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Review

Humoral immune profiling of mycobacterial antigen recognition in sarcoidosis and Löfgren's syndrome using high-content peptide microarrays

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SUMMARY

Introduction: Sarcoidosis is considered an idiopathic granulomatous disease, although similar immunological and clinical features with tuberculosis (TB) suggest mycobacterial involvement in its pathogenesis. High-content peptide microarrays (HCPM) may help to decipher mycobacteria-specific antibody reactivity in sarcoidosis.
Methods: Serum samples from patients with sarcoidosis, Löfgren's syndrome, and TB, as well as from healthy individuals (12/group), were tested on HCPM containing 5964 individual peptides spanning 154 *Mycobacterium tuberculosis* proteins displayed as 15-amino acid stretches. Inclusion/exclusion and significance analyses were performed according to published methods.
Results: Each study group recognized 68–78% *M. tuberculosis* peptides at least once. *M. tuberculosis* epitope recognition by sarcoidosis patient sera was 42.7%, and by TB patient sera was 39.1%. Seven and 16 peptides were recognized in 9/12 (75%) and 8/12 (67%) sarcoidosis patient sera but not in TB patient sera, respectively. Nine (75%) and eight (67%) out of twelve TB patient sera, respectively recognized *M. tuberculosis* peptides that were not recognized in sarcoidosis patient sera.
Conclusions: Specific IgG recognition patterns for *M. tuberculosis* antigens in sarcoidosis patients re-affirm mycobacterial involvement in sarcoidosis, providing biologically relevant targets for future studies pertaining to diagnostics and immunotherapy.

Contents

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Introduction

Sarcoidosis is a disorder of unknown aetiology, characterized by non-caseating granulomatous inflammation sustained by CD4 T-cell activation. It affects mainly lung and hilar lymph nodes, but has a propensity to affect every organ/tissue.[1] The disease is characterized by a wide spectrum of clinical manifestations, from the acute, self-limiting Löfgren's syndrome, to chronic fibrotic forms, suggesting a multifactorial pathogenesis. The correlation with specific major histocompatibility complex (MHC) class II molecules, in addition to recruitment and expansion of specific subsets of CD4 T-cells in the alveolar space in well-defined patient subgroups (i.e., Löfgren's syndrome), suggests that antigen-driven inflammation plays a critical role.[2–4]

Since sarcoidosis and pulmonary tuberculosis (TB) are both granulomatous diseases, and since they share a similar distribution of the lesions in the lung, mycobacterial species have been incriminated in the pathogenesis of sarcoidosis.[1,5,6] *Mycobacterium tuberculosis*-derived antigens and DNA have been isolated from lung granulomas,[1] while T-cell reactivity to several mycobacterial proteins has been demonstrated in subgroups of patients with sarcoidosis.[1,7–11] However, since the specificity and reproducibility of these observations are limited, the CD4/CD8 ratio in bronchoalveolar lavage fluid (BALF) remains the diagnostic benchmark. While patients with Löfgren's syndrome may clear mycobacterial antigens from the systemic circulation to resolve granulomatous inflammation, individuals with chronic fibrotic forms of systemic sarcoidosis may not be able to do so, succumbing to end-organ damage and fibrosis.[2]

Immunoglobulin G (IgG) molecules are increased in the blood and BALF of sarcoidosis patients, suggesting a potential role for antigen-specific humoral immune responses in triggering sarcoid inflammation.[1] The discovery that serum IgG from subgroups of patients with chronic sarcoidosis binds to *M. tuberculosis* proteins (catalase, purified protein derivatives) in the Kveim reagent (used for diagnosis), suggests mycobacterial involvement in granulomatous inflammation in sarcoidosis.[12,13] Nevertheless, due to technical constraints, it has not been possible to gauge the entire spectrum of antibody responses to mycobacterial antigens.

High-content peptide microarrays (HCPM) now offer the opportunity to test the presence of immunologically relevant epitopes with small quantities of clinical material.[14] Using this technique, the presence of IgG molecules specific for a high number of epitopes derived from human pathogens may be visualized and quantified, as has been reported previously for TB, influenza, cytomegalovirus infection, and pertussis.[15–19] This information can then be processed using statistical methods in order to generate an immune recognition landscape for each protein, based on the IgG recognition of individual epitopes.

This study using an HCPM platform customized with 154 *M. tuberculosis* antigens was performed to describe the differential humoral immune responses to a high number of *M. tuberculosis* antigens in serum from patients with Löfgren's syndrome, sarcoidosis, and active pulmonary TB, as well as in serum from healthy individuals, by means of qualitative and quantitative analyses. Individual *M. tuberculosis* epitopes with potential implications in the differential diagnosis of sarcoidosis, Löfgren's syndrome, and pulmonary TB are discussed.

Methods

Design

The study was designed as a cross-sectional comparison of 'reactosomes' (i.e., the overall immune profile detected on the peptide microarray, composed of the mean intensity of recognition and the number of recognitions over the specific peptides) in four groups of subjects: healthy controls (HTC), patients with active TB (TB), patients with sarcoidosis (SARC), and patients with Löfgren's syndrome (LOF). Every group comprised 12 serum samples, each from a single volunteer, matched by ethnic group (all the enrolled subjects were Swedish), age, and sex, in order to avoid recruitment biases in the data analysis. However, sex matching was not entirely possible for the Löfgren's syndrome and sarcoidosis groups.

Serum samples

Sera from 12 healthy subjects were obtained from collections from the Lung Research Laboratory and from the Department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Stockholm, Sweden. Sera from patients with sarcoidosis and Löfgren's syndrome were obtained from a collection of the Lung Research Laboratory, Karolinska Institutet, Stockholm, Sweden. Sera from patients with active TB were obtained from the Sahlgrenska Hospital, University of Goteborg, Goteborg, Sweden, and from the Clinic of Infectious Diseases, Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden. All samples were collected and used after the necessary institutional review board approval had been obtained (2005/1031-31 and 2009/20-32, approved by the Regional Ethics Review Board in Stockholm). All subjects who actually donated blood for the study signed an informed consent form.

Microarray slides and experiments

Peptide microarray slides were customized and manufactured by JPT (Berlin, Germany).[20] The slides contain three identical sub-arrays with 5964 unique peptides on each sub-array. Each sub-array contains 16 blocks arranged in a regular pattern, with spots arranged in a 16×15 matrix (a schematic representation of a microarray and a table containing a list of the proteins displayed on the chip are available in the **Supplementary Material** in the online version at doi: http://dx.doi.org/10.1016/j.ijid.2017.01.021; Figure S1, Table S1). Each sub-array contains positive controls, negative controls, the unique peptides spanning 154 *M. tuberculosis* proteins of interest (Table S1), totalling 17 892 spots per slide. The entire amino acid sequence of each *M. tuberculosis* protein was printed on the microarray as 15-mer amino acid peptides overlapping the

previous and next printed peptide by 11 amino acids; this allows for the identification of minimal amino acid epitopes of four amino acids per spot defined by antibody reactivity.

Experiments were performed following a standardized protocol[15]: 300 μl serum diluted 1:100 in washing solution (filtered phosphate-buffered saline (PBS), 3% foetal calf serum (FCS; Sigma, Germany), and 0.5% Tween (Sigma, Germany)) was added to the peptide microarray slide, covered with a cover slip (Gene-Frame, Abgene, UK) to distribute the dilution evenly over the slide, and incubated at 4 °C in a humid chamber for 16 h. After removal of the cover slip, the slides were washed five times on a shaker, 5 min each (2 × with washing solution, 2 × with sterile water, and 1 × with filtered Milli-Q water at the end). Just after the washing procedure, 300 μl of Cy5-labeled goat anti-human IgG (affinity-purified secondary antibody; Abcam, UK) diluted 1:500 in washing solution was added to the slides in the dark, and the slide covered with a cover slip and incubated in a dark, humid chamber at room temperature for 1 h. The washing steps were repeated after the incubation with the secondary antibody. Prior to scanning, the slides were dried with a spinner (Euro Tech, UK). Five additional slides were processed using only buffer in the first incubation step in order to detect false-positive spots due to non-specific binding of the secondary antibody. High-definition images from the slides were acquired with a GenePix 4000 B microarray scanner (Axon Instruments–Molecular Devices, Union City, USA) using wavelengths of 635 nm (red channel, for the specific IgG signal quantification) and 532 nm (green channel, positive controls for grid alignment and orientation). Data acquisition from the images was performed with the software GenePix 6 Pro (Axon Instruments–Molecular Devices, Union City, USA).

Peptide microarray data analysis

The data analysis consisted of four steps, as outlined below.

Quality control

All images and aligned files were inspected to ensure that artefacts were not included in the analysis and to detect spots not identified or erroneously flagged by the software. Images of background and foreground intensities were created from the original files of every sub-array with the open-source software Bioconductor (https://www.bioconductor.org/) to exclude signal artefacts. All spots or areas that did not represent a high quality signal were removed from the analysis. Further quality controls included the following: computation of the index value (log2 foreground/background); scatter plots (index vs. log background) for each slide to remove outliers and abnormal values; scatter plots (average index vs. average log background) for all slides in each group to address the efficacy of the negative and positive controls; check of flag distribution proportions (−100, −75, −50, 0) for all and each group.

False-positive and 'empty' spot removal, and exclusion of low intensity signal spots

All spots identified as false-positive on the buffer slides were removed from the analysis, as well as all spots that did not show any signal (as described in Reilly and Valentini[41]). Low response spots with a signal below a computed cut-off (μ + 2 SD, where SD is the standard deviation of μ, the mean value of negative controls in the slides of each study group), were also removed.

Normalization

The normalization process was performed using the simple linear model, as described previously[20,21]: $I = slide_i + subarray_j + block_k + \varepsilon$, where I is the measured intensity; $slide_i$ is the slide effect, i.e., the effect on the intensity due to the existence of the spot on a certain slide; $subarray_j$ is the sub-array effect, i.e., the effect on the intensity due to the position of the spot at one of the sub-arrays in the slide; $block_k$ is the block effect or the effect on the intensity due to the position of the spot in one of the blocks in the sub-array; ε is the residual composed of the biological interaction and slide and sub-array interaction ($slide_i$ * $subarray_j$). Data were fitted into the linear model and the estimated slide, sub-array, and block effects removed. The quality of the normalization was assessed by visual inspection of the normalized data plot in all of the study groups. To fit the model, the lm function of R was used.

Analysis and data mining

First, the exclusive recognition analysis (ERA) was performed to identify top peptides recognized in a specific study group but not in all the others.[19] Top peptides identified in each group were plotted according to the index value and number of times they were recognized in the group of interest. Second, a significance analysis of microarray (SAM)[22] was performed to assess the peptides differentially recognized in a study group vs. a reference group, for the following comparisons: (1) sarcoidosis, Löfgren's syndrome, and TB patients, respectively vs. healthy controls; (2) sarcoidosis and Löfgren's syndrome, respectively vs. TB patients; (3) sarcoidosis plus Löfgren's syndrome vs. healthy controls; (4) sarcoidosis plus Löfgren's syndrome vs. TB patients.

All pre-processing and statistical analyses were performed using in-house scripts and customized open-source packages of Bioconductor, R software (http://www.bioconductor.org/index.html).

Results

Two different means of immune recognition are formally possible when comparing serum samples from one patient group versus a control group: (1) stronger recognition ('highly recognized'), defined as a stronger immune-fluorescence intensity/peptide or number of peptides, and (2) reduced recognition ('under-recognized'), defined as a reduced fluorescence signal/individual peptide or reduced number of peptides.

The SAM analysis performed for the three disease groups (sarcoidosis, Löfgren's syndrome, TB) vs. healthy controls identified 258 differentially recognized peptides for sarcoidosis (97 highly and 161 under-recognized; q-value <0.001), 313 differentially recognized peptides for Löfgren's syndrome (160 highly and 153 under-recognized; q-value <0.001), and 147 differentially recognized peptides for TB (138 highly and nine under-recognized; q-value <0.001). The top 20 highly recognized peptides for each comparison are reported in Tables 1–3.

For the same comparisons, the ERA results are plotted in Figures 1–3. The two top peptides resulting from ERA in sarcoidosis vs. healthy controls (Figure 1) are also among the top 20 peptides in Table 1 (SELTRFTPEAVVEKY and ADMWGPSSDPAWERN, marked in bold; fold change 4.12 and 3.44, respectively). The same applies to the top four peptides (in bold in Table 2) in the ERA comparison of sera from patients with Löfgren's syndrome vs. healthy controls (Figure 2). In contrast, none of the top strongly recognized peptides in serum from TB patients vs. healthy controls is also present in the top strongly recognized peptides in Table 3. Of note, a single peptide (AGEHEAAAAGYVCAL) was always present among the top 20 strongly recognized peptides in the three comparisons (sarcoidosis/Löfgren's syndrome/TB vs. healthy controls).

The exclusive analysis showed specific patterns of recognition for sarcoidosis and TB: serum samples from patients with sarcoidosis exhibited IgG in at least one serum sample for 567 out of 1329 (42.7%) *M. tuberculosis* epitopes that were not recognized in any serum sample from TB patients, whilst the latter had 487 out of 1245 (39.1%) IgG for *M. tuberculosis* epitopes

Table 1
Significance analysis of microarrays (SAM): recognition of *Mycobacterium tuberculosis*. A peptides in sera of patients with sarcoidosis (SARC, $n = 12$) vs. healthy controls (HTC, $n = 12$). The top-responding *M. tuberculosis* peptides from ERA (exclusive recognition analysis) are indicated in bold.

Ranking	Peptide	Rv number	Description	Score	Fold change
1	TPAAGAAPSAGAAPA	Rv0286	Uncharacterized PPE family protein PPE4	4.89	2.72
2	**SELTRFTPEAVVEKY**	Rv1629	DNA polymerase I (POL I) (EC 2.7.7.7)	4.79	4.12
3	ARWGSLYDALYGTDV	Rv1837c	Malate synthase G (EC 2.3.3.9)	4.76	5.76
4	EDYTDAAQPIWWVVR	Rv0280	Uncharacterized PPE family protein PPE3	4.67	2.70
5	AGEHEAAAAGYVCAL	Rv3021c	Uncharacterized PPE family protein PPE47/PPE48	4.62	3.70
6	**ADMWGPSSDPAWERN**	Rv1886c	Diacylglycerol acyltransferase Ag85B	4.51	3.44
7	GSSAMILAAYHPQQF	Rv1886c	Diacylglycerol acyltransferase Ag85B	4.40	3.16
8	LTEIGYLLPEPDDFT	Rv1837c	Malate synthase G (EC 2.3.3.9)	4.39	2.71
9	NTGSFNAGNYNTGYF	Rv0305c	Uncharacterized PPE family protein PPE6	4.29	4.98
10	AALVGALSVPHSWTT	Rv0915c	Uncharacterized PPE family protein PPE14	4.14	2.14
11	GYATGGMSTAALSSG	Rv0442c	Uncharacterized PPE family protein PPE10	4.09	2.57
12	TFLAYLVLDPLIYFG	Rv2123	Uncharacterized PPE family protein PPE37	4.08	2.38
13	SAPVGGLDSGNPNPG	Rv0442c	Uncharacterized PPE family protein PPE10	4.03	2.96
14	MFSGFDPWLPSLGNP	Rv0256c	Uncharacterized PPE family protein PPE2	4.01	2.80
15	GQSVTGYNNSVSVTS	Rv0755c	Uncharacterized PPE family protein PPE12	4.01	2.05
16	EKYGLTPRQYPDFAA	Rv1629	DNA polymerase I (POL I) (EC 2.7.7.7)	4.00	3.60
17	NQSFPVTVNWSTPAV	Rv3347c	Uncharacterized PPE family protein PPE55	3.98	1.97
18	AQSAAIAHATGASAG	Rv1807	PPE family protein	3.95	4.46
19	KDSDSPDKLRRVVAH	Rv1802	Uncharacterized PPE family protein PPE30	3.95	2.50
20	LAIFASNLDEFYMVR	Rv2984	Polyphosphate kinase (EC 2.7.4.1)	3.94	2.62

Table 2
Significance analysis of microarrays (SAM): recognition of *Mycobacterium tuberculosis* peptides in sera of patients with Löfgren's syndrome (LOF, $n = 12$) vs. healthy controls (HTC, $n = 12$). The top-responding *M. tuberculosis* peptides from ERA (exclusive recognition analysis) are indicated in bold.

Ranking	Peptide	Rv number	Description	Score	Fold change
1	ARWGSLYDALYGTDV	Rv1837c	Malate synthase G (EC 2.3.3.9)	5.78	9.14
2	AGEHEAAAAGYVCAL	Rv3021c	Uncharacterized PPE family protein PPE47/PPE48	5.58	4.75
3	LTEIGYLLPEPDDFT	Rv1837c	Malate synthase G (EC 2.3.3.9)	5.31	3.59
4	MWAGYRWAMSVELTQ	Rv3369	F420-dependent oxidoreductase	5.04	5.15
5	TFLAYLVLDPLIYFG	Rv2123	Uncharacterized PPE family protein PPE37	4.76	3.00
6	**HDQFVHTLTAGAGSY**	Rv2396	PE-PGRS family protein PE_PGRS41	4.73	3.21
7	**ADMWGPSSDPAWERN**	Rv1886c	Diacylglycerol acyltransferase Ag85B	4.72	4.77
8	**SGFFNGGPGTVSGIA**	Rv0355c	PPE family protein PPE8	4.60	5.17
9	NTGSFNAGNYNTGYF	Rv0305c	PPE family protein PPE6 (uncharacterized protein)	4.58	5.76
10	YPTVDYAFQYDGVND	Rv2608	Uncharacterized PPE family protein PPE42	4.55	3.53
11	MDFGLQPPEITSGEM	Rv1809	Uncharacterized PPE family protein PPE33	4.52	4.52
12	SGYLNGDSRASGWIH	Rv1548c	Uncharacterized PPE family protein PPE21	4.50	2.57
13	GFGNFGSYNIGFGNV	Rv0304c	PPE family protein PPE5	4.42	2.57
14	**SELTRFTPEAVVEKY**	Rv1629	DNA polymerase I (POL I) (EC 2.7.7.7)	4.38	4.25
15	LEFVRPVAVDLTYIP	Rv3616c	ESX-1 secretion-associated protein EspA	4.28	3.32
16	AAHFDYGSALLSKTT	Rv1860	Alanine and proline-rich secreted protein Apa MPT-32	4.17	3.61
17	FLEETFAAYDQYLSA	Rv3018c	Uncharacterized PPE family protein PPE46	4.10	3.17
18	MFSGFDPWLPSLGNP	Rv0256c	Uncharacterized PPE family protein PPE2	4.10	3.58
19	TPAAGAAPSAGAAPA	Rv0286	Uncharacterized PPE family protein PPE4	4.10	2.94
20	KELSADIARRPMAKP	Rv2544	Putative lipoprotein LppB	4.05	3.16

Table 3
Significance analysis of microarrays (SAM): recognition of *Mycobacterium tuberculosis* peptides in sera of patients with tuberculosis (TB, $n = 12$) vs. healthy controls (HTC, $n = 12$). The top-responding *M. tuberculosis* peptides from ERA (exclusive recognition analysis) are indicated in bold.

Ranking	Peptide	Rv number	Description	Score	Fold change
1	FFQELADLDRQLISL	Rv3616c	ESX-1 secretion-associated protein EspA	3.76	4.34
2	LSTLTGEEWHGPASA	Rv1807	PPE family protein	3.37	3.62
3	FGELLFTNPTGAFQF	Rv1387	Uncharacterized PPE family protein PPE20	3.35	2.75
4	NLGTGNSGWGNSDPS	Rv2356c	Uncharacterized PPE family protein PPE40	3.18	3.47
5	FEAAFAMTVPPAEVA	Rv2768c	PPE family protein (PPE family protein PPE43)	3.16	2.55
6	IMQLTTEQWLGPASM	Rv2768c	PPE family protein (PPE family protein PPE43)	3.13	2.64
7	LAAAAAWDALAAELY	Rv1808	Uncharacterized PPE family protein PPE32	3.10	3.34
8	NANLGDYNVGSGNVG	Rv1917c	Putative uncharacterized protein Rv1917c	3.04	2.43
9	NSNTGGFNMGQYNTG	Rv0355c	PPE family protein PPE8	2.96	6.03
10	AGPPQRWFVVWLGTA	Rv1860	Alanine and proline-rich secreted protein Apa (45 kDa glycoprotein) MPT-32	2.83	2.14
11	AGEHEAAAAGYVCAL	Rv3021c	Uncharacterized PPE family protein PPE47/PPE48	2.83	3.66
12	TSIVPFVVYYGPVEL	Rv0305c	Uncharacterized PPE family protein PPE6	2.82	2.23
13	CGSAVAIGGDGGAGG	Rv2396	PE-PGRS family protein PE_PGRS41	2.82	2.92
14	FLEETFAAYDQYLSA	Rv3018c	Uncharacterized PPE family protein PPE46	2.79	3.96
15	GYATGGMSTAALSSG	Rv0442c	Uncharacterized PPE family protein PPE10	2.77	3.56
16	STKIVIAGGFGSGKT	Rv3362c	Uncharacterized protein	2.72	2.03
17	TVLVGGLRVLGANYK	Rv1908c	Catalase-peroxidase (CP) (EC 1.11.1.21) (Peroxidase/catalase)	2.72	2.12
18	SAYASPRIGQPVGSE	Rv0442c	Uncharacterized PPE family protein PPE10	2.72	2.47
19	PFNVNLKLQFLHDAF	Rv3347c	Uncharacterized PPE family protein PPE55	2.69	2.09
20	SSTAATFASGPSGLL	Rv1807	PPE family protein	2.68	2.69

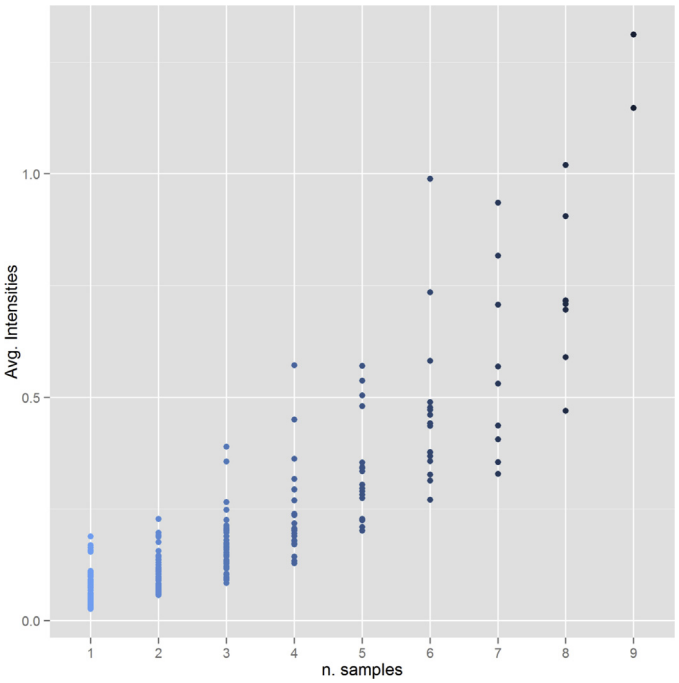

Figure 1. Exclusive recognition analysis (ERA) results plot for sarcoidosis vs. healthy control samples: average intensity of the peptides never recognized in serum from healthy controls by number of times they were recognized in serum from patients with sarcoidosis.

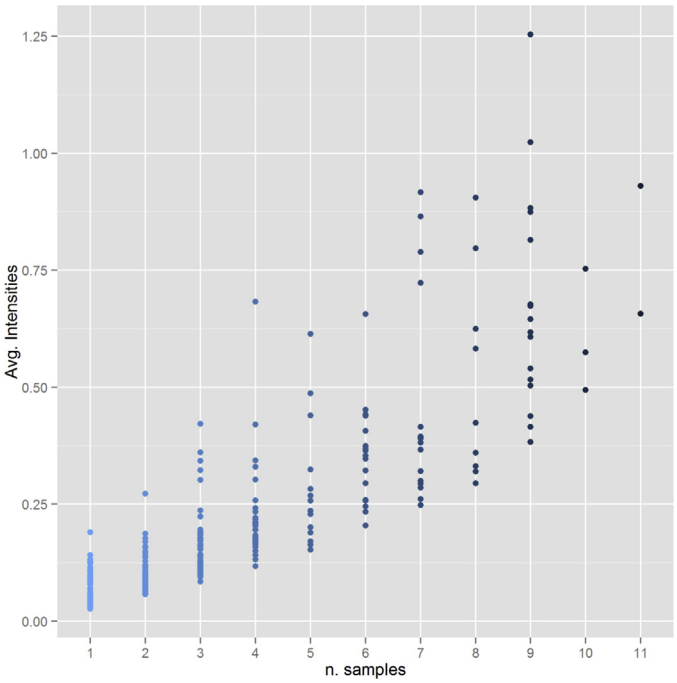

Figure 3. Exclusive recognition analysis (ERA) results plot for TB vs. healthy control samples: average intensity of the peptides never recognized in serum samples from healthy controls by number of times they were recognized in serum from TB patients.

not present in sarcoidosis. The top two strongly recognized *M. tuberculosis* peptides identified via this exclusive analysis (Figure 4) in the serum of patients with sarcoidosis as opposed to serum from TB patients were also found in the top position of the highly recognized peptides identified by the SAM analysis (Table 4).

A specific serum epitope recognition pattern could also be identified by comparing the sera of patients with Löfgren's syndrome to those of patients with TB: 380 of 1329 epitopes not recognized in any of the tested TB patient sera were found to be recognized in at least one sample from the Löfgren's syndrome group, while 557 of 1245 peptides not found to be recognized in

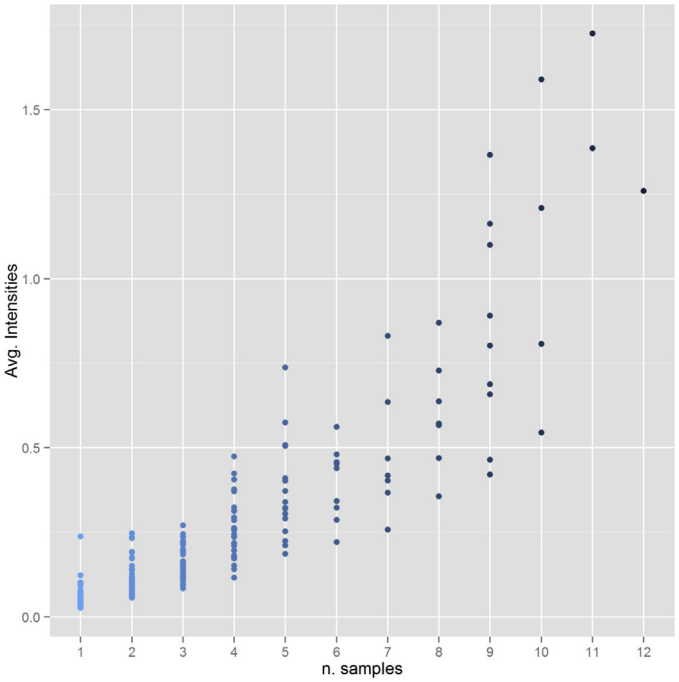

Figure 2. Exclusive recognition analysis (ERA) results plot for Löfgren's syndrome vs. healthy control samples: average intensity of the peptides never recognized in serum samples from healthy controls by number of times they were recognized in serum from patients with Löfgren's syndrome.

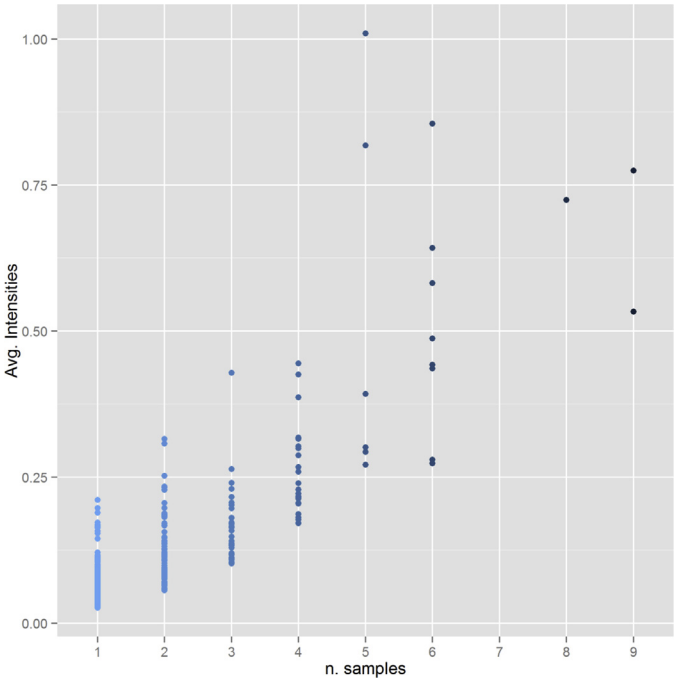

Figure 4. Exclusive recognition analysis (ERA) results plot for sarcoidosis vs. TB samples: average intensity of the peptides never recognized in serum samples from TB patients by number of times they were recognized in serum from patients with sarcoidosis.

Table 4
Significance analysis of microarrays (SAM): recognition of *Mycobacterium tuberculosis* peptides in sera of patients with sarcoidosis (SARC, $n = n = 12$) vs. patients with tuberculosis (TB, $n = 12$). The top-responding *M. tuberculosis* peptides from ERA (exclusive recognition analysis) are indicated in bold.

Ranking	Peptide	Rv number	Description	Score	Fold change
1	IFLIGIPFNAATLDA	Rv0355c	PPE family protein PPE8	3.51	3.00
2	RRWVDELTIVVGSTA	Rv0212c	Possible transcriptional regulatory protein NadR	3.41	2.86
3	LRGWLGMWSLRVAQT	Rv0272c	Alpha/beta hydrolase (uncharacterized protein)	3.39	3.02
4	**VWARREHPTYEDIVG**	Rv3881c	ESX-1 secretion-associated protein EspB (antigen MTB48)	3.28	2.49
5	**MRPVDEQWIEILRIQ**	Rv3472	Conserved protein (uncharacterized protein)	3.27	2.19
6	RGPGQMLGGLPVGQM	Rv1196	PPE family protein	3.25	3.25
7	GRTVVPVTATDIRAD	Rv0212c	Possible transcriptional regulatory protein NadR	3.20	2.60
8	**RDRLSTYFNEQVFPV**	Rv2984	Polyphosphate kinase (EC 2.7.4.1) (ATP-polyphosphate phosphotransferase)	3.06	2.57
9	APPPPVIAPNAPQPV	Rv1860	Alanine and proline-rich secreted protein Apa (45 kDa glycoprotein) MPT-32	3.02	2.14
10	LEPDTTDVERMYRRL	Rv0302	Transcriptional regulator, TetR family	3.01	3.20
11	VPWDADDGRCVPGAR	Rv0212c	Possible transcriptional regulatory protein NadR	2.97	2.30
12	SSSGAIGNSGLANAG	Rv0355c	PPE family protein PPE8	2.95	3.68
13	IDGPAPDGYPIINYE	Rv0934	Phosphate-binding protein PstS 1 (PBP 1) (PhoS1) (PstS-1) (P38) (antigen Ag78)	2.92	2.52
14	GDTVSGVFNTGIGAP	Rv0355c	PPE family protein PPE8	2.86	3.34
15	TGTPAEESGHILIHD	Rv0394c	Uncharacterized protein	2.84	2.18
16	TDNTGILNAGSYNTG	Rv0355c	PPE family protein PPE8	2.83	2.78
17	SLQGVLATRPDFVFG	Rv0212c	Possible transcriptional regulatory protein NadR (probably AsnC-family)	2.83	2.22
18	VEIPGVDTVRNQFDR	Rv3841	Ferritin BfrB (EC 1.16.3.1) (non-heme ferritin Ftn) (Nox19)	2.82	3.01
19	**REGPDGLRWGVESIC**	Rv0796	Putative transposase for insertion sequence element IS986/IS6110 (ORFB)	2.81	2.14
20	SPPAAAGDLVGPGCA	Rv2875	Immunogenic protein MPT70	2.81	2.01

sera from patients with Löfgren's syndrome were recognized in at least one sample from patients with TB. Interestingly, the top two peptides from this analysis (RDRLSTYFNEQVFPV and MRPVDEQWIEILRIQ), which compared *M. tuberculosis* peptides recognized in serum samples from Löfgren's syndrome patients to serum samples from patients with TB (Figure 5), also occupy the top two positions in the list of highly recognized peptides (Table 5).

The results from differential IgG recognition analyses in serum samples from patients with sarcoidosis or Löfgren's syndrome vs. patients with TB (Tables 4 and 5) showed that nine of the top 20 highly recognized peptides were shared between the two comparisons. The IgG reactivity profile in serum samples from patients with sarcoidosis combined with patients with Löfgren's syndrome vs TB-positive individuals identified the same peptides occurring in the topmost positions in Table 6 (positions 1 to 7, in addition to positions 11 and 13). Again, the top three peptides (LRGWLGMWSLRVAQT, RRWVDELTIVVGSTA, and RGPGQMLGGLPVGQM) identified in the corresponding ERA (Figure 6) were found among the top 20 *M. tuberculosis* peptides recognized by sarcoidosis as well as Löfgren's syndrome patients compared to patients with TB.

In a similar fashion, nine of the *M. tuberculosis* peptides were recognized by patients with sarcoidosis and patients with Löfgren's syndrome vs. healthy individuals, as reflected by the results of the analysis presented in Table 7. The top four *M. tuberculosis* peptides shown in the ERA (Figure 7) are also present

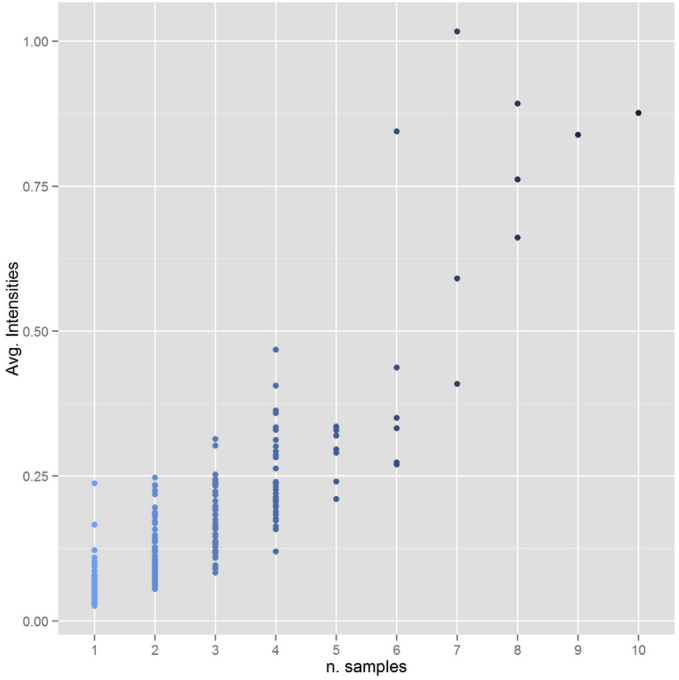

Figure 5. Exclusive recognition analysis (ERA) results plot for Löfgren's syndrome vs. TB samples: average intensity of the peptides never recognized in serum from patients with TB by number of times they were recognized in serum from patients with Löfgren's syndrome.

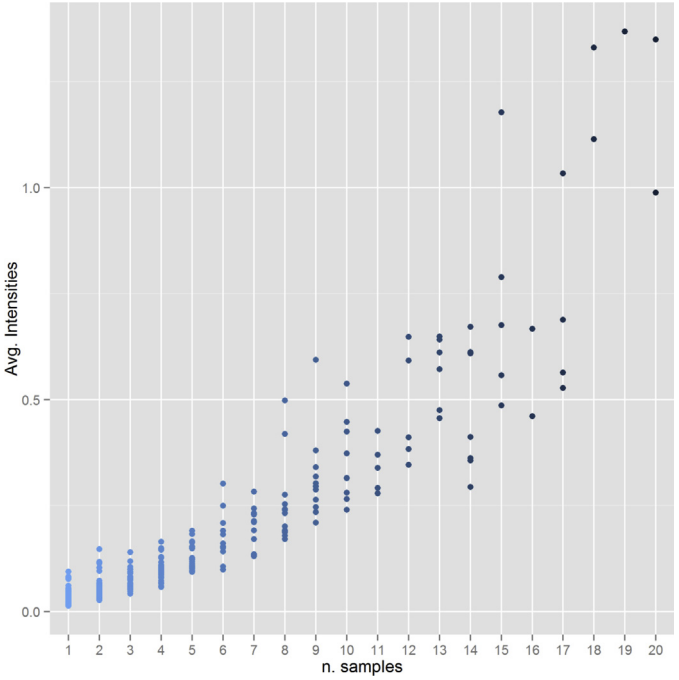

Figure 6. Exclusive recognition analysis (ERA) results plot for Löfgren's syndrome + sarcoidosis vs. TB patient samples: average intensity of the peptides never recognized in serum samples from patients with TB by number of times they were recognized in serum from patients with Löfgren's syndrome or sarcoidosis.

Table 5
Significance analysis of microarrays (SAM): recognition of *Mycobacterium tuberculosis* peptides in sera of patients with Löfgren's syndrome (LOF, $n = 12$) vs. patients with tuberculosis (TB, $n = 12$). The top-responding *M. tuberculosis* peptides from ERA (exclusive recognition analysis) are indicated in bold.

Ranking	Peptide	Rv number	Description	Score	Fold change
1	**RDRLSTYFNEQVFPV**	Rv2984	Polyphosphate kinase (EC 2.7.4.1)	4.45	3.04
2	**MRPVDEQWIEILRIQ**	Rv3472	Conserved protein (Uncharacterized protein)	4.02	2.45
3	SGFFNGGPGTVSGIA	Rv0355c	PPE family protein PPE8	3.89	4.63
4	RRWVDELTIVVGSTA	Rv0212c	Possible transcriptional regulatory protein NadR	3.78	3.11
5	**LRGWLGMWSLRVAQT**	Rv0272c	Alpha/beta hydrolase (uncharacterized protein)	3.74	3.39
6	RGPGQMLGGLPVGQM	Rv1196	Nucleoprotein	3.71	4.11
7	GDTVSGVFNTGIGAP	Rv0355c	PPE family protein PPE8	3.68	4.69
8	GRAGGGAALGGGGMG	Rv3881c	ESX-1 secretion-associated protein EspB (antigen MTB48)	3.50	2.66
9	VEIPGVDTVRNQFDR	Rv3841	Ferritin BfrB (EC 1.16.3.1) (non-heme ferritin Ftn) (Nox19)	3.29	4.52
10	**GSAATLGGFSAWQLG**	Rv0583c	Lipoprotein, MK35	3.24	2.15
11	GGRNGSGGGDLFGGF	Rv0538	Possible conserved membrane protein	3.22	2.50
12	VVVIREQPPPGNPPR	Rv0915c	Uncharacterized PPE family protein PPE14	3.14	3.11
13	VPPSWAAPSTRPVSA	Rv3136	Uncharacterized PPE family protein PPE51	3.10	2.21
14	**LDDVVEVSAGETIPS**	Rv1030	Potassium-transporting ATPase ATP-binding subunit (EC 3.6.3.12)	3.08	2.08
15	VGAHTSGWFNQSTQA	Rv3343c	Uncharacterized PPE family protein PPE54	3.07	2.86
16	GRTVVPVTATDIRAD	Rv0212c	Possible transcriptional regulatory protein NadR	3.04	2.35
17	AQDAMAMYGYAGSSA	Rv1705c	Uncharacterized PPE family protein PPE22	3.01	2.60
18	REVMRAASKVEPVPV	Rv0394c	Possible secreted protein (uncharacterized protein)	2.99	2.30
19	LEPDTTDVERMYRRL	Rv0302	Transcriptional regulator, TetR family	2.99	2.87
20	PGNVNTGVGNTGSIN	Rv0304c	PPE family protein PPE5	2.92	3.61

Table 6
Significance analysis of microarrays (SAM): recognition of *Mycobacterium tuberculosis* peptides in sera of patients with Löfgren's syndrome (LOF, $n = 12$) + patients with sarcoidosis (SARC, $n = 12$) vs. patients with tuberculosis (TB, $n = 12$). The top-responding *M. tuberculosis* peptides from ERA (exclusive recognition analysis) are indicated in bold.

Ranking	Peptide	Protein	Description	Score	Fold change
1	**LRGWLGMWSLRVAQT**	Rv0272c	Alpha/beta hydrolase (uncharacterized protein)	4.47	3.20
2	RRWVDELTIVVGSTA	Rv0212c	Possible transcriptional regulatory protein NadR	4.37	2.98
3	**RGPGQMLGGLPVGQM**	Rv1196	PPE family protein	3.90	3.65
4	**RDRLSTYFNEQVFPV**	Rv2984	Polyphosphate kinase (EC 2.7.4.1) (ATP-polyphosphate phosphotransferase)	3.88	2.79
5	**MRPVDEQWIEILRIQ**	Rv3472	Conserved protein (uncharacterized protein)	3.86	2.32
6	GRTVVPVTATDIRAD	Rv0212c	Possible transcriptional regulatory protein NadR	3.70	2.47
7	LEPDTTDVERMYRRL	Rv0302	Transcriptional regulator, TetR family	3.69	3.03
8	REVMRAASKVEPVPV	Rv0394c	Possible secreted protein (uncharacterized protein)	3.52	2.29
9	TGGWEERLSVSLRAV	Rv0212c	Possible transcriptional regulatory protein NadR	3.50	2.54
10	GRAGGGAALGGGGMG	Rv3881c	ESX-1 secretion-associated protein EspB (antigen MTB48)	3.49	2.46
11	GDTVSGVFNTGIGAP	Rv0355c	PPE family protein PPE8	3.47	3.96
12	SGFFNGGPGTVSGIA	Rv0355c	PPE family protein PPE8	3.40	3.49
13	VEIPGVDTVRNQFDR	Rv3841	Ferritin BfrB (EC 1.16.3.1) (non-heme ferritin Ftn) (Nox19)	3.31	3.69
14	RNHTITDWAESELKR	Rv0272c	Alpha/beta hydrolase (uncharacterized protein)	3.30	2.18
15	VQRARDSVDDIRVAR	Rv0379	Calcium dodecin	3.22	2.52
16	GGRNGSGGGDLFGGF	Rv0538	Possible conserved membrane protein	3.22	2.23
17	SLQGVLATRPDFVFG	Rv0212c	Possible transcriptional regulatory protein NadR	3.19	2.14
18	ASAAATQLTPFTEPV	Rv1807	PPE family protein	3.16	2.03
19	**GSAATLGGFSAWQLG**	Rv0583c	Lipoprotein, MK35	3.14	1.98
20	AAAGGHPCQGLYHHS	Rv0272c	Alpha/beta hydrolase (uncharacterized protein)	3.03	2.68

Table 7
Significance analysis of microarrays (SAM): recognition of *Mycobacterium tuberculosis* peptides in sera of patients with Löfgren's syndrome (LOF, $n = 12$) + patients with sarcoidosis (SARC, $n = 12$) vs. healthy controls (HTC, $n = 12$). The top-responding *M. tuberculosis* peptides from ERA (exclusive recognition analysis) are indicated in bold.

Ranking	Peptide	Protein	Description	Score	Fold change
1	ARWGSLYDALYGTDV	Rv1837c	Malate synthase G (EC 2.3.3.9)	6.49	7.26
2	AGEHEAAAAGYVCAL	Rv3021c	Uncharacterized PPE family protein PPE47/PPE48	5.90	4.19
3	TPAAGAAPSAGAAPA	Rv0286	Uncharacterized PPE family protein PPE4	5.82	2.83
4	LTEIGYLLPEPDDFT	Rv1837c	Malate synthase G (EC 2.3.3.9)	5.55	3.12
5	**SELTRFTPEAVVEKY**	Rv1629	DNA polymerase I (POL I)	5.36	4.19
6	TFLAYLVLDPLIYFG	Rv2123	Uncharacterized PPE family protein PPE37	5.34	2.67
7	EDYTDAAQPIWWVVR	Rv0280	Uncharacterized PPE family protein PPE3	5.22	2.59
8	**ADMWGPSSDPAWERN**	Rv1886c	Diacylglycerol acyltransferase/mycolyltransferase Ag85B (DGAT)	5.19	4.05
9	GSSAMILAAYHPQQF	Rv1886c	Diacylglycerol acyltransferase/mycolyltransferase Ag85B (DGAT)	5.19	3.07
10	AQSAAIAHATGASAG	Rv1807	PPE family protein	5.09	4.64
11	MFSGFDPWLPSLGNP	Rv0256c	Uncharacterized PPE family protein PPE2	5.08	3.17
12	AWWQDTVNGHTRIGL	Rv1242	Ribonuclease VapC33 (RNase VapC33)	4.96	2.62
13	MWAGYRWAMSVELTQ	Rv3369	F420-dependent oxidoreductase	4.95	4.02
14	AAAEGLSHEVGSGRL	Rv2462c	Trigger factor (TF) (EC 5.2.1.8) (PPIase)	4.87	2.76
15	NTGSFNAGNYNTGYF	Rv0305c	PPE family protein PPE6 (uncharacterized protein)	4.75	5.05
16	KDSDSPDKLRRVVAH	Rv1802	Uncharacterized PPE family protein PPE30	4.74	2.45
17	SAPVGGLDSGNPNPG	Rv0442c	Uncharacterized PPE family protein PPE10	4.74	2.91
18	AALVGALSVPHSWTT	Rv0915c	Uncharacterized PPE family protein PPE14	4.72	2.30
19	ACNMNHALITGVADA	Rv2618	Uncharacterized protein	4.63	2.62
20	**EKYGLTPRQYPDFAA**	Rv1629	DNA polymerase I (POL I) (EC 2.7.7.7)	4.60	3.88

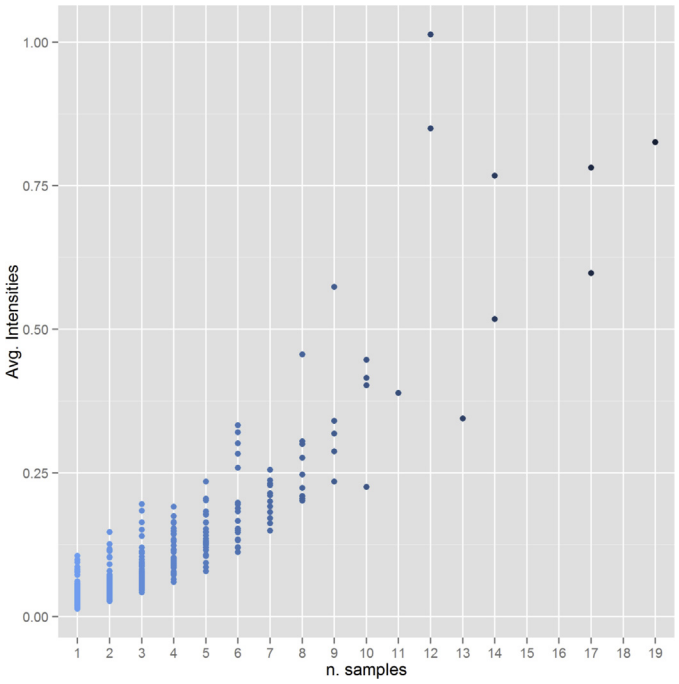

Figure 7. Exclusive recognition analysis (ERA) results plot for Löfgren's syndrome + sarcoidosis vs. healthy control samples: average intensity of the peptides never recognized in serum samples from healthy controls by number of times they were recognized in serum samples from patients with Löfgren's syndrome or sarcoidosis.

in Table 7 (see also Table 1 (sarcoidosis patients vs. healthy individuals) and Table 2 (Löfgren's syndrome patients vs. healthy individuals)).

Discussion

Immune response signatures among patients with sarcoidosis and patients with TB have been evaluated previously in the context of gene expression analysis and immunological mediators in samples from peripheral blood.[23] A differential gene expression pattern was seen between the two patient groups, despite much overlap (>90%). Pertinent to immunological mediators, proinflammatory cytokines such as vascular endothelial growth factor (VEGF), interferon gamma (IFN-γ), and interleukin (IL)-12 have been shown to be up-regulated in TB patients but not in sarcoidosis patients. The presence of T-cell 'centric' cytokines ties in with previous reports of the recognition of *M. tuberculosis*-derived early-secreted antigenic target 6 kDa (ESAT-6) and catalase-peroxidase (KatG) peptides in patients with Löfgren's syndrome recognized by T-cells isolated from peripheral blood or from BALF.[7,8] The present study is the first to explore and characterize *M. tuberculosis* epitope-specific humoral immune responses among patients with sarcoidosis, patients with Löfgren's syndrome, and patients with TB in relation to healthy controls.

The findings presented in this study demonstrate that specific antibody responses against *M. tuberculosis* antigens are present in serum from patients with sarcoidosis, regardless of HLA background and clinical disease. This lends support to the theory that exposure to mycobacterial antigens is involved in the pathogenesis of sarcoid inflammation and pathogenesis.[1,10] Furthermore, the IgG response observed in this study was found to be qualitatively different between patients with sarcoidosis and pulmonary TB, suggesting that individual genetic factors may influence the immunological course of the encounter with mycobacteria, and eventually determine the clinical outcome. This may rely on the individual's ability to control mycobacterial growth and to purge the remaining bacterial antigens, or conversely lead to progression to clinical disease due to 'immune incompetence'.[1,2,5–8,10,12,24]

It was found that peptides from the PE/PPE/PE_PGRS (proline–glutamic acid/proline–proline–glutamic acid/proline–glutamic acid_polymorphic guanine–cytosine-rich sequence) family in mycobacteria were well recognized by serum from patients with sarcoidosis, with Löfgren's syndrome, and with TB, suggesting a potential role of these epitopes in clinical disease manifestation. The PE/PPE/PE_PGRS proteins account for approximately 10% of the *M. tuberculosis* genome,[25] and have been studied in the context of cellular and humoral immune responses among patients with active pulmonary TB, as well as activation of human innate immune cells in vitro.[26–29] The results from these studies have attributed the PE/PPE/PE_PGRS proteins with the ability to induce strong proinflammatory immune responses (IFN-γ, tumour necrosis factor alpha (TNF-α) production; apoptotic pathway activation). Their presence in tissue is thus likely to amplify the local immune effector milieu, partly explaining the intense granulomatous response in sarcoidosis. In contrast, PPE18 is strongly recognized in serum samples from patients with sarcoidosis and Löfgren's syndrome as compared to TB patients. This protein has been implicated in the disruption of T-cell proliferation in response to purified protein derivative (PPD), as well as the potential skewing of immune responses to the Th2 phenotype in an IL-10-dependent manner,[30] which induces antibody production.

Of note, serum IgG from patients with sarcoidosis or Löfgren's syndrome, as compared to serum from healthy controls, recognized the secreted proteins antigen 85 B (Ag85B) and EspB. Ag85 B is an important enzyme involved in mycobacterial cell wall maintenance (mycolic acid transfer),[31] as well as an integral component of several TB vaccine candidates in clinical trials, due to its immunogenic potential in TB patients as well as protective efficacy in vaccination models.[31,32] Intriguingly, serum IgG from TB patients included in the study did not recognize Ag85 B epitopes. This is plausible, since Ag85 B has been more closely associated with potentially protective CD4 and CD8 T-cell responses in TB patients as well as preclinical models of vaccine efficacy studies.[33–37] Antibody responses to Ag85B, on the other hand, appear to be the result of a severe TB pathology in humans, and thus represent a marker of disease progression.[38,39] EspB is secreted by *M. tuberculosis* using the same injection machinery (esx1) as ESAT-6,[40] as well as several PE/PPE proteins that were not observed to be recognized in the present study.[25]

The antibody responses to the *M. tuberculosis* antigens discussed in this study have not been described before for patients with sarcoidosis or Löfgren's syndrome, which also largely applies to TB patients. Taken together, the findings presented in this report shed new light on an array of mycobacterial epitopes, i.e., antigens that may have significance in the pathogenesis of sarcoidosis and associated systemic inflammatory diseases. This, therefore, opens up new possibilities for improving our understanding of the immunology that sustains granulomatous inflammation and may also assist in the development of non-invasive diagnostics.

In conclusion, in this study it was possible to qualitatively and quantitatively gauge *M. tuberculosis* antigen-specific IgG responses in patients with sarcoidosis and Löfgren's syndrome for the first time. This study also sheds light on individual defined epitopes that are differentially recognized between the patient groups and highlights the major targets that could be useful in diagnosis as well as the development of immunotherapies for sarcoidosis and associated systemic inflammatory diseases.

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Conflict of interest

None of the authors has a conflict of interest to declare.

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