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Antigen Presentation Ability of *Salmonella* Carrying DNA Vaccine Model and *MCP-3* gene

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Abstract

The objective of this study is to determine the antigen presentation ability of a DNA vaccine model that is co-delivered with that of recombinant *Salmonella enterica serovar Typhimurium* (STM1) expressing chemokine macrophage chemotactic protein-3 (*MCP-3*). The DNA vaccine, pVROVA, was constructed by amplification of the ovalbumin coding region from sOVA-C1. Dendritic cells (DCs) were obtained from IL-4 and GMCSF stimulated mouse bone marrow stem cell. Cultured DCs were incubated with STM1 carrying a model ovalbumin gene (pVROVA). Furthermore, MHC class I antigen presentation of a dominant OVA peptide was assayed *in vitro.* The experiments were designed to determine the effect of co-delivering *MCP-3* with that of ovalbumin in STM1. Our results show that a plasmid pROVA-carrying ovalbumin gene was succesfully constructed and sequence analysis of the ovalbumin-coding revealed an identity match of 100% with that of the chicken ovalbumin DNA sequences from the GenBank database. We also found that the presence of the *MCP-3* encoding plasmid in STM1 or *E. coli* DH1 could increase the recovery of both STM1 and *E. coli* DH1 over those that carry the empty plasmids. Antigen presentation assay also indicates that *MCP-3* can positively influence the presentation of ovalbumin. Conclusion: the infection of DCs by STM1-carrying DNA vaccine and *MCP-3* results in an increase of processing and presentation of ovalbumin *in vitro.*

Keywords : DNA vaccine, *MCP-3*, APC, *Salmonella*, Dendritic cells

Introduction

Salmonella enterica serovar Typhimurium (STM-1) could infect dendritic cells (DCs) and stimulate these cells to present heterologous antigens. However, it is not yet known whether the DCs antigen presentation is a direct or indirect phenomenon. In a direct antigen presentation, STM1 is presumably internally ingested into the DCs and its peptide directly presented by the DCs themselves. Previous study have reported that DCs can process *Salmonella* and directly present peptides derived from *Salmonella*-encoded antigens to $CD4^+$ and $CD8^+$ T cells (Wick, 2002). Moreover, *Salmonella*-infected DCs that were not fixed in paraformaldehyde, could present foreign epitopes to T cell hybridoma as shown by the secretion of IL-2 from the T cell (Yrlid *et al*., 2001, Yrlid and Wick, 2000). This mode of presentation occurs when the bacteria does not induce cell death in the infected cells (Sundquist *et al*., 2004). However, *Salmonella* can be cytotoxic, thereby inducing apoptosis to the infected cells. In the indirect (cross-presentation) method of antigen presentation, bystander DCs could phagocytosise any infected or apoptotic cells to subsequently obtain and present the STM1-related antigens with the assistance of MHC class I molecules. In this case, the neighboring DCs may internalize apoptotic materials and cross-present *Salmonella* antigens to the T cells (Yrlid *et al*., 2001, Yrlid and Wick, 2000). Heat-shock proteins have been proposed to be involved in antigen presentation through

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the indirect, cross presentation pathway. This occurs as antigen presenting cells (APCs) take up peptides that are chaperoned by heat shock protein released upon cell death (Li *et al*., 2002). APCs then re-present these peptides with their MHC molecules (Li *et al*., 2002). It was previously found that intact protein, small peptide, or peptide-heat shock proteins complexes are essential for cross presenting presentation (Shen and Rock, 2004). Despite this knowledge however, the exact mechanism of antigen transfer from parenchymal cells and cross-presentation in vivo remains unclear and controversial.

Murine chemotactic protein, MCP3, activates a variety of inflamatory cells through a number of different receptors for C-C chemokines that are expressed on the target cells. This chemokine is a pluripotent CC chemokine, which is active on a spectrum of cells including monocytes, lymphocytes, granulocytes, natural killer (NK) and dendritic cells (DCs). MCP was shown to bind to at least three different receptors (CCR1, CCR2 and CCR3) that are present at these inflammatory cells (Wetzel *et al*., 2001). The biological properties of MCP3 offer possibilities for its use as an immunotherapy and adjuvant. Previous studies have found that the transduction of human MCP3 by a parvoral vector induces leucocyte infiltration and reduces growth of human cervical carcinoma cell xenografted into mice. Interestingly, the DCs, which are among the most potent antigen presenting cells, were also found to be attracted into MCP3-tranduced tumors (Wetzel *et al*., 2001). MCP3 has also been utilized as a genetic adjuvant when it was fused with a DNA vaccine and resulted in a broad immune response in mice (Biragyn *et al*., 2002, Rainczuk *et al*., 2003a, Rainczuk *et al*., 2003b).

To investigate whether STM1-infected DCs present STM1 antigen via direct presentation or through cross-presentation, an antigen cross-presentation assay using DCs with mismatched MHC I haplotypes was performed. In this experiment, we could also engineer a better immune response against a DNA vaccine that is delivered by STM1. We performed this with a DNA vaccine construct, pVROVA (encoding ovalbumin), which was co-delivered with recombinant *MCP-3* in STM1. STM1 expressing *MCP-3* was used in a variety of *in vitro* experiments to determine the effects of the chemokine on the immunogenicity of the passenger antigen.

As the pMO*MCP-3* and pVROVA plasmids confer ampicillin and kanamycin resistance, respectively, it was possible to use drugs selection method in ensuring that both plasmids were retained during culture. The pVROVA DNA vaccine plasmid will be express ovalbumin only in eukaryotic cells, and can only occur if there is a transfer of the DNA vaccine to the cell nucleus. The use of *MCP-3* molecule as an immunological adjuvant might be applied to any bacterial vaccine delivery strain. Furthermore, because *S. Typhimurium* is invasive in mice, an initial concern would be that *MCP-3* may only exert its effect due to this invasive property of *S. Typhimurium* itself. To investigate this, we thus utilised a non-invasive *E. coli* DH1 that might reveal the ability of *MCP-3* to augment the immunogenicity of bacteria that are normally non-invasive.

Materials and Methods

Plasmids and bacterial strain used in this study

Plasmids, bacterial strains and cell lines used in these experiments are listed in Table 1.

DNA vaccine construction

We have constructed a DNA vaccine encoding ovalbumin that was encoded by a plasmid selected for ampicillin resistance (Rainczuk *et al*., 2003a). It was necessary to re-clone the ovalbumin coding sequence into a vector with a different drug resistance marker, to enable dual selection with the *MCP-3* encoding plasmid. The DNA vaccine, pVROVA, was constructed by amplification of the ovalbumin coding region from sOVA-C1 (Figure 1) with primers (5'CGCGGATC CATGGGCTCCATCGGTGCAGC3') and (5'CGCGGATCC TTAAGGGGAAACACA

Table 1 Plasmids, bacterial strains and cell line used in this experiment

Bacterial strains and cell lines	Features, use, source
E. coli DH5α	Maintenance of plasmid DNA (Hanahan, 1983).
E. coli DH1	Vaccination of mice (Hanahan, 1983).
S. Typhimurium LT2-9121	Passage plasmid DNA (Reeves and Stevenson, 1989)
S. Typhimurium, STM1	Attenuated vaccine strain *(aroA⁻)*. Used for the vaccination of mice (Alderton *et al.*, 1991)
B3Z86/90.14	T cell hybridoma restricted to $CD8^+$ $H2^{Kb}$ OVA peptide SIINFEKL
Plasmids	
pMOhly1	Amp^r, prokaryotic expression vector with haemolysin secretor signal apparatus (Spreng *et al.*, 1999)
pMO*MCP-3*	pMOhly1 expressing *MCP-3*. (this study)
pVR1012	Kan^r, CMV promoter (DNA vaccine). VICAL Inc, USA
pVROVA	pVR1012 expressing chicken ovalbumin. (this study)

TCTGCC3').

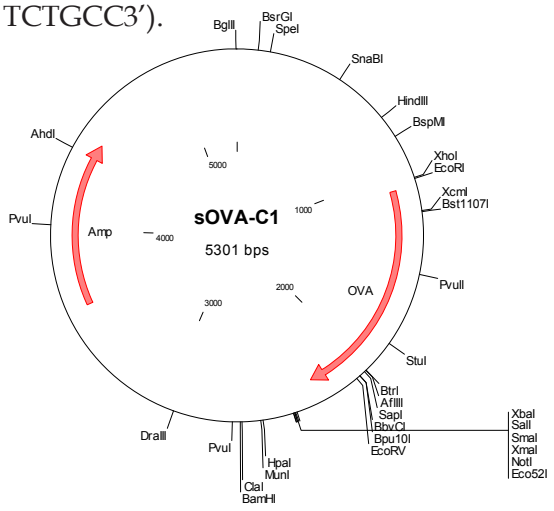

Figure 1. Plasmid map of the eukaryotic expression vector, sOVA-C1, which contains the ovalbumin (OVA) gene and ampicillin (Amp) resistance gene.

The PCR product was digested with *Bam*HI (sites underlined) and inserted into pVR1012 (Figure 2) that had been digested with the same enzyme.

The resultant plasmid, pVROVA (Figure 3) carries a CMV promoter that could direct ovalbumin expression into the cytoplasmic compartment of mammalian cells.

Bacteria were transformed with plasmid DNA as previously described (Bachtiar *et al.*, 2003). The plasmid was first transformed into LT2-9121, and plasmid DNA was subsequently isolated from this strain prior to electroporation into STM1. Passaging of plasmid DNA via LT2-9121 ensures stability of plasmid DNA in STM1.

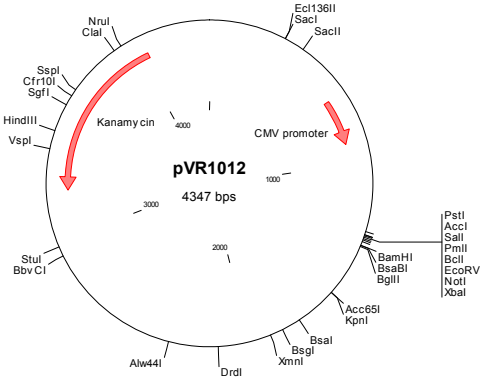

Figure 2. Plasmid pVR1012 harbouring the kanamycin resistance gene and CMV promoter.

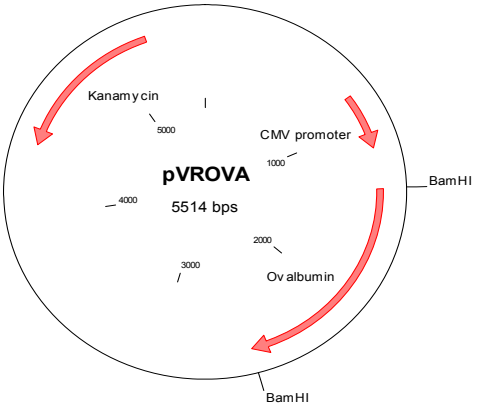

Figure 3. A schematic representative of pROVA, a VR1012 based DNA vaccine harbouring the chicken ovalbumin gene.

Sequence analysis of pVROVA

To ensure the new clone pVROVA is bearing an ovalbumin coding-DNA sequence, VROVA was subjected to DNA sequencing using either the forward primer (5'CG GATCCATGGGCTCCATCGGTGCA GC3') or reverse primer (5'CGCGGATCCTT AGGGAAACACATCTGCC3'), using the Big Dye termination kit.

Antigen presentation assay

Six to eight week old female C57BL/6 ($H2^{Kb}$) and Balb/C ($H2^{Kd}$) SPF mice were used in these experiments. Mice were obtained from the Animal Resource Centre, Perth, Australia and were housed at RMIT University animal facility, Melbourne Australia. Animal ethic experiment was approved by RMIT University Ethic Comission. Denditic cells were obtained from mouse femura bone marrow after incubation with GMCSF and IL-4 (Rainczuk *et al*., 2003b). The ability of mammalian cells infected with STM1 carrying the ovalbumin-expressing plasmid pVROVA to stimulate B3Z cells (B3Z cells are a murine T cell hybridoma specic for the dominant H2Kb MHC class I/ovalbumin epitope 257-264, of sequence SIINFEKL) was assessed. To do this, DCs were cultured and incubated with STM1 carrying pVROVA and MHC class 1 antigen presentation of a dominant OVA peptide was assayed as previously described in (Bachtiar *et al*., 2003). A cross-presentation assay was performed as follows. DCs with a B3Z cells-mismatched haplotype (i.e; $H2^{Kd}$) were set to ingest STM1. We then add the cells with a B3Z cells-matched haplotype to determine (using appropriate control groups) whether the DCs directly present the OVA peptide or if it does so through a cross-presentation pathway. In order to determine this, DCs of the first haplotype were incubated with STM1 for one hour. Antibiotic was then added to kill any extracellular STM1, and the second aliquot of DCs (of either matched or mismatched haplotype) was added. As all extracellular bacteria were killed prior to the addition of the second aliquot of DCs, they could only access STM1 antigen from the first batch of DCs that have taken up STM1. The cultures were incubated overnight, and B3Z cells were added. For quantification of antigen presentation, the same colorimetric assay as described in (Bachtiar *et al*., 2003) was performed the next day. Therefore, any presentation of OVA peptide by the second aliquot of DCs added was presumed to be by cross-presentation. Figure 4 summarises the three repetitions of this experiment.

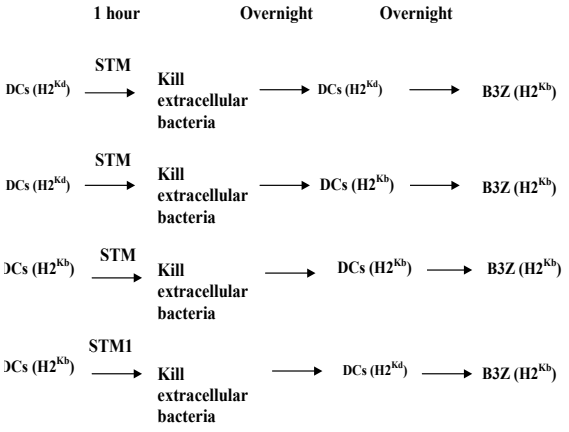

Figure 4. Schematic diagram of the cross-presentation assay

In vitro uptake of E. coli DH1 by cultured DCs

To evaluate the effect of Macrophages Chemotactic Protein, *MCP-3* on the uptake of *E. coli* DH1 by cultured DCs, we incubated either *E. coli* alone, *E. coli* with empty plasmid and *E. coli* carrying *MCP-3* encoding DNA together with the DCs at infection multiplicity of 100 as was previously conducted with STM1 (Bachtiar *et al*., 2003). The percentage uptake of bacterial cells (compared to the original inoculum) was analysed by T test.

Results

Construction of the pVROVA DNA vaccine

The chicken ovalbumin gene was successfully cloned into the pVR1012 vector and digestion using *Bam*H1 (the cloning sites) confirmed the resultant positive clones; (lanes 2 and 15) consist of a 1.3 kb DNA fragment (Figure 5).

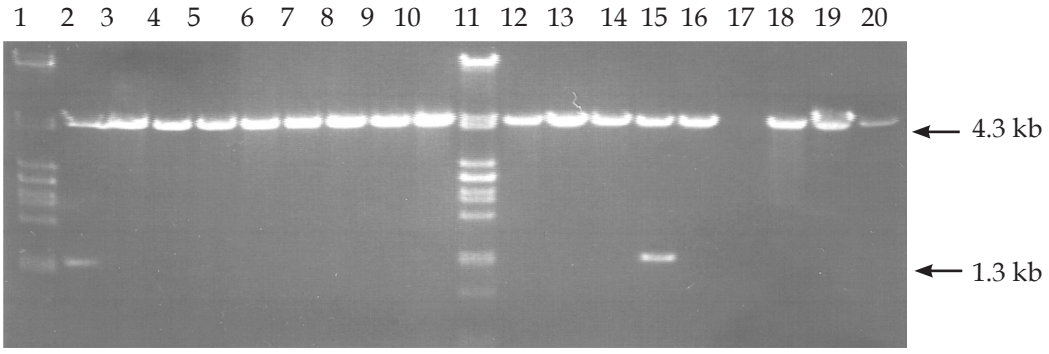

Figure 5. DNA gel electrophoresis of potential pVROVA clones. Lane 1 and lane 11, λ/*Pst*I. Restriction enzyme *Bam*H1 was used to digest plasmid DNA. Successful clones with two bands; 4.3 kb (pVR1012) and 1.3 kb (ovalbumin-coding sequence) were observed in lane 2 and lane 15.

Sequence analysis of the ovalbumin-coding region in clone 15 revealed an identity match of 100% with that of the chicken ovalbumin DNA sequences from the GenBank database. This clone was termed pVROVA and was used for subsequent experiments.

In vitro uptake of STM1 and E. coli by cultured DCs.

As a simple indicator of the effectiveness of secreted *MCP-3* to promote uptake by DCs, the bacterium with and without *MCP-3* encoding plasmid was incubated with murine bone-marrow cells that had been enriched for DCs with GMCSF and IL-4. Results for *E. coli* are shown in Figure 6 compared to those previously obtained for STM1.

This shows that the presence of the *MCP-3* encoding plasmid in STM1 or *E. coli* DH1 could increase the recovery of both STM1 and *E. coli* DH1 over those that carry the empty plasmids.

Antigen presentation and cross-presentation analysis

In addition to the plasmid directing *MCP-3* expression, a second plasmid encoding ovalbumin (pVROVA) was introduced into STM1. As pMO*MCP-3* and pVROVA plasmids confer ampicillin and kanamycin resistance

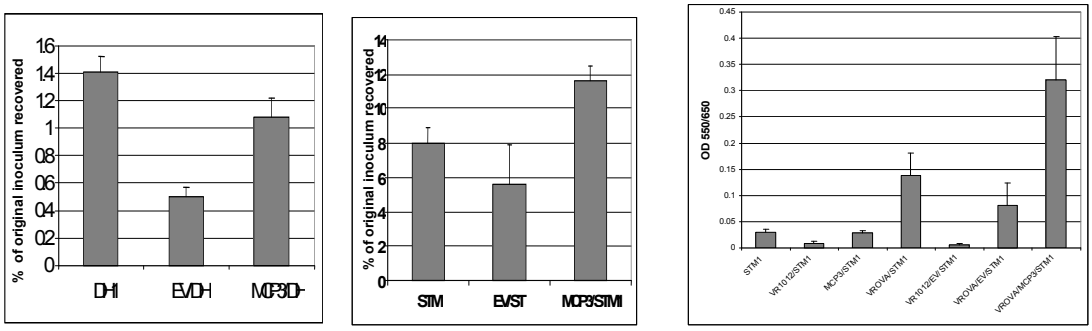

Figure 6. Uptake of *E. coli* DH1 (A) and STM1 (B) with and without *MCP-3*-expressing plasmid by cultured DCs. P values: DH1 vs. EV/DH1 ($P < 0.05$); *MCP-3*/DH1 vs. DH1 ($p > 0.05$); *MCP-3*/DH1 vs. EV/DH1 ($P < 0.05$); *MCP-3*/STM1 vs. STM1 ($P < 0.01$); *MCP-3*/STM1vs. EV/STM1, ($P < 0.05$). Note data of STM1 experiment was taken from previous experiment (see section 4.3.3). EV: Empty vector (pMOHly1). Figure 6 C. *In vitro* signalling of T cells by incubated with STM1 DCs STM1 was transformed with either empty plasmid (VR1012/STM1); *MCP-3*/STM1; DNA vaccine alone (VROVA/STM1) or DNA vaccine and chemokine (VROVA/*MCP-3*/STM1). STM1 was then incubated with cultured DCs. The signalling of B3Z cells was measured in colorimetric assay. Only those carrying VROVA should signal. Values obtained from OVA expressing plasmid with *MCP-3* were compared with those without *MCP-3*. VROVA/*MCP-3*/STM1 vs. VROVA/EV/STM1, $P<0.005$; VROVA/*MCP-3*/STM1 vs. VROVA/STM1, $P < 0.05$; VROVA/*MCP-3*/STM1 vs. *MCP-3*/STM1, $P < 0.001$. EV: Empty vector (pMOhly1).

respectively, using drug selection enabled both plasmids to be retained in STM1. The existence of plasmids in STM1 was confirmed by PCR amplification with the appropriate primers (i.e. *MCP-3* and ovalbumin primers) as described in (Bachtiar, 2005). It was found that under drug selection with ampicillin and kanamycin both plasmids were maintained. Plasmid VROVA will direct the expression of ovalbumin only in eukaryotic cells, and can therefore only occur if there is (1) uptake of STM1 by eukaryotic cells and (2) transfer of the DNA vaccine to the nucleus. These processes were monitored by analysing the presentation of the dominant $CD8^+$ epitope of ovalbumin, SIINFEKL, to T cells as previously described in (Bachtiar *et al*., 2003). Figure 6 B shows that maximal stimulation of the reporter CTL cell line B3Z86/90 was obtained after incubation with STM1 carrying the *MCP-3*-expressing plasmid (more than three-fold increase over STM1 carrying the empty vector, $P < 0.05$), indicating that *MCP-3* positively influenced the presentation of ovalbumin.

A cross presentation analysis indicated that DCs might predominantly involve in a direct presentation of the ovalbumin peptide rather than cross-presentation to T cell (Figure 6C). Firstly, the group of which only Balb/C DCs were added showed that this haplotype cannot present. When Balb/C are added first, followed by C57BL/6 (after elimination of extracellular bacteria), there is only a relatively minor increase in presentation. This is probably

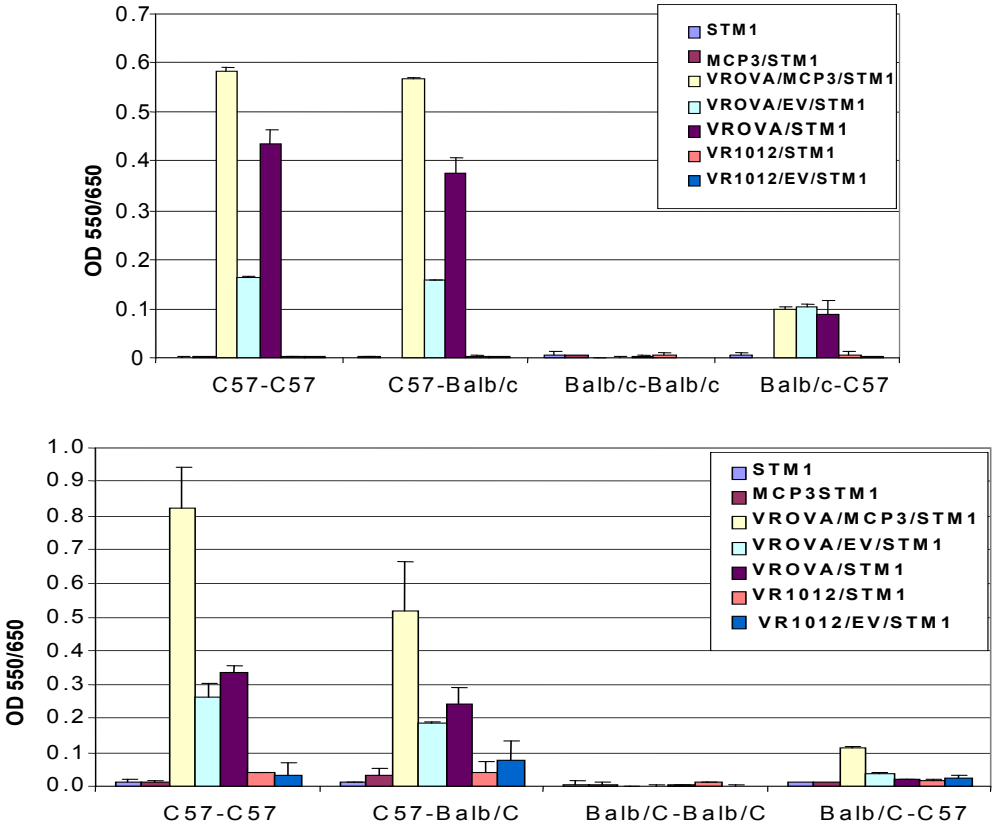

Figure 7. MHC class I presentation to dominant ovalbumin CD8+ epitope by DCs to B3Z T cell. Data from 2 independent experiments is shown (A and B). The assay was performed by sequential co-culture of DCs with a matched or mismatched haplotype to B3Z cells. STM1 carrying the indicated plasmid constructs was incubated with DCs from either Balb/C or C57BL/6 mice. After 1 h incubation and killing of the extracellular bacteria, each infected-DC culture was co-cultured with matched or mismatched DCs (C57-C57; C57-Balb/C; Balb/C-Balb/C or Balb/C-C57) followed by addition the responder cell (B3Z cells). The signalling of responder cells was then measured.

specific; however, as in the first experiment (Figure 7A and Figure 7B) the only increase was seen in cells that carried OVA sequences.

In the second experiment, this increase was only seen in the VROVA/*MCP-3*/STM1 group. Conversely, when C57BL/6 cells were added first, followed by either an aliquot of C57BL/6 or Balb/C presentation was at a high level.

Finally, this experiment demonstrates a positive effect of the presence of *MCP-3* to the magnitude of antigen presentation of SIINFEKL by DCs to T cells, as DCs incubated with STM1 expressing this chemokine increased antigen presentation over that of STM1 with the empty vector ($P<0.05$).

Discussion

The ability of APCs surveying the gut mucosa to take up *S. Typhimurium* is one requirement for efficient oral STM1-based DNA vaccination. Activated APCs migrate to the lymph nodes, then produce and process the passenger molecule for further presentation to immune cells. The results revealed that the signalling of B3Z cells (T cells receptors recognising MHC class I/SIINFEKL) could increase ovalbumin expressing plasmid with *MCP-3* over those without *MCP-3*. It indicated that *in vitro,* co-delivery of a DNA vaccine with a chemoattractant molecule *MCP-3*, enhanced the ability of cultured DCs to take up, process and present passenger antigens carried by STM1. This finding supports the previous study that *MCP-3* increased the immunogenicity of STM1 (Bachtiar, 2005), it is probable that increased antigen presentation might be due to increased uptake of *MCP-3*-secreting STM1 (Bachtiar *et al*., 2003). In future experiments, trafficking of DCs to *MCP-3*-secreting STM1 shall be investigated.

Infection of bone marrow-derived macrophages with wild-type *S. Typhimurium* 14028 results in presentation of epitopes derived from a bacterial encoded antigen on MHC class I (Catic *et al*., 1999, Svensson and Wick, 1999) and MHC class II molecules after internalisation of apoptotic macrophages by bystander DCs (Yrlid *et al*., 2000, Svensson *et al*., 1997). In this current study, an experiment was designed to determine if the presentation of antigen was performed by the APC's that actually take up the bacterial vaccine. The *in vitro* analysis was performed where cultured DCs were incubated with STM1 carrying a model ovalbumin gene and MHC class I antigen presentation of dominant OVA peptide was assayed. It was shown that STM1 could be taken up by the cells and enable heterologous antigen presentation (Bachtiar *et al*., 2003). An assay was performed in order to determine if this presentation is direct (i.e. the DCs that take up the STM1 present the peptide), or indirect (cross-presentation) where bystander DCs phagocytose infected cells and subsequently present the peptide by cross-presentation through MHC class I. The result from this assay indicated that the mode of antigen presentation occurred primarily via a direct antigen presentation, with minimal contribution of the cross-presentation method. In these experiments, the incubation with the second aliquot of DCs was done overnight. It may be that longer incubation would result in an increased of cross-presentation. This study suggests the possibility of vaccine development by STM1 carrying DNA vaccine and *MCP-3*.

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