Cancer Immunol Immunother (2004) 53: 963–977
DOI 10.1007/s00262-004-0542-8

ORIGINAL ARTICLE

Joanna Galea-Lauri · James W. Wells · David Darling
Phillip Harrison · Farzin Farzaneh

Strategies for antigen choice and priming of dendritic cells influence the polarization and efficacy of antitumor T-cell responses in dendritic cell–based cancer vaccination

Received: 19 November 2003 / Accepted: 17 March 2004 / Published online: 14 May 2004
© Springer-Verlag 2004

Abstract Dendritic cells (DCs) primed with tumor antigens (Ags) can stimulate tumor rejection. This study was aimed at evaluating the polarization of T-cell responses using various DC Ag-priming strategies for vaccination purposes. DCs cocultured with irradiated "apoptotic" tumor cells, DC-tumor fusions, and DCs pulsed with freeze-thaw tumor lysate Ags served as Ag-primed DCs, with EG7 tumor cells (class II negative) expressing OVA as the model Ag. DCs loaded with class I– and class II–restricted OVA synthetic peptides served as controls. Primed DCs were assessed by the in vitro activation of B3Z OVA-specific CD8 T cells and the proliferation of OVA-specific CD8 and CD4 T cells from OT-I and OT-II TCR transgenic mice, respectively. In vivo responses were measured by tumor regression following treatment with Ag-primed DCs and by CTL assays. Quantification of IL-2, IL-4, IL-5, IFN-γ, and TNF-α by cytometric bead array (CBA) assay determined the polarization of TH1/TH2 responses, whereas H-2 K^b /SIINFEKL tetramers monitored the expansion of OVA-specific T cells. DC-EG7 hybrids stimulated both efficient class I and class II OVA responses, showing that DC-tumor hybrids are also capable of class II cross-presentation. The hybrids also induced the most potent CTLs, offered the highest protection against established EG7 tumors and also induced the highest stimulation of IFN-γ and TNF-α production. DCs cocultured with irradiated EG7 were also effective at inducing OVA-specific responses, however with slightly reduced potency to those evoked by the hybrids. DCs loaded with lysates Ags were much less efficient at stimulating any of the OVA-specific T-cell responses, showed very little antitumor protection, and stimulated a weak TH1 response, overbalanced by an IL-5 TH2 response. The strategy of Ag-loading clearly influences the ability of DCs to polarize T cells for a TH1/TH2 response and thus determines the outcome of the elicited immune response, during various vaccination protocols.

Keywords CTL · Dendritic cell vaccination · Fusion · Tetramers · TH1/TH2 cytokines

Abbreviations

DC	Dendritic cell
FSC	Forward scatter
SSC	Side scatter
TC	Tumor cells

Introduction

Characterization of the immunobiology of dendritic cells (DCs) has established their critical role in antigen (Ag) presentation for the initiation and maintenance of immune responses (for reviews, see [4, 25, 31]). Several mouse tumor models show unequivocally the efficacy of Ag-bearing DCs as tumor vaccines [10, 20, 28, 53, 62, 64, 65]. A study using healthy recipients also proves the immunogenicity and safety of DCs in humans, and demonstrates that a single injection of a small number of DCs is sufficient to rapidly expand T-cell immunity for both naïve and recall Ags [16]. DCs are now also being investigated for their potential therapeutic ability for cancer immunotherapy in humans (reviews [13, 22, 27, 32, 51, 56, 60, 70, 71, 78, 81]) with over 30 clinical trials already submitted in the United States (for details browse http://clinicaltrials.gov). Data emerging from

This work was supported by Grant 9853 from the Leukaemia Research Fund, UK; a JRC studentship from GKT; and the Lewis Family Research Trust

J. Galea-Lauri (✉) · J. W. Wells · D. Darling · F. Farzaneh
Department of Molecular Medicine, GKT School of Medicine,
King's College London, The Rayne Institute,
123 Coldharbour Lane, London, SE5 9NU, UK
E-mail: joanna.galea-lauri@kcl.ac.uk
Tel.: +44-20-78485901
Fax: +44-20-77333877

P. Harrison
Institute of Liver Studies, GKT School of Medicine,
King's College London, Denmark Hill Campus,
London, SE5 9PJ, UK

some of these studies indicate that DC vaccination can induce both immunological and more importantly clinical responses albeit in a very small number of patients [5, 34, 52, 57, 59, 64, 67, 74, 76, 77]. However, for improved efficacy, several parameters still need to be optimized, such as DC preparation, choice of Ags, Ag-loading protocols, dosing, injection sites, and dose scheduling, as well as adequate immune monitoring. Some studies now suggest the use of mature, rather than immature DCs [15, 16, 39, 44], and the s.c. rather than i.v. route of administration [16, 18, 21, 45].

Different protocols have been described to deliver Ags to DCs. Foremost among these have been the pulsing of DCs with synthetic MHC class I–restricted tumor Ag–derived peptides [3, 5, 11, 12, 58]. However, using specific peptides can have certain disadvantages. For example, their application is limited in patients who express the HLA specific for that peptide and also ignores the important role of MHC class II T-helper responses. Additionally, peptides appear to induce weak CTL responses and often need to be modified for stronger binding affinity to MHC class I [54, 68]. Furthermore, peptides only reside on the DC surface for short periods of time [2, 48] and more importantly can be associated with Ag and/or MHC class I loss variants in vivo [37]. Therefore, to avoid such disadvantages, the strategy chosen for Ag formulation and delivery into the DCs must ensure that Ags have access to both pathways of MHC presentation—i.e., class I and class II—thus allowing a wide peptide repertoire restricted by multiple HLA alleles crucial for the polyvalent stimulation of both $CD8^+$ and $CD4^+$ T-cell responses [7, 31]. Such strategies include: pulsing DCs with full-length native or recombinant proteins; and infection with recombinant viruses, transfection with DNA or mRNA, or even transfection using whole tumor–derived RNA [26, 36, 38]. However, the ultimate strategy for DC Ag-loading also needs to be clinically practical and preferably with broad applicability. Therefore, other approaches utilizing whole tumors as source of Ag have been developed, including DCs loaded with tumor lysates [20, 59], dying tumor cells (apoptotic bodies and necrotic cells) [8, 14, 19, 33, 61], or DCs fused directly with tumor cells (see review [73]).

In the absence of adequate immune monitoring, it is unclear as to which Ag-priming stragey for DC vaccination would be of most therapeutic value. For example, we recently reported that the magnitude of induction of an MHC class I–restricted antitumor CTL response is dependent on the DC Ag-loading strategy [23]. However, due to lack of immune probes for the tumor model under investigation, we were unable to fully characterize the immune responses, thus impairing our ability to explain the differences in the efficacy of the immune responses induced by the different Ag-loading strategies.

In this study, we use the EL4 murine thymoma cell line (class I^+, class II^-) stably expressing chicken ovalbumin as a model Ag (E.G7-OVA cells) to evaluate the efficacy of bone marrow–derived DCs to induce class I and class II OVA-specific T-cell responses when loaded with E.G7-derived Ags and used as a vaccine. The immunological responses were monitored by quantitative analysis of MHC class I and MHC class II–restricted OVA-specific T-cell responses both in vitro and in vivo, as well as the overall balance of TH1/TH2 cytokine responses. We show that different strategies of Ag-loading into DCs can lead to the differential polarization of T-cell responses, thus suggesting that the choice of Ag-priming of DCs might be critical to the outcome of the immune response.

Materials and methods

Mice

C57/BL6 ($H\text{-}2^b$) female mice were purchased from Harlan, Oxford, UK. OT-I [17] and OT-II [6] TCR transgenic mice ($H\text{-}2^b$) were obtained from Prof. M. Kemeny (see ''Acknowledgements''). OT-I T cells recognize the OVA $peptide_{257\text{-}264}$ (SIINFEKL) in association with class I, whereas the OT-II T cells recognize the OVA $peptide_{323\text{-}339}$ (ISQAVHAAHAEINEAGR) in association with class II.

Cell lines

The tumor cells used were the Ia^- lines EL4 ($H\text{-}2^b$ thymoma; class II^-) and E.G-7 (abbreviated to EG7), a stable transfectant of the EL4-expressing OVA [55]. EG7 cells present OVA as H-2 K^b /SIINFEKL (also referred to as H-2 K^b /OVA). The B3Z T-cell line, used specifically to measure H-2 K^b /OVA responses, represents a hybridoma generated by the fusion of B3 cells (a $V_\beta 5$-expressing CTL clone specific for H-2 K^b /OVA) and Z.8 cells (NFAT- *lacZ*–inducible derivative of BW5147) [40]. The hybridoma 25-D1.16 which secretes a mAb specific for H-2 K^b /SIINFEKL [66] was provided by Dr R. Germain (see ''Acknowledgements''). All cell lines were cultured under normal tissue culture conditions using RPMI / 10% FCS in the presence of penicillin and streptomycin.

Dendritic cells

Dendritic cells were cultured from the bone marrow of 3-week-old C57/BL6 female mice as follows [49]. Bone marrow cells were seeded at 2×10^6 cells per 100-mm bacteriological plates in 10 ml of RPMI / 10% FCS / 50-μM 2-ME, supplemented with standard antibiotics. The cultures were initiated with rGM-CSF and rIL-4, both at 10 ng/ml (Peprotech, London, UK). At day 3, 10 ml of medium containing rGM-CSF (final concentration 10 ng/ml) was added to the cells, with a further 5 ml of medium containing rGM-CSF (final concentration 5 ng/ml) added at day 6. DCs, most of which were nonadherent, were used between days 7 and 10.

Other reagents

The class I and class II OVA peptides, sequences 257–264 and 323–339, respectively, were purchased from the Department of Molecular Biology and Biotechnology, University of Sheffield, UK. H-2 K^b/SIINFEKL–tetramer complexes (PE-conjugate) were purchased from PROIMMUNE, Oxford, UK. The following mAbs (all FITC-conjugates, unless otherwise stated) were obtained from BD Pharmingen (Oxford, UK): clone 28–8-6 (anti-H-2 K^b /H-2D^b unconjugated), clone AF6-120.1 (anti-I-A^b), clone HL3 (anti-CD11c), clone 16-10A1 (anti-B7.1), clone GL1 (anti-B7.2), clone RB6-8C5 (anti-Gr-1), clone 37.51 (anti-CD28:R-PE conjugate), clone 53–6.7 (anti-CD8a:Cy-Chrome conjugate). The following mAbs were obtained from Serotec (Oxford, UK): clone KAT-1 (anti-ICAM-1) and clone 3/23 (anti-CD40, unconjugated). Anti-CD40 and anti-H-2 K^b/H-2D^b were detected using appropriate secondary reagents which were FITC-conjugated. For immunophenotyping, DCs were preincubated for 15 min with FCBLOCK (anti-CD16/CD32; clone 2.4G2 from Pharmingen).

Loading DCs with OVA synthetic peptides

Dendritic cells were resuspended at 5×10^6 cells/ml in RPMI medium containing either the class I–restricted $OVA_{257\text{-}264}$ peptide or the class II $OVA_{323\text{-}339}$ peptide (range 1 nM–1 μM). The cells were left at 37°C for 4–6 h. Excess peptides were removed by washing the DCs twice in RPMI medium. The binding of SIINFEKL peptide to H-2 K^b was assessed by labeling the cells with the mAb 25-D1.16 mAb, using rabbit anti-mouse-Ig FITC for detection, and subjected to flow cytometry. DCs pulsed with the irrelevant RGYVYQGL peptide served as controls. Detection of the binding for the class II OVA peptide to DCs was not assessed as no mAb was available for this work.

Ag-loading strategies using whole EG7 cells

Ag-loading was performed over 4–6 h using day-8 DC cultures. This time point resulted in optimal DC viability and recovery, as determined previously [23].

Loading DC with EG7 lysate

Soluble EG7 lysate Ags were prepared by three freeze-thaw cycles (−140°C/37°C) of cells in 1 ml PBS, with removal of cell debris by centrifugation at 12,000 rpm for 5 min (Biofuge Pico Bench centrifuge; Heraeus Inst.) [59]. Supernatant proteins were then added to DC cultures at 100 μg/5×10^6 DCs in 1 ml and left at 37°C. Excess Ags were removed by washing the DCs as described above. The uptake of proteins present in the lysates was assessed indirectly by the uptake of albumin-FITC which was added as a tracer protein to the lysate preparations. The Ag-loaded DCs were then quantified using flow cytometry.

Loading DCs with apoptotic EG7 tumor cell fragments

"Necrotic apoptosis" in the EG7 cells was induced by UV irradiation (302 nm) as previously described [24]. Increasing numbers of apoptotic EG7 cells were then cocultured with DC cultures (2×10^6 DCs/ml), for 4–6 h at 37°C. Excess apoptotic cell fragments not taken up by the DCs were removed by Ficoll-Hypaque centrifugation. The uptake of apoptotic cells by the DCs was assessed by flow cytometry, by labeling the EG7 cells with PKH26 (FL2 dye), as described previously [23]. DCs were identified using an anti-MHC class II FITC-conjugated mAb, a marker absent on the EG7 cells. DCs which had taken up tumor cell fragments were identified as double-positive cells for both FITC (FL1 channel) and PKH26 (FL2 channel). Fluorescent microscopy was used to confirm the presence of apoptotic cell bodies within the DCs.

DC-EG7 hybrid formation

DCs and tumor cells were mixed together at different DC to tumor cell ratios, centrifuged, and washed in Mg^{2+} /Ca^{2+} free PBS (at 37°C). Fusion was induced by 50% PEG / 10% DMSO under identical conditions as described previously [23]. The cells were then cultured for 4 h to allow recovery from PEG/DMSO treatment. Unfused EG7 cells were removed by negative immunoselection: the mixture of cells (i.e., unfused DCs, DC-EG7 hybrids, and unfused EG7) was labeled using a biotinylated anti-CD11c mAb (a marker expressed by DCs and DC hybrids but not unfused EG-7 cells), with excess Ab removed by washing. The cells were then mixed with Streptavidin Magnesphere Paramagnetic Particles (1 μm in size; Promega), and isolated using a DYNAL MPC (Magnetic Particle Concentrator; Dynal, UK), discarding the unlabeled EG7 cells.

The efficiency of PEG/DMSO-mediated hybrid formation was assessed by flow cytometry using mAbs for Ags exclusively expressed by each of the fusion partners. These were CD28-PE and MHC class II–FITC for EG7 and DCs, respectively. The DC-EG7 hybrid cells would express both molecules, and thus be identified as double-labeled cells. Fluorescent microscopy was also used to visualize the hybrid cells.

In vitro induction of OVA-specific T-cell responses by Ag-loaded DCs

Activation of B3Z T cells assayed colorimetrically

B3Z (3×10^4) T cells were added to 5×10^4 Ag-loaded DCs in a 96-well plate, left to coculture for 16 h, and were then washed once with 100-μl PBS. Any lacZ activity

induced in the B3Z cells was measured by the addition of 100 µl of 5-mM ONPG in PBS / 0.5% NP-40 after 4 h at 37°C, with the absorbance being read at 415 nm, as described [72].

Proliferation of naïve OT-I and OT-II T lymphocytes

OT-I and OT-II lymphocytes were used to measure OVA-specific CD8 and CD4 proliferative responses, respectively, to the different Ag-loaded DCs. Lymphocytes, isolated from both the lymph nodes and spleens, were first depleted of RBCs by lysis in an ammonium chloride Tris buffer (Sigma) and also of B cells using mouse pan B–specific Dynabeads (B220), according to manufacturer's instructions. Lymphocytes (2×10^5) were then cocultured with the Ag-loaded DCs. DCs loaded with either class I or class II OVA peptide served as controls. After 5 days of coculture, cells were pulsed with ^{3}H-thymidine for 6 h, harvested using a Skatron Micro96 Harvester and counted in aqueous phase LSC cocktail, Ultima Gold Scintillant (Canberra-Packard), using a liquid scintillation analyzer (TRI-CARB 2200CA; Packard).

Induction of CTLs in mice injected with Ag-loaded DCs

Lymph nodes and spleen cells were collected and pooled from three mice which had been immunized 21 days earlier by i.p. injection of Ag-loaded DCs (5×10^5 DCs). The lymphocytes (3×10^7) were stimulated by coculture with irradiated (5,000 rads) EG7 cells (1.5×10^6) for 5 days in 10-ml RPMI / 10% FCS and 50-µM 2-ME in upright 25-cm^2 flasks, and cells tested for CTL activity against EG7 and EL4. The supernatants from these cocultures were kept for the analysis of TH1/TH2 cytokines (see details below). For the CTL assay, the target cells were labeled with ^{51}Cr and added to the responder T cells at various effector to target (E/T) ratios (performed in triplicates). Percentage lysis of targets was calculated as described previously [23].

Induction of in vivo OVA-specific T-cell responses by Ag-loaded DCs

Lymphocytes from mice immunized with Ag-loaded DCs were cocultured with irradiated EG7 cells for 48 h and tested for specific TCR recognition of H-2 K^b/OVA peptide. This was done by labeling cells for CD8 and TCR using anti-CD8a Cy-Chrome conjugated Ab and H-2 K^b/SIINFEKL tetramer complex (PE-labeled), respectively. The percentage of CD8 T cells with specific reactivity for OVA were quantified by FACS analysis using CellQuest software (BD).

Cytometric bead array (CBA) analysis of TH1/TH2 cytokine responses induced in the different immunized mice

Supernatants obtained from the 5-day cocultures of immunized lymphocytes with irradiated EG7 cells (described above), were analyzed for T-helper cytokines using CBA assays. The CBA assay, performed using a kit (BD Biosciences), allows the simultaneous detection and quantification of soluble murine IL-2, IL-4, IL-5, IFN-γ, and TNF-α in a single sample. The principle of the CBA assay is as follows: five bead populations of equal size but distinct fluorescence intensities (resolved in the FL3 channel of a flow cytometer) are coated with capture antibodies specific for the different cytokines and mixed together to form the CBA. The cytokine capture beads are mixed with recombinant cytokine standards or test samples and then mixed with the PE-conjugated detection antibodies, which are resolved in the FL2 channel. Following acquisition of sample data by flow cytometer, the results were analyzed using the BD CBA analysis software. Standard curves, plotted using the MFI of the beads for cytokine values ranging from 0 to 5,000 pg/ml, were used for quantifying the sample cytokines.

Treatment of EG7-tumors by vaccination using Ag-loaded DCs

Tumors were established in 6–8-week-old female C57/BL6 mice by s.c. injection of 4×10^5 EG7 cells, and treated on days 3 and 10 by the i.p. injection of 5×10^5 Ag-loaded DCs. All procedures were carried out in accordance with the guidelines set out for the welfare of animals under a project licence issued by the UK Home Office and under guidelines set out by the local ethical committee. Tumor growth was monitored at regular intervals and mice culled when tumor growth exceeded 21 mm. Data are presented as the percentage of mice surviving over time, and a column scatter graph (dot plot) showing each value for tumor size in a group using Graphpad Prism Version 3.0.

Results

Cell surface phenotype of BmDCs generated by GM-CSF and low-level IL-4

Bone marrow cells cultured for 6–8 days in high doses of GM-CSF and one initial low dose of IL-4 yielded a population of cells with 70–85% displaying typical DC markers as judged by expression of cell surface H-2 K/D, I-A, CD11c, B7.1, B7.2, ICAM-1, and CD40 (Fig. 1A). Under these culture conditions, the DCs expressed very low or no GR-1 (an early myeloid/granulocyte marker) and very low F4-80 (macrophage marker), suggesting that low-level IL-4 is necessary to prevent neutrophil and macrophage contamination, respectively (Fig. 1B). In

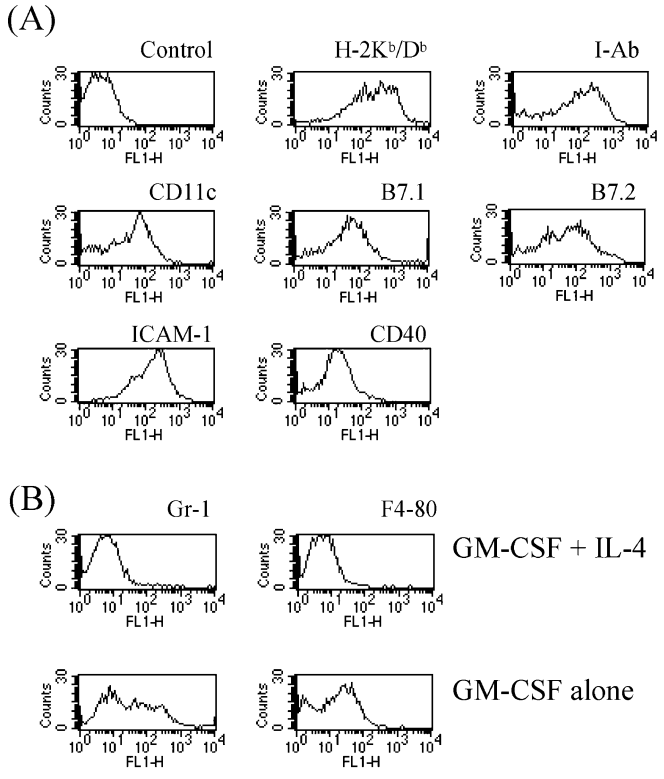

Fig. 1 **A** FACS profiles of day 7 bone marrow–derived DCs cultured in GM-CSF and low levels of IL-4, showing that the cells express high levels of H-2 K^b/D^b, I-A, CD11c, B7.1, B7.2, ICAM-1, and modest levels of CD40 consistent with DC phenotype. **B** FACS profiles showing that DCs cultured without the addition of IL-4 express significant levels of the neutrophil marker GR-1 and macrophage marker F4-80, indicating that IL-4 is essential for DC differentiation

addition, DCs had a spontaneous secretion of low levels of IL-12 (measured by ELISA) which increased significantly on exposure to LPS or TNF (data not shown), showing that the combination of cytokine concentrations used generates DCs which are responsive to maturation signals [50]. We also analyzed the DC phenotype after Ag-priming of the DCs by various loading strategies and found insignificant changes in the levels of B7.1, B7.2, CD40, H-2 K/D, I-A, and CD40, when compared together (data not shown).

Detection, quantification and optimization of Ag-loading of DCs

DC peptide pulsing

The amount of surface detectable H-2 K^b /SIINFEKL after peptide-pulsing was directly proportional to the initial peptide concentration (Fig. 2A) and was optimal at 37°C with very little binding at 4°C (data not shown). DCs pulsed with an irrelevant peptide were negative for 25-D1-16 staining (Fig. 2B). The amount of peptide used to obtain physiological levels of H-2 K^b /SIINFEKL on DCs was determined from the levels

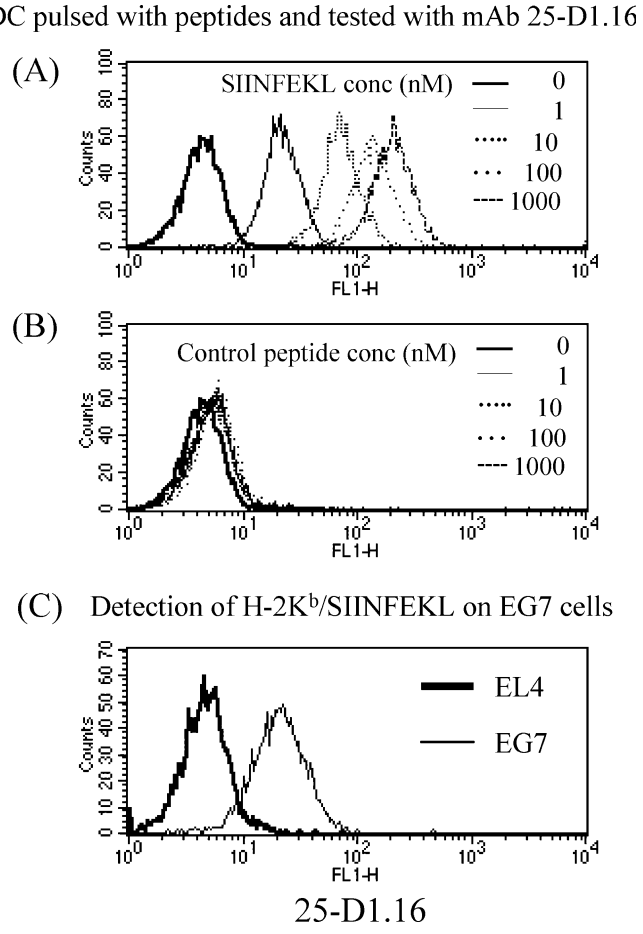

Fig. 2 **A** FACS profiles of DCs after being pulsed with SIINFEKL peptide and stained with the mAb 25-D1.16 specific for the H-2 K^b /SIINFEKL complex. Graphs show that the relative staining of the cells (i.e., binding of SIINFEKL to the H-2 K^b) is proportional to the initial dose of the peptide used. **B** DCs pulsed with an irrelevant peptide are negative for 25-D1.16 staining, showing that this mAb is only specific for H-2 K^b/SIINFEKL. **C** FACS analysis of the EG7 cells shows significant expression of surface H-2 K^b/SIINFEKL compared with the parental line EL4. On comparison to the DCs pulsed with SIINFEKL in **A**, the physiological levels of SIINFEKL on the EG7 cells are estimated to be 1 nM

expressed by EG7 and estimated by FACS analysis to be around 1 nM (Fig. 2C).

DCs loaded with EG7 lysate

The uptake of protein from the lysates by DCs was inferred indirectly from the simultaneous uptake of albumin-FITC. Although the uptake of albumin by DCs does not necessarily mean uptake of other proteins in the lysate preparation, it provides a means of assessing protein uptake by the DCs. After 4 h, the majority of the DCs were positive for the uptake of albumin-FITC (Fig. 3A), with the amount of albumin-FITC being directly proportional to the initial dose of albumin (range from 1 to 1,000 μg/ml; Fig. 3B). This suggests that over this time period and dose range, the DCs were not limited by the amount of Ag they could take up. Thus, in

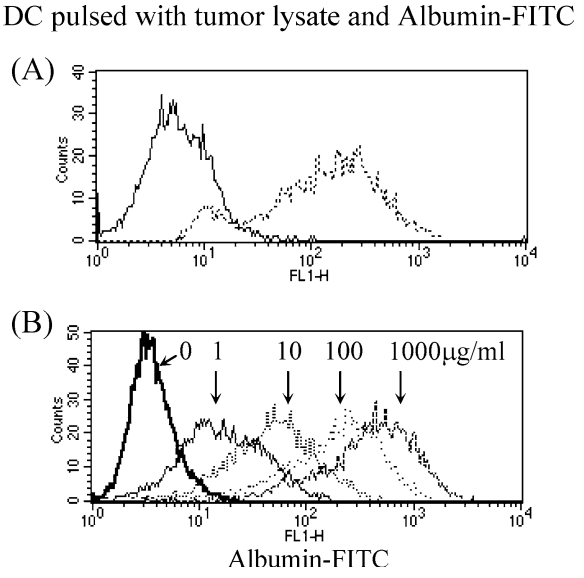

Fig. 3 **A** Histogram profiles of DCs after coculture with tumor-derived lysate proteins with (*dotted line*) or without (*solid line*) the addition of albumin-FITC protein used as a tracer for protein uptake. As shown in the overlay histograms, 90% of the DCs are positive for FITC-protein indicating active protein uptake by the DCs. **B** FACS analysis of DCs after being pulsed with increasing doses of albumin-FITC showing that at this dose range the amount of albumin-FITC taken up by the DCs is proportional to the intial protein concentration

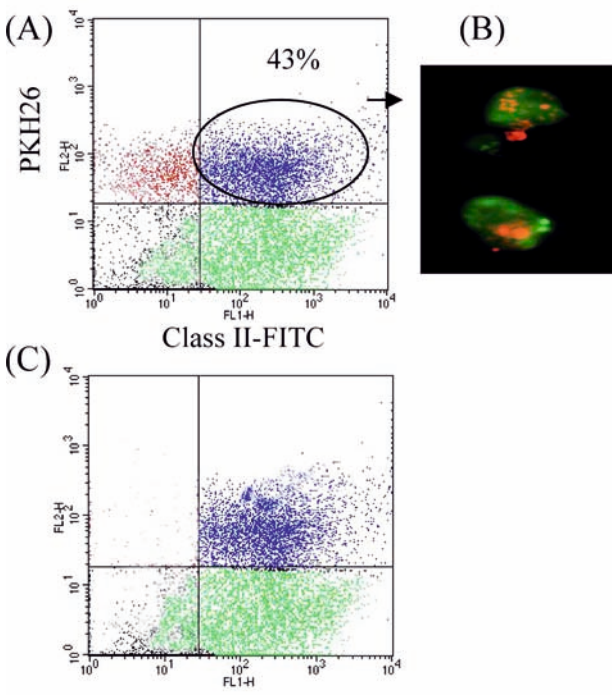

Fig. 4 **A** Dot plot showing FACS analysis of DCs (labeled with MHC class II–FITC, FL1 channel) after the uptake of apoptotic cell bodies (labeled with PKH26 red dye, FL2 channel) after a 6-h coculture of the DCs with apoptotic EG7 cells. The DCs that have taken up the apoptotic cells are fluorescent in both channels (shown as *blue pseudocolor dots*). **B** Flourescence microscopy confirms the presence of apoptotic cell bodies (*red*) in the DCs. **C** Dot plot showing the successful removal by Ficoll centrifugation of any apoptotic EG7 cells that are not taken up by the DCs

terms of determining the number of EG7 cells used for lysate preparation for DC loading, we used the highest number of cells required for the maximal DC loading determined by the other loading strategies (see below). This was calculated to be five EG7 cells per one DC.

DC loaded with apoptotic EG7 cells

The uptake of apoptotic cells by the DCs was monitored by PKH26-labeled EG7 cells. Within 4 h of coculture, apoptotic tumor fragments could be detected within 43% of the total DCs (DCs labeled with class II-FITC), as assessed by the percentage of double fluorescent cells (blue pseudocolor dots, Fig. 4A). Presence of apoptotic cells within the DCs was confirmed by fluoresence microscopy (Fig. 4B). Excess apoptotic cells that were not taken up by the DCs were successfully removed by Ficoll centrifugation (Fig. 4C). Maximal DC-loading was obtained with an optimal ratio of five EG7 cells to one DC (Fig. 4A). Increasing the number of apoptotic cells did not result in a significant increase of their uptake by DCs, as judged by either the number of DCs that take up the apoptotic cells or the amount of apoptotic cells taken up by the DCs (data not shown).

DC-EG7 hybrid formation

Hybrid cells, DC-EG7, were quantified by the coexpression of CD28-PE (EG7 marker, Fig. 5A) and MHC class II–FITC (DC marker, Fig. 5B). After PEG/DMSO treatment, the fusion efficiency at a DC/EG7 ratio of 1:1 was 31.7% of total DCs (double-labeled cells shown as pseudoblue dots, Fig. 5D). No significant numbers of double-labeled cells were present in a control mixture of EG7 and DCs lacking PEG/DMSO treatment (Fig. 5C). Increasing or decreasing the ratio of the fusion partners beyond 1:1 resulted in a significant decrease in fusion efficiency (data not shown). Unfused EG7 cells were successfully removed by immunomagnetic selection as shown by their absence after re-FACSing (Fig. 5E). Following fusion, the proportion of DCs staining positive for H-2 K^b/SIINFEKL was 29.6% of total DCs, which agreed with a fusion efficiency of 31.7% obtained for this experiment (Fig. 5F). The cells that did not stain positive for H-2 K^b/SIINFEKL in Fig. 5F represent unfused DCs.

In vitro induction of OVA-specific T-cell responses by Ag-loaded DCs

Colorimetric assay using B3Z T-cell line

Activation of B3Z T cells by H-2 K^b/SIINFEKL complex is measured by the induced expression of the reporter gene product β-galactosidase [72]. Addition of increasing doses of SIINFEKL peptide to DCs elicits a

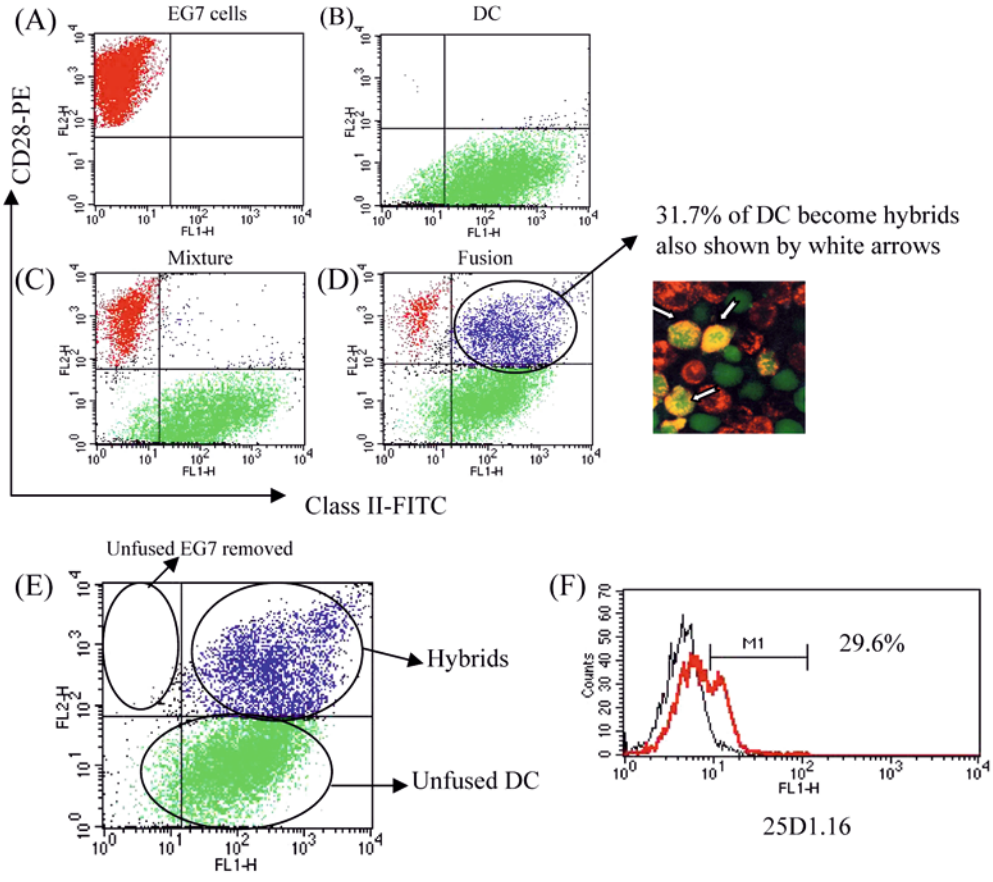

Fig. 5A–F FACS analysis for the detection and quantification of DC-EG7 hybrid cells. The fusion partners are detected by the unique expression of CD28 on EG7 cells marked by PE labeling in the FL2 channel (**A**) and Class II on DC marked by FITC labeling in the FL1 channel (**B**). Following PEG/DMSO treatment, a fusion efficiency of about 30% is obtained and the hybrid cells are detected as double fluorescent cells (shown as *pseudoblue dots* in **D**), which are not present in the control mixture (**C**). The hybrid cells are confirmed as double fluorescent by microscopy (*white arrows*). The successful removal of unfused EG7 cells by immunomagnetic depletion (**E**), and after fusion, the hybrid cells (marked as 29.6% of total DC), also express surface H-2 K^b/SIINFEKL which they obtained from the EG7 fusion partner (**F**)

dose-dependent B3Z response (data not shown), indicating that the B3Z T-cell response is sensitive to differences in levels of MHC/peptide expressed on the DCs. The level of activation of the B3Z cells by the Ag-loaded DCs was dependent on the Ag-priming strategy, as shown by the much higher level of activation of the B3Z by the DC-EG7 hybrid cells, or the DCs loaded with apoptotic EG7 cells, compared with DCs loaded with lysate Ags (Fig. 6A). The activation of B3Z cells was only marginally increased when the amount of lysates added to the DCs was increased by up to a 100-fold. In addition, lysate-pulsed DCs were never as efficient at B3Z activation as other Ag-loaded DCs. This suggests strongly that cell-associated ovalbumin is cross-presented much more efficiently than soluble Ag, a finding reported by other independent investigators [47]. DCs pulsed with 1-nM SIINFEKL peptide served as positive controls, whereas unloaded DCs provided negative controls (Fig. 6A). DCs loaded with EL4-derived Ags were incapable of stimulating the B3Z cells, thus showing that the activation was specific to the OVA Ag rather than the parental EL4-derived Ags (data not shown).

Proliferative responses using naïve OT-I lymphocytes

Responses to H-2 K^b/OVA by Ag-loaded DCs were also measured by the induction of a proliferative response in naïve OT-I T cells. As shown in Fig. 6B, the levels of T-cell activation paralleled the B3Z assay, again suggesting strongly that the source and strategy used for Ag-loading determines the efficiency of cross-presentation of OVA by the DCs.

Proliferative responses using naïve OT-II lymphocytes

Responses to OVA cross-presented by class II after DC Ag-loading were determined by the proliferation of naïve OT-II lymphocytes. DCs pulsed with 1-nM OVA peptide$_{323\text{-}339}$ served as positive controls. The levels of activation of the OT-II cells were dependent on the priming strategy for DCs, as shown by the much higher level of activation of the OT-II cells by the DC-EG7 hybrid cells and the DCs loaded with apoptotic EG7 cells, compared with DCs loaded with lysates Ags (Fig. 6C). The activation of the OT-II cells was only moderately increased when the amount of lysate added to the DCs was increased by up to a 100-fold (data not shown). This suggests that, similar to the cross-presentation by class I, soluble Ags are also cross-presented inefficiently by class II on the DCs when compared with cell-associated Ags, such as those obtained from apoptotic cells or hybrid cells.

Induction of CTLs in mice injected with Ag-loaded DCs

Unimmunized mice, mice immunized with SIINFEKL peptide alone or with a control mixture of DCs and EG7

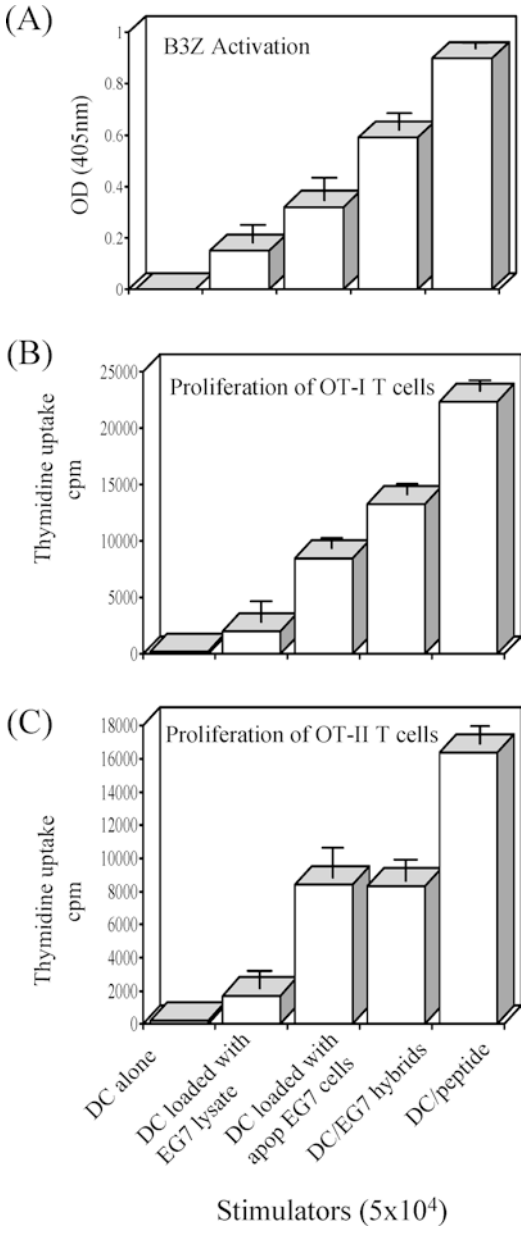

Fig. 6 **A** Activation of the B3Z T-cell hybridoma. **B** Proliferation of OT-I naïve T cells by DCs loaded with EG7-derived Ags, by various methods. DCs pulsed with SIINFEKL peptide served as positive control. The data shows that the methods used to load the DCs induce differential cross-presentation of OVA to the T cells; however, the fusion strategy gives the highest efficiency of T-cell activation, reflecting higher levels of H-2 K^b/SIINFEKL after loading. **C** Activation of naïve OT-II T cells by Ag-loaded DCs. The data show that the DC-EG7 hybrid cells and DCs cocultured with irradiated EG7 cells are much more efficient at cross-presentation of class II–OVA peptide and hence T-cell activation, than DCs pulsed with tumor lysate Ags

cells (i.e., no priming) gave no significant CTL responses against either EG7 or EL4 cells (Fig. 7). Mice immunized with irradiated tumor cells alone gave a very weak but similar CTL response against both EG7 and EL4, suggesting that there was no OVA-specific response. Mice immunized with DCs pulsed with the SIINFEKL peptide mounted a strong CTL response against EG7 cells, but as expected, not against the parental EL4 cells, showing that the response was OVA-specific, and that this particular OVA peptide, as shown by several others, is immunogenic.

In addition, all the other groups of mice immunized with the different Ag-primed DCs mounted CTL responses; however, the magnitude of the response was dependent on the Ag-loading strategy for the DCs. DCs loaded with lysate-derived Ags induced only a weak CTL response with equal potency against EG7 and EL4, thus the response was unlikely against OVA recognition. The DCs loaded with apoptotic EG7 cells induced efficient CTL responses which were both OVA-dependent and independent. The DC-EG7 hybrid cell vaccine induced the most potent CTL response and again this was both OVA dependent and independent.

Induction of in vivo OVA-specific T-cell responses by Ag-loaded DCs

The induction of an OVA-specific $CD8^+$ T-cell response in the immunized mice was analyzed by the use of tetrameric complexes, after 48-h coculture of the immunized lymphocytes with irradiated EG7 cells. H-2 K^b/SIINFEKL-specific T cells were only detected in mice vaccinated with DCs pulsed with SIINFEKL peptide, DCs loaded with apoptotic EG7 cells, and DC-EG7 hybrids (Fig. 8). The mice immunized with DCs loaded with EG7-derived lysate Ags did not show a significant OVA-specific T-cell response, as seen by the lack of $CD8^+$/$tetramer^+$ cells.

Analysis of TH1/TH2 cytokine responses induced in Ag-loaded DC vaccinated mice

Cytokine responses, analyzed by a CBA assay, were used to determine the effect of Ag-priming on the balance of TH1 and TH2 responses. Unimmunized naïve mice showed no cytokine response, and none of the immunized mice mounted a significant IL-4 response (Fig. 9 and Table 1). Mice which lacked a significant CTL response (i.e., those immunized with control mixture of DCs and EG7, tumor cells alone, or peptide alone) showed a high IL-5 response (TH2 cytokine) and a high-to-moderate TNF-α and IFN-γ (TH1) response, but no significant IL-2 (TH1) response (Fig. 9 and Table 1). Mice which gave good CTL responses (i.e., those immunized with DCs pulsed with either peptide, or apoptotic EG7 cells as well as DC-EG7 hybrids) showed a very low or no IL-5 response but a moderate-to-high IL-2 response and a moderate to very high IFN-γ and TNF-α response (Fig. 9 and Table 1). The mice which mounted weak CTL responses (i.e., those immunized with DCs pulsed with tumor lysates) showed a moderate IL-5 response, with a very weak IL-2, but a moderate IFN-γ and TNF-α, response (Fig. 9 and Table 1). Taken

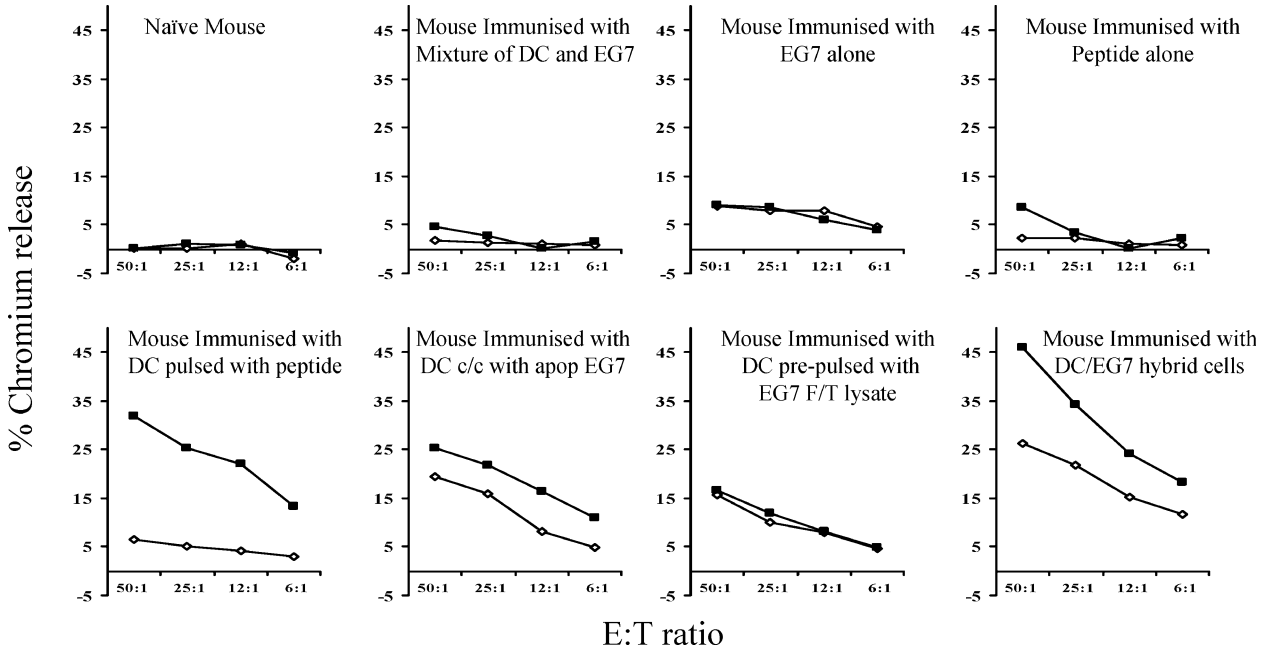

Fig. 7 Induction of CTL activity in mice immunized by different Ag-loaded DCs. After immunization, the lymphocytes were cocultured with irradiated EG7 cells and tested for CTL activity against EG7 (*solid squares*) and parental EL4 cells (*open diamonds*). Unimmunized mice, or mice injected with a control mixture of DCs and EG7, EG7 alone, or peptide alone, failed to give any significant CTL activity. Mice immunized with DCs pulsed with SIINFEKL peptide gave high levels of CTLs specific against the target cells that expressed that Ag, i.e., EG7 and not EL4. Mice immunized with the different Ag-loaded DCs showed a differential potency in the induction of CTLs, with the DCs pulsed with lysates showing the weakest response and the hybrid cells giving the strongest response. Additionally, the CTLs induced by the mice immunized with DCs cocultured with apoptotic cells or the hybrid cells also showed a higher CTL response against the EG7 cells compared with the EL4 cells, indicating that some of the CTLs were OVA specific

together, these data strongly suggest that the choice of strategy for Ag-loading of DCs can influence the overall TH1/TH2 cytokine balance and thus the polarization of a T-cell response. In addition, the data also suggest that IL-2 and IFN-γ are both necessary for CTL induction and that when the levels of IFN-γ are low to moderate, secretion of IL-5 can be detrimental to the induction of a successful CTL response.

Treatment of established EG7 tumors by immunization using Ag-loaded DCs

Ag-primed DCs were tested for their efficacy in the treatment of established tumors, induced by the subcutaneous injection of live EG7 cells. Untreated mice were culled by day 21, mice injected with control vaccine (DCs mixed with EG7 without loading) were all culled shortly afterward (day 28), whereas 4/8 mice vaccinated with DC-EG7 hybrid, 3/8 mice vaccinated with DCs cocultured with apoptotic EG7 cells, and 2/8 mice vaccinated with DCs pulsed with tumor lysates were tumor free until day 65 (Fig. 10A). The average size of tumors varied in the different groups of immunized mice, with control unimmunized mice showing the largest, fastest growing tumors, and mice immunized with DC-EG7

Table 1 Quantification of TH1/TH2 cytokines by cytometric bead array (CBA) assay on flow cytometry. Cytokines are expressed as pg/ml. Mice were immunized with DCs loaded with EG7-derived tumor antigens by four different strategies: DCs pulsed with the class I–restricted SIINFEKL peptide, DCs cocultured with irradiated apoptotic EG7 cells, DCs loaded with EG7-derived lysates antigens, and DC-EG7 hybrids. Naïve mice, mice immunized with a control mixture of DCs and EG7 cells, mice immunized with EG7 cells alone, or mice immunized with peptide alone served as "negative" controls. Lymphocytes from immunized mice were then cocultured with irradiated EG7 cells for 5 days. Supernatants from these cocultures were then used as cytokine sources for the CBA assay. Recombinant cytokines were used for standard curves used for the quantification of sample cytokines by software provided by BD Biosciences

Cytokine (levels in pg/ml)	Control mice	DCs mixed with control EG7 cells	EG7 alone	Peptide alone	DCs pulsed with peptide	DCs cocultured with irradiated apoptotic EG7	DCs loaded with lysates antigens	DC-EG7 hybrids
IL-2	2.2	10.7	2.8	6.0	138.7	146.6	26.7	36.1
IL-4	3.3	5.7	3.5	3.6	0.0	1.6	4.7	3.4
IL-5	3.7	530.2	28.7	81.6	3.1	2.1	20.3	29.9
IFN-γ	2.3	280.9	16.8	52.8	340.4	347.1	101.6	$>5{,}000$
TNF-α	5.6	186.9	91.5	117.4	28.7	30.5	136.6	533.9

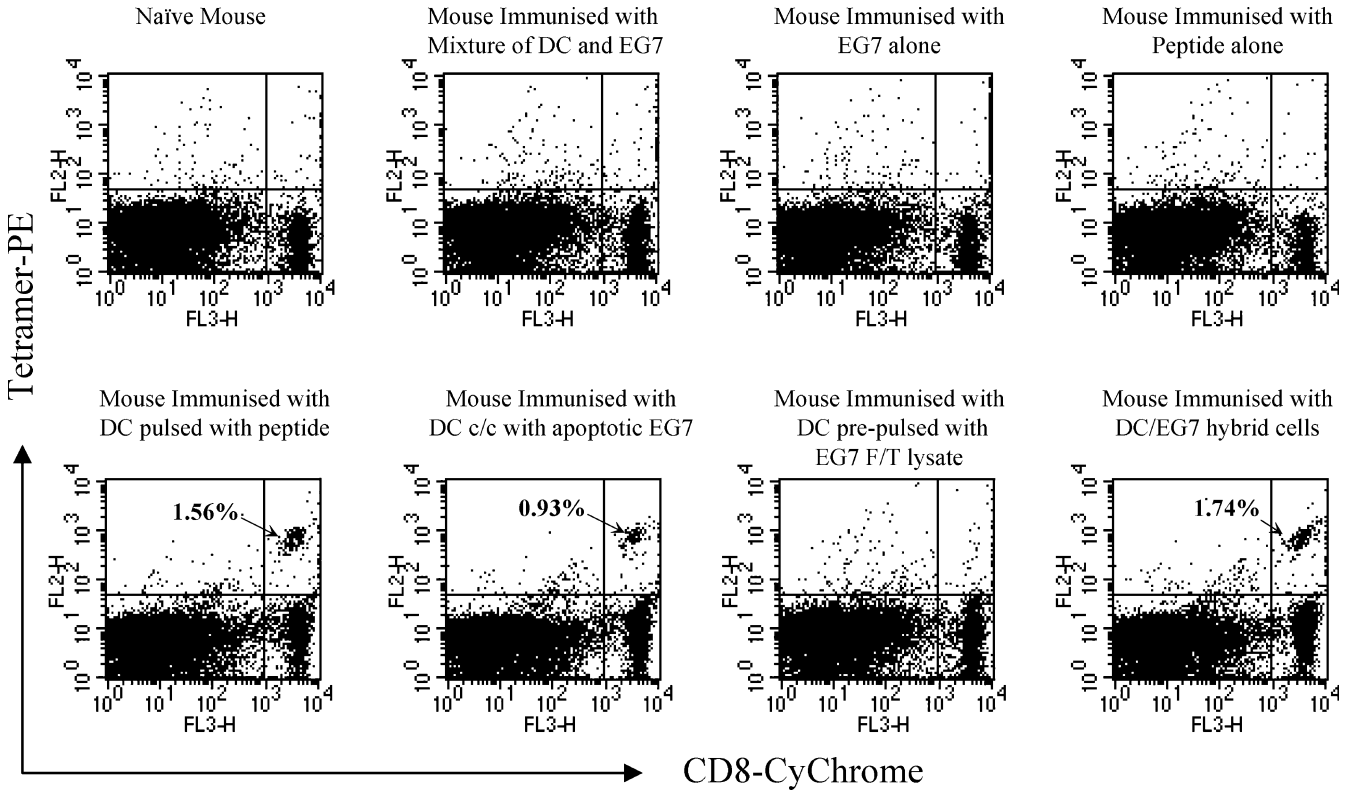

Fig. 8 FACS analysis of immunized mouse lymphocytes for the induction of Ag-specific T-cell response by the different Ag-loaded DCs. As shown in the figure, the only mice that induced an Ag-specific response (marked by tetramer staining of $CD8^+$ T cells) were the mice immunized with DCs pulsed with SIINFEKL peptide (used as a positive control), mice immunized with DCs cocultured with apoptotic cells and DC-EG7 hybrid cells. Values represent percentage number of total cells that stained positive for CD8 and H-2 K^b/SIINFEKL tetramers

hybrid tumors showing the least aggressive growth (Fig. 10B). In addition, in some of the mice, particularly those immunized with the DC-EG7 hybrids, some of the tumors initially started to grow but then subsequently regressed (Fig. 10C), indicating the induction of potent anti-EG7 responses by the Ag-loaded DCs.

Discussion

DCs primed with tumor Ags can stimulate an immunological as well as clinical response in selected patients [5, 34, 52, 57, 59, 64, 67, 74, 76, 77]. Despite such clinical efficacy, a significant proportion of patients with metastatic tumors remain unresponsive to immunomodulatory therapy, mandating improved understanding of DC function and Ag-priming as a means to enhance clinical efficacy. Multiple techniques for Ag-priming of DCs have been reported, as described above. However, it remains unclear as to which of these techniques would polarize the T cells for the most efficacious antitumor immunity. Both the mechanism of Ag presentation by DCs and the ability to stimulate antitumor immune T cells are most likely influenced by the Ag formulation [75]. The present study describes a model for the rapid assessment of different Ag-loading and priming of DCs in their ability to polarize T-cell responses, using OVA as a strong immunogen model Ag.

In a previous study, we have shown that different strategies for Ag-priming of DCs lead to different efficiencies of Ag-loading. For example during PEG-induced fusion only a small number of DCs become DC-tumor hybrids. Similarly during coculture of DCs with apoptotic tumor cells only a small number of DCs take up apoptotic tumor fragments [23]. In contrast, the present study shows that the majority of DCs become loaded when pulsed with peptide or soluble Ag, and additionally, do not seem to be limited by Ag uptake (Figs. 2 and 3). Therefore, in the present study, we first optimized the conditions for achieving the highest level of fusion efficiency and also the highest uptake of apoptotic cells by the DCs. We then used the highest tumor to DC ratios from these experiments and used this for the loading of DCs with tumor lysate Ags.

Results show that DCs loaded with EG7-derived Ags induce different T-cell responses depending on the Ag-loading strategy, as indicated by different potencies of CTL activity, and by differences in the profile of TH1 and TH2 cytokines in the immunized lymphocytes. Interestingly, the DCs which appeared to induce the most potent CTL activity—i.e., the DC-EG7 hybrids—also induced the strongest in vitro MHC class I OVA–T-cell response (B3Z assay and proliferation of OT-I T cells), the highest expansion of OVA-specific T cells (based on the number of Ag-specific T cells by tetramer staining [Fig. 8]), the strongest IFN-γ response,

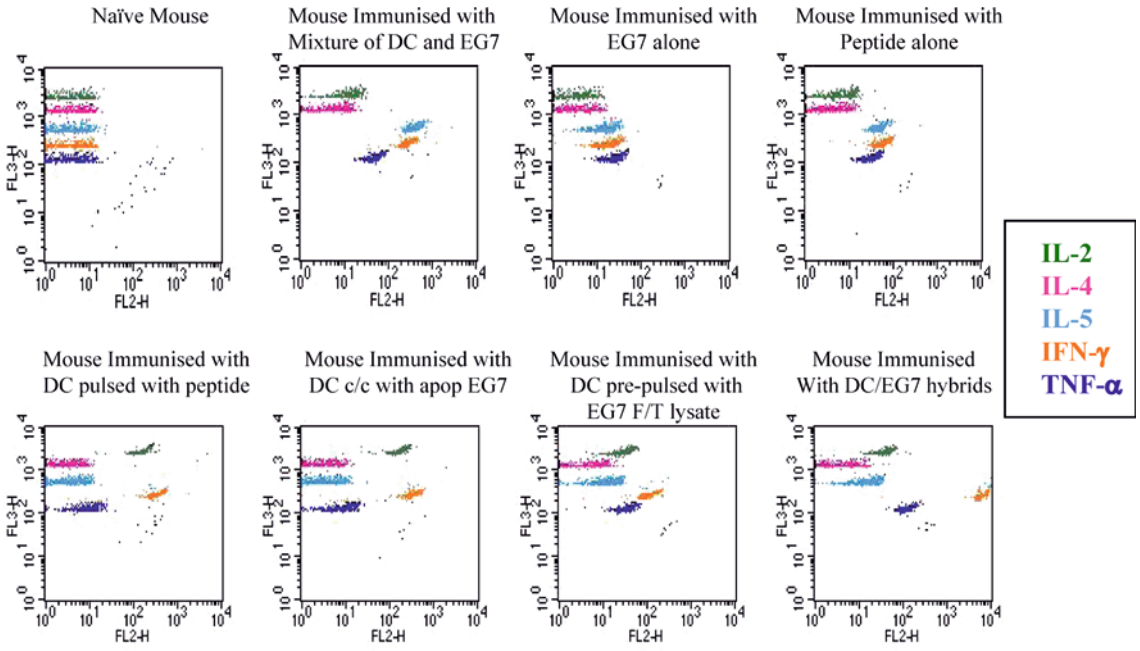

Fig. 9 Analysis of TH1/TH2 response by the CBA assay. This assay shows the relative amounts of the different cytokines secreted by the immunized lymphocytes after immunization by different Ag-loaded DCs. IL-4 was not detected during the assay; however, raised IL-5 levels (TH2 marker) and very low or absent IL-2 (TH1 marker) were present in the same mice that lacked CTL activity, i.e., mice immunized with control mixture of DCs and EG7, EG7 alone, or peptide alone. High IL-2, low or absent IL-5, and high IFN-γ were all present in the mice which gave significant CTL responses

and an overall TH1 cytokine response. In addition, the DC-EG7 hybrids were capable of inducing both class I and class II-OVA–specific responses, thus suggesting that in addition to class I presentation, hybrids are also capable of class II cross-presentation. The CBA data for the TH1/TH2 cytokines strongly suggest that different Ag-loaded DCs are capable of polarizing the T-cell response to a dominant TH1 or TH2 response, thus emphasizing the critical nature of the Ag-loading strategy to the vaccine design and the outcome of the clinical response.

The differential effect on T-cell responses by the different Ag-loaded DCs is unlikely to be due to differential effects on DC maturation, since the levels of B71, B72, CD40, class I, and class II on the DCs did not vary with the different Ag-priming strategies (data not shown). Furthermore, the data also suggest that the potency of CTL induction is not a function of the number of Ag-loaded DCs per se, especially since the number of DCs used for CTL induction was based on the total number of DCs and not on the percentage of DCs that become loaded with Ag. For example, from the albumin-FITC experiments we know that the large proportion of DCs (over 90%) become loaded with soluble Ag present in the lysates, but these DCs are poor stimulators of CTL activity. On the other hand, a much smaller number of DCs become loaded with apoptotic tumor cells, and an even smaller number of DCs are capable of becoming DC-tumor hybrids, but they do induce strong CTL responses. Thus, the potency of CTL induction appears to be related to the mechanisms by which the DCs acquire, process, and present Ags, with the ultimate outcome being dependent on the dose of Ag which is being cross-presented [43, 80].

The efficacy of DC-EG7 hybrids is likely to be related to the fact that the tumor cells supply ready-synthesised Ags in the context of appropriate MHC molecules and the DC provides all the requisite costimulatory molecules. In addition, the DC not only acquires ready-made MHC-Ag complexes already expressed on the surface of the tumor cell partner (as seen from the staining of the 25-D1.16 Ab on the hybrid cells), but any Ags expressed from the tumor partner will now function as endogenous Ags with full access to the classical pathway of MHC class I presentation. Therefore, the level of expression of the Ags and thus MHC class I–petide complexes in these hybrids is maintained at a high level. In fact, according to the B3Z assay, which is sensitive to the levels of H-2 K^b/SIINFEKL, the hybrids expressed the highest levels of H-2 K^b/OVA compared with the other Ag-loaded DCs. This study also shows activation of OT-II cells by the hybrid cells, thus suggesting that hybrids are capable of cross-presenting class II Ags. Thus DC-tumor hybrid cells are capable of simultaneous and efficient stimulation of both class I and class II T-cell responses.

The efficacy of DCs primed with irradiated "necrotic apoptotic" tumor cells is most likely due to efficient cross-priming of the Ags by the DCs. UV irradiation induces stress-induced death [24], a mechanism also thought to be important for DC maturation and enhancement of specific Ag presentation to CD8 cells [69, 75]. However, DCs cocultured with irradiated EG7 cells were not as potent in inducing antitumor responses

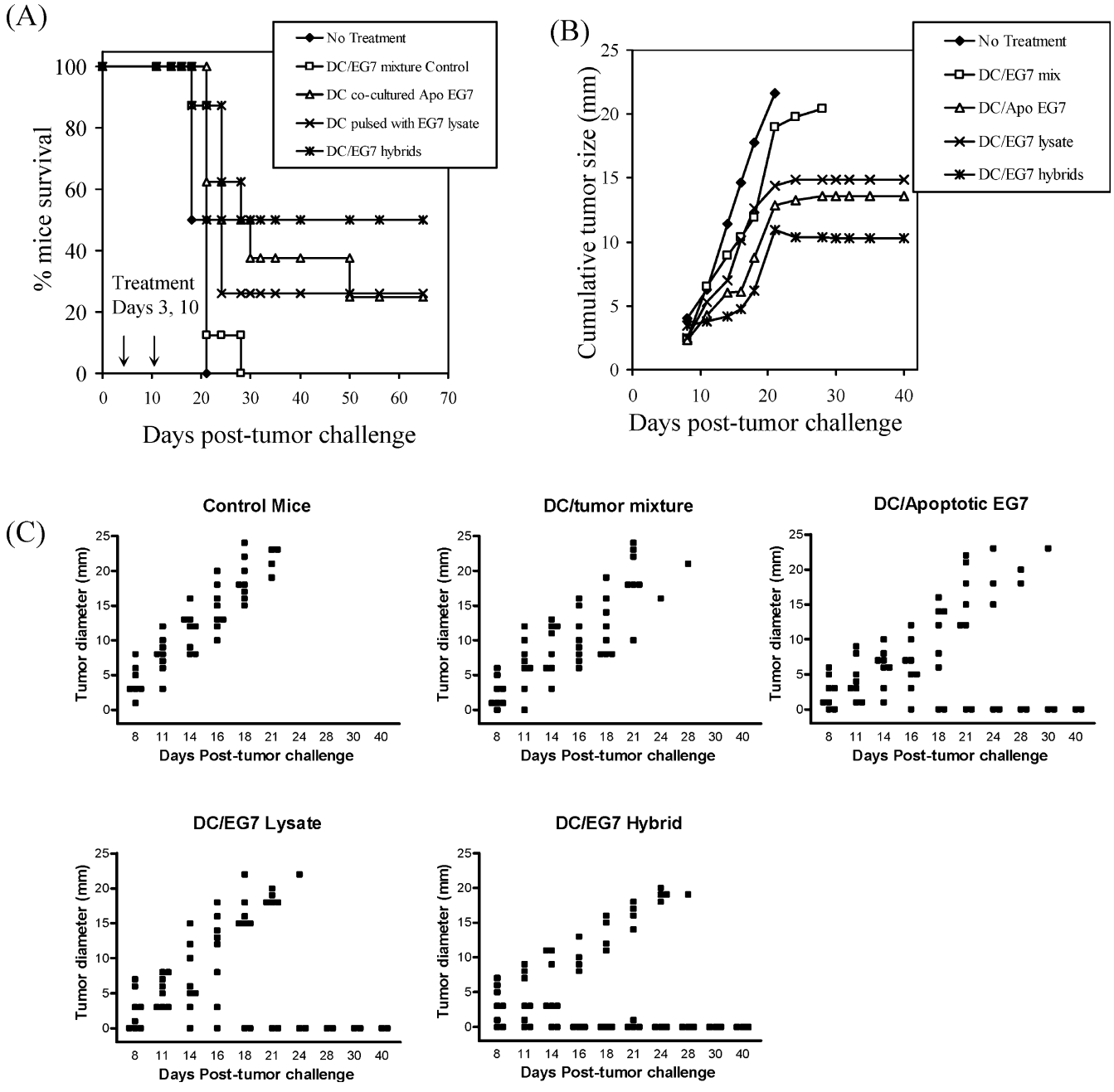

Fig. 10A–C Effect of Ag-loaded DCs on survival and treatment of EG7 tumors using eight mice/group. **A** Survival curve of mice treated with different Ag-loaded DCs after establishment of EG7 tumor. The DC-EG7 hybrid cells offered the highest protection in the immunized mice with 50% of the mice being long-term tumor free. The mice immunized with DCs pulsed with tumor lysates offered the least protection for mice survival. **B** The graph shows the relative size of the tumors in the different immunized mice. The mice treated with DC-EG7 hybrids showed the smallest tumors, control mice reached 23-mm sized tumors and were all culled by day 21. The data show that the different Ag-loaded DCs offer different degrees of protection to the mice. **C** Each mouse is plotted as an individual to emphasize that in some of the immunized mice (for example, the mice treated with the DC-EG7 hybrids) some of the mice never form any tumors, and some tumors that form also regress, indicative of a potent anti-EG7 immune response

as the DC-EG7 hybrids. This is probably related to the mechanism of uptake of exogenous Ags such as apoptotic cell fragments, which relies on phagocytosis. The data presented suggest that although coculture of apoptotic cell fragments provides an efficient method of loading Ags into DCs, it also limits the total amount of Ags that they can take up. Although the Ags from the irradiated EG7 cells appear to end up for processing on MHC class I as seen by the activation of B3Z and OT-I cells, a proportion of the exogenous Ags are also processed for MHC class II, as seen by the activation of OT-II cells, thus lowering the level of Ags available for MHC class I–restricted presentation. This was probably

reflected in the lower response by the B3Z and the OT-I cells when compared with the levels of T-cell activation by the DC-EG7 hybrid cells.

The inefficiency of tumor lysate–pulsed DCs to induce Ag-specific responses could be explained by the inefficient cross-presentation of soluble Ags by the DCs, and by a weak TH1 cytokine response. In a study documenting the quantitative analysis of cross-presentation, it was reported that cell-associated OVA is presented 500-fold more efficiently than soluble OVA to CD4 OT-II cells and 50,000-fold more efficiently to CD8 OT-I cells [47]. In the present study, DCs pulsed with EG7-derived lysate were very inefficient at both the stimulation of class I OVA T-cell responses, as seen by the inefficient stimulation of either B3Z or the OT-I cells, and the stimulation of class II OVA T-cell responses. The activation of B3Z cells was only marginally increased when the amount of lysate added to the DCs was increased a 100-fold, demonstrating the inefficiency of cross-presentation of soluble Ags. Additionally, this activation was never as efficient as the other loading strategies (data not shown), irrespective of the increased level of protein loading. We were also unable to detect any H-2 K^b / OVA peptide on the surface of the DCs after EG7-lysate uptake, irrespective of the DC to lysate ratio (data not shown). However, the fact that lysate-pulsed DCs were capable of stimulating the B3Z and the OT-I cells, suggests that some of the OVA Ag from the lysate is in fact cross-presented by MHC class I pathway of the DC; however, the level of H-2 K^b /OVA on the surface is too low based on lack of its detection by FACS, and probably too low for efficient CTL induction. The relative inefficiency of CTL stimulation by DCs pulsed with tumor lysates is in accord with other independent studies [19, 75], yet other reports have demonstrated that tumor lysate–priming of DCs can stimulate a tumor-specific T-cell response, both in a murine model [20] and in a human clinical trial for stage IV melanoma [59]. However, we wish to emphasize that the methods of lysate preparation and vaccination schedules in these studies were different from those employed in this study. It is possible that to enhance cross-presentation of exogenous soluble Ags by DCs, the Ags would need to be modified for uptake by phagocytosis, such as by linking to latex beads [42] or coating with immune complexes [9].

In conclusion, this study clearly shows that the strategy used for Ag-DC priming influences the outcome of the T-cell responses, with DC-tumor hybrids inducing the more potent responses. Although one could argue that this might only apply to strong immunogens such as OVA, our previous study also suggests the same outcome for weak self-Ags [23]. Thus, our data, together with several other independent studies, strongly suggest that DC-tumor hybrid vaccines are potent inducers of antitumor immune responses [1, 28—30, 35, 41, 46, 79]. DC hybrids offer several advantages over other Ag-bearing DCs. For example, the hybrid cells are likely to provide one of the best means of generating polyepitope vaccines, and thus polyvalent immune responses, by the expression of a much broader range of the TAA. Another major advantage for hybrid cell vaccination is the ease with which the hybrids can be generated. This is particularly relevant for cancers such as leukemia. Not only is it easy to obtain large numbers of blast cells from the patient but in addition there are no requirements for the mechanical or chemical disruption of tissue to obtain single cells as required for the generation of DC-hybrid cells for solid tumors. Ag-priming strategies that rely on a small number of defined peptides or single Ags leave too much to chance and run the risk of leaving out crucial antigenic components.

Acknowledgements We are grateful to Prof. Mike Kemeny, Department of Immunology, for use of the OT-I and OT-II transgenic mice, and to Dr Feyruz Rassool in the Department of Haematological Medicine for help with the use of the fluorescent microscope. We are also grateful to Dr J. Morrow from the Department of Immunology at St. Bartholomew's and the Royal London School of Medicine, London, UK, for the B3Z and EG7 cells and also useful discussions. Finally, we are indebted to Dr R. Germain from the Lymphocyte Biology Section, NIAID, NIH, USA, for his invaluable gift of the 25-D1.16 hybridoma.

References

1. Akasaki Y, Kikuchi T, Homma S, Abe T, Kofe D, Ohno T (2001) Antitumor effect of immunizations with fusions of dendritic and glioma cells in a mouse brain tumor model. J Immunother 24:106
2. Amoscato AA, Prenovitz DA, Lotze MT (1998) Rapid extracellular degradation of synthetic class I peptides by human dendritic cells. J Immunol 161:4023
3. Bakker ABH, Marland G, Deboer AJ, Huijbens RJF, Danen EHJ, Adema GJ, Figdor CG (1995) Generation of antimelanoma cytotoxic t-lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells in-vitro. Cancer Res 55:5330
4. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulandran B, Palucka K (2000) Immunobiology of Dendritic Cells. Annu Rev Immunol 18:767
5. Banchereau J, Palucka AK, Dhodapkar M, Burkeholder S, Taquet N, Rolland A, Taquet S, Coquery S, Wittkowski K, Bhardwaj N, Piniero L, Steinman R, Fay J (2001) Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res 61:6451
6. Barnden MJ, Allison J, Heath WR, Carbone FR (1998) Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol 76:34
7. Bennett SRM, Carbone FR, Karamalis F, Miller JF, Heath WR (1997) Induction of a CD8(+) cytotoxic T lymphocyte response by cross-priming requires cognate CD4(+) T cell help. J Exp Med 186:65
8. Berard F, Blanco P, Davoust J, Neidhart-Berard EM, Nouri-Shirazi M, Taquet N, Rimoldi D, Cerottini J, Banchereaux J, Palucka AK (2000) Cross-priming of naive CD8 T cells against melanoma antigens using dendritic cells loaded with killed allogeneic melanoma cells. J Exp Med 192:1535
9. Berlyn KA, Schultes B, Leveugle B, Noujaim AA, Alexander RB, Mann DL (2001) Generation of CD4(+) and CD8(+) T lymphocyte responses by dendritic cells armed with PSA/anti-PSA (antigen/antibody) complexes. Clin Immunol 101:276
10. Boczkowski D, Nair SK, Snyder D, Gilboa E (1996) Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J Exp Med 184:465

11. Brossart P, Heinrich KS, Stuhler G, Behnke L, Reichardt VL, Stevanovic S, Muhm A, Rammensee HG, Kanz L, Brugger W (1999) Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. Blood 93:4309
12. Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L, Brugger W (2000) Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. Blood 96:3102
13. Burdin N, Moingeon P (2001) Cancer vaccines based on dendritic cells loaded with tumor-associated antigens. Cell Biol Toxicol 17:67
14. Chevallier P, Spisek R, Morineau N, Milpied N, Avet-Loiseau H, Harousseau JL. Meflah K, Gregoire M (2001) Induction of leukemia-specific autologous CTLs by leukemia derived dendritic cells or dendritic cells pulsed with autologous killed leukemic blasts: potential implications for immunotherapy of acute myeloid leukemia. Blood 98:3013
15. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N (2001) Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med 193:233
16. Dhodapkar MV, Steinman RM, Sapp M, Desai H, Fossella C, Krasovsky J, Donahoe SM, Dunbar PR, Cerundolo V, Nixon DF, Bhardwaj N (1999) Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J Clin Invest 104:173
17. Dillon SR, Jameson SC, Fink PJ (1994) V-beta-5(+) T-cell receptors skew toward ova + H-2 k(b) recognition. J Immunol 152:1790
18. Eggert AAO, Schreurs MWJ, Boerman OC, Oyen WJC, de Boer AJ, Punt CJ, Figdor CG, Adema GJ (1999) Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. Cancer Res 59:3340
19. Ferlazzo G, Semino C, Spaggiari GM, Meta M, Mingari MC, Melioli G (2000) Dendritic cells efficiently cross-prime HLA class I-restricted cytolytic T lymphocytes when pulsed with both apoptotic and necrotic cells but not with soluble cell-derived lysates. Int Immunol 12:1741
20. Fields RC, Shimizu K, Mule JJ (1998) Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. Proc Natl Acad Sci U S A 95:9482
21. Fong L, Brockstedt D, Benike C, Wu L, Engleman EG (2001) Dendritic cells injected via different routes induce immunity in cancer patients. J Immunol 166:4254
22. Fong L, Engleman EG (2000) Dendritic cells in cancer immunotherapy. Annu Rev Immunol 18:245
23. Galea-Lauri J, Darling D, Mufti G, Harrison P, Farzaneh F (2002). Eliciting cytotoxic T lymphocytes against acute myeloid leukemia-derived antigens: evaluation of dendritic cell/leukemia cell hybrids and other antigen loading strategies for dendritic cell-based vaccination. Cancer Immunol Immunother 51:299
24. Galea-Lauri J, Richardson AJ, Latchman DS, Katz DR (1996) Increased heat shock protein 90 (hsp90) expression leads to increased apoptosis in the monoblastoid cell line U937 following induction with TNF-alpha and cycloheximide—a possible role in immunopathology. J Immunol 157:4109
25. Gallucci S, Lolkema M, Matzinger P (1999) Natural adjuvants: endogenous activators of dendritic cells. Nat Med 5:1249
26. Gilboa E, Nair SK, Boczkowski D (2000) Cancer immunotherapy with tumor RNA-transfected dendritic cells. Cancer Gene Ther 7:004
27. Girolomoni G, RicciardiCastagnoli P (1997) Dendritic cells hold promise for immunotherapy. Immunol Today 18:102
28. Gong JL, Chen DS, Kashiwaba M, Kufe D (1997) Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. Nat Med 3:558
29. Gong JL, Koido S, Chen DS, Tanaka Y, Huang L, Avigan D (2002) Immunization against murine multiple myeloma with fusions of dendritic and plasmacytoma cells is potentiated by interleukin 12. Blood 99:2512
30. Gong JL, Nikrui N, Chen DS, Koido S, Wu ZK, Tanaka Y, Cannistra S, Avigan D, Kufe D (2000) Fusions of human ovarian carcinoma cells with autologous or allogeneic dendritic cells induce antitumor immunity. J Immunol 165:1705
31. Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S (2002) Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol 20:621
32. Gunzer M, Janich S, Varga G, Grabbe S (2001) Dendritic cells and tumor immunity. Semin Immunol 13:291
33. Hoffmann TK, Meidenbauer N, Dworacki G, Kanaya H, Whiteside TL (2000) Generation of tumor specific T lymphocytes by cross-priming with human dendritic cells ingesting apoptotic tumor cells. Cancer Res 60:3542
34. Holtl L, Rieser C, Papesh C, Ramoner R, Herold M, Klocker H, Radmayr C, Stenzyl A, Bartsch G, Thurner M (1999) Cellular and humoral immune responses in patients with metastatic renal cell carcinoma after vaccination with antigen pulsed dendritic cells. J Urol 161:777
35. Homma S, Toda G, Gong JL, Kufe D, Ohno T (2001) Preventive antitumor activity against hepatocellular carcinoma (HCC) induced by immunization with fusions of dendritic cells and HCC cells in mice. J Gastroenterol 36:764
36. Irvine AS, Trinder PKE, Laughton DL, Ketteringham H, McDermott RH, Reid SC, Haines AM, Amir A, Husain R, Doshi R, Young LS, Mountain A (2000) Efficient nonviral transfection of dendritic cells and their use for in vivo immunization. Nat Biotechnol 18:1273
37. Jager E, Ringhoffer M, Karbach J, Arand M, Oesch F, Knuth A (1996) Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and CD8(+) cytotoxic-T-cell responses: evidence for immunoselection of antigen-loss variants in vivo. Int J Cancer 66:470
38. Jenne L, Schuler G, Steinkasserer A (2001) Viral vectors for dendritic cell-based immunotherapy. Trends Immunol 22:102
39. Jonuleit H, Giesecke-Tuettenberg A, Tuting T, Thurner-Schuler B, Stuge TB, Paragnik L, Kandemir A, Lee PP, Schuler G, Knop J, Enk JH (2001) A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. Int J Cancer 93:243
40. Karttunen J, Sanderson S, Shastri N (1992) Detection of rare antigen-presenting cells by the LacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. Proc Natl Acad Sci USA 89:6020
41. Kikuchi T, Akasaki Y, Irie M, Homma S, Abe T, Ohno T (2001) Results of a phase I clinical trial of vaccination of glioma patients with fusions of dendritic and glioma cells. Cancer Immunol Immunother 50:337
42. Kovacsovicsbankowski M, Clark K, Benacerraf B, Rock K (1993) Efficient major histocompatibility complex class-I presentation of exogenous antigen upon phagocytosis by macrophages. Proc Natl Acad Sci U S A 90:4942
43. Kurts C, Miller JFAP, Subramaniam RM, Carbone FR, Heath WR (1998) Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. J Exp Med 188:409
44. Labeur MS, Roters B, Pers B, Mehling A, Luger TA, Schwarz T, Grabbe S (1999) Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. J Immunol 162:168
45. Lappin MB, Weiss JM, Delattre V, Mai B, Dittmar H, Maier C, Manke K, Grabbe S, Martin S, Simon JC (1999) Analysis of mouse dendritic cell migration in vivo upon subcutaneous and intravenous injection. Immunology 98:181
46. Li J, Holmes LM, Franek KJ, Burgin KE, Wagner TE, Wei Y (2001) Purified hybrid cells from dendritic cell and tumor cell fusions are superior activators of antitumor immunity. Cancer Immunol Immunother 50:456
47. Li M, Davey GM, Sutherland RM, Kurts C, Lew AM, Hirst C, Carbone FM, Heath WR (2001) Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo. J Immunol 166:6099

48. Ludewig B, McCoy K, Pericin M, Ochsenbein AF, Dumrese T, Odermatt B, Toes REM, Melief CJ, Hengartner H, Zinkernagel RM (2001) Rapid peptide turnover and inefficient presentation of exogenous antigen critically limit the activation of self- reactive CTL by dendritic cells. J Immunol 166:3678
49. Lutz MB, Kukutsch N, Ogilvie ALJ, Rossner S, Koch F, Romani N, Schuler G (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 223:77
50. Lutz MB, Suri RM, Niimi M, Ogilvie ALJ, Kukutsch NA, Rossner S, Schuler G, Austyn JM (2000) Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. Eur J Immunol 30:1813
51. Luykx-de Bakker SA, de Gruijl TD, Scheper RJ, Wagstaff J, Pinedo HM (1999) Dendritic cells: a novel therapeutic modality. Ann Oncol 10:21
52. Mackensen A, Herbst B, Chen JL, Kohler G, Noppen C, Herr W, Spagnoli G, Cerundolo V, Lindemann A (2000) Phase I study in melanoma patients of a vaccine with peptide-pulsed dendritic cells generated in vitro from CD34(+) hematopoietic progenitor cells. Int J Cancer 86:385
53. Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, celluzzi C, Falo LD, Melief CJ, Ildstad ST, Kast WM, Deleo AB, Lotze MT (1995) Bone-marrow-derived dendritic cells pulsed with synthetic tumor peptides elicit protective and therapeutic antitumor immunity. Nat Med 1:1297
54. Minev BR, Chavez FL, Dudouet BM, Mitchell MS (2000) Synthetic insertion signal sequences enhance MHC class I presentation of a peptide from the melanoma antigen MART-1. Eur J Immunol 30:2115
55. Moore MW, Carbone FR, Bevan MJ (1988) Introduction of soluble-protein into the class-I pathway of antigen processing and presentation. Cell 54:777
56. Mule JJ (2000) Dendritic cells: at the clinical crossroads. J Clin Invest 105:707
57. Murphy GP, Tjoa BA, Simmons SJ, Ragde H, Rogers M, Elgamal A, Kenny GM, Troychak MJ, Salgaller ML, Boynton AL (1999) Phase II prostate cancer vaccine trial: report of a study involving 37 patients with disease recurrence following primary treatment. Prostate 39:54
58. Navabi H, Jasani B, Adams M, Evans AS, Mason M, Crosby T, Borysiewicz L (1997) Generation of in vitro autologous human cytotoxic T-cell response to E7 and HER-2/neu oncogene products using ex-vivo peptide loaded dendritic cells. Dendritic Cells in Fundamental and Clinical Immunology, vol 3. 417:583
59. Nestle FO, Alijagic S, Gilliet M, Sun YS, Grabbe S, Dummer R, Burg G, Schadendorf D (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 4:328
60. Nestle FO (2000) Dendritic cell vaccination for cancer therapy. Oncogene 19:6673
61. Nouri-Shirazi M, Banchereau J, Bell D, Burkeholder S, Kraus ET, Davoust J, Palucka KA (2000) Dendritic cells capture killed tumor cells and present their antigens to elicit tumor-specific immune responses. J Immunol 165:3797
62. Paczesny S, Beranger S, Salzmann JL, Klatzmann D, Colombo BM (2000) Protection of mice against leukemia after vaccination with dendritic cells loaded with apoptotic leukemia cells. Cancer Gene Ther 7:1402
63. Paglia P, Chiodoni C, Rodolfo M, Colombo MP (1996) Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. J Exp Med 183:317
64. Panelli MC, Wunderlich J, Jeffries J, Wang E, Mixon A, Rosenberg SA, Marincola FM (2000) Phase 1 study in patients with metastatic melanoma of immunization with dendritic cells presenting epitopes derived from the melanoma-associated antigens MART-1 and gp100. J Immunother 23:487
65. Porgador A, Snyder D, Gilboa E (1996) Induction of antitumor immunity using bone marrow-generated dendritic cells. J Immunol 156:2918
66. Porgador A, Yewdell JW, Deng YP, Bennink JR, Germain RN (1997) Localization, quantitation, and in situ detection of specific peptide MHC class I complexes using a monoclonal antibody. Immunity 6:715
67. Reichardt VL, Okada CY, Liso A, Benike CJ, Stockerl-Goldstein KE, Engleman EG, Blume KG, Levy R (1999) Idiotype vaccination using dendritic cells after autologous peripheral blood stem cell transplantation for multiple myeloma—a feasibility study. Blood 93:2411
68. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Einhom JH, White DE (1998) Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 4:321
69. Sauter B, Albert M, Francisco L, Bhardwaj N (1999) Consequences of cell death: exposure to necrotic but not apoptotic cells induces the maturation of immunostimulatory dendritic cells. J Invest Dermatol 113:035
70. Schreurs FWJ, Eggert AAO, Punt CJA, Figdor CG, Adema GJ (2000) Dendritic cell-based vaccines: from mouse models to clinical cancer immunotherapy. Crit Rev Oncogenesis 11:1
71. Schuler G, Steinman RM (1997) Dendritic cells as adjuvants for immune-mediated resistance to tumors. J Exp Med 186:1183
72. Shastri N, Gonzalez F (1993) Endogenous generation and presentation of the ovalbumin peptide/k(b) complex to T-cells. J Immunol 150:2724
73. Shu SY, Cohen P (2001) Tumor-dendritic cell fusion technology and immunotherapy strategies. J Immunother 24:99
74. Small EJ, Fratesi P, Reese DM, Strang G, Laus R, Peshwa MV, Valone FH (2000) Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells. J Clin Oncol 18:3894
75. Strome SE, Voss S, Wilcox R, Wakefield TL, Tamada K, Flies D, Chapoval A, Lu J, Kasperbauer JL, Padley D, Vile R, Gastineau D, Wettstein P, Chen LP (2002) Strategies for antigen loading of dendritic cells to enhance the antitumor immune response. Cancer Res 62:1884
76. Thurner B, Haendle I, Roder C, Dieckmann D, Keikavoussi P, Jonuleit H, Schuler G (1999) 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 190:1669
77. Timmerman JM, Czerwinski DK, Davis TA, Hsu FJ, Benike C, Hao ZM, Engleman EG, Levy R (2002) Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. Blood 99:1517
78. Timmerman JM, Levy R (1999) Dendritic cell vaccines for cancer immunotherapy. Annu Rev Med 50:507
79. Trefzer U, Weingart G, Chen YW, Herberth G, Adrian K, Winter H, Audring H, Guo YJ, Sterry W, Walden P (2000) Hybrid cell vaccination for cancer immune therapy: first clinical trial with metastatic melanoma. Int J Cancer 85:618
80. Watts C (1997) Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunol 15:821
81. Zitvogel L, Angevin E, Tursz T (2000) Dendritic cell-based immunotherapy of cancer. Ann Oncol 11:199