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Attenuated *Salmonella typhimurium* can be used to transduce human dendritic cells towards vaccination against HIV-1

A thesis presented to the faculty of
The Rockefeller University
in partial fulfillment of the requirements for
the degree of doctor of Philosophy

by

Deborah Gurner

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For my parents

In memory of my brother, Samuel Gurner (1967 – 1995),
who would have found this all pretty cool

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Abbreviations

AIDS: acquired immune deficiency syndrome

AP: alkaline phosphatase

APC: antigen-presenting cell

BMDC: bone marrow-derived dendritic cell (DC)

BSA: bovine serum albumin

CMV: cytomegalovirus

CTL: cytotoxic T lymphocyte

DC: dendritic cell

DEC-205: dendritic and epithelial cells, 205 kDa (glycoprotein)

DNA: deoxyribonucleic acid

ELISA: enzyme-linked immuno-sorbent assay

ELISpot: enzyme-linked immuno-spot

ER: endoplasmic reticulum

FCS: fetal calf serum

FDG: fluoroscein di-β-D-galactopyranoside

Flt-3L: Fms-like tyrosine kinase receptor-3 ligand

GFP: green fluorescent protein

GM-CSF: granulocyte-macrophage colony-stimulating factor

HAV: hepatitis A virus

HBSS: Hanks' balanced salt solution

HBV: hepatitis B virus

Hib: *Haemophilus influenzae* type b

HIV: human immunodeficiency virus

HRP: horseradish peroxidase

ICS: intracellular cytokine staining

IFN: interferon

IL: interleukin

IM: intramuscular

IN: intranasal

IP: intraperitoneal

LAMP: lysosomal-associated membrane protein

LB: Luria Bertani (broth)

LPS: lipo-polysaccharide

LTNP: long-term non-progressor

M1: matrix 1 (influenza protein)

MAGI: multinuclear-activation-of-a-galactosidase-indicator (assay)

MHC: major histocompatibility complex

MLN: mesenteric lymph node

MOI: multiplicity of infection

mRNA: messenger ribonucleic acid (RNA)

NK: natural killer

NRAMP: natural resistance-associated membrane protein

OD: optical density

OVA: ovalbumin

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered solution

PCR: polymerase chain reaction

PO: *per os*

PP: Peyer's patch

RNA: ribonucleic acid

RPMI: Roswell Park Memorial Institute (medium)

RT-PCR: reverse transcriptase polymerase chain reaction (PCR)

SC: subcutaneous

SCV: *Salmonella*-containing vacuole

SEB: staphylococcal enterotoxin B

SFC: spot-forming cell

Sif: *Salmonella*-induced filament

SHIV: simian-human immunodeficiency virus

SIV: simian immunodeficiency virus

SPI: *Salmonella* Pathogenicity Island

TAP: transporter-associated-with-antigen-presentation

TLR: toll-like receptor

TNF: tumor necrosis factor

TTSS: Type Three Secretion System

Abstract

The promise of DNA vaccination is limited in part by invasive, untargeted administration. We refined the strategy by incorporating attenuated bacteria to deliver plasmids directly to APCs.

Initial studies involved human macrophages, which were infected with aroA-*Salmonella typhimurium* transformed with eukaryotic vectors encoding β-galactosidase. Results of staining with x-gal demonstrated effective transfer of plasmids to the target cells, which expressed the reporter gene themselves. Similarly, immunocytochemical staining of macrophages infected with bacteria bearing a eukaryotic vector encoding HIV-1 *env* showed expression of the viral gene.

Macrophages were also infected with bacteria harboring vectors encoding SIV *env* and HIV-1 *tat*, then subjected to lysis, RNA extraction and RT-PCR. In this way, conceptual proof of the system was achieved at the level of transcription. Indeed, in the latter case, we recovered a splice product, which specifically confirms eukaryotic expression.

Further studies involved human DCs, cultivated from PBMCs *in vitro*. We first used flow cytometry to show that infection with *Salmonella* induces maturation, a state ultimately necessary for immunogenicity. Once again, bacteria carrying β-galactosidase vectors were used initially. DCs were also infected with bacteria carrying a vector encoding HIV-1 *gag*, then stained for expression of the viral protein. Both immunocytochemistry and flow cytometry confirmed Gag expression.

Finally, transduced DCs were co-cultivated with autologous T-cells in studies aimed at detecting interferon-γ expression as a gauge of antigen presentation. Bacteria were first transformed with eukaryotic vectors encoding influenza M1 protein. PBMCs were therefore obtained from donors known to have memory responses to influenza. Co-cultures yielded significant T-cell responses (by ELISpot and flow cytometry) to DCs transduced to express M1. We next obtained PBMCs from HIV-1-infected donors, setting up co-cultures involving DCs infected with bacteria carrying HIV-1 *gag* vector. Again, significant T-cell responses were detected when DCs were transduced to express the viral protein using *Salmonella*.

Thus, using attenuated *S. typhimurium*, we can deliver vectors encoding foreign antigens directly to human APCs. DCs transduced to express viral proteins can present antigen to autologous T-cells *in vitro*. As a vaccine tool, this strategy represents a potential way to achieve non-invasive, targeted delivery of DNA vaccines.

Chapter I:

Introduction

THE QUEST FOR A VACCINE AGAINST HIV-1

Despite over two decades of effort against it, the global HIV-1 epidemic continues to plague humanity (1). In the face of such an unprecedented medical challenge, the scientific community has made important advances in virology, immunology and pharmacology. Nonetheless, it has proven extremely difficult both to contain the spread of infection around the world, and to prevent disease progression in most infected individuals.

As a retrovirus, HIV-1 presents us with unique obstacles to vaccine design (2, 3). Unlike other viral pathogens, for example, HIV-1 integrates into the host cell genome and therefore remains hidden from the immune system as latent proviral DNA. Furthermore, viral particles are camouflaged by their heavily glycosylated envelopes. Those proteins that are effectively exposed as potential immune targets are highly variable. Indeed, HIV-1 has a substantial degree of genetic diversity, as well as antigenic heterogeneity. Additionally, the virus employs mechanisms towards immune evasion, including the shedding of "decoy" monomeric envelope proteins and the induction of MHC downregulation. Finally, and perhaps most key for viral persistence within the host, HIV-1 targets the very cells of the immune system that would otherwise be its foil: A definitive hallmark of AIDS pathogenesis is the destruction of CD4+ T-cells.

Apart from fundamental problems posed by the nature of the virus itself, we as scientists also face practical challenges imposed by the limitations in our own knowledge and technology. An accurate animal model for AIDS and realistic challenge virus are not available, for example, despite the significant utility of rhesus macaques and SIV/SHIV (4). More importantly, we have not yet defined the human responses that confer protection from HIV-1 disease: What are the immune correlates of protection in so-called "long-term non-progressors (LTNPs)" and exposed but uninfected individuals? After 20 years of careful scrutiny of these rare cases, we do not have an unequivocal answer. (5).

When HIV-1 was first identified, our understanding of immune responses was in fact relatively poor. Thus, early attempts to design a vaccine against the virus were, in retrospect, misguided, and certainly informed by empiricism above all else (6). More recent vaccine candidates represent technologies that have emerged only since the AIDS epidemic began (2). Despite reason for hope, we remain confronted by the essential question of whether an effective vaccine will be possible at all (6). In the case of previous viral pathogens, in contrast to HIV-1, it was always clear to vaccinologists that people who survived acute infection would go on to be immune to subsequent threat from the same virus. With very few examples of natural immunity to HIV-1, we lack absolute indicators to guide us in vaccine development (7). Furthermore, we must accept that sterilizing immunity against HIV-1 may never be possible (8). Rather, it is likely that the general goal of prophylactic immunization

should be to prime immune responses that will allow the host to contain the pathogen after initial infection (7).

Approaches to HIV-1 vaccine design, therefore, have been necessarily creative. Established strategies such as live attenuated viruses, inactivated viruses and subunit vaccines have proven inadequate or unsafe; thus, newer, innovative technologies continue to be actively investigated (2, 6-8). These experimental vaccines include live recombinant vectors (pox viruses, adenoviruses, etc.) and DNA plasmids. As a prime-boost regimen, in fact, the combination of these two novel approaches has proven especially hopeful (2, 7).

In and of itself, however, DNA vaccination seems a whimsical and unlikely strategy (9). Nonetheless, its great simplicity, practicality and potential for versatility have inspired a persistent effort to develop the technique for clinical use. Despite some promise of *in vivo* efficacy, there continues to be much speculation as to precise mechanism of action. Most studies suggest that injected vaccine plasmids are received and expressed by non-lymphoid tissue with subsequent antigen transfer to professional antigen-presenting cells (10-12). Limitations of the approach include invasive administration, untargeted delivery and only modest levels of *in situ* protein expression. Many groups have therefore undertaken to refine the strategy towards enhancing DNA vaccine-elicited immune responses (13, 14).

SALMONELLA AS A VACCINE TOOL

Despite the predominant tendency for infectious pathogens to be transmitted mucosally, most vaccines approved for human use in the United States must be administered invasively (Table 1). Indeed, since the discontinuation of the oral (Sabin) polio vaccine in 2000 (15), the only oral vaccine approved for use in the U.S. is live attenuated *Salmonella typhi*, which works against typhoid fever. Among the few newer vaccines that have been approved in recent years, there is one other mucosally-administered preparation: a live attenuated influenza virus for intranasal use. *Salmonella*, however, is the only live bacteria used as a vaccine (though *Vibrio* and *Mycobacteria* are employed in other parts of the world).

Perhaps, then, it is in part because *Salmonella spp.* were already in place as clinical agents that several groups undertook to explore their potential use as tools of vaccination against other pathogens. Attenuated *Salmonella* has, for example, been engineered to express heterologous antigens from HIV, SIV, influenza, *Listeria*, pertussis toxin and cholera toxin (16-25). It is clear, however, that serving as *in situ* protein-expression vectors is not the bacteria's greatest strength in this context. Rather, it is more important to consider the advantages of exploiting *Salmonella* as vaccine-delivery vehicles.

The need for effective new vaccine adjuvants for use in strategies against HIV-1 is well-established (26), and there is particular interest in the development of

TARGET	TYPE	ROUTE
diphtheria/tetanus	subunit	IM
meningococcus	subunit	IM
yellow fever virus	live attenuated	SC
Salmonella typhi (typhoid fever)	live attenuated	PO
rabies virus	inactivated	IM
hepatitis B virus (HBV)	subunit	IM
hepatitis A virus (HAV)	inactivated	IM
diphtheria/tetanus/pertussis	subunit	IM
diphtheria/tetanus/pertussis/HBV/poliovirus	subunit/inactivated	IM
HAV/HBV	subunit/inactivated	IM
measles	live attenuated	SC
Haemophilus influenzae type b (Hib)/meningococcus/HBV	subunit	IM
measles/mumps/rubella	live attenuated	SC
rubella	live attenuated	SC
mumps	live attenuated	SC
Hib/meningococcus	subunit	IM
pneumococcus	subunit	IM/SC
HBV	subunit	IM
HAV	inactivated	IM
Varicella	live attenuated	SC
influenza	live attenuated	IN
Hib	subunit	IM
pneumococcus	subunit	IM
influenza	subunit	IM

Table 1 Vaccines currently in use in the United States Source: *PDR*, 58th edition (2004) (27) IM=intramuscular, SC=subcutaneous, PO=*per os* (oral), IN=intranasal.

mucosal adjuvants (28-30). An adjuvant may enhance the efficacy of a vaccine in more than one way, including by modifying the micro-environment to render it more receptive to the immunogen (e.g., by serving as a chemoattractant for relevant cells), or by facilitating and targeting delivery of the vaccine itself. *Salmonella* as a vaccine carrier would therefore be functional as its own adjuvant on both levels. Lymphotropic, intracellular pathogens, the bacteria naturally home to gut-associated lymphoid tissue, where their endotoxin and invasive behavior make them a provocative immune stimulus *in situ*. In fact, other bacterial antigens (including cholera toxin and *E. coli* heat-labile enterotoxin) are widely used experimentally in animals as mucosal adjuvants, but generally deemed too toxic for human use (30). Importantly, *Salmonella* would also effectively overcome the baseline tolerogenic response of the gut towards antigen received into the lumen (31). Administration, like that of the existent mucosal vaccine against typhoid fever, would mimic natural *Salmonella* transmission through the oral route.

The current project, then, represents an attempt to build on the idea of using attenuated *Salmonella* as a vaccine vector by using the bacteria instead as vaccine vehicles. As an experimental approach, conventional DNA vaccination offers no possibility for non-invasive administration or guided delivery. Furthermore, the generation of mucosal immunity is very unlikely with direct intramuscular or subcutaneous injection of plasmids. If, however, we use *Salmonella* to carry and deliver our DNA vaccine vectors, we may circumvent these limitations, and further

enhance immunogenicity with built-in bacterial adjuvanticity. First, however, we must confirm the conceptual design of this model and evaluate its efficacy *in vitro*.

Chapter II:

Materials and Methods

BACTERIA

Growth

The attenuated *Salmonella typhimurium* aroA- strains 33275 and 14028 were kindly provided by Stanley Lin (Vion Pharmaceuticals, Inc., New Haven, CT). Bacteria were cultivated on solid and in liquid LB medium supplemented with antibiotics (tetracycline, ampicillin or kanamycin) 50-100μg per mL, as appropriate. Extended storage was in 10% glycerol solution at -70°C.

Transformation

Salmonella were grown to logarithmic phase then rendered electro-competent by chilling on ice and washing three times with sterile H_2O. Bacteria were then concentrated to $5.0 \times 10^{10} - 1.0 \times 10^{11}$/mL for transformation. Electroporation was carried out using the Gene Pulser II (Bio-Rad Laboratories, Inc., Hercules, CA) with cuvette gap 0.1cm, 1.8kV, 25μFD, 200Ω and a time constant of 4-6msec. Each reaction involved 50μL of bacterial solution and 0.5 – 2.0μg DNA. Following electroporation, bacteria were grown for 1 hour in SOC medium before being plated.

Quantification of bacterial density

Correlation of optical density (OD) and *Salmonella* concentration was achieved by serial dilutions and plating on solid medium. It was thereby concluded that an OD_{600} of 0.8 is approximately equivalent to 2×10^8 bacteria per mL.

Quantification of plasmid content

Salmonella transformed with plasmids of various sizes were grown to an OD_{600} of 0.8. The QIAprep Spin Miniprep Kit (Qiagen, Inc., Valencia, CA) was then used to extract extra-genomic DNA from bacterial solutions of different volumes. An OD_{260} of 1.0 was considered equivalent to 50μg/mL, and 1μg of 1000bp of DNA was equated with 9.1×10^{11} molecules (as per New England Biolabs, Beverly, MA). In this way, it was determined that each transformed bacterium carries 300-900 plasmids.

Infectious protocols

Salmonella were grown overnight at 37°C, then diluted ~1:20 and grown another 3-4 hours to desired OD. Cells to be infected were washed and incubated with antibiotic-free medium (RPMI 1640 supplemented with serum, L-glutamine and Hepes buffer). (Macrophages were left in their original wells in 6-well plates; dendritic cells were ultimately seeded at 2×10^6/500μL into Eppendorf® 1.5mL

tubes.) Cells were infected with the bacteria at desired multiplicities of infection, then incubated at 37°C for 1 hour. Post-infection, cells were washed 2-3 times with gentamicin-containing buffer (PBS or HBSS), then incubated in fresh culture medium as described below. (Dendritic cells were re-plated in medium containing cytokines at 10^6/2mL of 12-well plates.) When tetracycline-sensitive *Salmonella* (33275) were used, 10μg of tetracycline was added to each well.

CELL CULTURE

Human blood samples

The New York Blood Center supplied "Leukopacks" derived from anonymous donors. Whole blood samples were obtained from consenting adult volunteers, some of whom are part of a cohort of HIV-infected patients seen at the Rockefeller University Hospital outpatient clinic under the supervision of Dr. Martin Markowitz.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated as a buffy coat from human blood samples (or packed leukocytes) using Ficoll-Hypaque gradient (Robbins Scientific, Sunnyvale, CA).

Human macrophage cultivation

PBMCs were washed with HBSS and seeded at ~3 x 10^7 cells/well of 6-well plates in RPMI 1640 supplemented with 1% penicillin-streptomycin, 1% L-glutamine, 1% Hepes buffer and 5% heat-inactivated autologous serum. Medium was changed every two days. Adherent cells were infected as above on day 8 and assayed in the same wells.

Human dendritic cell cultivation

PBMCs were subjected to CD14+ selection using magnetic beads and columns in accordance with the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). CD14+ cells were then seeded at 2 x 10^6/well of 6-well plates in RPMI 1640 supplemented with 1% penicillin-streptomycin, 1% L-glutamine, 1% Hepes buffer, 1% heat-inactivated autologous serum (or 5% type AB human serum (Sigma, St. Louis, MO)), recombinant granulocyte-macrophage colony stimulating factor (100IU/mL; Leukine, Immunex, Seattle, WA) and recombinant IL-4 (20ng/mL; R&D Systems, Minneapolis, MN). Additional cytokines were added to the cultures at the same concentration every 2 days. Immature dendritic cells were infected as above on day 6-8. Mature dendritic cells were obtained by adding IL-1β, TNF-α and IL-6 (R&D Systems, Minneapolis, MN) at 10ng/mL, and prostaglandin E_2 (Sigma, St. Louis, MO) at 1μg/mL.

Dendritic cell/T-cell co-cultivation

After CD14+ selection, CD14- cells were cryopreserved in freezing medium (Gibco, Grand Island, NY) to be used as a source of autologous bulk T-cells once dendritic cell cultivation was complete. Cells were then defrosted and seeded into the wells of a 96-well plate at 2 x 10^5/100μL (in early studies) or into the wells of a 48-well plate at 1.2 x 10^6/500μL (in later studies).

Just after peptide pulsing or influenza infection, or approximately 40 hours after *Salmonella* infection, dendritic cells were added to achieve the desired ratio (1:30 in early studies, with final volume of 200μL in wells of 96-well plates; or 1:60/1:30 in later studies, with final volume of 1mL in wells of 48-well plates). Culture medium used in DC/T-cell co-cultures was RPMI 1640 supplemented with 1% penicillin-streptomycin, 1% L-glutamine, 1% Hepes buffer and 5% type AB human serum (Sigma, St. Louis, MO).

Cultivation of murine dendritic cells from bone marrow

Marrow from the femurs and tibias of mice were harvested by flushing the epiphyseal and medullary cavities with RPMI 1640 using a 3cc syringe fitted with a 25ga x 5/8" needle (BD, Rutherford, NJ). One volume of 1.66% aqueous NH_4Cl was added, followed by incubation on ice for 5 minutes. Antibodies to CD4 (TIB 207, clone GK 1.5, ATCC, Rockville, MD), CD8 (TIB 150, clone HO2.2, ATCC),

CD45RB (B220 isoform; TIB 146, clone RA3-3A 1/6.1, ATCC) and MHC II (TIB 229, clone B21-2, ATCC) were added (as hybridoma supernatants, at a 1:20 dilution) to encourage complement-mediated lysis of differentiated lymphocytes and class II MHC+ cells. Rabbit complement (Pel-Freez Clinical Division, Rogers, AR) was also added at a 1:15 dilution, and cells were incubated for 1 hour at 37°C. After washing, cells were plated in 24-well plates at ~1 x 10^6/well. Medium used was RPMI 1640 supplemented with L-glutamine, 5% heat-inactivated FCS (Gemini Bioproducts, Calabasas, CA), 1% Hepes buffer, 20μg/mL gentamicin, 50μM 2-mercaptoethanol and 666u/mL of recombinant murine granulocyte-macrophage colony stimulating factor (Kirin Brewery Co., Maebashi, Gunma, Japan). Medium was changed every two days.

ASSAYS

β-galactosidase detection with 5-bromo-4-chloro-3-indolyl galactopyranoside (x-gal)

Cells to be stained (macrophages or dendritic cells) were incubated with 1% formaldehyde and 0.2% glutaraldehyde in PBS for 5 minutes at room temperature, then washed twice with PBS. Staining for β-galactosidase expression was achieved by then incubating with 4mM potassium ferrocyanide, 4mM potassium ferricyanide, 1mM magnesium chloride, and 0.4mg/mL x-gal in PBS for 1 hour at 37°C in a non-CO_2 incubator. Staining was stopped by replacing the solution with PBS.

Macrophages were kept in their wells for this procedure. Dendritic cells were either kept in wells, or affixed to a slide using a "cytospin" technique. This preparation was achieved using "Cytofunnels" and a Cytospin II centrifuge (Shandon, Pittsburgh, PA). Briefly, $1.0 \times 10^4 - 1.0 \times 10^5$ cells in 100-150μL were spun onto glass slides at 900rpm for 3 minutes. The slides were air-dried for at least 1 hour, then fixed (stabilized) in 100% acetone for 10 minutes before being air-dried once again.

Immunocytochemistry

Cells were stained using the Vectastain® ABC-AP Kit (Vector Laboratories, Burlingame, CA). Briefly, cells (either in wells or on slides) were first incubated with blocking (goat) serum for 20 minutes, then with primary antibody (1:500 dilution of HIV-infected patient's serum) for 30 minutes. Cells were then washed 5-10 times with PBS, and incubated for 30 minutes with biotinylated secondary (goat anti-human) antibody. Finally, cells were incubated with the Vectastain® ABC-AP Reagent (avidin-biotinylated enzyme complex) for 30 minutes, then washed and incubated 20-30 minutes with an alkaline phosphatase substrate (Vector® Red).

Flow cytometry

β-galactosidase activity in dendritic cells was detected with the DetectaGene™ Green CMFDG lacZ Gene Expression Kit (Molecular Probes, Eugene

OR). Briefly, cells were hypertonically loaded with fluoroscein di-β-D-galactopyranoside (FDG), a fluorescent substrate of the enzyme. Additionally, chloroquine was added (to raise lysosomal pH, thereby inhibiting endogenous β-galactosidase activity), as well as propidium iodide (to identify and exclude dead cells) and verapamil (to enhance signal by blocking efflux of fluorescent reaction product).

All antibodies used in interferon-γ assays were obtained from BD-Pharmingen (San Diego, CA) at dilutions recommended by the supplier. Cells were collected from DC/T-cell co-cultures, pelleted and re-suspended in 1mL of medium in 5mL polypropylene tubes. In studies using cells derived from HIV-infected patients, the co-stimulatory antibodies αCD28 and αCD49d were added (0.5μL/mL), as well as relevant peptides or the superantigen staphylococcal enterotoxin B (SEB) (Sigma, St. Louis, MO) as a positive control for the assay. The cells were then incubated for 6 hours in the presence of 10μg/mL brefeldin A (Sigma, St. Louis, MO) at 37°C with tubes at a 5° angle. At the conclusion of this incubation, 2μL EDTA was added to each tube, followed by a 15 minute incubation at room temperature. Cells were then lysed by the addition of FACS Lysing Solution (BD-Biosciences, San Diego, CA) followed by another 15 minute room temperature incubation. Permeabilization was achieved by re-suspension and washing with 0.1% saponin and 0.1% bovine serum albumin in PBS. Staining with relevant antibodies was carried out at room temperature for 45 minutes, before washing with the permeabilization solution and fixation with 1% paraformaldehyde in PBS. Flow cytometry was performed using a

FACS Calibur instrument, and flow cytometric analysis using CELL-Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

ELISpot

MultiscreenHTS Filter Plates (Millipore, Billerica, MA) were used for both human and mouse assays. For the human interferon-γ ELISpot, plates were coated with anti-human-interferon-γ 1-D1K capture antibody (Diapharma, Mabtech, Stockholm, Sweden) at a 1:100 dilution in 0.1M $NaHCO_3$ buffer (pH=9.5), 100μL per well. For the mouse interferon-γ ELISpot, anti-mouse interferon-γ antibody (R&D Systems, Inc., Minneapolis, MN) was diluted 1:40 in 0.1M $NaHCO_3$ buffer, 100μL/well. Plates were then washed 4 times with 0.05% Tween 20 in PBS, then blocked with 10% FCS/RPMI 1640 (mouse assay) or 5% human serum/RPMI 1640 (human assay), 100μL/well for 1-2 hours at 37°C. Blocking medium was then removed, and wells were seeded with cells, 150-200 μL total volume per well (0.5-1.0 x 10^6 cells [e.g., splenocytes]/well for mouse assay; 1.0-2.0 x 10^5 CD14- T-cells for human assays, with the addition of dendritic cells for varying ratios). Plates were then incubated overnight at 37°C to allow for antibody capture. On the following day, plates were washed 4-5 times with 0.05% Tween 20/PBS, then incubated with detection antibody. For the human assay, biotinylated antibody (7-B6-1-biotin; Diapharma, Mabtech, Stockholm, Sweden) was diluted 1:1000 in PBS, then added 50μL/well; the mouse antibody (R&D Systems, Inc., Minneapolis, MN) was diluted 1:60 in 1.0% BSA/PBS and added 100μL/well. Human assays were then incubated at

37°C for 2 hours; mouse assays were incubated at this stage overnight at 4°C. Both types of assay were next washed again 4 times with 0.05% Tween 20/PBS, then developed using a blue color Development Module (R&D Systems, Inc., Minneapolis, MN). ELISpots were read using a plate-reader and ImmunoSpot® Software (Cellular Technologies Ltd., Cleveland, OH).

Peptides

A complete set of consensus subtype B HIV-1 Gag was obtained from the NIH AIDS Research & Reference Reagent Program as 15-mers overlapping by 11. Lyophilized protein was solubilized and pooled, to be ultimately used at 2μg/mL. In co-cultures involving cells from HIV-infected donors, dendritic cells used for positive control were first incubated with the Gag peptide pool for 1 hour at 37°C (in plain RPMI 1640), then seeded into wells already containing autologous CD14- T-cells. Additionally, for re-stimulation prior to intracellular cytokine staining and flow cytometry, Gag pool was added directly to co-culture cells and incubated with brefeldin A for 6 hours at 37°C as previously described.

Influenza virus

Purified human influenza virus (X:31, A/Aichi/68 [H3N2]) was obtained from Charles River Laboratories (North Franklin, CT). For interferon-γ ELISpot and

intracellular cytokine staining assays to detect memory T-cell responses to influenza matrix protein (M1), influenza-infected autologous dendritic cells provided both the positive control (in co-culture) and the source of re-stimulation (for all co-culture categories). Cells were thus infected at a multiplicity of infection approximately equal to 2 in RPMI 1640 at 37°C for 1 hour.

PLASMIDS

pTrc-β and pSLICK-z

β-galactosidase vectors were kindly provided by Stanley Lin (Vion Pharmaceuticals, Inc., New Haven, CT). pTrc-β encodes the reporter gene under the control of a prokaryotic Trp-lac hybrid promoter; pSLICK-z encodes the gene under a CMV promoter. Both plasmids also code for ampicillin resistance.

pVAX1/HIV*gag*

pVAX1/HIV*gag* was kindly provided by Yaoxing Huang (Aaron Diamond AIDS Research Center, The Rockefeller University, New York). It was constructed by first "codon optimizing" HIV-1 subtype C *gag* (32-34), then inserting the gene into pVAX1 (Invitrogen, Carlsbad, CA) along with leader sequences (35, 36). The

plasmid therefore encodes HIV-1 *gag* under a CMV promoter; it contains a pMB1 origin, BGH polyadenylation signal and kanamycin resistance gene.

p*env-gag*

p*env-gag* was kindly provided by Yaoxing Huang (Aaron Diamond AIDS Research Center, The Rockefeller University, New York). It was constructed by first modifying the pVAX1 vector (Invitrogen, Carlsbad, CA) as follows. The hEF1α promoter was PCR amplified from pBudCE4.1 (Invitrogen), then cloned into the EcoR1/Not1 site, such that a dual-promoter vector was created. "Codon-optimized" HIV-1 subtype C *env* and *gag* were then inserted, resulting in a vector encoding both viral genes under separate promoters. The vector also contains a pMB1 origin, BGH polyadenylation signal and kanamycin resistance gene.

M1 vector

A vector encoding influenza matrix protein (M1) was kindly provided by Thomas Moran (Mount Sinai School of Medicine, New York). It was constructed by inserting a gene encoding M1 (WSN strain source) into pCAGGS.MCS (37-40). The viral gene is therefore under the control of the chicken β-actin promoter; the vector also encodes an ampicillin resistance gene.

pCDNA3.1/SIV*env* and pCDNA1.1/HIV*tatrevenv*

Vectors encoding SIV *env* and HIV-1 *tat*, *rev* and *env* were kindly provided by Zhiwei Chen (Aaron Diamond AIDS Research Center, The Rockefeller University, New York). Construction of the vectors involved inserting the *env* gene from SIV_{mac239} into pCDNA3.1 (Invitrogen, Carlsbad, CA). Similarly, the region of the HIV-1 (subtype C) genome comprising *env*, as well as both *tat* exons and both *rev* exons was inserted into pCDNA1.1 (Invitrogen, Carlsbad, CA). Both vectors contain a CMV promoter and ampicillin resistance gene.

RT-PCR

SIV *env*

For RT-PCR amplification of SIV *env*, RNA was extracted from cells using the RNeasy® Mini Kit (Qiagen, Valencia, CA). The RNase-Free DNase Set (Qiagen, Valencia, CA) was incorporated into the protocol, as per manufacturer's instructions. Reverse transcription was then carried out using Superscript™ RNase H-Reverse Transcriptase (GibcoBRL Life Technologies, Invitrogen, Carlsbad, CA). Nested PCR was then performed using the following primers:

EF1 5'-ACATTCATGTGGACAAATTGCAGAGGAGA-3'

EF2 5'-TACTGCAAAATGAATTGGTTTCTTAATTGG-3'

ER1 5'-GGGAGGGGAAGAGAACACTGGCCTATA-3'

PCR was done in a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Foster City, CA) in 100μL volumes containing 0.5 to 1.0μg of DNA, 10mM Tris-HCl (pH=8.5), 50mM KCl, 1.5mM $MgCl_2$, 0.1% Triton X-100, 200 μM each dATP, dGTP, dCTP and dTTP, 20pmol of each primer and 2.5U of Taq polymerase. The first round of PCR consisted of an initial cycle at 95°C for 2 minutes, followed by 30 cycles of 95°C for 20 seconds, 45°C for 1.5 minutes and 72°C for 2 minutes, using primers EF1 and ER1. The second PCR round, using primers EF2 and ER1, consisted of another 30 cycles of 94°C for 20 seconds, 55°C for 1.5 minutes and 72°C for 8 minutes.

HIV-1 *tat*

For RT-PCR amplification of spliced HIV-1 *tat*, RNA was extracted from cells using the Oligotex Direct RNA Mini Kit (Qiagen, Valencia, CA). Reverse transcription was then carried out using Superscript™ II reverse transcriptase (GibcoBRL Life Technologies, Invitrogen, Carlsbad, CA). PCR was then performed using the following primers:

TE1 5'-ATGGAGCCAGTAGATCCTAACCTAGAGCC-3'

TE2 5'-CTAATCGAATGGATCTGTTTTTGTCTTGC-3'

PCR was done in an Eppendorf Mastercycler Gradient (Brinkmann, Westbury, NY) in 50μL volumes containing 0.5-1.0μg of DNA, 5μL PCR Buffer II (Perkin-Elmer, Foster City, CA), 1.5mM $MgCl_2$, 200μM each dATP, dGTP, dCTP and dTTP, 20pmol of each primer and 0.5μL Gold taq polymerase (Applied Biosystems, Foster City, CA). PCR consisted of an initial cycle at 95°C for 10 minutes, followed by 40

cycles of 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds, concluding with an incubation of 72°C for 10 minutes.

MICE

BALB/c and C57BL/6

BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Chapter III:

Human macrophages can be transduced *in vitro* using attenuated *Salmonella typhimurium*

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S. typhimurium*) is a facultative intracellular pathogen whose preferred host cell is the macrophage (41). Wild-type *Salmonellae*, in fact, persist and thrive within the cells (42), which is precisely what permits them to eventually cause systemic disease (43). The bacteria adhere to and actively invade macrophages, thereby triggering a cascade of intracellular events that are associated with cellular activation (44). Using type III pili, *Salmonellae* directly deliver effector proteins to the macrophage (41), a property apparently not shared by other species of gram-negative bacteria (44).

A block in the common aromatic biosynthesis pathway yields auxotrophic *Salmonellae* that require aromatic metabolites for survival. Two such metabolites, paraaminobenzoic acid and dihydroxybenzoic acid, are not available in mammalian tissues; thus, bacteria that are aroA- mutants, for example, cannot grow and are avirulent to their hosts (45). After only a few rounds of division, such auxotrophs die intracellularly because of their inability to synthesize the aromatic amino acids they need to replicate (46). Because they are attenuated and non-pathogenic, aromatic-dependent *Salmonellae* have been judged worthy of consideration as anti-bacterial vaccine candidates (45, 47).

RESULTS

Attenuated *Salmonella* can deliver a reporter gene vector to human macrophages: eukaryotic expression of β-galactosidase detected by incubation with a substrate

We were interested in developing *Salmonella* as an instrument with which to improve genetic immunization. We hypothesized that we could design a system wherein attenuated bacteria are used as plasmid-delivery vehicles. Rather than exploit the protein expression machinery of the bacteria themselves, we would harness auxotrophic *Salmonellae* to carry and deliver DNA vaccines to appropriate target cells (Figure 1).

We began, therefore, by using electroporation to transform aroA- *Salmonella* (strain 33275) with each of two vectors, "pTrc-β" and "pSLICK-z." Both encode the β-galactosidase gene, but the former has a prokaryotic (Trp-lac hybrid) promoter, while the latter uses a CMV promoter (See Chapter II). Observation of the transformed bacteria in different media confirmed promotional specificity in the context of these vector backbones: *Salmonellae* carrying pTrc-β can express β-galactosidase; the same strain of *Salmonella* carrying pSLICK-z cannot. AroA- *Salmonellae* were also transformed with other, unrelated vectors (e.g., pEGFP) or left untransformed altogether.

Primary human macrophages were cultivated *in vitro* from peripheral blood

Figure 1 How the system would work *We envisioned using attenuated* S. typhimurium *to deliver DNA vaccines directly to antigen-presenting cells (APCs). Bacteria invade the cell or are actively phagocytosed* ***(1)****. The auxotrophic mutant* Salmonellae *would then lyse within the phagosome* ***(2)****, or escape from the vesicle into the cytoplasm* ***(4)****, before disintegrating* ***(5)****. Either way, the plasmids would be liberated and imported into the APC nucleus* ***(3)****. With subsequent host cell expression, the foreign proteins can be processed and presented in the context of MHC class I (and possibly also MHC class II) molecules* ***(6)****.*

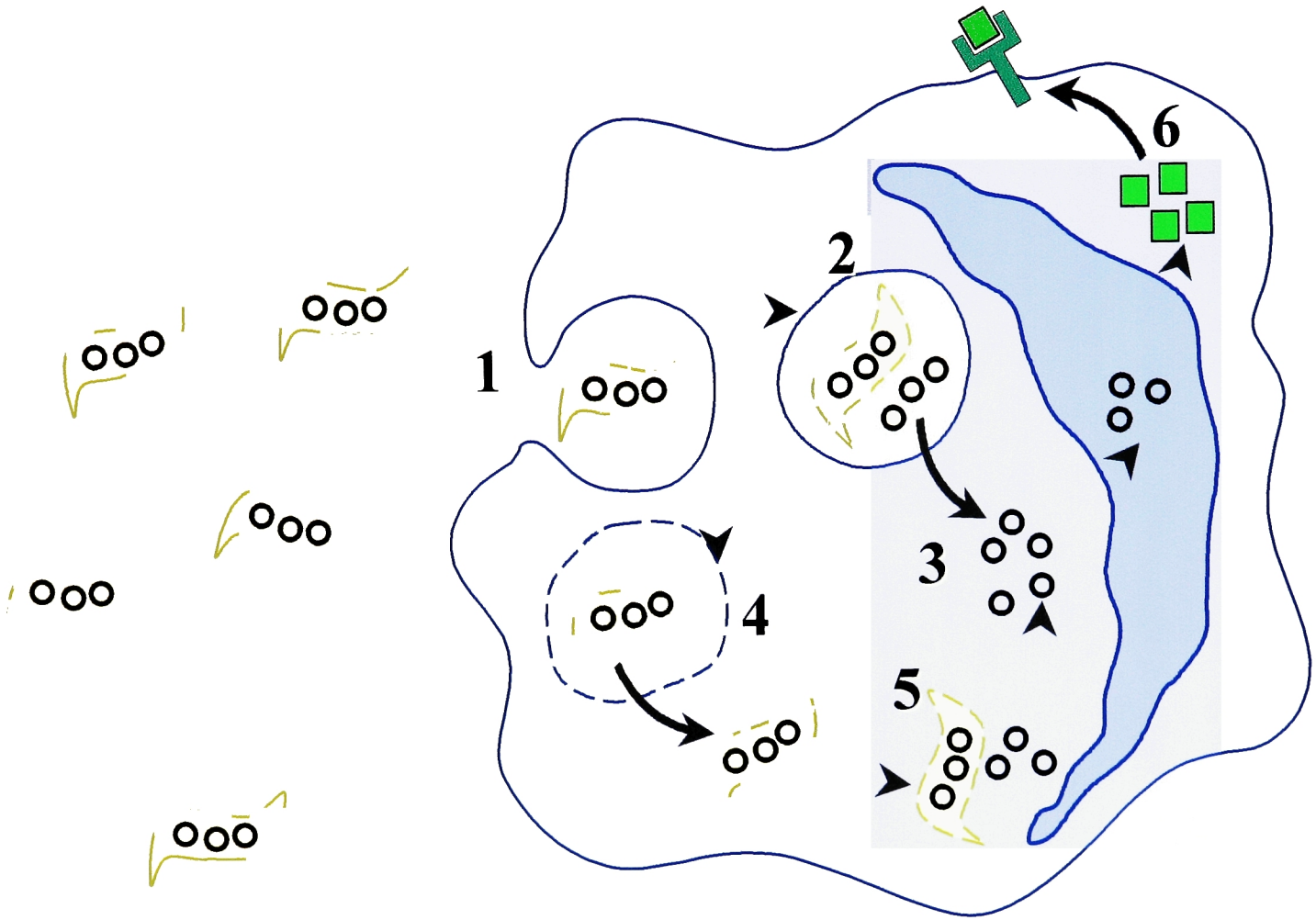


Figure 1

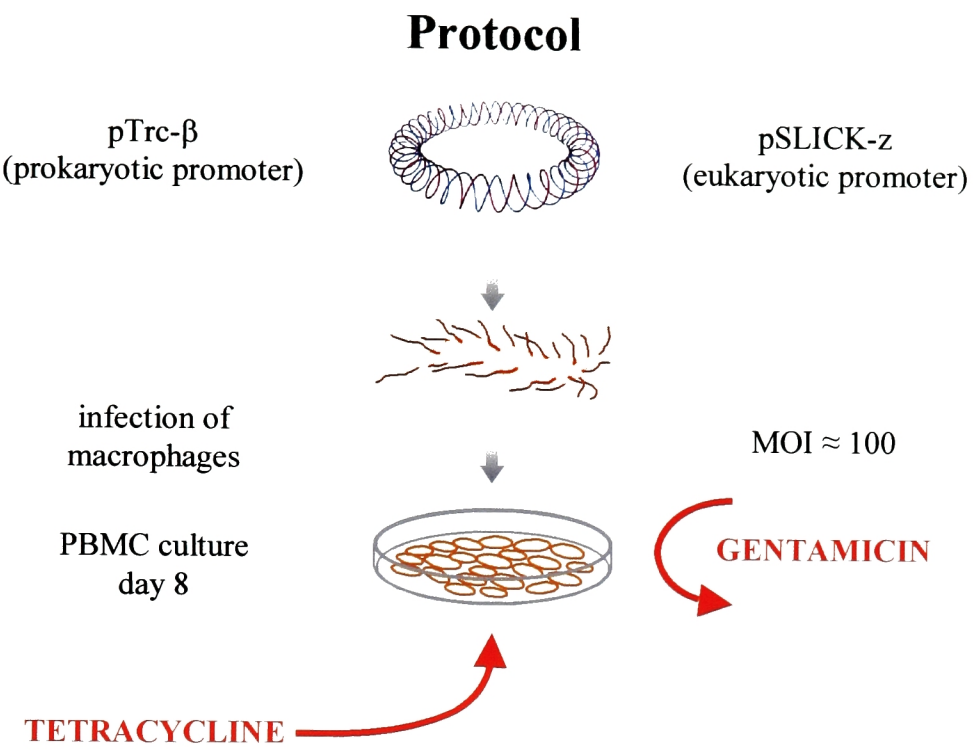
mononuclear cells (PBMCs), and infected with bacteria. In order to gauge optimal timing of infection, cell cultures were surveyed and manipulated at different time-points (day 6-day 8 post-isolation of PBMCs). It was thereby determined that macrophages (as adherent cells) are most numerous on day 8. Moreover, using *Salmonella* bearing prokaryotic vectors encoding GFP, as many as seven bacteria at once could be fluoroscopically visualized within a single cell following infection at a multiplicity of 100 (not shown).

Using the basic protocol described (see Chapter II), macrophages were infected with the various *Salmonellae*, then co-incubated with x-gal, the β-galactosidase substrate, at different time-points. Based on the degree of staining that resulted, it was determined that a delay of 36-48 hours post-infection yields the most extensive reporter gene expression.

Ultimately, we arrived at an effective, precise protocol with which to evaluate our first hypothesis (Figure 2). To further refine infectious conditions, we varied multiplicity of infection (MOI), and found manifest evidence of an infectious dose-dependency: More β-galactosidase activity is seen in cultures infected at higher MOI, with (MOI=100)>(MOI=50)>(MOI=25)>(MOI=1). At the highest MOI tested (100), the degree of x-gal staining suggested ~50% of macrophage expression of the reporter gene.

Overall staining patterns are described in Table 2. Negative controls included

Figure 2 The original infectious protocol *AroA-* Salmonella *was transformed using electroporation with one of two plasmids encoding β-galactosidase as shown. Control bacteria were either left untransformed or transformed with irrelevant ("pX") plasmids. Macrophages were cultivated from human peripheral blood mononuclear cells (PBMCs) using the adhesion method, then infected at MOI shown on day 8 post-isolation. Following infection, cells were washed with gentamicin-containing medium. Because at this time we were using a tetracycline-sensitive strain (33275), we also added this antibiotic to ensure intracellular bacterial death.*


Protocol
pTrc-β
(prokaryotic promoter)
pSLICK-z
(eukaryotic promoter)
infection of
macrophages
MOI ≈ 100
PBMC culture
day 8
GENTAMICIN
TETRACYCLINE

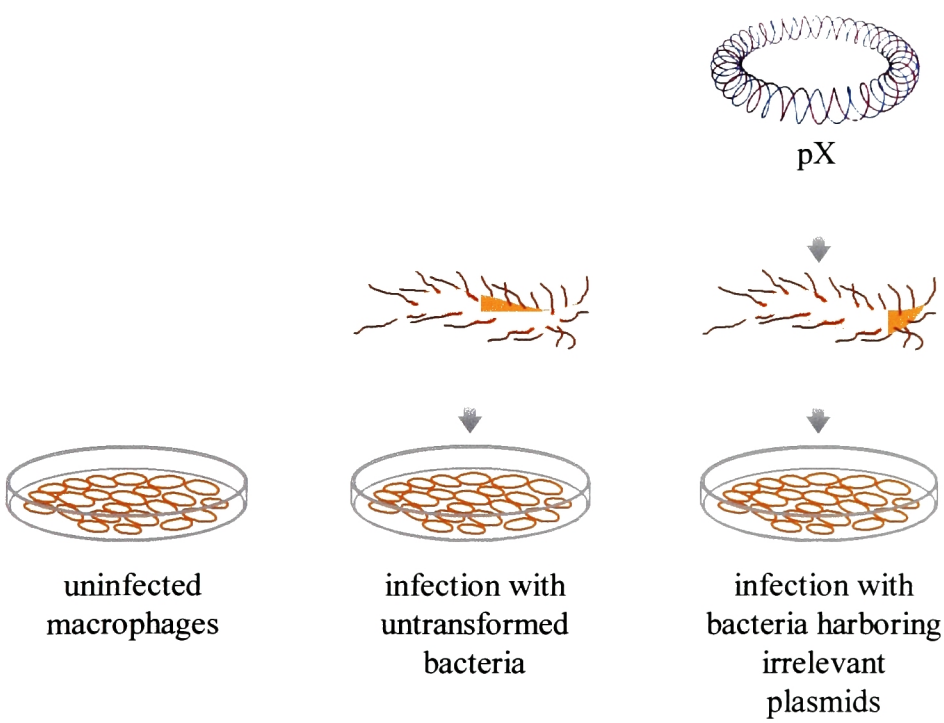

Controls
pX
uninfected
macrophages
infection with
untransformed
bacteria
infection with
bacteria harboring
irrelevant
plasmids

Figure 2

uninfected macrophages, cells infected with untransformed bacteria and cells infected with bacteria harboring irrelevant plasmids. In the presence of x-gal, these cultures showed no blue staining at all (Figure 3A).

Macrophages infected with bacteria bearing the pTrc-β vector, on the other hand, did show some, albeit minimal, staining in the presence of the reporter gene

MACROPHAGES INFECTED AT MOI=100 BY . . .	β-GALACTOSIDASE EXPRESSION (BLUE COLOR)	STAINING MORPHOLOGY
nothing	no	n/a
untransformed bacteria	no	n/a
bacteria transformed with irrelevant plasmids (e.g., pUC19, pEGFP)	no	n/a
bacteria transformed with pTrc-β (prokaryotic promoter)	<1%	scattered, punctuate
bacteria transformed with pSLICK-z (CMV promoter)	40-50%	diffuse, intracellular

Table 2

substrate. The intracellular morphology, however, was distinctive: These cells bore a scattered, punctate pattern, suggestive of vacuolized, contained bacteria that are actively expressing the β-galactosidase vector themselves (Figures 4A and 4B).

In contrast, cells infected with *Salmonellae* carrying the pSLICK-z vector stained in a diffusely intracellular way, suggesting that the macrophages are expressing the reporter gene themselves (Figures 3B, 5A and 5B).

In light of these staining patterns, we concluded that it was indeed possible to employ attenuated *S. typhimurium* as a plasmid-delivery vehicle. The results of this initial reporter gene study support the proposition that extra-genomic DNA can be transferred from an invasive bacterium to its target cell, which goes on to express the foreign vector itself.

Figures 3-5 ***Salmonella*** **can be used to transduce human macrophages** ***in vitro*** **such that they express a reporter gene** *Macrophages were infected with aroA-Salmonella (33275) at MOI≈100. Figure* ***3A*** *shows cells infected with bacteria carrying irrelevant plasmids, which yielded no visible blue staining. Figure* ***3B****, on the other hand, represents cells infected with pSLICK-z, which encodes β-galactosidase under the control of a eukaryotic promoter. Note multiple diffusely blue macrophages. Figures* ***4A*** *and* ***4B*** *show cells infected with* Salmonellae *bearing pTrc-β, which contains β-galactosidase under a prokaryotic promoter. Minimal, punctuate blue staining is evident, probably reflecting phagocytosed bacteria. Figures* ***5A*** *and* ***5B*** *are two more images of cells infected with* Salmonellae *carrying pSLICK-z.*

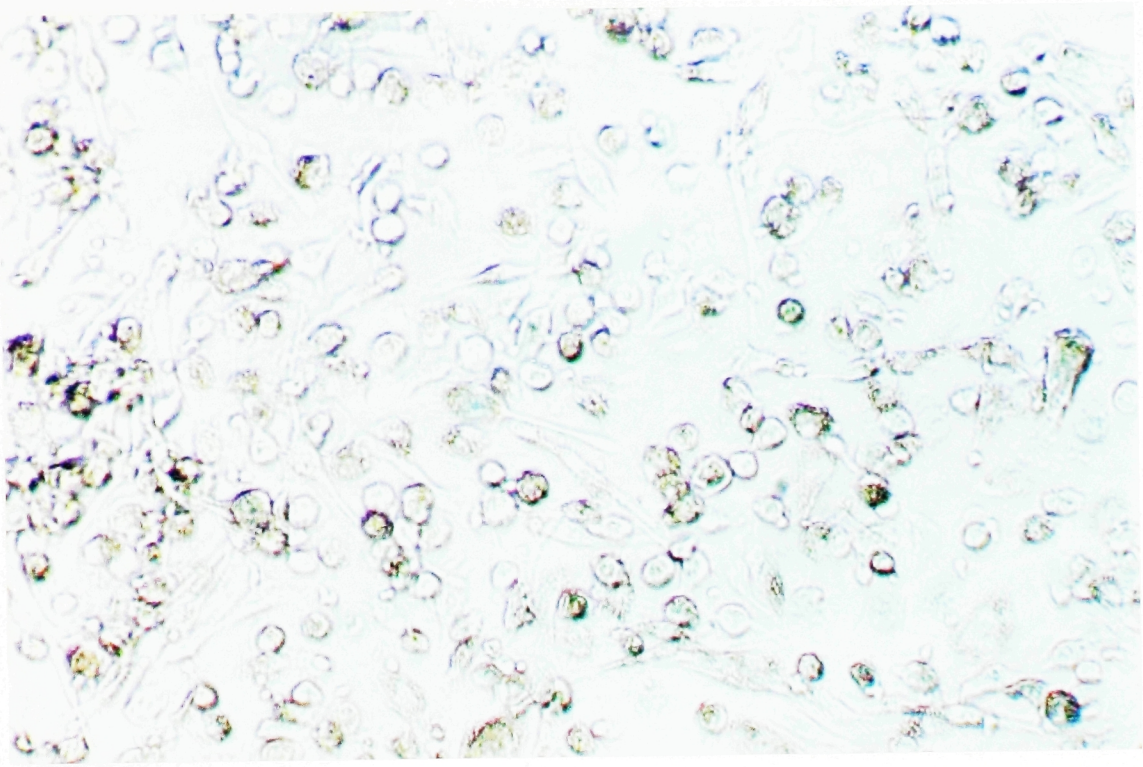


Figure 3A

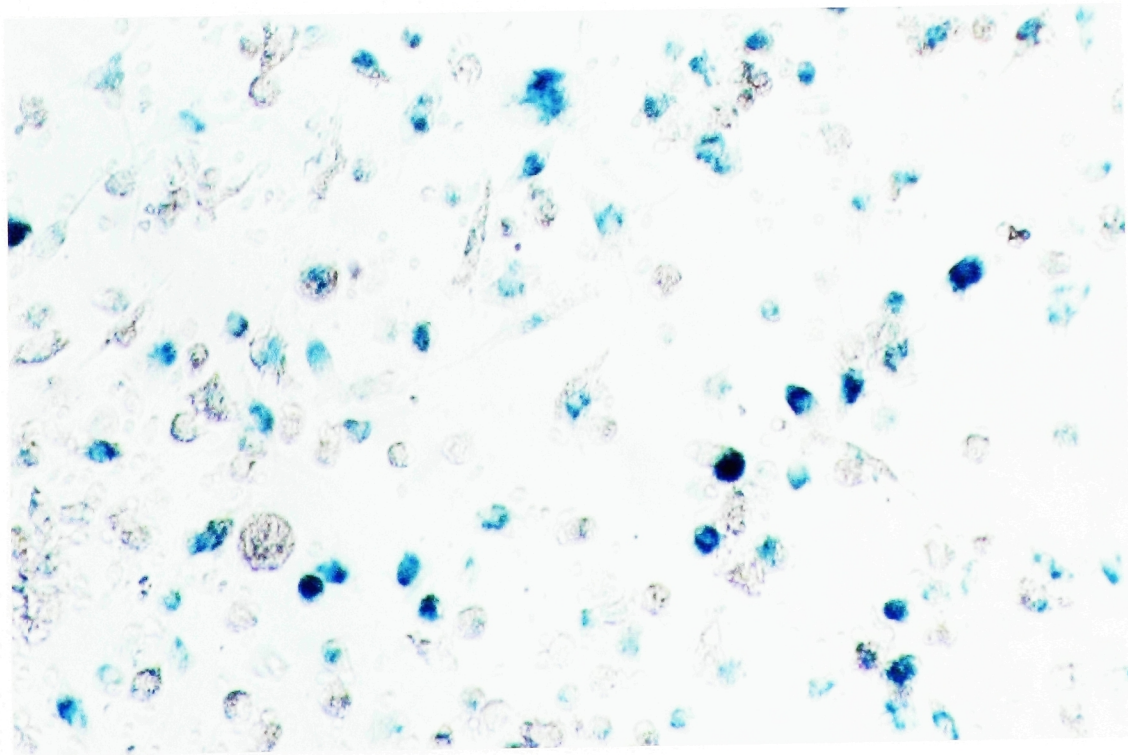


Figure 3B

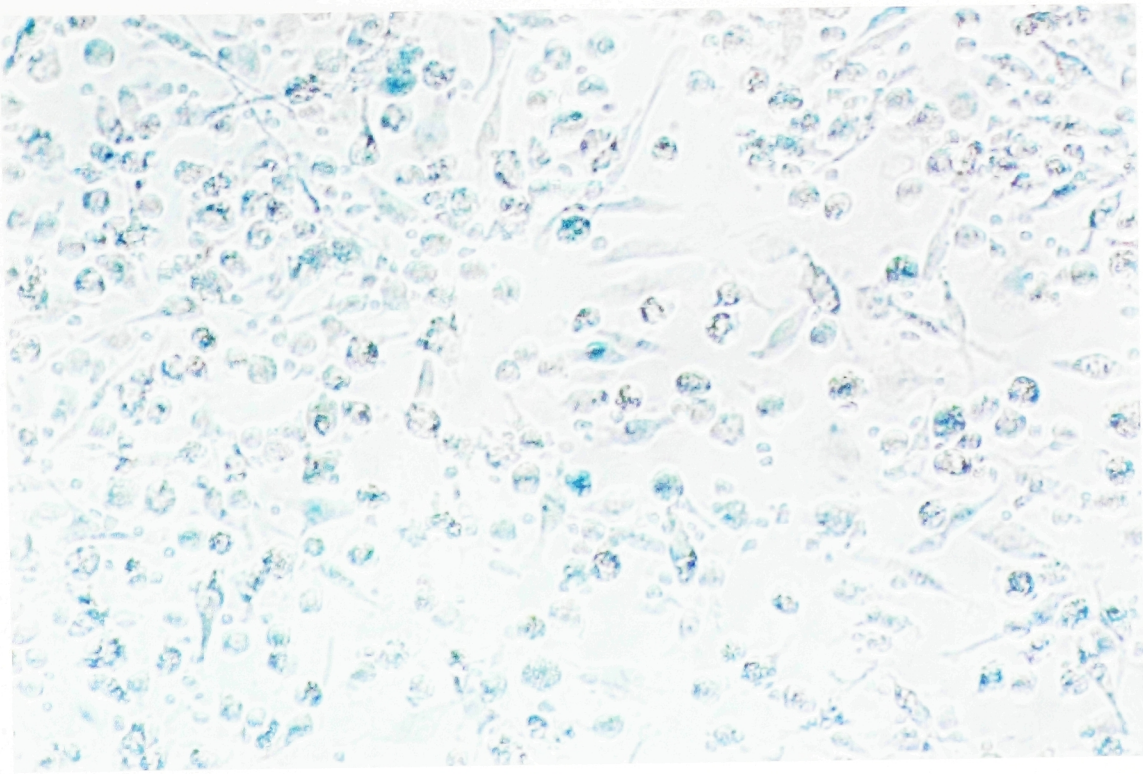


Figure 4A

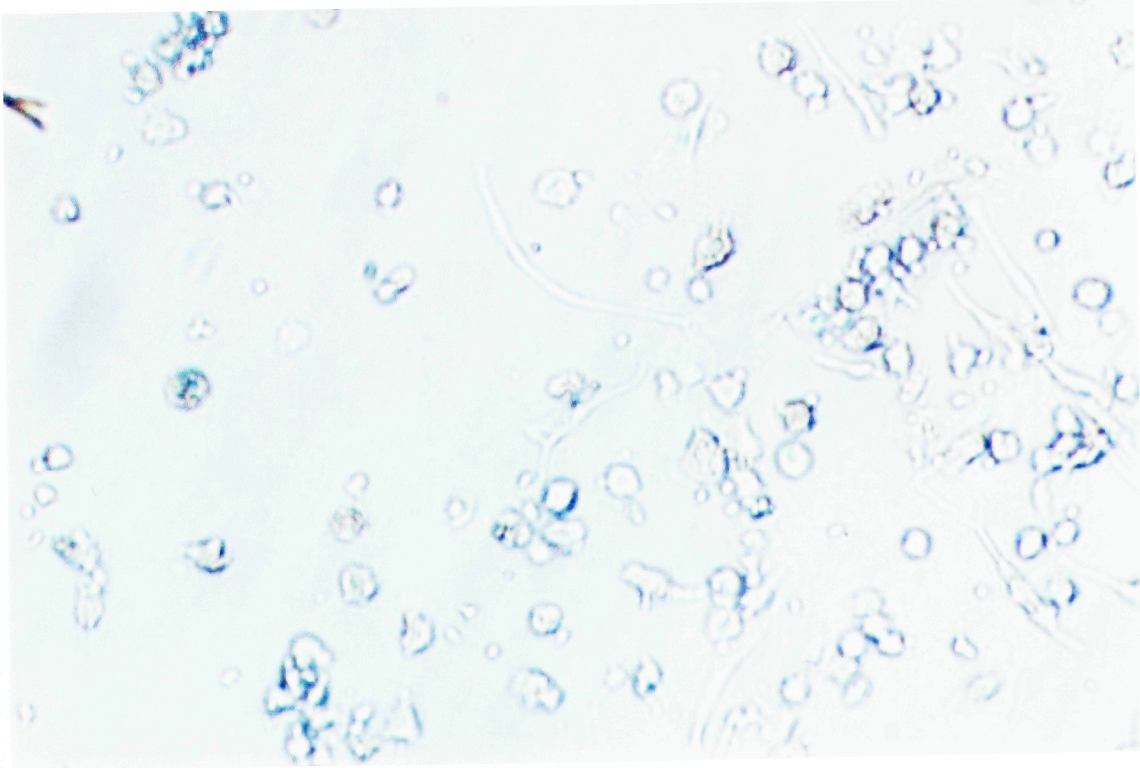


Figure 4B

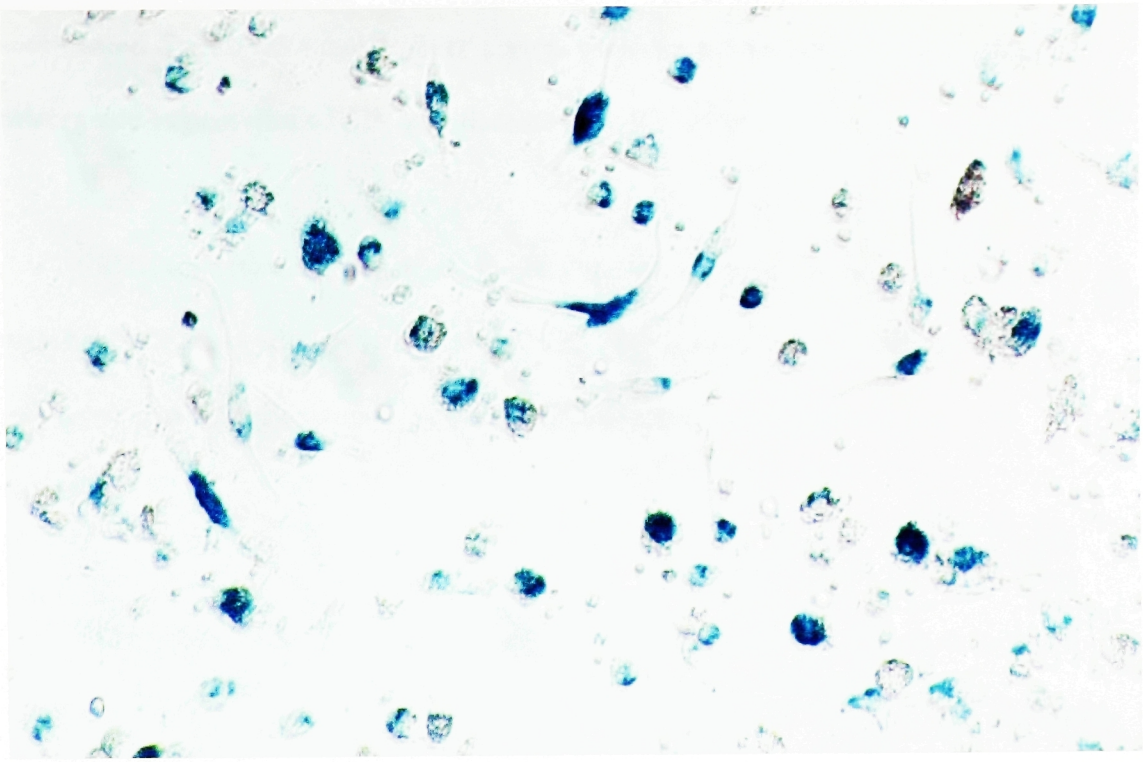


Figure 5A

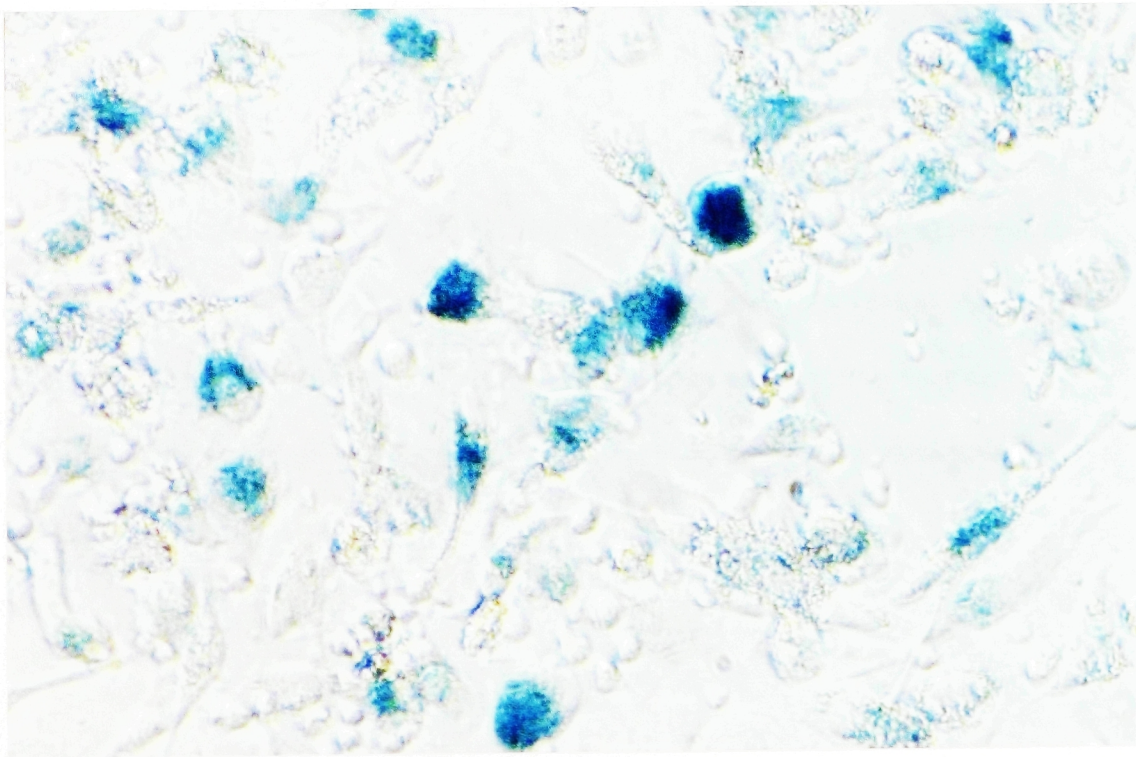


Figure 5B

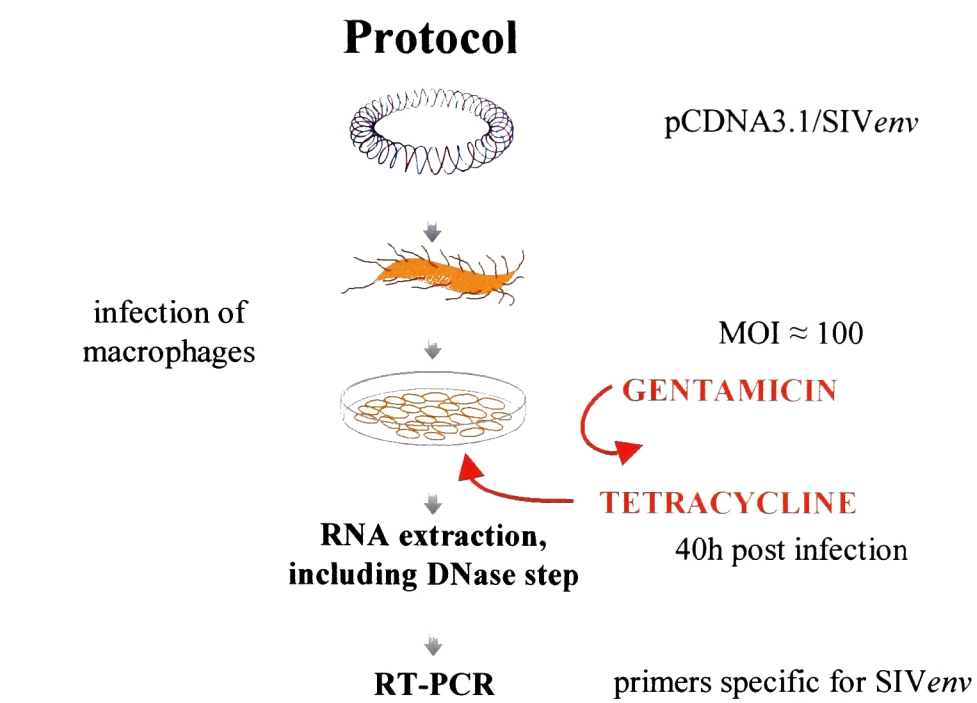
Attenuated *Salmonella* can deliver a viral gene vector to human macrophages: eukaryotic expression of SIV *env* detected by RT-PCR

With the vision of a vaccine delivery system in mind, we next chose to evaluate the system using a viral gene instead of a reporter gene. Furthermore, we undertook to confirm conceptual design at the level of transcription, instead of translation.

Therefore, once again we carefully chose our experimental vectors, including one encoding the *env* gene of SIV_{mac239} (pCDNA3.1/SIV*env*). Primers were designed towards recovery of the *env* gene transcript (See Chapter II for details). Again, we transformed aroA- bacteria (33275) and cultivated primary human macrophages from PBMCs.

After a sufficient period of time post-infection (36-48 hours, as determined earlier), macrophages were scraped to liberate them from the culture dish, then pooled and lysed. RNA was extracted from the cell lysates in a process involving the addition of DNase. Finally, RT-PCR was carried out to recover any viral gene transcripts (Figure 6).

Figure 6 The infectious protocol used towards RT-PCR Salmonella *was transformed with a eukaryotic vector encoding SIV*env *as shown. Macrophages were infected on day 8 following isolation of human PBMCs. Controls for this experiment included uninfected macrophages, macrophages infected with bacteria carrying irrelevant plasmids, and macrophages to which naked pCDNA3.1/SIV*env *was added. Approximately 40 hours post-infection and washing, the cells were harvested, pooled and lysed. RNA extraction included a DNase step. RT-PCR employed primers specific for SIV*env.


Protocol
pCDNA3.1/SIVenv
infection of
macrophages
MOI ≈ 100
GENTAMICIN
TETRACYCLINE
RNA extraction,
including DNase step
40h post infection
RT-PCR
primers specific for SIVenv

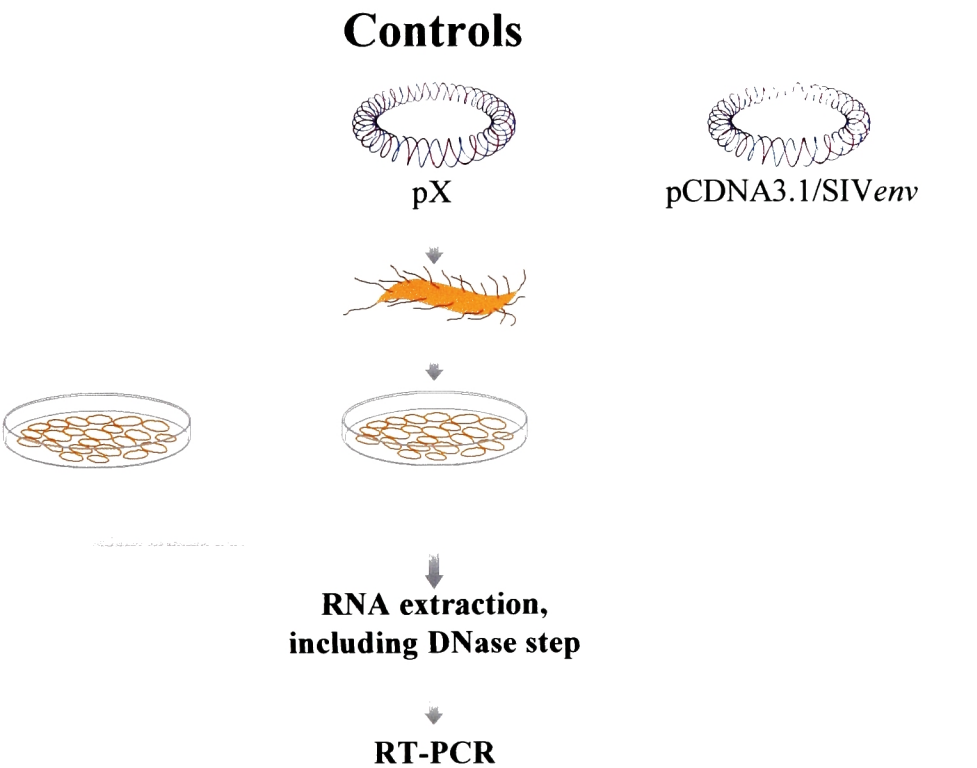

Controls
pX
pCDNA3.1/SIVenv
RNA extraction,
including DNase step
RT-PCR

Figure 6

Overall results of PCR are listed in Table 3. Controls once again included

	RT-PCR product (*env* transcript)?
reagent control	no
uninfected macrophages	no
macrophages infected with bacteria transformed by irrelevant plasmids (e.g., pSLICK-z)	no
macrophages infected with bacteria harboring pCDNA3.1/SIV*env*	yes
pure pCDNA3.1/SIV*env* post-DNase treatment used in extracting RNA from macrophages	no

Table 3

uninfected macrophages, macrophages infected with untransformed bacteria and macrophages infected with bacteria carrying irrelevant plasmids (including β-galactosidase vectors). Additionally, sham "pellets" comprising pure DNA were used as a control for the DNase treatment. PCR with the *env* primers yielded a product of the appropriate size (1500bp) only from the cells infected with *Salmonellae* harboring pCDNA3.1/SIV*env* (Figure 7).

Thus, we believe that we confirmed the efficacy of our plasmid-delivery system at the level of transcription. Recovery of the mRNA transcript of SIV*env* from infected macrophages suggests that the attenuated bacteria were able to deliver the plasmid to their target cells in such a way as to permit the latter to express the viral gene.

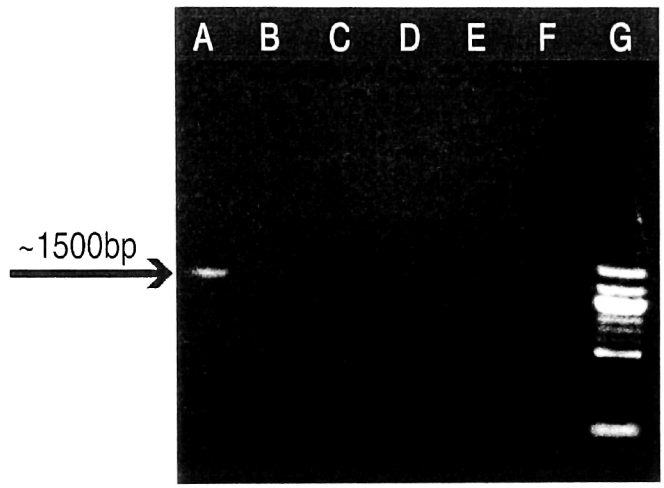


Figure 7 ***Salmonella*** **can be used to transduce macrophages** ***in vitro*** **such that they express a viral gene** *RT-PCR using primers specific for SIV*env *performed on RNA extracted from cell lysates yielded the following results:* **(A)** *macrophages infected with bacteria carrying pCDNA3.1/SIV*env, **(B)** *macrophages infected with bacteria carrying pSLICK,* **(C)** *macrophages infected with bacteria carrying pSLICK-z,* **(D)** *macrophages infected with untransformed bacteria,* **(E)** *uninfected macrophages,* **(F)** *reagent control, and* **(G)** *100bp ladder. Note that only in lane* **(A)** *do we see a band corresponding to the size of SIV* env. *Not shown are results of the same RT-PCR done on sham DNA "pellets" comprising 0.01μg, 0.1μg and 1.0μg of pCDNA3.1/SIV*env, *which yielded no bands.*

Attenuated *Salmonella* can deliver a viral gene vector to human macrophages: eukaryotic expression of HIV-1 *env* detected by immunocytochemistry

We continued studying our system of targeted plasmid delivery using viral vectors. In an attempt to once again prove efficacy at the level of translation, we undertook to detect expression of a viral protein using immunocytochemistry. We therefore transformed aroA- *Salmonella* with a eukaryotic promoter-driven expression vector that encodes the HIV-1 genes *tat*, *rev* and *env* (pCDNA1.1/HIV*tatrevenv*). At this time, however, we used a different auxotrophic *Salmonella* strain (14028), which is reportedly more invasive than 33275. Human macrophages were cultivated in the usual manner, then infected in a protocol based on our previous experience (Figure 8). Macrophages were fixed and blocked before staining using a Vectastain® alkaline phosphatase (AP)-based kit (See Chapter II for details).

Overall results of the staining are listed in Table 4. Initially, a horseradish

MACROPHAGES INFECTED AT MOI=100 BY . . .	Env STAINING?
nothing	no
untransformed bacteria	no
bacteria transformed with pCDNA1.1/HIV*tatrevenv*	yes (50-80%)

Table 4

Figure 8 The protocol used to infect human macrophages toward immunocytochemistry *Human macrophages were infected* in vitro *as usual, as shown. Approximately 40 hours post-infection and washing, macrophages were fixed and blocked in their wells. Serum from an individual infected with HIV-1 subtype C was used for primary antibody in order to be specific for the viral source of the gene encoded by the vector carried by the bacteria. Secondary antibody conjugated to alkaline phosphatase (AP) was added, then the cells were incubated with an AP substrate for development.*

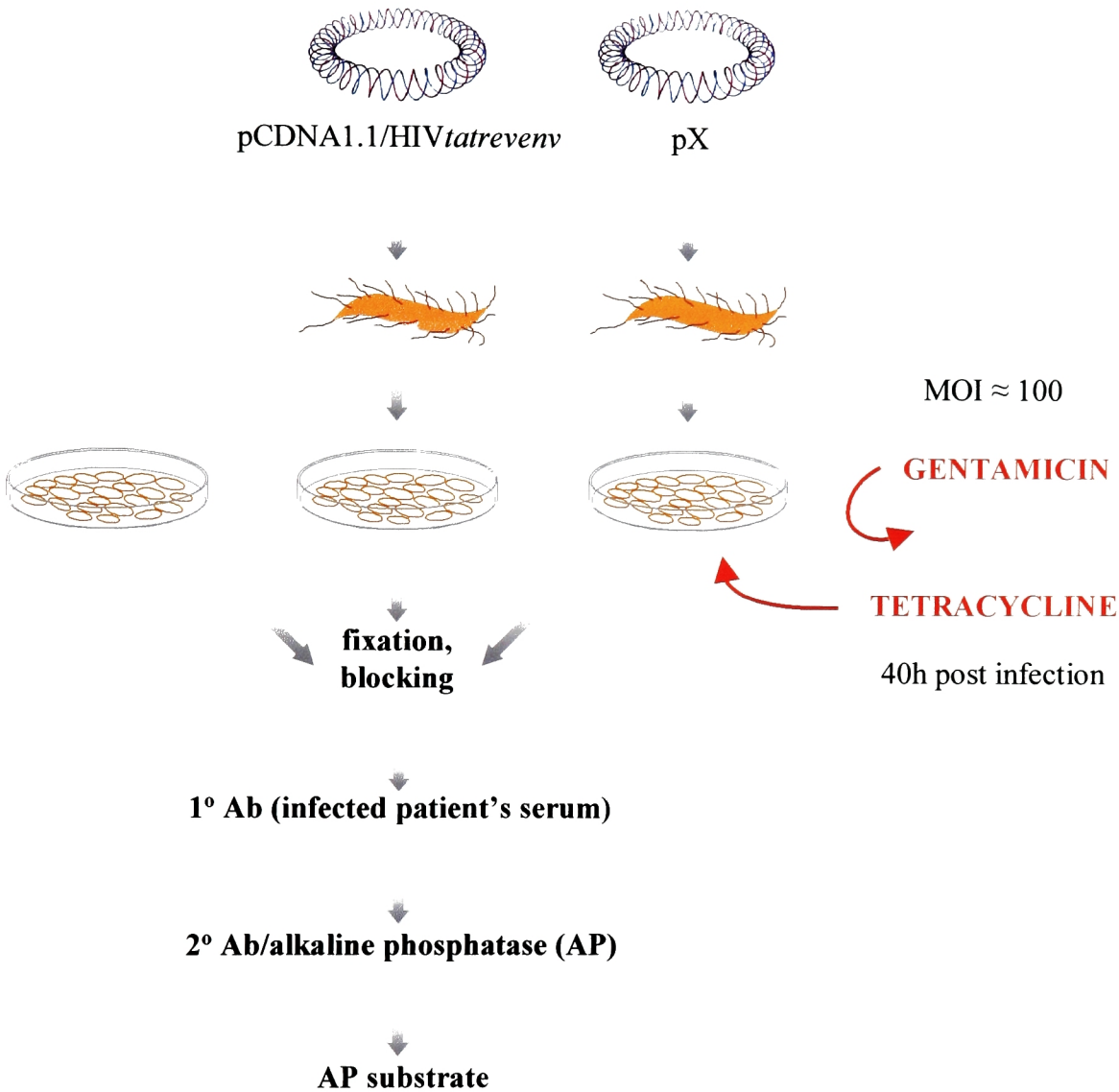

pCDNA1.1/HIVtatrevenv
pX
MOI ≈ 100
GENTAMICIN
TETRACYCLINE
40h post infection
fixation,
blocking
1º Ab (infected patient's serum)
2º Ab/alkaline phosphatase (AP)
AP substrate

Figure 8

peroxidase (HRP)-based system was used, but endogenous peroxidase activity within the macrophages proved difficult to fully neutralize, yielding muddy-appearing negative control cultures (not shown). Using AP instead, however, permitted very "clean" staining, with no visible background in the control cultures (Figures 9-11). Macrophages infected with *Salmonellae* bearing pCDNA1.1/HIV*tatrevenv* demonstrated widespread staining, a pattern consistent with the more invasive strain (14028) and the high MOI (100) used in this experiment (Figures 9-11). Also visible among the red-colored macrophages were two persistently colorless types of cells: T-cells (which appeared as smaller, more rounded non-adherent cells) and residual bacteria (which account for much of the apparent debris in the surrounding medium). These populations, therefore, functioned as an additional, internal control for the study.

Attenuated *Salmonella* can deliver a viral gene vector to human macrophages: eukaryotic expression of the HIV *tat* splice product detected by RT-PCR

Our next challenge was to confirm, unequivocally, that what we had detected thus far was evidence of eukaryotic, not prokaryotic, expression. Could the immunocytochemical staining, for example, simply reflect bacterially-expressed protein that had permeated the macrophage cytosol? Were the bacteria themselves actually capable of driving a CMV promoter through the use of a cryptic prokaryotic

Figures 9-11 ***Salmonella*** **can be used to transduce human macrophages** ***in vitro*** **such that they express a viral gene** *Macrophages were infected with aroA-Salmonella (14028) at MOI=100. The images represent cells infected with either untransformed bacteria (Figures* ***9A****,* ***10A*** *and* ***11A****) or* Salmonella *carrying pCDNA1.1/HIV*tatrevenv, *which encodes the viral genes under the control of a CMV promoter (Figures* ***9B****,* ***10B*** *and* ***11B****). Immunocytochemical staining for HIV Env reveals widespread color, suggesting effective receipt and expression of pCDNA1.1/HIV*tatrevenv *by the infected macrophages.*

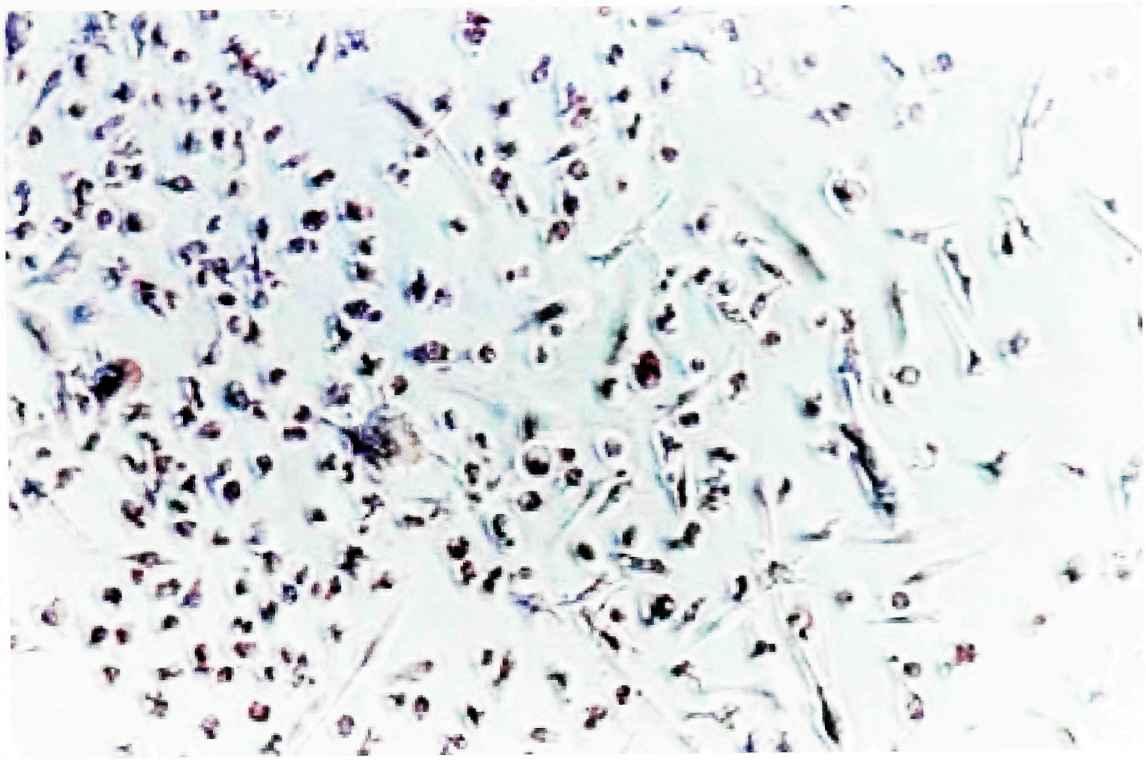


Figure 9A

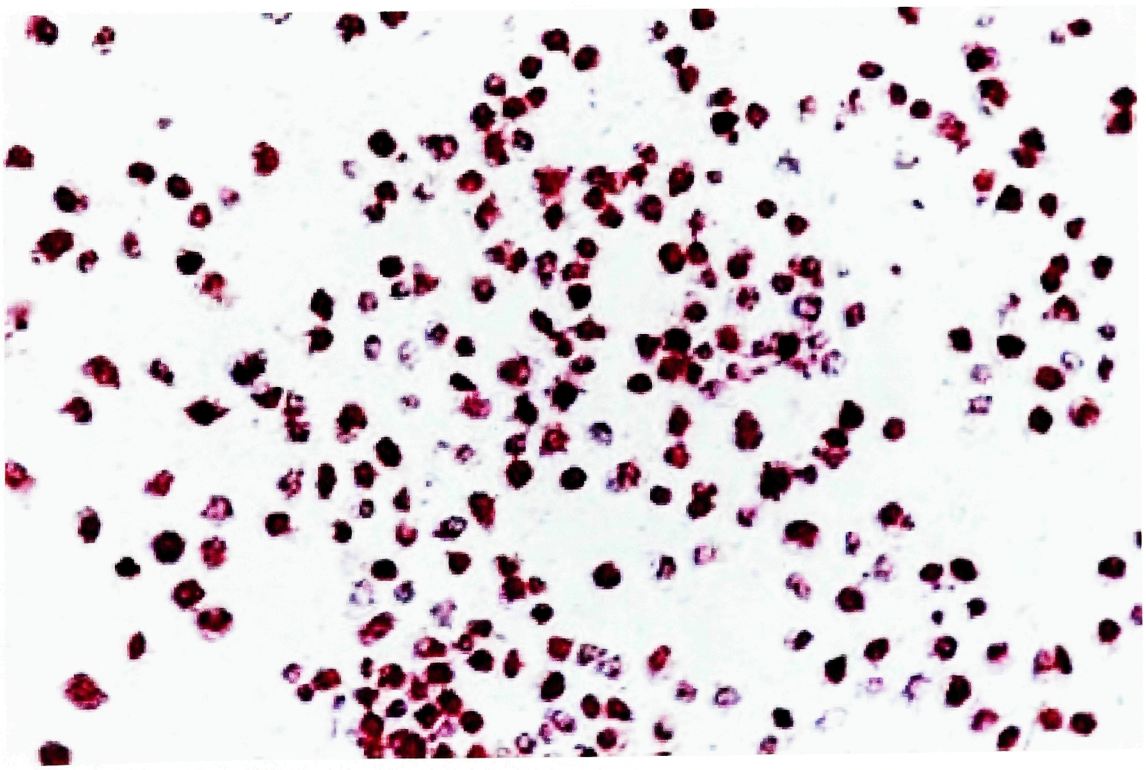


Figure 9B

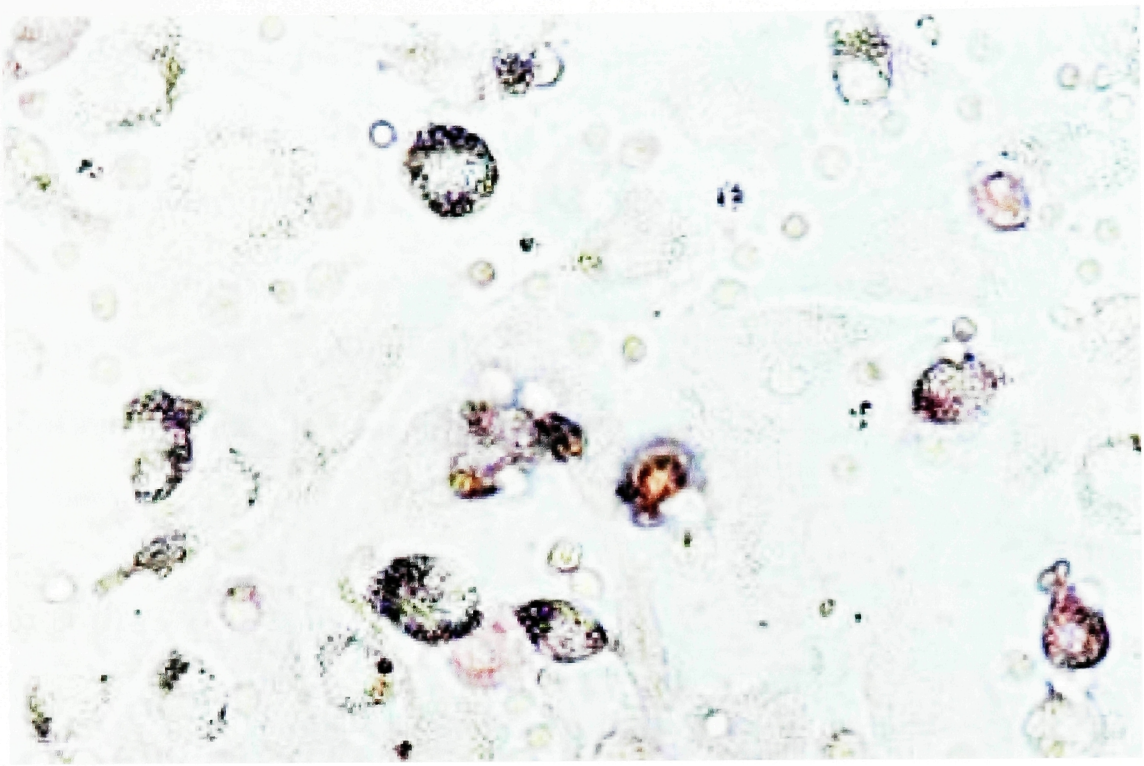


Figure 11A

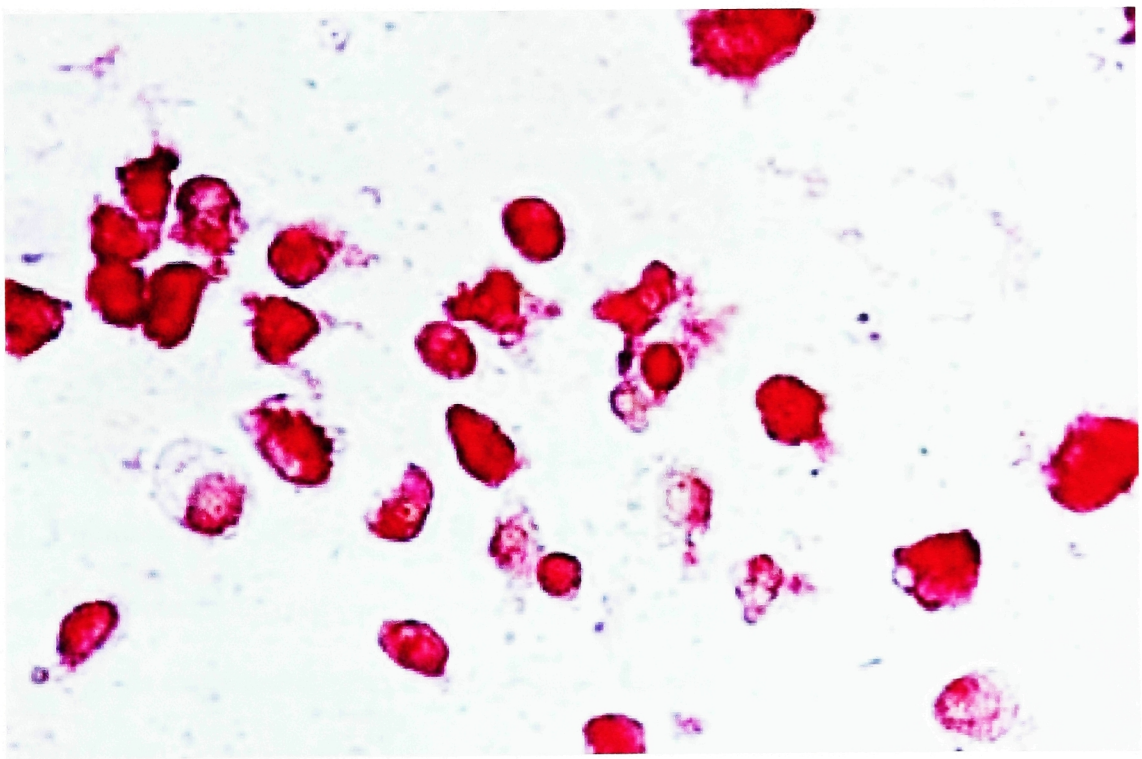


Figure 11B

enhancer, for example (48-53)? Although the β-galactosidase vectors we used in our original study, pTrc-β and pSLICK-z, appeared specific for prokaryotic or eukaryotic usage, respectively, we ultimately discovered that a CMV promoter can in fact be used by *Salmonella* in the context of certain plasmid backbones (e.g., many Invitrogen® vectors). A culture of *Salmonella* transformed to carry a vector (pVAX1) encoding HIV-1 *gag*, for example, tests positively for p24 content by ELISA (data not shown).

We sought, then, to prove the conceptual premise of our system by detecting evidence of RNA splicing, a uniquely eukaryotic phenomenon (54, 55). Initially, we took advantage of a HeLa cell-derived multinuclear-activation-of-a-galactosidase-indicator (MAGI) assay (56), which allows us to gauge the presence of HIV-1 Tat by measuring β-galactosidase activity as a surrogate marker. Though the assay worked to our satisfaction (not shown), we later learned that the gene product of the first *tat* exon alone is sufficient to function as a *trans* activator (57).

We then instead turned to PCR once again. Primers were carefully designed to flank HIV *tat* (Figure 12). After infecting human macrophage cultures as before (e.g., Figure 8) with *Salmonella* bearing pCDNA1.1/HIV*tatrevenv*, the cells were liberated, pooled and lysed (See Figure 6). RT-PCR was carried out after extraction of mRNA (as explained in Chapter II). Controls included macrophages to which pure pCDNA1.1/HIV*tatrevenv* was added. Results are listed in Table 5 and illustrated graphically in Figure 13.

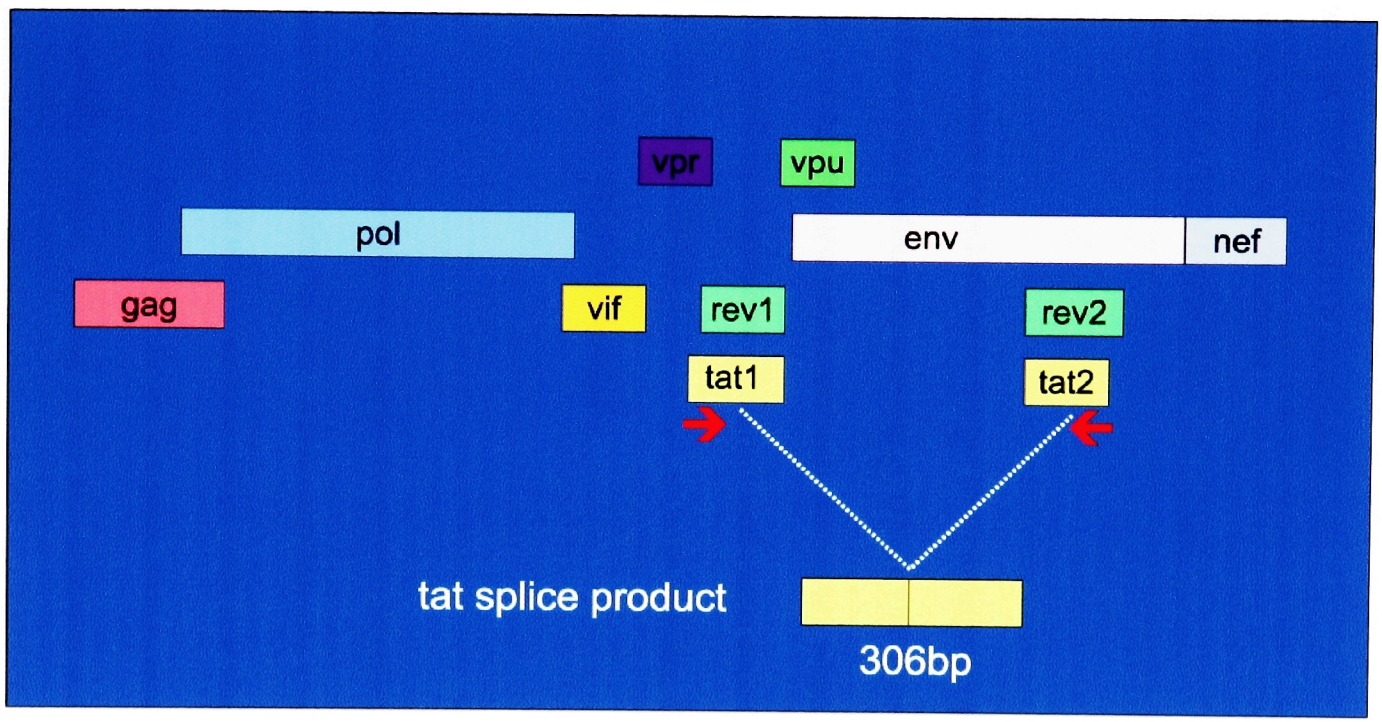

vpr
vpu
pol
env
nef
gag
vif
rev1
rev2
tat1
tat2
tat splice product
306bp

Figure 12

	SPLICE PRODUCT (*tat*) DETECTED BY RT-PCR?
reagent control	no
uninfected macrophages/PBMCs	no
macrophages + pCDNA1.1/HIV*tatrevenv*	no
macrophages infected with bacteria harboring pCDNA1.1/HIV*tatrevenv*	yes

Table 5

Our PCR products were limited to the gel lane corresponding to the lysate of macrophages infected with *Salmonella* carrying pCDNA1.1/HIV*tatrevenv*. We therefore believe that the brightest band represents spliced *tat*, which can only be a consequence of macrophage expression. Thus, we again have confirmation of the intended design, wherein attenuated *Salmonellae* serve as vehicles for delivery of eukaryotic vectors to their macrophage targets.

DISCUSSION

Using bacteria as DNA delivery vehicles for genetic immunization was first attempted by Jerald Sadoff's team (58, 59). Attenuated *Shigella* was employed to deliver a reporter gene vector *in vitro* to cultured BHK cells, and also generated *in vivo* lymphoproliferative responses specific for the same foreign antigen when administered intranasally to mice. Another group soon followed suit (46), exploring the possibility of using *Salmonella* as an orally-administered inoculation bearing vectors encoding two virulence factors of *Listeria*. Again, both direct evidence of

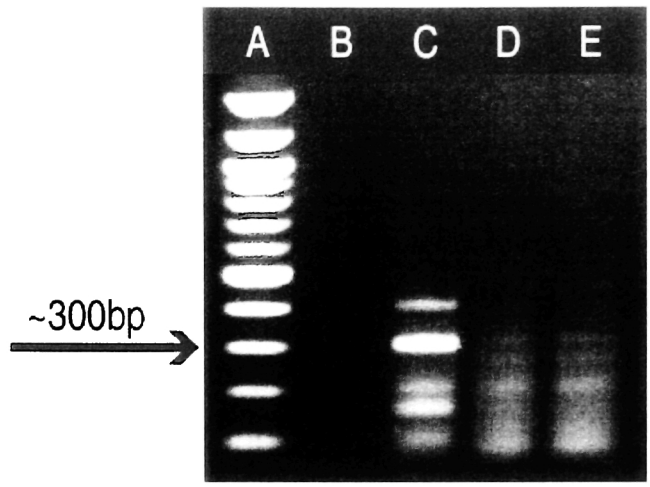


Figure 13 ***Salmonella*** **can be used to transduce human macrophages** ***in vitro*** **such that they express a viral gene** *RT-PCR using primers designed to recover spliced HIV-1* tat *performed on RNA extracts from cell lysates yielded the following results:* **(A)** *100bp ladder,* **(B)** *reagent control,* **(C)** *macrophages infected with* Salmonella *bearing pCDNA1.1/HIV*tatrevenv, **(D)** *uninfected cells, and* **(E)** *macrophages with pure pCDNA1.1/HIV*tatrevenv *added. Note that the brightest band in lane* **(C)** *corresponds to a size equivalent to that of full* tat *(both exons together). Recovery of a splice product confirms eukaryotic (and not prokaryotic) expression.*

genetic transfer to host cells (peritoneal macrophages) as well as systemic immune responses were documented.

Thus, when we began our investigation, there was a small but sufficiently promising precedent. As we undertook our own studies, several other groups continued to pursue the burgeoning model of bacteria as plasmid-delivery vehicles in vaccine design (60-68). All relevant studies published to date, however, have

involved either cell lines or murine cells (*in vitro* or *in vivo*); our experimentation is unique in demonstrating the efficacy of the system in primary human cells.

Designing such a system notwithstanding, we are confronted with the question of its potential utility as a successful vaccine strategy. Are macrophages, for example, truly the most desirable target cell for our purposes?

Before the primacy of dendritic cells (DCs) was recognized, macrophages were understood to be important as antigen-presenting cells (APCs) (69). At the very least, they were known to be capable of expressing MHC class II glycoproteins on their surface, processing endocytosed antigen and producing IL-1.

While we now appreciate that DCs are the most important APCs (especially given their role in priming naïve T-cells), we nonetheless must accept the complementary role in presentation played by macrophages. They are, for example, specialized in the phagocytosis of large particles, including antigens from bacteria and parasites, whether intracellular or extracellular pathogens. Indeed, their phagocytotic capacity is a first-line defense, and they are important in mediating innate immune responses (70).

More important towards our end, however, is their contribution to the effector phase of adaptive immunity. The ingestion of microorganisms, though a primitive, non-specific defense, also induces the expression of MHC class II and co-stimulatory

(e.g., B7) molecules. Furthermore, macrophages also produce cytokines that stimulate T-cell proliferation and differentiation (e.g., IL-12). In fact, the principal function of macrophages is ultimately the generation of CD4+ effector T-cells (71). Activated macrophages also become more efficient APCs, with increased cytokine production and expression of MHC class II and co-stimulatory molecules. Additionally, there is a positive feedback process, by which activated T helper cells produce interferon (IFN)-γ and CD40L, which, in turn, induce MHC class II and co-stimulatory molecules (71).

Thus, although macrophages are not necessarily our first choice as vaccine targets, they are not without relevance or utility in the current context. A more challenging issue, then, is the well-documented observation that *Salmonella* invasion of macrophages induces apoptosis. What if our vaccine kills the very APCs it targets?

Of the many studies that have characterized and explored *Salmonella*-induced cell death, the vast majority (72-82) exclusively involve murine macrophages or cell lines, while only three related studies (83-85) included human cells to any extent. Moreover, some have noted directly that *S. typhimurium* entry into murine macrophages ". . . causes considerably more eukaryotic cell death than does entry of the same *Salmonella* serovar into human macrophage cells" (86). This finding is consistent with our own work: Despite reports of murine macrophage death occurring within minutes of *Salmonella* invasion (72), we routinely surveyed our

human cells with trypan blue 36-48 hours post-infection, and found that <5% of cells were dead. One possible explanation for this apparent discrepancy is the difference across species in host resistance factors. Specifically, in the case of susceptibility to *Salmonella* virulence, it has become clear that genes mapping to the Bcg/Ity locus are important (87-95). There are, however, differences between mice and humans with respect to the encoded protein that has been identified, natural resistance-associated membrane protein (NRAMP) (89). Indeed, the Bcg/Ity locus displays considerable polymorphism, even within a species, yielding corresponding phenotypic variation in NRAMP. Even functional variability according to the sex of the host has been observed, suggesting a role for hormonal influences (91). The contribution of loci other than Bcg/Ity has yet to be fully elucidated (93). Importantly, however, it is known that the protein product of the mammalian suppressor of *svg1* (*mss1*) gene corresponds to the ATPase part of the 26S protease complex, which is believed to have a pivotal role in regulating apoptosis. The fact that human macrophages display a greater degree of *mss1* expression than murine macrophages (86) is further consistent with our observations regarding the relative resilience of human cells to *Salmonella* invasion.

Another factor to consider in a discussion of the possibility of macrophage apoptosis is bacterial strain choice. At least one recent study documents the substantial variability across *Salmonella* serovars in their cytotoxicity in human macrophages, including the extent of apoptosis induced (96).

The case for permitting a *Salmonella*-based vaccine to be invasively delivered to macrophages is strengthened by one final consideration: the potential for cross-presentation by DCs. While a more detailed discussion of DCs in the context of this system is forthcoming (See Chapters IV and V), it should be noted here that antigen from apoptotic cells can be acquired by bystander DCs and cross-presented to T-cells (97-100). In fact, Mary Jo Wick and colleagues specifically confirmed this process using murine macrophages rendered apoptotic by *Salmonella* (78). Thus, it may ultimately emerge that our method of *Salmonella*-facilitated genetic immunization is particularly effective *in vivo* because of an unintended toxicity to macrophage targets.

Chapter IV:

Infection of dendritic cells with *Salmonella typhimurium* induces maturation but is also cytotoxic

INTRODUCTION

The importance of mucosal immunogenicity in vaccine design has long been recognized (101, 102), especially in the case of combating HIV infection and disease (103-105). Mucosal surfaces are the primary sites of transmission for most infectious diseases, and over 75% of all HIV transmission, in particular, occurs by the mucosal route (105). If we can induce mucosal immunity with vaccination, we may be able to prevent pathogen entry altogether.

Using *S. typhimurium* to deliver a vaccine would permit mucosal involvement at the outset: Inoculation itself would be mucosal, through the oral route into the digestive tract. Furthermore, the bacteria are known to egress from the gut lumen via the M cells, which overlie Peyer's patches (106). Once these specialized epithelial cells are effectively traversed (through transcytosis), *Salmonellae* reach a large intraepithelial "pocket" containing T lymphocytes, B lymphocytes and macrophages. Below the epithelium, the bacteria reach DCs (106-108). Because of this convenient natural route of administration and targeted entry, live attenuated *Salmonella* has previously been proposed as a paradigm of mucosal vaccines (109). In fact, some have called specifically for vaccines targeting M cells (109-111), and at least one group has proceeded with an *in vivo* investigation of such a scheme using reovirus

vectors (112). Indeed, in order to fully and accurately evaluate the potential for mucosal as well as systemic immunogenicity, we must perform *in vivo* studies.

RESULTS

Taking advantage of the OVA/OT mouse model: an attempt to take the system *in vivo*

We were interested in exploring the potential strength of our vaccine delivery system *in vivo*. Specifically, we hoped to take advantage of so-called "OT I" and "OT II" mice, which are transgenic for T-cell receptors specific for ovalbumin (OVA) epitopes in the context of MHC class I and II restriction, respectively (113, 114). We felt that the murine model would not only enable us to assess the efficacy of oral inoculation, but also to examine the extent to which DCs are successfully targeted by the transformed bacteria.

We therefore began by constructing a plasmid vector encoding OVA under the control of a eukaryotic promoter. After sequencing the vector and confirming its expressive capacity, we transformed aroA- *Salmonella* (14028) with it, as before.

Next, we designed three different protocols involving OT mice and the new OVA vector delivery vehicles. The first of these (Figure 14) was intended simply to confirm DC maturation and T-cell proliferation following *in vitro* co-culture. Later

(Figure 15), we planned to actually inoculate our animals, and ultimately to look for more specific T-cell responses (Figure 16).

Thus, we began by cultivating murine bone marrow-derived DCs (BMDCs) *in vitro*. Based in part on our protocol for infecting human macrophages (See Chapter III), we similarly undertook to infect BMDCs with *Salmonella* using varying MOI (10-100).

Our plans for further manipulation of the cells were truncated, however, when we observed severe cytotoxicity, apparently as a direct consequence of exposure to the bacteria. Staining with trypan blue, for example, revealed almost wholesale death of the murine BMDCs (not shown), even at low MOI. Indeed, we are not the first to observe such toxicity in DCs: Yrlid et al. (115) saw similar rates of *Salmonella*-induced apoptosis of murine BMDCs, specifically when using the same strain (14028) that we used to infect the cells. Additionally, others have found *Salmonella* exposure to be toxic even to human DCs (116), apparently because of a virulence factor encoded by the bacterial *sipB* gene, which activates caspase-1 towards apoptosis (117). Rescigno and Borrow have therefore proposed, in fact, that such cytotoxicity may serve teleologically as a mechanism of immune evasion (118).

We found nonetheless that, 24 hours after infection at MOI=10, we were able to assay some surviving BMDCs for maturation markers, as planned. What cells

Figures 14-16 An attempt to take the system *in vivo* *Figures **14-16** illustrate schematically our original plans to take advantage of the transgenic "OT" mouse system using ovalbumin (OVA) as a model antigen.*

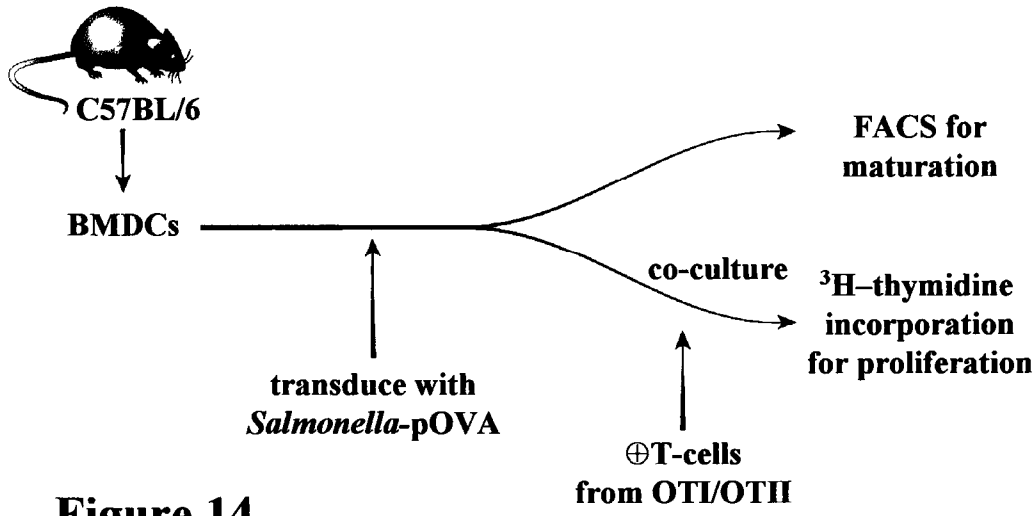


Figure 14

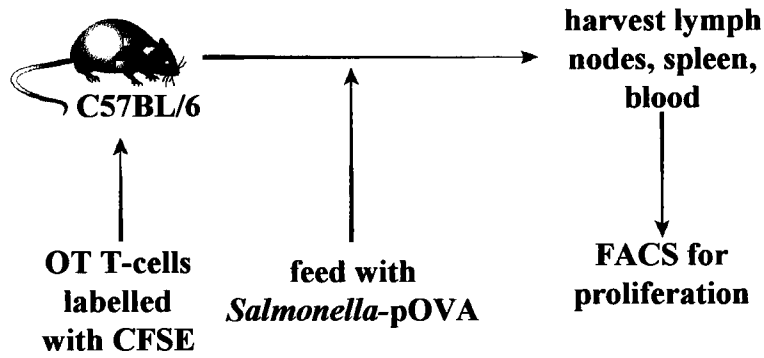


Figure 15

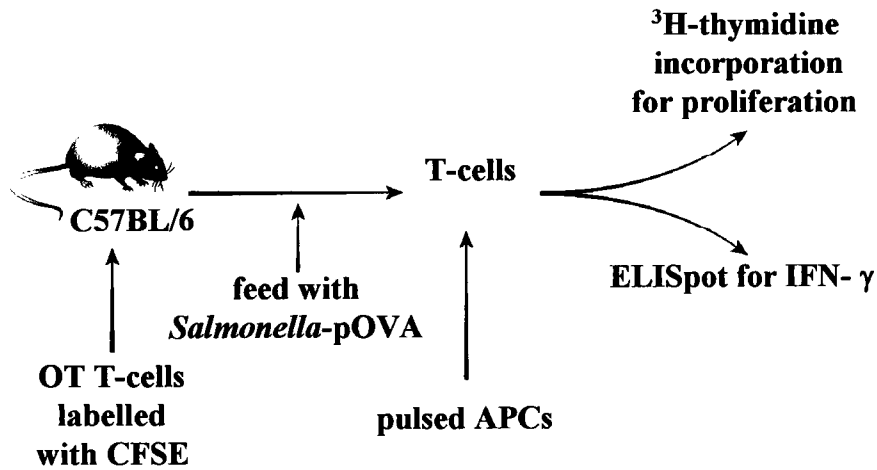


Figure 16

remained viable after bacterial infection, therefore, were stained for CD40, MHC class II and CD86. Flow cytometry data reveal that the *Salmonella*-exposed DCs did, in fact, mature. Moreover, they express relevant surface markers to a greater extent than DCs incubated with LPS as a positive control (Figure 17). This finding, too, is consistent with the observations of others (119, 120). Whether LPS-induced maturation occurs through the use of toll-like receptors (TLRs) (121, 122) or not (123, 124), it appears that *Salmonella*-induced NFκB activation is more pronounced than that by LPS alone (125).

Designing a vaccination trial to detect mucosal as well as systemic responses: another attempt to take the system *in vivo*

Despite our initial frustrations with the murine model, we persisted in hoping to achieve *in vivo* success with our DNA vaccine delivery system. Although we had observed evidence of substantial cytotoxicity on exposure of murine DCs to *Salmonella in vitro*, we hypothesized that there would be a greater chance of efficacious antigen presentation *in vivo*. Not only would the functional MOI be lower with indirect, oral administration, we would also likely benefit from the phenomenon of bystander DC presentation (78, 97-100). Furthermore, we were inspired by a number of publications documenting successful immunogenicity with *Salmonella* inoculation of mice (46, 60, 63, 65, 66, 126, 127), despite presumed toxicity towards DCs.

Figure 17 Infection of murine bone marrow-derived dendritic cells (BMDCs) with *S. typhimurium* induces maturation *Infection of BMDCs with* Salmonella *(at MOI=10) is seen to increase expression of relevant surface molecules. LPS was also added to a pool of uninfected cells as a positive control. Note that bacterial infection seems to yield a greater degree of maturation than LPS, as reflected in the relative degrees of expression of the maturation markers (CD-40, MHC-II and CD-86) detected in this study. "*Salm*/pVAX1" refers to DCs infected with bacteria harboring "empty" pVAX1 vector; "*Salm*/pOVA" refers to DCs infected with bacteria carrying pVAX1-*ova. *Not shown are the results of staining uninfected, unmanipulated BMDCs with the same antibodies as a negative control, which demonstrated minimal expression of the maturation markers studied.*

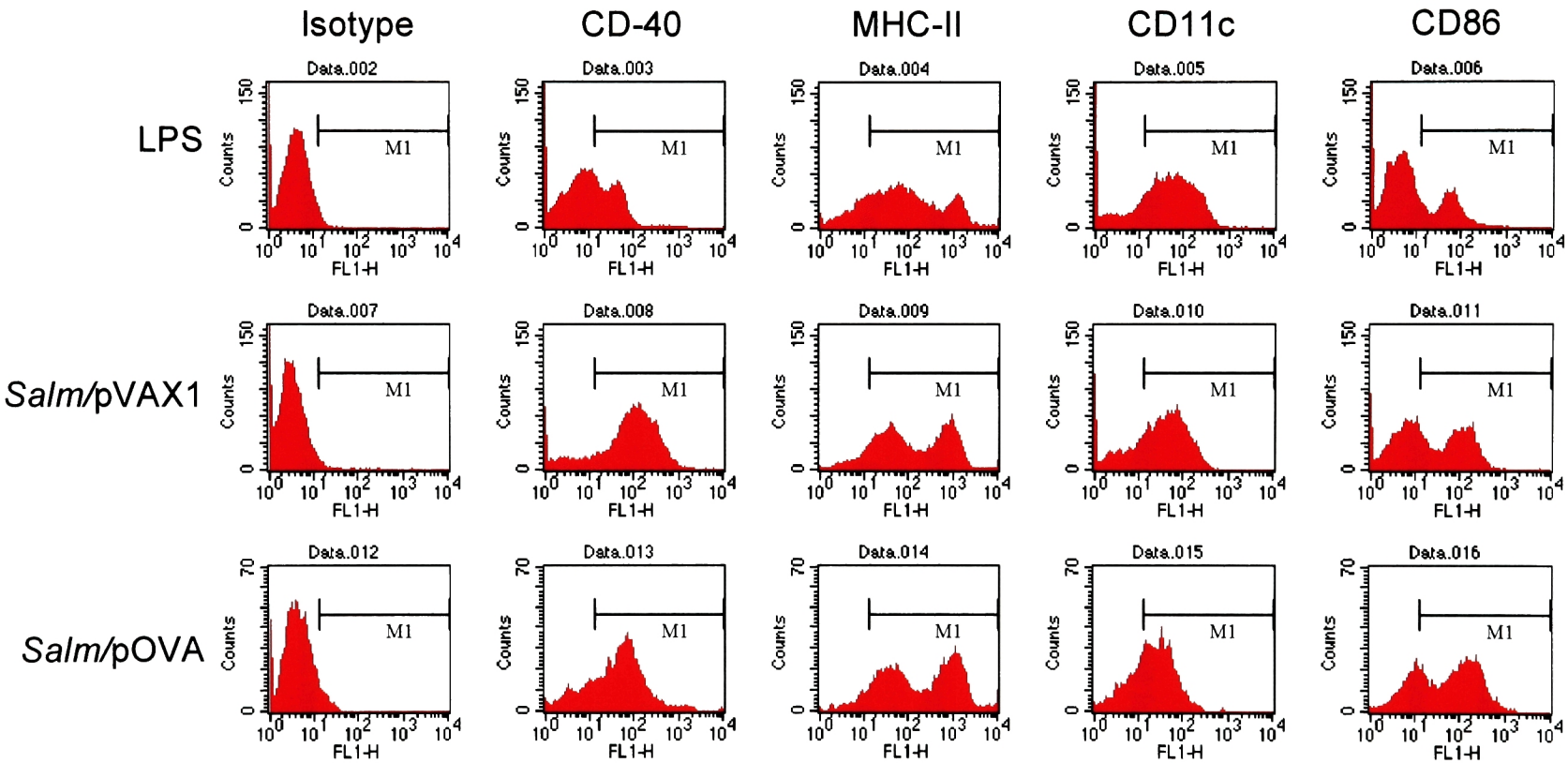

Isotype
CD-40
MHC-II
CD11c
CD86
LPS
Salm/pVAX1
Salm/pOVA
Data.002
Data.003
Data.004
Data.005
Data.006
Data.007
Data.008
Data.009
Data.010
Data.011
Data.012
Data.013
Data.014
Data.015
Data.016
M1
Counts
FL1-H

Figure 17

At a later date, we therefore proceeded to design and carry out an *in vivo* trial involving naïve BALB/c mice, which was the species available to us at this time (Figure 18). The plasmid vector with which we transformed our bacteria in this case encodes both HIV-1 *env* and *gag* under dual eukaryotic promoters. Mice were orally vaccinated three times over the course of 6 weeks using inocula of varying sizes. The group of mice serving as positive control was vaccinated with the same DNA vaccine vector intramuscularly, an approach already known to yield systemic humoral and cellular responses (128). Finally, both blood and feces were harvested for eventual ELISA, while splenocytes, mesenteric lymph nodes (MLNs) and Peyer's patches (PPs) were harvested for ELISpot assays. Once again, however, our efforts were limited by confounding circumstances: MLN cells proved too contaminated (by adipose tissue) to assay, and PP cells were too few. Splenocytes, on the other hand, were intact and usable but yielded no evidence of systemic immunity in response to Env or Gag re-stimulation *in vitro*. Similarly, humoral responses were seen by ELISA to be minimal or nil.

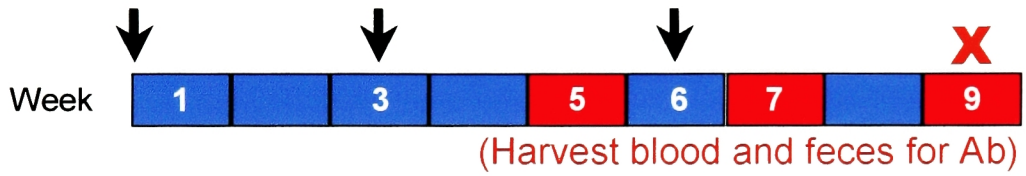
We were thus prompted to consider the basis for this apparent vaccination failure. Was there, for example, still too much APC death with the *Salmonella* doses we used? Additionally, we speculated about the possibility of tolerogenesis instead of immunogenesis: The gut, after all, is evolved for tolerance, and the mucosal immune system mounts tolerogenic responses most often, as it receives the myriad antigens associated with microbial flora or proteins in the food stream (31, 101, 129, 130). DCs are known to play a role in the mediation of tolerance (131, 132), and are

Figures 18 and 19 **A second attempt to take the system *in vivo*** *Two brief trials were designed wherein transformed bacteria were administered orally (PO) to BALB/c mice. Control groups received the vaccine vectors as naked DNA given intramuscularly (IM) (Figure **18**) or carried by* Salmonella *given intraperitoneally (IP) (Figure **19**). Doses were given as indicated, and the mice were sacrificed to permit tissue harvesting. Additionally, blood and feces were harvested during the course of the first trial (Figure **18**).*

Group (3 mice per group)

A	p *env-gag* IM (100μg)
B	*Salm*/pVAX1 PO (1×10^{10})
C	*Salm*/p*env-gag* PO (1×10^{10})
D	*Salm*/p*env-gag* PO (1×10^{9})
E	*Salm*/p*env-gag* PO (1×10^{8})
F	*Salm*/p*env-gag* PO (1×10^{7})
G	unvaccinated

Plan: 3 doses, 3 weeks apart
blood, feces – ELISA for Ab (IgG/A)
spleen, GALT – ELISpot for CMI



(Harvest blood and feces for Ab)

Figure 18

Group (3 mice per group)

A	*Salm*/p*gag* PO (1×10^{9})
B	*Salm*/p*gag* PO (1×10^{7})
C	*Salm*/p*gag* PO (1×10^{5})
D	*Salm*/p*gag* PO (1×10^{3})
E	*Salm*/p*gag* IP (1×10^{7})
F	*Salm*/p*gag* IP (1×10^{3})
G	unvaccinated

Plan: 1 dose, sacrifice after 2 weeks
spleen, GALT – ELISpot for CMI

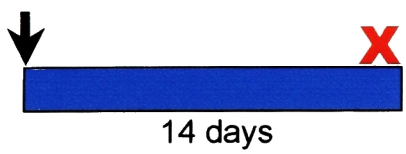


Figure 19

also known to be anatomically associated with both MLNs (133) and PPs (134). It would seem, however, that by virtue of their induction of DC maturation, our vaccine vehicles could eschew tolerogenesis (135, 136).

We proceeded by attempting a second, shorter *in vivo* trial involving a lower dose range and a simpler vector (encoding only HIV-1 *gag*). Additionally, we included a new group of mice that were inoculated via the intraperitoneal (IP) route (Figure 19). We were also sure to process our MLNs cleanly this time, by using a lymphocyte-separation medium formulated especially for murine cells.

Results of IFN-γ ELISpot assays revealed no spot-forming cells (SFC) from PPs, and only a moderate response (100 SFC/10^6 cells) from splenocytes in the group that had received the highest oral dose (10^9 bacteria). More significantly, the group that was vaccinated with the highest IP dose (10^7 bacteria) yielded ~300 SFC/10^6 from cells of the MLNs.

Thus, we see evidence of the system's efficacy *in vivo* only perhaps when the bacteria are delivered directly to APCs – in this case, within the peritoneum. Peritoneal macrophages, however, are known to be weak APCs (137), although they do produce a soluble factor that enhances the antigen-presenting capacity of DCs (138). While some groups have found that murine peritoneal macrophages do have the ability to prime T-cells (139-141), it may be more likely that DCs derived from peritoneal precursors did the effective presenting (142-145).

The weakness or absence of a response with oral administration, however, merits further consideration. One possible explanation is our *Salmonella* strain choice. – Is it so much more toxic than the various other strains used in effective murine trials (46, 60, 63, 65, 66, 68, 126, 127, 146)? One group, after all, used our strain (14028) specifically when apoptosis of murine APCs was the desired outcome (78). Similarly, different *Salmonella* strains may display distinct physiologic dynamics with respect to exiting the gut and reaching relevant lymphoid tissue. Thus, the timing of our sample harvesting and immunological assaying may have been sub-optimal (147).

Salmonella infection of human dendritic cells induces maturation

Because of the unique importance of DCs as priming APCs, we wanted to further confirm the ability of our *Salmonella*-based vaccine delivery system to reach and activate these cells. Rather than continue to use murine tissue, however, it was sensible instead to return to human cells.

We therefore undertook to isolate human PBMCs and cultivate DCs *in vitro* (See Chapter II for protocol details). Infection of the immature cells with *Salmonella* was carried out in a manner similar to that of our previous protocol involving macrophages (Figure 20). Although we saw considerably less cytotoxicity on

exposure to *Salmonella* than with murine DCs, we did appreciate a greater level of cell death relative to human macrophages infected with the same bacteria at the same MOI (not shown).

Approximately 36 hours post-infection, the DCs were pooled and stained for maturation markers. Flow cytometry results of the initial assay are shown in Table 6.

	MEAN VALUES			
	CD 80	CD83	CD 86	CD 25
UNINFECTED	71.45	45.05	1082.05	17.92
***SALMONELLA*-INFECTED**	101.02	61.84	1655.08	32.28

Table 6

Later, we also stained for DC-LAMP, with LPS added to one pool of cells as a positive control (Figure 21).

Figure 20 Infection of human dendritic cells (DCs) with *S. typhimurium* induces maturation *Human PBMCs were isolated, then subjected to CD14+ selection. A week-long cultivation of these cells in the presence of GM-CSF and IL-4 yielded immature DCs. Infection with* Salmonella *was performed at a lower MOI than macrophages (25), and washing was done carefully, so as not to lose these non-adherent cells.*

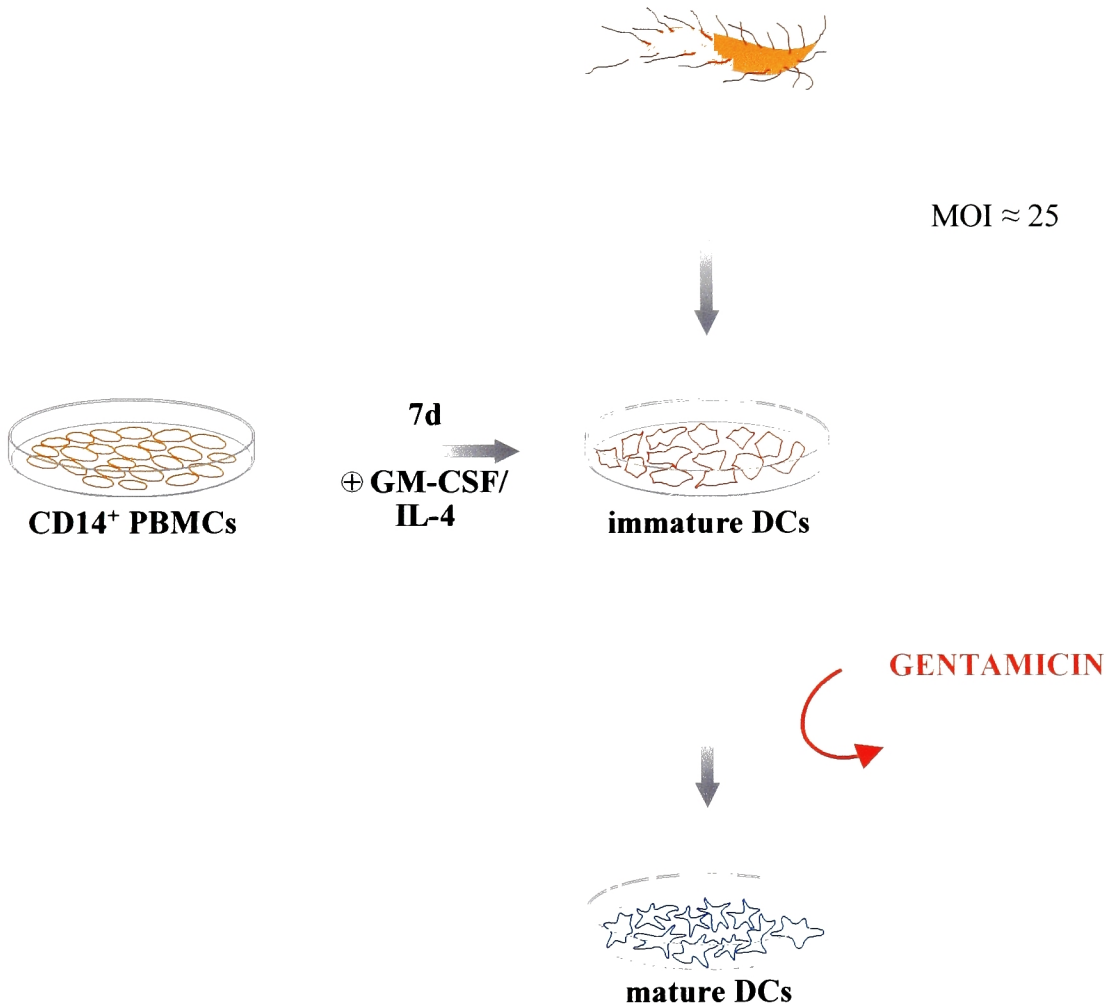

MOI ≈ 25
7d
⊕ GM-CSF/
IL-4
CD14+ PBMCs
immature DCs
GENTAMICIN
mature DCs

Figure 20

We see, therefore, that *Salmonella* infection of human DCs results in increased expression of molecules associated with DC maturity. Furthermore, in the context of vaccine design, DC maturation is a wholly desirable effect (135). Our observation that *Salmonella* induces efficient human DC maturation is consistent with the findings of other groups (119, 120, 135, 148). It should also be noted that, as a potential tool of vaccination, *Salmonella* compares favorably in this regard with other microbes, which have apparently evolved instead to interfere with DC maturation (118).

In addition to the up-regulation of known maturation markers, it has been observed that *Salmonella* infection of DCs also generally activates them, resulting in part in the production of chemokines that work to recruit macrophages, neutrophils, natural killer cells and other DCs (119, 149). At the level of activation of members of the NFκB family, in fact, *Salmonella* is seen to activate DCs better than pure LPS (125). Cytokines secreted by DCs as a consequence of *Salnonella* exposure include TNF-α, IL-12, IL-6 and IL-18 (116, 117, 150, 151).

Figure 21 Infection of human DCs with *S. typhimurium* induces maturation

Cells were stained with either an isotype control or antibodies against the maturation markers listed. Note that CD83 is a surface marker, while DC-LAMP requires that the cells be permeabilized. Control cells received the additional known maturation stimulus of pure LPS. Not shown here are similar flow images reflecting enhanced expression of CD80, CD83, CD86 and CD25 with Salmonella *infection of human DCs.*

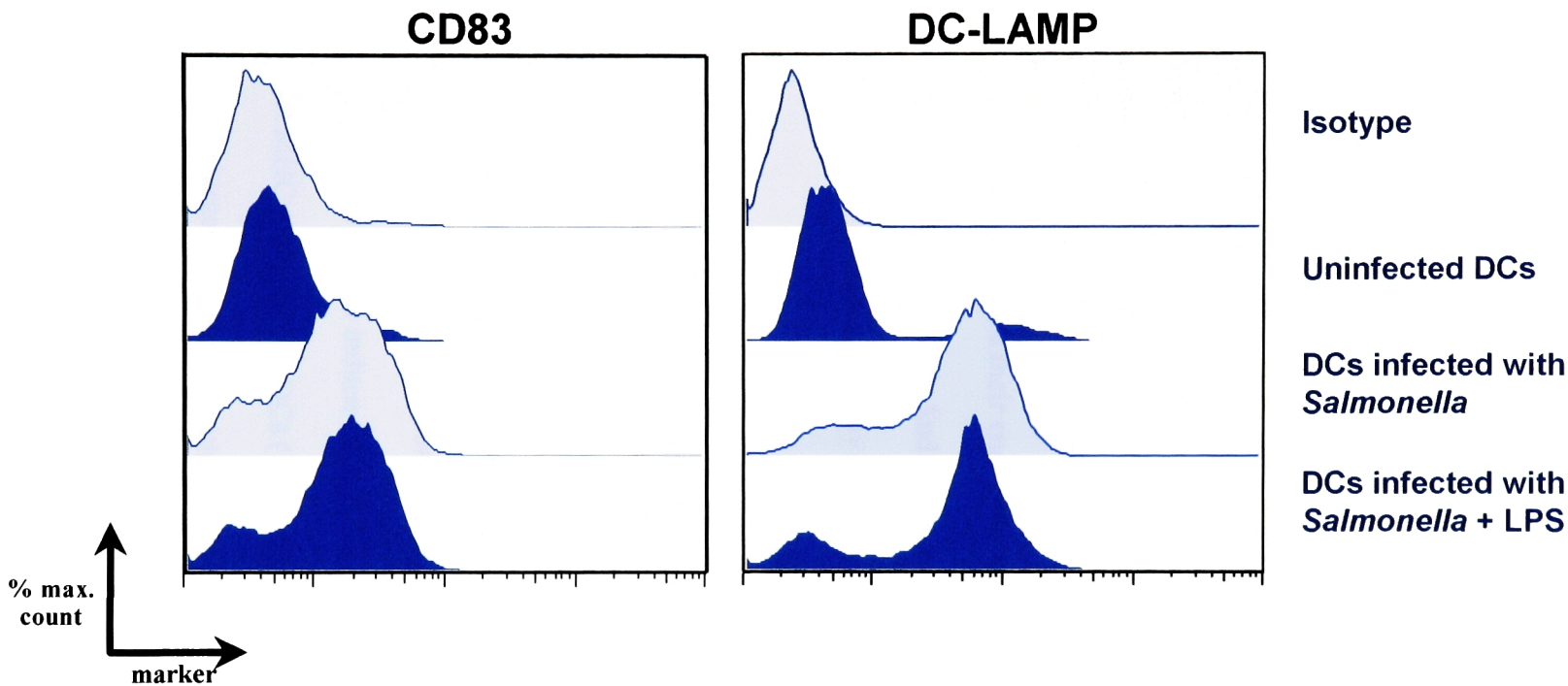

CD83
DC-LAMP
Isotype
Uninfected DCs
DCs infected with Salmonella
DCs infected with Salmonella + LPS
% max. count
marker

Figure 21

DISCUSSION

While immature DCs are specialized in capturing and processing antigens into peptides towards MHC presentation, mature DCs are initiators and modulators of the immune system (131, 152). Indeed, the maturation of DCs is a crucial step in the generation of an immune response.

The nature of possible interactions between DCs and *Salmonella*, therefore, is an important consideration for us. Will the attenuated bacteria be a hindrance or a help in our attempt to deliver DNA vaccines to APCs? Will *Salmonella* effectively target DCs *in vivo*? Will DCs survive their encounter with the bacteria, at least long enough to present to, and prime, T-cells? And will our plasmid-delivery vehicles themselves trigger maturation of DCs, thereby ensuring an APC that is ready for immunogenesis?

Although macrophages were long regarded as the main target of *Salmonella*, it is now known that the bacteria are first internalized by DCs. In fact, one study suggests that DCs actually represent more receptive host cells than macrophages (153). Furthermore, in addition to the induction of maturation and general activation described herein, bacteria are seen to induce the neo-biosynthesis, stabilization and surface expression of MHC molecules in DCs (148). Thus, evidence suggests that *Salmonella* would likely target and interact with DCs in a manner appropriate for vaccine delivery.

It has recently been observed by one group that encounters between *Salmonella* and monocytic precursors in the skin prevent differentiation into DCs (154). Nonetheless, the same investigators have found that encounters between the bacteria and differentiated DCs yield an effective immune response towards antigens expressed by the *Salmonella* (155). Furthermore, LPS and inflammatory cytokines associated with *Salmonella* infection are known to stimulate the mobilization of tissue-based DCs from the periphery to secondary lymphoid organs (156, 157). Thus, although *Salmonella* will not encourage the conversion of monocytes to dendritic cells, it is very likely that they will encounter existent dendritic cells in the course of infection (as with any microbe) or inoculation.

Interestingly, Svensson et al. note that the increased surface expression of MHC-I, MHC-II, CD86, CD40 and CD54 that they observe on DC encounter with *Salmonella* does not require bacterial viability or internalization (120). Thus, it may be speculated that bacterially induced maturation and activation on this level is purely a function of LPS exposure, and would therefore likely be a matter of TLR signaling (121, 122, 124). In this way, bacteria can be considered to harbor and deliver a built-in vaccine adjuvant (158).

On the other hand, the same group also notes that the up-regulation of CD80 expression does in fact require viable, invasive bacteria and internalization (120). Thus the mechanism of such up-regulation associated with the maturation process is unclear. It can be speculated, however, that the bacterially induced release of

cytokines such as TNF-α works in a positive feedback manner to encourage maturation.

If we are to undertake to use *Salmonella* to deliver vaccines to DCs then, we must also concern ourselves with the potential effects that bacterial virulence factors would have on their targets. The possible induction of apoptosis has been established (*vide infra*), although we may perhaps avoid this fate by choosing our strain carefully. But what of mechanisms the bacteria have evolved towards escaping immune surveillance as they persist within their host cells? Expression of many of the relevant virulence proteins are under the control of the transcription regulator *PhoP*. Studies show, however, that *PhoP* has no bearing on the ability of DCs to internalize *Salmonella*, or to go on to process and present antigens (153). Moreover, *PhoP* does not affect the maturation process induced in immature DCs that encounter *S. typhimurium*, specifically (120). Thus, it is unlikely that *Salmonella* virulence factors would interfere with the use of the bacteria in targeted vaccine delivery.

Hence we see that interaction with *Salmonella* stimulates DCs to mature, a critical step towards becoming activators and regulators of T-cells. Such an effect is precisely what is required of a vaccination tool.

Chapter V:

Human dendritic cells can be transduced *in vitro* using attenuated *Salmonella typhimurium*

INTRODUCTION

As the most potent and effective APCs known, DCs should be a central concern in vaccinology. They alone initiate immune responses by stimulating quiescent, naïve and memory T lymphocytes. Indeed, they serve as a unique liaison between antigen and T-cell; antigens that have no contact with DCs are ignored by T-cells (159). Furthermore, DCs are the sentinels in immune surveillance and can migrate when necessary to optimize clonal selection. Moreover, in addition to activating lymphocytes, they also work to tolerize T-cells to self-antigens. Thus, DCs can be a powerful tool for manipulating the immune system (131), and the potential for targeting DCs should be a major consideration in vaccine design.

Since the singular significance of DCs was recognized, in fact, many groups have indeed undertaken to target, or otherwise involve, DCs in their immunization strategies. Against infectious pathogens, in particular, DCs have been specifically targeted with adenovirus-based vectors (160-162), recombinant yeast vectors (163), liposome-encapsulated proteins (164), pseudo-typed virus (165) and synthetic peptide immunogens (166). DCs have also been effectively reached by DNA vaccines (167-169), including in the skin through the use of a topical adjuvant (170). Linking vaccines to Fms-like tyrosine kinase receptor-3 ligand (Flt-3L) (171) or to the

lysosomal-associated membrane protein 1 (LAMP1) targeting sequence (172) has also resulted in successful DC contact.

After designing our system of facilitated DNA vaccine delivery in the context of human macrophages, we went on to demonstrate that our aroA- bacteria can infect and mature human DCs. We were therefore inspired to continue working with human DCs, in order to verify that the strategy itself can be used to target these all-important cells. We were encouraged, too, by the work of other groups, who had successfully reached DCs with bacterially-based systems (60, 68, 127, 173-177).

RESULTS

Attenuated *Salmonella* can deliver a reporter gene vector to human dendritic cells: eukaryotic expression of β-galactosidase detected by incubation with substrates and flow cytometry

. We were interested in reproducing some of our early macrophage studies, this time in the context of primary human DCs. We therefore once again chose to work with reporter gene vectors in order to demonstrate manifestly that attenuated *Salmonella* can be used to deliver functional plasmids to target cells.

We began then by isolating human PBMCs, then cultivating DCs *in vitro*, as before (See Chapter II for procedural details). Using a protocol based on our

infection of macrophages, we infected immature DCs with aroA- *S. typhimurium* (33275) bearing either pTrc-β or pSLICK-z (Figure 22). After 1 hour of exposure to the bacteria, careful washing with antibiotic-containing medium and overnight incubation, the β-galactosidase substrate, x-gal, was added to the wells (See Chapter II for precise protocol). Overall results of the staining are listed in Table 7.

DENDRITIC CELLS INFECTED AT MOI=50 BY . . .	β-GALACTOSIDASE EXPRESSION (BLUE COLOR)	STAINING MORPHOLOGY
nothing	no	n/a
untransformed bacteria	no	n/a
bacteria transformed with irrelevant plasmids (e.g., pCDNA3.1/SIV*env*)	no	n/a
bacteria transformed with pTrc-β (prokaryotic promoter)	<1%	scattered, punctuate
bacteria transformed with pSLICK-z (CMV promoter)	30-50%	diffuse, intracellular

Table 7

As with macrophages that were infected with these same bacteria, the difference in staining morphology is clear: Cells infected with *Salmonella* carrying the prokaryotic vector show a spottiness suggestive of contained bacteria that are

Figure 22 ***Salmonella*** **can be used to transduce human DCs** ***in vitro*** **such that they express a reporter gene** *AroA- S. typhimurium (33275) bearing different β-galactosidase vectors, as shown, were used to infect immature human DCs (MOI=25). Approximately 40 hours post-infection, the cells were fixed, then incubated with x-gal, a β-galactosidase substrate.*

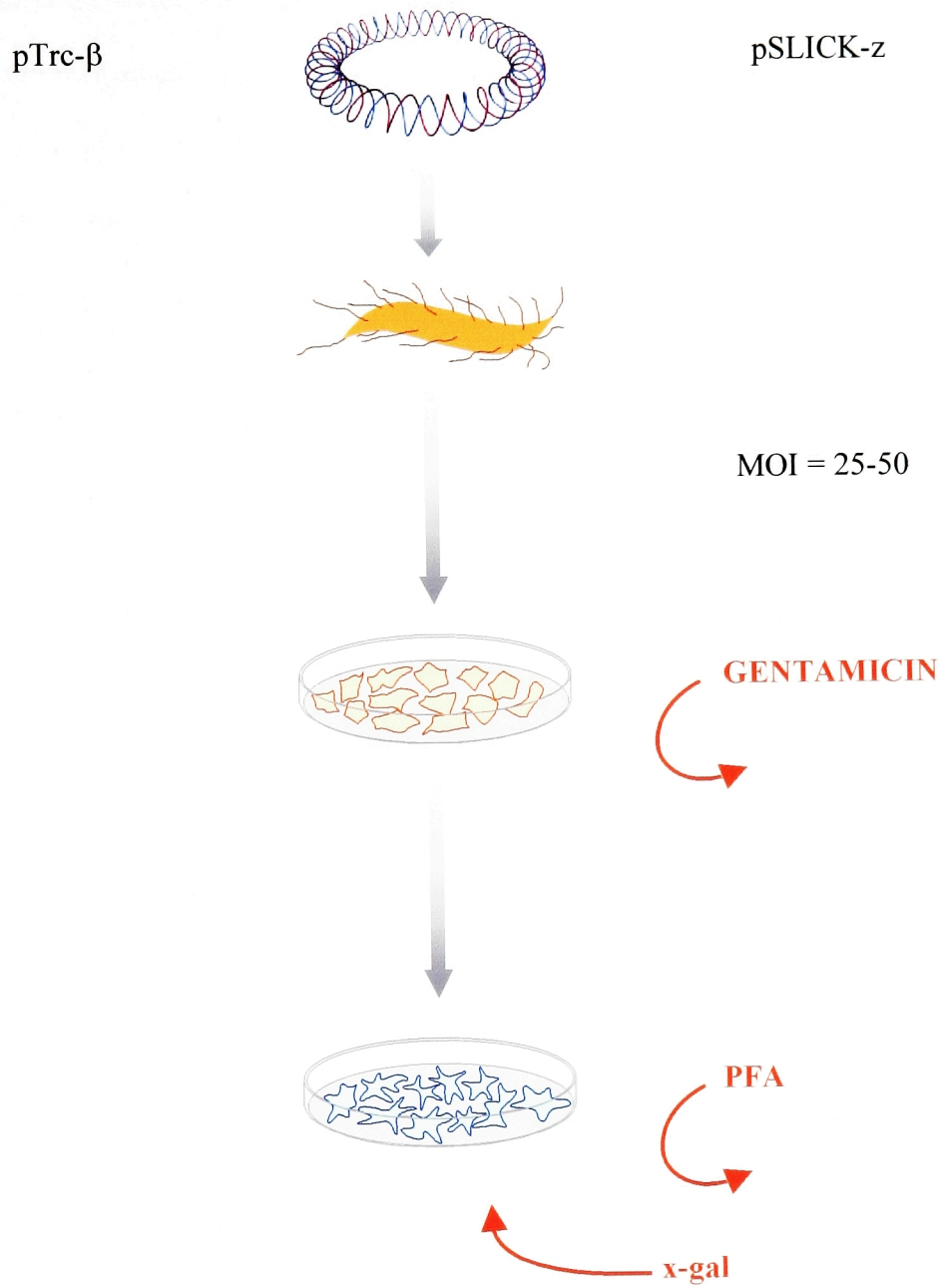


Figure 22

expressing the reporter gene themselves, while DCs infected with bacteria harboring the eukaryotic vector stain diffusely, apparently throughout the cytosol (Figures 23-25). At higher magnification, a mature DC phenotype is grossly evident (Figure 25).

As before, when we varied MOI, we appreciated a dose-dependency reflected in the relative number of cells staining blue per well (not shown). Staining 36-48 hours after infection, for example, we saw very few blue cells per field under low magnification with MOI=5 (0-4), in contrast to the number of blue cells per field with MOI=50 (60-70). We found, however, that human DCs (not unlike murine DCs) are more sensitive to the cytotoxic effects of the bacteria than human macrophages (as evident in trypan blue staining after exposure). Thus, optimal MOI for maximal staining of DCs is 35-50 (as opposed to 85-100 with macrophages).

Using the manifest quantification permitted by this reporter gene model, we also undertook to compare the ability of our bacteria to deliver pSLICK-z to immature DCs versus mature DCs. Staining with x-gal revealed that the attenuated *Salmonella* targets immature cells more effectively than mature cells (not shown), an observation that is not surprising given the antigen-capturing, endocytosing function of immature DCs *in vivo* (131).

We next chose to take advantage of the non-adherent nature of DCs, an experimental convenience that we did not have when working with macrophages. Thus, we could now manipulate and stain our target cells towards flow cytometric

Figures 23-25 ***Salmonella*** **can be used to transduce human DCs** ***in vitro*** **such that they express a reporter gene** *Immature DCs were infected at MOI=50 with bacteria carrying either pTrc-β or pSLICK-z. Approximately 40 hours post-infection, cells were incubated with x-gal, a β-galactosidase substrate. Cells exposed to bacteria carrying the prokaryotic promoter-driven vector (Figures* ***23A*** *and* ***24A****) show spotty, punctuate staining, suggestive of contained bacteria expressing the vector themselves within the cell. DCs infected, on the other hand, with* Salmonella *carrying the eukaryotic promoter-driven vector at the same MOI show more widespread color, with a diffusely intracellular pattern, suggesting DC expression (Figures* ***23B*** *and* ***24B****). At higher magnification (Figure* ***25****), a distinctly mature DC phenotype can be appreciated.*

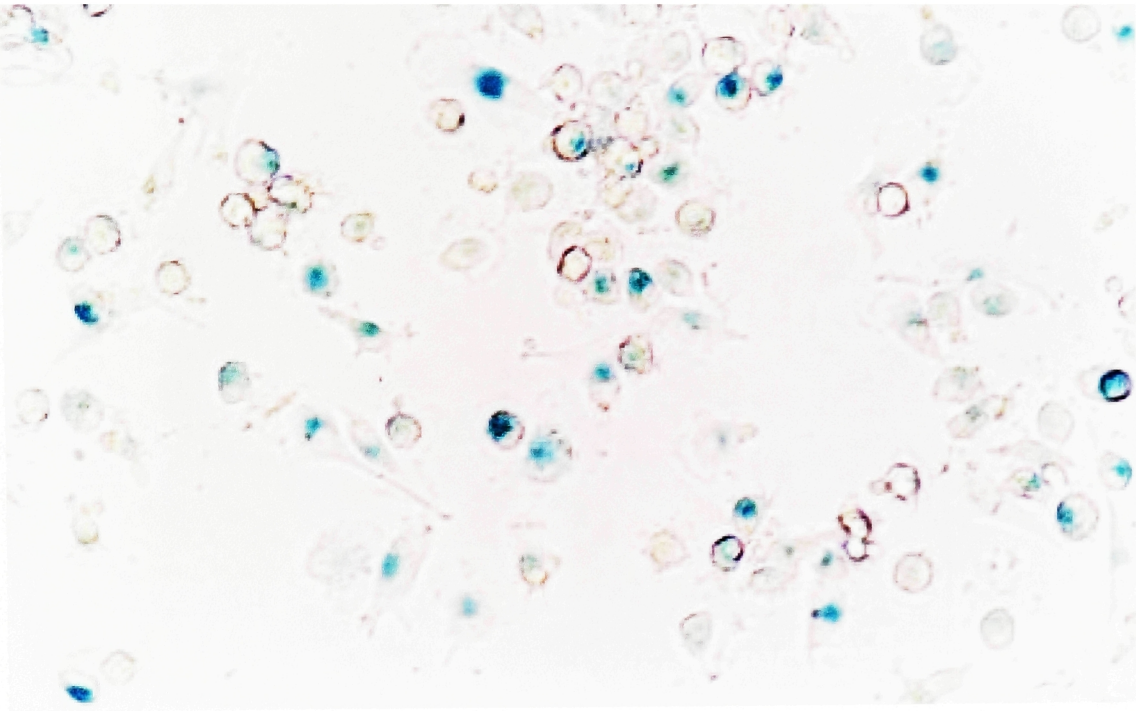


Figure 23A

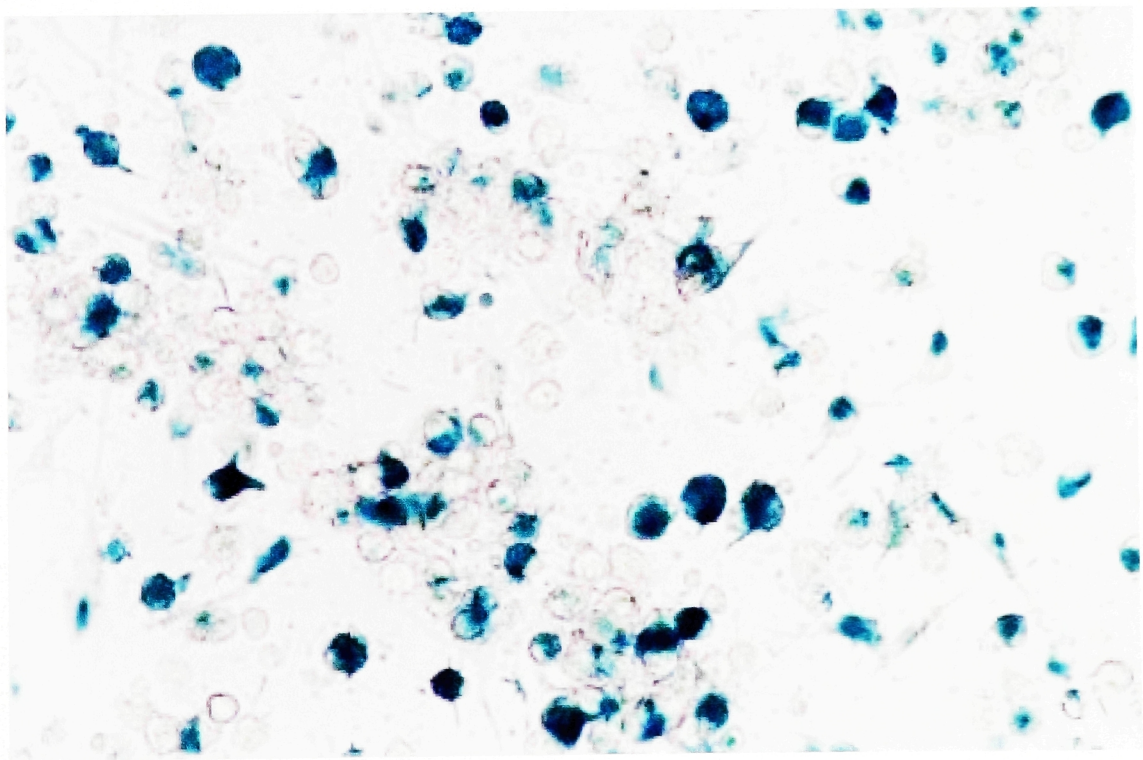


Figure 23B

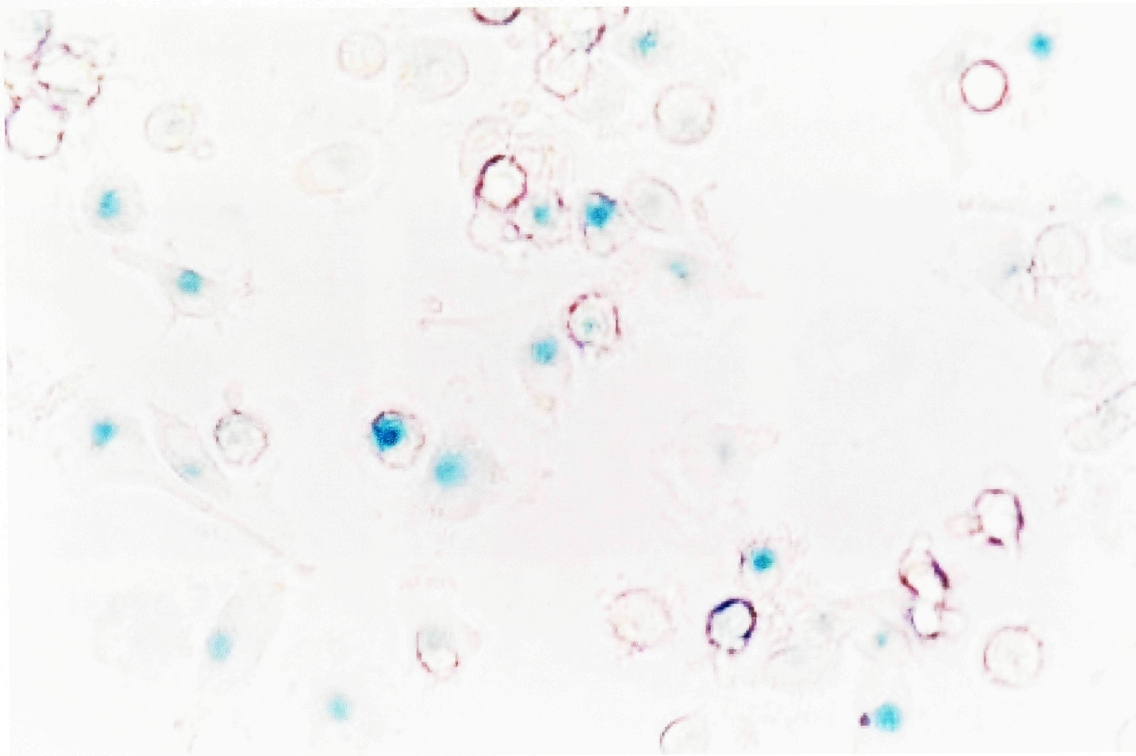


Figure 24A

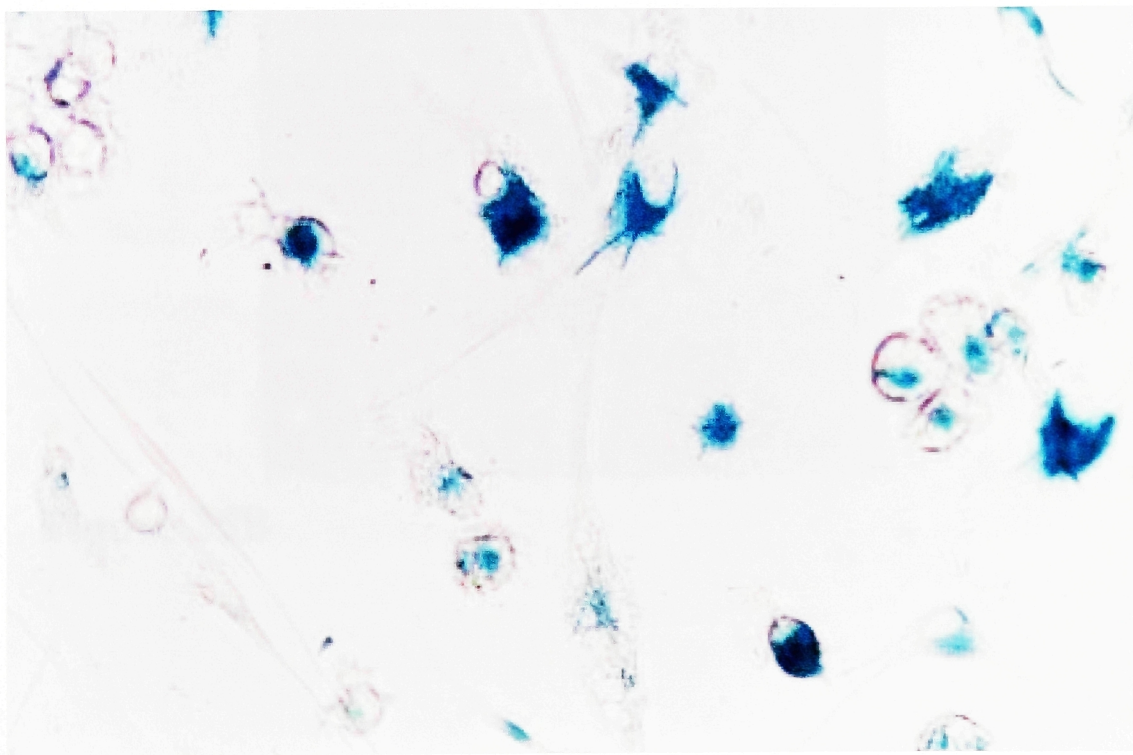


Figure 24B

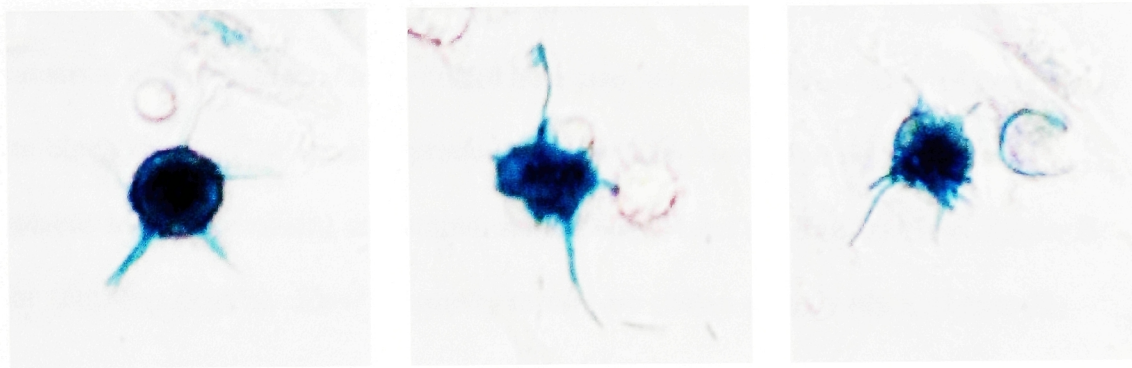


Figure 25A

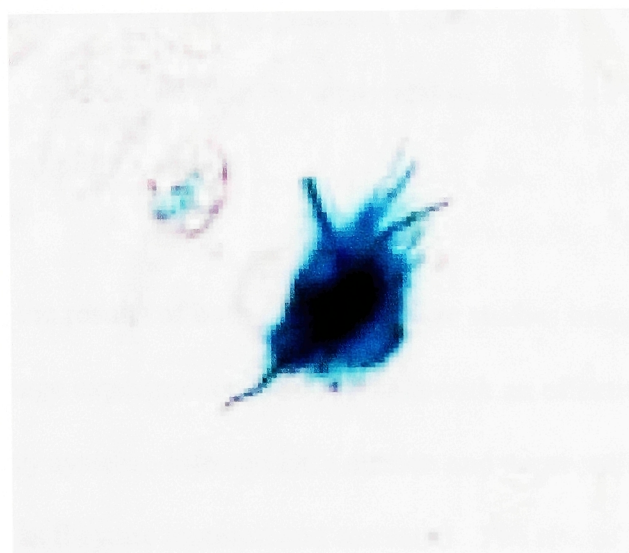


Figure 25B

analysis. Our initial use of the technology began once more with the β-galactosidase reporter gene vectors. Instead of incubating the cells with x-gal, however, we incubated them with fluoroscein di- β-D-galactopyranoside (FDG), a fluorescent substrate of the enzyme. Our protocol here also involved incubation with verapamil (to block efflux of the reaction product), chloroquine (to inhibit endogenous β-galactosidase expression) and propidium iodide (to label dead cells) (See Chapter II for complete details). Flow cytometry results are shown graphically in Figure 26.

Although we can appreciate the dose-dependency of protein expression seen with an increase in MOI, we may also note the limitation of cytotoxicity – evident, for example, in the relative scales of the y-axes in the two graphs. With MOI=50, there were far fewer viable cells within our analytical gate, which excluded DCs staining positive for propidium iodide.

Nonetheless, the results of both β-galactosidase studies involving DCs, like those of our macrophage experiments, are consistent with an effective *Salmonella*-based plasmid delivery system. Infected DCs receive and express the vector carried by the bacteria, even as they are matured and activated. The risk of cytotoxicity can apparently be minimized by decreasing MOI.

Figure 26 ***Salmonella*** **can be used to transduce human DCs** ***in vitro*** **such that they express a reporter gene** *Approximately 40 hours post-infection, DCs were incubated with fluoroscein di-β-D-galactopyranoside (FDG), a fluorescent substrate of β-galactosidase, as well as verapamil (to minimize efflux of the reaction product) and chloroquine (to inhibit endogenous β-galactosidase expression). Additionally, propidium iodide was added to allow us to gate specifically on living cells. Graphs on the left represent cells infected with bacteria carrying pTrc-β; graphs on the right reflect DCs infected with pSLICK-z. With the change in MOI, a dose-dependent effect can be appreciated. Furthermore, the change in y-axis scale suggests an increase in cytotoxicity with higher MOI.*

MOI = 25

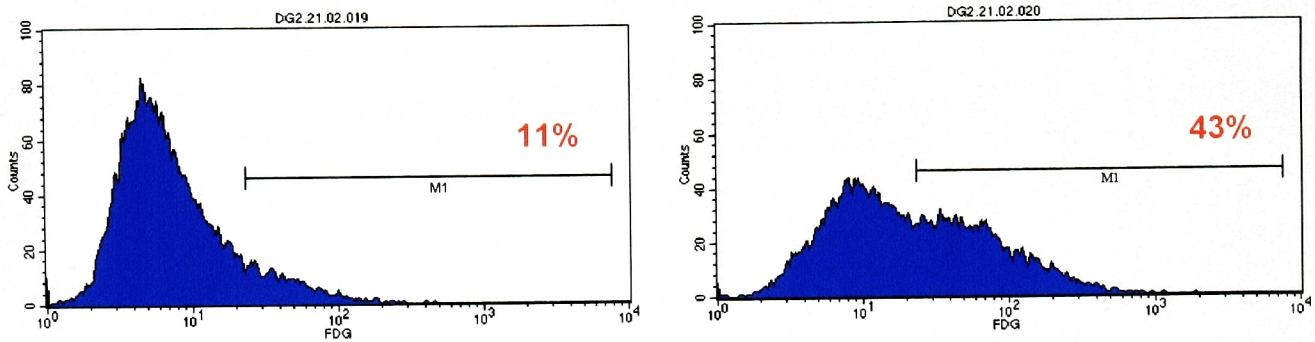


Figure 26A

MOI = 50

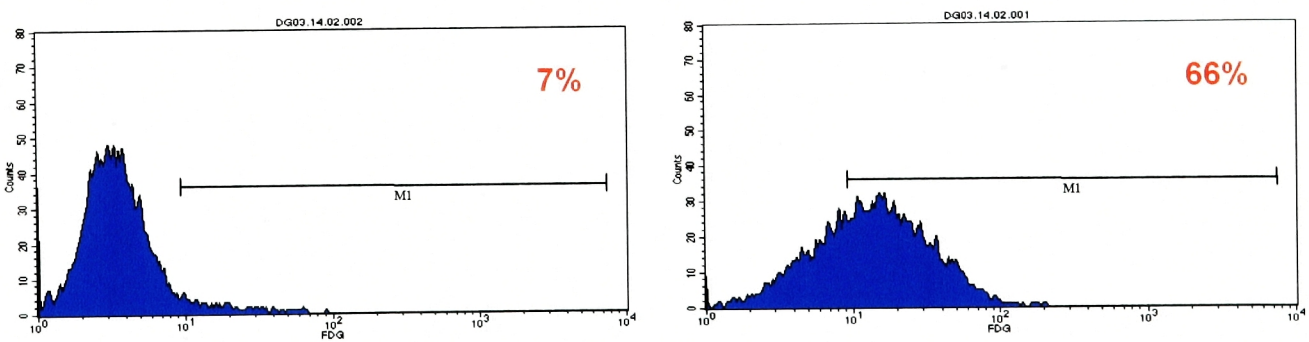


Figure 26B

Attenuated *Salmonella* can deliver a viral gene vector to human DCs: eukaryotic expression of HIV-1 *gag* detected by immunocytochemistry and flow cytometry

We continued to explore our strategy using human DCs because we felt that it was important to confirm efficacy with this cell population. Once we had evidence of successful bacterial-assisted transfer of the plasmid encoding β-galactosidase, however, we wanted to test the system using a vector containing a viral gene.

Thus, we began this set of experimentation again by transforming aroA- bacteria (14028) with a eukaryotic promoter-driven vector, this time a plasmid encoding HIV-1 *gag* (pVAX1/HIV*gag*). This vaccine vector is known to yield particularly high levels of expression in human cells in part because its genetic sequence was chosen on the basis of eukaryotic codon preference (See Chapter II regarding details of plasmid design and construction).

Human PBMCs were isolated in the usual way, and DCs were cultivated as before. At this time, however, it was determined that the DCs could be more easily infected and manipulated in small tubes, rather than in wells (Figure 27). In this way, fewer cells were lost in washing. Once infected (at MOI=50), the cells were re-plated for overnight incubation.

Figure 27 The new protocol used to infect human DCs with *Salmonella* more effectively *Cells were put into Eppendorf® tubes at 2 x 10^6/500μL, and incubated with loose caps. Spinning at 2000rpm x 5 minutes permitted more thorough washing, after which the cells could be re-plated at 10^6/2mL culture medium until time of assay.*

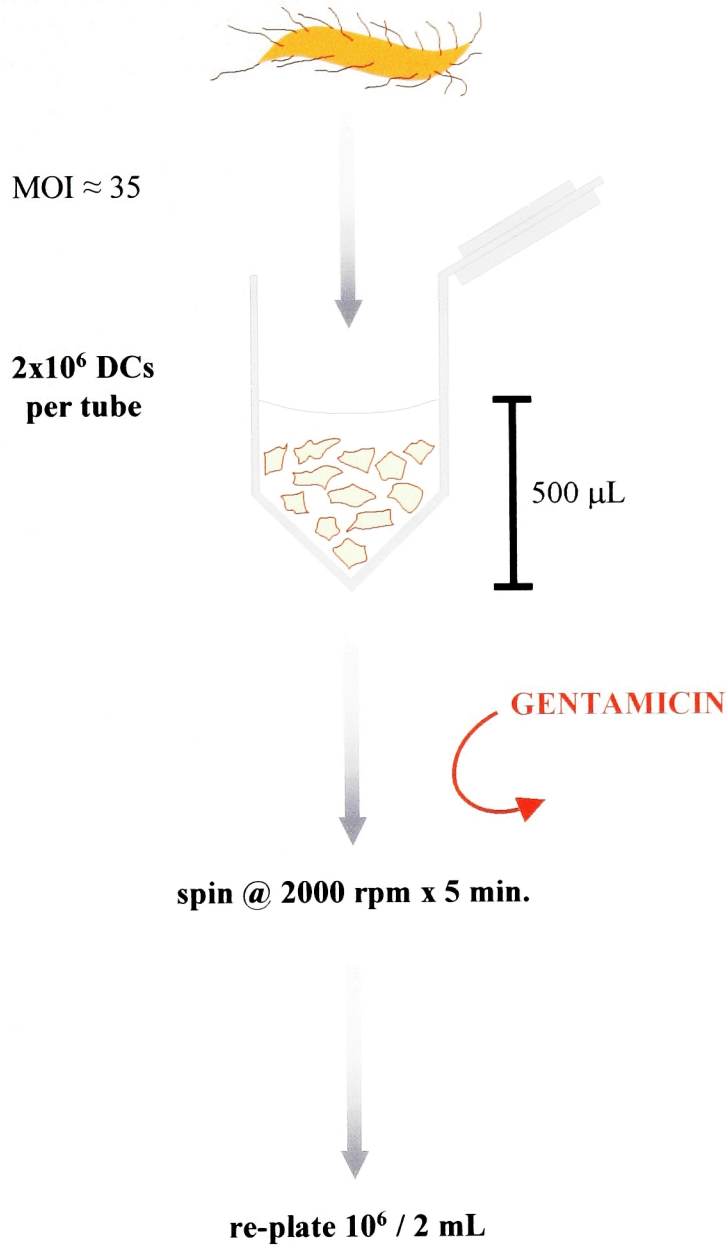

MOI ≈ 35
2x10⁶ DCs per tube
500 µL
GENTAMICIN
spin @ 2000 rpm x 5 min.
re-plate 10⁶ / 2 mL

Figure 27

The first way in which we chose to analyze the cells for viral gene expression was immunocytochemistry. For this purpose, we used a "cytospin" technique to mount DCs onto glass slides approximately 40 hours post-infection (See Chapter II for details). Cells were then fixed and blocked before being incubated with HIV-infected patient's serum as a source of primary antibody. (This approach was chosen to be specific for HIV-1 subtype C, the same clade as the genetic source of the vector's *gag* insert.)

Results of the staining are listed in Table 8 and illustrated photographically in Figures 28-30.

DENDRITIC CELLS INFECTED AT MOI=50 BY . . .	Gag STAINING
bacteria carrying an irrelevant plasmid (e.g., pSLICK-z)	~1%
bacteria transformed with pVAX1/HIV*gag*	~90%

Table 8

Control cells in this study were infected with bacteria carrying an irrelevant plasmid (e.g., pSLICK-z). Immunocytochemical staining revealed dramatic visible differences between the two infectious categories, reflecting what we must conclude is broad, effective uptake and expression of the vaccine vector by the appropriately-infected DCs. Indeed, on higher magnification (Figure 30), we can appreciate a certain deepening of the staining color in the perinuclear huff, suggesting a state of

active protein production (178), and consistent with the known trafficking and localization pattern of Gag in human cells (179). The high percentage (90%) of cells that stained positive for Gag expression is consistent with the relatively optimized conditions achieved in this study, including invasiveness of bacterial strain (14028) and expressiveness of vector (pVAX1 with "codon-optimized" insert).

We proceeded by evaluating the efficacy of bacterial pVAX1/HIV*gag* delivery in a second way, taking advantage once again of flow cytometry resources. In this study, we found that we could successfully employ a commercially-available antibody that was originally raised against HIV-1 subtype B p24. Despite clade differences, it seems that there is sufficient homology within *gag* across subtypes (180, 181).

Thus, we again infected (MOI=35) human DCs with *Salmonella* carrying different vectors, then incubated them with the fluorophore-conjugated antibody (See Chapter II for protocol). The cells were ultimately fixed and assayed for protein expression using flow cytometry. Results are represented graphically in Figure 31.

The flow cytometric measurement of relative levels of p24 (Gag) expression is consistent with our immunocytochemical results. We see that we can gauge DC expression of the foreign protein in both a gross histological way and in a more quantitative fashion.

Figures 28-30 ***Salmonella*** **can be used to transduce human DCs** ***in vitro*** **such that they express a viral gene** *DCs were infected at MOI=50 with bacteria carrying either an irrelevant plasmid (Figures* ***28A*** *and* ***29A****) or pVAX1/HIV*gag *(Figures* ***28B****,* ***29B*** *and* ***30****), which encodes the viral gene under a CMV promoter. Cells were mounted on glass slides using a "cytospin" approach (in contrast to the cells illustrated in Figures* ***23-25****). Hence, particulate matter seen represents an artifact of the technique, which affixes all contents of the medium to the slide at once. (Note that visible, unstained bacteria may serve as an additional control.) Immunocytochemical staining was carried out as previously, using patient serum as a source of primary antibody, and the Vectastain® ABC-AP kit for secondary antibody and substrate.*

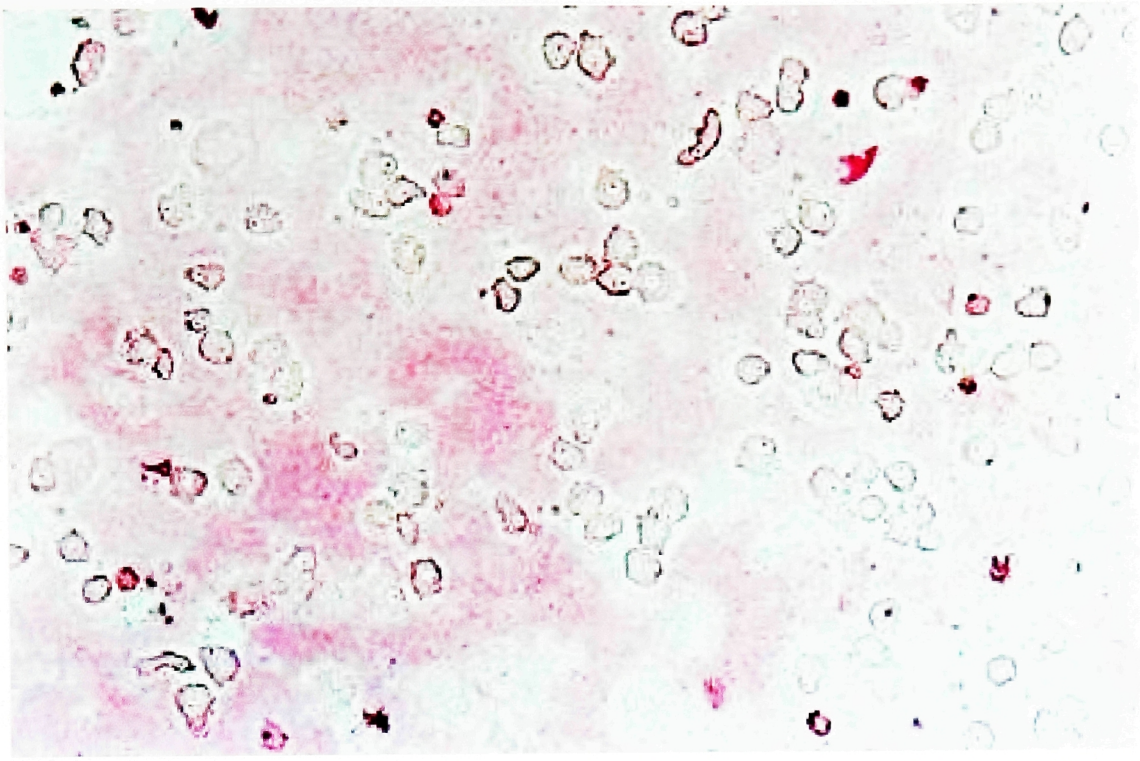


Figure 28A

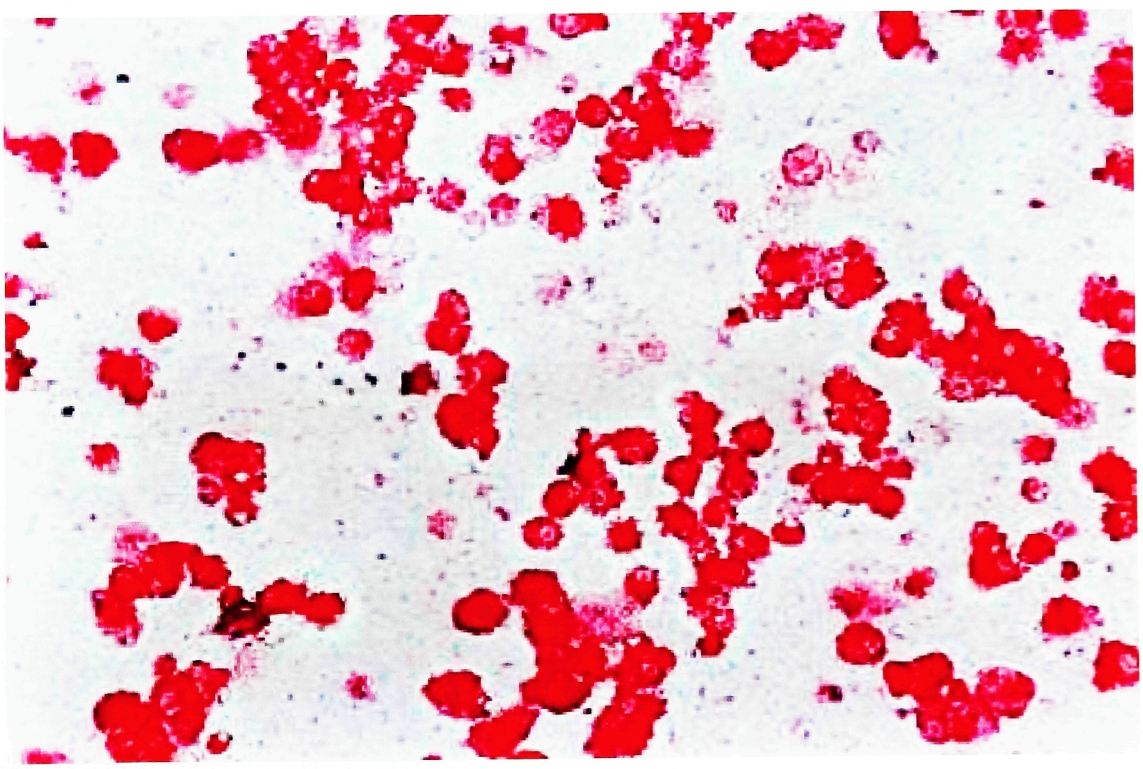


Figure 28B

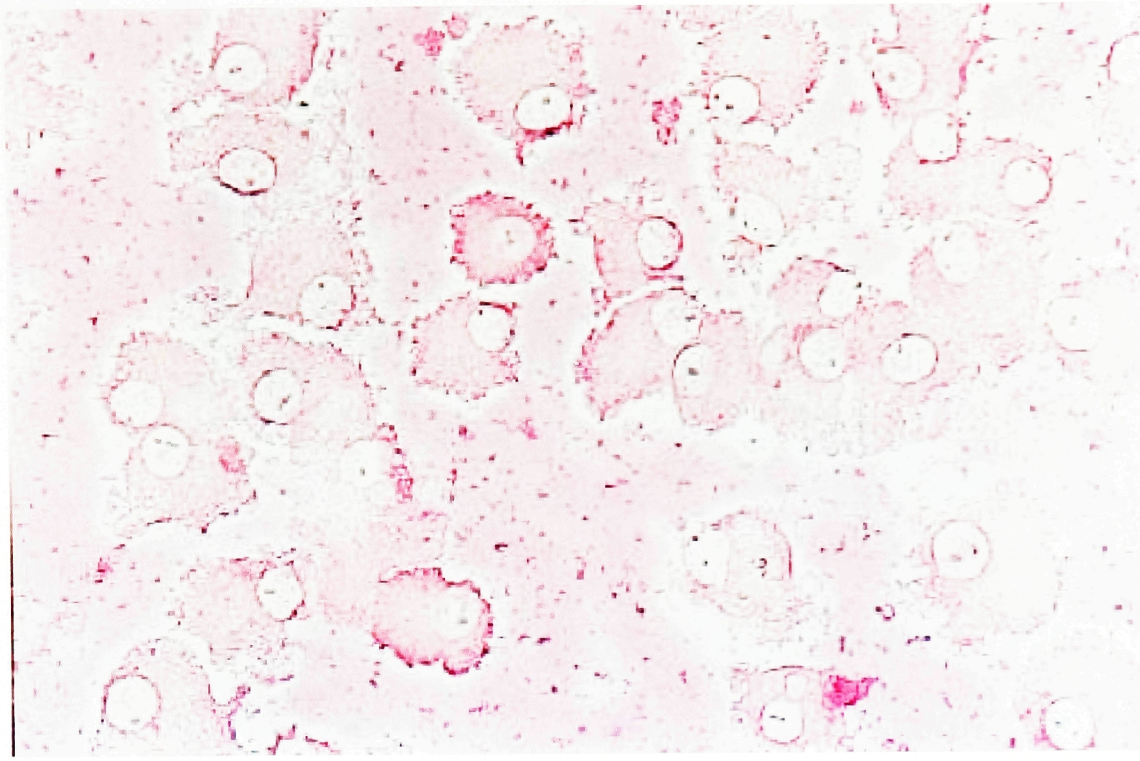


Figure 29A

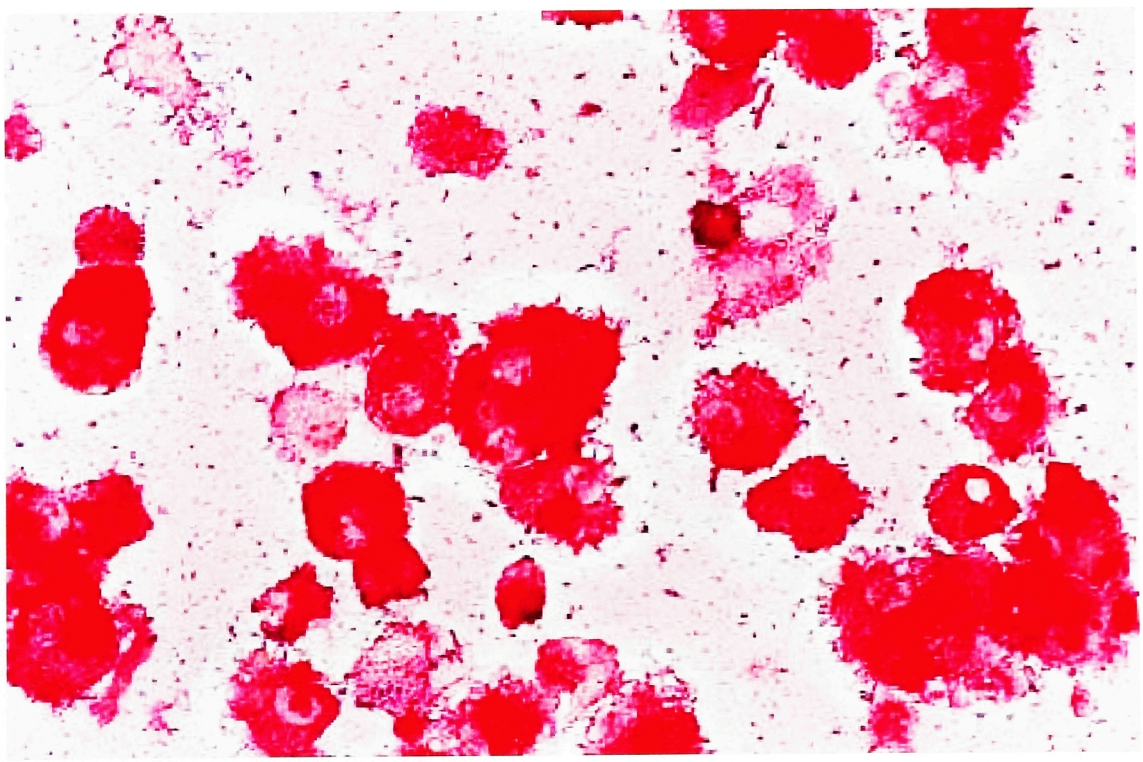


Figure 29B

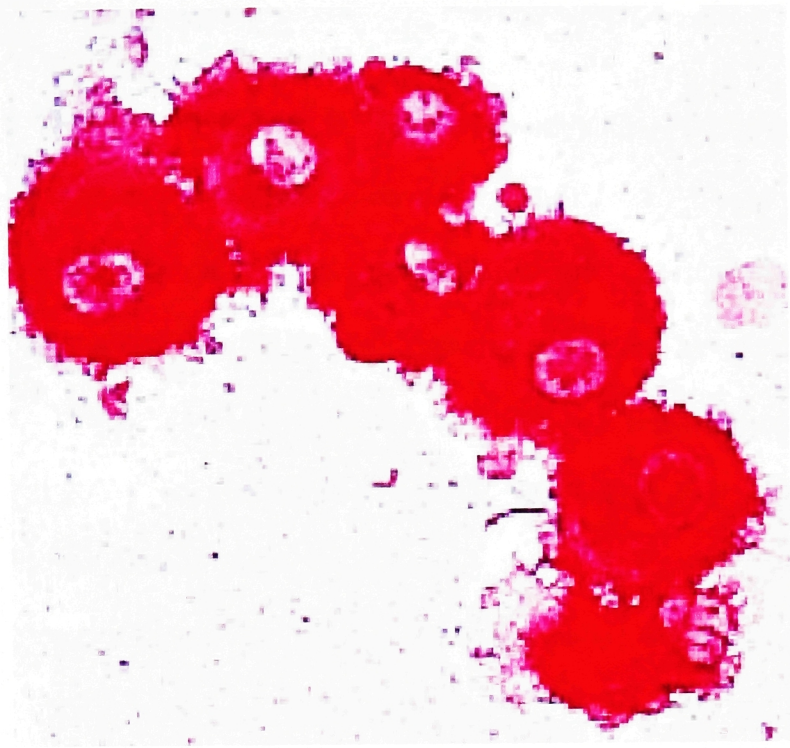


Figure 30A

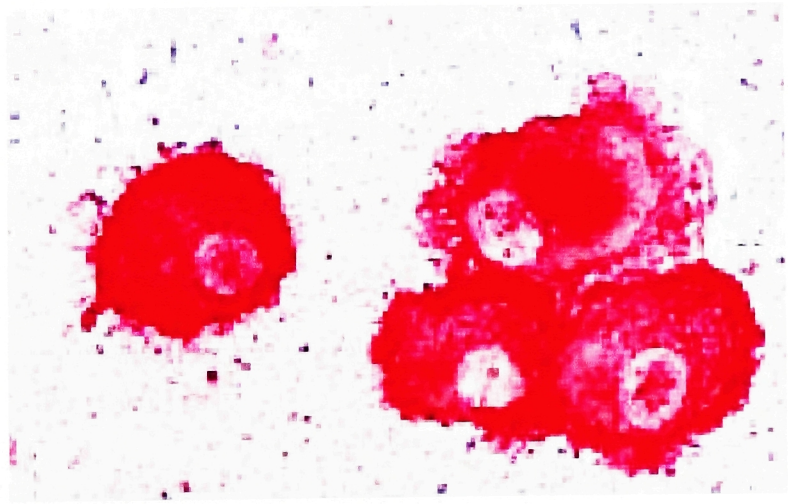


Figure 30B

DISCUSSION

As a general vaccine strategy, genetic immunization as it was originally conceived and tested requires augmentation and enhancement for true *in vivo* efficacy. With intradermal, subcutaneous or intramuscular administration of naked DNA, there is no targeted delivery to APCs (except perhaps Langerhans cells in the skin) (182-184). It has in fact been unclear as to which cells actually receive and express the genetic material, and which cells present the foreign antigens to T-cells. Indeed, much has been made of the "cross-priming" (185), or "direct" versus "indirect" priming (186) involved. Whatever the case, there seems to be a general consensus that steps must be taken towards improvement of the basic approach (182-184).

One obvious measure for strengthening the technique would be to ensure that DCs receive or manufacture the antigen themselves for presentation. A few groups have therefore already investigated the potential for DC delivery with DNA vaccination, whether intramuscular (168), subcutaneous (167, 168, 187) or intranasal (169). Intentional, directed targeting of DNA vaccines to immature DCs with a concomitant maturation stimulus, however, has not yet been achieved.

As we have already considered (*vide infra*), immature DCs would have to be reached and simultaneously matured for a vaccine to induce immunogenicity and prime naïve T-cells (131). It is therefore unlikely that any approach that falls short of this goal would yield protection from pathogenic challenge. In fact, in addition to the

Figure 31 ***Salmonella*** **can be used to transduce human DCs such that they express a viral gene** *DCs were infected at MOI=35 with bacteria carrying either an "empty" vector (pVAX1) or pVAX1/HIV*gag, *which uses a eukaryotic promoter to drive expression of the viral protein. Cells were then incubated with either an isotype control antibody or a commercially-available antibody against the Gag component, p24.*

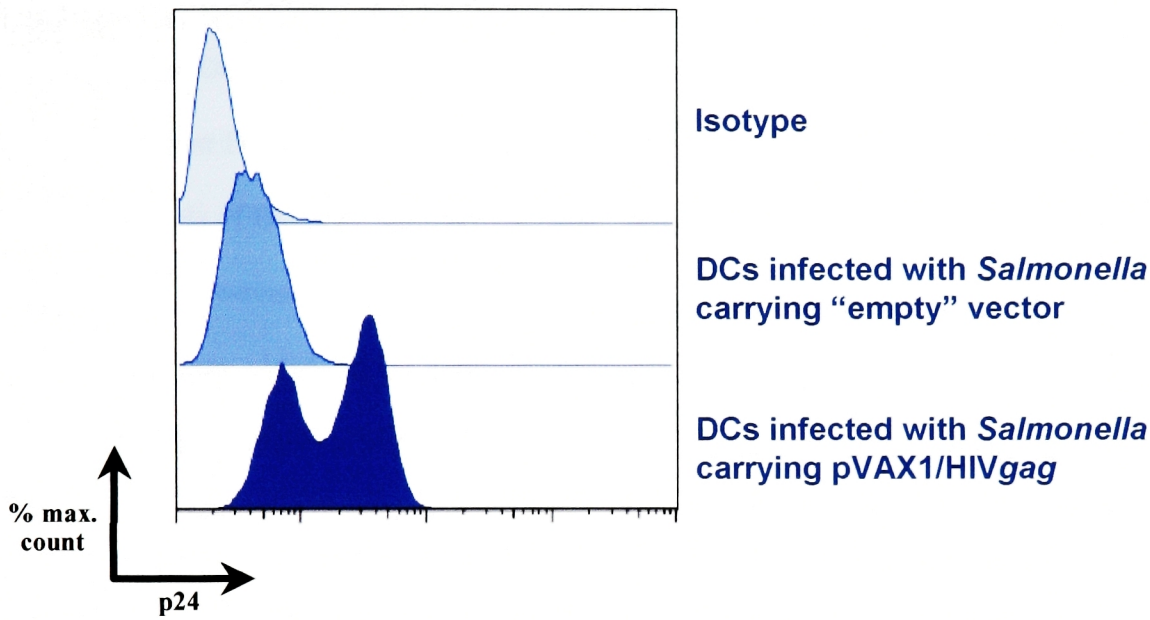

Isotype
DCs infected with *Salmonella* carrying "empty" vector
DCs infected with *Salmonella* carrying pVAX1/HIV*gag*
% max. count
p24

Figure 31

context of vaccination against infectious disease, DCs are also being actively investigated as tools of immuno-regulation for treating allograft rejection and autoimmune disease (188, 189). The need to provide a maturation stimulus for immunogenicity has also been illustrated in studies involving successful protein delivery to the DC receptor DEC-205. By conjugating a model antigen (OVA) to an antibody against DEC-205, Bonifaz et al. did effectively achieve significantly enhanced levels of DC presentation to T-cells. Without a maturation stimulus (in the steady-state), however, the effect of the DCs on the T-cells was tolerogenic; only when αCD40 was added did the delivery of antigen to DCs result in immunogenicity (190). Indeed, conjugation to αDEC-205 represents one promising strategy towards direct vaccine targeting to DCs *in vivo*.

Other approaches to immunization involving DCs have involved attempts to prime T-cells against tumor antigens. Some of these studies have in fact already progressed to clinical trials (191, 192), though they generally involve *ex vivo* manipulation of the cells before re-infusion.

Thus, a *Salmonella*-based carrier would certainly allow for some advantages over other vaccine strategies. We have shown here that the bacteria can not only deliver DNA vaccines directly to DCs, they also function as a maturation stimulus, themselves. The system would additionally almost certainly represent "direct" priming insofar as the APCs would be doing the expressing themselves – although

"indirect" priming would likely also be at play as a consequence of apoptosis and macrophage involvement.

In addition to its untargeted administration, conventional DNA vaccination also requires improvement towards the generation of mucosal immunity (182, 184, 193, 194), especially in challenging HIV-1 infection. Once again we must concern ourselves with DCs in this regard. Mucosal administration of a vaccine inoculum, alone, is insufficient to ensure mucosal responses. DCs are found throughout mucosal tissue, including in intranasal (195), buccal (196), tonsillar (197), tracheal (198), pulmonary (199), genital (200-202) and intestinal (102, 134, 203-207) regions. The possibility of tolerogenesis, however, must be avoided, as with any other route. In fact, oral administration in particular warrants caution in this regard, since the gut's natural function involves tolerogenic responses to antigens.

One group therefore undertook to develop an appropriate adjuvant for use with an oral inoculum that would target and mature DCs directly. Williamson et al. found that co-administration of Flt-3L (with or without IL-1) successfully converted a tolerogenic oral inoculation into an immunogenic one (130). The adjuvant, however, was itself administered through an IP injection.

More relevant to our purposes is a study that investigated the DC migration patterns within the gut in response to both cholera toxin (as an adjuvant candidate) and *S. typhimurium*. Both induced migration of DCs from the subepithelial dome

region of PP mucosa into underlying B- and T-cell rich follicles and adjacent parafollicular T-cell zones (208). Thus, our DNA vaccine delivery vehicles would likely permit appropriate and effective DC targeting, even within mucosal tissue following oral administration.

Chapter VI:

Human dendritic cells, transduced using attenuated *Salmonella typhimurium*, can present foreign antigens to autologous T-cells *in vitro*

INTRODUCTION

The effort to design a vaccine against HIV-1 has necessitated careful consideration of precisely which viral antigens are optimal for protective immunogenicity. Studies involving both CD4+ and CD8+ responses have concluded that Gag epitopes are important (209-211); therefore, this protein has generally been incorporated into HIV-1 vaccine designs (212-220).

Assays that have proven useful in evaluating the immunogenic strength of vaccine candidates include the ELISpot assay, as well as intracellular cytokine staining (ICS). Both approaches have been used to monitor the role of cytotoxic T lymphocytes (CTL) in viral infection, and can similarly be employed to quantify CD8+ T-cell responses to vaccines (221, 222), particularly in terms of IFN-γ expression.

When human subjects are not available to serve as donors of HIV-primed cells, it is more convenient to test vaccine strategies in the context of another viral infection. Influenza, therefore, represents a practical model system, as there is widespread exposure to (and vaccination against) this pathogen in the United States.

As a model antigen, then, influenza matrix protein (M1) is an appropriate choice for experimental vaccine design, since it is highly conserved across types and strains (223-226), and it is the most abundant protein in the viral particle (226, 227). Furthermore, CTL responses to M1 are seen to confer protection from viral challenge in mice (228, 229). Indeed, the influenza-M1 model system has been useful in evaluating DC presentation of antigen to T-cells both *in vitro* and *in vivo* (229-233). Although HIV-1 certainly represents a very different sort of pathogen than influenza, and while AIDS pathogenesis is very distinct from the course of influenza infection, it is not inappropriate to test HIV-1 vaccine strategies in an influenza-based system. T-cells that are primed against M1 are, for example, a practical tool for evaluating the basic potential for antigen presentation by autologous DCs targeted with a DNA vaccine.

RESULTS

Attenuated *Salmonella* can deliver a DNA vaccine vector to dendritic cells towards effective antigen presentation: autologous T-cell responses detected by ELISpot and flow cytometry

We were interested in evaluating the potential for antigen presentation by human DCs transduced to express a foreign protein using attenuated *S. typhimurium*. We therefore isolated PBMCs from HIV-1 infected donors, then set up co-cultures involving DCs and T-cells. Gag peptide pools were used to pulse DCs as a positive

control. Other DCs were infected at MOI=35 with aroA- *Salmonella* (strain 14028) bearing a vector encoding HIV-1 *gag* (pVAX1/HIV*gag*). T-cell responses were assessed by staining cells for IFN-γ and measuring expression using flow cytometry.

Our initial studies proved impractical, however, because Gag-specific CD8+ T-cells failed to proliferate over time *in vitro*, even in co-culture with Gag-pulsed DCs. Indeed, it has been seen by others that CD8+ T-cells specific for viral antigens from most HIV-1 infected donors do not proliferate in co-culture (234), even with mature DCs present (235).

We therefore turned to an alternative infectious context in which to monitor antigen presentation. It has previously been seen that most local healthy volunteers demonstrate CD8+ T-cell responses (by ELISpot and flow cytometry for IFN-γ expression) specific for influenza (236). Thus, we designed a protocol for setting up DC/T-cell co-cultures towards IFN-γ ELISpot and ICS analysis of T-cell responses (Figure 32). Blood samples from eight donors were ultimately processed as follows. PBMCs were isolated, then separated into CD14+ and CD14- populations (See Chapter II for details). The latter were kept frozen during the 6- or 7-day period required to cultivate DCs from the former. Before being seeded into a 96-well plate for co-culture (at a 1:30 ratio) with defrosted autologous T-cells, DCs were either infected with influenza as a positive control, infected with *Salmonella* (14028) bearing a vaccine vector (at MOI=35), or left unmanipulated as a negative control.

Figure 32 The basic protocol used to set up DC/T-cell co-cultures and detect effective antigen presentation *PBMCs were isolated from whole blood, then subjected to CD14+ selection using Miltenyi Biotec® magnetic beads (See Chapter II for details). CD14- cells were frozen during the 6-7 days it took to cultivate DCs from the CD14+ cells using the cytokines indicated. DCs were incubated with virus, peptides, bacteria or plain cytokines before being seeded into co-culture with autologous T-cells (in either a 96-well plate or a 48-well plate, at a 1:30 ratio or 1:60 ratio). Peptide and virus incubations were carried out just prior to seeding; infection with bacteria was carried out approximately 40 hours before seeding into co-culture. T-cell expression of IFN-γ was analyzed at different time-points using the ELISpot assay or intracellular cytokine staining (ICS) and flow cytometry.*

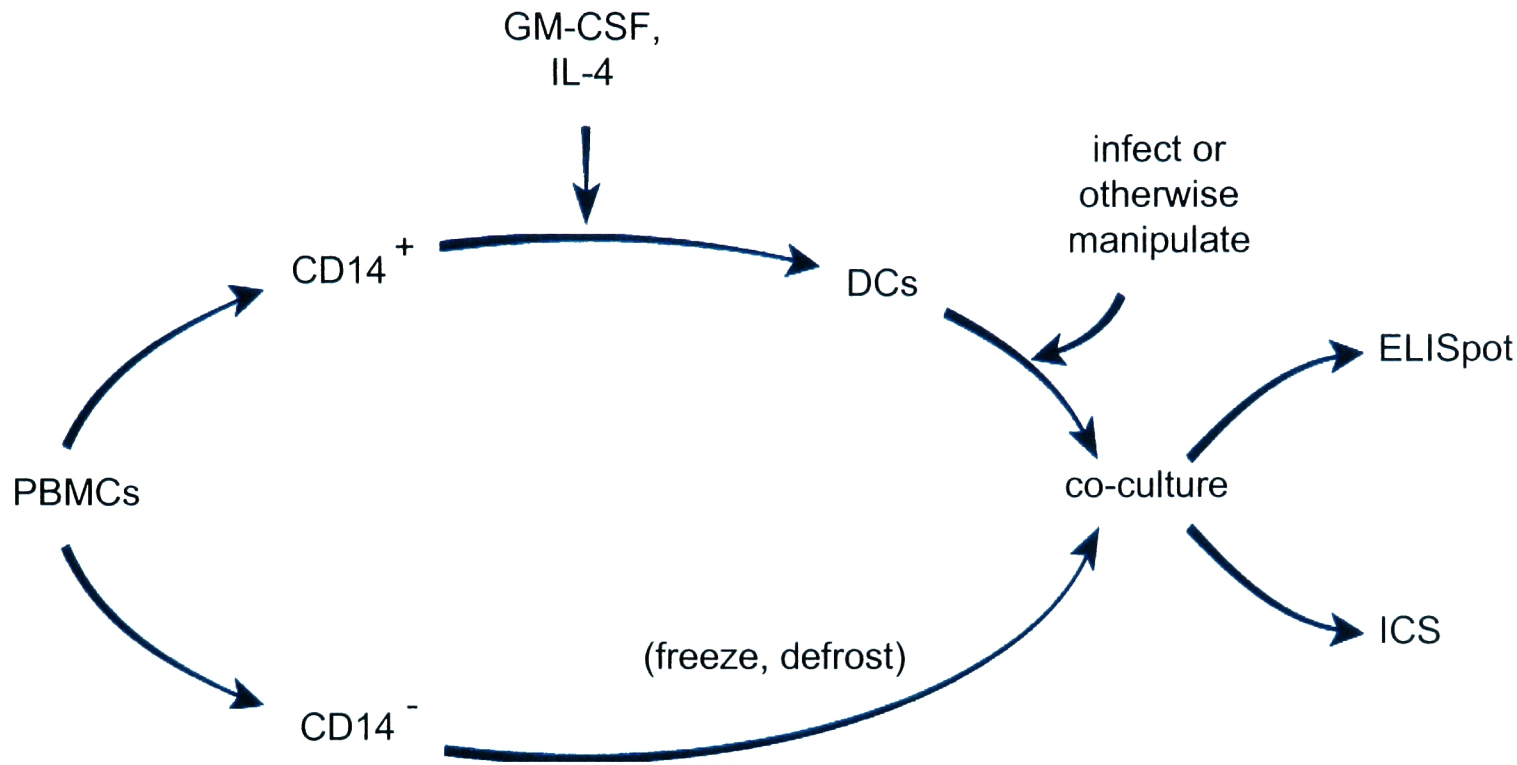

GM-CSF,
IL-4
infect or
otherwise
manipulate
CD14 +
DCs
ELISpot
PBMCs
co-culture
ICS
(freeze, defrost)
CD14 -

Figure 32

Vaccine plasmids employed were either "empty" pVAX1 (Invitrogen®) or an expression vector (pCAGGS) encoding influenza M1 protein. Assays for IFN-γ expression were carried out at day 0 and day 10 or 14.

Overall ELISpot results are shown in Figure 33. In general, co-cultures involving DCs that were infected with *Salmonella* carrying M1 vector yielded greater T-cell responses than co-cultures involving influenza-infected DCs. Co-cultures containing DCs infected with *Salmonella* bearing "empty" vector usually showed some T-cell responses at day 0, which diminished over time, revealing a significant gap between this category and co-cultures with DCs infected by M1 vector-bearing bacteria. Indeed, the initial IFN-γ expression detected in co-cultures involving DCs infected by *Salmonella* carrying pVAX1 could represent a memory response to the bacterial vehicle itself. In contrast to all three co-culture categories involving infected DCs, control DCs were seen to stimulate autologous T-cells to a minimal extent (Figure 33).

A subset of the same donor samples was stained for intracellular IFN-γ expression using a fluorophore-labeled antibody, then analyzed using flow cytometry. As with the ELISpot results, a greater difference between the IFN-γ expression from co-cultures containing DCs infected by pVAX1-bearing *Salmonella* and co-cultures containing DCs infected by M1 vector-bearing *Salmonella* was seen as the co-culture was carried out for 10-14 days (Figure 34A and B). Additionally, it was confirmed

Figure 33 ELISpot assay results from antigen presentation studies using the influenza model system *DC/T-cell co-cultures were set up at a 1:30 ratio in 96-well plates with DCs infected as indicated (MOI=35 for* Salmonella*). Cells were then assayed at different time-points using ELISpot, with re-stimulation (just prior to assaying) provided by the addition of autologous influenza-infected DCs, again at a 1:30 ratio. Results shown in* ***(A)*** *summarize data from eight different donors at sixteen different time-points.* ***(B)*** *represents a single donor, illustrating the general trends observed. Co-cultures involving DCs infected with* Salmonella *carrying "empty" vector, for example, tended to display IFN-γ T-cell responses early on, which would then diminish over time. (*N.B: *Values are normalized for occasional assay background.)*

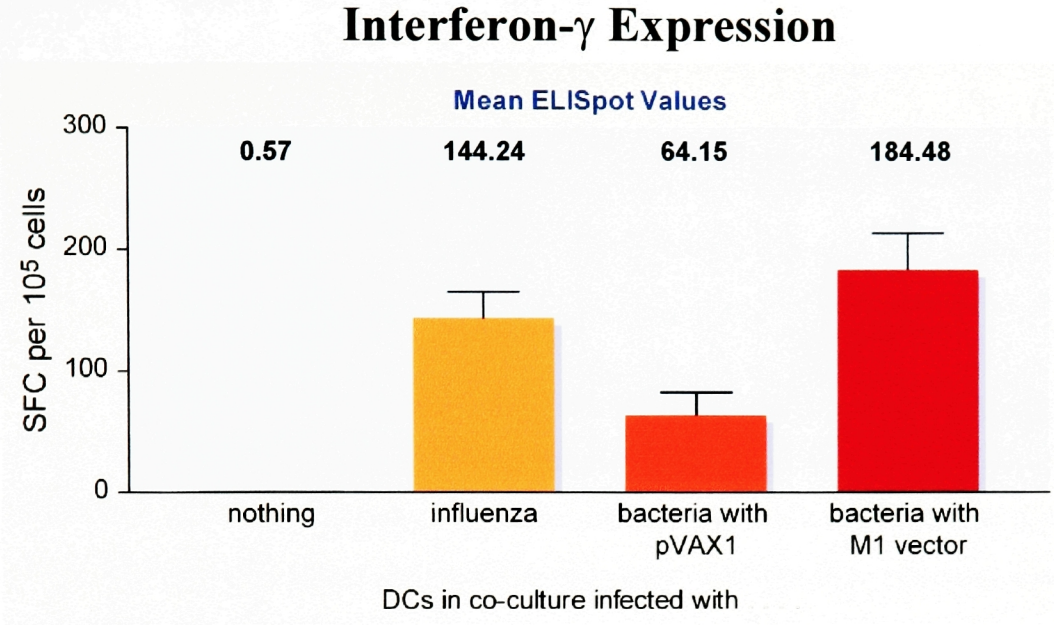


Figure 33A

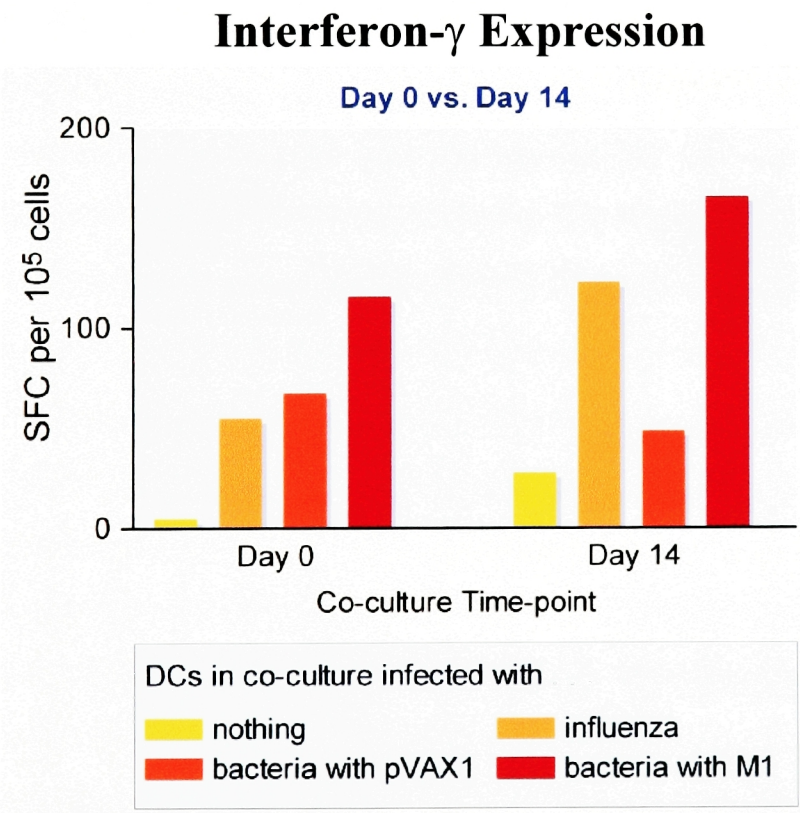


Figure 33B

Figure 34 Intracellular cytokine staining (ICS) results from antigen presentation studies using the influenza model system *DC/T-cell co-cultures were set up at a 1:30 ratio in 96-well plates with DCs infected as indicated. Cells were then assayed at different time-points using ICS and flow cytometry, with re-stimulation provided by the addition of autologous influenza-infected DCs.* ***(A)*** *shows data from one donor, illustrating the general pattern observed at early time-points (day 0 in this case), including relatively close levels of IFN-γ expression between the two co-culture categories involving* Salmonella*-infected DCs.* ***(B)*** *illustrates how the gap between the two categories broadens over time (after 10 days in this case), with co-cultures involving DCs infected with* Salmonella *carrying M1 vector yielding consistently higher levels of T-cell IFN-γ expression.* ***(C)*** *illustrates the dominance of CD8+ responses, even, as shown here, at day 0.*

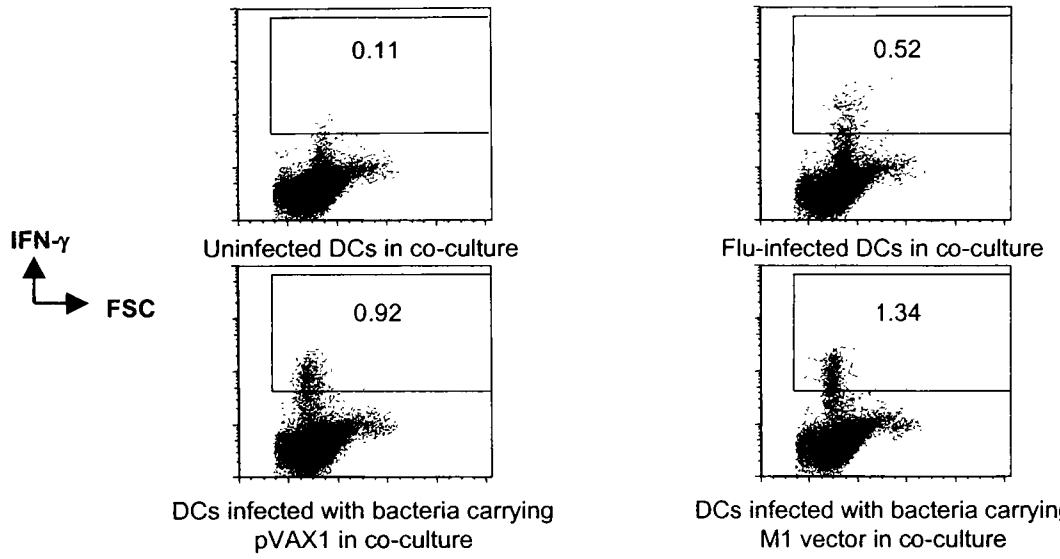


Figure 34A

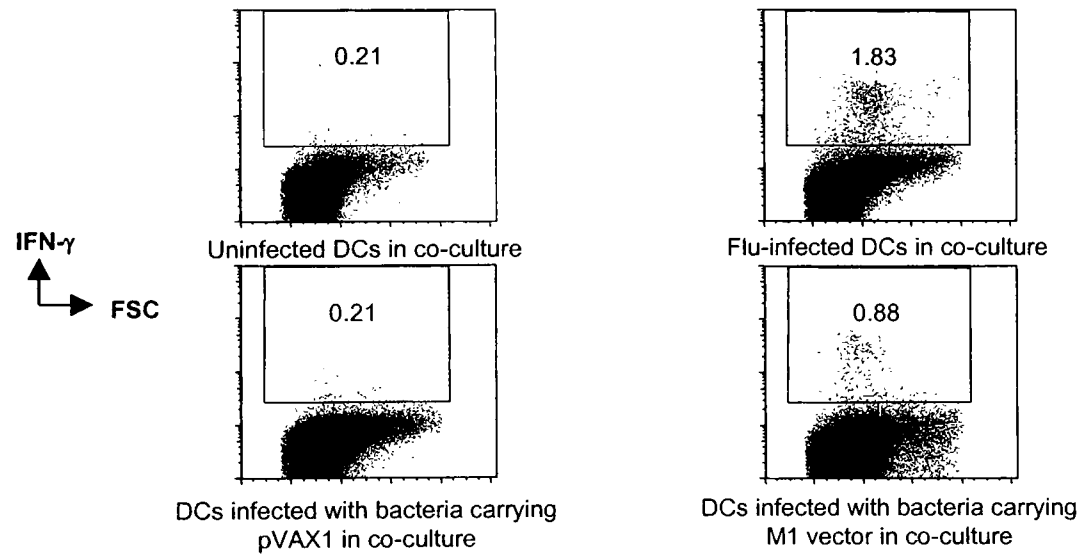


Figure 34B

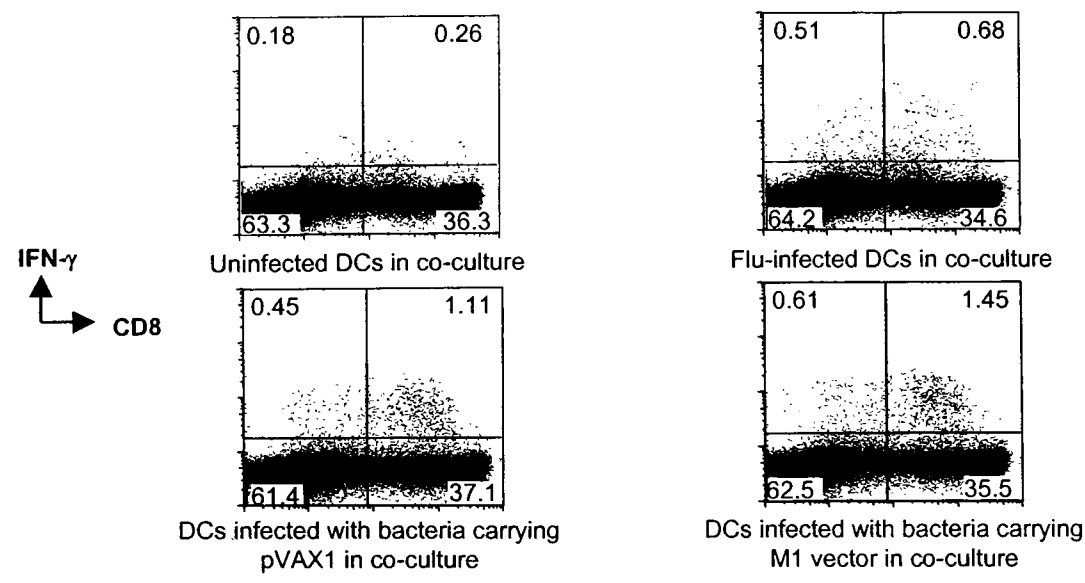


Figure 34C

that most of the IFN-γ expression was by CD8+ T-cells (Figure 34C). Neither the ELISpot data nor the ICS studies, however, revealed significant M1-specific T-cell proliferation over time (i.e., expansion) in response to DCs infected with *Salmonella* carrying M1 vectors (Figure 33B, e.g.).

In the course of working with the influenza/M1 model system, we came across an exceptional case that inspired us to explore innate immune responses in the context of the co-cultures (Figure 35). This anonymous donor displayed relatively robust M1-specific T-cell responses, especially in cultures containing DCs infected with *Salmonella* bearing M1 vectors (Figure 35A). In staining for CD4 and CD8, however, it appeared that the IFN-γ-expressing T-cells were neither CD4+ nor CD8+ (Figure 35B and C). With our subsequent samples, therefore, we undertook to rule out NK and γδ cells by staining for CD3 and γδ, respectively (Figure 36). Responding cells in these cases were thus seen to be CD3+ and γδ-. Nonetheless, closer scrutiny of CD3 patterns (Figure 37A) suggested that a more definitive investigation of the possible role of NK cells be undertaken. Subsequent samples were therefore stained for CD56 (Figure 37B). IFN-γ-expressing cells were thus consistently found to be CD56- (and γδ-), which confirms that they are not NK cells. Indeed, M1-specific IFN-γ-secreting T-cells were never again found to be CD8-/CD4-; the donor that triggered our exploration of innate immune responses proved to be anomalous.

Figure 35 An interesting case of apparent non-CD8+/CD4+ responses to presentation of M1 antigen *The graphs illustrate data derived (at day 14) from the cells of one of eight anonymous donors, which were used to analyze DC presentation of influenza M1 antigen to autologous T-cells. As seen in* ***(A)****, this sample yielded relatively high levels of IFN-γ expression in the co-culture involving DCs infected with* Salmonella *carrying M1 vector. Staining for CD4* ***(B)*** *and CD8* ***(C)****, however, suggested that the responses were from CD8-/CD4- cells, which was an unexpected finding.*

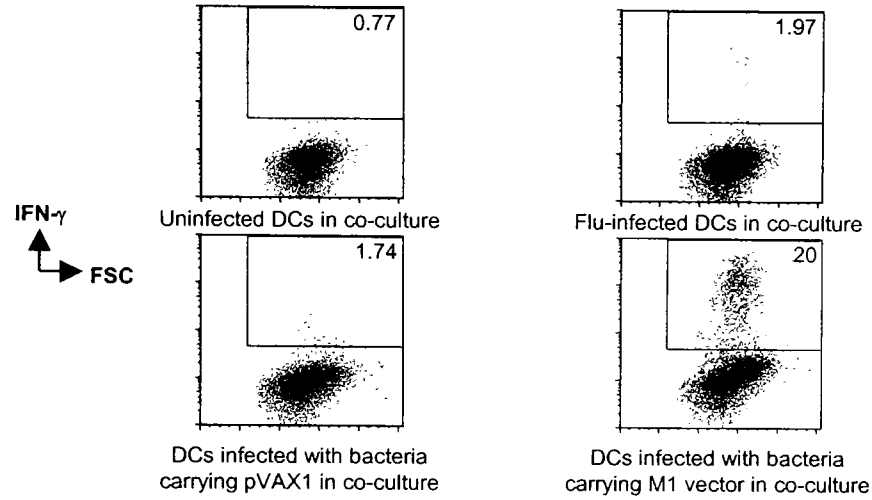


Figure 35A

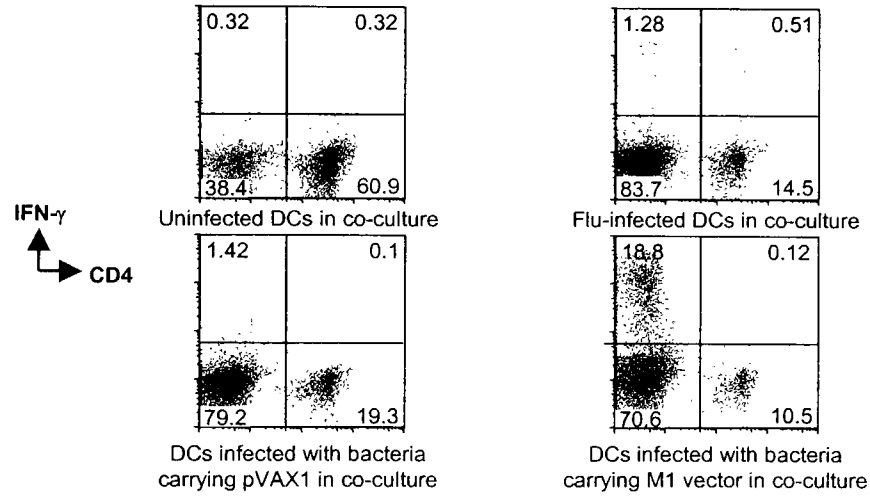


Figure 35B

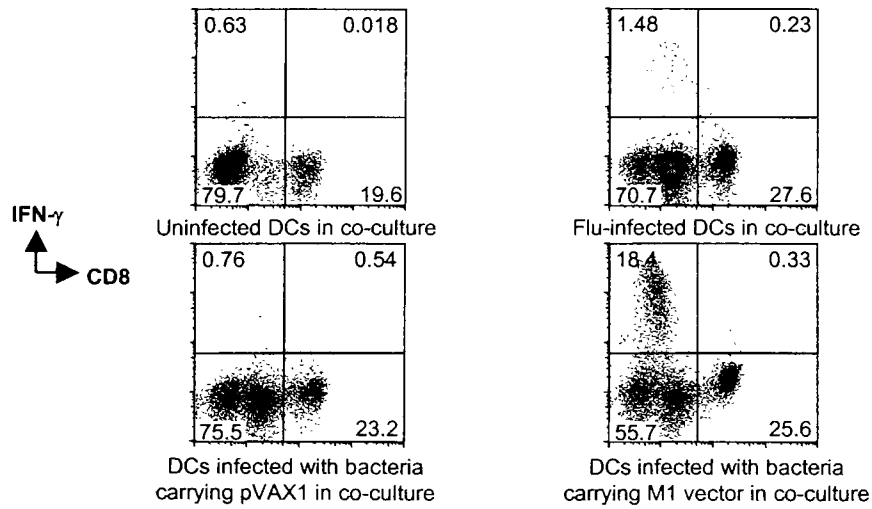


Figure 35C

Figure 36 Co-cultures stained for CD3 and γδ *The previous case (**Figure 35**) inspired an exploration of the possible role of the innate immune system in mediating responses to DC presentation of M1 antigen, particularly when* Salmonella *vehicles are involved. Co-culture cells were therefore stained for CD3 (to rule out NK cell activity) and γδ (to rule out γδ cell activity). As illustrated, responsive T-cells are clearly CD3- and γδ-.*

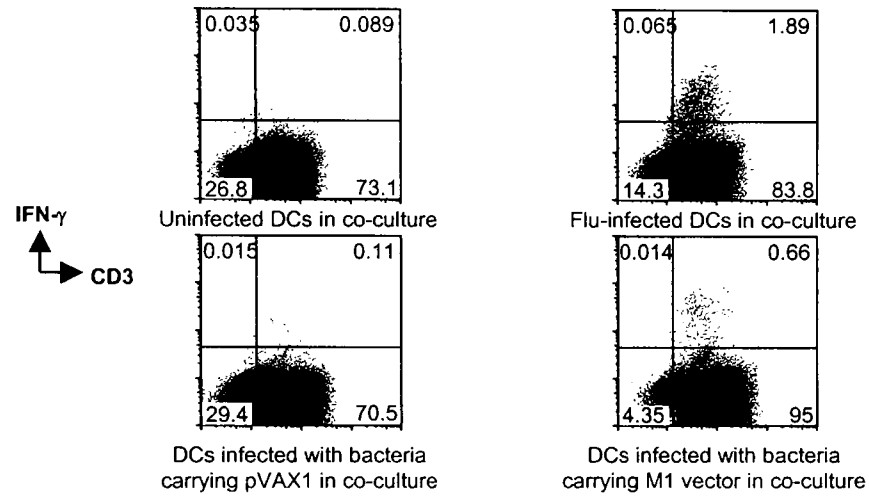


Figure 36A

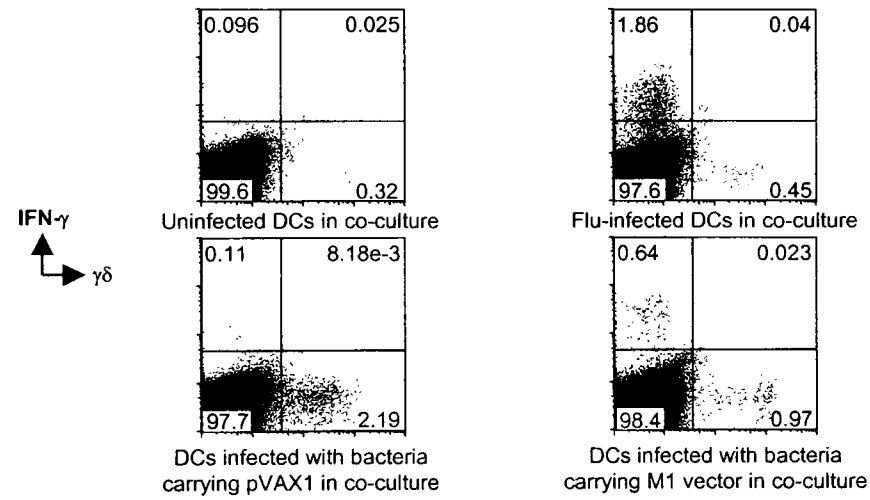


Figure 36B

Figure 37 Definitive flow cytometric exclusion of NK involvement in co-culture responses to DC presentation of M1 antigen *In comparing CD3 staining patterns between the two co-culture categories indicated* ***(A)****, it appeared that cells exposed to DCs infected with* Salmonella *carrying M1 vector expressed CD3 to a lesser extent than cells from co-cultures containing influenza-infected DCs. In order to definitively rule out NK cell involvement, therefore, cells were stained for CD56* ***(B)****. IFN-γ secreting cells were thus seen to be CD56-, confirming that they are not NKs. (Indeed, no IL-2 was added to these co-cultures, making it unlikely that any NK cells would survive extended cultivation.) Similarly, IFN-γ-secreting cells were consistently found to be γδ-* ***(C)****.*

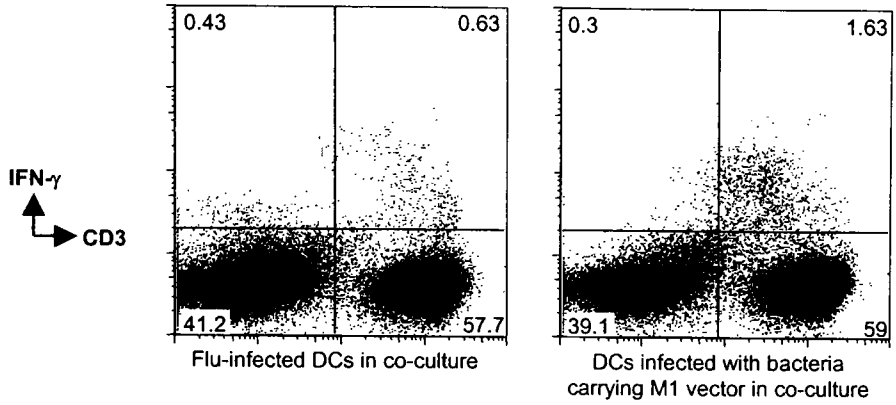


Figure 37A

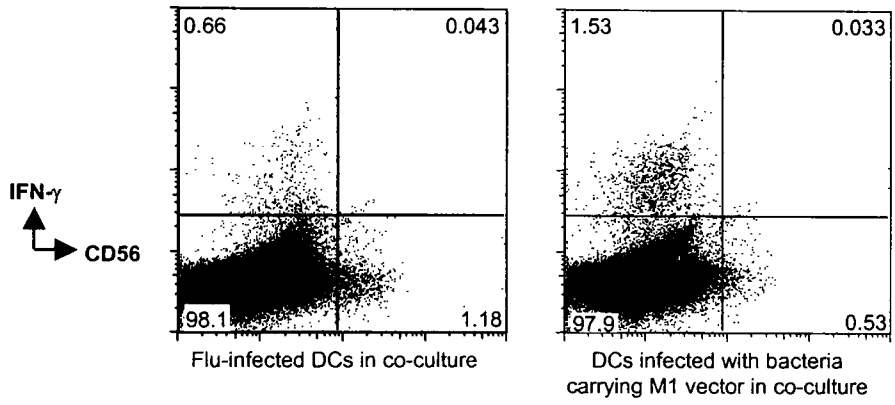


Figure 37B

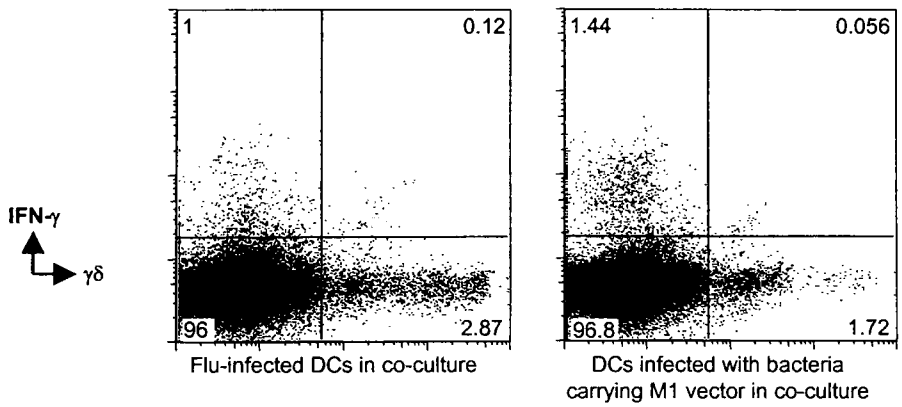


Figure 37C

Any distinction in CD3 expression between IFN-γ-expressing T-cells from co-cultures with influenza-infected DCs and T-cells from co-cultures with *Salmonella*-infected DCs (Figure 37A) can perhaps be explained by a qualitative difference in maturation between the two DC populations: DCs that were pulsed with virus were first exposed to a cytokine cocktail (IL-6, IL-1β, TNF-α and prostaglandin E_2) in order to mature them. In contrast, DCs infected with bacteria were stimulated to mature only by the *Salmonellae* themselves, presumably through a TLR (121, 122, 124). It is thus possible that the two pools of DCs attained different levels of maturation or activation, which, in turn, would have influenced the way in which they interacted with and activated T-cells.

We decided to return to the HIV model system to confirm the ability of DCs infected by *Salmonella* vehicles to present vaccine antigens. In order to have an effective positive control, however, it was necessary this time to obtain blood samples from HIV-1-infected LTNPs, since it has been established that HIV-1-specific CD8+ T-cells proliferate *in vitro* only when derived from LTNPs (234, 235). Thus, we can use the extent of expansion of LTNP T-cells in co-culture with autologous Gag-pulsed DCs as a standard of comparison (235) to T-cell proliferation in co-culture with DCs infected by *Salmonella* carrying DNA vaccine vectors. We therefore acquired blood samples from three known LTNPs, and proceeded to isolate PBMCs and set up co-cultures as before. Briefly, cells were subjected to CD14+ selection towards DC cultivation, while CD14- cells were frozen until the time of co-culture. Before the two populations of cells were united, immature DCs were either matured

with cytokines then left unmanipulated, matured with cytokines then incubated with Gag peptide pool, infected (at MOI=35) with *Salmonella* (14028) carrying pVAX1, or infected (at MOI=35) with *Salmonella* (14028) carrying pVAX1/HIV*gag*. The addition of maturation cytokines and infection with *Salmonella* were carried out approximately 40 hours prior to starting the co-culture; pulsing with Gag was carried out immediately prior to starting the co-culture. DCs were carefully washed (and re-plated, when appropriate) before being seeded into the wells of 48-well plates with T-cells at a ratio of 1:60.

T-cells were sampled for analysis early in the co-culture (day 2-4) and again later on (day 9-10). At these time-points, cells were stained with fluorophore-labeled anti-IFN-γ antibody and assayed by flow cytometry to quantify expression of the cytokine. Results of the analysis of co-cultures from all three donors are shown in Figures 38-40. Cells were simultaneously stained for CD69 expression to better focus on activated T-cells. Early time-points suggest a general state of activation and secretion in response to Gag re-stimulation *in vitro* from all co-culture categories, including those containing mature but otherwise unmanipulated DCs. Later in the course of co-culture, however, we see, as expected, expansion of the responsive T-cells in the positive control category, wherein DCs were pulsed with a Gag peptide pool. The T-cells from co-cultures involving DCs infected with *Salmonella* bearing *gag* vectors do not expand as much over time, though there still appear to be significant differences between this category and the T-cells from co-cultures involving DCs infected with bacteria bearing "empty" vector. On visual inspection of

Figures 38-40 Co-cultures derived from HIV-infected LTNPs *Data from three different LTNP donors are illustrated. Cells were processed as before towards separation of DCs from T-cells. DCs were either left unmanipulated, pulsed with a Gag peptide pool, or infected (at MOI=35) with* Salmonella *carrying one of two vectors, as labeled. Cultures were set up in 48-well plates, with a DC:T ratio of 1:60. Before assaying, all categories of cells were re-stimulated with Gag, plain medium or SEB, as indicated. Early co-culture time-points reflect general (evidently non-specific) T-cell activation and IFN-γ secretion. Later time-points, however, show a decrease in T-cell responses from co-cultures involving unmanipulated DCs, and DCs infected with* Salmonella *carrying "empty" vector. Although specific T-cell populations from co-cultures involving Gag-pulsed DCs are seen to expand over time, responding T-cells from co-cultures involving DCs infected with* Salmonella *carrying* gag *vector do not consistently proliferate* in vitro. *Nonetheless, there is a consistent gap between the two* Salmonella*-infected DC co-culture categories at the later time-points.*

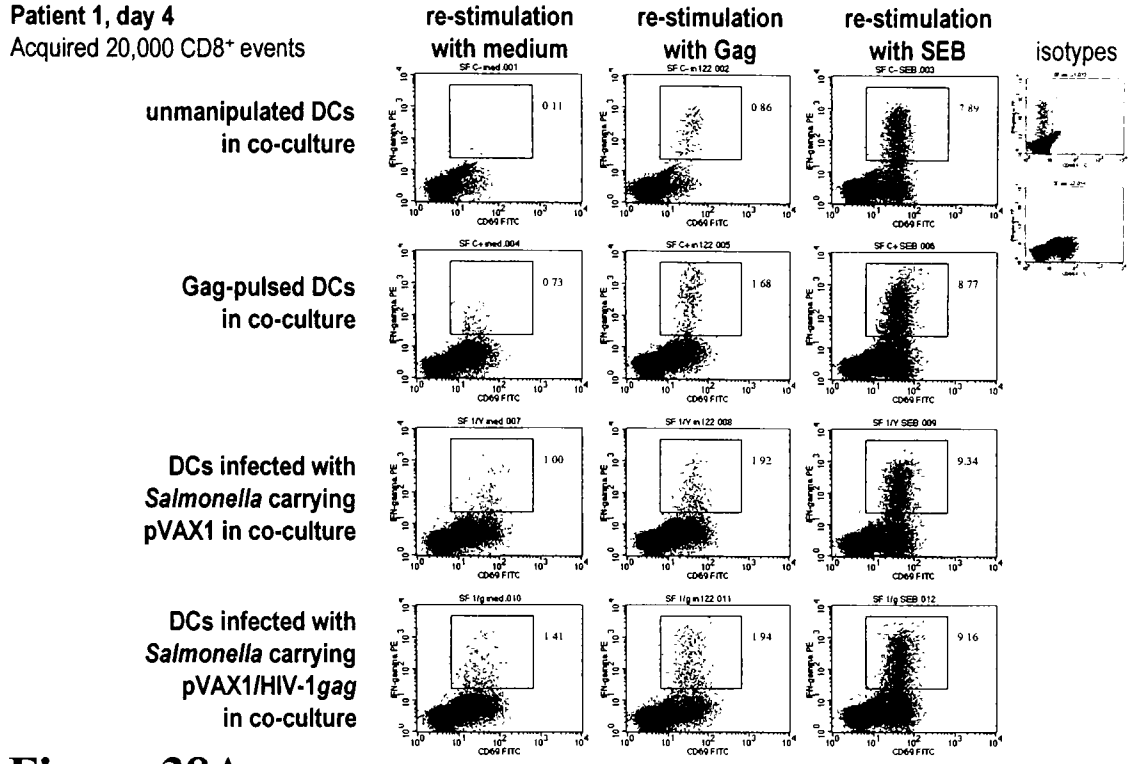


Figure 38A

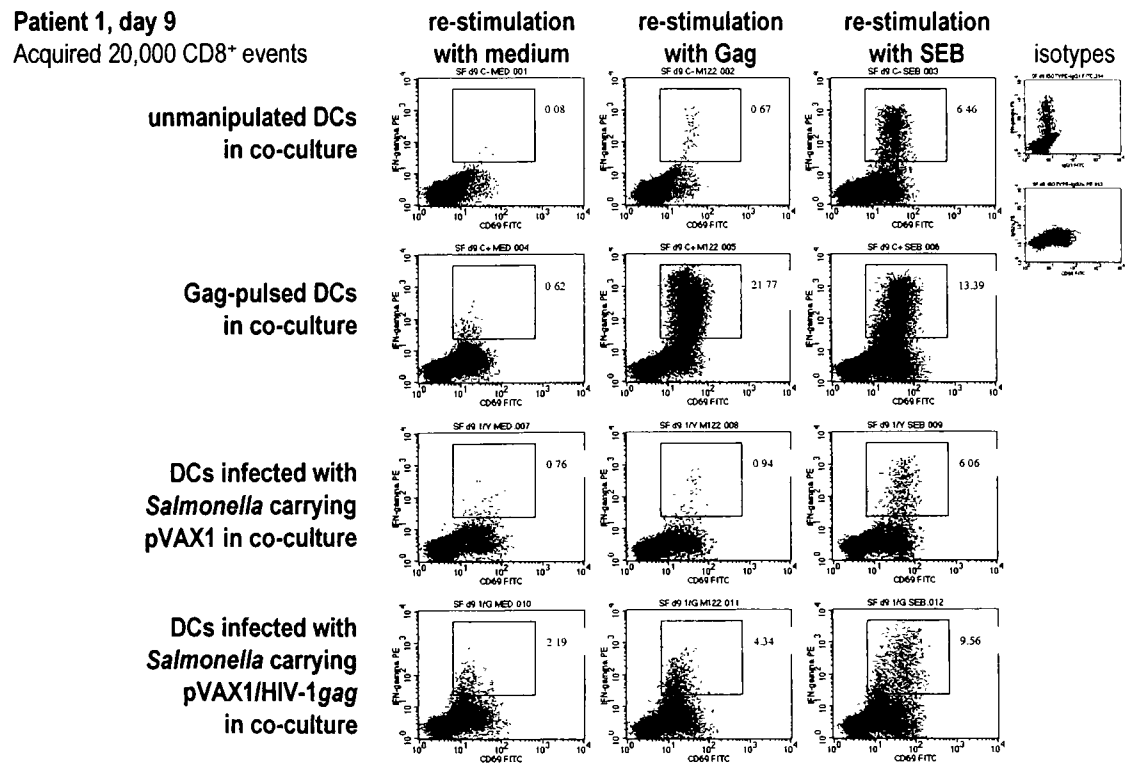


Figure 38B

Patient 2, day 3
Acquired 50,000 CD8+ events

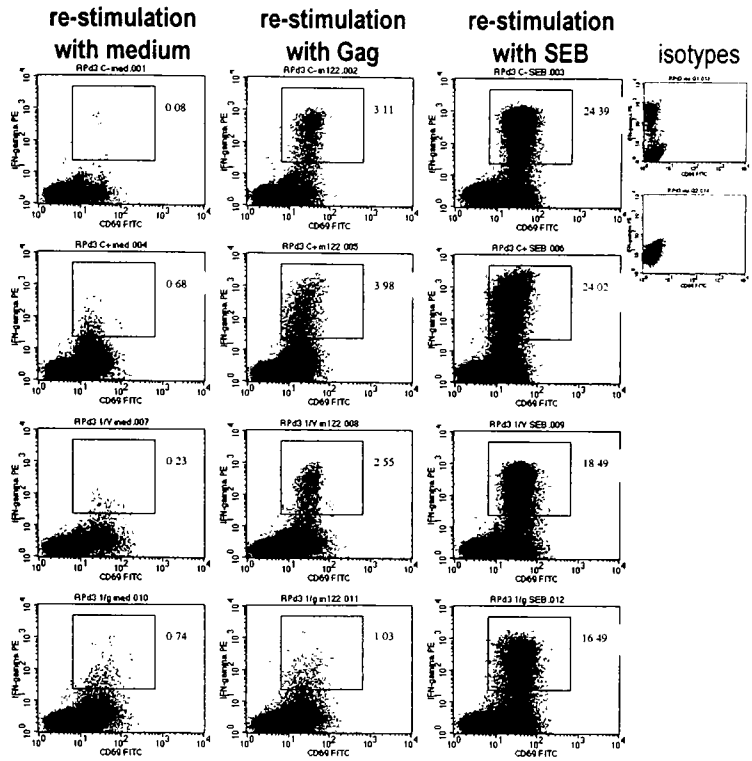


Figure 39A

Patient 2, day 9
Acquired 50,000 CD8+ events

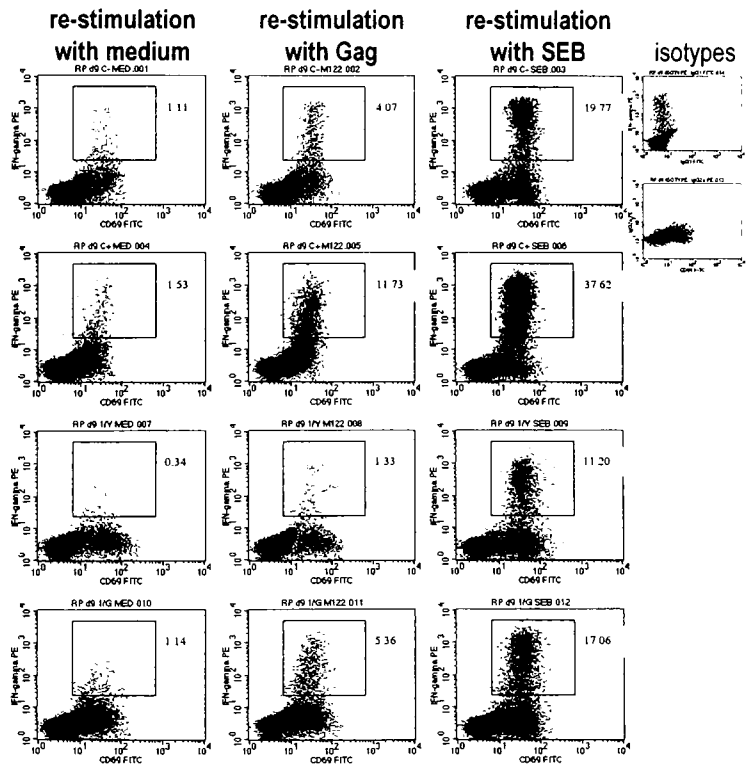


Figure 39B

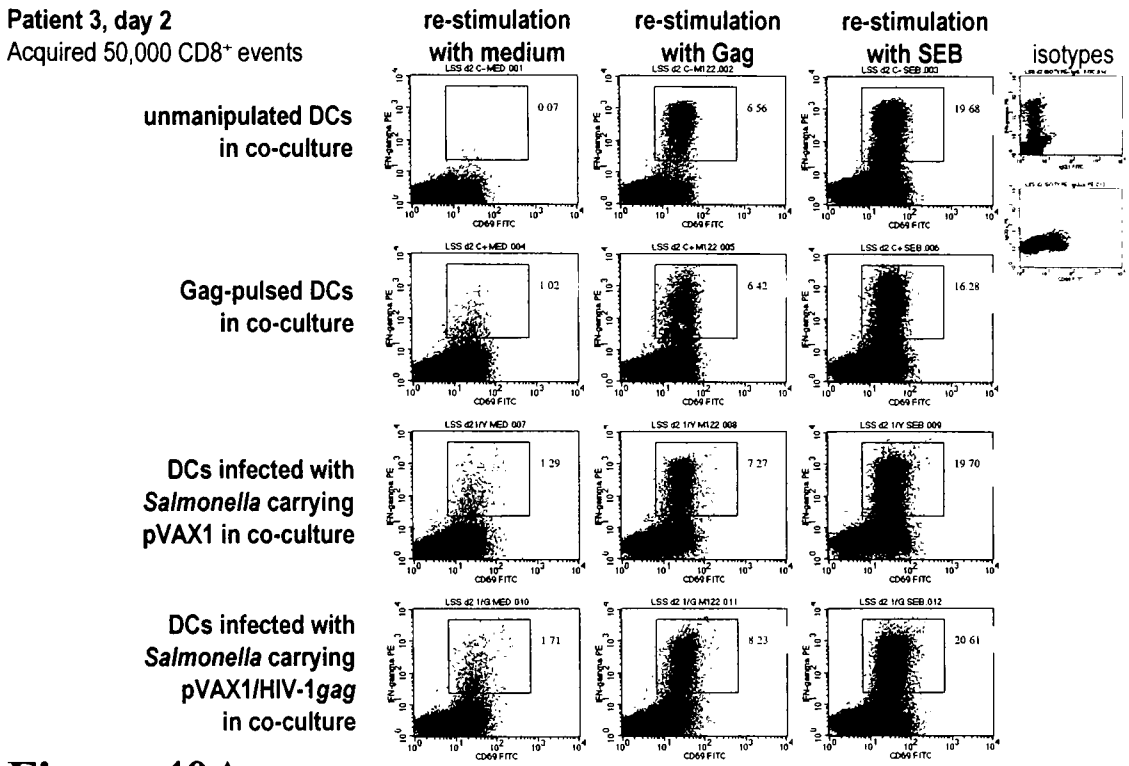


Figure 40A

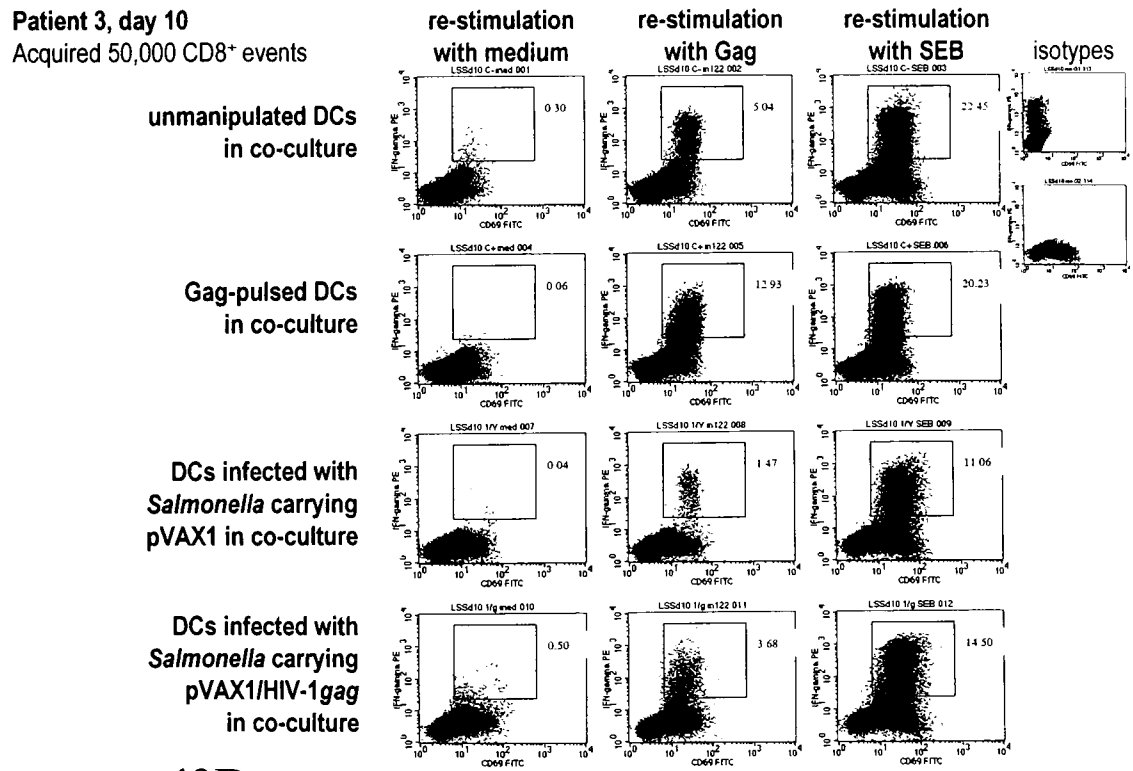


Figure 40B

the co-cultures, in fact, it could be appreciated that most of the DCs exposed to *Salmonella* were dying, and the corresponding co-cultures generally looked unhealthy.

We therefore concluded that bacteria-induced cytotoxicity was interfering with, and ultimately preventing, effective DC presentation and consequent T-cell proliferation over time. We thus decided to lower MOI with our next pool of DCs for co-cultivation with autologous T-cells. The DCs of our subsequent blood sample were therefore infected with *Salmonella* carrying pVAX1/HIV*gag* at three different MOIs: 30, 15 and 5. Results of staining co-culture T-cells for IFN-γ expression are shown in Figure 41. Though the absolute degree of expansion at day 10 is only moderate, there is a clear inverse dose-dependency (in contrast to assays for expression), indicating that increased MOI probably results in a counter-productive degree of DC cytotoxicity.

We proceeded to obtain four more blood samples from HIV-infected patients known to yield proliferative T-cell responses *in vitro* when restimulated with Gag-pulsed autologous DCs (237). We decided at this time not only to greatly reduce our MOI, but also to slightly modify our co-culture protocol, as follows. We performed day 0 whole blood screenings for T-cell (CD4+ and CD8+) responsiveness to Gag re-stimulation *in vitro* in order to establish a baseline potential for IFN-γ secretion. We then set up DC/T-cell co-cultures as before, but we now had sufficient cell numbers for a 1:30 ratio in the wells of 48-well plates. DCs were infected with *Salmonella* at

Figure 41 The effect of decreasing MOI on subsequent antigen presentation by DCs in co-culture *As before, DC/T-cell co-cultures were set up using cells from a known HIV-infected blood donor. Co-cultures were again carried out in 48-well plates with a DC:T-cell ratio of 1:60. DCs infected with* Salmonella *carrying* gag *vector, however, were infected at three different MOIs this time, as indicated. The data reveal progressively increasing IFN-γ T-cell responses as MOI decreases, suggesting more effective antigen presentation secondary to diminished cytotoxicity.*

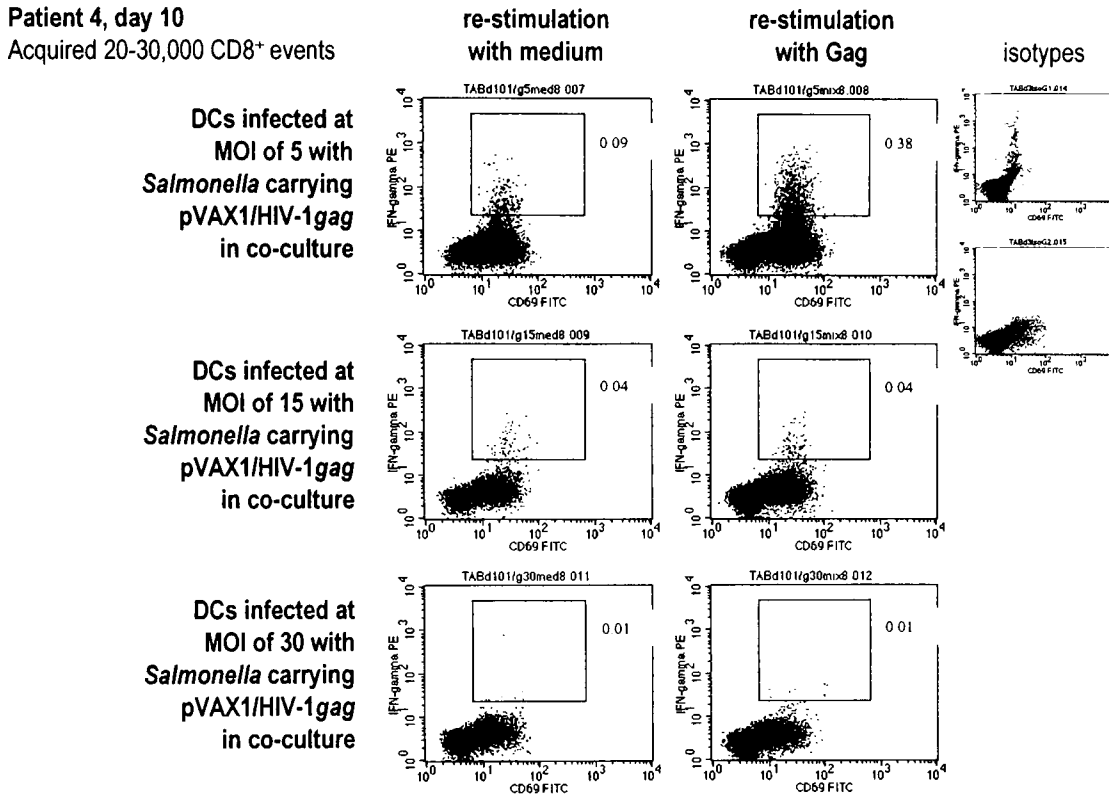

Patient 4, day 10
Acquired 20-30,000 CD8+ events
re-stimulation with medium
re-stimulation with Gag
isotypes
DCs infected at MOI of 5 with Salmonella carrying pVAX1/HIV-1gag in co-culture
DCs infected at MOI of 15 with Salmonella carrying pVAX1/HIV-1gag in co-culture
DCs infected at MOI of 30 with Salmonella carrying pVAX1/HIV-1gag in co-culture
TABd101/g5med8 007
TABd101/g5mix8 008
TABd101/g15med8 009
TABd101/g15mix8 010
TABd101/g30med8 011
TABd101/g30mix8 012
0 09
0 38
0 04
0 04
0 01
0 01
IFN-gamma PE
CD69 FITC

Figure 41

MOI=1, 2.5 and 5. Maturation cytokines were added to all DCs, including those infected with bacteria. Co-culture T-cells were analyzed for IFN-γ expression on day 6. Results of ICS and flow cytometry are shown in Figures 42-45.

The data suggest that an optimal balance was achieved between bacterial cytotoxicity and effective vaccine delivery towards antigen presentation. Indeed, the maximal degree of cytokine expression in response to DCs infected with *Salmonella* bearing *gag* vectors generally exceeds that in response to DCs pulsed with Gag peptide pool as a positive control. Additionally, the absolute peak levels of IFN-γ expression seem to be greater from co-cultures involving DCs infected with *Salmonella* carrying pVAX1/HIV*gag* than from co-cultures involving Gag-pulsed DCs.

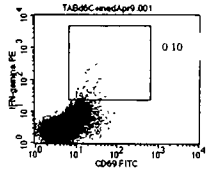
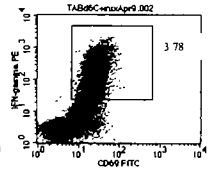
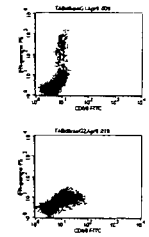
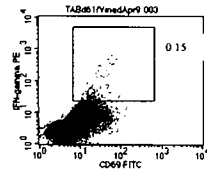
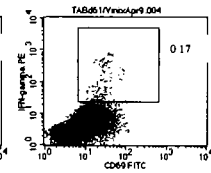
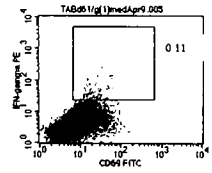
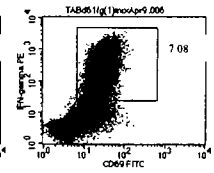
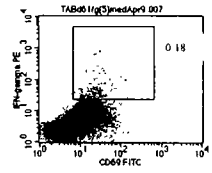
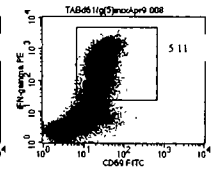
One possibly significant experimental detail to consider here, however, is the addition of maturation cytokines to all DCs destined for co-culture, including those infected with *Salmonella*. This modification, intended to better control experimental conditions, reflects an approach that is distinct from previous co-cultures. Thus, we were not in this instance dependent on bacterially induced maturation of DCs for immunogenic readiness. Indeed, it may be useful in the future to examine the degree of DC maturation possible with infection at the low MOIs used in these studies.

Although optimal responses appear on average to plateau with co-culture DCs infected at MOI ≈2.5, the results also seem to reflect qualitative and quantitative

Figures 42-45 Dramatic increase in effective antigen presentation with further reduction of MOI *Blood from HIV-1-infected donors was once again processed for co-culture and ICS. Studies were streamlined to eliminate redundant positive (SEB) and negative (unmanipulated DCs) controls, in order to increase cell number for analysis. More definitive controls were provided by DCs pulsed with Gag peptide pool (positive) and DCs infected with* Salmonella *bearing "empty" vector (negative). After manipulation, DCs were seeded into co-culture with autologous T-cells at a 1:30 ratio in 48-well plates. Cells from all four co-cultures were stained for IFN-γ expression and analyzed by flow cytometry on day 6. (Gag peptide pool provided the re-stimulation.) Results from all donors reveal significant degrees of IFN-γ expression from T-cells that were co-cultivated with DCs infected with* Salmonella *carrying* gag *vector. CD8+ responses are seen to plateau with MOI ≈2.5.*

Patient 5, day 6
Acquired 50,000 events

CD8+

	re-stimulation with medium	re-stimulation with Gag	isotypes
Gag-pulsed DCs in co-culture			
DCs infected with *Salmonella* carrying pVAX1 in co-culture			
DCs infected at MOI of 1 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture			
DCs infected at MOI of 5 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture			

Patient 5, day 6
Acquired 50,000 events

CD4+

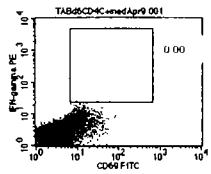
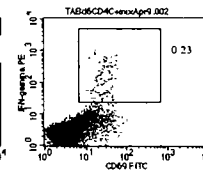
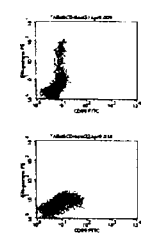
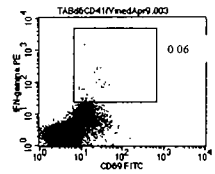
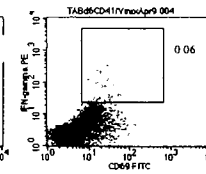
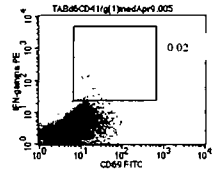
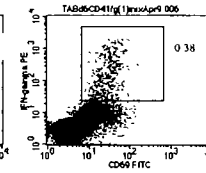
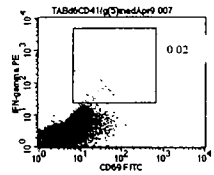
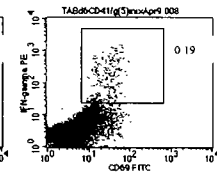
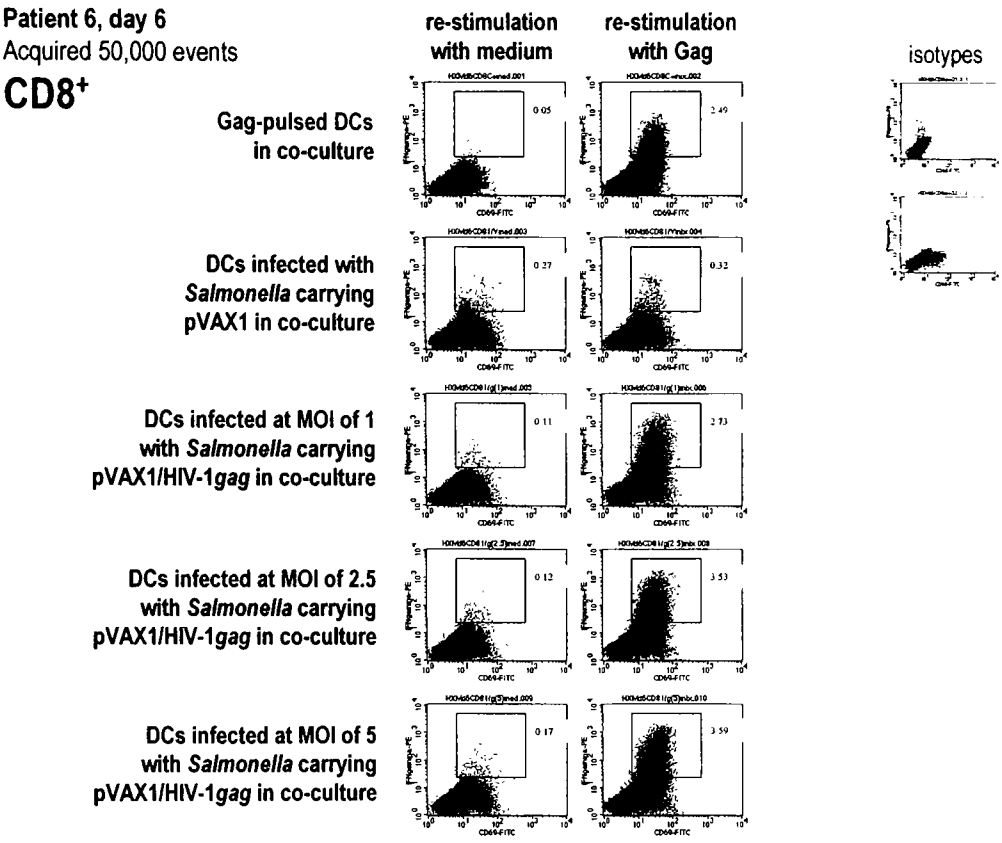
	re-stimulation with medium	re-stimulation with Gag	isotypes
Gag-pulsed DCs in co-culture			
DCs infected with *Salmonella* carrying pVAX1 in co-culture			
DCs infected at MOI of 1 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture			
DCs infected at MOI of 5 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture			

Figure 42

Patient 6, day 6
Acquired 50,000 events

CD8+



Patient 6, day 6
Acquired 50,000 events

CD4+

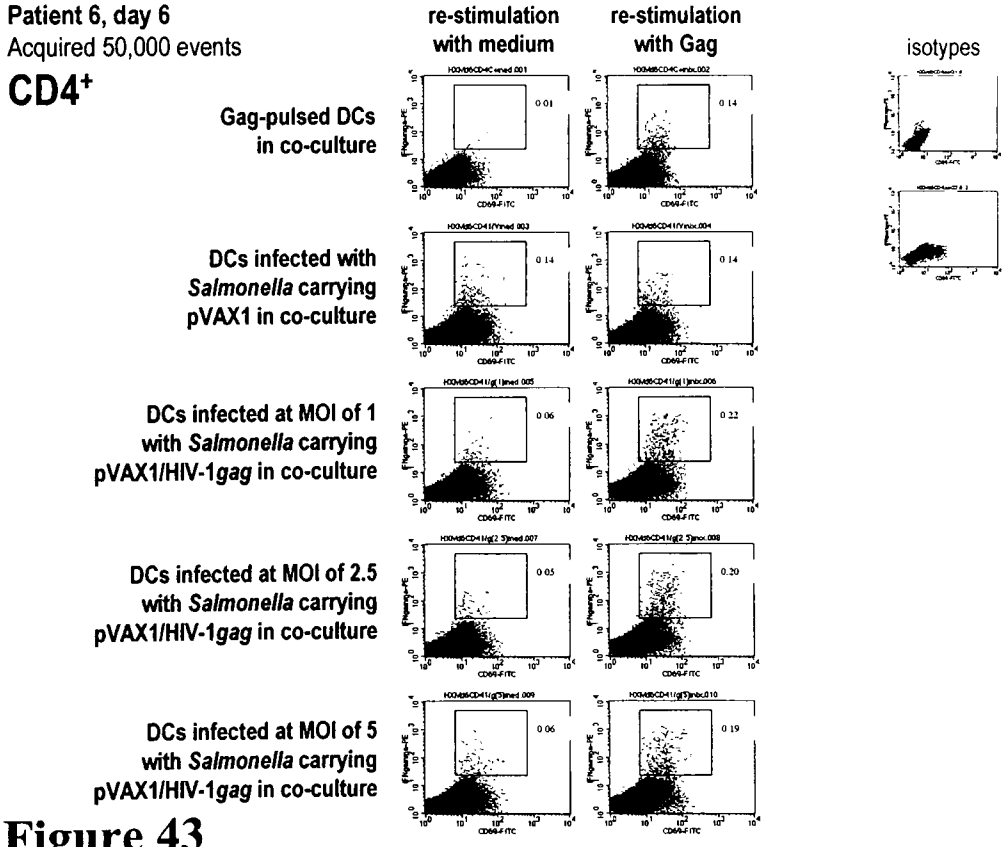


Figure 43

Patient 7, day 6
Acquired 50,000 events

CD8+

re-stimulation with medium | re-stimulation with Gag | isotypes

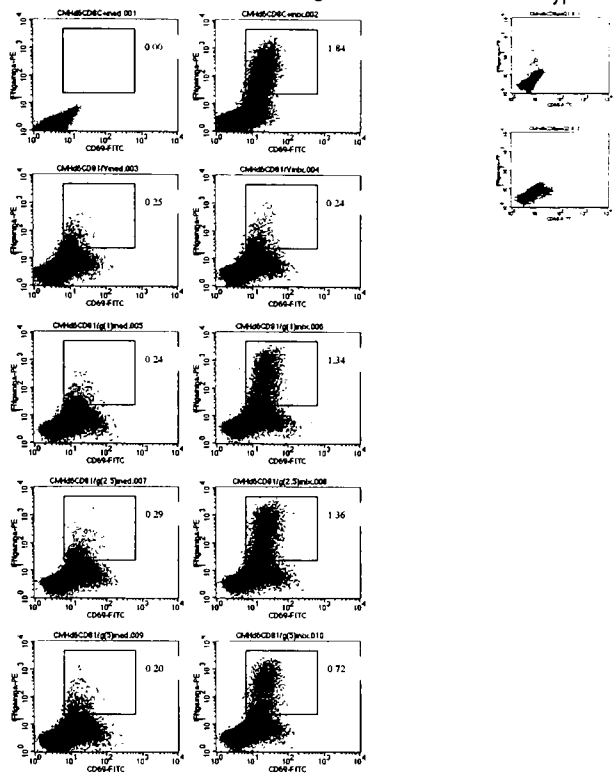
Gag-pulsed DCs in co-culture

DCs infected with *Salmonella* carrying pVAX1 in co-culture

DCs infected at MOI of 1 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture

DCs infected at MOI of 2.5 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture

DCs infected at MOI of 5 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture



Patient 7, day 6
Acquired 50,000 events

CD4+

re-stimulation with medium | re-stimulation with Gag | isotypes

Gag-pulsed DCs in co-culture

DCs infected with *Salmonella* carrying pVAX1 in co-culture

DCs infected at MOI of 1 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture

DCs infected at MOI of 2.5 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture

DCs infected at MOI of 5 with *Salmonella* carrying pVAX1/HIV-1*gag* in co-culture

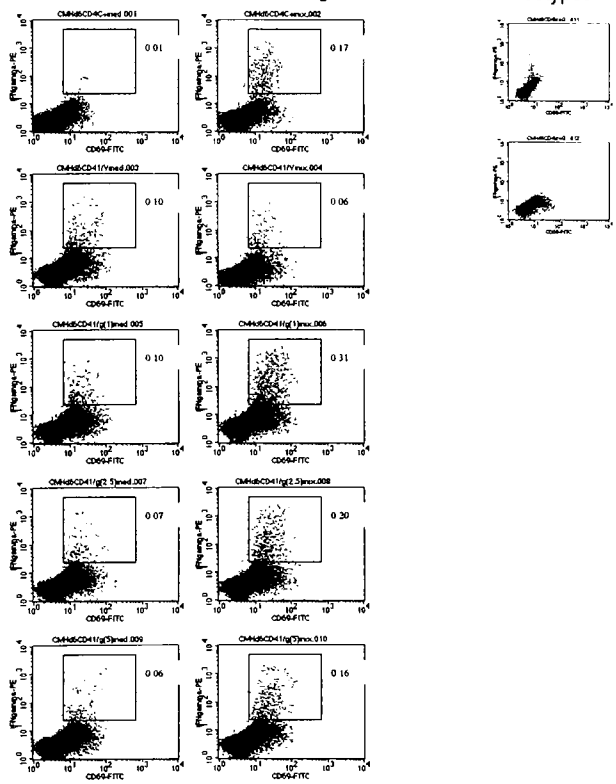


Figure 44

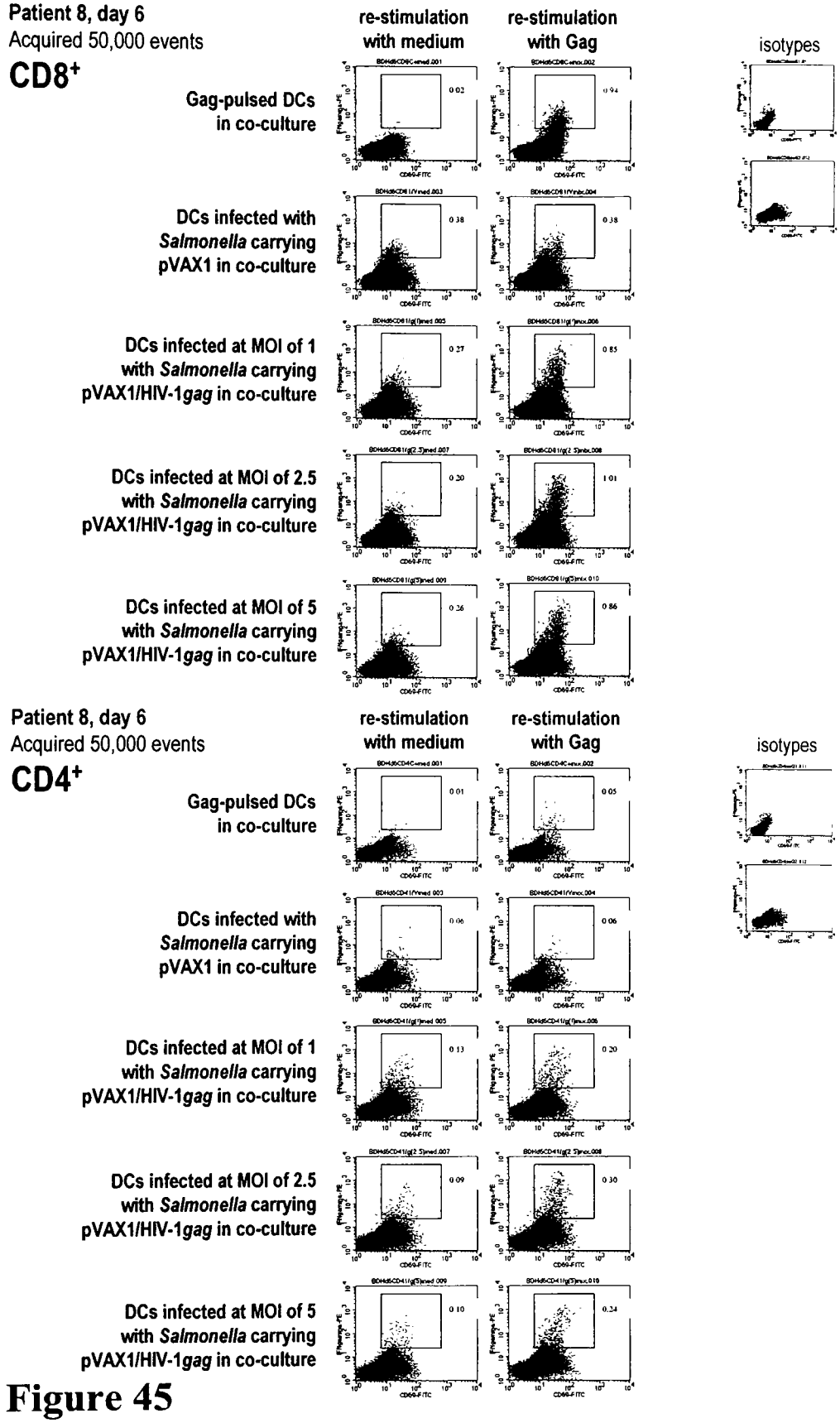

Patient 8, day 6
Acquired 50,000 events
CD8+
re-stimulation with medium
re-stimulation with Gag
isotypes
Gag-pulsed DCs in co-culture
DCs infected with Salmonella carrying pVAX1 in co-culture
DCs infected at MOI of 1 with Salmonella carrying pVAX1/HIV-1gag in co-culture
DCs infected at MOI of 2.5 with Salmonella carrying pVAX1/HIV-1gag in co-culture
DCs infected at MOI of 5 with Salmonella carrying pVAX1/HIV-1gag in co-culture
Patient 8, day 6
Acquired 50,000 events
CD4+
re-stimulation with medium
re-stimulation with Gag
isotypes
Gag-pulsed DCs in co-culture
DCs infected with Salmonella carrying pVAX1 in co-culture
DCs infected at MOI of 1 with Salmonella carrying pVAX1/HIV-1gag in co-culture
DCs infected at MOI of 2.5 with Salmonella carrying pVAX1/HIV-1gag in co-culture
DCs infected at MOI of 5 with Salmonella carrying pVAX1/HIV-1gag in co-culture

Figure 45

differences across individuals. One explanation for this phenomenon may simply be different medical histories of *Salmonella* exposure among the patients, since it is known that this may affect subsequent responses to the bacteria as vaccine delivery vehicles (238). Similarly, there are polymorphisms in the genes that encode host resistance factors in humans (89, 91, 93) such that individuals are likely to have distinct immunologic responses (and therefore vulnerabilities) to microbial pathogens, which would affect their reception to microbial vaccine vehicles.

Finally, it has emerged recently that there is a continuum of memory CD8+ T-cell subsets involving an eventual conversion of so-called "effector" memory T-cells to "central" memory T-cells. It is the latter subset that comprises cells that are capable of persisting and proliferating (239). We believe, therefore, that the expanding, IFN-γ–secreting T-cell populations that we detect in our ICS assays are central memory T-cells. Thus, because our individual patients were sampled at different points in their infection and disease course, it is possible that their T-cells were assayed at different points in the effector to central memory continuum, and, thus, with different proportions of proliferative subsets present.

DISCUSSION

We chose to measure T-cell IFN-γ expression as a gauge of the potential for DCs infected with *Salmonella* carrying DNA vaccine vectors to present antigen. Accepting IFN-γ as an appropriate cytokine marker of memory T-cell activation (and,

therefore, of effective DC antigen presentation) presumes that it is an accurate indicator of functional (central) memory T-cell activity. Recently, however, some investigators have questioned the use of IFN-γ assays as definitive measurements of the immunogenic potential of vaccine candidates.

Instead, a number of assays have been designed towards detecting actual cytolytic function, a return to the basis of the now largely disfavored ^{51}Cr-release assay. These include measurements of perforin (234, 240), granzyme (240, 241), caspase-3 activity (242) and CD107a and b as a reflection of degranulation (243). Additionally, several groups have explored the utility of monitoring the expression of other cytokines, including TNF-α (242, 244) and IL-2 (235). Indeed, it has been noted that expression of cytokines such as IL-2, MIP-1β, TNF-α and IL-4 dominate in response to effective vaccines against HBV and tetanus, not IFN-γ (245, 246).

Similarly, we must consider ultimately whether it is most appropriate to measure CD8+ T-cell responses in general as an indicator of immunogenicity. It has been demonstrated in the SIV/rhesus macaque model, for example, that viremia can be controlled even in the absence of CD8+ T-cells (247, 248). Furthermore, the recent prominent case of HIV-1 superinfection in the face of broad CD8+ T-cell responses (249) suggests that CD8+ T-cells may not play as critical a role in controlling infection as HIV vaccinologists previously thought.

Thus, it is perhaps relevant to note that immune responses detected in co-cultures involving DCs infected by *Salmonella* carrying *gag* vectors include CD4+ activity, as well as CD8+ (Figures 42-45). Given the likely mechanism of DNA vaccine delivery by the invasive, intracellular bacteria, it is provocative to consider how the system might yield MHC class II-restricted antigen presentation. We may note, for example, that bystander DCs that internalize apoptotic cells and process their antigens for presentation do so to both CD4+ and CD8+ T-cells (78, 99, 100). Additionally, it is possible that the bacteria themselves express the eukaryotic vaccine vectors to some extent (48-53), which would certainly result in MHC class II-restricted presentation by DCs.

Perhaps the most significant conclusion we can draw from the data herein, therefore, is about the potency of DCs as APCs. We must accept, for example, that very few DCs are actually reached with infection at MOIs of between 1 and 5. Indeed, in our early studies aimed at assessing the degree of target cell expression as a reflection of functional vector transfer (See Chapters III and V), MOI was kept as high as possible (e.g., 50-100) in order to presumably reach the maximal number of APCs. In carrying out studies aimed at confirming antigen presentation, however, it was appreciated that widespread expression is not equivalent to widespread presentation; rather, DCs are largely rendered apoptotic as a consequence of bacterial invasion. Thus, in reducing MOI, we can preserve the viability of the small fraction of DCs that are reached and invaded by the *Salmonella* carriers.

The fact that such a small population of DCs can have such a significant effect on T-cells is not surprising. It is well-established, in fact, that very few DCs are required in order to activate literally thousands of T-cells (131, 250). Furthermore, although DCs are the primary APCs of the body, they constitute <1% of the total cell population of lymphoid tissue (71). The system of vaccine delivery evaluated in the present study can thus serve in part to illustrate the relative functional importance of DCs among the cells of the immune system. Moreover, the apparently successful antigen presentation achieved here clearly suggests that targeting DCs may be generally fruitful as a vaccine design strategy.

Chapter VII:

Discussion

HORIZONTAL GENE TRANSFER

We have known for 60 years that functional DNA can be transferred from one organism to another, thereby changing the genetic character of the latter (251). The potential significance of horizontal gene transfer was not fully recognized until the 1950s, however, when bacterial multi-drug resistance emerged on a global scale. It is now understood that horizontal (or "lateral") gene transfer has played an integral role in the evolution of bacterial genomes (252). In eukaryotic phylogeny, too, horizontal gene transfer has apparently occurred with important evolutionary consequences. Indeed, in the years since the first full genome sequences were made available, there has been renewed interest in the topic (253-257).

Among unrelated species, gene transfer is infrequently observed. Rarely, for example, have we seen spontaneous genetic exchange between prokaryotes and eukaryotes. One well-documented case of such an anomaly is the induction of tumors in higher plants by *Agrobacterium tumefaciens*, wherein a bacterial plasmid is taken up and expressed by a plant cell (258).

The first documented evidence of direct transfer of cloned genes from bacteria to mammalian cells was reported in 1980 (259). Expression of the plasmid gene was

detected very infrequently, however, and appeared to be a consequence of sporadic phagocytosis. Many years later, another group reported genetic transfer from wild-type *Listeria* and, to a lesser extent, *Shigella* to mammalian cells *in vitro* (67).

It was not until 1995 that attenuated bacteria were intentionally manipulated towards the targeted delivery of DNA to eukaryotic cells (58). With this innovative approach, a new paradigm for vaccination was born: that of a facilitated genetic immunization, or *bacterial transduction*. Although integration into the target cell genome is not involved (as with a retrovirus), we feel that the use of a "third party" to deliver genetic material ultimately warrants such a term. Thus, the bacteria employed as transducers are "vehicles," rather than "vectors," as they are not intended to express the extra-genomic DNA they carry themselves.

BACTERIAL TRANSDUCTION

It has been known for decades by bacteriologists that intracellular bacteria (including *Mycobacteria*, *Chlamydia*, etc., as well as *Salmonella*) manage to survive within phagosomes (58, 260-263). When we first undertook to develop *Salmonella* as plasmid-delivery vehicles, however, we could only speculate as to precisely how the attenuated bacteria might accomplish the task (Chapter III, Figure 1). Although the mechanism of plasmid nuclear import remains a mystery, *Salmonella* persistence and avoidance of lysosomal degradation within its host cell is now a well-understood phenomenon.

Upon invasion, *Salmonella* immediately begins to shield itself from host cell defenses by using its virulence system. It controls its intracellular niche, the *Salmonella*-containing vacuole (SCV), through the activity of various bacterial proteins, particularly those encoded by the *Salmonella* Pathogenicity Island 2 locus (SPI-2). Thus, the SCV is effectively segregated from the cell's degradative pathway throughout the life-span of the bacteria (43).

SCVs are actively maintained by *Salmonella*-induced filament (Sif) A, which is expressed by the SPI-2 Type Three Secretion System (TTSS) (42, 43). Furthermore, the SCV is matured by the bacteria in a rab7 GTPase-dependent manner (264, 265).

Importantly, another SPI-2 TTSS protein, SpiC, interferes with intracellular trafficking (266), preventing phagosomal-lysosomal fusion (267). Bacterial Sif proteins work further to prevent SCVs from proceeding to late endosomes, and even interfere with the trafficking of non-*Salmonella*-containing vesicles (268, 269). Additionally, it has specifically been revealed that SPI-2 TTSS proteins prevent all contact between SCVs and NADPH oxidase-containing vesicles (270-272). In fact, *Salmonella* uses enzymes to defend itself from oxidative stress, including superoxide dismutase and glucose 6-phosphate dehydrogenase (43).

Remarkably, it has recently emerged that the bacteria restore cytoskeletal architecture once within their protected SCVs. Releasing a protein called SptP, *Salmonella* reverses the cellular changes induced by its invasion (273). Finally, it is also now known that most of the recently elucidated bacterial survival strategies probably apply to persistence within DCs, as well as macrophages (274), though most relevant studies have involved macrophages or macrophage cell lines.

Although we now understand that aroA- *Salmonella* probably remain within vesicles until they perish intracellularly (Chapter III, Figure 1), it remains unclear how the plasmids they deliver are imported into the eukaryotic nucleus. Assuming that the delivered DNA is released directly into the cytoplasm once the bacteria lyse and the vesicular wall dissipates, it must be transported into the nucleus for transcription to occur. With subsequent translation in the cytosol, the foreign protein would be degraded by the proteasome, and resulting peptides shuttled into the endoplasmic reticulum (ER) by transporters-associated-with-antigen-presentation (TAP) for MHC class I presentation (275). This endogenous pathway is, in fact, what is assumed to occur with conventional, untargeted DNA vaccination once the injected plasmids are taken up by APCs (276), although it is recognized in this case that the majority of DNA is rapidly degraded by nucleases (277).

A more appropriate, parallel model to consider would be the use of a so-called "gene gun" for DNA vaccination. This technique involves affixing the vaccine plasmids to gold particles before inoculation (278). Similarly, different kinds of

DNA microparticles have been produced for use in immunization (279, 280). It is thought that such a formulation permits direct delivery to APCs, which phagocytose the synthetic particulate matter. Furthermore, gold or microparticles would protect the DNA from extracellular degradation and possibly allow for mucosal administration (279, 280). Presumably, the DNA particles enter the phagosomal-lysosomal pathway towards intracellular degradation. While foreign non-DNA components could in theory be presented on MHC class II molecules, the DNA itself is apparently imported into host cell nuclei and expressed. Encoded proteins are then presented on MHC class I molecules (279).

The use of attenuated *Salmonella* to carry DNA vaccines, therefore, would allow for similar mechanistic advantages. As an adjuvant, *Salmonella* not only permits for targeted delivery through passive phagocytosis by APCs, but also by active invasion of them. Furthermore, like microparticles, bacterial vehicles protect DNA from premature extracellular degradation. Additionally, unlike synthetic formulations, *Salmonella* bears components that have naturally evolved as immunostimulants, including LPS as well as unmethylated sequences in bacterial DNA (281). Finally, as discussed previously (See Chapter IV), *Salmonella* vehicles allow for the mucosal administration of DNA vaccines directly to inductive sites.

In general, a system of bacterial transduction as a vaccine strategy offers considerable promise for *in vivo* use. Apart from the built-in adjuvant functions described, *Salmonella* is a practical tool because of its well-understood genetics and

physiology. Indeed, live attenuated *Salmonella typhi* is already in use as a vaccine against typhoid fever (27). Furthermore, infection with attenuated strains is easily controlled with antibiotics, which provide a "safety net" in the event of any systemic toxicity (e.g., from LPS) or reversion to wild-type virulence.

Nonetheless, certain potential complications and confounders of the approach must also be considered. Perhaps the greatest threat to *in vivo* efficacy that has emerged from *in vitro* experimentation is cytotoxicity: As we have seen, *Salmonella* infection can induce apoptosis of APCs. *In vivo*, however, this effect may not prove too much of a hindrance to effective antigen presentation, since uninfected, healthy bystander DCs can cross-present (97-100). Furthermore, we may conclude from the studies at hand (See Chapter VI) that there are sufficient numbers of viable DCs to effectively present vaccine antigens to autologous T-cells, even with a very low MOI.

Another theoretical obstacle to using a *Salmonella*-based vaccine strategy *in vivo* is the possibility of pre-existing immunity to the bacterial vehicles themselves. It is known, however, that immunity against one strain or serotype of *Salmonella* does not protect against infection with another (282, 283). Thus, an individual previously infected or inoculated with *S. typhi* would not raise a memory response against *S. typhimurium*. More recently, it has been seen in the context of recombinant vaccinia vectors that pre-existing systemic immunity can be overcome by mucosal immunization (193, 194, 284), a phenomenon that may also apply to *Salmonella*. More specifically, however, investigators have found that prior immunologic

experience with either a homologous or heterologous strain actually potentiates the subsequent serum and mucosal antibody responses directed against foreign antigens delivered by recombinant *Salmonella* (101, 238). Thus, prior exposure to *Salmonella* as a pathogen or a vaccine should not preclude its subsequent use as a carrier for DNA vaccines against other pathogens. In fact, immunologic experience with *Salmonella* may enhance the subsequent response to a foreign antigen encoded by a *Salmonella*-delivered vector (101).

Another concern regarding DNA vaccination in general is the risk of integration into the host cell genome, which may promote oncogenesis. While immunization with naked DNA has not been found to lead to genomic integration (285), delivery of eukaryotic expression vectors by invasive *E. coli* (286) and attenuated *L. monocytogenes* (61) has been reported to yield some chromosomal integration *in vitro*. This occurrence may be a consequence of the high plasmid copy numbers delivered to each cell by the bacteria (281). The risk for integration with use of bacterial vehicles *in vivo* is not yet known. It should be recalled, however, that transduced cells are probably fated to die, either from the apoptotic effects of *Salmonella* invasion, or from CTLs that respond to their presentation of foreign antigens (derived from the DNA vaccine or the bacterial carriers themselves). Thus, the risks associated with chromosomal integration may be averted *in vivo*.

FUTURE PROSPECTS

Developing the strategy of bacterial transduction as an approach to vaccination would require that we reach the optimal balance of maximal immunogenicity and minimal toxicity *in vivo*. The background of the carrier strain, the type of mutation selected to achieve attenuation, and the intrinsic properties of the immunogen itself would all have to be considered before proceeding to clinical trials. Any such aspect of the formulation could, alone, affect the extent and quality of the immune response elicited (287).

Salmonella spp. are not the only bacteria that could be used in the system. *Shigella*, *E. coli* and *Listeria*, for example, have also been investigated as potential vaccine carriers (58, 59, 61, 62, 64, 288), and the relative advantages of the different species should be thoroughly explored. More importantly, perhaps, genetic modifications to the carriers could achieve greater efficacy. While *Shigella* and *Listeria* are known to actively escape from phagosomes, *Salmonella* and other candidate carriers could possibly be improved by engineering them to express an enzyme with phagosomal escape function, such as listeriolysin (281).

In addition to strain choice, careful consideration must be given to the precise nature of the attenuating mutation, including the level of attenuation and stabilization of the attenuated phenotype. An auxotrophic mutation involving the biosynthesis pathway of purines or aromatic amino acids, for example, minimizes the virulence of

Salmonella (289). Other consequences of such mutations, however, should also be factored into the design of the system. It is known, in fact, that mutations affecting virulence properties of the bacteria tend to evoke different immune responses. Thus, different mutants may actually promote different T help patterns (287).

Nonetheless, minimizing toxicity to the host is certainly paramount in choosing a vaccine delivery vehicle. A gram negative bacteria such as *Salmonella* could theoretically induce septic shock if it is not carefully modified. Thus, as a tool in cancer therapy, *S. typhimurium* mutants have been engineered to avoid inducing a systemic TNF-α based inflammatory response while retaining hyperinvasive properties thought to be desirable for efficacy (290).

Dosing and vaccination schedule would also have to be experimentally determined. What is the minimum effective dose of bacteria needed to ensure *in vivo* transduction and antigen presentation? Does the priming need to be boosted? What are the *in vivo* kinetics of bacterial growth and persistence? (Is the administration of antibiotics necessary for timely elimination of the vehicles?)

More important, of course, is the need to choose antigens wisely. A DNA vaccine against HIV-1, for example, is a significant challenge to design, even without considering method of delivery (13, 14). What permutation of viral genes should be included? (Should they be "codon-optimized" for increased eukaryotic expression and Rev-independent nuclear export?) Which vector backbone should be employed,

and with which eukaryotic promoter? Is a leader sequence necessary? In general, steps should be taken to maximize gene expression *in situ* and augment DNA vaccine-elicited immune responses, in addition to the adjuvanticity provided by the bacterial carriers.

Development would also require *in vivo* studies prior to clinical trials. As we have seen, however, use of the murine model is limited and confounded by the cytotoxicity of *S. typhimurium* towards murine DCs. For the purpose of evaluating vaccine candidates against HIV-1, then, it would instead be more sensible to employ the rhesus macaque/SIV system. Although this non-human primate model has been very important for pre-clinical challenge studies of AIDS vaccines, it has recently been pointed out that improvements are required to more closely reflect the actual biological circumstances of HIV-1 infection in humans (4). Bearing this need in mind, however, it would indeed be useful to attempt *in vivo* immunization using bacterial transduction in macaques.

In general, we feel that the use of *Salmonella* carriers to deliver DNA vaccines constitutes a powerful tool with which to improve the efficiency and augment the immunogenicity of basic genetic immunization. Because of its simplicity and economy, DNA vaccination has been especially attractive as a strategy for use in the developing world. With the additional advantage of oral administration, therefore, its potential utility is enhanced even further. Indeed, the system explored in this report offers an excellent prototypical design from which to base other such technologies of

genetic delivery. As an approach to immunization, specifically, it may predict the general future direction of vaccinology.

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