Intranodal injection of semimature monocyte-derived dendritic cells induces T helper type 1 responses to protein neoantigen

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Dendritic cells (DCs) represent the most potent antigen-presenting cells of the immune system capable of initiating primary immune responses to neoantigens. Here we characterize the primary CD4 T-cell immune response to protein keyhole limpet hemocyanin (KLH) in 5 metastatic melanoma patients undergoing a tumor peptide–based dendritic cell vaccination trial. Monocyte-derived dendritic cells displaying a semimature phenotype, as defined by surface markers, were loaded ex vivo with antigen and injected intranodally at weekly intervals for 4 weeks. All patients developed a strong and long-lasting delayed-type hypersensitivity reactivity to KLH, which correlated with the induction of KLH-dependent proliferation of CD4 T cells in vitro. Secondary in vitro stimulation with KLH showed significant increase in interferon-γ and interleukin-2 (IL-2) but not IL-4, IL-5, nor IL-10 secretion by bulk T cells. On the single-cell level, most TH1 cells among in vitro–generated KLH-specific T-cell lines confirmed the preferential induction of a KLH-specific type 1 T helper immune response. Furthermore, the induction of KLH-specific antibodies of the IgG2 subtype may reflect the induction of a type 1 cytokine profile in vivo after vaccination. Our results indicate that intranodal vaccination with semimature DCs can prime strong, long-lasting CD4 T-cell responses with a TH1-type cytokine profile in cancer patients. (Blood. 2003;102:36-42)

Introduction

Dendritic cells (DCs) play a pivotal role in the initiation of T-cell–mediated immune responses, making them an attractive cellular adjuvant for use in cancer vaccines.[1] DCs have been shown to promote protective and therapeutic antitumor immunity in several animal models.[2-4] These studies have formed the basis for the clinical use of DCs in vaccination strategies against human tumor Ags. Current pilot DC vaccination studies have indeed been able to elicit specific antitumoral immune responses and some clinical responses. Nevertheless, differences in the study design, including the type of DC preparation such as DC origin, their maturation stage, the nature and duration of activation, remain a matter of debate.[5] Human myeloid DCs derived from monocytes,[6-8] $CD34^+$ stem cells,[9] and $CD11c^+$ cells[10] all have been able to induce some degree of immune response in vivo. Antigen-specific immunity also has been induced using DCs at different stages of their differentiation/maturation such as mature DCs,[7,8] semimature DCs,[6,9] and immature DCs.[11,12] However, a recent study showed inhibition of effector T-cell function using immature DCs in 2 volunteers.[13] In addition, the strength of the Ag-specific immune response induced also may depend on the route of vaccine administration and the vaccination schedule.[14] It is therefore of critical importance to test the immunogenicity of DC preparation in the context of the study protocol.

To this aim we added protein keyhole limpet hemocyanin (KLH) as helper epitope to our DC preparation by the exogenous pathway of Ag presentation. KLH is a snail-derived neoantigen and a large globular protein that consists of a wide array of immunogenic CD4 T-cell determinants and can therefore be used as a tracer molecule to easily and reproducibly monitor the induction of a primary immune response. Furthermore, as a helper protein in a peptide-based vaccination trial, KLH also may favor the induction of cytotoxic T lymphocyte (CTL) responses through the generation of bystander CD4 T-cell help.[15-18]

In the context of a peptide-based vaccination trial,[6] semimature monocyte-derived DCs generated in fetal calf serum–supplemented medium and pulsed with KLH were injected intranodally at weekly intervals. Here we present a quantitative and qualitative analysis of the primary immune response to KLH.

Patients, materials, and methods

Reagents

The complete medium (CM) used throughout consisted of RPMI 1640 (Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS, Life Technologies, Eggenstein, Germany), 10 U/mL penicillin/streptomycin (Gibco), and 50 μg/mL L-glutamine (Seromed, Basel, Switzerland). Keyhole limpet hemocyanin (KLH) from megathura crenulata (Calbiochem, Bad Soden, Germany) was solubilized and passed 4 times through a purification column to remove endotoxin contamination (Detoxigel column, Pierce, Rockford, IL). After purification less than 1 pg/mL endotoxin was detected, as assessed by the limulus amebocyte lysate (LAL) test (sensitivity level of 1 pg/mL). The procedure was defined according to the limit requested by our study protocol.

Patient selection

All metastatic melanoma patients treated with DC vaccination at our institution according to our clinical protocol during a defined period of time (June 1996 to January 1997) were included in this study. The DC

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Submitted July 26, 2002; accepted January 6, 2003. Prepublished online as *Blood* First Edition Paper, January 30, 2003; DOI 10.1182/blood-2002-07-2274.

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vaccination study was approved by the Zürich University Hospital institutional review board; informed consent was provided according to the Declaration of Helsinki. Inclusion criteria for the vaccination trial and patient's characteristics have been published previously.[6] Corresponding patient numbers to the previous publication are given in parenthesis: 1 = (5), 2 = (16), 3 = (9), 4 = (14), and 5 = (8). Clinical responses included 1 complete response (patient 3), 1 partial remission (patient 5), 1 minor response (patient 2), and 2 progressive diseases (patients 1 and 4).[6]

Generation of DCs

Peripheral blood mononuclear cells (PBMCs) were separated from fresh patient blood (100 mL) using Ficoll-Hypaque density centrifugation, resuspended in CM, and allowed to adhere to plastic dishes. After 2 hours at 37°C, the nonadherent cells were removed and the adherent cells were subsequently cultured in FCS containing CM supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) (800 U/mL, kindly provided by U. Haus, Sandoz, Nürnberg, Germany) and interleukin-4 (IL-4) (500 U/mL, PharMingen, Hamburg, Germany). After 7 days of culture nonadherent cells were collected.

Production of DC vaccine

DCs were pulsed for 2 hours at 37°C with 50 μg/mL KLH protein and either HLA-A2 binding melanoma-associated peptides for tyrosinase, Melan-A/melanoma antigen recognized by T cells (MART)–1, and gp100 (patients 1-4) or HLA-A1–binding peptides derived from melanoma antigen encoding gene (MAGE)–1 and MAGE-3 (patient 5), depending on the HLA type of the patient. Before injection 1×10^6 DCs were washed 3 times in sterile phosphate-buffered saline (PBS) and resuspended in a total volume of 0.5 mL PBS. Injection into an uninvolved inguinal lymph node was performed under ultrasound guidance as published.[6] All injected DC preparations were negative for bacterial and fungal contamination.

Immunization protocol

Antigen-loaded DCs were repetitively administered into the same uninvolved inguinal lymph node according to a schedule of 4 vaccinations at weekly intervals. At weeks 6 and 10, 2 additional immunizations were given.

Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) skin tests were performed before vaccination, at week 4 and week 10 of the vaccination protocol by intradermal injection of KLH (5 μg in 200 μL PBS) into the forearm. Negative control was PBS alone. A positive skin test was defined as erythema and induration of more than 5 mm at 48 hours after intradermal injection.

KLH-dependent proliferation in vitro

PBMCs from patients had been collected before vaccination, at week 4 and week 10 of the vaccination protocol and frozen. For the assays described in this paragraph, samples were thawed and assayed together. Recall proliferation was assessed by stimulating 1×10^5 peripheral blood leukocytes (PBLs) for 4 days with 20 μg/mL KLH. Phytohemagglutinin (PHA) stimulation (10 μg/mL, Gibco) was done in parallel to produce a positive control for cell viability and responsiveness. During the final 6 hours of the incubation period cells were pulsed with 1 μCi (0.037 MBq)/well of [^{3}H]-thymidine (Amersham, Little Chalfont, United Kingdom), harvested onto glass microfiber filter strips using a cell harvester, and placed in a liquid scintillation counter. Values are expressed as mean counts per minute (cpm) ± standard deviation (SD) of triplicate wells. Blocking of major histocompatibility complex (MHC) class II–mediated T-cell proliferation was performed by addition of human leukocyte antigen (HLA)–DR antibody-containing supernatants derived from cultured L243-hybridoma (ATCC HB-55) cells or the corresponding isotype control antibody.

Recall cytokine production

1×10^6 PBMCs were stimulated for 4 days with either KLH (20 μg/mL) or PHA (10 μg/mL, Gibco) in 1 mL CM at 37°C. After centrifugation, cell-free supernatants were collected and stored at −80°C until the interferon (IFN)-γ, IL-4, and IL-10 content were determined by commercially available enzyme-linked immunosorbent assay (ELISA) systems: human IFN-γ ELISA (Gibco) with a sensitivity level of 32.5 pg/mL (lowest standard used in the assay), human IL-4 ELISA (R&D Systems, Abington, United Kingdom) with a sensitivity level less than 3.25 pg/mL (lowest standard used in the assay), and human IL-10 ELISA (R&D Systems) with a sensitivity level less than 1.5 pg/mL.

Generation of KLH-specific T-cell lines and intracellular cytokine staining

PBMCs were cultured in 96-well flat-bottom plates (Costar, Cambridge, MA) in RPMI 1640 supplemented with 5% human serum (HS; Swiss Red Cross Laboratory, Bern, Switzerland) and 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 50 μg/mL kanamicin, and 5×10^{-5} M 2-ME (Gibco Laboratories, Grand Island, NY) in the presence of KLH (10 μg/mL). Recombinant IL-2 (20 U/mL, kindly provided by Dr A. Lanzavecchia, Basel Institute for Immunology, Switzerland) was added at day 6. T-cell lines were expanded in medium containing IL-2 and analyzed at day 20. For intracellular cytokine staining KLH-specific T cells (1×10^6) were stimulated with 10^{-7} M phorbol myristate acetate (PMA) and 1 μg/mL ionomycin (Sigma Chemicals, St Louis, MO) in 1 mL 10% FCS-RPMI in 24-well plates. After 2 hours of incubation 10 μg/mL brefeldin A (Sigma) was added to the culture for the last 2 hours. Cells were washed twice in PBS, fixed with PBS containing 2% paraformaldehyde (Merck, Darmstadt, Germany) for 15 minutes at 4°C, washed with PBS-1% FCS-0.5% saponin (Sigma), and incubated in the same buffer for 20 minutes at room temperature before adding fluorescein isothiocyanate–labeled anti–IFN-γ (IgG1) and phycoerythrin-labeled anti–IL-4 (IgG2b) antibodies (Becton Dickinson, Mountain View, CA). Before flow cytometry acquisition, stained cells were washed twice with PBS-1% FCS. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson); 2×10^4 events were acquired for each sample.

Detection of KLH-specific antibodies

Maxisorp 96-wells (Nunc Brand Products, Wiesbaden, Germany) were coated with 50 μg/mL KLH in sodium carbonate buffer, pH 9.6, overnight at 4°C. After washing (PBS/0.05 Tween 20) and blocking with PBS/1% bovine serum albumin (BSA) for 2 hours at room temperature, serial dilutions of serum were added to the wells and incubated overnight at 4°C. Biotin-conjugated monoclonal antihuman IgG1, IgG2, IgG3, and IgG4 (Sigma) were incubated for 2 hours at room temperature, followed by the addition of peroxidase-conjugated avidin (DAKO Diagnostics, Zug, Switzerland) for 30 minutes at 37°C. For color development tetramethylbenzidine (TMB) one-step substrate system (DAKO) was used. Plates were measured by photometer, and results were indicated as absorption (A) at wavelength 620 nm.

Results

Generation of CD1a$^+$ semimature DCs

DCs were differentiated from plastic-adherent peripheral blood monocytes in the presence of medium containing 10% FCS and supplemented with IL-4 and GM-CSF as described in "Patients, materials, and methods." After 7 days of culture, harvested nonadherent cells consisted routinely of more than 90% CD1a$^+$ DCs expressing high levels of MHC class II products and intermediate levels of CD86 and CD80 (Figure 1A). Consistent with a phenotype of intermediate maturity, generated DCs expressed low levels of maturation marker CD83 (Figure 1A). No expression of activation marker CD25 was detected (Figure 1). Generated DCs had the ability to produce large amounts of IL-12 upon stimulation with CD40-ligand transfected L cells (> 1000 pg/mL, n = 2). No marked difference in the maturation stage of

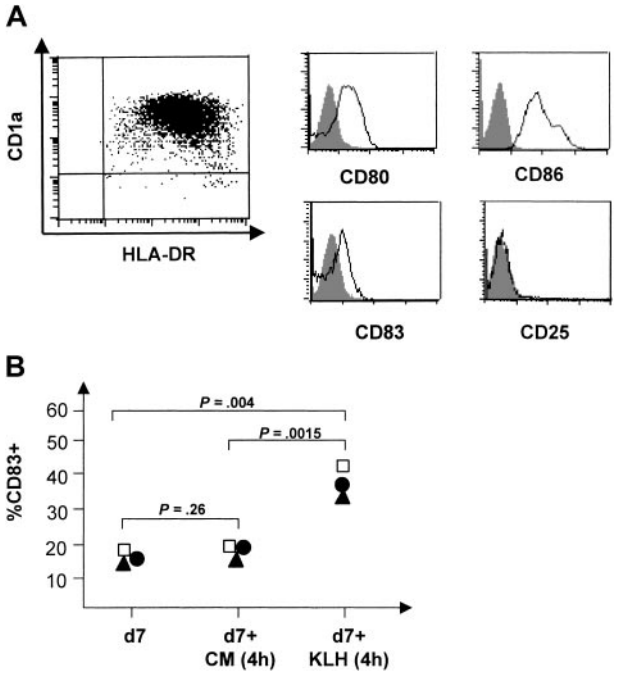

Figure 1. Monocyte-derived DCs generated in the presence of FCS are CD1a$^+$, exhibit a phenotype of intermediate maturity, and show a strong tendency to mature. (A) Cell surface marker expression by fluorescent flow cytometry analysis. Monocyte-derived DCs generated in the presence of FCS were more than 90% CD1a$^+$HLA-DR^{high}, expressed intermediate levels of CD80 and CD86 and low levels of maturation marker CD83, and lacked CD25 expression. Furthermore, all DCs expressed CD11c and lacked monocytic marker CD14 (data not shown). Data are representative of 6 independent experiments. (B) Four-hour pulsing of DCs with KLH increases the number of CD83$^+$ DCs. CD83$^+$ cells were assessed after 7 days of culture plus or minus an additional 4-hour culture with KLH or complete medium (CM) as control. Indicated *P* value was obtained by an impaired *t* test. Significance was defined as $P < .05$. Symbols represent independent experiments.

DCs derived from patients was detected, according to their surface expression of CD80 and CD83 (Table 1). Interestingly, an additional 4 hours' stimulation with soluble protein KLH (containing < 1 pg/mL LPS) but not with medium alone as control significantly enhanced the number of CD83$^+$ DCs (Figure 1C), indicating a strong tendency of generated DCs to mature. Therefore, the vaccine preparation for intranodal injection consisted of DCs exhibiting a semimature phenotype.

Intranodal injection of KLH-pulsed semimature DCs induces strong DTH reactivity to KLH

The standard in vivo assay for CD4 T-cell–mediated immunity is injection of Ag into the skin and assessment of the subsequent reaction. Ag-specific memory CD4 T cells will mediate an infiltration at site of challenge that is clinically detected within 24-72 hours as so-called delayed-type hypersensitivity (DTH) reaction. None of the patients showed DTH reactivity to KLH before vaccination (week 0). After 4 cycles of vaccination (week 4) intracutaneous challenge with soluble KLH in PBS but not PBS alone elicited strong DTH reactions, as shown by the erythematous induration more than 10 mm in diameter in all the patients (Figure 2). No significant changes in DTH diameter were observed at week 10 after 2 additional booster immunizations. Representative biopsies showing massive infiltration of CD45RO-positive memory T cells confirmed the presence of a DTH reaction. Comparable DTH reactions to KLH also were elicited in patients who were not challenged with soluble KLH at week 0 (n = 2, data not shown), confirming that the immune response to KLH was generated by the intranodal DC vaccination.

KLH-dependent T-cell proliferation in vitro is MHC class II restricted and correlates with the induction of DTH reactivity to KLH in vivo

Secondary proliferative T-cell responses in vitro were obtained by culturing prevaccination and postvaccination PBMCs in the presence or absence of KLH for 4 days. Before vaccination, KLH-dependent T-cell proliferation of patients 1, 2, 3, and 5 was not significantly different from background proliferation of PBLs cultured in CM alone (< 2000 cpm for all patients) (Figure 3A-B). Only in patient 5 was a PBL proliferation of 7825 ± 78 cpm before vaccination observed. At week 4 of the vaccination protocol, all patients demonstrated a significant increase of the proliferative activity to KLH stimulation (Figure 3A), while background proliferation in CM alone remained less than 2000 cpm (Figure 3B). No significant changes were observed at week 10 after 2 additional booster immunizations (Figure 3A). To assess the involvement of an MHC class II–restricted antigen presentation, we added HLA-DR–specific blocking antibody to the proliferation assay. Figure 3B shows that the KLH-specific PBL proliferation was indeed inhibited by anti–HLA-DR mAb, indicating that the KLH-specific response was predominantly mediated by MHC class II–restricted CD4 T cells. A highly significant correlation between the KLH-dependent T-cell proliferation in vitro and the diameter of the KLH-specific DTH reaction in vivo confirmed the validity of the in vitro assay to assess the KLH-specific immune response (coefficient of regression 0.976, $P < .01$, Figure 3C).

Generated KLH-specific T-cell immunity is long-lived

We assessed the duration of the KLH-specific immune response by testing DTH reactivity to KLH at various time points after the last DC vaccination. Patients 2, 3, and 5, who responded to therapy, were tested after 9, 4, and 5 months, respectively, before receiving additional booster immunizations. Progressing patient 4 was tested at 12 months, after 4 cycles of additional chemotherapy. Patient 1 died 6 months after vaccination and could not be tested. The DTH response persisted at all times tested, without evidence of a significant decline of the KLH-specific immunity (Figure 4). The in vivo data correlated with enhanced KLH-specific PBL proliferation in vitro (data not shown).

Preferential induction of a T helper 1–type cytokine profile

Next we analyzed the T-cell cytokine profile induced by vaccination with KLH-pulsed DCs. In vitro restimulation of total PBMCs with KLH demonstrated a preferential KLH-dependent TH1-type cytokine profile with enhanced secretion of IFN-γ and IL-2, while IL-4, IL-5, and IL-10 remained low (Figure 5A). To confirm that the cytokine production was mediated by KLH-specific T cells and not due to bystander T-cell activation, unspecific polyclonal restimulation with PHA was done in parallel. Whereas restimulation with KLH demonstrated an increase of the IFN-γ/IL-4 ratio,

Table 1. Differentiation stage of DCs derived from study patients

Patient no.	CD80$^+$, %	CD83$^+$, %
1	90	11
2	91	16
3	86	13
4	ND	ND
5	89	22

ND indicates not done.

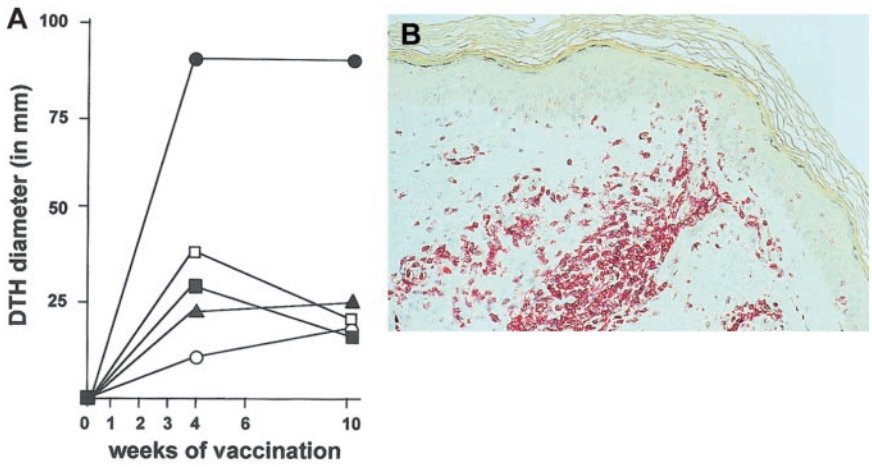

Figure 2. Intranodal vaccination with KLH-pulsed semimature DCs induces strong DTH reactivity to KLH. (A) Quantitation of the KLH-specific immune response by assessment of DTH reactivity to KLH in vivo. DTH reactivity to KLH was tested at week 0, 4, and 10 by intradermal injection of 5 μg KLH in 200 μL sterile PBS into the forearm. 48 hours after the challenge maximal diameter of the elicited indurated erythema was assessed. ■ indicates patient 1; ●, patient 2; ▲, patient 3; □, patient 4; and ○, patient 5. (B) Immunhistochemical characterization of the lymphocytic infiltrate at the DTH site. Alkaline-phosphatase-antialkaline phosphatase (APAAP) staining with a CD45RO mAb was performed, demonstrating a strong perivascular and epidermal infiltration of CD45RO$^+$ memory T cells. Original magnification, × 100.

no defined bias resulted from the polyclonal stimulation with PHA (Figure 5B), suggesting that the enhancement of TH1-type cytokine profile was mediated by KLH-specific effector T cells. In addition, KLH-specific T-cell lines were generated from postvaccination PBMCs of patients 1, 3, and 5. Intracellular cytokine staining of reactivated cell lines revealed that a large portion of the KLH-specific cells (42.5%-72.9%) were Th1 cells (production of IFN-γ but not IL-4); 8.5%-19.1% of the cells demonstrated a Th0 profile (production of IFN-γ and IL-4); and only 0.6%-5.5% KLH-specific Th2 cells were present (production of IL-4 but not IFN-γ). Remaining cells did not produce IFN-γ or IL-4 (Figure 5C). These data further confirm the preferential induction of a KLH-specific Th1-type immune response after intranodal vaccination with immature KLH-pulsed DCs.

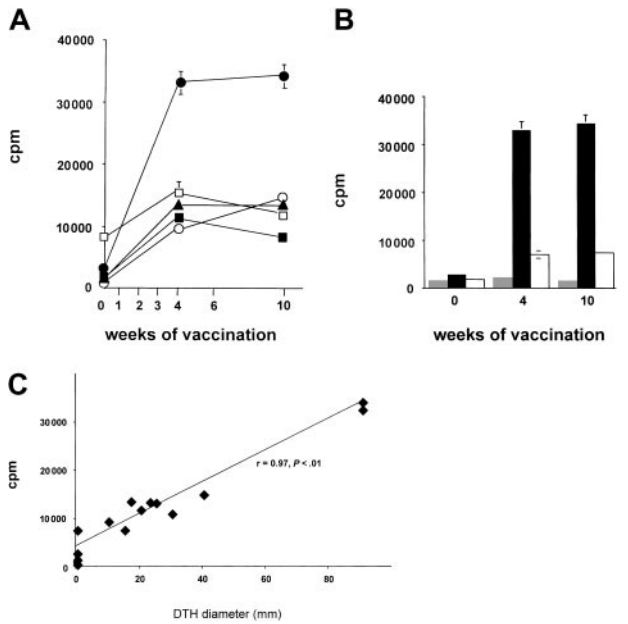

Figure 3. KLH-dependent MHC class II–restricted T-cell proliferation in vitro correlates with DTH reactivity to KLH in vivo. (A) In vitro quantification of the KLH-specific immune response by PBL proliferation to KLH. 10^5 PBLs (week 0, 4, and 10) were plated in 96 round-bottom culture plates with 200 μL CM and stimulated with 20 μg/mL KLH for 4 days. Proliferative activity was assessed after a final 6 hours' pulsing with 1 μCi (0.037 MBq)/well [^{3}H]TdR. Negative control using FCS-supplemented complete medium showed only background proliferation (< 2000 cpm). Data shown represent the mean of triplicate wells. Standard deviations (error bars) were less than 15% for all data points. ■ indicates patient 1; ●, patient 2; ▲, patient 3; □, patient 4; and ○, patient 5. (B) KLH-dependent T-cell proliferation is MHC class II restricted. PBLs (week 0, 4, and 10) were allowed to proliferate to KLH in the absence or presence of anti–HLA-DR mAb (L243 hybridoma culture supernatant 1/1 vol/vol) and its respective isotype control. Proliferation was gradually restored by serial dilution (1/1, 1/10, or 1/100 vol/vol) of the supernatant. Data shown (patient 2) are representative for all patients (n = 5). ▨ indicates CM; ■, KLH + isotype control; and □, KLH + anti–HLA-DR. (C) KLH-dependent T-cell proliferation correlates with the size of DTH to KLH (coefficient of regression r = 0.97, $P < .01$).

Production of KLH-specific IgG2 antibodies in vivo

Dendritic cells can retain native unprocessed antigen and transfer it to naive B lymphocytes to initiate class switching in a primary T-cell–dependent response.[19] It has been suggested that a difference in the balance of Th1 and Th2 cytokines at the site of B-cell activation accounts for a differential production of IgG subclasses. In rodents this relation is well defined: a switch of an antigen-specific antibody subclass in response to immunization can be used as a reliable indicator of the cytokines produced in vivo. Vaccination of KLH-pulsed murine DCs induced anti-KLH mAb of the IgG2a subtype, characteristic of a TH1 response.[20] In humans it has been reported that IgG2 production depends on the presence of IFN-γ,[21-23] while IgG1, IgG3, and IgG4[24,25] are rather related to Th2 cytokines. Patients 1, 3, 4, and 5 were tested for the presence of KLH-specific serum IgG antibodies. Before vaccination none of

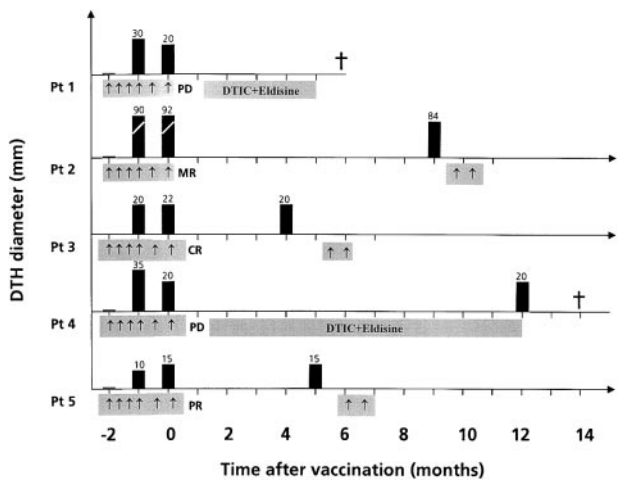

Figure 4. The immune response to KLH is long-lived. DTH diameter in millimeters before vaccination, after 4 and 6 cycles of vaccination, and at different time points after the last vaccination. Symbols: ↑ indicates DC vaccination cycle; †, time of death; CR, complete response, PR, partial response; MR, minor response; and PD, progressive disease.

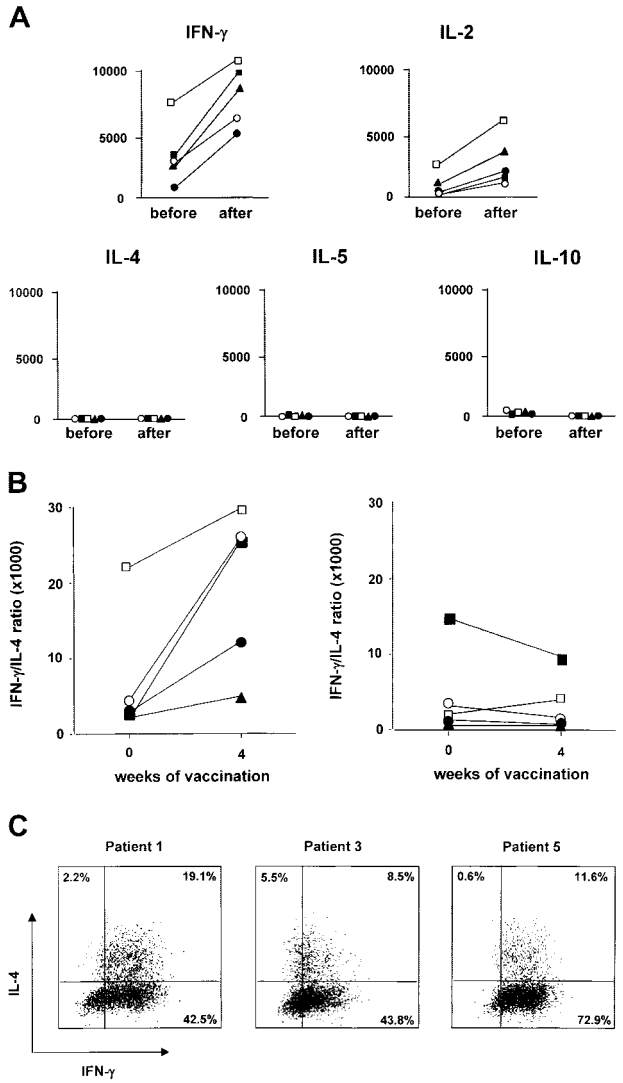

Figure 5. Preferential induction of a KLH-specific T helper 1–type cytokine profile. (A) IFN-γ, IL-2, IL-4, IL-5, and IL-10 cytokine secretion of PBLs after in vitro restimulation with KLH. Prevaccination and postvaccination PBLs were stimulated in vitro with KLH for 4 days, and cytokine levels were measured in cell-free supernatants by ELISA. Values represent cytokine concentrations in pg/mL produced by 10^6 cells in 1 mL complete medium. Negative control using FCS containing complete medium showed cytokine production above the detection limit of the ELISA. ■ indicates patient 1; ●, patient 2; ▲, patient 3; □, patient 4; and ○, patient 5. (B) IFN-γ/IL-4 ratio, as indicator of the preferential cytokine pattern, by PBLs after antigen-specific restimulation with KLH (left panel) or polyclonal stimulation with PHA (right panel). ■ indicates patient 1; ●, patient 2; ▲, patient 3; □, patient 4; and ○, patient 5. (C) Intracellular IFN-γ and IL-4 staining of long-term KLH-specific T-cell lines generated in patients 1, 3, and 5.

the patients had detectable serum IgG antibodies to KLH (Figure 6). After 4 cycles of vaccination, KLH-specific IgG2 antibodies but not of the IgG1, IgG3, or IgG4 subtype were induced in 3 of 4 patients (1, 4, and 5). Patient 3 did not develop any detectable IgG to KLH. In accordance with previous reports showing a correlation between IFN-γ and the production of IgG2 antibodies in humans, these results may indicate a Th1-type cytokine profile in the lymph node at the site of B-cell priming.

Discussion

In this study we demonstrate that repetitive intranodal injection of semimature human monocyte–derived DCs pulsed with protein neoantigen KLH induces strong and long-lasting cellular immune responses to KLH, as demonstrated by DTH reactivity in vivo and KLH-dependent T-cell proliferation in vitro. Interestingly, maximal KLH-specific T-cell responses were induced after the initial 4 cycles of vaccination, could not be further enhanced by additional immunizations (week 6 and 10), and persisted for several months without the need for additional boosters. Long-lived immune responses to protein Ags usually require the use of granuloma-forming complete Freund adjuvant by favoring the in vivo persistence of Ag and therefore keeping CD4 T cells on a constant level of activation.[26] Since in vivo persistence of KLH in our study is unlikely, the persistence of the response may reflect the magnitude of expanded KLH-specific T cells induced by DC vaccination.[27]

Several groups have reported that immunization with soluble KLH protein in adjuvant induces Th0/Th2-type immune response[28-30] independently of the route and the schedule of immunization.[31] To assess the role of DCs in the TH cell polarization in vivo, we performed a qualitative analysis of the KLH-specific CD4 T-cell immune response by a framework of assays, including the measurement of KLH-dependent cytokine production by bulk T cells in vitro, the cytokine profile of in vitro–generated KLH-specific T-cell lines, and the identification of KLH-specific antibody subclass induced in vivo. Assessment of the type of CD4 T-cell immune response is of key importance, since dendritic cells, in addition to their unique role in the generation of protective immunity, also have been implicated in the induction of T-cell tolerance by promoting TH2 responses, T regulatory cells, and anergic T cells. This functional plasticity among monocyte-derived DCs depends on their stage of maturation, the type, and the

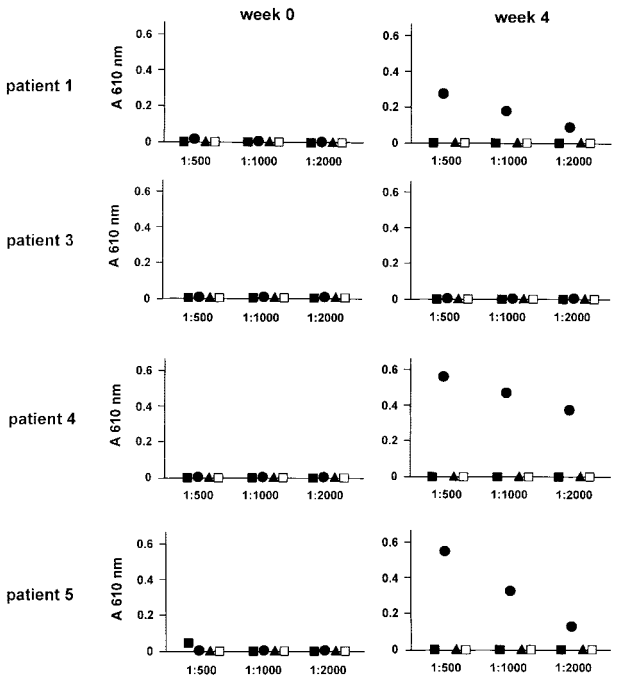

Figure 6. Production of KLH-specific IgG2 antibodies in vivo. Serially diluted prevaccination and postvaccination sera (1:500, 1:1000, 1:2000) were applied on microplates previously coated with KLH and allowed to react sequentially with the following reagents: murine biotinylated anti–human IgG subclass mAb (■, IgG1; ●, IgG2; ◆, IgG3; and □, IgG4) and peroxidase-conjugated avidin. Tetramethylbenzidine was added for color development, and the absorption (A) at 620 nm was read with an ELISA reader. Since serial dilution of the serum samples demonstrated that measurements were collected in the linear phase of the absorption curve, we subtracted the specific background value from the measured value. Mean background absorption in our assay was 0.5 (range, 0.3 to 0.7).

duration of activation. Mature CD40 ligand–activated monocyte-derived DCs secrete large amounts of IL-12, induce T helper (TH)–1 cell differentiation, and have therefore been called DC1.[32] DC1 also can be induced by activation signals such as LPS, proinflammatory cytokines IL-1, TNF-α, and IL-6, CpG, poly I:C, and viruses.[33-35] By contrast, resting or immature monocyte-derived DCs promote TH0/TH2-type cells or T-regulatory cells.[13,36] Anti-inflammatory signals such as IL-10, transforming growth factor-β (TGF-β), steroids, interfering either with DC maturation or their ability to produce IL-12, may lead to the induction of TH2 cells,[37] T-regulatory cells,[38] or anergic T cells.[39] In addition, prolonged activation signals may exhaust the capacity of DC1 to produce IL-12 and favor the induction of TH2 responses.[40,41] Our monocyte-derived DCs generated in culture with GM-CSF and IL-4 in the presence of FCS were $CD1a^+$ and showed a typical phenotype of intermediate maturity expressing low levels of CD83.[42,43] In addition, these DCs showed a strong tendency for further maturation as shown by the rapid increase in $CD83^+$ DCs after a 4-hour pulsing with KLH. Lipopolysaccharides (LPS), which are notably present in FCS, may contribute, through the engagement of pattern recognition receptor TLR-4, to drive resting monocyte-derived DCs into the DC1 development pathway. Indeed, intranodal injection of semimature FCS-derived DCs induced primary T-cell responses with a preferential type 1 cytokine bias. A recent study by Dhodapkar et al showed that a single subcutaneous injection of immature monocyte-derived DCs generated with GM-CSF and IL-4 over 7 days in autologous conditions down-regulated the immune response by inducing IL-10–producing CD8 T-regulatory cells.[13] DCs generated in the presence of autologous plasma instead of FCS are immature, completely lack CD83 expression, and express lower amounts of CD80 (data not shown). In addition, DCs generated in autologous conditions do not express CD1a. Previous reports showed that $CD1a^+$ monocyte-derived DCs were superior to $CD1a^-$ monocyte-derived DCs in their ability to produce IL-12 and to drive TH1 responses.[43] The direct delivery into the lymph node, an environment rich in proinflammatory cytokines released by trafficking memory T cells,[44] may represent an additional maturation stimulus for injected DCs. In addition, intranodal injection may ensure the presence of high DC numbers at the site of T-cell priming, whereas the number of subcutaneously injected DCs ultimately reaching the lymph node may be quite low, considering the poor migratory activity of resting DCs. High DC numbers are indeed necessary for TH1 differentiation, whereas low DC numbers at the priming site may induce TH0/TH2 differentiation.[45] Repetitive vaccination cycles may also promote full DC maturation through CD40-CD40 ligand interactions between CD40L-expressing memory T cells and injected semimature DCs.[16-18]

It has been proposed that bystander CD4 T helper response is an essential requirement for the induction of CD8 T-cell responses to weak antigens such as tumor antigens (which are normally encountered outside an inflammatory context). T helper cells may promote the induction of primary CD8 T-cell response either directly through the secretion of TH1 cytokines (such as IFN-γ and IL-2) or indirectly by promoting DC maturation through CD40-CD40 ligand interactions. A previous study showed the induction of an immune response against KLH in one renal cell carcinoma patient undergoing a partial response to vaccination with mature monocyte-derived DCs but not in 3 patients with either stable or progressive disease.[46] In our study a strong TH1-biased immune response against KLH was detected in all patients, which might also be relevant for the induction of cytotoxic T-cell responses in vivo. Indeed, strong Th1 responses to KLH were induced in all patients developing a tumor-specific CD8 T-cell response after vaccination.[6] Based on our data, the requirement for KLH as helper epitope to promote the generation of a CD8 T-cell response should be tested in controlled clinical trials.

In conclusion, we show that FCS-derived DCs are semimature, and we demonstrated in 5 melanoma patients that intranodal injection of semimature DCs induces a strong and long-lasting CD4 T-cell response with a preferential TH1-type cytokine profile. Since subtle differences in the study design are critical for the induction of a therapeutic immune response, the use of a protein neoantigen as helper epitope allows one to reproducibly trace and characterize the immune response elicited during DC vaccination trials.

References

1. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245-252.
2. Mayordomo JI, Zorina T, Storkus WJ, et al. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. Nat Med. 1995;1:1297-1302.
3. Porgador A, Snyder D, Gilboa E. Induction of antitumor immunity using bone marrow-generated dendritic cells. J Immunol. 1996;156:2918-2926.
4. Specht JM, Wang G, Do MT, et al. Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. J Exp Med. 1997;186:1213-1221.
5. Nestle FO, Banchereau J, Hart D. Dendritic cells: on the move from bench to bedside. Nat Med. 2001;7:761-765.
6. Nestle FO, Alijagic S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med. 1998;4:328-332.
7. Thurner B, Haendle I, Roder C, et al. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med. 1999;190:1669-1678.
8. Dhodapkar MV, Steinman RM, Sapp M, et al. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J Clin Invest. 1999;104:173-180.
9. Banchereau J, Palucka AK, Dhodapkar M, et al. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res. 2001;61:6451-6458.
10. Fong L, Hou Y, Rivas A, et al. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. Proc Natl Acad Sci U S A. 2001;98:8809-8814.
11. Lodge PA, Jones LA, Bader RA, et al. Dendritic cell-based immunotherapy of prostate cancer: immune monitoring of a phase II clinical trial. Cancer Res. 2000;60:829-833.
12. Murphy GP, Tjoa BA, Simmons SJ, et al. Infusion of dendritic cells pulsed with HLA-A2-specific prostate-specific membrane antigen peptides: a phase II prostate cancer vaccine trial involving patients with hormone-refractory metastatic disease. Prostate. 1999;38:73-78.
13. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med. 2001;193:233-238.
14. Fong L, Brockstedt D, Benike C, Wu L, Engleman EG. Dendritic cells injected via different routes induce immunity in cancer patients. J Immunol. 2001;166:4254-4259.
15. Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J Exp Med. 1997;186:65-70.
16. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature. 1998;393:478-480.
17. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature. 1998;393:480-483.
18. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature. 1998;393:474-478.
19. Wykes M, Pombo A, Jenkins C, MacPherson GG. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. J Immunol. 1998;161:1313-1319.

20. Timmerman JM, Levy R. Linkage of foreign carrier protein to a self-tumor antigen enhances the immunogenicity of a pulsed dendritic cell vaccine. J Immunol. 2000;164:4797-4803.
21. Kawano Y, Noma T, Yata J. Regulation of human IgG subclass production by cytokines: IFN-gamma and IL-6 act antagonistically in the induction of human IgG1 but additively in the induction of IgG2. J Immunol. 1994;153:4948-4958.
22. Inoue R, Kondo N, Kobayashi Y, Fukutomi O, Orii T. IgG2 deficiency associated with defects in production of interferon-gamma; comparison with common variable immunodeficiency. Scand J Immunol. 1995;41:130-134.
23. de Gruijl TD, Bontkes HJ, Walboomers JM, et al. Differential T helper cell responses to human papillomavirus type 16 E7 related to viral clearance or persistence in patients with cervical neoplasia: a longitudinal study. Cancer Res. 1998;58:1700-1706.
24. Briere F, Servet-Delprat C, Bridon JM, Saint-Remy JM, Banchereau J. Human interleukin 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG1 and IgG3. J Exp Med. 1994;179:757-762.
25. Spiegelberg HL, O'Connor RD, Falkoff RJ, Beck L. Interleukin-4 induced IgE and IgG4 secretion by B cells from atopic dermatitis patients. Int Arch Allergy Appl Immunol. 1991;94:181-183.
26. Kundig TM, Bachmann MF, Oehen S, et al. On the role of antigen in maintaining cytotoxic T-cell memory. Proc Natl Acad Sci U S A. 1996;93:9716-9723.
27. Sprent J. Immunological memory. Curr Opin Immunol. 1997;9:371-379.
28. Groves PL, Pech MH, Troutt AB, Kelso A. Limiting dilution analysis reveals the precursors of interleukin-4-producing CD4+ cells induced by protein immunization. Immunology. 1994;83:25-32.
29. Kelso A, Groves P, Troutt AB, Pech MH. Rapid establishment of a stable IL-4/IFN-gamma production profile in the antigen-specific CD4+ T cell response to protein immunization. Int Immunol. 1994;6:1515-1523.
30. Kelso A, Groves P, Troutt AB, Francis K. Evidence for the stochastic acquisition of cytokine profile by CD4+ T cells activated in a T helper type 2-like response in vivo. Eur J Immunol. 1995;25:1168-1175.
31. Sedlik C, Deriaud E, Leclerc C. Lack of Th1 or Th2 polarization of CD4+ T cell response induced by particulate antigen targeted to phagocytic cells. Int Immunol. 1997;9:91-103.
32. Rissoan MC, Soumelis V, Kadowaki N, et al. Reciprocal control of T helper cell and dendritic cell differentiation. Science. 1999;283:1183-1186.
33. Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc Natl Acad Sci U S A. 1999;96:9305-9310.
34. Cella M, Salio M, Sakakibara Y, Langen H, Julkunen I, Lanzavecchia A. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. J Exp Med. 1999;189:821-829.
35. Jonuleit H, Kuhn U, Muller G, et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur J Immunol. 1997;27:3135-3142.
36. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J Exp Med. 2000;192:1213-1222.
37. King C, Davies J, Mueller R, et al. TGF-beta1 alters APC preference, polarizing islet antigen responses toward a Th2 phenotype. Immunity. 1998;8:601-613.
38. Matyszak MK, Citterio S, Rescigno M, Ricciardi-Castagnoli P. Differential effects of corticosteroids during different stages of dendritic cell maturation. Eur J Immunol. 2000;30:1233-1242.
39. Steinbrink K, Wolfl M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. J Immunol. 1997;159:4772-4780.
40. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol. 2000;1:311-316.
41. Ebner S, Ratzinger B, Krosbacher M, et al. Production of IL-12 by human monocyte-derived dendritic cells is optimal when the stimulus is given at the onset of maturation, and is further enhanced by IL-4. J Immunol. 2001;166:633-641.
42. Xia CQ, Kao KJ. Heparin induces differentiation of CD1a+ dendritic cells from monocytes: phenotypic and functional characterization. J Immunol. 2002;168:1131-1138.
43. Chang CC, Wright A, Punnonen J. Monocyte-derived CD1a+ and CD1a- dendritic cell subsets differ in their cytokine production profiles, susceptibilities to transfection, and capacities to direct Th cell differentiation. J Immunol. 2000;165:3584-3591.
44. Kundig TM, Bachmann MF, DiPaolo C, et al. Fibroblasts as efficient antigen-presenting cells in lymphoid organs. Science. 1995;268:1343-1347.
45. Tanaka H, Demeure CE, Rubio M, Delespesse G, Sarfati M. Human monocyte-derived dendritic cells induce naive T cell differentiation into T helper cell type 2 (Th2) or Th1/Th2 effectors: role of stimulator/responder ratio. J Exp Med. 2000;192:405-412.
46. Rieser C, Ramoner R, Holtl L, et al. Mature dendritic cells induce T helper type-1-dominant immune responses in patients with metastatic renal cell carcinoma. Urol Int. 1999;63:151-159.