Therapeutic immunization strategies against cervical cancer
Bungener, Laura Barbara

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Chapter 3

Induction of cytotoxic T lymphocyte activity by immunization with recombinant Semliki Forest virus: indications for cross-priming

Anke Huckriede, Laura Bungener, Marijke Holtrop, Jacqueline de Vries-Idema, Barry-Lee Waarts, Toos Daemen and Jan Wilschut

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Abstract

For the rational design of vaccines capable of inducing CD8\(^+\) T cell responses knowledge of the identity of the antigen-presenting cell (APC) and the mechanism of antigen presentation is very important. Here, we address these issues for alphavirus-based immunization, in particular immunization with recombinant Semliki Forest virus (rSFV). Studies with dendritic cells (DC) from various origins revealed that rSFV has a very limited capacity to infect this cell type \textit{in vitro}. To further investigate \textit{in vivo} whether rSFV infects professional APC directly or whether the antigens reach APC via a mechanism of cross-priming we compared the immunological effects of three different SFV-constructs encoding the influenza nucleoprotein (NP). These constructs differ in the amount of NP produced per cell or in the stability of the NP, respectively. Induction of cytotoxic T lymphocytes (CTL) appeared to benefit from a large amount of stable antigen. In contrast, rapid antigen degradation, and thus availability of antigenic peptides in the infected cell, was found to be disadvantageous. Based on these \textit{in vitro} and \textit{in vivo} results, we hypothesize that antigen presentation after rSFV-based immunization proceeds via a mechanism in which APC are not infected directly but acquire antigen from other infected cells and present it to CTL in a process of cross-priming.
The mechanism underlying CTL induction by rSFV

Introduction

CD8⁺ cytotoxic T lymphocytes (CTL) play a vital role in clearing virus infections, are involved in tumor surveillance and can even eradicate existing tumors and metastases. Accordingly, there is a growing interest in inducing CTL responses by vaccination. Several methods to achieve this goal have been developed in recent years. Many of these techniques are based on the use of DNA or RNA vectors. Immunization with these so-called ‘genetic vaccines’ aims at expression of antigens in such a way that they get access to the MHC class I processing and presentation route in professional antigen-presenting cells (APC) [Leitner 1999, Whitton 1999, Shedlock 2000].

Alphavirus vectors are very attractive vaccine candidates that have shown superiority to other approaches of genetic immunization in several model systems [Brand 1998, Fleeton 2000]. The vectors are based on the single-stranded RNA genomes of Sindbis virus (SIN), Semliki Forest virus (SFV) or Venezuelan Equine Encephalitis virus (VEE), respectively [Xiong 1989, Liljeström 1991, Pushko 1997]. The general approach for constructing alphavirus vector systems (recently reviewed in [Hewson 2000]) involves transcription of the viral RNA genomes to cDNA. The sequence encoding the non-structural viral proteins is then cloned into a plasmid such that it is followed by a multiple cloning site for insertion of genes of interest. RNA transcribed from this plasmid spans the viral sequences as well as the transgene. Once introduced in the cytoplasm this RNA is self-replicating due to the encoded viral replicases. When in thus infected cells the genetic information encoding the structural virus proteins is provided in trans, the replicated RNA molecules can be packaged and recombinant virus particles are formed. These particles can efficiently infect various cell types in vitro and in vivo. Once in the cytosol, the vector RNA induces high expression of the encoded transgenes. The transgenes are expressed for approximately 48–72 h after which the infected cells die by apoptosis [Glasgow 1998, Murphy 2000]. These properties make recombinant alphaviruses safe and efficient vehicles to induce humoral as well as cellular immune responses [Zhou 1995, Berglund 1997, Schlesinger 1999, Daemen 2000].

So far, little data are available concerning the mechanism of CTL induction upon alphavirus-based immunization. It is now generally accepted that activation of naive T cells requires antigen presentation by professional APC, in particular dendritic cells (DC) (recently reviewed in [Banchereau 1998, Bhardwaj 2001, den Haan 2001, Mellman 2001, Théry 2001]). The DC have to present the relevant antigenic peptides on their surface in the context of MHC class I molecules. Moreover, they have to provide co-stimulatory activity by surface molecules like CD40, CD80, CD86 and others, which are expressed by activated DC [Banchereau 1998, Bhardwaj 2001].

Two alternative mechanisms may account for antigen presence in DC after recombinant SFV (rSFV) immunization: (i) recombinant alphaviruses could infect DC
Chapter 3

directly thereby inducing synthesis of the encoded antigen in the cytosol followed by processing of the protein in the MHC class I processing and presentation route (direct priming); (ii) the recombinant virus particles could primarily infect other cell populations. When these cells undergo apoptosis they could serve as a source of apoptotic bodies containing substantial amounts of the expressed antigen. Dendritic cells have been shown to take up apoptotic bodies and to efficiently present the enclosed antigens on MHC class I molecules in a process of so-called cross-priming [Albert 1998a, b].

We addressed the question of direct priming versus cross-priming in the context of rSFV-based immunization by two sets of experiments. First, using different populations of DC from murine and from human origin and β-galactosidase (LacZ) as reporter gene we investigated the capacity of rSFV to infect DC in vitro. Second, we evaluated the mechanism of rSFV immunization in vivo using constructs that differed in the stability of the expressed antigen, on the one hand, and in the amount of antigen expressed on a per cell basis on the other hand. In line with Whitton et al. [Whitton 1999], we argued that rapid antigen degradation should be beneficial if rSFV immunization proceeded via a mechanism of direct priming. Rapid degradation of the antigen provides a large pool of antigenic peptides, which is immediately available for presentation on MHC class I molecules. On the other hand, in a scenario where cross-priming accounts for antigen presentation rapid antigen degradation is expected to be disadvantageous since the antigen would simply not survive long enough to reach the DC. Consequently, cross-priming should be particularly enhanced by large amounts of stable antigen as provided by high expression plasmids.

We therefore produced three different rSFV-constructs encoding the influenza nucleoprotein (NP): a standard NP construct, a construct encoding an unstable NP variant, and a construct inducing enhanced NP expression. Accelerated degradation was achieved by generating an NP–ubiquitin fusion construct, the ubiquitin moiety targeting the protein for rapid degradation by proteasomes [Tanaka 1996, Rodriguez 1997]. Increased expression was obtained by cloning the NP sequence into the pSFV plasmid directly behind the first 102 base pairs of the SFV capsid gene, which are known to serve as a translational enhancer [Sjöberg 1994]. Recombinant SFV particles produced with these constructs were used for immunization of mice and the induction of CTL was evaluated using MHC class I tetramer technology, IFNγ ELISPOT, and 51Cr release assays.

Infection of DC with rSFV in vitro was very inefficient implying that DC are not a primary target for the recombinant virus particles. In vivo rSFV-based immunization clearly benefited from a large pool of stable antigen whereas rapid antigen degradation had an adverse effect on CTL induction. These results point to cross-priming as the primary mechanism of CTL induction involved in rSFV-based immunization.
Material and methods

Cells

Baby hamster kidney cells (BHK) were grown in GMEM (Life Technologies, Breda, The Netherlands) containing 2 mM glutamine, 5% fetal calf serum (FCS) and 10% tryptose phosphate broth. TC-1 cells (kindly provided by Dr. C Melief and Dr. R Offringa, Leiden, The Netherlands) were cultured in Iscove’s medium (IMDM, Life Technologies) supplemented with 10% FCS. EL-4 cells (H2b) were also grown in IMDM with 10% FCS.

For the culture of murine DC, bone marrow cells were isolated from femurs of C57BL/6, DBA/2, A/J or BALB/c mice, respectively. Cells were plated in 6-well plates at a density of 2x10⁶ cells/ml in β-IMDM (IMDM, 10% FCS, 50 µM β-mercaptoethanol) supplemented with 1000 U/ml recombinant murine GM-CSF (Peprotech, London, UK) and 20 ng/ml recombinant murine IL-4 (Peprotech). On days 2, 4, and 6, 75% of the medium was replaced by fresh medium (containing GM-CSF and IL-4). DC were harvested on days 6–8 and were used for infection experiments. Where indicated lipopolysaccharide (LPS) was added on day 6 to give a final concentration of 100 ng/ml.

For the culture of human DC, peripheral blood mononuclear cells (PBMC) were isolated and incubated for 2 h in a 6-well plate (5x10⁶ cells/ml in RPMI (Life Technologies) + 15% human pool serum (HPS)). Non-attached cells were removed and 1 ml RPMI/15% HPS supplemented with 300 ng/ml recombinant human GM-CSF (PBH, Hannover, Germany) and 1000 U/ml recombinant human IL-4 (PBH) was added. The medium was exchanged on day 3 and the cells were harvested and used for infection experiments on day 5.

For the investigation of fresh DC, human PBMC were stained directly after isolation with PE-labeled anti-CD33 and a mixture of FITC-labeled anti-CD14 and anti-CD16 (all antibodies from BD Pharmingen, San Diego, USA). Cells were subsequently washed and resuspended in RPMI, 20% FCS, 50 µM β-mercaptoethanol, 5 mM EDTA. Cells were filtered through a 30 µm filter to remove aggregates and then sorted on a Becton Dickinson Fluorescence Activated Cell Sorter (FACS). The CD33⁺CD14⁻CD16⁻ population represents immature and mature DC while granulocytes and monocytes are CD33⁺CD14⁺CD16⁻ and lymphocytes and NK cells are CD33⁻ [Lekkerkerker 1999].

Plasmids

The vectors pSFV1 and pSFV3 containing the LacZ sequence were purchased from Life Technologies [Liljestrom 1991]. The vectors pSFV Helper 1, pSFV Helper 2, pSFV Helper S1, and pSFV4.2 were kindly provided by Dr. Peter Liljestrom, Stockholm, Sweden [Liljestrom 1991, Berglund 1993, Smerdou 1999]. The plasmid pNP28 encoding the nucleoprotein of influenza strain A/NT/60/68 was kindly put at our disposal.
by Dr. George Brownlee, Oxford, UK. The plasmid pCMVub(A76) was kindly provided by Dr. Fernando Rodriguez and Dr. Lindsay Whitton, La Jolla, USA [Rodriguez 1997].

**Cloning of the NP constructs**
The plasmid pNP28 was used as a template for amplification of the NP sequence by PCR. The amplified sequence (with newly introduced Xmal restriction sites at both ends) was cloned into the multiple cloning site of pSFV3 (Figure 1). This plasmid, named pSFV-NP, encodes the full length 55 kDa NP protein.

In order to generate a high expression plasmid, NP was cloned behind the SFV enhancer sequence C' [Sjöberg 1994]. Using the pSFV Helper S1 plasmid as a template, the enhancer sequence located at the 5'-end of the capsid gene was amplified by PCR and BamHI restriction sites were introduced at both ends [Smerdou 1999]. The enhancer sequence was cloned into the BamHI site of pSFV1 rendering the plasmid pSFV1-enh. Successful insertion and proper orientation were verified by sequence determination. The NP sequence was excised from the pSFV-NP construct with Xmal and was cloned into the Xmal-linearised pSFV1enh plasmid resulting in the plasmid pSFV-enhNP (Figure 1). The C'-NP fusion protein (enhNP) has a molecular weight of approximately 60 kDa.
For construction of a ubiquitin–NP fusion protein we made use of the plasmid pCMV-ub(A76) [Rodriguez 1997]. The ubiquitin encoded in this plasmid carries an alanine at position 76 instead of the usual glycine. Due to this change in amino acid sequence ub-protein fusion products are no longer prone to degradation by ubiquitin-specific hydrolases [Rodriguez 1997]. For cloning, the NP sequence was amplified introducing BclI restriction sites at both ends. The fragment was inserted into pGEM T Easy T/A cloning vector (Promega, Madison, USA) and was further amplified in the non-methylating Escherichia coli strain JM110. Since NP contains an internal BclI restriction site, the fragment was cut out of pGEM T Easy by partial digestion (0.1 U enzyme per 50 µg plasmid DNA, 70 min, 37°C). The proper fragment of 1506 bp was excised from agarose gel and was eluted with the help of QIAEX II (QIAGEN, Hilden, Germany). This fragment was cloned into BclI-linearised pCMV-ub plasmid such that the ubiquitin sequence and the NP sequence are in frame. The ubiquitin–NP fusion sequence was cut out of the pCMV plasmid by NotI digestion and was cloned into NotI-linearised pSFV4.2 (Figure 1). This plasmid, named pSFV-ubNP, encodes the ubiquitin–NP fusion protein (ubNP) which has a molecular weight of approximately 63 kDa.

Production, purification, and titer estimation of rSFV
Production of rSFV was performed as described [Heikema 1997]. Briefly, RNA in vitro transcribed from pSFV-LacZ, pSFV-NP, pSFV-enhNP or pSFV-ubNP was mixed 1:1 with RNA transcribed from pSFV-Helper1 or pSFV-Helper2. 8x10⁶ BHK cells were infected with a total of 20 µg RNA mix by electroporation with a Biorad Gene Pulser II (2 pulses of 850 V/25 µF; Bio-Rad, Hercules, USA). After 24 h, the medium containing the recombinant virus was harvested and either snap-frozen directly in liquid nitrogen (for in vitro experiments) or further purified (for in vivo immunizations). Purification and titer determination were performed as described [Daemen 2000, Chapter 2].

Infection of DC with rSFV
To 6–8 day old DC cultures, rSFVLacZ particles were added at various multiplicities of infection (MOI) as indicated. Six, twenty-four, or forty-eight hours after addition of the particles the cells were washed, fixed, and incubated with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) as described to visualize LacZ activity [Schoen 1999]. For infection of freshly isolated DC, the FACS-sorted mixture of immature and mature DC (CD33CD14/CD16-) was plated directly in 96-well plates. rSFV encoding LacZ was added at MOI of 50 and 500, respectively. Thirteen hours later, cells were stained for LacZ activity.
Pulse-chase labeling of infected cells
1.5x10^6 TC-1 cells per well were plated in a 24-well tissue culture plate. The next day, cells were infected with rSFV Helper1 particles encoding the respective NP variants at a MOI of 50. After 5 h of infection, cells were washed with methionine-free DMEM (Life Technologies), incubated in this medium for 30 min to deplete internal methionine storage, and then labeled with 10 µCi ^35S-methionine (Amersham Pharmacia Biotech) per well for 10 min. Labeling was stopped by addition of unlabeled methionine and emetine (final concentrations 25 µM and 10 µg/ml, respectively). Cells were either harvested directly (0 min chase) or were chased for the given time periods. Where indicated lactacystin was present during labeling at a concentration of 50 µM. Cells were harvested in 150 µl TENT-SDS (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Triton-X 100, 1% SDS, pH 7.4). Protein concentrations were determined by the DC protein assay (Bio-Rad). Cell lysates were analyzed by SDS-PAGE followed by Coomassie Blue staining and autoradiography on Kodak XAR film.

Immunizations and harvest of spleen cells
C57Bl/6 mice were immunized once intraperitoneally with 4x10^5 purified recombinant SFV Helper 2 particles encoding the respective NP variants. Ten days later, spleen cells were isolated and depleted of erythrocytes by treatment with ACK buffer (150 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.2). When used for determination of bulk CTL activity, cells were cultured for 5 days in β-IMDM at a concentration of 2x10^6 cells/ml in upright 25 cm² flasks. For stimulation of NP-specific CTL the D^b-restricted NP epitope NP_366−374 (ASNENMDAM) was added to a final concentration of 20 ng/ml [Parker 1996].

MHC tetramer staining and flow cytometry
Freshly isolated, erythrocyte-depleted spleen cells were washed once in FACS buffer (PBS, 0.5% BSA, 0.02% sodium azide). 2x10^6 cells were then stained with FITC-conjugated anti-CD8 (BD Pharmingen, San Diego, USA) and PE-conjugated H-2D^b MHC tetramers carrying the NP_366−374 epitope [Haanen 1999]. Stained and washed cells were analyzed on an Epics Elite flowcytometer (Coulter, Miami, USA).

ELISPOT assay
Elispot assays for detection of IFNγ-producing cells were performed essentially as described by Hylkema et al. [Hylkema 2000]. Briefly, 4x, 2x, 1x, or 0.5x10^6 freshly isolated, erythrocyte-depleted spleen cells were plated in 96-well plates that had been coated with anti-IFNγ and blocked with BSA. NP_366−374 peptide was added to a final concentration of 50 ng/ml. Peptide was omitted from control wells to check for unspecific IFNγ-production. After overnight incubation cells were lysed and IFNγ-positive spots were detected with the help of biotinylated anti-IFNγ and alkaline
phosphatase-conjugated streptavidin (BD Pharmingen) as described [Hylkema 2000]. Spots were counted under a stereomicroscope.

\textbf{\textsuperscript{51}Cr release assay}

For use as target cells, EL-4 cells were left untreated (control) or were pulsed with NP\textsubscript{366-374} at a concentration of 15 µg/ml for 1 h. Cells were then labeled for 1 h with 50 µCi \textsuperscript{51}Cr/10^6 cells (ICN Biomedicals, Zoetermeer, The Netherlands), followed by extensive washing. \textit{In vitro} stimulated bulk CTL were harvested, washed once with β-IMDM and added to the target cells at effector to target cell ratios of 30:1, 10:1 and 3:1, respectively. Supernatants were harvested after 4 h and radioactivity was determined by γ-counting. Percent specific release was calculated as 100 x (experimental release - spontaneous release)/(maximal release - spontaneous release).

\textbf{Statistics}

The unpaired Student’s t-test (assuming unequal variances) was used to analyze differences in the amount of MHC tetramer-positive T cells among the experimental groups. Results were considered significant when P <0.05.

\textbf{Results}

\textbf{Infection of DC \textit{in vitro}}

The capacity of rSFV to infect DC directly was investigated \textit{in vitro} using recombinant virus particles that carry the LacZ reporter gene. Infection of DC derived from murine bone marrow and cultured for 8 days in the presence of GM-CSF and IL-4 was very inefficient. Even at a multiplicity of infection (MOI) of 1000 we found a maximum of 0.15% of LacZ-positive cells (Table 1). This percentage was attained 6 h after infection while at later time points the percentage of LacZ-positive cells was even lower. In contrast, BHK cells were infected with an efficiency of >90% (MOI 5) and LacZ expression was sustained for at least 48 h. Resistance of DC to infection was not mouse strain-specific since DC from C57BL/6, DBA, A/J, and BALB/c mice all showed the same very low infection efficiency (Table 1). Similarly, the maturation state of the DC had no effect on infection efficiency. DC cultured for 6 days are mainly immature, yet these cultures showed similar low infection rates as older cultures containing a higher proportion of mature DC. Activation of DC with lipopolysaccharide (LPS) did not affect infectability either (Table 1).
Although mice are a suitable host for SFV and most experiments addressing the issue of CTL priming by immunization with rSFV immunization have been performed in mice we considered the possibility that DC of other species are more susceptible to SFV infection. Human DC cultured from peripheral blood mononuclear cells in the presence of GM-CSF and IL-4 for 5 days were therefore included in the experiments. However, infection efficiencies were similarly low as those found for murine DC (Table 1).

In order to evaluate whether in vitro cultured DC are representative for DC in vivo, we repeated the infection experiments with DC freshly isolated from human blood [Lekkerkerker 1999]. CD14-CD16+CD33+ cells representing a mixture of immature and mature DC were infected with rSFV encoding LacZ at MOI of 50 and 500, respectively. Again, similar to the in vitro cultured DC, infection was very inefficient, indicating that resistance to rSFV infection is an intrinsic property of DC (results not shown).

### Expression and degradation kinetics of NP variants
In order to enable the analysis of antigen presentation after rSFV immunization in vivo, different constructs encoding influenza NP as a model antigen were generated. For this purpose the full-length NP gene, the NP gene with the SFV capsid-driven enhancer

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**Table 1. Efficiency of infection by rSFV**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>MOI</th>
<th>Infected cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK</td>
<td>-</td>
<td>5</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Murine DC (C57Bl/6)</td>
<td>-</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>50</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>500</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1000</td>
<td>0.14</td>
</tr>
<tr>
<td>LPS</td>
<td>500</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td>LPS</td>
<td>1000</td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Murine DC (DBA/2)</td>
<td>-</td>
<td>5</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>500</td>
<td>0.08</td>
</tr>
<tr>
<td>Murine DC (BALB/c)</td>
<td>-</td>
<td>50</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>500</td>
<td>0.08</td>
</tr>
<tr>
<td>Murine DC (A/J)</td>
<td>-</td>
<td>50</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>500</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1000</td>
<td>0.15</td>
</tr>
<tr>
<td>Human DC (monocyte-derived)</td>
<td>-</td>
<td>5</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>50</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>500</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Cells were infected with rSFV encoding LacZ at the MOI indicated. Six hours after infection cells were stained for LacZ activity and positive cells were scored by phase contrast microscopy.
sequence (enhNP), and a ubiquitin–NP fusion construct were cloned into SFV-based vectors as depicted schematically in Figure 1 and described in detail in Material and methods. These constructs encode NP variants with molecular weights of 55, 60 and 63 kDa, respectively. Expression of NP from all three constructs was verified by immunostaining of NP in cells infected with the respective rSFV particles (results not shown).

Expression levels and degradation kinetics of the NP variants were determined in pulse-chase experiments. Metabolic labeling of cells infected with the standard NP construct revealed substantial expression of NP 5 h after infection (Figure 2A, 0 min). Under the same conditions, expression of enhNP was about 8–10 times higher than that of NP (as demonstrated by running different amounts of cell lysate from NP- and enhNP-infected cells (Figure 2B)). In contrast, the amount of ubNP found after pulse labeling was very low. During the subsequent chase of 15 and 60 min, respectively, degradation of NP and enhNP was very slow, while ubNP was completely degraded after a 60 min chase (Figure 2A).

![Figure 2. Analysis of NP kinetics by metabolic labeling.](image)

Figure 2. Analysis of NP kinetics by metabolic labeling. (A) Evaluation of synthesis and degradation. TC-1 cells were infected with the different rSFV constructs as indicated. 5 h later cells were pulse-labeled with $^{35}$S methionine and chased for the periods indicated as described in Material and methods. Cell lysates were analyzed by SDS-PAGE followed by autoradiography. (B) Comparison of translation levels. Samples were prepared as in (A). The indicated amounts of sample were analyzed by SDS-PAGE and autoradiography to allow estimation of the efficiency of the enhancer sequence. (C) Investigation of immediate protein degradation. Cells were infected and labeled as in (A) in the absence or presence of the proteasome inhibitor lactacystin. Cell lysates prepared immediately after a 10 min pulse-labeling were analyzed as in (A).
The low amount of ubNP detected even at t=0 min might be caused by low expression of the protein or by extremely rapid degradation (induced by the ub moiety) as has been observed for other ub constructs [Rodriguez 1997]. In order to discriminate between these possibilities we compared the amount of newly synthesized protein in the absence and presence of the proteasome inhibitor lactacystin (Figure 2C) [Cerundolo 1997]. When the proteolytic activity of the proteasomes was inhibited the amount of protein expressed from rSFVNP but especially the amount of protein expressed from rSFVubNP was strongly increased. Under these conditions, ubNP was present at equal amounts as NP expressed from the standard construct. This observation shows that the NP- and the ubNP-vector exhibit similar expression levels and that the two protein constructs thus differ only in their degradation kinetics. Surprisingly, the amount of enhNP was consistently found to be decreased when cells were labeled in the presence of lactacystin. Discrete degradation products became visible on the fluorograms. This result indicates that protein degradation pathways independent of proteasome activity were activated in these cells, possibly triggered by the large amount of recombinant protein present.

Immunological effects of the different NP constructs
The constructs thus characterized were used for immunization of mice. Earlier observations had shown that a dose of 4x10⁵ rSFVNP particles induces a solid though suboptimal immune response (T. Daemen, A. Huckriede, unpublished results). This dose was chosen to allow for detection of improved as well as impaired responses as compared to those induced by the standard rSFVNP construct. Ten days after intraperitoneal injection of the different rSFV particles, frequencies of NP-specific CD8⁺ T cells were determined by flow cytometry using MHC tetramers carrying the NP₃₆₆−₃₇₄ peptide [Hylkema 2000]. All three constructs were capable of priming NP tetramer-reactive CD8⁺ T cells although to different extents (Figure 3A). The enhNP construct was clearly most effective (mean frequency: 1 NP₃₆₆−₃₇₄-specific CTL per 41 CD8⁺ T cells) followed by NP (1 in 76). Recombinant SFV encoding ubNP appeared to be least effective in precursor induction (mean frequency: 1 in 164). While enhNP- and NP-encoding virions-induced CTL in all mice tested, rSFVubNP failed to do so in three out of eight mice. As compared to the NP-immunized group, the mean number of MHC tetramer-positive cells was significantly higher in the enhNP-immunized group (one-tailed t-test, P=0.04) but significantly lower in the ubNP-immunized group (one-tailed t-test, P=0.02). In general, variation in the percentage of MHC tetramer-positive CD8⁺ cells within each experimental group was substantial as can be deduced from Figure 3B.
The mechanism underlying CTL induction by rSFV

Figure 3. MHC tetramer analysis of NP-specific CTL. Freshly isolated splenocytes from mice vaccinated with NP-, enhNP-, or ubNP-encoding rSFV were stained for CD8 (FITC) and epitope-specific T cell receptors with tetramers containing the NP366-374-peptide of influenza virus strain A/NT/60/68 (PE). (A) Dot plots of gated live (propidium iodide-negative) lymphocytes. The indicated percentages refer to double-positive cells among the lymphocytes. Outcome of a typical experiment is shown. (B) Summary of MHC tetramer analysis. Results are expressed as percentage of tetramer-positive cells among CD8+ T cells. Each dot represents one mouse. The stripe indicates the median of the respective experimental group.

Since MHC tetramer staining detects all NP366-374-specific CTL irrespective of their state of activation, we also performed an ELISPOT assay to determine the number of active CTL capable of producing IFNγ upon stimulation with the respective peptide. Again, the mean number of active NP-specific CTL was highest in mice injected with the rSFVenhNP and lowest in mice injected with rSFVubNP (Figure 4). The number of spleen cells producing IFNγ in the absence of peptide was similarly low in rSFV-injected mice and PBS-injected control mice (not shown).
Figure 4. Analysis of IFNγ-producing cells. Splenocytes from mice immunized with rSFV (NP, enhNP or ubNP, respectively) were cultured overnight on anti-IFNγ-coated ELISA plates in the presence of the NP\textsubscript{366-374} epitope of influenza virus strain A/NT/60/68. The next day, IFNγ-positive spots were developed as described in Material and methods and counted. Results are expressed as number of IFNγ spots per 10\textsuperscript{5} splenocytes. Again, each dot represents one mouse and the stripe indicates the group median. In the absence of NP peptide the mean number of IFNγ spots per 10\textsuperscript{5} cells was <3 for all groups.

Another indication for active CTL is lytic activity towards cells presenting the relevant peptide. When stimulated \textit{in vitro} for 5 days with NP\textsubscript{366-374}-peptide CTL from 9 out of 10 mice immunized with rSFVNP and all mice immunized with rSFVenhNP were able to lyse peptide-loaded target cells. In contrast, 3 out of 8 mice immunized with rSFVubNP failed to do so. In general, CTL from enhNP-vaccinated mice exhibited the highest activity, followed by CTL from NP-vaccinated ones. CTL from ubNP-immunized animals showed highly variable activities. Results of a typical experiment are shown in Figure 5.

In summary, immunological responses were highest with the rSFVenhNP construct indicating that the amount of antigen correlates positively with the number of induced precursors, the number of activated precursors and the lytic activity of the CTL. On the other hand, stimulation of protein degradation by expression of NP as a fusion product with ubiquitin had an adverse effect on the induction of CTL responses.
Figure 5. Determination of lytic activity of CTL. Splenocytes from rSFV-vaccinated mice (encoding NP, enhNP, or ubNP, respectively) were restimulated for 5 days in vitro with the NP366-374-epitope. These effector cells were then incubated for 4 h with ⁵¹Cr-labeled EL-4 cells that had been pulsed with medium (open symbols) or the NP366-374-epitope (filled symbols). Each symbol represents one mouse. Outcome of a representative experiment is shown.

Discussion

In this study, we elucidated the mechanism of antigen processing and presentation involved in immunization with rSFV vectors. To this end we first examined whether rSFV is capable of infecting DC directly. Using DC from murine as well as human origin and MOI up to 1000, we observed that infection of DC is highly inefficient in vitro. Our experiments with murine DC were restricted to myeloid DC grown from bone marrow cells while those performed with human DC included myeloid as well as lymphoid DC. We cannot exclude that mouse lymphoid DC are susceptible to rSFV infection yet the results obtained with human DC as well as observations of others (see below) make this rather unlikely. The maturation state of DC appeared to have no effect on their infectability: immature DC (6 day cultures), on the one hand, and DC activated with LPS, on the other hand, were both highly resistant to infection. These results are in line with data of Navas et al. [Navas 2002] who recently reported that rSFV (encoding Hepatitis C core protein) was unsuitable for infection of human DC.

It remains to be elucidated why DC cannot be infected by rSFV with higher efficiency. Preliminary experiments using radio-labeled wild type SFV imply that myeloid DC are capable of internalizing virus (Laura Bungener, unpublished observations). This would suggest that the blockade is on a post-entry level. Possibly, transgene expression is suppressed by interferon-induced pathways, which have also
been shown to affect replication of wild type SFV [Hefti 1999, Landis 1998]. On the other hand, infection of DC by SIN appeared to be determined by the amino acid composition of the E2 glycoprotein [Gardner 2000] (see below). Future research will have to clarify which mechanism(s) prevent(s) infection of DC by SFV replicons.

Earlier studies elucidating the fate of rSFV in vivo further support that DC are not the primary target for rSFV infection. Upon injection of rSFV into the peritoneal cavity the vast majority (approximately 99%) of infected cells were found to be located in the peritoneal lining while other organs like spleen and lungs showed comparatively low amounts of luciferase used as reporter enzyme in these experiments [Klimp 2001]. Intramuscular injection of rSFV encoding the enhanced green fluorescent protein (EGFP) revealed persistence of EGFP RNA at the injection site for at least 7 days. In contrast, EGFP RNA was detected in much lower amounts and only very transiently (≤24 h) in draining lymph nodes or the spleen [Morris-Downes 2001]. Taken together these results imply that the major targets for rSFV are non-lymphoid cells resident at the injection site rather than DC.

Similar to rSFV, rSIN has been described as being rather inefficient in infecting DC in vitro and in replicating on these cells [Gardner 2000, Ryman 2002]. Repeated passaging of SIN virus on human DC and use of the viral genes thus obtained in the rSIN system improved infection of human DC significantly (although these measures had much less pronounced effects on the infection of murine DC). Enhanced infection efficiency could be attributed to point mutations in the E2 spike protein as well as in non-structural virus proteins [Gardner 2000]. However, these observations demonstrate mainly the high plasticity of the viral RNA genome rather than being an indication for a natural tropism of SIN virus for DC.

The situation may be somewhat different for VEE vectors. Upon subcutaneous injection of GFP-encoding rVEE, Langerhans cells, the resident DC in the skin, were found to be primary targets of the vector [MacDonald 2000]. Upon infection, these cells accumulated in the draining lymph nodes. In fact, it was reported that the number of infected cells found in the local lymph nodes equaled 45% of the injected recombinant virus particles. This would indicate an exceptionally strong preference of rVEE to infect Langerhans cells rather than other resident cell populations.

Thus, while alphaviruses in general appear to differ in their cell tropism and single site mutations might further modify their capacity to infect certain cell types, data on SFV consistently suggest that DC are not a preferred target for this virus. Regarding the fact that a single injection of mice with as little as 100 rSFV particles is sufficient to induce vigorous CTL responses [Zhou 1995], it is therefore highly unlikely that direct infection of DC plays an important role in rSFV-based immunization.

For the further elucidation of the mechanism involved in rSFV-based immunization, we injected mice with three constructs that differed in the amount of stable antigen expressed (NP versus enhNP) or in the immediate availability of proteolytic antigenic
peptides (NP versus ubNP), respectively. High level expression (enhNP) and rapid degradation (ubNP) were verified by pulse-chase experiments. Efficient generation of the H-2D\(^b\)-restricted NP\(^{366-374}\) epitope from rapidly degraded NP had been shown earlier [Cerundolo 1997]. We argued that immunization with the enhNP construct should be beneficial if cross-priming was the mechanism of CTL activation after immunization with rSFV. Infection of cells with this construct leads to the accumulation of stable protein in the cytosol. Upon apoptosis (triggered by the rSFV infection), the protein ends up in apoptotic bodies, which in turn may be taken up by DC as has been described for other experimental systems [Albert 1998a, b, Guermonprez 2002, Larsson 2001]. In this scenario, rapid antigen degradation is supposed to be detrimental since the antigen may not survive long enough to allow apoptosis and cross-priming to occur [Whitton 1999]. On the other hand, rapid antigen degradation should be advantageous if the infected cells themselves would present the antigenic peptides to T cells. The ubiquitin tail ensures targeting of the protein to the proteasomes resulting in the presence of a large pool of antigenic peptides immediately available for presentation on MHC class I molecules. In several experimental systems, it has been shown that enhancement of antigen degradation can indeed lead to an increase in CTL precursor frequency [Rodriguez 1997, 1998, Tobery 1997, 1999, Velders 2001, Wu 1997]. These experiments involve intramuscular injection of naked DNA, application of DNA by gene gun and immunization with the help of recombinant vaccinia virus, respectively. For all of these techniques it has been shown that direct infection of APC does occur although it might not be the exclusive mechanism of T cell priming [Condon 1996, Corr 1999, Norbury 2002, Porgador 1998, Sbai 2002].

With respect to rSFV-based immunization, we found that the magnitude of the CTL response is high when a large amount of stable antigen is available, as is the case after immunization with the enhNP construct. In contrast, rapid degradation as achieved by fusing NP to ubiquitin had no beneficial effect (compared to unmodified NP) and even appeared to be disadvantageous for CTL induction by rSFV. These data confirm earlier observations with rSFV encoding the E6 and E7 proteins of human papillomavirus where high antigen stability also correlated positively with enhanced immunogenicity [Daemen 2002]. Following the argumentation outlined above, we consider these results as further indications for cross-priming as the mechanism of CTL induction involved in rSFV-based immunization.

Thus, several lines of evidence derived from in vitro and in vivo experiments presented here as well as from data generated by others [Klimp 2001, Morris-Downes 2001] indicate that CTL induction by immunization with rSFV proceeds predominantly via a mechanism of antigen transfer from initially infected cells to professional APC. Though convincing in our eyes, the evidence provided is indirect. Future experiments will therefore concentrate on studying the interaction of rSFV, infectable cells and DC in more detail in vitro as well as in vivo.
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The picture on the first page of this chapter is a negative stain electronmicroscopy picture of A/Panama influenza virus.
References


