

## REVIEW ARTICLE

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# Flow Cytometry Applications in the Study of Immunological Lung Disorders

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## ABSTRACT

The use of flow cytometry in the clinical laboratory has grown substantially in the past decade. Flow cytometric analysis provides a rapid qualitative and quantitative description of multiple characteristics of individual cells. For example, it is possible to detect the cell size and granularity, aspects of DNA and RNA content and the presence of cell surface and nuclear markers which are used to characterize the phenotype of single cells.

Flow cytometry has been used for the immunophenotyping of a variety of specimens including whole blood, bone marrow, serous cavity fluids, (cerebrospinal fluid) CSF, urine and all types of body fluids. The technique has also been applied to human bronchoalveolar lavage (BAL) fluid, peritoneal fluids and blood.

In this review, we describe the current status of the application of flow cytometry as a diagnostic tool in various lung diseases.

We focus on the analysis of BAL cell composition in chronic obstructive lung disease (COPD), asthma, lung cancer, sarcoidosis, tuberculosis and idiopathic eosinophilic pneumonia (IEP).

**Keywords:** Asthma; Broncho alveolar lavage (BAL); COPD; Flow cytometry; Lung disease; Sarcoidosis

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## INTRODUCTION

Bronchoalveolar lavage (BAL) provides an important various diffuse lung diseases. For example,

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BAL fluid diagnostic tool that can help facilitate the diagnosis of can be analyzed to determine the profile of white blood cells and to detect respiratory pathogens.<sup>1</sup> Human BAL is considered as a mirror of lung inflammation and is believed to provide insight into the underlying pathophysiology of many chronic and acute lung diseases. For example, numerous investigators have reported flow cytometric analysis of BAL cells and alterations in BAL composition, particularly in T-lymphocyte subsets, in sarcoidosis.<sup>2-4</sup> In addition to the presence of inflammatory mediators, the cellular components of the lungs and BAL are also involved in the pathogenesis of lung disease. Each disease has a specific BAL cellular composition, for example, in asthma the important cells in BAL and lungs are considered to be Th2 T-cells, eosinophils and dendritic cells whereas in COPD, neutrophils and macrophages are the predominant cells detected in BAL fluid. Thus, the application of flow cytometric analysis have proved highly informative in defining the details of cellular subsets, often by the use of specific antibodies against cell surface markers or cluster of differentiation (CD) markers, which help to differentiate between selective subsets of cells characteristic of a particular disease.

We discuss here the application of flow cytometry as a potentially useful tool in the diagnosis of various lung diseases including chronic obstructive lung disease (COPD), asthma, lung cancer, sarcoidosis, tuberculosis and idiopathic eosinophilic pneumonia (IEP).

### COPD

Cigarette smoking is the most important risk factor for COPD and is expected to emerge as the third most common cause of death by 2020.<sup>5,6</sup> Cigarette smoke induces both the release of numerous inflammatory mediators including chemokines from airway epithelial cells and alveolar macrophages. This results in the recruitment of neutrophils, monocytes, CD8+ and CD4+ cells into the lungs and induces the release of excessive amounts of proteases by macrophages and neutrophils.<sup>7</sup> Investigation of BAL fluid antigens and cells in COPD patients have added greatly to our understanding of this disease. In a similar manner, animal models of COPD have provided useful tools to investigate the mechanisms underlying cellular recruitment and their activation status. In one study, increased levels of BAL macrophages, neutrophils, and lymphocytes were reported in BAL fluid 24h after

exposure to mainstream and sidestream cigarette smoke.<sup>7</sup> This, in part, mimics the relationship found between the number of cytotoxic CD8+ T-cells in BAL and the decline in lung function in COPD patients<sup>8,9</sup> and the altered balance between CD4+ helper T cells and CD8+ cytotoxic T-cells in the lungs of COPD patients.<sup>9</sup> These types of studies indicate that analysis of the BAL cellular composition in combination with clinical phenotyping could help the physician to better characterize COPD patients. Unfortunately, currently fluorescence activated cell sorting (FACS) analysis alone cannot be used to clearly differentiate between the stages of COPD severity and this is an area that requires a greater research effort.

### Asthma

Asthma is characterized as a chronic inflammatory disease of the airways associated with increased numbers of Th2 cells and the concomitant recruitment of granulocytes. The clinical manifestations of asthma are associated with increased levels of the Th2 cytokines IL-4, IL-5 and IL-13 in the serum and lungs. Flow cytometry has been used in the determination of surface markers on eosinophils<sup>10</sup> and neutrophils in asthmatic patients.<sup>11</sup> A number of mouse models have been used in conjunction with human studies to further define the cellular subsets involved in asthmatic inflammation and airway hyperresponsiveness.<sup>12</sup> For example, Van Rijt et al. described a flow cytometric method for differential cell counts of murine BAL fluid cells by staining with a combination of commercially available antibodies for MHCII, CCR3, CD3, B220 and CD11c markers on T and B cells.<sup>12</sup> Using a combination of cell size, granularity and fluorescence, these authors were able to confirm that the eotaxin receptor (CCR3) was expressed on eosinophils, CD3 expressed on T cells, B220 expressed on B cells, MHCII expressed on B cells and DCs and CD11c was expressed on DCs.<sup>13</sup>

An allergic reaction is characterized by the synthesis of allergen-specific immunoglobulin of the IgE class and Th2 cytokines (IL-4, IL-5, and IL-13), which leads to the recruitment and sensitization of effector cells such as eosinophils, dendritic cells, basophils, and mast cells.<sup>14,16</sup> Thus, it is becoming increasingly possible to use flow cytometry as a diagnostic tool to aid the clinical characterization of patients by measuring intracellular cytokine and chemokine levels in combination with cell-specific CD markers.<sup>17</sup>

### Sarcoidosis

Sarcoidosis is a systemic disease characterized by the presence of non-caseating granulomas in affected organs, with the lung being the major diseased organ in more than 90% of patients.<sup>18,19</sup> Studies on BAL fluid in pulmonary sarcoidosis have demonstrated that sarcoid granuloma formation in the lung is preceded by an influx of mononuclear cells into the alveoli.<sup>20</sup> The presence of activated alveolar macrophages and CD4+ (helper/inducer) T-lymphocytes were shown as markers of alveolitis.<sup>21,22</sup> Furthermore, the increased numbers of BAL CD4+ T cells are considered a hallmark of pulmonary sarcoidosis.<sup>23,24</sup> The percentages of CD4+ and CD8+ T-cells in BAL correlate well with IHC methods in sarcoidosis patients.<sup>25</sup> These data suggest that FACS analysis of BAL CD4/CD8 ratios in conjunction with the determination of angiotensin-converting enzyme (ACE) and lung imaging (X-ray or HRCT) could provide better and earlier diagnosis of disease. That said, the BAL lymphocytosis, low or normal granulocytes and the increase in CD4+/CD8+ ratio seen in pulmonary sarcoidosis are not disease-specific and further more refined flow cytometric tests may be required to aid diagnosis.<sup>26-28</sup>

### Lung Cancer

Lung cancer remains the most lethal of all cancers worldwide with a dismal prognosis and 5-year survival rate of less than 15%.<sup>29</sup> Lung cancer is subdivided into two major subtypes based on their histology: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC includes adenocarcinoma, squamous cell carcinoma and large-cell carcinoma<sup>30</sup> and represents roughly 80% of all pulmonary cancers.<sup>31</sup> NSCLC is relatively insensitive to chemotherapy compared with the SCLC<sup>29</sup> and presents commonly as an incurable locally advanced or metastatic disease.<sup>29,30</sup> A variety of diagnostic tools are currently applied for the detection of lung cancers including immunohistochemistry (IHC), together with chromogenic in situ hybridisation (CISH) and fluorescence in situ hybridisation (FISH). Flow cytometric analysis of BAL and or lung homogenates could potentially add to the diagnostic tests available.

There are several antigens on tumor cells such as NYESO-1, WT1 antigen, MRP3, MAGE and BAGE family, gp100, SART-1, tyrosinase and MUC-1 which are implicated in the immune response to the tumor.<sup>32,33</sup> A number of groups have determined the difference of

abundance of CD4+, CD8+ and CD56+ BAL lymphocytes and their subpopulations between cancerous and healthy lung tissue from the same patient using flow cytometry.<sup>34-37</sup> CD27+28+ T-cells are immature memory T-cells whereas CD27-28- T-cells are mature forms of CD4+ and CD8+ T lymphocytes.<sup>38</sup> CD27-28- T-cells are more activated in cancerous lung compared to healthy lung whilst the CD27-28- T-cells are less activated in lung cancer. Overall, CD4+ lymphocytes are more activated in cancerous lung compared to the healthy lung, while the CD8+ forms are less activated in lung disease although the number of both CD4+ and CD8+ T-lymphocytes in lung cancer is significantly higher than in healthy tissue from the same patient.<sup>34</sup>

FACS analysis could also be used to determine the degree of apoptosis in lung cancer. Apoptosis is defined by characteristic morphological and biochemical changes.<sup>38</sup> Apoptotic pathways are often functionally inactive in malignant lung cells which results in increased cell survival.<sup>39</sup> For example, the Fas receptor (APO-1 or CD95) and its ligand play a key role in the initiation of one major apoptotic pathway in malignant tumors.<sup>40,41</sup> Loss of the Fas protein has been reported to induce resistance to apoptosis; however, apoptotic resistance in some Fas-expressing malignant cells has also been reported.<sup>42-44</sup> Nambu et al. analyzed the expression of Fas and sFas protein by FACS using anti-Fas Abs<sup>45,46</sup> and described the expression of sFas in human pulmonary adenocarcinoma. Thus, FACS analysis has the potential to play a central role in the evaluation of the Fas expression and in the diagnosis of adenocarcinoma and other NSCLCs. However, IHC, FISH and CISH still remain the gold standard for diagnosis of lung cancer.

### Mycobacterium Tuberculosis (Mtb)

Mtb is a highly successful human pathogen which causes ~10 million deaths worldwide each year.<sup>47</sup> Active tuberculosis occurs in people with apparently normal immune systems and in HIV-infected people before profound depletion of circulating CD4+ lymphocytes.<sup>47</sup> Mtb itself has evolved specific mechanisms for evading destruction by the human immune system. A hallmark stage for Mtb infection is the survival and persistence in macrophages during disease progression.<sup>48</sup> In most healthy people, adaptive T cell responses control but do not eradicate Mtb, resulting in a persistent mycobacterial infection that

can expand and cause disease when T cell immunity fails.<sup>48</sup> Healthy people with persistent infection have robust memory CD4<sup>+</sup> T cell responses, reflected in strong positive tuberculin skin test reactions and high frequencies of Mtb *s*-specific T cells. However, little is known about how Mtb evades and resists this active CD4<sup>+</sup> T cell response. Mtb-specific T cells produce IFN- $\gamma$  that is essential for T cell-mediated immunity.<sup>48-51</sup> IFN- $\gamma$  up-regulates MHC-II Ag processing in macrophages, propagating a protective immune response, but it is inefficient at directly activating human macrophages to kill intracellular bacilli.<sup>52,53</sup>

The heterogeneity of alveolar macrophages recovered from BAL of patients with TB has been investigated. A large percentage of alveolar macrophages were found in the lowest-density fraction in patients with TB.<sup>54</sup> The ability of flow cytometry to detect both intracellular cytokines and chemokines such as INF- $\gamma$  and IL-12 as well as cell surface markers suggest that this technique could be useful in diagnosis. This could be combined with labeling of Mtb with chromogens such as FITC to further improve diagnostic capabilities.<sup>55,56</sup>

### Idiopathic Eosinophilic Pneumonia (IEP)

IEP is characterized by the accumulation of eosinophils in the alveolar spaces and the interstitium of the lung, frequently accompanied by peripheral eosinophilia.<sup>57</sup> Recently, much attention has been focused on the importance of cell-cell interaction through adhesion molecules in the inflammatory process. Various adhesion molecules have been found to be involved in the migration of eosinophils to airway mucosa through vascular endothelial cells.<sup>58,59</sup> To clarify the roles of adhesion molecules of eosinophils in the pathogenesis of eosinophilic pneumonia, Azuma et al. analyzed their expression by eosinophil and T-lymphocyte populations in peripheral blood and BAL obtained in patients with IEP. They reported increased numbers of eosinophils expressing CD11a LFA-1, CD11b (Mac-1), CD18, CD49d (VLA-4), and CD62L (L-selectin) in BAL, but not in the blood, of IEP patients. Over expression of CD54 was seen only in BAL eosinophils which indicate local activation by stimulated T-cells.<sup>60</sup> Finally, measurements of adhesion molecule expression of infiltrated cells, T-lymphocyte activation and cytokines in BAL fluid may be helpful in evaluating disease activity in IEP for predicting the effects of treatment on the disease.

### Important Considerations

Before the implementation of flow cytometric analysis into general use in clinical practice in Iran several issues need to be addressed. It is important to integrate the standard operating procedures used for flow cytometric analysis worldwide with specific procedures used for BAL analysis to produce standardized protocols for the use of each specific flow cytometry machine for the detection of individual cells types or combination of cell types with BAL analysis. Only then can the investigation into how flow cytometric analysis varies with individual disease and severity/subtype of disease be studied and compared with current gold-standard approaches. These studies need to be conducted in designated specialist sites in order to provide the information required before the technology can be used outside of these centers. There are other local issues in Iran relating to problems with obtaining and transporting the correct Abs, which will be important to address before the regular use of flow cytometry can be performed in clinical diagnosis as an adjunct to the physician's clinical knowledge.

### Summary

In summary, although not currently used in routine diagnosis, there is a huge potential to use flow cytometric analysis of BAL cells in the differential diagnosis of many lung diseases. These analyses will initially need to be used in conjunction with current gold standard tests but the ease of use and rapid result time suggests that FACS should eventually be an important part of diagnosis, determination of disease severity and of drug responses.

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