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Enhanced LPS-induced activation of IL-27 signalling in sarcoidosis

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ABSTRACT

Rationale: Granulomas in sarcoidosis have recently been described as containing Interleukin (IL)-27, one of the members of the IL-12 family of cytokines, which also includes IL-35. Levels of these cytokines and the IL-27 receptor subunits were hypothesised to differ between patients with sarcoidosis compared to healthy controls in peripheral blood.
Methods: Using a cross-sectional study design, plasma and peripheral blood mononuclear cells (PBMC) were collected from patients and control subjects. Protein and mRNA (in PBMC) levels for IL-27 and IL-35 (*IL27*, *EBI3*, *IL12A* subunits) as well as IL-27 receptor (IL6ST and IL27RA subunits) were assessed spontaneously and following direct (LPS) and indirect (anti-CD3/28 activation beads) macrophage stimulation using RT- PCR, ELISA and flow cytometry.
Results: Following stimulation with LPS, PBMC of patients with sarcoidosis displayed significantly enhanced expression of *IL27* and *EBI3* mRNA ($p = 0.020$ and $p = 0.037$ respectively) compared to PBMCs from healthy controls. There was also significantly enhanced production of IL-27 by PBMC from patients with sarcoidosis compared to healthy controls in response to LPS stimulation ($p = 0.027$).
IL6ST mRNA and IL6ST protein were significantly lower in patients with sarcoidosis (mRNA $p = 0.0002$; MFI $p = 0.0015$) whilst IL27RA protein levels were significantly higher in patients with sarcoidosis compared to healthy controls (MFI $p < 0.0001$).
Plasma IL-35 protein levels did not differ between control and sarcoidosis subjects ($p = 0.23$).
Conclusion: These results suggest there may be exaggerated activation of IL-27 signalling in response to LPS in sarcoidosis.

1. Introduction

Sarcoidosis, a term first used in the late 19th century to describe granulomatous skin lesions, is currently recognised as a systemic granulomatous inflammatory disease predominantly affecting the lungs [1,2]. The non-caseating granulomas observed in affected tissues remain the key pathological feature [3]. The aetiology and the mechanisms of pathogenesis have not been fully elucidated despite more than a century of research. It is generally believed that sarcoidosis occurs in genetically susceptible individuals following exposure to disease associated antigens that trigger a Th1 dominated immune response ultimately leading to granuloma formation [4].

One putative sarcoid antigen is lipopolysachcharide (LPS), a component of the gram-negative bacterial cell wall. Polymorphisms of the LPS receptor components CD14 and toll like receptor 4 (TLR4) have been linked to sarcoidosis [5,6]. Likewise LPS has been identified in the lungs of patients with sarcoidosis and PBMC and BAL cells of patients with sarcoidosis show hypersensitivity reactions following stimulation with LPS [7]. In addition LPS is known to trigger a Th1 based immune response promoting interferon-γ (IFNγ) which is one of the key cytokines involved in granuloma formation in sarcoidosis [4,8]. In patients with sarcoidosis IFNγ is produced by Th1 cells in the presence of IL-12, which is another key cytokine involved in the pathogenesis of sarcoidosis [4,9]. However, a recent clinical trial found no benefit

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Abbreviations

ACE	angiotensin-converting enzyme
BAL	bronchoalveolar lavage
DLCO	diffusion capacity of the lungs for carbon monoxide
EBI3	Epstein-Barr virus induced-gene 3
FBS	fetal bovine serum
FEV1	forced expiratory volume in 1 s
FVC	forced vital capacity
FITC	fluorescein isothiocyanate
HPRT	hypoxanthine–guanine phosphoribosyl transferase
IFNγ	interferon gamma
IL	interleukin
IL6ST	interleukin 6 signal transducer (gp130)
IL12A	interleukin 12A
IL12RB1	interleukin 12 receptor beta 1
IL27	interleukin 27 (p28)
IL27RA	interleukin 27 receptor alpha
LPS	lipopolysachcharide
MFI	mean fluorescence intensity
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
PFA	paraformaldehyde
SD	standard deviation
Th1 cell	T helper cell type 1
TLR4	toll like receptor 4
Tregs	T regulatory cells
WCC	white cell count

using ustekinumab treatment (a monoclonal antibody blocking the IL-12/23 signalling pathway) in patients with chronic sarcoidosis [10].

Recently, a role for IL-27, an IL-12 related cytokine, has also been associated with granuloma formation after the cytokine was detected in granulomatous lesions of patients with sarcoidosis [11]. Our group previously detected elevated interleukin 27 (IL27, also known as p28) mRNA levels in peripheral blood of patients with sarcoidosis [12]. IL-27 is a heterodimeric cytokine comprised of two subunits, IL27 and Epstein-Barr virus induced-gene 3 (EBI3) [13]. This cytokine signals through a heterodimeric receptor formed from IL-27 receptor alpha (IL27RA) and interleukin 6 signal transducer (IL6ST, also known as gp130) and has been demonstrated to promote IFNγ release synergistic with IL-12 [13,14]. IL-27 could thus promote granuloma formation at early stages of the disease through promotion of Th1 cells and IFNγ. The cytokine might at the same time be involved in disease control, given that there is evidence that IL-27 down-regulates excessive immune responses [15].

IL-35 is yet another member of the IL-12 family of cytokines and comprises IL12A (p35) and EBI3 [16]. Similarly, the receptor comprises IL-12 receptor beta 1 (IL12RB1) and IL6ST [17]. It is believed to down-regulate excessive immune responses and has been shown to ameliorate airway inflammation in murine models [18,19]. Adoptive transfer of IL-35-induced T regulatory cells in a murine model of inflammatory bowel disease reverses the associated inflammation as long as both IL12A and EBI3 are active [20,21]. Since IL-35 shares one subunit with IL-12 (IL12A) and the other subunit with IL-27 (EBI3) and both IL-12 and IL-27 appear to be involved in the pathogenesis of sarcoidosis, we aimed to further characterise the expression of the IL-12 family cytokines IL-27 and IL-35 following direct stimulation of macrophages using LPS or indirect activation using anti-CD3/28 beads. We hypothesised that one or both of these cytokines may be dysregulated in patients with sarcoidosis compared to controls. We also aimed to characterise the expression of subunits of the IL-27 receptor (IL6ST and IL27RA) in PBMC and peripheral blood T helper cells in patients with sarcoidosis and healthy controls.

2. Methods

2.1. Subjects

This cross-sectional study was approved by the Human Research Ethics Committee at Prince of Wales Hospital (HREC Ref. NO 10/134) and informed, written consent was obtained from all patients and healthy control volunteers. Inclusion criteria for patients were a prior diagnosis of sarcoidosis according to the criteria established by the World Association of Sarcoidosis and Other Granulomatous diseases, the American Thoracic Society and the European Respiratory Society [22]. The final diagnosis was either clinical (Löfgren's syndrome or uveitis) or established by positive biopsies featuring non-caseating granulomas or typical bronchoalveolar lavage (BAL) lymphocytosis with CD4/CD8 ratios >3.5. Treatment status was recorded for all patients but immunosuppressive treatment was not an exclusion criterion in this study. Diffusion capacity of the lungs for carbon monoxide (DLCO), serum ACE levels and lymphocyte counts were recorded in most patients as they can be used in monitoring the disease. Spirometry was performed on healthy participants when possible and a significant history of lung, atopic, autoimmune or granulomatous disease was used as exclusion criteria.

2.2. PBMC and plasma isolation and cryopreservation

Approximately 50 mL of blood was collected in anticoagulant Vacutainer tubes (BD Biosciences, Australia). All samples were processed on the same day and whole blood cell count was performed using a Coulter cell counter (Beckman Coulter, California, USA). Plasma was separated and PBMC were isolated via density gradient centrifugation using Ficoll-Hypaque Lymphoprep (Stemcell, Australia). Total RNA was isolated from 5×10^6 PBMC using TriReagent (Sigma-Aldrich, Australia). Plasma and PBMC were stored at in vapour-phase nitrogen until further analysis.

2.3. Antigen stimulation assay

For the antigen stimulation assays, PBMC were thawed, washed and rested overnight. On Day 2, 5×10^5 PBMC were added to the wells of 96-well plates (Greiner Bio-One, Germany) in 250 μl of media containing FBS, Penicillin and Streptomycin. PBMC were stimulated with LPS (1 μg/mL, from *E. coli* strain 0111:B4 purified by phenol extraction, Sigma-Aldrich, Australia), anti-CD3/28 activation beads (Thermo Fisher Scientific, CA, USA) at a concentration of 1 bead/8.5 cells. Cells were lysed in TriReagent after 6 h for collection of total RNA. Cell culture supernatant was collected after 48 h for protein analysis.

2.3.1. RNA extraction and cDNA synthesis

Total RNA was extracted from PBMCs using TriReagent (Sigma-Aldrich) and the RNA concentration and 260/280 ratio were