1 SATB1 is required for the development of experimental autoimmune encephalomyelitis

2 to maintain T cell receptor responsiveness

3 Running title: EAE development requires SATB1

4 Immunology (Host Defense): Autoimmunity and immunodeficiency

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21

1   Abstract

2   The genome organizer special AT-rich sequence binding protein 1 (SATB1) regulates

3   specific functions through chromatin remodeling in helper T cells. We recently reported

4   that T cells from SATB1 conditional knockout (SATB1cKO) mice, in which the *Satb1*

5   gene is deleted from hematopoietic cells, impaired the phosphorylation of signaling

6   molecules in response to T cell receptor (TCR) crosslinking. However, the *in vivo* T cell

7   response upon antigen presentation in the absence of SATB1 remains unclear. We

8   show that SATB1 modulates the T cell antigen response during the induction and

9   effector phases. The expression of SATB1 was upregulated in response to TCR

10   stimulation, suggesting that SATB1 is important for the antigen response. Therefore,

11   we examined the role of SATB1 in the TCR response and induced experimental

12   autoimmune encephalomyelitis (EAE) using the myelin oligodendrocyte glycoprotein

13   peptide 35-55 (MOG35-55) and pertussis toxin. SATB1cKO mice were resistant to

14   EAE and showed defects in IL-17- and IFNγ-producing pathogenic T cells. Thus,

15   SATB1 expression appears necessary for T cell function in the induction phase. To

16   examine SATB1 function during the effector phase, we used a tamoxifen-inducible

17   SATB1 deletion system, SATB1cKO-ER-Cre mice. We transferred encephalitogenic T

18   cells from MOG35-55-immunized SATB1cKO-ER-Cre mice into healthy mice.

19   Tamoxifen-treated recipient mice before the onset of paralysis were resistant to EAE.

20   Furthermore, recipient mice treated with tamoxifen after the onset of EAE exhibited no

1   disease progression. Thus, SATB1 is essential for maintaining TCR responsiveness

2   during the induction and effector phases and may represent a novel therapeutic target

3   for T cell-mediated autoimmune diseases.

4   Key words: autoimmune disease, EAE model, SATB1 knockout, TCR response

1   Introduction

2   The chromatin structure modulates accessibility to target genes and is

3   important for the regulation of gene expression and cellular functions. Chromosomal

4   organizers regulate chromatin topology, forming transcription-regulatory compartments

5   (1, 2). Matrix attachment regions (MARs) or scaffold-associated regions in DNA

6   sequences are thought to mediate chromatin loop formation, which is important for the

7   compaction of genomic DNA and for the organization of chromatin into units of

8   genomic function (3, 4). MARs frequently colocalize with enhancer regions and several

9   MARs have been suggested to mediate the positive or negative regulation of gene

10   expression (5-7). Special AT-rich sequence binding protein 1 (SATB1) is a

11   MAR-binding protein (8) that is predominantly expressed in the thymus. SATB1 recruits

12   the ATP-utilizing chromatin assembly and remodeling factor (ACF) and the

13   nucleosome-remodeling factor (NuRD) complex to specific DNA sites and functions as

14   an epigenetic regulator that facilitates the functioning of several chromatin-remodeling

15   factors (9). A subsequent study demonstrated that SATB1 expression is highly

16   associated with thymocyte differentiation (10). In addition, SATB1 regulates the

17   expression of multiple helper T cells genes and modulates effector T cell function by

18   holding promoter regions of cytokine genes   in close proximity (11). Thus, chromatin

19   remodeling by SATB1 maintains the function of peripheral T cells.

20   Signals from T cell receptors (TCRs) are critical for the activation of T cells,

1   and together with those from cytokine receptors, activate diverse signaling pathways

2   that control the fate as well as the function of activated T cells (12). Naïve CD4 T cells

3   begin a process of differentiation into effector T cells upon stimulation via cell surface

4   receptors. Naïve CD4 T cells also have the capacity to differentiate into distinct T

5   helper (Th) subsets, such as Th1, Th2, Th17, and Tfh cells, as defined by the different

6   cytokines present in the microenvironment (13). In addition to the appropriate

7   cytokines, antigen recognition by TCRs is the most critical mediator for the functional

8   differentiation of Th subsets (14). These processes are transcriptionally regulated and

9   involve the induction of specific transcription factors (15). Chromatin remodeling, which

10   controls the accessibility of transcription factors to their target genes, represents the

11   next most critical regulatory mechanism in Th cell differentiation (16). We recently

12   reported that T cells from SATB1-deficient mice severely impaired proximal signaling

13   following TCR engagement *in vitro* (17), suggesting that SATB1 supports T cell

14   differentiation into Th subsets and/or the maintenance of Th subsets. However, the

15   contribution of SATB1 to supporting T cell function after antigen stimulation remains

16   unknown.

17   In this study, we examined *in vivo* T cell responses in conditional knockout mice

18   lacking SATB1 in hematopoietic cells (SATB1cKO$_V$) or in T cells (SATB1cKO$_L$). To this

19   end, we used experimental autoimmune encephalomyelitis (EAE). SATB1cKO$_V$ mice

20   and SATB1cKO$_L$ mice were resistant to EAE induced with myelin oligodendrocyte

1   glycoprotein peptide 35-55 (MOG35-55). We demonstrated that T cells derived from

2   both lines of SATB1cKO mice failed to proliferate and produce cytokines in response to

3   protein antigens. In the transfer EAE model, the induction of the *Satb1*-deletion after the

4   onset of paralysis prevented the disease progression. Our results suggest that SATB1

5   plays an important role in the priming and maintenance of T cell responses *in vivo*.

6

1   Materials and Methods

2   Mice

3   SATB1-floxed mice were generated as described previously (17). Vav-Cre mice,

4   ER-Cre mice, and OT-II TCR transgenic mice were purchased from the Jackson

5   Laboratory (Bar Harbor, ME, USA). Lck-Cre mice were obtained from the laboratory

6   animal resource bank at Nibiohn (Osaka, Japan). SATB1 conditional knockout mice

7   were generated by crossing Satb1-floxed with Vav-Cre mice, Lck-Cre mice, and ER-Cre

8   mice to generate SATB1cKO$_V$ mice, SATB1cKO$_L$ mice, and SATB1cKOe mice,

9   respectively. The estrogen receptor (ER) ligand-binding domain was fused to the Cre

10   recombinase (ER-Cre) in Cre expressing mice to generate tamoxifen-inducible

11   conditional knockout mice. Since the administration of tamoxifen can induce the nuclear

12   translocation of ER-Cre and the subsequent inactivation of target genes containing the

13   loxP sequence, we generated SATB1cKOe mice and used them to induce the deletion

14   of *Satb1* in CD4 T cells during the effector phase. OTII mice express transgenic TCRs

15   specific for the chicken ovalbumin (OVA) 323-339 peptide. Since these mice are useful

16   for analyzing antigen (OVA) specific T cell responses, we generated SATB1cKOe mice

17   and assayed the proliferation of T cells in the absence or presence of SATB1. C57BL/6

18   mice were purchased from the Charles River Laboratories (Kanagawa, Japan).

19   C57BL/6 CD45.1 mice and RAG2$^{-/-}$ mice were bred at the Toho University animal

20   facility under specific pathogen-free conditions in accordance with the institutional

1   guidelines (18). All experiments using mice received approval from the Toho University

2   Administrative Panel for Animal Care (17-53-311) and Recombinant DNA (17-53-303).

3   The mice used were aged 8−12 weeks.

4

5   Real-time PCR

6   Real-time PCR was performed as described previously (19). Total RNA was

7   isolated from cells using Isogen (Nippon Gene, Toyama, Japan). RNA (500 ng/reaction)

8   was reverse transcribed using a High-Capacity cDNA Archive kit (Applied Biosystems,

9   Foster City, CA, USA). For quantitative analysis, real-time PCR was conducted using

10 the TaqMan Gene Expression Assay kit (Applied Biosystems). Mm00487425_01 for

11 *Fos*, Mm01268940_m1 for *Satb1*, Mm00519943_m1 for *Il23r*, Mm01168134_m1 for

12 *Ifng*, Mm00475162_m1 for *Foxp3*, Mm01261022_m1 for *Rorc*, and Mm02619580_g1

13 for actin were used as primers on an Applied Biosystems 7500 Fast system. β-actin

14 was used as an endogenous reference for normalization. Quantitative real-time PCR

15 experiments were repeated twice in triplicate.

16

17 EAE induction

18   Mice were immunized s.c. in the flank on day 0 with 150 μg of MOG35-55

19 peptide in complete Freund's adjuvant (CFA) containing 5 mg/ml H37RA (Difco

20 Laboratories, Detroit, MI, USA), as previously described (20). Two-hundred nanograms

1 of pertussis toxin (PT; List Laboratories, Campbell, CA, USA) were injected

2 intraperitoneally on days 0 and 2. For passive transfer EAE, donor mice were

3 immunized as describe above. Ten days later, draining lymph node (DLN) cells were

4 cultured at 4 x 10^6 cells/ml with 10 mM MOG35-55 peptide for 3 days in RPMI1640

5 culture medium with IL-23, anti-IL-4, and anti-IFNγ antibodies, as previously described

6 (20). Then, 10^7 CD4 T cells were purified using negative selection kinetics on a MACS

7 system (Miltenyi Biotec, Bergisch Gladbach, Germany) and transferred intravenously

8 into naïve and 500-rad x-irradiated mice. Mice were graded for EAE on a clinical scale

9 of 0–6: 0, no disease; 1, complete loss of tail tone; 2, hindlimb weakness; 3, hindlimb

10 paralysis; 4, complete hind and partial forelimb paralysis; 5, hind and forelimb paralysis;

11 6, death.

12

13 Recall responses

14 DLN cells were prepared from immunized mice and cultured for 72 h with the

15 MOG35-55 peptide or OVA. They were pulsed for 6 h with 3H-thymidine (Amersham

16 Biosciences, Little Chalfont, UK) and assayed for the incorporation of 3H-thymidine

17 using Topcount (Perkin Elmer, Waltham, MA, USA), as previously described (21).

18 Supernatants were collected at 24 h and assayed for IL-2, or at 72 h for IL-17 and IFNγ,

19 with OptEIA ELISA kits (BD Biosciences, Franklin Lakes, NJ, USA). Cells were stained

20 with anti-mouse CD4 and CD8 antibodies. The cells were then washed with washing

1   buffer (1% FCS, 0.1% sodium azide in PBS) and fixed with Cytofix/Cytoperm kit (BD

2   Biosciences) for 20 min. Intracellular staining was performed using anti-mouse

3   IL-17-PE, IFNγ-PE, and Foxp3-FITC antibodies for 30 min. In some experiments,

4   isotype control antibodies used under similar conditions indicated the specific binding of

5   test antibodies. Cells were acquired on a BD LSRFortessa X-20 instrument using the

6   DIVA software. Data were analyzed using the FlowJo software.

7

8   Tamoxifen treatment

9   Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) was resuspended in ethanol,

10 diluted in corn oil to a final concentration of 10 mg/ml, and heated at 37 °C until

11 dissolved. 4-Hydroxytamoxifen (4-OHT, Sigma) was dissolved in ethanol. To delete

12 *Satb1* in donor CD4 T cells, 100 μL of the tamoxifen solution or oil were injected

13 intraperitoneally into recipient mice adoptively transferred with CD4 T cells from

14 SATB1cKOe mice once a day for three consecutive days, 3 days after cell transfer or 1

15 day after disease onset.

16   For the *in vivo* T cell proliferation assay, naïve CD4 T cells derived from

17 SATB1cKOe-OTII mice were labeled with the Cell Proliferation Dye eFluor 670

18 (eBioscience, San Diego, CA, USA). The labeled CD4 T cells were adoptively

19 transferred into Rag2$^{-/-}$ mice intravenously. Recipient mice were treated with tamoxifen

20 or oil for three consecutive days after cell transfer and were injected intravenously with

1   OVA on day 1 after the transfer. The proliferation of donor T cells was determined by

2   flow cytometry using a Cell Proliferation Dye. For the *in vitro* deletion of *Satb1*, 0.2 μM

3   of 4-OHT were administered into the culture medium for the induction of Cre-mediated

4   deletions.

5

6   Apoptosis assay

7   CD4 T cells prepared from WT or SATB1cKO$_L$ mice were stimulated with

8   plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) antibodies for the indicated

9   times to induce TCR-mediated apoptosis. Apoptosis was determined using the

10   MEBSTAIN apoptosis TUNEL kit (MBL, Nagoya, Japan).

11

12   Statistical analysis

13   Data are presented as means ± SEM. The statistical analysis was performed

14   using GraphPad Prism 6.0 (GraphPad Software, SanDiego, CA, USA), and a *p* value <

15   0.05 was considered significant. Statistical significance was assessed using the

16   unpaired Student's *t*-test or the Mann-Whitney U test.

17

18

1   Results

2   **TCR stimuli induce SATB1 expression**

3   T cells express numerous genes to gain effector function after antigen

4   stimulation. If SATB1 plays an essential role in T cell function, SATB1 expression

5   should be upregulated in response to TCR stimulation. Therefore, we assessed the

6   transcription of *Satb1* in naïve CD4 T cells from WT mice. In response to TCR

7   crosslinking with anti-CD3 and anti-CD28 antibodies, *Satb1* mRNA expression

8   gradually increased over 60 min, peaked at 120 min, and was substantially diminished

9   at 12 h (Figure 1A). Since transcription was induced, we assessed the SATB1 protein

10   levels by immunoblotting. As shown in Figure 1C, TCR crosslinking also led to an

11   increase in SATB1 and c-Fos protein levels. Although the changes in SATB1

12   expression were slightly delayed compared to those in the immediate early gene *c-Fos*

13   (Figure 1B), these results suggest that SATB1 plays a role in the regulation of T cell

14   function.

15

16   ***SATB1 conditional knockout mice are resistant to EAE induction***

17   We bred SATB1f/f mice with Vav-Cre or Lck-Cre mice to generate mice with

18   hematopoietic cell-specific (SATB1cKO$_V$) or T cell-specific (SATB1cKO$_L$) cKO of *Satb1*.

19   To examine the role of SATB1 in T cell responses, we generated C57BL/6 WT,

20   SATB1cKO$_V$, and SATB1cKO$_L$ T cell-mediated autoimmune disease mouse models of

1   EAE. These mice were immunized s.c. with the MOG35-55 peptide in CFA on day 0 and

2   intravenously injected with PT on days 0 and 2. As shown in Figure 2, WT mice

3   developed EAE with an incidence of 100% that arose on day 12, whereas SATB1cKO$_V$

4   and SATB1cKO$_L$ mice failed to develop EAE during the 35 days following immunization.

5   A histological analysis of the lumbar spinal cord from WT mice 20 days after

6   immunization clearly demonstrated cellular infiltration in the spinal parenchyma (Figure

7   2B). No such infiltration was observed in the spinal cords of SATB1cKO$_V$ mice,

8   consistent with the lack of EAE development (Figure 2B).

9   We recently reported that thymic development was impaired in SATB1cKO$_V$

10   mice. We propose that peripheral T cells may have been activated due to homeostatic

11   expansion, and were thus susceptible to apoptosis induced following TCR stimulation;

12   this, in turn, may lead to EAE resistance. Moreover, SATB1-deficient T cells may be

13   susceptible to cell death induced following TCR activation. To examine this possibility,

14   CD4 T cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1

15   μg/ml) antibodies to induce TCR-mediated apoptosis (activation-induced cell death,

16   AICD). We demonstrated that the percentage of CD4 T cells exhibiting TUNEL-positive

17   staining increased from 1 to 18.8% in a time-dependent manner. This result indicates

18   that WT T cells display AICD following TCR crosslinking (Figure 2C). In contrast,

19   SATB1-deficient T cells from SATB1cKO$_L$ mice were resistant to TCR-mediated cell

20   death. These results suggest that the hyperactivation of T cell does not occur, even in

1   the absence of SATB1.

2

3   ***SATB1 conditional knockout mice failed to generate IL-17 and IFNγ-producing T***

4   ***cells.***

5   To examine whether pathogenic T cells were generated in SATB1 conditional

6   knockout mice, we compared the *in vitro* recall responses of DLN cells from WT,

7   SATB1cKO$_V$, and SATB1cKO$_L$ mice. DLN cells were prepared 10 days after

8   immunization, when EAE symptoms were not observed in WT mice. Although the

9   proliferative recall responses to various doses of the MOG35-55 peptide were observed

10 in DLN cells from WT mice, cellular proliferation was not detected in DLN cells from

11 either of the SATB1 conditional knockout mice (Figure 3A). Next, we analyzed cytokine

12 production in response to treatment with the MOG35-55 peptide. IL-2 production in T

13 cells from DLN cells was much higher in WT mice than in SATB1cKO$_V$ and SATB1cKO$_L$

14 mice, indicating that the proliferation of SATB1-deficient T cells was defective (Figure

15 3A). A dose-dependent production of IFN-γ and IL-17 was detected in cultures of DLN

16 cells from WT mice and SATB1 conditional knockout mice, but the production of each of

17 these cytokines was severely diminished in SATB1cKO$_V$ and SATB1cKO$_L$ mice (Figure

18 3A). Since SATB1 regulates the thymic development of T cells (17), the failure of

19 inducing a T cell response can be attributed to the fact that SATB1 knockout mice

20 possess only small population of peripheral T cells. However, the number of CD4 T cells

1   prepared from SATB1cKO$_L$ mice represented 18 to 20% of the total number of lymph

2   node cells (Figure 3B). The CD4-positive T cell population in SATB1cKO$_L$ mice was

3   slightly reduced when compared to that in WT mice (where the number of CD4 T cells

4   represented approximately 25% of the number of total lymph node cells), suggesting

5   that the absence of a CD4 T cell population was not the conclusive cause underlying

6   the failure of the T cell response and the presence of EAE resistance in SATB1

7   conditional knockout mice. The reduced *in vitro* IL-17 and IFNγ production by DLN cells

8   derived from SATB1cKO$_V$ and SATB1cKO$_L$ mice suggested a defect in Th17 and Th1

9   generation. To examine this possibility, DLN cells were prepared 10 days after

10   immunization, incubated with the MOG peptide, and assessed for intracellular IL-17

11   and IFNγ staining. As shown Figure 3B, CD4$^+$ IL17$^+$ Th17 cells were found at a much

12   lower frequency in DLN cells from SATB1cKO$_L$ mice than in those from WT mice

13   (0.26% vs 2.1% of total cells, and 1.3% vs 8.3% of CD4 T cells). Moreover, the

14   abundance of CD4$^+$ IFNγ$^+$ Th1 cells was much lower in SATB1cKOL mice than that in

15   WT mice (0.17% vs 2.2% of total cells and 0.09% vs 9.1% of CD4 T cells).

16   To examine whether the abortive production of these cytokines by DLN cells

17   from both lines of SATB1cKO mice might represent a specific response to the MOG

18   peptide, we analyzed the recall responses after immunization with OVA emulsified in

19   CFA (Figure 4). DLN cells were prepared 10 or 28 days after immunization, incubated

20   with OVA, and the proliferation of T cells and cytokine production were assessed.

1   Similar to the T cell response against the MOG peptide, a reduction in cytokine

2   production, cell proliferation, and the generation of Th17/Th1 cells was observed in

3   cells from SATB1-deficient mice (Figure 4). These diminished recall responses in

4   SATB1cKO$_V$ and SATB1cKO$_L$ mice were maintained for at least four weeks, suggesting

5   that T cell priming requires SATB1 for the induction of antigen responses and that

6   SATB1 generally modulates T cell function in response to TCR signaling.

7   Mice lacking SATB1 displayed EAE resistance and a defective induction of

8   pathogenic T cells. These results led us to hypothesize that in SATB1-deficient mice an

9   immune regulatory microenvironment could be established. To evaluate this possibility,

10   we generated EAE pathogenic Th17 cells from WT mice and transferred them into

11   SATB1cKO$_V$ and SATB1cKO$_L$ mice. As shown in Figure 5, WT, SATB1cKO$_V$, and

12   SATB1cKO$_L$ mice receiving MOG-reactive CD4 T cells developed EAE at similar

13   severities. These data suggest that the attenuated EAE phenotype in SATB1-deficient

14   mice is T cell-mediated. Together, these findings suggest that T cells derived from

15   SATB1-deficient mice failed to differentiate into pathogenic T cells during the priming or

16   induction phases.

17

18   ***SATB1 maintains antigen responsiveness after T cell priming.***

19   Our findings suggest that pathogenic T cells are generated by a

20   SATB1-dependent mechanism. In addition to its regulatory function during the priming

1   phase, SATB1 may also regulate T cell function during the effector phase of EAE. To

2   investigate whether SATB1 is necessary for maintaining the self-reactivity of T cells

3   after the establishment of pathogenic responses, we generated SATB1cKOe mice by

4   crossing SATB1-floxed mice with ER-Cre mice to inducibly delete *Satb1*. Ten days after

5   s.c. immunization, DLN cells from SATB1cKOe mice were incubated for 3 days with the

6   MOG35-55 peptide and IL-23; then, CD4 T cells were adoptively transferred

7   intravenously into WT mice. All recipient control mice developed EAE with a disease

8   incidence of 100% and similar clinical scores and time courses (Figure 6A). These

9   results suggest that SATB1cKOe mice normally generate neuroreactive T cells. Three

10   days after the transfer, recipient mice were injected intraperitoneally with tamoxifen

11   every other day for a total of three injections (days 3, 5, and 7) to delete *Satb1* in donor

12   T cells. In contrast to control conditions, SATB1-deficient T cells could not induce

13   paralysis (Figure 6A).

14   To examine the role of SATB1 in cytokine production by T cells, we immunized

15   SATB1cKOe mice with the MOG peptide or OVA in CFA and induced the deletion of

16   *Satb1* in these mice in the presence or absence of tamoxifen on days 11 and 13 after

17   immunization. On day 14, CD4 T cells were prepared from the DLN cells of these mice

18   and were stimulated with antigen-loaded irradiated splenocytes from CD45.1 mice as

19   antigen-presenting cells. The recall cytokine production and intracellular staining

20   assays showed a dose-dependent production of IFNγ and IL-17 by cultures of

1   SATB1-sufficient CD4 T cells (Figure 6B). In contrast, cytokine production in response

2   to the MOG peptide or OVA was hardly detected in cultures of SATB1-deficient CD4 T

3   cells (Figure 6B). The defect in cytokine production was confirmed by intracellular

4   staining (Figure 6C), suggesting that pathogenic CD4 T cells have lost the ability to

5   produce cytokines in the absence of SATB1 expression. These results suggest that the

6   lack of SATB1 in CD4 T cells after priming suppresses the production of effector

7   cytokines for EAE induction.

8   We also assessed whether the proliferation of CD4 T cells depends on *Satb1*

9   expression. Similar to cytokine production, the proliferative recall responses were

10   diminished in cultures of SATB1-deficient CD4 T cells (Figure 6B). To investigate the

11   role of SATB1 in antigen-specific T cell responses *in vivo*, we use OTII mice and the

12   OVA 323-339 tetramer. OTII mice express T cell receptors specific for the OVA 323-339

13   peptide and the OVA tetramer is able to detect antigen-specific CD4 T cell responses.

14   We transferred OVA-specific CD4 T cells prepared from SATB1cKOe-OTII mice into

15   RAG2$^{-/-}$ mice. Donor CD4 T cells were labeled with the eFluor 670 cell

16   proliferation-specific dye and transferred into recipient mice. These mice were treated

17   with or without tamoxifen, and OVA was administered to stimulate donor OTII cells.

18   Three days after immunization, dye-division proliferation was assessed by flow

19   cytometry. As shown in Figure 6D, most tetramer-positive donor CD4 T cells (>80%)

20   without tamoxifen showed a divided profile. In contrast, SATB1-deficient donor CD4 T

1   cells failed to proliferate in response to OVA. These results using SATB1cKOe mice

2   suggest that: 1) SATB1 in CD4 T cells represents an essential factor for maintaining the

3   antigen receptor responses; 2) SATB1 plays an important role in T cell responses that

4   is independent of its roles in thymic development (17).

5

6   ***Encephalitogenic T cells lacking SATB1 partially retain the properties of Th17***

7   ***cells***

8   Th17 cells play an essential role in the induction of EAE (22, 23). The numbers of

9   Th17 cells were adequate for the induction of the adoptive transfer of EAE under

10   SATB1-sufficient conditions (Figure 6A). The same number of tamoxifen-mediated

11   SATB1-deficient CD4 T cells did not induce EAE paralysis. Moreover, the

12   encephalitogenic activity in T cells was nearly abolished following treatment with

13   tamoxifen (Figure 6). We suspected that the deletion of *Satb1* induces Th17 cells to

14   change their properties from pathogenic to suppressive. Furthermore, the loss of

15   SATB1 may induce the maturation of Treg cells, since SATB1 negatively regulates the

16   expression of the *Foxp3* transcription factor (24), which regulates the generation and

17   function of Treg cells. To investigate this possibility, 10 days after s.c. immunization,

18   DLN cells prepared from SATB1cKOe mice were incubated with the MOG35-55 peptide

19   and IL-23 (Th17 skew condition) for 3 days, and then CD4 T cells were adoptively

20   transferred intravenously into CD45.1 WT mice. Recipient mice were treated with or

1   without tamoxifen on day 2 after the transfer. Next, CD45.2 donor CD4 T cells were

2   FACS-sorted, and the expression of Th17 cell-related genes was analyzed. As shown

3   in Figure 7A, the level of *Satb1* was significantly reduced in the presence of tamoxifen.

4   The *Rorc* and *Il23r* genes were similarly expressed in SATB1-sufficient and

5   SATB1-deficient CD45.2 CD4 T cells, suggesting that CD4 T cells lacking SATB1

6   partially maintain encephalitogenic properties. In contrast, the transcription of the *Il17a*

7   and *Ifng* pathogenic cytokines was reduced in SATB1-deficient CD4 T cells. Flow

8   cytometry also showed that SATB1-deficient cells displayed a reduction in the

9   intracellular IL-17 and IFNγ-positive population (Figure 7B). We next assessed whether

10   CD4 T cells upregulate *Foxp3* expression in the absence of SATB1. In contrast to naïve

11   CD4 T cells, SATB1-sufficient Th17 cells exhibited a reduction in Foxp3 expression

12   (Figure 7A and 7B). Moreover, the Foxp3 mRNA and protein levels in SATB1-deficient

13   Th17 cells were reduced rather than increased, when compared with those in

14   SATB1-sufficient Th17 cells (Figure 7A and 7B). These results suggest that Th17 cells

15   induced following treatment with the MOG peptide and IL-23 partially retain Th17

16   properties, without undergoing reprogramming to suppressive Th lineages even in the

17   absence of SATB1 expression. Combined with the results in Figure 5, these data

18   indicate that SATB1 contributes to the antigen responses of CD4 T cells during the

19   effector phase.

20

1   ***The induction of the Satb1 deletion in CD4 T cells after the onset of EAE prevents***

2   ***disease progression***

3   Finally, to examine whether controlling SATB1 function in T cells has any

4   therapeutic potential for suppressing the progression of EAE, we immunized

5   SATB1cKOe mice with the MOG35-55 peptide in CFA. Ten days after immunization,

6   DLN cells were incubated for 3 days with the MOG peptide and IL-23, and then CD4 T

7   cells were adoptively transferred into WT mice to induce transfer EAE. After detecting

8   the onset of the limp tail phenotype (score 1 for EAE), diseased mice were treated with

9   tamoxifen to induce *Satb1* deletion. Control mice treated with tamoxifen-free oil

10   displayed disease progression with an increase in severity. In contrast, mice injected

11   with tamoxifen exhibited significantly alleviated EAE symptoms, including no

12   progression in the severity of the disease (Figure 8). These results suggest that T cells

13   lacking SATB1 attenuate antigen responsiveness during the effector phase, and that

14   controlling SATB1 activity in T cells allows the hosts to survive in the presence of

15   autoimmune disease.

16

1   Discussion

2

3   The expression of SATB1 in thymocytes is highest at the double-positive stage

4   and decreases during the progression of the maturation stage (17). We first attempted

5   to clarify the expression of SATB1 in T cells following the crosslinking of antigen

6   receptors. A quantitative analysis in CD4 T cells indicated that SATB1 was clearly

7   expressed in response to TCR stimulation. The elevation of SATB1 expression is

8   considered to induce changes in the structure of chromosomes, leading to an increase

9   in gene expression for factors involved in the effector function of the helper T cell subset.

10   These data suggest that SATB1 is induced by TCR signaling and that it plays important

11   roles in peripheral naïve T cells in regulating the generation of antigen-specific T cells

12   and maintaining the function of effector T cells. In general, CD4 T cells differentiate into

13   helper T cells after the stimulation of the TCR and that of the cytokine receptor. SATB1

14   plays an important role in Th2 cells by controlling chromosomal structure (11). The

15   differentiation of naïve T cells into Th2 cells (priming stage) is controlled by nuclear

16   events such as epigenetic modifications and transcription. In naïve T cells, the

17   transcription machinery prevents their spontaneous differentiation into Th2 cells. Upon

18   TCR stimulation, naïve T cells are stimulated with a polarizing signal through the IL-4

19   receptor pathway to upregulate the expression of GATA-3. GATA-3 drives Th2

20   differentiation in a cell-autonomous fashion. During the priming stage, the Th2-related

1   cytokine gene locus undergoes chromatin remodeling and epigenetic modifications,

2   which contribute to Th2 cell-related gene expression. SATB1 modulates the formation

3   of these chromosomal hubs (11). After differentiation, Th2 cells produce Th2-related

4   cytokines upon TCR stimulation and GATA-3 upregulation. Similar to that in Th2 cells,

5   chromatin remodeling may contribute to the regulation of encephalitogenic T cells (25).

6   SWI/SNF is an ATP-dependent chromatin-remodeling complex. SRG3 (SWI3-related

7   gene) enhances the function of the SWI/SNF complex by stabilizing it (25, 26).

8   SRG3-SWI/SNF-mediated chromatin remodeling in CD4 T cells facilitates EAE via the

9   induction of Th17 cells (25). In addition, the epigenetic modification of histones

10   following chromatin remodeling occurs during the generation of Th17 cells from naïve T

11   cells. A *cis* element, conserved non-coding sequence 2 (CNS2), interacts with the

12   promoters of *Il17* and *Il17f* (27, 28). CNS2 is bound by the p300 and JMJD3 (JmjC

13   domain-containing protein 3) histone remodeling enzymes, which modify the

14   acetylation status of histone H3 and inhibit the suppressive histone marker H3K27me3,

15   respectively, resulting in the increased accessibility of RORγt to the *Il17* and *Il17f* gene

16   promoters (28). These findings suggest that SATB1 activity manipulates chromatin

17   structure and leads to the development of Th17 cells. In this study, we demonstrated

18   that SATB1-deficient mice exhibited a severely impaired production of EAE-related

19   cytokines, such as IFNγ and IL-17. This indicates that SATB1 is necessary for the

20   generation of encephalitogenic Th17 cells. However, we did not determine how SATB1

1   regulates T cell responses by modulating chromatin structure in pathogenic T cells.

2   While it is still necessary to identify the molecular mechanism underlying the

3   SATB1-mediated differentiation of T cells, the results of this study suggest that SATB1

4   is an essential factor for the formation of the chromatin hub during the priming stage of

5   Th cells.

6   In this study, we showed that SATB1cKO$_V$ and SATB1cKO$_L$ mice are

7   completely resistant to the active induction of EAE. This suggests that SATB1 is

8   required for the generation of pathogenic Th cells. We also demonstrated that

9   pathogenic T cells lacking SATB1 expression, which were induced by tamoxifen

10   treatment on day 2 after transfer into WT mice, failed to induce EAE. Therefore, we

11   assessed the possibility that controlling SATB1 activity after the onset of symptoms

12   leads to the inhibition of disease progression. We prepared MOG-reactive Th17 cells

13   from SATB1cKOe mice and transferred them into healthy recipient mice. These Th17

14   cells induced paralysis and resulted in progressive symptom severity; cells without the

15   induction of *Satb1* deletion were used as a control. In contrast, mice injected with

16   tamoxifen to induce the deletion of SATB1 did not exhibit palsied tails and legs or

17   disease progression. These results suggest that SATB1 is necessary not only for the

18   induction of pathogenic gene expression during the priming phase, but also for

19   retaining the pathogenic activity of T cells during the effector phase. We reported that

20   peripheral T cells from SATB1-deficient mice showed weak TCR signaling (17). Since

1   TCR signaling is a common essential factor during the priming and effector phases of

2   EAE, SATB1 and its related molecules might regulate antigen responses. Our results

3   suggest that donor encephalitogenic T cells hardly respond to CNS-antigens and could

4   not exhibit enough pathogenicity to induce autoimmune disease. Although further

5   studies are required to reveal the molecular mechanism underlying SATB1-mediated T

6   cell responses, our findings have important implications for the regulation of SATB1

7   activity in the treatment of EAE and/or Th17-mediated diseases.

8

9   We and others investigators have reported that SATB1 is required for the

10   development of hematopoietic cells and T cells (17, 29). In the thymic stage, T cell

11   progenitors develop into not only conventional T cells but also thymic Treg cells.

12   SATB1 binds Treg cell-specific super-enhancers and upregulates the expression of

13   Treg-related genes, such as *Foxp3* (24). Thus, SATB1-dependent Treg

14   super-enhancer activation is crucial for Treg cell specification. In addition, SATB1

15   regulates *Foxp3* expression in conventional T cells (24). The deletion of *Satb1* in

16   mature CD4 T cells derepressed *Foxp3* expression; SATB1-deficient conventional T

17   cells expressed *Foxp3*. A part of these *Foxp3*-positive T cells converted from

18   conventional T cells on expressed CD25 on the cell surface and showed suppressive

19   activity (24). The treatment of donor T cells with tamoxifen reduced SATB1 expression

20   and EAE pathogenicity in our transfer EAE model. In contrast to a previous report,

1   *Foxp3* expression was decreased in SATB-deficient T cells. Based on these results,

2   we reasoned that the loss of pathogenicity in donor T cells in the absence of SATB1

3   expression is independently regulated from the SATB1-mediated derepression of

4   *Foxp3* expression.

5

6   In summary, this represents the first report on the role of SATB1 expression

7   in the induction of autoimmune diseases during the *in vivo* pathogenic T cell response.

8   Our work demonstrated that SATB1 regulates pathogenic Th17 cell generation and

9   effector function. Finally, the deletion of *Satb1* suppressed the self-reactive T

10   cell-mediated autoimmune disease, EAE, suggesting that SATB1 may represent a

11   promising therapeutic target for T cell-mediated autoimmune diseases.

12

13

14

15

1   Acknowledgments

16

17   Disclosure

18   The authors declare that there are no financial conflicts of interest.

19

20   Abbreviations used in this article:

21   ACF, ATP-utilizing chromatin assembly and remodeling factor

1   AICD, activation-induced cell death

2   CFA, complete Freund's adjuvant

3   CNS2, conserved non-coding sequence 2

4   DLN, draining lymph node

5   EAE, experimental autoimmune encephalomyelitis

6   ER, estrogen receptor

7   MARs, matrix attachment regions

8   MOG, myelin oligodendrocyte glycoprotein

9   NuRD, nucleosome-remodeling factor

10   OVA, ovalbumin

11   PT, pertussis toxin

12   RT, reverse transcription

13   SATB1, special AT-rich sequence binding protein 1

14   SRG3, SWI3-related gene

15   TCR, T cell receptor

16   Th, T helper cell

17   WT, wild-type

18

19

20

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1   Figure legends

2

3   Figure 1

4   Expression of SATB1 in T cells

5   (SATB1 is induced or expressed in murine T cells after TCR stimulation)

6   Naïve CD4 T cells prepared from WT mice were stimulated with anti-CD3 and

7   anti-CD28 antibodies for the indicated times. The mRNA expression of *SATB1* (A) and

8   *c-Fos* (B) in these cells was analyzed using real-time RT-PCR. The transcriptional

9   levels of these genes were analyzed relative to β-actin. * Values were significantly

10   different from those in control cells (0 min) under the same conditions ($p < 0.05$). These

11   results are representative of three independent experiments. (C) Protein expression for

12   SATB1 and c-Fos, determined by immunoblotting using anti-SATB1 and anti-c-Fos

13   antibodies. One experiment, representative of three independent experiments, is

14   shown.

15

16   Figure 2

17   Failure of SATB1 conditional knockout mice to develop EAE.

18   (A) Mice were s.c. immunized with the MOG peptide in CFA and injected intravenously

19   with PT on days 0 and 2, and assessed by clinical scores for 35 days after

20   immunization. * $p < 0.05$. (B) Spinal sections were stained with HE (day 20) after

21   immunization. Experiments were conducted three times, with essentially similar results.

22   (C) Apoptotic T cell induction by TCR crosslinking was detected by TUNEL staining.

23   Representative flow cytometry plots are shown.

24

25   Figure 3

1   *In vitro* recall response to the MOG peptide for DLN cells from WT, SATB1cKO$_V$, and

2   SATB1cKO$_L$ mice.

3   (A) Mice were immunized with MOG in CFA. DLN cells were prepared 10 or 28 days

4   after immunization and incubated with the MOG peptide at the indicated doses;

5   proliferation was assessed on day 3. The production of IL-2 (day 1), IL-17 (day 3), and

6   IFNγ (day 3) was determined by ELISA. (B) DLN cells were prepared from naïve WT

7   and SATB1cKO$_L$ mice, or from mice on day 10 after immunization, as described in the

8   legend of Figure 3 (A). DLN cells were incubated with the MOG peptide, after which the

9   intracellular expression of IL-17 and IFNγ were assessed. The numbers in the light

10   quadrants represent the percentages of the total cells. * $p < 0.05$. One representative

11   result of three independent experiments is shown.

12

13   Figure 4

14   *In vitro* recall response to OVA for DLN cells from WT, SATB1cKO$_V$, and SATB1cKO$_L$

15   mice.

16   (A) Mice were immunized with OVA in CFA. DLN cells were prepared 10 or 28 days

17   after immunization and incubated with OVA at the indicated doses; proliferation was

18   assessed on day 3. The production of IL-2 (day 1), IL-17 (day 3), and IFNγ (day 3) was

19   determined by ELISA. (B) DLN cells were prepared from naïve WT and SATB1cKO$_L$

20   mice or from mice on day 10 after immunization, as described in the legend of Figure 4

21   (A). DLN cells were incubated with OVA, after which the intracellular expression of IL-17

22   and IFNγ were assessed. The numbers in the light quadrants represent the

23   percentages of total cells. * $p < 0.05$. One representative result of three independent

24   experiments is shown.

25

26

27   Figure 5

1   The transfer of encephalitogenic T cells induces EAE in SATB1-deficient mice.

2   DLN cells were prepared from WT mice 10 days after immunization and incubated with

3   the MOG peptide and IL-23 for 3 days. CD4 T cells were transferred intravenously into

4   naïve WT, SATB1cKO$_V$, or SATB1cKO$_L$ mice (n = 10). Representative results of three

5   independent experiments are shown as the mean EAE scores ± SD.

6

7   Figure 6

8   SATB1 is an essential factor for T cell responses during the effector phase of EAE.

9   (A) Encephalitogenic Th17 cells were prepared from DLN cells derived from

10   SATB1cKOe mice after immunization and stimulated with the MOG peptide and IL-23.

11   CD4 T cells were transferred into naïve WT mice, and *Satb1* was deleted by tamoxifen

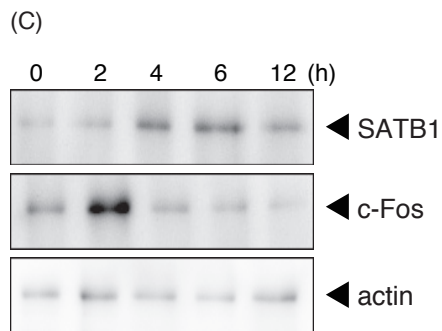12   injection. Clinical symptoms were monitored for 35 days. (B) SATB1cKOe mice were

13   immunized with the MOG peptide or OVA in CFA and administered tamoxifen on days

14   11 and 13 to delete *Satb1*. CD4 T cells were prepared from these mice on day 14 and

15   restimulated with the MOG peptide or OVA presented by irradiated splenocytes from

16   CD45.1 mice. Cytokine production and T cell proliferation were assessed. *, Values

17   were significantly different from those in control cells (SATB1-sufficient cells) under the

18   same conditions ($p < 0.05$). (C) IL-17$^+$ and IFNγ^+ CD4 T cells in (B) were analyzed by

19   intracellular staining for cytokines and flow cytometry. (D) CD4 T cells from

20   SATB1cKOe-OTII mice were labeled with Cell Proliferation Dye eFluor 670 and were

21   transferred into RAG2$^{-/-}$ mice. Recipient mice were treated with tamoxifen and then

22   injected with OVA. T cell proliferation was assessed via eFluor 670 dilution in treated

23   (right) versus untreated (left) mice. Plot areas gated on OVA 323-339 tetramer$^+$ CD4$^+$.

24

25   Figure 7

26   Encephalitogenic gene expression in Th17 cells not expressing SATB1.

1 To prepare Th17 cells, DLN cells (CD45.2) from MOG immunized SATB1cKOe mice

2 were skewed using the MOG peptide and IL-23 and were transferred into recipient mice

3 (CD45.1). These mice were treated with or without tamoxifen on days 1, 3, and 5 after

4 the transfer. On day 7, CD4$^+$ CD45.2$^+$ transferred donor T cells were sorted from the

5 spleen and lymph nodes of recipient mice using a FACS Aria III cell sorter. (A)

6 Quantitative PCR was used to measure the mRNA expression of *Il17A*, *Ifng*, *Rorc*, *Il23r*,

7 *Satb1*, and *Foxp3* in donor CD4 T cells. *Values were significantly different from those

8 in control cells (SATB1-sufficient Th17 cells) under the same conditions ($p < 0.05$).

9 Experiments were conducted three times, with essentially similar results. (B) CD45.2$^+$

10 CD4$^+$ Th17 cells from SATB1-deficient mice (with tamoxifen) or SATB1-sufficient mice

11 (without tamoxifen), or control CD4 T cells form naïve C57BL/6 mice were assessd for

12 intracellular IL-17 or Foxp3 expression. The numbers in the square represent the

13 percentage of CD45.2$^+$ CD4$^+$ cells.

14

15 Figure 8

16 *Satb1* deletion in Th17 cells after the onset of EAE alleviates clinical symptoms in EAE

17 mice.

18 Encephalitogenic Th17 cells were prepared from DLN cells derived from SATB1cKOe

19 mice after immunization and stimulated with the MOG peptide and IL-23. CD4 T cells

20 were transferred into naïve WT mice, and *Satb1* was deleted by tamoxifen injection

21 after the onset of EAE. Clinical symptoms were monitored for 30 days.
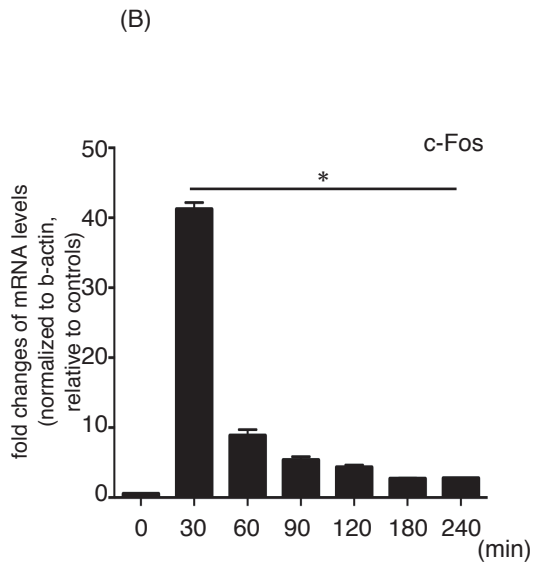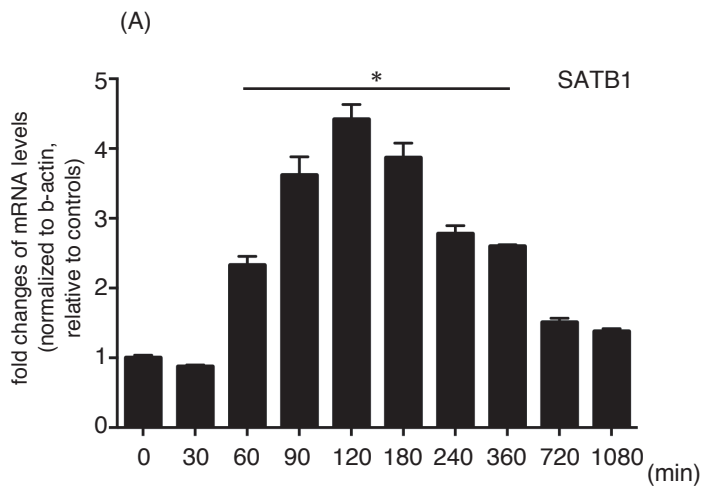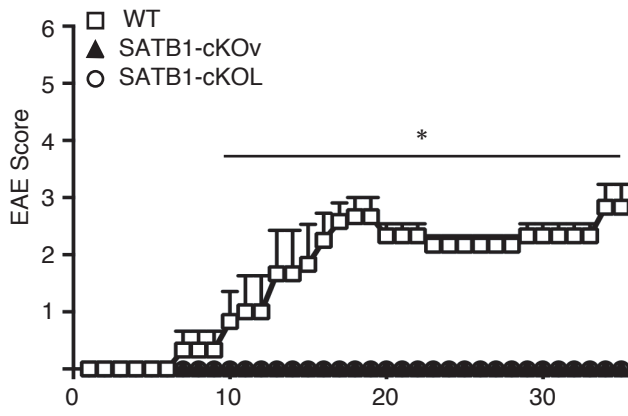
22

23

Figure 1

(A)

(B)

Wild type
(score 2)

SATB1-cKOv
(no symptom)

x 100

higher magnification

(C)

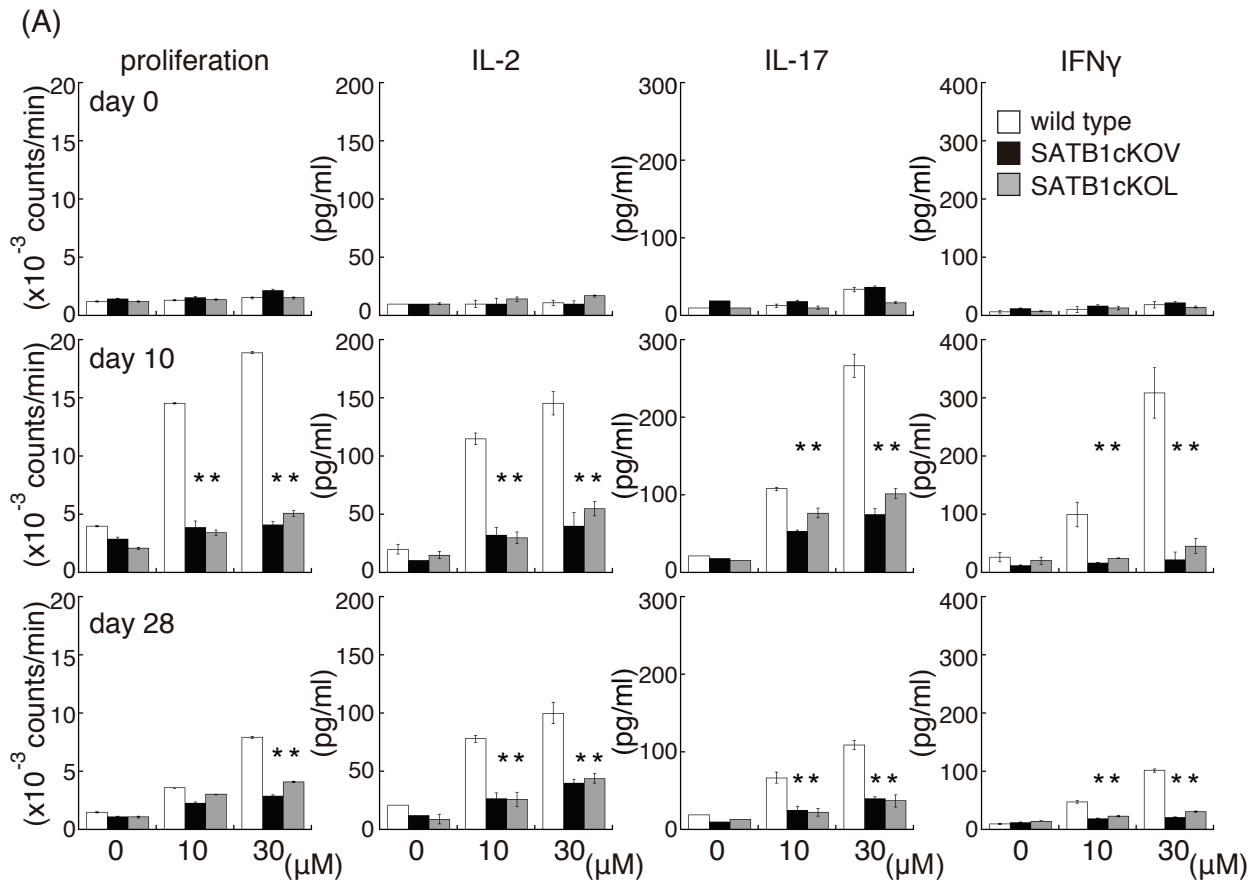|	0h	6h	12h	24h	48h	
cell number	1.00	3.45	11.3	15.5	18.8	wild type
	0.71	1.14	2.34	4.98	11.2	SATB1 cKOL

TUNEL positive
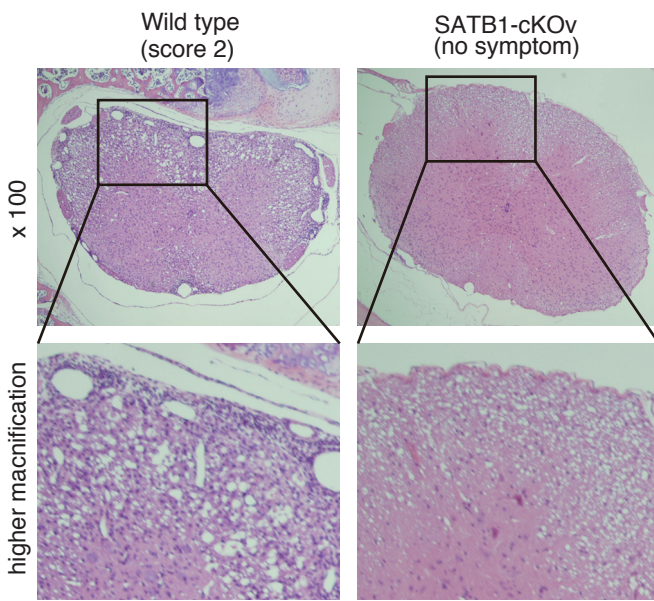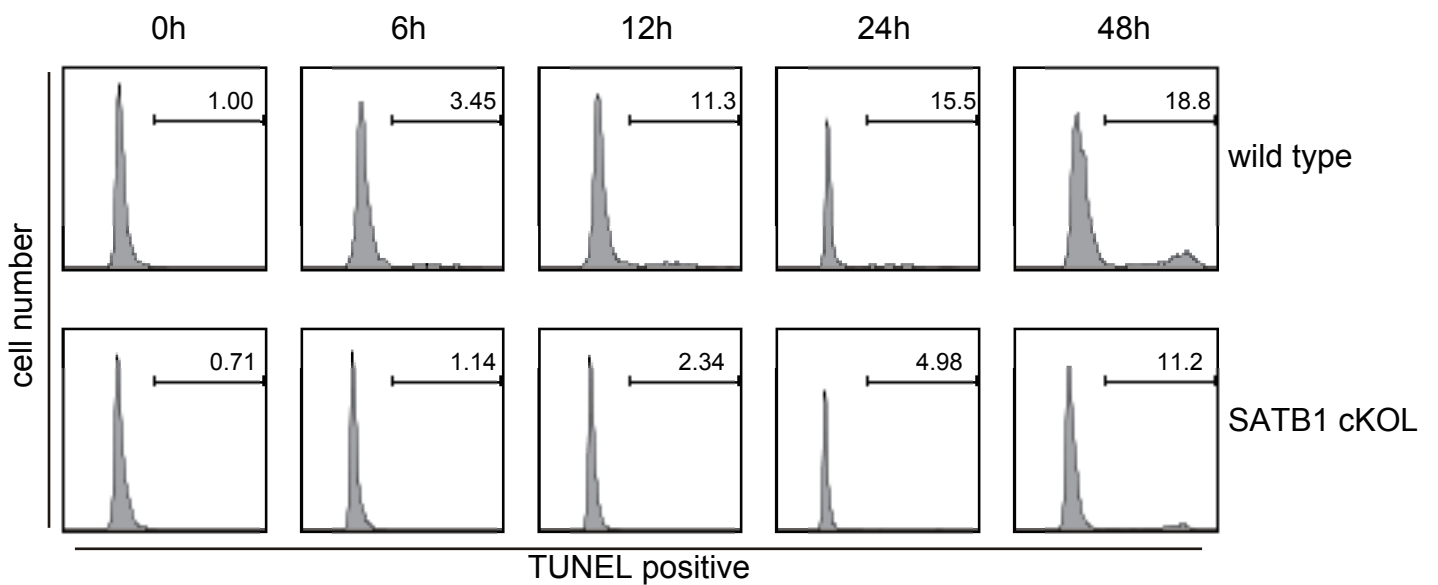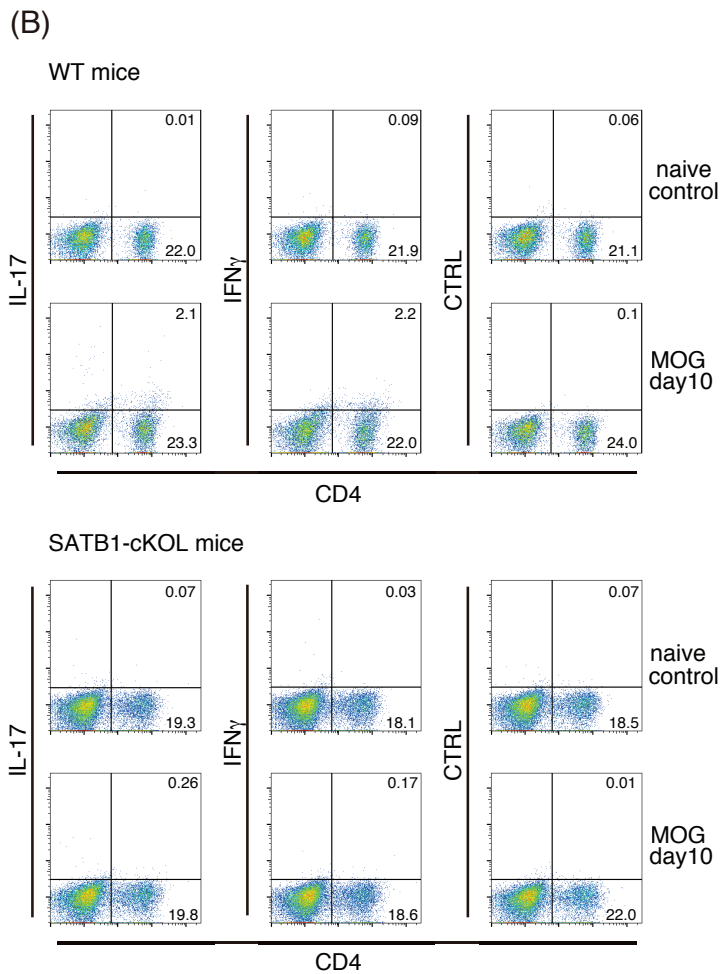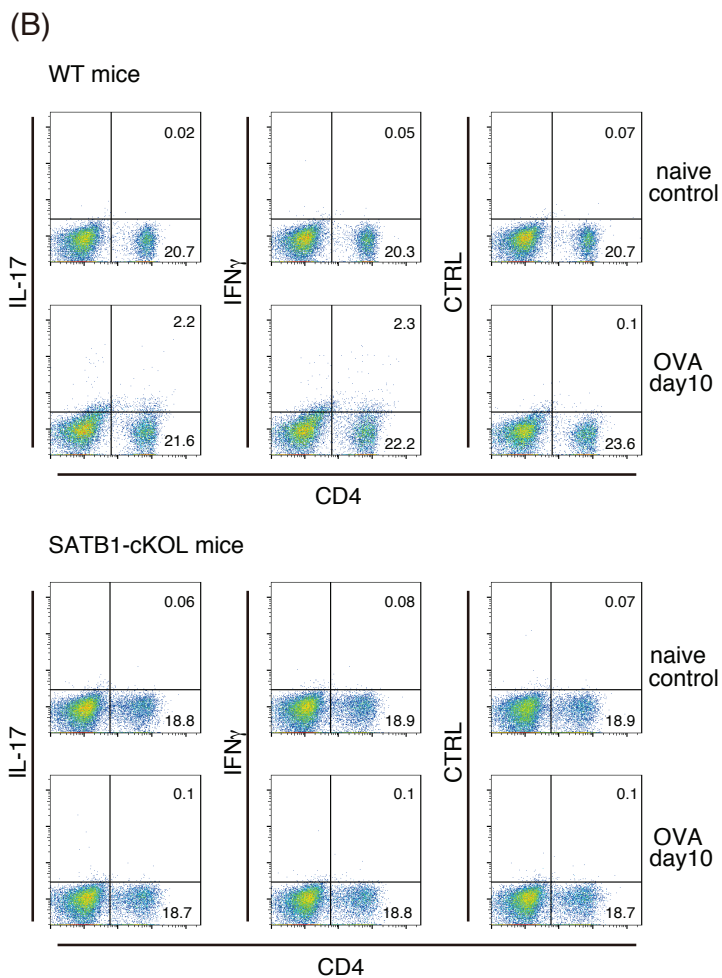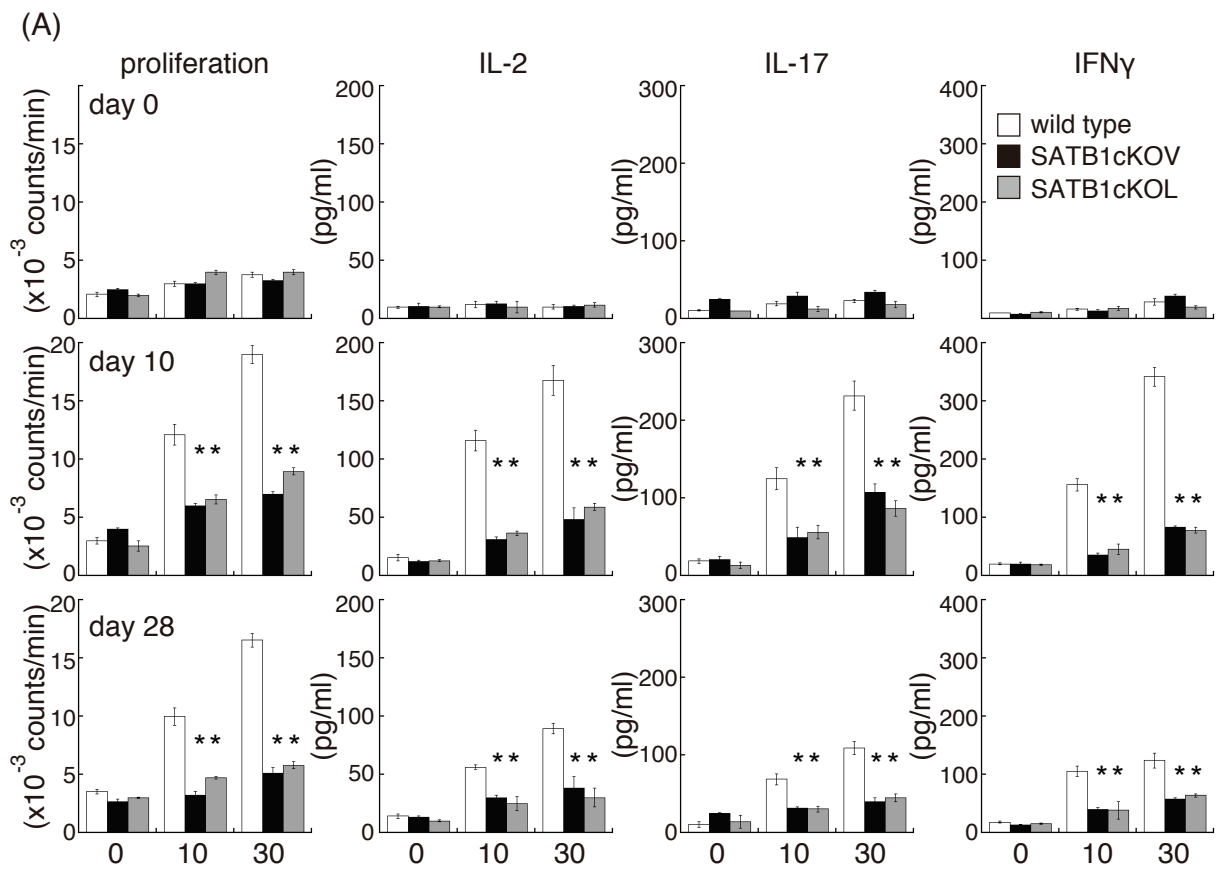
Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

(A)

Rorc

fold changes of mRNA levels (normalized to β-actin, relative to naive CD4 T cell controls)

40
30
20
10
0

Naive | − | +
tamoxifen

Il-23r

fold changes of mRNA levels (normalized to β-actin, relative to naive CD4 T cell controls)

20
15
10
5
0

Naive | − | +
tamoxifen

Foxp3

fold changes of mRNA levels (normalized to β-actin, relative to naive CD4 T cell controls)

1.5
1.0
0.5
0

Naive | − | +
tamoxifen

*

Il-17

fold changes of mRNA levels (normalized to β-actin, relative to naive CD4 T cell controls)

150
100
50
0

Naive | − | +
tamoxifen

*

SATB1

fold changes of mRNA levels (normalized to β-actin, relative to naive CD4 T cell controls)

2

1

0

Naive | − | +
tamoxifen

*
not detect

Ifn-gamma

fold changes of mRNA levels (normalized to β-actin, relative to naive CD4 T cell controls)

50
40
30
20
10
0

Naive | − | +
tamoxifen

*

(B)

Gated : CD45.2

Naive CD4 T (control) | - tamo (SATB1 suf) | + tamo (SATB1 def)

Foxp3
11.8 | 1.4 | 1.1

IL-17
0.11 | 10.6 | 0.25

IFNγ
0.13 | 10.3 | 0.23

CD4

Figure 7

Figure 8