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Induction of Human Dendritic Cell Maturation Using Transfection with RNA Encoding a Dominant Positive Toll-Like Receptor 4

Robin M. Cisco,* Zeinab Abdel-Wahab, Jens Dannull,* Smita Nair,* Douglas S. Tyler,* Eli Gilboa,* Johannes Vieweg,† Yehia Daaka,* and Scott K. Pruitt2*†

Maturation of dendritic cells (DC) is critical for the induction of Ag-specific immunity. Ag-loaded DC matured with LPS, which mediates its effects by binding to Toll-like receptor 4 (TLR4), induce Ag-specific CTL in vitro and in vivo in animal models. However, clinical use of LPS is limited due to potential toxicity. Therefore, we sought to mimic the maturation-inducing effects of LPS on DC by stimulating TLR4-mediated signaling in the absence of exogenous LPS. We developed a constitutively active TLR4 (caTLR4) and demonstrated that transfection of human DC with RNA encoding caTLR4 led to IL-12 and TNF-α secretion. Transfection with caTLR4 RNA also induced a mature DC phenotype. Functionally, transfection of DC with caTLR4 RNA enhanced allostimulation of CD4+ T cells. DC transfected with RNA encoding the MART (Melan-A/MART-1) melanoma Ag were then used to stimulate T cells in vitro. Cotransfection of these DC with caTLR4 RNA enhanced the generation of MART-specific CTL. This CTL activity was superior to that seen when DC maturation was induced using either LPS or a standard mixture of cytokines (TNF-α, IL-6, IL-1β, and PGE2). We conclude that transfection of DC with RNA encoding a functional signaling protein, such as caTLR4, may provide a new tool for studying TLR signaling in DC and may be a promising approach for the induction of DC maturation for tumor immunotherapy.


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Materials and Methods

Cell lines and reagents

HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 plus 10% FCS. The cell
line 293-luc-xB, a stable transfectant of HEK293 containing an NF-kB-dependent luciferase reporter plasmid, was created by Lipofectamine-mediated DNA transfection followed by geneticin (Invitrogen, Carlsbad, CA) selection. TAP-deficient, HLA-A0201 T2 cells were a gift from Dr. P. Cresswell (Yale University, New Haven, CT) and were maintained in serum-free AIM-V medium (Invitrogen). Melanoma cell lines DM6 (Melan-A/MART1+, HLA-A2+) and DM150 (Melan-A/MART1+, HLA-A2+) were derived from fresh surgical melanoma specimens and were gifts from Dr. H. Seigler (Duke University Medical Center, Durham, NC). Melanoma cell line 397mel (HLA-A1/A25, MART-1+) was a gift from Dr. S. Rosenberg (National Cancer Institute, Bethesda, MD). Hybridomas BB7.2 and MA9 (both anti-HLA-A1) Gap 3 (anti-A3), and A11.1 (anti-A11) mAbs were obtained from the American Type Culture Collection. A purified mAb against HLA-A1 was purchased from One Lambda (Canoga Park, CA).

**Monocyte-derived DC**

Human DC were generated from adherent monocytes as previously described (16, 19). Briefly, PBMC were isolated by Ficoll gradient centrifugation of buffy coat lymphocytes purchased from the American Red Cross. A limited HLA-A phenotype was determined by flow cytometry of PBMC using HLA-specific mAbs. PBMC not immediately used for DC production were cryopreserved in 90% human AB serum (Valley Labs, Winchester, VA)/10% DMSO. Fresh (or thawed) PBMC were plated in tissue culture flasks in AIM-V serum-free medium. After a 2-h plastic adherence step, nonadherent lymphocytes were gently washed away and adherent cells were used for all experiments in serum-free medium (X-VIVO-15; BioWhittaker, Walkersville, MD) supplemented with 1000 U/ml GM-CSF (Immunix, Thousand Oaks, CA) and 1000 U/ml IL-4 (R&D Systems, Minneapolis, MN). After 5–6 days in culture, the immature DC were evaluated by MOPS-formaldehyde agarose gel electrophoresis to confirm the generation of the chimeric caTLR4 protein. The plasmid used for the in vitro transcription of green fluorescent protein (GFP) RNA has been previously described (15, 16, 21). The plasmid encoding MART with an A27L aa mutation was created by overlapping PCR of cloned wild-type MART using primers containing the desired DNA mutation and confirmed by sequencing, Western blotting, and functional studies (22).

**DNA constructs**

A chimeric caTLR4 construct consisting of the nerve growth factor receptor leader sequence fused to a FLAG epitope tag followed by a truncated TLR4 molecule was created using PCR cloning and standard molecular biology techniques. The truncation of human TLR4 was located between aa 2–35. The TLR4 molecule was created using PCR cloning and standard molecular biology techniques. The generation of the chimeric caTLR4 protein. The plasmid used for the in vitro transcription of green fluorescent protein (GFP) RNA has been previously described (15, 16, 21). The plasmid encoding MART with an A27L aa mutation was created by overlapping PCR of cloned wild-type MART using primers containing the desired DNA mutation and confirmed by sequencing, Western blotting, and functional studies (22).

**In vitro transcription of RNA**

For the production of RNA, plasmid DNA was digested with the restriction enzyme SpeI at a site present at the 3’ end of the 64-adenosine segment of the modified pCDNA3 plasmid. After ethanol precipitation and resuspension in water, the digested DNA was used as template for in vitro RNA transcription using a commercial kit (mMessage machine; Ambion, Austin, TX). After DNase treatment, the resultant RNA was purified, quantitated, evaluated by MOPS-formaldehyde agarose gel electrophoresis to confirm purity containing a full-length RNA, and then stored at −80°C until use. LPS levels of the in vitro-transcribed caTLR4 RNA were below detection level (<0.5 endotoxin units/ml), as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Falmouth, MA).

In several experiments, caTLR4 RNA was digested with RNaseI, (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions and then boiled. This RNase-treated caTLR4 RNA was then used for electroporation of DC as described below.

**DC transfection**

Immature human DC were electroporated with RNA as previously described (16). Briefly, the immature DC were harvested and washed three times in cold PBS, then resuspended in cold Opti-MEM (Invitrogen) at 1 × 10^6/100 μl. Two hundred-microliter aliquots of cells were mixed in a 2-mm gap cuvette along with a total of 20 μg RNA. When a combination of two RNAs was used, 10 μg of each RNA was used. The cells and RNA were then subjected to 300 V for 500 μs in a square wave electrophorator (BTX, San Diego, CA). The electroporated DC were immediately transfected to tissue culture wells with fresh X-VIVO-15 medium containing IL-4 and GM-CSF. Control DC were electroporated with GFP RNA, which allowed for assessment of electroporation transfection efficiency using fluorescence microscopy.

**Induction of DC maturation**

Maturation agents were added to the DC culture medium —2–4 h after DC were electroporated with RNA. The maturation agents used included poly(lC) (12.5 μg/ml; Sigma-Aldrich, St. Louis, MO), LPS (5 μg/ml; Sigma-Aldrich), or cytokine mixture (18) consisting of TNF-α (5 ng/ml), rH-18 (5 ng/ml), rHL-6 (150 ng/ml) (all from R&D Systems), and PGE_2 (1 μg/ml; Sigma-Aldrich).

**Cytokine secretion**

DC culture supernatants were harvested 24 and 48 h after transfection and stored at −20°C until use. IL-12 p70 levels and TNF-α concentrations were assessed using commercially available ELISA kits (R&D Systems).

**Allosstimulatory capacity of RNA-transfected DC**

To evaluate the ability of DC electroporated with caTLR4 RNA to stimulate CD4+ T cells, we performed allogeneic MLRs using RNA-electroporated DC as stimulators. In these experiments, DC were electroporated with RNA, and after 1 h in culture, maturation stimuli were added as indicated. After an additional 3 h of incubation, the DC were washed and resuspended in medium and irradiated (3000 rad). Allogeneic CD4+ T cells (1 × 10^5), isolated by magnetic bead separation (Miltenyi Biotech, Auburn, CA) from PBMC, were then stimulated at a 1:10 (responder:stimulator) ratio with the RNA-electroporated DC. After 4 days in culture, 1 μCi of [3H]thymidine (NEN, Boston, MA) was added to each well and incorporated radioactivity was measured after 16 h using liquid scintillation counting. GFP RNA-electroporated DC were used to establish the baseline proliferation induced by immature electroporated DC. GFP RNA-electroporated DC treated with various maturation stimuli served as a positive control for the stimulation of T cell proliferation by mature DC. DC alone and T cells alone were used as negative controls.

**In vitro T cell cultures**

For T cell cultures, only DC from HLA-A2+ donors were used, as determined by flow cytometry of donor PBL using the anti-HLA mAbs. Approximately 4 h after transfection, DC were added to autologous nonadherent lymphocytes (as a source of T cells) at a 1:10 (stimulator:responder) ratio in tissue culture medium consisting of RPMI 1640 plus 10%FCS supplemented with antibiotics and 10 μM 2-ME. IL-2 at 20 U/ml was added beginning on day 7 and then twice per week thereafter. Each T cell culture was restimulated with RNA-transfected DC at 7- to 10-day intervals for a total of three to four stimulations. As a positive control for maturation, we used our current standard regimen consisting of exposure of RNA-electroporated DC to cytokine mixture beginning 1 h after transfection. After 4 h in culture, DC were washed and added to the T cell cultures. As a negative control for maturation, DC were left untreated after RNA transfection and added to T cell cultures in the same fashion. Beginning ~7 days after the final stimulation, Ag specificity and T cell effector function were evaluated.

**Evaluation of MART peptide/HLA-A2 tetramer-positive T cells**

A PE-labeled class I HLA-A2 tetramer loaded with the Melan-A23 A27L immunodominant peptide analog ELAGIGILTV, in combination with a FITC-labeled anti-CD8 was purchased from Beckman Coulter (San Diego, CA) and was used according to the manufacturer’s instructions. Staining was evaluated using flow cytometry.

**Ag-specific T cell IFN-γ release**

In vitro-stimulated T cells were evaluated for Ag-specific effector function using an IFN-γ ELISPOT assay as previously described (16). This assay was performed at a stimulator:responder ratio of 1:10 in triplicate. T2 cells were evaluated for Ag-specific effector function using an IFN-γ ELISPOT assay as previously described (16). This assay was performed at a stimulator:responder ratio of 1:10 in triplicate. T2 cells were obtained from the manufacturer’s instructions. Staining was evaluated using flow cytometry.
loaded exogenously with either MART A27L peptide or M1 peptide (influenza matrix protein 1 HLA-A2-restricted immunodominant peptide GILGFVFTL, as a negative control) were used as targets. In addition, melanoma cell lines, previously HLA-typed and evaluated for MART expression, were used as targets. Plates were scanned and images were counted by an ImmunoSpot Series Analyzer (Cellular Technology, Cleveland, OH).

Determination of cytotoxicity

$^{31\text{Cr}}$ release assays were performed in triplicate using established methods as previously described (23). Percent specific lysis was calculated from the equation: (experimental release $-$ spontaneous release)/maximum release $-$ spontaneous release (targets in medium)) $\times 100$. Spontaneous release was $<15\%$ of maximum release. SDs for triplicate wells were $<5\%$.

Results

Construction and evaluation of caTLR4

We wanted to demonstrate the functional consequences of transfecting cells with constitutively active chimeric TLR4. To generate a constitutively active molecule, we deleted the extracellular portion of TLR4 (aa 1–620) (5). This deletion was based on a mutational analysis of Drosophila Toll in which the deletion of the homologous portion of the molecule created a constitutively active Toll (20). After confirming the identity of our construct by DNA sequencing, we demonstrated that HEK293 cells transfected with this construct expressed a FLAG-tagged protein of appropriate molecular mass by Western blotting of cell lysates (data not shown). Transfection of HEK293 cells with the plasmid containing this caTLR4 construct led to the activation of NF-$\kappa$B, demonstrating that our chimeric caTLR4 DNA encoded a functionally active protein (data not shown).

Although these transfection experiments using plasmid DNA showed that the caTLR4 plasmid DNA had constitutive NF-$\kappa$B-activating activity, our main goal was to determine whether RNA transcribed from this plasmid DNA could be transfected into cells and be translated into a functionally active signaling protein. We therefore used the caTLR4 plasmid DNA as a template for in vitro RNA transcription and evaluated whether transfection with this caTLR4 RNA would activate NF-$\kappa$B. For these experiments, we created a stable transfectant of HEK293 cells containing a NF-$\kappa$B dependent luciferase reporter (293-luc- NF-$\kappa$B). When 293-luc-$\kappa$B cells were transfected with caTLR4 RNA, NF-$\kappa$B activation was induced (Fig. 1), thus confirming that transfected caTLR4 RNA was translated into a functional signaling protein.

Having shown that we could electroporate a cell line with caTLR4 RNA and generate a functional protein, we wanted to demonstrate activation of cellular signaling in human monocye-derived DC. To this end, we transfected immature DC with caTLR4 RNA and used Western blotting of DC cell lysates to evaluate activation of p38 MAPK, a signaling pathway known to be important in DC maturation (7). As shown in Fig. 2, p38 MAPK was phosphorylated to a greater extent after transfection with caTLR4 RNA, compared with the control GFP RNA. As a positive control for maturation in these experiments, DC were exposed to poly(I:C), a synthetic analog of dsRNA known to mediate maturation via TLR3 (24). These results further demonstrate that transfected caTLR4 RNA is translated intracellularly within DC into a functional signaling protein. Our next step was to evaluate the consequences of transfecting DC with caTLR4 RNA with respect to DC maturation.

Effect of caTLR4 RNA transfection on DC: phenotypic and functional analysis

DC maturation was evaluated by phenotypic analysis of cell surface markers after transfection with caTLR4 RNA. Fig. 3 shows that transfection of DC with caTLR4 RNA induced surface expression of the maturation marker CD83 and stimulated increased surface expression of CD40 and of costimulatory molecules CD80 and CD86. Expression of these phenotypic markers of maturation in caTLR4 RNA-transfected DC was comparable, or superior, to that induced by cytokine mixture or LPS in DC transfected with control RNA. In addition, Fig. 3 also demonstrates that treatment of caTLR4 RNA with RNase abolished its maturation-inducing effects on the DC phenotype, indicating that the effects observed after caTLR4 RNA transfection were not due to LPS contamination. These results confirmed that transfection of DC with caTLR4 RNA induced phenotypic maturation of DC. The next step was to measure the functional consequences of caTLR4 RNA-induced DC maturation. Various parameters of DC function were examined, starting with cytokine secretion.

The effect of caTLR4 RNA transfection on DC cytokine secretion was determined by measuring cytokine levels in culture supernatants using ELISA. As shown in Fig. 4A, transfection with caTLR4 RNA induced the secretion of both IL-12 (p70) and TNF-$\alpha$. As a control for DC maturation, we exposed the DC to either LPS or cytokine mixture. As demonstrated, DC transfected with caTLR4 RNA secreted TNF-$\alpha$ at levels comparable to those

![FIGURE 2](http://www.jimmunol.org/) Transfection of DC with caTLR4 RNA activates p38 MAPK. Human DC were transfected with the indicated RNA and left untreated or were exposed to poly(I:C) (PIC) to induce maturation. Forty-eight hours later, cell lysates were prepared, subjected to SDS-PAGE, and analyzed for expression of phospho-p38 (P-p38) and total p38 by Western blotting. Equal amounts of total protein were loaded in each lane. Positive staining is seen for DC samples treated with poly(I:C) as well as for DC transfected with caTLR4 RNA. The results are representative of two experiments.
induced by LPS (Fig. 4A). Similarly, as shown in Fig. 4B, LPS treatment and caTLR4 RNA transfection of DC induced comparable levels of IL-12. Thus, consistent with functional DC maturation, electroporation of caTLR4 RNA into DC induced the secretion of immunostimulatory cytokines. When caTLR4 RNA digested with RNase was used for DC electroporation, the induction of TNF-α (Fig. 4C) and IL-12 (Fig. 4D) was abolished, confirming that the cytokine secretion induced by caTLR4 RNA transfection was not due to contaminating LPS.

We next wanted to assess the effect of caTLR4 RNA transfection on the ability of DC to stimulate helper T cells. We therefore evaluated the ability of RNA-transfected DC to stimulate proliferation of allogeneic CD4+ T cells. As demonstrated in Fig. 5, caTLR4 RNA-transfected DC were more efficient stimulators of T
cell proliferation, when compared with DC transfected with GFP RNA. The stimulation of proliferation seen with caTLR4-transfected DC was comparable to and in some cases superior to that seen with DC stimulators matured with the cytokine mixture after transfection. In two additional experiments, the enhanced allostimulation observed for DC electroporated with caTLR4 RNA was abolished when the caTLR4 RNA was treated with RNase before electroporation (data not shown). This further confirmed that the effects observed after caTLR4 RNA transfection were not due to contaminating LPS. These results, along with the phenotype and cytokine secretion data, demonstrate that transfection of DC with caTLR4 RNA induced DC maturation that was comparable to or superior to that induced with the clinically used cytokine mixture or LPS.

A hallmark of mature DC function is the ability to stimulate naive CTLs, a function critical for generating antitumor immunity. We therefore evaluated whether transfection with caTLR4 RNA would enhance the ability of DC to stimulate an Ag-specific effector T cell response in vitro. T cells were stimulated with DC transfected with MART RNA, as a tumor Ag, and caTLR4 RNA. The highest levels of MART peptide-specific tetramer-positive CD8\(^+\) T cells were induced by MART RNA-transfected DC stimulators that were matured with caTLR4 RNA (Fig. 6 and Table I).

We further evaluated the function of DC transfected with MART RNA and caTLR4 RNA by measuring IFN-\(\gamma\) secretion and cytolytic activity of T cells stimulated with these DC. To measure IFN-\(\gamma\) secretion, we used the ELISPOT assay. DC transfected with MART RNA and caTLR4 RNA stimulated Ag-specific IFN-\(\gamma\) secretion at levels comparable to that induced by MART RNA-transfected DC stimulators matured with the cytokine mixture (Fig. 7). As shown, these T cells specifically recognized both T2 cells loaded with MART A27L peptide and DM6 cells, an HLA-A2\(^*\)MART\(^+\) melanoma cell line.

Finally, we wanted to assess the ability of the T cells stimulated with MART and caTLR4 RNA-transfected DC to lyse MART-expressing targets. As shown in Fig. 8, T cells stimulated with MART RNA-transfected DC and matured using caTLR4 RNA were the most effective at lysing target cell lines in a MART Ag-specific manner. These results suggest that the induction of DC maturation with caTLR4 RNA transfection is highly effective in stimulating the generation of Ag-specific effector T cells, and may be superior to maturation induced using the clinically utilized cytokine mixture or LPS.

**Discussion**

DC maturation is critical for effective stimulation of Ag-specific effector T cell responses (1). Experimentally, a variety of agents have been used to induce DC maturation, including monocyte-conditioned medium (25), cytokine mixture (consisting of TNF-\(\alpha\), IL-6, IL-1\(\beta\), and PGE\(_2\)) (18), poly(I:C) (a synthetic analog of dsRNA) (24), CpG oligonucleotides (26, 27), and LPS (6). These latter three agents mediate their effects on target cells through binding to endogenous TLRs (TLR3, 9, and 4, respectively) (5, 24, 28, 29).

The use of LPS for the induction of DC maturation in clinical immunotherapy is unlikely because of toxicity (potentially leading to toxic shock) and batch-to-batch variability. However, using in

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**FIGURE 5.** Transfection with caTLR4 RNA enhances the allostimulatory activity of DC. Human DC were transfected with either caTLR4 RNA or with GFP RNA as a control. The GFP RNA-transfected DC were then either left untreated (negative control) or exposed to a maturation stimulus (M, which for MLR-A was LPS and for B through E was the cytokine mixture). After a 4-h incubation, these DC were irradiated (3000 rad) and cultured with allogeneic CD4\(^+\) T cells. Proliferation was assessed 4 days later using \(^{3}H\)thymidine incorporation.

**FIGURE 6.** Enhanced generation of MART HLA-A2 tetramer-positive CD8\(^+\) T cells after stimulation with DC transfected with MART RNA plus caTLR4 RNA. The effector T cell populations generated after serial stimulation with RNA-transfected DC were stained with a MART peptide-loaded HLA-A2 tetramer. MART peptide-specific CD8\(^+\) T cells were then identified using flow cytometry. The percentages indicate the fraction of tetramer-positive cells within the entire population of T cells. The results are representative of five experiments.
vitro and in vivo animal models, DC induced to mature using LPS and then loaded with MHC-restricted antigenic peptides are highly effective in stimulating Ag-specific T cell responses (1, 10, 11). By using caTLR4 RNA to induce DC maturation, we sought to stimulate the pathways that mediate the effects of LPS on DC without using this exogenous potentially toxic agent.

When LPS binds to TLR4, the adaptor molecules myeloid differentiation factor 88, Toll-IL-1R domain-containing adapter protein/myeloid differentiation protein 88 adapter-like, and Toll-IL-1R domain-containing adapter inducing IFN-β are recruited to the intracellular Toll-IL-1R domain of TLR4, leading to activation of multiple signaling pathways, including NF-κB and p38 MAPK (30). Using inhibitors of these signaling pathways, Ardeshna et al. (6) showed that both are critically important for optimal DC maturation by LPS. As demonstrated in Figs. 1 and 2, transfection of cells with caTLR4 RNA activates both NF-κB and p38 MAPK, similar to the known effects of LPS on intracellular signaling.

The ability of LPS-matured DC to generate effector T cells may in part be mediated by LPS-induced IL-12 secretion. IL-12 has been demonstrated to stimulate Th cell proliferation and biasing the Th response toward Th1. As assessed by the ability to stimulate proliferation of allogeneic CD4+ T cells, DC transfected with caTLR4 were comparable, or even superior, to DC transfected with control RNA and then matured using either LPS or cytokine mixture (Fig. 5).

Although DC transfection with caTLR4 RNA clearly induces maturation, one of our most important findings is that MART RNA-transfected DC, when matured using caTLR4 RNA transfection, are superior inducers of anti-MART effector T cells. When compared with the use of cytokine mixture as a maturation inducer, caTLR4 RNA-transfected DC stimulated the generation of higher numbers of MART-specific effector T cells as assessed by both tetramer binding (Fig. 6 and Table I) and IFN-γ secretion (Fig. 7). In

*The effector T cell populations generated after serial stimulation with RNA-transfected DC were stained with a MART peptide-loaded HLA-A2 tetramer. MART peptide-specific CD8+ T cells were then identified using flow cytometry. The percentages indicate the fraction of tetramer-positive cells within the entire population of T cells.

![Table 1](https://example.com/table1)

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![Figure 7](https://example.com/figure7)

**FIGURE 7.** IFN-γ ELISPOT of T cells stimulated with DC transfected with MART and TLR4 RNA. Autologous T cells were serially stimulated with DC transfected with the indicated combinations of RNA. As a positive control for maturation induction, RNA-transfected DC were treated with cytokine mixture (CC) before being added to T cell cultures. After three serial stimulations, IFN-γ ELISPOT was performed using melanoma cell line targets DM6 (MART+, A2+) and 397mel (A3+, A2+, MART+) as well as T2 cells loaded with either the HLA-A2-restricted immunodominant MART A27L peptide or influenza M1 peptide (as a negative control). These results are representative of four experiments.

![Figure 8](https://example.com/figure8)

**FIGURE 8.** MART Ag-specific lytic activity of T cells stimulated with caTLR4-transfected DC. After three serial stimulations with DC transfected with the indicated RNA, T cells were evaluated for Ag-specific lytic activity using a ⁵¹Cr release assay. Targets included melanoma cell lines DM6 (MART+, A2+), DM150 (MART+, A2+), and DM150 loaded with MART A27L peptide, as well as T2 cells loaded with either MART A27L peptide or influenza M1 peptide (as a negative control). These results are representative of three separate experiments, each with similar results.
addition, T cells stimulated with DC transfected with MART RNA and induced to mature using caTLR4 RNA transfection had the highest lytic activity against melanoma cells expressing MART (Fig. 8). Thus, the induction of DC maturation using caTLR4 transfection might have potential clinical applications for the stimulation of anti-tumor effector T cell responses during immunotherapy.

The use of caTLR4 RNA transfection to induce DC maturation has several additional practical advantages with respect to clinical applications for DC-based tumor immunotherapy. First, RNA transfection using electroporation is a simple procedure that eliminates the need for potentially toxic transfection reagents (such as lipid modifiers) that have not been approved for clinical use. Second, RNA encoding tumor Ags can be cotransfected, along with caTLR4 RNA, into DC for tumor Ag loading. Third, RNAs encoding additional signaling molecules involved in specific signaling pathways could potentially be combined to enhance certain aspects of DC maturation. We are currently developing constitutively active mutants of other TLRs as well as of downstream signaling pathways that have not been approved for clinical use. Second, RNA encoding tumor Ags can be cotransfected, along with caTLR4 RNA, into DC for tumor Ag loading. Third, RNAs encoding additional signaling molecules involved in specific signaling pathways could potentially be combined to enhance certain aspects of DC maturation. We are currently developing constitutively active mutants of other TLRs as well as of downstream signaling pathways that have not been approved for clinical use.

The use of constitutively active signal molecule RNA also has potential applications for more fully elucidating the role of specific TLRs in the induction of DC maturation. Constitutively active forms of several other TLRs that we are currently developing will be ideal for this purpose. As an example, dsRNA, the ligand for TLR3, has many additional effects on cellular signaling that are not mediated directly through binding to TLR3 (33). An RNA encoding a constitutively active TLR3 will allow us to specifically evaluate and manipulate dsRNA-mediated maturation-inducing effects in DC that are TLR3 mediated.

In summary, we show that transfection of human monocyte-derived DC with caTLR4 RNA induces DC maturation. Both phenotype and based on cytokine secretion, as well as ability to stimulate proliferation of allogenic CD4+ T cells, the DC maturation induced with caTLR4 RNA transfection is comparable or even superior to that induced using either LPS or the clinically utilized cytokine mixture of TNF-α, IL-1β, IL-6, and PGE2. Most importantly, DC transfected with RNA encoding the MART melanoma Ag most effectively induced MART-specific effector T cells when maturation was induced by cotransfection with caTLR4 RNA. The induction of DC maturation using caTLR4 RNA transfection may therefore be a promising approach for DC-based clinical tumor immunotherapy.

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