Pulmonary sarcoidosis is associated with exosomal vitamin D–binding protein and inflammatory molecules

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Background: Sarcoidosis is an inflammatory granulomatous disorder characterized by accumulation of T_H1-type $CD4^+$ T cells and immune effector cells within affected organs, most frequently the lungs. Exosomes are extracellular vesicles conveying intercellular communication with possible diagnostic and therapeutic applications.

Objectives: We aimed to provide an understanding of the proinflammatory role of bronchoalveolar lavage fluid (BALF) exosomes in patients with sarcoidosis and to find candidates for disease biomarkers.

Methods: We performed a mass spectrometric proteomics characterization of BALF exosomes from 15 patients with sarcoidosis and 5 healthy control subjects and verified the most interesting results with flow cytometry, ELISA, and Western blot analyses in an additional 39 patients and 22 control subjects.

Results: More than 690 proteins were identified in the BALF exosomes, several of which displayed significant upregulation in patients, including inflammation-associated proteins, such as leukotriene A_4 hydrolase. Most of the complement-activating factors were upregulated, whereas the complement regulator CD55 was seen less in patients compared with healthy control subjects. In addition, for the first time, we detected vitamin D–binding protein in BALF exosomes, which was more abundant in patients. To evaluate exosome-associated vitamin D–binding protein as a biomarker for sarcoidosis, we investigated plasma exosomes from 23 patients and 11 healthy control subjects and found significantly higher expression in patients.

Conclusion: Together, these data contribute to understanding the role of exosomes in lung disease and provide suggestions for highly warranted sarcoidosis biomarkers. Furthermore, the validation of an exosome-associated biomarker in the blood of patients provides novel, and less invasive, opportunities for disease diagnosis. (J Allergy Clin Immunol 2017;139:1186-94.)

Key words: *Exosomes, extracellular vesicles, sarcoidosis, leukotrienes, vitamin D–binding protein, proteome, biomarkers, complement*

Sarcoidosis is an inflammatory granulomatous disorder affecting mostly the lungs. Spontaneous remission often occurs, although around one third of patients have chronic disease, which can be fatal.[1,2] The cause of sarcoidosis is not yet fully understood, but immunologic evidence and its geographic variation have suggested causes, including infection, occupational exposure, and genetic factors. The presence of clonal T_H1-like $CD4^+$ T cells, macrophages, and immune effector cells within affected organs suggests an antigen-driven autoimmune disease.[3] Autoantigens, such as vimentin and ATP synthase, have been identified as targets for expanded T-cell clones in the lung,[4] and other data suggest roles of microorganisms because of the T-cell reactivity against mycobacterium tuberculosis catalase-peroxidase (mKatG).[5]

Exosomes are 30- to 150-nm vesicles derived from endosomal compartments, which act as messengers between cells[6] and can either stimulate or inhibit immune cells, depending on their cellular origin.[7] Dendritic cell exosomes can stimulate T cells[8,9] and are promising cancer vaccine candidates,[10,11] but exosomes from the gut and cancer cells seem to inhibit the immune system.[12,13] Exosomes have been found in most body fluids, including breast milk[14] and plasma,[15] and are likely to play physiologic roles and have potential as disease biomarkers.

We have previously found exosomes in bronchoalveolar lavage fluid (BALF) of healthy subjects[16] and shown in functional studies that BALF from patients with sarcoidosis is enriched in proinflammatory exosomes and can induce production of IFN-γ and IL-8 *in vitro*.[17] In further functional studies we have shown that macrophage-and dendritic cell–derived exosomes carry leukotriene (LT) pathway components and have migration-inducing capacities,[18]

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Abbreviations used
BAL: Bronchoalveolar lavage
BALF: Bronchoalveolar lavage fluid
FITC: Fluorescein isothiocyanate
5-LO: 5-Lipoxygenase
LT: Leukotriene
LTA_4H: Leukotriene A_4 hydrolase
MAC: Membrane attack complex
NTA: Nanoparticle tracking analysis
VDBP: Vitamin D–binding protein

suggesting that exosomes might contribute to airway remodeling. We have also established that BALF exosomes in asthmatic patients carry LT-forming enzymes and functionally induce production of cytokines and LTs.[19] Recently, it was also found that exosomes promote the chemotaxis of neutrophils by transporting LTB_4.[20] Taken together, the accumulating evidence points to a role of exosomes in LT-mediated intercellular communication, possibly with implications in patients with airway disease.

In view of the latter evidence, we aimed to thoroughly dissect disease-associated components of BALF exosomes from patients with sarcoidosis. We conducted a full proteomic analysis of the exosomal contents, followed by validations of the most interesting results using flow cytometry, ELISA, Western blotting, and nanoparticle tracking analysis (NTA). We detected clear proteomic differences between exosomes from patients with sarcoidosis and control subjects, with proinflammatory and immune system activation pathway components more abundant in patients' exosomes. Several of the proteomic findings, including an increase in LT-forming enzymes, were successfully validated by using several techniques, as were a number of potential biomarkers. These findings indicate the importance of future investigations of the role of these proteins in exosomes in patients with sarcoidosis but also as disease biomarkers to support the invasive and circumstantial diagnostic procedures of patients with sarcoidosis.

METHODS

Study subjects

BALF from 44 patients with sarcoidosis (median age, 46 years; 52% male and 11% smokers), 3 patients with fibrosis, 2 asthmatic patients, and 1 patient with alveolitis was obtained as part of routine diagnostic investigations at Karolinska Hospital, Sweden. Sarcoidosis diagnoses were established according to World Association of Sarcoidosis and Other Granulomatous Disorders guidelines[21] based on typical clinical signs, biopsy specimens showing noncaseating granuloma formation, and chest radiographic findings compatible with sarcoidosis. The diagnoses were further supported by differential BALF cell counts and BALF CD4/CD8 ratios and by ruling out other causes of these observations. Patients with Löfgren syndrome (n = 9) were identified based on acute onset of the disease with fever, erythema nodosum, and/or ankle arthritis and bilateral hilar lymphomas with or without concomitant parenchymal infiltrates. None of the patients had received any kind of immunosuppressant therapy at the time of bronchoscopy. Healthy control subjects (n = 22; median age, 27 years; 68% male and 14% smokers) free of medication with normal chest radiographs, blood cell counts, and electrolytes volunteered for bronchoalveolar lavage (BAL). No participants had signs of respiratory tract infection at least 4 weeks before the bronchoscopy. All the subjects had normocalcemia at the time of bronchoscopy, which is reported together with the other characteristics of the patients, including smoking habits, x-ray stages, and serum calcium and angiotensin-converting enzyme levels, where available (see Table E1 in this article's Online Repository at www.jacionline.com). All subjects provided informed consent adhering to protocols approved by the regional ethics committee.

Exosome isolation from BALF and blood

BAL was performed, as described previously.[22] BALF was strained through a double layer of Dacron nets (Millipore, Bedford, Ireland) and centrifuged at 400*g* for 10 minutes at 4°C. Cell viability was determined by mean of Trypan blue exclusion and was always greater than 90%. BALF exosomes were isolated by means of differential centrifugation, as described elsewhere.[17] Plasma exosomes were isolated by centrifuging whole blood at 600*g* for 10 minutes, and the supernatant was further centrifuged at 20,000*g* for 20 minutes. This supernatant was filtered through a 0.22-μm filter and ultracentrifuged at 140,000*g* for 90 minutes, after which, the pellet was washed in PBS.

Sample preparation and isobaric tags for relative and absolute quantification labeling

Isobaric tags for relative and absolute quantification (iTRAQ) reagents and buffers were obtained from AB Sciex (Framingham, Mass). Additional reagents, including triethyl ammonium bicarbonate, liquid chromatography–mass spectrometry grade acetonitrile, and methyl methanethiosulfonate, were obtained from Sigma (St Louis, Mo). Sequence-grade trypsin was used for protein digestion (Promega, Madison, Wis). All aqueous solutions were prepared by using water from a Milli-Q water purification system (Merck Millipore, Billerica, Mass). BALF exosomes were prepared for proteomic analysis, as previously described,[14] with modifications to accommodate for labeling with 8-plex iTRAQ reagents (AB Sciex), as described in the Methods section in this article's Online Repository at www.jacionline.com.

Liquid chromatography–tandem mass spectrometry

An Orbitrap Velos coupled to an Easyn-LC (Thermo Scientific, Waltham, Mass) was used for liquid chromatography–tandem mass spectrometry (LC-MS/MS). Additional analyses were made with the QSTAR Elite (AB Sciex), together with an UltiMate 3000 capillary LC (Dionex, Sunnyvale, Calif). The data were directly analyzed with ProteinPilot software and the Paragon identification algorithm with a human Swiss-Prot database (release August, 18, 2011; 20,245 entries).

Flow cytometry

To characterize the exosomes based on original BALF volume, 4.5-μm anti-human HLA-DR beads (clone HKB-1; Dynal, Oslo, Norway) were coated with BALF exosomes corresponding to 6 mL of the original BALF volume per microliter of beads and stained with fluorescein isothiocyanate (FITC)–conjugated antibodies to HLA-DR, HLA-ABC, CD9, CD54, CD63, CD81, CD86, dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN) (BD Biosciences, San Diego, Calif), or Mucin 1, cell surface associated (MUC-1) (Abcam, Cambridge, Mass), as previously described.[17]

For validation of the iTRAQ results, where exosomal quantities were based on total protein contents, 2 μg of BALF exosomes was added per microliter of 4-μm latex beads (Molecular Probes, Paisley, United Kingdom) coated with anti-human CD63 (BD Biosciences) and stained with HLA-DR–FITC, CD63-phycoerythrin, CD55-FITC (BD Biosciences), or C3-phycoerythrin (LSBio LifeSpan Bioscience, Seattle, Wash) or with isotype-matched controls. Samples were analyzed with a BD Biosciences FACSCalibur and FlowJo software (TreeStar, San Carlos, Calif).

Western blotting for LT pathway proteins and complement C3

Methods for Western blotting for detection of LTs and complement C3 are found in the Methods section in this article's Online Repository.

ELISA for VDBP

Thirteen healthy control subjects and 6 patients with nonsarcoidosis lung disorders (3 with fibrosis, 2 with asthma, and 1 with alveolitis) were analyzed by using an ELISA kit, according to the manufacturer's protocols