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*J Immunol* 2003; 171:6275-6282; doi: 10.4049/jimmunol.171.11.6275

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Injection of Immature Dendritic Cells into Adjuvant-Treated Skin Obviates the Need for Ex Vivo Maturation

Smita Nair,* Catherine McLaughlin,* Alon Weizer,† Zhen Su,† David Boczkowski,* Jens Dannull,† Johannes Vieweg,† and Eli Gilboa2*

A key and limiting step in the process of generating human monocyte-derived dendritic cells (DC) for clinical applications is maturation. In the setting of immunotherapy, DC are matured ex vivo by culturing them with various agents that mimic the conditions encountered at a site of inflammation. This study examined whether the ex vivo DC maturation step could be replaced by maturing DC in situ by injecting immature DC into sites pre-exposed to agents that induce a microenvironment conducive to in situ maturation of the injected DC. The hypothesis was that recapitulation of the physiological conditions occurring during pathogen infection would lead to optimal conditions for DC maturation, migration, and function. Murine immature DC injected into adjuvant (Adjuprime, poly-arginine, or Imiquimod)-pretreated skin exhibited lymph node migratory capacity comparable to and immunostimulatory capacity equal to or exceeding that of ex vivo matured DC. Acquisition of migratory capacity did not always correlate with enhanced immunostimulatory capacity. Immunostimulatory capacity was not enhanced when mature DC were injected into adjuvant-prepared sites and remained below that seen with immature DC matured in situ. Immature DC injected into adjuvant-pretreated sites were more effective than mature DC in stimulating antitumor immunity in mice.11 Indium-labeled human monocyte-derived immature DC injected into adjuvant (Imiquimod)-pretreated sites in cancer patients acquired lymph node migratory capacity comparable to ex vivo matured DC. This study shows that in situ maturation offers a simpler and potentially superior method to generate potent immunostimulatory DC for clinical immunotherapy. The Journal of Immunology, 2003, 171: 6275–6282.

Effective immunization in the setting of cancer will require the induction of potent Th1 CD4⁺ and CD8⁺ CTL responses. Because dendritic cells (DC) are powerful APCs with unique ability to activate naive CD4⁺ and CD8⁺ T cells (1, 2), immunization with ex vivo Ag-loaded DC could represent a potentially powerful method of inducing antitumor immunity (3, 4).

The tissue resident DC, termed immature DC, are able to capture Ag from the environment, but are deficient in stimulating T cells. In response to pathogen infection and the ensuing inflammatory response, DC undergo a differentiation process called maturation, whereby they up-regulate the capacity to migrate to draining lymph nodes and present the captured Ags to T cells (1, 2). To activate Th1 CD4⁺ T cells and CTL, the DC has to integrate a number of maturation/differentiation stimuli. At the site of pathogen encounter, exposure to pathogen-derived determinants, proinflammatory cytokines, and/or cell debris induces the first steps in the maturation process (5). This includes the up-regulation of co-stimulatory molecules and chemokine receptors, whereby the DC acquire the ability to present Ags to T cells and migrate to the lymph node, respectively. At the lymph node, encounter of cognate CD4⁺ T cells provides additional differentiation stimuli to the DC, which regulate the survival of the activated T cells and the polarization of the CD4⁺ T cells (6).

Immunotherapy using ex vivo generated DC requires that the DC mature in culture to a stage in which they can migrate to the lymph nodes and activate the cognate T cells. Although the list of agents that can affect DC maturation is long and growing, the exact sequence of events and composition of factors required for the efficient differentiation of the DC to become potent stimulators of Th1 CD4⁺ T cells and CD8⁺ CTL are not known. The difficulties have been underscored by the recognition that, under certain conditions, in vitro generated DC can favor the induction of Th2 CD4⁺ T cell responses or tolerize the cognate T cells (1, 7, 8–13). One of the best-characterized protocols for generating mature human DC involves the differentiation of DC from monocytes. In this protocol, monocytes are cultured for 5–7 days in the presence of GM-CSF and IL-4 to generate immature DC, followed by 1–2 days of culture in the presence of TNF-α, IL-6, IL-1β, and PGE₂ to induce their maturation (14, 15). Yet, despite rigorous criteria used for their characterization in vitro, it is not clear whether the chosen conditions for maturation result in DC with optimal in vivo functions, including migration to lymph nodes and activation of Th1 CD4⁺ and CD8⁺ CTL. In addition, the reagents used for DC maturation using this or other protocols can be expensive and not always readily available for clinical use.

This study describes a strategy termed in situ maturation that eliminates the ex vivo DC maturation step. Instead, the Ag-loaded immature DC are injected into tissue that is pre-exposed to agents that favor the generation of a Th1-inducing environment. The hypothesis is that the use of appropriate agents will recapitulate the physiological conditions occurring during pathogen infection in a manner that will lead to the optimal conditions for the maturation,
migration, and function of the injected DC. Because maturation of DC in situ resembles more closely the physiological process involved in response to pathogen infection, in situ maturation may lead to enhanced T cell immunity. In addition, the in situ DC maturation approach eliminates an ex vivo cell culture step and dispenses with the use of expensive biologicals (maturation agents), which are not always available for clinical use.

In this study, we show that murine bone marrow-derived immature DC injected into skin exposed to adjuvants are as effective or superior to ex vivo matured DC in migrating to lymph nodes, stimulating CTL responses, and inducing tumor immunity. Furthermore, immature human monocyte-derived DC injected into adjuvant-treated skin of cancer patients acquire migratory capacity and migrate to the draining lymph node as effectively as ex vivo matured DC.

Materials and Methods

Mice
Four- to 6-wk-old C57BL/6 mice (H-2b) were obtained from The Jackson Laboratory (Bar Harbor, ME). In conducting the research described in this work, the investigators adhered to the “Guide for the Care and Use of Laboratory Animals” as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities at the Duke vivarium are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Reagents

Adjuvate was obtained from Pierce (Rockford, IL) and reconstituted as per manufacturer’s recommendation in PBS at 5 mg/ml. The reconstituted product was frozen at −20°C. Each mouse received a dose of 100 μg/ear of Adjuvate in 20 μl of PBS 20 min before DC injection. Imiquimod cream, 5%, is sold under the trade name Aldara by 3M Pharmaceuticals (St. Paul, MN). A single-use packet of Imiquimod cream, 5%, containing 0.25 g of cream (12.5 mg of Imiquimod) was applied with an applicator to 0.25 g of cream (12.5 mg of Imiquimod) was used for each application.

For experiments in cancer patients, Imiquimod cream, 5%, was applied three times on the injection site topically (every other day for 6 days) before injection of DC. A single-use packet containing 0.25 g of cream (12.5 mg of Imiquimod) was used for each application.

Murine cell lines

The F10.9 clone of the B16 melanoma of C57BL/6 origin is a highly metastatic, poorly immunogenic, and a low class I-expressing cell line. B16/F10.9 cells were transfected with chicken OVA cDNA to generate B16/F10.9-OVA. Other cell lines used were EL4 (C57BL/6, H-2b, thymoma) and E.G7-OVA (EL4-OVA, EL4 cells transfected with chicken OVA cDNA). Cells were maintained in DMEM supplemented with 10% FCS, 25 mM HEPE, 2 mM l-glutamine, and 1 mM sodium pyruvate. Murine precursor-derived DC were generated in the presence of GM-CSF supernatant harvested from F10.9 cells transfected with the GM-CSF cDNA. Actively growing F10.9/GM-CSF cells were cultured in RPMI 1640 containing 0% FCS, 1 mM Na pyruvate, 1 mM nonessential amino acids, 100 IU/ml penicillin, 100 mg/ml streptomycin, 5 × 10−8 M 2-ME, and 10 mM HEPES (complete RPMI) at 37°C and 5% CO2. GM-CSF-containing supernatant was harvested after 24 h of capillary culture. The GM-CSF supernatant was used to generate murine DC at a final dilution of 0.1%.

Generation of murine DC

Murine DC were generated from the bone marrow of mice in the presence of GM-CSF and IL-4 using established protocols (16), with slight modifications (17). Briefly, marrow from tibias and femurs of C57BL/6 mice were harvested, followed by treatment of the precursors with ammonium chloride Tris buffer for 3 min at 37°C to deplete the RBC. The precursors were plated in RPMI-5% FCS with GM-CSF (15 ng/ml) and IL-4 (10 ng/ml). GM-CSF-containing supernatant was harvested after 24 h capillary culture from GM-CSF-producing melanoma cell line (F10.9/GM-CSF), and IL-4 was obtained from Peprotech (Rocky Hill, NJ). The concentration of GM-CSF used was determined by ELISA. Cells were plated at 107/ml and incubated at 37°C and 5% CO2. Three days later, the floating cells (mostly granulocytes) were removed and the adherent cells were replated with fresh GM-CSF and IL-4-containing medium. Nonadherent cells were harvested on day 5, electroporated or untreated, and replated at 106/ml in GM-CSF- and IL-4-containing medium. Nonadherent cells were harvested on day 3 as immature DC. For mature DC, nonadherent cells were harvested on day 7, washed, and replated at 106/ml in GM-CSF- and IL-4-containing medium. After 1 day (day 8 DC), the nonadherent cells were harvested, washed, and electroporated with RNA, or just replated with no electroporation. Nonadherent cells were harvested on day 9 as mature DC. For migration studies, untreated day 6 and day 9 DC were washed and labeled with 1 μM CFSE (Molecular Probes, Eugene, OR), per manufacturer’s protocol.

Preparation of OVA, tyrosinase-related protein (TRP)2, and actin in vitro transcribed RNA

Cloning of pGEM4Z/OVA/A64. Chicken OVA cDNA in pUC18 was kindly provided by B. Rouse (University of Tennessee, Knoxville, TN). The 1.9-kb EcoRI fragment containing the coding region and 3′ untranslated region was cloned into the EcoRI site of pGEM4Z/A64 to generate plasmid pGEM4Z/OVA/A64. The plasmid was linearized with SpeI for use as a template for in vitro transcription.

Cloning of pSP73-Sph/TPR2/A64. Oligonucleotides containing 64 A-T bp followed by an SpeI restriction site were placed between the EcoRI and NruI sites of pGEM4Z (Promega, Madison, WI) to create the plasmid pGEM4Z/nTcN64. The HindIII-Ndel fragment of pGEM4Z/A64 was cloned into pSP73 (Promega) digested with HindIII and Ndel to create pSP73/A64. The plasmid pSP73-Sph was created by digesting pSP73/A64 with SphI, filling in the ends with T4 DNA polymerase, and religating. pSP73-Sph/A64/Not contains a Ndel restriction site adjacent to the SpeI site. Total RNA was isolated from actively growing B16/F10.9 cells. Reverse transcription was primed with an anchored oligo(dT) primer, and the TRP2-cDNA was amplified from the first strand using the forward primer 5′-GATGTTACCAAGCTGTCACCCGATGGGTGGATGG-3′ and the reverse primer 5′-GTGATGATCCGCGGCGTACGCGTTCGCGTGCATC-3′. The resulting product was digested with BglII and BamHI and cloned into the BamHI site of pSP73-Sph/A64.

Cloning of pGEM4Z/murine actin/A64. The forward primer, 5′-TTATGGAATCCCAGATGCATGACTGACTGACGCTC-3′; and the reverse primer, 5′-TTATTGATGATCCGCGGCGTACGCGTTCGCGTGCATC-3′ were used to amplify the actin coding sequence from the first-strand cDNA. The PCR fragment was cloned into the HindIII-BamHI sites of pGEM4Z/A64 to generate pGEM4Z/murine actin/A64. RNA transcription was conducted using T7 message machine kits (Ambion, Austin, TX) per manufacturer’s protocol.

Electroporation of murine DC with RNA

DC were electroporated with RNA, as described for human DC (18, 19). Nonadherent cells were harvested on day 5 or 8, washed, and gently resuspended in Opti-MEM (Life Technologies, Grand Island, NY) at 2.5 × 107/ml. The supernatant from DC culture was saved as conditioned medium for later use. Cells were electroporated in 2-mm cuvettes (200 μl of DC (5 × 105 cells) at 300 V for 500 μs using an Electro Square Porator ECM 830 (BTX, San Diego, CA). The amount of in vitro transcribed RNA used was 2 μg/107 DC. Cells were immediately transferred to 60-mm tissue culture petri dishes containing a 1:1 combination of conditioned DC growth medium and fresh RPMI with GM-CSF and IL-4. Transfected cells were incubated at 37°C, 5% CO2 overnight, washed twice in PBS, and then injected into mice.

Murine DC isolation from lymph nodes and flow cytometry

Immature and mature CFSE-labeled DC were injected intradermally in the ear pinna. After 18–20 h, the draining auricular lymph nodes were harvested and processed, as described by Vreem and Shortman (20). Lymph nodes were digested for 20 min at room temperature in RPMI 1640 with 5% FCS and 200 U/ml collagenase type II (Life Technologies, Grand Island, NY) and 200 U/ml DNase I (Sigma-Aldrich), and then treated for 5 min with 0.01 M EDTA to disrupt the T cell-DC complexes. A single cell suspension was generated, and the cells were washed before staining with mAbs. Cells were treated with Fc Block (anti-CD16/32; BD PharMingen, San Diego, CA). Cells were then labeled for 20 min on ice for each Ab, starting with biotin anti-mouse I-Aβ, followed by streptavidin-APC, followed by PE anti-mouse CD11c. All Abs were obtained from BD PharMingen. Cells were washed, fixed, and analyzed within 48 h.
**CTL induction in vivo and cytotoxicity assay**

Bone marrow precursor-derived DC were generated and transfected with RNA, as described above. Naïve, syngeneic mice were immunized intradermally in the ear pinna with 3–5×10^5 precursor-derived DC per mouse in 50 μl of PBS. Draining auricular lymph nodes were harvested 7 days later, and 10^7 cells (lymph node cells) were cocultured with 2×10^5 stimulator cells (DC electroproporated with RNA) in 5 ml of IMDM with 10% FCS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5×10^-5 M 2-ME per well in a six-well tissue culture plate. The responders were stimulated with the same Ag as used for the immunization. Cells were cultured for 5 days at 37°C and 5% CO2.

**Tumor challenge**

DC were transfected with the various RNA preparations, and naive, syngeneic mice were immunized intradermally in the ear pinna with 5×10^5 precursor-derived DC per mouse in 50 μl PBS. Mice were challenged with 1×10^7 B16/F10.9 or B16/F10.9-OVA cells s.c. (in the flank) 7 days after the immunization. Tumor growth was evaluated every other day, starting on day 10. Mice were sacrificed once the tumor size reached 20 mm. Effectors were harvested 30 min at 37°C. A total of 10^7 europium-labeled targets and serial dilutions of effectors were used for the cytotoxicity assay at a 100:1 E:T ratio. Data are represented as percentage specific release (% spontaneous release)/(total release - spontaneous release) x 100. Spontaneous release of the target cells was less than 25% of total release by detergent in all assays. SEs of the means of triplicate cultures were less than 5%.

**Generation of human DC**

All cellular material used in these experiments was obtained from human subjects following informed consent through protocols approved by the Duke University Investigational Review Board. DC were generated from monocytes in the presence of GM-CSF and IL-4, as previously described (21). PBMC were suspended at 6–7×10^6/ml in 30 ml of AIMV and transferred to tissue culture flasks for adherence. After 1 h of adherence, the nonadherent cells were removed, and the adherent cells were resuspended in 30 ml of X-VIVO 15 with 80 U/ml GM-CSF and 500 U/ml IL-4. Immature human DC were harvested after 6–7 days of culture at 37°C, 5% CO2. Cells were electroporated and cryopreserved in 10% FCS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5×10^-5 M 2-ME. A total of 10^5–10^6 electroporated immature DC were injected intradermally in the ear pinna of mice treated with Adjuprime. Adjuprime is a carbohydrate polymer, which functions by physically entrapping the Ag and targeting APCs via glucan receptors. Adjuprime also causes stimulation of T cells, the release of lysosomal enzymes, and inflammatory factors (Pierce, Rockford, IL). As controls, immature or mature DC were injected into untreated ear pinna. To monitor for the migration of DC to the lymph node, DC were stained with the vital dye CFSE. Twenty-four hours postinjection, the draining auricular lymph node was dissected and cell suspension was generated using a protocol that preserves the integrity of the DC population (Materials and Methods). A total of 5×10^5 immature DC were administered unilaterally in the upper right quadrant and the number of DC injected, it was possible to determine that 1% of the injected immature DC migrated to the draining lymph node. In contrast, 9.4% of the injected mature DC migrated to the draining lymph node. Notably, when immature DC were injected into the ear that was pretreated with Adjuprime, 9% of the injected DC migrated to the draining lymph node. Moreover, the migrated DC expressed high levels of MHC class II (upper right quadrant), despite the fact that the injected immature DC populations expressed low to intermediate levels of class II (Fig. 1). These observations are, therefore, consistent with the hypothesis that injection of immature DC into the Adjuprime-treated tissue results in their maturation, as judged by their up-regulation of MHC class II expression and capacity to migrate to the draining lymph nodes. The small, but detectable presence of class II^high^CFSE^+^ DC in the lymph nodes of mice injected with immature DC in the absence of adjuvant pretreatment (1%) could be due to either maturation of some immature DC in the process of injection, or more likely the

**Results**

Immature DC injected into adjuvant-treated skin acquire migratory capacity and exhibit enhanced immunostimulatory capacity in mice

Fig. 1 shows the cell surface phenotype of the immature and mature DC populations used in these studies. As we have previously shown, the day 6 DC exhibit typical immature phenotype characterized by low to intermediate levels of MHC class II expression (22). By contrast, in the mature day 9 DC population, expression of MHC class II and B7-1 is significantly up-regulated. Immature and mature DC also exhibit characteristic morphology, as revealed by microscopic analysis (data not shown).

We wanted to determine whether injection of immature DC into tissue that was pretreated with adjuvants induces their functional maturation (in situ maturation). Immature DC were injected into the ear pinna of mice treated with Adjuprime. Adjuprime is a carbohydrate polymer, which functions by physically entrapping the Ag and targeting APCs via glucan receptors. Adjuprime also causes stimulation of T cells, the release of lysosomal enzymes, and inflammatory factors (Pierce, Rockford, IL). As controls, immature or mature DC were injected into untreated ear pinna. To monitor for the migration of DC to the lymph node, DC were stained with the vital dye CFSE. Twenty-four hours postinjection, the draining auricular lymph node was dissected and cell suspension was generated using a protocol that preserves the integrity of the DC population (Materials and Methods). As shown in Fig. 2A, the injected and resident lymph node DC were identified as CD11c^+^class II^high^CFSE^+^ and CD11c^+^class II^high^CFSE^+^ cells, respectively. Based on the percentage of CD11c^+^class II^high^CFSE^+^ cells in the lymph node (shown in the upper right quadrant) and the number of DC injected, it was possible to determine that 1% of the injected immature DC migrated to the draining lymph node. In contrast, 9.4% of the injected mature DC migrated to the draining lymph node. Notably, when immature DC were injected into the ear that was pretreated with Adjuprime, 9% of the injected DC migrated to the draining lymph node. Moreover, the migrated DC expressed high levels of MHC class II (upper right quadrant), despite the fact that the injected immature DC populations expressed low to intermediate levels of class II (Fig. 1). These observations are, therefore, consistent with the hypothesis that injection of immature DC into the Adjuprime-treated tissue results in their maturation, as judged by their up-regulation of MHC class II expression and capacity to migrate to the draining lymph nodes. The small, but detectable presence of class II^high^CFSE^+^ DC in the lymph nodes of mice injected with immature DC in the absence of adjuvant pretreatment (1%) could be due to either maturation of some immature DC in the process of injection, or more likely the

**Human DC migration studies**

DC were thawed, washed, and recovered overnight in X-VIVO 15 medium supplemented with 800 U/ml GM-CSF. Cells were resuspended at 10^7/ml in X-VIVO 15. A total of 5×10^5 immature DC was loaded with 40 μCi or mature DC with 60 μCi111In (sp. act., 1.85 GBq/μg) and incubated for 30 min at 37°C. Cells were washed three times with 0.9% sodium chloride (saline) and resuspended in saline/5% autologous plasma for injection. Four injections (1.25×10^10 each) were administered intradermally in a diamond-shaped pattern (~2.5 cm apart and at a distance of 10 cm from the
presence of a small population of mature (class II<sup>high</sup>) DC in the day 6 population (see Fig. 1). Note that the apparent discrepancy between the percentage of migrated DC and percentage of DC in the lymph node (see also Fig. 4A) is due to the emigration of resident DC into lymph nodes following treatment with adjuvant.

To monitor immunostimulatory capacity, DC were electroporated with OVA mRNA 18–20 h before injection. Auricular lymph nodes were harvested 7–8 days post injection of the DC and assayed for the presence of OVA-specific CTL. As shown in Fig. 2B, mature DC were more potent than immature DC in stimulating a CTL response. However, when the immature DC were injected into the adjuvant-pretreated site, the CTL response generated was comparable to that seen with mature DC. Thus, judging from the migration and immunostimulatory capacities of the injected DC, injection of immature DC into Adjuprime-pretreated sites induced their functional maturation to become immunopotent APC.

To begin to assess the generality of the in situ maturation protocol, we tested two additional adjuvants, Imiquimod and poly-arg. Imiquimod, used as a 5% cream formulation under the trade name Aldara (3M Pharmaceuticals), has been recently approved for the treatment of external genital and perianal warts in humans (23, 24). In murine studies, Imiquimod given by topical or intravaginal routes exhibits antiviral (25, 26) and antitumor activity (27). Local administration of Imiquimod induces a Th1-conducive cytokine storm that includes IFN-α, TNF-α, IL-1α, IL-6, and IL-8 (28–30), and causes the emigration of resident Langerhans cells from the treated site (31). Poly-arg was shown to enhance the immunogenicity and anticancer activities of peptide Ags (32, 33). Fig. 3 confirms the results shown in Fig. 2B that immature DC injected into a site pretreated with Adjuprime stimulate a potent CTL response, comparable to the CTL response stimulated by mature DC. In addition, Fig. 3 shows that pretreatment of the injection site with either Imiquimod or poly-arg potentiates the CTL response stimulated by immature DC. Imiquimod was almost as effective as Adjuprime, whereas the immunostimulatory effect of poly-arg was small.

Local maturation of the injected DC will be influenced by the degree of inflammatory response, which depends on the choice of adjuvant as well as the experimental parameters used, such as dose and timing of adjuvant application. In the experiment shown in Figs. 2 and 3, the adjuvants were applied 15 min before DC injection. It is, therefore, not inconceivable that further delaying the injection of the immature DC will permit the development of a
more potent inflammatory response, which will result in a more complete and effective maturation of the injected DC. To test this possibility, in the experiment shown in Fig. 4, the interval between application of Imiquimod and DC injection was extended to 4 h, and the impact on DC maturation was tested, as described in Fig. 2. In this experiment, we also tested whether injection of mature DC into adjuvant-treated sites could further enhance their migratory and immunostimulatory potential. As shown in Fig. 4A, the percentages of DC injected into untreated skin that migrated to the draining lymph nodes were 1.8 and 10% for immature and mature DC, respectively, 6.5 and 10% for immature and mature DC injected into Imiquimod-treated site, and 6.2 and 12% for immature and mature DC injected into poly-arg-treated site, respectively. Thus, pretreatment with either Imiquimod or poly-arg had a similar enhancing effect on the migration of the immature DC, whereas neither agent enhanced further the migration of mature DC.

The immunostimulatory capacity of the injected DC is shown in Fig. 4B. In this experiment, immature DC injected into Imiquimod-pretreated sites stimulated a more potent CTL response than mature DC injected into untreated sites, whereas injection of immature DC into poly-arg-pretreated sites had no effect. Interestingly, injection of mature DC into either Imiquimod- or poly-arg-pretreated sites did not improve CTL induction over what was seen with mature DC injected into nontreated sites. These observations confirm and extend the observations shown in Figs. 2 and 3 and suggest that optimizing the conditions of adjuvant application can further enhance the in situ maturation and immunopotency of the injected immature DC. Importantly, this experiment also shows that enhanced lymph node migration of DC does not necessarily correlate with enhanced immunostimulatory capacity. Mature DC migrated more efficiently (10%) than immature DC injected into Imiquimod-pretreated sites (6.5%), yet the latter stimulated a more potent CTL response. In addition, despite the enhanced migration of immature DC injected into poly-arg-pretreated sites, no enhancement of CTL induction was seen. The observation that injection of mature DC into Imiquimod-pretreated sites did not enhance their immunostimulatory capacity and was inferior to injection of immature DC into Imiquimod-pretreated sites suggests that the ex vivo maturation protocol used in this study was suboptimal and irreversible, because the mature DC failed to respond to optimal stimuli when injected into the adjuvant-treated site.

**Immature DC injected into Imiquimod-treated skin stimulate potent antitumor immunity**

We next examined whether the enhanced migratory and immunostimulatory capacity of in situ matured DC also leads to improved antitumor immunity. The ear pinna of mice was pretreated with Imiquimod 4 h before injection of immature DC, which in the experiment shown in Fig. 4B led to superior CTL induction by the in situ matured DC compared with the ex vivo matured DC. Mice were immunized once with chicken OVA or murine TRP-2 mRNA-transfected DC and challenged 1 wk later with OVA-expressing B16/F10.9 or B16/F10.9 tumor cells, respectively. TRP-2 is an endogenous melanocyte-specific tumor Ag in the B16/F10.9 melanoma tumor (34). The control groups consisted of mice immunized with actin mRNA-transfected DC injected into Imiquimod-pretreated sites. As shown in Fig. 5, A and B, despite a single immunization, there was a significant delay in the appearance of palpable tumors in mice immunized with in situ matured DC compared with mice immunized with immature DC. Immunization against the foreign chicken OVA model Ag was not more effective than immunization against TRP-2, which is an endogenous nonmutated self Ag. In fact, immunization against TRP-2 appeared to be slightly superior because 40% (2/5) mice remained tumor free, although the difference was not statistically significant. A similar trend was seen in other experiments (Zhao et al., unpublished data). The experiments shown in Fig. 5 provide preliminary evidence that in situ matured DC are capable of inducing effective antitumor immunity and correlate with the CTL induction data (Fig. 4), underscoring the immunopotency of the in situ matured DC. However, additional studies using therapeutic models and multiple vaccination cycles will be required to determine the antitumor potential of in situ matured DC.

**Immature human DC injected into Imiquimod-treated skin of cancer patients acquire migratory capacity**

To test whether DC can be matured in situ in cancer patients, we measured the migration of indium-111 oxyquinoline (111In)-labeled DC to the draining lymph nodes. When indicated, the injection site was pretreated by topical application of Imiquimod in a 5% cream formulation (Aldara). Human immature DC were generated from monocytes in a 5-day culture in the presence of the
ve mice per group) were implanted s.c. with 1 fungigmized with actin or TRP-2 mRNA-transfected DC. One week later, mice three experiments. DC injected into Imiquimod-pretreated sites. Data are representative of 45 days for mice immunized with OVA mRNA-transfected immature or mature DC injected into Imiquimod-pretreated sites. The median time to tumor onset was 23 days for mice immunized with OVA mRNA-transfected immature DC injected into Imiquimod-pretreated sites. Relative to OVA mRNA-transfected immature DC, p value was .02 for mice immunized with OVA mRNA-transfected immature DC injected into Imiquimod-pretreated sites. The median time to tumor onset was 19 days for mice immunized with TRP-2 mRNA-transfected immature DC injected into Imiquimod-pretreated sites. Data are representative of two experiments. A B

FIGURE 5. Inhibition of tumor growth in mice immunized with mRNA-transfected DC. A, B16/F10.9-OVA tumor model: C57BL/6 mice were immunized with actin or OVA mRNA-transfected DC, as described in Fig. 4. One week later, mice (five mice per group) were implanted s.c. with 1 × 10⁶ B16/F10.9-OVA tumor cells, and tumor growth was monitored (see Materials and Methods). The log rank test (Mantel-Haenszel test) was used to determine the differences between individual groups. Relative to OVA mRNA-transfected immature DC, p value was .02 for mice immunized with OVA mRNA-transfected immature DC injected into Imiquimod-pretreated sites. The median time to tumor onset was 23 days for mice immunized with OVA mRNA-transfected immature or mature DC and 45 days for mice immunized with OVA mRNA-transfected immature DC injected into Imiquimod-pretreated sites. Data are representative of three experiments. B, B16/F10.9 tumor model: C57BL/6 mice were immunized with actin or TRP-2 mRNA-transfected DC. One week later, mice (five mice per group) were implanted s.c. with 1 × 10⁶ B16/F10.9 tumor cells, and tumor growth was monitored. Relative to TRP-2 mRNA-transfected immature DC, p value was .02 for mice immunized with TRP-2 mRNA-transfected immature DC injected into Imiquimod-pretreated sites. The median time to tumor onset was 19 days for mice immunized with TRP-2 mRNA-transfected immature and 24 days for mice immunized with TRP-2 mRNA-transfected immature DC injected into Imiquimod-pretreated sites. Data are representative of two experiments.

FIGURE 6. Imaging of ¹¹¹In-labeled, PSA RNA-transfected DC migration as a function of time in a cancer patient. A, Imaging of ¹¹¹In-labeled DC migration as a function of time in a patient injected simultaneously with PSA RNA-transfected, mature (left extremity), and immature (right extremity) DC. Migration of mature, but not immature DC could be observed 4 h following intradermal injection (arrow). Representative figure of six treated subjects. B, Mature DC were injected into untreated sites. Immature DC were injected into Imiquimod-treated skin sites, as indicated. Representative figure of two treated subjects.

cytokines GM-CSF and IL-4 and transfected with prostate-specific Ag (PSA) RNA, as previously described (21). To generate mature DC, the immature DC were cultured for an additional 24 h in the presence of the proinflammatory cytokines IL-1β, IL-6, TNF-α, and PGE₂ (15).

¹¹¹In-labeled DC were injected simultaneously at opposite skin sites located at the medial aspect of the patient’s upper thigh, an anatomic area drained by the inguinal and subinguinal lymph nodes (landing site). Four intradermal injections, each containing 1.25 × 10⁷ cells, were administered into each thigh according to an injection grid. Immediately after the injection, imaging via gamma camera was performed at defined time intervals, as shown below. When indicated, the injection site was treated with Imiquimod (three times) before DC injection, as described in Materials and Methods. Fig. 6A shows the imaging of DC migration in a patient injected with mature and immature DC at opposite skin sites in the upper thigh. As expected, DC migration to the inguinal lymph node was detected, as early as 4 h postinjection (arrow) only with the mature DC population. Twenty-one hours postinjection, spread of the migrated DC to a second lymph node became evident. Similar results were obtained in six of six patients analyzed in this manner, always demonstrating the superior migratory capacity of mature DC (data not shown). These observations are consistent with a recent report demonstrating the lymph node migration of ex vivo generated mature DC in cancer patients at similar efficiencies (35). It should be noted that the measured migration efficiency, which ranged after 24 h between 0.5 and 2.0%, is an underestimate of the actual migration efficiency of the injected DC because migration continues for additional 24–48 h (data not shown) and the ¹¹¹In-induced cell death during this period is substantial. Therefore, this analysis provides a relative, rather than absolute, measure of the migratory capacity of the injected DC. In Fig. 6B, the migration of mature DC injected into untreated skin was compared with immature DC injected into skin pretreated with Imiquimod. Migration of mature DC to the draining lymph node could be readily detected. Interestingly, the immature DC injected into the pretreated site also migrated to the lymph node with evidence of spread to a second lymph node (arrow). Similar results were obtained in two of two patients tested. Thus, consistent with the murine studies shown in Fig. 4, immature DC injected into adjuvant (Imiquimod)-pretreated skin acquire enhanced migratory and lymph node-homing capacity.


days to tumor onset
percent tumor-free mice


days to tumor onset
percent tumor-free mice

% Migration

% Migration

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Discussion

The purpose of this study was to determine whether the ex vivo DC maturation step can be replaced by injecting immature DC into sites that were exposed to agents that induce a microenvironment conducive to the functional in situ maturation of the injected DC. The criteria used to monitor the fate of the injected DC in the murine studies were migration, immunostimulatory capacity, and induction of tumor immunity. In the human studies, the ability of the injected DC to migrate and home to the draining lymph nodes was measured.

Our studies confirm and extend previous observations that ex vivo matured murine DC exhibit enhanced migratory and lymph node-homing potential (Figs. 2 and 3), superior immunostimulatory capacity (Figs. 2–4), and increased antitumor activity (Fig. 5) (35–39). The main finding of this study was that, when immature murine DC were injected into sites pretreated with either of two adjuvants, Adjuprime or Imiquimod, their migratory capacity (Figs. 2 and 3), immunostimulatory capacity (Figs. 2–4), and antitumor activity (Fig. 5) were enhanced to levels comparable to or exceeding that of the ex vivo matured DC. These observations support the central hypothesis of this study, that immature DC injected into the adjuvant-pretreated sites undergo an effective maturation process in situ. This conclusion is also supported by the observation that the majority of the injected immature class II<sub>intermediate</sub> DC (Fig. 1) that migrated to the lymph node expressed high levels of MHC class II<sub>high</sub> molecules comparable to that of the ex vivo matured DC (CD11<sup>+</sup>c<sup>+</sup> class II<sub>high</sub> CFSE<sup>-</sup> populations, Figs. 2A and 4A).

In situ maturation of injected DC recapitulates more closely the physiological process evolved in response to pathogen infection by eliminating the need to identify the optimal reagents and conditions for maturing DC ex vivo. Hence, immunization with in situ matured DC could lead to superior immunity. This was indeed suggested by the observations that immature DC injected into Imiquimod-treated skin stimulated a more potent CTL response (Fig. 4B) and were more effective at inhibiting tumor growth (Fig. 5) than mature DC injected into untreated sites. The conditions for in situ DC maturation (choice of adjuvant, dose, and timing of adjuvant application) have not been optimized, and it is conceivable that additional modifications will further augment the immunopotency of the ex vivo generated DC. This was indicated in this work by the fact that simply increasing the interval between adjuvant application and DC injection led to an enhanced immune response and tumor immunity.

Illustrating the potential limitations of ex vivo maturation protocols, injection of mature DC into Imiquimod-treated sites did not further potentiate their immunostimulatory capacity, which remained less than that of immature DC injected into Imiquimod-treated sites (Fig. 4). This observation suggests that the ex vivo maturation protocol used in this instance was suboptimal and led to a partially matured DC, which was refractory to optimal maturation stimuli encountered in situ.

Acquisition of migratory and immunostimulatory capacity is the hallmark of the DC maturation process. However, as shown in Fig. 4A, migration does not always correlate with immunopotency. For example, mature DC migrated more efficiently (10%) than immature DC injected into Imiquimod-pretreated sites (6.5%), yet the latter stimulated a more potent CTL response (Fig. 4B) and tumor immunity (Fig. 5). Furthermore, despite the enhanced migration of immature DC injected into poly-arg-pretreated sites (Fig. 4A), no enhancement of CTL induction was seen (Fig. 4B). A dichotomy between migration and maturation was also suggested from studies in patients with dermatopathic lymphadenitis. In these patients, DC with immature phenotype were found to accumulate in the draining lymph nodes, suggesting that the DC exposed to inflammatory stimuli characteristic to this disease have acquired the capability to migrate to lymph nodes without undergoing phenotypic maturation (40). These observations underscore the fact that dependent on the composition of the stimuli encountered, the DC can undergo partial maturation, whereby they up-regulate the migratory, but not immunostimulatory, capacity, and probably vice versa. This observation also suggests that assessing DC maturation and immunopotency by measuring DC migration in vivo should be interpreted with caution.

Ongoing and future studies examine in detail the mechanistic underpinning of the in situ maturation process, factors and cell types that accumulate at the site of adjuvant application, the characteristic of the DC migrating to the draining lymph nodes, and the impact of various genetic deficiencies. For example, Imiquimod was shown to be a ligand for Toll-like receptor 7 (TLR7) (41, 42), yet the bone marrow-derived DC used in this study do not express TLR7. It is therefore conceivable that Imiquimod acts on local cellular targets to induce an inflammatory response conducive for DC maturation. Whether this involves signaling via TLR7 will be tested in TLR7-deficient mice. Of particular interest would be to understand the difference between DC that acquired only migration capacity and DC that underwent a more complete maturation process acquiring also enhanced immunostimulatory capacity (Fig. 4).

The relevance to human clinical settings was suggested by the observation that human immature DC injected into Imiquimod-pretreated skin acquired enhanced migratory capacity comparable to that of ex vivo matured DC (Fig. 6). However, as noted above, additional studies using immunological and clinical endpoints will be required to determine the usefulness of this approach.

In summary, this study describes a protocol for using ex vivo generated DC for immunotherapy that dispenses with the need to mature the DC ex vivo. This protocol requires less manipulations ex vivo, replaces expensive and often difficult to obtain cytokines with readily available inexpensive reagents, and could be more effective in stimulating immunity in vivo.

References


