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A Novel Codon-optimized SIV Gag-pol Immunogen for Gene-based Vaccination

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Abstract

Simian immunodeficiency virus (SIV) is a robust pathogen used in non-human primates to model HIV vaccines. SIV encodes a number of potential vaccine targets. By far the largest and most conserved protein target in SIV is its gag-pol protein that bears many epitopes to drive multivalent immune T cell responses. While gag-pol is an attractive antigen, it is only translated after a frame shift between gag and pol with the effect that gag and pol are expressed at an approximate 10/1 ratio. The codon bias of native lentiviral genes are also mismatched with the abundance of tRNAs in mammalian cells resulting in poor expression of unmodified SIV genes. To provide a better SIV gag-pol immunogen for gene-based vaccination, we codon-optimized the full gag-pol sequence from SIVmac239. To increase pol expression, we artificially moved the pol sequence in frame to gag to bypass the need for a translational frame shift for its expression. Finally, we inserted four "self-cleaving" picornavirus sequences into gag p24, protease, reverse transcriptase, and into integrase to fragment the proteins for potentially better immune presentation. We demonstrate that these immunogens are well expressed *in vitro* and drive similar antibody and T cell responses with or without cleavage sequences.

Introduction

Vaccines are the most economical medical intervention to control infectious agents, yet traditional vaccine strategies have struggled to control some of the more difficult pathogens (reviewed in (Barry, 1999)). In the late 1980's, "genetic vaccines" were invented that circumvented the need to produce proteins in the laboratory and purify them, and instead uses the vaccinated person's own cells to produce the vaccines *in situ*. By this approach, single genes encoded in simple plasmids can be delivered directly into a person's cells *in*

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vivo using a gene gun or needle injection (Acsadi et al., 1991; Johnston et al., 1991; McCabe et al., 1988; Sanford et al., 1987; Wolff et al., 1992; Wolff et al., 1990; Wolff et al., 1991; Yang et al., 1990). Once delivered, the DNA is transcribed and translated into the pathogen protein that can generate vaccine responses as if the host has been infected. In essence, the host cell becomes a factory to produce the protein vaccine. Importantly, this *in situ* intracellular production of pathogen proteins from a plasmid not only produces antibodies, but also allows protein display on Major Histocompatibility Complex (MHC) I and II molecules on the host cells. This MHC I display of genetic vaccine proteins drives CD4 and CD8 T cell responses that are important to kill intracellular pathogens. Based on this, gene-based vaccines have been extensively applied against human immunodeficiency virus (HIV-1) and against its models simian immunodeficiency virus (SIV), and SIV-HIV chimeras (SHIVs) (reviewed in (Barry, 2012).

If one relies on a gene to express vaccine antigens, the genes that are used in a genetic vaccine must translate proteins efficiently. Early work using with HIV-1 envelope plasmids demonstrated that these were surprisingly poorly expressed considering that these are human viruses (Boyer, 1997; Fuller et al., 1997; Lu et al., 1995). This is due in part to the poor codon-bias of HIV genes relative to the tRNA pools that are present in mammalian cells (Haas et al., 1996).

To avoid this problem, one can "codon-optimize" genes by replacing their codons that are served by low abundance tRNAs with codons with better tRNA pools and that are better expressed. When applied for HIV envelope, codon-optimization increased expression 100-fold (Haas et al., 1996). Increasing antigen expression generally increases immune responses after genetic immunization (Andre et al., 1998), so this approach has become common practice for gene-based vaccines. These positive effects may not be due soley to increased protein translation. More recent studies suggest that codon-optimization can alter protein folding (Komar et al., 1999). Other work indicates that codon-optimized sequences may be less efficiently N-glycosylated (Honarmand Ebrahimi et al., 2014; Ujvari et al., 2001). For proteins like HIV envelope, this may have positive effects if lower glycosylation better reveals protein epitopes. Conversely, if too little of the protein is glycosylated this may impede secretion display on the cell surface for secreted proteins.

SIV and SHIV are important challenge viruses to test candidate HIV vaccines in non-human primates. Therefore, the vaccines that are tested in these models must match the final challenge virus. Most SIV or SHIV vaccines utilize the SIV gag protein and either SIV or HIV envelope depending on the challenge virus (reviewed in (Barouch and Korber)). While gag has many epitopes to be targeted by CD4 and CD8 T cell responses, it has less than half the size of pol. Therefore, expressing both gag and pol may supply a richer repertoire of T cell epitopes for multivalent T cell responses.

The gag-pol protein may be an attractive antigen. However, pol is out of frame with gag and is only expressed after a translational frame shift. This translates into the expression of approximately one molecule of pol for every 10 of gag. Given these issues, in this work, we codon-optimized the full gag-pol sequence from SIVmac239. To increase pol expression, we synthetically changed its reading frame to bypass the need for frame shifting for its

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expression. Finally, we inserted four "self-cleaving" picornavirus sequences into gag p24, protease, reverse transcriptase, and into integrase to fragment the proteins for potentially better immune presentation. Here we evaluate the *in vitro* expression of these novel immunogens and test their ability to drive antibody and T cell responses *in vivo* in mice.

Materials and Methods

Cell culture

A549 cells were purchased from (ATCC, Manassas, VA) and were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS; HyClone, Rockford, IL) and penicillin/streptomycin at 100 U/mL (Invitrogen).

Codon-optimized Gag-pol Fusion Protein Genes

The gag-pol sequence from SIVmac239 (accession number AY588946 (O'Connor et al., 2004)) was used to design the SIV gag-pol fusion protein. The 534 amino acids constituting the p55 gag protein were fused in frame *in silico* with the first amino acid of the 1138 amino acid pol protein that is produced by frame shift during gag-pol expression (Jacks et al., 1988). This 1672 amino acid sequence was also modified to mutate the second amino acid of gag from glycine to an alanine to prevent myristylation and membrane targeting to improve antigen degradation and presentation (Wong and Siliciano, 2005). In addition, a His6 tag was added to the c-terminus of the virtual protein for detection and purification. To improve the ability of the proteasome to cleave the proteins, four picornavirus 'self-cleaving' 2A peptides (Szymczak et al., 2004) were inserted into the middle of gag p24, protease (PRO), reverse transcriptase (RT), and integrase (INT) (Fig. 1 and Supplemental Figure 1). The inserted sequences included glycine-glycine-serine (GGS) flexibility spacers using native G or S amino acids where possible (Table 1). Insertion sites were selected to minimize disruption of native reading frame. The self-cleaving sequences were inserted into SIV gag-pol fusion with two identical restriction sites flanking each as follows: P2A was flanked by AfeI sites, E2A was flanked by BbvCI sites, T2A was flanked by SpeI sites, and F2A was flanked by XhoI sites. This *in silico* polypeptide called SIV gag-pol fusion cleaver (Fig. 1 and Supplemental Figure 1) was used to generate a virtual human codon-optimized cDNA sequence using the GeneBuilder program (UT Southwestern). This virtual sequence was used by Genscript (Piscataway, NJ) to synthesize the SIV gag-pol fusion cleaver cDNA. This sequence was then sequentially restriction digested and ligated with AfeI, BbvCI, SpeI, and XhoI to remove the cleavage sites and generate a parallel SIV gag-pol fusion protein without self-cleaving functions.

Adenovirus Vectors

The SIV gag-pol fusion cleaver and SIV gag-pol fusion cDNAs were cloned into pShuttle-CMV from the Ad-Easy system (Q Biogene) and this plasmid was recombined with the adenovirus serotype 5 (Ad5) plasmid pAd-Easy1. This plasmid was cut with PacI, the DNA purified, and used to transfect 293 cells. Both viruses were rescued and amplified with final production from a Cell Factory (Corning) as in (Parrott et al., 2003). Each virus was purified by double banding on CsCl gradients and viral particle (vp) concentration was quantitated by OD260.

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In Vitro Protein Expression

293 cells were transfected with 4 μg of each of the pShuttle-CMV plasmids described above using 40 μl of Polyfect Transfection Reagent (Qiagen) and western blotting was performed. To test expression from the vectors, A549 cells were infected with Ad5-CMV-SIV gag-pol fusion and gag-pol fusion cleaver at 1000 vp/cell. Lysates were harvested 48 hours after transfection or infection and proteins separated on 10% SDS-PAGE gels prior to transfer to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% dried milk in Tris-buffered saline with 0.1% Tween 20 (TBST). Blots were probed with a 1/1000 dilution of SIV_{mac}251 p17 Gag Monoclonal (KK59) antibody from the AIDS Reagent program for one hour at room temperature. Blots were washed in TBST and probed with a 1/2000 dilution of goat anti-mouse-horse radish peroxidase (HRP) secondary antibody (Pierce Chemical) for one hour. Blots were washed and HRP was detected with West Femto Chemiluminescent reagent (Pierce).

Immunizations

Female BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). They were housed in the Mayo Clinic Animal Facility under the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) guidelines with animal use protocols approved by the Mayo Clinic Animal Use and Care Committee. All animal experiments were carried out according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, the principles of the NIH Guide for the Care and Use of Laboratory Animals, and the policies and procedures of Mayo Clinic. Mice were immunized with 10^{10} vp of the indicated vectors or PBS by the intramuscular (i.m.) route, and boosted with the same amount after 3 weeks. Samples were collected and immune responses were measured at the indicated times.

Enzyme-linked immunosorbant assay (ELISA)

At indicated times, blood was collected in BD microtainer tubes with serum separator (Becton Dickinson and Company) from the submandibular vein. Samples were stored at −20°C until assayed. Immulon 4 HBX plates (Thermo, Milford, MA) were coated with 100 ng/well SIV gag p24 in PBS at room temperature at 4°C overnight. Wells were blocked with 3% BSA, 1xPBS at room temperature for 1 hour. Mouse serum was diluted 1:100 in 3% BSA, 1xPBS and applied to plates at room temperature 2 hours. Wells were washed with 1xPBS and rabbit anti-mouse IgG (Pierce Chemical) was added at a 1:4000 dilution for two hours. Wells were washed and the plates were developed with 100μL 1 step Ultra TMB ELISA (Thermo Fisher Scientific Inc, Rockford, IL). 50 μL 2N H_2SO_4 was added to each well to quench development and OD450 was measured using the Beckman Coulter DTX 880 Multimode Detector system.

SIV Gag-Specific IFN-γ ELISPOT

Fresh splenocytes were isolated after euthanasia of the mice as in (Weaver and Barry, 2008) and these were stimulated with select overlapping SIVmac239 gag peptide pools (MGVRNSVLSGKKADE, NSVLSGKKADELEKI, SGKKADELEKIRLRP, HAEEKVKHTEEAKQI, KVKHTEEAKQIVQRH, TEEAKQIVQRHLVVE,

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KQIVQRHLVVETGTT, QRHLVVETGTTETMP, VVETGTTETMPKTSR, DVKQGPKEPFQSYVD, GPKEPFQSYVDRFYK, PFQSYVDRFYKSLRA, YVDRFYKSLRAEQTD, CGKMDHVMAKCPDRQ, DHVMAKCPDRQAGFL, AKCPDRQAGFLGLGP, DRQAGFLGLGPWGKK) containing CD4 and CD8 T cell epitopes identified in BALB/c mice (Xu et al., 2009) or with Con A (5 μg/ml) as a positive control. 1×10^6 splenocytes were seeded in in 96-well PVDF-backed plates (MAIP S 45, Millipore, Bedford, MA) previously coated with anti-IFN-γ. The cells were incubated with antigens for 36 h at 37°C, were then removed, and the wells were washed prior to incubation with 100 μl of alkaline-phosphatase-conjugated anti-IFN-γ for 2 h at room temperature. Spots representing individual cells secreting IFN-γ were detected using BCIP/ NBT. The plates were washed and the spots were counted by Zellnet Consulting (New Jersey, NJ). Responses in terms of IFN-γ spot-forming cells (SFC) are represented for 10^5 total input PBMC were determined for individual macaques after subtracting background. 10 spots represent the background of the assay as it is twice the number observed in cells cultured in the medium.

Data Analysis

Graphs and statistical analyses were performed using Prism Graphical software.

Results

Codon-optimized Gag-pol Fusion Protein Genes

The SIVmac239 (Jacks et al., 1988) p55 gag open reading frame (ORF) was fused in frame *in silico* with the first amino acid of the pol protein. This produced a single open reading frame of 1672 amino acids. Since the gag and pol ORFs normally overlap by approximately 550 base pairs (bp), fusing them in frame would normally create a direct tandem sequence repeat that would be prone to rearrangement. However, when the sequence was codon-optimized, these previously overlapping identical sequences were wobbled at their third codon positions rendering them no longer homologous.

This virtual sequence was modified to improve immunogenicity and detection. First, a His6 tag was added to the c-terminus of the protein. The second amino acid of gag was mutated from glycine to alanine to prevent myristoylation to improve antigen presentation for CD8 T cell responses (Wong and Siliciano, 2005). Previous work has shown that expressing HIV gag as fragments improves proteasome degradation and MHC I presentation (Sykes and Johnston, 1999). To express one protein, but potentially cleave it into fragments, we introduced four different picornavirus 'self-cleaving' 2A peptides (Szymczak et al., 2004) into gag p24, protease (PRO), reverse transcriptase (RT), and integrase (INT) (Fig. 1, Supplemental Figure 1, Table 1).

This *in silico* polypeptide was used to generate a codon-optimized cDNA sequence. During this design, the cleavage sites were each flanked by two matching restriction endonuclease sites to allow removal of the cleavage sites (Fig. 1). After synthesis, SIV gag-pol fusion cleaver was sequentially digested removing the sites to produce the codon-optimized

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sequence SIV gag-pol fusion (Fig. 1). Both sequences were introduced into replication-defective adenovirus serotype 5 (Ad5) vectors for testing.

Protein Expression

293 cells were transfected with expression vectors carrying each of the fusion proteins and western blots were performed using anti-SIV gag p17 and anti-His6 (Fig. 2). Probing the n-terminus of the fusion protein with anti-p17 detected bands migrating approximately at molecular weights of 15, 18, and 25 kDa from for the gag-pol fusion construct. This construct expresses intact SIV protease (PRO), so normal protein processing is expected (Fig. 1). In contrast in the cleaver construct, anti-p17 detected these bands, a larger band of approximately 75 kDa, as well as a blob of over-exposed protein between (Fig. 2A). Since gag is 55 kDa, detection of the 75 kDa band suggests that at least part of the pol portion of the fusion protein is expressed. After Ad5 vectors were produced, A549 cells were infected with the vectors at 1,000 vp/cell and lysates were analysed with the anti-p17 antibody (Fig. 2B). The previously unresolved blob now resolved into two bands of at approximately 45 and 55 kDa for the cleaved construct (Fig. 2B). While SIV gag antibodies are readily available, the dearth of SIV pol antibodies prevented confirmation of pol expression. Probing with anti-His6 failed to detect bands negating the ability to detect c-terminal fragments (Fig. 2A).

The protease of the fusion cleaver protein is disrupted by insertion of the E2A sequence, so the detected bands may result from proteolysis of the fusion protein by cellular proteases or the inserted self-cleaving sequences. P2A self-cleavage would be predicted to generate a p17-tagged protein of approximately 29 kDa. E2A cleavage would generate a p17 containing protein of approximately 62 kDa if no other cleavage occurred. T2A and F2A would generate proteins of 96 and 145 kDa kDa with no other proteolysis. The cleaved construct expressed bands of approximately 15, 18, 25, 45, 55, and 75 kDa with p17 on their n-termini suggesting some may have been produced by 2A cleavage, perhaps combined with cellular protease activity.

Immunization of Mice Expressing SIV Gag-pol Constructs

Western blot analysis suggest that the SIV gag-pol fusion antigen is expressed and its cleavage is likely driven by SIV protease. In contrast, the fusion cleaver construct generated a different pattern of cleavage perhaps driven by the 2A sequences alone or in combination with cellular proteases. To test if these variations affected the immunogenicity of the constructs, each was cloned into replication-defective adenovirus serotype 5 (Ad5) vectors. These were then used to immunize groups of female BALB/c mice with 10^{10} vp by the intramuscular route.

Antibody Responses

Three weeks after priming immunization, sera were collected and assayed by ELISA for anti-gag p24 antibodies. Antibody levels were virtually identical for the two constructs (Fig. 3A). The animals were boosted with the same amount of vector and their sera were tested again 3 weeks later revealing similar antibody levels in the mice.

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T Cell Responses

At the six-week time point, the animals were sacrificed and their splenocytes were assayed for SIV-specific T cell responses by ELISPOT (Fig. 3B). Splenocytes from mice vaccinated with both constructs generated detectable IFN-γ spot forming cells (SFCs) after stimulation with SIV gag peptide pools. However, the fusion protein without cleavage sequences actually generated stronger T cell responses than the cleaved construct.

Discussion

This antigen engineering study was motivated by the pragmatic need for a codon-optimized SIV gag-pol expression construct for vaccine testing. Expressing both gag and pol was motivated by observations that vaccination with SIV gag alone can generate immunodominant T cell responses that can be easily escaped by the viral mutation (Barouch et al., 2003; Barouch et al., 2002). To maximize the likelihood of driving multivalent T cell responses in pol, its large reading frame were pulled into the same reading frame as gag. Given the expense of synthesizing a 5,000 base pair codon-optimized gene, we also explored if modulating protein cleavage might modify T cell and antibody responses against the antigen by inserting picornavirus self-cleavage sequences into the protein.

This work demonstrated that the two codon-optimized sequences were expressed and that use of native SIV polymerase or the 2A self-cleaving sequences produced differing patterns of protein fragments. These studies were facilitated by the availability of reagents against the gag domain, but were limited by a lack of antibody, protein, and peptide reagents. Data from the cleaved construct suggested that at least part of pol was translated in the large fusion, but lack of His6 antibody staining made it unclear if the full fusion was expressed. Considering that non-codon-optimized gag-pol is translated, it is likely that the full codon-optimized cDNA is as well.

When these genes were used in first generation Ad5 vectors in mice, both antigens generated immune responses against at least the gag domain of the fusion protein. Both vectors generated similar antibody responses against gag p24 antigen. The SIV gag-pol fusion construct appeared to generate somewhat stronger T cell responses against SIV gag peptide pools, although these were not significantly different from each other. These data suggest that both constructs may have utility in SIV protection studies and that further studies may be warranted to explore the utility of protein cleavage to drive immune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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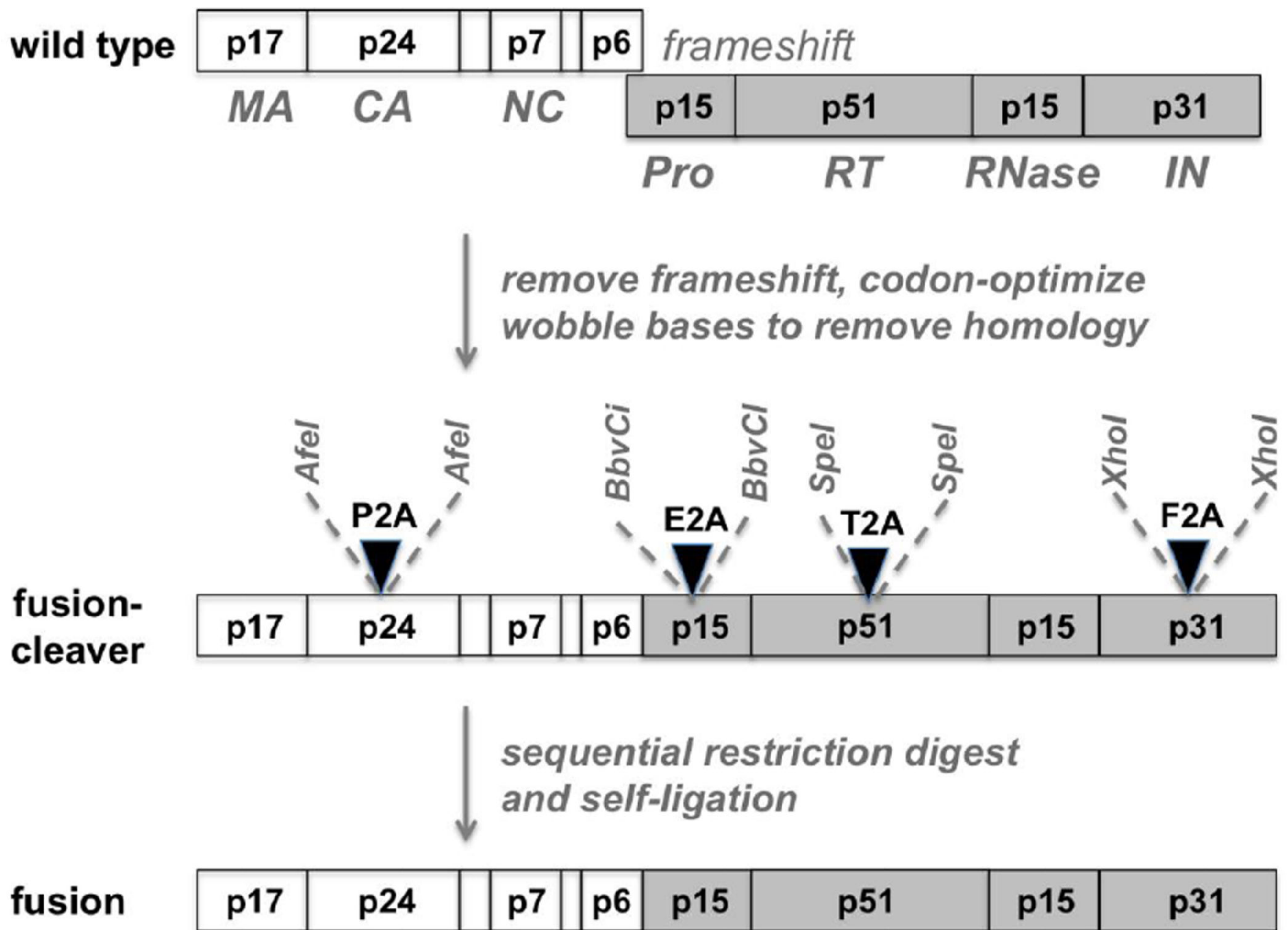

Figure 1. Schematic of wild type and codon-optimized SIV gag-pol expression cassettes

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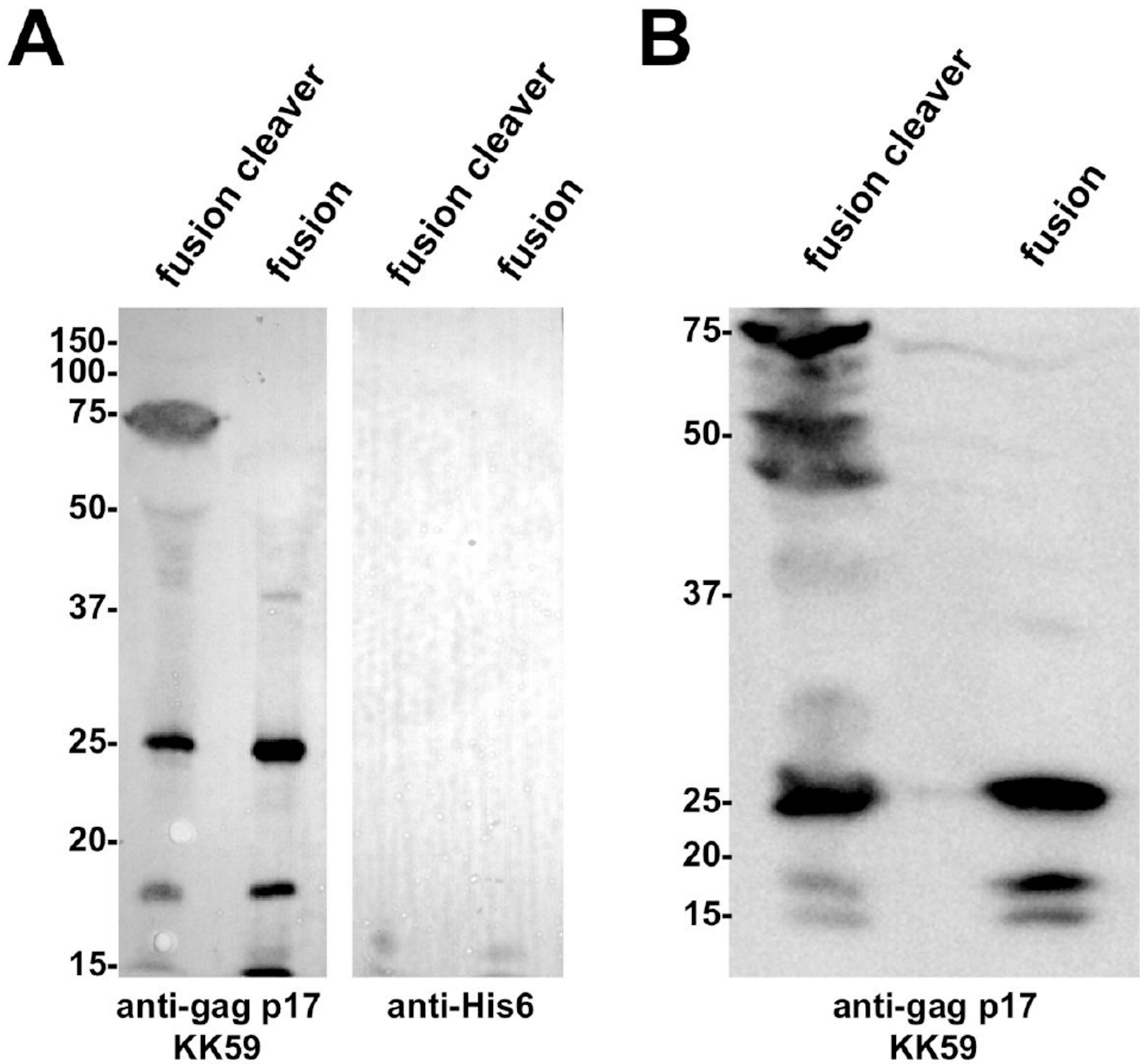

Figure 2. Western blot analysis of protein expression by SIV gag-pol fusion and SIV gag-pol fusion cleaver

A) 293 were transfected with plasmids expressing the indicated constructs and lysates were separated on SDS-PAGE gels prior to western blotting with anti-SIV gag p17 or anti-His6. **B)** 293 cells were infected with Ad5 vectors expressing the indicated constructs and western blotting was performed with anti-p17.

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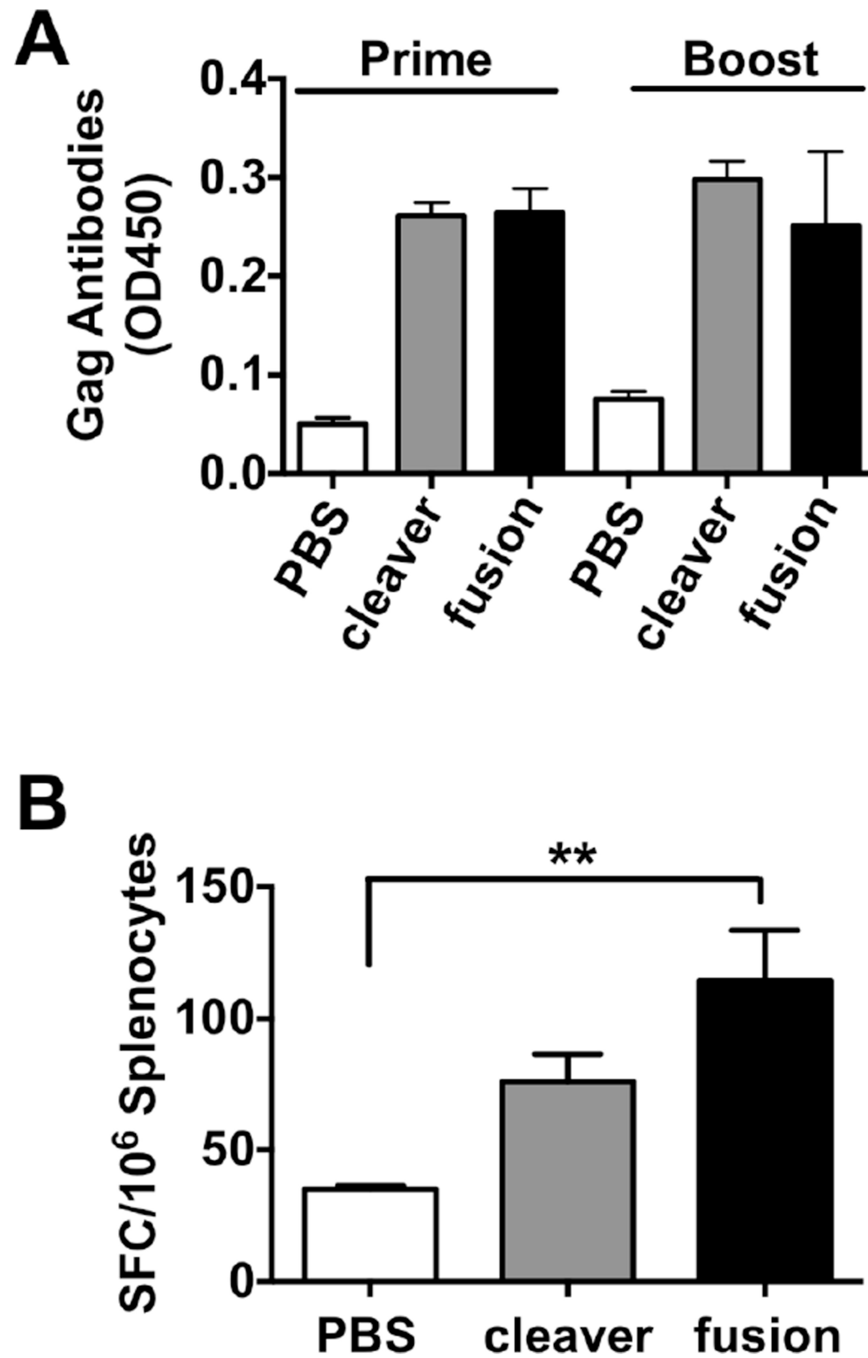

Figure 3. Immune responses generated by SIV gag-pol constructs

Groups of 5 BALB/c mice were immunized by the intramuscular route with 10^{10} vp of the indicated vectors or PBS once (prime) or twice (boost). **A)** Anti-SIV gag p24 ELISAs were performed. **B)** Anti-SIV gag IFN-g ELISPOTs were performed on splenocytes and responses are expressed as cytokine spot forming cells (SFCs) per million cells. One way ANOVA with Bonferroni multiple comparison post-test demonstrated **$p < 0.01$.

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Table 1

Self-Cleaving Insertions In Gag-Pol Fusion Cleaver Protein.

SIV Protein	Cleavage Site	Inserted Sequence[&]
p24	**P2A**	GGSATNFSLLKQAGDVEENPG*PGGS
PRO	**E2a**	GGSQCTNYALLKLAGDVESNPG*PGGS
RT	**T2A**	GGSEGRGSLLTCGDVEENPG*PGGS
INT	**F2A**	GGSVKQTLNFDLLKLAGDVESNPG*PGGS

[&]The full picornavirus cleavage sequence from (Szymczak et al., 2004) is underlined and the intended cleavage site between G and P is marked with an asterisk.

A Novel Codon-optimized SIV Gag-pol Immunogen for Gene-based Vaccination

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Abstract

Simian immunodeficiency virus (SIV) is a robust pathogen used in non-human primates to model HIV vaccines. SIV encodes a number of potential vaccine targets. By far the largest and most conserved protein target in SIV is its gag-pol protein that bears many epitopes to drive multivalent immune T cell responses. While gag-pol is an attractive antigen, it is only translated after a frame shift between gag and pol with the effect that gag and pol are expressed at an approximate 10/1 ratio. The codon bias of native lentiviral genes are also mismatched with the abundance of tRNAs in mammalian cells resulting in poor expression of unmodified SIV genes. To provide a better SIV gag-pol immunogen for gene-based vaccination, we codon-optimized the full gag-pol sequence from SIVmac239. To increase pol expression, we artificially moved the pol sequence in frame to gag to bypass the need for a translational frame shift for its expression. Finally, we inserted four "self-cleaving" picornavirus sequences into gag p24, protease, reverse transcriptase, and into integrase to fragment the proteins for potentially better immune presentation. We demonstrate that these immunogens are well expressed *in vitro* and drive similar antibody and T cell responses with or without cleavage sequences.

Introduction

Vaccines are the most economical medical intervention to control infectious agents, yet traditional vaccine strategies have struggled to control some of the more difficult pathogens (reviewed in (Barry, 1999)). In the late 1980's, "genetic vaccines" were invented that circumvented the need to produce proteins in the laboratory and purify them, and instead uses the vaccinated person's own cells to produce the vaccines *in situ*. By this approach, single genes encoded in simple plasmids can be delivered directly into a person's cells *in vivo* using a gene gun or needle injection (Acsadi et al., 1991; Johnston et al., 1991; McCabe et al., 1988; Sanford et al., 1987; Wolff et al., 1992; Wolff et al., 1990; Wolff et al., 1991; Yang et al., 1990). Once delivered, the DNA is transcribed and translated into the pathogen protein that can generate vaccine responses as if the host has been infected. In essence, the host cell becomes a factory to produce the protein vaccine. Importantly, this *in situ* intracellular production of pathogen proteins from a plasmid not only produces antibodies, but also allows protein display on Major Histocompatibility Complex (MHC) I and II molecules on the host cells. This MHC I display of genetic vaccine proteins drives CD4 and CD8 T cell responses that are important to kill intracellular pathogens. Based on this, gene-based vaccines have been extensively applied against human immunodeficiency virus (HIV-1) and against its models simian immunodeficiency virus (SIV), and SIV-HIV chimeras (SHIVs) (reviewed in (Barry, 2012).

If one relies on a gene to express vaccine antigens, the genes that are used in a genetic vaccine must translate proteins efficiently. Early work using with HIV-1 envelope plasmids demonstrated that these were surprisingly poorly expressed considering that these are human viruses (Boyer, 1997; Fuller et al., 1997; Lu et al., 1995). This is due in part to the poor codon-bias of HIV genes relative to the tRNA pools that are present in mammalian cells (Haas et al., 1996).

To avoid this problem, one can "codon-optimize" genes by replacing their codons that are served by low abundance tRNAs with codons with better tRNA pools and that are better expressed. When applied for HIV envelope, codon-optimization increased expression 100-fold (Haas et al., 1996). Increasing antigen expression generally increases immune responses after genetic immunization (Andre et al., 1998), so this approach has become common practice for gene-based vaccines. These positive effects may not be due soley to increased protein translation. More recent studies suggest that codon-optimization can alter protein folding (Komar et al., 1999). Other work indicates that codon-optimized sequences may be less efficiently N-glycosylated (Honarmand Ebrahimi et al., 2014; Ujvari et al., 2001). For proteins like HIV envelope, this may have positive effects if lower glycosylation better reveals protein epitopes. Conversely, if too little of the protein is glycosylated this may impede secretion display on the cell surface for secreted proteins.

SIV and SHIV are important challenge viruses to test candidate HIV vaccines in non-human primates. Therefore, the vaccines that are tested in these models must match the final challenge virus. Most SIV or SHIV vaccines utilize the SIV gag protein and either SIV or HIV envelope depending on the challenge virus (reviewed in (Barouch and Korber)). While gag has many epitopes to be targeted by CD4 and CD8 T cell responses, it has less than half the size of pol. Therefore, expressing both gag and pol may supply a richer repertoire of T cell epitopes for multivalent T cell responses.

The gag-pol protein may be an attractive antigen. However, pol is out of frame with gag and is only expressed after a translational frame shift. This translates into the expression of approximately one molecule of pol for every 10 of gag. Given these issues, in this work, we codon-optimized the full gag-pol sequence from SIVmac239. To increase pol expression, we synthetically changed its reading frame to bypass the need for frame shifting for its expression. Finally, we inserted four "self-cleaving" picornavirus sequences into gag p24, protease, reverse transcriptase, and into integrase to fragment the proteins for potentially better immune presentation. Here we evaluate the *in vitro* expression of these novel immunogens and test their ability to drive antibody and T cell responses *in vivo* in mice.

Materials and Methods

Cell culture. A549 cells were purchased from (ATCC, Manassas, VA) and were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS; HyClone, Rockford, IL) and penicillin/streptomycin at 100 U/mL (Invitrogen).

Codon-optimized Gag-pol Fusion Protein Genes. The gag-pol sequence from SIVmac239 (accession number AY588946 (O'Connor et al., 2004)) was used to design the SIV gag-pol fusion protein. The 534 amino acids constituting the p55 gag protein were fused in frame *in silico* with the first amino acid of the 1138 amino acid pol protein that is produced by frame shift during gag-pol expression (Jacks et al., 1988). This 1672 amino acid sequence was also modified to mutate the second amino acid of gag from glycine to an alanine to prevent myristylation and membrane targeting to improve antigen degradation and presentation (Wong and Siliciano, 2005). In addition, a His6 tag was added to the c-terminus of the virtual protein for detection and purification. To improve the ability of the proteasome to cleave the proteins, four picornavirus 'self-cleaving' 2A peptides (Szymczak et al., 2004) were inserted into the middle of gag p24, protease (PRO), reverse transcriptase (RT), and integrase (INT) (Fig. 1 and Supplemental Figure 1). The inserted sequences included glycine-glycine-serine (GGS) flexibility spacers using native G or S amino acids where possible (Table 1). Insertion sites were selected to minimize disruption of native reading frame. The self-cleaving sequences were inserted into SIV gag-pol fusion with two identical restriction sites flanking each as follows: P2A was flanked by AfeI sites,

E2A was flanked by BbvCI sites, T2A was flanked by SpeI sites, and F2A was flanked by XhoI sites. This *in silico* polypeptide called SIV gag-pol fusion cleaver (Fig. 1 and Supplemental Figure 1) was used to generate a virtual human codon-optimized cDNA sequence using the GeneBuilder program (UT Southwestern). This virtual sequence was used by Genscript (Piscataway, NJ) to synthesize the SIV gag-pol fusion cleaver cDNA. This sequence was then sequentially restriction digested and ligated with AfeI, BbvCI, SpeI, and XhoI to remove the cleavage sites and generate a parallel SIV gag-pol fusion protein without self-cleaving functions.

Adenovirus Vectors. The SIV gag-pol fusion cleaver and SIV gag-pol fusion cDNAs were cloned into pShuttle-CMV from the Ad-Easy system (Q Biogene) and this plasmid was recombined with the adenovirus serotype 5 (Ad5) plasmid pAd-Easy1. This plasmid was cut with PacI, the DNA purified, and used to transfect 293 cells. Both viruses were rescued and amplified with final production from a Cell Factory (Corning) as in (Parrott et al., 2003). Each virus was purified by double banding on CsCl gradients and viral particle (vp) concentration was quantitated by OD260.

***In Vitro* Protein Expression.** 293 cells were transfected with 4 µg of each of the pShuttle-CMV plasmids described above using 40 µl of Polyfect Transfection Reagent (Qiagen) and western blotting was performed. To test expression from the vectors, A549 cells were infected with Ad5-CMV-SIV gag-pol fusion and gag-

pol fusion cleaver at 1000 vp/cell. Lysates were harvested 48 hours after transfection or infection and proteins separated on 10% SDS-PAGE gels prior to transfer to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% dried milk in Tris-buffered saline with 0.1% Tween 20 (TBST). Blots were probed with a 1/1000 dilution of SIV_{mac}251 p17 Gag Monoclonal (KK59) antibody from the AIDS Reagent program for one hour at room temperature. Blots were washed in TBST and probed with a 1/2000 dilution of goat anti-mouse-horse radish peroxidase (HRP) secondary antibody (Pierce Chemical) for one hour. Blots were washed and HRP was detected with West Femto Chemiluminescent reagent (Pierce).

Immunizations. Female BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). They were housed in the Mayo Clinic Animal Facility under the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) guidelines with animal use protocols approved by the Mayo Clinic Animal Use and Care Committee. All animal experiments were carried out according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, the principles of the NIH Guide for the Care and Use of Laboratory Animals, and the policies and procedures of Mayo Clinic. Mice were immunized with 10^{10} vp of the indicated vectors or PBS by the intramuscular (i.m.) route, and boosted with the same amount after 3 weeks. Samples were collected and immune responses were measured at the indicated times.

Enzyme-linked immunosorbant assay (ELISA). At indicated times, blood was collected in BD microtainer tubes with serum separator (Becton Dickinson and Company) from the submandibular vein. Samples were stored at -20°C until assayed. Immulon 4 HBX plates (Thermo, Milford, MA) were coated with 100 ng/well SIV gag p24 in PBS at room temperature at 4°C overnight. Wells were blocked with 3% BSA, 1xPBS at room temperature for 1 hour. Mouse serum was diluted 1:100 in 3% BSA, 1xPBS and applied to plates at room temperature 2 hours. Wells were washed with 1xPBS and rabbit anti-mouse IgG (Pierce Chemical) was added at a 1:4000 dilution for two hours. Wells were washed and the plates were developed with 100µL 1 step Ultra TMB ELISA (Thermo Fisher Scientific Inc, Rockford, IL). 50 µL 2N H_2SO_4 was added to each well to quench development and OD450 was measured using the Beckman Coulter DTX 880 Multimode Detector system.

SIV Gag-Specific IFN-γ ELISPOT. Fresh splenocytes were isolated after euthanasia of the mice as in (Weaver and Barry, 2008) and these were stimulated with select overlapping SIVmac239 gag peptide pools (MGVRNSVLSGKKADE, NSVLSGKKADELEKI, SGKKADELEKIRLRP, HAEEKVKHTEEAKQI, KVKHTEEAKQIVQRH, TEEAKQIVQRHLVVE, KQIVQRHLVVETGTT, QRHLVVETGTTETMP, VVETGTTETMPKTSR, DVKQGPKEPFQSYVD, GPKEPFQSYVDRFYK, PFQSYVDRFYKSLRA, YVDRFYKSLRAEQTD, CGKMDHVMAKCPDRQ, DHVMAKCPDRQAGFL, AKCPDRQAGFLGLGP, DRQAGFLGLGPWGKK) containing CD4 and CD8 T cell

epitopes identified in BALB/c mice (Xu et al., 2009) or with Con A (5 µg/ml) as a positive control. 1 x 10^6 splenocytes were seeded in in 96-well PVDF-backed plates (MAIP S 45, Millipore, Bedford, MA) previously coated with anti-IFN-γ. The cells were incubated with antigens for 36 h at 37°C, were then removed, and the wells were washed prior to incubation with 100 µl of alkaline-phosphatase-conjugated anti-IFN-γ for 2 h at room temperature. Spots representing individual cells secreting IFN-γ were detected using BCIP/ NBT. The plates were washed and the spots were counted by Zellnet Consulting (New Jersey, NJ). Responses in terms of IFN-γ spot-forming cells (SFC) are represented for 10^5 total input PBMC were determined for individual macaques after subtracting background. 10 spots represent the background of the assay as it is twice the number observed in cells cultured in the medium.

Data Analysis. Graphs and statistical analyses were performed using Prism Graphical software.

Results

Codon-optimized Gag-pol Fusion Protein Genes. The SIVmac239 (Jacks et al., 1988) p55 gag open reading frame (ORF) was fused in frame *in silico* with the first amino acid of the pol protein. This produced a single open reading frame of 1672 amino acids. Since the gag and pol ORFs normally overlap by approximately 550 base pairs (bp), fusing them in frame would normally create a

direct tandem sequence repeat that would be prone to rearrangement. However, when the sequence was codon-optimized, these previously overlapping identical sequences were wobbled at their third codon positions rendering them no longer homologous.

This virtual sequence was modified to improve immunogenicity and detection. First, a His6 tag was added to the c-terminus of the protein. The second amino acid of gag was mutated from glycine to alanine to prevent myristoylation to improve antigen presentation for CD8 T cell responses (Wong and Siliciano, 2005). Previous work has shown that expressing HIV gag as fragments improves proteasome degradation and MHC I presentation (Sykes and Johnston, 1999). To express one protein, but potentially cleave it into fragments, we introduced four different picornavirus 'self-cleaving' 2A peptides (Szymczak et al., 2004) into gag p24, protease (PRO), reverse transcriptase (RT), and integrase (INT) (Fig. 1, Supplemental Figure 1, Table 1).

This *in silico* polypeptide was used to generate a codon-optimized cDNA sequence. During this design, the cleavage sites were each flanked by two matching restriction endonuclease sites to allow removal of the cleavage sites (Fig. 1). After synthesis, SIV gag-pol fusion cleaver was sequentially digested removing the sites to produce the codon-optimized sequence SIV gag-pol fusion (Fig. 1). Both sequences were introduced into replication-defective adenovirus serotype 5 (Ad5) vectors for testing.

Protein Expression. 293 cells were transfected with expression vectors carrying each of the fusion proteins and western blots were performed using anti-SIV gag p17 and anti-His6 (Fig. 2). Probing the n-terminus of the fusion protein with anti-p17 detected bands migrating approximately at molecular weights of 15, 18, and 25 kDa from for the gag-pol fusion construct. This construct expresses intact SIV protease (PRO), so normal protein processing is expected (Fig. 1). In contrast in the cleaver construct, anti-p17 detected these bands, a larger band of approximately 75 kDa, as well as a blob of over-exposed protein between (Fig. 2A). Since gag is 55 kDa, detection of the 75 kDa band suggests that at least part of the pol portion of the fusion protein is expressed. After Ad5 vectors were produced, A549 cells were infected with the vectors at 1,000 vp/cell and lysates were analysed with the anti-p17 antibody (Fig. 2B). The previously unresolved blob now resolved into two bands of at approximately 45 and 55 kDa for the cleaved construct (Fig. 2B). While SIV gag antibodies are readily available, the dearth of SIV pol antibodies prevented confirmation of pol expression. Probing with anti-His6 failed to detect bands negating the ability to detect c-terminal fragments (Fig. 2A).

The protease of the fusion cleaver protein is disrupted by insertion of the E2A sequence, so the detected bands may result from proteolysis of the fusion protein by cellular proteases or the inserted self-cleaving sequences. P2A self-cleavage would be predicted to generate a p17-tagged protein of approximately

29 kDa. E2A cleavage would generate a p17 containing protein of approximately 62 kDa if no other cleavage occurred. T2A and F2A would generate proteins of 96 and 145 kDa kDa with no other proteolysis. The cleaved construct expressed bands of approximately 15, 18, 25, 45, 55, and 75 kDa with p17 on their n-termini suggesting some may have been produced by 2A cleavage, perhaps combined with cellular protease activity.

Immunization of Mice Expressing SIV Gag-pol Constructs. Western blot analysis suggest that the SIV gag-pol fusion antigen is expressed and its cleavage is likely driven by SIV protease. In contrast, the fusion cleaver construct generated a different pattern of cleavage perhaps driven by the 2A sequences alone or in combination with cellular proteases. To test if these variations affected the immunogenicity of the constructs, each was cloned into replication-defective adenovirus serotype 5 (Ad5) vectors. These were then used to immunize groups of female BALB/c mice with 10^{10} vp by the intramuscular route.

Antibody Responses. Three weeks after priming immunization, sera were collected and assayed by ELISA for anti-gag p24 antibodies. Antibody levels were virtually identical for the two constructs (Fig. 3A). The animals were boosted with the same amount of vector and their sera were tested again 3 weeks later revealing similar antibody levels in the mice.

T Cell Responses. At the six-week time point, the animals were sacrificed and their splenocytes were assayed for SIV-specific T cell responses by ELISPOT (Fig. 3B). Splenocytes from mice vaccinated with both constructs generated detectable IFN-γ spot forming cells (SFCs) after stimulation with SIV gag peptide pools. However, the fusion protein without cleavage sequences actually generated stronger T cell responses than the cleaved construct.

Discussion

This antigen engineering study was motivated by the pragmatic need for a codon-optimized SIV gag-pol expression construct for vaccine testing. Expressing both gag and pol was motivated by observations that vaccination with SIV gag alone can generate immunodominant T cell responses that can be easily escaped by the viral mutation (Barouch et al., 2003; Barouch et al., 2002). To maximize the likelihood of driving multivalent T cell responses in pol, its large reading frame were pulled into the same reading frame as gag. Given the expense of synthesizing a 5,000 base pair codon-optimized gene, we also explored if modulating protein cleavage might modify T cell and antibody responses against the antigen by inserting picornavirus self-cleavage sequences into the protein.

This work demonstrated that the two codon-optimized sequences were expressed and that use of native SIV polymerase or the 2A self-cleaving

sequences produced differing patterns of protein fragments. These studies were facilitated by the availability of reagents against the gag domain, but were limited by a lack of antibody, protein, and peptide reagents. Data from the cleaved construct suggested that at least part of pol was translated in the large fusion, but lack of His6 antibody staining made it unclear if the full fusion was expressed. Considering that non-codon-optimized gag-pol is translated, it is likely that the full codon-optimized cDNA is as well.

When these genes were used in first generation Ad5 vectors in mice, both antigens generated immune responses against at least the gag domain of the fusion protein. Both vectors generated similar antibody responses against gag p24 antigen. The SIV gag-pol fusion construct appeared to generate somewhat stronger T cell responses against SIV gag peptide pools, although these were not significantly different from each other. These data suggest that both constructs may have utility in SIV protection studies and that further studies may be warranted to explore the utility of protein cleavage to drive immune responses.

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Table 1. Self-Cleaving Insertions In Gag-Pol Fusion Cleaver Protein.

SIV Protein	Cleavage Site	Inserted Sequence[&]
p24	**P2A**	GGSATNFSLLKQAGDVEENPG*PGGS
PRO	**E2a**	GGSQCTNYALLKLAGDVESNPG*PGGS
RT	**T2A**	GGSEGRGSLLTCGDVEENPG*PGGS
INT	**F2A**	GGSVKQTLNFDLLKLAGDVESNPG*PGGS

[&] The full picornavirus cleavage sequence from (Szymczak et al., 2004) is underlined and the intended cleavage site between G and P is marked with an asterisk.

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Figure Legends

Figure 1: Schematic of wild type and codon-optimized SIV gag-pol expression cassettes.

Figure 2: Western blot analysis of protein expression by SIV gag-pol fusion and SIV gag-pol fusion cleaver. A) 293 were transfected with plasmids expressing the indicated constructs and lysates were separated on SDS-PAGE gels prior to western blotting with anti-SIV gag p17 or anti-His6. **B)** 293 cells were infected with Ad5 vectors expressing the indicated constructs and western blotting was performed with anti-p17.

Figure 3: Immune responses generated by SIV gag-pol constructs. Groups of 5 BALB/c mice were immunized by the intramuscular route with 10^{10} vp of the indicated vectors or PBS once (prime) or twice (boost). **A)** Anti-SIV gag p24 ELISAs were performed. **B)** Anti-SIV gag IFN-g ELISPOTs were performed on splenocytes and responses are expressed as cytokine spot forming cells (SFCs) per million cells. One way ANOVA with Bonferroni multiple comparison post-test demonstrated **$p < 0.01$.