Cathepsin B Controls the Persistence of Memory CD8+ T Lymphocytes

Susan M. Byrne, Anne Aucher, Syarifah Alyahya, Matthew Elder, Steven T. Olson, Daniel M. Davis and Philip G. Ashton-Rickardt

_J Immunol_ 2012; 189:1133-1143; Prepublished online 27 June 2012;
doi: 10.4049/jimmunol.1003406
http://www.jimmunol.org/content/189/3/1133

Supplementary Material  http://www.jimmunol.org/content/suppl/2012/06/27/jimmunol.1003406.DC1

References  This article cites 61 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/189/3/1133.full#ref-list-1

Why _The JI_? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

Subscription  Information about subscribing to _The Journal of Immunology_ is online at: http://jimmunol.org/subscription

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

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

_J The Journal of Immunology_ is published twice each month by The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2012 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Cathepsin B Controls the Persistence of Memory CD8+ T Lymphocytes

Susan M. Byrne,* Anne Aucher, ‡ Syarifah Alyahya,* Matthew Elder,* Steven T. Olson,§ Daniel M. Davis,‡ and Philip G. Ashton-Rickardt*

The persistence of memory T lymphocytes confers lifelong protection from pathogens. Memory T cells survive and undergo homeostatic proliferation (HSP) in the absence of Ag, although the cell-intrinsic mechanisms by which cytokines drive the HSP of memory T cells are not well understood. In this study we report that lysosome stability limits the long-term maintenance of memory CD8+ T cell populations. Serine protease inhibitor (Spi) 2A, an anti-apoptotic cysteolic cathepsin inhibitor, is induced by both IL-15 and IL-7. Mice deficient in Spi2A developed fewer memory phenotype CD44hiCD8+ T cells with age, which underwent reduced HSP in the bone marrow. Spi2A was also required for the maintenance of central memory CD8+ T cell populations after acute infection with lymphocytic choriomeningitis virus. Spi2A-deficient Ag-specific CD8+ T cell populations declined more than wild-type competitors after viral infection, and they were eroded further after successive infections. Spi2A protected memory cells from lysosomal breakdown by inhibiting cathepsin B. The impaired maintenance of Spi2A-deficient memory CD8+ T cells was rescued by concomitant cathepsin B deficiency, demonstrating that cathepsin B was a physiological target of Spi2A in memory CD8+ T cell survival. Our findings support a model in which protection from lysosomal rupture through cytokine-induced expression of Spi2A determines the long-term persistence of memory CD8+ T cells.

Received for publication October 13, 2010. Accepted for publication August 22, 2011.

This work was supported by National Institutes of Health Grant AI45108, the Wellcome Trust, and Cancer Research UK (to P.G.-A.R.). S.M.B. was supported by a Molecular and Cell Biology training grant at the University of Chicago.

Address correspondence and reprint requests to Dr. Philip G. Ashton-Rickardt, Section of Immunobiology, Division of Immunology and Inflammation, Department of Medicine, Faculty of Medicine, Imperial College London, London W12 0NN, United Kingdom; or Committee on Immunology, University of Chicago, Chicago, IL 60637; or Section of Immunobiology and Infection, Division of Cell and Molecular Biology, Imperial College London, London SW7 2AZ, United Kingdom; and Center for Molecular Biology of Oral Diseases, University of Illinois at Chicago, Chicago, IL 60612.

The online version of this article contains supplemental material.

Abbreviations used in this article: Cath B KO, cathepsin B knockout; HSP, homeostatic proliferation; ILN, inguinal lymph node; KLRG1hi, killer cell lectin-like receptor G1hi [KLRG1hi]; LCMV, lymphocytic choriomeningitis virus; LMP, lysosomal membrane permeabilization; PCD, programmed cell death; p.i., postinfection; Spi, serine protease inhibitor; TCM, central memory T cell; WT, wild-type.

Copyright © 2012 by The American Association of Immunologists, Inc.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1003406
mitochondrial-induced apoptosis; however, the predominance of these different apoptotic pathways depends on the stimulus and cell type. The physiological downstream targets for cathepsins in vivo are not well understood.

We have identified serine protease inhibitor (Sp) 2A as a physiological inhibitor of the lysosomal pathway of death in mice (29). Sp2A, encoded by the SerpinA3g gene on mouse chromosome 12 (30), is unusual for a serine protease inhibitor (or serpin) in that it inhibits cysteine cathepsins and resides in the cytosol and nucleus (31). Sp2A was first identified in CD8+ T cells due to its particular gene expression pattern: it has a low level of expression in naive cells; it is upregulated in effector cells, especially in IL-7Rhi memory cell precursors; and it continues to be expressed in memory CD8+ T cells, months after the infection has subsided (29). Overexpressing Sp2A enhances the initial development of memory CD8+ T cells (29), but whether Sp2A plays a nonredundant role in maintaining long-term memory CD8+ T cells is unclear.

To examine the role of Sp2A in memory CD8+ T cell survival, we examined Sp2A knockout (KO) mice (A. Dev et al., submitted for publication) with C57BL/6, DBA/2, and Thy1.1+ congenic mice. C57BL/6 mice were purchased from The Jackson Laboratory via Charles River UK. Female mice were used at 16 wk old unless indicated otherwise. C57BL/6 KO mice were (A. Dev et al., submitted for publication) crossed with P14 transgenic mice (32) and Sp2A KO P14+ (CD45.2+) mice (>98% purity). The cells were also combined in equal amounts and 5000 P14+CD8+ cells were co-administered into WT recipients (CD45.1+2+). Mice were infected i.p. with 2 × 10⁷ PFU LCMV Armstrong. P14+ cells were identified by flow cytometry using Ab and gp33/H-2Db tetramer staining (5, 35). Secondary and tertiary adoptive transfers were performed >200 d p.i. using the same procedure with donor CD8+ splenocytes taken from the previous recipients.

Materials and Methods

Mouse strains

Sp2A KO mice were (A. Dev et al., submitted for publication) crossed with P14 transgenic mice (32) and cathepsin B–/– mice (33), all on the C57BL/6 background. CD45.1 congenic C57BL/6 mice (The Jackson Laboratory) were also crossed as described. C57BL/6, DBA/2, and Thy1.1+ congenic C57BL/6 mice were purchased from The Jackson Laboratory via Charles River UK. Female mice were used at 16 wk old unless indicated otherwise. All mice were maintained in accordance with the University of Chicago Institutional Animal Care and Use Committee and UK Home Office regulations.

Tissue preparation

Peripheral blood was drawn by tail vein puncture. Spleen, inguinal lymph nodes, and bone marrow from the femur were harvested using standard techniques. RBCs were lysed using Tris-buffered ammonium chloride. Intraphagocytic lymphocytes and lung lymphocytes were isolated by first perfusing the organs with PBS. These organs were ground through a 70-μm NaCl gradient from 0 to 0.2 M in buffer. Spi2A fractions were detected by SDS-PAGE. Original buffer with 0.1 M NaCl. The fusion protein was then eluted in buffer containing 15 mM reduced glutathione and detected by SDS-PAGE. After dialysis into 20 mM Tris buffer containing 15 mM reduced glutathione and detected by SDS-PAGE.

Flow cytometry

mAbs against mouse CD8, CD4, CD44, CD45.1, CD45.2, IL-7, R, and KLRG1 were used. All Abs were purchased from BD Biosciences or eBioscience. Stains were performed in PBS supplemented with 0.2% BSA (Sigma–Aldrich), 10% normal goat serum (Invitrogen), and anti-CD16/32 Fc block (eBioscience). Ag-specific T cells were stained using tetrants against the LCMV gp33 epitope on H-2Dβ (Beckman Coulter). BrdU incorporation was measured using the FITC BrdU flow kit (BD Biosciences). Flow cytometry was done on a CyAn ADP Analyzer (Beckman Coulter), and gating sorting was done on a FACSAria (BD Biosciences).

Real-time PCR

mRNA was isolated using the Qiagen microRNA kit. cDNA was prepared using the Invitrogen SuperScript III kit and oligo(dT). Real-time PCR reactions were run using the SYBR Green PCR Master mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Primer sequences for the genes SerpinA3g, Serpinaj3f, vimentin, and B2-microglobulin come from Winkler et al. (34).

Cytokine upregulation

CD44+CD8+ cells were purified by anti-CD8a magnetic microbeads (Miltenyi Biotec) and FACs purified from three pooled wild-type (WT) mice (>98% purity). Cells (10⁶) were cultured in 100 μl complete DMEM-10 without cytokine for 4 h and then cultured in 100 ng/ml IL-2, IL-7, or IL-15 (eBioscience) for the indicated periods.

Memory T cell assays

To measure T cell proliferation, mice were fed 0.8 mg/ml BrdU in drinking water for 7 d. For competition experiments, CD8+ T cells were purified using anti-CD8a magnetic microbeads (Miltenyi Biotec) from WT P14+ (CD45.1+) and Sp2A KO P14+ (CD45.2+) mice (>98% purity). The cells were expressed in equal amounts and 5000 P14+CD8+ cells were co-administered into WT recipients (CD45.1+2+). Mice were infected i.p. with 2 × 10⁷ PFU LCMV Armstrong. P14+ cells were identified by flow cytometry using Ab and gp33/H-2Db tetramer staining (5, 35). Secondary and tertiary adoptive transfers were performed >200 d p.i. using the same procedure with donor CD8+ splenocytes taken from the previous recipients.

Live cell imaging

Cells were imaged in eight-well chambered coverglasses (chambered borosilicate coverglass; Lab-Tek) precoated with 10 μg/ml fibronectin (Sigma–Aldrich). Cells were imaged at 37°C, 5% (v/v) CO₂ by resonance laser scanning confocal microscopy (TCS SPS RS; Leica) using an excitation wavelength of 488 nm with a ×63 water immersion objective (numerical aperture 1.2) and analyzed (Velocity and ImageJ; National Institutes of Health). Acidic lysosomes were visualized by staining with LysoTracker Green DND-26 (Molecular Probes) according to the supplier’s instructions.

Recombinant Sp2A expression and purification

Sp2A was expressed in Escherichia coli as a GST fusion protein with a factor Xa cleavage site. The protein was purified from cell extracts by batch adsorption to glutathione-Sepharose beads in 20 mM sodium phosphate buffer, 0.1 M NaCl, 0.1 mM EDTA containing 15 mM PMSF, 5 mM DTT, and 1% Triton X-100. Adsorbed beads were packed into a column and washed with 10 vol buffer containing 1 M NaCl followed by 10 vol original buffer with 0.1 M NaCl. The fusion protein was then eluted in buffer containing 15 mM reduced glutathione and detected by SDS-PAGE. The GST tag was cleaved by incubation of ~300 μg/ml protein with ~10 μg/ml factor Xa for 4 h in sodium phosphate buffer without PMSF, DTT, or Triton X-100 followed either by inactivation of the factor Xa with 100 μL Glu-Gly-Arg-chloromethylketone or adsorption of the enzyme with soybean trypsin inhibitor-agarose beads. After dialysis into 20 mM Tris buffer (pH 7.4) containing 1 mM EDTA and 1 mM DTT, the protein was loaded onto a Mono Q column and was eluted with a linear NaCl gradient from 0 to 0.2 M in buffer. Sp2A fractions were detected by SDS-PAGE and cathepsin L inhibitory activity, pooled, concentrated, and dialyzed into storage buffer consisting of 0.1 M HEPES, 0.1 M NaCl, 5 mM DTT (pH 7.4). Concentrations of Sp2A were determined from the 280 nm absorbance based on an extinction coefficient of 26,300 M⁻¹ cm⁻¹ calculated from the amino acid sequence (36).

Cathepsin activity assays

The buffers used for characterizing the kinetics and stoichiometry of Sp2A inhibition of cathepsins were based on those found to yield optimal enzyme stability: for cathepsins L, B, and papain, 20 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, 0.1% PEG 8000, 5 mM DTT (pH 6); for cathepsin S, 20 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, 0.1% PEG 8000, 5 mM DTT (pH 7.4); for cathepsins H, V, and K, 0.1 M sodium acetate, 1 mM EDTA, 0.1% PEG 8000, 5 mM DTT (pH 5.5). Note that the kinetics of cathepsin L inhibition by Sp2A were found to be independent of pH in the
Results

Reduced levels of endogenous memory phenotype CD44hi CD8+ T cells in Spi2A KO mice

We have reported the complete absence of Spi2A mRNA expression in Spi2A KO mice (A. Dev et al., submitted for publication), whereas the expression of the linked Serpina3f gene (30) was not affected. Spi2A KO mice are viable and exhibit no gross differences in the levels of erythroid, myeloid, or lymphoid cells and possess normal proportions of CD8+, CD4+, and B220+ lymphocytes (Supplemental Tables I, II). However, Spi2A KO mice develop fewer memory phenotype CD44hiCD8+ T cells with age. The activation marker CD44 is upregulated by memory phenotype T cells after the recognition of endogenous Ags (39). Among T cells, the proportion of CD44hi memory phenotype cells increases with age and Ag experience as the proportion of CD44hi naive cells declines (40). Twelve-week-old Spi2A KO mice possessed similar proportions of CD44hi peripheral blood leukocytes; however, at 16 wk, Spi2A KO mice developed a significantly lower CD44hi proportion of CD8+ peripheral blood leukocytes compared with WT mice (Fig. 1A, 1B). Spi2A KO mice also showed a significant reduction in the number of memory phenotype CD44hi CD8+ T cells in the bone marrow at 15 wk old (p = 0.0008) (Fig. 1C). Lower CD44hi proportions were also found in the spleen, lymph nodes, and intrahepatic lymphocytes, although these differences were not consistently statistically significant (data not shown). No differences were observed in younger mice (9 wk), demonstrating an age-dependent phenotype (Fig. 1C). Furthermore, the defect in memory phenotype T cell levels in Spi2A KO mice was specific to CD8+ T cells, as the level of CD44hiCD4+ cells was not affected (Fig. 1B, 1C). Thus, Spi2A deficiency results in an age-specific decrease in the level of memory phenotype CD44hiCD8+ T cells.

FIGURE 1. Spi2A-deficient mice develop fewer memory phenotype CD44hi CD8+ T cells. (A) Representative flow cytometry staining of CD44hiCD8+ and CD44hiCD4+ T cells in the blood of 16-wk-old WT or Spi2A KO mice. (B) Proportion of CD44hi cells in the CD8+ or CD4+ T cell populations. (C) Upper graph, Number of CD44hiCD8+ or CD44hi CD4+ T cells present in one femur of 9- and 15-wk-old mice. Lower graph, BrDU incorporation of CD44hiCD8+ and CD44hiCD4+ T cells after 1 wk BrDU administration in 9- and 15-wk-old mice. For (A)-(C) data show mean ± SEM of at least five mice. (D) CD44hiCD8+ cells were purified by magnetic microbeads and FACS sorting from three samples each consisting of three spleens from pooled WT mice. Cells were cultured in complete DMEM-10 without cytokine for 4 h and then cultured in 100 ng/ml cytokine for the indicated times. Serpina3f expression was assayed by real-time PCR and shown relative to B2-microglobulin expression levels. For all panels, significance values were calculated by two-way ANOVA with a Bonferroni posttest. Results are representative of at least two independent experiments. **p < 0.01, ***p < 0.001.

5.5–7.4 range. All cathepsins were activated by diluting stock solutions into buffer containing 5 mM DTT and incubating for 5–15 min on ice.

The molar concentrations of active cathepsins were determined by active-site titration with E64 (37). Briefly, ~50–100 nM solutions of enzyme were incubated with increasing molar ratios of E64 for a time shown to yield complete inhibition based on measured second order rate constants for E64 inhibition. Residual cathepsin activity was then measured in standard fluorogenic substrate assays (described below) and plotted as a function of E64 concentration. The x-intercept corresponding to complete inhibition yielded the active enzyme concentration. Turnover numbers for hydrolysis of substrates by cathepsins under standard conditions (described below) were calculated based on active-site titration data and served as calibrators of functional enzyme activity in all experiments. To measure the kinetics of inhibition, reactions were done at 25˚C under pseudo-first-order conditions in which the concentration of Spi2A was at least 5- to 10-fold greater than the concentration required to completely inhibit the enzyme activity (38). Reactions were initiated by adding a small aliquot of cathepsin to a solution of Spi2A in 100 µl reaction buffer and then quenched after varying reaction times by adding 900 µl 50 µM fluorogenic substrate in reaction buffer. The residual enzyme activity was measured from the initial linear rate of fluorescence increase (380 nm excitation, 440 nm emission) due to hydrolysis of the substrate. The time-dependent loss in enzyme activity was fit by a single exponential decay function to obtain the pseudo-first-order inhibition rate constant. This was divided by the Spi2A concentration to give the second-order inhibition rate constant. Observed pseudo-first-order rate constants were found to increase in proportion to the Spi2A concentration in all cases, confirming that all reactions were bimolecular.

To measure the stoichiometry of inhibition, 100 nM cathepsin was incubated with increasing molar ratios of Spi2A to cathepsin (0–500 nM) in 50 µl reaction volumes at 25˚C for a time that yielded complete reaction (>90%) based on measured second order rate constants and over which enzyme activity remained stable. Residual enzyme activity was then measured by adding 950 µl 50 µM substrate directly to the reaction mixture or to a suitable dilution of this mixture, and the initial rate of substrate hydrolysis was measured as in the kinetics experiments. A plot of residual enzyme activity versus molar ratio of Spi2A to enzyme concentration yielded the stoichiometry of inhibition from the x-axis intercept.
**Impaired homeostatic proliferation of Spi2A KO memory phenotype CD44hiCD8+ T cells**

Memory CD8+ T cells undergo a low rate of cell division in the absence of overt Ag stimulation, referred to as homeostatic proliferation. The bone marrow is the preferred site for the homing and HSP of memory CD8+ T cells (8–10). The CD44hiCD8+ T cells in the bone marrow of 15-wk-old Spi2A KO mice had significantly (p = 0.0008) lower levels of HSP measured by BrdU incorporation in vivo (39) (Fig. 1C). This requirement for Spi2A was also specific for CD8+ T cells, as CD44hiCD4+ T cells in the same mice showed no HSP difference (Fig. 1C). Naive CD44hiCD4+ T cells either CD8+ or CD4+) showed very little BrdU incorporation, consistent with previous reports (41). We conclude that Spi2A is required for the homeostatic proliferation of memory phenotype CD44hiCD8+ T cells.

Memory CD8+ T cells require the cytokines IL-7 and IL-15 for survival and HSP in the bone marrow; however, they do not require continued TCR stimulation (42). Spi2A expression is up-regulated upon NF-kB activation, yet Spi2A expression continues long after infection in memory CD8+ T cells (29, 31). We found that IL-7 and IL-15, as well as IL-2, induced the expression of Serpina3g mRNA in CD44hiCD8+ T cells in vitro in the absence of additional TCR stimulation (Fig. 1D). We conclude that Spi2A is upregulated by these cytokines to ensure the maintenance of memory CD8+ T cells.

**Spi2A is required for the long-term maintenance and HSP of LCMV-specific memory CD8+ T cells, but it is redundant for determining the level of memory cell precursors**

Although memory phenotype CD44hiCD8+ T cells, which are generally stimulated by environmental Ags, share many similarities with Ag-specific memory CD8+ T cells stimulated by experimental infections, they possess some differences in their maintenance requirements (42). To address the role of Spi2A in an Ag-specific memory CD8+ T cell response, we examined CD8+ T cells after acute infection with LCMV (29, 35). Tetramer staining was used to identify CD8+ T cells specific for the gp33 LCMV epitope after LCMV infection (Fig. 2A). We further examined these Ag-specific CD8+ T cells by examining their surface expression of phenotypic markers. These markers define CD8+ subsets that possess various functional properties: a memory precursor phenotype (IL-7RhiKLRG1lo) or a TCM (CD62L+) phenotype. With time, the long-term Ag-specific memory CD8+...
T cell population will gradually become CD62Lhi, IL-7Rhi, and KLRG1lo (4, 5, 43).

Both WT and Spi2A KO mice showed a similar expansion of Ag-specific CD8+ T cells 8 d p.i. (Fig. 2B). They also showed a similar subsequent contraction in the number of LCMV-specific CD8+ T cells (Fig. 2B). Additionally, we did not observe any difference in the proportion of IL-7RhiKLRG1lo memory precursor cells among the gp33-specific CD8+ population at any time between WT and Spi2A KO mice (Fig. 2B). Therefore, we found no evidence to indicate that Spi2A is solely required for the survival of early memory precursor cells. This contradicts an earlier report of decreased CTL levels after antisense knockdown of Spi2A mRNA (29). We have since discovered that other Serpina3 family genes are also upregulated in activated T cells (Supplemental Fig. 1). These genes are nearly identical to Spi2A (>90% homology in the case of Serpina3f) except for some variability in the region encoding the reactive center loop, which determines the specifity of protease inhibition (30). Therefore, the earlier antisense approach likely also reduced the expression of homologous, but distinct, mRNAs related to Spi2A. In Spi2A KO mice, Spi2A mRNA expression was specifically ablated by targeting Serpina3g exon 4 (A. Dev et al., submitted for publication). Our present study reveals redundancy between Spi2A and other serpins in determining the survival of memory precursor cells in the clonal burst phase of the CD8+ immune response.

However, from day 130 p.i. onwards, we observed significantly lower numbers of gp33-specific memory CD8+ T cells in Spi2A KO compared with WT mice (spleen, p = 0.05; inguinal lymph node [ILN], p = 0.04) (Fig. 2B). Throughout the Ag-specific CD8+ T cell response, the proportion of IL-7RhiKLRG1lo cells remained similar between WT and Spi2A KO mice, and by day 130, these cells were mainly IL-7RhiKLRG1lo (Fig. 2B). Therefore, the absolute number of IL-7RhiKLRG1lo memory gp33+CD8+ cells also decreased correspondingly in Spi2A KO mice (spleen, p = 0.02; ILN, p = 0.03).

However, in the long-term memory phase after infection, the proportion of central memory CD62Lhi cells in the gp33+CD8+ T cell population was significantly reduced in Spi2A KO mice compared with WT controls (spleen, p = 0.02; bone marrow, p = 0.02) (Fig. 2C). The absolute number of TCM gp33+CD8+ T cells was also significantly decreased in Spi2A KO mice compared with WT (spleen, p = 0.04; bone marrow, p = 0.04) (Supplemental Table III).

Furthermore, we observed a significant decrease in the percentage of BrdU+ memory gp33+CD8+ T cells in the bone marrow of Spi2A KO mice (p = 0.04) (Fig. 2D). This proliferation

FIGURE 3. Cell-intrinsic impaired maintenance of Spi2A-deficient CD8+ memory T cells after viral infection. CD8+ splenocytes from gp33 epitope-specific P14 TCR transgenic WT (CD45.1+) mice and Spi2A KO (CD45.2+) mice were isolated and mixed in an even ratio. Five thousand P14+CD8+ cells were adoptively transferred into CD45.1+2+ hybrid congenic recipients, which were subsequently infected with LCMV. (A) Competitive adoptive transfer experiment. (B) Flow cytometry analysis of gp33+CD8+ splenocytes showing CD45.1+ (WT) donor, CD45.2+ (KO) donor, and CD45.1+2+ endogenous populations. (C) Upper row: Total number of gp33+CD8+ cells in various organs after LCMV infection. ILN indicates all ILN; bone marrow is from one femur. IHL, intrahepatic lymphocyte. Lower row: Ratio of Spi2A KO to WT among donor P14+CD8+ cells. Initial ratio of 1 is indicated by dotted line. (D) BrdU incorporation of P14+CD8+ cells in bone marrow after 1 wk BrdU administration in mice 70–75 d p.i. Significance values were calculated by a paired Student two-tailed t test. Data show mean ± SEM of at least six mice. Results are representative of at least two independent experiments.
difference occurred even within the CD62L+gp33+CD8+ cell subset, demonstrating that this effect was not simply due to Spi2A KO mice having fewer TCM (Fig. 2D). Therefore, Spi2A is solely required for the full long-term maintenance and HSP of Ag-specific memory CD8+ T cells in vivo.

Cell-intrinsic effect of Spi2A on memory CD8+ T cells

To isolate the cell-intrinsic requirements for Spi2A in memory CD8+ T cell development, Spi2A-deficient gp33+CD8+ T cells were examined alongside competing WT gp33+CD8+ T cells in a shared WT environment. Naive Spi2A KO (CD45.2+) CD8+ T cells expressing the P14 transgenic TCR specific for the LCMV peptide gp33/H-2Db (5) were mixed with WT CD45.1+P14+CD8+ T cells in a 1:1 ratio. Five thousand P14+CD8+ cells were adoptively transferred into CD45.1+2+ hybrid congenic C57BL/6 recipients, which were infected with LCMV (Fig. 3A). Spi2A-deficient and WT donor P14+CD8+ T cells were identified by the expression of congenic markers (Fig. 3B). The kinetics of LCMV-specific CD8+ T cell numbers during the expansion (day 8 p.i.), contraction (day 15 p.i.), and memory phases (day 70 p.i. onwards) matched those previously reported for this virus model (29, 35) (Fig. 3C). The ratio of Spi2A KO to WT P14+ cells remained equivalent during the expansion phase; however, during the memory phase, the ratio of Spi2A KO to WT P14+ cells dropped to <1 in all of the lymphoid organs studied (Fig. 3C). Therefore, our findings indicate that although Spi2A was not solely required for CTL during the initial clonal expansion (5, 35), Spi2A was required for ensuring the continued maintenance of LCMV-specific memory CD8+ T cells. These findings match our earlier results in intact LCMV-infected Spi2A KO mice (Fig. 2B).

The ratio of P14+ cells in the bone marrow during the expansion and contraction phases differed from the other organs studied. It has since been reported that polymorphisms present in the CD45.1 congenic interval impair CD45.1+ hematopoietic stem cell homing to the bone marrow, independent of apoptosis or proliferation (44). Similarly, these polymorphisms also appear to impair CD45.1+ CD8+ T cell migration to the bone marrow during the contraction phase. However, even with such an impairment, CD45.1+ WT P14+CD8+ T cells still outcompeted their Spi2A KO CD45.2+ counterparts, as the Spi2A KO to WT ratio of P14+ cells eventually declined to <1 in the bone marrow during the memory phase (Fig. 3C).

As in complete Spi2A KO mice, the adoptively transferred Spi2A-deficient memory P14+CD8+ T cells underwent less homeostatic proliferation compared with their WT counterparts in the same mice (Fig. 3D). We conclude that Spi2A is required in a cell-intrinsic manner to ensure the maintenance and HSP of memory CD8+ T cells following an acute LCMV infection.

Spi2A deficiency leads to erosion of Ag-specific memory CD8+ T cell populations after successive infections

An important physiological requirement for memory T cell populations is persistence through multiple rounds of reinfection (2). Because Spi2A acted to ensure the maintenance and HSP of...
primary memory CD8+ T cells, we wanted to determine whether it was also required to ensure the survival of secondary and tertiary CD8+ memory cells. Memory P14+CD8+ splenocytes from an initial adoptive transfer (1˚, as described in Fig. 3) were isolated and retransplanted into naive CD45.1+2+ recipients (Fig. 4A, 4B). Upon this secondary adoptive transfer (2˚) and reinfection, the ratio of Spi2A KO to WT P14+CD8+ cells further decreased to 0.51 after 231 d (Fig. 4C). After a third successive adoptive transfer (3˚) into naive CD45.1+2+ recipients, the ratio of Spi2A KO to WT P14+CD8+ cells decreased again to 0.25 after 93 d (Fig. 4C). Control experiments transferring 5000 memory gp33+CD8+ cells from either WT or Spi2A KO mice into congenic recipients revealed equal amounts of re-expansion after LCMV infection (Supplemental Fig. 2). As we had observed with primary memory cells (Fig. 3), Spi2A is required to maintain secondary and tertiary memory CD8+ T cells. Therefore, Spi2A protects the Ag-specific memory CD8+ T cell population from erosion after successive rounds of viral infection.

Cathepsin B-driven lysosomal permeabilization in Spi2A KO memory cells

Lysosomal membrane permeabilization (LMP) results in the release of cathepsins into the cytosol and the induction of cell death (45). The release of cathepsin B also degrades and permeabilizes the lysosomal membrane in a positive feedback loop (31, 46). Lysosomes in live FACS-purified CD8+ splenocytes were marked with LysoTracker Green, a fluorescent probe that accumulates in intact acidic organelles, and then visualized by confocal fluorescence microscopy (Fig. 5A). LysoTracker labeling is lost upon LMP and the resulting increase in pH (47). WT memory phenotype CD44hiCD8+ cells possessed significantly greater LysoTracker fluorescence compared with naive CD44loCD8+ cells ($p = 0.0001$) (Fig. 5B) due to more acidic intact lysosomes indicated by a higher number of LysoTracker puncta (Fig. 5A). This indicates more secretory lysosomes and/or level of metabolic activity in memory phenotype versus naive CD8+ T cells (48).

Significantly fewer intact lysosomes were found in memory phenotype CD8+ cells from Spi2A KO mice ($p = 0.0001$) (Fig. 5B), with clearly fewer puncta of LysoTracker visible (Fig. 5A). To determine whether increased cathepsin B activity contributed to LMP in Spi2A KO memory phenotype CD8+ cells, we crossed Spi2A KO mice to cathespin B KO (Cath B KO) mice (33). In the absence of cathepsin B, the number of lysosomes in Spi2A KO × Cath B KO memory phenotype CD8+ cells returned to the WT level (Fig. 5). Thus, the reduced number of lysosomes in Spi2A KO mice was not due to impaired development or organelle organization. Rather, Spi2A ensures lysosomal integrity by preventing cathepsin B-mediated LMP and the resulting initiation of the lysosomal pathway of death.

**Spi2A is a direct inhibitor of cathepsin B**

To confirm that Spi2A is a direct inhibitor of cathepsin B, we showed that recombinant Spi2A produced from *E. coli* inhibited cathepsin B with a stoichiometry of 10:1 giving a corrected rate of $6 \times 10^3$ M$^{-1}$ s$^{-1}$ (Fig. 6A, 6B) (49). Examination of a panel of papain-like cysteine cathepsins gave a range of rates and stoichiometries of inhibition by Spi2A (Fig. 6C). SDS-PAGE revealed the presence of intact cathepsin B and cleaved Spi2A as the only visible products of the reaction because of the instability of serpin/cysteine protease complexes to SDS denaturation (Fig. 6D) (50). Therefore, as has been observed with other cross class-specific serpins, Spi2A inhibits cathepsin B by forming a serpin/protease inhibitory complex whose thioester linkage is unstable under reducing conditions (51).

**Cathepsin B deficiency restores the endogenous memory phenotype CD44hiCD8+ T cell population in Spi2A KO mice**

We determined whether cathepsin B was a physiological target for the Spi2A-mediated maintenance of memory CD8+ T cells in vivo. To examine this, we crossed Spi2A KO mice to Cath B KO mice (33). Cath B KO mice appear normal but are resistant to TNF-$
\alpha$-induced hepatocyte apoptosis (20). Flow cytometry staining revealed a similar number of memory phenotype CD44hiCD8+ T cells in the bone marrow of 15-wk-old Spi2A KO × Cath B KO mice compared with WT controls. Cath B KO mice also possessed WT levels of memory phenotype CD44hiCD8+ T cells. Therefore, cathepsin B deficiency alone did not produce a global increase in cell number (Fig. 7A). Rather, cathepsin B deficiency specifically rescued the memory phenotype CD44hiCD8+ T cell population in Spi2A KO × Cath B KO mice. In addition to rescuing the number of cells, cathepsin B deficiency also restored the impaired homeostatic proliferation of memory phenotype
CD44hiCD8+ cells in Spi2A KO mice (Fig. 7B). Therefore, the inhibition of cathepsin B is a physiological mechanism by which Spi2A ensures the maintenance and HSP of endogenous memory phenotype CD8+ T cells.

Cathepsin B deficiency rescues the LCMV-specific central memory CD8+ T cell population in Spi2A KO mice

Next, we determined whether cathepsin B inhibition was a mechanism by which Spi2A maintained the level and self-renewal of CD44hiCD8+ cells in Spi2A KO mice (Fig. 7B). Therefore, the inhibition of cathepsin B is a physiological mechanism by which Spi2A ensures the maintenance and HSP of endogenous memory phenotype CD8+ T cells.

FIGURE 6. Kinetics and stoichiometry of cathepsin inhibition by Spi2A. (A) Progress curve for the inhibition of cathepsin B (5 nM) by Spi2A (440 nM). The solid line is a fit by a single exponential decay function. (B) Cathepsin B (100 nM) was incubated with the indicated molar ratios of Spi2A for a time sufficient to reach the reaction endpoint and then assayed for residual enzyme activity as in the kinetics experiment. The x-axis intercept of the solid linear regression fit of the data represents the stoichiometry of inhibition. (C) Second-order rate constants and stoichiometries of inhibition (SI) for the reactions of Spi2A with different cathepsins. The corrected second-order rate constants for reaction through the inhibitory pathway were obtained by multiplying the apparent second-order rate constant by the stoichiometry of inhibition (50). (D) SDS-PAGE analysis (10% gel) of the reaction of Spi2A with cathepsin B. Shown are 0.5 μM cathepsin B alone (lane 1), 2 μM Spi2A with 0.5 μM cathepsin B reacted for 90 min (lane 2), and 2 μM Spi2A alone (lane 3). Intact cathepsin B and cleaved Spi2A are the only visible products of the reaction because of the instability of serpin/cysteine protease complexes to SDS denaturation.

FIGURE 7. Concomitant cathepsin B deficiency restores the reduced number and homeostatic proliferation of Spi2A-deficient memory phenotype CD44hiCD8+ and memory CD8+ T cells. A and B. Fifteen-week-old mice were dissected and stained as in Fig. 1. (A) Number of CD44hiCD8+ T cells present in one femur. (B) BrdU incorporation of CcsD44hiCD8+ T cells after 1 wk BrdU administration. Significance values were calculated by two-way ANOVA with a Bonferroni posttest. C and D. CD8+ splenocytes from gp33 epitope-specific P14 TCR transgenic WT (CD45.1+) mice and either Spi2A KO, Spi2A KO x Cath B KO, or Cath B KO (CD45.2+) mice were isolated and mixed in even ratios. Five thousand P14+CD8+ cells were adoptively transferred into CD45.1+2+ hybrid congenic recipients, which were subsequently infected with LCMV, as described in Fig. 3. (C) Ratio of Spi2A KO, Spi2A KO x Cath B KO, or Cath B KO to WT among donor P14+CD8+ cells in the spleen 70–76 d p.i. Significance values were calculated by one-way ANOVA with a Bonferroni posttest. (D) BrdU incorporation of P14+CD8+ cells in spleen and bone marrow after 1 wk BrdU administration in mice 70–75 d p.i. Significance values were calculated by a paired Student two-tailed t test. For all panels, data show mean ± SEM of at least five mice and are representative of at least two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
LCMV-specific memory CD8+ T cells. Spi2A KO × Cath B KO mice and Cath B KO mice were infected with LCMV and analyzed by flow cytometry after 130 d p.i. as in Fig. 2. Spi2A KO × Cath B KO mice possessed WT numbers of memory gp33 tetramer+ CD8+ T cells (Fig. 8A). As we observed for endogenous memory phenotype CD44hiCD8+ T cells (Fig. 7), cathepsin B deficiency itself did not increase the LCMV-specific memory CD8+ T cell population above WT levels (Fig. 8A).

Furthermore, the proportion of CD62L+ TCM within the Ag-specific memory CD8+ T cell population in both Spi2A KO × Cath B KO and Cath B KO mice remained at WT levels (Fig. 8B). In addition to restoring the level of LCMV-specific memory CD8+ cells, cathepsin B deficiency also corrected the defective homeostatic proliferation in the bone marrow (Fig. 8C). We conclude that inhibition of cathepsin B by Spi2A ensures the maintenance and HSP of long-lived LCMV-specific memory CD8+ T cells.

The inhibition of cathepsin B by Spi2A in memory CD8+ T cells was also cell-intrinsic, as demonstrated by the equivalent survival of Spi2A KO × Cath B KO compared with WT memory P14+CD8+ T cells in competitive adoptive transfer experiments >70 d after LCMV infection (Fig. 7C). Likewise, the ratio of Cath B KO to WT memory P14+CD8+ T cells also remained unchanged (Fig. 7C). The cell-intrinsic effect of Spi2A’s inhibition of cathepsin B extended to homeostatic proliferation, which was also restored to a WT level in Spi2A KO × Cath B KO P14+CD8+ T cells in the bone marrow (Fig. 7D). Therefore, we conclude that Spi2A can act directly within CD8+ T cells to inhibit cathepsin B and ensure the survival and self-renewal of long-term memory cells.

**Discussion**

Memory T lymphocytes persist for the life of the organism to retain the history of previous pathogen encounters. Survival cytokines (including IL-7 and IL-15) control the homeostasis of long-term memory CD8+ T cells, and yet the cell-intrinsic protective mechanisms that they trigger to regulate these populations are poorly understood. We present a model for the long-term maintenance of memory CD8+ T cell populations whereby these cytokines induce Spi2A to counteract the limitation placed on persistence by lysosomal permeabilization.

Previous work from our laboratory has shown that reducing Spi2A expression using an antisense message decreases the number of CTL and memory CD8+ T cells (29). This is in contrast with our current findings with Spi2A KO mice, which show that the level of CTL is not affected but that only memory CD8+ T cells are affected. Other homologous Serpina3 family genes, distinguished by their unique reactive centers (30), are also upregulated by activated T cells (Supplemental Fig. 1). Therefore, a major shortcoming of the earlier study was that the antisense approach most likely knocked down the expression of homologous, but distinct, mRNAs related to Spi2A. The knockout strategy in the current study specifically ablated Spi2A mRNA expression by targeting Serpina3g exon 4, and so resolved the shortcomings of the earlier approach.

Although Spi2A overexpression can increase the number of CTL during clonal expansion (29), we now show that Spi2A is uniquely required for the maintenance, although not the development, of memory CD8+ T cells. This requirement was most pronounced in TCM, which exhibit long-term persistence and homeostatic proliferation to provide lasting immunity. Adoptive
transfer competition experiments demonstrated that Spi2A not only controlled the survival of memory CD8+ T cells after an initial infection, but it also prevented the erosion of this population after additional challenges. This predicts that protection from cathepsin B may be important in preserving memory CD8+ T cells after repeated infection with heterologous viruses (2).

We show that Spi2A is required to maintain intact lysosomes in memory phenotype CD8+ cells. The rescue of the levels of intact lysosomes in Spi2A KO × Cath B KO CD44+CD8+ cells demonstrated that cathepsin B release is a cause as well as a consequence of LMP in T cells. Although it is well established that lysosomal cathepsin B triggers PCD (45), the anti-apoptotic Bcl-2 family members that can be cleaved and inactivated by isolated cathepsin B in vitro (28) have yet to be shown to be targets in vivo. Spi2A acts in the cytosol to inhibit cysteine cathepsins after lysosomal permeabilization, and it protects fibroblasts from downstream PCD (31). Although Spi2A KO memory phenotype CD8+ T cells possess fewer intact lysosomes, we did not observe any difference in the protein levels of Bcl-2, Bcl-XL, Mcl-1, or Bim (Supplemental Fig. 3A). Therefore, at this time, the downstream death pathways triggered by LMP in Spi2A KO memory CD8+ T cells are not clear. The decrease in the number of intact lysosomes could also limit the ability of Spi2A KO memory CD8+ T cells to stave off PCD through the degradation of the contents of autophagosomal vesicles (16, 17, 52).

The resulting cathepsin B amplification loop for LMP may explain why Spi2A deficiency results in impaired maintenance of memory CD8+ T cells despite the relatively slow kinetics for recombinant Spi2A inhibition of purified cathepsin B. Spi2A inhibition of cathepsin B in vivo may also occur through faster kinetics and a lower stoichiometry of protease inhibition than that measured in vitro because of unknown cofactor molecules that localize or activate the reacting proteins, a common feature of other serpin–protease reactions (49). Because Spi2A has the capacity to inhibit several cathepsins (Fig. 6), Spi2A may also be required to protect cells from other cathepsins. Spi2A overexpression in CD8+ T cells increases the proportion of surviving memory cells (29); however, cathepsin B-deficient memory CD8+ T cells only showed WT levels of survival (Figs. 7, 8). The balance of cathepsin activity, and not cathepsin B exclusively, could be responsible for the Spi2A KO phenotype. However, because cathepsin B deficiency was sufficient to restore the number and HSP of Spi2A-deficient memory CD8+ T cells, the inhibition of another cathepsin may not be physiologically relevant in this context.

Another anti-apoptotic serpin, Spi6, protects CTL from granzyme B by maintaining the integrity of lysosomal-like cytotoxic granules (53). Spi2A and Spi6 appear to be similar in the way they protect CD8+ T cells from the breakdown of lysosomal vesicles. However, Spi6 ensures CTL survival and thereby determines the size of the clonal burst (53), but it plays no role in continued memory CD8+ T cell survival (35). Conversely, Spi2A ensures memory CD8+ T cell maintenance and thereby determines the size of the memory pool, but it has no effect on CTL and the clonal burst. This division of labor between Spi6 and Spi2A presumably reflects the relative importance of different executioner proteases (granzyme B for CTL; cathepsin B for memory CD8+ cell) in controlling CD8+ T cell survival after viral infection.

Although the effector memory (CD62L−) CD8+ T cell population may expand after heterologous infections, the total number of central memory CD8+ T cells remains fixed (54). The cytokines IL-7 and IL-15 are important for the survival and homeostasis of TCM (54–57). However, the cell-intrinsic mechanisms by which these cytokines ensure memory CD8+ T cell survival have remained unclear. IL-7 and IL-15 signaling upregulates expression of other anti-apoptotic molecules, including Bcl-2 and Bcl-xL (42). However, overexpression of Bcl-2 or Bcl-xL did not affect the level of memory CD8+ T cells in vivo (58, 59). IL-7 also induces Mcl-1, which is also required for T cell survival (13), but a role in memory CD8+ T cell homeostasis has not been tested. We show that IL-7 and IL-15 signaling also upregulates Spi2A, and that Spi2A determines the level and HSP of TCM by protecting these cells from intracellular cathepsin B.

The greatest reduction of Spi2A-deficient memory CD8+ T cells occurred in the bone marrow, a major reservoir for TCM (10). The bone marrow is also the predominant site of memory CD8+ T cell homeostatic proliferation due to signals provided by the organ microenvironment (8, 9). For full turnover, memory CD8+ T cells must receive the IL-15 signal trans-presented by these niches (55, 60). Consistent with this, the decline and reduced homeostatic proliferation of memory CD8+ T cells seen in IL-15 KO mice resembles that observed in Spi2A KO mice (55, 57). Additionally, we did not observe a significant increase in the basal level of PCD in either memory phenotype or Ag-specific memory CD8+ T cells from Spi2A KO mice, as evidenced by staining with the apoptosis dyes annexin V or YoPro1 (Supplemental Figs. 3B, 3C). However, given the well-documented anti-apoptotic function of Spi2A (29, 31), a subtle increase in PCD (below our level of detection in ex vivo memory T cells) is still the most likely for the decreased HSP of Spi2A KO memory cells. Our findings support a model in which IL-7 and IL-15 control memory CD8+ T cell survival through protection by Spi2A from cathepsin B.

A variety of apoptotic stimuli can induce lysosomes to release their contents, including cathepsin B, into the cytosol, where they trigger PCD (27). Our results suggest that not only is the lysosomal pathway of cell death involved in pathological processes (27), but it also plays a physiological role in the resting homeostasis of central memory CD8+ T cells. The ability of Spi2A to inhibit cytosolic cathepsin B activity for full memory CD8+ T cell maintenance would be particularly important in multiple natural infections with viruses or certain vaccination regimes. Thus, the identification of Spi2A as a survival factor would predict that pharmacological inhibition of cathepsins (29, 61) could potentially increase the population of persisting long-lived memory CD8+ T cells, leading to less attrition over time and prevention of chronic viral infections.

**Acknowledgments**

We thank M. Birrell for hematology analysis and N. Liu, M. Zhang, and L. Levine for help with generating the Spi2A KO mice.

**Disclosures**

The authors have no financial conflicts of interest.

**References**

30. Forsyth, S., A. Horvath, and P. Coughlin. 2003. A review and comparison of the
31. Liu, N., S. M. Raja, F. Zazzeroni, S. S. Metkar, R. Shah, M. Zhang, Y. Wang,
10. Mazo, I. B., M. Honczarenko, H. Leung, L. L. Cavanagh, R. Bonasio,
11. Tokoyoda, K., S. Zehentmeier, A. N. Hegazy, I. Albrecht, J. R. Grün,
30. Forsyth, S., A. Horvath, and P. Coughlin. 2003. A review and comparison of the
30. Forsyth, S., A. Horvath, and P. Coughlin. 2003. A review and comparison of the
30. Forsyth, S., A. Horvath, and P. Coughlin. 2003. A review and comparison of the
30. Forsyth, S., A. Horvath, and P. Coughlin. 2003. A review and comparison of the
30. Forsyth, S., A. Horvath, and P. Coughlin. 2003. A review and comparison of the
30. Forsyth, S., A. Horvath, and P. Coughlin. 2003. A review and comparison of the
30. Forsyth, S., A. Horvath, and P. Coughlin. 2003. A review and comparison of the