TAK1 is indispensable for development of T cells and prevention of colitis by the generation of regulatory T cells

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Abstract
Transforming growth factor (TGF)-β-activating kinase 1 (TAK1) is critical for Toll-like receptor- and tumor necrosis factor-mediated cellular responses. In B cells, TAK1 is essential for the activation of mitogen-activated protein kinases (MAPKs), but not nuclear factor-κB (NF-κB), in antigen receptor signaling. In this study, we generate T cell-specific TAK1-deficient (LckCre+/Tak1flox/flox) mice and show that TAK1 is indispensable for the maintenance of peripheral CD4 and CD8 T cells. In thymocytes, TAK1 is essential for TCR-mediated activation of both NF-κB and MAPKs. Additionally, LckCre+/Tak1flox/flox mice developed colitis as they aged. In these mice, accumulations of activated/memory T cells as well as B cells were observed. Development of regulatory T (Treg) cells in thymus was abrogated in LckCre+/Tak1flox/flox mice, suggesting that the loss of Treg cells is the cause of the disease. Together, the results show that TAK1, by controlling the generation of central Treg cells, is important for preventing spontaneously developing colitis.

Introduction
Transforming growth factor (TGF)-β-activating kinase 1 (TAK1) is a serine/threonine kinase that belongs to the mitogen-activated protein kinase kinase family and plays crucial roles in signaling pathways initiated by IL-1β receptor (IL-1R), tumor necrosis factor-α receptor (TNFR) and Toll-like receptors (TLRs) (1, 2). IL-1β/TLR ligands and TNF stimulations lead to the rapid recruitment of TAK1 to TNFR-associated factor (TRAF)-6 and TRAF2, respectively, and TAK1 phosphorylates IκB kinase (IKK)-β and MKK6 (3). Activated IKK complex in turn phosphorylates IκB to initiate its degradation, and freed nuclear factor-κB (NF-κB) translocates into the nucleus and induces the expression of genes involved in inflammation (4). On the other hand, phosphorylation of MKK6 leads to the activation of mitogen-activated protein kinases (MAPKs) including c-Jun N-terminal kinase (JNK) and p38 (5, 6). TAK1-deficient cells fail to activate NF-κB and MAPK in response to these ligands, indicating that TAK1 is essential for the activation of signaling pathways in response to these stimuli (1, 2).

In addition to receptors belonging to the IL-1R/TLR and TNF superfamily, TAK1 is also involved in antigen receptor signaling in B lymphocytes (1). B cell receptor (BCR) stimulation triggers the rapid activation of protein kinase C (PKC)-β, and it transduces signals via a complex composed of CARD11, Bcl10 and MALT1, leading to the activation of the IKK complex (7). Although TAK1-deficient B cells develop normally, except for B-1 B cells, BCR-mediated proliferation is severely impaired in TAK1-deficient B cells (1). Interestingly, TAK1 is recruited to the Bcl10 complex and controls the activation of JNK, but not NF-κB, in BCR signaling (1).

TCR signaling utilizes mechanisms similar to those of BCR. The importance of NF-κB in T cell development and TCR signaling has been extensively studied. TCR signaling also activates the IKK complex via a complex of Bcl10, CARD11 and MALT1 downstream of PKCθ (7). In contrast to B cells, it has been shown that in vitro RNA interference-mediated knockdown of TAK1 in Jurkat cells results in diminished NF-κB
activation in TCR signaling (8). However, the role of TAK1 in T cells in vivo has yet to be clarified.

In the present study, we show that TAK1 plays indispensable roles in T cell development and the maintenance of tolerance by establishing and using T cell-specific TAK1-deficient (Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox}) mice. In contrast to BCR signaling, TCR-mediated NF-κB activation was dependent on TAK1. Surprisingly, T cell-specific TAK1-deficient mice developed severe colitis as they aged. Peripheral T cells isolated from old Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox} mice showed an activated phenotype, which could account for the observed pathological phenotypes. Furthermore, thymocytes and peripheral T cells from young Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox} mice did not contain the population of regulatory T (Treg) cells. Our study uncovers novel physiological roles for TAK1 in T cell regulation and functioning.

Methods

**Generation of T cell-specific TAK1-deficient mice**

Tak1\textsuperscript{floxed/flox} mice were generated as previously described (1). Lck-Cre mice (9) were bred with Tak1\textsuperscript{floxed/flox} mice to generate mice carrying Lck-cre and floxed Tak1 (Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox}) genes. These mice were then mated with Tak1\textsuperscript{floxed/flox} mice. Offspring carrying Lck-cre and floxed Tak1 genes (Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox} or Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox}) were used for analysis.

**Southern blot analysis**

Southern blot analysis for the deletion of floxed Tak1 allele was performed as described previously (1).

**Immunoblot analysis**

Cells were lysed in a lysis buffer containing 0.5% Nonidet-P40, 150 mM NaCl, 20 mM Tris-Cl (pH 7.5), 1 mM EDTA and a protease inhibitor cocktail (Complete; Roche Diagnostics, Indianapolis, IN, USA). Lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membrane was blotted with the indicated antibodies, and visualized with an enhanced chemiluminescence system (Perkin Elmer Life Sciences, Boston, MA, USA). Anti-JNK, ERK and phospho-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-JNK, ERK and IκBα were from SantaCruz (Santa Cruz, CA, USA).

**Flow cytometry**

Single-cell suspensions were prepared from thymus, spleen and lymph node. Cells were stained with FITC-, PE-, PerCP- or allophycocyanin (APC)-conjugated antibodies (PharMingen), and then analyzed on a FACS Calibur (Becton Dickinson, Lincoln Park, NJ, USA). For Foxp3 staining, we used an APC-conjugated anti-Foxp3 and Foxp3 staining buffer set (eBioscience, San Diego, CA, USA), and followed the manufacturer’s protocols.

**Proliferation assay**

Thymocytes (2 × 10\textsuperscript{5}) were cultured in 96-well plates for 36 h with the indicated concentration of anti-CD3ε with or without 2 μg ml\textsuperscript{-1} of anti-CD28 (PharMingen). One microcurie of [\textsuperscript{3}H]-thymidine ([\textsuperscript{3}H]TdR) was pulsed for the last 12 h, and then [\textsuperscript{3}H]TdR uptake was measured in a β-scintillation counter (Packard).

**Cell viability**

Thymocytes were treated with the indicated concentration of recombinant mouse TNF (R&D Systems, Minneapolis, MN, USA) for 24 h. Cell viability was examined using Annexin V–Cy3 (BioVision, Mountain View, CA, USA) and FACS Calibur.

**Electrophoretic mobility shift assay**

Thymocytes (2 × 10\textsuperscript{5}) were treated with stimuli for the indicated periods. Nuclear extracts were purified from cells and incubated with a specific probe for the NF-κB DNA-binding site, electrophoresed and then visualized by autoradiography as described previously (1).

**Histopathological analysis**

Paraffin-embedded colon samples were sectioned and stained with hematoxylin and eosin. Transverse sections are shown in the figures. Inflammation was graded semi-quantitatively from 0 (no change) to 5 (most severe colitis) for each region of the colon (cecum, and ascending, transverse, and descending colon, and rectum). The summation of the score for each segment of the colon provides a total disease score per mouse (0–25). The scoring was performed in a blinded manner by two independent investigators.

**Statistical analysis**

Data are expressed as the mean ± SD. P-values were determined using the Student’s t-test.

Results and Discussion

**TAK1 is required for T cell maturation and homeostasis**

To investigate the functional role of TAK1 in T cells, we generated T cell-specific TAK1-deficient mice by mating floxed Tak1 (Tak1\textsuperscript{floxed/flox}) mice with transgenic mice expressing Cre under the control of the proximal Lck promoter (9). Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox} and Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox} mice were born at the expected Mendelian ratios, and young mice (4- to 12-weeks old) showed no gross defects in growth and survival in specific pathogen-free (SPF) conditions. Southern blot analysis showed almost complete Cre-mediated deletion of Tak1 in thymocytes from Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox} mice (Fig. 1A). We also checked the deletion of Tak1 by immunoblotting using lysate prepared from thymocytes. Although Cre-mediated deletion led to the production of a truncated/mutated TAK1 lacking the ability to activate NF-κB and AP-1 (1), intact TAK1 disappeared in Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox} thymocytes (Fig. 1B).

First, we analyzed whether T cell-specific TAK1 deficiency affected T cell development. Flow cytometric analysis of thymocyte populations in Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox} mice revealed that single-positive (SP) cells, especially CD8 SP thymocytes, were markedly reduced (Fig. 1C and Table 1), although the population of CD4\textsuperscript{+}CD8\textsuperscript{+} double-positive (DP) thymocytes was not altered between the control and Lck\textsuperscript{Cre+} × Tak1\textsuperscript{floxed/flox} mice. The double-negative (DN) compartment was further
analyzed for the expression of CD44 and CD25, which can define four subsets of DN thymocytes (10). There were no differences in the DN1 to DN4 subsets between control Lck<sup>Cre/+</sup> Tak<sup>flox/+</sup> and Lck<sup>Cre/+</sup> Tak<sup>flox/flox</sup> mice (Fig. 1C). It has been reported that mature CD8 SP cells progressively down-regulate HSA (or CD24) surface expression when they go through their final maturation steps in the thymus (11). In both CD8 SP and CD4 SP cells in Lck<sup>Cre/+</sup> Tak<sup>flox/flox</sup> mice, we found a severe reduction of HSA<sup>low</sup> mature cells, whereas the number of HSA<sup>high</sup> immature cells was not affected (Fig. 1D). These results suggest that, in Lck<sup>Cre/+</sup> Tak<sup>flox/flox</sup> mice, SP thymocytes progressively disappear during their final maturation steps in the thymus. An analysis of splenocytes and lymph node cells showed that peripheral T cells were remarkably

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**Fig. 1.** T cell development in Lck<sup>Cre/+</sup> Tak<sup>flox/flox</sup> mice. (A) Southern blot analysis of genomic DNA from thymocytes for deletion of the floxed Tak1 allele. (B) Immunoblot analysis of thymocytes from Lck<sup>Cre/+</sup> Tak<sup>flox/+</sup> and Lck<sup>Cre/+</sup> Tak<sup>flox/flox</sup> mice. Cells were lysed and immunoprecipitated with anti-TAK1. Then immunoprecipitants were subjected to SDS-PAGE and immunoblotted with the anti-TAK1. H.C., heavy chain of antibody. (C–F) Flow cytometric analysis of T cell development in the thymus (C and D), spleen (E) and lymph node (F) from Lck<sup>Cre/+</sup> Tak<sup>flox/+</sup> and Lck<sup>Cre/+</sup> Tak<sup>flox/flox</sup> mice. Cells from 8-week-old mice were stained with the indicated antibodies. Percentages of cells within each quadrant are shown (C, E and F). The panels represent histograms of control cells (black line) and Lck<sup>Cre/+</sup> Tak<sup>flox/flox</sup> cells (filled line) (D). Results are representative of four different experiments.

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**Table 1.** Number of T and B cell subsets in 8-week-old control and Lck<sup>Cre/+</sup> Tak<sup>flox/flox</sup> mice

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<th>Thymocytes (× 10&lt;sup&gt;5&lt;/sup&gt;)</th>
<th>Splenocytes (× 10&lt;sup&gt;5&lt;/sup&gt;)</th>
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<td></td>
<td>Lck&lt;sup&gt;Cre/+&lt;/sup&gt; Tak&lt;sup&gt;flox/+&lt;/sup&gt;</td>
<td>Lck&lt;sup&gt;Cre/+&lt;/sup&gt; Tak&lt;sup&gt;flox/flox&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>750.8 ± 421.6</td>
<td>731.8 ± 299.4</td>
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<tr>
<td>DP</td>
<td>655.4 ± 354.1</td>
<td>688.8 ± 285.1</td>
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<tr>
<td>CD4 SP</td>
<td>69.2 ± 49.4</td>
<td>30.2 ± 13.2</td>
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<tr>
<td>CD8 SP</td>
<td>14.4 ± 16.6</td>
<td>2.6 ± 1.2</td>
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<tr>
<td>DN</td>
<td>11.9 ± 9.5</td>
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TAK1 is essential for TCR-mediated proliferation and survival after TNF stimulation

Since there were few peripheral T cells in Lck\textsuperscript{Cre/+} Tak\textsuperscript{flox/flox} mice, we examined T cell responses against TCR stimulation using thymocytes. As shown in Fig. 2(A), thymocytes prepared from Lck\textsuperscript{Cre/+} Tak\textsuperscript{flox/+} mice poorly proliferated in response to activation of anti-CD3 compared with control thymocytes, even in the presence of the co-stimulatory anti-CD28. We have previously shown that TAK1 is essential for the survival of fibroblasts in response to TNF stimulation by controlling NF-κB (1). We also investigated the viability of thymocytes in response to TNF. While thymocytes prepared from Lck\textsuperscript{Cre/+} Tak\textsuperscript{flox/+} mice were viable after TNF stimulation, this stimulation induced cell death in thymocytes from Lck\textsuperscript{Cre/+} Tak\textsuperscript{flox/flox} mice in a dose-dependent manner (Fig. 2B), indicating that anti-, but not pro-, apoptotic signal induced by TNF is diminished in Lck\textsuperscript{Cre/+} Tak\textsuperscript{flox/flox} mice. Taken together, these results indicate that TAK1 is required for TCR- and TNF-mediated cellular responses in T cells.

TCR-mediated activation of NF-κB and MAPKs is abrogated in Lck\textsuperscript{Cre/+} Tak\textsuperscript{flox/flox} thymocytes

We next examined the activation of signaling molecules. With TCR cross-linking or phorbol ester/calcium ionophore (PMA/iono) stimulation, JNK and ERK were activated in thymocytes prepared from control mice (Fig. 3A). In sharp contrast, activation of JNK, but not ERK, was abrogated in TAK1-deficient thymocytes. Then we examined activation of NF-κB in response to TCR or PMA/iono stimulation. TCR-mediated IκBα phosphorylation or PMA/iono-induced IκBα degradation was severely impaired in TAK1-deficient thymocytes (Fig. 3B). In accord with this, the induction of DNA–NF-κB binding activity was also impaired in TAK1-deficient thymocytes compared with control cells (Fig. 3C). These results indicate that TAK1 is indispensable for the activation of both NF-κB and JNK in TCR signaling.

Inflammatory bowel diseases in Lck\textsuperscript{Cre/+} Tak\textsuperscript{flox/flox} mice

Lck\textsuperscript{Cre/+} Tak\textsuperscript{flox/flox} mice appeared normal and healthy in the SPF facility until 3 months of age; however, we found that most Lck\textsuperscript{Cre/+} Tak\textsuperscript{flox/flox} mice began showing wasting and severe diarrhea from 4 to 6 months of age. These old Lck\textsuperscript{Cre/+}...
Tak1<sup>flox/flox</sup> mice also showed stiff colon, rectal prolapse and enlarged spleen and lymph nodes compared with littermate control mice. Upon histological examination of tissues from these mice, we observed prominent ulceration, loss of goblet cells in the epithelial layer and mononuclear cell infiltration of the colon (Fig. 4A and B). The small intestine was never affected (data not shown), and the littermate control mice showed no or only a few symptoms and histological change in their intestine.

Along with splenomegaly, total cell numbers of splenocytes were increased in old <sup>Lck</sup><sup>Cre/+</sup> Tak1<sup>flox/flox</sup> mice, and the percentage of myeloid lineage cells such as macrophages and neutrophils was up-regulated (data not shown). In addition, the percentage of peripheral T cells in old <sup>Lck</sup><sup>Cre/+</sup> Tak1<sup>flox/flox</sup> mice seemed to be slightly higher than that in young <sup>Lck</sup><sup>Cre/+</sup> Tak1<sup>flox/flox</sup> mice (data not shown). Flow cytometric analysis of the expression of CD44 and CD62L revealed that there were almost no CD62L<sup>high</sup>CD44<sup>low</sup> naive T cells remaining in the spleen and lymph nodes of old <sup>Lck</sup><sup>Cre/+</sup> Tak1<sup>flox/flox</sup> mice (Fig. 4C). Given that >90% of the peripheral T cells of <sup>Lck</sup><sup>Cre/+</sup> Tak1<sup>flox/flox</sup> mice were CD44<sup>high</sup>, most of these cells were considered as activated/memory T cells. To assess the Cre-mediated deletion of Tak1 in peripheral T cells of old mice, CD3<sup>+</sup> cells or B220<sup>+</sup> cells were sorted from spleen of control and <sup>Lck</sup><sup>Cre/+</sup> Tak1<sup>flox/flox</sup> mice. Southern blot analysis showed that there was no Δ allele in T cells from old <sup>Lck</sup><sup>Cre/+</sup> Tak1<sup>flox/flox</sup> mice, but there was in old <sup>Lck</sup><sup>Cre/+</sup> Tak1<sup>flox/+</sup> mice (Fig. 4D), indicating that the remained and/or up-regulated peripheral T cells were 'leaked' cells, which had had escaped from Cre-mediated deletion. These results suggest that TAK1 plays a critical role in preventing the development of colitis by controlling homeostasis of T cells.

**Role of Treg cells in the development of colitis in <sup>Lck</sup><sup>Cre/+</sup> Tak1<sup>flox/flox</sup> mice**

The development of autoimmune bowel disease is sometimes caused by the lack of suppression in immune responses. For instance, mice deficient in IL-2, IL-2RB or TGF-β1 and transgenic mice expressing dominant-negative TGFβRII spontaneously develop colitis with massive enlargement of the spleen and lymph nodes because of defects in the maintenance of Treg cells (12–19). These cells have been shown to play a central role in the maintenance of immune tolerance and the termination of immune responses (20–22). A subset of Treg cells develops intrathymically under the control of the specific transcription factor, Foxp3 (20–22). To investigate the role of TAK1 in the development of Treg cells, we...
checked the population of CD4+CD25+Foxp3+ T cells in LckCre+/+Takflox/+ mice. Thymocytes and splenocytes were prepared from 6-week-old LckCre+/+Takflox/+ and LckCre+/+Takflox/flox mice, stained with appropriate antibodies and then analyzed by flow cytometry. While Treg cells substantially existed in both thymus and spleen of control mice, these cells were not detectable in both organs of LckCre+/+Takflox/flox mice (Fig. 5A). Furthermore, the expression level of CD45RB in CD4 SP thymocytes of LckCre+/+Takflox/flox mice was similar to that of control mice (Fig. 5B). These results suggest that TAK1 is indispensable for development of thymocytes to Treg precursor cells, but not to CD4+CD45RBhigh cells, which are capable of inducing colitis when injected in SCID mice (23).

In summary, we showed that TAK1 is critical for the maintenance of mature T cells, and also for the prevention of spontaneous colitis. Furthermore, TAK1-deficient thymocytes showed impaired TCR-mediated responses, activation of NF-κB and MAPKs. The mice also showed severe defects in the maintenance of mature CD4 SP and CD8 SP thymocytes and of peripheral T cells. Nevertheless, the numbers of DN and DP thymocytes were not altered in LckCre+/+Takflox/flox mice. It is assumed that LckCre+/+-mediated deletion is initiated at the onset of CD44 in earlier DN stages of thymocyte development, suggesting that TAK1 is not critical for the development and maintenance of DP cells.

Involvement of TAK1 in the maintenance of SP thymocytes and peripheral T cells reminds us of the role of the components of the IKK complex in T cells. For instance, it is reported that T cell-specific deletion of NEMO/IKKγ or the expression of IKK-β without kinase activity results in the diminished generation of peripheral CD4+ and CD8+ T cells (24). These results suggest that TAK1 regulates appropriate T cell development by activating the NF-κB pathway. Whereas IKK activity is also essential for the maintenance of mature B cells, we have shown that B cells do not require TAK1 for their development and maintenance, except for peritoneal B-1 B cells (1). These results indicate that the requirement for TAK1 in the activation of IKK complex is determined in a cell type-specific fashion.

Unexpectedly, T cell-specific TAK1-deficient mice spontaneously developed colitis as they aged. Although TAK1-deficient thymocytes were hyporeactive to TCR stimulation, old T cell-specific TAK1-deficient mice showed splenomegaly, lymphadenopathy with full of B cells and a high percentage of activated/memory T cells. Further analysis of thymocytes revealed that intrathymic development of CD4+CD25+Foxp3+ Treg cells was totally dependent on TAK1. Since it has been reported that the expression of Foxp3 in CD4 SP thymocytes is mostly limited to HASAlow population (25), it may be possible that the defect in the development of Treg cells correlates with blockade in the maturation of SP thymocytes. Although cytokines such as IL-2 and TGF-β1 are critical for the maintenance of Treg cells, they were shown to be dispensable for the thymic development of natural Treg cells (26). The mechanisms for thymic development of natural Treg cells are not currently well understood. It has been shown that Bcl10 and PKCδ are critical for the development of thymic Treg cells (27), suggesting that TCR-signaling pathway leading to the activation of NF-κB is critical for natural Treg cells development.

Although LckCre+/+Takflox/flox mice also showed a defect in the maintenance of naive peripheral T cells, thymic SP cells appeared and the defect was more profound in the development of natural Treg cells. Therefore, we assume that the balance between effector and Treg cells is perturbed in the absence of TAK1, and that it is responsible for the spontaneously developed colitis. However, further studies are required to clarify the precise mechanisms for the development of colitis under conditions of TAK1 deficiency.

During the preparation of the manuscript, the generation of Cd4-Cre: Takflox/flox mice, in which TAK1 is deleted in T cells, was reported (28). In that study, development of any diseases was not reported, although the T cell maintenance was similarly defective. The difference may be because of the difference in the promoters regulating Cre expression. The Lck promoter induces expression of the gene early in T cell development, compared with the Cd4 promoter, which starts to express DP stage of thymocytes. Our mouse model appears quite useful for investigating the mechanisms of autoimmune
colitis. Future studies should clarify the detailed mechanisms of how TAK1 controls the development of various subsets of T cells and maintains immune tolerance.

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Abbreviations

APC: allophyococytin
BCR: B cell receptor
DN: double negative
SP: specific pathogen free
TAK1: transforming growth factor-β-activating kinase 1
TGF: transforming growth factor
TLR: Toll-like receptor
TNF: tumor necrosis factor
TRAF: TNFR-associated factor
Treg: regulatory T

References