Inhibition of p300 impairs Foxp3+ T regulatory cell function and promotes antitumor immunity

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Forkhead box P3 (Foxp3)+ T regulatory (Treg) cells maintain immune homeostasis and limit autoimmunity but can also curtail host immune responses to various types of tumors1,2. Foxp3+ Treg cells are therefore considered promising targets to enhance antitumor immunity, and approaches for their therapeutic modulation are being developed. However, although studies showing that experimentally depleting Foxp3+ Treg cells can enhance antitumor responses provide proof of principle, these studies lack clear translational potential and have various shortcomings. Histone/protein acetyltransferases (HATs) promote chromatin accessibility, gene transcription and the function of multiple transcription factors and nonhistone proteins3,4. We now report that conditional deletion or pharmacologic inhibition of one HAT, p300 (also known as Ep300 or KAT3B), in Foxp3+ Treg cells increased T cell receptor–induced apoptosis in Treg cells, impaired Treg cell suppressive function and peripheral Treg cell induction, and limited tumor growth in immunocompetent but not in immunodeficient mice. Our data thereby demonstrate that p300 is important for Foxp3+ Treg cell function and homeostasis in vivo and in vitro5–12, but there are limited data concerning HAT inhibitors and Treg cells. Of the three main HAT families (GNAT, p300-CBP and MYST13), the p300-CBP family, and p300 in particular, can acetylate and stabilize Foxp3 protein expression in transfected cells3,4. Here we assess whether p300 targeting can affect Treg homeostasis or function and thereby promote antitumor immunity.

We conditionally deleted Ep300 (which encodes p300) in Treg cells by crossing Ep300fl/fl and Foxp3YFP-Cre mice (Supplementary Fig. 1a). The resultant mice (hereafter Ep300−/− mice) are deficient for Ep300 only in Foxp3+ cells. Ep300−/− mice were born at expected Mendelian ratios, but from 10 weeks of age developed mild weight loss, dermatitis, lymphadenopathy and splenomegaly (Fig. 1a), and local mononuclear infiltrates in lung, liver and skin (Fig. 1b and Supplementary Table 1). They also showed mildly lower blood hematocrit and hemoglobin levels as compared to wild-type (WT) littermates (Supplementary Fig. 1b). Compared to WT littermates, Ep300−/− mice showed more basal T cell activation and production of proinflammatory cytokines, though overall T cell proportions were similar (Fig. 1c and Supplementary Fig. 1c,d). They also had higher serum IgG1 concentrations (Supplementary Fig. 1e), and in contrast to WT littermates, ~20% developed auto-antibodies (Supplementary Table 2). Microarray analysis showed that compared to WT Treg cells, Ep300−/− Treg cells overexpressed proapoptotic genes (Supplementary Fig. 2a,b). Although Ep300 deletion did not affect CD4+Foxp3+ cell numbers or expression of Foxp3 protein under basal conditions (Fig. 1d and Supplementary Figs. 1 and 3a), upon activation Ep300−/− Treg cells had increased Fasl mRNA (Fig. 1e, Supplementary Fig. 3b) and apoptosis (Fig. 1f). Likewise, Ep300−/− Treg cells adoptively transferred into immunodeficient but not immunocompetent mice survived for a shorter length of time compared to WT Treg cells (Fig. 1g). The suppressive functions of Ep300−/− Treg cells were modestly impaired and declined with age (Supplementary Fig. 4a). Hence, unlike scurfy mice, in which Foxp3 mutation disrupts DNA binding and causes lethal autoimmunity by 3 weeks of age, mice with Ep300 deletion have moderately affected Treg cell biology, comparable to that in other mice with conditional Treg cell targeting14–16.

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To assess T<sub>reg</sub> cell function in vivo, we transferred Ep300<sup>−/−</sup> or WT T<sub>reg</sub> cells plus conventional effector T (T<sub>eff</sub>) cells into immunodeficient mice. Compared to WT T<sub>reg</sub> Cells, Ep300<sup>−/−</sup> T<sub>reg</sub> cells were poor suppressors of homeostatic T<sub>eff</sub> cell proliferation (Fig. 1h) and had shorter cell survival (Supplementary Fig. 4h). In a second in vivo test of Ep300 deletion in T<sub>reg</sub> cells, we analyzed transplanted cardiac allografts. Immunodeficient recipients adoptively transferred with T<sub>eff</sub> cells alone, at the time of engraftment, developed acute rejection by 14 d after transplant, whereas mice receiving concurrent transfer of WT T<sub>eff</sub> and T<sub>reg</sub> cells maintained allografts long term (>100 d). However, mice given WT T<sub>eff</sub> cells and Ep300<sup>−/−</sup> T<sub>reg</sub> cells rejected their allografts by 25 d after transplant (Fig. 1i). In a third in vivo test, we transplanted hearts into WT or Ep300<sup>−/−</sup> mice and treated the recipients with CD154-specific monoclonal antibody (mAb) plus donor splenocytes (DST)<sup>17</sup>. This potent form of co-stimulation blocked (CD154 mAb/DST) (j). *P < 0.05 and **P < 0.01, and data are from two or three independent experiments. Error bars indicate S.E.M.

We next tested whether Ep300 deletion in T<sub>reg</sub> cells promotes antitumor immunity. Growth of TC1 lung adenocarcinomas, which express human papillomavirus E7 protein, was impaired in Ep300<sup>−/−</sup> mice compared to WT mice (Fig. 2a). Deletion of Ep300 did not affect lymphoid CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cell numbers (data not shown) but did lower their expression of CD103, which is important for T<sub>reg</sub> cell recruitment to tumor sites<sup>18,19</sup>, and increased the proportion of lymphoid CD8<sup>+</sup>T<sub>eff</sub> cells and interferon-γ (IFN-γ) production (Fig. 2b). Ep300 deletion also enhanced the beneficial effects of vaccination with adenosvirally expressed E7 protein (Ad.E7) on TC1 tumor growth (Fig. 2c). Likewise, Ep300 deletion impaired the growth of established ovalbumin (OVA<sup>+</sup>) AE17 mesotheliomas (Fig. 2d). Compared to WT mice bearing tumors, Ep300<sup>−/−</sup> mice bearing either tumor type had more mononuclear cell infiltration (Fig. 2e), higher intratumoral CD8<sup>+</sup>, granzyme B and IFN-γ<sup>+</sup> Teff cells (Fig. 2f), and immunohistology showed less T<sub>reg</sub> cell infiltration and high intratumoral CD8<sup>+</sup>T<sub>eff</sub> cell numbers (Supplementary Fig. 5). Likewise, compared to WT tumor-bearing mice, AE17 tumor–bearing Ep300<sup>−/−</sup> mice had fewer lymphoid CD4<sup>+</sup>Foxp3<sup>+</sup>CD110<sup>−</sup>T<sub>reg</sub> cells but more CD8<sup>+</sup>IFN-γ<sup>+</sup> and OVA-specific CD8<sup>+</sup>T<sub>eff</sub> cells (Fig. 2g). AE17 tumor–bearing Ep300<sup>−/−</sup> mice also had low numbers of intratumoral T<sub>reg</sub> cells and low amounts of both total and acetylated Foxp3 protein, but high numbers of CD8<sup>+</sup>OVA-specific T<sub>eff</sub> cells (Fig. 2h). Lastly, deletion of Ep300 reduced T<sub>reg</sub> cell proliferation in tumor-bearing mice (Fig. 2j) and Supplementary Fig. 6a. Hence, Ep300 conditional targeting can diminish T<sub>reg</sub> cell proliferation and accumulation within tumors and enhance antitumor immunity.
p300i decreased acetylation of Foxp3 by p300 (Fig. 3a) and decreased Treg cell expression of Foxp3 mRNA and protein (Supplementary Fig. 6b). Treg cells lacking Ep300 had lower levels of histone H3 acetylation at the Foxp3 promoter region compared to WT Treg cells, and p300i decreased the levels of acetylation at the Foxp3 promoter in WT but not Ep300−/− Treg cells, indicating the specificity of this approach (Fig. 3b). We also assessed effects of p300i on the transforming growth factor-β–induced conversion of naive CD4+CD25− T cells into Foxp3+ induced Treg (iTreg) cells; iTreg cell development was impaired, especially in the first 2 d of culture (Fig. 3c). Use of p300i reduced acetylation at the CNS1 enhancer, a region crucial to the process of peripheral tolerization of CD4+Foxp3+, CD8+ and CD8+IFN-γ cells in lymphoid tissues from Ep300−/− or WT TC1 tumor-bearing mice. (c) Tumor volume after Ad.E7 vaccination of Ep300−/− and WT mice bearing TC1 tumors. (d) Ep300 level in AE17 tumors of WT but not in Ep300−/− mice. (e) Mononuclear cell infiltration in Ep300−/− and WT mice bearing TC1 or AE17 tumors; scale bars, 100 μm. (f) Analysis of mRNA level of CD8, IFN-γ, granzyme-B (GrB) and Foxp3 in tumor samples from Ep300−/− and WT mice bearing TC1 or AE17 tumors (five per group). (g) Analysis of the percentage of CD4+Foxp3+CD103+, CD8+IFN-γ and OVA-specific CD8+ T cells in the spleens of Ep300−/− and WT mice bearing AE17 tumor-bearing mice. (h) Analysis of the frequency of CD4+Foxp3+, CD8+ and OVA-specific CD8+ T cells in tumor sites of Ep300−/− and WT mice bearing AE17 tumors. (i) Western blot showing Foxp3 and acetyl-Foxp3 (lys31) level in AE17 tumors of Ep300−/− versus WT tumor bearing mice (β-actin loading control). (j) Analysis of the percentage of Foxp3+ BrdU+ cells in Ep300−/− and WT mice bearing AE17 tumors. *P < 0.05, **P < 0.01, ***P < 0.001. Error bars indicate s.e.m.

We used a parent-to-F1 adoptive transfer model involving alloantigen-induced T cell activation, proliferation and cytokine production to assess the effects of p300i on conventional T cell functions in vivo. T cell activation and proliferation were comparable in groups receiving p300i or DMSO control (Supplementary Fig. 8a). We next evaluated the effects of the p300i, C646, on T cell responses in cardiac allograft recipients. Administration of C646 but not DMSO carrier abrogated Treg cell–dependent allograft survival, and we observed comparable effects using a peptidic p300i (Lys-CoA-Tat2)4 (Fig. 3j). To eliminate the possibility that p300i affects graft survival by altering CD4+CD25− T cell function, we adoptively transferred CD4+CD25− T cells into immunodeficient recipients and treated them with p300i or DMSO control. In both groups, acute rejection developed similarly (Supplementary Fig. 8b). We also tested whether p300i affected grafts directly by infusing p300i into isograft recipients; we did not observe any effects on long-term graft survival (>100 d survival, data not shown), which supports the idea that p300i acts preferentially on Treg cells. Moreover, p300i can prevent, or abrogate established, Treg cell–dependent immune responses, as shown by the ability of p300i, given from the day of surgery or from 30 d after transplant, to induce rejection in mice rendered tolerant by therapy with CD154 mAb and DST17 (Fig. 3j). Hence, p300i administration differentially affects Treg and Teff cell in vivo; notably, p300i use can decrease Treg cell suppressive function while simultaneously leaving intact protective Teff cell responses.

Lastly, we tested the effects of p300i on tumor growth. We found that p300i treatment significantly decreased growth of established TC1 tumors in normal (Fig. 4a) but not immunodeficient (Fig. 4b) mice.
The compound probably reached tumor sites in the immunodeficient mice, as shown by decreased acetylation of histone H3 in tumor cells, and we observed no effect on the acetylation of p300-independent targets (for example, α-tubulin) (Fig. 4c). p300i also significantly inhibited growth in Rag1−/− recipients treated with p300i, a second peptidic p300i (Lys-CoA-Tat peptide) or DMSO (five per group). Bottom, cardiac allograft survival in immunocompetent (B6) mice. Treg cell–dependent tolerance in WT recipients (four per group) was induced using CD154 mAb and DST, and then the mice were treated with p300i or DMSO (via 14-d Alzet pump delivery) begun at (red), or 30 days after (blue) transplantation (Tx). **P < 0.01 and ***P < 0.001 versus Teff, Treg and DMSO (top) or versus DMSO alone (bottom). Data from four independent experiments. Error bars indicate s.e.m.
Figure 4  p300i therapy impaired tumor growth in immunocompetent mice. (a, b) TC1 tumor growth in WT (day 18) (a) and Rag1−/− (b) mice treated with p300i or DMSO. (c) Western blotting assay showing α-tubulin and H3 acetylation in TC1 tumors in immunodeficient hosts (day 18) treated with p300i or DMSO. (d) AE17 tumor growth in WT mice (day 18) treated with p300i or DMSO. (e) Mononuclear cell infiltration and tumor necrosis in TC1 and AE17 models in WT mice (day 18) treated with p300i or DMSO. (f) Analyses of CD8+OVA+ cell percentage in mice bearing AE17 tumors treated with p300i or DMSO (day 18). *P < 0.05, **P < 0.01; data representative of four independent experiments, seven to ten mice per group. Error bars indicate s.e.m.

has potential therapeutic applications in cancer, and other conditions in which Foxp3+ Treg cells limit protective host immune responses.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The microarray data are deposited in the Gene Expression Omnibus under accession code GSE47989.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.L. and L.W. performed most studies, analyzed data and edited the manuscript. Y.L. wrote the manuscript. J.P., L.-C.S.W., V.K., S.S. and S.M.A. performed tumor studies and analyzed tumor data. R.H. undertook mouse breeding and performed histology. U.H.B. analyzed microarray data, analyzed data and edited the manuscript. T.R.B. performed histology. T.A. performed autoantibody screening, analyzed data and edited the manuscript. P.K.B. and P.A.C. provided unique mice and analyzed data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Ethics statement. Studies were approved by the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia (2008-7-746 and 2010-6-561).

Mice. We purchased male CD90.2+ C57BL/6 (B6), B6 CD90.1+, BALB/c and B6 Rag1−/− mice (The Jackson Laboratory), and Ep300fl/fl mice27 and Foxp3YFP-Cre mice28 were described previously. Mice were housed under pathogen-free conditions and used at 6–24 weeks of age.

Antibodies, flow cytometry and cell sorting. We purchased the following monoclonal antibodies (mAbs) for flow cytometry from eBioscience or BD Pharrmingen: Foxp3-specific antibody conjugated to phycoerythrin (PE) (NRFRF-30, 12-4771-82), Foxp3-specific antibody conjugated to allophycocyanin (APC) (FJK-16S, 17-5773-82), CD4-specific antibody conjugated to eFluor450 (RM-4.5, 48-0042-82), CD8ε-specific antibody conjugated to APC (53-6.7, 51-0081-82), CD44-specific antibody conjugated to APC (IM7, 17-0441-81), CD62L-specific antibody conjugated to PE-Cy7 (MEL-14, 25-0621-81), CD69-specific antibody conjugated to PE (H1.2F3, 12-0691-82), Ki67-specific antibody conjugated to PerCP-Cy5.5 (5B6, 561284), annexin V–specific antibody conjugated to eFluor450 (88-8006-72), CD103-specific antibody conjugated to PE (2E7, 12-1031-82), IFN-γ–specific antibody conjugated to APC (XMGI2.1, 557735), IL-2–specific antibody conjugated to PE (JES6-5H4, 554428), IL-17A–specific antibody conjugated to APC (TC11-18H10F, 560184), IL-4–specific antibody conjugated to PE (BV4-1D11, 554389), CD25-specific antibody conjugated to APC (PC61.5, 17-0251-81), GTR-specific antibody conjugated to APD (DTA-1, 17-5874-81) and CTLA4–specific antibody conjugated to PE (UC10-4E10-11, 557220). We undertook western blotting using antibodies to Foxp3 (FJK-16S, eBioscience, I:1,000), β-actin (4790, Cell Signaling, I:1,000), α-tubulin (2144, Cell Signaling, I:1,000), acetyl-α-tubulin (3971, Cell Signaling, I:500), p300 (C-20, Santa Cruz, I:1,000), histone H3 (05-499, Upstate, I:1,000) and actetyl-histone H3 (06-942, Upstate, I:1,000). Flow cytometry was performed using a Cyan flow cytometer (Beckman Coulter), and data were analyzed using FlowJo 8 software (Tree-Star). CD4+YFP+(Foxp3+) Flow cytometry was performed using a Cyan flow cytometer (Beckman Coulter), and data were analyzed using FlowJo 8 software (Tree-Star). CD4+YFP+(Foxp3+) cells were sorted from Foxp3YFP-Cre or Ep300+/−/− mice using a FACS Aria cell sorter (BD Bioscience, UPenn Cell Sorting Facility).

Plasmids and pS001. A p300 expression vector was provided by X.-J. Yang, and we purchased NFAT expression vectors and NFAT–IL-2 promoter Luciferase reporter (Addgene). Foxp3-MinR1 was described previously4, as were details of Plasmids and p300i.

Hematology and autoantibody detection. Citrated blood samples were tested using an automated hematology analyzer modified and calibrated for mouse blood samples. Pooled sera from male Ep300−/− or WT mice were diluted 1:5 and incubated with cryosections from normal male and female C57BL/6 mice, washed in PBS, followed by FITC-conjugated goat anti-mouse IgG secondary antibodies (Jackson ImmunoResearch Laboratories, 1:200). In addition to pooled WT healthy sera, controls included incubation with secondary antibody alone. Pooled sera from NZB mice with known autoantibodies served as positive controls. If any autoantibodies were detected, serum from each mouse was reanalyzed separately.

 Luciferase assays. 293T cells were maintained (37 °C, 5% CO2) in RPMI-1640 plus 10% heat-inactivated FBS, penicillin and streptomycin. Cells at 80–90% confluence were transfected with NFAT–IL-2 promoter luciferase reporter, plus NFAT, Foxp3, p300 expression vectors or empty vector, using Lipofectamine 2000 (Invitrogen). After 48 h, cells were treated with 6 ng mL−1 phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin (Sigma) in the absence or presence of p300i for 5–6 h, and luciferase activities of whole-cell lysates were analyzed with a dual-luciferase reporter assay kit (Promega).

Western blotting. Cell lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and subjected to immunoblotting6.

ChiP assays. For ChiP assays using EZ-Magna CHIP A Chromatin Immunoprecipitation Kit (17-408, Upstate), DNA-chromatin complexes were prepared from 2 × 106 Treg cells, and genomic DNA was precipitated using 10 μg of antibodies against p300 or acetylated histone H3 or control rabbit IgG (Upstate). Genomic DNA in precipitates was probed by RT-PCR for Foxp3 promoter using forward primer 5′ TCCCCCTAC1TAC CGGGGCCCTCTA 3′ and reverse primer 5′ TGAGATAACAGGGCTCATGAGAAACACA 3′, and for the Foxp3 CNS1 region using forward primer 5′ TAAAGGACCTGACGACCC ACACTGGA 3′ and reverse primer 5′ ATAGAAGACATAACACCAGCGG 3′.

Microarrays. RNA was extracted with RNeasy kits (Qiagen), and RNA integrity and quantity were analyzed by photometry (DU640; Beckman–Coulter). Microarray experiments were performed with whole-mouse-genome oligo-arrays (Mouse430a 2.0; Affymetrix), and array data were analyzed with MAYDAY 2.12 (ref. 29). Array data were subjected to robust multiamage average normalization. Normalized data were used for calculating fold changes of genes that were increased or decreased in expression with the Student's t-test, and data with >1.5-fold differential expression (P < 0.05 with Storey’s false discovery rate <0.3) were included in the analysis. Data underwent z-score transformation for display.

Homoeostatic proliferation assay. One million CD90.1+CD4+CD25+ T cells were mixed with half a million CD4+YFP+ Treg cells sorted from Foxp3YFP-Cre or Ep300+/−/− mice (CD25−) and adoptively transferred to Rag1−/− mice8. Spleen and lymph nodes were isolated after 7 d, and total CD90.1+CD4+ T cells were determined by flow cytometry analysis of Cell Trace Violet dilution.

Heterotopic cardiac allografts were performed with plate-bound CD3/CD28 mAbs (Invitrogen), IL-2 (10 U mL−1) and TGF-β (2 ng mL−1) for 3 d and analyzed by flow cytometry. Parent-to-F1 assay. CFSE-labeled single cells from C57BL/6 mice (H-2b) were injected into B6D2F1 mice (H-2d) (n = 3 per group), and recipients were treated with DMSO or p300i (C66/6, 8.9 mg per kg body weight per day) for 3 d. Thereafter, donor cells (H-2d negative) were analyzed for proliferation, cellular activation and cytokine production23. Data are representative of two independent experiments.

Cardiac transplantation. Heterotopic cardiac allografts were performed using BALB/c donors and B6 Rag1−/− recipients (n = 5 per group). In brief, B6 Rag1−/− mice engrafted with BALB/c hearts were adoptively transferred with 1 × 106 CD4+CD25+ T cells alone or along with 5 × 106 Foxp3YFP-Cre Treg cells or Ep300+/−/− Treg cells, and Alzet pumps were implanted subcutaneously to deliver p300i or DMSO infusions. In additional experiments, Treg cell–dependent allograft tolerance was induced by treatment of WT or Ep300+/−/− recipients at the time of engraftment with CD154 mAbs (Bix X Cell) plus 5 × 106 donor splenocytes17. Allograft survival was monitored daily, and rejection was defined as cessation of heartbeat.
BrdU staining. WT or Ep300−/− AE17 tumor-bearing mice were injected twice per day with BrdU (1 mg, intraperitoneally) for 3 d and then killed. Spleen cells were stained for CD4, Foxp3 and BrdU according to the manufacturer’s instructions (BrdU labeling kit, BD Bioscience) and analyzed by flow cytometry.

Cell lines and tumor models. 293T cells were obtained from American Type Culture Collection. TC1 cells, derived from mouse lung epithelial cells that were immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene, were provided by Y. Paterson. The murine AE17.ova mesothelioma cell line (D. Nelson, University of Western Australia) was derived from mesothelioma cells developing in mice treated intraperitoneally with asbestos and then stably transduced with chicken ovalbumin. Cells were grown in RPMI, 10% FBS (FBS), 2 mM glutamine, and 5 µg ml−1 penicillin and streptomycin. Each mouse was shaved on its right flank and injected subcutaneously with 1.2 × 10⁶ TC1 or 2 × 10⁶ AE17 tumor cells. For p300i experiments, mice received p300i or DMSO via Alzet pumps inserted 7 d after initial tumor injection. For p300i experiments, mice received the non-peptidic p300i (C646, 8.9 mg/kg/d) or DMSO via Alzet pumps, beginning 7 d after initial tumor injection, and continued for 14 d. Tumor volume was determined by the following formula: (3.14 × long axis × short axis × short axis)/6 (ref. 32). Tumors were paraffin-embedded and stained by H&E or snap-frozen and stained by immunoperoxidase.

Ad.E7 vaccination. 1 × 10⁶ TC1 (E7+) cells were injected subcutaneously into the right flanks of WT and Ep300−/− mice. One week later, mice bearing TC1 flank tumors (~100 mm³ in size) were either left untreated, or vaccinated subcutaneously in the left flank (contralateral to the tumor) with 1 × 10⁹ plaque forming units (PFU) of Ad.E7 vector, as previously described. Three days following initial vaccination, mice received a booster vaccine of 1 × 10⁹ PFU of Ad.E7 in the left flank. Tumor sizes were monitored twice per week.

OV A-tetramer staining. OVA-specific CD8⁺ T cells within tumors and spleens of experimental mice were determined by flow cytometry. Tumors were cut into small fragments and digested for 1 h at 37 °C with a cocktail of collagenase type I (0.1 U ml⁻¹, Worthington), collagenase type II (0.1 U ml⁻¹, Worthington), collagenase type IV (0.1 U ml⁻¹, Worthington), DNase I (0.05 U ml⁻¹, Worthington) and elastase (5 U ml⁻¹, Worthington) in L15-medium (Leibovitz). Single-cell suspensions were blocked for 30 min with anti-Fc receptor antibody (eBioscience, CD16/32, clone: 93, 14-0161-82, eBioscience, 1:50), washed (2% FBS in PBS), stained for 1 h with phycoerythrin-conjugated H-2Kb tetramer loaded with OVA peptide (SIINFEKL) (iTag MHC Tetramer; Beckman Coulter), washed and stained with allophycocyanin-conjugated anti-CD8α (clone 53-6.7; BD Bioscience, 1:50) for 30 min. A commercial kit (Fixable Aqua Dead Cell stain kit, Invitrogen) was used to exclude dead cells from data analyses. Cell acquisition was performed on a Cyan flow cytometer.

Statistics. Data were analyzed by GraphPad Prism 5.0d. All normally distributed data were displayed as mean ± s.e.m. Measurements between two groups were performed with a Student’s t-test or Mann-Whitney U test. Groups of three or more were analyzed by one-way analysis of variance (ANOVA) or the Kruskal-Wallis test. Survival analysis was calculated using log-rank (Mantel-Cox) test.

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