Highly efficient expression of transgenic proteins by naked DNA-transfected dendritic cells through terminal differentiation

Adriana T. Larregina, Adrian E. Morelli, Olga Tkacheva, Geza Erdos, Cara Donahue, Simon C. Watkins, Angus W. Thomson, and Louis D. Falo Jr

Dendritic cells (DCs) play a key role in the induction and control of immunity. Genetic engineering of DCs is a promising approach for the development of a broad range of immunomodulatory strategies, for purposes ranging from genetic immunization to tolerance induction. The development of DC-based immunotherapies is limited by the inability to efficiently transfect DCs using naked DNA. Here we demonstrate that after plasmid DNA delivery, the transgene expression level controlled by the human immediate-early cytomegalovirus promoter (hIE-CMVp) is higher in mature DCs than in immature DCs and is further increased after terminal differentiation of DCs by agonist anti-CD40 monoclonal antibody (mAb) or after DC interaction with CD4+ T cells. CD40 signaling of DCs resulted in nuclear translocation of the transcription factors nuclear factor-κB (NF-κB), activator of protein-1 (AP-1), and cyclic adenosine monophosphate (cAMP)-responsive element, necessary for the activation of hIE-CMVp. Transgene expression by DCs diminished after the inhibition of these transcription factors or the blockade of adhesion molecules involved in the DC–T-cell synapse. Importantly, CD40 signaling of DCs resulted in the highly efficient expression and presentation of transgenic antigens and the induction of “in vivo” cytotoxic T-cell (CTL) responses specific for transgenic antigen peptides, demonstrating the functional potential of genetically engineered DCs. (Blood. 2004;103:811-819)

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Introducetion

Dendritic cells (DCs) have a key role in initiating and controlling immune responses. In addition to being the most potent antigen-presenting cells (APCs), DCs determine the nature and magnitude of immune responses and provide a link between innate and acquired immunity.1,2 Genetic engineering of DCs offers potential for the development of immune-regulatory strategies for purposes ranging from immunization to tolerance induction. The capacity of DCs engineered to express transgenic antigens, cytokines, or T-cell costimulatory molecules to induce or bias T helpers 1 and 2 (Th1/Th2)–skewed immune responses, or to promote tolerance, is the subject of current investigation.3-5 DNA-based immunization has potential advantages over protein-based vaccines.5-8 These include the simultaneous delivery of transgenic antigens and immunomodulatory genes to DCs. Despite advances in the understanding of DC biology, the development of genetic immunization strategies using DCs transfected with plasmid DNA has been limited by low transfection efficiencies.9 Currently, the most efficient method for DC transfection is infection by recombinant adenovirus (rAd) at a relatively high multiplicity of infection (MOI). However, the applicability of viral vectors is limited by their potential to interfere with DC function and by the coadministration of viral antigens that elicit strong B- and T-cell responses in the host that may limit the readministration of viral vectors.10-12 Indirect evidence suggests that the signaling pathways involved in maturation/terminal differentiation of DCs may enhance the level of transgene expression driven by the human immediate-early cytomegalovirus promoter (hIE-CMVp). We and others have shown that rAd induces the maturation of mouse bone marrow (BM)–derived DCs (BMDCs) mainly by nuclear translocation of nuclear factor-κB (NF-κB).11 NF-κB is one of the factors necessary to initiate mRNA transcription by hIE-CMVp, and nuclear translocation of NF-κB occurs immediately after CMV infection.13,14 Furthermore, low doses of rAd, engineered to be internalized by cell surface CD40, resulted in enhanced hIE-CMVp–driven transgene expression by DCs.15 CD40 ligand (L) (CD154) is expressed transiently on the surfaces of activated T cells and mediates the terminal differentiation of DCs. This phenomenon takes place during formation of the immunologic synapse between DCs and CD4+ T cells.16,20 Therefore, we hypothesized that signaling DCs through CD40 may increase DC transgene expression controlled by the hIE-CMVp.

In this study we have compared the transfection efficiency of different methods to deliver naked DNA to murine DCs and have analyzed the mechanism(s) involved in transgene expression. We found that gene gun delivery of transgenes controlled by the hIE-CMVp was higher in mature DCs than in immature DCs and further increased after terminal differentiation of mature DCs by CD40 stimulation or interaction with CD4+ T cells. This enhanced...
transgene expression in mature DCs depended on nuclear translocation of the transcription factors NF-κB, activator of protein-1 (AP-1), and cyclic adenosine monophosphate (cAMP)-responsive element. Our results show that transfection with naked DNA of mature DCs signaled by CD40 or those adhesion molecules involved in DC–T-cell interactions resulted in the highly efficient expression of transgenic proteins to levels induced by rAds. Importantly, CD40-induced terminal differentiation of mature DCs resulted in efficient expression, processing, and major histocompatibility complex (MHC) class I–restricted presentation of transgenic antigens to T cells. Moreover, DCs transfected with the gene gun and signaled through CD40 induced a strong cytotoxic T-cell (CTL) response against DNA-encoded transgenic antigens in vivo, demonstrating the potential for genetically engineering DCs with naked DNA.

Materials and methods

Mice

Eight- to 12-week-old C57BL/6 mice or OT-1 mice (Jackson Laboratories, Bar Harbor, ME) were housed in the pathogen-free animal facility of the University of Pittsburgh and used according to institutional guidelines.

Genetic vectors

Plasmids encoding the reporter protein firefly luciferase, enhanced green fluorescence protein (EGFP), or β-galactosidase (pCMV-LacZ) were generated by subcloning the gene of interest into the pcDNA3.1+ plasmid vector (Invitrogen, Carlsbad, CA) at the XbaI–HindIII restriction site of the multiple cloning site. The plasmid pCMV-OVA-489 encodes a truncated form of ovalbumin (OVA) sequence 138 to 386 that remains intracytoplasmic. The plasmid pCMV-OVA–transmembrane (TR) encodes a membrane-bound form of the OVA sequence 138 to 386 linked to the sequence encoding the first 118 amino acids of the transferrin receptor subcloned into XbaI–HindIII restriction sites of pcDNA3.1+. All transgenes were under the control of the hIE-CMVp. Plasmids were amplified in Escherichia coli DH5α (Gibco BRL, Life Technologies, Gaithersburg, MD) and purified using Endofree Qiagen Maxi kits (Qiagen, Chatsworth, CA). Generation and purification of the E1-E3 deleted rAds encoding luciferase (rAd-Luc) and OVA-489 (rAd-OVA489) or β-galactosidase (rAd-LacZ) under the control of hIE-CMVp was performed as described. All rAds were used to infect DCs in vitro at an MOI of 100.

Generation, purification, and terminal differentiation and apoptosis analysis of DCs

DCs were generated after culturing BM precursors and used on day 6 after culture. Mature DCs were separated from immature DCs by 14.5% (wt/wt) metrizamide gradient centrifugation (Sigma, St. Louis, MO) (purity of mature DCs [CD11c+ CD86+] was 83% or higher). Terminal differentiation of DCs was performed with anti-CD40 monoclonal antibody (mAb) (BD Pharmingen, San Diego, CA) (10 μg/mL) as previously described. As control, DCs were incubated with low endotoxin/nonazide irrelevant hamster immunoglobulin M (IgM; 10 μg/mL) as previously described. As control, DCs were incubated with low endotoxin/nonazide irrelevant hamster immunoglobulin M (IgM; 10 μg/mL) as previously described. As control, DCs were incubated with low endotoxin/nonazide irrelevant hamster immunoglobulin M (IgM; 10 μg/mL) as previously described.

Transfection of DCs

DCs (10⁶) were transfected using gene gun, lipofection, CaPO₄ precipitation, electroporation, or rAd infection. For gene gun, naked DNA was precipitated into 1-μm gold particles (Bio-Rad Laboratories, Hercules, CA) as described. Cells were resuspended in 200 μL complete medium, and gene gun transfection was performed by delivering one shot using either a Helios (Bio-Rad Laboratories) or an Accel Gene Gun (PowderJect Vaccines, WI) device at a helium pressure of 250 psi. For lipofection, DCs were transfected using Lipofectamine-Plus reagent ( Gibco) according to the manufacturer’s protocols. Electroporation and CaPO₄ precipitations were performed as described. For rAd infection, pCMV-rAd was used at an MOI of 100, as described. After transfection, cells were cultured in complete medium without cytokines, and transgene expression was assessed at indicated time points. Luciferase expression was analyzed as described previously.

Transmission electron microscopy

DCs were gene gun–transfected with pCMV-OVA-TR, cultured for 24 hours, and labeled with anti-OVA mAb (Sigma) followed by bead-conjugated antimouse immunoglobulin G (IgG; Miltenyi Biotec, Auburn, CA) and immunobead–sorted by their surface expression of transgenic OVA (OVA+ DC purity 90% or greater). OVA+ or OVA– DCs were fixed in 2.5% glutaraldehyde and processed for transmission electron microscopy (TEM). Ultrathin sections were analyzed using a JEOL 1210 electron microscope (JEOL, Chicago, IL).

Quantification of transgenic proteins/peptides in transduced-DCs

Total DCs or mature DCs were transfected with plasmid DNA or rAd encoding pCMV-OVA 489, pCMV-OVA-TR, or pCMV-EGFP and were cultured in the presence or absence of agonist hamster (IgM) anti-CD40 mAb or irrelevant hamster IgM. Transgene expression was analyzed by flow cytometry or by immunofluorescence microscopy in cytopsins 24 hours later. For cell membrane staining, DCs were collected, rinsed in cold phosphate-buffered saline (PBS), and incubated with anti-OVA mAb (Sigma) for 1 hour at 4°C, followed by FITC F(ab')₂ antimouse IgG (Sigma). For intracytoplasmic detection of OVA, DCs were fixed in 2% paraformaldehyde and permeabilized in buffer containing 0.1% saponin; this was followed by 1-hour incubation with FITC-conjugated rabbit anti-OVA polyclonal antibody (Sigma) at 4°C.

We analyzed the loading of transgenic OVA peptide in H-2Kb molecules by transfected mature DCs by labeling with the mAb 25-D1.16 specific for the OVA257-264 (SIINFEKL) peptide–H2Kb complex, followed by FITC F(ab')₂ antimouse IgG (Sigma). As negative controls, we included DCs transduced with a plasmid encoding pCMV-LacZ as an irrelevant transgene, followed by labeling with anti-OVA antibody or 25-D1.16 mAb, as described. After immunostaining, DCs were fixed in 1% paraformaldehyde, and OVA expression was analyzed by flow cytometry.

OT-1 CD8+ CTL proliferation assays

Naive CD8+ T cells were obtained from transgenic OT-1 mice. Spleen and lymph node single-cell suspensions were depleted of red blood cells, and naive CD8+ T cells were purified using nylon wool columns followed by complement-mediated lysis of macrophages, CD4+ T cells, DCs, B cells, and memory T cells expressing LyC6 (purity of naive CD8+ OT-1 T cells 85% or greater by flow cytometry). Monoclonal antibodies included anti-F4/80 (macrophages), anti-I-A (M5114.15.2) (DCs), anti-B220 (B lymphocytes), anti-CD4 ( GK1.5) (ATCC, Rockville, MD), and anti-LyC6 (memory T cells) (BD Pharmingen). Mature or immature control or pCMV-OVA-TR–transfected DCs were cultured with or without agonist anti-CD40 mAb. After 24 hours, DCs (stimulators) were γ-irradiated (200 Gy) and cocultured with naive CD8+ OT-1 T cells at different stimulator/responder ratios in round-bottom, 96-well plates for 3 days. For the final 18 hours, wells were pulsed with 1 μCi (0.037 MBq) [3H] thymidine. The amount of radioisotope incorporated was determined using a β scintillation counter. Assays were performed in triplicate, and results are expressed as mean counts per minute ≥ 1 SD.

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Quantitative analysis of NF-κB, AP-1, and CREB translocation

Nuclear translocation of transcription factors was quantified in DC nuclear extracts using TransAM enzyme-linked immunosorbent assay (ELISA)-based kits specific for human-, mouse-, and rat-activated NF-κB, phosphorylated AP-1, or cAMP-responsive element binding protein (CREB) (Active Motif, Carlsbad, CA) according to the manufacturer’s protocols and was analyzed after 1, 3, or 18 hours. For NF-κB, nuclear extracts were plated on 96-well plates coated with the immobilized oligonucleotide containing the activated NF-κB consensus site (5′-GGGACTTTCCC-3′).26 For AP-1, nuclear extracts were placed in wells containing the immobilized DNA 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element oligonucleotide (5′-TGAGTCA-3′), which is bound specifically by AP-1 dimers,27 followed by incubation with an antibody recognizing phosphorylated c-Jun. For CREB, nuclear extracts were placed in wells coated with immobilized oligonucleotide containing the cAMP-responsive element site (5′-TGAAGTCA-3′), followed by incubation with an antibody recognizing the Ser133-phosphorylated CREB.26-30 Results are expressed as nuclear -fold increase of the 3 different factors and were calculated according to the formula [nuclear content in treated DCs/nuclear content in control DCs] at different time points.

Blocking assays of nuclear factor translocation and adhesion molecules

Nuclear translocation of NF-κB, AP-1, or cAMP-responsive element was blocked by preincubating DCs with one of the following inhibitory agents: (1) NF-κB SN50 cell-permeable inhibitor peptide (18 μM; Calbiochem-Novabiochem, San Diego, CA),31 (2) SP600125, a cell-permeable inhibitor of the c-Jun N terminal kinase (10 μM; Calbiochem)12,21,31; (3) cAMP-dependent protein kinase peptide inhibitor (10 μM; Promega, Madison, WI).10 After transfection with pCMV-Luc, DCs were cultured in medium containing the specific inhibitors or their solvents (negative controls) and agonist anti-CD40 mAb. Adhesion molecules were blocked with 25 μg/mL of the following LE/NA blocking mAbs: anti-CD54 (3E2), anti-CD102 (3C4), anti-CD11a (17/4), anti-CD11b (M1/70), or anti-CD18 (GAME-46) (BD PharMingen), or with 100 μg/mL mannan to block dendritic cell–specific ICAM-grabbing nonintegrin molecule (DC-SIGN).34 Controls included incubation of transfected-DCs with species- and isotype-matched irrelevant mAbs.

DC CD4+ T-cell cocultures

Naive CD4+ T lymphocytes were purified from naive C57BL/6 mice. Memory CD4+ T cells were isolated from C57BL/6 mice gene–immunized with pCMV-Luc on the abdominal skin (1 priming dose + 2 boosts 7 days apart). On day 21, naive or pCMV-Luc–immunized mice were killed, and naive or memory CD4+ T lymphocytes were isolated from lymph nodes and spleens using mouse naive or memory CD4+ T-cell purification column kits (R&D Systems) (purity 95% or greater by flow cytometry). Mature or immature DCs (106) were transfected with pCMV-Luc and cocultured with naive or memory CD4+ T cells at a 1:6 ratio. After 24 hours, luciferase expression was assessed on CD11c+ DCs purified by immunomagnetic bead sorting (Miltenyi Biotech). As controls, gene–transfected DCs cultured in medium with or without agonist anti-CD40 mAb were included. Controls included pCMV-Luc–transfected DCs cultured alone, with agonist anti-CD40 mAb, or in the presence of CD4+ T cells with or without species- and isotype-matched mAbs.

In vivo generation of OVA-specific CTL response

For OVA–specific CTL generation in vivo, C57BL/6 mice were immunized by intradermal injection of 3 × 106 mature DCs transfected with the different naked DNA delivery methods (gene gun, lipofection, CaPO4), or rAd encoding OVA489 followed by signaling through the CD40 molecule. Mature DCs were transfected in vitro and were cultured in the presence of agonist CD40 mAb for 24 hours. After culture, DCs were extensively washed in sterile PBS and were resuspended at a concentration of 3 × 106 cells/200 μL sterile PBS. C57BL/6 mice were immunized intradermally in the lower flanks (100 μL/dose). Mice were given a priming dose of transfected mature DCs on day 1 and boosted on days 7, 21, and 28. Thirty-five days after priming, the mice were humanely killed. Lymph nodes draining immunized skin and spleens were dissected to obtain single-cell suspensions, 3 × 105 cells were restimulated in culture in the presence of OVA protein (1 mg/mL) and SIINFEKL peptide (20 ng/mL), and CTL assays were performed as described.6 Positive controls included mice immunized with mature DCs pulsed with SIINFEKL peptide and signaled with agonist anti-CD40 mAb. Negative controls included groups of mice injected with mature DCs signaled with agonist anti-CD40 mAb and transfected with pCMV-LacZ or rAd LacZ and naive mice injected with PBS.

Statistical analysis

Comparisons of 3 or more means (±1 SD) were performed using analysis of variance (ANOVA) and then the Student Newman–Keuls test.35 Comparisons of 2 means (±1 SD) were performed using the Student t test. A P value of less than .05 was considered significant.

Results

Efficiency of transgene expression by BMDCs transfected with different methods

The expression of transgenic luciferase by murine BMDCs (day 6) DNA-transfected by lipofection, calcium phosphate precipitation (CaPO4), electroporation, and gene gun or infected with rAd was quantified by luminometry (Figure 1A). DCs expressed the highest levels of luciferase after rAd infection [211 676 ± 43 000 relative light unit (RLU)/μg protein]. Gene gun induced higher levels of transgene expression (74 718 ± 3400 RLU/μg protein) than lipofection, CaPO4, or electroporation (131 ± 12, 290 ± 9, and

![Figure 1](https://www.bloodjournal.org)
235 \pm 25 \text{ RLU/µg protein, respectively}) \text{ (Figure 1A). To determine}
the number of DCs that express the transgene, DCs were transduced with plasmid DNA or rAd encoding the intracytoplasmic form of chicken OVA (pCMV-OVA-489), and the percentage of DCs expressing the transgene was assessed by flow cytometry. Expression of OVA was detected in 62.4\% \pm 4\% of DCs infected with rAds and in 21.3\% \pm 3\% of DCs transfected with the gene gun. Transfection efficiencies induced by lipofection, Ca\textsubscript{2}PO\textsubscript{4}, or electroporation remained low (1.3\% \pm 0.3\%, 3.2\% \pm 0.2\%, and 2.0\% \pm 0.5\% of OVA\textsuperscript{+} DCs, respectively) \text{ (Figure 1B). The enhanced transgene expression observed after gene gun transfection compared with other methods may be ascribed to the direct delivery of DNA-loaded gold particles to the cytosol of DCs or, alternatively, to the uptake of free gold particles from culture medium by DCs. To address this question, the correlation between level of transgene expression and intracellular location of gold particles was analyzed ultrastructurally in DCs transfected with a plasmid encoding the transmembrane form of OVA (pCMV-OVA-TR). Twenty-four hours after transfection, DCs expressing OVA on their surfaces were separated from OVA-TR\textsuperscript{-} DCs by immunomagnetic bead sorting, and both DC populations were analyzed by TEM. OVA-TR\textsuperscript{-} DCs showed a high number of gold particles free in the cytosol (probably delivered directly by the gene gun shot), whereas OVA-TR\textsuperscript{+} DCs contained few gold particles inside membrane vesicles, suggesting bead internalization by endocytosis (Figure 1C-D).

Because rAd induces efficient transgene expression and activation/maturation in BMDCs, we investigated in BMDC cultures whether transgenes delivered by the gene gun were expressed preferentially by mature DCs. Our BMDC cultures were composed of immature DCs (CD11c\textsuperscript{+}, MHC 1\textsuperscript{b}, MHC 2\textsuperscript{lo}, CD80\textsuperscript{lo}, CD86\textsuperscript{lo}, CD40\textsuperscript{lo}) and mature DCs (CD11c\textsuperscript{+}, MHC 1\textsuperscript{hi}, MHC 2\textsuperscript{hi}, CD80\textsuperscript{hi}, CD86\textsuperscript{hi}, CD40\textsuperscript{hi}) \text{ (Figure 2A).} After transfection with pCMV-Luc, mature DCs expressed significantly higher levels of luciferase than immature DCs (73 435 \pm 6780 vs 5032 \pm 670 \text{ RLU/µg protein, respectively}; \textit{P} < .001 \text{ (Figure 2B).} To assess the percentage of DCs expressing the transgene according to the stage of DC maturation, immature DC and mature DC cultures were gene gun-transfected with pCMV-OVA-TR was analyzed by flow cytometry. Transgenic OVA expression was detected in 34\% \pm 4\% of mature DCs and 4\% \pm 1\% of immature DCs \text{ (Figure 2C).}

### CD40 signaling increases transgene expression by BMDCs

Based on the facts that mature DCs expressed higher levels of hE-CMVp-driven transgenes than immature DCs and that signaling by CD40 triggers terminal differentiation (maturation) of DCs, we tested whether CD40 signaling of mature DCs further enhanced transgene expression up to levels observed with rAds. For this purpose mature DCs were transduced with the gene gun or infected with rAds encoding luciferase and cultured with agonist (IgM) anti-CD40 mAb or with control IgM. Adding anti-CD40 mAb increased luciferase expression 3-fold in mature DCs transfected with the gene gun and 1.5-fold in mature DCs infected with rAd \text{ (Figure 3A).} We then assessed the enhancing effect of CD40 stimulation on transgene expression regarding the percentage of DCs expressing intracytoplasmic OVA. Mature DCs were gene gun-transduced with pCMV-OVA-489 or infected with rAd-OVA-489, cultured with or without anti-CD40 mAb and analyzed by flow cytometry. After CD40 ligation the number of OVA\textsuperscript{+} mature DCs increased from 40\% \pm 2\% to 79\% \pm 3\% in DCs transfected with the gene gun and from 73\% \pm 3\% to 88\% \pm 3\% in DCs infected with rAd \text{ (Figure 3B).} The results obtained with the gene gun were further confirmed in cytopsins of mature DCs gene gun-transfected with pCMV-OVA-TR or pCMV-E GFP \text{ (Figure 3C-H).}

Signaling by CD40 sustained transgene expression in DCs transfected with the gene gun for up to 72 hours \text{ (Figure 3I).} The enhanced transgene expression detected in mature DCs after CD40 stimulation correlated with the terminal differentiation of mature DCs \text{ (assessed by further up-regulation of CD86 and CD54) \text{ (Figure 3J).} Gene gun transfection by itself did not induce the terminal differentiation of mature DCs, but it did not abrogate CD40-mediated DC terminal differentiation \text{ (Figure 3J).} The effect of CD40 stimulation on transgene expression was not due to higher viability of DCs because apoptosis of mature DCs 24, 48, and 72 hours after gene gun transfection was not significantly different \text{ (5\%-10\% of apoptotic DCs) between experimental groups (data not shown).}

### CD40 signaling increases transgenic antigen presentation by BMDCs to T cells

To determine the effect of CD40 stimulation on the ability of BMDCs to process and present transgenic antigenic peptides in MHC 1 molecules, mature DCs were transfected with pCMV-OVA-TR by gene gun, and the level of expression of OVA\textsubscript{257-264} (SIINFEKL) peptide-H-2K\textsuperscript{b} was assessed on the cell surface by flow cytometry. Percentages of mature DCs expressing SIINFEKL-H-2K\textsuperscript{b} and the density of peptide-MHC 1 complexes \text{ (assessed by mean fluorescence intensity [MFI]) increased substantially after treatment with anti-CD40 mAb (34\%-78\% in DCs expressing SIINFEKL-H-2K\textsuperscript{b} and 23\%-68\% in MFI) \text{ (Figure 4A).} We then evaluated the ability of mature DCs transfected with pCMV-OVA-TR with the gene gun to present the SIINFEKL-H-2K\textsuperscript{b} complex to naive OT-1 CD8\textsuperscript{+} T cells, which bear transgenic
Figure 3. Signaling DCs through CD40 increases transgene expression to protein levels similar to those induced by rAd-Luc. (A) Transgene expression in mature DCs, gene gun-transfected with pCMV-Luc or infected with rAd-CMV-Luc and cultured in the presence or in the absence of the agonist anti-CD40 mAb. Mature DCs infected with rAd expressed significantly higher levels of luciferase compared with mature DCs transfected with gene gun (P < .01). The enhancing effect of CD40 signaling was observed in mature DCs transfected with gene gun or rAd by the induction of similar levels of luciferase expression (P > .05). Controls included mature DC transfected and incubated in the presence of hamster anti-immunoglobulin IgM. (B) The efficiency of transfection after CD40 ligation was determined by the expression of cytoplasmic OVA in mature DC gene gun-transfected with pCMV-OVA-489 or infected with rAd-OVA-489. Numbers in graphs are percentages and MFI of OVA expression of transmembrane OVA (D) or cytoplasmic EGFP (H) by mature DCs 24 hours after gene gun transfection. (F) As negative controls, mature DCs were transfected with pCMV-LacZ and labeled with anti-OVA mAb. (G-H) Enhanced expression of transmembrane OVA (G) or cytoplasmic EGFP (H) by mature DCs 24 hours after gene gun transfection plus agonist anti-CD40 mAb. Transgene expression is observed in DCs with gold particles in the cytoplasm (arrows). (C) Phase-contrast microscopy. (D-H) Fluorescence microscopy. Original magnification × 400. Bar = 10 μm. (I-J) Effect of CD40 signaling of mature DCs on gene expression is maintained for 72 hours and is attributed to the terminal differentiation of mature DCs. (I) Time-point curve showing that the high transgene expression observed in mature DCs transfected with gene gun and signaled through CD40 was sustained up to 72 hours. (J) DC terminal differentiation by CD40 signaling was assessed by the expression of the activation markers CD86 and CD54 in mature DCs, indicated by the significant increase of the MFI (numbers in graphs).

Mechanism(s) of CD40-dependent enhancement of hIE-CMVp-driven transgenes in DCs

Because all transgenes tested were driven by hIE-CMVp, we ascertained whether the increase in transgene expression by DCs after CD40 signaling was caused by nuclear translocation of the transcription factors NFκB, AP-1, or CREB, which are involved in initiation of hIE-CMVp–controlled transcription.13,14 Translocation of NFκBp65 and AP-1 was observed 1 hour after CD40 signaling, whereas translocation of CREB was detectable 3 hours later. The maximum level of translocation of the 3 factors occurred 3 hours later, with amounts still detected after 18 hours. The highest nuclear increase was observed for NFκB (3.69 ± 0.3-fold increase at 3 hours; P < .0001) and to a lower extent for AP-1 (2.7 ± 0.16; P = .0005) and CREB (2.29 ± 0.1; P = .0014) (Figure 5A). Gene gun treatment by itself did not induce significant nuclear translocation of transcription factors compared with nontreated cells (P > .05), and there was no difference between translocation induced by anti-CD40 mAb alone or in combination with the gene gun (P > .05) (not shown). Accordingly, the specific inhibition of nuclear translocation of NFκB, AP-1, and CREB significantly diminished hIE-CMVp–dependent luciferase expression in mature DCs (P < .0001) (Figure 5B).

Stimulation by CD40 induces DC clustering because of the up-regulation and increased affinity of adhesion molecules (ie, intracellular adhesion molecules [ICAMs], β2 integrins, and DC–SIGN).37–41Thus, we investigated whether the effect of CD40 on transgene expression by mature DCs was a combined effect of direct stimulation by CD40 and interaction of these adhesion molecules. Specific blockade of CD18, CD54, CD102, DC–SIGN (P < .0001), and, to a lesser extent, CD11a (P < .001) resulted in significant inhibition of transgene expression and DC aggregation (Figure 6A).

The adhesion molecules that mediate DC-DC interaction are also involved in the organization of the immunologic synapse between DCs and T cells. Thus, we tested whether transgene
expression by mature DCs increased during the formation of the synapse. For this, mature DCs or immature DCs were transfected with pCMV-Luc with the gene gun and cocultured with syngeneic C57BL/6 naive or memory CD4+ T cells. Interaction with either CD4+ T-cell population increased transgene expression similarly by mature DCs, with lower effect by immature DCs (Figure 6B). The increase in transgene expression was similar to that induced by CD40 signaling of mature DCs, with lower effect by immature DCs (Figure 6B).

**Discussion**

For the purpose of immune therapy using genetically engineered DCs, transfection with naked DNA may be desirable over recombinant viral vectors. So far, DCs have been shown to be exceedingly difficult to transfect with naked DNA, and the highest transfection efficiencies have been achieved using recombinant viral vectors. Previous reports have shown effective naked DNA transfection of primary cells such as CD34+ bone marrow stem cells and glial cells using the gene gun. Here we analyzed the transfection efficiencies induced by different naked DNA delivery methods in DCs and showed that gene gun induced significantly higher transgene expression. This effect was a consequence of shooting DNA-loaded gold particles directly into the cytosol of DCs rather than of the uptake of gold particles from the culture medium by macropinocytosis. By using naked DNA, we demonstrated that the efficient transfection of DCs was limited to the population of mature DCs and that transgene expression increased dramatically after DC terminal differentiation induced by signaling of DCs by CD40, which was responsible for the nuclear translocation of transcription factors required by hIE-CMVp. Importantly, when combined with CD40 ligation of mature DCs, gene gun and rAd induced similar levels of transgene expression and similar numbers of DCs expressing transgenic antigens. Our mechanistic study suggests that increased transgene expression was caused by the nuclear translocation of NF-κB, AP-1, and CRE. These transcription factors bind to sequences in the enhancer region of hIE-CMVp, contributing to the strength of the promoter.
We have previously described that the gene gun induces rapid activation and migration of skin DCs.5,21 Interestingly, under our experimental conditions, the gene gun did not induce further maturation or terminal differentiation of mature DCs, as demonstrated by unchanged levels of expression of the DC activation markers CD54 and CD86 and by the inability to translocate NF-kB and AP-1 to the DC nucleus. These opposite results can be explained by the fact that the gene gun induces the activation and migration of skin DCs transfected in vivo or in ex vivo models of skin explants. Under such experimental conditions, the activation of skin DCs can be ascribed to the damage caused to epidermal keratinocytes that secrete proinflammatory cytokines necessary to trigger DC maturation and migration.5,21

Signaling DCs through CD40 in vitro induces the formation of homotypic DC clusters. This effect is caused by an increase in the levels of ICAM-1 (CD54), ICAM-2 (CD102), and ICAM-3 (CD50) and to an enhanced affinity for their ligands, the β2 integrins, or DC-SIGN.37-41 Our results demonstrate that the blockade of CD54, CD11a, CD18, or DC-SIGN consistently diminishes transgene expression in terminal differentiated DCs. These observations strongly suggest that transgene expression controlled by the hIE-CMVp in DCs that are signaled by CD40 is regulated by a complex mechanism that also combines signaling through adhesion molecules. Importantly, the adhesion molecules involved in the formation of DC clusters are also implicated in the initial steps of the organization of the immunologic synapse between DC and CD4+ T cells. As a consequence of this interaction, DCs receive the necessary CD4+ T-cell “help” (by CD40-CD40L interaction) to initiate the CD8+ T-cell response.44-46 Therefore, we hypothesized that the DC-CD4+ T-cell interaction might affect the level of transgene expression by DCs in a similar fashion to CD40 signaling and homotypic DC aggregation. Our results demonstrated that coculture of transfected mature DCs with naive or antigen-specific memory CD4+ T cells induces transgene expression equivalent to that observed after CD40 signaling. This observation suggests that the interaction of maturing migratory DCs, transfected in peripheral tissues, with T cells in secondary lymphoid organs results in a further increase in transgene expression. This in turn facilitates the delivery of transgenic antigens and immunoregulatory factors by transfected DCs where antigen presentation occurs.

The enhanced transgene expression by DCs is functionally significant, as demonstrated by the efficient processing and presentation of a transgenic antigen-derived peptide by DCs. The success of preventive or therapeutic immunization against tumors or...
intranuclear pathogens depends on the generation of antigen-specific CTL responses. Effective CTL induction requires efficient antigen and adjuvant delivery to induce effective antigen presentation and T-cell costimulation by mature DCs. We have shown that CD40 signaling of transfected DCs induces efficient loading of transgenic antigen peptides into MHC class I and potent antigen presentation to naive CD8+ specific T cells. The increased expression of SIINFEKL–H2Kb complexes on the surfaces of DCs correlated with enhanced T-cell stimulatory capacity. Importantly, DCs that were transfected and terminally differentiated by CD40 ligation induced significantly higher antigen-specific T-cell proliferation than DCs loaded with saturating doses of synthetic peptide, even when the latter population was identically matured by CD40 ligation. This observation could be explained by a sustained synthesis of transgenic proteins, followed by sustained exportation of MHC class I peptide complexes to the DC surface in gene gun–transfected and CD40-activated mature DCs. Indeed the turnover of MHC class I molecules, together with a decreased concentration of OVA peptide in the culture wells over a period of 72 hours, may account for the different OT-1 proliferation activity observed in OT-1 assays stimulated with CD40-signalized gene gun–transfected mature DCs compared with OVA(SIINFEKL)–pulsed CD40-signalized mature DCs.

Importantly, the effect we observed in ex vivo OT-1 proliferation assays was further confirmed in “in vivo” CTL assays. As expected, gene gun transfection and rAd infection of mature DCs signaled with agonist CD40 mAb were similar, and both induced significantly higher CTL response than mature DCs transfected with other naked DNA methods. Furthermore the CTL response induced by gene gun–transfected or rAd-infected mature DCs was slightly higher than the CTL response induced by OVA257–264 (SIINFEKL)–peptide-pulsed mature DCs.

Taken together, our results demonstrate that high-level transgene expression can be achieved in DCs by combining an efficient DNA delivery method with an appropriate signaling strategy able to translocate to the DC nucleus those transcription factors required for functioning of the transpromoter used. The data presented here further elucidate the mechanisms regulating efficient transgene expression by DCs and contribute to the development of DC engineering strategies for the purpose of immunoregulation.

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References


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