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Structure and Immunogenicity of Alternative Forms of the Simian Immunodeficiency Virus Gag Protein Expressed Using Venezuelan Equine Encephalitis Virus Replicon Particles

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Abstract

Venezuelan equine encephalitis virus replicon particles (VRP) were engineered to express different forms of SIV Gag to compare expression *in vitro*, formation of intra- and extracellular structures and induction of humoral and cellular immunity in mice. The three forms examined were full-length myristylated SIV Gag (Gag^{myr+}), full-length Gag lacking the myristylation signal (Gag^{myr-}), or a truncated form of Gag^{myr-} comprising only the matrix and capsid domains (MA/CA). Comparison of VRP-infected primary mouse embryo fibroblasts, mouse L929 cells and primate Vero cells showed comparable expression levels for each protein, as well as extracellular virus-like particles (VRP-Gag^{myr+}), and distinctive cytoplasmic aggregates (VRP-Gag^{myr-}) with each cell type. VPR were used to immunize BALB/c mice, and immune responses were compared using an interferon (IFN)-γ ELISPOT assay and a serum antibody ELISA. Although all three VRP generated similar levels of IFN-γ-producing cells at 1 week post-boost, at 10 weeks post-boost the MA/CA-VRP-induced response was maintained at a significantly higher level relative to that induced by Gag^{myr+}-VRP. Antibody responses to MA/CA-VRP and Gag^{myr+}-VRP were not significantly different.

Keywords

SIV Gag; VEE replicon vectors; SIV Gag-specific immunity in BALB/c mice

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INTRODUCTION

Development of an effective vaccine against human immunodeficiency virus type 1 (HIV-1) remains a critical need, especially in areas of high incidence where access to treatment is limited. The effort to meet this need has involved new vaccine technologies, namely genetic vaccines, which combine the safety of a subunit vaccine with the ability to present endogenously produced proteins to the immune system, an advantage previously only associated with live virus vaccines. Vaccine vectors based on purified DNA, live viruses, or defective viruses differ in properties that may have a direct impact on their ability to induce protective immunity to HIV-1, such as the cells that are targeted, the amount of immunogen that is synthesized and persistence of the vector in the host (Amara and Robinson, 2002;Barouch et al., 2001;Schnell, 2001). Only head-to-head comparisons of vaccine vectors can reveal their relative effectiveness, and some such comparisons have been reported in the last few years (for examples, see Casimiro et al., 2003;Doria-Rose et al., 2003;Shiver et al., 2002).

The molecular form of the immunogen is another important factor in the ultimate success of a vaccine vector. The optimal configuration of a vaccine vector will require matching the specific vector biology with the most immunogenic form of the vectored protein. For example, studies of immunogen design have been performed in the context of DNA and canarypox vaccine vectors expressing SIV or HIV-1 group-specific antigen (Gag) protein with widely varying results. In the case of DNA, abrogation of Gag-directed budding increased the cellular immune response in some cases (Bu et al., 2003;Young and Ross, 2006), but not in another (Qiu et al., 2000), while the canarypox vector expressing budding-competent Gag was the most immunogenic (Chen et al., 2005). This suggests that the distinct biology and specific design of these genetic vaccines resulted in the determination of different optimal forms of Gag. It is in this context that defective Venezuelan equine encephalitis virus replicon particle vectors (VRP) expressing various forms of SIV Gag were compared both with respect to the properties of the expressed proteins in cell culture and the strength and persistence of the cellular responses induced in mice.

Several groups have demonstrated the importance of a cellular immune response in the natural control of both HIV-1 and SIV replication (Borrow et al., 1994;Brander and Walker, 1999;Davis et al., 2002;Edwards et al., 2002;Koup et al., 1994;Letvin et al., 2006;Ogg et al., 1998;Picker, 2000;Schmitz et al., 1999). For this reason, the Gag proteins of HIV-1 and SIV are key targets in vaccine studies. They are expressed at a relatively high level by the virus, are fairly conserved and carry a high density of CTL epitopes (Bertoletti et al., 1998;Johnson et al., 1991;Johnson and Walker, 1994;Nixon, 1988;Riviere et al., 1989).

Two lines of evidence form the basis for the use of the full-length unmutated *gag* gene in the majority of vaccine vectors. First, particulate antigens have been shown to be more immunogenic than soluble proteins, possibly reflecting a structure-related adjuvant effect (Michel et al., 1990;Paliard et al., 2000). Second, full-length myristylated Gag (Gag^{myr+}) expressed in the absence of any other viral proteins is able to self-assemble into immature virus-like particles (Delchambre et al., 1989;Gheysen et al., 1989;Wagner et al., 1992). It would be expected that cytoplasmic Gag^{myr+} would be processed for presentation on MHC class I molecules and Gag-containing VLPs would be taken up by dendritic cells for processing through both the MHC class I and class II pathways (Yewdell et al., 1999), thereby giving the maximum immune response. However, the Gag protein can be produced in the cell in any of several forms, which may influence the efficiency of antigen presentation. Bu, et al. (2003) demonstrated that assembly and budding of the Gag immunogen is disrupted by mutating the major homology region involved in multimerization or by inserting a protein destruction signal. An alternative method for blocking Gag release is to mutate the glycine at codon 2 that is part of the signal for the co-translational modification of Gag with the fatty acid myristic acid. Gag protein that lacks the myristic acid moiety (Gag^{myr-}) cannot target to the cell membrane and is retained in the cytoplasm (Bryant and Ratner, 1990;Gottlinger et al., 1989;Qiu et al., 2000;Rein et al., 1986). This cytoplasmic form of Gag is the immunogen that has been used in the first clinical trial of a VRP vaccine, and preliminary, still blinded results have been published (Chulay et al., 2006). Gag^{myr+} and Gag^{myr-} represent two forms of the Gag protein that could be processed through the MHC class I pathway to induce $CD8^+$ T-cell responses. Gag protein also has been expressed as a secreted unassembled protein by addition of the t-PA signal sequence to its N-terminus (Qiu et al., 2000). An alternative form of Gag, comprised of only the matrix and capsid (MA/CA) coding domain, has been shown previously to induce both humoral and cellular immune responses, either delivered by viral vectors without the myristylation signal (Caley et al., 1997;Davis et al., 2000), or by DNA vectors with the myristylation signal (Bråve et al., 2005)

Gag^{myr+} expressed in the context of the HIV genome assembles and forms extracellular, enveloped particles in cultured primate cell lines. However, it has been reported that in mouse cells that have been engineered to allow HIV entry and gene expression, HIV-1 Gag^{myr+} fails to localize to the plasma membrane or form virus particles (Chen et al., 2001;Mariani et al., 2000). In a system using an HIV-1/murine leukemia virus pseudotype to infect murine cells expressing human cyclin T1, assembly of infectious HIV-1 particles was demonstrated (Lund el al., 2004). In work with virus vectors or DNA (Chen et al., 2005;Young and Ross, 2006) and experiments presented here, Gag^{myr+} expressed alone from a heterologous delivery system can also overcome the block to assembly and efficiently produce extracellular Gag-containing particles in murine cells.

In this study we utilized the VRP vector system (Davis et al., 2002;Pushko et al., 1997) to produce phenotypically distinct forms of the SIVsmH-4i Gag protein in both primary and transformed murine cell lines, as well as in a primate cell line. We found that three forms of the Gag immunogen (Gag^{myr+}, Gag^{myr-} and MA/CA) were expressed to similar intracellular levels in these cell lines. However, each form of Gag gave rise to a distinct molecular structure that was consistently seen in all three cell types, including the budding of virus-like particles directed by expressed Gag^{myr+}. The effect of these structural differences on immunogenicity was tested in VRP-immunized BALB/c mice.

RESULTS

Expression of different forms of SIV Gag in VRP-infected monkey and murine cells

Figure 1 describes the different forms of the SIVsm H-4i Gag protein used in this study: i) full-length myristylated Gag (Gag^{myr+}), ii) full-length non-myristylated Gag (Gag^{myr-}) and iii) a non-myristylated fragment spanning the matrix and capsid domains (MA/CA). These were expressed from individual VEE replicon vectors and packaged into VEE replicon particles (VRP). The ablation of the myristylation site is predicted to prevent the association of Gag or MA/CA with cellular membranes (Bryant and Ratner, 1990;Gottlinger et al., 1989;Rein et al., 1986). The truncation of the polyprotein at the C-terminus of CA is expected to negatively affect RNA binding and Gag-Gag interactions due to deletion of the interaction (I) domain in NC (Bowzard et al., 1998;Dupraz and Spahr, 1992;Jowett, et al., 1992), as well as interactions with cellular components involved in budding, ubiquitylation and endosomal sorting due to deletion of the late (L) domain in p6 (reviewed in Morita and Sundquist, 2004).

The VRP were used to infect three different cell lines, Vero cells (a monkey kidney fibroblast cell line), L929 cells (a mouse fibroblast cell line), and primary murine embryonic fibroblasts (MEFs) from outbred CD-1 mouse embryos, to determine whether VRP-driven expression of different forms of SIV Gag was altered in murine cells. Expression was assayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (Fig. 2). Proteins with the expected apparent molecular weights were detected in each lysate: myristylated or nonmyristylated Gag (55kDa) or MA/CA (41kDa). The faster migrating bands in the Gag^{myr+} and Gag^{myr-} lanes likely represent products from a predicted internal initiation site near the MA/CA boundary (Nicholson et al., 2006).

The analysis of intracellular protein production in monkey and mouse cells was extended using the same assay, SDS-PAGE and Western blot, in a semi-quantitative comparison (Materials and Methods). The results of two experiments (Table 1) show that VRP-infected Vero cells and MEFs contain comparable amounts of each of the three forms of Gag.

The comparison of protein levels for a given form of Gag across cell types using the semi-quantitative Western blot protocol accurately reflects the relative levels of protein production in the primate and murine cells examined. However, when different forms of Gag are compared for a single cell type, this protocol necessarily gives only an estimate because of possible differences in accessibility of the capsid epitope to its cognate monoclonal antibody. For example, it appears that Gag^{myr+} is produced in greater amounts than Gag^{myr-} or MA/CA in both cell types, even when, as in this analysis, the extracellular, budded Gag^{myr+} is not included. It may be that Gag^{myr+}, when bound to a nylon membrane, more efficiently displays the capsid epitope, or is actually present in higher amounts due to greater stability in the cytoplasm of the VRP-infected cell.

Each of the three forms of Gag protein expressed from VRP was evaluated for its ability to direct budding of virus-like particles from monkey and mouse cell lines. As a first step, putative viral particles in the culture supernatants were partially purified and concentrated by pelleting through a 20% sucrose cushion. Proteins in the pellet were resuspended in PBS and examined by SDS-PAGE and Western blot analysis. A protein of the correct apparent molecular weight (55kDa) was detected in culture supernatants after expression of Gag^{myr+} in all three cell lines, but no supernatant viral proteins were seen after expression of Gag^{myr-} or MA/CA in any of the three cell lines (Fig. 3; myristylation-minus results shown only for Vero cells).

In an alternative assay for particle production, a commercial ELISA (Zeptometrix) was used to measure the amount of p55 Gag in high speed pellets of VRP-infected cell culture supernatants collected 18 hours post-infection. In the absence of SIV protease expression, any particles produced would be immature, containing the p55 precursor polyprotein. Using this assay, it was determined that Vero cells produced 0.17 pg of p55 per cell and MEFs produced 0.75 pg of p55 per cell. More p55 also was observed in culture supernatants of Gag^{myr+}-VRP-infected MEF cultures than Gag^{myr+}-VRP-infected Vero cells when assayed by Western blot (Fig. 3). The reason for higher level p55-containing particle production from the MEFs is not known, but taken together, these results demonstrate that VRP-Gag^{myr+}-infected primary mouse cells are fully capable of producing extracellular Gag-containing particles.

Characterization of different forms of Gag protein by thin-section transmission electron microscopy

To further evaluate the intracellular localization and morphology of each of the Gag proteins expressed from the VRP, we examined both cell monolayers and pelleted supernatants from each of the three cell lines by transmission electron microscopy (TEM). Particles corresponding to the size of an immature SIV virion were detected by TEM of negatively-stained pelleted culture supernatants from cells infected with VRP expressing myristy lated Gag but not Gag^{myr-} or MA/CA (data not shown). Thin-section TEM of cell monolayers showed budding particles at the surface of all three cell lines only when Gag^{myr+} was expressed from the VRP (Fig. 4A-C). Occasionally, Gag^{myr+} particles could be observed budding into internal membranes (data not shown). Expression of Gag^{myr-} unexpectedly produced stacked aggregates of Gag protein in the cytoplasm (Fig. 4D-F) that were not detected in any of the cell lines infected with either Gag^{myr+}-VRP or MA/CA-VRP, or in mock infected cells. Expression of the MA/CA form of Gag resulted in no discernable intracellular structures (MA/CA and mock not shown).

The identity of the budding particles and the intracellular aggregates was confirmed by immunogold labeling using an SIV Matrix-specific monoclonal antibody followed by TEM. Both the budding particles and the intracellular aggregates were efficiently labeled with the colloidal gold-conjugated anti-mouse IgG secondary antibody indicating that these two forms did contain Gag protein (Fig. 5A-C). These TEM results confirmed the Western blot and p27 ELISA results showing Gag-containing particle production by Gag^{myr+}-VRP-infected monkey and mouse cells, and revealed that high level expression from the VEE replicon vector of Gag^{myr-}, a form of Gag that cannot associate normally with the plasma membrane, but retains the I and L domains, leads to intracellular accumulation of distinctive aggregates. The formation of such distinct Gag-containing structures in VRP-infected murine cells suggested that changes in myristylation and aggregation might significantly affect the immunogenicity of Gag in the VRP-immunized BALB/c mouse.

Identification of SIV Gag H-2^{d}-restricted peptides

The ELISPOT assay for Gag-specific cellular immunity is a straightforward, quantitative method for comparing the immunogenicity of two vaccines. To establish this assay for different forms of Gag expressed by VRP in the BALB/c mouse, we tested a library of 125 peptides representing the entire SIV Gag protein and identified the most reactive peptides in an IFN-γ ELISPOT. Peptides spanning the Gag protein of SIVmac 239 (15-mers overlapping by 11) were obtained from the NIH AIDS Repository and selectively mixed using an overlapping checkerboard protocol. These mixtures were used in an ELISPOT assay to test their ability to stimulate IFN-γ secretion by splenocytes taken from Gag-VRP vaccinated BALB/c mice (H-2^{d}), and three reactive peptides were identified (amino acid sequences in Materials and Methods). Stimulation with any one of these peptides gave at least 70 spots per 1×10^6 cells after subtraction of the average background seen with the irrelevant influenza HA peptide and no peptide negative controls. All three reactive peptides map to the MA/CA region of Gag. Results to be described were obtained with a pool of all three positive peptides.

To determine whether this pool of peptides contains determinants that are presented in the context of class I or class II MHC antigens, or both, splenocytes were isolated from eight MA/CA-VRP-immunized mice at 3 weeks post-boost, stimulated with the pool of three reactive peptides and analyzed by intracellular cytokine staining (ICS) for IFN-γ. Flow cytometry was used to measure intracellular IFN-γ staining of spleen cells that also had been stained for both CD4 and CD8 (data not shown). Both CD4^{+} and CD8^{+} T cells expressed increased levels of intracellular IFN-γ following stimulation with the peptide pool compared to cells incubated with control peptides. Therefore, the pool of H-2^{d} reactive peptides contains both class I and class II determinants. This result is consistent with the induction of a balanced cellular immune response by the VRP vectors, including both CD8+ T cells and CD4+ T helper cells, which support T and B cell expansion.

Immunogenicity of the various forms of Gag in BALB/c mice

Having demonstrated that VRP-expressed forms of Gag give similar phenotypes in cultured monkey cells and in primary mouse cells, and that in both of these cell types the different forms of Gag are distinguished by their morphology and ability to form particles, groups of BALB/c mice were vaccinated with each of the three VRP to test whether the phenotypes observed in cell culture affect the immunogenicity of Gag.

In three separate experiments, groups of BALB/c mice were each inoculated with a different form of Gag-VRP at week 0 and boosted at week 4. At 5, 10 and 14 weeks (1, 6 and 10 weeks post-boost) a subset of mice from each immunized group was euthanized, and splenocytes were isolated and tested in an IFN-γ ELISPOT assay using a mixture of the three Gag-reactive peptides (Fig. 6). In panels 6A-C the number of IFN-γ-producing cells (spots) per 1×10^6 splenocytes for individual mice harvested at each time point are represented as the percent of the median number of spots detected at week 5 for each VRP group. This representation illustrates the relative decrease in the antigen-specific cellular response at later times post-boost for the three VRP groups. A comparison of the difference between the 100% level (equal to the median at week 5) and the median percent at weeks 10 and 14 for each VRP group (shown by the bars) shows that a higher number of Gag-specific IFN-γ-producing cells are maintained in the MACA-VRP group than in the Gag^{myr+}-VRP group at both later time points. The decrease in median spot numbers during the time course from the acute phase (5 weeks, set at 100%) to the memory phase (14 weeks) for each VRP group in the individual experiments is shown in Figure 6D-F.

Statistical comparisons of the number of IFN-γ-producing cells per 1×10^6 splenocytes were made among the three VRP groups at each time point, and between each VRP group at each time point, combining values for all three experiments (Table 2). This analysis showed a significant difference across all groups at week 10 (median values of 211, 319 and 305, $p = 0.03$) and at week 14 (median values of 163, 182 and 242, $p = 0.006$). When the three experiments were examined in pairwise comparisons, we observed a highly significant difference at week 14 between Gag^{myr+} and MA/CA both in terms of spots per 1×10^6 splenocytes (median values 163 and 242, $p = 0.0002$; Table 2) and percent of median spots per 1×10^6 cells at week 5 (median values = 0.377 and 0.670, $p < 0.0001$; Fig 6C). Taken together, these results show that immunizations with VRP expressing different forms of Gag give acute cellular immune responses in BALB/c mice that are statistically equivalent, but that at later times the response to MA/CA-VRP is maintained at a significantly higher level than that to Gag^{myr+}-VRP, with a 50% greater median number of IFN-γ-secreting cells at week 14 in the combined experiments. Although the cellular responses to all types of Gag-VRP decreased between 5 weeks and 14 weeks, as would be expected in a comparison of IFN-γ-secreting cells in the acute and memory phases of the response, in each case the decrease was less when MA/CA was the immunogen.

The humoral immune responses induced by Gag^{myr+}-VRP, Gag^{myr-}-VRP and MA/CA-VRP vaccination were compared using an ELISA assay with purified MA/CA protein as the antigen (Davis et al., 2000). Median titers at 10 weeks post-boost in the three separate mouse immunization experiments described above were 10,000, 20,000, and 7,500 for Gag^{myr+}, 20,000, 60,000, and 10,000 for Gag^{myr-} and 10,000, 20,000 and 5,000 for MA/CA, respectively. Two-group comparisons showed that Gag^{myr-} induced significantly more binding antibody than Gag^{myr+} in two of three experiments ($p = 0.002$, 0.02, and 0.46), and in one of three experiments induced more than MA/CA ($p = 0.001$, 0.10, and 0.12). ELISA titers induced by Gag^{myr+} and MA/CA were not significantly different ($p = 0.99$, 0.61, and 0.78).

DISCUSSION

The goal of this study was to determine the expression, morphologic and immunogenic phenotypes of three alternative forms of SIVsm H-4i Gag using the VRP vector in the mouse model. The result would be considered when choosing immunogens for ongoing primate studies in support of HIV-1 vaccine development for humans. These experiments also represent an example of an empirical head-to-head test of different forms of the same immunogen to determine the form that takes best advantage of a specific vector biology.

Two developments paved the way for the use of VRP in the BALB/c mouse in this study. First, using SIV Gag-VRP we demonstrated that three distinct forms of SIVsm H-4i Gag show similar *in vitro* phenotypes in both a primate cell line and primary mouse fibroblasts. Second, reactive SIV MA/CA peptides were identified in an IFN-γ ELISPOT assay for BALB/c mice. These combined results indicated that Gag-VRP vaccination of the BALB/c mouse would be a useful model in which to compare different forms of SIV Gag for their ability to induce a cellular immune response.

Although the very distinct molecular structures found in cultured monkey and murine cells suggested that the three forms of Gag might differ dramatically in immunogenicity, our results revealed no significant difference in the humoral responses or in the acute phase of the cellular response. However, in IFN-γ ELISPOT assays, the average number of Gag-reactive splenocytes was maintained at a 50% higher level in the memory phase when MA/CA was the immunogen.

The incremental improvement in maintenance of antigen-specific T cells, as opposed to any dramatic increase in the initial cellular response, may reflect the identity of the VRP-infected cell. Wild-type VEE and VRP packaged with wild-type glycoproteins are found very soon after infection in dendritic cells in the draining lymph node with the properties of antigen-presenting cells. However, mutations in the glycoproteins, such as those used in this study, direct the VRP to different cells in the draining lymph node (MacDonald and Johnston, 2000; West and Johnston, unpublished results), cells which may be involved in cross-presentation rather than direct presentation of antigens. In the context of a cross-presenting cell, the different molecular structures of Gag immunogen tested here may have only a small, but nonetheless significant, impact on the immune response induced by VRP vaccination. The further characterization of VRP target cells in the lymph node is currently underway.

The nature of the cellular target was addressed in a previous study of intramuscular DNA vaccination, in which the expressed Gag^{myr-} immunogen gave lower level CTL responses than expression of either particle-forming Gag^{myr+} or secreted, non-particle forming Sc-Gag (Qiu, et al., 2000). One interpretation of this result is that transfer of protein from transduced muscle cells to migratory dendritic cells (cross-presentation) is more efficient with the secreted forms of Gag.

At the outset of this study, we could not assume that different forms of Gag would behave in the mouse model the same way they behave in the more relevant primate background. There are numerous steps in the HIV-1 replication cycle that appear to be blocked in mouse cells (Atchison et al., 1996;Feng et al., 1996;Landau et al., 1988). It has been observed that assembly at the plasma membrane of murine cells occurs at very inefficient levels (Mariani et al., 2000). Using the VRP system, we were able to induce particle budding in a primate cell line (Vero) and in both transformed (L929) and primary (MEF) murine cells. It is likely that the overexpression of Gag from the strong 26S promoter of the VRP overcame the assembly block in the murine cells and allowed Gag^{myr+} to assemble and bud (Hatziioannou et al., 2005). Infection of a murine myocyte cell line, C2C12, with a canarypox vector expressing HIV Gag, protease and Env also showed budding of virus-like particles (Chen et al., 2005). It may be that expression levels in this vector system also were sufficiently high to overcome the block to assembly.

Unexpectedly, expression of Gag^{myr-} by the VRP vector resulted in accumulation in the cytoplasm of distinctive Gag-containing aggregates that often had the appearance of concentric arcs associated with small electron dense particles. It is possible that aggregation results from high level expression of full-length mutated Gag that cannot associate with the plasma membrane. Although purely speculative, it may be that the specific morphology shown by the

Gag^{myr-} aggregates represents an early stage in the development of an aggresome whose maturation has been aborted due to VRP effects on cellular biosynthesis (Kopito, 2000).

The canarypox vector expressing nonmyristylated Gag did not produce an intracellular aggregated form, perhaps because the intracellular concentration was not high enough, or because protease was also expressed from that vector (Chen et al., 2005). However, an aggregate with similar morphology was detected in insect cells infected with a baculovirus vector expressing SIV Gag^{myr+}-Pol (Yamshchikov et al., 1995). In this case, the level of Gag^{myr+} expression from the baculovirus vector may have saturated the limited sites for budding and raised the cytoplasmic concentration of Gag over the threshold for aggregation.

In the context of the VRP vector, our results show that delivery of MA/CA maintains a significantly greater number of antigen-specific IFN-γ-secreting splenocytes during the memory phase compared to Gag^{myr+}, suggesting that among the forms of Gag tested, MA/CA is the immunogen of choice for this vaccine vector. The modest improvement in the longevity of the response induced by this altered form of Gag may or may not translate into other vaccine vector systems. However, it is likely that the most effective HIV vaccine will combine several such incremental improvements, and with that in mind, the MA/CA form of Gag should be considered when testing vaccine vectors for efficacy against HIV.

MATERIALS AND METHODS

Cells and plasmids

Vero and L929 cells were obtained from the American Type Culture Collection and were maintained as recommended. BHK cells were maintained and prepared for electroporation as described previously (Davis et al, 2000;Liljeström and Garoff, 1991).

The SIVsm H-4i full-length clone (Hirsch et al., 1989) was used as the template for PCR to generate the *gag* genes that were inserted into the pVR21 vector (Balasuriya et al, 2000). Primers were designed to amplify from the 3’ end of the nsp^4 gene of VEE through the 26S promoter and the 5’ leader of the 26S mRNA. Overlapping PCR was used to join this VEE vector-derived fragment upstream of the following *gag* gene segments: (i) the full-length *gag* gene encoding the intact myristylation signal (encoding Gag^{myr+}), (ii) the full-length *gag* gene with a change in codon 2 from Gly to Ala to ablate the myristylation signal (encoding Gag^{myr-}), and (iii) the matrix-capsid region of *gag* also containing the change in codon 2 (encoding MA/CA). The overlapping PCR products spanned from either the AvrII site (nt 5952 from the VEE 5’ end) or the SwaI site (nt 6962) in nsP4, through the 26S mRNA promoter and the SIV gene to a unique PmeI site added at the 3’ end of the SIV sequence. This PCR product was cloned into the PCR-Blunt vector (Invitrogen) and validated by sequencing. Flanking upstream AvrII or SwaI sites and the downstream PmeI site were then used to subclone the 26S transcription units with the SIV genes into the p VR21 vector. For the first two mouse immunization experiments, a non-myristylated MA/CA-expressing p VR2 vector was used (Davis et al., 2000), which differs from the MA/CA-expressing p VR21 vector described above only in that a different cloning strategy for p VR2 necessarily omitted the downstream 14 nts of the 30 nt-long 26S mRNA 5’ leader. The MA/CA-expressing p VR21 vector containing the full leader sequence was compared to the p VR2 vector with respect to level of MA/CA expression in MEFs and induction of cellular immunity in the third mouse immunization experiment. The two MA/CA vectors gave comparable levels of expression *in vitro* and statistically equivalent numbers of interferon (IFN)-γ-secreting cells in the ELISPOT assay at both 1 and 10 weeks post-boost. Therefore, the results for both of these vectors were combined for statistical comparison to Gag^{myr-} and Gag^{myr+}.

Each of the VEE Gag expression vectors was used in an *in vitro* transcription reaction (mMessage mMachine, Ambion, Inc.) to generate VEE replicon RNA. Capsid helper RNA and V3014 glycoprotein helper RNA (encoding independently attenuating mutations in the E1 and E2 glycoproteins; Grieder et al., 1995) were transcribed *in vitro* from separate plasmids for co-electroporation with the *gag* replicon RNA to produce VEE replicon particles (VRP). VRP were partially purified, and the infectious units(IU)/ml were determined for each preparation on BHK cell monolayers as described previously (Davis et al, 2000;Pushko et al, 1997).

Preparation of primary murine embryo fibroblasts (MEFs)

Pregnant CD-1 female mice (E13) were obtained from Charles River Labs. Embryos were harvested at 15-16 days gestation, rinsed in PBS, then transferred to complete medium (Dulbecco's minimal essential medium with 10% FBS, 2mM L-glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin and 10mM HEPES). Tissue was minced with a sterile razor then homogenized by passing through a 16G needle several times. The homogenate was incubated with trypsin at 37°C for a total of 90 minutes, during which time additional trypsin was added to the cells at 30 minute intervals. Cells were incubated on ice for 10 minutes to allow large aggregates to settle out and then the supernatant was centrifuged for 10 min at 1000× g at 4°C. The resulting pellet was resuspended in complete medium, and the cells were plated, allowed to grow at 37°C, 5% CO_2 and passed twice to select for adherent fibroblasts before trypsinizing and freezing in DMSO-containing freezing medium. Prior to use, cells were thawed, plated and passed once.

Western blot analysis of cultured cell lysate and supernatants

Monolayers of each cell type were prepared and grown to ~80% confluence. Parallel cultures were infected with each VRP at a multiplicity of infection (MOI) of 10 (10 IU per cell) and incubated at 37°C for 16-18 hrs prior to harvest. Cell supernatants were collected and centrifuged at 18,000× g for 5 min to remove cell debris and nuclei. Supernatant Gag particles were then partially purified and concentrated by pelleting through a 20% sucrose cushion for 16-18hrs at 13,000× g at 4°C. The cell monolayer was rinsed with PBS and lysed in a buffer containing 0.05M Tris (pH 7.5), 0.1M NaCl, 0.1% EDTA, 0.2% NP40 and a protease inhibitor cocktail (Complete, Roche Applied Science). The cell lysate was collected and centrifuged at 18,000× g for 5 min to remove cell membrane fragments and nuclei. Aliquots of the pelleted culture supernatants and the clarified cell lysates were dissociated in 1% sodium dodecyl sulfate (SDS) and 2.75mM β-mercaptoethanol and their constituent proteins were resolved on a NuPAGE 4-12% Bis-Tris polyacrylamide gel (Invitrogen) and transferred to PVDF membrane (Amersham). After blocking overnight in 5% Amersham membrane blocking agent in Tris-buffered saline solution with Tween 20 [TBST: 0.1M Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20], the membrane was incubated with SIV capsid-specific monoclonal antibody KK64 (NIH AIDS Repository Cat.#2321, Kent et al., 1991) at a dilution of 1:1000 in blocking solution. As the epitope for this monoclonal antibody is in SIV capsid, it would react with all forms of Gag protein used in this study. The membrane was washed thoroughly and incubated with an HRP-conjugated horse anti-mouse IgG secondary antibody (Promega) at a 1:1000 dilution in TBST. Antibody-binding bands were detected using enhanced chemiluminescence (ECL-Amersham) and Kodak Biomax film.

A semi-quantitative Western blot was performed as follows. Any cells that had been released into the culture supernatant during the 18 hours of infection were collected by centrifugation, lysed and added to the lysate of attached cells. This was necessary because of the greater number of floating cells in the Gag^{myr+} cultures than in the Gag^{myr-} and MA/CA cultures. Also, a series of preliminary Western blots was performed to determine the amount of cell lysate and anti-capsid (CA) monoclonal antibody KK64 needed to give an excess of antibody over antigen, thereby detecting total antigen in each lysate. For the final Western blot, an equal number of lysed cell equivalents was applied to the gel for each cell line infected with each VRP, and an amount of antibody known to be in excess of each antigen was used for detection. Appropriate bands in the final blots were quantified using Image J software.

Transmission electron microscopy and immunogold labeling

Each of the three cell lines (Vero, L929 and MEF) was infected with each of the VRP at an MOI of 10. Cell supernatants were removed, and the cell monolayers were fixed with 3% glutaraldehyde in 0.15M sodium phosphate buffer and post-fixed with potassium ferrocyanide-reduced osmium tetroxide. Cells were embedded *in situ* in Polybed 812 epoxy resin and cut into ultrathin sections of 70 nm. Sections were post-stained in uranyl acetate and lead citrate, then photographed using a LEO EM-910 transmission electron microscope (LEO Electron Microscopy Inc. Thornwood, NY) at 80KV. All sections were cut parallel to the substrate.

For immunogold labeling of the Gag particles, cells were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde. Cells were embedded in L.R. White resin, sectioned parallel to the substrate at 80 nm, labeled using a 1:100 dilution of a monoclonal antibody to SIV Matrix (KK59 - NIH AIDS Repository Cat.# 2320, Kent et al., 1991), followed by a 1:50 dilution of a goat anti-mouse IgG secondary antibody conjugated to a 5 nm colloidal gold particle (Polysciences, Inc., Warrington, PA Cat.# 22730). KK59 monoclonal antibody, like KK64 described above, reacted with all forms of Gag used in this study and gave better binding than KK64 under the fixation conditions required for electron microscopy. Immunogold labeling was followed by staining in uranyl acetate and lead citrate and sections were photographed as above.

Quantitation of extracellular particle production by p27 ELISA

Pellets obtained by high speed centrifugation of culture supernatants were resuspended in a total volume of 200 μl and analyzed using a Retro-tek SIV p27 antigen ELISA kit (Zeptometrix) according to the manufacturer's recommendations. Determination of protein amount was made by comparison to an antigen standard curve using a p27 sample provided by the manufacturer. Duplicate values were averaged, and an average background value for mock-infected cells was subtracted. Final values (pg/ml) were multiplied by total volume and divided by the number of cells from which the supernatant was collected to derive pg of p27 per cell.

VRP vaccination of BALB/c mice

Five-week-old BALB/c mice were obtained from Charles River Labs and housed in accordance with NIH and institutional guidelines. Eight mice per group per time point were each vaccinated with VRP expressing either Gag^{myr+}, Gag^{myr-} or MA/CA at a total dose of 1×10^6 IU in 20 μl split equally between the rear footpads. Vaccinations were given at 0 and 4 weeks. Serum was collected one day prior to the priming dose, one day prior to the boost, and during the harvesting of the spleen at 1, 6 or 10 weeks post-boost.

Identification of SIV Gag $H\text{-}2^d$-restricted peptides

The SIVmac239 Gag peptide library (AIDS Research and Reference Reagent Program, Cat.# 6204) consists of 125 15-mer peptides that overlap by 11 amino acids. Peptides were pooled and arranged in an overlapping checkerboard as previously described (Kern et al., 1999; Guido Ferrari, personal communication). Each pool consisted of 11 consecutive peptides and 12 overlapping peptides. The checkerboard was constructed such that one peptide was present in each of two otherwise independent pools. Using the intersection of these pools, an individual reactive peptide was easily identified. The peptide pools were used in an IFN-γ ELISPOT assay (see below) at a final concentration of 2 μg/ml to stimulate splenocytes isolated from immunized BALB/c mice. The mice were given two doses of 1×10^6 IU of Gag^{myr+}-VRP at

0 and 4 weeks. An IFN-γ ELISPOT assay was performed 3 weeks post-boost. The initial ELISPOT assay identified several candidate pools. The individual peptides that comprised these pools were then tested in a second IFNγ ELISPOT assay, thereby identifying three reactive peptides: KQIVQRHL VVETETGTT (Cat.# 5236), NIYRRWIQLGLQKCV (Cat.#5276) and YVDRFYKSLRAEQTD (Cat.#5285). Stimulation of splenocytes from mice immunized with VRP-Gag^{myr+} with a pool of these three peptides gave 104, 158 or 74 spots per 10^6 cells, respectively, compared to 14 spots per 10^6 cells with a pool of three negative peptides (Cat.#'s 5211, 5233 and 5255).

Assay of cellular immune response by IFN-γ ELISPOT

Multiscreen 96-well filtration plates (Millipore MHABS4510) were incubated overnight at 4° C with 100 μl per well of a 5μg/ml solution of antibody to mouse IFN-γ (AN18 Mabtech) in sodium bicarbonate buffer (pH 9.6), washed with PBS and blocked with AIM V (Invitrogen), 5% FBS medium. After blocking, plates were washed again prior to adding peptides at a final concentration of 2 μg/ml in a volume of 50 μl.

Groups of vaccinated mice were euthanized according to institutional guidelines at week 1, 6 or 10 post-boost. Spleens were removed and disrupted with a syringe plunger. The single cell suspensions were then washed twice in a 1:10 dilution in PBS of Alsever's solution (8g dextrose, 5.5g citric acid, 4.2g sodium chloride in a final volume of 1 L, PBS/A). The red blood cells were lysed by adding 1 ml of sterile water, immediately followed by 9 ml of PBS/A. Cells were washed twice again, then resuspended in RPMI medium. Splenocytes were overlaid onto a Lympholyte M cushion (Cedarlane) and centrifuged for 30 min at 2500 rpm and 20°C. The buffy coat was removed, resuspended in PBS/A and washed twice. After the last wash, cells were resuspended in AIM V medium without added serum, counted and adjusted to 1×10^7 cells/ml and 50 μl were plated into wells of anti-IFN-γ antibody coated 96 well plates containing either 50 μl of peptide(s) to be tested, an irrelevant control influenza virus hemagglutinin (HA) peptide (IYSTVASSL), or a no peptide control. Three 15-mer SIV Gag peptides identified from the mapping IFN-γ ELISPOT assays, Cat.#5236, Cat.#5276 and Cat.#5285, were used individually and as a pool of 3 in this assay. The plates were incubated undisturbed for 20-24 hours at 37°C and 5% CO_2. Plates were washed with chilled distilled water, incubated on ice for 10 minutes, washed 10 times with wash buffer (PBS with 0.01% Tween-20) and then incubated with 100 μl per well of 1μg/ml of biotinylated antibody to mouse IFN-γ (R4-64A2, Mabtech) in PBS containing 0.01% Tween-20, 1% bovine serum albumin (BSA) for 16-18 hours at 4°C. After the incubation, plates were washed ten times with wash buffer. Strepavidin-alkaline phosphatase (Mabtech) was diluted 1:1000 in PBS/0.01% Tween-20/1% BSA, 100μl was added to each well and plates were incubated for 1-2 hours at 25°C. Plates were washed 10 times and spots were developed with 100 μl of BCIP/NBT substrate (Promega) prepared according to the manufacturer's instructions. Spots were counted using an automated plate reader with Immuno Spot software (Cellular Technology Ltd.).

Three non-reactive SIV Gag peptides were used as negative controls in addition to the irrelevant K^d HA peptide (IYSTVASSL) and no peptide sample. Averaged negative HA and no peptide control values for each spleen were subtracted from the individual samples. Concanavalin A at a concentration of 5 μg/ml was included in two wells of each splenocyte preparation as a positive control for the presence of viable T cells.

ELISA for anti-MA/CA antibody

Antisera were tested for antibody against MA/CA as described previously (Davis et al., 2000) except that six-His-tagged MA/CA protein was made in *Escherichia coli* strain BL21DE3 using the pET24a expression plasmid. Horseradish peroxidase-linked anti-mouse

IgG (Sigma) was used as the secondary antibody. Titers represent the highest serum dilution that gave an optical density at 450 nm of ≥ 0.2.

Statistical analysis of ELISPOT results

Nonparametric one-way analysis of variance (the Kruskal-Wallis test using Van der Waerden normal scores) was used to evaluate possible differences in ELISPOT and ELISA responses across the Gag^{myr+}, Gag^{myr-} and MA/CA groups. The Wilcoxon rank-sum test (using Van der Waerden normal scores) was used for the two-group comparisons of Gag^{myr+} to Gag^{myr-}, Gag^{myr+} to MA/CA, and Gag^{myr-} to MA/CA. Monte Carlo estimates of exact p-values were used. The Bonferonni method was used to adjust p-values to account for multiple comparisons. All of these statistical analyses were performed using SAS statistical software, Version 9.1, SAS Institute Inc., Cary, NC. The Mann-Whitney Test was used to compare values for the Percent of Median Spots per $1{\times}10^6$ Cells at Week 5 for the Gag^{myr+}-VRP and MA/CA-VRP groups at week 14 (Instat program, GraphPad Software).

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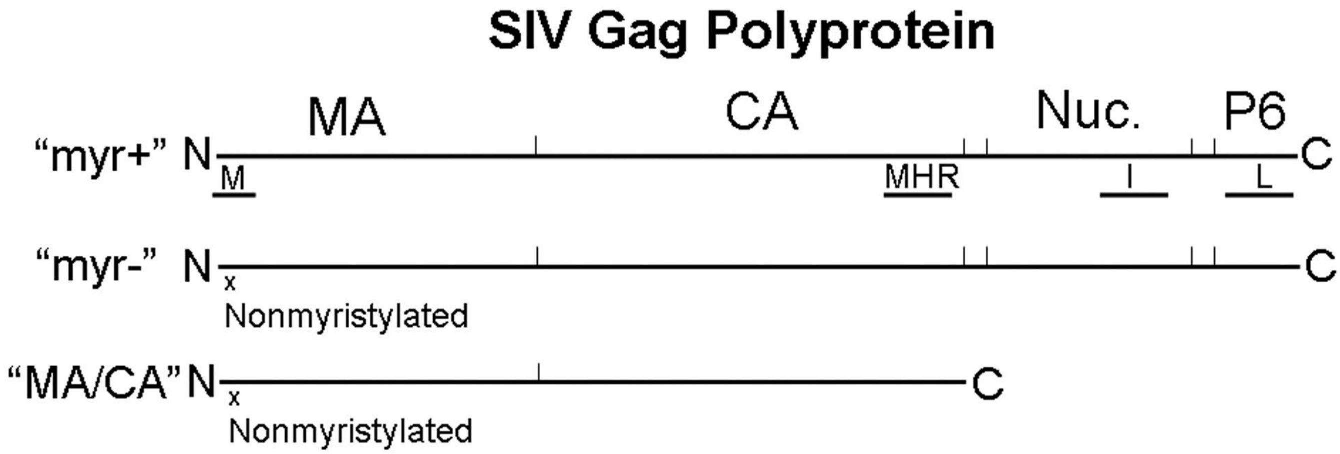

Figure 1.
Diagram of three forms of the SIV Gag protein expressed from the VEE replicon expression vector. The approximate locations of the M, membrane targeting domain, MHR, major homology region, I, interaction domain, L, late domains and x, mutant myristy lation site are shown.

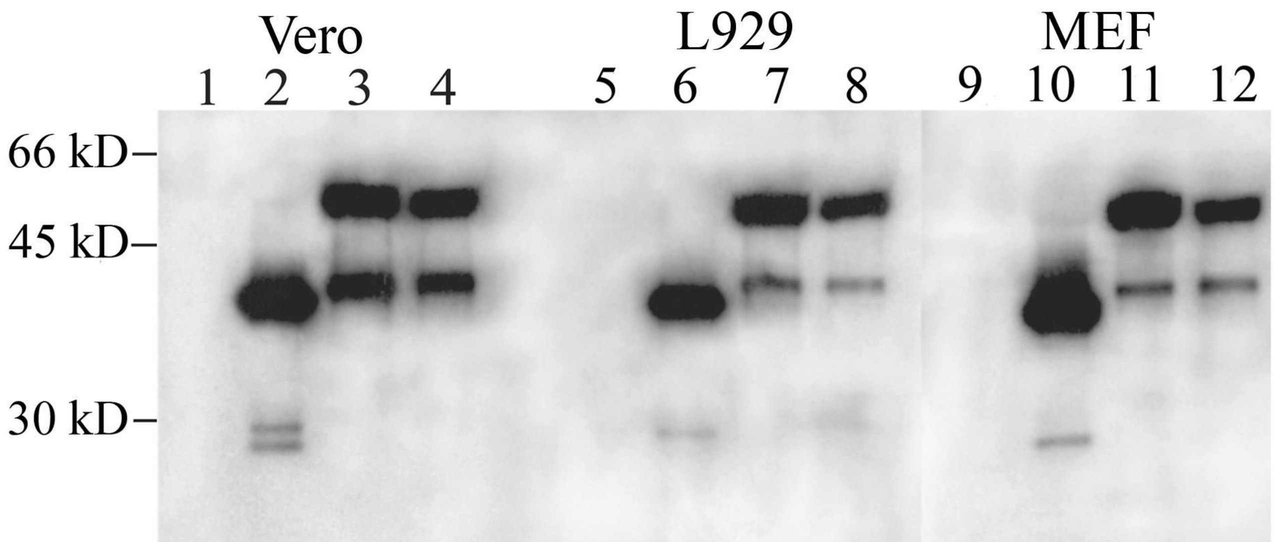

Figure 2.
Cell-associated Gag protein in VRP-infected cultured cells. Vero cells (lanes 1-4), L929 cells (lanes 5-8) and MEFs (lanes 9-12) were infected at a multiplicity of infection of 10 with VRP expressing either MA/CA (lanes 2,6,10), Gag^{myr-} (lanes 3,7,11) or Gag^{myr+} (lanes 4,8,12) or mock infected (lanes 1,5,9). At 18 hours post-infection monolayers were lysed with NP-40 and the lysate analyzed by SDS-PAGE on a 4-12% gradient polyacrylamide gel followed by Western blot with an anti-Capsid monoclonal antibody.

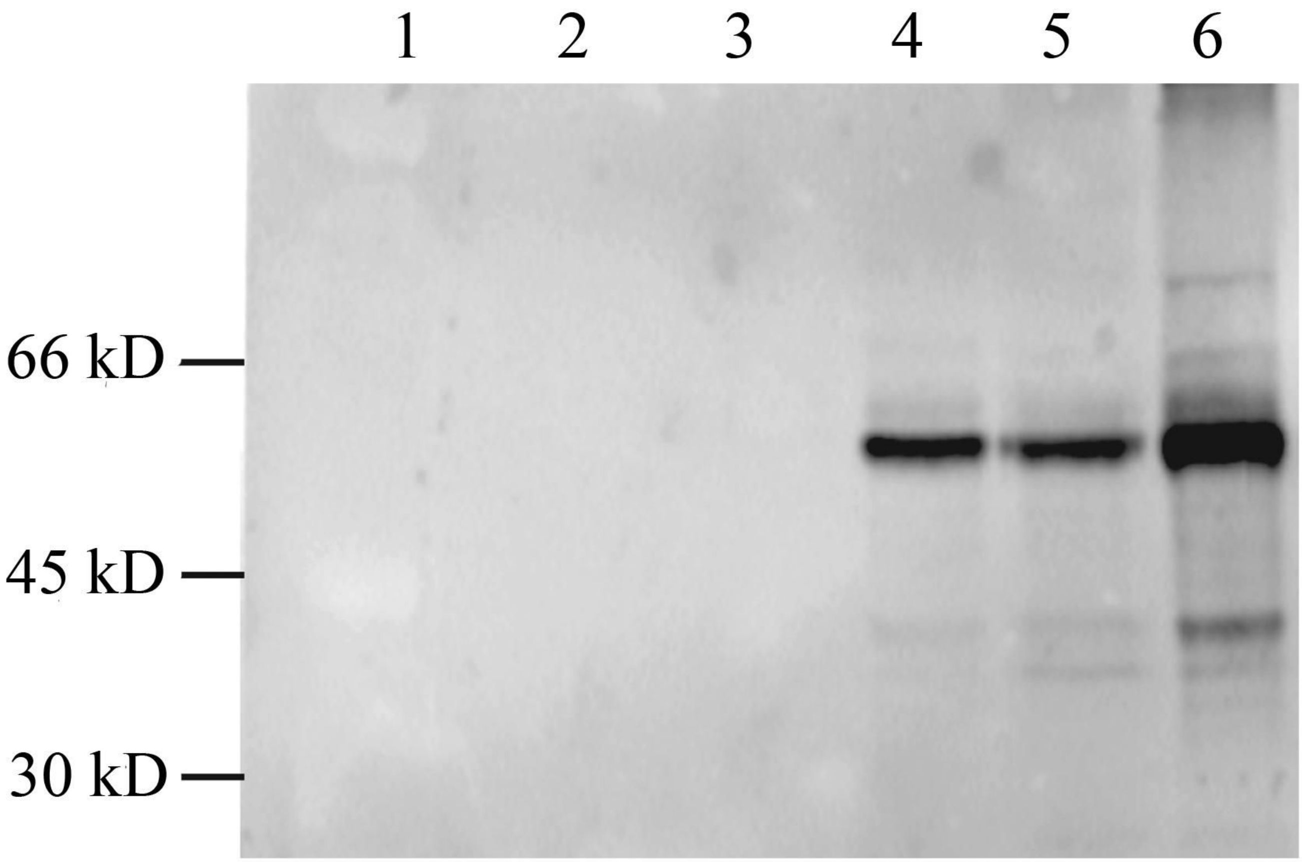

Figure 3.

Extracellular Gag protein in cultures of VRP-infected cells. Vero cells (lanes 1-4), L929 cells (lane 5) and MEFs (lane 6) were infected as described for Figure 2 with VRP expressing either: MA/CA (lane 2), Gag^{myr-} (lane 3) and Gag^{myr+} (lanes 4-6). Cell supernatants were collected at 18 hours post-infection, layered over a 20% sucrose cushion and centrifuged. Pellets were resuspended in PBS, dissociated with SDS and β-mercaptoethanol, and analyzed by SDS-PAGE and Western blot as described for Figure 1.

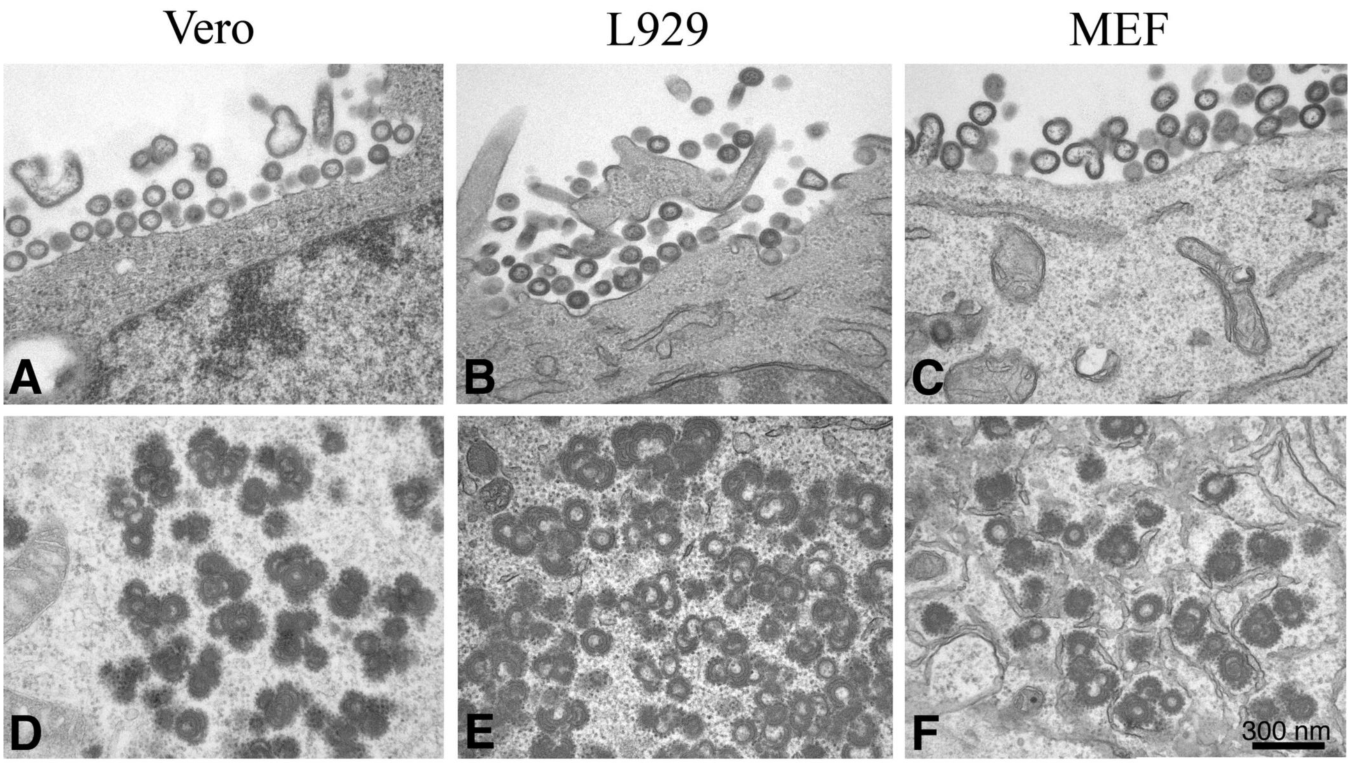

Figure 4.

Thin section electron microscopy of VRP-infected cultured cells. Vero cells (A,D), L929 cells (B,E) or MEFs (C,F) were infected as for Figure 2 with either Gag^{myr+}-VRP (A-C) or Gag^{myr-}-VRP (D-F). At 18 hours post-infection monolayers were fixed, embedded, sectioned and stained for TEM as described in Materials and Methods. Representative fields are shown for each culture at the same magnification.

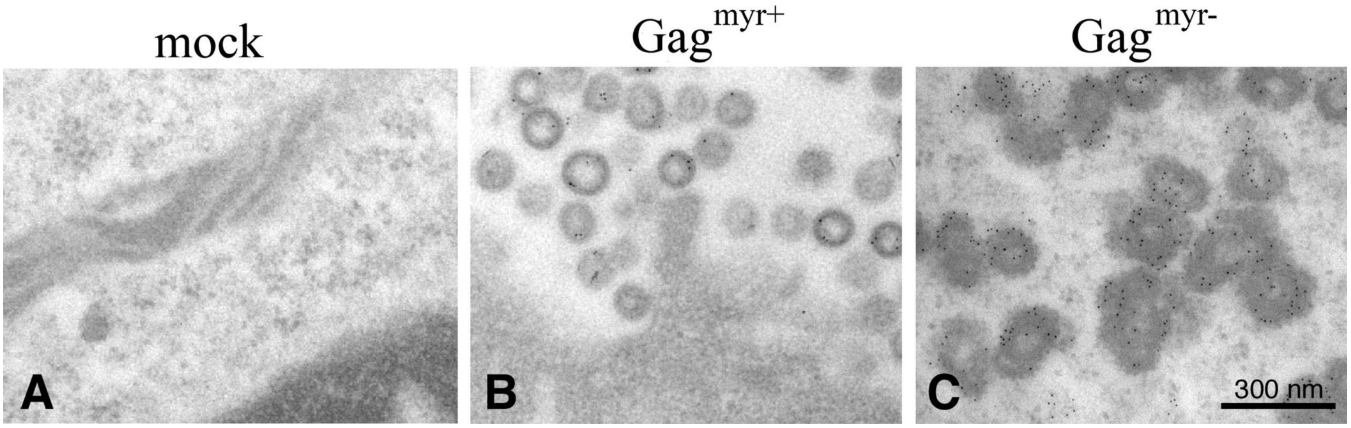

Figure 5.
Immunogold labeling followed by thin section electron microscopy of VRP-infected vero cells. Cells were infected as for Figure 2 and fixed at 18 hours post-infection. Monolayers were sectioned, treated with anti-Matrix monoclonal antibody followed by colloidal gold-conjugated anti-mouse IgG antibody then stained with uranyl acetate and lead citrate.

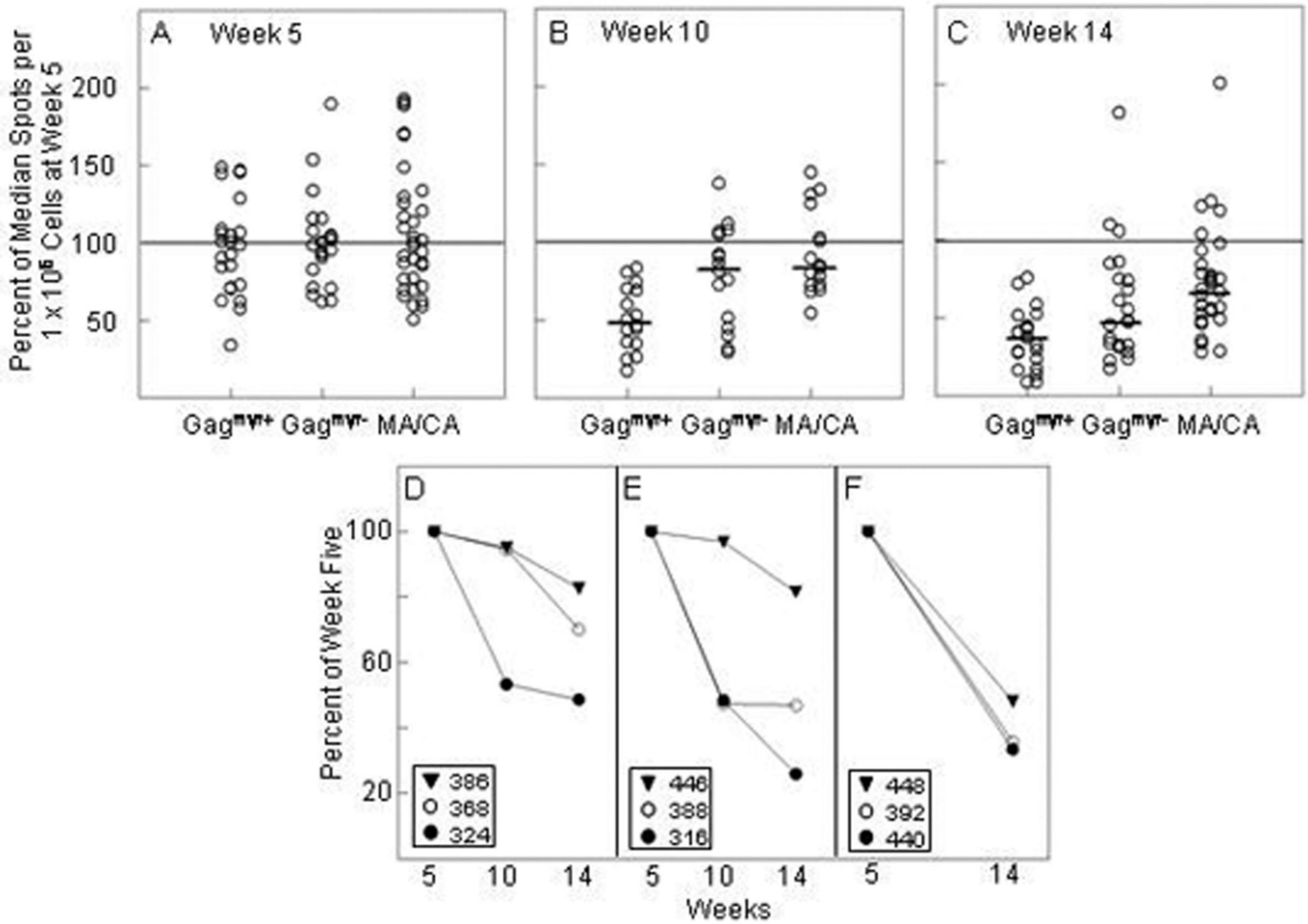

Figure 6.

ELISPOT assay of splenocytes from Gag-VRP immunized BALB/c mice. Mice were immunized with Gag^{myr+}-, Gag^{myr-}- or MA/CA-VRP at weeks 0 and 4. Splenocytes were harvested from groups of mice at either week 5, 10 or 14 and stimulated with a pool of Gag-reactive peptides in an IFN-γ ELISPOT assay (Materials and Methods). A: IFN-γ-secreting cells (spots) per 1×10^6 cells in individual spleens for the three independent experiments combined plotted as a percent of the median number of spots detected at 5 weeks (1 week post-boost). N = 24 for Gag^{myr+}, N = 24 for Gag^{myr-}, N = 34 for MA/CA. B: Spots per 1×10^6 cells at 10 weeks (6 weeks post-boost) plotted as a percent of the median number of spots detected at 5 weeks. Bars represent median percents for each group at 10 weeks. N = 16 for all groups. Experiment three did not include a 10 week time point. C: Spots per 1×10^6 cells at 14 weeks (10 weeks post-boost) plotted as a percent of the median number of spots detected at 5 weeks. Bars represent median percents for each group at 14 weeks. N = 24 for Gag^{myr+}, N = 24 for Gag^{myr-}, N = 31 for MA/CA. D.-F.: Median spots per 1×10^6 cells for each of three independent experiments represented as a percent of the median spots per 1×10^6 cells at week 5. Insets show the median spots per 1×10^6 cells at 5 weeks for each group in each experiment. Solid inverted triangles, MA/CA group; open circles, Gag^{myr-} group; solid circles, Gag^{myr+} group.

TABLE 1

Intracellular Protein Production (semi-quantitative Western blot)

Cell line	Protein					
	Gag^{myr+}		Gag^{myr-}		MA/CA	
	Exp. 1	**Exp. 2**	**Exp. 1**	**Exp. 2**	**Exp. 1**	**Exp. 2**
Vero	10,740	11,238	6578	9231	6992	8773
MEF	10,266	14,423	6213	10753	7856	8767

TABLE 2

Statistical Comparisons - IFN-γ ELISPOT Experiments

Comparison across all three groups: Week 5	Median spots per 1 × 10^6 cells	p-value	Two-group comparisons: Week 5	Median spots per 1 × 10^6 cells	p-value
Gag^{myr+}	432		Gag^{myr+}/Gag^{myr-}	432/378	**0.80**
Gag^{myr-}	378	**0.74**	Gag^{myr+}/MA/CA	432/361	**0.66**
MA/CA	361		Gag^{myr-}/MA/CA	378/361	**0.92**
Week 10					
Gag^{myr+}	211				
Gag^{myr-}	319	**0.03**			
MA/CA	305				
Week 14					
Gag^{myr+}	163		Gag^{myr+}/Gag^{myr-}	163/182	**0.16**
Gag^{myr-}	182	**0.006**	Gag^{myr+}/MA/CA	163/242	**0.0002**
MA/CA	242		Gag^{myr-}/MA/CA	182/242	**0.22**