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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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ASUNTO	Radicación	09-121156
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	
	Folios	015

E- 17303

Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (fase nacional) ☒ Cap.I ☒ Cap. II ☐

No. Sol. Internacional PCT/IB2008/051385
 Pub. Internacional WO 2008/126039

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

<u>Descripción:</u>	Folios: 85 a 139	28/10/2009
<u>Reivindicaciones:</u>	Folios: 147 a 162	28/10/2009
<u>Figuras:</u>	Folios: 164 a 173	28/10/2009
<u>Prioridad:</u>	AR P20070101520	11/04/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Líneas celulares, composiciones para el tratamiento de melanomas que las comprenden, procedimiento para preparar las composiciones y métodos de tratamiento".

El objeto de la presente solicitud es proveer líneas celulares de melanoma humano para el tratamiento de enfermedades malignas.

El problema planteado en esta solicitud es la necesidad de obtener líneas celulares que induzcan actividad en células T y funcionen como inmunomoduladores.

Expediente 09-121156 1er Art.45

Para solucionar este problema técnico la solicitud en estudio suministra cuatro líneas celulares de melanoma humano usadas para el tratamiento de enfermedades malignas.

El objeto de la solicitud definida anteriormente se clasificó en la IPC: A61K 39/00; C12N 5/06.

4. Excepción a la patentabilidad (*Artículo 20 D486 literal d*)

El objeto de las reivindicaciones 59 a 77 es un método de tratamiento aplicado a pacientes humanos o animales. Sin embargo, de acuerdo con el artículo 20 literal d), esta materia no se considera patentable.

5. Reivindicaciones relativas a un uso o a un segundo uso (*Art 14 y 21 D 486*)

Las reivindicaciones 38 y 47 reclaman el uso de la composición obtenida, dichas reivindicaciones no son patentables, de acuerdo con el Art 14.

6. De la solicitud de Patente

Reivindicaciones (*artículo 30 D486*)

El capítulo reivindicatorio presentado carece de concisión y claridad por cuanto:

- 1) La línea celular reclamada en la reivindicación 4 puede ser de cuatro tipos de cultivos diferentes, denominados Mel-XY#. Sin embargo, la denominación de cada cultivo es particular del inventor y no permite establecer las características de cada uno de ellos. Se recomienda definir las líneas celulares de manera que sea evidente la diferencia entre cada una de ellas, indicando por ejemplo el tipo de célula, el porcentaje de células apoptóticas o necróticas, medio de cultivo, etc.;
- 2) Características como tipo de irradiación realizada, tipo de células, condición fisiológica de la célula son importantes para la definición de las mismas, así como para la definición del procedimiento de preparación de estas. Se recomienda incluir esta información en las reivindicaciones independientes;
- 3) La composición reclamada en la reivindicación 12 no se diferencia de la materia reclamada en la reivindicación 1 puesto que ambas poseen los mismos elementos caracterizantes. Se recomienda modificar esta reivindicación de manera que se indique cada uno de sus elementos definidos y se indique las proporciones de los mismos, de acuerdo con la materia sustentada en la descripción;
- 4) Existen numerosas reivindicaciones que reclaman la misma materia lo cual hace que el capítulo reivindicatorio carezca de concisión. Se recomienda dejar una única reivindicación independiente de cada categoría (reivindicaciones 12, 25, 40, 44 para composición, 39 y 43 para tipo de células, 48 y 54 para procedimiento, entre otras);

De esta manera, el capítulo reivindicatorio presentado no cumple con los requisitos establecidos en el art. 30 de la Decisión 486.

7. Determinación del Estado de la Técnica

Para determinar el estado de la técnica se ha tenido en cuenta la fecha de presentación de la solicitud prioritaria reivindicada con la solicitud: 11/04/2007.

Teniendo en cuenta la fecha indicada se realizó búsqueda en el estado de la técnica de documentos relacionados con la solicitud, encontrándose los siguientes documentos relevantes a la misma:

Nº	Documento	Título	Fecha de publicación
D1	Thumann P., et.al. Journal of Immunological Methods 277 (2003): 1 – 16	"Antigen loading of dendritic cells with whole tumor cell preparations"	2003
D2	Longhi P. et.al., Proc Amer Assoc Cancer Res, Volume 45, 2004	"Preclinical studies of a human vaccine based on dendritic cells loaded with apoptotic/necrotic melanoma cells"	2004
D3	Goldszmit et.al., J Immunol, 2003; 171:5940-5947;	"Dendritic Cells Charged with Apoptotic Tumor Cells Induce Long-Lived Protective CD4+ and CD8+ T cell immunity against B16 melanoma"	2003
D4	WO 02/053176	"An autologous anti-cancer vaccine"	11/07/2002

Resumen de los documentos citados:

D1: Vacunación basada en células dendríticas (DC) ha sido ampliamente usada para la inducción de inmunidad anti-tumoral en estudios clínicos. La carga de antígenos de DC con preparaciones de células tumorales es un método atractivo cuando el material celular tumoral esta disponible (resumen, Fl. 258).

D2: Las células dendríticas son potentes células presentadoras de antígenos capaces de tomar eficientemente, procesar y presentar antígenos a las células T ayudadoras para iniciar una respuesta inmune (resumen, Fl. 265).

D3: Las células dendríticas son potentes células presentadoras de antígenos y vectores atractivos para inmunoterapia en cáncer. Usando el melanoma B16, un tumor experimental pobremente inmunogénico que expresa bajos niveles de productos MHC clase I, se investiga si las DC cargadas ex-vivo con células apoptóticas podrían elicitar células T dependientes CD4+ and CD8+, e inmunidad a largo término siguiendo la aplicación de la composición vacunal en ratones (resumen, Fl. 266).

D4: La invención se relaciona con una composición farmacéutica para inducir una respuesta inmune dirigida contracélulas malignas en un sujeto mamífero. La composición comprende como ingrediente activo células presentadoras de antígenos autólogos (APC) las cuales fueron cargadas con una mezcla de al menos dos lisados de líneas celulares tumorales alogénicas (resumen).

<http://www.wipo.int/patentscope/search/en/search.jsf> ingresar WO2002053176.

8. Análisis de patentabilidad (artículo 14 D486)

Novedad (artículo 16 D486)

La reivindicación 39 reclama una composición para el tratamiento de melanomas humanos caracterizada porque comprende células dendríticas autólogas maduras, células dendríticas autólogas cargadas con células de al menos una línea celular de melanoma humano heterólogo, células apoptóticas de dicha al menos una línea celular de melanoma humano heterólogo y células necróticas de dicha al menos una línea celular de melanoma humano heterólogo.

La reivindicación 43 igualmente reclama una composición para el tratamiento adyuvante de melanomas humanos, caracterizada porque comprende células dendríticas maduras, células dendríticas cargadas con células de al menos una línea celular de melanoma humano heterólogo, células apoptóticas de una línea celular de melanoma humano heterólogo y células necróticas de una línea celular de melanoma humano heterólogo.

Tal como mencionó la autoridad de búsqueda internacional, los documentos D1 a D3 divulgan composiciones elaboradas con este tipo de células. El documento D1 divulga un protocolo para la carga de células dendríticas con preparaciones de células tumorales, tanto necróticas como apoptóticas o lisados tumorales, usando células de melanoma (Pág. 3 col. izq párrafo 1). Este documento usa la línea celular de melanoma MEL-526 (Pág. 3 col. izq párrafo 3). Tal como divulga D1, el materia necrótico tiene la capacidad de inducir la maduración de células dendríticas (Pág. 2 col. der párrafo 2) por lo que las células DC usadas también son maduras. Además se utilizaron células de melanoma apoptóticas las cuales fueron irradiadas con 1.0 J/cm^2 UV-B (Pág. 4 col izq, párr. 2) y células necróticas de melanoma que fueron sometidas a ciclos de calentamiento y enfriamiento (Pág. 4 col izq, párr. 3).

De esta manera, el documento D1 divulga todas las características de la invención reclamada en las reivindicaciones 39 y 43 de la solicitud.

El documento D2 divulga una composición usada para vacunación en sujetos que sufren de melanoma. Se usa una composición con células dendríticas, se utiliza una mezcla de células de melanoma apoptóticas y necróticas (resumen). Se identifica el efecto de las células necróticas en la maduración de las DC y el efecto de las células apoptóticas en la activación de células T.

De esta manera, el documento D2 divulga todas las características de la invención reclamada en las reivindicaciones 39 y 43 de la solicitud.

El documento D3 divulga una composición que comprende células dendríticas cargadas con células de melanoma de la línea B16-F1 (Pág. 5940 col der último párr), células en apoptosis que fueron irradiadas y células necróticas que habían sido sometidas a ciclos de calentamiento/enfriamiento (Pág. 5941 col izq).

De esta manera, el documento D2 divulga todas las características de la invención reclamada en las reivindicaciones 39 y 43 de la solicitud.

Nivel inventivo (Artículo 18 D486)

La reivindicación 1 reclama una línea celular de melanoma humano para el tratamiento de enfermedades malignas caracterizada porque dicha línea celular es seleccionada del grupo comprendido por a) Mel-XY1, b) Mel-XY2, c) Mel-XY3, d) Mel-XX4 y e) sub-poblaciones de las mismas;

Las reivindicación 12 reclama una composición para el tratamiento del melanoma, caracterizada porque comprende al menos una línea celular de melanoma alogénica seleccionada del grupo comprendido a) Mel-XY1, b) Mel-XY2, c) Mel-XY3, d) Mel-XX4 y combinaciones de las mismas, en donde dichas líneas celulares son incapaces de proliferar. La reivindicación 25 es similar y la reivindicación 48 reclama el procedimiento para la preparación de la composición.

En reivindicaciones dependientes se indica el carácter de células apoptóticas y necróticas de las células de melanoma, las proporciones de estas y la forma de inactivación de las mismas.

Tal como se manifestó arriba los documentos D1 a D3 divulgan también líneas celulares de melanoma, de acuerdo con los apartes ya citados. El documento D1 por ejemplo indica en la forma de generación de apoptosis de las células el uso de irradiación con 1.0 J/cm² UV-B (Pág. 4 col izq, párr. 2) y células necróticas de melanoma que fueron sometidas a ciclos de calentamiento y enfriamiento (Pág. 4 col izq, párr. 3). Igualmente, indica que la composición tiene un porcentaje de células apoptóticas/necróticas y células dendríticas 1:1 (Pág. 3 col izq párr.2).

La diferencia entre la materia reclamada en la solicitud y D1 puede estar dada por la línea celular de melanoma específica empleada en la solicitud.

El efecto que produce esta diferencia no resulta aparente puesto que ambos documentos indican que se tratan de líneas celulares de melanoma con células apoptóticas y necróticas.

El problema técnico objetivo que resuelve la solicitud es proveer líneas celulares de melanoma alternativas.

Sin embargo, las modificaciones realizadas en la solicitud tales como el porcentaje de células apoptóticas/necróticas, corresponde a las modificaciones que puede realizar la persona del oficio en sus pruebas experimentales de cultivo celular y formulación de composiciones. Igualmente, las líneas celulares obtenidas por la solicitud no parecen tener un efecto sorprendente sobre la respuesta inmune generada por la composición.

De esta manera, la materia reclamada en la solicitud resulta obvia frente a la divulgación hecha en D1 e incumple el artículo 18 de la D486.

De manera similar a la expuesta para el documento D1, las divulgaciones realizadas en D2 ó D3 daban a la persona del oficio la información sobre la composición de células dendríticas y células de melanoma que podían ser usadas como composición vacunal.

El documento D4 por su parte, también divulgaba composiciones de células dendríticas que incluye el uso de células autólogas para mejorar la respuesta inmune generada por la composición (resumen). Este documento también revelaba el uso de células de melanoma en estado apoptótico (Pág. 13 párrafo 1). Igualmente, las células dendríticas maduras incluyen los fenotipos CD14+ y CD83 (Pág. 39).

Dado que las reivindicaciones adicionales 2 a 77 no aportan características esenciales diferenciables de los aportes del estado de la técnica, dichas reivindicaciones no se consideran inventivas.

De esta manera, a partir de la información divulgada en D2 y D4 ó D3 y D4, la persona del oficio podía llevar a cabo la invención reclamada en la solicitud, por lo que esta carece de nivel inventivo e incumple el artículo 18 de la D486.

9. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Thumann P., <i>et al.</i> Journal of Immunological Methods 277 (2003): 1 – 16	39, 43 1-77	Novedad Nivel inventivo
D2	Longhi P. <i>et al.</i> , Proc Amer Assoc Cancer Res, Volume 45, 2004	39, 43 1-77	Novedad Nivel inventivo

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D3	Goldszmit et.al., J Immunol, 2003; 171:5940-5947;	39, 43 1-77	Novedad Nivel inventivo
D4	WO 02/053176	1-77	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de 60 días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.



JOSE LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 02 OCT. 2012

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Antigen loading of dendritic cells with whole tumor cell preparations

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Abstract

Dendritic cells (DC) based vaccinations have been widely used for the induction of anti-tumoral immunity in clinical studies. Antigen loading of DC with whole tumor cell preparations is an attractive method whenever tumor cell material is available. In order to determine parameters for the loading procedure, we performed dose finding and timing experiments. We found that apoptotic and necrotic melanoma cells up to a ratio of one-to-one, equivalent to 1 mg/ml protein per 1×10^6 DC, can be added to monocyte derived DC without effecting DC recovery extensively. Using the isolated protein content of tumor cells (lysate) as a parameter, up to 5 mg/ml protein per 1×10^6 DC can be added. To achieve significant protein uptake at least 1 mg/ml of protein have to be added for more than 24 h as tested with FITC-labelled ovalbumin. Maturation inducing cytokines can be added simultaneously with the tumor cell preparations to immature DC without affecting the uptake. Furthermore, we tested the feasibility of cryopreservation of loaded and matured DC to facilitate the generation of ready to use aliquots. DC were cryopreserved in a mix of human serum albumin, DMSO and 5% glucose. After thawing, surface expression of molecules indicating the mature status (CD83, costimulatory and MHC molecules), was found to be unaltered. Furthermore, cryopreserved DC kept the capability to stimulate allogenic T-cell proliferation in mixed leukocyte reactions at full level. Loaded and matured DC pulsed with influenza matrix peptide (IMP) retained the capacity to induce the generation of IMP-specific cytotoxic T-lymphocytes after cryopreservation as measured by ELISPOT and tetramer staining. The expression of the chemokine receptor CXCR-4 and CCR-7 remained unaltered during cryopreservation and the migratory responsiveness towards MIP-3 β was unaltered as measured in a migration assay. Thus we conclude that the large scale loading and maturation of DC with whole tumor cell preparations can be performed in a single session. These data will facilitate the clinical application of DC loaded with whole tumor cell preparations.

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Keywords: Dendritic cells; Immunotherapy; Tumor-associated antigens; Cryopreservation; Melanoma; Antigen loading

Abbreviations: DC, dendritic cells; TAA, tumor-associated antigens.

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1. Introduction

Dendritic cells (DC) constitute a specialized system of antigen presenting cells (APC) that are initiators and modulators of immune responses against microbial, tumoral and self antigens (Steinman,

1991). Their unique capacity to induce and boost immunity makes them an attractive tool for immunotherapy, particularly for the induction of anti-tumoral immunities (Steinman and Dhodapkar, 2001; Schuler and Steinman, 1997). Methods that allow for the large scale *ex vivo* generation of DC from peripheral blood monocytes (Thurner et al., 1999) largely facilitate the introduction of DC in clinical vaccination trials. *Ex vivo* manipulation has certain advantages: it allows for the control of DC quality (i.e. maturation status, DC subset) and expression level of desired antigens. Furthermore, injection of the prepared DC can be performed at anatomical sites of interest (i.e. lymph nodes or tumors). Numerous DC based immunotherapeutic trials with *ex vivo* generated DC, aiming for the initiation or amelioration of an anti-tumoral T-cell immunity, have been performed for a wide range of tumors (recently reviewed in (Fong and Engleman 2000; Banchereau et al., 2001; Jenne and Bhardwaj, 2001). In some of these trials clinical responses were reported and in a few selected ones the induction or enhancement of tumor specific T-cells ("proof of concept") has been demonstrated. The antigen loading methods applied in most trials to achieve the MHC-restricted presentation of tumoral antigen were either peptide pulsing, involving immunodominant sequences of defined tumor associated antigens (TAA), or different whole tumor cell preparations. The synthesis of large quantities of clinical grade 8–10 amino acid long peptides that fit into the MHC class-I groove is technically rather easy and peptide pulsing of DC populations is thus an elegant way to achieve the desired TAA presentation. It has been shown that peptide pulsed DC expand peptide specific CTL in healthy subjects (Dhodapkar et al., 1999) and melanoma patients (Schuler-Thurner et al., 2000). However, there are certain caveats with this approach. The longevity of MHC-peptide complexes *in vivo* is unknown, the affinity of peptides for their various HLA molecules varies, competition between peptides may affect immunogenicity, MHC class II restricted epitopes for activation of CD4⁺ T cells are still scarce and the approach is inherently tailored for individuals as it is dependent upon the HLA type.

In contrast to peptide pulsing, using whole tumor cell preparations for DC loading avoids the need for detailed tumor analysis and individual HLA-typing, as it is assumed that tumoral antigens, including as yet

undefined TAA and rare mutations, will be presented on MHC class-I and -II molecules by autologous DC. The latter argument is of special importance as in principle it is desirable to aim for the parallel presentation of HLA class I and II restricted antigens, as the absence of CD4⁺ helper cells affects the generation of long term CD8⁺ T-cell memory (Zajac et al., 1998) and CD4⁺ helper T-cells are considered important for effective anti-tumor immune responses (Toes et al., 1999).

The disadvantage of using whole tumor cell preparations includes the difficult validation of such a vaccine, the potential capacity for the induction of autoimmunity via the presentation of non-tumor-antigens (Gilboa, 2001) and the necessity to obtain a sufficient number of autologous tumor cells by invasive procedures. Furthermore, tumor metastases may have a different antigen profile than the one expressed by primary tumor cells or the cells obtained for antigen loading. The preparations used for antigen loading are usually mechanically or thermally disrupted and thus necrotic tumor cells. Necrotic tumor cell material has the capacity to induce DC maturation when given to immature DC (Sauter et al., 2000), but this is variable so that the induction of further maturation of DC prior to clinical use is desirable. This is probably critical in order to avoid a "semi-mature" maturation status of the antigen loaded DC which is associated with a tolerogenic antigen presentation (Lutz and Schuler, 2002; Jonuleit et al., 2001; Dhodapkar et al., 2001). Clinical trials have already been performed using DC loaded with tumor cell lysates (Nestle et al., 1998; Thurner et al., 1998; Geiger et al., 2000). However, little is known about the efficacy of antigen loading and the antigen concentrations required to achieve antigen presentation using such loading techniques and usually no preclinical optimization has been reported. Soluble antigen, such as tumor derived protein, is taken up by macro-pinocytosis and processed into the class II pathway if maturation is induced. However, uptake of cell associated antigen appears to result in far more efficient cross-presentation (Li et al., 2001). For tumor antigens it has been shown that cross-presentation of melanoma derived TAA is less effective for single TAA than peptide pulsing, but the overall efficiency of killing tumor cells is better with cross-primed CTL (Jenne et al., 2000).

We have worked out here a protocol for the loading of DC with tumor cell preparations, either necrotic, apoptotic or tumoral lysate, by use of a melanoma cell line. We then probed the feasibility to cryopreserve DC loaded by these protocols and matured by maturation inducing cytokines and PGE₂. The cryopreservation of aliquots of “ready to use” loaded and matured DC is of special interest as it circumvents the need for repetitive preparations of the DC vaccine for an individual patient.

We found that loading of DC up to a ratio of 1:1 followed by cryopreservation using our recently developed protocol (Feuerstein et al., 2000) is possible without loss of function. This protocol will facilitate the use of DC loaded with whole tumor cell preparations in DC-based immunotherapy trials.

2. Materials and methods

2.1. Cell lines and culture media

A melanoma cell line, MEL-526 (HLA: A2, A3, B50, B62) was kindly provided by Dr. M. T. Lotze, University of Pittsburgh, USA. This cell line expresses MelanA/MART1, tyrosinase, MAGE-3 and gp-100 (Tuting et al., 1998). It was cultured in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine (Bio Whittaker), penicillin–streptomycin mixture with 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco-Invitrogen, Karlsruhe, Germany) and 10% heat-inactivated fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). Cells were sub-cultured every 3 days after treatment with trypsin-EDTA (Sigma, St. Louis, MO, USA).

2.2. Antibodies and reagents

The following monoclonal antibodies (mAb) were used. FITC-labeled anti-human CD86 (BU63) from Cymbus (Chandlers Ford, Great Britain), HLA-DR (L243) and HLA-ABC (G46-2.6) from BD Pharmingen (Hamburg, Germany), PE-conjugated murine CD80 (L307.4) and CXCR-4 (12G5) from BD Pharmingen, CD83 (Hb15a) mAb from Immunotech (Marseille, France). A rat anti-human CCR-7 Ab was kindly provided by Reinhold Förster and Markus Lipp from the Department of Molecular Tumor Genetics

and Immunogenetics, Max-Delbrück-Center for Molecular Medicine (MDC), Berlin, Germany. For this Ab a FITC-labeled rabbit anti-rat IgG (Jackson Immuno Research, Westgrove, USA) acted as secondary Antibody. We used purified murine control IgG1-FITC, IgG2b-PE and IgG2a from Cymbus as well as rat IgG2a (R35-95) from Jackson Immuno Research.

2.3. Flow cytometric analysis

Cultured cells were washed, suspended at 3×10^5 in 50 µl of cold facs solution (DPBS, Bio Whittaker) containing 0.1% sodium azide (Sigma) and 10 mg/ml human serum albumin (HAS) and incubated with labeled mAb or appropriate isotypic controls for 30 min. Cells were then washed twice and resuspended in 300 µl of cold FACS solution. Stained cells were analyzed for two-color immunofluorescence with a FACStar cell analyzer (Becton-Dickinson, Mountain View, CA). Cell debris was eliminated from the analysis using a gate on forward and side scatter. A minimum of 10^4 cells was analyzed for each sample. Results were processed using Cellquest software (Becton-Dickinson).

2.4. DC generation from buffy coats and leukaphereses

Leukaphereses of healthy donors were obtained according to institutional guidelines. Peripheral blood mononuclear cells (PBMC) were prepared by density centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). PBMC were resuspended (50×10^6 cells/well) and brought to cell factories (Nunc, Roskilde, Denmark). Cells were incubated at 37 °C to allow for adherence. After 1 h, non-adherent cells were removed and remaining cells were fed with RPMI 1640 medium (Bio-Whittaker, Walkersville, MD, USA) containing 1% of heat-inactivated autologous plasma, 10^3 IU GM-CSF/ml, and 10^3 IU IL-4/ml (Novartis Pharma, Nuremberg, Germany). Cells were substituted with 10% of fresh medium containing 10^3 IU GM-CSF and 10^3 IU IL-4 per ml on days 2 and 4. On day 5, DC maturation was induced with a cocktail of cytokines as published (Jonuleit et al., 1997). At the same time cells were loaded with tumor cell material as described below. The following cytokines were added: IL-4, 1000 U/ml; IL-1β, 10 ng/ml; IL-6,

1000 U/ml (all from Strathmann, Hamburg, Germany), GM-CSF, 1000 U/ml (Leukomax®, Novartis, Basel, Switzerland, PGE₂, 1 µg/ml (Minprostin®, Pharmacia & Upjohn); TNF-α, 10 ng/ml (Bender, Vienna, Austria). Cells were harvested after 2 days and used for experiments.

2.5. Induction of apoptosis in MEL-526 cells

To induce apoptosis, MEL-526 cells were irradiated with 1.0 J/cm² UV-B (UV 3003 K, Waldmann Medizintechnik, Villingen-Schwenningen, Germany). After irradiation, MEL-526 cells were kept for 8 h in culture to allow apoptosis to occur. Apoptosis was measured using an annexin-V kit (Pharmingen). The UV-B dose necessary to induce apoptosis in more than 60% of the melanoma cells 8 h after irradiation was tested to be 0.5 J/cm².

2.6. Generation of necrotic tumor material and tumor lysate of MEL 526 cells

MEL 526 were detached by using trypsin-EDTA (Gibco-BRL) and resuspended in RPMI 1640 (Bio-Whittaker) at a concentration of 10⁷ cells per ml. Cells were subsequently treated with five cycles of heating and freezing stored in a 15-ml centrifuge tube (Nunc): one cycle consist of 10 min in a water quench at 42 °C and successional 90 s in liquid nitrogen. After this procedure we used an Ultrasonic Homogenizer (Sonicifier 250 from Branson, Danbury, CT, USA) to dissolve remaining clots of cell components. The now attained suspension was put through a syringe-driven 0.22 µm filter (Millex-GV from Millipore, Bedford, USA) and used as necrotic cell material; to receive tumor lysate further work steps were performed as recently described (Herr et al., 2000). Briefly, the necrotic cell material was centrifuged for 15 min at 15,000 × g and then centrifuged in Centriprep centrifugal filter units (Centriprep YM-10 from Millipore, Bedford, USA) to attain the protein-fraction greater than 10 kDa.

2.7. Determination of FITC-ovalbumin uptake

In order to measure the efficacy of protein uptake by immature DC we added fluorescein (FITC) conjugated ovalbumin (FITC-OVA, Molecular Probes,

Leiden, the Netherlands) to DC cultures and performed time courses of FITC-OVA. To measure the protein content in the extracellular fluid we took samples at different time points, centrifuged gently to remove cells and stored it at –20 °C prior to analysis. After all supernatants had been collected we transferred them to a 96-well ELISA-Plate (Nunc) and FITC-OVA content was quantified by fluometry. Fluorescence was measured with an excitation wavelength of 490 nm and an emission wavelength of 535 nm using a Victor 1420 plate reader (PerkinElmer, Rodgau-Jügesheim, Germany).

To calculate the uptake of FITC-OVA by DC we analysed the cells at comparable time points and performed FACS analysis. Semi-quantitatively the mean fluorescence intensity (MFI) was determined.

2.8. Cryopreservation

Cryopreservation of loaded and matured DC was performed as recently described (Feuerstein et al., 2000). In short, cells were taken up in 20% human serum albumin (Pharmacia & Upjohn) at a concentration of 20 × 10⁶ cells/ml, transferred to 1.8 ml cryotubes (Nunc) and stored for 10 min on ice. Afterwards an equal volume of cryopreservation medium was added to the cell suspension. This medium consists of 55% human serum albumin 20% (Pharmacia & Upjohn), 20% dimethyl sulfoxide (Sigma) and 25% glucose 40% (Glucosteril™, Fresenius, Bad Homburg). Cells were then frozen at –1 °C/min in a cryo freezing container (Nalgene cryo 1 °C freezing container, Nalgene, Roskilde, Denmark) down to –80 °C. Cells were kept frozen for approximately 90 min. Thawing was also performed as described recently (Feuerstein et al., 2000). Briefly, cryotubes were taken out of the fridge and thawed in a water bath with 37 °C till detachment of the cells was visible and added to a cell culture dish containing prewarmed RPMI medium supplemented with 1% autologous plasma, 1000 U/IL-4/ml and 1000 U/GM-CSF/ml. Cells were kept in the incubator for 1–2 h prior to further analysis.

2.9. Mixed leukocyte reaction

Tests were performed in 96 well round bottom plates, as medium we used the same mixture that

served for T-cell culture: RPMI 1640 (Bio Whittaker) supplemented with 2 mM L-glutamine (Bio Whittaker), penicillin–streptomycin mixture with 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco-BRL) and 5% heat-inactivated human serum (self-processed, pooled human plasma from co-workers of our laboratory).

T-cell enriched fractions were obtained as 1 h non-adherent fraction of PBMC prepared from buffy-coats or leukaphereses.

T-cells were brought to a concentration of 2×10^6 cells/ml, DC were used at 2×10^5 cells/ml. A ratio of 30:1 of T-cells to DC and dilution up to 1000:1 was performed, whereas triplicates were carried out. T-cells without DC served as control.

Cells were pulsed after 4 days of incubation at 37 °C with 1 µCi/well of ^3H -TdR (Amersham, Buckinghamshire, England), incubated for further 12 h and frozen at –20 °C until analysis with a liquid scintillation counter (Wallac 1450 Mikrobeta plus, PerkinElmer Wallac, Freiburg, Germany).

2.10. Pulsing with influenza matrix peptide

To demonstrate the capacity of cryopreserved DC to efficiently induce the generation of specific CTLs, we pulsed loaded and matured DC with influenza matrix peptide (IMP) (Clnalfa, No. C:S-029, Läufelfingen, Switzerland) prior to cryopreservation. We used a concentration of 20 µg/ml to pulse 1×10^6 DC/ml for a period of 2 h. DC were washed after these 2 h in order to remove unbound peptide. Directly after the pulsing DC were cryopreserved or remained in the dish as control.

2.11. Tetramer analysis and ELISPOT assays

Soluble IMP HLA A2.1 tetramers were prepared and binding to T cells was analysed by flow cytometry at 37 °C as described (Whelan et al., 1999). In short, 1 µl of tetramer (concentration 0.5–1 mg/ml) was added to 2×10^6 cells in about 60 µl (volume remaining in the tube after spinning and dropping off the supernatant) medium, consisting of RPMI 1640, supplemented with gentamicin, glutamine and 5% allogenic heat-inactivated human serum (pool serum) for 15 min at 37 °C. Cells were then cooled (without washing) and incubated for 15 min on ice with tri-

color conjugated mAb to human CD8 (Caltag Laboratories, Burlingame, CA). After three washing cycles, cells were analysed on a FACScan (Becton Dickinson).

The ELISPOT assay was used as described to quantitate antigen-specific IFN γ release of effector T-cells. 1×10^5 /well CD8 $^+$ T-cells or 2×10^4 /well were added in triplicates to nitrocellulose-bottomed 96 well plates (MAHA S4510) pre-coated with the primary anti-IFN γ mAb (1-D1K, Mabtech, Stockholm) in 50 µl ELISPOT medium (RPMI1640, 5% heat-inactivated human serum) per well. After addition of influenza matrix peptide-pulsed DC and incubation for 20 h, wells were washed six times, incubated with biotinylated second mAb to IFN γ (7-B6-1, Mabtech) for 2 h, washed and stained with Vectastain Elite kit (Vector Laboratories, Burlingame, CA, USA). Spots were evaluated and counted using a special computer assisted video imaging analysis system (Carl-Zeiss Microscopie, Göttingen, Germany).

2.12. Migration assays

In order to test the migratory properties of cryopreserved and loaded DC migration assays were performed using a chemotaxis chamber designed for 96 well plates (Chemo TX system MBA96 from Neuro Probe, Gaithersburg, MD, USA). A total of 405 µl of chemokine containing solution or negative control (RPMI 1640) were placed in the lower wells, on which we adjusted a polycarbonate filter (5 µm pore size, Neuro Probe). As chemokine we used MIP-3 β (PeproTech, London, UK) in a concentration of 50 ng/ml. After closing the lid of the Chemo TX system, we loaded 100 µl of a cell suspension containing 0.33×10^6 DC/ml into the upper wells over the filter. The complete chamber was kept at 37 °C in the incubator for 90 min. Thereafter the cell suspensions in the upper wells were removed by suction before removing the filter. Cells that migrated to the lower chambers were counted by FACS analysis on a FACScan (Becton Dickinson). Briefly, each sample was counted for 20 s. Cell number of a whole sample is calculated by multiplying this cell number with the time necessary to count the whole volume of a sample.

3. Results

3.1. Determination of loading parameters

The uptake of the protein ovalbumin by monocyte derived DC is a receptor independent mechanism (pinocytosis) and thus high protein concentrations are necessary to yield a substantial protein uptake. We measured the uptake of FITC-OVA by using a fluometric analysis to determine the extracellular protein concentration in the culture medium (Fig. 1a) and in parallel FACS to measure the FITC intensity in DC cultured with FITC-OVA (Fig. 1b). We found that after the addition of 2 mg/ml FITC labeled ovalbumin to 1×10^6 immature DC in 1 ml culture medium, an uptake of 1 mg/ 1×10^6 DC was achieved after 36 h at 37 °C, while no uptake was measured at 4 °C, indicating an active process rather than the attachment of the protein to the cells (data not shown). The amount of protein (ovalbumin) taken up per DC was calculated to be 1 ng. About 1 ng ovalbumin protein contains 1.35×10^{13} molecules, and this number is comparable to the number of TAA peptides when the pulsing is done with 10 µg peptide per 1×10^6 DC in 1 ml. The simultaneous addition of maturation inducing cytokines did not reduce the uptake of the soluble protein (Fig. 1a) or the medium fluorescence intensity (MFI) achieved in DC (Fig. 1b). Comparable to these findings the positivity of DC after the uptake of PKH-67 labeled apoptotic cells and necrotic cellular fragments derived from melanoma cells was also not affected by the presence of the maturation inducing cytokines but reached high levels ($\geq 80\%$) after 14 h of cultivation (data not shown). We next used the FITC-OVA to determine the protein concentration necessary to yield an elevation of the FITC-intensity when added to the culture of DC. When a low concentration of FITC-OVA (100 µg/ml) was added, no increase of the medium fluorescence intensity was measured (Fig. 1b). Only higher concentrations (1 mg/ml) yielded a linear increase of the MFI. Again, the rise of the MFI was only seen at 37 °C while at 4 °C no such increase was measurable.

The recovery of mature, antigen-loaded DC is one important parameter to be optimized when large scale DC are being generated for numerous sequential

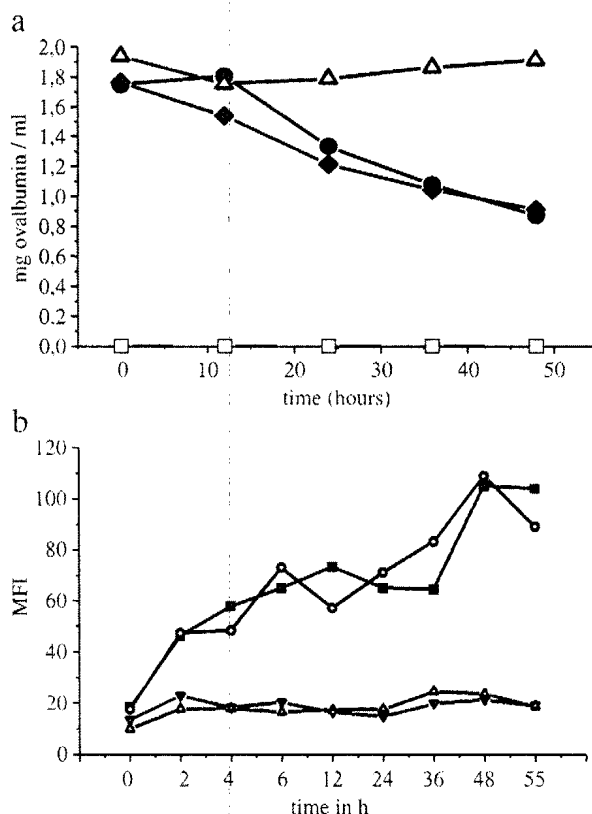


Fig. 1. Uptake of soluble ovalbumin by immature DC. To measure the uptake of soluble protein we added different concentrations of FITC-conjugated ovalbumin to cultures of 1×10^6 DC per ml in 3 ml in six well plates. At the indicated time points we harvested 100 µl and separated cells and medium by centrifugation. Medium was analysed for FITC-OVA content (a) by measuring fluorescence in a spectrometer. Maturation inducing cytokines and PGE₂ (cocktail) were added ($\blacklozenge$) to compare the uptake of FITC-OVA by maturing DC with immature DC ($\bullet$). To test the stability of FITC-OVA, we measured simultaneously the protein content in a well without DC (Δ). In order to proof that the protein uptake is an active process and the measured protein reduction in the supernatant is not an artefact associated with adherence of protein to DC, we performed the same experiments at 4 °C. No uptake was found in these experiments. The data shown in (a) are one representative out of three experiments. To demonstrate the uptake of FITC-OVA by DC directly we analysed the cells for FITC expression by FACS (b). For these experiments we reduced the FITC-OVA concentration to 1 mg/ml as this concentration was the highest we used for the large scale loading. 1 mg/ml FITC-OVA was added to immature DC in the absence ($\square$) or presence ($\circ$) of maturation inducing cytokines. Similarly, 100 µg FITC-OVA was added to immature DC in the presence (Δ) or absence ($\blacklozenge$) of maturation inducing cytokines. As for (a), no uptake of FITC-OVA was observed at 4 °C.

vaccinations. In order to determine the maximum possible tumor cell concentrations that can be applied without significant cell loss, we performed dose-finding studies for the loading with apoptotic and necrotic melanoma cells as well as lysate derived thereof. First of all we noted that the simultaneous addition of the maturation inducing cocktail increases the DC recovery, as less DC were lost due to re-adherence to the dish and cellular apoptosis determined by trypan blue staining (data not shown). Based on the uptake experiments and the increased recovery, we continued the

experiments with simultaneous addition of maturation inducing cytokines.

We found that up to a ratio of one tumor cell to one DC only minor reductions of DC recovery occurred. Whenever higher concentrations were introduced recovery was reduced substantially (Fig. 2a). The best recovery of loaded DC at higher concentrations was achieved with melanoma cell lysate, as the cell loss reached only 40% with 5 mg/ml per 1×10^6 DC, reflecting the protein content of approximately 5×10^6 tumor cells.

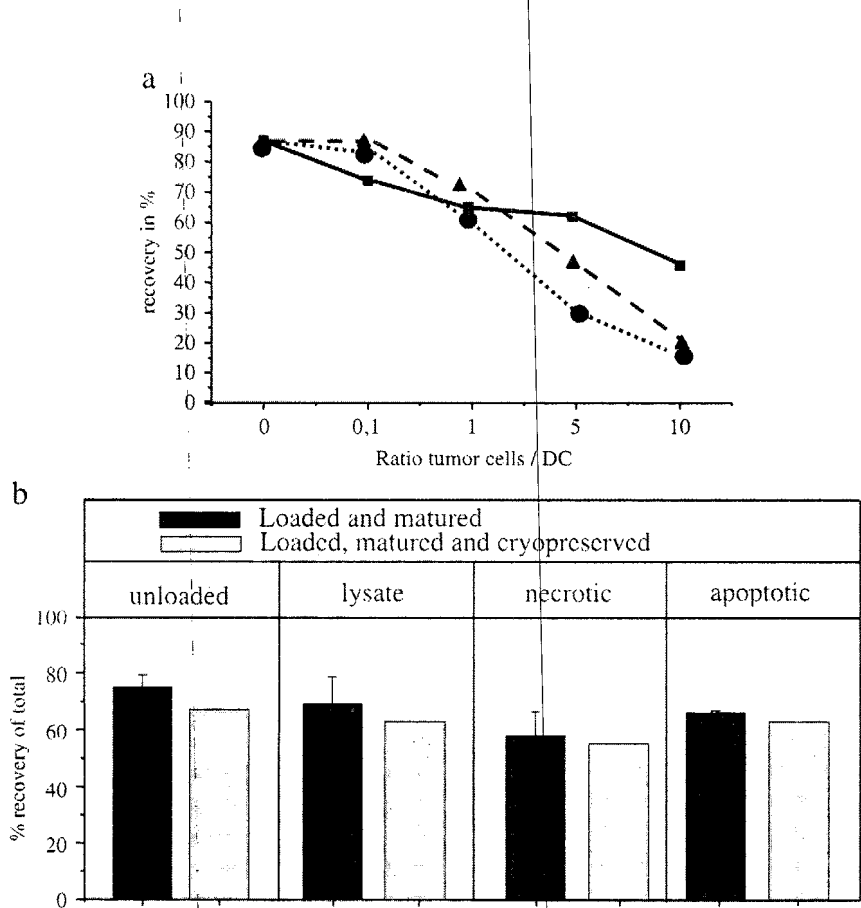


Fig. 2. Recovery of DC after loading with different tumor cell preparations. 3×10^6 immature DC were cultured in 3 ml medium in a six-well plate. Different concentrations of the melanoma cell preparations were added together with maturation inducing cytokines (a). After 36 h we counted recoverable viable DC by trypan blue staining using Neubauer counting chambers. Tumor cells were added as lysate (-●-), necrotic tumor cell material (-●-) or as apoptotic tumor cells (-▲-). The experiment shown in (a) represents one out of five experiments with similar results. In one experiment, we found a better recovery for necrotic tumor cell loading than for apoptotic cell loading. To analyse the recoverability of antigen loaded and matured DC after the cryopreservation we loaded 10×10^6 in 10 ml medium in tissue culture plates with one-to-one ratios of the indicated tumor cell preparation and cryopreserved the cells after 36 h of incubation (b). For loaded and matured DC two plates were counted and results are given in mean + standard deviation. One of the plates was cryopreserved as described and counted after thawing and 2 h of cultivation in a dish. One out of three independent experiments with comparable results is shown.

3.2. Recovery after cryopreservation

Based on these findings, we next determined the practicability of cryopreservation of DC loaded with the described tumor cell preparations (apoptotic, necrotic or lysate) for 36 h at a one-to-one ratio with simultaneous addition of maturation inducing cyto-

kines. Loaded and matured DC were harvested and frozen according to the protocol described. After 2 h of cryopreservation at -80°C cells were thawed as described and kept for 1 h at 37°C prior to further analysis. The recovery of cryopreserved DC was generally between 60% and 70% (Fig. 2b). Cell loss was attributed to handling rather than cell death, as

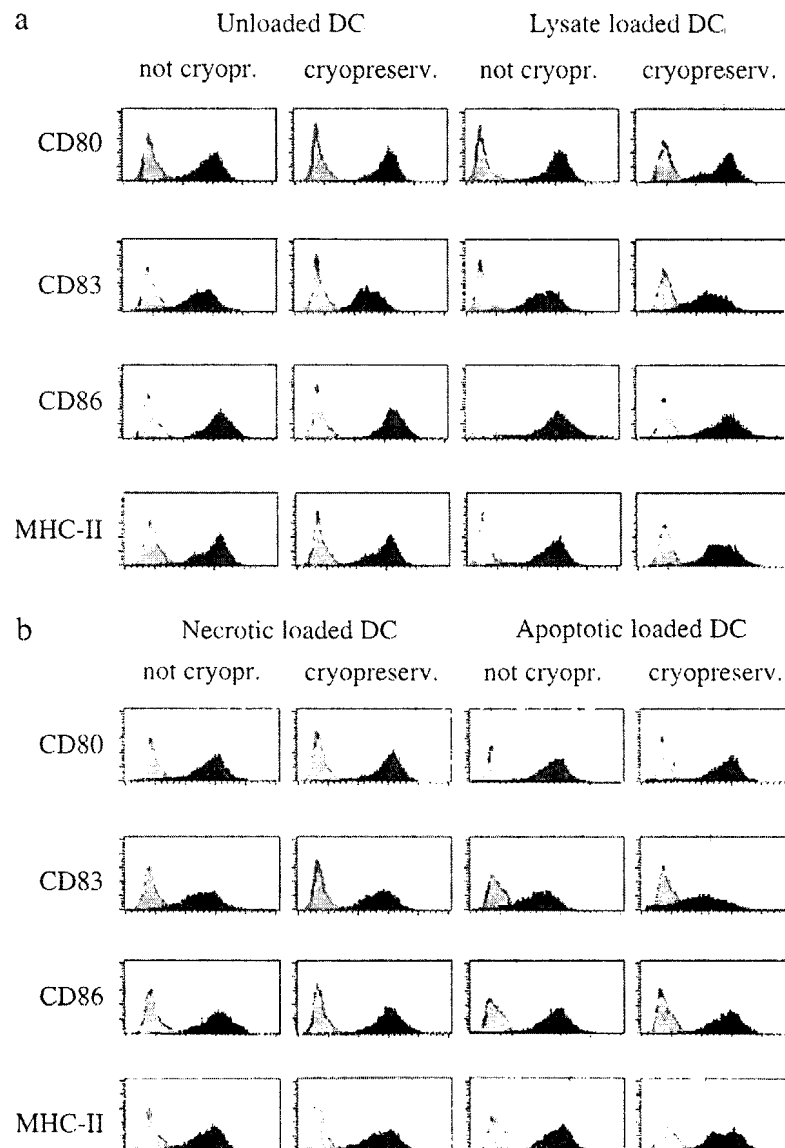


Fig. 3. Surface marker expression of loaded, matured and cryopreserved DC. DC were loaded and matured as described in Fig. 2b and analysed for expression of surface markers as described in Section 2. To exclude the presence of remaining apoptotic or viable melanoma cells when DC were loaded with apoptotic melanoma cells we gated on the MHC-II expressing cells (the melanoma cells used for loading were MHC-II negative).

trypan blue staining of cryopreserved cells was not elevated as compared to unpreserved cells (data not shown). Nevertheless, a cell loss of up to 40% of the initial DC number should be calculated when DC therapy with loaded and cryopreserved DC is planned. In four out of five experiments performed we found the lowest rate of recovery for DC loaded with necrotic cell material.

We have recently shown that the ratio of one apoptotic tumor cell to one dendritic cell is sufficient to induce the generation of anti-tumoral and, to a lesser extent, anti-TAA CTL (Jenne et al., 2000). We thus conducted the following experiments with this cellular ratio as it represents a compromise between necessity (high dose of antigen) and feasibility (recovery/amount of available tumor cell material), although presumably higher tumor cell ratios might yield higher antigen presentation. The feasibility of a simultaneous addition of maturation inducing cytokines,

which was described above, further facilitates the handling of large scale loading of DC.

3.3. Unaltered surface marker expression after loading, maturation and cryopreservation

As the T-cell stimulation capacity of DC heavily depends on the expression of MHC and costimulatory molecules we probed for the expression of such markers by using FACS. No difference in the expression of CD80, CD83, CD86 and MHC molecules was detected when antigen loading and maturation was done as described above for the different antigen loading conditions. After cryopreservation the surface expression levels of these molecules remained unaltered (Fig. 3a–b). Based on these findings it can be speculated that a similar capacity of cryopreserved cells to stimulate the generation of antitumoral immunities is retained after cryopreservation.

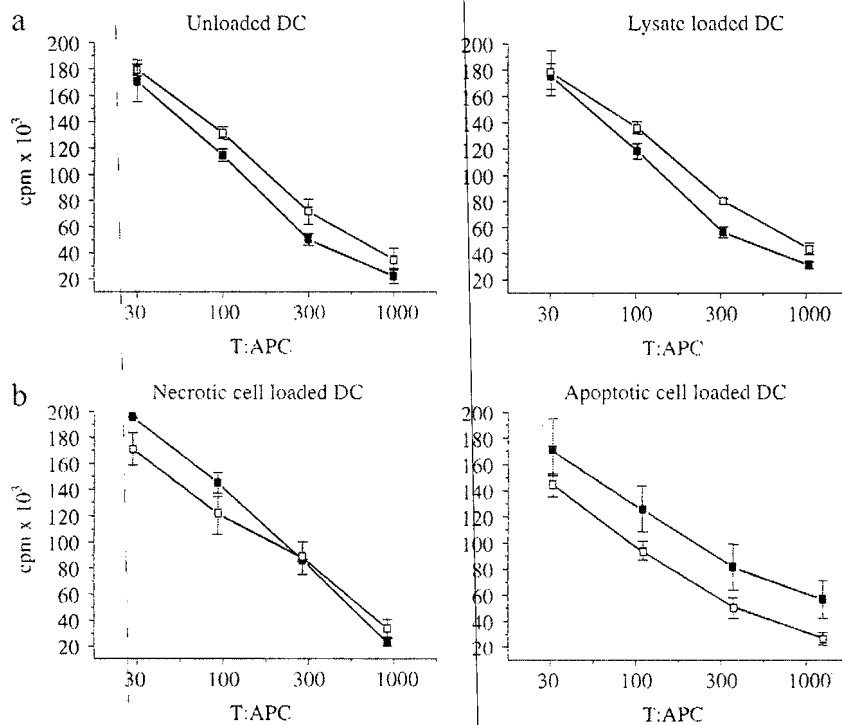


Fig. 4. Allostimulatory capacity of loaded, matured and cryopreserved DC. Again, immature DC were cultured with the different tumor cell preparations as described in Fig. 2b and their allostimulatory capacity was probed in MLR as described. Loaded and matured DC (■) were compared with cryopreserved (□) DC. About 1 μ Ci/well of 3 H-TdR was added to the cultures for the last 12–14 h. Data are given in mean of triplicates $\pm$ S.D. Counts per minute (cpm) of control T-cells was below 1000 in all experiments performed. One out of three experiments with similar results is shown in this figure.

3.4. Cryopreserved antigen loaded DC retain their capability to stimulate allogenic T-cell proliferation in mixed lymphocyte reactions

To determine the capacity of loaded and cryopreserved DC to stimulate allogenic T-cell proliferation, as a marker of the stimulatory capacity of DC, we performed MLR with unloaded, loaded and cryopreserved DC. We found that all loading methods together with the simultaneous induction of maturation by using the described cytokines and PGE₂, yielded comparable

levels of T-cell stimulatory capacities (Fig. 4a–b). This finding indicates that the loading with all tumor cell preparations with the described loading parameters is possible without affecting DC function. When cryopreserved DC were used for the MLRs we found stimulatory capacities equal to the capacities of unpreserved DC (Fig. 4a–b). In accordance with the finding of unaltered MHC-molecule and costimulatory molecule expression as seen in the FACS analysis the method used here for cryopreservation is not effecting the general capacity of DC to stimulate T-cells.

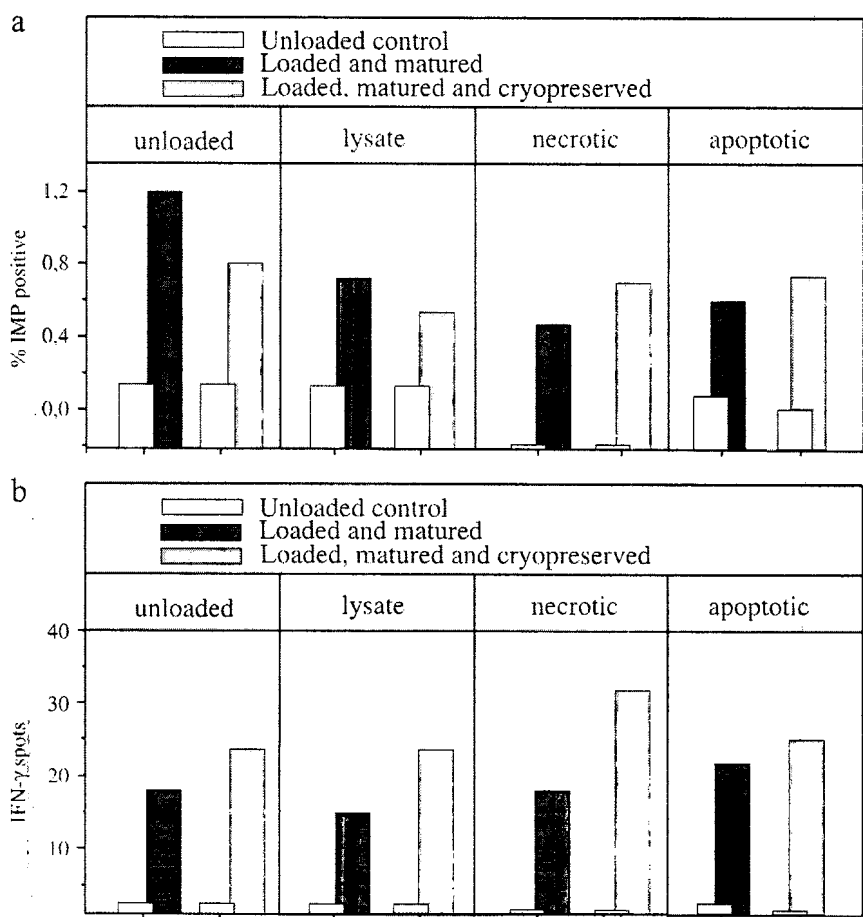


Fig. 5. Induction of Influenza matrix peptide specific T-cells by loaded, matured and cryopreserved DC. To demonstrate the unaltered expression of MHC-I bound peptide after cryopreservation and the capacity of cryopreserved DC to induce specific T-cells, we pulsed HLA-A2 positive matured and loaded DC with influenza matrix peptide (IMP) presented by HLA-A2. After thawing we cultured the DC together with autologous CD8⁺ T-cells for one week. A total of 30 IU IL-2 per ml were added every second day. We measured the induction of IMP-specific T-cells by tetramer (a) and ELISPOT analysis (b). Unpulsed DC of each loading condition served as control. The figures represent one out of three experiments with comparable results.

3.5. Comparable induction of IMP specific T-cells by cryopreserved DC

In order to compare the capacity of cryopreserved DC to induce antigen specific T-cell immunity we have chosen to measure the induction of anti-IMP CTL by using influenza matrix peptide-pulsed DC. As

for the other experiments we compared loaded with unloaded and cryopreserved with unpreserved DC. As cryopreserved DC were pulsed with the IMP before undergoing the cryopreservation procedure, these experiments also provides informations concerning the stability of the MHC-peptide complex during the cryopreservation procedure. We measured the induc-

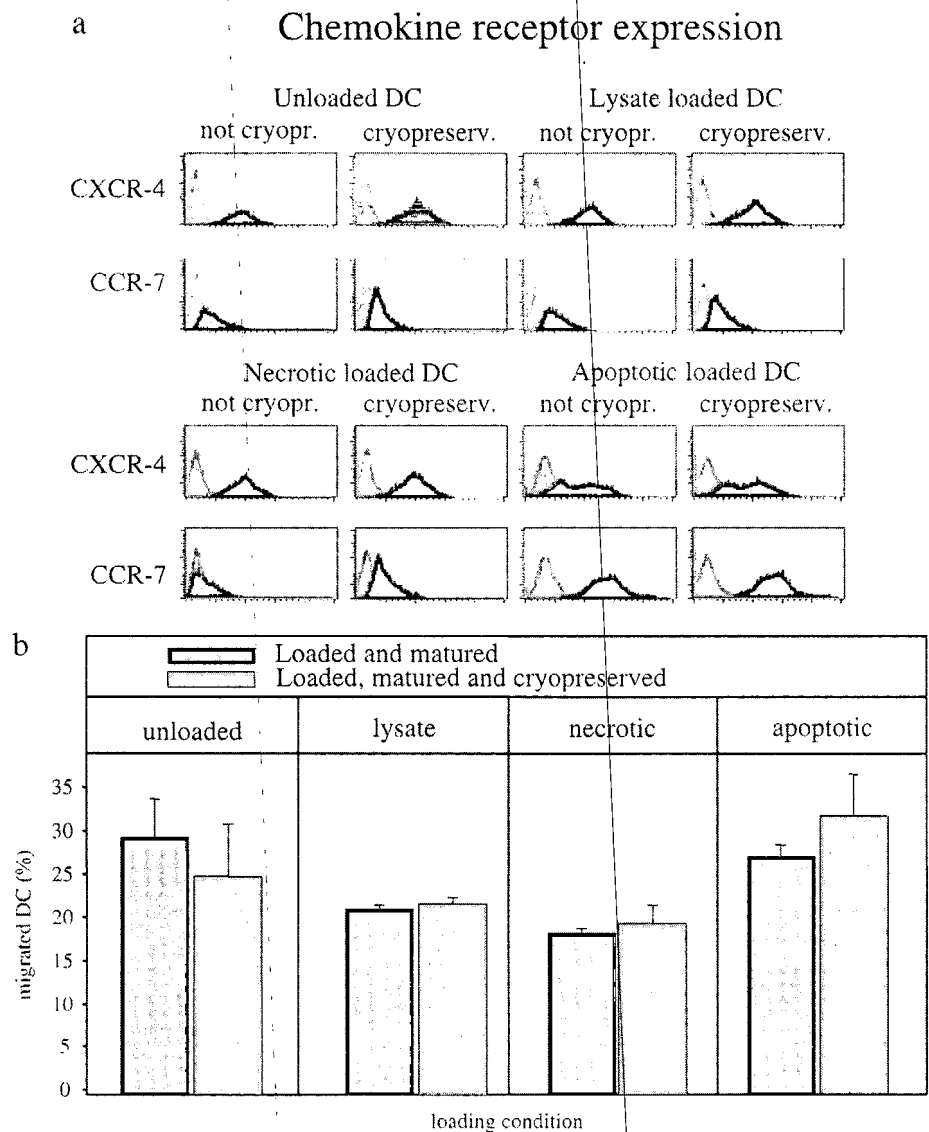


Fig. 6. Chemokine receptor expression and migration of loaded, matured and cryopreserved DC. Again, immature DC were cultured with the different tumor cell preparations as described. Chemokine receptor expression of each condition after 36 h of loading and maturation was measured by FACS (a). Isotype controls were measured in parallel. To test whether the expression of chemokine receptors goes along with migratory capacities we performed migration assays (b). Migration of DC against MIP-3 β and medium alone was counting cells in the lower chamber by FACS as described. Migration of DC against medium alone was below 5% in all experiments performed. Two experiments with similar results were performed.

tion of IMP-specific CTL by using ELISPOT and tetramer analysis. We found comparable levels of induced IMP specific T-cells by both methods (Fig. 5a–b). Unloaded DC were most efficient in the induction of IMP specific T-cells, possibly due to an increased expression of MHC-I molecules with low affinity binding peptides as for the DC loaded with tumoral antigen. In contrast to tetramer staining, where the frequency of IMP specific T-cells was generally slightly lower (in three experiments) when cryopreserved DC were used for the stimulation of the T-cells, we found higher frequencies of IMP-specific IFN- γ producing T-cells for the cryopreserved DC in ELISPOT analysis.

3.6. Chemokine receptor expression

The capacity of antigen loaded DC to migrate to regional lymph nodes after the vaccination is thought to be one of the most important parameters for the efficacy of a DC based vaccination. To assess the potential *in vivo* capacity of antigen loaded and cryopreserved DC to migrate, we measured the expression of chemokine receptors characteristic for mature DC. Mature DC express CXCR-4, for which SDF-1 is a chemoattractant, and CCR-7 with a chemoattractant activity against MIP-3 β . We found an unaltered expression of both chemokine receptors in DC loaded with each of the loading condition and in cryopreserved DC (Fig. 6a).

3.7. Migration of cryopreserved and antigen loaded DC

To probe the functional consequences of the chemokine receptor expression, we performed migration assays. As chemoattractant we used MIP-3 β as described above. As negative control, lower chambers of the migration assay's chambers were filled with medium alone. We found no major differences in the migration of all three loading techniques in comparison with unloaded mature DC (Fig. 6b). Furthermore, the cryopreservation procedure had no negative effect on the migratory potential of antigen loaded DC (Fig. 6b). We thus conclude that neither antigen loading nor the procedure of cryopreservation has a negative effect on capacity of DC to migrate towards MIP-3 β . Presumably, these findings indicate that antigen

loaded, cryopreserved DC migrate to an extent comparable to unpreserved DC *in vivo*.

4. Discussion

Using whole tumor cells for the loading of DC has several potential advantages. The whole antigen profile of a given tumor cell can in principle be presented in a MHC-II and via cross-presentation also a MHC-I (Carbone et al., 1998). The simultaneous presentation of antigen by both pathways is desirable as antigen specific CD4 $^{+}$ helper cells promote the generation of long term CD8 $^{+}$ T-cell memory (Zajac et al., 1998) and is critical for an effective CD4 $^{+}$ helper T-cells are essential for an anti-tumor immune responses via several other mechanisms (Toes et al., 1999). Furthermore, when tumor material is accessible there is no need to determine the antigenic profile or the HLA-type of a patient before the beginning of a DC-based immunotherapy. On the other hand, a given tumor cell expresses about 30,000 genes at a given time of which only about 30 are of tumoral origin (Velculescu et al., 1999), thus the density of tumoral antigen in tumor cell preparations is presumably low. This might be an advantage as well, as it has previously been shown in a murine model that priming of T-cells with high levels of peptide selects for low affinity/avidity T-cells whereas low levels of peptide on antigen presenting cells selects for high affinity/avidity T-cells (Zeh et al., 1999; Alexander-Miller et al., 1996). The high percentage of non-tumoral antigens in tumor cells bears the risk of inducing autoimmunity against self-antigens presented in an immunostimulatory context (Gilboa, 2001). However, although in transgenic and thus artificial mouse models immunity against tumoral antigen expressed at high levels in the pancreatic island was inducible by DC vaccination (Ludewig et al., 2000), so far no induction of autoimmunity (except vitiligo) was reported in DC vaccination studies neither in mouse nor human to the best of our knowledge.

Here we focus on three methods to prepare melanoma cells for an uptake by immature monocyte derived DC: necrotic melanoma cell material, generated by repetitive freeze–thaw cycles, melanoma cell lysate, which can be generated from necrotic melanoma cells by additional ultracentrifugation steps, and

apoptotic melanoma cells, generated by irradiating melanoma cells with UV-B light.

Apart from pure proteins, necrotic tumor cell material contains a crude mixture of all kinds of cellular components, i.e. fragments of the destroyed cellular membrane, intracellular organelles and cellular RNA and DNA. Necrotic tumor cells have been shown to induce maturation in DC without further addition of maturation inducing cytokines (Sauter et al., 2000) probably by heat shock proteins which are found abundantly in necrotic tumor cell material (Somersan et al., 2001). The presence of RNA and DNA in necrotic tumor cell material might contribute to the efficacy of DC loading with necrotic tumor cells as RNA can be used to load DC (Boczkowski et al., 1996), although the release of intracellular RNases by disrupting the cellular integrity is likely to limit the efficacy of this mechanism. Due to the induction of cell death in DC necrotic cellular material of melanoma cells can not be given to DC in great abundance. We found that the upper limit of necrotic cell material loading from melanoma cell lines is a ratio of 1:1. The simultaneous addition of maturation inducing cytokines did not affect the uptake of necrotic tumor cell material but increased the recovery of loaded DC substantially.

Higher tumor cell concentrations can be applied for the loading of DC if all cellular fragments are removed by centrifugation and only tumor protein (lysate) is used for the loading procedure. For the uptake of soluble protein, DC can only use the mechanism of macro-pinocytosis (Watts, 1997) and no receptor mediated uptake occurs. This leads to a substantial lower cross-presentation of soluble ovalbumin as compared to cell-associated ovalbumin. Li et al. (2001) found a 50,000-fold lower cross-presentation (MHC-I restricted) of soluble ovalbumin together with a 500-fold lower MHC-II restricted presentation. Together with the finding that 100 µg to 1 mg/ml of ovalbumin have to be fed to DC before ovalbumin specific clones are activated (Brossart and Bevan, 1997), and with regard to the low frequency of tumoral proteins in whole tumor cell preparations, only a low level of antigen presentation can thus be achieved by lysate loading. In order to determine a loading parameter for the application of lysate we performed systematic uptake studies with ovalbumin. We found that at least 1 mg/ml medium and 1×10^6

should be present for 36 h to allow a substantial uptake of soluble ovalbumin as a model protein. The simultaneous addition of maturation inducing cytokines did not alter the uptake of ovalbumin.

Although several studies have reported some induction of anti-tumoral immunity by DC loaded with tumor cell lysate even when very low tumor protein concentrations were applied for a short period of time (120 µg/3 h, Schnurr et al., 2001; 100 µg/12 h, Bachleitner-Hofmann et al., 2002; 100 µg/ml for 6 days, Wen et al., 2002; 10 µg/24 h, Holtl et al., 2002), lysate loading was less efficient in trials when compared to apoptotic pancreatic tumor cells (Schnurr et al., 2002) or for acute myeloid leukemia (Galea-Lauri et al., 2002) and failed to elicit an anti-EBV T-cell immunity when lysates from Epstein-Barr virus-transformed lymphoblastoid cell lines were used to load DC (Ferlazzo et al., 2000).

During apoptosis, the asymmetry of plasma membrane phospholipids is lost, which exposes phosphatidylserine (PS) externally and PS receptors of the DC have been reported to be critical in mediating uptake of apoptotic cells (Fadok et al., 2000). The receptor mediated antigen uptake and the assumed high access of the antigen to the cross-presentation pathway led to speculations of a very efficient cross-presentation after the phagocytosis of apoptotic tumor cells (for review, see (Larsson et al., 2001; Jenne and Sauter, 2002)). While the efficacy of influenza virus infected (and thus apoptotic) monocytes to serve as antigen loading agent is very high with one monocyte per 100 DC (Albert et al., 1998), we found a less efficient presentation of TAA when apoptotic melanoma cells were used to generate an anti-TAA immunity (Jenne et al., 2000). This probably reflects the above-mentioned fact that TAA are only a small fraction of the total antigen of a tumor cell. However, in these studies we were able to generate an anti-tumoral immunity with a ratio of one apoptotic melanoma cell to one DC. Comparative studies of necrotic vs. apoptotic cell loading yielded no difference in the efficacy of both loading methods to generate an anti-tumoral immunity (Kotera et al., 2001; Lambert et al., 2001). Thus the choice whether or not apoptotic tumor cells should be used for the large scale loading of DC largely depends on the handling. In contrast to the rather simple induction of necrosis (repetitive freeze thaw cycles) viable tumor cells have to be in order to use apoptotic

tumor cells as loading agent. Furthermore, it is difficult to induce a constant percentage of apoptosis in tumor cells from patients especially as viable tumor cells have to be excluded with extremely high accuracy from being injected into cancer patients in order to avoid the generation of new metastasizes. We thus argue that the use of apoptotic cells for the ex vivo loading of DC might be best suited when tumor cell lines are used to load DC.

In our experiments we tested important functions of loaded, matured and cryopreserved DC that might be important for the induction of anti-tumoral immunities, i.e. viability, expression of MHC- and costimulatory-molecules, induction of allogenic T-cell proliferation and specific induction of T-cells specific for a peptide pulsed on the DC before performing the cryopreservation, the expression of chemokine receptors characteristic for mature DC and the migratory properties of DC against Mip-3 β . We were able to demonstrate that no alterations occur due to the cryopreservation.

We have tested here systematically parameters for loading of monocyte derived DC with various forms of total tumor cell preparations and have established that such DC can be successfully cryopreserved after loading. Although we have used melanoma cells as a model based upon our experience the experiments described here form a guideline for choosing the most appropriate loading method for a given tumor type. Our findings are useful to establish large-scale preparations of monocyte derived DC aliquots loaded with either apoptotic, necrotic or lysates of tumor cells.

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Immunology/Tumor Immunobiology 3: Specific Immunomechanisms and Cancer Vaccines: Basic Studies I**Abstract #1277****Preclinical studies of a human vaccine based on dendritic cells loaded with apoptotic/necrotic melanoma cells****Paula Longhi, Leticia Machiavelli, Laura Bover, Marcela Barrio, Erika Von Euw and José Mordoh**Centro de Investigaciones Oncológicas FUCA - Instituto Alexander Fleming, Buenos Aires, Argentina
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Dendritic cells (DCs) are potent APC able to efficiently take up, process and present antigens to naive T helper cells to initiate an immune response. Recently, Goldszmid et al. (J.Immunol. 171(11), 2003) described that vaccination of C57BL6 mice with DCs loaded with apoptotic B16 melanoma cells induced a long lasting protection in 80 % of vaccinated animals. The aim of the present study was to analyze in the human setting DC maturation and T cell stimulation capacity obtained after DC phagocytosis of human melanoma cells. Human monocyte-derived DCs were coincubated either with apoptotic (70 Gy irradiated, +72 hs culture) or necrotic (50 Gy irradiated + 3 freeze-thaw cycles) IIB-MEL-IAN human melanoma cells. Phagocytosis was monitored by FACS after PKH26 and PKH67 staining of tumor cells and DCs. After phagocytosis, DCs maturation was measured by up-regulation of co-stimulatory molecules CD86, CD80, CD83 and MHC-II. More than 50 % of immature DCs phagocytosed apoptotic cells (78% Annexin + / IP-) and over 60% phagocytosed necrotic cells (88% Annexin+ / IP+). Necrotic cells were more effective to induced DCs maturation than apoptotic cells (6 ± 0.5 % FITC-Dextran-uptake vs 40 ± 7.5 %, respectively). DCs cell surface expression of CD80, CD86, MHC-II and CD83 was clearly up-regulated upon coincubation with apoptotic cells, comparable to LPS-induced maturation. Although the addition of LPS, TNF- α , IL-1 β , and MCM (Monocyte Conditioned Medium) during the coincubation improved the maturation of DCs that had taken up apoptotic cells (DC-Apo), it was lower than that obtained with DCs that had captured necrotic cells (DC-Nec). T cell proliferation was determined by H3-dThd incorporation and T cell stimulation was measured by detection of intracellular IFN- γ . Both T-cell proliferation and stimulation induced by DC-Apo were greater than those obtained with DC-Nec. We conclude that both apoptotic and necrotic melanoma cells could be used as a source of Ags for DCs since they are taken up almost equally by DCs, but necrotic tumor cells appear to induce better DC maturation than apoptotic cells, according to the FITC-Dextran uptake assay. However, phagocytosis of apoptotic cells may play a more important role in T-cell activation. These results could be relevant for the design of antimelanoma vaccines with DCs, and these studies will be extended to tumor cells of distinct origin.

Dendritic Cells Charged with Apoptotic Tumor Cells Induce Long-Lived Protective CD4⁺ and CD8⁺ T Cell Immunity against B16 Melanoma¹

Romina S. Goldszmid,^{*†} Juliana Idoyaga,[‡] Alicia I. Bravo,[‡] Ralph Steinman,^{2§} José Mordoh,^{*} and Rosa Wainstok^{2†}

Dendritic cells (DCs) are potent APCs and attractive vectors for cancer immunotherapy. Using the B16 melanoma, a poorly immunogenic experimental tumor that expresses low levels of MHC class I products, we investigated whether DCs loaded ex vivo with apoptotic tumor cells could elicit combined CD4⁺ and CD8⁺ T cell dependent, long term immunity following injection into mice. The bone marrow-derived DCs underwent maturation during overnight coculture with apoptotic melanoma cells. Following injection, DCs migrated to the draining lymph nodes comparably to control DCs at a level corresponding to ~0.5% of the injected inoculum. Mice vaccinated with tumor-loaded DCs were protected against an intracutaneous challenge with B16, with 80% of the mice remaining tumor-free 12 wk after challenge. CD4⁺ and CD8⁺ T cells were efficiently primed in vaccinated animals, as evidenced by IFN- γ secretion after in vitro stimulation with DCs loaded with apoptotic B16 or DCs pulsed with the naturally expressed melanoma Ag, tyrosinase-related protein 2. In addition, B16 melanoma cells were recognized by immune CD8⁺ T cells in vitro, and cytolytic activity against tyrosinase-related protein 2_{180–188}-pulsed target cells was observed in vivo. When either CD4⁺ or CD8⁺ T cells were depleted at the time of challenge, the protection was completely abrogated. Mice receiving a tumor challenge 10 wk after vaccination were also protected, consistent with the induction of tumor-specific memory. Therefore, DCs loaded with cells undergoing apoptotic death can prime melanoma-specific helper and CTLs and provide long term protection against a poorly immunogenic tumor in mice. *The Journal of Immunology*, 2003, 171: 5940–5947.

Melanoma is the cancer with the fastest increasing incidence (1), and survival of patients with distant metastases is generally <1 year. With increased understanding of the requirements for initiating immunity, dendritic cells (DCs)³ have become attractive vectors for immunotherapy of this and other cancers (2–9). DCs are proving to be effective in initiating and expanding immune responses in humans (10, 11), providing a rationale for their use as adjuvants for active immunization against melanoma (12–16). Different strategies have been developed to load DCs with tumor Ags, including MHC-restricted synthetic peptides derived from the Ag (12–17), tumor RNA (18,

19), tumor lysates (20), tumor-derived exosomes (21), and dying tumor cells (22–26); these have been assessed in preclinical models and, in some cases, clinical trials (12–26). Tumor cells (dying cells or Ab-coated tumor cells) are of interest as an antigenic source, because the DCs would have the potential to present a broad range of tumor-associated Ags and on both MHC class I and II products regardless of the MHC haplotype of the patient (27–30).

To test whether combined CD4⁺ and CD8⁺ long term immunity could be elicited by DCs loaded with tumor cells, we have conducted preclinical analyses to immunize mice with DCs previously exposed to apoptotic melanoma cells ex vivo. There are already some examples, using DCs bearing defined tumor Ag or tumor cells engineered to secrete GM-CSF, where T cell-based resistance to melanoma has been achieved in animals (31–33), but there is no precedent for the induction of combined MHC I- and II-restricted immunity in vivo with DCs loaded with tumor cells. We will show that DCs, after phagocytosis of apoptotic tumor cells, elicit long-lived combined CD4⁺ and CD8⁺ immunity against the MHC class I low B16 murine melanoma.

Materials and Methods

Animals and cell lines

Six- to 8-wk-old male C57BL/6 (HH-2^b, CD45.2⁺) mice were obtained from the Facultad de Ciencias Veterinarias, University of La Plata (Buenos Aires, Argentina) and from Taconic Farms (Germantown, NY), and CD45.1⁺ B6.SJL-Ptprc were obtained from Taconic Farms. Mice were maintained in pathogen-free conditions, and studies were performed in accordance with local ethical guidelines. B16 (HH-2^b) is a poorly immunogenic murine melanoma of spontaneous origin (34). The B16-F1 line (a gift from Dr. A. Vecchi, Istituto Mario Negri, Milan, Italy) was maintained in culture in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FCS (Natocor, Córdoba, Argentina), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin (Life Technologies,

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³ Abbreviations used in this paper: DC, dendritic cell; Apo, apoptotic B16 cells; DC-Apo, DCs cocultured with Apo; DC-Apo, DCs and Apo without coculture; DC-Nec, DCs cocultured with Nec; DC-Spleen, DCs cocultured with apoptotic splenocytes; Nec, necrotic B16 cells; PI, propidium iodide; TRP-2, tyrosinase-related protein-2.

Gaithersburg, MD). The EL-4 thymoma (American Type Culture Collection, Manassas, VA) was used as a control tumor. Cell lines were periodically tested to be mycoplasma-free.

Abs and reagents

We purchased anti-CD16/32, FITC-conjugated anti-CD86, I-A^b, rat IgG, mouse IgG, PE-anti-CD11c, biotinylated anti-CD45.2, and allophycocyanin-streptavidin (all from BD Biosciences, Mountain View, CA); anti-CD8 and CD4 MACS microbeads (Miltenyi Biotec, Auburn, CA); CFSE (Molecular Probes, Eugene, OR); and ACK buffer (BioSource International, Camarillo, CA).

Induction and detection of apoptosis and necrosis

After reaching 70–80% confluence, tumor cells were harvested with 0.5 mM EDTA, washed with PBS (HyClone, Logan, UT), and resuspended for irradiation (70 Gy, linear accelerator; Siemens, New York, NY). Irradiated cells were washed twice, cultured for 48 h, and harvested by pipetting for coculture with DCs or for detection of apoptosis using the annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA) on a FACS Vantage SE (BD Biosciences). UV-irradiated (100 J/m², UV Stratalinker 2400; Stratagene, La Jolla, CA) syngeneic splenocytes were used as control apoptotic cells. Necrosis of B16-F1 cells was induced by four rapid cycles of freeze/thaw, as evidenced by combined annexin V/propidium iodide (PI) staining.

Generation of bone marrow-derived DCs and culture with apoptotic cells

As previously described (35), bone marrow cells were harvested from femurs and tibias of normal C57BL/6 mice and washed with PBS. Cells were resuspended in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamicin (Life Technologies), 50 µM 2-ME (Sigma-Aldrich), and 10% supernatant from CHO cells transfected with the plasmid pCDNA3 containing the recombinant mouse GM-CSF gene (the pCDNA3-rmGM-CSF plasmid was provided by Dr. O. Burrone, International Center for Genetic Engineering and Biotechnology, Trieste, Italy) and cultured in 100-mm diameter plates. On day 3 the cells were refed with fresh medium. On day 5 of culture immature DCs were harvested and used for *in vitro* phenotypic analysis and for coculture with apoptotic cells (Apo). For coculture, 2×10^5 DCs were incubated with Apo at a 1/1 ratio for 24 h, washed twice with PBS, and used for vaccination. Coculture with necrotic cells or syngeneic splenocytes was performed at a 1/1 ratio.

Phagocytosis of irradiated B16 melanoma cells

After irradiation, apoptotic B16 cells were dyed red using PKH26-GL (Sigma-Aldrich) and cocultured with immature DCs that were dyed green with PKH67-GL (Sigma-Aldrich) at a 1/1 ratio. After 24 h, flow cytometric analysis was performed, and phagocytosis of Apo was defined by the percentage of double-positive cells. Alternatively, phagocytosis was observed by confocal microscopy (LSM 510; Zeiss, New York, NY).

*Surface phenotype and *in vivo* migration of DCs*

The surface phenotype of bone marrow-derived DCs was analyzed before and after coculture with apoptotic tumor cells. DCs were double-stained with PE-conjugated anti-CD11c and FITC-conjugated anti-CD86 or anti-MHC II (I-A^b). B6 bone marrow-derived DCs (CD45.2⁺) with and without previous coculture with apoptotic B16 were injected s.c. into CD45.1⁺ recipient mice, and draining lymph nodes were removed 24 h later. Single-cell suspensions were prepared with 400 U/ml collagenase (Roche, Indianapolis, IN) for 25 min. Cells were then incubated with PE-conjugated anti-CD11c, allophycocyanin-conjugated anti-CD45.2, and FITC-conjugated anti-CD86 or MHC II. Flow cytometric data were analyzed with FlowJo (Tree Star software, San Carlos, CA).

Vaccination of mice

Mice were injected s.c. with 2×10^5 DCs cocultured with Apo (DC-Apo), DCs alone, Apo alone, mixtures of DCs plus Apo without previous coculture (DC + Apo), DCs cocultured with necrotic cells (DC-Nec), necrotic cells alone (Nec), DCs cocultured with apoptotic syngeneic splenocytes (DC-Spleen), or vehicle (PBS) weekly for 4 wk. One week after the last injection, mice received a tumor challenge with 1.3×10^4 live B16 cells. Animals were monitored for tumor growth every other day by palpation, and diameters were measured using a Vernier caliper. Tumor-bearing animals were sacrificed when the tumors displayed severe ulceration or

reached a size of 250 mm². For evaluation of immunological memory, mice were challenged 10 wk after the last vaccination. In the case of tumor-free animals, experiments were terminated 12 wk after tumor challenge, and autopsies were performed. All experiments included 10–13 mice/group.

In vivo depletion of CD4⁺ and CD8⁺ T lymphocytes

Mice immunized as described were injected with murine ascites containing 1 mg of rat monoclonal anti-CD4 (clone GK 1.5) or anti-CD8 (clone 53-6.72; hybridomas were provided by Dr. A. Vecchi). Three consecutive injections were performed i.v. on the day before tumor challenge, followed by two i.p. injections on the day of and the day after tumor challenge. Control experiments were performed by injecting the same amount of standard rat IgG (Sigma-Aldrich). Depletion of CD4⁺ and CD8⁺ T cells from spleen was monitored using anti-CD4 and anti-CD8 mAb.

IFN-γ production by CD4⁺ and CD8⁺ T cells

Four weeks after vaccination, CD4⁺ and CD8⁺ T cells were isolated from spleen of vaccinated or control mice using MACS microbeads. Purified CD4⁺ or CD8⁺ T cells were subsequently cocultured in 96-well, flat-bottom microtiter plates (3×10^5 cells/well) in the presence of B16 cells, DC-Apo, or DCs that had been pretreated with tyrosinase-related protein 2 (TRP2)_{180–188} peptide (SVYDFVFWL; a gift from Dr. A. Kalergis, Rockefeller University, New York, NY) or OVA (SIINFEKL) as a control peptide (1 h 37°C, 1 µM) for 48 h in Click's medium containing 0.75% mouse serum. The titers of IFN-γ in the culture supernatants were determined by ELISA (OptEIA kit; BD Pharmingen) according to the manufacturer's protocol.

In vivo cytotoxicity assay

C57BL/6 splenocytes were divided into two populations and labeled with either a high concentration (5 µM) or a low concentration (0.5 µM) of CFSE for 10 min at 37°C, then washed twice with PBS containing 0.1% BSA. CFSE^{high} cells were pulsed with 1 µM TRP2_{180–188} peptide, while CFSE^{low} remained nonpulsed. After washing, CFSE^{high} cells were mixed with equal numbers of CFSE^{low} cells, and 10^7 or 5×10^6 of this cell suspension was injected i.v. into DC-Apo- or DC-vaccinated mice. Spleens and lymph nodes from recipient mice were taken 10 h later for flow cytometric analysis. *In vivo* killing was indicated by loss of CFSE^{high} peptide-pulsed cells relative to CFSE^{low} nonpulsed cells. The percentage of specific lysis was calculated according to the formula: $(1 - (\text{ratio unprimed}/\text{ratio primed}) \times 100)$, where ratio unprimed = % CFSE^{low}/CFSE^{high} cells remaining in control mice, and ratio primed = % CFSE^{low}/CFSE^{high} cells remaining in vaccinated mice.

Statistical analysis

Statistical analyses were performed using the Wilcoxon rank test.

Results

Apoptotic B16 melanoma cells are efficiently phagocytosed by DCs

Whole tumor cells are an advantageous source of Ags for DC vaccination because they provide a wide range of tumor Ags that can be processed onto both MHC class I and class II products by DCs. To induce apoptosis, B16 cells were irradiated (70 Gy) and cultured for 48 h. The tumor cells then failed to grow in culture and could not induce tumors in mice at high doses (10^6 cells). Apoptosis was documented by annexin V/PI staining (Fig. 1). After 48 h, cultured unirradiated cells contained 10–12% early apoptotic cells characterized by annexin V⁺/PI[−] staining (Fig. 1A, lower right). In contrast, 70–80% of irradiated cultured tumor cells had undergone early apoptotic death (Fig. 1B). Twelve to 19% of the cells displayed annexin V⁺/PI⁺ staining, indicating secondary necrosis in both unirradiated and irradiated cultures (Fig. 1, A and B, upper right). Eighty percent of freeze/thawed necrotic cells displayed annexin V⁺/PI⁺ staining (data not shown). Then PKH67 green-labeled DCs (Fig. 1C) were cultured with PKH26 red-labeled, apoptotic tumor cells (Fig. 1D) for 24 h, and phagocytosis was documented by the percentage of double-positive cells. At 37°C, >40% of immature DCs phagocytosed apoptotic material (Fig. 1E), and this was confirmed by confocal fluorescence microscopy and electron microscopy (data not shown). Freeze/

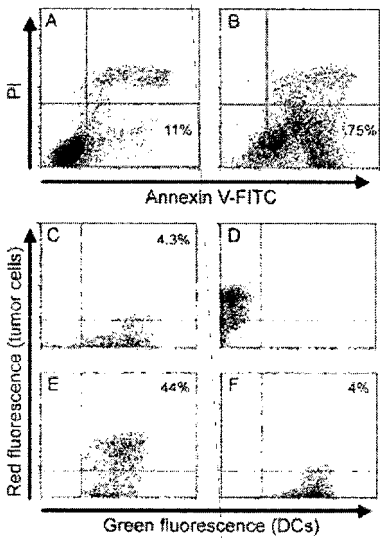


FIGURE 1. Apoptosis of irradiated B16 melanoma cells and phagocytosis by DCs. B16-F1 melanoma cells were cultured for 48 h, either untreated (A) or after irradiation (B), and stained with annexin V-FITC and PI. Early apoptotic cells were defined as annexin V-FITC⁺, but PI⁻, while necrotic cells were double-positive. To demonstrate phagocytosis (C–F), DCs were labeled green with PKH67-GL fluorescent cell linker (C), and apoptotic cells were labeled red with PKH26-GL (D); these were cultured together at a 1/1 ratio for 24 h at 37°C (E) or 4°C (F). The frequency of double-positive phagocytic DCs (gated as green profiles) is shown in the upper right quadrants.

thawed necrotic cells were also phagocytosed by DCs to a comparable extent as apoptotic cells (not shown). To rule out the possibility of nonspecific tumor cell binding, coculture of apoptotic tumor and DCs was performed at 4°C; the percentage of double-positive cells was only 4% (Fig. 1F). DCs also efficiently phagocytosed apoptotic syngeneic splenocytes (data not shown).

DCs loaded with apoptotic B16 cells migrate to lymph nodes and up-regulate CD86 and MCH II expression

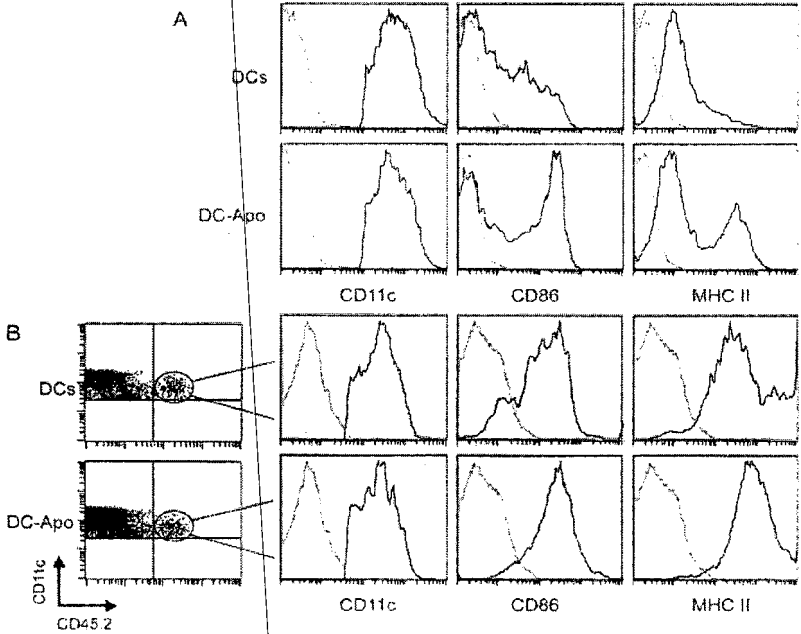
To induce a primary immune response, DCs have to undergo a maturation process that involves up-regulation of MHC and co-

stimulatory molecules, and the DCs must also migrate to lymph nodes to select infrequent naive T cell clones for activation. To assess these attributes in our system, we cultured irradiated B16 cells for 24 h with DCs and double-stained with PE-anti-CD11c and either FITC-anti-CD86 or FITC-anti-MHC II. While the expression of CD11c remained similar to that of DCs that were not cultured with tumor cells, a large fraction of the tumor-exposed DCs up-regulated CD86 as well as MHC II (Fig. 2A). We then analyzed the migratory capacity of these cells. CD45.2⁺ DCs that had been pulsed with apoptotic B16 cells or left unpulsed were injected s.c. into CD45.1⁺ recipient mice. After 24 h the draining lymph nodes were obtained, and the surface phenotype of the injected CD45.2⁺ cells was analyzed. CD11c⁺ cells from both tumor-pulsed and unpulsed DCs cultures were found in the draining lymph nodes, and both showed an up-regulation of CD86 and MHC II molecules (Fig. 2B). The number of injected CD45.2⁺ cells in the lymph nodes corresponded to ~0.5% of the administered inoculum, consistent with prior data (36). Therefore, a s.c. injection of DCs, with or without exposure to apoptotic tumor, leads to the residence of comparable, but small, numbers of mature DCs in the draining lymph nodes, setting the stage for testing their immunizing capacity against B16 melanoma challenge.

DCs loaded with apoptotic B16 cells induce specific resistance to B16 tumors including long term memory

To evaluate whether DCs cocultured with apoptotic B16 cells (DC-Apo) were able to elicit resistance to challenge with tumor, mice were vaccinated s.c. weekly for 4 wk with 2×10^5 DC-Apo and challenged with 1.3×10^4 B16 cells 1 wk later. The efficiency of vaccination by DC-Apo was compared with that in several other types of DCs controls: DCs plus Apo without previous coculture (DC+Apo), DCs alone, apoptotic tumor cells alone (Apo), DCs cocultured with necrotic cells (DC-Nec), necrotic cells alone (Nec), and vehicle (PBS; Fig. 3.4). Also, to rule out the possibility of a nonspecific activation by DCs phagocytosing apoptotic cells, a group of mice that received DCs cocultured with syngeneic apoptotic splenocytes (DC-Spleen) was included. All mice were monitored every other day after tumor challenge, and the tumor volumes were measured.

FIGURE 2. Phenotype of DCs before and after coculture with apoptotic B16 and migration into the lymph nodes. A, Surface phenotype of DCs without (DCs) and with (DC-Apo) coculture with apoptotic B16 cells. The DCs and DC-Apo were stained with PE-conjugated anti-CD11c, followed by FITC-conjugated anti-CD86 or MHC II (I–A^b). CD86 and MHC II expression was evaluated on CD11c⁺-gated cells. B, CD45.2⁺ DCs or CD45.2⁺ DC-Apo were injected s.c. into CD45.1⁺ mice. Twenty-four hours later, draining lymph nodes were harvested, and single-cell suspensions were obtained. Cells were then incubated with allophycocyanin-conjugated anti-CD45.2, PE-conjugated anti-CD11c, followed by FITC-conjugated anti-CD86 or MHC II. The expression of CD86 and MHC II was analyzed on CD45.2⁺, CD11c⁺-gated cells, which were not found in uninjected CD45.1⁺ mice. Gray lines show staining with isotype-matched control Abs. One representative experiment of three is shown.



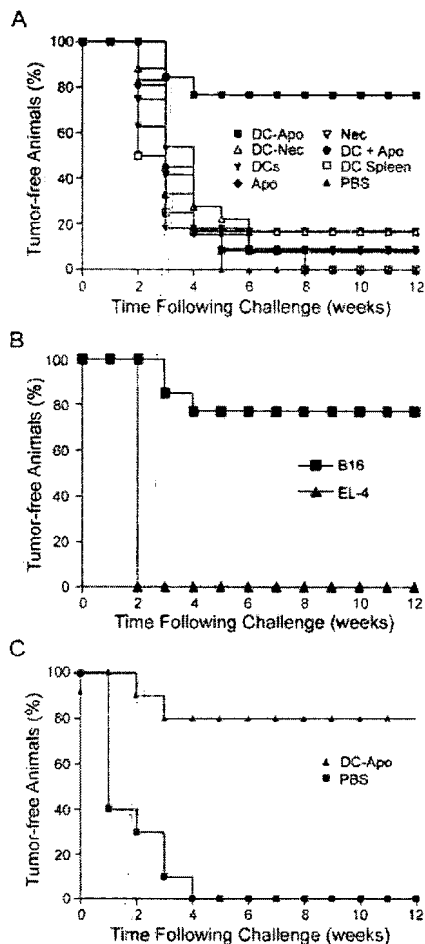


FIGURE 3. Protection against tumor development after vaccination with DCs loaded with apoptotic B16 melanoma. *A*, Mice (10–13/group) were vaccinated with DCs that had phagocytosed apoptotic tumor cells for 24 h (DC-Apo), DCs alone, apoptotic tumor cells alone (Apo), a mixture of DCs and tumor cells (DC+Apo), DCs that had phagocytosed apoptotic spleen cells (DC-Spleen), PBS, DCs that had phagocytosed necrotic B16 melanoma cells (DC-Nec), or necrotic tumor cells alone (Nec) weekly for 4 wk and challenged with 1.3×10^4 B16 cells 1 wk later. *B*, Mice (10/group) were vaccinated with DC-Apo and challenged with either B16 or EL-4 cells 1 wk after immunization. *C*, Mice (10/group) were vaccinated with DC-Apo or PBS weekly for 4 wk and challenged 10 wk later with 1.3×10^4 B16 cells. In *A–C*, mice were monitored every other day for tumor growth and were scored positive when tumors were palpable. One representative experiment of three is shown.

Eighty percent of the mice vaccinated with DC-Apo remained tumor-free 12 wk after challenge relative to 0% in the PBS controls (Fig. 3*A*). None of the control groups showed protection relative to the nonvaccinated animals. When mice were injected with Apo only or with DC+Apo without previous coculture, only 8% ($p < 0.01$) remained tumor-free at wk 12, indicating that DCs are necessary to induce strong protection and that they need to be previously cocultured with Apo. In mice injected with DCs only, 9% ($p < 0.01$) of the mice were tumor-free after 12 wk, ruling out the possibility that tumor rejection was solely due to NK cell expansion, which can be induced by DCs (37–39) or to immunity in response to culture medium proteins presented by DCs. It has been shown that DCs cultured in the presence of FCS are capable of inducing CD4⁺ T cell help (40, 41) and to prime CTLs more efficiently than DCs cultured in FCS-free conditions (40). Therefore, the presence of FCS-derived peptides loaded onto the DCs

might have contributed to the observed antitumor response. When mice were vaccinated with DCs loaded with syngeneic apoptotic splenocytes, 0% remained tumor-free ($p < 0.01$), indicating that the induction of immunity was not due to a nonspecific activation of DCs after phagocytosis of dying cells. In contrast to DC-Apo, only 17% ($p < 0.01$) of the mice injected with DC-Nec remained tumor-free, demonstrating that Apo are a better source of Ag for DCs priming. In summary, only mice vaccinated with DC-Apo showed strong protection against the poorly immunogenic B16 melanoma (Table 1).

To analyze the specificity of this antitumor response, mice vaccinated with DCs loaded with apoptotic B16 cells were challenged with the EL-4 lymphoma, but all the mice developed tumor within 2 wk (Fig. 3*B*). This indicates that vaccination with DCs loaded with apoptotic B16 cells induces a strong protection against B16 challenge, but does not protect against a nonrelated syngeneic tumor.

To test whether vaccinated mice could acquire immunological memory, mice were vaccinated with DC-Apo and challenged with B16 10 wk later. Strong protection was again achieved after vaccination with DC-Apo. Twelve weeks after tumor challenge, 80% of the mice remained tumor-free vs 0% of control mice ($p < 0.01$; Fig. 3*C*). These data indicate that DC-Apo vaccination imparts considerable memory to B16.

Both CD4⁺ and CD8⁺ T cells are required for the antitumor response

We studied the role of CD4⁺ and CD8⁺ T cells in the observed resistance to B16, which, as mentioned, has not previously been shown to elicit combined CD4⁺ and CD8⁺ immunity in mice. Mice vaccinated with DC-Apo received anti-CD4, anti-CD8, or control rat IgG on the day before tumor challenge, followed by two injections on days 0 and 1 after tumor challenge. In vivo depletion of either CD4⁺ or CD8⁺ T cells by Ab treatment completely abrogated the protection from tumor challenge (Fig. 4). Since these depletions were performed after DC-Apo vaccination, the results suggest that both CD4⁺ and CD8⁺ T cells play a crucial role in resistance to B16 melanoma following vaccination with DC-Apo.

DCs charged with apoptotic B16 efficiently prime both CD4⁺ and CD8⁺ T cells

To verify that both CD4⁺ and CD8⁺ T cells could be primed to produce IFN- γ after immunization with DC-Apo, CD4⁺ and CD8⁺ T cells were purified by positive selection from spleens

Table 1. Tumor-free survival after vaccination with DCs loaded with apoptotic B16 melanoma^a

Expt. No.	DC-Apo	DC	Apo	PBS
1	10/13 (77%)	1/11 (9%)	1/12 (8%)	0/12 (0%)
2	8/10 (80%)	1/11 (9%)	1/12 (8%)	0/10 (0%)
3	8/10 (80%)	1/10 (10%)	1/10 (10%)	0/10 (0%)
	DC + Apo	DC-Nec	Nec	DC-Spleen
1	1/12 (8%)	2/13 (15%)	1/8 (12%)	0/12 (0%)
2	1/11 (9%)	2/11 (18%)	2/11 (18%)	0/12 (0%)

^a C57BL/6 mice were vaccinated s.c. with 2×10^5 DCs loaded with apoptotic B16 cells (DC-Apo), DCs alone, apoptotic tumor cells alone (Apo), a mixture of DCs and tumor cells (DC + Apo), DCs that had phagocytosed apoptotic spleen cells (DC-Spleen), PBS, DCs that had phagocytosed necrotic B16 melanoma cells (DC-Nec), or necrotic tumor cells alone (Nec) weekly for 4 wk and challenged with 1.3×10^4 B16 cells 1 wk later. Results are presented as the number of tumor-free survivors over the total number of animals 12 wk following tumor challenge.

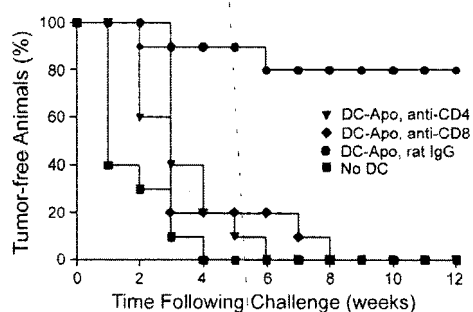


FIGURE 4. In vivo depletion of either CD4⁺ or CD8⁺ T cells ablates protective antitumor immunity. Mice (10/group) were vaccinated on days 0, 7, 14, and 21 with DC-Apo or PBS. Vaccinated animals were divided into groups treated with anti-CD4, anti-CD8, or normal rat IgG on days 27, 28, and 29. Then mice were challenged with 1.3×10^4 B16 cells on day 28. Mice were monitored every other day and were scored positive when the tumors were palpable. One representative experiment of two is shown.

obtained from mice injected with DC-Apo or control mice 4 wk after immunization. The T cell fractions were stimulated *in vitro* for 48 h with intact B16 cells or with DCs that had been pretreated with TRP-2 peptide or OVA-derived control peptide, and the medium was then assessed for the production of IFN- γ (Fig. 5). TRP-2 is a tissue differentiation Ag expressed by normal melanocytes and melanoma cells in both humans and mice (31, 42–45) and was used as a model tumor Ag. As shown in Fig. 5, only CD8⁺ T cells from DC-Apo-vaccinated mice were able to respond to intact B16 cells. However, both subsets responded to DC-TRP-2

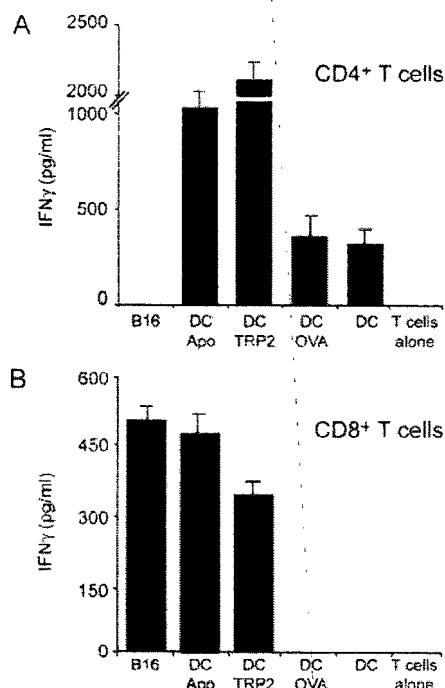


FIGURE 5. CD4⁺ or CD8⁺ T cells are primed by DC-Apo to secrete IFN- γ . CD4⁺ (A) or CD8⁺ (B) T cells were purified using MACS beads from spleens obtained from DC-Apo-vaccinated or control mice 4 wk after immunization. These cells were stimulated with intact B16 cells, DC-Apo, DC pulsed with TRP-2 peptide, or DC pulsed with OVA peptide, as described in *Materials and Methods*. After 48 h culture supernatants were harvested, and IFN- γ release was measured by ELISA. No IFN- γ was produced by the PBS control mice (not shown). The mean $\pm$ SD of triplicate determinations are shown.

and DC-Apo, with the CD4⁺ T cell responses being more vigorous (Fig. 5). As controls, CD8⁺ T cells from mice vaccinated with DC-Apo made <30 pg/ml without challenge or following challenge with DCs only or DC-OVA peptide (Fig. 5B). CD4⁺ T cells from mice injected with DC-Apo also did not release IFN- γ in the absence of DCs, but did form small amounts of cytokine following culture with DCs only or with DCs loaded with the control peptide (Fig. 5A). Although these assays were performed using mouse serum, the DCs used as APCs had been cultured in 10% FCS, and the presence of FCS-derived peptides could explain the control CD4⁺ T cell response observed against DC-OVA or DCs alone (40, 41). T cells from control nonvaccinated mice made <30 pg/ml of IFN- γ with or without challenge (data not shown). Our data indicate that IFN- γ -producing, TRP-2-specific, CD4⁺ and CD8⁺ T cells are primed when mice are vaccinated with DCs loaded with B16 melanoma.

In vivo cytotoxic effector activity induced after vaccination with DC-Apo

To evaluate whether Ag-specific cytolytic activity could be detected *in vivo* following vaccination with DC-Apo, an *in vivo* cytotoxicity assay was performed. Syngeneic splenocytes were labeled with a high concentration of CFSE and loaded with the TRP-2 peptide. These were coinjected *i.v.* with equal numbers of non-TRP-2-loaded splenocytes, which were labeled with a lower concentration of CFSE. The mice had been vaccinated with DC-Apo or DCs 4 wk earlier, and the spleens were harvested 10 h later after injection of CFSE^{high} and CFSE^{low} cells. The specific cytolytic activity was measured by the disappearance of TRP-2-loaded CFSE^{high} target cells. No cytolytic activity was observed in mice injected with DCs, but in mice injected with DC-Apo, the specific cytolytic activity was 75% when 10^7 cells were transferred and went up to 92% when 5×10^6 cells were transferred (Fig. 6). We did not determine whether CD4⁺ or CD8⁺ T cells were responsible for this lytic activity, but nevertheless, our data indicate that vaccination with DC-Apo leads to the production of effector T

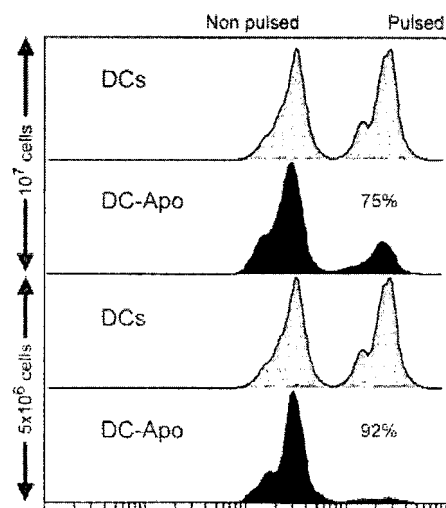


FIGURE 6. In vivo cytotoxic activity. CFSE^{high} TRP-2_{180–188}-pulsed splenocytes and equal numbers of CFSE^{low} nonpulsed splenocytes were coinjected *i.v.* into mice vaccinated with DC-Apo or DCs 4 wk after immunization. Ten hours later spleens were harvested, and *in vivo* cytotoxicity of transferred cells was measured by the selective disappearance of the TRP-2-loaded CFSE^{high} cells. Specific lysis was calculated as described in *Materials and Methods*.

cells that both secrete IFN- γ (Fig. 5) and have cytolytic activity (Fig. 6).

Discussion

DCs-based immunotherapy is being developed as a novel approach to vaccination against cancer. DCs have the potential to be loaded with a spectrum of tumor Ags and to prime both CD8 $^{+}$ and CD4 $^{+}$ T cells. However, the immune response to DCs loaded with different sources of tumor Ag requires investigation. In this study we demonstrate that B16 melanoma cells undergoing apoptotic death as a result of irradiation and culture are an effective source of Ag for DCs-based vaccines, including the induction of both CD8 $^{+}$ - and CD4 $^{+}$ -dependent, long-lived antitumor responses. The B16 melanoma model has been a valuable model for the study of tumor immunity, but previous reports using apoptotic/necrotic cells to load DCs (46, 47) have not documented combined CD4 $^{+}$ and CD8 $^{+}$ dependent long term immunity.

In addition to being a source of tumor Ags for MHC class I and II products, dying cells may influence the maturation state of the DCs and thereby the quantity and quality of the ensuing immune response. There are conflicting reports about whether apoptotic or necrotic cells are capable of inducing DC maturation. Sauter et al. (48) found that incubation of DCs with necrotic, but not apoptotic, tumor cell lines induce maturation, while other reports concluded that incubation with apoptotic cells is sufficient (25, 26, 46, 49). Our results show that after coculture with gamma irradiation induced apoptotic B16 melanoma cells, a high proportion of DCs phagocytose the apoptotic cells and up-regulate the expression of CD86 and MHC class II molecules (Fig. 2A). However, it is possible that the presence of necrotic cells (<20%) in our apoptotic cell preparation was relevant to the observed DCs maturation.

We also characterized the DCs that migrate to the draining lymph nodes following s.c. injection, comparing DCs that had been pulsed or not pulsed with apoptotic cells. The migrants uniformly expressed high levels of CD86 and MHC II molecules (Fig. 2B) comparable to fully mature and immunogenic DCs. However, when we enumerated the migrating DCs only ~0.5% of the original inoculum was found in the draining lymph nodes 1 day after injection.

Our current vaccine model uses four weekly injections of DC-Apo, and we have used this to prevent rather than treat B16 melanomas. Eighty percent of the mice remained tumor-free 12 wk following challenge with B16 cells (Fig. 3A). No protection was observed when DC-Apo-vaccinated mice were challenged with the syngeneic EL-4 lymphoma (Fig. 3B), suggesting that the immune response was directed against B16 melanoma Ag. Moreover, vaccination with DC-Apo induced immunological memory, since, again, 80% of mice remained tumor-free when they received the tumor challenge 10 wk after immunization (Fig. 3C). It will be important to use DCs in the therapeutic setting, but at the tumor cells doses we have used, large (~1-cm diameter) tumors are evident by 14 days, too quickly to study therapy. However, a critical limitation in the current use of DC-Apo, as evident in Fig. 2B, is the low number of migrating DCs 1 day after injecting 200,000 cells in each of the four paws, since a total of only ~4,000 DCs were found in the draining lymph nodes. This suggests that with current methods the injected DCs are functioning far below their potential. This will need to be addressed to achieve strong T cell immune responses and to approach therapeutic models.

When either CD4 $^{+}$ or CD8 $^{+}$ T cell subsets were depleted by Ab treatment of vaccinated mice, the immune protection was completely abrogated, indicating that both T cell subsets were necessary for effective, *in vivo* vaccination with DC-Apo (Fig. 4). *In vitro* T cell assays also indicated that both CD4 $^{+}$ and CD8 $^{+}$ T cells

were primed to produce IFN- γ after restimulation with DCs loaded with apoptotic B16 cells or DCs loaded with the TRP-2 melanoma Ag (Fig. 5). In addition, the CD8 $^{+}$ cells were able to produce IFN- γ upon coculture with B16 melanoma cells, and the immunized mice were able to kill TRP-2-coated splenocytes *in vivo* (Fig. 6). However, NK cells as well as other innate pathways, such as phagocytes, NKT cells, and complement components, could also contribute to the resistance that takes place in the presence of tumor-specific T cells that are elicited by DC-Apo. In the results reported by Hung et al. (33), the immune response induced by vaccination with B16 cells engineered to secrete GM-CSF required CD4 $^{+}$ T cells at the effector phase of the antitumor response. In contrast, their data showed that depletion of CD8 $^{+}$ T cells only partially reverted the protective response, suggesting that vaccination with B16:GM-CSF-secreting cells did not induce effective CD8 $^{+}$ immunity. Similarly, Shibagaki et al. (32) showed that vaccination with B16:GM-CSF-secreting cells was relatively inefficient with regard to CTL and memory induction. Our data indicate that DC-Apo have an advantage in eliciting combined helper and cytotoxic T cell immunity.

Although the CD4 $^{+}$ helper cells did not recognize B16 melanoma cells directly, because the latter do not express MHC class II, the immune CD4 $^{+}$ T cells did recognize DCs loaded with tumor (Fig. 5). Ossendorp et al. (50) have previously shown that optimal CTL induction requires cognate CD4 $^{+}$ T cell help even for MHC class II-negative tumors. In that study the authors used a peptide vaccination strategy to show that depletion of CD4 $^{+}$ T cells during either the induction or effector phase of the immune response abrogates the survival of preimmunized mice. Likewise, Mumberg et al. (51) showed that adoptively transferred CD4 $^{+}$ T cells eliminate MHC class II-negative tumor cells in SCID mice. IFN- γ was required, but it did not induce MHC class II expression on the tumor, suggesting that tumor Ag were being cross-presented by MHC class II $^{+}$ host cells. Our data together with others (31, 33, 50) indicate that tumor-specific CD4 $^{+}$ T cells are required upon tumor challenge for the effective antitumor response in immunocompetent mice. Such T cells may have several functions: to help CD8 $^{+}$ T cell function in the draining lymph node, to contribute to CTL infiltration into the tumor bed (52), to maintain *in situ* CTL effector functions (53), to release factors that more directly provide resistance against the tumor, i.e., IFN- γ (54–56), and/or to recruit other effector cells (33).

We found that the TRP-2_{180–188} peptide was recognized by both CD4 $^{+}$ and CD8 $^{+}$ T cells. This peptide has been identified as a relevant MHC class I (K b) epitope (43, 44), but Wang et al. (31) have reported that immunization with DCs loaded with TRP-2_{180–188} peptide fused to a cell-penetrating peptide induce a CD4 $^{+}$ T cell response as well, and that this is required for antitumor immunity, as evidenced by CD4 $^{+}$ T cell depletion and the use of CD4 $^{+}$ knockout mice. Peptides are known to bind to MHC class II molecules via a nine amino acid core sequence (57–59), and this 9-aa core also is recognized by the TCR (60). Therefore, the strong CD4 $^{+}$ T cell response to TRP-2 that we observed is not totally surprising.

When DCs are loaded with TRP-2 peptide only (using peptide itself or a cell-penetrating fusion TRP-2 peptide, TRP-2 protein transduction) and used to vaccinate mice against B16 melanoma, the survival was found to be lower than that observed with B16:GM-CSF-secreting cells, presumably because TRP-2 is but one of many Ags in B16 cells (31–33). We also suspect that our use of DC-Apo immunizes CD4 $^{+}$ or CD8 $^{+}$ T cells to many more B16 melanoma Ags than TRP-2, but these remain to be identified.

A perplexing finding in our study was that DCs loaded with apoptotic cells, but not necrotic cells, were immunogenic, even though both sources of dying cells were phagocytosed well by the

DCs. Shimizu et al. (61), using lysates or peptide-pulsed DCs vaccines, reported that CD4⁺ T cell protection required the addition of IL-2 and the addition of the immunogenic protein keyhole limpet hemocyanin to the immunizing protocol. Kotera et al. (46) have reported that DCs pulsed with highly enriched apoptotic or necrotic (lysates) B16 cells have equivalent antitumor activity, although their experiments were only followed for 18 days after challenge, and the immunization conditions only allowed for a delay in tumor growth. However, there are other examples showing that DCs loaded with apoptotic cells are more efficient than DCs loaded with necrotic cells in activating T cells (24, 27, 62, 63). In the results reported by Albert et al. (27), Ag acquired from apoptotic cells were efficiently cross-presented by DCs, while DCs carrying Ag derived from necrotic cells were inefficient at inducing CTLs. Strome et al. (62) found that DCs loaded with irradiated tumor cells were effective stimulators of OVA-specific CTL clones in vitro, whereas DCs loaded with freeze-thawed tumor cells failed to stimulate IFN- γ secretion. Furthermore, they reported an inhibitory effect of freeze-thawed tumor cells on the in vitro CTL activity. Similar results were observed when they used human melanoma cell lines and gp100-specific CTL clones. Then the authors assessed the two Ag-loading strategies in vivo, and again only DCs loaded with irradiated tumor cells were effective in preventing the outgrowth of the OVA-expressing E.G7 thymoma. Schnurr et al. (63) have found that DCs bearing apoptotic pancreatic tumor cells were better suited than tumor-lysate-loaded DCs to activate both CD8⁺ and CD4⁺ T cells in vitro. Using a squamous cell carcinoma model, Hoffmann et al. (24) have reported that apoptotic cell-loaded DCs likewise induce stronger CTL responses compared with cell lysate-loaded DCs. Perhaps necrotic cell lysates are adequate for loading DCs with tumor-associated Ags only when the latter are more resistant to proteolysis or when the lysates provide additional stimulatory stimuli for the DCs.

Many studies have been conducted using dying tumor cells as a source of Ags (22–26, 46, 62, 63). However, none of these has shown in vivo induction of combined CD4⁺ and CD8⁺ antitumor immunity. In the present study we found that DCs phagocytosing apoptotic tumor cells elicit long-lived combined CD4⁺ and CD8⁺ immunity against the poorly immunogenic B16 melanoma. DC-Apo have been shown by Berard et al. (22) to be able to prime naive human T cells in vitro to melanoma Ags. These findings together with our preclinical model set the stage for comparable studies in humans with DC-Apo as adjuvants for active immunization against cancer.

Acknowledgments

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