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ORIGINAL RESEARCH

Pharmacological Inhibition of Chitotriosidase (CHIT1) as a Novel Therapeutic Approach for Sarcoidosis

Barbara Dymek[1,2], Piotr Sklepkiewicz[1], Michal Mlacki[1], Nazan Cemre Güner[1], Patrycja Nejman-Gryz[3], Katarzyna Drzewicka[1], Natalia Przysucha[3], Aleksandra Rymaszewska[1], Magdalena Paplinska-Goryca[3], Agnieszka Zagozdzon[1], Małgorzata Proboszcz[3], Łukasz Krzemiński[1], Jan H von der Thüsen[4], Katarzyna Górska[3], Karolina Dzwonek[1], Zbigniew Zasłona[1], Pawel Dobrzanski[1], Rafał Krenke[3]

[1]Molecure SA, Warsaw, 02-089, Poland; [2]Postgraduate School of Molecular Medicine, Medical University of Warsaw, Warsaw, 02-097, Poland; [3]Department of Internal Medicine, Pulmonary Diseases and Allergy, Medical University of Warsaw, Warsaw, 02-097, Poland; [4]Department of Pathology, Erasmus Medical Center, Rotterdam, 3015 GD, the Netherlands

Correspondence: Barbara Dymek, Żwirki i Wigury 101, Warsaw, 02-089, Poland, Tel +48 22 552 67 24, Email b.dymek@molecure.com

Introduction: Sarcoidosis is a systemic disease of unknown etiology characterized by granuloma formation in the affected tissues. The pathologically activated macrophages are causatively implicated in disease pathogenesis and play important role in granuloma formation. Chitotriosidase (CHIT1), macrophage-derived protein, is upregulated in sarcoidosis and its levels correlate with disease severity implicating CHIT1 in pathology.

Methods: CHIT1 was evaluated in serum and bronchial mucosa and mediastinal lymph nodes specimens from sarcoidosis patients. The therapeutic efficacy of OATD-01 was assessed ex vivo on human bronchoalveolar lavage fluid (BALF) macrophages and in vivo in the murine models of granulomatous inflammation.

Results: CHIT1 activity was significantly upregulated in serum from sarcoidosis patients. CHIT1 expression was restricted to granulomas and localized in macrophages. Ex vivo OATD-01 inhibited pro-inflammatory mediators' production (CCL4, IL-15) by lung macrophages. In the acute model of granulomatous inflammation in mice, OATD-01 showed anti-inflammatory effects reducing the percentage of neutrophils and CCL4 concentration in BALF. In the chronic model, inhibition of CHIT1 led to a decrease in the number of organized lung granulomas and the expression of sarcoidosis-associated genes.

Conclusion: In summary, CHIT1 activity was increased in sarcoidosis patients and OATD-01, a first-in-class CHIT1 inhibitor, demonstrated efficacy in murine models of granulomatous inflammation providing a proof-of-concept for its clinical evaluation in sarcoidosis.

Keywords: chitinase, OATD-01, granuloma, macrophages, interstitial lung disease

Introduction

Sarcoidosis is a complex, multisystem granulomatous disease of unknown etiology that affects individuals worldwide.[1] More than 90% of sarcoidosis patients develop intrathoracic disease with lymph node or lung involvement. A histological hallmark of sarcoidosis is the accumulation of macrophages and CD4+ T cells forming non-caseating granulomas.[2,3] The immune cells accumulation strongly suggests that the inflammatory response is induced by specific auto- or exogenous antigens.[4] Several antigens of microbial and non-microbial origin together with some self-antigens have been linked to sarcoidosis immunopathogenesis.[4] These antigens are believed to stimulate an abnormal response of antigen-presenting cells.[4] The pathologically activated macrophages release proinflammatory mediators which promote T cell activation and initiation of granuloma formation, depending on genetic background and environmental factors.[5,6] Macrophages can further transform into epithelioid cells, which fuse forming multinucleated giant cells within granulomatous structures. Mature granulomas are composed of a central cluster containing macrophages and macrophage-derived giant cells

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surrounded by lymphocytic infiltrate.[6] The dynamic interplay between macrophages and T lymphocytes is believed to determine the outcome of disease: from mild and self-resolving to progressive and recurrent, leading to lung fibrosis.[7]

There is no specific targeted treatment for sarcoidosis and current therapeutic approaches aim at the reduction of the burden of granulomatous inflammation in symptomatic patients. Systemic corticosteroids (CS) remain the standard of treatment. Although short-term benefits of CS have been documented, this class of drugs is known from a significant potential for serious side effects.[3,8] Clearly, there is a strong unmet medical need to develop new therapies for sarcoidosis with improved clinical benefit and safety profile compared to the currently available therapeutic options.

Since studies in patients with sarcoidosis demonstrated highly elevated (>5 fold) serum activity of chitotriosidase (CHIT1) and correlation between CHIT1 serum activity and disease severity, progression and clinical prognosis,[9,10] inhibition of this enzyme seems to be a novel and promising therapeutic approach in sarcoidosis. CHIT1 is a myeloid cell-specific protein and one of the two enzymatically active members of the chitinase family, which have been implicated in the pathology of several lung diseases.[11–13] Literature evidence implicates CHIT1, rather than the other active chitinase-acidic mammalian chitinase (AMCase), as the primary active chitinase in human lungs.[14] Additionally, it has been demonstrated that peripheral blood mononuclear cells (PBMCs) isolated from sarcoidosis patients specifically form granuloma-like multi-cellular aggregates, when incubated with purified protein derivative (PPD)-coated beads and CHIT1 is one of the most highly upregulated genes in ex vivo granulomas, but also in the lung tissue from patients with sarcoidosis.[15]

In our earlier paper, we reported development of OATD-01, which is a small-molecule chitinase inhibitor, and presented its pharmacokinetic and pharmacodynamic features.[16] Recently, we have shown that OATD-01 is effective in the bleomycin-induced pulmonary fibrosis model in mice and that its activity is mediated by suppression of the profibrotic macrophages.[17] The general objective of the current study was to comprehensively evaluate CHIT1 as a novel therapeutic target in sarcoidosis. The specific aims were to measure CHIT1 activity and expression in sarcoidosis patients as well to evaluate the effects of the pharmacological inhibition of CHIT1 with small-molecule inhibitor OATD-01 ex vivo on sarcoidosis macrophages and in vivo in the murine models of granulomatous inflammation in mice.

Materials and Methods

General Study Design

The study was designed as a translational research and included the analysis of material collected from sarcoidosis patients and control subjects followed by the application of murine models of granulomatous inflammation. A scope of human involvement included a collection of biological samples, ie serum, bronchoalveolar lavage fluid (BALF) and specimens of the bronchial mucosa and mediastinal lymph nodes. Additionally, BALF macrophages isolated from sarcoidosis patient were used to study effects of chitinase inhibitor OATD-01 in vitro. Murine models of granulomatous inflammation induced by multiwall carbon nanotubes (MWCNT) and ESAT-6 peptide instillation were used to study the therapeutic efficacy of OATD-01 in vivo.

Human Material Collection

A cohort of newly diagnosed sarcoidosis patients who had not been treated previously (n = 60) as well as control group (n = 29) were recruited in the Department of Internal Medicine, Pulmonary Diseases and Allergy of the Medical University of Warsaw, Poland between 2016 and 2018. The following criteria based on ATS guidelines[18] had to be met to be included in the sarcoidosis group: 1) diagnosis of sarcoidosis supported by the results of histopathological examination of lymph nodes or lung biopsies showing non-caseating granulomas, 2) exclusion of other granulomatous pulmonary diseases, mainly tuberculosis. None of the sarcoidosis patients presented with any acute condition or exacerbation of concomitant diseases. Patients did not present signs of respiratory tract infection in the preceding 6 weeks. The control group comprised volunteers with no history of chronic lung diseases and without any symptoms of respiratory tract infection in the preceding 6 weeks. Normal lung function tests were a mandatory inclusion criterion in this group. Those volunteers were responding to a request for study participation provided via public notice. The characteristics of control and sarcoidosis groups is provided in Table 1. Our study presented in the manuscript has been approved by the Local

Table 1 Characteristics of Sarcoidosis Patients and Control Subjects

	Control	Sarcoidosis	*P* value
Number	29	60	NA
Sex (male, female)	11, 18	32, 28	0.1847
Age (in years) median (min, max)	32 (22, 68)	41 (22, 68)	0.0020
BMI median (min, max)	24.9 (17.6, 44.1)	28 (20.5, 39.4)	0.0011
Race	29 Caucasian	60 Caucasian	NA
Smoker (n/%):			0.0831
No	15 (52%)	40 (67%)	
Yes	9 (31%)	7 (12%)	
Ex	5 (17%)	13 (22%)	
Smoking (packs per year):			0.3791
0	15	40	
0–10	7	9	
>10	7	11	
Scadding sarcoidosis stage (1–4)	NA		NA
1		19	
2		38	
3		3	
4		0	
DLCO (% predicted) mean (standard deviation)	NA	96.0% (17.3)	NA

Abbreviation: NA, not applicable.

Bioethics Committee at the Medical University of Warsaw, Poland (No. of approval: KB/236/2015) and was in full compliance with the Declaration of Helsinki. The consent was obtained from the study participants prior to study commencement. Blood, bronchoalveolar lavage fluid (BALF) and the specimens of bronchial mucosa and mediastinal lymph nodes were collected and processed as previously described.[19,20]

Human Samples Analyses

Human CHIT1 Protein

The CHIT1 protein concentration in human serum samples was evaluated using Human Chitotriosidase ELISA Kit (CycLex) and the measurements were performed according to the protocol. The range and the sensitivity of the applied kit were 56.25–3600 pg/mL and 48.3 pg/mL, respectively.

Chitinolytic Activity in Human Serum

The enzymatic activity of chitinases in human serum was measured as previously described.[16]

CHIT1 Immunohistochemical Staining of BALF Cells

The immunocytochemical staining of BALF cell smears was performed as previously.[17] In brief, anti-CHIT1 antibodies (Biorbyt, orb377995; Lot# CQ2228) were used followed by donkey anti-rabbit-horseradish peroxidase (HRP) secondary antibodies (Jackson ImmunoResearch, 711–035-152, Lot# 126333). The percentage of CHIT1-positive macrophages was determined based on a microscopic examination of the cell morphology (300 cells from various fields).

BALF Macrophages Culture and Treatment

Macrophages from BALF of sarcoidosis patients (n = 6) were isolated by adhesion to plastic as described previously.[17] The cells were treated with OATD-01 at 1 μM or vehicle (0, 1% dimethyl sulfoxide [DMSO]) for 24h and cell supernatants were collected for multiplex analysis of cytokines and chemokines (Bio-plex assay, Bio-rad).

Animal Studies

All in vivo experiments were approved by the Local Ethics Committee for Animal Experimentation, Warsaw, Poland (No. of approval: WAW1/798/2018) and followed the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care. C57BL/6 mice were purchased from Charles River Laboratory (Germany). There were two animal models applied for studying OATD-01 efficacy in vivo: a 10-day-long acute model and a 40-day-long model of persistent granulomatous inflammation.

Animal Models of Granulomatous Inflammation Induced by Multiwall Carbon Nanotubes (MWCNT) and ESAT-6 Peptide Instillation

Granulomatous inflammation in murine lungs was induced by a triple oropharyngeal administration of MWCNT and ESAT-6 peptide to 8-week-old C57Bl/6 female mice. MWCNT with outer diameter of 10–30 nm and length of 5–15 μm (SES Research; Catalog# 900–1201; Lot GS-1815) at the dose of 0.1 mg/mouse and ESAT-6 peptide (Innovagen; NNALQNLARTISEAG; Acetate) at the dose of 20 μg/mouse was administered in a volume of 50 μL/mouse in a vehicle (Dipalmitoylphosphatidylcholine [DPPC] 0.1 mg/mL; mouse serum albumin 0.2 mg/mL in phosphate buffered saline [PBS]) at 3 consecutive days under 5% isoflurane anesthesia. OATD-01 in 0.5% carboxymethylcellulose was administered orally at 100 mg/kg dose from day 0 to 10 in the 10-day-long model of acute granulomatous inflammation and from day 10 to 40 in the therapeutics scheme of treatment in the 40-day-long chronic model. In acute model mice were sacrificed on day 10 after the initial MWCNT/ESAT-6 administration and the BALF was collected for subsequent analyses with flow cytometry and biochemical methods. In the chronic model, mice were sacrificed on day 40 and lungs were collected from each mouse for histopathological evaluation (left lung), gene expression analysis and chemokine concentrations (right lung).

Bronchoalveolar Lavage Fluid Collection

At the end of the study, murine lungs were washed with 1 mL of PBS via trachea and then the collected BALF was centrifuged (10 min, 2000 rpm, 4°C). Supernatant was collected for further analysis and cells pellet was used for flow cytometry analysis.

Chitinolytic Activity in Murine BALF

The enzymatic activity of chitinases in murine BALF was measured as described previously.[17]

BALF Cells Preparation and Flow Cytometry Analysis

The pellet of BALF cells was resuspended in 300 μL of PBS with bovine serum albumin (BSA) and used for flow cytometry analysis. The cells were pipetted through cell strainer cap directly to the well of 96-well plate and were centrifuged (5 min, 1500 rpm, 4°C). Then supernatant was discarded and cells were resuspended in 54 μL of 5% normal rat plasma in PBS with BSA and blocked for 15 minutes on ice. Then, the cells were divided into two parts and to each 3 μL of antibody cocktail was added (according to Table 2). The cells were stained on ice for 30 minutes in dark, washed twice in PBS with BSA and resuspended in PBS with BSA and NaN_3 for the flow cytometry analysis (Guava, Merck).

Table 2 Antibodies Used for Flow Cytometry Analysis

Antibody	Cat. No.
Anti-CD11b-AF488 (clone M1/70)	Biolegend, 101217
Anti-CD45.2-PE (clone 104)	eBiosciences, 12–0454
Anti-Ly6G-PerCP-Cy5.5 (clone 1A8)	Biolegend, 127616
Anti-CD11c-APC (clone N418)	eBiosciences, 17–0114–82

Lung Collection and Histological Evaluation

Firstly, the lungs were perfused with PBS via the right ventricle and then right upper airway was closed and the right lung was dissected for further RNA and protein analysis. The left lung was instilled with 10% neutral buffered formalin (NBF) via a catheter in trachea and infused with 1 mL of NBF. Then left lung was removed *en block* with heart, thymus and trachea and kept in NBF for 48h. Lung was sliced sagittally. To facilitate handling in the next steps, halves of lobes were pre-embedded in melted agarose solution (2% in PBS). The blocks were processed according to a standard protocol, embedded in paraffin, cut into 5 µm sections and mounted on glass slides. Slides were then stained with hematoxylin–eosin (HE) using a standard protocol. Histological analysis of granulomatous inflammation on day 40 was assessed on 3 sets of sections per animal separated by 100 µm. Lung granulomatous inflammation was evaluated in a blinded fashion using the established scoring system (0–5, where 0 – no MWCNT particles, 1 – free uninvolved MWCNT particles, 2 – MWCNT particle accompanied with few cells, 3 – 1 layer of cells around MWCNT particle, 4 – 2–3 layers of cells around MWCNT particle, 5 – small organized granulomatous structure).

Real-Time PCR

One lobe from the right lung of each mouse was stored in RNALater buffer (Invitrogen). Total RNA was isolated using RNeasy MiniKit (Qiagen) applying the manufacturer's protocol. An equal amount of RNA was used for reverse transcription reaction (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems) according to the manufacturer's protocol. For real-time PCR the murine gene specific TaqMan Assays together with TaqMan Gene Expression Master Mix (Applied Biosystems) and Real-time PCR Detection System CFX-384 (BIO-RAD) were used. The relative gene expression was calculated based on the $2^{-\Delta\Delta CT}$ method.[21]

Lung Homogenates Preparation for ELISA

The snap-frozen lobes of the right lung were thawed, weighed and transferred to ice-cold tubes with PBS with protease inhibitor cocktail (Sigma). The lung tissue was homogenized and centrifuged at 10,000 g for 10 minutes at 4°C. Collected supernatants were then used for ELISA assays.

CCL2 and CCL4 Protein Concentration

CCL2 and CCL4 in BALF and lung homogenates were quantified by ELISA as per the manufacturer's protocol (R&D System). The range and the sensitivity of the CCL2 detecting kit were 7.8–500 pg/mL and 0.666 pg/mL, respectively. The range and the sensitivity of the CCL4 detecting kit were 7.8–500 pg/mL and 3 pg/mL, respectively.

Statistical Analysis

Data are shown as means ± standard error of mean (SEM). GraphPad Prism v. 8.0 was used to perform statistical analyses. For statistical analyses of two groups paired two-tailed *t*-test was used. To evaluate the differences between the groups with normal distribution (verified with D'Agostino & Pearson omnibus normality test) parametric one-way ANOVA with the Dunnett's test for multiple comparisons was utilized. In the case of non-normal distribution, a non-parametric Kruskal–Wallis test with Dunn's multiple comparison test was used to evaluate the differences between the groups. The differences between groups were tested using Pearson's chi-squared test or Fisher's test (for categorical variables) and Mann–Whitney *U*-test (for comparisons between two independent groups). P-values <0.05 were considered statistically significant.

Results

CHIT1 is Highly Upregulated in Sarcoidosis and Activated Macrophages in the Human Lung of Sarcoidosis Patients are a Main Source of CHIT1

We first analyzed the chitinolytic activity and CHIT1 concentration in serum from patients with sarcoidosis and control subjects. Results confirmed the highly elevated chitinolytic activity (>75-fold) and CHIT1 concentration (>13-fold) in sarcoidosis serum compared to control samples. (Figure 1A and B). Moreover, no AMCase enzymatic activity was detected in those samples (the activity below level of detection), and chitinolytic activity highly correlated with CHIT1

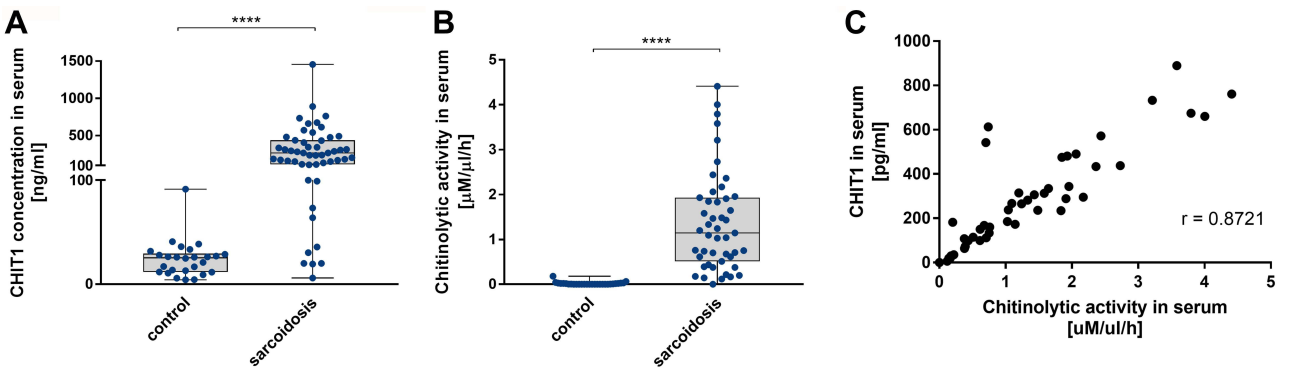

Figure 1 CHIT1 activity and concentration in sarcoidosis patients' serum is highly upregulated. (**A**) Chitinolytic activity in serum and (**B**) CHIT1 concentration from control donors and sarcoidosis patients. Data presented as box and whiskers plot **** P<0.0001. (**C**) The correlation of the chitinolytic activity and CHIT1 concentration in serum of patients with sarcoidosis. Correlation coefficient r = 0.8721, p<0.000001.

levels in serum indicating that CHIT1 comprises the main source of chitinolytic activity in sera of sarcoidosis patients (Figure 1C). These results were expanded by the immunocytochemistry of BALF cell smears from sarcoidosis patients which demonstrated abundant CHIT1-positive cells, mostly macrophages, while no AMCase signal was detected (Figure 2A). Moreover, the immunohistochemical evaluation demonstrated robust expression of CHIT1, but not AMCase, in the bronchial mucosa and mediastinal lymph nodes from the same sarcoidosis patients. CHIT1 expression was restricted to the tissue affected by the granulomatous disease (Figure 2B), with no expression in the granuloma-unaffected areas (Figure 2C). The most significant CHIT1 expression was detected in activated macrophages and granuloma-specific macrophage-derived cells like epithelioid and giant cells. Of note, neither AMCase expression in diseased tissue (Figure 2B and C) nor its activity in samples was detectable, confirming that CHIT1 is the dominant chitinase in human lungs.

OATD-01 Inhibits Upregulated Chitinolytic Activity in Sarcoidosis Patients' Serum and Has a Direct Effect on Patients' BALF Macrophages

To confirm the activity of OATD-01, we have evaluated its ex vivo ability to inhibit chitinolytic activity in BALF and serum samples of sarcoid patients. The analysis revealed that OATD-01 inhibited chitinolytic activity with IC_{50} values within a nanomolar range (Figure 3A and B). Furthermore, we conducted experiments to establish a functional link between CHIT1 and sarcoidosis pathology. The Bio-plex analysis of the levels of inflammatory mediators in the supernatants from sarcoidosis patients' BALF macrophages and treated with OATD-01 revealed that OATD-01 reduced levels of several pro-inflammatory chemokines and cytokines implicated in the development of granulomatous inflammation, including CCL4, IL-15 and IL-1 antagonist receptor (Figure 3C–H). Interestingly, no inhibitory effects on the expression of other inflammatory cytokines (IL-6, IL-17) were observed. These results demonstrated the ability of OATD-01 to potently suppress the inflammatory phenotype of sarcoidosis-activated macrophages.

OATD-01 Demonstrates Anti-Inflammatory Activity in an Acute, 10-Day-Long MWCNT + ESAT-6 Murine Model of Granulomatous Inflammation

In order to evaluate the therapeutic potential of inhibition of chitinases with OATD-01, the acute, 10-day-long murine model of granulomatous inflammation (Figure 4A) was applied that was shown to be driven by either innate or adaptive immune response following MWCNT and ESAT-6 instillation. OATD-01 treatment at 100 mg/kg/dose in a preventive treatment approach for 10 consecutive days resulted in the suppression of inflammatory response as determined by a decreased efflux of pathogenic neutrophils to lungs and increased number of protective resident alveolar macrophages (Figure 4B–D). We have identified sarcoidosis-associated CCL4 chemokine as one of the pro-inflammatory mediators that was reduced in murine BALF following OATD-01 treatment suggesting that the anti-inflammatory effects of CHIT1 inhibition are exerted via modulation of lung macrophages. Specifically, as evidenced in this experiment, inhibition of the

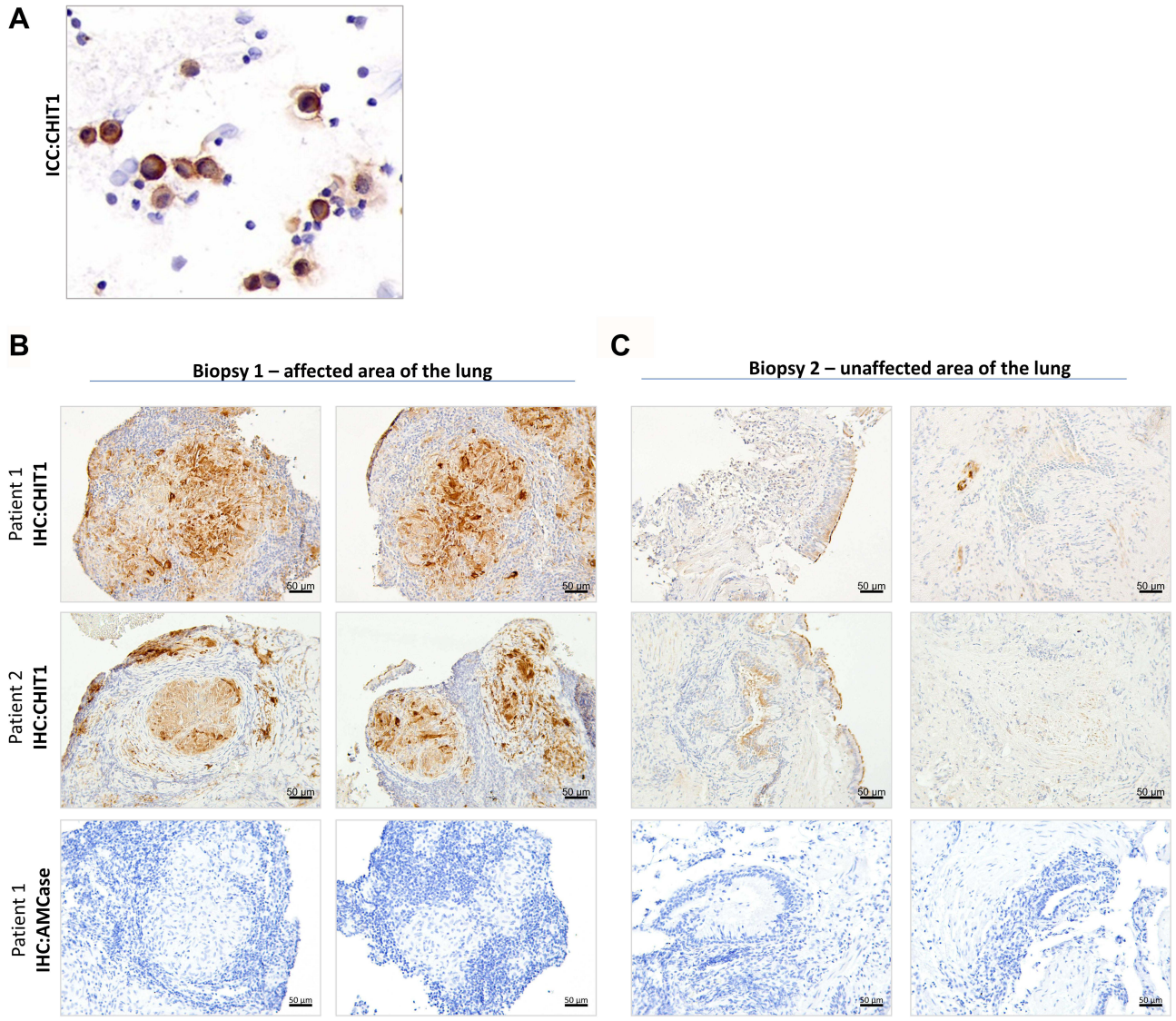

Figure 2 Pathological macrophages are a source of CHIT1 in sarcoidosis. (**A**) Representative immunocytochemistry for CHIT1 in BALF cells smears from sarcoidosis patients (n = 12); (**B** and **C**) Representative images for CHIT1 and AMCase immunohistochemistry of the bronchial mucosa and mediastinal lymph nodes specimens of sarcoidosis patients (n = 15).

critical pro-inflammatory chemokine (Figure 4E and F) leads to the inhibited recruitment of pro-inflammatory cells. Concurrently, we have confirmed target engagement in the lungs by showing a significant reduction of the MWCNT-induced chitinolytic activity in murine BALF after OATD-01 treatment (Figure 4G).

OATD-01 Shows Therapeutic Efficacy in a Chronic 40-Day-Long MWCNT + ESAT-6 Murine Model of Sarcoidosis

To further evaluate the therapeutic potential of OATD-01, we have utilized the chronic 40-day-long model of persistent granulomatous inflammation in mice induced by MWCNT and ESAT-6 peptide (Figure 5A) with confirmed expression of CHIT1 in the murine lungs (Supplemental Figure S1). OATD-01, administered at 100 mg/kg dose in the therapeutic scheme of treatment starting from day 10 when the presence of granulomatous inflammation was confirmed, significantly affected the granulomatous inflammation. The histological assessment of murine lungs after 40 days post MWCNT + ESAT6 administration revealed that OATD-01 treatment decreased the number of organized granulomatous lesions (Score 3–4) compared to the vehicle-treated group without altering total MWCNT incidence in the lungs (Figure 5B–H).

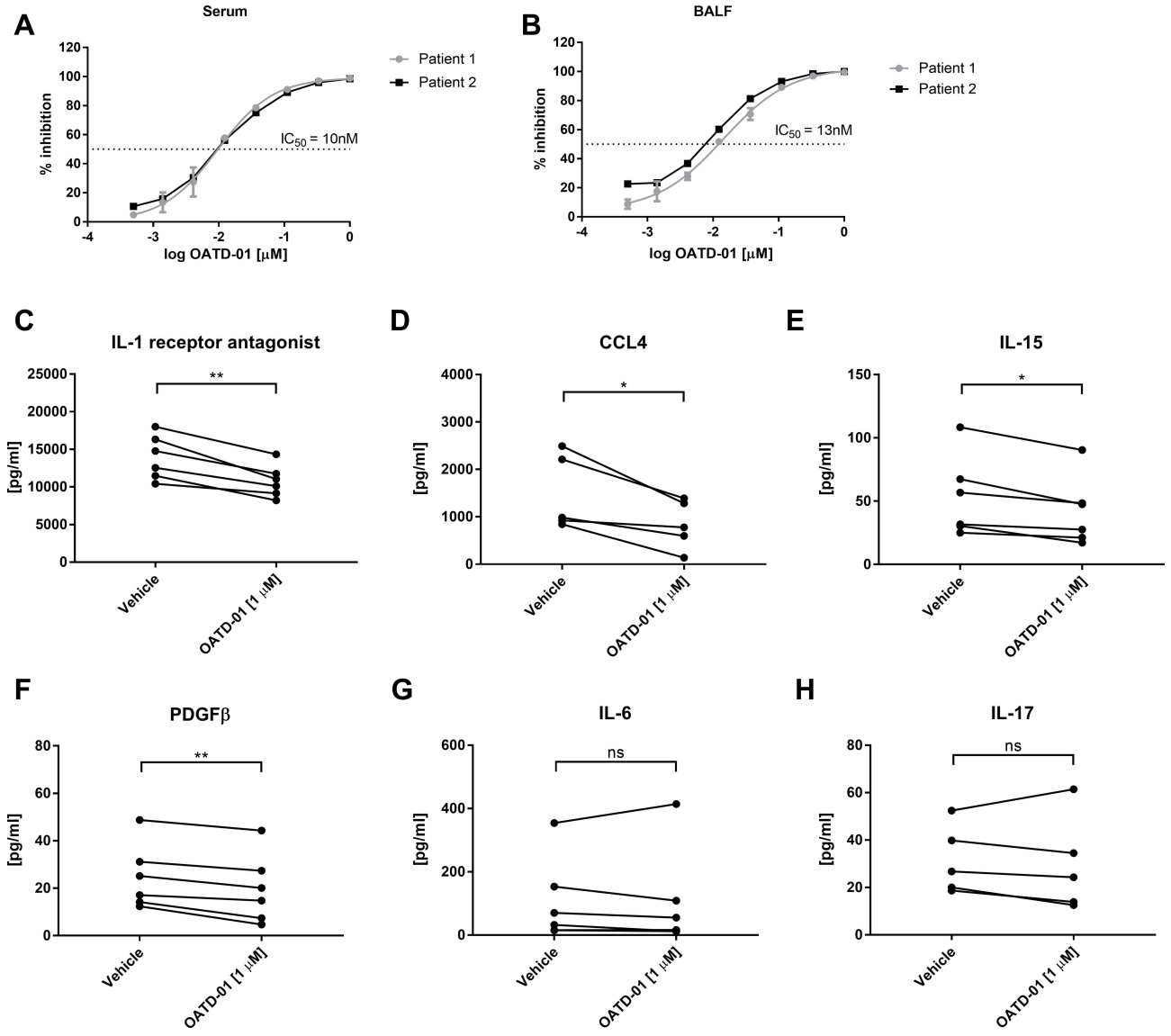

Figure 3 OATD-01 inhibits upregulated chitinolytic activity in serum from sarcoidosis patients and has a direct effect on patients' BALF macrophages. (**A**) Ex vivo inhibition of the chitinolytic activity in sarcoidosis patients serum samples (n = 2) by OATD-01. (**B**) Ex vivo inhibition of the chitinolytic activity in sarcoidosis patients BALF samples (n = 2) by OATD-01. (**C**–**H**) The levels of immune responses' modulators (CCL4, IL-15, PDGFβ, IL-1 receptor antagonist, IL-6, IL-17) in sarcoidosis BALF macrophages supernatants (n = 6) after OATD-01 treatment. The concentration of analytes in supernatants from untreated and OATD-01-treated macrophages were compared using two-tailed paired *t*-test. *P<0.05, **P<0.01.

Moreover, gene expression analysis in the lungs demonstrated that OATD-01 downregulated the expression of genes associated with macrophage activation (*Chi3l1, Ccl2, Spp1*) (Figure 5I–L). On the protein level, OATD-01 treatment significantly reduced CCL4 expression in lung homogenates (Figure 5M). Increased levels of CCL4 have been associated with pulmonary sarcoidosis suggesting its role in disease pathology and granuloma formation and persistence. These results are in accordance with the data obtained in BALF macrophages from sarcoidosis patients. In summary, OATD-01 administered in the therapeutic regimen demonstrated therapeutic efficacy in the chronic MWCNT + ESAT-6-induced mouse model of granulomatous inflammation suggesting its potential as a novel, CS-sparing therapy for sarcoidosis.

Discussion

In the current study, we have reported a comprehensive analysis of activity and expression of CHIT1 in sarcoidosis patients showing that pathological macrophages are the main source of CHIT1. Importantly, AMCase expression is limited in sarcoidosis patients. Moreover, we have demonstrated that pharmacological inhibition of CHIT1 with OATD-

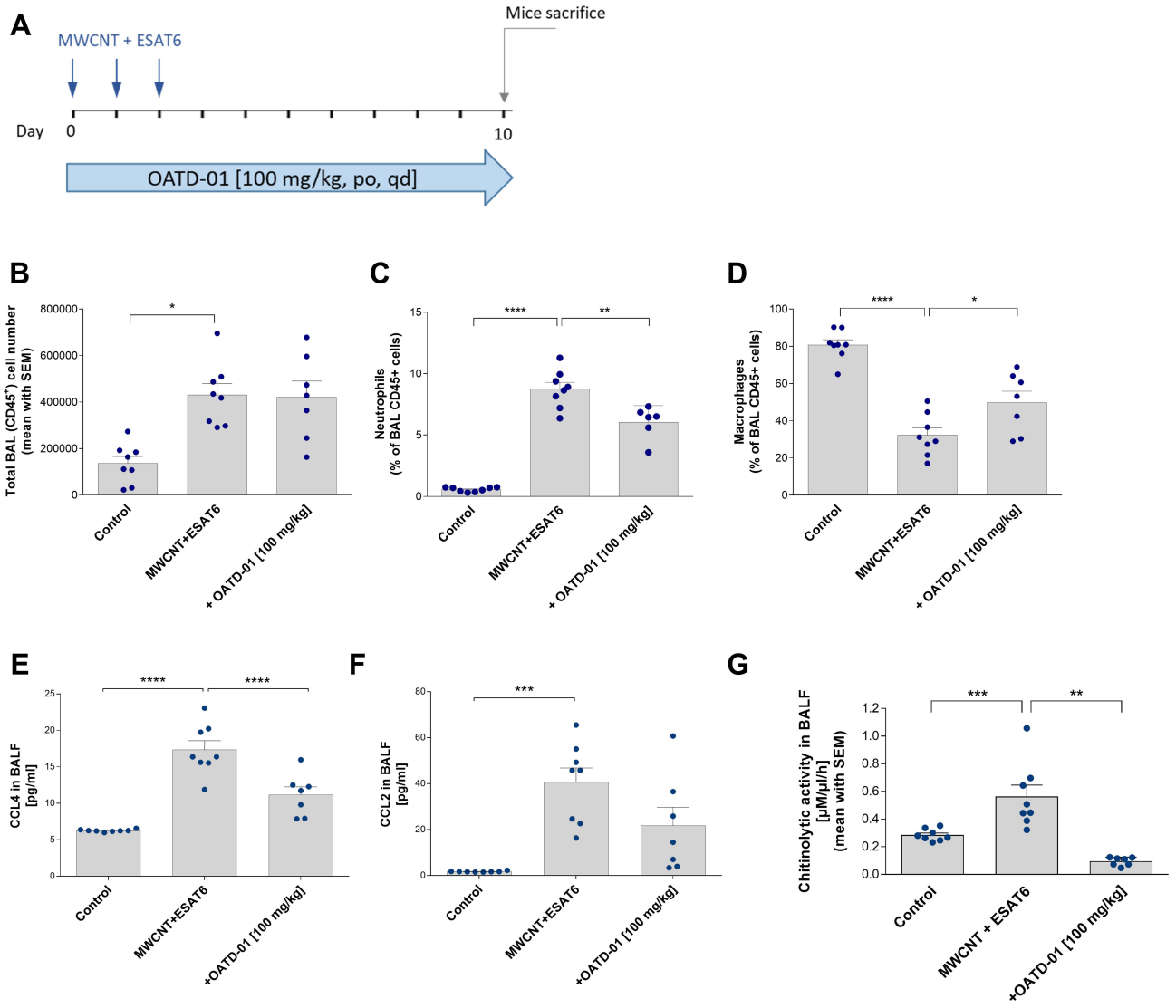

Figure 4 OATD-01 shows the anti-inflammatory effects in 10-day-long MWCNT + ESAT-6 model of granulomatous inflammation in mice. (**A**) A scheme of the in vivo experiment. MWCNT + ESAT-6 was administered oropharyngeally on 3 consecutive days and OATD-01 was administered orally, once a day for 10 days at 100 m/kg dose. (**B**) Analysis of CCL2 levels in murine BALF following OATD-01 administration in comparison to the vehicle-treated animals. (**C**) Analysis of CCL4 levels in murine BALF following OATD-01 administration in comparison to the vehicle-treated animals. (**D**) Chitinolytic activity in BALF following OATD-01 administration as compared to the vehicle-treated animals. (**E**–**G**) Flow cytometry analysis of BALF leukocyte subpopulations such as alveolar macrophages and neutrophils following 10-day treatment with OATD-01 as compared to the vehicle-treated mice. Data presented as mean ± SEM; *p<0.05, **p<0.01, ***P<0.001, ****P<0.0001.

01 resulted in the attenuated inflammatory response in the MWCNT + ESAT-6 induced model when administered in both acute and chronic scheme. In an acute 10-day long model, OATD-01 changed the ratio of immune cell subpopulations, namely decreasing the percentage of pro-inflammatory neutrophils while increasing the percentage of anti-inflammatory alveolar macrophages. In the chronic, 40-day-long murine model of granulomatous inflammation, the suppression of the chitinolytic activity by OATD-01 resulted in the significant attenuation of pathological granulomas in the mouse lungs. Additionally, ex vivo OATD-01 reduced the production of the pro-inflammatory mediators, such as CCL4, IL-15 and IL-1 receptor antagonist by BALF macrophages obtained from sarcoidosis patients. To our knowledge, our study is the first one providing the preclinical proof-of-concept data showing that inhibition of CHIT1 may represent a novel therapeutic approach in sarcoidosis acting via inhibition of alveolar macrophage responses, which leads to a decreased inflammation and subsequently reduced granuloma formation in the lungs.

We have shown elevated chitinolytic activity in serum of more than 85% of sarcoidosis patients presenting it as a common event in sarcoidosis pathology both systemically and locally in the diseased lungs. Interestingly, no

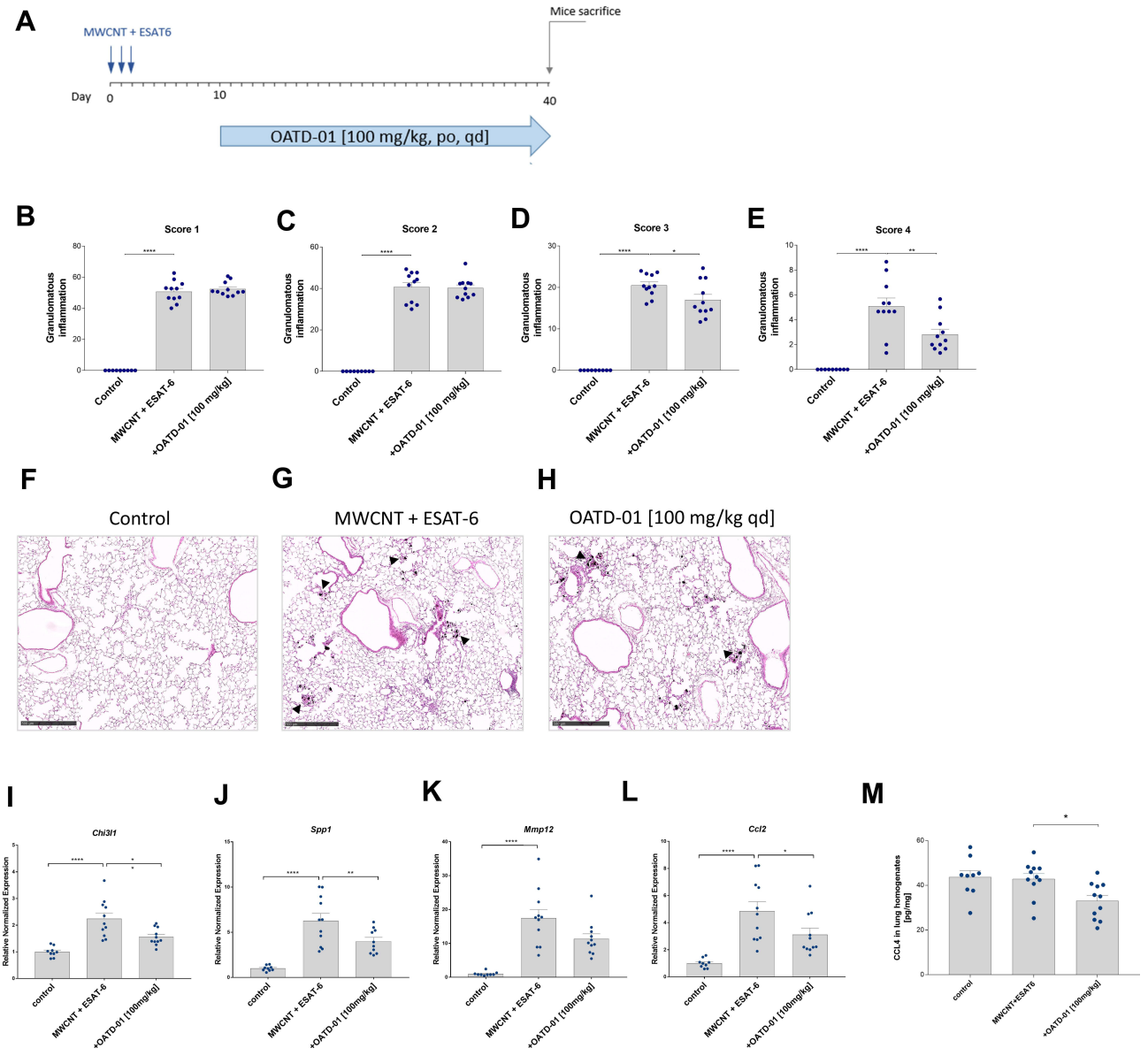

Figure 5 OATD-01 demonstrates therapeutic effects in 40-day-long MWCNT + ESAT-6 murine model of granulomatous inflammation affecting granuloma maturation and pro-inflammatory mediators expression. (**A**) A scheme of the in vivo experiment. MWCNT + ESAT-6 was administered oropharyngeally on 3 consecutive days and OATD-01 was dosed in a therapeutic treatment regimen, once a day, for 30 days at 100 mg/kg dose. (**B**–**E**) Analysis of pulmonary granulomatous inflammation by histological assessment in the lung sections (n = 3 per animal) in animals following OATD-01 administration in comparison to the vehicle-treated control group. (**F**–**H**) Representative images of lung granulomatous changes visualized by the HE staining in control animals, mice instilled with MWCNT + ESAT-6 only or dosed also with OATD-01. The arrows indicate granuloma-like changes. (**I**–**L**) The relative expression of pro-inflammatory mediators (*Chi3l1, Spp1, Mmp12, Ccl2*) in the lungs in vehicle- and OATD-01-treated animals evaluated by qtPCR. (**M**) Analysis of CCL4 levels in lung homogenate following OATD-01 treatment in comparison to the vehicle-treated animals. Data presented as mean ± SEM; *p<0.05, **p<0.01, ***P<0.001, ****P<0.0001.

chitinolytic activity at pH 2 was detected, proving that CHIT1 and not AMCase is the active chitinase in sarcoidosis. CHIT1 protein and activity was detected in all tested serum samples (either control or sarcoidosis group), suggesting that among the individuals in the study there were no carriers of inactivating *CHIT1* gene duplication, although no genetic study was applied to confirm that. Expression profile of chitinases in sarcoidosis was verified by the immunohistochemical analysis of bronchial mucosa and mediastinal lymph nodes specimens. Robust CHIT1 expression was limited to the granulomas, sparing the disease-unaffected tissue. In contrast, we were not able to demonstrate AMCase expression in the sarcoidosis patients' lungs. The most significant CHIT1 expression was detected in activated macrophages and macrophage-derived epithelioid and giant cells. This observation validates immunocytochemical analysis of BALF cells

smears which demonstrated macrophage-specific CHIT1 staining. Our data expands on the previous reports, which show elevated CHIT1 expression and activity in sarcoidosis.[9,10] We have specified previous findings regarding the CHIT1 localization and limited AMCase expression in sarcoidosis. Overall, we demonstrate that CHIT1 is the pathological, predominant chitinase in sarcoidosis with expression limited to macrophages in sarcoid lung granulomas.

Recently, we have shown that OATD-01 selectively inhibits chitinases with no significant interactions with off-targets and it has a favorable pharmacokinetic profile in rodents and higher species proving its excellent druglike properties.[16] OATD-01 administered once daily orally in doses between 30 and 100 mg/kg demonstrated significant antifibrotic efficacy in a preclinical murine model of bleomycin-induced pulmonary fibrosis.[17]

Having identified alveolar macrophages as the main source of CHIT1 in sarcoid lungs, we evaluated the effects of OATD-01, a highly potent and selective chitinase inhibitor, on the BALF macrophages from sarcoidosis patients ex vivo. The inhibitor has affected macrophage secretome, including the reduction of the level of a pro-inflammatory mediator such as CCL4 (MIP-1β), IL-15 and IL-1 receptor antagonist. CCL4 is a CC chemokine that is involved in the recruitment of T cells, in particular CD4+ T cells, to the site of inflammation.[22] Importantly, it was shown that the concentration of CCL4 in BALF was significantly increased in patients with sarcoidosis, regardless of the stage of the disease.[23] Levels of CCL4 correlated with the number of CD4+ and CD8+ T cells, indicating the role of this mediator in the recruitment of immune cells.[23] This proves an indirect effect of OATD-01 on adaptive immunity as a consequence of modulation of macrophage secretome.

Another important cytokine inhibited by OATD-01 is IL-15 - A pleiotropic, pro-inflammatory signaling protein that has an impact on various immune cells. In particular, it was shown that it promotes the survival of T cells and B cells.[24] It has been reported that IL-15 plays a role in the modulation of Th1-type immune response.[25] Of note, IL-15 has been demonstrated to be highly expressed in the lung tissue[26] and BALF fluid[27] of patients with sarcoidosis. Moreover, it has been also implicated in the formation of multinucleated giant cells,[28] showing even broader therapeutic potential for OATD-01. Finally, we have shown that OATD-01 modulated IL-1 receptor antagonist, which is a member of the IL-1 cytokine family and is secreted by immune and epithelial cells. IL-1 receptor antagonist modulates immune responses related to IL-1 signaling via prevention of binding of IL-1α and IL-1β to IL-1 receptor.[29] Moreover, anakinra - The recombinant IL-1 receptor antagonist, is used for the treatment of inflammatory disorders, eg rheumatoid arthritis.[30] In sarcoidosis, it was proposed that the IL-1 receptor antagonist to IL-1β ratio in BAL fluid macrophage culture supernatants can serve as a prognostic factor in patients with pulmonary sarcoidosis.[31] In the future we propose to further explore CHIT1 inhibition as a therapeutic strategy to modulate macrophage fate in sarcoidosis pathology. Our hypothesis is based on the direct effects of OATD-01 on the expression of pro-inflammatory mediators by BALF macrophages. This suggests that inhibition of CHIT1 may represent a novel therapeutic approach in sarcoidosis acting via modulation of macrophages and affecting the migration of the inflammatory cells to the lung.

The fact that macrophages are at the core of granuloma formation processes, prompted us to evaluate the therapeutic potential of OATD-01 in the murine models of granulomatous inflammation. There is no gold standard preclinical animal model of sarcoidosis and several models have been proposed to mimic granulomatous inflammation observed in humans.[32–34] The mouse model induced by MWCNT and ESAT-6 administration allows for the evaluation of both acute inflammatory response and the chronic persistent granuloma formation processes in murine lungs. OATD-01 treatment resulted in the downregulation of the expression of activated macrophages associated genes: *Chi3l1, Ccl2* and *Spp1*. Chitinase-3 like-protein-1 (CHI3L1), which is believed to be an activation marker for macrophages, has been strongly associated with inflammatory and fibrotic lung diseases[35–37] including sarcoidosis, where it serves as a disease biomarker.[38] CCL2 is a pleiotropic, pro-inflammatory chemokine acting via several mechanisms, including the recruitment of immune cells to the site of inflammation and T cells polarization modulation.[39] A body of evidence exists which indicates CCL2 as an important mediator in various inflammatory and fibrotic lung disorders, including sarcoidosis.[40,41] Osteopontin (*Spp1* gene product) is a secreted protein expressed by a variety of different cell types that regulates diverse biological functions.[42] In sarcoidosis patients osteopontin is highly upregulated in plasma[43] and more importantly, it is expressed by epithelioid and multinucleated giant cells in lung granulomas and was implicated in granuloma formation.[44] Additionally, the inhibition of chitinases in either acute or chronic model of granulomatous inflammation in mice led to the decreased levels of CCL4 – the pro-inflammatory chemokine that was also reduced in human BALF macrophages'

supernatant from sarcoidosis patients following OATD-01 treatment ex vivo. Taken together OATD-01 inhibited secretion of inflammatory mediators relevant in various lung disorders.

There are some limitations of this study that need to be considered. Firstly, the human material originated from a single-center, cohort study with a limited number of participants. Secondly, although there were attempts to match the sarcoidosis and control groups, in particular based on sex, age and smoking status, the sarcoidosis and control groups were not age-matched, and it cannot be excluded that age has an impact on the activity and expression of CHIT1. Thirdly, because sarcoidosis' triggering factors are not fully defined, there is no widely accepted animal model of this disease that would fully resemble sarcoidosis pathology.[32] Although MWCNT along with ESAT-6 peptide instillation to mice has been shown to evoke persistent granulomatous inflammation in murine lungs with immune cell infiltration,[33,34] the mechanisms by which carbon nanotubes induce inflammatory responses in murine lungs remain unknown. Despite these limitations, we believe that the results of this study present important additions to the current knowledge on CHIT1 being a potential therapeutic target.

Conclusion

To conclude, the clinical data from patients with sarcoidosis, the effects of OATD-01 on BALF macrophages and the results from animal models of acute and chronic granulomatous inflammation support the role of CHIT1 in sarcoidosis development and progression and provide a rationale for targeting CHIT1 as a novel therapeutic approach.

Institutional Review Board Statement

The study involving human samples was approved by the Local Bioethics Committee at the Medical University of Warsaw, Poland, No. of approval: KB/236/2015. The animal studies were approved by the First Local Ethics Committee for Animal Experimentation, Warsaw, Poland, No. of approval: WAW1/798/2018.

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Disclosure

At the time of the study BAD, PS, MM, NCG, KD, AR, AZ, ŁK, KDz, ZZ and PD were employees and shareholders of Molecure SA (previously OncoArendi Therapeutics SA), which develops inhibitors of chitinases. JvdT received research funding from BMS, OncoArendi Therapeutics and Roche, and consulting fees from Boehringer Ingelheim, MSD and Roche. RKr received fees for lectures and/or travel expenses from AstraZeneca, Chiesi, Polpharma and Roche. KG received fees for lectures and/or travel expenses from Boehringer Ingelheim and Roche. The authors report no other conflicts of interest in this work.

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