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Quantitative intact proteomics investigations of alveolar macrophages in sarcoidosis

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ABSTRACT: **Alveolar macrophages are important for granuloma formation, which is the histological hallmark in sarcoidosis. Their function as antigen-presenting cells in sarcoidosis is also believed to be relevant to the outcome of disease, resulting either in remission or a prolonged chronic inflammation in the lungs.**

Our aim was to study alterations in the expression levels of the soluble proteome of alveolar macrophages in pulmonary sarcoidosis as compared with healthy controls, with the goal of identifying specific proteins and pathways important for the mechanisms of disease and/or disease phenotype.

Quantitative proteomics approach using two-dimensional difference gel electrophoresis coupled to mass spectrometry was applied. Data was evaluated using multivariate modelling and pathway analyses. 69 protein spots were found to be significantly altered between sarcoidosis and healthy controls. Among these, 25 unique proteins were identified. Several of the identified proteins were related to key alveolar macrophage functionality, including the Fcγ-mediated phagocytosis and clathrin-mediated endocytosis pathways.

Global proteomics analysis provided identification of alterations of a subset of proteins not previously reported in sarcoidosis. These alterations primarily affect biological pathways related to phagocytic macrophage functionality. These findings provide important insights into the role of macrophages in sarcoidosis pathogenesis.

KEYWORDS: **Differential gel electrophoresis, endocytosis, mass spectrometry, multivariate analysis, phagocytosis, proteomics**

Sarcoidosis is a systemic granulomatous disease of unknown aetiology involving the lungs in ~90% of patients [1]. Immunologically, pulmonary sarcoidosis is characterised by an increased number of activated alveolar macrophages and CD4+ T-cells with a T-helper (Th) type 1 cytokine profile in the lungs [2]. Alveolar macrophages are involved both in innate [3] and adaptive immune responses [4]. They produce a range of pro-inflammatory cytokines and are a main source for tumour necrosis factor-α production, which plays an important role in the hallmark granuloma formation in sarcoidosis [5–7]. In addition, alveolar macrophages have an important function as antigen-presenting cells (APCs) in sarcoidosis, interacting with T-cell lymphocytes *via* human leukocyte antigen (HLA) molecules and T-cell receptors, respectively. A number of studies have shown that genetic polymorphisms in the HLA complex are of relevance to the disease outcome [3, 8]. Our group has previously shown a strong association between sarcoidosis patients with Löfgren's syndrome and the HLA-DRB1*03 allele. This group of patients often has a good prognosis with spontaneous resolution, and shows well-defined characteristics including acute onset, bilateral hilar adenopathy, ankle arthritis and/or erythema nodosum. In contrast, the group of patients having the HLA-DRB1*14/15 allele are associated with a more persistent inflammatory phenotype [9].

Sarcoidosis is a complex disease where environmental and genetic factors are important for the disease outcome. Reductionist research focusing on single biomolecules or pathways may provide some limitations in the understanding of the pathogenesis in multifactorial disorders, such as

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TABLE 1 Subject characteristics

Subject	Diagnosis	Chest radiograph stage#	Löfgren's syndrome	Smoking history	Cell viability	Total cells $\times 10^6$	Alveolar macrophages %	Lymphocytes %	CD4/CD8	FVC %	FEV_1 %
H1	Healthy	0	NA	Never	91	13.6	86	11	1.6	126	109
H2	Healthy	0	NA	Never	86	17.5	82	14	ND	104	100
H3	Healthy	0	NA	Never	94	18.8	89	11	ND	114	119
H4	Healthy	0	NA	Never	88	18.6	94	4	ND	116	107
H5	Healthy	0	NA	Never	86	19.9	76	23	1.8	132	111
H6	Healthy	0	NA	Never	82	12.8	90	6	ND	113	105
H7	Healthy	0	NA	Never	92	5.8	87	12	ND	ND	ND
S1	Sarcoidosis	I	Yes	Never	79	65.4	62	36	9.6	72	69
S2	Sarcoidosis	I	Yes	Never	93	39.8	87	13	6	83	87
S3	Sarcoidosis	III	No	Never	92	24.4	93	7	4.7	99	102
S4	Sarcoidosis	II	No	Ex	96	47.5	47	52	8.9	ND	67
S5	Sarcoidosis	II	No	Never	98	61.5	73	25	4.9	81	80
S6	Sarcoidosis	I	Yes	Ex	95	33	67	29	4.8	97	99
S7	Sarcoidosis	I	Yes	Ex	92	21.9	70	29	13	94	82

FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; H: healthy; S: sarcoidosis; NA: not applicable; Never: never-smoker; Ex: ex-smoker (>1 year); ND: not determined. #: chest radiograph stage: 0=normal, I=bilateral hilar lymphoma (BHL), II=BHL with infiltrates, and III=parenchymal infiltration.

sarcoidosis. Complementary systems level approaches, including quantitative proteomics, give the opportunity to study changes in the expression profile of hundreds of proteins and their corresponding pathways simultaneously. The number of proteomics studies investigating the characteristics of human macrophages is very limited [10, 11]. JIN *et al.* [12] performed a thorough investigation of the differences between the proteomes of circulating blood monocytes and resident alveolar macrophages, revealing differential protein expression profiles particularly in proteinases (for example, cathepsins) and actin regulatory elements involved in, *e.g.* phagocytosis and cytokine release, as well as up-regulations of oxidant defence systems required for adaptation to the oxidative environment of the lung. While these investigations provide an important basis for understanding of macrophage differentiation and polarisation occurring in the lung, global proteomics studies investigating phenotypic alterations of human primary alveolar macrophages in response to respiratory disease are scarce. Exceptions include investigations of alveolar macrophage proteome responses to infection by porcine reproductive and respiratory syndrome virus [13] and influenza A virus [14]. Previous proteomics studies on sarcoidosis have been performed mainly on bronchoalveolar lavage (BAL) fluid and serum [15–22], thereby primarily reflecting proteins actively secreted or exudated into the bronchoalveolar lumen, such as plasma proteins and antioxidant proteins. In the current study, we have focused on the intracellular proteome of alveolar macrophages and performed differential proteomics investigations using two-dimensional differential gel electrophoresis (DIGE) with the aim to identify specific proteins and pathways of importance for alveolar macrophage involvement in sarcoidosis. To our knowledge, this is the first clinical quantitative proteomic study performed on isolated alveolar macrophages from pulmonary sarcoidosis patients.

MATERIAL AND METHODS

Study subjects

Bronchoscopy was performed as previously described on seven healthy control subjects and seven sarcoidosis patients as part of the initial diagnostic routine investigation [9]. The diagnostic criteria for sarcoidosis patients were in accordance with the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) criteria [23], including chest radiographic changes and altered pulmonary function. Also, granulomas were present in airway epithelial biopsies, and the CD4/CD8 ratio was increased ($\geqslant$3.5) in BAL T-cell populations. Patients (four males and three females, median age 30 (range 21–45) years) had either a sudden onset with fever, erythema nodosum and/or arthritis (Löfgren's syndrome, n=4), or an insidious onset of symptoms with pronounced fatigue and unproductive cough (non-Löfgren's, n=3). The patients' chest radiography changes were classified into stages I–III. None of the patients included was under any immunosuppressive treatment. Three of the patients were ex-smokers (>1 year since last cigarette), and four were never-smokers. All healthy subjects (one male and six females, median age 25 (range 19–28) years) were never-smokers and free from any symptoms indicating respiratory disease and all had normal chest radiographs and lung function (table 1). None of the individuals had had signs of respiratory infection within the last 4 weeks prior to inclusion in the study. The study was approved by the Stockholm Local Ethical Committee (case number 02–427).

Sample preparation and quantitative intact proteomics analysis

Alveolar macrophages were isolated from other BAL cells by Percoll density gradient (GE Healthcare, Uppsala, Sweden) as previously described [24]. The inertia of the Percoll reagents in terms of inducing activation during Percoll separation has been verified on a number of antigen presenting cell types, including alveolar macrophages [25–27]. BAL cells were cytospun and stained with May–Grünwald–Giemsa stain, followed by differential counting of macrophages, lymphocytes, eosinophils, neutophils and mast cells both prior to and following Percoll separation. Percoll separation resulted in >95% purity of alveolar macrophages. Subsequently, alveolar macrophages were solubilised and the total protein content was fractionated by ultracentrifugation into soluble and membrane fractions (online supplementary material). The soluble protein fractions were labelled using minimal DIGE [20] according to the manufacturer's instructions (GE Healthcare). In brief, a pooled internal standard was created from aliquots of all samples (Cy2-labelled), co-separated on two-dimensional electrophoresis gels with one sarcoidosis and one healthy sample (Cy3 or Cy5

labelled) and used for normalisation. The isoelectric focusing was performed on 24 cm pH4-7 linear gradient immobilised pH gradient strips, run on IPGphor II (GE Healthcare) for 53.7 kVh, followed by a second dimension SDS-PAGE on 10% large format gels (20 × 24 cm), thereby focusing the proteome analysis to the acidic proteins with molecular weight <100 kDa. Image acquisition was performed using a Typhoon-9410 fluorescence scanner (GE Healthcare) and subsequent image analysis was performed using Progenesis SameSpots V3.2 (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) (online supplementary material).

Protein identification

Altered protein-spots (ANOVA; $p<0.05$) were trypsin digested, and further analysed by matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) (Micromass-R MALDI-TOF, San Diego, CA, USA) or MALDI-TOF/TOF (Applied Biosystems, Framingham, MA, USA) mass spectrometry (MS). Database searching for peptide mass fingerprinting was performed using ProFound (http://prowl.rockefeller.edu) for MALDI/MS data, and peptide mass mapping was performed using Mascot for MALDI-TOF/TOF data (www.matrixscience.com/search_form_select.html) on the NCBI nonredundant protein sequence database (version 2009/03/01).

Statistical analysis

In DIGE image analysis, ANOVA ($p<0.05$) was applied on log-transformed protein volumes normalised by Cy2 internal standard intensities using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). Multivariate analysis using principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) analysis were performed using SIMCA P+12.0.1 (Umetrics, Umeå, Sweden).

Pathway analysis

Ingenuity pathway analysis (Ingenuity Systems Inc., Redwood City, CA, USA) was used for pathway analysis of the differentially

TABLE 2 Protein identities

Protein name	Analysis	Accession#	p-value	Score¶	Theoretical and experimental values		Observed		Cov. %	Matched peptides
					Mw kDa	pI	Mw kDa	pI		
Sly1p	MALDI-TOF	AAP97146	<0.0001		70.3	5.6	82	5.3	12	6
EPHB1	MALDI-TOF	AAD02030	<0.0001		111.5	5.9	103	6.0	12	7
MyD88	MALDI-TOF	AAB49961	0.0064		33.7	5.6	38	5.3	34	7
DGKB isoform 1	MALDI-TOF	NP_690874	<0.0001		130.1	5.9	87	5.2	9	7
DPP	MALDI-TOF	AAQ83119.1	<0.0001		99.1	6.0	87	6.2	7	5
ZFP1 isoform 1	MALDI-TOF	NP_991331	<0.0001		60.5	5.4	88	6.2	19	9
PLD	MALDI-TOF	AAA36444	<0.0001		93.3	5.9	95	5.4	9	6
GSS	MALDI-TOF	NP_000169	0.0068		52.5	5.7	78	5.8	25	10
VIM	MALDI-TOF	AAC05002	<0.0001		53.7	5.1	55	5.6	31	16
RAB11B	MALDI-TOF	AAQ18786	<0.0001		137.6	5.3	115	5.2	10	7
ORP1	MALDI-TOF	AAG53407	0.009		107.1	6.1	98	5.9	14	9
AP2B1	MALDI-TOF	AAH06201	<0.0001		106.6	5.2	110	5.1	11	8
GSN	MALDI-TOF	NP_937895	<0.0001		80.9	5.6	95	5.9	22	13
DYNC1LI1	MALDI-TOF	AAD44481	<0.0001		56.9	6.1	58	6.2	12	7
VCP	MALDI-TOF	NP_009057	<0.0001		89.9	5.2	94	5.3	19	12
TOP2B	MALDI-TOF	AAB01982	0.009		38.6	5.1	34	5.3	31	6
ALDHA1	MALDI-TOF	AAC51652	<0.0001		55.4	6.3	85	6.7	20	7
ATP1A1	MALDI-TOF	NP_000692	<0.0001		114.2	5.3	93	5.1	11	6
AK5	MALDI-TOF	AAH36666	0.0012		63.8	5.0	69	4.9	14	4
RhoA	MALDI-TOF	AAAH12860	<0.0001		53.6	5.3	114	5.2	22	7
GOLIM4	MALDI-TOF	NP_055313	<0.0001		81.9	4.7	87	5.1	18	9
β-actin	MALDI-TOF/TOF	CAA45026		54	41.8	5.2	43	6.0	5	2
β-actin	MALDI-TOF/TOF	AAH08633		173	41.3	5.6	43	6.1	11	5
γ-interferon thiol reductase	MALDI-TOF/TOF	AF097362		92	28.5	4.7	25	5.5	4	1
Keratin 10, type I, cytoskeletal	MALDI-TOF/TOF	KRHU0		173	41.3	5.6	43	6.1	5	3
HSP70 (mortalin)	MALDI-TOF/TOF	AAH00478		494	73.7	5.9	90	5.7	13	6
VIM	MALDI-TOF/TOF	AAA61279		69	53.7	5.0	81	5.1	5	8
VIM	MALDI-TOF/TOF	CAG28618		301	53.6	5.1	81	5.1	14	5
VIM	MALDI-TOF/TOF	AAA61279		557	53.7	5.0	75	4.6	25	8

Mw: molecular weight; pI: isoelectric point; EPHB1: Eph-like receptor tyrosine kinase; MALDI-TOF: matrix-assisted laser desorption ionisation-time of flight; MyD88: myeloid differentiation protein 88; DGKB: diacylglycerol kinase; DPP: dipeptidylpeptidase; ZFP1: zinc finger protein; PLD: phospholipase D; GSS: glutathione synthetase; VIM: vimentin; RAB11B: Rab11 family-interacting protein 1; ORP1: OSBP-related protein 1; AP2B1: adaptor-related protein complex 2, β1 subunit; GSN: gelsolin isoform b; DYNC1LI1: dynein light chain-A; VCP: valosin-containing protein; TOP2B: topoisomerase II β isozyme; ALDHA1: aldehyde dehydrogenase 1; ATP1A1: Na^+/K^+ ATPase α1 subunit; AK5: adenylate kinase isoenzyme 5; RhoA: RhoA/RAC/CDC42 exchange factor; GOLIM4: Golgi integral membrane protein 4; HSP70: heat shock 70 kDa protein 9. #: National Center for Biotechnology Information accession number; ¶: Mascot score is shown.

TABLE 3 Statistics associated with individual proteins

Protein name	Sarcoidosis#	Healthy¶	Sarcoidosis/control+	ANOVA (p<0.05)
Sly1p	1.56	0.56	2.8	0.02
EPHB1	1.09	0.45	2.4	0.05
MyD88	1.36	0.65	2.1	0.02
DGKB isoform 1	1.21	0.60	2.0	0.04
DPP	1.15	0.60	1.9	0.04
ZFP1	1.13	0.64	1.8	0.05
PLD	1.47	0.91	1.6	0.01
GSS GSH-S	1.37	0.86	1.6	0.04
VIM	1.61	1.07	1.5	0.03
RAB11B	1.23	0.88	1.4	0.004
ORP1	1.06	0.76	1.4	0.04
AP2B1	1.22	0.88	1.4	0.04
GSN	1.11	0.84	1.3	0.04
DYNC1LI1	1.26	0.97	1.3	0.01
VCP	1.07	0.83	1.3	0.007
TOP2B	1.14	0.89	1.3	0.03
ALDHA1	1.01	0.80	1.3	0.04
ATP1A1	0.82	0.99	0.8	0.02
AK5	0.80	1.35	0.6	0.05
RhoA	0.67	1.14	0.6	0.02
GOLIM4	0.48	1.18	0.4	0.02
β-actin	1.43	0.58	2.5	0.01
β-actin	1.19	0.68	1.7	0.05
γ-interferon lysosome transferase	1.10	0.91	1.2	0.05
Keratin 10, type I	1.03	1.32	0.8	0.01
VIM	0.75	1.08	0.7	0.02
HSP70	0.76	1.34	0.6	0.04
VIM	0.53	1.02	0.5	0.04
VIM	0.64	1.39	0.5	0.01

EPHB1: Eph-like receptor tyrosine kinase; MyD88: myeloid differentiation protein 88; DGKB: diacylglycerol kinase; DPP: dipeptidylpeptidase; ZFP1: zinc finger protein; PLD: phospholipase D; GSS: glutathione synthetase; VIM: vimentin; RAB11B: Rab11 family-interacting protein 1; ORP1: OSBP-related protein 1; AP2B1: adaptor-related protein complex 2, β1 subunit; GSN: gelsolin isoform b; DYNC1LI1: dynein light chain-A; VCP: valosin-containing protein; TOP2B: topoisomerase II β isozyme; ALDHA1: aldehyde dehydrogenase 1; ATP1A1: Na^+/K^+ ATPase α1 subunit; AK5: adenylate kinase isoenzyme 5; RhoA: RhoA/RAC/CDC42 exchange factor; GOLIM4: Golgi integral membrane protein 4; HSP70: heat shock 70 kDa protein 9. #: median of log-transformed normalised protein volumes in sarcoidosis (n=7); ¶: median of log-transformed normalised protein volumes in controls (n=7); +: fold changes between groups, values >1 upregulated in sacoidosis and <1 downregulated.

expressed proteins identified in the DIGE analysis. Protein gene names and normalised, log-transformed expression levels of selected proteins were loaded into ingenuity pathway analysis, and adjusted Fisher's exact test was used for statistical testing in the pathway analyses.

RESULTS

Proteomics analysis of alveolar macrophages

Semi-quantitative DIGE analysis of the soluble protein fraction from alveolar macrophages resulted in 648 protein-spots correctly matched across all 21 gel images and of sufficient quality for subsequent quantitative uni- and multivariate analyses. Univariate one-way ANOVA analysis ($p>0.05$) showed that 69 of the analysed protein spots were significantly altered between sarcoidosis and healthy control groups. The use of an internal standard for correction of the technical variability in conjunction with fourth generation two-dimensional electrophoresis analysis software [24] provided a high overall statistical power (0.84), despite the low number of subjects included, thereby allowing detection of significant alterations in protein abundances down to 1.2-fold changes. A total of 25 unique proteins were identified, of which the expression levels were up-regulated in 17 and downregulated in eight in sarcoidosis compared with healthy controls. The proteins' identities and associated statistics are presented in tables 2 and 3, respectively.

Multivariate modelling

PCA was initially applied to provide an overview of the general trends of the dataset. The PCA scores-plot showed a clear clustering of sarcoidosis and healthy groups, respectively (fig. 1). The PCA also revealed one outlier among the healthy individuals (H7), located outside the Hotelling T^2 95% confidence area. The H7 sample contained a large number of erythrocytes due to bleeding during the BAL procedure, and an additional hypotonic sample-treatment step was required before use in the DIGE analysis. This may have caused the different attributes of H7. Exclusion of the H7 individual did not, however, alter the overall ranking of the variable contribution in either the unsupervised PCA analyses or the subsequent supervised (OPLS) analyses, and the individual was therefore included in all further analyses. In order to further evaluate the predictive power of the 69 significantly altered proteins with regards to patient diagnosis, supervised OPLS analysis was performed with diagnosis as the qualitative response variable. The result was a robust model with good group separation ($R^2=0.86$, $Q^2=0.41$) (fig. 2). The loadings as well as the variable importance on the projections plots showed that valosin-containing protein (VCP), rab-11, keratin 10 and topoisomerase 2b were the most important variables in driving the group separation. Further OPLS analysis based on sarcoidosis phenotype (Löfgren's or non-Löfgren's sarcoidosis) gave a highly predictive model, with a predictive power of 72% ($Q^2=0.72$) based on seven-fold cross-validation (fig. S1).

Pathway and network analyses

The 25 identified proteins were further investigated with pathway analyses using ingenuity pathway analysis software.

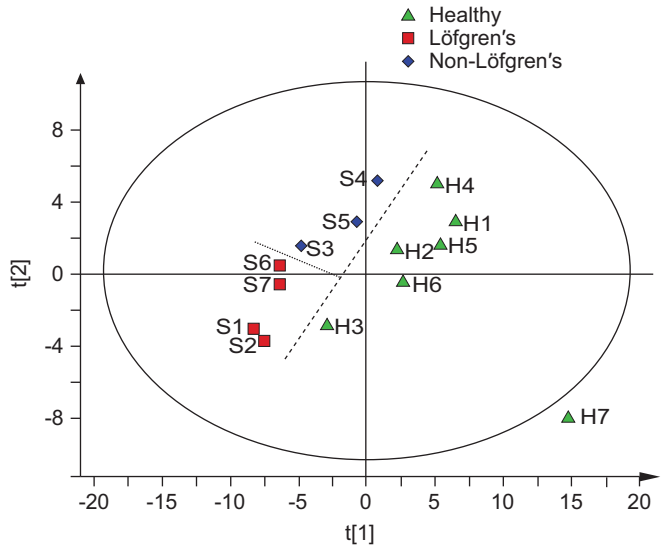

FIGURE 1. Scores plot of principal component analysis. This unsupervised statistical model revealed a separation between sarcoidosis (S) and healthy (H) subjects. In addition, a subclustering of the two clinical phenotypes of sarcoidosis (Löfgren's and non-Löfgren's) can be discerned primarily along the second principal component (y-axis). The circle indicates the Hotelling's T^2 95% confidence interval.

Two macrophage-related pathways were found to be significantly upregulated in sarcoidosis; the clathrin-mediated endocytosis pathway ($p=0.0019$) and the Fcγ receptor-mediated phagocytosis pathway ($p=1.3\times10^{-5}$). Additional network analysis revealed that 11 of the 25 proteins were associated to the nuclear factor (NF)-κB network (fig. 3). These proteins were the myeloid differentiation factor 88 (MyD88), Rab11 family-interacting protein 1 (RAB11A), adaptor protein complex 2, β1 subunit (AP2B1), gelsolin (GSN), phospholipase D (PLD), Eph-like receptor tyrosine kinase (EPHB1), VCP, topoisomerase IIb (top2b), vimentin (VIM), Na^+/K^+ ATPase α1 subunit (ATP1A1) and RhoA/RAC/CDC42 exchange factor GEFT (RhoA).

DISCUSSION

In this study, we investigated the expression levels of the soluble proteome of alveolar macrophages in sarcoidosis patients as compared with healthy controls, with the purpose of identifying specific proteins and pathways involved in the pathogenesis of sarcoidosis. Of the 69 protein species found to be significantly altered between groups, we were able to identify 25 unique proteins by mass spectrometry (tables 2 and 3). Subsequent pathway analyses revealed that a number of these proteins are associated with two complementary pathways central for macrophage-related immunofunctionality, Fcγ-mediated phagocytosis pathway and clathrin-mediated endocytosis pathway. Both pathways were upregulated in sarcoidosis. Phagocytosis of exogenous pathogens and associated endosome-vesicle networking represent the key initial steps in the antigen processing and presentation cascade, a central functionality of alveolar macrophages. In sarcoidosis, the subsequent steps involve antigen presentation on major histocompatibility complex (MHC) class II molecules in the plasma membrane to $CD4^+$ T-cells for the induction of an adaptive immunoresponse with a classic Th type 1 cytokine profile [4].

Multiple complementary modes of phagocytosis are active in macrophages, and the Fcγ receptor-mediated pathway represents one central mechanism in the innate response. The binding of the Fc portion of IgG to Fcγ receptors on the surface of macrophages activates a range of antimicrobial responses

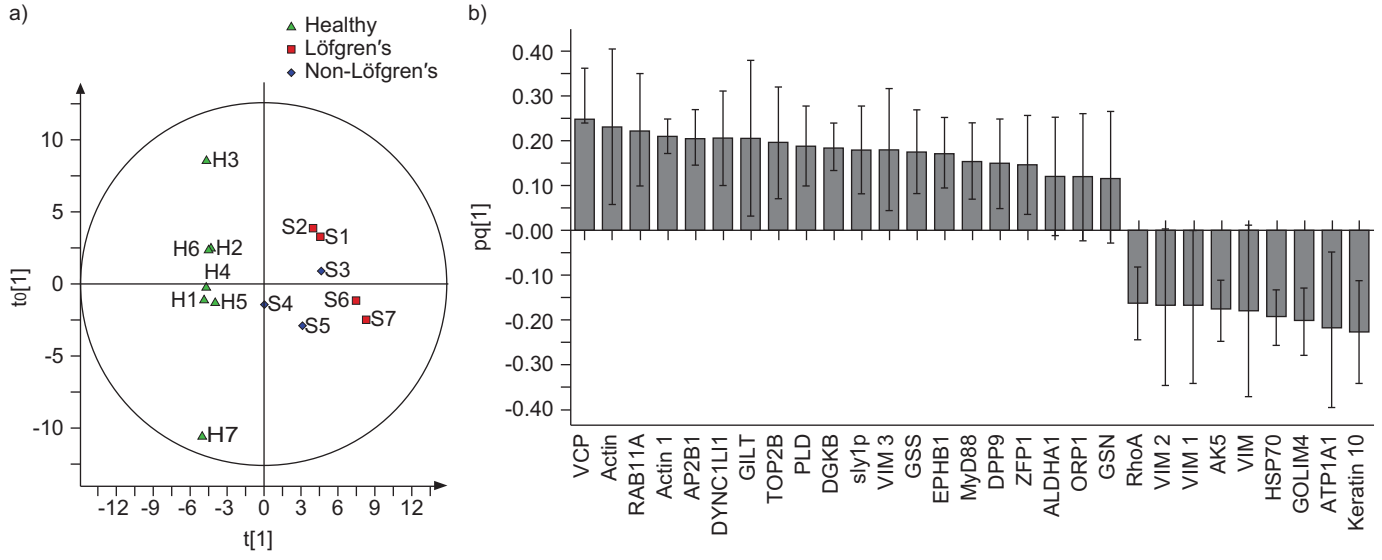

FIGURE 2. Orthogonal projections to latent structures (OPLS) analysis based on diagnosis. a) The OPLS scores plot illustrates a clear separation between the sarcoidosis (S) and healthy (H) groups, respectively ($R^2=0.86$, $Q^2=0.41$). b) The column loadings plot displays the most prominent proteins/protein spots driving the group separation. VCP: valosin-containing protein; RAB11A: Rab11 family-interacting protein 1; AP2B1: adaptor-related protein complex 2, β1 subunit; DYNC1LI1: dynein light chain-A; GILT: γ-interferon-lysosomal thiol reductase; TOP2B: topoisomerase II β isozyme; PLD: phospholipase D; DGKB: diacylglycerol kinase eta; VIM: vimentin; GSS: glutathione synthetase; EPHB1: ephrin type-B receptor 1; MyD88: myeloid differentiation protein 88; DPP9: dipeptidylpeptidase 9 short form; ZFP1: zinc finger protein; ALDHA1: aldehyde dehydrogenase 1; ORP1: OSBP-related protein 1; GSN: gelsolin isoform b; RhoA: RhoA/RAC/CDC42 exchange factor GEFT; AK5: adenylate kinase isoenzyme 5; HSP70: heat shock 70 kDa protein 9; GOLIM4: Golgi integral membrane protein 4; ATP1A1; Na^+/K^+ ATPase α1 subunit.

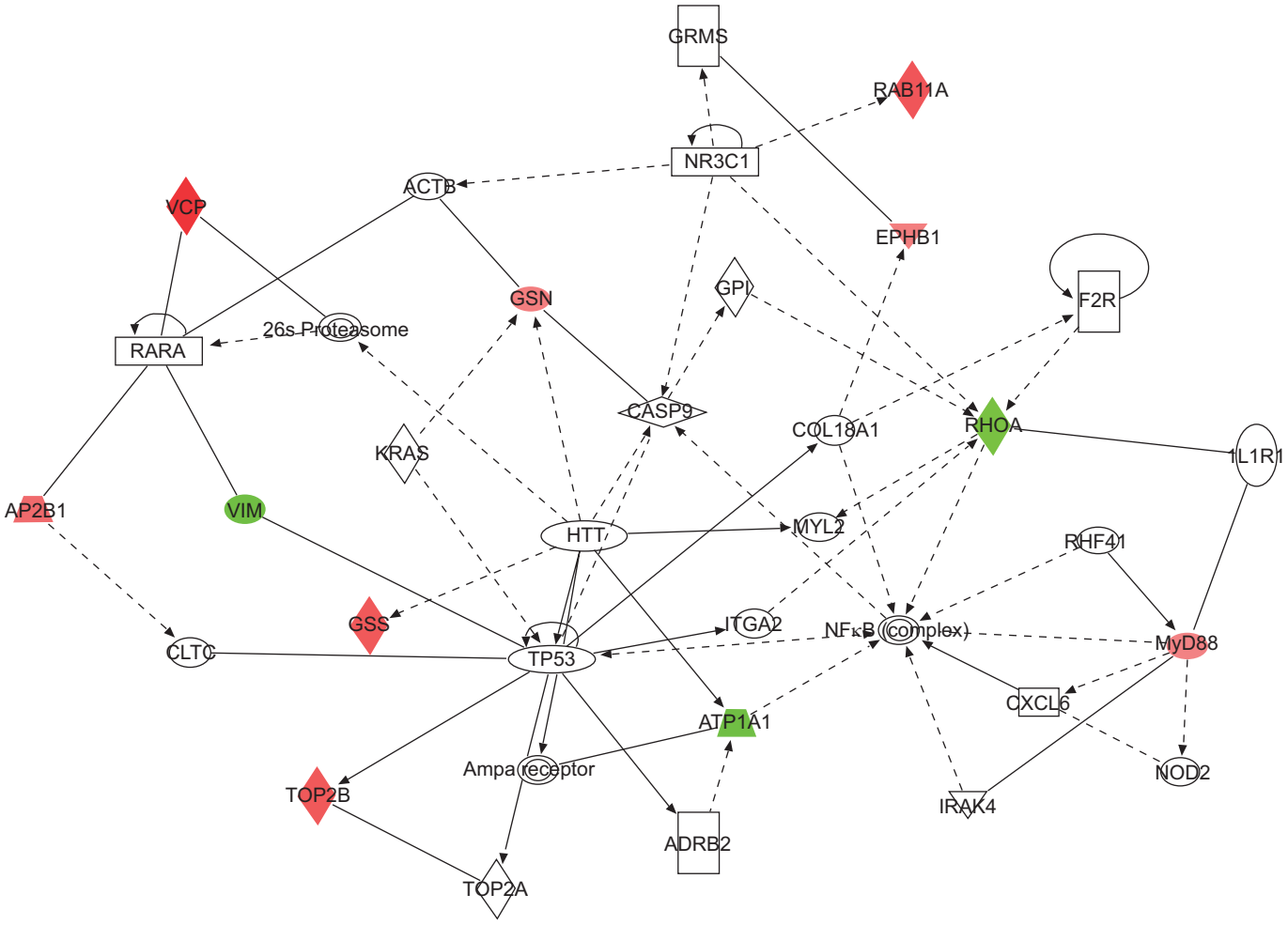

FIGURE 3. Network analysis displaying proteins associated with the nuclear factor (NF)-κB-node network. Proteins identified as upregulated (red) or downregulated (green) in sarcoidosis are indicated in the network. Continuous and dashed arrows show direct or indirect correlation between proteins, respectively. RAB11A: Rab11 family-interacting protein 1; VCP: valosin-containing protein; EPHB1: ephrin type-B receptor 1; GSN: gelsolin isoform b; AP2B1: adaptor-related protein complex 2, β1 subunit; TOP2A: topoisomerase II α isozyme; TOP2B: topoisomerase II β isozyme; RhoA: RhoA/RAC/CDC42 exchange factor GEFT; VIM: vimentin; GSS: glutathione synthetase; MyD88: myeloid differentiation protein 88; ATP1A1; Na^+/K^+ ATPase α1 subunit.

including phagocytosis, inflammatory cytokine secretion and generation of reactive oxidant species [8, 28]. PLD, found to be upregulated in sarcoidosis, has been shown to be of important for the regulation of phagocytosis in primary human macrophages [29, 30]. Another pathway associated with the phagocytotic mechanism, the clathrin-mediated endocytosis pathway, was also found to be altered in sarcoidosis, including upregulation of adaptor protein complex-2 (AP-2) and RAB11A. Internalised macromolecules, such as bacteria, are carried into the cell in clathrin-coated membrane vesicles derived from invagination of plasma membrane by endocytosis. Endocytosis is also a central step in Fcγ-mediated phagocytosis, as well as in the MHC class II antigen processing, which is an integral part of the alveolar macrophage functionality as APCs. AP-2 is a core protein of the clathrin-mediated endocytic pathway and has been shown to be highly active in endosomal trafficking of the MHC class II antigen presentation. RAB11A has been shown to regulate membrane traffic [31] during phagocytosis in human macrophages, (26)as well as in the fusion of intracellular transport vesicles [32, 33]. Finally γ-interferon lysosomal thiol reductase (GILT), also found to be upregulated in sarcoidosis, is a soluble thiol reductase constitutively expressed in APCs, and important for the antigen presentation and subsequent $CD4^+$ T lymphocytes activation in sarcoidosis [34]. This enzyme reduces disulfide bonds of processed proteins, thereby facilitating peptide loading into the peptide-binding groove of the HLA-molecule, and it was recently shown that GILT is required for correct MHC I presentation of viral glycoproteins [30]. Taken together, these results show an upregulation of a number of proteins related to the antigen presentation mechanisms in alveolar macrophages in patients with pulmonary sarcoidosis compared with control subjects.

The large number of protein variables investigated through a relatively small number of subjects poses a challenge in proteomics analyses, and results from traditional univariate statistics that may be unreliable due to a high number of false positives. Multivariate analyses provide a good complement in this context, as the issue of multiple testing can be avoided and, at the same time, the large degree of covariance inherent in

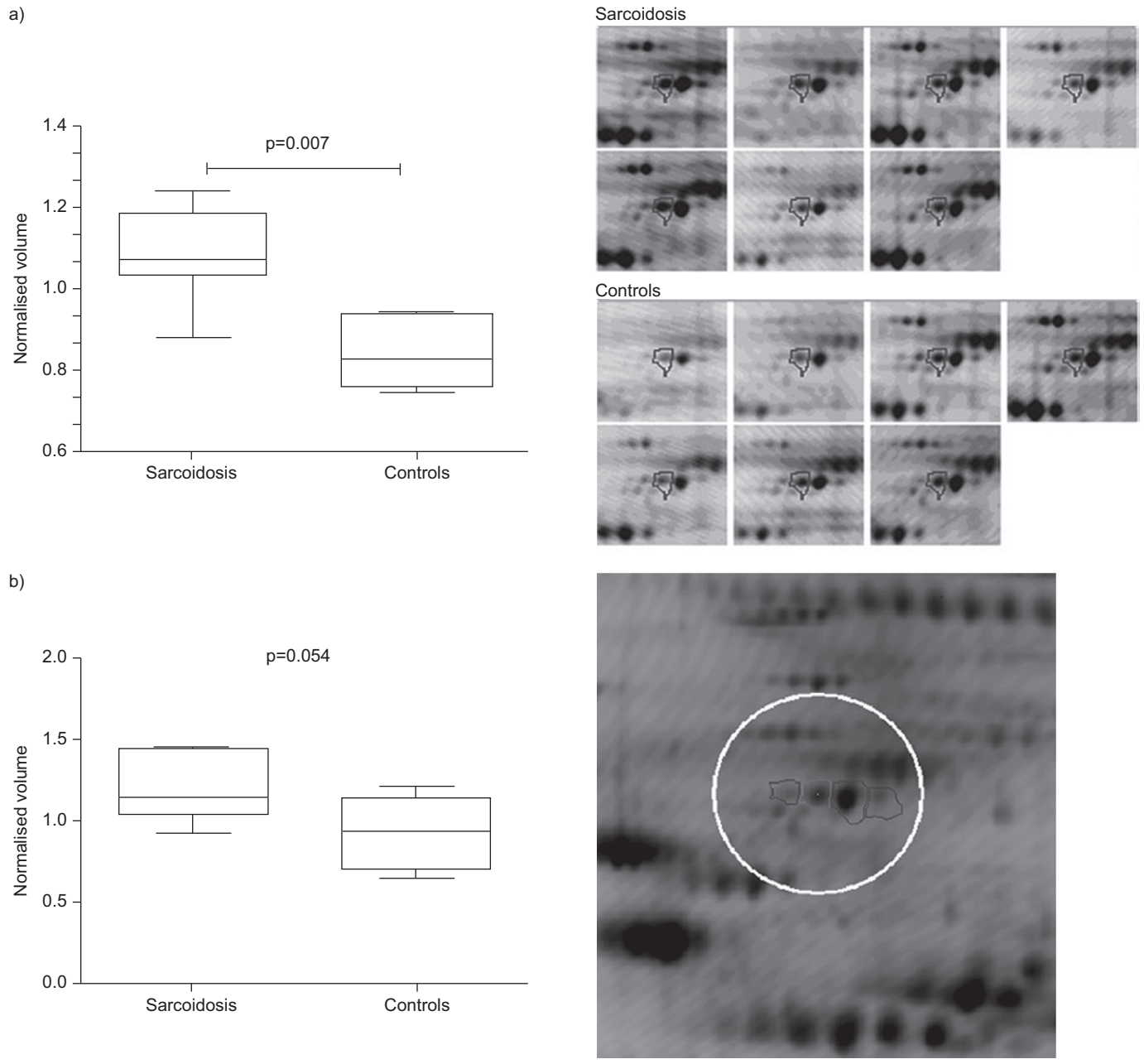

FIGURE 4. Valosin-containing protein protein-spot matching from the differential gel electrophoresis image analysis. The significantly altered protein spot was located in a spot train consisting of four protein spots detected above the method limit of quantifiction (a). Statistical analysis of the merged normalised spot volumes for the spot train failed to reach significance (b).

omics analyses is taken into consideration in the statistical analysis. In contrast to the more commonly used PCA modelling, OPLS analysis is a supervised method designed to pull out the predictive variance of interest from the variance unrelated (orthogonal) to the hypothesis of interest, thereby acting as a noise filter that improves the interpretability of the multivariate model, particularly in relating the observed group separation to specific protein biomarkers [35]. OPLS thus serves to focus the predictive information into a single component, thereby giving easier identification of biomarkers of interest, as well as an improved and more accurate assessment of the predictive power of the selected biomarkers. The resulting OPLS model revealed a strong separation between groups ($R^2Y=0.86$) with a good predictive power ($Q^2=0.41$), particularly when considering the limited number of individuals included in the study (fig. 2a). The same protein variables that were significantly altered by univariate statistical measures were driving the OPLS model, thereby providing an alternate means of validating these findings. VCP was the top ranked variable for discriminating between sarcoidosis patients and healthy (fig. 2b). VCP, also known as p97, is a transitional endoplasmic reticulum ATPase involved in multiple mechanisms, including membrane fusion [36], regulation of transcriptional activation of NF-κB *via* inhibitor (I)κB degradation [37] and apoptosis [6], as well as the

endoplasmic reticulum-associated degradation system [38]. The VCP network is complex, regulating multiple signalling pathways through 40 different specific adaptor proteins [39]. VCP is subject to a number of post-translational modifications, resulting in a train of spots detected by two-dimensional electrophoresis gels. Given that only one of the protein spots (fig. 4a) and not the entire spot train (fig. 4b) was altered, VCP is not conducive to traditional validation by Western blotting. As such, alternate bioinformatics approaches to validation were performed, including cross-validation of the multivariate models described above, as well as pathway and network analysis to investigate the biological feasibility of the observed alterations. Network analysis revealed that in addition to VCP, 10 of the 25 proteins identified as significantly altered in sarcoidosis were associated with the extended NF-κB-node network (fig. 3).

Clinically, lung sarcoidosis can be divided into patients with Löfgren's syndrome and those without. Löfgren's syndrome patients present an acute onset with an elevated CD4/CD8 T-cells ratio in the lungs, bilateral hilar adenopathy, ankle arthritis and/or erythema nodosum. This patient group presents spontaneous resolution within a span of 2 years without any treatment. On the contrary, patients without Löfgren's syndrome generally present with a less distinct clinical phenotype and frequently develop a persistent inflammation and lung fibrosis (~20%). As such, it is of great interest to identify molecular markers and molecular pathways that can discriminate between the two vastly different disease courses. OPLS analysis to investigate differences between Löfgren's and non-Löfgren's patients provided a very strong model with a clear separation between groups (fig. S1), where PLD and RhoA, both associated with phagocytosis, were the most driving variables. One important observation was the tight clustering of Löfgren's patients (S1, S2, S6 and S7), in contrast to the more dispersed group of non-Löfgren's patients (S3, S4 and S5), reflective of the clinical phenotypes where patients with Löfgren's show a distinct and typical acute onset whereas non-Löfgren's patients have a more heterogeneous clinical phenotype. Given the limited number of individuals in the respective patient subgroups, interpretation should be performed with some caution. However, the robustness of the model with a predictive power of 72% ($Q^2=0.72$, based on seven-fold cross-validation) indicates some validity to these findings.

To our knowledge, this is the first quantitative proteomic study performed on isolated alveolar macrophages from sarcoidosis patients. By combining global proteomics analysis with pathway analysis tools, a more systems level understanding of how the observed alterations in the soluble proteome of alveolar macrophages relate to intracellular signalling pathways involved in sarcoidosis could be achieved. These findings represent a step forward in understanding the complexity of sarcoidosis pathogenesis, where several proteins linked to complementary phagocytotic and antigen presentation mechanisms appear to be of importance.

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STATEMENT OF INTEREST

None declared.

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