Activation of Dendritic Cells That Cross-Present Tumor-Derived Antigen Licenses CD8$^+$ CTL to Cause Tumor Eradication[1]

Geertje J. D. van Mierlo,* Zita F. H. M. Boonman,† Hélène M. H. Dumortier,* Annemieke Th. den Boer,* Marieke F. Fransen,* Jan Nouta,* Ellen I. H. van der Voort,*‡ Rienk Offringa,* René E. M. Toes,*‡ and Cornelis J. M. Melief[2]*

The fate of naive CD8$^+$ T cells is determined by the environment in which they encounter MHC class I presented peptide Ags. The manner in which tumor Ags are presented is a longstanding matter of debate. Ag presentation might be mediated by tumor cells in tumor draining lymph nodes or via cross-presentation by professional APC. Either pathway is insufficient to elicit protective antitumor immunity. We now demonstrate using a syngeneic mouse tumor model, expressing an Ag derived from the early region 1A of human adenovirus type 5, that the inadequate nature of the antitumor CTL response is not due to direct Ag presentation by the tumor cells, but results from presentation of tumor-derived Ag by nonactivated CD11c$^+$ APC. Although this event results in division of naive CTL in tumor draining lymph nodes, it does not establish a productive immune response. Treatment of tumor-bearing mice with dendritic cell-stimulating agonistic anti-CD40 mAb resulted in systemic efflux of CTL with robust effector function capable to eradicate established tumors. For efficacy of anti-CD40 treatment, CD40 ligation of host APC is required because adoptive transfer of CD40-proficient tumor-specific TCR transgenic CTL into CD40-deficient tumor-bearing mice did not lead to productive antitumor immunity after CD40 triggering in vivo. CpG and detoxified LPS (MPL) acted similarly as agonistic anti-CD40 mAb with respect to CD8$^+$ CTL efflux and tumor eradication. Together these results indicate that dendritic cells, depending on their activation state, orchestrate the outcome of CTL-mediated immunity against tumors, leading either to an ineffective immune response or potent antitumor immunity. *The Journal of Immunology,* **2004, 173: 6753–6759.**

The coexistence of tumor immunogenicity with persistent tumor growth indicates that tumor-specific T cells have not been properly activated in vivo or that tumor immune evasion mechanisms operate. Although tumor-specific T cells can often successfully prevent tumor outgrowth after preventive vaccination (1–4), boosting an effective immune response against established tumors is much more challenging. The development of successful therapeutic vaccination requires detailed insight into the mechanisms leading to proper T cell immunity in tumor-bearing hosts. The dominant mechanism of Ag presentation to CTL has been argued to be mediated by tumor cells themselves that have reached tumor draining lymph nodes (DLNs)[3] via lymphatic channels (5). Because most tumors lack the costimulatory makeup required for CTL induction, it has been proposed that Ag presentation by tumor cells is inefficient, and therefore cannot elicit protective antitumor immunity (5). Alternatively, it has been postulated that the primary route of Ag presentation to tumor-specific CTL in tumor-bearing hosts involves professional APC, most likely dendritic cells (DCs), that cross-present tumor-derived Ag after uptake and processing (6, 7). Inefficient antitumor immunity would result, in this scheme, from the fact that the Ag-presenting professional APC have not been alarmed to a state conducive for CTL-priming. Because tumor-growth is initially not accompanied by the proper inflammatory stimuli and many inflammatory stimuli lead to activation of professional APC as recognized by up-regulation of their costimulatory capacity, it is thought that Ag is presented by nonactivated professional APC (8). This does not lead to proper CTL priming, allowing uncontrolled tumor growth (9). In this scenario, Ag processing and presentation is not inefficient. Rather the lack of activation of the APC in tumor-bearing hosts precludes proper activation of tumoricidal CTL.

For optimal tumor-specific CTL immunity, help from CD4$^+$ T cells is required. Previously, it was shown that help for CTL priming is mediated via CD40-CD40 ligand interactions (10, 11), and that "help" provided via CD40 signaling in vivo is a powerful way to install successful treatment of tumor-bearing mice through the induction of potent tumor-specific CTL immunity (12). Triggering of CD40 might lead to activation of APC endowing them with the capacity to activate CTL, as shown by the observation that CD40-matured DC in contrast to immature DC, are able to mount CTL immunity (13). Alternatively, CD40 triggering in vivo might act directly on tumor-specific CTL, as it was recently published that the help provided by CD4$^+$ T cells to achieve CD8$^+$ T cell memory is not routed through the APC, but results from a direct interaction between CD4$^+$ T cells and CD8$^+$ T cells (14).

Departments of *Immunohematology and Bloodtransfusion, †Ophthalmology, and ‡Rheumatology, Leiden University Medical Center, Leiden, The Netherlands

Received for publication February 5, 2004. Accepted for publication September 21, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by European Community Grant QZK 3-T-1999-00064 and by Dutch Cancer Foundation Grants RUL 99-2025 and RUL 97-1450. R.E.M.T. is funded for research by a fellowship from the Royal Academy of Arts and Sciences.

[2] Address correspondence and reprint requests to Dr. Cornelis J. M. Melief, Department of Immunohematology and Bloodtransfusion, Postal Zone E3-Q, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, The Netherlands. E-mail address: C.Melief@lumc.nl

[3] Abbreviations used in this paper: DLN, draining lymph node; Ad5E1A, early region 1A of human adenovirus type 5; DC, dendritic cell; NDLN, nondraining lymph node; ODN, oligodeoxynucleotide.

0022-1767/04/$02.00

To gain more insight into the mechanisms underlying CTL priming in tumor-bearing hosts, we took advantage of a well-defined tumor model expressing an Ag derived from the early region 1A of human adenovirus type 5 (Ad5E1A). Eradication of this tumor is CD8-mediated because administration of in vitro activated $CD8^+$ clonal T cells leads to clearance of the tumor (15). Moreover, tolerization of E1A-specific CTL by the E1A-derived CTL peptide epitope resulted in the inability of the mice to eradicate E1A-expressing tumors (16). Also, in this tumor model CD40 ligation in vivo leads to the systemic appearance of E1A-specific CTL that eradicate established tumors. When CD8 cells were depleted, treatment with the agonistic CD40 mAb did not lead to tumor eradication, but tumors continued growing (12).

We now show that tumor Ags are presented to CTL by cross-presentation of Ag by $CD11c^+$ cells. In tumor-bearing animals, tumor specific CTL do arise, but these are not endowed with efficient effector function, appearing only as "poised" CTL in tumor DLNs. In vivo activation of DC by treatment with a DC-activating agent results in gain of effector function of tumor-specific CTL leading to eradication of established tumors. Clearly, the antitumor response elicited by anti-CD40 mAb treatment operates via activation of $CD11c^+$ cells, as anti-CD40 mAb treatment of tumor-bearing CD40-deficient mice harboring CD40-proficient CTL was not successful. Furthermore, for production of an efficient antitumor immune response, the expression of CD40 on $CD8^+$ T cells is not required, as DC activation by CpG1826 treatment led to effective tumor eradication.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Breeding Laboratories (Maastricht, The Netherlands). $TAP^{-/-}$ mice and $CD40^{-/-}$ mice (both on C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). Strain 42 mice, bred at Netherlands Central Organization for Applied Scientific Research (TNO) Prevention and Health (Leiden, The Netherlands) are TCR transgenic mice expressing the TCR α-chain and β-chain derived from the H-2^b-restricted, Ad5E1A$_{234-243}$-specific CTL clone 5 (15, 16). Strain 42*$CD40^{-/-}$ mice were also bred at TNO Prevention and Health. Mice were kept at the Leiden University Medical Center animal facility and used at 7–13 wk of age in accordance with national legislation and under supervision of the animal experimental committee of the University of Leiden.

Tumor cells

Mouse embryo cells transformed by Ad5E1A plus EJ-*ras* (16) were cultured in IMDM (Invitrogen Life Technologies, Rockville, MD) supplemented with 8% (v/v) FCS, 50 μM 2-ME, glutamine, and penicillin, as described (16).

Tumor experiments

CD40-negative E1A-expressing tumor cells (1×10^7) were injected s.c. into 7- to 13-wk-old male mice in 200 μl of PBS. Tumor size was measured twice weekly with calipers in three dimensions. Treatment was started 20–30 days after tumor inoculation, when palpable tumors were present. Mice were sacrificed when tumor size exceeded 1 cm^3 to avoid unnecessary suffering.

Treatments

The FGK-45 hybridoma cells producing a stimulatory anti-CD40 Ab were provided by A. Rolink (Basel Institute for Immunology, Basel, Switzerland) (17). Mice received 100 μg of the anti-CD40 mAb given either i.v. (days 0, 1, and 2 of treatment) in 200 μl of PBS or intratumorally (days 0 and 3 of treatment) in 40 μl of PBS. As a control, mice received 100 μg of rat-IgG specific for human CD40 (6E9) (18) in the same volume of PBS. The CpG1826 oligodeoxynucleotides (ODN), which is a 20-mer containing two CpG motifs (TTCATGA**CG**TTCCTGA**CG**TT; the bold nucleotides represent the immunostimulatory CpG sequences) was provided by Coley Pharmaceutical (Langenfeld, Germany) and used at their suggested optimal working concentration of 50 μg/injection, intratumorally in 40 μl of PBS at days 0 and 3 of treatment. MPL (detoxified LPS) was provided by Corixa (Seattle, WA) and used at the suggested optimal concentration of 10 μg/injection, intratumorally in 40 μl of PBS at day 0 and 3 of treatment.

CFSE labeling and adoptive transfer

Single cell suspensions were made from spleen and peripheral lymph nodes of strain 42 mice. Erythrocytes were depleted by ammonium chloride treatment (2 min on ice). Cells were washed once in cold medium and once in cold PBS, after which they were resuspended in PBS at 1×10^7 cells/ml and incubated with 0.5 μM CFSE (Molecular Probes, Eugene, OR) for 30 min at 37°C. FCS was added to a concentration of 5% FCS, and the cells were washed in PBS. TCR transgenic $CD8^+$ T cells (3×10^6) were injected into the tail veins of tumor-bearing mice in 200 μl of PBS.

Flow cytometry

Single cell suspensions of spleens and lymph nodes were prepared by mechanical disruption. Blood samples and cell suspensions of spleens were depleted of erythrocytes by ammonium chloride treatment for 5 min at room temperature. Cells were stained with directly allophycocyanin-conjugated mAb against CD8 (clone 53-6.7; BD Pharmingen, San Diego, CA) combined with PE-conjugated E1A$_{234-243}$-loaded H-$2D^b$ tetramers (E1A-TM) or, after CD11c-enrichment, with directly allophycocyanin-conjugated mAb against CD11c (clone HL3; BD Pharmingen) combined with stainings for CD80 (clone 16-10A1; BD Pharmingen), CD86 (clone GL1; BD Pharmingen), CD40 (clone 3/23; BD Pharmingen), I-A/I-E (clone M5/114.15.2; BD Pharmingen), or H-$2K^b$ (clone AF6-88.5; BD Pharmingen). Data acquisition and analysis was done on a BD Biosciences FACScan (San Jose, CA) with CellQuest software.

Intracellular IFN-γ staining

Single cell suspensions of lymph nodes were prepared by mechanical disruption. Intracellular staining was performed using BD Cytofix cytoperm kit with BD Golgiplug (BD Pharmingen), according to the manufacturer's protocol. During the 6-h incubation with BD Golgiplug, 4.5 μg/ml of the E1A-peptide or a control peptide was added. Stainings were performed with directly allophycocyanin-conjugated mAb against CD8 (clone 53-6.7; BD Pharmingen), PE-conjugated E1A-TM and FITC-labeled IFN-γ (clone XMG1.2; BD Pharmingen). Data acquisition and analysis was done on a BD Biosciences FACScan with CellQuest software.

Separation of $CD11c^+$ and $CD11c^-$ populations

Peripheral lymph nodes of tumor-bearing mice were treated with collagenase (250 U/ml; Sigma-Aldrich, St. Louis, MO) and DNase (50 μg/ml, Sigma-Aldrich) for 30 min at 37°C. $CD11c^+$ cells were positively selected using magnetized Ab for CD11c (N418; Miltenyi Biotec, Bergisch Gladbach, Germany). The cell populations were analyzed by FACS, showing the $CD11c^+$ population circa 90% pure, and >95% of the $CD11c^-$ population was cleared of $CD11c^+$ cells.

Proliferation assay and IFN-γ ELISA

The $CD11c^+$ and $CD11c^-$ cell populations were incubated at graded doses with 0.1×10^6 spleen cells of TCR transgenic strain 42 mice. E1A-specific proliferation was measured after 3 days. At 8 h before termination, 0.5 μCi of [^{3}H]thymidine per well was added. Supernatant taken after a 20-h incubation was analyzed for IFN-γ production by a standard sandwich ELISA.

E1A-PCR

DNA from the tissues was isolated with High Pure PCR Template Preparation kit (Roche, Basel, Switzerland), as recommended by the manufacturer, and was amplified by PCR for 30 cycles using the primers for E1A: 5′-GCAGGAAGGGATTGACTTACTCAC-3′ (sense) and 5′-CTCAGGT TCAGACACAGGACCTTT-3′ (antisense). PCR products of 467 bp were observed after separation by electrophoresis in a 1% agarose gel.

Results

Tumor Ags are cross-presented to $CD8^+$ T cells

$CD8^+$ CTL-mediated immunity is crucial for eradication of E1A-expressing tumors (12). Cross-priming as well as direct priming have been postulated as a mechanism to induce tumor-protective CTL immunity (5–7, 19). For priming of CTL in a direct fashion, tumor cells have to migrate to the tumor DLN. To study whether

E1A-specific CTL could be primed directly by tumor cells in tumor DLNs or by tumor Ag-presenting professional APC, we analyzed whether tumor cells can be found in the secondary lymphoid organs of tumor-bearing animals. Twenty-five days after s.c. injection of E1A-expressing tumor cells, secondary lymphoid organs were examined. Both PCR amplification of the DNA encoding E1A (Fig. 1*A*) and selective in vitro outgrowth of tumor cells from lymph node cultures in medium containing G-418 (data not shown) revealed that the tumor cells had migrated to lymph nodes draining the tumor, but not to other lymph nodes or spleen. Subsequently we investigated whether these tumor cells, being able to reach the lymphoid organs, were capable of presenting tumor Ag in vivo. Therefore, we injected E1A-expressing tumor cells into wild-type and $TAP^{-/-}$ mice, the APC of the latter being incapable of cross-presenting tumor-derived Ags because of defective MHC class I-loading in a TAP-dependent fashion. When a palpable tumor had developed, CFSE-labeled transgenic, E1A-specific $CD8^+$ T cells were injected. Three days later, division of tumor-specific T cells in different lymphatic organs was analyzed. As shown in Fig. 1*B*, the tumor Ag is not presented in the DLNs of $TAP^{-/-}$ mice, contradicting direct Ag presentation by the tumor cells themselves. In contrast, proliferating E1A-specific CTL were found in tumor DLNs of TAP-competent C57BL/6 mice. Together, these findings indicate that the tumor Ag is presented to the immune system by cells derived from the host, even though tumor cells are detectable in the tumor DLN.

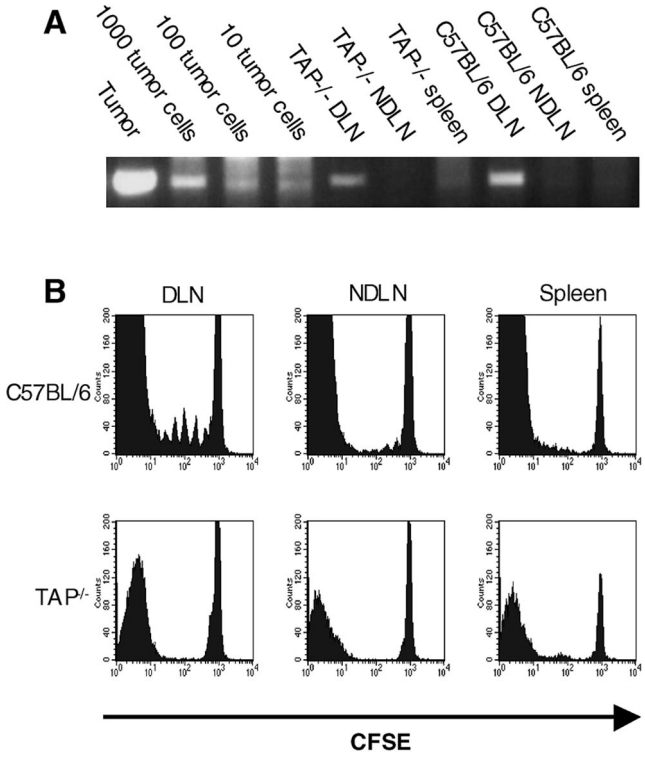

FIGURE 1. Host cells are required for presentation of tumor Ags. $TAP^{-/-}$ mice or wild-type mice were injected s.c. with 10×10^6 E1A-expressing tumor cells. *A*, At the time when palpable tumors had developed (25 days after tumor inoculation), DNA was extracted out of tumor DLN, nondraining lymph node (NDLN), and spleen and analyzed by PCR for presence of tumor cells. *B*, Likewise, when palpable tumors had developed, 3×10^6 CFSE-labeled E1A-specific transgenic $CD8^+$ T cells were injected i.v. After 3 days mice were sacrificed and division of the CFSE-labeled cells was analyzed in spleens and lymph nodes. Plots shown are gated on $CD8^+$ cells. One representative experiment of six is shown.

Tumor Ags are presented by $CD11c^+$ cells

These results indicate that the predominant cell responsible for Ag presentation to naive CTL is of host origin. Therefore we wished to determine the identity of this APC because this cell is likely to be responsible for the inadequate immune reactivity in tumor-bearing hosts. To this end, we separated cells from tumor DLNs in a $CD11c^+$ (DC-enriched) and a $CD11c^-$ (DC-depleted) fraction. The $CD11c^+$ and $CD11c^-$ populations were incubated directly ex vivo with E1A-specific $CD8^+$ T cells derived from TCR transgenic mice, and proliferation of the $CD8^+$ T cells was determined. As shown in Fig. 2, the population that best stimulated the TCR transgenic T cells was found in the $CD11c^+$ population isolated from the tumor DLNs. These findings indicate that $CD11c^+$ cells, most likely DC, are important for Ag presentation to CTL in otherwise naive tumor-bearing mice. Because $E1A^+$ tumor cells do not express CD11c (data not shown) and the CD11c-negative population of TAP knockout mice did not stimulate TCR transgenic T cells, these findings confirm that Ag presentation is not mediated by tumor cells that have traveled to the DLN.

In vivo activation of $CD11c^+$ cells by anti-CD40 treatment leads to improved immunity

Although tumor Ags are presented to the immune system in vivo, no effective antitumor immune response can be found in most of the tumor-bearing animals. We demonstrate that $CD11c^+$ APC are responsible for tumor Ag presentation. Because activation of these cells is considered crucial for induction of CTL immunity, this observation prompted us to examine the effect of the anti-CD40 agonist on $CD11c^+$ cells in vivo. For this purpose, tumor DLNs of anti-CD40 mAb and untreated C57BL/6 mice bearing an E1A-expressing tumor were analyzed 3 days after the first anti-CD40 injection. In Fig. 3*A* it is shown that $CD11c^+$ cells isolated from anti-CD40-treated mice had up-regulated their surface expression of CD80, CD86, CD40, MHC class I, and MHC class II in comparison with the $CD11c^+$ cells isolated from untreated tumor-bearing mice. Thus, anti-CD40 treatment led to an activated phenotype of the $CD11c^+$ cells present in tumor DLNs.

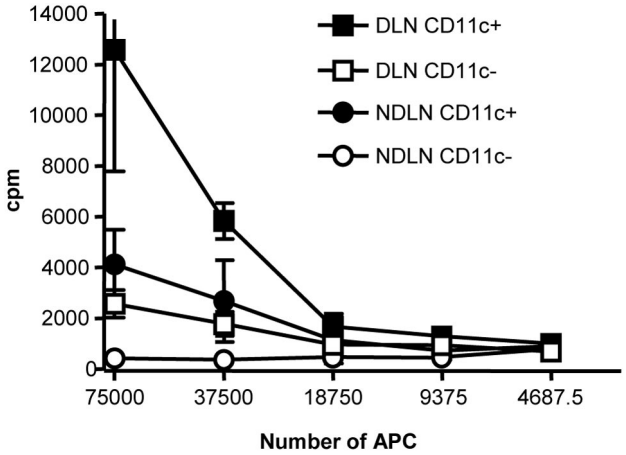

FIGURE 2. $CD11c^+$ cells are important for presentation of tumor Ags. C57BL/6 mice were injected s.c. with 10×10^6 E1A-expressing tumor cells. When palpable tumors had developed, DLN and NDLN were analyzed. Lymph nodes of eight mice were pooled. $CD11c^+$ and $CD11c^-$ cell populations were separated. The different cell populations were incubated for 3 days with E1A-specific transgenic $CD8^+$ T cells. Proliferation of the CTL was determined. One representative experiment of three is shown.

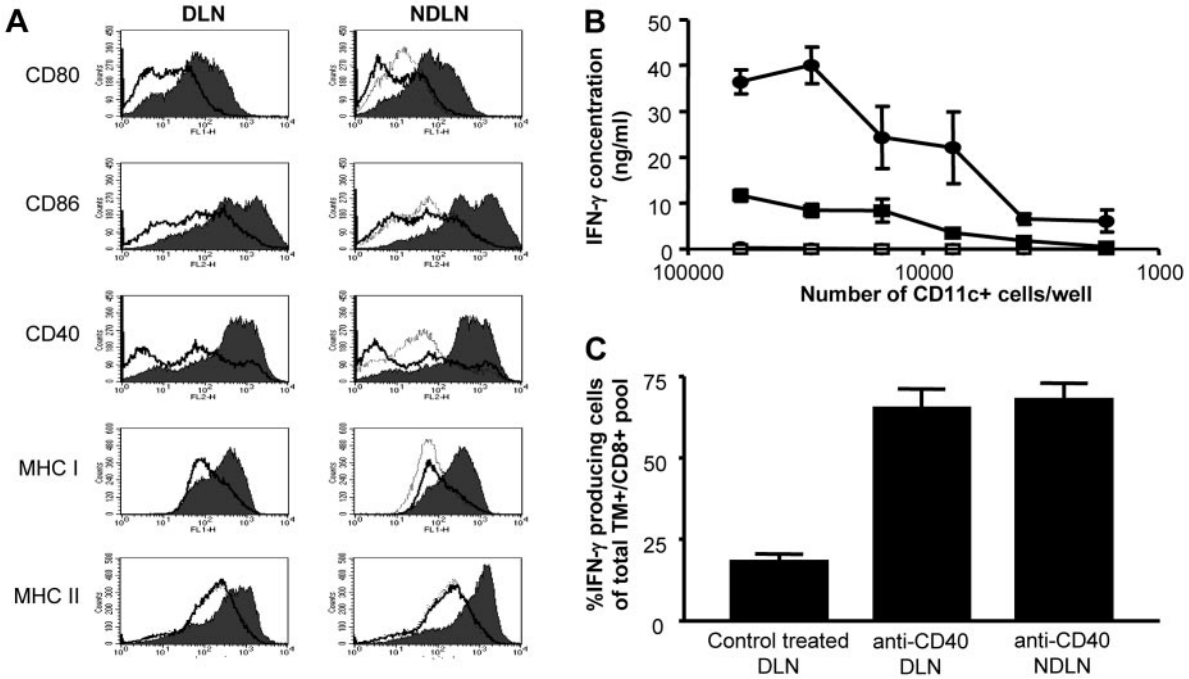

FIGURE 3. In vivo activation of $CD11c^+$ cells by anti-CD40 treatment leads to improved $CD8^+$ T cell activation. *A*, C57BL/6 mice were injected s.c. with 10×10^6 E1A-expressing tumor cells. When palpable tumors had developed, mice were treated i.v. with anti-CD40 mAb (shaded histogram) or control Ab (thick histogram). At day 3 after start of treatment mice were sacrificed, $CD11c^+$ cells were isolated out of the lymph nodes and analyzed directly ex vivo. Expression of CD80, CD86, CD40, MHC class I, and MHC class II are shown. Naive mice without tumors were included in the experiment (thin histogram). One representative experiment of three is shown. *B*, $CD11c^+$ cells were isolated out of LNs of C57BL/6 mice (not bearing a tumor) either treated with anti-CD40 mAb (circles) or control Ab (squares), and were directly ex vivo loaded with the E1A-peptide (■ and ●), or a control peptide (RAHYNIVTF, □ and ○). IFN-γ production by E1A-specific transgenic T cells after 20 h of incubation with the $CD11c^+$ cells was assessed by ELISA. *C*, C57BL/6 mice were injected s.c. with 10×10^6 E1A-expressing tumor cells. When palpable tumors had developed (25 days after tumor inoculation), mice were treated i.v. with anti-CD40 mAb or control mAb. Six days after start of treatment, effector function of T cells, specific for the tumor Ag, located in tumor DLN and NDLNs was assessed by intracellular IFN-γ staining after a short peptide restimulation in vitro. Percentages of tumor-specific $CD8^+$ T cells producing IFN-γ are shown (Mean ± SD, $n = 8$). Results shown are derived from one of two experiments with similar results.

To investigate whether these $CD11c^+$ cells next to their activated phenotype indeed were superior for inducing T cell activation we isolated $CD11c^+$ cells of tumor-free anti-CD40 and control-treated animals, loaded the cells ex vivo with peptide, and incubated them with transgenic T cells specific for the tumor Ag E1A. We intentionally did not use tumor-bearing animals, as tumor burden and, as a consequence, availability of Ag is higher in untreated animals than in animals treated with anti-CD40 mAb. Therefore, comparison of T cell activating capacity of DC in this system is not necessarily a faithful reflection of DC activation. As shown in Fig. 3*B*, T cells incubated with $CD11c^+$ cells isolated out of anti-CD40-treated animals produced a higher amount of IFN-γ than T cells incubated with $CD11c^+$ cells isolated out of untreated animals. Negligible IFN-γ production by the tumor-specific T cells was seen after incubation with $CD11c^+$ cells loaded with a control peptide (Fig. 3*B*). Thus, next to their activated phenotype, the $CD11c^+$ cells of anti-CD40 treated animals are also functionally superior for priming tumor-specific T cells.

In untreated tumor-bearing animals, $CD8^+$ T cells that recognize the tumor Ag arise and reside in the tumor DLN. Because it is not known whether these CTL also acquire effector function, we explored the capacity of tumor-specific T cells to produce IFN-γ, as one parameter of effector phenotype, after encounter of tumor Ag in untreated and anti-CD40-treated tumor-bearing animals. As shown in Fig. 3*C*, only a small proportion (17%) of the tumor-specific T cells found in the tumor DLNs of untreated mice produced IFN-γ directly ex vivo, whereas after treatment with the anti-CD40 mAb >65% of the tumor-specific T cells produced IFN-γ. Hence, as was the case upon peptide loading ex vivo (Fig. 3*B*), also in vivo the activated $CD11c^+$ APC were better capable of turning naive tumor-specific $CD8^+$ T cells into effector cells.

Other DC-activating agents have similar effects on antitumor immune responses

Treatment of tumor-bearing animals with the anti-CD40 mAb leads to systemic spread of tumor-specific CTL resulting in tumor eradication (12). To analyze whether other professional APC-activating agents can induce similar effects, we treated otherwise naive tumor-bearing mice intratumorally with the TLR4 and TLR9 ligands, MPL, and CpG1826 respectively. These agents are known to activate DC both in vivo and in vitro (20–25 and data not shown). Like in vivo CD40 triggering, intratumoral treatment with these agents was sufficient for eradication of the E1A-expressing tumors (Fig. 4*A*). The antitumor effect of CpG1826 could not be explained by a toxic effect of the CpG1826 on the tumor cells themselves, as in CD8-depleted animals the rate of tumor growth was comparable in untreated and CpG1826 treated animals (data not shown). In addition, the activation of host $CD11c^+$ cells was strongly associated with systemic spread of endogenously formed CTL in these mice (Fig. 4*B*).

Antitumor immunity provoked by anti-CD40 mAb treatment requires CD40 expression by host APC

To analyze whether CD40 expression by $CD11c^+$ cells and/or by $CD8^+$ T cells is required for the induction of effective CTL immunity in tumor-bearing mice after treatment with the anti-CD40

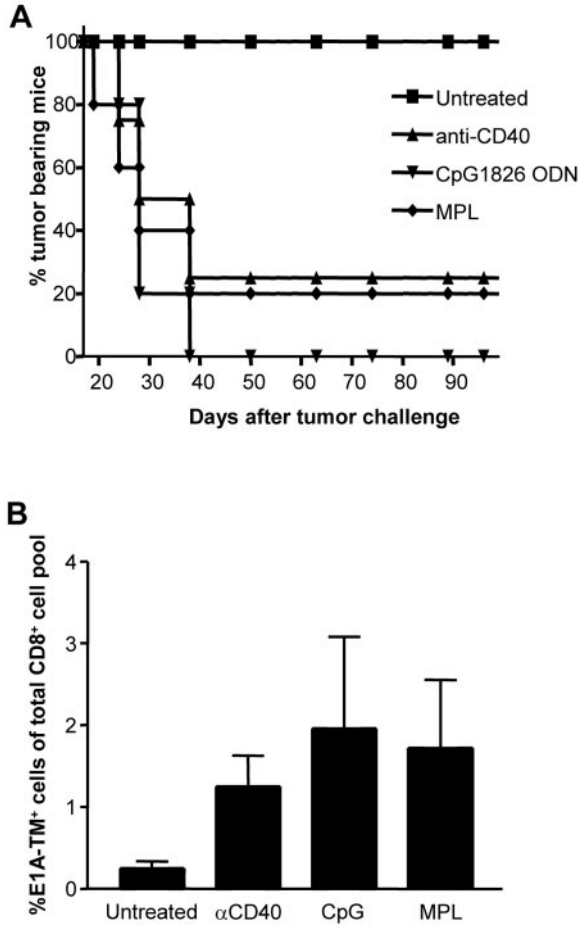

FIGURE 4. DC-activating agents elicit an effective immune response. C57BL/6 mice were injected s.c. with 10×10^6 E1A-expressing tumor cells. When palpable tumors had developed (after 21 days), mice were treated intratumorally with anti-CD40 mAb, CpG1826, or MPL. *A*, Percentage of tumor-bearing mice after treatment with anti-CD40 mAb, CpG1826, MPL, or no treatment are shown. *B*, Percentage of endogenously formed E1A-TM$^+$ T cells of total CD8$^+$ T cell pool in blood 10 days after start of treatment (Mean ± SD, $n = 5$). Results shown are derived from one of two experiments with similar results.

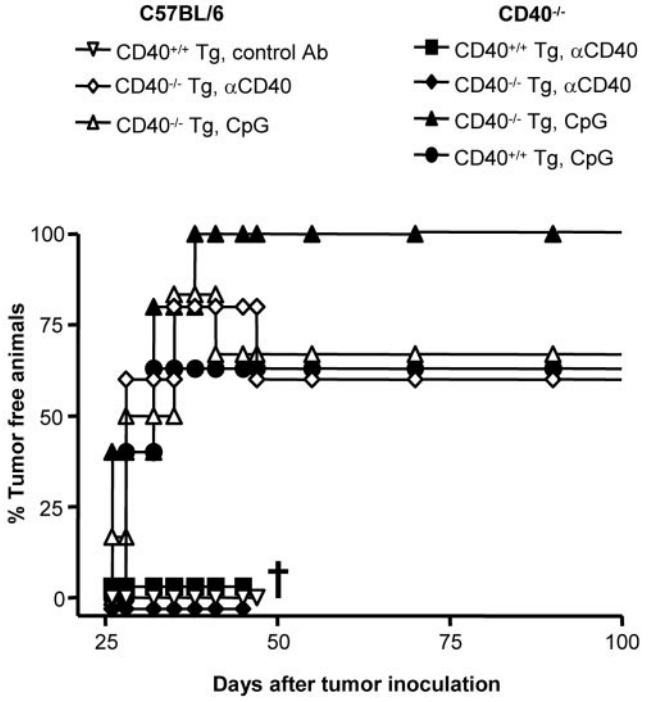

FIGURE 5. CD40 expression on CD8$^+$ T cells is not required for effective antitumor immunity. A total of 3×10^6 E1A-specific, CD40-proficient or CD40-deficient naive TCR transgenic CD8$^+$ T cells were adoptively transferred into CD40$^{-/-}$ mice (filled symbols) or C57BL/6 mice (open symbols). Three days later these mice were injected s.c. with 10×10^6 E1A-expressing tumor cells. When palpable tumors had developed (21 days after tumor inoculation), mice were treated intratumorally with anti-CD40 mAb, CpG1826, or control mAb. To avoid unnecessary suffering, mice were killed when the size of the tumors exceeded 1 cm^3. The mice that were still alive at day 100 after tumor challenge had all rejected their tumors completely and remained tumor-free for at least 50 more days. The experiment was performed twice with similar results.

mAb, we adoptively transferred CD40-proficient or CD40-deficient E1A-specific TCR transgenic CD8$^+$ T cells into tumor-bearing CD40$^{-/-}$ mice or normal C57BL/6 mice. As shown in Fig. 5, treatment with anti-CD40 mAb led to tumor eradication only in wild-type mice, but not in CD40$^{-/-}$ mice, irrespective of CD40 expression by the transfused transgenic T cells.

In contrast, treatment of tumor-bearing CD40$^{-/-}$ mice with the TLR9 stimulating agent CpG1826 was associated with clearance of the s.c. growing tumor, indicating that triggering of CD40-deficient DC with other stimuli endows them with the capacity to generate efficient CTL immunity, without the need of CD40 expression on CD8$^+$ T cells. Indeed, tumor eradication correlated strongly with the expansion of the E1A-specific CTL population in peripheral blood of CpG1826-treated CD40$^{-/-}$ mice whereas no expansion of this population was observed in tumor-bearing CD40$^{-/-}$ mice treated with anti-CD40 mAb (data not shown). Also, rechallenge with tumor cells of the CpG-treated CD40$^{-/-}$ mice that had eradicated their primary tumors, did not lead to tumor outgrowth, indicating T cell memory formation in the absence of CD40. Thus, together these data show that CD40 expression on host APC is crucial for CTL expansion and tumor eradication in tumor-bearing mice after anti-CD40 treatment. Furthermore, CD40 expression on CD8$^+$ T cells is not sufficient or necessary for eliciting effective antitumor immune responses.

Discussion

The outcome of CTL-mediated immunity is dictated by the environment in which first Ag encounter takes place. In this study we show, using a model tumor Ag derived from Ad5E1A, that not tumor cells themselves, but rather CD11c$^+$ host cells are responsible for Ag presentation to tumor-specific CTL, despite the fact that tumor cells can be detected in tumor DLNs. Although CD11c$^+$ cells, most likely DC, are exquisitely capable of activating Ag-specific T cells, no systemic and effective CTL response is induced in tumor-bearing mice. This is most likely a consequence of Ag-presentation by immature DC, as CD40 ligation or triggering of TLR4 or TLR9, which are associated with activation of CD11c$^+$ cells (23, 24), resulted in a powerful immune response. Indeed, the CD40-mediated signal has to be directed toward host APC because tumor-bearing CD40$^{-/-}$ mice harboring CD40-proficient CTL do not eradicate E1A-expressing tumors after anti-CD40 mAb treatment. The observation that CD11c$^+$ cells present tumor-derived Ag to tumor-specific CTL without inducing a systemic antitumor response is intriguing, as it indicates that tumor-specific T cells do not necessarily ignore the tumor. Instead, they are activated leading to clonal expansion in tumor DLNs. However, they are not programmed to full effector function, as evidenced by the observation that these CTL do not produce IFN-γ, when analyzed directly ex vivo. Also, they cannot be detected

outside the DLN. We refer to these T cells as "poised" T cells, ready for action, not deleted or anergized, but also not properly activated by DC to migrate and exert peripheral effector function (see also Ref. 26). Interestingly, effective, systemic CTL-mediated immunity is induced when $CD11c^+$ cells are activated through CD40. This identifies the DC as the central cell orchestrating the outcome of tumor-specific immunity, determining tolerance or effective antitumor immunity.

These findings are in line with recent publications, using DEC205-targeted Ag presentation to $CD8^+$ T cells in immature or mature DC (27), or the effect of inducible expression of a model Ag in DC in vivo (28). When the Ag in these studies was presented by DC under "steady state" conditions, Ag-specific CTL tolerance was readily induced. In contrast, when CD40-activated DC presented the Ag, a powerful CTL response was the result. Our data indicate that similar outcomes of CTL-mediated immunity are seen when the Ag presented by the DC has been acquired exogenously from progressively growing tumors.

The data presented in this study stand in marked contrast to findings made in another tumor model, describing that not host-derived APC, but rather tumor cells themselves are responsible for Ag presentation to naive CTL (5). Although not well understood, several explanations can account for these contrasting observations. For example, the stability of the tumor Ag may play an important role in determining whether a tumor Ag is cross-presented to CTL. In case the Ag is highly unstable, it is likely that a strong flow of antigenic peptides makes it to the endoplasmic reticulum of tumor cells, leading to a high peptide-MHC density on the surface of tumor cells. In this scenario, a much lower amount of protein Ag is available for uptake by DC, keeping the Ag outside the sophisticated mechanisms for efficient cross-presentation (29, 30). In this case, indirect presentation of tumor Ags by DC is inefficient, allowing direct presentation of Ag by tumor cells that display a sufficient high peptide-MHC density on their cell surface. Also, it has been shown that the availability of a CTL epitope for biosynthetic processing or cross-presentation depends on the position of the epitope in the protein (31).

Alternatively, the type of tumor might be important with respect to its ability to directly present to naive T cells. For example, it is feasible that lymphoma cells, due to their chemokine receptor and homing-receptor makeup, can readily enter the T cell zone of DLNs, whereas other types of tumor, although capable of entering the DLN, do not make it into the zone in which priming of naive T cells occurs. The latter notion could explain the lack of CTL activation in TAP-deficient mice as the used E1A-expressing tumor cells are positive for MHC class I expression and are capable of activating naive TCR transgenic cells when analyzed directly ex vivo (data not shown), but apparently not in vivo despite the presence of tumor cells in tumor DLNs. However, these observations can also be explained by the superior efficiency of DC compared with tumor cells to communicate with CTL because semiquantitative analysis showed the presence of a substantial number of tumor cells in tumor DLNs (Fig. 1*A*).

Our data and those of others (32–34) show that administration of DC stimulating agents can be a powerful tool to evoke antitumor immunity under conditions of sufficient cross-presentation. It will be important to gain a detailed understanding of the mechanisms that govern tumor Ag presentation in vivo. Chemotherapy with gemcitabine increases cross-presentation leading to growth delay, but not complete eradication of tumors in a model involving mesothelioma transfected with the influenza hemagglutinin gene (35). Combination of gemcitabine with anti-CD40 mAb led to complete cure of these tumors (36). Moreover, not only expression of costimulatory molecules, but also Ag presentation by DC is enhanced after activation of the DC by CpG sequences (37). In addition, a combination of an Ag with CpG sequences was shown to lead to $CD11c^+$ cells that were capable of eliciting protective immunity in naive mice in the absence of further Ag or adjuvant (38). Although we did not study the exact mode of action of treatment with CpG or MPL, it is conceivable that also in our study these agents act through the activation of DC. Together, these findings indicate that DC-activating agents have multiple effects on DC that are all beneficial to their capacity to prime efficient CTL responses.

Recently, it was shown that $CD8^+$ T cells can express CD40 and that this CD40 expression is essential for $CD8^+$ T cell memory formation (14). Our results clearly demonstrate that CD40 expression by host APC, but not by $CD8^+$ CTL precursors is crucial for induction of CTL immunity following CD40 triggering in vivo. The potential of $CD8^+$ T cells to express CD40 is, as such, not sufficient to allow productive CTL activation. Although we observed that, after adoptive transfer of transgenic T cells, the transgenic T cells dominate the response against the specific Ag (data not shown and Ref. 39), no CTL immunity could be induced by treatment with the anti-CD40 mAb when $CD8^+$ T cells from CD40-competent donors were transferred into CD40-deficient mice (Fig. 5). DC activation via TLR9 by CpG1826 ODN was sufficient to mount a strong antitumor CTL response, independent of possible CD40 expression on the transfused $CD8^+$ T cells. Moreover, like CD40 signaling, also signaling via TLR4 and TLR9 led to CTL effector function and memory formation as mice rejected the initial tumor and were resistant to a subsequent rechallenge with tumor cells (data not shown). This was also evident in $CD40^{-/-}$ mice after tumor eradication in response to treatment with CpG1826 ODN, indicating that, in contrast to previously published data (14), CD40 expression on $CD8^+$ T cells is not necessary for formation of CTL memory (40).

Together these observations strongly indicate that effective CTL activation and memory formation are dependent on proper activation of DC. Provided that human cancers have shed enough protein Ags for cross-presentation by tumor-associated DC, all that may be required for effective tumor immunotherapy might be proper DC activation by suitable DC triggers, such as CD40 agonists and TLR ligands.

Acknowledgments

We thank M. S. J. Mulders for biotechnical help and A. M. Bakker for helping us designing the primers used for the E1A-PCR.

References

1. Toes, R. E. M., R. J. J. Blom, E. van der Voort, R. Offringa, C. J. M. Melief, and W. M. Kast. 1996. Protective antitumor immunity induced by immunization with completely allogeneic tumor cells. *Cancer Res. 56:3782*.
2. Vierboom, M. P., M. C. Feltkamp, A. Neisig, J. W. Drijfhout, J. ter Schegget, J. J. Neefjes, C. J. Melief, and W. M. Kast. 1998. Peptide vaccination with an anchor-replaced CTL epitope protects against human papillomavirus type 16-induced tumors expressing the wild-type epitope. *J. Immunother. 21:399*.
3. Feltkamp, M. C., H. L. Smits, M. P. Vierboom, R. P. Minnaar, B. M. de Jongh, J. W. Drijfhout, J. ter Schegget, C. J. M. Melief, and W. M. Kast. 1993. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur. J. Immunol. 23:2242*.
4. Nanni, P., G. Nicoletti, C. De Giovanni, L. Landuzzi, E. Di Carlo, F. Cavallo, S. M. Pupa, I. Rossi, M. P. Colombo, C. Ricci, et al. 2001. Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in HER-2/neu transgenic mice. *J. Exp. Med. 194:1195*.
5. Ochsenbein, A. F., S. Sierro, B. Odermatt, M. Pericin, U. Karrer, J. Hermans, S. Hemmi, H. Hengartner, and R. M. Zinkernagel. 2001. Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. *Nature 411:1058*.
6. Sotomayor, E. M., I. Borrello, F. M. Rattis, A. G. Cuenca, J. Abrams, K. Staveley-O'Carroll, and H. I. Levitsky. 2001. Cross-presentation of tumor antigens by bone marrow-derived antigen-presenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression. *Blood 98:1070*.

7. Huang, A. Y., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky. 1994. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science 264:961*.
8. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu. Rev. Immunol. 12:991*.
9. Fuchs, E. J., and P. Matzinger. 1996. Is cancer dangerous to the immune system? *Semin. Immunol. 8:271*.
10. Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. M. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature 393:480*.
11. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478*.
12. van Mierlo, G. J., A. T. den Boer, J. P. Medema, E. I. van der Voort, M. F. Fransen, R. Offringa, C. J. M. Melief, and R. E. Toes. 2002. CD40 stimulation leads to effective therapy of $CD40^-$ tumors through induction of strong systemic cytotoxic T lymphocyte immunity. *Proc. Natl. Acad. Sci. USA 99:5561*.
13. Schuurhuis, D. H., S. Laban, R. E. Toes, P. Ricciardi-Castagnoli, M. J. Kleijmeer, E. I. van der Voort, D. Rea, R. Offringa, H. J. Geuze, C. J. Melief, and F. Ossendorp. 2000. Immature dendritic cells acquire $CD8^+$ cytotoxic T lymphocyte priming capacity upon activation by T helper cell-independent or -dependent stimuli. *J. Exp. Med. 192:145*.
14. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on $CD8^+$ T cells in the generation of $CD8^+$ T cell memory. *Science 297:2060*.
15. Kast, W. M., R. Offringa, P. J. Peters, A. C. Voordouw, R. H. Meloen, A. J. van der Eb, and C. J. Melief. 1989. Eradication of adenovirus E1-induced tumors by E1A-specific cytotoxic T lymphocytes. *Cell 59:603*.
16. Toes, R. E., R. Offringa, R. J. Blom, C. J. Melief, and W. M. Kast. 1996. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. *Proc. Natl. Acad. Sci. USA 93:7855*.
17. Rolink, A., F. Melchers, and J. Andersson. 1996. The SCID but not the RAG-2 gene product is required for Sμ-Sϵ heavy chain class switching. *Immunity 5:319*.
18. Mittler, R. S., T. S. Bailey, K. Klussman, M. D. Trailsmith, and M. K. Hoffmann. 1999. Anti-4-1BB monoclonal antibodies abrogate T cell-dependent humoral immune responses in vivo through the induction of helper T cell anergy. *J. Exp. Med. 190:1535*.
19. Zinkernagel, R. M. 2002. On cross-priming of MHC class I-specific CTL: rule or exception? *Eur. J. Immunol. 32:2385*.
20. Behboudi, S., D. Chao, P. Klenerman, and J. Austyn. 2000. The effects of DNA containing CpG motif on dendritic cells. *Immunology 99:361*.
21. Lore, K., M. R. Betts, J. M. Brenchley, J. Kuruppu, S. Khojasteh, S. Perfetto, M. Roederer, R. A. Seder, and R. A. Koup. 2003. Toll-like receptor ligands modulate dendritic cells to augment cytomegalovirus- and HIV-1-specific T cell responses. *J. Immunol. 171:4320*.
22. Pulendran, B., P. Kumar, C. W. Cutler, M. Mohamadzadeh, T. Van Dyke, and J. Banchereau. 2001. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. *J. Immunol. 167:5067*.
23. Schwarz, K., T. Storni, V. Manolova, A. Didierlaurent, J. C. Sirard, P. Rothlisberger, and M. F. Bachmann. 2003. Role of Toll-like receptors in costimulating cytotoxic T cell responses. *Eur. J. Immunol. 33:1465*.
24. Sparwasser, T., E. S. Koch, R. M. Vabulas, K. Heeg, G. B. Lipford, J. W. Ellwart, and H. Wagner. 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol. 28:2045*.
25. Sparwasser, T., R. M. Vabulas, B. Villmow, G. B. Lipford, and H. Wagner. 2000. Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins. *Eur. J. Immunol. 30:3591*.
26. Nguyen, L. T., A. R. Elford, K. Murakami, K. M. Garza, S. P. Schoenberger, B. Odermatt, D. E. Speiser, and P. S. Ohashi. 2002. Tumor growth enhances cross-presentation leading to limited T cell activation without tolerance. *J. Exp. Med. 195:423*.
27. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman. 2002. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral $CD8^+$ T cell tolerance. *J. Exp. Med. 196:1627*.
28. Probst, H. C., J. Lagnel, G. Kollias, and M. van den Broek. 2003. Inducible transgenic mice reveal resting dendritic cells as potent inducers of $CD8^+$ T cell tolerance. *Immunity 18:713*.
29. Guermonprez, P., L. Saveanu, M. Kleijmeer, J. Davoust, P. Van Endert, and S. Amigorena. 2003. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature 425:397*.
30. Houde, M., S. Bertholet, E. Gagnon, S. Brunet, G. Goyette, A. Laplante, M. F. Princiotta, P. Thibault, D. Sacks, and M. Desjardins. 2003. Phagosomes are competent organelles for antigen cross-presentation. *Nature 425:402*.
31. Wolkers, M. C., N. Brouwenstijn, A. H. Bakker, M. Toebes, and T. N. Schumacher. 2004. Antigen bias in T cell cross-priming. *Science 304:1314*.
32. Krepler, C., V. Wacheck, S. Strommer, G. Hartmann, P. Polterauer, K. Wolff, H. Pehamberger, and B. Jansen. 2004. CpG oligonucleotides elicit antitumor responses in a human melanoma NOD/SCID xenotransplantation model. *J. Invest. Dermatol. 122:387*.
33. Labeur, M. S., B. Roters, B. Pers, A. Mehling, T. A. Luger, T. Schwarz, and S. Grabbe. 1999. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J. Immunol. 162:168*.
34. Lonsdorf, A. S., H. Kuekrek, B. V. Stern, B. O. Boehm, P. V. Lehmann, and M. Tary-Lehmann. 2003. Intratumor CpG-oligodeoxynucleotide injection induces protective antitumor T cell immunity. *J. Immunol. 171:3941*.
35. Nowak, A. K., R. A. Lake, A. L. Marzo, B. Scott, W. R. Heath, E. J. Collins, J. A. Frelinger, and B. W. Robinson. 2003. Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J. Immunol. 170:4905*.
36. Nowak, A. K., B. W. Robinson, and R. A. Lake. 2003. Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. *Cancer Res. 63:4490*.
37. Datta, S. K., V. Redecke, K. R. Prilliman, K. Takabayashi, M. Corr, T. Tallant, J. DiDonato, R. Dziarski, S. Akira, S. P. Schoenberger, and E. Raz. 2003. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J. Immunol. 170:4102*.
38. Shah, J. A., P. A. Darrah, D. R. Ambrozak, T. N. Turon, S. Mendez, J. Kirman, C. Y. Wu, N. Glaichenhaus, and R. A. Seder. 2003. Dendritic cells are responsible for the capacity of CpG oligodeoxynucleotides to act as an adjuvant for protective vaccine immunity against *Leishmania major* in mice. *J. Exp. Med. 198:281*.
39. Kedl, R. M., W. A. Rees, D. A. Hildeman, B. Schaefer, T. Mitchell, J. Kappler, and P. Marrack. 2000. T cells compete for access to antigen-bearing antigen-presenting cells. *J. Exp. Med. 192:1105*.
40. Lee, B. O., L. Hartson, and T. D. Randall. 2003. CD40-deficient, influenza-specific CD8 memory T cells develop and function normally in a CD40-sufficient environment. *J. Exp. Med. 198:1759*.