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A 28KDa recombinant outer membrane protein of *Brucella melitensis* down-regulates type-1 immune response in mice

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The genus *Brucella*, as α2- proteobacteria, is a gram negative facultative intracellular zoonotic bacterium that causes brucellosis, a chronic disease of cattle, human and other species (Fernandes and Baldwin 1995) causing serious economic loss. Because of intracellular localization, it has been suggested that cell mediated immune (CMI) response is crucial in the control of infection implying that Th1 response is required (Splitter *et al.*1996). *B. abortus* S-19 and RB51 are the 2 WHO recommended vaccines against brucellosis in cattle, however, they may induce abortion in pregnant animals (Corbel 1997). Besides, they also interfere during serodiagnosis to identify vaccinated and infected animals (Mallick *et al.* 2007). Hence, attempts were made to develop alternative vaccines using several *Brucella* antigens such as p39 (Al-Mariri *et al.*2001), OMP31 (Cassataro *et al.*2005), L7/L12 (Mallick *et al.*2007), and OMP28 (Kaushik *et al.*2010). Accordingly, the present study demonstrates the down-regulation of Th1 immune response in mice vaccinated with r-omp28 of *Brucella*.

Animals: Inbred female Swiss albino mice, aged 4–6 weeks, were procured from the Laboratory Animal Resources (LAR) of the institute and they were housed under standard conditions having free access to feed and water *ad lib.*

PCR amplification and cloning: *B. melitensis* 16M DNA encoding 28kDa omp gene was amplified by PCR, using a set of primers selected on the basis of published nucleotide sequence (Chaudhuri *et al.*2005). To overexpress the 28 kDa outer membrane protein (omp28) of *B. melitensis*, the amplified DNA was cloned into the expression vector, pPROExHTb. Restriction sites for BamH I and Hind III were introduced to oligonucleotides to facilitate cloning. PCR was performed for 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR amplified product was eluted from agarose gel and ligated to the pPROExHTb vector with T4 DNA ligase. The newly constructed plasmid was designated as pOMP28 and was transformed into *E. coli* DH 5α cells.

Purification of r-omp28: *Escherichia coli* DH5α cells harboring recombinant plasmid pOMP28 were grown in LB medium till optical density (600nm) reaches to 0.5. The cells were then induced with 1 mM IPTG and allowed to grow further for 6 h at 37°C. Polyhistidine tagged recombinant omp28 was purified under denaturation condition by Ni-NTA affinity chromatography. The purity of the protein was confirmed by SDS-PAGE analysis.

Immunization of mice: The mice were divided into 2 groups with 20 mice in each group. The mice were injected intramuscularly with 30µg of omp28 in 200µl and PBS (control) and boosted subsequently on day 21 with the same dose and route.

Collection of serum: Mice were bled by retro-orbital puncture with capillary tubes and blood was collected in sterile microfuge tubes. Blood was incubated at room temperature for an hour and subsequently kept at 4°C for retraction. Finally, the serum was collected and centrifuged at 5000×g for 10 min at 4°C to remove residual blood cells. The final preparation was kept at –20°C till use.

Determination of antigen specific immunoglobulin isotypes: Sera of immunized mice were collected at weekly interval to monitor the antibody response by iELISA as per Cassataro *et al*.(2004) using 96-well microtitre plate coated

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overnight with 100 µl of purified r-omp28 antigen (1.25 µg/ml) in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at 4°C. The titer was expressed as optical density (OD) units, which was obtained by multiplying the reciprocal dilution of the serum by the OD (A492) at that dilution (Bhattacharjee *et al.*2006).

Lymphocyte proliferation assay: The lymphocyte proliferation was measured after 7 days of booster vaccination according to Mosmann (1983). Mice (5) from each group were randomly taken and rest of the mice were used for other studies (data not shown). Cells were stimulated *in-vitro* with r-omp28 (1 µg/ml), concanavalin-A (ConA) (2.5 µg/ml) or medium alone in triplicate.

In vitro *assay for IFN-γ, IL-2 and IL-10 production by spleen cells*: Gamma interferon (IFN-γ), interleukins IL-2 and IL-10 were estimated by antigen capture ELISA using cytokine assay kit. The assay was made in the culture supernatant of spleen cells collected after 72 h of antigen stimulation. The concentration of IFN-γ, IL-2 and IL-10 in the culture supernatants were calculated for each experimental group using a linear-regression equation obtained from the absorbance values of the standards.

Statistical analysis: The data were analysed and expressed as the mean±SD. Results of iELISA were analyzed by ANOVA and Dunnet's and Tukey's post hoc test using Prism 3.0 software.

YajC (Vemulapalli *et al.*2000), p39 (Al-Mariri *et al.*2001), omp31 (Cassarato *et al.*2005) and L7-L12 (Mallick *et al.*2007) could induce a protective humoral or/ and cellular immune response.

The cytokine profile in this study demonstrated the induction of B-cells by r-omp28 in immunized mice with significantly higher (P<0.05) production of IL-10 (Fig.3) affecting shift of immune response towards Th2 type. The later was supported by class switching response of B cells by producing significantly higher (P<0.05) IgG1 response in r-omp28 vaccinated mice (Fig.1), indicated downregulation of Th1 cytokines (Jegerlehner *et al.*2007). Since the IgG isotype response is determined by the pattern of cytokines secreted by CD4 helper T cells, the titre of IgG1 and IgG2a against r-omp28 was measured. A significantly higher titre of IgG1 compared to IgG2a (Figs 1, 2) was observed in the mice immunized with r-omp28 antigen. It indicated the induction of Th2 type of immune response (Coban *et al.*2004) with significant production of cytokine IL-10. This was further confirmed in the study by spleenocyte proliferative response and production of IFN-γ and IL-2 after *in vitro* stimulation of spleenic cells with r-omp28. Spleenocytes from vaccinated mice did not show significant proliferation and produced low level of IFN- γ and IL-2, in contrast, the production of IL-10 was significantly (P<0.05) higher (Fig.3). Since IFN- γ and IL-2 are the major cytokines responsible for induction of cell mediated immune response (Jacques *et al.*2007) against intracellular organisms, therefore the pattern of cytokines produced in the present study suggest the suppression of Type-1 immune response by r-omp28 (Diptee *et al.*2005). Upregulation of IL-10 by omp28 might

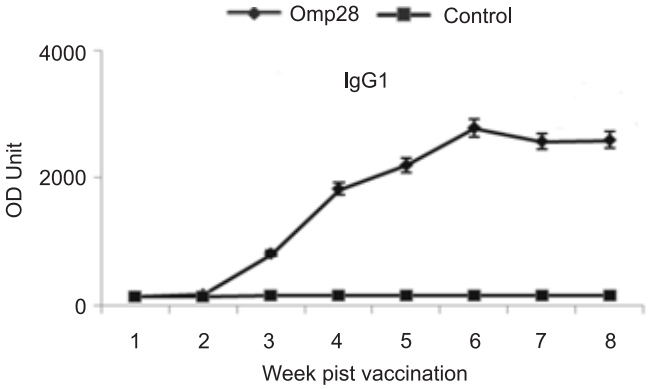

Fig.1. r-OMP28 specific antibody isotype IgG1 responses in pooled sera of mice vaccinated with r-OMP28 and PBS (control). OD unit represents reciprocal dilution of serum multiplied by A492 at that dilution.

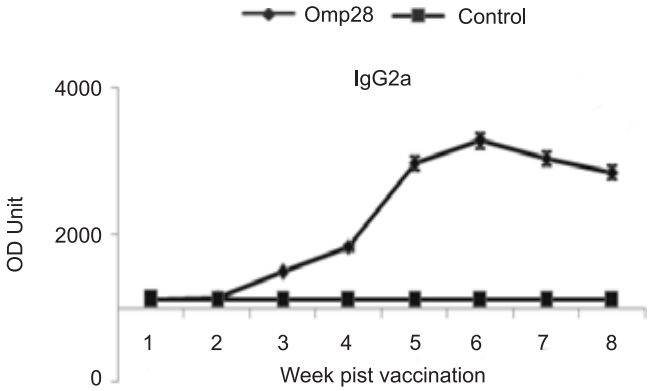

Fig. 2. r-OMP28 specific antibody isotype IgG2a responses in pooled sera of mice vaccinated with r-OMP28 and PBS (control). OD unit represents reciprocal dilution of serum multiplied by A492 at that dilution.

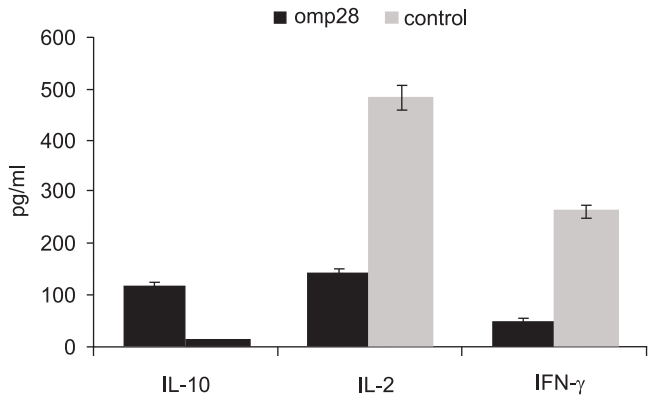

Fig. 3. Quantitative estimation of cytokines IL-10, IL-2 and IFN-g secreted by spleenocytes upon *in vitro* stimulation with r-OMP28.

have resulted in low production of IFN-γ indicating the suppression of Type-1 immune response (Huang *et al.*1999).

In conclusion, the results indicated that r-omp28 induced Th2 type of immune response with increased IL-10 production. The antigen, downregulates the production of IFN- γ and IL-2 resulting in poor or no Th1 type of immune response in mice.

SUMMARY

A 28 kDa recombinant outer membrane protein (r-omp28) of *Brucella melitensis* was evaluated for the induction of immune response in mice. The study demonstrated the downregulation of type-1 immune response in mice vaccinated with r-omp28 of *B. melitensis*. The level of IgG2a was observed significantly lower than IgG1 in mice immunized with r-omp28. The cytokine profile and proliferation of cells was characteristic of Th2 type as we detected significantly high level of IL-10 but no or significantly low level of IL-2 and IFN-γ. Further, the production of gamma-interferon (IFN-γ) was downregulated by r-omp28. Therefore, it implies that *B. melitensis* r-omp28 might down-regulates type-1 immune response in vaccinated mice.

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