Induction of HIV-1-Specific Immunity After Vaccination with Apoptotic HIV-1/Murine Leukemia Virus-Infected Cells

Anna-Lena Spetz, Anna Smed Sörensen, Lilian Walther-Jallow, Britta Wahren, Jan Andersson, Lars Holmgren and Jorma Hinkula

*J Immunol* 2002; 169:5771-5779; doi: 10.4049/jimmunol.169.10.5771

http://www.jimmunol.org/content/169/10/5771

References

This article cites 53 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/169/10/5771.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Induction of HIV-1-Specific Immunity After Vaccination with Apoptotic HIV-1/Murine Leukemia Virus-Infected Cells

Anna-Lena Spetz, Anna Smed Sörensen, Lilian Walther-Jallow, Britta Wahren, Jan Andersson, Lars Holmgren, and Jorma Hinkula

Ag-presenting dendritic cells present viral Ags to T cells after uptake of apoptotic bodies derived from virus-infected cells in vitro. However, it is unclear whether apoptotic virus-infected cells are capable of generating immunity in vivo. In this study, we show that inoculation of mice with apoptotic HIV-1/murine leukemia virus (MuLV)-infected cells induces HIV-1-specific immunity. Immunization with apoptotic HIV-1/MuLV-infected syngeneic splenocytes resulted in strong Nef-specific CD8+ T cell proliferation and p24-induced CD4+ and CD8+ T cell proliferation as well as IFN-γ production. In addition, systemic IgG and IgA as well as mucosa-associated IgA responses were generated. Moreover, mice vaccinated with apoptotic HIV-1/MuLV cells were protected against challenge with live HIV-1/MuLV-infected cells, whereas mice vaccinated with apoptotic noninfected or MuLV-infected splenocytes remained susceptible to HIV-1/MuLV. These data show that i.p. immunization with apoptotic HIV-1-infected cells induces high levels of HIV-1-specific systemic immunity, primes for mucosal immunity, and induces protection against challenge with live HIV-1-infected cells in mice. These findings may have implications for the development of therapeutic and prophylactic HIV-1 vaccines. The Journal of Immunology, 2002, 169: 5771–5779.

A
poptosis, or programmed cell death, is a process char-
acterized by expression of phosphatidylserine on the cell surface, caspase activation, DNA fragmentation, and formation of apoptotic bodies. During development and maintenance of tissue homeostasis, apoptosis is an inconspicuous process in vivo due to rapid clearance of dead cells by cells specialized in phagocytosis preventing induction of immune responses (1). Hence, APC that ingest Ag-containing structures by phagocytosis require additional stimulation, apart from the uptake of apoptotic bodies per se, to induce primary T cell activation. Matzinger (2–3) hypothesized that apoptotic cell death of virus-infected or other-
wise damaged cells may provide a signal that alerts the immune system. The exposure of Ag-presenting dendritic cells to signals such as inflammatory cytokines, necrotic cells, CpG oligonucleo-
tides, double-stranded RNA, or bacterial components may provide the necessary cue for immune activation (4–9). However, the number of apoptotic cells may be another factor contributing to the immunological outcome. High numbers of apoptotic cells, resulting in a slow clearance, may also induce dendritic cell maturation and presentation of intracellular Ags from apoptotic cells (10).

In vitro studies have shown that the uptake of apoptotic bodies derived from influenza-, EBV-, or CMV-infected cells by dendritic cells results in efficient MHC class I-restricted presentation of viral epitopes (11–13). Ags contained in these apoptotic bodies are introduced into MHC class I presentation pathways for cross-pre-
sentation and stimulation of Ag-specific CD8+ T cells (14). DNA that is present in apoptotic bodies can also be transferred into the APC and subsequently expressed within the APC (15–17).

The generation of cytotoxic T cells is associated with the control of viremia in HIV-1-infected patients, but CD4 Th responses and neutralizing Ab responses are also likely to be involved in the immune control of HIV-1 (18). Antiretroviral treatment results in immune reconstitution but does not improve HIV-1-specific immunity. On the contrary, HIV-1-specific CD4 and CD8 T cell-mediated responses decline after introduction of antiretroviral treatment (19–21). Thus, the reduction of HIV-1-specific cellular immunity is likely to contribute to treatment failure and viral rebound after interruption of therapy. Therapeutic vaccinations that are capable of reconstituting HIV-1-specific immune responses may prove to be an alternative strategy to obtain control of virus replication (22–23).

Persistent HIV-1 infection occurs only in humans and primates. Therefore, vigorous efforts are made to develop small-animal models (24). To overcome the cellular tropism of HIV-1, which is a major obstacle in small-animal models, we have used a pseudotype virus composed of the envelope of murine leukemia virus (MuLV) and the HIV-1 LAI genome (25). Using such a pseudovirus, we have previously shown that it is possible to deliver HIV-1 genes into murine splenocytes (26). We have also demonstrated that HIV-1/MuLV-infected splenocytes can provide a continuous release of infectious HIV-1 in vivo and in vitro (27). HIV-1 RNA and isolation of infectious HIV-1 could be demonstrated up to 14 days after inoculation with live HIV-1/MuLV-infected cells. HIV-1 DNA was also detectable in splenocytes, at a somewhat lower frequency compared with cells isolated from the peritoneal cavity. However, there were no indications of reactivation of new
murine cells in vivo. It was necessary to inoculate live HIV-1/MuLV-infected cells, because cell-free HIV-1/MuLV did not establish an infection (4).

In this study, we raised the question of whether apoptotic HIV-1-infected cells are capable of eliciting HIV-specific immune responses in vivo. Mice were vaccinated with apoptotic HIV-1/MuLV-infected syngeneic splenocytes before challenge with live HIV-1/MuLV-infected cells. We show that HIV-1-specific cellular and humoral immune responses were induced after vaccination with apoptotic HIV-1/MuLV-infected cells. Furthermore, immunization with apoptotic HIV-1-infected cells also conferred protection against challenge with live HIV-1/MuLV-infected cells.

Materials and Methods

Production of pseudotype virus

Preparation of the HIV-1 LAI/MuLV pseudotype virus was performed as previously described (25–26). In brief, amphotropic MuLV (A4070) in the neomycin-resistant amphot-CEM-1B cell line (kindly provided by Drs. D. K. and S. A. Speck, University of California at San Diego, La Jolla, CA) was used to prepare pseudovirus with the HIV-1 LAI strain. CEM-1B cells were cultured in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with antibiotics and 10% (v/v) FCS at a cell concentration of 5 × 10^6 cells/ml and infected with cell-free HIV-1 LAI stock virus (0.5 ml of HIV-1 containing 1–3 mg/ml p24). After 24 h of infection, the CEM-1B cells were washed and resuspended in fresh RPMI medium containing G418 (400 μg/ml). The supernatant was collected 5–6 days later, and cell-free supernatants were quantified after centrifugation for production of HIV-1 p24 Ag and infectious HIV-1 particles in primary murine spleen cells and peripheral blood lymphocytes as well as in T cell lines (26). Aliquots were stored at −70°C until use.

Production of syngeneic apoptotic HIV-1/MuLV-infected cells

Infection of mouse splenocytes was performed as previously described (26). In brief, C57BL/6 splenocytes were cultured in RPMI 1640 medium and activated with Con A (Sigma-Aldrich, St. Louis, MO) for 24 h. After washes, cells were infected with HIV-1/MuLV or MuLV for 24 h and washed twice in RPMI medium containing 10% FCS. Cells were cultured in RPMI medium containing 10% FCS, and every third day, 50% of the medium was harvested for viral titration and replaced with new medium.

ELISA was used to quantify the p24 content in cell-free supernatants at days 1, 3, and 6 after infection, and tissue culture ID50 was calculated. The efficiency of infection was also measured by intracellular p24 staining. Approximately 5 × 10^5 splenocytes were fixed in 3.7% formaldehyde (Sigma-Aldrich) and permeabilized with 0.1% saponin (Riedel-de Haen, Seelze, Germany) dissolved in PBS, followed by incubation with the intracellular HIV-1-specific anti-p24 Ab (PE-conjugated KC57; Coulter, Miami, FL). Cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA) (28). Stocks of the virus-infected or non-infected cells were frozen in 10% DMSO until use. Cells were thawed and washed before apoptosis induction by gamma irradiation. The apoptotic process induced by gamma irradiation (150 Gy) has previously been demonstrated by morphological changes, flow cytometry of annexin V binding, and propidium iodide staining and DNA fragmentation on agarose gels (15–17).

Immunization and HIV-1/MuLV challenge

C57BL/6 mice from the Embryo and Genome Research core facility at Karolinska Institutet (Stockholm, Sweden) were kept under germ-free pathogen-defined barrier conditions. The local animal research ethical committee approved animal care and experimental procedures. Syngeneic apoptotic HIV-1/MuLV-infected cells (dose equivalent of 0.84 ± 0.15 μg p24) were inoculated i.p. Mice were sacrificed after one or two immunizations with 3-week interval. 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. Cell-free HIV-1/MuLV was inoculated i.p. using 8 × 10^5 tissue culture ID50. Mice were sacrificed 8–10 days after challenge. HIV-1 isolation was routinely performed from 10^6 peritoneal cells, and p24 secretion was measured from PHA-stimulated murine 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 (29). DNA corresponding to 100,000 mouse spleen cells was run in each PCR, and DNA from each mouse was tested five times.

T cell assays

Splenocytes (2 × 10^6 cells/well) were cultured for 2–6 days in RPMI 1640 supplemented with 2 mM l-glutamine, 5 × 10^−5 M 2-ME, 10 mM HEPES, antibiotics, and 10% FCS. Ags were purified recombinant proteins: Nef (0.6 μg/ml; kindly provided by Drs. B. Kohleisen and V. Erle, National Research Center for Environment and Health, Neuberger, Germany), p24 (2 μg/ml; Protein Sciences, Meriden, CT), control protein (2 μg/ml; Protein Sciences), and Con A (2 μg/ml; Sigma-Aldrich). Proliferation was measured using [3H]thymidine (1 μM; Sigma-Aldrich) in PBS for 15 min according to the manufacturer’s instructions. After 4 days of culture (2 × 10^5 cells/well; Costar, Corning, NY), cells were collected, washed in PBS containing 2% FCS, and stained with anti-CD3-APC, anti-CD8-PerCP, and anti-CD4-PE (BD Biosciences). Flow cytometry analyses were performed using a FACS-Calibur, and data was analyzed using CellQuest software (BD Biosciences). IFN-γ release into the supernatants of Ag-stimulated splenocytes after 48 h was measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Ab assays

The levels of HIV-1-specific Ab in serum, bronchoalveolar lavage, and fecal pellets were quantified using ELISA, as previously described (30–32). Briefly, the recombinant HIV-1 Ags used to coat the plates (1 μg/ml) were Tat (kindly provided by Dr. J. Karn, Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.), Nef (kindly provided by Drs. B. Kohleisen and V. Erle, National Research Center for Environment and Health), Rev (Swedish Institute for Infectious Disease Control, Stockholm, Sweden), p24 and gp160 (Protein Sciences) as well as baculovirus-derived control protein (CAG). For epitope analyses, plates were coated with specific peptides from clade B/LAI gp41 (NEQLLELDKWASLWN), clade A/UG03 gp41 (EKDDLALKWNLWN), or clade C/BR25 gp41 (NEGDDLADSNLWN) peptides (10 μg/ml; Thermo Hybaid, Ulm, Germany). Mouse sera were diluted 1/50, 1/250, 1/1250, and 1/6250 in PBS with 0.5% BSA and 0.05% Tween 20 (Sigma-Aldrich). HRP-labeled goat anti-mouse IgG and anti-IgA using o-phenylene diamine as a substrate were used to reveal the presence of Ab by a color reaction.

Statistical Analysis

Statistical significance was assessed by the nonparametric Mann-Whitney U test for T cell responses and Ab levels. Fisher’s exact test was used to evaluate significance regarding protective immunity and was considered significant at p < 0.05.

Results

Immunization

To investigate whether HIV-1-infected apoptotic cells are capable of inducing immune responses in vivo, we prepared stocks of HIV-1/MuLV-infected or noninfected splenocytes (26). A high frequency of murine splenocytes expressed intracellular HIV-1 p24 Ag at low levels after infection, as detected by flow cytometry

FIGURE 1. Intracellular HIV-1 p24 expression in murine splenocytes used for production of apoptotic cells. Flow cytometry of intracellular p24 staining in control cells (dotted line) and HIV-1/MuLV-infected cells (solid line). Filled histogram shows isotype control of HIV-1/MuLV-infected cells. Data show log of fluorescence intensity. A total of 30,000 cells per sample were analyzed.
(Fig. 1). Due to the relatively low p24 expression, an exact frequency of p24-expressing cells could not be measured in the murine cells. Instead, a dose equivalent of 0.84 ± 0.15 μg p24 measured after cell lysis (1–2 × 10^6 cells) was used for each immunization. DNA-damaging agents such as gamma irradiation induce apoptosis through extensive involvement of the mitochondrial pathway (reviewed in Ref. 33). We have previously demonstrated that 150 Gy of gamma irradiation induces apoptosis in noninfected and HIV-1-infected cells with morphological changes and formation of apoptotic bodies. In this study, C57BL/6 mice were immunized i.p. with gamma-irradiated HIV-1/MuLV-infected or noninfected syngeneic splenocytes. The i.p. route was chosen because we aimed to target Ags to the spleen.

Inoculation i.p. with live HIV-1/MuLV-infected splenocytes can provide release of infectious HIV-1 in vivo. HIV-1 virus isolation was positive from the peritoneal cavity in 43 of 44 animals and from the spleen in 10 of 22 mice, while only 1 of 22 was virus positive in the blood. Because the peritoneal cavity has the highest incidence of virus-isolation positive results in this murine model, in this study, we assessed whether immunization with lethally irradiated (150 Gy) HIV-1/MuLV cells led to release of infectious HIV-1 in the peritoneal cavity. We were unable to recover infectious HIV-1 from mice immunized with irradiated cells, while immunization with live HIV-1/MuLV-infected cells led to isolation of virus (Table I). Inoculation with cell-free HIV-1/MuLV did not establish an infection (Table I). Three weeks after the first apoptotic cell immunization, the first groups of mice were sacrificed while the remaining mice received a booster vaccination. After an additional three weeks, the second groups of mice were sacrificed and the remaining mice were challenged with live HIV-1/MuLV-infected cells.

**HIV-1-specific T cell responses**

To assess the capacity of apoptotic syngeneic HIV-1/MuLV-infected cells to induce cell-mediated HIV-1-specific immune responses, we analyzed lymphocyte proliferation and IFN-γ production after restimulation with HIV-1 Ags. Data from six individual mice in each experimental group are shown (Fig. 2). HIV-1 p24-specific lymphocyte proliferation was induced in four of six mice after one inoculation with apoptotic HIV-1/MuLV-infected spleen cells but was more pronounced after a second boost (six of six) (Fig. 2A). Mice immunized with apoptotic control cells did not show any p24-specific lymphocyte proliferation (0 of 12) (Fig. 2B). Both groups showed p24-specific proliferation after challenge with live HIV-1/MuLV-infected spleen cells. However, the values obtained in mice immunized with apoptotic HIV-1/MuLV cells were significantly higher (p = 0.04) compared with the group that received apoptotic control cells. Mice immunized with apoptotic HIV-1/MuLV-infected cells showed a strong Nef-specific proliferation after the first immunization in contrast to the control group (Fig. 2, C and D). A second group of mice immunized twice with apoptotic HIV-1/MuLV-infected cells had a lower Nef-induced proliferation (p = 0.003) compared with the first vaccination group (Fig. 2C). However, the Nef-specific responses were significantly higher (p = 0.002) in the groups that were immunized with apoptotic HIV-1/MuLV-infected cells compared with controls. To assess whether Ag delivered by using apoptotic cells were presented to CD4+ and/or CD8+ T cells, splenocytes from mice immunized once 6 wk before sacrifice were labeled with CFSE and thereafter restimulated with Ag in vitro for 4 days. Recovered cells were stained with anti-CD3, anti-CD4, and anti-CD8 mAbs before analyses by flow cytometry. To reflect the actual numbers of cells collected from each well after in vitro culture, data were acquired during 3 min for each sample. The acquired events were gated on live CD3+ cells (Fig. 3). Fewer live CD3+ cells were collected

### Table 1. Lack of detectable virus in mice after immunization with apoptotic HIV-1/MuLV cells

<table>
<thead>
<tr>
<th>Immunizations</th>
<th>Number of Virus-Positive Samples</th>
<th>Total Number of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Apoptotic HIV-1/MuLV (one time)</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>B. Apoptotic noninfected (one time)</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>C. Live HIV-1/MuLV (one time)</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>D. Cell-free HIV-1/MuLV</td>
<td>0/4</td>
<td></td>
</tr>
</tbody>
</table>

* HIV-1 virus isolation was performed from 1 × 10^6 peritoneal cells/sample. Peritoneal cells were isolated 8 days after immunization and cultured with activated human T cells. ELISA was used to measure p24 content in cell supernatants collected every 3–4 days until 21 days of culture. In A and B, all peritoneal cells were collected from three mice/group and divided into eight samples. In C and D, one sample was analyzed per mouse.
from the wells containing cells from the control group in accordance with the cpm data. There was a striking CD8\(^+\) Nef-specific response detected in mice immunized with apoptotic HIV-1/MuLV cells (Fig. 3). Frequency analyses revealed that 68.6% ± 13.3% of the CD8\(^+\) CD3\(^+\) T cells had proliferated after Nef restimulation in the group that received an immunization with apoptotic HIV-1/MuLV cells compared with 15.1% ± 17.3% in the control group. In addition, the total number of cells recovered was higher in the group that received apoptotic HIV-1/MuLV cells compared with 15.1% ± 17.3% in the control group (Mann-Whitney test, \(p \leq 0.05\)) was also detected after immunization with apoptotic HIV-1/MuLV cells. Analyses of the p24-specific response revealed significant proliferation of both CD4\(^+\) (8.6% ± 1.8%) and CD8\(^+\) (47.5% ± 9.9%) \(T\) cells after immunization with apoptotic HIV-1/MuLV cells compared with the CD4\(^+\) (2.1% ± 0.9%) and CD8\(^+\) (5.9% ± 3.9%) \(T\) cells in the control group (Mann-Whitney test, \(p \leq 0.05\)).

ELISA was used to detect the presence of IFN-\(\gamma\) in the tissue culture supernatants from the p24- and control protein-stimulated splenocytes (Fig. 4). One immunization with apoptotic bodies derived from HIV-1/MuLV-infected splenocytes resulted in p24-specific induction of IFN-\(\gamma\) in three of six mice analyzed, and after two immunizations, in six of six mice. The control groups did not show any IFN-\(\gamma\) release. These data showed induction of T cell responses after immunization with apoptotic HIV-1/MuLV-infected splenocytes.

**HIV-1-specific humoral immune responses**

To assess the systemic and mucosa-associated humoral responses after vaccination, we used ELISA to quantify the levels of Ab to HIV-1 Ags in sera, feces, and bronchoalveolar lavage specimens (Figs. 5–7). Immunization with apoptotic HIV-1/MuLV-infected splenocytes resulted in a significant rise in the levels of anti-HIV-1-specific Ab of the IgG type against p24, gp160, Rev, and Tat (Fig. 5). This was further boosted after challenge with live HIV-1/MuLV-infected cells (Fig. 5). Immunization with apoptotic noninfected splenocytes did not result in significant rises in anti-HIV-1-specific Ab levels. There was no induction of Ab directed against the control protein CAG after vaccination with apoptotic cells (Fig. 5, I and J). The level of anti-Nef IgG in sera was not significantly induced after immunization with apoptotic HIV-1/MuLV-infected splenocytes compared with the corresponding control group. Increased levels of anti-Nef IgG in sera were instead detected after apoptotic cell immunization in both groups (data not shown). To further study the extent of the humoral response induced after immunization with apoptotic HIV-1/MuLV-infected splenocytes, the levels of HIV-1-specific IgA Ab in sera were also measured. There was a significant rise in serum IgA Ab directed against p24 and gp160 after vaccination with apoptotic HIV-1/MuLV-infected cells (Fig. 6, A and B). Furthermore, inoculation with apoptotic HIV-1/MuLV-infected cells provided an unusually effective sensitization to mucosal IgA induction by the challenge. Mucosal (gut)-associated IgA Abs against p24, gp160, Nef, and Tat were significantly induced, as measured in fecal pellets (Fig. 6, C–K). In addition, in bronchoalveolar lavage specimens from mice vaccinated with apoptotic HIV-1/MuLV-infected splenocytes and challenged with HIV-1/MuLV, a significant induction of anti-Nef IgA Abs was detected (Fig. 7). These data show the induction of a broad systemic and mucosa-associated humoral HIV-1-specific immune response after immunization with apoptotic HIV-1/MuLV-infected splenocytes.

To further characterize the HIV-1 envelope protein specificity of the serum IgG, we performed ELISA using the gp41 clade B/LAI peptide containing the ELDKWASLWN epitope, which is well documented as being part of a highly conserved broad HIV-1 subtype neutralizing epitope (34, 35). We were unable to measure reactivity against the clade B/LAI epitope in the control group. This was also true 10 days after challenge (Fig. 8A). Inoculation two times with apoptotic HIV-1/MuLV cells resulted in reactivity against the B/LAI epitope, which also remained after challenge. To investigate whether cross-clade reactivity was obtained, sera from one animal was also incubated with a gp41 clade A/UG031 and gp41 clade C/BR25 peptides (Fig. 8B). Before immunization, this animal did not show any gp41 reactivity, but after two inoculations
FIGURE 5. Animals immunized with apoptotic HIV-1/MuLV-infected syngeneic cells elicit systemic IgG responses against HIV-1. Mice were immunized with apoptotic HIV-1/MuLV-infected (A, C, E, G, and I) or noninfected (B, D, F, H, and J) syngeneic cells. Mice were immunized once, twice, or twice plus a challenge dose with live HIV-1/MuLV-infected cells as indicated by arrows. Ab responses (IgG) in sera to p24 (A and B), gp160 (C and D), Rev (E and F), Tat (G and H), or control (rCAG) (I and J) proteins were measured. Results shown are for six individual mice in each group. OD values from 1/50 dilution are shown. Significant differences between the corresponding groups that were immunized with infected or noninfected apoptotic cells by the nonparametric Mann-Whitney test are indicated by *, p ≤ 0.05, and **, p ≤ 0.01, respectively.

Induction of protective immunity

Inoculation with live HIV-1/MuLV infected cells in naive mice leads to recovery of HIV-1 from the peritoneal cavity and the spleen between 8 and 14 days postinoculation; thereafter, the infection is cleared. Inoculation has to be performed with live HIV-1/MuLV-infected cells, because challenge with cell-free HIV-1/MuLV has not resulted in any established infection

Discussion

An increasing number of viral Ags have been shown to be cross-presented after uptake of apoptotic bodies, including Ags from influenza, vaccinia, cytomegalovirus, EBV, and HIV-1 (11–13, 36–39). In addition, measles virus and canarypox virus infection have been shown to result in uptake of apoptotic bodies and maturation of bystander dendritic cells (40–41). Although these studies have examined Ag presentation in vitro, none of them have investigated the immunogenicity of virus-infected apoptotic cells in vivo. The importance of the cross-priming phenomenon in viral infections has been demonstrated in vivo for poliovirus, which infects nonhematopoietic cells (36). In addition, Ag-laden apoptotic bodies created by vectors coexpressing influenza virus hemagglutinin or nucleoprotein genes and mutant caspase genes have been shown to induce T cell responses (42). The present study is the first demonstration of inoculation with apoptotic HIV-1-infected cells resulting in HIV-1-specific immunity. It also demonstrates that immunizations with apoptotic cells induce systemic and mucosa-associated Ab in addition to having adjuvant properties for induction of T cell responses. Moreover, vaccination with lethally irradiated syngeneic HIV-1/MuLV-infected splenocytes conferred protection against viral challenge. Serum IgG was induced against p24, gp160, Rev, and Tat HIV-1 Ags in all immunized animals at frequencies of 83–100% after at least two i.p. immunizations. Serum IgA responses were detected already after one i.p. immunization toward the structural HIV-1 Ags gp160 and p24.

In the present study, a striking Nef-specific CD8+ T cell response was observed already after one inoculation of apoptotic HIV-1/MuLV-infected cells. The response was significantly reduced after a booster immunization. Additional experiments are required to more closely characterize the Nef-specific responses.
The present study showed that i.p. immunization with irradiated syngeneic HIV-1-infected cells resulted in effective induction of mucosal HIV-1-specific IgA. The lowest proportion of responders (two of six) was seen toward the regulatory protein Rev, while all mice responded against the other four HIV-1 Ags tested. We have recently found a poor frequency and low magnitude of HIV-Ag-specific mucosal fecal IgA after a single i.p. injection of viable HIV-1/MuLV-infected syngeneic spleen cells. In only 20% of the animals could a p24- or Nef-Ag-specific fecal IgA be detected, while in bronchoalveolar lavage, 60–80% of the mice developed IgA against p24, gp160, or Nef. Thus, the present study showed that the i.p. route of immunization with apoptotic HIV-1-infected cells could efficiently prime the local mucosa of the intestines, even though it seems as if at least two immunizations are required to obtain detectable mucosa-associated IgA (Fig. 6). Other studies

The present study showed that i.p. immunization with irradiated syngeneic HIV-1-infected cells resulted in effective induction of mucosal HIV-1-specific IgA. The lowest proportion of responders (two of six) was seen toward the regulatory protein Rev, while all mice responded against the other four HIV-1 Ags tested. We have recently found a poor frequency and low magnitude of HIV-Ag-specific mucosal fecal IgA after a single i.p. injection of viable HIV-1/MuLV-infected syngeneic spleen cells. In only 20% of the animals could a p24- or Nef-Ag-specific fecal IgA be detected, while in bronchoalveolar lavage, 60–80% of the mice developed IgA against p24, gp160, or Nef. Thus, the present study showed that the i.p. route of immunization with apoptotic HIV-1-infected cells could efficiently prime the local mucosa of the intestines, even though it seems as if at least two immunizations are required to obtain detectable mucosa-associated IgA (Fig. 6). Other studies

or noninfected (D, F, H, and J) syngeneic cells. Mice were immunized once, twice, or twice plus a challenge dose with live HIV-1/MuLV-infected cells as indicated by arrows. Ab responses (IgA) in sera to p24 (A) and gp160 were measured. Results from individual mice. Mean OD values ± SD from six mice vaccinated with apoptotic noninfected splenocytes twice and then challenged with HIV-MuLV are depicted (C). Significant differences in the groups that received immunizations with HIV-1/MuLV-infected apoptotic cells as compared with values obtained in the apoptotic noninfected control group by the nonparametric Mann-Whitney test are indicated by *, p ≤ 0.05, and **, p ≤ 0.01, respectively. Ab responses (IgA) in feces to p24 (C and D), gp160 (E and F), Rev (G and H), Nef (I and J), and Tat (K) proteins were measured. Each dot represents results from individual mice. OD values from 1/2 dilutions are shown. Significant differences between the corresponding groups that were immunized with infected or noninfected apoptotic cells by the nonparametric Mann-Whitney test are indicated by *, p ≤ 0.05, and **, p ≤ 0.01, respectively. The significance of values regarding Tat reactivity was compared with prevaccination values. Tat reactivity in mice immunized with apoptotic noninfected cells was not determined.
The feasibility of using apoptotic HIV-1/MuLV-infected cells for induction of immunity without the use of an adjuvant implies that necessary signals for immune activation are provided in vivo after immunization with apoptotic HIV-1-infected cells (8). Successful immunization with a concentrate of lethally irradiated cells, which have initiated the apoptotic cell death program, is consistent with effective uptake by phagocytes, such as Ag-presenting dendritic cells. After uptake of Ag in the periphery, dendritic cells undergo maturation and migrate to secondary lymphoid organs where recruitment of naive T cells occurs (45). For effective Ag presentation, dendritic cells require costimulation, and it is conceivable that such a signal is provided by secondary necrotic events after inoculation with a bolus of lethally irradiated cells and/or by viral motifs (4–5, 8–9, 14, 46). However, it should be noted that at present we have no data to support uptake by dendritic cells after inoculation i.p. Induction of protective immunity demonstrated in the present report required that the apoptotic cells were HIV-1/MuLV infected indicating that altered cell-surface Ags were not involved in protection.

We were unable to isolate virus after immunization with lethally irradiated cells in the present study indicating that the immune responses observed were not due to virus replication. However, the question of whether immunization with irradiated HIV-1-infected cells leads to virus replication in vivo needs to be addressed in a highly pathogenic animal model due to the limited capacity of HIV-1/MuLV to replicate in mice as compared with replication of SIV or SHIV in macaques. Additional experiments are also required to formally demonstrate whether the immune responses

| Immunizations                        | Number of Virus Isolation-Positive Mice/
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Apoptotic HIV-1/MuLV (two times plus challenge)</td>
<td>0/12b</td>
</tr>
<tr>
<td>B. Apoptotic noninfected (two times plus challenge)</td>
<td>8/10</td>
</tr>
<tr>
<td>C. Apoptotic MuLV (two times plus challenge)</td>
<td>3/4</td>
</tr>
<tr>
<td>D. Nonimmunized (plus challenge)</td>
<td>4/4</td>
</tr>
</tbody>
</table>

- HIV-1 virus isolation was performed from 1 × 10⁸ peritoneal cells/mouse. Peritoneal cells were isolated 10 days after challenge with live HIV-1/MuLV-infected cells and cultured with activated human T cells. ELISA was used to measure p24 content in cell supernatants collected every 3–4 days until 21 days of culture.
- Statistically significant difference compared to the other groups: A/B (p < 0.001), A/D (p < 0.001), and A/C (p < 0.008) (Fisher’s exact test).


definition of columns: 

- **Immunizations**: A. Apoptotic HIV-1/MuLV (two times plus challenge)
- **Number of Virus Isolation-Positive Mice**: 0/12
- **Total Number of Mice**: 12
- **Immunizations**: A. Apoptotic HIV-1/MuLV (two times plus challenge)
- **Number of Virus Isolation-Positive Mice**: 8
- **Total Number of Mice**: 10

![Image](http://www.jimmunol.org/DownloadedFrom/83x315to263x741)_

FIGURE 8. Reactivity against the gp41 cross-clade epitope ELKDW ASLWN after inoculation with apoptotic HIV-1/MuLV-infected cells. Sera from control animals before and 10 days after challenge (■) as well as sera from mice immunized two times with apoptotic HIV-1/MuLV cells before and after challenge (□) were analyzed for reactivity against the clade B/LAI peptide NEQLLELDKWASLWN (4). Serum IgG OD_{450} values at 1/50 dilution mean ± SD from six mice is shown. Preimmunization serum and sera after two inoculations with apoptotic HIV-1/MuLV-infected cells, as well as serum obtained 10 days after challenge of live HIV-1/MuLV-infected cells, were analyzed for reactivity against clade B/LAI peptide NEQLLELDKWASLWN, clade A/UG031 EKDLLALDKWASLWN, and clade C/BR25 NEQDLALDSWNLWN (8). Serum IgG OD_{450} values at 1/50 dilution from one animal are shown.

using recombinant proteins or peptides delivered i.p. indicate that this route of immunization followed by a mucosal booster results in significant vaccine Ag-specific fecal IgA responses (43–44). In this study, we show for the first time that mucosal IgA can be obtained against several HIV-1 Ags by inoculation of apoptotic HIV-1-infected cells to the local intestinal mucosa. Analysis of the HIV-1 envelope protein specificity in the serum IgG revealed that the Ab specifically reacted with the transmembrane protein gp41. The IgG were found to recognize the gp41_{651–670} peptide (ELDKW ASLWN), well documented as being part of a highly conserved broadly reactive HIV-1 subtype neutralizing epitope (34–35).
measured in the present report were the result of cross-presentation of Ags.

If inoculation with apoptotic HIV-1-infected cells confers immunity, why is effective immunity often not obtained during HIV-1 infection? First, it is unclear whether HIV-1-infected cells undergo apoptosis in vivo so as to provide the necessary cues for immune activation. The viral dose, the site of infection, the type of cell death of the infected cell, as well as apoptosis in noninfected bystander cells may vary during HIV-1 infection, thereby influencing the outcome of immune responses. An intriguing question is whether individual variations in induction of apoptotic cell death in infected and noninfected cells during early HIV-1 infection influence disease progression. Second, viral proteins may inhibit effective Ag presentation after uptake of apoptotic HIV-1-infected cells. HIV-1 Tat has been shown to prevent phagocytosis of apoptotic bodies by dendritic cells (47). It is also noteworthy that HIV-1 Nef, like many other microbial proteins, has evolved multiple ways to protect HIV-1-infected cells from apoptosis (48–51).

Third, the immune-evasion mechanisms induced by HIV-1 replication may overcome or prevent effective induction of any immune response. However, antiretroviral treatment leads to reconstitution of immunity to many pathogens and also allows patients to respond to immunization using recall Ags and neo-Ags (52). Therapeutic vaccination during effective antiretroviral therapy is a strategy that may give the immune system the power to combat immune-evasion factors, such as Nef and Tat, and also enable control of virus replication after discontinuation of antiretroviral therapy. We speculate that it may be feasible to induce an immune response by therapeutic vaccination with apoptotic autologous HIV-1-infected cells while maintaining effective antiretroviral treatment. A further advantage of autologous vaccination is that HIV-1 strains with variant sequences are included in the composition (53).

Acknowledgments
We thank Reinhard Benthin for skilful handling of animals and Hernan Concha for flow cytometry.

References


