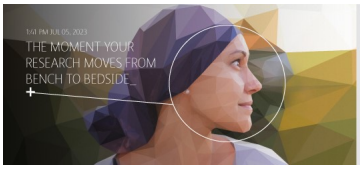

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Long Peptide Vaccination Can Lead to Lethality through CD4+ T Cell-Mediated Cytokine Storm

Hiroshi Kitamura,*,†,‡,§ Christine Sedlik,*,†,‡,§,¶ Alexandra Jacquet,*,†,‡,§ Bruno Zaragoza,*,†,‡ Mathilde Dusseaux,*,†,‡,§ Virginie Premel,*,†,‡,§,¶ Xavier Sastre-Garau,‖ and Olivier Lantz*,†,‡,§,¶

The optimization of anticancer therapeutic vaccines can lead to better efficacy but also to stronger adverse effects. In a mouse model of antitumor vaccination using a long peptide (LP), which included MHC class I- and II-restricted male (H-Y) epitopes, we observed unexpected mortality. Mice with an increased frequency of anti–H-Y CD4 T cells were primed with LP+CpG and boosted 10 d later. Within hours of boost, they displayed shock-like signs with high mortality. Serum cytokine levels were high. TNF-α secreted by the CD4 T cells was identified as the key effector molecule. Priming with a short peptide (SP), which included the MHC class II-restricted epitope, was a more efficient primer than LP, but did not lead to mortality when used as boost. The high mortality induced by LP compared with SP was probably related to its specific ability to be presented by B cells. Finally, targeting the LP sequence to dendritic cells allowed tumor protection without side effects. Our data: 1) confirm that the immune system can be very dangerous; 2) caution against the use of systemic activation of high-frequency Ag-specific T cells as induced by high doses of LP; and 3) underline the benefit of targeting Ag to dendritic cells. ***The Journal of Immunology*****, 2010, 185: 892–901.**

Exploitation of the adaptive immune system to fight tumors is attractive in that the targeted mechanism of action would be expected to induce a minimum of side effects (1). Adoptive transfer of immune effectors has demonstrated the capacity of the immune system to destroy large tumors (1, 2). Potential effectors are directed against molecules expressed or overexpressed by tumor cells and can be Abs (for example, HER-2 for breast cancer or CD20 for B cell lymphoma) (3, 4) or T cells, either CD8 (1, 2, 5–7) or, more recently, CD4 (8, 9). As adoptive transfer of effector T cells is labor intensive and expensive, efficient vaccination strategies are needed.

Although CD8 T cells have attracted the most attention, CD4 T cells are also important for tumor immunotherapy (10–14): CD4 T cells help CD8 T cells during both the priming and effector phases of the immune response (15). Cytotoxic CD8 cell priming is improved by the inclusion of MHC class II (MHC II) epitopes in peptide-based vaccines (16–19), showing stronger antitumor activity even toward MHC II-negative tumors (20). CD4 T cells can also display antitumor activity by themselves (14, 21, 22), either directly or by secreting lymphokines that recruit and activate innate effector cells: macrophages (14), NK cells (22), and eosinophils (21).

Strong priming strategies are required to generate large numbers of high-quality effector T cells (1, 2). We previously demonstrated that stronger stimulation is necessary for intratumoral CD4 T cell accumulation than for proliferation or lymphokine secretion (23). Thus, both CD8 and CD4 T cells specific for tumor Ag must be strongly activated to achieve tumor destruction (10, 24).

Inexpensive and easy to use, synthetic peptides are attractive Ags for vaccination (25). The average sizes of the short peptides (SPs) that bind to MHC I and II molecules are 8–10 and 13 and 14 aas, respectively. SPs have a high affinity for MHC, but their priming efficiency is low due to poor pharmacodynamics (26). Vaccines with SP CD8 T cell epitopes can also induce CD8 T cell tolerance (27, 28), with low response rates in clinical trials against malignant solid tumors (29, 30). Because SPs are presented by specific MHC alleles, patients should be selected according to HLA haplotype for inclusion in clinical trials. Long peptides (LPs) (15–35 aa) bind more efficiently to MHC II molecules for induction of optimal T cell responses (31, 32). Long peptides may be preferentially presented by professional APCs, as, at least for MHC I-restricted epitopes, they require endocytosis and processing (33, 34).

In a mouse tumor model using human papillomavirus (HPV) 16 E6 protein transfected cell lines, vaccination with an LP including CD4 and CD8 T cell epitopes displayed markedly improved antitumor activity as compared with an MHC I-restricted SP vaccine (33). In humans, vaccination of patients with advanced cervical cancer with LPs containing HPV16 E6 and E7 oncoprotein sequences was well tolerated and strongly immunogenic (35) (C. Sedlik, H. Kitamura, and O. Lantz, unpublished observations). In different trials, the same vaccine induced tumor-specific CD4 and CD8 T cell immunity in patients with resected HPV16-positive cervical cancer (36) and cured a large proportion of patients with HPV16-positive vulvar intraepithelial neoplasia (37).

The absence of toxicity observed with a variety of older vaccine strategies may be related to their poor efficacy with the generation of a small number of effector cells. The new stronger immunization protocols may generate a high number of effector cells but lead to severe adverse events. Anti-CTLA4 blockade induced high-grade autoimmunity including dermatitis, enterocolitis, hepatitis, and

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Abbreviations used in this paper: B, boosted; DC, dendritic cell; HPV, human papillomavirus; IP-10, IFN-γ–inducible protein 10; LP, long peptide; MHC I, MHC class I; MHC II, MHC class II; MS, male splenocytes; P, primed; SP, short peptide; T, tumor; Tg, transgenic; UD, undetectable level.

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hypophysitis (38). Massive cytokine storm and multiorgan failure were observed in six healthy volunteers who received a single infusion of superagonist anti-CD28 Ab (39).

SP treatment can also be dangerous. Administration of viral peptide to virus-immune mice can lead to rapid activation of pre-existing virus-specific CD8 T cells with high lethality (40), induced by TNF-α secreted by the peptide-specific CD8 T cells. To our knowledge, there have been no reports that activation of Ag-specific CD4 T cells can lead to serious or lethal consequences. In this study, we show that optimized vaccination with LP induces proliferation and activation of Ag-specific CD4 T cells to such an extent that the mice die within hours of the first boost. TNF-α was identified as the key effector of a cytokine storm that induced death and was related to rapid Ag presentation by B cells. Our data indicate that the immune system should be used with caution to fight tumors because improvements in T cell priming methods may lead to serious side effects and even death postboost.

Materials and Methods

Mice

Female C57BL/6 (B6) mice were purchased from Charles River Laboratories (L'Arbresle, France). Marilyn TCR transgenic (Tg) *RAG2*$^{-/-}$ mice are specific for the Dby–H-Y Ag presented by H-2A^{b} and are either on the CD45.1 or CD45.2 background (41). Female B6 mice deficient in IFN-γR1 and μMT (devoid of B cells) B6 mice were provided by C. Soudais (U932, Institut Curie, Paris, France) and B. Ryffel (Unité Mixte de Recherche 6218, Centre National de la Recherche Scientifique, Orléans, France), respectively. All of the mice were bred and housed in our specific pathogen-free animal facility. Live animal experiments were performed in accordance with the guidelines of the French Veterinary Department.

Tumor experiments

The MCA-101 (MCA) cell line does not have a Y chromosome. The MCA–H-Y cell line was derived from MCA-Dby cell line (23, 42) by a second transfection with a Uty expression vector. A total of 10^5 MCA or MCA–H-Y cells in 50 μl PBS were injected s.c. into the left flank. Tumor size was measured every 3 d. Mice were sacrificed when tumor volume reached 1000 mm^3.

H-Y peptides and vaccination

LP S-54-T, containing Dby and Uty epitopes (SSGSANAGFNSNRANSSRSSGSSHNRGFGGGGYGGFYQQLGWMHHNMDLIGDNT) and Dby SP (NAGFNSNRANSSRSS) were purchased from NeoMPS (Strasbourg, France) at 98% purity grade. Peptides were dissolved in 0.5% DMSO PBS. On days 0 (priming) and 10 (boost), mice were vaccinated i.p. with 9 nmol LP/SP in PBS, 40 μg endotoxin-free CpG-ODN 1018 (sequence 5′-TGACTGTGAACGTTCGAGATGA-3′; TriLink Biotechnologies, San Diego, CA), and emulsified in 50% IFA (Sigma-Aldrich, St. Louis, MO). For male cell vaccination, 3×10^6 male splenocytes in 100 μl 0.5% BSA-PBS and 40 μg CpG were injected i.p. Where indicated, mice were treated with mAbs specific for TNF-α (clone XT3.11; BioXCell, West Lebanon, NH) or an isotype-matched control (clone HRPN). mAbs (1 mg) were administered i.p. 1 d preboost.

DNA vaccination

Anesthetized (Imalgen and Rompun, 2%) mice were immunized by i.m. injection into the tibial cranial muscle of plasmid DNA (30 μg/30 μl) encoding anti-DEC205–Dby-Uty (anti–DEC205-LP). Anti-human–Bcl1-Dby-Uty (anti–Bcl1-LP) was used as control. After DNA injection, transcutaneous electric pulses were applied by electrodes placed 5 to 6 mm apart (8 pulses, 125 V/cm, 20 ms) using an ECM830 generator (Harvard Apparatus, Holliston, MA).

Flow cytometry

Cells were incubated with anti-FcγIII/IIR–specific mAb (2.4G2). Six- to eight-color flow cytometry was performed with directly conjugated Abs from BD Pharmingen (San Diego, CA), Biolegend (San Diego, CA), or eBioscience (San Diego, CA): anti–Vβ6-FITC (RR4-7), anti–CD45.2-PE (104), anti–CD4-PerCP–Cy5.5 (L3T4), anti–CD45.1-PE–Cy7 (A20), anti–CD19-APC (1D3), and anti–TCRβ-APC–Alexa 750 (H57-597). Data was acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA), and analyzed using FlowJo software (Tree Star, Ashland, OR).

In vitro stimulation and analysis of cytokine production

A total of 10^6 splenocytes were incubated with or without 400 nM SP at 37°C for 48 h. Cytokine levels (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IFN-γ–inducible protein 10 [IP-10], IFN-γ, and TNF-α) were measured using Luminex assays (Invitrogen, Carlsbad, CA).

Histology

Lung, heart, kidney, spleen, liver, jejunum, ileum, and colon were harvested. Intestines were gently flushed with PBS. The harvested organs were fixed in 10% neutral-buffered formalin overnight; 3-μm paraffin-embedded sections were stained with H&E (all tissues) and Giemsa or PAS (intestinal samples).

Intracellular cytokine staining for TNF

A total of 2×10^6/well splenocytes were cultured with Ag and GolgiPlug (BD Biosciences) for 4 h. The cells were then stained with Abs against CD4, CD8, CD11b, CD11c, CD19, CD45.1, CD45.2, and TNF using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences). The LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells.

Proliferation assay

Mice were primed i.p. with 9 nmol LP plus 40 μg CpG and IFA and injected i.v. with 9 nmol LP or SP 10 d later. Spleens harvested 30 min after peptide injection were incubated with 1 mg/ml collagenase D (Roche, Basel, Switzerland) and 25 μg/ml DNase I (Sigma-Aldrich) for 30 min at 37°C. After filtration through a 40-μm grid, CD11c$^+$ and CD11c$^-$ fractions were isolated with anti-CD11c magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and labeled with anti–CD8α-FITC, anti–CD11b-FITC, and anti–CD19-PE. Cells were sorted on an FACSAria (BD Biosciences). Serial dilutions of the five sorted APC fractions were cultured for 1–3 d with 5×10^4 CFSE-labeled Marilyn T cells.

Statistical analysis

Statistical analysis of survival time was performed by the log-rank Kaplan-Meier test. Other statistical analyses were calculated using the Student *t* test. Results were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Vaccination with LP causes lethal shock

We previously showed that after adoptive transfer into *RAG2*$^{-/-}$ mice, anti-male Ag (H-Y) TCR Tg Marilyn CD4 T cells, which are specific for the MHC II-restricted Dby epitope, can induce the regression of H-Y-expressing tumors, whereas anti–H-Y TCR Tg MataHari CD8 T cells, specific for the Uty MHC I-restricted epitope, cannot (22). In a different tumor model, prophylactic male cell priming was required to slow the growth of a Dby-expressing MCA-101 fibrosarcoma implanted into Marilyn TCR Tg *RAG2*$^{-/-}$ hosts (42). To test the role of Ag presentation by MHC I and II molecules in vaccination against tumor-expressing epitopes restricted by both kinds of MHC molecules, MCA-101 tumor was transfected with Dby and Uty constructs. Several Ag sources were used as antitumor vaccines: LP in CpG /IFA, SP in CpG /IFA, and male cells in CpG as a positive control.

A prophylactic regimen with two injections 10 d apart (Fig. 1*A*) followed by tumor cell implantation 7 d later was used. This regimen did not prolong the survival of mice inoculated with the parental H-Y–negative MCA cell line (Fig. 1*B*). In contrast, in mice inoculated with MCA–H-Y tumors, vaccination with male splenocytes slowed the tumor growth and prolonged survival, whereas vaccination with either SP or LP did not (Fig. 1*C*).

To reproduce situations found after therapeutic transfer of T cells in humans or during primary responses (8, 9, 43, 44), in which the number of Ag-specific T cells can reach 1–5% of total T cells, we artificially increased the frequency of specific CD4 T cells by transferring 10^5 to 10^6 Marilyn T cells into the B6 hosts. In this

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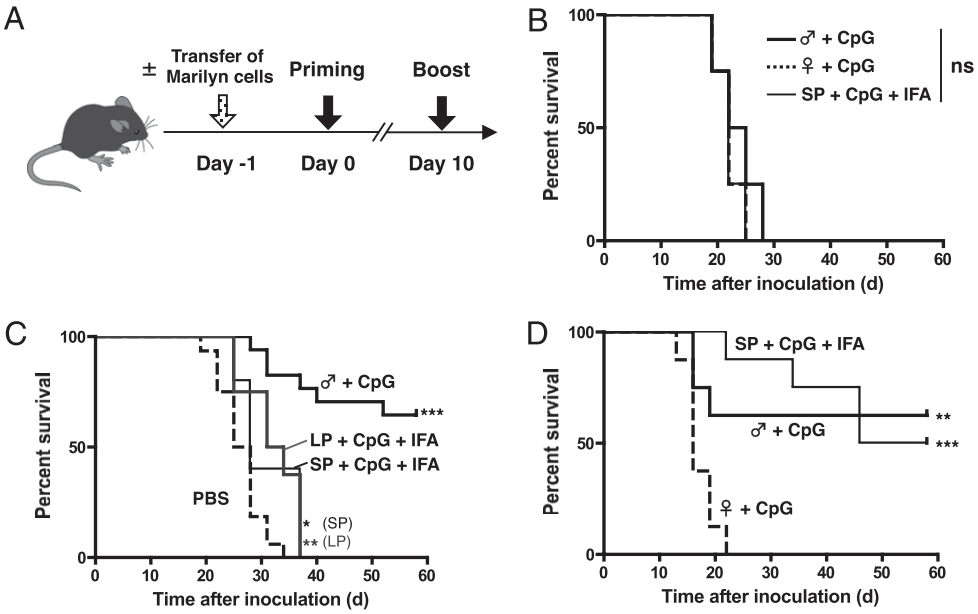

FIGURE 1. Survival curves of mice inoculated with MCA or MCA–H-Y tumor cells. *A*, Generic experimental schema. A total of 10^6 Marilyn T cells were (*B* and *D*) or were not (*C*) transferred into B6 mice, which then were vaccinated twice i.p. with male or female splenocytes, Dby SP, or LP according to the protocol shown in *A*. Seven days after the second vaccination, 10^5 MCA (*B*) or MCA–H-Y tumor cells (*C* and *D*) were inoculated s.c. into the left flank. Pooled data from one (*B*), three (*C*), and two (*D*) experiments with a total of 4 (*B*), 18 (*C*), and 8 (*D*) mice per group. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ by log-rank test.

setting, vaccination with either SP or male cells prolonged survival (Fig. 1*D*). In case of LP vaccination, 76% (16 out of 21) of the mice unexpectedly died within 72 h after LP boost (Fig. 2*A*, 2*B*), precluding the assessment of antitumor activity. The mice developed shock-like symptoms before dying: hunched backs, apparent hypothermia, reduced mobility, unresponsiveness, tremor, and death (40) within hours after the boost (Fig. 2*A*). No symptoms or deaths were observed in the absence of Marilyn CD4 T cell transfer, in mice with MataHari Uty-specific CD8 T cell transfer, or in mice vaccinated with SP or male splenocytes (data not shown). No mice showed shock-like symptoms with adjuvant only, whereas 87% (14 out of 16) of the mice vaccinated with LP without adjuvant died after the boost (data not shown).

The same experiments were performed using female *Rag2*$^{-/-}$ mice as hosts. Ninety-five percent (19 out of 20) of the mice injected with Marilyn CD4 T cell died within 8 h after the LP boost (Fig. 2*C*, 2*D*). As few as 10^4 Marilyn cells were sufficient to induce high mortality after LP boost. These results indicate that the humoral response is not involved in the LP-induced mortality. Thus, LP vaccination leads to lethal adverse effects in mice when the frequency of specific CD4 T cells has been artificially increased. This toxicity appears to be related to CD4 T cell triggering.

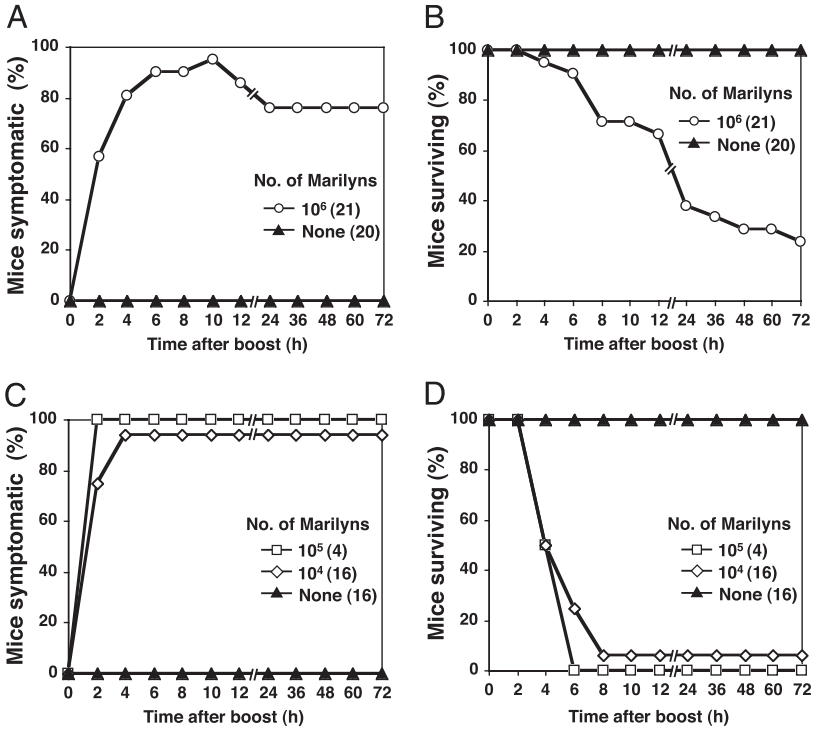

FIGURE 2. Vaccination with long peptides can cause shock-like symptoms and death. Symptoms and survival of B6 (*A*, *B*) or RAG2$^{-/-}$ (*C*, *D*) mice with or without Marilyn cell transfer. The indicated numbers of Marilyn T cells were transferred into C57BL/6 (B6) or *RAG2*$^{-/-}$ mice, which were then i.p. primed with 9 nmol LP+CpG in IFA and boosted with LP 10 d later according to Fig. 1*A*. Data from a total of 41 mice in five experiments (*A*, *B*) and 36 mice in four experiments (*C*, *D*) as indicated.

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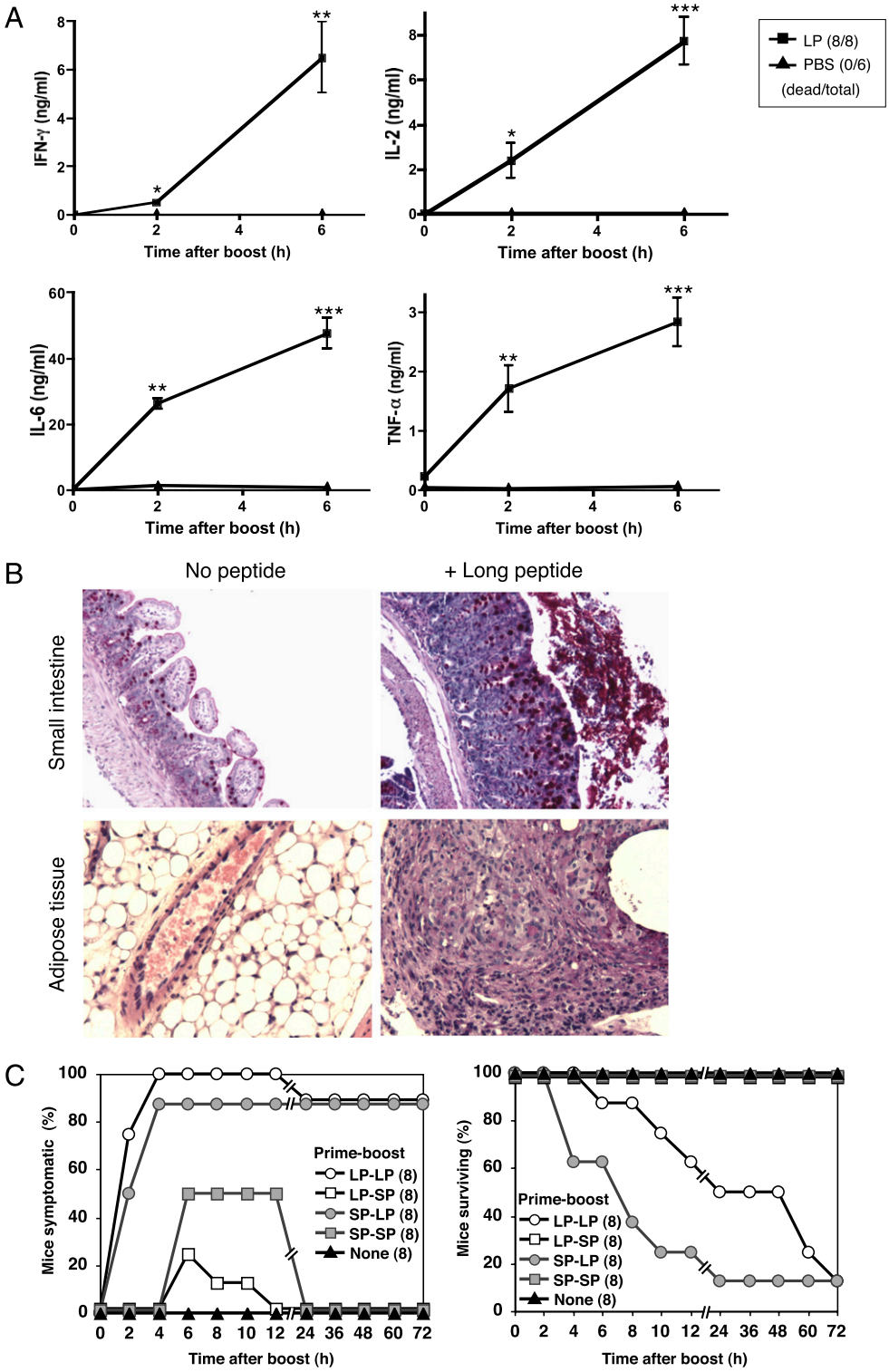

FIGURE 3. Lethal shock is induced by cytokine storm. *A*, Cytokine levels of mice primed and boosted with LP. Posttransfer of 10^6 Marilyn T cells, B6 mice were vaccinated i.p. with LP according to Fig. 1*A*. Cytokine levels in serum were determined preboost and at 2 and 6 h later. Pooled data from two independent experiments. *B*, Histopathological findings 6 h postboost in the small intestine (PAS; original magnification ×100) and peri-intestinal adipose tissue (H&E; original magnification ×200). *C*, Boost with MHC II-restricted SP induces shock-like signs, but does not kill. A total of 10^6 Marilyn cells were transferred into B6 mice, which were then primed and boosted as indicated. Nine nanomoles LP or SP were i.p. administered with CpG and IFA. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ by Student *t* test.

Lethal shock is induced by cytokine storms after LP boost

Before death, the mice displayed shock-like signs highly suggestive of cytokine storm. Serum cytokine levels were measured before and after LP boost (Fig. 3*A*). In all of the LP boosted mice, IFN-γ, IL-2, IL-6, and TNF-α levels were high (Fig. 3*A*).

A similar phenomenon was described in a CD8 T cell system in which boost of lymphocytic choriomeningitis virus-immune mice with an immunodominant epitope peptide induced death (40). Pathological modifications were seen in the gut with mucus expulsion by the goblet cells. We therefore looked at histopatho-

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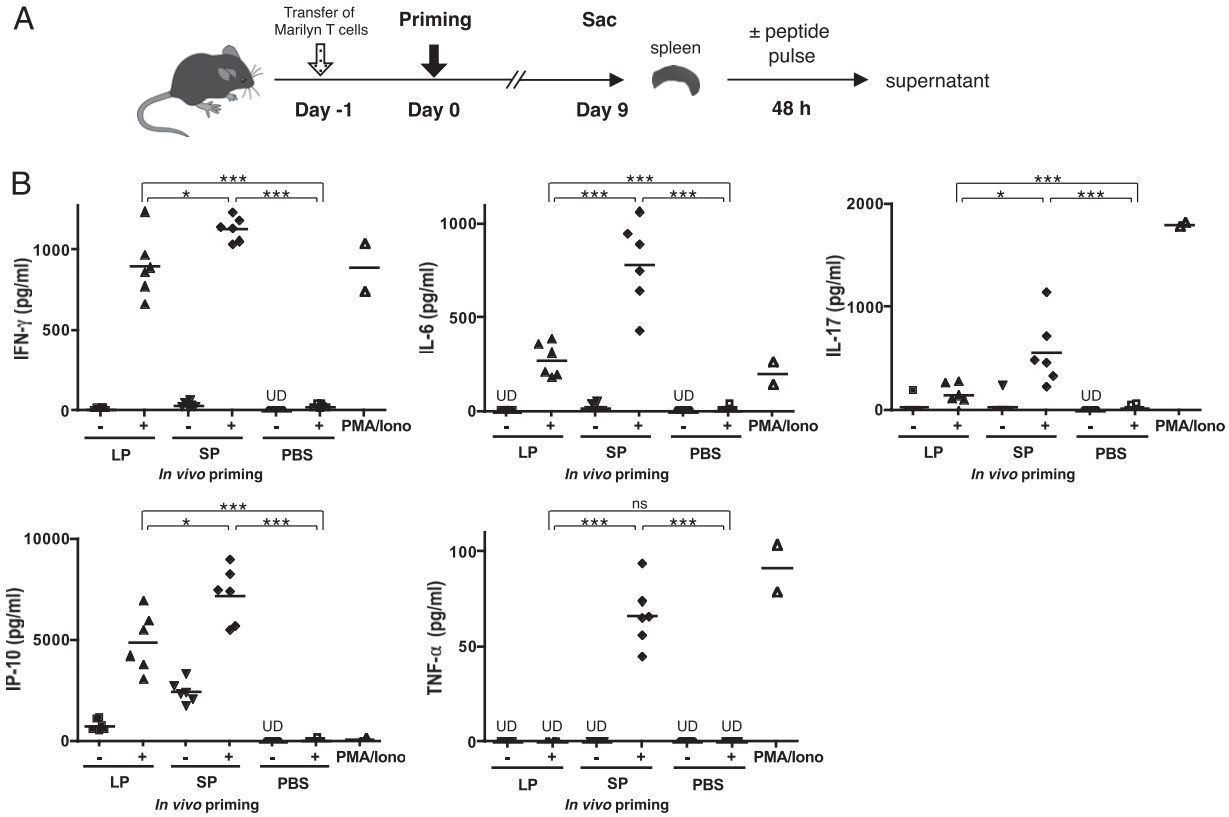

FIGURE 4. In vivo SP priming induces more in vitro cytokine production by splenocytes than LP. *A*, A total of 10^6 Marilyn cells were transferred into B6 mice, which were then primed with LP or SP. After 9 d, the spleens were harvested and cultured with or without 100 nM SP peptide for 48 h. *B*, Cytokine levels in the supernatant. $*p < 0.05$; $***p < 0.001$ by Student *t* test. UD, undetectable level.

logical changes in the small intestine after the LP boost. The small intestine of the LP-vaccinated mice displayed blunted villi with denudation of the basement membrane. Goblet cells were enlarged and located near the intestinal lumen (Fig. 3*B*). Detached cells and expelled mucus in the lumen were apparent. No histopathological changes were observed in other organs. These data suggest that the symptoms and death induced by LP vaccination are caused by a cytokine storm with associated immunopathology (40, 45).

Vaccination with SP causes shock-like signs but not death

To determine whether the different effects of LP versus SP were caused during the priming or the boost phase of our vaccination scheme, the four possible prime-boost combinations of LP and SP were tested. Fifty percent of mice primed and boosted with SP (SP-SP) displayed shock-like signs postboost, but all recovered from the symptoms within 18 h (Fig. 3*C*). Only 25% of mice in the LP-SP group displayed shock-like signs, and they all recovered. The SP-LP group displayed the same mortality (87%), but the deaths were earlier than the LP-LP group, suggesting that the SP priming induces more active (or numerous) effector cells than LP, but that only LP boost causes lethal shock.

To compare the priming efficacy of LP versus SP, the number of Marilyn cells in the peripheral blood was quantified just before boost. The number of Marilyn T cells was highly increased in comparison with the PBS vaccinated controls ($p < 0.001$) but was the same after LP or SP priming (data not shown). No correlation was found between the number of Marilyn cells measured pre-boost and death.

CD4 T cell priming is stronger with SP than with LP

To investigate whether LP and SP would have differential priming capacity, we measured lymphokine secretion by splenocytes from mice with Marilyn cell transfer, primed with LP or SP (with CpG/IFA) postrestimulation in vitro 9 d later (Fig. 4*A*). Cytokine levels of IFN-γ, IL-6, IL-17, IP-10, and TNF-α were higher after SP than LP priming (Fig. 4*B*). Thus, SP is a more efficient primer than LP.

TNF-α is essential for LP-mediated death

As lymphokine storm has been implicated in the death induced by peptide injection of lymphocytic choriomeningitis virus-immune mice (40), we assessed the serum cytokine levels 6 h postboost. High levels of IFN-γ, IL-2, IL-6, IP-10, and TNF-α were found in mice boosted with LP (Fig. 5*A*). Significant but lower levels of IFN-γ, IL-6, and IP-10 were found in mice boosted with SP. High levels of serum IL-2 and TNF-α were seen only in mice boosted with LP. Of the cytokines measured, high levels of serum IFN-γ, IL-2, and TNF-α seemed to be correlated with death.

To determine which cytokine is the major mediator of the lethality, we used an IFN-γR1–deficient host. B6 mice were also treated with anti–TNF-α neutralizing mAbs. The high morbidity (80%) and mortality (80%) were the same for the IFN-γR1 knockout mice, the untreated B6 or isotope control group excluding the involvement of type 2 IFN. In contrast, only 17.5% (2 out of 12) mice treated with anti–TNF-α mAbs died after LP boost (Fig. 5*B*), indicating a requirement for TNF-α, which was produced by the Marilyn CD4 T cells (Fig. 5*C*).

Intravenous administration of LP but not SP as boost induces high mortality in a dose-dependent manner

The differences in timing and occurrence of side effects after SP or LP boost (Fig. 3*C*) suggest variations in LP and SP pharmacokinetics (e.g., slower availability or faster degradation of SP). To control the biodisponibility and the dose of the peptides, the mice were boosted i.v. instead of i.p. with varying doses of LP or SP. All

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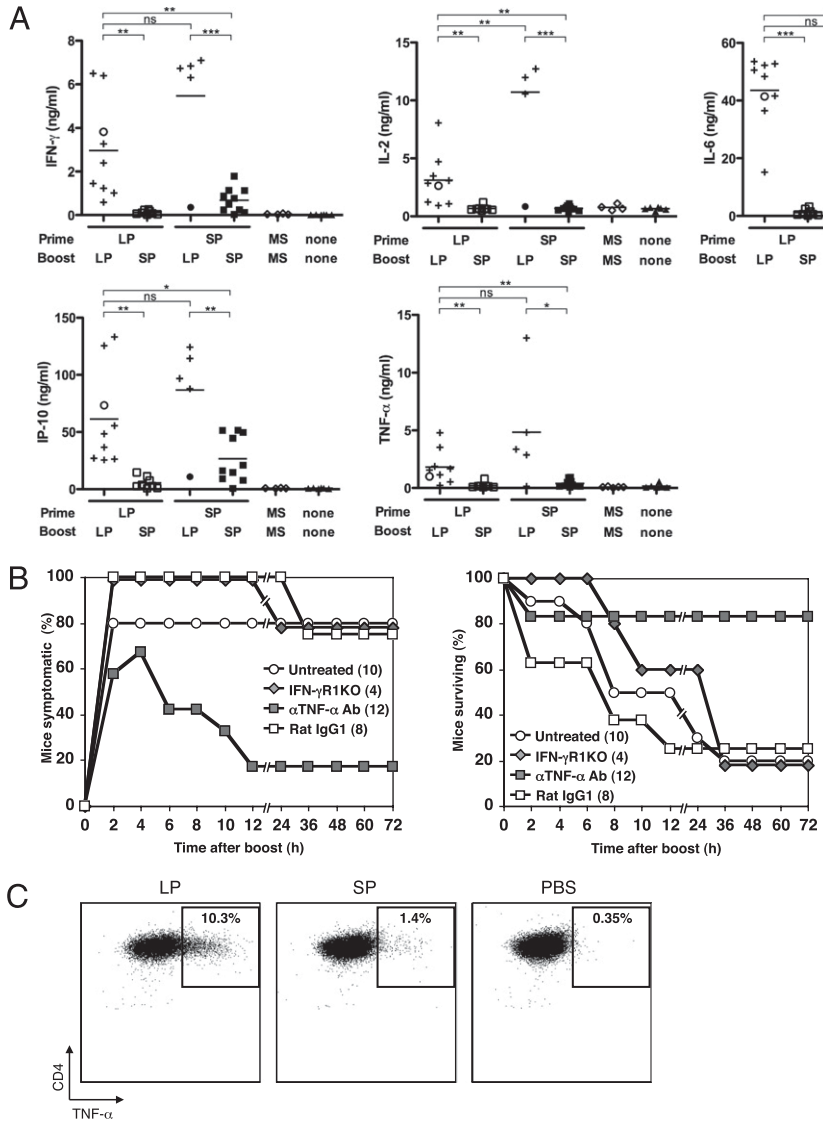

FIGURE 5. TNF-α is essential for LP-mediated deaths. *A*, A total of 10^6 Marilyn cells were transferred into B6 mice, which were then primed and boosted with LP or SP as indicated. Serum cytokine levels were measured 6 h after the boost. Crosses indicate dead mice. *B*, A total of 10^6 Marilyn cells were transferred into wild-type B6 or IFN-γR1–knockout mice, which were then primed i.p. with LP. Nine days later, wild-type mice were treated with neutralizing mAbs specific for TNF-α or isotype control rat IgG1 and were boosted i.p. with LP on the following day. The data summarized a total of 24 mice from two independent experiments. *C*, Marilyn cells boosted with LP produced more TNF-α than with SP. A total of 10^6 Marilyn cells were transferred into 9 B6 mice, which were then primed i.p. with LP/CpG/IFA and, 10 d later, were i.v. boosted with 9 nmol LP, SP, or PBS (three mice/group). Thirty minutes later, the spleens were harvested and analyzed using intracellular cytokine staining as described in *Materials and Methods*. Representative dot plot in each group is shown (gated on $CD4^+/CD45.1^+$ cells). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ by Student *t* test. MS, male splenocytes.

of the mice i.v. injected with 9 nmol LP immediately exhibited shock-like signs and died within 30 min (Fig. 6*A*, 6*B*). The mice i.v. injected with 9 nmol SP displayed symptoms 4 h postboost but recovered without any death. The death rate was dose dependent for LP boost (Fig. 6*C*). As little as 1 nmol LP induced high mortality, whereas doses as high as 27 nmol SP did not. The difference in mortality between LP and SP boosts is thus not related to low in vivo availability of SP (34).

Higher stimulation of APCs after LP versus SP administration

To examine the effect of the amount of Ag on APCs as well as the type of APC involved after i.v. boost, spleens were harvested 30 min after peptide injection. Five types of APCs were sorted by FACS and used to stimulate CFSE-labeled Marilyn T cells in vitro (Fig. 7*A*). Fewer APCs were required to induce a given level of stimulation after LP boost than after SP boost, except for macrophages ($CD11b^+CD11c^-$) (Fig. 7*B*, 7*C*). This suggests that the number of MHC–peptide complexes on APCs is higher after an LP than after an SP boost.

Stronger activation of Marilyn cells by B cells ($CD19^+CD11c^-$) was seen after LP than after SP boost. To examine B cell implication in the differential mortality observed after LP versus SP boosts, B6 and μMT (devoid of B cells) mice were i.p. primed with LP/CpG/IFA and i.v. boosted 10 d later. The μMT mice showed shock-like signs at 4 h after LP boost but recovered. In contrast, B6 mice boosted with LP died within 2 h postboost (Fig. 7*D*). These data suggest that LP is rapidly presented by B cells to Marilyn CD4 T cells, inducing the toxic shock.

To overcome the toxicity due to Ag presentation by B cells and still keep the putative antitumor effect induced by a vaccine incorporating both MHC I and II epitopes, we constructed a DNA vaccine targeting the Ag to dendritic cells (DCs) by fusing the Uty and Dby epitopes to an anti-DEC205 single chain Ab (46).

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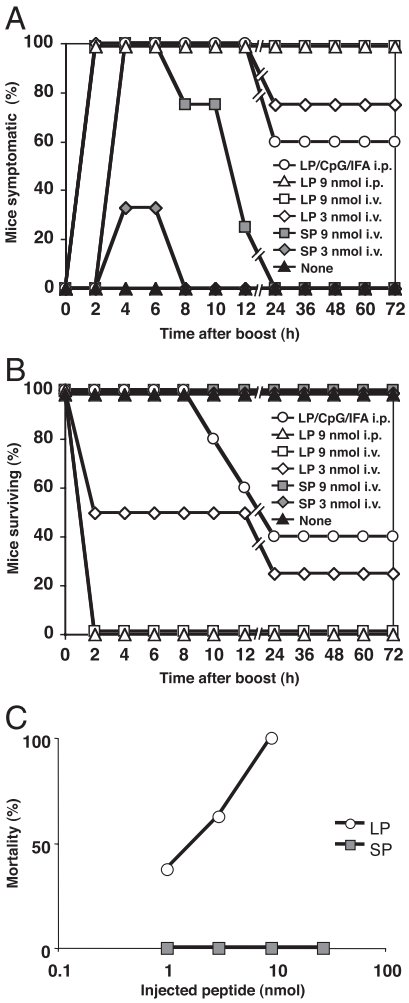

FIGURE 6. LP but not SP boost induces a high mortality in a dose-dependent manner. *A* and *B*, A total of 10^6 Marilyn cells were transferred into B6 mice, which were then primed i.p. with LP/CpG/IFA and, 10 d later, were boosted i.v. with LP or SP at the indicated dose. These figures show one experiment representative of three including a total of 89 mice. *C*, Relationship between mortality and LP or SP dose used for the boost.

According to the schedule shown in Fig. 1*A*, Marilyn T cells were injected into mice that were then immunized twice with 30 μg of anti–DEC205-LP plasmid, control irrelevant anti-human Bcl1-LP or the LP. Postinoculation of MCA–H-Y tumor cells, two of 8 mice surviving after LP boost were not protected against tumor growth. No protection was also observed in mice vaccinated with the anti–Bcl1-LP plasmid. In contrast, mice vaccinated with anti–DEC205-LP plasmid displayed no shock-like signs or death and 8 of 13 mice did not show any tumor growth (Fig. 8). Thus, vaccination with a chimeric MHC I and II antigenic sequence targeted to DCs induces a good antitumor protection without side effects.

Discussion

In this study on peptide vaccination, we show that boosting with LP can activate epitope-specific effector CD4 T cells generated by previous priming, which then leads to a lethal cytokine storm. No mortality was observed with much higher boost doses of SP despite similar priming. TNF-α secreted by the primed CD4 T cells is the major mediator. The adverse effects observed after LP boost are related to a better presentation in comparison with SP of the MHC II-restricted epitope by B cells in addition to DCs. Finally, targeting Ag presentation to DCs allowed efficient antitumor activity without side effects.

Our system is rather artificial in that we increased the number of responding CD4 T cells by adoptive transfer of monoclonal naive CD4 T cells. However, we also observed significant adverse effects including lethality posttransfer of as low as 3×10^4 Marilyn CD4 T cells into B6 mice (data not shown) and even 10^4 cells into *RAG2*$^{-/-}$ hosts. In the absence of adoptively transferred CD4 T cells, some symptoms and inconstant lethality were observed especially when regulatory T cells were depleted (data not shown). The number of specific CD4 T cells in our system attains levels found after adoptive transfer of T cells in humans or during a primary response (8, 9, 44). The findings in this study are thus relevant to potential therapeutic situations in humans: the low incidence of adverse effects observed during current therapeutic anticancer vaccine regimens may be related to their poor efficacy. As manipulation of the immune system (use of adjuvant, depletion of regulatory T lymphocytes, etc.) improves these regimens, vaccination may become more efficient against tumor but increasingly dangerous.

The dying mice showed symptoms consistent with TNF-α–related toxicity, as was observed after peptide restimulation of viral or tumoral peptide specific memory CD8 T cell (40, 47). In contrast to septic shock syndrome (48), serum levels of IL-1 after LP boost did not increase (data not shown), whereas TNF-α and IL-6 levels were very high. The mice with the highest IL-2 levels were the ones that subsequently died (Fig. 5*A*), and a correlation was seen between serum TNF-α and IL-2 levels ($R^2 = 0.6785$; $p = 0.0018$). Serum IL-2 levels after LP boost in mice treated with anti–TNF-α Ab were lower than in untreated or isotype Ab-treated mice (data not shown), suggesting that IL-2 production is a consequence rather than a cause of TNF-α secretion. The toxicity of TNF-α is known when used as an antitumor agent (49, 50) or when endogenously released after a treatment by a superagonistic anti-CD28 Ab, TGN1412 (39). Our results are consistent with the clinical experience: high morbidity can be observed in any situation in which high levels of TNF are released.

Why would LP, but not SP, boost induce lethal adverse effects when SP is as efficient at priming as LP? A 27-fold higher dose of SP does not induce death despite a high incidence of symptoms (Fig. 6*C*), thus this difference is unlikely due to faster degradation. This is not due to an unexpected direct adjuvant effect of the LP on the APC, as no maturation was induced in vitro by the LP on purified DCs (C. Sedlik, H. Kitamura, and O. Lantz, unpublished observation). The type of APC presenting the Dby epitope after LP versus SP boost appears to be crucial. On a per-cell basis, all of the APC subsets were more powerful at inducing CD4 T cell activation after LP boost. Surprisingly, B cells could stimulate CD4 T cells only after LP, but not SP, boost (Fig. 7*B*, 7*C*). Although B cells were less efficient than DCs, they are much more numerous, leading to more rapid and stronger CD4 T cell activation after LP boost. Confirming this interpretation, no death was observed in B cell-deficient mice, although the symptoms were similar in intensity and kinetics to those observed in mice boosted with SP (Fig. 7*D*). However, B cells are not always necessary, as lethality is also observed in RAG-deficient hosts harboring monoclonal CD4 T cells. One possible explanation for this apparent discrepancy is the very high number of effector cells generated in these empty hosts in the absence of regulatory T cells and of competition with polyclonal T cells.

In addition to the potent antitumor effect observed in preclinical models (33) and clinical protocols (35, 36, 51), LP use in therapeutic cancer vaccines is of interest due to the requirement for Ag processing by professional APCs to generate MHC I-restricted

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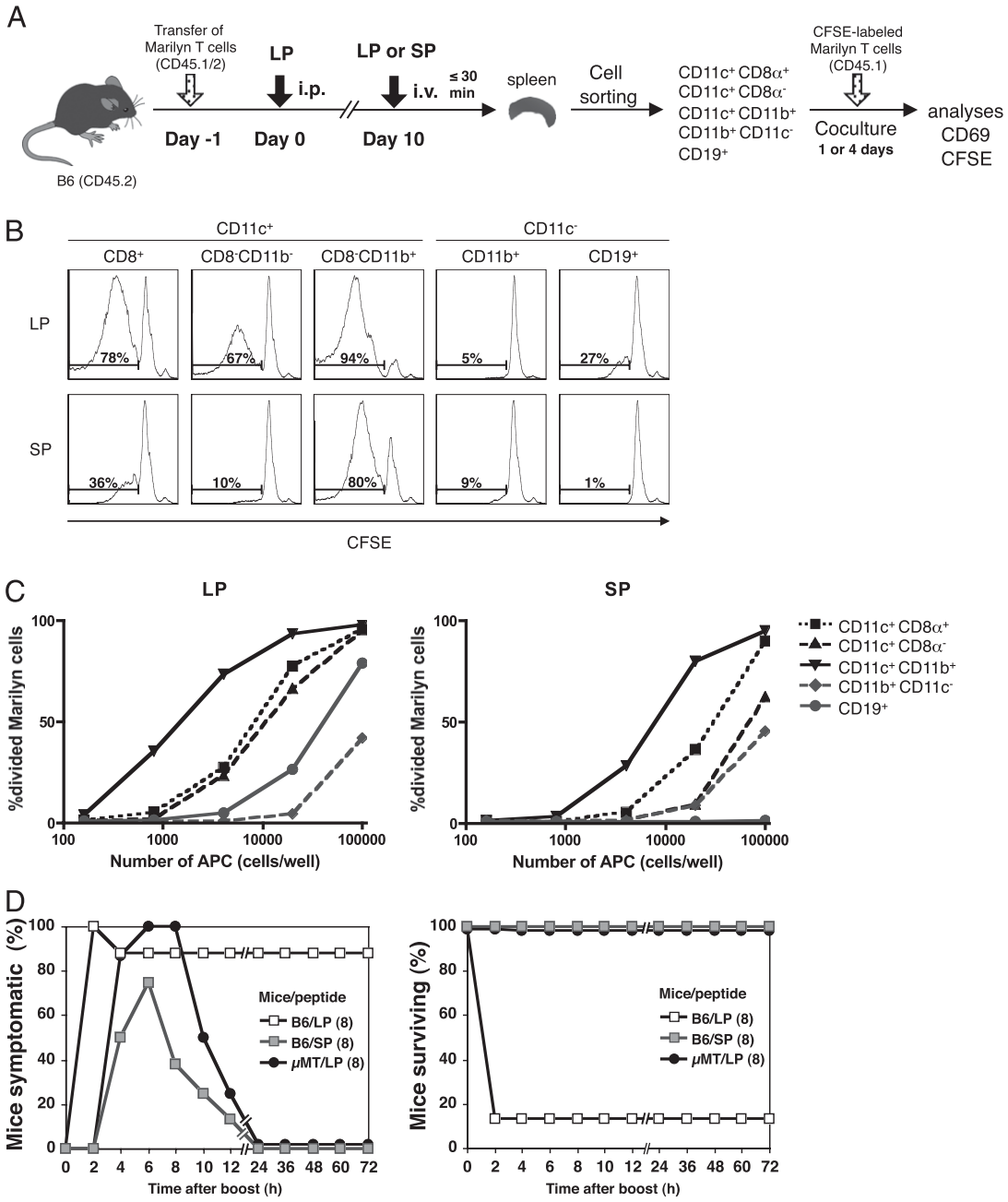

FIGURE 7. Administration of LP induces more potent APCs to present Ag to Marilyn T cells than SP. *A*, Marilyn cells were transferred into B6 mice, which were primed i.p. with LP, and, 10 d later, were boosted with LP or SP. Thirty minutes (or just before death) after the peptide injection, the spleens were harvested. The APCs were sorted as $CD8\alpha^+CD11c^+$, $CD8\alpha^-CD11b^-CD11c^+$, $CD8\alpha^-CD11b^+CD11c^-$, $CD11b^+CD11c^-$, or $CD19^+CD11c^-$ cells and used at the indicated cell concentration in an in vitro proliferation assay using CFSE-labeled CD45.1 Marilyn T cells as responding cells. *B*, Representative profiles are shown for the 2×10^4 APC/well point. *C*, Percentage of Marilyn divided cells on day 4 according to the number of APCs. *D*, A total of 10^6 Marilyn cells were transferred into B6 or μMT mice, which were then i.p. primed with LP/CpG/IFA and, 10 d later, were boosted with LP or SP. The data summarized a total of 24 mice from two independent experiments.

epitopes. In an OVA model system, only $CD11c^+$ cells presented Ag to OT-1 CD8 T cells after LP vaccination, whereas $CD11c^+$, $CD19^+$, and even $CD3^+$ cells in the draining lymph nodes presented Ag postvaccination with minimal MHC I-restricted peptide, but the CD4 T cell response was not evaluated (34). Our results confirm that the MHC II epitopes in LP are presented differently from the MHC I epitope derived from LP after processing (32, 52). This may explain why CD4 T cells responses were better than CD8 T cell responses during clinical trials using overlapping HPV16 E6 and E7 LPs to treat HPV neoplasia (36, 37) (X. Sastre-Garau and O. Lantz, unpublished observations). Using the Dby/Uty chimeric LP, Marilyn CD4 T cells were more strongly stimulated than MataHari CD8 T cells (data not shown). Although this finding should be confirmed with other Ags, LP containing both MHC I and II epitopes may induce stronger CD4 than CD8 responses. Finally, a vaccine strategy relying on Ag presentation to CD4 T cells only by DCs may be important to prevent adverse effects. Although no such side effects were found in the clinical trials using HPV LP (35–37), increasing the LP dose or other concomitant immune modulations aimed at increasing vaccine

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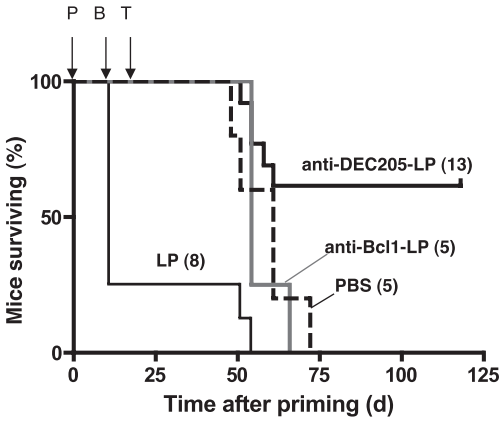

FIGURE 8. Survival curves of mice vaccinated with anti–DEC205-LP plasmid, targeting only DC, and inoculated with MCA–H-Y tumor cells. A total of $\times 10^5$ Marilyn T cells were transferred into B6 mice, which were then primed and boosted with LP or by electroporation with anti–DEC205-LP plasmid, anti–Bcl1-LP plasmid, or PBS according to the protocol shown in Fig. 1*A*. Tumor was inoculated 7 d later. Pooled data of two independent experiments. B, boosted; P, primed; T, tumor.

potency (e.g., depletion of regulatory T cells, for instance) may increase the risk of toxicity, and this should be taken into account for the clinical use of such vaccines.

In conclusion, our study shows that rapid Ag presentation by B cells after LP vaccination activates pre-existing Ag-specific CD4 T cells, which can induce lethal effects. In the context of clinical applications, our data indicate that when enough effector cells have been generated beforehand by a good priming, the boost should be carried out with caution to avoid a synchronized triggering of CD4 T cells such as that caused by i.v. injection and/or high Ag dose. In addition to strategies targeting Ag presentation to DCs, such as the anti-DEC205 single-chain Ab used in this study, one way to prevent this problem would be to use LP covalently coupled to adjuvants that would allow the injection of smaller Ag doses, as these adjuvant/LP combinations are more active (53).

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Disclosures

The authors have no financial conflicts of interest.

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