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Advances in Brief

α-Type-1 Polarized Dendritic Cells: A Novel Immunization Tool with Optimized CTL-inducing Activity

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Abstract

Using the principle of functional polarization of dendritic cells (DCs), we have developed a novel protocol to generate human DCs combining the three features critical for the induction of type-1 immunity: (*a*) fully mature status; (*b*) responsiveness to secondary lymphoid organ chemokines; and (*c*) high interleukin-12p70 (IL-12p70)-producing ability. We show that IFN-α and polyinosinic:polycytidylic acid (p-I:C) synergize with the "classical" type-1-polarizing cytokine cocktail [tumor necrosis factor α (TNFα)/IL-1β/IFNγ], allowing for serum-free generation of fully mature type-1-polarized DCs (DC1). Such "α-type-1-polarized DC(s)" (αDC1) show high migratory responses to the CCR7 ligand, 6C-kine but produce much higher levels of IL-12p70 as compared to TNFα/IL-1β/IL-6/prostaglandin E_2 (PGE_2)-matured DCs (sDC), the current "gold standard" in DC-based cancer vaccination. A single round of *in vitro* sensitization with αDC1 (*versus* sDCs) induces up to 40-fold higher numbers of long-lived CTLs against melanoma-associated antigens: MART-1, gp100, and tyrosinase. Serum-free generation of αDC1 allows, for the first time, the clinical application of DCs that combine the key three features important for their efficacy as anticancer vaccines.

Introduction

Dendritic cells (DCs) are increasingly applied as vaccines for cancer patients (1, 2). Several features of DCs, including their maturation status, migratory potential, and cytokine production, were shown important for the ability of DC-based cancer vaccines to induce high numbers of Th1-type $CD4^+$ T cells and $CD8^+$ CTLs. Effective induction of antitumor CTL responses requires fully mature DCs that express high levels of costimulatory molecules (3, 4) and that can migrate in response to lymph-node-produced CCR7 ligands (5). In addition, high interleukin-12p70 (IL-12p70) secretion dramatically enhances the ability of DCs to induce tumor-specific Th1 cells and CTLs, and promotes tumor rejection in therapeutic mouse models (6–8). Unfortunately, because the maturation stage of DCs obtained in the current protocols inversely correlates with their ability to produce IL-12p70 (9, 10), the desirable combination of all of the above three features: high immunostimulatory function, high migratory activity, and high capacity to produce IL-12p70, could not be attained by any previous DC-based vaccines. We have reported that the presence of IFN-γ during the IL-1β/tumor necrosis factor α(TNFα)–induced DC maturation overcomes such maturation-associated "exhaustion," yielding stable type-1 polarized DC(s) (DC1) that produce up to 100-fold higher levels of IL-12p70 (*versus* control DCs) on interaction with CD40L-expressing $CD4^+$ Th cells, and that exhibit a dramatically improved capacity to induce Th1-type responses (11). Here, we report that the inclusion of IFN-α and polyinosinic: polycytidylic acid (p-I:C) to our original DC1-inducing cytokine cocktail, composed of IL-1β, TNFα, and IFN-γ (11), allows for the generation of DC1 in clinically relevant serum-free AIM-V medium. For the first time, this allows for the clinical use of DCs combining a fully mature status and high migratory functions with a strongly elevated, instead of exhausted, ability to produce IL-12p70. When directly compared with the current "gold standard" DCs (sDCs matured by IL-1β/TNFα/IL-6/prostaglandin E_2 (PGE_2); ref. 12), such "α-type-1-polarized DC" (αDC1) induce up to 40-fold higher numbers of melanoma-specific CTLs in a single round of *in vitro* sensitization.

Materials and Methods

Media and Reagents. Iscove's modified Dulbecco's medium with 10% fetal calf serum (both from Life Technologies, Inc., Grand Island, NY) or 2% human serum (Atlanta Biologicals, Norcross, GA) or serum-free AIM-V medium (Life Technologies, Inc., Grand Island, NY) were used to generate DCs. In preliminary experiments (over 30 experiments with blood of different donors), we have also tested (with similar results) 1, 2, 5, and 10% concentrations of fetal calf serum and human serum from different suppliers. The following factors were used to generate mature DCs: *rhu* granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (gifts from Schering-Plough, Kenilworth, NJ); IFN-α (Intron A- IFN-α-2b, Schering-Plough); rhuTNFα, rhuIL-1β, and rhuIFN-γ (all Strathmann Biotech, Hannover, Germany); rhuIL-6 (Genzyme Cambridge, MA); lipopolysaccharide (*Escherichia coli* 011:B4, Sigma, St. Louis, MO); PGE_2 (Sigma); and p-I:C (Sigma). To assure that endotoxin contamination did not contribute to the observed activity of p-I:C, we performed control experiments with END-X B15 endotoxin removal columns (Seikagaku America, Falmouth, MA), with no differences observed. IL-2 (gift from Chiron Corp. Emeryville, CA) and rhuIL-7 (R&D Systems, Minneapolis, MN) were used to support T-cell expansion in *in vitro* sensitization cultures. Flow cytometry analyses were performed with Beckman Coulter Epics XL, after labeling with CD86, CD3, CD8, CD4, CD14, CD1a, and isotype control monoclonal antibodies (all BD-PharMingen, San Jose, CA), CD83 (Coulter, Miami, FL), and CCR7 (R&D).

Generation of Dendritic Cells. Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors or melanoma patients (all stage IV, apart from a single stage-II donor) were isolated with lymphocyte separation medium (Cellgro Mediatech, Herndon, VA). Monocytes were isolated on density gradients, with Percoll (Sigma; refs. 9, 11) or Isolate (Irving Scientific, Santa Ana, CA), followed by plastic adherence, or with $CD14^+$ magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany), in all cases with similar results. Monocytes were cultured for 6 days in 24-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at 5×10^5 cells per well in *rhu* GM-CSF and IL-4 (both 1,000 IU/mL). On the basis of preliminary experiments (data not shown), we have used the following optimal concentrations of the maturation factors: IL-1β (25 ng/mL); TNFα (50 ng/mL), IFNγ

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(1,000 units/mL); IL-6 (1,000 units/mL); PGE_2 (10^{-6} mol/L); p-I:C (20 μg/mL); IFNα (3,000 units/mL); lipopolysaccharide (250 ng/mL), all added at day 6 until day 8.

Interleukin-12p70 Production. Dendritic cells were harvested, washed, and plated in 96-well plates at 2×10^4 cells/well. To mimic the interaction with CD40L-expressing Th cells, CD40L-transfected J558 cells [a gift from Dr. P. Lane, University of Birmingham, United Kingdom, that in previous studies proved equivalent to activated $CD4^+$ T cells and soluble CD40L (9–11, 13)] were added at 5×10^4 cells/well. Twenty-four-hour supernatants were analyzed by IL-12p70 ELISA (Endogen, Woburn, MA).

Chemotaxis. Dendritic cell migration, induced by 6C-kine (Biosource, Camarillo, CA), was measured in 96-well 5 μm pore ChemoTx system (Neuro Probe, Gaithersburg, MD). DCs (25×10^3 in 25 μL of AIM-V medium) were placed on the membrane surface and incubated for 90 min at 37°C, before the enumeration of migrated DCs in bottom chambers (in four random areas). Results were expressed as mean DC numbers ± SEM in the four areas in duplicate wells. To determine the IL-12p70-producing ability of migrated DCs, CD40L-J558 cells were added directly to the bottom chambers, containing the migrated DCs, for 24 hours (see above).

CTL Induction. $CD8^+$ T cells (96–98% pure) from $HLA\text{-}A2^+$ donors were negatively isolated with the StemSep system (StemCell Technologies Inc., Vancouver, British Columbia, Canada). $CD8^+$ T cells (5×10^5 cells) were sensitized by autologous αDC1 or sDC (5×10^4 cells) pulsed with the HLA-A2-restricted peptides MART-1 (27–35), gp100 (209–217 and 154–162), and tyrosinase (368–376D). 3,000 Rad γ-irradiated CD40L-J558 cells (5×10^4) were added as surrogate of CD40L-expressing $CD4^+$ Th cells (9, 11, 13). In preliminary experiments, we have also used (with similar results; data not shown) the Staphylococcus Enterotoxin B (SEB)-driven model (9, 11) of CTL induction, with SEB-pulsed DC1 (or sDCs), $CD8^+$ T cells, and irradiated $CD4^+$ T cells as an alternative source of CD40L-mediated "helper signals." In all cases, rhuIL-2 (50 units/mL) and IL-7 (10 ng/mL) were added at day 4. $CD8^+$ T-cell cultures were expanded by an additional stimulation (day 14) with irradiated peptide-pulsed autologous PBMCs. At day 28, the differentially induced $CD8^+$ T-cell lines were stimulated with peptide-pulsed $HLA\text{-}A2^+$ T2 cells to monitor the frequency of melanoma-specific $CD8^+$ T cells with IFN-γ enzyme-linked immunospot (ELISPOT). The numbers of nonspecific spots, obtained with unpulsed T2 cells (nonspecific controls) were subtracted. CTL activity was determined by ^{51}Cr-release assays by using T2 cells, pulsed with individual peptides, with unpulsed T2 cells as nonspecific controls.

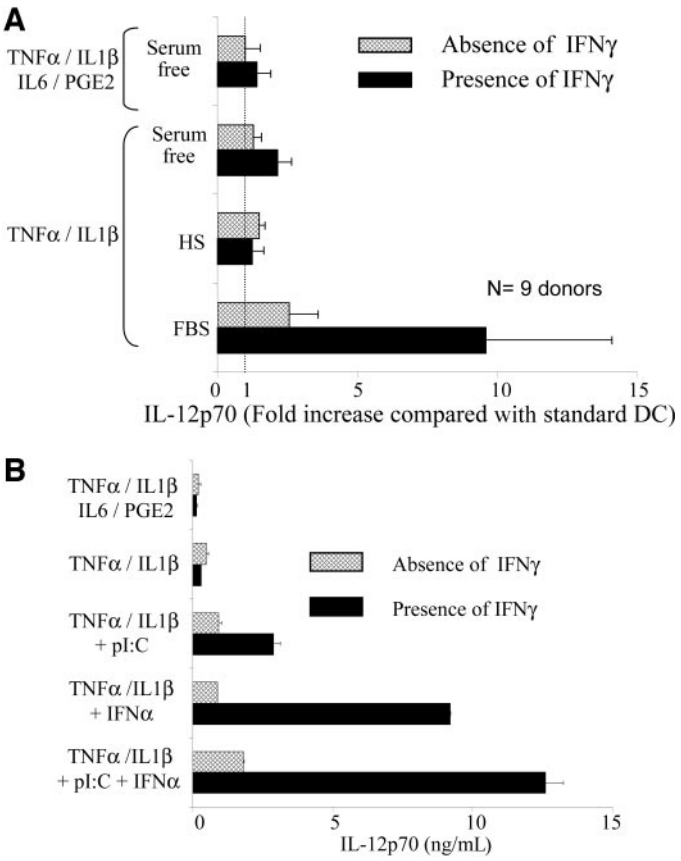

Fig. 1. IFNα and pI:C support DC1 induction in serum-free medium. In *A*, DCs generated from healthy donors in serum-free or serum-supplemented medium and matured by the combination of IL-1β and TNFα (11) or the combination of IL-1β/TNFα/IL-6/PGE_2 (sDCs; ref. 12), in the absence or presence of IFNγ, were harvested and stimulated with CD40L. The data from nine donors are expressed as a fold-increase in IL-12-producing capacity, compared with sDCs generated in parallel cultures from each individual donor (mean ± SEM). In *B*, DC1 from a healthy donor, generated in serum-free medium with the type-1 polarizing cocktail (IL-1β/TNFα/IFNγ) supplemented with IFNα, pI:C, or their combination, are superior producers of IL-12p70. Similar data were obtained when we used the blood of seven additional donors.

Results

IFNα and p-I:C Support the Generation of Fully Mature DC1 in Serum-free Conditions. As we reported previously (11), the inclusion of IFNγ in the maturation cocktail containing IL-1β and TNFα resulted in the development of stable type-1-polarized DC (DC1) in fetal calf serum-supplemented medium, characterized by high ability to produce IL-12p70 upon subsequent stimulation (Fig. 1). However, this "traditional" type-1-polarizing cocktail (IL-1β, TNFα, and IFNγ) was ineffective in serum-free AIM-V medium, or in human serum-supplemented medium (Fig. 1*A*). Similarly, the addition of IFNγ to a widely used "complete cytokine cocktail" (IL-1β/TNFα/IL-6/PGE_2; ref. 12) also proved ineffective in inducing DC1 in serum-free conditions.

Whereas neither IFNα nor P-I:C alone (nor in combination with IL-1β and TNFα) were priming DCs for subsequent production of high levels of IL-12p70 (Fig. 1*B*; refs. 11, 13), the addition of IFNα to the cocktail of IL-1β, TNFα, and IFNγ allowed for the generation of DC1 that produced very high levels of IL-12p70 from normal donors (Fig. 1*B*) and from patients with advanced melanoma (Fig. 2). Additional exposure to p-I:C further improved the IL-12p70-producing ability.

α-Type-1-polarized DCs (αDC1), generated in serum-free conditions in the presence of all five factors (IL-1β, TNFα, IFNγ, IFNα, and p-I:C), demonstrated a fully mature surface phenotype, similar to IL-1β/TNFα/IL-6/PGE_2-matured standard DC (sDCs), expressing similar levels of the maturation-associated CD83 and CD86, and of the predictive marker of lymph-node-migratory ability of DCs, (5, 14, 15), CCR7 (Fig. 2; please note that sDC show higher autofluorescence). The induction of a fully mature DC phenotype (αDC1) was consistently observed only when the complete α-type-1-polarizing cocktail, containing both IFNα and p-I:C, was used (Fig. 2*A*). Interestingly, although p-I:C was inducing only incremental enhancement of the IL-12-producing function and of the expression of CD83, and CD86, it was critical for the expression of high levels of CCR7 on maturing DCs.

***α* Type-1 DC (*αDC1*) Combine Fully Mature Status with the Ability to Produce Elevated Levels of IL-12p70 after Migrating in Response to CCR7 Ligand.** Because PGE_2, the critical component of the complete cytokine cocktail (12), has been shown important not only for the expression of CCR7 but also for the responsiveness of DCs to CCR7 ligands (14, 15), we tested whether αDC1 can migrate in response to the CCR7 ligand, 6C-kine. As shown in Fig. 2*B*, αDC1 efficiently (although slightly less efficient than sDCs) migrated in response to 6C-kine, indicating that IFNα and p-I:C can provide an alternative (to PGE_2) signal instructing DC to migrate to the lymph nodes.

Importantly, subsequent CD40L stimulation of αDC1 that had migrated in response to the 6C-kine gradient, revealed the persistence of their high ability to produce IL-12p70 (Fig. 2*C*). These data demonstrate that the migratory and IL-12-producing DC functions are expressed by the same individual cells and predict high ability of αDC1 to produce IL-12p70 *in vivo*, on migration to vaccine site-draining lymph nodes.

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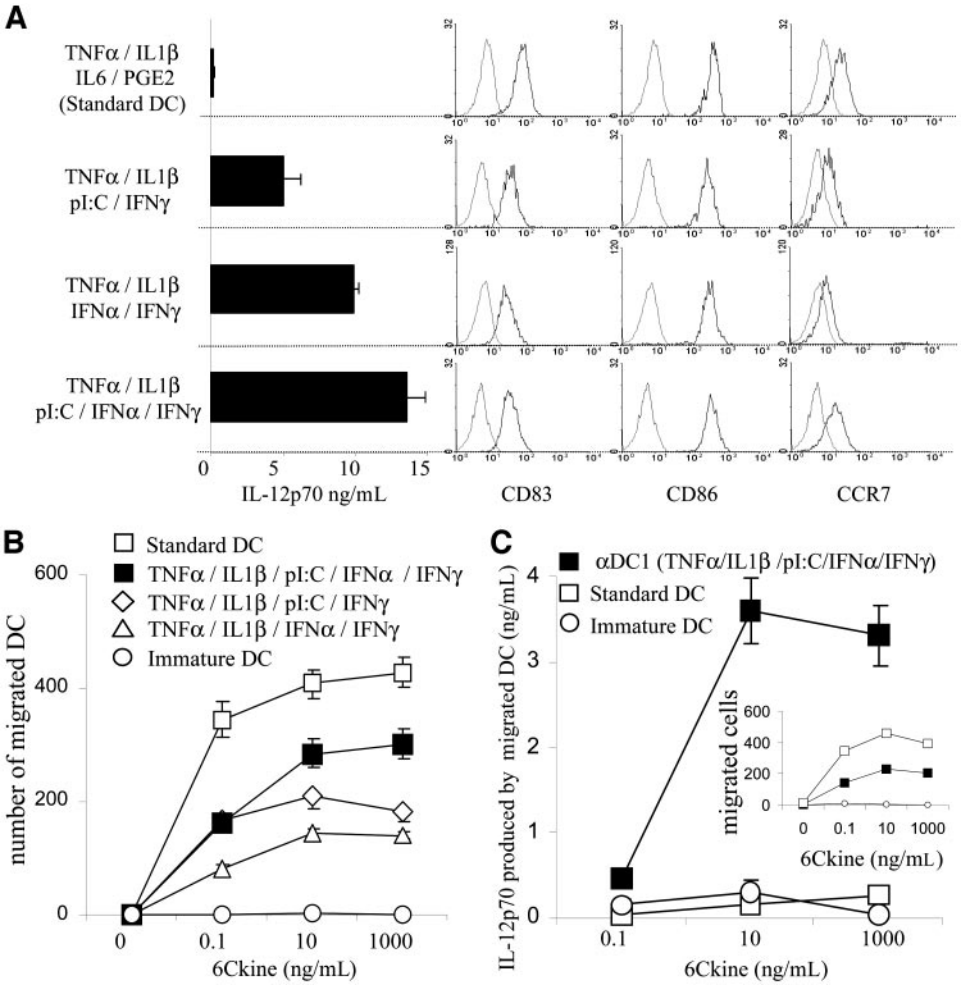

Fig. 2. αDC1 from melanoma patients are fully mature and retain high IL-12-producing capacity after the 6C-kine-induced migration. *A*, production of IL-12p70 and expression of the maturation-associated markers on sDC and DC1 induced in serum-free cultures. The data from a representative of 12 experiments, that all gave comparable results. The optimal induction of CCR7 on αDC1 required the inclusion of all five factors. *B*, 6C-kine-induced migration of differentially matured DCs. *C*, αDC1 (but not sDCs nor immature DC) retain their ability to produce high levels of IL-12p70 in response to CD40L, after migration in 6C-kine gradients. *Inset*, the corresponding relative numbers of migrated DCs.

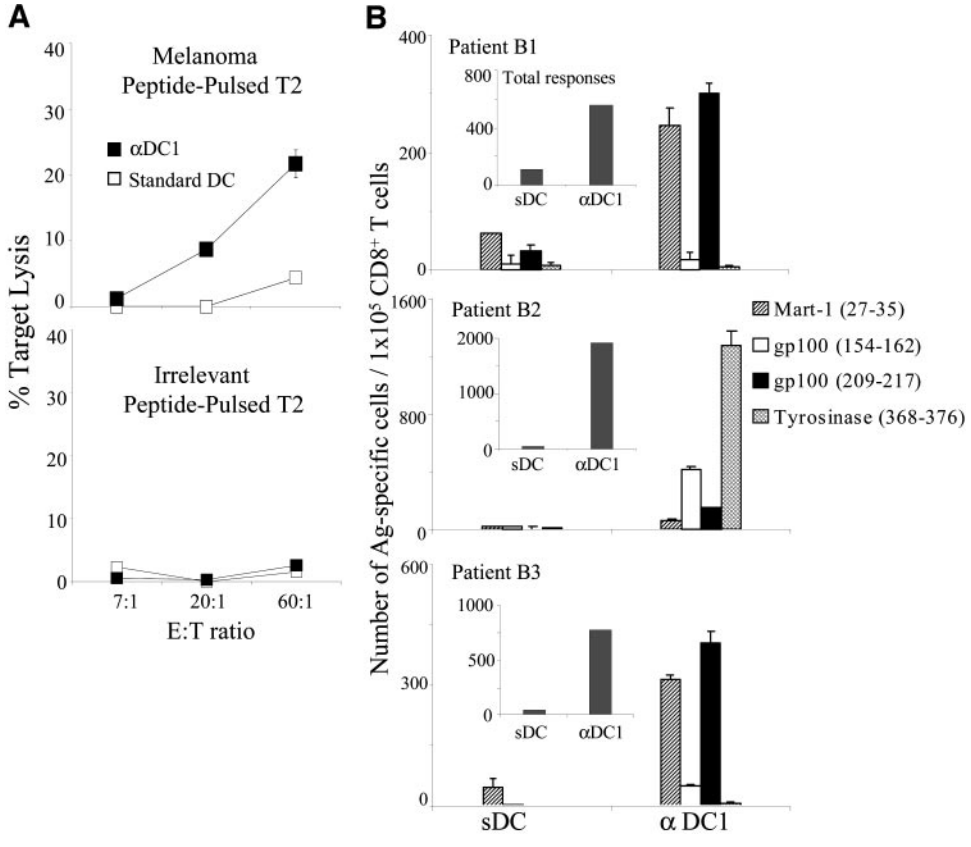

Fig. 3. αDC1 exhibit superior capacity to induce $CD8^+$ T-cell responses against melanoma. αDC1 or sDC from HLA-$A2^+$ melanoma patients were pulsed with HLA-A2-presented melanoma-associated CTL epitopes and were used to sensitize autologous $CD8^+$ T cells. *A*, cytotoxic activity of the CTLs induced by standard DC or by αDC1 against control or peptide-pulsed T2 cells. Data from one of three experiments with the blood from melanoma donors that each gave similar results. *B*, frequencies of $CD8^+$ T cells responsive to individual peptides in three HLA-$A2^+$ melanoma patients (Patient B1, stage II; 14 months after resection. Patients B2 and B3, stage IV). The level of non-specific background (obtained with nonpulsed T2 cells) was subtracted in all cases. *Insets*, total numbers of melanoma peptide-specific $CD8^+$ T cells induced by *in vitro* vaccination with sDC or αDC1 in each of the patients; respectively, 101 *versus* 564 (Patient B1), 51 *versus* 1902 (Patient B2), and 66 *versus* 774 (Patient B3). Similar differences between αDC1- and sDC-sensitized cultures have also been observed in eight experiments with the blood of HLA-$A2^+$ healthy donors, which demonstrated an advantage of αDC1 in inducing MART-1-specific responses.

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αDC1 Show Strongly Elevated Ability to Induce Melanoma-specific CTL Responses. To analyze the comparative ability of αDC1 to promote the development of tumor antigen-specific CTLs, αDC1 or sDCs were pulsed with melanoma-associated antigenic peptides and were used as an *in vitro* vaccine to stimulate autologous peripheral blood $CD8^+$ T cells from HLA-$A2^+$ melanoma patients. Long-term $CD8^+$ T cell lines obtained by further expansion with autologous PBMCs were harvested at day 24 and were used as responder cells against HLA-$A2^+$ T2 cells pulsed with individual peptides. As shown in Fig. 3*A*, when compared with sDCs, αDC1 proved superior in the induction of the melanoma-specific responses of cytotoxic T cells (CTLs). The superior activity of αDC1, was evident in case of each of the HLA-A2-restricted melanoma-associated CTL epitopes (MART-1-, gp100-, and tyrosinase-specific), and was observed in each of the three melanoma patients evaluated (Fig. 3*B*), as well as in eight healthy donors (in whom MART-1-specific responses were analyzed; data not shown). Whereas sDCs were also effective in sensitizing the melanoma-specific responses, αDC1 induced an average of 20-fold higher CTL levels (see the legend to Fig. 3 for the overall numbers of sDC-induced and αDC1-induced CTLs).

Discussion

In attempt to boost the ability of DCs to induce anticancer responses, we have developed a novel serum-free culture procedure yielding DCs that combine, within a single cell, three features important for their efficacy as carriers of anticancer vaccines: (*a*) fully mature status; (*b*) high migratory responsiveness to lymph-node-associated chemokines (CCR7 ligands); and (*c*) ability to produce high levels of IL-12p70, after the migration in response to 6C-kine. αDC1 efficiently migrate in response to 6C-kine, produce high levels of IL-12p70 after migration and subsequent CD40 ligation, and promote superior CTL induction *in vitro*. Although the sensitization with standard mature DCs (12), commonly used in current clinical trials, was also clearly effective, the data obtained with the cells from three melanoma patients (stage II-IV), indicate that one round of *in vitro* sensitization with αDC1 yields, as an average, 20-fold higher numbers of long-lived melanoma-specific CTLs.

The present data demonstrate that DC maturation does not necessarily need to be associated with the exhaustion of their ability to produce IL-12 (9, 10), and opens the possibility of clinical application of fully mature DCs with elevated IL-12-producing capacity, obviating the inclusion of either fetal calf serum (16) or PGE_2 (12), used in the currently available protocols. In contrast to previously applied DC-based vaccines, which relied on either immature DCs (with high ability to produce IL-12 but low stimulatory and migratory capacities) or on mature DCs (with high stimulatory and migratory functions, but reduced IL-12 production), the currently described αDC1 protocol allows us for the first time to combine all of these desirable features within a single DC.

The present data show that IFNα and p-I:C (an IFNα-inducing factor) synergize with the IFN-γ-based type-1-polarizing cocktails in promoting the development of fully mature DC1 under serum-free conditions. The ability of p-I:C to supplement the action of recombinant IFNα suggests that its activity is partially independent from the induction of IFNα/β, in accord with observations that, in IFNα/β receptor-deficient mice, p-I:C fails to induce several maturation-associated DC markers, but does induce CCR7 (17). The molecular mechanism(s) of the synergism of IFNα, p-I:C, and IFNγ in the induction of DC1, is a subject of our ongoing analysis. By analogy with other cell systems, it is likely to involve mitogen-activating protein kinase (MAPK)-, extracellular signal-regulated protein kinase (ERK)-, signal transducers and activators of transcription (STAT)-, and interferon regulatory factor (IRF)-signaling pathways (18–19).

Although high IL-12p70 production plays a key role in the ability of DC1 to induce Th1 responses (8, 11, 13), other factors may be also involved. DCs matured with p-I:C alone (without IFNγ) show increased ability to induce polyclonal Th1 responses, in the absence of an elevated IL-12p70 production (11, 13), suggesting that additional, as-yet-unidentified factors, may also contribute to high CTL-inducing ability of αDC1.

The *in vivo* effectiveness of αDC1, as prospective cancer vaccines, will need to be evaluated in comparative clinical trials. Superior CTL-inducing activity of αDC1 *in vitro*, combined with their high *in vitro* migratory function (60–95% of the migratory potential of sDCs in different donors) suggests their high *in vivo* efficacy. However, we need to test which $CD4^+$ T-cell antigens, including "heterologous helper antigens" such as KLH (2, 16), are best suited to provide the optimal level of $CD4^+$ T-cell help to assure the optimal performance of αDC1.

In addition to their prospective use as vaccine carriers, αDC1 may also be used to develop additional therapies for cancer and chronic infections with pathogens resistant to standard treatments, such as HIV. Their superior ability to activate Ag-specific T cells *in vitro* may also allow the use of αDC1 as *ex vivo* inducers of tumor-specific T cells for adoptive immunotherapy, or as a tool to identify new factors and mechanisms involved in the development of type-1 immunity.

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