Epstein-Barr virus-induced gene 3-deficiency leads to impaired antitumor T-cell responses and accelerated tumor growth

Zhenzhen Liu1, Jin-Qing Liu1, Yun Shi1,2, Xiaotong Zhu1,2, Zhihao Liu1,3, Ming-Song Li2, Jianhua Yu1, Lai-Chu Wu4, Yukai He5, Guoqiang Zhang6,*, and Xue-Feng Bai1,*

1Department of Pathology and Comprehensive Cancer Center; Ohio State University; Columbus, OH USA; 2Department of Gastroenterology; Guangdong Provincial Key Laboratory of Gastroenterology; Nanfang Hospital; Southern Medical University; Guangzhou, China; 3Department of Hepatobiliary Surgery; Nanfang Hospital; Southern Medical University; Guangzhou, China; 4Davis Medical Center; Department of Molecular and Cellular Biochemistry; Ohio State University; Columbus, OH USA; 5Cancer Immunology; Inflammation, and Tolerance Program; Georgia Regents University Cancer Center; Augusta, GA USA; 6Department of Thoracic Surgery; Xinqiao Hospital; Third Military Medical University; Chongqing, China

**Correspondence to:** Xue-Feng Bai; Email: Xue-Feng.Bai@osumc.edu; Guoqiang Zhang; Email: zhang006006@163.com

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Epstein-Barr virus-induced gene 3 (EBI3) encoded protein can form heterodimers with IL-27P28, and IL-12P35 to form IL-27, and IL-35. However, IL-27 stimulates, whereas IL-35 inhibits antitumor T-cell responses. IL-27 also limits the Foxp3+ regulatory T cell (Treg) population, whereas IL-35 has been shown to expand Tregs and foster Treg suppressive functions. It remains unclear which group of forces are dominant during antitumor T-cell responses. In this study, we evaluated the tumor growth and antitumor T-cell responses in EBI3-deficient mice that lack both IL-27 and IL-35. We found that injecting B16 melanoma cells into EBI3-deficient C57BL/6 mice, or J558 plasmacytoma cells into EBI3-deficient BALB/c mice resulted in significantly increased tumor growth relative to those implanted in wild-type control mice. Tumors from EBI3-deficient mice contained significantly decreased proportions of CD8+ T cells and increased proportions of CD4+Foxp3+ Treg cells as compared to those from EBI3-intact mice. Tumor-infiltrating T cells from EBI3-deficient mice were impaired in their capacity to produce IFNγ. Phenotypically, Tregs from EBI3-deficient mice were highly suppressive and produced IL-10 in the tumor microenvironment. Depletion of Tregs or inactivation of the IL-10 pathway significantly abrogated tumor growth enhancement in Ebi3−/− mice. Finally, we showed that Ebi3−/− mice administered a melanoma vaccine failed to mount a CD8+ T-cell response and the vaccine failed to confer tumor rejection in EBI3-deficient mice. Taken together, these results suggest that Ebi3−/− mice show a phenotype of IL-27-deficiency rather than IL-35-deficiency during anti-tumor T-cell responses. Thus, our results suggest that endogenous IL-27 is critical for both spontaneous and vaccine-induced antitumor T-cell responses.

**Introduction**

Epstein-Barr virus-induced gene 3 (EBI3) encoded protein can form heterodimers with IL-27P28 and IL-12P35 to form IL-27, and IL-35. IL-27 is mainly produced by antigen-presenting cells (APCs) and signals through a heterodimeric receptor (IL-27R) consisting of the IL-27Rα (WSX-1) and the gp130 subunits. IL-35 has been shown to be secreted by FoxP3+CD4+CD25+ regulatory T cells (Tregs) in mice or "iTreg" cells, a regulatory T-cell population induced by IL-35. IL-35 signals through a unique heterodimer of receptor chains IL-12Rβ2 and gp130 or the homodimers of each chain in target cells.

Functionally, IL-27 and IL-35 share some similar properties. IL-27 was first described as a cytokine promoting T helper (Th) type 1 (Th1) responses, but later pieces of evidence suggested that IL-27 is a negative regulator of Th1, Th2 and Th17 responses. IL-27 is also a potent inducer of IL-10 production by T cells. Consequently, IL-27 has been shown to inhibit inflammation in a number of autoimmune disease models. IL-35 has been shown to suppress T-cell proliferation, Th17 and Th2 immune responses, and inhibit experimental arthritis, airway inflammation and diabetes mellitus in non-obese diabetic (NOD) mice. IL-27 and IL-35 appear to have opposing roles in regulating CD4+CD25+Foxp3+ Treg responses. In vitro, IL-27 has been shown to inhibit the conversion of inducible T regulatory cells (iTreg) and the expression of Foxp3, CD25 and the immune checkpoint cytokine T lymphocyte associated protein 4 (CTLA4). IL-27Rα-deficient (Wsx-J−/−) mice exhibit increased Treg conversion and expansion during autoimmune inflammation.
responses. IL-27 transgenic mice are deficient in Treg cells and develop systemic inflammation at 8-11 weeks of age. In contrast, IL-35 has been shown to expand Foxp3+ Tregs. IL-35 itself has been shown to be required for Treg suppressive functions as IL-35-deficient (either Ebi3 or P33) Treg cells have been shown to exhibit reduced regulatory activity in vitro and fail to control homeostatic proliferation or cure inflammatory bowel disease in vivo.

IL-27 and IL-35 also play opposing roles in the induction of cytotoxic T lymphocyte (CTL) responses. In tumor models, IL-27 can promote CTL accumulation in tumors and inhibit tumor growth by a number of mechanisms including promotion of CTL survival and effector functions. By contrast, IL-35 inhibits antitumor CTL responses and promotes tumor growth. We recently convincingly demonstrated the opposing roles of IL-27 and IL-35 in antitumor CTL responses using the mouse plasmacytoma J558 model. We found that J558 cells expressing IL-27 stimulate potent antitumor CTL responses and IL-27 expressing J558 tumors fail to grow in BALB/c mice whereas J558 cells expressing IL-35 inhibit CTL responses and exhibit accelerated tumor growth in BALB/c mice.

Ebi3−/− mice are deficient for both IL-27 and IL-35; thus, the deficiency of EBI3 can lead to reduced or increased antitumor T-cell responses and tumor rejection, depending on the balance of the 2 groups of forces mediated by IL-27 and IL-35. Given the potential importance of these 2 cytokines in the regulation of tumor immunity, evaluation of antitumor T-cell responses in Ebi3−/− mice may offer a unique opportunity to reveal the relative importance of the 2 cytokines in tumor immunity. In this study, we evaluated antitumor T-cell responses in Ebi3−/− mice. We found that injection of B16 melanoma or J558 plasmacytoma cells into Ebi3−/− mice resulted in significantly increased tumor growth relative to those grown in EBI3-intact controls. Tumors from EBI3-deficient mice contained significantly decreased percentages of interferon γ (IFNγ) producing CD8+ T cells and increased percentages of CD4+FoxP3+ Tregs. Tregs from EBI3-deficient mice were highly suppressive and produced IL-10 in the tumor microenvironment, and displaying suppressive functions largely dependent upon IL-10. Finally, we showed that a melanoma vaccine failed to induce an effective CD8+ T-cell response and confer tumor rejection in Ebi3−/− mice. These results suggest that Ebi3−/− mice show a phenotype of IL-27-deficiency. Our results suggest that the endogenous IL-27 is critical for the generation of both spontaneous and vaccine-induced antitumor T-cell responses.

**Results**

**EBI3-deficiency enhances tumor growth and impairs antitumor T-cell responses**

To determine the effect of EBI3-deficiency on tumorigenesis and tumor growth, we injected B16.F10 melanoma cells into Ebi3−/− and wild-type (WT) C57BL6 mice subcutaneously (s.c.). As shown in Fig. 1A, tumors grew significantly faster in Ebi3−/− mice than in WT mice. When B16.F10 cells were given intravenously (i.v.) to Ebi3−/− and WT mice, we found that more melanoma colonies formed in the lungs of Ebi3−/− mice than in WT mice, which leads to increased lung weights in Ebi3−/− mice compared to WT mice (Fig. 1B). To determine if adaptive immunity plays a role in determining tumor growth in Ebi3−/− mice, we s.c. or i.v. injected B16.F10 cells into Rag1−/− or Ebi3−/−Rag1−/− mice. Similar tumor growth kinetics (Fig. 1C) and lung tumor foci formation (Fig. 1D) were observed in these 2 distinct recipient mice. Thus, the tumor growth difference between Ebi3−/− and WT mice was caused by differential adaptive immunity.

To determine whether T-cell responses differ between WT and Ebi3−/− mice, we used immunostaining and cytotoxicity assays to detect tumor-infiltrating CD4+ and CD8+ T cells. As shown in Figure 2A, more tumor-infiltrating CD8+ T cells were detected in tumors from WT mice than in tumors from Ebi3−/− mice. The proportion of infiltrating CD4+ T cells, on the other hand, were similar in tumors from Ebi3−/− mice as compared to tumors from WT mice. Tumor-infiltrating CD8+ (Fig. 2B) and CD4+ T cells (Fig. 2C) produced less IFNγ in tumors from Ebi3−/− mice than in tumors from WT mice. However, higher percentages of Foxp3+CD4+ Treg cells were detected in tumors from Ebi3−/− mice (Fig. 2D).

To determine if these findings can be validated in another tumor model, EBI3-deficient BALB/c mice and control BALB/c mice were s.c. injected with J558 plasmacytoma cells. As shown in Fig. 3A, significantly increased tumor growth was observed in Ebi3−/− BALB/c mice. J558 tumors from Ebi3−/− mice contained relatively fewer CD8+ T cells and relatively higher percentages of CD4+ T cells (Fig. 3B). The infiltrating CD8+ and CD4+ T cells in tumors from EBI3-deficient mice were also less capable of producing IFNγ (Fig. 3C). However, a larger population of Foxp3+CD4+ Tregs was detected in tumors from EBI3-deficient mice than from tumors from BALB/c mice (Fig. 3D). Thus, EBI3-deficiency impairs antitumor T-cell responses and enhances tumor-specific Treg responses in both tumor models tested.

**Increased Treg response in Ebi3−/− mice inhibits antitumor T-cell responses and enhances tumor growth**

To further delineate the impacts of increased Treg responses on tumor immunity in EBI3-deficient mice, we depleted Tregs in Ebi3−/− and C57BL/6 mice using anti-CD25 antibody. B16. F10 cells were s.c. injected into anti-CD25-treated Ebi3−/− and WT C56BL/6 mice. As shown in Fig. 4A, anti-CD25 treatment more significantly impacted melanoma tumor growth in Ebi3−/− mice than in WT mice, and Treg-depletion resulted in increased numbers of IFNγ-producing CD8+ T cells in both Ebi3−/− and WT tumor-bearing mice.

In the B16.F10 i.v. injection model, depletion of Tregs particularly in Ebi3−/− mice also significantly reduced melanoma foci in the lungs (Fig. 4B). To test if Tregs from Ebi3−/− mice had comparative suppressive functions with Tregs from WT mice, we i.v. injected B16.F10 cells into Ebi3−/−Rag1−/− mice. Meanwhile, these mice either received EBI3-deficient CD25+ T cells alone or EBI3-deficient CD25- T cells plus Tregs from either WT mice or from Ebi3−/− mice. By day 21, mice were sacrificed and lung tumor foci formation was examined. As demonstrated
in Fig. 4C, mice receiving Tregs from Ebi3−/− mice had the highest numbers of melanoma foci in the lungs and heavier lungs, whereas mice receiving WT Tregs had similar numbers of melanoma foci and lung weights as mice receiving no Tregs. Thus, Tregs from Ebi3−/− mice are more suppressive to antitumor T cell responses as compared to WT Tregs that enhance tumor growth.

**IL-10 pathway is largely responsible for increased Treg function and enhanced tumor growth in Ebi3−/− mice**

IL-10 is a known cytokine that is produced by Treg cells and exerts inhibitory effects to T cell priming. By comparing IL-10 production among various tumor-infiltrating leukocytes, we found that tumor-infiltrating Tregs were the major source of IL-10 in tumors, and tumor-infiltrating Tregs from Ebi3−/− mice produced relatively more IL-10 (Fig. 4A). To determine if elevated IL-10 production by Treg cells in Ebi3−/− mice drives increased Treg suppressive functions, we generated Ebi3−/− IL-10−/− mice by breeding Ebi3−/− mice with IL-10−/− mice, and we purified CD4+CD25+ Tregs from WT, Ebi3−/− and Ebi3−/− IL-10−/− mice, and co-cultured them with CD25 T cells from EBI3-deficient mice. We consistently found that EBI3-deficient Tregs are more suppressive than EBI3-sufficient Tregs, and IL-10 deficiency largely abrogated the suppressive functions of Tregs from Ebi3−/− mice (Fig. 5B). To test if enhanced tumor growth in Ebi3−/− mice was also affected by IL-10-deficiency, B16.F10 tumor cells were i.c. or i.v. injected into WT, Ebi3−/− and Ebi3−/− IL-10−/− mice. We found that the IL-10 deficiency in Ebi3−/− mice significantly inhibited subcutaneous tumor growth (Fig. 5C) and attenuated the increased lung metastases seen in Ebi3−/− mice (Fig. 5D). Thus, IL-10 is responsible for the observed increased Treg suppression and enhanced tumor growth in Ebi3−/− mice.

**EBI3-deficiency impairs the efficacy of tumor antigen vaccination**

A lentiviral vector expressing a mutated tyrosinase related protein 1 (TRP1-lv) has been shown to elicit potent CD8+ T-cell responses against multiple TRP1 epitopes. Importantly, the activated CD8+ T cells effectively recognize wild-type TRP1 antigen and protect against B16 melanoma challenge. To determine if EBI3-deficient mice are also defective in induced antitumor T-cell responses, Ebi3−/− C57BL/6 and WT mice were immunized with 2.5 × 105 TU of TRP1-lv. On day 14 after TRP1-lv immunization, we detected higher numbers of TRP1-lv-specific CD8+ T cells in the blood of WT mice (Fig. 6A). Upon challenge with B16.F10 melanoma cells, TRP1-lv-immunized WT mice showed markedly reduced tumor growth relative to that occurring in Ebi3−/− mice (Fig. 6B). Consistent with the tumor growth curve, tumors from TRP1-lv-immunized EBI3−/− mice contained fewer tumor-infiltrating CD8+ T cells as compared to WT mice (Fig. 6C). Tumors from TRP1-lv-vaccinated EBI3−/− mice also contained fewer peptide-specific CD8+ IFNγ+ T cells as compared to tumors from WT mice (Fig. 6D). Thus, EBI3-deficient mice are highly resistant to TRP1-lv vaccination-induced antitumor T-cell responses.

**Discussion**

In this study, we investigated tumor-specific T-cell responses in 2 strains of Ebi3−/− mice, namely C57BL/6 and BALB/c,
using the B16 melanoma and the J558 plasmacytoma tumor models. We made 2 surprising discoveries that are of importance for understanding the regulation of tumor-specific T-cell responses. First, EBI3-deficient mice generate extremely poor spontaneous and induced antitumor T-cell responses and exhibit accelerated tumor growth. Second, Tregs from spontaneous and induced antitumor T-cell responses and exhibit impaired antitumor T-cell responses. This observation is surprising, since it differs from the previous report that shows B16 melanomas grow relatively slower and stimulate increased T-cell responses in EBI3-deficient mice. However, our results are consistent with other studies showing that mice deficient for IL-27Rα or IL-27Rβ generate deficient antitumor T-cell responses and exhibit accelerated tumor growth.

We have previously reported that Ebi3−/− mice exhibit increased Treg responses that downregulate autoimmune inflammation in the central nervous system. In this study, we found that tumors from Ebi3−/− mice possess higher numbers of Tregs as compared with their WT counterparts. Decreased CTL responses and increased Treg responses in Ebi3−/− mice are reminiscent of IL-27−/−, but not IL-35-deficiency, since IL-35 has been shown to expand Tregs. A few lines of evidence support this notion. First, IL-27Rα-deficient mice have been shown to exhibit increased Treg conversion/expansion and display increased tumor growth with reduced antitumor T-cell responses. Second, experiments in vitro have revealed that IL-27 inhibits the conversion of inducible T regulatory cells and the expression of Foxp3, CD25 and CTLA4.18,19 Third, IL-27 transgenic mice are

![Figure 2. EBI3-deficiency impairs antitumor T-cell responses. Immunostaining and cytometric analysis of tumor-infiltrating T cells from melanoma tumors grown in Ebi3−/− and wild-type (WT) mice. (A) Flow cytometry analysis of CD4+ and CD8+ T cells in the B16.F10 tumors from WT and Ebi3−/− mice. Data gated on CD4+ tumor infiltrating lymphocytes (TILs). (B) Flow cytometry analysis of IFNγ+ CD8+ T cells in the tumors from WT and Ebi3−/− mice. Data gated on CD8+ TILs. (C) Flow cytometry analysis of IFNγ+ CD4+ T cells in the tumors from WT and Ebi3−/− mice. Data gated on CD4+ TILs. (D) Flow cytometry analysis of Foxp3+ CD4+ T cells in the tumors from WT and Ebi3−/− mice. Data gated on CD4+ TILs. Statistical analysis was performed by Student’s t test; **p < 0.01; ***p < 0.001. Bars indicate SD from 5 mice per group. Data shown represents 3 experiments with similar results.](image-url)
deficient in Tregs and develop systemic inflammation at 8-11 weeks of age. Fourth, while expression of IL-27 in B16 melanoma cells inhibited tumor growth, we have recently shown that expression of IL-35 in B16 melanoma cells leads to tumor growth enhancement and reduced tumor T-cell infiltration, with Treg responses unaffected. Thus, the tumor enhancement and Treg expansion observed in Ebi3¡⁄¡ mice can be explained by IL-27, but not IL-35-deficiency. In addition to indirectly regulating Treg homeostasis, recent studies have also shown that IL-27 signaling is required for Treg cell survival, and can program Tregs into a unique T-betCXCR3 phenotype, specialized for regulating Th1 responses. However, the relevance of these observations in cancer immunity remains untested.

EBI3 and IL-12p35 form a novel cytokine IL-35, which has been shown to contribute to Treg suppressive functions, and EBI3-deficient Tregs have been shown to be unable to suppress autoimmune T-cell responses. However, in this study we found that Ebi3¡⁄¡ Tregs not only suppress antitumor T-cell responses, but also exhibit stronger immunosuppressive functions as compared to their WT counterparts. This conclusion is supported by the following 2 lines of evidences. First, depletion of Tregs in Ebi3¡⁄¡ mice significantly inhibited tumor growth and lung metastasis in Ebi3¡⁄¡ mice. Second, co-adoptive transfer of Ebi3¡⁄¡ Tregs with T cells into Rag1¡⁄¡ Ebi3¡⁄¡ mice significantly enhanced melanoma lung tumor formation compared with mice receiving WT Tregs and mice receiving no Tregs. The IL-10 pathway has long been implicated in the Treg-mediated suppression of autoimmunity and tumor immunity. Conditional deletion of IL-10 in Foxp3¡Tregs has revealed that Treg-derived IL-10 is particularly relevant to the suppression of inflammation in the environmental interfaces including the gut, lung and skin. Since IL-10-deficiency abrogated Treg suppressive activity and lung melanoma enhancement in Ebi3¡⁄¡ mice, these results suggest that Treg cells can suppress antitumor T-cell responses in the absence of IL-27 and IL-35 via the IL-10 pathway.

The potent antitumor activity of IL-27 has been well established in a variety of tumor models. Several mechanisms to account for this phenomenon have been proposed, including the

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**Figure 3.** Enhanced tumor growth and impaired T cell responses in EBI3-deficient BALB/c mice. (A) 5 × 10⁶ J558 plasmacytoma cells were s.c. injected into each wild-type (WT) or Ebi3¡⁄¡ BALB/c mice. The sizes of tumors were measured over time using calipers. (B-D) Immunostaining and cytofluorimetric analysis of infiltrating cells in tumors isolated from mice shown in A. Quantitative analysis of total CD4+ and CD8+ lymphocytes (B) IFNγ positive CD4+ and CD8+ T cells (C) and FoxP3+ CD4+ T cells (D) in tumors from WT and Ebi3¡⁄¡ BALB/c mice. Statistical analysis was performed by Student’s t test; *p < 0.05; **p < 0.01. Bars indicate SD of 5 mice in each group. Data shown represent 2 experiments with similar results.
induction of antitumor T-cell responses. Recently, we have shown that IL-27-stimulated CD8+ T cells up-regulate survival molecules and exhibit a survival advantage, which could be a mechanism by which IL-27 enhances antitumor CTL responses. Although our findings have revealed a predominant role for IL-27 in limiting Treg responses in this study, depletion of Treg cells in Ebi3−/− mice did not lead to complete tumor protection, suggesting that lack of direct stimulation of CD8+ T cells by IL-27 could also play a role in the accelerated tumor growth observed in Ebi3−/− mice.

Taken together, we have found that Ebi3−/− mice exhibit accelerated tumor growth and show diminished antitumor T-cell responses. Tregs from Ebi3−/− mice show increased, rather than decreased immunosuppressive activity. These results also indicate that Treg cells suppress antitumor T-cell responses using the IL-10 pathway in the absence of IL-27 and IL-35. Thus, this study has revealed a significant mechanism by which IL-27 mediates its antitumor activity, i.e., by limiting Treg responses. Our results also reveal the importance of endogenous IL-27 for vaccine-induced antitumor T-cell responses.

**Materials and Methods**

**Mice**

C57BL/6, IL-10−/−, Rag1−/−, C57BL/6, BALB/c mice were purchased from The Jackson Laboratory. Ebi3−/− C57BL6 and Ebi3−/− Rag1−/− mice have previously been described. Ebi3−/− IL-10−/− mice were generated through breeding Ebi3−/− with IL-10−/− mice for 2 generations. PCR was used for the identification of mice genotypes. The primers used were: EB13 (forward) 5′-CTG CGT CAC TAA CTC GGA-3′, Ebi3 (reverse) 5′-ACG ACA TCA GGG TCT GAT-3′, ATC AAC-3′; and IL-10 (forward) 5′-CTG CAC TAC CAT CAA AGG-3′ and IL-10 (reverse) 5′-CTG TTC CCT TCT AGG ACT CTG TA-3′. All mice were

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**Figure 4.** Tregs from Ebi3−/− mice are highly immunosuppressive. (A) 1 × 10⁵ B16F10 cells were subcutaneously (s.c.) injected into each C57BL/6 and Ebi3−/− C57BL/6 mouse and anti-CD25 or control IgG antibodies (400 μg/mouse) were intraperitoneally (i.p.) administrated to each mouse every 4 days for a total of 3 times. Twenty-one days later mice were sacrificed, and subcutaneously grown tumors were isolated and weighed. Average weights of the tumors from each group of mice (n = 5) are shown (left panel). Intracellular interferon γ (IFNγ) staining and cytotoxicity analysis were performed on tumor-infiltrating lymphocytes and quantified (right panel). Statistical analysis was performed by Student’s t test; ***p < 0.001; **p < 0.01. (B) 1 × 10⁵ B16F10 cells were intravenously (i.v.) injected into each Ebi3−/− C57BL/6 mouse and anti-CD25 or control IgG antibodies (400 μg/mouse) were administrated i.p. to each mouse every 4 days for a total of 3 times. Twenty-one days later mice were sacrificed, and tumor foci in the lungs were evaluated. The average weight of the lungs from each group of mice are shown in the right panel. Statistical analysis was performed by Student’s t test; **p < 0.01. (C) 1 × 10⁵ B16F10 cells were i.v. injected into Ebi3−/− Rag1−/− C57BL/6 mice. On the same day, mice either received 5 × 10⁵ CD25+ T cells (from Ebi3−/− mice) alone, or 5 × 10⁵ CD25+ T cells plus 5 × 10⁵ Tregs from WT mice, or 5 × 10⁵ CD25+ T cells plus 0.5 × 10⁵ Treg cells from Ebi3−/− mice. Twenty-one days later mice were sacrificed, and tumor growth in the lungs were evaluated. Average weights of lungs from each group of mice are shown in the right panel. Statistical analysis was performed by Student’s t test; *p < 0.05; **p < 0.01.
maintained in the animal facilities of The Ohio State University. The animal facilities are fully accredited by American Association for Accreditation of Laboratory Animal Care.

Cancer cell lines and tumor establishment in mice
B16.F10 melanoma cells and mouse plasmacytoma J558 cells have been described previously. 50-52 The tumor cells were maintained in RPMI 1640 medium (Gibco) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, and 5% FBS.

To establish tumors in mice, 1 × 10^5 B16.F10 cells or 5 × 10^6 J558 cells were injected subcutaneously (s.c.) into recipient mice at the flank in 200 μL of PBS. The length (a) and width (b) of tumors were measured using a digital caliper every 2 or 3 days. The tumor volume was calculated according to the formula V = ab^2/2 as described. 50 To establish melanoma lung metastasis, each mouse was injected with 1 × 10^5 B16.F10 cells via the tail vein. Mice were monitored for up to 3-4 weeks. At the end of the experiments, mice were sacrificed, and their lungs were weighed and examined for tumor metastasis, as previously described. 50

Antibodies and flow cytometry
FITC-, PE-, APC- or PerCP-labeled antibodies to CD4, CD8α, CD45, FoxP3, IFNγ and isotype-matched control antibodies were purchased from BD Biosciences (San Diego, CA) or eBiosciences (San Diego, CA). For staining of cell surface markers, disassociated cells from tumors were stained with various antibodies in staining buffer (PBS with 1% FCS) and incubated on ice for 30 min. After washing with staining buffer, cells were fixed in 1% Paraformaldehyde in PBS. For intracellular cytokine staining, cells were stimulated in culture medium for 4 h with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL of ionomycin in the presence of Golgistop (1:1500; BD Biosciences). Viable cells were then fixed in IC fixation buffer (eBioscience), permeabilized with 1 × permeabilization buffer (eBioscience) and stained with respective antibodies. Staining for Foxp3 was performed according to manufacturer’s protocol (BD Biosciences). Stained cells were analyzed on an FACSCalibur flow cytometer, and data were analyzed using the Flowjo software (Tree Star, Inc., OR).

Isolation of CD4^+CD25^+ cells from spleens
Mononuclear cells were prepared from spleens, and CD4^+ T cells were isolated by negative selection. Briefly, splenocytes were first incubated with anti-CD8 (TIB210) and anti-Fc receptor (2.4G2) antibodies on ice for 30 min, followed by incubation with sheep anti-Rat IgG Dynabeads (Invitrogen). CD25^+ cells were isolated from CD4^+ cells by first staining them with PE-anti-CD25 mAb (BD Biosciences), followed by magnetic
antibody cell separation using anti-PE microbeads (Miltenyi Biotec). The isolated cells were of >90% purity.

**T cell adoptive transfer**

Purified CD25⁺ CD4⁺ and CD8⁺ T cells from Ebi3⁻/⁻ mice were mixed with CD4⁺CD25⁺ Tregspurified from WT or Ebi3⁻/⁻ mice, and were i.v. injected into Ebi3⁻/⁻ Rag1⁻/⁻ mice followed by i.v. inoculation of B16.F10 cells.

**CD25⁺ T cell depletion**

To deplete CD25⁺ T cells, mice received i.p. injection of 400 µg IgG1 isotype control mAb (anti-HRPN, BioXcell) and antibody cell separation using anti-PE microbeads (Miltenyi Biotec). The isolated cells were of >90% purity.

**Treg-mediated suppression assay**

$1 \times 10^6$/mL purified CD4⁺ CD25⁺ T cells from Ebi3⁻/⁻ mice were cocultured with graded numbers of CD4⁺CD25⁺ Treg cells from WT, Ebi3⁻/⁻ or Ebi3⁻/⁻ IL-10⁻/⁻ mice in the presence of irradiated splenocytes ($2 \times 10^6$/mL) from Ebi3⁻/⁻ Rag1⁻/⁻ mice and 0.1 µg/mL anti-CD3 mAb (2C11). After 48 h, 1 µCi/well [³H]-Thymidine was pulsed into the cultures, and incorporation of [³H]-Thymidine was measured in a liquid scintillation plate counter 12 h later.

**Mice vaccination, tumor cell challenge and CTL response to the vaccine**

A lentivector expressing a mutated tyrosinase-related protein 1 (TRP1-Lv) was injected into the footpads of each WT or Ebi3⁻/⁻ mouse. Two weeks after TRP1-Lv injection, mice were bled, and peripheral blood cells were stimulated with 1 µg/mL TRP1-455 peptide for 3 h, and peptide-specific IFNγ-producing CD8⁺ T cells were quantified by cytofluorimetric analysis. Two weeks after TRP1-Lv immunization, $1 \times 10^5$ B16 cells were s.c. injected into each mouse. Tumor growth was monitored over time using calipers. Twenty-one days after tumor cell injection, mice were sacrificed, and the numbers of CD8⁺ T cells among CD45⁺ tumor infiltrating lymphocytes (TILs) were quantified by flow cytometry. Disassociated cells from tumors were stimulated with peptide TRP1-455 for 4 h and stained for CD8 and IFNγ, followed by flow cytometry analysis. Peptide-specific, IFNγ-producing CD8⁺ T cells were quantified. Statistical analysis was performed by Student’s t test; *p < 0.05; **p < 0.001. Bars indicate 4 mice per group. Data represent 2 experiments with similar results.

![Figure 6](image_url)

**Statistical Analysis**

Data are expressed as mean ± SD. Two-tailed Student’s t test or paired t test was used for statistical analysis. p < 0.05 was considered significant.
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