Linköping University Post Print

Neutralizing activity and cellular immune responses induced in mice after immunization with apoptotic HIV-1/murine leukemia virus infected cells

Jorma Hinkula, Lilian Walther-Jallow, Anna Lauren, Barbro Makitalo, Monica Oberg, Britta Wahren, Eva-Maria Fenyo and Anna-Lena Spetz

N.B.: When citing this work, cite the original article.

Original Publication:

Jorma Hinkula, Lilian Walther-Jallow, Anna Lauren, Barbro Makitalo, Monica Oberg, Britta Wahren, Eva-Maria Fenyo and Anna-Lena Spetz, Neutralizing activity and cellular immune responses induced in mice after immunization with apoptotic HIV-1/murine leukemia virus infected cells, 2009, VACCINE, (27), 46, 6424-6431.

<http://dx.doi.org/10.1016/j.vaccine.2009.06.016>

Copyright: Elsevier Science B.V., Amsterdam.

<http://www.elsevier.com/>

Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-52391>

Neutralizing activity and cellular immune responses induced in mice after immunization with apoptotic HIV-1/Murine Leukemia virus infected cells

Jorma Hinkula^{a,b,c}, Lilian Walther-Jallow^d, Anna Laurén^e, Barbro Mäkitalo^d Monica Öberg,^e Britta Wahren^{a,b}, Eva-Maria Fenyo^e and Anna-Lena Spetz^{d*}

^aMicrobiology and Tumorbiology Center, Karolinska Institutet, Sweden

^bSwedish Institute for Infectious Disease Control, Department of Virology, Stockholm, Sweden

^cDepartment of Molecular and Clinical Medicine, Linköping University, Linköping, Sweden

^dCenter for Infectious Medicine, Department of Medicine, Karolinska University Hospital Huddinge, Karolinska Institutet, Sweden

^eDepartment of Laboratory Medicine, Division of Medical Microbiology/Virology, Lund University, Sweden

***Corresponding author:** Anna-Lena Spetz, Center for Infectious Medicine, F59, Karolinska Institutet, Karolinska University Hospital Huddinge, S-141 86, Stockholm, Sweden Phone; +46 8 58582272, Fax; +46 8 7467637, e-mail; anna-lena.spetz@ki.se

Abstract

Dendritic cells present microbial antigens to T cells after uptake of apoptotic vesicles from infected cells. We previously reported that immunizations with apoptotic HIV-1/Murine Leukemia virus (MuLV) infected cells lead to induction of both cellular and humoral immune responses as well as resistance to mucosal challenge with live HIV-1/MuLV infected cells.

Here we extended those studies and investigated whether apoptotic cells from HIV-1/MuLV infected cells stimulate the production of HIV-1 neutralizing activity.

We compared different routes of administration and were able to induce p24- and Nef-specific cellular proliferation after intraperitoneal (i.p.), intranasal (i.n.), subcutaneous (s.c) and intramuscular (i.m.) immunizations. Serum IgG and IgA antibodies directed against gp160, p24, or Nef were also produced regardless of immunization route used. However, the induction of mucosa associated IgAs from faeces or vaginal secretions were detected only after either i.p. or i.n. immunizations. We were able to measure neutralizing activity in sera of mice after i.p and i.n. immunization. Neutralizing reactivity was also detected after s.c and i.m. immunizations in the presence of the cytokine adjuvant granulocyte macrophage-colony stimulating factor (GM-CSF).

Conclusively we show induction of cellular and humoral immune responses including neutralizing activity after immunization with apoptotic HIV-1/MuLV infected cells in mice.

The results from this study support further evaluations using apoptotic cells as antigen delivery system for vaccination against HIV-1 in other animal models.

Keywords: HIV-1, virus neutralization, apoptosis, T cells, B cells, immunization routes

Running title: Apoptotic cells as antigen delivery system

1. Introduction

In the present study we used apoptotic cells as an antigen delivery system, with the aim to investigate whether immunizations with infected apoptotic cells are able to induce cellular and humoral immune responses in sera and at mucosal sites as well as neutralizing activity in sera. Microbial infected cells that undergo apoptosis can be taken up by neighboring antigen presenting cells such as dendritic cells and allow for efficient antigen presentation on major histocompatibility complex (MHC) class I and II molecules without infecting the antigen presenting cells [1]. This phenomenon termed cross-presentation was first coined studying minor histocompatibility antigens [2]. Cross-presentation of microbial antigens has since then been shown for many pathogens such as influenza virus, HIV-1, Vaccinia virus, Canary pox virus, Epstein Barr virus, Cytomegalovirus, Salmonella and tuberculosis [1, 3, 4]. The term cross-presentation implies that exogenous protein or peptide antigens are taken up by the antigen presenting cell leading to antigen-presentation on MHC class I molecules. The molecular mechanisms for this pathway are not fully understood [5]. In addition to transfer of proteins, we previously demonstrated transfer of DNA between eukaryotic cells after uptake of apoptotic cells [6-8]. Transfer of DNA led to production of proteins synthesized in the recipient cell provided that the DNA was integrated in the donor genome [6]. The infected apoptotic cell that carries integrated DNA can thus be viewed as an antigen delivery system that carries both microbial DNA and proteins from the dying cell. In addition, certain apoptotic cells have intrinsic adjuvant activity and capacity to induce dendritic cell maturation [9-14]. In contrast, other apoptotic cells are rather tolerogenic or null to the immune system [15-17].

In a previous mouse study we raised the question of whether apoptotic HIV-1 infected cells were capable of eliciting HIV-specific immune responses *in vivo* [18]. To overcome the

cellular tropism of HIV-1, which is a major obstacle in small animal models, we used a pseudotyped virus generated by using the amphotropic MuLV and HIV-1_{LAI} [19, 20] . This pseudovirus can infect and replicate in murine cells leading to production of gp120 and gp160 HIV-1 proteins [19] . We were able to show that inoculation of mice with apoptotic HIV-1/MuLV infected cells induces HIV-1 specific immunity [18] . We used i.p vaccination in the previous study because it allows for induction of immune responses in the spleen of mice. We also reasoned that the close proximity to lymphoid compartments of the gut could potentially be beneficial for induction of gut and mucosa associated immune responses. HIV-1 infection is characterized by a heavy viral load burden in lymphoid organs including lymphoid compartments at mucosal sites such as the gut [21, 22] . The most common routes of transmission world-wide are via mucosal routes in the genital and rectal regions. It is therefore very likely that both prophylactic and therapeutic HIV-1 vaccines should be able to mount HIV-1 specific immune responses able to clear virus and virus infected cells at mucosal sites. There are still many unresolved questions regarding which route of administration that would be required in order to mount effective HIV-1 specific immune responses in the genital-rectal area and in the gut-associated lymphoid compartment. The present study was undertaken to compare different routes of administration after vaccination with apoptotic HIV-1/MuLV infected cells and in addition to measure whether neutralizing activity could be detected in these mice.

2. Materials and Methods

2.1 Mice and immunizations

C57BL/6 mice were bred and kept at the animal facility at MTC, Karolinska Institutet. Mice were immunized i.p, s.c, i.m. or i.n. with either apoptotic HIV-1/MuLV infected or MuLV infected cells as previously reported [18]. In brief, human CEM-1B cells containing the complete murine leukaemia virus A4070 genome were infected with the human immunodeficiency virus type 1 IIIB [23]. The supernatant the infected cultures contained HIV-1/MuLV pseudovirus, which was then used to infect Concanavalin A (ConA)/rIL-2 activated syngeneic murine spleen cells. The content of HIV-1 p24 antigen was analysed by lysis of 1×10^6 HIV-1/MuLV infected splenocytes [20]. A total dose equivalent of 1ng of p24 was given on each day of immunization and this amount corresponded to $1-2 \times 10^6$ cells. Two groups of mice immunized s.c or i.m also received recombinant murine rGM-CSF, Prospecc-Tany Ltd., Israel) as adjuvant ($1 \mu\text{g}/\text{immunization}$). The obtained cells were frozen in 10% DMSO until the day of immunization. The day of immunization cells were thawed, washed two times in PBS and exposed to gamma-irradiation (150 Gy) for apoptosis induction, as previously described [18]. Animals were immunized two times with 3 weeks between immunizations. Two weeks after the last immunization the mice were bled and sera were analysed for antibody content. In challenge experiments, mice were immunized twice before receiving the infectious dose of 1×10^5 tissue culture ID₅₀ HIV-1/MuLV contained in 10^6 live cells i.p. Mice were sacrificed 8-10 days after challenge. HIV-1 isolation was routinely performed from 106 peritoneal cells and p24 secretion was measured from PHA-stimulated human T cells at days 4, 7, 10, 13, 18 and 21. HIV-1 proviral DNA was detected by nested PCR using pol primers JA79-JA82 [24]. DNA corresponding to 100,000 mouse spleen cells was run in each PCR and DNA from each mouse was tested five times. Faeces and vaginal IgA was collected as previously described [25-27]. In brief, fresh fecal pellets were collected and weighted. The faeces was dissolved in PBS containing 1% protease inhibitors (100 mg/1mL PBS, Sigma-Aldrich, St Louis, MA). The faeces debris were removed by centrifugation $1200 \times g$ for 20 min at $+4^\circ\text{C}$ and the IgA containing supernatants were collected and frozen in -70°C until assayed by ELISA.

2.2 Cellular immune responses

Splenocytes (2×10^5 cells/well) were cultured for 3-6 days in RPMI 1640 supplemented with 2mM L-glutamine, 5×10^{-5} M 2-ME, 10mM Hepes, 50 IU/ml penicillin and $50 \mu\text{g}/\text{ml}$

streptomycin as well as 10% FCS (GIBCO, Life Technologies, Paisley, United Kingdom). Antigens were purified recombinant proteins; Nef (0.6µg/ml) (kindly provided by Drs. B. Kohleisen and V. Erfle, GSF, Neuherberg Germany), p24 (2,5µg/ml) (Protein Sciences, Meriden, CT), control protein (2,5µg/ml) (Protein Sciences, Meriden, CT) and Con A (2µg/ml) (Sigma). Proliferation was measured using ³H-thymidine (1µCi/well) (Amersham, Pharmacia, Uppsala, Sweden). Liquid scintillation was used to reveal counts per minute (cpm). IL-2 and IFN-γ released into the supernatants of antigen-stimulated splenocytes after 48 hours were measured using ELISA kits (MabTech, Nacka, Sweden) according to the manufacturer's instructions.

2.3 ELISA

ELISA was carried out essentially as previously described [26] . Briefly, ELISA plates (Nunc Maxisorp; Odense, Denmark) were coated with recombinant subtype B gp160, p24 (1µg/ml) (Protein Sciences Corp., Meriden, CT, USA) or recombinant subtype B p55 (1 µg/ml) (Aalto, Dublin, Ireland) and *E. Coli*-expressed recombinant proteins Nef (GSF, Munchen, Germany.) as well as control antigen baculovirus lysate, (1ug/mL) [28] . Briefly, plates were blocked with 5% fat-free milk in PBS and serum was diluted in 2,5% milk in PBS with 0.05% Tween 20 and added 100ul/well. HRP labeled goat anti-mouse IgG (Bio-rad laboratories, Richmond) or IgA (Southern Biotech, Birmingham, AL), using o-phenylene diamine as a substrate was used to reveal the presence of antibodies by a color reaction. Plates were then developed for 30 min by adding O-phenylene diamine buffer (Sigma). The colour reaction was stopped with 2.5 M H₂SO₄ and the optical density (OD) was read at 490 nm. Absorbance values higher than twice the pre-immunization value were considered positive.

2.4 Plaque reduction neutralization assay

Three different HIV-1 isolates were used in the neutralization assays: HIV-1_{IIIB} (the immunogen) and two primary HIV-1 isolates of subtype B (SE1991:1541 and SE1838:3995) collected in Sweden [29, 30] . Virus stocks were prepared on peripheral blood mononuclear cells (PBMC) as described previously [31] .

The GHOST(3) cell line-based plaque assay is a single cycle infectivity assay for HIV and SIV, where green fluorescent protein (GFP) expression is a hallmark of infection [29, 32, 33]. The assay was performed in 96-well microtiter plates (TRP, Switzerland) where infected single cells or syncytia appear as distinct green fluorescent plaques and are counted as plaque-forming units (PFU). To determine an appropriate virus concentration for the neutralization assays the virus was first titrated on the GHOST(3) cells. For the neutralization assay, heat inactivated sera and the virus were diluted and mixed in culture medium (DMEM, (Sigma, UK) supplemented with 7.5% FCS (Hyclone, Argentina) and 50U/ml penicillin and 50ug/ml streptomycin as well as 2ug/ml polybrene (Sigma, UK)), to give a final 1:40 serum dilution and as a virus dilution to yield between 20 and 100 PFU/well. The virus and serum mixtures were incubated at 37 °C for one hour. After incubation, the mixtures were further diluted in two 5-fold steps and distributed to triplicate wells in a volume of 150 µl per well. The virus and virus-serum mixtures were titrated in parallel to allow determination of the percentage of neutralization. The day after infection the virus-serum mixtures were replaced with fresh medium. The cultures were checked for expression of GFP using fluorescence microscopy three days after infection. Virus titres were calculated as PFU/ml: (average number of plaques in triplicate wells × virus dilution)/volume in the well. The neutralizing property of the serum was calculated as percentage plaque reduction of the virus titration by the formula $1 - (\text{PFU with serum} / \text{PFU without serum}) \times 100$. The assay has a cut-off for neutralization at 3 SD (30%,) that is, values below 30% are considered as negative for neutralization.

Results

3.1 Lymphocyte proliferation and cytokine production after immunization with apoptotic HIV-1/MuLV infected cells.

To compare different routes of administration using apoptotic cells as an antigen delivery system, we immunized mice two times with three weeks interval before sacrifice and measured the capacity of splenocytes to respond to *in vitro* restimulation. We were able to induce significant proliferation against both rNef and rp24 after immunization i.p, s.c, i.m. and i.n. with apoptotic HIV-1/MuLV cells compared with control apoptotic cells (Fig. 1 A and B). The addition of the pro-inflammatory cytokine GM-CSF as an adjuvant did not further improve the HIV-1 specific lymphocyte proliferation. To measure the overall capacity of the splenocytes to proliferate, we measured ConA induced proliferation among the different groups of mice tested. There was a significantly reduced ConA induced response in the mice immunized with apoptotic HIV-1/MuLV s.c in the presence of GM-CSF (Fig. 1C). This group of mice was nevertheless able to mount both HIV-1 Nef and p24 specific responses.

We collected supernatants from the antigen-stimulated splenocytes cultures and assessed IL-2 and IFN- γ content after 48 hours of restimulation. We could detect significant levels of IFN- γ after restimulation with Nef in cultures obtained from mice immunized with apoptotic HIV-1/MuLV infected cells immunized i.p., i.m., and i.n (Fig. 2A). The addition of GM-CSF was necessary for Nef-induced proliferation after s.c and resulted in increased IFN- γ production after i.m. immunization. Low but significant p24 induced IFN- γ production was detected after i.p and i.n immunization. However, the addition of GM-CSF as an adjuvant resulted in p24 induced IFN- γ production also after s.c and i.m immunization (Fig. 2B). The ConA induced IFN- γ responses were similar in all groups of mice (Fig. 2C).

The IL-2 responses mirrored the proliferative responses (Fig 3). Hence, all groups of mice that received apoptotic HIV-1/MuLV infected cells, regardless of immunization route,

produced IL-2 in vitro after re-stimulation with Nef and p24. The ConA induced IL-2 production was similar in all groups of mice. The quantities of IL-2 detected after ConA stimulation was comparable or even lower than the HIV-1 antigen stimulated cultures, which is likely to reflect differences in the kinetics of mitogen induced IL-2 production compared with antigen induced.

3.2 Induction of HIV-1 reactive antibodies in sera and at mucosal sites after immunization with apoptotic HIV-1/MuLV infected cells

The presence of HIV-1 reactive IgG and IgA was measured in mice after different routes of administration with apoptotic infected cells (Table 1). The mice were immunized two times with three week interval and sera were collected two weeks after the last immunization. There was significant induction of HIV-1 specific serum IgG and IgA after immunization with apoptotic HIV-1/MuLV infected cells i.p. s.c, or i.n. However, the i.m route required the addition of GM-CSF in order to induce detectable titres against p24 of IgG and IgA classes and IgG against gp160. The addition of GM-CSF resulted in significantly increased IgG titres against gp160, p24 and Nef as well as IgA p24 after s.c. immunization. Overall the most robust responses, defined as significant reactivity against all antigens tested (gp160, p24, and Nef), were induced after i.p immunization without any adjuvant required or after s.c immunization with addition of GM-CSF. The mice immunized i.n also had relatively high titres against all antigens tested. However, due to higher inter individual variation in the i.n. group not all values reached significance.

Because HIV-1 is transmitted mostly via mucosal surfaces and is likely to persist also at these sites after infection, we measured the presence of HIV-1 specific IgA isolated from faeces and vaginal lavage (Table 2). We were able to detect significant induction of faecal IgA against

gp160 and p24 after immunization with apoptotic HIV-1/MuLV infected cells i.p. We were also able to detect measurable responses of Nef-reactive IgA in faeces and against gp160, p24 and Nef in vaginal lavage after i.p. immunizations but these values did not reach significance. In immunization with apoptotic HIV-1/MuLV infected cells resulted in significant titres of faecal and vaginal IgA against p24. There were no detectable mucosa associated IgA detected after immunization s.c or i.m even if GM-CSF were added.

3.3 Neutralizing activity detected in sera after immunization with apoptotic HIV-1/MuLV infected cells.

The induction of neutralizing antibodies is a major goal for the development of a prophylactic vaccine but it may also be of importance for a therapeutic HIV-1 vaccine because some data support the presence of persistent neutralizing antibodies in long-term non-progressors [34-37] . We have previously reported reactivity in sera against the gp41 cross-clade epitope ELDKWASLWN after immunization with apoptotic HIV-1/MuLV infected cells [18] . We therefore decided to investigate whether it was possible to detect neutralizing activity using a standardized assay after immunization with infected cells [29] .

In the first set of experiments mice were immunized i.p either one or two times before sera were collected and analyzed for neutralizing activity against autologous virus (Table 3). Mice were also challenged with live HIV-1/MuLV infected cells after immunizations and sera were collected after challenge. The control group of mice immunized with non-infected cells did not mount any neutralizing antibodies, not even ten days after challenge with live HIV-1/MuLV infected cells. Immunization once with apoptotic HIV-1/MuLV cells did not result in detectable neutralizing activity. However, we were able to reveal values above the cut-off of 30% neutralization after two immunizations with apoptotic HIV-1/MuLV cells i.p. in all

experiments performed. The presence of neutralizing activity persisted but did not increase after challenge with live HIV-1/MuLV infected cells (Table 3). All mice that were immunized with apoptotic HIV-1/MuLV infected were virus isolation negative after challenge, while the majority of mice immunized with apoptotic MuLV cells were virus isolation positive (Table 3). However, we could not detect neutralization against two primary isolates SE1991:1541 or SE1838:3995 (data not shown).

To further evaluate requirements for induction of neutralizing antibodies using apoptotic HIV-1/MuLV infected cells as immunogen, we compared different routes of immunizations (Table 3). Inoculation with apoptotic MuLV infected cells were control groups for each administration route and these values were set to 0% neutralization. Sera from three-six mice were pooled to have enough material for testing and data after two immunizations are shown. We could detect neutralization in the groups of mice that had received s.c immunizations in the presence of GM-CSF. However, s.c immunization without addition of GM-CSF did not provide detectable neutralizing activity. Similar results were obtained with the i.m. route. Hence, i.m immunization with HIV-1/MuLV infected cells did not show any neutralization while one of the two groups of mice immunized i.m in the presence of GM-CSF displayed neutralizing activity. There was also some variation in the results obtained from the group immunized i.n where one group of mice displayed neutralizing activity while the other group did not.

4. Discussion

In the present report we show that it is possible to induce neutralizing activity in mice after immunization with apoptotic HIV-1/MuLV infected cells. We compared different routes of immunizations and found that the most robust neutralizing activity was induced after i.p immunization and after s.c administration in the presence of GM-CSF. However, neutralizing

activity was also detected in the groups of mice immunized i.m in the presence of GM-CSF and i.n. without any adjuvant. In our first report, using apoptotic HIV-1 infected cells as immunogen, we could detect antibodies directed against Env and against a linear peptide spanning the gp41 cross-clade epitope ELDKWASLWN [18] . Here, we can confirm induction of both IgG and IgA antibodies directed against gp160 after immunization with apoptotic HIV-1/MuLV infected cells i.p. without any adjuvant and after s.c. or i.m. injection in the presence of GM-CSF. The pseudovirus HIV-1/MuLV is composed of a MuLV envelope and has the complete HIV-1 LAI genome inserted [19] . Infection with HIV-1/MuLV can be neutralized by MuLV-Env-specific antibodies but not by the HIV-1LAI neutralizing mAb P4/D10 [23] . In general when two unrelated viruses infect the same cell, there is phenotypic exchange of the envelope viral glycoproteins and the pseudotype progeny commonly contains a mosaic of the glycoproteins of both viruses [19] . However, the finding that HIV-1/MuLV is not neutralized by the P4/D10 mAb questions whether HIV-1 Env exists on the surface of the pseudotype virus. In addition, it was shown that removal of the carboxyterminal domain of the transmembrane HIV-1 Env protein was required to obtain pseudotype virus with a MuLV Env negative virus [38] . The finding that we can detect neutralizing activity in sera from mice immunized with HIV-1/MuLV infected cells poses the question of immunogen source and specificities of the neutralizing activity. Upon uptake the phagocytosed apoptotic vesicles are being degraded and antigen can be presented by both MHC class I and class II molecules [5] . In addition, we have shown transfer of DNA from the apoptotic cells to the phagocyte leading to de novo protein synthesis [6-8] . Hence, it can be hypothesized that the induction of apoptosis in the HIV-1/MuLV infected cells allowed for presentation of epitopes with neutralizing activity. It should be noted that challenge with live HIV-1/MuLV cells did not further boost the neutralizing responses. The observation of neutralizing antibodies in these mice was a serendipity finding and we now aim to

characterize these antibodies further preferable in a larger animal model more suitable for HIV-1 vaccine testing enabling sampling of larger quantities of sera. Immunization of monkeys using apoptotic SIV infected cells could reveal whether the MuLV part of the pseudovirus is required for the conformation leading to induction of neutralizing antibodies or whether apoptotic HIV-1/SIV infected cells allows for induction of neutralizing activity.

The induction of mucosa associated IgA is a desirable component for a prophylactic HIV-1 vaccine and may also have a role to play in therapeutic vaccinations. The results presented here show that it was only the i.n. and i.p. routes that gave detectable mucosa-associated IgAs after immunization with apoptotic HIV-1/MuLV infected cells. The intranasal route of immunization resulted in induction of IgA recovered from both faeces and vaginal secretions. We previously reported that i.p. immunization with apoptotic HIV-1/MuLV infected cells can lead to resistance to challenge with live HIV-1/MuLV infected cells [18]. In two independent experiments, all twelve animals that received two i.p. immunizations with apoptotic HIV-1/MuLV and displayed mucosa associated antibodies (Table 2) and neutralizing activity (Table 3) as well as being resistant to mucosal challenge [18] and Table 3). The frequency virus isolation positive animals immunized with control apoptotic cells were 11/14 [18]. Hence, the mucosal challenge experiments performed suggests that the immune responses induced by vaccination with apoptotic HIV-1/MuLV infected cells may have functional implications.

We report here that the induction of cellular immune responses, measured as splenocyte proliferation and IL-2 production, was less dependent upon the vaccination route used. Hence, all different vaccinations routes tested here (s.c., i.m., i.n., and i.p.) resulted in significant induction of proliferation and IL-2 production after restimulation with Nef or p24. The

addition of GM-CSF as an adjuvant did not further improve the magnitude of proliferation. However, the addition of GM-CSF increased the Nef and p24-induced IFN- γ production as well as antibody production after s.c and i.m. immunization.

The rationale behind the addition of GM-CSF was to facilitate the recruitment of dendritic cells to the site of immunization [39]. GM-CSF is able to induce differentiation of monocytes to immature dendritic cells with capacity to phagocytose apoptotic cells in vitro. In addition, immunization with irradiated, GM-CSF transfected tumor cells was previously shown to stimulate a local inflammatory reaction consisting of dendritic cells, macrophages and granulocytes [40, 41]. As the name implies, GM-CSF has the ability to generate granulocytes and macrophage lineage populations of cells from precursors.

We could not detect any additional effect in terms of splenocyte proliferation after using GM-CSF as adjuvant, reflecting that it did not promote T cell proliferation either directly or indirectly. However, the addition of GM-CSF resulted in increased HIV-specific IFN- γ production and antibody production in sera. This finding is in line with previous reports showing augmented CD4⁺ T cell responses after vaccination with a bicistronic HIV-1 DNA vaccine expressing gp120 and GM-CSF [42]. Furthermore, GM-CSF converted an autoimmune response to a self-antigen into an anti-tumor response by increasing the density of dendritic cells, increasing the frequency of antigen-specific T cells and the amount of IFN- γ produced [42].

In summary, we have shown that immunization with apoptotic HIV-1/MuLV infected cells via the i.p. or i.n. route induced mucosa-associated IgA. Furthermore, we could detect HIV-1

neutralizing activity in sera after immunization with apoptotic HIV-1/MuLV infected cells.

These findings support the exploration of apoptotic cells as an antigen delivery system.

Acknowledgements

Grants were received from the Swedish Research Council, the Swedish International Development Cooperation Agency/Department for Research Cooperation (SIDA/SAREC) and the European Commission (LSHP-CT-2005--018953).

Figure legends**Figure 1. HIV-1 specific proliferation induced after immunization with apoptotic HIV-1/MuLV infected cells.**

The HIV-1 induced proliferation after restimulation in vitro of splenocytes with recombinant Nef and p24 protein was measured by ³H-thymidine uptake after four days of culture (a and b). The overall capacity of the T cells to proliferate was estimated by stimulation with the lectin ConA (c). The assays were set up in triplicates and the values in counts per minute (cpm) are shown. The graph shows the average proliferation $\pm$ standard deviation from six mice in each group. Levels of significance between the groups immunized with either apoptotic MuLV- or HIV-1/MuLV- infected cells were evaluated by non-parametric Mann-Whitney test (p-values <0.05 are indicated with * and p-values <0.01 with **) for each immunization route analyzed (i.p, s.c., i.m, or i.n.).

Figure 2. HIV-1 specific IFN- γ production after immunization with apoptotic HIV-1/MuLV infected cells.

The HIV-1 induced IFN- γ release in supernatants after restimulation in vitro of splenocytes with recombinant Nef and p24 protein was measured by ELISA after 48 hours of culture (a and b). The overall capacity of the cells to produce IFN- γ was estimated by stimulation with the lectin ConA (c). The assays were set up in duplicates and the values in pg/ml of detectable IFN- γ are shown. The graph shows the average value $\pm$ standard deviation from six mice in each group. Levels of significance between the groups immunized with either apoptotic MuLV- or HIV-1/MuLV- infected cells were evaluated by non-parametric Mann-Whitney test (p-values <0.05 are indicated with * and p-values <0.01 with **) for each immunization route analyzed (i.p, s.c., i.m, or i.n.).

Figure 3. HIV-1 specific IL-2 production after immunization with apoptotic HIV-1/MuLV infected cells.

The HIV-1 induced IL-2 release in supernatants after restimulation in vitro of splenocytes with recombinant Nef and p24 protein was measured by ELISA after 48 hours of culture (a and b). The overall capacity of the T cells to produce IL-2 was estimated by stimulation with the lectin ConA (c). The assays were set up in duplicates and the values in pg/ml of detectable

IL-2 are shown. The graph shows the average value $\pm$ standard deviation from six mice in each group. Levels of significance between the groups immunized with either apoptotic MuLV- or HIV-1/MuLV- infected cells were evaluated by non-parametric Mann-Whitney test (p-values <0.05 are indicated with * and p-values <0.01 with **) for each immunization route analyzed (i.p, s.c., i.m, or i.n.).

References

- [1] Larsson M, Beignon AS, Bhardwaj N. DC-virus interplay: a double edged sword. *Semin Immunol* 2004;16(3):147-61.
- [2] Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* 1976;143(5):1283-8.
- [3] Yrlid U, Wick MJ. Salmonella-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells. *J Exp Med* 2000;191(4):613-24.
- [4] Schaible UE, Winau F, Sieling PA, Fischer K, Collins HL, Hagens K, et al. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat Med* 2003;9(8):1039-46.
- [5] Cresswell P, Ackerman AL, Giodini A, Peaper DR, Wearsch PA. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol Rev* 2005;207:145-57.
- [6] Holmgren L, Szeles A, Rajnavolgyi E, Folkman J, Klein G, Ernberg I, et al. Horizontal transfer of DNA by the uptake of apoptotic bodies. *Blood* 1999;93(11):3956-63.
- [7] Spetz AL, Patterson BK, Lore K, Andersson J, Holmgren L. Functional gene transfer of HIV DNA by an HIV receptor-independent mechanism. *J Immunol* 1999;163(2):736-42.
- [8] Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz AL, et al. Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc Natl Acad Sci U S A* 2001;98(11):6407-11.
- [9] Hoffmann TK, Meidenbauer N, Dworacki G, Kanaya H, Whiteside TL. Generation of tumor-specific T-lymphocytes by cross-priming with human dendritic cells ingesting apoptotic tumor cells. *Cancer Res* 2000;60(13):3542-9.
- [10] Feng H, Zeng Y, Graner MW, Katsanis E. Stressed apoptotic tumor cells stimulate dendritic cells and induce specific cytotoxic T cells. *Blood* 2002;100(12):4108-15.
- [11] Goldszmid RS, Idoyaga J, Bravo AI, Steinman R, Mordoh J, Wainstok R. Dendritic cells charged with apoptotic tumor cells induce long-lived protective CD4⁺ and CD8⁺ T cell immunity against B16 melanoma. *J Immunol* 2003;171(11):5940-7.
- [12] Ignatius R, Marovich M, Mehlhop E, Villamide L, Mahnke K, Cox WI, et al. Canarypox virus-induced maturation of dendritic cells is mediated by apoptotic cell death and tumor necrosis factor alpha secretion. *J Virol* 2000;74(23):11329-38.

- [13] Ishii S, Hiroishi K, Eguchi J, Mitamura K. Dendritic cell maturation induced by delivery of ultraviolet-mediated apoptotic colorectal cancer cell lines. *Anticancer Res* 2003;23(3B):2457-63.
- [14] Johansson U, Walther-Jallow L, Smed-Sorensen A, Spetz AL. Triggering of dendritic cell responses after exposure to activated, but not resting, apoptotic PBMCs. *J Immunol* 2007;179(3):1711-20.
- [15] Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J Exp Med* 2000;191(3):411-6.
- [16] Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest* 2001;108(7):957-62.
- [17] Ferguson TA, Herndon J, Elzey B, Griffith TS, Schoenberger S, Green DR. Uptake of apoptotic antigen-coupled cells by lymphoid dendritic cells and cross-priming of CD8(+) T cells produce active immune unresponsiveness. *J Immunol* 2002;168(11):5589-95.
- [18] Spetz AL, Sorensen AS, Walther-Jallow L, Wahren B, Andersson J, Holmgren L, et al. Induction of HIV-1-specific immunity after vaccination with apoptotic HIV-1/murine leukemia virus-infected cells. *J Immunol* 2002;169(10):5771-9.
- [19] Spector DH, Wade E, Wright DA, Koval V, Clark C, Jaquish D, et al. Human immunodeficiency virus pseudotypes with expanded cellular and species tropism. *J Virol* 1990;64(5):2298-308.
- [20] Andang M, Hinkula J, Hotchkiss G, Larsson S, Britton S, Wong-Staal F, et al. Dose-response resistance to HIV-1/MuLV pseudotype virus ex vivo in a hairpin ribozyme transgenic mouse model. *Proc Natl Acad Sci U S A* 1999;96(22):12749-53.
- [21] Belyakov IM, Berzofsky JA. Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. *Immunity* 2004;20(3):247-53.
- [22] Haase AT. Perils at mucosal front lines for HIV and SIV and their hosts. *Nat Rev Immunol* 2005;5(10):783-92.
- [23] Hinkula J, Rollman E, Lundholm P, Benthin R, Okuda K, Wahren B. Genetic immunization with multiple HIV-1 genes provides protection against HIV-1/MuLV pseudovirus challenge in vivo. *Cells Tissues Organs* 2004;177(3):169-84.
- [24] Lyamuya E, Bredberg-Raden U, Albert J, Grankvist O, Msangi V, Kagoma C, et al. Comparison of in-house and commercial sample preparation and PCR amplification systems for detection of human immunodeficiency virus type 1 DNA in blood samples from Tanzanian adults. *J Clin Microbiol* 1997;35(1):278-80.
- [25] VanCott TC, Kaminski RW, Mascola JR, Kalyanaraman VS, Wassef NM, Alving CR, et al. HIV-1 neutralizing antibodies in the genital and respiratory tracts of mice intranasally immunized with oligomeric gp160. *J Immunol* 1998;160(4):2000-12.
- [26] Lundholm P, Asakura Y, Hinkula J, Lucht E, Wahren B. Induction of mucosal IgA by a novel jet delivery technique for HIV-1 DNA. *Vaccine* 1999;17(15-16):2036-42.
- [27] Devito C, Zuber B, Schroder U, Benthin R, Okuda K, Broliden K, et al. Intranasal HIV-1-gp160-DNA/gp41 peptide prime-boost immunization regimen in mice results in long-term HIV-1 neutralizing humoral mucosal and systemic immunity. *J Immunol* 2004;173(11):7078-89.
- [28] Kjerrstrom A, Hinkula J, Engstrom G, Ovod V, Krohn K, Benthin R, et al. Interactions of single and combined human immunodeficiency virus type 1 (HIV-1) DNA vaccines. *Virology* 2001;284(1):46-61.

- [29] Shi Y, Albert J, Francis G, Holmes H, Fenyo EM. A new cell line-based neutralization assay for primary HIV type 1 isolates. *AIDS Res Hum Retroviruses* 2002;18(13):957-67.
- [30] Karlsson A, Parsmyr K, Sandstrom E, Fenyo EM, Albert J. MT-2 cell tropism as prognostic marker for disease progression in human immunodeficiency virus type 1 infection. *J Clin Microbiol* 1994;32(2):364-70.
- [31] Bjorndal A, Deng H, Jansson M, Fiore JR, Colognesi C, Karlsson A, et al. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J Virol* 1997;71(10):7478-87.
- [32] Morner A, Bjorndal A, Albert J, Kewalramani VN, Littman DR, Inoue R, et al. Primary human immunodeficiency virus type 2 (HIV-2) isolates, like HIV-1 isolates, frequently use CCR5 but show promiscuity in coreceptor usage. *J Virol* 1999;73(3):2343-9.
- [33] Nordqvist A, Fenyo EM. Plaque-reduction assays for human and simian immunodeficiency virus neutralization. *Methods Mol Biol* 2005;304:273-85.
- [34] Carotenuto P, Looij D, Keldermans L, de Wolf F, Goudsmit J. Neutralizing antibodies are positively associated with CD4+ T-cell counts and T-cell function in long-term AIDS-free infection. *Aids* 1998;12(13):1591-600.
- [35] Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF, et al. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N Engl J Med* 1995;332(4):209-16.
- [36] Pilgrim AK, Pantaleo G, Cohen OJ, Fink LM, Zhou JY, Zhou JT, et al. Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection. *J Infect Dis* 1997;176(4):924-32.
- [37] Lauren A, Thorstensson R, Fenyo EM. Comparative studies on mucosal and intravenous transmission of simian immunodeficiency virus (SIVsm): the kinetics of evolution to neutralization resistance are related to progression rate of disease. *J Gen Virol* 2006;87(Pt 3):595-606.
- [38] Schnierle BS, Stitz J, Bosch V, Nocken F, Merget-Millitzer H, Engelstadter M, et al. Pseudotyping of murine leukemia virus with the envelope glycoproteins of HIV generates a retroviral vector with specificity of infection for CD4-expressing cells. *Proc Natl Acad Sci U S A* 1997;94(16):8640-5.
- [39] Hamilton JA. GM-CSF in inflammation and autoimmunity. *Trends Immunol* 2002;23(8):403-8.
- [40] Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, et al. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A* 1993;90(8):3539-43.
- [41] Mach N, Dranoff G. Cytokine-secreting tumor cell vaccines. *Curr Opin Immunol* 2000;12(5):571-5.
- [42] Barouch DH, Santra S, Tenner-Racz K, Racz P, Kuroda MJ, Schmitz JE, et al. Potent CD4+ T cell responses elicited by a bicistronic HIV-1 DNA vaccine expressing gp120 and GM-CSF. *J Immunol* 2002;168(2):562-8.

Table 1

HIV-1 antibody titres in serum after immunization with apoptotic HIV/MuLV infected cells^a

Immunogen	GM-CSF	Route of admin	Serum IgG			Serum IgA		
			gp160	p24	Nef	gp160	p24	Nef
MuLV ^b	-	i.p.	<100	<100	<100	<100	<100	<100
HIV/MuLV ^b	-	i.p.	165 (100-400)	770 (170-6000)	135 (100-330)	200 (100-800)	1200 (250-2200)	110 (100-340)
MuLV ^b	-	s.c.	<100	<100	<100	<100	<100	<100
HIV/MuLV ^b	-	s.c.	105 (100-300)	470 (210-5100)	100 (100-180)	100 (100-210)	605 (200-1900)	105 (100-240)
MuLV ^c	+	s.c.	<100	<100	<100	<100	<100	<100
HIV/MuLV ^b	+	s.c.	540 (280-1200) ^d	6050 (1600-12500) ^d	475 (110-1400) ^d	185 (100-900)	1150 (560-3900) ^d	130 (100-400)
MuLV ^c	+	i.m.	<100	<100	<100	<100	<100	<100
HIV/MuLV ^c	-	i.m.	100 (100-130)	315 (100-490)	115 (100-600)	100 (100-150)	225 (100-450)	100 (100-190)
HIV/MuLV ^c	+	i.m.	225 (100-660)	3100 (1300-5100) ^d	175 (100-230)	125 (100-400)	770 (340-1300) ^d	125 (100-440)
MuLV ^c	-	i.n.	<100	<100	<100	<100	<100	<100
HIV/MuLV ^c	-	i.n.	145 (100-320)	475 (160-880)	200 (100-660)	185 (100-410)	560 (380-1300)	130 (100-450)

^a Female C57Bl/6 mice were immunized at 0 and 3 weeks with syngeneic apoptotic HIV-1/MuLV infected cells either with or without GM-CSF (1µg). Serum was collected two weeks after the last immunization and were analysed for presence of HIV-1 IgG and IgA binding antibodies. The data are expressed as the reciprocal of serum antibody titre (median and range).

Levels of significance between the groups immunized with either apoptotic MuLV- or HIV-1/MuLV- infected cells were evaluated by Wilcoxon signed rank test (p-values <0.05 were considered significant; in bold). Control antigen OD values at the titre 1/100 ranged between 0.011-0.044 and did not significantly differ between groups of mice.

^b n=12

^c n=6

^d Significant differences between the groups immunized with apoptotic HIV-1/MuLV infected cells either with or without GM-CSF were evaluated by non-parametric Mann-Whitney test (p<0.05 were considered significant).

Table 2

HIV antibody titres at mucosal sites after immunization with apoptotic HIV/MuLV infected cells^a

Immunogen	GM-CSF	Route of Admin.	Faecal IgA				Vaginal IgA			
			Total IgA ug/ml	gp160	p24	Nef	Total IgA ug/ml	gp160	p24	Nef
MuLV ^b	-	i.p.	59.0±13.1	<4	<4	<4	9.5±3.1	<2	<2	<2
HIV/MuLV ^b	-	i.p.	54.5±14.7	10 (4-32)	16 (4-24)	4 (4-16)	13.2±3.4	2 (2-4)	2 (2-4)	6(2-8)
MuLV ^b	-	s.c.	57.0±12.5	<4	<4	<4	9.5±2	<2	<2	<2
HIV/MuLV ^b	-	s.c.	68.8±16.5	<4	<4	<4	11.8±4.2	<2	<2	<2
MuLV ^c	+	s.c.	58.8±17.0	<4	<4	<4	11±2	<2	<2	<2
HIV/MuLV ^b	+	s.c.	62.5±12.6	<4	<4	<4	8.6±4	<2	<2	<2
MuLV ^c	+	i.m.	52.3±12.2	<4	<4	<4	7.2±2.4	<2	<2	<2
HIV/MuLV ^c	-	i.m.	56.0±11.0	<4	<4	<4	8.7±4.2	<2	<2	<2
HIV/MuLV ^c	+	i.m.	66.8±20.4	<4	<4	<4	13.3±5.4	<2	<2	<2
MuLV ^c	-	i.n.	70.3±17.4	<4	<4	<4	12.2±5.6	<2	<2	<2
HIV/MuLV ^c	-	i.n.	57.5±13.8	8 (4-16)	64 (32-128)	14 (4-32)	12.3±4.6	4 (2-8)	8 (4-16)	2 (2-4)

^aFemale C57Bl/6 mice were immunized at 0 and 3 weeks with syngeneic apoptotic HIV-1/MuLV infected cells either with or without GM-CSF (1µg). Faecal and vaginal Ig A were isolated two weeks after the last immunization and were analysed for presence of HIV-1 binding antibodies. The specific antibody binding data are expressed as the median and range and the total IgA data are expressed as the arithmetic mean± SD.

Levels of significance between the groups immunized with either apoptotic MuLV- or HIV-1/MuLV- infected cells were evaluated by Wilcoxon signed rank test (p-values <0.05 were considered significant; in bold)

^b n=12

^c n=6

Table 3

HIV Neutralizing activity in serum after immunization with apoptotic HIV/MuLV infected cells^a

Immunogen	GM-CSF	Route of administration	Number of immunizations	Number of experiments	Challenge	% neutralization
MuLV	-	Intraperitoneal	1 or 2	4	-	0-22
MuLV	-	“	2	1	+ ^c	12
HIV/MuLV	-	“	1	1	-	29
HIV/MuLV	-	“	2	4	-	40-93
HIV/MuLV	-	“	2	2	+ ^d	50-69
MuLV	+	Subcutaneous	2	1	-	0
HIV/MuLV	-	“	2	2	-	15-22
HIV/MuLV	+	“	2	2	-	39 - 87
MuLV	+	Intramuscular	2	1	-	0
HIV/MuLV	-	“	2	2	-	20 - 33
HIV/MuLV	+	“	2	2	-	8 - 97
MuLV	-	Intranasal	2	2	-	0
HIV/MuLV	-	“	2	2	-	23 – 86
Positive control serum from HIV-1-infected patient	n.a. ^b	n.a.	n.a.	5	n.a.	81 - 99

^a Female C57Bl/6 mice were immunized at 0 and 3 weeks with syngeneic apoptotic HIV-1/MuLV infected cells either with or without GM-CSF (1µg). In some experiments challenge with live infected cells was performed. Neutralization against HIV_{IIIB} was measured in sera obtained from pools of 3-6 mice isolated two weeks after last immunization or challenge. The data are expressed as % neutralization compared with the control group in a serum dilution of 1:40. Each lane represents data sets from pools of 3-6 mice. Three independent experiments using different batches of cellular vaccine preparations were performed for the i.p. route, while the other administration routes represent data obtained from one or two experiments but neutralization assay was set up using at least two different

pools of sera. Totally 108 mice were used in the above experiments. A fluorescence plaque reduction assay using GHOST cells expressing CD4 and CXCR4 as well as the GFP marker was used. Neutralizing activity against the HIV_{IIIB} isolate was analysed and was considered positive above 30% neutralization (3SD above control).

^bn.a., not applicable

^c Mice were challenged with live HIV-1/MuLV and three out of four mice were virus isolation positive

^d Mice were challenged with live HIV-1/MuLV and zero out of twelve mice were virus isolation positive [18]