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Alterations in the membrane-associated proteome fraction of alveolar macrophages in sarcoidosis

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Abstract. *Background*: Alveolar macrophages are implicated in the pathogenesis of lung sarcoidosis. Their interaction with T-cells leads to an inflammatory response that may either resolve within 2 years, or become chronic with an increased risk to develop lung fibrosis. *Objective*: To perform quantitative profiling of the membrane-associated proteome of alveolar macrophages in sarcoidosis patients and healthy individuals to identify specific proteins and pathways involved in sarcoidosis pathology. *Methods*: Differential proteomic analysis was performed on iTRAQ (isobaric Tag for Relative and Absolute Quantitation) labeled samples using tandem mass spectrometry. Subsequently, uni- and multivariate statistical analyses and pathway- and network analyses were performed. *Results*: Eighty proteins were differentially expressed between healthy and sarcoidosis patients. Down-stream pathway analysis confirmed our recent reports of up-regulation of two phagocytotic pathways: Fcγ receptor-mediated phagocytosis and clathrin-mediated endocytosis signaling. An additional pathway, pyruvate metabolism, was found to be up-regulated in sarcoidosis patients. The oxidative phosphorylation pathway was differentially expressed in subgroups of sarcoidosis, with up-regulation in Löfgren's patients and down-regulation in non-Löfgren's patients. *Conclusion:* This unprecedented proteome profiling of the membrane-associated fraction of alveolar macrophages confirmed previous findings of alterations in phagocytotic pathways due to sarcoidosis, as well as indicated a differential dysregulation of the oxidative phosphorylation pathway related to disease outcome in sarcoidosis. *(Sarcoidosis Vasc Diffuse Lung Dis 2016; 33: 17-28)*

Key words: alveolar macrophages, proteomics, sarcoidosis, membrane-associated proteins

Introduction

Sarcoidosis is a systemic granulomatous inflammatory disease often affecting the lungs (1). It is a multi-factorial disease where genetic predisposition and environmental exposure both play important roles (2, 3). The clinical manifestations are heterogeneous and can be classified into two main groups: Patients with Löfgren's syndrome, representing 35% of all patients in Scandinavia, have an acute onset including erythema nodosum, ankle arthritis, and bilateral hilar lymphadenopathy which typically resolve spontaneously within a period of two years (4). In contrast, non-Löfgren's patients usually have an insidious onset of disease with persistent chronic lung

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inflammation and an increased risk of developing fibrosis (5). Our group has previously identified a genetic predisposition for the respective disease courses with strong associations between HLA-DRB1-03 and good prognosis especially in Löfgren's syndrome, and between HLA-DRB1-14/15 and a more chronic disease course, respectively (4). HLA-DR molecules are membrane-spanning proteins localized to the surface of antigen presenting cells (APC) including alveolar macrophages (AMs). AMs carry out multiple functions associated with the innate immune response, and also act as a link to the adaptive immune response. In addition to expressing HLA class II molecules, they also display co-stimulatory CD40, CD80 and CD86 required for interaction with CD4^{pos} T-cells, and activation of an adaptive (Th1) response (6). The subsequent TNF-α production by AMs is essential for the formation of non-caseating granulomas, which are the histological hallmark of sarcoidosis (7). As such, it is evident that a number of membrane-associated proteins (MAPs) are of importance in the pathology of sarcoidosis, yet no global investigations of this specific sub-proteome have been undertaken in AMs to date.

The number of proteomics studies investigating the characteristics of human macrophages is very limited (for review, see (8, 9)). Jin et al. performed a thorough investigation of the differences between the proteomes of circulating blood monocytes and resident AM (10), revealing differential protein expression profiles particularly in proteinases (e.g. 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 polarization occurring in the lung, global proteomics studies investigating phenotypic alterations of human primary AM in response to respiratory disease are scarce. Exceptions include investigations of AM proteome responses to infection by Porcine reproductive and respiratory syndrome virus (11), influenza A virus (12), and COPD (13). Previous proteomics studies on sarcoidosis have mainly been performed on bronchoalveolar lavage (BAL) fluid and serum (14-21), thereby primarily reflecting proteins actively secreted or exudated into the bronchoalveolar lumen, such as plasma proteins and anti-oxidant proteins.

We recently performed quantitative profiling of the soluble proteome by means of gel-based proteomics approaches of primary AMs in sarcoidosis (22). In the current study we performed complementary proteome profiling of the membrane-associated fraction of AMs from sarcoidosis patients versus healthy controls using mass-spectrometry based shotgun proteomics approach, with the purpose of identifying additional, yet undiscovered proteins and pathways of relevance for sarcoidosis pathology.

Materials and Methods

Study Population

The study was approved by the Regional Ethical Review Board in Stockholm, Sweden (case number 02-427) and all subjects gave their written consent. Bronchoscopy was performed on six healthy control subjects and on eight sarcoidosis patients as part of the initial diagnostic routine investigation within three months after onset of symptoms (4, 23). The patients were included in a consecutive way after referral to the Department of Respiratory Medicine at the Karolinska University Hospital, Stockholm, Sweden. The diagnostic criteria for sarcoidosis patients was in accordance with the WASOG criteria (1), including typical chest radiographic changes. Also, granulomas were present in airway epithelial biopsies and the CD4/CD8 ratio was increased (≥ 3.5) in bronchoalveolar lavage (BAL) T-cell populations. Patients (5 males and 3 females, median age 38 (23-73) 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 patients, n=4). Extra-thoracic involvement, apart from ankle arthritis and/or erythema nodosum in Löfgren's syndrome patients, was observed in two of the non-Löfgren's patients (S3: superficial lymphnodes; S5: CNS, uveitis). The patients' chest radiographic changes were classified into stage I-III. None of the patients included were under any immunosuppressive treatment > 1 month prior to BAL (S7 received a single infusion of etanercept >1 month prior to BAL*)*. Three of the patients were ex-smokers (>1 year since last cigarette), and four were never-smokers, and one reported

Table 1. Clinical data

Subject ID	Diagnosis	Chest - X ray stage	Löfgren's syndrom	Smoking history	Cell Viability	Total cells x106	AMs %	Lympho-cytes %	CD4/ CD8	FVC X	FEV1 %	TLC %	DLco %
H1	Healthy	0	NA	No	91	13.6	86	11	1.6	126	109	ND	ND
H2	Healthy	0	NA	No	86	17.5	82	14	ND	104	100	ND	ND
H4	Healthy	0	NA	No	88	18.6	94	3.8	ND	116	107	ND	ND
H5	Healthy	0	NA	No	86	19.9	76	23	1.8	132	111	ND	ND
H6	Healthy	0	NA	No	82	12.8	90	6.4	ND	113	105	ND	ND
H7	Healthy	0	NA	No	92	5.8	87	12	ND	ND	ND	ND	ND
S2	Sarcoidosis	I	yes	No	93	39.8	87	13	6	83	87	84	100
S3	Sarcoidosis	III	No	No*	92	24.4	93	6.8	4.7	99	102	89	107
S4	Sarcoidosis	II	No	Ex	96	47.5	47	52	8.9	88	85	96	85
S5	Sarcoidosis	II	No	No	98	61.5	73	25	4.9	81	80	ND	ND
S6	Sarcoidosis	I	yes	Ex	95	33.0	67	29	4.8	97	99	97	77
S7	Sarcoidosis	I	yes	Ex	92	21.9	70	29	13	94	82	91	87
S8	Sarcoidosis	I	yes	Ex	75	58.7	65	34	9	89	88	91	77
S9	Sarcoidosis	II	No	No	94	29.9	87	11	2.5	91	80	79	102

X-ray stage (0): Normal; (I): Bilateral hilar lymphoma (BHL); (II): BHL w ith infiltrates; (III):parenchymal infiltration
Smoke: non-smoker (no), ex-smoker (ex) [>1 year]
NA: not applicable; ND: not determined; AM: alveolar macrophages
FVC = forced vital capacity (% predicted), FEV1 = forced expiratory volume in one second (% predicted), TLC = total lung capacity,
DLco = diffusion capacity for carbon monoxide
* Occasional use of w ater-pipe reported

recreational use of water-pipe. All healthy subjects (1 male and 5 females, median age 28 (21-35) years) were never-smokers and free from any symptoms indicating respiratory disease and all had normal chest x-ray and lung function (see table 1). None of the individuals had any signs of respiratory infection within the last four weeks prior to BAL. The majority of the subjects listed in table 1 were also included in our recent gel-based proteomics characterizations of the soluble macrophage proteome (S1-S7, H2-H7), and the Subject IDs correspond to the ones used in our previous publication (22).

Peptide labeling and LC-MS/MS analysis

Macrophages were isolated from bronchoalveolar lavage (BAL) cells using a Percoll™ density gradient as previously described (22, 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). Cells were then lysed and proteins were subfractionated through ultracentrifugation as previously described (22, 28) (see Figure S1[1] for experimental workflow). Briefly, protein homogenates were subjected to ultracentrifugation at 100 000 x g and the resulting pellets were suspended in 500 μL 2.5 M NaBr for 45 min on ice with shaking. Another ultracentrifugation was performed at 4°C for 1 hour at 100,000 x g. The supernatant containing membrane-associated proteins (MAPs) was stored at -80°C until further analysis. MAP proteins were precipitated with acetone, solubilized in 50 μL 1% SDS, after which additional 50 μL water was added (final SDS concentration 0.5%), and protein concentrations were determined using the Bio-Rad D_C protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The samples were then diluted to 0.1% SDS using water and 1 M TEAB buffer (final concentration TEAB buffer 0.025 M). 75 μg of protein from the MAP fraction of each sample was reduced (5 mM DTT, 30 min at 56°C), alkylated (0.015 M iodoacetamide, 30 min), and digested using Trypsin (modified sequencing grade, Promega, Madison, WI, USA) overnight at 37°C. A pooled internal standard was created by pooling 12 μg from each sample. Subsequently, 20 μg of each sample was labeled with iTRAQ reagents (8-plex; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The pooled sample was cleaned using an SCX-cartridge (StrataSCX, Phenomenex, Torrence, CA, USA). iTRAQ-labeled tryptic peptide samples

[1] To view supporting figures and tables, see additional files online version

were dissolved in 200 μL 8 M urea. Narrow range IPG-strips (pH 3.5 - 4.5, 24 cm long, GE Healthcare Bio-Sciences, Uppsala, Sweden) were rehydrated in 8 M Urea and 1% Pharmalyte™ 2.5-5 (GE Healthcare, Bio-Sciences) over night. Dry sample application gels (33×3×2 mm) were rehydrated in sample over night. The IPG strips were put in the focusing tray and the application gels containing the samples were placed on the anodic end of the IPG strips with filter paper between the application gels and the electrodes. The strips were covered with mineral oil and the focusing was performed on an Ettan™ IPGphor™ (GE Healthcare Bio-Sciences) until 100 kVh had been reached. After focusing, the strips were passively eluted into 72 continuous fractions using milliQ water using an IPG extractor. Samples were then freeze dried in a vacuum centrifuge and kept at -20°C until use. Prior to analysis, each fraction was re-suspended in 8 μl 3% acetonitrile, 0.1% formic acid.

LC-ESI-LTQ-Orbitrap analyses

Thirty-six of totally 72 IPG fractions were analyzed by LC-MS/MS. 3 μl from each fraction was injected into online HPLC-MS connected to a hybrid LTQ-Orbitrap Velos mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). An Agilent HPLC 1200 system (Agilent Technologies, Santa Clara, CA, USA) was used to provide the gradient for online reversed-phase nano-LC at a flow of 0.4 μl/min. Solvent A was 97% water, 3% ACN, 0.1% formic acid; and solvent B was 5% water, 95% ACN, 0.1% formic acid. The curved gradient went from 2% B up to 40% B in 45 min, followed by a steep increase to 100% B in 5 min. The sample was injected on a C18 guard desalting column (Agilent Technologies) prior to a 15 cm long C18 picofrit column (100 μm internal diameter, 5 μm bead size, Nikkyo Technos Co., Tokyo, Japan) installed on to the nano electrospray ionisation (NSI) source of the Orbitrap Velos instrument. Acquisition proceeded in ~3.5 s scan cycles, starting by a single full scan MS at 30000 resolution (profile mode), followed by two stages of data-dependent tandem MS (centroid mode): the top 5 ions from the full scan MS were selected firstly for collision induced dissociation (CID, at 35% energy) with MS/MS detection in the ion trap, and finally for higher energy collision dissociation (HCD, at 45% energy) with MS/MS detection in the orbitrap. Precursors were isolated with a 2 m/z width and dynamic exclusion was used with 60 s duration.

Protein identification and quantification

The MS/MS spectra were searched by Mascot 2.2 (Matrix Science Limited, London, UK) under the software platform Proteome Discoverer 1.1 (Thermo Fischer Scientific, San Jose, CA, USA) against the IPI human target decoy (update 100524) protein sequence database. A precursor mass tolerance of 10 ppm and product mass tolerances of 0.02 Da for HCD-FTMS and 0.8 Da for CID-ITMS were used. Quantitation of iTRAQ 8-plex reporter ions was done by Proteome Discoverer on HCD-FTMS tandem mass spectra using an integration window tolerance of 20 ppm. Results were limited to ≥ 2 high confident peptides/protein for quantification using a false discovery rate of <5%.

Statistical Analysis

Univariate data analysis was carried out using Significance Analysis of Microarrays (SAM) (29), version 3.09. The data was log transformed (base 2) and the parameters were as follows: response type: two-class unpaired, analysis type: standard, test statistic: T-statistic. The normality of the data was tested by means of Shapiro-Wilk test, and >95% of the proteins displayed a normal distribution in all three groups following log transformation. Multivariate analysis using principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) analysis were performed using SIMCA P 13.0 (Umetrics, Umeå, Sweden). Model performance is reported as cumulative correlation coefficients for the model (R^2) and predictive performance based on seven-fold cross validation calculations (Q^2), as well as cross-validated ANOVA (CV-ANOVA) p-values for the OPLS models.

Pathway- and Network Analyses

Ingenuity Pathway software (Ingenuity Systems Inc., Redwood City, CA, USA) was used for the pathway- and network analysis on differentially expressed proteins. Protein gene names and log-transformed expression levels of selected proteins were

loaded into IPA, and adjusted Fisher's exact test was used for statistical testing in the pathway analyses.

Results

In the current study, quantitative proteomics investigations of a fraction enriched for membrane-associated proteins was performed on AMs from Healthy subjects (n=6) compared to sarcoidosis patients (n=8), subdivided into non-Löfgren's (n=4) or Löfgren's syndrome (n=4). The majority of these subjects (all of the Healthy, 6 of the sarcoidosis patients) were also included in our recently reported gel-based proteomics profilings of the soluble fraction of AMs.

Alterations in AM Proteome Profiles due to Sarcoidosis

A total of 1656 proteins were identified; 423 with at least two high confidence (95%) peptides and detection levels above the limit of quantification (LOQ) across all 16 samples by means of 8-plexed iTRAQ (isobaric Tag for Relative and Absolute Quantitation) labeling (table S1)[1]. Applying univariate statistical analysis with a false discovery rate of 5% (FDR<0.05, Figure S2)[1], 80 proteins were differentially expressed between controls and sarcoidosis patients (table S2)[1]. Notably, only two of these proteins overlapped with the findings from our previously performed characterizations of the soluble AM proteome from primarily the same subjects using gel-based proteomics approaches (22), thereby confirming the complementary nature of the two studies. Subsequent multivariate analysis using unsupervised principal component analysis (PCA) showed a separation of sarcoidosis patients and controls driven by both the 1st and 2nd principal components (Figure 1). No strong outliers were identified in the data set, and all subjects were included in the downstream supervised multivariate modeling using orthogonal projections to latent structures (OPLS). In contrast to the more commonly used PCA modeling, OPLS analysis is a supervised method designed to pull out the predictive variance of interest from the variance unrelated to the hypothesis of interest (e.g. Löfgren's vs. non-Löfgren's patients), thereby acting as a noise filter which improves the interpretability of the multivariate model, particularly in relating the observed group separation to specific protein biomarkers (30). OPLS thus gives easier identification of biomarkers of interest as well as an improved and more accurate assessment of the predictive power of the selected biomarkers.

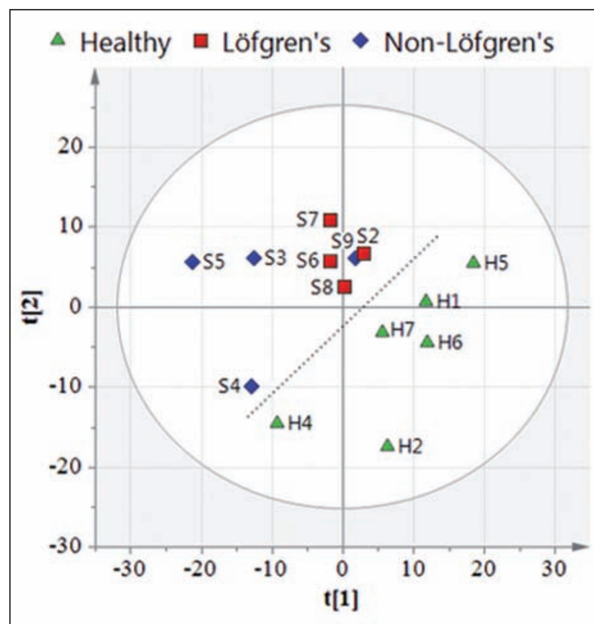

Fig. 1. Multivariate data analysis: PCA. Scores plot of PCA using all 423 proteins (R^2=0.44; Q^2=0.16). The sarcoidosis patents separate well from the healthy individuals (dotted line). A tight clustering of patients with Löfgren's disease (red) can be observed, while non-Löfgren's patients (green) are more spread out, reflecting the more heterogeneous phenotype of these patients

OPLS was performed to evaluate the predictive power to separate the sarcoidosis subjects from healthy controls (Figure 2). An optimized model was created through iterative variable selection using a Variable Influence on the Projection (VIP) score cutoff of 1.0. The selected subset of 13 putative biomarker proteins resulted in a highly significant separation between healthy and sarcoidosis patients (p[CV-ANOVA]=0.0009, 1 predictive and 0 orthogonal components, R^2=0.76) with 72% predictive power (Q^2 = 0.72) based on 7-fold cross validation (Figure 2 and table 2). Three of the 13 proteins are involved in endocytic transport; MP1 (mitogen-activated protein kinase scaffold protein 1), C11orf59 (regulator complex protein PDRO) and RAB7A (ras related protein Rab-7a). MP1 and C11orf59 are part of a protein complex called the Ragulator complex. This complex is anchored to lipid rafts in late

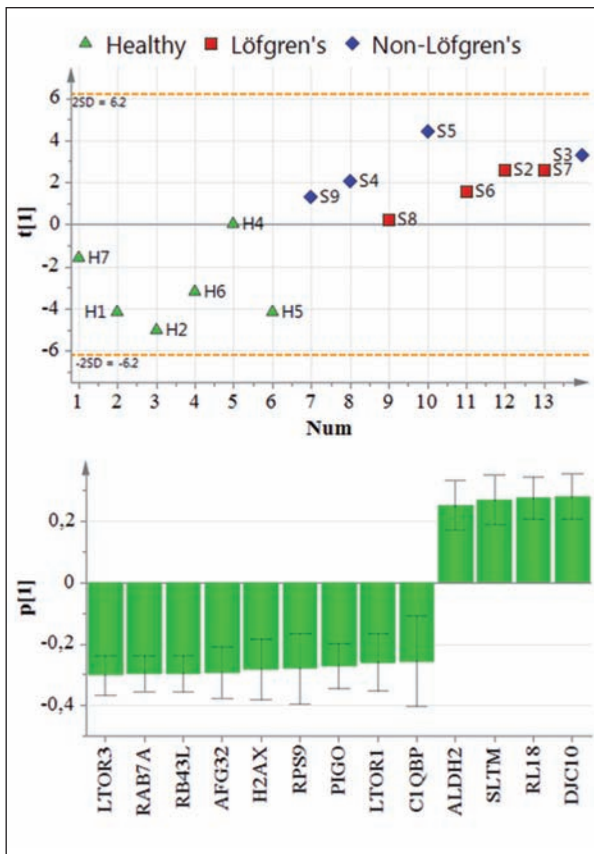

Fig. 2. Multivariate data analysis: OPLS. OPLS analysis of sarcoidosis patients versus control subjects (scores plot, top panel) resulted in a robust separation between groups (R^2=0.76; Q^2=0.72, p= 0.0009), with primarily 13 proteins driving the separation between the groups (displayed in the column loading plot, bottom panel)

endosome membranes via C11orf59, recruits mTORC1 to lysosomal membranes in amino acid signalling and is also involved in MAPK signalling (31). Additionally, RAB7A has a role in the maturation of phagosomes (32). One of the 13 model proteins, a mitochondrial protein termed the complement component 1 Q subcomponent-binding protein, is involved in host-virus interactions (33). The remaining 9 proteins in the final OPLS model are involved in DNA repair, alcohol metabolism, protein folding and ribosomal activity (table 2).

Pathway and Network Analyses

Downstream pathway analysis performed on the 80 proteins differentially expressed (FDR<0.05) in AM of sarcoidosis patients compared to healthy controls showed significant alteration of four pathways. In concordance with the parallel study performed by means of gel-based proteomics on the soluble fraction from AMs (22), we found an up regulation of two pathways associated with phagocytosis; the Fcγ receptor-mediated phagocytosis (p-value $1.5*10^{-5}$) and the clathrin-mediated endocytosis signaling ($p=9.6*10^{-9}$). Through the complementary proteome profiling approaches used in the current study, significant alterations of two additional pathways was identified; up-regulation of the pyruvate metabolism pathway ($p=7.5*10^{-9}$), and down-regulation of the oxidative phosphorylation pathway (Figure 3; $p=1.0*10^{-10}$) in the sarcoidosis group compared

Table 2. Proteins included in the final OPLS predictive model. The upward arrow indicates up-regulation in sarcoidosis patients

Protein name	Acronym	Function
Aldehyde dehydrogenase, mitochondrial ↑	ALDH2	Alcohol metabolism
Histone	H2A.x H2AFX	DNA repair
Mitogen-activated protein kinase scaffold protein 1	MAPKSP1	Late endosome membrane, crucial signal transduction
60S ribosomal protein L18 ↑	RPL18	Protein synthesis
Ribosomal protein S9, isoform CRA.c	RPS9	Protein synthesis
Putative uncharacterized protein RAB7A	RAB7A	Maturation of phagosomes
AFG3-like protein 2	AFG3L2	ATP-dependent protease
Putative Rab-43-like protein ENSP00000330714	N/A	Membrane network
Isoform 2 of DnaJ subfamily C member 10 ↑	DNAJC10	Protein folding, ER
RhoA activator C11orf59	C11ORF59	Endosome system
Complement component 1 Q subcomponentbinding protein	C1QBP	Host-virus interaction
Isoform 2 of GPI ethanolamine phosphate transferase 3	PIGO	Glycosylphosphatidylinositolanchor biosynthesis
Putative uncharacterized protein SLTM ↑	SLTM	Inhibition of transcription

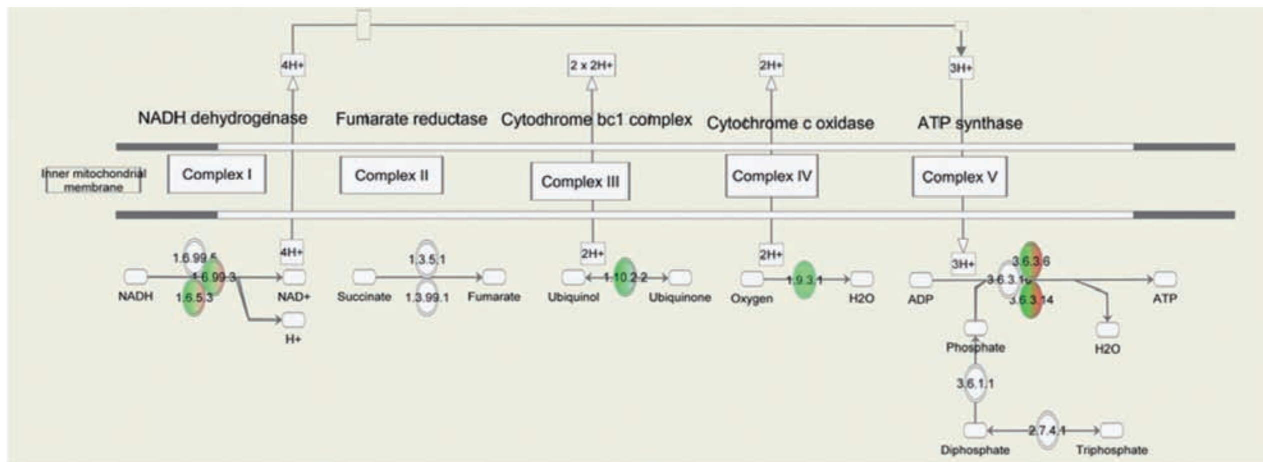

Fig. 3. Pathway analysis. Proteins in the mitochondrial respiratory chain were down-regulated in sarcoidosis patients. This alteration was driven completely by patients with non-acute (non-Löfgren's syndrome) sarcoidosis

to healthy. Notably the latter was completely driven by the non-Löfgren group, as no alterations were observed in patients with Löfgren's syndrome.

Differences in AM Proteome Expression of Löfgren's and Non-Löfgren's Sarcoidosis sub-phenotypes

OPLS modeling with respect to Löfgren's syndrome revealed a highly robust separation between patients with Löfgren's syndrome from the non-Löfgren's group. The selected subset of 14 putative biomarker proteins resulted in a highly significant separation between subjects with Löfgren's syndrome and non-Löfgren's sarcoidosis patients (p[CV-ANOVA]=0.008, 1 predictive and 0 orthogonal components, R^2=0.90) with 85% predictive power (Q^2 = 0.85) based on 7-fold cross validation (Figure S3)[1]. Three of the 14 proteins are key components of the oxidative phosphorylation pathway.

Discussion

Here we present a quantitative investigation of alterations in the expression levels of the membrane-associated fraction of AMs of patients with lung sarcoidosis compared to healthy controls. In contrast to our recently reported gel-based proteomics investigation of the soluble fraction of AMs from primarily the same subjects (22) the current study applied mass spectrometry based shotgun proteomics approaches, which has been shown in numerous studies to target a complementary subsection of the cellular proteome (34, 35). Traditional univariate statistical analysis showed that the expression levels of 80 proteins were significantly altered due to sarcoidosis. Downstream pathway analysis revealed that the observed changes were associated with significant alterations of four biological pathways, including a significant up-regulation of two phagocytosis-related pathways in sarcoidosis; the Fcγ receptor-mediated phagocytosis in macrophages ($p=1.5*10^{-5}$) and clathrin-mediated endocytosis signaling ($p= 9.6*10^{-9}$; Figure 4). These results confirm our observations in a previous investigation focusing on the soluble proteome of alveolar macrophages (22). The fact that these fundamentally different proteomics approaches performed on different fractions of AMs identified the same two pathways emphasizes the potential importance of these two pathways in sarcoidosis.

Phagocytosis is a central process in macrophage functionality, associated with both innate and adaptive immune responses, where the Fcγ receptor-mediated phagocytotic pathway is one of the main activation pathways. This mechanism mediates internalization of pathogens (bacteria, virus and parasites) into membrane-derived vacuoles and phagosome system which finally leads to degradation of pathogens and the presentation of specific antigens to memory T-cells in the lungs (36). The FcR also mediates signal transductions that regulate the production of reactive oxygen species (ROS). These re-

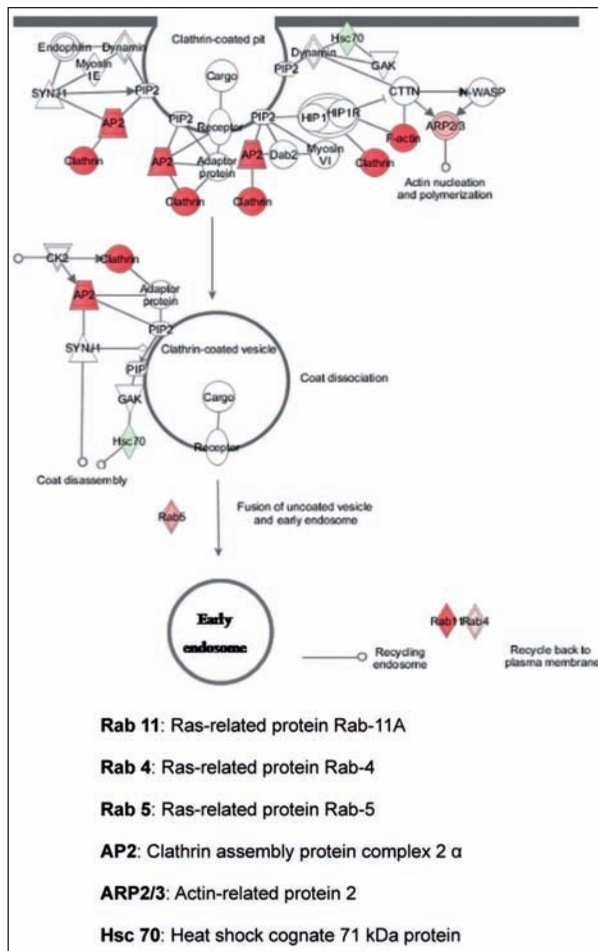

Fig. 4. Pathway analysis. Proteins part of the Clathrin-mediated endocytosis signaling were up-regulated in sarcoidosis patients

active oxygen species, including superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2), are important in the innate response for degradation of pathogens by alveolar macrophages. However, excessive levels of ROS lead to oxidative stress and cause tissue damage. The mitochondria represent the main potential source of non-specific endogenous ROS generation (37, 38), with defective oxidative phosphorylation releasing large amounts of ROS (39).

In addition to alterations in phagocytotic pathways, results from pathway analyses in the current study also showed a significant down-regulation of the oxidative phosphorylation pathway (Figure 3; $p=1.0*10^{-10}$), as well as significant up-regulation of pyruvate metabolism ($p=7.5*10^{-9}$). Interference in the mitochondrial electron transport and the resulting ROS production has been associated with increased inflammation in the airways (40, 41). High exposure to ROS and the resulting redox imbalance in the cell has also been shown to cause damage to cellular and mitochondrial proteins, leading to mitochondrial dysfunction (42-44). Redox imbalance has been associated with a number of chronic inflammatory lung diseases including COPD (45), asthma (46), lung fibrosis and cancer (47). In sarcoidosis, AMs have been shown to produce high levels of superoxide anion, (48, 49), and specific protein markers for oxidative stress have been reported in BAL fluid from sarcoidosis patients (16). Also systemic oxidative stress has been reported in pulmonary sarcoidosis (50).

High concentrations of ROS can in addition to tissue damage also trigger changes in signal transduction. Studies have shown that imbalance in redox signaling leads to activation of NFκβ where ROS are directly implicated as second messengers by regulating ubiquitination and degradation of Ik (51). In the network analysis from this study (Figure 5) we also observed alterations in the expression of proteins related to the extended NFKβ signaling network responsible for the activation of several inflammatory genes, also in concordance with our previous findings (22). Pyruvate has been shown to act as an endogenous anti-inflammatory molecule by protecting cells from ROS and suppressing both TNF-α secretion and NF-κB expression (52). The up-regulation of pyruvate metabolism observed in sarcoidosis could thus represent a general protective response to counterbalance the increased oxidative stress.

The down regulation observed at three key sites in the mitochondrial electron transport chain (Complexes I, III and IV, Figure 3) may lead to a mitochondrial dysfunctionality of AMs which can be of importance in sarcoidosis pathogenesis and the subsequent development of fibrosis. Interestingly, the observed alterations in the oxidative phosphorylation pathway were completely driven by the non-Löfgren's patients, down-regulation of proteins from the mitochondrial electron transport pathway occurring in non-Löfgren's sarcoidosis patients alone. Furthermore, multivariate modeling by OPLS showed a highly significant group separation between Löfgren's and non-Löfgren's patients (p(CV-ANOVA)=0.008), with 3 of the 14 proteins driving the

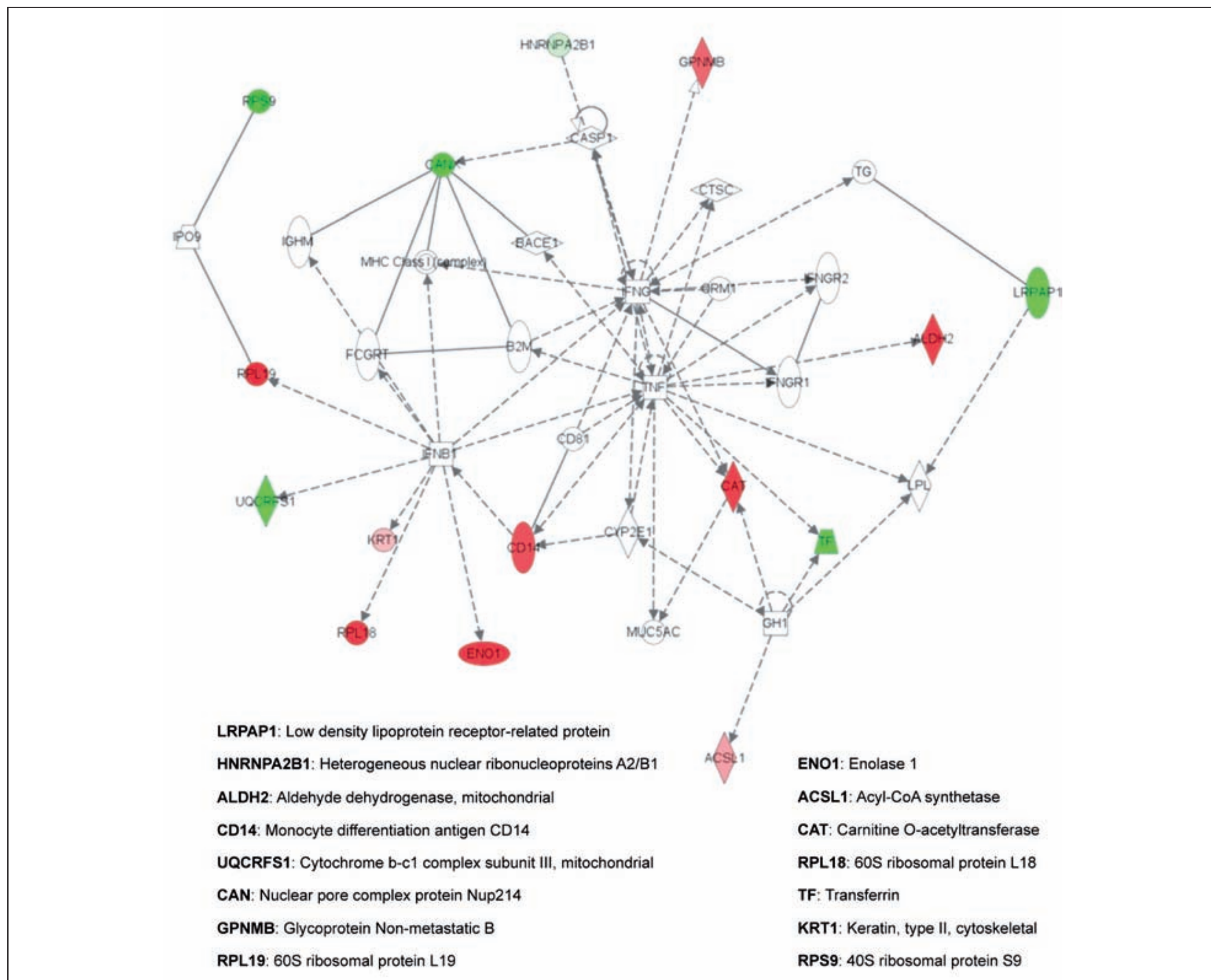

Fig. 5. Network analysis. Network analysis revealed a number of the significantly altered proteins to be associated with the extended NFkB regulatory network.

separation being key components in oxidative phosphorylation.

Accordingly, the observed alterations in the oxidative phosphorylation pathway may be of clinical relevance for non-Löfgren's patients, which accounts for the majority of all sarcoidosis patients. Indeed, this subset of sarcoidosis patients is characterized by a tendency to present an unresolved chronic inflammation, frequently leading to fibrosis. In lung sarcoidosis, where about 20% of non-Löfgren's patients may develop some degree of fibrosis; there is a need for new treatments in addition to standard corticosteroids, and anti-oxidant might be a possible complementary alternative. Demedts and coworkers found that treatment with N-acetylcysteine anti-oxidative therapy as a supplement to traditional corticosteroids treatment reduced fibrosis progression in patients with idiopathic pulmonary fibrosis (53). Based on the findings presented here, it may be relevant to investigate whether similar regimens may have a protective effect also in non-Löfgren's sarcoidosis patients. It should be highlighted that the number of subjects included in the current study is very limited, and the results should be interpreted with some caution. However, the fact that all three bioinformatics-based validation approaches utilized (cross-validation of the multivariate models, pathway- and network analysis) all point to the same re-

sults provides validity to the biological relevance of the findings.

In summary, two phagocytotic pathways were found to be up-regulated in AMs of sarcoidosis patients; the Fcγ receptor-mediated phagocytosis and the clathrin-mediated endocytosis signaling, thereby verifying our previous findings from proteome characterizations of the soluble proteome of AMs. The fact that these fundamentally different proteomics approaches performed on different fractions of AMs identified alterations in the same two pathways, with minimal overlap in the specific proteins identified, emphasizes the potential importance of these two pathways in sarcoidosis. In addition, these complementary studies revealed down-regulation of pathways related to mitochondrial respiration, which could lead to an imbalance in the oxidative homeostasis, in turn triggering increased levels of ROS formation. These alterations were observed exclusively in non-Löfgren's sarcoidosis patients, and could thereby represent a piece in the puzzle explaining their unresolved inflammation and prolonged disease course. This imbalance in oxidative homeostasis could potentially be associated with the increased risk of development of fibrosis observed in this sarcoidosis phenotype.

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