Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12799

Recombinant *Salmonella typhimurium* outer membrane protein A is recognized by synovial fluid CD8 cells and stimulates synovial fluid mononuclear cells to produce interleukin (IL)-17/IL-23 in patients with reactive arthritis and undifferentiated spondyloarthropathy

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Accepted for publication 4 April 2016
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Summary

In developing countries, one-third of patients with reactive arthritis (ReA) and undifferentiated spondyloarthropathy (uSpA) are triggered by *Salmonella typhimurium*. Synovial fluid mononuclear cells (SFMCs) of patients with ReA and uSpA proliferate to low molecular weight fractions (lmwf) of outer membrane proteins (Omp) of *S. typhimurium*. To characterize further the immunity of Omp of *Salmonella*, cellular immune response to two recombinant proteins of lmwf, OmpA and OmpD of *S. typhimurium* (rOmpA/D-sal) was assessed in 30 patients with ReA/uSpA. Using flow cytometry, 17 of 30 patients' SF $CD8^+$ T cells showed significant intracellular interferon (IFN)-γ to Omp crude lysate of *S. typhimurium*. Of these 17, 11 showed significantly more $CD8^+CD69^+$ IFN-γ T cells to rOmpA-sal, whereas only four showed reactivity to rOmpD-sal. The mean stimulation index was significantly greater in rOmpA-sal than rOmpD-sal [3·0 (1·5–6·5) *versus* 1·5 (1·0–2·75), $P < 0·005$]. Similarly, using enzyme-linked immunospot (ELISPOT) in these 17 patients, the mean spots of IFN-γ-producing SFMCs were significantly greater in rOmpA-sal than rOmpD-sal [44·9 (3·5–130·7) *versus* 19·25 (6–41), $P < 0·05$]. SFMCs stimulated by rOmpA-sal produced significantly more proinflammatory cytokines than rOmpD-sal: IFN-γ [1·44 (0·39–20·42) *versus* 0·72 (0·048–9·15) ng/ml, $P < 0·05$], interleukin (IL)−17 [28·60 (6·15–510·86) *versus* 11·84 (6·83–252·62) pg/ml, $P < 0·05$], IL-23 [70·19 (15–1161·16) *versus* 28·25 (> 15–241·52) pg/ml, $P < 0·05$] and IL-6 [59·78 (2·03–273·36) *versus* 10·17 (0·004–190·19) ng/ml, $P < 0·05$]. The rOmpA-sal-specific $CD8^+$ T cell response correlated with duration of current synovitis ($r = 0·53$, $P < 0·05$). Thus, OmpA of *S. typhimurium* is a target of SF $CD8^+$ T cells and drives SFMC to produce increased cytokines of the IL-17/IL-23 axis which contribute to the pathogenesis of *Salmonella*-triggered ReA.

Keywords: antigens, arthritis, bacterial, cytotoxic T cells, cytokines

Introduction

Reactive arthritis (ReA) presents as acute asymmetrical lower limb oligoarthritis with or without enthesitis, conjunctivitis or urethritis within 2–4 weeks of enteric or genitourinary infections. It belongs to a heterogeneous group called seronegative spondyloarthropathies (SSA), which have a strong association with human leucocyte antigen (HLA)-B27 [1,2]. The various microbes implicated are *Salmonella typhimurium*, *Shigella*, *Campylobacter jejuni*, *Yersinia enterocolitica* and *Chlamydia trachomatis* in ReA. Several patients who have a similar clinical picture but do not have a history of preceding infection and do not fulfil the criteria for other members of SSA are labelled as undifferentiated spondyloarthropathy (uSpA) [3]. A raised level of sera antibodies to enteric bacteria responsible for ReA was observed in patients with uSpA [4]. T cells play an important role in mediating synovial inflammation, as evidenced by the presence of both $CD4^+$ and $CD8^+$ cells in the synovial fluid (SF) [5,6]. The antigenic targets recognized by T cells in *Chlamydia*-induced arthritis are Chlamydial heat shock protein (Hsp)60, histone-derived protein Hc1 and outer membrane protein 2 (Omp 2) [7], whereas the β subunit of urease (19 kDa protein), 32-kD

ribosomal L2 protein and Hsp60 are the major antigenic proteins in *Yersinia*-induced ReA [8]. Although HLA-B27-restricted antigen-specific CD8 T cells are reported in SF of *Salmonella*-induced ReA patients [9,10], the antigenic targets have not been defined. We have reported previously that SFMCs of one-third of patients with ReA/uSpA show a significant lymphoproliferative response to the crude lysate of *S. typhimurium* [11]. Further, Omp was found to be more immunogenic than the cytosolic constituents of *S. typhimurium* [12]. SF T cells proliferated more significantly in the low molecular weight fractions (< 22–40 kDa) than the high molecular weight fractions of Omp. Proteomic analysis of the low molecular weight fractions by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis predicted 10 proteins: 37 kDa OmpA, 33 kDa TsX, 28 kDa putative Omp, 28 kDa Vac J, 39 kD OmpD, 18 kDa OmpX, 23 kDa OmpW, 43 kDa OmpS1 and 19 kDa peptidoglycan-associated lipoprotein. OmpA was present in most of the low molecular weight fractions, whereas OmpD was present in one of the stimulatory fractions [13]. Therefore, we sought to investigate whether there is an antigen-specific T cell response to recombinant *Salmonella* OmpA or D and whether these recombinant proteins stimulate SFMC to produce proinflammatory cytokines in the SF of patients with ReA and uSpA.

Material and methods

Patients and controls

Thirty patients with ReA/uSpA were recruited into the study. ReA was defined as the presence of acute or subacute onset asymmetrical lower limb oligoarthritis, preceded by a diarrhoeal illness within the past 4 weeks, whereas patients with uSpA satisfied the European seronegative spondyloarthropathy group (ESSG) criteria [14]. Clinical variables, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and radiological sacroiliitis were assessed. HLA-B27 analysis was performed by polymerase chain reaction (PCR) using sequence-specific primers [15]. Ten patients with rheumatoid arthritis (RA) fulfilling American College of Rheumatology (ACR) criteria [16] were included as disease control. The study was approved by the Institute Ethics Committee and written informed consent was taken from all subjects. Peripheral blood (PB) and SF was collected during therapeutic paracentesis and synovial fluid and peripheral blood mononuclear cells (SF/PBMCs) were isolated by density gradient centrifugation.

Synthesis of *Salmonella* Omp crude lysate and recombinant OmpA and OmpD

Crude Omp lysate of *S. typhimurium* (ATCC 22733) (Sal-crude OMP) was prepared by ultracentrifugation as described previously [17]. A pellet containing Omp was dissolved in sodium lauryl sarcosine for the removal of lipids, incubated for 30 min and centrifuged again at 105 000 ***g*** to acquire the crude lysate of Omp.

DNA from *S. typhimurium* was isolated using the CTAB (Cetyl tri-methylammonium bromide) method. OmpA and OmpD genes were amplified by PCR, using the following primers: OmpA, forward CGAATTCATGCC-GAAA GATAA-CACCTGGT and reverse GCTCGAGAAGCCTG CGGCTGAGTTA; for OmpD, forward CGGATCCATGGA GGTATATAACAAA and reverse CAAGCTTGG-AACTGG TA-GTTCAG. Cloning of amplified OmpA and D genes was performed using the pGEMT kit (Promega, Madison, WI, USA) in *Escherichia coli* DH5-alpha strain (gift from CIMAP, Lucknow, India) as per the manufacturer's protocol. In brief, the amplified fragments were ligated into pGEMT vector; the positive clones were selected and confirmed by sequencing. The desired EcoR1-digested fragments were gel eluted (Qiagen gel elution kit; Limburg, the Netherlands) and cloned again in a pET 28a (Noavagen, Darmstadt, Germany) expression vector and confirmed by sequencing. Expression of the cloned gene was induced by addition of 0·5 mM isopropyl-P-D- thiogalactopyranoside (IPTG) (Sigma, St Louis, MO, USA) for 6 h at 37°C in the BL-21 strain of *E. coli*. Cells were harvested, and recombinant protein tagged to Histidine was purified using Ni-NTA resin affinity columns (Merck, New York, NY, USA), according to the manufacturer's protocol (Supporting information, Fig. S1)

Enumeration of antigen-specific frequency of SF T cells

Flow cytometry. The frequency of antigen-specific SF T cells producing interferon (IFN)-γ and T helper type 17 (Th17) cells was analysed by flow cytometry. Cells (1×10^6) were stimulated with 10 μg/ml of rOmpA-sal, rOmpD-sal, Sal-OMP crude and phorbol myristate acetate (PMA) (50 ng/ml) + ionomycin (1·0 μg/ml) (Sigma) as positive control for 6 h. Golgi plug monensin (2 μM; Sigma) was added in the last 4 h of stimulation. Surface staining of cells was performed with anti-CD8 allophycocyanin (APC) (clone RPA-T8), anti-CD4 phycoerythrin (PE) cyanin 7 (Cy-7) (clone SK3) and anti-CD69 PE (clone FN50). Cells were fixed and permeabalized using the cytoperm/cytofix kit (AbD Serotec, Kidlington, Oxford, UK) and stained with anti-IFN-γ fluorescein isothiocyanate (FITC) (clone RPA-T8) and anti-interleukin (IL)−17 PE (clone SCPL 1362). All monoclonal antibodies were obtained from BD Biosciences (San Diego, CA, USA) except for antibodies to IFN-γ FITC, which was procured from Beckman Coulter (Pasadena, CA, USA). PBMCs of three patients were analysed for antigen-specific responses, as for SFMCs, by flow cytometry. Acquisition of cells was performed using the Beckman Coulter flow cytometer. $CD8^+/CD4^+CD69^+$ IFN-γ^+ T cells were

assigned as antigen-specific, while $CD4^+IL\text{-}17^+$ T cells were defined as Th17 cells.

Enzyme-linked immunospot (ELISPOT) assay. IFN-γ ELISPOT assay was performed as per the manufacturer's protocol (BD Biosciences, San Diego, CA, USA). For stimulation, $1{\cdot}0 \times 10^5$ SFMCs were taken and cultured with 10 μg/ml of rOmpA-sal and rOmpD-sal for 24 h at 37°C and 5% CO_2. Spots were read on a Biosys 4000 instrument (Miami, FL, USA).

Definition of a positive response by flow cytometry and ELISPOT. Using flow cytometry, a positive response was defined if the stimulation index (SI) (frequency of antigen-specific $CD8/CD4^+$ ($CD69^+IFN\text{-}\gamma^+$) T cells in antigen-stimulated/frequency of $CD8/CD4^+$ ($CD69^+IFN\text{-}\gamma^+$) T cells without stimulation was ≥ 3 in response to Sal-crude OMP or rOmpA-sal and rOmpD-sal [18]. Similarly for ELISPOT, a positive response was defined if the number of IFN-γ-producing cells (spots) in response to antigens was more than three times the spots without the antigens.

Estimation of cytokines in culture supernatant of rOmpA-sal- and D-sal protein-stimulated cultures. SFMCs, $1{\cdot}0 \times 10^5$/ml, were stimulated with 10 μg/ml of recombinant antigen in complete RPMI-1640 medium for 48 h at 37°C in a 5% humidified CO_2 incubator. Culture supernatant was collected and stored at −40°C until analysis. Cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) with IFN-γ and IL-6 kits (BD Biosciences, San Diego, CA, USA) and IL-17 and IL-23 kits (e-Biosciences, San Diego, CA, USA), according to the manufacturer's instructions. The detection limit for IFN-γ and IL-6 was 4·7–300 pg/ml, while for IL-17 and IL-23 it was 4–500 pg/ml and 15–2000 pg/ml, respectively. Absorbance was read using an ELISA plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Non-parametric tests were used for intergroup comparisons. Paired analysis between responses to rOmpA-sal and rOmpD-sal was performed by Wilcoxon's matched-pairs test. The flow cytometry and ELISPOT data were represented as median with interquartile range, while for ELISA the data are represented as median and range. Spearman's correlation was used to analyse the correlation between different disease parameters with antigen-specific immune response. A P-value < 0.05 was taken as significant. All analysis was performed using SPSS version 16 and GraphPad Prism version 6 (trial version) software.

Table 1. Demographic features of reactive arthritis/undifferentiated spondyloarthopathy (ReA/uSpA) patients

Clinical details	ReA/uSpA ($n = 30$)
M:F	25:5
ReA/uSpA	16/14
Median age (years, range)	27·5 (15–65 years)
Duration of current episode (range)	30 days (3 days–1·5 years)
Erythrocyte sedimentation rate	59·5 (5–122)
Median mm/h (range)	
C-reactive protein (CRP) (mg/dl)	3·4 (0·33–9·65)
HLA-B27 positivity	22/29

M = male; F = female; HLA = human leucocyte antigen.

Results

Patients

Thirty patients (25 males, five females) with a median age of 27·5 years (15–65 years) were recruited. Sixteen patients had ReA and 14 had uSpA. The median duration of the current episode was 30 days (3 days–1·5 years). Twenty-two of the 29 patients tested were HLA-B27-positive (Table 1). Ten patients with RA (seven females, three males) with a median age of 45·5 (38–57) years were recruited as disease controls. All patients were positive for RF. The median disease duration in RA was 3 years (3 months–11 years) and median disease activity score (DAS ESR) of patients with RA was 4·06 (3·5–6·52).

Patients with immunity to Salmonella. Seventeen (12 ReA and 5 uSpA) of 30 patients were positive for SF CD8 T cell against Sal-crude OMP by flow cytometry and were categorized as patients with immunity to *Salmonella*. Of these 17 patients, 14 (82%) were positive for HLA-B27. These patients were analysed further for rOmpA-sal and rOmpD-sal-specific responses by flow cytometry, ELISPOT and ELISA.

Antigen-specific T cells by flow cytometry

Of the 17 who showed an immune response to Salmonella SF $CD8^+$ T cells, 11 (64·7%) responded to rOmpA-sal, whereas only five (29·4%) patients' $CD8^+$ T cells showed a response to rOmpD-sal. The median SI was significantly higher for rOmpA-sal compared to rOmpD-sal [3·0 (1·5–6·5) *versus* 1·5 (1·0–2·75), $P < 0{\cdot}005$; Fig. 1a,b]. There was a significant positive correlation between duration of current episode of knee joint arthritis with $CD8^+$ T cell response (SI) to Sal-crude OMP ($r = 0{\cdot}58$, $P < 0{\cdot}05$), as well as with rOmpA-sal ($r = 0{\cdot}53$, $P < 0{\cdot}05$), Fig. 1c. There was no difference between the response to either rOmpA or rOmpD between ReA and uSpA patients. Of 10 patients with RA, only one patient showed a $CD8^+$ T cell response to Sal-crude OMP, and no patients showed responses to rOmpA-sal or rOmpD-sal.

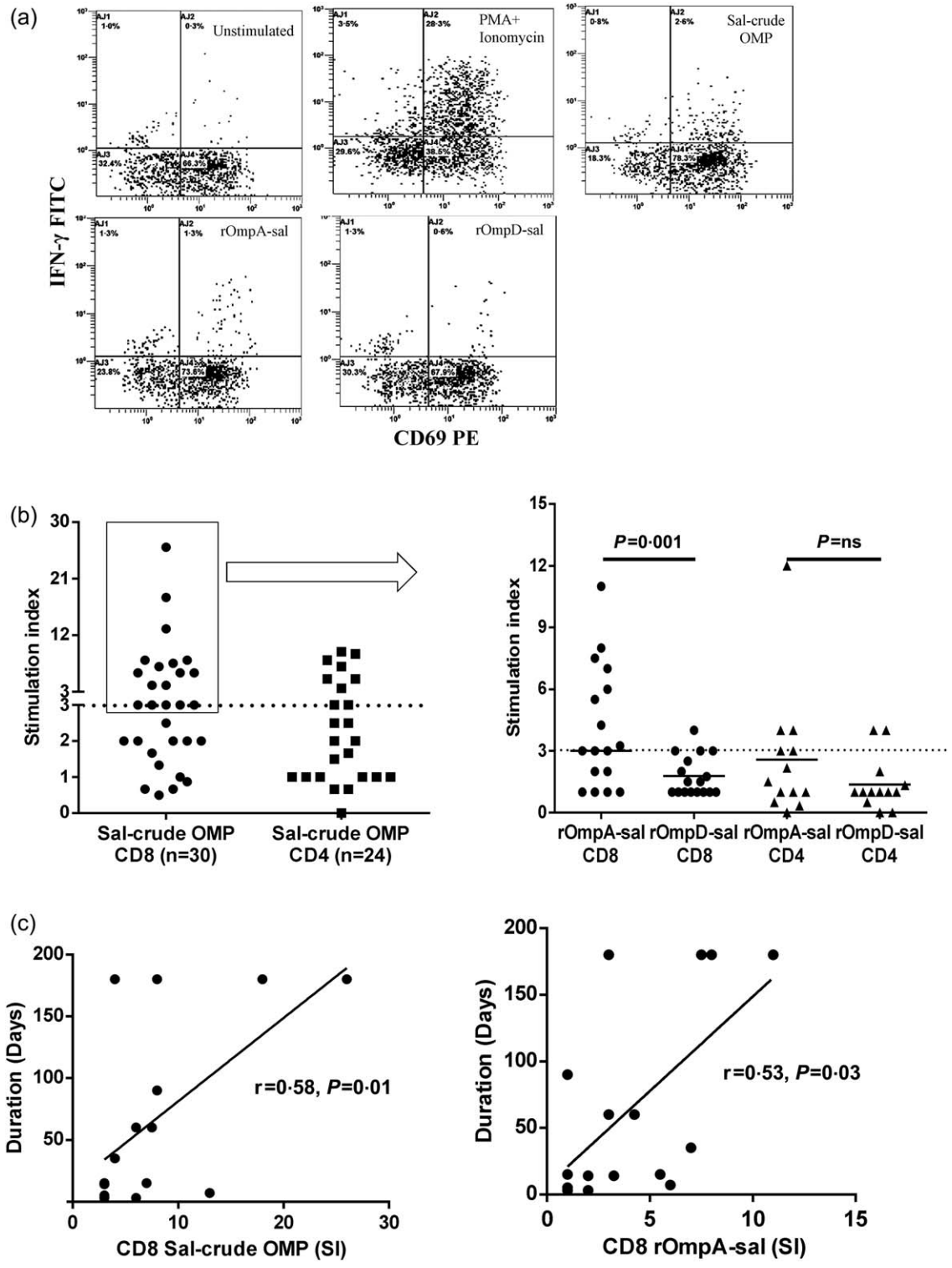

Fig. 1. Recombinant (r) outer membrane proteins A and D of *Salmonella typhimurium* (rOmpA/D-sal)-specific SF T cell response by flow cytometry. (a) Representative flow cytometry plot of synovial fluid (SF) CD8 T cell response to Sal-crude Omp, phorbol myristate acetate (PMA) + ionomycin (positive control), rOmpA-sal and rOmpD-sal. $CD69^+$interferon (IFN)-γ^+ CD8 T cells are taken as antigen-specific cells. (b) Scatter-plot of stimulation index (SI) of CD8 and CD4 SF T cells to Sal-crude OMP. SF CD8 T cells from 17 patients showing immunity to Sal-crude OMP (in square box) were analysed for rOmpA-sal- and rOmpD-sal-specific $CD8^+$ T ($n = 17$) and $CD4^+$ T ($n = 13$ of 17) cell responses. The *y*-axis represents the SI. The horizontal bar represents the median, $P < 0{\cdot}05$ was taken as significant, n.s. = not significant. (c) Graph showing Spearman's correlation between $CD8^+$ T cell responses (stimulation index) to Sal-crude OMP and rOmpA-sal with duration of current episode (days). $P < 0{\cdot}05$ was taken as significant.

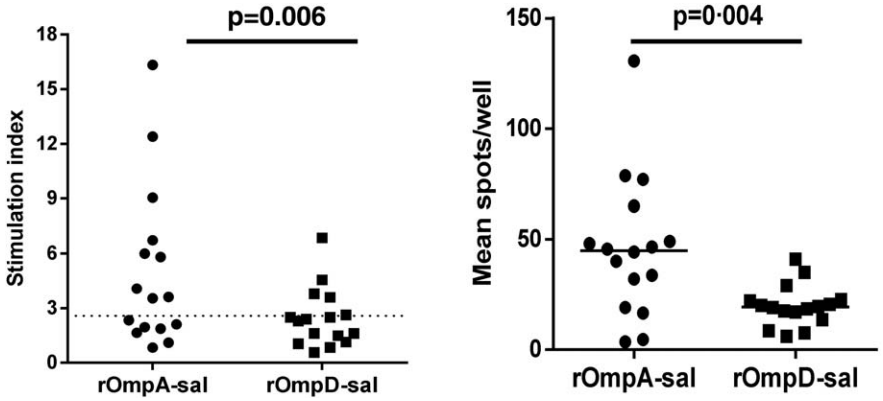

Fig. 2. Recombinant (r) outer membrane proteins A and D of *Salmonella typhimurium* (rOmpA/D-sal)-specific response by enzyme-linked immunosorbent assay (ELISPOT) assay. Scatter-plot of rOmpA-sal- and rOmpD-sal-stimulated interferon (IFN)-γ-producing synovial fluid mononuclear cells (SFMCs) in 16 reactive arthritis/undifferentiated spondyloarthopathy (ReA/uSpA) patients' synovial fluid showing immunity to *Salmonella* by ELISPOT assay. The *y*-axis represents the stimulation index and mean spots/well in response to antigen used (*x*-axis). The horizontal bar represents the median. $P < 0{\cdot}05$ was taken as significant.

Thirteen of 17 SFMC patients were tested for $CD4^+$ T cell response. A CD4 response to rOmpA-sal was seen in five of 13 patients, while only two showed a response to rOmpD-sal. No difference was found in SI for rOmpA-sal and rOmpD-sal, 1·5 (0·75-3·5) *versus* 1·0 (0·75-1·66), $P =$ n.s. (Fig. 1b).

Antigen-specific $CD4^+IL\text{-}17^+$ T cells were not increased in both recombinant antigens in all 11 patients who were analysed (data not shown). None of the RA patients showed $CD4^+$ response to Sal Omp crude and to recombinant antigens.

Antigen-specific T cells frequency by ELISPOT assay

Sixteen of 17 patients showing immunity to *Salmonella* were tested for antigen-specific IFN-γ-producing SFMCs by ELISPOT; nine (56.2%) of the patients' SFMCs showed a positive response to rOmpA-sal, compared to only 4 (25%) to rOmpD. Stimulation index and number of spots (IFN-γ-producing cells) per well in these 16 patients was significantly higher to rOmpA-sal than rOmpD-sal, 3.59 (1.9–6.5) versus 2.35 (1.24–3.35), $p < 0.05$ and 44·9 (3·5–130·7) *versus* 19·25 (6–41), $P <$ 0·05 respectively; Fig. 2, Supporting information, Fig. S2. Only the SF cells of one patient with RA showed a positive response to Sal-crude Omp and to rOmpA-sal protein.

Production of proinflammatory cytokines

SFMCs of all 17 patients with evidence of *Salmonella* immunity were stimulated with rOmpA-sal and rOmpD-sal, and the levels of proinflammatory cytokines in culture supernatant were compared. Upon stimulation, with rOmpA-sal SFMCs produced significantly more IFN-γ [1·44 (0·39–20·42) ng/ml *versus* 0·4 ng/ml (0·041–4·43) ng/ml, $P < 0{\cdot}05$], IL-17 [28·60 (6·15–510·86) pg/ml *versus* 14·17 pg/ml (> 4–179·15) pg/ml, $P < 0{\cdot}05$], IL-23 [70·19 (15–1161·16) pg/ml *versus* 30·04 pg/ml (> 15–197·15) pg/ml, $P < 0{\cdot}05$] compared to unstimulated SFMCs. IL-6 levels were significantly higher upon stimulation with both rOmpA-sal [59·78 ng/ml (2·03–273·36) ng/ml *versus* 7·82 ng/ml (1·40–153·80) ng/ml, $P < 0{\cdot}0001$] and rOmpD-sal [10·17 (1·72–190·2) ng/ml *versus* 7·82 ng/ml (1·40–153·80) ng/ml, $P < 0{\cdot}05$] when compared with unstimulated SFMCs. However, SFMCs produced significantly more IFN-γ [1·44 (0·39–20·42) ng/ml *versus* 0·72 (0·048–9·15) ng/ml, $P < 0{\cdot}05$], IL-17 [28·60 (6·15–510·86) pg/ml *versus* 11·84 (6·83–252·62) pg/ml, $P < 0{\cdot}05$], IL-23 [70·19 (15–1161·16) pg/ml *versus* 28·25 (> 15–241·52) pg/ml, $P <$ 0·05] and IL-6 [59·78 (2·03–273·36) ng/ml *versus* 10·17 (0·004–190·19) ng/ml, $P < 0{\cdot}05$] in response to rOmpA-sal compared to rOmpD-sal (Fig. 3). Cytokine levels were not found to be elevated significantly in culture supernatant with SFMC from patients with RA either with rOmpA-sal or rOmpD-sal compared to unstimulated ones.

Discussion

The present study is a continuation of our previous investigations to define the antigenic targets of *S. typhimurium* of T cells in ReA/uSpA, and the data suggest that rOmpA-sal is more immunogenic than rOmpD to SF T cells by elucidation of the antigen-specific T cell response by fluorescence activated cell sorter (FACS) and IFN-γ-producing SFMC by ELISPOT. Further, rOmpA-sal stimulates SF cells to produce proinflammatory cytokines.

The presence of increased antigen-specific $CD8^+$ T cell frequency to whole Omp of *S. typhimurium* was used to identify patients with ReA/uSpA with previous exposure to *Salmonella*. As a greater number of patients showed a significant response to $CD8^+$ T cells than $CD4^+$ T cells, we took this parameter to identify patients for further studies with recombinant bacterial antigens. In previous reports,

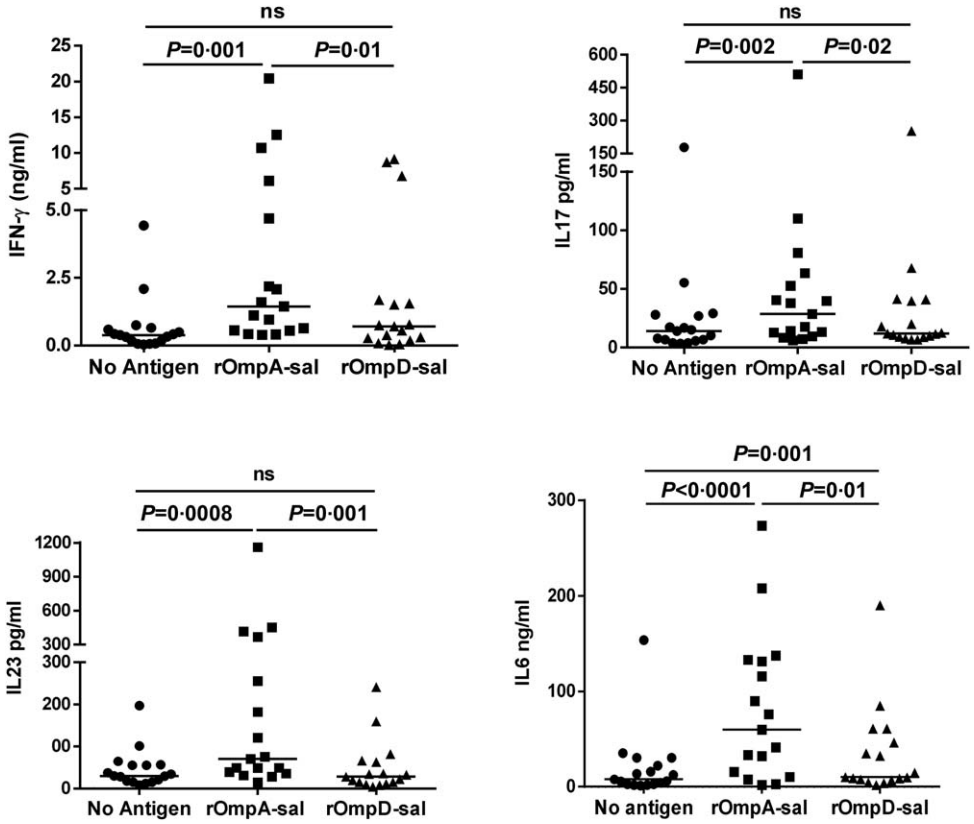

Fig. 3. Recombinant (r) outer membrane proteins A and D of *Salmonella typhimurium* (rOmpA/D-sal)-stimulated production of proinflammatory cytokines. Significantly higher levels of Interferon (IFN)-γ (a), interleukin (IL)−17 (b), IL-23 (c) and IL-6 (d) in culture supernatant of synovial fluid mononuclear cells (SFMCs) upon stimulation with rOmpA-sal as compared to no antigen and rOmpD-sal, in 17 reactive arthritis/ undifferentiated spondyloarthopathy (ReA/uSpA) patients showing immunity to *Salmonella*. The horizontal bar represents the median. $P < 0{\cdot}05$ was taken as significant.

nearly one-third of patients with ReA/uSpA showed SFMC proliferation by lymphocyte transformation testing (LTT) to crude whole bacterial lysate [11–13], suggesting a trigger by *Salmonella*. Flow cytometry provides rapid detection of the antigen-specific response [19,20] without need of radiolabelled compounds such as tritiated thymidine. Using the Omp-specific SF T cell response, a higher proportion of patients [17 of 30 (56.7%)] showed evidence of previous exposure to *Salmonella*. It cannot be inferred that ReA/uSpA were triggered by *S. typhimurium*. In western countries, elevated levels of serum immunoglobulin (Ig)G and IgA antibodies to enteric bacteria are used more commonly to identify sporadic cases of *Salmonella* induced ReA/uSpA; however, due to the high incidence of gut infection in our country [11] this is not reliable. Cellular responses in peripheral blood to bacterial antigens are usually negative or weak. We tested three patients' PBMC for antigen-specific T cell response (data not shown) and found that they were all negative to Sal-crude OMP.

A higher $CD8^+$ T cell response (57%) in the SF compared to $CD4^+$ T cells to *Salmonella* whole Omp crude is expected because ReA/uSpA are associated strongly with HLA-B27, as the arthritogenic peptide is presented by antigen-presenting cells in the context of major histocompatibility complex (MHC) Class I molecules to $CD8^+$ T cells. A mitogenic response was more evident in $CD8^+$ T cells than $CD4^+$ T cells, showing a more activated status of the former. A predominant $CD8^+$ T cell response has been reported in patients with *Yersinia*- and *Salmonella*-induced ReA in the synovial compartment [9,10,21]. In HLA-B27 transgenic mice, as well as in synovial fluid of *Chlamydia*-induced ReA patients, peptides derived from different *C. trachomatis* proteins stimulated CD8 T cells in an HLA-B27-restricted manner [22]. The pathogenic potential of HLA-B27-restricted $CD8^+$ T cells in mediating the pathogenesis of arthritis and bowel diseases has been doubtful [23]. The presence of homodimeric forms of HLA-B27 on antigen-presenting cells provides an expansion of antigen-specific $CD4^+$ T in transgenic mice [24]. Antigen-specific $CD4^+$ T cells have been shown in *Chlamydia*- [7] and *Yersinia*-induced ReA to Hsp60 protein [25]. The presence of $CD4^+$ T cells, even if fewer in number, licences APC and facilitates $CD8^+$ T cell killing of target cells. A higher number of $CD69^+CD8^+$ and $CD4^+$ T cells as well as cytokine production without antigen stimulation *ex vivo* was observed, implying preactivation of SFMCs by *in-vivo* bacterial antigenic exposure, and seen in an earlier report on the SF [26]. A positive correlation between the SI of CD8 T cells in response to Sal-crude OMP and rOmp-A but not with rOmpD-sal was seen with the duration of the current episode of synovitis, implying a higher number of memory $CD8^+$ T cells with persistent disease.

There have been no reports of T cell or humoral response to *Salmonella* rOmpA-sal or D in patients with typhoid fever or *Salmonella*-induced ReA. However, immunogenicity to OmpA has been studied in murine or human

macrophages and dendritic cells (DCs). OmpA of *Klebsiella pneumoniae* binds and activates human macrophages to produce IL-1β, IL-8, IL-10, IL-12 and tumour necrosis factor (TNF)-α and nitric oxide by murine macrophage cell lines [27], as well as eliciting cytotoxic T lymphocyte (CTL) responses to human DCs [28], while OmpA of *S. typhimurium* activates mouse DC cells and helps in Th1 polarization [29]. In line with these observations, a higher SF CTL response in patients with ReA/uSpA to rOmpA-sal was observed. OmpA is a transmembrane protein consisting of an N-terminal eight-transmembrane amphipathic β-barrel region, while the C-terminal region is retained in the periplasm. OmpA maintains cell integrity, bacterial conjugation and bacteriophage binding. It also facilitates Gram-negative bacteria to invade mammalian cells [30]. Most of the immunogenic residues lie near the C-terminal region [31].

A smaller number of patients' T cells responded to rOmpD-sal compared to rOmpA-sal by both flow cytometry and ELISPOT. With the exception of IL-6, no other cytokine production was increased in response to OmpD-sal compared to non-treated cells. Like OmpA, there have been no studies in humans investigating the immune response to OmpD, which is the major constituent of the porins present in the cell membrane of *S. typhimurium* and plays a major role in facilitating bacterial survival. It causes efflux of toxic compounds generated during infection in the host and serves to bind the bacteria to human macrophages and intestinal cells [32]. In mice immunized with porins (containing mainly OmpD), IgM antibodies are produced by B1b cells and these antibodies reduce *S. typhimurium* bacteraemia, suggesting a protective role for OmpD [33]. In RAW264.7, a mouse macrophage cell line, OmpD inhibits replication of *S. typhimurium* and reduces systemic dissemination by neutralizing reactive oxygen species [34].

OmpA activates both human and murine DCs through Toll-like receptor (TLR)−2 and TLR-4 and its signalling pathways [mitogen-activated protein kinase (MAP) kinase and extracellular-regulated kinase (ERK)] to produce proinflammatory cytokines such as IL-12, TNF, IL-6 and IL-18 [27–29]. In this study we found higher IL-6, IL-17, IL-23 and IFN-γ levels in response to rOmpA-sal compared to rOmpD-sal, although we failed to detect significant intracellular IL-17 production in CD4 T cells by FACS. This could be related to only 6 h stimulation compared to 48 h stimulation for the former assay. Alternatively, the source of IL-17 could be CD8 T cells (Tc17 cells) [35] or innate cells such as natural killer (NK) cells and γδ-T cells in SFMC [36]. Increased levels of IL-23 and IL-6 in response to rOmpA-sal may lead to the induction of IL-17-producing cells. Mice infected with *Salmonella enterica* express IL-17 in draining lymph nodes and develop arthritis early after onset of enterocolitis [37]. We and others have shown that patients with ReA/uSpA have increased IL-17 and IL-6 in the synovial fluid compared to patients with rheumatoid arthritis [38,39], but IL-23 was not detected in our previous study [39]. Here we have shown that rOmpA-sal could induce IL-23 production in SFMCs. The IL-17/IL-23 axis has an important pathogenic role in patients with B27-related spondyloarthritis, such as ankylosing spondylitis and psoriasis. The use of anti-IL-17 antibodies has shown excellent benefit in ameliorating the skin and joint symptoms of psoriatic arthritis [40]. The success of the monoclonal antibody tocilizumab, which targets the receptor for IL-6 (IL-6R) in the treatment of inflammatory arthritis and a subset of other immunological conditions, has established IL-6 as a major player in such inflammatory conditions [41].

Therefore, our data suggest that the dominant antigenic epitopes in *Salmonella*-induced ReA/uSpA probably lie within OmpA. By using smaller fragments of OmpA or performing peptide mapping in future we can define the exact T cell epitope of OmpA in patients with *Salmonella*-triggered ReA. This may help in devising a diagnostic test and advancing our understanding of the pathogenesis of *Salmonella*-induced ReA/uSpA. The strength of our study is the use of robust techniques such as flow cytometry and ELISPOT to demonstrate rOmpA-sal CD8/CD4$^+$ T in SF for the first time in the ReA synovial fluid compartment, although HLA-B27 restriction and intracellular signalling pathways have yet to be explored.

In conclusion, our data suggest that in ReA/uSpA patients' OmpA is immunogenic to synovial fluid mononuclear cells. It is recognized mainly by CD8$^+$ T cells of SF and induced proinflammatory cytokine in the SF of ReA/uSpA patients.

Acknowledgements

This work was supported by extramural fund awarded to R. M. by Department of Biotechnology, New Delhi, India. S. C. was supported by a Fellowship from the University Grant Commission, New Delhi, India.

Disclosure

The authors have declared no disclosures.

Author contributions

S. C. performed all experimental work and manuscript writing. R. M. performed the study design, patient recruitment and manuscript editing. A. K. S. conducted the experimental guidance of recombinant protein expression. A. A. contributed to the study design, patient recruitment, experimental trouble shooting and manuscript editing.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel showing the recombinant (r) outer membrane proteins A and D of *Salmonella typhimurium* (rOmpA/D-sal). Transformation of the BL-21 strain of *Escherichia coli* was performed with pET vector only, pET-OmpA gene and pET-OmpD gene. Lane 1 is the molecular weight marker; I (lanes 3, 5 and 8) denotes induced with 0·5 mM isopropyl-P-D- thiogalactopyranoside; UI (lanes 2, 4 and 7) is for the uninduced BL-21 strain of *E. coli*; purified (lanes 6, 9) denotes the recombinant protein after Ni-His column purification.
Fig. S2. Representative picture of interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay of the recombinant (r) outer membrane proteins A and D of *Salmonella typhimurium* (rOmpA/D-sal)-specific response to reactive arthritis (ReA) patients' synovial fluid mononuclear cells (SFMCs). SFMCs stimulated with well 1: no antigen treatment; well 2: phorbol myristate acetate (PMA) + ionomycin treatment; well 3: crude OMP-sal; well 4: rOmpA-sal-treated; well 5: rOmpD-sal. Each spot represents a single IFN-γ-producing cell.