Ontogeny and phagocytic function of baboon lung dendritic cells

Shanjana Awasthi¹, Roman Wolf² and Gary White²

Dendritic cells (DCs) are the most potent antigen-presenting cells and have been implicated in the ontogeny of lung DCs and immune response against pathogens. The DCs are activated after coming in contact with pathogens, pathogen-derived ligands, foreign particles and endogenous stress signals. The activated DCs move to local lymph nodes where they stimulate naive T cells, B cells or other immune cells, and regulate the stress-, antigen- or pathogen-specific adaptive immune response.

Although several studies have confirmed the immunomodulatory function of lung DCs against respiratory pathogens in adult humans and animal models¹¹–¹⁶, the ontogeny of lung DCs and immune functions remain unclear in preterm infants who are highly susceptible to respiratory infections. Thus, it is important to investigate the ontogeny, phenotypic characteristics and functions of pulmonary DCs during prenatal life.

A few studies on fetal rat and mouse lung DCs¹¹,¹⁷,¹⁸ mainly focus on histological examination of fetal lung tissues for cell staining with major histocompatibility complex (MHC) class II antibody. The staining with antibodies against MHC class II or a single-cell-surface marker is not helpful in identifying DCs, because DCs are identified based on the expression of a set of markers, for example, MHC, T-cell costimulatory molecules. As such, the pulmonary epithelial cells and macrophages also express MHC class II molecules on their cell surface.¹⁹ The expression of a combination of markers only determines the presence and type of DCs. Thus, for the phenotyping and functional studies, it is important to first isolate the lung DCs. One recent study was conducted on neonatal lamb model of respiratory syncytial virus infection¹² in which the functions of isolated neonatal lung DCs were elucidated in vitro. However, there was no information on the phenotype and functions of lung DCs from fetal/preterm counterparts. From other smaller animal models (for example, rat and mouse pups), it is very difficult to culture enough number of cells because of the smaller organ size. In this study, we investigated the phenotypic and phagocytic ability of the isolated lung DCs from fetal/preterm counterparts. Because of ethical reasons, these studies are not possible in humans. Baboons are evolutionary close to humans, and their immune system is quite similar to the humans.¹⁰,²¹

Keywords: dendritic cells; innate immunity; phenotype; phagocytosis

The preterm infants are very susceptible to respiratory infection during the first year of their life.¹–⁴ The increased susceptibility of preterm infants to respiratory infections is associated with immature lung and poorly developed immune system. The developmental aspects of various immune components and their involvement in infection remain to be understood. Specifically, at the onset of infection, the antigen-presenting cells are the first type of immune cells that encounter the pathogens and define the downstream host defense mechanisms. Among various types of antigen-presenting cells, dendritic cells (DCs) have been recognized as the most potent antigen-presenting cells.²–⁸ The DCs are present in both lymphoid and nonlymphoid organs of the body, and are important in mounting immune response against pathogens. The DCs are activated after coming in contact with pathogens, pathogen-derived ligands, foreign particles and endogenous stress signals.⁹ The activated DCs move to local lymph nodes where they stimulate naive T cells, B cells or other immune cells, and regulate the stress-, antigen- or pathogen-specific adaptive immune response.¹⁰

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RESULTS
Morphologic characteristics of fetal and baboon lung cells with variable density separated on OptiPrep density gradient

After density gradient separation, we collected lung cells from three distinct fractions: one at the bottom (density $>1.085 \text{ g ml}^{-1}$), second in the middle (density $1.065–1.085 \text{ g ml}^{-1}$) and third on the top (density $<1.065 \text{ g ml}^{-1}$). The lung cells with heavier density ($>1.085 \text{ g ml}^{-1}$) settled at the bottom. Morphologically, these cells appeared to be epithelial cells, macrophages and red blood cells (RBCs). The cells in the middle fraction showed morphology similar to lymphocytes. In top fraction, the floating lung cells of adult baboon appeared as mature DCs. In contrast, the fetal baboon lung cells in top fraction were round, had vacuolar cytoplasm and did not possess the dendrites (Figure 1). We did not detect mature lung DCs in any of the fetal lung cell population obtained from top, bottom or middle fractions of density gradient. The fetal baboon lung cells collected from top of the density gradient were unique to fetal baboons, and were not identified in adult baboon. The morphologic features of lung cells were maintained, whether cells were harvested on day 0 or 1, after 16–18 h of culture (Figure 1).

Yield of fetal and adult baboon lung cells

Most of the cells (90–95%) settled in the bottom fraction after density gradient separation. Only 3–7% and 0.2–1% of the total lung cells were isolated in the middle and top fractions, respectively (Table 1). The yield of adult and fetal baboon lung cells isolated in the top fraction varied from $0.1 \times 10^6$–$1.3 \times 10^6$ per gram lung tissue. Approximately, $0.1$–$0.7 \times 10^6$, $0.5$–$1.3 \times 10^6$ and $0.5$–$1 \times 10^6$ cells were harvested in the top fraction per gram lung tissue of 125–140 days of gestation age (dGA) fetal, 165–175 dGA fetal and adult baboons, respectively.

Immunophenotype of lung cells isolated in middle and bottom fractions after density gradient centrifugation

For immunophenotyping of lung cells in different fractions, the flow cytometry was performed. Flow cytometry analysis of antibody-stained lung cells in bottom fraction show that both adult and fetal baboon cells were negative for CD2, CD20, CD1a, CD11c, DC-SIGN (CD209), CMKLR1, ILT7 (CD85g), CD40 and CD86. Only very few adult baboon lung cells were positive for HLA-DP, DQ, DR (up to 5%) as compared to negligible staining by fetal baboon lung cells. Morphologically, most of the cells showed features similar to RBCs, macrophages and large epithelial cells.

In the middle fraction, however, 80% of the adult baboon lung cells with small forward and side scatter were positive for CD2 (lymphocyte cell marker). In contrast, only 11–12% of fetal baboon lung cells isolated in the middle fraction were positive for CD2 marker. The morphology of these cells was similar to the normal typical lymphocytes: condensed nucleus and scant cytoplasm.

The immunophenotype of the lung cells obtained from bottom or middle fractions of density gradient was same on day 0 and 1 of harvesting.

Immunophenotype of lung cells separated in the top fraction

The adult and fetal baboon lung cells separated in the top fraction were always negative for CD2 (T-cell marker), CD20 (B-cell marker) and CD14 (monocyte-macrophage marker) (Figure 2). These cells were also negative for plasmacytoid DC markers: CMKLR1,22 CD20923 and ILT7.24 The flow-activated cell sorting (FACS) analysis of cells with large forward and side scatter shows that as compared to adult baboon lung DCs (∼40% positive for HLA-DP, DQ, DR), only 5.5% fetal lung cells were positive for HLA-DP, DQ and DR markers (Table 2). Thirty-one and 15% of adult baboon lung DCs were positive for CD86 and CD11c, respectively, as compared to 10.8% and 5.8% of fetal baboon lung cells separated in the top fraction (Table 2). Only 9% of adult baboon lung DCs expressed CD80 as

| Table 1 | Percent fetal and adult baboon lung cells obtained from top, middle and bottom fractions after density gradient separation |
|--------|---------------------------------------------------------------|
| Fractions of OptiPrep density gradient | Percent cells (harvested on day 0 and 1) per total lung cell number\(^a\) |
|        | Fetal baboon | Adult baboon |
| Top fraction | Day 0 | Day 1 | Day 0 | Day 1 |
| 0.9 (0.2) | 0.5 (0.04) | 0.8 (0.5) | 0.3 (0.2) |
| Middle fraction | 6.7 (0.2) | 2.5 (0.5) | 4.8 (0.8) | 4.3 (0.3) |
| Bottom fraction | 92.4 (0.4) | 97.0 (0.5) | 94.4 (1.3) | 95.4 (0.5) |

\(^a\)The values are mean (s.e.m.) percent number of total lung cells from 125–165 dGA fetal (n=3) and adult (n=4) baboons. The cells were subjected to density gradient separation either immediately (day 0) or after overnight culture (day 1).
compared to 18% of fetal baboon lung cells (Table 2). Both fetal and adult baboon lung cells with small forward and side scatter stained with low mean fluorescent intensity (MFI; negligible staining) for these markers.

The MFI of HLA-DP, DQ, DR (MHC II), CD1a, CD14, CD11c, CD40, CD80 and CD86 markers was further analyzed by plotting the FACS histograms (Figure 3). The results suggest that the MFI values for HLA-DP, DQ, DR, CD11c and CD86 were higher for adult baboon lung DCs as compared to fetal baboon lung cells obtained from the top fraction. However, the MFI value for CD80 was low in lung DCs of adult baboons as compared to the fetal baboon lung cells. The immunophenotype of fetal and adult baboon lung cells harvested on day 0 was not different from those harvested on day 1 after 16–18 h of culture (data not shown).

On the basis of the morphologic, immunophenotypic and isolation characteristics, it is clear that the adult baboon lung DCs are separated as low-density cells in the top fraction of OptiPrep density gradient. Previous literature reports in different adult animal models also support this observation.15,25–27 A significant number of fetal baboon lung cells with low-density are also isolated in the same top fraction of OptiPrep density gradient. These fetal baboon lung cells are morphologically different from mature adult baboon lung DCs, and show unique morphologic and phenotypic characteristics distinct from macrophages, monocytes, lymphocytes, epithelial cells and other kind of immune cells. Fetal baboon lung cells separated in the top fraction stained low for the typical DC markers and morphologically appeared similar to the immature DCs without dendrites on their cell surface. On the basis of these results, we believe that these fetal baboon lung cells probably represent the DC-precursor cells that may transform into mature DCs after birth and during adulthood.

Phagocytosis of heat-killed, FITC-labeled Escherichia coli K12 bioparticles by DCs

After the isolation of lung DCs or DC-precursor cells from adult and fetal baboons, we confirmed their viability and phagocytic function. Approximately 90–95% cells were viable. Phagocytic capacity of lung DCs or DC-precursor cells was determined by flow cytometric dot-plot analysis (Figure 4). The phagocytosis assay was performed using

Table 2 Percent numbers of cell-surface marker positive lung cells harvested in top fraction of density gradient tube after overnight culture (day 1)

| Cell-surface marker | 125–140 dGA baboon | 175 dGA baboon | Adult baboon (10–35 years age) |
|---------------------|---------------------|----------------|-----------------------------|
| CD11c               | 5.8 (4.2)           | 2.8 (0.9)      | 11.4 (3.8)                  |
| HLA-DP, DQ, DR      | 5.5 (1.5)           | 8.0 (2.0)      | 34.4 (9.2)                  |
| CD40                | 0.7 (0.7)           | 0.3 (0.1)      | 4.7 (2.3)                   |
| CD80                | 18.0 (13.1)         | 2.3 (1.1)      | 9.0 (6.5)                   |
| CD86                | 10.8 (7.3)          | 13.0 (5.0)     | 31.6 (9.5)                  |

The values are mean (s.e.m.) percent positive lung DCs from 125–140 dGA (n=3), 175 dGA (n=3) and adult (n=4) baboons. The isolated cells were negative for plasmacytoid DC markers: DC-SIGN (CD209), ILT7 and CMKLR1. There was no change in percent numbers of cell-surface marker positive lung cells isolated on day 0 or 1.
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serum-opsonized, fluorescent-labeled, heat-killed E. coli bioparticles. The x axis represents the green fluorescence (FL-1 channel) and y axis represents the red fluorescence (FL-3 channel; Figure 4). When extracellular fluorescein isothiocyanate (FITC)-labeled bacteria are present on the surface of the cells, trypan blue quenches the green fluorescence of FITC and provides red fluorescence to the cells. The cells with surface-adherent bacteria (red fluorescence) are shown in upper quadrants. The cells with phagocytosed FITC-labeled bacteria (green fluorescence) are shown in right quadrants. When the reaction was performed on ice (negative control), the lung DCs were found distributed in only two left quadrants without any phagocytosed particles (Figure 4). In phagocytosis assay tubes (performed at 37°C), cell populations were identified distributed in all four quadrants (Figure 4). Lower left quadrant contains nonfluorescent events corresponding to cells without any associated particles (no interaction), upper left quadrant contains cells with only membrane-bound E. coli bacteria (adherence), upper right quadrant contains cells with both internalized and surface-associated E. coli bioparticles (adherence and ingestion) and lower right quadrant corresponds to green fluorescent events of cells with ingested E. coli bioparticles (phagocytosis). The percent phagocytosis of E. coli bioparticles was further confirmed by fluorescence microscopy. Representative photomicrograph is shown in Figure 4. There was significant difference in phagocytic ability of fetal baboon lung DC-precursor cells from that of adult baboon lung DCs (P<0.05, Table 3, Figure 4). The fetal baboon lung DC-precursor cells were 3 (175 dGA),

![Image] Figure 3 Flow cytometric histogram charts of fetal (a) and adult (b) baboon lung cells obtained from the top fraction of density gradient solution on day 1. The cells with large forward and side scatter were gated within the circle. The dark line shows the histogram for isotype control antibody-stained cells, and the light line shows the histogram for marker-specific antibody-stained cells. The histogram of unstained cells always showed pattern similar to the histogram of isotype control antibody-stained cells. Data on x axis show the fluorescence intensity. Percent numbers of cells positive and mean fluorescence intensity (MFI) values for the specific marker are shown within the histogram charts for the isotype control antibody-stained (I) and marker-specific antibody-stained (M) cells under marked area (\textsuperscript{\textcircled{M}}). The area was selected based on absence of fluorescence by isotype control antibody-stained cells and unstained cells.

![Image] Figure 4 Phagocytosis of Escherichia coli K12 bioparticles by fetal and adult baboon lung dendritic cells (DCs). The lung DCs from fetal (125–140 dGA, n=3, 165 dGA, n=3), close-to-term (175 dGA, n=3) and adult (n=3) baboons were incubated with serum-opsonized, heat-killed, fluorescein isothiocyanate (FITC)-labeled E. coli bioparticles at 37°C. The cells with internalized FITC-labeled E. coli bioparticles show green fluorescence and collected on FL-1 channel (on x axis). The fluorescence of cells with surface-adhered extracellular bioparticles (quenched with trypan blue) was collected on FL-3 channel (on y axis). The cells with ingested (right lower quadrant), adhered-ingested (right upper quadrant), adhered (left upper quadrant) E. coli bioparticles were analyzed by flow cytometry. Panel (a) shows the dot-plot charts of lung DCs incubated with E. coli bioparticles on ice (negative control) and panel (b) shows the dot-plot charts of lung DCs incubated with E. coli bioparticles at 37°C (phagocytosis or ingestion). Dot-plot charts are from one representative animal of each type. The percent numbers of cells in each quadrant are shown within the dot-plot charts. Panel (c) shows the fluorescent micrograph of a representative cell (stained blue with Hoechst 33342 stain) with ingested FITC-labeled E. coli bioparticles (green fluorescence).
10 (165 dGA) and 150 (125–140 dGA)-fold less capable of phagocytosing *E. coli* bioparticles as compared to the adult baboon lung DCs (Table 3).

**DISCUSSION**

Very few published studies are available in the literature that focus on the functions of *in vitro* neonatal-macrophages and cord blood...
or blood monocyte-derived DCs. Results of our earlier study on fetal baboon bone marrow-derived DCs provide evidence that the DC functions (phagocytosis and cytokine secretion) are impaired during prenatal fetal life in baboons. In our previous study, we had cultured DCs using bone marrow samples of fetal, close-to-term and adult baboons in presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. The fetal baboon bone marrow-derived DCs were morphologically and phenotypically similar to adult baboon bone marrow-derived DCs. Irrespective of gestation age of fetal baboons or age of the adult baboon, the cell-surface expression of all the DC markers was found to increase as the duration of incubation increased in culture medium containing GM-CSF and IL-4. The phenotypes of in vitro bone marrow-derived DCs are different from lung DCs as evident from these studies. Thus, it is difficult to extend the results from in vitro derived DCs to resident DCs in various tissues. To study the importance of DCs in pulmonary innate immunity, it is important to conduct focused study on resident lung DCs. Such studies with fetal lung DCs are very difficult to perform because the lung size of fetal rodents is very small to obtain sufficient number of cells. Moreover, these investigations cannot be conducted in human fetuses due to ethical reasons. Because the immunology, anatomy and physiology of nonhuman primate are very similar to humans and preterm baboon model mimics the clinical situation of preterm infants, studies in preterm baboons are highly relevant.

The lung DCs make only 1% of total lung cell population; results of our earlier study on fetal baboon bone marrow-derived DCs provide evidence that the DC functions (phagocytosis and cytokine secretion) are impaired during prenatal fetal life in baboons. In our previous study, we had cultured DCs using bone marrow samples of fetal, close-to-term and adult baboons in presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. The fetal baboon bone marrow-derived DCs were morphologically and phenotypically similar to adult baboon bone marrow-derived DCs. Irrespective of gestation age of fetal baboons or age of the adult baboon, the cell-surface expression of all the DC markers was found to increase as the duration of incubation increased in culture medium containing GM-CSF and IL-4. The phenotypes of in vitro bone marrow-derived DCs are different from lung DCs as evident from these studies. Thus, it is difficult to extend the results from in vitro derived DCs to resident DCs in various tissues. To study the importance of DCs in pulmonary innate immunity, it is important to conduct focused study on resident lung DCs. Such studies with fetal lung DCs are very difficult to perform because the lung size of fetal rodents is very small to obtain sufficient number of cells. Moreover, these investigations cannot be conducted in human fetuses due to ethical reasons. Because the immunology, anatomy and physiology of nonhuman primate are very similar to humans and preterm baboon model mimics the clinical situation of preterm infants, studies in preterm baboons are highly relevant.

The lung DCs make only 1% of total lung cell population; therefore, it is difficult to isolate these cells in sufficient quantity for functional studies. In the past, lung DCs have been cultured using collagenase and trypsin enzymatic treatment, or in combination with positive selection using antibody (for example, CD11c)-bound columns or Percoll density gradient. There are reasons to believe that the collagenase, trypsin and osmotic shock treatment may disrupt the cell-surface receptors. Because there is no lung DC-specific marker known, and antibody-based positive selection method for isolating DCs has its own limitations, it is difficult to interpret the immunophenotype of the natural resident lung DCs from the published studies. We simplified the isolation procedure in this study and utilized only slow mechanical disruption and gentle processing. We believe that the DCs harvested using this method closely represent the unmodified, natural primary DC population present in vivo in lung. However, the yield of lung DCs from adult baboons was found much lower (1 × 10^6 cells per gram wet lung) compared to that from adult humans as published earlier. This discrepancy could be due to the differences in methods for isolation and collection. In the study by Masten et al., the lung DCs were collected from the density range (1.030–1.075 g ml^{-1}) of Percoll-gradient, which may have led to harvesting of increased number of cells including non-DCs. In this study, however, we collected the lung DCs only from the top of 11.5% OptiPrep solution (density < 1.065 g ml^{-1}; Accurate Chemicals, Westbury, NY, USA). Earlier, we have been successful in using OptiPrep density gradient solution to harvest in vivo bone marrow-derived DCs of baboons and obtained good yield. Previously, the OptiPrep density gradient solution has also been used successfully for purification of DCs from liver, lung, lymph, lymph node, spleen, and other tissues by other investigators.

This is the first study when isolation, phenotypic and functional characteristics of lung DCs or DC-precursor cells have been studied to compare adult and fetal DC population. On the basis of phenotypic characteristics, such as absence of CD2, CD20, CD14, positive (but low) staining with HLA-DR, DQ, DR, CD11c, CD80 and CD86 and similar cellular density, we identify that these fetal baboon lung cells probably represent the DC-precursor cells. The fetal baboon lung DC-precursor cells are more vacuolar and do not present the tentacles on their cell surface (characteristic of mature DCs). As compared to the fetal baboon lung DCs, the adult baboon lung DCs exhibited the long dendrites (Figure 1). Because the fetal baboons are not exposed to external stimuli (for example, dust, pathogens), which are inhaled continuously in lung after birth, we would expect the DCs or DC-precursor cells to be present in immature form in lungs of fetuses or preterm animals. It is possible that same DC-precursor cells would develop into mature DCs during the process of attaining adulthood. A recent study suggests that the lung DCs of both neonatal and adult sheep are phenotypically and functionally similar. It is conceivable that preterm/fetal lung DCs are immature and immunodefective as compared to the lung DCs from full-term neonates as well.

The number of positive cells and MFI values for CD11c, HLA-DR, DQ, DR (MHC II) and CD86 markers was found increased in adult baboon lung DCs. On the other hand, the expression of CD80 was increased on cell surface of fetal baboon lung DC-precursor cells as compared to the adult baboon lung DCs (Figure 3, Table 2). The expression pattern of CD40, CD80 and CD86 by adult baboon lung DCs. The fetal baboon lung DCs, the adult baboon lung DCs exhibited the long dendrites (Figure 1). Because the fetal baboons are not exposed to external stimuli (for example, dust, pathogens), which are inhaled continuously in lung after birth, we would expect the DCs or DC-precursor cells to be present in immature form in lungs of fetuses or preterm animals. It is possible that same DC-precursor cells would develop into mature DCs during the process of attaining adulthood. A recent study suggests that the lung DCs of both neonatal and adult sheep are phenotypically and functionally similar. It is conceivable that preterm/fetal lung DCs are immature and immunodefective as compared to the lung DCs from full-term neonates as well.

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To our knowledge, this is the first report focused on studying the phenotypic and phagocytic ability of isolated fetal lung DC-precursor cells. Most of the studies available in literature are focused on the localization of lung DCs or DC-precursor cells in fetal lung tissues of rodents and humans by immunohistology after staining with la (MHC class II, HLA-DR) and DC-SIGN (C-type lectin expressed on...
Results from these studies suggest that Ia + cells are present even at day 17 of gestation in fetal rats (77% of full term, term period 17–22 days), but these cells were only 40–60% effective in stimulating T-cell proliferation as compared to adult lung Ia + cells.17 The Ia + cells were also present at 12 weeks of gestation in human fetal lung, kidney, heart and pancreas (31% of full term, term period 12–38 weeks).22 In an ensuing study by Soilleux et al.,23 a plasmacytoid DC marker, DC-SIGN or CD209, was used to characterize the density and distribution of DCs in fetal and adult human lung tissues by immunohistology; the fetal lung tissue stained positive for DC-SIGN (CD209) and cells looked similar to non-dendritic macrophages, positive for CD14. In contrast, in the present study, the isolated fetal baboon lung DC-precursor cells or adult baboon lung DCs did not stain with CD14 (Figure 2) or CD209-specific antibody (data not shown). The isolated lung cells also did not stain with any of the other plasmacytid DC-markers (CMKLR1 and IL17)-specific antibody. These results suggest that the isolated fetal and adult baboon lung DC populations are mainly of myeloid type.

In conclusion, we report that the fetal lung DCs are immature and functionally deficient in responding to Gram-negative bacterial stimuli. We validated that these results in nonhuman primate model will be translatable to humans. Future studies with isolated fetal baboon lung DC population would be very useful in understanding the immunostimulatory functions and their importance in early childhood diseases.

**METHODS**

**Baboon lung tissues**

The animal studies were approved by Institutional Animal Care and Use Committees, Environmental Health and Safety or Institutional Biosafety Committee of the University of Texas Health Science Center at San Antonio (UTHSCSA), TX, USA and University of Oklahoma Health Science Center (OUHSC), Oklahoma City, OK, USA. Baboon (Papio cynocephalus and P. anubis) colonies are maintained at Southwest Foundation for Biomedical Research, San Antonio, TX, USA and Baboon Resources, OUHSC, Oklahoma City, OK, USA. At the time of necropsy, the whole fresh lung or a lobe of lung from fetal (delivered at 125, 140, 165, 175 days of gestation) and adult baboons (age range 10–35 years, mean age 22.3 years) was collected in RPMI 1640 medium containing 2 mM glutamine, 10% FBS, cut and minced into small pieces using sterile scissors and forceps. Freshly collected lobe of the lung or whole lung samples were transported on ice in RPMI 1640 medium containing 2 mM glutamine, 100 U ml−1 penicillin, 100 µg ml−1 streptomycin and 100 U ml−1 gentamicin, 1 mM EDTA, 0.5% bovine serum albumin and 10 mM HEPES) and centrifuged. The lung cells were separated in different fractions (top, middle and bottom) of OptiPrep density gradient solution with different density ranges were collected after centrifugation. The cell counts were taken, and viability was confirmed by trypan blue dye exclusion method. Because mature lung is assumed to have DC population,14,24 which are isolated as low-density cells on top of the OptiPrep density gradient solution,25-27 first we confirmed the isolation of adult baboon lung DCs from other lung cells in the top fraction by immunophenotyping and microscopy. Similar method was then employed to characterize the fetal baboon lung DC population separated in the top fraction. The morphology and phenotype of lung cells collected from bottom and middle fractions of OptiPrep density gradient were also characterized.

**Culture and isolation of lung DCs**

Freshly collected lobe of the lung or whole lung samples were transported on ice in RPMI 1640 medium containing 2 mM glutamine, 10% FBS, 10 mM HEPES, 10 µg ml−1 gentamicin, 100 U ml−1 penicillin, 100 µg ml−1 streptomycin and 10% FBS, cut and minced into small pieces using sterile scissors and forceps. We used only mild mechanical disruption to dissociate the tissue. Collagenase or trypsin enzyme treatment and osmotic shock were not used because these treatments may lead to loss of surface receptors and proteins.41-43 The finely minced pieces of lung tissue were gently in-and-out pipetted using sterile transfer pipette and washed several times with culture medium. The single-cell suspension was obtained after passing the minced lung tissue through nylon mesh. The fibrous material was washed several times with culture medium on top of the nylon mesh. The single-cell suspension was seeded in tissue culture flask (Nalge Nunc International Corp., Rochester, NY, USA) at a density of 30–50×10⁶ leukocytes per 175 cm² flask in RPMI 1640 medium containing 2 mM glutamine, 10% FBS, 10 µg ml−1 gentamicin, 100 U ml−1 penicillin, 100 µg ml−1 streptomycin and 10% FBS. The DCs were either harvested the next day or incubated overnight (16–18 h; day 1) at 37 °C in 5% CO₂ atmosphere. The DC population was separated from RBCs, lymphocytes and granulocytes using OptiPrep cell-separation solution (density 1.32 g ml−1, Accurate Chemicals) as described earlier.13 Briefly, 3–5×10⁹ nonadherent cells were suspended in 3 ml of Hanks balanced salt solution (Invitrogen) and 1 ml of OptiPrep solution (density 1.085 g ml−1). The cell suspension was overlaid with 11.5% OptiPrep solution (density 1.065 g ml−1) prepared in endotoxin-free diluent (0.88% NaCl, 1 ×EDTA, 0.5% bovine serum albumin and 10×mH HEPES) and centrifuged. The lung cells separated in different fractions (top, middle and bottom) of OptiPrep density gradient solution with different density ranges were collected after centrifugation. The cell counts were taken, and viability was confirmed by trypan blue dye exclusion method. Because mature lung is assumed to have DC population,14,24 which are isolated as low-density cells on top of the OptiPrep density gradient solution,25-27 first we confirmed the isolation of adult baboon lung DCs from other lung cells in the top fraction by immunophenotyping and microscopy. Similar method was then employed to characterize the fetal baboon lung DC population separated in the top fraction. The morphology and phenotype of lung cells collected from bottom and middle fractions of OptiPrep density gradient were also characterized.

**FACS analysis**

The harvested baboon lung cells from top (with density <1.065 g ml−1), bottom (with density >1.085 g ml−1) and middle (with density range 1.065–1.085 g ml−1) fractions of density gradient were washed twice with Dulbecco’s phosphate-buffered saline (D-PBS) and suspended in D-PBS containing 1% FBS (10⁹ cells per ml). The cells were incubated on ice for 30 min in dark withfluorochrome-conjugated antibodies (1 µg per 1 million cells): FITC, phycoerythrin (PE), allophycocyanin (APC) or biotin-conjugated, anti-human HLA-DR, CD20 (clone RPA 2.10, reactive with baboon B cells) and PE-conjugated anti-human CD20 (clone 2H7, reactive with baboon T cells) and anti-human HLA-DP, DQ, DR (clone TU39), CD1a (clone SK9), CD40 (clone 5C3), CD86 (clone FUN-1), CD80 (clone BBI), CD11c (clone M5E2), CD11c (clone S-HCL-3), CMKLR1 (clone BZ332), CD209 or DC-SIGN (eb-H209) antibodies (purchased from BD Biosciences, San Diego, CA, USA or eBioscience, San Diego, CA, USA) as described earlier.24 The cells were stained with cell-surface marker antibodies in the following combinations: (1) CD11c, CD14, (2) CD1a, HLA-DR, DQ, (3) CD40, CD80, CD86 and (4) CMKLR1, CD209, IIT7. To rule out the presence of lymphocytes, the harvested lung cells were also stained with FITC-conjugated anti-human CD2 (clone RPA 2.10, reactive with baboon T cells) and PE-conjugated anti-human CD20 (clone 2H7, reactive with baboon B cells) antibodies. Antibodies 60–70% of the baboon monocytes and macrophages express CD14 as detected by flow cytometry (personal communication, Dr Krishna K Murthy, Department of Virology and Immunology, SFBR, San Antonio, TX, USA); thus, we included CD14-specific antibody to identify monocytes or macrophages. The lung cells stained with biotinylated antibody were washed and further incubated with 1 µg of streptavidin-APC conjugate (BD Biosciences) for 20 min. The appropriate isotype-matched control antibodies were used to determine the levels of background staining. Unstained cells were used as negative control to rule out autofluorescence. Fluorescence analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The histogram and dot-plot data were collected and analyzed using CellQuest Version 3.1 (Becton Dickinson Immunocytometry Systems), winMDI 2.8 (Joseph Trotter, The Scripps Research Institute, San Diego, CA) and Summit version 4.3 (Daio Colorado Inc., Fort Collins, CO, USA) software programs. We gated the positive cells in the histogram charts and obtained MFI values.

**Phagocytosis assay**

The phagocytic ability of DCs obtained from top fraction of the density gradient was studied using heat-killed, FITC-labeled, 0.2–2 µm size, E. coli K12 bioparticles (Molecular Probes, Eugene, OR, USA).55 First, the bioparticles were incubated with 0.9% NaCl solution supplemented with 1 mM CaCl₂, 1 mM
MgCl₂ and 25% pooled healthy adult baboon sera for 30 min at 37 °C in a shaking water bath. The opsonized bioparticles were washed twice, suspended in HEPS buffer containing 1 mM CaCl₂ and 1 mM MgCl₂ and sonicated (Braun Labsonic 2000 sonicator) for 3 cycles of 10 s each just before use. The isolated cells (5 × 10⁶) were suspended in 1 mM HEPS buffer containing 1 mM CaCl₂ and 1 mM MgCl₂ and incubated at 37 °C in a shaking water bath with serum-opsonized E. coli K12 bioparticles (1 cell: 10 E. coli K12 bioparticles). Another set of tubes was incubated on ice to serve as negative controls. To stop phagocytosis, incubation mixtures were withdrawn from water bath and stored on ice. An equal volume of trypan blue solution (250 μg/ml diluted in 0.1% trypan blue, pH 4.0) was added to the tubes to quench the green fluorescence of extracellular bacteria attached on the cell surface. The quenching with trypan blue provides red fluorescence that can be detected by flow cytometry. After 1 min incubation in ice, the samples were subjected to flow cytometry using a FACSCalibur flow cytometer at FACS Core Facility, UTHSCSA. The lung tissues of adult baboons were obtained from Southwest Foundation of Biomedical Research (Director: Dr Peter W Nathanielsz, UTHSCSA). The lung tissues of newborn and preterm fetuses were obtained from Baboon Resources (Director: Jacqueline J Coalson, UTHSCSA) and Center for Pregnancy and Newborn Research (Director: Dr Peter W Nathanielsz, UTHSCSA). The lung tissues of adult baboons were obtained from Southwest Foundation of Biomedical Research, San Antonio, TX, USA or at FACS and Imaging Core Facility, OUHSC, Oklahoma City, OK, USA. The cells were illuminated using 15 mW of 488 nm argon ion laser light. FITC and red fluorescence were collected through a 530/30 nm (FL-1) and 670 nm (FL-3) band-pass filters, respectively. The data were collected and analyzed using CellQuest Version 3.1 or winMDI2.8 software program.

Different stages of phagocytosis and percent phagocytosis were further confirmed by fluorescence microscopy. After the incubation of cells with FITC-labeled E. coli bioparticles, the cells were stained with 1 μg/ml Hoechst 33,342 dye (Invitrogen-Molecular Probes, CA, USA). After staining for 1 h at room temperature, the cells were fixed and observed under Leica 4000B fluorescence microscope for green (FITC) and blue (Hoechst 33,342) fluorescence. The imaging of cells with ingested fluorescent bioparticles was taken into account.

**Statistical analysis**

The results were analyzed by Student’s t-test for statistical significance using Prism software (GraphPad, San Diego, CA, USA). P < 0.05 was considered significant.

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