Promoter Knock-In Mutations Reveal a Role of Mcl-1 in Thymocyte-Positive Selection and Tissue or Cell Lineage-Specific Regulation of Mcl-1 Expression[1]

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We previously demonstrated that IL-3 stimulates transcription of the antiapoptotic gene *mcl-1* via two promoter elements designated as the SIE and CRE-2 sites. To further study the functional role of these two DNA elements, mutant mice with targeted mutations of both SIE and CRE-2 sites (SC mutants) were generated. Homozygous SC mutants manifested a markedly reduced level of Mcl-1 in thymus but not in other major organs such as spleen, liver, lung, or heart. Reduced expression of Mcl-1 in SC mutant thymus resulted in attenuated positive selection of double-positive thymocytes into both CD4 and CD8 lineages, a result likely due to reduced survival of SC mutant double-positive thymocytes that were supposed to be positively selected. In contrast, in the peripheral lymphoid organs, only $CD8^+$ but not $CD4^+$ T cells were significantly reduced in homozygous SC mutant mice, a result consistent with a more dramatic decrease both of Mcl-1 expression and cell viability in mutant $CD8^+$ compared with mutant $CD4^+$ T cells. Impaired T cell development and peripheral $CD8^+$ lymphopenia in homozygous SC mutant mice were both cell autonomous and could be rescued by enforced expression of human Mcl-1. Together, the promoter-knock-in mouse model generated in this study not only revealed a role of Mcl-1 in thymocyte-positive selection, but also uncovered that Mcl-1 expression is regulated in a tissue or cell lineage-specific manner. *The Journal of Immunology,* **2009, 182: 2959–2968.**

Mcl-1, a member of the Bcl-2 family protein, was originally identified in the ML-1 human myeloid leukemia cell line that underwent phorbol ester-induced differentiation (1). Mcl-1 is widely expressed in various tissues and cell lineages, and a great number of studies indicate that Mcl-1 plays an apical role in many cell survival and death pathways (2). Mcl-1 deficiency results in embryonic lethality at the peri-implantation stage (3). Conditional knock-out approaches have thus been used to assess Mcl-1 functions in other tissues. Using this approach, Mcl-1 has been shown to be essential for the survival of many cell types in the hematopoietic system, including hematopoietic stem cells, lymphoid precursors, mature T and B lymphocytes, and neutrophils (4–6).

T cell progenitors arise from the bone marrow and migrate into the thymus to become early committed T cells that lack expression of TCR, CD4, and CD8. These cells are termed double-negative (DN)[3] thymocytes (7). DN thymocytes then develop in an ordered progression and mature into the $CD4^+CD8^+$ double-positive (DP) stage (8). At this stage, immature DP thymocytes expressing unique TCRs are tested individually by two selection processes that eliminate cells whose TCRs either cannot recognize self peptide/MHC complex (positive selection) or react too strongly (negative selection). These two selection processes ensure that most DP thymocytes selected to differentiate into CD4 single-positive (SP) or CD8SP thymocytes, and eventually peripheral T cells, can respond properly when encountering foreign peptides in the context of self MHC in the periphery (8–10). Many Bcl-2 family members have been shown to play a role in T cell development. Deletion of Bcl-2 resulted in rapid loss of thymocytes and naive T cells (11, 12); Bcl-xL is required for the survival of DP thymocytes (13–15) and Bim is essential for thymocyte negative selection (16, 17). Mcl-1 plays an important role in the survival of DN and SP thymocytes (6, 18). Whether Mcl-1 is also involved in other stages of T cell development, e.g., the DP to SP transition, is still not clear.

Mcl-1 expression is stimulated by a variety of cytokines (IL-3, IL-5, IL-6, IL-7, IL-15) and growth factors (GM-CSF, vascular endothelial growth factor, epidermal growth factor) (6, 19–23). Activation of Mcl-1 expression by IL-3 is mainly regulated at the transcriptional level (20). Further analysis indicated that IL-3-stimulated transcription of the *mcl-1* gene in a bone marrow-derived cell line is mediated through two upstream DNA motifs located at positions −87 (the SIE site) and −70 (the CRE-2 site), respectively (24). Interestingly, these two promoter elements can each confer IL-3 inducibility on a heterologous promoter, but work additively in mediating IL-3 response via two distinct signaling pathways (24). The CRE-2-binding complex containing the CREB protein is induced and activated by IL-3 via activation of the PI3K (PI3K)/Akt-dependent pathway, whereas IL-3 stimulation of *mcl-1* gene transcription through the SIE motif involves phosphorylation of a hematopoietic cell-specific transcription factor PU.1 by a $p38^{MAPK}$-dependent pathway (25). The initial aim of this study

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[3] Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive; WT, wild type; hMcl-1, human Mcl-1; LN, lymph node; HPRT, hypoxanthine phosphoribosyltransferase; CMFDA, 5-chloromethylfluorescein diacetate.

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was to investigate the functional roles of these two DNA motifs inside an animal system. Unexpectedly, mutations of these two DNA elements turned out to generate mutant mice (hereinafter referred to as SC mutant mice) that manifested a hypomorphic phenotype only in selective cell types such as those in the thymus. The markedly reduced expression of Mcl-1 in the thymus of the SC mutant mice provided a novel mouse model to study detailed roles of Mcl-1 during thymocyte development, which cannot be achieved with previously published Mcl-1 knock-out models (3, 6, 18). In this study, we demonstrate that Mcl-1 plays a role in thymocyte-positive selection. In addition, we demonstrate that Mcl-1 expression is regulated in a tissue or cell type-specific manner.

Materials and Methods

Generation of SC mutant mice

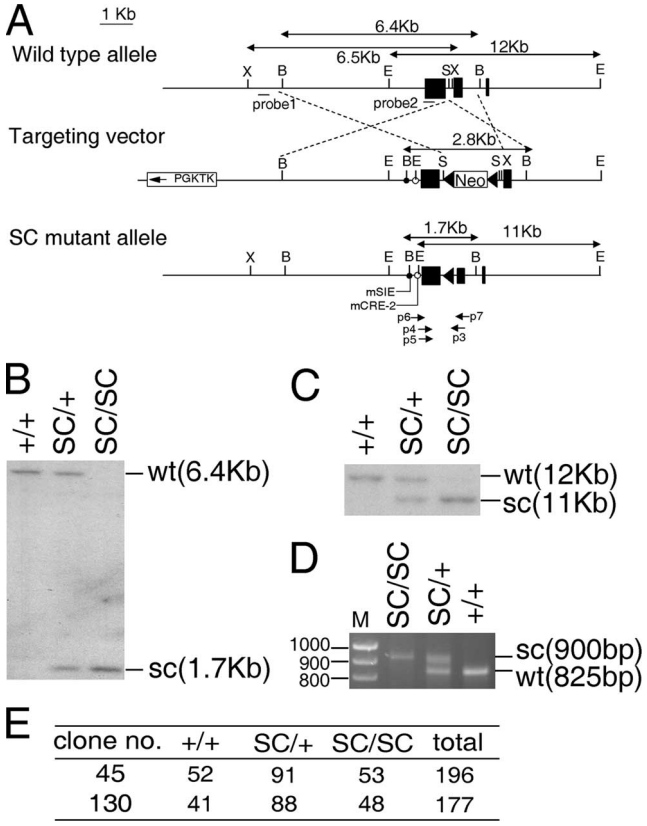
To generate SC mutant mice, two overlapping genomic fragments harboring the *mcl-1* gene locus were isolated from a 129/Svj mouse genomic library and used to construct the targeting vector. This vector, after homologous recombination, introduced a floxed Neo marker and two clustered mutations into the SIE ($^{-94}$TTACGGGAAGTC^{-83}) and CRE-2 sites ($^{-77}$CTGCGTCAGCAC^{-66}) of the *mcl-1* gene locus (24). For the CRE-2 motif, the introduced mutation generated a new *Eco*RI site ($^{-77}$CTG GAATTCCAC^{-66}), whereas that of the SIE site became a *Bam*HI recognition sequence ($^{-94}$TTAGGATCCGTC^{-83}). This targeting vector was electroporated into R1 embryonic stem cells, and Southern blotting using probe 1 or 2 as depicted in Fig. 1*A* was conducted to select clones that had undergone homologous recombination at the *mcl-1* locus. Two positive ES cell clones (no. 45 and no. 130) were microinjected into C57BL/6 blastocysts to generate chimeric mice. Male chimeric mice were backcrossed with C57BL/6 females to generate heterozygous mice. To remove the Neo cassette in the targeted allele, heterozygous mice were mated with EIIa-CRE transgenic mice (26) (originally in FVB/N background but backcrossed to C57BL/6 for more than seven generations, a gift provided by Ying-Hue Lee at IMB, Academia Sinica, Taipei, Taiwan). Mice used in this study were backcrossed to C57BL/6 for at least seven generations after removal of the Neo marker. To genotype mice with a PCR method, common primer 3 (p3), wild-type (WT)-specific primer 4 (p4) and mutant-specific primer 5 (p5) were mixed together in the same reaction, which would generate 825- and 900-bp DNA fragments for the WT and SC mutant alleles, respectively. The extra 75 bps for the mutant allele was due to the presence of a 34-bp loxP site and some remaining restriction sites after removal of the Neo marker. In some cases, primers 6 and 7 (p6 and p7) were used to genotype both WT and SC mutant alleles (WT, 850 bps; SC, 925 bps). The sequences of primers mentioned above are as follows: P3, 5′-TCTTC CCTCTTCCCGCACGG-3′; P4, 5′-GGAAGTCCTCGCCTGCGTCA-3′; P5, 5′-GATCCGTCCTCGCCTGGAAT-3′; P6, 5′-CTGCGGCGTC CGACCATG-3′, and P7, 5′-CTCTCCCGGCTCTAGAGCAG-3′. All animal experiments were performed in accordance with the guidelines set by Academia Sinica Institutional Animal Care and Utilization Committee.

Flow cytometry

Thymocytes, splenocytes, or cells from lymph nodes were separated into single-cell suspensions and one million cells were used for each set of staining using various combinations of cell surface markers. Data were collected on either FACSCalibur or LSRII with CellQuest or FACSDiva software, respectively (BD Biosciences) and analyzed using FlowJo software (TreeStar). The following mAbs conjugated to PE, FITC, allophycocyanin, PE-Cy7, or pacific blue (from eBioscience or BD Pharmingen) were used for cell surface staining: CD4 (GK1.5), CD8α (53-6.7), TCR-β (H57-597), CD69 (H1.2F3), CD5 (53-7.3), HSA (30-F1), B220 (RA3-6B2), CD3ε (145-2C11), TCR-α2 (B20.1), TCR-β5 (MR9-4), NK1.1 (PK136), TER119 (TER-119), Gr-1 (RB6-8C5), CD11b (M1/70), H-YTCR (T3.70), CD25 (PC61.5), and CD44 (IM7).

Human Mcl-1 (hMcl-1) transgenic mice

hMcl-1 transgenic mice (in C57BL/6 background) were generated by a standard protocol using a transgenic construct in which the human Mcl-1 cDNA was driven by the RNA polymerase II promoter (27). Line no. 30, which manifested the highest level of expression among various lines, was used in this study.



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clone no.	+/+	SC/+	SC/SC	total
45	52	91	53	196
130	41	88	48	177

FIGURE 1. Targeted mutation of the mouse *mcl-1* genomic locus. *A*, The structures of the WT, targeting vector, and recombinant allele are shown together with some relevant restriction enzyme sites (E, *Eco*RI; B, *Bam*HI; X, *Xba*I; S, *Sma*I). Mutant SIE (mSIE) and CRE-2 sites (mCRE-2), probes 1 and 2 and the predicted length of *Eco*RI or *Bam*HI restriction fragments in Southern blot analysis are as indicated. *B* and *C,* Southern blot analysis of genomic DNA extracted from the indicated mouse tail and digested with *Bam*HI (*B*) or *Eco*RI (*C*) and probed with the probe 2. *D*, Representative PCR genotypic analysis using primers p3 and p4 for the WT (wt, 825 bp), and p3 and p5 for the SC mutant allele (SC, 900bp). *E*, Genotype analysis of offspring from Mcl-1$^{+/SC}$ intercrosses.

Immunoblotting

Thymocyte or lymph node subpopulations were purified by cell sorter or magnetic beads. Purified subsets were lysed and analyzed by immunoblotting using Ab specific to mouse Mcl-1 (28), hMcl-1, Bcl-xL, Bax, Bcl-2 (all from Santa Cruz Biotechnology), Bim (ProSci), or β-actin (Sigma-Aldrich).

RNA isolation and quantitative real time RT-PCR

RNA was extracted from sorted cell populations using TRIzol reagent (Invitrogen). One to two micrograms of RNA was used as template for the production of cDNA through reverse transcription. Quantitative real-time PCR was performed in triplicate with TaqMan universal PCR mastermix and mouse mcl-1 or hypoxanthine phosphoribosyltransferase (HPRT)-specific primers and analyzed on an ABI Prism 7900 sequence detector (Applied Biosystems). Data were captured and analyzed using Sequence Detector software. *Mcl-1* mRNA levels were standardized to that of the housekeeping control gene HPRT.

Bone marrow chimeras

Bone marrow cells from WT or SC mutant mice were depleted of B220$^+$ and CD3$^+$ subpopulations and three million of these cells were injected into the lateral tail vein of sex-matched, 6- to 8-wk-old C57BL/6J-Thy1.1 recipient mice that were lethally irradiated with 1000 rad gamma-irradiation. Chimeric mice were analyzed 8–10 wk after reconstitution.

Determination of the homing efficiency of CD8SP or lymph node CD8$^+$ T cells

Homing efficiency was determined according to a published protocol (29) with some modifications. In brief, mature CD8 SP thymocytes or lymph node CD8$^+$ T cells purified from WT or SC mutant mice were labeled with 0.1 μM 5-chloromethylfluorescein diacetate (CMFDA), 20 μM 7-amino-4-chloromethylcoumarin (Molecular Probes), or 2 μM PKH-26 (Sigma-Aldrich) according to the manufacturer's protocol. Labeled cells from each group were mixed in equal numbers in PBS and an aliquot of this mixture was analyzed to confirm the preinjection ratio of SC mutant to WT cells. Two to four million cells from this cell mixture were injected into the tail vein of normal mice. Sixteen hours after injection, lymph nodes and spleens from recipients were analyzed by flow cytometry for the presence of SC or WT cells. The ratio of SC mutant to WT cells recovered from each organ was normalized to the ratio of cells in the preinjection mixture. To minimize any possible bias from a specific labeling dye, control and mutant cells reverse-labeled with the indicated two dyes were always included in every set of experiment.

In vivo cell survival assay

Mature SP thymocytes or lymph node T cells (CD4 or CD8 lineage) were purified from WT or SC mutant mice and labeled with CFSE (carboxyfluorescein diacetate, succinimidyl ester) (Molecular Probes) for 10 min at 37°C. Two to four millions of labeled cells were i.v. injected into the tail vein of normal recipient mice. Five days after injection, lymph nodes and spleens from the recipient mice were analyzed for the presence of CFSE-labeled cells. In some cases, purified control or SC mutant lymph node T cells (both were Thy1.2$^+$) were transferred into C57BL/6J-Thy1.1 recipient mice. Five days after injection, the peripheral lymphoid organs of recipient mice were analyzed for the presence of Thy1.2$^+$, donor CD4$^+$, or CD8$^+$ T cells.

Statistical analysis

Statistical analysis was performed with two-tailed Student's *t* test using the GraphPad Prism 4.0 software.

Results

Targeted mutation of the mouse mcl-1 *genomic locus*

To understand the functional roles of the SIE and CRE-2 motifs located in the mouse *mcl-1* gene promoter inside an animal system, mutant mice carrying an *mcl-1* mutant allele (SC mutant) with both motifs mutated were generated in this study (see *Materials and Methods*). Mutant mice generated in this way harbored a mutant *mcl-1* allele whose SIE and CRE-2 sites were replaced with *Bam*HI and *Eco*RI sites, respectively, and a loxP site plus a few restriction enzyme sites (41-bp long) inserted into the 1st intron of the *mcl-1* gene locus (Fig. 1*A*). As a control, we also generated another *mcl-1* mutant allele (termed wt-loxP) whose structure is identical with the SC mutant allele except that both SIE and CRE-2 sites were still in a WT configuration (see supplemental Fig. S1).[4] Southern blotting and PCR analysis were used to confirm the genotypes of offspring from intercrosses between heterozygous SC mutant mice (Fig. 1, *B–D*) or between heterozygous wt-loxP mice (supplemental Fig. S1).

Defective T cell development in SC mutant mice

Homozygous SC mutant mice (hereinafter, all such homozygous mutants were referred to as SC mutant mice) were born at a ratio that was close to the Mendelian frequency (Fig. 1*E*) and survived to the adulthood without any obvious physical abnormalities. Immunoblotting analysis revealed that Mcl-1 expression in SC mutant mice was markedly reduced (~90%) in thymus (Fig. 2*A* and supplemental Table I), but was only marginally or not affected in various other major organs examined, including liver, heart, lung and spleen (Fig. 2*A*). The tissue-specific reduced expression of Mcl-1 in SC mutant mice was due to the introduced mutations at the *mcl-1* gene promoter, because Mcl-1 expression was not disturbed in the thymus of homozygous wt-loxP control mice (supplemental Fig. S1D).

Immunoblotting analysis indicated that Mcl-1 expression was reduced in all thymocyte subsets (DN, DP, CD4SP, and CD8SP) sorted from SC mutant mice, compared with those from control littermates (Fig. 2*B*). We next examined whether there might be any defect in the thymic development of SC mutant mice. Total thymic cellularity was similar in WT and SC mutant mice. Flow cytometric analysis indicated that there was a slight but consistent decrease of DN, CD4SP, and CD8SP cells in these mutant mice (Fig. 2, *C* and *D*). Further analysis of lineage-negative (CD4$^-$ CD8$^-$ B220$^-$ CD3$^-$ NK1.1$^-$ TER119$^-$ Gr-1$^-$ CD11b$^-$) DN thymocytes indicated that both DN1 (CD25$^-$CD44$^+$) and DN2 (CD25$^+$CD44$^+$) subpopulations were significantly reduced in SC mutant mice, whereas a marginal or no significant reduction was observed for mutant DN3 (CD25$^+$CD44$^-$) and DN4 (CD25$^-$CD44$^-$) subsets (Fig. 2, *E* and *F*).

Mcl-1 is essential for positive selection of both CD4 and CD8 lineages

Next, we examined whether the reduction of SP thymocytes in SC mutant thymus was due to a defect in positive selection. To address this issue, we first examined whether the proportion of DP thymocytes undergoing positive selection, i.e., expressing high levels of maturation markers, such as TCR-β and CD69, was altered in mutant mice. As shown in Fig. 3*A*, 3.48 $\pm$ 0.08% mutant DP thymocytes expressed high levels of TCR-β (TCR-β^{high}), compared with 4.30 $\pm$ 0.05% in control cells ($n = 9$). Similar reduction was also observed for another maturation marker CD69 (6.26 $\pm$ 0.09% in SC mutant v.s. 8.38 $\pm$ 0.10% in WT). These results suggest that the proportion of DP thymocytes that undergo positive selection is reduced in SC mutant compared with WT mice. Of note, those DP cells that were positively selected and matured into TCR-β^{high}CD4SP or TCR-β^{high}CD8SP thymocytes in SC mutant mice had normal expression levels of all maturation markers examined including CD5, HSA, CD3, and CD69 (data not shown).

To further address the role of Mcl-1 in positive selection, control or SC mutant mice were bred to express transgenic TCRs that recognize specific Ags presented in the context of MHC class I or II. To examine positive selection of the CD4 lineage, control or SC thymocytes expressing the OT-II TCR were analyzed. The OT-II TCR (using the Vα2 and Vβ5 gene segments) recognizes the OVA peptide 323–339 presented by MHC class II I-A^b (30). As shown in Fig. 3, *Bi* and *Bii*, SC mutant mice expressing OT-II TCR (SC/SC-OT-II) had reduced percentages and absolute numbers of TCRVα2 TCRV$\beta5^{high}$ DP and CD4SP thymocytes, compared with control mice expressing the same TCR (+/+-OT-II). Lymph node TCRVα2 TCRV$\beta5^{high}$ CD4$^+$ T cells were also significantly reduced in SC/SC-OT-II mice (Fig. 3*Biii*). These results suggest that positive selection of the CD4 lineage is defective in SC mutant mice.

We next examined whether there was any defect in positive selection of the CD8 lineage. To address this issue, control or SC mutant mice were crossed with transgenic mice expressing an $\alpha\beta$TCR that recognizes a peptide from the male Ag H-Y, presented by H-2D^b class I MHC molecules (31). Positive selection of the CD8 lineage in the H-Y TCR transgenic mice was evaluated in the female mice. As shown in Fig. 3, *Ci* and *Cii*, SC mutant females expressing the H-Y TCR (SC/SC-HY) had reduced percentages and absolute numbers of DP and CD8SP thymocytes in the T3.70^{high} (H-Y TCR^{high})-gated population, compared with control mice expressing the same TCR (+/+-HY). Lymph node T3.70^{high}

[4] The online version of this article contains supplemental material.

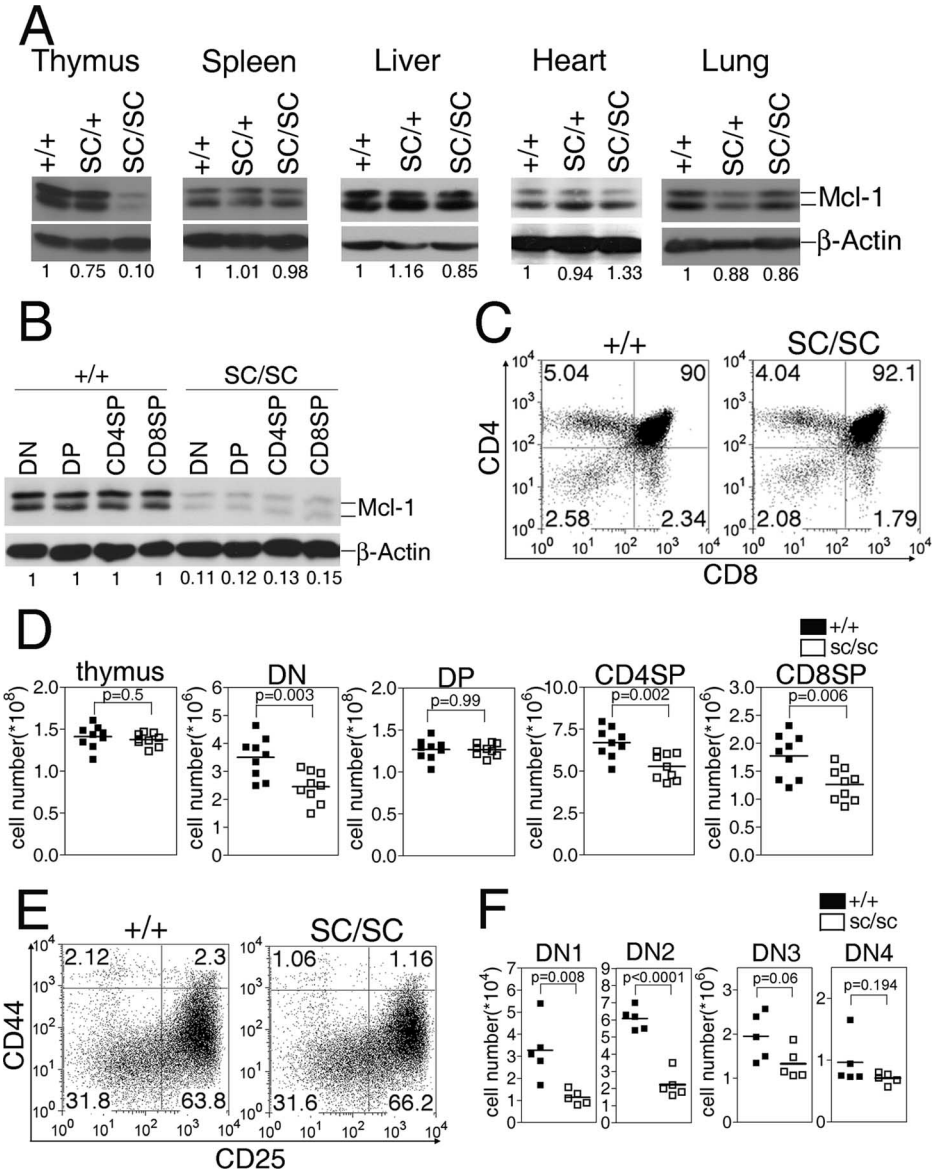

FIGURE 2. SC mutation selectively attenuates Mcl-1 expression in thymus and affects T cell development. *A*, Immunoblotting analysis of total protein extracts lysed from thymus, spleen, liver, heart, or lung from mice with the indicated genotypes (Mcl-$1^{+/+}$, Mcl-$1^{SC/+}$ or Mcl-$1^{SC/SC}$) using Ab specific to Mcl-1 or β-actin. β-actin was included as a loading control. *B*, Immunoblotting analysis of Mcl-1 protein from sorted subsets of thymocytes as indicated from control (+/+) and SC mutant (SC/SC) mice. Relative Mcl-1 protein levels in each indicated tissue or cell type, compared with that in controls (+/+), are as indicated at the bottom of each immunoblot. *C*, Thymocytes from control and SC mutant mice were stained for CD4 and CD8 and analyzed by flow cytometry. Percentage of cells residing within each quadrant is shown on the dot plots. *D*, Number of total thymocytes was determined by trypan blue exclusion. Absolute numbers of DN, DP, and mature (TCR-β^{high}) CD4SP and CD8SP thymocytes were calculated based on the percentage of each population. Nine mice were analyzed for each genotype, each represented by a square (solid or open). *E*, Lineage negative ($CD4^-$ $CD8^-$ $B220^-$ $CD3^-$ $NK1.1^-$ $TER119^-$ $Gr\text{-}1^-$ $CD11b^-$) DN cells were stained for CD25 and CD44 and analyzed by flow cytometry. Numbers indicate percentage of cells in each marked gate. *F*, Graphical representation of DN subset numbers. Subsets are classified as $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-$ $CD25^+$ (DN3), and $CD44^-CD25^-$ (DN4).

$CD8^+$ T cells were also significantly reduced in SC/SC-HY females (Fig. 3*Ciii*). These results suggest that positive selection of the CD8 lineage is also defective in SC mutant mice.

Next, we examined what role Mcl-1 may play in positive selection. We first examined whether Mcl-1 might affect thymocyte proliferation. BrdU pulse-labeling experiment indicated that reduced Mcl-1 expression in SC mutant thymus did not impair thymocyte proliferation (supplemental Fig. S2). In fact, a slightly increased proliferation rate was observed in mutant thymocytes, a result likely due to compensation for peripheral lymphopenia in SC mutant mice (see Fig. 4). We next examined whether Mcl-1 might affect TCR-α rearrangements. To address this issue, we measured the relative level of Jα61 signal end break in control and SC mutant DP thymocytes as a way to assess their endogenous TCRα rearrangements (32). The result shown in supplemental Fig. S3 indicated that the level of Jα61 signal end break was not significantly affected in SC mutant mice with or without expressing OT-II or H-Y TCR, suggesting that decreased Mcl-1 expression in SC mutant thymocytes does not affect TCR-α rearrangements per se.

We next examined the possibility that defective positive selection was due to increased apoptosis of SC mutant DP thymocytes that were supposed to be positively selected. It is difficult to compare a subtle difference in the apoptotic rate between control and SC mutant DP thymocytes that are undergoing positive selection, as apoptotic cells are rapidly cleared by resident macrophages in the thymus. We therefore took an indirect approach to address this issue. First, control or SC mutant mice were injected with anti-CD3 Ab to activate TCR on DP thymocytes. The results shown in supplemental Fig. S4*A* indicate that SC mutant DP thymocytes were much more susceptible to anti-CD3-induced cell death than control thymocytes in vivo. Next, an in vitro "die of neglect" assay was conducted, and the result showed that SC mutant DP thymocytes lost viability significantly faster than control DP cells in a growth medium supplemented with serum only (Fig. S4*B*). Third, we examined the deletion of H-Y TCR transgenic thymocytes in control or SC mutant males expressing the H-Y TCR (+/+-HY or SC/SC-HY). As shown in Fig. S4*C*, a slightly but consistently more reduction both in the percentage and the absolute number of $T3.70^+$ DP as well as CD8SP thymocytes was observed in SC/SC-HY compared with control males. This result indicates that deletion of H-Y TCR transgenic DP thymocytes is slightly more prominent in SC/SC-HY than in control males. Taken together, these results support the possibility that defective positive

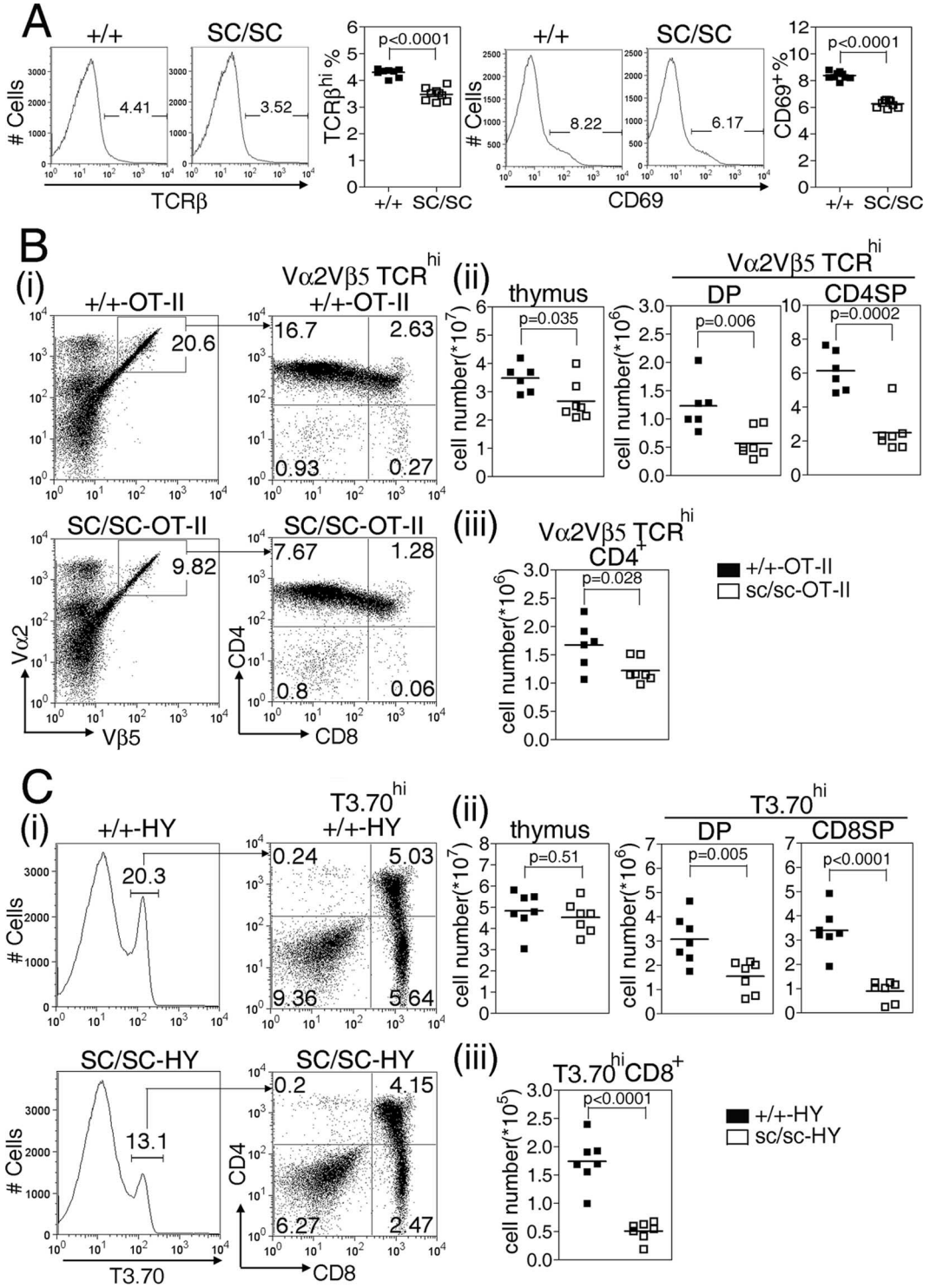

FIGURE 3. Defective positive selection in SC mutant mice. *A*, Thymocytes from Mcl-1$^{+/+}$ or Mcl-1$^{SC/SC}$ mice were stained for CD4, CD8, and TCR-β or CD69 and analyzed by flow cytometry. Histograms show representative TCR-β and CD69 staining profiles on gated DP thymocytes. Numbers indicate percentage of cells in the gated population. *Right panels* next to each pair of histograms are results from analysis of nine mice for each genotype. *B*, Positive selection of the CD4 lineage was analyzed in mice expressing OT-II TCR. Thymocytes from WT or SC mutant mice expressing OT-II TCR (+/+-OT-II or SC/SC-OT-II) were stained for CD4, CD8, TCRVα2, and TCRVβ5. *i*, Surface expression of TCRVα2 and TCRVβ5 on +/+-OT-II or SC/SC-OT-II thymocytes was analyzed by flow cytometry (*left*). Surface expression of CD4 and CD8 on gated TCRV$\alpha2^{high}$/TCRV$\beta5^{high}$ (Vα2Vβ5TCR^{high}) thymocytes was as indicated (*right*). *ii*, Absolute numbers of total, TCRV$\alpha2^{high}$/TCRV$\beta5^{high}$ DP or TCRV$\alpha2^{high}$/TCRV$\beta5^{high}$ CD4 SP thymocytes. Absolute numbers were calculated based on the percentage of each population as analyzed in *i*. *iii*, Absolute number of TCRV$\alpha2^{high}$/TCRV$\beta5^{high}$ CD4$^+$ T cells in lymph nodes from +/+-OT-II or SC/SC-OT-II mice. *C*, Positive selection of the CD8 lineage was analyzed in female mice expressing H-Y TCR. Thymocytes from WT or SC mutant mice expressing H-Y TCR (+/+-HY or SC/SC-HY) were stained for CD4, CD8, and T3.70 TCR. *i*, Surface expression of T3.70 TCR on +/+-HY or SC/SC-HY thymocytes was analyzed by flow cytometry (*left*). Surface expression of CD4 and CD8 on gated T3.70 TCR^{high} thymocytes was as indicated (*right*). *ii*, Absolute numbers of total, T3.70 TCR^{high} DP or T3.70 TCR^{high} CD8 SP thymocytes. Thymic cellularity was calculated as described in *B*. *iii*, Absolute number of T3.70 TCR^{high} CD8 T cells in lymph nodes from +/+-HY or SC/SC-HY mice.

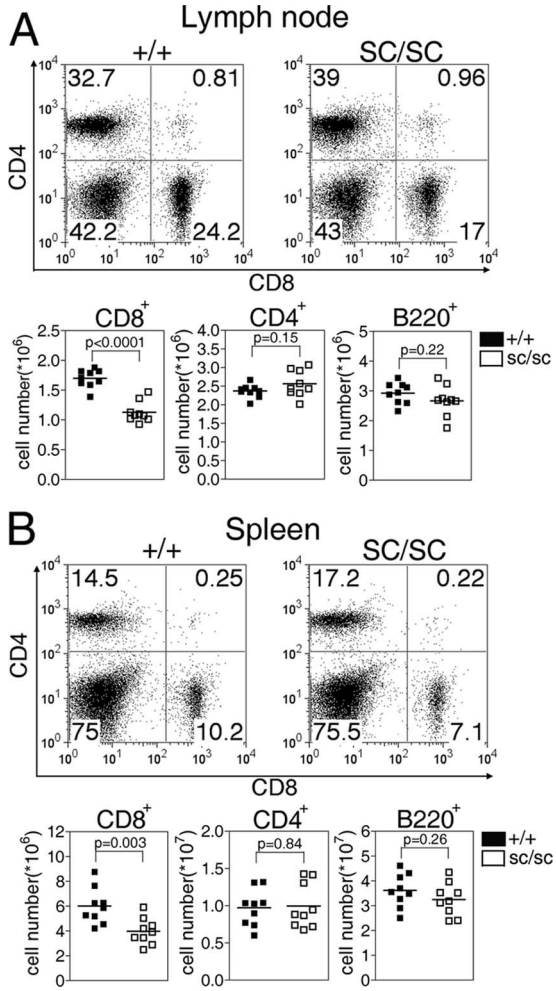

FIGURE 4. Peripheral $CD8^+$ T cell deficiency in SC mutant mice. Lymph nodes (*A*) and spleens (*B*) from WT and SC mutant mice were stained for CD4, CD8, and B220 (data not shown) and analyzed by flow cytometry. Percentage of cells residing within each quadrant is shown on the dot plots. Absolute numbers of $CD8^+$, $CD4^+$, and $B220^+$ lymphocytes were calculated based on the percentage of each population in lymph nodes (*A*) or spleen (*B*).

selection in SC mutant mice was due to increased apoptosis of DP thymocytes that were supposed to be positively selected.

Reduced survival of $CD8^+$ T but not $CD4^+$ T cells in the peripheral lymphoid organs of SC mutant mice

As we observed a role of Mcl-1 in positive selection of thymocytes, we next examined the phenotype of SC mutant mice in the peripheral lymphoid organs. As shown in Fig. 4, a significant reduction of $CD8^+$ T cells (~35%) was observed in lymph nodes (Fig. 4*A*) and spleens (Fig. 4*B*) of SC mutant mice. However, these mutant mice appeared to have a nearly normal number of $CD4^+$ T as well as B ($B220^+$) cells in these two peripheral lymphoid organs. Given that Mcl-1 is involved in positive selection of both CD4 and CD8 SP thymocytes, the specific reduction of $CD8^+$ but not $CD4^+$ T cells in the peripheral tissues of mutant mice is somewhat surprising. One possibility is that mutant $CD4^+$ T cells may undergo lymphopenia-driven proliferation with a better efficiency than mutant $CD8^+$ T cells. However, analysis of the activation state of these two cell lineages in the peripheral tissues of SC mutant mice did not support this possibility (supplemental Fig. S5). Another possibility is that the partial defect in maturation of both CD4 and CD8 SP thymocytes in SC mutant thymus did not really result in decreased egress of mature SP cells into peripheral tissues, i.e., only certain proportions of mature SP cells are normally transported to the peripheral tissues and the amounts generated in SC mutant mice are already enough for this purpose. If this is the case, the observed reduction of peripheral $CD8^+$ but not $CD4^+$ T cells in SC mutant mice would be due to differential susceptibility to cell death of these two mutant T cell lineages. To examine this possibility, the in vitro "die of neglect assay" was first conducted. For this experiment, splenocytes from control or SC mutant mice were isolated and cultured in growth medium without any supplemented cytokines. At various times after culturing, splenic $CD4^+$ or $CD8^+$ T cells that underwent apoptosis were analyzed by flow cytometry using annexin V and specific cell surface markers. As shown in Fig. 5*A*, under such in vitro assay system, a markedly increased apoptotic percentage was observed in SC mutant than in control $CD8^+$ T cells (e.g., 75 v.s. 50% at 36 h time point). However, under the same experimental conditions, the apoptotic percentage of SC mutant $CD4^+$ T cells was only marginally increased (<10%), compared with that of control cells.

Next, we examined whether the in vivo survival of $CD4^+$ and $CD8^+$ T cells was also differentially affected by the SC mutation. To address this issue, lymph node $CD4^+$ and $CD8^+$ T cells were purified from control or SC mutant mice, labeled with CFSE, and injected into WT recipient mice. Five days after transfer, the number of CFSE-labeled donor cells in the lymph node or spleen of the recipient mice was analyzed by flow cytometry. The results shown in Fig. 5*B* indicated that, while the number of donor $CD4^+$ T cells from WT or SC mutant mice detected in peripheral tissues of recipients was quite similar, the number of donor $CD8^+$ T cells from SC mutant mice was markedly reduced compared with that of donor $CD8^+$ T cells isolated from the control littermates (*i*). Similar results were obtained when the in vivo survival assay was conducted by transferring control or SC mutant donor T cells (Thy 1.2^+) into C57BL/6J-Thy1.1 recipient mice (see *Materials and Methods*), i.e., the in vivo viability of SC mutant $CD8^+$ T cells was reduced significantly more than that of mutant $CD4^+$ T cells (Fig. 5*Bii*). Next, the same experiments were conducted using mature ($TCR\beta^{high}$) CD8SP and CD4SP thymocytes as donor cells and the results indicated that the number of donor CD8SP but not CD4SP thymocytes from SC mutant mice detected in the recipients was markedly reduced, compared with that of the same donor cell type from control mice (Fig. 5*C*). We next examined whether the reduced number of mutant donor CD8SP or $CD8^+$ T cells detected in recipient's spleen or lymph node was due to impaired homing of these cells to these tissues. Homing assay was conducted using control and SC mutant cells labeled with two different fluorescent dyes (see *Materials and Methods*). The results shown in Fig. 5*D* indicated that the homing efficiency of WT or SC mutant CD8SP thymocytes (or lymph node $CD8^+$ T cells) to lymph node (LN) or spleen of recipient mice was quite similar (sc/wt = 0.9–1.1), suggesting that the results shown in Fig. 5, *B* and *C* indicate that SC mutant $CD8^+$ T cells (or their precursors CD8SP thymocytes) were much more susceptible to apoptosis than control $CD8^+$ T cells (or their precursors CD8SP thymocytes) in vivo.

Next, we examined whether such difference in apoptosis susceptibility between peripheral $CD4^+$ and $CD8^+$ T cells in SC mutant mice could be due to differentially attenuated expression of Mcl-1 in these two cell lineages. To address this issue, we analyzed Mcl-1 expression both at the mRNA and protein levels in lymphocytes isolated from control or SC mutant mouse lymph nodes. Quantitative RT-PCR analysis indicated that, in control mice, the relative levels of mcl-1 mRNA

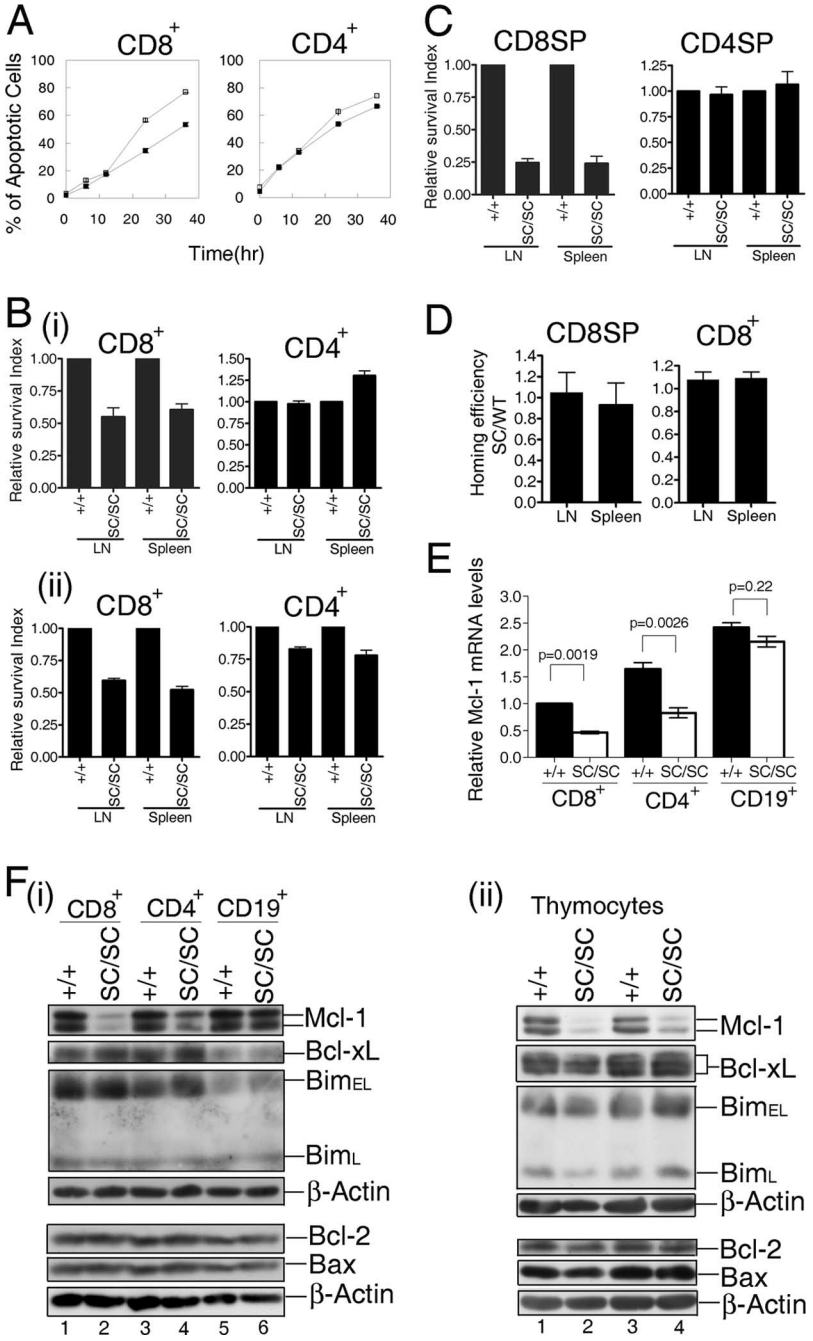

FIGURE 5. Reduced survival of $CD8^+$ T but not $CD4^+$ T cells in SC mutant mice. *A*, In vitro survival assay. Splenocytes isolated from control or SC mutant mice were cultured in RPMI 1640 containing 10% FBS. At various times after culturing, the percentage of $CD4^+$ or $CD8^+$ T cells undergoing apoptosis (Annexin V-positive) was determined by flow cytometry. Shown here is one representative result from three independent experiments with very similar results. *B*, In vivo survival assay. *i*, Peripheral $CD8^+$ or $CD4^+$ lymphocytes purified from control or SC mutant mice were labeled with CFSE and transferred into WT recipient mice. Five days after transfer, the number of CFSE-labeled donor cells detected in the LN or spleen of recipients were analyzed by flow cytometry. Results are plotted as "relative survival index" where the detected number of mutant donor cells was plotted as a relative number to that of wt donor cells, which was set as 1. $n = 2$. *ii*, Same as in *i* except that donor T cells ($Thy1.2^+$) were transferred into C57BL/6J-Thy1.1 recipients. Five days after adoptive transfer, donor T cells present in LN or spleen of the recipients were analyzed based on their expression of Thy1.2 and specific cell surface markers. $n = 3$. *C*, Same as in *Bi* except that TCR-β^{high}CD8 SP or TCR-β^{high}CD4 SP thymocytes purified from control or SC mutant mice were analyzed in the in vivo survival assay. *D*, Homing assay was conducted using control and SC mutant TCRβ^{high}CD8SP thymocytes (labeled with PKH26 or CMFDA) or lymph node $CD8^+$ T cells (labeled with CMFDA or 7-amino-4-chloromethylcoumarin) (see *Materials and Methods*). Sixteen hours after transfer, the number of labeled control or mutant cells in LN and spleens of recipients was analyzed by flow cytometry. Homing efficiency was plotted as the ratio of the SC mutant to wt cell numbers in the specified organ. $n = 2$ for CD8SP and 8 for $CD8^+$ T cells. *E*, Relative *mcl-1* mRNA levels in purified $CD8^+$, $CD4^+$ and $CD19^+$ lymphocytes were determined by quantitative RT-PCR. All *mcl-1* mRNA levels in cells as indicated were plotted as a relative amount to that present in +/+ $CD8^+$ T cells, which was set as 1. $n = 3$. *F*, Immunoblotting analysis of Mcl-1, Bcl-2, Bcl-xL, Bim, Bax, and β-actin expressions in $CD8^+$, $CD4^+$ T, or $CD19^+$ B lymphocytes (*i*) or thymocytes (*ii*) from control or SC mutant mice. For both panels, the same cell lysates were analyzed by two immunoblots (*top* and *bottom*) using specific Abs as indicated. One representative result from at least three independent experiments with very similar results was shown for each panel. See Supplemental Table I for relative protein levels of the indicated protein in each genotype ($n = 3$–8).

in $CD8^+$, $CD4^+$ T, and $CD19^+$ B cell lineages were 1:1.6:2.4 (normalized to the house keeping gene hprt; $p < 0.05$ for both the differences between $CD8^+$ and $CD4^+$ and between $CD8^+$ and $CD19^+$ cells, $n = 3$) (Fig. 5*E*). However, such difference did not result into any significant difference in Mcl-1 protein levels in these three cell lineages (Fig. 5*Fi* and supplemental Table I; equal cell number was loaded in each lane). In contrast, in SC mutant mice, the SC mutation resulted into differential attenuation of *mcl-1* mRNA expression in T and B cell lineages compared with their normal counterparts in WT mice (~50% reduction for both $CD4^+$ and $CD8^+$ T cells, and less than 15% for the $CD19^+$ B cell lineage, Fig. 5*E*), and such difference resulted into differential degree of attenuation of Mcl-1 protein expressions in these three mutant cell lineages, i.e., a moderate attenuation for $CD4^+$ T cells (~40%), a prominent reduction for mutant $CD8^+$ T cells (~70%), and a more variable but generally minor effect on $CD19^+$ B cells (average reduction ~26%, but $p > 0.05$) (Fig. 5*Fi* and supplemental Table I). Taken together, the prominent reduction of Mcl-1 expression in $CD8^+$ T cells but much less in $CD4^+$ T or B cell lineages correlated well with the specific reduction of $CD8^+$ but not $CD4^+$ T or B cell numbers in peripheral lymphoid organs of SC mutant mice.

Given that Bcl-2 family members function by forming complexes with selective members, we next examined the possibility that decreased expression of Mcl-1 may indirectly affect the steady-state levels of other Bcl-2 family members, which may partially contribute to some phenotypes observed in the SC mutant mice. As shown in Fig. 5*F* and supplemental Table I, no significant influence on the expression levels of Bcl-2, Bcl-xL, Bim, or Bax proteins could be observed in thymocytes, $CD4^+$, or $CD8^+$ T cells from the SC mutant mice. Although additional Bcl-2 family members other than the four mentioned above have not been extensively analyzed in this study, these results suggest that it is unlikely

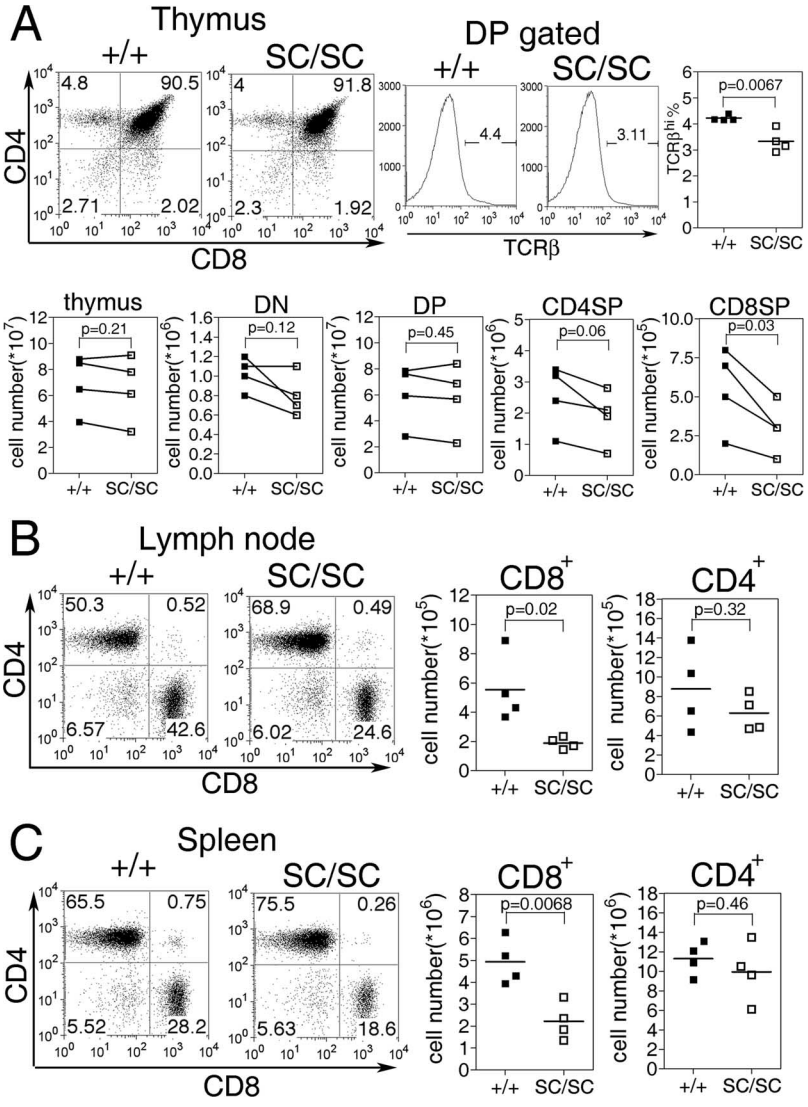

FIGURE 6. Impaired T cell development and peripheral CD8$^+$ lymphopenia in SC mutant mice are both cell autonomous. Lethally irradiated C57BL/6J-Thy1.1 mice were reconstituted with WT or SC mutant bone marrow cells (Thy1.2$^+$). Eight to ten weeks after reconstitution, donor-derived cells in the thymus (*A*), lymph node (*B*), or spleen (*C*) of recipient mice were stained for Thy1.2, CD4, CD8, and TCR-β and analyzed by flow cytometry. *A–C*, Surface expression of CD4, CD8, or TCR-β on gated Thy1.2$^+$ thymocytes or peripheral T cells. Numbers represent the percentage of cells in the indicated population. Absolute numbers of donor-derived thymocyte subsets or CD4$^+$ or CD8$^+$ T cells in the specified organs of recipients are as indicated. Histograms shown in *A* are representative TCR-β^{high} staining profiles on donor-derived DP thymocytes. Shown here are results from four independent experiments, each with a pair of recipient mice reconstituted with control or SC mutant bone marrow cells.

that the SC mutation would affect the expression of other Bcl-2 family members and contribute to the phenotypes observed in the SC mutant mice.

Impaired T cell development and peripheral CD8$^+$ lymphophenia in SC mutant mice are both cell autonomous and can be rescued by overexpression of human Mcl-1

Given that Mcl-1 is widely expressed in many cell types, we next examined whether defective T cell development and peripheral CD8$^+$ lymphophenia in SC mutant mice were due to an intrinsic reduction of Mcl-1 in SC mutant T cell lineages. To address this issue, we reconstituted irradiated Thy1.1$^+$ recipients with bone marrow cells from WT or SC mutant mice (both were Thy1.2$^+$). Eight weeks after reconstitution, we assessed the composition of donor-derived cells in the recipient mice. Fig. 6 shows that the SC mutant phenotype was basically recapitulated in chimeric mice reconstituted with SC mutant bone marrow. That is, compared with those in chimeric mice reconstituted with wt bone marrow, there was a significant reduction in the proportion of DP thymocytes expressing high levels of TCR-β and in the number of mature CD4SP, CD8SP thymocytes, and peripheral CD8$^+$ T cells in chimeric mice reconstituted with SC mutant bone marrow (Fig. 6, *A–C*). Together, these results indicate that impaired T cell development and peripheral CD8$^+$ lymphopenia in SC mutant mice are both cell autonomous.

Last, we examined whether defective thymocyte development and CD8$^+$ lymphopenia observed in the SC mutants were indeed due to reduced expression of Mcl-1 in these mice. To address this issue, transgenic mice carrying a hMcl-1 transgene under the control of RNA polymerase II promoter were first generated (see *Materials and Methods* and Fig. 7*A*). One such transgenic line (no. 30) expressing a prominent level of hMcl-1 (Fig. 7*B*) was then mated to the SC mutant background. Although control mice overexpressing hMcl-1 itself did not manifest any obvious phenotype in the hematopoietic system (Fig. 7, *C–E* and data not shown), overexpression of hMcl-1 significantly reversed the decrease in the proportion of DP thymocytes expressing high levels of maturation markers (TCR-β^{high} and CD69) and the percentage of peripheral CD8$^+$ T cells in SC mutant mice (Fig. 7, *C–E*). Together, these results indicated that the observed phenotypes in the SC mutants were indeed due to reduced expression of Mcl-1 in the T cell lineage of these mice.

Discussion

In this study, a new mouse model to study the role of Mcl-1 during T cell development has been generated. This new mouse model took advantage of a promoter knock-in allele of the *mcl-1* gene (the SC mutant allele), which manifests a hypomorphic phenotype only in selected tissues like thymus. In thymus, all four subsets of thymocytes express Mcl-1. Conditional knock-out of Mcl-1 at the

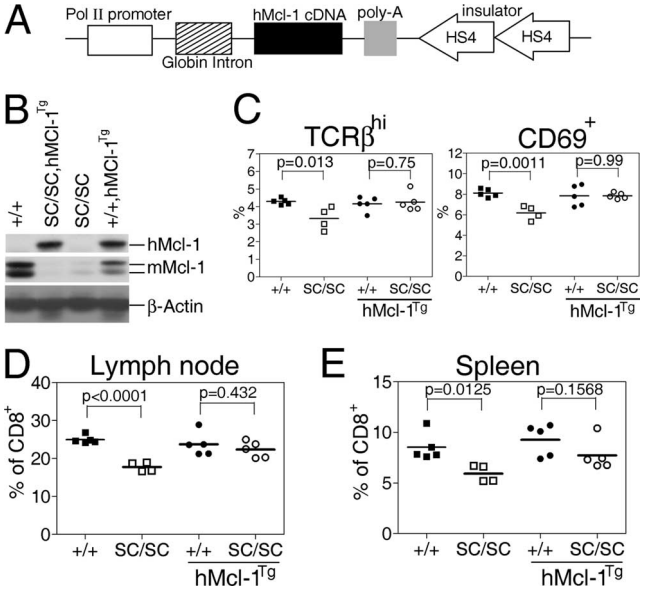

FIGURE 7. Enforced expression of hMcl-1 restores frequency of DP thymocytes undergoing positive selection and peripheral $CD8^+$ T cell percentages in SC mutant mice. *A*, Schematic representation of the RNA Pol II-hMcl-1 transgenic construct. The human (h) Mcl-1 cDNA was driven by the mouse RNA Pol II promoter. Two copies of the chicken β-globin insulators (HS4) were placed downstream of the hMcl-1 cDNA in a reverse orientation (27). An intron segment and poly-A signal from the rabbit globin gene were also included in this construct at positions as indicated. *B*, Immunoblotting analysis of hMcl-1 protein expression in thymocytes from control or SC mutant mice carrying or not carrying the hMcl-1 transgene ($hMcl\text{-}1^{Tg}$). The blot was probed with Abs specific to hMcl-1, mouse (m) Mcl-1 or β-actin. *C*, Thymocytes from mice to be analyzed were stained for CD4, CD8, and TCR-β or CD69 and analyzed by flow cytometry. Percentages of DP thymocytes expressing high levels of TCR-β or CD69 from mice with the indicated genotypes were plotted in this figure. *D* and *E*, Same as in *C* except lymph node cells or splenocytes were stained for CD4 and CD8. Shown here are percentages of $CD8^+$ T cells in lymph nodes (*D*) or spleens (*E*) of mice with the indicated genotypes. ($n = 4$–5 for each genotype).

DN stage by using Cre driven by the Lck promoter prevents thymocytes from differentiating into the DP stage, which precludes any further study on the role of Mcl-1 during later stages of T cell development (6). A recent study using CD4 promoter-driven Cre to delete Mcl-1 at the DN4 or later stages indicated that Mcl-1 is required for the maturation of CD4SP and CD8SP thymocytes (18). However, the detailed role of Mcl-1 during the transition from the DP to SP stage is still unclear.

In SC mutant mice, the markedly reduced Mcl-1 expression in thymus still allowed T cell development to progress from the DN to SP stage, albeit with a slightly reduced efficiency. However, it is the latter characteristics, i.e., reduced efficiency, that allowed us to identify a role of Mcl-1 during thymocyte-positive selection. In SC mutant mice, the percentage of DP thymocytes expressing markers for cells undergoing positive selection, i.e., TCR-β and CD69, was reduced by ~25% compared with that in control mice. We demonstrated that such reduction was not due to decreased proliferation of mutant thymocytes. In addition, the TCR-α-chain rearrangement appeared to be normal in SC mutant DP thymocytes. These results together with the observed increased sensitivity of SC mutant DP thymocytes to at least three death stimuli strongly suggest that defective positive selection in SC mutant mice is due to loss of viability of those DP thymocytes that are supposed to be positively selected. This study, together with earlier findings (6), indicate that Mcl-1 plays a crucial role throughout all stages of T cell development, which is distinct from two other antiapoptotic proteins, Bcl-2 and Bcl-x, whose functions are more restricted to the DN and SP (11, 12) or to the DP stage of T cell development (13–15), respectively. How Mcl-1 collaborates with Bcl-2 or Bcl-x during each distinct T cell differentiation stage remains to be determined.

In this study, we also unexpectedly identified that *mcl-1* promoter elements are differentially required in different tissues or cell lineages, albeit Mcl-1 is a widely expressed protein. The prominent reduction of Mcl-1 expression in thymus but not other major organs in SC mutant mice and the specific attenuation of mcl-1 mRNA expression in SC mutant peripheral T but not B lymphocytes suggest that the SIE and/or CRE-2 motifs are more critical for *mcl-1* transcription in the T cell lineage than other cell lineages inside an organism. Of note, no significant reduction of Mcl-1 protein levels was observed in the tissue extracts made from SC mutant spleens. This is likely because ~75% of cells in this organ are non-T cell lineages, which would mask the SC mutation effect on the remaining ~25% of cells that are T cell lineages.

In this study, we notice that in SC mutant mice, the SC mutation attenuated mcl-1 transcription to a similar extent in both $CD4^+$ and $CD8^+$ T cells, i.e., both were reduced to ~50% of that found in their control counterparts. However, the Mcl-1 protein expression was less attenuated in mutant $CD4^+$ than in $CD8^+$ T cells. The likely explanation for this result is that differential amounts of mcl-1 mRNA were synthesized in these two cell lineages. In control mice, the relative mcl-1 mRNA level in $CD4^+$ or B lymphocytes was higher than that in $CD8^+$ T cells. However, the steady-state Mcl-1 protein level in these three lymphocyte lineages was quite similar, suggesting that Mcl-1 protein synthesis is under some kind of regulation that would limit further translation from mRNA that is beyond certain threshold levels, like that in $CD8^+$ T cells. In contrast, in SC mutant mice, the mcl-1 mRNA levels in mutant $CD4^+$ and $CD8^+$ T cells were both lower than the "threshold level" in control $CD8^+$ T cells, suggesting that the amount of Mcl-1 protein to be synthesized in these two mutant cell lineages would still be proportional to the amount of available mRNA. If such scenario is correct, mutant $CD4^+$ T cells would have more Mcl-1 proteins synthesized than mutant $CD8^+$ T cells, because slightly higher levels of mcl-1 mRNA were still made in mutant $CD4^+$ than in mutant $CD8^+$ T cells.

Last, although Mcl-1 protein levels correlated with the apoptosis susceptibility of mutant $CD4^+$ and $CD8^+$ T cells, we cannot completely rule out the possibility that more reduction of steady-state Mcl-1 levels in mutant $CD8^+$ T cells is secondary to the decreased viability of these cells. Alternatively, our results also pointed to another possibility that, in $CD4^+$ T cells, Mcl-1 might not be as important for cell survival as in $CD8^+$ T cells. More experiments would be required to address the latter possibility.

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Disclosures

The authors have no financial conflict of interest.

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