionnaire,<sup>13</sup> normal spirometric values (Vitalograph Compact machine), and chest radiography

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Infection and inflammation of the lung are a common cause of morbidity and mortality in patients with AIDS.<sup>1</sup> We and others have shown previously that HIV infected individuals with pneumonitis have identifiable abnormalities in lung cells obtained by bronchoalveolar lavage (BAL).<sup>2,3</sup>

underwent bronchoscopy with bronchoalveolar lavage. All patients were Centers for Disease Control (CDC) HIV stage II (asymptomatic) or III (persistent generalised lymphadenopathy) (12 stage II, 10 stage III)<sup>14</sup> and had peripheral blood CD4 T lymphocyte counts repeatedly of >400/ $\mu$ l. None of this group was taking antiretroviral therapy and there was no history of injecting drug use.<sup>15</sup>

Ten healthy adult volunteers with no past history of lung disease or symptoms suggesting viral infection in the last three months acted as controls.

The study was approved by the local ethical committee and all subjects gave written consent before taking part.

#### BRONCHOALVEOLAR LAVAGE (BAL)

A standardised BAL was performed on all subjects using an Olympus P20 fiberoptic bronchoscope. Following sedation with 5 mg intravenous midazolam the vocal cords were anaesthetised under direct vision with 4% lignocaine. Further lignocaine (2 ml aliquots of a 2% solution) was used in the airways as necessary as the bronchoscope was advanced. The tip of the bronchoscope was wedged in a subsegmental bronchus of the right middle lobe and a total of 180 ml of warmed, sterile 0.9% normal saline was instilled in 20 ml aliquots. The BAL fluid was aspirated under low pressure wall suction into a sterile, siliconised glass bottle kept on ice as recommended in the report of the European Society of Pneumology Task Group on BAL.<sup>16</sup> Recovery was between 80 and 150 ml. There was no difference in this between HIV and control populations.

At the time of BAL, blood was taken for CD4 count,  $\beta_2$ -microglobulin determined by radial immunodiffusion (Binding Site, Birmingham, UK), and HIV (p24) antigen measured using enzyme immunoassay (Du Pont, Stevenage, UK).

#### CYTOSPIN PREPARATION

A cell count was performed on the BAL fluid which was then filtered through a single layer of coarse gauze to remove excess mucus. The cells were washed three times in phosphate buffered saline (PBS). Cell concentration was adjusted to  $3-5 \times 10^5$ /ml and cytocentrifuge preparations were made (Cytospin II, Shandon Instruments, UK) with 50  $\mu$ l aliquots. The cytospin preparations were air dried at room temperature for 60 minutes, fixed for 10 minutes in a 1:1 mixture of chloroform and acetone, wrapped in plastic film and stored at  $-20^\circ\text{C}$  until used. One cytospin from each BAL sample was stained with a modified May-Grunwald Giemsa stain to record the differential cell count.

#### STAINING AND MONOCLONAL ANTIBODIES

The cytospin preparations were stained with monoclonal antibodies (MoAbs) to identify all macrophages (MoAb EBM11/CD68; Dako, High Wycombe, UK),<sup>17</sup> monocytes (MoAb

UCHM1/CD14; Dako),<sup>18</sup> and HLA-DR (RFDR1; Royal Free Hospital School of Medicine, London, UK)<sup>19</sup> using an indirect immunoperoxidase method.<sup>20</sup> The preparations were incubated with mouse antihuman monoclonal antibody for 55 minutes at room temperature, rinsed with PBS, and then incubated with a rabbit antimouse peroxidase conjugate for 45 minutes. After rinsing with PBS the preparations were developed in a solution containing 0.01% hydrogen peroxide and 0.6% 3,3'-diaminobenzidine tetrahydrochloride. Preparations for CD14 and CD68 were counterstained in Harris' haematoxylin, dehydrated, and mounted in DPX (Merck, Dagenham, UK) for microscope viewing by one investigator. In all cases non-specific staining by monoclonal antibodies was controlled at standardisation by comparison with staining by isotype matched irrelevant monoclonal antibodies. Negative controls were also included omitting the primary antibody. For CD68 and CD14 any macrophages exhibiting identifiable staining differing from that of the negative control were scored as positive. At least 150 macrophages were counted for each preparation and the percentage of positive macrophages was recorded.

HLA-DR expression was quantified on each preparation stained with monoclonal antibody RFDR1 by measurement of optical density using a monochrome video camera attached to a microscope with a  $\times 40$  objective. The relative density of the reaction product was recorded using a computerised image analyser (Seescan, Cambridge, UK). No counterstaining was used in this preparation. Optical density measured per unit area was directly proportional to the intensity of the staining.

The measurements were carried out on multiple cells randomly selected from at least six high power fields from each cytospin preparation including negative controls. The density of HLA-DR antigens was determined by the difference between the median optical density of a given sample and its negative control. The reading thus obtained was expressed on an arbitrary linear scale. To allow for day to day variation in measured optical density a cytospin preparation from one specific control was always stained and counted within every batch of optical density measurements. Thresholds were then adjusted each time to this standard preparation. Results between different batches could then be related to each other using the result of the specific constant control.

The relative proportions of macrophage subsets was determined using monoclonal antibodies RFD1 and RFD7 (Royal Free Hospital School of Medicine, London, UK) in "double immunofluorescence" methods.<sup>21</sup> Such analysis has been shown to discriminate between antigen presenting cells (RFD1 + RFD7 -), mature phagocytes (RFD1 - RFD7 +), and suppressive macrophages (RFD1 + RFD7 +).<sup>7</sup> Here an initial layer of a mixture of monoclonal antibodies RFD1 (IgM) and RFD7 (IgG) at appropriate concentration was applied to the cytospin preparations for 45 minutes. After washing in PBS a second layer containing a

Table 1 Demographic data of study populations

	HIV (n=22)	Control (n=10)
Male sex	90% (20/22)	80% (8/10)
Median age (years)	29 (21-42)	24 (21-30)
Median blood CD4 count (cells/ $\mu$ l)	545 (410-1450)	855 (360-1520)
Median blood $\beta_2$ -microglobulin (mg/ml)	2.5 (1.3-3.7)	1.5 (1.3-2.3)
Proportion of smokers	73% (16/22)	30% (3/10)
Median duration of smoking (pack years)	5 (0.5-30)	2.5 (0.5-5)

Median (range) values given where appropriate.

mixture of affinity purified goat antimouse IgM (conjugated to fluorescein isothiocyanate, FITC) and a goat antimouse IgG (conjugated to tetramethylrhodamine isothiocyanate, TRITC) (both from Southern Biotechnology, Birmingham, Alabama, USA) was applied for 30 minutes. The cytopsin preparations were then washed and mounted in PBS/glycerol (9:1) and examined under a Zeiss fluorescence microscope equipped with epi-illumination and barrier filters for FITC and TRITC. Positive cells were counted in multiple high power fields (minimum of 100 cells counted). The proportion of each phenotypic subset was determined by the formula:

$$\text{proportion} = \frac{\text{no. specific phenotype} \times 100}{(\text{RFD1} + \text{RFD7} -) + (\text{RFD1} - \text{RFD7} +) + (\text{RFD1} + / \text{RFD7} +)}$$

#### DATA ANALYSIS

Median percentages of positive cells for each monoclonal antibody were compared between the different groups of subjects using the Mann-Whitney test. Spearman's rank correlation was used to assess the correlation between the expression of macrophage markers and blood immune parameters.

## Results

### CHARACTERISTICS OF STUDY POPULATION

The demographics of the study population are shown in table 1. A difference in the proportion

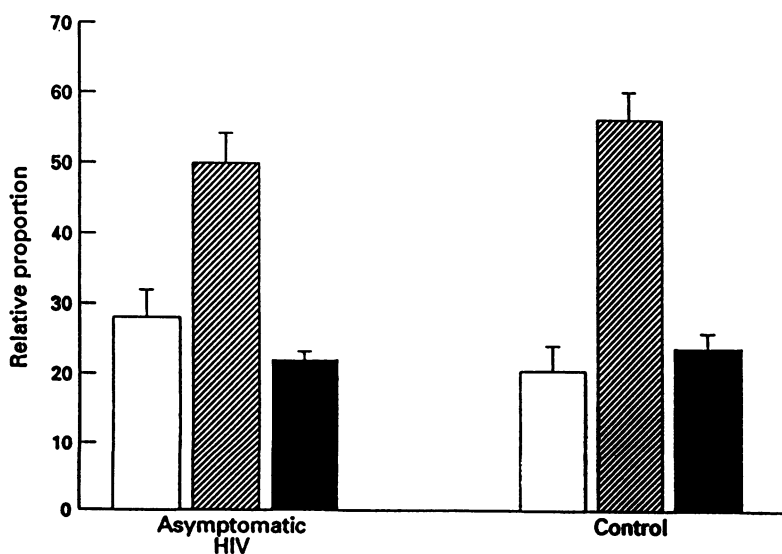


Figure 1 Comparison of macrophage subpopulations in bronchoalveolar lavage fluid from asymptomatic HIV infected subjects and controls. The macrophage subpopulations are distinguished phenotypically by monoclonal antibodies RFD1 and RFD7. Relative proportions of each subpopulation (mean and standard error) are shown. There are similar proportions of RFD1+D7- (□), RFD1-D7+ (▨), and RFD1+D7+ (■) between the two groups.

of HIV positive and control smokers was seen, although when smokers and non-smokers within the two groups were compared no difference in any measured parameter was noted.

Two of the 22 patients had detectable levels of HIV antigen in their serum. There was, however, no difference in immunological analysis of BAL fluid between these and the other HIV patients.

### ABSOLUTE AND DIFFERENTIAL CELL COUNTS

There was no statistical difference in differential cell counts in BAL fluid between HIV infected and control populations. Median differentials expressed as percentage of macrophages, lymphocytes, and granulocytes were 94%, 5%, and 0.5% in the HIV population compared with 94%, 5%, and 0.5% in the controls. Three of the 22 HIV positive subjects and two of the 10 controls had a lymphocyte differential count of >10%. One HIV positive patient had a 16% neutrophilia.

The median absolute alveolar macrophage cell count (determined by pre-filter cell count  $\times$  volume of BAL return  $\times$  macrophage differential count) was raised in the HIV positive group ( $15.4 \times 10^6$  (range  $2.3-58.3 \times 10^6$  cells) compared with  $9.0 \times 10^6$  (range  $1.4-38.8 \times 10^6$  cells) in the controls), but this did not reach statistical significance ( $p=0.1$ ).

### IMMUNOLOGICAL ANALYSIS

The median proportion of alveolar macrophages expressing the monotype marker CD14 did not differ between the HIV and control groups (2.5% (range 1-15%) compared with 2% (range 1-5%),  $p>0.5$ ). There was no difference in the proportions of CD68+ cells found in the BAL fluid of the two populations: the median proportion staining positive in the HIV group was 97% (range 85-99%) compared with 95% (range 85-98%) in the controls ( $p>0.2$ ).

No difference was seen between the two groups in the relative proportions of macrophages expressing antigen presenting cell (RFD1+RFD7-), phagocytic cell (RFD1-RFD7+), and suppressive cell (RFD1+RFD7+) phenotypes. Expressed as RFD1+:RFD7+:RFD1+/RFD7+ the mean values were 28:50:22 and 20:56:24 for HIV positive subjects and controls, respectively (fig 1).

Median HLA-DR expression on alveolar macrophages as measured by optical density readings was increased by 60% in the HIV positive group ( $p<0.005$ ) (fig 2). The distribution of this expression showed greater variation in the HIV positive group than in the controls. The overall range of expression by cells of the control group fell within that of the HIV positive group. Thus, only a proportion of HIV positive alveolar macrophages showed increased expression (fig 3).

### SIGNIFICANCE OF SMOKING

The comparison of 16 smokers and six non-smokers within the HIV positive group revealed

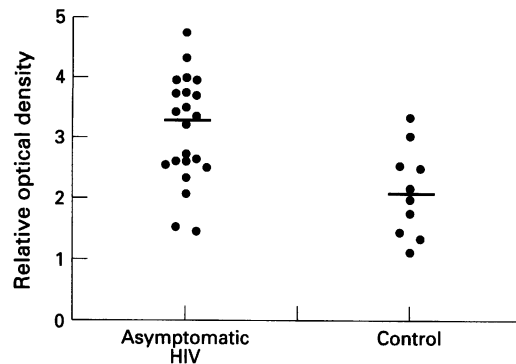


Figure 2 Expression of HLA-DR on alveolar macrophages from asymptomatic HIV infected subjects and controls. Each dot represents median HLA-DR expression of the alveolar macrophages of one subject. Overall median values for the two populations are denoted by the horizontal bars ( $p < 0.005$ ).

no significant effect of smoking on HLA-DR expression or alveolar macrophage sub-population phenotypes (table 2). In a similar manner the results of alveolar macrophage analysis in the three control subjects who smoked was no different from the other volunteers (data not shown). When the non-smokers in both groups were compared no statistically significant difference was found: median expression of HLA-DR of 2.88 (range 1.4–4.3) in HIV positive non-smokers and 1.97 (range 1.1–3.2) in controls. This result substantiates our finding that the statistical difference seen between the whole groups (HIV positives versus controls) reflects an increase in a proportion of HIV positive subjects as shown in fig 3.

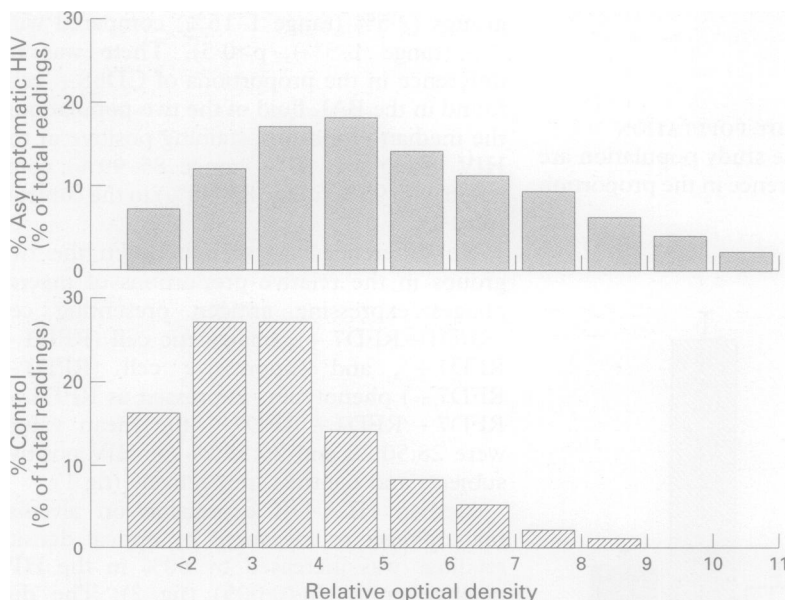


Figure 3 Expression of HLA-DR on alveolar macrophages for the asymptomatic HIV positive and control groups. All readings for all subjects have been used and represented as a frequency histogram.

Table 2 Comparison of HIV positive smokers and non-smokers

	Smokers	Non-smokers	p value
Median (range) HLA-DR	3.37 (2.06–4.74)	2.88 (1.44–4.32)	NS
Macrophage subsets (RFD1+:D7+:D1+D7+)	31:47:22	19:60:22	NS

NS = not significant.

#### RELATIONSHIP WITH CLINICAL STATUS AND BLOOD IMMUNE PARAMETERS

Immunological analysis of BAL fluid was unaffected by the presence (CDC group III) or absence (CDC group II) of clinically detectable lymphadenopathy. Using Spearman's rank correlation no relationship was found between blood CD4 counts,  $\beta_2$ -microglobulin levels, or CD4/ $\beta_2$ -microglobulin and macrophage HLA-DR expression. (CD4:  $\rho = 0.19$ ,  $p = 0.39$ ;  $\beta_2$ -microglobulin:  $\rho = 0.17$ ,  $p = 0.45$ ; CD4/ $\beta_2$ -microglobulin:  $\rho = 0.07$ ,  $p = 0.74$ ).

#### Discussion

Our study reveals that asymptomatic HIV infection is associated with significantly raised levels of alveolar macrophage MHC class II expression. However, this change in DR expression is not associated with any detectable change in the proportions of alveolar macrophage subsets.

The observation of increased levels of HLA-DR is in agreement with Buhl and coworkers who found increased expression of class II MHC molecules DR, DQ, and DP on alveolar macrophages from asymptomatic HIV infected patients.<sup>22</sup> A similar picture of increased MHC class II expression, albeit confined to tissue macrophages, is seen in maedi visna virus infection in sheep (a lentiviral illness producing multisystemic disease through persistent infection of the monocyte/macrophage lineage).<sup>23</sup>

Few human studies have investigated alveolar macrophage antigen expression in asymptomatic HIV infection. Results from patients with pneumonitis have suggested that there is a loss of HLA-DR expression with advancing disease.<sup>22,24</sup> This loss also occurs from skin Langerhans cells in AIDS patients,<sup>25</sup> to a variable degree in blood monocytes,<sup>26–28</sup> but not in gut macrophages.<sup>29</sup> Heagy and others have also shown that progressive clinical disease was associated with a loss of MHC class II expression which could be restored by incubation with interferon  $\gamma$ .<sup>26,30,31</sup> Indeed, the increase in HLA-DR observed in our study may be a reflection of increased interferon  $\gamma$  production in these asymptomatic patients as previously suggested by Buhl.<sup>22</sup>

Our finding of increased HLA-DR expression on alveolar macrophages is consistent with the enhanced ability of such cells to stimulate concanavalin A and pokeweed mitogen lymphocyte proliferation assays as previously shown.<sup>32</sup> Taken together with the recent demonstration that, even in patients with respiratory symptoms and low blood CD4 counts there is an increased ICAM-1 expression on alveolar macrophages,<sup>33</sup> the data suggest that sustained interactions between macrophages and lymphocytes may be contributing to the inflammatory response at all stages of HIV infection.

The central role of alveolar macrophages as inductive, suppressive, and effector cells means that interaction between alveolar macrophages and HIV can affect both lymphocyte function (through failure of antigen presentation<sup>11,34</sup>) and phagocyte function.<sup>35</sup> In our study we

found no evidence for alterations in the proportions of alveolar macrophages expressing phenotypic markers of antigen presenting, phagocytic, and suppressive cell subpopulations compared with controls. This contrasts with findings in both the lung<sup>2</sup> and gut mucosa<sup>29</sup> of AIDS patients where there was a significant reduction in the accessory cell phenotypic marker RFD1, implying that such reductions are associated with advancing disease.

Differential cell populations in the BAL fluid appear to have a degree of prognostic significance in HIV patients with pneumonitis.<sup>36</sup> An alveolar CD8 lymphocytosis has been described in patients both with and without respiratory symptoms.<sup>37,38</sup>

In our study group we found lymphocytosis to be no more common than in the control population. This may reflect the patient selection in that all patients studied had peripheral blood CD4 counts within the normal range.

More of the HIV positive group were cigarette smokers, which has been shown to increase HLA-DR expression.<sup>39</sup> However, we could find no evidence to suggest that smoking was responsible for our results.

In conclusion, we found significant increases in HLA-DR expression on alveolar macrophages in the lungs of asymptomatic HIV patients that were unrelated to smoking. This was not explained by an overall change in the proportion of phenotypically distinct macrophage subpopulations. Whether this finding reflects an HIV-induced functional alteration in the immune response is the subject of ongoing investigation.

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