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14. ABSTRACT

To enhance DC-based vaccines, we used the combination of a synthetic ligand-inducible CD40 receptor (iCD40) along with TLR-4 ligation in human monocyte-derived DCs. The iCD40 receptor permits targeted, reversible activation of CD40 in vivo, potentially bypassing the essential role of CD4+ T cells for activation of DCs. As a rigorous preclinical study of this approach, we evaluated key parameters of DC activation and function. While neither iCD40 nor TLR4 signaling alone led to high levels of IL-12p70 and IL-6, using iCD40 in combination with lipopolysaccharides (LPS) led to strongly synergistic production of both. Furthermore, this approach led to high expression of DC maturation markers, epitope-specific cytotoxic T cell and TH1 responses, as well as DC migration in vitro. Moreover, use of iCD40-modified and LPS-stimulated DCs led to targeted expansion of autologous T cells against the attractive tumor-associated antigen, prostate-specific membrane antigen (PSMA), supporting this technology as a potent strategy for DC-based prostate cancer immunotherapy.

15. SUBJECT TERMS
Dendritic Cell Vaccine, Prostate Specific Membrane Antigen, Prostate Cancer

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Introduction

We recently developed a novel DC activation system based on the CD40 signaling pathway to extend the pro-stimulatory state of DCs within lymphoid tissues by providing DC-targeted functionality, temporal control, and resistance to CD40 regulatory mechanisms. This engineered recombinant receptor was comprised of the cytoplasmic domain of CD40 fused to ligand binding domains and a membrane-targeting sequence. Administration of a lipid-permeable, dimerizing drug intraperitoneally led to the potent induction of CD40-dependent signaling cascades and greatly improved immunogenicity against both defined antigens and tumors in vivo relative to other activation modalities(*1*). Hence the chimeric CD40 was named inducible CD40 (iCD40). The high utility of iCD40-activated DCs (iCD40-DCs) in mice, suggested that methods to stabilize (or de-repress) endogenous CD40 signaling might also enhance the potency of DC vaccines.

Our recent published (*1*) studies have suggested a new method to promote DC function *in vivo*, manipulation of a chimeric inducible CD40. While we have accumulated significant supporting data in mice for the iCD40 approach, human monocyte-derived DCs are not identical to murine bone marrow-derived DCs. In particular, we found that in human DCs iCD40 synergized with Toll-like receptor-4 (TLR-4) signaling for IL-12 production in DCs. In our phase I/II clinical trial we will use prostate specific membrane antigen (PSMA) as a prostate-specific antigen to activate prostate cancer specific autologous T cell response. PSMA is a unique membrane-bound glycoprotein, which is overexpressed on prostate cancer as well as the neovasculature of most solid tumors, such as renal, bladder, pancreatic, and lung carcinoma, and sarcoma(*2*), but not in the vasculature of normal tissues(*3, 4*). Therefore PSMA-targeted therapies can destroy or prevent formation of prostate tumors as well as of new blood vessels. Clinical trials using PSMA as a tumor antigen showed no adverse effects in hormone–refractory prostate cancer patients, had favorable PSMA-specific cellular immune responses with significant decreases in PSA levels (*5-7*).
        During the last year of DOD award we preclinically evaluated the PSMA-specific immunogenicity and *in vitro* migratory abilities of our newly developed DC vaccine, i.e. iCD40-DCs loaded with PSMA protein. In preparation to the phaseI/II clinical trial, we have produced and evaluated the integrity of recombinant PSMA protein. Using cytotoxic assay with LNCaP cells, we have found that iCD40-DCs induced enhanced PSMA-specific CTL responses. We used overlapping PSMA library for direct measurements of PSMA-specific Th and CTL responses by IFN-γ ELISpot assay and performed low-resolution genotyping of the HLA-DRB1, HLA-A, B, C loci of healthy volunteers. We have observed enhanced and dimerizer drug dependent migratory abilities of iCD40-DCs *in vitro*.

Production and evaluation of recombinant PSMA protein

The baculovirus transfer vector, pAcGP67A (BD Biosciences) containing the cDNA of extracellular portion of PSMA (residues 44-750) was kindly provided by Dr Pamela J. Bjorkman (Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA). PSMA was fused with a hydrophobic secretion signal, Factor Xa cleavage site, and N-terminal 6x-His affinity tag. High titer baculovirus was produced by the Baculovirus/Monoclonal antibody core facility of Baylor College of Medicine. PSMA protein was produced in High 5 cells infected with recombinant virus for 48-hours, and protein was purified from cell supernatants using Ni-NTA affinity columns (Qiagen, Chatsworth, CA) as previously described (8). Before purification the expression of ~100 kDa solitary band of PSMA protein was detected by western blot (**Figure 1**). Briefly, supernatants of High 5 cells were separated in 12% SDS-PAGE gels, and proteins were transferred to nitrocellulose membranes (Bio-Rad). Blots were hybridized with mouse-anti-human PSMA antibody (Y-PSMA1, Anogen-YES Biotech Laboratories Ltd, Ontario, Canada) followed by goat anti-mouse IgG-HRP (Santa Cruz Biotechnology). Blots were developed using the SuperSignal West Dura Stable substrate system (Pierce, Rockford, Illinois). After purification the presence of hPSMA was additionally confirmed (data not shown) by silver staining using Imperial Protein Stain kit (Pierce, Rockford, IL).
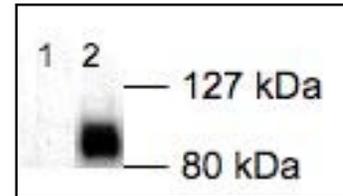

Figure 1. Expression of hPSMA in the supernatants of High five cells. Lane 1, supernatants from non-transduced cells (negative control); lane 2, High 5 cells transduced with baculovirus expressing hPSMA

*iCD40-DCs induce enhanced MHC class I and II- restricted T cell responses to PSMA*

PSMA is a target antigen in vaccine for phase I/II clinical trial. Therefore, we tested the optimum antigen dose to achieve maximum cytotoxic activity of cytotoxic lymphocytes (CTLs) induced by autologous iCD40-DCs activated with lipopolysaccharides (LPS) and AP20187 dimerizer drug in PSMA-specific cytotoxic chromium-release assay. iCD40-DCs of HLA-A2[+] donor were pulsed with 0.1, 1, 10 and 50 or 100 μg/ml PSMA and mixed with autologous T cells. DCs pulsed with 10 or 50 μg/ml PSMA induced potent CTLs capable to recognize and kill tumor cells in an antigen-specific manner, i.e. able to kill the PSMA-expressing prostate cancer
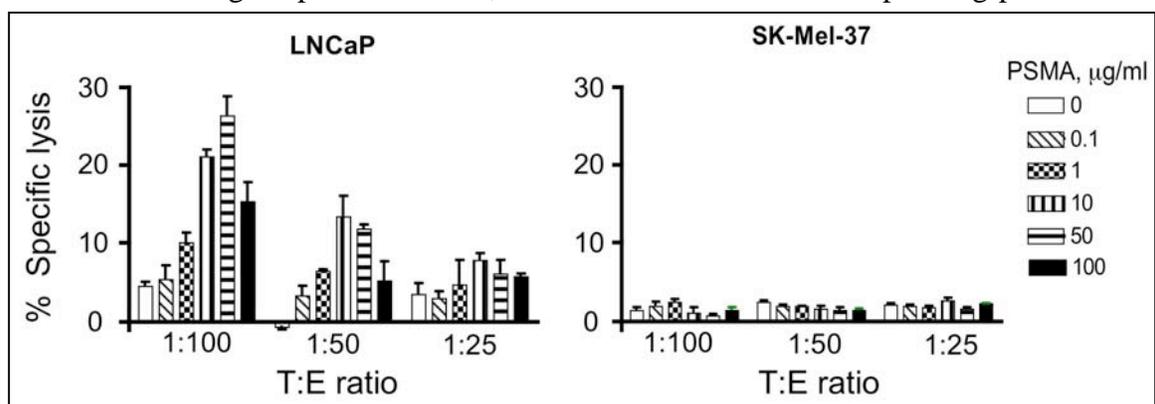
Figure 2. PSMA-specific lysis of LNCaP (PSMA[+]/HLA-A2[+]) prostate cancer cells by HLA-A2[+] CTLs expanded by autologous BP-GMAX-CD1 cells pulsed with dilutions of hPSMA. On Day 4 of culture, DCs from HLA-A2[+] healthy donors were pulsed with 0.1, 1, 10, 50, 100 μg/ml of hPSMA protein or left untreated. On Day 5 of culture, cells were transduced with Ad5f35-ihCD40 at 10,000 viral particle VP/cell and stimulated with 1 μg/ml LPS and 100 nM AP20187. T cells were cocultured with DCs at a 1:3 ratio for 7 days and then restimulated with fresh DCs on Day 14. T cells were evaluated for antigen-specific lytic activity using a ^{51}Cr-release assay. T:E, target: effector ratio.

cell line LNCaP (HLA-A2$^+$), but not the PSMA-negative SK-Mel-37 (HLA-A2$^+$) melanoma cell line. Because the difference in efficacy between 10 μg/ml and 50 μg/ml doses of PSMA was not statistically significant (p>0.05), the 10 μg/ml dose of PSMA was selected for *ex vivo* pulsing of DCs (**Figure 2**).

Moreover, we compared the PSMA-specific CTL activity induced by autologous iCD40-DCs activated with LPS and AP1903 (dimerizer drug) and DCs stimulated with control stimuli, such as CD40L+LPS and maturation cocktail (MC) (**Figure 3**). In this experiment HLA A2-positive human ihCD40-DCs were pulsed with 10 μg/ml PSMA protein and activated with 1 μg/ml LPS and 100 nM AP1903 in order to induce killing of HLA-A2$^+$ LNCaP cells (expressing PSMA) but not HLA-A2$^+$ SK-Mel-37 (PSMA-negative cells) by cytotoxic T cells. AP1903 and LPS treated ihCD40-expressing DCs induced the highest antigen-specific CTL activity among the groups tested. Killing of LNCaP cells was significantly (p < 0.001) higher for the Ad-ihCD40+AP1903+LPS group compared to control groups. This study provides *in vitro* proof of concept for the use of iCD40-DCs pulsed with PSMA and activated with LPS and AP1903 in the treatment of prostate cancer.
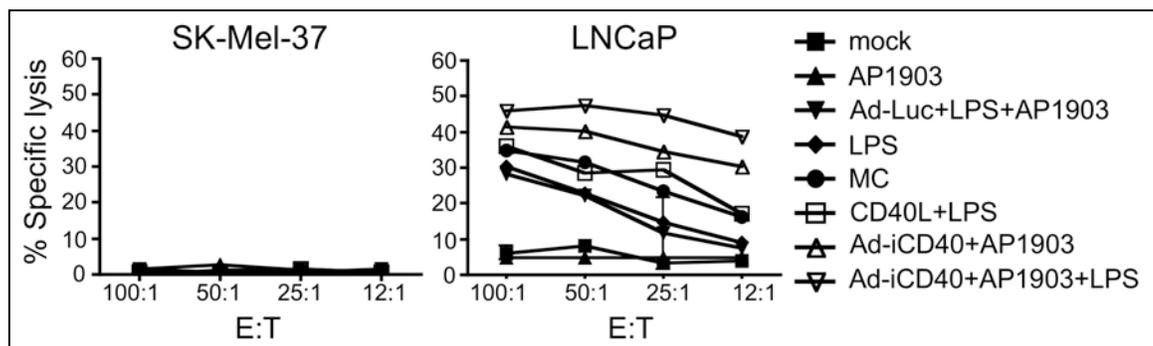

Figure 3. Enhanced PSMA-specific CTL function induced by LPS and AP1903-activated ihCD40-expressing DCs. On Day 5 of culture, DCs from HLA-A2-positive healthy donors were pulsed with 10 μg/ml human PSMA protein. On day 6 cells were transduced with Ad5f35-ihCD40 at 10,000 VP/cell and activated with 1 μg/ml LPS and 100 nM AP1903. T cells were colcultured with DCs at 1:10 ratio for 7 days and then restimulated with fresh DCs on days 14 and 20. T cells were evaluated for antigen-specific lytic activity using a ^{51}Cr-release assay. E:T , effector: target ratio.

Although some PSMA class-I and II epitopes are described in the literature, there are remaining discrepancies concerning whether they are functional in immunoassays, such as IFN-γ ELISpot (*9*). A broad panel of epitopes must be identified from PSMA to optimize vaccination strategies and facilitate monitoring of tumor-specific T cell populations after immunotherapeutic interventions. To date, PSMA epitopes have been identified for only a limited number of HLA alleles using peptide candidate approach. This precluded the development of immunoassays for direct measurements of PSMA-specific Th and CTL responses. Therefore, in order to develop new PSMA epitopes for IFN–γ ELISpot assay we have generated the library covering the complete sequence of PSMA and consisting of 184 15-mer peptides with 11-aa overlap pooled into 28 pools of 14 peptides (**Figure 4**).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
15	1	2	3	4	5	6	7	8	9	10	11	12	13	14
16	15	16	17	18	19	20	21	22	23	24	25	26	27	28
17	29	30	31	32	33	34	35	36	37	38	39	40	41	42
18	43	44	45	46	47	48	49	50	51	52	53	54	55	56
19	57	58	59	60	61	62	63	64	65	66	67	68	69	70
20	71	72	73	74	75	76	77	78	79	80	81	82	83	84
21	85	86	87	88	89	90	91	92	93	94	95	96	97	98
22	99	100	101	102	103	104	105	106	107	108	109	110	111	112
23	113	114	115	116	117	118	119	120	121	122	123	124	125	126
24	127	128	129	130	131	132	133	134	135	136	137	138	139	140
25	141	142	143	144	145	146	147	148	149	150	151	152	153	154
26	155	156	157	158	159	160	161	162	163	164	165	166	167	168
27	169	170	171	172	173	174	175	176	177	178	179	180	181	182
28	183	184												

Figure 4. PSMA peptide library. All peptides were divided into 28 pools in such a way that each peptide is present in 2 pools (in bold). This method allows determining those single peptides that most likely induced responses to the peptide pools. Thus, responses to pools 9, 20, 24 and 26 may be induced by single peptides 79, 135 and 163 respectively.

We have enrolled healthy volunteers into the study approved by Baylor College of Medicine Institutional Review Board on Human Subjects (protocol # H-18532). In order to evaluate the PSMA library for direct measurement of PSMA-specific Th and CTL responses and also identify the HLA-class-I/II-restricted PSMA epitopes for IFN-γ ELISpot assay, we have performed low-resolution HLA-DRB1 and HLA-A, B and C genotyping of the donors using low-resolution Fastype™ System (Bio-Synthesis, Inc.). Using autologous iCD40-DCs pulsed with extracellular part of PSMA protein, PSMA-specific T cells from prostate cancer patients and healthy volunteers were reactivated and expanded *in vitro*. PSMA-specific T cells were detectable in an IFN-γ ELISpot assay after overnight incubation with each of the peptide pools (**Figure 5**). In donor with the
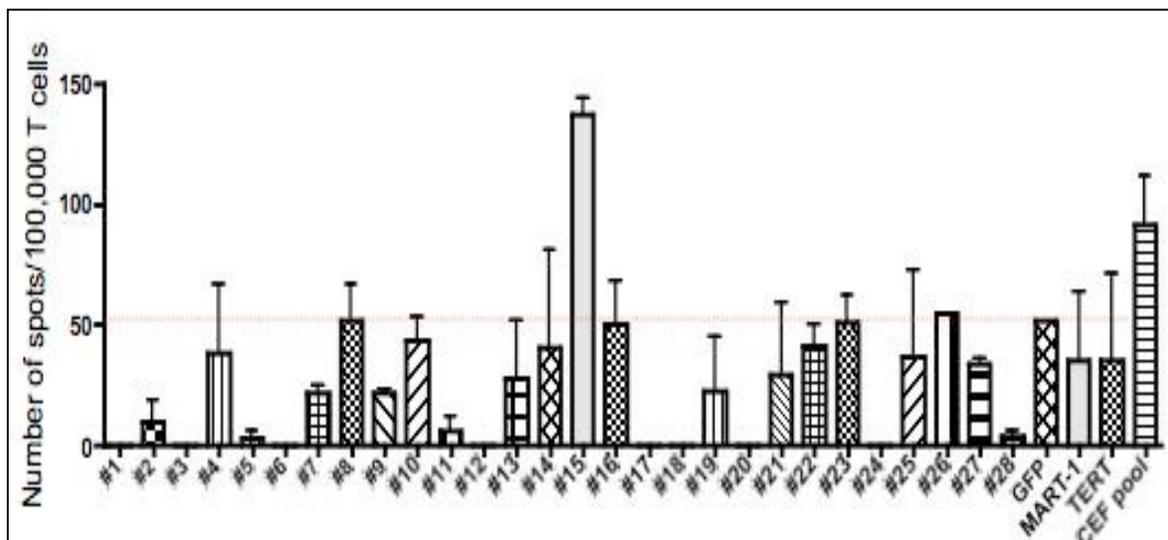

Figure 5. Induction of PSMA-specific T cell responses using PSMA peptide library. Autologous T cells were purified from PBMCs by negative selection using the Pan-T Cell Isolation kit (Miltenyi Biotec, Auburn, CA) and mixed with DCs at a DC:T cell ratio of 1:5. Cells will be incubated in complete RPMI with 20 U/ml hIL-2 (R&D Systems, Minneapolis, MN). T cells will be restimulated at Day 7 and assayed at Day 14 of culture. The IFN-γ ELISpot assay was performed using PSMA peptide pools (#1-#28), GFP, MART-1 and TERT overlapping peptide pools were used as negative controls and CEF pool was used as positive control.

genotype HLA-DRB1*1301/1401, HLA-A3/ 32, HLA-B*08/B72 (70) and Cw*12, peptide pool #15 demonstrated induction of IFN-γ levels indicating the potential presence of immunodominant peptides in this pool (**Figure 5**). The individual peptides from pool #15 were selected for further screening and work is on-going. Following this initial screening with each of the PSMA peptide pools, the minimal recognized T cell epitopes will be identified and used in the analysis of PSMA-specific Th/CTL responses (secondary end-points) in phase I/II clinical trial.

Comparison of migratory capacity of human iCD40-DCs in vitro

We previously identified that CCR7 expression is induced in iCD40-DCs, we assessed the migratory capacities of iCD40-DCs *in vitro*.

We tested whether human iCD40-DCs could migrate *in vitro* towards chemokine MIP-3β (CCL19) in transwell assays upon dose escalation of AP1903 dimerizer drug (**Figure 6**). iCD40-DCs treated with 100 nM AP1903 dimerizer had migration levels comparable to DCs stimulated with CD40L+LPS or maturation cocktail (MC). Moreover, iCD40-DC migration was significantly increased by addition of LPS. Here we show that migration of iCD40-expressing DCs is dimerizer drug-dependent and peaks at 100 nM AP1903. Thus, 100nM AP1903 was the selected dose for *ex vivo* stimulation of iCD40-DCs in clinical protocol.
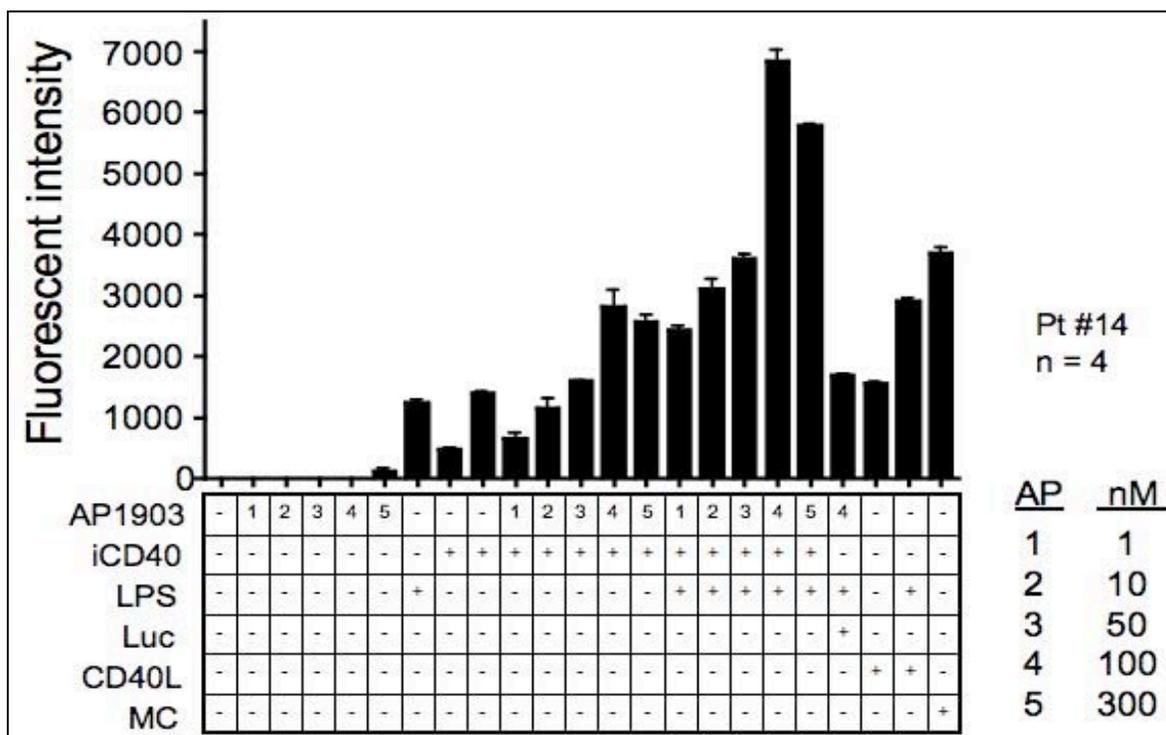
Figure 6. AP1903-dependent migration of human DCs. Chemotaxis of DCs was measured by migration through a polycarbonate filter with 8 μm pore size in 96-Multiwell HTS Fluoroblok plates. Assay medium (250 μL) containing 100 ng/ml CCL19 or assay medium alone (as control for spontaneous migration) were loaded into the lower chamber. DCs (50,000) were labeled with Green-CMFDA cell tracker (Invitrogen) and placed into the upper chamber in a total volume of 50 μL for 1 hour at $37\,^{0}C$ and 5% CO_2. Migrated cell fluorescence within the lower chamber was measured using the FLUOstar OPTIMA reader (excitation 485 nm, emission 520 nm). The mean fluorescence of spontaneously migrated cells was subtracted from the total number of migrated cells towards CCL19 for each condition. Pt: patient, AP: AP1903.

Key Research Accomplishments

> ➢ Purified and verified the integrity of Prostate Specific Membrane Antigen (PSMA) protein

> ➢ Performed low-resolution genotyping of the HLA-DRB1, HLA-A, B, C loci in healthy donors

> ➢ Established optimum antigen dose to achieve maximum PSMA-specific cytotoxic activity of CTLs induced by autologous iCD40-DCs in PSMA-specific cytotoxic chromium-release assay

> ➢ iCD40-DCs improved the generation of MHC class I–restricted CTL responses to PSMA in PSMA-specific cytotoxic chromium-release assay

> ➢ Developed and evaluated a peptide library for direct measurements of PSMA-specific Th/CTL responses in IFN-γ ELISPOT assays

> ➢ iCD40 was sufficient to induce the migratory abilities of human DCs *in vitro* and migration was dimerizer drug dependent

Reportable Outcomes

➢   Lapteva N, Seethammagari MR, Hanks B, Jiang J, Levitt JM, Slawin KM, Spencer DM Enhanced Activation of Human Dendritic Cells by Inducible CD40 and TLR4 Ligation **Cancer Research**. 2007, 67 (21), p 10528-10537

➢   Lapteva N, Seethammagari MR, Hanks B, Jiang J, Levitt JM, Slawin KM, Spencer DM Preclinical Evaluation of an iCD40-Dendritic Cell Vaccine for Prostate Cancer April 14-18th 2007, **AACR Annual Meeting**, Los Angeles CA

➢   Aldrich MB, Sanders D, Lapteva N, and SY Chen SOCS1 Downregulation in Dendritic Cells Promotes Memory T Cell Responses. **Vaccine** *in press*.

➢   Lapteva N, Seethammagari MR, Hanks BA, Jiang J, Levitt JM, Slawin KM, Spencer DM Enhancement of Immunostimulatory Functions of Human Dendritic Cells by Inducible CD40 and TLR4 Ligation 5th **Cancer Center Symposium**, 2007 Cancer Center, Texas Medical Center, Houston, TX

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Conclusion

The proposed study is directly translatable into the clinic and will serve as the final preclinical step preceding application for a phase I/II clinical trail for hormone-refractory metastatic prostate cancer patients, which will be conducted partly within the Scott Department of Urology at Baylor College of Medicine, Houston, TX. We have expressed and purified PSMA protein, which served as a prototype for production of clinical GMP grade protein for pulsing iCD40-DCs. Using CTL assays against LNCaP (prostate cancer) cells, we have established the dose of the PSMA antigen to pulse human iCD40-DCs in clinical protocol and provided the *in vitro* proof of concept for use of these cells in clinical trial. In addition, we evaluated the PSMA library for the direct analysis of PSMA-specific T cell responses (secondary end-points) in phase I/II clinical trial.

Appendices

Reprint of the manuscript resulted from the accomplished work (Lapteva et al, Cancer Research, 2007) with the detailed information on materials and methods used in this work.

Enhanced Activation of Human Dendritic Cells by Inducible CD40 and Toll-like Receptor-4 Ligation

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Abstract

Despite the potency of dendritic cells (DC) as antigen-presenting cells for priming adaptive immunity, DC-based cancer vaccines have been largely insufficient to effectively reduce tumor burden or prevent tumor progression in most patients. To enhance DC-based vaccines, we used the combination of a synthetic ligand-inducible CD40 receptor (iCD40) along with Toll-like receptor-4 (TLR-4) ligation in human monocyte-derived DCs. The iCD40 receptor permits targeted, reversible activation of CD40 *in vivo*, potentially bypassing the essential role of CD4^{+} T cells for activation of DCs. As a rigorous preclinical study of this approach, we evaluated key parameters of DC activation and function. Whereas neither iCD40 nor TLR-4 signaling alone led to high levels of interleukin (IL)-12p70 and IL-6, using iCD40 in combination with lipopolysaccharide (LPS) or monophosphoryl lipid A led to strongly synergistic production of both. Furthermore, this approach led to high expression of DC maturation markers, epitope-specific CTL and T helper 1 responses, as well as DC migration *in vitro* and *in vivo*. Moreover, use of iCD40-modified and LPS-stimulated DCs led to targeted expansion of autologous T cells against tumor-associated antigens, including prostate-specific membrane antigen, and elimination of preestablished tumors, supporting this technology as a potent strategy for DC-based cancer immunotherapy. [Cancer Res 2007;67(21):10528–37]

Introduction

Due to their unique antigen processing and presentation and high-level expression of costimulatory and cytokine molecules, dendritic cells (DC) are by far the most potent antigen-presenting cells for priming and activating naïve T cells (1). This property has led to their use as a cellular platform for vaccination in several encouraging anticancer clinical trials with regards to enhanced tumor antigen–specific immune responses (2). Nevertheless, clinical efficacy of DC vaccines with regards to overall patient survival has been disappointing. This is likely due to several key deficiencies, including suboptimal activation, limited migration to draining lymph nodes, and insufficient life span for optimal T-cell activation in the lymph node environment.

A critical variable in optimization of DC-based cancer vaccines is interaction of DCs with immune effector cells such as CD4^{+} and CD8^{+} and regulatory T cells. In these interactions, DC maturation state is a key factor in determining the resulting effector functions (3). To maximize CD4^{+} and CD8^{+} T-cell priming while minimizing regulatory T-cell expansion, DCs need to be fully activated or "licensed" (4), expressing high levels of costimulatory molecules like CD40, CD80, and CD86 and proinflammatory cytokines like interleukin (IL)-12p70 and IL-6. Equally important, DCs must migrate efficiently from the site of vaccination to draining lymph nodes to initiate T-cell interactions (5).

In humans and mice, several DC subtypes have been characterized by surface markers and function. Monocyte-derived DCs (MoDC) are used in most antitumor vaccine clinical trails due to easy preparation of large batches of these cells from peripheral blood precursors (6). For *ex vivo* maturation of MoDCs, the majority of DC-based trials have used a standard maturation cytokine cocktail (MC), composed of tumor necrosis factor (TNF)-α, IL-1β, IL-6, and prostaglandin E_2 (PGE_2). The principal function of PGE_2 in this context is to sensitize chemokine receptor 7 (CCR7) to its ligands, CC chemokine ligand 19 (CCL19) and CCL21, thereby enhancing migration to draining lymph nodes (7). However, PGE_2 has also been reported to have immunosuppressive activities including suppression of T-cell proliferation (8), inhibition of proinflammatory cytokine production (e.g., IL-12p70 and TNF-α; ref. 9), and down-regulation of MHC II surface expression (10). Therefore, maturation protocols that circumvent PGE_2 while promoting migration may significantly improve the therapeutic efficacy of DC-based vaccines.

We have recently developed a novel DC activation system based on temporal control of the CD40 signaling pathway to extend the prostimulatory state of DCs within lymphoid tissues. DC functionality was improved by increasing both the amplitude and duration of CD40 signaling (11). To accomplish this, CD40 was reengineered by fusing the cytoplasmic domain of CD40 to synthetic ligand binding domains along with a membrane-targeting sequence. Administration of the lipid-permeable chemical inducer of dimerization, AP20187 (12), led to *in vivo* induction of CD40-dependent signaling cascades in murine DCs. This strategy significantly enhanced the immunogenicity against both defined antigens and tumors *in vivo* well beyond that achieved with other widely accepted activation modalities (11). The robust potency of this ligand-inducible CD40 (named iCD40) in mice suggested that this method might also enhance human DC vaccines. Nonetheless, iCD40 signaling alone in MoDCs was insufficient for full activation.

Toll-like receptor (TLR) signaling also plays a critical role in DC maturation and activation, and DCs express multiple distinct TLRs (13). The 11 mammalian TLRs respond to various pathogen-derived macromolecules, contributing to the activation of innate immune responses along with initiation of adaptive immunity. Lipopolysaccharide (LPS) and clinically relevant analogues, like monophosphoryl lipid A (MPL), bind to cell-surface TLR-4 complexes (14), leading to signaling that culminates in the induction of

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transcription factors such as NF-κB and IRF3, along with mitogen-activated protein kinases p38 and c-Jun NH_2-terminal kinase (15, 16). During this process, DCs mature and partially up-regulate surface proteins such as CD40, CD80, and MHC molecules and proinflammatory cytokines like IL-6, IL-12, and type I IFNs (17). LPS-induced maturation enhances the ability of DCs to stimulate antigen-specific T-cell responses *in vitro* and *in vivo* (18). Nevertheless, TLR-4 signaling alone is also not sufficient to elicit a functional antitumor immune response in most models.

In this study, we adapted iCD40 for application to human MoDCs, showing that combining iCD40 signaling with TLR-4 ligation leads to persistent and robust MoDC activation. These activated, enhanced DCs not only possess high migratory capacity *in vitro* and *in vivo* but also produce very high levels of IL-12p70, which is not seen with either stimulus alone. Further, enhanced DCs potently activate antigen-specific T helper 1 (Th1) and CTLs, including specificity for the weakly immunogenic self antigen prostate-specific membrane antigen (PSMA). To our knowledge, this is the first demonstration that potent DC activation and migratory capacity can be achieved in the absence of maturation cocktails that contain PGE_2. Moreover, iCD40/TLR-4–activated DCs show potent antitumor activity against large, preestablished tumors. These studies form the pharmacologic foundation for improved cancer immunotherapy.

Materials and Methods

Tumor cell lines and peptides. NA-6-Mel, T2, SK-Mel-37, LNCaP, and EG.7-OVA cell lines were purchased from American Type Culture Collection. HLA-A2–restricted peptides MAGE-3-A2.1 p271-279 (FLWGPRALV), influenza matrix p58-66 (GILGFVFTL), and HIV-1 gag p77-85 (SLYNTVATL) were used to analyze $CD8^+$ T-cell responses. In Th cell polarization experiments, HLA-DR11.5–restricted tetanus toxoid peptide TTp30 FNNFTVSF-WLRVPKVSASHLE was used. All peptides were synthesized by Genemed Synthesis, Inc., with a high-performance liquid chromatography–determined purity of >95%.

Generation and stimulation of human DCs. Peripheral blood mononuclear cells from healthy donors were isolated by density centrifugation of heparinized blood on Lymphoprep (Nycomed), washed with PBS, resuspended in CellGenix DC medium, and allowed to adhere in culture plates for 2 h at 37°C and 5% CO_2. Nonadherent cells were removed by extensive washings, and remaining monocytes were cultured for 5 days in 500 units/mL hIL-4 and 800 units/mL human granulocyte macrophage colony-stimulating factor (R&D Systems). As assessed by morphology and flow cytometry, resulting immature DCs were MHC class I^{hi}, MHC class II^{hi}, $CD40^{lo}$, $CD80^{lo}$, $CD83^{lo}$, $CD86^{lo}$. The immature DCs were $CD14^-$ and contained <3% contaminating $CD3^+$ T cells, $CD19^+$ B cells, and $CD16^+$ natural killer (NK) cells.

Cells (2×10^6/mL) were cultured in 24-well dishes and transduced with adenoviruses at 10,000 viral particles/cell [~160 multiplicity of infection (MOI)] for 90 min at 37°C and 5% CO_2. Immediately after transduction, DCs were stimulated with MPL, FSL-1, Pam3CSK4 (InvivoGen), LPS (Sigma-Aldrich), AP20187 [or AP1903 (ARIAD Pharmaceuticals)], or MC [10 ng/mL TNF-α, 10 ng/mL IL-1β, 150 ng/mL IL-6 (R&D Systems), and 1 μg/mL PGE_2 (Cayman Chemicals)]. Alternatively, DCs were stimulated with 1 μg/mL CD40L (Alexis Biochemicals) with 1 μg/mL enhancer. For T-cell assays, DCs were pulsed with 50 μg/mL PSMA or MAGE-3 peptide 24 h before and after adenoviral transduction.

Surface markers and cytokine production. Cell surface staining was done with fluorochrome-conjugated monoclonal antibodies (mAb; BD Biosciences). Cells were analyzed on a FACSCalibur cytometer (BD Biosciences). Cytokines were measured in culture supernatants using ELISA kits for human IL-6 and IL-12p70 (BD Biosciences).

DC migration assay. Chemotaxis of DCs was measured by migration through a polycarbonate filter (8 μm pore size) in 96-Multiwell HTS

Fluoroblok plates (BD Biosciences). Assay medium (250 μL) containing 100 ng/mL CCL19 (R&D Systems) or assay medium alone (as a control for spontaneous migration) was loaded into the lower chamber. DCs (50,000) were labeled with Green-CMFDA Cell Tracker (Invitrogen), unstimulated or stimulated for 48 h with indicated reagents, and added to the upper chamber in a total volume of 50 μL for 1 h at 37°C and 5% CO_2. Cells that had migrated through the membrane were measured with a FLUOstar OPTIMA reader (BMG Labtech, Inc.). Mean fluorescence of spontaneously migrated cells was subtracted from the total.

Tetramer staining. HLA-A2 tetramers assembled with MAGE-3 peptide were from Baylor College of Medicine Tetramer Core Facility. Presensitized $CD8^+$ T cells in 50 μL of PBS containing 0.5% FCS were stained with phycoerythrin-labeled tetramer for 15 min on ice before addition of FITC-CD8 mAb (BD Biosciences). After washing, results were analyzed by flow cytometry.

Polarization of naïve Th cells. Naïve $CD4^+CD45RA^+$ T cells from HLA-DR11.5-positive donors (genotyped using FASTYPE HLA-DNA SSP typing kit; BioSynthesis) were isolated by negative selection using naïve $CD4^+$ T-cell isolation kit (Miltenyi Biotec). T cells were stimulated with autologous DCs pulsed with tetanus toxoid (5 flocculation units/mL) and activated with various stimuli at stimulator/responder ratio of 1:10. After 7 days, T cells were restimulated with autologous DCs pulsed with TTp30 and transduced with Ad-iCD40. Cells were stained with phycoerythrin-anti-CD4 antibody (BD Biosciences), fixed, and permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences), and then stained with human IFN-γ mAb (eBioscience) and analyzed by flow cytometry. Supernatants were analyzed using human Th1/Th2 BD Cytometric Bead Array Flex Set on BD FACSArray Bioanalyzer (BD Biosciences).

Migration of human DCs in mouse host. To assess migration of human DCs *in vivo*, we developed an adenovector, Ad5-CBR, expressing red-shifted (emission peak, 613 nm) luciferase from *Pyrophorus plagiophalamus* click beetles (Promega). MoDCs were transduced with ~50 MOI of Ad5-CBR and 160 MOI of Ad5/f35-iCD40. DCs were then matured with MC or 1 μg/mL LPS (Sigma-Aldrich). Mouse bone marrow–derived DCs were obtained as described before (11) and were matured with LPS. DCs (2×10^6) were injected into the left and right hind footpads of irradiated (250 rad) BALB/c mice (three mice per group, $n = 6$). Mice were i.p. injected with D-Luciferin (~1 mg) and imaged over several days using an IVIS 100 imaging system (Xenogen Corp.). Luminescent signal was measured, and popliteal and inguinal lymph nodes were removed at day 2 after DC inoculation. The lymph node signals were measured and background was subtracted for each group ($n = 6$).

Data analysis. Results are expressed as the mean ± SE. Sample size was determined with a power of 0.8, with a one-sided α-level of 0.05. Differences between experimental groups were determined by Student's t test.

IFN-γ ELISpot and chromium release assays, PSMA purification, and *in vivo* tumor vaccination studies are described in detail in Supplementary Methods.

Results

Expression of iCD40 and induction of DC maturation. To investigate whether iCD40 signaling can enhance the immunogenic functions of human MoDCs, we generated an adenovirus, Ad5/f35-ihCD40 (simplified to Ad-iCD40), expressing chemical inducer of dimerization–inducible human CD40 receptor (analogous to previously described mouse iCD40 vector) under cytomegalovirus (CMV) promoter control (11). The cytoplasmic signaling domain from human CD40 was subcloned downstream of a myristoylation-targeting domain and two tandem AP20187-binding $FKBP12_{v36}$ domains (ref. 19; Fig. 1A). Immature DCs expressed endogenous CD40, which was highly induced by LPS (Fig. 1B). Transduction of Ad-iCD40 led to expression of distinctly sized iCD40, which did not affect endogenous CD40 expression. Interestingly, LPS also significantly increased iCD40 expression. When DCs from four different donors were transduced with control vector expressing
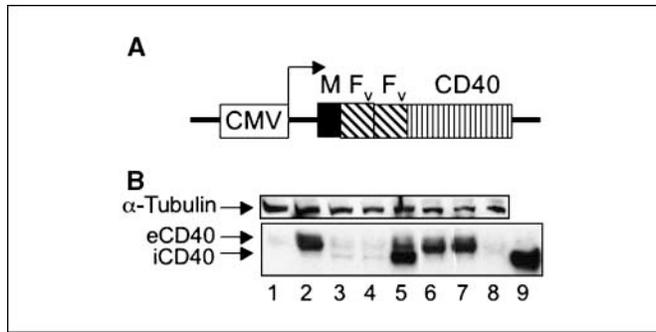
Figure 1. Schematic diagram of iCD40 and expression in human DCs.
A, schematic showing orientation of CD40 cytoplasmic domain, FKBP12$_{v36}$ domains (F_v; ref. 19), and myristoylation-targeting domain (*M*). Expression of M-F$_v$2-CD40 chimeric protein (iCD40) is under CMV promoter control. *B,* expression of endogenous (*eCD40*) and recombinant inducible (*iCD40*) forms of CD40 assessed by Western blot. *Lane 1,* wild-type DCs (endogenous CD40 control); *lane 2,* DCs stimulated with LPS (1 μg/mL); *lanes 3 and 4,* DCs transduced with 10,000 viral particles/cell (MOI ∼ 160) of Ad5/f35-iCD40 (iCD40-DCs) with or without AP20187 dimerizer drug, respectively; *lane 5,* iCD40-DCs stimulated with LPS and AP20187; *lane 6,* DCs stimulated with CD40L and LPS; *lane 7,* DCs transduced with Ad5/f35-GFP (GFP-DCs) at MOI 160 and stimulated with AP20187 and LPS; *lane 8,* GFP-DCs stimulated with AP20187; *lane 9,* 293 T cells transduced with Ad5/f35-iCD40 (positive control). Levels of α-tubulin served as internal control. Representative of three different donors.

CMV promoter–driven enhanced green fluorescent protein (eGFP), Ad5/f35-eGFP, the percentage of transduced cells was similar between nonactivated and LPS-activated cells, whereas eGFP expression levels were significantly higher (∼ 3.5-fold) in LPS-activated DCs (Supplementary Fig. S1). This is consistent with induction of the CMV promoter by LPS (20), and interpretations of any additive effects of iCD40 and LPS must take this into account.

We previously showed that mouse variant iCD40 signaling can induce murine bone marrow–derived DC maturation (11), which is critically linked to the transition from a tolerogenic to an activating, immunogenic state (3, 11). To determine whether humanized iCD40 also affects expression of DC maturation markers, we transduced

MoDCs with Ad-iCD40 (iCD40-DCs) and evaluated expression of maturation markers CD40, CD80, CD83, and CD86 following various stimuli. As expected, LPS (or derivative MPL)-mediated TLR-4 signaling led to up-regulation of all four markers (Fig. 2; refs. 16, 21, 22). Each marker was also induced by iCD40 signaling, albeit to a lesser extent. However, both stimuli cooperated to induce the highest levels of maturation markers, including activation marker CD83, at levels comparable to the potent combination of enhanced CD40L and LPS (or MC; data not shown). In contrast, control Ad-Luc alone, expressing Renilla luciferase, provided only incidental activation (data not shown) and revealed no additive effects when combined with LPS. These results show that the combination of iCD40 and TLR-4 ligands provides sufficient stimulation for potent DC maturation. Moreover, the effects of LPS are unlikely to be due simply to the up-regulation of iCD40 because of the already substantial effects of LPS monotreatment.

Inducible CD40 signaling and TLR-4 ligation synergize for IL-12p70 and IL-6 production in human DCs. IL-12p70 heterodimer, composed of p35 and p40 chains, activates T-cell and NK cell responses, leading to IFN-γ production. IL-12 also favors Th1 differentiation and acts as a vital link between innate and adaptive immunity (1, 23). Therefore, induction of biologically active IL-12p70 is likely to be critical for optimum DC-based vaccines. Nonetheless, current DC vaccination protocols that include PGE$_2$ suppress p35 and thus IL-12 production (24). Previously, we reported that iCD40 signaling promotes p35 expression in mouse bone marrow–derived DCs (11). Because TLR-4 ligation can promote p40 expression (25), we investigated whether iCD40 and TLR-4 signaling can cooperate for IL-12p70 production by culturing iCD40-DCs in the presence of LPS (or MPL) and measuring IL-12p70 production in cell supernatants.

Although neither LPS (or MPL) nor iCD40 signaling alone could induce significant (>30 pg/mL) IL-12 levels, the combination led reproducibly to robust (>10 ng/mL) IL-12 levels, well beyond that achieved with cross-linking–enhanced CD40L and LPS (Fig. 3A). Predictably, MC did not trigger IL-12 production or synergize with
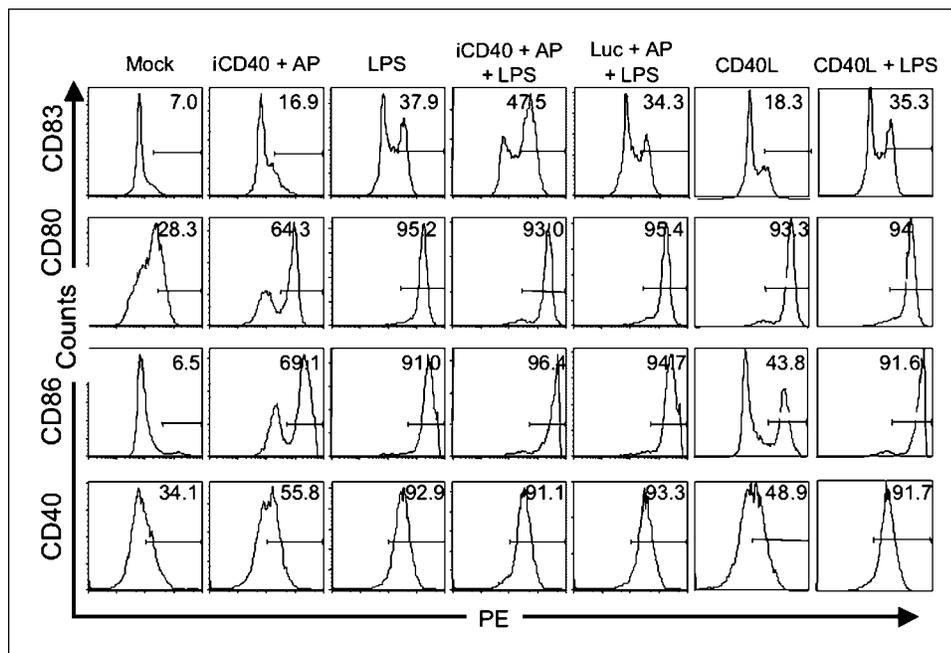
Figure 2. Enhanced maturation status of iCD40-DCs stimulated with LPS. Immature MoDCs were transduced with Ad5/f35-iCD40 (*iCD40*) or Ad5/f35-Luc (*Luc*) and stimulated with LPS (1 μg/mL) for 48 h in the presence of 100 nmol/L AP20187 (*AP*). Alternatively, DCs were matured with LPS alone or 1 μg/mL enhanced CD40L (Alexis Biochemicals). Percentage of DCs expressing CD83, CD80, CD86, and CD40 was determined with phycoerythrin (*PE*)-conjugated antihuman mAbs (BD Biosciences) by flow cytometric analysis. Fluorescence-activated cell sorting histograms from one donor (of at least three) experiment. Representative of three different donors.

Figure 3. Synergism of iCD40 and TLR-4 for IL-12p70 and IL-6 production. *A,* immature human MoDCs (5×10^5) were transduced with Ad5/f35-iCD40 or Ad5/f35-Luc (*Ad-Luc*) and stimulated with LPS or MPL (1 μg/mL) for 48 h. Alternatively, DCs were stimulated with standard maturation cocktail (*MC*) or with PGE$_2$-free MC (*MC w/o PGE$_2$*). After 48 h, supernatants were assayed for IL-12p70. Additionally, DCs were matured in the presence of LPS with enhanced CD40L (1 μg/mL; *right;* data from an independent experiment). *B,* MoDCs were transduced with Ad5/f35-iCD40 and stimulated with indicated concentrations of FSL-1, Pam3CSK4, or MPL for 48 h. Supernatants were assayed 48 h after stimulation. *C,* MoDCs were transduced with Ad5/f35-iCD40 and stimulated with LPS (1 μg/mL) or CD40L (1 μg/mL). IL-12p70 production was monitored at various time points post-stimulation (*top*). In parallel, cells of the same donor were washed thrice at 24 h post-stimulation and cultured in medium without stimulatory factors for the indicated time points before IL-12p70 ELISA (*bottom*). *D,* DCs were transduced with Ad5/f35-iCD40 or Ad5/f35-Luc and stimulated with LPS or MPL (1 μg/mL) and CD40L (1 μg/mL) for 48 h. After 48 h, supernatants were assayed for IL-6. The IL-12p70 and IL-6 expression levels were measured from at least five different donors and all experiments included duplicate samples.
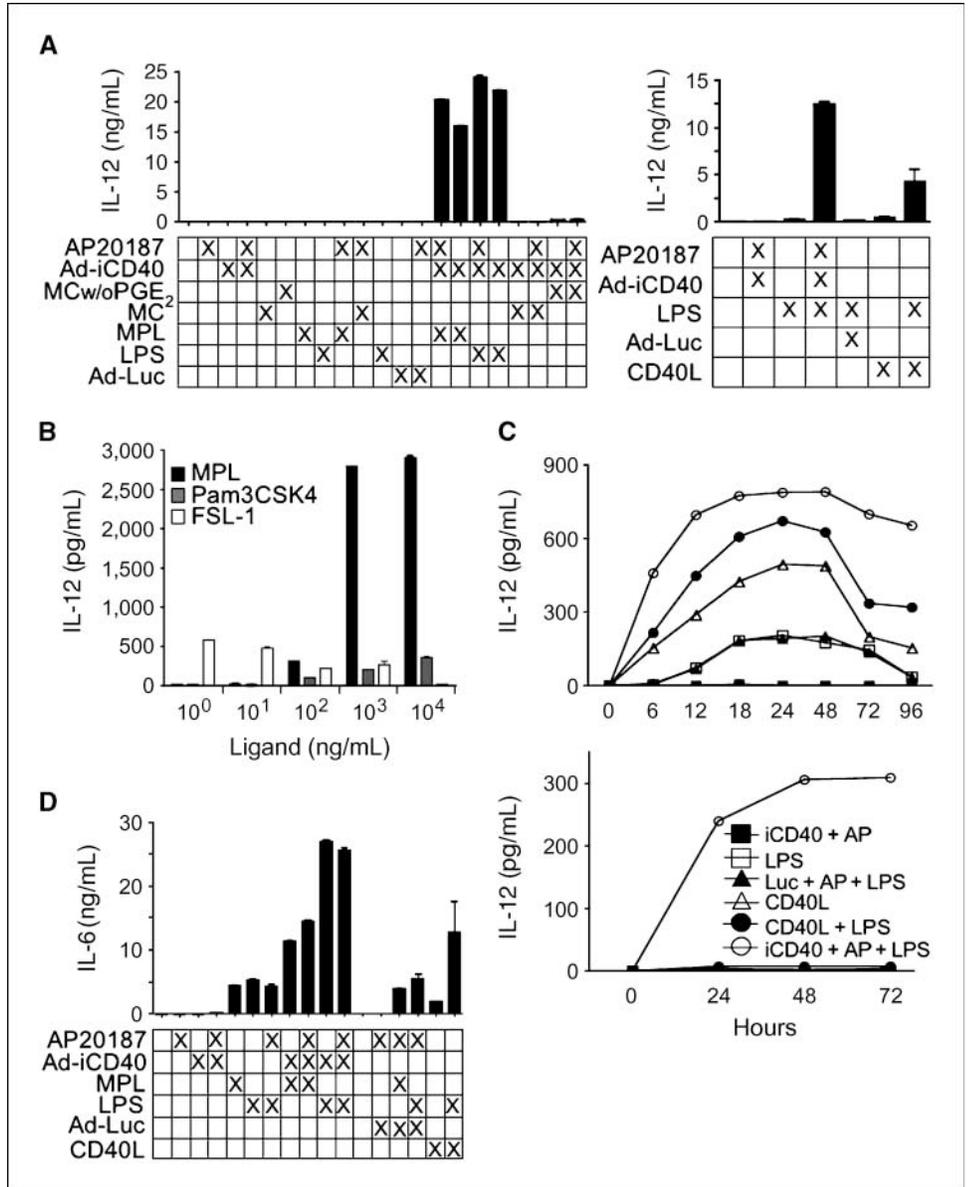
iCD40, presumably due to PGE$_2$. However, MC lacking PGE$_2$ did synergize somewhat with iCD40, but ~25-fold less than with LPS or MPL (Fig. 3*A, left*), consistent with the reported deleterious role for PGE$_2$ in DC activation.

The augmented activation potential of iCD40 versus endogenous CD40 suggested that iCD40 might bypass some negative feedback mechanisms induced by CD40 signaling. To test this hypothesis, we examined the up-regulation of a known inhibitory protein, suppressor of cytokine signaling (SOCS1), by various stimuli in MoDCs (Supplementary Fig. S2). Strikingly, whereas SOCS1 mRNA was highly induced by CD40L and LPS as previously reported (26, 27), the combination of iCD40 signaling and LPS triggered 3-fold lower levels of SOCS1. Moreover, iCD40 signaling alone did not induce SOCS1, unlike CD40L-mediated signaling.

To determine whether other TLRs could also synergize with iCD40, we tested ligands for TLR-1, TLR-2, TLR-4, and TLR-6 for IL-12 production. FSL-1 (ligand for TLR-2/TLR-6) and Pam3CSK4 (ligand for TLR-1/TLR-2) induced only low levels of IL-12p70 in

iCD40-DCs (Fig. 3*B*). As before, TLR-4 ligation with MPL synergized with CD40 signaling.

Because CD40 signaling is normally restricted to a relatively short time period (28), which potentially limits stimulation of adaptive immunity, we examined whether iCD40 could induce both enhanced and prolonged expression of IL-12p70 in TLR-4–stimulated DCs. To evaluate the kinetics of IL-12 expression, we compared LPS-treated iCD40-DCs with LPS- and/or CD40L-stimulated DCs. We observed that LPS-treated iCD40-DCs were able to produce IL-12p70 for over 72 h after stimulation compared with CD40L- or control vector–transduced DCs (Fig. 3*C*) in which IL-12p70 expression ceased shortly after (within 24 h) LPS stimulation was removed. These results indicate that both the amount and duration of IL-12p70 production are increased by the combination of TLR-4 and iCD40 signaling.

In addition to IL-12, IL-6 plays an important role in cell survival and resistance to regulatory T cells (17, 29). IL-6 expression was enhanced when iCD40-DCs were stimulated with LPS or MPL
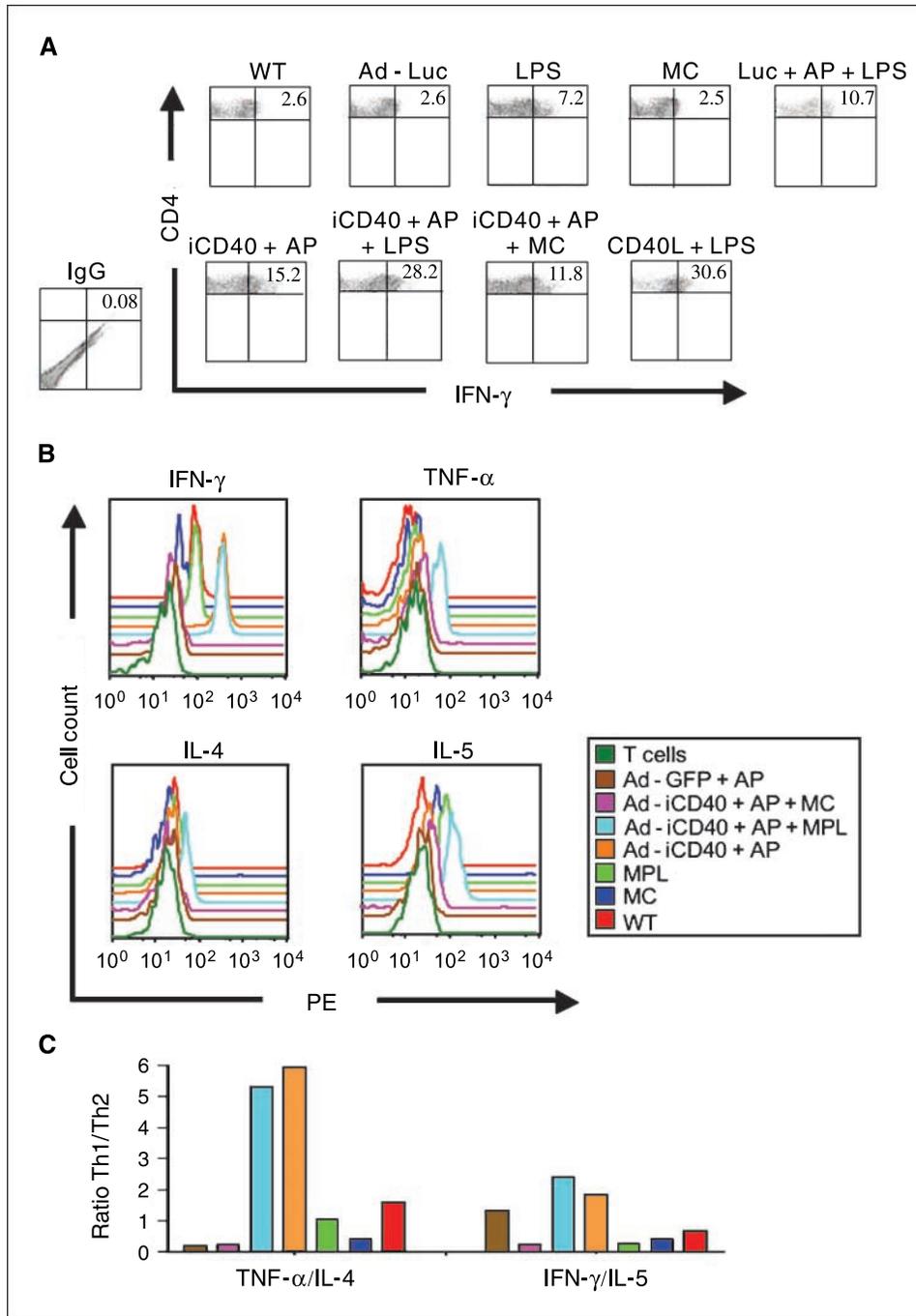
Figure 4. iCD40-DCs significantly induce Th1-polarized antigen-specific CD4$^+$ T cells. A, DCs from HLA-DR11.5 donor were pulsed with tetanus toxoid and treated with the listed reagents. Autologous CD4$^+$CD45RA$^+$ T cells were cocultured with DCs (at DC/T-cell ratio of 1:10) for 7 d and restimulated at day 8 with DCs pulsed with TTp30 peptide (FNNFTVSFWLRVPKVSASHLE). T cells were double stained with anti-IFN-γ-FITC and anti-CD4-phycoerythrin antibodies. Percentage of CD4$^+$/IFN-γ^+ T cells is indicated. B, supernatants were harvested and analyzed by BD Cytometric Bead Array Flex Set for expression of IFN-γ, TNF-α, IL-4, and IL-5. C, ratios of Th1/Th2 cytokines were calculated by division of Th1 cytokine concentration (pg/mL) by Th2 cytokine concentration (pg/mL). Representative of two experiments.

(Fig. 3D). Similarly to IL-12p70, the synergism for IL-6 production between iCD40 and TLR-4 was independent of dimerizer drug.

iCD40- and TLR-4–stimulated DCs enhance antigen-specific Th1 polarization. To further investigate whether iCD40-DCs matured with TLR-4 ligands can effectively prime CD4$^+$ T helper (Th) cells, we analyzed whether they can augment CD4$^+$ epitope-specific T-cell responses *in vitro*. Naïve CD4$^+$CD45RA$^+$ T cells were stimulated for 7 days in the presence of autologous iCD40-DCs pulsed with the model antigen, tetanus toxoid. At day 7, T cells were stimulated with the MHC class II–restricted tetanus toxoid (TT) epitope, TTp30. The production of IFN-γ was significantly increased in the CD4$^+$ T cells cocultured with iCD40-DCs and

iCD40-DCs stimulated with either LPS or MC. IFN-γ production was iCD40 dependent because it was only weakly induced by LPS alone and uninduced by control virus Ad-Luc or MC stimulation alone (Fig. 4A and data not shown). Interestingly, the level of IFN-γ production by Th cells was comparable between iCD40 + LPS–treated DCs and CD40L + LPS–stimulated DCs. We analyzed T-cell polarization by assessing Th1/Th2 cytokine levels in the supernatants of T cells using a cytometric bead array (Fig. 4B and C and Supplementary Fig. S3). The levels of IFN-γ, TNF-α, IL-4, and IL-5 secreted cytokines were increased in helper T cells stimulated by iCD40-DCs, indicating the expansion of both Th1- and Th2-polarized T cells. However, the levels of Th1 cytokines were

significantly higher than Th2-associated cytokines, indicating a predominant expansion of Th1 cells. In contrast, induction of TT-specific CD4$^+$ T-helper cells from naïve CD4$^+$CD45RA$^+$ cells using MC-matured DCs led to only a modest bias in TT epitope–specific Th1 differentiation. These results suggest that iCD40 signaling in DCs enables them to effectively induce antigen-specific Th1 differentiation, possibly due to higher IL-12 production.

TLR-4-stimulated iCD40-DCs induce strong tumor antigen–specific CTL responses. We further investigated whether iCD40 and TLR-4 ligation could enhance CTL responses to poorly immunogenic melanoma self antigens such as MAGE-3. iCD40-DCs from HLA-A2$^+$ donors were pulsed with class I HLA-A2.1–restricted MAGE-3–derived immunodominant peptide, FLWGPRALV, and cocultured with autologous T cells. After two stimulations, the frequency of antigen-specific T cells was assessed by IFN-γ–specific ELISpot assay (Fig. 5A). iCD40-DCs stimulated with MPL led to a 50% increase in MAGE-3–specific T cells relative to iCD40-DCs stimulated with MC and an ∼5-fold increase in antigen-specific T cells compared with control nontransduced (wild-type) DCs, consistent with potent expansion of CD8$^+$ T cells to weak immunogens.

Finally, we investigated whether iCD40-DCs were capable of enhancing antigen-specific CTL killing of tumor cells. Immature DCs from HLA-A2$^+$ volunteers were transduced with Ad-iCD40, pulsed with MAGE-3 peptide, and used as stimulators to generate CTLs *in vitro*. As targets, we used SK-MEL-37 cells (HLA-A2$^+$, MAGE-3$^+$) and T2 cells pulsed with MAGE-3 A2.1 peptide (HLA-A2$^+$, MAGE-3$^+$). NA-6-MEL cells (HLA-A2$^+$, MAGE-3$^-$) and T2 cells (HLA-A2$^+$) pulsed with an irrelevant A2.1-restricted influenza matrix peptide served as negative controls. CTLs induced by iCD40-DCs were capable of efficiently recognizing and lysing their cognate targets (SK-MEL-37; *top left*) and also T2 cells pulsed with MAGE-3 A2.1 peptide (*bottom left*), indicating the presence of MAGE-3–specific CTLs (Fig. 5B). In contrast, control targets were lysed at significantly lower levels (*right*). Moreover, we consistently observed improved lytic activity when MPL- or MC-treated iCD40-DCs were used as stimulators compared with nontransduced MPL- or MC-treated DCs. These results were consistent with the observed significant expansion of HLA-A2/MAGE-3–specific, tetramer-positive CD8$^+$ CTLs when incubated with MPL-treated iCD40-DCs (Fig. 5C).

As a second test of enhanced CTL expansion to weak immunogens, we tested whether TLR-4– and iCD40-stimulated DCs could enhance CTL lytic activity to the self protein PSMA. DCs generated from healthy HLA-A2$^+$ volunteers were pulsed with PSMA (residues 44–750), transduced with Ad-iCD40 or control Ad-Luc, and cocultured with autologous T cells. After three rounds of stimulation, antigen-specific CTL activity was measured using LNCaP cells (HLA-A2$^+$PSMA$^+$) as targets and SK-Mel-37 (HLA-A2$^+$PSMA$^-$) as control cells for PSMA-pulsed DCs (Fig. 5D). These data show that iCD40-transduced DCs are capable of inducing significantly more potent PSMA-specific CTL responses *in vitro* than MC-treated or CD40L + LPS–stimulated DCs.

Inducible CD40 enhances CCR7 expression in MoDCs and PGE$_2$-independent migration. In addition to other surface markers, CCR7 is up-regulated on mature DCs and is required for migration to draining lymph nodes (30). Recently, several reports have indicated that, apart from chemotaxis, CCR7 also affects DC "cytoarchitecture," the rate of endocytosis, survival, migratory speed, and maturation (31). Along with costimulatory molecules and Th1 cytokines, iCD40 signaling also strongly up-regulates CCR7

expression in human DCs in the presence (by ∼100-fold) or absence (by ∼10-fold) of TLR-4 signaling (Fig. 6A). Moreover, CCR7 expression correlated with Ad-iCD40 viral dose escalation (Supplementary Fig. S4). These data correspond to previously described observations that CD40L induces CCR7 expression on human monocyte-derived DCs (32).

Because CCR7 expression levels correlate with enhanced migration toward MIP-3β (CCL19) *in vivo*, we tested whether human iCD40-DCs could migrate *in vitro* toward MIP-3β in transwell assays (Fig. 6B and Supplementary Figs. S5 and S6). Figure 6B shows that iCD40-DCs treated with AP20187 dimerizer have migration levels comparable to that with MC stimulation. Moreover, iCD40-DC migration was further increased by MPL. In contrast, CD40L alone or in combination with MPL or MC was not able to induce high levels of migration. These highly reproducible data indicate that iCD40 is sufficient to induce CCR7 expression and DC migration *in vitro*, in contrast to the widely held belief that PGE$_2$ is essential for lymph node homing of human DC.

Chemokines and chemokine receptors share a high degree of sequence identity within a species and between species (33). On this basis, we developed a novel xenograft model for monitoring the migration of human DCs *in vivo*. Human MoDCs were transduced with iCD40 and matured with LPS or MC. Control mouse DCs were matured with LPS. To visualize migration *in vivo*, MoDCs were cotransduced with Ad5-CBR, expressing red-shifted luciferase (Fig. 6C and Supplementary Fig. S7). As expected, following injection into footpads, immature DCs did not migrate to the draining popliteal lymph nodes. However, iCD40-DCs matured with LPS or MC were detectable in the xenogeneic popliteal lymph nodes within 2 days after inoculation (Fig. 6C). The migration of iCD40-DCs stimulated with LPS was significantly ($P = 0.036$) higher than that of nonstimulated DCs and was comparable to mouse DC migration (Fig. 6C). Moreover, at day 2, the iCD40-DCs were detected in inguinal lymph nodes whereas MC-stimulated DCs were undetectable, suggesting higher migratory abilities of iCD40-DCs than MC-stimulated DCs. Collectively, these results indicate that iCD40 signaling in DCs plays a critical role in controlling CCR7 expression and is sufficient for DC migration to lymph nodes. The migration of iCD40-DCs is further enhanced when the cells are stimulated with LPS, correlating with enhanced CCR7 expression.

Enhancement of antitumor immunity mediated by LPS-treated iCD40-DCs. These studies clearly show the enhancement of DC immunostimulatory properties *in vitro*. To confirm that this strategy can also translate into an antitumor therapeutic effect *in vivo*, we vaccinated mice bearing preestablished EG.7-OVA tumors. Mice (six per group) were vaccinated once with iCD40-DCs, which were pulsed with SIINFEKL and activated with LPS and AP1903 (clinical analogue of AP20187). Control mice were vaccinated with DCs stimulated with LPS alone, iCD40-DCs activated with AP1903 alone, or Ad-Luc–transduced DCs activated with LPS and AP1903 (DC-Luc + LPS + AP1903). As shown in Supplementary Fig. S8, the combination of iCD40 and TLR-4 ligation significantly ($P < 0.05$) enhanced the antitumor efficacy of the vaccine. Moreover, this was accompanied by a significant expansion of antigen-specific CD8$^+$ T cells. Thus, TLR-4 stimulation can significantly augment the immunostimulatory antitumor effects of iCD40-DCs.

Discussion

Given the preeminent role of DCs as antigen-presenting cells, their exploitation as natural adjuvants in vaccination protocols for the treatment of various malignancies is predictable (34, 35). The
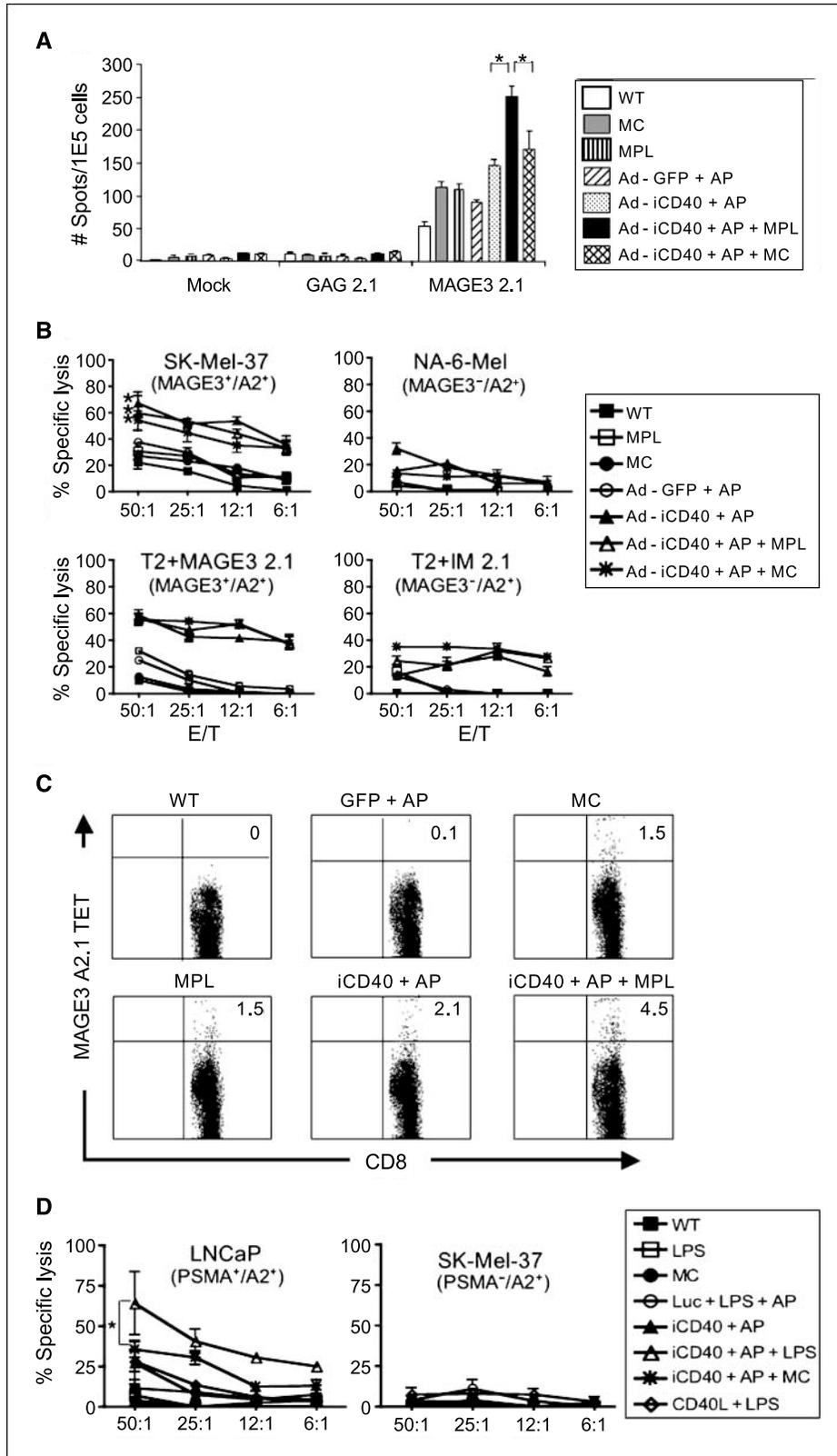
Figure 5. Enhanced induction of antigen-specific CTLs by iCD40-DCs. MoDCs derived from HLA-A2[+] donors were transduced with the indicated reagents and pulsed with MAGE-3 peptide (25 μg/mL). DCs were cultured with autologous T cells (1:3 DC/T-cell ratio) for 7 d in complete RPMI supplemented with hIL-2 (20 IU/mL). T cells were restimulated with DCs at day 7. *A,* frequency of MAGE-3 2.1 peptide–specific T cells was determined by IFN-γ ELISpot analysis. T cells (10[5] per well) were stimulated with MAGE-3 2.1 or GAG 2.1 [(negative control)/irrelevant peptide] or cultured without stimulation (*Mock*). *, $P < 0.05$. *B,* DCs from HLA-A2[+] donors were cocultured with autologous T cells. After three serial stimulations with DCs, T cells were evaluated for antigen-specific lytic activity by ^{51}Cr release assays. Assays were done in triplicate. *IM,* influenza matrix peptide. *, $P < 0.05$, relative to wild-type DCs. *C,* effector T-cell populations generated after serial stimulation with DCs were stained with MAGE-3 2.1 peptide–loaded HLA-A2 tetramer. MAGE-3 peptide–specific CD8^{+} T cells were identified by flow cytometry. Numbers indicate the percent of tetramer-positive cells within the entire population of CD8^{+} T cells. Representative of three experiments. *D,* enhanced cytolytic function of PSMA-specific CTLs induced by iCD40-DCs. MoDCs generated from HLA-A2[+] male volunteers were pulsed with 50 μg/mL PSMA protein and transduced with Ad-iCD40 or Ad-Luc. DCs were cultured with MC or LPS (1 μg/mL) with or without CD40L (1 μg/mL). Antigen-specific CTL activity was assessed by ^{51}Cr release assay. Similar results were obtained in three independent experiments. *, $P < 0.05$, relative to wild-type DCs.

results of numerous DC-based vaccine clinical trials have been published and show their immunostimulatory capacities in patients. In each case, DC efficacy depends on many variables, especially maturation status and efficient migration to lymph nodes. Several clinical trials in cancer patients showed the potency of DCs to induce adaptive immunity to tumor-specific antigens (34). However, clinical responses were transient and warrant further improvement in DC vaccine design (36).

The key limitations of current DC-based vaccines are the transient activation state within lymphoid tissues, low induction of CD4^{+} T-cell immunity, and impaired ability to migrate to the draining lymph nodes (37). Less than 24 h following exposure to LPS, DCs terminate synthesis of the Th1-polarizing cytokine IL-12 and become refractory to further stimuli (38), limiting their ability to activate Th cells and CTLs. Other studies indicate that <5% of intradermally administered mature DCs reach the lymph nodes, showing inefficient homing (33). These findings underscore the

need for either prolonging the activation state and migratory capacities of the DCs or temporally coordinating the DC activation "window" with engagement of cognate T cells within lymph nodes.

Recently, we developed a new method to promote mouse DC function *in vivo* by manipulation of a chimeric inducible CD40 receptor (11). We observed that the inducible CD40 approach is also effective in enhancing the immunostimulatory function of human DCs. Consistent with previous reports of the synergistic

Figure 6. Up-regulation of CCR7 expression and enhanced migratory capacities of iCD40-DCs. *A,* human MoDCs were transduced with Ad-iCD40 (iCD40) or Ad-Luc (*Luc*) and cultured for 48 h with AP20187 (100 nmol/L), MC and LPS or MPL (1 μg/mL). CCR7 expression was measured using phycoerythrin-conjugated antihuman CCR7 mAb. Representative of five different donors. *B,* human DCs were transduced with 10,000 viral particles/cell of Ad5/f35-iCD40 (iCD40) or Ad-Luc and incubated for 48 h with MPL (1 μg/mL), MC, CD40L (1 μg/mL), and AP20187 (100 nmol/L). DCs were labeled with Green-CMFDA Cell Tracker and added to upper chamber. Fluorescence of cells that migrated through the microporous membrane was measured. Each experiment (including the control spontaneous migration to the medium) was done in triplicate for at least four different donors with similar results. *C,* human DCs were transduced with Ad-CBR-Luc with or without Ad-iCD40 and stimulated as indicated. Mouse DCs (*mDC*) were transduced with Ad-CBR-Luc and stimulated with LPS. DCs (2 × 10^{6}) were injected into both hind footpads of three mice per group (*n* = 6). Lymph nodes were removed at day 2 after DC inoculation. Mean luminescent signal from the dissected popliteal and inguinal lymph nodes was measured and normalized by background subtraction. *, *P* < 0.05; ***, *P* < 0.001, relative to mock DCs.
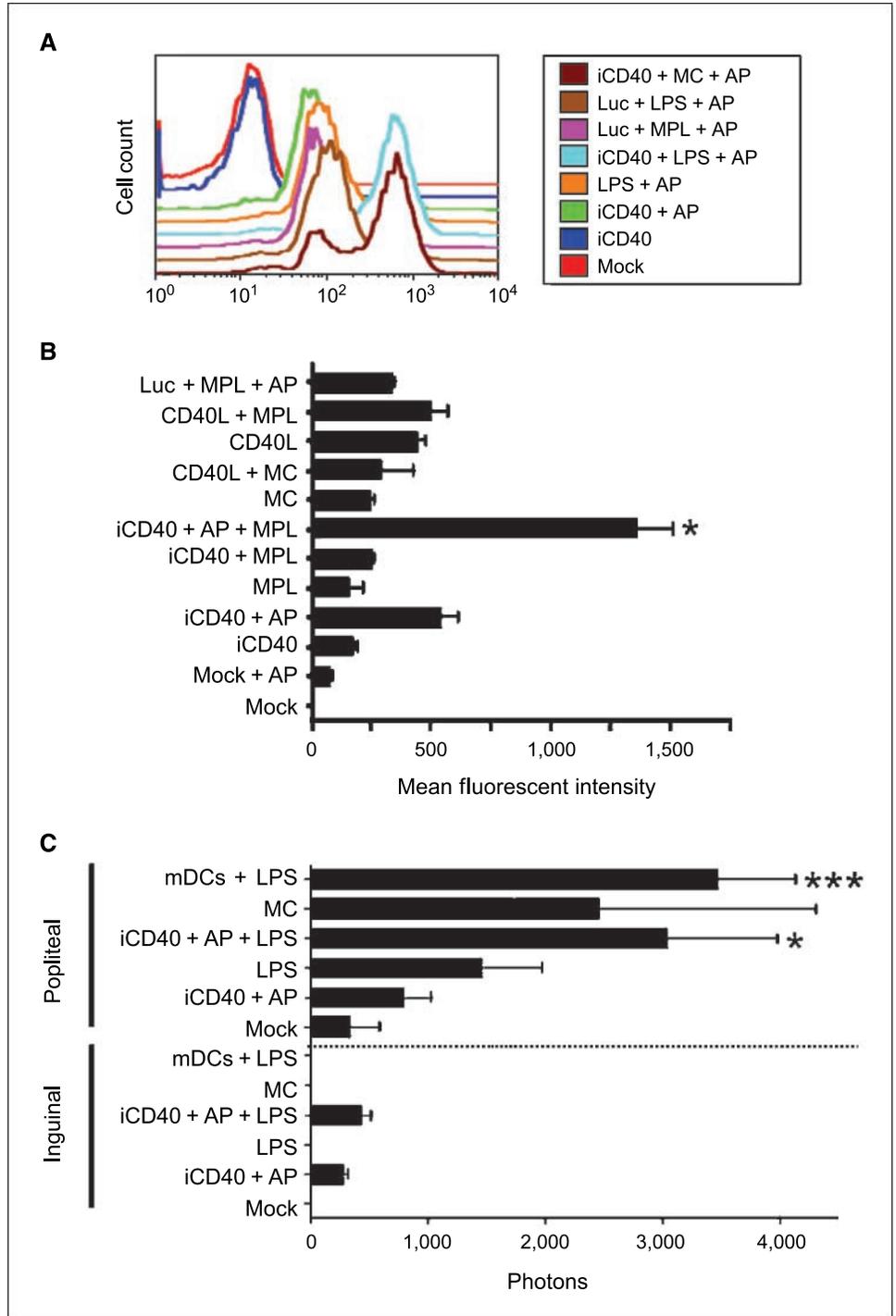
activity of combining TLR and CD40 signaling for IL-12p70 secretion, iCD40 plus TLR-4 signaling induced high-level IL-12 secretion, DC maturation, T-cell stimulatory functions, and extensive migratory capacities (18, 39). It was also recently shown that increased and prolonged secretion of IL-12p70 in DCs could break self-tolerance, which likely is attributable, in part, to overriding the production of SOCS1, which inhibits IL-12 signaling (27). We found that although endogenous CD40 signaling stimulated by soluble CD40L leads to SOCS1 up-regulation, iCD40 activates MoDCs without significant SOCS1 induction. Additionally, iCD40 signaling unleashes high and prolonged expression of IL-12p70 in DCs, which exhibit enhanced potency in stimulating $CD4^+$ T cells and CTLs.

IL-6 is implicated in the survival of many different cell types by activation of antiapoptotic pathways such as p38 mitogen-activated protein kinase, extracellular signal–regulated kinases 1 and 2 (47), and phosphatidylinositol 3-kinase (40). We also found the induction of IL-6 expression by iCD40 and TLR-4 signaling in DCs. This finding could partly explain the prolonged survival of DCs we previously described (11). Furthermore, IL-6 expression is critical in the ability of DCs to inhibit the generation of $CD4^+CD25^+$ T regulatory cells (29). In this context, an iCD40-DC–based vaccine could potentially suppress negative regulators *in vivo*, inhibiting peripheral tolerance to targeting antigens.

One major focus of cancer immunotherapy has been the design of vaccines to promote strong tumor antigen–specific CTL responses in cancer patients (41). However, accumulating evidence suggests that $CD4^+$ T cells also play a critical role in antitumor immunity because they contribute to the induction, persistence, and expansion of $CD8^+$ T cells (42). Our study showed that iCD40-DCs could effectively prime naïve T cells and effectively expand antigen-specific cells representing both arms of the immune response (i.e., MAGE-3– and PSMA-specific CTLs and TT-specific $CD4^+$ T cells). We further showed that Th1 (IFN-γ and TNF-α) cytokines were produced predominantly in the milieu of iCD40-DC–stimulated $CD4^+$ T cells, indicating expansion of Th1 cells. As expected, these cytokines were not detected when T cells were stimulated with MC-treated DCs, likely because PGE_2 (a key MC component) is a powerful suppressor of Th1 responses (9).

Interestingly, synergistic IL-12 production by iCD40 and TLR-4 signaling was primarily chemical inducer of dimerization independent (Fig. 3A). This suggests that relatively high basal iCD40 signaling is sufficient for some activation signals. In contrast, dimerization of iCD40 was necessary for optimum DC migration (Fig. 6B and Supplementary Fig. S6). These contradictory effects imply that distinct signaling domains within the relatively small cytoplasmic domain of CD40 may respond differently to over-expression at the membrane or enhanced synthetic dimerization. Nevertheless, the net effect is greatly enhanced antitumor immunity in the presence of TLR-4 ligation (Supplementary Fig. S8 and data not shown).

Recent mouse studies have shown that DC migration directly correlates with T-cell proliferation (43). Therefore, the increase in migration should enhance efficacy of DC-based vaccines (37). Current DC vaccine protocols include preconditioning the vaccine injection site with inflammatory cytokines or *ex vivo* stimulation of DCs with TLR ligands and proinflammatory cytokines, consisting primarily of MC constituents (43, 44). Despite its numerous immunosuppressive functions (8–10, 45, 46),

PGE_2 has been used for the past few years as an indispensable component of the DC maturation cocktail because it stimulates the migratory capacity of DCs by up-regulating CCR7 and sensitization to its ligands. Alternative approaches enhancing DC migration without PGE_2 should be beneficial for DC-based vaccine improvement.

The results of our study show that iCD40 signaling not only up-regulates CCR7 expression on MoDCs but also stimulates their chemotaxis to CCL19 *in vitro*. Additionally, immature DCs transduced with iCD40 were able to migrate as efficiently as MC-stimulated DCs both *in vitro* and *in vivo*. Moreover, migration of iCD40-DCs was further induced when cells were stimulated with TLR-4 ligands. It was recently shown that stimulation of CCR7 increases the migratory rate of DCs, indicating that this receptor can regulate DC locomotion and motility (47). Finally, it has been shown that stimulation of CCR7 enhances the mature phenotype of DCs (48). Thus, by transduction of DCs with iCD40, we have enhanced CCR7 expression, along with DC migration and maturation status, obviating the need for PGE_2. However, further studies are necessary to identify the specific mechanisms of iCD40 on DC migration.

In DC-based studies, the method of DC modification is critical because DCs are relatively resistant to physical methods of transfection. Viruses, such as poxviruses, herpes simplex virus, and influenza virus, induce a cytopathic effect on target DCs and inhibit T-cell activation or induce Th2 skewed responses (49). In contrast, adenoviral transfection is an efficient method for delivery of genes to DCs. Therefore, it is currently widely used in clinical trials. However, together with the desired transgene, adenoviral proteins could be efficiently processed and presented on the DC surface, leading potentially to dilution of desired immunity. Here, we show that DCs do not lose the capacity of processing, presenting, and inducing T-cell responses to weak tumor antigens such as PSMA and MAGE-3 after they have been transduced with adenovirus. Nonetheless, more studies on epitope competition should be done to rule out the possibility of dominant presentation of adenoviral proteins by adenovirus-transduced DCs.

Overall, iCD40 stimulation of DCs was capable of inducing a potent CTL response to the prostate-specific antigen PSMA, which was capable of significantly increased killing of target LNCaP cells. These observations form part of the basis for a planned clinical trial based on iCD40-transduced, PSMA protein–loaded DCs, in combination with dimerizer drug, in patients with advanced, hormone-refractory prostate cancer. Ultimately, by expanding antigen-specific T cells, our DC-based vaccine approach could complement recently described techniques that are based on the expansion of tumor-derived T cells or the genetic modification of polyclonal endogenous T cells to antigen specificity (50).

Acknowledgments

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