CD14 and LBP as Novel Biomarkers for Sarcoidosis by Proteomics of Extracellular Vesicles

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Abstract

Sarcoidosis is an inflammatory granulomatous multi-organ disease of unknown cause. The granulomatous inflammation in sarcoidosis is driven by the interplay between T cells and macrophages. Extracellular vesicles (EVs) play important roles in intercellular communication. Isolated serum EVs from seven patients with sarcoidosis and five control subjects were subjected to non-targeted proteomics analysis. Then 2,292 proteins were detected; 42 proteins were upregulated in patients with sarcoidosis relative to control subjects, and 324 proteins were downregulated. The protein signature of EVs from patients with sarcoidosis reflected disease characteristics such as antigen presentation and immunological disease. Candidate biomarkers were further verified by targeted proteomics analysis in 46 patients and 10 control subjects. CD14 and lipopolysaccharide-binding protein (LBP) were validated by targeted proteomics analysis and upregulation of them was confirmed by immunoblotting. Their expression was also upregulated in macrophages of lung granulomatous lesions. Consistent with these findings, CD14 levels were increased in lipopolysaccharide-stimulated macrophages during multinucleation, concomitant with increased levels of CD14 and LBP in EVs. The area under the curve values of CD14 and LBP were 0.81 and 0.84, respectively. Thus CD14 and LBP in serum EVs, which are associated with granulomatous pathogenesis, can improve the diagnostic accuracy in patients with sarcoidosis.

Background

Sarcoidosis is a systemic granulomatous disease associated with T-lymphocyte and macrophage activation and migration of these cells into affected organs. There is heterogeneity in disease manifestation, severity, and clinical course.^{1–3} To better understand this disease, we need effective biomarkers for diagnosis and prognosis. Unfortunately, the ideal biomarker for sarcoidosis has not yet been discovered. Among identified serum biomarkers, angiotensin-converting enzyme (ACE) and soluble interleukin-2 receptor (sIL-2R) are most relevant but lack sensitivity and specificity.⁴ Roughly 30–80% of patients with sarcoidosis have increased ACE levels; sensitivity ranges between 22 and 86% and specificity between 54 and 95%.⁵ On the other hand, sIL-2R levels are proposed as a marker of disease activity in sarcoidosis. But elevated sIL-2R levels are not specific for sarcoidosis and can be found in other granulomatous diseases, hematological malignancies, and various autoimmune disorders.⁴ The use of sIL-2R as a diagnostic marker for sarcoidosis remains a matter of debate.

In light of progress in mass spectrometry (MS) technology, a great deal of attention has been paid to omics approaches for the study of heterogeneous diseases.⁶ Although advances in proteomics have facilitated the discovery of protein biomarkers, the application of proteomics, which measures features that are closer to the phenotype, has been limited in comparison with genomics and transcriptomics.⁷

Although serum is regarded as an ideal source of marker molecules due to high reproducibility and minimal invasiveness, the masking of small amounts of key proteins is unavoidable because of the wide dynamic range.⁸ To solve this problem, we focused in this study on extracellular vesicles (EVs) in the

serum. EVs are increasingly appreciated as important carriers of biologic cargo that play key roles in intercellular communication by transferring contents such as mRNA, microRNA, and proteins between neighboring cells. EV constituents play a variety of pathological roles in diseases including malignancies, inflammatory disorders, and infections.^{7,9,10} From these backgrounds, EV sandwich ELISA system has been developed to diagnose lung cancer for clinical usage.¹¹ Although ultracentrifugation has been widely regarded as the gold standard to isolate EVs, size exclusion chromatography (SEC)-based EV isolation yields much higher purity through MS analysis than ultracentrifugation.⁹ Despite progress in exosome research, no standard method exists for providing exact quantitative and qualitative analyses of exosomes.¹² Some methods have been developed for the isolation of exosomes, including ultracentrifugation, affinity-based methods, and SEC. Some comparative studies showed that the isolation of EVs by SEC retains the biophysical properties of EVs, resulting in a higher yield.¹³ Tandem mass tag-based non-targeted proteomics analysis of serum EVs isolated by ultracentrifugation revealed that fibulin-3 in EVs may serve as a novel diagnostic biomarker for chronic obstructive pulmonary disease and might be closely related to its pathophysiology.¹⁴ With this background, we isolated serum EVs using SEC, which can detect less abundant serum proteins, thereby applying label-free proteomics strategies to discover novel biomarkers for sarcoidosis.

Methods

Study Design.

Participants were recruited for a discovery and a validation phase from the database of patients diagnosed with sarcoidosis at Osaka University Hospital (2013–2019). All patients with sarcoidosis were diagnosed according to the international ATS/ERS/WASOG criteria.¹ Serum samples were separated by centrifugation and stored frozen at -80°C for further analyses. To sustain the sample quality, the procedure such as freezing and thawing of serum was avoided as much as possible. For the discovery cohort, 5 control subjects and 7 patients with sarcoidosis were selected (Table S1). Patients with sarcoidosis were untreated when the samples were collected. The samples were subjected to quantitative high-throughput proteomics using liquid chromatography-mass spectrometry (LC-MS/MS). The validation cohort included 10 control subjects and 46 patients with sarcoidosis. For validation, candidate proteins identified in the discovery cohort were quantified by targeted proteomics using MS (selected reaction monitoring [SRM]) as described previously.¹⁵ The clinical information of participants is shown in Table S2. This study was performed according to the guidelines described in the Declaration of Helsinki for medical research involving human subjects. This study protocol was approved by the Ethics Committee of Osaka University (no. 17148), and all study participants gave written informed consent.

EV Isolation.

Serum EVs were isolated by size-exclusion chromatography, using an EV Second L70 SEC column (GL Science, Tokyo, Japan).¹⁶ After blocking with fetal bovine serum (FBS) and washing with phosphate-buffered saline (PBS), 400 µl of serum was loaded onto the column and eluted with PBS. The first 300 µl of the eluate was discarded; thereafter, the eluate was collected in three fractions of 100 µl each. The isolation of EVs was confirmed according to the guidelines delineated in Minimal information for studies of extracellular vesicles 2018 (MISEV2018).¹⁷ Size distributions and numbers were confirmed by NanoSight nanoparticle tracking analysis (Malvern Instruments, Malvern, UK). The processing of the EV proteins for proteomic analysis was performed as indicated in the Supplementary Information.

Non-Targeted Proteomics and Targeted Proteomics (Selected Reaction Monitoring).

In the discovery phase, quantitative proteomics was performed using an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) combined with UltiMate 3000 RSLC nano-flow high-performance liquid chromatography (HPLC) (Thermo Scientific). In the validation phase, protein abundance was measured by selected reaction monitoring (SRM) on a TSQVantage triple quadrupole mass spectrometer (Thermo Fisher Scientific) as described.^{18–20} SRM data were analyzed using the Skyline software (MacCoss Lab Software, Seattle, WA, USA).²¹ Peak area ratios of endogenous light (L) peptides and their heavy (H) isotope-labeled internal standards were used for accurate quantification. Peptide light/heavy ratios were log2 transformed and median-centered.

Bioinformatics Analysis of the Proteome With Version Information.

To identify biologically relevant molecular networks and pathways in the proteome, upregulated protein IDs were used as input data. The following tools were used for analysis: Ingenuity Pathways Analysis (IPA) (ver 01.13, Qiagen N.V.) for enrichment analysis and upstream analysis, Clue GO/Clue Pedia plugin (ver 2.5.7) from Cytoscape (ver 3.8.0)(5) for enrichment analysis, and KeyMolnet (ver 6.2, KM Data Inc., <https://www.km-data.jp>) for network analysis.

Statistical Analysis.

Pearson's chi-square test or Welch's t-test were used to compare healthy control subjects and patients with sarcoidosis. Correlations between two parameters were calculated using Spearman's rank correlation coefficients. Differences were considered statistically significant at $P < 0.05$. Receiver operating characteristic curves were constructed using the SRM results. Area under the curve (AUC) values were used to evaluate the diagnostic value of each marker. Multiple logistic regression analysis was applied to

calculate the predictive probability of a multimarker for the diagnosis of sarcoidosis. Statistical analysis were conducted using JMP Pro v. 14.3.0 (SAS Institute, Cary, NC, USA).

Transmission Electron Microscopy.

Samples of EVs (10 µg) were adsorbed onto a formvar/carbon-coated nickel grid for 1 h. EVs were fixed with 2% paraformaldehyde and then incubated with the following primary antibodies: anti-CD9 (MM2/57; Thermo Fisher Scientific), anti-CD14 [EPR3653] (ab133335; Abcam, Cambridge, UK), and anti-lipopolysaccharide-binding protein (LBP) polyclonal (PA5-21642; Invitrogen, USA). Immunoreactive EVs were visualized using anti-mouse IgG(H+L) (EMGMHL10) and anti-rabbit IgG(H+L) (EMGFAR10; BBI Solutions, UK) antibodies preabsorbed with 10-nm gold particles.

Cell Culture and Lipopolysaccharide Stimulation.

RAW 264.7, a murine macrophage/monocyte lineage cell line, obtained from ATCC (ATCC no.: TIB-71), was cultured in DMEM containing 0.5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. For stimulation with lipopolysaccharide (LPS), RAW 264.7 cells (1×10^6 cells/ml) were seeded into 6-well plates in DMEM with exosome-free FBS and stimulated with 10 ng/ml LPS for 6 h. EVs in the supernatant (650 µl/well) were collected using size exclusion chromatography column. EVs were then concentrated by ultracentrifugation (100,000×g, 4°C, 70 min), dissolved in RIPA buffer, and evaluated by blotting. RAW 264.7 cells were fixed and stained with Diff-Quick stain (Sysmex, Kobe, Japan).

Results

Proteomics for discovery of novel biomarkers for sarcoidosis.

To discover a novel biomarker for sarcoidosis, we performed quantitative high-throughput proteomics using LC-MS/MS, followed by SRM verification (Fig. 1A). EVs were isolated by SEC from serum samples of control subjects and patients with sarcoidosis (Table S1).¹⁶ The isolation of EVs was confirmed according to the MISEV2018 guidelines.¹⁷ EVs from both groups expressed the EV marker protein CD9 and were similar in shape and size, less than 100 nm (Fig. 1B). EVs were positive for flotillin-1, CD63, and CD9 and negative for calnexin and haptoglobin (Fig. 1C). In the nanoparticle tracking analysis, serum EVs from both groups were indistinguishable in size and number (Fig. 1D, E and F).

The non-targeted proteomics analysis of EVs identified 2,292 proteins. Of those, 42 proteins were significantly upregulated in patients with sarcoidosis, and 324 were downregulated (Fig. 2A, Table 1, and Table S3). A principal component analysis of EV protein abundance partially separated control subjects from patients with sarcoidosis (Fig. 2B). Identified proteins were present in the cytoplasm (51%), plasma

membrane (29%), and extracellular space (6%; Fig. 2C). Notably, the Ingenuity Pathway Analysis (IPA) of the protein signature of sarcoidosis EVs revealed factors involved in antigen presentation, immune response, and inflammatory response (Fig. 2D). Both tumor necrosis factor- α and transforming growth factor β 1 pathways ranked highly as upstream signaling factors, suggesting that the protein fingerprints of serum EVs from patients with sarcoidosis reflect not only disease characteristics, but also its pathogenesis (Fig. 2E). We visualized the network of functions and pathways of the 42 upregulated proteins using the Clue GO/Clue Pedia plugin from Cytoscape. The nominal significant ($P < 0.05$) pathways and associated proteins are shown in Fig. 2F. Protein-protein interaction analyses revealed that the 42 upregulated proteins were clustered in six functional groups including transfer of LPS from the LBP carrier to CD14 (37.5%) and antigen processing cross-presentation (12.5%).

Table 1

Significantly Upregulated Proteins in Extracellular Vesicles From Patients With Sarcoidosis Compared to Those From Healthy Subjects

Uniprot ID	Description	Fold change	P-value
P36222	Chitinase-3-like protein 1	∞	0.032
O60704	Protein-tyrosine sulfotransferase 2	∞	0.033
Q9NZM1	Myoferlin	∞	0.004
Q7RTS3	Pancreas transcription factor 1 subunit alpha	∞	0.024
Q9Y230	RuvB-like 2	∞	0.034
Q8TAY7	Protein FAM11 D	∞	0.047
Q92522	Histone H1x	∞	0.038
P01040	Cystatin-A	∞	0.040
P06732	Creatine kinase M-type	∞	0.049
P98171	Rho GTPase-activating protein 4	∞	0.014
P36269	Glutathione hydrolase 5 proenzyme	∞	0.007
A8MVU1	Putative neutrophil cytosol factor 1C	∞	0.040
P14598	Neutrophil cytosol factor 1	∞	0.040
A6NI72	Putative neutrophil cytosol factor 1B	∞	0.040
Q8NCG7	Sn1-specific diacylglycerol lipase beta	∞	0.004
Q8N5C1	Protein FAM26E	∞	0.048
O15357	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2	∞	0.019
Q75V66	Anoctamin-5	∞	0.045
Q14406	Chorionic somatomammotropin hormone-like 1	12.92	0.015
A5YKK6	CCR4-NOT transcription complex subunit 1	11.77	0.027
Q9BYX4	Interferon-induced helicase C domain-containing protein 1	7.52	0.008
Q6A163	Keratin, type I cytoskeletal 39	5.78	0.019
P20963	T-cell surface glycoprotein CD3 zeta chain	5.53	0.030
Q15256	Receptor-type tyrosine-protein phosphatase R	5.22	0.007
O14815	Calpain-9	4.22	0.032
P42574	Caspase-3	4.15	0.001

Uniprot ID	Description	Fold change	P-value
Q10588	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2	3.83	0.018
A0A075B6K4	Immunoglobulin lambda variable 3 – 1	3.74	0.013
Q92928	Putative Ras-related protein Rab-1C	3.38	0.048
Q3MII6	TBC1 domain family member 25	3.24	0.025
P24941	Cyclin-dependent kinase 2	2.72	0.039
Q8IU81	Interferon regulatory factor 2-binding protein 1	2.55	0.038
Q96JQ0	Protocadherin-16	2.26	0.044
Q9Y5X9	Endothelial lipase	2.26	0.024
P58166	Inhibin beta E chain	2.26	0.042
P15291	Beta-1,4-galactosyltransferase 1	1.91	0.024
P26572	Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase	1.89	0.012
P18428	Lipopolysaccharide-binding protein	1.79	0.004
P02766	Transthyretin	1.78	0.027
P08571	Monocyte differentiation antigen CD14	1.71	0.019
P04003	C4b-binding protein alpha chain	1.67	0.041
Q9UK55	Protein Z-dependent protease inhibitor	1.58	0.019

Subsequently, we selected 25 proteins, taking into consideration of previous reports, in the validation cohort using SRM (Fig. 3A, Table S2, and Table S4). Targeted proteomics enables the efficient and specific verification of biomarker candidates without requiring antibodies and is, thus, a powerful tool for biomarker validation.^{14,15,20} Among 25 biomarker candidates, the expression levels of LBP and the monocyte differentiation antigen CD14 were markedly elevated in patients with sarcoidosis (Fig. 3B). Consistent with these findings, LPS signaling is highly ranked in the IPA upstream analysis (Fig. 1F), suggesting that these proteins are involved in sarcoidosis pathogenesis. Although serum levels of CD14 were elevated in patients with sarcoidosis,²² we did not observe this difference in free serum levels of both CD14 and LBP, in contrast to the EV levels, measured by ELISA (Fig. 3C). Analyses in KeyMolnet revealed that both CD14 and LBP were closely linked upstream and downstream to key molecules related to granuloma formation (Fig. 3D).

The presence and upregulation of these proteins were subsequently confirmed by western blotting (Fig. 4A) and immunoelectron microscopy (Fig. 4B), respectively. Furthermore, we confirmed the presence of

CD14 and LBP in serum EVs isolated by both ultracentrifugation and the phosphatidylserine affinity method (data not shown).

The expression of CD14 and LBP in vivo and in vitro.

We also assessed the expression levels of these proteins *in vivo* by immunostaining of tissue samples. CD14 and LBP were weakly expressed in mononuclear cells in the lung and lymph nodes of healthy control subjects. By contrast, the expression of CD14 and LBP was strikingly increased in granulomatous lesions, especially in multinucleated giant cells (MGCs) and surrounding mononuclear cells (Fig. 5A and B). MGCs, the hallmarks of granuloma, are generated by monocytes in response to various stimuli, including LPS.²³ To observe the dynamic changes of CD14 and LBP during MGC formation *in vitro*, we stimulated RAW 264.7 cells with LPS. Consistent with the *in vivo* results showing that CD14 was upregulated in macrophages, CD14 levels in EVs from cell culture supernatants were significantly elevated (Fig. 5C). Moreover, the LBP levels in EVs were also elevated, although these levels were unaltered in LPS-stimulated macrophages. Taken together, both CD14 and LBP were upregulated in the process of granuloma formation *in vitro* and *in vivo*. Moreover, given that in macrophages, the tetraspanin CD9 is closely colocalized with CD14, thereby inhibiting LPS-induced signaling,²⁴ and that CD9 is strongly involved in macrophage fusion into MGCs,²⁵ CD14 in macrophages and EVs might actively participate in the pathogenesis of granulomatous diseases (Fig. 5D).

Diagnostic potential of CD14 and LBP in serum EVs for sarcoidosis.

To further evaluate the diagnostic potential of the identified biomarkers, we analyzed their AUC values. The AUC values for CD14 and LBP were 0.81 and 0.84, respectively (Fig. 6A). Considering that most of the previously reported biomarkers appear to have some limitations in terms of sensitivity and specificity, it is intriguing to investigate different combinations. By combining the novel biomarkers with ACE, sIL-2R, and both, the AUC values could be increased further to 0.96, 0.96, and 0.98, respectively (Fig. 6B and Figure S1). Although LBP was weakly correlated with clinical parameters such as ACE ($r: 0.45$; $P < 0.01$), Krebs von den Lungen-6 ($r: 0.35$; $P < 0.05$), and C-reactive protein ($r: 0.60$; $P < 0.01$), the level of CD14 was not correlated with any parameters including monocyte number (Table S5). Although 59.6% of patients were ACE-negative, as many as 45.1% and 48.3% of ACE-negative patients could be further diagnosed by CD14 and LBP, respectively. Similarly, 46.8% of patients were sIL-2R-negative, and 40.8% and 45.5% of sIL-2R-negative patients could be further diagnosed by CD14 and LBP, respectively (Fig. 6C). These findings suggest that our novel biomarkers possess distinct properties in comparison to conventional biomarkers.

To further assess the potential of the novel biomarkers to monitor treatment response, we compared their levels in three patients with sarcoidosis before and after steroid administration. The CD14 and LBP levels

in serum EVs of these patients decreased remarkably after therapy (Figure S2), suggesting that they could also serve as therapeutic biomarkers.

Discussion

Because both ACE and sIL-2R have several limitations such as insufficient sensitivity and specificity, there is an urgent need to develop better biomarkers for sarcoidosis. Although peripheral blood may be regarded as an ideal source of biomarkers, MS-based serum proteomics is exceptionally challenging due to its broad dynamic range with abundant proteins such as albumin.⁸ To overcome these problems, we focused on serum EVs and identified new biomarkers for sarcoidosis by employing both non-targeted, label-free proteomics and targeted proteomics. Although the importance of EVs, especially exosomes, has been increasingly demonstrated in cancer and immune diseases,^{11,26–28} proteomics-based discovery of biomarkers for inflammatory diseases is in its infancy. Considering that isolating EVs can reduce the complexity of serum protein analysis and that EVs reflect both immune response and granuloma formation, serum EVs could become an ideal source of novel biomarkers, providing liquid biopsy samples for personalized medicine approaches. While EVs have advantages such as stability and accessibility, they also have disadvantages such as difficulties in both isolation and quantitation.¹⁷ Although ultracentrifugation is widely regarded as the gold standard, SEC-based EV isolation yields much higher purity through MS analysis than ultracentrifugation.⁹ For this reason, we isolated EVs using SEC instead of ultracentrifugation. Previous studies leveraging MS-based proteomics to examine bronchoalveolar lavage fluid, alveolar macrophages, plasma, and EVs in sarcoidosis identified several differentially expressed proteins, including pulmonary surfactant A2, vitamin D-binding protein, and amyloid P.⁷ Regarding serum EVs, the vitamin D-binding protein levels in EVs derived from plasma samples or bronchoalveolar lavage fluid are elevated in patients with sarcoidosis.²⁹ Although we detected a higher number of proteins, we could not confirm this finding, which was presumably caused by differences in isolation and MS methods.

The verification of many biomarker candidates by conventional immunoblotting would have been time-consuming and less specific; the targeted proteomics approach allowed us to verify biomarker candidates specifically and efficiently without using any antibody. Combining non-targeted and targeted proteomics approaches, we identified CD14 and LBP as potential biomarkers of sarcoidosis, followed by their confirmation in western blots and immunohistochemical tissue staining.

CD14 is a myeloid differentiation antigen mainly expressed on monocytes and macrophages. Both CD14 and LBP are required for recognition of LPS by Toll-like receptor (TLR)4.³⁰ LPS is a major component of the gram-negative bacterial cell wall and a typical example of a pathogen-associated molecular pattern. To facilitate microbial recognition and to amplify cellular responses, certain TLRs require additional proteins, such as LBP, CD14, CD36, and high mobility group box 1. As several infectious organisms like viruses, *Mycobacterium spp.*, and *Propionibacterium acnes* have been implicated in the pathogenesis of sarcoidosis,¹³¹³² the newly identified biomarkers CD14 and LBP may support an infectious etiology of

sarcoidosis. Previous studies also showed that the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway was more activated in patients with sarcoidosis, and particularly STAT1 and STAT3 played a role in granuloma formation.^{33–35} Some EV proteins detected in the discovery phase were correlated with CD14 and LBP through TLRs and the STAT pathway (Fig. 3D). Given that CD14 and LBP were upregulated in the process of granuloma formation *in vitro* and *in vivo* (Fig. 5) and that CD14-positive monocytes promote MGC formation,³⁶ both CD14 and LBP in serum EVs could be involved in the pathophysiology of sarcoidosis.

Considering the limited sensitivity and specificity of the conventional biomarkers ACE and sIL-2R and the positive correlations between various biomarkers, different combinations might be warranted. Our novel biomarkers possess both distinct properties and higher diagnostic potential in comparison to conventional biomarkers. Although LBP levels were weakly correlated with some clinical parameters, the CD14 levels were not correlated with any parameter. Several studies have demonstrated that a combination of different biomarkers increases both sensitivity and specificity.³⁷ Hence, a combination of our novel biomarkers with either ACE or sIL-2R may strikingly improve their diagnostic potential in patients with sarcoidosis.

Despite the great advantages of SRM to verify many biomarkers efficiently, our approach has a couple of limitations. First, we examined our new biomarkers regarding organ specificity and disease severity but observed no significant correlation (Figure S3). This might have been caused by the relatively small sample size, thus further studies are warranted. Moreover, the expression levels of the novel biomarkers were not increased in inflammatory lung disease, including COPD, bronchial asthma, and lung fibrosis (data not shown), implying their disease specificity. Next, given that genome, transcriptome, and metabolome networks are also important for precision medicine, it would be intriguing to aggregate our data by integrating multiple omics approaches.^{6,38} A system biology platform using clinical data, omics, and bioinformatics may lead to a better understanding of sarcoidosis.⁶

Declarations

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Author contributions

Y.F., Y.T., and A.K. contributed to study design, data analysis, data interpretation, and manuscript writing. K. U. performed label-free proteomics and analyzed data. R.N., M.I., J. A., and T.T. performed targeted proteomics and analyzed data. Y. N. and M. I. contributed to bioinformatics analysis. And all authors reviewed the manuscript.

Conflict of Interest

There are no conflicts of interest to declare.

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Figures

Figure 1

Figure 1

Strategy for discovery of novel biomarkers for sarcoidosis. A, In the discovery phase, serum EVs from patients with sarcoidosis and healthy control subjects were isolated using SEC and analyzed. In the validation phase, biomarker candidates were quantified using targeted proteomics SRM. Finally, the biomarkers were confirmed by immunoblotting. B, Transmission electron microscopy images of serum EVs from a healthy control subject and a patient with sarcoidosis (CD9 immunogold labeling). C,

Comparison of serum EVs with serum and A549 cell lysates. Immunoblots of flotillin-1, CD63, CD9, haptoglobin, and calnexin are shown. D, Representative distribution curves showing the particle sizes of serum EVs from a healthy control subject and a patient with sarcoidosis, analyzed using NanoSight. E and F, The mean diameters and numbers of serum EVs from patients with sarcoidosis and healthy control subjects, analyzed using NanoSight, are not significantly different. Error bars represent means $\pm$ standard deviations. ESI = electrospray ionization; EV = extracellular vesicle; HPLC = high-performance liquid chromatography; N.S. = not significant; SEC = size exclusion chromatography; SRM = selected reaction monitoring.

Figure 2

Figure 2

The proteomic profile of serum EVs reflects sarcoidosis characteristics and pathogenesis. A, A volcano plot of all identified 2,292 serum EV proteins by non-targeted proteomic analyses in patients with sarcoidosis and healthy control subjects. A total of 42 proteins were significantly upregulated, and 324 proteins were significantly downregulated in EVs of patients with sarcoidosis compared to those of healthy control subjects. Red line, P-value = 0.05. B, Healthy control subjects and patients with

sarcoidosis are separated by principal component analysis using all EV proteins identified by non-targeted proteomics analysis. C, Localization of all identified proteins in the Ingenuity Pathway Analysis. D and E, Pathways (D) and upstream molecules (E) determined in the Ingenuity Pathway Analysis to be over- ($\times 1.5$ or more) or under- ($\times 0.8$ or less) -represented in non-targeted proteomic analyses of serum EVs from patients with sarcoidosis compared to those from healthy control subjects. Red line, P-value = 0.05. F, A protein-protein network constructed with enrichment analysis based on the Reactome database reveals the interactions between the 42 upregulated proteins. EV = extracellular vesicle.

Figure 3

Figure 3

Validation of the selected reaction monitoring results. A, A flow chart of this study and representative SRM Figure. The quantitation of each endogenous protein in serum EVs was performed by comparing the brown area of an endogenous peptide (Light) to the corresponding synthetic peptide (Heavy). For further information, please see section Targeted Proteomics (Selected Reaction Monitoring) in the Supplementary information. B, CD14, LBP and CD9 levels in serum EVs are upregulated in patients with sarcoidosis compared to healthy control subjects, as determined by targeted proteomics (SRM). C, No significant difference in the serum levels of soluble CD14 and LBP can be detected between healthy control subjects and patients with sarcoidosis. D, A highly complex network of targets with significant relationships, generated by KeyMolnet. Red ellipses: upregulated proteins. Blue ellipses: downregulated proteins. ESI = electrospray ionization; EV = extracellular vesicle; HPLC = high-performance liquid chromatography; LBP = lipopolysaccharide-binding protein; N.S. = not significant; SRM = selected reaction monitoring.

Figure 4

A

B

Figure 4

Expression of CD14 and LBP in serum EVs. A, A representative immunoblot comparing CD14 and LBP in serum EVs of healthy control subjects and patients with sarcoidosis. B, Transmission electron microscopy images showing the CD14 and LBP expression in EVs labeled with primary antibody and anti-IgG gold-labeled secondary antibody. Data are representative of three independent studies with similar results. EV = extracellular vesicle; LBP = lipopolysaccharide-binding protein.

Figure 5

Figure 5

Expression of CD14 and LBP in vivo and in vitro. A and B, Immunohistochemical stainings of the lung and lymph node in a patient with sarcoidosis. CD14 (A) and LBP (B) are highly expressed in the granuloma (black arrows). These markers are also expressed in macrophages present in the lung and lymph node of the healthy control subject. C, Dynamic CD14 and LBP changes in EVs during macrophage multinucleation. Data are representative of three independent studies with similar results. D, The

upregulation of CD14 in serum EVs is associated with granuloma formation in patients with sarcoidosis. EV = extracellular vesicle; LBP = lipopolysaccharide-binding protein.

Figure 6

Figure 6

Diagnostic accuracy of the novel biomarkers for sarcoidosis. A, ROC curves of CD14 and LBP levels in serum EVs to diagnose sarcoidosis. B, ROC curves of CD14 and LBP combined with conventional biomarkers. C, CD14 and LBP in serum EVs can partially identify sarcoidosis patients without elevation of

serum ACE or sIL-2R level. ACE = angiotensin-converting enzyme; AUC = area under the curve; EV = extracellular vesicle; LBP = lipopolysaccharide-binding protein; ROC = receiver operating characteristics; sIL-2R = soluble interleukin-2 receptor.

Supplementary Files

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