Cell-Associated Double-Stranded RNA Enhances Antitumor Activity through the Production of Type I IFN

Sara McBride, Kasper Hoebe, Philippe Georgel and Edith Janssen

*J Immunol* 2006; 177:6122-6128; doi: 10.4049/jimmunol.177.9.6122

http://www.jimmunol.org/content/177/9/6122

**References** This article cites 46 articles, 17 of which you can access for free at: http://www.jimmunol.org/content/177/9/6122.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

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

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Cell-Associated Double-Stranded RNA Enhances Antitumor Activity through the Production of Type I IFN

Sara McBride,* Kasper Hoebe,† Philippe Georgel,† and Edith Janssen2*

The efficacy of tumor cell vaccination largely depends on the maturation and activation status of the dendritic cell. Here we investigated the ability of soluble and tumor cell-associated dsRNA to serve as an adjuvant in the induction of protective adaptive antitumor responses. Our data showed that cell-associated dsRNA, but not soluble dsRNA, enhanced both tumor-specific CD8+ and CD4+ T cell responses. The cell-associated dsRNA increased the clonal burst of tumor-specific CD8+ T cells and endowed them with an enhanced capacity for expansion upon a secondary encounter with tumor Ags, even when the CD8+ T cells were primed in the absence of CD4+ T cell help. The adjuvant effect of cell-associated dsRNA was fully dependent on the expression of TLR3 by the APCs and their subsequent production of type I IFNs, as the adjuvant effect of cell-associated dsRNA was completely abrogated in mice deficient in TLR3 or type I IFN signaling. Importantly, treatment with dsRNA-associated tumor cells increased the number of tumor-infiltrating lymphocytes and enhanced the survival of tumor-bearing mice. The data from our studies suggest that using cell-associated dsRNA as a tumor vaccine adjuvant may be a suitable strategy for enhancing vaccine efficacy for tumor cell therapy in cancer patients. The Journal of Immunology, 2006, 177: 6122–6128.

The use of autologous tumor vaccines is a promising method to target the greatest number of potential tumor Ags without requiring their individual identification. Dendritic cells (DCs) are the most potent APCs to take up, process, and present tumor vaccine Ags to naive tumor-specific CD4+ and CD8+ T cells (1–4). However, the efficacy of these treatment strategies largely depends on the maturation and activation status of the DC. Immature DCs exhibit potent phagocytic but poor stimulatory capabilities and generally mediate T cell tolerance, whereas mature DCs conversely exhibit poor phagocytic but potent T cell activation capacities (5). Current efforts toward enhancing the efficacy of tumor vaccine research have thus sought to either increase the number of Ag-specific DCs or selectively induce their activation and maturation (1–4, 6). An important pathway for DC maturation involves the detection of specific molecules of microbial origin through their cognate receptors, which include the TLR family (7, 8). Upon TLR engagement, DCs mature and up-regulate MHC-peptide complexes and costimulatory molecules on the cell surface as well as the secretion of cytokines and chemokines required for T cell activation (9, 10).

DCs are comprised of a heterogeneous family of leukocytes, and the DC subsets known to cross-present cellular Ags are derived from both the myeloid and lymphoid lineage; in mice, the cross-priming DC subsets are restricted to CD8+T populations (11, 12).

These cross-priming DC are endowed with specific TLR repertoires and expression patterns that change upon maturation or activation (9). It has recently been shown that the DC subsets that respond to TLR3, TLR4, and TLR9 ligands can enhance T cell priming in vitro (13). In vivo studies have demonstrated that co-administration of dsRNA with Ag enhances expansion of CD8+ T cells in a murine transfer model (14). Additional in vivo models have shown that dsRNA enhances endogenous CD8+ T cell responses upon treatment with peptide-pulsed DCs or Ag-pulsed cell lines (14–16). Despite similarities in TLR signaling pathways, engagement of some specific TLRs induces distinct activation states and cytokine secretion patterns by DCs and thereby different environments in which T cells become activated, as well as possibly influencing T cell function and survival.

We have investigated the ability of dsRNA to serve as an adjuvant in the induction of protective antitumor CD4+ and CD8+ T cell responses in normal and immunocompromised mice. We focused on those effector molecules that convey the dsRNA-mediated adjuvant effect and the subsequent changes in T cell proliferation, survival, and cytokine capacity.

Materials and Methods

Mice and cell lines

C57BL/6, B6/129 F1, B6/129S2-IL6tm1Kopf/J (IL-6−/−), and B6/129S-Tnfrsf1atm1IncTnfrsf1Btm1Inc/J (TNFRp55/p75) were obtained from The Jackson Laboratory, IL-12p40−/−, IL-10−/−, OT-1 Rag−/−, HY Rag−/−, TRIFtm2pm2 (17), TRIFtm2pm2MyD88−/− (all backcrossed to the C57BL/6 background), and B6/129TRIFtm2pm2 mice were bred in-house. Type I IFN receptor-deficient (IFNαBR−/−) mice were kindly provided by Dr. J. Sprent (The Scripps Research Institute, La Jolla, CA). Mice were maintained under specified pathogen-free conditions in accordance with the guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care International.

The previously described cell lines EL-4, EL-4mOVA, and MEC.B7.Sig-OVA were cultured in IMDM (Invitrogen Life Technologies) supplemented with 10% FCS, 50 μM 2-ME, 2 mM L-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin (18, 19).

Association of TLR ligands to cells

Irradiated (3000 rad) EL-4 and EL-4mOVA cells were loaded with synthetic dsRNA (polyinosinic-polycytidylic acid (poly(I:C)); Amersham Biosciences) via electroporation as previously described (16). Briefly, cells...
were electroporated (950 farads, 0.250 V) in the presence or absence of poly(UC) (10 μg in a 200-μl volume). This protocol has been shown to result in ~1 ng of poly(UC) per 10^5 cells (16). At the time of injection the irradiated and electroporated EL-4 and EL-4-mOVA cells are early apoptotic as determined by annexin V expression and do not express type I IFN or IL-2.

**Immunization and tumor challenge**

Mice of indicated strains were immunized s.c. with 20 x 10^5 irradiated and electroporated EL-4-mOVA cells reseeded in PBS. After 7 days, mice were sacrificed and isolated splenocytes were analyzed for OVA-specific T cell responses. In cases where B6/129 mice were used, age- and sex-matched littermates were used to control for minor histocompatibility differences between EL-4-mOVA cells and the B6/129 mice. Depletion of CD4^+ T cells in vivo was performed by i.p. administration of 150 μg of the mAb (mAb) GK1.5 on the first 3 days before immunization (18, 20). In tumor challenge experiments, CD4-depleted mice were immunized with 5 x 10^6 live EL-4-mOVA cells. As soon as palpable tumors had formed, 20 x 10^5 irradiated electroporated EL-4-mOVA cells were administered s.c. to the mice, and tumor growth was monitored daily with a vernier caliper. Mice were euthanized when tumors reached 1 cm^3.

**Function and enumeration of tumor-specific T cells**

For tumor-specific CD8^+ T cell responses in the periphery, splenocytes were stimulated for 6 days in vitro with irradiated syngeneic MEC.B7.Sig-OVA. Following restimulation, viable cells were collected over Ficoll gradient (Lymphocyte-M; Cedarlane Laboratories). Cells were incubated either directly ex vivo or following in vitro restimulation with the OVA 257–264 peptide (SIINFEKL; 5 μg/ml) or control peptide GP33–41 (KAVYNFATC; both from A & A Laboratories) for 5 h in the presence of brefeldin A. Surface staining for CD8 and intracellular cytokine staining for IFN-γ was performed using a Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions. The fold expansion of specific CD8^+ cells was calculated by dividing the absolute number of IFN-γ^+ CD8^+ cells after in vitro culture by the absolute number of IFN-γ^+ CD8^+ cells at the start of the culture (12, 18, 20). The cytolytic activity of restimulated splenocytes was evaluated by a JAM test as previously described using [3H]thymidine-labeled EL-4 cells loaded with OVA 257–264 or GP33–41 peptide (21). Specific killing was calculated as ((spontaneous cpm – experimental cpm) x 100)/spontaneous cpm.

OVA-specific CD4^+ TILs and Th2 cells were enumerated by ELISPOT after a 48-h in vitro stimulation with OVA257–264 (ISOVHAAHA EINAEGR; 10 μg/ml), control peptide LLO190–201 (NEKYAQAYPNVS; 10 μg/ml), or Con A (2 μg/ml; positive control) as previously described (22). For proliferative responses, 2 x 10^5 cells were cultured in 96-well plates in the presence of titrated doses of OVA257–264. After 72 h of culture, cells were pulsed with 0.2 μCi of [3H]thymidine, and [3H]thymidine incorporation was determined 16 h later.

**Tumor-infiltrating lymphocytes (TILs)**

Tumor cells and TILs were isolated as described previously (23). The degree of lymphocyte infiltration was determined by FACS analysis after staining for CD8α, TCR-αβ, CD19, and CD11b. For further quantitative enumeration of CD8^+ TILs, quantitative PCR was performed on restected tumors using SYBR Green and the following primers: mL32, 5'-GAAACTGCGGAAACCACC-3' (forward) and 5'-GGATCCTGCCCT TGAACCTT-3' (reverse); β-actin, 5'-CTGGAATGCCCCAGGCTTCA-3' (forward) and 5'-CATGCCGCTCCAGTCACAC-3' (reverse); CD8α, 5'-CCATGACCCTGCTGTGCTG-3' (forward) and 5'-GCGGCTCCATTT TCTTTGGA-3' (reverse); and OVA, 5'-GACTGACGCAAAGGCAAACCTGTT-3' (forward) and 5'-TGTTCCACCTGGAATGT GAAG-3' (reverse). The ratio of CD8α mRNA to OVA mRNA was determined after normalization to β-actin and L32 for each sample.

**In vitro DC assays**

Bone marrow (BM)-derived DCs from male mice of the indicated strains were generated with GM-CSF (BD Biosciences) as previously described (24). To evaluate cytokine production, DC activation, and phagocytotic capacity, 10^6 DCs were cultured in 96-well plates with 10^6 irradiated and electroporated EL-4-mOVA cells in the absence or presence of dsRNA. Supernatants were collected at different time points for IL-6, TNF-α, IL-10, and IL-12 analysis by cytometric bead array (BD Pharmingen). The concentration of type I IFN was determined by using a cell line co-cultured with the IFN-stimulated response element luciferase reporter construct discussed elsewhere (25). DC activation was determined by expression of the surface molecules MHC II, CD40, CD80, and CD86 in combination with CD11c and CD11b (all from eBioscience) by flow cytometric analysis. For Ag presentation assays, 5 x 10^5 DCs were incubated with 2 x 10^5 irradiated electroporated EL-4-mOVA cells. Purified OT-1 or HY CD8 T cells (Milenyi Biotec) were labeled with CFSE and added to the cultures after 36 h. Cell proliferation was determined by flow cytometric analysis of CFSE intensity in combination with staining for CD8α, Vα2, and HY TCR, respectively, at the indicated time points (19).

**Statistics**

Unless stated otherwise, the data are expressed as means ± SEM and evaluated using ANOVA followed by a Dunnett test. A probability value of p < 0.05 was considered statistically significant.

**Results**

**Cell-associated dsRNA, but not soluble dsRNA, enhances CD4^+ and CD8^+ T cell responses**

To test the adjuvant effect of synthetic dsRNA provided in the form of poly(I:C) on the adaptive antitumor response, wild-type mice were immunized with syngeneic thymoma cells expressing full-length OVA on their membrane (EL-4-mOVA). The EL-4-mOVA cells were either associated with dsRNA via electroporation or mixed with soluble dsRNA before injection. After 7 days, the frequencies and functions of OVA-specific CD4^+ and CD8^+ T cells were determined.

Immunization with cell-associated Ags generally results in poor CD4^+ T cell responses but strong CD8^+ T cell responses (26). Splenocytes from EL-4-mOVA-immunized mice did not show a proliferative response upon in vitro stimulation with their cognate ligand, whereas splenocytes from mice immunized with dsRNA-associated EL-4-mOVA demonstrated a small but significant response (Fig. 1A). To dissect whether this augmentation resulted from an increased number of Ag-specific CD4^+ T cells or an increased proliferative capacity per cell, ELISPOT assays were performed to enumerate the OVA-specific CD4^+ T cells. EL-4-mOVA immunization induced OVA-specific CD4^+ T cells that predominantly produced IFN-γ. Association of dsRNA with the EL-4-mOVA cells significantly enhanced the number of OVA-specific CD4^+ T cells producing IFN-γ, but not IL-4, indicating that dsRNA enhanced the induction OVA-specific CD4^+ T cells and skewed the response to a Th1 cell phenotype (Fig. 1A). No effect of soluble dsRNA was seen on the induction of the OVA-specific CD4^+ T cells, and the observed responses were comparable to immunization with EL-4-mOVA cells only (data not shown).

Cellular association of dsRNA with EL-4-mOVA cells induced comparable adjuvant effects in the OVA-specific CD8^+ T cell population. Cell-associated dsRNA, but not soluble dsRNA, doubled the CD8^+ T cell response (control, 1.39 x 10^4 ± 0.47 x 10^4; soluble poly(I:C), 1.08 x 10^4 ± 0.36 x 10^4; and cell-associated poly(I:C), 3.28 x 10^4 ± 0.62 x 10^4) (Fig. 1B). Therefore, the result of greater clonal expansion rather than increased expansion kinetics (data not shown). The phenotypic cytokine profile and cytokolytic capacity of the OVA-specific CD8^+ T cells was comparable on a per cell basis within all groups (data not shown). However, cell-associated dsRNA endowed the CD8^+ T cells with a greater capacity for secondary expansion when cultured in vitro with OVA-expressing fibroblasts (Fig. 1, D and E).

**Cell-associated dsRNA potentiates immune responses in CD4-depleted mice**

CD4^+ T cell help during CD8^+ T cell priming plays an important role in clonal expansion, cytokine production, capacity for secondary expansion, and generation of memory CD8^+ T cells (20, 27, 28). To dissect whether the enhanced CD8^+ T cell response was
an intrinsic trait of the CD8+ T cells or resulted from the enhanced CD4+ T cell response, we used mice that were depleted of CD4+ T cells before immunization. Although CD8+ T cells responses were induced in the absence of CD4+ T cells, their clonal expansion was diminished along with their capacity for secondary expansion upon re-encounter with cognate Ag (Fig. 2, A and B) (20, 27, 28). Cell-associated dsRNA, but not soluble dsRNA, enhanced the clonal expansion of CD8+ T cells (control, 5.5 × 10^3 ± 1.1 10^3; soluble poly(I:C), 4.2 10^3 ± 1.7 10^3; and cell-associated poly(I:C), 11.3 10^3 ± 1.8 10^3) SIINFEKL-specific CD8+ T cells/spleen (Fig. 1, C and D). Importantly, these CD8+ T cells regained their capacity for secondary expansion and were able to kill OVA-pulsed target cells in vitro (Fig. 2, B and C). These data indicate that cell-associated dsRNA intrinsically alters both the Ag-specific CD4+ and CD8+ T cell responses, resulting in a more robust anti-tumor response.

dsRNA sensing is essential for the adjuvanticity

Different pathways of sensing dsRNA have been suggested to exert adjuvant effects on the adaptive immune response. 129/TRIF<sup>−/−</sup> mice were immunized s.c. with 2 × 10^7 EL-4-mOVA cells that were electroporated (e.p.) in the presence or absence of synthetic dsRNA (poly(I:C)). As a control, EL-4-mOVA cells were mixed with dsRNA before immunization. After 7 days, splenocytes were isolated and Ag-specific CD4+ and CD8+ T cell functions were determined. A, Proliferation of Ag-specific CD8+ T cells after a 3-day stimulation with the OVA<sub>323-339</sub> epitope in vitro in splenocytes derived from mice immunized with EL-4-mOVA cells in the absence (■) or presence (▲) of dsRNA. B, The frequency of Ag-specific IFN-γ and IL-4 production by splenic CD4+ T cells was determined by ELISPOT upon stimulation with the MHC class II-restricted OVA<sub>323-339</sub> epitope (■) and control peptide LL0190-201 (▲). C, The frequency of IFN-γ Ag-specific CD8+ T cells in spleens 7 days after immunization upon a 5-h stimulation with OVA<sub>257-264</sub>-peptide (filled bars) as determined by intracellular cytokine staining. GP<sub>33-41</sub> peptide stimulation (open bars) was used as control stimulation. D, Flow cytometric analysis of the frequency of IFN-γ<sup>+</sup> OVA<sub>257-264</sub>-specific CD8+ T cells among total splenocytes before and after a 6-day in vitro restimulation with MEC.B7.Sig-OVA cells. The value in the left panel represents the percentage of total splenocytes that are CD8+ IFN-γ<sup>+</sup>. E, Fold expansion of OVA<sub>257-264</sub>-specific CD8+ T cells after in vitro stimulation with the absolute numbers of OVA<sub>257-264</sub>-specific CD8+ T cells at the start of the culture. Values represent mean ± SEM (n = 4) and are representative of three experiments.

The adjuvant effect of dsRNA is mediated through type I IFN

When BM-derived DCs were cultured with dsRNA they showed signs of maturation through up-regulation of MHC class I and class II as well as the costimulatory molecules CD40, CD80, and CD86 (data not shown) (10). In addition, these DCs produced various cytokines, including TNF-α, IL-6, IL-10 IL-12, and type I IFNs (10). In line with our previously published findings, the sensing of apoptotic cells in the form of irradiated, electroporated EL-4-mOVA induced small amounts of type I IFN in BM-derived DCs (12). Importantly, EL-4-mOVA cell-associated dsRNA but not soluble dsRNA, in a concentration similar to the cell-associated amount, significantly increased production of type I IFN by the DC. Comparable results were found for TNF-α, IL-6, and IL-12 (Fig. 4A and data not shown). To dissect which cytokines were elemental in conferring the dsRNA adjuvant effect, BM-DCs from cytokine and cytokine receptor-deficient mice were cultured with EL-4-mOVA cells (either normal or associated with dsRNA), and the proliferation of naïve OVA-specific OT-1 CD8+ T cells was evaluated. DCs from all animals induced comparable CD8+ T cell proliferation in the absence of dsRNA; 50–60% of the OT-1 cells showed signs of division based on their CFSE dilution profile. In all DC subsets except those generated from IFN-αβR<sup>−/−</sup> mice, cross-presentation of dsRNA-associated EL-4-mOVA resulted in enhanced proliferation, evident in both the percentages of dividing cells and the rounds of successful division (Fig. 4B).
Absence of dsRNA-mediated adjuvant effect in IFN-γ deficient mice. Wild-type mice were depleted of CD4+ T cells before immunization with 2 × 10^4 EL-4-mOVA cells that were electroporated (e.p.) in the presence of absence of synthetic dsRNA (poly(I:C)) as described in the Fig. 1 legend. A. The frequency of IFN-γ + Ag-specific CD8+ T cells in spleens 7 days after immunization upon a 5-h stimulation with OVA257–264 peptide (■) as determined by intracellular cytokine staining. GP33–41 peptide stimulation (□) was used as a control. B. Fold expansion of OVA257–264-specific CD8+ cells in vitro stimulation with MEC.B7.Sig-OVA cells. The fold expansion is calculated by dividing the absolute number of OVA257–264-specific CD8+ cells after in vitro stimulation by the absolute numbers of OVA257–264-specific CD8+ at the start of the culture. C. In vitro cytolytic activity of restimulated splenocytes from mice 7 days after immunization. Various E:T ratios were tested for killing of syngeneic target cells pulsed with OVA257–264 (■) or GP33–41 (□). Values represent mean ± SEM (n = 4) and are representative of four experiments.

As observed in vivo, the effect of cell-associated dsRNA on T cell proliferation was greater that that of soluble dsRNA. OT-I cells demonstrated greater proliferation when stimulated by dsRNA-associated EL-4-mOVA-fed DCS than normal EL-4-mOVA-fed DCS (Fig. 4C). The addition of soluble dsRNA resulted in only a small increase in OT-I proliferation, whereas recombinant IFN-α strongly enhanced the proliferative capacity, thus confirming a role for type I IFN in the adjuvant effect of dsRNA. Interestingly, HY CD8+ T cells, which recognize male self-Ag consistently expressed by the DC, showed no difference in proliferation when stimulated with BM-derived DCS that were fed dsRNA-associated EL-4-mOVA or EL-4-mOVA in the presence of soluble dsRNA. These observations suggest that the adjuvant effect of dsRNA sensing during presentation of self-Ags by cells differs from the mechanism observed in cross-presentation.

Absence of dsRNA-mediated adjuvant effect in IFN-αβR−/− mice

To confirm the role of type I IFN in the cell-associated dsRNA adjuvant effect in vivo, CD4-depleted wild-type and IFN-αβR−/− mice were immunized with EL-4-mOVA cells (normal or dsRNA associated). IL-12p40−/− mice, deficient in IL-12 and IL-23, both of which are cytokines with known adjuvant effects on T cell priming (29), were used as an additional control. Comparable to observations in CD4-depleted wild-type mice, cell-associated dsRNA enhanced primary expansion and restored both secondary expansion and the cytolytic capacity of OVA-specific CD8+ T cells in IL-12p40−/− mice, exclusive of IL-12 and IL-23, in the adjuvant effect of cell-associated dsRNA (Fig. 5 and data not shown). Importantly, cell-associated dsRNA did not enhance the OVA-specific CD8+ T cell responses in CD4-depleted IFN-αβR−/− mice, nor did it restore either cytotoxicity or secondary expansion (Fig. 5 and data not shown).

dsRNA-associated tumor vaccines inhibit tumor growth and increase TILs

To test whether dsRNA association enhanced the therapeutic potential of a tumor vaccine, we developed a model in which CD4-depleted wild-type mice were inoculated with live EL-4-mOVA cells and treated with irradiated EL-4-mOVA cells (normal or dsRNA-associated) once palpable tumors had formed. Treatment of mice with irradiated EL-4-mOVA cells (with or without soluble dsRNA) failed to inhibit tumor growth or enhance survival compared with untreated mice, as did dsRNA-associated EL-4 cells that lack the major rejection Ag OVA (Fig. 6, A and B, and data not shown). In contrast, increased survival (>40 days after tumor inoculation) and tumor growth inhibition were observed among mice treated with dsRNA-associated EL-4-mOVA cells. Analysis of dissected tumors 14 days postinoculation revealed increased TIL differences. Tumors from dsRNA-associated EL-4-mOVA-treated mice contained nearly 2-fold greater TILs (absolute numbers) than tumors derived from control, EL-4, and EL-4-mOVA-treated mice. However, in a sample size of n = 8, this increase was not significant (p < 0.56, data not shown). Subsequent FACS analysis showed that the percentage of CD8+ T cells among the TILs was significantly increased in dsRNA-associated EL-4-mOVA-treated mice (41.2 ± 11.8%) in comparison with control, EL-4-mOVA-treated, and dsRNA-associated EL-4 treated mice (20.3 ± 4.6%, 19.8 ± 7.2%, and 21.83 ± 5.2%, respectively). To obtain a more quantitative assessment of the CD8+ T cell population infiltrating the tumor, quantitative RT-PCR was performed and the ratio of CD8α mRNA to tumor Ag (OVA) mRNA was determined. No significant difference in CD8α:OVA mRNA ratio was observed between control, EL-4-mOVA, and dsRNA-associated EL-4 treated mice. Corresponding with the flow cytometric data, dsRNA-associated EL-4-mOVA treatment significantly increased the CD8α:OVA mRNA ratio, demonstrating increased CD8+ expressing cells infiltrating into the tumor.

These data show that cell-associated dsRNA can be used to induce a protective CD8+ T cell response to tumor Ags, even in immunocompromised mice.
FIGURE 4. The adjuvant effect of cell-associated dsRNA is mediated by type I IFN. A. Wild-type (WT) BM-derived DCs were cultured with irradiated EL-4-mOVA cells-associated to synthetic dsRNA (pIC), EL-4-mOVA cells with soluble (sol.) dsRNA, EL-4-mOVA cells, or dsRNA alone in a concentration representative of the amount of cell-associated dsRNA. After 24 h, type I IFN concentrations were determined using a cell line containing an IFN-stimulated response element-luciferase reporter construct (nd, not detectable). B. BM-derived DCs from indicated strains were cultured for 24 h with EL-4-mOVA cells electroporated (e.p.) in the presence or absence of synthetic dsRNA (poly(I:C)). Purified OVA-specific OT-1 CD8+ T cells labeled with CFSE were added for 64 h, and proliferation was determined by the CFSE dilution profile of the CD8α+ Vε2- cells. C. Timing of dsRNA availability affects DC presentation of exogenous proteins but not self-proteins. BM-derived DCs from wild-type male mice were cultured with EL-4-mOVA cells electroporated in the presence or absence of synthetic dsRNA (poly(I:C)) under the indicated conditions. After 30 h, purified OT-1 or HY-specific CD8+ T cells labeled with CFSE were added. Proliferation was determined after a 64-h culture by the CFSE dilution profile of the CD8α+ TCR-αβ+ cells. Representative histograms are shown of triplicate cultures and are representative of two experiments.

Discussion
In this study we have shown that tumor cell vaccination with cell-associated dsRNA, but not soluble dsRNA, enhances both tumor-specific CD8+ and CD4+ T cell responses and inhibits tumor growth in a therapeutic fashion when administered after tumor inoculation. The cell-associated dsRNA not only increased the clonal burst of tumor-specific CD8+ T cells but also endowed them with an enhanced capacity for expansion upon secondary encounter with tumor Ags, even when the CD8+ T cells were primed in the absence of CD4+ T cell help. The observed adjuvant effect of cell-associated dsRNA was fully dependent on the expression of TLR3 by the APCs and their subsequent production of type I IFNs.

Because dsRNA is produced by all viruses at some point during their replication phases, the immune system has developed many pathways to sense dsRNA. Interaction of dsRNA with TLR3, PKR, RNaseL, or RIG-I has been shown to induce type I IFNs that affect viral replication and function as adjuvants in the priming of virus-specific adaptive immune responses (30–33). In APCs such as DCs, PKR, RIG-I, and RNaseL are expressed in the cytoplasm and likely recognize dsRNA produced in the context of viral replication (31, 32). In contrast, TLR3 localizes to an intracellular vesicular compartment that has been suggested to be endosomal, because inhibiting the acidification of endosomes abrogates poly(I:C) signaling (34). TLR3 is believed to encounter dsRNA in these vesicles through the phagocytosis of apoptotic infected cells. Only nonplasmacytoid DCs of the myeloid lineage express TLR3; in the mouse, CD8α+ DCs that facilitate cross-priming constitutively express TLR3, and the expression level is up-regulated upon DC activation and maturation. These observations coincide with our finding that the adjuvant effect of cell-associated dsRNA is completely abolished in 29/TRIF−/− mice deficient in TLR3 signaling but normal for PKR, RIG-I, and RNaseL signaling.

Although type I IFNs were initially characterized as potent antiviral factors, they have also been shown to directly suppress tumor cell replication, induce tumor cell apoptosis, and reduce tumor growth via their anti-angiogenic properties (15, 35–37). In addition, type I IFNs possess antitumor properties through activation of the innate immune system by stimulating NK cell-mediated tumor lysis and enhancing the tumoricidal properties of macrophages (38, 39). Indeed, many studies showing a therapeutic antitumor effect of soluble dsRNA-induced type I IFNs failed to enhance tumor-specific adaptive responses, indicating that the effect resulted instead from either a direct impact on the tumor or activation of the innate immune response (15, 37). Our data show that only cell-associated, not soluble, dsRNA enhances tumor-specific T cell induction that results in an antitumor effect in vivo. This observation indicates that the production of type I IFN was insufficient alone to generate protective innate and adaptive antitumor responses, even when the tumor vaccine was administered simultaneously with soluble dsRNA. However, using IFN-αβR−/− DC and IFN-αβR−/− mice, we demonstrate that the adjuvant effect of cell-associated dsRNA is mediated through the production of type I IFN by the cross-priming DCs upon sensing dsRNA. Although the direct effect of type I IFNs on T cells is controversial (40, 41), in our studies the type I IFNs affected DC function in an autocrine fashion, because adding either dsRNA or recombinant type I IFN to IFN-αβR−/− DCs did not specifically affect CD8+ T cell proliferation. Various studies have shown that type I IFNs induce up-regulation of MHC-peptide complexes, costimulatory molecules (including CD40 and CD80/86), and the production of cytokines and chemokines by DCs. Notably, type I IFNs can induce full activation of DCs, transforming a DC with potent phagocytic capacity into a mature DC with poor phagocytic but potent T cell activation capacities (42, 43). This finding demonstrates why the correct timing of dsRNA sensing...
and type I IFN production is crucial in the adjuvant effect mediated by dsRNA. Our studies show that activation of OVA-specific CD8\(^+\) T cells in vitro is enhanced when dsRNA or recombinant type I IFN is introduced after the DC has taken up the OVA-expressing tumor cells but not when soluble dsRNA is administered before the tumor cells. Additional studies showed that DCs previously exposed to dsRNA have reduced phagocytosis of the tumor vaccine in vitro (data not shown). Importantly, HY-specific CD8\(^+\) T cells that recognized self-protein on DCs exhibited enhanced proliferation regardless of when the dsRNA was added to the culture. Because epitopes of self-protein are constitutively presented by the MHC in the absence of cross-priming, the dsRNA enhanced the T cell response by increasing the expression of costimulatory molecules by the DC. Our observations also explain why soluble dsRNA treatment together with peptide-pulsed DCs enhanced Ag-presenting capacity of the DCs, which resulted from up-regulation of MHC class I-peptide complexes and the costimulatory molecules CD80 and CD86 by the type I IFNs. In addition, cytokines and chemokines induced by the type I IFNs may have contributed to a more favorable environment for T cell recruitment and retention of the cells in the lymph nodes and their subsequent activation.

Although it is not clear what signals are required to induce memory CD8\(^+\) T cells in the absence of Th cells, the induction of CD8\(^+\) T memory in our studies is most likely mediated by the enhanced Ag-presenting capacity of the DCs, which resulted from up-regulation of MHC class I-peptide complexes and the costimulatory molecules CD80 and CD86 by the type I IFNs. In addition, cytokines and chemokines induced by the type I IFNs may have contributed to a more favorable environment for T cell recruitment and retention of the cells in the lymph nodes and their subsequent activation.

In light of the fact that many tumor-bearing patients have compromised immune systems as a direct result of treatment regimens including chemotherapeutic agents and/or radiotherapy, incorporation of dsRNA in tumor vaccines represents a feasible approach to amplifying antitumor CD8\(^+\) T cell responses. The data from our studies collectively suggest that using dsRNA as a tumor vaccine adjuvant may be a suitable strategy for enhancing vaccine efficacy by combining the advantages of autologous
tumor cells with a stimulus for an innate receptor expressed by all patients. Such an approach would deliver the dsRNA to DCs in an optimal manner because, after phagocytosis of the tumor vaccine, the dsRNA becomes available in endosomes that express the cognate receptor. Importantly, we have shown in a preclinical therapeutic protocol that this approach is able to enhance the survival of tumor-bearing immunocompromised mice. We believe that the results described herein provide a strong foundation for the future development of more clinically effective strategies for tumor cell therapy in cancer patients.

Acknowledgments

We thank K. R. Prilliman and S. P. Schoenberger for advice and critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References