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Chemoimmunotherapy of Tumors: Cyclophosphamide Synergizes with Exosome Based Vaccines[1]

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Dendritic cell-derived exosomes (DEX) are nanomeric vesicles harboring MHC/peptide complexes capable of promoting primary T cell responses and tumor rejection in the presence of adjuvants. In this study, we show that, in the absence of adjuvants, DEX mediate potent Ag-dependent antitumor effects against preestablished tumors in mice pretreated with immunopotentiating dosing of cyclophosphamide. Cyclophosphamide could 1) abolish the suppressive function of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells, 2) markedly enhance the magnitude of secondary but not primary CTL responses induced by DEX vaccines, 3) synergize with DEX in therapy but not prophylaxis tumor models. Therefore, therapeutic vaccines such as DEX aimed at boosting tumor-primed effector T cells could benefit procedures that minimize the effects of CD4$^+$CD25$^+$ regulatory T cells. *The Journal of Immunology,* **2006, 176: 2722–2729.**

Efficient immunization against cancer requires a vaccine capable of eliciting potent primary and secondary CD4$^+$ and CD8$^+$ T cell immune responses. We have reported that dendritic cells (DC)[4] secrete DC-derived exosomes (DEX), which are Ag-presenting vesicles originating from multivesicular endosomes. These nanomeric membrane vesicles harbor functional MHC/peptide complexes that elicit potent CD4$^+$ (1) and CD8$^+$ (2, 3) T cell responses directed against the immunizing peptides in vivo. Moreover, DEX pulsed with tumor peptides generate T cell-dependent antitumor effects (4) that were dramatically enhanced by adjuvants such as mature DC and TLR-3 or -9 ligands (3).

However, tumors have evolved several mechanisms to escape immune surveillance, including immune tolerance involving immunosuppressive T lymphocytes (5–8). Indeed, as observed in autoimmunity assuming that most tumor Ags are self Ags, antitumor immunity is controlled by mechanisms maintaining immunologic tolerance to self constituents, such as T cell control of self-reactive T cells. Tumors have been shown to induce rapid expansion of CD4$^+$CD25$^+$ regulatory T cells (Treg) in humans and mice, leading to delayed rejection of immunogenic tumors (9, 10). Conversely, elimination of these Treg, which constitute 1–3% of the peripheral CD4$^+$ T cell pool in naive mice, elicited potent antitumor immune responses leading to tumor eradication (7, 11–13). Thus, blocking Treg cell migration or function through immunotherapeutic approaches may help to defeat human cancer.

Cyclophosphamide (CTX) is known to reverse immunological tolerance and to facilitate adoptive immunotherapy through inhibition of suppressor cell activity (14–16). In a rat tumor model, Ghiringhelli et al. (10) reported that CTX or methotrexate induced a significant decrease in the CD4$^+$CD25$^+$/CD4$^+$ splenic T cell ratio and a suppression of Treg functions in tumor-bearing rats, restoring antitumor immune responses. Recently, Lutsiak et al. (17) reported that immunopotentiating dosing of CTX electively decreases Treg numbers and abolishes their regulatory functions in mice.

In the present study, we addressed the capacity of CD4$^+$CD25$^+$ Treg to restrict primary and secondary CD8$^+$ T cell responses elicited by DEX vaccines in tumor-bearing mice. We found that CTX did not promote DEX-mediated primary CD8$^+$ T cell responses, but dramatically boosted tumor or peptide-induced secondary CD8$^+$ T cell responses leading to potent synergistic effects against preestablished tumors. Altogether, CTX combined to DEX vaccine could be of great interest for the design of peptide-based vaccines.

Materials and Methods

Reagents

Cyclophophamide was obtained from Baxter (ENDOXAN), whereas mafosfamide was provided by F. Martin (Faculty of Medicine, Institut National de la Santé et de la Recherche Médicale Unité 517, Dijon, France). Mart-1_{26-35} (ELAGIGILTV), gp$100_{154-162}$ (KTWGQYWQV), gp$100_{209-217}$ (IMDQVPFSV), and Flu (GILGFVFTL) peptides were purchased from (Neosystem). Anti-NK1.1 mAb used for the NK cell depletion was produced from PK136 hybridoma (ATCC HB-191); anti-CD4 mAb used for the CD4 cell depletion was produced from YTS169 hybridoma

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[4] Abbreviations used in this paper: DC, dendritic cell; CTX, cyclophosphamide; Treg, CD4$^+$CD25$^+$ regulatory T cell; Tconv, CD4$^+$CD25$^-$ T; DEX, DC-derived exosome; LN, lymph node; HHD2, human D^b; BM-DC, bone marrow-derived DC.

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(given by F. Lemonnier, Institut Pasteur, Paris, France). CpG oligomeric sequences (5′-TCC ATG ACG TTC CTG ACG TT-3′) were given by Dr. A. Carpentier (Department of Neurology, Groupe Hospitalier Pitié Salpétrière, Paris, France).

Flow cytometry analyses

Tetramer staining. Lymph node or spleen mononuclear cells were first stained with 0.2 μg of HLA-A2/Mart1 or A2/HIVgag fluorescent (PE) soluble tetramers (Immunomics; Beckman Coulter) for 30 min at room temperature in 20 μl of PBS 1× 0.5% BSA (Sigma-Aldrich), then with anti-CD3 FITC mAb and anti-CD8 APC mAb (BD Pharmingen) for 30 min at room temperature before washing and analysis in a FACSCalibur (BD Biosciences).

Foxp3 staining. Foxp3 staining was realized according to manufacturer protocol (Foxp3 anti-mouse/rat Foxp3 staining set; eBioscience). Briefly, mononuclear cells were first stained with surface molecule Abs and washed with cold PBS. Cells were resuspended in the fix/perm buffer (eBioscience) and incubated at 4°C overnight in the dark. The next day, cells were first washed in cold PBS and then washed twice in permeabilization buffer (eBioscience). Fc block was then performed using anti-CD16/anti-CD32 Abs (24G2 clone; BD Pharmingen) for 15 min. Anti-mouse/rat Foxp3-PE was next added, and cells were incubated for 30 min. Cells were then washed twice in permeabilization buffer, resuspended in PBS 3% FCS, and analyzed in a FACSCalibur (BD Biosciences).

Mice

Human D^b (HHD2) mice derived from a strain deficient for mouse β_2-microglobulin and H-$2D^b$ molecules and transgenic for a chimeric MHC class I molecule, HLA-A0201/D^b, linked to the human β_2-microglobulin (18), were provided by F. Lemonnier. Female BALB/c (H-2^d) wild type were obtained from the "Centre d' Elevage Janvier" (Le Genest St Isle, France) and the "Centre d' Elevage Iffa Credo" (L'Arbresle, France) and maintained in the animal facility of the Gustave Roussy Institute according to the Animal Experimental Ethics Committee Guidelines.

DC culture

Mouse bone marrow-derived DC (BM-DC) were cultured as previously described (1). Briefly, bone marrow progenitor cells were grown in IMDM culture medium (Sigma-Aldrich) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10% decomplemented FCS (Invitrogen Life Technologies), 50 μM 2-ME (Sigma-Aldrich), and 30% J558-mGM-CSF culture supernatants for 10–12 days. At day 10, BM-DC were propagated in ultrafiltrated or ultracentrifuged medium (depleted in serum exosomes) as previously described (1, 19), and at day 12, the culture supernatant was collected for exosome preparation. The phenotype of BM-DC was analyzed by flow cytometry using anti-mouse CD11c, I-A^b, CD80, CD86, and CD40 mAbs (BD Pharmingen), and H-$2K^b$ and H-$2D^b$ at days 10 and 12. In addition, BM-DC propagated from HHD2 mice were stained with MA2.1 Ab-containing ascites.

Exosome production, purification, and loading

Exosomes were derived according to a process of ultrafiltration/diafiltration from Lamparski et al. (19). Briefly, 2–4 liters of DC culture medium was microfiltrated (3 μm/0.8 μm) and then ultrafiltered through a 500-kDa filter up to a final volume of 50 ml. These 50 ml of exosome containing medium was supplemented with up to 1 liter of PBS and a second step of 500=kDa ultrafiltration was performed, leading to a final volume of 20–50 ml. This preparation was ultracentrifuged at 100,000 × g onto a D20/30% sucrose gradient density cushion (d = 1.217 g/cm^3). The exosomal pellet recovered in the cushion was diafiltrated for sterilization and will be referred to as "DEX" henceforth. To elute the endogenous MHC class I peptides bound to exosomes, 100 μl of exosomes were treated with an equal volume of acetate buffer (pH 5.1), containing the synthetic CTL epitope MelanA/Mart1 at 10 μM at 4°C for 30 min (DEX/Mart1). After such an acidification, the preparation was neutralized with a Tris buffer (pH 11) on ice for 15 min to allow reformation of the trimolecular MHC class I/peptide complexes. Then, unbound peptides and debris were removed using an ultracentrifugation (100,000 × g/min, 40 min) step on a D2O/30% sucrose gradient density cushion. The exosomes recovered in the cushion were subsequently ultracentrifuged (100,000 × g, 1 h). The pellet was resuspended in PBS 1× and stored at −80°C. Unbound peptides could not exceed a final concentration of 1–7 nM (3, 19). The process was similar for the loading of $gp100_{154-162}$ and $gp100_{209-217}$ or Flu peptide (DEX/gp100; DEX/Flu).

Regulatory T cell purification and MLR

CTX or PBS were injected i.p in HHD2 mice. At day 6, spleen cells were collected. $CD4^+CD25^-$ T (Tconv) and $CD4^+CD25^+$ T (Treg) lymphocytes were purified using a $CD4^+CD25^+$ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. For MLR cocultures, 1 × 10^5 Tconv from PBS-treated HHD2 were cultured alone or with 2 × 10^4 irradiated allogeneic BALB/c splenocytes during 4 days in the presence or absence of $CD4^+CD25^+$ Treg (derived from CTX or PBS-treated HHD2 mice) at different Tconv to Treg ratio (2:1, 4:1, 8:1, and 16:1). One microcurie per well of [^{3}H]thymidine was added during the last 16 h. [^{3}H]Thymidine incorporation was measured by liquid scintillation counting after harvesting the cells on glass fibber filters using an automatic cell harvester (Tomtec). The same procedure was used to purify Treg and Tconv for adoptive transfer in tumor-bearing mice.

Tumor models

B16F10 cotransfected with the human HLA-A2 and gp100 encoding cDNA (B16A2/gp100) was provided by the Department of Tumor Immunology, Center for Molecular Life Science, Radbourg University Center, Nijmegen, The Netherlands (Dr. G. Adema). A total of 3 × 10^4 tumor cells was inoculated at day 0 in the right abdominal flank, and tumor growth was monitored biweekly using a caliper. The murine hepatoma MM45T-Li cell line was given by Dr. J. M. Perron (Liver Unit, Digestive Disease Federation, Clinique Dieulatoy, Centre Hospitalier Régional Universitaire Purpan, Toulouse, France) and used as irrelevant control for in vitro stimulation experiments.

MelanA/Mart-1 specific $CD8^+$ T cell induction in HHD2 mice

Transgenic mice were immunized in the footpad with 50 μl of the vaccine consisting of either DEX/Mart-1 (10 μg) or Mart-1 peptide (50 μg). When DEX/Mart-1 was admixed with endotoxin-free ODN-CpG oligomeric sequences, the dose of ODN-CpG was 20 μg of ODN-CpG per mouse in a total volume of 50 μl for footpad inoculation.

For priming studies, 2 mg per mouse of CTX was injected i.p. at day 0, followed, at day 6, by inoculation of the vaccines, and mice were sacrificed at day 11 for harvesting of popliteal and inguinal draining lymph nodes (LN). For prime-boost studies, a boost was performed at day 13 with the different vaccines, and mice were sacrificed at day 18 to analyze LN cells. Lymph node and spleen mononuclear cells were first stained with A2/Mart-1 or A2/HIVgag fluorescent (PE) soluble tetramers (0.2 μg), then with anti-CD3 FITC mAb and anti-CD8 APC mAb (BD Pharmingen) and analyzed in a FACSCalibur (BD Biosciences). LN and spleen cells were subjected to in vitro restimulation with Mart1 or irrelevant peptides for 48 h. Supernatants of these cultures were collected at 48–72 h to evaluate IFN-γ in EIA (BD Pharmingen).

Statistical analyses

Results were expressed as means ± SEM, or as ranges when appropriate. Groups were compared by using ANOVA followed by multiple comparison of means with Fisher's least significance procedure. When the variables studied were not normally distributed, nonparametric statistical methods were used. The Wilcoxon two-sample rank sum test was used to compare the values of continuous variables between two groups. When three or more groups were compared, the Kruskal-Wallis test was used. Values of $p < 0.05$ were considered significant at 95% confidence interval.

Results

CTX dramatically enhanced DEX-mediated antitumor effects

We have reported previously that DEX pulsed with HLA-A2.1-restricted synthetic tumor/self peptides (Mart-1/gp100) induced the differentiation of primary $CD8^+$ Tc1 lymphocytes in HLA-A2 transgenic mice (HHD2) mostly when combined to natural adjuvants (mature DC) or to TLR-3 and -9 ligands (dsRNA or ODN-CpG) (3). gp100 peptides presented by the DEX A2.1 molecules combined to ODN-CpG adjuvants were far more efficient than free peptides to reduce the growth of B16F10 melanoma coexpressing HLA-A2/gp100 in HHD2 mice (3). However, when DEX/gp100 vaccines in ODN-CpG adjuvants were used to immunize the host against bulky B16A2/gp100 tumors, DEX did not exhibit marked antitumor efficacy (Fig. 1*A*). Enhanced antitumor effects were achieved by pretreating the tumor-bearing hosts with immunopotentiating dosing of CTX. Indeed, CTX alone also promoted some tumor growth retardation (Fig. 1*A*), but markedly augmented the DEX/ODN-CpG-mediated antitumor effects against established tumors (Fig. 1*A*). Pretreatment of tumor-bearing mice with CTX before DEX/CpG vaccines promoted up to 30 ± 10% long-lasting complete tumor eradication (as compared with 10 ± 10% with DEX/ODN-CpG or CTX alone). Tumor-free animals were able to reject rechallenge with 10 times the minimal tumorigenic dose of B16A2/gp100 in 66% of cases, suggesting that long-term protective immune responses can be achieved using DEX/ODN-CpG combined with CTX (data not shown).

It is noteworthy that mafosfamide, the active metabolite compound mediating the alkylating bioactivity of CTX, had no direct cytotoxic effects onto B16A2/gp100 tumor cells in vitro (Fig. 1*B*), suggesting a noncell autonomous mode of action. Accordingly, CTX could enhance primary (Fig. 1*C*) and secondary (Fig. 1*D*) Mart-1 peptide-specific $CD8^+$ T cell responses elicited by DEX/ODN-CpG by 2- and 10-fold, respectively.

Furthermore, ODN-CpG were dispensable for the antitumor effects mediated by DEX in combination with CTX. Indeed, DEX/gp100 acquired, in hosts pretreated with CTX, a significant and long-lasting therapeutic efficacy against established B16A2/gp100 tumors (Fig. 2*A*). In similar settings, substitution of DEX/gp100 by high doses of synthetic gp100 peptides did not induce significant tumor growth retardation in the presence of CTX (Fig. 2*B*).

Altogether, CTX can boost DEX-mediated antitumor effects in the absence of adjuvants.

CTX inhibited Treg functions in vivo

Because immunopotentiating dosing of CTX is known to decrease absolute numbers and functions of Treg (17), we investigated the capacity of CTX to induce such effects in HHD2 mice bearing established B16A2/gp100 tumors. First, CTX significantly reduced the whole spleen cellularity ($75 \pm 8.7 \times 10^6$ vs $52 \pm 10 \times 10^6$ in

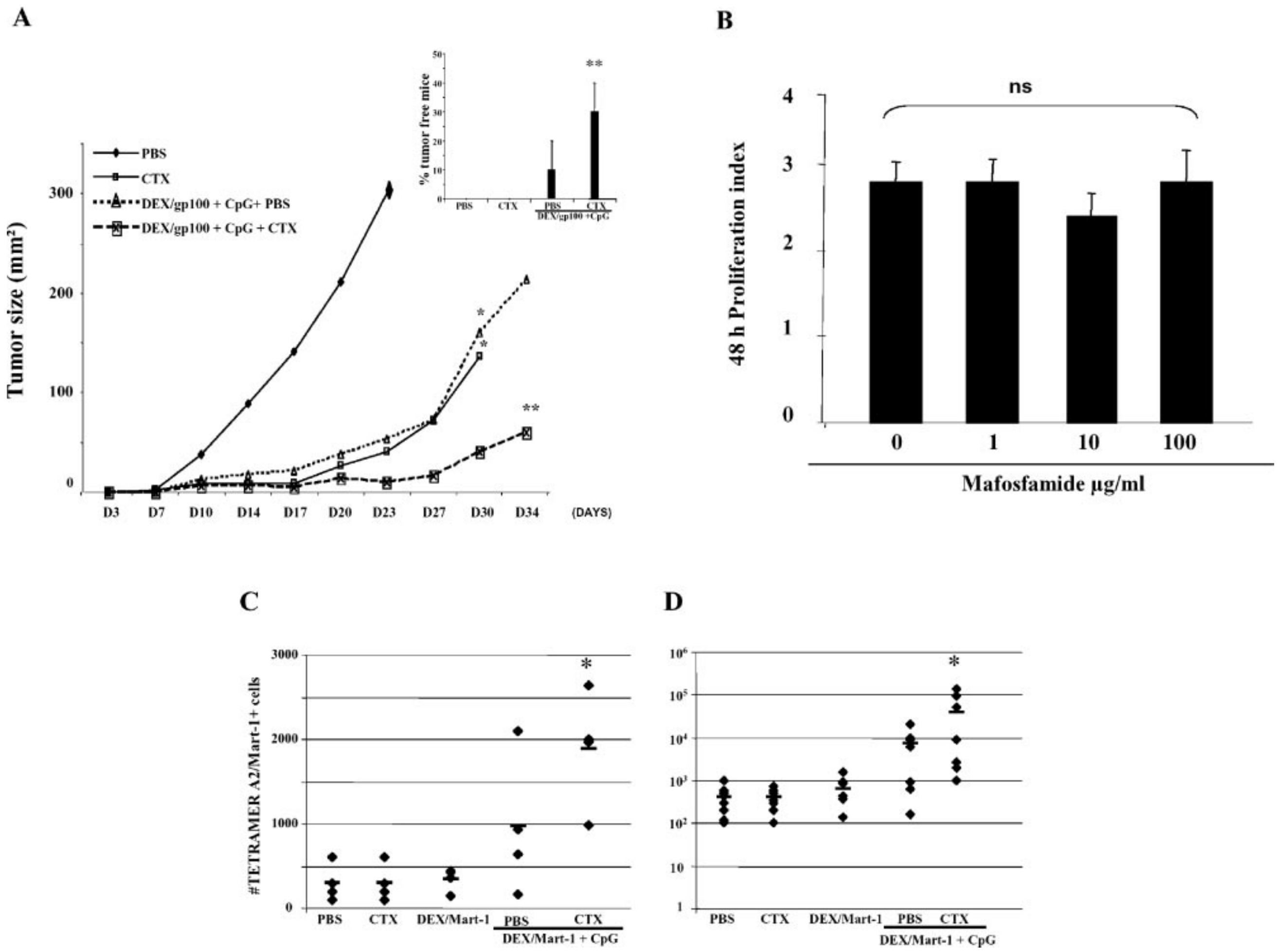

FIGURE 1. CTX enhanced DEX/ODN-CpG antitumor effects. *A*, Additive antitumor effects of two components, CTX and DEX/ODN-CpG. HHD2 mice were inoculated with 3×10^4 B16A2/gp100 cells at day 0, then treated i.p. with CTX (2 mg) or PBS at day 6. gp100-loaded DEX (10 μg) was injected in the footpad at day 12 together with 20 μg of ODN-CpG. Tumor growth was monitored over time. $*$, $p < 0.05$ as compared with the PBS group. $**$, $p < 0.05$ as compared with the DEX/ODN-CpG, DEX, and PBS groups. *B*, Mafosfamide did not inhibit tumor growth in vitro. B16A2/gp100 were cultured (5×10^4 cells/well) with complete medium alone or supplemented with 1–100 μg/ml mafosfamide. The proliferation index of B16A2/gp100 cells, defined as the number of living cells after a 48-h culture/5×10^4, remained stable. *C*, CTX enhances the CTL priming elicited by DEX/ODN-CpG. PBS or 2 mg/mouse of CTX were injected i.p. at day 0. At day 6 PBS, Mart1-loaded DEX (10 μg) alone or with 20 μg of ODN-CpG were injected in the footpad. Popliteal and inguinal draining LN cells were collected at day 11 for A2-Mart-1-specific tetramer staining. $*$, $p < 0.05$ as compared with the DEX/Mart1-CpG, DEX/Mart-1, CTX, and PBS groups. *D*, CTX allowed secondary T cell responses elicited by DEX/ODN-CpG. Same as *C*, but at day 13, a boost was performed with the different vaccines or PBS, and mice were sacrificed at day 18. All experiments included three to five mice per group and were performed twice with identical results. $*$, $p < 0.05$ as compared with DEX/Mart1-CpG, DEX/Mart-1, CTX, and PBS groups.

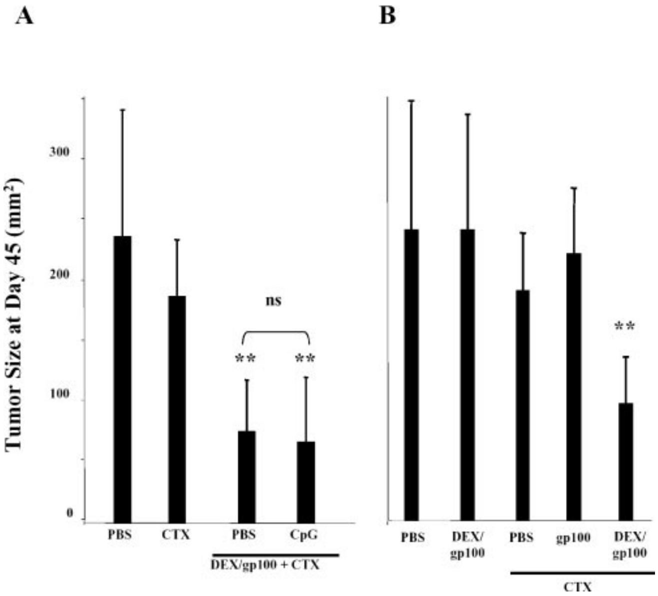

FIGURE 2. CTX promoted DEX-mediated antitumor effects. *A*, DEX-mediated antitumor effects when mice received CTX. Same as Fig. 1*A* but also in the absence of ODN-CpG. **, $p < 0.05$ as compared with CTX and PBS groups. *B*, Synthetic peptides could not substitute for DEX. Same as *A*, but gp100-loaded DEX (10 μg) or gp100 peptides (50 μg) were injected in the footpad at day 12. **, $p < 0.05$ as compared with CTX, DEX/gp100, gp100 + CTX, and PBS groups.

PBS- vs CTX-treated mice, respectively, $p < 0.05$). Second, CTX significantly decreased the $CD4^+CD25^+$ to $CD4^+$ ratio (8 ± 1.2% vs 15 ± 2.6% in CTX- vs PBS-treated mice, respectively, $p < 0.05$). Third, although pathognomonic of Treg function, expression of Foxp3 remained stable on $CD4^+CD25^+$ cells purified with magnetic beads after treatment with CTX (76.8 vs 74.4% of $CD4^+CD25^+$ T cells purified from PBS- or CTX-treated mice, respectively, NS) (Fig. 3*A*). However, $CD4^+CD25^+Foxp3^+$ cells derived from CTX-treated hosts lost their inhibitory functions in mixed allogeneic reactions in vitro (Fig. 3*B*).

To confirm that CTX facilitated DEX immunotherapy by eliminating tumor-induced Treg, we adoptively transferred $CD4^+CD25^+$ Treg derived from naive HHD2 mice into CTX-pretreated tumor-bearing mice before vaccination with DEX. The administration of Treg but not Tconv completely abrogated the therapeutic benefit of the combination of CTX and DEX vaccines, suggesting that Treg were indeed inhibited by CTX (Fig. 3*C*).

$CD8^+$ and $CD4^+$ T cells, but not NK cells, were required for DEX/CTX efficacy

To investigate the role of NK cell effectors in the DEX/CTX-mediated antitumor effects, we depleted HHD2 mice from NK cells using the anti-NK1.1 depleting Ab before vaccination. The combination of DEX/CTX remained efficient at preventing tumor growth in the absence of $NK1.1^+$ cells (Fig. 4*A*). In parallel, we failed to show that DEX loaded with an irrelevant CTL epitope (Flu peptides) or unloaded DEX (data not shown) could synergize with CTX to eradicate established tumors (Fig. 4*B*), suggesting that the therapeutic efficacy of DEX combined with CTX is dependent on HLA-A2-restricted $CD8^+$ T cells. The role of conventional $CD4^+$ T cells has also been assessed by treating HHD2 mice with the depleting YTS169 mAb anti-$CD4^+$ T cells (which depleted both conventional and Treg). In the absence of $CD4^+$ lymphocytes, the combination of CTX/DEX was significantly less efficient at preventing tumor growth than in the absence of depletion. It is interesting to note that in the absence of $CD4^+$ T cells, CTX

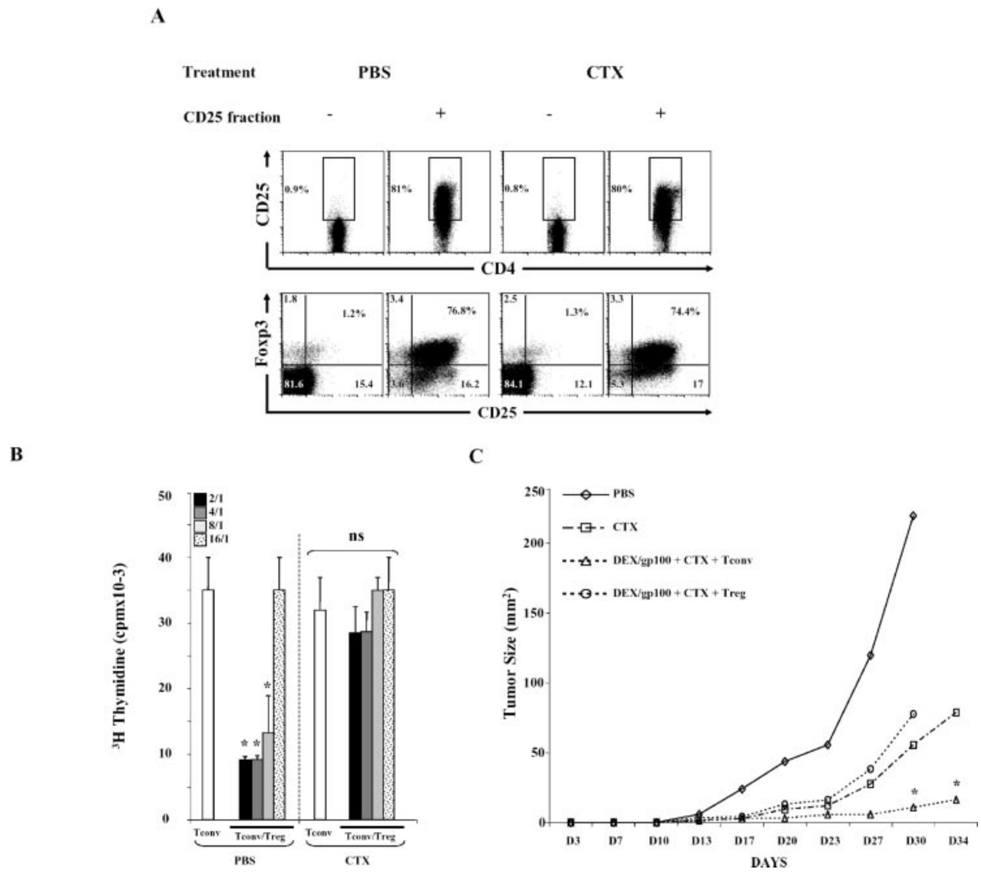

FIGURE 3. CTX inhibited Treg functions. *A*, $CD4^+CD25^+$ Treg exhibited a similar phenotype in CTX- and PBS-treated mice. Splenocytes were stained with anti-Foxp3 mAb after magnetic purification of $CD4^+$ $CD25^+$ T cells. These cells were used for MLR and adoptive transfer experiments. *B*, CTX suppressed the inhibitory effects of Treg. Tconv and Treg purified from PBS- or CTX-treated HHD2 mice were used in MLR (ratio of BALB/c splenocytes to Tconv was 1:5). *, $p < 0.05$ as compared with Tconv alone. *C*, Adoptive transfer of Treg abrogated the synergistic antitumor effects between DEX and CTX. Same as Fig. 2, but DEX/gp100-immunized mice pretreated with CTX were adoptively transferred with either 10^6 $CD4^+CD25^+Foxp3^+$ (Treg) or $CD4^+CD25^-Foxp3^-$ (Tconv) T lymphocytes at day 11. *, $p < 0.05$ as compared with PBS, CTX, or DEX/gp100 + CTX + Treg.

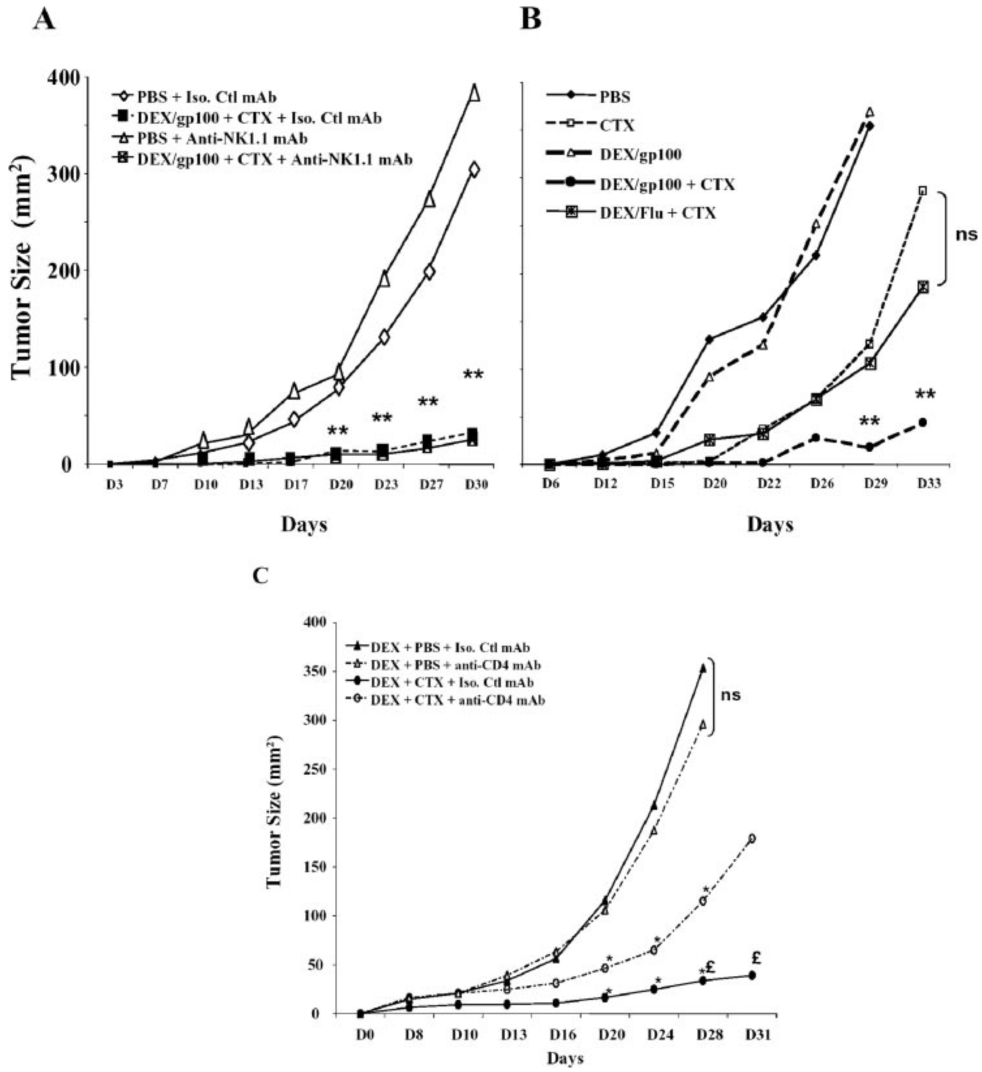

FIGURE 4. $CD4^+$ and $CD8^+$ T cells, but not NK cells, were required for DEX/CTX efficacy. *A*, The synergistic effects between DEX and CTX are NK cell independent. The same experimental settings as in Fig. 2 were used. NK cells were depleted using 200 μg/mouse of anti-NK1.1 mAb (PK136) given i.p. at day −7, −3, and 0, then once a week. Control mice were injected with irrelevant isotype control Ab. **, $p < 0,01$ as compared with PBS and anti-NK1.1 mAb alone. *B*, The synergistic effects between DEX and CTX are peptide dependent. gp100 or Flu-loaded DEX (10 μg) were injected in the footpad at day 12. **, $p < 0.01$ as compared with the DEX/Flu + CTX, DEX/gp100, CTX, and PBS groups. *C*, $CD4^+$ T cells are required for optimal efficacy of the combination of DEX/CTX. $CD4^+$ cells were depleted using 200 μg/mouse of YTS169 mAb given i.p. at day −7, −3, and 0, then once a week. Control mice were injected with irrelevant isotype control Ab. *, $p < 0.05$ as compared with DEX; £, $p < 0.05$ as compared with DEX + CTX + YTS169. All experiments were performed twice with similar results. A representative experiment containing five mice per group is depicted.

still mediated antitumor effects in addition to DEX, suggesting that another suppressor cell type is inhibited by CTX (Fig. 4*C*).

CTX promoted DEX-mediated secondary $CD8^+$ T cell responses

We hypothesized that CTX could either promote the capacity of DEX to prime naive $CD8^+$ T cells or to boost tumor-primed effector T cells in vivo. We failed to show that CTX allowed efficient priming by DEX in the absence of adjuvants. Indeed, in CTX-treated animals, DEX pulsed with Mart-1 peptides did not elicit the expansion and differentiation of Mart1-specific $CD8^+$ Tc1 cells in draining lymph node or spleen, as shown in flow cytometry using specific Mart-1 tetramers and in IFN-γ secretion (Fig. 5).

When CTX and DEX were administered in prophylaxis 3–10 days (data not shown) before tumor inoculation, no synergistic antitumor effects could be observed (Fig. 6). To formally demonstrate that CTX enabled DEX to boost secondary $CD8^+$ T cell responses, we immunized tumor-free hosts with Mart-1 peptides in adjuvant (ODN-CpG) (at day 0) and challenged mice (at day 12) using either Mart-1 peptides or DEX/Mart-1 6 days after administration of CTX (Fig. 7*A*). Administration of CTX after Mart-1 priming allowed the expansion of Mart-1-specific CTLs after a boost with either Mart-1 peptides (7 ± 0.16% of $CD3^+CD8^+$A2/Mart1$^+$ cells in draining LN vs 0.5 ± 0.35% in contralateral LN, $p < 0.05$) or DEX/Mart-1 (4 ± 1.5% in draining LN vs 2.3 ± 0.5% in contralateral LN, $p < 0.05$). However, CTLs were able to produce high amounts of IFN-γ, only when mice were challenged with DEX/Mart1 but not Mart-1 peptides alone (Fig. 7*B*). In the absence of CTX, no CTL differentiation was observed. The DEX capacity to boost peptide-specific $CD8^+$ Tc1 lymphocytes in the presence of CTX was not further enhanced by CpG (data not shown).

This result suggests that in the presence of CTX, DEX can boost effector T cell functions. Tumor cells should be able to prime specific effector T cells in vivo. Thus, we confirmed that tumor growth is able to promote the expansion of tumor-specific T cells because splenic T cells from tumor-bearing HHD2 mice produced tumor-specific IFN-γ in mixed B16A2/gp100 tumor lymphocyte cultures when mice were treated with CTX (Fig. 7*C*). As expected, no IFN-γ secretion was observed in PBS-treated animals or when splenocytes were stimulated with irrelevant tumor cells (Fig. 7*C*). Thus, DEX vaccines were able to boost secondary immune responses primed by tumor growth or peptides in the absence of functional $CD4^+CD25^+$ Treg cells.

Discussion

These experiments show for the first time 1) the ability of DC-derived exosomes to boost secondary immune responses induced by immunogenic tumors or peptides in adjuvants, 2) the inhibitory effects induced by Treg on DEX-mediated secondary immune responses, and 3) therefore, the synergistic antitumor effects of the combination of CTX and DEX.

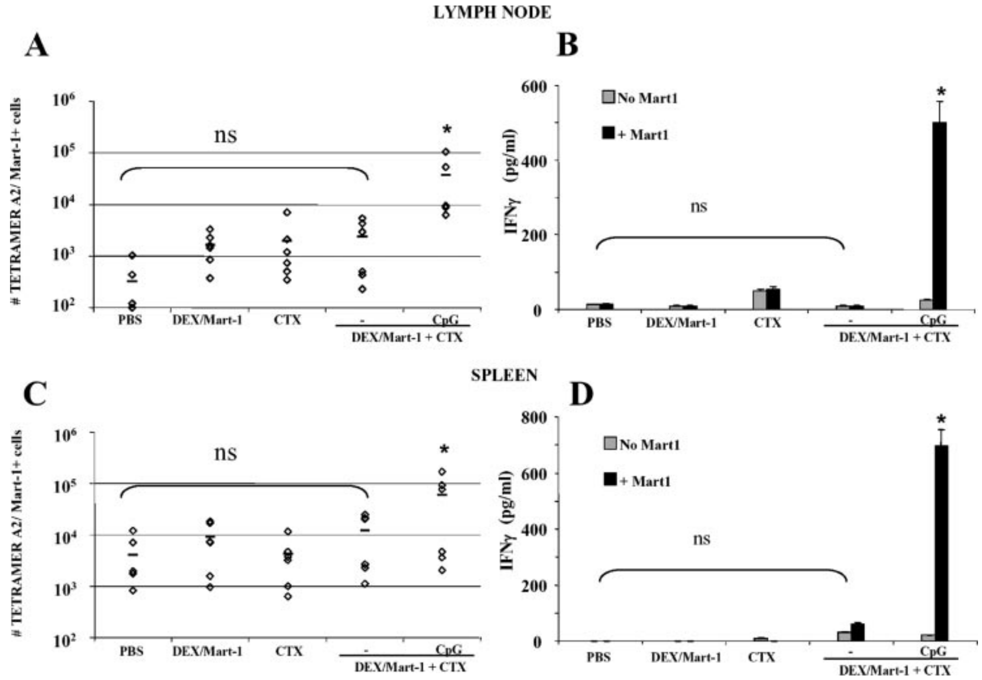

FIGURE 5. CTX did not allow DEX-mediated CTL priming. PBS or 2 mg/mouse of CTX were injected i.p. at day 0. At day 6, priming using various vaccine designs was performed. Popliteal and inguinal draining LN and spleen cells were then collected for A2-Mart-1-specific tetramer staining and in vitro stimulation with or without Mart1 peptides. This experiment included five to six mice per group and was performed twice with identical results. *, $p < 0.05$ as compared with all the other groups and same group with no Mart-1 in vitro stimulation.

CTX is an alkylating agent directly cytotoxic on various tumor cell types but also used as an immunosuppressive agent in organ transplants or in the management of rheumatoid arthritis, systemic lupus erythematosus, scleroderma, glomerulonephritis, chronic hepatitis, and other diseases. It is also known for decades that at low doses, CTX exhibits immunopotentiating properties (20–22). In various animal models, CTX augments delayed type hypersensitivity responses (20–22), increases Ab production, abrogates tolerance, and potentiates antitumor immunity (23–28). The mechanism of CTX immunopotentiation involves inhibition of a suppressor function (22). The demonstration has been made that tumor regression caused by the combination of CTX together with immune T cells could be inhibited by infusing CTX-sensitive L3T4$^+$ T cells from tumor-bearing hosts and not normal donors (29, 30). Thus, CTX facilitated the effectiveness of adoptive transfer of immune cells against tumor cells by eliminating tumor-induced suppressor T cells. Moreover, Ibe et al. (31) have also shown that CTX resulted in tumor regression by modulating the capacity of tumor-infiltrating lymphocytes to switch IL-10-producing tumor-infiltrating macrophages into IFN-γ producers, leading to destruction of the tumor vasculature. Interestingly, in a model of mouse diabetes depending on Treg and synchronously induced by a single injection of CTX, a time course analysis of the gene expression profiles of purified islet cells using microarrays revealed surprising findings. There was no reduction in the expression of genes characteristic of regulatory T cells but instead, a marked decrease in transcripts of genes specific to B cells followed by an increase in transcripts of chemokine (CXCL1, CXCL5, CCL7) and IFN-γ-regulated genes (32). Thus, the involvement of Treg in CTX-induced immunopotentiation has not been fully demonstrated, specifically during tumor regression. In our model system, we showed a 2-fold decrease in CD4$^+$CD25$^+$Foxp3$^+$ to CD4$^+$ ratio in spleens of B16A2/gp100-bearing HHD2 mice 6 days after CTX administration (but not at later time points). It is noteworthy that Foxp3$^+$ Treg are more susceptible to mafosfamide-induced cell death than conventional T cells in vitro (F. Ghiringhelli, unpublished data). We also showed that the suppressive function of purified Foxp3$^+$ Treg was lost 6 days after CTX administration using mixed allogeneic lymphocyte reactions (Fig. 3*B*), suggesting than molecular events downstream of Foxp3 transcription might influence Treg inhibitory function. Importantly, Treg could curtail the DEX-mediated antitumor effects because adoptively transferred Treg completely abrogated the synergistic tumoricidal activity elicited by CTX plus DEX (Fig. 3*C*).

Treg can restrict both priming of CD4$^+$ and CD8$^+$ T cell immune responses as well as memory responses. Treg control Th1 responses to foreign Ags induced by mature DC in vivo and contribute to Th2 responses (33). Similarly, CD4$^+$CD25$^+$ T cells promoted Th2 polarization during helminth infection by suppressing

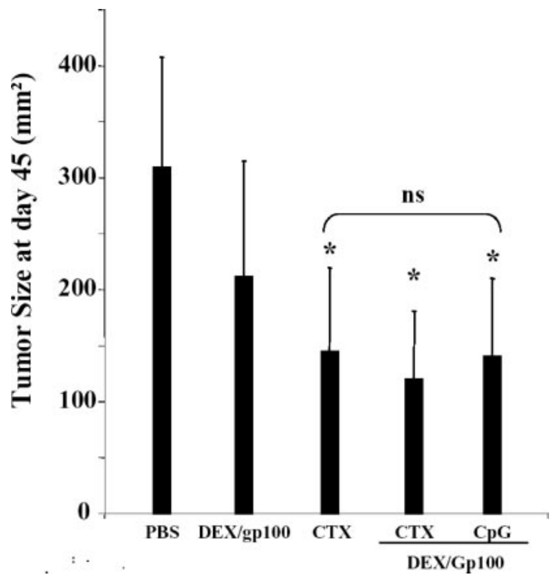

FIGURE 6. CTX did not synergize with DEX when tumors were not established. PBS or 2 mg/mouse of CTX were injected i.p. at day −10. gp100-loaded DEX (10 μg) was injected in the footpad at day −5 alone or together with 20 μg of ODN-CpG, and PBS was injected in control mice. Mice were then inoculated with 3 × 10^4 B16A2/gp100 cells at day 0. Tumors were measured bidimensionally twice a week. This experiment included five mice per group and was performed twice with identical conclusions. **, $p < 0.05$ as compared with DEX and PBS groups.

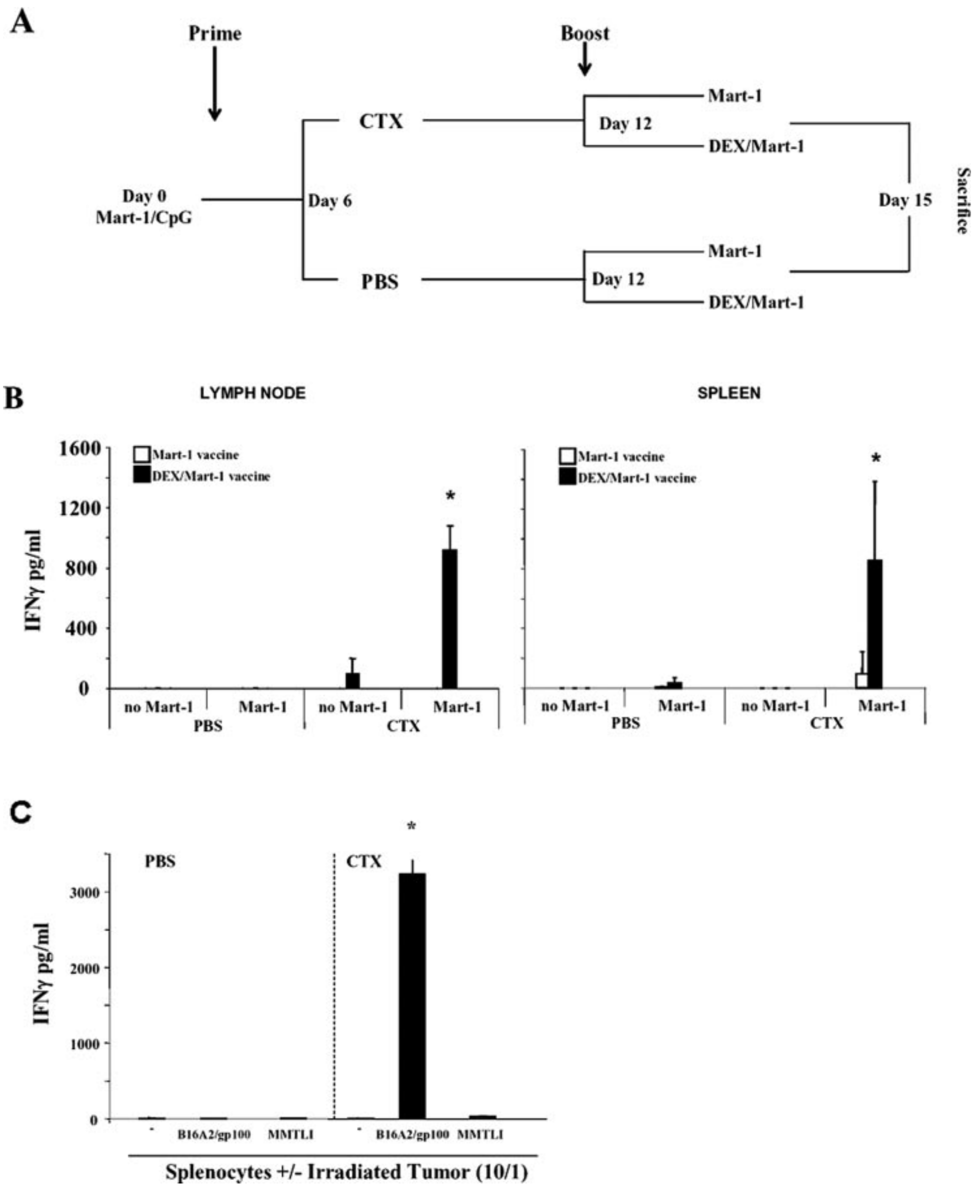

FIGURE 7. CTX allowed DEX-mediated secondary immune responses. *A*, Experimental setting. Mice were primed with Mart-1 peptides (50 μg/mouse) in ODN-CpG adjuvants (20 μg/mouse) at day 0. At day 6, CTX (2 mg/mouse) or PBS was administered i.p. At day 13, a boost consisting of either 50 μg of Mart-1 peptides or 10 μg of DEX/Mart1 was inoculated. *B*, DEX, but not synthetic peptides, boost secondary immune responses elicited by peptides/ODN-CpG in CTX-treated mice. At day 18, LN or spleen mononuclear cells were harvested for in vitro stimulation with or without Mart-1 peptides. Dosage of IFN-γ was performed in the culture supernatant 48 h later. $*$, $p < 0.05$ as compared with cells from PBS-treated mice and cells from Mart-1-vaccinated mice. *C*, Tumor cells elicited tumor-specific T cells. A total of 3×10^4 B16A2/gp100 tumor cells was injected at day 0 in the right abdominal flank; at day 6, PBS or CTX was injected i.p. to mice (five mice per group); at day 12, mice were sacrificed. Spleen cells were then harvested alone or in the presence of irradiated B16 or irrelevant tumor cell line MMTLi or medium alone. Dosage of IFN-γ was performed in the culture supernatant 48 h later. $*$, $p <$ 0.05 as compared with all PBS-treated groups and medium and MMTLi CTX-treated groups.

the development of Th1 responses (34). In tumor settings, a critical role of Treg to prevent elicitation of tumor-specific immunity in various tumor models has been described (7, 13). Depletion of $CD4^+CD25^+$ T cells enhanced the strength of memory $CD8^+$ T cell responses elicited after secondary *Listeria monocytogenes* infection or after boost immunization with LLO peptides or a DNA vaccine containing the listeriolysin gene (35). Suvas et al. (36) also showed, in a model of HSV1 viral infection, that depletion of Treg prolonged the maintenance of responding $CD8^+$ T cells. Preclinical studies in mice using DEX loaded with tumor peptides highlighted the priming capacity of these nanomeric vesicles harboring MHC class I/peptide complexes only in the presence of mature bone marrow DC or TLR-3 and -9 ligands (3). In this study, we show that, in the absence of adjuvants, DEX can boost peptide-induced $CD8^+$ Tc1 immune responses (Fig. 7) and presumably tumor-induced $CD8^+$ T cell responses (Fig. 7). This study clearly demonstrates that both primary immune responses induced by DEX/ODN-CpG and secondary immune responses induced by DEX alone are controlled by Treg, limiting the antitumor efficacy of the DEX vaccines in tumor-bearing hosts. Indeed, the combinations of DEX/CTX or DEX/CTX/ODN-CpG were efficient at promoting up to 30% tumor eradication and marked tumor growth retardation in most animals. However, ODN-CpG did not add any clinical benefit in the presence of CTX. This is surprising because suppressor T cells restrict the ability of DEX/ODN-CpG to induce expansion of peptide-specific primary and secondary $CD8^+$ T cell immune responses. Indeed, CTX could enhance by 2-fold (priming experiment) to 10-fold (prime boost experiment) the number of T cells binding to specific tetramers in draining lymph nodes of normal mice (Fig. 1*C*). It is possible that CpG might have induced the development of Treg in the B16 tumor model. Indeed, TLR-9 stimulation could promote plasmacytoid DC-mediated generation of Treg exhibiting strong immune suppressive function (37). Similarly, Treg are not only induced by viral infection (36) and tumor growth (9, 10) but also by active immunization as reported in clinical studies (38, 39). Supporting this view, investigators showed that DC from autoimmune mice can increase the number and function of Ag-specific Treg (40). Whether DEX could also induce Treg remains to be determined and compared with alternate vaccine strategies. However, IL-2 and/or strong $CD4^+$ Th responses generated by tumor vaccines could overcome the deleterious suppressive effects of Treg (13, 39).

It is important to bear in mind that vaccine strategies might not prime naive T cells but rather boost preexisting $CD4^+$ and $CD8^+$

T cell responses elicited by the tumor/host itself. Several lines of evidence point to the natural immunogenicity of certain tumor types that could be revealed after immunotherapy (41). Our data clearly indicate that tumor-induced priming is required for the efficacy of DEX/CTX (Fig. 7). However, the mechanisms by which DEX/CTX switched the balance from tumor-induced tolerance toward tumor-induced immunogenicity remains unclear. It is conceivable that in the absence of functional Treg, effector $CD4^+$ T cells might activate tumor-infiltrating DC through a CD40L-dependent pathway (42, 43). In this scenario, DEX could be efficiently presented by mature DC and promote T cell activation (3).

Preliminary studies using oral administration of low doses of CTX in cancer-bearing patients suggest the feasibility to inhibit Treg functions (our unpublished data) in vivo. Our data suggest that the combination of CTX and DEX represents a valuable combination therapy for immunotherapy against cancer.

Disclosures

The authors have no financial conflict of interest.

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CORRECTIONS

Caprio-Young, J. C., J. J. Bell, H.-H. Lee, J. Ellis, D. Nast, G. Sayler, B. Min, and H. Zaghouani. 2006. Neonatally primed lymph node, but not splenic T cells, display a Gly-Gly motif within the TCR β-chain complementarity-determining region 3 that controls affinity and may affect lymphoid organ retention. *J. Immunol.* 176**:** 357–364.

In **Results**, in the penultimate sentence of the second paragraph under the heading *A Gly-Gly motif is conserved within the CDR3 of lymph node T cell hybridomas,* reference to Figure 7*a* and 7*b* are reversed. The corrected sentence is shown below.

The lymph node TCR contains a rigid loop with an extended planar surface (Fig. 7*b*), whereas the splenic Th1 TCR presents a round shape with a less extended surface (Fig. 7*a*).

Taieb, J., N. Chaput, N. Schartz, S. Roux, S. Novault, C. Ménard, F. Ghiringhelli, M. Terme, A. F. Carpentier, G. Darrasse-Jèse, F. Lemonnier, and L. Zitvogel. 2006. Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. *J. Immunol.* 176: 2722–2729.

The tenth author's last name is incorrect. The correct name is Guillaume Darrasse-Jèze.

Swiecki, M. K., M. W. Lisanby, F. Shu, C. L. Turnbough, Jr., and J. F. Kearney. 2006. Monoclonal antibodies for *Bacillus anthracis* spore detection and functional analyses of spore germination and outgrowth. *J. Immunol.* 176: 6076–6084.

In Table II, the data reported for GA2–3[b] in column six (Δ*rml*D spores) should be negative (−) not 2-log shift (++). The corrected table is shown below.

Table II. *mABs raised against irradiated* B. anthracis *spores or purified* B. anthracis *exosporium*

mAbs	Western: Anti-*E. coli* Bc1A	Western: Anti-Deglyco, Bc1A	FACS: WT Sterne spores	FACS: Δ*bc1A* spores	FACS: Δ*rmlD* spores
AB2	ND[a]	ND	+	−	++
AF10[b]	+	+	+	−	++
AH8[8]	+	−	+	−	++
BD8[b]	+	+	+	ND	ND
BE12[b]	+	+	+	−	++
BF1-4[b]	+	+	+	−	++
BF12	ND	ND	+	−	++
BG11[b]	+	−	+	ND	ND
CA3*	+	−	+	−	++
DE3-1[b]	+	+	+	−	++
DE12	−	−	+	−	++
FD3-4[b]	+	+	+	ND	ND
EF12[b]	+	+	+	ND	ND
AA2-1	ND	ND	+	−	++
BA10-1[b]	+	+	+	−	++
DH4-1[b]	−	−	+	++	++
EA2-1	ND	ND	+	−	++
EA4-10[b]	+	+	+	−	++
EA4-10-4	+	+	+	−	++
EG4-4[b]	+	+	+	−	++
FH6-1[b]	−	−	+	++	++
GA2-3[b]	−	−	+	−	−
GB4-4[b]	−	−	+	ND	ND
GB4-6-2	ND	ND	+	−	++
HB2-2	ND	ND	+	−	++
IC801[b]	−	−	+	−	++
JB5-1[b]	+	+	+	−	++
JC8-5[sb]	+	+	+	−	++

[a] ND, Not determined, −, negative by Western or FACS; +, positive by Western or l-log shift by FACS; ++, 2-log shift by FACS ABS-EF12, mAbs raised against spores; AA2-I-JC8-5, mAbs and raised against exosporium.
[b] Included in Ref. 9.

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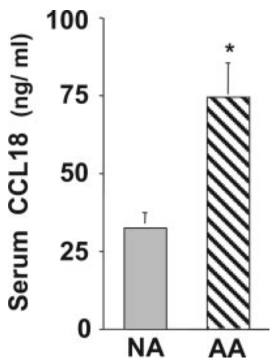
de Nadaï, P., A.-S. Charbonnier, C. Chenivesse, S. Sénéchal, C. Fournier, J. Gilet, H. Vorng, Y. Chang, P. Gosset, B. Wallaert, A.-B. Tonnel, P. Lassalle, and A. Tsicopoulos. 2006. Involvement of CCL18 in allergic asthma. *J. Immunol.* 176: 6286–6293.

In **Results**, in the last sentence of the paragraph under the heading *CCL18 is up-regulated in BAL and sera from AA patients,* and in Figure 4C, the concentration of serum CCL18 is expressed incorrectly as "pg/ml" instead of "ng/ml." The corrected sentence and figure are shown below.

CCL18 was significantly elevated in AA (73.9 ± 11.2 ng/ml) compared with NA (31.7 ± 5 ng/ml) subjects (Fig. 4*C*).



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The third author's first name is incorrect. The correct name is Kaihong Su.

Vaknin-Dembinsky, A., K. Balashov, and H. L. Weiner. 2006. IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production. *J. Immunol.* 176**:** 7768–7774.

During production, the figure from an unrelated article was inadvertently inserted as the image for Figure 8. The correct figure is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.