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Article Oncology

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Critical role of EBNA1-specific CD4+ T cells in the control of mouse Burkitt lymphoma in vivo

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CD4+ T cells play important roles in orchestrating host immune responses against cancer and infectious diseases. Although EBV-encoded nuclear antigen 1–specific (EBNA1-specific) CD4+ T cells have been implicated in controlling the growth of EBV-associated tumors such as Burkitt lymphoma (BL) in vitro, direct evidence for their in vivo function remains elusive due to the lack of an appropriate experimental BL model. Here, we describe the development of a mouse EBNA1-expressing BL tumor model and the identification of 2 novel MHC H-2I-A^b–restricted T cell epitopes derived from EBNA1. Using our murine BL tumor model and the relevant peptides, we show that vaccination of mice with EBNA1 peptide–loaded DCs can elicit CD4+ T cell responses. These EBNA1-specific CD4+ T cells recognized peptide-pulsed targets as well as EBNA1-expressing tumor cells and were necessary and sufficient for suppressing tumor growth in vivo. By contrast, EBNA1 peptide–reactive CD8+ T cells failed to recognize tumor cells and did not contribute to protective immunity. These studies represent what we believe to be the first demonstration that EBNA1-specific CD4+ T cells can suppress tumor growth in vivo, which suggests that CD4+ T cells play an important role in generating protective immunity against EBV-associated cancer.

Introduction

EBV is a human gammaherpesvirus with tropism for B cells and has been associated with several types of malignant tumors, including Burkitt lymphoma (BL), post-transplant lymphoproliferative disorder (PTLD), nasopharyngeal carcinoma (NPC), and Hodgkin disease (HD) (1–3). Although a subset of genes is responsible for the growth-transforming function of EBV, *EBV-encoded nuclear antigen 1* (*EBNA1*) is the only viral gene that is regularly detected in all EBV-associated tumors (BL, NPC, PTLD, and HD) and is required for the long-term persistence of EBV as well as the pathogenesis of EBV-associated cancers (3–5). Increasing evidence indicates that T cell responses to EBNA1 are important in controlling EBV infection (3, 6, 7), which suggests that EBNA1 is an important target for immunotherapy of EBV-associated malignancies.

However, the presence of the glycine and alanine repeat (GAr) domain within EBNA1 not only blocks its proteasomal degradation for the MHC class I antigen processing pathway, but also inhibits its own mRNA translation (8–11). Although we have recently identified a naturally processed HLA-B8–restricted epitope from EBNA1 (12), the overall capacity of MHC class I antigen processing and presentation in BL cells is significantly impaired, making EBNA1-positive tumor cells invisible to the host CD8+ T cells. By contrast, the EBNA1 protein can be normally processed and presented through the MHC class II processing pathway and elicits consistent CD4+ T cell immune response (7, 13–16). As a result, several MHC class II–restricted EBNA1 peptides have been identified (13, 17–19). These observations imply that EBNA1-specific CD4+ T cells may play a role in controlling tumor growth in vivo. However, due to the lack of a reliable animal model for EBV-associated tumors, the role of EBNA1-specific CD4+ T cells in antitumor immunity in vivo remains to be defined.

In this article, we describe the establishment of a murine BL model and the identification of EBNA1-derived T cell peptides for recognition by CD4+ T cells. We show that immunization of mice with EBNA1-derived T helper peptides can elicit potent CD4+ T cell responses and inhibit tumor growth following subsequent tumor challenge. More importantly, EBNA1-specific CD4+ T cells, but not CD8+ T cells, contributed to the observed antitumor immunity. These results suggest that EBNA1-specific CD4+ T cell response elicited by DC loaded with EBNA1 peptide (DC/EBNA1 peptide) vaccination plays an important role in inhibiting in vivo growth of EBNA1-expressing B6-BL tumor cells.

Results

Establishment and characterization of BL cell lines. The B6-BL murine cell line, initially generated from a human *Igλ-MYC*–transgenic mouse, shares many characteristics with human BL (20). The B6-BL cell line expressing EBNA1 (B6-BL/EBNA1) was generated from *Igλ-MYC* × EBNA1 double-transgenic mice, but the EBNA1 expression level could not be detectable by Western blot analysis with an EBNA1-specific antibody (data not shown). To make certain that EBNA1 was properly expressed in the murine BL cells, we successfully transduced B6-BL cells with a retroviral vector encoding EBNA1-GFP and designated the resultant cell line B6-BL/EBNA1-GFP. Expression of *EBNA1-GFP* fusion gene allowed us to monitor EBNA1 expression in the cells. B6-BL cell line expressing GFP (B6-BL/GFP) served as a control. EBNA1 expression in the B6-BL/EBNA1-GFP tumor cells was confirmed by Western blot analysis (Figure 1A). Further characterization of the B6-BL/EBNA1-GFP and B6-BL/GFP cell lines by FACS analysis with a panel of anti-

Nonstandard abbreviations used: Burkitt lymphoma (BL); 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT); EBV-encoded nuclear antigen 1 (EBNA1); GAr-deleted-EBNA1 (GAr-del-EBNA1); glycine and alanine repeat (GAr); Hodgkin disease (HD); nasopharyngeal carcinoma (NPC); post-transplant lymphoproliferative disorder (PTLD).

Conflict of interest: The authors have declared that no conflict of interest exists.

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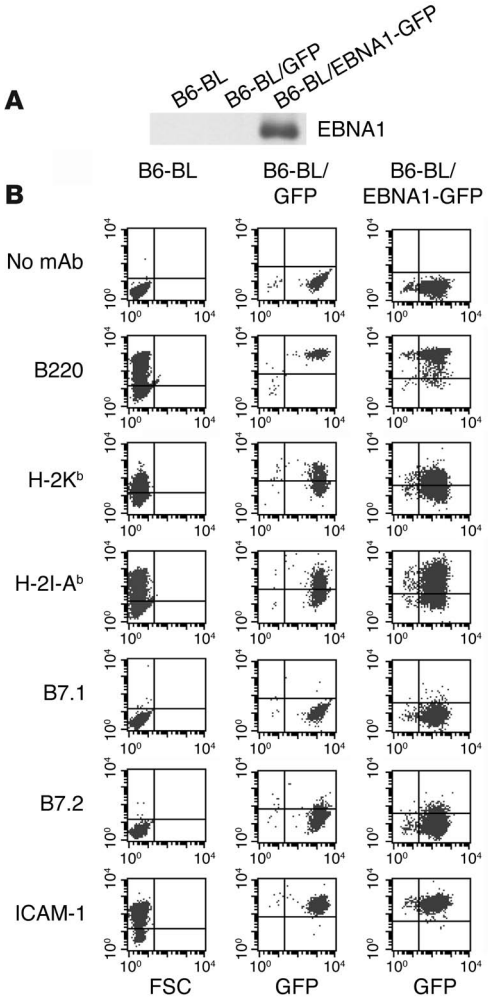

Figure 1
Generation and characterization of an EBNA1 expressing BL cell line. (**A**) BL cell lines were transduced to express the full-length *EBNA1-GFP* fusion gene. Expression of GFP served as a control. The expression of full-length EBNA1 protein in the B6-BL/EBNA1-GFP cells was determined by Western blot analysis using anti-EBNA1 mAb (1H4). (**B**) Expression patterns of cell-surface molecules and GFP on these tumor cell lines were analyzed by FACS, combined with a panel of mAb's, which are labeled on the left. FSC, forward scatter.

bodies revealed uniform expression of B220 B cell marker and of H-2K^b, I-A^b, and ICAM-1 molecules but little or no expression of CD80 (B7.1) or CD86 (B7.2) (Figure 1B). Thus, the B6-BL/EBNA1-GFP line was considered to closely resemble human EBNA1-positive BL cells, although some human BL cells do not express MHC class I and ICAM-1 molecules.

Immunogenicity of B6-BL/EBNA1-GFP cells. To test whether the expression of EBNA1-GFP or GFP in B6-BL cells might affect tumor immunogenicity as determined by growth properties, we examined the proliferation of BL cell lines both in vitro and in vivo. As shown in Figure 2A, the B6-BL, B6-BL/GFP, and B6-BL/EBNA1-GFP cells exhibited similar or identical growth activities in vitro by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The immunogenicity of B6-BL/EBNA1-GFP and B6-BL/GFP was assessed in vivo by subcutaneously injecting tumor cells into syngeneic B6 mice in different doses (from 2.5×10^5 to 1×10^6 tumor cells). All injections resulted in tumor growth, which became detectable 6–12 days after inoculation, depending on the number of tumor cells injected (data not shown). In a subsequent experiment, we subcutaneously injected mice with 5×10^5 tumor cells and measured tumor growth every 2 days. All 3 tumor cell lines had similar growth properties in vivo (Figure 2B), which suggests that neither EBNA1 nor GFP expression in B6-BL cells affected tumor cell immunogenicity.

Identification of EBNA1-specific T cell epitopes. Having established a BL mouse model with characteristics similar to human BL, we sought to identify EBNA1-derived T cell epitopes presented by murine MHC class II molecules. We first evaluated whether EBNA1 could stimulate T cell responses in B6 mice immunized with full-length or truncated forms of EBNA1 (GAr-deleted EBNA1, or GAr-del-EBNA1). T cells from splenocytes of the immunized mice were stimulated in vitro with 10 EBNA1 peptides, as previously described (19). After 6 days of stimulation, T cells from the draining lymph nodes of B6 mice vaccinated with the full-length EBNA1 protein showed strong reactivity against the EBNA1-P$_{607\text{-}619}$ peptide as compared with results for the 9 remaining peptide candidates (Figure 3A, upper panel). Similar results were obtained with T cells derived from B6 mice immunized with GAr-del-EBNA1 protein (Figure

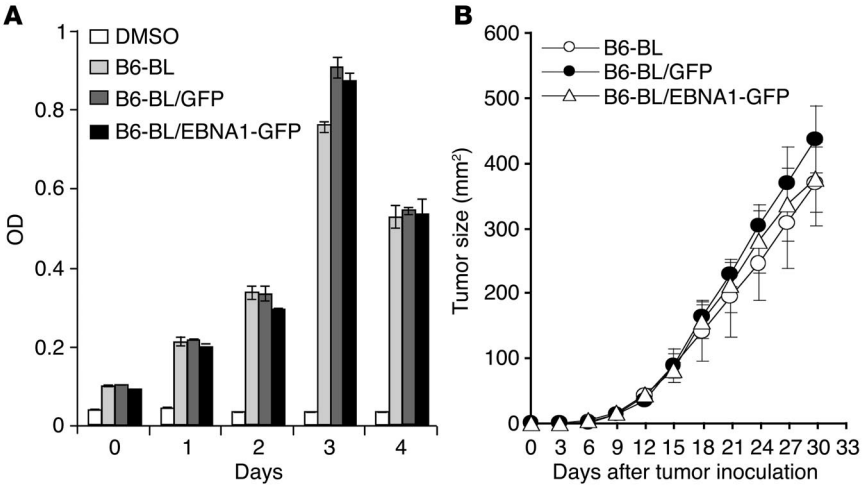

Figure 2
Immunogenicity of BL cells. (**A**) Comparison of in vitro growth of BL cell lines expressing GFP or EBNA1-GFP using MTT assay. Data represent mean ± SEM of triplicate cultures. There were no significant differences in tumor growth among the cell lines. (**B**) The growth of tumor cell lines in vivo. Mice were subcutaneously injected with 5×10^5 of B6-BL, B6-BL/GFP, or B6-BL/EBNA1-GFP tumor cells at day 0. Tumor size was recorded in mm^2 every 3 days. The results, reported as means ± SEM for 5 mice, indicate that neither EBNA1 nor GFP affected the immunogenicity of B6-BL tumor cells.

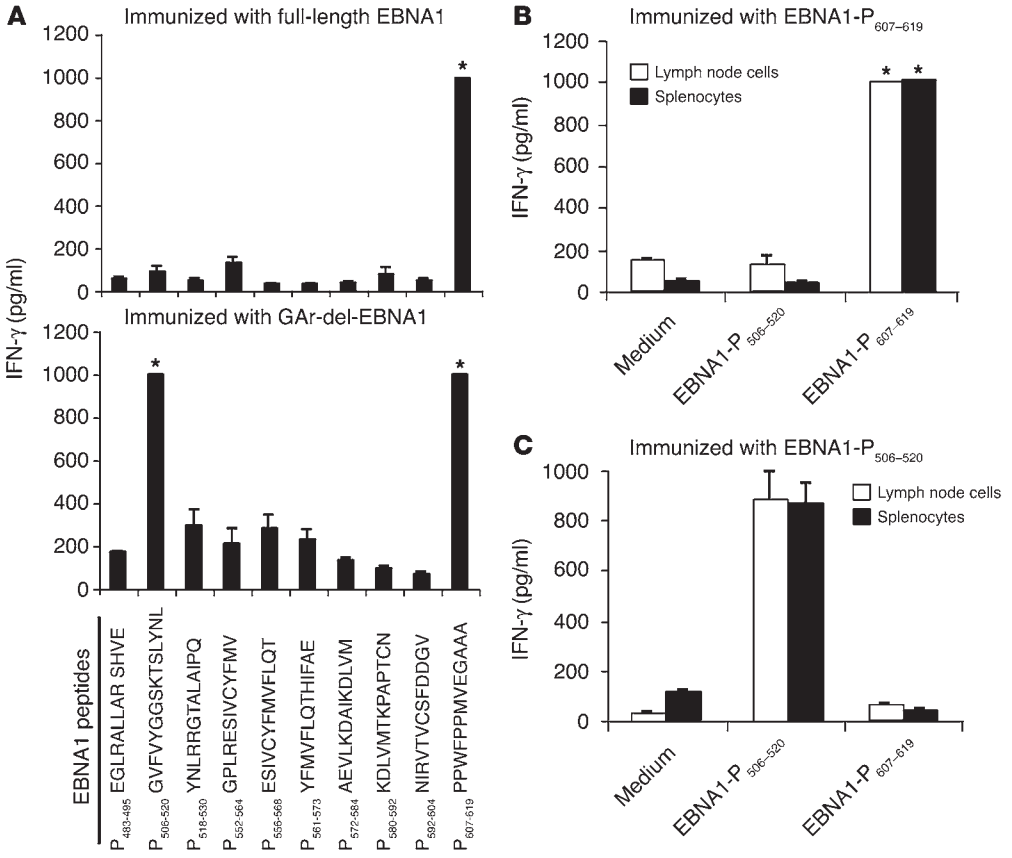

Figure 3

Identification of new EBNA1-specific T cell epitopes presented by murine I-A^b molecules. (**A**) EBNA1 peptides recognized by T cells from the immunized mice. B6 mice were immunized with 50 μg of full-length EBNA1 protein per mouse (upper panel) or GAr-del-EBNA1 (lower panel) in CFA. Eleven days later, T cells from draining lymph nodes of the mice were prepared, and 5×10^5 cells were stimulated in vitro in the presence of 10 μM of various synthetic peptides derived from EBNA1. After overnight culturing, the supernatants were tested for IFN-γ release by ELISA. (**B** and **C**) Generation of EBNA1-specific T cells after vaccination of mice with the newly identified T cell peptides. Both splenocytes (black bars) and lymph node cells (white bars) from mice immunized with 100 μg/mouse of EBNA1-$P_{607–619}$ (**B**) or EBNA1-$P_{506–520}$ (**C**) were stimulated in vitro with the corresponding as well as control peptides, and IFN-γ secretion was determined. Asterisks indicate that the readings at OD 450 nM for IFN-γ release were higher than those at the highest concentration (1,000 pg/ml) of the IFN-γ standards.

3A, lower panel). Besides the EBNA1-$P_{607-619}$ peptide, T cells from B6 mice immunized with the GAr-del-EBNA1 protein recognized a new EBNA1-$P_{506–520}$ peptide (Figure 3A, lower panel). To further test the immunogenicity and specificity of the peptides, we immunized mice with either EBNA1-$P_{607–619}$ or EBNA1-$P_{506–520}$ peptide. T cells from B6 mice immunized with EBNA1-$P_{607–619}$ recognized the same EBNA1-$P_{607–619}$ peptide, but not the EBNA1-$P_{506–520}$ peptide (Figure 3B). Conversely, T cells from B6 mice immunized with EBNA1-$P_{506–520}$ recognized the EBNA1-$P_{506–520}$ peptide, but not the EBNA1-$P_{607–619}$ peptide (Figure 3C). Taken together, these results suggest that while both EBNA1-$P_{506–520}$ and EBNA1-$P_{607–619}$ are capable of stimulating EBNA1-specific T cell responses, only the EBNA1-$P_{607–619}$ peptide is naturally processed and presented to T cells. Hence, all further studies to elucidate the role of EBNA1-specific T cells in the induction of antitumor immunity were conducted with this antigenic EBNA1-$P_{607–619}$ peptide.

Induction of both $CD4^+$ and $CD8^+$ T cell responses by DC/EBNA1-$P_{607–619}$ peptide vaccination. Since DCs are the most effective APCs for the induction of T cell–mediated immune responses (21), we next asked whether T cell responses could be elicited by DC/peptide vaccination. DCs generated from the bone marrow cells of B6 mice and pulsed with the EBNA1-$P_{607–619}$ peptide were used to immunize syngeneic B6 mice. Spleen cells isolated from these mice were stimulated in vitro with the EBNA1-$P_{607–619}$ peptide for 6 days and tested against 293I-A^b cells pulsed with EBNA1-$P_{607–619}$ peptide or the control EBNA1-$P_{572–584}$ peptide. IFN-γ release from T cells was not observed when T cells were stimulated with 293I-A^b cells alone or after pulsing with a control EBNA1-$P_{572–584}$ peptide. By contrast, significant amounts of IFN-γ were detected in the supernatants of T cells stimulated with 293I-A^b cells pulsed with the EBNA1-$P_{607–619}$ peptide (Figure 4A), which suggests that these $CD4^+$ T cells are capable of recognizing the EBNA1-$P_{607–619}$ peptide. To test whether EBNA1-$P_{607–619}$–specific $CD8^+$ T cells were elicited, we used EL-4, a murine T lymphoma cell line that expresses MHC class I (K^b) but not class II molecules. Little or no IFN-γ release from T cells was detectable after coculturing of T cells with EL-4 alone or EL-4 pulsed with EBNA1-$P_{572–584}$ control peptide. However, T cells from the immunized mice could respond to EL-4 cells pulsed with the EBNA1-$P_{607–619}$ peptide (Figure 4B). To further confirm these results, we performed intracellular cytokine staining of T cells after stimulation with EBNA1 peptides. As shown in Figure 4C, $CD4^+$ T cells from the EBNA1-$P_{607–619}$ peptide–immunized mice could produce IFN-γ upon stimulation with the same peptide. The percentage of T cells double positive for CD4 and IFN-γ was 0.94, compared with 0.02% after stimulation with the control EBNA1-$P_{572–584}$ peptide. Similarly, EBNA1-$P_{607–619}$ stimulation resulted in 1.79% of the T cells becoming double positive for CD8 and IFN-γ, compared with 0.03% following stimulation with the control EBNA1-$P_{572–584}$ peptide. These results suggest that EBNA1-$P_{607–619}$ peptide vaccination activates both $CD4^+$ and $CD8^+$ T cells.

Endogenous processing and presentation of EBNA1-$P_{607–619}$ peptide. Although T cell responses against peptides could be induced from human PBMCs or mice, in many cases the T cells fail to recognize antigen-expressing targets or tumor cells due to either the low affin-

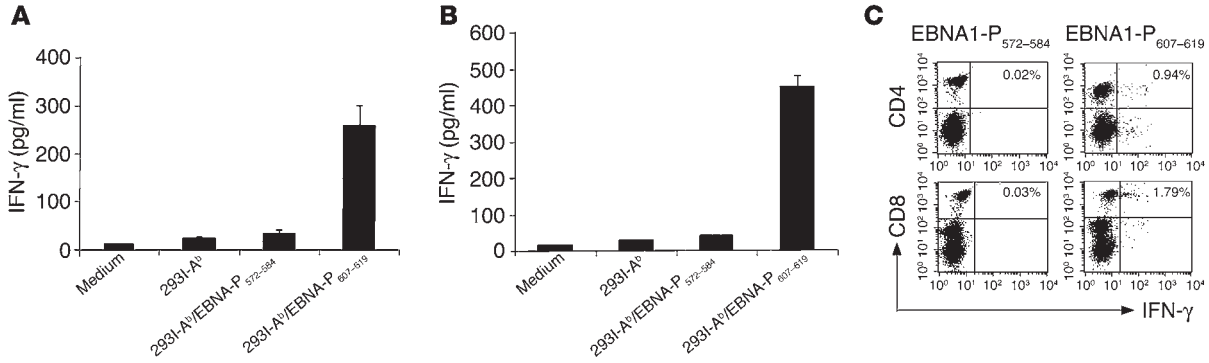

Figure 4
Characterization of EBNA1-$P_{607–619}$ peptide–specific CD4$^+$ and CD8$^+$ T cells. (**A**) Recognition of EBNA1-$P_{607–619}$ peptide by CD4$^+$ T cells. Mice were immunized with EBNA1$P_{607–619}$ peptide loaded onto bone marrow cell–derived DCs. After 2 weeks, splenocytes were prepared and stimulated in vitro with the peptide for 6 days, then tested against the same peptide-pulsed 293I-A^b cells for T cell recognition. EBNA1-$P_{572–584}$ peptide was used as a control. Data are means ± SEM of triplicate cultures. (**B**) Recognition of peptide-pulsed EL-4 target cells by CD8$^+$ T cells. (**C**) Intracellular staining of EBNA1 peptide–specific T cell responses. For intracellular IFN-γ staining, splenocytes were stimulated in vitro with EBNA1$P_{607–619}$ or EBNA1-$P_{572–584}$ (control) peptide overnight and stained with anti-CD4 and anti-CD8 mAb, respectively, followed by intracellular IFN-γ staining. The double-positive T cells were identified by FACS analysis. The percentage of double-positive cells is given in the upper right of each panel.

ity of the T cells or to the inability of tumor/target cells to present naturally processed peptides on their surface. Thus, we asked whether EBNA1-$P_{607–619}$-specific T cells could recognize target cells expressing EBNA1. 293I-A^b cells and a murine prostate tumor cell line expressing H-2Kb but not I-A^b (RM1) were transfected with plasmid DNAs carrying the full-length or GAr-del-*EBNA1* and used to stimulate T cells from EBNA1-$P_{607–619}$–immunized mice. As shown in Figure 5, little or no T cell activity was detected after stimulation with target cells transfected with the empty vector. However, T cells strongly recognized 293I-A^b cells transfected with either GAr-del-EBNA1 or full-length EBNA1, whereas only weak or negligible T cell activity was observed against RM1 cells transfected with the same constructs, which suggests that the EBNA1-specific MHC class II–restricted EBNA1-$P_{607–619}$ peptide is naturally processed and presented to T cells, while the MHC class I–restricted peptides are not naturally processed.

Inhibition of tumor growth by EBNA1-$P_{607–619}$ immunization. We next tested whether immunization of mice with DCs pulsed with EBNA1-$P_{607–619}$ could inhibit tumor growth upon tumor challenge. B6 mice were immunized by a single i.v. injection of 3×10^5 syngeneic DCs loaded with the EBNA1-$P_{607–619}$ peptide or a control EBNA1-$P_{572–584}$ peptide. Two weeks later, they were challenged by subcutaneous injection of B6-BL/EBNA1-GFP cells or control cell lines B6-BL and B6-BL/GFP. Immunization of mice with DC/EBNA1-$P_{607–619}$ peptide resulted in significant inhibition of B6-BL/EBNA1-GFP tumor growth but did not affect the growth of B6-BL or B6-BL/GFP tumor cells, which suggests that antitumor immunity is specific for EBNA1-expressing tumor cells (Figure 6A). Furthermore, immunization of mice with DC/EBNA1-$P_{572–584}$ control peptide failed to inhibit the growth of B6-BL/EBNA1-GFP tumor cells. Similar results were obtained in subsequent experiments that included additional controls for the specificity of antitumor immunity (Figure 6B). These findings indicate that DC/ EBNA1-$P_{607–619}$ immunization elicited antigen-specific immunity, leading to significant inhibition of the growth of B6-BL/EBNA-GFP tumor cells.

Tumor reactivity of EBNA1-$P_{607–619}$–specific CD4$^+$ T cells. To determine whether the EBNA1-$P_{607–619}$–elicited T cells were responsible for the observed inhibition of tumor growth in vivo, we first tested whether T cells elicited from DC/EBNA1-$P_{607–619}$–immunized mice were capable of recognizing B6-BL/EBNA1-GFP tumor cells. T cells were generated from the immunized mice and then tested against B6-BL, B6-BL/GFP, B6-BL/EBNA1-GFP, and tumor cells expressing cancer-testis antigen NY-ESO-1–fused GFP (RM1/NY-ESO-GFP tumor cells). T cells strongly recognized B6-BL/EBNA1-GFP tumor cells but did not respond to B6-BL, B6-BL/GFP, or RM-1/NY-ESO-1-GFP, as determined by IFN-γ release in ELISA and ELISPOT assay (Figure 7, A and B), which suggests that T cells were specific for tumor cells expressing EBNA1 but not GFP. We next determined the relative contribution of CD4$^+$ and CD8$^+$ T

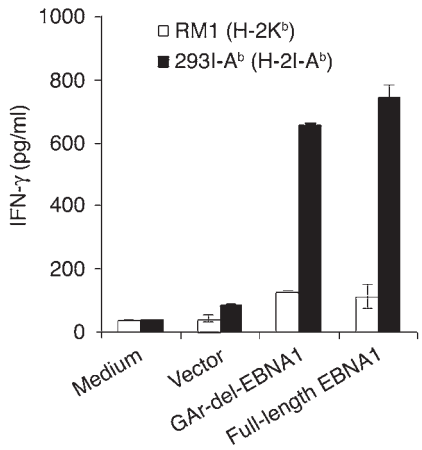

Figure 5
Endogenous presentation of EBNA1-$P_{607–619}$ epitope for T cell recognition. T cells from the immunized mice were tested for their ability to recognize 293I-A^b (black bars) and RM1 (white bars) cells transfected with vectors encoding full-length EBNA1, GAr-del-EBNA1 cDNA, or an empty vector. T cells recognized I-A^b–positive 293 cells transfected with vectors encoding full-length EBNA1 or GAr-del-EBNA1 cDNA, but not with empty vector; they did not recognize I-A^b–negative RM1 cells transfected with the full-length or GAr-del-EBNA1 cDNAs.

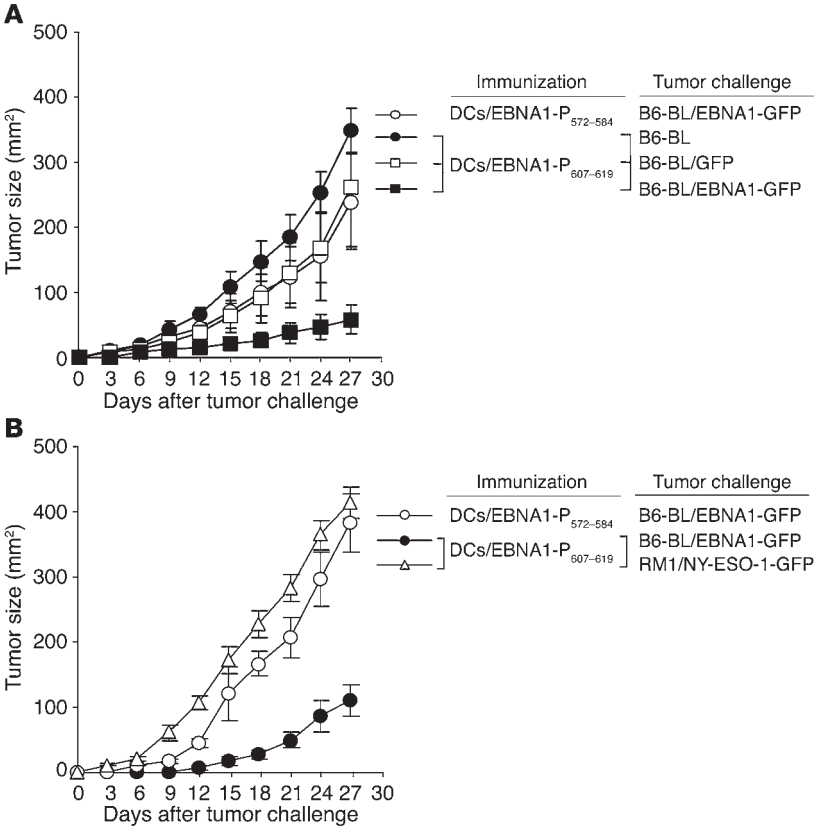

Figure 6
Inhibition of BL tumor growth by EBNA1-specific T cells in vivo. (**A**) Inhibition of tumor growth in DC/EBNA1-vaccinated mice. Two weeks after immunization with DCs pulsed with EBNA1-$P_{607–619}$ peptide or a control EBNA1-$P_{572–584}$ peptide, mice were challenged with 5×10^5 B6-BL/EBNA1-GFP cells or control tumor cell lines B6-BL, B6-BL/GFP. Tumor growth was measured every 2 days. Growth of B6-BL/EBNA1-GFP tumor cells was significantly inhibited in the mice immunized with DC/EBNA1-$P_{607–619}$ peptide compared with other control groups ($P = 0.0174$). (**B**) Specific suppression of B6-BL/EBNA1-GFP tumor cells. To further demonstrate specific inhibition of EBNA1-expressing tumor cells, we immunized mice with DCs loaded with EBNA1-$P_{607–619}$ peptide or a control peptide and challenged them with B6-BL/EBNA1-GFP or RM1/NY-ESO-1-GFP cells. Tumor size was measured every 2 days. Data are means ± SEM ($P = 0.0295$).

cell responses to B6-BL/EBNA1-GFP cells through T cell assay in the presence of anti-CD4 or anti-CD8 mAb. As shown in Figure 7C, IFN-γ release by T cells was not affected by the addition of anti-CD8 mAb's but was markedly reduced when anti-CD4 mAb's were added. These results suggest that EBNA1-specific $CD4^+$ T cells contributed to the inhibition of B6-BL/EBNA1-GFP tumor growth observed in vivo.

$CD4^+$ T cells are responsible for the inhibition of BL growth in vivo. To gain direct evidence for the role of $CD4^+$ T cells in antitumor immunity, we immunized CD4 KO, CD8 KO, and wild-type mice (B6) with DC/EBNA1-$P_{607–619}$. Two weeks later, these mice were challenged with 5×10^5 viable B6-BL/EBNA1-GFP tumor cells. As shown in Figure 8A, tumors grew rapidly in mice immunized with DC/EBNA1-$P_{572–584}$ control peptide and challenged with B6-BL/EBNA1-GFP cells. However, in both wild-type and CD8 KO mice immunized with DC/EBNA1-$P_{607–619}$, B6-BL/EBNA1-GFP tumor growth was significantly inhibited. By contrast, B6-BL/EBNA1-GFP tumor growth was not affected in CD4 KO mice. In fact, tumor growth in these mice was even faster than in wild-type mice immunized with a control EBNA1-$P_{572–584}$ peptide. Similar results were obtained in several independent experiments (data not shown).

To obtain further evidence for the role of EBNA1-specific $CD4^+$ T cells in the control of BL development, MHC class I (deficient in $CD8^+$ T cells) and class II (deficient in $CD4^+$ T cells) KO mice were immunized with DC/EBNA1-$P_{607–619}$ peptide and challenged with B6-BL/EBNA1-GFP. As shown in Figure 8B, tumor growth was remarkably inhibited in $CD4^+$ T cell–intact class I KO mice but not in $CD4^+$ T cell–deficient class II mice. We also performed T cell depletion experiments by intraperitoneal injection of the immunized mice with anti-CD4 and anti-CD8 mAb 1 day before tumor challenge and on days 1, 3, and 7 after challenge. Mice depleted of $CD8^+$ T cells retained the ability to control tumor growth, while those depleted of $CD4^+$ T cells failed to inhibit tumor growth (Figure 8C). Taken together, our results strongly suggest that EBNA1-$P_{607–619}$–specific $CD4^+$ T cells, but not $CD8^+$ T cells, are responsible for the observed antitumor immunity in vivo.

Discussion

Transgenic mice generated from a human *Igλ-MYC* fusion construct developed transgenic lymphoma with a pathology similar to BL (20). Because all BL tumors carry a reciprocal chromosomal translocation between immunoglobulin loci and *MYC* gene, reconstitution of B6-BL tumor cells (containing a human *Igλ-MYC* transgene) with EBNA1 mimics human EBV-associated BL. Human EBV-positive BL cells express EBNA1 but small or undetectable levels of other viral antigens. Thus, the new mouse EBNA1-expressing B6-BL tumor model established in this study reiterates many characteristics of human EBV-positive BL. Coexpression of *MYC* and EBNA1 in double-transgenic mice has been reported to promote lymphomagenesis (22). Since EBV does not infect murine B cells, it is difficult to generate such a model that completely recapitulates human EBV-positive BL at the present time. The purpose of this study was to establish an EBNA1-expressing B6-BL tumor model that would allow us to define the role of EBNA1-specific $CD4^+$ T cells in T cell–mediated antitumor immunity in vivo. Interestingly, EBNA1 expression in B6-BL tumor cells did not change the immunogenicity of B6-BL/EBNA1 cells compared with the growth property of the parental B6-BL cell line. This may be explained by the fact that the GAr domain in EBNA1 not only inhibits the translation of its own mRNA, but also blocks the degradation of

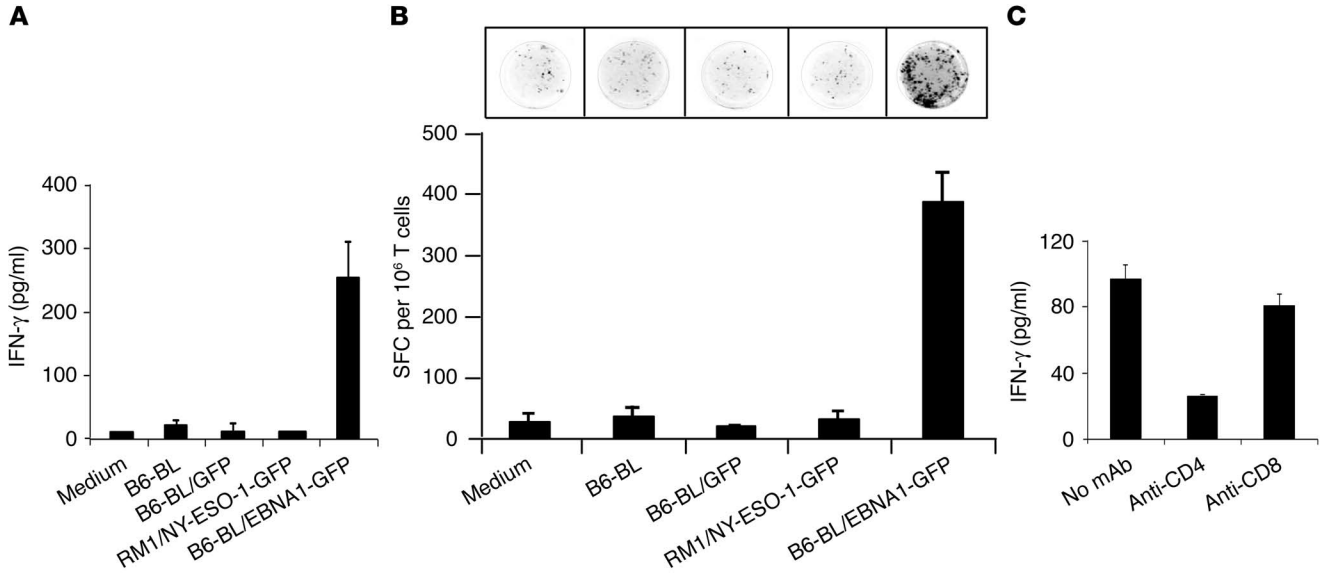

Figure 7

Correlation of T cell activity with the inhibition of BL. (**A**) T cell recognition of B6-BL/EBNA1-GFP cells. T cells from EBNA1-$P_{607–619}$ peptide–immunized mice were tested against a panel of tumor cell lines for tumor reactivity. IFN-γ release was determined by ELISA. (**B**) Tumor reactivity of T cells from the immunized mice as determined by ELISPOT. The antigen-irrelevant tumor cell line RM1/NY-ESO-1-GFP was used as a control. SFC, spot forming cells. (**C**) Identification of the T cell population ($CD4^+$ or $CD8^+$) responsible for tumor reactivity. We tested the ability of T cells to respond to B6-BL/EBNA1-GFP tumor cells in the presence of anti-CD4 and anti-CD8 mAb. Addition of anti-CD4 mAb abolished tumor cell recognition, while the presence of anti-CD8 mAb had no effect.

EBNA1 by proteasomes, thus significantly reducing its capacity to generate MHC class I–restricted peptides (8, 11).

To further assess antigen-specific antitumor immunity in the B6-BL/EBNA1 animal model, we identified two EBNA1-derived T cell epitopes that are presented by murine MHC I-A^b molecules to $CD4^+$ T cells and are capable of eliciting anti-EBNA1 immune responses in EBNA1-immunized B6 mice. Of particular interest is that the EBNA1-$P_{607–619}$ peptide could be processed and presented by murine I-A^b molecules. Although the EBNA1-$P_{607–619}$ peptide induced both $CD4^+$ and $CD8^+$ T cell responses against the peptide-pulsed target cells (Figure 4), $CD8^+$ T cells failed to recognize RM1 cells expressing EBNA1, which suggests that MHC class I–restricted EBNA1 epitopes are not naturally processed and presented on the tumor cell surface because of the presence of GAr domain within EBNA1 (8, 9). We further showed that, consistent with this notion, the $CD4^+$ T cell response was responsible for T cell–mediated inhibition of BL growth in vivo (Figure 7C). These results suggest that $CD4^+$ T cells, after activation by EBNA1-$P_{607–619}$ peptide, play an important role in the inhibition of BL tumor growth in vivo. More importantly, the BL grew progressively in EBNA1-$P_{607–619}$–immunized CD4 KO mice but were significantly inhibited in the immunized CD8 KO mice (Figure 8A). Experiments with MHC class I KO and class II KO mice further confirmed the role of EBNA1-specific $CD4^+$ T cells in antitumor immunity (Figure 8B). Taken together, these findings suggest that the induction of EBNA1-$P_{607–619}$ peptide–specific $CD4^+$ T cells by DC/peptide vaccination leads to significant inhibition of B6-BL/EBNA1 tumor growth in vivo. The new findings represent what we believe to be the first direct evidence that $CD4^+$ T cells are primarily responsible for the rejection of BL tumor expressing EBNA1 in vivo.

Although we recently demonstrated that HLA-B8–restricted EBNA1-specific $CD8^+$ T cells could be elicited from human PBMCs after multiple peptide stimulation (12), $CD4^+$ T cell response against EBNA1 is dominant (15, 17–19). Thus, $CD4^+$ T cell response in our tumor model resembles that in patients with EBV-associated tumor. Several mechanisms of $CD4^+$ T cell–mediated antitumor immunity have been proposed. Studies of EBNA1-specific $CD4^+$ T cell lines established from healthy human donors have shown that some $CD4^+$ Th1 cells can directly kill BL cells in an 18-hour ^{51}Cr release assay (18), which suggests that $CD4^+$ T cells might inhibit EBV-infected cells through cytotoxicity mediated by perforin or Fas ligand expressed by $CD4^+$ effector cells. Both perforin- and Fas-mediated cytotoxicity have been implicated in the clearance of murine gammaherpesvirus–68 (MHV-68), which has been used as a model of human EBV infection (23, 24). However, our EBNA1-$P_{607–619}$–activated T cells did not show any cytotoxic activity against B6-BL/EBNA1-GFP cells in either a 4-hour or a 16-hour ^{51}Cr release assay. Alternatively, $CD4^+$ T cells might indirectly kill target cells through the production of cytokines, such as IFN-γ, which have been shown to have inhibitory activity in EBV-induced B cell growth (25, 26). The control of tumor growth by IFN-γ in other animal models, including models for MHC class II–negative tumors (27, 28), is well established (29–31), although conflicting results have also been reported (32–34). Inhibition of angiogenesis rather than direct arrest of tumor cell proliferation has been attributed to IFN-γ–mediated antitumor immunity (35). We are currently investigating these possibilities using various types of KO mice.

It has been suggested that immunocompromised individuals such as HIV-infected patients have increased risk of developing BL, which is strongly associated with EBV (36–38). These studies suggest that the host immune system plays an important role in con-

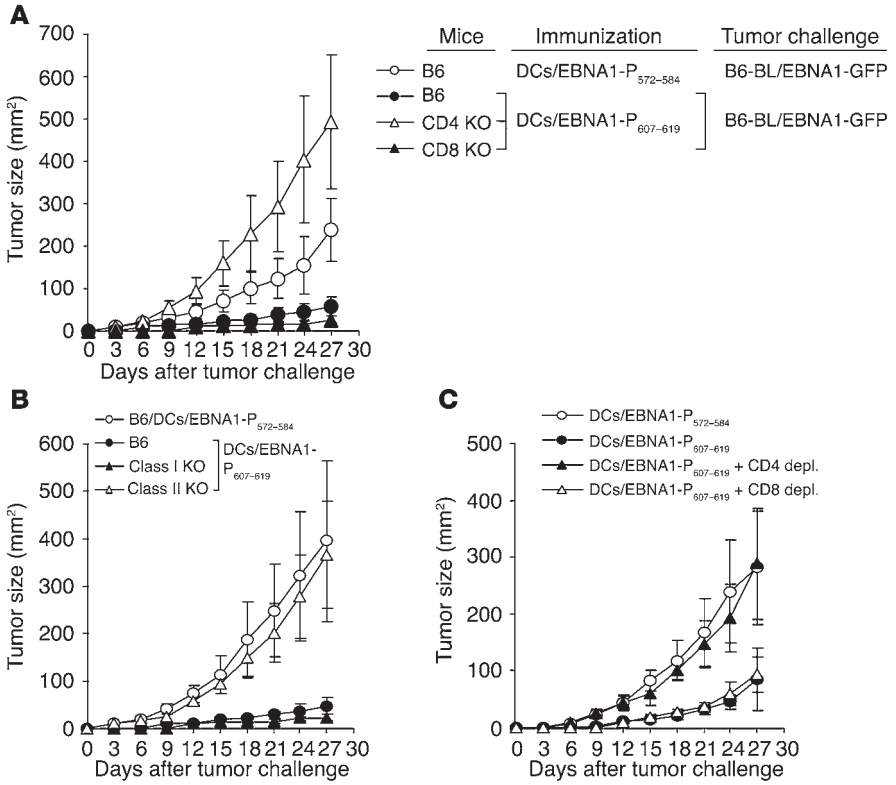

Figure 8

CD4+ T cells are responsible for the inhibition of BL cells in vivo. (**A**) Determination of T cell subsets responsible for the observed antitumor immunity. Wild-type, CD4 KO, and CD8 KO mice were immunized with DCs/EBNA1-$P_{607–619}$ peptide, and 2 weeks later were challenged with 5×10^5 B6-BL/EBNA1-GFP tumor cells. The tumor sizes were measured every 2 days after tumor challenge. Significant inhibition of tumor growth was observed in wild-type and CD8 KO mice immunized with DCs/EBNA1-$P_{572–584}$ peptide compared with other groups ($P = 0.005$), Similar results were obtained in 3 repeated experiments. (**B**) Antitumor immunity elicited in B6, MHC class I KO, but not in MHC class II KO mice. B6, class I, and class II KO mice were immunized with DCs/EBNA1-$P_{607–619}$ and were then challenged with B6-BL/EBNA1-GFP cells. DC/EBNA1-$P_{572–584}$ served as a specificity control. Significant suppression of tumor growth was observed in B6 and MHC class I KO mice immunized with DC/EBNA1-$P_{607–619}$ peptide compared with other groups ($P = 0.0065$). (**C**) Depletion (depl.) of the subset of CD4+ T cells abolished their ability to suppress tumor growth. The immunized mice were treated with anti-CD4 (GK1.5) or anti-CD8 (2.43) mAb's (200 μg in 500 μl volume) 1 day before tumor challenge and on days 1, 3, and 5 after challenge. Tumor growth was not inhibited in mice with depletion of CD4+ T cells, while depletion of CD8+ T cells did not affect antitumor immunity ($P = 0.0127$). B6 mice immunized with DC/EBNA1-$P_{607–619}$ or DC/EBNA1-$P_{572–584}$ peptide served as positive and negative controls, respectively.

trolling the development of BL. However, the relationship between immunocompromise and EBV-associated cancer is still poorly understood. In particular, it is not known whether the number and function of CD4 T cells are correlated with the development of EBV-associated BL. Since our results suggest that the induction of EBNA1-specific CD4+ T cells is critical in controlling the growth of EBNA1-expressing BL tumor model in vivo, it is important to identify MHC class II–restricted EBNA1 epitopes recognized by CD4+ T cells. Such epitopes could be used to stimulate CD4+ T cells specific for EBNA1 and then adoptively transferred along with EBV-specific CD8+ T cells into patients with EBV-associated cancer. Alternatively, these MHC class II–restricted EBNA1 peptides could be used in a vaccine in combination with MHC class I viral peptides to elicit both CD4+ and CD8+ T cell responses. It has been demonstrated that both CD4+ and CD8+ T cell responses are required for controlling the outgrowth of EBV-transformed B cells in seropositive donors (39). Thus, it is critical to include both MHC class I– and class II–restricted peptides from EBV antigens in cancer vaccines for recruiting and activating CD4+ and CD8+ T cell responses in clinical setting, ultimately leading to tumor destruction.

Methods

Mice. C57BL/6 (B6, H-2^b) female mice were obtained from the National Cancer Institute. CD4 KO and CD8 KO mice in a B6 background were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and had been backcrossed for more than 10 generations. MHC class I– and class II–deficient ($\beta 2m^{-/-}$ and I-$A^{b-/-}$, respectively) mice that had been backcrossed to the B6 background for more than 12 generations were purchased from Taconic (Germantown, New York, USA). All mice were maintained in the animal facility at Baylor College of Medicine under specific pathogen–free conditions and were used at 8–12 weeks of age. All studies were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine regarding the use of laboratory animals.

Cell lines. A murine BL cell line, designated B6-BL, was initially derived from human *Igλ-MYC* transgenic mice in a C57BL/6 background (20). The B6-BL/EBNA1 cell line was derived from *Igλ-MYC* × EBNA1 double-transgenic mice. Both B6-BL and B6-BL/EBNA1 cell lines were kindly provided by Herbert. C. Morse III and Ted Torrey at the National Institute of Allergy and Infectious Diseases, NIH. EBNA1 transgenic mice were independently generated using the method and constructs similar to one previously described (ref. 40; Ted Torrey, personal communication), and were used to cross with the *Igλ-MYC* transgenic mice. However, the EBNA1 expression in B6-BL/EBNA1 cell line could be detected by RT-PCR but not by Western blot analysis. Therefore, we introduced EBNA1 into B6-BL cells by a retroviral vector encoding the *EBNA1-GFP* gene. Retroviral EBNA1 constructs and viral supernatant preparation were conducted as previously described (12, 41). Expression of EBNA1-GFP was under the control of viral long-terminal repeat promoter. The resultant cell line was designated B6-BL/EBNA1-GFP. As a control, we generated a B6-BL/GFP cell line. We also generated an additional tumor cell line, RM1/NY-ESO-1-GFP, by introducing the *NY-ESO-1-GFP* fusion gene into the murine RM1 prostate cell line. The human embryo kidney 293 cell line expressing mouse MHC class II (I-A^b) molecules was previously described (42). These cell lines were maintained in RPMI 1640 medium (GIBCO; Invitrogen Corp., Carlsbad, California, USA) supplemented with 10% FBS (Gemini Bio-Products, Woodland, California, USA), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

EBNA1 peptides. The peptides were synthesized by a solid-phase method using a peptide synthesizer (model AMS 422; Gilson Co., Worthington, Ohio, USA) and were purified by HPLC and were more than 98% pure. The mass of some peptides was confirmed by mass spectrometry analysis. EBNA1 peptide sequences were identical to those previously described (19).

DC preparation and immunization. Bone marrow–derived DCs from C57BL/6 mice were prepared as previously described (42). In some experiments, B6 mice were immunized with 50 μg of full-length EBNA1 (19) or GAr-del-EBNA1 protein (a kind gift of Jindong Wang, University of Wisconsin, Madison, Wisconsin, USA) or 100 μg of EBNA1 peptides emulsified in an equal volume of CFA (Sigma-Aldrich, St. Louis, Missouri, USA) in a total volume of 50 μl.

T cell stimulation, cytokine release, and ELISPOT assay. Two million splenocytes were freshly prepared from the immunized mice (2 per group) and incubated with various EBNA1 peptides at a final concentration of 10 μM in RPMI 1640/5% mouse serum (Valley Biomedical Inc., Winchester, Virginia, USA) and cultured in a 24-well plate (Corning, Corning, New York, USA) at 37°C in 5% CO_2 for 6 days. These T cells were tested for their ability to recognize several tumor target cells or peptide-pulsed targets. In some experiments, T cells from splenocytes or draining lymph node cells of the immunized mice were directly tested for their ability to recognize target cells. Murine IFN-γ release was determined with ELISA kits (Endogen Inc., Woburn, Massachusetts, USA) according to the manufacturer's instructions. T cell activities against tumor target cells were also determined by ELISPOT, as previously described (43).

Intracellualr cytokine staining and flow cytometric analysis. Spleen cell cultures stimulated with EBNA1 peptides for 18 hours were established as described above. Cytokine secretion from T cells was then blocked by the addition of brefeldin A (10 μg/ml; Sigma-Aldrich) for 3 hours before harvesting. Cells were washed once in FACS buffer (1% FCS-PBS) and adjusted to 0.5×10^6/tube and stained for expression of CD4 and CD8 by phycoerythrin-conjugated (PE-conjugated) anti-CD4 (GK1.5) and anti-CD8 (53-6.7), respectively. After 30 minutes on ice, the cells were washed twice, then fixed with 2% paraformaldehyde-PBS for 20 minutes at 4°C, followed by intracellular staining in permeabilization buffer containing 0.5% saponin and 1% BSA in PBS. After incubation with 1 μg/tube FITC-conjugated anti–IFN-γ (XMG1.2; BD Biosciences — Pharmingen, San Diego, California, USA) for 45 minutes at 4°C. and the cells were washed, resuspended in FACS buffer, and analyzed by flow cytometry.

B6-BL, B6-BL/GFP, and B6-BL/EBNA1-GFP tumor cells were washed once in FACS buffer, adjusted to 0.2×10^5/tube, and stained for cell surface markers by incubation with 1–2 μg/tube of PE-conjugated anti-mouse mAb's (all from BD Biosciences — Pharmingen): anti-B220 (RA3-6B2), anti–H-2K^b (AF6-88.5), anti–H-2I-A^b (AF6-120.1), anti-B7.1 (16-10A1), anti-B7.2 (GL1), and anti–ICAM-1 (3E2). After 30 minutes on ice, cells were washed twice with FACS buffer and analyzed with a FACScan flow cytometer (BD, San Jose, California, USA).

MTT assay. Cells were seeded in a flat-bottomed, 96-well plate at 2×10^4 cells/well in RPMI-1640 plus 10% FCS. Before harvesting, 50 μl of the vital dye MTT (Sigma-Aldrich) in PBS (5 mg/ml) was added to the cultures. The blue dye taken up by the cells after 4 hours of incubation was dissolved in DMSO (100 μl/well). Readouts were taken at a 550 nm wavelength using an automated microplate reader.

Animal study. B6 mice and mice deficient in CD4, CD8, or MHC class I or class II molecules were immunized with EBNA1 peptides pulsed on B6 DCs (3×10^5/mouse) through tail veins. Two weeks later, mice were challenged with various tumor cells by subcutaneous injection of 5×10^5 of tumor cells. In some experiments, CD4$^+$ and CD8$^+$ T cells were depleted by intraperitoneal injection of 500 μl (containing 200 μg) of anti-CD4 (GK1.5) and anti-CD8 (2.43) mAb's, respectively, as previously described (42). Tumor growth was measured with a caliper every 2–3 days and the results described as tumor area in mm^2. Statistical significance was calculated with the two-sided Student's *t* test.

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