The Immunogenicity of Dendritic Cell-Based Vaccines Is Not Hampered by Doxorubicin and Melphalan Administration[1]

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Immunization of cancer patients is most effective in tumor-free conditions or in the presence of minimal residual disease. In the attempt to develop new strategies able to control tumor recurrence while allowing the development of protective immunity, we have investigated the immunogenic potential of two distinct vaccine formulations when provided alone or upon single and repeated treatment with chemotherapeutics drugs. Vaccine-induced T cell responses were first investigated by tracing Ag-specific T cell responses in mice bearing detectable frequencies of Ag-specific TCR transgenic CD4 and CD8 T cells. These studies indicated that immunization with peptide-pulsed dendritic cells and soluble Ag plus adjuvant elicited a comparable expansion and differentiation of CD4 and CD8 effector cells in the peripheral lymphoid tissues when provided alone or shortly after Doxorubicin or Melphalan administration. We also analyzed the potency of the combined vaccination in transgenic adenocarcinoma mouse prostate mice, which develop spontaneous prostate cancer. Dendritic cell-based vaccination elicited potent tumor-specific cytotoxic responses in mice bearing prostate intraepithelial neoplasia both in the absence and in the presence of Doxorubicin. Together our results indicate that Doxorubicin- or Melphalan-based chemotherapy and Ag-specific vaccination can be combined for adjuvant treatments of cancer patients. *The Journal of Immunology,* **2005, 174: 3317–3325.**

Conventional anti-cancer therapies (surgery and radio- and chemotherapy) have gained a considerable clinical success over the past years. Because of limitations imposed by the given current treatments, tumor-free survival is not always accomplished. For instance, surgery and radiotherapy are quite effective in the treatment of localized tumors, but they usually play a palliative role in the treatment of disseminated diseases. Chemotherapy in these cases is the treatment of choice, but severe toxic effects toward normal tissues often limit its use. The identification of tumor-specific Ag, tumor-specific lymphocytes, and tumor-specific T cell responses in cancer patients led to the development of immunotherapies aimed at augmenting antitumor immune responses. Studies mostly performed in preclinical studies have indicated that both active and adoptive immunotherapies are quite effective against small tumor burdens, but seem to be incapable of controlling large tumor masses (1, 2). The results generated in clinical trials supported these findings and suggest that immunotherapy is most effective in adjuvant settings (3, 4).

Together the available information indicates that both conventional treatment and immunotherapeutic strategies might benefit from combined treatments aimed at controlling tumor growth, while allowing vaccine-induced immune responses to develop and eliminate the minimal residual disease. The major limitation for combining antiblastic chemotherapy and immunotherapy is that cytotoxic drugs are generally regarded as immunosuppressive because of toxicity to the dividing immune cells in the bone marrow and peripheral lymphoid tissues (5). However, certain chemotherapeutic agents have shown immunomodulatory activities (6), and several combined approaches have already been attempted. For instance, chemotherapy has been proven to enhance the efficacy of tumor cell vaccines and immune cytokines, and to favor the activity of adoptively transferred tumor-specific T cells (7–18). A number of mechanisms have been proposed for the chemotherapy-triggered enhancement of immunotherapy response. For instance, chemotherapy may favor tumor cell death, and by that enhance tumor-Ag cross-presentation in vivo (19). Furthermore, drug-induced myelosuppression may induce the production of cytokines favoring homeostatic proliferation, and/or inhibit the activity of T regulatory cells (7, 10).

Although supporting the feasibility of combining chemotherapy and immunotherapy, previous studies did not provide qualitative and quantitative information on vaccine-induced T cell responses during chemotherapy administration. Thus, in this study, we have quantified by ex vivo flow cytometry analyses the potency of Ag-specific vaccination when performed in combination with antiblastic chemotherapy in different animal models that allow the tracing of Ag-specific CD4 and CD8 T cell responses in the context of peripheral lymphoid tissues. In the first animal model, we took advantage of the adoptive transfer (AT)[5] of traceable population of OVA-specific TCR transgenic (Tg) CD4 and CD8 T cells (20). We measured the expansion and differentiation of T lymphocytes by flow cytometry analyses and functional assays performed on cells

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[5] Abbreviations used in this paper: AT, adoptive transfer; DC, dendritic cell; DOXO, Doxorubicin; LACK, *Leishmania* receptor for activated C kinase; LN, lymph node; MEL, Melphalan; mPIN, mouse prostate intraepithelial neoplasia; OVAp, chicken OVA-derived peptide; Tag, SV40 large T Ag; Tg, transgenic; TRAMP, transgenic adenocarcinoma mouse prostate; WT, wild type.

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obtained from the peripheral lymphoid tissues of AT mice vaccinated in the absence or in the presence of antiblastic chemotherapy. In the second animal model, which allows the tracing of endogenous CD4 T cells specific for the model Ag *Leishmania* receptor for activated C kinase (LACK) (21), we investigated the possibility to perform vaccination upon repeated cycles of chemotherapy. Finally, we traced tumor-specific cytotoxicity in transgenic adenocarcinoma mouse prostate (TRAMP) mice. TRAMP mice are transgenic for the SV40 large T Ag (Tag) under the control of the rat probasin regulatory element. In the periphery, Tag is selectively expressed in prostate epithelial cells under the influence of sexual hormones (22). TRAMP mice remain Tag negative and healthy until puberty (i.e., wk 4–5) and then progressively develop mouse prostate intraepithelial neoplasia (mPIN) and invasive carcinoma of epithelial origin (23). In this model, the immune response against Tag has been well characterized, and it is dominated by CTLs specific for the sequence 404–411 (24, 25). At 7 wk of age, TRAMP mice are partially tolerant against Tag, but a Tag-specific CTL response can be induced by vaccination with peptide-pulsed dendritic cells (DC) (26).

As model vaccination strategies, we chose cell-associated (peptide-pulsed DC) and soluble (Ag plus adjuvant) Ag formulation. These can induce potent Ag- and tumor-specific CD4 and CD8 T cell responses in preclinical animal models, and are currently accepted for clinical trials (27–34).

Doxorubicin (DOXO) and Melphalan (MEL) were chosen as model chemotherapeutic agents, because of their different mechanism of action and because of their use in both preclinical and clinical studies (35). DOXO is a member of the anthracyclin family, known to exert cytotoxicity by a number of intracellular reactions, which include free radical formation, DNA intercalation, inhibition of topoisomerase II, disturbance of helicase function, and inhibition of signal transduction (36). It is used as a single agent, and also in combination with other chemotherapeutic agents (37). In humans, DOXO is mostly used at 45–60 mg/m^2 every 3–4 wk, at lower dosage every week or as continuous infusion (35). Mice tolerate higher doses of this drug, and DOXO has been demonstrated to reduce tumor growth at the dose of 4–15 mg/kg (14, 36, 38). MEL is an alkylating agent believed to exert its cytotoxic effects through the covalent linkage of alkyl groups to DNA. This leads to cross-linking of DNA strands or appearance of breaks into the DNA that hampers normal DNA replication and cell division. MEL is used at 8 mg/m^2 in patients and at 2.5 mg/kg in mouse models (35, 39). The most relevant side effects for these two drugs are myelosuppression, alopecia, mucositis, and pulmonary fibrosis. Depending on the schedule of administration, DOXO has also been shown to induce cardiomyopathy (35).

Our results indicate that a single administration of DOXO or MEL, as well as the repeated administration of DOXO before vaccine injection, did not hamper the immunogenicity of peptide-pulsed DC and soluble Ag vaccine formulations, and that potent T cell responses to the model Ags OVA and LACK and to the tumor-associated Ag Tag IV were indeed induced by the combined strategy. These data support the introduction of combined therapies in the adjuvant treatment of cancer patients.

Materials and Methods

Mice, cells, and reagents

Eight- to 10-wk-old C57BL/6 (Ly-5.2$^+$) and BALB/c mice were purchased from Charles River Laboratories. The TCR Tg mice DO11.10 (40) and OT-I (41) mice were bred to the homozygous condition. DO11.10 mice express a Tg $\alpha\beta$ TCR specific for the OVA-derived peptide (OVAp) 323–339 presented in the context of I-A^d molecules. OT-I mice express a Tg Vα2Vβ5 TCR specific for the OVAp$_{257–264}$ presented in the context of H-2^b molecules. The OT-I mice were further bred with congenic Ly-5.1$^+$ C57BL/6 mice (H-2^b) to distinguish donor CD8$^+$ T cells from host cells after their AT into Ly-5.2$^+$ C57BL/6 (H-2^b) recipients, as similarly described elsewhere (42). The frequency of Tg T cells in these mice was analyzed by flow cytometry analysis, which indicated that up to 97% of Ly-5.1$^+$ CD8$^+$ T cells expressed the Vα2 Vβ5 Tg TCR (data not shown). The 16.2β mice were previously described (21). T cells from these mice express a TCR transgenic β-chain derived from a LACK-specific hybridoma, and exhibit an increased frequency of LACK-specific CD4 T cells (21). Heterozygous TRAMP mice on a pure C57BL/6 background were typed for Tag expression by PCR-based screening assay, as described in ⟨www.jax.org⟩. All mice were housed and bred in a specific pathogen-free animal facility, treated in accordance with the European Union guidelines, and with the approval of the Institutional Ethical Committee.

All of the in vivo experiments were performed according to the Interdisciplinary Principles and Guidelines for the Use of Animal in Research, Marketing, and Education, and approved by Ethical Committee of the San Raffaele Scientific Institute.

DOXO (Pharmacia & Upjohn) was diluted in sterile 0.9% NaCl and was injected i.v. (6 mg/kg body weight). MEL (Alkeran) was diluted in PBS and injected i.v. (2.5 mg/kg). Chicken OVA was obtained from Sigma-Aldrich. The synthetic OVAp$_{323–339}$ (CD4 restricted) or OVAp$_{257–264}$ (CD8 restricted) were obtained from EspiKem. The Tag IV 404–411 peptide was obtained from Research Genetics. All of the mAb used in the study (with the exception of the KJ1-26 mAb, which was obtained from Caltag Laboratories) were obtained from BD Pharmingen.

Adoptive transfer

Single cell suspensions of spleen and lymph nodes (LN) derived from DO11.10 or OT-I mice were prepared as previously described (20, 42). Flow cytometry was used to determinate the percentage of DO11.10 and OT-I Tg cells. A total of 2.5–5 $\times$ 10^6 Tg CD4 or CD8 T cells was resuspended in 300 μl of PBS and injected in the tail vein of BALB/c or C57BL/6 recipient mice, respectively.

Immunization

Recipient mice were immunized s.c. in two sites with 100 μg of chicken OVA, dissolved in 100 μl of PBS, and emulsified with 100 μl of CFA. In the indicated experiments, mice were s.c. vaccinated in the right flank by injecting 2 $\times$ 10^5 peptide-pulsed DC resuspended in 100 μl of PBS. DC were prepared from bone marrow, as previously described (34). Briefly, bone marrow-derived cells were cultured with recombinant GM-CSF and IL-4 for 7 days. Thereafter, nonadherent cells were stimulated with 1 μg/ml LPS for 8 h and then incubated for 1 h at 37°C with 2 μM synthetic peptides OVAp$_{323–339,}$ OVAp$_{257–264}$, or Tag IV$_{404–411}$. Before injection into the mice, DC were characterized by flow cytometry after staining with anti-CD11c, I-A/I-E, CD80, CD86, and anti-CD40 mAbs. Generally, CD11c$^+$ cells represented 80% of the preparation and expressed a mature phenotype (data not shown) (34).

Flow cytometry analysis

Mice were sacrificed, and the blood, the peripheral LN (axiliar, brachial, and inguinal), and the spleen were collected by retro-orbital bleeding and surgical resection. LN and spleen were homogenized into a single cell suspension, while blood cells were isolated by Lympholyte (Cedarlane Laboratories) gradients. The cells were then incubated with a blocking buffer (5% rat serum and 95% culture supernatant of 2.4G2 anti-FcR mAb-producing hybridoma cells) for 20 min to saturate the FcRs. Viable lymphocyte counts were obtained by trypan blue exclusion. The frequency of lymphocytes was determined by flow cytometry analysis after staining of the cells with anti-CD4, anti-CD8, and anti-B220 mAb. The identity of the lymphocyte subpopulation was further confirmed by additional staining with anti-CD3 (CD3$^+$CD4$^+$; CD3$^+$CD8$^+$) and anti-CD19 mAb (CD19$^+$B220$^+$) (data not shown). Tg DO11.10 T cells were detected by staining with anti-CD4 mAb, and with the anti-clonotypic KJ1-26 mAb. Tg OT-I T cells were detected after staining with anti-CD8 and anti-Ly-5.1 mAb. The total number of DO11.10 and OT-I T cells was calculated by multiplying the total number of viable cells (obtained by counting viable cells by trypan blue exclusion) by the percentage of CD4$^+$, KJ1-26$^+$, or CD8$^+$, Ly-5.1$^+$ cells obtained by flow cytometry. In selected experiments, the phenotype of the cells was determined after staining with anti-CD44, anti-CD45RB, anti-CD62L, and anti-CD25 mAb. One thousand CD4$^+$, KJ1-26$^+$, or CD8$^+$, Ly-5.1$^+$ T cells were generally collected (FACSCalibur; BD Biosciences) and analyzed using the CellQuest software.

In some experiments, intracellular cytokine levels were determined, as previously described (43). Briefly, 10^6 LN or spleen cells derived from control and immunized AT mice were stimulated with 5 $\times$ 10^6 syngeneic splenocytes previously pulsed with the relevant OVAp (2 μM). After 2 h in culture at 37°C, brefeldin A (5 μg/ml) was added. After an additional

2 h, the cells were stained with either anti-CD4 and KJ1-26 mAb, or with anti-CD8 and Ly-5.1 mAb, fixed in 2% formaldehyde, and permeabilized in PBS containing 2% FCS, 0.5% saponin, 2% rat serum, and 0.2% sodium azide. The cells were then stained with anti-IL-2 and anti-IFN-γ mAb in permeabilization buffer. One thousand $CD4^+$, KJ1-26^+, or $CD8^+$, Ly 5.1^+ events were generally collected.

LACK-specific T cells were identified in 16.2β mice by staining with I-A^d/LACK fluorescent multimers (21). I-A^d/LACK multimers were obtained by incubating I-A^d/LACK dimers (3 μg/sample) with Alexa 488-coupled protein A (Molecular Probes; 0.3 μg/sample) in PBS for 30 min at room temperature. Free protein A binding sites were saturated by the addition of total IgG (1 μg/sample). A total of 6×10^5 LN cells was first incubated with a blocking buffer (5% rat serum and 95% culture supernatant of 2.4G2 anti-FcR mAb-producing hybridoma cells) for 20 min to saturate the Fc receptors and then stained with I-A^d/LACK multimers for 1 h on ice in PBS supplemented with 0.5% BSA. Thereafter, the cells were stained with PE- or PerCP-labeled anti-CD4 and anti-CD44 mAb and with allophycocyanin-labeled anti-CD8a, anti-CD11b, anti-B220 mAb (BD Pharmingen). TOPRO-3 (1 nM final; Molecular Probes) was added to the sample just before flow cytometry analysis to discriminate viable and dead cells. $CD8a^+$, $CD11b^+$, $B220^+$, $TOPRO^+$ cells were excluded by electronic gating during the acquisition. A total of $50–100 \times 10^3$ $CD4^+$ T cells was acquired using a FACSCalibur flow cytometer (BD Biosciences). To determine the frequency of LACK-specific cytokine-producing cells, 1×10^6 LN cells were cultured with unpulsed or LACK peptide-pulsed splenocytes derived from D011.10 TCR Tg mice for 4 h. During the last 2 h, brefeldin A (10 μg/ml; Sigma-Aldrich) was added to the cultures. Cells were then stained with anti-CD4 mAb and anti-KJ1-26 mAb (to exclude splenic DO11.10 CD4 T cells), fixed, permeabilized, and further stained with anti-IL-2 and anti-IFN-γ mAb. Cytokine release was determined in $CD4^+$, KJ1-26^- cells by flow cytometry.

The significance of differences between the experimental groups was tested by statistical analysis with an unpaired two-tailed Student's *t* test.

ELISA

Anti-OVA Abs were detected in the serum of mice 15 days after immunization, as previously described (44). Briefly, chicken OVA (20 μg/ml in PBS) was coated on 96-well plates. The plates were then blocked with PBS-BSA 1% and washed with PBS-Tween 20 0.05%. Thereafter, diluted serum samples were added and incubated overnight at 4°C. OVA-specific IgG were detected with HRP-conjugated anti-mouse IgG1 or IgG2a (Valter Occhiena). The reaction was developed by the addition of *o*-phenylenediamine and quantified at 490 nm.

In vivo cytotoxic assay

The in vivo cytotoxic activity of OT-1 T cells was determined, as previously described (34). Briefly, 20×10^6 C57BL/6-Ly-5.2 spleen cells were resuspended in 1 ml of PBS and labeled with the CFSE fluorescent dye at the final concentrations of 0.125 μM ($CFSE^{dim}$) or 1.25 μM ($CFSE^{bright}$) for 8 min at room temperature. Thereafter, the reaction was blocked by the addition of an equal volume of FCS. The cells were washed extensively with RPMI 1640–10% FCS, and resuspended at $20–30 \times 10^6$ cells/ml. The $CFSE^{bright}$ cells were pulsed with $OVAp_{257–264}$ (10 μg/ml) for 1 h at 37°C. Thereafter, the cells were washed, and an equal number of $CFSE^{bright}$-pulsed and $CFSE^{dim}$-unpulsed cells ($5–10 \times 10^6$ cells) was injected in the tail vein of control or vaccinated adoptively transferred mice. Sixteen hours later, the mice were sacrificed, and the presence of $CFSE^+$ cells in LN and spleen was quantified by flow cytometry.

In vitro cytotoxicity assay

Splenocytes were restimulated in vitro (34) in the presence of 1 μM Tag IV peptide. Day 5 blasts were tested for cytolytic activity in a standard 4-h ^{51}Cr release assay, as previously described (34), using as targets RMA cells (45) left unpulsed or pulsed with 10 μM Tag IV. ^{51}Cr release of target cells alone was always <25% of maximal ^{51}Cr release (target cells in 0.25 M HCl). LU were determined as the number of effector cells capable of killing 50% (LU-50) of target cells, and were expressed/10^6.

Results

Effect of DOXO and MEL administration on lymphocyte counts

The possibility to combine active vaccination with antiblastic chemotherapy relies on the absence of severe effects of the chemotherapeutic agents on the immune cell representation in secondary lymphoid tissues. Thus, we set to determine the effect of DOXO and MEL administration on lymphocyte distribution shortly after drug administration. DOXO and MEL were, respectively, used at 6 mg/kg (14, 36, 38) and 2.5 mg/kg (39). At these doses, DOXO and MEL have antitumor activity and are well tolerated by the mice (35). The frequency of CD4, CD8, and B cells was determined in the LN, in the spleen, and in the blood at different time points by flow cytometry analysis. Twelve, 24 (data not shown), and 48 h (Fig. 1) after drug injection, the numbers of CD4, CD8, and B cells were found to be reduced in the blood of DOXO-treated mice (Fig. 1*C*). In contrast, the number of cells in the LN and the spleen appeared to be unchanged at all the times (Fig. 1, *A* and *B*). Similarly, the administration of MEL appeared to be tolerated by the lymphoid tissues, as shown by the normal representation of $CD4^+$, $CD8^+$, and $B220^+$ lymphocytes (Fig. 1, *D–F*). Because the amount of active DOXO has been reported to halve 3 and 30 h after i.v. administration (35), we set out to avoid peak plasma concentration of the drugs and vaccinated mice 48 h after DOXO and MEL injection in all subsequent experiments. A similar administration schedule was previously adopted for cyclophosphamide and paclitaxel (7, 14).

DOXO administration does not prevent Ag-induced CD4 T cell expansion and differentiation

To trace vaccine-induced Ag-specific T cell responses, we took advantage of the AT of TCR Tg T cells (20). A representative experiment is reported in Fig. 2. TCR Tg T cells were derived from DO11.10 and OT-I TCR Tg mice, and adoptively transferred into the tail vein of syngenic BALB/c (Fig. 2, *A* and *B*) and congenic C57BL/6 mice (Fig. 2, *C* and *D*), respectively. Adoptively transferred DO11.10 cells were traced after staining with anti-CD4 and anti-TCR (KJ1-26) mAbs, while adoptively transferred OT-I cells were traced after staining with anti-CD8 and anti-Ly-5.1 mAbs.

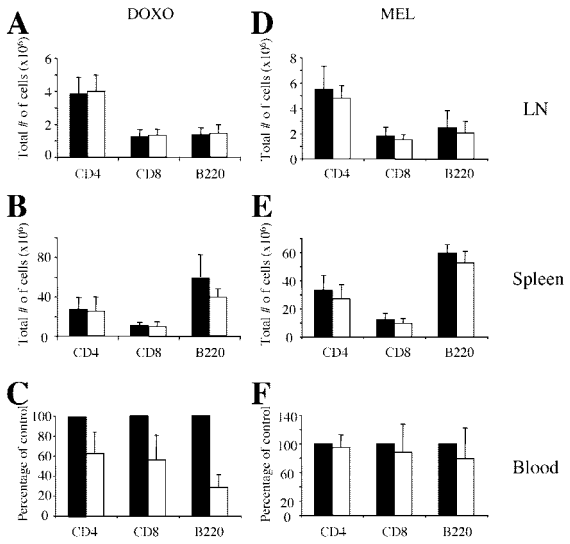

FIGURE 1. Effect of DOXO and MEL on the cellular composition of LN, spleen, and blood. BALB/c mice received an i.v. injection of PBS (■) or DOXO (6 mg/kg) (□; *A–C*) or MEL (2.5 mg/kg) (*D–F*). Forty-eight hours after drug injection, the mice were sacrificed, and cells derived from the LN (*A* and *D*), the spleen (*B* and *E*), and the blood (*C* and *F*) were analyzed by flow cytometry after staining with anti-CD4, anti-CD8, and anti-B220 mAb. The percentage of positive cells was multiplied by the total number of viable cells to obtain the total CD4, CD8, and B220 cell number that is reported in *A*, *B*, *D*, and *E*. In *C* and *F*, the number of lymphocytes obtained from 150 μl of blood was expressed as percentage of control (untreated mice). Each point is the average of six mice analyzed in two independent experiments.

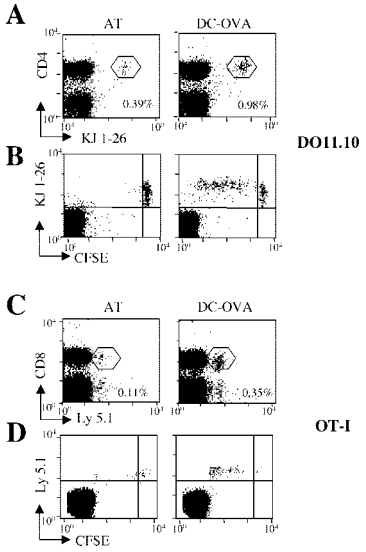

FIGURE 2. Visualization of Ag-specific CD4 and CD8 T cell responses. *A* and *B*, Five million CD4 T cells recovered from DO11.10 TCR Tg donor mice were labeled with CFSE and transferred via tail vein injection in syngenic BALB/c recipient mice. *C* and *D*, Five million CD8 T cells recovered from Ly-5.1$^+$ OT-I TCR Tg donor mice were labeled with CFSE and transferred via tail vein injection in Ly-5.2$^+$ congenic C57BL/6 recipient mice. Twenty-four hours after the transfer of the cells, mice were either left untreated (AT) or immunized with bone marrow-derived, LPS-matured, and OVAp-pulsed DC (DC-OVA). LN cells were recovered and analyzed by flow cytometry after staining with anti-CD4 and KJ1-26 mAb (*A* and *B*) or with anti-CD8 and anti-Ly-5.1 mAb (*C* and *D*). The frequency and the CFSE content of CD4$^+$, KJ1-26$^+$ (*A* and *B*), or CD8$^+$, Ly-5.1$^+$ (*C* and *D*) T cells within the viable lymphocyte gate are reported in the figure.

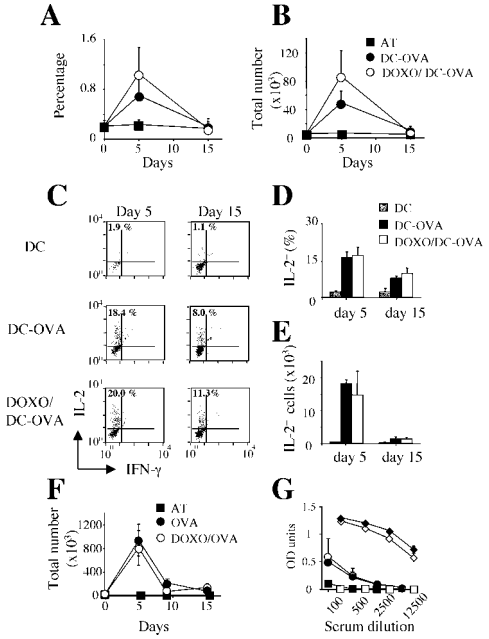

FIGURE 3. CD4 T cells expand and differentiate to DC-OVA and OVA/CFA vaccination during DOXO administration. BALB/c mice were adoptively transferred with DO11.10 TCR Tg T cells, as described in Fig. 2. Twenty-four hours after cell transfer, a group of mice received an i.v. injection of DOXO (6 mg/kg). After an additional 48 h, control and DOXO-treated mice were immunized with either OVAp$_{323–339}$-pulsed LPS-matured bone marrow-derived DC (DC-OVA; *A–E*) or OVA/CFA (OVA; *F* and *G*). Cells were recovered 5 and 15 days after immunization from the LN draining the site of vaccine injection and analyzed by flow cytometry after staining with anti-CD4 and KJ1-26 mAb. Mean percentages (*A*) and mean total numbers (*B* and *F*) ± SD of CD4$^+$, KJ1-26$^+$ T cells calculated over 8 (day 5) and 6 (day 15) mice analyzed are reported. *C*, LN cells were recovered from DC, DC-OVA, or DOXO/DC-OVA mice 5 and 15 days after DC challenge. The cells were restimulated with OVAp$_{323–339}$-pulsed splenocytes for 4 h at 37°C and examined for IL-2 and IFN-γ contents by intracellular staining after surface marking with anti-CD4 and KJ1-26 mAbs. Representative dot plots reporting cytokine production of restimulated cells after gating on CD4$^+$, KJ1-26$^+$ T cells are shown. The percentages indicated in the plot refer to CD4$^+$, KJ1-26$^+$, IL-2$^+$ cells. Percentage (*D*) and total number (*E*) of CD4$^+$, KJ1-26$^+$, IL-2$^+$ cells calculated with the data obtained from three independent mice are reported (▨, DC; ■, DC-OVA; □, DOXO/DC-OVA). These data are representative of two independent experiments performed with at least three mice per group. *F*, Mean total numbers ± SD of CD4$^+$, KJ1-26$^+$ T cells calculated over 6–12 mice analyzed are reported. *G*, The presence of OVA-specific IgG1 (diamonds) and IgG2a (circles) Abs in the preimmune serum (squares) and immune of OVA (filled symbols) and DOXO/OVA (open symbols) mice was determined in blood samples by ELISA 15 days after immunization. The experiment reported in the figure refers to one of five similar independent experiments.

Immunization of AT mice with OVAp$_{323–339}$- or OVAp$_{257–264}$-pulsed LPS-matured bone marrow-derived DC resulted in an increase in the frequency (Fig. 2) and total number (Figs. 3 and 4) of OVA-specific CD4$^+$, KJ1-26$^+$, and CD8$^+$, Ly-5.1$^+$ T cells. The increased frequency was attributable to Ag-induced clonal expansion, as demonstrated by CFSE dilution of TCR Tg T cells upon DC-OVA injection (Fig. 2, *B* and *D*).

We thus investigated the effect of DOXO administration on Ag-driven T cell responses. First, we analyzed CD4 T cell activation. To this aim, recipients of DO11.10 T cells were either left untreated or treated with DOXO, and then vaccinated with either control or OVAp$_{323–339}$-pulsed LPS-matured bone marrow-derived DC (34). LNs draining the site of DC injection were recovered 5 and 15 days after vaccination, and analyzed for the presence of DO11.10 T cells by flow cytometry after staining with anti-CD4 and KJ1-26 mAb (Fig. 3). Five days after immunization, a similar frequency of CD4$^+$, KJ1-26$^+$ T cells increased in size in DC-OVA and DOXO/DC-OVA mice, showing that T cell activation occurred to similar extent in the absence or in the presence of DOXO administration (not shown). Furthermore, both the frequency (Fig. 3*A*) and the total number (Fig. 3*B*) of Tg CD4 T cells increased, and soon after decreased to similar extents with indistinguishable kinetics in immunized mice. Thus, while the frequency of CD4$^+$, KJ1-26$^+$ T cells 5 days after immunization raised to 0.70 ± 0.28% and 1.05 ± 0.45% in DC-OVA- and DOXO/DC-OVA-immunized mice, respectively, at 15 days it was 0.20 ± 0.12% and 0.16 ± 0.03%, respectively. This indicated that the administration of DOXO did not alter either the expansion or the contraction of the Ag-specific T cell population following Ag encounter.

The surface and functional phenotype of DC-primed T cells was next investigated. LN cells were stained with anti-CD4, KJ1-26, anti-CD44, anti-CD25, anti-CD45 RB, and anti-CD62L mAb, and analyzed by flow cytometry. Comparable frequencies of CD4$^+$, KJ1-26$^+$ T cells derived from 5 or 15 days immunized DC-OVA and DOXO/DC-OVA mice up-regulated CD44 and CD25 and down-regulated CD45RB and CD62L as compared with CD4$^+$, KJ1-26$^+$ T cells derived from control (DC) mice (data not shown). LN cells were then restimulated in vitro with unpulsed (data not shown) or OVAp$_{323–339}$-pulsed syngeneic splenocytes. Intracellular IL-2 and IFN-γ (Fig. 3*C*) or IL-4 (data not shown) release was determined by intracytoplasmic staining after surface labeling of the cells with anti-CD4 and KJ1-26 mAbs. In the absence of Ag restimulation, cytokine production was undetectable (data not shown). Following Ag restimulation, a fraction of CD4$^+$, KJ1-26$^+$

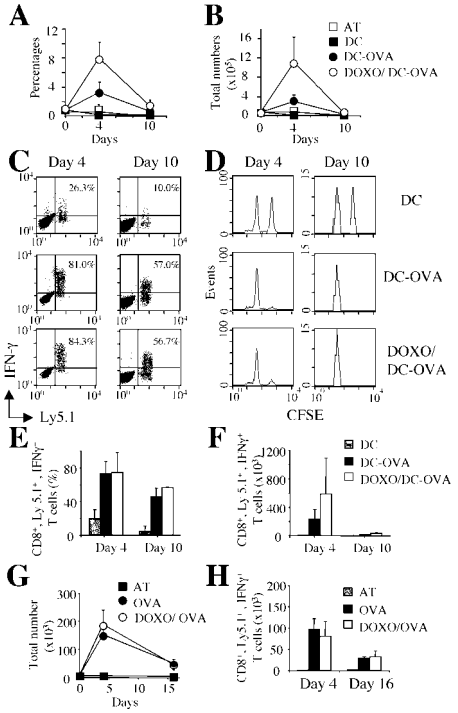

FIGURE 4. CD8 T cells proliferate and differentiate in response to DC-OVA and OVA/CFA immunization despite DOXO administration. OT-I T cells were transferred via tail injection in congenic C57BL/6 mice, as described in Fig. 2. Twenty-four hours later, a group of mice received DOXO i.v. (6 mg/kg). After an additional 48 h, mice were immunized with unpulsed DC (DC) or with $OVAp_{257–264}$-pulsed DC (DC-OVA and DOXO/DC-OVA) (*A–F*) or with OVA/CFA (OVA) (*G* and *H*). Mice were then sacrificed at the indicated times after immunization, and LN cells were analyzed by flow cytometry after staining with anti-CD8 and anti-Ly-5.1 mAb. Mean percentages ± SD (*A*) and total numbers ± SD (*B*) of $CD8^+$, Ly-5.1^+ T cells in draining LN of three mice per group are shown. The experiment is representative of three independent experiments. *C*, *E*, and *F*, LN cells derived from DC-, DC-OVA-, and DOXO/DC-OVA-immunized mice were restimulated with $OVAp_{257–264}$-pulsed splenocytes. The cells were then surface stained with anti-CD8, anti-Ly-5.1 mAb, fixed, permeabilized, and further stained with anti-IFN-γ mAb. Representative dot plots are shown in *C* after gating on $CD8^+$ T cell. The percentages reported in the plots refer to IFN-γ-producing cells within the $CD8^+$, Ly-5.1^+ population. The graphics in *E* and *F*, respectively, report the percentage and total number of $CD8^+$, Ly-5.1^+, IFN-γ^+ cells in DC (▨), DC-OVA (■), and DOXO/DC-OVA (□) mice. *D*, On day 4 after immunization, a mixture of $CFSE^{bright}$-labeled, $OVAp_{257–264}$-pulsed, and $CFSE^{dim}$-labeled, unpulsed spleen cells was injected in the tail vein. Sixteen hours later, cells from LN were examined by FACS. Histograms depict the relative frequency of $OVAp_{257–264}$-pulsed $CFSE^{bright}$ target cells and unpulsed $CFSE^{dim}$ cells, derived from one of three similar animals. *G*, Total numbers ± SD of $CD8^+$, Ly-5.1^+ T cells derived from OVA- and DOXO/OVA-treated mice calculated over six mice per time point (analyzed in two independent experiments) are reported. *H*, Total number of $CD8^+$, Ly-5.1^+, IFN-γ^+ T cells in AT (▨), OVA (■), and DOXO/OVA (□) mice.

T cells recovered from DC-OVA- and DOXO/DC-OVA-treated mice, but not from control DC-primed mice, produced IL-2. The frequency of $CD4^+$, KJ1-26^+ T cells capable of producing IL-2 was comparable in DC-OVA- and DOXO/DC-OVA-immunized mice (Fig. 3, *D* and *E*). The total number of IL-2-secreting cells was maximal after 5 days, and declined to similar extents by day 15. IFN-γ was mostly undetectable at any time in all groups of mice (Fig. 3*C*), as was IL-4 (data not shown).

We next investigated whether DOXO could be combined to a different vaccination strategy, such as Ag in adjuvant. To this aim, DO11.10 AT mice were treated with DOXO, and immunized 48 h later with OVA in CFA. Tg T cells were then recovered from the LN of immunized mice and analyzed by flow cytometry after staining with anti-CD4 and KJ1-26 mAb (Fig. 3*F*) or restimulated and analyzed for intracellular cytokine contents (data not shown). Comparable CD4 T cell expansion (Fig. 3*F*) and differentiation (data not shown) were measured in OVA- and DOXO/OVA-immunized mice. We also measured the presence of anti-OVA Ab in the sera of immunized mice by ELISA (Fig. 3*G*), as additional measure of T and B cell function. Immunization of the mice resulted in the appearance of OVA-specific IgG1 and IgG2a, whose production was not inhibited by DOXO administration.

Together these results indicated that vaccine-induced CD4 T cell responses could be elicited when vaccination was performed shortly after DOXO administration.

CD8 T cell priming occurs in the presence of DOXO administration

Protective immune responses require both CD4 and CD8 effector lymphocytes. Although CD4 T cells are required for proper CD8 T cell priming and the induction of CD8 T cell memory and for providing cell help to B cells and to cells of the innate response, CD8 T cells are primarily responsible for rejection of tumor cells (46). Thus, we felt it was important to investigate whether chemotherapy could hamper vaccine-induced CD8 T cell responses.

We then investigated the effect of DOXO on DC-induced CD8 T cell responses in mice adoptively transferred with traceable numbers of OT-I TCR Tg T cells (as depicted in Fig. 2*B*). Mice were either left untreated (AT) or treated with DOXO. Forty-eight hours later, mice were vaccinated with either control or $OVAp_{257–264}$-pulsed LPS-matured bone marrow-derived DC. LN draining the site of DC injection were recovered 4 and 10 days upon vaccination and analyzed for the presence of $CD8^+$, Ly-5.1^+ T cells by flow cytometry after staining with anti-CD8 and anti-Ly-5.1 mAb (Fig. 4). Four days after immunization, a similar frequency of $CD8^+$, Ly-5.1^+ T cells was found to be larger in size and to have up-regulated the surface expression of CD44 in DC/OVA and DOXO-DC/OVA (data not shown). This indicated that, as shown for CD4, also in the case of CD8, previous administration of DOXO does not prevent T cell activation. Furthermore, both the frequency (Fig. 4*A*) and the total number (Fig. 4*B*) of Tg $CD8^+$, Ly-5.1^+ T cells changed to similar extent and with comparable kinetics in DC/OVA and DOXO-DC/OVA mice. Thus, while the frequency of $CD8^+$, Ly-5.1^+ T cells in the LN of DC-OVA was 3.23 ± 1.4% and 0.63 ± 0.16% 4 and 10 days after immunization, it was 7.96 ± 2.16% and 1.42 ± 0.51% in the LN DOXO/DC-OVA mice, respectively.

The ability of DC-primed $CD8^+$, Ly-5.1^+ T cells to exert effector function was then further determined. As in the case of CD4 T cells, similar frequencies of $CD8^+$, Ly-5.1^+ T cells up-regulated the expression of CD44 and down-regulated CD45RB or CD62L in OVA and DOXO/OVA mice (data not shown). The ability of CD8 T cells to produce IFN-γ was then determined upon ex vivo Ag ($OVAp_{257–264}$) restimulation and intracellular staining. Only background levels of intracellular IFN-γ were detected in the absence of restimulation (data not shown), and in Ag-restimulated LN cultures derived from control (DC-primed) mice. Ag restimulation of LN cells derived from either DC-OVA- or DOXO/DC-OVA-primed mice elicited IFN-γ production by the vast majority of the $CD8^+$, Ly-5.1^+ T cells (72.7 ± 14.9% and 74.2 ± 24.2% at day 5, and 45.1 ± 10.7% and 56.1 ± 0.8% at day 10, respectively) (Fig. 4, *C* and *E*). Moreover, also, the total numbers of IFN-γ-producing $CD8^+$, Ly-5.1^+ T cells in cultures derived from DC-OVA-immunized mice were

comparable to the one found in the LN cultures derived from DOXO/DC-OVA-immunized mice (Fig. 4*F*).

As an independent measure for effector function, the cytotoxic activity of CD8 T cells was investigated by in vivo cytotoxic assay (34, 47). $CFSE^{bright}$ splenocytes were pulsed with the CD8-restricted OVAp and injected together with equal numbers of $CFSE^{dim}$-unpulsed cells into AT mice and into DC-OVA and DOXO/DC-OVA mice (Fig. 4*D*). Sixteen hours later, the LN and the spleen of these mice were harvested and analyzed by flow cytometry for $CFSE^{+}$ cells. Although both $CFSE^{bright}$ and $CFSE^{dim}$ cells could be found in equal proportion in the lymphoid organs of control mice, $CFSE^{bright}$ cells were only barely detectable in the LN of DC-OVA- and DOXO/DC-OVA-immunized mice, indicating comparable OVA-specific cytotoxic activity.

Similar results were obtained using soluble Ag in CFA as vaccine. Thus, comparable CD8 T cell expansion (Fig. 4*G*), IFN-γ production (Fig. 4*H*), and cytotoxic activities (data not shown) were measured in adoptively transferred OVA- and DOXO/OVA-immunized mice. Together these data indicated that DOXO administration 48 h before vaccination did not prevent vaccine-induced CD8 T cell activation, proliferation, and differentiation.

MEL-based chemotherapy does not prevent T cell priming

To investigate whether, in addition to DOXO, other chemotherapeutic agents could be combined to vaccination strategies, we analyzed the effect of MEL on DC (Fig. 5)- and Ag (data not shown)-mediated CD4 and CD8 T cell priming. As in the case of DOXO, we choose to first treat the mice with MEL, and 48 h later to immunize the mice. Thus, DO11.10 and OT-I AT mice were treated with MEL and 48 h later immunized with $OVAp_{323–339}$- or $OVAp_{257–264}$-pulsed DC, respectively. LN cells were then recovered from immunized mice and analyzed by flow cytometry after staining with anti-CD4 and KJ1-26 mAb or with anti-CD8 and Ly-5.1 mAb. Both the frequency (data not shown) and the total number of $CD4^{+}$, KJ1-26^{+} (Fig. 5*A*) and $CD8^{+}$, Ly-5.1^{+} (Fig. 5*C*) T cells increased to similar extent and with indistinguishable kinetics in the peripheral LN and the spleen (data not shown) of mice immunized in the absence or in the presence of previous MEL administration. Furthermore, increase in cell size, up-regulation of CD44 (data not shown), and ex vivo OVA-induced cytokine releases was comparable on cells derived from DC-OVA- and MEL-DC-OVA-treated mice (Fig. 5, *B* and *D*). Finally, comparable cytotoxic activity was detected in mice vaccinated with DC-OVA in the absence or in the presence of MEL (Fig. 5*E*). Together these data indicate that the administration of MEL before DC vaccination does not hamper the vaccine immunogenicity.

Multiple rounds of DOXO administration do not impair DC-induced T cell priming

Chemotherapeutic regimens usually require multiple administration of the drug, which could have a more profound effect on the development of vaccine-induced immune responses. To address this point, we took advantage of 16.2β TCR transgenic mice, which bear a detectable frequency of CD4 T cells specific for the model Ag LACK (21). Thus, at difference with the AT model, in this model we can trace an endogenous and renewable population of Ag-specific T cells (21).

To mimic the repeated cycle of chemotherapy performed in human infusion (35), 16.2β mice received an i.v. injection of DOXO (6 mg/kg) once per week for 3 consecutive wk. A group of mice received control (PBS) injections. Forty-eight hours after the last administration, mice were vaccinated with unpulsed and LACK-pulsed DC. Six days after immunization, the LNs and the spleen were recovered, counted, and analyzed by flow cytometry after staining with anti-CD4, anti-CD8, and anti-B220 mAb. Although the frequency of B220 and CD45 precursors was decreased in the bone marrow of treated mice, and the total number of $CD4^{+}$, $CD8^{+}$, and $B220^{+}$ lymphocytes in the spleen of DOXO-treated mice was 50% of the one of control mice, no differences were observed in the peripheral LN (data not shown). The LN draining the site of DC injection were thus analyzed for the presence of LACK-specific T cells after staining with fluorescent peptide MHC class II multimers (I-A^{d}/LACK) (21) and with anti-CD4 and anti-CD44 (Fig. 6*A*). DC-LACK injection resulted in comparable activation and enrichments for LACK-specific T cells. Thus, by 6 days after immunization, the total number of $CD4^{+}$, I-A^{d}/$LACK^{+}$ T cells in DC, $DOXO^{3}$, DC-LACK, and $DOXO^{3}$/DC-LACK was, respectively, $18.58 \pm 1.45 \times 10^{3}$, $21.6 \pm 14.28 \times 10^{3}$, $150.96 \pm 3.31 \times 10^{3}$, and $139.07 \pm 6.73 \times 10^{3}$. Moreover, LN cells were restimulated in vitro with unpulsed (data not shown), LACK-pulsed DO11.10 syngeneic splenocytes and intracellular IL-2 and IFN-γ (Fig. 6*B*), or IL-4 (data not shown) release was determined by intracytoplasmic staining. In the absence of Ag restimulation,

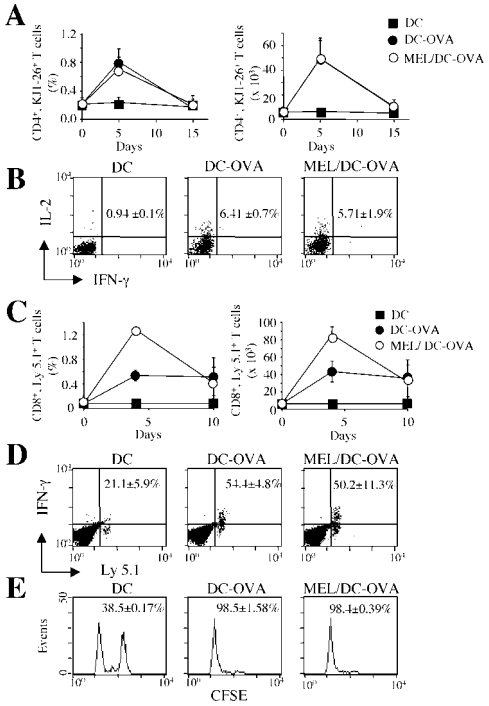

FIGURE 5. MEL does not prevent T cells priming when administered 48 h before vaccination. BALB/c and C57BL/6 mice were adoptively transferred with DO11.10 (*A*) and OT-I (*B*) TCR Tg T cells. Twenty-four hours after cell transfer, a group of mice received an i.v. injection of MEL (2.5 mg/kg). After an additional 48 h, mice were immunized with unpulsed (DC) or $OVA_{323–339}$-pulsed DC (DC-OVA; *A* and *B*) or $OVAp_{257–264}$-pulsed DC (DC-OVA; *C–E*). LN cells were recovered and analyzed by flow cytometry after staining with anti-CD4 and KJ1-26 mAb (*A* and *B*) or with anti-CD8 and anti-Ly-5.1 mAb (*C* and *D*). The mean percentages and mean total number ± SD of $CD4^{+}$, KJ1-26^{+} T cells and $CD8^{+}$, Ly-5.1^{+} T cells derived from three mice per group are reported in *A* and *C*, respectively. LN cells were also restimulated in vitro with OVAp, and intracellular cytokine release was determined, as described in Fig. 4 and this figure. Representative dot plot of data obtained at 15 and 10 days after vaccination is depicted (*B* and *D*). The frequencies ± SD of IL-2^{+} (*B*) and of IFN-γ^{+} (*D*) cells within the $CD4^{+}$, KJ1-26^{+} T cells and $CD8^{+}$, Ly-5.1^{+} population are reported. *E*, The in vivo $OVAp_{257–264}$-specific cytotoxic activity of mice vaccinated 10 days before was determined, as described in *D*. The percentage ± SD of specific lysis is reported. Four independent experiments were performed with comparable results.

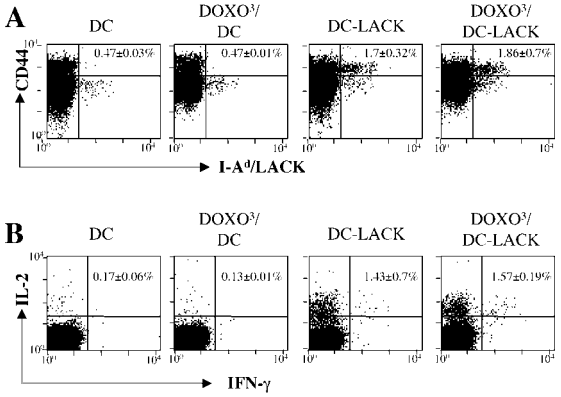

FIGURE 6. Repeated DOXO administration does not impair DC-induced CD4 T cell responses. The 16.2β mice were treated with DOXO once per week for 3 consecutive wk. Forty-eight hours after the last injection, control and DOXO-treated mice were challenged with unpulsed (DC) or LACK-pulsed (DC-LACK) LPS-matured bone marrow-derived DC. Six days later, draining LN were recovered and cells were stained with I-A^d/LACK multimers, anti-CD4, anti-CD44, anti-B220, anti-CD8a, anti-CD11b mAb, and with TOPRO-3, and analyzed by flow cytometry. *A*, Representative flow cytometry profiles are shown after gating on viable CD4$^+$, B220$^-$, CD8$^-$, CD11b$^-$, TOPRO-3$^-$ cells. The frequency ± SD of I-A^d/LACK$^+$ CD4$^+$ cells calculated over three mice per group is indicated. *B*, LN cells were stimulated in vitro with unpulsed or LACK-pulsed DO11.10 splenocytes. Thereafter, the cells were stained with anti-CD4 mAb and KJ1-26 mAb (to exclude DO11.10 T cells), fixed, permeabilized, further stained with anti-IL-2 and anti-IFN-γ mAb, and analyzed by flow cytometry. Representative dot plots showing IL-2 and IFN-γ production by CD4$^+$, KJ1-26$^-$ T cells are shown. The frequency ± SD of cytokine-producing cells calculated with three mice per group is reported. One additional experiment was performed with comparable results.

cytokine production was undetectable (data not shown). Upon Ag restimulation, a similar frequency (Fig. 6*B*) and total number (data not shown) of CD4$^+$ T cells recovered from DC-LACK- and DOXO/DC-LACK-treated mice produced IL-2 and/or IFN-γ.

This finding indicates that vaccination can be combined to chemotherapy, even after repeated cycles of DOXO administration.

DOXO and DC vaccination can be combined in spontaneously developing tumor TRAMP mice

We then investigated whether DOXO and vaccination could be combined to elicit CD8 T cells specific for a tumor-specific Ag in mice developing a tumor disease. To this aim, we used 7-wk-old TRAMP mice bearing mPIN (22, 26, 48), and DOXO, which although not a drug of first choice, is currently used in association with other drugs to treat androgen-independent prostate cancer (49, 50).

Seven-week-old TRAMP mice were thus treated with DOXO, and 48 h later received a s.c. injection of Tag-pulsed DC. As control, wild-type (WT) littermates were vaccinated with Tag-pulsed DC. One week later, animals were killed, and their genital apparatus was collected for morphology and histology examination. As expected (48), the genital organs of TRAMP and WT mice did not differ macroscopically (data not shown). Conversely, scattered foci of mPIN (i.e., cells with nuclear elongation, altered nucleus-to-cytoplasm ratio, and micropapillary projections) were evident at H&E staining only in TRAMP mice. Expression of Tag had a dim and patchy distribution in TRAMP mice and nicely overlapped pathologic foci (data not shown). Splenocytes from vaccinated mice were stimulated in vitro with Tag, and tested 5 days later in ^{51}Cr release assays (Fig. 7). As expected, a strong cytolytic activity against Tag-pulsed RMA target cells was found in cultured splenocytes from WT mice (Fig. 7*A*) and from TRAMP mice (Fig. 7*B*),

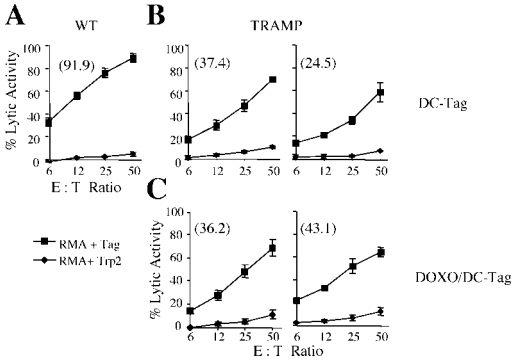

FIGURE 7. DOXO does not reduce the immunogenic potential of DC vaccination in tumor-bearing mice. Seven-week-old WT (*A*) and TRAMP male littermates either left untreated (*B*) or treated 48 h before with DOXO (*C*) were challenged with 2×10^5 Tag IV-pulsed DCs. After 1 wk, the mice were sacrificed and splenocytes were stimulated in vitro with Tag IV. Five days later, the cultures were harvested and tested in ^{51}Cr release assays against unpulsed (diamonds) or Tag IV$_{404-411}$-pulsed (squares) RMA cells. The percentage (mean ± SD of triplicates) of the specific lysis at the indicated E:T ratio of individual mice is shown. The numbers in the graphs refer to the LU-50. Two independent experiments were performed with comparable results.

although the lytic activity, measured by LU-50, was consistently lower in vaccinated TRAMP mice when compared with WT mice (26). Previous administration of DOXO did not hamper the ability of DC-Tag to prime a potent CTL response. Indeed, the mean LU-50 ± SD of DC- and DOXO/DC-Tag-vaccinated mice was 30.95 ± 9.12 and 39.65 ± 4.87, respectively (Fig. 7, *B* and *C*). This indicated that antiblastic chemotherapy is able to elicit a potent T cell response even in mice developing prostate cancer and partially tolerant to the tumor-associated Ag.

Discussion

In this study, we have investigated whether the administration of antiblastic chemotherapy impairs the immunogenicity of Ag-specific vaccination. This is an important issue in cancer treatment for the development of combined therapies able to control tumor growth, while allowing vaccine-induced immune responses.

Although the feasibility of combining chemotherapy and immunotherapy in tumor-bearing mice was supported by the findings that combined treatments in several cases ameliorated antitumor protection (7–10, 13, 16, 19), quantitative and qualitative information on vaccine-induced T cell responses were missing. In this study, we have analyzed vaccine-induced Ag-specific T cell responses at the single cell level in mice subjected to antiblastic chemotherapy.

Our experiments indicated that at the concentration used in these studies, chosen because well tolerated and capable of antitumor activity (35), DOXO and MEL did not perturb the normal distribution of the major lymphocyte subsets in peripheral lymphoid organs. Indeed, while a moderate effect was observed in the blood within the first 48 h after drug administration, a normal lymphocyte distribution was revealed at the time of sacrifice. We reasoned that the reduction in lymphocyte counts mostly seen in the blood could be explained by peak plasma concentration, which halves 3 and 30 h after i.v. administration (35). The toxic effect, however, was rapidly lost, allowing the re-establishment of a normal repertoire of lymphocyte in the peripheral lymphoid tissues soon after a single administration of DOXO or MEL. This is important in light of the administration of the combined therapy to patients with minimal residual disease, better managed by a tight therapeutic schedule. Repeated administration of DOXO also did not significantly affect the lymphocyte distribution in the LN. It is interesting

that while the number of B and T cells was reduced in the bone marrow and in the spleen of mice treated with three cycles of DOXO, lymphocytes were normally represented in the LN. Even though similar studies will have to be confirmed in patients, these results indicate that in the absence of other concomitant factors, the immune system remains competent to respond to vaccination after single or multiple administrations of DOXO- and MEL-mediated antiblastic chemotherapy.

To obtain quantitative and qualitative information on T cell responses induced by the combination of chemotherapy and immunotherapy, we used three different animal models that allow tracing of Ag-specific T cell function in peripheral lymphoid organs. In the first model, we took advantage of the AT of traceable population of DO11.10 or OT-I TCR Tg T cells specific for the model Ag OVA and addressed whether vaccination could be performed shortly after antiblastic chemotherapy. AT mice were immunized with OVA either as a cell-associated Ag (OVAp-pulsed DC) or as a soluble recombinant Ag plus adjuvant (CFA). Comparable CD4 and CD8 T cell activation was detected in the LN draining the site of DC-OVA and OVA immunization, whether the vaccine was provided in the absence or in the presence of previous DOXO or MEL administration. Thus, a similar frequency of OVA-specific cells increased in size (indicative of active T cell proliferation in vivo) and expressed augmented levels of CD44, CD25, and down-regulated CD45RB and CD62L shortly after immunization. Furthermore, a comparable frequency of OVA-specific T cells acquired effector function, as revealed by the ability of the cells to produce cytokines upon Ag restimulation in vitro or to lyse peptide-pulsed target cells in vivo. Thus, the administration of DOXO or MEL in close proximity to either DC-OVA or OVA vaccination does not hamper Ag presentation and T cell activation in the peripheral lymphoid tissue.

Ag-induced T cell clonal activation in vivo results in clonal proliferation, clonal differentiation, and clonal contraction (51). Our results indicate that DOXO and MEL administration did not hamper either of these phases. Indeed, both the frequency and the total number of Ag-specific T cells peaked within 1 wk and returned to baseline levels by 2 wk after vaccination in either control or chemotherapy-treated mice. This indicated that the kinetics of clonal expansion and clonal contraction are comparable in the absence or in the presence of DOXO and MEL. Furthermore, a similar frequency of CD4 T cells capable of OVA-specific IL-2 secretion, and of CD8 T cells capable of OVA-specific IFN-γ secretion, and cytotoxicity were found in control and DOXO- or MEL-vaccinated mice. Finally, anti-OVA Ab were detected in both OVA- and DOXO/OVA-treated mice, suggesting that DOXO did not hamper CD4 helper function or plasma cell differentiation in vivo. In this model system, OVA was more potent than DC-OVA in inducing CD4 T cell expansion. This is most likely due to the different amount of Ag being presented to naive T cells, and to the different Ag persistence. Indeed, while vaccination with Ag in CFA results in long-term Ag persistence (52), peptide-pulsed DC elicit transient T cell activation shortly after s.c. injection (53, 54) (our unpublished data). Despite these differences, DOXO and MEL failed to hamper DC-based vaccination, and comparable CD4 and CD8 T cell clonal expansion and differentiation were observed in mice immunized with peptide-loaded DC regardless of DOXO and MEL administration. This was a critical point that needed to be addressed because DC vaccination is one of the most potent vaccines in eliciting T cell priming in vivo (31) and currently used in clinical trials (55).

In the second animal model, we addressed whether vaccination could be performed after repeated administration of DOXO. This was important to determine, because most chemotherapeutic regimens require multiple administration of the drug. The model system we used allowed tracing of endogenous and renewable population of Ag-specific CD4 T cells (21), and thus we were able to take into consideration also possible effects due to bone marrow toxicity. Our experiments indicate that vaccination of untreated mice and of mice treated for 3 consecutive wk with DOXO elicited comparable CD4 T cell responses, in terms of proliferation and differentiation. Together with the findings obtained in the AT model, these data strongly support the possibility that the peripheral lymphoid tissues remain competent to respond to DC and soluble Ag vaccination after the recent administration of a single or repeated cycle of chemotherapy.

Finally, we determined the immunogenicity of Ag-pulsed DC vaccine combined to antiblastic chemotherapy in the TRAMP mouse model of tumor disease. TRAMP mice develop spontaneous prostate cancer because of the expression of SV40 Tag Ag, and allow tracing of CTL specific for the tumor-expressed Tag Ag (22). We have used 7-wk-old TRAMP mice, which all have mPIN (26, 48), and are partially tolerant to Tag (22), but are still able to respond to DC-mediated vaccination (26). The administration of DOXO and DC-Tag vaccines in close proximity did not prevent the ability of Tag-pulsed DC to prime potent Tag-specific CTLs. These data thus indicate that the combination of DOXO and DC-based vaccination can be performed despite the presence of partial T cell tolerance and of a developing tumor. Experiments are now ongoing in TRAMP mice to evaluate the therapeutic efficacy of the combined vaccination. These will determine whether the combination of chemotherapy and vaccination should be considered also a therapeutic, rather than only an adjuvant strategy.

The possibility to combine antiblastic therapy with Ag-specific vaccination is most likely not restricted to the chemotherapeutic agents used in this study. Indeed, previous reports indicated that the administration of cyclophosphamide enhanced the therapeutic efficacy of passive and active immunotherapy (8–10, 13), while gemcitabine enhanced anti-CD40 mAb immunotherapy in vivo (16, 19). The ability of these drugs to enhance immune responses to tumor was in some cases attributed to the elimination of suppressor T cells (7), while in other to the increase in tumor-Ag cross-presentation (16, 19). Although not statistically significant, in many of our experiments, administration of either DOXO or MEL before vaccination enhanced vaccine-induced T cell clonal expansion. Preliminary analyses indicated that, at difference with cyclophosphamide (8–10, 13), neither DOXO nor MEL changed the frequency of $CD4^+$, $CD25^+$ T cells, among which are found lymphocytes with suppressive activity (56). It is, however, possible that a synergistic effect is only revealed in the presence of a sizable tumor. Experiments are being performed in TRAMP mice to address this possibility.

In conclusion, our results demonstrate that DOXO and MEL administration can be combined with Ag-specific vaccination without adverse effect. These data provide the rationale for attempting the combined therapy in the adjuvant treatment of cancer patients.

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Disclosures

The authors have no financial conflict of interest.

References

1. Pardoll, D. M. 1998. Cancer vaccines. *Nat. Med. 4:525.*
2. Rosenberg, S. A. 2001. Progress in human tumor immunology and immunotherapy. *Nature 411:380.*
3. Greenberg, P. D. 1991. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. *Adv. Immunol. 49:281.*
4. Yu, Z., and N. P. Restifo. 2002. Cancer vaccines: progress reveals new complexities. *J. Clin. Invest. 110:289.*

5. Wijermans, P. W., W. B. Gerrits, and H. L. Haak. 1993. Severe immunodeficiency in patients treated with fludarabine monophosphate. *Eur. J. Haematol. 50:292*.
6. Ehrke, M. J., E. Mihich, D. Berd, and M. J. Mastrangelo. 1989. Effects of anticancer drugs on the immune system in humans. *Semin. Oncol. 16:230*.
7. Glaser, M. 1979. Regulation of specific cell-mediated cytotoxic response against SV40-induced tumor associated antigens by depletion of suppressor T cells with cyclophosphamide in mice. *J. Exp. Med. 149:774*.
8. Greenberg, P. D., and M. A. Cheever. 1984. Treatment of disseminated leukemia with cyclophosphamide and immune cells: tumor immunity reflects long-term persistence of tumor-specific donor T cells. *J. Immunol. 133:3401*.
9. Mastrangelo, M. J., D. Berd, and H. Maguire, Jr. 1986. The immunoaugmenting effects of cancer chemotherapeutic agents. *Semin. Oncol. 13:186*.
10. Proietti, E., G. Greco, B. Garrone, S. Baccarini, C. Mauri, M. Venditti, D. Carlei, and F. Belardelli. 1998. Importance of cyclophosphamide-induced bystander effect on T cells for a successful tumor eradication in response to adoptive immunotherapy in mice. *J. Clin. Invest. 101:429*.
11. Garaci, E., F. Pica, G. Rasi, and C. Favalli. 2000. Thymosin α1 in the treatment of cancer: from basic research to clinical application. *Int. J. Immunopharmacol. 22:1067*.
12. Schiavoni, G., F. Mattei, T. Di Pucchio, S. M. Santini, L. Bracci, F. Belardelli, and E. Proietti. 2000. Cyclophosphamide induces type I interferon and augments the number of $CD44^{hi}$ T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer. *Blood 95:2024*.
13. Emens, L. A., J. P. Machiels, R. T. Reilly, and E. M. Jaffee. 2001. Chemotherapy: friend or foe to cancer vaccines? *Curr. Opin. Mol. Ther. 3:77*.
14. Machiels, J. P., R. T. Reilly, L. A. Emens, A. M. Ercolini, R. Y. Lei, D. Weintraub, F. I. Okoye, and E. M. Jaffee. 2001. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/*neu* tolerized mice. *Cancer Res. 61:3689*.
15. Dudley, M. E., J. R. Wunderlich, P. F. Robbins, J. C. Yang, P. Hwu, D. J. Schwartzentruber, S. L. Topalian, R. Sherry, N. P. Restifo, A. M. Hubicki, et al. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science 298:850*.
16. 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*.
17. Hermans, I. F., T. W. Chong, M. J. Palmowski, A. L. Harris, and V. Cerundolo. 2003. Synergistic effect of metronomic dosing of cyclophosphamide combined with specific antitumor immunotherapy in a murine melanoma model. *Cancer Res. 63:8408*.
18. Belardelli, F., M. Ferrantini, G. Parmiani, J. Schlom, and E. Garaci. 2004. International meeting on cancer vaccines: how can we enhance efficacy of therapeutic vaccines? *Cancer Res. 64:6827*.
19. 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*.
20. Pape, K. A., E. R. Kearney, A. Khoruts, A. Mondino, R. Merica, Z. M. Chen, E. Ingulli, J. White, J. G. Johnson, and M. K. Jenkins. 1997. Use of adoptive transfer of T-cell-antigen-receptor-transgenic T cell for the study of T-cell activation in vivo. *Immunol. Rev. 156:67*.
21. Malherbe, L., C. Filippi, V. Julia, G. Foucras, M. Moro, H. Appel, K. Wucherpfennig, J. C. Guery, and N. Glaichenhaus. 2000. Selective activation and expansion of high-affinity $CD4^+$ T cells in resistant mice upon infection with *Leishmania major*. *Immunity 13:771*.
22. Greenberg, N. M., F. DeMayo, M. J. Finegold, D. Medina, W. D. Tilley, J. O. Aspinall, G. R. Cunha, A. A. Donjacour, R. J. Matusik, and J. M. Rosen. 1995. Prostate cancer in a transgenic mouse. *Proc. Natl. Acad. Sci. USA 92:3439*.
23. Shappell, S. B., G. V. Thomas, R. L. Roberts, R. Herbert, M. M. Ittmann, M. A. Rubin, P. A. Humphrey, J. P. Sundberg, N. Rozengurt, R. Barrios, et al. 2004. Prostate pathology of genetically engineered mice: definitions and classification: the consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. *Cancer Res. 64:2270*.
24. Mylin, L. M., R. H. Bonneau, J. D. Lippolis, and S. S. Tevethia. 1995. Hierarchy among multiple H-2b-restricted cytotoxic T-lymphocyte epitopes within simian virus 40 T antigen. *J. Virol. 69:6665*.
25. Mylin, L. M., T. D. Schell, D. Roberts, M. Epler, A. Boesteanu, E. J. Collins, J. A. Frelinger, S. Joyce, and S. S. Tevethia. 2000. Quantitation of $CD8^+$ T-lymphocyte responses to multiple epitopes from simian virus 40 (SV40) large T antigen in C57BL/6 mice immunized with SV40, SV40 T-antigen-transformed cells, or vaccinia virus recombinants expressing full-length T antigen or epitope minigenes. *J. Virol. 74:6922*.
26. Degl'Innocenti, E., M. Grioni, A. Boni, A. Camporeale, M. T. S. Bertilaccio, M. Freschi, A. Monno, C. Arcelloni, N. M. Greenberg, and M. Bellone. 2004. Peripheral T cell tolerance occurs early during spontaneous prostate cancer development and can be rescued by dendritic cell immunization. *Eur. J. Immunol. 35:66*.
27. Mayordomo, J. I., T. Zorina, W. J. Storkus, L. Zitvogel, C. Celluzzi, L. D. Falo, C. J. Melief, S. T. Ildstad, W. M. Kast, A. B. Deleo, et al. 1995. Bone marrow-derived dendritic cells pulsed with synthetic tumor peptides elicit protective and therapeutic antitumor immunity. *Nat. Med. 1:1297*.
28. Rosenberg, S. A., J. C. Yang, D. J. Schwartzentruber, P. Hwu, F. M. Marincola, S. L. Topalian, N. P. Restifo, M. E. Dudley, S. L. Schwarz, P. J. Spiess, et al. 1998. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med. 4:321*.
29. Marchand, M., N. van Baren, P. Weynants, V. Brichard, B. Dreno, M. H. Tessier, E. Rankin, G. Parmiani, F. Arienti, Y. Humblet, et al. 1999. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int. J. Cancer 80:219*.
30. Bellone, M., D. Cantarella, P. Castiglioni, M. C. Crosti, A. Ronchetti, M. Moro, M. P. Garancini, G. Casorati, and P. Dellabona. 2000. Relevance of the tumor antigen in the validation of three vaccination strategies for melanoma. *J. Immunol. 165:2651*.
31. Schuler, G., B. Schuler-Thurner, and R. M. Steinman. 2003. The use of dendritic cells in cancer immunotherapy. *Curr. Opin. Immunol. 15:138*.
32. Ribas, A., L. H. Butterfield, J. A. Glaspy, and J. S. Economou. 2003. Current developments in cancer vaccines and cellular immunotherapy. *J. Clin. Oncol. 21:2415*.
33. Finn, O. J. 2003. Cancer vaccines: between the idea and the reality. *Nat. Rev. Immunol. 3:630*.
34. Camporeale, A., A. Boni, G. Iezzi, E. Degl'Innocenti, M. Grioni, A. Mondino, and M. Bellone. 2003. Critical impact of the kinetics of dendritic cells activation on the in vivo induction of tumor-specific T lymphocytes. *Cancer Res. 63:3688*.
35. Chabner, B. A., C. J. Allegra, G. A. Curt, and P. Calabresi. 1996. Anti-neoplastic agents. In *The Pharmacological Basis of Therapeutics*. J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Ruddon, and A. G. Gimlan, eds. McGraw-Hill, New York, p. 1233.
36. Synold, T. W., and J. H. Doroshow. 1996. Anthracycline dose intensity: clinical pharmacology and pharmacokinetics of high-dose doxorubicin administered as a 96-hour continuous intravenous infusion. *J. Infus. Chemother. 6:69*.
37. Tan, C., H. Tasaka, K. P. Yu, M. L. Murphy, and D. A. Karnofsky. 1967. Daunomycin, an antitumor antibiotic, in the treatment of neoplastic disease: clinical evaluation with special reference to childhood leukemia. *Cancer 20:333*.
38. Candussio, L., G. Decorti, E. Crivellato, M. Granzotto, A. Rosati, T. Giraldi, and F. Bartoli. 2002. Toxicologic and pharmacokinetic study of low doses of verapamil combined with doxorubicin. *Life Sci. 71:3109*.
39. Gorelik, L., and M. B. Mokyr. 1995. Low-dose-melphalan-induced up-regulation of type-1 cytokine expression in the s.c. tumor nodule of MOPC-315 tumor bearers and the role of interferon γ in the therapeutic outcome. *Cancer Immunol. Immunother. 41:363*.
40. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes in vivo. *Science 250:1720*.
41. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell 76:17*.
42. Shrikant, P., and M. F. Mescher. 1999. Control of syngeneic tumor growth by activation of $CD8^+$ T cells: efficacy is limited by migration away from the site and induction of nonresponsiveness. *J. Immunol. 162:2858*.
43. Khoruts, A., A. Mondino, K. A. Pape, S. L. Reiner, and M. K. Jenkins. 1998. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J. Exp. Med. 187:225*.
44. Pape, K. A., A. Khoruts, A. Mondino, and M. K. Jenkins. 1997. Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigen-activated $CD4^+$ T cells. *J. Immunol. 159:591*.
45. Ljunggren, H. G., and K. Karre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants: analysis of the mechanism. *J. Exp. Med. 162:1745*.
46. Pardoll, D. 2003. Does the immune system see tumors as foreign or self? *Annu. Rev. Immunol. 21:807*.
47. Hernandez, J., S. Aung, W. L. Redmond, and L. A. Sherman. 2001. Phenotypic and functional analysis of $CD8^+$ T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J. Exp. Med. 194:707*.
48. Gingrich, J. R., R. J. Barrios, B. A. Foster, and N. M. Greenberg. 1999. Pathologic progression of autochthonous prostate cancer in the TRAMP model. *Prostate Cancer Prostatic Dis. 2:70*.
49. Bagley, C. M., Jr., R. F. Lane, J. C. Blasko, P. D. Grimm, H. Ragde, O. E. Cobb, and R. K. Rowbotham. 2002. Adjuvant chemohormonal therapy of high risk prostate carcinoma: ten year results. *Cancer 94:2728*.
50. Millikan, R., P. F. Thall, S. J. Lee, D. Jones, M. W. Cannon, J. P. Kuebler, J. Wade III, and C. J. Logothetis. 2003. Randomized, multicenter, phase II trial of two multicomponent regimens in androgen-independent prostate cancer. *J. Clin. Oncol. 21:878*.
51. Jenkins, M. K., A. Khoruts, E. Ingulli, D. L. Mueller, S. J. McSorley, R. L. Reinhardt, A. Itano, and K. A. Pape. 2001. In vivo activation of antigen-specific CD4 T cells. *Annu. Rev. Immunol. 19:23*.
52. Pape, K. A., R. Merica, A. Mondino, A. Khoruts, and M. K. Jenkins. 1998. Direct evidence that functionally impaired $CD4^+$ T cells persist in vivo following induction of peripheral tolerance. *J. Immunol. 160:4719*.
53. Ingulli, E., A. Mondino, A. Khoruts, and M. K. Jenkins. 1997. In vivo detection of dendritic cell antigen presentation to $CD4^+$ T cells. *J. Exp. Med. 185:2133*.
54. Ludewig, B., W. V. Bonilla, T. Dumrese, B. Odermatt, R. M. Zinkernagel, and H. Hengartner. 2001. Perforin-independent regulation of dendritic cell homeostasis by $CD8^+$ T cells in vivo: implications for adaptive immunotherapy. *Eur. J. Immunol. 31:1772*.
55. Figdor, C. G., I. J. de Vries, W. J. Lesterhuis, and C. J. Melief. 2004. Dendritic cell immunotherapy: mapping the way. *Nat. Med. 10:475*.
56. Sakaguchi, S. 2004. Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol. 22:531*.