Retrovirally Transduced Mouse Dendritic Cells Require CD4+ T Cell Help to Elicit Antitumor Immunity: Implications for the Clinical Use of Dendritic Cells

Stefan Schnell, James W. Young, Alan N. Houghton and Michel Sadelain

_J Immunol_ 2000; 164:1243-1250; doi: 10.4049/jimmunol.164.3.1243

http://www.jimmunol.org/content/164/3/1243

References

This article cites 38 articles, 26 of which you can access for free at: http://www.jimmunol.org/content/164/3/1243.full#ref-list-1

Subcription

Information about subscribing to _The Journal of Immunology_ is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Retrovirally Transduced Mouse Dendritic Cells Require CD4⁺ T Cell Help to Elicit Antitumor Immunity: Implications for the Clinical Use of Dendritic Cells

Stefan Schnell,*‡ James W. Young,† Alan N. Houghton,‡† and Michel Sadelain*§‡

Presentation of MHC class I-restricted peptides by dendritic cells (DCs) can elicit vigorous antigen-specific CTL responses in vivo. It is well established, however, that T cell help can augment CTL function, raising the question of how best to present tumor-associated MHC class I epitopes to induce effective tumor immunity. To this end, we have examined the role of MHC class II peptide-complexes present on the immunizing DCs in a murine melanoma model. To present MHC class I- and II-restricted Ags reliably on the same cell, we retrovirally transduced bone marrow-derived DCs with the model Ag OVA encoding well-defined class I- and II-restricted epitopes. The importance of CD4⁺ T cells activated by the immunizing DCs in this model is demonstrated by the following findings: 1) transduced DCs presenting class I and class II epitopes are more efficient than class I peptide-pulsed DCs; 2) MHC class II-deficient DCs fail to induce tumor protection; 3) CD4⁺ T cell depletion abolishes induction of tumor protection; and 4) DCs presenting bovine serum Ags are more effective in establishing tumor immunity than DCs cultured in syngeneic serum. When MHC class II-deficient DCs were directly activated via their CD40 receptor, we indeed observed a moderate elevation of OVA-specific CTL activity. However, this increase in CTL activity was not sufficient to induce in vivo tumor rejection. Thus, our results demonstrate the potency of genetically modified DCs that express both MHC class I and II epitopes, but caution against the use of DCs presenting only the former.

Dendritic cells (DC) are potent immunostimulatory cells capable of inducing vigorous immune responses in vitro and in vivo. Their remarkable effectiveness is in large part due to their efficiency in capturing, processing, and presenting Ags along with costimulatory signals (1). DCs are able to stimulate both CD4⁺ and CD8⁺ T lymphocytes (1). The ability of DCs to activate CD8⁺ CTL directly has prompted many investigators to use DCs pulsed with tumor-derived MHC class I-restricted peptides to elicit antitumor immune responses (2). It has thus been demonstrated that tumor-specific CD8⁺ CTLs and protective immunity against a subsequent tumor challenge can be induced in mice by administration of syngeneic DCs pulsed with defined MHC class I epitopes (3–5). These results provide the experimental support for many current clinical trials based on the administration of autologous DCs pulsed with defined peptides known to bind to HLA class I molecules. Although there are clear examples where cytotoxic activity can be induced without T cell help (6, 7), strong and sustained CD8⁺ CTL responses typically depend on the provision of T cell help (8, 9). Indeed, there are numerous indications that it is important, if not necessary, to activate T helper cells to establish effective antitumor immunity (10–12). However, there has been less focus on the requirements for inducing T cell help to augment antitumor responses, owing to the emphasis placed until recently on identifying target epitopes recognized by cytotoxic T cells and Abs.

To investigate the requirement for interactions between immunizing DCs and T helper cells, we examined the function of MHC class II-peptide complexes on adoptively transferred DCs. These complexes govern interactions of the immunizing DCs with host MHC class II-restricted T cells. We used retroviral-mediated gene transfer to transduce a well-defined Ag encoding H-2Kb and I-Ab-restricted epitopes (13, 14) and thereby to coexpress MHC class I- and II-restricted epitopes in individual cells. To investigate further the importance of class II-restricted Ag presentation to recipient CD4⁺ helper T cells, we used DCs that lack MHC class II molecules on their surface. We demonstrate that autologous DCs lacking MHC class II molecules retain the ability to elicit strong CTL responses in vivo but, unlike MHC II⁺ DCs, fail to establish tumor immunity. Following the recent demonstration that CD40 activation of APCs can mimic the CD40/CD40 ligand (CD40L) interactions occurring between CD4⁺ T cells and DCs (15–17), we investigated whether CD40 activation of DCs lacking MHC II would alleviate the dependence of tumor rejection on DC-T helper cell interactions. To investigate what Ags could play a role in DC-T helper cell interactions, we compared DCs cultured in the presence of xenogeneic or syngeneic serum. We found that the latter are less potent in inducing tumor immunity. Taken together, our findings illustrate the value of enrolling CD4⁺ T cells to achieve antitumor immunity and underline the need to combine class I and class II epitopes appropriately to maximize the effectiveness of adoptively transferred DCs.

*Department of Human Genetics; †Department of Medicine; ‡Immunology Program, Memorial Sloan-Kettering Cancer Center and Weill Medical College of Cornell University, New York, NY 10021

Received for publication August 30, 1999. Accepted for publication November 16, 1999.

Copyright © 2000 by The American Association of Immunologists

0022-1767/00/$02.00
Materials and Methods
Mice and cell lines
Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female MHC class II−/− backcrossed to the C57BL/6 genetic background (N13) were purchased from Taconic (Germantown, NY) (18). All mice were used at the age of 5–8 wk and housed at the Central Animal Facility of Memorial Sloan-Kettering Cancer Center (MSKCC). EL4, a C57BL/6 thyoma, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). MO4 (19), a chicken OVA-expressing subclone of B16, itself a C57BL6-derived melanoma, was kindly provided by Dr. P. Srivastava, University of Connecticut Health Center, Farmington, CT.

Antigens
The H-2Kb-restricted peptide SIINFEKL, corresponding to amino acid residues 257–264 of chicken OVA (13) was synthesized in the Peptide Synthesis Facility at MSKCC and highly purified (>99%) as assessed by HPLC and amino acid analysis.

Retroviral vector construction
The retroviral vector SFG is derived from the Moloney murine leukemia virus (20). The vector SFG-OVA encoding the chicken OVA cDNA was constructed in several steps. First, the plasmid pAC-neo-OVA containing the OVA cDNA was cut partially with Ncol and then with PstI (419 bp). Another fragment was prepared by digesting pAc-neo-OVA with PstI and AlwNI (587 bp). A PCR reaction was performed to yield the 3′ fragment from the AlwNI site (forward primer: 5′-CACATGCAGAAAATCAATG-3′) to adherent cells from day 2 cultures were collected and transferred onto GM-CSF and 50 U/ml recombinant murine IL-4 (Genzyme, Cambridge, MA). Following 2 days incubation, adherent cells were collected and transferred onto Xhol and BamHI sites (reverse primer: 5′-CCCGGCCATCTCGAGTTTAAAAAGGGGAACACATC-3′). The PCR product (190 bp) was digested with AlwNI and BamHI. The retroviral backbone was generated by cutting SFG-NTP (21) with Ncol and BamHI. The final product was sequenced to ascertain correct ligation of all the fragments. An ecotropic producer cell line was generated from the gp-E86 packaging cell line (22) as previously described (23).

Generation of bone marrow DCs and retroviral transduction
The procedure used for the generation of DCs in the presence of FBS (DC+FBS) was that described by Inaba et al. (24) with some minor modifications. Briefly, cells expressing CD8, CD4, B220, and I-Aβ were depleted from bone marrow cells by two rounds of affinity depletion using anti-CD8, GK1.5 (anti-CD4), RA3-3A1/6.1 (anti-B220), and M5/114.15.2 (anti-I-A). Mice were injected i.v. with 104 DCs without peptide (DC FBS) or OVA-transduced DCs (II−/−DC FBS/OVA), or DCs pulsed with SIINFEKL (DC+FBS/DC+FBS/SIINFEKL), or MHC II−/−DCs pulsed with SIINFEKL (II−/−DC+FBS/SIINFEKL). In some experiments, transduced II−/−DCs were activated with the agonistic activating anti-CD40 Ab IC10 or isotype-matched control Ab (25). Naïve adult C57BL/6 mice were immunized once with 104 DCs and challenged 10 days later with a s.c. injection of 1 × 105 MO4 melanoma cells. Tumor growth and survival were monitored three times per week.

In vivo depletion of CD4+ T cells
Mice were injected i.v. with 250 µg of anti-CD4 mAb GB1.5 or control anti-DR-5 Ab (SFR3-DR-5). The first injection was given 6 days before inoculation of DCs, followed by three other injections at 3-day intervals. The specific depletion was >99% as determined by flow cytometry (data not shown).

CD40 activation of DCs
Day 6 DC cultures (1 × 106/ml) were treated with control Ig or the agonistic activation Ab IC10 (10 µg/ml; kindly provided by Dr. H.-C. Liou, Cornell University, New York, NY) for 24 h (24). DCs treated with IC10 formed densely packed clusters, whereas control-treated cultures exhibited relatively sparse aggregates. For surface phenotyping, cells were stained in PBS-2% FBS for 30 min at 4°C. CD40 activation was detected by Western blot analysis. Cells were lysed, and 50 µg of protein from each sample was resolved on a 15% SDS-PAGE gel, probed with a monoclonal antibody (Becton Dickinson, Mountain View, CA), using forward- and side-scatter charac-

teristics to exclude dead cells. Data were analyzed using CellQuest (Becton Dickinson).

Mixed leukocyte reaction
Responder splenocytes from H-2Kb (CBA) mice were enriched for T cells by an initial plastic-adherence for 1 h at 37°C. The nonadherent cells were then opsonized with anti-B220 and I-A mAbs and removed using immune-magnetic beads (Dynal, Oslo, Norway). Responder T cells were cultured at 4 × 105 105 cells with 0.5 × 105.125 × 105 irradiated DCs for 4 days in flat-bottom microtiter plates. Proliferation of the responding T cells was based on the incorporation of 1[3H]thymidine (1 µCi/well) during the last 18 h of culture and measured in a Wallac (Gaithersburg, MD) scintillation counter.

CTL induction in mice
Naïve adult C57BL/6 mice were injected i.v. with 104 SIINFEKL-pulsed or OVA-transduced DCs. DCs were washed extensively with HBSS before i.v. injection. Splenocytes were harvested 7 or 30 days later and restimulated in vitro for 5 days with irradiated SIINFEKL-pulsed C57BL/6 splenocytes in RPMI 1640, 10% heat-inactivated FCS, 50 µM 2-ME, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cytolytic activity was next measured in a standard 4-h 51Cr release assay.

Cell-mediated cytotoxicity assays
In vitro restimulated lymphocytes were tested for cytolytic activity in a standard 4-h 51Cr release assay against the C57BL6 thyoma EL4, either alone or pulsed with SIINFEKL. Target cells were radiolabeled with 100 Ci/well 51Cr/105 cells for 1 h at 37°C. Cell-pulsed EL4 cells were prepared by incubating EL4 cells with the radiolabel and SIINFEKL peptide at a concentration of 10 µg/ml. Maximum, spontaneous, and specific 51Cr release were determined in standard fashion.

Immunization of mice with DCs
Bone marrow-derived DCs were generated as described above. Donors were either syngeneic wild-type C57BL6 mice or congenic MHC II−/− mice. Recipient mice were treated with either PBS; DCs without peptide (DC FBS) or DC+FBS); DCs transduced with the OVA cDNA (DC+FBS/OVA), or OVA-transduced MHC II−/− DCs (II−/−DC+FBS/OVA); DCs pulsed with SIINFEKL (DC+FBS/DC+FBS/SIINFEKL), or MHC II−/− DCs pulsed with SIINFEKL (II−/−DC+FBS/SIINFEKL). In some experiments, transduced II−/− DCs were activated with the agonistic activating anti-CD40 Ab IC10 or isotype-matched control Ab (25). Naïve adult C57BL/6 mice were immunized once with 104 DCs and challenged 10 days later with a s.c. injection of 1 × 105 MO4 melanoma cells. Tumor growth and survival were monitored three times per week.

Embryo transfer of CD4+ T cells
To detect class II-restricted T cell response, EL4 cells were pulsed with OVA-derived SIINFEKL peptide and i.v. injected into syngeneic wild-type C57BL6 mice or congenic MHC II−/− mice. Recipient mice were injected i.v. with 104 DCs without peptide (DC FBS) or DC+FBS); DCs transduced with the OVA cDNA (DC+FBS/OVA), or OVA-transduced MHC II−/− DCs (II−/−DC+FBS/OVA); DCs pulsed with SIINFEKL (DC+FBS/DC+FBS/SIINFEKL), or MHC II−/− DCs pulsed with SIINFEKL (II−/−DC+FBS/SIINFEKL). In some experiments, transduced II−/− DCs were activated with the agonistic activating anti-CD40 Ab IC10 or isotype-matched control Ab (25). Naïve adult C57BL6 mice were immunized once with 104 DCs and challenged 10 days later with a s.c. injection of 1 × 105 MO4 melanoma cells. Tumor growth and survival were monitored three times per week.

In vivo depletion of CD4+ T cells
Mice were injected i.v. with 250 µg of anti-CD4 mAb GB1.5 or control anti-DR-5 Ab (SFR3-DR-5). The first injection was given 6 days before inoculation of DCs, followed by three other injections at 3-day intervals. The specific depletion was >99% as determined by flow cytometry (data not shown).

CD40 activation of DCs
Day 6 DC cultures (1 × 106/ml) were treated with control Ig or the agonistic activation Ab IC10 (10 µg/ml; kindly provided by Dr. H.-C. Liou, Cornell University, New York, NY) for 24 h (24). DCs treated with IC10 formed densely packed clusters, whereas control-treated cultures exhibited relatively sparse aggregates. For surface phenotyping, cells were stained in PBS-2% FBS for 30 min at 4°C, using fluorochrome-conjugated mAbs specific for B7.1 (16-10A1), B7.2 (GL-1), and ICAM-1 (3E2) (PharMin
gen). Isotype-matched mAbs were used as controls. Fc receptors were blocked with anti-CD16/CD32 mAb (2.462, PharM
gen) before staining. Acquisition was performed on a FACScan (Becton Dickinson) using forward- and side-scatter parameters to exclude dead cells. Bcl-xL expression was detected by Western blot analysis. Cells were lysed, and 50 µg of protein from each sample was resolved on a 15% SDS-PAGE gel, probed for Bcl-xL (2E20; Santa Cruz, Santa Cruz Biotechnology, CA), and detected with the appropriate HRP-conjugated secondary Ab.

Statistical analyses
Recipient mice were randomly assigned to the different treatment groups. The survival rates for the different immunizations affected their survival rates, the nonparametric Wilcoxon rank sum test procedure was employed (26). Animals free of tumors by day 50 were then followed through days 80–100.
FIGURE 1. OVA-transduced DCs confer better tumor protection than SIINFEKL peptide-pulsed DCs. Naive adult C57BL/6 mice were immunized once with either PBS (PBS, n = 10), DCs without peptide (DC, n = 10), DCs transduced with the OVA cDNA (DC-OVA, n = 10), DCs pulsed with SIINFEKL (DC-SIINFEKL, n = 9), or mock-transduced DCs pulsed with SIINFEKL (DC-SIINFEKL mock-transduced, n = 9) (Wilcoxon rank sum test, p < 0.02, DC-OVA vs. DC-SIINFEKL). They were challenged 10 days later with a single s.c. injection of 1 × 10⁵ MO4 melanoma cells (day 0 on C). All mice given OVA-transduced DCs were protected up to day 100 (data not shown). About half of the animals immunized with peptide-pulsed DCs were protected when given an equal number of DCs (see also Fig. 2 and Fig. 7). No protection was achieved in immunized mice challenged with the parental B16 melanoma that does not express OVA (data not shown). To demonstrate efficient transduction of the bone marrow-derived DCs, B7.2 bright cells were sorted to high purity (A) and analyzed by Southern blot (B), which shows on average 1 vector copy/cell of the OVA cDNA. The OVA/EB ratio indicates the average vector copy number in control and DC samples. The retroviral producer cell line (E86OVA) bears four copies per cell.

Results

Genetically modified DCs that express the OVA cDNA are more potent inducers of tumor immunity than peptide-pulsed DCs

Expression of the cDNA encoding an Ag in DCs was compared with the loading of DCs with a class I-restricted peptide for stimulation of Ag-specific T cell responses and tumor immunity. We transduced bone marrow-derived DCs with the cDNA of chicken OVA or pulsed them with the OVA-encoded, Kβ-restricted peptide SIINFEKL. The former were transduced with a retroviral vector that placed OVA expression under the transcriptional control of the retroviral long terminal repeat. Mammalian cells transfected with the chicken OVA cDNA secrete OVA (27–29), which is therefore available for endocytic uptake and presentation by MHC class II molecules during and after retroviral transduction. DCs sorted to high purity based on their B7.2 marker expression (Fig. 1A) bore on average one vector copy per cell as shown by Southern blot analysis (Fig. 1B). Peptide-loaded DCs were pulsed with peptide immediately before injection and administered in an amount equal to the transduced DCs. Transduced and peptide-loaded DCs showed the same phenotypic characteristics in terms of cell surface expression of MHC class I, class II, B7.1, B7.2, and ICAM-1 (data not shown). We consistently found that syngeneic C57BL/6 mice immunized with a single dose of 10⁵ OVA-transduced DCs were all protected against a subsequent challenge with the OVA-transfected B16 melanoma MO4 (Fig. 1C). By comparison, about half of the animals immunized with DCs pulsed with SIINFEKL were protected. Mock-transduced DCs pulsed with the SIINFEKL peptide before injection achieved the same result (Fig. 1C), establishing that retroviral transduction did not in itself enhance DC function. Under these conditions, the transduced DCs bearing the OVA cDNA encoding both MHC class I and class II epitopes were therefore more potent in establishing tumor protection than DCs presenting only the Kβ-restricted epitope.

MHC II^−/− DCs, either transduced or peptide-pulsed, are less potent in inducing immunity than MHC II^+/− DCs

To address whether enhanced tumor protection was conferred by the class II-restricted epitope encoded in the OVA cDNA, we transduced DCs lacking MHC class II molecules on their surface (II^−/− DCs). As shown in Fig. 2A, the activity of DCs lacking MHC class II was not only reduced but practically abolished with respect to induction of tumor protection. The same was observed when mice were immunized with SIINFEKL peptide-pulsed DCs lacking MHC class II (Fig. 2B). MHC class II^−/− DCs either transduced with the OVA cDNA (Fig. 2A) or pulsed with the SIINFEKL peptide (Fig. 2B) showed comparable effectiveness, suggesting that presentation of the MHC class I epitope was not functionally different. MHC class II molecules expressed on the immunizing DCs were therefore essential to achieve tumor immunity, suggesting that CD4^+ lymphocytes engaged by these MHC molecules play a key role in establishing tumor protection. The involvement of CD4^+ T cells was confirmed by in vivo depletion of CD4^+ T lymphocytes. Administration of a CD4-specific mAb (GK1.5) during the induction phase abolished tumor protection induced by peptide-pulsed DCs (6) as well as by genetically-modified DCs (Fig. 2C). On the other hand, MHC II^−/− DCs either transduced with the OVA cDNA (Fig. 2A) or pulsed with the SIINFEKL peptide (Fig. 2B) showed comparable effectiveness, indicating that presentation of the MHC class I epitope was not functionally different in these two cell populations.

MHC II^−/− DCs elicit SIINFEKL-specific CTLs in vivo

We next examined whether DCs lacking MHC class II could not induce tumor immunity because of a failure to induce SIINFEKL-specific CTLs. Mice were immunized with SIINFEKL-pulsed II^+/− or II^−/− DCs, and spleens were harvested 7 days later to measure CTL activity. Mice immunized with DCs lacking or expressing MHC class II displayed high levels of SIINFEKL-specific
CTLs (Fig. 3). Interestingly, we consistently found that mice immunized with wild-type DCs had a high level of background cytotoxic activity that was not observed in mice immunized with DCs lacking MHC class II. These data indicate that DCs lacking MHC class II molecules induce tumor-specific CTLs, but nonetheless fail to establish protective immunity. When examining CTL activity on day 30 after immunization, it was apparent that mice immunized with II\(^{-/-}\) DCs still had substantial levels of SIINFEKL-specific CTLs, whereas mice immunized with II\(^{+/+}\) DCs did not (Fig. 3). Combined with the observations shown in Fig. 2, these findings suggested a correlation between absence of CD4 activation, decreased long-term CTL activity, and lack of tumor immunity.

**CD40 activation of DCs lacking MHC class II does not restore induction of antitumor immunity**

To investigate further the basis for suboptimal CTL activity in mice immunized with II\(^{-/-}\) DCs that could account for the lack of tumor immunity, we activated the immunizing DCs via their CD40 receptor. Such activation has indeed been shown to support CTL induction in a CD4-independent fashion (9, 16, 17). Thus, CD40 activation would be expected to increase CTL activity and perhaps overcome the lack of a concomitant CD4 response. DCs treated with the agonistic anti-CD4 mAb IC10 were efficiently activated as determined by up-regulation of cell-surface expression of B7.1, B7.2, and ICAM-1 (from 64 to 120, 60 to 105, and 90 to 120, respectively, with regard to mean log fluorescence) as well as expression of Bcl-xL (Fig. 4A). The successful activation of the II\(^{-/-}\) DCs was also reflected in a substantial increase in SIINFEKL-specific CTL activity (Fig. 4B). Mice given CD40-activated DCs showed SIINFEKL-specific CTL activity in their spleen by day 30, whereas mice given DCs treated with a control Ab did not (Fig. 4B). However, as shown in Fig. 4C, CD40 activation of DCs lacking MHC class II did not improve their ability to induce antitumor immunity. This indicated that mimicry of CD40/CD40L interactions occurring between DCs and T helper lymphocytes was not sufficient to restore tumor immunity.

**DCs generated in syngeneic serum are less potent in vivo than DCs generated in xenogeneic serum**

The observation that antitumor immunity conferred by DCs is dependent on host CD4\(^{+}\) T cells and MHC class II Ags expressed by DCs and host CD4\(^{+}\) cells raised a paradox: How can DCs pulsed only with an MHC class I-restricted peptide be effective? This prompted an investigation of the nature of Ags presented by MHC class II molecules in that setting. The possible role of Ags to
which DCs are exposed during their culture before infusion was examined. DCs are typically cultured in FBS, a rich source of xenogeneic Ags. Conditions were established to generate bone marrow-derived DCs in syngeneic C57BL/6 serum (B6S). DCs generated in the presence of FBS or B6S, respectively DCFBS and DCB6S, had identical profiles in terms of expression of I-A<sup>b</sup>, B7.1, B7.2, ICAM-1, and CD11c (Fig. 5). Additionally DCFBS and DCB6S showed equal stimulatory capacity in allogeneic MLRs (Fig. 6). Furthermore, peptide-pulsed DCFBS and DCB6S were equally capable of inducing a specific CTL response against SIINFEKL, but DCB6S did not generate the elevated background cytotoxicity caused by DCFBS (data not shown). By independent criteria, these DC populations appeared equivalent in inducing proliferative and Ag-specific CTL responses. However, as shown in Fig. 7, DCs grown in syngeneic serum showed significantly decreased potency in the induction of antitumor immunity (p < 0.01). This finding indicates that FBS Ags presented by MHC class II molecules of cultured DCs play an important role in establishing successful antitumor immunity, likely via induction of CD4<sup>+</sup> T cell help and further activation of DCs.

Discussion

We show that DCs transduced with a cDNA encoding both MHC class I and class II epitopes are more potent in the induction of antitumor immunity than DCs loaded with the MHC class I-restricted peptide alone. Immunization of mice with MHC class II-deficient DCs, whether transduced with OVA cDNA or pulsed with SIINFEKL peptide, could generate SIINFEKL-specific CTLs B7.2, ICAM-1, and CD11c (Fig. 5). Additionally DCFBS and DCB6S showed equal stimulatory capacity in allogeneic MLRs (Fig. 6). Furthermore, peptide-pulsed DCFBS and DCB6S were equally capable of inducing a specific CTL response against SIINFEKL, but DCB6S did not generate the elevated background cytotoxicity caused by DCFBS (data not shown). By independent criteria, these DC populations appeared equivalent in inducing proliferative and Ag-specific CTL responses. However, as shown in Fig. 7, DCs grown in syngeneic serum showed significantly decreased potency in the induction of antitumor immunity (p < 0.01). This finding indicates that FBS Ags presented by MHC class II molecules of cultured DCs play an important role in establishing successful antitumor immunity, likely via induction of CD4<sup>+</sup> T cell help and further activation of DCs.
but failed to induce effective long-term immunity. Immunization with wild-type DCs cultured in syngeneic serum had similar results. Because the different treatment groups received the same number of syngeneic DCs pulsed with the same amount of SIINFEKL peptide, the outcome in terms of tumor immunity was not determined by the amount of Ag or DC dosage, but rather by cellular interactions in which the MHC class II molecules of the DCs played a critical role. OVA-transfected cells indeed secrete OVA (27, 28), which is therefore available for endocytic uptake and after retroviral transduction. Presentation of OVA-encoded MHC class II-restricted epitopes has been shown in transfected B cells (29) as well as retrovirally transduced A20 B cells (R. Brentjens, I. Riviere, S. Schnell, A. Turham, M. Saddein, unpublished observations). In this tumor model, the importance of the CD4+ T cells activated by MHC class II-peptide complexes presented by the immunizing DCs is demonstrated by the following findings: 1) transduced DCs presenting MHC class I and II epitopes at the same time are more effective than class I peptide-pulsed DCs; 2) MHC class II-deficient DCs fail to induce tumor protection; 3) CD4+ T cell depletion abolishes tumor protection induced by MHC class II-bearing DCs; and 4) DCs cultured in FBS, unavoidably loaded with xenogeneic MHC class II-restricted helper peptides, are more efficacious than DCs cultured in syngeneic serum.

Further investigation of the SIINFEKL-specific CTL activity generated by MHC class II−/− DCs shows that these CTLs are short-lived compared with their counterparts induced by MHC class II+/+ DCs. The presence of memory CTLs correlated with tumor immunity. Indeed, mice immunized with MHC class II+/+ DCs were protected against tumor challenges (about one-half of them when treated with peptide-pulsed DCs and all of them when given genetically modified DCs, Fig. 1) and displayed substantial SIINFEKL-specific CTLs 30 days after primary immunization (Fig. 3). Mice given MHC class II−/− DCs did not develop tumor immunity (Fig. 2) and did not show SIINFEKL-specific CTLs 30 days after primary immunization (Fig. 3). These data suggest that conditions leading to the activation of CD4+ cells facilitated the generation of memory CTLs and tumor immunity. This correlation, however, does not imply that the only function of the activated CD4+ T cells is to provide some form of help to CTL.

To begin to address the function of host CD4+ T cells, we investigated whether their function is to activate DCs via CD40/CD40L interactions. Indeed, it has been shown that T helper cells can activate DCs via CD40 and increase their ability to stimulate CTLs (15–17). It is therefore possible that CD40 activation of immunizing DCs could reduce the CD4+ T cell dependence of CTL responses against tumors. We asked whether CD40 activation could enhance the function of DCs that could not engage CD4+ T cells via their TCR. After ex vivo CD40 activation, however, we found that II−/− DCs still failed to establish tumor immunity (Fig. 4C), but the CD40-activated II−/− DCs were now able to induce SIINFEKL-specific CTLs that persisted to day 30 (Fig. 4B). The level of CTL activity was about 8-to 16-fold less than that found in mice given an equal number of II+/+ DCs (see Figs. 3 and 4B). This observation does not dismiss the critical role played by CD40/CD40L interactions, but rather suggests that the full effect of CD40 activation is dependent on active CD4+ T cell-DC interactions and/or that CD4+ T cells exert other important effects that may or may not be mediated via the DC. For example, CD4+ T cells could sustain CTL function by secreting IL-2 or by activating other effector cell types (12, 30). However, CD4+ T cells have been shown not to be required in the effector phase of tumor immunity against different B16 melanoma sublines (31, 32).

In the first interpretation, CD40 activation does not result in maximal DC activation. It is noteworthy that, in other studies investigating the role of CD40 in tumor immunity, CD40 activation was performed in the presence of CD4+ cells (33–38). Thus, when CD40−/− mice immunized against a tumor failed to develop tumor immunity, CD40+/+ DCs restored immunity (38). However, this

**FIGURE 6.** Comparison of allogeneic stimulatory capacity of bone marrow-derived DCs generated either in FBS or syngeneic serum containing medium to stimulate an allogeneic MLR. Purified CBA/J T cells were cocultured with irradiated spleen or DCs at different stimulator to responder ratios in 96-well flat-bottom microtiter plates. Cell proliferation was measured on day 4 by counting the overnight incorporation of [3H]thymidine (mean ± SE in triplicate wells). CBA/J T cells responded equally to C57BL/6 DCs generated in syngeneic serum. Recipient mice were treated with DC FBS (n = 22), DC B6S (n = 16), or DC B6S-SIINFEKL (n = 19) and challenged 10 days later (day 0 on the figure) with a s.c. injection of 1 × 106 MO4 melanoma cells.

The difference in tumor protection between peptide-pulsed DCs was measured on day 4 by counting the overnight incorporation of [3H]thymidine (mean ± SE in triplicate wells). CBA/J T cells responded equally to DC FBS-SIINFEKL, but failed to induce effective long-term immunity. Immunization with wild-type DCs cultured in syngeneic serum had similar results. Because the different treatment groups received the same number of syngeneic DCs pulsed with the same amount of SIINFEKL peptide, the outcome in terms of tumor immunity was not determined by the amount of Ag or DC dosage, but rather by cellular interactions in which the MHC class II molecules of the DCs played a critical role. OVA-transfected cells indeed secrete OVA (27, 28), which is therefore available for endocytic uptake during and after retroviral transduction. Presentation of OVA-encoded MHC class II-restricted epitopes has been shown in transfected B cells (29) as well as retrovirally transduced A20 B cells (R. Brentjens, I. Riviere, S. Schnell, A. Turham, M. Saddein, unpublished observations). In this tumor model, the importance of the CD4+ T cells activated by MHC class II-peptide complexes presented by the immunizing DCs is demonstrated by the following findings: 1) transduced DCs presenting MHC class I and II epitopes at the same time are more effective than class I peptide-pulsed DCs; 2) MHC class II-deficient DCs fail to induce tumor protection; 3) CD4+ T cell depletion abolishes tumor protection induced by MHC class II-bearing DCs; and 4) DCs cultured in FBS, unavoidably loaded with xenogeneic MHC class II-restricted helper peptides, are more efficacious than DCs cultured in syngeneic serum.

Further investigation of the SIINFEKL-specific CTL activity generated by MHC class II−/− DCs shows that these CTLs are short-lived compared with their counterparts induced by MHC class II+/+ DCs. The presence of memory CTLs correlated with tumor immunity. Indeed, mice immunized with MHC class II+/+ DCs were protected against tumor challenges (about one-half of them when treated with peptide-pulsed DCs and all of them when given genetically modified DCs, Fig. 1) and displayed substantial SIINFEKL-specific CTLs 30 days after primary immunization (Fig. 3). Mice given MHC class II−/− DCs did not develop tumor immunity (Fig. 2) and did not show SIINFEKL-specific CTLs 30 days after primary immunization (Fig. 3). These data suggest that conditions leading to the activation of CD4+ cells facilitated the generation of memory CTLs and tumor immunity. This correlation, however, does not imply that the only function of the activated CD4+ T cells is to provide some form of help to CTL.

To begin to address the function of host CD4+ T cells, we investigated whether their function is to activate DCs via CD40/CD40L interactions. Indeed, it has been shown that T helper cells can activate DCs via CD40 and increase their ability to stimulate CTLs (15–17). It is therefore possible that CD40 activation of immunizing DCs could reduce the CD4+ T cell dependence of CTL responses against tumors. We asked whether CD40 activation could enhance the function of DCs that could not engage CD4+ T cells via their TCR. After ex vivo CD40 activation, however, we found that II−/− DCs still failed to establish tumor immunity (Fig. 4C), but the CD40-activated II−/− DCs were now able to induce SIINFEKL-specific CTLs that persisted to day 30 (Fig. 4B). The level of CTL activity was about 8-to 16-fold less than that found in mice given an equal number of II+/+ DCs (see Figs. 3 and 4B). This observation does not dismiss the critical role played by CD40/CD40L interactions, but rather suggests that the full effect of CD40 activation is dependent on active CD4+ T cell-DC interactions and/or that CD4+ T cells exert other important effects that may or may not be mediated via the DC. For example, CD4+ T cells could sustain CTL function by secreting IL-2 or by activating other effector cell types (12, 30). However, CD4+ T cells have been shown not to be required in the effector phase of tumor immunity against different B16 melanoma sublines (31, 32).

In the first interpretation, CD40 activation does not result in maximal DC activation. It is noteworthy that, in other studies investigating the role of CD40 in tumor immunity, CD40 activation was performed in the presence of CD4+ cells (33–38). Thus, when CD40−/− mice immunized against a tumor failed to develop tumor immunity, CD40+/+ DCs restored immunity (38). However, this

**FIGURE 7.** DCs generated in syngeneic serum (B6S) are substantially less active than DCs generated in FBS in the induction of tumor protection in vivo. Naïve adult C57BL/6 mice were immunized with equal numbers of DCs grown in FBS (DC FBS) or grown in syngeneic serum (DC B6S). Recipient mice were treated with DC FBS (n = 22), DC B6S-SIINFEKL (n = 22), DC B6S (n = 16), or DC B6S-SIINFEKL (n = 19) and challenged 10 days later (day 0 on the figure) with a s.c. injection of 1 × 106 MO4 melanoma cells.

The difference in tumor protection between peptide-pulsed DCs was highly significant (Wilcoxon rank sum test, p < 0.01).
rescue took place in the presence of recipient CD4+ T cells, and thus suggests that CD40/CD40L interactions were necessary for tumor immunity, but not that they were sufficient. Likewise, when administration of anti-CD40 mAb in vivo was shown to reverse tolerance induction by a tumor-related class I peptide (37), this result was achieved in the presence of recipient CD4+ cells and tumor Ags, including tumor-derived MHC class II-restricted epitopes that could have been presented by cross-priming. In both instances, other molecular interactions between CD4+ T cells and DCs might have contributed to the immune responses in addition to the CD40/CD40L interactions. In one instance, CD40 treatment has been shown to delay tumor progression in mice immunized with IIβ-DCs (38). However, a majority of animals rejected tumors without CD40 treatment in that particular model, suggesting that even a small increase in tumor-specific CTL activity may have been sufficient to prolong the delay in tumor progression. In our model, which uses an aggressive melanoma, the increase in CTL activity secondary to ex vivo CD40 treatment was not sufficient to overcome a lethal tumor challenge. Taken together, these results are consistent with an important role for molecular interactions in addition to those between CD40 and its ligand, e.g., those between MHC II and the TCR or between TRANCE and RANK (39, 40), in achieving maximal DC activation in vivo.

These findings have practical implications for the use of DCs to immunize against tumor cells. The data imply that the effectiveness of adoptively transferred DCs presenting a class I epitope is strongly enhanced by the MHC class II epitopes borne by the immunizing cells. In this interpretation, Ags present in FBS enable the infused DCs to interact with host CD4+ lymphocytes, which in turn increase the immunostimulatory function of the infused DCs and/or exert other antitumoral functions. The lesser tumor protection achieved with DCs cultured in C57BL/6 serum could be explained by the presentation of fewer and/or less antigenic class II-restricted helper epitopes (e.g., alternatively processed self Ags; Ref. 41). The exact nature of the bovine Ags active in our model and their relationship to the MO4 melanoma are difficult to specify. However, responses initiated against these Ags did not mediate tumor rejection, because immunized mice that rejected MO4 did not reject the parental B16 melanoma (data not shown). On the other hand, mice immunized with SIINFEKL-pulsed DCs were fully capable of rejecting MO4 cells that were passaged in mice and briefly cultured in serum-free medium before inoculation (data not shown). These findings argue against a role for CTL responses against FBS Ags in tumor rejection and are consistent with a predominant role for the OVA-specific CTLs (4, 5). These results further caution against the clinical use of MHC class I peptide-pulsed DCs generated under serum-free or autologous serum-containing conditions in the absence of MHC class II-restricted helper epitopes.

These data demonstrate that immunization protocols using DCs are more powerful when not only MHC class I but also MHC class II molecules are Ag loaded. One effective approach is to transduce the entire cDNA of the Ag with a retroviral vector (42). An essential question awaiting resolution is to identify what MHC class II-restricted epitopes are best suited to sustain antitumoral cytotoxic responses, whether they need to be expressed by the tumor, or whether unrelated recall Ags or superantigens would be equally or more effective.

Acknowledgments

We thank C. May for excellent technical assistance with animal studies, Dr. Y. Mori for helpful discussion, Dr. G. Heller for assistance with statistical analyses, and Dr. I. Riviere for reviewing the manuscript.
31. van Elsas, A., A. A. Hurwitz, and J. P. Allison. 1999. Combination immuno-
therapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and meta-
Enhanced therapeutic efficacy of tumor RNA-pulsed dendritic cells after genetic 
33. Mackey, M. F., J. R. Gunn, P. P. Ting, H. Kikutani, G. Dranoff, R. J. Noelle, and 
R. J. Barth, Jr. 1997. Protective immunity induced by tumor vaccines requires 
34. Mackey, M. F., J. R. Gunn, C. Maliszewsky, H. Kikutani, R. J. Noelle, and 
R. J. Barth, Jr. 1998. Dendritic cells require maturation via CD40 to generate 
35. Labeur, M. S., B. Roters, B. Pers, A. Mehling, T. A. Lugter, T. Schwarz, and 
S. Grabbe. 1999. Generation of tumor immunity by bone marrow-derived den-
evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell 
37. Diehl, L., A. T. den Boer, S. P. Schoenberger, E. I. van der Voort, 
T. N. Schumacher, C. J. Melief, R. Offringa, and R. E. Toes. 1999. CD40 acti-
vation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tol-
1999. Impaired ability of MHC class II−/− dendritic cells to provide tumor pro-
39. Bachmann, M. F., B. R. Wong, R. Josien, R. M. Steinman, A. Oxenius, and 
Y. Chos. 1999. TRANCE, a tumor necrosis factor family member critical for 
and G. Schuler. 1996. High level IL-12 production by murine dendritic cells: 
upregulation via MHC class II and CD40 molecules and downregulation by IL-4 
of a self-antigen can be processed into forms that are recognized by self-T cells. 
Retrovirally transduced human dendritic cells express a normal phenotype and 