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T Cell Responses to Mycobacterial Catalase-Peroxidase Profile a Pathogenic Antigen in Systemic Sarcoidosis

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Sarcoidosis is a systemic granulomatous disease associated with local epithelioid granulomas, CD4\(^+\) T cells, and Th1 cytokines. The tissue Ags that drive this granulomatous inflammation are uncertain. In this study, we used IFN-\(\gamma\)-ELISPOT assays and flow cytometry to assess lung and blood T cell responses to the candidate pathogenic Ag, Mycobacterium tuberculosis catalase-peroxidase (mKatG) in patients with sarcoidosis from two centers. Despite differences in patient phenotypic, genetic, and prognostic characteristics, we report that T cell responses to mKatG were remarkably similar in these cohorts, with higher frequencies of mKatG-reactive, IFN-\(\gamma\)-expressing T cells in the blood of sarcoidosis patients compared with nontuberculosis sensitized healthy controls, and (in a subset) in greater numbers than T cells reactive to purified protein derivative. In sarcoidosis, mKatG-reactive CD4\(^+\) Th1 cells preferentially accumulated in the lung, indicating a compartmentalized response. Patients with or without Löffgren syndrome had similar frequencies of mKatG-specific IFN-\(\gamma\)-expressing blood T cells. Circulating mKatG-reactive T cells were found in chronic active sarcoidosis but not in patients with inactive disease. Together, these results demonstrate that T cell responses to mKatG in sarcoidosis fit a profile expected for a pathogenic Ag, supporting an immunotherapeutic approach to this disease. The Journal of Immunology, 2008, 181: 8784–8796.

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antigenic specificities of the biased, oligoclonal TCR specific populations in sarcoidosis have been unsuccessful to date.

Recently, using a novel proteomic approach to identify potential pathogenic tissue Ags, we identified a specific mycobacterial protein, the catalase-peroxidase protein (mKatG), in 55% of sarcoidosis tissues and that was the target of circulating IgG in 48% of sarcoidosis patients (22). This approach capitalized on the assumption that a pathogenic "sarcoidosis" Ag would be a poorly soluble component of the sarcoidosis granulomas but otherwise required no a priori hypothesis regarding whether disease relevant Ags would be derived from foreign or endogenous proteins. The identification of mKatG protein as a candidate pathogenic Ag followed multiple studies that reported the presence of mycobacterial DNA in 0–80% of sarcoidosis tissues using PCR technologies (23). The conclusion that mycobacterial organisms are etiologically linked to sarcoidosis has remained controversial despite these studies due to the marked variability in results from different centers and the lack of corroborating evidence for specific mycobacterial Ags involved in driving the granulomatous inflammation (24). Recently, several reports have documented T cell responses to select mycobacterial proteins or peptides including a single mKatG peptide in peripheral blood of subsets of patients with sarcoidosis (25–27). Whether these mycobacterial peptides are relevant to the pathogenesis of sarcoidosis remains uncertain given the lack of evidence that the respective proteins or peptides are present in sarcoidosis tissues and whether T cell responses to these proteins are regulated in a manner expected for pathogenic Ags.

In this study, we set out to test the hypothesis that mKatG is a specific pathogenic Ag in sarcoidosis by assessing the T cell responses to this Ag in two cohorts of sarcoidosis patients (from the U.S. and Sweden) characterized by markedly different phenotypic, genetic, and prognostic profiles. Despite these differences, our studies reveal that T cell responses to mKatG are remarkably similar in both cohorts, with mKatG reactive, IFN-γ-expressing T cells circulating in subsets of patients in higher proportions than in non-Mycobacterium tuberculosis sensitized control subjects, in higher frequency than T cells reactive to PPD, with the preferential accumulation of mKatG-reactive CD4+/TH1 cells in lung, and with persistent mKatG specific T cell responses in patients with active disease but not in those patients with inactive disease. Together, these findings demonstrate that T cell responses to mKatG are regulated in a manner expected for a pathogenic Ag in sarcoidosis, providing a scientific foundation to consider Ag-specific immunotherapeutic approaches to this disease.

Materials and Methods

Study population

Clinical samples were obtained from patients with sarcoidosis recruited from specialized clinics or hospitals of the Johns Hopkins University in the U.S. or the Karolinska Institutet in Sweden. A diagnosis of sarcoidosis was established either by tissue biopsy or by initial manifestations consistent with Löfgren syndrome (erythema nodosum and/or acute arthritis, hilar adenopathy) without alternative diagnoses according to world-wide consensus criteria (1). Demographic characteristics of these patient groups are summarized in Table I. Based on clinical manifestations, chest radiograph, and pulmonary function tests, patients were classified as having active sarcoidosis or “inactive” disease, defined by resolution of disease manifestations or absence of disease progression off all therapy for at least 1 year (28). Untreated patients were those who had not received systemic therapy within 3 mo of the time of study. Control subjects included healthy individuals with documented skin testing to purified protein derivative (PPD) within the past year or with a self-reported prior history of vaccination with Bacillus Calmette-Guérin (BCG). PPD skin testing was performed in accordance with accepted criteria used in the respective countries. In the U.S., subjects received an intradermal injection of five tuberculin units of PPD (Tubersol, Aventis Pasteur); induration of ≥10 mm at 48–72 h was considered a positive skin test response (29). In Sweden, subjects received an intradermal injection of two tuberculin units of PPD; induration ≥6 mm at 72 h was considered a positive test. All study subjects participated voluntarily and provided informed consent under protocols approved by the local institutional review board. HLA class II (HLA-DR) genotyping of Swedish patients was determined by PCR using sequence-specific primers as previously described (30).

Reagents

Recombinant Mtb KatG protein was isolated and prepared using an Escherichia coli UM255 strain overexpression system carrying a plasmid construct pYZ256 containing the wild-type M. tuberculosis katG gene in a 2.9 kD EcoRV-KpnI fragment in pUC19 vector (31). The culture was grown in LB medium containing 100 µg/ml ampicillin and agitation overnight at 37°C. The cells were harvested by centrifugation at 4000 g for 30 min. The pellet was resuspended in 100 ml of 10 mM phosphate buffer (Na2HPO4 and NaH2PO4 and 0.5 mM EDTA) (pH 6.0) and sonicated with a Branson probe for 30 min. The supernatant was harvested by centrifugation at 12000 g for 30 min. The supernatant was harvested for further purification by ammonium sulfate precipitation, and the protein was harvested by centrifugation at 12000 × g for 30 min. The pellet was resuspended in the phosphate buffer and dialyzed against the same buffer at 4°C overnight and then assayed for peroxidase and catalase activity. The active fractions were further purified by gel filtration chromatography. A Superdex 200 gel filtration column (Pharmacia) was equilibrated with the phosphate buffer overnight. The catalase containing fractions were loaded onto the column with a flow rate of 0.2 ml/min. Fractions (1 ml) were collected and assayed for peroxidase and catalase activity. Active fractions were assessed for purity by SDS-PAGE, pooled, and then dialyzed against the above phosphate buffer at 4°C overnight (32). The purified KatG protein was at least 95% pure. The protein was kept at −80°C for long term storage and −20°C for short term (<2 mo) between immunological studies.

Human cell preparations

PBMC were prepared according to established protocols used by each institution (33, 34). In the U.S., PBMCs isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation were resuspended in complete medium (RPMI 1640, 10% pooled human AB serum (Sigma-Aldrich), 1% penicillin-streptomycin (Biosource)). In Sweden, Ficoll-isolated PBMCs were resuspended in complete tissue culture medium (CTCM) (RPMI 1640 with HEPES, 10% FCS, 1% penicillin-streptomycin, and 1% l-glutamine), (PPD) within the past year or with a self-reported prior history of vaccination with Bacillus Calmette-Guerin (BCG). PPD skin testing was performed in accordance with accepted criteria used in the respective countries. In the U.S., subjects received an intradermal injection of five tuberculin units of PPD (Tubersol, Aventis Pasteur); induration of ≥10 mm at 48–72 h was considered a positive skin test response (29). In Sweden, subjects received an intradermal injection of two tuberculin units of PPD; induration ≥6 mm at 72 h was considered a positive test. All study subjects participated voluntarily and provided informed consent under protocols approved by the local institutional review board. HLA class II (HLA-DR) genotyping of Swedish patients was determined by PCR using sequence-specific primers as previously described (30).

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Lung mononuclear cells obtained from bronchoalveolar lavage (BAL) were prepared according to established protocols used by each institution as approved by their respective institutional review board, with differences as noted (8, 35). Flexible bronchoscopy was performed using topically applied lidocaine after premedication with i.v. fentanyl and midazolam (U.S.) or morphine-hyoscine (Morfim-skopolamin, Meda) i.m. (Sweden). BAL was initiated by wedging the bronchoscope in one of the subsegments of the right middle lobe or lingula, and lavage performed with five 30 ml U.S.) or five 50 ml aliquots (Sweden). Excess BAL fluid not needed for clinical purposes was immediately transported to the laboratory. BAL cells were washed with PBS, resuspended in complete medium and counted by hemocytometer. Total cell viability was determined by Trypan blue exclusion. Differential counts were established from cytospin preparations (Shandon) using a modified Giemsa stain, with a minimum of 500 cells counted per sample.

ELISPOT assay
Ag-specific stimulation of T cells was assessed by an ELISPOT assay specific for human IFN-γ with technical differences as noted between the two sites. In the U.S., assays were performed using reagents and methods as recommended by the manufacturer (Cell Sciences). In brief, 96-well polyvinylidene fluoride-bottomed-well plates (Millipore) were coated with capture Ab overnight at 4°C and then blocked with 1% BSA in PBS for 2 h at room temperature. Equal numbers of PBMC (5 × 10^5 cells/well) or BAL cells (1 × 10^6 cells/well) were loaded into duplicate wells. Cells were incubated at 37°C in a humidified CO2 incubator for 18–22 h in either complete medium alone, mKatG (10 μg/ml), PPD (10 μg/ml), or positive control PHA (10 μg/ml). The number of spot forming cells (sfc) was detected using BCIP/NBT substrate and enumerated using an AID ELISPOT Reader system with AID ELISPOT Software 2.1 (AID Diagnostika).

At both sites, results were calculated as a mean value from replicate wells, and normalized to sfc per 10^6 cells. Ag-specific responses (PPD or mKatG) were calculated by subtracting sfc of medium control wells from the specific Ag result. For each subject, a “positive” ELISPOT response in the blood required that the number of Ag specific sfc was greater than or equal to twice the background (medium) sfc and that the difference of Ag specific sfc minus background sfc was greater than the upper 95th percentile of Ag specific responses from all PPD- subjects from the respective sites.

Flow cytometry
Intracellular cytokine staining for IFN-γ expression was performed to characterize Ag-specific lymphocyte responses. In brief, PBMC or BAL cells were incubated in polystyrene tubes for 6 h at 37°C in complete medium with or without Ag (mKatG, 20 μg/ml) or staphylococcal enterotoxin B (SEB, 1 μg/ml) as positive control (Toxin Technology). Brefeldin A (10 μg/ml) (Sigma-Aldrich) was added for the final 4 h of culture as described (30). Cells were harvested on day 6, stained with lymphocyte surface markers (CD3, CD4, CD8; see above), and cell fluorescence intensity was immediately measured within T cell subsets using a FACSCalibur cytometer. Ag-specific proliferation was indicated by decreased CFSE fluorescence intensity. Percentages of T cell proliferation were calculated from gates demarcating CFSE** (undivided parent population) and CFSE+ (divided progeny population) using positive (SEB) and negative (medium) controls. Results were normalized against spontaneous proliferation in medium alone (Sweden), as percent proliferation to mKatG minus percent proliferation to medium alone.

BAL cells from sarcoidosis patients from Sweden were stained with TCR-AV2S3-specific Ab clone F1 (Pierce Biotechnology) as previously described (30).

Statistics
Statistical analyses were performed using either JMP software version 5.0.1 (SAS Institute) or GraphPad Prism 5 (GraphPad Software). Group comparisons of unpaired quantitative data were performed using the Kruskal-Wallis test followed by individual comparisons using the Mann-Whitney U test. Paired analyses were performed by the paired Mann-Whitney U test. Results of nonparametric correlation analysis are expressed using the Spearman coefficient (rs) with p values determined by a 2-tailed test. Categorical data were analyzed using the 2-tailed Fisher’s exact test. A p value of <0.05 was considered significant.

Results
Circulating T cells reactive to mKatG are found in sarcoidosis patients from the U.S. and Sweden
To determine the profile of Ag-specific T cell responses in sarcoidosis patients to the candidate pathogenic Ag mKatG, T cell reactivity to recombinant mKatG was determined by ELISPOT using PBMC isolated from untreated patients with active sarcoidosis, healthy volunteers with a negative PPD skin test or a history of BCG vaccination (PPD–/BCG+, hereafter called PPD– subjects) or volunteers with a positive PPD skin test or a history of BCG vaccination (PPD+/BCG−, hereafter called PPD+ subjects). In the U.S. center, we found a significantly higher proportion of mKatG-reactive T cells in PBMC from patients with sarcoidosis compared with PPD– subjects (median frequency: 14 vs 2 sfc per 10^6 cells, p = 0.026) (Fig. 1A). There was no difference in the proportion of mKatG-reactive T cells in sarcoidosis compared with a group of PPD+ subjects (median freq. 14 vs 20 sfc/10^6 cells, p = 0.899). In Sweden, the median proportion of mKatG-reactive T cells from sarcoidosis patients was higher than that from PPD– subjects from this site, though this difference was not statistically different (median freq. 28 vs 33 sfc/10^6 cells, p = 0.697). Overall, pair-wise analyses demonstrated that the different subject groups (PPD–, sarcoidosis, PPD+) from the respective sites were not significantly different from each other. When subjects from both sites were combined, the difference between PPD– subjects and sarcoidosis patients was highly significant (p = 0.008) whereas there was no difference between sarcoidosis and PPD+ subjects (p = 0.545, Fig. 1B).

Given the differences in patient demographics and clinical phenotypes between the two sites (Table I), we assessed the operating characteristics of an mKatG ELISPOT test by defining a “positive” mKatG responder as a subject with a frequency of mKatG reactivity greater than two times media control and greater than the upper 95th confidence interval of the PPD– control groups from the respective sites (U.S. >13 sfc/10^6 PBMC; Sweden >30 sfc/10^6 PBMC). Using this criteria, we found that the overall proportions of positive mKatG responders in sarcoidosis patients from both the U.S. and Sweden were strikingly similar (both groups 50%, p = ns) and significantly higher than the proportion of responders in sarcoidosis patients from the U.S. and Sweden.
PPD− subjects from the U.S. or Sweden (p < 0.05, each comparison) (Fig. 1). T cell reactivity to mKatG is not correlated with T cell reactivity to PPD in sarcoidosis

The increased frequency of mKatG reactive, IFN-γ-producing T cells in the blood of sarcoidosis patients suggests prior exposure to mycobacterial organisms. To investigate whether mKatG is a dominant Ag or is accompanied by responses to other mycobacterial Ags, we analyzed the ex vivo proportions of IFN-γ-producing PBMC responsive to PPD in which mKatG is a minor component. As expected, PPD+ subjects were characterized by higher proportion of IFN-γ-producing PBMC in response to PPD compared with PPD− subjects from both the U.S. (p = 0.002) and Sweden (p = 0.019) (Fig. 2A). There were no significant differences between PPD responses in the sarcoidosis patients compared with PPD− subjects from the individual sites. In the U.S., the median T cell response to PPD was higher in the PPD+ subjects than in sarcoidosis patients (p = 0.004); in contrast, there was no difference in the T cell reactivity to PPD between sarcoidosis patients and PPD+ subjects from Sweden (p = 0.107) (Fig. 2A). Sarcoidosis patients from

**FIGURE 1.** Ex vivo T cell reactivity to mKatG in blood from patients with active untreated sarcoidosis and healthy PPD− or PPD+ subjects. PBMC were stimulated with recombinant mKatG and the number of IFN-γ-producing cells was measured by ELISPOT (see Materials and Methods). A, Dot plots represent mean number of IFN-γ-producing cells in mKatG wells minus negative control wells per 10^6 PBMC from individual subjects from the U.S. or Sweden as detailed in Materials and Methods; group medians indicated by horizontal lines. B, Dot plots of combined data from the U.S. (filled symbols) and Sweden (open symbols). C, Graph showing proportion of “positive” mKatG responders for each site, defined by samples with a frequency of mKatG-reactive T cells greater than two times media control wells and the absolute number of mKatG-reactive T cells (minus background) exceeding the upper 95% confidence interval level of mKatG responses from PPD− subjects from their respective sites (U.S. >13 sfc/10^6 PBMC; SE >30 sfc/10^6 PBMC).

**FIGURE 2.** Ex vivo T cell reactivity to PPD in blood from patients with active sarcoidosis and healthy PPD− or PPD+ subjects. PBMC were stimulated with PPD and the number of IFN-γ-producing PBMC was measured by ELISPOT. A, Dot plots represent mean number of IFN-γ-producing cells in PPD wells minus negative control wells per 10^6 PBMC from individual subjects from the U.S. or Sweden; group medians indicated by horizontal lines. B–D, Paired analysis of mKatG (filled symbols) and PPD (open symbols) responses for individual subjects. Data are grouped for patients with sarcoidosis and positive mKatG responses as defined in Fig. 1 and Materials and Methods (B), sarcoidosis patients with negative mKatG responses (C), or PPD+ subjects (D).
Sweden were characterized by higher T cell reactivity to PPD than in patients from the U.S. \((p = 0.0005)\).

When paired mKatG and PPD responses in both patient cohorts were examined, a dichotomy between T cell reactivity to mKatG and PPD was seen in sarcoidosis patients (Fig. 2, B and C). Within the group of sarcoidosis patients with a “positive” mKatG ELISPOT (as defined in Fig. 1, Materials and Methods), 14/21 (67%) patients had higher proportions of circulating T cells reactive to mKatG than PPD suggesting that mKatG is a dominant mycobacterial Ag in this subset of patients (Fig. 2B). The remaining sarcoidosis patients in this group (7/21) had higher T cell reactivity to PPD than mKatG, though they also had positive mKatG responses. Within the group of sarcoidosis patients with a “negative” mKatG ELISPOT, 16/21 (76%) patients had higher proportions of circulating T cells reactive to PPD than mKatG suggesting that mycobacterial Ags other than mKatG elicit dominant T cell responses in this subgroup of patients (Fig. 2C). These differential responses to mKatG and PPD resulted in an overall lack of correlation between PPD responses and mKatG responses in the positive mKatG responder subgroup or when all sarcoidosis patients were grouped together (Spearman coefficient \(r_s = 0.190, p = 0.408\)). Overall, 34/42 (85%) of sarcoidosis patients had T cell responses to either mKatG or PPD, suggesting an association with mycobacterial exposure in a majority of sarcoidosis patients. PPD\(^+\) subjects were uniformly (13/13) characterized by greater T cell reactivity to PPD than mKatG \((p = 0.0002)\), suggesting that mKatG is a minor recall Th1 Ag in these individuals (Fig. 2D).

**Compartmentalized enhanced mKatG responses in the lung of patients with pulmonary sarcoidosis**

To determine whether mKatG-reactive T cells localize to areas of granulomatous inflammation, we examined lung cells recovered from bronchoalveolar lavage fluid (BAL) in patients with sarcoidosis, over 90% of whom have pulmonary involvement. Overall, we found significantly increased proportions of IFN-\(\gamma\)-producing BAL cells in response to mKatG compared with PBMC from sarcoidosis patients from both the U.S. (median 60 vs 14 sfc/10\(^6\) cells, \(p = 0.002\)) and Sweden (median 270 vs 28 sfc/10\(^6\) cells, \(p = 0.013\)). The median frequency of mKatG-reactive BAL T cells in Swedish patients was greater than that in the U.S., though not statistically different \((p = 0.151)\) (Fig. 3A). When U.S. and Swedish patient groups were combined, the difference between BAL cells and PBMC responses to mKatG was highly significant (median BAL 70 vs PBMC 14 sfc/10\(^6\) cells, \(p < 0.0001\)). In 16 patients with concomitant BAL and PBMC analysis, a paired analysis confirmed the greater frequency of mKatG-reactive T cells in the lung compared with blood \((p = 0.0009)\), consistent with compartmentalization of mKatG-reactive T cells to the lung in sarcoidosis (Fig. 3B). In contrast to the BAL T cell reactivity to mKatG in sarcoidosis, there was no evidence for significant numbers of IFN-\(\gamma\)-producing mKatG-reactive BAL cells above media control wells from three nonsarcoidosis subjects undergoing diagnostic bronchoscopy who had at least 10% lymphocytes in their BAL fluid (mean \(\pm\) SE: 12 \(\pm\) 6 sfc/10\(^6\) cells). The median frequency of T cell reactivity to PPD was also higher in BAL cells compared with PBMC among sarcoidosis patients from both sites, with a higher frequency of
PPD-reactive BAL cells from sarcoidosis patients from Sweden compared with the U.S. (255 vs 50 sfc/10^6 cells, \(p = 0.009\)) (Fig. 3C). However, unlike the response to mKatG, T cell responses to PPD did not show a significant difference between the lung and blood (\(p = 0.168\)), suggesting T cells reactive to mycobacterial Ags other than mKatG have a reduced potential to localize in the lung compared with mKatG (Fig. 3D).

Both CD4^+ and CD8^+ T cells from lung and blood express IFN-\(\gamma\) in response to mKatG

To assess the relative contribution of CD4^+ vs CD8^+ T cells in the IFN-\(\gamma\)-expressing mKatG specific responses enumerated by ELISPOT assay, PBMC and BAL cells from sarcoidosis patients and healthy control subjects (U.S. only) were stained for intracellular IFN-\(\gamma\) expression after 6 h incubation with mKatG (Fig. 4, A–D). PBMC from PPD^- subject did not show a significant difference between the lung and blood (\(p = 0.168\)), suggesting T cells reactive to mycobacterial Ags other than mKatG have a reduced potential to localize in the lung compared with mKatG (Fig. 3D).

\begin{figure}
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\caption{IFN-\(\gamma\)-expressing mKatG-reactive lung and blood T cells derive from both CD4 and CD8 subsets in sarcoidosis. PBMC or BAL cells were incubated for 6 h with medium alone, mKatG, PPD, or SEB, and then intracellularly stained with anti-IFN-\(\gamma\) mAb or isotype control (see Materials and Methods). Dot plots represent percentage of cells expressing IFN-\(\gamma\) within electronically gated populations of CD3^+CD4^+ and CD3^+CD8^+ T cells. A, Gating strategy and representative examples of IFN-\(\gamma\) expression in PBMC from a healthy PPD^- subject, healthy PPD^+ subject, and two patients with sarcoidosis. B, Gating strategy and representative examples of IFN-\(\gamma\) expression in stimulated BAL cells from a control subject with hypersensitivity pneumonitis and two sarcoidosis patients. C, Graph represents percentage of IFN-\(\gamma\)^+ T cells in response to mKatG minus media control for PBMC or BAL cells within CD3^+CD4^+ and CD3^+CD8^+ subsets. D, Graph represents relative proportion of CD4^+ or CD8^+ T cells expressing IFN-\(\gamma\) in response to mKatG minus media control calculated as a proportion of total CD3^+ T cells reactive to mKatG. Horizontal lines depict median percentage of IFN-\(\gamma\)^+ T cells reactive to mKatG.
\end{figure}
blood T cells) \((p = 0.002)\) after accounting for the relative CD4\(^+\) lymphopenia typical in sarcoidosis patients (Fig. 4D). In BAL from sarcoidosis patients, both CD4\(^+\) and CD8\(^-\) T cells reactive to mKatG were detected, with 40% (4 of 10) of patients demonstrating over 0.5% of their total CD3\(^+\) T cell repertoire reactive to this Ag (Fig. 4, B and C). We found greater proportions of mKatG-reactive CD4\(^+\) T cells in the lung compared with blood \((p = 0.05)\), indicating compartmentalization of mKatG-reactive Th1
cells to the lung (Fig. 4C). This compartmentalization did not extend to the CD8+ T-cell subset as there was no difference in the proportions of CD8+ T cells reactive to mKatG in the lung compared with blood \( (p = 0.655) \). T-cell populations recovered by BAL in sarcoidosis are typified by greater numbers of CD4+ than CD8+ T cells \( (1, 6) \). When accounting for the increased CD4+ compared with CD8+ T cells in the lungs of our sarcoidosis patients, the proportion of total CD3+ T-cells reactive to mKatG was dominated in all cases by CD4+ Th1 cells (Fig. 4D).

**Ag specific lung and blood T-cell proliferation to mKatG is dominant in the CD4 T-cell subset**

To assess the proliferative potential of mKatG-reactive T cells in sarcoidosis, PBMC and BAL T cells were analyzed by flow cytometry for proliferation by monitoring CFSE dilution after 6 days (Fig. 5, A and B). To assess background levels of mKatG proliferation in a control population, eight PPD+ subjects from the U.S. were analyzed. These PPD+ subjects had low or undetectable levels of CD3+ T-cell proliferation to mKatG compared with control conditions \( (\text{mean } 0.13 \pm 0.09\% \text{, median } 0.015\%, \text{maximum } 0.72\% \text{ proliferation calculated by subtracting media control from mKatG proliferation}) \). Using this upper range \( (\geq 0.72\%) \) as a threshold for determining a significant proliferative response to mKatG, we found that 21/44 \((48\%)\) sarcoidosis patients from the U.S. had significant CD3+ blood T-cell proliferation to mKatG \( (\text{median } 0.615\% \text{ proliferation}) \). Proliferation to mKatG was greatest in the CD3+CD4+ compared with CD3+CD8+ T-cell subset \( (\text{median } 0.895\% \text{ vs } 0.010\%, p < 0.0001) \) (Fig. 5C).

The proliferative responses of Ag-stimulated BAL T cells in sarcoidosis are in general diminished compared with the proliferative responses in blood, likely reflective of a more differentiated memory phenotype for lung T cells \( (39, 40) \). In this context, we next determined whether mKatG induces proliferation of BAL T cells in patients with active pulmonary sarcoidosis. In 5 of 14 \((36\%)\) patients from the U.S. undergoing diagnostic bronchoscopy, we detected significant proliferation of CD3+ BAL T cells to mKatG \( (\geq 0.72\%) \) (Fig. 5D). In contrast to circulating blood T cells, there was no difference in the proportion of CD3+CD4+ BAL T cells that were proliferating to mKatG compared with CD3+CD8+ BAL T cells in sarcoidosis patients from the U.S. \( (p = 0.448, p = 0.018) \) or PPD \( (rs = 0.413, p = 0.028) \).

**Higher proportions of mKatG-reactive BAL cells are present in DRB1*0301+ Swedish sarcoidosis patients and correlate with numbers of AV2S3 BAL T cells**

In Sweden, DRB1*0301+ patients are characterized by expansions of TCR-specific AV2S3+ T cells in the lung and a higher likelihood of undergoing disease remission than DRB1*0301- patients \( (41) \). DRB1*0301+ sarcoidosis patients from Sweden were found to have higher frequencies of BAL cells producing IFN-γ in response to mKatG than DRB1*0301- patients \( (\text{median } 315 \text{ vs } 55 \text{ sfc/10}^6 \text{ PBMC}, p = 0.041) \) (Fig. 6A). In contrast, there was no significant difference noted between these groups in the proportions of mKatG-reactive PBMC in their blood \( (\text{median } 40 \text{ vs } 27 \text{ sfc/10}^6 \text{ PBMC}, p = 0.311) \) (Fig. 6A). Responses to mKatG were significantly higher in BAL cells than PBMC in DRB1*0301+ patients, consistent with compartmentalization of mKatG responses to the lung in these patients \( (\text{Fig. 6A}) \). In contrast to mKatG, there was no significant difference in the frequencies of T cells reactive to PPD within either BAL or PBMC in DRB1*0301+ compared with DRB1*0301- patients \( (\text{Fig. 6B}) \). We found a significant correlation between the numbers of AV2S3+ BAL T cells and BAL T cells reactive to mKatG \( (rs = 0.448, p = 0.018) \) or PPD \( (rs = 0.413, p = 0.028) \).
T cell reactivity to mKatG decreases with treatment or disease inactivity but not disease chronicity

To analyze whether mKatG responses correlate with disease manifestations that are associated with different probabilities of remission, we grouped patients into those with or without manifestations of Löfgren syndrome at time of diagnosis (2). We found no significant difference in the median mKatG responses in either BAL (\(p = 0.236\)) or blood (\(p = 0.378\)) between these groups (Fig. 7A). Both groups manifested higher median T cell reactivity to mKatG in the lung than blood, though this was not statistically significant in the smaller Löfgren group (\(p = 0.112\)).

To examine whether treatment, disease inactivity, or chronicity influences the frequency of circulating mKatG-reactive T cells, we analyzed PBMC from sarcoidosis patients within these groups. Systemic treatment with corticosteroid or immunosuppressive therapy was associated with reduced proportions of mKatG-reactive, IFN-\(\gamma\)-producing blood T cells compared with untreated patients from the U.S. or the combined U.S. and Swedish cohort (\(p = 0.031\)) (Fig. 7B). Strikingly, all sarcoidosis patients who had an absence of manifestations of clinically active disease for at least 1 year were characterized by a lack of circulating mKatG-reactive T cells, suggesting that sarcoidosis patients who have undergone disease remission have few or no mKatG-reactive T cells in their blood (\(p = 0.027\)) (Fig. 7B). Because >90% of sarcoidosis patients who undergo disease remission do so within the first 3 years of known disease (1), we analyzed mKatG responses in U.S. patients grouped according to their years of known diagnosis. Importantly, we found no significant differences in the median mKatG responses in the blood of patients with chronic sarcoidosis (known disease >3 years) compared with those patients with <3 yrs of active disease and a higher likelihood of remission (Fig. 7C). The proportion of “positive” mKatG responders remained constant regardless of years of active disease (40–45%), including those with active disease for >10 years (Fig. 7C), consistent with the premise that ongoing mKatG-reactive, IFN-\(\gamma\)-expressing T cell responses are pathogenic in a subset of patients with chronic sarcoidosis.

Discussion

The pathobiologic processes that result in systemic granulomatous inflammation in sarcoidosis remain poorly understood. Immunologic hallmarks of sarcoidosis, featured by the accumulation of CD4\(^+\) T cells, oligoclonal TCR-\(\alpha\beta\)\(^+\) expansions and Th1 cytokine production at sites of inflammation provide prima facie evidence that the development of sarcoidosis depends on Ag specific Th1 cells driving this pathologic response (1, 5). By using a novel proteomic approach using sarcoidosis tissues and circulating immunoglobulins, we previously identified the mycobacterium-derived protein, mKatG, as a candidate pathobiologically relevant Ag in this disease (22). In this study, we present direct evidence that the T cell responses to mKatG in sarcoidosis are regulated in a manner expected for a pathogenic Ag. Using IFN-\(\gamma\)-ELISPOT assays, we found striking similarities in the magnitude and prevalence of T cell responses among sarcoidosis patients with active disease from the U.S. and Sweden despite marked differences in underlying disease prevalence, clinical manifestations, and expected clinical outcomes typified by sarcoidosis patients from these two sites. These similarities provide robust validation that mKatG-reactive, IFN-\(\gamma\)-producing effector T cells play a role in a subset of sarcoidosis patients from the U.S. and northern Europe.

Importantly, several findings suggest mKatG is a pathogenic Ag of special relevance in a subset of sarcoidosis patients. First, a subset of patients was characterized by higher proportions of IFN-\(\gamma\)-expressing T cells reactive to mKatG compared with PPD, suggesting that mKatG is a dominant Ag within the T cell repertoire of anti-mycobacterial responses in these patients. Second, CD4\(^+\) Th1 cells reactive to mKatG accumulated in the lungs of patients with pulmonary involvement, indicating the trafficking of these Ag specific T cells to a site of granulomatous inflammation. In contrast, there was no apparent selective accumulation of PPD-reactive, IFN-\(\gamma\)-expressing T cells in the lungs of sarcoidosis patients, suggesting that T cells reactive to mycobacterial Ags other than mKatG have a reduced potential to localize to sites of inflammation. Finally, the persistence of mKatG-reactive, IFN-\(\gamma\)-producing T cells in patients with chronic active disease but not in patients who had undergone remission or had inactive disease, provides additional support for the premise that mKatG-specific, IFN-\(\gamma\)-producing T cells are important effector cells that participate in driving chronic granulomatous inflammation.

The finding that a separate subset of sarcoidosis patients had strong ex vivo responses to PPD but lower or absent numbers of mKatG-reactive, IFN-\(\gamma\)-expressing T cells in the blood suggests that mKatG is not the only relevant mycobacterial Ag in sarcoidosis. PPD-reactive T cells in the blood have been previously noted in sarcoidosis patients, with overall weaker proliferative responses in sarcoidosis patients than in healthy control subjects (39, 42). T cell responses to select mycobacterial proteins or derived peptides (ESAT-6, CFP-10, Ag-85, heat shock protein-70) have been recently documented in subgroups of sarcoidosis patients, and it
seems likely that some of these Ags could contribute to the repertoire of PPD-reactive, IFN-γ-expressing T cell responses in the blood of patients from our study (25–27). The relevance of these latter mycobacterial proteins in sarcoidosis remains less certain, because they have not been demonstrated in sarcoidosis tissues, nor is it known that CD4⁺ T cells reactive to these proteins selectively accumulate at sites of inflammation in sarcoidosis or persist in chronic disease.

Our finding that >80% of sarcoidosis patients possess significant numbers of IFN-γ-expressing T cells reactive to either mKatG or PPD indicates that most sarcoidosis patients from our centers were sensitized to mycobacterial Ags before the onset of their disease. Given this high prevalence, it is important to consider possible differences in the repertoire of T cell responses to specific mycobacterial Ags that may contribute to different outcomes following exposure to mycobacterial organisms. Studies of T cell responses to selected sets of mycobacterial Ags in M. tuberculosis infected individuals have usually involved proteins that are highly expressed and secreted (e.g., ESAT-6, CFP-10, Ag-85) and that elicit strong T cell responses in most individuals with active tuberculosis (43). To our knowledge, T cell responses to mKatG have not been evaluated in active tuberculosis. Our finding that PPD⁺ subjects had circulating mKatG-reactive T cells suggests that T cell memory to mKatG occurs following M. tuberculosis infection or BCG vaccination. However, a paired analysis revealed that PPD⁺ subjects had greater numbers of T cells reactive to PPD Ags than to mKatG, suggesting that mKatG is a minor T cell Ag in these individuals. The fact that a subset of sarcoidosis patients had significantly greater T cell reactivity to mKatG with little or no reactivity to PPD Ags highlights at least one immune response difference between sarcoidosis and individuals with latent M. tuberculosis infection or those vaccinated with BCG. Conceivably, mKatG-reactive, IFN-γ-producing effector T cells in sarcoidosis may contribute to the overall greater Th1 polarization seen in sarcoidosis compared with tuberculosis (44).

The reasons why mKatG may be an immunodominant T cell Ag in sarcoidosis but not in latent M. tuberculosis infection or BCG vaccination remain unclear. One striking difference between individuals with latent M. tuberculosis infection and sarcoidosis is the lack of evidence that sarcoidosis patients are susceptible to reactivation of live mycobacteria from a dormant state, despite years of corticosteroid or immunosuppressive therapy. This clinical observation leads to the hypothesis that viable mycobacterial organisms are no longer present in sarcoidosis tissues, with only select remnant mycobacterial proteins such as mKatG remaining that could drive differential immune responses (22). Additional possibilities include differences between these groups in mKatG expression within tissue mycobacteria, differences in Ag processing of mKatG by dendritic cells or macrophages depending on their state of activation, or the presence of pre-existing cross-reactive (to other microbial derived KatG) or autoreactive mKatG reactive T cells. For example, clinical isolates of M. tuberculosis reveal extensive strain variation in the katG region that could contribute to differences in expression or in coding sequences that affect immune responses (45). Because infection-induced inflammation may break tolerance either by cross-reactivity or from exposure to sequestered autoantigens, differences in the inflammatory host response during the course of M. tuberculosis infection and sarcoidosis could also lead to differences in cross (auto)-reactivity to mycobacterial Ags or autoimmunity (46). Our data are also consistent with the premise that active sarcoidosis could be triggered by non-M. tuberculosis mycobacterial organisms. In our original study that identified M. tuberculosis KatG as a candidate pathogenic Ag, mass spectrometry and peptide fingerprinting of a poorly soluble Ag in one tissue sample matched M. smegmatis KatG, consistent with the premise that KatG proteins from non-M. tuberculosis mycobacteria may also be relevant pathogenic Ags in sarcoidosis (22).

In this study, the anti-M. tuberculosis KatG mAb used to detect mKatG in sarcoidosis tissues by protein immunoblotting was demonstrated to cross-react with KatG from a subset of non-M. tuberculosis mycobacteria such as Mycobacterium smegmatis, but not others (e.g., Mycobacterium chelonae), and thus could not discriminate the precise mycobacterial species from which the tissue KatG was derived (22).

Experimental models of mycobacterial infection and autoimmunity indicate that T cells reactive to microbial Ags or pathogenic tissue autoantigens accumulate at sites of antigenic exposure where they can effect an inflammatory response (47–49). As expected for a pathogenic Ag in sarcoidosis, our study found that CD4⁺ IFN-γ-expressing Th1 cells reactive to mKatG accumulate in the lungs of patients with pulmonary involvement, with fewer circulating mKatG specific CD4⁺ Th1 cells in the blood. ELISPOT assays have previously been shown to preferentially detect effector memory T cells in tuberculosis, malaria, and other infections (50–53). Given that >95% of T cells in the sarcoidosis lung have a CD45RA⁻/CD45RA⁺ memory phenotype (40, 54), our current results suggest there is an accumulation of mKatG specific effector memory CD4⁺ Th1 cells in the sarcoidosis lung. Proliferation to mKatG was also dominant within the CD4⁺ T cell subset, suggesting central memory T cells reactive to mKatG are present in sarcoidosis in varying degrees in different patients. This possibility may parallel findings in chronic beryllium (Be) disease in which Be-sensitized individuals develop granulomatous pneumonitis histologically identical to pulmonary sarcoidosis and in whom circulating Be-specific CD4⁺ T cells with proliferative capacity are dominantly of a central memory T cells phenotype (55).

Importantly, circulating mKatG-reactive, IFN-γ-expressing T cells persisted with chronic disease, but were not present in those patients who had inactive disease suggesting these effector T cells play a role in ongoing granulomatous inflammation. The finding that there were more CD8⁺ than CD4⁺ mKatG-reactive T cells expressing IFNγ in the blood and the presence of CD8⁺ mKatG-reactive T cells in the lung suggest that a significant contribution to chronic IFN-γ-mediated granulomatous inflammation in these patients may derive from CD8⁺ mKatG specific T cells. In both sarcoidosis and tuberculosis, CD4⁺ T cells are found scattered throughout the granuloma, with CD8⁺ T cells dominantly around the periphery of the granuloma (1, 56, 57). Both human and experimental models of mycobacterial infection have demonstrated the importance of both CD4⁺ and CD8⁺ T cells in host defense against these organisms, and a similar interplay between CD4⁺ and CD8⁺ mKatG-specific T cells could also be relevant in sarcoidosis (58–61).

The mechanisms that promote the persistence of Th1 cells at site of inflammation in sarcoidosis despite years of disease remain unclear. One possibility discussed above is that there is persistence of pathogenic Ags such as mKatG at sites of granulomatous inflammation as suggested by our prior study that documented the presence of mKatG protein and DNA in ~50% of sarcoidosis tissues (22). The fact that a subgroup of patients had higher responses to mKatG than PPD suggests mKatG may be a more persistent Ag compared with other Ags contained within PPD preparations. In chronic Be disease, CD4⁺ Be-specific T cells in blood persist for many years of disease duration even after decades removed from last known occupational exposure, likely in part related to the persistence of Be in the lung (62). Because granulomas may serve as an impediment to removal of Ags by the presence of cells and...
matrix designed to wall off offending poorly soluble antigenic material, it is possible that mKatG is complexed with other proteins or sequestered within the granuloma in a manner that permits only a slow removal of this Ag (63). A second possibility is that cross-reactivity between microbial and autoAgs could play a role in chronic active sarcoidosis. For example, Ab responses to human and microbial heat shock proteins are commonly observed in patients with sarcoidosis and other inflammatory diseases (64). Alternatively, mKatG-reactive T cells may persist due to cross-reactivity with homologous peptides from environmental exposure to related mycobacterial organisms. Because it has been shown that granulomas serve as traps for superinfecting mycobacterial organisms, it is also conceivable that environmentally derived mycobacterial proteins may replenish a supply of existing mycobacterial Ags at sites of granulomas (65). A final possibility is that mKatG-reactive effector T cells persist despite the gradual removal of the pathogenic antigenic burden in tissues. Several studies indicate that effector memory T cells are capable of longstanding survival in absence of Ag (66–68).

A genetic basis for sarcoidosis risk and clinical phenotype is centered primarily on the MHC locus and HLA alleles, though the molecular mechanisms that determine different outcomes remain undefined (1, 69). The finding that DRB1*0301+ sarcoidosis patients had higher frequencies of mKatG-reactive lung T cells compared with DRB1*0301− patients, suggests a direct influence of this MHC genotype on mKatG Th1 responses. Because DRB1*0301 has been strongly associated with Löfgren syndrome and good prognosis in sarcoidosis (41), it is possible that DRB1*0301+ could directly affect localized Th1 responses by binding relevant peptides derived from this (or other) pathogenic Ags and influence clinical course. This possibility is enhanced by the finding that no difference in mKatG responses was seen in the blood of DRB1*0301+ compared with DRB1*0301− patients, suggesting that mKatG responses are manifested dominantly in tissues such as the lung where there is active inflammation. Of interest, it has been reported that DRB1*03 is associated with protection from tuberculosis, and with a favorable disease course (70). The finding that both the frequency of mKatG-reactive and PPD-reactive IFN-γ expressing T cells in the lung was higher in DRB1*0301+ patients is in keeping with premise that DRB1*0301 alleles may influence clinical outcomes by affecting local immune responses to mycobacterial Ags. Future studies will be necessary to determine whether the association of DRB1*0301 and good prognosis with Löfgren syndrome in sarcoidosis reflect similar mechanisms that may operate to contribute to a favorable outcome in active tuberculosis.

Intriguingly, we found a significant correlation between the proportion of mKatG and PPD responsive BAL cells and the number of AV2S3+ BAL T cells in DRB1*0301+ Swedish patients, allowing the speculation that AV2S3+ BAL T cells could contain T cells reactive to mKatG or other mycobacterial Ags. In line with this, a previous study showed that in vitro stimulation of peripheral blood cells from BCG vaccinated DRB1*0301+ healthy Swedish volunteers with Mycobacterium tuberculosis extracts gave rise to AV2S3+ T cell expansions (71). In a recent study, the present Swedish authors and their colleagues described a novel technique that allowed them to identify 78 peptides that were eluted from HLA-DR molecules expressed on sarcoidosis BAL cells from DRB1*0301+ sarcoidosis patients and thus, could potentially stimulate AV2S3+ T cells (72). These peptides included autoantigens such as llysyl-tRNA synthase and vimentin. Some of these peptides can induce IFN-γ expression in blood and BAL T cells from sarcoidosis patients, but so far any role for AV2S3+ T cells in such responses remains unknown (72). One could speculate that AV2S3+ T cells could be crossreactive and recognize both some autoantigens and mKatG or other mycobacterial Ags. However, we found no obvious sequence homologies between the HLA-DR-binding peptides and mKatG. Another possibility could be that AV2S3+ BAL T cells in DRB1*0301+ Swedish patients have specificities to mKatG or other mycobacterial Ags and are expanded in parallel with autoreactive T cells, expressing other TCR components with AV2S3 in this lung T cell subset.

The determinants of remitting vs chronic sarcoidosis remain uncertain but are of critical interest given that insights might allow new therapeutic approaches to this disease. Our finding that there was no distinction between the percentages of mKatG-reactive, IFN-γ-producing cells in either the lung or blood of patients with or without Löfgren syndrome at time of diagnosis implies that mKatG-reactive Th1 cells circulate at times of active inflammation and disease regardless of the prognosis or subsequent clinical course. This common thread strengthens the case that mKatG is a pathogenic Ag by providing a unifying trigger in sarcoidosis but mandates that other factors are critical in determining clinical outcome. The present Swedish authors have described a reduced Th1 phenotype for BAL cells in AV2S3+/DRB1*0301+ sarcoidosis patients that have a favorable prognosis with remission >80% of time (30). Other studies have linked persistent expression of TNF from BAL cells in sarcoidosis patients with chronic disease (73). Whether mKatG-reactive T cells will be shown to have a reduced Th1 phenotype in those with Löfgren syndrome or express greater amounts of TNF in those with chronic sarcoidosis remains to be determined. The possibility that some mKatG reactive CD4+ T cells express IL17 (i.e., Th17 effector cells) should also be considered given recent data that indicate Th17 cells may have an important role in host defense against specific pathogens and in organ specific autoimmune (74, 75). In this context, future studies are clearly indicated to determine whether mKatG specific T cells produce TNF or IL17 and more generally, whether differences exist in the profiles of cytokine producing mKatG reactive effector T cells in different subgroups of patients. It is also conceivable that mKatG-reactive T cells with regulatory properties (Tregs) may influence disease outcome. In this context, pathogen-specific Tregs have been shown to regulate immunity to foreign Ags and regulate host responses in experimental models of tuberculosis (76–78). Recent studies report that Tregs may be either defective in number or function in sarcoidosis, suggesting that Ag-specific Tregs could contribute to disease pathogenesis or influence clinical outcome (79, 80).

In summary, this study demonstrates that T cell responses to mKatG are regulated in a manner expected for a pathogenic Ag. The presence, persistence and dominance of mKatG-reactive, IFNγ-expressing T cells in a subset of patients together with the prior demonstration of mKatG in sarcoidosis tissues provides a foundation to consider mKatG specific responses as part of an immunophenotyping scheme that could assist in clinical evaluation or course prediction. It is also possible that an immunotherapeutic strategy using mKatG could be derived to benefit some patients with sarcoidosis.

Disclosures

Johns Hopkins University with D.R.M. and Y.Z. have a patent pending related to this work. Other co-authors have no financial conflict of interests with this study.

References

1. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by


