

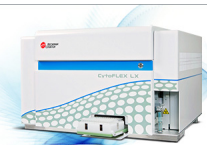


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## Potential of Transfected Muscle Cells to Contribute to DNA Vaccine Immunogenicity

Hidekazu Shirota, Lev Petrenko, Choongman Hong and  
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# Potential of Transfected Muscle Cells to Contribute to DNA Vaccine Immunogenicity<sup>1</sup>

Hidekazu Shirota,<sup>2</sup> Lev Petrenko, Choongman Hong, and Dennis M. Klinman<sup>3</sup>

The mechanism(s) by which DNA vaccines trigger the activation of Ag-specific T cells is incompletely understood. A series of *in vivo* and *in vitro* experiments indicates plasmid transfection stimulates muscle cells to up-regulate expression of MHC class I and costimulatory molecules and to produce multiple cytokines and chemokines. Transfected muscle cells gain the ability to directly present Ag to CD8 T cells through an IFN-regulatory factor 3-dependent process. These findings suggest that transfected muscle cells at the site of DNA vaccination may contribute to the magnitude and/or duration of the immune response initiated by professional APCs. *The Journal of Immunology*, 2007, 179: 329–336.

**D**eoxyribonucleic acid (DNA) vaccines provide a unique method for inducing cellular and humoral immune responses against infectious pathogens and tumor Ags. A strong promoter drives expression of an Ag-encoding gene, so that the relevant protein is transcribed and translated *in vivo* (1, 2). Intramuscular delivery of plasmid DNA vaccines leads to the expression of the encoded protein by a variety of cell types (1, 3–5). Evidence suggests that professional APCs play a dominant role in the induction of immunity. Some APCs are directly transfected, which causes them to rapidly migrate to the draining lymph nodes and initiate an immune response (5–7). Dendritic cells (DCs)<sup>4</sup> play a further role in cross-presenting Ag produced by transfected non-immune cells (such as muscle cells) (8–12). Both of these pathways are known to contribute to the activation of MHC-matched CD8 T cells.

Uncertainty remains concerning the ability of nonprofessional APCs to facilitate in maintaining and magnifying the induced response. Previous studies suggest that local somatic cells (such as myocytes, keratinocytes, and fibroblasts) may be involved in the direct presentation of Ag to T cells (13–15). This mechanism of Ag presentation may be of particular relevance when DNA vaccines are introduced via electroporation, as evidence suggests that electroporation leads to the more efficient transfection of muscle cells and improved immune responses (16–20). To investigate this issue, the Ag-presenting capacity of transfected muscle cells was examined. Following plasmid transfection *in vitro* and *in vivo*, both primary muscle cells and muscle cell lines up-regulated expression of MHC class I and costimulatory molecules. These cells gained the ability to present immunogenic peptides and DNA-en-

coded Ag to CD8 T cells, suggesting they may contribute directly to the induction and/or perpetuation of the Ag-specific immune response following DNA vaccination.

## Materials and Methods

### Animals and cell lines

C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD) and studied at 6–10 wk of age. OT-1 mice carrying OVA-TCR-transgenic T cells specific for the H-2K<sup>b</sup>-restricted CTL epitope on a RAG-1<sup>-/-</sup> background were obtained from The Jackson Laboratory. All studies were approved by the Center for Biologics Evaluation and Research (CBER) Animal Care and Use Committee. The NOR-10 (H-2<sup>b</sup>) mouse skeletal muscle cell line, Sol8 (H-2<sup>b</sup>) mouse skeletal muscle cell line, and CMT-93 (H-2<sup>b</sup>) mouse rectal carcinoma cell line were purchased from American Type Culture Collection. Primary human skeletal muscle cells from a single donor (BD Clonetics) were maintained in SkBM basal medium with BulletKit containing growth factors (BD Clonetics).

### Reagents

An OVA-encoding plasmid, pOVA, was prepared by cloning the OVA cDNA fragment obtained by digestion with *EcoRI* and *HindIII* (Toyobo) from pAc-neo-OVA into pcDNA6 (Invitrogen Life Technologies). Plasmid DNA was purified using an EndoFree plasmid mega kit (Qiagen) according to the manufacturer's protocol. These lysates were shown to be endotoxin-free before being dissolved in sterile PBS for injection. The peptide SIINFEKL (H-2K<sup>b</sup>-restricted OVA peptide 257–264) was synthesized at the CBER Core Facility.

### *In vivo* electroporation and gene delivery

Plasmid vectors were delivered *in vivo* by electroporation-mediated *i.m.* injection (16). Hair around the quadriceps femoris muscle of anesthetized mice was removed. The muscle was then injected with 20  $\mu$ g of plasmid DNA in 20  $\mu$ l using the TriGrid Electroporation Delivery System (Ichor Medical Systems). The electroporation protocol involved rectangular-wave, direct-current pulses applied at 220 V/cm peak amplitude and 8% duty cycle over 0.5 s. The TriGrid electroporation system consists of an electric pulse generator and a TriGrid array comprising four electrodes 2 mm in length with 2.5-mm interelectrode spacing.

### Cell preparation

Muscle tissue at the vaccination site was removed, sliced into fragments, and treated with 2 mg/ml collagenase type II (Worthington) for 60 min at 37°C. The resultant preparation was gently agitated to generate a single-cell suspension. To deplete APCs, these cells were resuspended in RPMI 1640 supplemented with 5% FCS and incubated for 2 h to remove plastic adherent cells. Nonadherent cells were recovered and used in the experiments.

OT-1 cells were isolated from the spleen of OT-1/RAG1<sup>-/-</sup> mice by negative selection using a combination of anti-MHC class II, anti-DX5, anti-CD11c, and anti-CD11b microbeads (Miltenyi Biotec). The resulting cell population was composed of 90  $\pm$  5% Va2V $\beta$ 5<sup>+</sup> cells and <0.5% I-A<sup>+</sup> cells, as measured by flow cytometry.

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<sup>1</sup> The assertions herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the Food and Drug Administration at large.

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<sup>4</sup> Abbreviations used in this paper: DC, dendritic cell; RNAi, RNA interference; siRNA, small-interfering RNA; IRF3, IFN-regulatory factor 3; pOVA, OVA-encoding plasmid; LMP, low m.w. protein; IP-10, IFN- $\gamma$ -inducible protein 10.

Table I. PCR primers used in this study

Gene	Sense/Antisense	Primers
<b>Mouse</b>		
<i>mB7.1</i>	S:	TGCTGTCTGTGCTATTGCTGGGAAACT
	AS:	CCCAGGTGAAGTCCCTCTGACACGTG
<i>mB7.2</i>	S:	GGGGATCCATGGGCTTGGCAATCCCTTAT
	AS:	TCCGGTGACCTTGCTTAGACGTGCAGG
<i>mTAP1</i>	S:	GTCCAGATGCCTTCGCTATCAG
	AS:	GTTGGCTGTGTCCTCAGTCAC
<i>mLMP2</i>	S:	GTTCCGGACGGAAGAAGTCC
	AS:	GCAGCTCATCTCCCAGGATG
<i>mLMP7</i>	S:	GCTCCGGAGCTCGCACTTC
	AS:	TAGTTGTCTCTGTGGGTAG
<i>mRANTES</i>	S:	ATGAAGATCTCTCTGCAGCTGCCCTCAC
	AS:	CTAGCTCATCTCCAATAGTTGATG
<i>mIP-10</i>	S:	ACCATGAACCCAAAGTGCTGCCGTC
	AS:	GTTTCACTCCAGTTAAGGAGCCCT
<i>mIFN<math>\beta</math></i>	S:	CCACAGCCCTCTCCATCAACTATAAGC
	AS:	AGCTCTCAACTGGAGAGCAGTTGAGG
<i>H-2K<sup>b</sup></i>	S:	GAGCCCCGGTACATGGAA
	AS:	CAGGTAGGCCCTGAGTCT
<i>m-actin</i>	S:	GACATGGAGAAGATCTGGCAACCACA
	AS:	ACGTCCACCCTGAGTGATTC
<b>Human</b>		
<i>huTAP1</i>	S:	ATCTCTGCTCGAAGTCTAGAGCAA
	AS:	AGCTTTTCCCTAAACTTCTGGG
<i>huLMP2</i>	S:	TTGTGATGGGTTCTGATTCCCG
	AS:	CAGAGCAATAGCGTCTGTGG
<i>huLMP7</i>	S:	TCGCCTTCAAGTCCAGCATGG
	AS:	CCAACCATCTTCCCTCATGTGG
<i>huRANTES</i>	S:	ATGAAGGCTCCTCCGGCCACGCCTCGC
	AS:	CTAGCTCATCTCCAAGAGTTGATG
<i>huIP-10</i>	S:	AGAGGAACCTCCAGTCTCAGC
	AS:	CCTCTGTGTGGTCCATCCTT
<i>huIFN<math>\beta</math></i>	S:	GAACCTTGACATCCCTGAGGAGATTAAGCAGC
	AS:	GTTCCTTAGGATTTCCACTCTGACTATGGTCC
<i>hu-actin</i>	S:	TTCAACTCCATCATGAAGTGTGACGTG
	AS:	TAAGTCATAGTCCGCTAGAAGCATT

### Transfection

DNA cytofectin complexes were prepared according to the manufacturers' instructions. Briefly, DNA was mixed 1:1 with the Fugene 6 transfection reagent (Roche Molecular Biochemicals) in 1 ml of serum-free OptiMEM (Invitrogen Life Technologies) for 15 min at room temperature and then added to cells (21).

### In vitro stimulation of OT-1 cells

Cells ( $2 \times 10^6$ /well in 2 ml) were transfected with 10  $\mu$ g/ml plasmid in 24-well plates. After 1 day of culture, cells were fixed with 0.5% paraformaldehyde for 15 min at 37°C (22). After extensive washing, cells ( $2 \times 10^5$  cells/well) were cocultured with  $3 \times 10^4$  OT-1 cells in 96-well plates. Supernatants were recovered for ELISA analysis after 24 h. Cells were then pulsed with [<sup>3</sup>H]Tdr (1.0  $\mu$ Ci/well) for another 12 h to monitor proliferation. Tritium incorporation was quantified using a  $\beta$ -scintillation counter.

### CFSE labeling

Purified OT-1 cells were labeled with CFSE according to the manufacturers' instructions. Briefly, OT-1 cells were suspended in PBS at  $2 \times 10^6$  cells/ml and incubated in 0.5  $\mu$ M CFSE (Molecular Probes) solution for 15 min at 37°C. After incubation, cells were washed twice with PBS.

### FACS

Cells were washed with PBS, fixed with 4% paraformaldehyde for 5 min at 37°C, and stained with Abs for 30 min at room temperature. Stained cells were washed, resuspended in PBS/0.1% BSA plus azide, and analyzed by FACSsort (BD Biosciences). Abs used (all from BD Pharmingen) included: PE-labeled anti-B7.1, anti-B7.2 and isotype control Abs, FITC-labeled anti-H2K<sup>b</sup>, anti-BB-1 and isotype control Abs, biotin-conjugated anti-mouse V $\alpha$ 2V $\beta$ 5 Ab, followed by avidin-conjugated CyChrome.

### ELISA

Cytokine levels in culture supernatants were measured by ELISA, as previously described (23). Ninety-six-well Immulon H2B plates (Thermo

LabSystems) were coated with first-stage IFN $\gamma$ -specific Ab and then blocked with PBS 1% BSA. Culture supernatants were added and bound cytokine was detected by the addition of biotin-labeled secondary Ab (all Abs obtained from BD Pharmingen), followed by phosphatase-conjugated avidin and a phosphatase-specific colorimetric substrate (Pierce). Standard curves were generated using recombinant cytokines purchased from R&D Systems. The concentrations of IFN- $\beta$  were determined using a mouse IFN- $\beta$  ELISA kit (PBL Bio-medical Laboratories). All assays were performed in triplicate.

### RNA interference (RNAi)

Three sets of Stealth RNAi duplexes and corresponding Stealth controls were synthesized by Invitrogen Life Technologies. Stealth RNAi compounds are 25 mer dsRNA containing proprietary chemical modifications that enhance nuclease stability and reduce off-target effects. RNA oligonucleotides used for targeting mouse IFN-regulatory factor 3 (IRF3) were as follows: IRF3 small-interfering RNAs (siRNA) 1: CCUAUCUCCUU ACCUCUGACCAGU, ACUGGUCAGAGGUAAGGGAGAUAGG; IRF3 siRNA2: GACUCCAGGCCACUGGAAUAUCU, AGAUUUUCCA GUGGCCUGGAAGUC; IRF3 siRNA2: CCAGGUCUCCAGCAGACA CUCUUU, AAAGAGUGUCUGCUGGAAGACCUGG. Cells were transfected with 40 nM siRNA using Lipofectamine RNAiMAX (Invitrogen Life Technologies) according to the manufacturers' instructions. Forty-eight hours after transfection, cells were used in additional experiments. Knockdown of IRF3 expression was verified by Western blotting.

### RT-PCR

Total RNA was extracted from target cells using TRIzol reagent (Invitrogen Life Technologies) as recommended by the manufacturer. One microgram of total RNA was reverse-transcribed in first strand buffer (50 mM Tris-HCl (pH 7.5), 75 mM KCl, and 2.5 mM MgCl<sub>2</sub>), containing 25  $\mu$ g/ml oligo(dT)<sub>12-18</sub>, 200 U of Malony leukemia virus reverse transcriptase, 2 mM dNTP, and 10 mM DTT. The reaction was conducted at 42°C for 1 h. A standard PCR was performed on 1  $\mu$ l of the cDNA synthesis using the primer pairs in Table 1. Aliquots of the PCR were separated on a 1.5% agarose gel and visualized with UV light after ethidium bromide staining.

### Histology and immunohistochemistry

Muscle from the site of plasmid electroporation was flash-frozen, sectioned in a cryostat (Histoserv), and stained with H&E for visualization of cellular inflammation. Sections were fixed in 0.3% H<sub>2</sub>O<sub>2</sub> methanol, then stained with FITC-conjugated anti-H-2K<sup>b</sup>, anti-B7.1, and biotinylated-anti CD8 $\alpha$  (Ly-2) Abs (BD Pharmingen). Biotinylated Ab was detected using avidin-biotin coupled to the chromogenic substrate 3,3'-diaminobenzidine (DakoCytomation). Sections were counterstained with hematoxylin and mounted. FITC signals were detected using a laser scanning microscope (LSM5 Pascal; Carl Zeiss).

### Statistical analysis

The Student *t* test was used to analyze all results. To facilitate comparisons when an experiment was repeated multiple times, results were standardized by calculating the fold change vs the control group in each individual experiment.

## Results

### Cells from DNA-vaccinated muscle tissue activate Ag-specific CD8 T cells

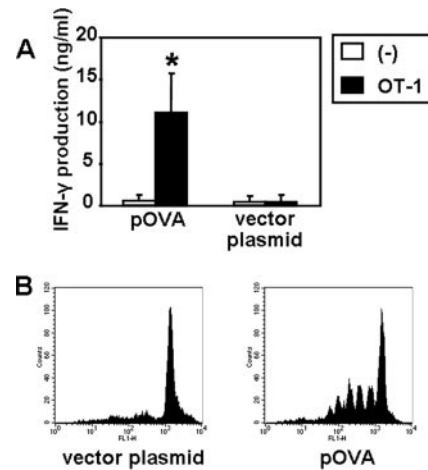
Previous studies established that cells from the site of DNA vaccination can contribute to the induction of both humoral and cell-mediated immune responses (13, 15). To confirm and extend these observations, an OVA-encoding plasmid (pOVA) was administered via electroporation to the muscle of C57BL/6 mice. Two days later (after professional APCs had migrated from the injection site to the draining lymph nodes; Ref. 6), the injected muscle was removed, digested with collagenase to form a single-cell suspension, and cultured with purified OVA-specific CD8 T cells from OT-1 mice.

Whereas muscle cells from mice treated with vector plasmid alone had no effect on the OT-1 cells, muscle cells from pOVA electroporated mice reproducibly stimulated the OT-1 cells to proliferate and secrete IFN- $\gamma$  (Fig. 1). This finding has several possible interpretations: 1) muscle cells at the site of vaccination might directly present Ag to CD8 T cells, 2) professional APCs might remain at the site of vaccination and mediate the stimulation of OT-1 cells, or 3) OVA produced by transfected muscle cells might be taken up by naive (nontransfected) professional APCs remaining in the muscle and cross-present this Ag to OT-1 cells.

### A muscle cell line transfected with pOVA activates Ag-specific CD8 T cells

Tissue from the site of DNA vaccination contains muscle cells, fibroblasts, fat cells, macrophages, and DCs. Despite rigorous efforts to completely purify muscle cells from this tissue, a low level of APC contamination persisted. Thus, to determine whether muscle cells devoid of APCs could present Ag to CD8 T cells, the H-2K<sup>b</sup> NOR-10 murine muscle cell line was used. NOR-10 cells transfected with pOVA stimulated purified OT-1 cells to produce IFN- $\gamma$  and proliferate, similar to the effect observed when purified muscle cells were studied. The level of activation observed following transfection of the NOR-10 muscle cell line was similar to that induced by pOVA transfection of a murine macrophage cell line (Fig. 2). No stimulation was observed when NOR-10 cells were transfected with vector plasmid alone (Fig. 2).

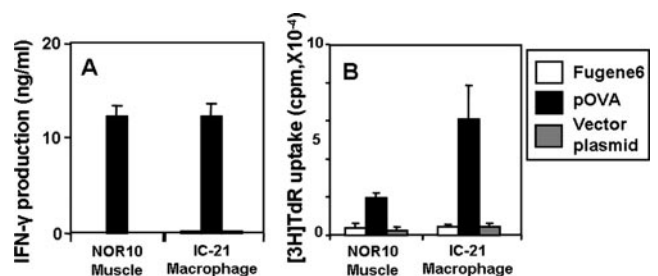
These findings suggested that pOVA-transfected murine muscle cells had the ability to directly stimulate Ag-specific CD8 T cells. However, it remained a formal possibility that OT-1 cells isolated from donor mice contained some contaminating APCs, and that these APCs were cross-presenting OVA produced by transfected NOR-10 cells. To evaluate this concern, whole mitomycin C-treated spleen cells, NOR-10 cells, and OT-1 cells were cultured with exogenous OVA. As seen in Fig. 3A, APC present in the spleen were able to present free OVA to OT-1 cells, whereas OT-1 cells alone or combined with untransfected NOR-10 cells were



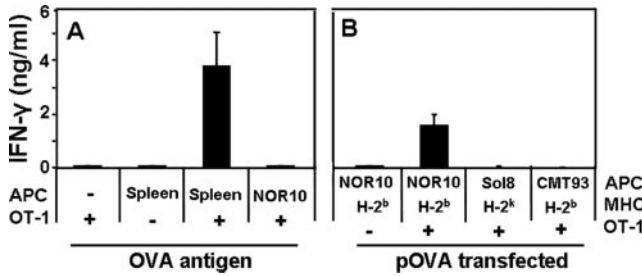
**FIGURE 1.** Muscle tissue from the site of pOVA vaccination stimulates OT-1 cells. A total of 20  $\mu$ g of pOVA or vector plasmid was delivered i.m. by electroporation to C57BL/6 mice. A single-cell suspension was prepared from muscle tissue removed from the site of vaccination 2 days later by digestion with collagenase type II. *A*, The muscle cell suspension was cultured with purified OT-1 cells from the spleen of OT-1/RAG1<sup>-/-</sup> mice for 48 h. Results represent the average  $\pm$  SD level of IFN- $\gamma$  in culture supernatants from four independently studied muscle cell samples per group. The experiment was repeated once with similar results. *B*, The muscle cell suspension was cultured with CFSE-labeled OT-1 cells for 60 h. The cells were stained with anti-V $\alpha$ 2V $\beta$ 5 Ab, and FACS was used to monitor CFSE levels in V $\alpha$ 2V $\beta$ 5<sup>+</sup> cells. Results are representative of four experiments. \*, *p* < 0.005 (compared with the pOVA-vaccinated muscle cell group).

unable to respond to free OVA. This finding suggests that the OT-1 population was not contaminated by APCs capable of presenting free Ag.

Studies of the MHC-mismatched muscle cell line provided further evidence against cross-presentation. As seen in Fig. 3B, pOVA-transfected NOR-10 cells stimulated H-2-matched OT-1 cells whereas similarly transfected Sol8 murine muscle cells (which expresses H-2<sup>k</sup>) did not (Fig. 3B). Of interest, the H-2<sup>b</sup>-expressing murine colon carcinoma cell line CMT93 and LL/2 mouse Lewis lung carcinoma cells also failed to stimulate OT-1 cells following pOVA transfection (Fig. 3B and data not shown). All three transfected cell lines produced similar levels of OVA



**FIGURE 2.** Activation of OT-1 cells by pOVA-transfected cell lines. The NOR-10 muscle cell line and the IC-21 macrophage cell line were transfected with 20  $\mu$ g of pOVA in Fugene6 for 24 h. The transfected cells were fixed with paraformaldehyde and added to purified OT-1 cells from the spleen of OT-1/RAG1<sup>-/-</sup> mice. *A*, IFN- $\gamma$  production after 24 h of culture. *B*, Proliferation monitored by adding [<sup>3</sup>H]TdR to culture for 12 h. All data in this figure represent the mean  $\pm$  SD of at least three independently treated cell populations/group and were confirmed in three additional experiments. \*, *p* < 0.01 (compared with Fugene6 alone).

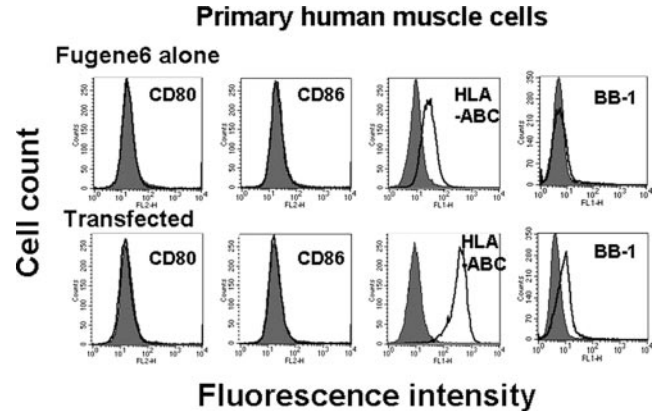


**FIGURE 3.** Activation of OT-1 cells by pOVA-transfected NOR-10 cells. *A*, Mitomycin C-treated spleen cells or NOR-10 cells were cultured with purified OT-1 cells in the presence of 100  $\mu\text{g}$  of OVA protein for 24 h. Results represent the average  $\pm$  SD level of IFN- $\gamma$  in culture supernatants from four independently studied samples per group. *B*, The cell lines shown were transfected with 20  $\mu\text{g}$  of pOVA in Fugene6 for 24 h. Transfected cells were then fixed and cultured with purified OT-1 cells. Results represent the average  $\pm$  SD of three independent cultures per group and repeated experiments yielded similar findings. \*,  $p < 0.05$  (compared with OT-1 and OVA culture group); \*\*,  $p < 0.005$  (compared with pOVA-transfected NOR-10 alone group).

(based on mRNA analysis, data not shown). These findings suggest that transfected NOR-10 cells directly activated Ag-specific CD8 T cells and that no indirect mechanism involving cross-presentation was involved.

#### Effect of plasmid transfection on MHC and costimulatory molecule expression by muscle cells

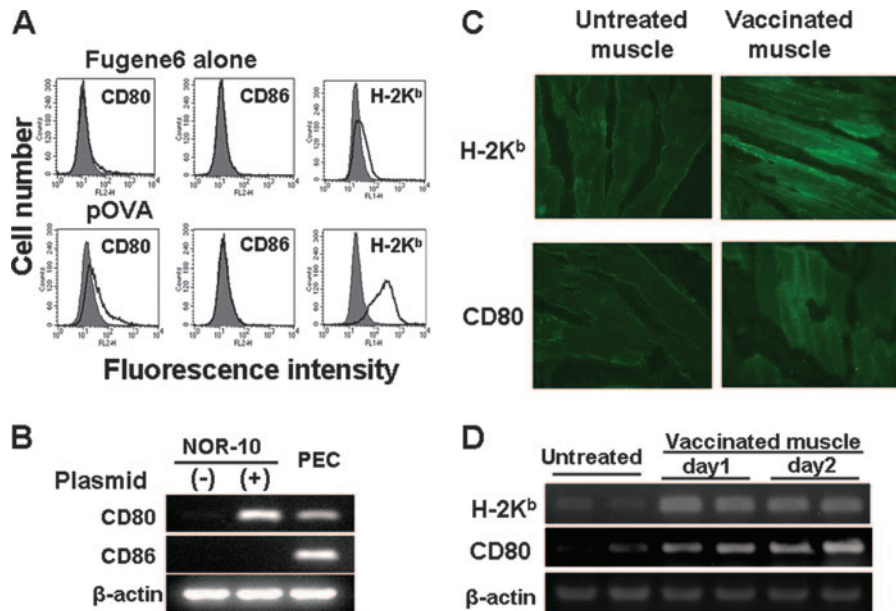
To efficiently activate CD8 T cells, Ag must be presented in the context of self MHC class I plus costimulatory molecules. Consistent with previous reports (24, 25), MHC class I and costimulatory molecule (B7.1 and B7.2) expression by the NOR-10 muscle cell line was low or undetectable by FACS and immu-



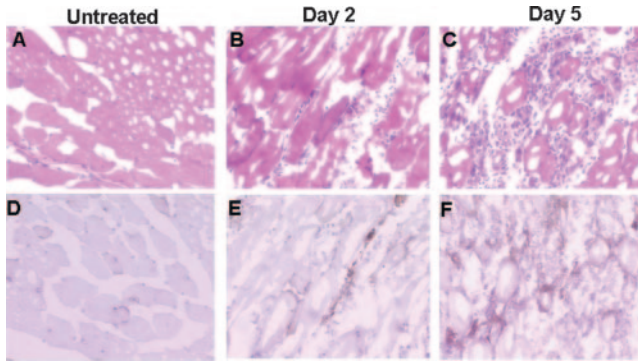
**FIGURE 5.** Effect of transfection on MHC and costimulatory molecule expression by human muscle cells. Primary human muscle cells were treated with Fugene6 alone (*upper panel*) or transfected with 20  $\mu\text{g}$  of vector plasmid in Fugene6 for 36 h (*lower panel*). Cells were stained with PE- or FITC-labeled anti-B7.1, B7.2, HLA-ABC, or BB-1 (open line) or isotype-matched control (gray), and analyzed by FACS. All experiments were repeated three times with similar results.

nohistochemical analysis (Fig. 4, *A* and *C*). However, when transfected with plasmid DNA, this muscle cell line significantly up-regulated expression of MHC class I and B7.1 (an effect not observed following treatment with Fugene 6 alone; Fig. 4*A*). These findings were confirmed in studies of B7.1 mRNA expression of plasmid-transfected NOR-10 cells, and immunohistochemistry and RT-PCR analysis of murine muscle cells at the site of DNA vaccination (Fig. 4, *B–D*).

The effect of plasmid transfection on primary human muscle cells was also examined. As in mice, unmanipulated human muscle cells had low or undetectable levels of MHC class I or



**FIGURE 4.** Effect of transfection on MHC and costimulatory molecule expression by NOR-10 and muscle cells. *A*, NOR-10 cells were treated with Fugene6 alone (*upper panels*) or 20  $\mu\text{g}$  of pOVA in Fugene6 for 36 h (*lower panels*). Cells were stained with PE- or FITC-labeled anti-B7.1, B7.2, or H-2K<sup>b</sup> Ab (open line) or isotype matched control (gray), and analyzed by FACS. *B*, NOR-10 cells were transfected with 20  $\mu\text{g}$  of plasmid in Fugene6. mRNA expression 12 h later was monitored by RT-PCR. Unstimulated peritoneal macrophages (PEC) acted as positive controls. C57BL/6 mice were immunized with 20  $\mu\text{g}$  of pOVA i.m. by electroporation. Muscle from the site of vaccination was removed. *C*, Sections at day 2 were stained for expression of H-2K<sup>b</sup> and B7.1. Binding of FITC-labeled Ab was examined by confocal microscopy ( $\times 25$ ). *D*, mRNA was extracted from muscle samples at days 1 and 2. mRNA expression was monitored by RT-PCR (two samples each group). All experiments were repeated three times with similar results.

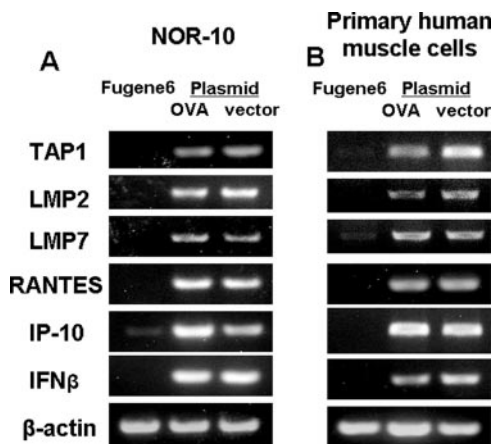


**FIGURE 6.** Histological analysis of muscle tissue from the site of pOVA electroporation. C57BL/6 mice were immunized with 20  $\mu\text{g}$  of pOVA i.m. by electroporation. Two and five days later, muscle from the site of vaccination was removed and stained with H&E (upper panels) or anti-CD8 (developed with 3,3'-diaminobenzidine and counterstained hematoxylin, lower panels). Magnification,  $\times 100$ . Data are representative of four independent experiments.

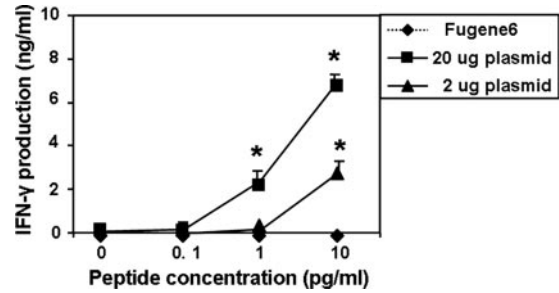
costimulatory molecule (B7.1, B7.2, and BB-1) expression (Fig. 5, upper panels). However, when transfected with plasmid, their expression of MHC class I and the alternative costimulatory molecule BB-1 increased significantly (Fig. 5, lower panels). These findings suggest that plasmid transfection may significantly improve the ability of both murine and human muscle cells to act as APCs.

*CD8<sup>+</sup> cells infiltrate DNA vaccinated muscles*

To examine whether plasmid-transfected muscle cells have the opportunity to come into contact with CD8 T cells in vivo, the site of vaccine electroporation was examined histologically. Infiltration of the vaccination site by inflammatory mononuclear cells was detected on day 1, with small numbers of CD8<sup>+</sup> T cells being observed by day 2 (Fig. 6, B and E, and data not shown). The inflammatory response increased substantially by day 5 and included large numbers of CD8<sup>+</sup> cells (Fig. 6, C and F). These findings are consistent with transfected muscle cells having the opportunity to directly activate CD8<sup>+</sup> T cells.



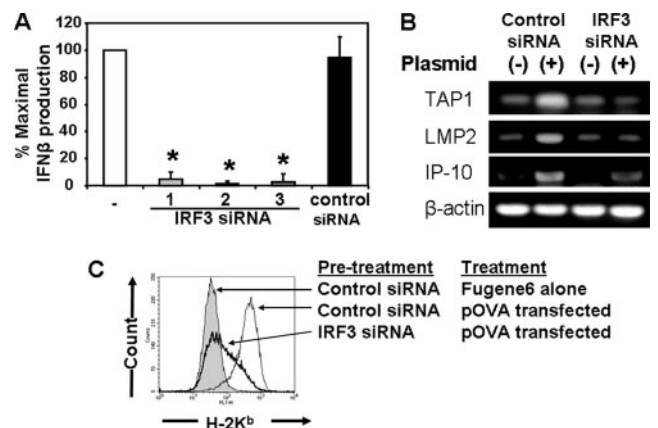
**FIGURE 7.** Gene expression induced by plasmid transfection. NOR-10 (A) and primary (B) human muscle cells were transfected with Fugene 6 alone or combined 20  $\mu\text{g}$  of pOVA or vector plasmid. mRNA levels were monitored 12 h later by RT-PCR. All experiments were repeated three times with similar results.



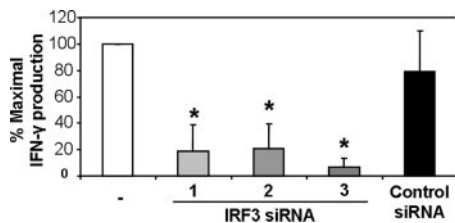
**FIGURE 8.** Activation of OT-1 cells by peptide-pulsed NOR-10 cells. NOR-10 cells were transfected with Fugene 6 plus 2 or 20  $\mu\text{g}$  of vector plasmid for 24 h. Increasing amounts of SIINFEKL peptide were added during the last 2 h of culture. The cells were fixed in paraformaldehyde and then cultured with OT-1 cells for 24 h. Results represent the average  $\pm$  SD of IFN- $\gamma$  (levels in culture supernatants from three independently evaluated cell populations per group and were confirmed in two additional experiments). \*,  $p < 0.005$  (compared with Fugene 6 alone in the correspondence peptide concentration).

*Effect of plasmid transfection on the expression of genes involved in Ag processing and presentation*

To present an endogeneously produced protein in the context of self MHC class I requires the expression of genes involved in Ag processing and presentation (26). Plasmid transfection of NOR-10 cells and primary human muscle cells resulted in a significant increase in the expression of the Ag-processing/presentation genes TAP1 and low m.w. proteins 2/7 (LMP2/7), genes encoding the cytokines/chemokines IFN- $\beta$ , IFN- $\gamma$ -inducible protein 10 (IP-10), and RANTES, and the VCAM-1 adhesion gene (Fig. 7 and data not shown). These effects were mediated by plasmid DNA transfection with either vector or pOVA and were also observed following transfection of the H-2<sup>k</sup>-expressing Sol8 muscle cell line. Consistent with the inability of pOVA-transfected CMT93 and LL/2 cells to stimulate OT-1 cells, transfection of those cell lines did not up-regulate expression of these genes (data not shown).



**FIGURE 9.** Activation of NOR-10 cells by plasmid transfection requires IRF3. NOR-10 cells were transfected with IRF3 or control siRNA in Lipofectamine RNAiMAX. Two days later, these cells were transfected with Fugene 6 plus 20  $\mu\text{g}$  of vector plasmid. A, Relative IFN- $\beta$  levels compared with cells pretreated with Lipofectamine RNAiMAX alone (mean  $\pm$  SD of four independent experiments, average IFN- $\beta$  production by cells pretreated with Lipofectamine RNAiMAX alone was 455 pg/ml). B, Gene expression levels determined by RT-PCR of mRNA isolated after 12 h of culture. C, Cells were stained after 36 h of culture for expression of H-2K<sup>b</sup> and analyzed by FACS. \*,  $p < 0.001$  (compared with Lipofectamine RNAiMAX alone).



**FIGURE 10.** IRF3 expression by transfected NOR-10 cells is required for OT-1 activation. NOR-10 cells were transfected with IRF3 or control siRNA in Lipofectamine RNAiMAX. Two days later, these cells were transfected with 20  $\mu$ g of pOVA in Fugene 6 for 24 h and then fixed in paraformaldehyde. OT-1 cells were added and IFN- $\gamma$  production was examined 24 h later. Relative IFN- $\gamma$  levels compared with cells pretreated with Lipofectamine RNAiMAX alone (average IFN- $\gamma$  production by cells pretreated with Lipofectamine RNAiMAX alone was 8.0 ng/ml). Results represent the mean  $\pm$  SD of four independent experiments. \*,  $p < 0.001$  (compared with Lipofectamine RNAiMAX alone group).

Thus, the introduction of plasmid DNA into the cytoplasm of muscle cells had pleiotropic effects that together improved their ability to present Ag to CD8 T cells.

This finding was further investigated by analyzing the ability of NOR-10 cells to present SIINFEKL peptide to OT-1 cells. As seen in Fig. 8, transfected NOR-10 cells presented SIINFEKL to Ag-specific CD8 cells in a concentration-dependent manner. This activity could not be attributed to APC contamination of the OT-1 population, because 1) mock-transfected NOR-10 cells did not present peptide when mixed with OT-1 cells and 2) the efficiency of peptide presentation increased with the concentration of transfecting plasmid.

#### *Muscle cell activation by plasmid DNA involves IRF3*

Stetson et al. (27) demonstrated that intracytoplasmic dsDNA triggered the production of IFN- $\beta$  by murine embryonic fibroblasts through an IRF3-dependent process. To determine whether the same pathway contributed to improving Ag presentation by NOR-10 cells, the effect of reducing IRF3 expression by treatment with siRNA was examined. Each of three different siRNA constructs designed to reduce IRF3 gene expression significantly impaired the production of IFN- $\beta$  and the up-regulation of class I and costimulatory molecules induced by plasmid transfection (Fig. 9). Concomitantly, the ability of NOR-10 cells transfected with pOVA to stimulate OT-1 cells was significantly inhibited by the siRNA-dependent reduction in IRF3 (Fig. 10). “Control” siRNA had no effect on any of these processes.

## Discussion

Early studies demonstrated that professional APCs (such as DCs) play a critical role in DNA vaccine-mediated immunity (5–12, 28). Subsequent research indicated that transfected DCs alone account for only a fraction of the total induced response, with nonprofessional APCs at the site of DNA vaccination (including keratinocytes and muscle cells) impacting the magnitude, duration, and nature of humoral and cellular response (13, 15, 29). One widely accepted interpretation of these findings holds that Ag released by transfected muscle cells or keratinocytes is taken up and cross-presented by professional APCs (8–12). However, there is an alternative explanation: that transfected muscle cells directly present endogenously produced Ag to immune cells. Such a role for transfected muscle is consistent with the observation that electroporation, which increases muscle cell transfection efficiency by  $\sim$ 100-fold, also induces significantly stronger immune responses (16–20). A direct role for transfected muscle cells is also supported

by studies indicating that certain nonimmune cells develop the ability to present Ag when dsDNA is introduced into their cytoplasm (21, 30, 31).

Current in vitro and in vivo studies support the concept that plasmid transfection induces muscle cells to increase their expression of MHC class I and costimulatory molecules and to up-regulate the machinery needed to process and present Ag (Figs. 4 and 5). As a result, pOVA-transfected muscle cells (and the NOR-10 muscle cell line) gain the ability to activate Ag-specific CD8 T cells (Figs. 1–3). These events proceed via an IRF3-dependent pathway and involve the up-regulation of IFN- $\beta$  (Figs. 9 and 10). Earlier studies showed that introducing dsDNA into the cytoplasm of many cell types (including fibroblasts, hepatocytes, pancreatic cells, and muscle cells) stimulated the production of IFN- $\beta$  (21, 30, 31). IFN- $\beta$  serves to up-regulate the expression of TAP, LMP, and MHC class I molecules through both autocrine and paracrine pathways, thereby improving the ability of even nonprofessional APCs to process and present Ag (32, 33). We postulate that these events, observed following DNA vaccination, actually evolved to bolster the host’s response to viral infection, because recognition of viral DNA would improve the infected cell’s ability to present foreign Ag to host T cells and thus eradicate the pathogen.

It is well-established that Ag produced by transfected muscle cells can be taken up and cross-presented by professional APCs (8–12). However, current experiments suggest that this mechanism may not account for all of the immune activation observed following transfection of nonprofessional APCs. Specifically: 1) pOVA-transfected NOR-10 cells effectively stimulate MHC-matched OT-1 cells to proliferate and secrete cytokine (Figs. 2 and 3); 2) OT-1 cells (alone or in combination with nontransfected NOR-10 cells) do not respond when cultured with free OVA (Fig. 3A); 3) pOVA-transfected Sol8 and NOR-10 muscle cells produce similar levels of OVA, but the Sol8 cells cannot activate OT-1 cells across an MHC barrier (Fig. 3B); and 4) NOR-10 cells transfected with vector alone presented SIINFEKL peptide to OT-1 cells in a concentration-dependent manner, whereas nontransfected NOR-10 cells could not (Fig. 8). These findings are consistent with transfected muscle cells directly mediating OT-1 cell activation without the necessity of secreted OVA being cross-presentation by contaminating DCs.

The literature suggests that muscle cells do not normally express costimulatory molecules and thus cannot mediate Ag-specific immunity (24, 25, 34). Consistent with this premise, we found that unmanipulated human and murine muscle cells have low or undetectable levels of MHC class I and costimulatory molecule (Figs. 4 and 5). However, conditions have been reported under which muscle cells are triggered to express such molecules. For example, the expression of MHC class I, B7.1, B7.2, CD40, and BB-1 has been observed in vitro following cytokine stimulation of primary muscle cells and in vivo in patients with myositis (an autoimmune disease of the muscle) (25, 34–37). Current findings indicate that plasmid transfection also up-regulates MHC class I expression by human and murine muscle cells. Of interest, transfection induced the expression of B7.1 by murine muscle but of BB-1 by human muscle cells (Figs. 4 and 5). Although BB-1 can function as costimulatory molecule (it induces IL-2 production by T cells), the precise function of this molecule is not well-established (38, 39). Whether this difference in costimulatory molecule expression underlies the greater immunogenicity of DNA vaccines in mice vs humans requires further examination.

Previous reports indicate that certain somatic cell types not typically considered to be elements of the immune system possess the ability to present Ag to T cells. These include fibroblasts, keratinocytes, Schwann cells, endothelial cells, and vascular smooth muscle

cells (33, 40–46). When placed in a suitable cytokine environment (characteristic of an inflammatory response, viral infection, autoimmune syndrome, or cancer and typified by the presence of type 1 IFNs), these cells can be converted into facultative APCs. For example, the MC57g fibroblast cell line acquires the ability to present Ag to T cells (41, 47) and produce IFN- $\beta$  when transfected with pOVA (data not shown).

The mechanism by which DNA transfection activates nonimmune cells is poorly understood. Current results are consistent with IRF3 being involved in this process, because treatment of NOR-10 cells with siRNA targeting IRF3 significantly reduced the ability of transfected cells to up-regulate MHC and costimulatory molecule expression or present Ag to OT-1 cells (Figs. 9 and 10). Of interest, we found that several different human and murine muscle cell lines (including NOR-10, skmc, Sol8, and C2C12) responded similarly to plasmid transfection, whereas nearly two-thirds of other cell types did not (data not shown). This observation may explain why i.m. injection is one of the more effective routes for DNA vaccine delivery.

Muscle cells transfected with an Ag-encoding DNA plasmid transcribe and translate the encoded gene (48). Current results indicate that transfection also stimulates muscle cells to 1) up-regulate expression of MHC class I and costimulatory molecules (genes needed to process and present the endogenously produced Ag; Figs. 4, 5, and 7) and 2) produce chemokines and adhesion molecules that impact the migration and activation of T cells (Fig. 7). As CD8<sup>+</sup> T cells as well as other inflammatory cells are present at the site of plasmid electroporation, opportunity for transfected muscle cells to interact with these T cells is present in vivo (Fig. 6 and Refs. 49–52). Taken together, these findings support the possibility that DNA-vaccinated muscle cells participate directly in the activation of Ag-specific CD8 T cells. However, these studies cannot eliminate the alternative: that professional APCs cross-present muscle-derived Ag under physiologic conditions in vivo, because such APCs are needed to initiate the immune response and thus cannot be removed from the system (8–12). Hopefully, further study will identify the receptor(s) and signaling cascade(s) involved in these processes and clarify the degree to which transfected muscle cells contribute to the overall immunogenicity of DNA vaccines.

## Disclosures

The authors have no financial conflict of interest.

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