Identification of Immunogenic HLA-B*27:05 Binding Peptides of Salmonella Outer Membrane Protein in Patients with Reactive Arthritis and Undifferentiated Spondyloarthropathy

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ABSTRACT. ***Objective.*** Salmonella outer membrane proteins (OMP) are major immunogenic targets to synovial fluid lymphocytes of patients with reactive arthritis (ReA)/undifferentiated spondyloarthropathy (uSpA). Because these patients have genetic predisposition to HLA-B*27 and its subtype HLA-B*27:05, we sought to identify immunogenic HLA-B*27:05-binding salmonella OMP peptides in patients with ReA/uSpA.

Methods. A total of 125 HLA-B*27:05-binding salmonella OMP peptides identified using ProPred-I software were synthesized and grouped in 23 pools. The peptide pools, along with crude enteric bacterial lysates and salmonella OMP, were cultured with synovial fluid (SF) or peripheral blood mononuclear cells (PBMC) from 23 patients with ReA/uSpA, 10 with rheumatoid arthritis (RA), and 10 healthy individuals in 96-well culture plates. Proliferation was measured by tritiated thymidine uptake and interferon-γ (IFN-γ) levels in culture supernatant. Individual peptides from pools having significant responses were retested with cryopreserved cells. Immunogenic peptides thus identified were further tested in 5 additional new patients with ReA/uSpA by flow cytometry. A Basic Local Alignment Search Tool program was used to search for similar peptides from a protein bank of arthritogenic bacteria and human protein.

Results. Nineteen of 23 SFMC from ReA/uSpA showed a significant proliferative response to salmonella OMP, with minimal response of PBMC (1/10) from ReA/uSpA, SFMC from RA (1/10), or PBMC from controls (1/10). Nine salmonella OMP peptides, QRAEMLPTL, SRSGLNIAL, LRFLYAKSL, RLEGTWVKL, ARCIAPYAL, KLFLTTAAL, YRNSDFFGL, QRPAVRVKL, and YRVGPGDVL, were identified. Response to QRAEMLPTL was seen in 6/7 HLA-B*27:05-positive patients. All immunogenic peptides had sequence similarity with peptides from arthritogenic bacterial proteins, while 5 had similarity with peptides from human proteins.

Conclusion. Nine novel immunogenic OMP peptides binding to HLA-B*27:05 were identified that showed sequence similarity with other arthritogenic bacteria. (J Rheumatol First Release Jan 25 2013; doi:10.3899/jrheum.110849)

Key Indexing Terms:
REACTIVE ARTHRITIS SALMONELLA OMP HLA-B*27:05
EPITOPE MAPPING PEPTIDE LYMPHOCYTE TRANSFORMATION TEST

Reactive arthritis (ReA) belongs to a heterogeneous group of diseases called spondyloarthritis. ReA has a strong association with HLA-B*27[1] and is triggered by a distant mucosal infection of either gastrointestinal[2] or genitourinary tract by enteric bacteria or *Chlamydia trachomatis*[3], respectively. Major enteric bacteria responsible for ReA are *Salmonella typhimurium*, *Shigella flexneri*, *Campylobacter jejuni*, and *Yersinia enterocolitica*[4]. Salmonella is a major cause for sporadic ReA in India, whereas yersinia and chlamydia are the common organisms in Western countries[5].

The relationship of HLA-B*27 subtypes with spondyloarthropathies is variable; however, HLA-B*27:05 and HLA-B*27:04 are associated with seronegative spondyloarthropathies in all ethnic groups including Asian Indians[6]. Peptides derived from arthritogenic bacteria presented by HLA-B*27 have been studied in patients with ReA triggered by chlamydia and yersinia. Studies have shown that outer membrane proteins (OMP) of gram-negative bacteria are target antigens for the humoral immune response in patients with ReA[7]. Indeed, synovial

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mononuclear cells proliferate vigorously in response to salmonella OMP in patients with salmonella-induced ReA[5]. Further, we have shown that the immunogenicity lies in the low molecular weight fractions of salmonella OMP[8].

OMP peptides have been identified in patients with chlamydia- and yersinia-induced ReA[9], but the immunogenic peptides of salmonella OMP in patients with ReA have not been delineated. In this study, we sought to identify immunogenic HLA-B*27:05-binding peptides from salmonella OMP in patients with ReA/uSpA. Subsequently, the identified immunogenic peptides of salmonella OMP were analyzed for their similarity to other arthritogenic bacterial and human proteins using bioinformatics tools. Identification of immunogenic peptides from salmonella OMP may assist in designing better diagnostic tools and understanding the basic pathogenesis of ReA/uSpA.

MATERIALS AND METHODS

Patients. Patients with ReA or uSpA who had active arthritis in a knee joint and agreed to participate were studied. Patients were identified with having ReA if they had asymmetrical oligoarthritis mainly of the lower limb joints preceded by diarrhea within 4 weeks. Patients who had a clinical picture similar to ReA but did not have any history of genitourinary or gastrointestinal infections and who fulfilled the European Spondylarthropathy Study Group criteria[10] were categorized as having uSpA. The latter group could not be categorized as having a well-defined disease such as ankylosing spondylitis, psoriatic arthritis, or arthritis associated with inflammatory bowel disease. Paired samples of heparinized peripheral blood (PB) and synovial fluid (SF) were collected from 23 patients with ReA/uSpA (Table 1). For controls, paired PB and SF were collected from 10 patients with rheumatoid arthritis (RA) and PB from 10 healthy volunteers. Among 10 patients with RA, 8 were female, median age was 49 years (range 32–54 yrs), and all were rheumatoid factor-positive. Among 10 healthy controls, 9 were male, with a median age of 28 years (range 24–31 yrs). Synovial fluid mononuclear cells (SFMC)/peripheral blood mononuclear cells (PBMC) were separated by Histopaque (Sigma) density-gradient centrifugation, and were used immediately or cryopreserved in freezing media (Sigma).

The study was approved by the institutional ethics committee.

*HLA-B*27 typing*. HLA-B*27 typing was done by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) using B*27-specific primers (IDT), as described[11]. Further, HLA-B*27-positive samples were subtyped for HLA-B*27:02–HLA-B*27:09 using subtype-specific primers (IDT) as described[12]. Briefly, PCR with primer sets 3' and 5' (Appendix 1) was carried out in first-round screening and then other primer sets were tested in patients who were negative in first-round screening.

Table 1. Demographic details of 23 patients with reactive arthritis/undifferentiated spondyloarthropathy.

Characteristics	
Male:female ratio	21:2
Median age, yrs (range)	21.5 (12–42)
Median disease duration, mo (range)	24 (0.5–48)
Median duration of current episode, days (range)	20 (5–300)
No. patients with inflammatory backache	14
No. patients with enthesitis	16
No. patients with positive family history	2
Erythrocyte sedimentation rate, median mm/h (range)	56 (8–150)

*HLA-B*27:05-binding peptides from OMP of salmonella*. Because the whole genome sequence of *S. typhimurium* strain LT2 is available[13], FASTA format sequences of all 251 OMP were obtained from the US National Center for Biotechnology Information and used for further analysis in peptide prediction. The promiscuous MHC Class I binding peptide prediction server, ProPred-I[14], was used for identification of HLA-B*27:05-binding peptides. The threshold for proteosome and immune-proteosome filters was set to 4%. Peptides with log score ≥ 6 were used for synthesis and further experiments. All predicted peptides were synthesized commercially (Mimotopes) with immunological grade purity of 75%–80%. These peptides were dissolved in DMSO (Sigma) and distributed into 23 pools (Table 2). Details of these peptides are provided in Appendix 2.

Mononuclear cell response to bacterial lysates and peptide pools. Crude bacterial lysates of salmonella, *S. flexneri*, yersinia O:3, and *Escherichia coli* were prepared as described[5]. OMP from salmonella were isolated by ultracentrifugation and lipids were dissolved in sodium lauryl sarcosine as described[15]. Lymphocyte transformation test (LTT) was done by stimulating mononuclear cells (SFMC and/or PBMC; 1×10^5 cells) from subjects with crude bacterial lysates (5 and 10 μg/ml) and peptide pools (10 μg/ml of each peptide in the pool) in 5-day cultures in 96-well U-bottom culture plates (Nunc). Stimulation with 5 μg/ml phytohemagglutinin (Sigma) in 3-day cultures was carried out as a positive control. Proliferation was measured by incorporation of radiolabeled ^{3}H-thymidine added in the terminal 18 h of culture. The uptake of ^{3}H-thymidine was measured using a beta-counter (Tri-Carb Liquid Scintillation Analyzer; Perkin-Elmer) as counts per minute (cpm) and the results were expressed as stimulation index (SI: mean cpm of antigen or mitogen/mean cpm of control). SI > 2.5 was taken as a significant proliferation[5]. Interferon-γ (IFN-γ) was measured by ELISA in the above LTT culture supernatants obtained after 48 h of culture, using the IFN-γ ELISA kit (Becton-Dickinson), whose sensitivity was 7 pg/ml. Any detectable amount of IFN-γ was taken as a stimulation response.

Identification of immunogenic peptides. To identify the individual immunogenic peptides, a second round of proliferation assays and IFN-γ ELISA was done with each individual peptide (10 μg/ml) from the responding pool using stored SFMC/PBMC. Before the culture setup procedure, cryopreserved cells were kept for stabilization for 4–6 h. In some cases where the availability of viable SFMC/PBMC was limited, peptides of pools with higher SI values were tested (Appendix 3). SI > 2.5 with a detectable IFN-γ level was selected as a significant response. Further, these peptides were tested by flow cytometry in 5 newly enrolled patients with ReA/uSpA. SFMC of these patients were stimulated *in vitro* with either individual peptides (10 μg/ml) or phorbol myristate acetate/ionomycin (10 ng/ml; Sigma) for 6 h. As well, SFMC were left untreated in c-RPMI for estimation of basal level activation. Brefeldin A (Sigma) was added for the last 4 h. Cells were surface-stained with CD8-APC (clone RPA-T8; BD Biosciences) and CD69-PE (clone TP1.55.3; Beckman-Coulter). After fixation/permeabilization with a Leukoperm kit (Serotech AbD), intracellular staining for IFN-γ-FITC (clone RPA-T8; Beckman-Coulter) was carried out and cells were acquired on a Beckman-Coulter Navios instrument. Results were analyzed by FlowJo (TreeStar). Subpopulations of CD8+ T cells double-positive for CD69/IFN-γ were analyzed.

Search for similar peptides across bacteria and human proteins. To identify any similarity across arthritogenic bacteria and molecular mimicry against human protein, the BLASTp procedure (Basic Local Alignment Search Tool; National Library of Medicine/National Institutes of Health, Bethesda, MD, USA; Website: http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp) was performed for each peptide in a nonredundant database. Organisms selected were *Homo sapiens* (taxid 9606) and all known major arthritogenic bacteria that can trigger ReA, such as *S. typhimurium* (taxid 90371), *Shigella sonnei* (taxid 624), *Y. enterocolitica* (taxid 630), *S. flexneri* (taxid 623), *C. jejuni* (taxid 197), *E. coli* (taxid 562), *C. trachomatis* (taxid 813), *Clostridium difficile* (taxid 1496), *Ureaplasma*

Table 2. Distribution of 125 salmonella outer membrane protein peptides in 23 pools. Peptides were distributed randomly. Each peptide is shown by peptide no. Bold type indicates peptide pools and peptides that stimulated synovial fluid mononuclear cells from patients with reactive arthritis/undifferentiated spondyloarthropathy. Numbers in parentheses are no. responders.

	Peptide No.											
Pools	12	**13**	14	**15**	16	17	18	**19**	**20**	**21**	**22**	**23**
1	2	6	7	**14**	16	18	20	23	25	26	27	28
2	30	**33 (3)**	42	59	64	65	92	85	98	103	116	120
3	122	3	4	5	8	9	10	11	12	13	15	18
4	19	21	22	24	29	31	32	34	35	36	37	38
5	39	40	41	**43 (3)**	44	45	46	**47 (3)**	48	50	51	52
6	53	**55 (2)**	56	58	60	61	62	63	66	67	68	69
7	70	72	73	74	75	76	77	78	79	80	81	82
8	83	84	86	87	88	89	90	91	92	94	95	97
9	99	**100 (7)**	101	102	104	105	106	107	**108 (2)**	**109**	**110 (2)**	111
10	112	113	114	115	117	118	119	**121 (2)**	123	124	**125 (2)**	96
11	1	49	54	57	71							

urealyticum (taxid 2130), and *S. enteritidis* (taxid 149539). The algorithm was automatically set for short peptide sequence BLAST.

Statistical analysis. Data are shown as median (range). Statistical analyses were performed using SPSS version 16 (SPSS). Correlation between groups was by Spearman rank correlation and Fisher exact test was used for proportion analysis.

RESULTS

*HLA-B*27 subtypes in patients with ReA/uSpA*. HLA-B*27 was present in 16 (68%) among 23 patients with ReA/uSpA (Table 3a), whereas none of the patients with RA or healthy controls were positive for HLA-B*27. Subtyping was done for 16 HLA-B*27-positive subjects: 11 (70.2%) were positive for HLA-B*27:05 and 1 patient each was positive for HLA-B*27:02, B*27:04, and B*27:07 (Figure 1; Table 3a). Two patients could not be subtyped.

The LTT response to crude bacterial lysates and salmonella OMP. SFMC of 7 of 23 (30%) patients with ReA/uSpA showed an antigen-specific proliferative response to salmonella, compared to 1 of 23 response to *S. flexneri* ($p < 0.05$) and none out of 10 patients with RA ($p < 0.07$). SFMC of 11 (47.8%) patients with ReA/uSpA showed cross-reactive responses, compared to 2/10 (20%) in RA controls, as the SI values were > 2.5 for more than 1 bacterial antigen (Table 3a and data not shown). SFMC from 3 patients showed no response to any bacterial lysates and were grouped as nonresponders. In contrast to whole bacterial extract, response to salmonella OMP was present in 19/23 (82%) SFMC samples, as compared to 1/10 in RA samples ($p < 0.001$). This response to salmonella OMP correlated significantly ($r = 0.7$, $p = 0.004$) with response to salmonella crude lysates in patients with ReA/uSpA. Response with PBMC of patients with ReA/uSpA was comparatively less; out of 10 PBMC samples only 2 (20%) responded to bacterial lysates and salmonella OMP (Table 3b). PBMC from 8 patients did not respond to any crude bacterial lysates and were nonresponders. None of the PBMC samples from patients with RA responded to bacterial lysates or salmonella OMP. Among PBMC of

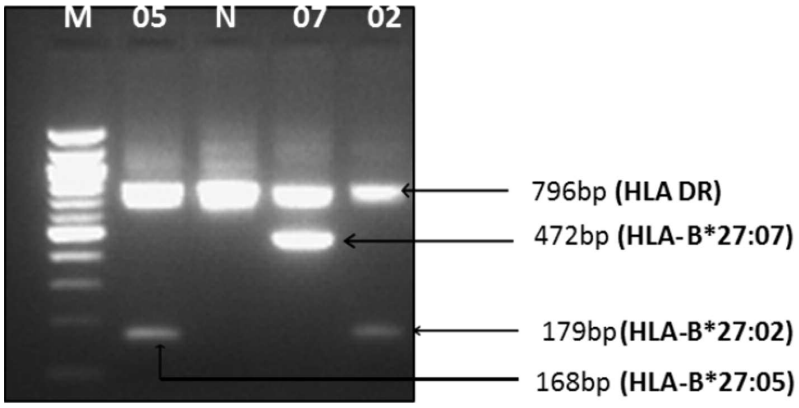

Figure 1. Gel photograph of HLA-B*27 subtyping by allele-specific primers. Lane M: 100-bp marker; lane 05: band at 168 bp for HLA-B*27:05; lane N: negative control DNA sample; lane 07: band at 472 bp for HLA-B*27:07; lane 02: band at 179 bp for HLA-B*27:02. 796-bp band is the internal control using primers for conserved HLA-DR.

Table 3a. Stimulation index (SI) values of lymphocyte transformation test in 23 synovial fluid mononuclear cell samples of patients with reactive arthritis/undifferentiated spondyloarthropathy; i.e., response to bacterial crude lysates and *Salmonella typhimurium* outer membrane protein (OMP). SI values > 2.5 (shown in bold type) were considered significant.

Patient ID	HLA-B*27	HLA-B*27 Subtype	PHA	ESC	YER	SHI	SAL	OMP	Response
01	+	NA	60.0	0.5	**4.7**	**3.6**	1.29	**5.63**	CR, OMP
02	+	B27*05	8.40	2.4	**3.2**	**26.8**	**6.6**	**2.6**	SHI, OMP
03	–	—	29.0	0.83	1.35	1.39	**3.11**	**2.53**	Sal, OMP
04	–	—	28.8	0.82	**3.35**	**2.9**	0.83	**3.35**	CR, OMP
05	+	B27*05	6.08	2.0	**9.19**	**14.2**	**13.6**	**9.8**	CR, OMP
06	+	B27*05	30.0	1.9	1.24	1.45	1.6	2.0	NR
07	+	B27*04	10.0	1.92	1.14	1.45	**3.6**	**6.0**	SAL, OMP
08	–	—	12.0	2.1	**3.3**	**10.2**	**11.6**	**4.2**	CR, OMP
09	–	—	25.0	2.3	**3.6**	**2.7**	**3.9**	**14.6**	CR, OMP
10	–	—	23.0	2.0	2.3	**4**	**3.6**	**5.4**	CR, OMP
11	+	B27*05	17.0	1.5	2.3	**2.8**	**4.3**	2.3	CR
12	+	B27*07	5.00	1.3	2.1	2.3	1.98	2	NR
13	+	B27*05	22.0	2	2.3	**5.7**	**3.6**	**11**	CR, OMP
14	+	B27*05	18.0	2.1	2.2	2.2	**6.2**	**11.2**	SAL, OMP
15	+	B27*05	23.0	**4.4**	**3.8**	**4.4**	**7.4**	**5.8**	CR, OMP
16	–	—	16.0	2.2	**2.57**	2.3	**8.7**	**5.5**	SAL, OMP
17	+	NA	29.0	**5**	**6.1**	**3.1**	**6.8**	**7.8**	CR, OMP
18	+	B27*05	5.0	2.3	2.4	2.3	**3.9**	**4.3**	SAL, OMP
19	+	B27*02	6.5	**2.6**	**3.25**	1.8	**4.75**	**6.25**	CR, OMP
20	+	B27*05	6.2	2.2	1.77	1.3	1.63	2.2	NR
21	–	—	12.0	2.1	2.3	2.1	2.1	**3.2**	OMP
22	+	B27*05	29.0	2.4	1.4	2.2	**5.4**	**6.4**	SAL, OMP
23	+	B27*05	6.80	1.2	1.2	2.2	**3.45**	**3.0**	SAL, OMP

PHA: phytohemagglutinin; ESC: *Escherichia coli;* YER: *Yersinia enterocolitica;* SHI: *Shigella flexneri;* SAL: *Salmonella typhimurium;* CR: cross-reactive; NR: nonresponder. NA: not available.

Table 3b. Stimulation index (SI) values of lymphocyte transformation test response in 10 peripheral blood mononuclear cell samples of patients with reactive arthritis/undifferentiated spondyloarthropathy; i.e., response to bacterial crude lysates and *Salmonella typhimurium* outer membrane protein (OMP). SI values > 2.5 (shown in bold type) were considered significant.

Patient ID	HLA-B*27	HLA-B*27 Subtype	PHA	ESC	YER	SHI	SAL	OMP	Response
07	+	B27*04	26.4	1.4	1.9	1.87	1.9	2.1	NR
08	–	—	126.0	1.2	1.42	1.1	1.85	2.23	NR
09	–	—	47.0	1.48	1.6	1.2	1.1	1.89	NR
10	–	—	126.0	2.1	2.2	1.9	2.0	2.1	NR
11	+	B27*05	114.0	1.3	1.8	1.0	1.3	1.6	NR
13	+	B27*05	37.0	2.1	1.8	1.6	2.0	2.1	NR
19	+	B27*02	21.0	2.0	2.1	1.8	1.8	2.1	NR
21	–	—	22.0	1.8	2.1	1.86	2.1	2.2	NR
22	+	B27*05	42.0	1.3	1.57	**3.1**	**2.89**	**2.6**	CR, OMP
23	+	B27*05	35.0	1.68	**2.71**	**2.8**	1.5	1.8	CR

PHA: phytohemagglutinin; ESC: *Escherichia coli;* YER: *Yersinia enterocolitica;* SHI: *Shigella flexneri;* SAL: *Salmonella typhimurium;* CR: cross-reactive; NR: nonresponder.

healthy individuals only 1 (10%) sample showed a cross-reactive response and it also showed response to salmonella OMP.

*LTT response to HLA-B*27:05-binding salmonella OMP peptide pool*. Among 23 SFMC of patients with ReA/uSpA, 9 (39%) showed proliferative responses to Pool-13 and Pool-22, whereas 8 (35%) patients' samples responded to Pool-5 and Pool-19, and 6 samples to Pool-9 and Pool-20. Thus, these pools were thought to be immunodominant (Figure 2). PBMC responses of these patients were very low (data not shown). The IFN-γ ELISA, however, suggested more immunodominant pools, as 9 PBMC responded for Pool-13 and Pool-7, and 6 PBMC responded to Pool-22 and Pool-11. PBMC of 5 patients each responded to Pools 1, 5,

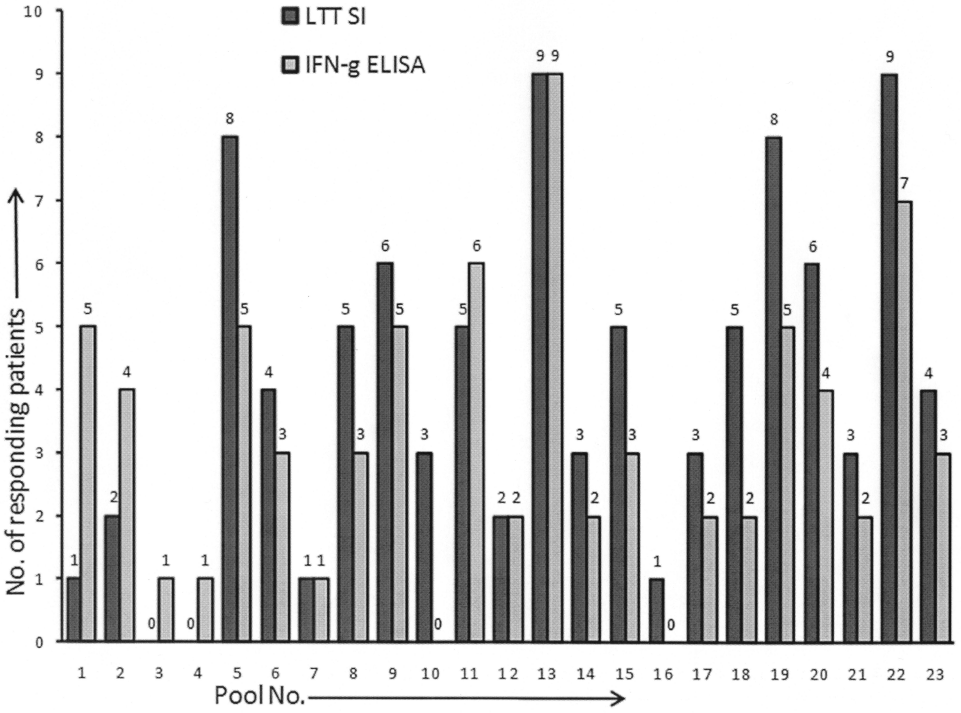

Figure 2. Numbers of patients showing synovial fluid mononuclear cell lymphocyte transformation test (n = 23) and IFN-γ ELISA (n = 20) responses to peptide pools among 23 patients with ReA/uSpA. Majority of patients (n = 9) showed response to Pool 13 and Pool 22. SI: stimulation index; IFN: interferon; ReA/uSpA: reactive arthritis/undifferentiated spondyloarthropathy.

9, and 19 (Figure 2). Taking account of results from both LTT and IFN-γ ELISA, Pools 5, 13, 19, and 22 were identified as consensus immunodominant peptide-containing pools (Figure 2). The LTT response in RA and healthy control samples was found with only 1 RA patient SFMC sample (Pools 7 and 14) and 1 healthy control PBMC sample (Pools 6, 11, 14, 15, and 19).

LTT response to individual peptides (second round). The final-round LTT and IFN-γ ELISA were carried out in 14 SFMC and 1 PBMC sample of patients with ReA/uSpA (Appendix 3). A proliferative response to peptide QRAEMLPTL (no. 100) from OprM, one of the OMP proteins, was present in 7 (50%) patients with ReA/uSpA (Table 2). All patients responding to QRAEMLPTL were HLA-B*27-positive and 6 were HLA-B*27:05-positive. A total of 9 peptides from salmonella OMP were predicted to be immunodominant peptides in patients with ReA/uSpA (Table 4).

Flow cytometric testing for immunogenic peptides. These 9 immunogenic salmonella OMP peptides were further tested with flow cytometry in 5 new patients. CD8+ T cells for 3 patients were IFN-γ and CD69-double-positive with peptide QRPAVRVKL (no. 108; Figure 3). Details of responses are given in Table 5. However, none of the 5 patient samples responded to peptide SRSGLNIAL (no. 47).

Table 4. Details of identified immunodominant peptides.

No. Patients Responded	Peptide No.	Pool No.	Sequence	Protein Details
7	100	13, 9	QRAEMLPTL	Outer membrane efflux-like protein, OprM (53 kDa) (485 aa) NP-459345
3	47	5, 19	SRSGLNIAL	Putative outer membrane protein (205 kDa) (1869 aa) NP-461450
3	43	5, 15	LRFLYAKSL	Outer membrane usher protein, FimD (95 kDa) (870 aa) NP-459541
3	33	13, 2	ARCIAPYAL	Outer membrane usher protein, FimD (95 kDa) (870 aa) NP-459541)
2	55	13, 6	LRLEGTWVK	Putative outer membrane protein (51 kDa) (468 aa) NP-459672
2	108	9, 20	QRPAVRVKL	Multidrug efflux system subunit MdtB (1040 aa) NP-461072
2	110	9, 22	KLFLTTAAL	Putative outer membrane protein (11 kDa) (94 aa) NP-459160
2	121	19, 10	YRNSDFFGL	Outer membrane protein F (40 kDa) (368 aa) NP-459974
2	125	22, 2	YRVGPGDVL	Putative outer membrane polysaccharide export protein (41 kDa) (379aa) NP-461063

Table 5. Response of synovial fluid CD8-positive T cells after stimulation with immunogenic salmonella outer membrane protein (OMP) peptides in 5 patients with ReA/uSpA. Percentages of CD8-positive T cells that were double-positive for CD69/interferon-γ (IFN-γ) were counted as positives (shown in bold type).

		Total Frequency of CD8-positive Cells Responding to Immunogenic Salmonella OMP Peptides																					
		Media		PMA/Iono		33		43		47		55		100		108		110		121		125	
ID	HLA-B*27 Status	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+	CD 69+	IFN-γ+ CD 69+
ReA-1	Pos	59.0	0.42	25.3	66.3	48.0	0.38	47.2	0.48	48.7	0.49	46.6	0.6	46.3	0.4	83.2	**2.65**	48.8	0.23	49.1	0.23	45.3	0.45
ReA-2	Pos	69.8	0.16	28.5	67.6	53.0	**1.1**	50.1	**0.78**	51.9	0.21	53.2	**0.38**	62.0	0.19	67.7	**0.4**	63.1	0.32	62.4	**0.52**	66.3	0.27
ReA-3	Pos	20.2	0.03	54	36.7	16.8	0.0	16.5	0.03	14.3	0.0	12.8	0.03	34.0	0.01	14.3	0.0	12.8	0.3	34.0	0.01	9.0	0.3
ReA-4	NA	29.5	1.7	23.6	25.4	31.4	2.0	29.1	**2.5**	30.1	2.3	34.4	0.9	28.6	1.8	29.4	0.93	32	**2.67**	30.7	1.68	33.7	0.7
ReA-5	Pos	0.0	0.01	29.2	37.7	0.0	**0.12**	0.8	**0.37**	0.0	0.0	0.5	0.0	26.1	**0.5**	15.4	**1.6**	22.1	**1.9**	23.6	**1.9**	11.7	**0.95**

PMA: phorbol myristate acetate; Iono: ionomycin; ReA: reactive arthritis; uSpA: undifferentiated spondyloarthropathy.

Similar peptides from other arthritogenic bacteria. Upon testing with BLASTp, all the 9 peptides that stimulated SFMC showed similarity with peptides from a panel of arthritogenic bacteria (Table 6). Peptide YRNSDFFGL (no. 121) showed similarity with 5 other bacterial peptides, while SRSGLNIAL (no. 47) and KLFLTTAAL (no. 110) showed similarity with only 1 peptide of other bacteria implicated in ReA.

Molecular mimicry between immunogenic salmonella OMP peptides and human proteins. Five immunogenic salmonella OMP peptides showed significant similarity with human peptides. The most immunogenic peptide QRAEMLPTL (no. 100) showed sequence similarity with peptides from 2 human proteins, coproporphyrinogen oxidase (EC 1.3.3.3) and RNA helicase. All human proteins having peptides similar to immunogenic bacterial OMP peptides were evolutionarily conserved enzymes (Table 7).

DISCUSSION

Using in silico methods, we identified 125 HLA-B*-27:05-binding nonamer peptides from 251 possible salmonella OMP. These peptides were subsequently tested for their immunogenicity using lymphocyte proliferation assays and IFN-γ production by PBMC and SFMC from patients with ReA/uSpA. Nine immunogenic peptides were thus identified. Peptide QRAEMLPTL from OprM was the most immunogenic, followed by SRSGLNIAL from putative OMP and LRFLYAKSL from FimD. In BLASTp analysis these immunodominant peptides were found to be cross-reactive with peptides from other "arthritogenic bacteria." Further, 5 immunogenic salmonella OMP peptides revealed similarity with peptides derived from human proteins.

This work is a continuation of earlier efforts to identify immunodominant epitopes of salmonella in patients with ReA/uSpA. Previous work had revealed that in one-third of patients with ReA/uSpA, salmonella was the trigger and the stimulation index with the OMP fraction was higher than that with the cytosolic fraction[5,14]. Subsequently, low molecular weight fractions were found to contain the immunodominant antigens[8]. We recently reported a significant proliferative response to salmonella OMP in patients with the enthesitis-related arthritis subtype of juvenile idiopathic arthritis, a disease similar to adult spondyloarthropathy[16]. Even in chlamydia- and yersinia-induced ReA, the immune response is targeted mostly against the OMP[17,18]. Apart from ReA/uSpA, OMP are immunogenic targets for both T and B cells in other *Enterobacteriaceae*-related diseases[19]. Thus we chose salmonella OMP proteins to identify their immunogenic epitopes in patients with ReA/uSpA.

HLA-B*27:05 was selected for peptide restriction because this subtype is the ancestor allele[20] and had good association with spondyloarthropathy in our cohort[6], and the peptide-binding algorithms are well established. In the present study HLA-B*27 was present in 70% of patients, similar to our earlier reports[8,15], and HLA-B*27:05 was the most common subtype (68%) in these patients.

With the availability of the whole genome sequence of salmonella[13], the 251 proteins of salmonella OMP were used as a substrate for proteosomal analysis and generation of HLA-B*27:05-binding peptides, using ProPred-I software[14]. The advantage of using ProPred-I is its reliability, because matrices are obtained from the Bioinformatics and Molecular Analysis Section server and from the literature. An initial run resulted in 641 peptides, too many to test by functional assays, and thus a threshold of log-score ≥ 6 was set and 125 peptides were selected. Another reason for choosing a log-score ≥ 6 was to include the bacterial peptide that mimics self-peptides, because peptides mimicking self-peptides have low to moderate binding affinity for HLA class I molecules[21]. Using similar approaches, Kuon, *et al* predicted 199 peptides from the proteome of chlamydia[22]. Compared to the prediction servers used by Kuon, *et al*, ProPred-I is more recent and

Table 6. Peptides similar to immunodominant salmonella OMP peptides from other "arthritogenic bacteria." Amino acid similarity match shown by "I" for exact match, "+" similar amino acid, "–" mismatch. OMP: outer membrane proteins; BLASTp: Basic Local Alignment Search Tool program.

Peptide No.	Immunogenic *Salmonella* OMP Peptide	BLASTp Alignments	Bacterial Protein (having subject sequence)
100	QRAEMLPTL	1: Query 1 QRAEMLPTL 9 Sbjct101 QRAEMLPTL 109	Putative outer membrane efflux lipoprotein [*Salmonella enterica* subsp. enterica serovar Enteritidis str. P125109]
		2: Query 1 QRAEMLPT 8 Sbjct 871 HREEMLPT 878	Putative sigma-54 interacting transcription anti-terminator [*Clostridium difficile* QCD-23m63]
		3: Query 1 QRAEMLPTL 9 Sbjct626 QRAELLETL 634	Hypothetical protein Ctra70_03825 [*Chlamydia trachomatis*]
47	SRSGLNIAL	1: Query 1 SRSGLNIAL 9 Sbjct1571 SRSGLNIAL1579	RatA [*Salmonella enterica* subsp. enterica serovar Typhimurium]
43	LRFLYAKSL	1:Query 1 LRFLYAKSL 9 Sbjct456 LRFLYAKSL 464	Fimbrial usher protein FimD [*Salmonella enterica* subsp. enterica serovar Enteritidis]
		2: Query 2 RFLYAKSL 9 Sbjct447 RFLYAKTL 454	Putative outer membrane protein [*Shigella flexneri* 5 str. 8401]
55	LRLEGTWVK	1: Query 1 LRLEGTWVK 9 Sbjct 308 LRLEGTWVK316	Hypothetical protein SSON_0635 [*Shigella sonnei*]
		2: Query 1 LRLEGTWVK 9 Sbjct308 LRLEGTWIK 316	Porin D [*Shigella flexneri*]
33	ARCIAPYAL	1: Query 1 ARCIAPYAL 9 Sbjct841 ARCIAPYAL 849	FimD [*Salmonella enterica*]
108	QRPAVRVKL	1: Query 1 QRPAVRVKL 9 Sbjct 190 QRPAVRVKL 198	1: Multidrug resistance protein mdtB [*Shigella flexneri*] 2: Multidrug efflux system subunit MdtB [*Yersinia enterocolitica*]
110	KLFLTTAAL	1: Query 1 KLFLTTAAL 9 Sbjct 2 KLFLTTAAL 10	Probable secreted protein [*Salmonella enterica* subsp. enterica serovar Enteritidis str. P125109]
121	YRNSDFFGL	1: Query 1 YRNSDFFGL 9 Sbjct 154 YRNSDFFGL 162	Outer membrane porin protein [*Yersinia enterocolitica* subsp. enterocolitica 8081]
		2: Query 1 YRNSDFFGL 9 Sbjct159 YRNSDFFGL 167	Outer membrane protein F precursor [*Salmonella enterica* subsp. enterica serovar Enteritidis str. P125109]
		3:Query 1 YRNSDFFGL 9 Sbjct154 YRNTDFFGL 162	Outer membrane protein S1 [*Salmonella enterica* subsp. enterica serovar Typhimurium str. D23580]
		4: Query 1 YRNSDFFGL 9 Sbjct155 YRNTDFFGL 163	Outer membrane porin protein (ompD) [*Salmonella enterica* subsp. enterica serovar Enteritidis str. P125109] CAR33062.1
		5: Query 1 YRNSDFFGL 9 Sbjct161 YRNSNFFGL 169	1: Outer membrane protein F [*Shigella flexneri* 2a str. 2457T] 2: Outer membrane protein F [*Shigella sonnei* Ss046]
125	YRVGPGDVL	1: Query 1 YRVGPGDVL 9 Sbjct 78 YRVGPGDVL 86	Putative polysaccharide export protein [*Shigella flexneri* 2002017] ADA74500.1
		2: Query 1 YRVGPGDVL 9 Sbjct 80 YRVGPGDVL 88	Putative polysaccharide export protein [*Shigella flexneri* 5 str. 8401] YP_689551.1

Table 7. Peptides similar to immunodominant salmonella OMP peptides from human proteins. Amino acid similarity match shown by "|" for exact match, "–" mismatch. OMP: outer membrane proteins; BLASTp: Basic Local Alignment Search Tool program.

Peptide No.	Immunogenic *Salmonella* OMP Peptide	BLASTp Alignments	Human Protein (having subject sequence)
100	QRAEMLPTL	1: Query 1 QRAEMLP 7 ||||||| Sbjct 97 QRAEMLP 103	Coproporphyrinogen oxidase
		2: Query 1 QRAEMLPTL 9 ||||||--| Sbjct 92 QRAEMLQKL 100	ATP-dependent RNA helicase DHX37
47	SRSGLNIAL	1: Query 1 SRSGLNIAL 9 |||-|-||| Sbjct 466 SRSSLSIAL 474	RAB11 family interacting protein 5 (class I), CRA_a
43	LRFLYAKSL	—	—
55	LRLEGTWVK	—	—
33	ARCIAPYAL	—	—
108	QRPAVRVKL	—	—
110	KLFLTTAAL	1: Query 2 LFLTTAAL 9 ||||-||| Sbjct 1434 LFLTMAAL 1441	DENN/MADD domain containing 4B
121	YRNSDFFGL	1: Query 1 YRNSDFF 7 |||-||| Sbjct 38 YRNGDFF 44	Tyrosinase
125	YRVGPGDVL	1: Query 2 RVGPGDVL 9 |-||||| Sbjct 639 RIGPGDVL 646	Seizure protein 6-homolog isoform 1

offers the immuno-proteosome filter. In 91% of the 125 peptides identified, the second position was occupied by arginine (R), which is a characteristic feature of HLA-B*27:05-binding peptides[23]. Further, 70% of predicted peptides had lysine (L) at their carboxyl terminus, showing the importance of proteosomal trimming in HLA-B*27 peptide interaction.

The panel of peptides had a variable binding score between 6 and 10 that suggests the variable binding affinity with HLA-B*27. Thus it is recommended to use the proliferation assay with ^{3}H-thymidine incorporation, which can identify a moderate to high response[24]. These peptides were distributed in pools; because these pools had nonoverlapping peptides, we screened with individual peptides to identify immunogenic peptides.

From the proliferation assay, 4 pools (13, 22, 5, and 19) were shown to be immunogenic, whereas 7 pools (13, 22, 11, 5, 1, 9, and 19) were identified by IFN-γ ELISA. On the other hand, the PBMC samples from patients with ReA/uSpA, disease controls, and healthy controls as well as SFMC from patients with RA did not show significant cellular proliferation or increased IFN-γ in culture supernatants on stimulation with peptides. This was similar to the response seen with bacterial crude lysates and salmonella OMP. This further substantiates that salmonella OMP-specific T cells are mainly present in the synovial compartment of patients with ReA/uSpA only[15].

Cryopreserved cells were stabilized after thawing in c-RPMI-10 for 4–6 hours[25]. Such strategies have proved superior for both the proliferation assays and IFN-γ secretion by cryopreserved T cells[26]. Using this strategy, 9 immunogenic salmonella OMP peptides were identified: QRAEMLPTL, SRSGLNIAL, LRFLYAKSL, RLEGTWVKL, ARCIAPYAL, KLFLTTAAL, YRNSDFFGL, QRPAVRVKL, and YRVGPGDVL. The peptide response was vigorous for SFMC and/or PBMC from patients with ReA/uSpA, as only peptides KRSTLALAI (no. 42) and SRWFAPVVA (no. 58) elicited responses in SFMC of a patient with RA and PBMC of a healthy control, respectively. None of the controls was HLA-B*27-positive.

T cell proliferation and IFN-γ production assays were employed to measure antigen-specific T cell stimulation; however, the cytokine secretion assay has now been replaced by IFN-γ ELISPOT or flow-based enumeration of IFN-γ-producing T cells. Further testing of these peptides with flow-based assays identified 8 out of 9 peptides as immunogenic.

These peptides have not previously been reported to be

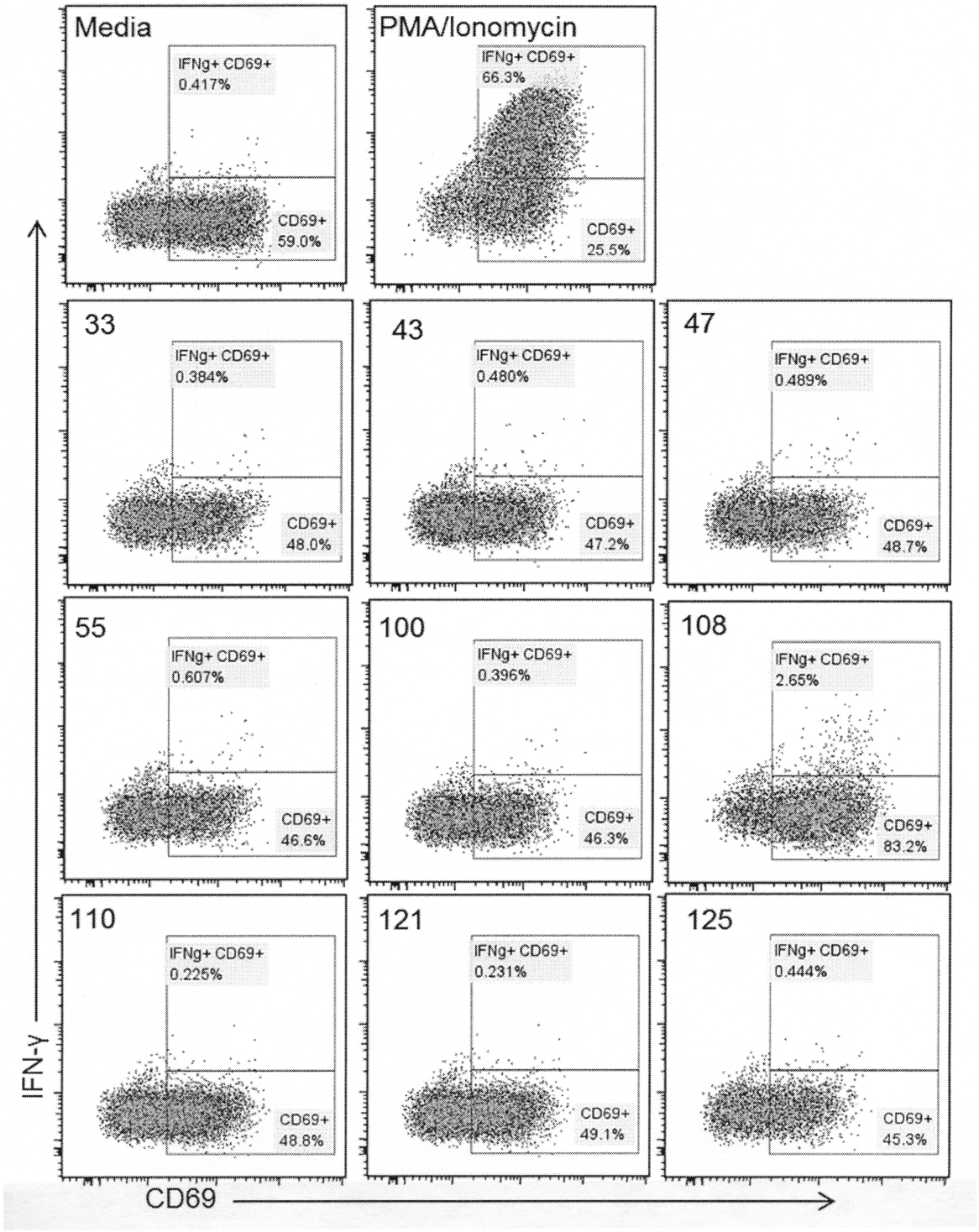

Figure 3. Dot plot illustrates CD8+ T cell response by flow cytometry. After staining for T cell surface markers and intracellular cytokines, a gate for CD8+ T cells was set to obtain the percentage of IFN-γ/CD69-double-positive cells. The percentages of CD69-single-positive and IFN-γ/CD69-double-positive cells are indicated inside the gates. IFN: interferon.

immunogenic and are not available in the 2 largest epitope databases, Immune Epitope Database[27] and SYFPEITHI[28], which have information on all known HLA-binding peptides, with or without T cell immunogenicity potential. Thus, these HLA-B*27:05-binding immunodominant peptides from salmonella OMP are new additions to the

immunogenic epitope database. Previously, 2 peptides, RNTDFFGL and TRVAFAGL, from OMP-C of salmonella OMP were identified in murine studies using CD8+ T cell responses[29].

Peptide response was not restricted by B*27:05 subtype even though all these peptides were generated using B*27:05-based algorithms. Peptides QRAEMLPTL and ARCIAPYAL were immunogenic in HLA-B*27-positive individuals only, and the majority of them were B*27:05 subtype-positive. In contrast, other immunogenic peptides did not follow any strict rule. HLA-B*27:05 shares 33% of its T cell epitopes with HLA-B*27:02, a mere 12% with HLA-B*27:04[30], and 59% with HLA-B*27:07[31]. Interestingly, peptide-sharing among these subtypes is quite high compared to the T cell epitope-sharing; for example, it is 73% between HLA-B*27:05 and B*27:02[32]. In our study, major overlap was seen between HLA-B*27:05 and HLA-B*27:04 subtypes; unfortunately, we had only a small number of patients with other subtypes for comparison. However, there was response to peptides LRFLYAKSL (no. 43), SRSGLNIAL (no. 47), YRNSDFFGL (no. 121), and YRVGPGDVL (no. 125) in patients who were HLA-B*27-negative, suggesting the promiscuous expression of these peptides.

The limitations of our study are that the specificity of these peptides for binding to HLA-B*27:05 was not examined by blocking with antibodies to HLA-B*27:05. Salmonella OMP-driven T cell lines generated from SFMC from patients with ReA/uSpA could be tested against these peptides as has been done in chlamydia- and yersinia-triggered ReA[33,34]. The response in HLA-B*27-positive healthy subjects needs further evaluation to determine their pathogenic potential[35], because all our control subjects were negative for HLA-B*27.

All peptides identified were nearly 100% similar to peptides from other enteric bacteria. In addition, peptide QRAEMLPTL (no. 100) was also similar to peptides of *C. difficile* and chlamydia. Most proteins from other bacteria containing these peptides were OMP, which further suggests that OMP are evolutionarily conserved and may act as potent immune targets for T cells[36].

The immunodominant peptide QRAEMLPTL from salmonella OprM was similar to human protein coproporphyrinogen oxidase. Coproporphyrinogen oxidase (EC 1.3.3.3) is a mitochondrial enzyme[37]; an autoimmune response to mitochondrial proteins is well documented in patients with primary biliary cirrhosis[38]. Further, peptide QRAEMLPTL (no. 100) also matched with 7 amino acids of a peptide from human protein ATP-dependent RNA-helicase DHX37; the 2 mismatches were present at positions 7 and 8, which are not crucial for the HLA-B*27-binding sites. Of note, RNA-helicase A has been found, in lupus, to be an autoantigen that is cleaved during apoptosis, eliciting autoantibodies against itself[39].

Besides peptide QRAEMLPTL (no. 100), 4 others [SRSGLNIAL (no. 47), KLFLTTAAL (no. 110), YRNSDFFGL (no. 121), and YRVGPGDVL (no. 125)] showed sequence similarity with peptides from the following proteins: RAB11 family-interacting protein 5 isoform CRA-a, DENN/MADD domain-containing 4B, seizure protein 6 homolog isoform 1, and tyrosinase, respectively. There is no report of any immune response against these

Appendix 1. Allele-specific primers used for HLA-B*27 subtype analysis. Subtypes were defined on the basis of presence or absence of corresponding polymerase chain reaction (PCR) band in 1.2% agarose gel electrophoresis.

Primer Set ID	Primer	Primer Sequence	Annealing Sites	PCR Band
1	<B*2701 to B*2709> B27-1F B27-1R	 5'-GCT ACG TGG ACG ACA CGC T-3' 5'-CTC GGT CGG TCT GTG CCT T-3'	 Exon 2, 76-94 Exon 2, 207-225	 149 bp
2	<B*2702> B27-2F B27-2R	5'-CTA CGT GGA CGA CAC GCT-3' 5'-TGT AGT AGC GGA GCG CGA-3'	Exon 2, 77-94 Exon 2, 238-255	 179 bp
3	<B*2703> B27-2F B27-3R	5'-CTA CGT GGA CGA CAC GCT-3' 5'-TGT CTC CCG GTC CCA ATG-3'	Exon 2, 77-94 Exon 2, 174-191	 115 bp
4	<B*2704> B27-2F B27-4R	5'-CTA CGT GGA CGA CAC GCT-3' 5'-TCT CAG CTG CTC CGC CT-3'	Exon 2, 77-94 Exon 3, 184-200	 394 bp
5	<B*2703, B*2705 > B27- 2R B27-5R	 5'-CTA CGT GGA CGA CAC GCT-3' 5'-AGC AGG GTC CGC AGG TC-3'	 Exon 2, 77-94 Exon 2, 228-244	 168 bp
6	<B*2706> B27-2F B27-6R	5'-CTA CGT GGA CGA CAC GCT-3' 5'-CGC TGG GTG ATC TGA GCT-3'	Exon 2, 77-94 Exon 3, 146-163	 357 bp
7	<B*2707> B27-2F B27-7R	5'-CTA CGT GGA CGA CAC GCT-3' 5'-ACG TCG CAG CCG TAC ATG-3'	Exon 2, 77-94 Exon 3, 20-37	 472 bp
8	<B*2708> B27-2F B27-8R	5'-CTA CGT GGA CGA CAC GCT-3' 5'-CGC AGG TTC CGC AGG C-3'	Exon 2, 77-94 Exon 2, 229-244	 168 bp
9	<B*2709> B27-2F B27-9R	5'-CTA CGT GGA CGA CAC GCT-3' 5'-TTG CCG TCG TAG GCG TG-3'	Exon 2, 77-94 Exon 3, 75-91	 285 bp

Appendix 2. Details of 125 predicted peptides by ProPred-I.

Peptide No.	Peptide Sequence	Hydro-phobicity	Molecular Wt. (Da)	Log Score	Protein (Acc. No.)
2	H- KRKDSAVSV –OH	0.26	989.14	7.4955	NP_460359
6	H- RQRMSAEGL –OH	-0.13	1,047.21	6.3969	NP_461117
7	H- ARANAGAGL –OH	-0.04	799.89	7.6009	AAL23066
14	H- RRPLNAQTT –OH	-0.12	1,056.19	6.3969	NP_459278
16	H- KRPLLADEA –OH	-0.04	1,012.18	6.3969	NP_461449
18	H- ARDRNALEL –OH	-0.16	1,057.18	7.6009	NP_459345
20	H- RRQGAELAL –OH	-0.06	1,013.17	8.6995	NP_460546
23	H- RRQVLKTGL –OH	-0.01	1,070.31	8.6995	NP_461382
25	H- KRRDLSIEL –OH	0.09	1,129.33	8.6995	NP_461382
26	H- RRSDISVDV –OH	-0.11	1,046.15	7.4955	AAL21904
27	H- ARGRGEHAL –OH	-0.18	966.07	7.6009	NP_490502
28	H- KRRLTKVAL –OH	-0.05	1,084.38	8.6995	NP_461451
30	H- ARGVELEAK –OH	-0.14	972.11	7.6009	NP_459196
33	H- ARNARNVGI –OH	-0.12	970.10	6.3969	AAL20585
42	H- ARTGNATQL –OH	-0.07	931.02	7.6009	NP_459345
59	H- SRWTHRGEL –OH	-0.01	1,141.26	9.2103	NP_460546
64	H- LRPGEEKKL –OH	-0.17	1,069.27	6.3969	NP_459278
65	H- TRDNPGTEL –OH	-0.17	1,002.05	7.6009	NP_463009
92	H- VRAADSDTL –OH	-0.05	947.02	7.6009	NP_461450
85	H- NRDLRKAGL –OH	-0.22	1,042.21	7.6009	NP_459345
98	H- VRQGEGKDL –OH	-0.22	1,001.11	6.3969	NP_460566
103	H- QRAVEASQI –OH	-0.02	1,001.11	6.3969	NP_459345
116	H- KRAAEVAAL –OH	-0.02	928.10	8.6995	AAL19239
120	H- RQGGDKHRL –OH	-0.36	1,066.19	6.3969	NP_463118
122	H- KRHLEGEPL –OH	-0.07	1,078.24	8.6995	NP_463090
3	H- RQMPLAEVL –OH	0.30	1,056.30	6.3969	NP_460359
4	H- RRLNEAVTL –OH	0.02	1,071.25	8.6995	NP_459345
5	H- KRKILAAVI –OH	0.22	1,011.32	7.4995	NP_459974
8	H- KRKVLALVI –OH	0.29	1,039.38	7.6009	NP_460434
9	H- RQTATLATL –OH	0.16	974.13	6.3969	NP_459345
10	H- ARAQYDTVL –OH	0.09	1,036.16	7.6009	AAL22060
11	H- KRLIPVALL –OH	0.49	1,022.35	8.6995	NP_462302
12	H- ARCIAPYAL –OH	0.44	977.20	7.6009	NP_45954
13	H- KRLLLITAL –OH	0.49	1,040.36	8.995	NP_462453
15	H- ARDIGGTGL –OH	0.05	858.95	7.6009	NP_463090
18	H- RRPYLKAEL –OH	0.00	1,145.38	8.6995	NP_461450
19	H- KRPLLRAEL –OH	0.07	1,095.36	8.6995	NP_461449
21	H- ARGDLATTL –OH	0.09	917.03	7.6009	NP_461449
22	H- KRPLLSAEI –OH	0.16	1,026.25	7.495	NP_461449
24	H- ARGFQAAVF –OH	0.25	966.11	6.907	NP_459562
29	H- RRSSFLVFL –OH	0.39	1,124.36	9.2103	NP_459341
31	H- KRSTLALAI –OH	0.21	972.20	7.495	NP_459672
32	H- SRAALYPTL –OH	0.29	991.16	7.6009	NP_463124
34	H- KRWWAGDSL–OH	0.18	1,118.27	10.309	NP_461450
35	H- SRDGGTVPL –OH	0.03	900.99	7.6009	NP_461072
36	H- ARQLEGLKI –OH	0.08	1,027.24	6.3969	NP_461449
37	H- LQYCAKQKL –OH	0.18	1,094.35	6.9078	NP_463401
38	H- SRFIAAVNA –OH	0.21	948.09	6.9078	NP_461819
39	H- ARSPLLPQL –OH	0.35	994.21	7.6009	NP_462101
40	H- LRDDAWVTL –OH	0.28	1,088.23	7.6009	NP_462091
41	H- SRKTKILPL –OH	0.13	1,055.33	7.6009	NP_463270
43	H- LRFLYAKSL –OH	0.40	1,110.37	8.0064	NP_459541
44	H- SRLYPLPFL –OH	0.59	1,105.35	7.6009	NP_459372
45	H- ARVLACAAL –OH	0.42	887.12	7.6009	NP_461819
46	H- LRGLLPATL –OH	0.48	953.20	8.0064	NP_462842
47	H- SRSGLNIAL –OH	0.19	930.08	7.6009	NP_461450
48	H- ARVPHGKSL –OH	0.03	964.14	7.6009	NP_461819
50	H- SRWFAPVVA –OH	0.46	1,032.22	6.9708	AAL22507
51	H- ARVSHADAL –OH	0.04	939.04	9.2103	NP_461449
52	H- LRKEFGTKL –OH	0.01	1,091.32	7.6009	NP_461943
53	H- SRWNLGDAL –OH	0.16	1,031.14	9.2103	NP_460174
55	H- LRLEGTWVK –OH	0.25	1,101.32	7.6009	NP_459672
56	H- SRWSDGVTA –OH	0.04	978.03	7.6009	NP_461945
58	H- LRMTPIASL –OH	0.45	1,001.26	7.6009	NP_461094
60	H- FRQKIATAI –OH	0.21	1,047.27	6.3969	NP_461382
61	H- LRNTLVANL –OH	0.27	1,013.21	7.6009	NP_460039
62	H- TQWQLTQKL –OH	0.25	1,145.33	6.9078	NP_460546
63	H- FRYLCNQKA –OH	0.16	1,142.35	6.9078	NP_490502
66	H- GQFFTSGPL –OH	0.39	953.07	6.9078	AAL21103
67	H- LRPSLGYVL –OH	0.47	1,017.24	7.6009	NP_459317
68	H- TRISLADPL –OH	0.26	985.15	7.6009	NP_460174
69	H- GRATLIPAV -OH	0.32	897.09	6.3969	NP_461073
70	H- MRAGPATVR –OH	0.02	958.16	7.6009	NP_460691
72	H- GRAYIQDEL –OH	0.03	1,064.17	7.6009	NP_459971
73	H- MRARIMLFL –OH	0.54	1,150.53	7.6009	NP_463250
74	H- TRLVGVVFL –OH	0.58	1,003.26	7.6009	NP_461073
75	H- GRGEHALMV –OH	0.11	969.14	6.3969	NP_490502
76	H- MRKSLFLLL –OH	0.55	1,120.47	7.6009	NP_462932
77	H- TRPAIGTVL –OH	0.32	927.12	7.6009	NP_463270
78	H- HRASLPWFL –OH	0.53	1,126.33	7.6009	NP_490502
79	H-MRQMEGAWL –OH	0.28	1,121.35	7.6009	NP_459229
80	H- TRQANFTVI –OH	0.19	1,049.20	6.3969	NP_461943
81	H- HRFANGVGF –OH	0.17	1,004.12	8.5172	NP_462932
82	H- MRTHTLFKV –OH	0.26	1,132.40	7.6009	NP_462932
83	H- TRQLLASGV –OH	0.20	944.10	6.3969	NP_461117
84	H- HRYAAIAAL –OH	0.28	985.16	9.2103	NP_460177
86	H- TRTAINATL –OH	0.14	960.10	7.6009	NP_461450
87	H- IREAIGKLL –OH	0.26	1,012.27	6.3969	AAL21578
88	H- NRFGVGVVS –OH	0.19	934.07	6.9103	NP_461945
89	H- TRTLDMEAL –OH	0.12	1,049.22	7.6009	NP_462557
90	H- IRHFSNGSL –OH	0.18	1,030.16	6.3969	NP_461188
91	H- NRVDLKLVF –OH	0.23	1,103.33	7.6009	NP_459229
93	H- IRMSAGIAL –OH	0.39	931.17	7.6009	NP_459206
94	H- NRYLYSIDK –OH	0.02	1,171.32	9.1203	NP_460488
95	H- VRLDDVSKL –OH	0.05	1,044.22	7.6009	NP_460546
97	H- NRYVKSINL –OH	0.06	1,106.29	9.2103	NP_461449
99	H- IRSDITGRL –OH	0.09	1,030.20	7.6009	NP_461063
100	H- QRAEMLPTL –OH	0.21	1,058.27	7.6009	NP_459345
101	H- VRSLSDQAL –OH	0.10	988.11	7.6009	NP_459345
102	H- IRTMPGVNL –OH	0.32	1,000.23	7.6009	NP_459577
104	H- VRVMNAPQL –OH	0.26	1,027.26	7.6009	NP_460775
105	H- IRYGLTDRL –OH	0.17	1,106.29	9.2103	NP_461968
106	H- QRLYPALLL –OH	0.53	1,086.35	7.6009	NP_460546
107	H- VRVVPVAGL –OH	0.44	909.14	7.6009	NP_461945
108	H- QRPAVRVKL –OH	0.02	1,066.32	7.6009	NP_461072
109	H- YRFNDYVSL –OH	0.22	1,176.30	9.2103	NP_459341
110	H- KLFLTTAAL –OH	0.48	977.22	6.1023	NP_459160
111	H- QRQPGANII –OH	0.07	996.14	6.3969	NP_461072
112	H- YRIQRAEML –OH	0.12	1,179.41	7.6009	NP_459345
113	H- KLFQIIGRL –OH	0.44	1,087.38	8.6679	AAL19239
114	H- QRQVAERIL –OH	0.02	1,112.30	6.3969	NP_461072
115	H- YRLPAIPEL –OH	0.41	1,071.29	7.6009	NP_460174
117	H- QRSITLGAL –OH	0.25	958.13	7.6009	NP_459562
118	H- YRNNDFFGL –OH	0.14	1,145.24	7.6009	NP_460490
119	H- KRFAFALWL –OH	0.55	1,151.43	10.309	NP_459533
121	H- YRNSDFFGL –OH	0.19	1,118.22	7.6009	NP_459974
123	H- RQKIGVMRL –OH	0.09	1,100.40	6.3969	NP_461819
124	H- YRNTDFFGL –OH	0.21	1,132.25	7.6009	NP_461210
125	H- YRVGPGDVL –OH	0.21	975.12	7.6009	NP_461063
96	H- IRQLCWVSL –OH	0.65*	1,117.39	7.6009	NP_463270
1	H- KLFLFIVML –OH	0.93*	1,123.52	6.1092	NP_461093
49	H- LRILLVGSL –OH	0.64*	983.27	7.6009	NP_459409
54	H- FRLIRLLPL –OH	0.67*	1,140.49	7.6009	NP_460734
57	H- FRPILMTTL –OH	0.61*	1,091.39	7.6009	NP_461072
71	H- TRLAILITL –OH	0.61*	1,013.29	7.6009	NP_461945

proteins in any autoimmune disease. However, none of these peptides figures in a dataset of HLA-B*27:05-binding peptides having similarity to human protein peptides[40].

Using a bioinformatics approach, our study revealed a set of novel HLA-B*27:05-binding peptides of salmonella OMP that were immunogenic to T cells from patients with ReA/uSpA. Cell-mediated immune response to this set of peptides identified in our study could be evaluated as a diagnostic assay to identify patients with ReA/uSpA in the setting of early undifferentiated arthritis. It will be interesting to investigate whether there is a cross-reactive immune response to peptides derived from human proteins showing similarity in patients with spondyloarthropathy that may suggest mimicry at the molecular level in the pathogenesis of this group of diseases.

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Appendix 3. Details of peptide pools and peptides used in second-round lymphocyte transformation test (LTT). PBMC: peripheral blood mononuclear cells.

Patient ID	Responding Pools	Selected Pools	Peptides in Selected Pools (peptide no.)	Immunogenic Peptides
02	5,7,10,11,13,14, 15,16,17,18,19	—	56, 52, 124	56
05	9, 22	9	99, 100, 102, 104, 105, 106, 107, 108, 109, 110, 111	100, 106
07	5, 10, 11, 12, 13, 15	5, 11, 13, 15	39, 43, 44, 45, 46, 47, 48, 50, 51, 52, 6, 33, 3, 21, 40, 55, 72, 84, 100, 113, 49, 14, 59, 5, 24, 43, 58, 74, 87, 102, 115, 57, 1, 49, 54, 57, 71	6, 33, 3, 40, 55, 72, 39, 41, 100, 113, 43, 46, 47, 59, 58, 87
08	17,18, 19	19	23, 85, 10, 34, 47, 63, 78, 90, 107, 121	121
10	5, 6, 11, 22	22	27, 116, 15, 37, 51, 68, 81, 95, 110, 125	125
13	13, 14, 18, 19, 22	13, 22	6, 33, 3, 21, 40, 55, 72, 84, 100, 113, 49, 27, 116, 15, 37, 51, 68, 81, 95, 110, 125	55, 100
14	5, 9, 20	5, 9, 20	39, 43, 44, 45, 46, 47, 48, 50, 51, 52, 99, 100, 102, 104, 105, 106, 107, 108, 109, 110, 111, 25, 98, 12, 35, 48, 66, 79, 93, 108	47, 101, 105, 106, 108, 110
15	8, 15, 22	15	14, 59, 5, 24, 43, 58, 74, 87, 102, 115, 57	43
18	6, 9, 11, 13, 21, 22	9	99, 100, 102, 104, 105, 106, 107, 108, 109, 110, 111	100, 108, 110
19	20, 22	22	27, 116, 15, 37, 51, 68, 81, 95, 110, 125	125
20	8, 13, 20	13	6, 33, 3, 21, 40, 55, 72, 84, 100, 113, 49	33, 100
21	5, 18	5	39, 43, 44, 45, 46, 47, 48, 50, 51, 52	43, 47
22*	5, 13, 22	5, 13	39, 43, 44, 45, 46, 47, 48, 50, 51, 52 6, 33, 3, 21, 40, 55, 72, 84, 100, 113, 49	100
23	13, 15	13	6, 33, 3, 21, 40, 55, 72, 84, 100, 113, 49	33, 100

*Second-round LTT with PBMC of this patient was done with peptides from Pool 19; peptide no. 121 was identified as immunogenic peptide.

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