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A Role for SATB1, a Nuclear Matrix Association Region-Binding Protein, in the Development of CD8SP Thymocytes and Peripheral T Lymphocytes

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Studies have suggested that binding of the SATB1 protein to L2a, a matrix association region located 4.5 kb 5′ to the mouse CD8α gene, positively affects CD8 expression in T cells. Therefore, experiments were performed to determine the effect on T cell development of reduced expression of SATB1. Because homozygous SATB1-null mice do not survive to adulthood due to non-thymus autonomous defects, mice were produced that were homozygous for a T cell-specific SATB1-antisense transgene and heterozygous for a SATB1-null allele. Thymic SATB1 protein was reduced significantly in these mice, and the major cellular phenotype observed was a significant reduction in the percentage of CD8SP T cells in thymus, spleen, and lymph nodes. Mice were smaller than wild type but generally healthy, and besides a general reduction in cellularity and a slight increase in surface CD3 expression on CD8SP thymocytes, the composition of the thymus was similar to wild type. The reduction in thymic SATB1 does not lead to the variegated expression of CD8-negative single positive thymocytes seen upon deletion of several regulatory elements and suggested by others to reflect failure to activate the CD8 locus. Thus, the present results point to an essential role for SATB1 late in the development and maturation of CD8SP T cells. The Journal of Immunology, 2005, 174: 4745–4752.

The development of lymphocytes from their progenitor cells is a highly ordered and coordinated process. Each developmental stage is characterized by a specific pattern of gene expression. On the basis of their expression of CD4 and CD8 accessory molecules, developing thymocytes can be subdivided into four populations. Early thymocytes express neither CD4 nor CD8 and are known as double-negative (DN) thymocytes. DN cells give rise to CD4+CD8+ double-positive (DP) cells from which the process of thymocyte development culminates in the maturation of CD4+CD8+ (CD4SP) cells for class II MHC and CD4+CD8− (CD8SP) cells specific for class I MHC. Although the major elements involved in the transcriptional regulation of CD4 (1, 2) and CD8 expression (3–9) have been identified, the mechanisms by which lineage-specific cell surface expression of CD8 is achieved is still largely unknown.

Nuclear matrix-associated regions (MARs) are short AT-rich DNA sequences that are widespread throughout the eukaryotic genome and have a great affinity for the nuclear matrix in vitro. MARs have been postulated to mediate chromatin loop formation important for compaction of genomic DNA and also to organize chromatin into units of genomic function (10, 11). MARs are frequently colocalized with enhancers or with the boundaries of genes. Together with the enhancers, several MARs have been suggested to mediate positive or negative regulation of gene expression (12–17).

The MAR-binding protein, SATB1, expressed predominantly in thymocytes, recognizes an ATC sequence context with high potential for unwinding by base unpairing (18). SATB1 has a MAR-binding domain and an atypical homeodomain, both of which are necessary for recognition of the core-unwinding element within a MAR (19, 20). In addition to the binding sites in the MARs of the IgH gene enhancer (18), SATB1 has been found to interact with the MARs of other Ag receptor and coreceptor genes, including the Igs, TCR-α, and CD8α genes (14, 17, 21, 22). Consistent with the idea that MAR-binding proteins participate in cell type-specific gene regulation at the level of higher-order chromatin structure, SATB1 was found to bind in vivo to genomic sequences that are tightly associated with the nuclear matrix at the bases of the chromatin loops (23). Recent studies suggest that SATB1 binds to DNA and recruits ATP-dependent chromatin remodeling complexes that modify histone acetylation and nucleosome placement over long distances in the IL-2Rα gene (24). The binding of SATB1 to genomic sites mediated the formation of a particular region-specific histone code (25).

Previous studies identified a MAR element (L2a) located ~4.5 kb upstream of the mouse CD8α gene that appears to play a role in regulation of the CD8α gene expression (16, 17). A number of enhancers have been described in DNase I hypersensitive regions within the 36-kb interval separating the mouse CD8β and CD8α genes (3–6, 8), and some interaction has been demonstrated between one of these (E8I) and a 4-kb DNA fragment containing the L2a element (6). Targeted deletion of the 4-kb fragment containing the L2a element leads to abnormal thymocyte development characterized by a large population of thymocytes that never turn on CD8 gene expression, leading to a decrease in thymic and peripheral CD8SP T cell populations (7).

We have shown that the L2a element interacts with the MAR-binding proteins SATB1 and CDP (17). CDP belongs to a family of proteins whose role as a transcriptional repressor has been demonstrated in a number of systems (13, 26, 27). On the basis of our
earlier studies of the cell type distributions of SATB1 and CD3 and their interaction with the L2a element (16, 17), we hypothesized that SATB1 and CD3 play positive and negative roles, respectively, in CD8α gene regulation. We have suggested that binding of SATB1 displaces the CD3 repressor from the L2a element and favors CD8α expression, probably by altering chromatin structure and/or allowing the L2a element and nearby CD8α gene to associate with the nuclear matrix (17).

Analysis of SATB1-null mice generated by targeted gene inactivation has demonstrated that the absence of SATB1 results in a severe impairment in T cell development characterized by arrest of thymocyte development at an abnormal DP stage (28). The SATB1-null model is complicated by the finding that although SATB1-null mice die at 3–6 wk of age, apparently due to lack of an essential function of SATB1 in nonthymic tissues, including brain. Therefore, to test our hypothesis that SATB1 plays a positive role in CD8 expression in vivo, we have generated mice in which expression of SATB1-antisense transcripts is targeted specifically to T lymphocytes and have bred these with mice heterozygous for a SATB1-null allele. The consequence of reducing thymus and T cell expression of SATB1 expression in these mice is a significant reduction in the percentage of CD8SP T cells present in the thymus and the periphery.

Materials and Methods

Generation of SATB1-antisense transgenic mice

To make the pCD2VA-SATB1-AS vector used for generating the SATB1-antisense transgenic mice, a 3-kb EcoRI fragment containing the mouse SATB1 5′ untranslated region was excised from the pmAT vector (19) and subcloned, in antisense orientation with respect to the promoter, into the EcoRI site of the pCD2VA plasmid (29). pCD2VA is a minigene cassette that contains regulatory sequences of the human CD2 gene, including promoter, locus control region, the first intron of the gene, and two polyadenylation signals found in the 3′-untranslated region. A 15-kb Kpn1-XhoI fragment containing the sequence of interest was excised from pCD2VA-SATB1-AS and injected into the pronucleus of (C57BL/6 J × DBA/2) F2 single-cell embryos as described by Brinster et al. (30).

Founder animals were identified by Southern hybridization of tail DNA and backcrossed (to C57BL/6 J × DBA/2) F2 mice to generate transgenic lines. Heterozygous offspring of the founders (+/Tg) were crossed to generate homozygous mice (Tg/Tg) that were identified by Southern blot analysis of tail DNA from mice heterozygous for the SATB1-null allele. Once identified by PCR analysis of SATB1-KO mice, a 4-kb NcoI fragment containing the 3′ untranslated region of the SATB1 gene (4.07 kb) was subcloned, and a 3-kb BamHI probe (mSATB1cDNA 2170-2556) that was derived from the pmAT vector (20) was used for PCR amplification of SATB1-antisense mice, a 3-kb EcoRI fragment containing the mouse SATB1 5′ untranslated region. A 15-kb Kpn1-XhoI fragment containing the sequence of interest was excised from pCD2VA-SATB1-AS and injected into the pronucleus of (C57BL/6 J × DBA/2) F2 single-cell embryos as described by Brinster et al. (30).

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Production of SATB1-antisense mice heterozygous for a SATB1-null allele

Generation of the SATB1-knockout (KO) mouse strain has been described previously (28). The targeted allele contains a deletion of the translation start site and the first five exons encoding the first 213 amino acids of SATB1. A neo resistance gene directed opposite to the direction of transcription of SATB1 is present in place of the excised segment. Because mice homozygous for the targeted allele did not survive to breeding age, the targeted allele was maintained by breeding heterozygotes. Homozygous SATB1-antisense transgenic mice were crossed with mice heterozygous for the targeted allele to produce SATB1-null mice. Fifty percent of the progeny of such backcrosses were homozygous SATB1-antisense and heterozygous for the SATB1-null allele.

Extraction and digestion of DNA from mouse tails

Mouse tails were digested overnight at 55°C in tail buffer (50 mM Tris (pH 8.0), 100 mM EDTA, and 1% SDS) containing 0.15 mg/ml proteinase K (Ambion). Samples were extracted sequentially with phenol, phenol-chloroform (25:24), and chloroform, and the DNA was precipitated with a solution containing 30% polyethylene glycol and 1.5 M NaCl. DNA pellets were recovered and resuspended in 100 μl of TE.

Southern blot analysis

The isolated DNA (12 μg) was digested to completion with EcoRI, and fragments were separated by electrophoresis in a 0.7% agarose gel and transferred to a nylon membrane (microtiter membranes (Schleicher & Schuell Microscience). Western blotting was performed using a SATB1 mouse mAb (BD Transduction Laboratories) and probed with a goat anti-actin polyclonal Ab (Santa Cruz Biotechnology). HRP-conjugated mouse-anti and goat-anti Abs were used with an ECL detection system (Amersham Biosciences) to visualize the proteins. To quantitate the relative amount of SATB1, films were scanned, and the intensity of the bands corresponding to SATB1 and actin was compared using National Institutes of Health image 1.62.

T cell proliferation assay

Spleen cells were isolated by passage through a 70-μm cell strainer (Falcon no. 2350). Following 5 min of incubation with RBC lysis buffer, splenocytes were rinsed twice, then 5 × 10^6 cells were cultured with 10 ng/ml

Polymerase chain reaction

The isolated DNA (12 μg) was digested to completion with EcoRI, and fragments were separated by electrophoresis in a 0.7% agarose gel and transferred to a nylon membrane (microtiter membranes (Schleicher & Schuell Microscience). Western blotting was performed using a SATB1 mouse mAb (BD Transduction Laboratories) and probed with a goat anti-actin polyclonal Ab (Santa Cruz Biotechnology). HRP-conjugated mouse-anti and goat-anti Abs were used with an ECL detection system (Amersham Biosciences) to visualize the proteins. To quantitate the relative amount of SATB1, films were scanned, and the intensity of the bands corresponding to SATB1 and actin was compared using National Institutes of Health image 1.62.
PMA and 500 ng/ml ionomycin in each well of a 24-well culture plate. Cell numbers were counted at different time points with a hemacytometer.

**Statistical analysis**

ANOVA was performed to compare the sample means of a set of parameters in groups of mice with six different genotypes (see below). The significance level was set at $\alpha = 0.05$. When the one-way ANOVA F test was significant ($p < 0.05$), a pairwise multiple comparison test (Tukey honestly significantly different) was used to study the difference between all possible pairs of means. ANOVA statistical analyses were performed using SYSTAT (version 10.0; SPSS). For Pearson correlation analyses, the significance level was set at $\alpha = 0.01$, and SPSS (version 10.1.0, 1999–2000; SPSS) software was used.

**Results**

**Generation of SATB1-antisense transgenic mice**

To produce mice that specifically express SATB1-antisense RNA in T lymphocytes (addressed below), the SATB1 cDNA sequence was cloned in the antisense orientation into a human CD2 minigene-based vector (29) previously shown (31) to confer position-independent, transgene copy number-dependent expression of the included gene in transgenic mice (Fig. 1A). Two founders were identified (Fig. 1B); one of which contained two copies of the transgene with an extra hybridizing band that cosegregated with the band of the expected size. Descendants of this founder were inbred to generate the homozygous SATB1-antisense strain 1 (abbreviated AS1). The second founder carried one copy of the SATB1 transgene, and surprisingly, its homozygous descendants, referred to as the AS2 strain, were found to have kinky tails, likely due to disruption of a nonessential gene involved in axis formation.

![FIGURE 1. Strategy for generation of SATB1-antisense mice. A. Diagram of the SATB1-antisense minigene structure. A plasmid, pHCD2VA-SATB1-AS, was constructed and used to generate transgenic mice as described in Materials and Methods. The probe used in Southern blot analysis to identify the transgenic mice is shown below the restriction map of the mSATB1 cDNA sequence. B. Southern blot analysis of EcoRI-digested genomic DNA isolated from tails of heterozygous (Tg/Tg), homozygous (Tg/Tg), and wild-type (+/+) mice bred from founders AS1 and AS2. A probe specific for SATB1 was used for Southern blot analysis and resulted in a 3.1-kb transgene fragment and a 4.1-kb endogenous SATB1 gene fragment. The additional 6-kb transgene band present in AS1 mice is discussed in the text and is included in the copy number figures. The numbers at the bottom of the gel indicate the total number of transgene copies present in the mice within corresponding lanes.](http://www.jimmunol.org/)

**Generation of mice homozygous for SATB1-antisense transgenes and heterozygous for a SATB1-null allele**

Although antisense approaches have been used successfully in many systems to down-regulate protein levels in a tissue-specific fashion (32, 33), they generally do not result in complete absence of the protein of interest. This proved to be the case in the present studies as well (see below). Therefore, we crossed AS1 and AS2 mice with heterozygous SATB1-KO mice (28) to try to obtain maximal reduction in thymic SATB1 protein. Progeny were intercrossed to generate mice homozygous for the SATB1-antisense transgene and heterozygous for the SATB1-null allele (referred to as AS1-KO and AS2-KO mice, respectively). We reasoned that reduction in SATB1 expression resulting from the presence of a single functional SATB1 allele (28) would be augmented by the presence of homozygous AS1 or AS2 transgenes.

**T lineage specificity of antisense transgene expression generates reduction of SATB1 protein in the thymus**

The protein product of the human CD2 minigene has been observed to be expressed uniformly in virtually all thymocytes as well as in peripheral T cells and megakaryocytes (34, 35). As shown in the RT-PCR analyses of Fig. 2A, antisense SATB1 mRNA accumulates predominantly in T cell-containing tissues (thymus, spleen, and lymph nodes). In wild-type mice, SATB1 protein was detected mainly in thymus, spleen, and lymph nodes with a lower level observed in the brain (Fig. 2B). The levels of SATB1 protein relative to actin were determined using National Institutes of Health image analysis. As shown in a representative gel (Fig. 2C) and summarized in Table I, the ratio of SATB1 to actin was reduced substantially in the thymus of experimental mice as compared with wild type. Statistically significant reductions in SATB1:actin ratios were observed in thymocytes of AS1-KO and AS2-KO, as well as in nontransgenic KO mice ($p < 0.0001$, $p = 0.002$, and $p = 0.01$, respectively, in comparison with wild-type mice). All transgenic mice (AS1 or AS2) or mice bearing a SATB1-null allele were significantly smaller than wild-type mice (Table II), but little, if any, difference was seen by 4–5 wk of age (data not shown). Life spans of transgenic mice were indistinguishable from wild-type mice (data not shown). However, significant reduction in total thymocyte numbers was observed in comparison with wild-type mice for AS2-KO, AS2, and antisense Tg-negative, KO mice (Table II). Neurological defects, such as the incomplete eye opening and the clasping reflex, observed in SATB1-null mice (28), were not observed in the knockdown strains, supporting the view that these symptoms were due to the absence of SATB1 protein from nonthymus tissues, such as brain. The slight reduction (7.1%) of SATB1 protein in the brain of AS1-KO mice (Fig. 2D) is much less than the reduction observed in thymus, spleen, and lymph nodes (58.9, 37.5, and 55.5%, respectively).

**Characterization of thymocytes in SATB1 homozygous-antisense and/or SATB1-null allele heterozygous KO mice**

The CD4 vs CD8 profiles of thymocytes from representative wild-type, AS1- KO, and AS2-KO mice are shown in Fig. 3. A statistically significant decrease in the percentage of CD8SP thymocytes was observed in mice of these and other genotypes tested when compared with wild type (Table II). The percentage of DN, DP, and CD4SP thymocytes appeared unaffected by the SATB1-antisense transgene or by the SATB1-KO allele (Table II). Reduction in the percentage of CD8SP thymocytes in mice bearing SATB1-antisense transgenes and SATB1-null alleles resulted in a statistically significant increase in thymic CD4SP:CD8SP ratios (Table II). Transgene-negative KO mice barely missed significance at $p = 0.05$.
role for SATB1 in CD8SP T cell development

The numbers below refer to control for protein loading. NIH Image 1.62 was used to measure the intensity of individual bands. The numbers below were scanned using NIH Image 1.62.

The number of CD8SP thymocytes in SATB1-antisense transgenic and/or KO mice is negatively correlated with thymic SATB1 protein expression.

A Pearson correlation analysis showed that SATB1 protein levels and percentage of CD8SP cells in individual mice showed a significant positive correlation (p = 0.003), whereas SATB1 protein level and the CD4SP:CD8SP ratio exhibited a significant negative correlation (p < 0.0001; Fig. 4). No correlation was observed between the SATB1 protein levels and the percentage of thymic CD4SP cells (data not shown). These results indicate that the percentage of CD8SP cells in the thymus is positively correlated with the amount of SATB1 expression in thymocytes.

Reduction of percentage of CD8SP cells in the periphery of SATB1-antisense, SATB1-null heterozygous mice

As shown in Fig. 5, the percentage of CD8SP cells in spleens of AS2-KO mice was reduced significantly relative to wild-type mice, resulting in a significant increase in CD4SP:CD8SP ratio. In lymph nodes, significant increases and decreases in CD4SP and CD8SP cells, respectively, resulted in a significant increase in CD4SP:CD8SP ratio.

Levels of cell surface CD3 on CD4SP cells are higher in mice with reduced amounts of thymic SATB1

Because we had hypothesized that SATB1 has an effect on expression of CD8, we tested whether reduction in thymic SATB1 expression resulted in a decrease in the level of surface CD8 as measured by mean fluorescence intensity (MFI). No significant decrease in CD8 MFI on CD8SP thymocytes or any other thymic subpopulations was detected. However, surprisingly, the levels of cell surface CD3 on CD8SP thymocytes of AS1-KO and AS2-KO mice were significantly higher (8–11 channels) than those of wild-type mice (Fig. 6 and Table III). Examination of CD3 MFI of CD8SP cells and of DP thymocytes revealed no significant differences among AS1-KO, AS2-KO, and wild-type mice. Thus, the effect of SATB1 protein reduction on cell surface CD3 expression appeared to be specific for cells of the CD8SP lineage.

Reduction of thymic SATB1 has no apparent effect on expression of several parameters characteristic of thymocyte maturity

It is generally accepted that CD3, CD5, and CD69 are up-regulated with reduced amounts of thymic SATB1 protein expression. The number of CD8SP thymocytes in SATB1-antisense transgenic and/or KO mice is negatively correlated with thymic SATB1 protein expression. A Pearson correlation analysis showed that SATB1 protein levels and percentage of CD8SP cells in individual mice showed a significant positive correlation (p = 0.003), whereas SATB1 protein level and the CD4SP:CD8SP ratio exhibited a significant negative correlation (p < 0.0001; Fig. 4). No correlation was observed between the SATB1 protein levels and the percentage of thymic CD4SP cells (data not shown). These results indicate that the percentage of CD8SP cells in the thymus is positively correlated with the amount of SATB1 expression in thymocytes.

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Reduction of thymic SATB1 has no apparent effect on expression of several parameters characteristic of thymocyte maturity

It is generally accepted that CD3, CD5, and CD69 are up-regulated in CD4SP and CD8SP cells following positive selection and that heat stable Ag is down-regulated (36, 37). Unlike the increase in MFI seen for CD3 on CD8SP thymocytes of AS1-KO and AS2-KO mice, CD3 levels on CD4SP thymocytes of these mice were indistinguishable from those of wild type. Moreover, the patterns of CD5, CD69, and heat stable Ag expression in the CD4SP and DP population did not differ significantly from those of wild-type mice (data not shown). Thus, in the presence of reduced amounts of thymic SATB1 protein, the CD4SP and DP subsets of AS1-KO and AS2-KO mice appear to be indistinguishable from wild type with respect to cell surface maturation.

It is generally agreed that T cell development within the DN population of the thymus progresses in order from CD25+CD44− to CD25+CD44+ to CD25−CD44− to CD25−CD44+ (38). Previous studies of SATB1-null mice demonstrated multiple defects
in T cell development that included a marked reduction in the CD3^+CD4^-CD8^- triple-negative population and expression of an intermediate level of CD25 on a CD4^+CD8^- subset (28). However, FACS analysis of thymocytes showed that none of the four major DN subsets was altered significantly in any of the five genotypes indicated above (data not shown).

**SATB1 reduction leads to impaired proliferation of peripheral T cells**

To address the mechanism that links reduced SATB1 expression with the reduced thymocyte populations, we used pharmacologic agents (PMA, a protein kinase C activator, and ionomycin, a calcium ionophore) to mimic TCR signaling and to activate T cells. As shown in Fig. 7, there were no obvious differences in cell numbers until after 48 h, when both stimulated AS1-KO and wild-type spleen cells began to proliferate robustly. However, AS1-KO splenocytes achieved only ~50% of the number of wild-type cells 72 or 96 h after stimulation. Therefore, peripheral T cells of SATB1-reduced mice have an impaired proliferative response to mitogenic stimuli.

**Discussion**

Results of previous studies detailed in the Introduction suggested that binding of SATB1 to L2a, a MAR element located ~4.5 kb upstream of the mouse CD8α gene, plays a positive role in CD8 expression and development of CD8-positive T cells (16, 17). Consistent with this hypothesis, mice homozygous for SATB1-null alleles lacked both CD8SP and CD4SP T cells and contained thymic anomalies, suggesting a role for SATB1 in normal T cell development (28). However, because these animals died between 2 and 3 wk of age, most likely because of nervous system impairment (28), their abnormal thymus phenotype might be attributed to non-T cell autonomous causes.

The present approach was designed to reduce SATB1 expression specifically in T lymphocytes by expressing transgenic SATB1-antisense RNA under the control of a human CD2 cassette. Furthermore, by making the SATB1-antisense transgenic mice heterozygous for a SATB1-null allele, we sought to reduce SATB1 expression in the thymus below a threshold level that might prove to be limiting for normal thymocyte development. This appears to have been successful for both AS1-KO and AS2-KO mice of different genotypes (see footnotes to Table I) were analyzed with respect to mean body weight, thymus lymphocyte cellularity, and percentage of CD4SP, CD8SP, DP, and DN thymocytes. Statistical analyses were performed by ANOVA as described in Materials and Methods.

### Table II. Mean body weight, thymus cellularity, and thymocyte subset composition of mice with different SATB1 antisense transgene and SATB1-null allele genotypes^a^

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Weight (g)</th>
<th>Total thymocytes (×10^6)</th>
<th>CD4SP (%)</th>
<th>CD8SP (%)</th>
<th>CD4SP:CD8SP</th>
<th>DP (%)</th>
<th>DN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (n = 17)</td>
<td>10.77 ± 2.05</td>
<td>47.09 ± 2.31</td>
<td>10.22 ± 3.74</td>
<td>10.41 ± 1.81</td>
<td>2.62 ± 0.63</td>
<td>82.41 ± 6.31</td>
<td>3.26 ± 1.31</td>
</tr>
<tr>
<td>AS1 (n = 11)</td>
<td>7.40 ± 1.59^b</td>
<td>50.53 ± 2.16</td>
<td>9.80 ± 2.60</td>
<td>2.84 ± 1.04^d</td>
<td>3.61 ± 0.82</td>
<td>84.50 ± 3.80</td>
<td>2.86 ± 0.63</td>
</tr>
<tr>
<td>AS1-KO (n = 13)</td>
<td>7.17 ± 1.03^b</td>
<td>43.50 ± 2.02</td>
<td>10.90 ± 2.88</td>
<td>2.21 ± 0.33^d</td>
<td>4.97 ± 1.21^b</td>
<td>84.10 ± 3.52</td>
<td>2.82 ± 0.82</td>
</tr>
<tr>
<td>AS2 (n = 9)</td>
<td>6.11 ± 1.63^b</td>
<td>19.83 ± 12.06^d</td>
<td>10.80 ± 4.31</td>
<td>2.70 ± 0.82^d</td>
<td>4.29 ± 1.86^d</td>
<td>83.40 ± 4.99</td>
<td>3.16 ± 1.00</td>
</tr>
<tr>
<td>AS2-KO (n = 14)</td>
<td>6.86 ± 1.33^b</td>
<td>24.14 ± 15.66^d</td>
<td>8.65 ± 1.93</td>
<td>2.06 ± 0.75^b</td>
<td>4.71 ± 1.83^b</td>
<td>86.66 ± 3.16</td>
<td>2.63 ± 0.91</td>
</tr>
<tr>
<td>KO (n = 9)</td>
<td>7.42 ± 1.12^b</td>
<td>24.50 ± 9.25^d</td>
<td>11.00 ± 0.97</td>
<td>2.76 ± 0.72</td>
<td>4.22 ± 1.18</td>
<td>83.80 ± 1.55</td>
<td>2.46 ± 0.39</td>
</tr>
</tbody>
</table>

^a^ Three-week-old mice of different genotypes were analyzed with respect to mean body weight, thymus lymphocyte cellularity, and percentage of CD4SP, CD8SP, DP, and DN thymocytes. Statistical analyses were performed by ANOVA as described in Materials and Methods.

^b^ Values of p < 0.0001 compared with WT group.

^c^ Values of p < 0.01 compared with WT group.

^d^ Values of p < 0.05 compared with WT group.
As2-KO mice, which demonstrate a significant reduction in the percentage of thymic and peripheral CD8SP cells, thereby increasing the CD4SP/CD8SP ratios (Table II). Some reduction in CD8SP thymocytes was also seen in As1, As2, and Ko mice, but the level of significance was strongest in As1-KO and As2-KO mice. Reduction in mouse weight and thymocyte numbers of mice heterozygous for a SATB1-null allele could reflect nonthymus autonomous effects of SATB1 reduction. However, the reduced mouse weight and numbers of thymocytes also were observed in homozygous As1 and As2 that lacked a SATB1-null allele. This raised the possibility that their mixed genetic background had an effect, i.e., mixtures of C57BL/6, DBA/2, and C57BL/6 were used in experimental and wild-type groups, respectively. The fact that the 10th generation of As1-KO backcrossed to C57BL/6 had indistinguishable body weights from wild-type C57BL/6 mice supports this possibility (data not shown). Alternatively, because SATB1 regulates multiple genes during T cell development, the T cell-specific effect of reduced SATB1 could have an indirect effect, which results in reduction of the body weight and/or the number of thymocytes. Importantly, we observed that the differences in body weight between the transgenic and wild-type mice became invisible by 4–5 wk of age, while the other phenotypes remained stable.

The basis for the significant increase in surface CD3 expression observed on CD8SP thymocytes of As1-KO and As2-KO mice compared with wild-type mice (Fig. 6) is unclear. This difference was confirmed using anti-TCR-αβ but not anti-TCR-γδ reagents (data not shown). This enhanced CD3 expression is not seen on CD4SP or DP thymocytes or on CD8SP T cells in lymph nodes and spleens of experimental and wild-type mice. It is possible that the high level of CD3 and αβ-TCR results from a perturbation in the requirements for positive selection imposed by reduction of thymic SATB1 protein. Alternatively, it may reflect a role for

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** A, Flow cytometric analysis of lymphocytes from wild-type (WT) and As2-KO mice at 3 wk of age using anti-CD4 and anti-CD8 Abs. Data are representative of spleens from five WT and six As2-KO mice. Percentages of lymphocyte subsets are shown in each quadrant. B, Bar graphs of least square means of percentages of CD4SP, CD8SP, and DN subsets and ratios of CD4SP vs CD8SP T cells in spleen and lymph nodes of WT (□) and As2-KO (■) mice. Values of *p* from statistical analysis are indicated below each graph. The y error bars of SD are shown.

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Levels of cell surface CD3 on CD8SP cells are higher in mice with reduced amounts of thymic SATB1. A, Thymocytes from wild-type (WT) (*n* = 11), As1-KO (*n* = 13), and As2-KO (*n* = 10) mice were stained with anti-CD4, anti-CD8, and anti-CD3 Abs as described in Materials and Methods. Dot plots of CD4 vs CD8 were gated as shown in the left panel, and CD3 expression in thymocyte subsets was quantified. Histograms showing CD3 expression in CD8SP thymocytes from individual mice of the As1-KO, As2-KO, and WT genotypes are shown in the right panel. The numbers in the histograms indicate the MFI of CD3 expression within the gated populations. B, MFI of CD3 expression in gated thymocyte subsets from groups of As1-KO, As2-KO, and WT mice (see A above). The error bars represent the SD based on the mean of CD3 MFI. The value shown below each graph is the *p* value obtained from one-way ANOVA statistical analysis.

<table>
<thead>
<tr>
<th>Thymocyte Subset</th>
<th>WTb</th>
<th>AS1-KOb</th>
<th>AS2-KOb</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td>2.65 ± 0.27</td>
<td>2.74 ± 0.26</td>
<td>2.85 ± 0.24</td>
</tr>
<tr>
<td>CD4lowCD8*</td>
<td>29.88 ± 4.41</td>
<td>38.44 ± 4.82</td>
<td>41.83 ± 6.86</td>
</tr>
<tr>
<td>CD4 “CD8*”</td>
<td>24.69 ± 4.30</td>
<td>34.80 ± 3.88</td>
<td>35.29 ± 7.23</td>
</tr>
<tr>
<td>Total CD8SP</td>
<td>27.47 ± 4.79</td>
<td>37.01 ± 4.71</td>
<td>39.79 ± 7.40</td>
</tr>
<tr>
<td>CD4SP</td>
<td>33.24 ± 4.78</td>
<td>32.16 ± 4.36</td>
<td>34.40 ± 3.31</td>
</tr>
</tbody>
</table>

a Thymocytes from WT, As1-KO, and As2-KO mice were isolated and stained with anti-CD4, anti-CD8, and anti-CD3 Abs. Thymocyte subsets observed in CD4 vs CD8 dot plots were gated and analyzed for CD3 expression. CD4lowCD8* and CD4 “CD8*” subsets represent subgates of the total CD8SP population. The values of MFI were obtained from the CD3 histograms.

b For genotypes, see footnotes to Table I.
SATB1 in regulating expression of genes encoding the αβ-TCR and/or CD3 components in the thymic CD8SP subset.

In a previous study (7), targeted deletion of a 4-kb HindIII fragment containing L2a and upstream hypersensitive sites within the CD8α enhancer resulted in a reduction of CD8SP thymocytes and in the appearance of a large CD8-negative single positive (SP) thymocyte population. We also found that reduction in thymic SATB1 results in a decrease in CD8SP thymocytes. However, it did not lead to the large degree of variation in CD8 expression represented by the CD8-negative SP thymocytes observed in the KO report (7). This suggests that reduction of SATB1 may interfere with a late role played by that protein in the maturation of CD8SP thymocytes. The absence of atypical CD8-negative SP thymocytes from AS1-KO and AS2-KO thymuses may indicate that sufficient SATB1 is present to permit a normal DN to DP transition. That the DP thymocytes obtained appear phenotypically normal as opposed to those seen in SATB1-null mice (28) supports a role for SATB1 in this transition. The variagated population of CD8-negative SP thymocytes (7) may result from the deletion within the L2a region of binding sites for other factors, such as Ikaros (39), required for the transition to DP thymocytes.

In the SATB1-reduced mice, we observed reduction exclusively within the CD8SP population. One explanation is that SATB1-reduced CD8SP cells are susceptible to apoptosis. Derepression of apoptosis-related genes in SATB1-null thymocytes, such as PD-1, RIP, and c-myc, may partially explain why SATB1-reduced thymocytes are vulnerable to this mechanism (28). SATB1-deficient CD8SP numbers may be affected by proliferation. In support of this hypothesis, we observed that the proliferative response to PMA plus ionomycin was impaired in SATB1-reduced mature T cells (Fig. 7).

Although these effects may contribute to CD8SP reduction, the mechanism by which SATB1 appears to regulate transcription prompts consideration of its involvement at an earlier commitment step in T cell maturation. SATB1 has been shown to regulate re-initiation of CD8SP T cells. We speculate that reinitiation of CD8 transcription requires interaction of SATB1 with the L2a element, thereby establishing the permissive chromatin configuration required for expression of CD8 in mature T cells. Recruitment by SATB1 of the CHREC/ACF complex (24, 25) to the L2a site of intermediate CD4SP thymocytes at the time of receptor reversal could position nucleosomes in a way favorable for re-expression of CD8α. Reduction in the amount of SATB1 protein at this step might result in failure to reinitiate CD8α expression, and cells bearing MHCl-specific αβ-TCRs may be lost possibly by apoptosis. Further study of the coreceptor reversal hypothesis using thymocytes deficient in SATB1 or containing altered L2a elements should shed light on the role of SATB1 in CD8SP T cell development.

Acknowledgments

We are grateful to Dr. Terumi Kohwi-Shigematsu for providing mouse breeders containing a targeted SATB1 allele, to Kent Clappay for FACS analysis, to June Harriss and Thomas Bardenat for help in the laboratory, to Ellen Richie for critical reading of the manuscript, to Robert J. Sims III for advice and help in preparing the manuscript, and to Ingrid C. Rojas for help with phenotyping of transgenic mice.

Disclosures

The authors have no financial conflict of interest.

References


