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High-Level Antigen Expression and Sustained Antigen Presentation in Dendritic Cells Nucleofected with Wild-Type Viral mRNA but Not DNA▽

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Dendritic cells (DC) are potent antigen-presenting cells that hold promise as cell-based therapeutic vaccines for infectious diseases and cancer. Ideally, DC would be engineered to express autologous viral or tumor antigens to ensure the presentation of relevant antigens to host T cells in vivo; however, expression of wild-type viral genes in primary cell lines can be problematic. Nucleofection is an effective means of delivering transgenes to primary cell lines, but its use in transfecting DNA or mRNA into DC has not been widely investigated. We show that nucleofection is a superior means of transfecting human and monkey monocyte-derived DC with DNA and mRNA compared to lipofection and conventional electroporation. However, the delivery of DNA and mRNA had significantly different outcomes in transfected DC. DC nucleofected with DNA encoding green fluorescent protein (GFP) had poor antigen expression and viability and were refractory to maturation with CD40 ligand. In contrast, >90% of DC expressed uniform and high levels of GFP from 3 h to 96 h postnucleofection with mRNA while maintaining a normal maturation response to CD40 ligation. Monkey DC nucleofected with wild-type, non-codon-optimized mRNA encoding simian immunodeficiency virus Gag stimulated robust antigen-specific effector T-cell responses at 24 h and 48 h postnucleofection, reflecting sustained antigen presentation in transfected DC, whereas no detectable T-cell response was noted when DC were nucleofected with DNA encoding the same Gag sequence. These data indicate that mRNA nucleofection may be an optimal means of transfecting DC with autologous tumor or viral antigen for DC-based immunotherapy.

Monocyte-derived dendritic cells (DC) are currently being used as therapeutic vaccines for cancer and infectious diseases, and approaches to express tumor-associated or viral antigens in DC are being widely sought (3, 16, 32, 38, 39). Viral vectors based on adenovirus, poxviruses, and lentivirus induce high-level antigen expression in DC but may be associated with safety concerns and generally require DNA codon optimization to overcome poor gene expression (11, 12, 14, 23, 35, 49). An attractive alternative to vector-mediated delivery of antigens into DC is nonviral gene transfer based on DNA or mRNA. DNA transfection of DC has been used successfully in some studies but is limited by poor expression levels and toxicity (2, 29, 41, 45, 49), although methods to enhance expression based on DC maturation have been proposed (28). mRNA transfection of DC is being increasingly utilized for cancer immunotherapy and in vitro stimulation of virus-specific T cells, but approaches to deliver mRNA into primary DC cultures have been limited (16, 18, 25, 39, 40, 43, 48, 50). Transfection of DC with mRNA offers the potential for high-level expression of wild-type viral transgenes that have poor expression in mammalian cells when traditional methods of gene delivery are used (8, 26). This is advantageous since expression of autologous viral or tumor genes in a patient-specific, DC-based vaccine would ensure stimulation of relevant T-cell responses in vivo (42, 48).

Recently, nucleofection has emerged as a superior method for the delivery of transgene to primary cell lines, including cytokine-induced killer cells, neurons, keratinocytes, macrophages, and DC (9, 15, 21, 27, 29, 33, 37, 47). Nucleofection has been used primarily as a method of introducing DNA into cells as it is reported to deliver DNA directly into the nucleus, enhancing gene expression (17). However, recent studies suggest that nucleofection of DC with mRNA is an effective means of inducing high-level antigen expression (27, 33). While conventional methods of gene delivery have been compared using monocyte-derived DC (41, 51), a limited number of studies have employed the more-efficient process of nucleofection to evaluate DNA and mRNA delivery into these cells (27), and none to our knowledge have compared the capacity for mRNA- and DNA-nucleofected DC to stimulate antigen-specific T-cell responses.

In the present study, we did a comprehensive analysis of DNA and mRNA transfection of human and monkey monocyte-derived DC, comparing liposomal transfection methods and conventional electroporation with nucleofection. We confirm that nucleofection is a superior method for the delivery of both DNA and mRNA into primary DC lines, although the outcome of nucleofection differed substantially depending on the source of genetic material. DC nucleofected with mRNA encoding the marker gene green fluorescent protein (GFP) had rapid and sustained antigen expression with limited toxicity and were responsive to maturation stimuli, whereas DC nucleofected with GFP DNA had limited antigen expression and poor viability and were refractory to maturation with CD40 ligand (CD40L). Notably, monkey DC nucleofected with

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wild-type simian immunodeficiency virus (SIV) *gag* mRNA induced a robust virus-specific T-cell response in cells isolated from immune monkeys, whereas DC nucleofected with viral DNA were unable to stimulate detectable T-cell responses.

MATERIALS AND METHODS

Plasmid and mRNA generation. Generation of the pSP73/GFP/A64 and pSP73/SIVmac239Gag/A64 plasmids and in vitro transcription of mRNA were performed as described previously (33). pSP73/SIVmac239Gag/A64 encodes a wild-type, non-codon-optimized Gag sequence isolated from a rhesus macaque 2 weeks postinfection with the SIVmac239 SIV isolate (33). pSP73/SIVmac239Gag/A64 was used as a template for the amplification of Gag by PCR using the following forward and reverse primers, respectively: pGagN1F, 5′GCGCTCGAGGCCAC CATGGGCGTGAG3′; and pGagN1R, 5′CGCGCGGCCGCTTACTTGCCCA ACTGCATGTAG 3′. pEGFP-N1 DNA was digested with XhoI and NotI, and the larger vector band was retrieved by gel purification. XhoI- and NotI-digested *gag* PCR product was inserted to the XhoI- and NotI-digested pEGFP-N1 DNA to generate pSIVmac239Gag-N1 encoding the wild-type sequence.

Cells. DC were cultured from the purified blood monocytes of SIV-naïve rhesus macaques or healthy human volunteers as described previously (6). In some experiments, DC were matured for 24 h or 48 h with 3 μg/ml recombinant trimeric CD40L (Immunex, Seattle, WA) as described previously (6). Approval was obtained from the institutional review board prior to experiments involving human samples and from the institutional animal use and care committee for all experiments involving rhesus macaque samples. K562 cells were grown in Iscove's modified Dulbecco's medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum.

Transfection of K562 cells and DC. K562 cells were washed twice with Opti-MEM (Gibco Invitrogen Corporation, Frederick, MD) and transfected with TransFast transfection reagent (Promega, Madison, WI) or TransMessenger reagent (Qiagen, Valencia, CA) by adding 2 or 4 μg GFP mRNA or DNA in Opti-MEM with other reagents provided by the manufacturers to 1×10^6 cells as described previously (33). For electroporation, 1×10^6 K562 cells were electroporated with 10 μg mRNA or DNA in a total volume of 250 μl in a 0.4-cm cuvette using the Gene Pulser II electroporation system (Bio-Rad, Hercules, CA) with voltage/capacitance settings of 260 V/150 μF or 300 V/150 μF, respectively (50). Immediately after electroporation, cells were incubated for 5 min on ice. For nucleofection, 10 μg of DNA or mRNA was added to 1×10^6 K562 cells in Opti-MEM in a final volume of 100 μl and transfected in a 2-mm-wide electroporation cuvette (BTX, San Diego, CA) using the T-16 program of the amaxa nucleofector (Amaxa, Koln, Germany). Smaller amounts of mRNA and DNA were used for lipofection than for electroporation and nucleofection as toxicity was noted at higher amounts with the former methods (data not shown). K562 cells were cultured in Iscove's modified Dulbecco's medium for 24 h at 37°C following transfection. DC were transfected as immature cells (day 5 of culture) or as mature cells after a 24-h incubation with CD40L (day 6 of culture). Lipofection using TransFast or TransMessenger reagents was done as for K562 cells. For electroporation, 1×10^6 to 2×10^6 DC in Opti-MEM were mixed with 5 μg to 20 μg mRNA or DNA, depending on the experiment, and electroporated as for K562 cells using voltage/capacitance settings of 300 V/150 μF and 250 V/300 μF, respectively, as described previously (33, 41). Nucleofection of DC was done as described for K562 cells using 5 μg to 20 μg mRNA or DNA and the U-02 or T-01 Amaxa program. Details relating to the electrical parameters of electroporation using the Amaxa nucleofector are proprietary. Nucleofection was performed according to the manufacturer's instructions, with some modifications. In preliminary studies, we determined that nucleofection of cells in Opti-MEM and buffers provided by the manufacturer resulted in similar efficiencies of transfection (data not shown), and we therefore used Opti-MEM for all transfection experiments. Following transfection, DC were cultured in prewarmed complete RPMI medium supplemented with granulocyte-macrophage colony-stimulating factor and interleukin-4 for 24 h or 48 h at 37°C, with and without CD40L, as described previously (33).

Confocal microscopy. DC nucleofected with pEGFP-N1 DNA or *gfp* mRNA 24 h previously were harvested using 20 mM EDTA and resuspended in phosphate-buffered saline prior to being settled onto glass slides at 37°C for 1 h. Adhered cells were fixed with 2% paraformaldehyde for 15 min and washed in phosphate-buffered saline. Gelvatol was used to apply coverslips to slides. DC were imaged for GFP and differential interference contrast using an Olympus FluoView 500 laser scanning confocal microscope (Olympus, Center Valley, PA). Images were collected using MetaMorph software (Molecular Devices, Sunnyvale, CA).

Flow cytometric analysis. GFP expression in transfected K562 cells and DC and expression of HLA-DR, CD80, CD83, CD86, and CD40 on transfected DC were done as described previously (33).

ELISPOT assay. Gag-specific gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assays were done as described previously (33). Briefly, immature monkey DC were nucleofected with 10 μg pSIVmac239Gag-N1 DNA or with *gag* mRNA transcribed from pSP73/SIVmac239Gag/A64 and simultaneously matured with CD40L (3 μg/ml) for 24 h or 48 h prior to incubation with autologous peripheral blood mononuclear cells (PBMC) at a 1:10 ratio. Control cells were nucleofected with pEGFP-N1 DNA or *gfp* mRNA transcribed from pSP73/GFP/A64 or mock transfected. IFN-γ spot-forming cells were developed and enumerated as described previously (4).

RESULTS

Nucleofection with mRNA is a superior method of introducing transgene into primary human and monkey monocyte-derived DC. We performed a side-by-side comparison of TransFast and TransMessenger lipofection methods along with electroporation and nucleofection using a GFP reporter construct. Cells were transfected with either pEGFP-N1 DNA or in vitro-transcribed *gfp* mRNA that was generated from the plasmid pSP73/GFP/A64 (33) and monitored for GFP expression at 24 h by flow cytometry. For lipofection methods, we used 4 μg DNA or mRNA as toxicity was noted at higher amounts (not shown), whereas for electroporation and nucleofection we used 10 μg DNA or mRNA. In initial experiments, we used the chronic myelogenous leukemic cell line K562 that is readily transfected with plasmid DNA or mRNA (7, 50). Transfection of K562 cells with EGFP-N1 DNA using the two lipofection methods produced no detectable GFP fluorescence, whereas transfection via electroporation resulted in 16% GFP-expressing cells at 24 h. In contrast, 60% of K562 cells expressed GFP following nucleofection with EGFP-N1 DNA using the T-16 program of the nucleofector device, which was shown in preliminary experiments to generate the highest efficiency of transfection with this cell line (Fig. 1a and data not shown). Transfection with mRNA was significantly more effective than transfection with DNA by all methods, with 8% to 16% GFP expression following lipofection and 86% and 91% of K562 cells expressing GFP following electroporation and nucleofection, respectively (Fig. 1a).

We next tested the capacities of lipofection, electroporation, and nucleofection to transfect human and monkey immature monocyte-derived DC. In addition to measuring GFP expression at 24 h posttransfection, we assessed the effect of transfection on DC viability by using the standard approach of trypan blue exclusion. Transfection of DC with DNA using lipofection or electroporation produced negligible GFP expression and significant cell death, with viability ranging from 40% to 57% (Fig. 1b and c) (Table 1). Nucleofection with DNA using the U-02 program was more effective at generating GFP-expressing DC than the other methods but was associated with enhanced cell death, with only 24% of DC remaining viable at 24 h posttransfection (Fig. 1b and c) (Table 1), similar to other reports (29). Switching the nucleofector program to T-01 as favored by others (29) did not enhance transfection efficiency, and increasing the quantity of DNA to 20 μg was associated with a significant increase in toxicity (data not shown). Lipofection with mRNA resulted in variable transfection of DC, with maximum expression noted in monkey DC following TransMessenger lipofection, although the propor-

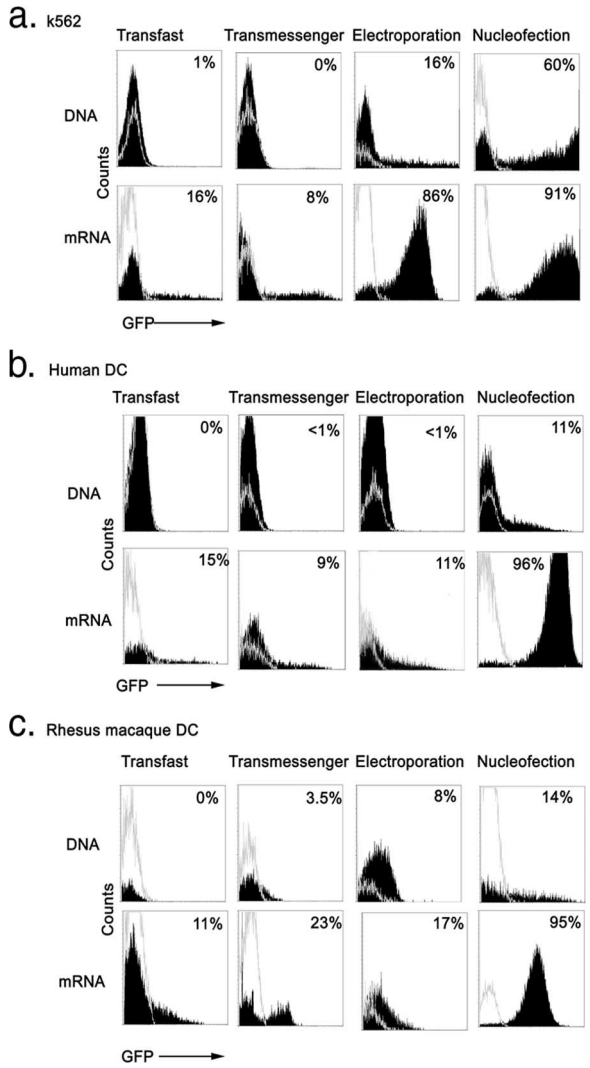

FIG. 1. Evaluation of DNA and mRNA transfection of K562 cells and human and monkey DC. K562 cells (a) or immature human (b) or rhesus macaque (c) monocyte-derived DC were transfected with pEGFP-N1 DNA (top row) (filled histograms) or *gfp* mRNA (bottom row) (filled histograms) or were mock transfected (empty histograms) using the methods indicated and analyzed by flow cytometry for GFP expression 24 h later. A total of 4 μg DNA or mRNA was used for lipofection with TransFast and TransMessenger reagents, whereas 10 μg DNA or mRNA was used for electroporation and nucleofection. Numbers represent the percentage of cells expressing GFP based on mock transfection.

tion of viable cells was only 52% to 55% (Fig. 1b and c) (Table 1). In contrast to K562 cells, electroporation of DC with mRNA was inefficient, with expression levels not exceeding 17% and viability averaging only 46% (Fig. 1b and c) (Table 1). However, nucleofection with mRNA using the U-02 program was a highly effective means of transfecting human and monkey monocyte-derived DC, with the proportion of DC expressing GFP reaching 96% at 24 h posttransfection (Fig. 1b and c) (Table 1). Nucleofection with mRNA maintained the highest cell viability of all methods, although the proportion of viable cells at 24 h posttransfection was still only 68% (Table 1), consistent with results of other reports (27). Similar transfection efficiency and DC viability were noted when nucleofection was performed with a range of mRNA from 5 μg to 20 μg (data not shown).

TABLE 1. Gene expression and cell viability following transfection of immature monkey DC

Method	DNA[a]		RNA[b]	
	GFP expression[c]	Viability[d]	GFP expression	Viability
TransFast[e]	0	57 (3)	7 (3)	52 (4)
TransMessenger[e]	2 (1)	49 (1)	14 (8)	55 (4)
Electroporation[f]	4 (7)	40 (2)	9 (7)	46 (3)
Nucleofection[g]	10 (4)	24 (5)	91 (3)	68 (6)

[a] EGFP-N1 DNA.
[b] In vitro-transcribed *gfp* mRNA.
[c] Percentage of cells expressing GFP at 24 h posttransfection. Mean (SEM) of two to five experiments.
[d] Percentage of viable cells at 24 h posttransfection. Mean (SEM) of two to five experiments.
[e] A total of 4 μg DNA or mRNA was used with both lipofection methods.
[f] Cells were electroporated with 10 μg DNA or mRNA using 250 V/300 μF or 300 V/150 μF settings, respectively.
[g] Cells were nucleofected with 10 μg DNA or mRNA using the U-02 program.

Flow cytometric analysis of nucleofected cells indicated that GFP expression varied in intensity and uniformity depending on whether DNA or mRNA was used, with DNA producing a wide range of fluorescence and mRNA generating uniform expression in almost all cells (Fig. 1b and c). To evaluate this in more detail, we examined immature monkey DC by confocal microscopy 24 h after DNA and mRNA nucleofection. The majority of mRNA-transfected DC showed a similar intensity of GFP expression when examined individually by microscopy, whereas GFP expression in DNA-transfected DC was highly variable, with some cells having intense fluorescence and a majority having weak or undetectable fluorescence (Fig. 2). Expression of GFP in DNA- or mRNA-transfected DC was cytoplasmic in distribution, as expected (Fig. 2). Similar results were found using human monocyte-derived DC (data not

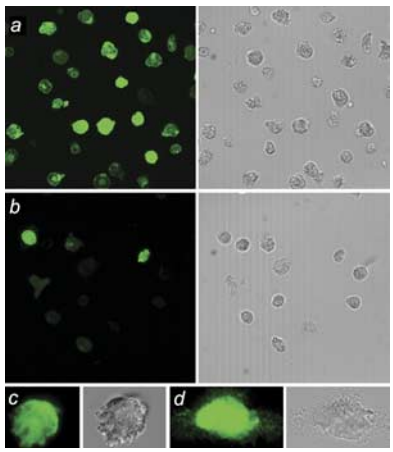

FIG. 2. Expression of GFP in mRNA- and DNA-nucleofected DC. Immature monkey monocyte-derived DC were nucleofected with *gfp* mRNA (a and c) or pEGFP-N1 DNA (b and d) and examined by confocal microscopy 24 h later. Shown are GFP expression (left) and differential interference contrast (right). (a and b) Original magnification, ×200. (c and d) Original magnification, ×1,500.

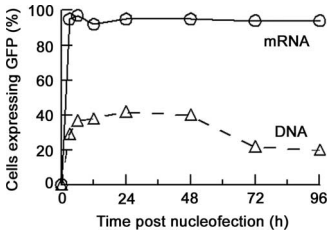

FIG. 3. High-level and durable GFP expression in monkey DC nucleofected with *gfp* mRNA but not with pEGFP-N1 DNA. Immature monkey monocyte-derived DC were nucleofected with 10 μg *gfp* mRNA or pEGFP-N1 DNA, and GFP expression was determined at various intervals posttransfection by flow cytometry.

shown). Taken together, these data indicate that nucleofection with mRNA is a superior method of introducing transgenes into DC, both with respect to antigen expression and to cell viability.

mRNA nucleofection of DC produces rapid and sustained expression of transgene. A potential limitation of mRNA transfection of DC for immunotherapy is that mRNA is labile in cells and may be degraded rapidly, resulting in limited duration of antigen expression. We therefore assessed the durability of transgene expression in immature monkey DC by harvesting cells at various intervals after DNA or mRNA transfection and determining the proportion of cells expressing GFP by flow cytometry. For these and all other experiments, we focused on nucleofection as the preferred method of transfection. GFP expression following nucleofection of DC with pEGFP-N1 DNA was detectable at 3 h posttransfection and maintained relatively constant levels of expression from 6 h to 48 h posttransfection, after which the proportion of GFP-expressing cells markedly declined (Fig. 3), similar to other reports (29, 45). In contrast, the proportion of monkey DC expressing GFP following nucleofection with in vitro-transcribed *gfp* mRNA reached near-maximal levels by 3 h and stayed at this high level for 96 h, the duration of the experiment (Fig. 3). Similar rapid and sustained kinetics of transgene expression were noted in other studies following mRNA delivery to human DC using either electroporation or nucleofection (27, 31, 33, 46, 50). These findings indicate that the delivery of mRNA rather than DNA generates a more-durable expression of transgene in monocyte-derived DC.

Relationship between DNA and mRNA nucleofection and DC maturation. Immature DC are specialized in antigen uptake, and as such, transfection of DC with either DNA or mRNA is traditionally done at the immature stage of differentiation. However, electroporation of mature human DC with mRNA is reported to be efficient (22, 31), and in the murine system, DNA transfection may be enhanced in mature DC (28). To determine the influence of DC maturation on transfection efficiency, we nucleofected human monocyte-derived DC with DNA and mRNA encoding *gfp* with and without prior treatment of cells with CD40L for 24 h to induce maturation. Maturation was confirmed by phenotypic analysis using flow cytometry (data not shown). Prior DC maturation did not enhance GFP expression, as nucleofection of DC with DNA or mRNA generated similar levels of transgene expression regardless of the maturation state at the time of gene delivery (Fig. 4a).

We next evaluated the responsiveness of DNA- and mRNA-nucleofected DC to maturation. Human immature DC were nucleofected with pEGFP-N1 DNA or *gfp* mRNA or were mock nucleofected and either left untreated or treated immediately with CD40L. Cells were harvested and their phenotype was analyzed 24 h postnucleofection by flow cytometry. To ensure that only the phenotype of transgene-expressing cells was analyzed, cells were gated based on GFP expression. Mock-nucleofected immature DC expressed relatively high levels of HLA-DR, CD86, and CD40 and lacked expression of CD80 and CD83, as expected (Fig. 4b and c) (6). Nucleofection of immature DC with pEGFP-N1 DNA did not induce maturation and in fact resulted in a modest downregulation in CD40 expression compared to that of mock-nucleofected cells (Fig. 4b). Moreover, immature DC nucleofected with DNA were refractory to CD40L, as GFP-expressing DC had negligible increases in CD83, CD86, and CD80 expression following CD40 ligation compared to those of mock-nucleofected controls (Fig. 4b). In contrast, immature DC nucleofected with *gfp* mRNA had a minor shift in expression of CD83 and CD80 compared to that of mock-nucleofected DC and responded fully to subsequent CD40 ligation, with increases in CD83, CD86, and CD80 expression, similar to those of mock-nucleofected cells (Fig. 4c). These data indicate that mRNA-nucleofected DC are responsive, whereas DNA-nucleofected DC are refractory to CD40L-mediated maturation.

mRNA- but not DNA-nucleofected DC stimulate robust virus-specific T-cell responses when expressing wild-type virus genes. A key issue in DC transfection for immunotherapy applications is the capacity to stimulate T-cell responses to the specific transgene being introduced. This has been evaluated in the past using lipofection and electroporation approaches to deliver DNA or mRNA into monocyte-derived DC (41, 51); however, the ability to stimulate T-cell responses following DNA and mRNA transfection of DC via nucleofection, which we have shown is a superior method of transfection, has not been compared. To evaluate this, we cultured monocyte-derived DC from a rhesus macaque that had a robust $CD8^+$ T-cell response to SIV Gag through vaccination (33). Immature DC were nucleofected with pGag-N1 DNA encoding a SIVmac239 *gag* sequence isolated from an SIV-infected animal, or the corresponding *gag* mRNA (33), and immediately matured with CD40L for various intervals to induce maturation. To ensure applicability to immunotherapeutic vaccine strategies designed to stimulate T-cell responses to autologous viral sequences (48), we used mRNA and DNA encoding wild-type, non-codon-optimized *gag* sequences. The transfected DC were then cultured with autologous PBMC at a 1:10 ratio in a short-term IFN-γ ELISPOT assay to determine their capacity to stimulate Gag-specific effector T cells (33). DC that had been nucleofected with *gag* mRNA 24 h earlier induced robust Gag-specific T-cell responses with a frequency of greater than 5,000 IFN-γ spot-forming cells per million PBMC (Fig. 5). Importantly, DC that had been nucleofected with *gag*-expressing mRNA 48 h previously induced reduced but still strong T-cell responses, supporting the notion that antigen expressed following the mRNA delivery of transgene is processed and presented in DC for extended periods. In contrast to DC

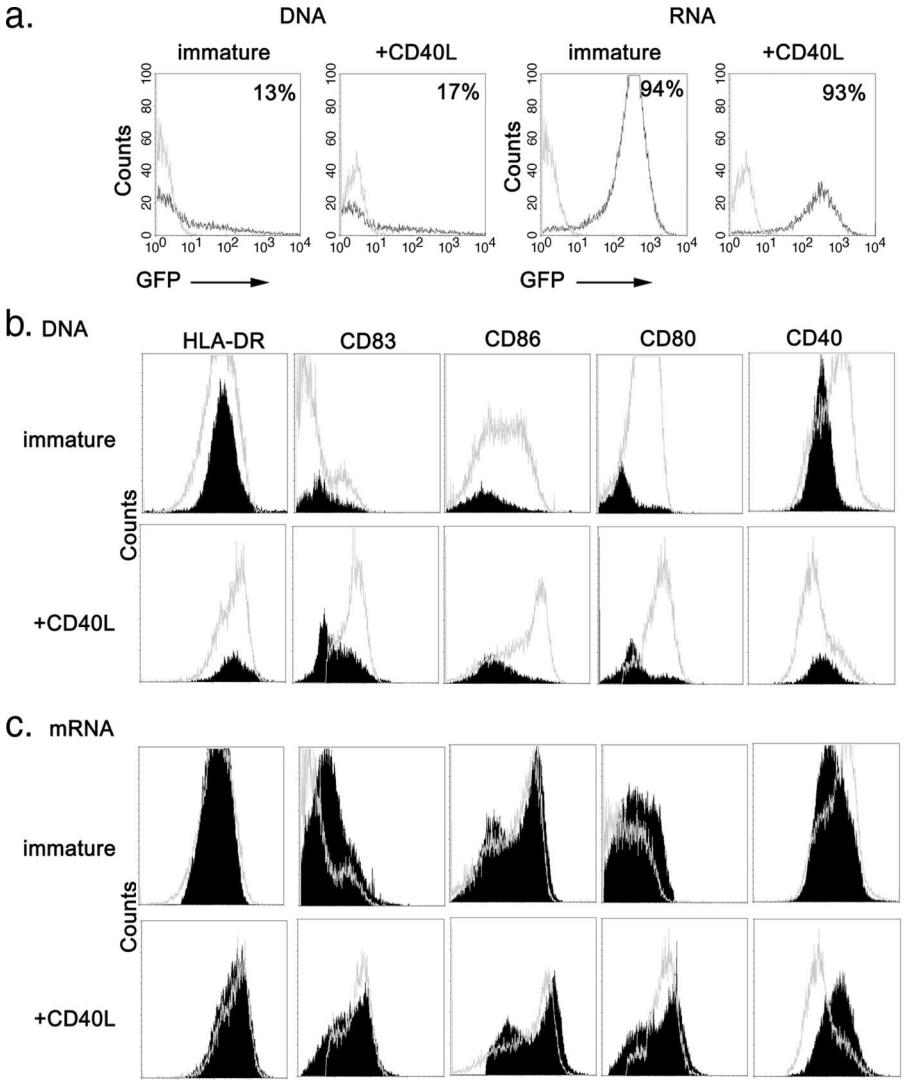

FIG. 4. Differential responsiveness of human DC nucleofected with *gfp* mRNA and pEGFP-N1 DNA to CD40L. (a) Human monocyte-derived DC were nucleofected with 10 μg pEGFP-N1 DNA or 10 μg *gfp* mRNA (solid line) or mock nucleofected (dotted line) with and without prior treatment of cells with 3 μg/ml CD40L as indicated for 24 h, and GFP expression was determined by flow cytometry. Numbers represent the percentage of cells expressing GFP based on mock transfection. (b and c) Immature human DC were nucleofected with DNA (b) (solid histograms) or mRNA (c) (solid histograms) or mock transfected (open histograms) and cultured with or without 3 μg/ml CD40L for 24 h as indicated. Cells were labeled with phycoerythrin-conjugated antibodies as indicated and analyzed by flow cytometry. Of the cells nucleofected with DNA or mRNA, only GFP-expressing DC are shown based on gating.

nucleofected with mRNA, DC nucleofected with DNA encoding wild-type *gag* generated no detectable responses above the nonspecific responses generated by DC nucleofected with pEGFP-N1 DNA at either 24 h or 48 h postnucleofection (Fig. 5). These data indicate that DC nucleofected with wild-type viral mRNA have a durable presentation of antigen to T cells and stimulate robust virus-specific T-cell responses, whereas DC nucleofected with a comparable DNA construct are ineffective.

DISCUSSION

Non-viral-based approaches for delivering genes to DC for cancer immunotherapy have distinct advantages over recombinant virus-based strategies, in particular because DNA or RNA can be amplified directly from a tumor and serve as a source of polyvalent patient-specific antigens (2, 19, 42). This strategy is being employed in the design of therapeutic DC-based vaccines for human immunodeficiency virus (HIV)-infected individuals as well, as viral mRNA expressing patient-derived sequences or sequences from SIV-infected monkeys can readily be introduced into monocyte-derived DC for stimulation of autologous T cells (33, 48). Our studies confirm that nucleofection is superior to conventional electroporation and lipofection for introducing DNA and mRNA into human and monkey monocyte-derived DC, although nucleofection offered no advantage over electroporation when mRNA was introduced into the K562 cell line. However, mRNA was a significantly better source of genetic material than DNA for nucleofection of DC, producing higher-level, more-uniform, and

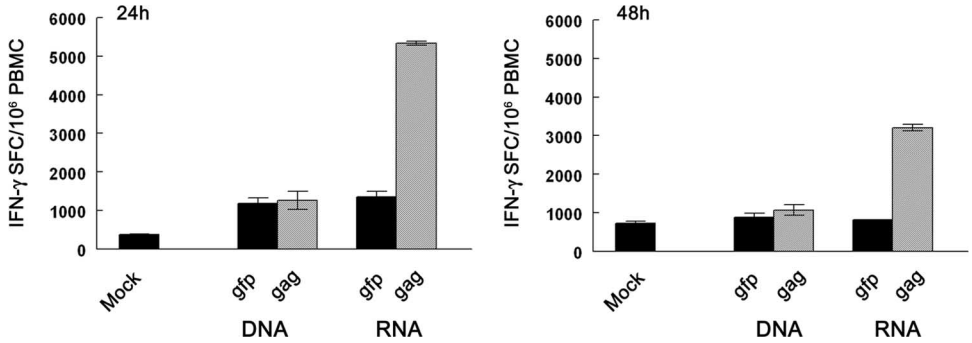

FIG. 5. Monkey DC nucleofected with non-codon-optimized mRNA but not with DNA encoding SIV *gag* stimulate potent Gag-specific effector T-cell responses. Immature DC propagated from an SIV Gag-vaccinated monkey were mock nucleofected (Mock) or nucleofected with DNA or mRNA encoding GFP or SIVmac239 Gag as indicated and matured with CD40L for 24 h (left) or 48 h (right) prior to incubation with autologous PBMC in an IFN-γ ELISPOT assay. IFN-γ spot-forming cells (SFC) were measured 24 h later. Data shown are means ± standard errors of the means (SEM) of triplicate determinations.

more-durable antigen expression with limited toxicity. The results we observed following transfection of DC with *gfp* mRNA using the standard methods of electroporation and lipofection were consistent with those of previous reports (41). Moreover, our results are similar to those observed by others following electroporation of DC with DNA encoding GFP (27, 41). However, a number of studies reported electroporation to produce at least 50% GFP-positive DC following mRNA transfection (25, 27, 39, 40, 50). This discrepancy between our results and those of others using electroporation might be explained by the use of different electroporation devices leading to differences in the delivery of electric current to cells.

In our studies, the efficiency of nucleofection was such that as little as 5 μg mRNA resulted in near-total transfection of DC, yet at least 50 μg mRNA can be safely delivered to DC without apparent toxicity (22). This provides the advantage of being able to introduce relatively small amounts of mRNA encoding multiple genes into DC simultaneously, such as different patient-derived viral genes for immunotherapy of HIV infection. Alternatively, mRNA encoding individual virus or tumor antigens could be introduced together with genes designed to enhance DC function and Th1-stimulating capacity, such as OX40 ligand (10) or interleukin-12 p70 (24), or to prolong DC survival, such as the gene encoding the antiapoptotic protein Bcl-xl (20). In contrast, increasing the amount of DNA had a negative impact on DC viability (data not shown), limiting the number of DNA species that could potentially be expressed. The toxicity of DNA is most pronounced when delivered to DC via nucleofection as we and others have shown (29), presumably because greater quantities of DNA enter cells using this method.

Prior or concurrent maturation of human monocyte-derived DC with CD40L did not alter nucleofection efficiency with either DNA or mRNA in our studies, similar to results in previous reports (29, 34), although in other studies, electroporation of mRNA into mature DC led to a modest increase in transgene expression (22). Larregina et al. reported that simultaneous transfection and CD40-mediated maturation of murine bone marrow-derived DC significantly enhanced DNA transfection efficiency (28), suggesting that maturation may have a greater effect on gene expression in the murine system. In our studies, human DC nucleofected with DNA were refractory to maturation with CD40L, as has previously been shown using lipopolysaccharide (29), which is presumably associated with DNA toxicity. In contrast, DC nucleofected with mRNA were fully responsive to CD40 ligation, similar to results of other reports (29, 33). These studies suggest that the mRNA-based delivery of genes will retain DC viability and responsiveness to maturation stimuli that are critical for T-cell stimulatory capacity, whereas the delivery of antigen via DNA could potentially be deleterious.

A critical factor in gene transfection of DC for immunotherapy is the capacity for the sustained presentation of antigen to tumor- or virus-specific T cells. We found that DC nucleofected with non-codon-optimized SIV *gag* mRNA up to 48 h earlier were capable of stimulating robust antigen-specific effector T-cell responses in PBMC isolated from a vaccinated macaque, equivalent to levels previously shown using peptide-pulsed DC (33), although responses were somewhat diminished from those induced 24 h following nucleofection. In contrast, DC nucleofected with DNA encoding non-codon-optimized Gag were unable to induce detectable Gag-specific T-cell responses even at 24 h posttransfection. Similarly, B lymphoblastoid cells transfected with mRNA encoding HIV *nef* protein were capable of stimulating Nef-specific T cells for at least 72 h posttransfection (25), and mature DC transfected with mRNA encoding the MART-1 tumor-associated antigen remained effective targets for MART-1-specific cytotoxic T cells for at least 96 h postelectroporation (31). Other comparative studies have shown that human DC expressing mRNA-encoded influenza virus matrix protein by electroporation were far superior in their capacity to stimulate M1-specific cytotoxic T cells than DC expressing DNA-encoded antigen (41). The durable antigen presentation in mRNA-nucleofected DC is likely to be a function of sustained protein expression and presentation rather than the persistence of intracellular mRNA, as introduced mRNA is rapidly degraded in cells (13, 22).

It is notable that we used a wild-type sequence of SIV *gag* for DC transfection rather than a codon-optimized sequence to reflect the conditions of DC-based immunotherapy using viral antigens derived from autologous virus sequences (33, 48). SIV and HIV *gag* mRNA are known to have multiple inhibitory/instability elements that substantially limit viral protein expres-

sion (1), yet Gag protein was readily detected in DC 24 h post-mRNA nucleofection when monkey-derived viral sequences were used (33). We do not have data on the long-term expression of Gag within nucleofected DC, and it is possible that expression does not persist as long as that of GFP, which is known to be highly stable (30). Nevertheless, the relative stability of antigen presentation should allow mRNA-transfected DC to traffic to lymph nodes and engage antigen-specific T cells following intradermal or subcutaneous delivery to patients (5, 36, 44). It is likely that the use of codon-optimized as opposed to wild-type DNA sequences would allow for sufficient antigen expression in DC for detectable T-cell responses to be elicited (28).

In summary, these data indicate that nucleofection of primary DC cultures with wild-type mRNA is an effective and nonperturbing means of delivering antigen for DC-based immunotherapy of cancer or infectious diseases, providing uniform and sustained antigen expression and presentation for stimulation of antigen-specific T cells. In contrast, while nucleofection is a more-effective means of introducing DNA into DC than other methods, the resulting low viability, refractoriness to maturation, and poor T-cell-stimulating capacity of transfected DC make this approach unfavorable for autologous DC-based vaccination.

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