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Uptake of Particulate Antigens in a Nonmammalian Lung: Phenotypic and Functional Characterization of Avian Respiratory Phagocytes Using Bacterial or Viral Antigens

Eveline D. de Geus, Christine A. Jansen, and Lonneke Vervelde

Major distinctive features of avian lungs are the absence of draining lymph nodes and alveoli and alveolar macrophages (MPhs). However, a large network of MPhs and dendritic cells (DCs) is present in the mucosa of the larger airways and in the linings of the parabronchi. For the modulation of respiratory tract immune responses, for example, by vaccination, a better understanding of Ag uptake in the chicken respiratory tract is needed. In this study, we provide detailed characterization of APCs in chicken lungs, including their functional in vivo activities as measured by the uptake of fluorescently labeled 1-μm beads that are coated with either LPS or inactivated avian influenza A virus (IAV) mimicking the uptake of bacterial or viral Ag. We identified different subsets of MPhs and DCs in chicken lungs, based on the expression of CD11c, activation markers, and DEC205. In vivo uptake of LPS- and IAV-beads resulted in an increased percentage MHC class II+ (MHC II+) cells and in the upregulation of CD40. The uptake of LPS-beads resulted in the upregulation of CD80 and MHC II on the cell surface, suggesting either uptake of LPS- and IAV-beads by different subsets of phagocytic cells or LPS-mediated differential activation. Differences in phagosomal acidification indicated that in chicken lungs the MHC II+ and CD80+ bead+ cell population includes DCs and that a large proportion of beads was either LPS or inactivated avian influenza A virus (IAV) mimicking the uptake of bacterial or viral Ag. We identified different subsets of MPhs and DCs in chicken lungs, based on the expression of CD11c, activation markers, and DEC205. In vivo uptake of LPS- and IAV-beads resulted in an increased percentage MHC class II+ (MHC II+) cells and in the upregulation of CD40. The uptake of LPS-beads resulted in the upregulation of CD80 and MHC II on the cell surface, suggesting either uptake of LPS- and IAV-beads by different subsets of phagocytic cells or LPS-mediated differential activation. Differences in phagosomal acidification indicated that in chicken lungs the MHC II+ and CD80+ bead+ cell population includes DCs and that a large proportion of beads was taken up by MPhs. LPS-bead+ cells were present in BALT, suggesting local induction of immune responses. Collectively, we characterized the uptake of Ags by phagocytes in the respiratory tract of chickens. The Journal of Immunology, 2012, 188: 4516–4526.

The avian RT differs significantly from the mammalian RT in morphology and airflow as well as immunologically. The mammalian lung has bidirectional airflow, whereas the avian lung has unidirectional airflow (reviewed in Ref. 6), and birds do not have a diaphragm but use air sacs to ventilate their lungs. In birds, the trachea bifurcates into two primary bronchi (7) that enter the lung and branch into secondary bronchi. Branches of the secondary bronchi are the tertiary bronchi or parabronchi that, like the alveoli in mammals, are surrounded by a network of blood capillaries and are the functional units of gas exchange (6, 8). A parabronchus is surrounded by the interparabronchial septa. Air enters through the lumen of a parabronchus, passes through the atria into the infundibula, where the air capillaries are located. Major immunological differences between mammalian and avian lung are the lack of draining lymph nodes (DLNs) and the lack of alveoli and of alveolar MPhs on the surfaces of the air capillaries in avian lung. However, in avian species, a large network of MPbs and DCs is present in the tissue of the larger airways and in the linings of the parabronchi, the subepithelial phagocytes, and the interstitial macrophages, indicating that the phagocytic cells are located strategically to clear the air of inhaled particles (9, 10).

In mammals, numerous studies have been performed on the uptake of Ags by airway DCs and MPhs (11–15). After intratracheal bead instillation into mouse lung, bead+ cells were found to be CD11c+CD11b+ MPhs, CD11b+CD11c− granulocytes, or CD11c−MHC II+ DCs (15). In the steady state, some migration of CD11c−MHC I+high cells to the mediastinal lymph nodes (MLNs) occurs, and this migration is increased upon stimulation (12, 15). After the ingestion of an Ag in the presence of danger signals, DCs migrate to the MLNs and upregulate the expression of MHC II and costimulatory molecules (4, 16). It has been shown that, after influenza infection, the langerin+CD103+CD11c− conventional dendritic cells (cDCs) that migrated to the MLNs presented Ag ex vivo to both CD4 and CD8 T cells (4). Upon OVA-bead administration, the DCs in the MLNs are CD11c+CD103+...
MHC II^{high} and induce high levels of OVA-specific T cell proliferation (15).

Few studies in chickens have addressed the uptake of particles by RT cells. It was shown that MPs present in infundibula and the atrial lining do not migrate to the respiratory surface but that particles are ferried from the air spaces to the MPs by the epithelial layer (10). The unidirectional airflow and the anatomy of the avian lung affect the deposition of respiratory Ags. Upon aerosol inoculation, large beads (3.7 μm and up) were deposited mainly in the anterior portion of the RT, whereas smaller particles were distributed more evenly (17–19). The heaviest deposition was observed at the bifurcations of the primary to secondary bronchi (19). A similar deposition was found after aerosol spray with avian influenza virus (20). In chickens, the phenotype and function of MPs and DCs are poorly defined because of a lack of defined markers, but progress has been made, and in vitro bone marrow-derived DCs (BMDCs) can be cultured and, like mammalian DCs, were shown to express surface CD11, the activation markers MHC II and CD40, and the C-type lectin DEC205 after LPS maturation (21).

In this study, we provide a detailed analysis of MPs and DCs in chicken lungs and characterize the phagocytic cells by using fluorescently labeled 1-μm beads that are coated with either LPS or inactivated avian influenza A virus (IAV) mimicking the uptake of bacterial or viral Ag, respectively. Chicken in commercial facilities are exposed constantly to high concentrations of airborne bacteria. LPS is a major constituent of the cell walls of Gram-negative bacteria and therefore was chosen as a model for the uptake of bacterial Ag. The size of *Escherichia coli* is ~0.5–2 μm (22); therefore, LPS-beads resemble a bacterium in size. Influenza virus has a size of ~70 nm (23) and is much smaller than an IAV-bead. However, because airborne influenza virus can be found in aerosols and dust particles and, when chickens are infected experimentally via the tracheal route, droplets containing virus are administered, an IAV-bead does resemble the size of a droplet or particle at the moment of initial deposition.

In summary, we determined the phenotypes of respiratory MPs and/or DCs in avian lung in the steady state and identified different populations based on marker expression and anatomical location. We then characterized phagocytic cells in the lung and show that the uptake of LPS- and IAV-beads resulted in an activated phenotype of different subsets of phagocytic cells in chicken lungs. Furthermore, in chicken lungs, the MHC II and CD80* bead* cell population contains DCs, based on the absence of phagosomal acidification. A large proportion of beads was taken up by MPs, which are located within the KUL01* (recognizes chicken mononuclear phagocytes), CD11*, and CD40* bead* population. Future experiments will address the APC/T cell interaction, especially in the BALT areas, to identify the RT DC subsets that are responsible for the priming of T cells and that can be targeted for vaccination purposes.

Materials and Methods

**Chickens**

One-day-old Lohmann Brown chickens were purchased from Verbeek Broederij and from the faculty’s Brown Leghorn layer flock. Animals were housed in groups and received food and water ad libitum. In compliance with Dutch law, all of the experiments were approved by the Animal Experimental Committee of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands, in accordance with the Dutch regulations on experimental animals.

**Influenza virus**

H9N2 A/Chicken/Saudi Arabia/SP02525/G3AV/2000 (Animal Health Service, Denter, The Netherlands) was inactivated using β-propiolactone (BPL) (Acros Organics). In brief, a 10% BPL solution was prepared in a 125 mM sodium citrate and 150 mM sodium chloride buffer, and 10 μl/ml was added to the virus. Virus-BPL solution then was incubated for 24 h at 4°C under continuous stirring. Inactivation was confirmed by inoculation in embryonated chicken eggs. Before being coupled, the virus first was centrifuged for 30 min at 3,000 × g, and then the supernatant was concentrated by ultracentrifugation for 1 h at 100,000 × g. The supernatant was discarded, and the pellet was resuspended in PBS. Protein content was determined using a bicinchoninic acid protein assay kit (Pierce).

**Coating of beads with LPS, H9N2 IAV, or FITC**

Carboxylated crimson fluorescent 1-μm beads (Molecular Probes) were coated with LPS from *E. coli* 0127:B8 (Sigma-Aldrich) and with inactivated H9N2 IAV according to manufacturers’ procedures. All of the steps were performed in glass tubes. LPS and IAV were dissolved at a concentration of 2.5 mg/ml in 50 mM MES buffer (pH 6), and equal volumes of beads were added to each type of Ag. The solution was incubated for 15 min at room temperature, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added at a concentration of 5 mg/ml. After the pH was adjusted to 6.5 using 1 M NaOH, the solution was incubated for 2 h at room temperature on a rocker. To label the coated beads with FITC, fluorescein-5-EX, succinimidyl ester (Molecular Probes) was added to the mix right before the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

To quench the reaction, glycine was added to a final concentration of 100 mM, and the solution was incubated for 30 min at room temperature. Coated beads were washed three times and resuspended in PBS with 1% BSA and 2 mM NaN₃.

Coating of H9N2 to beads was determined using FACS analysis. In brief, IAV-beads and uncoated beads were incubated with rabbit anti-H9 (Immune Techonologies) for 30 min on ice, followed by staining with Alexa Fluor 488-labeled donkey anti-rabbit IgG (H+L; Invitrogen) for 30 min on ice. Coating of FITC to beads was checked using flow cytometry by comparing the FITC signals of FITC-coated and uncoated beads.

Beads were analyzed using a FACS Calibur or FACS Canto (BD Biosciences), and data were analyzed using the software program FlowJo (Tree Star).

**DC culture and stimulation**

DCs were cultured from bone marrow (BM) collected from femurs and tibias of embryonation day 18 embryos as described previously (21), with modifications. In short, cells were cultured at a concentration of 2.5 × 10⁶/ml in six-well plates in RPMI 1640 (Life Technologies) complete medium containing 5% chicken serum (Life Sciences). Isolated RNA was used for quantitative reverse transcription PCR

Total RNA was isolated from BMDCs using the RNeasy Mini Kit and DNase-treated with the RNase-free DNase Set following the manufacturer’s instructions (Quagen Benelux). Purified RNA was eluted in 30 μl RNase-free water and stored at −80°C. All of the RNA samples were checked for quantity and quality using a spectrophotometer (ND-1000; Isogen Life Sciences). Isolated RNA was used for quantitative reverse transcription PCR.
PCR (qRT-PCR) analysis, qRT-PCR was performed to analyze the expression of IL-1β, IL-10, and IFN-γ in BMDCs. cDNA first was generated from 500 ng RNA with reverse transcription using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Then qRT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems). Primers (Invitrogen) and probes (Applied Biosystems) were designed according to previously published sequences (24, 25). RNA from Con A-stimulated splenocytes was used for standard curves. Chicken rRNA (28S) was used as a reference gene.

**Intratracheal inoculation of beads**

Four-week-old chickens were intratracheally (i.t.) administered 100 μl bead solution containing 3.6 × 10⁶ beads using a flexible oral gavage needle (Instech Solon). The chickens either received control beads, LPS-beads, or IAV-beads. At 2, 6, and 24 h post inoculation (hpi), chickens were sacrificed, and lungs, spleen, BM, and blood were collected for the analyses of the phenotype and migration patterns. To study the acidification of the endosomal compartment of the phagocytic cells, chickens were inoculated with FITC-LPS- or FITC-IAV-beads (200 μl bead solution containing 7.2 × 10⁹ beads). These chickens were sacrificed 1 hpi. Part of one lung containing the primary bronchus was snap-frozen for histology. That part was chosen because the highest deposition of beads was expected at the bifurcations of the primary to secondary bronchi (19). Remaining parts of lungs and other organs were used for flow cytometry analyses.

**Preparation of cells**

Lungs were cut into small pieces and digested with 2.4 mg/ml collagenase A (Roche) and 1 mg/ml DNAase I (Roche) solution for 30 min at 37°C as described previously (26). BM was flushed out from femurs and tibias using PBS. Single-cell suspensions of spleen, BM, and lung were prepared by gently squeezing through a 70-μm cell strainer (BD Biosciences). Leukocytes were isolated by density gradient centrifugation for 20 min at 1000 × g using Ficoll-Paque (GE Healthcare) and washed twice with PBS.

**Flow cytometry**

The uptake of beads by BMDCs and the effect on their phenotype were analyzed by flow cytometry. DCs were resuspended in PBS supplemented with 0.5% BSA and 0.005% NaN₃ (FACS buffer) and incubated with mouse anti-chicken CD40 (AbB Serotec; Table I) for 30 min on ice, followed by staining with PE-labeled goat anti-mouse IgG2a for 30 min on ice.

For phenotypical analyses of lung cells and for analyses of in vivo uptake, cells were resuspended in FACS buffer and stained using the mouse mAbs described in Table I. The putative CD11c mAb 6B5 recognizes the same cells in h BMDC cultures and in lymphoid tissues stained using immunocytochemical techniques described below as the previously published mAb SF2 (21). FITC-labeled goat anti-mouse IgG1 (Southern Biotechnology Associates), PE-labeled goat anti-mouse IgG2a (Southern Biotechnology Associates), DyLight 405-labeled goat anti-mouse IgG1 (Biolegend), and Pacific blue-labeled streptavidin (Invitrogen) were used as secondary Abs. On the basis of the number of cells isolated between 0.5 × 10⁶ and 1 × 10⁶ live cells [based on forward light scatter (FSC) profile] were taken up per sample. Cells were analyzed using a FACS-Calibur or a FACSCan (BD Biosciences), and data were analyzed using the software program FlowJo (Tree Star).

**Immunocytochemistry**

For fluorescent microscopy, cryostat sections (6 μm) were fixed in 2% paraformaldehyde, washed in PBS, and incubated for 1 h with optimally diluted (Department of Biological Sciences, Hybridoma Bank, Department of Biology, University of Iowa, Iowa City, IA; Table I) in PBS with 0.5% BSA and 0.01% NaN₃ (staining buffer). Sections were washed three times and incubated with Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) in staining buffer for 1 h. Nuclei were visualized using DAPI (Invitrogen). Pictures were taken using a Leica DMRE fluorescence microscope with a Photometrics CoolSNAP charge-coupled device digital camera and analyzed using the Image-Pro laboratory image analysis software.

For light microscopy, cryostat sections (6 μm) were fixed in acetone, and endogenous peroxidase treatment was performed in PBS with 0.3% H₂O₂. Slides were washed in PBS and incubated for 1 h with optimally diluted primary Ab or isotype controls (Table I) in staining buffer. Sections were washed three times and blocked with normal horse serum diluted in PBS followed by incubation with biotinylated horse anti-mouse IgG and avidin-biotinylated enzyme complex (VECTASTAIN Elite) as described previously (27). Color development was done using 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Slides were washed with PBS and counterstained with hematoxylin (Sigma-Aldrich).

**Data analysis**

The ratio of FITC/allophycocyanin geometric fluorescent intensities (GeoMFIs) was determined as follows: GeoMFIs of FITC (pH sensitive) and allophycocyanin (pH insensitive) signals of the beads themselves were determined within cells that had ingested one bead, and the FITC signal was divided by the allophycocyanin signal.

All of the data were analyzed using the Kruskal–Wallis or Mann–Whitney U test with SPSS 16.0 software (IBM). Graphs were prepared using Prism 4 (GraphPad).

**Results**

**Identification of different populations of respiratory MPhs and/or DCs in avian lung**

We first determined the phenotypes of chicken lung MPhs and DCs in the steady-state situation, using the markers that were found to be expressed on chicken BMDCs (21, Table I). The expression of CD11, KUL01, which characterizes chicken mononuclear phagocytes, DEC205, and the activation markers MHC II, CD40, and CD80 is shown in Fig. 1A. Using double staining (Fig. 1B), we show that the KUL01+ cells express high levels of MHC II, CD40, and CD80. The phenotypes of the CD11⁺ and MHC II⁺ cells indicated that different subpopulations were found in the lung. A subpopulation of CD11⁺ cells expressed MHC II, and ~50–70% of the CD11⁺ cells expressed CD40 and CD80 at slightly lower levels than the KUL01⁺ cells did. The remaining CD11⁺ cells expressed low levels CD40 and/or CD80. These are partially overlapping populations. A subpopulation of MHC II⁺ cells expressed KUL01, CD40, CD11, and low or intermediate CD80. When CD11⁺ or MHC II⁺ cells with low and high FSC were analyzed separately, we only found differences for CD80 expression within the MHC II⁺ cells between low and high FSC cells. The cells with higher FSC had higher levels of CD80 expression. This shows that different subsets of chicken MPhs and DCs are present in RT, based on marker expression.

In the avian lung, a network of MPhs and DCs, identified on the basis of morphology using light and electron microscopy (10, 28), can be found to be strategically located to ingest inhaled particles and pathogens. Sections of the lung revealed the abundant presence of CD11⁺ and KUL01⁺ cells in the interstitial tissue of the parabronchial wall (Fig. 2A, 2B). These cells also were located in the interstitial septa, in contrast to the DEC205⁺ cells that were located mainly in the interstitial tissue of the parabronchial wall (Fig. 2C).

**Coated beads can be used to mimic the uptake of bacterial and viral particulate Ag**

Corbanie et al. (18) showed previously that 1-μm beads could be recovered from the entire chicken RT. To mimic the spread of respiratory pathogens through the entire RT, we therefore decided to use 1-μm beads in these experiments. To determine which fluorescent color of beads would be suitable, we first compared the uptake of beads in vitro cultured BMDCs. Cells were cultured for 2 h with beads at a 10:1 ratio, and uptake was determined using flow cytometry. Yellow/green, red, and crimson beads were taken up efficiently (Supplemental Fig. 1A), but yellow/green and crimson beads were easier to distinguish from the background, and this feature makes them better suited for use in vivo (Supplemental Fig. 1B).

We coated crimson beads with LPS or IAV to use as a model for bacterial or viral Ag, respectively. We first determined whether the uptake of coated beads by chicken BMDCs differed from the uptake of uncoated beads. Coated beads were taken up efficiently by BMDCs, and the percentage of bead⁺ cells increased over time during the first 16 h of culture. However, the uptake of coated beads was decreased when compared with that of control beads (Fig. 3A). Although we observed decreased uptake, when we...
compared the proportion of cells that had taken up one bead/all bead+ cells, we observed that DCs that ingested coated beads had taken up more beads per cell when compared with uncoated beads (Fig. 3B). The uptake of higher numbers of beads per cell could be explained by the formation of bead aggregates because of the coating procedure; however, as determined by light microscopy,
for all types of beads, the majority of the beads were in a mono-

for all types of beads, the majority of the beads were in a mono-
disperse state (data not shown). LPS-beads, but not IAV-beads,

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II expression levels on lung-resident cells. Minimal bystander activation was observed after inoculation with LPS-beads, because CD80 expression was upregulated at 24 hpi with LPS-beads, but the expression was significantly lower than that in LPS-bead+ cells.

To determine which phagocytic cell subpopulations were activated after the ingestion of beads, we analyzed CD40 and CD80 expression on KUL01+, MHC II+, and CD11+ cells. Uptake of both LPS- and IAV-beads resulted in the upregulation of CD40 on KUL01+, MHC II+, and CD11+ cells (Fig. 5A, 5B). CD40 was upregulated at 2 and 6 h and back to control levels at 24 hpi. LPS-bead+ cells tended to have higher expression of CD40 than IAV-bead+ cells.

CD80 expression on CD11+ cells was much lower than that in the KUL01+ and MHC II+ population but still significantly increased when compared with that of control bead+ cells (Fig. 5B). CD80 expression was increased at 24 hpi after the ingestion of LPS-beads in both KUL01+ and MHC II+ cells. Administration of IAV-beads resulted in a small, but significant increase of CD80 expression within MHC II+ cells at 6 hpi. As observed for CD40, the expression of CD80 was low on CD11+ cells; however, cell surface expression was significantly increased in IAV-bead–inoculated birds at 2 and 6 hpi (Fig. 5C). These data indicate the activation of both KUL01+ and/or MHC II+ phagocytes after the ingestion of both LPS- and IAV-beads.

**FIGURE 4.** Activation of respiratory DCs and MPhs by the ingestion of coated beads. Control beads, LPS-beads, and IAV-beads were administered i.t., and the uptake and phenotype of bead+ cells in the lungs were determined at different time points postadministration. (A) Representative examples of the uptake of the different beads. (B) CD45 expression within bead+ cells. (C) MHC II expression within bead+ cells. Left panel shows the percentage expression; right panel shows MHC II fluorescent intensity. (D) CD40 expression levels within bead+ cells. (E) CD80 expression levels within bead+ cells. Data are depicted as mean ± SEM, and each group consisted of four chickens. *p < 0.05 when compared with the indicated group.
**FIGURE 5.** Activation of KUL01⁺, MHC II⁺, and CD11⁺ phagocytes. (A) FACS plots showing KUL01⁺ cells within the bead⁺ cell population at 2 hpi. Histogram of CD40 expression within the bead⁺ KUL01⁺ population is shown in the right panel. (B) Expression levels of CD40 within the KUL01⁺, MHC II⁺, and CD11⁺ bead⁺ cells. (C) Expression levels of CD80 within the KUL01⁺, MHC II⁺, and CD11⁺ bead⁺ cells. Data are depicted as mean ± SEM, and each group consisted of four chickens. *p < 0.05 when compared with the indicated group.

**LPS- and IAV-beads are taken up by both MPhs and DCs**

In chickens, markers to discriminate MPhs from DCs are lacking. To discriminate the uptake of the different beads by MPhs and DCs, we made use of the pH sensitivity of FITC-coated beads. DCs adapted their endocytic and phagocytic pathways to limit proteolytic activity, and as a result, their phagosomal compartment has a near neutral pH (29, 30), whereas MPhs have a much lower phagosomal pH and high proteolytic activity (31), resulting in the quenching of the FITC signal.

We set up the system in vitro and measured the uptake of FITC beads using cultured BMDCs and the chicken Mph cell line HD11. BMDCs express CD11, KUL01, MHC II, and DEC205 (21), whereas unstimulated HD11 cells all express KUL01 but are negative for CD11, MHC II, and DEC205 (data not shown). Cells were cultured with FITC beads for different time points, and FITC intensity was measured, normalized to the fluorescent intensity of the crimson beads. The data were analyzed as follows: GeoMFI of FITC (pH sensitive) and GeoMFI of allophycocyanin (pH-insensitive) of the bead itself were determined within cells that had ingested one bead, and the FITC signal was divided by the allophycocyanin signal (F/A ratio). To be able to compare results obtained with both BMDCs and HD11, F/A ratios were determined for individual beads before adding them to cell cultures. Next, the F/A ratios for BMDCs and HD11 were normalized using the F/A ratios of the individual beads. This means that, with a relative F/A ratio of 1, the FITC signal had not changed. Within 30 min after the addition of FITC beads, the F/A ratio of the phagosomal compartment of HD11 cells decreased, indicating a decrease in pH. The decrease in pH was transient, and pH was increased again at 3 h after the addition of beads. In contrast, in...
BMDCs, the F/A ratio was stable from 30 min until 3 h after the addition of beads, indicating a stable pH, without phagosomal acidification (Fig. 6).

We then administered the FITC-LPS- and the FITC-IAV-beads to chickens in vivo and analyzed the phenotype and F/A ratio of bead+ cells in the lung. For both types of beads, bead+ cells were detected in the lungs at 1 hpi (data not shown). As shown previously with the IAV-beads, a significantly higher percentage of FITC-IAV-bead+ cells expressed MHC II than LPS-bead+ cells (Fig. 7A). A significantly lower percentage of FITC-IAV-bead+ cells expressed CD80 as compared with FITC-LPS-bead+ cells. For both types of beads, hardly any beads were taken up by DEC205+ cells (Fig. 7A). No differences in the activation status of cells were observed as shown by cell surface expression of MHC II, CD40, and CD80 (data not shown).

To determine the F/A ratios, cells that had ingested one bead were gated, and FITC signals were analyzed in marker+ cells as shown in Fig. 7B. To be able to compare the results obtained with FITC-LPS-beads with those obtained with FITC-IAV-beads, the F/A ratios were calculated as follows: F/A ratios of individual beads were determined for both types of coated beads before administering them to chickens and used to normalize F/A ratios obtained in vivo. Similar to the in vitro experiment, this means that, with a relative F/A ratio of 1, the FITC signal was not changed. When FITC-LPS- and FITC-IAV-beads were analyzed separately, no significant differences between F/A ratios were observed, probably due to a lack of statistical power. We therefore analyzed data for both types of beads pooled together and found that the relative F/A ratio was decreased significantly in CD11+ (p = 0.012), KUL01+ (p = 0.017), and in CD40+ (p = 0.017) bead+ cells but not in CD80+ cells when compared with the F/A ratio in MHC II+ cells (Fig. 7C). We conclude that both types of beads are taken up by both MPhs and DCs, where the bead+ MHC II+ and CD80+ populations contain DCs and not the MPh population, because F/A ratios were not decreased in those populations.

Presence of LPS-beads in BALT areas

To determine whether cells migrate after the uptake of different beads, chickens were given uncoated beads, LPS-beads, or IAV-beads i.t., and blood and spleen were analyzed 2, 6, and 24 hpi using flow cytometry. After the uptake of uncoated beads, no bead+ cells were found in blood or spleen. LPS-bead+ cells were found in blood in 2 of 12 chickens and in spleen of 1 of 12 chickens. With IAV-beads, IAV-bead+ cells were only found in blood in 1 of 12 chickens (data not shown). When chickens were inoculated with FITC-LPS- and FITC-IAV-beads, a higher concentration of beads was used. To determine whether this higher dose would result in more bead+ cells in blood and spleen or possibly BM, we also measured the frequency of bead+ cells in those organs. With both types of beads, bead+ cells were found in blood of 4 of 8 of chickens, in spleen of 2 of 8 chickens, and never in BM (data not shown).

Using histology, we determined the location of RT bead+ cells in situ. Only when LPS-beads were given, bead+ cells were observed in BALT areas (Fig. 8). IAV-bead+ cells were not detected in BALT areas; however, we cannot exclude the possibility that these cells can reach the BALT area.

Discussion

Understanding DC biology has been fundamental to the study of immune responses in mammals. A heterogeneous group of APCs is present in rodent and human RT, including different DC populations, MPhs, and B cells. In this study, we provide a detailed analysis of MPhs and DCs in nonmammalian (chicken) lung and characterize the phagocytic cells by using fluorescently labeled beads that are coated with either LPS or IAV mimicking the uptake of bacterial and viral Ags.

We identified different subsets of chicken RT cells based on the expression of CD11 (putative CD11c), DEC205, KUL01, and the activation markers MHC II, CD40, and CD80, although their functional differences remain unknown.

Before the characterization of RT phagocytic cells, we first validated the use of the different beads in vitro. The in vitro culture of chicken BMDCs has been described previously (21), and these cells resemble mammalian DCs in morphology. As shown previously (21), BMDCs readily ingested polystyrene beads, and here we show that they also efficiently ingest LPS- and IAV-beads, although at lower levels than uncoated beads. Differences in the uptake and numbers of beads per cell might indicate different uptake mechanisms of uncoated and coated beads. Uncoated beads most likely were only taken up by phagocytosis, whereas coated beads could be taken up by receptor-mediated endocytosis. In mouse, it has been shown that the route of Ag uptake can impact Ag presentation by BMDCs (32). In our experiments, the uptake of LPS-beads by BMDCs resulted in CD40 upregulation, which resembled the upregulation of costimulatory molecules on chicken BMDCs after LPS stimulation (21) and on mouse splenic DCs after the uptake of LPS-beads (33). Also, cytokine mRNA expression after the ingestion of LPS-beads resembled the responses in chicken BMDCs after LPS stimulation (21), and therefore these LPS-beads are a useful tool to mimic bacterial uptake and stimulation by bacterial Ags.

In chicken, it is known that i.t. administration of LPS results in increased numbers of phagocytes in lung lavage (34). In humans, LPS inhalation induces the maturation of lung cells, with the upregulation of MHC II and CD80/86 (35). Likewise, in our study, chicken RT phagocytes acquired costimulatory molecules after the uptake of LPS-beads as indicated by the upregulation of MHC II, CD40, and CD80 cell surface expression. CD40 expression had returned to control levels at 24 hpi, which indicates transient ac-
tivation of RT phagocytes. The apparent downregulation of CD40 expression at 24 hpi is probably not caused by the cells that have just entered the lung and have ingested a bead, because CD40 upregulation was observed already at 2 hpi.

In contrast to the LPS-beads, the ingestion of IAV-beads by BMDCs did not result in CD40 upregulation or cytokine expression in vitro, but in vivo uptake of these beads did result in the upregulation of CD40 and, to a much lower extent, CD80. This is in line with previously published results showing a lack of acute responses in in vitro IAV-infected chicken tracheal organ cultures as compared with in vivo infected trachea (36). Furthermore, responses depend on the DC subset analyzed. Subpopulations of mouse respiratory DCs, CD103+, plasmacytoid DCs (pDCs), or CD103^2CD11b^high, differed in their in vitro susceptibilities to IAV infection, and pDCs were the least susceptible but produced the highest levels of IFN-α and upregulated costimulatory molecules. CD103^+ cells were the most susceptible but lacked the expression of costimulatory molecules, whereas the CD103^-CD11b^high subset acquired CD86, CD80, and CD40, but infection did not enhance cytokine production (37). Furthermore, Diebold et al. (38) observed significant differences in IFN-α responses after in vitro infection of mouse pDCs and cDCs, where pDCs produced high levels of IFN-α and cDCs showed no detectable IFN-α production. Responses were similar when live and 56°C heat-inactivated virus was used, and therefore IFN-α responses depend on the uptake of IAV by the different DC subsets and not on infection. When administered in vivo, the cells that ingested an IAV-bead reacted in a similar manner to in vivo influenza-infected mouse RT cells, with upregulation of costimulatory molecules (4, 39). Inoculation with coated beads also affected the bead^+ cells, probably because naive cells enter the lung (Supplemental Fig. 2).

In mouse, it was shown previously that the endosomal compartment of DCs has a near neutral pH (29, 30), whereas MPhs have a much lower phagosomal pH (31). In chickens, markers to discriminate MPhs from DCs are lacking, and we therefore made use of this difference between MPhs and DCs. The difference in phagosomal pH first was confirmed for chicken cells, using the HD11 MPh cell line and BMDCs. Using the combination of available markers and acidification of the endosomal compartment, we set out to identify respiratory DCs and MPhs in chicken lungs. After the uptake of a coated bead, MHC II^+ and CD80^+ cells did not acidify their endosomal compartment and therefore most likely include the DC population. The phenotype that we found for respiratory DCs (MHC II^+ and CD80^+), is comparable to the phenotype described in mouse lung parenchyma, where the CD11c^+ cells express MHC II and CD80 and...
are negative for CD40. In mouse, these cells are potent activators of naïve T cells (5). In chickens, it is not known which cell type is responsible for the priming of naïve T cells and whether a functional distinction between MPVs and DCs in priming capacity exists. In mouse, decreased phagosomal acidification in DCs reduces Ag proteolysis to a level that allows processing but does not fully destroy antigenic peptides. This contributes to the high Ag-presenting capacity of DCs (40, 41). MPVs have much higher levels of proteolysis than DCs, which limit their efficiency as APCs (42). In chicken lungs, we identified cells that decreased the pH of their endosomal compartment after the uptake of a bead (CD11+, KUL01+, and CD40+ phagocytes) and cells that did not (MHC II+ and CD80+ phagocytes), suggesting that in chicken MPVs also are functionally distinct from DCs. A large proportion of beads was taken up by MPVs, as also was published for the uptake in mouse RT (15). MPVs and DCs had different anatomical locations, with CD11+ and KUL01+ cells, the populations containing the MPVs, located in the interstitial tissue of the parabronchial wall and in the interatrial septa. However, DEC205+ cells were located mainly in the interstitial tissue of the parabronchial wall. After the uptake of Ag, DCs mature and migrate toward immune inductive sites. In chickens, the location where the cells migrate to and interact with T cells is unclear, because of the lack of DLNs. Possible locations where APCs could migrate are the BALT at the junctions between primary and secondary bronchi, the follicles present in the interstitium between parabronchi, or the spleen. In chickens, BALT is constitutively present and is thought to compensate for the lack of DLNs (43). After the inoculation with LPS-beads, bead+ cells were present in BALT areas (Fig. 8). We occasionally observed bead+ cells in blood and spleen after the inoculation with coated beads but not after the inoculation with uncoated beads. This could indicate that, after the uptake of Ag, APCs migrate mainly locally toward the BALT area and not systemically toward the spleen. This suggests local induction of RT immune responses as was suggested already in a previous study addressing RT humoral immune responses (44). Alternatively, we cannot exclude the possibility that inoculation with an even higher number of beads might allow consistent detection of beads in spleen and blood.

In summary, we determined the phenotypes of lung cells in the steady state and identified different subsets of RT MPVs and DCs. Furthermore, we show that the uptake of LPS- and LAV-beads resulted in the upregulation of costimulatory molecules of different subsets of phagocytic cells, with the MHC II+ and CD80+ bead+ cell population containing DCs based on the absence of phagosomal acidification. Future experiments will address the APC/T cell interaction, especially in the BALT areas, to identify the RT DC subsets that are responsible for the priming of T cells and that can be targeted for vaccination purposes.

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Disclosures

The authors have no financial conflicts of interest.

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