

**Figure 3** | FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells produce T<sub>H</sub>2 cytokines and support B1 cell proliferation. **a**, FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells (5,000 cells per well) were cultured with the indicated cytokines (10 ng ml<sup>-1</sup>) for 8 days. Bottom panels show flow cytometry of cultured FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells on day 8. **b**, FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells, and CD4<sup>+</sup> T cells from MLN or spleen (1 × 10<sup>4</sup> cells per well) were cultured with the indicated reagents for 3 days, and culture supernatants were analysed in triplicate by ELISA for the indicated cytokines. ConA, concanavalin A; P+I, PMA plus ionomycin. **c**, FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells (5,000 cells per well) were cultured with the indicated cytokines (10 ng ml<sup>-1</sup>) for 5 days, and culture supernatants were analysed in triplicate by ELISA. **d**, Intracellular cytokine staining of FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells stimulated with the indicated cytokines, and T<sub>H</sub>1 and T<sub>H</sub>2 cells stimulated with PMA plus ionomycin. **e**, CFSE-labelled B cells (5 × 10<sup>5</sup>) isolated from the peritoneal cavity were cultured with 1.8 × 10<sup>5</sup> FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells (red line) or 10 ng ml<sup>-1</sup> IL5 (blue line) as described in Methods. CFSE dilution patterns were examined on CD19<sup>+</sup> CD23<sup>-</sup> B1 cells and CD19<sup>+</sup> CD23<sup>+</sup> B2 cells. **f**, CFSE-labelled B1 cells were transferred intraperitoneally into Rag2<sup>-/-</sup> or gc<sup>-/-</sup> Rag2<sup>-/-</sup> mice (2.5 × 10<sup>5</sup> per mouse) with the indicated cells. CFSE dilution patterns of B1 cells in recipient mice (three mice per group) were examined on day 9 (top panels). Percentages of cells dividing 0, 1, 2 or 3 times were calculated and the statistics of differences of undivided cells are shown (bottom panel). **g**, Reduction of FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells (left) and peritoneal B1 cells (right) after administration of anti-IL7Rα as described in Methods. PEC, peritoneal exudate cells. All results are representatives of two to three independent experiments. Data in bar charts are shown as the mean and s.e.m.

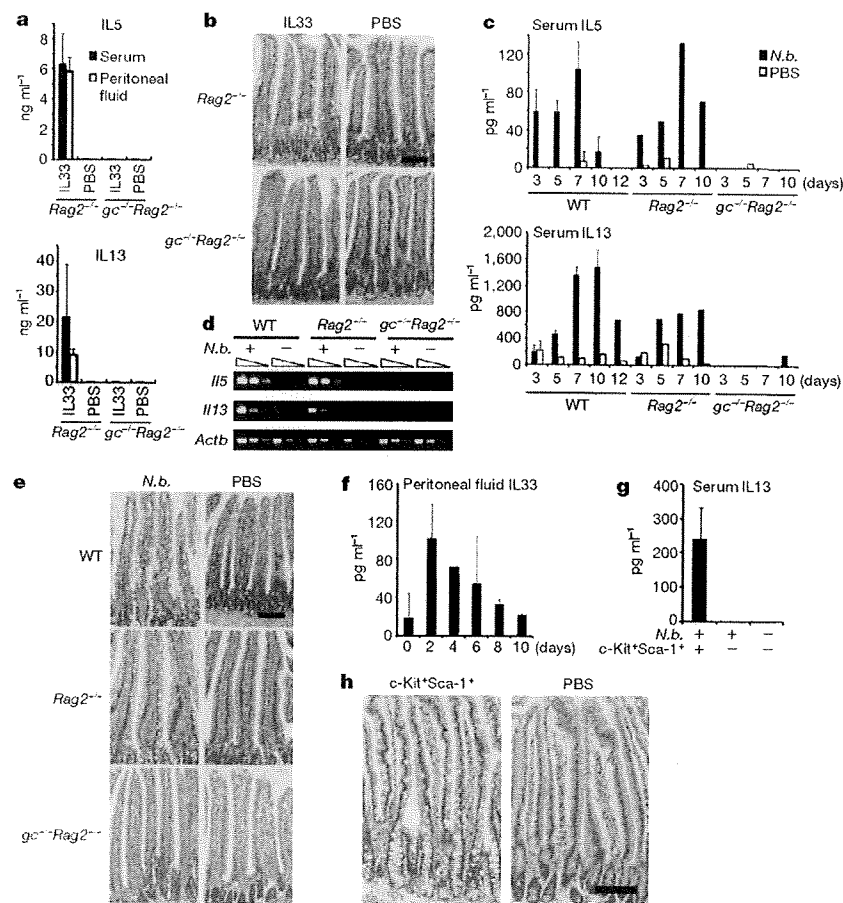
but the amounts produced were considerably lower (Supplementary Fig. 7).

IL5 is a critical growth factor for B1 cells, which are abundant in the peritoneal cavity and have a crucial role in innate-type immune responses by producing natural antibodies<sup>7</sup>. Peritoneal cavity B cells comprised of both B1 and B2 cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) and cultured with or without FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells. As shown in Fig. 3e, FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells induced the division of CD23<sup>-</sup> B1 cells, as did the addition of recombinant IL5, whereas CD23<sup>+</sup> B2 cells barely divided. Such B1 cell division was blocked by the addition of anti-mouse IL5 neutralizing antibody (TRFK5) (data not shown). Comparison of cell division of CFSE-labelled B1 cells transferred into Rag2<sup>-/-</sup> mice bearing FALCs or gc<sup>-/-</sup> Rag2<sup>-/-</sup> mice devoid of FALCs demonstrated that B1 cells transferred into Rag2<sup>-/-</sup> mice divided more (Fig. 3f). Co-transfer of FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells but not CD4<sup>+</sup> T cells into gc<sup>-/-</sup> Rag2<sup>-/-</sup> mice induced B1 cell division (Fig. 3f). The administration of anti-IL7Rα but not anti-c-Kit monoclonal antibody resulted in the reduction of FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells and peritoneal B1 cells (Fig. 3g and data not shown). These results collectively indicate that FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells support the self-renewal of B1 cells *in vivo*.

IL5 and IL6 regulate B-cell antibody production<sup>5,6</sup>. When FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells were co-cultured with splenic B cells, IgA production was induced (Supplementary Fig. 8), suggesting that FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells have a helper function in IgA secretion. Because there was no increase in the number of surface IgA<sup>+</sup> cells after the cultivation of B cells with FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells (data not shown), FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells probably accelerate the secretion of IgA from a small population of IgA<sup>+</sup> cells present in the culture rather than inducing class switch recombination.

IL5 and IL13 induced by IL25 or IL33 are also important for the mediation of allergic inflammation and protection against helminth infection<sup>8,9,20</sup>. In particular, IL13 has a critical role in goblet cell hyperplasia, which is crucial for mucin secretion leading to helminth expulsion<sup>8,9</sup>. Although IL25 produced by eosinophils and basophils<sup>21</sup> and IL33 produced by endothelial cells, epithelial cells and adipocytes<sup>22,23</sup> induce IL13, the identity of cells producing IL13 has been obscure<sup>9,21,24</sup>. Administration of IL33 to Rag2<sup>-/-</sup> but not gc<sup>-/-</sup> Rag2<sup>-/-</sup> mice induced the production of IL5 and IL13 and goblet cell hyperplasia (Fig. 4a, b). Because gc<sup>-/-</sup> Rag2<sup>-/-</sup> mice have mast cells and basophils, the above results indicate that FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells are the major cell population producing IL5 and IL13 *in vivo* in response to IL33. After *Nippostrongylus brasiliensis* infection, IL5 and IL13 were readily detected in the sera of wild-type and Rag2<sup>-/-</sup> but not gc<sup>-/-</sup> Rag2<sup>-/-</sup> mice (Fig. 4c). Induction of messenger RNAs for IL5 and IL13 in the mesentery and goblet cell hyperplasia were observed in wild-type and Rag2<sup>-/-</sup> but not gc<sup>-/-</sup> Rag2<sup>-/-</sup> mice (Fig. 4d, e). *N. brasiliensis* infection induced sufficient IL33 in the peritoneal cavity of Rag2<sup>-/-</sup> mice to induce IL13 from FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells (Fig. 4f and Supplementary Fig. 9). Adoptive transfer of FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells into the peritoneal cavity resulted in the production of serum IL13 and goblet cell hyperplasia in helminth-infected gc<sup>-/-</sup> Rag2<sup>-/-</sup> mice (Fig. 4g, h). CD4<sup>+</sup> T-cell transfer did not induce serum IL13 until 2 weeks later when 100–300 pg ml<sup>-1</sup> IL13 was detected in the sera. These results indicate that the contribution of FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells to the production of IL13 and goblet cell hyperplasia after *N. brasiliensis* infection is independent of T cells.

FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> IL7Rα<sup>+</sup> T1/ST2<sup>+</sup> cells proliferate in response to IL2 and produce large amounts of T<sub>H</sub>2 cytokines, which support the self-renewal of B1 cells and goblet cell hyperplasia after helminth infection. Such IL2-dependent growth and innate-type effector functions are shared by NK cells, a T<sub>H</sub>1 type innate lymphocyte subset. Hence, we propose that FALC c-Kit<sup>+</sup> Sca-1<sup>+</sup> IL7Rα<sup>+</sup> T1/ST2<sup>+</sup> cells are T<sub>H</sub>2 type innate lymphocytes and that this population therefore be called natural helper cells.



**Figure 4 | FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells produce IL5 and IL13 in response to IL33 and induce goblet cell hyperplasia after helminth infection.** **a, b**, IL33 (0.125 µg in PBS) or PBS alone was administered intraperitoneally to *Rag2*<sup>-/-</sup> or *gc*<sup>-/-</sup>*Rag2*<sup>-/-</sup> mice (two mice per group) every 2 days three times. **a**, Cytokine levels in the serum and peritoneal fluid were determined in triplicate by ELISA on day 7. **b**, Goblet cell hyperplasia in the small intestine was examined on day 7. Goblet cells are seen as white round circles along the surface of villi. **c–e**, The indicated strains of mice were infected by subcutaneous injection of 500 *N. brasiliensis* (*N.b.*) larvae. **c**, Serum cytokine levels were analysed in triplicate by ELISA on the indicated days after infection. The numbers of wild-type (WT), *Rag2*<sup>-/-</sup> and *gc*<sup>-/-</sup>*Rag2*<sup>-/-</sup> mice analysed each day were 2, 1 and 1, respectively. Experiments were

performed twice and similar results were obtained. **d**, Expression of *Il5* and *Il13* mRNAs by mesenteric cells was determined by RT-PCR 14 days after infection. **e**, Goblet cell hyperplasia in the small intestine on day 10 after infection. **f**, Production of IL33 in the peritoneal cavity after *N. brasiliensis* infection. The peritoneal cavity of two mice was washed with 300 µl PBS on each day and assayed for IL33 in triplicate. **g, h**, FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells (2.5 × 10<sup>5</sup>) or PBS alone were transferred intraperitoneally into *gc*<sup>-/-</sup>*Rag2*<sup>-/-</sup> mice 1 month after *N. brasiliensis* infection (two mice per sample). One week after cell transfer, serum IL13 levels (**g**) and goblet cell hyperplasia in the small intestine (**h**) were examined. All scale bars, 200 µm. Results are representative of two to three independent experiments. Data in bar charts are shown as the mean and s.e.m.

**METHODS SUMMARY**

**Preparation of FALC cells.** Mesenteries separated from intestine and mesenteric lymph nodes were cut into small fragments with scissors, and digested in DMEM containing 2 mg ml<sup>-1</sup> collagenase type I and 4% BSA. The supernatant was aspirated off after centrifugation to remove adipocytes. Finally, cells were suspended in HBSS containing 10% FCS after filtration through a 32-µm nylon mesh.

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Author Contributions** K.M. conceived the study, performed experimental work, and wrote the paper; T.Y. performed the pathological work; M.T. and T.T. performed the helminth infection experiments; T.I. and H.K. performed the lymphoid progenitor assay; J.-i.F., M.O. and H.F. performed experiments, interpreted data and provided intellectual input; and S.K. conceived the study and wrote the paper.

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## METHODS

**Mice.** Mice used in this study were on a C57BL/6 background unless otherwise stated, and maintained at Taconic or in our animal facility under specific pathogen-free conditions. Wild-type C57BL/6 mice were purchased from Japan SLC. *W/W<sup>w</sup>* mice<sup>25</sup>, *S/S<sup>s</sup>* mice<sup>26</sup> and *aly/aly* mice<sup>27</sup> were obtained from CLEA Japan. *W/W<sup>w</sup>* and *S/S<sup>s</sup>* mice were on a WBB6F1 background and we used wild-type mice on a WBB6F1 background as control mice. *Rag2<sup>-/-</sup>* (stock no. RAGN12)<sup>28</sup>, *g<sup>c</sup>-/- Rag2<sup>-/-</sup>* (stock no. 4111)<sup>10,28</sup>, *Il15<sup>-/-</sup>* (stock no. 4269)<sup>29</sup>, and *nu/nu* (stock no. B6NU) mice were purchased from Taconic. *g<sup>c</sup>-/-* mice were obtained by mating *g<sup>c</sup>-/- Rag2<sup>-/-</sup>* mice with wild-type C57BL/6 mice. *Il15<sup>-/-</sup> Rag2<sup>-/-</sup>* mice were obtained by crossing *Il15<sup>-/-</sup>* and *Rag2<sup>-/-</sup>* mice. Inhibitor of DNA binding 2 (*Id2<sup>+/-</sup>* and *Id2<sup>-/-</sup>* mice<sup>16</sup> on a mixed genetic background (129/Sv × NMRI) were provided by H. Kiyono. *Rorc<sup>GFP/GFP</sup>* mice<sup>17</sup>, *Il2/15rb<sup>-/-</sup>* (also known as *Il2rb<sup>-/-</sup>*) mice<sup>30</sup> and *Il7<sup>-/-</sup>* mice<sup>31</sup> were provided by S. Fagarasan, T. W. Mak and K. Ikuta, respectively. All experiments were approved by the Animal Care and Use Committee of the Keio University and were performed in accordance with the institutional guidelines.

**Human samples.** The use of human samples from autopsies was generously permitted by the bereaved families.

**Antibodies and reagents.** Monoclonal antibodies specific for mouse c-Kit (2B8), Sca-1 (E13-161), CD25 (PC61), CD38 (90), CD44 (IM7), CD69 (HL2F3), Flt3 (A2F10.1), Thy-1.2 (CD90.2), FcγR (2.4G2), CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), TCRβ (H57-597), TCRδ (GL3), Gr-1 (RB6-8C5), erythroid cell marker (TER-119), CD19 (1D3), CD11c (HL3), Mac-1 (M1/70), NK1.1 (PK136), CD49b/pan-NK cells (Dx5), CD45 (104), B220 (RA3-6B2), CD40 (HM40-3), CD28 (37.51), IL4 (11B11), IL12 (C17.8), IFNγ (XMG1.2) and anti-human c-Kit monoclonal antibody (104D2) were purchased from BD Pharmingen. Monoclonal antibodies against mouse IL5 (TRFK5), c-Kit (ACK2), IL7Rα (A7R34), CD27 (LG.7F9), GITR (DTA-1), FcεRIα (MAR-1), IL13 (eBio13A) and anti-human IL7Rα monoclonal antibody (eBioRDR5) were purchased from eBioscience. Anti-mouse CD23 (B3B4) and anti-mouse T1/ST2 monoclonal antibodies were purchased from Caltag Laboratories and MD Bioscience, respectively. Allophycocyanin (APC)-conjugated streptavidin was used to reveal staining with biotinylated monoclonal antibodies. Four-colour flow cytometry was performed on a FACSCalibur (BD Bioscience). Cells were gated through a lymphocyte gate defined by FSC and SSC, and dead cells were gated out by propidium iodide.

mIL3, mIL4, mIL5, mIL6, mIL7, mIL9, mIL12, hFlt3l (also known as hFLT3LG), mSCF, mM-CSF, mGM-CSF and hTGFβ1 were purchased from PeproTech, hIL2 from Shionogi Pharmaceutical Co., mBAFF (also known as mTnfsf13b), mIL15, mTNFα, mIL25 and mIL33 were from R&D systems, LPS (*Escherichia coli* 055: B5), concanavalin A and PMA from Sigma, and ionomycin from Calbiochem.

**Preparation of cell suspensions.** Mice were transcardially perfused with 10 U ml<sup>-1</sup> heparin in PBS under nembutal anaesthesia. Mesenteric tissues were carefully separated from the intestines, and mesenteric lymph nodes were removed. Samples were cut into small fragments with scissors, transferred to a 15-ml tube in 5 ml of DMEM containing 2 mg ml<sup>-1</sup> collagenase type I (GIBCO) and 4% BSA (Sigma), then digested at 37 °C by shaking at 1g for 45 min. We added 10 μg ml<sup>-1</sup> DNase I (Roche) for human samples. The supernatant was aspirated off after centrifugation to remove adipocytes. Finally, cells were suspended in HBSS containing 10% FCS after filtration with a 32-μm nylon mesh. To purify Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells, cells were enriched for Lin<sup>-</sup> cells by negative sorting on an AutoMACS (Miltenyi Biotec GmbH) with a combination of magnetic beads conjugated with monoclonal antibodies against CD4, CD8α, CD11c, B220, NK1.1, Mac-1, Gr-1, TER119 and FcεRIα. Subsequently, samples were stained with monoclonal antibodies against other Lin markers (CD3, CD5, CD19, TCRβ, TCRδ), c-Kit and Sca-1, and sorted on a FACS Aria (BD Bioscience).

Bone-marrow-derived mast cells and basophils were prepared by culturing bone marrow cells with 20% WEHI-3 culture supernatant for 7 days, stained with monoclonal antibodies against c-Kit, FcεRIα and CD49b followed by sorting c-Kit<sup>+</sup>FcεRIα<sup>+</sup>CD49b<sup>-</sup> (mast cells) and FcεRIα<sup>+</sup>CD49b<sup>+</sup> cells (basophils). Thymic DN2 cells were prepared from adult thymocytes. Thymocytes were stained with magnetic beads conjugated with anti-CD4 and anti-CD8α monoclonal antibodies and CD4<sup>+</sup>CD8<sup>-</sup> double negative (DN) cells were negatively sorted by AutoMACS. Double-negative cells were further stained with anti-CD25 and anti-CD44 monoclonal antibodies, and CD25<sup>+</sup>CD44<sup>+</sup> cells were sorted as DN2 cells on a FACS Aria. Thy-1<sup>+</sup>CD4<sup>-</sup>LTi cells were prepared from fetal liver cells as described previously<sup>32</sup>. c-Kit<sup>+</sup>αβ<sub>2</sub><sup>+</sup>IL7Rα<sup>+</sup> fetal liver cells from day 13 embryos were cultured on TSt-4 cells for 17 days.

**Electron microscopy.** Sorted Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells were centrifuged, and the cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer,

pH 7.2, for 1 h. After washing, cells were post-fixed in 1% osmium tetroxide for 2 h, stained en bloc with uranyl acetate, dehydrated in a series of graded ethanol solutions, and embedded in an Epon/Araldite mixture. Ultrathin sections were stained with lead citrate and examined under an electron microscope (Nippon Denshi EX-200).

**Microarray analysis.** Total RNA was extracted from 5 × 10<sup>5</sup> FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells, LTi cells and DN2 cells after direct sorting into a vial containing ISOGEN LS (Nippon Gene). Total RNA was further purified using an RNeasy Micro Kit (QIAGEN) and amplified by a Two-Cycle Target Labelling method (Affymetrix). Microarray processing was done by the Central Research Laboratory, Keio University School of Medicine. The cRNA was hybridized to GeneChip mouse Genome 430 2.0 Array chips (Affymetrix). Hybridized chips were stained, washed and then scanned with a GeneChip Scanner 3000 7G (Affymetrix). Silicon Genetics software GeneSpring (Tomy Digital Biology) was used for data analysis.

**Measurement of cytokines.** FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells or CD25<sup>+</sup>CD11c<sup>-</sup>CD49b<sup>-</sup>CD4<sup>+</sup> cells sorted from the spleen and MLN were seeded at 1 × 10<sup>4</sup> cells per well or 5 × 10<sup>3</sup> cells per well into 96-well round bottom tissue culture plates in RPMI-1640 complete medium (RPMI-1640 medium, Sigma) containing 10% FCS, 50 μM 2-mercaptoethanol (GIBCO), 100 U ml<sup>-1</sup> penicillin and 100 μg ml<sup>-1</sup> streptomycin (GIBCO), 1 × non-essential amino acids (Sigma), 10 mM HEPES (Sigma), and 1 mM sodium pyruvate (GIBCO) with various stimulants including IL7 (10 ng ml<sup>-1</sup>), IL2 (10 ng ml<sup>-1</sup>), PMA (30 ng ml<sup>-1</sup>) plus ionomycin (500 ng ml<sup>-1</sup>), concanavalin A (10 μg ml<sup>-1</sup>), or LPS (5 μg ml<sup>-1</sup>). Supernatants were collected on day 3 or 5. Cytokine levels in culture supernatants were determined in triplicate by ELISA using Quantikine kits (R&D). Immunoglobulin concentrations were measured by Clonotyping System-HRP (SouthernBiotech). T<sub>H</sub>1 and T<sub>H</sub>2 cells were prepared by stimulating splenic CD4<sup>+</sup> cells in anti-CD3ε-coated plates (5 μg ml<sup>-1</sup> for 5 h) with anti-CD28 (1 μg ml<sup>-1</sup>) in the presence of a combination of IL12 (10 ng ml<sup>-1</sup>) and anti-IL4 (10 μg ml<sup>-1</sup>), or a combination of IL4 (10 ng ml<sup>-1</sup>), anti-IL12 (10 μg ml<sup>-1</sup>) and anti-IFNγ (10 μg ml<sup>-1</sup>), respectively, for 5 days. Intracellular cytokine staining was performed as follows. T<sub>H</sub>1 and T<sub>H</sub>2 T cells were stimulated for 3 h with PMA (30 ng ml<sup>-1</sup>) and ionomycin (500 ng ml<sup>-1</sup>) in the presence of Brefeldin A at the recommended concentrations (eBioscience). FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells were stimulated with IL33 (10 ng ml<sup>-1</sup>), IL2 (10 ng ml<sup>-1</sup>) or a combination of IL2 (10 ng ml<sup>-1</sup>) and IL25 (10 ng ml<sup>-1</sup>) for 4 days in the absence of Brefeldin A. Cells were fixed and permeabilized with IntraPrep (Beckman Coulter) and then stained intracellularly with FITC-conjugated anti-IFNγ (XMG1.2), phycoerythrin (PE)-conjugated anti-IL5 (TRFK5) and APC-conjugated anti-IL13 (eBio13A).

**Immunofluorescence staining.** Mesenteric tissues were embedded in OCT compound (Sakura Fine Tec Japan) and quickly frozen in liquid nitrogen. Frozen sections were cut using a cryostat into 9-μm slices, and were immediately fixed in acetone for 10 min, washed with TBS, pretreated with Block-ace (Dainippon Pharmaceutical) for 15 min at 37 °C, incubated with a 0.3% H<sub>2</sub>O<sub>2</sub>/0.1% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution for 10 min at room temperature, and washed three times. Rat anti-c-Kit (ACK-2) and hamster anti-CD3ε (145-2C11) monoclonal antibodies were diluted in TBS containing 5% goat serum (Nichirei Biosciences), incubated for 2 h at room temperature, and washed three times with TBS. Subsequently, sections were incubated with Histofine Simple Stain Mouse MAX-PO (Rat) (Nichirei Biosciences) for 1 h at room temperature and washed three times with TBS. Sections were incubated with FITC-conjugated anti-HRP and Alexa fluor 568-conjugated anti-hamster IgG for 2 h at room temperature. PE-conjugated anti-B220 monoclonal antibody was used to stain B cells. Samples were then incubated with 1 μg ml<sup>-1</sup> DAPI (Invitrogen) for 10 min. Finally, slides were mounted in Fluorescence Mounting Medium (DAKO). Images were acquired under a LSM510META confocal microscope (Carl Zeiss) and images edited with Photoshop software (Adobe Systems).

**Giemsa staining.** Cytospin specimens of sorted FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells were fixed in methanol for 30 s, dried, and stained with 10% Giemsa stain solution (Wako) for 10 min, then flushed with water.

**RT-PCR.** Total RNA was purified using the RNeasy Plus Mini kit (QIAGEN). RNA was reverse-transcribed with the Ready-to-go T-primed first-strand kit (Amersham). Primer pairs for *Id2*, *Rorc*, *Lta*, *Ltb*, *Ltrb*, *Maf*, *Gata3*, *Junb*, *Stat4*, *Stat6*, *Tbx21* and *Actb* (Supplementary Table 1) and Taq polymerase (BioLine) were used for PCR. After incubation at 95 °C for 5 min, products were amplified by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and then 72 °C for 5 min. In a different set of experiments, primer pairs for *Il5*, *Il13* and *Actb* (Supplementary Table 1) and Taq polymerase (Takara) were used for PCR. After incubation at 95 °C for 5 min, products were amplified by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 15 s and then 72 °C for 5 min. Experiments were performed on fivefold dilutions of cDNA and the amount of cDNA was normalized by *Actb*.

**CFSE labelling and adoptive transfer of B1 cells.** CD3<sup>-</sup>B220<sup>+</sup> B cells or CD3<sup>-</sup>B220<sup>lo</sup> B1 cells sorted from peritoneal cavity cells were labelled with 2.5  $\mu$ M CFSE (Invitrogen) at 37 °C for 5 min and washed twice, then co-cultured with BAFF (50 ng ml<sup>-1</sup>) alone or with BAFF plus 1.8  $\times$  10<sup>5</sup> FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells or BAFF plus 10 ng ml<sup>-1</sup> IL5 for 3 days. Cells were then stained with anti-CD19 and anti-CD23 monoclonal antibodies, and cell division patterns as demonstrated by CFSE dilution were examined on CD19<sup>+</sup>CD23<sup>-</sup> B1 cells and CD19<sup>+</sup>CD23<sup>+</sup> B2 cells. For *in vivo* proliferation assays, CFSE-labelled B1 cells were transferred into recipient mice with PBS, 2  $\times$  10<sup>5</sup> CD4<sup>+</sup> T cells or FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells. After 9 days, peritoneal cells were collected and stained with anti-B220 and anti-Mac-1, and CFSE dilution of B220<sup>+</sup>Mac-1<sup>+</sup> B1 cells was analysed by flow cytometry. Percentages of cells dividing 0, 1, 2 and 3 times were calculated.

**Administration of anti-IL7 receptor antibodies.** PBS or anti-IL7R $\alpha$  monoclonal antibody (1 mg per mouse) in PBS was administered to wild-type mice on days 0 and 2, and FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells and peritoneal B1 cells were analysed on day 5. The results shown in Fig. 3g are representative of three independent experiments with similar results.

**Helminth infection.** Mice were inoculated subcutaneously with 500 viable third-stage *N. brasiliensis* larvae in 500  $\mu$ l PBS<sup>33</sup>. Animals were euthanized 3, 5, 7, 10 and 12 days after infection, and serum for ELISA, mesentery cells for RT-PCR, and small intestines for H&E staining collected. Peritoneal fluids were also collected by washing the peritoneal cavity with 700  $\mu$ l PBS except that 300  $\mu$ l PBS were used to wash the peritoneal cavity when measuring IL33. For adoptive transfer, 2.5  $\times$  10<sup>5</sup> FALC c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells cultured with 10 ng ml<sup>-1</sup> IL2 for 4 days or splenic CD4<sup>+</sup> T cells were transferred intravenously into *gc*<sup>-/-</sup> *Rag2*<sup>-/-</sup> mice that had been

infected with *N. brasiliensis* for 33 days. One week after cell transfer, serum for ELISA and small intestines for H&E staining were isolated.

**Statistical analysis.** Data are shown as the mean and s.e.m. Statistical analysis was performed using the Mann-Whitney *U*-test.

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# Thymoproteasome Shapes Immunocompetent Repertoire of CD8<sup>+</sup> T Cells

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## SUMMARY

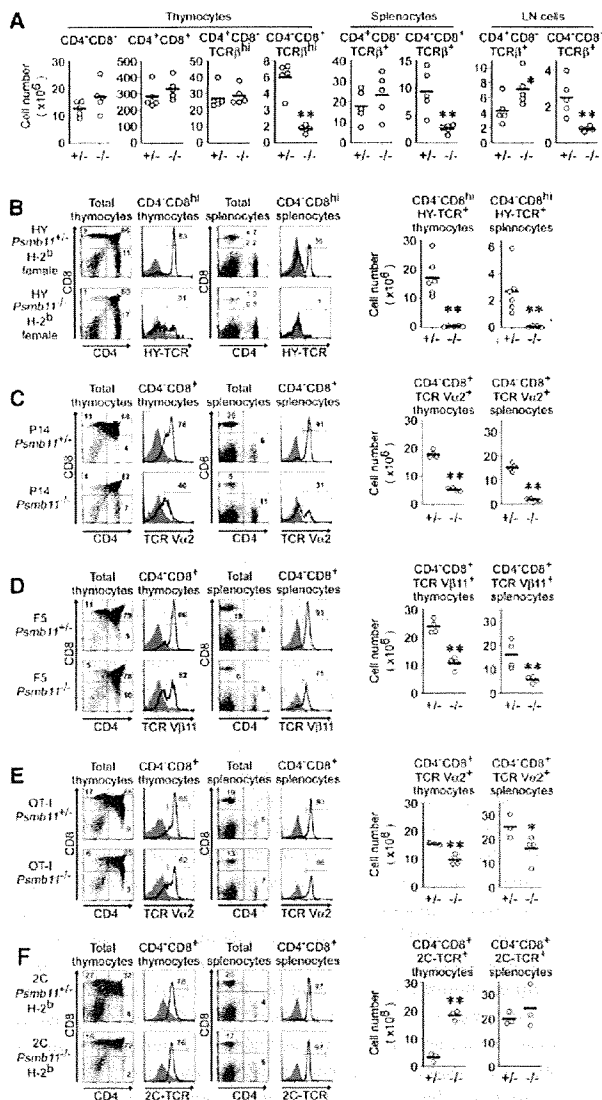
How self-peptides displayed in the thymus contribute to the development of immunocompetent and self-protective T cells is largely unknown. In contrast, the role of thymic self-peptides in eliminating self-reactive T cells and thereby preventing autoimmunity is well established. A type of proteasome, termed thymoproteasome, is specifically expressed by thymic cortical epithelial cells (cTECs) and is required for the generation of optimal cellularity of CD8<sup>+</sup> T cells. Here, we show that cTECs displayed thymoproteasome-specific peptide-MHC class I complexes essential for the positive selection of major and diverse repertoire of MHC class I-restricted T cells. CD8<sup>+</sup> T cells generated in the absence of thymoproteasomes displayed a markedly altered T cell receptor repertoire that was defective in both allogeneic and antiviral responses. These results demonstrate that thymoproteasome-dependent self-peptide production is required for the development of an immunocompetent repertoire of CD8<sup>+</sup> T cells.

## INTRODUCTION

Most T lymphocytes are generated in the thymus. By entering the thymus and interacting with the microenvironment of the thymic cortex, lymphoid progenitor cells are induced to develop into thymocytes that express T cell receptor (TCR), as well as coreceptors CD4 and CD8 (double-positive, DP) (Scollay et al., 1988). Newly generated DP thymocytes that express a virgin set, or the germline repertoire, of TCRs are motile, seeking TCR engagement by interacting with peptide-major histocompatibility complex (MHC) expressed in the cortical microenvironment (Bousso et al., 2002; Li et al., 2007). DP thymocytes that receive weak signals of low-avidity (i.e., affinity × number per cell) TCR engagement are induced to survive and further develop into mature T cells that express large amounts of TCRs and

either one of CD4 or CD8 (single-positive, SP) (Ashton-Rickardt et al., 1993, 1994; Hogquist et al., 1994; Sebzda et al., 1994; Takahama et al., 1994; Alam et al., 1996). This process is referred to as positive selection and is assumed to contribute to the enrichment of an immunocompetent, i.e., useful and self-protective, repertoire of self-MHC-restricted foreign-antigen-reactive T cells (Kisielow et al., 1988; von Boehmer, 1994; Allen, 1994; Starr et al., 2003). In contrast, DP thymocytes that receive strong signals of high-avidity TCR engagement are deleted, a process referred to as negative selection (Kappler et al., 1987; Palmer, 2003). It is well appreciated that negative selection is essential for eliminating self-reactive T cells and thereby preventing autoimmunity (Strasser, 2005; Siggs et al., 2006).

Unlike negative selection, the physiological and pathological importance of positive selection is still controversial. Positive selection was originally identified as the thymic process that determines the MHC-restriction specificity of T cells (Bevan, 1977; Zinkernagel et al., 1978) and is assumed to contribute to enriching an inherently rare T cell repertoire that is useful in the body harboring a given combination of MHC polymorphisms. However, it was shown that the germline TCR repertoire before positive and negative selection is inherently conserved to be MHC reactive (Zerrahn et al., 1997; Merckenschlager et al., 1997). It was also shown that a single MHC-peptide ligand identified in B lymphoma cells could induce positive selection of a diverse repertoire of T cells (Ignatowicz et al., 1996; Fukui et al., 1997). Based on these results, along with the structural analysis of TCR-MHC-peptide interactions, it is proposed that the specificity of TCR for peptides is not demanding during positive selection and that rather than positive selection, it is the subsequent negative selection that establishes the MHC-restriction specificity and the peptide specificity of peripheral T cells (Marrack and Kappler, 1997; Huseby et al., 2005; Dai et al., 2008; Huseby et al., 2008). However, those T cells generated in mice expressing single MHC-peptide ligands show markedly reduced cellularity and an unusual TCR repertoire that occasionally causes autoimmunity (Ignatowicz et al., 1996; Huseby et al., 2005; Oono et al., 2001). Thus, it is unclear whether the positive selection detectable in those single MHC-peptide-expressing mice represents positive selection occurring in the normal body. More importantly, it remains unanswered whether



**Figure 1.  $\beta 5t$  Regulates Positive Selection of Diverse, but Not All, TCR Specificities of  $CD8^+$  T Cells**

(A) Numbers (per mouse) of thymocytes, splenic T cells, and lymph node T cells in indicated populations were determined by flow cytometry in 3- to 6-week-old *Psmb11*<sup>-/-</sup> or *Psmb11*<sup>+/-</sup> mice. Data of individual mice (circles) and means (bars) are shown (n = 5).

(B–F) Thymocytes and splenocytes from HY-TCR-transgenic H-2<sup>b</sup> female mice (B), as well as P14-TCR- (C), F5-TCR- (D), OT-I-TCR- (E), and 2C-TCR- (F) transgenic H-2<sup>b</sup> mice, were analyzed by flow cytometry for CD4 and CD8. Histograms show TCR expression profiles (solid lines) obtained by staining with an antibody specific for HY-TCR (T3.70) (B), TCR V $\alpha$ 2 (C), TCR V $\beta$ 11 (D), TCR V $\alpha$ 2 (E), or 2C-TCR (1B2) (F), overlaid with control staining profiles (shaded lines), of the indicated cell populations. Numbers indicate percentage of cells within indicated areas. Graphs indicate cell numbers (per mouse) of indicated populations in individual mice (circles) and their means (bars) (n = 3 to 6). \*p < 0.05; \*\*p < 0.01. See also Figure S1.

self-peptides displayed in the thymus play a role in positive selection of an immunocompetent repertoire of T cells.

We previously identified  $\beta 5t$ , a proteasome subunit that is specifically expressed in thymic cortical epithelial cells (cTECs) (Murata et al., 2007). Proteasomes are multicatalytic protease complexes that are responsible for the degradation of cytoplasmic proteins and the production of antigen peptides presented by MHC class I molecules (Brown et al., 1991; Rock et al., 1994). The  $\beta 5$  catalytic subunits of the proteasome are responsible for its chymotrypsin-like activity, producing peptides that possess at their carboxyl termini hydrophobic residues that can bind efficiently to MHC class I molecules (Heinemeyer et al., 1993; Fehling et al., 1994; Rock and Goldberg, 1999).  $\beta 5t$ -containing proteasomes, termed thymoproteasomes, exhibit low chymotrypsin-like activity compared with the other types of proteasomes, i.e.,  $\beta 5$ -containing standard proteasomes or  $\beta 5i$ -containing immunoproteasomes (Murata et al., 2007). Interestingly,  $\beta 5t$ -containing cTEC-specific thymoproteasomes are essential for the generation of the optimal cellularity of  $CD8^+$  T cells (Murata et al., 2007). However, the mechanism by which thymoproteasomes regulate T cell development has yet to be disclosed.

In this study, we examined how thymoproteasomes regulate T cell development. Our results showed that thymoproteasomes were essential for the positive selection of major and diverse, but not all, repertoire of  $CD8^+$  T cells. We also found that thymoproteasomes conferred on cTECs the ability to express MHC class I-peptide complexes that were capable of generating major repertoire of  $CD8^+$  T cells. cTECs in thymoproteasome-deficient mice compensatively assembled immunoproteasomes and expressed an altered set of MHC class I-peptide complexes that fail to positively select most repertoires of  $CD8^+$  T cells. In addition,  $CD8^+$  T cells generated in the absence of thymoproteasomes displayed an altered TCR repertoire that was defective in allogeneic and antiviral responses. Thus, this study reveals a unique role of cTEC-specific protein degradation that is essential for the cTEC-specific production of self-peptide-MHC class I complexes, and these complexes are required for the development of an immunocompetent and self-protective repertoire of  $CD8^+$  T cells.

**RESULTS**

**$\beta 5t$  Regulates Positive Selection of Major Repertoire of  $CD8^+$  T Cells**

In  $\beta 5t$ -deficient (*Psmb11*<sup>-/-</sup>) mice, the numbers of  $CD8^+$  T cells in the spleen and lymph nodes were markedly reduced to 27% and 31% (ratio between averages, n = 5), respectively, of those in normal mice (Figure 1A). The number of  $CD4^+CD8^+$  single-positive ( $CD8SP$ ) thymocytes was also reduced to 21% (n = 5) of the control, whereas the numbers of  $CD4^+CD8^-$  double-negative (DN),  $CD4^+CD8^+$  DP, and  $CD4^+CD8^-$  ( $CD4SP$ ) thymocytes were unchanged (Figure 1A), indicating that  $\beta 5t$  specifically regulates the  $CD8$  lineage, but not the  $CD4$  lineage of T cell development beyond the DP stage. Other lineages of immune cells, including TCR $\gamma\delta^+$  cells, NK cells, NKT cells, macrophages, dendritic cells, B cells, and  $CD8\alpha\alpha^+$  intraepithelial lymphocytes, showed no decreases in their numbers in *Psmb11*<sup>-/-</sup> mice (Figure S1A available online). In order to examine how  $\beta 5t$  specifically affects  $CD8^+$  T cell development, *Psmb11*<sup>-/-</sup> mice were crossed with TCR-transgenic mice. In HY-TCR-transgenic

mice, MHC class I H-2D<sup>b</sup>-restricted male-antigen-specific TCR drives the positive and negative selection of CD8-lineage T cells in female and male H-2<sup>b</sup> mice, respectively. We found that the generation of CD8SP thymocytes and splenic CD8<sup>+</sup> T cells in H-2<sup>b</sup> female HY-TCR-transgenic mice was severely impaired by the lack of  $\beta$ 5t (to 2.2% and 2.6%, respectively, of control cell numbers,  $n = 4-6$ ) (Figure 1B). In contrast,  $\beta$ 5t deficiency has no effect on the decreased numbers of DP thymocytes and CD8<sup>hi</sup> splenic T cells in H-2<sup>b</sup> male HY-TCR-transgenic mice (Figure S1B). Furthermore, the arrested T cell development at DP stage in null-selector H-2<sup>d</sup> HY-TCR-transgenic mice was also unaltered by the absence of  $\beta$ 5t (data not shown). Together, the results indicate that  $\beta$ 5t affects positive selection rather than negative or null selection of HY-TCR-transgenic T cells. In contrast, the generation of CD4SP thymocytes and splenic CD4 T cells in I-A<sup>b</sup>-restricted pigeon cytochrome c-specific AND-TCR-transgenic mice and I-A<sup>b</sup>-restricted ovalbumin-specific OT-II-TCR-transgenic mice was not diminished in the absence of  $\beta$ 5t (Figures S1F and S1G), indicating that  $\beta$ 5t is dispensable for the positive selection of MHC class II-restricted TCR-transgenic T cells.  $\beta$ 5t deficiency neither affected the negative selection of thymocytes in two additional MHC class I-restricted TCR-transgenic models nor caused any signs of autoimmune diseases in various organs (Figure S1C-S1E). These results indicate that the  $\beta$ 5t-containing thymoproteasome specifically regulates positive selection, rather than negative selection, of CD8<sup>+</sup> T cells, rather than CD4<sup>+</sup> T cells.

Similar to HY-TCR-transgenic T cells, the numbers of splenic T cells in two other MHC class I-restricted TCR-transgenic mice, namely, lymphocytic choriomeningitis virus-specific P14-TCR-transgenic mice and influenza virus-specific F5-TCR-transgenic mice, were markedly reduced in the absence of  $\beta$ 5t (12%,  $n = 4$  and 36%,  $n = 4$ , respectively, relative to control, i.e.,  $\beta$ 5t<sup>+</sup> mice, Figures 1C and 1D). Accordingly, the generation of CD8SP thymocytes in these TCR-transgenic mice was severely impaired by the lack of  $\beta$ 5t (Figures 1C and 1D), indicating that  $\beta$ 5t regulates the positive selection of CD8<sup>+</sup> T cells with multiple TCR specificities. Interestingly, however, MHC class I-restricted ovalbumin-specific OT-I-TCR-transgenic and allogeneic H-2L<sup>d</sup>-specific 2C-TCR-transgenic CD8<sup>+</sup> T cells were less severely affected in *Psmb11*<sup>-/-</sup> mice (Figures 1E and 1F). The number of OT-I-TCR-transgenic splenic CD8<sup>+</sup> T cells in *Psmb11*<sup>-/-</sup> mice was reduced to 64% ( $n = 3$  to 4) of that in control OT-I-TCR-transgenic mice (Figure 1E). Most notable was that the number of 2C-TCR-transgenic splenic CD8<sup>+</sup> T cells in *Psmb11*<sup>-/-</sup> mice was 122% ( $n = 3$ ) of that in control 2C-TCR-transgenic mice carrying  $\beta$ 5t (Figure 1F). These results indicate that the development of CD8<sup>+</sup> T cells that express individual specificities of MHC class I-restricted TCRs is differentially dependent on  $\beta$ 5t. Along with the finding that the majority of CD8<sup>+</sup> T cells are lost in *Psmb11*<sup>-/-</sup> mice (Figure 1A), these results also indicate that the  $\beta$ 5t-containing thymoproteasome regulates the positive selection of major and diverse, but not all, repertoires of MHC class I-restricted CD8<sup>+</sup> T cells.

#### **$\beta$ 5t Regulates cTEC-Mediated Positive Selection of CD8<sup>+</sup> T Cells within Thymic Cortex**

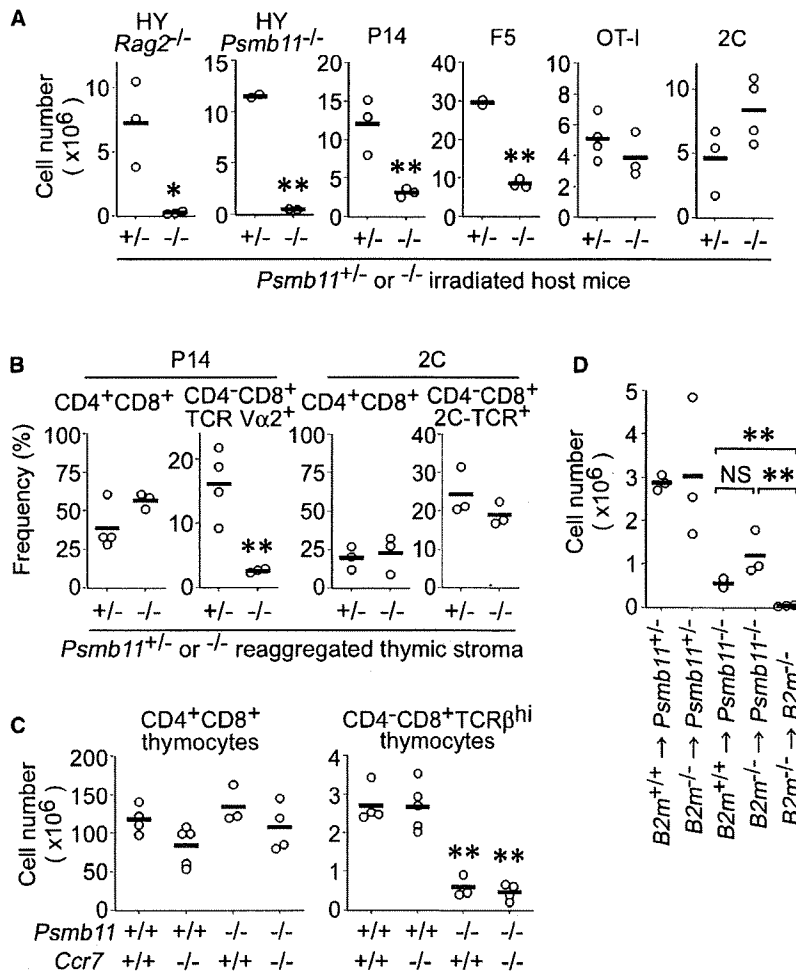
To determine the cells that are responsible for the  $\beta$ 5t-mediated regulation of T cell development, we examined positive selection

among various TCR specificities in bone marrow chimeras (Figure 2A). To do so, hematopoietic progenitor cells from TCR-transgenic mice were reconstituted in irradiated *Psmb11*<sup>-/-</sup> mice. In the thymus of  $\beta$ 5t-deficient mice, positive selection of HY-TCR-, P14-TCR-, and F5-TCR-transgenic T cells was markedly diminished, whereas positive selection of OT-I-TCR-transgenic and 2C-TCR-transgenic T cells was affected little (Figure 2A). Thus, the differential  $\beta$ 5t dependence of positive selection among various TCR specificities was reproduced in these bone marrow chimeras reconstituted in the thymus of irradiated *Psmb11*<sup>-/-</sup> mice (Table 1). In contrast,  $\beta$ 5t deficiency in bone marrow donor cells did not diminish the positive selection of HY-TCR-transgenic T cells in the thymus of  $\beta$ 5t-sufficient mice (Figure 2A), indicating that  $\beta$ 5t in nonhematopoietic stromal cells, but not bone marrow-derived hematopoietic cells, is responsible for positive selection of the repertoire of CD8SP thymocytes. Furthermore, the specific deficiency of  $\beta$ 5t in cTEC-enriched thymic stromal cells markedly diminished the capability to induce the generation of CD8<sup>+</sup> T cells from isolated DP thymocytes of P14-TCR-transgenic mice, but not 2C-TCR-transgenic mice, in reaggregated fetal thymus organ culture (Figure 2B). Along with the results showing that  $\beta$ 5t is exclusively expressed in cTECs (Murata et al., 2007 and data not shown), these results indicate that thymoproteasome-expressing cTECs regulate the positive selection of major and diverse, but not all, repertoire of CD8<sup>+</sup> T cells. The  $\beta$ 5t independency of positive selection weakly correlated with the signaling intensity of transgenic TCR, which was measured by the amount of surface CD5 and CD8 expression (Figure S5).

In thymic medulla, newly generated SP thymocytes interact with various cells, including mTECs, which promiscuously express various tissue-specific antigens (Derbinski et al., 2001; Anderson et al., 2002; Kyewski and Derbinski, 2004). The CCR7-mediated migration of SP thymocytes to the medulla is essential to trim the cortically generated T cell repertoire to establish self-tolerance (Takahama, 2006; Kurobe et al., 2006; Nitta et al., 2009). Thus, it is possible that the  $\beta$ 5t regulation of repertoire formation may involve the migration of positively selected thymocytes to the medulla and the negative selection induced in thymic medulla. However, we found that the development of CD8SP thymocytes was defective even in *Psmb11*<sup>-/-</sup> *Ccr7*<sup>-/-</sup> mice, similar to *Psmb11*<sup>-/-</sup> mice (Figure 2C), indicating that defective positive selection in the absence of  $\beta$ 5t reflects neither CCR7-mediated migration of positively selected thymocytes nor negative selection in thymic medulla. We also found that the formation of thymic medulla including Aire-expressing medullary epithelial cells was not impaired in  $\beta$ 5t-deficient mice (Figure S2). Thus,  $\beta$ 5t-containing thymoproteasome regulates the positive selection of major CD8<sup>+</sup> T cell repertoires within thymic cortex without the contribution of subsequent negative selection in thymic medulla.

The reduced number of CD8<sup>+</sup> T cells in bone marrow chimeras reconstituted in irradiated *Psmb11*<sup>-/-</sup> mice was not markedly altered when bone marrow cells were isolated from mice deficient for  $\beta$ 2-microglobulin ( $\beta$ 2m), a component of MHC class I molecules (Figure 2D). Thus, the impaired but detectable positive selection in  $\beta$ 5t-deficient mice is not due to MHC class I-restricted antigen presentation by bone marrow-derived cells, including dendritic cells and T-lineage cells, which were





**Figure 2. β5t in cTECs Regulates Positive Selection of Major TCR Specificities of CD8<sup>+</sup> T Cells**

(A) T cell-depleted bone marrow cells from indicated donor mice were transferred into lethally irradiated *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> H-2<sup>b</sup> host mice. Thymocytes were analyzed 5 weeks after the reconstitution. Graphs indicate numbers (per mouse) of CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>β</sup><sup>hi</sup> thymocytes in individual mice (circles) and their means (bars) (n = 2 to 4).

(B) CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were isolated from P14-TCR-transgenic (left) or 2C-TCR-transgenic (right) mice with a cell sorter and reaggregated with *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> H-2<sup>b</sup> fetal thymic stromal cells that contained 30%–40% cTECs, <3% mTECs, and 60%–70% non-TEC stromal cells, isolated from 2-deoxyguanosine-treated fetal thymus lobes. CD4<sup>+</sup>CD8<sup>+</sup> thymocytes before the culture had >99% purity. The indicated cell populations were analyzed 4 days after reaggregated thymus organ culture. Graphs indicate percentages of indicated populations in individual reaggregated thymuses (circles) and their means (bars) (n = 3 to 4).

(C) Numbers of CD4<sup>+</sup>CD8<sup>+</sup> or CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>β</sup><sup>hi</sup> thymocytes (per thymus lobe) in 4- to 6-week-old *Psmb11*<sup>+/+</sup>*Ccr7*<sup>+/+</sup>, *Psmb11*<sup>+/+</sup>*Ccr7*<sup>-/-</sup>, *Psmb11*<sup>-/-</sup>*Ccr7*<sup>+/+</sup>, or *Psmb11*<sup>-/-</sup>*Ccr7*<sup>-/-</sup> mice.

(D) Thymocytes isolated from indicated bone marrow chimera mice were analyzed by flow cytometry for the expression of CD4, CD8, and TCRβ. Shown are the numbers of CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>β</sup><sup>hi</sup> thymocytes (n = 2 to 3). The number of CD8SP thymocytes in *Psmb11*<sup>-/-</sup> mice reconstituted with B2m<sup>-/-</sup> bone marrow cells (*B2m*<sup>-/-</sup> → *Psmb11*<sup>-/-</sup>) was not significantly (p ≥ 0.05) different from that in *Psmb11*<sup>-/-</sup> mice reconstituted with B2m<sup>+/+</sup> bone marrow cells (*B2m*<sup>+/+</sup> → *Psmb11*<sup>-/-</sup>) but was significantly (p < 0.01) larger than that in B2m<sup>-/-</sup> mice reconstituted with B2m<sup>-/-</sup> bone marrow cells (*B2m*<sup>-/-</sup> → B2m<sup>-/-</sup>), indicating that the generation of CD8SP thymocytes in *Psmb11*<sup>-/-</sup> mice was independent of bone-marrow-derived MHC class I molecules. \*p < 0.05; \*\*p < 0.01; NS, not significant. See also Figure S2.

previously shown to be capable of inducing positive selection under certain experimental conditions (Zinkernagel and Althage, 1999; Li et al., 2005; Choi et al., 2005; Kirberg et al., 2008). Rather, the reduced production of CD8<sup>+</sup> T cells in *Psmb11*<sup>-/-</sup> mice is likely due to the defective and inefficient capability of thymoproteasome-deficient cTECs to induce positive selection.

#### cTECs Display a Thymoproteasome-Specific Set of MHC Class I-Associated Peptides

The surface expression of H-2K and H-2D MHC class I molecules, as well as I-A MHC class II molecules, on cTECs in *Psmb11*<sup>-/-</sup> mice was not markedly diminished (Figure 3A), although the surface expression of K<sup>b</sup>, D<sup>b</sup>, and L<sup>d</sup> on β5t-deficient cTECs was slightly altered to 93% (n = 5), 89% (n = 5), and 103% (n = 3) of that in control cTECs carrying β5t (Table 2). Similar to cTECs from normal mice, β5t-deficient cTECs were fully capable of presenting SIINFEKL antigen peptide to stimulate OT-I-TCR-transgenic CD8<sup>+</sup> T cells (Figure 3B). Thus,

β5t-deficient cTECs are competent in presenting antigen peptides to MHC class I-restricted T cells. The majority of proteasomal β5-type subunits expressed in β5t-deficient cTECs were β5i, rather than β5 (Figure 3C), whereas β1i and β2i, rather than β1 or β2, were predominant in cTECs (Murata et al., 2007). Thus, unlike normal cTECs that predominantly express β5t-containing thymoproteasomes, β5t-deficient cTECs compensatively assemble β1i-, β2i-, and β5i-containing immunoproteasomes. Indeed, most MHC class I H-2L<sup>d</sup> molecules expressed by cTECs exhibited the peptide-bound "folded" form (as detected by 30-5-7 antibody), rather than the peptide-empty "open" form (as detected by 64-3-7 antibody), irrespective of the presence or absence of β5t (Figure 3D). Thus, most MHC class I molecules expressed by cTECs in the presence or absence of β5t are associated with peptides, similarly to those expressed by other cells, such as mTECs.

In order to explore the nature of MHC class I-associated peptides in cTECs, we then examined the expression of epitopes

**Table 1. Development of CD8<sup>+</sup> T Cells that Express Individual Specificities of MHC Class I -Restricted TCR Is Differentially Dependent on  $\beta$ 5t**

TCR	V $\alpha$ and V $\beta$	MHC Restriction Peptide <sup>a</sup>	Antigen	% <i>Psmb11</i> <sup>-/-</sup> / <i>Psmb11</i> <sup>+/-b</sup>			
				Thymocytes		Splenocytes	
				Mice <sup>c</sup>	BMC <sup>c</sup>	Mice	BMC
Bulk	Bulk	Bulk	Bulk	21.0	19.6	27.3	17.8
HY	V $\alpha$ 17 V $\beta$ 8.2	D <sup>b</sup>	Smcy	2.2	3.7	2.6	5.8
P14	V $\alpha$ 2 V $\beta$ 8.1	D <sup>b</sup>	LCMV gp33	29.7	26.1	12.4	12.3
F5	V $\alpha$ 4 V $\beta$ 11	D <sup>b</sup>	Flu NP68	45.3	28.9	35.5	14.9
OT-I	V $\alpha$ 2 V $\beta$ 5	K <sup>b</sup>	Ovalbumin	63.4	76.0	63.9	56.0
2C <sup>d</sup>	V $\alpha$ 3 V $\beta$ 8.2	K <sup>b</sup>	(L <sup>d</sup> + $\alpha$ -KGDH)	553.2	181.1	121.7	47.7

<sup>a</sup> Smcy is a Y-chromosome-encoded H-Y antigen. LCMV gp33 is glycoprotein 33 of lymphocytic choriomeningitis virus. Flu NP68 is influenza virus nucleoprotein 68.

<sup>b</sup> 100  $\times$  (average CD8<sup>+</sup> SP T cell number in *Psmb11*<sup>-/-</sup> mice/average CD8<sup>+</sup> SP T cell number in *Psmb11*<sup>+/-</sup> mice).

<sup>c</sup> Mice, data obtained from TCR-transgenic mice as shown in Figure 1. BMC, data from bone marrow chimera mice as shown in Figure 2A.

<sup>d</sup> 2C-TCR-transgenic T cells in H-2<sup>b</sup> mice are positively selected by K<sup>b</sup> and are reactive to allogeneic L<sup>d</sup> molecules that are associated with the endogenous protein  $\alpha$ -ketoglutarate dehydrogenase ( $\alpha$ -KGDH).

produced by the complexes of MHC class I molecules and limited varieties of peptides. We found that the expression of one of these epitopes directly detected by the TCR-like monoclonal antibody 25-D1.16 (Porgador et al., 1997; Mareeva et al., 2008), which recognizes a fraction of H-2K<sup>b</sup> molecules associated with a population of peptides (Porgador et al., 1997), was markedly higher in cTECs from *Psmb11*<sup>-/-</sup> mice than in those from control mice (Figures 3E–3G). The surface expression of this epitope on mTECs, thymocytes, B cells, and dendritic cells was not different between *Psmb11*<sup>-/-</sup> and control mice (Figures 3E and 3F and Figure S3A), indicating that  $\beta$ 5t specifically regulates the surface expression of this epitope on cTECs. Likewise, the cell-surface expression of another epitope of the H-2K<sup>b</sup> complex associated with a different population of peptides, detected by 22-C5.9 (Porgador et al., 1997), was specifically altered in cTECs from *Psmb11*<sup>-/-</sup> mice compared to those from control mice (Table 2 and Figure S3B). The cTEC-specific difference in the expression of these epitopes between *Psmb11*<sup>-/-</sup> and control mice suggests that the repertoire of MHC class I-associated peptides expressed by cTECs is specifically regulated by  $\beta$ 5t-containing thymoproteasomes and that cTECs in normal mice display a repertoire of MHC class I-associated peptides that are uniquely generated by thymoproteasomes.

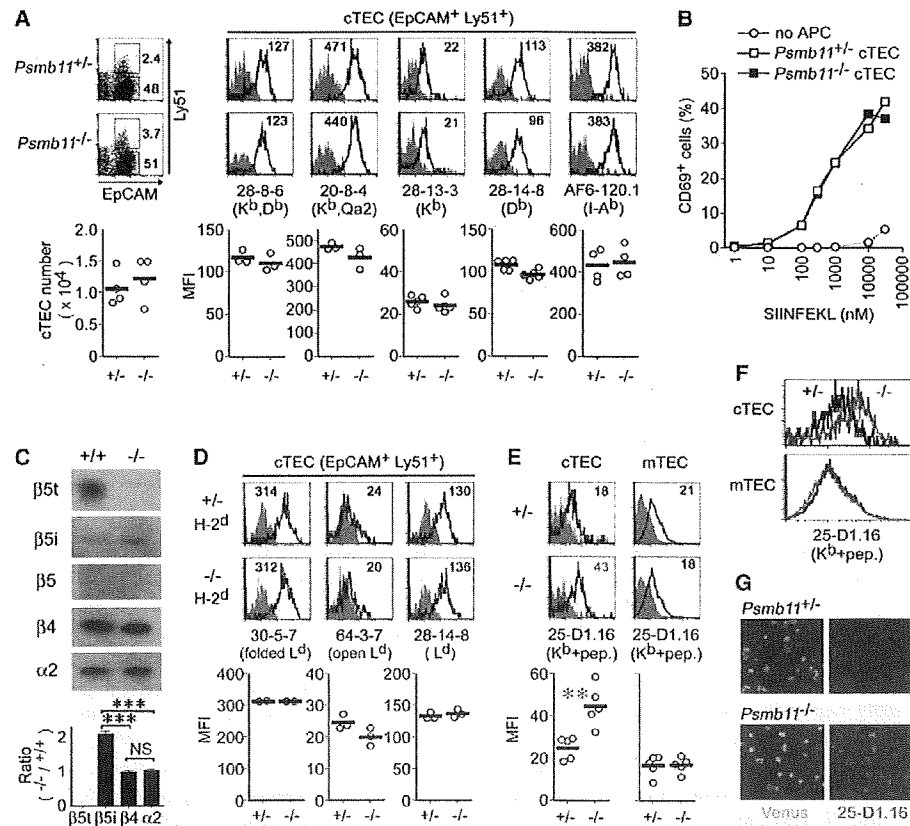
#### Functionally Incompetent Repertoire of CD8<sup>+</sup> T Cells in $\beta$ 5t-Deficient Mice

Finally, we examined the function and repertoire of lymphopenic CD8<sup>+</sup> T cells that were generated in the absence of  $\beta$ 5t. We found that CD8<sup>+</sup> T cells generated in *Psmb11*<sup>-/-</sup> mice were capable of proliferation and granzyme B production in response to TCR and CD28 stimulation (Figures 4A and 4B). OT-I-TCR-transgenic CD8<sup>+</sup> T cells generated in *Psmb11*<sup>-/-</sup> mice were fully capable of proliferating in the presence of SIINFEKL peptide (Figure 4C). Homeostatic proliferation of CD8<sup>+</sup> T cells isolated from *Psmb11*<sup>-/-</sup> mice was not impaired in vivo in both irradiated mice and RAG2-deficient (*Rag2*<sup>-/-</sup>) mice (Figure 4D). Thus, CD8<sup>+</sup> T cells generated in the absence of thymoproteasomes are functionally potent to maintain in vivo survival in lymphopenic

environments and to proliferate and become cytotoxic T-lymphocytes (CTL) in response to TCR stimulation.

CD8<sup>+</sup> T cells in *Psmb11*<sup>-/-</sup> mice exhibited increased frequency of cells that highly expressed CD44 and Ly6C (Figure 4E). The frequency of CD44<sup>hi</sup> CD8<sup>+</sup> T cells in *Psmb11*<sup>-/-</sup> mice increased with the ontogeny (Figure 4F) and this increase coincided with the severer decrease in absolute number of CD44<sup>lo</sup> CD8<sup>+</sup> T cells than CD44<sup>hi</sup> CD8<sup>+</sup> T cells (Figure 4G). Both CD44<sup>lo</sup> and CD44<sup>hi</sup> CD8<sup>+</sup> T cells in *Psmb11*<sup>-/-</sup> mice were capable of proliferative response to TCR and CD28 stimulation (Figure 4H). However, CD44<sup>lo</sup> CD8<sup>+</sup> T cells isolated from wild-type mice did not undergo excessive homeostatic expansion upon intravenous administration in *Psmb11*<sup>-/-</sup> mice, unlike in lymphopenic *Rag2*<sup>-/-</sup> mice (Figure 4I), suggesting that the increase in frequency of CD44<sup>hi</sup> cells is unlikely due to the homeostatic expansion of CD44<sup>lo</sup> cells and subsequent phenotype conversion into CD44<sup>hi</sup> cells in *Psmb11*<sup>-/-</sup> mice. Thus, the marked in vivo persistence of CD44<sup>hi</sup> memory-type CD8<sup>+</sup> T cells compared to CD44<sup>lo</sup> naive CD8<sup>+</sup> T cells (Tanchot et al., 1997; Murali-Krishna et al., 1999) and possible defect in the maintenance of CD8<sup>+</sup> T cells generated in *Psmb11*<sup>-/-</sup> mice (for example, by defective TCR interactions with self MHC class I-peptide complexes because of the alteration in TCR repertoire) may contribute to the increased ratio of CD44<sup>hi</sup> cells over CD44<sup>lo</sup> cells in CD8<sup>+</sup> T cells of *Psmb11*<sup>-/-</sup> mice and the slightly elevated responses of CD8<sup>+</sup> T cells from *Psmb11*<sup>-/-</sup> mice.

Indeed, the distribution of TCR-V $\beta$  and TCR-V $\alpha$  in CD8<sup>+</sup> T cells was altered in *Psmb11*<sup>-/-</sup> mice (Figure 5A), whereas no substantial difference was detected in the TCR-V distribution in CD4<sup>+</sup> T cells between *Psmb11*<sup>-/-</sup> and control mice (Figure 5A), indicating that the T cell repertoire was altered specifically in CD8<sup>+</sup> T cells. Thus, CD8<sup>+</sup> T cells generated in *Psmb11*<sup>-/-</sup> mice carry undiminished TCR responsiveness but an altered TCR repertoire. The functional consequence of the altered repertoire of CD8<sup>+</sup> T cells in *Psmb11*<sup>-/-</sup> mice was examined by measuring immune response to foreign antigens. We found that CD8<sup>+</sup> T cells isolated from *Psmb11*<sup>-/-</sup> mice showed markedly diminished proliferative responses to allogeneic antigens (Figure 5B), which were dependent on  $\beta$ 2 m-associated MHC class I



**Figure 3. cTECs Produce  $\beta 5t$ -Specific Peptide-MHC Class I Complexes**

(A) Surface expression of MHC class I and class II molecules on cTECs from *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> H-2<sup>b</sup> mice. CD45<sup>-</sup>TER119<sup>-</sup> thymic stromal cells from 2-week-old mice were stained for EpCAM, Ly51, and indicated antibodies specific for MHC class I or class II molecules. Histograms show staining profiles (solid lines) overlaid with control staining profiles (shaded histograms) of EpCAM<sup>+</sup>Ly51<sup>+</sup> cTECs. Numbers in histograms indicate mean fluorescence intensity (MFI). Graphs show MFI of the histograms with indicated antibodies in individual measurements (circles) and their means (bars). Shown on the left are representative dot plots for EpCAM and Ly51 expression in CD45<sup>-</sup> thymic stromal cells and the number of EpCAM<sup>+</sup>Ly51<sup>+</sup> cTECs in *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> mice. Numbers in dot plots indicate the frequency of cells within the box. The expression of D<sup>b</sup> was significantly ( $p < 0.05$ ) reduced in *Psmb11*<sup>-/-</sup> mice.

(B) CD8<sup>+</sup> T cells from the spleen of OT-I-TCR-transgenic mice were cocultured in the absence or presence of CD45<sup>-</sup>I-A<sup>b</sup>Ly51<sup>+</sup> cTECs from *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> H-2<sup>b</sup> mice. cTECs were pretreated with indicated concentrations of SIINFEKL peptide. Twenty hours later, the frequency of CD69<sup>+</sup> cells in CD8<sup>+</sup> T cells was analyzed by flow cytometry.

(C) Proteasomal components in  $\beta 5t$ -deficient cTECs. Ly51<sup>+</sup> cells were purified from the thymus of *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> mice, lysed, and subjected to immunoblot analysis with the indicated antibodies.  $\beta 4$  and  $\alpha 2$  were used as controls. Means and standard errors ( $n = 3$ ) of the ratios of the chemiluminescence signals in *Psmb11*<sup>-/-</sup> cTECs to those in *Psmb11*<sup>+/-</sup> cTECs are also shown. \*\*\* $p < 0.001$ ; NS, not significant. The signals for  $\beta 5$  were not detectable in cTECs from either *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> mice.

(D–F) CD45<sup>-</sup>TER119<sup>-</sup> thymic stromal cells were prepared from 2-week-old *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> H-2<sup>d</sup> (D) or H-2<sup>b</sup> (E) mice (F) and stained for EpCAM, Ly51, and indicated MHC class I molecules. Monoclonal antibodies used were 30-5-7 (specific for the peptide-bound “folded” form of L<sup>d</sup>), 64-3-7 (specific for the peptide-empty “open” form of L<sup>d</sup>), 28-14-8 (specific for L<sup>d</sup> irrespective of peptide binding), and 25-D1.16 (specific for K<sup>b</sup> associated with a limited variety of peptides). Histograms show staining profiles (solid lines) overlaid with control staining profiles (shaded histograms) of EpCAM<sup>+</sup>Ly51<sup>+</sup> cTECs or EpCAM<sup>+</sup>Ly51<sup>-</sup> mTECs (D and E). Staining profiles of EpCAM<sup>+</sup>Ly51<sup>+</sup> cTECs and EpCAM<sup>+</sup>Ly51<sup>-</sup> mTECs isolated from *Psmb11*<sup>-/-</sup> (red lines) and *Psmb11*<sup>+/-</sup> (black lines) mice are also indicated (F). Numbers in histograms indicate MFI. Graphs show the MFI of the histograms with indicated antibodies in individual measurements (circles) and their means (bars). \*\* $p < 0.01$ .

(G) Venus-expressing (green) cTECs isolated from  $\beta 5t$ <sup>+/-</sup> or  $\beta 5t$ <sup>-/-</sup> H-2<sup>b</sup> mice were stained with 25-D1.16 (red).

See also Figure S3.

molecules (Figure 5C). The decrease in allogeneic response was not limited to a single combination of allogeneic stimulation but was shared by various allogeneic combinations (Figures 5B and 5C and Figure S4A). However, a certain combination of allogeneic response (H-2<sup>d</sup> anti-H-2<sup>b</sup>) of CD8<sup>+</sup> T cells was not severely defective in *Psmb11*<sup>-/-</sup> mice (Figure S4A), in agreement

with the possibility that CD8<sup>+</sup> T cells generated in the absence of thymoproteasomes are functionally potent in response to TCR engagement but are defective in the formation of a functionally competent TCR repertoire. The allogeneic response of CD8<sup>+</sup> T cells from *Psmb11*<sup>-/-</sup> mice was reduced even in isolated CD44<sup>lo</sup> naive T cells (Figure S4B), suggesting that the defective

**Table 2. Surface Expression of MHC Molecules on cTECs and mTEC in  $\beta 5t$ -Deficient Mice**

Mouse Examined	Target Molecule	Detecting Antibody	% <i>Psmb11</i> <sup>-/-</sup> / <i>Psmb11</i> <sup>+/-</sup> <sup>a</sup>	
			cTECs	mTECs
H-2 <sup>b</sup>	K <sup>b</sup> , D <sup>b</sup>	28-8-6	94.4	101.5
	K <sup>b</sup> , Qa2	20-8-4	89.9	93.5
	K <sup>b</sup>	28-13-3	93.4	105.0
	D <sup>b</sup>	28-14-8	89.1	100.1
	K <sup>b</sup> + peptides <sup>b</sup>	25-D1.16	180.2 <sup>b</sup>	101.3
	K <sup>b</sup> + peptides <sup>b</sup>	22-C5.9	139.1 <sup>b</sup>	95.4
	I-A <sup>b</sup>	AF6-120.1	103.3	102.6
H-2 <sup>d</sup>	L <sup>d</sup>	28-14-8	102.8	100.3
	L <sup>d</sup> (folded) <sup>c</sup>	30-5-7	99.8	95.6
	L <sup>d</sup> (open) <sup>c</sup>	64-3-7	81.5	92.0

<sup>a</sup>MFI values of indicated molecules with indicated antibodies were measured by flow cytometry as shown in Figure 3. Shown are 100 × (average MFI in *Psmb11*<sup>-/-</sup> mice/average MFI in *Psmb11*<sup>+/-</sup> mice). For the analysis with 22-C5.9, cTECs and mTECs were *Psmb11*-driven Venus<sup>+</sup> and Venus<sup>-</sup> CD45<sup>-</sup> thymic cells.

<sup>b</sup>25-D1.16 and 22-C5.9 are specific for K<sup>b</sup> associated with a limited and mutually different variety of peptides (Porgador et al., 1997). The MFI values were significantly ( $p < 0.05$ ) larger in *Psmb11*<sup>-/-</sup> cTECs than in *Psmb11*<sup>+/-</sup> cTECs.

<sup>c</sup>30-5-7 is specific for the peptide-bound folded form of L<sup>d</sup>, whereas 64-3-7 is specific for the peptide-empty open form of L<sup>d</sup> (Lie et al., 1991).

CD8<sup>+</sup> T cell response in *Psmb11*<sup>-/-</sup> mice is due to the defective repertoire of naive CD8<sup>+</sup> T cells rather than the reduced frequency of naive cells in CD8<sup>+</sup> T cells. Finally, we found that upon influenza virus infection under the conditions where control mice could survive due to CD8<sup>+</sup> T cell responses, *Psmb11*<sup>-/-</sup> mice exhibited severe lethality (Figure 5D). These results indicate that CD8<sup>+</sup> T cells generated in the absence of thymoproteasomes are defective in mounting immune responses to allogeneic and viral antigens.

## DISCUSSION

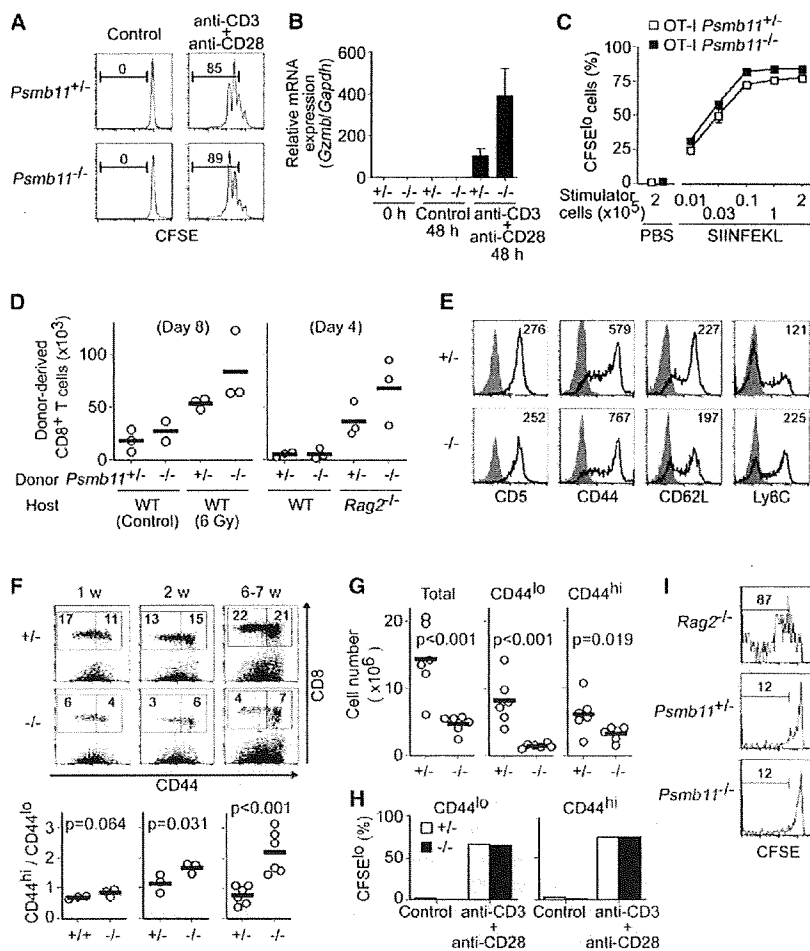
The present results demonstrate that cTECs display thymoproteasome-specific MHC class I-peptide complexes that are essential for the development of major and diverse repertoire of CD8<sup>+</sup> T cells. Thymoproteasome-deficient cTECs displayed altered MHC class I-peptide complexes that generated an altered TCR repertoire that was defective in allogeneic and antiviral responses. These results suggest that cTEC-specific production of MHC class I-associated self-peptides due to thymoproteasome-mediated protein degradation is essential for the development of an immunocompetent and self-protective CD8<sup>+</sup> T cell repertoire.

Chymotrypsin-like activity mediated by the  $\beta 5$  subunits of proteasomes is responsible for the production of peptides that carry carboxyl-terminal hydrophobic residues that efficiently associate with MHC class I molecules (Fehling et al., 1994; Rock and Goldberg, 1999). No carboxypeptidases other than proteasomes are detectable in the cells except lysosomes, whereas various aminopeptidases in the cytoplasm and the

endoplasmic reticulum are involved in trimming the amino termini of proteasome-generated peptides (Reits et al., 2003; Yewdell et al., 2003). Indeed, naturally processed self-peptides eluted from many alleles of MHC class I molecules are highly biased to possess hydrophobic amino acids, and basic residues to a less extent, at the carboxyl terminus (Falk et al., 1991; Hunt et al., 1992; Young et al., 1995). On the other hand,  $\beta 5t$ -containing thymoproteasomes exhibit selectively reduced chymotrypsin-like activity but normal trypsin-like and caspase-like activities, compared with other types of proteasomes, i.e.,  $\beta 5$ -containing standard proteasomes and  $\beta 5i$ -containing immunoproteasomes (Murata et al., 2007). It is therefore reasonable to speculate that the specifically reduced chymotrypsin-like activity in thymoproteasomes is responsible for the generation of the cTEC-specific repertoire of cytoplasmic peptides. Even though the TAP complex, which is responsible for transporting cytoplasmic peptides into the lumen of the endoplasmic reticulum, prefers the hydrophobic carboxyl termini of the peptides (Momburg et al., 1994; Uebel et al., 1997; Burgevin et al., 2008), our results show that most of the MHC class I molecules expressed on the surface of cTECs irrespective of  $\beta 5t$  expression are associated with the peptides rather than being peptide-empty. Thus, the thymoproteasome-mediated production of a cTEC-specific repertoire of cytoplasmic peptides likely results in the production of a unique repertoire of MHC class I-associated peptides specifically displayed by cTECs.

With regard to the nature of MHC class I-bound peptides expressed by cTECs, current technology of mass spectrometry analysis (which requires 10<sup>8</sup> to 10<sup>9</sup> cells) does not readily allow us to directly identify the sequences of MHC-bound peptides expressed by isolated cTECs (approximately 1 × 10<sup>4</sup> cells can be isolated per mouse). However, the present results obtained by the two different TCR-like monoclonal antibodies show that the expression of the epitopes, likely produced by the complexes of MHC class I molecules and endogenously produced peptides, was markedly and specifically altered in cTECs from  $\beta 5t$ -deficient mice compared to those from control mice, suggesting that cTECs display a thymoproteasome-specific set of MHC class I-associated peptides. The peptides expressed by cTECs may be unique to cTECs and different from the peptides expressed by other cells because of the unique enzymatic activity of cTEC-specific thymoproteasome. Alternatively, the decreased chymotrypsin-like activity of thymoproteasomes may uniquely limit the variety of MHC class I-associated peptides displayed by cTECs. It is also possible that these antibodies may recognize a conformational status of K<sup>b</sup>, which may be increased specifically in cTECs in the absence of  $\beta 5t$  but be independent of the variety of associated peptides.

Our results show that the positive selection of various transgenic TCR is differentially affected by  $\beta 5t$  deficiency in multiple magnitudes rather than in an all-or-none manner. It is thus speculated that the positive selection of a single TCR specificity is induced by multiple (thymoproteasome-dependent and thymoproteasome-independent) peptides associated with MHC class I molecules. A major fraction of positively selecting peptides may be uniquely generated by thymoproteasomes in cTECs, whereas some positively selecting peptides may be additionally generated in a thymoproteasome-independent manner.



**Figure 4. CD8<sup>+</sup> T Cells Generated in  $\beta$ 5t-Deficient Mice Are Functionally Potent to Proliferate and Become Cytotoxic T-Lymphocytes in Response to TCR Stimulation In Vitro and to Maintain Survival In Vivo**

(A and B) Splenic CD8<sup>+</sup> T cells were purified from *Psmb11<sup>+/-</sup>* or *Psmb11<sup>-/-</sup>* mice, labeled with CFSE, and cultured with or without plate-bound anti-CD3 and anti-CD28 for 48 hr. (A) Histograms show CFSE fluorescence profiles, and numbers indicate the frequency of CFSE<sup>lo</sup> cells. (B) mRNA expression of granzyme B determined by quantitative RT-PCR analysis was normalized to GAPDH mRNA expression, and those in *Psmb11<sup>+/-</sup>* CD8<sup>+</sup> T cells before the culture were set to 1.

(C) Splenic CD8<sup>+</sup> T cells ( $1 \times 10^5$ ) from *Psmb11<sup>+/-</sup>* or *Psmb11<sup>-/-</sup>* OT-I-TCR-transgenic H-2<sup>b</sup> mice were labeled with CFSE and cultured with PBS- or SIINFEKL-loaded C57BL/6 splenocytes for 40 hr. Graphs show means  $\pm$  standard errors of the frequency of CFSE<sup>lo</sup> cells ( $n = 3$ ).

(D) Splenic CD8<sup>+</sup> T cells from *Psmb11<sup>+/-</sup>* or *Psmb11<sup>-/-</sup>* mice were labeled with CFSE and intravenously injected into nonirradiated (control) or irradiated (6 Gy) wild-type mice or nonirradiated *Rag2<sup>-/-</sup>* mice ( $1 \times 10^6$  cells/host mouse). Graphs indicate numbers of CD8<sup>+</sup>CFSE<sup>+</sup> splenocytes in individual mice (circles) and their means (bars) ( $n = 2$  to 3).

(E) The expression of CD5, CD44, CD62L, and Ly6C in CD8<sup>+</sup>TCR $\beta^+$  spleen cells from  $\beta$  *Psmb11<sup>+/-</sup>* or *Psmb11<sup>-/-</sup>* mice was analyzed by flow cytometry. Numbers in histograms indicate MFI.

(F) The expression of CD44 and CD8 in TCR $\beta^+$  spleen cells from *Psmb11<sup>+/-</sup>* or *Psmb11<sup>-/-</sup>* mice at indicated ages was analyzed by flow cytometry. Numbers in dot plots indicate the frequency of cells within the box. Graphs indicate the ratios of the numbers of CD44<sup>hi</sup> CD8<sup>+</sup> T cells over CD44<sup>lo</sup> CD8<sup>+</sup> T cells in individual mice (circles) and their means (bars,  $n = 3-6$ ). \* $p < 0.05$ .

(G) Absolute numbers (circles) and their means (bars,  $n = 6$ ) of total, CD44<sup>lo</sup>, and CD44<sup>hi</sup> subsets of CD8<sup>+</sup>TCR $\beta^+$  spleen cells in *Psmb11<sup>+/-</sup>* or *Psmb11<sup>-/-</sup>* mice at 6 to 7 weeks old were analyzed by flow cytometry.

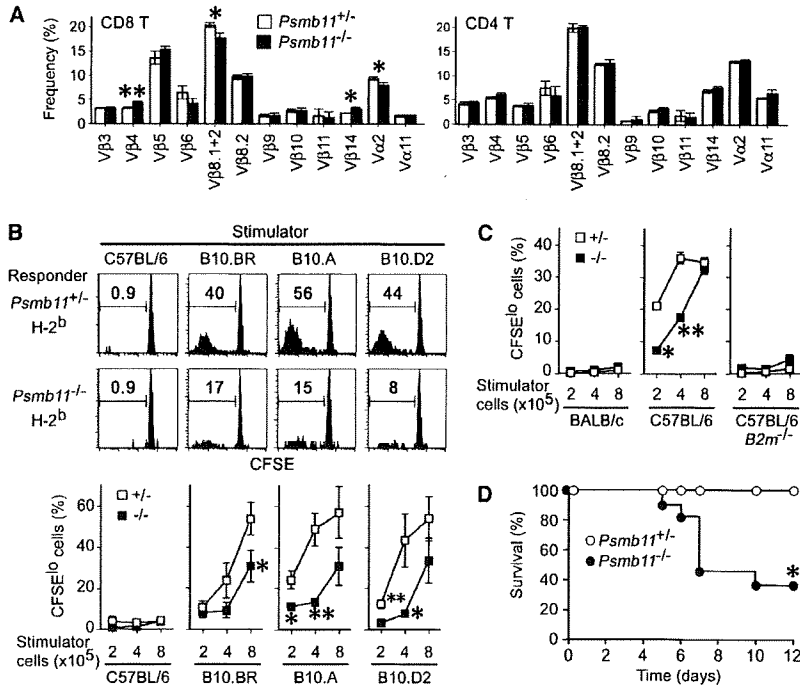
(H) CD44<sup>lo</sup> and CD44<sup>hi</sup> CD8<sup>+</sup> T cells isolated from the spleen of *Psmb11<sup>+/-</sup>* (open bars) or *Psmb11<sup>-/-</sup>* (closed bars) mice were labeled with CFSE and cultured with or without plate-bound anti-CD3 and anti-CD28 antibodies for 48 hr. Graphs indicate the frequency of CFSE<sup>lo</sup> cells.

(I) CD44<sup>lo</sup> CD8<sup>+</sup> T cells ( $5 \times 10^5$ ) from wild-type mice were CFSE-labeled and intravenously administered into nonirradiated *Rag2<sup>-/-</sup>*, *Psmb11<sup>+/-</sup>*, or *Psmb11<sup>-/-</sup>* mice. Histograms show representative CFSE fluorescence profiles ( $n \geq 5$ ), and numbers indicate the frequency of CFSE<sup>lo</sup> cells.

It is also interesting to note that the signaling intensity of transgenic TCR, which is measured by the amount of surface CD5 and CD8 expression (Park et al., 2007), weakly correlated with the presumable  $\beta$ 5t independency of positively selecting ligands, suggesting that positively selecting peptides generated in cTECs by thymoproteasomes tend to exhibit low TCR signaling intensity. It is possible that the MHC class I-associated self-peptides uniquely displayed by cTECs may be rich in peptides that exhibit low-avidity TCR engagement, as previously suggested (Ashton-Rickardt et al., 1994). It should be emphasized that the order of  $\beta$ 5t independency among the TCR-transgenic models (HY < P14 < F5 < OT-I < 2C) is not identical to the order of the TCR signaling intensity (HY < F5 < 2C  $\leq$  P14 < OT-I) (Figure S5; Ernst et al., 1999; Ge et al., 2004; Park et al., 2007; Agenès et al., 2008), arguing against the possibility that  $\beta$ 5t regulates the production or activity of a general costimulus for positive selection. Indeed,

our results showing that  $\beta$ 5t-deficient cTECs are fully capable of presenting various concentrations of antigen peptide to stimulate CD8<sup>+</sup> T cells contradict such possibility.

The present results also show that  $\beta$ 5i-containing immunoproteasomes appear to be the dominant cellular proteasome in cTECs of  $\beta$ 5t-deficient mice. Immunoproteasomes are able to produce a set of peptides that efficiently associate with MHC class I molecules at the carboxyl terminus (Rock and Goldberg, 1999). Indeed, our results suggest that cTECs in  $\beta$ 5t-deficient mice express the MHC class I-peptide complex, whereas the altered set of MHC class I-peptide complex expressed by thymoproteasome-deficient and thereby immunoproteasome-dominant cTECs is inefficient in inducing the positive selection of major CD8<sup>+</sup> T cell repertoires. Thus, in order to generate an optimum CD8<sup>+</sup> T cell repertoire, cTECs may have to display a repertoire of MHC class I-bound peptides that inefficiently



**Figure 5. Altered TCR Repertoire and Defective Immune Responses of CD8<sup>+</sup> T Cells in  $\beta$ 5t-Deficient Mice**

(A) TCR-V $\beta$  and TCR-V $\alpha$  distribution in CD8<sup>+</sup>TCR $\beta$ <sup>+</sup> spleen cells (CD8<sup>+</sup> T cells) or CD8<sup>-</sup>TCR $\beta$ <sup>+</sup> spleen cells (CD4 T cells) from *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> mice was determined by flow cytometry. Graphs show means  $\pm$  standard errors of three independent measurements. (B and C) Splenic CD8<sup>+</sup> T cells ( $1 \times 10^5$ ) from *Psmb11*<sup>+/-</sup> or *Psmb11*<sup>-/-</sup> H-2<sup>b</sup> (B) or H-2<sup>d</sup> (C) mice were labeled with CFSE and cocultured with irradiated splenocytes ( $2 \times$ ,  $4 \times$ , or  $8 \times 10^5$ ) from indicated mouse strains for 4 days. Histograms show representative CFSE fluorescence profiles of CD8<sup>+</sup> T cells cultured with  $4 \times 10^5$  stimulator cells. Numbers in histograms indicate the frequency of CFSE<sup>0</sup> cells. Graphs show means  $\pm$  standard errors of at least three independent measurements. (D) Eleven *Psmb11*<sup>-/-</sup> mice (closed circles) and 6 *Psmb11*<sup>+/-</sup> mice (open circles) were infected with 1,000 PFU of A/PR8 virus. Survival was monitored up to 12 days after the infection. Data were pooled from two experiments. The Mann-Whitney nonparametric *U*-test was used to compare survival between groups of mice. \**p* < 0.05; \*\**p* < 0.01. See also Figure S4.

associate with MHC class I molecules and tend to provide low TCR signaling intensity.

Our results show that CD8<sup>+</sup> T cells in  $\beta$ 5t-deficient mice are defective in allogeneic and antiviral responses. Upon TCR stimulation, CD8<sup>+</sup> T cells in  $\beta$ 5t-deficient mice were capable of proliferation and granzyme B production. On the other hand, the TCR repertoire in V regions of CD8<sup>+</sup> T cells was altered in  $\beta$ 5t-deficient mice. Allogeneic responses of CD8<sup>+</sup> T cells in  $\beta$ 5t-deficient mice were defective in various, but not all, allogeneic combinations and were defective even when CD8<sup>+</sup> T cells were isolated into naive T cells. Thus, we think that the defect in immune responses of  $\beta$ 5t-deficient mice is due to an altered repertoire of CD8<sup>+</sup> T cells generated in the absence of  $\beta$ 5t-containing thymoproteasome. It is remarkable that the thymoproteasome, which may affect the MHC class I-associated peptide repertoire specifically in cTECs, is essential for shaping the immunocompetent TCR repertoire of CD8<sup>+</sup> T cells.

We found that the immune protection of  $\beta$ 5t-deficient mice from influenza virus infection is defective. Cytotoxic T lymphocyte (CTL) activity was observed in mice from day 5 to day 15 after influenza virus infection (Kedzierska et al., 2006; Stambas et al., 2007) and that virus-specific CD8 CTLs are the key effectors of virus clearance in mice infected with influenza virus (Bender et al., 1992; Mozdzanowska et al., 1997; Doherty et al., 1997). Thus, we think that the death of  $\beta$ 5t-deficient mice infected with influenza virus is a result of incompetent CTL responses in the mice due to an altered repertoire of CD8<sup>+</sup> T cells.

Based on the analysis of mice expressing B cell lymphoma-derived single MHC peptides, it was previously assumed that any peptide that causes low-avidity TCR engagement can trigger positive selection of thymocytes and that rather than

positive selection, subsequent negative selection establishes repertoire formation of T cells (Ignatowicz et al., 1996; Huseby et al., 2005). It was additionally shown that the ability to induce positive selection experimentally was not limited to cTECs but also included fibroblasts and T-lineage cells (Pawlowski et al., 1993; Hugo et al., 1993; Zinkernagel and Althage, 1999; Martinic et al., 2003; Choi et al., 2005; Li et al., 2005). In fact, if positive selection should select developmental thymocytes solely according to low-avidity TCR engagement, any MHC-peptide complex expressed by any cell type could support the generation of a full repertoire of T cells. However, our results demonstrate that the development of an immunocompetent T cell repertoire requires positive selection by thymoproteasome-dependent MHC class I-peptide complexes specifically expressed by cTECs. Thus, the small number and unusual repertoire of T cells positively selected by the single MHC-peptides (Ignatowicz et al., 1996; Fukui et al., 1997) may resemble abnormal positive selection and incompetent T cell development detectable in  $\beta$ 5t-deficient mice. Accordingly, similar to  $\beta$ 5t-deficient cTECs, fibroblasts and hematopoietic cells can only induce positive selection of only a limited repertoire of T cells, such as 2C-TCR-transgenic T cells, but not HY-TCR-transgenic T cells (Zerrahn et al., 1999; Lilić et al., 2002). Instead, our results support the idea that self-peptides expressed by cTECs critically contribute to the establishment of an immunocompetent T cell repertoire (Singer et al., 1986).

The unique peptide-producing activity of cTECs may not be limited to MHC class I-associated peptides but also occur in MHC class II-associated peptides (Takahama et al., 2008), since cathepsin L and thymus-specific serine protease, which are lysosomal proteases that are highly expressed by cTECs, are required for the optimal generation of the CD4 T cell repertoire

(Nakagawa et al., 1998; Honey et al., 2002; Gommeaux et al., 2009). A recent report also showed that thymic epithelial cells, including cTECs, have high constitutive activity of autophagy, which contributes to MHC class II peptide-mediated T cell repertoire formation and self-tolerance (Nedjic et al., 2008). The unique features of cTECs in protein degradation and self-peptide presentation may govern the positive selection of T cells in both CD4 and CD8 lineages.

The present study reveals that positive selection induced by thymoproteasome-expressing cTECs is essential for the formation of an immunocompetent and self-protective repertoire of CD8<sup>+</sup> T cells. In the absence of thymoproteasome-mediated positive selection, mice exhibited severe defects in the allogeneic responses of CD8<sup>+</sup> T cells and in the survival to influenza virus infection. On the other hand, we detected no signs of autoimmunity in thymoproteasome-deficient mice. Thus, positive selection induced by thymoproteasomes and cTECs specifically governs the installment of diverse repertoire of CD8<sup>+</sup> T cells, which is important for rejecting allogeneic tissues and eradicating virus-infected cells, rather than the establishment of a self-tolerant T cell repertoire, which is separately governed by other mechanisms, including Aire-associated promiscuous gene expression by mTECs (Derbinski et al., 2001; Anderson et al., 2002). Together, this study suggests that positive selection induced by a thymoproteasome-specific repertoire of self-peptides is essential for the installment of an immunocompetent and self-protective repertoire of CD8<sup>+</sup> T cells. We propose that the cTEC expression of thymoproteasomes be considered for the therapeutic reconstitution of T cells by transplantation or regeneration of thymic stromal cells in the clinical setting.

#### EXPERIMENTAL PROCEDURES

##### Mice

The list of mice used in this study and the procedure for preparation of irradiated bone marrow chimeras are described in Supplemental Experimental Procedures. Animal experiments were performed after obtaining approval from the Animal Experimentation Committee of the University of Tokushima.

##### Flow Cytometry Analysis and Cell Sorting

Multicolor flow cytometry analysis and cell sorting were performed with FACS-Calibur and FACS-Vantage (BD Bioscience), as described previously (Ueno et al., 2005). Thymic stromal cells were prepared by digesting thymic fragments with collagenase, dispase, and DNase I (Roche), as described previously (Gray et al., 2006; Hikosaka et al., 2008). To sort thymic epithelial cells, thymic stromal cells were enriched by depleting CD45<sup>+</sup> cells and TER119<sup>+</sup> cells using magnetic-bead-conjugated antibodies specific for CD45 and TER119 (Miltenyi Biotec) prior to FACS cell sorting.

##### Reaggregated Thymus Organ Culture

The procedures for fetal thymus organ culture and reaggregated thymus organ culture were described previously (Ueno et al., 2005). DP thymocytes were sorted from adult TCR transgenic mice (purity >99%). Thymic stromal cells were prepared from E15.5 fetal thymus lobes that were cultured for 5 days in the presence of 2-deoxyguanosine. DP thymocytes ( $5 \times 10^5$ ) and thymic stromal cells ( $5 \times 10^4$ ) were reaggregated and organ-cultured for 4 days.

##### Analysis of Proteasomal Subunits

Ly51<sup>+</sup> cells were magnetically enriched from adult thymus using biotin-conjugated antibody specific for Ly51 and streptavidin-conjugated magnetic beads. Cells with >90% purity were lysed and subjected to immunoblot analysis.

#### Additional Experimental Procedures

See Supplemental Experimental Procedures for additional experimental procedures.

#### SUPPLEMENTAL INFORMATION

The Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2009.10.009.

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# Milk fat globule epidermal growth factor-8 blockade triggers tumor destruction through coordinated cell-autonomous and immune-mediated mechanisms

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**Carcinogenesis reflects the dynamic interplay of transformed cells and normal host elements, but cancer treatments typically target each compartment separately. Within the tumor microenvironment, the secreted protein milk fat globule epidermal growth factor-8 (MFG-E8) stimulates disease progression through coordinated  $\alpha_v\beta_3$  integrin signaling in tumor and host cells. MFG-E8 enhances tumor cell survival, invasion, and angiogenesis, and contributes to local immune suppression. We show that systemic MFG-E8 blockade cooperates with cytotoxic chemotherapy, molecularly targeted therapy, and radiation therapy to induce destruction of various types of established mouse tumors. The combination treatments evoke extensive tumor cell apoptosis that is coupled to efficient dendritic cell cross-presentation of dying tumor cells. This linkage engenders potent antitumor effector T cells but inhibits FoxP3<sup>+</sup> T reg cells, thereby achieving long-term protective immunity. Collectively, these findings suggest that systemic MFG-E8 blockade might intensify the antitumor activities of existing therapeutic regimens through coordinated cell-autonomous and immune-mediated mechanisms.**

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Abbreviations used: 5-FU, 5-fluorouracil; BMDC, bone marrow-derived dendritic cell; MFG-E8, milk fat globule epidermal growth factor-8; NOD-SCID, nonobese diabetic-severe combined immunodeficiency; VEGFR-2, antivascular endothelial growth factor receptor-2.

Cancer pathogenesis involves not only the cell-autonomous defects that arise from alterations in oncogenes and tumor suppressors but also the impact of host antitumor responses (1). Cancer cells that have escaped immune control are selected for the ability to exploit factors present in the tumor microenvironment to further disease progression (2–4). Among this array of soluble moieties, inflammatory cytokines including TNF- $\alpha$ , IL-6, and IL-1 $\beta$  play key roles through triggering NF- $\kappa$ B-, STAT-3-, and MyD88-dependent pathways (5–8).

GM-CSF is another cytokine frequently produced in the tumor microenvironment, where it may contribute to either tumor protection or promotion (9). Through studies of GM-CSF-deficient mice, we identified milk fat globule epidermal growth factor-8 (MFG-E8) as a critical determinant of the pro- and antiinflammatory activities of the cytokine (10). MFG-E8 is a secreted

phosphatidylserine-binding protein that signals through  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins (9–12). Under steady-state conditions, GM-CSF induces MFG-E8 expression in mononuclear phagocytes, enabling the efficient uptake of apoptotic cells, the production of TGF- $\beta$  and CCL22, and the maintenance of FoxP3<sup>+</sup> T reg cells (10). Under conditions of cellular stress, however, the ligation of Toll-like receptors dampens MFG-E8 expression, whereupon GM-CSF elicits CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells through an MFG-E8-independent pathway. Thus, the levels of MFG-E8 present in the tumor microenvironment might modulate the functions of GM-CSF during carcinogenesis.

In malignant melanoma, MFG-E8 expression is increased in tumor cells and/or infiltrating

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myeloid elements upon progression to the vertical growth phase, the stage in which melanoma cells acquire the competence for invasion and dissemination (12, 13). In a mouse melanoma model, MFG-E8 augmented tumorigenicity and metastatic capability through Akt- and Twist-dependent mechanisms (12). MFG-E8 enhanced melanoma cell resistance to apoptosis, induced an epithelial-to-mesenchymal transition, and stimulated invasion and angiogenesis. MFG-E8 also contributed to local immune suppression by evoking FoxP3<sup>+</sup> T reg cell infiltrates and suppressing Th1 reactions and NK and CD8<sup>+</sup> T cell cytotoxicity.

Because MFG-E8 is expressed at high levels in diverse tumor types (14, 15), including melanoma, this soluble protein might serve as a general target for cancer therapy. In contrast to most oncologic treatments, which primarily address either the tumor or host separately, MFG-E8 antagonists might affect both compartments. Indeed, shRNA knockdowns of MFG-E8 sensitized tumor cells to cytotoxic agents and small molecule inhibitors of receptor tyrosine kinases *in vitro*, whereas MFG-E8 blockade with a dominant-negative mutant potentiated tumor immunity generated with irradiated, GM-CSF-secreting tumor cell vaccines (10, 12). Based on these results, we hypothesized that systemic targeting of MFG-E8 might contribute to tumor destruction in several complementary ways. In this paper, we show that antibodies to MFG-E8 cooperate with conventional cancer therapies to effectuate sustained control of established mouse tumors through the coupling of cell-autonomous and host-mediated pathways.

## RESULTS

### Combinatorial therapy with systemic MFG-E8 blockade

To explore the therapeutic potential of anti-MFG-E8 antibodies, we first characterized the moderately immunogenic MC38 colon carcinoma model that is syngeneic to C57BL/6 mice. At 10 d after intradermal inoculation, when tumors were well established (~25 mm<sup>2</sup>), the systemic administration of gemcitabine, a cytotoxic agent with modest activity in patients with colon carcinoma (16), afforded a small delay in MC38 tumor growth in a dose-dependent fashion (Fig. 1 A). Although a blocking anti-MFG-E8 mAb (17) displayed minimal antitumor activity when infused alone, combination treatment with gemcitabine resulted in tumor regressions that were sustained throughout the duration of the study (4 mo). A rabbit polyclonal anti-MFG-E8 serum (18) showed comparable efficacy (Fig. 1 B), whereas an isotype control mAb was inactive (not depicted), establishing the specificity of the response. Synergistic antitumor effects were also obtained when the anti-MFG-E8 antibodies were administered beginning 3 or 7 d after gemcitabine but not when infused before chemotherapy, indicating that the sequence of the combination was important for activity (Fig. S1).

Systemic anti-MFG-E8 antibodies similarly enhanced the therapeutic potency of 5-fluorouracil (5-FU) and CPT-11 (Fig. 1 C), two agents frequently used in the treatment of advanced colon carcinoma patients (16). As with gemcitabine, these combination therapies achieved prolonged tumor con-

trol, in contrast to the limited impact of individual agents. MFG-E8 blockade also intensified the activity of an epidermal growth factor receptor tyrosine kinase inhibitor and an anti-vascular endothelial growth factor receptor-2 (VEGFR-2) mAb (Fig. 1 D). Additionally, a short course of radiation therapy directed toward subcutaneous MC38 lesions was rendered more efficacious when combined with anti-MFG-E8 antibodies (Fig. 1 E). Synergistic effects of anti-MFG-E8 antibodies and chemotherapy were similarly observed in the poorly immunogenic B16 melanoma model. In this system, combinations of MFG-E8 blockade with doxorubicin, etoposide, or dacarbazine achieved significant tumor control, although gemcitabine proved inactive (Fig. 1 F and not depicted). Moreover, the combined administration of anti-MFG-E8 antibodies and doxorubicin also triggered the destruction of established EL-4 thymomas (Fig. S2). Collectively, these experiments reveal the ability of systemic MFG-E8 blockade to intensify the antitumor effects of conventional oncologic therapies in diverse tumor models.

### MFG-E8 blockade enhances drug-induced apoptosis

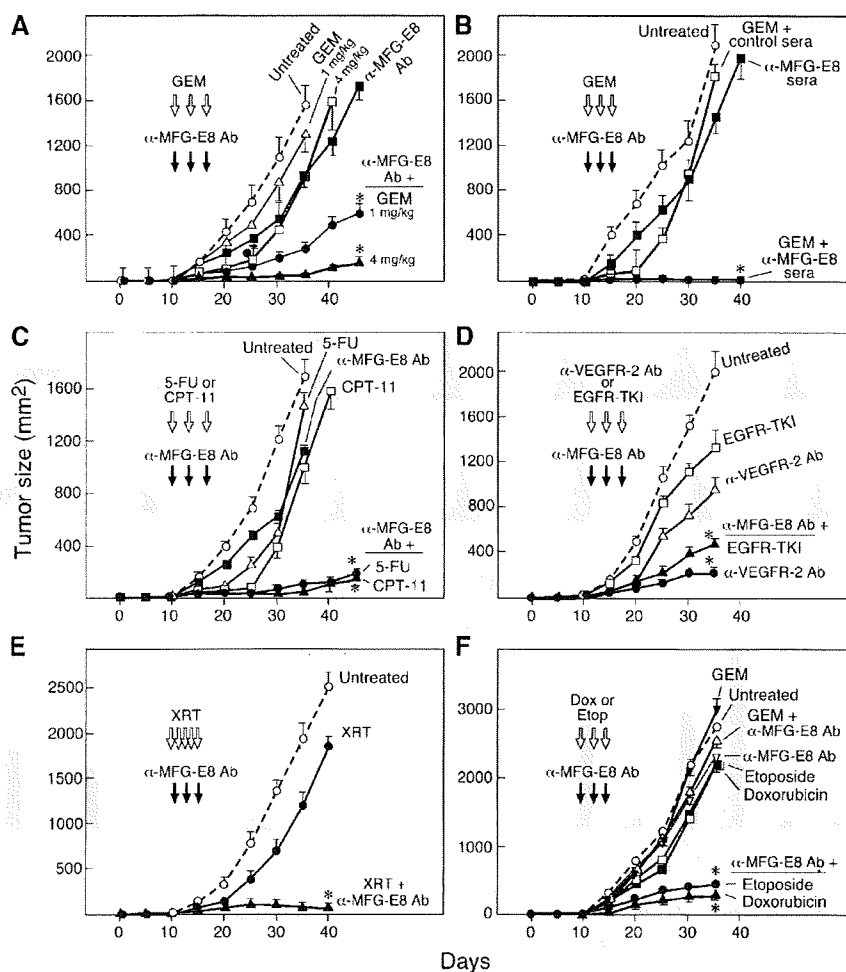
Because standard cancer treatments displayed only modest single-agent activity against various types of tumors, we wondered whether the anti-MFG-E8 antibodies might modulate tumor cell killing. In this context, we previously showed that MFG-E8 triggered Akt activation in tumor cells through  $\alpha_v\beta_3$  integrin signaling, which resulted in an attenuation of etoposide-induced death (12). In accordance with these findings, MFG-E8 was not detectable in MC38 and B16 cells at baseline, whereas cells that survived an overnight exposure to diverse chemotherapeutic drugs manifested significant MFG-E8 expression, which was evident intracellularly, at the surface membrane, and in culture supernatants (Fig. 2, A and B; and not depicted).  $\gamma$  irradiation also induced MFG-E8 surface expression on EL-4 thymoma (EG.7-OVA) cells (Fig. 2 C). Furthermore, stable drug-resistant variants of MC38 cells, which were generated through prolonged exposure to escalating concentrations of gemcitabine, CPT-11, or 5-FU *in vitro*, and similarly derived doxorubicin-resistant B16 cells demonstrated much higher levels of MFG-E8 compared with the parental tumor cells (Fig. 2 D and not depicted).

Consistent with an antiapoptotic function for surface and/or secreted MFG-E8, the addition of anti-MFG-E8 mAbs but not irrelevant control antibodies potentiated the killing of MC38 cells with gemcitabine and 5-FU, as revealed by the enhanced expression of annexin V (Fig. 2 E). The addition of rabbit polyclonal anti-MFG-E8 serum manifested comparable effects (unpublished data). The induction of apoptosis with the combined therapy also resulted in a loss of mitochondrial membrane potential (unpublished data). Moreover, the combination therapies triggered increased levels of tumor apoptosis *in vivo*. MC38 tumors harvested from mice that received gemcitabine plus anti-MFG-E8 antibodies showed enhanced caspase 3 activation as compared with tumors isolated from mice treated with either agent alone (Fig. 2 F). Similarly,

B16 melanomas manifested elevated caspase 3 activation after combined dacarbazine and MFG-E8 blockade. These results support the idea that MFG-E8 blockade compromises tumor cell viability in a cell-autonomous fashion, although the increased caspase activation in vivo might partially reflect the death of stromal elements within the tumor microenvironment. The anti-MFG-E8 antibodies may also attenuate tumor angiogenesis (18), which might contribute to tumor cell death as well. Collectively, these results reveal a role for MFG-E8 in mediating resistance to cytotoxic therapy and suggest that anti-MFG-E8 antibodies might serve as a complementary strategy to intensify drug-induced tumor cell killing.

### Combinatorial therapy with MFG-E8 blockade stimulates T cell immunity

Because MFG-E8 plays a key role in T reg cell homeostasis (10), we investigated whether the antitumor activities of combined chemotherapy and MFG-E8 blockade might also involve host immunity. In accordance with this idea, anti-MFG-E8 antibodies failed to increase the minimal killing of MC38 cells achieved with gemcitabine in immunodeficient nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice (Fig. 3 A). Moreover, antibody depletion experiments established that CD8<sup>+</sup> and to a lesser extent CD4<sup>+</sup> T cells, but not NK cells, were required in wild-type



**Figure 1. MFG-E8 antibody blockade synergizes with cytotoxic therapies to mediate tumor destruction.** (A) Established MC38 carcinomas (25 mm<sup>2</sup>) were treated with systemic gemcitabine (GEM) and/or an anti-MFG-E8 mAb, as indicated. (B) Same conditions as in A, but with rabbit anti-MFG-E8 sera. (C) Established MC38 carcinomas were treated with 5-FU or CPT-11 with or without anti-MFG-E8 mAb. (D) Established MC38 carcinomas were treated with anti-VEGFR-2 mAb DC101 or EGFR-TKI AG490 with or without anti-MFG-E8 mAb. (E) Established MC38 tumors were treated with local irradiation (XRT) with or without systemic anti-MFG-E8 mAb. (F) Established B16 melanomas (25 mm<sup>2</sup>) were treated with systemic doxorubicin, etoposide, or GEM with or without anti-MFG-E8 mAb. Each experiment was performed with five mice per group, and similar results were observed for each panel in five independent experiments. Shown are the means  $\pm$  SEM per cohort for a representative experiment. \*,  $P < 0.05$  between a treatment group and control.