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Prime boost HIV vaccination with recombinant influenza virus vectors stimulates specific and mucosal CD8$^+$ T cell immune response in BALB/c mice

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Abstract

HIV/AIDS continues to be a significant medical problem worldwide. An effective and safe vaccine remains a high priority. Most HIV vaccine candidates to date have failed to elicit effective immune responses that are necessary to control HIV infection. The results of a promising phase III trial conducted in Thailand using a recombinant canarypox vector vaccine (ALVAC) expressing HIV Gag in combination with recombinant HIV-Env glycoprotein gp120 (AIDSVAX), showed 31.2% efficacy in humans and raised the prospect of a protective vaccine. The most recommended HIV vaccines are focusing on inducing specific CD8+ T as a critical immune response to control progression and dissemination of HIV virus from the site of infection into different mucosal compartments of the body.This study project used influenza viruses as a mucosal live vaccine vector to stimulate effective CD8+ T cell immunity. Recombinant influenza A viruses, H3N2 (HK-X31) and H1N1 (A/PR8/8/34) expressing defined mouse HIV-1 CD8+ T cell epitopes (H-2Kd Gag197-205 and H-2Kd Tat17-25) in the neuraminidase (NA) stalk were generated using reverse genetics and administered as a prime-boost vaccine within various mucosal routes of vaccination, intranasal-intranasal, intravaginal-intravaginal, intranasal-intravaginal and intravaginal-intranasal vaccination in BALB/C mice. Following those prime-boost vaccinations, tetramer and intracellular cytokine staining assays used for the detection of specific CD8+ T cell immune response in harvested organs, spleen, bronchoalveolar lavage (BAL), mediastinal and inguinal lymph nodes. In addition, mucosal HIV-specific CD8+ T cells were detected using specific anti-mouse CD8α antibodies directed against specific integrins (LPAM-1 and CD103). Moreover, the level of specific cytokines, such as interleukin-15 (IL-15) detected within specific mucosal CD8+ T cells for the detection of the migrated HIV-1 Gag+ CD8+ T cells.Our result showed there was an induction of CD8+ T cells targeted H-2KdGag197-205, compared to no CD8+ T cell responses specific for H-2Kd Tat17-25 in recombined influenza-HIV vaccinated BALB/c mice. Also, comparable HIV and endogenous influenza-specific CD8+ T cell responses following intranasally-intranasally prime-boost vaccination in harvested lymphoid tissues, spleen, bronchoalveolar lavage, and mediastinal lymph nodes compared to inguinal lymph nodes which included a high proportion of specific CD8+ T cell immune response following intravaginal-intravaginal prime-boost infection. Moreover, a proportion of these cells isolated from mice infected with recombinant influenza-HIV vaccine intranasally-intranasally prime-boost expressed mucosal surface integrins, especially LPAM-1(α4β7) of local and distal lymph nodes higher than the levels observed following intravaginal vaccination. In addition, mucosal LPAM-1+HIV-Gag197-205+ CD8+ T cells harvested of intranasal prime-boost vaccinated mice were recognized by a high expression of IL-15 compared to LPAM-1-HIVGag197-205+ CD8+ T cells.We conclude that the intranasal prime-boost vaccination as one of the mucosal routes of vaccination using recombinant influenza viruses as mucosal viral vectors of HIV vaccine in BALB/c mice has an important role in stimulating both specific and mucosal CD8+ T cells within a high level and these cells would be important for migration of mucosal specific CD8+ T cells given the mucosal acquisition of HIV infection and control of HIV-1 virus dissemination through mucosal compartments.

Keywords: *Prime boost HIV vaccination, recombinant influenza virus vectors, specific and mucosal CD8$^+$ T cell, immune response, BALB/c mice*

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Abbreviations

HLA, Human leukocyte antigen; MHC-1, major histocompatibilty complex class I; Env, HIV envelope; Gag, HIV group specific antigen; Tat, HIV transcriptional transactivator NP, viral nucleoprotein;NP147, TYQRTALV peptide spanning residues 147–155 of viral nucleoprotein; Gag197, AMQMLKETI peptide spanning residues 197-205 of viralGroup-associated antigen, Tat17, QPKTACTNC peptide spanning residues 17–25 of viral transcriptional transactivator protein; H or HA, hemagglutinin; N or NA, neuraminidase;PR8, A/PR8/34;X31,

A/HK/x31;;BAL, bronchoalveolar lavage; ICS, intracellular cytokine staining; MDCK, Madin–Darby canine kidney; X31 NA-Gag197, recombinant X31 influenza virus expressing the AMQMLKETI peptide in the NA stalk; PR8 NA-Gag197, recombinant PR8 influenza virus expressing the AMQMLKETI peptide in the NA stalk; X31 NA-Tat17, recombinant X31 influenza virus expressing the QPKTACTNC peptide in the NA stalk; PR8 NA-Tat17, recombinant PR8 influenza virus expressing the QPKTACTNC peptide in the NA stalk.

Introduction

Since the first identification of HIV/AIDS, it has become a serious threat to humanity. More than 60 million individuals have been infected in this epidemic, with 34 million infected persons are living with HIV infection in 2010. Moreover, more than 2.6 million new cases of infection are reported every year (Kaufman et al. 2008; Barouch 2008; UNAIDS/WHO 2009; UNAIDS 2012). Most cases of infection are located in the developing world, and nearly half of these individuals have died, especially in under developing countries (Duerr, Wasserheit& Corey 2006; Mathers & Loncar 2006; Al-Shareefi & Cotter 2018). The generation of a safe and effective HIV-1 vaccine remains the most likely approach to control HIV infection in humans (Johnston &Fauci 2008). There have been many trials to develop new HIV vaccine candidates during the last 30 years, including DNA vaccines, recombinant vectored and subunit vaccines, mostly in non-primate animals, such as macaques. However, four candidate vaccines have been tested in humans with no significant efficacy for 3 of these, but one Thailand in 2009 (RV144: phase III) elicited a moderate level of protection against HIV (31%). As mucosal surfaces of the human and animal body are considered the main portal of entry for most pathogenic viruses, therefore the main target to control the mucosal transmission of these viral infections is the induction of mucosal immune response. For example, HIV is transmitted into the body through gastrointestinal and genitor-rectal mucosa, while other viruses, influenza, and poliovirus are penetrating through the respiratory tract mucosa. All these mucosal areas are armed by specific cytotoxic CD8+ T cells as the direct line of the immune response against mucosal viruses (Belyakov, IM et al. 2004). Moreover, it is highly recommended for effective HIV vaccines to be able to induction of specific CD8+ T cells responses by targeting highly conserved regions of HIV-1 virus, which would be more effective in control virus replication.

Also, HIV-1 specific CD8+ T lymphocytes play an effective role in preventing replication of viruses through mucosal surfaces, such as genital mucosa, resulting in control of HIV infection. For example, seronegative, resistant Kenyan sex workers have a high level of mucosal cytotoxic CD8+ T cells, compared to infected groups of sex workers (Kaul et al. 2000). Also, it is indicated that HIV-1 resistant individuals are similarly characterized by high levels of granzymes and perforin, leading to the high cytolytic activity of cytotoxic CD8+ T cells. Additionally, there is a negative correlation between HIV-1 RNA copy number and the level of specific HIV-1 CD8+ T cells (Hersperger 2010).

Moreover, there is a highly significant proportion of HIV-1 Gag+ mucosal CD8+ T cells detected in rectal mucosa of a nonviremic group of HIV-1 patients compared to another group of HIV-1 chronic patient groups. This confirmed that the broadly mucosal HIV-1 Gag CD8+ T cells in mucosal tissues induced against conserved regions of the HIV Gag epitope have a critical role to control HIV-1 viral load compared to other parts of the body (Ferre et al., 2010). Also, as a response to high conserved regions of HIV-1 Gag epitopes, broadly Gag+ CD8+ T cells are controlling viremia in HIV-1 infected individuals compared to CD8+ T cells induced in response to variable regions of HIV-1peptides (Kunwar et. al., 2013; Al Shareefi et al. 2019a, 2019b).

According to all previous mentioned roles of HIV CD8+ T cell in control of HIV-1 replication and transmission, therefore our project's goal was to design new mucosal $CD8^+$ T cells based HIV vaccine using recombinant influenza viruses to deliver HIV-1 $Gag_{197-205}$ and Tat_{17-25} epitopes in order induce specific mucosal HIV-1 $CD8^+$ T cells to control HIV-1 replication and dissemination through mucosal compartment preferred by HIV virus entry.

Materials and methods

Rescue of recombinant influenza-HIV $Gag_{197-205}$ and influenza-HIV Tat_{17-25}

Two strains of laboratory influenza viruses, and X31 (H3N2) and PR8 (H1N1) have been used in the reverse genetics strategy. These strains are adapted in mice and so are ideal for the expression of HIV-1 $gag_{197-205}$ and tat_{17-25} epitopes in the mouse models used in this study. Live influenza vectors are created by reverse genetics using a viral genome including eight segments of the virus. First step generation of plasmids containing NA segments from these inserted with specific HIV-epitopes ($Gag_{197-205}$ and Tat_{17-25}), by providing whole amount of negative sense of RNA converted from the cloned cDNA, utilizing specific enzymes, such as RNA polymerase 1, and combined with the other seven plasmids to form the new generated virus after transfection of all eight plasmids into HEK cells and amplification of whole virus in MDCK cells.

Cloning work included the design of specific forward and reverse primers necessary for the generation of HIV epitopes, such as $gag_{197-205}$ AMQMLKETI (5'GCC ATG CAA ATG TTA AAA GAG ACC ATC 3') and tat_{17-25} QPKTACTNC (5'CAG CCT AAA ACT GCT TGT ACC AAT TGC 3', and amplification of new PR8-NA and X31-NA gene segments inserted with the generated HIV-1 epitopes ($Gag_{197-205}$ and Tat_{17-25}), ligated with pHW2000 plasmid and used as one segment of the eighth segments of new modified X31 or PR8 influenza viruses.
The cloning work involved amplification with PCR for cloning sequences of Gag197-205 into NA segment using specific designed primers, included forward primer for short fragment, Bm-NA-1 –F : 5'TAT TCG TCT CAG GGA GCA AAA GCA GGA GT-3'; Reverse primer for short fragment , X31-NA197(45)R: 5'-GAT GGT CTC TTT TAA CAT TTG CAT GGC GGA GTC GCA CTC ATA TTG CTT AAA ATG CAA TG-3'; forward primer for large fragment, X31-NA197(45)F: 5'-GCC ATG CAA ATG TTA AAA GAG ACC ATC CCC GCG AGC AAC CAA GTA ATG CCG TGT GAA-3'; and reverse primer for large fragment ,Bm-NA-1413R: 5'ATA TCG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT-3'.

The amplification work included two PCR reactions, the first aimed to generate two overlapping fragments (short & large fragments) of X31-NA and PR8-NA with the inserted HIV-CD8+ T cell epitopes ($gag_{197-205}$& tat_{17-25}). For example, the two primers, Bm-NA-1 (5'-TAT TCG TCT CAG GGA GCA AAA GCA GGA GT-3') as the forward primer and X31-NA197(45)R (5'-GAT GGT CTC TTT TAA CAT TTG CAT GGC GGA GTC GCA CTC ATA TTG CTT AAA ATG CAA TG-3') as the reverse primer to amplify the short fragment of the X31-NA with Gag epitope, with the other two primers X31-NA197(45)F (5'-GCC ATG CAA ATG TTA AAA GAG ACC ATC CCC GCG AGC AAC CAA GTA ATG CCG TGT GAA-3') as the forward primer and Bm-NA-1413R (5'-ATA TCG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT-3') as the reverse primer for amplification of large fragment of the X31-NA-$gag_{197-205}$. The second recombinant PCR reaction involved two primers, Bm-NA-1 (5'-TAT TCG TCT CAG GGA GCA AAA GCA GGA GT-3') as the forward primer and Bm-NA-1413R (5'-ATA TCG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT-3') as the reverse primer. This recombinant PCR2 amplified a single entire NA segment containing the Gag epitope that can be inserted into the reverse genetics plasmid pHW2000. All of the previous steps were followed for the amplification of X31 and PR8-NA-Tat17-25. Then, the generated NA plasmid has been transformed into E. coli JM109 competent cells for growing up and then purified and digested using specific restriction endonuclease enzymes and then sequenced for detecting the inserted HIV epitopes. Finally, the generated NA plasmid used combined with the other 7 plasmids of the influenza virus to generate the recombinant influenza-HIV virus for use as a vaccine.

After that, the ability of modified recombinant influenza viruses for haemagglutination assay by chicken red blood cells (RBCs), which were washed twice with PBS and RBCs diluted with PBS to an approximate concentration of 0.5%. A 50 µl aliquot of PBS was placed into each well of 96 well (V bottom) plate, and 100 µl of each influenza virus stock added into the first column of the plate, using a wild type virus as a positive control. Two-fold dilutions of the virus were made by transferring 50 µl between wells and disposing of the final 50 µl from the last well. After that, 50 µl of the standard chicken RBCs were added to all of the wells and then mixed by gentle tapping, followed by incubation for 30 min at room temperature after which agglutination was scored. Also, recombinant influenza viruses were amplified in 10 day old fertilized eggs. The virus stock of each rescued virus was diluted via a series of 1:10 fold dilutions ranging from 10-1 to 10-5 in sterile PBS. After that, the 10 days old embryonated eggs were injected with 0.1 ml of 10-5 diluted virus and incubated for 2 days in a 35 °C incubator, with the allantoic fluids harvested into 50 ml Falcon tube on ice.

Detection of recombinant influenza viruses in harvested allantoic fluids
RNAs were extracted using the RNeasy Mini Kit (Qiagen) as follows. Firstly, 150 µl of harvested allantoic fluid was mixed well with 500 µl of 70% (v/v) ethanol, and then 500 µl of RLT buffer added, with mixing. A 750 µl aliquot of the mixture was transferred to the purification column, followed by centrifugation for 30 seconds at 1500×g, with the eluate discarded and the previous step repeated with the rest of the mixture. After that, 500 µl of RW1 buffer was added to the wash column with centrifugation at 1500×g for 30 seconds and discarding of the eluate. The column was washed twice using 500 µl RPE buffer in a similar manner. The column was then dried by centrifugation for 1 min at 1500×g, followed by elution with 20 µl of nuclease-free water. This was allowed to incubate at room temperature for 1 min in order to dissolve the extracted RNA, followed by centrifugation for 30 seconds at 1500×g to elute the purified RNA product. After that, extracted RNAs were treated with RQ1 DNase (Promega) before converted to cDNA in order to avoid any genomic DNA of interference with IL-15 expression. Firstly, 8 µl of extracted RNA added to 1 µl of 10x DNase buffer and 1 µl of DNase and then samples were pulse spin and incubated for 30 min at 37 0C. Then, 1 µl of DNase stop solution was added to each sample and incubated for 10 min at 65 0C and finally hold on ice for 5 min to be ready for reverse transcription.

Furthermore, purified RNA of modified recombinant influenza viruses were reverse transcribed by adding the extracted RNA to an RNase- free PCR tube in a volume of 10 µl and to this was added 2 µl of 10× reverse transcriptase buffer (RT buffer), 2 µl dNTP mix, 2 µl of 10 µM oligo-dT primer (or BmNA-1 forward primer),

and 1µl of Omniscript reverse transcriptase), and 3 µl RNA mixed well, before incubation for 60 min at 37 0C. An aliquot of the resultant cDNA (5 µl) was mixed with 10 µl of 1× Phusion master mix, 2 µl of each forward and reverse primer (BmNA-1 and BmNA-1413), and 1 µl of nuclease-free water, and subject to the PCR2 cycling conditions (Table 1), then electrophoresed on 0.8% Agarose gel, as previously described. Also, for accurate titration of new generated recombinant influenza viruses, a plaque assay was used. This involved trypsinization of MDCK cell culture and then re-suspended in 5 ml of MDCK cell plaque assay media (500 ml of Gibco RPMI 1640) containing (2 mM L-glutamine (5 ml of 200mM stock), 1 mM MEM sodium pyruvate (5 ml of 100 mM stock), 100 U/ml penicillin/streptomycin (5 ml of 10,000 u/ml sock), 12 mg/ml Gentamycin (300 µl of 40 mg/ml stock) and 45 ml heat-inactivated fetal calf serum (FCS). After counting, the concentration was adjusted to 3.8× 105 cells/ml and 3 ml added to each well of a 6 well plate. The media was removed from wells after cell adherence and each well inoculated with 150 µl of ten-fold dilution of virus stock (10-1-10-7), in addition to no virus-added cells (negative control) and then plates incubated at 37°C, 5% CO2 for 45 min. A 200 µl aliquot of Trypsin Worthington (TW) was added to 50 ml of 2×L 15 medium (Invitrogen) and then mixed with 50 ml of 1.8% Agarose (A-6013 Agarose, Sigma-Aldrich) with 3 ml used to overlay the infected cells per well, followed by incubation at 37°C, 5% CO2 for 3 days. After that, Plaques were counted as plaque forming units (pfu) per milliliter in order to be used for titration of the generated modifies viruses before used in the mouse study.

Vaccination and infection of BALB/C mice

Six-eight weeks old BALB/c mice were purchased from the Animal Resources Centre (ARC) and then housed under daily observation and monitoring program until adaptation before they inoculated with the recombinant influenza vaccine strains following environmental adaptation. All of the animal experiments were completed according to protocols approved by the AAHL Animal Ethics Committee. The vaccination program followed various vaccine strategies to stimulate CD8 T cell responses. i) Intranasal administration of 30 µl (1 × 104 PFU) recombinant influenza vaccine (X31-NA-Gag 197-205) as a prime dose, followed 6 weeks later by intranasal immunization with 30 µl (50 PFU) PR8-NA-Gag197-205. ii) Intravaginal administration of 30 µl (1 × 104 PFU) recombinant influenza vaccine (X31-NA-Gag197-205) as a prime dose, followed 6 weeks later by intravaginal immunization with 30 µl (50 PFU) PR8-NA-Gag197-205. iii) Intranasal administration of 30 µl (1 × 104 PFU) recombinant influenza vaccine (X31-NA-Gag197-205) as a prime dose, followed 6 weeks later by intravaginal immunization with 30 µl (50 PFU) PR8-NA-Gag197-205, and iv) Intravaginal administration of 30 µl (50 PFU) PR8-NA-Gag197-205 as a prime dose, followed 6 weeks later by intranasal immunization with 30 µl (1 × 104 PFU) recombinant influenza vaccine (X31-NA-Gag197-205).

Preparation of single cells of lymphoid tissues

Spleens, bronchoalveolar lavage (BAL) and lymph nodes, inguinal (ILN) and mediastinal (MedLN) lymph nodes were harvested from different groups of BALB/C infected mice. These were used for the preparation of single cells suspension of lymphocytes, which were treated with anti-mouse IgM + IgG in a petri dish for 45 min for panning of B lymphocytes and enrichment of T cells. Lymphocytes were counted using a hemocytometer, and the cell concentration brought to 1-2 × 106 cells/ml by adding an appropriate amount of complete RPMI for T cell phenotype, intracellular cytokine staining (ICS) and tetramer staining following flow cytometry techniques.

Tetramer staining

Tetramer staining includes adding aliquots (100 µl) of enriched T cells to a 96 well round bottom plate, followed by centrifugation at 1600 rpm for 4 min at 4°C, with the pellet resuspended in 50 µl of the appropriate tetramer, H2-KdNP147-155-PE (TYQRTRALV) or H2-KdGag197-205-APC (AMQMLKETI) at 1/100 dilution. These specific tetramers were prepared by complexing MHC-1 molecules with the specific CD8 T cell receptor and conjugated with specific peptides (glycoprotein H2-KdNP147-155 or KdGag197–205) and Streptavidin-phycoerythrin treated with Fluorophore. The 96 well plate was covered with foil and incubated at room temperature for 1 hr, following which the cells were washed two times with 150 µl FACS buffer, and resuspended in 50 µl diluted antibodies of interest: anti-CD8-PERCP (1/200 dilution), anti-CD62L-FITC (1/400 dilution), anti-CD44-APC (1/400 dilution), anti-LPAM-1-PE (1/400 dilution), anti-CD103-FITC (1/400 dilution) and anti-CCR9-FITC (1/400 dilution). Afterward, the plate of stained cells was incubated for 30 min on ice. Samples were then washed twice with 150 µl FACS buffer and cells resuspended in a final volume of 200 µl FACS buffer before analysis using a BD-FACS-LSR II.

Intracellular cytokine staining (ICS)

For intracellular cytokine staining, 100 µl of enriched T cells (depleting B cells by incubation at 37 0C for 1 h in the mixed solution of anti-mouse IgG/IgM (Jackson ImmunoResearch, CA, USA), added to a 96 well round bottom plate, followed by adding 100 µl stimulation mixture solution, containing the following items i) NP+ flu or Gag+ HIV peptide in dilution of 1/500 of stock concentration 1mg/ml (final concentration 2µg/ml); ii) IL-2 within a dilution 1/20 of the stock concentration 1000u/ml (final concentration 50u/ml); Brefeldin A (Golgi Plug in a final dilution 1/500 of stock concentration 5 mg/ml (final concentration 10 µg/ml), and then incubated for 5

hr at 37oC. The plate was then centrifuged at 1600 rpm for 4 min at 4⊥C, the supernatant discarded and cells washed twice with 150 µl FACS buffer. Then the supernatant was discarded and cells resuspended in 50 µl FACS buffer included anti-CD8-α-PerCP 5.5 (1/200), in addition to 50 µl of anti-CD8-PerCP (1/200) as a single color control, and then incubated for 20 min on ice, and then washed twice by centrifugation at 1600 rpm for 4 min at 4⊥C after adding 200 µl FACS buffer. Cells were then resuspended in 100 µl of Golgi-plug solution, containing 2 µl of Golgi-plug in 98 µl PBS, and then 100 µl of 0.5% paraformaldehyde was added, before initial incubation for 15 min at room temperature. The plate was then covered in foil and incubated overnight at 4⊥C. On the next day, the plate was centrifuged and the cells resuspended in 150 µl of Perm wash solution (10%) and incubated for 20 min at 4⊥C for permeabilizing the cytoplasm membrane. After that, cells were resuspended in 50 µl Perm wash solution (10%) containing a cocktail of intracellular cytokines (anti-mouse IFNγ-FITC; IL2-PE and TNF-α-APC antibodies of a final concentration (1/100), in addition to 50 µl of single color controls, anti-CD8-FITC, anti-CD8-APC, and anti-CD8-PE of a final concentration (1/100). The plate was then incubated for 30 min on ice and cells washed twice with 150 µl of 10% Perm Wash buffer and then re-suspended in a final volume of 200 µl FACS buffer for analysis by FACS using the BD-FACS LSR II. All steps of intracellular staining followed the protocol recommended by the Cytofix/Cytoperm Plus kit: PharmingenTM, CA.

Results

Rescue the characterised recombinant influenza-HIV viruses

In order to generate new modified live recombinant influenza viruses expressing HIV-CD8+ T cell epitopes, specific HIV-CD8 T cell epitopes, Gag197-205, (AMQMLKETI) and Tat17-25, (QPKTACTNC) were cloned in the NA segments of X31 and PR8 after residue 44 following a two-step PCR reaction. The first set of PCR reactions generated short (200 bp) and large (1200 bp) amplified fragment for each of X31-NA-Gag197-205 and Tat17-25 and PR8-NA-Gag197-205 and Tat17-25. While in the second step, the recombinant PCR2 reaction joined the two amplified fragments into one (1400 bp), resulting in the generation of recombinant X31-NA-Gag197-205 and Tat17-25 and PR8-NA-Tat17-25 sequences. The recombinant influenza NA segments (X31-NA & PR8-NA) expressing HIV-1 epitopes (gag197-205 & tat17-25) were cloned into plasmid pHW2000. This resulted in the generation of the recombinant plasmids: pHW2000-X31-NA-Gag197-205, pHW2000-X31-NA-Tat17-25, and pHW2000-PR8-NA-Tat17-25. And then, the generation of influenza viruses including the modified NA genes was achieved using reverse genetics, involving the transfection of these seven genes along with the wild respective type strains of influenza viruses (X31 or PR8) into HEK293T cells co-cultured with MDCK cells for amplification of the generated viruses. Virus supernatants were subjected to Haemagglutination assay to confirm the rescue of the viruses with similar HA titer, PR8-NA-Tat and X31-NA-Tat of 1/256 as an HA titer, while the titer of X31-NA-Gag is 1/128.

Following that, three rescued influenza viruses were amplified by the inoculation of the viral supernatant into 10 days old embryonated eggs. After two days, eggs were placed at 4 0C to inactivate the embryos and then the allantoic fluids containing the amplified viruses were harvested and kept at -800C. Also, in order to confirm the correct sequences of three modifies generated recombinant influenza viruses, the viral RNAs extracted from harvested allantoic fluids were reverse transcribed into cDNA and the NA gene then amplified using recombinant PCR2. This produced a band of the appropriate size for the NA segment (1.4 Kb). This was excised and purified to be sent for sequencing, and the results of sequences were accurate as similar as previously mentioned. This confirmed that the NA gene with inserted epitopes had been faithfully maintained in the amplification process.

Novel vaccine strain of recombinant influenza viruses expressing HIV-1 Gag197-205

Many previous trials of vaccination procedures in mice and other laboratory animals have been applied using live recombinant vectors expressing specific genes inserted into these vectors by a novel strategy named reverse genetics reverse genetics. As one of the most recommended viruses used in this strategy, influenza A viruses were used to generate recombinant influenza viral vectors, in which specific functional exogenous and endogenous nucleotides of HIV CD8+ T cell epitopes inserted into the NA stalk of influenza viruses, such as PR8 (H1N1) and X31 (H3N2). In order to evaluate CD8+ T cell immune response induced in mice as a result of stimulated NP+ and HIV specific epitopes, the amplified new generated recombinant influenza viruses expressed specific HIV-Gag197-205 epitopes need to be titred for detection of the viral dose in order to use in primary and secondary influenza infection of BALB/C mice. Therefore, a sample of the harvested allantoic fluids was subjected to an HA assay to confirm the successful amplification of the rescued viruses. Also, all of these rescued viruses used in a plaque assay in order to detect the optimum dose of these viruses used in the infection of mice.

In our study, influenza viruses were used as a live vector for delivering HIV-1 Gag197-205 into mucosal areas through a prime-boost infection in BALB/c mice in order to induce both HIVGag197-205 and influenza NP147-155 specific CD8+ T cell response. For evaluation of immuno-efficacy of the recombinant modified vaccine and its ability to induce a specific immune response against HIV and influenza epitopes, various procedures of

vaccination were performed in a mouse model in order to obtain which one of these procedures is the best procedure to enhance HIV specific and mucosal immune response. Accordingly, BALB/c mice were infected intranasally (i.n) as a primary infection using the modified X31-NA-Gag197-205 and X31-NA-Tat17-25 individually in order to test the ability of single dose of the recombinant influenza-HIV vaccine to stimulate specific CD8+ T cell immune responses and according to results of induced CD8+ T cell immune response against HIVGag197-205 following primary intranasal infection, there was no detected HIVGag197-205+ CD8+ T cells response in the group of BALB/c mice of intranasally infected with X31-NA-Tat17-25 compared to a clearly detected immune response induced for HIVGag197-205 in the intranasal primary infected mice with X31-NA-Gag197-205 (data not shown). Therefore, we continue our plan for the next experiment with X31-NA-Gag197-205 and PR8-NA-Gag197-205 only. While the next step was the prime-boost infection of BALB/c mice using the modified recombinant influenza-HIV vaccines within different routes of infection, intranasally or intravaginally as previously mentioned in the method section.

To characterize the quality of cellular immune response, two immune assays were used for the detection of both influenza NP147-155 and HIVGag197-205 specific CD8+ T cells induced by different mucosal routes of vaccination including primary and secondary infection of BALB/C mice. These two immunological methods were included intracellular cytokine (ICS) and tetramer staining assays.

Comparison of weight change following infection among BALB/c mice

6-8 week old BALB/c mice were primed intranasal or intravaginally with a dose of 1×104 p.f.u. of X31-NA-Gag197-205 influenza virus and then monitored daily for body weight until the 10th day of infection in order to compare the change of weight of the two infected groups of infected mice. Following the first and second day of intranasal infection, infected mice were maintaining their normal weight average, but they started losing their weight gradually in the third day of intranasal infection and continue with increasing the weight loss clearly during the fourth day of infection and reaching to the highest loss of weight in the fifth and sixth day with a statistically significant difference compared to the first two days following intranasal infection. After that, the weight loss stopped and gradually and slowly started gaining weight until the complete recovery of infected mice in the 10th day of infection as an indication of influenza virus clearance (Fig. 1, A). While in the intravaginal infected group of mice on the seventh day of infection, there was no detected loss weight during the 10th day period of infection (Fig. 1, B). Weight loss of intranasal infected mice from 3rd to 6th day of infection is evident that Viral replication was suspected as it is an evident with ability of influenza virus for replication in the mucosal region of pulmonary tract and the gradual related to the highest titer of the replicated virus in mice in compared to no loss of weight in the intravaginal infected group of mice as evidence that influenza virus could not replicate normally in the urogenital mucosa as there is no presence of a protease in the vaginal mucosa as it is necessary which need for cleavage of hemagglutinin (HA) gene of influenza viruses increasing their pathogenicity in the mucosal compartments (Garulli, Kawaoka & Castrucci 2004).

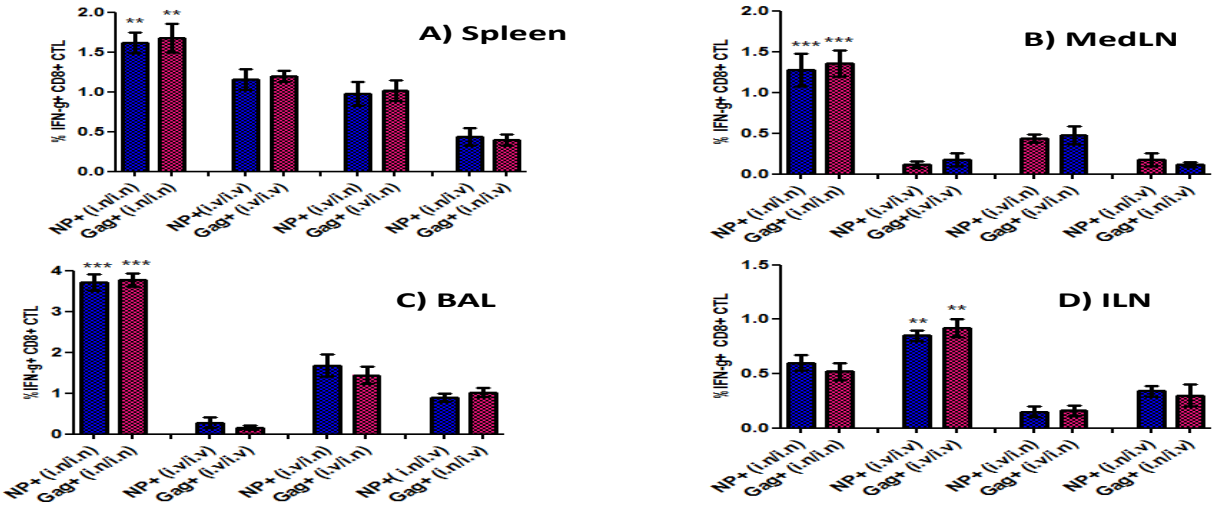
Induced CD8$^+$ T cells detected by tetramer staining

In order to detect both HIVGag197-205 and influenza NP147-155 CD8+ T cell immune responses induced following primary and secondary infection in mice, Primary infection has been used first to check the ability of the generated recombinant influenza-HIV viruses to induce specific CD8+ T cell immune response (data not shown). 6-8 week old BALB/c mice were infected individually with recombinant influenza viruses X31-NA-Gag197-205 or X31-NA-Tat17-25 of dose 1× 104 pfu, different organs of the infected mice were harvested to prepare T cells. Spleen, BAL, MedLN, and ILN of the vaccinated mice were used for isolation T lymphocytes and then stained with Kd-HIV-Gag and Kd-flu-NP labeled tetramers. The characterized induced CD8+ T cells specific for Gag197-205 and NP147-155 were equivalent as a proportion (Figure 1-A) and number of specific CD8+ T cells (Figure 1-B) and the higher level of induced cells was detected following each last intranasal dose of infection, within a highly significant difference following two intranasal doses of vaccination which was clearly detected in spleen (A), BAL (B) and mediastinal lymph nodes (C), compared to another routes of infection especially intravaginal route of vaccination, although there was a detected level of CD8+ T cells immune response in urogenital draining lymph nodes following intranasal infection.

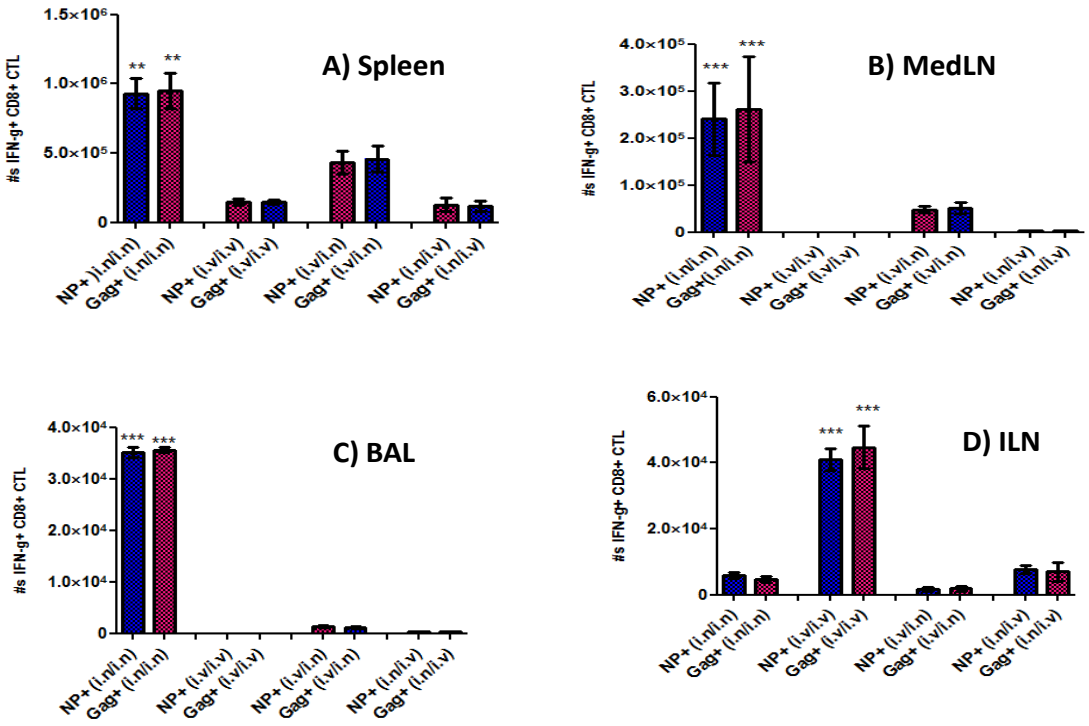
However, the best level of stimulated CD8+ T cell immune response in ILN was detected after intravaginal infection (D), particularly the prime-boost intravaginal vaccination and this can be explained as influenza virus has a high opportunity to replicate in lung and respiratory system draining lymph node, mediastinal lymph nodes (MedLN) as a result of high level of protease enzyme need for cleavage of HA1-HA2 of influenza virus increasing the infectivity and replication of the virus compared to the uro-vaginal area of low or non-detected titer of influenza virus as it is not containing protease enzyme (Garulli, Kawaoka&Castrucci 2004; Lu et al. 2012), although the specific viral antigen has entered into urogenital mucosa without replication of the viral vector and then recognized specific influenza NP147-155 and HIV Gag197-205 epitopes by antigen-presenting cells (APCs) and then detected by specific proliferated CD8+T cells inducing low detected level of immune

response against the HIV-Gag specific peptide and as can be seen in the same data detected compared to the intranasal infection.

Also, there was an equivalent proportion and number of specific tetramer+ HIVGag197-205 and influenza NP147-155 CD8+ T cells induced in spleen and another pulmonary inflammatory regions or draining lymph nodes (as shown in Figure 2-A and Figure 2- B) which also showed detected levels of HIVGag197-205 tetramer+CD8+ T cells induced as a response to different prime-boost vaccination strategies, however, the Intranasal prime-boost administration of our vaccine gave the strongest responses in spleen (A), BAL (B) and MedLN (C) than other routes of vaccination. While intravaginal prime-boost vaccination induced the highest responses in ILN (D) only.



(Figure 1-A): This figure showed that induced CD8+ T cells specific for Gag197-205 and NP147-155 were equivalent as a proportion of specific CD8+ T cells (Figure 1-B) and the higher level of induced cells was detected following each last intranasal dose of infection, within a highly significant difference following two intranasal doses of vaccination which was clearly detected in spleen (A), BAL (B) and mediastinal lymph nodes (C), compared to another routes of infection especially intravaginal route of vaccination, although there was a detected level of CD8+ T cells immune response in urogenital draining lymph nodes following intranasal infection.



(Figure 1-B): This figure showed that induced CD8+ T cells specific for Gag197-205 and NP147-155 were equivalent as number of specific CD8+ T cells and the higher level of induced cells was detected following each last intranasal dose of infection, within a highly significant difference following two intranasal doses of vaccination which was clearly detected in spleen (A), BAL (B) and mediastinal lymph nodes (C), compared to another routes of infection especially intravaginal route of vaccination, although there was a detected level of CD8+ T cells immune response in urogenital draining lymph nodes following intranasal infection.

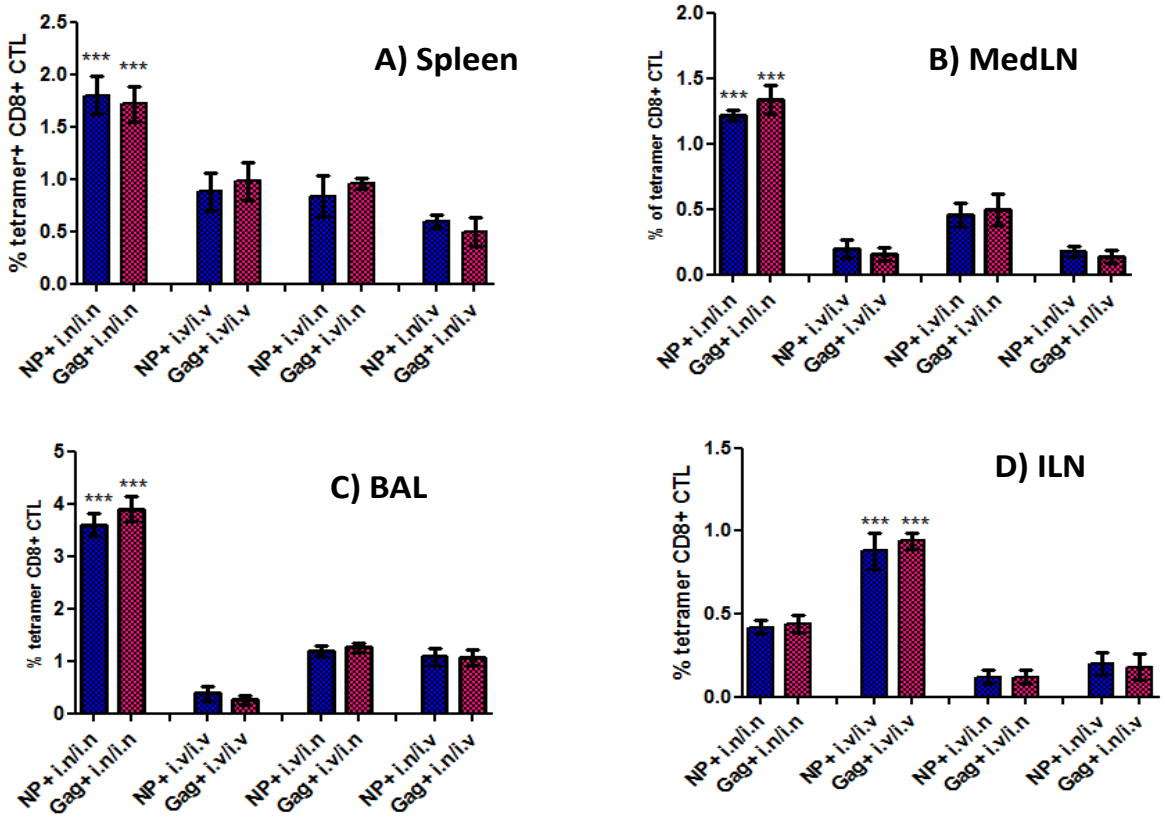


Figure 2-A: In this figure, there was an equivalent proportion of specific tetramer+ HIVGag197-205 and influenza NP147-155 CD8+ T cells induced in spleen and another pulmonary inflammatory regions or draining lymph nodes (as shown in Figure 2-A and Figure 2- B) which also showed detected levels of HIVGag197-205 tetramer+CD8+ T cells induced as a response to different prime-boost vaccination strategies, however, the Intranasal prime-boost administration of our vaccine gave the strongest responses in spleen (A), BAL (B) and MedLN (C) than other routes of vaccination. While intravaginal prime-boost vaccination induced the highest responses in ILN (D) only.

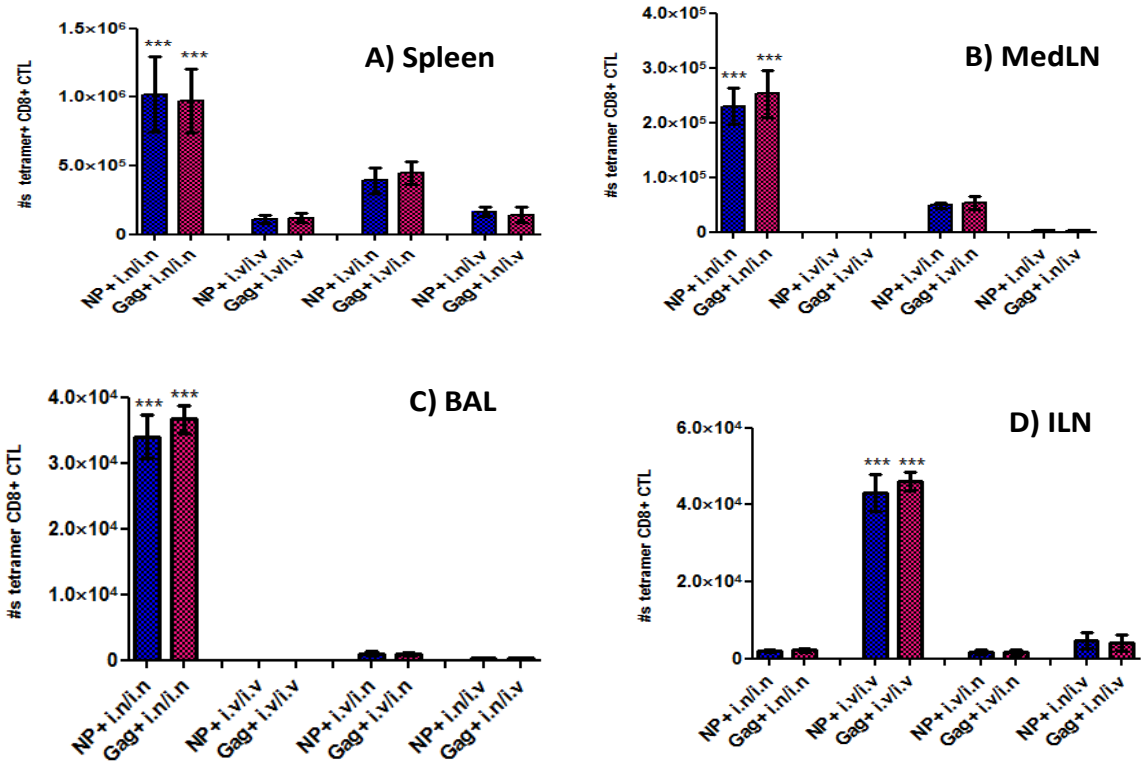


Figure 2-B: In this figure, there was an equivalent proportion of specific tetramer+ HIVGag197-205 and influenza NP147-155 CD8+ T cells induced in spleen and another pulmonary inflammatory regions or draining lymph nodes (as shown in Figure 2-A and Figure 2- B) which also showed detected levels of HIVGag197-205 tetramer+CD8+ T cells induced as a response to different prime-boost vaccination strategies, however, the Intranasal prime-boost administration of our vaccine gave the strongest responses in spleen (A), BAL (B) and MedLN (C) than other routes of vaccination. While intravaginal prime-boost vaccination induced the highest responses in ILN (D) only.

Detected functional specific CD8+ T cells by intracellular cytokine assay (ICS)

As a result of using various mucosal routes for primary and secondary infection of BALB/c mice, T cells harvested of the spleen, BAL, MLN and ILN of the vaccinated mice and treated with stimulation for five hours with each of Kd-HIV-Gag and Kd-flu-NP labeled peptides separately. For example, following influenza infection in mice, a number of specific T lymphocytes were detected by measuring the expressed IFN-γ+/CD8+ T cell-induced in a response to specific influenza peptide stimulation, whereas the function of T lymphocytes immune response is detected by measuring both TNF-α+ and IL-2+ expressed CD8+ T cells. As subsets of functional T cells, TNF-α+/CD8+ T cell population is a subset of IFN-γ+/CD8+ T cell and subsequently IL-2+ CD8+ T cells is a smaller subset of TNF-α+ CD8+T cells population (La Gruta, Turner & Doherty 2004). As mentioned in previous mice studies, it has been demonstrated that a number of antigen-specific CD8+ T cells producing IFN-γ+ are in a high similarity with a number of antigen-specific tetramer + cells. In our resent study, data were shown that there was an equivalent comparison for proportions and number of IFN-γ+/CD8+ T cells induced against both HIV-Gag197-205 and NP147-155 epitopes following recombinant HIV-influenza virus vaccine. Also, these data shown a high increase of IFN-γ+/CD8+ T cell against both Gag and NP+ peptides following prime-boost intranasal infection in various lymphoid organs, spleen (Fig. 3, A), BAL (Fig. 3, B), MedLN (Fig. 3, C), compared to another procedures of prime-boost infection. While in ILN the intravaginal infection was the only procedure that increases antigen-specific CD8+ T cells producing IFN-γ+ as the genital tract draining lymph nodes (Fig. 3, D).

Discussion

In our study, two HIV-1 epitopes were used to be delivered by two kinds of influenza viruses, X31 and PR8. First HIV-1 epitope derived from a highly conserved regions of group-specific antigen, Gag197-205 as a structural protein necessary for assembly of newly generated virion during life cycle of HIV-1 in infected individuals (Bryant & Ratner 1990), and the another one is a Tat17-25 as a regulatory protein with the ability to transmit virus from infected cell to non-infected cell (Gavioli, R et al. 2008; Gavioli, Riccardo et al. 2004) which would be targeted. The conserved nine amino CD8 T cell epitopes, specific of each of these HIV proteins were used, Gag197-205 (AMQMLKETI) and Tat17-25 (QPKTACTNC). These HIV epitope sequences as cloned cDNA was inserted into the respective NA molecules, and reverse genetics used to generate recombinant virus from these cloned cDNA ligated into pHW2000 plasmid, and then combined with another plasmids including the seventh cloned cDNA represented influenza gene segments (PA, NP, NS, M, HA, PB1 and PB2) to transfected into HEK cells and amplified into MDCK cells. The role of the plasmid is to provide the ability of these gene segments to be involved in transcription and translation resulting in the synthesis of viral mRNA and viral RNA.

It is anticipated that targeting these two different aspects of viral replication independently is likely to be synergistic in eliciting a stronger response. To allow for different vaccination strategies, two strains of influenza viruses were used, X31(H3N2) and PR8 (H1N1). Both are adapted in mice and share the same six internal genes (PB1, PB2, PA, M, NP, and NS segments), but differ from each other in both external segment genes HA, (hemagglutinin) and NA, (neuraminidase). Use of these two strains of influenza viruses as vectors should, therefore, avoid neutralizing antibodies being generated during secondary infections used for boosting and increasing the induction of CD8+ T cell immune response for influenza and HIV epitopes. Importantly, these two strains of laboratory influenza viruses have been used previously to express HIV-CD8 T cell epitopes in mice for stimulation of specific CD8 T cell immune response against specific HIV genes (Cukalac et al. 2009).

In our study, Influenza virus type A has been used as a live vector as it is considered one of the most attractive candidates of efficient viral vector used for expressing viral antigens, especially those targeting respiratory viral infections and other mucosal transmitted diseases (Marsh &Tannock 2005; Satterlee 2008; Smith 2007). This is because of the ability of influenza virus to infect through the mucosal regions, resulting in a broad and long term immune responses (Fodor et al. 1999; Mueller et al. 2010; Neumann &Kawaoka 2001; Yao 2008). It is also relatively easy to manipulate gene segments of influenza virus by reverse genetics to generate novel recombinant viruses for use as vaccine vectors (Palese et al., 1996; Steinhauer&Skehel 2002; Steinhauer 2009). Most of the recombinant influenza viruses generated by reverse genetics have involved manipulation of HA and NA genes (Honda et al. 1990; Hwang et al. 2000; Kobayashi, M et al. 1992; Poon et al. 1998; Yamanaka, Ishihama& Nagata 1990). Indeed, recombinant influenza viruses manipulated in this way have been used as vaccine vectors expressing HIV CD8+ T cell epitopes to stimulate a mucosal cytotoxic T cell response that is necessary to control viremia (Casimiro et al. 2005; Hoffmann et al. 2002; Liu, J et al. 2009; Pachler 2010; Rimmelzwaan et al. 2007; Sexton et al. 2009; Wit et al. 2004). For example, a recombinant influenza virus expressing HIV Gag epitope (P17) inserted into the NA segment could stimulate both humoral and cellular responses (de Goede et al. 2009; Fiorentini et al. 2010.; Pal et al. 2002; Siegismund et al. 2009), while an alternate HIV-1 vaccine candidate used HIV Envproteins inserted into the NA stalk of recombinant influenza vector to induce a robust cytotoxic CD8+ T cell response (Cukalac et al. 2009). In this case, the vaccine included two parts, a prime dose containing X31-NA-Env given intranasally and boost dose of PR8-NA-Env administered intraperitoneally, inducing CD8+ T cell response represented by specific HIV-1 Env cytokines, such as IFN-γ, IL-2, and TNF-α (Cukalac et al. 2009).

Also, recombinant influenza virus vectors (H1N1 and H3N2) expressing SIV CD8+ T cell epitopes (Gag or Tat) used as recombinant HIV vaccine in mouse models induced mucosal cellular immune response. For example, specific cytotoxic CD8+ and CD4+ T lymphocyte immune responses were stimulated in the vaccinated group of macaques promising to be protective against SIV infection in the future (Sexton et al. 2009). Also, the intranasal vaccination of macaques by the HIV recombinant vaccine has led to stimulating a strong mucosal immune response in vaginal mucosa of macaques protected infected macaques by virulent SHIV virus administered intravaginally (Barnett et al. 2008). Also, various trials of HIV-1 vaccination procedures based on the mucosal model of immunization were highly effective to induce both systematic and mucosal immune response against HIV-1. For instance, intranasal immunization by recombinant influenza vector expressing HIV-1 Env protein (gp41) has stimulated humoral immune response represented by HIV-1 specific antibodies in lung, spleen and urogenital mucosa in mice model (Ferko et al. 1998) This is because the ability of recombinant influenza viruses to replicate in upper respiratory tract leading to induce immune response in different parts, such as lung and urogenital tract (Ferko et al. 2004). In addition, live recombinant influenza virus expressed HIV-1 Env311 peptide inserted into NA stalk of influenza viruses, has been successfully used in mice model to stimulate HIV specific cytotoxic T lymphocytes immune response in mouse model trial.

According to the recent studies, both Gag and Tat have the ability to promote or inhibit replication of HIV-1 by their regulation of some cellular enzymes called helicases. These specific enzymes are hydrolyzing the nucleotide triphosphates (NTPs) and in a way of modifying the viral nucleic acids, and then modulating HIV-1 replication (Lorgeoux, Guo and Liang 2012). Also, Gag-specific CD8+ T lymphocytes are highly migrated to the different effector mucosal sites following intramuscularly infection with a recombinant adenovirus vector expressing Gag in mice and macaques (Kaufman et al. 2008).

This is because of the ability of HIV Gag peptide to stimulate specific HIV-1 CD8+T cell immune response in mice and other animal models (Faul et al. 2009; Lawrence et al. 2013; McGettigan et al. 2001; McGettigan et al. 2003). Moreover, there were highly specific conserved regions of HIV-1 Gag peptides detected on the wild type virus genome, in which it induces a robust HIV-1 CD8+ T cell immune response in the infected mice. For example, detected immune response against HIV-1 Gag H2d restricted AMQMLKETI epitopes were a mostly dominant following infection of BALB/c mice using DNA vaccine expressing these HIV-1 epitopes (Lawrence et al. 2013; Wallace A. 2013).

Previous studies of HIV vaccines in animal models were used different vectors to deliver specific HIV epitopes or proteins. For example, adenovirus, vaccinia virus, fowl pox viruses were used as vectors in various previous HIV-1 vaccine studies. All of these viral vectors used induced immune response in different levels; however, they were not protected against HIV. This is because they were not concentrated on the stimulation of mucosal immune response, which is considered the direct way to control HIV transmission through mucosal surfaces. Therefore, in our study, we focused on mucosal viruses to be used as a vector to deliver HIV epitopes. For this purpose, we chose the influenza virus, this is because of the ability of the influenza virus to induce a strong mucosal immune response particularly in the mucosal regions of the upper respiratory tract, which would be very sensitive to the influenza virus infection. Moreover, there is a high link and strong connection between the mucosa of the upper respiratory tract and of urogenital tract mucosa, with mucosally-administered antigens able to the illicit specific mucosal immune response in different mucosal surfaces (Holmgren and Czerkinsky, 2005). Importantly, the two strains of laboratory influenza viruses were used previously to express HIV-1 CD8+ T cell epitopes in mice for stimulation of specific CD8 T cell immune response against specific HIV Env epitope (Cukalac et al. 2009).

Following intranasal dose of infection, X31-NA-Gag197-205 influenza infected BALB/c mice were showing a significant difference loss of weight started from the third day until the sixth day of infection which was suspected followed replication of influenza virus in respiratory tract mucosa as a result of protease enzyme in pulmonary mucosal area increasing the pathogenicity of influenza virus infection in compared to the intravaginal infected group of mice which not shown any detected loss of weigh as there is no enzyme affecting the pathogenicity of infected influenza virus, however, this does not mean there was no induced immune response in the urogenital mucosa following influenza infection. Different parameters were highly recommended to use for the detection of characterized and magnitude of specific CD8+ T cells following infection or vaccination. For example, IFN-γ+ production measured by intracellular cytokines and cytotoxicity assay, and the peptide tetramer staining assay are the most dominant methods used for measurement of the immune response (Altman et al. 1996; Heeney&Plotkin 2006; Pantaleo&Koup 2004).

In order to compare of different mucosal routes for prime-boost infection in BALB/c mice, lymphocytes harvested from spleen, bronchoalveolar lavage, respiratory draining lymph nodes (mediastinal) and genital draining lymph nodes (inguinal) lymph nodes of various mucosal routes of immunized BALB/c mice were tested for production of cytokines, such as IFN-γ+, TNF-α, and IL-2 specific for HIV-Gag197-205 and influenza-NP147-155 epitopes of harvested CD8+ T cells stimulated five hours with each of Kd-HIV-Gag and Kd-flu-NP labeled peptides separately. Our results indicated that intranasal prime-boost infection of BALB/c group of mice with the recombinant influenza viruses expressing HIV-Gag197-205 induced a significant increase of producing IFN-g CD8+ T cells as proportion and number ($P < 0.05$) in respiratory lymphoid regions, BAL and MedLN in addition to spleen in comparison to that of another groups of mice immunized by intravaginal–intravaginal, or by combination of intranasal-intravaginal prime-boost vaccination. While in ILN, there is a significant increase in IFN-γ+ produced specific HIV-Gag and NP+ CD8+ T cells after intravaginal-intravaginal prime-boost infection compared to another route of immunization.

In our study, following influenza infection in mice, the number of specific CD8+ T lymphocytes was detected by measuring the expressed IFN-γ+/CD8+ T cell-induced in a response to specific influenza peptide stimulation, whereas the function of T lymphocytes immune response is detected by measuring both TNF-α+ and IL-2+ producing CD8+ T cells. As subsets of functional T cells, TNF-α+/CD8+ T cell population is a subset of IFN-γ+/CD8+ T cell and subsequently IL-2+ CD8+ T cells is a smaller subset of TNF-α+ CD8+T cells population (La Gruta, Turner & Doherty 2004).

As mentioned in previous mice studies, it has been demonstrated that a number of antigen-specific CD8+ T cells producing IFN-γ are in a high similarity with the number of antigen-specific tetramer + cells. In our resent study, data were shown that there was an equivalent comparison for proportions and the number of tetramer+ CD8+ T cells and IFN-γ+/CD8+ T cells induced against both HIV-Gag197-205 and NP147-155 epitopes following recombinant HIV-influenza virus vaccine. Also, these data showed a high increase of IFN-γ+/CD8+ T cell against both Gag and NP+ peptides following prime-boost intranasal infection in various lymphoid organs, spleen, BAL, MedLN compared to another procedure of prime-boost infection. While in ILN the intravaginal infection was the only procedure that increases antigen-specific CD8+ T cells producing IFN-γ+ as the genital tract draining lymph nodes. As mentioned previously, both tetramer and ICS indicated to a detected HIVGag197-205+ and influenza NP147-155+ CD8+ T cells responses in urogenital draining lymph nodes in addition to secondary lymphoid tissues, spleen is an evident that both influenza virus and HIVGag197-205 antigens were recognized by specific CD8+ T cells and this is also supported by ability of influenza virus to replicate in the genital mucosa of progesterone treated mice following infection with a higher dose of influenza virus-induced detected cytotoxic T cells in genital mucosa compared to lower dose of influenza virus led to low or not detected titer of replicated influenza virus with detected CTL response (Garulli, Kawaoka&Castrucci 2004). While intranasal prime-boost vaccination was the best procedure of infection-induced a higher specific and mucosal HIVGag197-205+ CD8+ T cells response in primary and secondary lymphoid tissues detected by both tetramer staining and intracellular cytokine assay showing the high number of tetramer+ and IFN-γ+/HIVGag+ or influenza NP+ CD8+ T cells.

Also, induction of mucosal CD8+ T cell immune responses through HIV vaccines aims to prevent virus transmission and progression (Bretscher 1999; Caley 1997; Gardner &Luciw 2008; Gherardi& Esteban 2005; Im&Hanke 2004). There are many routes to induce the mucosal immune response, but HIV mucosal vaccines are most effective when primed boost administrated intravaginally and intranasally, leading to a high mucosal and systemic immune responses compared to the other routes of vaccination (Belyakov, IM, Derby, M. A., Ahlers, J. D., Kelsall, B. L., Earl, P., Moss, B., Strober, W. and Berzofsky, J. A. 1998; Duerr 2010; Pialoux et al. 2008). In previous studies of HIV vaccines in mice, influenza viruses were used in the CTL based vaccines as a live vector expressing HIV-1 Env311 epitope induced a high response of specific CD8+ T cells against HIV-Env peptide in the spleen following a prime-boost intranasal infection, in addition to influenza specific CD8+ T cell response. These immune responses were included both IFN-γ+ and tetramer specific CD8+ T cell response (Cukalac et al. 2009). This is because influenza viruses used as vaccine vectors have the ability to replicate in pulmonary mucosa stimulating the mucosal immune response for influenza antigens epitopes in addition to the delivered HIV Gag epitope. While, using influenza virus intravaginally would stimulate a mucosal immune response in the genital area although there is no detected replicated influenza virus as the influenza antigen and HIV-1 epitope have been recognized by antigen presenting cells (APCs) inducing a specific and mucosal immune response in the genital mucosa (Garulli, Kawaoka&Castrucci 2004).

Also, the ability of previous trials of vaccines to induce a high cellular immune response and then migration of Ag-specific cytotoxic T cells to mucosal sites is depending on the route of Ag's delivery into the body. Additionally, the initial site of Ag's administration will affect the level of primary and secondary immune response for different strategies of vaccines, such as vector-based vaccines (Belyakov, IM et al. 1998a; Gallichan, WSaR, K.L. 1996; Kantele et al. 1999; Offit 1991). One of these vaccines is the recombinant adenovirus vector expressing SIV-Gag delivered intramuscularly, stimulated a highly detectable mucosal CD8+ and CD4+ T cells migrated from systemic sites in mice and rhesus monkeys (Kaufman et al. 2008).

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