Recombinant *Salmonella enterica* serovar Typhimurium vaccine vector expressing green fluorescent protein as a model antigen or human immunodeficiency virus type 1 subtype C Gag

by

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AUTHOR'S FOREWORD

HIV-1 vaccinology is still one of those fields of biomedical research where even angels fear to tread and we (PhD candidates) enter at our own peril. Although almost two and half decades have passed since the first report of HIV/AIDS, a protective vaccine against the virus has not successfully being developed. No candidate HIV vaccines have even reached Phase IV clinical trials. The key challenges to the HIV-1 vaccine development still remain. Most scientists have even speculated that a successful HIV-1 vaccine will never be developed. With all the challenges and loss of hope, there is a need to come up with more radical and pragmatic approaches to HIV vaccine development, if the tables are to be turned. The use of *Salmonella* bacterial vaccines expressing HIV antigens offers novel strategies for activation and induction of all arms (innate, mucosal, systemic, CD4+ T-cell, CD8+ T-cell and humoral responses) of the immune system against the virus. Regarding the research reported here, I have excluded the bulk of my work that seemed to yield little contribution to knowledge in the field of HIV vaccinology. I spent most of my time attempting to express HIV-1 Gag antigens using strong promoters in *Salmonella* bacterial vaccine vectors with fruitless and poor immunogenicity results in mice. It is beyond the scope of the thesis to include all that work. Although such work will not be reported herein, it is important to point out that it set up some groundwork and direction to the approach reported here. The thesis focuses mainly on the development and use of a novel approach to expression and delivery of foreign antigens by a recombinant *Salmonella* vaccine vector. The *Salmonella* expression system was based on the use of the *Escherichia coli lac* operon sequences to express green fluorescent protein (model) and HIV-1 Gag antigens. The antigens were fused downstream the first 40 amino acid residues of the *E. coli* β-galactosidase α-peptide with expression driven by the *lac* promoter. Oral vaccination of mice with the recombinant *Salmonella* vaccine vector induced systemic antigen-specific CD8+ Tc1/Tc2-, CD4+ Th1/Th2-cell cytokine and IgG1/IgG2a antibody responses. Such multi-pronged vaccine-induced immune responses are critical in prevention or control of most mucosal pathogens. It is my hope that scientists in the field of *Salmonella* vector vaccinology will find great interest in employing such a simple but ingenious approach used in this study for their own endeavours in developing vaccines for human or veterinary use.

Nyasha Chin'ombe, September 2006

DECLARATION

The work described in the thesis was performed in the Division of Medical Virology, Department of Clinical Laboratory Sciences of the University of Cape Town, under the general supervision of Professor **Anna-Lise Williamson**, Professor **Enid G Shephard** and Dr **William Bourn**. This is my own work and where the use of others is included, their contributions have been acknowledged.

Signed by candidate

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Nyasha Chin'ombe

September 2006

DEDICATION

In memory of

my late brother, **Boas "Pinto" Chin'ombe** (1958-1999), who died after a battle with HIV/AIDS,

my late father, **Mamvura "Njore"Chin'ombe** *(*1901-1984)

&

the "lost generations" of Masikiri Village, Bikita, Zimbabwe

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- My brothers (Norman, Stephen and Hallmark) and sisters (Yeukai and Ellen) in Zimbabwe, for moral support.
- My mother, Pfudzai, for always reminding me of the biblical and inspirational quote from Psalms 126v5, "he that goes forth weeping, bearing the seed for sowing, shall come home singing with joy as he brings the harvest".
- All my friends and colleagues, Phelex Manyanga, Nandipha Mahlulo, Gerald Chege, Unity Magosa, Siphiwo Mabija, Andile Ngwane, Wangari Serah Kimani, Collet Dandara, Patience Basvi, Daniswa Pupa, Zoleka Skepu, Denis Chopera, Nani Khanyiswa, Everisto Munani, Ludwe, Thabisa Mupakata, Lazarus Matizirofa, Juao Perraire, Lufeyo Banda and others, for company, encouragements and fun.
- My ancestors and/or God, for making me sail successfully through the turbulent sea of research.
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- International AIDS Vaccine Conference Committee, for 2004 and 2005 International Scholarships to attend and present some of my work at the AIDS Vaccine conferences held in Lausanne, Switzerland, September 2004 and Montreal, Canada, September 2005.
- South African AIDS Vaccine Initiative (SAAVI), for funding and bursary.

ABRIDGED TABLE OF CONTENTS

LIST OF ABBREVIATIONS

'	prime
α	alpha
β	beta
γ	gamma
2 YT	2x yeast-tryptone
A	adenine
a.a	amino acid
AIDS	acquired immunodeficiency syndrome
bp	base pair(s)
C	cytosine
CA	capsid
CBA	cytometric bead array
CD	cluster of differentiation
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cfu	colony-forming units
Con A	Concanavalin A
C-terminal	carboxyl terminal
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
Env	envelope
G	guanine
g	standard gravitational acceleration
Gag	group antigen
GFP	green fluorescent protein
hr	hour(s)
HIV-1	human immunodeficiency virus type 1
HRP	horseradish peroxidase
IFN-γ	interferon gamma
IgG	immunoglobulin G
IL	interleukin
IN	integrase
IPTG	isopropyl-thio-β-galactosidase
Kb	kilobase(s)
kDa	kilo-Dalton
lac	lactose
LPS	lipopolysaccharide
LTR	long terminal repeat
MA	matrix
min	minute(s)
ml	millilitre(s)
mRNA	messenger RNA
MW	molecular weight
NC	nucleocapsid
ND	not determined
ng	nanogram(s)
nm	nanometer(s)
N-terminal	amino terminal
°C	degree celcius
OD	optical density (absorbance)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RBS	ribosome-binding site
PR	protease
RNA	ribonucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SFUs	spot-forming units
SI	stimulation index
T cell	T lymphocyte
T	thymine
Tc1 cell	T cytotoxic type 1 cell
Tc2 cell	T cytotoxic type 2 cell
Th1 cell	T-helper type 1 cell
Th2 cell	T-helper type 2 cell
TNF-α	tumour necrosis factor alpha
U	uracil
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-galactosidase
μ	micro

Note: abbreviations for amino acids are listed in **Appendix C**

PUBLICATIONS AND CONFERENCE ABSTRACTS/PRESENTATIONS

1 **Chin'ombe N**, Williamson A-L, Shephard EG and Bourn W. Development of recombinant *Salmonella* vectors for HIV-1 subtype C vaccine for Southern Africa. *Cent Afr J Med. (Supplement)* 2003 49 (1-2):21-22

2 **Chin'ombe N**, Williamson A-L, Shephard EG and Bourn W. Development of recombinant *Salmonella* vectors for HIV-1 subtype C vaccine for Southern Africa. 5th Congress of the Federation of African Immunological Societies (FAIS), Victoria Falls, Zimbabwe, 27 April/May 2003 (abstract/oral presentation)

3 **Chin'ombe N**, Williamson A-L, Shephard EG and Bourn W. Immunogenicity of a recombinant *Salmonella* vaccine vector expressing HIV-1 Gag and GFP antigens. 3RD Annual Africa Genome Conference, 22-24 March 2005, Nairobi, Kenya (Abstract/oral presentation)

4 **Chin'ombe N**, Shephard EG, Williamson A-L, Makhubela S and Bourn W. Systemic CD4+ T cell responses to an oral recombinant *Salmonella Typhimurium* vector expressing codon-optimized HIV-1 subtype C Gag. AIDS VACCINE 2005 Congress, 6-9 September 2005, Montreal, Canada (Abstract/poster presentation)

5 **Chin'ombe N**, Shephard EG, Williamson A-L and Bourn W. Systemic CD4+ T cell responses to an orally-delivered recombinant *Salmonella* vector expressing codon-optimized HIV-1 subtype C Gag. VIROLOGY AFRICA Congress, 8-11 November 2005, Cape Town South Africa (Abstract/Poster presentation)

6 **Chin'ombe** N, Shephard E, Bourn W and Williamson A-L, Recombinant *Salmonella* vaccine vector expressing foreign antigens, 7th WHO-IVR Global Vaccine Research Forum, 03-06 December, 2006, Bangkok, Thailand (Abstract/Poster presentation)

7 **Chin'ombe N**, Shephard EG, Williamson A-L and Bourn W. Induction of mixed CD8+ Tc1/Tc2 cytokine responses to an orally-delivered recombinant *Salmonella* vaccine vector in mice. (manuscript)

8 **Chin'ombe N**, Williamson A-L, Shephard EG and Bourn W. Induction of mixed CD4+ Th1/Th2 cytokine responses to an orally-delivered recombinant *Salmonella enterica* serovar Typhimurium vaccine vector expressing codon-optimized HIV-1 subtype C Gag. (manuscript)

9 **Chin'ombe N**, Williamson A-L, Shephard EG and Bourn W. Localization of HIV-1 Gag in a recombinant *Salmonella* vaccine vector: short communication. (manuscript)

ABSTRACT

Salmonella bacteria and human immunodeficiency virus type 1 (HIV-1) infect their hosts via the mucosal routes. They replicate in the mucosa-associated lymphoid tissue before they spread to systemic sites. To prevent systemic infection with these pathogens, it is critical to develop effective vaccines that induce both cellular and humoral immune responses in the mucosal and systemic compartments. Recombinant live attenuated *Salmonella* expressing foreign antigens offer a great potential as effective oral vaccines that are capable of inducing both mucosal and systemic responses. The overall objective of the study reported here was to design, construct and evaluate cellular and humoral immune responses of a recombinant attenuated *Salmonella enterica* serovar Typhimurium vaccine vector expressing *Aequorea victoria* green fluorescent protein (GFP) model or HIV-1 subtype C Gag antigens. Recombinant prokaryotic expression plasmids, pGEM+GFP, pGEM+wtGag and pGEM+Salmgag, harbouring *gfp*, *wtgag* (wild-type HIV-1 *gag)* and *salmgag* (codon-optimized HIV-*1 gag*) genes respectively, were developed. Each of the genes was fused in-frame with the 5'-domain (first 40 codons) of the *E. coli* β-galactosidase α-gene fragment (*LacZ*α), with expression under the relevant *Escherichia coli lac* operon transcription and translation sequences. Two more expression plasmids, pGEM+P1724 and pGEM+24D were further designed and constructed, in which codon-optimized HIV-1 *p41* or *p24* genes were embedded in-frame between first the 40 and last 87 codons of *E. coli* β-galactosidase α-gene fragment and with expression under the *Escherichia coli lac* operon transcription and translation sequences. The five recombinant plasmids were used to genetically transform an *AroC Salmonella enterica* serovar Typhimurium vaccine mutant, thereby generating five recombinant vaccines: AroC+GFP, AroC+wtGag, AroC+Salmgag, AroC+P1724 and AroC+P24D. High-level antigen expression of recombinant GFP, wtGag, Salmgag, P41 and P24D by the *Salmonella* vaccine vector was demonstrated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. It was concluded that the fusion of the antigens to the amino and/or carboxyl terminal domains of the β-galactosidase α-peptide and the use of natural *E. coli lac* operon sequences potentially enhanced the high expression of the heterologous antigens by the bacterial vector.

To investigate the potential of the recombinant *Salmonella* as a vaccine vector, BALB/c mice were orally vaccinated with AroC+GFP, AroC+wtGag, AroC+Salmgag, AroC+P1724 or AroC+P24D and systemic antigen-specific CD8+ Tc1/Tc2, CD4+ Th1/Th2 cytokine and IgG1/IgG2a antibody responses were evaluated. One group of mice (for each vaccine) was vaccinated on Day 0 and sacrificed on Day 28. The effect of multiple vaccinations was investigated by inoculating another group of mice with the same vaccines on Days 0, 28 and 56 with sacrifice on Day 84. The animals were bled before each vaccination and sacrifice and serum processed for antibody analyses. At sacrifice, spleens were harvested and the frequency of antigen-specific CD4+ T- and CD8+ T cells secreting cytokines assessed by interferon-gamma and interleukin-4 enzyme-linked immunospot (IFN-γ and IL-4 ELISPOT) assays. The cytokines, tumour necrosis factor alpha (TNF-α), IFN-γ, IL-4 and interleukin-5 (IL-5) secreted by the splenocytes

into supernatant after stimulation with various antigen-specific epitopes were quantified by cytometric bead array (CBA) assay.

Splenocytes from mice vaccinated once with AroC+GFP did not elicit detectable CD8+ T-cell cytokine response after *ex vivo* stimulation with an H-2K^d restricted class I GFP epitope (HYLSTQSAL). Analysis of immunogenicity in mice that received two booster vaccinations showed induction of high frequencies of GFP-specific CD8+ T cells (226 net IFN-γ and 132 IL-4 spot-forming units (SFUs)/10^6 splenocytes). The result was further confirmed by CBA assay which showed that the splenocytes secreted into the supernatant elevated levels of IFN-γ (65-fold above background) and IL-4 (5-fold above background) cytokines after stimulation for 48 hours with the GFP peptide. GFP-specific serum IgG1 (suggesting CD4+ Th2) and IgG2a (suggesting CD4+ Th1) immune responses were induced after a single inoculation and the responses were enhanced after the two booster immunizations. The findings suggested that the recombinant *Salmonella* vaccine was capable of vectoring the GFP model antigen in mice for induction of systemic antigen-specific CD8+ Tc1/Tc2, CD4+ Th1/Th2 cytokine and antibody immune responses.

Mice vaccinated once with recombinant *Salmonella* expressing wtGag did not induce detectable HIV-1 Gag-specific CD8+ T cell cytokine responses after single or triple inoculations. However, HIV-1 Gag-specific CD4+ Th2 (IL-4), but not Th1 (IFN-γ) cytokine response were detected on Day 84. The stimulation of the splenocytes from the boosted mice with a H-2K^d restricted class II Gag epitope (MRC-13) resulted in secretion into supernatant of elevated levels of IL-4 cytokine (5-fold above the background). The vaccinated mice did not elicit Gag-specific IgG antibody response by Day 28 or Day 84. The reason(s) for the imprinting of only a Gag-specific CD4+ Th2 (IL-4)-biased response by the AroC+wtGag were not clear. However, the lack of codon-optimization of *wtgag* might have affected its expression and subsequent delivery by the *Salmonella* vector. Mice vaccinated with the *Salmonella* vector expressing Salmgag (codon-optimized Gag) did not induce specific CD8+ or CD4+ T-cell cytokine responses after single inoculation. However after three inoculations, low HIV-1 Gag-specific CD8+ Tc1 (IFN-γ) cytokine response was induced. IFN-γ induced after stimulation of splenocytes with an H-2K^d restricted class I Gag epitope (AMQMLKDTI) was 3-fold above the background. Splenocytes from these mice also secreted elevated HIV-1 Gag-specific CD4+ Th1 (TNF-α (29-fold above background) and IFN-γ (8-fold above background)) and Th2 (IL-4 (26-fold above background) and IL-5 (>89-fold above background)) cytokines after stimulation with the H-2K^d restricted class II Gag epitope, MRC-13. Serum Gag-specific IgG1 and IgG2a were detected in vaccinated mice on Day 84 and this further confirmed the elicitation of mixed Gag-specific CD4+ Th1 and Th2 responses. The codon-optimization of *salmgag* potentially enhanced its stable expression *in vivo* by the recombinant *Salmonella* vaccine vector and subsequently improved the nature, quality and magnitude of the HIV-1 Gag-specific immune responses.

Mice vaccinated with AroC+p1724 and AroC+p24D elicited HIV-1 Gag-specific CD4+ Th2 (IL-4) responses after single inoculation. The responses improved after three inoculations for only the AroC+P1724 vaccine, but not AroC+P24D. Gag CD8+ Tc2 (IL-4) response was induced only by the vaccine AroC+P24D after three inoculations. However, no Gag-specific IgG antibody responses were detected in mice vaccinated with these two vaccines. Although no Gag-specific humoral responses were detected, the study demonstrated that cellular responses could be elicited to the embedded antigens that were expressed by the *Salmonella* vector. Sub-cellular localization of GFP and Salmgag in recombinant *Salmonella enterica* serovar Typhimurium vaccine vector was finally investigated using fluorescence microscopy. GFP was homogenously distributed throughout the vector. In contrast, Salmgag was confined predominantly at the poles of the bacterial cells. The difference in the properties of the two antigens probably affected their localization inside the bacterial cells.

In conclusion, a recombinant *Salmonella enterica* serovar Typhimurium vaccine vector expressing high levels of GFP model or HIV-1 Gag antigens using the *E. coli lac* operon system was developed. Furthermore, it was also demonstrated that, using the expression system, an orally-delivered recombinant *Salmonella* vaccine vector expressing GFP or HIV-1 Gag could induce systemic antigen-specific CD8+ Tc1/Tc2, CD4+ Th1/Th2 cytokine or IgG1/IgG2a antibody profiles in mice. Future studies should further exploit the strategy employed in this study for high-level expression of heterologous antigens in *Salmonella* and development of the next generation of mucosal vaccines for human and animal uses.

CHAPTER 1: INTRODUCTION TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 VACCINES

"In literature as in love, we are astounded at what is chosen by others" Andre Maurois (1885-1967)

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CHAPTER 1: INTRODUCTION TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 VACCINES

1.1 SCOPE OF THE HIV/AIDS PROBLEM

More than two decades ago, the human immunodeficiency virus (HIV) was recognized as the etiologic agent for acquired immunodeficiency syndrome (AIDS) (Gallo *et al.*, 1983; Gelmann *et al.*, 1983). Since then, the virus has diversified genetically and spread globally. To date, there are no prophylactic or therapeutic vaccines to mitigate the ever-expanding global HIV/AIDS pandemic. According to the latest available UNAIDS/WHO report, the scope of problem has reached unprecedented proportions (Table 1.1). The numbers of people who were living or newly infected with the virus at the end of 2005 were high. Many people have also died of AIDS-related diseases in the same year. Analyses of global trends

Table 1.1: The global scope of the HIV/AIDS problem based on 2005 estimates (UNAIDS/WHO, 2005)

Number of people living with HIV in 2005	**Total**	**40.3 million**
	Adults	38.0 million
	Children under 15 years	2.3 million
People newly infected with HIV in 2005	**Total**	**4.9 million**
	Adults	4.2 million
	Children under 15 years	700 000
AIDS deaths in 2005	**Total**	**3.1 million**
	Adults	2.6 million
	Children under 15 years	570 000

indicate that the developing countries have been the worst affected by the HIV/AIDS epidemic (Table 1.2). Of the more than 40 million people living with HIV globally at the end of 2005, the majority were found in Sub-Saharan Africa. Other regions that include developing countries where the HIV/AIDS epidemic is also severe are South/South East Asia, Latin America, East Asia, North America and Eastern Europe/Central Asia. The global nature of the problem calls for the development of effective prophylactic vaccines against the HIV.

Although sub-Saharan Africa has the majority of global HIV/AIDS cases, the molecular epidemiology of the virus varies with sub-regions. The Southern African countries have the worst AIDS epidemic in the sub-Saharan African region, with more than 94% of infections caused by HIV-1 Subtype C (Osmanov *et al.*, 2002). The greatest number (more than 5 million) of people living with the virus in the world is found in South Africa (Shisana and Simbayi, 2002; UNAIDS/WHO, 2005; Department of Health, South Africa, 2005). Other countries in Southern Africa (Botswana, Swaziland, Lesotho, Zimbabwe, Zambia, Namibia) also have a high incidence of HIV-1 subtype C. There is therefore an urgent need for the development of a safe, effective, affordable and easy-to-deliver prophylactic vaccine for HIV-1 subtype C. The approach of developing a specific vaccine for Southern Africa region is likely to be beneficial

because of the high levels of homogeneity of HIV-1 subtype C viruses circulating in the region (Osmanov *et al.*, 2002; Williamson *et al.*, 2003). The lack of access to effective anti-retroviral therapy in the region further supports the need for the development of a prophylactic or therapeutic vaccine for use in mitigating the HIV/AIDS epidemic.

Table 1.2: The scope of the HIV/AIDS problem in Sub-Saharan Africa (compared to other regions) in 2005 (UNAIDS/WHO, 2005)

Region	Adults & children living with HIV (end of 2005)	Adults & children newly infected with HIV in 2005	Adult prevalence (%) in 2005	Adult and child deaths due to AIDS in 2005
Sub-Saharan Africa	25.8 million	3.2 million	7.2%	2.4 million
South/SE Asia	7.4 million	990,000	0.7%	480,000
Latin America	1.8 million	200,000	0.6%	66,000
East Asia	870,000	140,000	0.1%	41,000
North America	1.2 million	43,000	0.7%	18,000
Eastern Europe/Central Asia	1.6 million	270,000	0.9%	62,000
Western/Central Europe	720,000	22,000	0.3%	12,000
Caribbean	300,000	30,000	1.6%	24,000
North Africa/Middle East	510,000	67,000	0.2%	58,000
Oceania	74,000	8,200	0.5%	3,600
Global	**40.3 million**	**4.9 million**	**1.1%**	**3.1 million**

1.2 HIV-1 GENOMIC ORGANIZATION AND GENE TARGETS FOR VACCINES

HIV-1 is a Lentivirus which belongs to the Family *Retroviridae*. The virus has an RNA genome of 9.8 kb (Muesing *et al.*, 1985). The basic genetic and replication cycle of HIV-1 have recently been reviewed in Freed *et al.*, (2004) and Sierra *et al.*, (2005). The HIV-1 genome encodes nine open reading frames (Figure 1.1). The HIV-1 virion structure is shown in Figure 1.2. The virion is spherical in shape and is composed of a host-derived lipid envelope. The core of the virion is made up of Gag proteins and contains two RNA molecules and other HIV-1 proteins. The different proteins encoded by the HIV-1 genome play important roles in the life cycle of the virus. The nine proteins encoded by the HIV-1 genome are classified into four groups as: (a) Structural proteins (Gag and Env), (b) Enzyme proteins (Pol), (c) Regulatory proteins (Tat and Rev) and (d) Accessory (auxiliary) proteins (Vpu, Vpr, Vif and Nef). The different HIV-1 antigens are all targets for vaccine development (Figure 1.2). The HIV-1 envelope glycoprotein (gp160) is encoded by the *env* gene, and is cleaved into gp120 (SU) and gp41 (TM) glycoproteins by a cellular protease (Crise *et al.*, 1990). The gp120 has both the CD4 receptor and co-receptor binding domains (Feng *et al.*, 1996). The binding of CD4 to gp120 enhances its interactions with the chemokine receptors (reviewed in Galanakis *et al.*, 2005). This interaction is essential for viral attachment and entry during infection. Gp160 glycoproteins are therefore there prime targets for

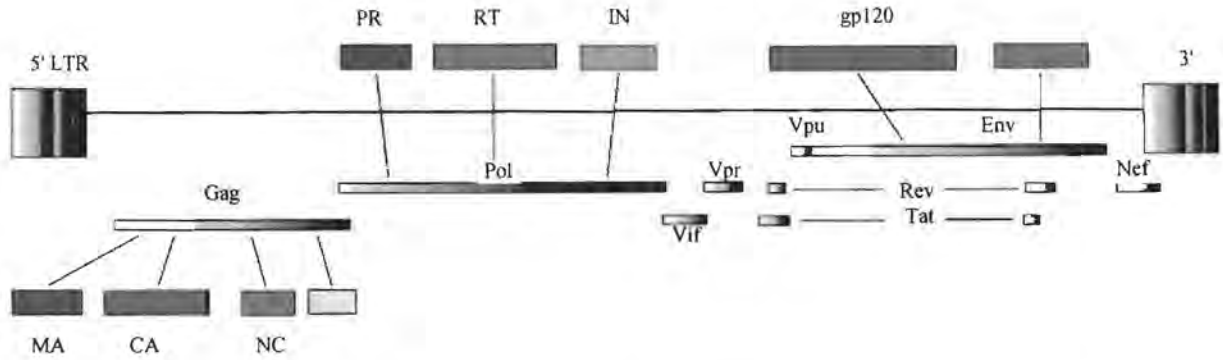

Figure 1.1: The genetic organization of the HIV-1 genome (adapted from Freed, 2004). The long terminal repeats (LTRs) flank the HIV-1 genes. Gag precursor is cleaved into matrix (MA), capsid (CA), nucleocapsid (NC) and p6. The Pol is cleaved into viral protease (PR), reverse transcriptase (RT) and integrase (IN). The Env glycoprotein (gp160) generates surface glycoprotein gp120 and the transmembrane glycoprotein gp41. The HIV-1 genome also encodes Vif, Vpr, Nef, Tat, Rev and Vpu.

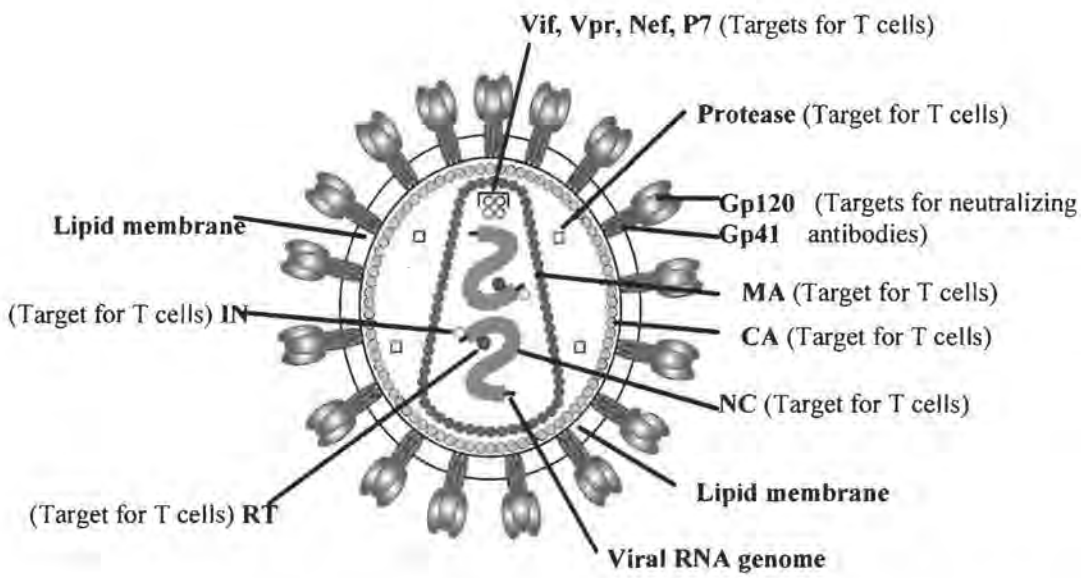

Figure 1.2: Schematic structure of a mature HIV-1 virion structure and gene targets for vaccine development (modified from from Giri *et al.*, 2004).

neutralizing antibodies which can potentially block HIV-1 entry into the cell. However, HIV-1 Env can also be utilized in vaccines that are expected to induce T cell immune responses. Most of the current HIV-1 subunit vaccines are based on Gp160.

With the exception of Env, other HIV-1 antigens are not suitable targets for vaccines that are designed to induce neutralizing antibodies. However, these antigens can be targeted for vaccines that are expected to induce HIV-1-specific cellular immune responses (Figure 2.2). The *gag* gene encodes a 55 KDa polyprotein (P55) which is cleaved by the viral PR into MA (p17), CA (p24) and NC (p15). P15 is further processed into smaller fragments, p2, p7, p1 and p6 (Cherry and Wainberg, 2002). The MA is the

N-terminal component of Gag and initiates virion assembly by targeting Gag and Gag-Pol polyprotein to the plasma membrane of the infected cell (Freed, 1998). The functions of the components of NC (P15) are poorly understood. However, the NC components are thought to be important for virus infectivity and have numerous other vital functions in the virus life cycle such as packaging (Feng *et al.*, 1996; Ott *et al.*, 2005). Gag is one of the highly conserved HIV-1 antigens with several immunodominant T cell epitopes (Cao *et al.*, 1997; Ferrari *et al.*, 2000; Novitsky *et al.*, 2001; Currier *et al.*, 2006; Newberg *et al.*, 2006).). This has made it a prime target for T cell vaccines that are expected to control primary HIV-1 viremia and disease progression.

The *pol* gene is initially expressed as a Gag-Pol fusion polyprotein (p160) by a translational frameshifting mechanism (Jacks *et al.*, 1988). The precursor is cleaved to produce protease (PR, p10), reverse transcriptase (RT, p66/51) and integrase (IN, p32) (Figure 1.1). RT is responsible for the reverse transcription of viral RNA into DNA (Gotte *et al.*, 1999; Jonckheere *et al.*, 2000). The HIV-1 integrase (IN) is critical for the incorporation of viral DNA into the chromosomal DNA of the target cell (Turner and Summers, 1999). Tat (viral transactivator) protein is a regulatory RNA-binding protein and activates viral gene transcription (Li *et al*; 2005a; Brady and Kashanchi, 2005; Hetzer *et al.*, 2005; Gibellin *et al.*, 2005). The Tat protein increases production of viral mRNAs and enhances viral replication. Rev protein is a regulator of virion protein expression (Turner and Summers, 1999). Nef (negative factor) plays important roles in the downregulation of the cell surface expression of CD4 and major histocompatibility complex class 1 molecules (MHC-I), increasing the infectivity of viral particles, and affecting signal transduction pathways of the infected cell, thereby promoting viral replication and spread (Das and Jameel, 2005; Li *et al.*, 2005). The importance of Vif, Vpu and Vpr has been reviewed recently (Anderson and Hope, 2004; Yu *et al.*, 2005). Vif functions during viral assembly by promoting the formation of competent particles and to initiate new infections. Vpu has a role in the enhancement of virion maturation and release. Vpr is believed to be important in assisting the transport of the pre-integration complex from the cytoplasm into the nucleus. Although regulatory and accessory antigens are also good targets for T cell vaccines (Yu *et al.*, 2005), few programs have utilized them.

1.3 HIV-1 GENETIC DIVERSITY AND EPIDEMIOLOGY

One of the key characteristics of HIV-1 is its high genetic variability, which emanates mainly from the failure of RT to proof-read while creating the full-length RNA transcript (Peeters and Sharp, 2000; Thomson *et al.*, 2002). In addition to having a high error rate, HIV-1 RT has recombinogenic properties which facilitate the generation of new viral genetic recombinants (Peeters *et al.*, 2003). These two factors work in concert to generate extreme genetic diversity in the HIV-1 genome. With such high diversity of the virus, vaccine development and therapeutic management due to emerging drug resistance, have been a great challenge up to this day (reviewed in Lal *et al.*, 2005; Julg and Goebel, 2005; Geretti, 2006).

The HIV-1 has been divided into groups, sub-types and circulating recombinant forms (CRF) and unique recombinants (Robertson *et al.*, 2000). Three distinct HIV-1 groups of viruses exist. They are M (main),

O (outlier) and N (new or non-M/non-O). Group M viruses are responsible for the majority of HIV-1 infections worldwide. Group O isolates are highly divergent from group M, their prevalence is low compared to other viruses and infection is confined to W. African countries (Peeters and Sharp, 2000). Group N viruses have only been identified and isolated from Cameroon. Phylogenetic analysis of the *env* and *gag* genes of the group M has established 9 distinct subtypes (subtypes A, B, C, D, F, G, H, J and K) (Peeters *et al.*, 2003; Geretti, 2006). The global distribution and prevalence of the individual HIV-1 subtypes and their recombinants vary extensively (Table 1.3; Figure 1.3), and this is a great challenge to the development of vaccines.

Table 1.3: Geographical distribution of HIV-1 subtypes (adapted from Geretti *et al.*, 2006)

Subtype	**Prevalence in relation to other subtypes**	**Main geographical distribution** (see also Figure 1.3)
A	High	Eastern Africa, Eastern Europe and Central Asia
B	High	Americas, Western Europe, Australia and Japan
C	High	Southern Africa, Eastern Africa, India and China
D	High	Eastern Africa
F	Low	South America, Central Africa and Eastern Europe
G	Low	Central Africa
H	Low	Central Africa
J	Low	Central Africa
K	Low	Central Africa
Recombinants	Low	Found normally in area where there are more than one subtype

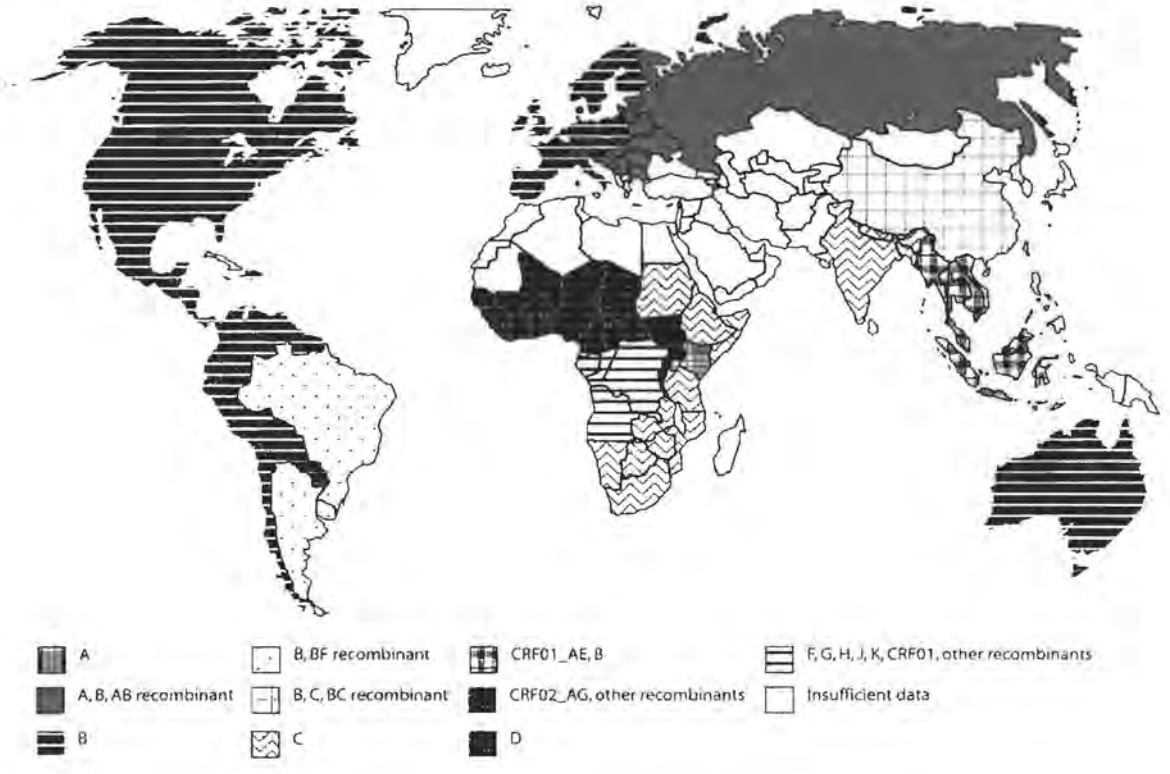

Figure 1.3: The epidemiology of HIV-1 subtypes and their recombinants (adapted from Duerr *et al.*, 2006).

Subtype C has become the most prevalent in the HIV-1 pandemic, as it accounts for the majority of all circulating viruses in the world. In East Africa, where other subtypes are circulating, recombinants of subtype C have been isolated. Sub-type C is also responsible for the most serious epidemic currently occurring in southern African countries. The genetic diversity of HIV-1 subtypes and emergence of new recombinants remains one of the key challenges in vaccine development. However, the high prevalence of HIV-1 subtype C in Southern Africa may allow for development of vaccines specifically directed against the subtype, for use specifically in this region.

1.4 IMMUNE TARGETS FOR HIV-1 VACCINE DEVELOPMENT FOR SOUTHERN AFRICA

The main mode of HIV-1 infection in Southern Africa is through sexual transmission. It is therefore critical that an HIV-1 vaccine for the region induce both mucosal and systemic innate and adaptive immune responses against the virus (Figure 1.4). Although the immune correlates of HIV-1 protection have not yet been defined, HIV-1 vaccines are expected to specific CD8+ T-, CD4+ T and B- cell immune responses, all of which are fundamental in containing HIV-1 infection or replication (Letvin, 2002, 2005; Paranjape, 2005; Nigam and Kerketta, 2006). Bacterial vaccine vectors such as *Salmonella* vaccines offer great potential as delivery systems for delivery of HIV-1 antigens to the immune system through mucosal surfaces (Chapter 2). They can be used to induce appropriate immune responses that may control HIV-1 infection.

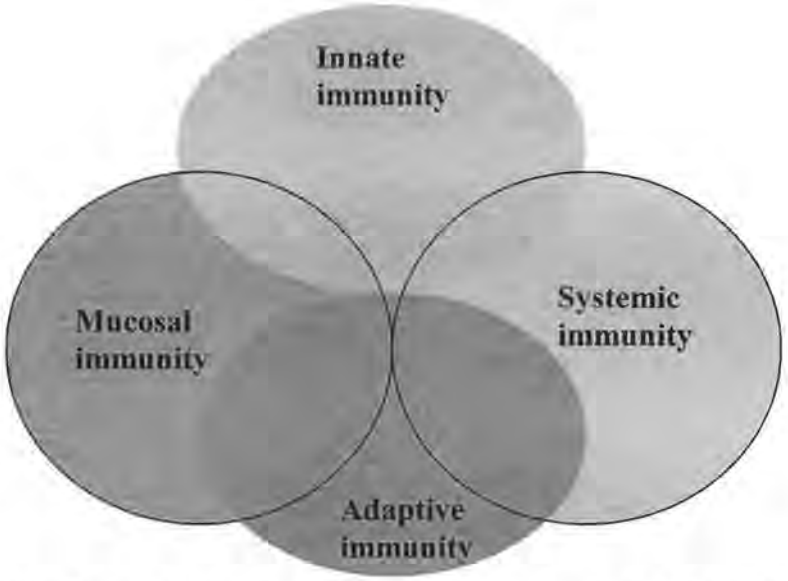

Figure 1.4: Immune targets for an ideal HIV-1 vaccine for Southern Africa. The vaccine needs to stimulate all arms of the immune system. Recombinant *Salmonella* vaccine vectors are likely to provoke such immune responses (Chapter 2).

1.4.1 Innate immune responses against HIV-1

In HIV-1 infection, cells of the innate system play an important role especially in the early stages soon after transmission (Lehner, 2003; Ahmed *et al.*, 2005). The cells secrete a number chemokines and cytokines which may potentially block viral transmission and replication at mucosal sites. For instance,

NK (natural killer) cells are important cells of the innate immune system and they kill virus-infected cells either directly or through antibody-dependent cell-mediated cytotoxicity (ADCC) and they can produce IFN-γ and β-chemokines, which have anti-HIV activities (Forthal *et al.*, 2001; Lodoen and Lanier, 2006). This results in suppression of HIV-1 replication during the early stages of infection before the HIV-1-specific adaptive immune responses are elicited. β-chemokine-specific responses inhibit viral entry only to R5 viruses (Cocchi *et al.*, 1995). Studies by Giavedoni *et al* (2000) have demonstrated antiviral activities of natural killer (NK) cells soon after SIV infection in rhesus macaques. Apart from NK cells, other cells of the innate system, such as macrophages and dendritic cells also secrete a number of chemokines and cytokines which can contribute in controlling of HIV-1 infection during the early stages (Guidotti and Chisari, 2001). Furthermore, non-cytolytic CD8+ T cells may also produce cell antiviral factor (CAF) which potentially has anti-HIV-1 activity and there may be some correlation with asymptomatic infection and slower disease progression (Ahmed *et al.*, 2005). In many ways, innate immune response controls the disease and regulates the nature and quality of the subsequent adaptive immune responses (Pulendran and Ahmed, 2006).

1.4.2 HIV-1-specific mucosal immunity

Most pathogens infect their hosts through the mucosal surfaces (Neutra *et al.*, 1996). These surfaces are found in the gastro-intestinal, urogenital, and the respiratory (nasal) tracts. The mucosal immune system, also called the mucosal associated lymphoid system (MALT), is found in these tracts (McGhee *et al.*, 1999). One of the key features of these inductive sites is the presence of M (microfold) cells. The M cells play important roles in uptake and transport of pathogens or antigens (Neutra *et al.*, 1996). HIV-1 utilizes these M cells in the mucosal surfaces for systemic transmission (Amerongen *et al.*, 1991). Besides mucosal transmission, HIV-1 replication also occurs in the mucosal lymphoid tissue before systemic spread (Mehandru *et al.*, 2004, 2005). The CD4+ T cells of mucosal lymphoid tissues are also the targets of HIV-1 throughout infection, leading to their depletion (Brenchley *et al.*, 2004; Mehandru *et al.*, 2004; Li *et al.*, 2005). Mucosal plasma cells synthesize secretory immunoglobulin A (IgA) which has the potential of neutralizing pathogens such as HIV-1 (Devito *et al.*, 2002; Dumais *et al.*, 2002). The MALT is rich in immune cells such as dendritic cells, macrophages, CD4+ and CD8+ T cells which can play important roles in provoking mucosal immunity to a variety of pathogens (Neutra and Kozlowski, 2006). Studies have also shown that there are SIV- and HIV-specific CD8+ T cell responses in the genital tracts of SIV-infected macaques (Lohnman *et al.*, 1995; Jordan *et al.*, 1999). Targeting HIV-1 vaccines for mucosal delivery with *Salmonella* vaccine vectors could potentially reduce such depletion by induction of strong mucosal immunity which limits the replication of the virus in the MALT. Inducing such CD8+ T cell responses with a recombinant *Salmonella* bacterial vector could also potentially control HIV-1 replication in the MALT.

The rationale of using *Salmonella* as a vaccine vector for HIV-1 is based on the fact that the two pathogens use the M cells as their gateway to systemic infection. Infection with *Salmonella* stimulates strong mucosal immune responses and the bacteria can therefore be used to deliver HIV-1 antigens to the

mucosal inductive sites (Chapter 2). One of the key advantages of mucosal vaccination against HIV-1 is that mucosal immunity also protects systemic infection, whereas systemic immunity poorly protects against mucosal infection (Lekkerkerker *et al.*, 2004; Neutra and Kozlowski, 2006). The other advantage is that antigenic exposure at one mucosal site activates B and T cells to emigrate and home to other mucosal surfaces, thereby conferring protection at these sites (Rosenthal and Gallichan, 1997; Iijima *et al.*, 2001; Ogra *et al.*, 2001; Kunisawa *et al.*, 2005). Protective vaccine-induced mucosal immunity against SHIV has been demonstrated in animal models (Crotty *et al.*, 2001). Therefore *Salmonella* vectors can be exploited to deliver HIV-1 antigens for induction of protective mucosal and systemic immune responses.

1.4.3 HIV-1-specific CD8+ T cell immune responses

CD8+ T cell responses are an important arm of adaptive immunity and can play a major role in controlling of viral infections. In the case of HIV-1, CD8+ cytotoxic T lymphocytes (CTLs) have the potential to destroy virally-infected cells by lysis. In HIV-1 infection, antigen-specific CD8+ T cells control infection by a number of mechanisms (Yang *et al.*, 1997). Firstly, the binding of HIV-1-specific CD8+ T cells to viral peptides presented by human leukocyte antigens (HLAs) on the surface of infected cells can trigger lysis of HIV-1 infected cells (Hadida *et al.*, 1999). The lysis of the virus-infected cells occurs through the production of perforin and granzymes, which penetrate into the cells and induce apoptosis (Berke *et al.*, 1993; McMichael and Rowland-Jones, 2001). Secondly, HIV-1-specific CD8+ T cells produce cytokines such as interferon gamma (IFN-γ and tumour necrosis factor alpha (TNF-α), which have antiviral activities. Thirdly, CD8+ T cells produce chemokines which block HIV entry by binding to HIV-1 coreceptors (Cocchi *et al.*, 2000). The cytolytic activities of CD8+ T cells can further be enhanced by these chemokines (Hadida *et al.*, 1998).

There is mounting evidence that CD8+ T cell responses play an important role in HIV-1 infection. In primary infection, HIV-1-specific CD8+ T-cells have been found to control viral replication (Walker *et al.*, 1987; Koup *et al.*, 1994; Yang *et al.*, 1997). After infection, the emergence of HIV-1-specific CD8+ T-cells is associated with a decrease in viraemia in plasma (McMichael and Rowland-Jones, 2001; Douek, 2003). Strong HIV-1-specific CD8+ T cell responses in chronic infection are also correlated with low viremia and slow disease progression (Musey *et al.*, 1997; Migueles *et al.*, 2002; Betts *et al.*, 2006). In animal models of HIV-1 infection, it has been shown that depletion of CD8+ T lymphocytes in SIV-infected macaques would lead in failure to control viral replication (Schmitz *et al.*, 1999; Madden *et al.*, 2004). *In vitro* studies have further demonstrated that HIV-1-specific CTLs could efficiently kill infected cells and inhibit viral replication (Yang *et al.*, 1996). It has also been noted that highly HIV-1-exposed seronegative individuals had detectable HIV-1-specific CTLs (Kaul *et al.*, 2001; Fowke *et al.*, 2000). The emergence of CD8+ T cell epitope mutants has been found to be associated with rapid disease progression (Trachtenberg *et al.*, 2003; Allen *et al.*, 2004). Furthermore, vaccine-induced CD8+ T cell responses have been shown to protect macaques from developing AIDS after challenge with simian-

human immunodeficiency virus (Amara *et al.*, 2001). All these studies clearly demonstrate that protection or control of HIV-1 infection require cellular (CD8+ T cell) immune responses.

Although HIV-1 infection induces vigorous CD8+ T cells in HIV-infected individuals, clearance of the virus is never achieved (Betts *et al.*, 2001a). The emergence of viral CTL escape mutations always occurs and renders virus-infected cells undetectable to host cytotoxic T-lymphocytes (Pitcher *et al.*, 1999; Trimble *et al.*, 2000; Goulder and Watkins, 2004; Peyerl *et al.*, 2004). These mutations change the epitopes or regions recognized by the CD8+ T cells such that they can no longer be recognized. It seems, therefore that vaccines inducing CD8+ T cells are not going to prevent infection, but will only control infection. In addition, vaccine-induced CD8+ T cell responses might decrease viral load in infected individuals, thereby making them less infective. This may prevent individuals progressing into disease rapidly as well as preventing them from spreading the virus, given their low viral load. In Southern Africa, a vaccine that can control HIV-1 infection could go a long way in reducing the disease burden by slowing down disease severity and progression. Recombinant *Salmonella* vaccine vectors can potentially induce antigen-specific CD8+ T cell responses and the bacteria can therefore potentially be used to deliver HIV-1 antigens for human vaccinations in Southern Africa.

1.4.4 HIV-1-specific CD4+ T cell immune responses

HIV-1 infects CD4+ T cells and this subsequently leads to their depletion and onset of disease (Ho *et al.*, 1995; Alimonti *et al.*, 2003). Although HIV-1 targets these cells, there is mounting evidence to support that CD4+ T helper (Th) responses also play a critical role in the control of HIV-1 infection and replication (Kalams *et al.*, 1999; Norris *et al.*, 2004; Jansen *et al.*, 2006). HIV-infected individuals who are long-term non-progressors (LTNPs) have been found to have strong CD4+ T cell responses to HIV-1 antigens (Kalams *et al.*, 1999). CD4+ T cell responses were also found to be associated with control of HIV viremia. It was noted that patients with the highest CD4+ T cell responses had the lowest viral loads, whereas patients with the lowest CD4+ T cell responses had the highest viral loads (Rosenberg *et al.*, 1997). In some patients, strong CD4+ T helper responses were found to be associated with strong CD8+ CTL responses (Kalams *et al.*, 1999). Recent studies have further suggested that HIV-specific CD4+ Th1 cells producing INF-γ and IL-2, together with IgG2 were important in long-term control of HIV-1 infection and reduced viremia (Emu *et al.*, 2005; Martinez *et al.*, 2005). Thus, CD4+ T cells provide immunological help to CD8+ T cell responses. CD4+ T cells also produce cytokines such as IFN-γ and TNF-α which have antiviral activities. CD4+ T cells further provide help for antibody responses that may be critical for neutralization of the virus. There have been recent reports of the cytotoxic CD4+ T-cells detected in HIV-1 infection (Zaunders *et al.*, 2004).

Infection with *Salmonella* induces predominantly antigen-specific CD4+ T cells (Chapter 2). The bacteria can therefore potentially be exploited in delivery of HIV-1 antigens to the immune system for induction of virus-specific CD4+ T cell responses (Chapter 2). Such an immune response is expected to enhance HIV-1 specific CD8+ T cell responses together with virus-specific antibodies.

1.4.5 HIV-1-specific humoral immune responses

Humoral immune response plays a protective role against many pathogens (Robbins *et al.*, 1995). It is mediated by antibodies which are produced by B-lymphocytes. The antibodies offer protection by binding (neutralizing) pathogen antigens, thereby preventing infection. Infection with HIV-1 also induces virus-specific antibody responses (Letvin and Walker, 2003). HIV-1-infected individuals elicit high levels of antibodies against different viral proteins during primary infection (Nabel and Sullivan, 2000). The antibodies that have the capacity to neutralize HIV-1 (neutralizing antibodies) are directed against Env (Parren *et al.*, 1999) and they can potentially block HIV-1 replication or infection by virus neutralization or antibody-dependent cellular cytotoxicity of infected cells (Pantaleo and Koup, 2004; Reymolds *et al.*, 2006).

The role of antibodies in preventing or controlling HIV-1 infection or/and disease is still uncertain. However, it is known that the glycoprotein subunits, gp120 and gp41 are the key targets for neutralizing antibodies (Pantophlet and Burton, 2006). Neutralizing antibodies are normally correlated with significant decline of the primary viremia (Koup *et al.* 1994). Furthermore, some infected individuals with strong neutralizing antibody responses control their viremia for a long time (Harrer *et al.*, 1996). Experimental studies in animal (SHIV) models have shown that passive immunization with monoclonal antibodies could protect monkeys from challenge infections (Mascola *et al.*, 2000; Baba *et al.*, 2000). A number of monoclonal antibodies generated from HIV-infected individuals have been described (Table 1.4). These monoclonal antibodies are capable of neutralizing primary and laboratory isolates of HIV-1. Although vaccine candidates targeting induction of neutralizing antibodies have been developed, it seems

Table 1.4: Examples of human monoclonal antibodies for the envelope protein that have been found to neutralize HIV-1 (adapted from McMichael, 2006; Pantophlet and Burton, 2006)

Name of antibody	Specificity	Key characteristics	References
IgG1b12	CD4-binding domain of gp120	-Long CDR3 (complementarity-determining region 3) region; highly immunogenic and broadly neutralizing.	Sapphire *et al.*, 2001.
X5	Coreceptor binding site	Fab only effective	Labrijn *et al.*, 2003.
2G12	Complex mannose residues of gp120	-Antibody has VH domain swap; -poorly immunogenic, but broadly neutralizing	Calarese *et al.*, 2003.
2F5	Conserved membrane proximal domain of gp41	-IgG3 with long CDR3; -cross-reacts with cardiolipin; -poorly immunogenic, but broadly neutralizing	Haynes *et al.*, 2005; Zwick *et al.*, 2004.
4E10	Conserved membrane	-IgG3 with long CDR3; -proximal domain of gp41 cross-reacts with cardiolipin; - poorly immunogenic, but broadly neutralizing	Cardoso *et al.*, 2005; Haynes *et al.*, 2005.

none can elicit broad and potent neutralizing antibody responses. It is therefore the current goal to produce vaccines which can induce HIV-1-specific neutralizing mucosal IgA and serum IgG for

prevention of HIV infection. This target should be possible, given the fact that secretory IgA (SigA) antibodies to HIV have been detected in vaginal secretions of sero-negative women who have been highly exposed to HIV infection (Mazzoli *et al.*, 1999; Devito *et al.*, 2000a; Devito *et al.*, 2000b). IgA purified from the vaginal secretions were found to neutralize HIV-1 and to block HIV-1 transcytosis across the mucosa (Mazzoli *et al.*, 1999; Devito *et al.*, 2002). IgA was also detected in the seminal fluid sero-negative men who have been exposed to HIV infection (Lo Caputo *et al.*, 2003). These studies further support the role of mucosal neutralizing antibodies in protection against HIV-1 infection.

The use of oral recombinant *Salmonella* bacterial vectors for HIV-1 is expected not only to induce mucosal HIV-1-specific IgA, but also serum IgG antibody responses (Chapter 2). However, HIV-1 Env and gp120 antigens expressed in *Salmonella* vectors are not expected to induce neutralizing antibodies because they lack relevant post-translational modifications. On the contrary, recombinant *Salmonella* vectors carrying HIV-1 Env DNA vaccines bacteria have the potential of inducing strong HIV-1-specific broadly neutralizing antibodies in the mucosal and systemic immune compartments (Devico *et al.*, 2002; Fouts *et al.*, 2003).

1.4.6 Immune correlates of HIV-1 protection

To date, the exact immune correlates of protection against natural HIV-1 infection are not known. However, it is generally known that HIV-1 protective immune responses should comprise of CD8+ T, CD4+ T cell and humoral responses (Sections 1.4.3-1.4.5). Various components of the immune system may control HIV-1 infection in different ways with varying efficiencies (Figure 1.5). Whereas neutralizing antibodies can block HIV-1 infection, they are not effective against cells which are already infected (Pantaleo and Koup, 2004). Cellular immune responses can control HIV-1 infection, but they cannot prevent infection. The two components of the immune response cannot target latently HIV-infected cells. The key challenge is to develop vaccines that can induce multiple forms of immune

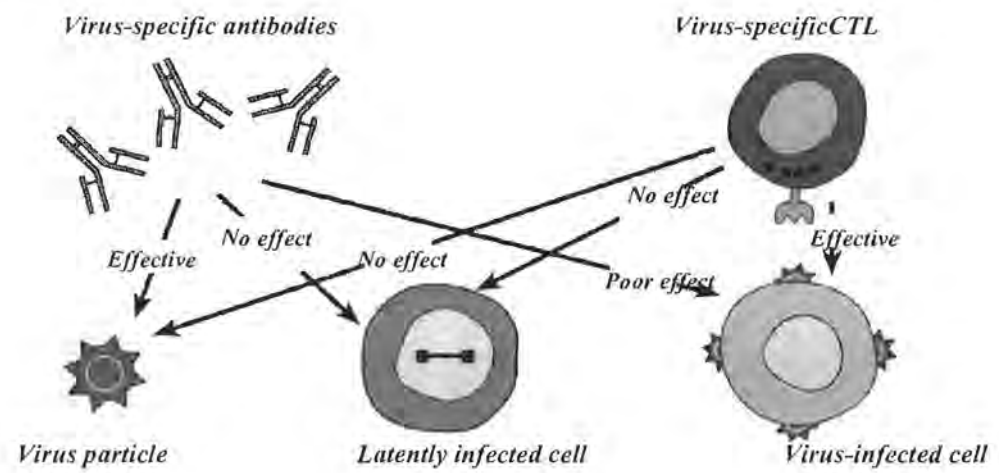

Figure 1.5: The effectiveness of different components of the anti-HIV immune response against various forms of virus (Pantaleo and Koup, 2004).

responses against the virus. Mucosal immune responses may potentially be required to control the early stages of HIV-1 infection and replication. Mucosal immunity may also delay systemic infection by the virus. To date, we do not know the exact nature, quality and magnitude of immune responses that should be elicited by the HIV-1 or vaccine for prevention of AIDS. Nevertheless, recent animal studies have suggested that vaccine-induced SIV-specific central memory CD8+ T and CD4+ T cells may correlate with protection against simian AIDS disease in monkeys (Sun *et al.*, 2005; Vaccari *et al.*, 2005; Acierno *et al.*, 2006; Letvin *et al.*, 2006; Sun *et al.*, 2006). These studies suggest that effective vaccines against HIV-1 need to generate long-term protective central memory CD8+ and CD4+ T cell responses.

1.5 SCIENTIFIC OBSTACLES TO HIV-1 VACCINE DEVELOPMENT

Current efforts in development of HIV vaccines are marred by a number of scientific challenges (recently reviewed in Egan, 2004; Garber *et al.*, 2004; Sahni and Nagendra, 2004; Alcami *et al.*, 2005; Burgers and Williamson, 2005; Excler, 2005; Duerr *et al.*, 2006). In brief, these obstacles to vaccine developments include the genetic diversity and hypervariability of the virus (Section 1.3), mucosal transmission and replication of the virus in the gut, transmission of the virus by infected cells, resistance of wild-type viruses to antibody neutralization, genomic integration of virus into host genome (latent infection), clonal T cell exhaustion after infection, intrinsic immunosuppressive properties of several HIV-1 proteins, rapid emergence of viral escape mutants, down-regulation of MHC class I antigens, lack of ideal animal models for HIV-1, our poor understanding of the immunological correlates of HIV-1 protection. All these challenges make the development of prophylactic (or therapeutic) vaccines a difficult task.

1.6 HIV-1 VACCINE DEVELOPMENT STRATEGIES

To date, there is no HIV-1 vaccine for Southern Africa or elsewhere. However numerous different efforts to develop candidate HIV-1 vaccines are underway. A variety of approaches have been employed in the development of these HIV-1 vaccine candidates (Nabel, 2002; Bojak *et al.*, 2002; Hanke, 2001; Alcami *et al.*, 2005; Excler, 2005; Letvin, 2005; Girard *et al.*, 2006; Duerr *et al.*, 2006 and summarized in Table 1.5).

Table 1.5: Key approaches to HIV-1 vaccine development

Vaccine strategy	Description of the strategy	Are candidates using HIV-1 Subtype C from Southern Africa being developed?
Live attenuated vaccines	Genetically attenuated HIV-1 vaccines	No
Inactivated vaccines	Inactivated whole HIV-1 virions	No
Subunit vaccines	Recombinant HIV-1 antigens such as gp120	No
Virus-like particles	Recombinant HIV-1 Gag virus-like particles	Yes
DNA vaccines	Plasmid DNA expressing HIV-1 antigens	Yes
Viral vaccine vectors	Recombinant viruses expressing HIV-1 antigens	Yes
Bacterial vaccine vectors	Recombinant bacteria expressing HIV-1 antigens	Yes
Combined vaccines	Combination of any of the above strategies	Yes

1.6.1 Live attenuated HIV-1 vaccines

It is possible to genetically attenuate HIV-1 by the tools of genetic engineering whereby mutations are introduced in specific viral genes. This generates genetically attenuated HIV-1 vaccines, which are expected to confer immunogenicity without causing AIDS. The prospects and challenges in development of these live attenuated HIV-1 vaccines have recently been reviewed (Voltan and Robert-Guroff; 2003; Whitney and Ruprecht, 2004). Although some of the live attenuated vaccines under development have shown a degree of protective efficacy in animal models (Koff *et al.*, 2006), this approach has not attracted much attention because of safety concerns in humans. People vaccinated with a *nef*-deleted HIV-1 vaccine had a decline in their CD+ T-cell counts 12 years after vaccination and most of them developed AIDS (Learmont *et al.*, 1999; Greenough *et al.*, 1999). Other potential pitfalls of live attenuated HIV-1 vaccines include potential integration of viral genome into the host chromosome, viral persistence and transmissibility, generation of immune escapes, recombination with other viruses and host immune exhaustion (Whitney and Ruprecht, 2004). To date, no live attenuated HIV-1 vaccine candidates specifically targeted for Southern Africa or anywhere else have been developed or tested in clinical trials.

1.6.2 Inactivated HIV-1 vaccines

It has been a classical approach to use inactivated viruses as vaccines. This strategy has also been explored for HIV-1 vaccine development (reviewed in Sheppard, 2005). Although the HIV-1 vaccine candidates may be safe, the approach is not advocated for due to poor immunogenicity elicited by these vaccines. The vaccines normally fail to induce broad and protective immune responses in animal models (Lifson *et al.*, 2004). Such HIV-1 vaccines would not be very useful for Southern Africa, given their poor immunogenicity.

1.6.3 Subunit HIV vaccines

Subunit HIV-1 vaccine candidates against HIV-1 have been developed. These vaccines are based on purified HIV-1 antigens such as envelope glycoprotein. The vaccines are typically designed to induce predominantly neutralizing antibodies. Early studies in which gp120 was used as a subunit vaccine, showed some protection in vaccinated animals after viral challenge (Berman *et al.*, 1990). One of the first HIV-1 vaccines that entered Phase I trial in the early 1990s was also based on envelope subunit (Kovacs *et al.*, 1993; Keefer *et al.*, 1994). Since then, a number of additional Phase I trials have been performed in which gp160 or gp120 subunit vaccines were used. Some of the vaccines have already been evaluated in Phase II clinical trials. The results of the first phase III study of the recombinant gp120 (rgp120), by VaxGen (Brisbane, CA, USA) showed that there was no statistically significant reduction of HIV-1infection within the study population (Watanabe, 2003). Other phase III trials are still underway and the results are not yet available (IAVI, 2006). There are still key challenges in developing HIV-1 Env subunit vaccines that elicit protective immunity. Although subunit vaccines have tested in other parts of the world, none has been developed specifically for testing and use in Southern Africa.

1.6.4 HIV-1 Virus-like particles as vaccines

The use of virus-like particles (VLPs) as immunogens is well established (Noad and Roy, 2003). Structural proteins of most viruses are able to self-assemble into VLPs, which can then be used as subunit vaccines. They are highly immunogenic when used for vaccinations. To date, a number of candidate vaccines based on HIV-1 VLPs have been developed (reviewed in Doan *et al.*, 2005). Most of these vaccines have been found to induce both strong humoral and cellular immune responses when administered animals and humans. The University of Cape Town HIV-1 Vaccine research group, supported by the South African AIDS Vaccine Initiative (SAAVI), has developed Gag VLPs, based on the South African HIV-1 Subtype C Du_{422} isolate. The VLPs have been shown to be highly immunogenic in preclinical studies (Jaffray *et al.*, 2004). The VLPs were also found to dramatically boost HIV-1-specific cellular immune responses when used in conjunction with matched DNA vaccine in a prime-boost strategy (Jaffray *et al.*, 2004).

1.6.5 DNA vaccines for HIV-1

The use of recombinant DNA plasmids that carry HIV-1 genes, which are expressed after vaccination, is one of the latest approaches in vaccine development (Giri *et al.*, 2004). The plasmids, when used as DNA vaccines induce immune responses specific to the antigen genes carried (reviewed in Donnelly *et al.*, 2005). The first HIV-1 DNA vaccine to go into human trials was based on Rev and Env and was shown to be safe when administered intra-muscularly (MacGregor *et al.*, 1998; Ugen *et al.*, 1998). In the study, non-neutralizing antibody responses and specific proliferation were induced, but no CTL activity was demonstrated. Boyer *et al.*, (1997) had previously demonstrated protection in chimpanzees against a heterologous HIV after vaccination with a DNA vaccine. Studies by Barnett *et al.*, (1997) had also demonstrated that both CD8+ CTL and humoral immune responses could be induced in animals primed with gp120 DNA vaccine and boosted with gp120 subunit vaccine. Since then, a number of candidate DNA vaccines for HIV-1 have been developed and tested (Estcourt *et al.*, 2004; Giri *et al.*, 2004; Girard *et al.*, 2006). Two DNA vaccines, pThgagC and SAAVI DNA-C have been developed for Southern Africa, and were shown to be highly immunogenic in preclinical trials in mice and baboons (van Harmelen *et al.*, 2003; Burgers *et al.*, 2006). One of the vaccines, SAAVI DNA-C is earmarked for Phase I clinical trials in South Africa and USA in 2008. There is also a great potential of using recombinant *Salmonella* in delivery of some of these DNA vaccines developed for Southern Africa.

1.6.6 Viral vaccine vectors for HIV-1

Viruses can be exploited as vaccine vectors for heterologous antigens (Monahan and Salgaller, 1999; Polo *et al.*, 2002; Dudek and Knipe, 2006). The key advantage of viral vectors is that they can generate strong antigen-specific protective CD8+ T cell responses (Truckenmiller and Norbury, 2004). They can also induce strong humoral immune responses, especially neutralizing antibodies to the coat proteins. Thus just like HIV-1, most of the viral vectors can target both the innate and adaptive immune responses at both the mucosal and systemic compartments. A number of viral vaccine vectors for HIV-1 antigens have so far been developed (Table 1.6). These viral vaccine vectors have been genetically engineered to

express different HIV-1 antigens. In most of the studies, both strong humoral and cell-mediated immune responses were induced against the recombinant HIV-1 antigens. For example, modified vaccinia Ankara (MVA) vaccine vector has been shown to induce protective virus-specific mucosal and systemic humoral and cellular immunity in a SHIV DNA prime-MVA boost vaccination protocol (Wang *et al.*, 2004). Effective anti-HIV immunity, sometimes protective, has been observed in a number of preclinical studies

Table 1.6: Examples of viruses used as live vaccine vectors for SIV or HIV-1 vaccines

Viruses	References
Pox viruses such as vaccinia, canarypox, fowlpox, MVA	Rolph and Ramshaw, 1997; Essajee and Kaufman, 2004; Franchini *et al.*, 2004; Im and Hanke, 2004.
Adenoviruses	Gomez-Roman and Robert-Guroff , 2003; Barouch and Nabel, 2005; Vanniasinkam and Ertl, 2005.
Alphaviruses such as Venezuelan equine encephalitis, Sindbis, and Semliki Forest virus	Davis *et al.*, 2002; Williamson, 2002; Sundback *et al.*, 2005; Perri *et al.*, 2003.
Rhabdoviruses such as vesicular stomatitis and rabies viruses	McKenna *et al.*, 2003; Egan *et al.*, 2004; McGettigan *et al.*, 2006.
Paramyxoviruses such as Sendai virus, measles virus	Takeda *et al.*, 2003; Lorin *et al.*, 2004; Kato *et al.*, 2005
Picorvirus such as poliovirus, rhinovirus	Moldoveanu *et al.*, 1995; Crotty and Andino, 2004.
Herpes simplex virus	Murphy *et al.*, 2000.

in which vectors such as adenovirus, alphavirus, sendai virus, herpes simplex virus, human rhinovirus and polio virus were used to express HIV-1 antigens (Berglund *et al.*, 1997; Shiver *et al.*, 2002; Murphy *et al.*, 2000; Matano *et al.*, 2001; Arnold *et al.*, 2005; Crotty *et al.*, 2001). The utility of these viral vectors in humans is still uncertain. Most of the recombinant vectors are still in phases I, II and III human clinical trials (IAVI, 2006). In Southern Africa, VEE replicon vector expressing HIV-1 subtype C Gag is already in Phase I clinical trials in South Africa and Botswana (Excler, 2005; Nkolola and Essex, 2006).

1.6.7 Bacterial vaccine vectors for HIV-1

Attenuated bacteria can be used to deliver heterologous bacterial, viral or parasitic antigens to the host's immune system (Shata *et al.*, 2000; Mollenkopf *et al.*, 2001; Garmory *et al.*, 2003; Roland *et al.*, 2005). Their potential use as candidate HIV-1 vaccine vectors to deliver either HIV-1 antigens or HIV-1 DNA vaccines is being increasingly studied (Table 1.7). Most of these bacterial vectors are capable of inducing strong humoral and cellular immune responses to antigens expressed or DNA vaccines carried. They can also activate both the innate and adaptive immune responses. BCG expressing SIV or HIV antigens can induce antigen-specific immune responses in vaccinated animals (Chujoh *et al.*, 2001; Kawahara *et al.*, 2002; Kawahara *et al.*, 2006). BCG is generally an attractive vector for HIV-1, because its safety record is known and can be delivered orally. The *Mycobacterium smegmatis* is a nonpathogenic, rapidly growing mycobacterium that is related to BCG and has also recently been used to deliver HIV-1 antigens for induction of HIV-1-specific immune responses (Cayabyab *et al.*, 2006; Yu *et al.*, 2006). Another attractive bacterial vaccine vector for HIV-1 is *Listeria monocytogenes* (Lieberman and Frankel, 2002; Peterson and Johnson, 2004). Its key advantage as a vaccine vector is that it replicates in the cytosol,

Table 1.7: Examples of bacteria used as live vaccine vectors for HIV-1 vaccines

Bacterium	References
Salmonella	Shata *et al.*, 2001; Devico *et al.*, 2002; Hone *et al.*, 2002; Fouts *et al.*, 2003; (see also Chapter 2).
Mycobacterium bovis bacille-Calmette-Guerin (BCG) or *M. smegmatis*	Ferrari *et al.*, 2000; Chujoh *et al.*, 2001; Kawahara *et al.*, 2002; Kanekiyo *et al.*, 2005; Cayabyab *et al.*, 2006; Yu *et al.*, 2006.
Shigella species	Vecino *et al.*, 2002; Shata and Hone, 2002; Xu *et al.*, 2003.
Listeria monocytogenes	Lieberman and Frankel, 2002; Paterson and Johnson, 2004; Mata *et al.*, 2001.

thereby inducing both strong CD8+ and CD4+ T cell responses. *Shigella*, like *L. monocytogenes* is capable of escaping from the endosome into the cytosol, resulting in strong cellular immune responses.Attenuated *Shigella* strains have already been successfully used to deliver HIV-1 DNA vaccines, resulting in induction of HIV-1-specific CD8+ T responses (Fennelly *et al.*, 1999; Shata and Hone, 2001; Xu *et al.*, 2003). The potential use of *Salmonella* as vaccine vectors for heterologous antigens, including HIV-1 antigens is the subject of this dissertation (see Chapter 2 for review of the literature).

1.7 CONCLUSIONS AND PROJECT MOTIVATION

The gravity of the HIV/AIDS epidemic in Southern Africa calls for an urgent development of safe, effective, affordable and easy-to-deliver vaccines to mitigate the ever-expanding problem. Although the immunological correlates of protection of HIV-1 are still poorly understood, tremendous efforts have already been made towards the development of these vaccines. Most of the vaccines currently under development for use in Southern Africa are based on the HIV-1 subtype C *gag* gene (Williamson *et al.*, 2003). There are a number of reasons why the *gag* has been selected for vaccine development. HIV-1 Gag is one of the most relatively conserved antigens and can therefore be targeted for the development of a vaccine for diverse HIV-1 subtypes for the region (Novitsky *et al.*, 2001; Williamson *et al.*, 2003, Thomas, 2005). In natural infection, HIV-1 Gag-specific CD8+ T cells play an important role in controlling primary HIV-1 viremia and slowing disease progression (Rinaldo *et al.*, 1995; Riviere *et al.*, 1995; Buseyne *et al.*, 2002; Wagner *et al.*, 1999; Gupta *et al.*, 2006; Turnbull *et al.*, 2006). Furthermore, Gag-specific CD8+ T cell responses have broad cross-reactivity for diverse HIV-1 strains (Cao *et al.*, 1997; Betts *et al.*, 2005). In addition, Gag contains a number of immunodominant epitopes which are conserved among HIV-1 subtypes (Ferrari *et al.*, 2000; Novitsky *et al.*, 2001; Currier *et al.*, 2006; Newberg *et al.*, 2006). Thus, targeting conserved HIV-1 proteins such as Gag for development of vaccines for Southern Africa is logical. The overall goal of the current proposal would be to develop a recombinant oral bacterial vaccine vector for HIV-1 Gag derived from Southern Africa. No such work has previously been done.

CHAPTER 2: INTRODUCTION TO RECOMBINANT *SALMONELLA* VACCINE VECTORS

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CHAPTER 2: INTRODUCTION TO RECOMBINANT *SALMONELLA* VACCINE VECTORS

2.1 *SALMONELLA* BACTERIA

The Salmonellae belong to the family *Enterobacteriaceae* and are enteric, gram-negative, facultatively anaerobic, flagellated and non-lactose fermenting bacteria (Brenner, 1984; Farmer, 2003). They cause salmonellosis, which has symptoms that range from mild gastroenteritis to fatal systemic fevers in mammals, birds, reptiles, birds and insects (Hess and Kaufmann, 1996; Ohl and Miller, 2001). Typhoid fever is the life-threatening form of salmonellosis in humans and is caused by *Salmonella enterica* serovar Typhi. The source of infection by *Salmonella enterica* serovar Typhi is usually contaminated food or water. Approximately 16 million new cases of the disease and about 600 000 deaths are reported annually worldwide (Ivanoff, 1995; Parry, 2004). The disease is more prevalent in developing than in developed countries due to inadequate sanitation and healthcare. The incidence of the disease is estimated to be high in south-central Asia, south-east Asia and southern Africa (>100 cases per 100 000 people per year), while in the rest of Asia, Africa and Latin America, the incidence ranges from 10 – 100 cases per 100 000 people per year (Bhan *et al.*, 2005).

The nomenclature of *Salmonella* has evolved over time. The original classification of the bacteria was based on various aspects such as clinical symptoms, epidemiology, host and biochemical aspects (Farmer, 1985; 2003). This classification was however confusing and flawed. A new system was later proposed (Brenner *et al.*, 2000; Heyndrickx *et al.*, 2005; Tindall *et al.*, 2005). Under the new system, the genus *Salmonella* is divided into two species, namely *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further classified into 6 subspecies, namely *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica* and *hautenae*, also known as subspecies I, II, IIIa, IIIb, IV and VI respectively. The subspecies classification is based on factors such as chromosomal DNA hybridization and multilocus enzyme electrophoresis (Miller and Pegues, 2000; Farmer, 2003). *S. Typhi* and *S. Typhimurium* are now classified as *Salmonella enterica* subspecies *enterica* serovar Typhi and *Salmonella enterica* subspecies *enterica* serovar Typhimurium or simply referred to as *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Typhimurium respectively. Most members of the *Salmonella enterica* species are associated with warm-blooded vertebrates (Uzzau *et al.*, 2000). More than 99% of the *Salmonella* serovars identified to date belong to *Salmonella enterica* (Yan *et al.*, 2003). Serovars in *Salmonella bongori* comprises very few serovars and they are usually isolated from cold-blooded animals (Brenner *et al.*, 2000). Most serovars of *Salmonella* are host adapted, while others are host-restricted (Kingsley and Baumler, 2000; Uzzau *et al.*, 2000). Examples of such serovars are An example of each are *S. enterica* serovar Typhi and *S. enterica* serovar Typhimurium which cause typhoid in humans and mice respectively. Whereas *S. enterica* serovar Typhimurium causes mild gastroeroentritis in humans, it causes fatal typhoid in mice. *S. enterica* serovar Typhi is host-restricted to humans where it causes typhoid but does not infect mice or other animals. *S. enterica* serovar Typhimurium has therefore been used as a mouse model for the human typhoid disease. Some host-restricted salmonellae include *S. enterica* serovar Gallinarum (poutry), *S. enterica* serovar Abortus-ovis (sheep), *S. enterica* serovar

Abortus-equi (horses) and *S. enterica* serovar Paratyphi (human) (Uzzau *et al.*, 2000). These host-restricted serovars cause typhoid fever-like symptoms in these respective animals. *S. enterica* serovar Dublin (cattle), *S. enterica* serovar Choleraesuis (swine) are also examples of host-adapted serovars which can occasionally infect humans and ovine (Uzzau *et al.*, 2000). The host adaptation and differences in pathogenesis of various *Salmonella* serovars is probably due to the differences in evolution and presence of specific virulence factors (Kingsley and Baumler, 2002; Amavisit *et al.*, 2003). Attenuated strains of host-restricted or host-adapted *Salmonella* serovars can be targeted for use as vaccine vectors for specific organisms.

2.2 MOLECULAR PATHOGENESIS OF *SALMONELLA*

2.2.1 *Salmonella* pathogenicity islands

One of the key characteristics of the genus *Salmonella* is the clustering of the majority of virulence genes within regions around the chromosome. These gene clusters are called *Salmonella* pathogenicity Islands (SPIs) and are critical for the molecular pathogenesis of salmonellosis (Figure 2.1). A number of SPIs have been characterized and have been found in a range of *Salmonella enterica* serovars (reviewed in Hansen-Wester and Hensel, 2001; Hensel, 2004; Schmidt and Hensel, 2004; van Asten and van Dijk, 2005). In brief, SPI1 plays a role in the invasion, inflammation and macrophage apoptosis. Some of the

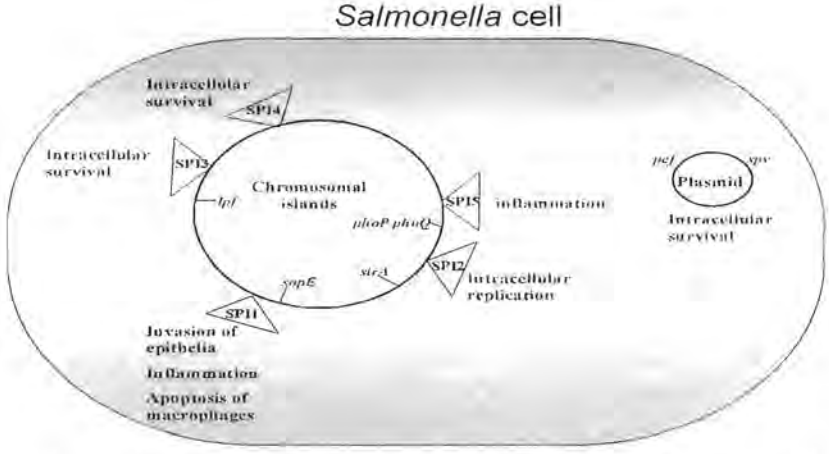

Fig. 2.1: The key *Salmonella* pathogenicity islands (SPIs) that are known to play critical roles in pathogenesis (adapted from Sirard *et al.*, 1999). The physical distribution of some SPIs (triangles) and major virulence factors are shown. The proposed function(s) of each SPI are indicated below it. The relative location of each island on the bacterial chromosome is also indicated.

genes found in SPI1 encode the type III secretion systems (TTST) which are mainly composed of effector proteins that are translocated out of the bacteria into the extracellular environment. The SPI2, which also encodes some TTST effector proteins, is essential for systemic infection and the proliferation within host organs by the bacteria. The expression of effector molecules on SPI2 is induced when the bacteria are internalized in the phagocytes. Bacteria with mutations in SPI2 are unable to replicate efficiently inside the phagocytes (Michael *et al.*, 2004) and can therefore potentially be used as attenuated vaccines. SPI3, SPI4, and SPI5 are also important pathogenicity islands that play various roles

in intracellular survival of the bacteria in the intracellular environment. SPI5 encodes factors that are key in fluid secretion and inflammatory reaction in the intestinal mucosa (Sirard *et al.*, 1999). Other SPIs (SPI6-10) also have been identified, but their roles in molecular pathogenesis of *Salmonella* have not been defined. The modern tools of recombinant DNA technology are now available to delete or modify genes within these pathogenicity islands in order to generate attenuated *Salmonella* vaccines.

2.2.2 *Salmonella* infection cycle

Infection by *Salmonella* occurs mainly through the oral/gastric route after taking contaminated food or water. The bacteria are capable of resisting the acidic conditions of the stomach and reach small and large intestines. The invasion of the mucosa-associated lymphoid tissue by *Salmonella* occurs via the M cells (Figure 2.2), which are specialized epithelial cells found in the Peyers patches (Bradley *et al.*, 1994;

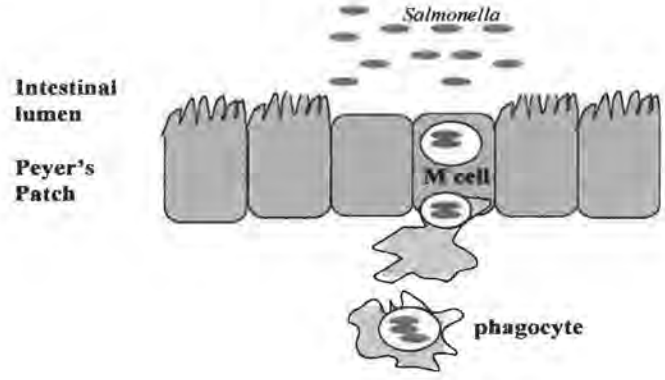

Figure 2.2: The invasion of the M cell by *Salmonella* (modified from Guiney and Lesnick, 2005). The invasion of the M cells is facilitated by effector proteins encoded by SPI1 and SPI2.

Clack *et al.*, 1994; Jepson and Clark, 1998). The bacteria initially replicate directly in Peyers patches and subsequently disseminate via the mesenteric lymph nodes to systemic sites such as the spleen and liver (Bradley *et al.*, 1994). Virulent *Salmonella* such as serovar Typhi undergo two episodes of infection, the transient primary bacteraemia and secondary bacteraemia (Everest *et al.*, 2001) (Figure 2.3). Clinical disease in secondary bacteraemia has symptoms such as inflammation, ulcerations and necrosis. Serovars of *Salmonella* which are less virulent or which are genetically attenuated are unable to cause these clinical symptoms because of reduced capacity to invade or replicate.

Salmonella reside inside the phagocytes in specialized structures called *salmonella*-containing vacuoles (SCV) and are incapable of undergoing phagosome-lysosome fusion and causing subsequent degradation of the bacteria (Buchmeier and Heffron, 1991, Oh *et al.*, 1996). The intracellular *Salmonella* may cause apoptosis of infected cells and this may result in further spread of the bacteria via the mesenteric lymph nodes to the blood (Jarvelainen *et al.*, 2003). The mechanism of *Salmonella*-induced cell death (apoptosis) and its significance to *Salmonella* pathogenesis have been reviewed (Knodler and Finlay,

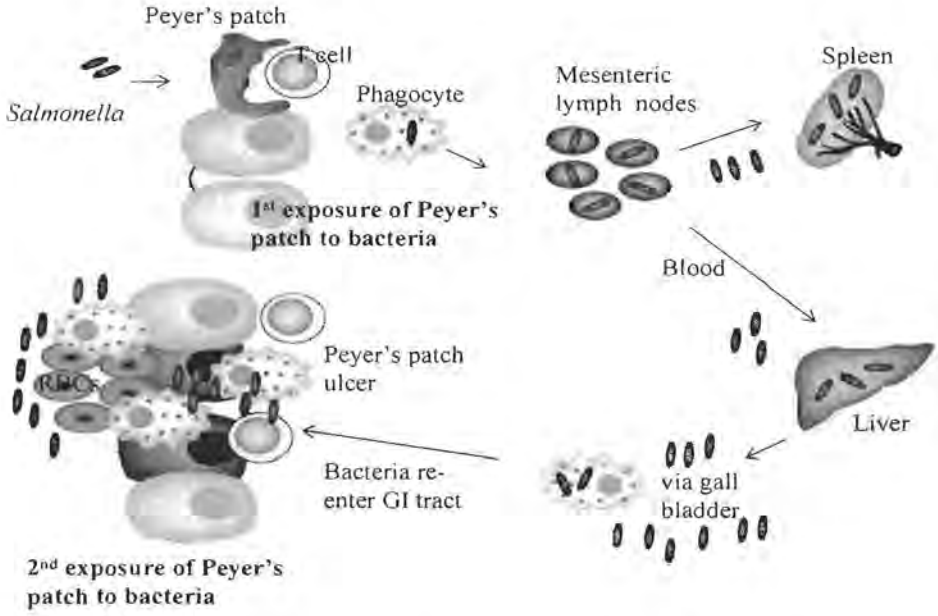

Figure 2.3: The natural cycle of *Salmonella enterica* serovar Typhi infection in the human host (adapted and modified from Everest *et al.*, 2001). The *Salmonella* bacteria infect via the Peyer's patches, migrate to mesenteric lymph nodes and reach the systemic organs. The replication in these sites results in a re-infection episodes in which the bacteria re-enter the GALT through the gall bladder. It is the re-infection of the Peyer's patches which results symptoms such as tissue damage, ulcerations, bleeding and necrosis.

2001; Hueffer and Galan, 2004). Apoptosis of infected cells may be an evolutionary mechanism for the bacteria to spread and infect more cells (Haimovich and Venkatesan, 2006). The ability of *Salmonella* to colonize and invade the mucosa-associated lymphoid tissue (MALT) and spread to distal sites such as liver and spleen makes them potential candidates for delivery of mucosal vaccines.

2.3 IMMUNE RESPONSES TO *SALMONELLA* INFECTION

Infection with bacteria normally activates both the innate and the adaptive immune responses. Host responses to *Salmonella* infection have been found to involve both the innate and adaptive arms of the immune system (Jones and Falkow, 1996; Mastroeni, 2002; Eckmann, 2004; Wick, 2004; Bueno *et al.*, 2005; Hancock *et al.*, 2005; Kalupahana *et al.*, 2005; Salazar-Gonzalez and McSorley, 2005).

2.3.1 Innate immune response

Soon after *Salmonella* infection, the innate immune system is triggered by the host's recognition of pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides (LPS), flagellin, polycytosine guanine (CpG) motifs (bacterial DNA) and peptidoglycan (Wick, 2004; Salazar-Gonzalez and McSorley, 2005). The PAMPs are recognized by host pattern-recognition receptors such as toll-like receptors (TLRs), thereby facilitating immunostimulation (Eckmann *et al.*, 2004). For example, TLR4 recognizes bacterial LPS and is expressed in the intestinal epithelial cells (Backhed *et al.*, 2003). TLR5 is associated with the recognition of bacterial flagella (mainly made up of flagellin). It is also expressed by intestinal epithelial cells, thereby making the cells responsive to flagellin stimulation by producing

proinflammatory cytokines, which can control the *Salmonella* infection (Gewirtz *et al.*, 2003). In the early phases of infection, the NADPH oxidase-dependent anti-microbial functions of phagocytes and the innate resistance gene *Nramp1* (natural resistance-associated macrophage protein 1) also play the cumulative control of bacterial growth (Mastroeni *et al.*, 2000b; Mastroeni, 2002; Zaharik *et al.*, 2002). Nramp1 is believed to play a role in the generation of oxygen- and nitrogen-dependent antimicrobial activities (Wigley *et al.*, 2004). Nramp1$^{-/-}$ mice succumb to *Salmonella* infection while Nramp1$^{+/+}$ mice can control primary *Salmonella* infection (Caron *et al*, 2002; Monack *et al.*, 2004). Defensins (peptides) produced by phagocytes have been implicated in killing of *Salmonella* during infection (Groisman *et al.*, 1992; Salzman *et al.*, 2003). Cytokines such as TNF-α and IFN-γ produced by the cells of the innate immune system such as dendritic cells have antimicrobial activities and are also involved control of *Salmonella* infection (Sebastiani *et al.*, 2002). The innate immune system can, therefore, control early *Salmonella* infection by phagocytosis and production of antimicrobial molecules. However, the innate immunity alone cannot clear virulent *Salmonella* infection without the adaptive (cellular and humoral) immune system (Lalmanach and Lantier, 1999).

2.3.2 Cellular immune responses

CD4+ and CD8+ T lymphocytes are crucial for protective immune responses against many intracellular bacterial pathogens such as *Salmonella* (Kerksiek and Pamer, 1999). In most cases these cells are critical for sterilizing immunity against bacterial infection (Shen *et al.*, 1998; Lo *et al.*, 1999). The major histocompatibilty complex class I and class II antigen-processing pathways are responsible for the activation of antigen-specific CD8+ and CD4+ T lymphocytes respectively (Lanzavecchia, 1996; Trombetta and Mellman, 2005). CD8+ T cells are able to recognize peptides bound to the MHC class I molecules while CD4+ T cells recognize peptides bound to the MHC class II molecules. CD4+ and CD8+ T cell responses target most of the *Salmonella* antigens such as protein antigens, porins, flagellin, pilin, LPS and Vi surface polysaccharides (Mastroeni and Menager, 2003). The recognition of peptides derived from these antigens results in induction of antigen-specific CD4+ T- or CD8+ T cell immune responses.

Few studies have extensively investigated the role of both CD4+ and CD8+ cell immune responses to *Salmonella* infection. However, it is generally known that infection with *Salmonella* induces adaptive immunity, which can potentially contribute to protection (Ravindran and McSorley, 2005 McSorley *et al.*, 2000). After phagocytosis by macrophages or dendritic cells, the bacteria replicate in the *Salmonella*-containing vacuoles. *Salmonella* antigens or peptides are predominantly presented by MHC class II molecules to the CD4+ T cells. A number of studies have used proliferative assays to detect CD4+ T cells after *Salmonella* infection in animals. Using such assays, *Salmonella*-specific CD4+ T cell responses have been observed in mice, calves and humans (Sztein *et al.*, 1994; Harrison *et al.*, 1997; Villarreal-Ramos *et al.*, 1998). It has also been observed that CD4+ Th1 cytokines (IL-2 and IFN-γ) were induced predominantly upon *in vitro* restimulation of immune cells from *Salmonella*-vaccinated mice (Harrison *et al.*, 1997). Recent studies by Kirby *et al.*, (2004) have shown a higher percentage of memory

CD4+ Th1 cells producing IFN-γ and TNF-α in mice immunized with *Salmonella*. The involvement of both CD4+ Th1 and Th2 cells against *Salmonella* porins has been demonstrated. Bergemen *et al.*, (2005) studied the generation of CD4+ T cell responses directed against epitopes of *Salmonella* FliC antigen vaccinated mice. It was shown that macrophages and dendritic cells infected with *Salmonella* could process and present FliC epitopes, resulting in stimulation of antigen-specific CD4+ T cell proliferation and IFN-γ secretion (Bergemen *et al.*, 2005). It has also been shown that MHC class II knockout and CD4 knockout mice are highly susceptible to *Salmonella* (Hess *et al.*, 1996), underlining the critical role of CD4+ T cell responses in protection. IFN-γ knockout mice have also been shown to fail to be susceptible to disseminated septicaemia after *Salmonella* infection (Bao *et al.*, 2000). From all these studies, it is clear that the CD4+ T cell responses (especially Th1) play a key role in controlling *Salmonella* infection. How CD4+ T cell responses regulate other aspects of the immune system such as CD8+ T cell and humoral responses during *Salmonella* infection is still not clear.

The role of CD8+ T cells in controlling intracellular pathogens such as *Salmonella* is well recognized (White *et al.*, 1996; Harty and Bevan, 1999; Kerksiek and Pamer, 1999; Ravindran and McSorley, 2005). Since *Salmonella* reside and replicate in the SCVs (Oh *et al.*, 1996), it is not obvious that processing and presentation of exogenous antigens by the classical MHC class I pathway for induction of CD8+ T cell responses will occur. However, recent studies have recorded the induction of *Salmonella*-specific CD8+ T cells after bacterial infection in humans and mice (Lundin *et al.*, 2002; Salerno-Goncalves *et al.*, 2002; Diaz-Quinonez *et al.*, 2004; Lo *et al.*, 2004). Studies by Lo *et al.*, (1999) have shown MHC class Ib-restricted responses after infection with wild-type *Salmonella* bacteria. Later studies further showed that a CD8+ epitope derived from *Salmonella* HSP-60 could also be processed and presented to CD8+ T cells (Lo *et al.*, 2004). The critical role of CD8+ T cells was also previously demonstrated by Lo *et al.*, (1999), who showed that Class I-deficient beta2-microglobulin mice were susceptible to *Salmonella* infection. Studies by Pasetti *et al.*, (2002) confirmed that *Salmonella* vaccine vectors could elicit antigen-specific CD8+ T cell responses in mice. The mechanisms by which exogenous antigens (from the SCVs) are cross-presented by the MHC class I molecules to give rise to CD8+ T cell responses are not clear. However, studies have suggested that apoptotic cells infected with antigens could be an important source for cross-priming in such situations (Albert *et al.*, 1998). *Salmonella*-infected cells undergo bacterial-induced apoptosis and the apoptic blebs could be the main sources of antigens for the generation of *Salmonella*-specific CD8+ T cells (Yrlid and Wick, 2000; Wijburg *et al.*, 2002). Bystander dendritic cells, not macrophages, have been suggested to be the antigen-presenting cells that engulf the *Salmonella*-infected apoptotic cells for induction of CD8+ T cells (Wijburg *et al.*, 2002; Sundquist *et al.*, 2004). It is also now known that dendritic cells are capable of processing and cross-presenting exogenous antigens for induction of CD8+ T cell responses (Brode and Macary, 2004; Heath *et al.*, 2004). Other models by which exogenous antigens could directly access the cytosol for induction of CD8+ have recently been reviewed (Rock and Shen, 2006). It is not clear as to which of these models is dominant in cross-presentation of *Salmonella* antigens for induction of CD8+ T cell responses. Despite our poor understanding of cross-presentation, the fact that *Salmonella* induce CD8+ T cell immune responses

means that the attenuated bacteria could be usefully exploited as vaccine vehicles that carry recombinant antigens (Section 2.4).

2.3.3 Humoral immune responses

Salmonella infection provokes humoral immune responses which contributes to successful control of infection (Mastroeni *et al.*, 2000a; Mittrucker *et al.*, 2000). Mice challenged with *Salmonella* elicit antibody responses to several antigens such as LPS, flagella, fimbriae, porins, lipoproteins, heat-shock proteins and other bacterial proteins such as outer membrane proteins (Pasette *et al.*, 2003; Secundino *et al.*, 2006). Although antibodies are produced against several *Salmonella* antigens, their general role in preventing or controlling infection is unclear. Studies in humans have shown that high antibody titres specific to *Salmonella* surface antigens correlated with protection against infection (Isibasi *et al.*, 1988). Passive transfer of immune serum or B cells has been found to be protective against *Salmonella* infection in mice (Hochadel and Keller, 1977). B-cell deficient mice have increased susceptibility to *Salmonella* infection (Mastroeni *et al.*, 2000a; 2003). Recent work has shown that *Salmonella* porins induce lifelong bactericidal antibody memory responses in mice (Secundino *et al.*, 2006). Attenuated *Salmonella* vaccines can therefore be used as recombinant vectors that are capable of inducing protective antigen-specific antibody responses.

2.3.4 Mucosal immune responses

Pathogens such as *Salmonella*, which invade at mucosal surfaces, provoke mucosal and systemic immune responses. At mucosal compartments, the expected B cell immunity comprises mainly secretory immunoglobulin A (s-IgA), while serum IgG immune response is expected in the systemic compartments (Chen and Schifferli, 2000; Dietrich *et al.*, 2003; Wyszynska *et al.*, 2004). Experimental evidence shows that mucosal secretory IgA correlates with resistance to infection by bacteria (Rosenthal and Gallichan, 1997; McCluskie and Davis, 1999; Ogra *et al.*, 2001). The two types of antibodies (IgA and IgG) possibly neutralize the pathogens and control infection in the mucosal and systemic compartments respectively. T cell-mediated immune responses can also control *Salmonella* infection at both the mucosal and systemic compartments. It has been documented that T cells produced at one mucosal surface are capable of homing and offer protection at other mucosal surfaces (Rosenthal and Gallichan, 1997; Ogra *et al.*, 2001). This is one of the key advantages of oral vaccines such as attenuated *Salmonella*.

2.4 LIVE ATTENUATED *SALMONELLA* VACCINES

A number of vaccine strategies to reduce the burden of salmonellosis (especially typhoid fever) have been developed. They include the use of inactivated whole cell vaccines, Vi-based subunit vaccines and live attenuated vaccines. Guzman *et al.*, (2006) have enlisted some of the pros and cons of using each of the three strategies of vaccination against *Salmonella* (Table 2.1). From this table, it can be seen that there are many advantages of the use of live vaccines. For safety reasons, the live vaccines must genetically be attenuated by mutation.

Table 2.1: Comparisons of different vaccination strategies for typhoid fever (adapted from Guzman *et al.*, 2006).

Characteristics	**Whole cell vaccine**	**Vi vaccine**	**Live attenuated (Ty21a)**
Vaccination route	Parenteral	Parenteral	Oral
Adverse systemic reactions	10-20%	2%	<1%
Adverse local reactions	10-50%	10-40%	NA
Protective efficacy	60-80%	64-72%	60-80%
Duration of protection	7 years	17-21 months	4-7 years
Booster doses/revaccination	3 years	2-3 years	1-7 years
Medical professional needed for vaccination	Yes	yes	No

A number of genes have been targeted in generating live attenuated vaccines. Some of these genes are listed in Table 2.2. Currently, the genes targeted for attenuation and generation of *Salmonella* vaccines have been those involved in biosynthesis, regulation and virulence pathways (Doggett and Brown, 1996; Mastroeni *et al.*, 2001). Recent advances in genetic engineering has enabled the creation precise deletions of *Salmonella* genes for the generation of safer, defined, live attenuated vaccines. Genetically

Table 2.2: Examples of genes that have been targeted for attenuating *Salmonella* in development of live vaccines. The list is not meant to be comprehensive, but to show examples of genes that can be mutated or deleted to decrease virulence or invasiveness of the bacteria

Gene mutated	*Gene function*
aroA, aroC	Aromatic amino acid biosynthesis (Hoiseth and Stocker, 1981; Stocker, 1988, 2000).
galE	Conversion of UDP-galactose to UDP-glucose (Germanier and Furer, 1971).
pur	Purine biosynthesis (Edwards and Stocker, 1988).
htrA	Heat and oxidative stress protection (Mutunga *et al.*, 2004).
cya/crp	Biosynthesis of cAMP and AMP receptor expression (Curtiss, 1993, Stocker, 1988).
phoP/phoQ	Phosphate metabolism (Castelli *et al.*, 2000; Chamnongpol *et al.*, 2003).
dam	Expression of DNA adenine methylase (Ritchie *et al.*, 1986; Ritchie, 1996; Heithoff *et al.*, 2001).
ompR	Porin regulation (Pickard *et al., 1994*; Fernandez-Mora *et al.*, 2004).
SifA	Vacuolar membrane maintenance (Bacrout *et al.*, 2003).
SsaV	SPI-2 gene (Michael *et al.*, 2004).

defined, non-reverting mutations into these genes found on the *Salmonella* chromosome (especially on pathogenicity islands) can now also be targeted by the technologies in order to generate attenuated vaccines. Methods such as signature-tagged mutagenesis, STM (Auret and Charbit, 2005; Saenz and Dehio, 2005) can be used to completely delete single or multiple genes so as to guarantee complete safety of the vaccines in humans or animals.

A number of attenuated *Salmonella* vaccine candidates for prevention of typhoid fever have been developed. Ty21a was the first attenuated typhoid fever vaccine and was generated by chemical and UV mutagenesis of the *galE* gene (Germainer and Furer, 1971; Mitov et al., 1992). It is the only licensed vaccine (as Vivotif, Berna Biotech, Switzerland). It is taken orally and is generally effective in protection against typhoid fever (Table 2.1). It has been demonstrated that *Salmonella* protection by this vaccine is mediated by mucosal (IgA) and serum (IgG) antibodies as well as CD8+ T cell responses (Salerno-Goncalves *et al.*, 2002). It was also shown that Ty21a induced systemic CD4+ T cells secreting IFN-γ and antibody responses in vaccinated individuals (Viret *et al.*, 1999). Human trials in Egypt showed protective efficacy of 96% and the period of protection was 3 years after vaccination with Ty21a (Wahdan *et al.*, 1982). Recent studies have further confirmed that immunization of humans with Ty21a induced both CD4+ and CD8+ T-cell responses in peripheral blood, together with mucosal IgA and serum IgG antibody responses (Kilhamn *et al.*, 2003). The study demonstrated that despite being attenuated, Ty21a vaccine could still induce immune responses.

There are still other live attenuated vaccines under development. Examples of these live attenuated *Salmonella* vaccines with known genetic mutations include *aro* mutants such as *Salmonella enterica* serovar Typhi CVD906 and CVD908 (Hone *et al.*, 1991), *cya/crp* mutants such as *Salmonella enterica* serovar Typhi Chi3927 (Curtiss *et al.*, 1993) and *Pho*P/Q mutants such as *Salmonella enterica* serovar Typhi Ty800 (Hohmann *et al.*, 1995; 1996a). Highly novel mutants include *Salmonella* strains attenuated in *ssa*V and *sif*A genes (McKelvie *et al.*, 2004; Michael *et al.*, 2004). Humans vaccinated with CVD 906 have developed strong immune responses against LPS, although there were some adverse symptoms such as fever and bacteraemia in some volunteers (Hone *et al.*, 1992). Studies with CVD 908 showed that the vaccine was highly immunogenic, with induction *Salmonella* LPS-specific IgG and IgA antibodies (Tacket *et al.*, 1992). The two vaccines were further attenuated by introducing mutations in the *htr* gene to create double mutants, CVD 906-*htrA* and CVD 908-*htrA*) were well-tolerated and immunogenic in Phase I and II clinical trials (Tacket *et al.*, 1997; 2000). Furthermore, recent studies by Salerno-Goncalves *et al.*, (2003) have shown that CVD 908-*htrA* vaccine could induce both CD4+ and CD8+ T cell responses in vaccinated volunteers. In this study, IFN-gamma-specific responses to *Salmonella* flagella were induced in some of the vaccinees (Salerno-Goncalves *et al.*, 2003). Ty800 (*aroA phoP* mutant) was shown to be safe and immunogenic by inducing IgA and serum IgG antibody responses in Phase I clinical trials (Garmory *et al.*, 2002). Recent studies of another oral typhoid vaccine, M01ZH09 (Microscience Ltd, UK), which has non-reverting mutations in *aroC* and *ssaV* genes, have shown that it is well tolerated and very immunogenic even after a single vaccination (Kirkpatrick *et al.*, 2005, 2006).

Not all attenuated *S. enterica* serovar Typhi strains that have been developed have shown favourable immunogenicity results in human trials. Ty445 (*aroA phoP phoQ* mutant) is such an example; it has not shown any reactogenicity in humans even after two immunizations with a high dose of 5×10^{10} cfu (Hohmann *et al.*, 1996b). This could be due to over-attenuation of the vaccine, which may have rendered it to poorly invade the GALT of the vaccinated individuals. Live attenuated *S. enterica* serovar Typhi

χ8110 vaccine was also shown to be poorly immunogenic in healthy adult volunteers (Frey *et al.*, 2001). However, the ability of some attenuated *Salmonella* vaccines to induce both cellular and humoral immune responses, the ease of their delivery through the oral route to induce both mucosal and systemic immunity and their low cost of production make them good candidates for use in delivery of heterologous antigens.

2.5 RECOMBINANT *SALMONELLA* VACCINE VECTORS EXPRESSING HETEROLOGOUS ANTIGENS: PRECLINICAL STUDIES

Recent years have seen exploration of attenuated live *Salmonella* vaccines as vectors for the delivery of heterologous antigens (reviewed in Hormaeche, 1991; Doggett and Brown, 1996; Kraehenbuhl *et al.*, 1998; Sirard *et al.*, 1999; Stocker, 2000; Shata *et al.*, 2000; Bumann *et al.*, 2000; Gentschev *et al.*, 2001; Medina and Guzman, 2001; Bachtiar *et al.* 2003; Garmory et al., 2003b; Roland *et al.*, 2005; Atkins *et al.*, 2006; Spreng *et al.*, 2006). These live attenuated *Salmonella* vaccines have several advantages for use as delivery systems for heterologous antigens: (i) They mimic the natural infection of most pathogens such as HIV-1 which infect their host through mucosal surfaces. (ii) They are intracellular pathogens which are capable of surviving and replicating inside antigen-presenting cells (dendritic cells and macrophages) (Santos and Baumler, 2004). This facilitates continual processing and presentation of antigens to the immune system. (iii) They are relatively inexpensive to produce or manufacture for large-scale immunizations. In addition, no highly trained medical professionals are required for immunizations, further reducing the cost of delivery. (iv) Non-reverting live attenuated *Salmonella* strains can now be developed by recently developed technologies in genetic engineering. These mutants cannot revert to wild-type and can therefore be used even in immunosuppressed animals/humans. This property may make it possible for *Salmonella* vaccines to be used for patients infected with HIV-1. (v) The bacterial vaccines are treatable with antibiotics should adverse effects occur during vaccinations. (vi) The oral route is more practical, socially acceptable and reliable in developing countries where resources may be scarce. The oral (mucosal) vaccination also results in induction of both mucosal and systemic immune responses, unlike systemic vaccination which does not normally elicit mucosal immunity. (vii) They have adjuvant activities (viii) They can hold large amount of foreign DNA (large multivalent antigen capacity). (ix) The molecular tools and techniques developed over the years for genetic manipulation of *E. coli* can easily be applied to *Salmonella* vaccine manipulation. All these advantages make *Salmonella* vaccines attractive for use as recombinant vectors for vaccines against other pathogens, including HIV-1.

However, despite all the advantages mentioned above, live attenuated *Salmonella* may have a number of potential pitfalls as vaccine vectors (Galen and Levine, 2001; Kotton and Hohmann, 2004; Detmer and Glenting, 2006). The problems that may be encountered include (i) high instability especially when high copy number plasmids are used, (ii) segregational plasmid loss during cell division, (iii) poor immunogenicity if the antigen is not expressed at high levels, (iv) metabolic burden to the bacterial vector if antigen is expressed at high levels, (v) post-translational cellular proteolytic degradation of heterologous antigens and (vi) no post-translational modification of expressed proteins. Although there

are drawbacks, a number of viral, bacterial, parasitic and other antigens have been successfully expressed in *Salmonella* vaccine vectors. Vaccination of animals or human volunteers with some of the recombinant *Salmonella* has resulted in immune responses directed against the heterologous antigens. Most of the preclinical studies have been conducted using attenuated *Salmonella enterica* serovar Typhimurium in mice, as *Salmonella enterica* serovar Typhimurium infection in mice is a model for *Salmonella enterica* serovar Typhi infection in humans. The other reason for use of *Salmonella enterica* serovar Typhimurium in preclinical studies is that *Salmonella enterica* serovar Typhi is host-restricted to humans only and does not naturally infect mice through the oral route.

2.5.1 Recombinant *Salmonella* vectors expressing viral antigens

Recombinant live attenuated *Salmonella* vaccine vectors expressing viral antigens have been developed. The presence of a wide array of mucosal and/or systemic immune responses against the live vector and foreign antigens has been demonstrated in a number of different studies. One of the earliest studies by Schodel *et al.*, (1990) showed that aromatic amino acid-dependent attenuated *Salmonella enterica* serovar Typhimurium could express a hybrid Hepatitis B virus (HBV) nucleocapsid-preS fusion antigen. Oral vaccination of mice with the recombinant *Salmonella* vaccine vector induced high serum IgG antibody titres against the HBV antigens. Further studies have been done in which the recombinant *Salmonella* vaccine vector expressing HBV nucleocapsid fused to *Plasmodium yoelii* circumsporozoite protein was developed (Schodel *et al.*, 1997). Mice vaccinated with the vector were protected from malarial challenge. In another study, Shams *et al.*, (2001) demonstrated that *Salmonella enterica* serovar Typhimurium vectors expressing lymphocytic choriomeningitis virus (LCMV) nucleoprotein could induce specific antiviral CD8+ CTL responses after oral vaccination of mice. Long-lived protective immunity was also shown and vaccinated mice survived challenge with a lethal dose of LCMV.

There has been a single report in which attenuated *Salmonella enterica* serovar Typhimurium SL3261 was used to express feline immunodeficiency virus Gag (FIV Gag) antigen (Tijhaar *et al.*, 1997). Two intra-peritoneal immunizations of cats induced weak FIV Gag-specific antibodies. No FIV Gag-specific antibodies were detected when the cats were orally vaccinated twice. No protection was conferred when the cats were challenged with a lethal FIV using either route (Tijhar *et al.*, 1997). This showed that protection against infection can sometimes fail if a poor immune response is induced after vaccination. Later studies by Steger *et al.*, (1999) investigated immune responses in rhesus macaques orally vaccinated with recombinant S. serovar Typhimurium expressing SIV capsid protein (p27). The recombinant *Salmonella* vaccine induced p27-specific lymphoproliferative responses together with low p27-specific humoral immune responses. The immune responses did not suppress responses to subsequent parenteral vaccination. In another study, Coste *et al.*, (2001) developed *Salmonella enterica* serovar Typhimurium vaccine vectors expressing rotavirus VP2 and VP6 antigens. Unlike as in eukaryotic cells, the VP2 and VP6 did not form virus-like particles (VLPs) in the *Salmonella* cells. However, intra-nasal vaccination of female mice with the recombinant *Salmonella* vaccines elicited VP2 and VP6-specific antibody responses in both serum and milk. When the mice were challenged with a

lethal dose of rotavirus, there was no protection, indicating that the antibody responses were not protective.

Human papillomaviruses (HPVs) are a leading cause of cervical cancer and other anogenital cancers (recently reviewed in DiMaio and Liao, 2006). There is, therefore, a general urgency for the development of second generation vaccines against these viruses (Schiller and Nardelli-Haefliger , 2006). Few studies have investigated the possibility of developing mucosal vaccines against these viruses using *Salmonella* as delivery vectors. Nardelli-Haeflinger *et al.*, (1997) developed a recombinant *Salmonella enterica* serovar Typhimurium (*Pho*P mutant) expressing the HPV-16 LI antigen. The assembling of L1 VLPs in the vector was demonstrated and intranasal vaccination of mice with the bacterial vaccine vector elicited HPV L1-specific (IgA and IgG) antibodies in serum, saliva and vaginal secretions. Using the same model, mucosal vaccination of mice with a recombinant *Salmonella* expressing HPV L1 induced anti-tumour activity (Revaz *et al.*, 2001). In a similar study, oral vaccination of mice with recombinant *Salmonella enterica* serovar Typhimurium expressing codon-optimized HPV-16 L1 resulted in better humoral immune responses when compared to a vector expressing wild-type HPV-16 L1 (Baud *et al.*, 2004b). Not only anti-HPV-16 L1 humoral responses, but also anti-HPV-16 L1 neutralizing antibodies were induced after vaccination with the bacterial vector expressing the codon-optimized L1. This study showed that codon-optimization of antigens for expression in *Salmonella* and formation of VLPs could be a critical factor in determining the quality and magnitude of immune responses elicited.

A recombinant *Salmonella enterica* serovar Typhimurium SL3261 vaccine vector expressing HIV-1 gp120 antigen has been constructed by Fouts *et al.*, (1995). Although high level expression of the antigen was achieved, vaccination of mice with the vector did not induce HIV-1 gp120-specific antibodies. The failure of post-translational modification of gp120 into its native form could be the reason for the failure of the *Salmonella* vector in inducing antibody responses. However induction of HIV gp120-specific cellular proliferation and a CD4+ Th1 cytokine profile were demonstrated.

In another study, Evans *et al.*, (2003) developed a recombinant *Salmonella enterica* serovar Typhi that expressed a fragment of SIV Gag fused to the *Salmonella* type III-secreted SopE protein. Oral vaccination of rhesus macaques by the vector induced Gag-specific CD8+ T cell responses. Another recent study used the same strategy to express *Listeria* p60 antigen in a recombinant *Salmonella enterica* serovar Typhimurium (Panthel *et al.*, 2005). Vaccination of mice with the vector induced antigen-specific CD4+ and CD8+ T cell responses. Kotton *et al.*,(2006) also expressed HIV-1 Gag fused to SopE in *Salmonella enterica* serovar Typhimurium and assessed the safety of the vector in human subjects. Although the vector was safe, Gag-specific immune responses were poor in vaccinated people.

Most of the examples cited above illustrate the potential of using attenuated *Salmonella* in development of viral novel vaccines. In some of the studies, protective immunity was induced, suggesting that the bacterial vector activated the relevant immune responses against the heterologous antigens. Such studies

should provide insights into how best a vaccine can be developed, especially for viral pathogens such as HIV-1 in which the immune correlates of protection are poorly understood.

2.5.2 Recombinant *Salmonella* vectors expressing bacterial antigens

Heterologous antigens from other bacterial pathogens have been cloned and expressed in recombinant *Salmonella* vaccine vectors and their immunogenicity has been evaluated. Live attenuated *Salmonella enterica* serovar Typhimurium (*Pho*P mutant) expressing *Helicobacter pylori* antigens, A and B subunits of urease (ureAB) under the constitutive *tac* promoter were developed (Corthesy-Theulaz *et al.*, 1998). Intranasal vaccination of mice resulted in the induction of ureAB-specific IgG1 and IgG2a antibodies, thereby suggesting that both CD4+ Th1 and Th2 type immune responses had been induced. Splenic and Peyer's patches CD4+ lymphocytes were also shown to proliferate *in vitro* after stimulation with urease antigen (Corthesy-Theulaz *et al.*, 1998). Upon challenge of vaccinated mice with *Helicobacter pylori* bacterium, there was 60% protection. In another study, Kozarov *et al.*, (2000) expressed *Porphyromonas gingivalis* hemagglutinin A (HagA) antigen in *Salmonella enterica* serovar Typhimurium vector. The *hagA* gene was expressed by the vector using its natural promoter. The study demonstrated that purified HagA could be recognized by sera of vaccinated mice. The HagA antigen expressed by the vector was also found to be recognized by serum from a periodontal patient (that is, a patient infected with the *Porphyromonas gingivalis* bacterium).

Chabalgoity *et al.*, (2001) developed a recombinant attenuated *Salmonella enterica* serovar Typhimurium vector for immunization of dogs against *Echinococcus granulosus*. The *Salmonella* vector constitutively expressed a *E. granulosus* fatty acid binding protein (FABP). The vector was well-tolerated in orally vaccinated dogs. Both humoral (IgG1 and IgG2) and cellular immune responses against the heterologous antigen were induced after the vaccination.

Garmory *et al.*, (2003) used the attenuated *Salmonella enterica* serovar Typhimurium (SL3261) vaccine strain to express *Yersinia pestis* V antigen. When mice were vaccinated with the recombinant vector, V antigen-specific serum antibody responses were induced. The vaccinated animals survived *Y. pestis* challenge. This was the first study to demonstrate that vaccination of mice with recombinant *Salmonella* vaccine vector expressing V antigen could result in protective immunity against plague. In another study, Morton *et al.*, (2004) developed a recombinant *Salmonella enterica* serovar Typhi vaccine (*aroA aroC htrA* mutant) surface-expressing *Yersinia pestis* F1 antigen. Intranasally vaccinated mice elicited F1 antigen-specific IgG2a (Th1) and IgA antibody responses and were protected (60%) from a lethal challenge with plague. This study showed that *Salmonella* could potentially be used to develop vaccines against agents for bioterrorism. Efforts are already underway to develop these vaccines (Atkins *et al.*, 2006; Calhoun and Kwon, 2006).

The effect of heterologous antigen location in recombinant attenuated *Salmonella enterica* serovar Typhimurium vector on immune responses in orally-vaccinated mice was investigated by Kang and

Curtiss (2003). *Streptococcus pneumoniae* surface protein A (PspA) was expressed either intracellularly or secreted. Orally delivered recombinant *Salmonella* vector secreting PspA induced far better PspA-specific IgG than one expressing the antigen cytoplasmically. It was concluded that recombinant *Salmonella* vector secreting PspA enhanced antigen-specific systemic immune responses compared to one in which the antigen was retained in the cytoplasm. The reason for such differences could be that intracellular expression causes metabolic burden to the vector, while secretion of antigen does not profoundly affect the capacity of the vector to deliver the antigen to the immune system. In another study, Mollenkopf *et al.*, (2001) showed that an attenuated recombinant *Salmonella typhimurium* vaccine vector secreting the immunodominant antigen, ESAT-6 of *Mycobacterium tuberculosis* via the hemolysin secretion system of *E. coli* induced a protective immune response in mice. The numbers of tubercle bacilli in challenged mice were reduced after a vaccination with a single dose (Mollenkopf *et al.*, 2001). This study further confirmed that secretion of antigens was better at inducing a protective immune response than intracellular expression of the antigens by the bacteria.

McKelvie *et al.*, (2004) expressed *E. coli* heat labile toxin B subunit (LT-B) using an *in vivo* inducible promoter, *ssaG*, in a *Salmonella enterica* serovar Typhimurium vector. Anti-LT-B humoral immune responses were detected after oral vaccination of mice with the recombinant *Salmonella* vector. This clearly demonstrated the importance of using promoters which are upregulated when the vector is inside the phagocytes. The metabolic burden placed onto the vector by expression of heterologous antigens during vaccine manufacture is also reduced drastically by use of such inducible promoters (Galen and Levine, 2001).

It has further been demonstrated that attenuated *Salmonella enterica* serovar Typhi CVD 908-*htrA* or *Salmonella enterica* serovar Typhimurium SL3261 expressing tetanus toxin (TT) fragment C (frag C) could provoke antigen-specific mucosal and systemic IgA and IgG antibody secreting cells as well as T cell-mediated immunity in neonatal mice (Caputoozzo *et al.*, 2004). The study was the first to show the ability of recombinant *Salmonella* vaccine vectors to induce both strong humoral and cellular immune responses early in life (neonates). The results from this study are significant, given the fact that most vaccines are targeted for administration to neonates.

In a study by Wyszynska *et al.*, (2004) *Campylobacter jejuni* genes, *cjaA*, *cjaC* and *cjaD* were cloned and expressed in attenuated *Salmonella enterica* serovar Typhimurium (x4550). Oral vaccination of chicken with the *Salmonella* vectors induced serum IgG and mucosal IgA antibody responses against the *Campylobacter* antigens. The vaccination resulted in protection against *Campylobacter* bacterial colonization of the cecum, thereby demonstrating the effectiveness of the elicited immune responses. This study indicated that *Salmonella* could also be used in the development of vaccines of veterinary importance. Other approaches of using *Salmonella* to vector *Campylobacter jejuni* antigens have recently been demonstrated by Sizemore *at al.*, (2006).

The above studies clearly illustrate that recombinant *Salmonella* vaccines can be exploited as vectors for other bacterial antigens. Since most bacterial pathogens of human and veterinary importance infect through mucosal surfaces (like *Salmonella*), it is logical to use *Salmonella* vectors to deliver antigens for induction of protective mucosal and systemic immune responses.

2.5.3 Recombinant *Salmonella* vectors expressing parasitic antigens

Recombinant *Salmonella* vaccine vectors that express parasitic antigens have also been developed. The first demonstration of the use of attenuated *Salmonella* as a vector for a parasitic antigen was developed in the early 1990s by Flynn *et al.*, (1990). These researchers developed a recombinant *Salmonella enterica* serovar Typhimurium chromosomally expressing circumsporozoite (*CSP*) gene of the mouse malaria *Plasmodium yoelii*. Orally vaccinated mice developed CSP-specific CTL responses. Gonzalez *et al.*, (1994) later used *Salmonella enterica* serovar Typhi to chromosomally express the *Plasmodium falciparum* CSP. Vaccination of human volunteers induced serologic and CTL responses to the foreign antigen (Gonzalez *et al.*, 1994).

Cieslak *et al.*, (1993) expressed a major surface antigen, SREHP (serine rich *Entamoeba histolytica* protein) in an attenuated *Salmonella enterica* serovar Typhimurium vaccine. They showed that the vector could successfully infect the gerbil, a rodent model for amoebic liver abscess and intestinal amoebiasis. Later studies by Zhang and Stanley (1996) showed that *Salmonella enterica* serovar Typhimurium expressing the SREHP antigen was immunogenic in both mice and girbels. The vaccinated gerbils were protected against amoebiasis. Further studies by Zhang and Stanley (1997) showed that *Salmonella enterica* serovar Typhi expressing SREHP antigen was also immunogenic in mice.

Xu *et al.*, (1995) constitutively expressed *Leishmania major* promastigote surface glycoprotein, gp63 in *Salmonella enterica* serovar Typhimurium BRD509 mutant under the *tac* promoter. Mice orally vaccinated with the vector resisted infection after challenge with lethal *L. major*. Together with proliferative T-cell responses, mice also displayed leishmanial specific IgG2a, which further suggested that CD4+ Th1 cell responses were induced. No IgG1 antibody responses were detected suggesting that that vaccine only imprinted a Th2 response. Later studies by McSorley *et al.*, (1997) investigated the use of different promoters (*nirB* and *osmC*), to express the gp63 in the *Salmonella* vector. It was demonstrated that immunogenicity to the heterologous antigen differed due to differences in the strength of the inducible promoters.

Lattermann *et al.*, (1999) managed to constitutively express the extracellular copper/zinc superoxide dismutase (AVSOD2) of the filarial parasite*Acanthocheilonema viteae* in attenuated *Salmonella enterica* serovar Typhimurium vaccine strain SL3261. Jirds orally immunized with the recombinant vaccine produced strong AVSOD2-specific humoral immune responses. Upon challenge of vaccinated jirds with *A. viteae*, there was a reduction of the worm burden by 30% in these rodents. In another study, Pogonka *et al.*, (2003) demonstrated that a single oral dose of recombinant *Salmonella enterica* serovar

Typhimurium SL3261 vector expressing *Eimeria tenella* SO7 and TA4 antigens induced antigen-specific humoral immune responses in chicken. The stable expression of the antigens in the vector was achieved by the use of the pTECH2 expression vector with antigens fused to the C-terminus of tetanus toxin fragment C. Recent studies have further demonstrated that protective CD8+ T cell responses could be induced in chicken vaccinated with a recombinant *Salmonella enterica* serovar Typhimurium expressing *Eimeria* antigens (Konjufca *et al.*, 2006). These studies further showed the utility of *Salmonella* vaccines in delivering parasitic antigens to the immune system of different animals.

2.5.4 Recombinant *Salmonella* vectors expressing other antigens

One of the most common applications of the *Salmonella* vaccine vectors has been the delivery of cancer or tumor antigens to the immune system. One of the earliest investigations of the use of *Salmonella* vaccines as potential vectors for cancer antigens was conducted by Medina *et al.*, (1999). In their study, these researchers used attenuated *Salmonella enterica* serovar Typhimurium SL7207 vector to trigger anti-tumor immunity to beta-galactosidase (β-gal) when expressed as a model tumor-associated antigen (TAA). Oral vaccination of mice with the vector induced β-gal-specific humoral and CD8+ CTL immune responses. Upon challenge of vaccinated mice with an aggressive fibrosarcoma expressing β-gal, the animals showed a significant reduction in tumor take and growth.

Feng *et al.*, (2004) used recombinant attenuated *Salmonella enterica* serovar Typhimurium SL7207 vector to express the VEGFR2 (*flk-1*) gene. The aim of the study was to investigate the anti-vasculature and anti-glioma effects of the recombinant vector in mouse model of intracranial G1261 glioblastoma. The study demonstrated that the *Salmonella* vector expressing the *flk-1* gene could significantly inhibit glioblastoma growth, prolong the survival period and improve the survival rate of orally vaccinated mice. Flk-1 specific CD8+ CTL responses were demonstrated after vaccination. The authors urged that orally administered recombinant *Salmonella* vaccine could potentially be used for both prophylactic and therapeutic purposes.

Gentschev *et al.*, (2005) also investigated the potential use of *Salmonella* in delivering another cancer antigen. The recombinant *AroA Salmonella enterica* serovar Typhimurium strains expressing the C-Raf antigen using *E. coli* hemolysin secretion system were developed. Immunization of mice with the vaccine vector induced C-Raf-specific antibody and T-cell responses. Furthermore, therapeutic vaccination of tumor-bearing mice significantly reduced tumour growth in two transgenic mouse models of Raf oncogene-induced lung adenomas. In a similar study, Lee *et al.*, (2005) developed an attenuated *S. choleraesuis* expressing thrombospondin-1 (TSP-1) gene and the vector was used for therapeutic treatment of primary melanoma and experimental pulmonary metastasis in the syngeneic mouse B16F10 melanoma model. There was significant inhibition of tumour growth in mice after the vaccination with the vector. It was concluded that *Salmonella* vectors could be used for effective treatment of both primary and metastatic melanomas (Lee *et al.*, 2005). These studies suggest that the use of *Salmonella* vectors in development of prophylactic and therapeutic vaccines for cancer may be feasible.

Bradley *et al.*, (1997) developed a recombinant *Salmonella* vector expressing fox anti-sperm antigens. Oral vaccination of the foxes with the vector induced antigen-specific mucosal immune responses in the female genital tract, together with systemic IgG responses. Such studies demonstrate that recombinant *Salmonella* may be used as immuno-contraceptive vaccines in both humans and animals

A recent study by Goni *et al.*, (2005) involved the expression of mouse prion protein (PrP) by an attenuated recombinant *Salmonella* vector. Mucosal (oral) vaccination of mice with the recombinant bacterial vector induced gut anti-PrP IgA and systemic anti-PrP IgG. The study demonstrated that oral vaccination with the vector delayed or prevented prion disease in mice exposed orally to the 139A scapie strain. There is, therefore, a possibility of using recombinant *Salmonella* vectors in preventing prion disease in both domestic animals and humans. From the above cited examples, it is clear that *Salmonella* can also be used for delivery of other antigens which are not necessarily of viral, bacterial and parasitic origins.

2.6 RECOMBINANT *SALMONELLA* EXPRESSING HETEROLOGOUS ANTIGENS: HUMAN TRIALS

Most studies of the potential of using *Salmonella* as vaccine vectors are still in the preclinical stage (Section 2.5). Such studies are usually conducted in a murine model, with *Salmonella enterica* serovar Typhimurium as a vector. To investigate, the potential of *Salmonella* as vaccine vectors in humans, *Salmonella enterica* serovar Typhi, is usually used. Few studies that utilize this species as a carrier for recombinant antigens in humans have so far been conducted (Table 2.3). Most of these studies are still in the early phases of clinical human trials and are based on the licensed vaccine, Ty21a. Other vaccines based on different *Salmonella enterica* serovar Typhi strains in clinical trials.

Tacket *et al.*, (1997), developed a recombinant *Salmonella enterica* serovar Typhi mutant (*cya/crp/cdt*) expressing the hepatitis B core (HBc) and pre-S genes (see also Table 2.3). Oral vaccination of human subjects in a Phase I trial showed that the vaccine was well-tolerated and no adverse symptoms, such as

Table 2.3: Examples of human (clinical) studies in which recombinant *Salmonella enterica* serovar Typhi expressing heterologous antigens were used as oral vaccines

Mutant	Antigen expressed	Antigen-specific immune responses	Reference
Ty21a (*galE, rpoS*)	*Shigella sonnei* LPS	IgA or IgG antibody	Black *et al.*, 1987.
Ty21a (*galE, rpoS*)	*Vibrio cholerae* LPS	IgG antibody	Forest *et al.*, 1989.
Ty2 (*aroC/D, rpoS*)	*P.falciparum* CSP	IgG antibody, CD8+ T cell	Gonzalez *et al.*, 1994.
Ty2 (*cya, crp, cdt, rpoS*)	Hepatitis B	No IgG antibody	Tacket *et al.*, 1997.
Ty2 (*aroC/D, htrA*)	tetanus toxin	IgG antibody, proliferative CD4+ T cell	Tacket *et al.*, 2000.
Ty2 (*aroC/D, htrA*)	Vi capsular polysaccharide	IgG and IgA antibody	Tacket *et al.*, 2004.
Ty800 (*aroA, phoP/Q*)	*H. pylori* urease subunits A & B	No serum IgG or mucosal IgA antibody	DiPetrillo *et al.*, 1999.
Ty21a (*hyA*)	*H. pylori* urease subunits A & B	CD4 Th1 cell, No serum IgG antibody	Bumann *et al.*, 2001.
Ty21a	*H. pylori* urease subunits A & B	T cell	Metzger *et al.*, 2004.

fever, were reported. The vaccinees developed *Salmonella*-specific IgG responses, but no response to hepatitis B antigens were recorded. In a separate study, recombinant *Salmonella enterica* serovar Typhi CVD909 overexpressing the Vi capsular polysaccharide (Vi), was used in vaccination health adults (n=32) (Tacket *et al.*, 2004). Mucosal Vi-specific antibody responses were detected in the vaccines. However, very poor Vi-specific IgG was detected in the serum.

Metzger *et al.*, (2004) studied volunteers vaccinated with recombinant *Salmonella enterica* serovar Typhi Ty21a expressing subunits A and B of *Helicobacter pylori* urease. Some of the volunteers (56%) developed cellular immune responses to *H. pylori* urease. No severe adverse effects were noted in the volunteers after the vaccination. In another Phase I trial, volunteers were vaccinated with recombinant *Salmonella enterica* serovar Typhi M01ZH09 (*aro*C, *ssa*V mutant) (Microscience Pty, UK) that expressed *E. coli* LT-B (Microscience, unpublished data). The vaccine was also well tolerated and immunogenic after two doses. A recombinant M01ZH09 expressing hepatitis B core antigen was also recently found to elicit antigen-specific CD4+ T cell responses in vaccinated volunteers (Microscience, unpublished data).

All these few studies clearly demonstrate that recombinant *Salmonella enterica* serovar Typhi vaccines may be used as vectors for foreign antigens. Although this can be achieved, a lot of work still needs to be done to optimize expression of the foreign antigens in the vectors.

2.7 RECOMBINANT *SALMONELLA* AS VECTORS FOR DNA VACCINES

The use of naked DNA as a vaccine was originally demonstrated by Wolff and collegues in 1990 (Wolff *et al.*, 1990). Since then, naked DNA vaccines have been used for induction of potent immune responses, especially cell-mediated (reviewed in Ulmer *et al.*, 1996; Liu *et al.*, 1998; Donnelly *et al.*, 2005). In more recent years, the use of attenuated *Salmonella* vaccines as delivery vectors for these DNA vaccines has been explored (Shata *et al.*, 2000; Dietrich *et al.*, 2003; Xu and Ulmer, 2003; Loessner and Weiss, 2004; Schoen *et al.*, 2004). The actual mechanisms by which *Salmonella* deliver DNA vaccine to elicit immune responses are not yet clear. It has, however, been hypothesized that the DNA vaccine is first delivered specifically into antigen-presenting cells such as macrophages and dendritic cells, which can then express, process and present the antigen peptides for induction of an immune response (Schoen *et al.*, 2004). A number of studies have been conducted to demonstrate the utility of the *Salmonella* bacterial vectors carrying DNA vaccines in inducing immunogenicity (Table 2.4). Almost all this work is in the pre-clinical stage and therefore the mouse model (*Salmonella enterica* serovar Typhimurium as a vector) is being used. Several studies have also demonstrated that recombinant *Salmonella enterica* serovar Typhimurium vaccines carrying DNA vaccines can be delivered *in vivo* to host cells such as macrophages (Darji *et al.*, 1997; Medina *et al.*, 2000; Paglia *et al.*, 2000; Zheng *et al.*, 2001; Cochlovius *et al.*, 2002). In spite of *Salmonella*-delivered DNA vaccines still being in pre-clinical, they are likely to offer a novel approach to vaccination in humans.

Table 2.4: Examples of pre-clinical studies in mice in which recombinant *Salmonella enterica* serovar Typhimurium vectors were used as vaccine vectors for DNA vaccines

Route	Antigen/gene on DNA plasmid	Immune responses/ efficacy	Reference
Oral	Listeriolysin	Humoral, CD4+ Th and CTL; Protection from L. monocytogenes challenge	Darji *et al.*, 1997
Oral	transcription factor Fos-related antigen 1 and endoglin	CD8+ T cell; Effective suppression of breast tumor growth and metastasis	Mizutani *et al.*, 2004; Lee *et al.*, 2006
Oral	TAA (b-gal)	Humoral, CD4+ Th1, CTL; Reduction in tumour take	Paglia *et al.*, 1998; Medina *et al.*, 2000)
Oral	HIV-1 Env	CD8+ T cell	Shata *et al.*, 2001
Oral	tyrosine hydroxylase antigen	CD8+ T cytokine; complete protection against metastatic neuroblastoma	Pertl *et al.*, 2002
Oral	CEA	CD8+ T cytokine; protective against tumour	Xiang *et al.*, 2001
Oral/others	HIV-1 Env	Humoral, CD8+ T	Vecino *et al.*, 2002
Oral	HbsAg	CD8+ T, CD4+ T, humoral	Woo *et al.*, 2001
Oral	HIV-1 Env	Humoral, CD8+ T	Vecino *et al.*, 2002
Oral	Melanoma hgp100 and epitopes	CD8+ T cell; Protective immunity	Xiang *et al.*, 2000 ; Cochlovius *et al.*, 2002
Oral	HSV Glycoproteins D and B	CD4+, Humoral; protection (vaginal challenge)	Flo *et al.*, 2001

2.8 EFFECT OF PRE-EXISTING *SALMONELLA* VECTOR IMMUNITY

The impact of pre-existing anti-vector immunity on the efficiency of recombinant *Salmonella* vaccine vectors in delivering foreign antigens is still poorly understood. It is currently not clear whether prior exposure to *Salmonella* enhances or decreases the immune response to vectored antigens. In early studies, Bao and Clements (1991) found that pre-existing *Salmonella* immunity in mice improved their subsequent serum and mucosal responses. These observations were supported by the findings of Whittle and Verma (1997) who demonstrated that antibody responses to a viral antigen expressed by a recombinant *Salmonella* were enhanced in mice that were previously primed with the vector alone. A recent study by Jespersgaard (2001) further supported these results by showing that secondary responses to a recombinant *Salmonella* expressing *S. mutans* glucan-binding domain (GLU) of the enzyme glucosyltransferase was not affected by pre-existing immunity. In another study in which serovar Typhi Ty21a expressing *H. pylori* ureases A and B was used to vaccinate volunteers, it was noted that prior immunity enhanced immune response to the foreign antigen (Bumann *et al.*, 2001). These studies, though limited, suggest that prior exposure to the vector enhances immunogenicity to the heterologous antigens. The mechanisms of this enhancement of immune responses are not yet clear. However, it could be due enhanced uptake of antibody-coated recombinant bacteria by antigen-presenting cells or by alteration of cytokine profile during antigen presentation (Igietseme *et al.*, 2004; Kotton and Hohmann, 2004).

Other research has suggested that pre-existing vector immunity affected negatively the delivery of heterologous antigens by recombinant *Salmonella* vaccine vectors. Studies have been conducted by

Attridge *et al.*, (1997), Roberts *et al.*, (1999) and Kohler *et al.*, (2000), who all found that pre-existing immunity against *Salmonella* lowered memory serum responses and interfered with the response against the foreign antigen. Further studies have shown that hypo-responsiveness to foreign antigens occurred when they were delivered by recombinant *Salmonella* vectors (Attridge *et al.*, 1997; Vindurampule and Attridge, 2003a; Domenech *et al.*, 2005). Studies by Domenech *et al.*, (2005) have shown that subsequent rapid clearance of the vector was due to better *Salmonella*-specific CD8+ T cell responses induced after initial priming. After first booster vaccination, the vector was cleared within 7 days, and after the second booster, the clearance was within 4 days. It has also been argued that the nature of the heterologous antigen and the type of *Salmonella* vector used also determine whether preexisting immunogenicity to vaccine will be affected (Vindurampule and Attridge, 2003b; Attridge and Vindurampule, 2005). The mechanism of the poor immune responses due to pre-existing vector immunity is still not clear. However, it has been suggested that the clonal dominance of the bacteria vector B and T epitopes may impair the presentation of epitopes of the heterologous antigen to the immune system (LeClerc *et al.*, 1990). Further research needs to be done to clarify whether pre-existing immunity will have any impact on the use of *Salmonella* bacterial vaccines vectors, especially in areas such as Southern Africa, where the wild-type *Salmonella* serovars are endemic.

2.9 CONCLUSIONS AND PROJECT OBJECTIVES

There is a growing literature on the use of live attenuated recombinant *Salmonella* bacterial vaccines as potential delivery vectors for heterologous viral, bacterial, parasitic and other antigens. The primary rationale of using *Salmonella* vaccines as vectors is that they have a great potential of inducing both antigen-specific mucosal and systemic immune responses. Other advantages of the vaccines include, easy laboratory manipulations, inexpensive to manufacture for large-scale vaccinations and easy route of administration.

No previous studies have explored the potential use of *Salmonella* vaccines in delivering viral antigens derived from Southern Africa HIV-1 Subtype C. Since the majority of HIV-1 infections in Southern Africa occur by sexual transmission via mucosal surfaces, it is a logical proposal to develop a recombinant bacterial vaccine vector that would be administered orally for induction of both mucosal and systemic immune responses. The overall goal of the research project was, therefore, to investigate the potential use of an orally-delivered live attenuated *Salmonella enterica* serovar Typhimurium as a vaccine vector for HIV-1 subtype C Gag from Southern Africa. This goal was to be achieved by first designing an efficient prokaryotic expression plasmid for cloning of heterologous antigens. The jellyfish *Aequorea victoria* green fluorescent protein was initially to be used as a model antigen. The gene was to be expressed by the recombinant *Salmonella* vector and immune responses to orally vaccinated mice evaluated. Based on this model, a recombinant *Salmonella* vaccine vector expressing wild-type or codon-optimized HIV-1 subtype C Gag was also to be developed. Furthermore, the sub-cellular localization of the antigens (GFP and HIV-1 Gag) in the recombinant *Salmonella* vector was to be investigated.

CHAPTER 3: DEVELOPMENT AND IMMUNOGENICITY OF A RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR EXPRESSING *AEQUOREA VICTORIA* GREEN FLUORESCENT PROTEIN AS A MODEL ANTIGEN

"Either I will find a way, or I will make one" Sir Philip Sidney (1554-1586)

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CHAPTER 3: DEVELOPMENT AND IMMUNOGENICITY OF A RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR EXPRESSING *AEQUOREA VICTORIA* GREEN FLUORESCENT PROTEIN AS A MODEL ANTIGEN

3.1 SUMMARY

The use of jellyfish *Aequorea victoria* green fluorescent protein (GFP) as a model antigen in vaccine research and development has not previously been explored widely. No studies have investigated the cellular and humoral immunogenicity of GFP when delivered by attenuated *Salmonella* vaccine vectors. The current study, therefore, set out to develop and evaluate cellular and humoral immunogenicity of an attenuated recombinant *Salmonella enterica* serovar Typhimurium vaccine expressing GFP model antigen. A recombinant GFP expression plasmid, pGEM+GFP, was constructed in which the *gfp* gene was fused in-frame with the first 40 codons (5' domain) of the *E. coli* β-galactosidase α-gene fragment. The expression of the gene was under the *lac* promoter. The recombinant *Salmonella* carrying the plasmid expressed high levels of GFP. Immunogenicity was investigated in two groups of mice orally vaccinated with 10^8 colony forming units (CFUs)/mouse of the *Salmonella* vaccine. One group was sacrificed after 4 weeks (Day 28) and the other boosted twice (Day 28 and Day 54) with the same amount of vaccine and sacrificed on Day 84. At sacrifice, splenocytes were stimulated *ex vivo* in the presence of an H-2K^d-restricted Class I GFP peptide (HYLSTQSAL). Mice primed with the recombinant *Salmonella* vector did not elicit detectable GFP-specific CD8+ T-cell cytokine response. However, a strong immune response was elicited after two booster inoculations. It was demonstrated that 226 IFN-γ and 132 IL-4 GFP-specific net SFUs/10^6 splenocytes were formed. The induction of mixed GFP-specific CD8+ Tc1 and Tc2 cell cytokine responses were further confirmed by a CBA assay, which showed that high levels of IFN-γ (65.2-fold above background), TNF-α (4.5-fold above background) and IL-4 (10.4-fold above background) were secreted by 1.5 x 10^8 splenocytes after 48 hours of stimulation with the GFP peptide. The results suggested that, *in vivo*, the GFP antigen expressed by the recombinant *Salmonella* vaccine vector could be processed and cross-presented to MHC-I molecules for induction of both CD8+ Tc1 (IFN-γ and TNF-α) and Tc2 (IL-4) cytokine responses. The study also showed that GFP-specific serum IgG1 and IgG2a immune responses were induced in mice after single or booster vaccinations. The presence of both GFP-specific IgG1 and IgG2a antibodies in the serum suggested the induction of mixed GFP-specific CD4+ Th1 and Th2 responses. The findings in this study, therefore, demonstrated the effectiveness of a recombinant *Salmonella* as a mucosal vaccine vector that could induce mixed CD8+ Tc1/Tc2 and CD4+ Th1/Th2 cytokine responses, together with serum IgG1 and IgG2a antibody responses. Based on this model, other foreign antigens could potentially be expressed and delivered by the bacterial vaccine vector.

3.2 INTRODUCTION

Green fluorescent protein (GFP) was initially identified in 1962 in the jellyfish *Aequorea victoria* by Shimomura (historical review in Shimomura, 2006). Its cDNA was cloned and sequenced by Prasher and collegues three decades later (Prasher *et al.*, 1992). Subsequent studies by various groups demonstrated

that GFP was fluorescent when expressed in both prokaryotic and eukaryotic cells (Inouye and Tsuji, 1994; Chalfie *et al.*, 1995). Wild-type GFP is a 27-kDa protein with 238 amino acids and has a major absorbance peak at 395 nm, a minor absorbance peak at 470 nm and can be excited by both standard long-wave UV lamps and fluorescein isothiocyanate (FITC) filter sets (Margolin, 2000). Wild-type GFP emits green light after absorption of UV or blue light. The GFP chromophore responsible for the fluorescence is made up a cyclic tripeptide (Ser-Try-Gly, amino acids 65-67) and is only fluorescent when embedded within the complete GFP protein (Cody *et al.*, 1993). Only amino acids 7-229 are required for GFP fluorescence (Li *et al.*, 1997a). To date, there are a number of GFP mutants which have been generated and are also fluorescent (Tsien, 1998).

Some publications have recently reviewed the applications of GFP in various areas of the biological sciences (Tsien, 1998; Phillips, 2001. Zimmer, 2002; Lippincott-Schwartz *et al.*, 2003; March *et al.*, 2003; Hynes *et al.*, 2004; Stewart, 2006). These applications include, among others, the use of GFP as a reporter of gene transcription, as a protein fusion tag for protein expression or localization, as an active indicator of expression, for whole organism visualization and as a folding marker. GFP has also found use in the study of a variety of bacterial cellular processes such as cytokinesis, chromosomal replication and partitioning, sporulation and signal transduction. Furthermore, GFP can be used to study cells by flow cytometry (Galbraith *et al.*, 1999).

Although GFP has found widespread applications in many fields of the biological sciences, its use in vaccine research and development is sketchy. Very limited studies have investigated the immunogenicity of GFP in mice. Two initial reports have demonstrated GFP immunogenicity in mice that were vaccinated with GFP-expressing leukemia, T cell lymphoma, or sarcoma cells (Stripecke *et al.*, 1999; Gambotto *et al.*, 2000). Cellular and humoral immune responses against GFP were also observed in rhesus macaques that underwent haematopoietic stem cell transplantation with GFP-transduced CD34+ cells (Rosenzweig *et al.*, 2001). Since the identification of a GFP peptide, HYLSTQSAL ($GFP_{a.a.200-208}$) peptide as a mouse H-$2K^d$-restricted Class I epitope (Gambotto *et al.*, 2000), no further studies have explored GFP's potential use as a model antigen in development of vaccination protocols especially for T-cell vaccines. No studies have also investigated the use of oral attenuated recombinant *Salmonella enterica* serovar Typhimurium as vaccine vectors for GFP model antigen. The current study set out to develop a novel recombinant GFP-expression plasmid cassette and to investigate the potential of an oral attenuated recombinant *Salmonella enterica* serovar Typhimurium carrying the plasmid as a vaccine vector for the GFP model antigen in mice. In brief, the main objectives of the study were:

1. to design and construct a prokaryotic expression plasmid capable of expressing GFP model antigen under suitable *lac* operon transcriptional and translational domains.
2. to use the expression cassette in genetic transformation of *E. coli* and *Salmonella enterica* serovar Typhimurium, and characterize the GFP antigen expression in the two Gram negative bacteria.

3. to investigate induction of both GFP-specific systemic cellular (CD8+ Tc1 and Tc2 cytokine) and humoral (IgG1 and IgG2a) immune responses in mice after oral vaccination with the recombinant *Salmonella* vaccine vector expressing the GFP antigen.
4. to use the *Salmonella*-GFP vaccine model to pave and chart the way forward for the development of recombinant *Salmonella* vaccine vectors for human immunodeficiency virus subtype C Gag antigens (Chapters 4, 5 and 6).

3.3 MATERIALS

Materials used in the study are given in Appendices D, E, F, G and I. They included bacterial strains, oligonucleotides for polymerase chain reaction and DNA sequencing, plasmids, restriction enzymes, solutions, buffers and media.

3.4 GENERAL METHODS

General microbiological (bacteriological), molecular biology and immunological methods used in the study were as described in Appendices A and B.

3.5 EXPERIMENTAL DESIGN AND PROTOCOLS

3.5.1 Construction of a recombinant pGEM+GFP expression plasmid: molecular cloning of *gfp* gene

The recombinant plasmid, pGEM+GFP (Appendices G4 and H1) was constructed by PCR-mediated mutagenesis in which primers used for PCR had mismatches to the DNA template, which contained the *gfp* gene. The *gfp* gene, encoding GFP, was amplified with GFP2 (forward) and GR (reverse) (Appendix F) synthetic oligonucleotides as primers. PEHAOGFP plasmid (Appendix G1) kindly provided by Dr W Bourn (University of Cape Town) was used as template DNA. The primers were rationally designed so that GR could have a *Hind*III site, 5'-AAGCTT-3', and a Gram-negative bacterial stop codon, 5'-TAAG-3' at its end. The GR primer was also designed so that after cloning of the *gfp* PCR product, there would be a second stop codon, TAAT, one codon downstream of TAAG. The primer GFP2 was designed so that the *gfp* gene to be amplified by PCR would be in-frame with the 5' domain (first 40 codons) of β-galactosidase α-gene in pGEM-Teasy vector (Appendix G2). Restriction site for *Nar*I, *Ehe*I and *Bbe*I, 5'-GGCGCC-3', was incorporated in the GFP2 primer. The two primers had few base mismatches with their respective target DNA sequences in *gfp* template (PEHAOGFP).

The PCR reaction was conducted in a 50 µl volume with 4.5 units AmpliTaq Gold™ DNA Polymerase, 1x PCR buffer, 1.5 µM of each primers (GFP2 and GR), 0.2 mM dNTPs, 1.5 mM magnesium chloride and 10 ng of PEHAOGFP plasmid DNA template. The PCR cycling conditions were as follows: 1 cycle of 95°C for 5 min, 5 cycles of 95°C for 45 s, 55°C for 30 s, 72°C for 2 min, 25 cycles of 95°C for 45 s, 64°C for 30 s, 72°C for 2 min, and a final extension of 72°C for 7 min. Analysis of the *gfp* amplicon aliquot (5 µl) was done by agarose gel electrophoresis (Appendix B1.4). An aliquot (1 µl) of remaining

amplicon was ligated into a linearized pGEM-T Easy plasmid (Appendix B1.7). The ligation reaction was used in the genetic transformation of competent *E. coli* SCS110 cells (Appendix A1.4). The recombinant SCS110 clones harbouring the recombinant plasmid (hereby designated pGEM+GFP) were screened for presence of *gfp* fragment and its orientation by blue-white screening procedure (Appendix A1.1) and UV-illumination. The white and fluorescing (candidate) clones were cultured using standard protocols (Appendix A1.2). To investigate the presence of the recombinant *gfp* gene in plasmids, restriction mapping (Appendix B1.5) was performed initially with *Eco*R1 followed by double digestion with *Nar*I and *Hind*III. Restriction mapping of the plasmids was followed by maximum plasmid isolation (Appendix B1.2) from one of the candidate clones. The *gfp* gene in the candidate pGEM+GFP plasmid was sequenced (Appendix B1.8) to further confirm whether the gene was still in-frame with the 5' end of β-galactosidase α-gene in pGEM-T Easy vector or whether it did not acquire point mutations from polymerase chain reaction or genetic deletions. The analysis of sequencing data was performed using the DNAMAN and ABI Prism Automated DNA Analyzer (Chromas) (Applied Biosystems, Foster City, USA) softwares.

3.5.2 Development of recombinant *E. coli* and *Salmonella* and assessment of GFP expression

To investigate the expression of recombinant GFP, pGEM+GFP and pGEM (negative control) plasmids (Appendix G) were used in the genetic transformation of competent *E. coli* and *Salmonella enterica* serovar Typhimurium AroC mutant. IPTG, X-gal and ampicillin (Appendix D) were included in the 2x YT agar plates (Appendix A1.2). The plates for *Salmonella* were further supplemented with Aromix and Tryosine (Appendix D). Plates were incubated overnight and fluorescence of colonies viewed under UV light (Appendix A4) the following morning. Single colonies were cultured in 100 ml 2x YT liquid broth with or without IPTG. The aim of including or excluding IPTG in the broth was to establish if GFP expression (under the *lac* promoter) was inducible or constitutive in a *Salmonella* vaccine vector. To determine GFP expression by recombinant *E. coli* and *Salmonella enterica* serovar Typhimurium mutants, total protein was extracted from each culture (Appendix B2.1) and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Appendix B4), followed by Coomassie blue staining of the gel (Appendix B5). Western blotting (Appendix B6) was performed to identify and confirm the specificity and integrity of the GFP protein band seen on the SDS-PAGE after Coomassie blue staining. A mixture of two anti-GFP mouse monoclonal antibodies (Clones 7.1 and 13.1) (Roche) was used as a primary antibody (diluted at 1.1000). Goat-anti-mouse immunoglobulins conjugated to horseradish peroxidase, diluted at 1.1000 were used as secondary antibody. The blot was developed by enhanced chemiluminescence.

Solubility of GFP expressed in recombinant *Salmonella* vaccine vector was evaluated by SDS-PAGE (Appendix B) analysis of total, soluble or extracellular (media) fractions. The soluble fraction was prepared by the Fast-Prep method of protein isolation (Appendix B2.2). The soluble fraction was analyzed using 12.5 % SDS-PAGE (Appendix B4). To check if there was any secretion of the GFP out of cells, culture medium (extracellular fraction), which was used for the growing of bacteria, was also

loaded onto the SDS-PAGE. Total protein fraction prepared by the SDS-lysis method (Appendix B2.1) was also loaded on the SDS-PAGE. The prediction of solubility of GFP when overexpressed in *E. coli* (or *Salmonella*) was based on Wilkinson-Harrison solubility model (Wilkinson and Harrison, 1991). The protein amino acid sequences were "cut and pasted" into an input window at the web site www.biotech.ou.edu and the solubility probabilities were calculated automatically.

The colony morphology and growth of the recombinant *Salmonella* vaccine vectors were noted. The degree of fluorescence, size and uniformity of colonies on plates with or without selection were also noted as suggestive surrogate markers for stable expression of GFP.

3.5.3 Vaccination of mice with recombinant *Salmonella* vaccine vector expressing GFP

Recombinant *Salmonella enterica* serovar Typhimurium vaccine vectors were prepared as described in Appendix A2.1. The vaccines were designated AroC+GFP (recombinant *Salmonella enterica* serovar Typhimurium AroC mutant expressing GFP) and AroC+pGEM (recombinant *Salmonella enterica* serovar Typhimurium mutant not expressing GFP; used as negative control). To prepare heat-killed AroC+GFP (HK-AroC+GFP), the live bacterial culture (AroC+GFP) was heated in a 65 °C water bath for 20 min. The inclusion of HK-AroC+GFP was to check if the bacteria that could not invade the gut-associated lymphoid tissue would elicit systemic immune responses. Female H-2^d BALB/c mice (n=5) were purchased from South Africa Vaccine Producers Pty Ltd (Johannesburg, South Africa), housed at the University of Cape Town Animal Unit and allowed to adapt for a minimum of 10 days before vaccinations. All the animal procedures were approved by the University of Cape Town Animal Ethics Committee. The vaccination protocols (blood collection and sacrifice schedules) for two separate experiments (Experiments 1 and 2) are given in Tables 3.1 and 3.2 respectively. The sacrifice was performed 28 days after the last inoculation because that is the time when all the bacterial vaccines are expected to be cleared by the mouse immune system. The blood was processed by centrifugation (4 000 rpm for 3 minutes) and sera were stored at -20 °C until assessment of humoral immune responses (Appendix B10). The spleens from each group were pooled and processed as described in Appendix B7.

Table 3.1: Vaccines and vaccination protocol for Experiment 1. Mice were inoculated ONCE by intragastric gavage (Appendix A2.3) with *Salmonella* vaccines. The mice were bled prior to inoculation and sacrifice. The blood was processed by centrifugation and sera were stored until evaluation of humoral immune responses as described in Appendix B10. At sacrifice (on Day 28) the spleens from each group of mice were processed as described in Appendix B7.

Inoculum (vaccine)	Inoculum/animal	Inoculation date	Sacrifice date
AroC+GFP	10^8 cfu	Day 0	Day 28
AroC+pGEM	10^8 cfu	Day 0	Day 28
HK-AroC+GFP	10^8 cfu	Day 0	Day 28

Table 3.2: Vaccines and vaccination protocol for Experiment 2. Mice were inoculated THREE times by intragastric gavage (Appendix A2.3) on Days 0, 28 and 56 with *Salmonella* vaccines (AroC+GFP and AroC+pGEM). The mice were bled prior to inoculation and sacrifice. The blood was processed by centrifugation and sera were stored until evaluation of humoral immune responses as described in Appendix B10. At sacrifice (on Day 84), the pooled spleens from each group of mice were pooled and processed as described in Appendix B7.

Inoculum (vaccine)	Inoculum/animal	Inoculation date	Sacrifice date
AroC+GFP	10^8 cfu	Day 0, Day 28, Day 56	Day 84
AroC+pGEM	10^8 cfu	Day 0, Day 28, Day 56	Day 84
HK-AroC+GFP	10^8 cfu	Day 0, Day 28, Day 56	Day 84

3.5.4 Phenotype of splenocytes

Cell surface markers on splenocytes were determined using Flow Cytometry as described in Appendix B11. The markers determined were CD3, CD4, CD8 and CD19. The CD4/CD8 ratios were calculated for each group of mice.

3.5.5 Assessment of CD8+ Tc1/Tc2 cell responses in the spleen

The frequencies of antigen-specific T cells in the spleen were determined by determined using **IFN-γ** and **IL-4 ELISPOT** assays (Appendix B8). The following peptides/antigens were used as stimulants in the assays:

(1) Media only (No peptide/antigen) (100 µl/well R10 medium) (background control)

(2) Irrelevant peptide (TYSTVASSL), H-2K^d binding peptide (100 µl/well, final concentration of 2 µg/ml) (negative peptide control)

(3) GFP CD8+ T cell peptide HYLSTQSAL, H-2K^d binding peptide (100 µl/well, final concentration of 2.0 µg/ml)

(4) Con A (Sigma, MO, USA) (100 µl/well, final concentration of 0.5 µg/ml) (Assay control). The Con A was used as a positive assay control.

The peptides/antigens were prepared as described in Appendix D.

The plating of stimulants into ELISPOT plates was done in triplicate, followed by addition of 500 000 splenocytes in 100 µl media to each well. The frequencies of IFN-γ and IL-4 spot-forming units were normalized to 1x10^6 splenocytes and means (±SD) were calculated. The background spots in the absence of stimuli were subtracted to give net SFUs/10^6. The cut-off for a positive ELISPOT response was arbitrarily defined as at least three times the no-peptide (medium) SFU number and the number of specific gross SFUs being > 50 /10^6 cells (and non-specific SFUs <50/10^6 cells).

The level of cytokines (**IFN-γ, TNF-α, IL-4** and **IL-5**) produced by splenocytes during stimulation with the GFP peptide was determined by **Cytometric Bead Array** (CBA) (Appendix B9). Splenocytes from

each group of mice were cultured in 96-well, round-bottomed culture plates at a density of 1.5 x 10^6 splenocytes per well in a fixed volume of 200 µl of culture medium which contained specific stimulant. Plates were incubated at 37 °C, 5.0% CO_2, 90% humidity. After 48 hrs (which has been shown to be the time for maximum cytokine secretion), 150 µl of cell free culture supernatant was collected from the wells and kept at −20 °C until assayed for cytokines using the CBA. The principles and concepts behind the CBA assay have been reviewed in Morgan *et al.*, (2004). Stimulation index (SI) for a specific stimulant was calculated from the CBA data as: cytokine level (pg/ml) in peptide-stimulated culture divided by cytokine level (pg/ml) in unstimulated culture. SI>2 indicated the induction of antigen-specific cytokine response.

3.5.6 Assessment of humoral immune responses

The ELISA protocol was used to determine GFP- or LPS-specific serum IgG, IgG1 and IgG2a antibodies as described in Appendix B10. ELISA plates were coated with 5 µg/ml of GFP or LPS. GFP- and LPS-specific IgG was determined for pools of sera for each group of mice at various dilutions (1/100, 1/1000, 1/10 000, 1/100 000). Total GFP-specific IgG was also determined in serum for individual mice vaccinated with AroC+GFP. GFP and LPS-specific IgG1/IgG2a profiles for vaccinated mice were determined at Day 28 (Experiment 1) and Day 84 (Experiment 84). The GFP IgG1/IgG2a profile of 1 mouse vaccinated with AroC+GFP was monitored at Days 28, 56 and 84 (Experiment 2). Antibody responses were defined as positive when specific mean OD_{405nm} >2-fold that of the negative control or when the mean OD_{405nm} ratio to prebleed >2.

3.6 RESULTS

3.6.1 Development of a recombinant expression plasmid vector, pGEM+GFP: molecular cloning of *gfp* gene

To express high levels of GFP antigen in *Salmonella*, a prokaryotic expression cassette was rationally designed. The *E. coli lac* operon sequences in pGEM-Teasy vector (Promega, USA) were exploited in designing the vector. The structural domains of the engineered expression plasmid (pGEM+GFP) developed by this study are illustrated in Figure 3.1A and DNA and amino acid sequences of the GFP as a fusion with LacZα are shown in Figure 3.1B. The *gfp* gene was successfully amplified (Figure 3.2A), ligated into linearized pGEM-Teasy plasmid and transformed into *E coli* SCS110 cells. Clones which

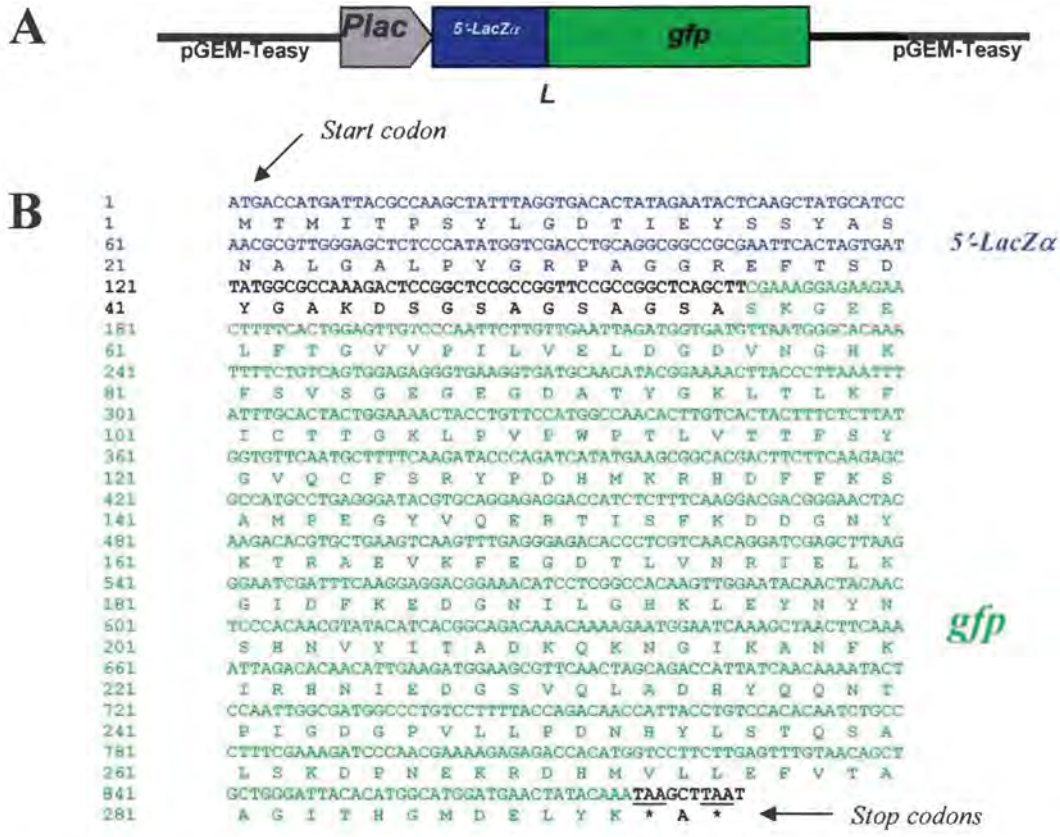

Figure 3.1: The general structural components of the GFP expression construct (pGEM+GFP) developed by the study. (**A**) A schematic representation of the GFP expression plasmid. The GFP was fused with the N-terminal domain (first 40 amino acids) of the *β*-galactosidase *α*-fragment in pGEM-Teasy plasmid. A small linker (L) was included (in-frame) between the two gene fragments. The expression was under the *E. coli lac* promoter and other *lac* operon transcription and translation domains such as the Shine-Dalgarno and transcription start sequences found in pGEM-Teasy plasmid (Appendix G2). (**B**) The DNA and amino acid sequences of the resultant *gfp* gene and GFP protein as a fusion gene/protein with the *β*-galactosidase *α*-fragment. The whole fusion protein was 32 KDa. Two preferred stop codons (TAAG and TAAT) were included at the end of the *gfp* gene to prevent translation of the whole *LacZα* gene. The main features and complete DNA sequence of pGEM+GFP plasmid are given in Appendices G4 and H1 respectively.

were white and fluorescing under UV were taken for *gfp* screening as they had the gene in the correct orientation and potentially in-frame with *LacZα*. One clone with the gene in-frame with the *LacZα* was chosen (Figure 3.2B) and recombinant plasmid was hereby designated pGEM+GFP (Appendices G4 and H1) and the clone was named SCS110+GFP. Another clone (blue) with recircularized pGEM-Teasy plasmid was chosen for future use as negative control and was designated SCS110+pGEM, the plasmid was named pGEM (Appendix G3).

The cloned *gfp* gene was sequenced and found not to have acquired point mutations during PCR amplification. Analysis of sequence data indicated that the cloned *gfp* gene was successfully fused to the *β*-galactosidase *α*-fragment in-frame (Figure 3.1B). The *β*-galactosidase *α*-fragment had 120 codons, 40 amino acids and was 4.239 kDa in size. A small linker (spacer) sequence with 45 codons was successfully cloned between the *β*-galactosidase *α*-fragment and *gfp*. The small linker peptide had only 15 amino acids (YGAKDSGSAG SAGSA) and a molecular weight of 1.266 kDa. The intervening sequence (linker) was designed to include a restriction site for *Nar*I and this site was critically important for subsequent sub-cloning of other genes downstream the *LacZα* and in-frame. The *gfp* gene had 711 codons, 237 amino acids and a molecular weight of 26.617 kDa. The whole *β*-galactosidase-GFP fusion protein, which was generated by the study, was 32.122 kDa. A preferred translation stop codon (TAAG) that was incorporated into the PCR primer, GR, was found at the end of the *gfp* gene. An extra stop codon, TAAT, was successfully incorporated one codon downstream the first one.

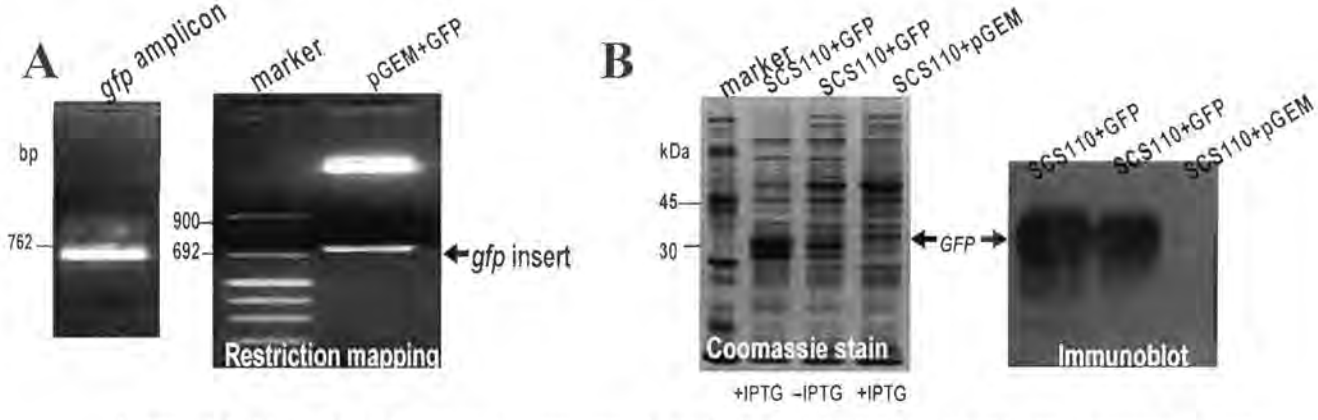

Figure 3.2: Molecular cloning and expression of the GFP in *E. coli*. **(A)** The *gfp* gene was amplified by polymerase chain reaction and ligated into a linearized pGEM-T Easy vector. The ligation reaction was used to transform competent SCS110 *E. coli* cells and recombinant clones were screened. One of the candidate clones was digested with *Nar*I and *Hind*III restriction endonuclease enzymes to screen for the presence of the *gfp* insert. **(B)** SDS-PAGE and Western blotting analysis of recombinant *E. coli* SCS110 cells expressing GFP. Total lysates of *E. coli* harbouring pGEM+GFP plasmid were analyzed by SDS-PAGE and stained by Coomassie brilliant blue. SCS110 cells expressed higher levels of GFP when IPTG was added into the media than when IPTG was not added into the media. SCS110 cells not expressing GFP (negative control, with pGEM plasmid) were used as a negative control for GFP expression. Western blot (Immunoblot) analysis further showed that the bacteria expressed the GFP antigen at very high levels. Total bacterial lysates were transferred to nitrocellulose membranes and probed with GFP antibody.

The expression of GFP protein in *E. coli* was evaluated by growing the recombinant bacteria with or without IPTG. It was noted the recombinant SSC110 cells fluoresced under UV when IPTG was added in the growing media. The fluorescence of the bacteria was reduced when IPTG was not added into the media. SDS-PAGE analysis of bacteria grown overnight with or without IPTG showed that very high levels of GFP were expressed (Figure 3.2B). The presence of two GFP fragments (a large and a small) on the SDS-PAGE and Western blot suggested that there was either aborted translation at a specific mRNA motif or post-translational proteolytic processing or degradation of the protein at specific site.

3.6.2 Recombinant *Salmonella* expressing GFP antigen

To develop a recombinant vaccine vector expressing GFP, the prokaryotic expression plasmid, pGEM+GFP, was used to transform an aromatic amino acid dependent (*AroC*) mutant, *Salmonella enterica* serovar Typhimurium vaccine mutant (Appendix E). The *Salmonella* clone harbouring the plasmid was designated AroC+GFP. As a negative control, the *Salmonella enterica* serovar Typhimurium mutant was also transformed with pGEM plasmid to generate AroC+pGEM, which was to be used as a negative control. Investigation of GFP expression was initially determined by viewing the bacterial liquid cultures and colonies over UV light. It was observed that the AroC+GFP colonies were highly fluorescent under UV light (Figure 3.3A). The AroC+GFP liquid cultures were also fluorescent under UV and slightly under normal light and this suggested that very high levels of the GFP were expressed by the recombinant bacteria (Figure 3.3B).

To further investigate whether the *lac* promoter was constitutive or inducible in recombinant *Salmonella enterica* serovar Typhimurium, total bacterial proteins from cultures of AroC+GFP and AroC+pGEM grown with or without IPTG were analyzed by SDS-PAGE and Western Blotting. High levels of expression of the GFP antigen by the recombinant *Salmonella* bacteria were demonstrated (Figure 3.3C). Two GFP fragments (a large and a small) were also observed on the SDS-PAGE and Western blot. The study therefore showed that *E. coli lac* promoter could constitutively drive GFP expression in *Salmonella* without the need for IPTG induction as in *E coli*.

To investigate GFP solubility in the *Salmonella* vector, the soluble and total fractions were prepared and analyzed by SDS-PAGE. It was demonstrated that the GFP protein was soluble when expressed in the bacteria (Figure 3.3D). A GFP band was visible on SDS-PAGE after Coomassie blue staining in both the total and soluble fractions. The GFP was the most abundant bacterial protein. There was no evidence to show that the GFP was secreted into the media, as no 32 KDa protein band was visible after Coomassie blue staining of the SDS-PAGE. Theoretical prediction of the solubility of GFP when overexpressed in the bacterial cells (*E. coli* or *Salmonella*) was done using Wilkinson and Harrison (1991). The model showed that GFP fused to the LacZα peptide was 66% insoluble (44% soluble) when expressed in the two bacteria.

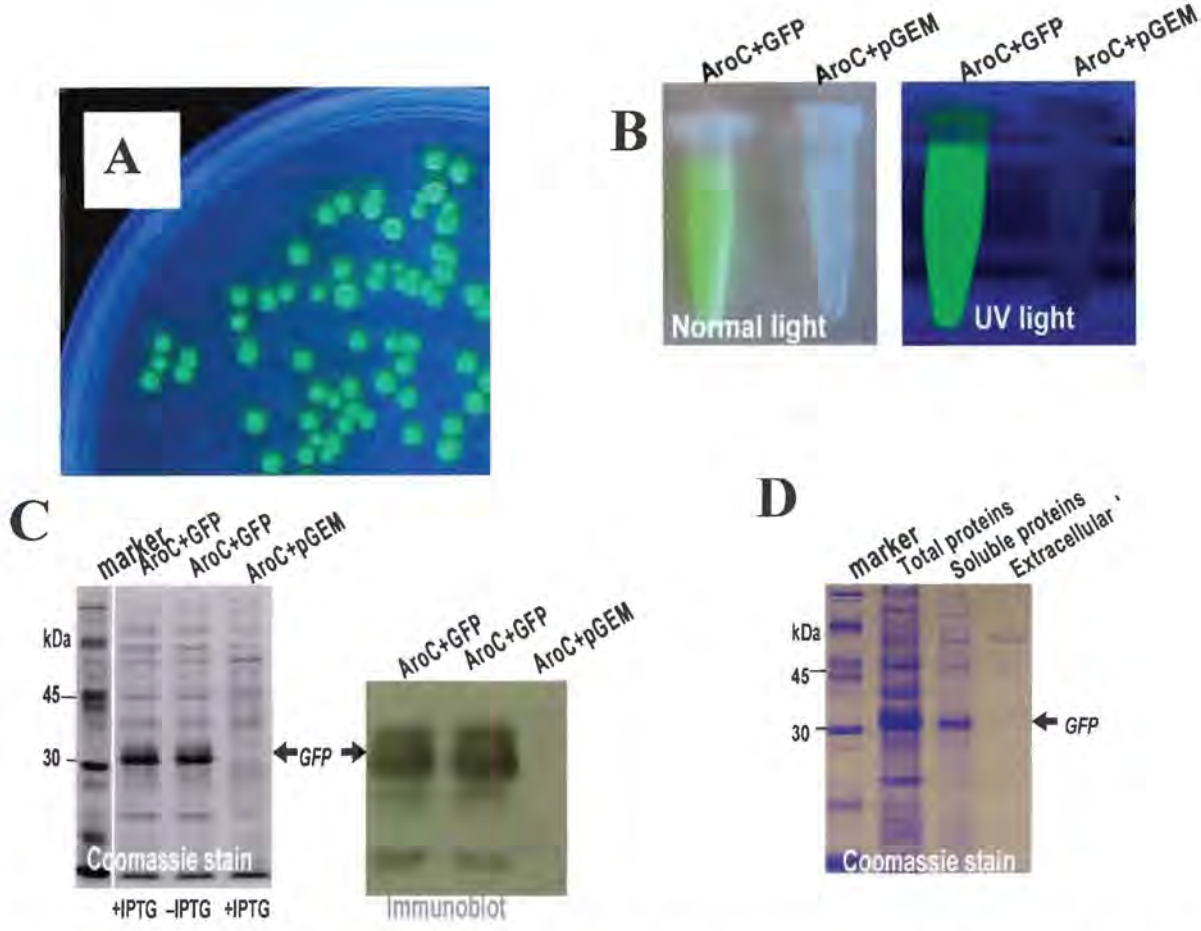

Figure 3.3: Expression of GFP antigen by recombinant *Salmonella enterica* serovar Typhimurium vaccine vector. (**A**) The recombinant *Salmonella* (AroC+GFP) bacterial colonies were fluorescent under UV light. (**B**) The bacterial liquid cultures expressing GFP (AroC+GFP) were also fluorescent under UV light. The bacterial cultures were also slightly fluorescent even under normal light. The bacteria (AroC+pGEM) were not fluorescent under normal or UV light since they were not expressing GFP. (**C**) SDS-PAGE and Western blotting analysis of recombinant *Salmonella enterica* serovar Typhimurium expressing GFP. GFP bands were visible on the Coomassie blue stained SDS-PAGE. The GFP antigen was the most abundantly expressed protein by the bacteria with or without IPTG induction. The expression of GFP was confirmed by immunoblotting. (**D**) SDS-PAGE analysis of the solubility of GFP when expressed in recombinant *Salmonella enterica* serovar Typhimurium vector. The GFP protein band was visible in the lanes loaded with soluble fraction and total proteins. The GFP antigen was not exported out of the bacteria.

General analyses of bacterial colonies from AroC+GFP and AroC+pGEM showed that they had a healthy morphology. The colonies were uniform and almost of the same size (Figure 3.3A). The colonies could also continue to fluoresce and maintain the plasmid for some time when grown without selection.

3.6.3 Immunogenicity of a recombinant *Salmonella* vaccine vector expressing GFP

3.6.3.1 Lymphocyte phenotype after vaccination

The effect of either a single or three inoculations with the *Salmonella* vaccines on the composition of the lymphocyte population in the spleen of vaccinated mice was investigated. The proportion of lymphocytes in the lymphocyte gate from vaccinated mice was almost the same as that for naïve mice (Table 3.3).

Table 3.3: The proportion (as % of events in the lymphocyte gate) of B and T cells in the spleens of naïve mice and mice given recombinant *Salmonella* vaccines (see Tables 3.1 and 3.2). The lymphocyte phenotype was determined as described in Appendix B11.

Vaccine group	CD3/CD19 Negative	CD3+	CD4+	CD8+	CD4+/CD8+ ratio	CD19+
Day 28 (Expt. 1)						
Aroc+GFP	14.4	43.0	31.0	10.9	2.85	42.6
AroC+pGEM	12.2	46.1	31.4	11.8	2.66	41.7
HK-AroC+GFP	12.3	46.1	32.1	12.3	2.61	41.6
Day 84 (Expt. 2)						
AroC+GFP	15.0	39.4	28.7	9.7	2.98	45.6
AroC+pGEM	10.8	50.0	35.8	12.9	2.78	39.2
HK-AroC+GFP	9.3	49.5	35.1	12.6	2.79	41.2
Naïve mice	15.9	41.4	28.3	12.3	2.30	42.7

3.6.3.2 GFP-specific CD8+ Tc1/Tc2 cytokine responses to recombinant *Salmonella* expressing GFP

Specific CD8+ T cell responses induced in mice after oral vaccination of mice with the *Salmonella* vaccine vectors were evaluated. The responses were measured on Day 28 (Experiment 1) and Day 84 (Experiments 2). IFN-γ and IL-4 ELISPOT assays were used to evaluate the frequencies of *Salmonella* vaccine-induced systemic GFP-specific CD8+ T cells secreting IFN-γ and IL-4 respectively. No GFP-specific IFN-γ responses in the spleen were detected after a single inoculation in mice with any of the *Salmonella* vaccines (Figure 3.4A). However, on Day 84, GFP-specific IFN-γ responses were detected in mice vaccinated with AroC+GFP (Figure 3.4A). The responses were more than three times greater than the background (medium only in the absence of peptide). No GFP-specific IFN-γ responses were elicited by the AroC+pGEM (negative control) and heat-killed AroC+GFP on Day 28 or Day 84 (Figure 3.4A).

Vaccine-induced GFP-specific IL-4 responses were also investigated. On Day 84, 132 net IL-4 SFUs/10^6 splenocytes for the vaccine were measured (Figure 3.4B). The number of IL-4 SFUs for vaccines, AroC+pGEM and heat-killed AroC+GFP on Day 84 (Figure 3.4B) were below 10, indicating that no non-specific GFP IL-4 response were induced. In a nutshell, this study showed that more GFP-specific IFN-γ SFUs were produced than GFP-specific IL-4 SFUs on Day 84 in mice vaccinated with live AroC+GFP. The IFN-γ SFUs to IL-4 SFUs ratio was 1.71.

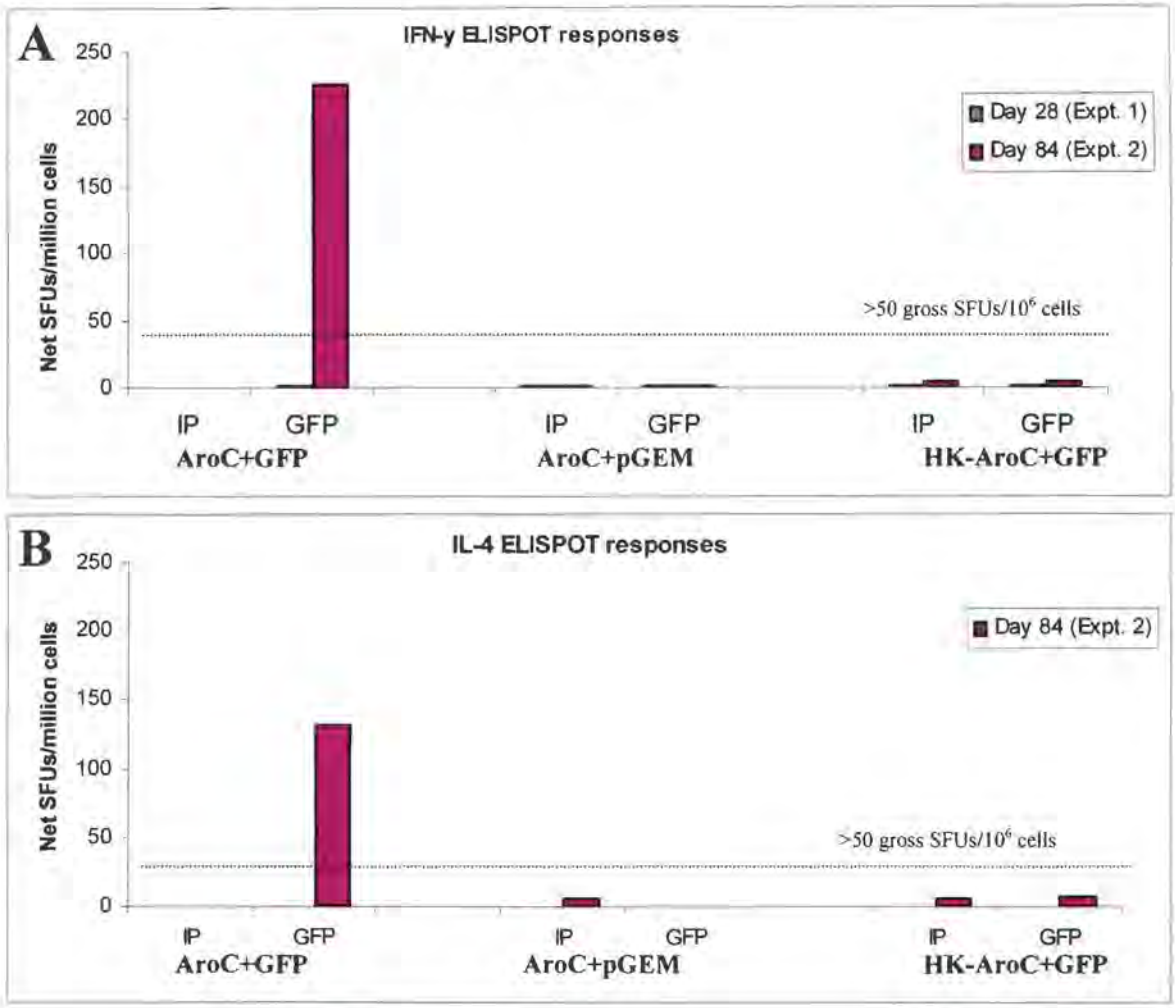

Figure 3.4: The magnitude of **GFP-specific CD8+ T cell responses** as measured by IFN-γ (**A**) and IL-4 (**B**) ELISPOT assays. Groups of mice were vaccinated ONCE (Experiment 1, Table 3.1) or THREE times (Experiment 2, Table 3.2) with live *Salmonella* vaccine expressing GFP, AroC+GFP; a negative *Salmonella* control vaccine, AroC+pGEM or a heat-killed *Salmonella* vaccine expressing GFP, HK-AroC+GFP. On Days 28 (Experiment 1) and 84 (Experiment 2) splenocytes from the vaccinated mice were incubated with medium only (negative assay control), or stimulated with irrelevant peptide (IP) TYSTVASSL or the GFP CD8+ T cell peptide HYLSTQSAL in IFN-γ and IL-4 ELISPOT assays. IFN-γ ELISPOT assay was performed on Day 28 (Experiment 1) and Day 84 (Experiment 2) (**A**), while IL-4 ELISPOT assay was performed only on Day 84 (Experiment 2) (**B**). Each bar in the graphs represents the net SFUs/10^6 cells. A positive response was arbitrarily defined as at least three times the no-peptide (medium) SFU number and the number of specific SFUs being > 50 /10^6 cells.

Splenocytes from vaccinated mice were also stimulated with Con A, to check if the cells were viable and could secrete IFN-γ and IL-4 cytokines. The cells produced >50 IFN-γ or IL-4 SFUs after Con A stimulation (Figure 3.5) and this showed that the cells were viable and the assays were working. It was however observed that (CD8+ Tc1) IFN-γ SFUs > (CD8+ Tc2) IL-4 SFUs.

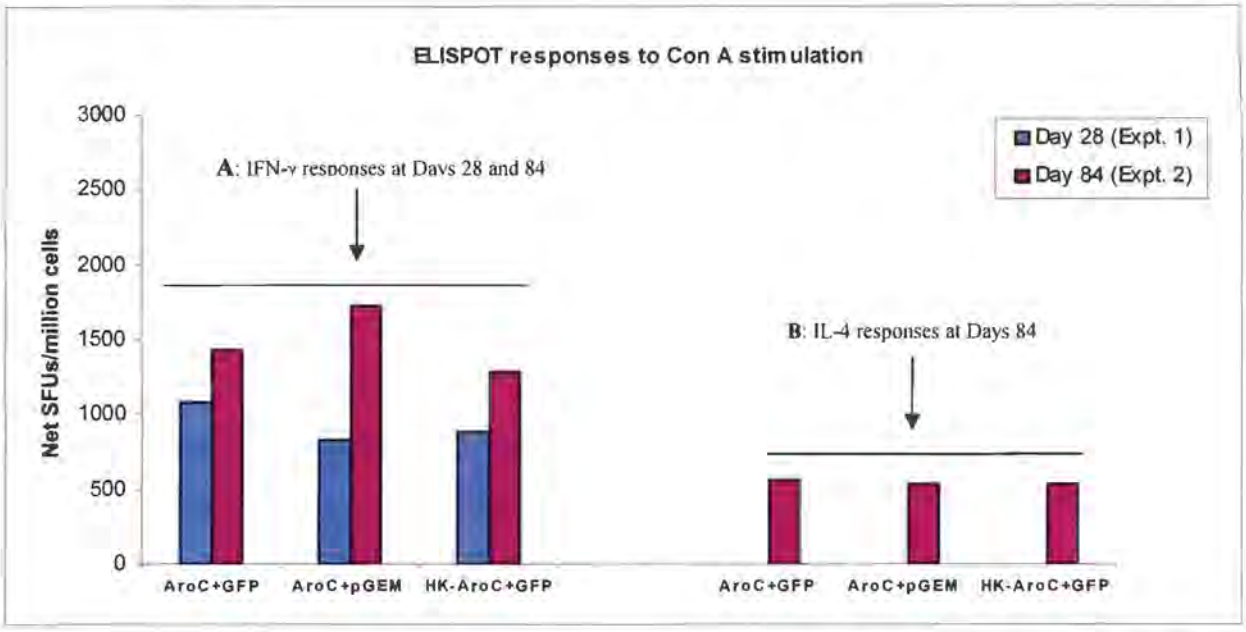

Figure 3.5: Con A-mediated responses of splenocytes as determined by IFN-γ (**A**) and IL-4 (**B**) ELISPOT assays. Groups of mice were vaccinated ONCE (Experiment 1, Table 3.1) or THREE times (Experiment 2, Table 3.2) with AroC+GFP; AroC+pGEM or HK-AroC+GFP. On Days 28 (Experiment 1) and 84 (Experiment 2) splenocytes from the vaccinated mice were incubated with Con A in IFN-γ and IL-4 ELISPOT assays. IFN-γ ELISPOT assay was performed on Day 28 (Experiment 1) and Day 84 (Experiment 2) (**A**), while IL-4 ELISPOT assay was performed only on Day 84 (Experiment 2) (**B**). Each bar in the graphs represents the net SFUs/10^6 cells. A positive response was arbitrarily defined as at least three times the no-peptide (medium) SFU number, and the number of specific SFUs being > 50 /10^6 cells.

The CBA assay was used to quantify the cytokines, IFN-γ, TNF-α, IL-4 and IL-5, produced by splenocytes after stimulation with the GFP H-2K^d binding peptide (HYLSTQSAL). On day 28, the levels of the CD8+ Tc1 (IFN-γ and TNF-α) and Tc2 (IL-4 and IL-5) cytokines were not elevated above the background (Figure 3.6). Analysis of cytokine responses on Day 84 showed that the amount of IFN-γ, TNF-α and IL-4 (but not IL-5) produced by the splenocytes from AroC+GFP-vaccinated mice were elevated above the background (Figure 3.6). The GPF peptide SI for AroC+GFP vaccine were higher than those from the negative control vaccine (AroC+pGEM) for these three cytokines. The heat-killed AroC+GFP vaccine induced no GFP-specific cytokine responses.

The CBA assay, therefore, further confirmed that more GFP-specific CD8+ Tc1 cytokine response than GFP-specific CD8+ Tc2 cytokine response was induced in AroC+GFP-vaccinated mice (Day 84). The pg/ml ratio of IFN-γ and TNF-α to IL-4 and IL-5 at Day 84 was 3.2.

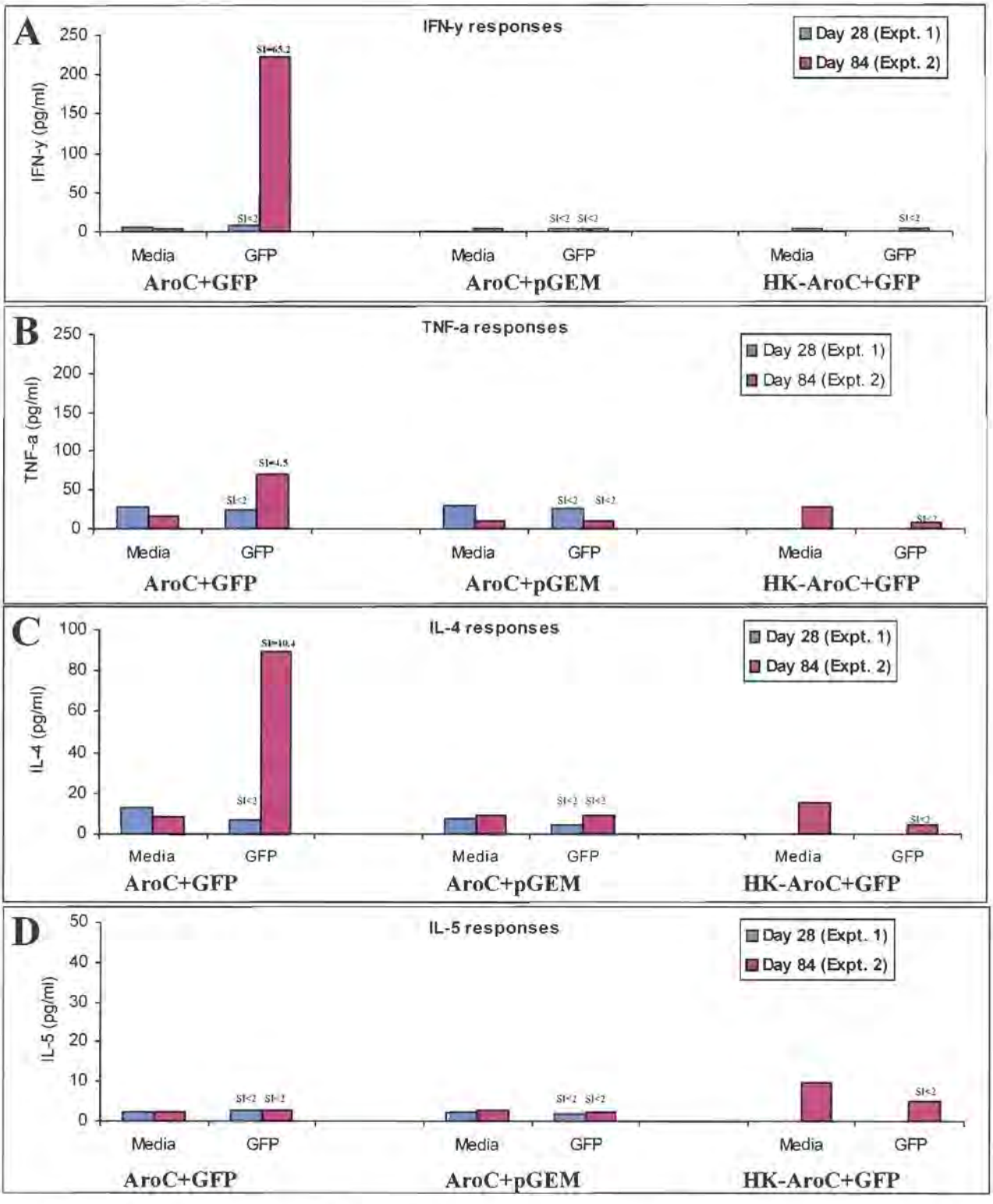

Figure 3.6: The magnitude of **GFP-specific CD8+ T cell responses** as determined by quantification of IFN-γ (**A**), TNF-α (**B**), IL-4 (**C**) and IL-5 (**D**) cytokines (in pg/ml) secreted by splenocytes during stimulation for 48 hours in the absence or presence of GFP CD8+ T cell peptide, HYLSTQSAL, using CBA assay. Groups of mice were vaccinated ONCE (Experiment 1, Table 3.1) or THREE times (Experiment 2, Table 3.2) with live *Salmonella* vaccine expressing GFP, AroC+GFP, a negative *Salmonella* control vaccine, AroC+pGEM or a heat-killed *Salmonella* vaccine expressing GFP, HK-AroC+GFP. On Days 28 (Experiment 1) and 84 (Experiment 2), splenocytes from the vaccinated mice were incubated with medium only (negative assay control), or stimulated with the GFP peptide HYLSTQSAL and the amounts of IFN-γ (**A**), TNF-α (**B**), IL-4 (**C**) and IL-5 (**D**) measured by CBA assay. (The cytokine levels were not determined on Day 28 for the vaccine, HK-AroC+GFP). Each bar in the graphs represents pg of cytokine produced per ml in 48 hrs. Responses are positive if the SI is greater than 2 and is indicated.

3.6.3.3 GFP-specific humoral immune responses to recombinant *Salmonella* vaccine

To evaluate GFP antigen-specific humoral immune responses in mice after vaccination with recombinant *Salmonella* vaccine vectors, the ELISA protocol was used. The mice elicited GFP-specific IgG antibodies after both single and booster vaccinations with AroC+GFP (Figure 3.7A). On Day 28, GFP-specific antibody response was 3.38-fold above the prebleed background for the vaccine AroC+GFP. After booster vaccinations (three inoculations) with the vaccine, the GFP IgG response increased several

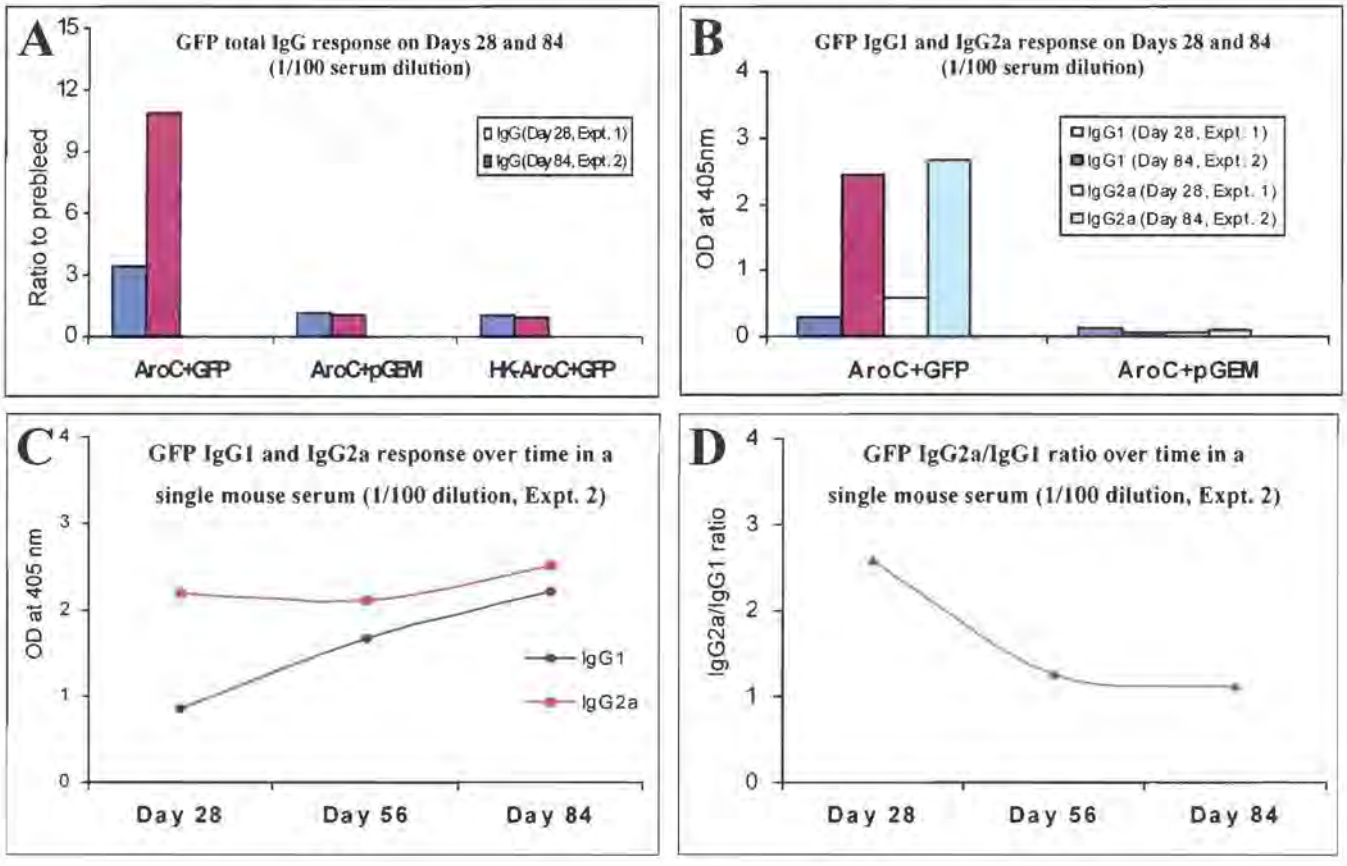

Figure 3.7: Serum GFP-specific IgG responses in mice vaccinated with *Salmonella* expressing a GFP antigen. Groups of mice were vaccinated ONCE (Table 3.1) or THREE times (Table 3.2) with *Salmonella* vaccines, AroC+GFP, heat-killed AroC+GFP, or AroC+pGEM. Prior to inoculation, the mice were bled and pools of serum made. On Days 28 (Experiment 1) and 84 (Experiment 2) the mice were bled and serum made. (**A**) The GFP-specific IgG was measured serum in pools of each group of mice for Day 28 or Day 84 in 1/100 serum dilution. The data are represented as the ratios of the mean OD_{405nm} of duplicates to the mean OD_{405nm} prebleed duplicates. (**B**) The GFP-specific IgG1 and IgG2a were measured in serum pools of each group of mice for Day 28 or Day 84 in 1/100 serum dilution. Each bar represents the mean OD_{405nm} value of duplicates. (**C**) GFP-specific IgG1 and IgG2a was measured for one individual mouse vaccinated with the recombinant *Salmonella* expressing GFP (AroC+GFP) at Day 28, Day 56 and Day 84. Each data point represents the mean OD_{405nm} value of duplicates. (**D**) The IgG2a/IgG1 ratios for the single mouse vaccinated with the recombinant *Salmonella* expressing GFP (AroC+GFP) at Day 28, Day 56 and Day 84 were plotted. Antibody responses were defined as positive when the OD_{405nm} >2-fold that of the negative control (AroC+pGEM) or when the mean OD_{405nm} ratio to prebleed >2.

fold above the prebleed. It was also noted that GFP-specific IgG response could be detected in 1/10 000 dilution of serum pools from mice vaccinated three times, but no response could be detected from the same dilution for the mice vaccinated once (results not shown). The OD_{405} ratio to prebleed of mice vaccinated with AroC+pGEM or HK-AroC+GFP was less than 2 and this indicated that no GFP antibody responses was elicited (Figure 3.7A).

To further understand the nature of humoral immune responses, GFP antigen-specific IgG1 and IgG2a isotypes in the sera of vaccinated mice were also determined. After a single inoculation with AroC+GFP, both IgG1 (Th2) and IgG2a (Th1) responses were induced (Figure 3.7B). The IgG2a responses were greater than IgG1 response. There were variations in the GFP IgG subclass responses among the individual mice. Three mice elicited both IgG1 and IgG2a (IgG2a>IgG1), while the other two mice did not elicit detectable GFP-specific serum IgG1 and IgG2a (results not shown).

After three inoculations (Day 84), the levels of both GFP-specific IgG1 (Th2) and IgG2a (Th1) in the serum pool were boosted in all the mice vaccinated with AroC+GFP (Figure 3.7B). The levels of IgG2a were greater than that of IgG1, with the difference between the two isotypes narrower than after single inoculation of mice with AroC+GFP.

To have a better understanding of changes in GFP-specific IgG isotypes over time, the levels of IgG1 and IgG2a antibodies were followed for a single mouse after AroC+GFP inoculation. It was noted that both IgG antibody isotypes increased over time with IgG2a dominating over IgG1 (Figure 3.7C). There was a degree of convergence of the two isotypes as the number of inoculations increased. The IgG2a to IgG1 ratio decreased over time (Figure 3.7D).

3.6.3.4 LPS-specific humoral immune responses to recombinant *Salmonella*

To determine the success of mucosal invasion and possible systemic response of vaccinated mice to *Salmonella*, *Salmonella*-specific LPS IgG immune responses were investigated. After a single inoculation, all the vaccines (live or heat-killed recombinant *Salmonella*) produced LPS-specific IgG antibody responses (Figure 3.8A). The LPS IgG antibody response from AroC+pGEM was slightly higher than from AroC+GFP vaccine and HK-AroC+GFP. Three inoculations with the three *Salmonella* vaccines slightly boosted LPS IgG response (Figure 3.8A). Heat-Killed (HK)-AroC+GFP vaccine also induced LPS antibody response on Day 84, but this was lower than the response induced by live *Salmonella* vaccines. Further analysis of LPS IgG subclass response in mice vaccinated with AroC+GFP and AroC+pGEM showed the dominance of IgG2a to IgG1 antibodies (Figure 3.8B). *Salmonella* LPS-specific IgG2a (Th1) was greater than *Salmonella* LPS-specific IgG1 (Th2) after single inoculation and the gap between the two isotypes increased after three inoculations.

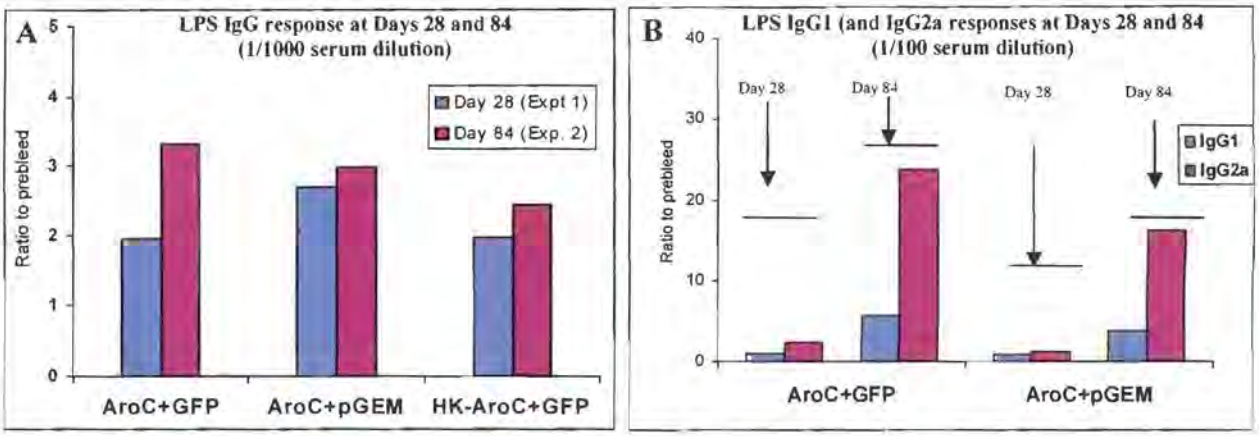

Figure 3.8: Serum LPS-specific IgG responses in mice vaccinated with *Salmonella* expressing GFP antigen. Groups of mice were vaccinated ONCE (Table 3.1) or THREE times (Table 3.2) with *Salmonella* vaccines, AroC+GFP, heat-killed AroC+GFP or AroC+pGEM. Prior to inoculation, the mice were bled and pools of serum made. On Days 28 (Experiment 1) and 84 (Experiment 2) the mice were bled and serum made. (**A**) The LPS-specific IgG was measured in serum pools of each group of mice for Day 28 or Day 84 in 1/1000 serum dilution. The data are represented as the ratios of the mean OD_{405nm} of duplicates to the mean OD_{405nm} prebleed duplicates. (**B**) The LPS-specific IgG1 and IgG2a were measured in serum pools of each group of mice for Day 28 or Day 84 in 1/100 serum dilution. The data are represented as the ratios of the mean OD_{405nm} of duplicates to the mean OD_{405nm} prebleed duplicates. Antibody responses were defined as positive when the mean OD_{405nm} ratio to prebleed >2.

3.7 DISCUSSION

The key challenge with *Salmonella* bacterial vaccine delivery systems is to optimize the expression of high levels of the foreign antigens for presentation to the immune system. In the current study, a new strategy based on *E. coli lac* operon control sequences was designed and tested for expression of a model foreign antigen, *Aequorea victoria* green fluorescent protein, in *AroC Salmonella enterica* serovar Typhimurium mutant. Oral vaccination of mice with the bacterial vector elicited GFP-specific systemic CD8+ (Tc1 and Tc1) cytokine and humoral (IgG1/IgG2a) immune responses. This is the first reported study in which the expression of GFP fused to LacZα peptide and driven by the *lac* promoter, in orally delivered recombinant attenuated *Salmonella enterica* serovar Typhimurium elicited both cellular and humoral immune responses.

3.7.1 Development of a recombinant *Salmonella* expression cassette

The success of the *lac*-GFP fusion strategy was attributed immensely to the rational design of the prokaryotic expression plasmid, pGEM+GFP (Figure 3.1). Previous work in which unfused GFP was expressed under a strong prokaryotic constitutive promoter (*mtr*) failed to give high-level accumulation of the antigen in bacteria (Chin'ombe, unpublished results). One of the potential reasons for the failure to overexpress GFP under such a strong promoter could be the formation of unfolded GFP inclusion bodies, post-transcriptional mRNA instability or post-translational GFP proteolytic degradation by bacterial proteases. It was later hypothesized that using a weaker *E. coli lac* promoter to express the GFP, but fused in-frame to the N-terminal domain of the *E. coli* β-galactosidase α-fragment could result in better expression of the antigen in both *E. coli* and *Salmonella*. The inclusion of the N-terminal domain of the β-galactosidase α-gene fragment, which itself is an *E. coli* bacterial peptide might have contributed immensely to the high expression of GFP observed in both *E. coli* and *Salmonella enterica* serovar Typhimurium vector (Figures 3.2 and 3.3). Fusing foreign proteins to other prokaryotic peptides has the potential to enhance expression of the cloned genes (Jacquet *et al.*, 1999). In such situations, translational efficiency improves and problems associated with translational initiation are resolved by construction of such fusion genes. Furthermore, fusion proteins have been shown to be, in most cases, resistant to proteolytic degradation, thereby overcoming the problems of instability normally associated with foreign proteins (Itakura *et al.*, 1977; Martinez *et al.*, 1995; Jacquet *et al.*, 1999). The fusion of *gfp* to the 5'-domain of LacZα also potentially stabilized GFP mRNA of the antigen gene and increased its half-life. In a similar study, it was shown that fusing genes to the 5' UTR (untranslated region) of *ompA* was effective in stabilizing the mRNA transcripts (Hansen, 1994).

There were other considerations borne in mind in rationally designing pGEM+GFP for high-level expression of the GFP antigen in *Salmonella* vaccine vector. They included the ribosome-binding site, the nature of origin of replication (ori), promoter (*lac*) properties, and translation termination sequences. Most of these transcriptional and translational domains are present in the pGEM-Teasy vector (Promega, U.S.A). Although pGEM-T easy plasmid was originally designed as a cloning vector, this study designed a novel strategy of using it as both a cloning and protein expression vector for the two Gram-negative

bacteria, *E. coli* and *Salmonella.* Cloning of PCR products in T/A vectors such as pGEM-Teasy plasmid exploits the characteristic of some of the DNA polymerases such as *Taq* DNA polymerase which preferentially add a single 3'-A overhang to blunt-ended, double-stranded DNA via terminal transferase-like activity (Hu, 1993). The current study rationally designed the primers so that the amplified *gfp* gene (with the 3'-A overhangs introduced by the *Taq* DNA polymerase) could be cloned in-frame with the 5'-domain of LacZα in pGEM-T easy plasmid. The study also capitalized on the presence of these various transcriptional and translational structures in pGEM-Teasy vector, which was designed as a cloning vector, not an expression vector. The origin of replication in pGEM-Teasy vector allowed for high copy number of the plasmid (300-400 copies per cell) in *Salmonella* vector, thereby increasing the *gfp* gene dosage and high expression of the antigen. Initiation of translation of the cloned antigen gene involves a specific ribosome-binding site (RBS) just upstream from the translation start codon. It has also been shown that the efficiency of translation initiation depends on the distance between the Shine-Dalgano (SD) sequence and the initiation codon (Jay *et al.,* 1982; Chen *et al.,* 1994). In the current study, the natural SD sequence for the *LacZα* gene in the pGEM-T easy plasmid was used for efficient ribosome binding.

The *lac* promoter was constitutive in *Salmonella* because of the absence of the *lac* repressor as in *E. coli* (Figures 3.2 and 3.3). Although the weak *lac* promoter was used, the high plasmid copy number was expected to increase the gene dosage for high expression. The choice of stop codons used also affects the efficiency of translation termination. Analysis of how frequently the three alternative translation termination codons (TAA, TAG and TGA) are used in *E. coli* has shown a very strong bias towards the use of stop codon TAA in genes that show high levels of expression (Poole *et al.,* 1995). In this study, the stop codon, TAA(G) was used in the pGEM+GFP plasmid to increase efficiency of translation termination. To prevent read-through by ribosomes during gene translation, an extra stop codon, TAA(T) was added one codon downstream of the first stop codon (Figure 3.1). With all these considerations, overexpression of GFP in the recombinant *Salmonella* vaccine vector was achieved (Figure 3.3). This was expected to facilitate the delivery of sufficient GFP antigen to the immune system by the *Salmonella* vector after vaccination. No adverse effects of GFP overexpression on *Salmonella* vaccine vector were observed and this indicated that the metabolic burden was minimal. The continued fluorescence of the GFP showed that the upstream LacZα leader sequence (40 amino acids in length) did not affect its folding.

In the study, it was noted that the GFP expressed in recombinant *Salmonella enterica* serovar Typhimurium was soluble (Figure 3.3). However, another proportion of the GFP was expressed as insoluble inclusion bodies. This was predicted by the Wilkinson and Harrison model (Wilkinson and Harrison, 1991). To improve the solubility of the GFP, two approaches can also be employed. One is to rationally add some amino acid residues at the N- or C-terminal ends of the GFP, which improve its solubility. The second approach would be to fuse and co-express GFP with soluble antigens such as NusA (95% soluble), GrpE (93% soluble), and BFR (95% soluble) proteins (Davis *et al.,* 1999). NusA

has already been evaluated in its ability to improve solubility of human interleukin 3, bovine growth hormone and tyrosine (Davis *et al.*, 1999; Davis and Harrison, 1998). It was anticipated that the solubility of GFP in recombinant *Salmonella* vector would influence its antigen processing and presentation to the immune system.

3.7.2 GFP-specific CD8+ Tc1 and Tc2 cytokine responses

For pathogens that infect their hosts through the oral route, preventative vaccines should induce both mucosal and systemic CD8+ T cells. One of the key advantages of oral delivery by *Salmonella* is that both mucosal and systemic immune responses can be induced, unlike systemic vaccination, which does not induce a mucosal response (Medina and Guzman, 2001; Ogra *et al.*, 2001). In the current study, systemic T cell responses were investigated in mice after oral delivery of recombinant *Salmonella* vaccine vector expressing the GFP model antigen. The vaccination of the mice with the *Salmonella* vaccines did not result in profound changes in the splenocyte population of cells (Table 3.4). The rapid clearance (about 14 days after infection) of the attenuated *Salmonella* bacteria potentially causes the population of splenocytes to revert to normal proportions by the time the mice are sacrificed (day 28 after the last vaccination).

Antigenic exposure of naïve CD8+ T cells results in their differentiation into distinct subsets secreting different cytokine profiles (Li *et al.*, 1997; Sad *et al.*, 1997; Cerwenka *et al.*, 1998). These subsets are mainly the CD8+ T cytotoxic-1 (Tc1) cells that secrete IL-2, TNF-α and IFN-γ; and the CD8+ T cytotoxic-2 (Tc2) cells that secrete IL-4, IL-5, IL-6 and IL-10. Both CD8+ Tc1 and Tc2 cell populations have been suggested to be cytolytic (Cerwenka *et al.*, 1998). In the present study, the existence of GFP specific CD8+ T cells secreting both Tc1 and Tc2 cytokine profiles in mice orally vaccinated with *Salmonella* vaccine vector expressing GFP was demonstrated by IFN-γ and IL-4 ELISPOT assays (Figure 3.4). The bias was however towards the Tc1 response. The demonstration of the simultaneous production of high levels of GFP peptide-specific IFN-γ, TNF-α and IL-4 cytokines after 48 hours of *ex vivo* stimulation of the splenocytes from vaccinated mice (Figure 3.5) further supported that both CD8+ Tc1 and Tc2 cell immune responses were induced, but here again, the response was predominantly a Tc1 response. The failure of heat-killed AroC+GFP to induce these GFP-specific cytokine responses suggested that mucosal invasion and possibly systemic spread of the recombinant *Salmonella* vector was necessary to provoke GFP-specific response.

Antigens expressed by *Salmonella* are expected to be presented mainly by the MHC-II molecules to give predominantly antigen-specific CD4+ T cell responses. The failure by *Salmonella* to have access to the cytosolic MHC-I presentation pathway poses the key question as to how CD8+ T cells encounter antigens. *Salmonella* have a high tropism for dendritic cells (Santos and Baumler, 2004) and these cells are now known to have the capacity of cross-priming exogenous antigens (Albert *et al.*, 1998; Brode and Macary, 2004; Heath *et al.*, 2004). Dendritic cells can also engulf the *Salmonella*-infected apoptotic cells which may be an important source of antigens that can be processed for induction of CD8+ T cell

responses (Wijburg *et al.,* 2002; Sundquist *et al.,* 2004). However, it seems that the high-level expression of the GFP antigen shown in this study facilitated antigen processing and cross-presentation for induction of CD8+ T cell responses. The high amounts of the antigen also potentially improved the immunodominance of GFP CD8+ epitopes over *Salmonella* vector epitopes (Rollenhagen *et al.,* 2004). It has been demonstrated that antigen abundance (antigen dose) is one of the key factors that determine CD8+ T cell immunodominance (La Gruta *et al.,* 2006). However, besides the abundance of the antigen or peptide, there are other factors that may affect immunodominance. In the current study, it was difficult to establish whether the strong CD8+ T cell responses demonstrated were CD4+ T-cell dependent or independent. However, the high levels of both GFP-specific IgG1 and IgG2a (Section 3.7.4) suggested that both GFP-specific CD4+ Th1 and Th2 responses were elicited in vaccinated mice.

CD8+ T cells control many viral infections and should be targets for vaccine-induced antiviral immunity (Yewdell and Haeryfar, 2005). The polarized pattern of secreted cytokines by CD8+ T cells observed in the current study might have a great relevance to immune responses against many viral pathogens including HIV-1, and might further determine whether immune responses would be successful or not. Although CD8+ Tc2 cells have been shown to display cytotoxic potential just like the CD8+ Tc1 cells (Sad *et al.,* 1995), their immunological and clinical relevance is poorly defined. Some reports showed that Tc2 cells could provide B cell help by secretion of IL-4 (Maggie *et al.,* 1994). These cells display cytotoxicity function just like the Tc1 cells (Sad *et al.,* 1995). High numbers of Tc2 cells have also been found to be correlated with better antibody immune responses in old age (Schwaiger *et al.,* 2003; Yen *et al,.* 2000). High numbers of CD8+ T cells (Tc2) producing IL-4, but not INF-γ have been found in blood and skin of AIDS patients (Maggi *et al.,* 1995). Dobrzanski *et al.,* (1999) have also established that Tc2 cells reduce lung metastasis in a mouse tumour model. Although the factors that influence the differentiation of antigen-specific CD8+ Tc1 and Tc2 cytokine responses *in vivo* are have not yet been clear, development of vaccines that induce such immune responses should be targeted. *Salmonella*-based vaccine technology offers that hope for development of such T-cell vaccines.

3.7.3 Humoral immune responses

The current study further investigated vaccine-induced antibody (IgG) responses to the orally delivered recombinant *Salmonella* expressing GFP model antigen after both primary and booster vaccinations. It was demonstrated that GFP-specific IgG responses could be induced after both primary and booster immunizations of mice (Figures 3.7 and 3.8). In natural oral infection with *Salmonella*, both mucosal IgA and serum IgG are induced and these also play a critical role in controlling of infection (Dietrich *et al.,* 2003). The antibody response is normally induced against the most abundant and surface antigens such as LPS, flagellin, porins and outer membrane proteins (Chapter 2). The mechanism by which foreign antigens expressed intracellularly by *Salmonella* induce humoral immune response is less clear. However, it is known that *Salmonella* infection may induce cross-presentation of infected cells (Yrlid and Wick, 2000; Wijburg *et al.,* 2002; Sundquist *et al.,* 2004). The death of infected cells may result in presentation of the expressed antigen present in apoptotic bodies to the B cells, thereby resulting in

induction of antibody response. This encounter with the antigen may result in the generation of memory B cells and plasma cells secreting the antigen-specific antibody response. Booster vaccinations with the *Salmonella* expressing the same antigen would result in rapid and improved antibody response.

The induction of LPS-specific antibodies after both single and triple inoculations (Figure 3.8) suggested successful vaccination and mucosal invasion of lymphoid tissues by the bacterial vectors. The higher LPS-antibody responses to AroC+pGEM indicated that the negative control was better at invading the mucosal surfaces than the vaccine, AroC+GFP. The failure of heat-killed AroC+GFP to induce GFP-specific immune responses indicated that invasion of the gut-associated lymphoid tissues was critical for the induction of humoral immune responses.

The relation of the levels of the GFP-specific IgG1 to IgG2a isotypes was used as a surrogate marker for the CD4+ Th1 and Th2 balance. In murine B cells, the cytokines IFN-γ, secreted by Th1 cells, and IL4, secreted by Th2 cells, have been shown to induce an immunoglobulin subclass switch to IgG2a and IgG1, respectively (Isakson *et al.*, 1982; Snapper and Paul, 1987). GFP-specific antibodies of all IgG1 and IgG2a subclasses in the sera of mice immunized with AroC+GFP were detected (Figure 3.8) and this suggested the existence of both GFP-specific CD4+ Th1 and Th2 cell immune responses. Interestingly, it was noted that after single inoculation, IgG2a > IgG1 showing the dominance of Th1 over Th2 responses. However, after three inoculations, the IgG2a/IgG1 profile was almost the same (although with slightly more of IgG2a than IgG1) thereby suggesting a balance in the Th1/Th2 responses. These observations allowed the speculation that both Th1 and Th2 responses could be found in an individual mouse at balanced levels. In conclusion, a recombinant prokaryotic expression system for efficient expression of heterologous antigens in a recombinant *Salmonella enterica* serovar Typhimurium vaccine vector was developed. The expression system used the *E. coli lac* operon control sequences and the with *gfp* model gene under the *E. coli lac* promoter. The GFP antigen was fused in-frame with the LacZα peptide and this potentially enhanced expression of the antigen in the recombinant *Salmonella enterica* serovar Typhimurium vaccine vector. The inclusion of the LacZα peptide did not have any functional consequences on GFP since its folding, fluorescence and immunogenicity were not affected. Vaccination of mice with recombinant *Salmonella* expressing the GFP model antigen induced GFP-specific CD8+ cytokine and humoral responses. Both GFP-specific IgG1 and Ig2a isotypes were induced suggesting the provocation of both GFP-specific Th1 and Th2 immune responses. Therefore, it has been shown that the strategy developed in this study could be used to test the cloning of other foreign antigens for induction of antigen-specific T and B cell responses. HIV-1 Gag is an example of such foreign antigens which may be targeted for delivery using the approach developed in this study (Chapters 4, 5 and 6). The GFP can further be used in tagging other antigens for determination of their subcellular localization in the bacteria (Chapter 7). In future, the approach may also be used to compare different attenuated vaccine mutants using GFP as a model for export either by type III or tat export systems and the effect on the immune responses.

CHAPTER 4: DEVELOPMENT AND IMMUNOGENICITY OF A RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR EXPRESSING WILD-TYPE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C GAG

"They are ill-discoverers who think there is no land when they see nothing but sea" Francis Bacon (1561-1626)

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CHAPTER 4: DEVELOPMENT AND IMMUNOGENICITY OF A RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR EXPRESSING WILD-TYPE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C GAG

4.1 SUMMARY

No previous studies have investigated cellular and humoral immune responses of wild-type South African HIV-1 subtype C Gag when delivered by attenuated recombinant *Salmonella* vaccine vectors. The objective of the current study was to develop a recombinant *Salmonella enterica* serovar Typhimurium expressing HIV-1 Subtype C Gag and evaluate its immunogenicity in mice. Wild-type HIV-1 SubtypeC *gag* (*wtgag*) gene derived from South Africa HIV-1 isolate (Du_{422}), was cloned into the backbone of pGEM+GFP plasmid (Chapter 3) and a recombinant expression plasmid, pGEM+wtGag was generated. A recombinant *Salmonella enterica* serovar Typhimurium vector expressing wtGag (AroC+wtGag) was developed. The expression of high level of wtGag antigen by the recombinant *Salmonella* was demonstrated. To evaluate immunogenicity of the recombinant *Salmonella* vaccine vector, groups of mice (n=5) were orally vaccinated once or boosted twice. No systemic HIV-1 Gag-specific CD8+ T cell cytokine responses were detected after both primary and booster vaccinations of mice. However, systemic HIV-1 Gag-specific CD4+ Th2 (IL-4), but not Th1 (IFN-γ) cytokine response was induced only after booster vaccinations. The splenocytes stimulated with an $H\text{-}2^d$-restricted Class II Gag epitope (MR13, NPPIPVGDIYKRWIILGLNK) for 48 hours produced elevated IL-4 cytokine level 4.5-fold above the background. HIV-1 Gag-specific IgG antibody responses were not detected in vaccinated mice on both Day 28 and Day 84 in 1/100 serum dilution. It was recommended that the wild-type HIV-1 *gag* gene be codon-optimized for expression by the *Salmonella* as this could potentially improve the immune responses of the antigen.

4.2 INTRODUCTION

One novel approach for the generation of mucosal and systemic immunity against HIV-1 would be the use of recombinant attenuated recombinant bacterial pathogens such as *Salmonella* expressing HIV-1 antigens in vaccinations. *Salmonella* vectors have already been used as delivery vectors for other heterologous antigens for induction of mucosal and systemic immune responses (Chapter 2). However, no studies have previously investigated the use recombinant *Salmonella* as potential vaccine vectors for HIV-1 Gag derived from Southern Africa viral isolates.

The characterization and selection of HIV-1 Subtype C *gag* gene for use in vaccine development for Southern Africa has been published (Williamson *et al.*, 2003, Thomas, 2005). The HIV-1 Subtype C *gag* gene was isolated from Du_{422}, a sex-worker from a Durban (Du) cohort and the full-length gene was generated by reverse-transcription from plasma RNA (Williamson *et al.*, 2003). Comparative phylogenetic analyses of the Du422 *gag* with other HIV-1 Subtype C *gag* sequences from other Southern African countries and the reference Subtype C showed a high degree of relatedness (Williamson *et al.*, 2003, Thomas, 2005). Because of such relatedness, the Du_{422} HIV-1 *gag* has been selected for

development of an HIV-1 Subtype C vaccine for Southern Africa. Prior to cloning Du_{422} HIV-1 *gag* gene into vectors reported in the thesis, early extensive studies were done that involved cloning of the *gag* gene into two *Salmonella* expression plasmids to generate pGagmtr and pGagssa (Appendices G11 and g12). The plasmids were used to develop two recombinant *Salmonell*a vaccines (AroC+pGagmtr and AroC+pGagssa). Oral vaccination of mice with the *Salmonella* vaccine vectors failed to induce any detectable HIV-1 Gag-specific cellular and antibody immune responses. The recombinant bacterial vectors were highly unstable and Gag antigen expression was very low (Chin'ombe, unreported work). Although these early studies are not included in the current reported study, they will be referred in some sections of this thesis as unpublished work. Some of the plasmids generated by these early studies were further used in some cloning manipulations. For example, the *gag* in recombinant plasmid, pGagssa was used in the current studies (Chapter 4) for generation of the recombinant plasmid, pGEM+wtGag.

The successful oral delivery of GFP model antigen expressed by recombinant *Salmonella enterica* serovar Typhimurium (Chapter 3) in generating systemic immune responses in mice demonstrated the utility of the bacteria. This prompted investigations into the use of the same strategy for HIV-1 vaccine development with the hope of inducing HIV-1 Gag-specific CD8+ T-, CD4+ T- cell and humoral immune responses. Therefore the main objectives of the current study were:

1. to develop a recombinant expression plasmid with wild-type (Du_{422}) HIV-1 *gag* gene (full-length or truncated). The *gag* gene was to be cloned into the backbone of the prokaryotic expression plasmid, pGEM+GFP (developed in Chapter 3)
2. to use the recombinant expression plasmid, pGEM+wtGag for the development of a recombinant *Salmonella enterica* serovar Typhimurium vaccine vector expressing wtGag
3. to investigate the induction of HIV-1 Gag-specific systemic cellular and humoral immune responses in mice after oral vaccination with the recombinant *Salmonella* vaccine vector.

4.3 MATERIALS

The materials (such as *E. coli* and *Salmonella* bacterial strains, oligonucleotides, plasmids, restriction enzymes, solutions, buffers, media and HIV-1 Gag peptides) used in the current study are given in Appendices D, E, F and I). The type of mice used in the study has been described previously (Chapter 3). The DNA vaccine, pTHgagC used as a positive control for (ELISPOT and CBA) immunoassays was kindly provided by Dr J van Harmelen (University of Cape Town). The development and immunogenicity of pTHgagC DNA vaccine has previously been reported (van Harmelen *et al.*, 2003).

4.4 GENERAL METHODS

Bacteriological, molecular and immunological methods used in the study have been described in great detail elsewhere (Appendices A and B).

4.5 EXPERIMENTAL DESIGN AND PROTOCOLS

4.5.1 Construction of recombinant pGEM+wtGag expression plasmid: molecular cloning of wild-type HIV-1 *gag* gene

To construct a recombinant prokaryotic HIV-1 Gag expression plasmid, pGEM+wtGag (Appendices G5 and H2), a single sub-cloning step was employed (Figure 4.1). Briefly, wild-type HIV-1 subtype C *gag* gene fragment was digested out (with *Nar*I and *Hind*III) from Pgagssa (see Appendix G12 for map), separated by gel electrophoresis (Appendix B1.4), gel purified (Appendix B1.6) and ligated (Appendix B1.7) to a linealized pGEM+GFP plasmid backbone which was also generated by double restriction digestion with *Nar*I and *Hind*III (Chapter 3). The ligation reaction was used in the genetic transformation of competent SCS110 cells (Appendix A1.4). Recombinant SCS110 clones with pGEM+wtGag plasmid were screened by restriction enzyme mapping (Appendix B1.5), initially with *Eco*RI and followed by *Nar*I and *Hind*III. The candidate *gag* gene in pGEM+wtGag plasmid was sequenced (Appendix B1.8).

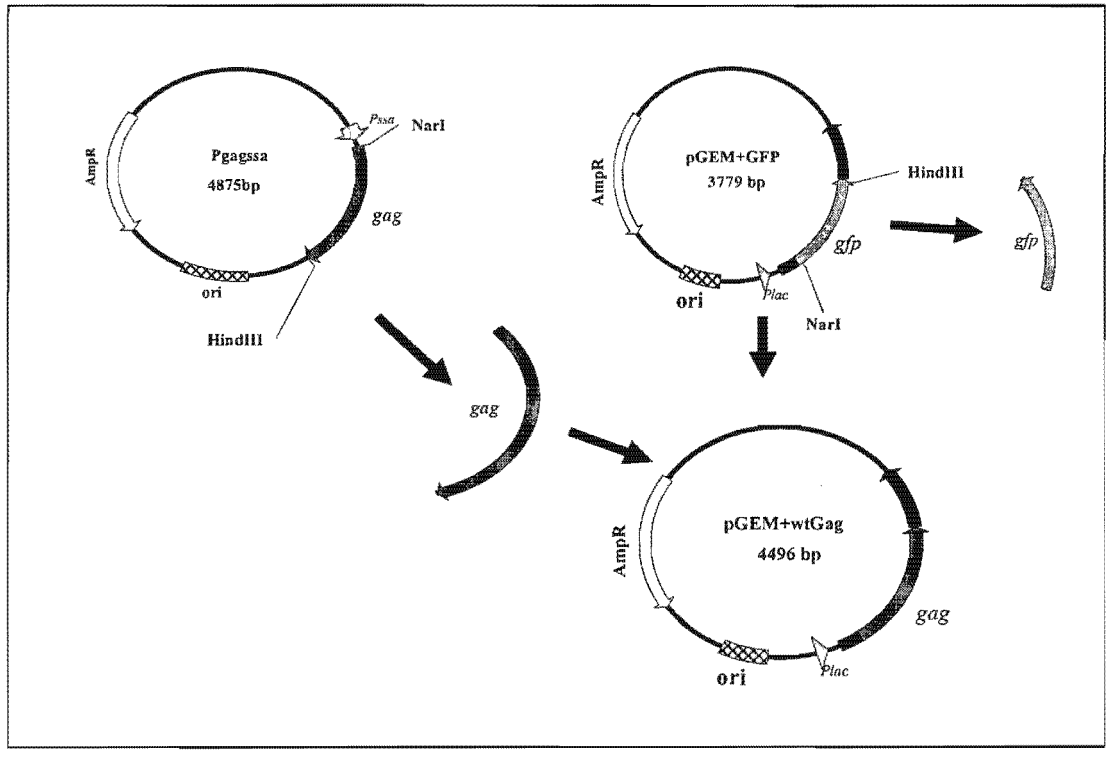

Figure 4.1: Molecular cloning strategy of wild-type HIV-1 subtype C *gag*. The *wtgag* gene from Pgagssa was cloned into the backbone of pGEM+GFP to generate pGEM+wtGag. Details on the generation of Pgagssa are given in Appendix G12.

4.5.2 Construction of recombinant pGEM+GagK expression plasmid: molecular cloning of truncated wild-type HIV-1 *gag* gene

A truncated wild-type *gag* (*gagK*) was cloned. The construction of a recombinant prokaryotic expression plasmid (designated pGEM+GagK) with the 3'-end of the wild-type HIV-1 *gag* gene was performed with polymerase chain reaction (Figure 4.2). The oligonucleotide primers, designated, K1 and K2 (Appendix

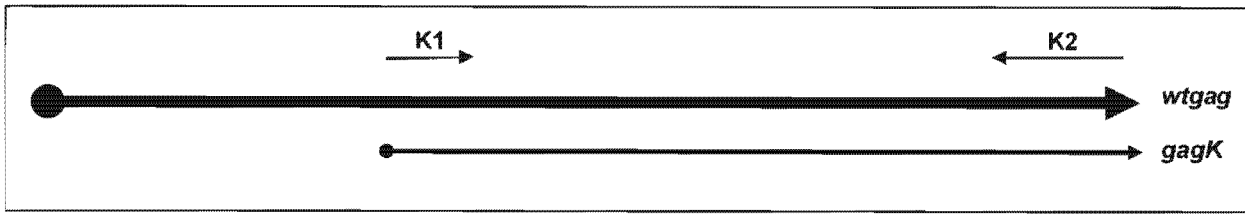

Figure 4.2: Schematic representation of the strategy employed in amplification of *gag*K from wild-type HIV-1 subtype C *gag* by polymerase chain reaction. The oligonucleotides, K1 and K2 (Appendix F) were used as primers in amplification of *gag*K from *wtgag* DNA template.

F) were used for PCR and were rationally designed so that when cloned into pGEM-T Easy plasmid, GagK (truncated HIV-1 Gag) would be in-frame with the N-terminal domain of the β-galactosidase. A stop codon, TAA, was included at the end of the reverse primer, K2. The PCR reaction was conducted in a 50 µl volume with 4.5 units QIAGEN Taq DNA Polymerase, 1x PCR buffer, 1.5 µM of each primers (K1 and K2), 0.2 mM dNTPs, 1.5 mM magnesium chloride and 10 ng of Pgagmtr (Appendix G11) DNA template. (Pgagmtr and Pgagssa had the same Du_{422} HIV-1 Subtype C *gag* gene (Appendix G1)). The PCR cycling conditions were as follows: 1 cycle of 95°C for 5 min, 5 cycles of 95°C for 45 s, 45°C for 30 s, 72°C for 1 min, 25 cycles of 95°C for 45 s, 55°C for 30 s, 72°C for 2 min, and a final extension of 72°C for 7 min. An aliquot (10 µl) of the PCR amplicon was analysed by agarose gel electrophoresis (Appendix B1.4). The amplicon (1 µl) was ligated into linearized pGEM-T Easy plasmid as described in Appendix B1.7. The ligation reaction was used in the transformation of competent *E. coli* cells as described in Appendix B1.4. Screening of recombinant bacterial clones with pGEM+gagK was done using restriction mapping procedure as described in Appendix B1.5.

4.5.3 Development of recombinant *Salmonella* expressing wtGag and assessment of antigen expression

Transformation of competent *AroC Salmonella enterica* serovar Typhimurium mutant with pGEM+wtGag and pGEM+GagK plasmids was performed as previously described (Chapter 3). Expression of the wtGag by recombinant *Salmonella enterica* serovar Typhimurium vector (AroC+wtGag) was checked using standard 12.5 % SDS-PAGE (Appendix B4) and Western blotting (Appendix B6). Western blots were probed with anti-p24 antibodies. The presence of wtGag or GagK antigen in culture lysates was further determined by the Roche Elecsys® HIV p24 Ag assay (Roche Diagnostics Boehringer Mannheim GmbH, Germany) according to manufacturer's instructions. The Roche Elecsys® HIV p24 Ag assay is a semi-quantitative assay and cannot be used with accuracy to quantify the amount of Gag present in a sample. The bacterial protein lysates were diluted (1/100 or 1/1000) in water and the Elecysys® 2010 analyzer used to determine the relative amount of the antigen. Expression of GagK by recombinant *Salmonella enterica* serovar Typhimurium AroC mutant (AroC+GagK) was checked by 12.5 % SDS-PAGE (Appendix B4) by running three concentrations (5µg, 2µ and 0.5µg) of total bacterial lysate of AroC+GagK). The probability of solubility of wtGag and gagK

when overexpressed in *E. coli* or *Salmonella* was predicted using the Wilkinson-Harrison solubility model (Wilkinson and Harrison, 1991) as described in Chapter 3.

4.5.4 Vaccination of mice with recombinant *Salmonella* vaccine vector expressing wild-type HIV-1 Gag

Stocks of recombinant *Salmonella enterica* serovar Typhimurium vaccine vector, AroC+wtGag and negative control vaccine, AroC+pGEM were prepared as described in Appendix A2.1. The vaccination protocol employed in Chapter 3 was followed. In brief, groups of mice were vaccinated by intragastic gavage (Appendix A2.3) with AroC+wtGag and AroC+pGEM (Tables 4.1 and 4.2). A positive control vaccine, a naked DNA vaccine, pTHgagC, carrying Du_{422} HIV-1 *gag* gene was included, for intramuscular vaccination (single immunization) of mice. A single inoculation of pTHgagC has been shown to induce high levels of Gag-specific cellular responses that can be detected 12 days after vaccination (van Harmelen *et al.*, 2003). The DNA vaccine was included as a positive control for assays to assess cellular immune responses.

Table 4.1: Vaccines and vaccination protocol for Experiment 1. Mice were inoculated ONCE by intragastric gavage (Appendix A2.3) with *Salmonella* vaccines (AroC+wtGag and AroC+pGEM), and a DNA vaccine, pTHgagC (100μg) was given by intramuscular inoculation on Day 16. The mice were bled prior to inoculation and sacrifice. The blood was processed by centrifugation and sera were stored until evaluation of humoral immune responses as described in Appendix B10. At sacrifice on Day 28, the spleens from each group of mice were pooled and processed as described in Appendix B7.

Inoculum (vaccine)	Inoculum/animal	Inoculation date	Sacrifice date
AroC + wtGag	10^9 cfu	Day 0	Day 28
AroC + pGEM	10^9 cfu	Day 0	Day 28
pTHgagC	100 μg	Day 16	Day 28

Table 4.2: Vaccines and vaccination protocol for Experiment 2. Mice were inoculated THREE times by intragastric gavage (Appendix A2.3) on Days 0, 28 and 56 with *Salmonella* vaccines (AroC+wtGag and AroC+pGEM). A DNA vaccine (pTHgagC) was given by intramuscular inoculation on Day 72. The mice were bled prior to inoculation and sacrifice. The blood was processed by centrifugation and sera were stored until evaluation of humoral immune responses as described in Appendix B10. At sacrifice, the spleens from each group of mice were pooled and processed as described in Appendix B7.

Inoculum (vaccine)	Inoculum/animal (Day 0, Day 28, Day 56)	Inoculation date	Sacrifice date
AroC + wtGag	*$10^9 \rightarrow 10^8 \rightarrow 10^8$cfu	Day 0, Day 28, Day 56	Day 84
AroC + pGEM	*$10^9 \rightarrow 10^8 \rightarrow 10^8$cfu	Day 0, Day 28, Day 56	Day 84
pTHgagC	100 μg	Day 72	Day 84

*the dosage was reduced from 10^9 to 10^8 cfu after few animals had died after the first inoculation.

4.5.5 Assessment of T cell responses in the spleen

The pools of spleens (in 10 ml RPMI with 10% FCS) from each group of mice were processed as described in Apendix B7. The frequencies of HIV-1 Gag-specific T cells secreting cytokines (IFN-γ and IL-4) were determined by IFN-γ and IL-4 ELISPOT assays (Appendix B8). Splenocytes (500 000/well) were stimulated in ELISPOT plates with Du_{422} HIV-1 Gag CD4+ T and CD8+ T cell peptides, an irrelevant peptide (Table 4.3) or Con A (see Appendix D for preparation of all the stimulants). Each stimulant was added into three wells (triplicate). The stimulants used were:

(1) Media with no peptide (100 µl/well R10 medium) (medium background control)

(2) AMQ Gag peptide (100 µl/well, final concentration of 2.0 µg/ml)

(3) TTST Gag peptide (100 µl/well, final concentration of 2.0 µg/ml)

(4) MRC2 Gag peptide (100 µl/well, final concentration of 2.0 µg/ml)

(5) MRC13 Gag peptide (100 µl/well, final concentration of 2.0 µg/ml)

(6) MRC17 Gag peptide (100 µl/well, final concentration of 2.0 µg/ml)

(7) Irrelevant peptide (IP), H-2K^d binding peptide (100 µl/well, final concentration of 2 µg/ml)

(8) Con A (100 µl/well, final concentration of 0.5 µg/ml)).

Table 4.3: Amino acid sequence of HIV-1 Gag peptides used in the ELISPOT and CBA assays

Gag peptide	Description	Complete amino acid sequence
AMQ	H-2^d-restricted class I peptide (CD8+ peptide)	AMQMLKDTI
TTST	H-2^d-restricted class I peptide (CD8+ peptide)	TTSTLQEQI
MRC2	H-2^d-restricted class II peptide (CD4+ peptide)	VHQAISPRTLNAWVKVIEEK
MRC13	H-2^d-restricted class II peptide (CD4+ peptide)	NPPIPVGDIYKRWIILGLNK
MRC17	H-2^d-restricted class II peptide (CD4+ peptide)	FRDYVDRFFKTLRAEQATQE
Irrelevant peptide (IP)	H-2K^d binding peptide (CD8+ peptide)	TYSTVASSL

Splenocytes were plated in triplicates for each stimulant and 500 000 cells/well were used. The frequencies of IFN-γ and IL-4 spot-forming units were normalized to $1x10^6$ splenocytes and means (±SD) were calculated. The background spots in the absence of stimuli were subtracted to give net SFUs/10^6. The cut-off for a positive ELISPOT response was arbitrarily defined as at least three times the no-peptide (medium) SFU number and the number of specific gross SFUs being > 50 /10^6 cells (and non-specific SFUs <50/10^6 cells).

CD4+ Th1/Th2 and CD8+ Tc1/Tc2 cytokines produced during stimulation with peptides/antigens were determined by CBA assay (Appendix B8). Splenocytes from each group of mice were cultured in 96-well, round-bottomed culture plates at a density of 1.5 x 10^6 splenocytes per well in a final volume of 200 µl of culture medium. Plates were incubated at 37 °C, 5% CO_2, 90% humidity for 48 h after which 150 µl of cell free culture supernatants were collected and kept at −20 °C until assayed for cytokines (IFN-γ, TNF-α, IL-4 and IL-5) using a CBA assay as described in Appendix B8. Stimulation index (SI)

for a specific stimulant was defined as: cytokine level (pg/ml) in peptide-stimulated culture divided by cytokine level (pg/ml) in unstimulated culture. A specific response was arbitrarily defined as positive when SI>2.

4.5.6 Assessment of humoral immune responses to recombinant *Salmonella* vaccine

HIV-1 Gag or LPS-specific IgG in sera of vaccinated mice was determined using an ELISA protocol (Appendix B10) for serum pools collected on Day 28 (Experiment 1) and Day 84 (Experiment 2). The ELISA plates were coated with HIV-1 subtype B Pr55 antigen (HIV-1 BH10 expressed in baculovirus, Quality Biologicals) or *Salmonella* LPS (Sigma) at a final concentration of 5 ug/ml. Mouse serum pools were diluted accordingly (1/100 and 1/1000). Antibody responses were defined as positive when specific mean OD_{405nm} >2-fold that of the negative control or when the mean OD_{405nm} ratio to mean OD_{405nm} of prebleed >2.

4.6 RESULTS

4.6.1 Development of HIV-1 Gag expression plasmid vectors: molecular cloning of Du_{422} *gag* gene

The Du_{422} *gag* gene was successfully cloned into the backbone of pGEM+GFP (previously developed Chapter 3). The general structural components of the recombinant HIV-1 *gag* expression plasmid (designated pGEM+wtGag) are illustrated in Figure 4.3A and Appendix G5. The cloning step involved restriction of wild-type HIV-1 Subtype C *gag* from Pgagssa (Appendix G) with *NarI*/*Hind*III and ligation of the gene into the backbone of pGEM+GFP plasmid vector (linearized with same enzymes. Just as with the *gfp* gene in pGEM+GFP plasmid (Chapter 3), the *gag* gene in pGEM+wtGag was in-frame with the 5'-domain of the *E. coli* β-galactosidase α-gene fragment and expression was under the prokaryotic promoter, P*lac*. Unlike the *gfp* in pGEM+GFP, which had TAAG as stop codons, the *gag* in pGEM+wtGag had a more powerful and efficient prokaryotic Gram-negative stop codon, TAAT, which was successfully incorporated at the end of the gene. Analysis of the *wtgag* revealed the presence of many rare *Salmonella* codons and Shine-Dalgarno-like motifs.

To further investigate whether a smaller fragment of wtGag would also be expressed at high levels, a recombinant expression plasmid, pGEM+GagK, which contained the truncated *gag* gene was constructed (Figure 4.3B). The truncated gene spanned the 3' end of the full-length *gag*. The gene was successfully amplified by PCR and cloned. All the necessary transcriptional and translational functions required for antigen expression where successfully incorporated into the vector. The plasmid backbones of pGEM+GagK and pGEM+wtGag were the same. The DNA and amino acid sequences of the full-length wild-type HIV Gag developed by this study are shown in Figure 4.3C.

To evaluate expression of wild HIV-1 Gag in *Salmonella*, the two plasmid vectors, pGEM+wtGag and pGEM+gagK were used in generating recombinant *Salmonella enterica* serovar Typhimurium by genetic transformation. The expression of the wtGag and GagK by the recombinant *Salmonella* vaccine vectors, AroC+wtGag and AroC+GagK was assessed. Studies by SDS-PAGE and Western Blot analysis (Figure 4.4) showed that the recombinant *Salmonella* (AroC+wtGag) constitutively expressed high levels of full-length wild-type HIV-1 Gag (59.423 kDa) in the presence or absence of IPTG. The Gag protein band was visible on both Commassie blue-stained SDS-PAGE and Western blot. The Western blot further showed the existence of several minor HIV-1 Gag-specific bands, some of which were less than 59.423 KDa in size.

Expression of truncated version (GagK) of wild-type HIV-1 Gag by the recombinant *Salmonella*, AroC+GagK was also investigated. Very high expression of GagK by the *Salmonella* vaccine was demonstrated (Figure 4.4B). It was shown that GagK was the most highly expressed and abundant protein in the recombinant *Salmonella*. By serial dilution of total protein lysate loaded on SDS-PAGE, it was demonstrated that GagK was the most abundant protein in the bacteria (Figure 4.10). The high level expression of HIV-1 wtGag and GagK by the recombinant *Salmonella* bacteria was further confirmed by

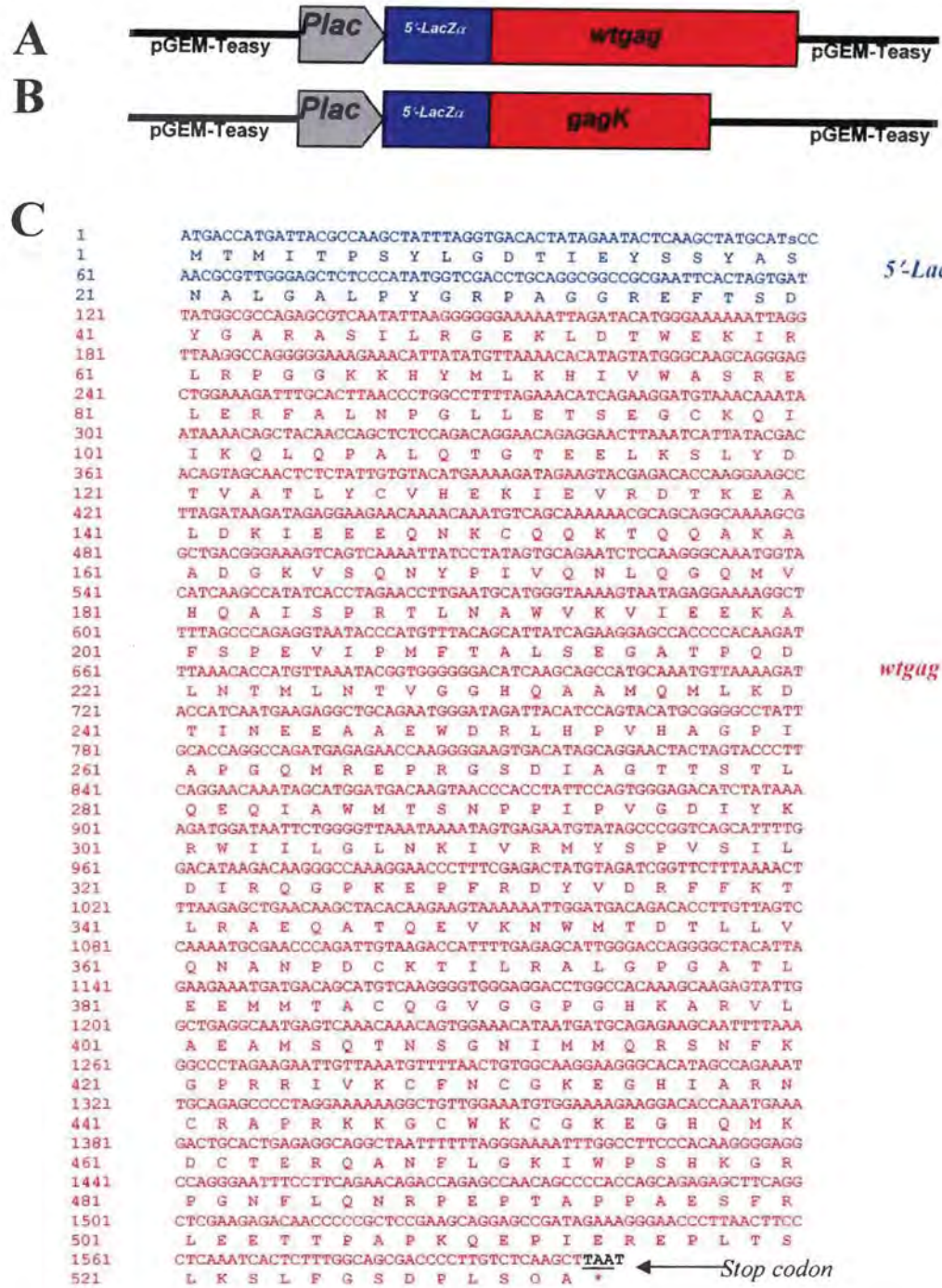

Figure 4.3: The general structural components of the wild-type HIV-1 Gag expression constructs (pGEM+wtGag and pGEM+GagK) developed by the study. (**A**) A schematic representation of the wtGag expression plasmid (pGEM+wtGag). The wtGag was fused with the N-terminal domain (first 40 amino acids) of the *β*-galactosidase *α*-fragment in pGEM-Teasy plasmid. The expression was under the *E. coli lac* promoter and other *lac* operon transcription and translation domains found in pGEM-Teasy plasmid. (**B**) A schematic representation of the GagK expression plasmid (pGEM+GagK). The transcription and translational components were the same as those found in pGEM+wtGag. (**C**) The DNA and amino acid sequences of the resultant *wtgag* gene and wtGag protein as a fusion gene/protein with the *β*-galactosidase *α*-fragment. The whole fusion protein was 59.423 KDa. A preferred stop codon (TAAT) was included at the end of the *wtgag* gene to prevent translation of the whole *LacZα* gene. The key features and complete DNA sequence of pGEM+wtGag plasmid are given in Appendices G5 and H2 respectively.

the Roche Elecsys® HIV p24 Ag assay (Figure 4.4C). Both antigens were shown to be expressed at very high levels, detectable in 1 in 100 dilution of the total bacterial protein lysates.

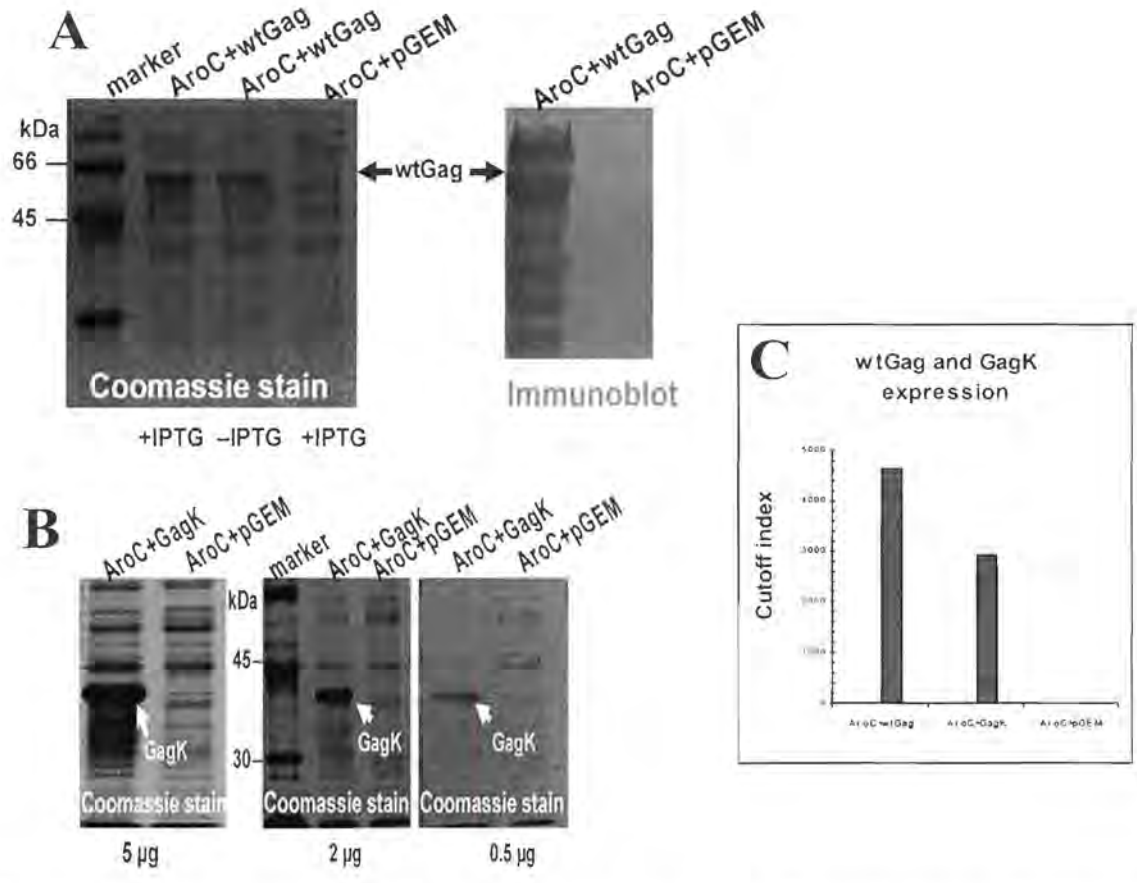

Figure 4.4: Expression of wild type HIV-1 Gag in recombinant *Salmonella enterica* serovar Typhimurium vaccine vector. (**A**) SDS-PAGE and Western blotting analysis of recombinant *Salmonella enterica* serovar Typhimurium (AroC+wtGag) expressing wtGag. The wtGag protein bands were visible on the Coomassie blue stained SDS-PAGE and the antigen was expressed in the bacteria with or without IPTG added into the growth media. Western blot showed that there were other smaller protein bands that reacted with Gag antibodies. (**B**) SDS-PAGE analysis of recombinant *Salmonella enterica* serovar Typhimurium (AroC+GagK) expressing GagK. Diluted amounts of bacterial protein lysates of AroC+GagK and AroC+pGEM were loaded on gels so that only the most expressed and abundant proteins could be viewed. The GagK protein band (arrow) was distinctively visible on all the three gels. (**C**) The relative expression of wtGag and GagK by recombinant *Salmonella* vaccine (AroC+wtGag and AroC+GagK) cultures as determined by Roche Elecsys® HIV p24 Ag assay. The total bacterial protein lysate was diluted 1/100 in water. Protein lysate from the negative control, AroC+pGEM was also included. The cut-off index was calculated by the Elecsys® 2010 analyzer using readings from the negative and positive calibrators

The solubility of Gag when expressed in *E. coli* or *Salmonella* was predicted by the Wilkinson and Harrison solubility model (Wilkinson and Harrison, 1991). The model predicted that wild-type Gag and its truncated version, GagK were highly insoluble when overexpressed in the bacteria. WtGag and GagK

had insolubility probabilities of 85.3% and 91.8% respectively. However, the model also showed that if the truncated 5'-end of the wtGag were to be overexpressed in *E. coli* or *Salmonella*, it would be more soluble than GagK (3'-end of the wtGag).

Although AroC+wtGag and AroC+GagK were successfully developed, a very high degree of instability of the vectors was observed especially for AroC+GagK bacteria which could not be grown viably to stationary phase. Mutants always arose during culturing of AroC+GagK with antibiotic selection. It was also noted that bacterial cells were poorly viable after growth beyond the logarithmic phase. Attempts to prepare vaccine stocks of recombinant *Salmonella* expressing GagK failed because of the high degree of instability. The plasmids were being lost rapidly from the bacteria as ampicillin selection pressure was declining. The AroC+GagK vaccine seemed not suitable for animal vaccination and all further investigations were discontinued. Only *Salmonella* expressing the full-length wtGag was used for animal vaccinations. It was however also noted that AroC+wtGag bacteria grew slowly. Growing bacteria into the stationary phase further indicated that there were fast-growing mutants arising.

4.6.2 Immunogenicity of a recombinant *Salmonella* vaccine vector expressing wild-type Gag

4.6.2.1 CD8+ Tc1/Tc2 and CD4+ Th1/Th2 cytokine responses to recombinant *Salmonella* expressing wild-type HIV-1 Gag

ELISPOT assays were used to enumerate the frequencies of HIV-1 Gag-specific CD4+ or CD8+ cells secreting IFN-γ and IL-4 cytokines during stimulation with specific peptides or antigens. On Day 28 (Experiment 1) and Day 84 (Experiment 2), the frequencies of splenocytes from mice vaccinated with AroC+wtGag secreting IFN-γ after stimulation with AMQ, TTST, MRC2, MRC13 or MRC17 were not above background (Figure 4.5A). The splenocytes from mice vaccinated with the DNA positive control vaccine (pTHgagC), when stimulated with the Gag CD8+ T cell and CD4+ T cell Gag peptides, produced high numbers of specific IFN-γ SFUs as expected (Figure 4.5A).

Gag-specific ELISPOT IL-4 response to the vaccines was determined on Day 84 only. The number of specific IL-4 responses in AroC+wtGag-vaccinated mice was below the 50 SFUs/million cells cut-off (Figure 4.5B). Two of the Gag peptides, AMQMLKDTI and MRC13, each induced 26 IL-4 SFUs and this was not considered as a specific response. The frequencies of splenocytes secreting specific IL-4 were high after stimulation with both CD8+ and CD4+ Gag peptides (except for TTST peptide) for the DNA vaccine (Figure 4.5B).

To check if the cells were viable and assays working, splenocytes from vaccinated mice were also stimulated with Con A. As previously observed (Chapter 3), the cells produced very high numbers of IFN-γ or IL-4 SFUs after Con A stimulation (Figure 4.6).

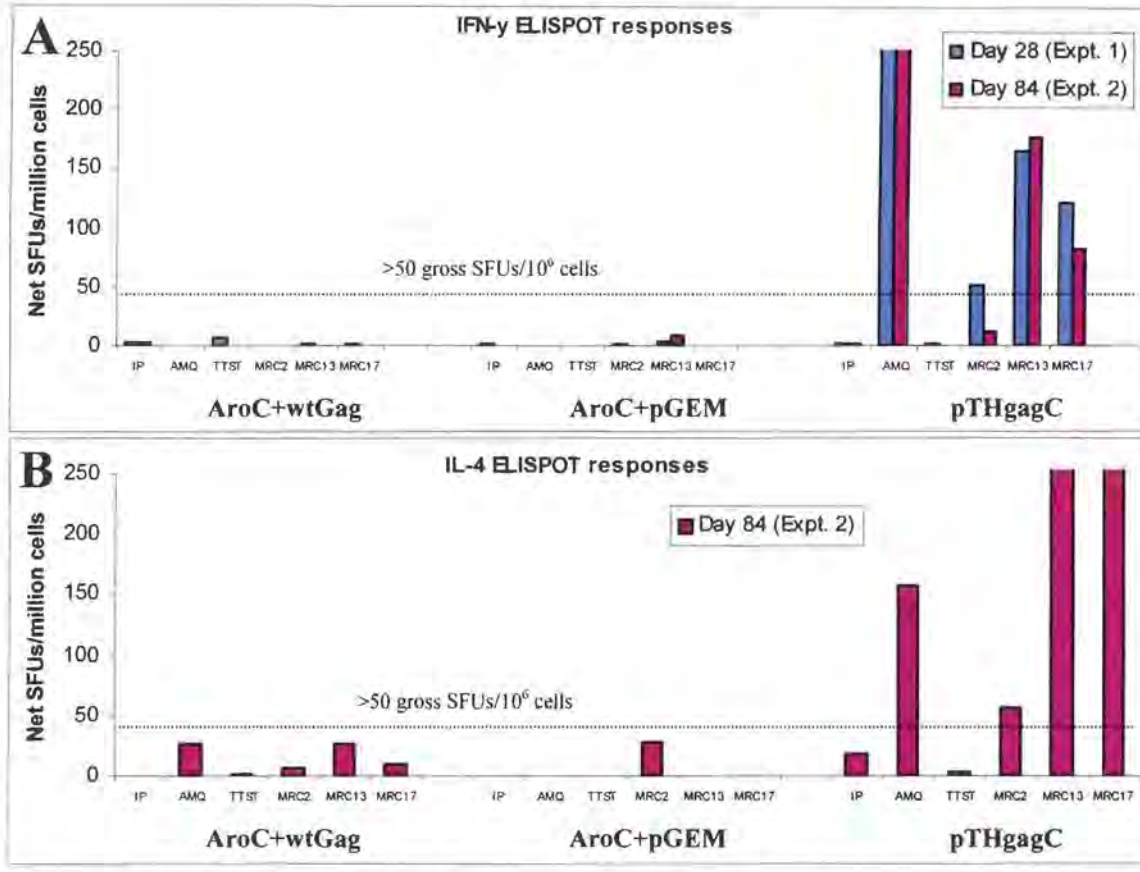

Figure 4.5: The magnitude of **HIV-1 Gag-specific CD8+ and CD4+ T cell responses** as measured by IFN-γ (**A**) and IL-4 (**B**) ELISPOT assays. Groups of mice were vaccinated ONCE (Experiment 1, Table 4.1) or THREE times (Experiment 2, Table 4.2) with *Salmonella* vaccine expressing wtGag, AroC+wtGag, a negative *Salmonella* control vaccine, AroC+pGEM or a DNA vaccine, pThgagC. On Days 28 (Experiment 1) and 84 (Experiment 2) splenocytes from the vaccinated mice were incubated with medium only (negative assay control), or stimulated with irrelevant peptide (IP) TYSTVASSL or the Gag CD8+ T cell peptides (AMQ = AMQMLKDTI and TTST= TTSTLQEQI) or Gag CD4+ T cell peptides (MRC2, MRC13 and MRC17) in IFN-γ and IL-4 ELISPOT assays. IFN-γ ELISPOT assay was performed on Day 28 (Experiment 1) and Day 84 (Experiment 2) (**A**), while IL-4 ELISPOT assay was performed only on Day 84 (Experiment 2) (**B**). Each bar in the graphs represents the net SFUs/10^6 cells. A positive response was arbitrarily defined as at least three times the no-peptide (medium) SFU number, and the number of specific SFUs being > 50 /10^6 cells.

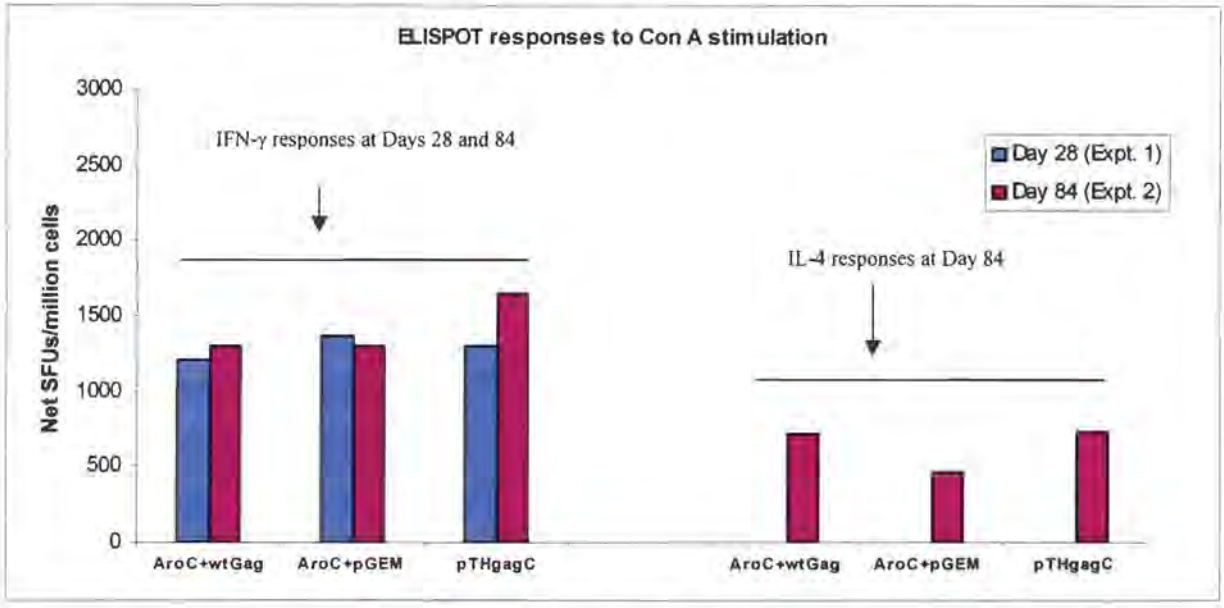

Figure 4.6: Con A-mediated responses of splenocytes as determined by IFN-γ (**A**) and IL-4 (**B**) ELISPOT assays. Groups of mice were vaccinated ONCE (Experiment 1, Table 4.1) or THREE times (Experiment 2, Table 4.2) with AroC+wtGag, AroC+pGEM or pTHgagC. On Days 28 (Experiment 1) and 84 (Experiment 2) splenocytes from the vaccinated mice were incubated with Con A in IFN-γ and IL-4 ELISPOT assays. IFN-γ ELISPOT assay was performed on Day 28 (Experiment 1) and Day 84 (Experiment 2) (**A**), while IL-4 ELISPOT assay was performed only on Day 84 (Experiment 2) (**B**). Each bar in the graphs represents the net SFUs/10^6 cells. A positive response was arbitrarily defined as at least three times the no-peptide (medium) SFU number, and the number of specific SFUs being > 50 /10^6 cells.

The CBA assay was used to quantify IFN-γ, TNF-α, IL-4 and IL-5 cytokines produced by splenocytes during stimulation with the Gag peptides. On Day 28 (Experiment 1), splenocytes from mice vaccinated with AroC+wtGag did not produce elevated cytokines after stimulation for 48 hrs with Gag-specific CD8+ and CD4+ peptides (Figure 4.7A-D). This suggested that single inoculation of mice with the *Salmonella* vaccine could not induce detectable CD8+ Tc1/Tc2 and CD4+ Th1/Th2 cell responses. On the other hand, splenocytes of mice vaccinated with the DNA positive control vaccine, pTHgagC responded well to both Gag CD8+ and CD4+ peptides (especially AMQ, TTST, MRC13 and MRC17) (Figure 4.7A-D).

Splenocytes of mice boosted with the AroC+wtGag (Experiment 2) only responded to Gag peptide MRC13, with production of IL-4 only and a SI of 4.5 (Figure 4.7C). Few cells producing IL-4 in response to MRC13 stimulation had been detected previously with IL-4 ELISPOT assay. The high level of IL-4 secreted by the splenocytes suggested that only a Gag-specific CD4+ Th2 cytokine response was induced on Day 84 after three inoculations with the recombinant *Salmonella* vector expressing wtGag.

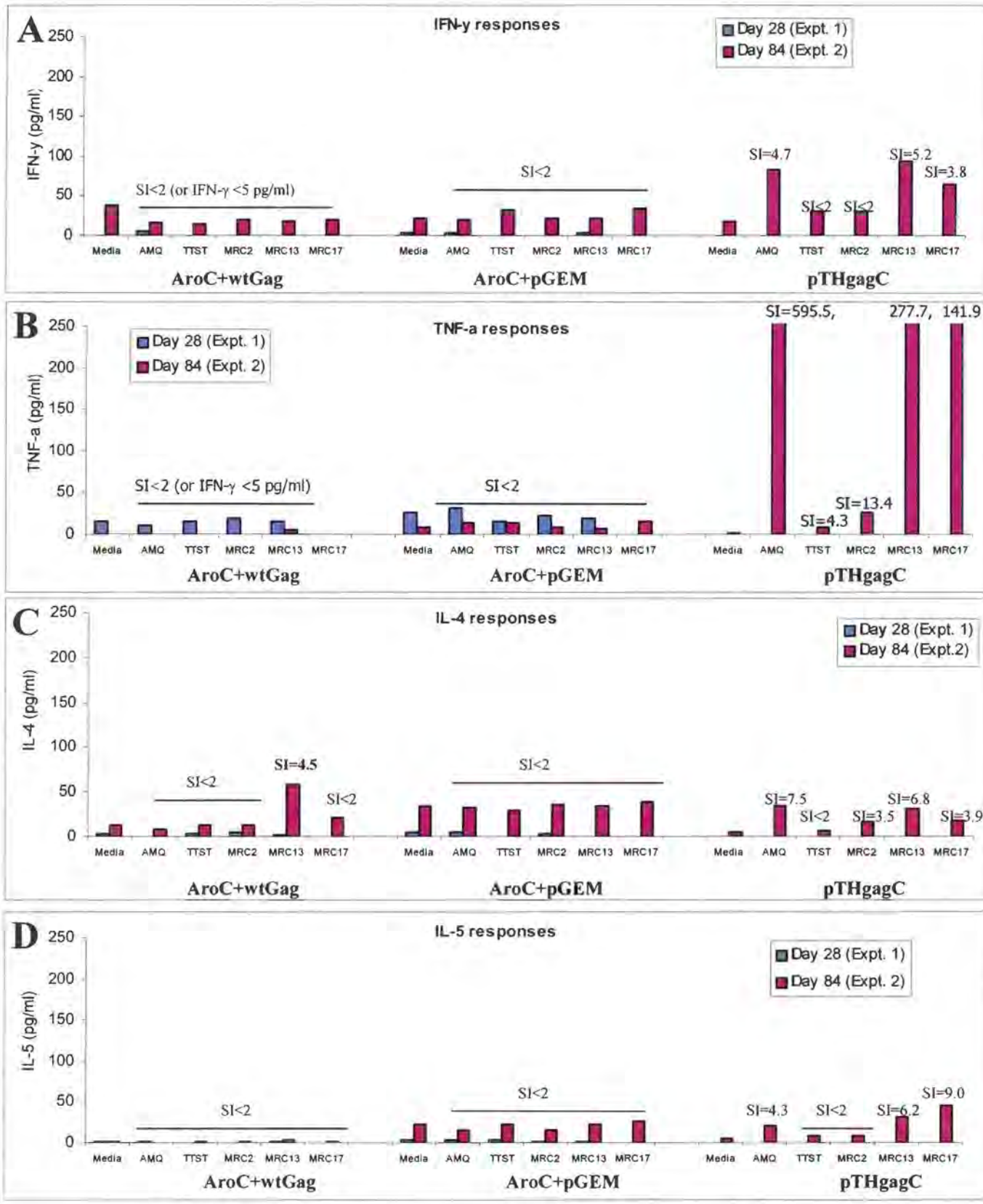

Figure 4.7: The magnitude of **Gag-specific CD8+ T cell responses** as determined by quantification of IFN-γ (**A**), TNF-α (**B**), IL-4 (**C**) and IL-5 (**D**) cytokines (in pg/ml) secreted by splenocytes during stimulation for 48 hours in the absence or presence of Gag peptides, using CBA assay. Groups of mice were vaccinated ONCE (Experiment 1, Table 4.1) or THREE times (Experiment 2, Table 4.2) with AroC+wtGag, AroC+pGEM or pTHgagC. On Day 28 (Experiment 1) and Day 84 (Experiment 2), splenocytes from the vaccinated mice were incubated with medium only (negative assay control), or stimulated with the Gag CD8+ T cell peptides (AMQ = AMQMLKDTI and TTST= TTSTLQEQI) or Gag CD4+ T cell peptides (MRC2, MRC13 and MRC17) and the amounts of IFN-γ (**A**), TNF-α (**B**), IL-4 (**C**) and IL-5 (**D**) measured by CBA assay. Each bar in the graphs represents pg of cytokine produced per ml in 48 hrs. Responses are positive if the SI is greater than 2 and is indicated.

4.6.3.2 HIV-1 Gag- and LPS-specific humoral immune responses

Induction of Gag-specific antibody (IgG) responses in mice vaccinated with AroC+wtGag, AroC+pGEM and pTHgagC was evaluated. On Day 28 (Experiment 1), no HIV-1 Gag-specific antibody responses were detectable. The OD_{405} ratio to prebleed of AroC+wtGag and AroC+pGEM (negative control) were below 2 (Figure 4.8A). On Day 84 (Experiment 2) there was no boosting of the Gag IgG antibody response (Figure 4.8A). As expected, the pTHgagC DNA vaccine did not also elicit antibody response in vaccinated mice (Figure 4.8A).

Salmonella LPS-specific IgG antibody response was further analyzed in vaccinated mice. All the *Salmonella* vaccines induced LPS-specific antibody responses on Day 28 (Figure 4.8B). AroC+wtGag induced LPS IgG response that was almost twice as much as in mice vaccinated with AroC+pGEM. On Day 84, the LPS IgG responses increased by a very small margin in mice vaccinated with AroC+wtGag, but the response increased almost twice in mice vaccinated with AroC+pGEM (Figure 4.8B).

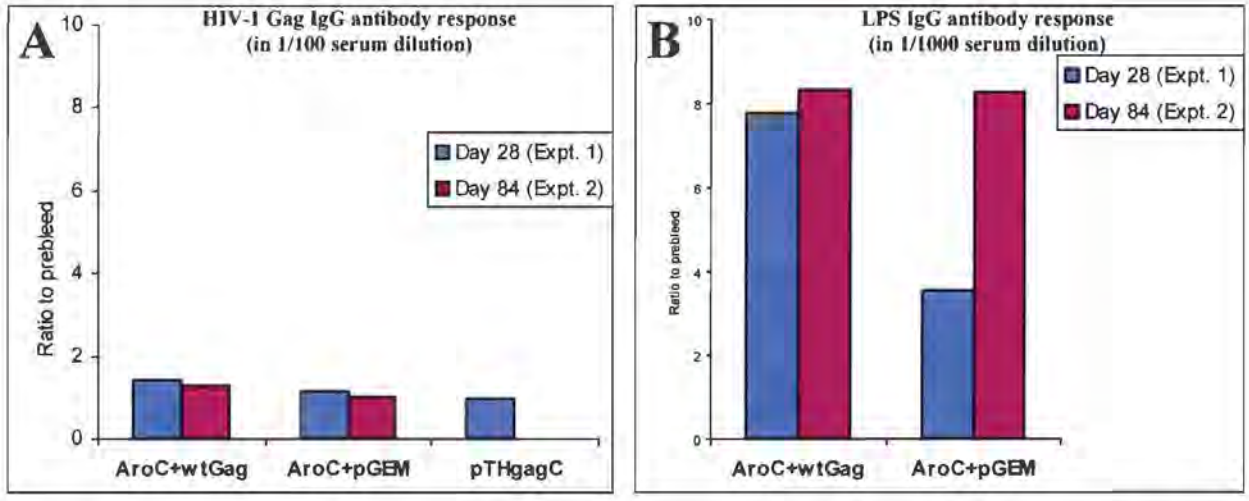

Figure 4.8: Serum HIV-1 Gag- and *Salmonella* LPS-specific IgG responses in mice vaccinated with *Salmonella* expressing wtGag. Groups of mice were vaccinated ONCE (Table 4.1) or THREE times (Table 4.2) with *Salmonella* vaccines, AroC+wtGag, AroC+pGEM or DNA vaccine (pTHgagC). Prior to inoculation, the mice were bled and pools of serum made. On Days 28 (Experiment 1) and 84 (Experiment 2) the mice were bled and serum made. (**A**) The HIV-1 Gag-specific IgG was measured serum in pools of each group of mice for Day 28 or Day 84 in 1/100 serum dilution. The data are represented as the ratios of the mean OD_{405nm} of duplicates to the mean OD_{405nm} prebleed duplicates. (**B**) The LPS-specific IgG was measured in serum pools of each group of mice for Day 28 or Day 84 in 1/1000 serum dilution. The data are represented as the ratios of the mean OD_{405nm} of duplicates to the mean OD_{405nm} prebleed duplicates. Antibody responses were defined as positive when the mean OD_{405nm} ratio to prebleed was >2.

4.7 DISCUSSION

Delivery of heterologous antigens to the mucosal surface by recombinant *Salmonella* vectors is probably one of the best strategies for inducing both mucosal and systemic immune responses (Sirard *et al.*, 1999). After oral vaccination, the *Salmonella* bacteria invade the mucosal surfaces and spread through the mesenteric lymph nodes to distal sites such as spleen and liver (Bradley *et al.*, 1994; Everest *et al.*, 2001). This is expected to result in induction of both mucosal and systemic cellular and humoral immune responses (Chen and Schifferli, 2000; Huang *et al.*, 2001; Chen and Schifferli, 2003; Salam *et al.*, 2006). The current study investigated the development and systemic immunogenicity of a recombinant attenuated live *Salmonella enterica* serovar Typhimurium vaccine vector expressing wild-type Du_{422} HIV-1 Subtype C Gag in mice after oral immunization.

4.7.1 Development of a recombinant *Salmonella* expressing wild-type HIV-1 Subtype C Gag

This study employed the strategy reported in Chapter 3 to develop efficient HIV-1 Gag expression plasmids, pGEM+wtGag and pGEM+GagK. Very high levels of expression of the antigens (wtGag and GagK) in recombinant *Salmonella* vaccine vectors were achieved (Figure 4.3). The key reasons for the successful overexpression of wtGag in the recombinant *Salmonella enterica* serovar Typhimurium vaccine vectors are the same as those outlined previously in Chapter 3 for GFP expression. The rational design of the expression plasmids was a critical factor in successful expression of the antigens in *Salmonella* (discussed in Chapter 3). Unlike GFP (Chapter 3), wtGag and GagK expression seemed to be toxic to bacterial cells, especially during stationary phase. The Gag expression also seemed to cause metabolic burden to the bacterial vector and this was anticipated to affect the efficiency of the vector in presenting sufficient foreign antigen to the immune system. Glick (1995) previously defined metabolic burden as the "amount of resources (raw material and energy) that is withdrawn from host's metabolism to the foreign DNA". The compounded effects of metabolic burden brought about by wtGag expression and its possible toxicity effects had potential negative effects on recombinant *Salmonella enterica* serovar Typhimurium growth and viability *in vivo* after vaccination of mice. The negative effects normally observed in such situations include reduced growth rate, reduced cell viability, vector instability and plasmid loss resulting in the emergence of mutants that overtake cultures (Villaverde and Carrio, 2003; Ventura and Villaverde, 2006). Corchero and Villaverde (1998) have also shown that the severity of the metabolic burden induced by the expression of heterologous antigens is specific for a given antigen. Some viral structural proteins antigens have domains that are toxic to bacterial vector and they include E1 from HCV, M2 from influenza virus, 3AB from polio virus, gp41 from HIV and they have stretches of hydrophobic domains, thought to cause pore formation in biological membranes (Ciccaglione *et al.*,1998, 2004; Pinto *et al.*, 1992; Towner *et al.*, 2003). The nature of HIV-1 Gag toxicity to bacterial cells is poorly understood.

Expression of heterologous antigens in recombinant *Salmonella enterica* serovar Typhimurium can also potentially result in degradation of the recombinant proteins by bacterial proteases. Although full-length Gag could be visible on SDS-PAGE, Western blotting analysis indicated that the Gag protein was

degrading (Figure 4.4). The several bands on the Western blots could also probably be due to the high frequency of Shine-Dalgarno (SD)-like sequences (which included sequences such as 5'-AGGA-3') and several start (ATG) codons in the *gag* gene. Gag expression could potentially be initiated at clusters of codons (AGG and AGA) that mimicked the Shine-Dalgarno sequences. The extent to which the *wtgag* mRNA could be degraded by the bacterial RNA degradosome was not investigated and it was not clear whether the inclusion of the *LacZα* leader sequence would reduce the degradation. Most studies to explain mRNA degradation have only been conducted in *E. coli* and the mechanisms are poorly understood. However, it has been shown that fusing some heterologous antigens to some 5' untranslated regions such as the highly structured *E. coli ompA* leader sequence could increase the stability of mRNA transcripts by preventing degradation (Emory *et al.*, 1992; Hansen *et al.*, 1994; Kushner, 2002).

The presence of rare codons such as AGG, AGA, TGT, CGA, CTA, TGC, CCA, CCC and CCU in the *wtgag* gene (Figure 4.3) could also play an important role in affecting expression of full-length Gag. Premature termination of translation at rare codons could result in production of several truncated Gag polypeptides of different molecular weight as observed in the current study. One of the possible reasons for the presence of several truncated Gag bands observed on SDS-PAGE and Western blot could be the lack codon-optimization for optimal expression in *Salmonella*. Since the *wtgag* gene was not optimized for codon usage in *Salmonella enterica* serovar Typhimurium, a number of problems of antigen expression were likely to be encountered. The utilization of rare codons in different species has been found to have biological significance in determining the rates of translation, protein folding, and degradation (Saier, 1995, Komar *et al* 1999; Roche and Sauer, 1999). In *Escherichia coli* and *Salmonella,* codon usage can affect the rate of protein translation and rare codons cause ribosome stalling during translation (Zhang *et al.*, 1994; Kupust *et al.*, 2002). Studies by Kupust *et al.*, (2002) have suggested that pausing of the ribosome at rare arginine codons (such as AGA, AGG) triggers degradation of partially synthesized recombinant polypeptides in *E. coli.* This could account for the presence of several bands observed on Gag Western blot. It was therefore proposed that the *gag* gene be optimized for codon usage in *Salmonella enterica* serovar Typhimurium as this was though to improve stable expression of full-length Gag (Chapter 5). Therefore, despite the high expression, the toxicity to the vector and the lack of codon-optimization of the gene were likely to have a negative impact on the delivery of the HIV-1 Gag antigen to the immune system by the recombinant *Salmonella* vaccine vector.

4.7.2 HIV-1 specific CD8+ Tc1/Tc2 cytokine responses

Systemic cellular immunogenicity of recombinant *Salmonella enterica* serovar Typhimurium expressing full-length wild-type HIV-1 Gag was evaluated after primary and secondary vaccination of mice intragastrically. No HIV-1 Gag-specific CD8+ Tc1 and Tc2 cytokine responses were detectable in vaccinated mice (Figures 4.5 and 4.7). A number of possible factors could explain the failure for the delivery of the Gag antigen to the MHC-1 molecules for induction of strong CD8+ T cell cytokine responses. First, the phenomenon of immunodominance could be at play. The two H-2K^d restricted Class I Gag peptides (AMQMLKDTI and TTSTLQEQI) were probably sub-dominant when presented together

with the whole plethora of *Salmonella* antigenic peptides. The second factor could be poor cross-presentation of the Gag peptides to the MHC-1 molecules. Cross-presentation of antigens to the MHC-1 molecules is a very inefficient process and it is regulated by many aspects such as dose and route of immunization (Maecker *et al.*, 2001). The third factor could be the formation of inclusion bodies in the bacterial vector. Particulate antigens (inclusion bodies in this case) cannot be efficiently cross-presented to the MHC-1 molecules to give CD8+ T cell responses, compared to soluble antigens such as GFP which was soluble in the vector. Predictions using Wilkinson and Harrison model indicated that wtGag and GagK antigens were highly insoluble. A study by Hone *et al.*, (1996) showed that when HIV-1 gp120 was expressed cytoplasmically in a *Salmonella* vaccine vector, it formed inclusion bodies. Oral vaccination of mice with the recombinant vector failed to induce systemic HIV-1 gp120-specific CD8+ CTLs (Hone *et al.*, 1996). It was suggested that the solubility of the antigen could affect its cross-presentation to the MHC-I molecules when expressed in *Salmonella*. Another possible reason for failure to detect Gag-specific CD8+ T cell responses could be the lack of CD4+ T help, although in human HIV-1 infection, Gag responses are known to be CD4+ T-cell dependent as contrasted to Env responses which are T-cell independent (Binley *et al.*, 1997). The instability of the *Salmonella* vector expressing wtGag potentially account for the lack of its immune response in mice.

4.7.3 HIV-1 specific CD4+ Th1/Th2 cytokine responses

In the current study, Gag-specific CD4+ Th cell responses were investigated. No HIV-1 Gag-specific Th1 or Th2 cytokine responses were induced in mice after single oral vaccination with the recombinant *Salmonella* vaccine vector. Multiple booster vaccinations were necessary for induction of detectable immune responses. After two booster vaccinations of mice no CD4+ Th1 cytokine responses were observed by both ELISPOT and CBA assays (Figures 4.5 and 4.7). However, HIV-1 Gag-specific Th2 cytokine (IL-4) responses were observed. The reasons for induction of only Gag-specific IL-4, but not IFN-γ responses remained unclear. However, there are a number of factors that may affect the differentiation of CD4+ T cells into either Th1 or Th2 and they include dose, form of antigen, the affinity of the peptide–T-cell-receptor interaction, route of vaccination and the cytokine milieu (Constant and Bottomly, 1997; Constant *et al.*, 2001). The failure by the *Salmonella* vector, AroC+Gag to induce Th1 responses could explain why no HIV-1 Gag-specific CD8+ T cell responses were generated. Th1 responses have been known to provide help for activation of CD8+ T cells and macrophages. It seemed in this study that such help was not provided for the induction of HIV-1 Gag-specific CD8+ T cell responses. The induction of IL-4, the cardinal cytokine of Th2 responses, promotes B-cell activation and humoral immunity (Spellberg and Edwards, 2001). However, HIV-1 Gag-specific antibody response was not induced (Section 4.7.4). The instability of the *Salmonella* vector might also have affected the delivery of the Gag to the immune system.

The scientific and clinical relevance of vaccine-induced (Th2) IL-4 response in the field of HIV vaccinology is still not well understood. Studies by Clerici and Shearer (1994) have proposed a Th1-to-Th2 switch hypothesis in which the response shifts from CD4+ Th1 to Th2 during AIDS disease

progression in HIV-infected individuals. Studies in both children and adults indicate that HIV-1 infection is associated with higher levels of IL-4 production (Clerici *et al.*, 1993; Clerici and Shearer, 1994). On the other hand, some studies have suggested a suppressive effect of IL-4 on HIV-1 replication (Wang *et al.*, 1998). In the study reported in this Chapter, it was not clear whether there was any shift from Th1 to Th2 responses, as no Th1 responses were ever detected with the assays employed. It was also not clear whether such vaccine-induced CD4+ Th2 responses demonstrated in the study would promote disease progression or antibody responses. In HIV-1 vaccinology, the induction of CD4+ Th1 is more critical than the Th2 response (Chapter 1). Therefore the importance of a vaccine that imprints Th2-biased responses needs to be investigated in future studies.

4.7.4 HIV-1 specific humoral immune responses

The failure of the recombinant *Salmonella* vaccine (AroC+wtGag) to induce HIV-1 Gag-specific antibody responses (Figures 4.8A) could be attributed to a number of reasons. The metabolic burden placed on the bacteria by the Gag expression could result in reduced amount of antigen being delivered to the immune system as bacteria lost plasmid *in vivo* after vaccination. It has been shown by other studies that the nature of the immune response induced may correlate positively with antigen abundance (Rollenhagen, *et al.*, 2004). Due to metabolic burden, the recombinant bacterial vector may not deliver enough antigen to the immune system after vaccination (Galen and Levine, 2001; Knodler *et al.*, 2005). The cellular toxicity of Gag might also have caused reduced the viability of the bacteria in invading the mucosal system of mice for induction of high serum antibodies. Another potential reason for failure to induce antibody response was that the wtGag was potentially forming insoluble particles. Although particulate antigens can induce antibody responses, the efficiency cannot be compared with soluble antigens that are capable of inducing high titres of antibodies when delivered with *Salmonella* vectors. The post-translation proteolytic degradation of full-length Gag potentially affected its humoral immune responses. In spite of the absence of Gag-specific IgG, the vaccinated mice elicited high levels of *Salmonella* LPS-specific IgG antibody responses (Figures 4.8). This suggested that the live vaccine vectors successfully invaded the mucosa-associated lymphoid tissues after single inoculation.

In conclusion, full-length wild-type HIV-1 *gag* and its truncated version, *gagK* sequences were successfully cloned and expressed at high levels in *Salmonella enterica* serovar Typhimurium vaccine vector using the *E. coli lac* promoter. Despite the high expression of the wild-type Gag by the recombinant *Salmonella* vector, only HIV-1-specific CD4+ Th2 cytokine responses were induced in vaccinated mice. The reasons for the imprinting of only Gag-specific Th2 responses by the vector were not clear. The lack of codon-optimization of *wtgag* for expression in the *Salmonella* vector was potentially one of the factors that affected the nature, quality and magnitude of the immune responses. It was recommended that the gene be optimized for expression in *Salmonella enterica* serovar Typhimurium (Chapter 5).

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CHAPTER 5: DEVELOPMENT AND IMMUNOGENICITY OF A RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR EXPRESSING CODON-OPTIMIZED HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C GAG

"Change is not made without inconvenience, even from worse to better" Richard Hooker (1554-1600)

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CHAPTER 5: DEVELOPMENT AND IMMUNOGENICITY OF A RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR EXPRESSING CODON-OPTIMIZED HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C GAG

5.1 SUMMARY

No previous studies have interrogated the impact of codon optimization of HIV-1 subtype C Gag on its cellular and humoral immunogenicity in mice when delivered by a recombinant *Salmonella* vaccine vector. It was therefore the objective of the current study to develop and investigate the immunogenicity of a recombinant *Salmonella enterica* serovar Typhimurium vaccine expressing codon-optimized HIV-1 subtype C Gag (Salmgag). Du_{422} (wild-type) HIV-1 *gag* gene (reported in Chapter 4) was codon-optimized to include the most frequently used *Salmonella enterica* serovar Typhimurium codons. The artificially synthesized *salmgag* gene was cloned into the backbone of pGEM+GFP plasmid (reported in Chapter 3) to generate pGEM+Salmgag plasmid. The recombinant plasmid was used to generate a recombinant *AroC Salmonella enterica* serovar Typhimurium vaccine, AroC+Salmgag. Expression of high levels of Salmgag antigen by the vaccine was demonstrated. To investigate the immunogenicity of the vaccine, groups of mice (n=5) were orally vaccinated once or boosted twice with the vaccine. Mice primed with the *Salmonella* vaccine did not elicit HIV-1 Gag-specific CD8+ or CD4+ T-cell cytokine responses as determined by ELISPOT and CBA assays. However, mice which received booster vaccinations elicited HIV-1 Gag-specific CD8+ Tc1 cytokine (IFN-γ) responses (2.6-fold above background). The mice also elicited mixed HIV-1 Gag-specific CD4+ Th1 (TNF-α and IFN-γ) and Th2 (IL-4 and IL-5) cytokine responses. The CD4+ Th cytokines levels secreted by stimulated splenocytes were 29.1-fold (TNF-α), 7.5-fold (IFN-γ), 26.2-fold (IL-4) and ≥89.3-fold (IL-5) above the background. Vaccinated mice elicited low HIV-1 Gag-specific IgG responses by Day 28 (1.5-fold above prebleed in 1/100 serum dilution). The humoral responses were improved by Day 56 (5.2-fold above prebleed in 1/100 serum dilution) and further increased by Day 84 (22.2-fold above prebleed in 1/100 serum dilution). Further analysis of the Gag-specific IgG subclasses in mice showed the presence of both IgG1 and IgG2a with IgG2a being greater than IgG1, and this further confirmed that mixed HIV-1 Gag-specific CD4+ Th1 and Th2 responses were elicited. Codon optimization of the HIV-1 *gag* gene, therefore, potentially enhanced its immunogenicity when expressed by a recombinant *Salmonella* vaccine vector.

5.2 INTRODUCTION

Codon usage bias occurs during heterologous gene expression in both prokaryotic and eukaryotic systems (Grantham *et al.*, 1980; Ermolaeva, 2001; Sinclair and Choy, 2002; Gustafsson *et al.*, 2004; Sorensen and Mortensen, 2005). The bias affects optimal expression of the heterologous genes which depends mostly on the abundance of cognate tRNAs within the cell (Lithwick and Margalit, 2003). When foreign genes are not codon-optimized, there are a number of translational hurdles associated with the presence of rare codons. These hurdles include mistranslational amino acid substitutions, frame-shifting events or premature translational termination (Kane, 1995; McNulty *et al.*, 2003). The presence of the

rare codons may further affect foreign protein accumulation, mRNA and plasmid stability, and in some cases, inhibit protein synthesis and cell growth (Zahn, 1996; Ejdeback *et al.*, 1997; Baneyx, 1999; Wu *et al.*, 2004; Kim and Lee, 2006). Protein quality can also be affected by codon bias as it has been observed in *E. coli* that insertion of lysine for arginine at the AGA rare codon could happen (Seetharam *et al* 1988). However, some studies have found weak correlation between codon optimization and gene expression (Ikemura, 1981; Duret and Mouchiroud, 1999; Urrutia and Hurst, 2003). In *Salmonella* vaccine vectors, Baud *et al.*, (2004b) found that the levels of HPV L1 expression decreased when the gene was codon-optimized. The reasons for the decrease in L1 were not clearly defined by the study. However, it was noted that the recombinant *Salmonella* vector expressing the codon-optimized L1 gave better immune responses in mice than the vector expressing the wild-type L1 (Baud *et al.*, 2004b).

In previous studies, the fusion of wild-type HIV-1 Subtype C *gag* with the 5' domain of the *E coli* β-galactosidase α-gene fragment as a strategy to improve expression in *Salmonella* vector was reported (Chapter 4). Although expression of Gag was improved by the strategy, only CD4+ Th2 immune responses were generated in mice vaccinated with the recombinant *Salmonella* vector. One of the key factors that could affect the *in vivo* expression of wtGag by the *Salmonella* vector after vaccination was lack of codon-optimization of the gene. The *wtgag* gene had a number of codons which were rare in *Salmonella* (see Table 5.1 for codon frequencies). Examples of such rare codons included AGG, AGA, TGT and TGC. Some of these rare codons were present in *wtgag* at very high frequencies (Figure 4.3). It was therefore critically important to investigate in this study the impact of codon optimization of the

Table 5.1: Codon frequencies in *Salmonella enterica subsp. enterica serovar Typhimurium*. The triplet codons and their respective frequencies per thousand codons are shown. Rare (minor) codons have lower frequencies than major (commonly used) codons.

UUU 20.8	UCU 10.0	UAU 16.0	UGU 5.2
UUC 14.5	UCC 11.3	UAC 12.3	UGC 6.9
UUA 12.8	UCA 10.4	UAA 1.5	UGA 1.3
UUG 12.2	UCG 9.2	UAG 0.4	UGG 14.9
CUU 13.6	CCU 8.2	CAU 11.4	CGU 13.4
CUC 11.6	CCC 8.4	CAC 10.5	CGC 20.4
CUA 6.4	CCA 7.4	CAA 14.1	CGA 5.5
CUG 46.9	CCG 20.5	CAG 27.6	CGG 10.5
AUU 24.8	ACU 10.0	AAU 18.7	AGU 9.3
AUC 23.3	ACC 22.1	AAC 20.7	AGC 17.5
AUA 8.8	ACA 9.1	AAA 29.3	AGA 6.1
AUG 22.6	ACG 16.3	AAG 14.9	AGG 3.9
GUU 17.5	GCU 17.4	GAU 32.2	GGU 18.4
GUC 16.5	GCC 32.2	GAC 20.8	GGC 30.3
GUA 10.9	GCA 18.2	GAA 34.5	GGA 11.6
GUG 21.2	GCG 31.0	GAG 21.6	GGG 12.1

(Reference: http://www.kazusa.or.jp/codon).

gene on its immunogenicity when delivered by a recombinant *Salmonella enterica subsp. enterica serovar Typhimurium* vaccine vector. In brief, the objectives of this study were:

1. to construct a recombinant expression plasmid, pGEM+Salmgag with the full-length codon-optimized wild-type HIV-1 *gag* gene (*salmgag*) for expression in *Salmonella enterica* serovar Typhimurium.
2. to develop a recombinant *Salmonella* vaccine vector expressing Salmgag.
3. to evaluate the induction of HIV-1 Gag-specific immune responses (both humoral and cellular) in mice orally vaccinated with the recombinant *Salmonella* vaccine vector expressing Salmgag.

5.3 MATERIALS

Materials used in the study have been given in the Appendices and Chapters 3 and 4.

5.4 GENERAL METHODS

All the microbiological, molecular and immunological methods used in the study have been described in Appendices A and B. Comparative sequence analyses of wild-type HIV-1 *gag* and *salmgag* were performed by GeneOptimizer™ software program (Geneart, USA).

5.5 EXPERIMENTAL DESIGN AND PROTOCOLS

5.5.1 Codon optimization of wild-type HIV-1 *gag* gene to *salmgag*

The *salmgag* gene was assembled from synthetic oligonucleotides (Geneart, USA). The gene was received from Geneart (USA) cloned into pPCR-script (Stratagene, U.S.A) at *Xho*I and *Sac*I restriction sites to give the recombinant plasmid pSCRIPT+salmgag (Appendix G10).

5.5.2 Construction of recombinant pGEM+Salmgag expression plasmid: molecular cloning of *salmgag* gene

The construction of recombinant *salmgag* expression plasmid, pGEM+Salmgag (Appendices G6 and H3) involved the same cloning strategy described in Chapter 4 for pGEM+wtGag. The *salmgag* gene was cloned into the backbone of pGEM+GFP (diagrammatically summarized in Figure 5.1). The pGEM+GFP vector backbone was prepared by double restriction digestion with *Nar*I/*Hind*III (to release out the *gfp* fragment). The *salmgag* gene in Pscript+salmgag (Appendix G10) was also digested with *Nar*I/*Hind*III and the gene fragment separated by agarose gel electrophoresis (Appendix B1.4) and purified from the agarose (Appendix B1.6). The *salmgag* gene fragment was ligated to the linearized pGEM+GFP backbone as described in Appendix B1.7. The ligation reaction was used in transforming competent SCS110 cells (Appendix A1.4). Candidate recombinant SCS110 clones with pGEM+Salmgag plasmid were screened using restriction enzyme mapping (Appendix B1.5) after mini-plasmid isolation (Appendix B). Maximum plasmid isolation (Appendix B1.5) was performed on a selected candidate clone. The *salmgag* gene in pGEM+Salmgag plasmid was sequenced as described in Appendix B1.8.

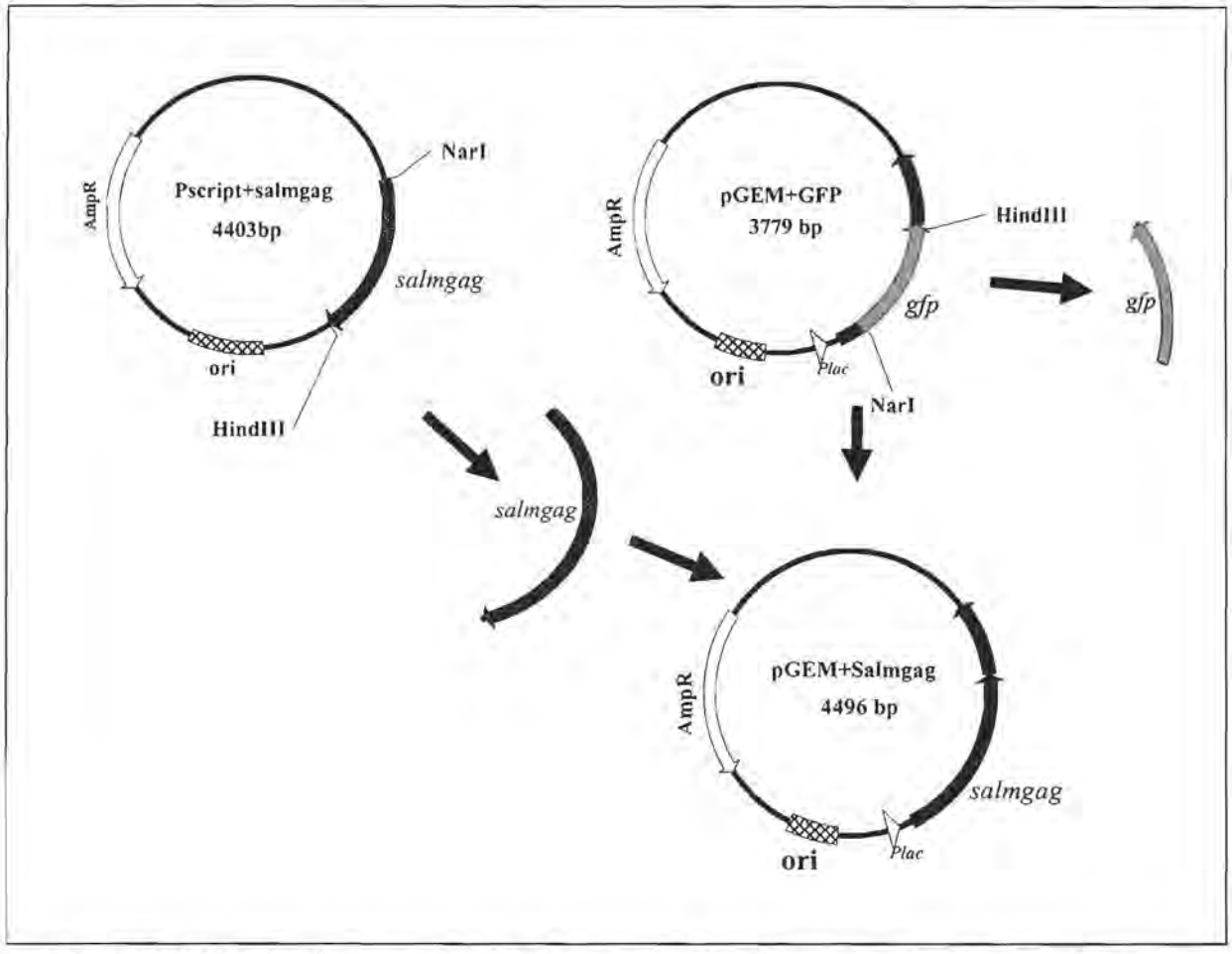

Figure 5.1: Molecular cloning strategy of *salmgag* gene. The *salmgag* gene from Pscript+salmgag was cloned into the backbone of pGEM+GFP to generate pGEM+Salmgag.

5.5.3 Development of recombinant *Salmonella* expressing Salmgag

The genetic transformation of competent *AroC Salmonella enterica* serovar Typhimurium mutant was performed as described in Appendix A1.4 with recombinant plasmid, pGEM+Salmgag. This resulted in the generation of a *Salmonella* vaccine clone, AroC+Salmgag. Salmgag expression by recombinant *E. coli* SCS110 clone, SCS110+Salmgag was assessed by 12.5 % SDS-PAGE (Appendix B4) and Western blotting (Appendix B6). Salmgag antigen expression by AroC+Salmgag was assessed by 12.5 % SDS-PAGE (Appendix B4). The relative amount of Salmgag antigen in culture lysates was further determined by the Roche Elecsys® HIV p24 Ag assay according to manufacturer's instructions. The protein lysates were diluted (1/100 or 1/1000) in water and expression of Salmgag evaluated by the Elecysys® 2010 analyzer.

5.5.4 Vaccination of mice with recombinant *Salmonella* vaccine vector

AroC+Salmgag and AroC+pGEM (negative control) were prepared as described in Appendix A2.1. Two groups (n=5) of mice were vaccinated by the oral route as described in Appendix A2.3 with AroC+Salmgag and AroC+pGEM (Tables 5.1 and 5.2). Blood was collected from mice prior to every

vaccination and sacrifice and processed for ELISA. At sacrifice, the pooled spleens for each group of mice were processed for ELISPOT and CBA assays as described in Apendix B7.

Table 5.2: Vaccines and vaccination protocol for Experiment 1. Mice were inoculated ONCE by intragastric gavage (Appendix A2.3) with *Salmonella* vaccines, AroC+Salmgag and AroC+pGEM. The mice were bled prior to inoculation and sacrifice. The blood was processed by centrifugation and sera were stored until evaluation of humoral immune responses as described in Appendix B10. At sacrifice on Day 28, the spleens from each group of mice were pooled and processed as described in Appendix B7.

Inoculum (vaccine)	Inoculum/animal/ inoculation	Inoculation date	Sacrifice date
AroC + Salmgag	10^8 cfu	Day 0	Day 28
AroC + pGEM	10^8 cfu	Day 0	Day 28

Table 5.3: Vaccines and vaccination protocol for Experiment 2. Mice were inoculated THREE times by intragastric gavage (Appendix A2.3) on Days 0, 28 and 56 with *Salmonella* vaccines, AroC+Salmgag and AroC+pGEM. The mice were bled prior to inoculation and sacrifice. The blood was processed by centrifugation and sera were stored until evaluation of humoral immune responses as described in Appendix B10. At sacrifice, the spleens from each group of mice were pooled and processed as described in Appendix B7.

Inoculum (vaccine)	Inoculum/animal/ inoculation	Inoculation date	Sacrifice date
AroC + Salmgag	10^8cfu	Day 0, Day 28, Day 56	Day 84
AroC + pGEM	10^8cfu	Day 0, Day 28, Day 56	Day 84

5.5.5 Assessment of T cell responses in the spleen

HIV-1 Gag-specific CD4+ Th1/Th2 and CD8+ Tc1/Tc2 cytokine responses in the spleen were determined by ELISPOT and CBA assays as previously described (Chapter 4).

5.5.6 Assessment of HIV-1 Gag-specific humoral immune responses

The evaluation of HIV-1 Gag-specific IgG antibody responses to the *Salmonella* vaccine vector expressing Salmgag was performed as described in Chapter 4. HIV-1 Gag-specific IgG1 and IgG2a isotype responses were also determined by p55 ELISA for serum pools as described in Appendix B10. The NEW LAV BLOT kit (Appendix B6.3) was used to confirm the presence of HIV-1 Gag-specific antibodies in sera of mice from the vaccine group, AroC+Salmgag from Experiment 2.

5.6 RESULTS

5.6.1 Comparative HIV-1 *gag* (*wtgag*) and *salmgag* gene sequence analysis

The wild-type HIV-1 *gag* (*wtgag*) gene, originally cloned into pGEM+Gag (Chapter 4), was codon-optimized (*salmgag*) for expression in *Salmonella enterica subsp. enterica serovar* Typhimurium. Analyses of the wild-type *gag* (*wtgag*) gene had previously indicated the presence of several rare *S. enterica subsp. enterica serovar* Typhimurium codons such as AGA, AGG, CGA, CUA, CUG and AUA (Chapter 4). Comparative analysis of the two genes (*wtgag* and *salmgag*) showed that most of the codons in *salmgag* were now those frequently found in *Salmonella enterica subsp. enterica serovar* Typhimurium, unlike the *wtgag* which also had several rare codons (Figure 5.2A and 5.2B).

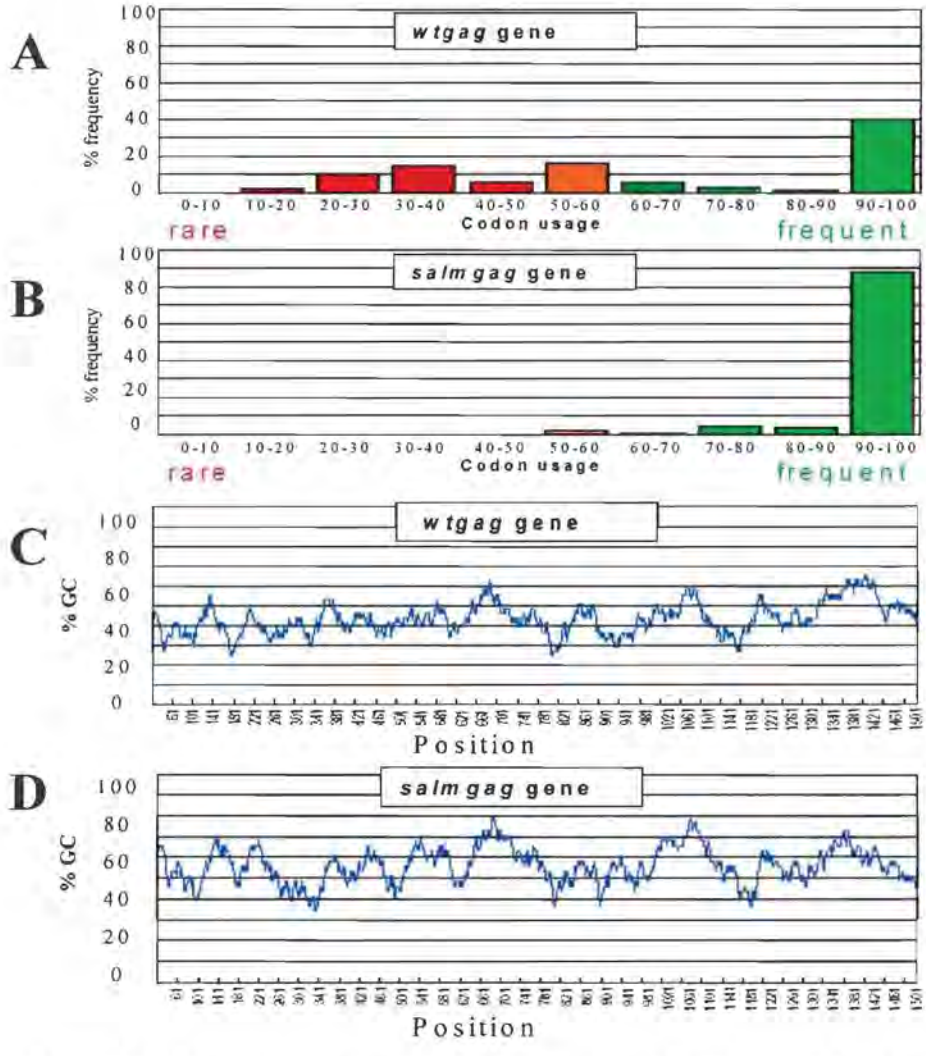

Figure 5.2: Comparative analysis of codon usage and G+C content in *Salmonella enterica* serovar Typhimurium of *wtgag* and *salmgag*. **(A)** Codon usage of *wtgag* and *salmgag* in *Salmonella enterica* serovar Typhimurium of *wtgag* and *salmgag* was analyzed. The most frequently used codon was set to 100 and the remaining were scaled accordingly. The histogram represents the overall codon frequency within the coding region; for example red bars indicated that the frequency of extremely rare codons and the green bars indicated the most frequently used codons in *Salmonella enterica* serovar Typhimurium. **(B)** Comparative analysis of G+C content within the coding regions of wild-type *wtgag* and *salmgag*. The plots represent the %G+C content at the indicated positions.

There were some sequence motifs in *wtgag* which could negatively influence the expression of the gene in the *Salmonella* vaccine vector. These motifs included internal Shine-Dalgarno-like sequences, TATA - boxes, Chi recombination sites and a -35 box. The motifs were removed during codon optimization and were not therefore present in *salmgag*. A+T-rich or G+C-rich sequence stretches, repeat sequences and RNA secondary structures were also avoided during codon optimization. The strong deviations in G+C content (>80%; <30%) were avoided where possible and the total G+C content was adjusted to ~50% (Figure 5.2C and D).

5.6.2: Development of *salmgag* expression plasmid vectors: molecular cloning of *salmgag* gene

The *salmgag* gene was successfully cloned to generate a recombinant expression plasmid, pGEM+Salmgag (Appendices G6 and H3). The generalized structure of the recombinant plasmid with the full-length *salmgag* gene is shown in Figure 5.3A. All the necessary transcriptional and translational structures for efficient gene expression were included in the expression plasmid. The recombinant *salmgag* gene in pGEM+Salmgag was sequenced and was found to have all the necessary domains. The DNA and amino acid sequences of the full-length *salmgag* gene fused to the first 40 first codons of the β-galactosidase α-fragment are shown in Figure 5.3B.

A

B

```
1     ATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCC      5'-LacZα
1      M  T  M  I  T  P  S  Y  L  G  D  T  I  E  Y  S  S  Y  A  S
61    AACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGAT
21     N  A  L  G  A  L  P  Y  G  R  P  A  G  G  R  E  F  T  S  D
121   TATGGCGCCCGCGCGAGCATTCTGCGCGGCGAAAAACTGGATACCTGGGAAAAAATTCGC
41     Y  G  A  R  A  S  I  L  R  G  E  K  L  D  T  W  E  K  I  R
181   CTGCGCCCGGGTGGCAAAAAACATTATATGCTGAAACACATCGTTTGGGCGAGCCGCGAA
61     L  R  P  G  G  K  K  H  Y  M  L  K  H  I  V  W  A  S  R  E
241   CTGGAACGCTTTGCGCTGAACCCGGGCCTGCTGGAAACCAGCGAAGGCTGTAAACAGATC
81     L  E  R  F  A  L  N  P  G  L  L  E  T  S  E  G  C  K  Q  I
301   ATCAAACAGCTGCAGCCGGCGCTGCAGACCGGCACCGAAGAACTGAAGAGCCTGTACGAT
101    I  K  Q  L  Q  P  A  L  Q  T  G  T  E  E  L  K  S  L  Y  D
361   ACCGTTGCGACCCTGTATTGTGTGCATGAAAAAATCGAAGTGCGCGATACCAAAGAAGCC
121    T  V  A  T  L  Y  C  V  H  E  K  I  E  V  R  D  T  K  E  A
421   CTGGATAAAATCGAAGAAGAACAGAACAAATGTCAGCAGAAAACGCAGCAGGCGAAAGCG
141    L  D  K  I  E  E  E  Q  N  K  C  Q  Q  K  T  Q  Q  A  K  A
481   GCGGATGGCAAAGTCAGCCAGAATTATCCGATCGTGCAGAATCTGCAGGGCCAGATGGTT
161    A  D  G  K  V  S  Q  N  Y  P  I  V  Q  N  L  Q  G  Q  M  V
541   CATCAGGCCATTAGCCCGCGCACCCTGAATGCGTGGGTGAAAGTGATCGAAGAAAAAGCG
181    H  Q  A  I  S  P  R  T  L  N  A  W  V  K  V  I  E  E  K  A
601   TTTAGCCCGGAAGTCATTCCGATGTTTACCGCGCTGAGCGAAGGCGCGACCCCGCAGGAT
201    F  S  P  E  V  I  P  M  F  T  A  L  S  E  G  A  T  P  Q  D      salmgag
661   CTGAACACCATGCTGAATACGGTGGGCGGCCATCAGGCGGCCATGCAGATGCTGAAAGAT
221    L  N  T  M  L  N  T  V  G  G  H  Q  A  A  M  Q  M  L  K  D
721   ACCATCAATGAAGAAGCGGCGGAATGGGATCGCCTGCATCCGGTTCATGCGGGCCCGATT
241    T  I  N  E  E  A  A  E  W  D  R  L  H  P  V  H  A  G  P  I
781   GCGCCGGGCCAGATGCGCGAACCGCGCGGCAGCGATATTGCGGGCACCACGAGCACCCTG
261    A  P  G  Q  M  R  E  P  R  G  S  D  I  A  G  T  T  S  T  L
841   CAGGAACAGATTGCCTGGATGACCAGCAACCCGCCGATTCCGGTGGGCGATATCTATAAA
281    Q  E  Q  I  A  W  M  T  S  N  P  P  I  P  V  G  D  I  Y  K
901   CGCTGGATCATTCTGGGCCTGAATAAAATCGTGCGCATGTATAGCCCGGTCAGCATTCTG
301    R  W  I  I  L  G  L  N  K  I  V  R  M  Y  S  P  V  S  I  L
961   GATATTCGCCAGGGCCCGAAAGAACCGTTTCGCGATTATGTCGATCGCTTTTTTAAAACC
321    D  I  R  Q  G  P  K  E  P  F  R  D  Y  V  D  R  F  F  K  T
1021  CTGCGCGCGGAACAGGCGACCCAGGAAGTCAAAAATTGGATGACCGATACCCTGCTGGTC
341    L  R  A  E  Q  A  T  Q  E  V  K  N  W  M  T  D  T  L  L  V
1081  CAGAATGCGAACCCGGATTGTAAAACCATTCTGCGCGCGCTGGGCCCGGGTGCGACCCTG
361    Q  N  A  N  P  D  C  K  T  I  L  R  A  L  G  P  G  A  T  L
1141  GAAGAAATGATGACCGCCTGCCAGGGCGTGGGCGGTCCGGGCCACAAAGCGCGCGTTCTG
381    E  E  M  M  T  A  C  Q  G  V  G  G  P  G  H  K  A  R  V  L
1201  GCGGAAGCGATGAGCCAGACCAACAGCGGCAACATCATGATGCAGCGCAGCAACTTTAAA
401    A  E  A  M  S  Q  T  N  S  G  N  I  M  M  Q  R  S  N  F  K
1261  GGCCCGCGTCGCATTGTGAAATGTTTTAACTGTGGTAAAGAAGGCCACATTGCCCGCAAT
421    G  P  R  R  I  V  K  C  F  N  C  G  K  E  G  H  I  A  R  N
1321  TGCCGCGCACCGCGCAAAAAAGGCTGTTGGAAATGCGGCAAAGAGGGCCACCAGATGAAA
441    C  R  A  P  R  K  K  G  C  W  K  C  G  K  E  G  H  Q  M  K
1381  GATTGCACCGAACGCCAGGCCAATTTTCTGGGCAAAATTTGGCCGAGCCACAAAGGCCGC
461    D  C  T  E  R  Q  A  N  F  L  G  K  I  W  P  S  H  K  G  R
1441  CCGGGCAATTTTCTGCAGAACCGCCCGGAACCGACCGCACCGCCGGCGGAATCATTTCGC
481    P  G  N  F  L  Q  N  R  P  E  P  T  A  P  P  A  E  S  F  R
1501  CTGGAAGAAACCACCCCGGCCCCGAAACAGGAACCGATTGAACGCGAACCGCTGACCAGC
501    L  E  E  T  T  P  A  P  K  Q  E  P  I  E  R  E  P  L  T  S
1561  CTGAAATCACTGTTTGGCAGCGATCCGCTGAGCCAAGCTTAAT                    ← Stop codon
521    L  K  S  L  F  G  S  D  P  L  S  Q  A  *
```

Figure 5.3: The general structural components of the Salmgag expression construct developed by the study. (**A**) A schematic representation of the Salmgag expression plasmid. The Salmgag was fused with the N-terminal domain (first 40 amino acids) of the β-galactosidase α-fragment in pGEM-Teasy plasmid. The expression was under the *E. coli lac* promoter and other *lac* operon transcription and translation domains found in pGEM-Teasy plasmid. (**B**) The DNA and amino acid sequences of the resultant *salmgag* gene and Salmgag protein as a fusion gene/protein with the β-galactosidase α-fragment. The whole fusion protein was 59.423 KDa. A preferred stop codon (TAAT) was included at the end of the *salmgag* gene to prevent translation of the whole *LacZα* gene. The main features and complete DNA sequence of pGEM+Salmgag plasmid are given in Appendices G6 and H3 respectively.

5.6.3 Recombinant *E. coli* and *Salmonella* vector expressing Salmgag

The expression of Salmgag in recombinant *E. coli* or *Salmonella enterica* serovar Typhimurium vaccine vector harbouring the recombinant expression plasmid, pGEM+Salmgag was explored. In *E. coli*, the

expression of Salmgag was determined by SDS-PAGE and Western blotting. High level antigen expression was demonstrated by SDS-PAGE (Figure 5.4A). The Salmgag protein band was visible on the Commassie-stained PAGE. The protein band was confirmed by Western blotting (Figure 5.4A) Analysis of protein expression by recombinant *Salmonella* vaccine vector by SDS-PAGE also showed that the Salmgag antigen was being expressed at high levels (Figure 5.4B). The Salmgag protein band was also visible on the Coomassie blue stained SDS-PAGE gel and was therefore not confirmed by

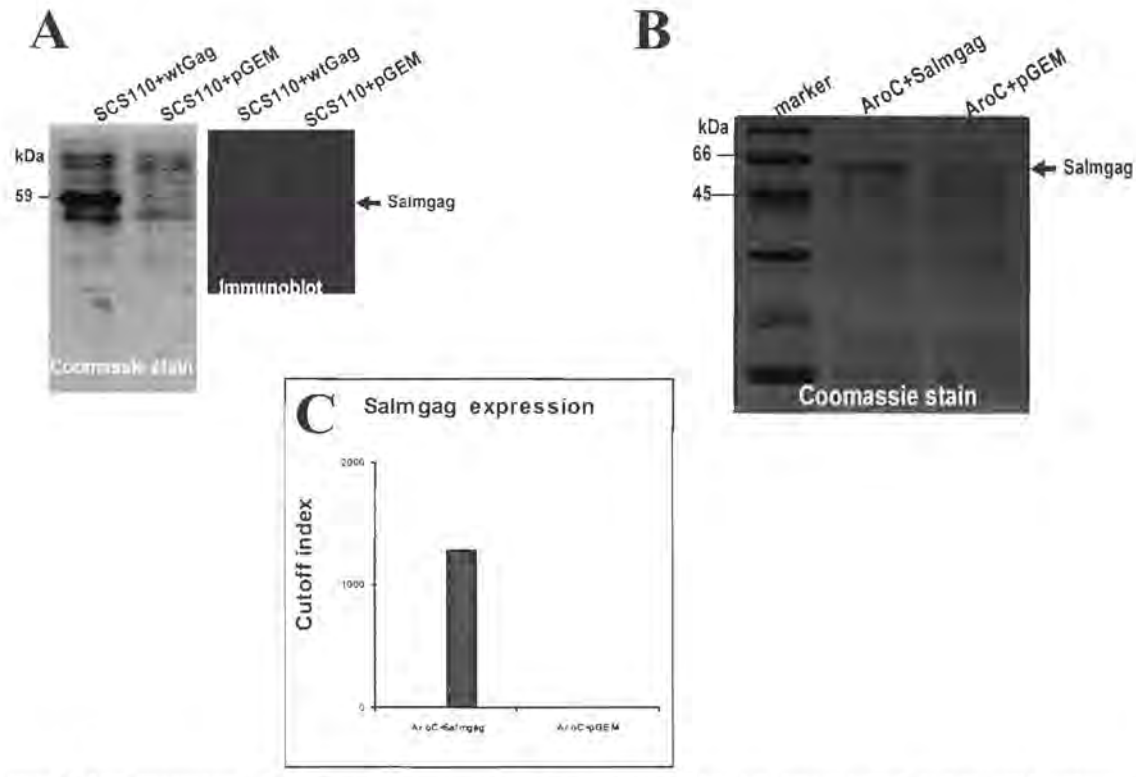

Figure 5.4: Expression of Salmgag in recombinant *E. coli* (SCS110+Salmgag) and *Salmonella enterica* serovar Typhimurium (AroC+Salmgag). (**A**) SDS-PAGE and Western blotting analysis of recombinant *E. coli* (SCS110+Samgag) expressing Salmgag. The Salmgag protein band was visible on both a Coomassie blue-stained SDS-PAGE and Western blot. (**B**) SDS-PAGE analysis of recombinant *Salmonella enterica* serovar Typhimurium (AroC+Salmgag) expressing Salmgag. The Salmgag protein band was visible on the Coomassie blue-stained SDS-PAGE. (**C**) The relative expression of Salmgag by recombinant *Salmonella* vaccine (AroC+Salmgag) as determined by Roche Elecsys® HIV p24 Ag assay. The total bacterial protein lysate was diluted 1/1000 in water. Protein lysate from the negative control, AroC+pGEM was also included. The cutoff-off index was calculated by the Elecsys® 2010 analyzer using readings from the negative and positive calibrators.

Western blotting. The Salmgag antigen was the most abundantly expressed protein in the bacterial vector. The expression of Salmgag by AroC+Salmgag was further demonstrated by the Roche Elecsys® HIV p24 Ag assay. It was noted that expression of Salmgag led to lysis of *Salmonella* bacterial cells. The bacterial colonies were translucent and this suggested that the bacteria were dying especially in the stationary phase of growth (results not shown)

5.6.4 Immunogenicity of a recombinant *Salmonella* vaccine vector expressing Salmgag

5.6.4.1 CD8+ Tc and CD4+ Th cytokine responses to recombinant *Salmonella* expressing Salmgag

The nature of HIV-1 Gag-specific systemic CD8+ Tc1/Tc2 and CD4+ Th1/Th2 cytokine responses to recombinant *Salmonella enterica* serovar Typhimurium vaccine vector expressing Salmgag were evaluated. The analysis of ELISPOT results from mice vaccinated with AroC+Salmgag showed that there were no HIV-1-Gag specific CD4+ Th1 or CD8+ Tc1 splenocytes secreting IFN-γ on Day 28 (single inoculation, Experiment 1) (Figure 5.5A). The frequencies of IFN-γ SFUs formed after stimulation of splenocytes from mice vaccinated with AroC+Salmgag with the H-2K^d restricted Class I

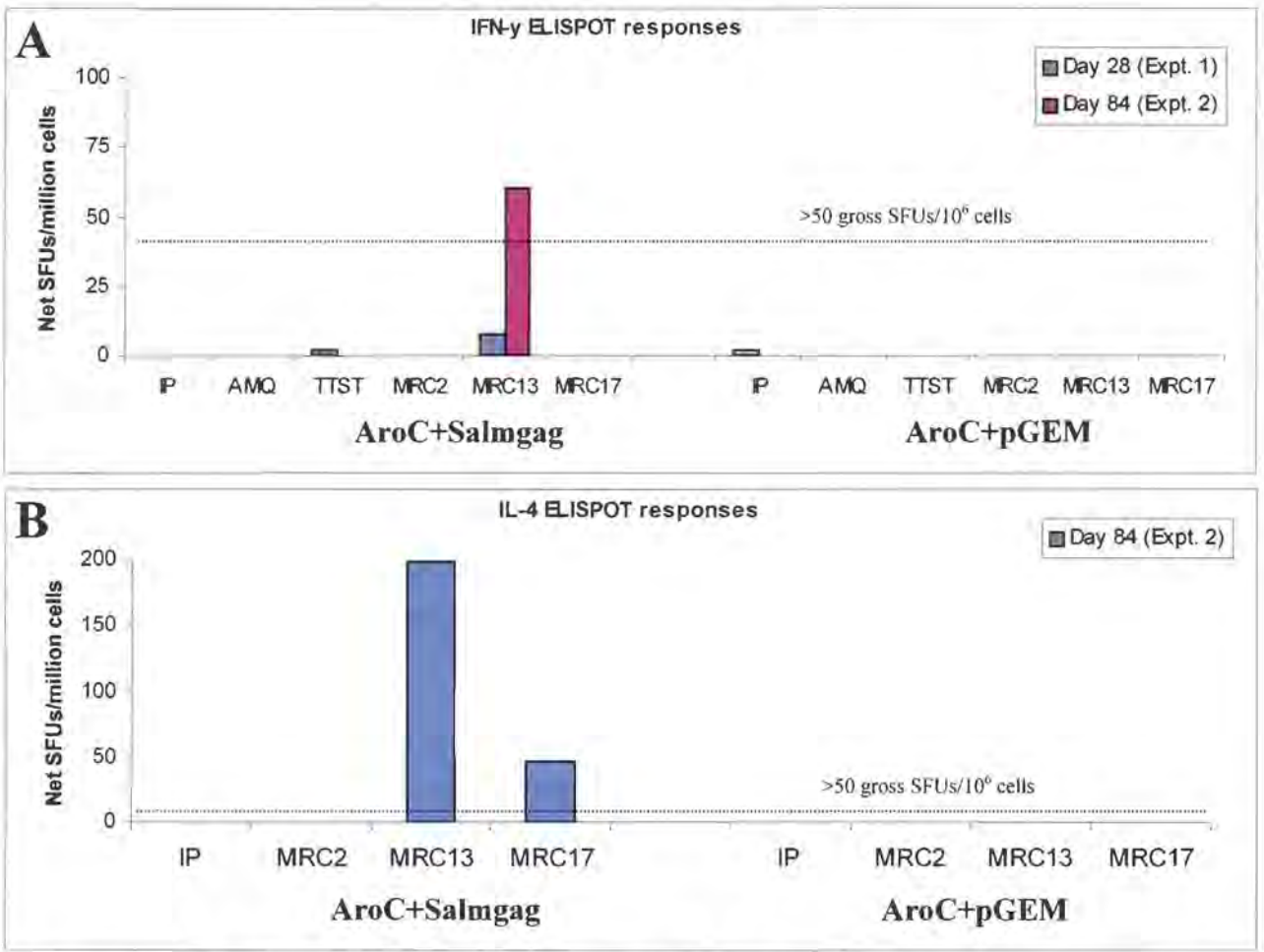

Figure 5.5: The magnitude of **HIV-1 Gag-specific CD8+ and CD4+ T cell responses** as measured by IFN-γ (**A**) and IL-4 (**B**) ELISPOT assays. Groups of mice were vaccinated ONCE (Experiment 1, Table 5.2) or THREE times (Experiment 2, Table 5.3) with *Salmonella* vaccine expressing Salmgag, AroC+Salmgag, or a negative *Salmonella* control vaccine, AroC+pGEM. On Days 28 (Experiment 1) and 84 (Experiment 2) splenocytes from the vaccinated mice were incubated with medium only (negative assay control), or stimulated with irrelevant peptide (IP) TYSTVASSL or the Gag CD8+ T cell peptides (AMQ = AMQMLKDTI and TTST= TTSTLQEQI) or Gag CD4+ T cell peptides (MRC2, MRC13 and MRC17) in IFN-γ and IL-4 ELISPOT assays. IFN-γ ELISPOT assay was performed on Day 28 (Experiment 1) and Day 84 (Experiment 2) (**A**), while IL-4 ELISPOT assay was performed only on Day 84 (Experiment 2) (**B**). Each bar in the graphs represents the net SFUs/10^6 cells. A positive response was arbitrarily defined as at least three times the no-peptide (medium) SFU number, and the number of specific SFUs being > 50 /10^6 cells.

Gag peptides (AMQ and TTST) and H-2K^d restricted Class II Gag peptides (MRC2, MRC13 and MRC17) were not above the background. However, on Day 84 (Experiment 2, 3 inoculations), IFN-γ SFUs were observed from splenocytes stimulated with MRC13 peptide (Figure 5.5A), indicating HIV-1 Gag-specific CD4+ Th1 response.

On Day 84, mice vaccinated with AroC+Salmgag elicited HIV-1 Gag-specific CD4+ Th2 (IL-4) responses. The frequencies of MRC13- and MRC17-specific IL-4 SFUs were above the background (Figure 5.5B). The frequencies of CD8+ Tc2 cells secreting IL-4 were not determined.

HIV-1 Gag-specific responses in mice vaccinated with the recombinant *Salmonella* vaccine expressing Salmgag were further investigated by quantifying the cytokines, TNF-α, IFN-γ, IL-4 and IL-5, secreted by splenocytes after stimulation with specific peptides. On Day 28 (Experiment 1), splenocytes did not respond to stimulation with the irrelevant or Gag CD8+ T cell and CD4+ T cell peptides (Figures 5.6A-D). The cytokines secreted after stimulation with the peptides were not elevated above the background.

On Day 84 (Experiment 2), HIV-1 Gag-specific cytokine responses were observed in mice vaccinated with AroC+Salmgag. Splenocytes responded to stimulation with AMQ peptide by secreting elevated IFN-γ (SI=2.6) and this indicated that borderline HIV-1-specific CD8+ Tc1 cell response was elicited (Figure 5.6A). This response was not previously detected by IFN-γ ELISPOT assay (Figure 5.5A). The other CD8+ peptide, TTST did not stimulate any elevated IFN-γ response (Figure 5.6A). HIV-1-specific CD4+ Th1 IFN-γ cytokine response was detected on Day 84 in mice vaccinated with AroC+Salmgag. There were high levels of IFN-γ produced by the splenocytes after stimulation with MRC13 peptide (Figure 5.6A). The IFN-γ SI for this peptide was high (7.5). The MRC13 peptide also induced the secretion of high levels TNF-α (SI=29.1) (Figure 5.6B) and this further confirmed the existence of Gag-specific CD4+ Th1 responses in vaccinated animals. Analysis of IL-4 and IL-5 (CD4+ Th2) responses in mice showed that only the MRC13 peptide could stimulate the splenocytes from AroC+Salmgag-vaccinated mice to secrete the cytokines (Figures 5.6 C and 5.6D). Elevated levels of IL-4 (SI=26.2) and IL-5 (SI≥89.3) were secreted. The unstimulated cells did not produce any IL-5 (value = 0 pg/ml) and that was the reason why the MRC13 had an IL-5 SI of greater than or equal to 89.3. There were no substantial peptide-specific cytokine responses to MRC-2 and MRC-17 by the splenocytes from AroC+Salmgag-vaccinated mice (Figures 5.6A-D).

The Th1 (IFN-γ and TNF-α)/Th2 (IL-4 and IL-5) ratio was calculated based on the cytokines secreted by cells after stimulation with MRC13 peptide. The ratio was 2.6, indicating the dominance of the Th1 response. Although the Th1 response based on this ratio was dominating, the background response for Th1 cytokines was about 10-fold above the Th2 background response.

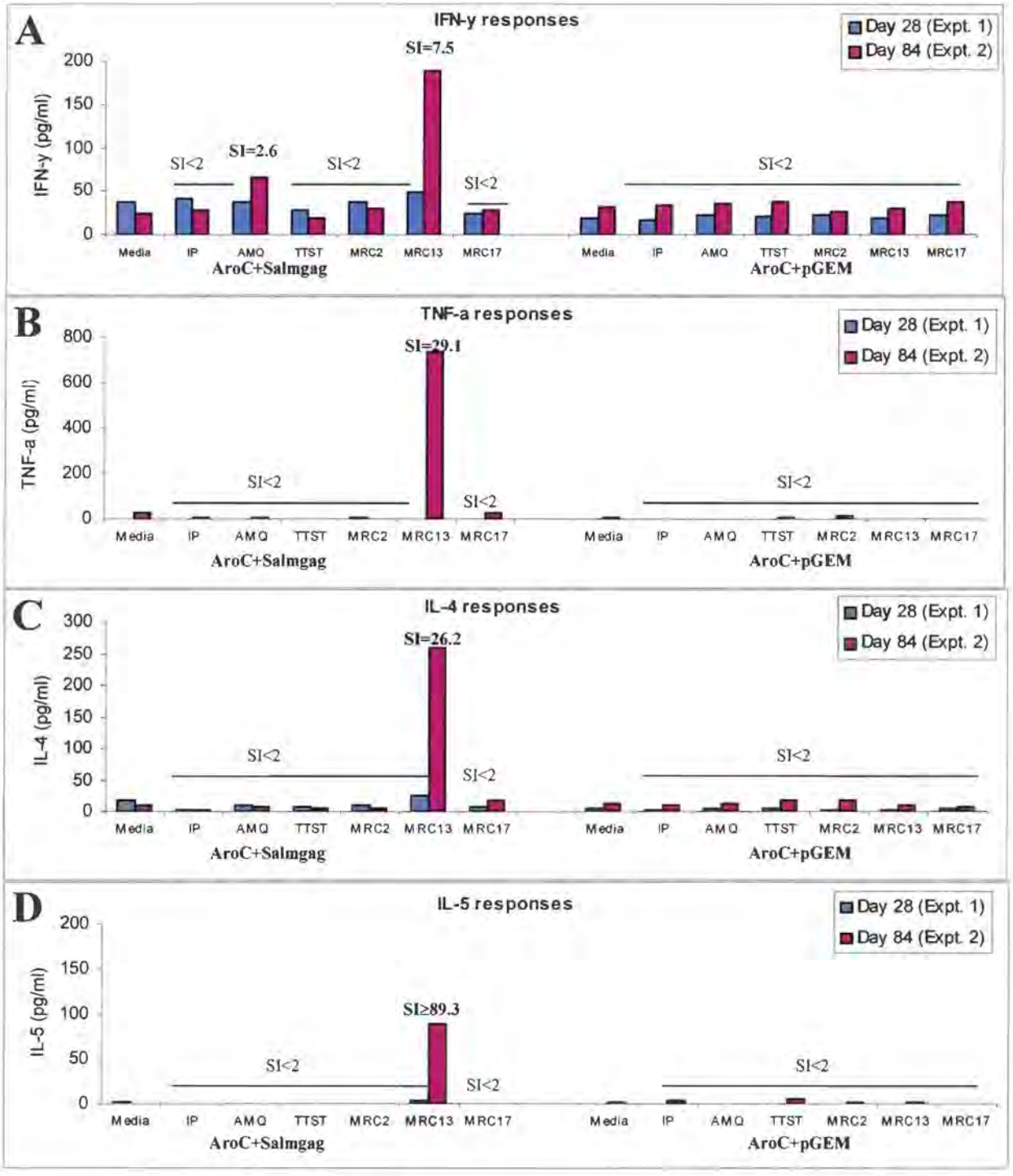

Figure 5.6: The magnitude of **HIV-1 Gag-specific CD8+ T cell responses** as determined by quantification of IFN-γ (**A**), TNF-α (**B**), IL-4 (**C**) and IL-5 (**D**) cytokines (in pg/ml) secreted by splenocytes during stimulation for 48 hours in the absence or presence of Gag peptides, using CBA assay. Groups of mice were vaccinated ONCE (Experiment 1, Table 5.2) or THREE times (Experiment 2, Table 5.3) with AroC+Salmgag or AroC+pGEM. On Day 28 (Experiment 1) and Day 84 (Experiment 2), splenocytes from the vaccinated mice were incubated with medium only (negative assay control), or stimulated with irrelevant peptide (IP) TYSTVASSL, the Gag CD8+ T cell peptides (AMQ = AMQMLKDTI and TTST= TTSTLQEQI) or the Gag CD4+ T cell peptides (MRC2, MRC13 and MRC17) and the amounts of IFN-γ (**A**), TNF-α (**B**), IL-4 (**C**) and IL-5 (**D**) measured by CBA assay. Each

5.6.4.2 HIV-1 Gag-specific humoral immune responses to recombinant *Salmonella*

The induction of HIV-1 Gag-specific humoral immune responses in mice after vaccination with a recombinant *Salmonella* vaccine expressing Salmgag (AroC+Salmgag) was evaluated. No or very low serum HIV-1 Gag specific antibody response (1.52-fold OD_{405} reading above prebleed) was detected on Day 28 in serum (1/100 dilution) of mice vaccinated with AroC+Salmgag (Figure 5.7A). On Day 56, the

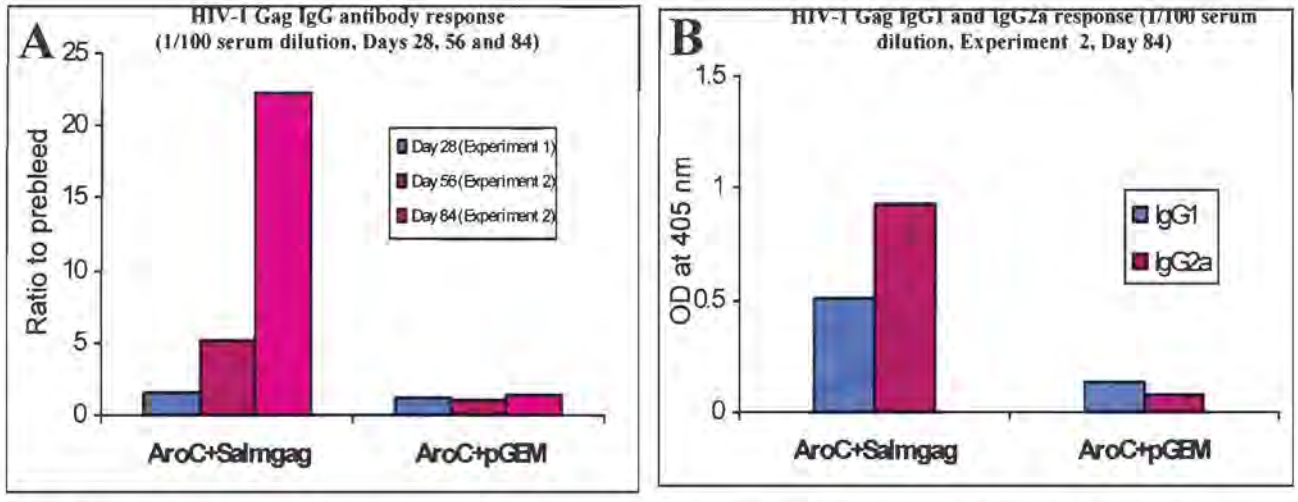

Figure 5.7: Serum HIV-1 Gag-specific IgG responses in mice vaccinated with *Salmonella* expressing a Salmgag antigen. Groups of mice were vaccinated ONCE (Table 5.1) or THREE times (Table 5.2) with *Salmonella* vaccines, AroC+Salmgag or AroC+pGEM. Prior to inoculation, the mice were bled and pools of serum made. On Days 28 (Experiment 1), 56 and 84 (Experiment 2) the mice were bled and serum made. (**A**) The HIV-1 Gag-specific IgG was measured serum in pools of each group of mice for Day 28, 56 or Day 84 in 1/100 serum dilution. The data are represented as the ratios of the mean OD_{405nm} of duplicates to the mean OD_{405nm} prebleed duplicates. (**B**) The HIV-1 Gag-specific IgG1 and IgG2a were measured in serum pools of each group of mice for Day 84 in 1/100 serum dilution. Each bar represents the mean OD_{405nm} value of duplicates. Antibody responses were defined as positive when the mean OD_{405nm} ratio to prebleed >2 or the OD_{405nm} >2-fold that of the negative control (AroC+pGEM).

antibody response was boosted (5.21-fold OD_{405} reading above prebleed) (Figure 5.7A). The Gag-specific antibody response was further boosted by Day 84 (22.23-fold OD_{405} reading above prebleed) (Figure 5.7A). The nature of IgG responses in mice vaccinated three times (Day 84) with AroC+Salmgag was further investigated by analyzing Gag-specific IgG2a (Th1) and IgG1 (Th2) in the serum. Both Gag specific IgG subclasses (Ig2a and IgG1) were detected with Ig2a > IgG1 (Figure 5.8B). HIV-1 Gag-specific antibody responses were confirmed using the New LAV Blot I HIV-1 Western blotting kit (results not shown). The serum from the AroC+Salmgag vaccinated mice reacted specifically with Gag bands (P55, P40, P24/25 and P17/18) on the blot.

5.7 DISCUSSION

5.7.1: Codon optimization and expression of HIV-1 subtype C *gag*

It is critical to consider codon optimization of the viral genes for optimal expression in *Salmonella* bacterial vaccine vectors if a strong immune response is to be induced. The current study investigated the development and immunogenicity of recombinant *Salmonella* vector expressing Salmgag (optimized Du_{422} HIV-1 Gag). The wild-type *gag* gene was optimized for expression by *Salmonella enterica* serovar Typhimurium codons without affecting the primary amino acid sequence of the final protein. Comparative analyses of wild-type *gag* and *salmgag* indicated a number of variations in the two genes. The most frequently used *Salmonella enterica* serovar Typhimurium codons were found in *salmgag* than in wild-type *gag* (Figure 5.2). The wild-type gag had many *Salmonella enterica* serovar Typhimurium rare codons which could have a negative impact on gene expression. The exclusion of such codons in *salmgag* was hoped to improve stable expression by the *Salmonella* vaccine vector after vaccination of mice. Replacement of rare HIV-1 codons with major ones has a significant effect on translation (Haas *et al.*, 1996). In *Escherichia coli* and *Salmonella,* codon usage can affect the rate of protein translation, with low usage or rare codons causing the ribosome to pause during translation (Zhang *et al.*, 1994). Replacement of these rare with major codons has also been suggested to improve stable expression of genes (Gustafsson *et al.*, 2004). Several studies have shown that codon-optimization of heterologous genes improved their expression in bacterial systems (Makoff *et al.*, 1989; Apeler *et al* 1997; Lakey *et al.*, 2000; Hu *et al.*, 2003; Yadava and Ockenhouse, 2003). However, some studies have shown that expression of rare tRNAs or codon optimization of genes did not result in a marked improvement in heterologous antigen expression (Andrews *et al.*, 1996; Alexeyev and Winkler, 1999; Baud *et al.*, 2004), thereby suggesting that other factors other than codon usage could affect heterologous protein expression levels. These factors include the strength of the promoter, the nucleotides at the 5'-end of the gene, the nature of the Shine-Dalgarno sequence, mRNA stability and susceptibility of the expressed protein to post-translational degradation (Deana *et al.*, 1996, 1998; Stenstrom *et al.*, 2001; Al-Zarouni and Dale, 2002; Griswold *et al.*, 2003; Stenstrom and Isaksson, 2002; Jin *et al.*, 2006).

HIV-1 genes are mainly A+T-rich (Kypr and Mrazek, 1987), as opposed to *Salmonella* genome, which has a high G+C content of 52% (Groisman and Ochman, 1997). The A+T-rich stretches in wild-type *gag* were therefore avoided during codon optimization and this improved the G+C-content of *salmgag* (Figure 5.2). It has also been shown that some HIV-1 viral mRNAs contain instability sequences that require the *rev* regulatory gene product for mRNA stabilization in eukaryotic cells (Maldarelli *et al.*, 1991). These sequences are present in *gag-pol* region. The extent of the effect of these sequences on Gag expression in *Salmonella* vaccine vectors has not been unraveled. It was not clear in this current study whether the sequences could affect the stability of *gag* mRNA or whether the rev-dependent expression of Gag was a phenomenon only found in eukaryotic cells. It has therefore been suggested that removal of these instability sequences (INS) during codon optimization could resolve the problem (Kotsopoulou *et al.*, 2000). The exclusion of sequence domains such as internal RBS sites, TATA -boxes, Chi

recombination sites and a -35 boxes in *salmgag* was also further expected to contribute to its optimal expression in *Salmonella* vaccine.

Expression of high levels of Salmgag was achieved in recombinant *E. coli* and *Salmonella* vaccine (Figure 5.4). It was difficult to assess whether the overexpression of the antigen was entirely attributable to codon-optimization, since previous studies had also shown that (codon) unoptimised antigens (GFP and wtGag, Chapters 3 and 4) could still be overexpressed with this *lac* operon system. It seemed therefore, several factors, some of which have previously been outlined (Chapter 3) were potentially responsible for the overexpression of the Salmgag antigens in the recombinant *Salmonella enterica* serovar Typhimurium vaccine vector.

It was noted that the codon-optimized Gag seemed to be more toxic to *Salmonella* than the wild-type Gag as it caused bacterial lysis especially in the stationary phase. This aspect has also been observed in a study by Covone *et al.*, (1998) who showed that expression of the heat-labile enterotoxin LTK63 could be toxic to *Salmonella* vector during static growth. Although there was cell toxicity and cell death (lysis) of *Salmonella* vector expressing Salmgag, it seemed that the arising of new mutants without plasmid was minimal compared to those which arose for recombinant *Salmonella enterica* serovar Typhimurium vector was expressing wild-type HIV-1 Gag (results not shown).

Codon optimization of genes for expression in recombinant *Salmonella* vaccine vectors seem to have an impact on the nature, breadth and magnitude of the immune responses induced after vaccination. Baud *et al* (2004b) showed improved antigen-specific immune responses against a *Salmonella*-based vaccine expressing human papillomavirus type 16 L1 after codon-optimization. In the current study, implementation of codon optimization strategy resulted in the recombinant vector giving better immunogenicity readouts than the vector expressing the wild-type Gag (Chapter 4). This is line with previous studies in which Spreng *et al.*, (2000) showed that expression of measles virus (MV) epitopes in *Salmonella* vaccine vector could be enhanced by codon optimization. Oral vaccination of MV-susceptible C3H mice with the recombinant *Salmonella* vector induced MV-specific serum antibodies and CD4+ T cell response (Spreng *et al.*, 2000). Challenging of the mice with rodent-adapted, neurotropic MV strain resulted in 30% protection (Spreng *et al.*, 2000). This demonstrated the critical importance of antigen codon optimization for optimal expression in recombinant *Salmonella* vectors.

5.7.2 HIV-1 specific CD8+ Tc cytokine responses

Despite the high expression of Salmgag, it was not clear why HIV-1 Gag-specific CD8+ T cell responses were very low after vaccination of mice with the recombinant *Salmonella* vaccine vector. Some of the possible reasons for this have already been outlined in Chapter 4. The CD8+ epitopes could be sub-dominant when presented to the immune system together with *Salmonella* vector-derived epitopes. Salmgag probably formed insoluble particles (as suggested by the Wilkinson and Harrison model), which could not be cross-presented efficiently by the MHC-1 molecules for induction of CD8+ T cell

responses. The presentation of Gag peptides/antigen together with a plethora of *Salmonella* antigens could affect their immunodominance. After vaccination, the immune system has to respond not only to the HIV-1 Gag peptides, but also to peptides generated from the *Salmonella* bacterial proteins. Immunodominance would operate at the level of all the peptides per given time. Some of the Gag peptides could easily become sub-dominant in such a situation in which T cells compete against each other to respond to antigens. CD8+ T cells sometimes compete for access to antigen-bearing APCs (Kedl *et al.*, 2003). T cells may also compete to respond to both the same and different antigens (Kedl *et al.*, 2003). These competitions could affect the way in which the Salmgag antigens carried by the recombinant *Salmonella* were cross-presented to the immune system. The presentation of vector antigens may also potentially lead to non-specific suppression of the presentation of Gag peptides. One of the possible reasons for failure to detect Gag-specific CD8+ T cell responses could be the absence of CD4+ T help. Although both Gag-specific CD4+ Th1 and Th2 responses were induced, it was not clear whether this was sufficient for provision of the required help. However, in human infection, HIV-Gag responses are known to be CD4+ T-cell dependent as contrasted to Env responses, which are T-cell independent (Binley *et al.*, 1997).

5.7.3 HIV-1 specific CD4+ Th cytokine responses

In the current study, the production of high levels of both IFN-γ and IL-4 cytokines after stimulation of AroC+Salmgag splenocytes with an H-2K^d restricted Class II epitope (MRC13) demonstrated that mixed HIV-1 Gag-specific CD4+ Th1 and Th2 cell responses were induced. Such biphasic responses in which both Th1 and Th2 cytokines are induced after vaccination with *Salmonella* have been observed in other studies (Galdiero *et al.*, 1998; Pascual *et al*, 1999; Kang *et al.*, 2003; Jun *et al.*, 2005). There were differences in the bias of the responses in these studies. To date, the mechanisms that determine whether an antigen-specific Th1 or Th2 response is induced to *Salmonella*-delivered heterologous antigens are still not understood. Nevertheless, these two types of immune response induced by the recombinant *Salmonella* vector are critical targets for vaccines that are required to induce both cell-mediated and antibody responses for protection against infection by a number of pathogens. The Th1 cytokines promote CD8+ T cell responses and antibody class-switching to IgG2a, while Th2 cytokines promote humoral immune responses and class-switching to IgG1 and IgA (O'Garra and Arai, 2000; Spellberg and Edwards, 2001).

5.7.4 HIV-1 specific humoral immune responses

The development of an HIV-1 vaccine that elicits potent humoral immune responses is still a challenge to the scientific community. Such antibody responses should be able to neutralize many strains of the virus if they are to be useful. Although Gag is not a target for neutralizing antibodies, the current study investigated whether *Salmonella* expressing codon-optimized Gag could induce HIV-1 specific antibodies. Gag-specific IgG responses were induced in mice on Day 28 and the responses were boosted by Day 56 and Day 84 (Figure 5.7). This contrasted previous studies (Chapter 4) in which no Gag-specific antibody responses were induced after vaccination of mice with recombinant *Salmonella*

expressing wild-type HIV-1 Gag. Codon optimization therefore potentially facilitated the optimal expression of Salmgag *in vivo* after vaccination and subsequent presentation of the antigen to the B cells. This is consistent with previous studies have also shown that codon-optimized antigens could give better antibody responses than unoptimized ones when delivered by recombinant *Salmonella* vector (Baud *et al.*, 2004b).

Further characterization of the anti-HIV-1 Gag antibody responses induced after secondary vaccinations with AroC+Salmgag showed the presence of both IgG1 and IgG2a subclasses (Figure 5.7). However, there was a bias towards the production of IgG2a at the expense of IgG1 indicating more of a Th1-type than a Th2-type of helper responses. This result was supported by the finding that AroC+Salmgag induced Gag-specific CD4+ Th1 and Th2 cytokines (IFN-γ and IL-4) (Section 5.7.3). These cytokines produced by the Th1 and Th2 cells were most probably responsible for the induction of heavy-chain isotype switching to both IgG2a and IgG1 respectively (Spellberg and Edwards, 2001).

It was not clear whether full-length Salmgag formed virus-like particles (VLPs) inside the *Salmonella* vector or not. Studies on other viral structural genes such as human papillomavirus *L1* have shown formation of VLPs inside the *Salmonella* bacteria after cytoplasmic expression (Nardelli-Haefliger *et al.*, 1997; Baud *et al.*, 2004a). Other studies have also shown that full-length viral nucleocapsid proteins derived from Nipah virus, measle virus and Newcastle disease virus could self-assemble into VLPs when expressed in *E. coli* (Campbell and Vogt, 1995; Warness *et al.*, 1995; Kho *et al.*, 2003; Tan *et al.*, 2004). Since *Salmonella* and *E. coli* are phylogenetically related, these viral proteins should also form VLPs if expressed in *Salmonella* vaccine vectors. In contrast, rotavirus VP6 and VP2 failed to form VLPs when expressed in *Salmonella* vaccine vector (Coste *et al.*, 2001). The current studies did not determine whether Salmgag VLPs were formed inside the bacterial vector or not. Generally Gag VLPs have been known to activate the innate immune system, thereby orchestrating cytokine responses that determine the quality of cellular and/or humoral immune responses induced (Deml *et al.*, 2005). The aggregations, whether as misfolded proteins or VLPs provoked the humoral immune responses observed in this study.

In conclusion, the results from this study demonstrated that a recombinant *Salmonella* expressing a codon-optimized Gag elicited mixed HIV-1-specific Th1 (TNF-α, IFN-γ and IgG2a) and Th2 (IL-4, IL-5 and IgG1) immune responses after oral vaccination of mice. The codon optimization of the HIV-1 Subtype C Gag had a profound impact on its immunogenicity when expressed and delivered by the recombinant *Salmonella* vaccine vector. In developing recombinant *Salmonella* vaccines for HIV-1, it is therefore critical to optimize the viral genes for expression by the vector as this improves the nature, quality and magnitude of the immune responses elicited.

CHAPTER 6: RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR EXPRESSING HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C P41 OR P24 EMBEDDED IN LACZα

"If at first, the idea is not absurd, then there is no hope for it" Albert Einstein (1879-1955)

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CHAPTER 6: RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR EXPRESSING HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C P41 OR P24 EMBEDDED IN LACZα

6.1 SUMMARY

Embedding heterologous antigens into other proteins for expression in recombinant *Salmonella* vaccine vectors is an unexplored phenomenon in the field of vaccinology. No previous studies have investigated the immunogenicity of foreign antigens embedded in other bacterial proteins and delivered by recombinant *Salmonella* vaccine vectors. It was the objective of the current study to develop a recombinant *Salmonella enterica* serovar Typhimurium vaccine vector expressing HIV-1 subtype C P41 or P24 when embedded in the *E. coli* β-galactosidase α-protein. Truncated *salmgag* gene fragments, *p1724* (*p41*) and *p24D* were amplified by polymerase chain reaction and cloned in-frame between the 5' and 3' domains of the LacZα gene fragment in pGEM-Teasy plasmid. The two recombinant expression plasmids, designated pGEM+p1724 and pGEM+p24D were used in the generation of recombinant *Salmonella* vaccine vectors, AroC+p1724 and AroC+p24D. Both *E. coli* and *Salmonella* vaccines expressed high levels of P41 or P24D. Gag-specific immune responses against the recombinant *Salmonella* vaccines were investigated in orally vaccinated mice. HIV-1 Gag-specific CD4+ Th2 (IL-4) cytokine responses were induced by Day 28 in mice vaccinated with AroC+P1724 (MRC13 SI=3.3) or AroC+P24D (MRC13 SI=3.7). Two booster vaccinations improved this response in mice vaccinated with AroC+P24D (MRC13 SI=15.9), but the response was maintained in mice vaccinated with AroC+P1724 (MRC13 SI=2.1). HIV-1 Gag-specific CD8+ Tc1 (IFN-γ) response was induced by Day 84 in mice vaccinated with AroC+P1724 (TTST SI=4.4). AroC+P24D elicited HIV-1 Gag CD8+ Tc2 (IL-4) response by Day 84 (AMQ SI=3.0). No Gag-specific antibody responses were detected in vaccinated mice by Day 28, 56 and 84. This is the first study to demonstrate that embedded HIV-1 subtype C P41 and P24 would give antigen-specific CD8+ and CD4+ T cell cytokine responses when delivered by a recombinant *Salmonella* vaccine vector.

6.2 INTRODUCTION

Previous studies demonstrated that high level expression of GFP, Gag or Salmgag in recombinant *Salmonella* could be achieved by fusing the antigens in-frame with the N-terminal domain of the LacZα (Chapters 3, 4 and 5). Despite high-level expression being achieved, post-translational proteolytic degradation of the antigens was evident. Furthermore, evidence of instability and cellular toxicity of full-length Salmgag on *Salmonella* bacterial vector were noted. Instead of fusing the antigen with only the N-terminal domain of the LacZα, another strategy for foreign antigen expression in *Salmonella* vaccine vector was employed in this study. The strategy involved embedding truncated Salmgag fragments (P41 and P24) into a prokaryotic protein, the *E. coli* β-galactosidase α-gene peptide. It was also speculated that embedding the antigens would circumvent the difficulties always encountered in attempting to express short viral antigens such as HIV-1 P24 in bacteria (Murby *et al.*, 1996). P41 and P24 were

selected in this study because they contain all the relevant Gag CD4+ and CD8+ T cell epitopes to evaluate immune responses in mice. Thus the objectives of the study were:

1. to construct recombinant expression plasmids (pGEM+P1724 and pGEM+P24D) with truncated *salmgag* gene fragments, *p41* and *p24* embedded into the *LacZα*.
2. to develop a recombinant *Salmonella enterica* serovar Typhimurium vaccine vector expressing the embedded P41 or P24D.
3. to evaluate the induction of HIV-1 Gag-specific immune responses in mice after oral vaccination with the recombinant *Salmonella* vaccine vector expressing P41 or P24D.

6.3 MATERIALS

Materials used in the study have been given Appendices and previous chapters.

6.4 GENERAL METHODS

Some of the microbiological, molecular and immunological methods used in the study have been described in Appendices A and B.

6.5 EXPERIMENTAL DESIGN AND PROTOCOLS

6.5.1 Construction of recombinant pGEM+P1724 and pGEM+P24D expression plasmids: molecular cloning of *p41* and *p24* genes

To construct recombinant plasmids, pGEM+P1724 (Appendix G8) and pGEM+P24 (Appendix G7), polymerase chain reaction was used to amplify the HIV-1 *p41* (*p1724*) and *p24* genes from the *salmgag* gene template in Pscript+Salmgag (Figure 6.1). The primers (Appendix F), CA1 (forward) and CA2 (reverse) were used to amplify *p24*, while MA1 (forward) and CA2 (reverse) were used to amplify *p1724* (*p41*). The primers were rationally designed so that the genes could be cloned in-frame with both the 5'

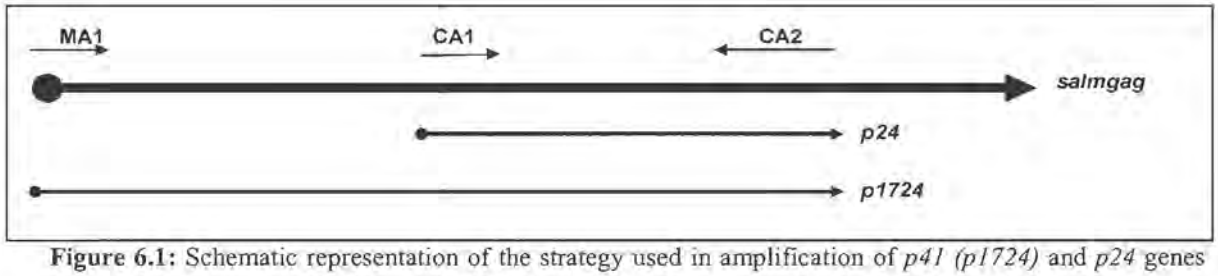

Figure 6.1: Schematic representation of the strategy used in amplification of *p41 (p1724)* and *p24* genes from *salmgag* template. The primers, MA1, CA1 and CA2 were used for the amplification of the genes.

and 3' domains of the *LacZα* gene fragment in the pGEM-Teasy plasmid (Appendix G2). The PCR was conducted in a 50 µl volume with 3 units QIAGEN Taq DNA Polymerase, 1x PCR buffer, 1.5 µM of each primers, 0.2 mM dNTPs, 1.5 mM magnesium chloride and 10 ng of Pscript+salmgag template. The PCR cycling conditions were: 1 cycle of 95°C for 2 min, 5 cycles of 95°C for 30 s, 45°C for 30 s, 72°C for 1 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 2 min, and a final extension of 72°C for 7 min. The PCR products were analysed by agarose gel electrophoresis. Amplicons (3 µl of each) were

ligated into pGEM-T Easy plasmid as described in Appendix B1.7. The ligation reaction was used in the transformation of competent *E. coli* SCS110 cells as described in Appendix A1.4. Screening of recombinant clones with pGEM+p1724 and pGEM+p24 was performed using restriction mapping protocols as described in Appendix B1.5.

6.5.2 Development of recombinant *Salmonella* expressing P41 and P24

The genetic transformation of competent *AroC Salmonella enterica* serovar Typhimurium mutant was performed as described in Appendix A1.4 with recombinant plasmids, pGEM+p1724 and pGEM+p24D (with p24 gene partially deleted). This resulted in the generation of the following *Salmonella* vaccine clones: AroC+P1724 and AroC+P24D. The expression of P41 and P24D antigen in recombinant *E. coli* SCS110 clones (SCS110+P1724 and SCS110+P24D) was assessed by plating the bacteria on plates with IPTG and X-gal and development of the blue colour was noted. The expression of the two antigens was further assessed by 12.5 % SDS-PAGE (Appendix B4) and Western blotting (Appendix B6). P1724 and P24D antigen expression by the recombinant *Salmonella enterica* serovar Typhimurium vaccine vectors was assessed by 12.5 % SDS-PAGE (Appendix B4) and the Roche Elecsys® HIV p24 Ag assay (Roche Diagnostics Boehringer Mannheim GmbH, Germany) according to manufacturer's instructions. The protein lysates were diluted (1/100 or 1/1000) in water and analyzed by the Elecysys® 2010 analyzer.

The solubility of only the recombinant P24D (from AroC+P24D) and LacZα (from AroC+pGEM) antigens was investigated as described in Appendix B2.2. The bacterial (overnight) cultures were sonicated and the total, soluble or extracellular fractions analysed by SDS-PAGE. The solubility of the antigens when overexpressed in *Salmonella* was estimated using the Wilkinson and Harrison Model as previously described (Chapter 3).

6.5.3 Vaccination of mice with recombinant *Salmonella* vaccines

The recombinant *Salmonella* vaccine vectors, AroC+P1724, AroC+P24D and AroC+pGEM were prepared as described in Appendix A2.1. Two groups of mice (Tables 6.1 and 6.2) were vaccinated using the same protocol described in Chapter 5.

Table 6.1: Vaccines and vaccination protocol for Experiment 1. Mice were inoculated ONCE by intragastric gavage (Appendix A2.1) with *Salmonella* vaccines (AroC+P1724, AroC+P24D and AroC+pGEM). The mice were bled prior to inoculation and sacrifice. At sacrifice, blood and spleens were collected. The blood was processed by centrifugation and sera were stored until evaluation of humoral immune responses as described in Appendix B10. The spleens from each group of mice were pooled and processed as described in Appendix B7.

Inoculum (vaccine)	Inoculum/animal/ inoculation	Inoculation date	Sacrifice date
AroC + P1724	10^8 cfu	Day 0	Day 28
AroC + P24D	10^8 cfu	Day 0	Day 28
AroC + pGEM	10^8 cfu	Day 0	Day 28

Table 6.2: Vaccines and vaccination protocol for Experiment 2. Mice were inoculated THREE times by intragastric gavage (Appendix A2.3) on Days 0, 28 and 56 with *Salmonella* vaccines (AroC+P1724, AroC+P24D and AroC+pGEM). The mice were bled prior to inoculation and sacrifice. The blood was processed by centrifugation and sera were stored until evaluation of humoral immune responses as described in Appendix B10. At sacrifice (on Day 84), the spleens from each group of mice were pooled and processed as described in Appendix B7.

Inoculum (vaccine)	Inoculum/animal/ inoculation	Inoculation date	Sacrifice date
AroC + P1724	10^8cfu	Day 0, Day 28, Day 56	Day 84
AroC + P24D	10^8cfu	Day 0, Day 28, Day 56	Day 84
AroC + pGEM	10^8cfu	Day 0, Day 28, Day 56	Day 84

6.5.4 Assessment of T cell immune responses in the spleen

HIV-1 Gag-specific CD4+ Th1/Th2 and CD8+ Tc1/Tc2 cytokine responses were evaluated using (IFN-γ and IL-4) ELISPOT and CBA assays as previously described in Chapter 4.

6.5.5 Assessment of humoral immune responses

HIV-1 Gag- or LPS-specific IgG humoral immune responses by the recombinant *Salmonella* vaccines were evaluated as described in Appendix B10. The determination of serum HIV-1 Gag and LPS-specific IgG responses in serum pools of vaccinated mice was performed using an ELISA protocol (Appendix B10) at Day 28 (Experiment 1), Day 56 and Day 84 (Experiment 2). The ELISA plates were coated with HIV-1 subtype B Pr55 antigen or LPS at a final concentration of 5 ug/ml. Mouse serum pools were diluted accordingly (1/100 and 1/1000).

6.6 RESULTS

6.6.1 Development of a recombinant expression plasmid vectors: molecular cloning of *p41 and p24D*

Truncated *salmgag* gene fragments (*p1724* and *p24D*) were cloned. The key structural components of the two recombinant plasmids (designated pGEM+p1724 and pGEM+p24D) developed by this study are given in Figures 6.2 and 6.3). Polymerase chain reaction was successfully used to amplify the genes, *p1724* and *p24* (results of PCR products not shown). The PCR products were successfully ligated into linearized pGEM-Teasy vector. Screening of recombinant plasmids from recombinant *E. coli* SCS110

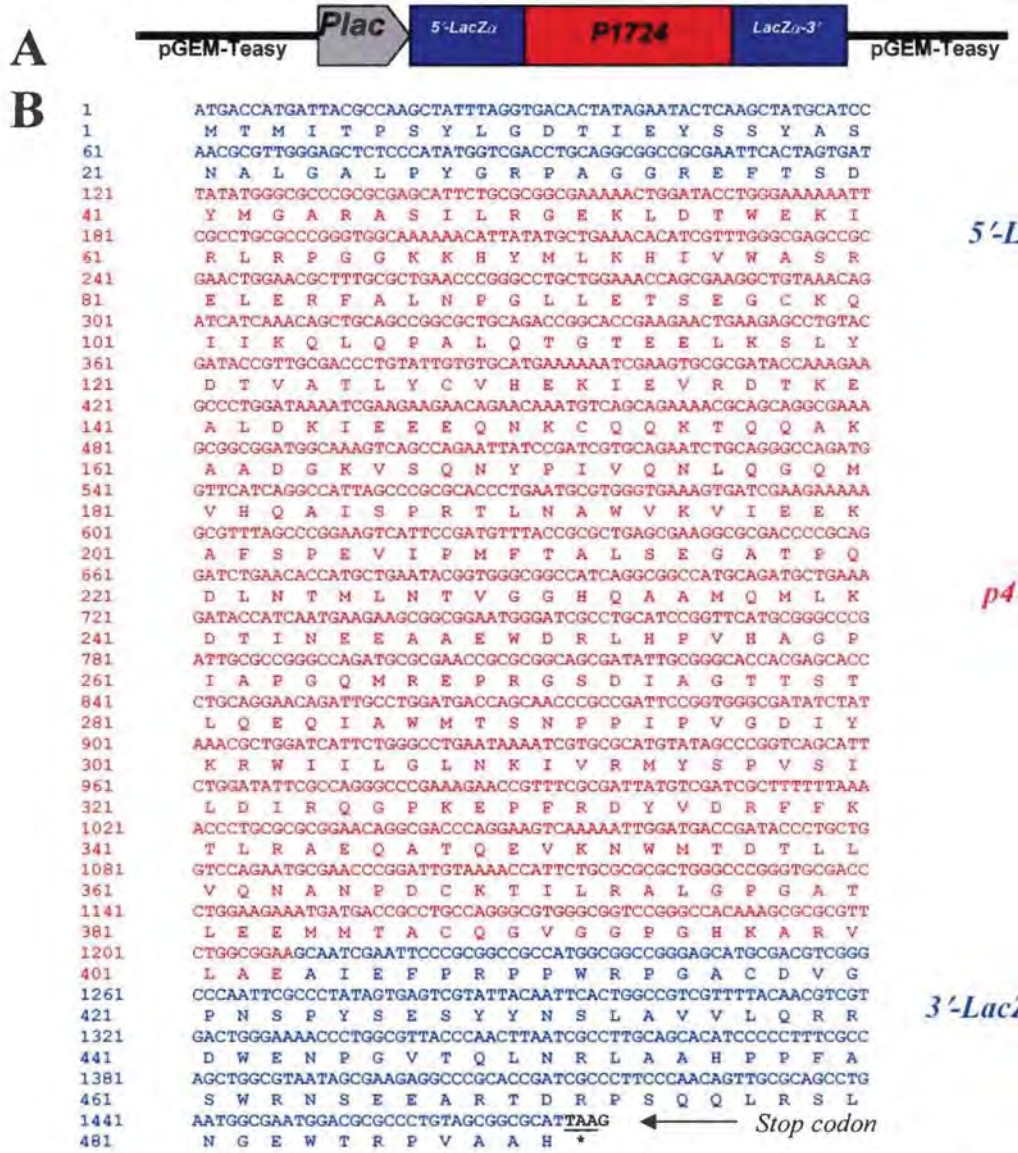

Figure 6.2: The general structural components of P41 expression construct (pGEM+P1724) developed by the study. (**A**) A schematic representation of the P41 expression plasmid. The P41 (made up of P17 and P24) was fused in-frame to the N- and C-terminal domains of the *β*-galactosidase *α*-fragment in pGEM-Teasy plasmid. The expression was under the *E. coli lac* promoter and other *lac* operon transcription and translation domains. (**B**) The DNA and amino acid sequences of the resultant *p1724 (p41)* embedded in *β*-galactosidase *α*-fragment. The fusion protein had 1473 ORFs, 491 amino acids and a molecular weight of 54.865 KDa. The main features and complete DNA sequence of pGEM+P1724 plasmid are given in Appendices G8 and H5 respectively.

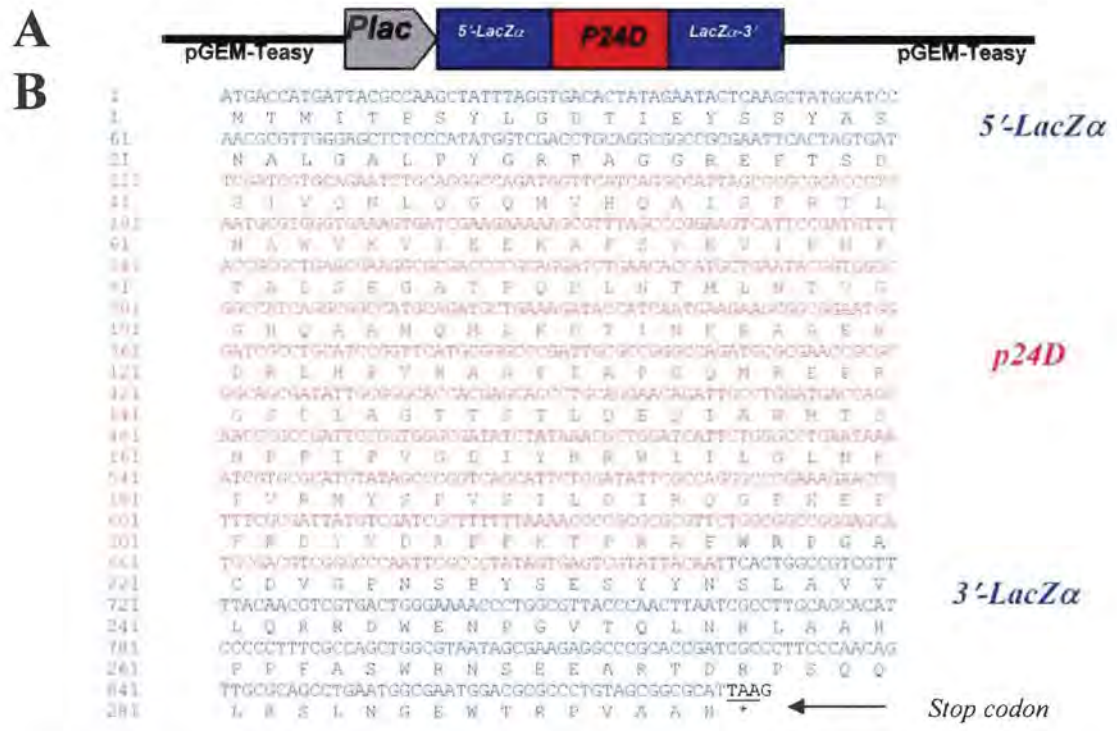

Figure 6.3: The general structural components of P24D expression construct (pGEM+P24D) developed by the study. (**A**) A schematic representation of the P24D expression plasmid. The P24D was fused in-frame to the N- and C-terminal domains of the *β*-galactosidase *α*-fragment in pGEM-Teasy plasmid. The expression was under the *E. coli lac* promoter and other *lac* operon transcription and translation domains. (**B**) The DNA and amino acid sequences of the resultant *p24d* embedded in *β*-galactosidase *α*-fragment. The fusion protein had 885 ORFs, 295 amino acids and a molecular weight of 32.910 KDa. The main features and complete DNA sequence of pGEM+P24D plasmid are given in Appendices G7 and H4 respectively.

clones was pursued. Candidate clones with pGEM+p1724 plasmids were found and one clone was selected for further studies. It was noted with restriction mapping that some of the clones had lost the *p1724* gene while other had short deletions within the gene (results not shown).

Screening of candidate pGEM+p24 plasmids (expected to carry the full-length recombinant *p24* gene) was also performed initially using *EcoR*I restriction mapping. Most clones had lost the gene, while some had deletions within the *p24* gene or part of the *LacZα* gene in pGEM-Teasy plasmid. This was shown by restriction mapping that some of the unique restriction enzyme sites had been lost. Mapping of one of the clones (named clone D) showed that a major deletion had occurred at the 3′-end of the *p24* gene and the *lacZα* gene, but still the gene was in the correct orientation. The plasmid was designated pGEM+p24D (where D stood for deletion). Practically, no clone was isolated with recombinant pGEM+p24 plasmid with full-length *p24* gene. All the plasmids had deletions or the gene had lost frame (results not shown).

The genes (*p41* and *p24d*) in candidate plasmids, pGEM+p1724 and pGEM+p2D were confirmed by sequencing using M13 primers and primers used for PCR. The sequencing also re-confirmed the presence of the relevant transcriptional and translational structures and the orientation of the genes. The whole *p41* gene was found to be in correct reading frame with the whole of the *LacZα* gene (Figure 6.2B). The protein was embedded between the N-terminal domain of LacZα, MTMITPSYLG DTIEYSSYAS NALGALPYGR PAGGREFTSD and the C-terminal domain of LacZα, IEFPRPPWRP GACDVGPNSP YSESYYNSLA VVLQRRDWEN PGVTQLNRLA AHPPFASWRN SEEARTDRPS QQLRSLNGEW TRPVAAH. The *p24d* gene was also embedded the *LacZα* gene (Figure 6.3B). The deletion in *p24* in pGEM+p24D (previously shown by restriction mapping) was further confirmed by sequencing (Figure 6.3B). Only part (WRP GACDVGPNSP YSESYYNSLA VVLQRRDWEN PGVTQLNRLA AHPPFASWRN SEEARTDRPS QQLRSLNGEW TRPVAAH) of the C-terminal domain of the LacZα fragment was present. The amino acid sequence of the deleted part was IEFPRPP. Despite the deletion, the remaining *p24* gene was still in-frame with both the 5'-end and the remaining 3'-end of the *LacZα*. It was therefore logical to proceed with this clone (with pGEM+p24D) for further investigations since the mouse CD4+ T and CD8+ T cell epitopes of interest were still present in the cloned *p24d* gene.

6.6.2 Recombinant *E. coli* and *Salmonella* vectors expressing P41 or P24D

Expression of P41 and p24D antigens in recombinant *E. coli* or *Salmonella enterica* serovar Typhimurium vaccine vectors harbouring pGEM+p1724 and pGEM+P24D was explored. The expression of P1724 and p24D by *E. coli* SCS110 clones (SCS110+P1724 and SCS110+p24D) was initially investigated colorimetrically by the inclusion of the X-gal in plates and visualizing if the colonies turned blue. The colorimetric method also showed that the genes were cloned in frame with the 5' and 3' ends of the *LacZα*. Since the two genes *(p1724* and *p24d*) were cloned in-frame with both the N- and C-terminal domains of LacZα, the full functionality (α-complementation) of β-galactosidase was expected to be minimally affected (Figure 6.4A). The bacterial colonies of recombinant *E. coli* cells expressing P24D were almost as dark blue as colonies from the positive control (recombinant *E. coli* cells expressing the full-length β-gal α-fragment. The bacterial colonies expressing P41 were light blue. Colonies expressing Salmgag were white since no α-complementation took place. The colony sizes from the different recombinant bacteria were the same for SCS110+p24D, SCS110+P1724 and SCS110+pGEM. Colonies from SCS110+Salmgag were slightly smaller than other recombinants.

The expression of P41 and P24D antigens by recombinant *E. coli* was further investigated using SDS-PAGE and Western blotting. Very high-level expression of the two antigens was observed (Figure 6.4B) after IPTG induction. P41 protein band was visible on SDS-PAGE stained with Coomassie brilliant blue, but the band for P24D was too faint to be visible with naked eye after staining the electrobloted SDS-PAGE. However, Western blot analysis confirmed the expression of both the antigens by the bacteria (Figure 6.4B).

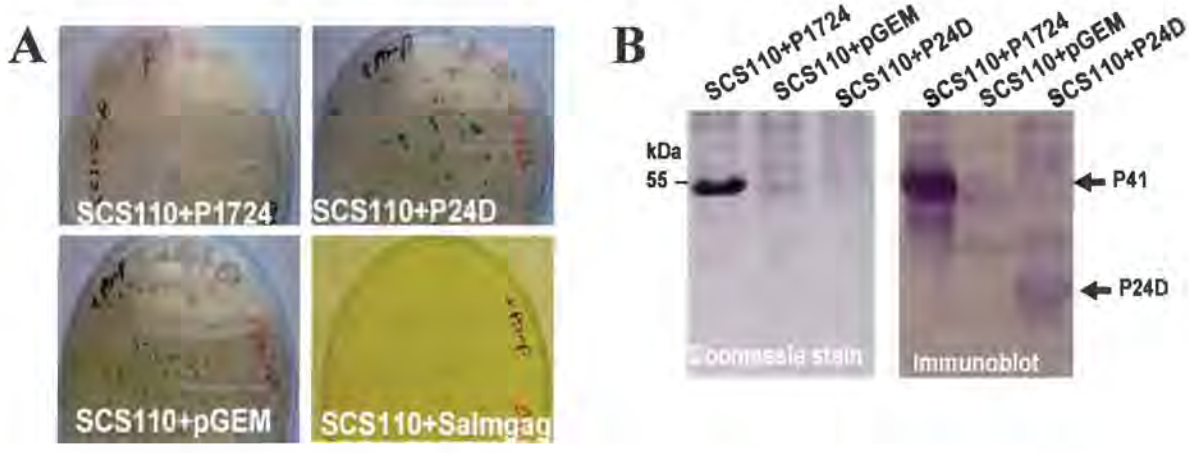

Figure 6.4: Expression of P41 and P24D in *E. coli* SCS110 cells. (**A**) Expression of P41 and P24D as determined by alpha-complementation. The clones were grown on plates with IPTG and X-gal (**B**) SDS-PAGE and Western blotting analysis of protein analysis of P41 and P24D expression in *E. coli* SCS110 cells. Total protein lysate of overnight recombinant *E. coli* SCS110 cells expressing P1724, LacZα (negative control) and p24D were run on 12.5% SDS-PAGE. The proteins were electrobloted onto a nitrocellulose membrane and probed with anti-P55 antibodies. The SDS-PAGE was stained after electrobloting of the proteins onto membrane.

Recombinant *Salmonella enterica* serovar Typhimurium vaccine vectors expressing P41 and P24D were developed by genetic transformation with the respective recombinant expression plasmids, pGEM+p1724 and pGEM+p24D to generate oral vaccines, AroC+P1724 and AroC+P24D. A negative control vaccine, AroC+pGEM (expressing LacZα) was also generated by transformation of *Salmonella enterica* serovar Typhimurium with the pGEM plasmid as previously described (Chapters 3, 4 and 5).

The expression of P41 and P24D in recombinant *Salmonella* vaccines was evaluated by SDS-PAGE. As observed in recombinant *E. coli,* very high levels of antigen expression were observed in recombinant *Salmonella* vaccine vectors (Figure 6.5). The P41 and P24D protein bands were clearly visible on the Coomassie-stained SDS-PAGE and this indicated that these antigens were the most expressed and abundant proteins in the vaccines (Figure 6.5A). The expression of P41 and P24D in recombinant vaccines was further confirmed by Roche Elecsys® HIV p24 Ag assay (Figure 6.5B).

Further analysis of recombinant P24D (from AroC+P24D) and LacZα (from AroC+pGEM) by SDS-PAGE showed that the antigens were insoluble when expressed in the bacteria (Figure 6.5C). The antigens were also not exported out of the bacteria as it was noted that the extracellular (media) fraction did not have any visible bands (results not shown). To further determine the solubilities of the recombinant P41 and P24D when overexpressed in *E. coli* and *Salmonella* vaccine vectors, the

previously described Wilkinson and Harrison Model (Wilkinson and Harrison, 1991) was used. The model showed that the antigens had high probabilities (P41, 72.2%; P24D, 81.8% and LacZα, 93.0%) of being insoluble when overexpressed by the bacteria.

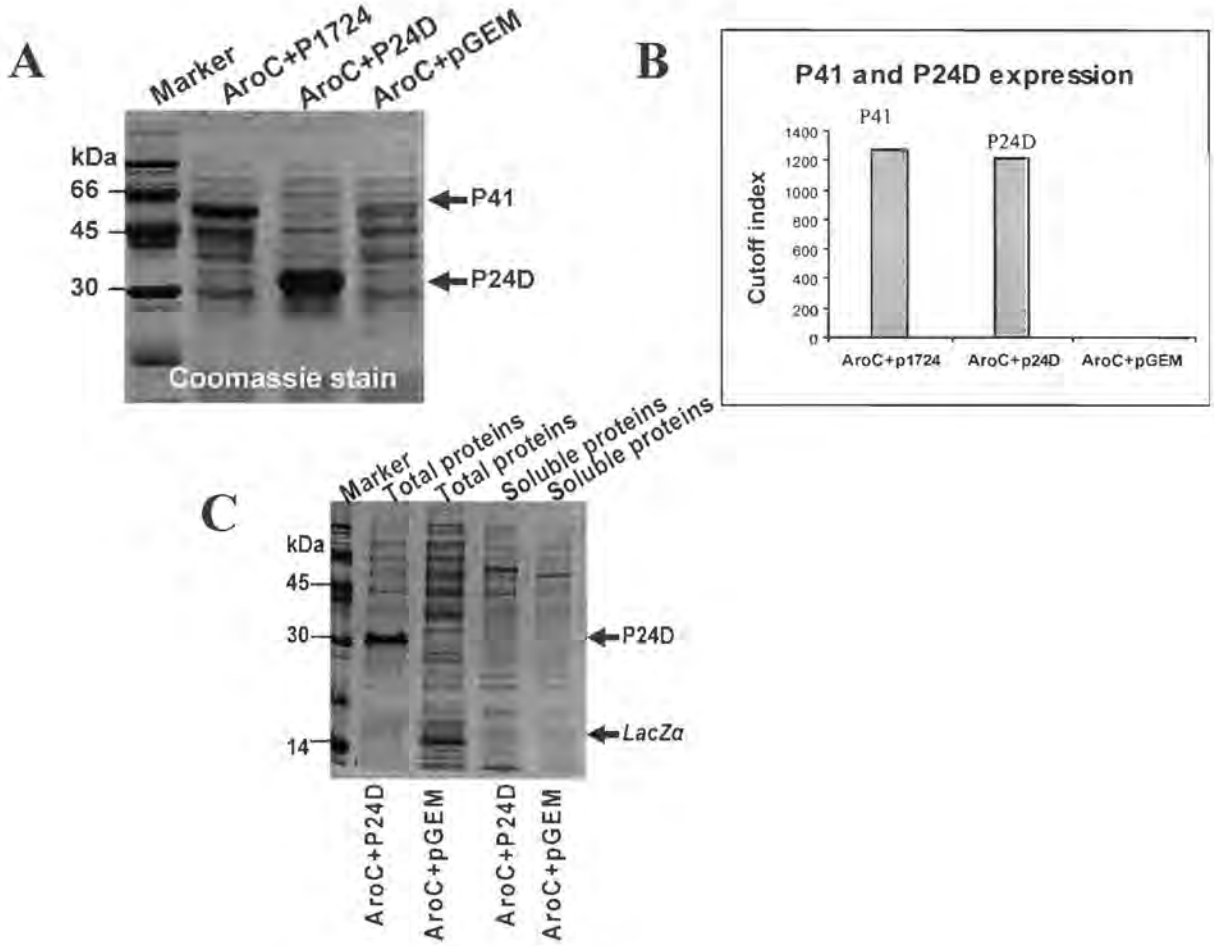

Figure 6.5: Expression of P41 and P24D in recombinant *Salmonella enterica* serovar Typhimurium vaccine vectors. (**A**) SDS-PAGE analysis of recombinant *Salmonella* expressing P41 or P24D (AroC+P1724 or AroC+P24D). The antigen bands were visible on the Coomassie-stained gel. (**B**) The relative expression of P41 and P24D by the recombinant *Salmonella* vaccine cultures as determined by Roche Elecsys® HIV p24 Ag assay. The total bacterial protein lysate was diluted 1/1000 in water. Protein lysate from the negative control, AroC+pGEM was also included. The cut-off index was calculated by the Elecsys® 2010 analyzer using readings from the negative and positive calibrators. (**C**) SDS-PAGE analysis of solubility of p24D and LacZα antigens as expressed in the recombinant *Salmonella* vaccine vectors. AroC+P24D and AroC+pGEM total and soluble fractions were analyzed.

6.6.3 Immunogenicity of a recombinant *Salmonella* vaccine vector expressing P41 or P24D

6.6.3.1 HIV-1 Gag-specific CD8+ and CD4+ T cell cytokine responses

To evaluate HIV-1 Gag-specific CD8+ and CD4+ T cell immunogenicity of recombinant *Salmonella enterica* serovar Typhimurium vaccine vector expressing P41 and P24D in vaccinated mice, IFN-γ and IL-4 ELISPOT and CBA assays were employed. On Day 28 (single inoculation) and Day 84 (three

inoculations), no HIV-1 Gag-specific IFN-γ and IL-4 ELISPOT responses to AroC+P1724 or AroC+P24D were observed. The number of IFN-γ SFUs produced after stimulation of splenocytes with Gag peptides were below the cut-off value.

Cellular immunogenicity in mice vaccinated with AroC+P1724 and AroC+P24D was further evaluated by quantifying IFN-γ and IL-4 produced by splenocytes stimulated with Gag peptides using the CBA assay. The SI in response to the Gag peptides were indicated (Figure 6.6). AroC+P1724 vaccine induced

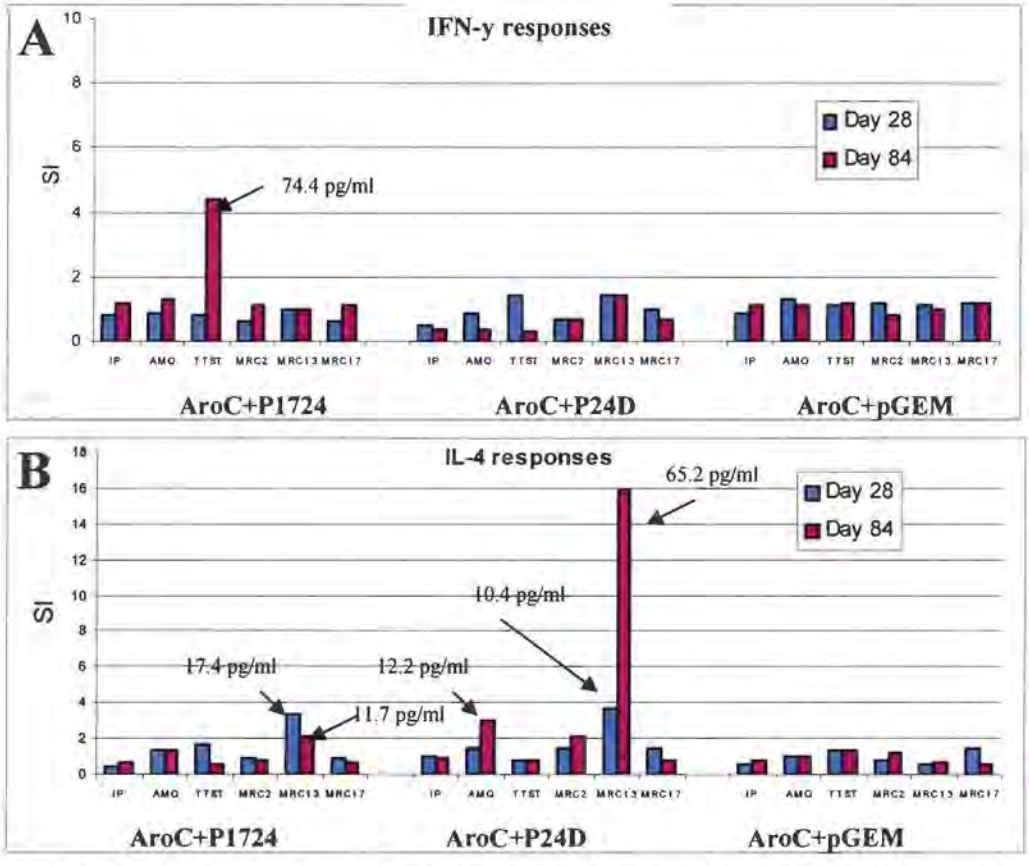

Figure 6.6: The magnitude of **Gag-specific CD8+ T cell responses** as determined by quantification of IFN-γ (**A**) and IL-4 (**B**) cytokines (in pg/ml) secreted by splenocytes during stimulation for 48 hours in the absence or presence of Gag peptides, using CBA assay. Groups of mice were vaccinated ONCE (Experiment 1, Table 6.1) or THREE times (Experiment 2, Table 6.2) with AroC+P1724, AroC+P24D or AroC+pGEM. On Day 28 (Experiment 1) and Day 84 (Experiment 2), splenocytes from the vaccinated mice were incubated with medium only (negative assay control), or stimulated with the Gag CD8+ T cell peptides (AMQ = AMQMLKDTI and TTST= TTSTLQEQI) or the Gag CD4+ T cell peptides (MRC2, MRC13 and MRC17) and the amounts of IFN-γ (**A**) and IL-4 (**B**) measured by CBA assay. Each bar in the graphs represents SI for each peptide. Responses are positive if the SI is greater than 2 and the pg of cytokine produced per ml in 48 hrs is indicated.

a Gag-specific CD8+ Tc1 cytokine response after three inoculations (Figure 6.6A). The IFN-γ SI of other Gag peptides were <2 and this showed that no Gag-specific CD8+ Tc1 and CD4+ Th1 cytokine

responses could be detected. Mice vaccinated with AroC+P24D did not induce Gag-specific cytokine responses after both single and triple inoculations (Figure 6.6A).

A single inoculation of mice with AroC+P1724 resulted in only MRC13-specific cells producing elevated levels of IL-4 (Th2) cytokine (Figure 6.6B). The response was not boosted in mice by Day 84, but almost maintained (Figure 6.6B). The other peptides did not induce the secretion of IL-4 from splenocytes vaccinated with AroC+P1724 (Figure 6.6B). In mice vaccinated with AroC+P24D, AMQ specific IL-4 cytokine (CD8+ Tc2) response was induced only on Day 84. MRC13-specific IL-4 (CD4+ Th2) response was detected on Day 28 in mice vaccinated with AroC+P24D and the response was boosted strongly by Day 84 (Figure 6.6B). More IL-4 than IFN-γ was produced by these MRC13-specific cells.

6.6.3.2 HIV-1 Gag-specific humoral immune responses

HIV-1 Gag-specific serum IgG immune response was evaluated on Days 28, 56 and 84 in mice vaccinated with the recombinant *Salmonella* vaccine vector expressing P41 or P24D. The two *Salmonella* vaccines did not elicit Gag-specific antibody responses at the three time points (Figure 6.19A). However IgG response against the *Salmonella* LPS were very high on Day 84 (Figure 6.19B), and this suggested that successful vaccination of the mice occurred.

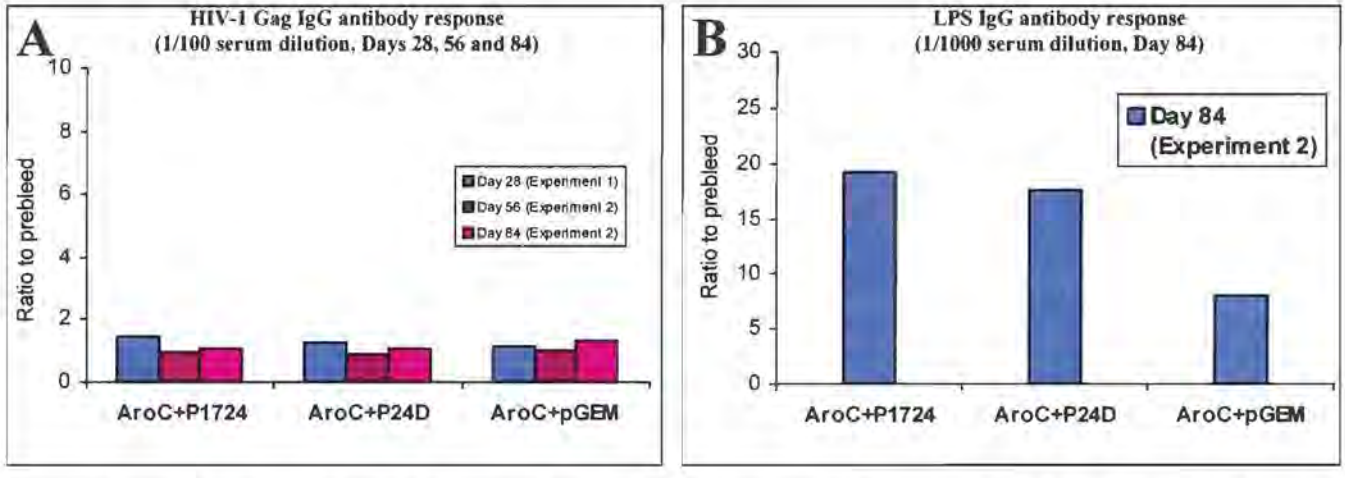

Figure 6.7: Serum HIV-1 Gag- and *Salmonella* LPS-specific IgG responses in mice vaccinated with *Salmonella* expressing P41 or P24D. Groups of mice were vaccinated ONCE (Table 6.1) or THREE times (Table 6.2) with a *Salmonella* vaccines, AroC+P1724, AroC+P24D and AroC+pGEM. Prior to inoculation, the mice were bled and pools of serum made. On Days 28 (Experiment 1), 56 and 84 (Experiment 2), the mice were bled and serum made. (**A**) The HIV-1 Gag-specific IgG was measured serum in pools of each group of mice for Days 28, 56 and 84 in 1/100 serum dilution. (**B**) The LPS-specific IgG was measured in serum pools of each group of mice for Day 84 in 1/1000 serum dilution. The data are represented as the ratios of the mean OD_{405nm} of duplicates to the mean OD_{405nm} prebleed duplicates. Antibody responses were defined as positive when the mean OD_{405nm} ratio to prebleed was >2.

6.7 DISCUSSION

6.7.1: Development of recombinant *Salmonella* expressing P41 and P24

A new approach to expression of heterologous antigens in recombinant *E. coli* and *Salmonella* vaccine vector was employed. The strategy involved cloning of foreign antigen genes (*p41* and *p24*) in-frame between the 5' and 3' domains of the *E. coli* LacZα gene fragment. The rationale of taking such an approach was based on a postulation that "embedding" the foreign antigens into a bacterial protein would reduce the post-transcriptional degradation of the mRNA transcripts by the RNA degradosome or prevent post-translational proteolytic degradation of the antigens by bacterial proteases. It was also speculated that "embedding" the antigens into a prokaryotic antigen would reduce the metabolic burden and the bacteria would potentially not recognize the whole polyproteins as "foreign". It was also anticipated that "embedding" the truncated Gag antigens (P41 and P24) would further improve their expression, while reducing the cytopathogenicity observed with full-length Salmgag in Chapter 5. P41 and P24 antigens were selected because they contain the necessary CD4+ and CD8+ T cell Gag epitopes for assessment of immunogenicity in mice (Mata *et al.*, 1998).

The truncated *salmgag* genes (*p41* and *p24d*) were successfully cloned in-frame with both 5' and 3' ends of LacZα (Figures 6.3). The cloning of full-length *p24* gene was however problematic in *E. coli*. All the clones screened had the gene either completely lost or had some sections deleted, potentially by genetic recombination. The reason for loss or deletion of *p24* could be attributed to sequence homologies between the gene and the bacterial chromosome or pGEM plasmid, which facilitated genetic recombination. One clone which had only a small deletion in the gene (P24D) was used in the current studies since the gene was still in-frame with both the N- and C-terminal domains of *LacZα* gene and had the Gag CD4+ and CD8+ T cell epitopes required to test immunogenicity.

The cloning of both the P41 and P24D in-frame with the 5' and 3' ends of the *LacZα* was expected to minimally affect α-complementation phenomenon in *E. coli* SCS110 cells. X-gal could still be hydrolysed by the β-galactosidase enzyme to yield a blue precipitate (Figure 6.4). The colonies of recombinant *E. coli* cells expressing P24D were as dark blue as colonies from the positive control, indicating no interference of α-complementation. Conversely, the colonies expressing P41 were light blue and this indicated that although the antigen was in-frame with the β-gal α-fragment, there was some interference to α-complementation. Longer polypeptides such as P41 are likely to have more interference than shorter ones such as P24D. Most of the original cloning vectors based on α-complementation were developed by Messing and have been called pUC vectors (Vieira and Messing, 1982). The current cloning vectors such as pGEM-Teasy and pBluescript plasmids were developed based on the pUC vectors. Alpha-complementation phenomenon was not noted in recombinant *Salmonella enterica* serovar Typhimurium vaccine vectors expressing P41 and P24D. This is because *Salmonella* do not naturally have the *lac* operon as with *E. coli* which overexpressed the chromosomally cloned *lac* repressor.

The growth of the recombinant *E. coli* was not affected by the expression of foreign antigens. This suggested that the expression of the antigens did not induce adverse metabolic burden on the bacteria. The high-level expression of the antigens (Figures 6.4 and 6.5) also suggested that there was limited degradation of the antigens in bacteria. As in *E. coli*, very high level of expression of the antigens was observed in recombinant *Salmonella* vaccine vectors (Figures 6.4 and 6.5). The embedding of the antigens into the LacZα seemed to have even improved their stable expression. However, the embedding of the antigens made them to be highly insoluble in bacteria (Figure 6.14 and Table 6.3). The formation of inclusion bodies was dictated by the amino acid residue composition of β-galactosidase α-fragment, which when theoretically expressed alone was highly insoluble. The improvement in expression of the antigens also potentially facilitated formation of inclusion bodies. It has been established that overexpression of heterologous genes promoted formation of such inclusion bodies (Schlieker *et al.*, 2002; Baneyx and Mujacic, 2004). The impact of insolubility or formation of inclusion bodies of P41 and P24D in recombinant *Salmonella enterica* serovar Typhimurium vaccine vectors on immunogenicity in mice remained unclear.

6.7.2 CD8+ T and CD4+ T cell cytokine responses to recombinant *Salmonella* expressing P41 and P24D

Despite very high expression of P41 and P24D in recombinant *Salmonella* vaccine vectors, CD8+ Tc1 and Tc2 cytokine responses were only observed after booster vaccination of mice. The stimulation index > 4 for IFN-γ was observed from AroC+P1724 after stimulation of splenocytes with TTST peptide (Figure 6.6) suggested induction of HIV-1-specific CD8+ Tc1 cytokine response. The stimulation index of 3 for IL-4 observed from AroC+P24D vaccine after stimulation of splenocytes with AMQ peptide (Figure 6.6) suggested the induction of HIV-1-specific CD8+ Tc2 cytokine response. Although these responses were low, they suggested that oral vaccination of mice with recombinant *Salmonella* expressing foreign antigens embedded in prokaryotic proteins would give antigen-specific CD8+ T cell immune responses. Theoretically fusing antigens or their epitopes inside other proteins should not affect CD8+ T cell responses since such responses depend on epitopes, and not the whole antigen.

Gag-specific CD4+ T cell responses in mice vaccinated with the *Salmonella* vaccines were also evaluated. Single vaccination of mice with recombinant *Salmonella* vaccine vectors expressing P41 and P24D induced antigen-specific IL-4 (Figure 6.6). This suggested that Gag-specific CD4+ Th2 responses were elicited. The responses were maintained after booster vaccination with AroC+P1724, but improved drastically with AroC+P24D. The improvement in IL-4 responses for AroC+P24D could be a result of the high expression of the antigen by the vaccine. Only IFN-γ responses were observed after stimulation of the cells with the Gag CD4+ T cell epitopes. The production of only IL-4 showed that AroC+P1724 and AroC+P24 were Th2-biased vaccines. The factors that facilitated the imprinting of a strong Th2-biased response were not clear. However such results have previously been observed in which vaccination of mice with a *Salmonella* vector induced a strong Th2-biased response (Jun *et al.*, 2005; Wu *et al.*, 2006).

6.7.3 HIV-1 specific humoral immune responses

Despite the high level expression of P41 and P24D by the recombinant *Salmonella* vaccines, HIV-1 Gag-specific humoral responses were not detected on Days 28, 56 and 84 (Figure 6.7). The failure to induce the antibody responses could be a result of embedding the Gag fragments into *E. coli* LacZα gene fragment. The embedding potentially affected the native structure of the proteins, thereby affecting immune responses to the Gag B-cell epitopes. The B-cell epitopes of P41 and P24D could also be lost due to poor folding of the proteins. Compared to Salmgag (Chapter 5), there were also possibly fewer B-cell epitopes on P41 and P24D (due to their smaller sizes) and this could potentially affect the detection of antibody responses by ELISA.

In conclusion, a method for expression of foreign antigens in a recombinant *Salmonella* vaccine vector was developed. The method involved fusing the antigens, P41 or P24 between the N- and C- terminal domains of *E. coli* LacZα. Oral vaccination of mice with the recombinant *Salmonella* vaccines could still induce HIV-1 Gag-specific cellular immune responses. Approaches to improve the induction of antigen-specific cellular and humoral immune responses against recombinant *Salmonella* vaccines expressing embedded antigens or epitopes need future investigations.

CHAPTER 7: SUB-CELLULAR LOCALIZATION OF GREEN FLOURESCENT PROTEIN OR HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C GAG IN RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR

"The picture lives only within the frame we have invented for it" (Holmes, 1995)

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CHAPTER 7: SUB-CELLULAR LOCALIZATION OF GREEN FLOURESCENT PROTEIN OR HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C GAG IN RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM VACCINE VECTOR

7.1 SUMMARY

The sub-cellular compartmentalization of HIV-1 Subtype C Gag in recombinant *Salmonella* vaccine vectors has not previously been investigated. The objective of this study was to determine the localization of HIV-1 Gag and GFP in a recombinant *Salmonella enterica* serovar Typhimurium vaccine vector using GFP as a fluorescent tag. The *salmgag* (codon-optimized HIV-1 *gag*) gene was cloned between the first 40 codons of *E. coli* β-galactosidase α-gene and full-length *gfp* gene to generate a recombinant expression plasmid, pGEM+Salmgag-GFP with expression under the *lac* promoter. A recombinant *Salmonella enterica* serovar Typhimurium vector (AroC+Salmgag-GFP) expressing Salmgag-GFP was developed. The expression of Salmgag-GFP in recombinant *E. coli* and *Salmonella* was demonstrated. Evaluation of subcellular localization by fluorescence microscopy showed that the Salmgag was localized mainly to the polar regions of the bacterial cell. However, this was in contrast to GFP that was homogenously distributed within the bacterial cell when expressed alone. The mechanism underlining the bipolar localization of Salmgag in the recombinant *Salmonella* was unclear. The phenomenon could be attributed to bacterial stress response induced by the constitutive overexpression of the antigen. It was not clear whether the differences in the subcellular localization of Salmgag and GFP in the bacterial vector affected the nature and magnitude of immune responses previously observed (Chapters 3 and 5).

7.2 INTRODUCTION

One of the key applications of GFP in bacteriology is its use as a fusion tag for monitoring the expression and subcellular localization of other proteins (March *et al.*, 2003; Hynes *et al.*, 2004; Giepmans *et al.*, 2006). The expression and localization of fusion proteins can be monitored in live bacterial cells (Margolin, 2000). The advantage of using GFP over other reporter proteins is that localization is monitored without the need for substrate or cofactors (Cubitt *et al.*, 1995; Tsien, 1998). GFP fusions have already been made in which bacterial proteins involved in various processes were tracked (Phillips, 2001; Meile *et al.*, 2006; Miller *et al.*, 2006).

One of the recent applications of GFP is its use as a protein folding reporter (Waldo *et al.*, 1999; Waldo, 2003; Cabantous *et al.*, 2005; Pedelacq *et al.*, 2006). In Gram-negative bacteria such as *Salmonella* and *E. coli*, foreign proteins tend to form misfolded aggregations called inclusion bodies when overexpressed (Villaverde and Carrio, 2003; Baneyx and Mujacic, 2004). The rationale of using GFP as a folding reporter is because its fluorescence depends on correct folding of the whole fusion protein. Therefore when GFP is fused downstream of a protein that folds correctly, fluorescence is not affected. However, when the fusion partner misfolds or forms inclusion bodies, the GFP fluorescence is affected drastically (Waldo *et al.*, 1999; Pedelacq *et al.*, 2006).

No previous studies have investigated the cellular localization of full-length HIV-1 subtype C Gag antigen in live *Salmonella* vaccine vectors. In previous work (Chapter 3, 4 and 5), it was demonstrated that there were some differences in the nature and magnitude of antigen-specific immune responses induced by a *Salmonella* vaccine vector expressing GFP or HIV-1 Gag antigen. The studies also suggested that GFP was largely soluble, while HIV-1 Gag antigens were predominantly insoluble when expressed in the vector. It was therefore important to investigate the localization of these antigens in the *Salmonella* vector. Thus the main objectives of the current research were:

1. to construct a recombinant expression plasmid with *salmgag* fused in-frame with the 5' end (first 40 codons) of the *E. coli* β-galactosidase α-gene fragment and the *gfp* with expression under the *lac* promoter.
2. to develop recombinant *Salmonella enterica* serovar Typhimurium vaccine vector expressing the Salmgag-GFP fusion antigen
3. to investigate on differences in the sub-cellular localization of the Salmgag and GFP antigens in the recombinant *Salmonella* vector.

7.3 MATERIALS

Materials used in this study have been described in Appendices.

7.4 GENERAL METHODS

General methods were used as described in Appendices A and B.

7.5 EXPERIMENTAL DESIGN AND PROTOCOLS

7.5.1 Construction of recombinant pGEM+Salmgag-GFP expression plasmid: molecular cloning of *salmgag-gfp* fusion gene

The construction of recombinant plasmid, pGEM+Salmgag-GFP (Appendices G9 and H6) with recombinant *salmgag-gfp* fusion gene involved two cloning steps (Figures 7.1 and 7.2). The first step involved replacement of wild-type Du_{422} HIV-1 *gag* in Pgagmtrgfp (Appendix G1) with *salmgag* gene. The Pgagmtrgfp was doubly digested with restriction enzymes, *Nar*I and *Hind*III, to excise out the wild-type HIV-1 *gag*. The digestion reaction was run on agarose gel and the vector (larger fragment) excised from the gel and purified as described in Appendix B1.6. Pscript+Salmgag plasmid (Appendix G10) was also doubly digested with *Nar*I/*Hind*III and DNA fragments separated by agarose gel electrophoresis. The *salmgag* DNA insert was isolated from the gel, purified and ligated to the linearized vector to generate Psalmgagmtrgfp plasmid. The ligation reaction was used for the transformation of competent SCS110 cells (Appendix A1.4). Recombinant clones were screened using restriction enzyme mapping (Appendix B1.5). However, there was a stop codon, TGA at the end the *gfp* gene in Psalmgagmtrgfp. Such a stop codon, which is not efficient in translation termination in *Salmonella* was to be replaced with an efficient one, TAA. I also wanted the expression of Salmgag-GFP to be under the *lac* promoter. This was to be achieved in the second cloning step (Figure 7.2).

The second cloning step involved cloning of the *salmgag-gfp* fusion gene from Psalmgagmtrgfp plasmid into the plasmid vector backbone of pGEM+GFP (Figure 7.2). Psalmgagmtrgfp plasmid was doubly

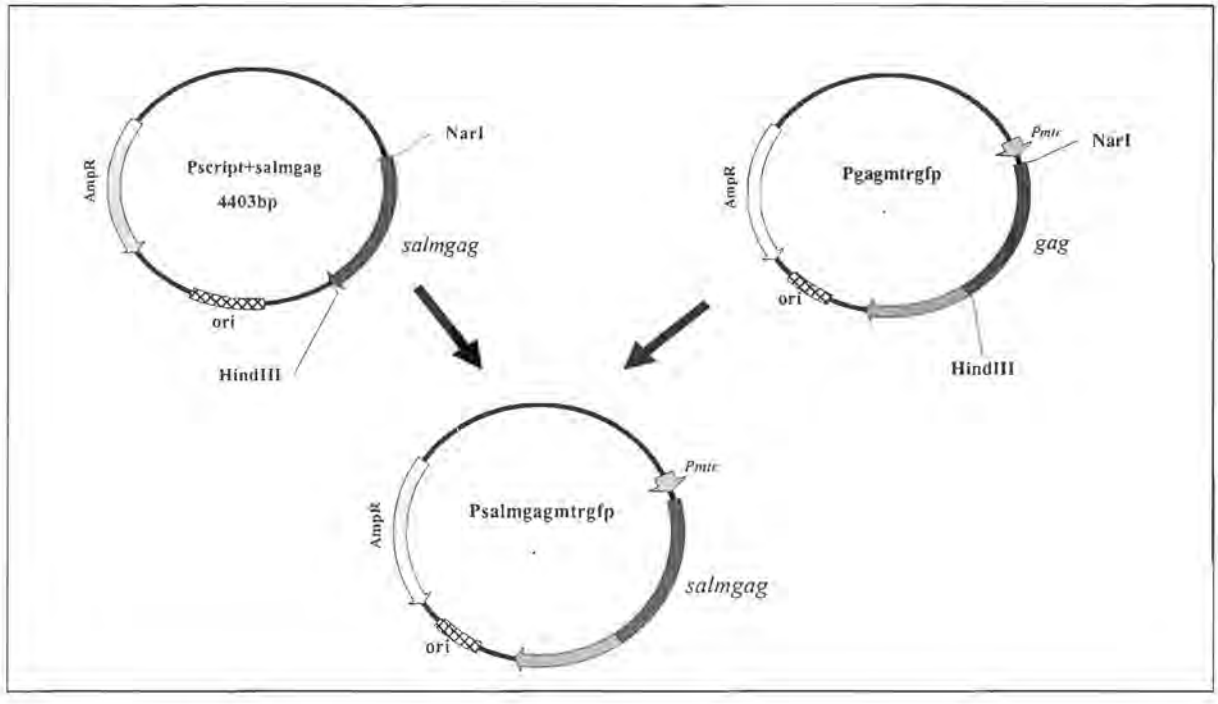

Figure 7.1: Molecular cloning of *salmgag* gene. The *salmgag* gene from Pscript+Salmgag was cloned into the backbone of Pgagmtrgfp to yield Psalmgagmtrgfp plasmid.

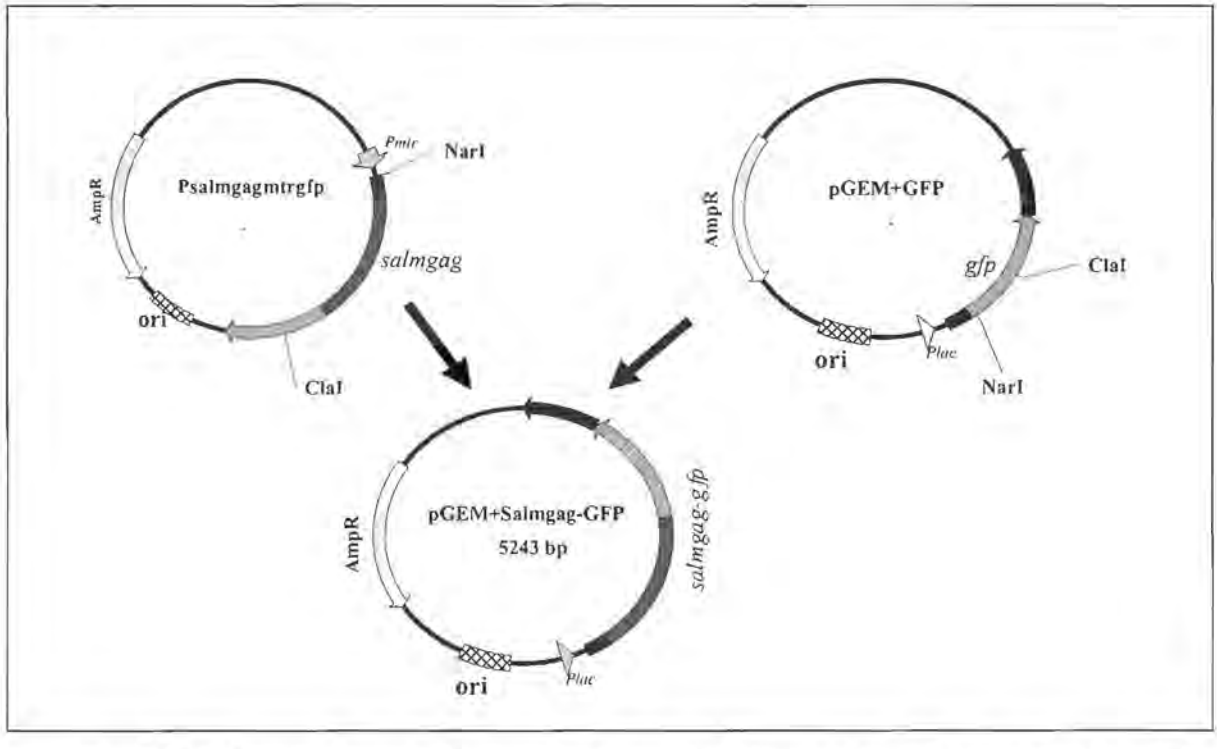

Figure 7.2: Molecular cloning of *salmgag-gfp* gene. The *salmgag-gfp* gene fragment was cloned from from Psalmgagmtrgfp into the backbone of pGEM+GFP expression plasmid to yield pGEM+Salmgag-GFP plasmid.

digested with *Nar*I and *Cla*I to release the *salmgagag* and a part of *gfp* gene and the insert was cut from the gel and purified. The pGEM+GFP plasmid was also doubly digested with the same two enzymes to release out part of the *gfp* gene. The DNA band for the vector was cut from the gel, purified and ligated to the *salmgag-gfp* insert. The ligation reaction was used in the transformation of competent *E coli* SCS110 cells and screening of candidate clones was pursued using restriction mapping. Maximum plasmid preparation (Appendix B1.2) of pGEM+Salmgag-GFP from the candidate clone was performed.

7.5.2 Development of recombinant *Salmonella* expressing Salmgag-GFP and assessment of sub-cellular localization of the fusion antigen

The transformation of competent *AroC Salmonella enterica* serovar Typhimurium mutant with pGEM+Salmgagfp was performed using standard protocol (Appendix A1.4). Salmgag-GFP expression by recombinant *E. coli* SCS110 was determined by SDS-PAGE (Appendix B4). The selected bacterial clone was grown in 2 YT liquid medium with IPTG overnight at 37°C. Expression of Salmgag-GFP by the recombinant *Salmonella* was evaluated by UV fluorescence. The UV fluorescence of AroC+Salmgag-GFP clones on agar plates was compared with that of AroC+ GFP (developed previously, Chapter 3). The determination of sub-cellular localization of Salmgag-GFP fusion protein in the recombinant *Salmonella* vaccine vector was performed using fluorescence microscopy (Appendix A3). The sub-cellular localization of Salmgag-GFP (in AroC+Salmgag-GFP) was compared with that of GFP expressed by the vaccine AroC+GFP (developed in Chapter 3).

7.6 RESULTS

7.6.1 Development of a recombinant expression plasmid vector, pGEM+Salmgag-GFP: molecular cloning of *salmgag-gfp* fusion gene

A recombinant expression plasmid, pGEM+Salmgag-GFP, with *salmgag* fused to *gfp* was successfully constructed. The general structure of pGEM+Salmgag-GFP is given in Figure 7.3A. The structure of the fusion protein (Salmgag-GFP) consisted of *salmgag* and *gfp* genes fused in-frame with the β-galactosidase α-fragment (Figure 7.3B). The expression of the 87 KDa fusion protein was under the *lac* promoter.

7.6.2 Cellular localization of Salmgag-GFP and GFP in recombinant *Salmonella* vaccine

Expression of Salmgag-GFP by recombinant *E. coli* SCS110 clone with the recombinant pGEM+Salmgag-GFP plasmid was successfully demonstrated. The recombinant SCS110 bacterial colonies were fluorescing under ultraviolet light indicating that the *gfp* gene was cloned in frame with the *salmgag*. The Expression of the 87 KDa Salmgag-GFP protein in the SCS110 cells was demonstrated by SDS-PAGE (Figure 7.4A). The protein band of the antigen was visible on the Coomassie-stained SDS-PAGE. The expression of the Salmgag-GFP protein in recombinant *Salmonella enterica* serovar Typhimurium was assessed by UV fluorescence. The colonies and cultures of the recombinant *Salmonella* expressing Salmgag-GFP were fluorescing under UV light (Figure 7.4B). It was noted that the fluorescence of these colonies was less than the fluorescence of colonies expressing GFP only (Figure 7.4C).

To investigate sub-cellular compartmentalization of GFP or Salmgag-GFP in the recombinant *Salmonella*, fluorescence microscopy was used to analyze live bacterial cells. It was observed that when expressed alone, the GFP was evenly distributed inside the live bacterial cell (Figure 7.5A). This was in contrast to the localization of Salmgag-GFP fusion antigen, which was predominantly confined to the poles of the bacteria (Figure 7.5B). The motility of the live bacteria could be observed under fluorescent microscopy. It was noted that the bacteria from AroC+GFP were more actively motile than those from AroC+Salmgag-GFP. The AroC+Salmgag-GFP bacteria maintained their fluorescence in stationary phase and dead cells (those which lost motility) continued to be fluorescent and some showed evidence of bursting and releasing of their fluorescent cellular contents (Figure 7.5C). The subcellular compartmentalization of Salmgag-GFP during bacterial cytokinesis was noted (Figure 7.5D). At resting stage (Figure 7.5 D1), the Salmgag-GFP protein exhibited a bipolar localization. During bacterial cytokinesis, the Salmgag-GFP was confined to the poles as well as the mid-centre (Figure 7.5 D2). The daughter cells received equal amounts of the Salmgag-GFP antigen (Figures 7.5D3 and 7.5D4).

B

```
1     ATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCC
1      M  T  M  I  T  P  S  Y  L  G  D  T  I  E  Y  S  S  Y  A  S
61    AACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGAT
21     N  A  L  G  A  L  P  Y  G  R  P  A  G  G  R  E  F  T  S  D
121   TATGGCGCCAGAGCGTCAATATTAAGGGGGGAAAAATTAGATACATGGGAAAAAATTAGG
41     Y  G  A  R  A  S  I  L  R  G  E  K  L  D  T  W  E  K  I  R
181   TTAAGGCCAGGGGGAAAGAAACATTATATGTTAAAACACATAGTATGGGCAAGCAGGGAG
61     L  R  P  G  G  K  K  H  Y  M  L  K  H  I  V  W  A  S  R  E
241   CTGGAAAGATTTGCACTTAACCCTGGCCTTTTAGAAACATCAGAAGGATGTAAACAAATA
81     L  E  R  F  A  L  N  P  G  L  L  E  T  S  E  G  C  K  Q  I
301   ATAAAACAGCTACAACCAGCTCTCCAGACAGGAACAGAGGAACTTAAATCATTATACGAC
101    I  K  Q  L  Q  P  A  L  Q  T  G  T  E  E  L  K  S  L  Y  D
361   ACAGTAGCAACTCTCTATTGTGTACATGAAAAGATAGAAGTACGAGACACCAAGGAAGCC
121    T  V  A  T  L  Y  C  V  H  E  K  I  E  V  R  D  T  K  E  A
421   TTAGATAAGATAGAGGAAGAACAAAACAAATGTCAGCAAAAAACGCAGCAGGCAAAAGCG
141    L  D  K  I  E  E  E  Q  N  K  C  Q  Q  K  T  Q  Q  A  K  A
481   GCTGACGGGAAAGTCAGTCAAAATTATCCTATAGTGCAGAATCTCCAAGGGCAAATGGTA
161    A  D  G  K  V  S  Q  N  Y  P  I  V  Q  N  L  Q  G  Q  M  V
541   CATCAAGCCATATCACCTAGAACCTTGAATGCATGGGTAAAAGTAATAGAGGAAAAGGCT
181    H  Q  A  I  S  P  R  T  L  N  A  W  V  K  V  I  E  E  K  A
601   TTTAGCCCAGAGGTAATACCCATGTTTACAGCATTATCAGAAGGAGCCACCCCACAAGAT
201    F  S  P  E  V  I  P  M  F  T  A  L  S  E  G  A  T  P  Q  D
661   TTAAACACCATGTTAAATACGGTGGGGGGACATCAAGCAGCCATGCAAATGTTAAAAGAT
221    L  N  T  M  L  N  T  V  G  G  H  Q  A  A  M  Q  M  L  K  D
721   ACCATCAATGAAGAGGCTGCAGAATGGGATAGATTACATCCAGTACATGCGGGGCCTATT
241    T  I  N  E  E  A  A  E  W  D  R  L  H  P  V  H  A  G  P  I
781   GCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAACTACTAGTACCCTT
261    A  P  G  Q  M  R  E  P  R  G  S  D  I  A  G  T  T  S  T  L
841   CAGGAACAAATAGCATGGATGACAAGTAACCCACCTATTCCAGTGGGAGACATCTATAAA
281    Q  E  Q  I  A  W  M  T  S  N  P  P  I  P  V  G  D  I  Y  K
901   AGATGGATAATTCTGGGGTTAAATAAAATAGTGAGAATGTATAGCCCGGTCAGCATTTTG
301    R  W  I  I  L  G  L  N  K  I  V  R  M  Y  S  P  V  S  I  L
961   GACATAAGACAAGGGCCAAAGGAACCCTTTCGAGACTATGTAGATCGGTTCTTTAAAACT
321    D  I  R  Q  G  P  K  E  P  F  R  D  Y  V  D  R  F  F  K  T
1021  TTAAGAGCTGAACAAGCTACACAAGAAGTAAAAAATTGGATGACAGACACCTTGTTAGTC
341    L  R  A  E  Q  A  T  Q  E  V  K  N  W  M  T  D  T  L  L  V
1081  CAAAATGCGAACCCAGATTGTAAGACCATTTTGAGAGCATTGGGACCAGGGGCTACATTA
61     Q  N  A  N  P  D  C  K  T  I  L  R  A  L  G  P  G  A  T  L
1141  GAAGAAATGATGACAGCATGTCAAGGGGTGGGAGGACCTGGCCACAAAGCAAGAGTATTG
381    E  E  M  M  T  A  C  Q  G  V  G  G  P  G  H  K  A  R  V  L
1201  GCTGAGGCAATGAGTCAAACAAACAGTGGAAACATAATGATGCAGAGAAGCAATTTTAAA
401    A  E  A  M  S  Q  T  N  S  G  N  I  M  M  Q  R  S  N  F  K
1261  GGCCCTAGAAGAATTGTTAAATGTTTTAACTGTGGCAAGGAAGGGCACATAGCCAGAAAT
421    G  P  R  R  I  V  K  C  F  N  C  G  K  E  G  H  I  A  R  N
1321  TGCAGAGCCCCTAGGAAAAAAGGCTGTTGGAAATGTGGAAAAGAAGGACACCAAATGAAA
441    C  R  A  P  R  K  K  G  C  W  K  C  G  K  E  G  H  Q  M  K
1381  GACTGCACTGAGAGGCAGGCTAATTTTTTAGGGAAAATTTGGCCTTCCCACAAGGGGAGG
461    D  C  T  E  R  Q  A  N  F  L  G  K  I  W  P  S  H  K  G  R
1441  CCAGGGAATTTCCTTCAGAACAGACCAGAGCCAACAGCCCCACCAGCAGAGAGCTTCAGG
481    P  G  N  F  L  Q  N  R  P  E  P  T  A  P  P  A  E  S  F  R
1501  CTCGAAGAGACAACCCCCGCTCCGAAGCAGGAGCCGATAGAAAGGGAACCCTTAACTTCC
501    L  E  E  T  T  P  A  P  K  Q  E  P  I  E  E  R  E  P  L  T  S
1561  CTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAAGCTTCCGGCTCCGCCGGTTCCGCC
521    L  K  S  L  F  G  S  D  P  L  S  Q  A  S  G  S  A  G  S  A
1621  GGCTCAGCTTCGAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTA
541    G  S  A  S  K  G  E  E  L  F  T  G  V  V  P  I  L  V  E  L
1681  GATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACA
561    D  G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G  D  A  T
1741  TACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCA
581    Y  G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V  P  W  P
1801  ACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCAAGATACCCAGATCATATG
601    T  L  V  T  T  F  S  Y  G  V  Q  C  F  S  R  Y  P  D  H  M
1861  AAGCGGCACGACTTCTTCAAGAGCGCCATGCCTGAGGGATACGTGCAGGAGAGGACCATC
621    K  R  H  D  F  F  K  S  A  M  P  E  G  Y  V  Q  E  R  T  I
1921  TCTTTCAAGGACGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAGGGAGACACC
641    S  F  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E  G  D  T
1981  CTCGTCAACAGGATCGAGCTTAAGGGAATCGATTTCAAGGAGGACGGAAACATCCTCGGC
661    L  V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N  I  L  G
2041  CACAAGTTGGAATACAACTACAACTCGCACAACGTATACATCACGGCAGACAAACAAAAG
681    H  K  L  E  Y  N  Y  N  S  H  N  V  Y  I  T  A  D  K  Q  K
2101  AATGGAATCAAAGCTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTA
701    N  G  I  K  A  N  F  K  I  R  H  N  I  E  D  G  S  V  Q  L
2161  GCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAAC
721    A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L  P  D  N
2221  CATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATG
741    H  Y  L  S  T  Q  S  A  L  S  K  D  P  N  E  K  R  D  H  M
2281  GTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAA
761    V  L  L  E  F  V  T  A  A  G  I  T  H  G  M  D  E  L  Y  K
2341  TAAGCTTAAT
781    *  A  *
```

5'-LacZα

salmgag

linker

gfp

Figure 7.3: The general structural components of the Salmgag-GFP expression construct developed by the study. (**A**) A schematic representation of the Salmgag-GFP expression plasmid. The *salmgag* gene was fused in-frame between the *5'-LacZα* and the *gfp* gene. The expression of Salmgag-GFP was under the *E. coli lac* promoter and other *lac* operon transcription and translation domains found in pGEM-Teasy plasmid. (**B**) The DNA and amino acid sequences of the resultant *salmgag-gfp* gene and its Salmgag-GFP protein. The whole fusion protein was 86.843 KDa. The main features and complete DNA sequence of pGEM+Salmgag-GFP are given in Appendices G9 and H6 respectively.

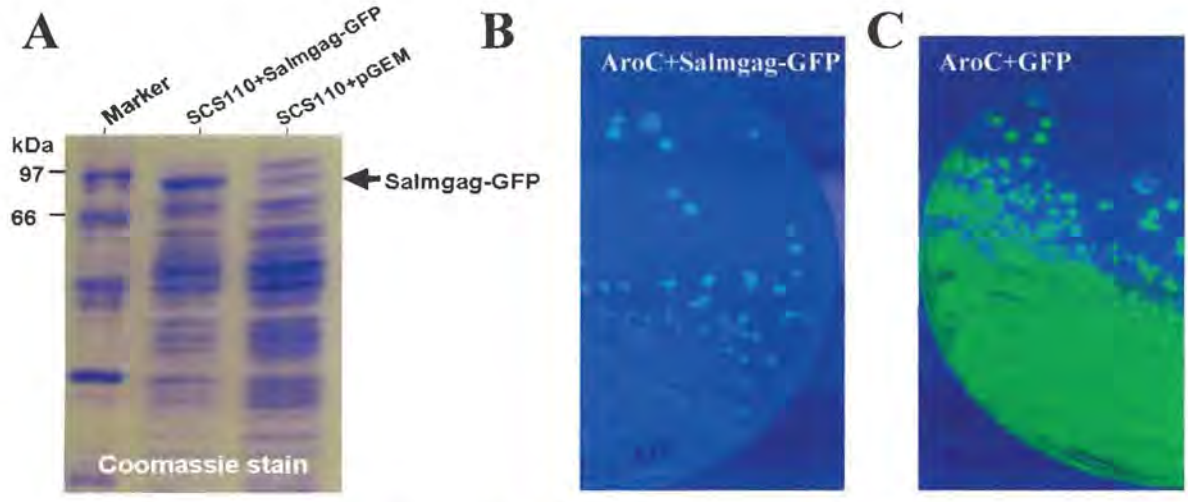

Figure 7.4: Expression of Salmgag-GFP fusion antigen in recombinant *E. coli* and *Salmonella* vaccine vector (**A**) SDS-PAGE analysis of recombinant *E. coli SCS110* cells expressing Salmgag-GFP. Total protein lysates of *E. coli* harbouring the pGEM+Salmgag-GFP or pGEM were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. SCS110 cells expressed high levels of Salmgag-GFP when IPTG was added into the media. The Salmgag-GFP protein band was visible on the gel. (**B**) Colonies (streak) of recombinant *Salmonella* vaccine vector expressing Salmgag-GFP. (**C**) Colonies (streak) of recombinant *Salmonella* vaccine vector expressing GFP only. It was noted that colonies from AroC+Salmgag-GFP were less fluorescent under UV light than colonies from AroC+GFP.

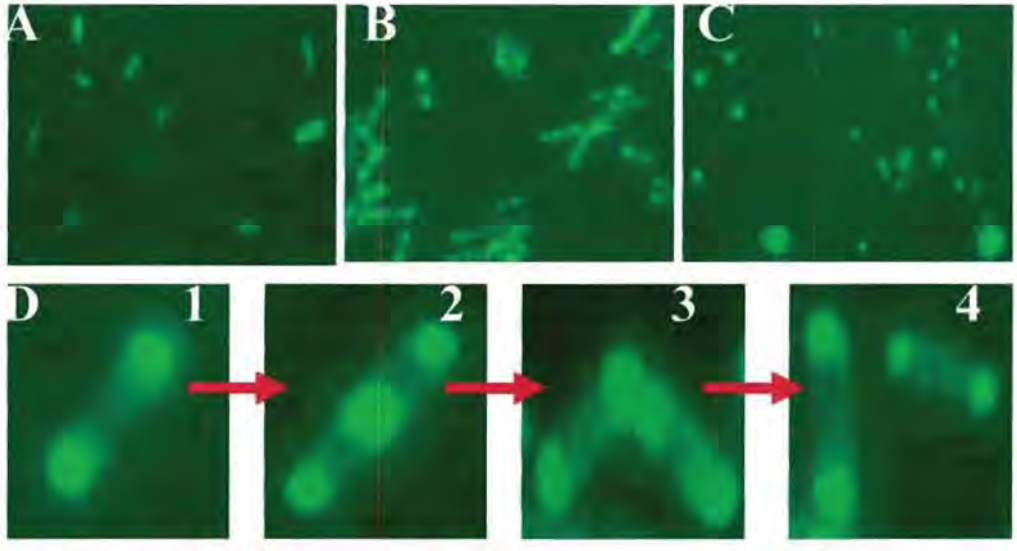

Figure 7.5: Sub-cellular localization of GFP and Salmgag-GFP in recombinant *Salmonella* vaccine vector as determined by fluorescence microscopy. Comparison of cellular localization of GFP (**A**) and Salmgag-GFP (**B**) in live recombinant *Salmonella*. The images show actively growing bacteria and how the antigens localized during this growth. (**C**) AroC+Salmgag-GFP bacteria in the stationary/dying phases of growth. Dying bacteria seemed to burst and release their fluorescent contents. The Salmgag-GFP maintained its fluorescence in these cells. (**D**) Sub-cellular localization of Salmgag-GFP in the live recombinant *Salmonella* expressing Salmgag-GFP at different stages of bacterial cytokinesis. Undividing cells had the Salmgag-GFP confined to the bacterial poles (**D1** and **D4**). When cell division began, half of the Salmgag-GFP migrated to the mid-centre while a quarter of the protein remained on each of the poles (**D2**). The Salmgag-GFP was equally distributed to daughter cells (**D3** and **D4**).

7.7 DISCUSSION

7.7.1 Molecular cloning and expression of *salmgag-gfp*

The current research study investigated the localization of Salmgag (codon-optimized HIV-1 Gag) as well as GFP in recombinant *Salmonella enterica* serovar Typhimurium vector. GFP was used as a molecular tag to monitor the localization of the Salmgag antigen in the bacterial vector. The high-level expression of the 87 KDa Salmgag-GFP fusion protein in the bacteria was potentially attributed to the rational design of the expression plasmid backbone (discussed in detail in Chapter 3). Cultures of *Salmonella* vector expressing Salmgag-GFP were fluorescent under UV (Figure 7.4) and this suggested that a certain proportion of the expressed Salmgag-GFP folded correctly. The fluorescence of the clones also indicated that Salmgag was successfully cloned in the correct reading frame with the N-terminal domain of β-galactosidase α-fragment sequence and the C-terminal domain of the GFP. It was noted that the degree of UV fluorescence of cultures from the vector expressing GFP (32 Kda) was more than that of cultures from the vector expressing Salmgag-GFP (87 KDa) (Figure 7.4). The amount of GFP in bacteria expressing the fusion protein could be less than the amount from bacteria producing GFP only. The formation of Salmgag inclusion bodies could also affect the degree folding and solubility of GFP downstream the Salmgag protein. This is in line with studies by Drew *et al.*, (2001) which demonstrated the utility of GFP in predicting solubility and folding of fusion proteins in *E. coli*. When a protein fused to GFP forms inclusion bodies, the GFP does not become fluorescent at all (Drew *et al.*, 2001). The reduced fluorescence of Salmgag-GFP fusion suggested that a certain proportion of the antigen was expressed in inclusion bodies. When an upstream antigen misfolds in GFP fusions, the fluorescence is normally affected (Waldo *et al.*, 1999; 2003).

7.7.2 Localization of *salmgag-gfp* and GFP in recombinant *Salmonella* vector

The study further demonstrated that Salmgag-GFP was localized at the poles of undividing live *Salmonella* cells, whereas GFP was homogenously distributed throughout the bacterial cells (Figure 7.5). The homogeneous distribution of the GFP antigen suggested that it was expressed as a soluble protein (Chapter 3), which could easily diffuse throughout the bacterial cytoplasm (Mullineaux *et al.*, 2006). On the other hand, the Salmgag-GFP was likely to form insoluble particles/inclusion bodies that could not easily diffuse throughout the bacterial cell. This is not the only study that has demonstrated the polar localization of a viral protein in a gram-negative bacterium. Studies by Giantini and Shatkin (1987) showed that reovirus major capsid localized on the polar regions when expressed in *E. coli*. The mechanisms underlying the bi-polar localization of Salmgag-GFP or other viral antigens remain unclear. A number of speculations were put forward to explain this sub-cellular distribution observed in the current study. The localization of the fusion protein to the polar regions was an attribution of environmental stress to the bacterial vector. It has been shown by other studies that environmental stress such as osmotic shock could lead to polar localization of the expressed protein (Santini *et al.*, 2001). The polar localization of the antigen in the bacteria could also be dependent on the properties of the expressed protein (Zhao J and Lambowitz, 2005). Specific (unknown) properties of the Salmgag could make it to be localized to the poles. It has been demonstrated that some bacterial proteins such as *E. coli* F plasmid

SopB protein naturally localizes to the polar regions because of their physical properties (Kim and Wang, 1998). It was not clear whether the Salmgag-GFP had such physical properties to confine itself at the poles. Another speculation was that the polar localization suggested that some protein-protein interactions between Salmgag and bacterial protein(s) at the poles could be occurring. Those bacterial proteins located at the poles could act as receptors for the Salmgag protein. The proteins could be important in division or replication of the bacterial cell. On cytokinesis, it was noted that part of the fusion protein relocated to the mid- centre of the cell, suggesting a role in cell division by such proteins.

The bright polar ends of the bacteria further suggested that the Salmgag-GFP fusion protein could be forming aggregates through Gag-Gag interactions. Studies by Lee and Linial (2004) have demonstrated that the basic residues in Gag nucleocapsid (NC) play critical roles in Gag-Gag and Gag-RNA interactions. In nature, it is the Gag-Gag interactions with RNA that result in formation of virions (Ma and Vogt, 2002). Whether such interactions occurred inside the *Salmonella* bacterial cells for formation of Gag VLPs remained unclear. However, early studies by Campbell and Vogt (1995) presented evidence that CA-NC from RSV or HIV-1 expressed in *E. coli* system could form VLPs. If Gag VLPs could be assembled after expression from *E. coli* as demonstrated by Campbell and Vogt's study, it is also likely that full-length HIV-1 Gag expressed in *Salmonella* would form particles, since the two bacterial systems are phylogenetically related. It was also noted that the Salmgag-GFP fusion protein seemed to affect the integrity of the bacterial membrane. Bacterial cells appeared translucent, indicating lysis or membrane perforations (results not shown). It has been shown that some viral proteins (especially structural) have highly hydrophobic transmembrane domains that could form hydrophilic pores in biological membranes (Carrasco *et al.*, 1995; Gonzalez and Carrasco, 2003; Ciccaglione *et al.*, 2004; Liao *et al.*, 2006). In nature, membrane binding of Gag is an essential step in assembly and budding of the virus during infection (Dalton *et al.*, 2005). The MA component of Gag has also been shown to have some lipid membrane binding properties (Zhou *et al.*, 1994; Hermida-Matsumoto and Resh, 2000). It was unclear in this study whether the (protein-lipid) interaction of Gag with the bacterial membrane was responsible for the translucence observed on colonies or the polar localization. Further future studies are necessary to unravel, in biochemical terms, whether there are any interactions between HIV-1 Gag and bacterial membranes. The implications of the localization of HIV-1 Gag (Salmgag) and GFP on their immunogenicity when delivered by the bacterial vector in mice were unclear. Perhaps the differences in the nature and magnitude of antigen-specific immune responses observed in Chapters 3 and 5 was, to some extent, a result of differences in the localization of the two antigens inside the bacterial vaccine vector. Future studies should further investigate the implications of heterologous antigen compartmentalization on antigen-specific immune responses of recombinant of *Salmonella* vaccine vectors.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

"Why is this thus? What is the reason of this thusness?" Artemus Ward (1834-1867)

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CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

8.1 Introduction

The development of a safe, effective and affordable HIV-1 Subtype C vaccine remains a grand challenge, not only for Southern Africa, but for the whole world at large. In 1999, the South African AIDS Vaccine Initiative (SAAVI) was established to coordinate efforts in the development of an HIV-1 vaccine for the region (Tucker and Mazithulela, 2004). Since then, various novel approaches have been undertaken by the various research teams involved in the development of the vaccine for Southern Africa under the auspice of SAAVI (reviewed in Williamson, 2002). The University of Cape Town (UCT) team has been investigating the use of (1) HIV-1 Gag viral-like particles being produced in baculoviruses (insect viruses) or plant expression systems (Jaffray *et al.*, 2004), (2) HIV-1 DNA vaccines carried on plasmid (van Harmelen *et al.*, 2003; Burgers *et al.*, 2006), (3) recombinant viral vectors for HIV-1 antigens (Davis *et al.*, 2002), (4) BCG as a vaccine vector for HIV-1 Gag (Thomas, 2005) and (5) recombinant *Salmonella* as a bacterial vaccine vector for HIV-1 Gag (Chin'ombe, work reported in this thesis). The main goal of this study was to set up some groundwork for the development of oral attenuated recombinant *Salmonella* vaccine vectors for HIV-1 antigens. The first step in the execution of this goal was to rationally design a prokaryotic expression vector for efficient expression of foreign antigens in recombinant *Salmonella* vaccine vector. The jellyfish GFP was used as a model antigen to investigate if a *Aro*C *Salmonella* bacterial vaccine strain could be used as a delivery system. This was followed by the cloning of HIV-1 Gag antigens (wild-type and codon-optimized) for expression and delivery by the vaccine vector. The systemic immune responses of the recombinant bacterial vector were evaluated in mice after oral vaccinations.

8.2 Rational design and development of *Salmonella* expression plasmid

After oral ingestion, *Salmonella* bacteria invade the gut-associated lymphoid tissue through the M cells (reviewed in Chapter 2). The bacteria spread to systemic organs such as liver, spleen and bone marrow and are internalized by the professional phagocytes, mainly macrophages and dendritic cells (Oh *et al.*, 1996). This characteristic feature makes the bacteria good candidates for delivery of antigens for induction of both the innate and adaptive arms of the immune system in both the mucosal and systemic compartments (Chen and Schifferli, 2000; Linehan and Holden, 2003; Wyszynska *et al.*, 2004; Kalupahana *et al.*, 2005). The development of recombinant *Salmonella* vaccine vectors for heterologous antigens is often hampered by problems of stable expression or toxicity to vectors of the antigens when overexpressed (Tijhaar *et al.*, 1994). The high constitutive expression of heterologous antigens may also affect the effectiveness of the *Salmonella* vectors in the delivery of antigens to the host's immune system (Galen and Levine, 2001). To circumvent these problems, the current study rationally designed and developed a versatile prokaryotic expression plasmid system for cloning of GFP or HIV-1 Gag antigens for their overexpression in a recombinant *Salmonella enterica* serovar Typhimurium vaccine vector. The subsequent successful induction of GFP- or Gag-specific systemic immune responses in mice after vaccination with recombinant *Salmonella enterica* serovar Typhimurium vaccine vectors may have been attributed to this rational design of the expression plasmids. The recombinant expression plasmids were

based on the *E. coli lac* operon sequences in pGEM+Teasy plasmid. Various aspects, such as origin of replication, *lac* promoter properties, ribosome binding site, translation start and termination codons and the inclusion of *LacZα* peptide linked in-frame with the antigens, were taken into considerations when the expression plasmids were constructed. The relevance of these aspects in influencing high-level expression of antigens has already been discussed in detail in Chapter 3. The high-level expression of the foreign antigen in the recombinant *Salmonella* vaccine may be required for induction of potent mucosal and systemic immune responses (Ogra *et al.,* 1999; Didierlaurent *et al.,* 2002). The high-level expression *in vivo* after vaccination may also facilitate successful delivery of the antigens by the bacterial vector to the host immune system and subsequent induction of strong immune responses (Medina *et al.,* 2000; Bumann, 2001; Rollenhagen *et al.,* 2004).

8.3 Development of recombinant *Salmonella* vaccine vectors

Although the original *E. coli lac* promoter system is simple and weak, the current study demonstrated that it could be used to constitutively drive high-level antigen expression in *Salmonella enterica serovar* Typhimurium vaccine vectors. In *E. coli,* the *lac* operon is transcriptionally regulated. In the genus *Salmonella*, the *lac* operon is non-existent, which explains why the bacteria cannot ferment lactose. The study also demonstrated that other domains of the operon such natural Shine-Dalgarno (AGGA), transcription start, -10 and -35 sequences were recognised by the bacteria. There were two start codons (ATG) separated by one none-start codon. This potentially increased the efficiency of antigen expression in *Salmonella* vaccine vectors. Two stop codons (TAA) separated by a none-stop codon were incorporated for the GFP expression plasmid. This powerful stop codon might have increased efficiency of antigen translation termination. The second stop codon prevented possible read-through by ribosomes during antigen translation by the *Salmonella* vector. The nature of the stop codon and the fourth base on the stop codon have been found to influence the efficiency of translational termination in *E. coli* (Poole *et al.,* 1995) and this may apply to *Salmonella* vaccine vectors too. Poole *et al.,* 1995 found out that in terms of translational efficiency, TAAN was the best stop codon, followed by TGAN and finally TAGN with efficiency ranging from 80% to 30%. In this study, TAA followed by the T or G base was preferred as stop codon for efficient translational termination

Studies in our laboratory independent of this study have shown that unfused GFP or Gag antigens could not be overexpressed in *Salmonella* vaccine vectors despite using strong promoters such as *mtr* promoter. The current study demonstrated that fusing the heterologous antigens to a prokaryotic peptide (LacZα) enhanced expression their expression (Chpaters 3, 4, 5, 6 and 7). The reasons underlying this enhanced antigen expression could be multifactorial. First, the fusion of prokaryotic peptide sequences to heterologous antigen potentially improved transcription of the whole fusion gene. Second, the stability of the mRNA transcripts is improved and are resistant to degradation. Third, the translational efficiency of the antigen mRNA transcripts was improved. The fusion of the antigens to the natural ribosome-binding site and N-terminus of β-galactosidase could have facilitated mRNA folding that promoted strong

translation. Finally, fusion proteins are likely to be resistant to post-translational proteolytic degradation (Butt *et al.*, 2005).

Overexpression of antigens may lead to cellular toxicity and this may depend on the nature of the expressed protein (Chapters 3, 4 and 5). Li *et al.*, (1999) have demonstrated that apoptosis of mammalian cells occurred when GFP was overexpressed. The cellular toxicity of recombinant GFP has also been demonstrated in plants (Rouwendal *et al.*, 1997) and in recombinant *Salmonella* (Wendland and Bumann (2002). In the current study, it was noted that GFP was not toxic to *E. coli* and *Salmonella* despite its massive antigen expression (Chapter 3). This could be due to the inclusion of the LacZα peptide sequence motif to the N-terminal end of the GFP. However, the study demonstrated that HIV-1 Gag and Salmgag were, to some extent, toxic to be bacterial cells (Chapter 4). The mechanisms of HIV-1 Gag toxicity to bacteria such as *Salmonella* vaccine vectors or other microorganisms such as BCG have not yet been elucidated. However, it has been speculated that the cell death by lysis (in the stationary phase) was possibly due to membrane destabilization and leakage or pore formation. Reduction of this toxicity could be achieved by insertion of a leader peptide (Donnelly *et al.*, 2001, 2006). In the current study, the fusion of HIV-1 Gag or Salmgag to with the N-terminal domain of β-galactosidase α-fragment potentially reduced the toxic effects of the antigen. Previous studies demonstrated that expression of unfused Gag or GFP under the strong constitutive promoter, *mtr* had detrimental effects on the *Salmonella* bacterial growth (results not reported). The insertion of lacZα peptide upstream of wild-type Gag and Salmgag was speculated to have reduced the toxicity of the antigens. This is consistent with findings from other studies. Donnelly *et al.*, (2001) have shown that inclusion of a leader peptide derived from the GroES Cochaperone drastically reduced toxicity and increased the expression of human apoptosis modulator protein Bax in *E. coli*. *Bax* protein product is highly toxic and leads to bacterial lysis when expressed even at low levels in *E. coli* (Asoh *et al.*, 1998). Montigny *et al.*, (2004) demonstrated that upstream insertion of Asp-Pro sequence peptide into hepatitis C virus E1 and E2 antigens reduced their cellular toxicity when expressed in bacteria. Corchero and Villaverde (1998) suggested that the features of the encoded antigens influenced plasmid maintenance in recombinant bacteria. It was noted that plasmids carrying toxic antigens were lost faster than those carrying non-toxic antigens (Corchero and Villaverde, 1998).

The current study also provided fundamental insights into differences in subcellular localization of GFP model and HIV-1 Gag antigens in the *Salmonella* vaccine vector (Chapter 7). Whereas GFP localized homogenously in the bacterial cells, HIV-1 Salmgag was confined only to the polar regions. The differences in the localization of the two antigens could be attributed to their differences in intrinsic properties of the two antigens (Corchero and Villaverde, 1998). Alternatively, Salmgag expression resulted in more metabolic burden to the vector than GFP expression. This might have resulted in osmotic shock which pushed the antigen to be confined to the polar regions of the bacterial cell. In addition to tagging HIV-1 Salmgag to monitor localization, the study demonstrated that GFP could be used as a predictive folding reporter. It has been demonstrated by other studies that when GFP fusion

proteins are overexpressed as inclusion bodies in *E. coli,* the GFP lost its fluorescence (Waldo *et al.,* 1999; Drew *et al.,* 2001). This explains why there was reduced fluorescence of Salmgag-GFP, which suggested that a huge proportion of the antigen formed inclusion bodies.

Codon optimization of genes did not have an impact on the *in vitro* expression of antigens by the *Salmonella* vaccine. High levels of GFP and HIV-1 Gag antigens were achieved despite their genes being unoptimzed or optimized for expression in *Salmonella.* The fusion of the LacZα to antigens and other factors were potentially responsible for their overexpression. However, codon-optimization had a profound impact on the nature and magnitude of the antigen-specific immune responses against HIV-1 Gag when delivered by the *Salmonella* vector (Chapter 4 and 5). The *Salmonella* vaccine expressing the native Gag only induced Gag-specific Th2 response after oral vaccination of mice (Chapter 4). In contrast, codon-optimized Gag induced CD8+ Tc1, CD4+ Th1 and Th2, IgG1 and IgG2a responses after vaccination of mice (Chapter 5).

The impact of formation of inclusion bodies on immunogenicity was not clear in this study. However, a study by Kesik *et al.,* (2004) demonstrated that inclusion bodies were immunogenic. The oral vaccination of mice with inclusion bodies carrying viral antigens could induce both mucosal and systemic immune responses (Kesik *et al.,* 2004). Previous studies by Brett *et al.,* (1993a) have also shown that antigen processing of a viral nucleoprotein (NP) expressed as inclusion bodies in *Salmonella* required at least 6 h after bacterial infection before macrophages could present the NP motifs effectively. In comparison, soluble NP only required 2 to 4 h before motifs were presented on macrophage cell surfaces (Brett *et al.,* 1993b). This difference could be due to the greater stability of a polypeptide in insoluble aggregates compared to that of a soluble protein by reason of resistance to degradative enzymes present in phagolysosomes (Jespersgaard *et al.,* 2001). It was therefore anticipated that even if the Gag or Salmgag were expressed as inclusion bodies in the *Salmonella* vector, they would be immunogenic.

8.4 Overall immunogenicity to recombinant *Salmonella* vaccine vectors

Although the correlates of protection of HIV-1 are still poorly understood, a successful vaccine against HIV-1 needs to stimulate the innate immune system and generate high neutralizing antibody levels and strong cellular immune responses in the mucosal and systemic compartments (Haynes *et al.,* 1996; Pantaleo and Koup, 2004; McMichael *et al.,* 2006). There is already evidence indicating that mucosal immunity against HIV-1 plays a critical role in antiviral protection (Belyakov *et al.,* 1998). Studies by Wu *et al.,* (1997) have demonstrated that live oral *Salmonella* vaccines expressing HIV-1 gp120 could induce mucosal immune responses in form of gp120-specific IgA antibody-secreting cell responses after oral vaccination of mice. Splenic gp120-specific CD4+ T cell responses were also observed. However no measurable HIV-1-specific CD8+ T cell immune responses were induced by the bacterial vector (Wu *et al.,* 1997). The current study investigated induction of only systemic GFP- and HIV-1 Gag-specific immune responses after oral delivery of recombinant *Salmonella* bacteria. T cell and serum antibody responses were monitored. Analysis of all the results concluded that oral vaccination of mice with the

recombinant *Salmonella* vaccine vectors could result in induction of systemic antigen-specific CD4+ T-, CD8+ T- and B-cell immune responses. The nature and magnitude of such responses depended on the antigen and its properties. Recombinant GFP was soluble and evenly distributed when expressed in the *Salmonella* vector and induced CD8+ Tc1 and Tc2, together with IgG1 (CD4+ Th2) and IgG2a (Th1) responses. Wild-type HIV-1 Gag was mainly expressed as inclusion bodies and induced only Th2 responses when delivered by the *Salmonella* vector. Codon-optimized HIV-1 Gag (Salmgag) was also predominantly expressed as inclusion bodies, localized to the bacterial poles and induced mainly mixed CD4+ Th1 and Th2, together with IgG1 and IgG2a responses. Some of these multi-pronged responses are important targets for vaccines that are required for prevention of infection by HIV-1 in Southern Africa.

8.4.1 CD8+ T cell cytokine responses

The study was able to demonstrate that oral vaccination of mice with recombinant *Salmonella* expressing GFP or HIV-1 Gag could result in generation of antigen-specific CD8+ T cell responses. Whereas recombinant *Salmonella* vectors expressing GFP induced both GFP-specific CD8+ Tc1 and Tc2 cytokine responses after three inoculations, the vectors expressing the wild-type or codon-optimzed HIV-1 Gag elicited no or very low detectable CD8+ T cell cytokine response. It was hypothesized that the solubility of GFP and stability of its expression *in vivo* in the *Salmonella* vectors could be some of the reasons for strong CD8+ T cytokine responses found. On the other hand, HIV-1 Gag expression was relatively toxic to the vector and the antigens were insoluble and this could be the reasons for the development of weak CD8+ T cell responses. Soluble and particulate antigens are also presented differently to the immune system.

Although infection with *Salmonella* is expected to induce predominantly CD4+ T cell responses since the bacteria reside in the phagosomes of the APCs, APCs such as macrophages and dendritic cells are capable of cross-priming and this may result in the induction of CD8+ T cell immune responses (Harding, 1996; Oh *et al.*, 1997; Heath *et al.*, 2004). The cross-presentation of antigens has been shown to be a very inefficient process and is affected by a number of factors such as dose and time (Maecker *et al.*, 2001). This could be the main reason why no or poor antigen-specific CD8+ T cell responses were detected after single vaccination of mice with the recombinant *Salmonella* vectors. It was not clear why no or poor HIV-1 Gag-specific CD8+ T cell responses were detected even after booster immunizations with the recombinant *Salmonella* vectors (Chapters 4 and 5). One of the possible reasons for the inefficient cross-presentation of the Gag or Salmgag antigens could be its insolubility. Hone *et al.*, (1996) showed that when HIV-1 gp120 was overexpressed cytoplasmically in a *Salmonella* vaccine vector, inclusion bodies were formed. Oral vaccination of mice with the recombinant vector did not induce systemic HIV-1 gp120-specific CD8+ CTLs. It was concluded that the failure to express a soluble gp120 in the vector was one of the reasons for the failure of the vector to evoke CD8+ T cell immunogenicity (Hone *et al.*, 1996). The poor CD4+ T help could also be one of the reasons for poor Gag-specific CD8+ T cell responses observed in the current study. This CD4+ help is known to be

critical for CD8+ T cell priming and maintenance of memory CD8+ T cells (Sun and Bevan, 2003; Sun *et al.,* 2004). Future studies need to focus on ways of developing *Salmonella*-based vaccines that induce strong memory CD8+ T cell responses, since this arm of the immune system plays a fundamental role in controlling HIV-1 infection or disease (Chapter 1). Recent studies on vaccine-induced correlates of protection have revealed that SIV-specific memory CD8+ T cells secreting Tc1 cytokine profile are fundamental in protection against AIDS disease in monkeys (Sun *et al.,* 2005; Acierno *et al.,* 2006; Sun *et al.,* 2006).

8.4.2 CD4+ T cell cytokine responses

The current study demonstrated that systemic GFP- and HIV-1 Gag-specific CD4+ T cell responses by the recombinant *Salmonella* vaccine vectors expressing heterologous antigens could be induced in orally vaccinated mice. Direct evidence of GFP-specific CD4+ T cell responses was not available as there were no known GFP-specific CD4+ peptides for use in stimulating the splenocytes. However, the presence of both GFP-specific IgG1 and IgG2a provided indirect evidence of the induction both CD4+ Th1 and Th2 responses. Vaccination of mice with recombinant *Salmonella* expressing wild-type Gag of Salmgag provided direct evidence of CD4+ T cell responses (Chapters 4 and 5). Whereas *Salmonella* expressing wild-type Gag induced only Th2 response, *Salmonella* expressing Salmgag induced mixed CD4+ Th1/Th2 cytokine responses. The reasons for these differences were unclear. It was however speculated that the codon-optimization of the *gag* enhanced the broad CD4+ T cell responses found in this study. The *Salmonella* expressing the wild-type Gag could not perhaps efficiently invade the mucosal lymphoid tissues so as to elicit both Th1 and Th2 responses. It is known that exogenous soluble and particulate antigens (such as GFP and HIV-1 Gag respectively) can be taken up by APCs, processed into peptides and presented by MHC-II molecules to CD4+ T cells (reviewed in Unanue, 2002; Veeraswamy *et al.,* 2003). Live *Salmonella* can also be taken by APCs by phagocytosis and the bacteria reside and replicate in phagosomes, with antigens being presented mainly by the MHC-II molecules to the CD4+ T cells (Svensson *et al.,* 1997; Yrlid and Wick, 2000; Kalupahana *et al.*, 2005).

The pivotal role of CD4+ T cell responses in vaccine development has always been recognized. CD4+ Th1 cytokines such as IFN-γ and TNF-α provide protective immunity against intracellular pathogens such as viruses and promote B cell class-switching to complement-fixing antibodies such as IgG2a in mice (Finkerlman *et al.,* 1990; Seder and Paul, 1994; Spellberg and Edwards, 2001). On the other hand, CD4+ Th2 cytokines such as IL-4 promote B cell class switching to neutralizing antibodies such as IgG1 in mice or IgG4 in humans and to regulate the intensity of Th1 cytokine responses (Stavnezer, 1996; Hsieh *et al.,* 1993; O'Garra and Arai, 2000; Spellberg and Edwards, 2001). In HIV-1 infection, the critical regulatory roles of CD4+ helper T cells on CD8+ T-cell and humoral responses have been documented (Chapter 2). The CD4+ Th1/Th2 balance has been documented to be an important determinant in HIV-1/AIDS disease pathogenesis and progression. Th1 cytokines promote long-term disease non-progression, while a switch from Th1 to Th2 cytokine profile contributed to AIDS disease progression (Clerici and Shearer, 1994; Clerici, 2002). On the contrary, a recent study did not find such

polarization of the Th1/Th2 cytokine profile in HIV patients (Fakoya *et al.*, 1997). However, the induction of mixed Th1/Th2 responses (as observed in Chapters 3 and 5) may be necessary for HIV-1 vaccines that need to induce both humoral and cellular responses. Future studies should attempt to unravel the best *Salmonella* vaccine-induced CD4+ Th1/Th2 profiles which correlate with protection against HIV-1 infection or disease. Some recent studies in monkeys have already shown that vaccine-induced memory CD4+ T cells secreting Th1 cytokines correlate with protection against AIDS disease (Letvin *et al.*, 2006; Sun *et al.*, 2006).

8.4.3 Humoral immune responses

The study demonstrated that vaccination of mice with recombinant *Salmonella* expressing GFP or Salmgag could elicit mixed antigen-specific IgG2a (Th1) and IgG1 (Th2) responses. It was likely that the Salmgag particles (whether folded correctly or incorrectly) could activate the B cells to proliferate and produce HIV-1-specific antibodies. Viral structural proteins such as rabies virus nucleocapsid or HPV L1 are capable of forming virus-like particles in bacteria and induce long-lasting humoral immune responses through activation of B cell and T cell helper responses (Nardelli-Haefliger *et al.*, 1997; Koser *et al.*, 2004). The lack of codon-optimization of wtGag was possibly responsible for the failure of *Salmonella* expressing the antigen to induce HIV-1-specific antibody response. The embedding of P41 and P24 into the LacZα perhaps destroyed the necessary antibody epitopes (by not folding into their native forms) and this could be one of the reasons why the recombinant *Salmonella* expressing the antigens could not elicit antigen-specific IgG after oral vaccination of mice.

The results reported in this study demonstrated the induction of mixed CD4+ Th1 and Th2 responses (Chapter 3 and 5). Characterization of the anti-GFP (Chapter 3) and anti-Salmgag (Chapter 5) antibody responses in the sera of mice vaccinated with recombinant *Salmonella* vaccine vectors showed a strong bias towards the production of IgG2a subclass at the expense of IgG1 subclass. Other studies have also found that predominantly antigen-specific IgG2a (Th1-type) responses were induced after oral vaccination of animals with recombinant *Salmonella* vaccines (Huang *et al.*, 2001; Kang *et al.*, 2002; Wu *et al.*, 2006). It is known that IgG subclasses have the capacity to neutralize toxins and viruses, opsonize particles for ingestion by phagocytes (opsonophagocytosis), or when complexed to antigens, activate the classic complement pathway (Charalambous and Balakrishnan, 2004). Some of the antibody isotypes such as IgG2a may play a key role in killer-cell antibody-dependent cell-mediated cytotoxicity (ADCC) (Snapper and Paul, 1987: Ahmad and Menezes, 1996). In HIV-1 vaccinology, the Gag is not targeted for induction of HIV-1 neutralizing antibodies. The primary target has been the development of neutralizing antibodies against HIV-1 envelope glycoprotein.

8.5 Novelty and relevance of the findings

- The work reported in the thesis is the first to explore the potential of using attenuated *Aro*C *Salmonella enterica* serovar Typhimurium mutant to deliver GFP and Southern Africa HIV-1

Subtype C Gag to the immune system through the oral route. This is a good starting point in the development of effective, safe and affordable vaccines for HIV-1 for the region.

- The work is the first to exploit the *E. coli lac* operon system to develop a system for overexpression of heterologous antigens (GFP and HIV-1 Subtype C Gag) in recombinant *Salmonella enterica serovar* Typhimurium vaccine vectors. The physiology of the *lac* operon was unravelled about half a century ago by Jacob and Monad and this operon is not present in Salmonellae. Most researchers emphasize on the use of stronger promoters such as *Tac* or *Trp*, believing that they can achieve better expression of heterologous antigens in *Salmonella* vectors. The *lac* promoter can also be used for the overexpression of antigens by *Salmonella* as demonstrated in this study.
- The phenomenon of fusing heterologous antigens to the N- and/or C-terminal domains of *E. coli* β-galactosidase α-fragment to enhance expression of recombinant *Salmonella enterica* serovar Typhimurium was original. Fusion of the antigens to the C-terminus of the β-galactosidase α-fragment did not affect their function and folding, but enhanced their expression. However, embedding the antigens in the β-galactosidase α-fragment (Chapter 6) affected their folding and immunogenicity, but further enhanced their expression.
- The work is one of the first to evaluate simultaneously the secretion of vaccine-induced antigen-specific CD4+ Th1/Th2 or CD8+ Tc1/Tc2) cytokines by splenocytes from mice vaccinated orally with recombinant *Salmonella enterica* serovar Typhimurium expressing heterologous antigens. Most of the current studies in HIV-1 vaccinology only evaluate the induction of IFN-γ cytokine without the considerations of the role of other cytokines such as TNF-α, IL-4 and IL-5. It should be critical to evaluate the simultaneous secretion of the whole array of cytokines induced after vaccinations. This would give some ideas on whether the vaccine would be protective or not.
- The work is one of the first to show that *Salmonella*-based vaccines could induce both antigen-specific memory CD8+ Tc1 and Tc2 cytokines. The study also showed that both antigen-specific memory CD4+ Th1 and Th2 cytokine responses could be induced after oral vaccination of mice with codon-optimized Gag expressed by a *Salmonella* vector.
- The study was the first to investigate the sub-cellular compartmentalization of HIV-1 Gag in a *Salmonella* vaccine vector. The localization of the antigens inside the bacterial vector could potentially affect their immunogenicity.

8.6 Perspectives for the future

The findings reported in the thesis call for further investigations into the use of oral attenuated *Salmonella* as vaccine vectors for HIV-1 antigens. The demonstration that GFP or HIV-1 Gag could be constitutively expressed at high levels by the *Salmonella* bacterial vector using the simple *E. coli lac* promoter was novel. Future studies should therefore further investigate the use of the *E. coli lac* or other operon systems such as *trp* operon for the expression of HIV-1 Gag and other antigens such as Tat, Rev, Nef, Pol and RT derived from circulating HIV-1 strains in Southern Africa by recombinant *Salmonella* vaccine vectors. The expression plasmid systems developed by the study can still be improved. Future

investigations need to look at ways of making such improvements. A number of strategies may be used to fine-tune the expression of HIV-1 antigens in the *Salmonella* vectors. A number of different constitutive promoters with different strengths such as *trp* and *mtr* may be tried by replacing the *lac* promoter. Levels of antigen expression may be also be tuned by changing the spacing between the -10 and -35 regions of the *lac* promoter. Different origins of replication may be used in the expression plasmids so as to influence plasmid copy number and therefore gene dosage. The ribosome-binding site sequences may be mutated so as to increase or decrease the levels of antigen expression. Therefore, the whole backbone of expression plasmids can potentially be re-engineered. Besides antigen expression, other key aspects such as alternative immunization strategies such as intranasal, vaginal and rectal routes, prime-boost strategies may also need to be investigated in future studies. Furthermore, since *Salmonella enterica* serovar Typhimurium is only a typhoid model in mice, future studies also need to investigate the use of recombinant *Salmonella enterica* serovar Typhi such as Vivotif® (Berna Biotech, Berne, Switzerland) and ZH07 (Microscience Ltd, UK) as vaccine vectors for HIV-1.

Intracellular overexpression of heterologous antigens by recombinant *Salmonella* has a number of problems such as induction of metabolic burden (Galen and Levine, 2001). Future studies should look at other ways of avoiding such burden and this can be achieved by expressing the HIV-1 antigens in different extra-cytoplasmic compartments (such as periplasm, outer membrane or extracellularly). It is further worthwhile to investigate displaying of HIV-1 antigens on the surface of *Salmonella* vaccine vectors. To achieve this, HIV-1 antigens or peptides may be fused to outer membrane proteins such as OmpC of *E. coli,* OmpB of *Vibrio cholerae* or OprI of *Pseudomonas aeruginosa* in an expression plasmid. OprI has already been used to surface display a foot and mouth disease virus epitope in a *Salmonella* vector (Cote-Sierra *et al.,* 1998). Hepatitis B and C viral antigens have successfully been displayed on the surface of *Salmonella* vaccine vectors using *P. syringae* Inp (Lee *et al.,* 2000). Future studies should also explore the secretion of HIV-1 antigens from *Salmonella* vaccine vectors. The *E. coli*-derived HlyA hemolysin secretion system (Gentschev *et al.,* 2002) can be engineered for secretion of HIV-1 antigens by *Salmonella* vectors. The type III secretion systems can also be used to translocate heterologous antigens out of the vector into the host cytosol for induction of strong CD8+ T cell responses (Ho and Starnbach, 2005). Integration of HIV-1 genes into the chromosome of *Salmonella* vectors could be a powerful alternative for overcoming problems of metabolic burden and plasmid stability and this needs future investigations. Chromosomal integration of a heterologous gene can potentially result in complete stabilization of expression (Galen and Levine, 2001; Garmory *et al.,* 2003c; Stratford *et al.,* 2005). The only drawback for chromosomal integration may be low gene dosage that may result in induction of poor immunogenicity after vaccination. This can potentially be circumvented by the use of very strong promoters or by integration of several copies of the HIV-1 *gag* gene into the *Salmonella* chromosome. Future studies should explore the use of strong, but regulated *in vivo* inducible promoters, such as *E. coli NirB* (Xu *et al.,* 1998), that would ensure low activity *in vitro* but would be increased once the bacterial vectors reach the appropriate locations in the tissues.

An alternative strategy to genetic stability of *Salmonella* vector that needs future studies is the use of balanced-lethal plasmid stabilization systems. This involves the insertion of the gene encoding the heterologous antigen into a plasmid containing a gene that complements a metabolic defect in the *Salmonella* vector (Galan *et al.,* 1990). A number of studies have already successfully utilized this system, in which the *asd* gene is inactivated in *Salmonella* (Tacket *et al.,* 1997b; Chen and Schifferli, 2000; Wyszynska *et al.,* 2004; Xu *et al.,* 2006). In *asd Salmonella* mutants the loss of the plasmid carrying the *asd* gene *in vivo* is lethal and only *Salmonella* harbouring the plasmid survive (Galan *et al.,* 1990). Using this system, plasmid instability associated with foreign viral antigen expression in *Salmonella* vaccine vectors may be circumvented.

The work reported here should be used as a foundation for future studies in the development of *Salmonella*-based vaccines for HIV-1 and other human pathogens. Foreign antigens can be overexpressed using the prokaryotic expression strategy developed by this study. The antigens can be delivered orally to the immune system using attenuated *Salmonella* vaccine vectors. It should be borne in mind that the nature and magnitude of the antigen-specific immune responses induced by the recombinant *Salmonella* vectors would depend on many factors, which include, the properties of the heterologous antigen to be expressed, the design of the expression prokaryotic plasmid, the nature of attenuating mutation of the vector, dose of vector administered and the vaccination route (Benyacoub *et al.,* 1999; Nardelli-Haefliger *et al.,* 2001; Curtiss, 2002).

APPENDICES

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APPENDIX A: MICROBIOLOGICAL (BACTERIOLOGICAL) METHODS

A1: PROTOCOLS FOR BACTERIAL CULTURES

A1.1. Preparing plates for Blue-White Color Screening (α-Complementation) of recombinant *E. coli* SCS110 cells

The agar plates were prepared as described in Sambrook *et al.*, (1989). Briefly, 2x YT agar (Appendix D) was prepared. Prior to pouring of the plates, ampicillin (to a final concentration of 100 μg/ml), 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (Appendix D) (X-gal) (to a final concentration of 80 μg/ml) and isopropyl-1-thio-β-Dgalactopyranoside (IPTG) (Appendix D) (to a final concentration of 20 mM) were added. Alternatively, 100 μl of 10 mM IPTG and 100 μl of 2% X-gal were spread on the solid agar prior to plating bacteria.

A1.2. Culture conditions for *E coli* and *Salmonella* bacteria

Standard bacteriological conditions for the culture of *E. coli* (and *Salmonella*) have been described in Sambrook *et al.*, (1989). Briefly, *E. coli* was cultured at 37 °C in 2x YT broth (Appendix D) with vigorous shaking. If the bacteria harboured a recombinant plasmid (encoding ampicillin resistance gene), ampicillin was added to a final concentration of 100 μg/ml. An overnight starter culture of 5 - 10 ml 2x YT broth was prepared by inoculating a single bacterial colony and this was grown for 12 – 18 hrs at 37 °C with vigorous shaking. The culture was diluted 1 in 100 in 100 ml 2x YT broth and grown with shaking at 37 °C. To grow *E. coli* on plates, 2x YT with agar (Appendix D) was used to prepare solid medium. The bacteria cultures were spread or streaked on the 2x YT agar plates with or without ampicillin (100 μg/ml) as appropriate. The plates were incubated overnight at 37 °C with plates inverted. The culture conditions for were the same as those outlined above, but with minor modifications. The 2x YT medium for growing *Salmonella enterica* serovar Typhimurium, AroC mutant was supplemented with aro mix and tyrosine (Appendix D). Storage of cultures was at -80 °C in 10% glycerol.

A1.3. Procedure for making competent *E. coli* and *Salmonella*

Competent *E. coli* and *Salmonella* cells were prepared using calcium chloride according to Sambrook *et al.*, (1989) with modifications. The bacteria were grown for 12 – 18 hrs at 37^{0}C with vigorous shaking in a starter culture of 5-10ml in 2x TY broth (supplemented for *Salmonella*) by inoculating a single colony from previous bacterial streak. The cultures were diluted 1 in 100 in 100 ml in 2x YT broth (supplemented for *Salmonella*) and grown with shaking at 37^{0}C. The bacteria were harvested when growth reached logarithmic phase (when OD_{600} was between 0.4 - 0.6) by centrifugation (5000 rpm for 5 min) at 4 °C. The cells were resuspended in 1/10th culture volume of 0.1M ice-cold calcium chloride and held on ice for 1-2 hours. The cells were centrifuged as before and resuspended in 1/10th culture volume of 0.1M ice-cold calcium chloride with a final concentration of 10% sterile glycerol. The cells were stored at -80^{0}C in 200 μl aliquots.

A1.4. Transformation of *E. coli* and *Salmonella* with plasmid vectors

The genetic transformation of competent *E. coli* or *Salmonella* cells with plasmid vectors was performed using the heat-shock procedure as described by Sambrook *et al.*, (1989), but with some modifications. The stored aliquots of competent cells were thawed on ice. Plasmid DNA (10 - 100 ng) or ligation reactions (1 - 10 μl) were added to the cells and incubated on ice for 10 - 20 min. The cells were heat-shocked in a 42 °C water-bath for 1 - 2 min and 0.9 ml of 2x YT broth (supplemented for *Salmonella* only) without antibiotic was added. The cells were allowed to grow at 37 °C water-bath for 60 min. After the incubation, 100 μl of the transformed cell culture was plated on 2x

YT Agar (supplemented for *Salmonella* only) plates with ampicillin and incubated at 37°C for 12 – 18 hrs. Verification of the presence of plasmids in recombinant bacterial clones was performed by restriction endonuclease mapping after mini-plasmid isolation (Appendix B).

A2: PROTOCOLS FOR MAKING *SALMONELLA* VACCINES AND VACCINATION OF MICE

A2.1. Production of vaccine stocks

Recombinant *Salmonella enterica* serovar Typhimurium vaccines were prepared by first transforming the competent bacteria with respective expression plasmids (as described in Appendix A1.4). A single colony was then inoculated into 100 – 200 ml of 2x YT media supplemented with ampicillin (100 μg/ml), aro mix and tyrosine ((Appendix D). The bacteria were cultured and harvested when they reached logarithmic phase (when OD_{600} was 0.8 – 1.0) by centrifugation at 5000 rpm for 10 min at 4 °C. The bacterial cells were suspended in $1/25^{th}$ culture volume of 15% sterile glycerol. The cells were stored at -80 °C in about 200 - 400 μl aliquots until required for animal vaccination.

A2.2. Bacterial counts and determination of plasmid loss in vaccine stocks

The stored aliquots (Appendix A2.1) of recombinant *Salmonella enterica* serovar Typhimurium vaccines were thawed and serial dilutions ($1/10^2$, $1/10^4$, $1/10^6$, $1/10^8$ and $1/10^{10}$) made in water or 2x YT. The last three dilutions ($1/10^6$, $1/10^8$ and $1/10^{10}$) were plated (100 μl) on 2x YT plates with or without ampicillin. The plates were incubated 12 – 18 hrs at 37 °C. The colonies were counted and the numbers used to determine the bacterial colony-forming units in original vaccine stocks.

A2.3. Animal vaccination

The vaccine stocks (Appendix A2.1) were thawed at room temperature and diluted in PBS to a required concentration of bacterial colony forming units. Prior to vaccination, food and water were removed from mice for at least 2 hours. The mice were anesthetized via an intraperitoneal injection of ketamine/xylazine and each mouse was then administered 100 μl of bacterial suspensions by intragastric gavage.with a feeding needle.

A3. FLUORESCENCE MICROSCOPY OF GFP OR SALMGAG-GFP EXPRESSING BACTERIA

Bacterial culture volume of 1 ml were pelleted by centrifugation at 5 000 rpm for 1 min at ambient temperature. The pellet was suspended in 1 ml of PBS and re-pelleted by centrifugation as before. The bacterial cells were suspended in 30% glycerol solution. The cell suspension (5 - 10 μl) was placed on a microscope slide with a coverslip. The sample was investigated as described by Lun and Wilson, (2004) using a Carl Zeiss Axiovert 200 M inverted epifluorescence microscope. The excitation and detection of GFP were performed using a FITC filter set. Digital images were captured with a standard-scan charge-coupled-device camera (AxioCam HR; Carl Zeiss) controlled by AxioVision software.

A4. ULTRAVIOLET (UV) FLUORESCENCE GFP-EXPRESSING BACTERIA

To check the fluorescence of bacterial colonies, Pedri dishes or liquid cultures in 2-ml eppendorf tubes were exposed to ultraviolet (UV) light (256 nm) on top of UV transilluminator box. Results were recorded.

APPENDIX B: MOLECULAR BIOLOGY AND IMMUNOLOGICAL METHODS

B1. DNA ISOLATION AND MANIPULATION PROTOCOLS

B1.1. Small-scale plasmid DNA isolation (mini-prep) from recombinant *E. coli* and *Salmonella*

The small-scale plasmid DNA isolation was performed using an alkaline/SDS procedure from Sambrook *et al.*, (1989). Briefly, 300 µl of the culture (recombinant *E. coli* or *Salmonella*) was centrifuged at 10 000 rpm for 5 min and 200 µl of Solution 1 (50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA) was added and mixed. Solution 2 (0.2M NaOH, 1% SDS) (400 µl) was then added and mixed gently. The tubes were incubated at ambient temperature for 10 minutes. Solution 3 (5 M Potassium Acetate, pH 5.2) (300 µl) was added and mixed well. The tubes were centrifuged at 14 000 rpm at ambient temperature for 5 min. The supernatant (650 µl) was taken into fresh tubes and 650 µl of isopropanol added. Plasmids were pelleted by centrifugation at 14 000 rpm for 15 min. The pellets were each washed with 500 µl of 70% ice-cold ethanol. After drying of the pellets, they were suspended in 50 µl of distilled water.

B1.2. Large-scale plasmid DNA isolation from recombinant *E. coli* and *Salmonella*

To prepare large amounts of plasmid DNA from recombinant *E. coli* or *Salmonella*, the Plasmid DNA Purification NucleoBond® AX100 PC Kit (Macherey-Nagel Inc. Germany) which employs a modified alkaline/SDS method from Sambrook *et al.*, (1989) was used according to manufacturer's instructions. Recombinant bacteria were grown in 50 – 100 ml 2x TY as described in Appendix A. The cells were harvested by centrifugation at 5000 rpm for 3 - 5 min at 4 °C. The pellet was suspended in 4 ml of S1 (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNase A, pH 8.0). 4 ml of S2 (200 mM NaOH, 1% SDS) was added to the re-suspended cells and incubated at ambient temperature for 5 min for cells to lyse. Buffer S3 (2.8 M KAc, pH 5.1) (4 ml) was then added to the suspension and incubated for 5 min on ice. The suspension was centrifuged at 12 000 rpm to pellet the cellular debris. The supernatant (contained the plasmid) was loaded onto AX100 column and was allowed to flow. The columns were washed twice with 5 ml of with N3 (10 mM Tris, 15% ethanol, 1 M KCl, pH 6.3). The plasmid DNA was eluted from the column with 2.5 ml of N5 (10 mM Tris, 15% ethanol, 1 M KCl, pH 8.5). The DNA was precipitated with 0.7 volumes of isopropanol and collected by centrifugation (15 000 rpm for 30 min). The pellets were washed with ice-cold 70% ethanol, dried and suspended in appropriate volume of water or TE buffer.

B1.3. DNA concentration and purity determination

DNA was quantified spectrophotometrically. The DNA dissolved in water or TE was diluted. The absorbance of the DNA was read at 260 nm (A_{260}) and 280 nm (A_{280}) with a spectrophotometer using UV light. The concentration of DNA in the original sample was calculated based on the fact that 1 OD at 260 nm corresponds to approximately 50 µg/ml double-stranded DNA (Sambrook *et al.*, 1989). To determine the purity of the DNA preparation, the A_{260}/A_{260} ratio was calculated. The ratio of 1.8 -2.0 indicated a high purity DNA sample.

B1.4. Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was carried out as described by Sambrook *et al.*, (1989). Depending on the sizes of DNA, agarose gel concentrations ranging from 0.8 to 2% were used in 1X TBE or TAE buffers (Appendix D). DNA samples (and molecular weight marker) to be run were mixed with 6X Loading buffer (Appendix D) and loaded on polymerized gel. The electrophoresis was performed using DC Power Supply apparatus connected to the electrophoresis unit as described in Sambrook *et al.*, (1989). After electrophoresis, the gel was strained with

Ethidium bromide and visualized using UV transilluminator. The gels were photographed using Kodak DC120 digital camera.

B1.5. Restriction endonuclease digestions and mapping

Restriction digestion of plasmid DNA was performed using standard suggested protocols (Samrook *et al.*, 1989). For restriction mapping, generally 1 - 5 µl (200 ng-1 µg) of DNA was digested in a total volume of 20 µl with 5 units of respective enzyme, 2 µl of 10X enzyme buffer (supplied by enzyme manufacturer) and water. Where more that one enzyme was used, the volume of water was adjusted appropriately. If the enzymes did not have compatible buffers, the digestion was done separately. To digest large amounts of DNA for cloning, the units of enzyme(s), the amount of buffer(s), DNA and water were adjusted appropriately. The restriction digest reactions were incubated at 37 °C for 1-4 hours or overnight, depending on the experiment. To check if digestion was complete, 5 µl of the digest was analyzed by gel electrophoresis (Appendix B1.4).

B1.6. Extraction of DNA from agarose gels

DNA was purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN, Germany). Briefly, DNA fragments were separated on an agarose gel (Appendix B1.4) in duplicates. After running the gel, half of the gel was removed and stained with ethidium bromide in order to locate the position of the required DNA fragments on the other half of the gel. The relevant DNA band was then cut from the unstained gel and 3 volumes of Buffer QG added. To solubilize, the gel was incubated at 50 °C. The solubilized sample was passed through the QIAGEN column by centrifugation. The column was washed with 70% ethanol and the DNA eluted in 20-50 µl of distilled water.

B1.7. DNA ligation

DNA ligation reactions were carried out in 10-20 µl volumes. Unless otherwise, ligation reactions were carried out using the Rapid Ligation Kit (Roche, Germany) reagents or the Promega pGEM-T easy Kit (Promega, USA) reagents according to manufacturer's recommendations. For the ligation of the PCR products using pGEM Teasy plasmid, the reagents supplied with the kit (Promega) were used. Ligation reactions contained 1-2 µl (20 - 60 ng) linearized plasmid vector, 5-10 µl of 2X Rapid Ligation Buffer (Roche or Promega), 5-10 µl (50 - 150 ng) insert DNA, 1 µl T4 DNA Ligase and appropriate volume of water. Depending on the experiment and nature of the ligation, the reactions were incubated at room temperature for 1-5 hours or 12 - 16 hours at 16°C before genetic transformation of competent *E. coli* cells.

B1.8. DNA sequencing

Sequencing of DNA was performed on automated sequencer at the University of Stellenbosch's Central DNA Sequencing Facility on an ABI-Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The ABI-Prism BigDye Terminator Cycle sequencing is a variation of the Sanger method. The analysis of DNA sequence data was done using the DNAMan software (Lynnon Biosoft, Canada) and ABI Prism Chromas software (Applied Biosystems, Foster City, CA)

B2. PROTEIN ISOLATION PROTOCOLS

B2.1. Total bacterial (*E. coli* and *Salmonella*) protein isolation procedure

Protein lysate was prepared from bacterial cultures (normally stationary phase overnight cultures) by the SDS lysis method. Bacterial cells were cultured in 50 - 100 ml 2x YT liquid broth (supplemented for *Salmonella*), with ampicillin selection. Culture volumes of 2 – 4 ml were pelleted at 14 000 rpm for 5 min. The pellets were washed once with equal volumes of ice-cold PBS and suspended in 250 µl lysis buffer (3% SDS, 10% Glycerol, 0.0625 M Tris-HCl, pH 6.8) and incubated in boiling water bath for 30 min. The tubes were centrifuged at 14 000 rpm for 10 min at ambient temperature and supernatants placed in fresh eppendorf tubes. The protein concentration was assayed using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Germany) (Appendix B3). The samples were stored at -20 °C until required.

B2.2. Soluble bacterial protein isolation procedure

Bacterial cells were cultured and harvested as in Appendix B2.1. The bacterial pellets were washed once with PBS and re-suspended in 1.5 ml of fresh PBS. Silica spheres/beads (200 µg) were added into the tubes. The tubes were placed onto a FastPrep FP120 machine (Bio 101, USA) and processed for 45 seconds at maximum speed. The tubes were centrifuged at 15 000 rpm for 10 min. The supernatants, which contained the soluble bacterial protein fractions, were placed in new tubes. Samples were stored at -20 °C until required.

B3. PROTEIN CONCENTRATION DETERMINATION BY BIO-RAD *DC* PROTEIN ASSAY

The concentration of bacterial protein lysates was determined by the Bio-Rad *DC* Protein Assay kit (Bio-Rad, Germany) according to manufacturer's recommendations. The *DC* Protein Assay is for protein concentration determination following detergent solubilization and the reaction is based on the well-documented Lowry assay (Lowry *et al.*, 1951). To prepare a protein standard curve, several dilutions of bovine serum albumin (BSA) were prepared ranging from 100 µg/ml to 2000 µg/ml. Reagent A (167 µl) was added to 33 µl of each sample or standard followed by addition of 1333 µl of Reagent B. The mixtures were vortexed and after 15 min incubation, absorbance was read at 750 nm. The concentrations of proteins in samples were determined using the BSA standard curve.

B4. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with modifications as previously described by Laemmli, (1970) and with recommendations from Sambrook *et al.*, (1989). Acrylamide (10% or 12.5%) running gels were prepared (Appendix D) and allowed to polymerize in gel moulds at ambient temperature for 4 hours or overnight at 4 °C. The gels were overlaid with water-saturated butanol to create a flat uniform surface. A 1 - 2 cm deep 4% acrylamide stacking gel (Appendix D) was poured and after polymerization, samples loaded and run as described in Sambrook *et al.*, (1989). After the run, the gels were stained with Coomassie Brilliant blue solution (Appendix D) or processed for Western blotting.

B5. COOMASSIE BRILLIANT BLUE STAINING AND DESTAINING

The SDS-PAGE was covered with Coomassie Brilliant blue solution (Appendix D) and incubated at ambient shaking gently for about 2 – 16 hrs. To destain the protein gel, the staining solution was removed and the gel was washed 5 - 10 times with Destaining solution I (Appendix D). Further washes were performed with Destaining solution II (Appendix D). The destained gel was then placed on a light box and photographed with Kodak DC120 digital camera.

B6. WESTERN BLOTTING AND IMMUNODETECTION PROTOCOLS

B6.1. Western blotting of proteins from gel to membrane

After separation of bacterial proteins by SDS-PAGE (**Appendix B4**), the gel was equilibrated in transfer buffer (Appendix D) for 10-15 min. The Hybond-P membrane (Amersham Pharmacia Bioteck, UK) was soaked in methanol for 2 min followed by rinsing in water and then in transfer buffer for 5 min each. The proteins were transferred to the membrane by electroblotting at 15 volts for 30 – 45 min. The membrane was rinsed in TBS-Tween (Appendix D) and soaked in 1% blocking buffer (Appendix D) for 1 hr at ambient temperature or 12 – 16 hrs at 4 °C.

B6.2. Immuno-detection of GFP or HIV-1 Gag antigens on Western blot

Immunodetection of GFP or HIV-1 Gag antigens from Western blots (Appendix B6.1) was carried out using reagents from BM Chemiluminescence Blotting Substrate (POD) Kit (Roche, Germany) according to the manufacturer's recommendations. Briefly, the membrane was blocked with 1% blocking solution (Appendix D) for 2 hrs at ambient temperature or 16 hrs at 4 °C. The blocking solution was removed and the primary antibody (GFP- or HIV-1 P55- or P24-specific) at the specified dilution (in 0.5% blocking solution) (Appendix D) was added. The membrane was incubated at ambient temperature for 2 hours. The primary antibody was removed and membrane washed twice with 10 – 20 ml TBS-Tween (Appendix D) for 10 min. The membrane was further washed once with 10 – 20 ml 1% blocking solution for 10 min. The blocking solution was removed and specific dilution (0.5% blocking solution) of secondary antibody added. After incubation for 30 - 60 min, the membrane was washed three times with 10 – 20 ml TBS-Tween (Appendix D). Three different detection methods were used. When secondary antibodies were HRP conjugated, the BM Chemiluminescence Blotting Substrate (POD) Kit (Roche, Germany) detection reagents were used according to manufacturer's instruction. The detection reagents, A and B (mixed at 100:1 ratio in water) were added onto the membrane. The membrane exposed to X-ray film and developed. Alternatively for HRP-conjugated secondary antibodies, the Nova Red Substrate solution (Vector Laboratories, CA, USA) was used. The membrane was covered with the solution and removed when the protein bands had developed. It was rinsed in water and dried. For alkaline phosphatase-conjugated secondary antibodies, visualization of proteins was achieved by means of BCIP-NBT (5 Bromo-4 Chloro-3 Indolyl Phosphate /NitroBlue Tetrazolium) substrate. The solution was also poured directly onto the membrane and colour developed within 10 min. The membrane was rinsed in water and dried.

B6.3. Immuno-detection of HIV-1 Gag antibodies using HIV NEW LAV Blot

The NEW LAV-BLOT I kit (Bio-Rad, France) (normally used for human HIV diagnostic detection) was used to detect HIV-1 Gag-specific antibodies in mouse serum with modifications of the recommented protocol. The NEW LAV-BLOT I kit reagents (strips and washing solution) were equilibrated at room temperature for 30 minutes. Each strip was covered with 2 ml of the reconstituted buffer solution/diluent (1 in 5 dilution with water). and 20 µl (1 in 100 dilution) of serum sample was then added. The strips were incubated at ambient temperature with slight shaking for 2 hrs. The strips were washed twice for 5 min with reconstituted buffer solution/diluent and then 2 ml of the secondary anti-mouse antibody conjugated to alkaline phosphatase or horseradish peroxidase (diluted appropriately) was added. This was followed by 1-hour incubation at room temperature. The strips were washed twice as before and 2 ml of development solution (Nova Red Substrate or BCIP/NBT depending on conjugate used) was added. The colour development was stopped by rinsing the strips in water. The strips were dried and HIV-1 Gag-specific bands evaluated against the reference strip.

B7. PREPARATION OF SPLENOCYTES

The spleens were meshed using a rubber stopper and metal grid (Sigma) placed in a petri dish to generate a single cell suspension. The cell suspension was transferred to a 50 ml conical centrifuge tube. The petri dish was washed with 10 ml RPMI to collect residual cells. The volume was made up to 50 ml with RPMI. The cell suspension was centrifuged at 1500 rpm for 5 minutes to pellet the cells. The pellet was re-suspended in 50 ml of RPMI and centrifuged as before. The pellet was then washed twice with 50 ml of RPMI (before centrifuging the cells at the last wash at which any extracelluar matter was removed using a Pasteur pipette. The cells were re-suspended in 50 ml RPMI complete medium (Appendix D). To count the cells and determine viability, 1/10 dilution of the suspension was made in Trypan Blue and counted in a Neubauer counting chamber. Cell concentration in suspension was calculated and adjusted to appropriate volume.

B8. DETECTION OF CYTOKINE-SECRETING SPLENOCYTES BY ENZYME LINKED IMMUNOSPOT (ELISPOT) ASSAY

(IFN-γ and IL-4 ELISPOT assays were carried out using BD Biosciences kits according to manufacturer's recommendations. Briefly, plates were coated with capture antibody (IFN-γ or IL-4 antibody at 5 μg/ml in PBS). The plates were sealed and incubated for 16 hr at 4°C. The coating buffer was discarded and wells were washed once with 200 μl/well blocking solution. Blocking solution (200 μl/well) was added and plates incubated for 2 hrs at ambient temperature. Peptides were diluted to the required working concentrations in R10 (RPMI 1640 + 10% FBS + Pen/Strep + 2ME). The splenocytes (Appendix B7) were suspended in 5 ml lysis buffer (5 mM Tris-HCl, 140 mM NH_4Cl, pH 7.3) for lysis of erythrocytes for 2 min and recentrifuged and resuspended in 2 ml of R10 (RPMI 1640 with Glutamax, Penicillin-Streptomycin, FBS, 2-ME), The cells were counted and prepared to a final concentration of 5 x 10^6 cells/ml in R10 medium. After 2 hr of incubation of plates, the blocking solution was discarded and wells were plated in triplicate with stimulants (100 μl per well) (Appendix D). This plating was followed by addition of splenocytes (100 μl/well of the cell suspension, that is, 500 000 cells/well). After incubation for 24 hr (IFN-γ ELISPOT plate) or 48 hr (IL-4 ELISPOT plate), plates were processed for to detect IFN-γ- or IL-4-spot-forming units. The cell suspensions were discarded and wells were washed twice with water and three times with Wash buffer I (1x PBS-0.05 Tween 20, Ph 7.4 (Sigma)). Detection Antibody (biotinylated anti-IFN-γ/IL-4) was diluted in dilution buffer (PBS with 10% FBS) and added (100 μl/well) to the plates. The plates were sealed and incubated at ambient temperature for 2 hr. The detection antibody solution was then discarded and the wells were washed three times with Wash Buffer I. Avidin-horseradish peroxidase (Avidin-HRP) was diluted in dilution buffer and 100 μl/well added to the plates. The plates were incubated for 1 hr at ambient temperature. The avidin-HRP solution was discarded and plates washed three times with Wash buffer I followed by another three washes with Wash Buffer II (1 X PBS (Sigma). The Nova Red Substrate solution (Southern Cross) was prepared by adding 3 drops of Reagent 1, 2 drops Reagent 2, 2 drops Reagent 3 and 2 drops of H_2O_2 to 15 ml of water. The solution (100 μl/well) was added to the plates and spots allowed to develop for 5 - 10 min in the dark. The development of spots was stopped by washing wells with cold running tap water. The plates were air-dried at room temperature overnight and stored in the dark until analysis. The plates were scanned on the ELISPOT CTL Analyser (Series 3B) and spots enumerated using a computerized ImmunoSpot Image Analyzer (Cellular Technology Ltd, Cleveland Ohio) with ImmunoSpot Version 3.2 software. The mean number of spots in triplicate wells was calculated and background subtracted. The values were expressed as net antigen-specific IFN-γ or IL-4 spot forming units (net SFUs) per million splenocytes.

B9. QUANTIFICATION OF CYTOKINES SECRETED BY SPLENOCYTES BY CYTOMETRIC BEAD ARRAY (CBA) ASSAY

The amounts of CD8+ Tc1 and Tc2 or CD4+ Th1 and Th2 cytokines (INF-γ, TNF-α, IL-4 and IL-5) secreted into the supernatants upon antigenic stimulation were quantified simultaneously using a mouse Th1/Th2 cytokine cytometric bead array (CBA) kit (BD Pharmingen) according to manufacturer's recommendations. The CBA employs a number of particles with different fluorescence intensities and can be used to simultaneously detect multiple cytokines in a single specimen (reviewed in Morgan *et al.*, 2004; Elshal and McCoy, 2006). Five bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for INF-γ, TNF-α, IL-4 and IL-5 proteins and have been mixed together to form the CBA, which is resolved in the FL3 channel of the BD FACSCalibur™ flow cytometer. Standards were prepared for each cytokine from 20 - 5000 pg/ml by serial dilutions. Capture beads (50 µl/sample) were added to the appropriate test or standard tubes. 50 µl of test sample or standard was then added and to the capture beads. This was followed by addition of 50 µl of the Mouse Th1/Th2 PE detection reagent. The assay tubes were incubated for 2 hr at ambient temperature in the dark. Wash buffer (1 ml) was added to each assay tube and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and bead pellets suspended in 300 µl of the wash-buffer. The samples were analyzed on the FACSCalibur flow cytometer (BD Pharmingen) with a BD CBA software. The standard curves for the cytokines (IFN-γ, TNF-α, IL-4 and IL-5) were generated.

B10. DETECTION OF ANTI-GFP, ANTI-HIV-1 GAG OR ANTI-LPS ANTIBODIES BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Determination of GFP, HIV-1 Gag (P24 or P55) or *Salmonella* LPS-antibody-specific responses (total IgG, IgG1 and IgG2a) was determined using a standard enzyme-linked immunosorbent assay (ELISA). In brief, flat-bottom, 96-well MaxiSorp ELISA plates (AEC-Amersham) were coated with 50 µl of antigen (GFP, HIV-1 Gag P24 or P55 or LPS) diluted in coating buffer, (Appendix D) at a concentration of 1-5 µg/ml. Plates were sealed and incubated overnight at 4°C, then washed three times with PBS-Tween (PBS containing 0.3% Tween 20, Merck) and blocked with 200 µl/well of blocking solution (Appendix D) and incubated overnight at 4°C.

Mouse serum samples were diluted (normally 1/10, 1/100 and 1/1000 or 1/10 000) using dilution buffer (Appendix D). The blocking buffer from the plates was decanted and 100 µl/well of diluted sera (in duplicate) added. The plates were sealed and incubated overnight at 4°C. The plates were washed six times with PBS-Tween (with 1% goat serum, 1% milk powder). 100 µl/well of biotinylated goat anti-mouse IgG (ICN/Cappel), diluted 1/2000 in dilution buffer, was added and plates sealed and incubated for 1-2 hours at 37°C. The plates were washed five times with PBS-Tween. Streptavidin-AP (Appendix D) (50 ul/well) diluted 1/1000 in dilution buffer was added and the plates were sealed and incubated for 1 hr at 37°C. The plates were washed five times with PBS-Tween and 50 µl/well of PNP (Appendix D) diluted to a final concentration of 1mg/ml in substrate buffer was then added. The plates were incubated in the dark for approximately 30 min at ambient temperature for colour development. The optical density was read at 405 nm (ref filter 492nm) and data analyzed. The means were calculated for the readings from the duplicate wells. To detect GFP-, P55- or LPS-specific IgG1 and IgG2a isotypes from sera, phosphatase-conjugated antibodies specific for IgG1 or IgG2a) (Serotec) at a dilution of 1/2000, were used instead of the biotinylated goat anti-mouse IgG and Streptavidin-AP (steps described above). All the other steps were the same as described above.

B11. STAINING OF CELL SURFACE MARKERS

To label cell surface receptors (CD3, CD4, CD8 and CD8) with fluorescent antibodies for analysis by flow cytometry, 1 x 10^6 splenocytes were added into tubes. This was followed by addition of 25 µl of blocking solution (Appendix D). The tubes were incubated for 20 min on ice in the dark. To wash the cells, 1 ml FACS Buffer (PBS containing 1% FCS and 0.1% NaN_3) was added and tubes centrifuged at 1500 rpm for 5min. The supernatant was discarded and the cells were re-suspended in the residual FACS buffer. The relevant antibody (Iso-APC, Iso-FITC, CD3-APC, CD4-FITC, CD8-FITC, CD19-FITC) (25 µl) was added. The tubes were incubated for 30 min in the dark. The cells were washed once with 2 ml of FACS buffer and cells were suspended in the residual volume of FACS Buffer. 1 ml of diluted FACS Lyse solution was added to each test tube and tubes incubated for 10 min at room temperature. The cells were washed once in 2 ml of FACS buffer and cells suspend in 400 µl FACS Buffer. The FACS Calibur was then run and data analyzed.

APPENDIX C: GENETIC AND AMINO ACID CODES

C1: Genetic code and amino acids encoded

First codon position (5')	Second codon position				Third codon position (3')
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	*	*	A
	Leu	Ser	*	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

* translation termination codon

C2: Amino acid letter codes

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asp	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX D: RECIPES OF MEDIA, SOLUTIONS AND BUFFERS USED IN THIS STUDY

D1. *E. COLI* AND *SALMONELLA* GROWTH MEDIA , SOLUTIONS AND BUFFERS

D1.1. Recipe for aro mix solution (x100) (for supplementing *Salmonella* growth media)

4-amino benzoic acid (Merck)	200 mg
2,3-Dihydroxy benzoic acid (Sigma)	200 mg
L-Phenylalanine (Sigma)	800 mg
L-Tryptophan (Sigma)	800 mg
dH_2O up to	200 ml

Filter-sterilise (0.22um pore size) & dispense into 10 ml aliquots. Store at –20 °C (for up to 6 months)

D1.2. Recipe for tyrosine solution (x100) (for supplementing *Salmonella* growth media)

L-tyrosine (Sigma)	800 mg
0.1M HCl up to	200 ml

Filter sterilise and dispense into 10 ml aliquots. Store at –20 °C (for up to 6 months)

D1.3. 2x YT (2x Yeast Tryptone) Liquid broth (for growing *Salmonella* or *E. coli*)

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
dH_2O up to	1000 ml

Adjust pH to 7.0 with 5N NaOH and sterilize by autoclaving

D1.4. 2x YT (Yeast Tryptone) Agar (for growing *Salmonella* or *E. coli*)

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Agar	15 g
dH_2O up to	1000 ml

Adjust pH to 7.0 with 5N NaOH (and sterilize by autoclaving)

D1.5. 1M $CaCl_2$ Stock solution (per 200 ml water) (for preparation of competent *Salmonella* or *E. coli*)

$CaCl_2.6H_2O$	54 g
dH_2O up to	200 ml (and filter with 0.22-micron filter)

D1.6. IPTG (for induction of *lac* operon in growing *E. coli*)

IPTG	2 g
dH_2O up to	10 ml

Filter sterilize with 0.22-micron filter and store at -20° C in aliquots

D2. 1M TRIS BUFFER

Tris	121.1 g
dH_2O up to	1000 ml

Adjust pH to 7.4, 7.6 or 8.0 with HCl, aliquot and sterilize by autoclaving

D3. 0.5M EDTA

EDTA	186.1 g
dH_2O up to	1 000 ml
Adjust pH to 8.0 with NaOH pellets	

D4. TAE, 50x Stock solution (for agarose gel electrophoresis)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA, pH 8.0	100 ml
dH_2O up to	1000 ml

D5. TBE, 5x Stock solution (for agarose gel electrophoresis)

Tris base	54 g
Boric acid	27.5 g
0.5M EDTA, pH 8.0	20 ml
dH_2O up to	1000 ml

D6. Loading buffer (for agarose gel electrophoresis)

Bromophenol blue	25 mg
Sucrose	4 g
0.5M EDTA, pH 8.0	0.4 ml
dH_2O up to	10 ml

D6. SOLUTIONS AND BUFFERS FOR SDS-PAGE

D6.1. Monomer solution (30% Acryramide stock solution)

Acrylamide	29.2 g
Bis-acrylamide	0.8 g
dH_2O up to	100 ml

The solution was filter-sterilized and stored in the dark at 4°C.

D6.2. 4X Running gel buffer

Tris	36.3 g
pH adjusted to 8.8 with HCl	
dH_2O up to	200 ml

D6.3. 4X stacking gel buffer (per 50 ml dH_2O r)

Tris	3 g
pH adjusted to 6.8 with HCl	
dH_2O up to	50 ml

D6.4. Separating gel (10% and 12.5% in 20 ml dH_2O

	10%	12.5%
Monomer solution	20 ml	25 ml

4X Running gel buffer	15 ml	15 ml
10% SDS	600 ul	600 ul
10% APS	300 ul	300 ul
TEMED	20 ul	20 ul
dH_2O	24.1 ml	19.1 ml

D6.5. Stacking gel

Monomer solution	750 ul
Stacking gel buffer	1.25 ml
10% APS	15 ul
TEMED	10 ul
dH_2O	3.0 ml

D6.6. SDS/Electrophoresis buffer (Tank buffer)

Tris	30.28
Glycine	1.44.13 g
SDS	10 g
dH_2O up to	10 litres

D6.7. 10% SDS

SDS	10 g

Dissolve at 68° C, allow to cool and adjust pH to 7.2 with concentrated HCl

dH_2O up to	100 ml

D6. 8. 10% Ammonium persulphate

Ammonium persulphate	0.5 g
dH_2O up to	5 ml

D6. 9. 2x Treatment buffer

4x stacking gel buffer	2.5 ml
10% SDS	4 ml
Glycerol	2ml
Bromophenol blue	2 mg
Dithiothreitol	0.31 g
dH_2O up to	10 ml

D7. COOMASSIE BRILLIANT BLUE STAINING AND DESTAINING SOLUTIONS

D7.1. Comassie brilliant blue staining solution

Coomassie Brilliant blue R	0.5 g
Methanol	800 ml
Acetic acid (glacial)	140 ml
dH_2O up to	2000 ml

D7.2. Destaining solution I

Methanol	400 ml
Acetic acid (glacial)	70 ml
dH_2O up to	1000 ml

D7.3. Destaining solution II

Methanol	50 ml
Acetic acid (glacial)	70 ml
dH_2O up to	1000 ml

D8. 6X AGAROSE GEL LOADING BUFFER/DYE

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in dH_2O	30%

D9. WESTERN BLOTTING AND IMMUNODETECTION BUFFERS

D9.1. Transfer buffer

Tris	5.82 g
Glycine	2.93 g
Methanol	200 ml
10% SDS	3.75 ml
dH_2O up to	1000 ml

D9.2. Tris-buffered saline (TBS)

Tris	12.11 g
Add 900 ml distilled water, pH 7.5 with HCl	
NaCl	9 g
dH_2O up to	1000 ml

D9.3. TBS-Tween

TBS with 0.1% Tween-20

D9.4. Blocking solution

Dilute the stock blocking buffer supplied with the Roche kit using TBS.

1%	10 ml stock blocking buffer + 90 ml TBS
0.5%	5 ml stock blocking buffer + 95 ml TBS

D10. SOLUTIONS, REAGENTS AND BUFFERS FOR ELISA PROTOCOLS

D10.1. Coating Buffer (Carbonate buffer, Ph 9.6 (Sigma)): One carbonate buffer capsule was dissolved in 100ml of de-ionised H_2O (not necessary to adjust pH. - usually about 9.6 to 9.8).

D10.2. PBS: 10X PBS liquid, pH 7.4 (Adcock Ingram): PBS (100 ml) was diluted into water and pH adjusted to 7.4.

D10.3. PBS-Tween: As in ELISPOT Solutions, Reagents and Buffers **(Appendix D11)**

D10.4. Blocking buffer: PBS containing 0.3% Tween 20, 1% goat serum (Southern Cross Biotechnology), 3% milk powder (Spar instant fat-free milk powder).

D10.5. Dilution buffer: PBS containing 1% goat serum, 1% milk powder was prepared.

D10.6. Biotinylated goat anti-mouse IgG (ICN/Cappel): Dilute 1/2000 in dilution buffer.

D10.7. Streptavidin-AP (Scientific Group): Dilute 1/1000 in Streptavidin HRP dilution buffer.

D10.8. Streptavidin-AP Dilution Buffer: 1% BSA (Roche), 0.02% $NaNa_3$ (Merck) in PBS was prepared.

D10.9. 4-Nitrophenyl Phosphate (PNP) Substrate (Sigma): Dilute to a final concentration of 1mg/ml in substrate buffer.

D10.10. Substrate buffer: 0.02% $NaNa_3$ (0.2g/L), 97ml di-ethanolamine (Merck), 0.8g Mg $CL_2.6H_2O$ (Merck). Add 700ml de-ionised H_2O and adjust the pH to 9.8 with 10M HCl. Make up to 1L. Store at 4°C.

D11. SOLUTIONS, REAGENTS AND BUFFERS FOR USED FOR ELISPOT PROTOCOLS

D11.1. Capture antibody: Purified Anti-mouse IFN-γ/IL-4: To coat the elispot plates, antibody stock at 1 mg/ml was diluted 1:200 (5 µg/ml final concentration), that is, for 1 plate: 60 µl antibody + 12 ml coating buffer (PBS, pH 7.2)

D11.2. Detection antibody: Biotinylated anti-mouse IFN-γ/Il-4: Stock antibody at a 0.5 mg/ml concentration was diluted 1:250 (2 µg/ml final concentration) in dilution buffer (1 X PBS with 10 % FBS). For 1 plate: 48 µl antibody + 12 ml 10% FBS 1X PBS was made.

D11.3. Streptavidin Horseradish Peroxidase: The stock concentration at 0.5 mg/ml was diluted 1:100 in dilution buffer (1 X PBS with 10% FBS). For 1 plate: 120 µl Avidin-HRP + 12 ml 10% FBS 1X PBS was used.

D11.4. Coating buffer: 1 X PBS (Gibco, pH 7.2) was used

D11.5. R10 medium: To prepare 100 ml R10 solution, 88.9 ml RPMI 1640 with Glutamax + 1 ml Penicillin-Streptomycin + 10 ml FBS + 100 µl 2-ME (Sigma). To make a stock of 50 mM 2-ME: 5 µl of 2-ME + 1.425 ml RPMI

D11.6. Wash buffer I: 1 X PBS/Tween (Sigma): PBS + 0.05% Tween 20 – one sachet makes 1 l solution, pH 7.4.

D11.7. Wash Buffer II: 1 X PBS (Sigma): One sachet makes 1 l solution, pH = 7.4.

D11.8. Dilution Buffer: 1 X PBS, pH 7.2 + 10% FBS

D11.9. Nova Red Substrate (Southern Cross): To 15 ml dH_2O, 3 drops Reagent 1 were added and mixed well, followed by 2 drops Reagent 2 and mixing well. 2 drops Reagent 3 were added and mixed well followed by 2 drops H_2O_2 and mixing well.

D12. PEPTIDES/ANTIGENS FOR ELISPOT AND CBA ASSAYS

D12.1. Con A (Sigma, stock at 1 mg/ml): The stock was diluted 1:100 to give 10 µg/ml concentration. 2 ml working stock was prepared, that is, 2 µl + 1980 µl in R10 medium. 100 µl/well was added to plate to get the final concentration of 0.5 µg/ml in each well after adding 100 µl of cells.

D12.2. Irrelevent peptide, TYSTVASSL (stock at 4 mg/ml): To prepare 2 ml working stock of 4 µg/ml: 2 µl + 1998µl R10 medium were mixed. 100 µl/well was added to plates to make a final concentration of 2 µg/ml after adding 100 µl of cells.

D12.3. AMQM CD8+ T cell peptide, AMQMLKDTI (stock at 4 mg/ml): To prepare 2 ml working stock of 4 µg/ml: 2 µl + 1998µl R10 medium were mixed. 100 µl/well was added to plates to make a final concentration of 2 µg/ml after adding 100 µl of cells.

D12.4. TTST Gag CD8+ T cell peptide, TTSTLQEQI (stock at 4 mg/ml): To prepare 2 ml working stock of 4 µg/ml: 2 µl + 1998µl R10 medium were mixed. 100 µl/well was added to plates to make a final concentration of 2 µg/ml after adding 100 µl of cells.

D12.5. GFP CD8+ T cell peptide, HYLSTQSAL (stock at 4 mg/ml): To prepare 2 ml working stock of 4 µg/ml: 2 µl + 1998µl R10 medium were mixed. 100 µl/well was added to plates to make a final concentration of 2 µg/ml after adding 100 µl of cells.

D12.6. MRC 2 CD4+ T cell Gag peptide, VHQAISPRTLNAWVKVIEEK (in powder): 2 mg powder was dissolved in 500 µl PBS to make stock concentration of 4 mg/ml and stored at –20°C Freezer. To prepare 2 ml working stock of 4 µg/ml: 2 µl + 1998µl R10 medium were mixed. 100 µl/well was added to plates to make a final concentration of 2 µg/ml after adding 100 µl of cells.

D12.7. MRC 13 Gag CD4+ T cell peptide, NPPIPVGDIYKRWIILGLNK (in powder): 2 mg powder was dissolved in 500 µl PBS to make stock concentration of 4 mg/ml and stored at –20°C Freezer. To prepare 2 ml working stock of 4 µg/ml: 2 µl + 1998µl R10 medium were mixed. 100 µl/well was added to plates to make a final concentration of 2 µg/ml after adding 100 µl of cells.

D12.8. MRC 17 Gag CD4+ T cell peptide, FRDYVDRFFKTLRAEQATQE (in powder): 2 mg powder was dissolved in 2000 µl in PBS to make stock concentration of 1mg/ml and stored at –20°C Freezer. To prepare 2 ml working solution of 4 µg/ml: 8µl + 1996 µl R10 medium were mixed. 100 µl/well was added to plates to make a final concentration of 2 µg/ml after adding 100 µl of cells.

APPENDIX E: BACTERIAL STRAINS USED AND DEVELOPED BY THIS STUDY

Table E1: *E. coli* and *Salmonella* bacterial strains used in this study

Strain	Genotype	Source
E. coli JM110	*rpsL (Str^r) thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI^{q}ZΔM15*]	Stratagene
E. coli SCS110	*rpsL (Str^r) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI^{q}ZΔM15*]	Stratagene
Salmonella enterica serovar Typhimurium vaccine vector	ΔAroC	Microscience, UK

Background information on JM110 and SCS110 cells: JM110 and SCS110 are deficient for the two methylases, Dam and Dcm. The Dam methylase recognizes the DNA sequence GATC and methylates the adenine residue and Dcm methylase recognizes the DNA sequence CCAGG and CCTGG and methylates the internal cytosine (Allamane *et al.*, 2000). The, SCS110 strain is an *endA*- derivative of the JM110 strain and are endonuclease mutants (*endA*). The quality of plasmid DNA prepared in SCS110 cells is better than DNA prepared in JM110 cells (Stratagene). Since JM110 and SC110 cells contain the *lacIqZ.M15* gene on the F′ episome, the blue-white screening for recombinant plasmids can be used.

Background information on *S. enterica* serovar Typhimurium AroC mutant : The ΔAroC *Salmonella enterica* serovar Typhimurium vaccine strain (WT05) is an auxotroph that was derived from *Salmonella enterica* serovar

Typhimurium vaccine TML strain (Khan *et al.*, 2003). The strain has a deletion in the *aroC* gene. The *aroC* gene encodes chorismate synthase, a key enzyme required in the biosynthesis of aromatic compounds, tryptophan, tyrosine, phenylalanine, para-aminobenzoic acid and 2,3-dihydroxybenzoate (Khan *et al.*, 2003). *Salmonella* strains with a mutation in the gene are attenuated when used in vaccination of animals or humans.

Table E2: Recombinant *Salmonella* (ΔAroC) vaccine vectors developed by this study

Name given	**Plasmid carried**	**Antigen expressed**	**Antigen-specific immunogenicity in mice**
AroC+Pgagmtr	Pgagmtr	wtGag	Not detectable (unreported results)
AroC+Pgagssa	Pgagssa	wtGag	Not detectable (unreported results)
AroC+GFP	pGEM+GFP	GFP	Detectable (see Chapter 3)
AroC+pGEM	pGEM	LacZα	Negative control (see Chapters 3, 4, 5, 6)
AroC+wtGag	pGEM+wtGag	WtGag	Detectable (see Chapter 4)
AroC+GagK	pGEM+GagK	GagK	Not determined
AroC+ pGagmtrGFP	pGagmtrGFP	wtGag-GFP	Not determined
AroC+Salmgag	pGEM+Salmgag	Salmgag	Detectable (see Chapter 5)
AroC+P1724	pGEM+P1724	P41 (P17+P24)	Detectable (see Chapter 6)
AroC+P24D	pGEM+P24D	Truncated P24	Detectable (see Chapter 6)
AroC+SalmgagGFP	pGEM+SalmgagGFP	Salmgag-GFP	Not detectable (see Chapter 7)

APPENDIX F: OLIGONUCLEOTIDES (PRIMERS) USED IN THIS STUDY

Table H1: Primers used in this study

Primer name	**Sequence (5'-3')**	**Gene amplified**
GFP2 (forward) GR (reverse)	ATG GCG CCA AAG ACT CCG GCT CCG AAG CTT ATT TGT ATA GTT CAT CCA TGC	*Gfp*
K1 (forward) K2 (reverse)	CCA CCC CAC AAG ATT TAA ACA C TTA AGC TTG AGA CAA GGC GTC	*GagK*
MA1 (forward)	ATA TGG GCG CCC GCG CGA GC	*P41*
CA1 (forward) CA2 (reverse)	CGA TCG TGC AGA ATC TGC AGG G GCT TCC GCC AGA ACG CGC GC	*P24, P41*
M13F M13R	G CCAGGGTTTT CCCAGTCACG AC TCATAGCTGTTTCCTGTGTGA	For universal sequencing from any vector with LacZ gene

*Nar*I, *Ehe*I and *Bbe*I restriction site: **GGCGCC**

*Hind*III restriction site: **AAGCTT**

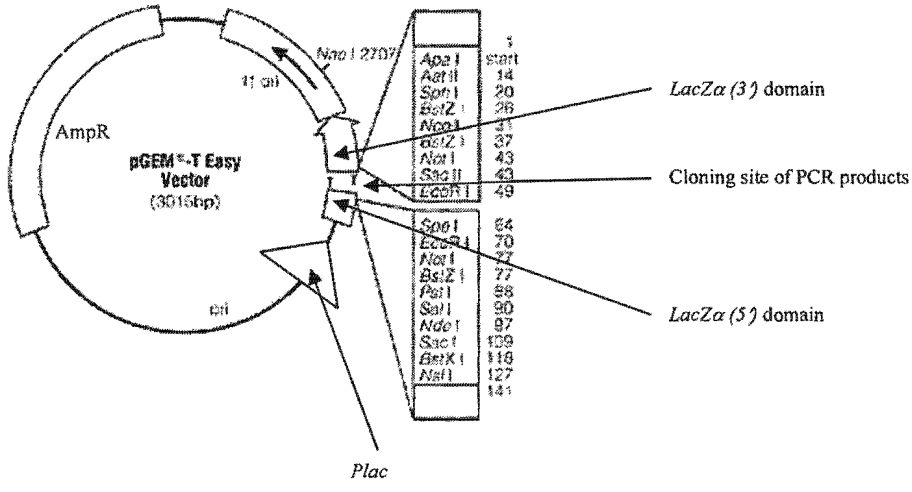
APPENDIX G: PLASMID VECTOR DESCRIPTIONS AND RESTRICTION MAPS

G1. RECOMBINANT PLASMIDS DEVELOPED USED OR DEVELOPED BY THIS STUDY

Plasmid name	Description	Source/origin
pGEM Teasy	Normally used as cloning vector for PCR products (Appendix G2)	Promega
pEHa0TGLF	Carries *gfp*, *tat*, *gag* genes for expression in BCG vaccine vector	Dr W Bourn, UCT
Pgem	Generated by self-ligation of pGEM Teasy with LacZα in-frame. Used as a negative control (Appendix G3)	This study (Chapter 3)
pGEM+GFP	Carries *gfp* fused in-frame with the 5' end of LacZα (Appendices G4 and H1)	This study (Chapter 3)
pGEM+wtGag	Carries wild-type HIV-1 *gag* fused in-frame with the 5' end of LacZα (Appendices G5 and H2)	This study (Chapter 4)
pGEM+GagK	Carries the 3' end of wild-type HIV-1 *gag* fused in-frame with 5' end of LacZα	This study (Chapter 4)
Pscript+salmgag	Carries the codon-optimized *gag*, that is, *salmgag* (Appendix G10)	Geneart, USA (Geneart, USA)
pGagmtr	Carries wild-type HIV-1 *gag* under strong constitutive *mtr* promoter (Appendix G11)	This study (unreported results)
pGagmtrGFP	Carries wild-type HIV-1 *gag* fused to *gfp* under strong constitutive *mtr* promoter	This study (unreported results)
pGagssa	Carries wild-type HIV-1 *gag* under strong *in vivo* induced *mtr* promoter (Appendix G12)	This study (unreported results)
pGEM+Salmgag	Carries *salmgag* fused in-frame with 5' end of LacZα (Appendices G6 and H3)	This study (Chapter 5)
pGEM+P1724	Carries *p41* fused in-frame between the 5' and 3' ends of LacZα (Appendices G8 and H5)	This study (Chapter 6)
pGEM+P24D	Carries truncated *p24* fused in-frame between the 5' and 3' ends of LacZα (Appendix G9)	This study (Chapter 6)
pGEM+Salmga-GFP	Carries *salmgag* and *gfp* fused in-frame with 5' end of LacZα (Appendices G9 and H6)	This study (Chapter 7)

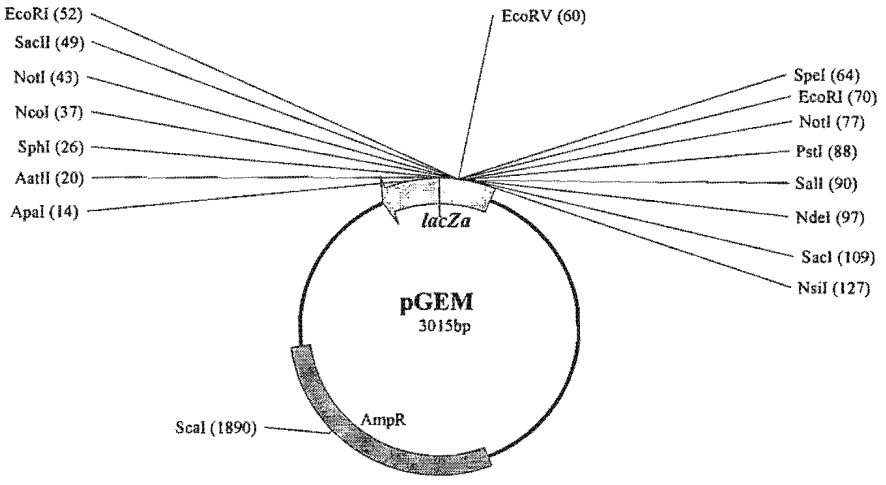
G2. pGEM-TEASY VECTOR MAP AND BACKGROUND INFORMATION

(adapted from Promega, USA catalogue)



Background information: The pGEM(R)-T Easy Vector has been linearized with *EcoR*V at Base 60 of this sequence and a T added to both 3' -ends. This vector is designed for cloning PCR products, and not as an expression vector. The pGEM-T Easy vector has a mutated version *pMB1* origin of replication and this makes the plasmid to have a copy number of 300-400 per bacterial cell. The pMBI origin of replication is closely related to that of *ColE1*. The *ColE1* origin of replication makes a plasmid to have a copy number of 300-500 per cell.

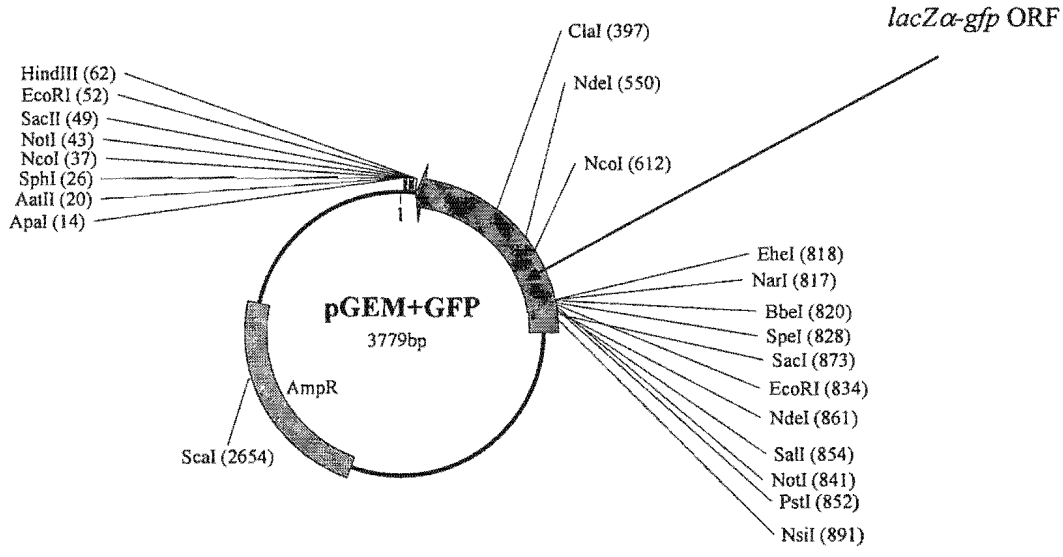
G3. pGEM VECTOR SKETCKY MAP AND LANDMARKS



pGEM Vector Sequence landmarks

Feature	location
multiple cloning region	10-128
pUC/M13 Reverse Sequencing Primer binding site	176-197
pUC/M13 Forward Sequencing Primer binding site	2949-2972
lacZα start codon (ATG)	178-180
lacZα stop codon (TAA)	2812-2814
lacZα coding region (β-galactosidase α-fragment)	1-180 2815-3015
lacZα N terminal region	60-180
lacZα C terminal regions	1-60, 2811-3015
lacZα operon sequences	2836-2996, 166-395
Beta-lactamase coding region	1337-2197
Lac promoter (*Plac*) region	181-302
-10 Sequence for lacZα operon	225-230
-35 Sequence for lacZα operon	249-253
Transcription start for lacZα operon	218
Shine-Dalgarno (AGGA) for lacZα	187-191

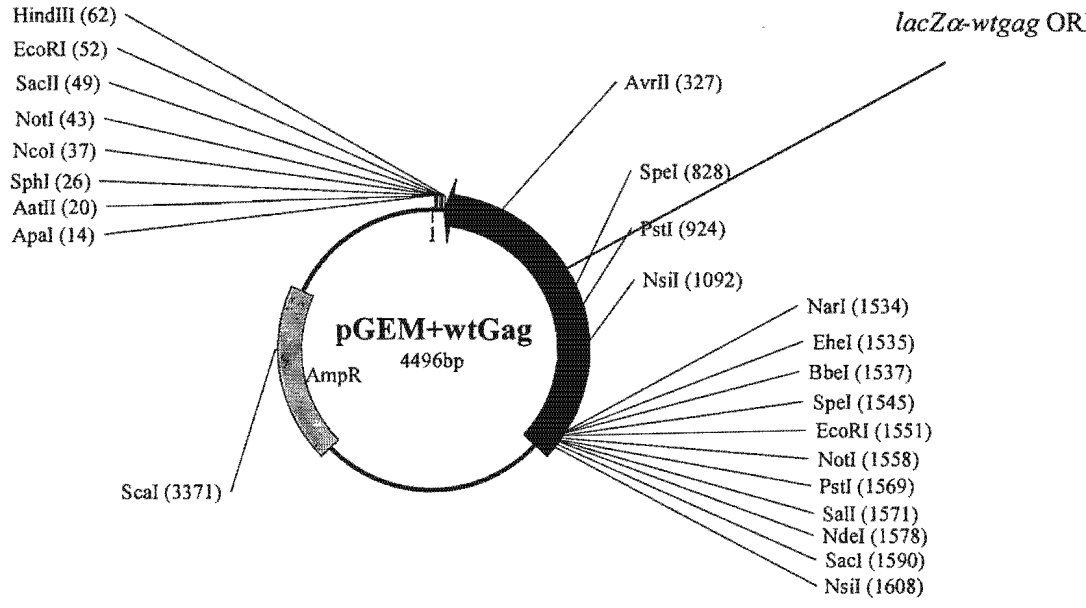
G4. pGEM+GFP VECTOR SKETCKY MAP AND LANDMARKS



pGEM+GFP Vector Sequence landmarks

Feature	**location**
pUC/M13 Reverse Sequencing Primer binding site	940-961
pUC/M13 Forward Sequencing Primer binding site	3713-3736
GFP2 primer binding site	800-823
GR primer binding site	62-88
lacZα-gfp start codon (ATG)	942-944
lacZα-gfp stop codon (TAA)	66-68
lacZα-gfp coding region	69-944
lacZα N terminal region	824-944,
Beta-lactamase coding region	2101-2961
Lac promoter (P*lac*) region	945-1066
-10 Sequence for lacZα operon	1013-1018
-35 Sequence for lacZα operon	982
Transcription start for lacZα operon	952-954
Shine-Dalgarno (AGGA) for lacZα-GFP	952-954

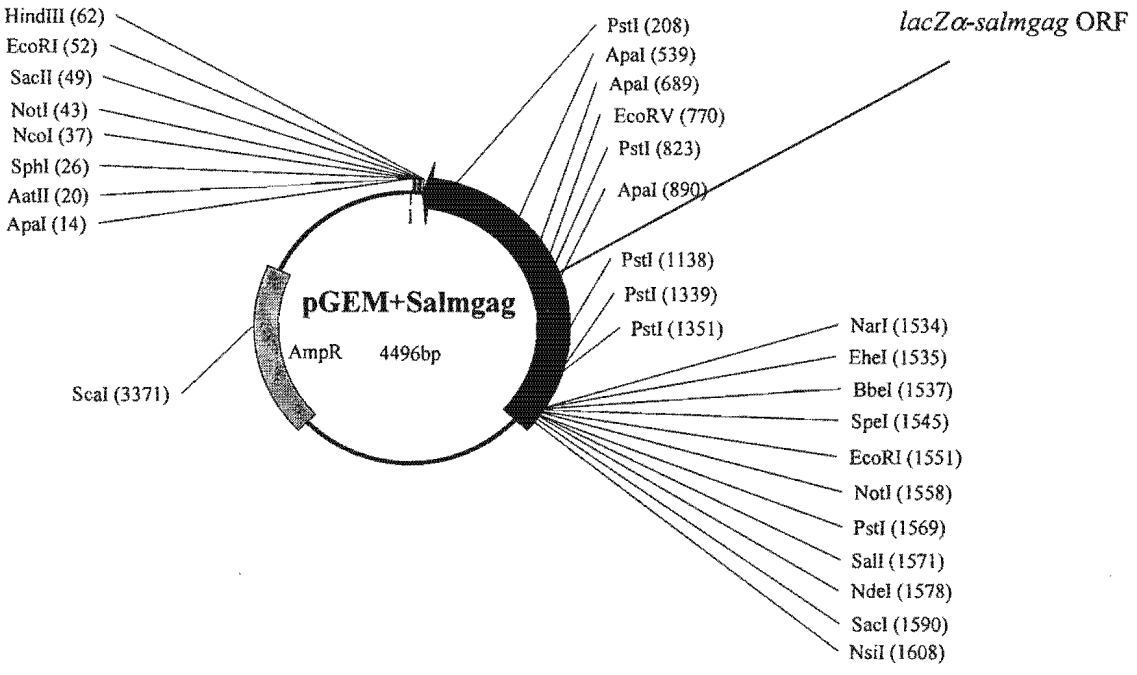
G5. pGEM+wtGag VECTOR SKETCKY MAP AND LANDMARKS



pGEM+wtGag Vector Sequence landmarks

Feature	location
pUC/M13 Reverse Sequencing Primer binding site	1658-1678
pUC/M13 Forward Sequencing Primer binding site	4431-4453
lacZα-wtgag start codon (ATG)	1659-1661
lacZα-wtgag stop codon (TAA)	59-62
lacZα-wtgag coding region	63-1661
lacZα N terminal region	1542-1661
Beta-lactamase coding region	2819-3678
Lac promoter (P*lac*) region	1662-1783
-10 Sequence for lacZα operon	1806-1811
-35 Sequence for lacZα operon	1730-1735
Transcription start for lacZα operon	1799
Shine-Dalgarno (AGGA) for lacZα-Gag	1678-1682

G6. pGEM+Salmgag VECTOR SKETCKY MAP AND LANDMARKS

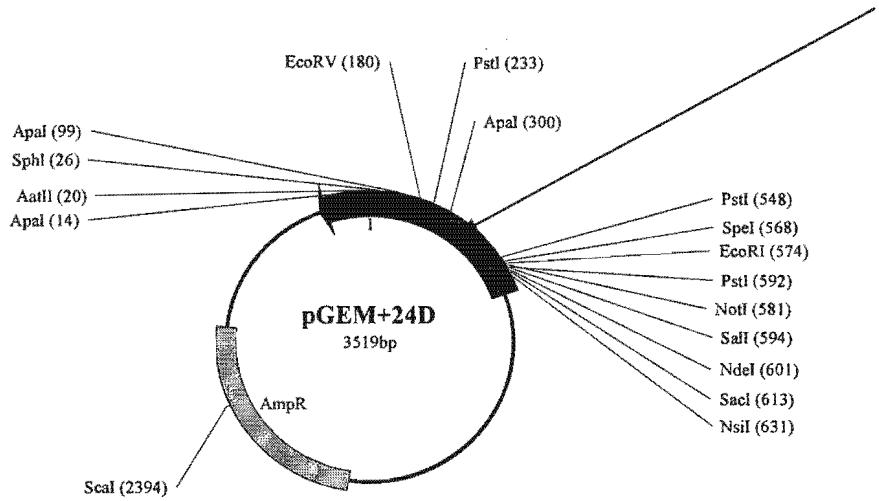


pGEM+Salmgag Vector Sequence landmarks

Feature	location
pUC/M13 Reverse Sequencing Primer binding site	1658-1678
pUC/M13 Forward Sequencing Primer binding site	4431-4453
lacZα-salmgag start codon (ATG)	1659-1661
lacZα-salmgag stop codon (TAA)	59-62
lacZα-salmgag coding region	63-1661
lacZα N terminal region	1542-1661
Beta-lactamase coding region	2819-3678
Lac promoter (P*lac*) region	1662-1783
-10 Sequence for lacZα operon	1806-1811
-35 Sequence for lacZα operon	1730-1735
Transcription start for lacZα operon	1799
Shine-Dalgarno (AGGA) for lacZα-Salmgag	1678-1682

G7. pGEM+24D VECTOR SKETCKY MAP AND LANDMARKS

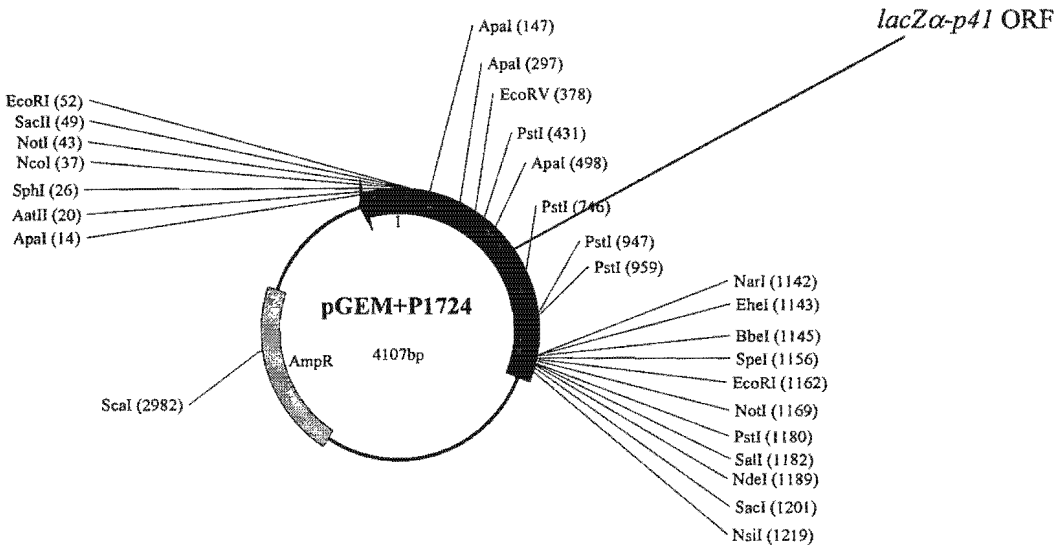
lacZα-p24d ORF



pGEM+P24D Vector Sequence landmarks

Feature	location
pUC/M13 Reverse Sequencing Primer binding site	681-701
pUC/M13 Forward Sequencing Primer binding site	3454-3476
lacZα-p24d start codon (ATG)	682-684
lacZα-p24d stop codon (TAA)	3316
lacZα-p24d coding region	1-684, 3319-3519
lacZα N terminal region	565-684
lacZα C terminal regions	0-39 (part deleted), 3316-3519
Beta-lactamase coding region	1842-2701
Lac promoter (P*lac*) region	685-806
-10 Sequence for lacZα operon	729-734
-35 Sequence for lacZα operon	753-758
Transcription start for lacZα operon	722
Shine-Dalgarno (AGGA) for lacZα-P24D	691-695

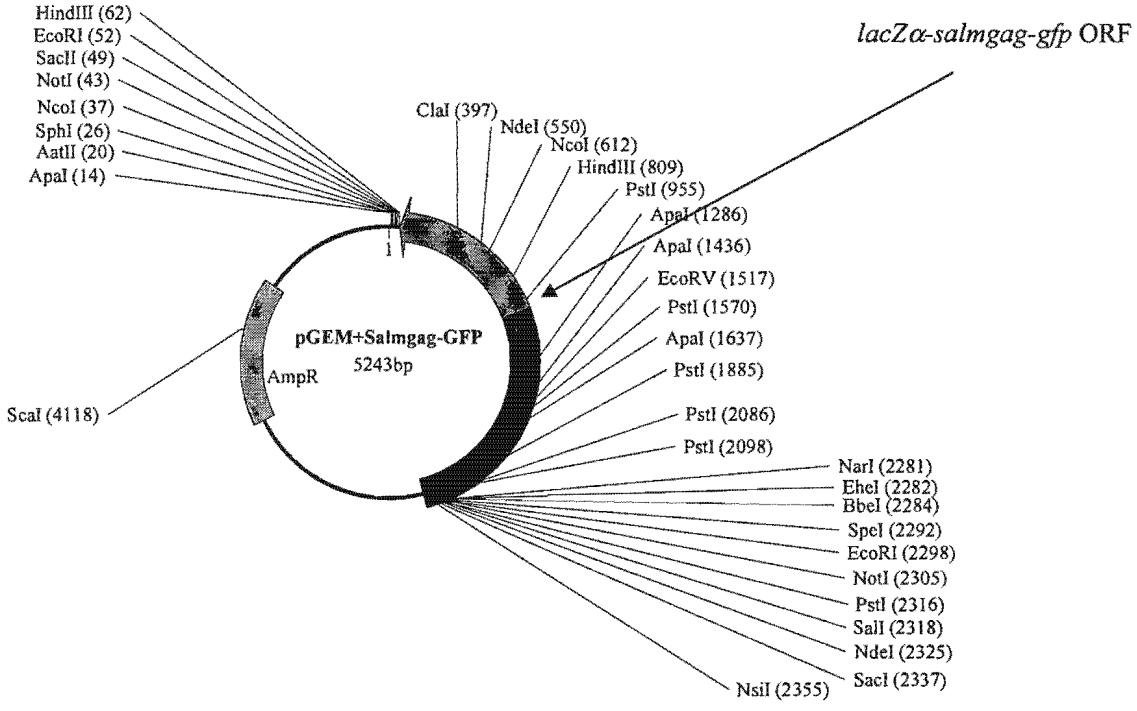
G8. pGEM+P1724 VECTOR SKETCKY MAP AND LANDMARKS



pGEM+P1724 Vector Sequence landmarks

Feature	location
pUC/M13 Reverse Sequencing Primer binding site	1269-1289
pUC/M13 Forward Sequencing Primer binding site	4042-4064
lacZα-p1724 start codon (ATG)	1270-1272
lacZα-p1724 stop codon (TAA)	3904-3906
lacZα-p1724 coding region	1-1272, 3907-4107
lacZα N terminal region	1153-1272
lacZα C terminal regions	1-60, 3904-4107
Beta-lactamase coding region	2430-3289
Lac promoter (P*lac*) region	1273-1394
-10 Sequence for lacZα operon	1317-1322
-35 Sequence for lacZα operon	1341-1346
Transcription start for lacZα operon	1309
Shine-Dalgarno (AGGA) for lacZα-P1724	1279-1283

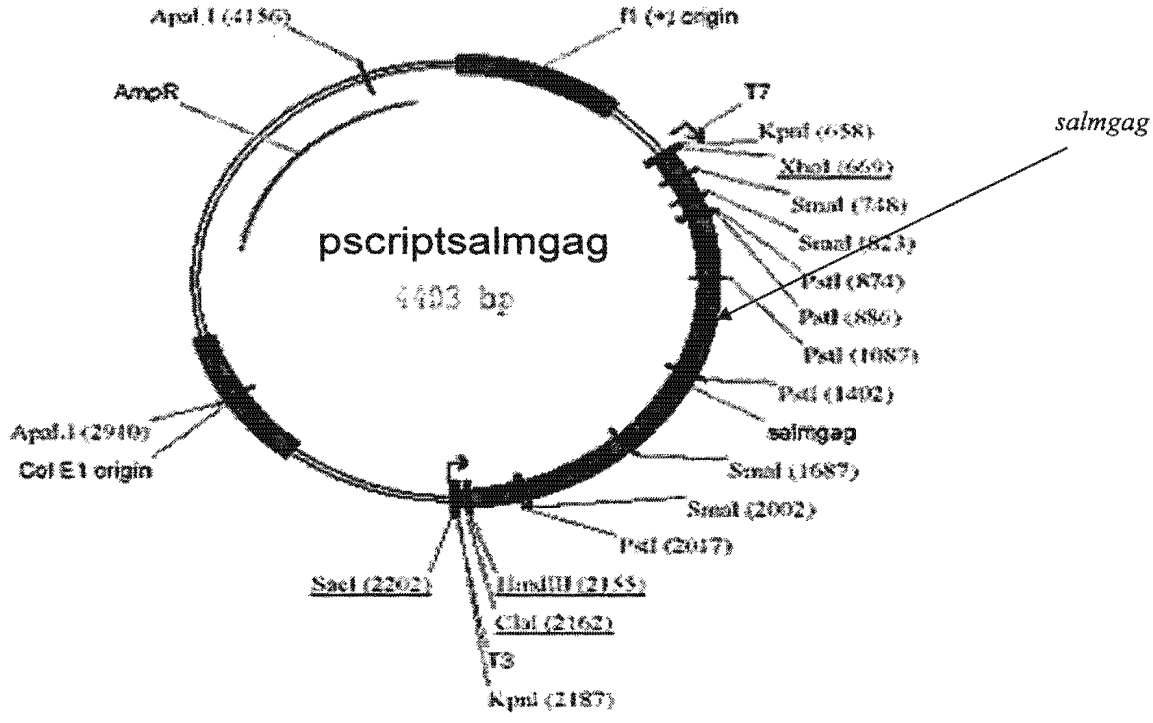
G9. pGEM+Salmgag-GFP VECTOR SKETCKY MAP AND LANDMARKS



pGEM+Salmgag-GFP Vector Sequence landmarks

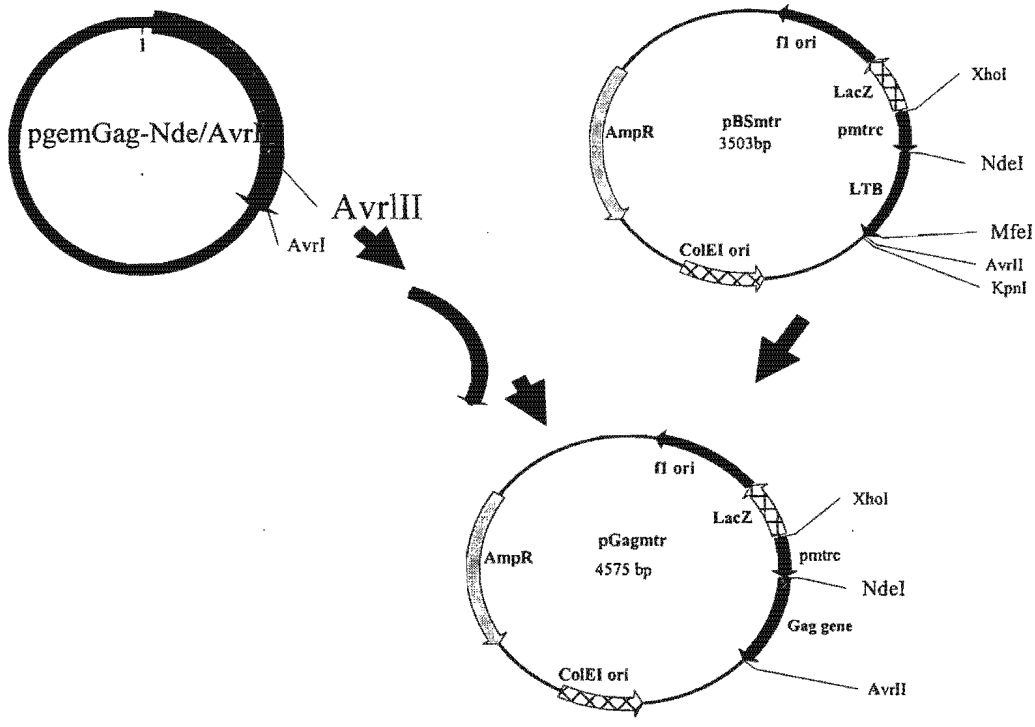
Feature	location
lacZα-salmgagfp start codon (ATG)	2406-2408
lacZα-salmgafpg stop codon (TAA)	66-68
lacZα-salmgagfp coding region	69-2408
lacZα N terminal region	2289-2408
Beta-lactamase coding region	3566-4425
Lac promoter (P*lac*) region	2409-2530
-10 Sequence for lacZα operon	2453-2458
-35 Sequence for lacZα operon	2477-2482
Transcription start for lacZα operon	2446
Shine-Dalgarno (AGGA) for lacZα-Salmgag	2416-2419

G10. PSCRIPT+SALMGAG PLASMID AND ITS SKETCKY MAP

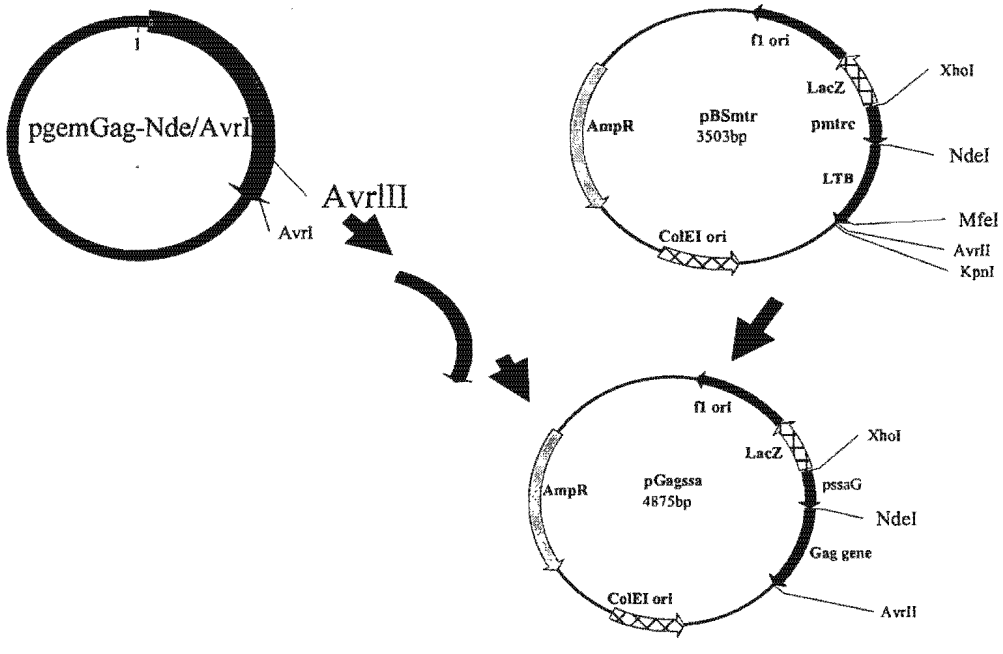


The synthetic gene, *salmgag* was assembled from synthetic oligonucleotides and cloned into pPCR-Script using XhoI and SacI restriction sites (Geneart, U.S.A). The plasmid DNA was purified (Qiagen midi-prep) from transformed bacteria. The final construct was verified by sequencing.

G11. CONSTRUCTION OF PGAGMTR PLASMID AND ITS SKETCKY MAP



G12. CONSTRUCTION OF PGAGSSA PLASMID AND ITS SKETCKY MAP



APPENDIX H: COMPLETE DNA SEQUENCS OF KEY EXPRESSION PLASMIDS DEVELOPED BY THIS STUDY

(see Appendix G for landmark features of each plasmid)

H1. pGEM+GFP complete DNA sequence

```
1       GGGCGAATTG GGCCCGACGT CGCATGCTCC CGGCCGCCAT GGCGGCCGCG GGAATTCGAT
61      TAAGCTTATT TGTATAGTTC ATCCATGCCA TGTGTAATCC CAGCAGCTGT TACAAACTCA
121     AGAAGGACCA TGTGGTCTCT CTTTTCGTTG GGATCTTTCG AAAGGGCAGA TTGTGTGGAC
181     AGGTAATGGT TGTCTGGTAA AAGGACAGGG CCATCGCCAA TTGGAGTATT TTGTTGATAA
241     TGGTCTGCTA GTTGAACGCT TCCATCTTCA ATGTTGTGTC TAATTTTGAA GTTAGCTTTG
301     ATTCCATTCT TTTGTTTGTC TGCCGTGATG TATACGTTGT GGGAGTTGTA GTTGTATTCC
361     AACTTGTGGC CGAGGATGTT TCCGTCCTCC TTGAAATCGA TTCCCTTAAG CTCGATCCTG
421     TTGACGAGGG TGTCTCCCTC AAACTTGACT TCAGCACGTG TCTTGTAGTT CCCGTCGTCC
481     TTGAAAGAGA TGGTCCTCTC CTGCACGTAT CCCTCAGGCA TGGCGCTCTT GAAGAAGTCG
541     TGCCGCTTCA TATGATCTGG GTATCTTGAA AAGCATTGAA CACCATAAGA GAAAGTAGTG
601     ACAAGTGTTG GCCATGGAAC AGGTAGTTTT CCAGTAGTGC AAATAAATTT AAGGGTAAGT
661     TTTCCGTATG TTGCATCACC TTCACCCTCT CCACTGACAG AAAATTTGTG CCCATTAACA
721     TCACCATCTA ATTCAACAAG AATTGGGACA ACTCCAGTGA AAAGTTCTTC TCCTTTCGAA
781     GCTGAGCCGG CGGAACCGGC GGAGCCGGAG TCTTTGGCGC CATAATCACT AGTGAATTCG
841     CGGCCGCCTG CAGGTCGACC ATATGGGAGA GCTCCCAACG CGTTGGATGC ATAGCTTGAG
901     TATTCTATAG TGTCACCTAA ATAGCTTGGC GTAATCATGG TCATAGCTGT TTCCTGTGTG
961     AAATTGTTAT CCGCTCACAA TTCCACACAA CATACGAGCC GGAAGCATAA AGTGTAAAGC
1021    CTGGGGTGCC TAATGAGTGA GCTAACTCAC ATTAATTGCG TTGCGCTCAC TGCCCGCTTT
1081    CCAGTCGGGA AACCTGTCGT GCCAGCTGCA TTAATGAATC GGCCAACGCG CGGGGAGAGG
1141    CGGTTTGCGT ATTGGGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT
1201    TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT CCACAGAATC
1261    AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA
1321    AAAGGCCGCG TTGCTGGCGT TTTTCCATAG GCTCCGCCCC CCTGACGAGC ATCACAAAAA
1381    TCGACGCTCA AGTCAGAGGT GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC
1441    CCCTGGAAGC TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC
1501    CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCATAGC TCACGCTGTA GGTATCTCAG
1561    TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC GAACCCCCCG TTCAGCCCGA
1621    CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC CCGGTAAGAC ACGACTTATC
1681    GCCACTGGCA GCAGCCACTG GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC
1741    AGAGTTCTTG AAGTGGTGGC CTAACTACGG CTACACTAGA AGAACAGTAT TTGGTATCTG
1801    CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT CCGGCAAACA
1861    AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG CAGATTACGC GCAGAAAAAA
1921    AGGATCTCAA GAAGATCCTT TGATCTTTTC TACGGGGTCT GACGCTCAGT GGAACGAAAA
1981    CTCACGTTAA GGGATTTTGG TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT
2041    AAATTAAAAA TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG
2101    TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC GTTCATCCAT
2161    AGTTGCCTGA CTCCCCGTCG TGTAGATAAC TACGATACGG GAGGGCTTAC CATCTGGCCC
2221    CAGTGCTGCA ATGATACCGC GAGACCCACG CTCACCGGCT CCAGATTTAT CAGCAATAAA
2281    CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG TGGTCCTGCA ACTTTATCCG CCTCCATCCA
2341    GTCTATTAAT TGTTGCCGGG AAGCTAGAGT AAGTAGTTCG CCAGTTAATA GTTTGCGCAA
2401    CGTTGTTGCC ATTGCTACAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA TGGCTTCATT
2461    CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC CCCATGTTGT GCAAAAAAGC
2521    GGTTAGCTCC TTCGGTCCTC CGATCGTTGT CAGAAGTAAG TTGGCCGCAG TGTTATCACT
2581    CATGGTTATG GCAGCACTGC ATAATTCTCT TACTGTCATG CCATCCGTAA GATGCTTTTC
2641    TGTGACTGGT GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC GACCGAGTTG
2701    CTCTTGCCCG GCGTCAATAC GGGATAATAC CGCGCCACAT AGCAGAACTT TAAAAGTGCT
2761    CATCATTGGA AAACGTTCTT CGGGGCGAAA ACTCTCAAGG ATCTTACCGC TGTTGAGATC
2821    CAGTTCGATG TAACCCACTC GTGCACCCAA CTGATCTTCA GCATCTTTTA CTTTCACCAG
2881    CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC
2941    ACGGAAATGT TGAATACTCA TACTCTTCCT TTTTCAATAT TATTGAAGCA TTTATCAGGG
3001    TTATTGTCTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC AAATAGGGGT
3061    TCCGCGCACA TTTCCCCGAA AAGTGCCACC TGATGCGGTG TGAAATACCG CACAGATGCG
3121    TAAGGAGAAA ATACCGCATC AGGAAATTGT AAGCGTTAAT ATTTTGTTAA AATTCGCGTT
3181    AAATTTTTGT TAAATCAGCT CATTTTTTAA CCAATAGGCC GAAATCGGCA AAATCCCTTA
3241    TAAATCAAAA GAATAGACCG AGATAGGGTT GAGTGTTGTT CCAGTTTGGA ACAAGAGTCC
3301    ACTATTAAAG AACGTGGACT CCAACGTCAA AGGGCGAAAA ACCGTCTATC AGGGCGATGG
3361    CCCACTACGT GAACCATCAC CCTAATCAAG TTTTTTGGGG TCGAGGTGCC GTAAAGCACT
3421    AAATCGGAAC CCTAAAGGGA GCCCCCGATT TAGAGCTTGA CGGGGAAAGC CGGCGAACGT
3481    GGCGAGAAAG GAAGGGAAGA AAGCGAAAGG AGCGGGCGCT AGGGCGCTGG CAAGTGTAGC
3541    GGTCACGCTG CGCGTAACCA CCACACCCGC CGCGCTTAAT GCGCCGCTAC AGGGCGCGTC
3601    CATTCGCCAT TCAGGCTGCG CAACTGTTGG GAAGGGCGAT CGGTGCGGGC CTCTTCGCTA
3661    TTACGCCAGC TGGCGAAAGG GGGATGTGCT GCAAGGCGAT TAAGTTGGGT AACGCCAGGG
3721    TTTTCCCAGT CACGACGTTG TAAAACGACG GCCAGTGAAT TGTAATACGA CTCACTATA
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H2. pGEM+wtGag complete DNA sequence

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1       GGGCGAATTG GGCCCGACGT CGCATGCTCC CGGCCGCCAT GGCGGCCGCG GGAATTCGAT
61      TAAGCTTGAG ACAAGGGGTC GCTGCCAAAG AGTGATTTGA GGGAAGTTAA GGGTTCCCTT
121     TCTATCGGCT CCTGCTTCGG AGCGGGGGTT GTCTCTTCGA GCCTGAAGCT CTCTGCTGGT
181     GGGGCTGTTG GCTCTGGTCT GTTCTGAAGG AAATTCCCTG GCCTCCCCTT GTGGGAAGGC
241     CAAATTTTCC CTAAAAAATT AGCCTGCCTC TCAGTGCAGT CTTTCATTTG GTGTCCTTCT
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301    TTTCCACATT TCCAACAGCC TTTTTTCCTA GGGGCTCTGC AATTTCTGGC TATGTGCCCT
361    TCCTTGCCAC AGTTAAAACA TTTAACAATT CTTCTAGGGC CTTTAAAATT GCTTCTCTGC
421    ATCATTATGT TTCCACTGTT TGTTTGACTC ATTGCCTCAG CCAATACTCT TGCTTTGTGG
481    CCAGGTCCTC CCACCCCTTG ACATGCTGTC ATCATTTCTT CTAATGTAGC CCCTGGTCCC
541    AATGCTCTCA AAATGGTCTT ACAATCTGGG TTCGCATTTT GGACTAACAA GGTGTCTGTC
601    ATCCAATTTT TTACTTCTTG TGTAGCTTGT TCAGCTCTTA AAGTTTTAAA GAACCGATCT
661    ACATAGTCTC GAAAGGGTTC CTTTGGCCCT TGTCTTATGT CCAAAATGCT GACCGGGCTA
721    TACATTCTCA CTATTTTATT TAACCCCAGA ATTATCCATC TTTTATAGAT GTCTCCCACT
781    GGAATAGGTG GGTTACTTGT CATCCATGCT ATTTGTTCCT GAAGGGTACT AGTAGTTCCT
841    GCTATGTCAC TTCCCCTTGG TTCTCTCATC TGGCCTGGTG CAATAGGCCC CGCATGTACT
901    GGATGTAATC TATCCCATTC TGCAGCCTCT TCATTGATGG TATCTTTTAA CATTTGCATG
961    GCTGCTTGAT GTCCCCCCAC CGTATTTAAC ATGGTGTTTA AATCTTGTGG GGTGGCTCCT
1021   TCTGATAATG CTGTAAACAT GGGTATTACC TCTGGGCTAA AAGCCTTTTC CTCTATTACT
1081   TTTACCCATG CATTCAAGGT TCTAGGTGAT ATGGCTTGAT GTACCATTTG CCCTTGGAGA
1141   TTCTGCACTA TAGGATAATT TTGACTGACT TTCCCGTCAG CCGCTTTTGC CTGCTGCGTT
1201   TTTTGCTGAC ATTTGTTTTG TTCTTCCTCT ATCTTATCTA AGGCTTCCTT GGTGTCTCGT
1261   ACTTCTATCT TTTCATGTAC ACAATAGAGA GTTGCTACTG TGTCGTATAA TGATTTAAGT
1321   TCCTCTGTTC CTGTCTGGAG AGCTGGTTGT AGCTGTTTTA TTATTTGTTT ACATCCTTCT
1381   GATGTTTCTA AAAGGCCAGG GTTAAGTGCA AATCTTTCCA GCTCCCTGCT TGCCCATACT
1441   ATGTGTTTTA ACATATAATG TTTCTTTCCC CCTGGCCTTA ACCTAATTTT TTCCCATGTA
1501   TCTAATTTTT CCCCCCTTAA TATTGACGCT CTGGCGCCAT AATCACTAGT GAATTCGCGG
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1681   TTGTTATCCG CTCACAATTC CACACAACAT ACGAGCCGGA AGCATAAAGT GTAAAGCCTG
1741   GGGTGCCTAA TGAGTGAGCT AACTCACATT AATTGCGTTG CGCTCACTGC CCGCTTTCCA
1801   GTCGGGAAAC CTGTCGTGCC AGCTGCATTA ATGAATCGGC CAACGCGCGG GGAGAGGCGG
1861   TTTGCGTATT GGGCGCTCTT CCGCTTCCTC GCTCACTGAC TCGCTGCGCT CGGTCGTTCG
1921   GCTGCGGCGA GCGGTATCAG CTCACTCAAA GGCGGTAATA CGGTTATCCA CAGAATCAGG
1981   GGATAACGCA GGAAAGAACA TGTGAGCAAA AGGCCAGCAA AAGGCCAGGA ACCGTAAAAA
2041   GGCCGCGTTG CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCG
2101   ACGCTCAAGT CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC
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2221   CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCATAGCTCA CGCTGTAGGT ATCTCAGTTC
2281   GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCG
2341   CTGCGCCTTA TCCGGTAACT ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC
2401   ACTGGCAGCA GCCACTGGTA ACAGGATTAG CAGAGCGAGG TATGTAGGCG GTGCTACAGA
2461   GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGA ACAGTATTTG GTATCTGCGC
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2581   CACCGCTGGT AGCGGTGGTT TTTTTGTTTG CAAGCAGCAG ATTACGCGCA GAAAAAAAGG
2641   ATCTCAAGAA GATCCTTTGA TCTTTTCTAC GGGGTCTGAC GCTCAGTGGA ACGAAAACTC
2701   ACGTTAAGGG ATTTTGGTCA TGAGATTATC AAAAAGGATC TTCACCTAGA TCCTTTTAAA
2761   TTAAAAATGA AGTTTTAAAT CAATCTAAAG TATATATGAG TAAACTTGGT CTGACAGTTA
2821   CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT CTATTTCGTT CATCCATAGT
2881   TGCCTGACTC CCCGTCGTGT AGATAACTAC GATACGGGAG GGCTTACCAT CTGGCCCCAG
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3001   GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT TTATCCGCCT CCATCCAGTC
3061   TATTAATTGT TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA GTTAATAGTT TGCGCAACGT
3121   TGTTGCCATT GCTACAGGCA TCGTGGTGTC ACGCTCGTCG TTTGGTATGG CTTCATTCAG
3181   CTCCGGTTCC CAACGATCAA GGCGAGTTAC ATGATCCCCC ATGTTGTGCA AAAAAGCGGT
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3361   GACTGGTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT ATGCGGCGAC CGAGTTGCTC
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3481   CATTGGAAAA CGTTCTTCGG GGCGAAAACT CTCAAGGATC TTACCGCTGT TGAGATCCAG
3541   TTCGATGTAA CCCACTCGTG CACCCAACTG ATCTTCAGCA TCTTTTACTT TCACCAGCGT
3601   TTCTGGGTGA GCAAAAACAG GAAGGCAAAA TGCCGCAAAA AAGGGAATAA GGGCGACACG
3661   GAAATGTTGA ATACTCATAC TCTTCCTTTT TCAATATTAT TGAAGCATTT ATCAGGGTTA
3721   TTGTCTCATG AGCGGATACA TATTTGAATG TATTTAGAAA AATAAACAAA TAGGGGTTCC
3781   GCGCACATTT CCCCGAAAAG TGCCACCTGA TGCGGTGTGA AATACCGCAC AGATGCGTAA
3841   GGAGAAAATA CCGCATCAGG AAATTGTAAG CGTTAATATT TTGTTAAAAT TCGCGTTAAA
3901   TTTTTGTTAA ATCAGCTCAT TTTTTAACCA ATAGGCCGAA ATCGGCAAAA TCCCTTATAA
3961   ATCAAAAGAA TAGACCGAGA TAGGGTTGAG TGTTGTTCCA GTTTGGAACA AGAGTCCACT
4021   ATTAAAGAAC GTGGACTCCA ACGTCAAAGG GCGAAAAACC GTCTATCAGG GCGATGGCCC
4081   ACTACGTGAA CCATCACCCT AATCAAGTTT TTTGGGGTCG AGGTGCCGTA AAGCACTAAA
4141   TCGGAACCCT AAAGGGAGCC CCCGATTTAG AGCTTGACGG GGAAAGCCGG CGAACGTGGC
4201   GAGAAAGGAA GGGAAGAAAG CGAAAGGAGC GGGCGCTAGG GCGCTGGCAA GTGTAGCGGT
4261   CACGCTGCGC GTAACCACCA CACCCGCCGC GCTTAATGCG CCGCTACAGG GCGCGTCCAT
4321   TCGCCATTCA GGCTGCGCAA CTGTTGGGAA GGGCGATCGG TGCGGGCCTC TTCGCTATTA
4381   CGCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA GTTGGGTAAC GCCAGGGTTT
4441   TCCCAGTCAC GACGTTGTAA AACGACGGCC AGTGAATTGT AATACGACTC ACTATA
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H3. pGEM+Salmgag complete DNA sequence

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1      GGGCGAATTG GGCCCGACGT CGCATGCTCC CGGCCGCCAT GGCGGCCGCG GGAATTCGAT
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121    TCAATCGGTT CCTGTTTCGG GGCCGGGGTG GTTTCTTCCA GGCGAAATGA TTCCGCCGGC
181    GGTGCGGTCG GTTCCGGGCG GTTCTGCAGA AAATTGCCCG GGCGGCCTTT GTGGCTCGGC
241    CAAATTTTGC CCAGAAAATT GGCCTGGCGT TCGGTGCAAT CTTTCATCTG GTGGCCCTCT
301    TTGCCGCATT TCCAACAGCC TTTTTTGCGC GGTGCGCGGC AATTGTGGCC AATGTGGCCT
361    TCTTTACCAC AGTTAAAACA TTTCACAATG CGACGCGGGC CTTTAAAGTT GCTGCGCTGC
421    ATCATGATGT TGCCGCTGTT GGTCTGGCTC ATCGCTTCCG CCAGAACGCG CGCTTTGTGG
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481   CCCGGACCGC CCACGCCCTG GCAGGCGGTC ATCATTTCTT CCAGGGTCGC ACCCGGGCCC
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601   ATCCAATTTT TGACTTCCTG GGTCGCCTGT TCCGCGCGCA GGGTTTTAAA AAAGCGATCG
661   ACATAATCGC GAAACGGTTC TTTCGGGCCC TGGCGAATAT CCAGAATGCT GACCGGGCTA
721   TACATGCGCA CGATTTTATT CAGGCCCAGA ATGATCCAGC GTTTATAGAT ATCGCCCACC
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901   GGATGCAGGC GATCCCATTC CGCCGCTTCT TCATTGATGG TATCTTTCAG CATCTGCATG
961   GCCGCCTGAT GGCCGCCCAC CGTATTCAGC ATGGTGTTCA GATCCTGCGG GGTCGCGCCT
1021  TCGCTCAGCG CGGTAAACAT CGGAATGACT TCCGGGCTAA ACGCTTTTTC TTCGATCACT
1081  TTCACCCACG CATTCAGGGT GCGCGGGCTA ATGGCCTGAT GAACCATCTG GCCCTGCAGA
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1501  TCCAGTTTTT CGCCGCGCAG AATGCTCGCG CGGGCGCCAT AATCACTAGT GAATTCGCGG
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1621  TCTATAGTGT CACCTAAATA GCTTGGCGTA ATCATGGTCA TAGCTGTTTC CTGTGTGAAA
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1981  GGATAACGCA GGAAAGAACA TGTGAGCAAA AGGCCAGCAA AAGGCCAGGA ACCGTAAAAA
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2221  CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCATAGCTCA CGCTGTAGGT ATCTCAGTTC
2281  GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCG
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3001  GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT TTATCCGCCT CCATCCAGTC
3061  TATTAATTGT TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA GTTAATAGTT TGCGCAACGT
3121  TGTTGCCATT GCTACAGGCA TCGTGGTGTC ACGCTCGTCG TTTGGTATGG CTTCATTCAG
3181  CTCCGGTTCC CAACGATCAA GGCGAGTTAC ATGATCCCCC ATGTTGTGCA AAAAAGCGGT
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3721  TTGTCTCATG AGCGGATACA TATTTGAATG TATTTAGAAA AATAAACAAA TAGGGGTTCC
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4201  GAGAAAGGAA GGGAAGAAAG CGAAAGGAGC GGGCGCTAGG GCGCTGGCAA GTGTAGCGGT
4261  CACGCTGCGC GTAACCACCA CACCCGCCGC GCTTAATGCG CCGCTACAGG GCGCGTCCAT
4321  TCGCCATTCA GGCTGCGCAA CTGTTGGGAA GGGCGATCGG TGCGGGCCTC TTCGCTATTA
4381  CGCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA GTTGGGTAAC GCCAGGGTTT
4441  TCCCAGTCAC GACGTTGTAA AACGACGGCC AGTGAATTGT AATACGACTC ACTATA
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H4. pGEM+P24D complete DNA sequence

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121   GACCGGGCTA TACATGCGCA CGATTTTATT CAGGCCCAGA ATGATCCAGC GTTTATAGAT
181   ATCGCCCACC GGAATCGGCG GGTTGCTGGT CATCCAGGCA ATCTGTTCCT GCAGGGTGCT
241   CGTGGTGCCC GCAATATCGC TGCCGCGCGG TTCGCGCATC TGGCCCGGCG CAATCGGGCC
301   CGCATGAACC GGATGCAGGC GATCCCATTC CGCCGCTTCT TCATTGATGG TATCTTTCAG
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421   GGTCGCGCCT TCGCTCAGCG CGGTAAACAT CGGAATGACT TCCGGGCTAA ACGCTTTTTC
481   TTCGATCACT TTCACCCACG CATTCAGGGT GCGCGGGCTA ATGGCCTGAT GAACCATCTG
541   GCCCTGCAGA TTCTGCACGA TCGAATCACT AGTGAATTCG CGGCCGCCTG CAGGTCGACC
601   ATATGGGAGA GCTCCCAACG CGTTGGATGC ATAGCTTGAG TATTCTATAG TGTCACCTAA
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661     ATAGCTTGGC GTAATCATGG TCATAGCTGT TTCCTGTGTG AAATTGTTAT CCGCTCACAA
721     TTCCACACAA CATACGAGCC GGAAGCATAA AGTGTAAAGC CTGGGGTGCC TAATGAGTGA
781     GCTAACTCAC ATTAATTGCG TTGCGCTCAC TGCCCGCTTT CCAGTCGGGA AACCTGTCGT
841     GCCAGCTGCA TTAATGAATC GGCCAACGCG CGGGGAGAGG CGGTTTGCGT ATTGGGCGCT
901     CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT TCGGCTGCGG CGAGCGGTAT
961     CAGCTCACTC AAAGGCGGTA ATACGGTTAT CCACAGAATC AGGGGATAAC GCAGGAAAGA
1021    ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT
1081    TTTTCCATAG GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT
1141    GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC TCCCTCGTGC
1201    GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC CGCCTTTCTC CCTTCGGGAA
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1381    ACTATCGTCT TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA GCAGCCACTG
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3241    AAGCGAAAGG AGCGGGCGCT AGGGCGCTGG CAAGTGTAGC GGTCACGCTG CGCGTAACCA
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3361    CAACTGTTGG GAAGGGCGAT CGGTGCGGGC CTCTTCGCTA TTACGCCAGC TGGCGAAAGG
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3481    TAAAACGACG GCCAGTGAAT TGTAATACGA CTCACTATA
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H5. pGEM+P1724 complete DNA sequence

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781     GCCATCCGCC GCTTTCGCCT GCTGCGTTTT CTGCTGACAT TTGTTCTGTT CTTCTTCGAT
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1021    GCGTTCCAGT TCGCGGCTCG CCCAAACGAT GTGTTTCAGC ATATAATGTT TTTTGCCACC
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1681    TCCGCCCCCC TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA
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2581    CACCGGCTCC AGATTTATCA GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG
2641    GTCCTGCAAC TTTATCCGCC TCCATCCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA
2701    GTAGTTCGCC AGTTAATAGT TTGCGCAACG TTGTTGCCAT TGCTACAGGC ATCGTGGTGT
2761    CACGCTCGTC GTTTGGTATG GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA
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3841    CGGGCGCTAG GGCGCTGGCA AGTGTAGCGG TCACGCTGCG CGTAACCACC ACACCCGCCG
3901    CGCTTAATGC GCCGCTACAG GGCGCGTCCA TTCGCCATTC AGGCTGCGCA ACTGTTGGGA
3961    AGGGCGATCG GTGCGGGCCT CTTCGCTATT ACGCCAGCTG GCGAAAGGGG GATGTGCTGC
4021    AAGGCGATTA AGTTGGGTAA CGCCAGGGTT TTCCCAGTCA CGACGTTGTA AAACGACGGC
4081    CAGTGAATTG TAATACGACT CACTATA
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H6. pGEM+Salmgag-GFP complete DNA sequence

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841     GATTTCAGGC TGGTCAGCGG TTCGCGTTCA ATCGGTTCCT GTTTCGGGGC CGGGGTGGTT
901     TCTTCCAGGC GAAATGATTC CGCCGGCGGT GCGGTCGGTT CCGGGCGGTT CTGCAGAAAA
961     TTGCCCGGGC GGCCTTTGTG GCTCGGCCAA ATTTTGCCCA GAAAATTGGC CTGGCGTTCG
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5161  CGATTAAGTT GGGTAACGCC AGGGTTTTCC CAGTCACGAC GTTGTAAAAC GACGGCCAGT
5221  GAATTGTAAT ACGACTCACT ATA
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APPENDIX I: RESTRICTION ENZYMES USED IN THIS STUDY

Enzyme	Recognition sequence	Source
*Eco*RI	G↓AATTC	Roche SA
*Hind*III	A↓AGCTT	Roche SA
*Nar*I	GG↓CGCC	Roche SA
*Bbe*I	GGCGC↓C	Roche SA
*Sfo*I	GGC↓GCC	New England Labs
*Cla*I	AT↓CGAT	Roche SA
*Nco*I	C↓CATGG	Roche SA
*Eco*RV	GAT↓ATC	Roche SA
*Nde*I	CA↓TATG	Roche SA

*The arrow (↓) indicates the recognition site

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