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## Activated and memory alveolar T-lymphocytes in idiopathic eosinophilic pneumonia

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*Activated and memory alveolar T-lymphocytes in idiopathic eosinophilic pneumonia. C. Albera, P. Ghio, P. Solidoro, I. Mabritto, L. Marchetti, E. Pozzi. ©ERS Journals Ltd, 1995.*

**ABSTRACT:** To investigate the possible involvement of T-lymphocytes in the immunopathogenesis of idiopathic eosinophilic pneumonia (IEP), we have evaluated the phenotypic characteristics both of peripheral blood and alveolar lymphocytes in six patients with symptomatic IEP, and in 24 healthy nonsmokers as controls, by employing bronchoalveolar lavage (BAL), monoclonal antibodies, and flow-cytometry.

In IEP, total and differential cell counts showed a mild alveolitis with an increase of eosinophil percentage and number; the alveolar lymphocyte count was also increased. In BAL, the total number both of CD4+ and CD8+ lymphocytes was significantly raised; CD4+ cells expressed early (CD25) activation antigens. The analysis of CD45R0, CD45RA and CD62L coexpression in IEP patients, when compared to healthy controls, revealed an accumulation of alveolar CD4+ cells showing phenotypic repertoire usually expressed by memory T-cells (CD45R0+, CD45RA-, CD62L-). CD8+ alveolar lymphocytes did not show any significant increase of activation antigen coexpression. Circulating lymphocytes were not significantly increased and showed only a significantly higher CD25 expression.

These data suggest that a pivotal role is played by activated and memory CD4+ alveolar lymphocytes in IEP patients.

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Eosinophilic lung disorders are characterized by the accumulation of eosinophil leucocytes in pulmonary structures (alveolar spaces, interstitium) and, frequently, also in the peripheral blood [1]. In patients with idiopathic eosinophilic pneumonia (IEP), bronchoalveolar lavage (BAL) shows an increase of the total cell count, with a marked increase of eosinophils; the percentage of macrophages is consequently reduced, whereas lymphocytes do not show significant changes [1]. Eosinophilic lung disorders include Loeffler's syndrome [2], Aspergillus infection [3], eosinophilic pneumonia in bronchial asthma [4], drug-induced pneumonia [5], tropical eosinophilia [6], chronic eosinophilic pneumonia [7], acute eosinophilic pneumonia with respiratory failure [8], Churg-Strauss syndrome [9], and idiopathic eosinophilic pneumonia [10].

Mechanisms leading to the parenchymal injury in these diseases are mainly mediated by factors contained in eosinophil granules [11]. Some useful information on the immunopathogenesis of idiopathic eosinophilic lung disease could be obtained by the analysis of processes physiologically involved in eosinophil maturation and traffic. In fact, in normal conditions, lymphocytes, recruited and activated by antigen-presenting cells, release lymphokines, such as interleukin-3 (IL-3) [12] granulocyte/macrophage colony-stimulating factor (GM-CSF)

[13], interleukin-4 (IL-4) [14] and interleukin-5 (IL-5) [13, 15], regulating eosinophil differentiation, maturation, proliferation, recruitment and activation [16]. These physiological activities, if upregulated, may have a central role in the modulation of eosinophil-mediated tissue damage within the alveolar microenvironment [17]. On the other hand, the accumulation of eosinophils in the lung, observed in experimental conditions in mice, appears to be lymphocyte-dependent, mast-cell-independent and mainly IL-5 mediated [15]. The aim of this study was to investigate a possible involvement of T-lymphocytes in the pathogenesis of IEP [18], by employing BAL, monoclonal antibodies and flow-cytometry.

### Patients and methods

#### Study population

Six symptomatic patients with IEP were studied. They included two males and four females, aged 37–58 yrs (mean±SD 43±9 yrs, one smoker and five nonsmokers).

Diagnosis, in all patients, was made on the basis of clinical, laboratory and chest radiographic data, and was

confirmed by histological findings on specimens from transbronchial lung biopsies performed during bronchoscopy, after bronchoalveolar lavage, in the same lung lobe or segment showing radiological abnormalities. Histological analysis showed eosinophilic infiltration of lung parenchyma without vasculitis, fibrosis, granulomas or necrosis; no evident increase of either S100+ or CD1+ cell was found either in tissues or in BAL. No parasitic infestation, exposure to chemicals, concomitant infection, drug intake or abnormal immunoglobulin E (IgE) levels were present in the population studied. No patient had a medical history of asthma, and pulmonary function tests excluded actual obstruction; in all cases, the disease was symptomatic (fever, cough, dyspnoea); elevation of erythrocyte sedimentation rate and resting hypoxaemia were evident (arterial oxygen tension ( $P_{a,O_2}$ ) <8 kPa). All patients studied had abnormal chest radiographs, showing peripheral nonsegmental infiltrates. In two patients, the clinical presentation was acute (disease duration 1–4 weeks), and in four it was subacute or chronic (disease duration more than 12 weeks). No patients had received steroids or any other therapy at the time of the study, and all showed a dramatic improvement of clinical signs and symptoms, and of radiological opacities after corticosteroid treatment (prednisone 1 mg·kg<sup>-1</sup> q.d. for 2–4 weeks).

As a control group, 24 healthy nonsmokers were studied; this group consisted of 14 males and 10 females, aged 37–58 yrs (mean±SD 56±14 yrs).

#### Cell isolation and preparation

Bronchoalveolar lavage was performed using the method described previously [19]. Briefly, informed consent was obtained from each subject; after a pretreatment with parental atropine sulphate (0.5 mg), the airways were anaesthetized with 2% lidocaine. A fiberoptic bronchoscope (Olympus BF-1T10) was advanced and wedged into either the middle lobe (controls) or into the radiologically affected segments or lobes (IEP patients) under direct visualization. Lavage was carried out with 200

mL of prewarmed (37°C) sterile saline solution in ten 20 mL aliquots, with immediate gentle vacuum (syringe) aspiration after each injection. The total amount of fluid recovered was measured (always >60% of injected saline); the first aliquot recovered was examined separately for cytological and bacteriological studies, and the rest were pooled. Immediately after lavage, the fluid was filtered through two layers of sterile surgical gauze. Cells were separated from the lavage fluid by centrifugation in a Haeraeus Minifuge-T refrigerated centrifuge (4°C, 20 min, 900 rpm) and washed in balanced salt solution (phosphate buffered saline (PBS) Serva). Total cell count and viability evaluation (trypan blue dye exclusion test) were performed on a Bürker haemocytometer; differential cell count was carried out on Diff-Quick (Don Baxter) stained cytospin smears, counting at least 400 cells. Immediately before BAL, peripheral blood samples were obtained from each patient and control subject by venipuncture: 10 mL vacuum sterile test tubes containing lithium heparin (Vacutainer, BD) were employed. Lymphocytes were separated by centrifugation of blood samples after stratification on density gradient (Lymphoprep, Nyegaard) for 20 min at 1,000 rpm. Simultaneously, haemochromocytometric assay was performed (Technicon Hematology System H3) to evaluate total and differential count of white blood cells.

#### Phenotypic studies

Nonadherent cells were obtained by 1 h incubation at 37°C in humidified 5% CO<sub>2</sub> atmosphere (Haeraeus auto-zero incubator), either from whole cellular BAL pellet or mononuclear cells from peripheral blood, washed twice in PBS and resuspended in sterile RPMI 1640 (Serva) in sterile 250 mL polystyrene flasks (Falcon). Phenotypic characteristics were evaluated by employing a panel of commercially available monoclonal antibodies (table 1) and flow-cytometry (Facsan, Becton-Dickinson). Standard staining procedure [20] and single or two-colour analysis with Facsan software (Becton-Dickinson) were used (at least 5,000 events were assumed for each sample).

Table 1. – CD, characteristics, specificity, and source of monoclonal antibodies employed

CD	Commercial	Specificity
CD3	Anti-Leu 4 *	Mature T-lymphocytes
CD4	Anti-Leu 3 *	Helper-inducer T-lymphocytes
CD8	Anti-Leu 2 *	Suppressor-cytotoxic T-lymphocytes
CD25	Anti-IL2R *	p55 IL-2 receptor
CD49a	Anti-VLA-1 #	β-intergrin; late-activated, not proliferating T-lymphocytes
CD45RA	Anti-Leu18 *	Isoform of common leucocyte antigen; naive T-lymphocytes
CD45R0	Anti-UCHL1 *	Isoform of common leucocyte antigen; memory T-lymphocytes
CD62L	Anti-Leu 8 *	L-selectin; adhesion molecule
CD45	Anti-HLA-DR *	MHC class II-related antigen
CD14	Anti-HLe-1 *	Pan-leucocyte
	Anti-LeuM3 *	Monocytes

\*: Source Becton-Dickinson (Mountain View, CA, USA); #: source T-Cell Science (Cambridge, MA, USA). IL-2: interleukin-2; VLA-1: very late activation antigen-1; HLA-DR: human leucocyte antigen-DR; MHC: major histocompatibility complex.

Lymphocyte gates were obtained by using linear forward angle light scatter; the lymphocyte gates were validated by employing CD45-fluorescein-conjugated (pan-leucocyte) and CD14 phycoerythrin-conjugated (monocyte) monoclonal antibodies [20]. The lymphocyte population was defined as CD45<sup>bright</sup> CD14<sup>negative</sup>; more than 95% of all lymphocytes in each sample was contained within the boundaries of the lymphocyte light-scatter gate.

### Statistical analysis

Data are expressed as mean±standard error of the mean (SEM); results obtained were analysed by Mann-Whitney-Test. A p-value of less than 0.05 was considered statistically significant.

## Results

All samples from IEP patients showed no growth of Gram-positive or Gram-negative bacteria, mycobacteria, or fungi. More than 95% of cells recovered were viable in all samples studied. Total and differential cell counts in patients with IEP showed, when compared to healthy controls, a mild alveolitis ( $2.9\pm 0.3$  vs  $1.8\pm 0.1 \times 10^5$  cells·mL<sup>-1</sup> p<0.001), an increase both of eosinophil percentage ( $49\pm 1$  vs 0%; p<0.001) and eosinophil absolute number ( $141.2\pm 34.4$  vs  $0 \times 10^3$  cells·mL<sup>-1</sup>; p<0.001). Neutrophil percentage, as well as total count, showed a mild but significant increase in IEP patients when compared to healthy controls ( $3.0\pm 0.5$  vs  $0.9\pm 0.4\%$ , respectively; p<0.008; and  $11.1\pm 1.6$  vs  $1.1\pm 0.4 \times 10^3$  cells·mL<sup>-1</sup>, respectively; p<0.001). No significant changes in lymphocyte mean percentage were seen ( $9.7\pm 1.8$  vs  $6.7\pm 1.3\%$ ; p=NS). The absolute number of alveolar lymphocytes was significantly increased, being about three times more than in healthy controls ( $27.7\pm 5.1$  vs  $10.8\pm 2.3 \times 10^3$  cells·mL<sup>-1</sup>; p<0.03).

CD3+, CD4+ and CD8+ alveolar lymphocytes were evaluated either as a percentage of positive cells or as absolute number of positive cells per millilitre fluid (table 2). When data were expressed as percentage of positive cells, CD3+ lymphocytes were higher in healthy controls

Table 2. – Percentage and absolute number of BAL CD3+, CD4+, CD8+ lymphocyte subsets in IEP patients and in control group

	IEP patients	Healthy controls	p-values
CD3+			
Cells %	83±1	91±1	<0.003
Cells ×10 <sup>3</sup> ·mL <sup>-1</sup>	25.0±7.5	9.9±1.8	<0.01
CD4+			
Cells %	53±6	51±2	NS
Cells ×10 <sup>3</sup> ·mL <sup>-1</sup>	16.7±6.8	5.5±1.0	<0.02
CD8+			
Cells %	31±11	37±2	NS
Cells ×10 <sup>3</sup> ·mL <sup>-1</sup>	8.4±1.9	4.2±0.8	<0.04
CD4/CD8 ratio	2.1±0.7	1.5±0.2	NS

Data are presented as percentage of all lymphocytes and as number of positive lymphocytes ×10<sup>3</sup>·mL<sup>-1</sup> (mean±SEM). BAL: bronchoalveolar lavage; IEP: idiopathic eosinophilic pneumonia; NS: nonsignificant.

Table 3. – Percentage of BAL CD3+, CD4+, and CD8+ lymphocytes expressing HLA-DR, CD25, CD49a, CD45R0, CD45RA, and CD26L in IEP patients and in control group

	IEP patients	Healthy controls	P-values
CD3+/HLA-DR+	25±5	16±2	NS
CD4+/CD25+	16±3	3±1	0.001
CD4+/CD49a+	13±2	7±2	NS
CD4+/CD62L+	2±1	3±0.6	NS
CD4+/CD62L-	55±8	45±1.9	NS
CD4+/CD45R0+	55±8	47±3	NS
CD4+/CD45RA+	2±1	6±1	NS
CD8+/CD25+	2±1	2±1	NS
CD8+/CD49a+	13±1	16±1	NS

Data are presented as percentage of positive lymphocytes (mean±SEM). For abbreviations see legend to table 2.

than in IEP patients, whereas no significant differences of CD4+ and CD8+ subsets were observed. Consequently, no significant changes of the CD4/CD8 ratio were observed, even in the presence of a slight increase of this ratio in IEP patients. When results were expressed as number of positive cells ×10<sup>3</sup>·mL<sup>-1</sup> recovery fluid, an increase in CD3+ cells, related to a high number both of CD4+ and CD8+ lymphocytes could be seen in IEP patients

The coexpression of activation antigens were referred and analysed either as percentage of all lymphocytes (table 3) or as absolute count (cells ×10<sup>3</sup>·mL<sup>-1</sup> recovered fluid) (table 4). When data were expressed as percentage of positive cells of all lymphocytes an increase of CD25 expression on CD4+ lymphocytes was observed ( $16.1\pm 2.7$  vs  $3.3\pm 1\%$ ; p<0.001). When data were expressed as a number of positive cells ×10<sup>3</sup>·mL<sup>-1</sup> recovered fluid we found a similar increased coexpression of CD25 on CD4+ lymphocytes ( $2.8\pm 0.7$  vs  $0.7\pm 0.4 \times 10^3$  cells·mL<sup>-1</sup>; p<0.05). CD8+ lymphocytes, on the contrary, did not show significant differences regarding the expression both of CD25 and CD49a activation antigens (percentage and total cell number). The evaluation of coexpression on CD4+ alveolar lymphocytes of CD45R0 and CD45RA (tables 3 and 4) showed the percentage and, in particular,

Table 4. – Absolute number of BAL CD3+, CD4+, and CD8+ lymphocytes expressing HLA-DR, CD25, CD49a, CD45R0, CD45RA, and CD62L in IEP patients and in control group

	IEP patients	Healthy controls	P-values
CD3+/HLA-DR+	4±1.3	2.5±0.7	NS
CD4+/CD25+	2.8±0.7	0.7±0.4	<0.05
CD4+/CD49a+	2.8±2	1.2±0.8	NS
CD4+/CD62L+	0.4±0.3	0.3±0.1	NS
CD4+/CD62L-	15.8±8.7	4.1±0.6	<0.02
CD4+/CD45R0+	15.9±8.6	4.7±1	<0.03
CD4+/CD45RA+	0.6±0.3	0.8±0.2	NS
CD8+/CD25+	0.4±0.2	0.2±0.1	NS
CD8+/CD49a+	2.8±1.2	2.8±0.1	NS

Data are presented as number of positive lymphocytes ×10<sup>3</sup>·mL<sup>-1</sup> (mean±SEM). For abbreviations see legend to table 2.

Table 5. – Absolute number of peripheral blood CD3+, CD4+ and CD8+ lymphocyte subsets in IEP and in control group

	IEP patients	Healthy controls	p-values
CD3+	2.1±0.3	2.1±0.3	NS
CD4+	1.3±0.2	1.3±0.1	NS
CD8+	0.9±0.1	0.9±0.1	NS
CD4/CD8 Ratio	1.5±0.1	1.4±0.1	NS

Data are presented as number of positive lymphocytes  $\times 10^3 \cdot \mu\text{L}^{-1}$  (mean $\pm$ SEM). For abbreviations see legend to table 2.

Table 6. – Absolute number of peripheral blood cells CD3+, CD4+ and CD8+ lymphocytes expressing HLA-DR, CD25, CD49a, CD45RO, CD46RA and CD62L in IEP patients and in control group

	IEP patients	Healthy controls	P-values
CD3+/HLA-DR+	0.12±0.03	0.07±0.02	NS
CD4+/CD25+	0.20±0.05	0.11±0.01	<0.002
CD4+/CD49a+	0.001±0.001	0.001±0.001	NS
CD4+/CD62L+	1.00±0.10	1.10±0.20	NS
CD4+/CD62L-	0.28±0.13	0.18±0.40	NS
CD4+/CD45RO+	0.57±0.11	0.40±0.80	NS
CD4+/CD45RA+	0.70±0.21	0.82±0.13	NS
CD8+/CD25+	0.01±0.004	0.02±0.004	NS
CD8+/CD49a+	0.005±0.003	0.004±0.002	NS

Data are presented as number of positive lymphocytes  $\times 10^3 \cdot \mu\text{L}^{-1}$  (mean $\pm$ SEM). For abbreviations see legend to table 1 and 2.

the number ( $15.9 \pm 8.6$  vs  $4.7 \pm 1 \times 10^5$  cells $\cdot\text{mL}^{-1}$ ;  $p < 0.03$ ) of memory (CD45RO+) T-cells in IEP was higher than in controls. Both low percentage and number of CD4+/CD45RA+ lymphocytes was observed in patients with IEP. In patients with IEP, an increase of CD4+/CD62L- lymphocytes, which was significant when evaluated as absolute cell number ( $15.8 \pm 8.7$  vs  $4.1 \pm 0.6 \times 10^5$  cells $\cdot\text{mL}^{-1}$ ;  $p < 0.03$ ), was observed (table 4).

In the peripheral blood of IEP patients, eosinophils were significantly higher than in controls ( $3.2 \pm 0.9$  vs  $0.4 \pm 0.1 \times 10^3$  cells $\cdot\mu\text{L}^{-1}$ , respectively;  $p < 0.006$ ) and only a significant increase of CD4/CD25+ lymphocytes was observed ( $0.2 \pm 0.05$  vs  $0.11 \pm 0.01 \times 10^3$  cells $\cdot\mu\text{L}^{-1}$ ;  $p < 0.02$ ) (table 5–6).

### Discussion

We demonstrated that patients with symptomatic IEP, when compared to healthy nonsmoking subjects, were characterized by an increase in total cells, eosinophils and lymphocytes in BAL fluid. The higher number of alveolar CD3+ cells was related both to CD4+ and CD8+ subsets. The analysis of expression of CD45RO and CD45RA, the two isoforms of a common leucocyte antigen preferentially expressed, respectively, on primed (*via* T-cell receptor) and on unprimed T-lymphocytes, and of L-selectin CD62L, which enables T-cells to adhere to a ligand expressed on high endothelium venules (HEV),

showed that the alveolitis of IEP patients was characterized by an accumulation of CD4+ lymphocytes expressing a memory phenotypic repertoire (CD45RO+, CD45RA-, CD62L-) [21–23]. In addition, accumulation of CD4+ alveolar lymphocytes showing a phenotypic pattern typical of lymphokine-secreting cell, characterized by the expression of early (CD25) activation antigens, was observed in IEP patients.

In the peripheral blood, only a slight increase of eosinophils was observed, accompanied by a less relevant accumulation of CD4+/CD25+ lymphocytes. These differences may be explained either by the preferential accumulation of different cell populations in different tissues and organs [18], or by the ability of activated eosinophils to act as antigen presenting cells for T-lymphocytes [24], leading to a self-maintaining mechanism of lung eosinophilic inflammation.

A similar increase in CD4+/CD25+ lymphocytes both in BAL and in peripheral blood has been observed [21] in allergic asthma, an eosinophil-mediated airway disease, and the number of alveolar CD4+/CD25+ cells was shown to correlate with the number of eosinophils and the severity of disease [25].

As far as the CD8+ lymphocyte subsets were concerned, there was a significant increase of total number of CD8+ cells, without any significant difference of activation antigen expression, suggesting that suppressor/cytotoxic T-lymphocytes are not actively involved in immunomodulation of idiopathic eosinophil-mediated lung disease.

In summary, we found in patients with symptomatic idiopathic eosinophilic pneumonia, an accumulation of alveolar CD3+ T-lymphocytes, both of CD4 and CD8+ subsets. The CD4+ helper/inducer T-lymphocytes exhibited the surface antigens typically associated with either activated or memory T-lymphocytes. Both activated and memory T-lymphocyte subsets may be involved in the modulation of eosinophilic inflammation in the alveolar spaces.

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