

Robust CD4⁺ and CD8⁺ T cell responses to SIV using mRNA-transfected DC expressing autologous viral Ag

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A potentially powerful strategy for therapeutic HIV vaccination is the use of DC transfected with mRNA encoding autologous viral Ag, as epitopes presented by transfected DC would exactly reflect those expressed by infected cells in the individual. Using human and rhesus macaque monocyte-derived DC, we show that nucleofection is a superior method for mRNA transfection, resulting in high-level protein expression and DC maturation. DC transfected with SIV *gag* isolated from an infected monkey stimulated robust Ag-specific recall T cell responses of similar magnitude to those induced by peptide-pulsed PBMC that were predominantly CD8⁺ T cell mediated. Enhanced CD4⁺ T cell responses were stimulated when Gag was redirected into the lysosomal pathway *via* the targeting signal derived from lysosome-associated membrane protein-1 (LAMP-1). Rhesus DC transfected with lysosome-targeted *gag* encoding an escape mutation in an immunodominant CTL epitope stimulated CD4⁺ and CD8⁺ T cell responses of almost equivalent magnitude directed towards undefined epitopes outside of the mutated region. Finally, *gag*-transfected DC from SIV-infected monkeys stimulated significant Ag-specific recall T cell responses in an entirely autologous system. These findings demonstrate that mRNA-transfected DC expressing SIV Ag derived from infected monkeys stimulate broad and relevant T cell responses, supporting this approach for therapeutic HIV vaccine development.

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Introduction

It is well appreciated that strong CD4⁺ and CD8⁺ T cell immunity is required for control of HIV infection [1–5], and therapeutic vaccines designed to promote broad cellular immune responses are being actively pursued [6]. However, an important challenge in the develop-

ment of vaccines for HIV is the diversity of clinical isolates [7] coupled with the propensity for the virus to undergo escape mutations in T cell epitopes through selective pressure *in vivo* [8]. Hence, CTL induced in response to the infecting strain may be incapable of controlling circulating virus as infection progresses [9–12]. To account for this complexity, a therapeutic vaccine should be tailored towards the autologous virus present in an individual at the time of immunotherapy.

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Abbreviation: **LAMP-1:** lysosome associated membrane protein-1

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DC are professional APC capable of Ag uptake, processing and presentation and are attractive candidates in the development of vaccines [13, 14]. *In vitro* studies have demonstrated that DC transduced *via* viral vectors or pulsed with inactivated viral particles are effective at stimulating HIV-specific CTL responses [15, 16]. Moreover, vaccination of rhesus macaques with DC expressing SIV or HIV Ag induces broad virus-specific cellular responses that promote protection against pathogenic virus challenge [17–21]. These prophylactic vaccine approaches have been based on well-characterized strains of virus that may not be suitable for therapeutic vaccination, as epitopes expressed by DC may not represent those in the infected individual. Efforts to include autologous viral Ag in therapeutic vaccines have relied on inactivated virus particles propagated from individual patients [22]; however, this procedure is labor-intensive and may not uniformly lead to sufficient virus recovery for DC loading in all patients.

An attractive alternative source of viral Ag for therapeutic DC-based vaccination is mRNA, an approach that has been developed in the cancer immunotherapy field [23, 24]. DC transfected with codon-optimized HIV mRNA have been shown to induce strong T cell immune responses *in vitro* [25, 26] with enhanced CD4⁺ T cell responses arising from DC expressing lysosome-targeted Ag [27]. DC transfected with autologous viral mRNA isolated from HIV-infected individuals induce significant virus-specific T cell responses [25]. The rhesus macaque SIV model provides an ideal preclinical setting to test the therapeutic potential of DC-based vaccines using virus-derived mRNA. Here, we evaluated the capacity of mRNA-transfected DC to stimulate T cell responses against SIV using an *in vitro* system. We adopted a new electroporation method to generate high level expression of *gag* mRNA isolated during infection, and assessed the efficacy of mRNA-transfected DC bearing the lysosomal-associated membrane protein-1 (LAMP-1) targeting signal to induce both Ag-specific CD8⁺ and CD4⁺ T cell responses. To provide a realistic test of the therapeutic potential of viral mRNA as an Ag source, we measured T cell immunity induced by mRNA-transfected DC expressing a virus escape mutation acquired during infection.

Results

Human monocyte-derived DC express high levels of Ag following nucleofection

Given the variety of approaches that have been devised for DC transfection, from lipid-based to electroporation-based methods, we first sought to optimize mRNA transfection of DC. In initial experiments, we used

human monocyte-derived DC transfected with *gfp* mRNA generated from the linearized plasmid pGEM4Z/GFP/A64 [28]. DC were propagated from purified monocytes [29] and cultured for 5 days at which time they were transfected by standard electroporation, TransFastTM lipofection, TransMessenger lipofection and nucleofection. Expression of GFP was measured 24 h later by flow cytometry. Transfection efficiency ranged from no to moderate expression of GFP following the standard methods of electroporation and lipofection consistent with earlier reports [30] (Fig. 1A). In contrast, 92% of DC expressed GFP following nucleofection with mRNA, similar to findings in murine macrophages [31] (Fig. 1A). GFP expression was detected as soon as 3 h and peaked at 24 h post nucleofection (Fig. 1B). At 48 h post nucleofection, 70% of human DC still expressed GFP, similar to previous findings [32].

Efficient mRNA transfection of DC from SIV-infected monkeys

We next evaluated the capacity of nucleofection to transfect rhesus macaque monocyte-derived DC. As for human DC, DC cultured from blood of SIV-naïve monkeys were readily transfected with mRNA, with 73% of cells expressing GFP protein within 24 h (Fig. 2A). Given that our goal is to use mRNA-transfected DC during SIV infection, we next determined whether

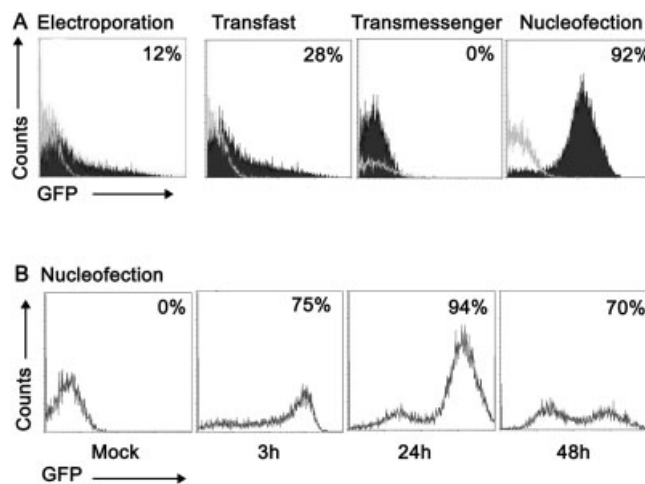


Figure 1. Efficient mRNA transfection of human monocyte-derived DC via nucleofection. (A) Immature human monocyte-derived DC were transfected with *gfp* mRNA (filled histogram) or mock transfected (empty histogram) using electroporation, TransFastTM lipofection, TransMessenger Transfection reagent or nucleofection, and GFP expression determined 24 h later. (B) Immature human monocyte-derived DC were nucleofected with *gfp* mRNA or mock-nucleofected and expression of GFP determined at 3, 24 and 48 h post nucleofection. Numbers represent the percent of DC transfected based on mock transfection.

SIV infection itself affects the capacity for DC to be transfected with mRNA. DC were propagated from pooled cryopreserved monocytes taken between 3 and 45 weeks post infection of a rhesus macaque with SIV/DeltaB670 (virus load ranged from 10^2 to 5×10^5 RNA copies/mL plasma over this time), and from monocytes collected at 27 weeks post infection from a macaque with neuroAIDS following SIVmac251 infection (virus load 1.2×10^6 RNA copies/mL plasma), and nucleofected with *gfp* mRNA as above. There was no evidence for reduction in mRNA transfection in either

sample, as evidenced by high level GFP expression (Fig. 2A). These data indicate that SIV infection does not impact the capacity for monocyte-derived DC to be transfected, even when cells are propagated from animals in the advanced stages of disease. We next evaluated the effect of diverting Ag into the lysosomal/endosomal pathway on protein expression in transfected DC. We first subcloned *gfp* from pGEM4Z/GFP/A64 into the pSP73/gp96ss/LAMP-1/A64 vector encoding the targeting signal of LAMP-1. SIV-naïve rhesus macaque monocyte-derived DC were then nucleofected with mRNA expressing GFP with and without LAMP-1 targeting signal and analyzed at 24 h post nucleofection. GFP expression was substantially decreased in DC transfected with endosome-targeted GFP as compared to controls, dropping from 88% to 15% (Fig. 2B). These data are consistent with a rapid processing of endogenously produced protein when it is directed into the endosome [33].

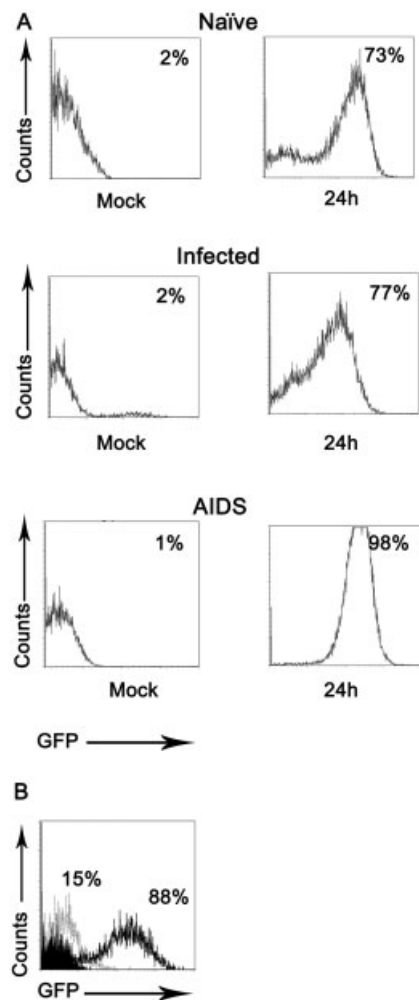


Figure 2. Efficient mRNA transfection of DC propagated from monkeys with and without SIV infection. (A) DC were propagated from an SIV-naïve macaque (top), from pooled monocytes taken from a macaque at 3–45 weeks post SIV infection (middle) or from monocytes taken 27 weeks post SIV infection from a monkey with neuroAIDS (bottom), and nucleofected with *gfp* mRNA or mock-nucleofected. Expression of GFP was determined 24 h later. (B) Monocyte-derived DC propagated from an SIV-naïve rhesus macaque were nucleofected with *gfp* mRNA (bold line), *gfp*-LAMP-1 mRNA (dotted line) or mock-nucleofected (filled histogram) and expression of GFP determined 24 h later. Numbers represent the percent of DC nucleofected based on mock transfection.

mRNA nucleofection induces phenotypic DC maturation

Transfection of immature human DC with mRNA *via* lipofection but not electroporation has been reported to induce maturation [26, 32, 34–36]. To test the effect of mRNA nucleofection on maturation of DC, we analyzed the phenotype of nucleofected monkey DC with and without stimulation with CD40L. Nucleofection of immature DC with *gfp* mRNA, but not mock nucleofection, led to expression of CD80 and CD83 and up-regulation of CD86 and CD40 indicative of maturation (Fig. 3). Expression of CD83 was moderately enhanced when mock- or mRNA-transfected DC were exposed to CD40L based on a more pronounced shift in CD83 expression (Fig. 3). These data indicate that mRNA transfection induces DC maturation, but that other exogenous stimuli known to induce maturation may provide additional benefits.

Efficient T cell stimulation using DC expressing wild-type *gag* mRNA

We next sought to determine whether wild-type, non codon-optimized SIV *gag* mRNA could be efficiently expressed in monkey DC, which is an important criterion for using autologous virus as a source of Ag. To test for expression of Gag protein in mRNA-transfected DC, we isolated PBMC from a rhesus macaque 2 weeks post infection with SIVmac239 and amplified *gag* from cellular DNA. Virus replication *in vivo* over this 2-week period had no impact on the sequence of the *gag* gene, as no mutations were detected when compared to the published SIVmac239 Gag sequence (data not shown). *gag* cDNA was subsequently cloned into pSP73-Sph/A64

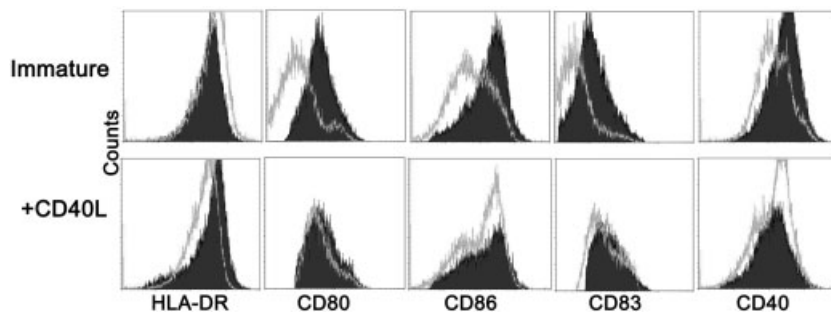


Figure 3. Nucleofection of monkey monocyte-derived DC results in phenotypic maturation. Day 5 immature monocyte-derived DC propagated from an SIV-naïve rhesus macaque were nucleofected with *gfp* mRNA (filled histogram) or mock-nucleofected (empty histogram), and then cultured without CD40L (upper row) or with the immediate addition of 3 $\mu\text{g}/\text{mL}$ CD40L (lower row). At 24 h post nucleofection, cells were stained with PE-labeled mAb as shown and analyzed by flow cytometry.

and mRNA generated for nucleofection of immature DC. At 24 h post nucleofection, Gag protein was readily detected in cell lysates as determined by Western blotting with SIV p17-specific mAb KK59 (Fig. 4A). Similarly, Gag protein could be detected in lysates following transfection of cells with *gag* mRNA linked to an influenza hemagglutinin (HA) tag and labeling with an HA-specific mAb (Fig. 4A).

To determine the capacity of mRNA-transfected monkey DC to stimulate Gag-specific T cell responses, we nucleofected SIVmac239 *gag* mRNA into DC propagated from a healthy monkey (R187) that had been immunized with a recombinant adenovirus-based vaccine [37]. This monkey expressed the MHC class I allele Mamu-A*01 and had a robust CTL response to the immunodominant SIV Gag epitope CM9_{181–189} [38] (data not shown). SIV *gag* mRNA-transfected DC stimulated a strong IFN- γ response from autologous PBMC that was Gag specific; negligible T cell reactivity was noted when PBMC were stimulated either with *gfp* mRNA-transfected or mock-transfected DC (Fig. 4B). This level of T cell reactivity was similar to that induced when PBMC were pulsed with peptides spanning the entire SIVmac239 Gag protein, the standard method for inducing virus-specific T cell responses [37] (Fig. 4B). DC pulsed with Gag peptides, but not a control panel of HA peptides, generated slightly more robust responses (Fig. 4B). We next addressed whether these recall responses were CD4⁺ or CD8⁺ T cell mediated and the effect of redirecting Gag to the lysosome on these responses. DC from R187 nucleofected with SIVmac239 *gag* mRNA induced Gag-specific T cell responses that were almost exclusively CD8⁺ T cell mediated based on cell depletion experiments (Fig. 4C). In contrast, DC nucleofected with *gag-LAMP-1* mRNA produced similarly strong CD8⁺ T cell mediated responses but greater CD4⁺ T cell responses to Gag (Fig. 4C). The enhanced T cell response noted with either *gag* or *gag-LAMP-1* transfection when CD4⁺ T cells were depleted is likely due to the concurrent enrichment of CD8⁺ T cells in

these conditions, as found in our previous studies [17, 37]. Together, these data demonstrate that DC nucleofected with wild-type SIV *gag* mRNA efficiently present Ag to virus-specific T cells and that redirecting Gag Ag to the lysosomal pathway enhances CD4⁺ T cell responses, consistent with previous reports [27, 39, 40].

T cell stimulation using DC expressing *gag* encoding a CTL escape mutation

The advantage of using autologous virus as a source of Ag in a therapeutic HIV vaccine is the capacity to present relevant epitopes to the immune system, particularly in the event of virus escape. To evaluate this capacity in our system, we next expressed *gag* isolated from a Mamu-A*01-expressing macaque M2201 that contained a threonine to isoleucine escape mutation at amino acid 182 within the immunodominant CM9 epitope 15 weeks after infection with the uncloned pathogenic isolate SIV/DeltaB670 [37]. To provide a relevant comparison to previous experiments, we continued to use DC and PBMC from the healthy Gag-vaccinated Mamu-A*01-expressing macaque R187. As expected, DC nucleofected with mRNA encoding SIVmac239 *gag* or *gag* cloned from SIV/DeltaB670 inoculum (B670 *gag*) stimulated robust Gag-specific recall T cell responses (Fig. 5A). In contrast, DC transfected with *gag* mRNA isolated from animal M2201 at 15 weeks post infection and encoding the CM9 epitope escape mutation [referred to as B670 *gag* (*T182I*)], induced substantially reduced T cell responses (Fig. 5A). *gfp* mRNA-transfected or mock-transfected DC stimulated background IFN- γ release from PBMC, as expected (Fig. 5A). To document that the T cell response to DC transfected with B670 *gag* (*T182I*) mRNA was directed to epitopes other than CM9, we generated shorter sequences of mRNA from the inoculum and escape viruses. DC transfected with B670 *gag*_{159–252} mRNA, encoding intact CM9 but no other known Mamu-A*01-restricted CTL epitopes, stimulated strong T cell responses, whereas DC trans-

fectured with B670 *gag*_{159–252}(T182I) mRNA encoding mutant CM9 induced no detectable response, similar to *gfp*- or mock-transfected DC (Fig. 5A). Similar results were obtained when DC were pulsed with wild-type

CTPYDINQM peptide as compared to peptide CIPYDINQM containing the threonine to isoleucine mutation at position 2 (data not shown), indicating that the difference in T cell response was not due to differential expression of the various mRNA constructs. These findings confirm that DC transfected with *gag* mRNA containing an escape mutation in the immunodominant

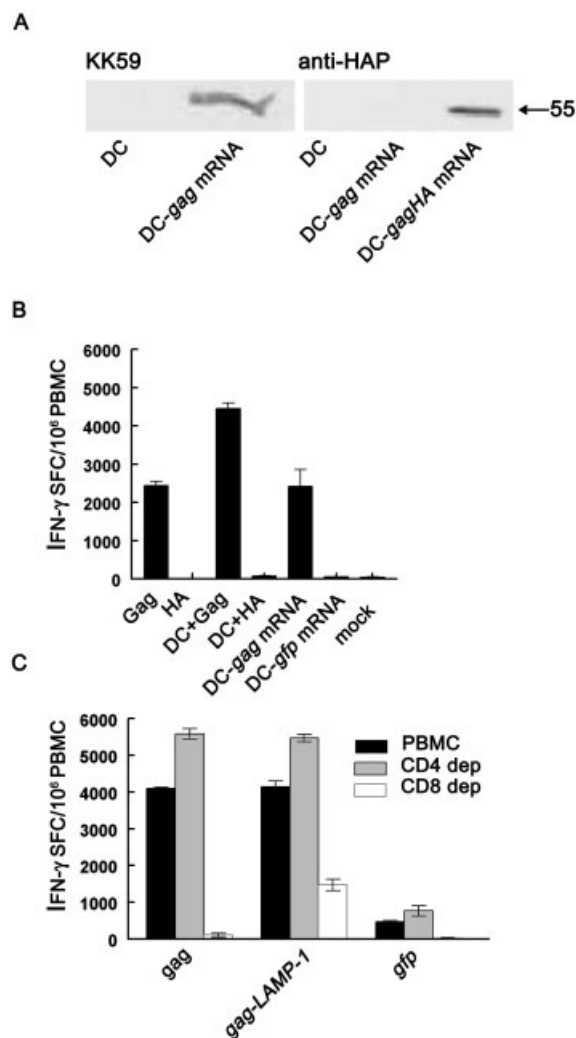


Figure 4. DC nucleofected with wild-type SIV *gag* mRNA stimulate Ag-specific T cells. (A) Left panel: Immature monkey monocyte-derived DC were nucleofected with SIV *gag* mRNA isolated from an infected monkey or mock-nucleofected and cell lysates probed with p17-specific mAb KK59 24 h later. Right panel: Immature monkey monocyte-derived DC were nucleofected with SIV *gag* mRNA, SIV *gag*-HA mRNA or mock-nucleofected and cell lysates probed with anti-HA mAb 24 h later. (B) PBMC from a healthy Gag-vaccinated Mamu-A*01-expressing rhesus macaque (R187) were pulsed with pools of Gag or HA peptides, or incubated with DC pulsed with Gag or HA peptides, or DC nucleofected with SIV mac239 *gag* mRNA, *gfp* mRNA or mock-nucleofected, and IFN- γ production detected 24 h later by ELISPOT. Shown are mean \pm SEM of triplicate determinations. (C) DC from R187 were nucleofected with mRNA encoding SIVmac239 *gag*, *gag*-LAMP-1 or *gfp* and incubated with autologous PBMC with or without Ab-mediated depletion of CD4⁺ or CD8⁺ T cells in an ELISPOT assay. Shown are mean \pm SEM of triplicate determinations. SFC: spot-forming cells, dep: depleted.

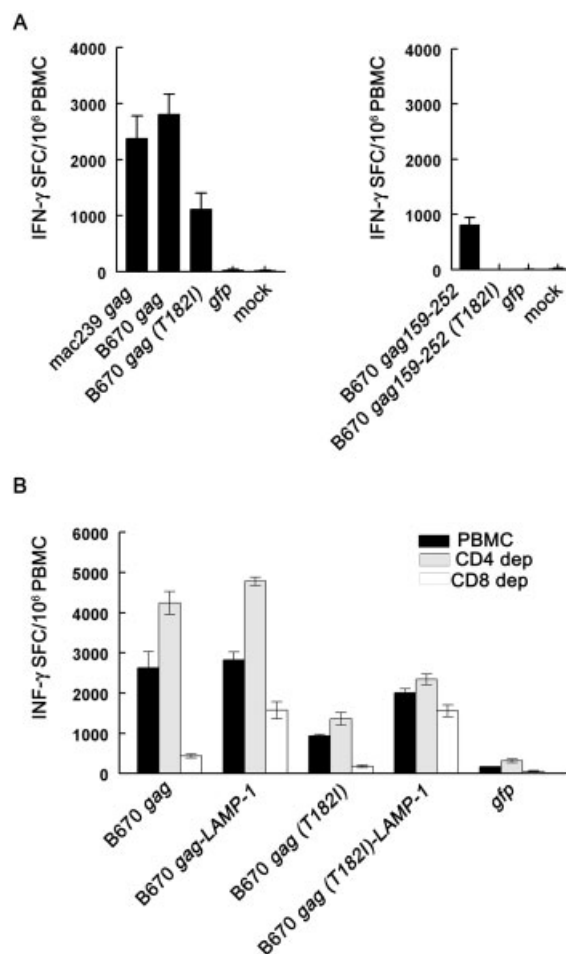


Figure 5. DC transfected with SIV *gag* or *gag*-LAMP-1 mRNA encoding a mutated immunodominant CTL epitope induce T cell responses to undefined subdominant epitopes mediated by both CD4⁺ and CD8⁺ T cells. (A) Left: DC from a healthy Gag-vaccinated Mamu-A*01-expressing rhesus macaque (R187) were nucleofected with mRNA encoding SIVmac239 *gag*, SIV/DeltaB670 *gag* (B670 *gag*), SIV/DeltaB670 *gag* with T182I mutation [B670 *gag* (T182I)], or *gfp* or mock-nucleofected and used in an ELISPOT assay with autologous PBMC. IFN- γ -producing cells were enumerated 24 h later. Right: DC from R187 were nucleofected with mRNA encoding SIV/DeltaB670 *gag*_{159–252} (B670 *gag*_{159–252}), SIV/DeltaB670 *gag*_{159–252} with T182I mutation [B670 *gag*_{159–252} (T182I)], or *gfp* or mock-nucleofected and used in an ELISPOT assay as above. (B) DC from R187 were nucleofected with B670 *gag*, B670 *gag*-LAMP-1, B670 *gag* (T182I), B670 *gag*(T182I)-LAMP-1 or *gfp* mRNA and used with autologous PBMC with and without Ab-mediated depletion of CD4⁺ or CD8⁺ T cells in an ELISPOT assay as above. Shown are mean \pm SEM of triplicate determinations. SFC: spot-forming cells, dep: depleted.

CM9 epitope presented intact subdominant epitopes for stimulation of T cells.

We next determined the impact of lysosome targeting of Gag protein on the resulting T cell responses to mutant Gag. DC from R187 nucleofected with B670 *gag* but not *gfp* induced Gag-specific T cell responses that were primarily CD8⁺ T cell mediated, as expected. In contrast, both CD8⁺ and CD4⁺-mediated T cell responses were induced by DC nucleofected with B670 *gag-LAMP-1* mRNA (Fig. 5B). The effect of lysosome targeting on the CD4⁺ T cell response was even greater when *gag* mRNA encoding the mutated CM9 epitope was expressed as a lysosomal protein via B670 *gag (T182I)-LAMP-1* mRNA, with almost equivalent CD4⁺ and CD8⁺ T cell responses being generated (Fig. 5B).

DC expressing autologous *gag* mRNA stimulate Ag-specific T cell responses

We next tested the ability of mRNA-transfected DC to stimulate virus-specific T cell responses in an entirely autologous system. We first determined the ease with which *gag* could be amplified from blood of SIV-infected monkeys. We were able to recover *gag* from cell-free plasma from 11/11 SIV-infected monkeys with virus loads as low as 10² RNA copies/mL plasma (data not shown), indicating that recovery of *gag* from infected plasma is remarkably efficient. DC were subsequently propagated from 3 macaques infected with SIV/DeltaB670 (M2201, M2301, and M7801) and 2 macaques infected with SIVmac251 (R484, R486). Plasma harvested at week 15 post infection from the first group and week 4 post infection from the second group was used to amplify the predominant *gag* sequence from viruses present in each animal. *gag* was then subcloned into the pSP73-Sph/A64 vector for mRNA generation and DC transfection. mRNA- or mock-transfected DC were then incubated with autologous PBMC from each animal in an ELISPOT assay. DC transfected with autologous *gag* mRNA induced greater T cell responses than DC transfected with *gfp* mRNA in each animal (Fig. 6A), and this difference was statistically significant when compared across all animals combined ($p=0.043$; Fig. 6B). Notably, in animals M7801 and M2201 that provided sufficient DC for additional control conditions, *gfp*- and mock-transfected DC stimulated similar background levels of IFN- γ release from autologous PMBC (Fig. 6A). This finding confirms that mRNA transfection in itself did not negatively or positively impact the capacity of DC to stimulate T cells. The magnitude of individual responses to Gag was not associated with virus load (data not shown). These data confirm that DC transfected with mRNA encoding Gag Ag isolated during infection can stimulate significant autologous T cell responses.

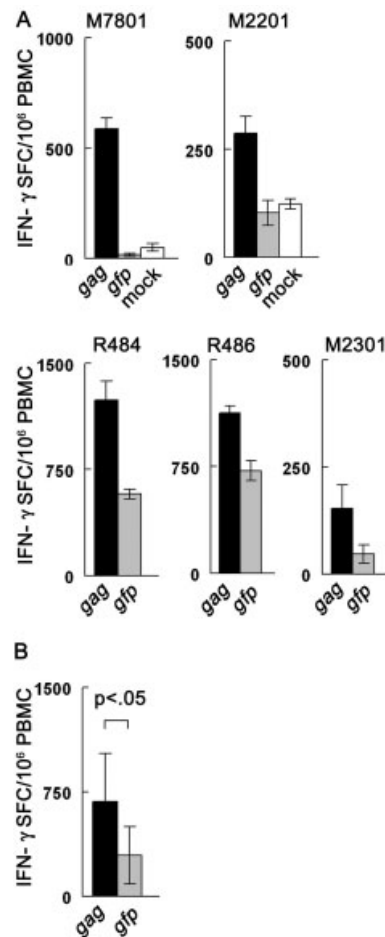


Figure 6. DC nucleofected with *gag* mRNA isolated from infected monkeys induce significant Gag-specific T cell responses from autologous PBMC. (A) Immature DC propagated from SIV-infected rhesus macaques were nucleofected with mRNA encoding autologous *gag* or *gfp* (five animals) or mock nucleofected (two animals) and used as stimulators of autologous PBMC in an IFN- γ ELISPOT assay. Shown are mean \pm SEM of triplicate determinations. (B) Comparison of IFN- γ release from autologous PBMC in response to *gag*- or *gfp*-transfected DC for all five animals combined. Shown are mean \pm SEM. SFC: spot-forming cells.

Discussion

The need for a therapeutic vaccine for HIV is apparent given the inability of antiretroviral therapy to eradicate virus in infected individuals [41, 42]. However, virus diversity coupled with emergence of escape mutants during the course of infection in infected individuals suggests that the source of Ag in such vaccines is critical [8, 43, 44]. Here we complement recent *in vitro* studies in humans [25, 27] by showing that monkey monocyte-derived DC expressing viral mRNA isolated during SIV infection stimulate strong and broad virus-specific T cell responses even in the face of virus escape, with an enhancement of the CD4⁺ T cell response seen when Ag is targeted to the lysosome.

We found that nucleofection was a superior method to introduce mRNA into monkey and human monocyte-derived DC, with electroporation and lipofection providing relatively poor levels of Ag expression. Nucleofection has been used for DNA transfection of human DC and cytokine-induced killer cells [45, 46] as well as mRNA transfection into primary rat neurons and murine macrophages cell lines [31, 47]. Other reports have indicated that electroporation is relatively efficient at transfecting human DC with mRNA, although results have been somewhat variable [25, 27, 32, 48]. mRNA transfection of immature monocyte-derived DC by nucleofection induced significant maturation as evidenced by expression of CD83 and CD80 and up-regulation of CD40 and CD86. Similarly, DC transfected with mRNA *via* lipofection or simple pulsing undergo phenotypic maturation [26, 34–36]. DC maturation is thought to be mediated by mRNA binding to endosomal TLR3 by regions of double-stranded secondary structures [35, 36]. In contrast, mRNA transfection of human monocyte-derived DC *via* electroporation does not appear to induce maturation [32], although the reason for this discrepancy is unclear. Despite the maturation induced through mRNA nucleofection, we found a modest enhancement of expression of CD83 following ligation of CD40, suggesting that maturation *via* mRNA transfection can be augmented by other stimuli. It is likely that agonists of other TLR such as TLR8 may synergize with mRNA to activate DC, as combined ligation of TLR3 and TLR8 appears to produce optimal maturation of myeloid DC [49].

We found that recovery of virus cDNA for mRNA generation is an efficient process even when samples are isolated from monkeys with very low virus loads, indicating that this approach may be preferable to cell culture techniques for the generation of autologous virus Ag [22]. However, the use of autologous virus Ag in itself is technically demanding, requiring the recovery of virus sequences from each individual. Nevertheless, autologous virus Ag does offer several potential advantages for immunotherapy over alternatives based on artificial sequences encoding a centralized (consensus or ancestral) gene, which have been put forward as a vaccine strategy to address HIV diversity [7]. Firstly, using DC transfected with autologous virus Ag expressing mutated immunodominant CTL epitopes leads to stimulation of CTL specific for subdominant epitopes, as we have shown, and such epitopes are known to be important in virus control [50]. While conserved subdominant epitopes would also be expressed by centralized sequences, using the latter in therapeutic vaccines in the face of immune escape could have a negative effect, as CTL specific for immunodominant epitopes present in the consensus sequence will be preferentially expanded [51]. This may occur even as

long as 4 weeks following complete replacement of circulating virus with escape mutants [37]. Secondly, *de novo* generation of novel CTL epitopes can arise from mutated immunodominant epitopes [52], and these novel epitopes would not be presented by DC transfected with centralized sequences. The value of reagents based on autologous virus over consensus sequences in stimulating enhanced CTL responses to variable regions of HIV has been demonstrated *in vitro* using comprehensive panels of overlapping peptides [53]. Whether autologous virus Ag provides a clinical advantage over centralized Ag awaits comparative DC-based immunotherapy studies in HIV-infected individuals.

It is important in therapeutic vaccination for HIV that virus-specific CD4⁺ T cell responses be expanded, as these are critical in maintenance of CTL [5, 54, 55]. Our results indicate that lysosome targeting of protein leads to reduced expression of the intact protein in transfected cells, but enhanced stimulation of virus-specific CD4⁺ T cells, consistent with previous findings [27]. The effect of targeting viral Ag to the lysosome on the relative magnitude of the CD4⁺ T cell responses was amplified with the loss of an immunodominant CTL epitope naturally acquired during SIV infection. Overall, these data support the use of lysosome-targeted autologous viral Ag in DC-based therapeutic vaccination for HIV.

Materials and methods

Plasmid and mRNA generation

gfp was subcloned from pGEM4Z/GFP/A64 [28] into pSP73-Sph/A64 [56, 57] using BamHI to generate pSP73/GFP/A64. Similarly, *gfp* was subcloned into pSP73/gp96ss/LAMP-1/A64 using SfiI to generate pSP73/GFP-LAMP-1/A64. For the initial generation of Gag-encoding plasmids, PBMC were isolated from a rhesus macaque 2 weeks post infection with SIVmac239 and cellular DNA extracted using DNeasy tissue kit (Qiagen, Chatsworth, CA). For all other Gag-expressing plasmids, cell-free plasma was isolated from monkeys infected with SIVmac251 or SIV/DeltaB670 and viral RNA isolated using QIAMP viral RNA kit (Qiagen). Samples from 12 infected monkeys with virus loads ranging from 10² to 5.5 × 10⁶ RNA copies/mL plasma were used over the course of the study. Approval was obtained from the institutional animal use and care committee for all experiments involving rhesus macaque samples. The *gag* gene from each virus was amplified by PCR using gene-specific primers and subsequently subcloned into the pSP73 vector to generate pSP73/mac239Gag/A64, pSP73/mac251Gag/A64, pSP73/B670Gag(T182I)/A64 and pSP73/B670Gag/A64, depending on the particular virus isolate. Influenza HA was tagged to SIVmac239 Gag to generate pSP73/Gag-HA/A64. *gag* from SIVmac239, SIV/DeltaB670 inoculum and SIV/DeltaB670 Gag (T182I) were additionally cloned into the pSP73-LAMP-1 vector to generate pSP73/GAG-LAMP-1/A64, pSP73/B670GAG-LAMP-1/A64 and pSP73/

B670GAG (T182I)-LAMP-1/A64, respectively. *gag* sequences encoding amino acids 159–252 from SIV/DeltaB670 encompassing either wild-type or mutated CM9 sequences were subcloned into pSP73 as above to generate pSP73/B670Gag159–252/A64 and pSP73/B670Gag159–252(-T182I)/A64, respectively. For generation of mRNA, pSP73/GFP/A64 was linearized with SpeI, whereas all Gag-expressing plasmids were linearized using NotI. *In vitro* transcription was done with T7 polymerase using the mMessage Machine kit (Ambion, Austin, TX). Purification of *in vitro* transcribed mRNA was performed by DNase I digestion followed by LiCl precipitation and a 70% ethanol wash. The quality of mRNA was checked by agarose formaldehyde gel electrophoresis. mRNA concentration was assayed by spectrophotometry (GeneQuant pro, Amersham Pharmacia Biotech, Little Chalfont, UK) at OD₂₆₀.

Propagation and transfection of DC

Approval was obtained from the institutional review board prior to experiments involving human samples. DC were cultured from purified blood monocytes of SIV-naïve or SIV-infected rhesus macaques or healthy human volunteers using 1000 U/mL GM-CSF (Berlex Laboratories, Inc., Richmond, CA) and 1000 U/mL IL-4 (Schering-Plough, Kenilworth, NJ) as described [29]. DC were transfected at day 5 of culture. For transfection using the TransFast™ Transfection reagent (Promega, Madison, WI) or TransMessenger reagent (Qiagen, Valencia, CA), 4 µg GFP mRNA was used with Opti-MEM (Gibco Invitrogen Corporation, Frederick, MD) and other reagents provided by the manufacturers and 10⁶ DC transfected according to each manufacturer's instructions. For electroporation, 2 × 10⁶ DC in Opti-MEM were mixed with 10 µg GFP mRNA and electroporated in a 0.4-cm cuvette using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA) at a voltage of 300 V and a capacitance of 150 µF. For nucleofection using Amaxa nucleofector (Amaxa, Köln, Germany), 10–20 µg mRNA was added to 1 × 10⁶–2 × 10⁶ DC in Opti-MEM and transfected in a 2-mm-wide electroporation cuvette (BTX, San Diego, CA) using the U-02 program. Smaller amounts of mRNA were used for lipofection as compared to electroporation and nucleofection as toxicity was noted at higher amounts with the former methods (data not shown). Following transfection with the various methods, cells were cultured in fresh RPMI with 10% FCS, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin-streptomycin and 10 mM HEPES buffer (complete media) supplemented with GM-CSF and IL-4 for 24 h. In some experiments DC were matured for 24 h immediately following transfection with 3 µg/mL recombinant trimeric CD40L (Immunex, Seattle, WA) as described [29].

Flow cytometric analysis

GFP expression was measured in transfected DC by flow cytometry at various intervals after transfection using a Beckman Coulter cytometer (Miami, FL). For immunophenotyping of DC, cross-reactive mAb to human HLA-DR, CD80, CD83, CD86 (all from BD Pharmingen, San Diego, CA) and CD40 (Ancell, Bayport, MN) were used as described [29].

Detection of Gag expression

At 24 h post transfection with *gag* or *gag-HA* mRNA, monkey DC were lysed and lysates were separated by gel electrophoresis and transferred to nitrocellulose membrane or polyvinylidene difluoride membrane (Bio-Rad), respectively. Gag p17 was detected by probing cell lysates with p17-specific mAb KK59 followed by HRP-conjugated anti-mouse IgG (Bio-Rad). Gag-HA was detected by mAb anti-HA-peroxidase (12CA5) (Roche Applied Science, Indianapolis, IN). Development was performed using the Immun-Star™ HRP Substrate kit (Bio-Rad).

ELISPOT assay

Previously frozen PBMC were pulsed with pools of 15-mer peptides spanning the entire sequence of SIVmac239 Gag protein or influenza HA (Sigma Genosys, Woodlands, TX) and IFN-γ production detected 24 h later by ELISPOT assay as described [37]. For assays using peptide-pulsed DC, monkey DC were matured with CD40L for 24 h as above and incubated with Gag or HA peptides (5 µg/mL) for 1 h at 37°C. Cells were washed and incubated with PBMC at a 1:10 ratio in the ELISPOT assay. For experiments using mRNA transfected DC, DC were transfected with *gag* or *gfp* mRNA or mock transfected (nucleofected in the absence of mRNA) and matured with CD40L for 24 h prior to incubation with PBMC. Where indicated, transfected DC were incubated with PBMC depleted of CD4⁺ T cells (CD4 microbeads, Miltenyi Biotec, Auburn, CA) or CD8⁺ T cells (CD8 microbeads, Miltenyi Biotec), as described [17]. Depletions were >90% efficient for each T cell subset [17]. The same total number of cells was added to wells irrespective of depletion conditions.

Statistics

We used non-parametric tests (Wilcoxon) to test the difference in median between autologous Gag-specific T cell responses and GFP-induced T cell responses.

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