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Original Article

# CD14 Positive Selection Displays an Edge in the Isolation of Macrophages from Induced Sputum of COPD Patients Using Immunobead Technology

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## Abstract

**Objectives:** Macrophages plays an important role in the pathophysiology of COPD. Sputum induction is a safe and non-invasive method for evaluation of airway inflammation in COPD. The present study aims to evaluate the yield of macrophage isolation from induced sputum of COPD patients by using commercially available immunomagnetic bead based approaches.

**Methods:** Sputum induction was done in COPD patients (n=13). Cell pellets obtained after the processing of sputum samples (n=11) were subjected to different isolation kits. Macrophages were isolated from cell pellet using positive and negative selection strategies. CD66abce microbead kit, PAN monocyte isolation kit and CD14 microbeads were used in three different combinations for obtaining pure and enriched macrophages from sputum.

**Results:** The results obtained from all sets of experiments were compared and per cent purity and enrichment of macrophages was calculated. CD14 positive selection kit when used for isolation yielded maximum enrichment (> 20-folds) and yielded greater purity as compared to negative selection strategies.

**Conclusion:** CD14 microbeads based positive selection appeared to be the method of choice for isolating macrophages from induced sputum of COPD patients for various downstream experimental processes.

## Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of the lungs to smoke, dusts and other air pollutants (1). It is characterized by increased numbers of macrophages, neutrophils and cytotoxic T-lymphocytes in airways and the lung parenchyma (2). The number of macrophages are increased in patients of COPD. Although macrophages appear to play a pivotal role in the pathophysiology of COPD as these cells may be activated by cigarette smoke to release several cytokines and chemokines (3), our present knowledge

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about the patho-physiological basis of COPD is limited (4). There are several difficulties in exploring the underlying process of COPD. The small airways and surrounding lung parenchyma which are the sites for functional inflammatory response associated with COPD are difficult to assess as they are situated in the lung periphery. Sputum induction is currently used as a direct, non-invasive method for the evaluation of airway inflammation in COPD (5). Induction of sputum is relatively safe and well-tolerated in patients with advanced stages of COPD or exacerbations (6). It provides an alternative to collecting the expectorated sputum or performing fiberoptic bronchoscopy. With sputum induction, samples can be obtained from the lower airways with minimal discomfort to the patient and is also suitable for repeated measures in most patients (7). The proportion of viable cells is higher in induced sputum as compared to the spontaneously generated sputum (8, 9). Thus, it has become a well validated research tool and is used as a diagnostic technique for evaluating a variety of indices of inflammation. It is inexpensive and is preferred by patients and thus represents an alternative to standard methods of sampling the airways (10). Although macrophages isolated from induced sputum appear to be a useful model for studying the pathophysiological basis of COPD, different macrophage subpopulations are reportedly present in the lung parenchyma (11, 12). Furthermore, sputum contains heterogeneous population of cells. To isolate the target population, remaining contaminating cells have to be removed. To this effect, it remains as yet to be examined as to which immune-epitope may be used for isolation of macrophages from sputum samples in highly precise and specific manner. In the present study, we have examined and compared among different immune-epitope based isolation of macrophages from sputum sample of COPD patients, as shown in Figure 1, and observed that CD14 based magnetic separation indeed yielded highly pure population of macrophages.

## Materials and Methods

### Details of the patients

COPD patients (n=13, aged 30-70 years) recruited from Department of Pulmonary and Sleep Medicine, All India Institute of Medical Sciences (AIIMS), New Delhi, were either smokers (n=5) or ex-smokers (n=8) having a smoking history of more than 10 pack-years. Patients who ceased smoking for more than 1 year were considered as ex-smokers. Table I provides the details of the patients. Stable COPD patients (stages 2 and 3) according to the GOLD guidelines (13) with an evidence of airflow limitation on spirometry $FEV_1$/forced vital capacity (FVC) ratio of <70% were included in the present study. Patients who had suffered from exacerbation and/or those who had taken steroids, had a history of any active inflammatory disease, had a lung disorder besides COPD were excluded from the study. Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of AIIMS, New Delhi.

### Sputum induction and processing

Sputum induction in patients (n=13) was performed using Ultrasonic Nebuliser, Omron NE-U17 (Omron Healthcare Co. Ltd, Japan). Only 11 subjects could successfully follow the given instructions as described previously (7). Briefly, three $FEV_1$ manoeuvres were measured 15 minutes after inhalation of 200 µg salbutamol. Highest value was taken as baseline. Subjects were then instructed to inhale freshly prepared hypertonic saline (4%, w/v) for a total duration of 15 minutes. In initial experiments patients could not expectorate adequate amount of sputum even after repeating the procedure three times with 2-3% (w/v) hypertonic saline and 4% hypertonic saline was found to be the optimal for induction. Again, in initial experiments with a higher concentration (5%, w/v) patients had bronchoconstriction. Hence, freshly prepared hypertonic 4% (w/v) saline was used in the present study. After 5 minutes of nebulisation, spirometric tests were performed to detect broncho-constriction and the nebulisation was continued if the $FEV_1$ had not fallen by more than 20%. The induction was stopped, if $FEV_1$ decreased by more than 20% compared with post-salbutamol baseline (14). After 5 minutes and at subsequent intervals, subjects were asked to rinse their mouth and blow their noses to avoid contamination with postnasal drip and saliva.

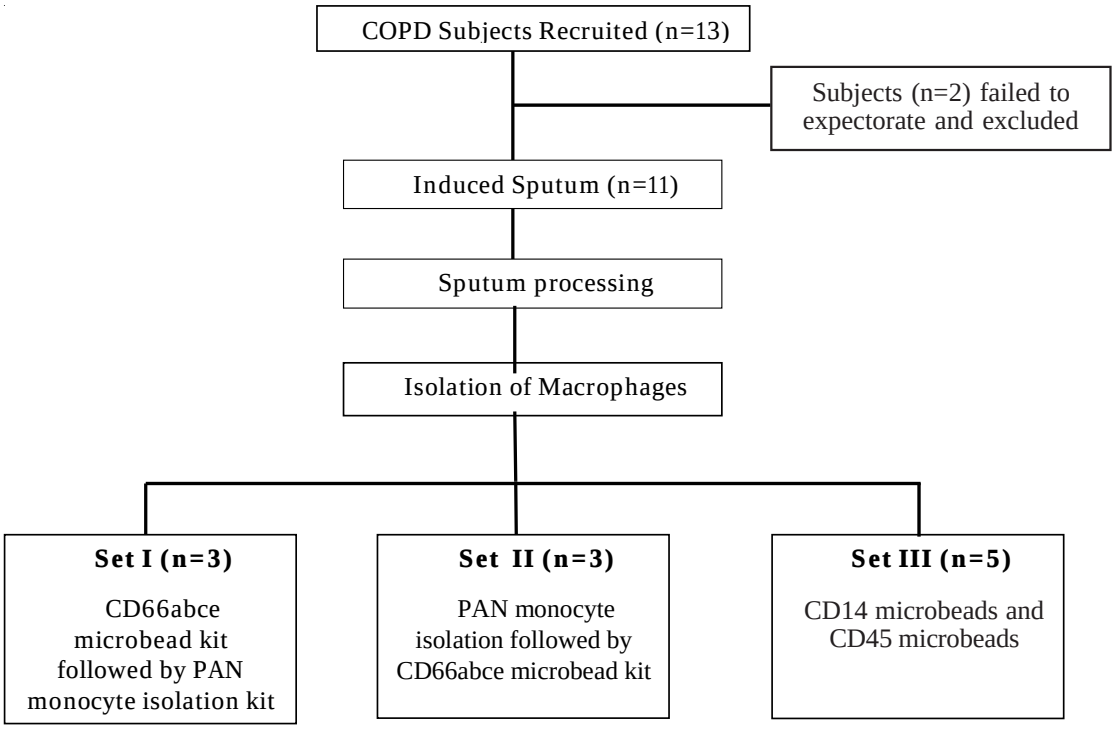


Fig. 1: Consort diagram showing various combination of kits used for experiments.

TABLE I: Showing details of COPD patients (n=13).

| S. No. | Differential Leucocyte count (DLC) (%) | | | | Pulmonary Function Test (PFT) (%) | | | Stages of Copd | Age | Gender | Smoking status | Pack Years | Ex–smoker (years) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Neutro-phils | Macro-phages | Eosino-phils | Lympho-cytes | FEV1 (%) | FVC (%) | FEV1/ FVC (%) | | | | | | |
| 1 | 63.7 | 12.13 | 8.31 | 8.79 | 31 | 44 | 53 | III | 48 | M | Smoker | 14 | – |
| 2 | 71.56 | 9.47 | 6.87 | 4.02 | 37 | 65 | 53 | III | 69 | M | Ex–smoker | 12 | 7 |
| 3 | 70.78 | 14.61 | 7.97 | 6.64 | 46.24 | 64.16 | 55.41 | III | 53 | M | Smoker | 10 | – |
| 4 | 63.35 | 7.36 | 3.86 | 4.23 | 66 | 93 | 56.08 | II | 61 | M | Smoker | 13 | – |
| 5 | 62.45 | 11.89 | 5.57 | 6.69 | 33 | 54 | 44.21 | III | 66 | M | Ex–smoker | 20 | 4 |
| 6 | 51.34 | 4.84 | 6.46 | 5.38 | 56 | 71 | 61.89 | II | 63 | M | Ex–smoker | 11 | 3 |
| 7 | 80.83 | 6 | 2.26 | 5.55 | 50 | 58 | 65.72 | II | 65 | M | Ex–smoker | 10 | 2.5 |
| 8 | 88 | 3.84 | 2.59 | 5.56 | 37 | 56 | 68 | III | 48 | M | Ex–smoker | 15 | 3 |
| 9 | 74.58 | 6.77 | 6.44 | 12.2 | 51.14 | 81.88 | 65.48 | II | 62 | M | Ex–smoker | 11 | 2 |
| 10 | 84.48 | 4.73 | 3.91 | 5.28 | 45 | 61 | 62 | III | 61 | M | Smoker | 16 | – |
| 11 | 44.85 | 10.51 | 12.85 | 25.42 | 61 | 84 | 57.91 | II | 50 | M | Ex–smoker | 12 | 2 |
| 12* | 69.2 | 11.8 | 7.16 | 10.84 | 35 | 56 | 53.24 | III | 43 | M | Ex–smoker | 10 | 5 |
| 13** | 58.60 | 12.64 | 12.30 | 16.46 | 30 | 52 | 51.67 | III | 49 | M | Smoker | 11 | – |

* and ** shows the details of subjects not included in the study. Sputum Induction was discontinued due to complain of dizziness after two rounds of sputum induction and failure to expectorate sputum, respectively.

Then the subjects were encouraged to cough and expectorate sputum into a sterile-pot.

Collected sputum was immediately kept on ice and then processed immediately as described by Bhowmik et al. (9). To reduce the salivary contamination, sputum plugs were selected and transferred into an eppendorf tube. Since the minimum sample weighing 100 mg was sufficient to carry out further steps, no sample pooling was required. The sputum was treated with freshly prepared 0.1% (w/v) dithiotrietol (DTT) solution. The

selected plugs were treated with a volume (in microlitres) equal to two times the weight of sputum portion (in milligrams). The tube containing sputum plugs and DTT was vortex mixed and was placed in roller incubated for 15 min to ensure complete homogenisation. The liquid was further diluted with phosphate buffer saline (PBS, pH 7.2) in a volume equal to that of 0.1% DTT and vortex mixed. The suspensions were double filtered through 70 µm (Falcon cell strainer, BD sciences) and 48 µm nylon mesh (SEFAR Nitex, India) to remove mucus and debris epithelial and squamous cells. Weight of the filterate was obtained. Cell suspension was centrifuged at 480 xg for 10 min at 4°C. The supernatant was aspirated and the cell pellets were re-suspended in PBS. The cell pellet was further used for isolation of macrophages using commercially available immunomagnetic beads as described below.

#### Isolation of macrophages using different immune-bead kits

Various commercially available immunomagnetic microbead based kits (CD66abce microbeads, PAN monocyte isolation kit and CD14 microbeads from Miltenyi Biotech, BergischGladbach, Germany; CD14 positive selection kit and RosetteSep Human monocyte enrichment negative selection cocktail from Stem Cell Tech (Vancouver, Canada Inc.) were used for isolation of macrophages from sputum. Since these kits were primarily validated and recommended for isolation of monocytes from blood, the efficacy of kits were first checked for isolating monocytes from blood before using these kits for sputum samples. All the kits yielded satisfactory levels of enriched population of monocytes from blood, however, RosetteSep negative selection kit yielded high neutrophil contamination. CD14 microbead kit gave the maximum yield of monocytes from blood and sputum samples. As neutrophil count is generally high in the sputum samples of COPD patients, we adopted a strategy of depleting granulocytes using CD66abce microbead kit followed by PAN monocyte isolation kit. In order to compare, we have also employed only PAN monocyte isolation which is a negative selection kit as well as CD14 microbeads kit which is a positive selection kit for isolation of macrophages from sputum samples.

#### Isolation of macrophages using CD66abce microbead kit followed by PAN monocyte isolation kit

Target cell isolation in sputum samples was carried out as per the manufacturer's instruction. Briefly, cell pellet was re-suspended in sterile and degassed phosphate buffer saline (PBS, pH: 7.2-7.4) with 2 mM EDTA and 0.5% (w/v) bovine serum albumin (BSA) and CD66abce-biotin antibody and anti-biotin beads were added. The column was rinsed with MACS buffer before proceeding to magnetic separation. After incubation, cells were applied to MACS-MS columns that were placed in mini MACS separation unit (MiltenyiBiotec) to undertake magnetic separation. Cells in the pooled flow-through represented unlabelled cells devoid of $CD66a^+$, $CD66b^+$, $CD66c^+$ and $CD66e^+$ cells. The eluate containing the unlabelled cells was centrifuged so as to obtain the cell pellet. Supernatant was discarded and the cell pellet was further subjected to PAN monocyte isolation kit. Cells were resuspended in the MACS buffer and FcR blocking reagent and biotin-antibody cocktail was added and it was incubated 15 minutes. Cells were then re-suspended in the buffer and anti-biotin microbeads were added. After incubation, cells were proceeded to magnetic separation. Cells in the flow-through were collected as untouched macrophages. Thus, cells which were magnetically labelled by antibodies were depleted as they were retained in the MACS column. All steps were carried out at 4°C. Cytospin smears were prepared at room temperature and smears were stained as described below at each stages of the experiment to determine the percentage of inflammatory cells present.

#### Isolation of macrophages using PAN monocyte isolation kit followed by CD66abce microbead kit

The pellet obtained after sputum processing was subjected to PAN monocyte isolation kit. Cocktail of biotin-conjugated antibodies and anti-biotin microbeads was added and same protocol as mentioned above was followed according to manufacturer's instructions. After magnetic separation, the unlabeled cells that passed through, represented the enriched monocyte cells. The eluate

obtained was centrifuged and the pellet was further subjected to CD66abce microbead kit, as described above, in order to rid of contaminating granulocytes, if any. Cytospin smears were prepared and smears were stained as described below at each stages of the experiment to determine the percentage of inflammatory cells present.

#### Isolation of macrophages using CD14 microbeads

Macrophages were enriched by positive selection by using CD14 microbeads (MiltenyiBiotec, BergischGladbach, Germany) as per the manufacturer's instruction. Briefly, cells obtained from processed sputum were re-suspended in the above buffer and CD14 microbeads were added to it. After incubation, the cell suspension was loaded in MACS column placed in a MACS separator for magnetic separation. After removing the column from the magnetic-field, the magnetically labelled CD14+ cells which were stuck to the column were eluted as positively selected cell fraction by firmly pushing the plunger onto the column. Cytospin smears were prepared and smears were stained as described below to determine the percentage of inflammatory cells present.

#### Differential staining

Total cell counts and assessment of viability was carried out using routine procedure of trypan blue and Neubauer hemocytometer (15). Viability was found to be greater than 90% in all cases. The cell suspension was first mixed with PBS (pH 7.4) to obtain a count of $1.0 \times 10^6$ cells/ml of the suspension. 75 µl of cell suspension was used for preparing cytospin slides at 500 rpm for 4 minutes using a cytocentrifuge obtained from Medilab Solutions (Gurgaon, Haryana, India), which were then air dried and stained with Diff-Quick (Siemens. Healthcare Diagnostics Inc., Deerfield, IL, USA) for overall differential cell count using the routine procedure. Blind-fold counting of 400 non-squamous cells were performed by two investigators for all individual cytospin slides prepared at various stages of every experiment (16). Three slides were prepared at each stage of experiment. Samples containing more than 80% non-squamous cells was considered satisfactory for undertaking further steps of isolation of macrophages. Further, readings with inter-observer and intra-observer differences of $\geq 10\%$ were considered unacceptable. Using these cut-off, all reported values appeared acceptable. Removal of microbeads was not required as they did not interfere with the down-stream processes since immunobeads reportedly do not activate cells or saturate cell surface epitopes (17).

#### Data analysis

Total and differential cell counts were obtained from cytospin smears of cells obtained from all sets of experiments. Percentage of inflammatory cells were calculated at each stages of experiment and presented as means±SDs. Enrichment of macrophages was also calculated from each set of experiment performed as the fold increase in the percent purity. Overall enrichment was calculated by multiplication of enrichment obtained in each of the sub-steps and the results were expressed as means±SDs. Kruskal-Wallis H-test was used for comparisons of overall enrichment for strategies I, II and III, followed by Dunn's test of multiple comparison with Benjamini-Hochberg FDR.

## Results

Table II shows the purity (percent) of inflammatory cells like macrophages, neutrophils and eosinophils retrieved at the end stage of every experimental approach, which were identified based on microscopic characteristics under Romanowsky staining. Table III shows the enrichment of macrophages from sputum sample of COPD patients in various sets of experiments. Percent purity of the inflammatory cells i.e., macrophages were enriched in various stages, however in one of the experiments in which CD66abce isolation was followed by PAN monocyte isolation, there was generally substantial (~50%) loss of the target cells. On the contrary, consistently high (~20-fold) degree of enrichment was seen in experiments with CD14 column. Figure 2 shows the cells obtained at various stages of experiments using the above mentioned kits.

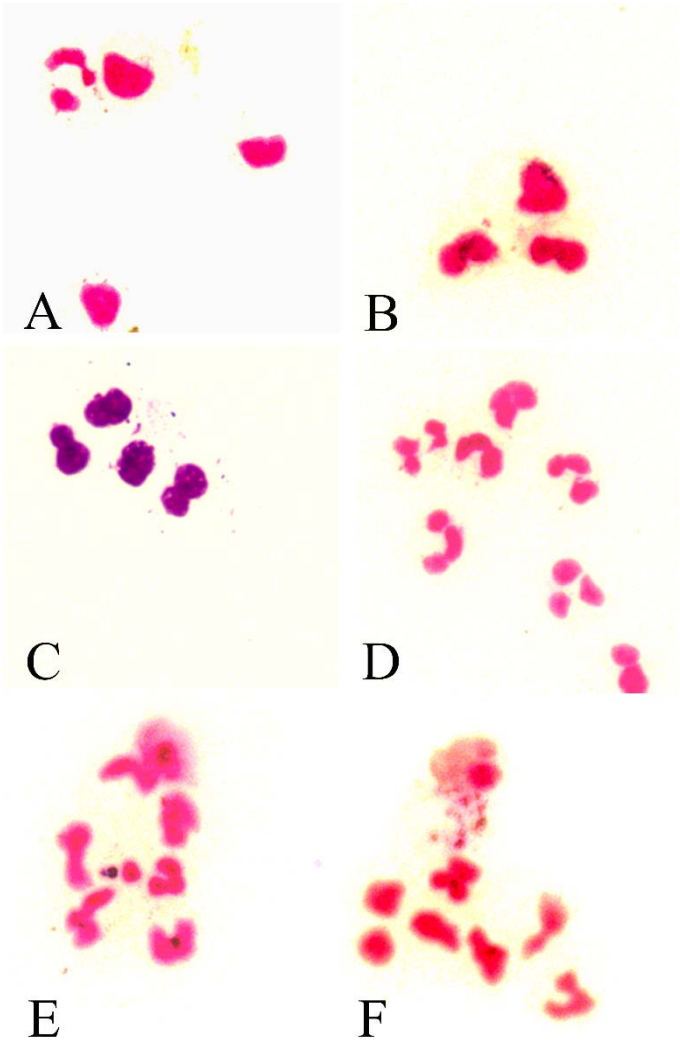


Fig. 2 : Cells obtained from sputum as seen in CD14 eluate (A), CD14 flow through (B), CD66 eluate (C), CD66 flow-through (D), PAN eluate (E) and PAN flow-through (F) stained with Giemsa. x40

TABLE II : Purity of macrophages, neutrophils and eosinophils retrieved from variousimmunobead based experiments to isolate macrophages from sputum samples.

| *S. No.* | *Experimental details (n)* | *Cell types retrieved (Percent)* | | |
|---|---|---|---|---|
| | | *Macrophages* | *Neutrophils* | *Eosinophils* |
| 1 | CD66 abce kit followed by PAN Monocyte isolation kit (3) | 1.9±1.6 | 22.8±19.8 | 0.1±0.1 |
| 2 | PAN Monocyte isolation kit followed by CD66 abce kit (3) | 4.8±8.3 | – | – |
| 3 | CD14 Microbeads (5) | 77.1±15.0 | 27.6±22.9 | 5.9±13.3 |

Values expressed as means±SDs.

TABLE III : Enrichment of macrophages observed in various sets of experimental approaches.

| *Procedure no.* | *Details of procedure* | *Overall enrichment* |
|---|---|---|
| 1 | CD66 abce → PAN monocyte | 9.4±7.7* |
| 2 | PAN monocyte → CD66 abce | 0.6±1.7** |
| 3 | CD14 microbeads | 20.1±4.1 |

Values expressed as means±SDs. Step 1 input for each procedure was $10^5$ inflammatory cells in sputum, and flow through obtained from step 1 was used as step 2 input. *p<0.05, **p<0.02 as compared to procedure no. 3.

In the first set of experiments (n=3) in which CD66abce microbead kit was followed by PAN monocyte isolation kit, the total cell count ranged from 1.2 – 17.9 × $10^6$ cells. PAN flow-through had high percentage (22.8%) of neutrophils followed by macrophages (2%) and very small numbers of eosinophils (0.1%). Although no frothy cell was seen in the cytospin prepared from the original cell pellet, small percentage of these cells could be seen in CD66 flow-through (2.5%) and in PAN eluate (0.8%). CD66 flow-through had 5.4% of band cells while PAN eluate had 0.002% of these cells.

In the second set of experiments (n=3) in which PAN monocyte isolation kit was followed by CD66abce isolation kit, the total cell count ranged from 2.0 – 6.9 × $10^6$ cells. Macrophages obtained were only 4.8% in the final CD66 flow-through; neutrophils and eosinophils could not be seen in CD66 flow-through. Other cells such as lymphocytes (1.4%) and karyorrhectic cells (0.02%) were also seen in the cytospin prepared from CD66 flow-through. PAN eluate had neutrophils in a highly dynamic ranges (33-90%) in different experiment sets. Unexpectedly, PAN monocyte eluate also hadmonocytes. Again, in PAN flow-through, percentage of lymphocytes (31%) was higher than that of the macrophages (18%). CD66 flow-through, which was supposed to contain maximum percentage of macrophages, had only 4.8% of these cells. Small percentage (1.4%) of lymphocytes could also be seen. Mast cells (1.6%) were also present in PAN eluate and 1.9% of these cells were also present in CD66 flow-through in one set of experiment. Frothy cells were not present initially but these cells were seen in varying ranges in PAN eluate (1.2%), PAN flow-through (12.3%) and CD66 eluate (17.2%).

Karyorrhectic cells were present in small percentage in PAN flow-through (0.2%) and CD66 flow-through (0.02%). Interestingly, the mean value of overall enrichment was seen < 1, for the fact two samples out of the three failed to yield any macrophages.

In the third set of experiments (n=5) in which CD14 microbeads was used, total cell count varied from 0.9 – 7.0 × $10^6$ cells. CD14 eluate had around 77.1% macrophages as expected, however, it also had 27.6% neutrophils. CD14 eluate also had a few eosinophils (1.6%). Unexpectedly, mast cells which were initially not seen were also recovered (2.2%) in CD14 eluate. CD14 flow-through contained 27.8% macrophages. Other cells such as neutrophils (71.5%), lymphocytes (3.7%) and eosinophils (2.9%) were also obtained in cytospin prepared from CD14 flow-through.

## Discussion

Macrophages play a pivotal role in the pathophysiology of COPD. These are found to be elevated in the airways, lung parenchyma, Bronchoalveolar lavage fluid and sputum in COPD patients (18). Previous studies have focussed on isolation of macrophages from bronchoalveolar lavage fluid and bronchoscopy which are invasive techniques and hence cannot be used repeatedly. In COPD, there is an accumulation of airway macrophages (19) and therefore airway inflammation may be evaluated by using a safe and non-invasive method of sputum induction (5). There are only a few studies so far documenting the isolation of macrophages from induced sputum of COPD patients. In order to separate the inflammatory cells in induced sputum of COPD patients, RosetteSep technique has been used earlier for enriching macrophages from induced sputum. On the other hand, CD14 microbeads were used for separation of monocytes from blood sample (20). Per cent purity of monocytes obtained from blood sample by using CD14 microbeads, which was found to be greater than 80%, was in the line with the results obtained from study by Mayer et al. (21). In the present study, different strategies were examined and compared using microbeads for obtaining macrophages from sputum sample of COPD patients. These macrophages could be further used for various downstream experimental processes such as for ex-vivo stimulation, culture experiments, RNA or protein expression analysis.

Generally, negative selection is considered as the primary choice for isolation of macrophages in order to obtain untouched cells. However, in the present study, available negative selection kits meant for common target tissues like blood, lymph nodes and spleen did not work satisfactorily for the sputum samples. It is also difficult to design a perfect depletion cocktail to target all cells that do not carry any cluster of differentiation (CDs), also present on macrophages. The overall enrichment of macrophages obtained by using CD66abce microbead kit followed by PAN monocyte isolation kit was less than 10-fold. When the same two kits were used inversely by using PAN monocyte isolation kit first followed by CD66abce microbead kit, the mean enrichment of macrophages obtained was <1 fold. However, it is to be noted that no enrichment was obtained in two samples, resulting in fall in mean retrieval data with very high coefficient of variation (283.3%). On the other hand, positive selection kit using robust selection marker (CD14) present on macrophages (22, 23) yielded satisfactory yield of macrophages. No lymphocytes were held by CD14 column. Thus, positive selection strategy using CD14 increased the specificity and yield of macrophages by about 20-fold. Although we could satisfactorily isolate the pro-inflammatory type of macrophages which were strongly positive for CD14 cell surface marker (24, 14), the subset of macrophages with very low expression of CD14 could have been missed out. As sputum induction yields cells from lower airways, it is not possible to comment whether the macrophages obtained are alveolar or bronchial macrophages nor does it gives us any idea about these being small or large macrophages.

Cell counting was done by using haemocytometer and no other automated methods was used to keep the experiments cost effective. Previous studies have shown that manual counts are not typically higher or lower than machine counts and it is an accurate method for estimating cell numbers when compared to similar estimates determined using other methodologies (25). Though microscope counting

done by using a Neubauer chamber remains to be a gold standard for cell counting, techniques like flow cytometry improves precision and speed in retrieving CD labelled cells with fluorphore. This remains as a limitation in the present study. Moreover, it is to be noted that the serum levels of alpha 1-antitrypsin, IgG and IgA, were not checked which are differentially affected in COPD (26-30) and might have discrete interaction with different processes of macrophage isolation undertaken in the present study. This issue was not addressed in the present study.

In conclusion, sputum induction is a safe and non-invasive method for evaluation of airway inflammation. It is evident from the results of different sets of experiments of this study that CD14 microbeads based positive separation yielded maximum enrichment of macrophages from the sputum samples obtained from COPD patients as compared to other kits individually and in combinations. Thus CD14 microbeads can be used efficiently for isolating macrophages from induced sputum of COPD patients.

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