

Regulation of T helper type 2 cell differentiation by murine Schnurri-2

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Schnurri (Shn) is a large zinc finger protein implicated in cell growth, signal transduction, and lymphocyte development. Vertebrates possess at least three Shn orthologues (Shn-1, Shn-2, and Shn-3), which appear to act within the bone morphogenetic protein, transforming growth factor β , and activin signaling pathways. However, the physiological functions of the Shn proteins remain largely unknown. In *Shn-2*-deficient mice, mature peripheral T cells exhibited normal anti-T cell receptor-induced proliferation, although there was dramatic enhancement in the differentiation into T helper type (Th)2 cells and a marginal effect on Th1 cell differentiation. *Shn-2*-deficient developing Th2 cells showed constitutive activation of nuclear factor κ B (NF- κ B) and enhanced GATA3 induction. *Shn-2* was able to compete with p50 NF- κ B for binding to a consensus NF- κ B motif and inhibit NF- κ B-driven promoter activity. Thus, *Shn-2* plays a crucial role in the control of Th2 cell differentiation by regulating NF- κ B function.

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Abbreviations used: BMDC, bone marrow-derived DC; CFSE, carboxyfluorescein diacetate succinimidyl ester; EMSA, electrophoretic mobility shift assay; Shn, Schnurri; Tg, transgenic; TRAF, TNF receptor-associated factor.

CD4 T cell-dependent immune responses are controlled by the balance of antigen-specific Th1 and Th2 cells (1–3). IL-12-induced activation of STAT4 is crucial for Th1 cell differentiation, whereas IL-4-induced STAT6 activation is crucial for Th2 cell differentiation (4–8). In addition to cytokine-induced signals, the activation of the TCR-mediated signaling is indispensable for both Th1 and Th2 cell differentiation. In particular, Th2 cell differentiation is largely dependent on the activation of p56^{lck}, calcineurin, and the Ras-ERK MAPK signaling cascade (9–11). A negative regulator of the above signaling pathways, SHP-1, also controls the efficiency of Th2 cell differentiation and Th2 cell-dependent immune responses (12). Master transcription factors for Th1 and Th2 cell differentiation have been revealed, i.e., GATA3 for Th2 cells and T-bet for Th1 cells (13–17).

In *Drosophila*, Mad-Medea and Schnurri (Shn), a large zinc finger protein, are reported to interact with each other and act as nuclear targets in the *Drosophila* decapentaplegic signaling pathway (18–20). In vertebrates, the *Drosophila* decapentaplegic signaling pathway

may equate to the bone morphogenetic protein/TGF- β /activin signaling pathways that play diverse roles in developmental processes (21). Vertebrates have at least three orthologues of Shn: Shn-1 (also known as HIV-EP1, MBP-1, PRDII-BF1, and α A-CRYBP1), Shn-2 (also known as HIV-EP2, MBP-2, AGIE-BP1, and MIBP1), and Shn-3 (also known as HIV-EP3, KRC, and ZAS3). The mRNA expression of Shn-2 was detected mostly in the brain, heart, and spleen (22–24). Recently, the requirement of Shn-2 in positive selection of thymocytes was reported (25) and *Shn-3*-deficient CD4⁺ CD8⁺ thymocytes were shown to exhibit a defect in cell survival (26). However, the precise physiological roles of these Shn family members remain largely unknown.

NF- κ B is a critical transcription factor that regulates Th2 cell differentiation and Th2 cell-dependent airway inflammation (27–29). NF- κ B-deficient (p50 subunit-deficient) mice were unable to mount OVA-induced airway inflammation (28). The lack of inflammation was not due to defects in T cell priming, T cell proliferation, or expression of important cell adhesion molecules, but rather to the impaired induction of GATA3 (30). Recently, one of the Shn family members, Shn-3 (KRC), was

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reported to associate with TNF receptor-associated factor (TRAF)2 to repress nuclear translocation of NF- κ B (31). In addition, Shn-3 (ZAS3) was shown to bind to the NF- κ B

motif directly, and the competition with NF- κ B binding resulted in the repression of transactivation of the NF- κ B target gene (32, 33). A direct repressive activity of Shn-3 was

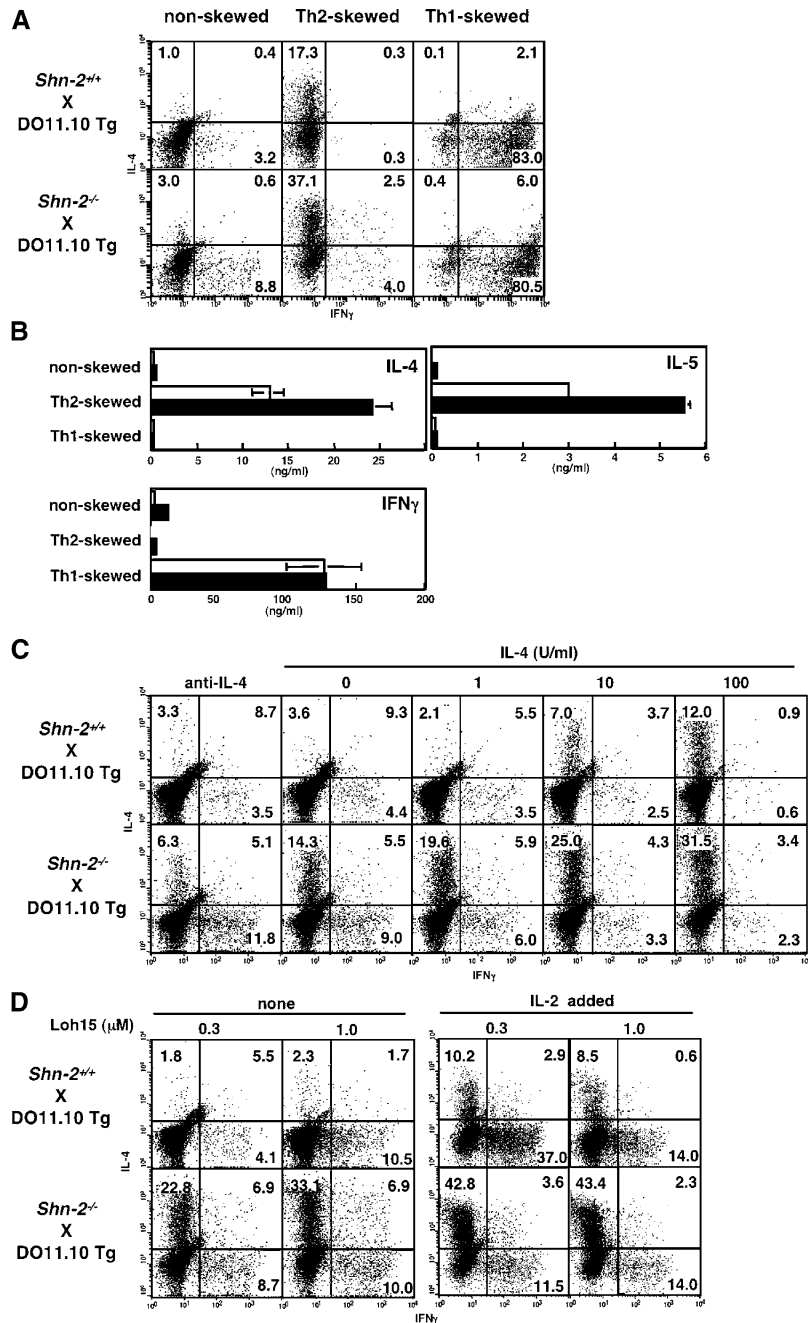


Figure 1. Enhanced Th2 cell differentiation in *Shn-2*^{-/-} × DO11.10 TCR Tg T cells. (A) Naive (CD44^{low}) CD4 T cells from *Shn-2*^{-/-} × DO11.10 Tg mice were purified by cell sorting and stimulated with antigenic OVA peptide (Loh15: 0.1 μ M) and irradiated BALB/c APCs for 5 d. Th2 cell-skewed (IL-4 with anti-IL-12 mAb and anti-IFN- γ mAb), Th1 cell-skewed (IL-12 with anti-IL-4 mAb), and non-skewed (IL-2 with anti-IL-4 mAb, anti-IL-12 mAb, and anti-IFN- γ mAb) conditions were used. Intracellular staining was performed with FITC-conjugated anti-IFN- γ mAb and PE-conjugated anti-IL-4 mAb. Under the typical Th2 cell-skewed conditions, the levels of Th2 cell differentiation in five experiments were 17.5 \pm 2.9% in *Shn-2*^{+/+} cultures and 38.0 \pm 11.8% in *Shn-2*^{-/-} cultures. P < 0.006. (B) A portion of

the same differentiated cell cultures used in A were restimulated with antigenic peptide and APCs for 24 h, and the concentrations of cytokines (IL-4, IL-5, and IFN- γ) in the culture supernatant were determined by ELISA. (C) Naive (CD44^{low}) CD4 T cells from *Shn-2*^{-/-} × DO11.10 Tg mice were stimulated with a specific OVA peptide (Loh15: 0.1 μ M) and irradiated normal BALB/c APCs in the presence of indicated doses of IL-4. Intracellular staining profiles of IFN- γ and IL-4 are shown with percentages of cells in each area. (D) Purified naive (CD44^{low}) CD4 T cells were stimulated with indicated doses of specific OVA peptides (Loh15: 0.3 and 1.0 μ M) and irradiated BALB/c APCs in the presence or absence of 30 U/ml of exogenous IL-2. Two independent experiments were performed with similar results.

also demonstrated (33). These emerging findings suggest that the Shn protein is involved in NF- κ B activation and/or NF- κ B-mediated gene transactivation.

Here, we investigate the role of Shn-2 in Th1/Th2 cell differentiation by using Shn-2-deficient (*Shn-2*^{-/-}) mice. Our results suggest that Shn-2 plays crucial roles in the control of Th2 cell differentiation by regulating NF- κ B activation and GATA3 expression.

RESULTS

Phenotypic and functional characterization of peripheral CD4 T cells in *Shn-2*-deficient (*Shn-2*^{-/-}) mice

Shn-2^{-/-} mice were previously shown to have a defect in T cell generation due to the impairment of positive thymic selection (25). However, we found that moderate numbers of CD4 and CD8 T cells were present in *Shn-2*^{-/-} mice of a BALB/c background (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20040733/DC1>). The cell surface expression of TCR- β , CD3 ϵ , common γ (γ C), IL-4R α , CD25, CD69, CD44, and CD62L on splenic CD4 T cells was found to be comparable to those of controls (Fig. S1 B). The TCR V β chain repertoire in the peripheral *Shn-2*^{-/-} T cells was similar to that of *Shn-2*^{+/+} T cells (Fig. S1 C). Anti-TCR- β - or anti-CD3 ϵ -induced proliferative responses were the same in *Shn-2*^{+/+} and *Shn-2*^{-/-} CD4 T cells (Fig. S2 A). Furthermore, the phosphorylation status of MAPKs (Erk1 and Erk2) after TCR cross-linking in *Shn-2*^{-/-} CD4 T cells was comparable to that of controls (Fig. S2 B). Thus, no obvious defect in the phenotype or the activation of *Shn-2*^{-/-} splenic CD4 T cells was noted. The expression of Shn-2 mRNA was detected in freshly prepared CD4 T cells, and it was decreased after anti-TCR stimulation in Th1 and Th2 cell differentiation cultures (Fig. S3).

Enhanced Th2 cell differentiation in *Shn-2*^{-/-} \times DO11.10 transgenic (Tg) naive CD4 T cells

Next, we assessed the capability of *Shn-2*^{-/-} CD4 T cells to differentiate into Th1/Th2 cells. Naive splenic CD4 T cells (CD4⁺ CD44^{low}) were purified by cell sorting (purity >98%) and then subjected to in vitro Th1/Th2 cell differentiation culture and analyzed by intracellular cytokine staining and ELISA. Naive CD4 T cells from *Shn-2*^{-/-} \times OVA-specific TCR- $\alpha\beta$ Tg (DO11.10 Tg) mice were stimulated with 0.1 μ M of antigenic OVA peptide and irradiated BALB/c splenocytes in the presence of appropriate cytokines and anti-cytokine mAbs under three different conditions: Th2-skewed (IL-4 with anti-IL-12 mAb and anti-IFN- γ mAb), Th1-skewed (IL-12 with anti-IL-4 mAb), and non-skewed (IL-2 with anti-IL-4 mAb, anti-IL-12 mAb, and anti-IFN- γ mAb) conditions. As shown in Fig. 1 A, Th2 cell differentiation was enhanced substantially in *Shn-2*^{-/-} T cells (17.3 vs. 37.1%), whereas Th1 cell differentiation was unaffected (83.0 vs. 80.5%). Under non-skewed conditions, the baseline levels of IL-4-producing and IFN- γ -producing cells were increased in *Shn-2*^{-/-} T cell cultures (Fig. 1 A, left). Portions of the same cell cul-

tures that were used for intracellular cytokine staining were restimulated, and cytokine production was measured by ELISA. Basically, a similar pattern among cultures was observed (Fig. 1 B). The production of IL-4 and IL-5 was increased about twofold in *Shn-2*^{-/-} Th2 cells, whereas similar levels of IFN- γ production in Th1 cells were detected. The levels of IL-4 and IFN- γ production in non-skewed cultures were very low, but they were slightly increased in the *Shn-2*^{-/-} cultures.

To examine further the effect on Th2 cell differentiation, naive CD4 T cells from *Shn-2*^{-/-} \times DO11.10 Tg mice were stimulated with a minimum dose of antigenic peptide (0.1 μ M) and APCs in the presence of graded doses of exogenous IL-4. As shown in Fig. 1 C, IL-4-producing Th2 cells were generated in an exogenous IL-4 dose-dependent manner in wild-type *Shn-2*^{+/+} T cell cultures. As expected, the generation of Th2 cells in *Shn-2*^{-/-} mice was dramatically enhanced at all doses of exogenous IL-4. The number of IL-5-producing cells was also enhanced in *Shn-2*^{-/-} T cell cultures (not depicted). Also, we detected increased numbers of IFN- γ -producing cells at all groups. Next, we examined the effect of the concentration of antigenic peptide and IL-2 in the culture. Naive CD4 T cells purified by cell sorting were stimulated with two different doses of antigenic peptide (0.3 or 1.0 μ M) and irradiated APCs from normal BALB/c mice in the absence of exogenous IL-4. A dramatic increase in the numbers of IL-4-producing Th2 cells was observed in *Shn-2*^{-/-} cultures at either dose of antigenic peptide (Fig. 1 D, left). In the presence of exogenous IL-2, Th2 cell generation was enhanced in both *Shn-2*^{+/+} and *Shn-2*^{-/-} T cell cultures, although the levels were substantially higher in *Shn-2*^{-/-} groups as compared with *Shn-2*^{+/+}.

Enhanced Th2 cell differentiation in *Shn-2*^{-/-} splenic CD4 T cells after anti-TCR mAb stimulation

To further assess the efficiency of Th2 cell differentiation in the splenic *Shn-2*^{-/-} T cells, purified splenic naive CD4 T cells (purity >98%) were stimulated with immobilized anti-TCR- β mAb in the presence of graded doses of exogenous IL-4. No APCs were added. 30 U/ml of exogenous IL-2 was added to the differentiation cultures. As shown in Fig. 2 A, IL-4-producing Th2 cells were generated in an exogenous IL-4 dose-dependent manner in the *Shn-2*^{+/+} wild-type T cell cultures, and as expected, the generation of Th2 cells in *Shn-2*^{-/-} mice was substantially enhanced in the cultures at all doses of exogenous IL-4 (Fig. 2 A). These results suggest that the ability to differentiate into Th2 cells was enhanced in *Shn-2*^{-/-} splenic CD4 T cells. The number of Th2 cells generated in *Shn-2*^{-/-} \times *STAT6*^{-/-} T cell cultures was insignificant, suggesting that the enhanced Th2 cell differentiation in *Shn-2*^{-/-} T cell cultures was STAT6 dependent (not depicted).

To assess the effect on Th1 cell differentiation in a non-TCR Tg system, *Shn-2*^{-/-} splenic CD4 T cells were stimulated with anti-TCR- β mAb in the presence of various doses of exogenous IL-12 and anti-IL-4 mAb (Fig. 2 B).

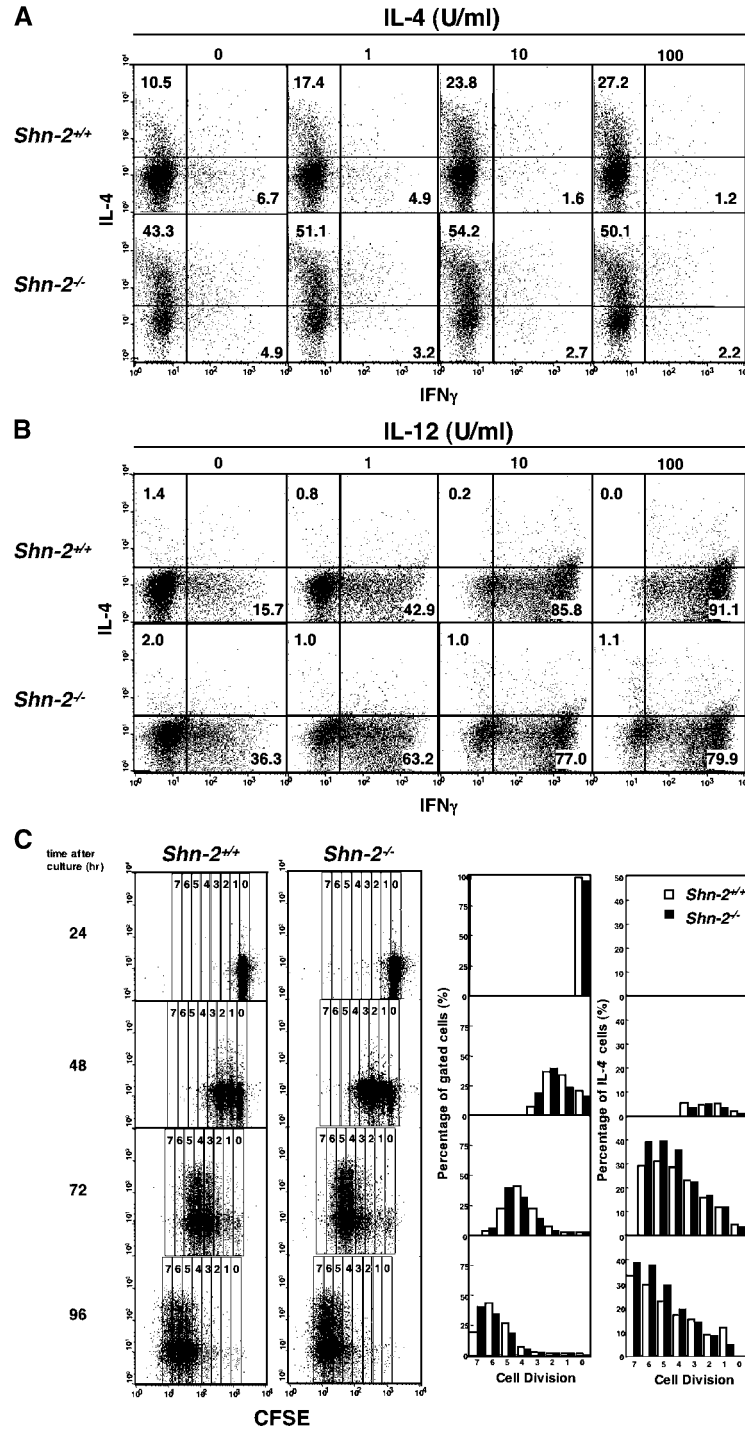


Figure 2. Enhanced Th2 cell differentiation in *Shn-2^{-/-}* T cells. (A) Splenic naive CD4 T cells from *Shn-2^{-/-}* mice were stimulated with immobilized anti-TCR- β mAb, 30 U/ml of exogenous IL-2, and indicated concentrations of IL-4 for 5 d. Intracellular staining profiles of IFN- γ and IL-4 are shown with percentages of cells in each area. The absolute numbers of cells harvested in these cultures were similar. Three independent experiments were performed with similar results. (B) Splenic naive CD4 T cells from *Shn-2^{-/-}* mice were stimulated with immobilized anti-TCR- β mAb in the presence of indicated doses of IL-12 and anti-IL-4 mAb. Intracellular staining profiles of IFN- γ and IL-4 are shown with percentages of cells in each area. The absolute numbers of cells harvested in these cultures were

similar. Three independent experiments were performed with similar results. (C) Splenic naive CD4 T cells were labeled with CFSE and stimulated with immobilized anti-TCR- β mAb in the presence of IL-4 and IL-2. After culturing for the indicated times, cells were restimulated and subjected to intracellular staining with APC-conjugated anti-IL-4 mAb. Percentages of the cells in the gates representing numbers of cell division (0–7) and percentages of IL-4-producing Th2 cells in each gate are shown in the right panels. Percentages of IL-4-producing cells in total (without gating) are 0.7% (24 h), 5.4% (48 h), 28.6% (72 h), and 29.3% (96 h) in *Shn-2^{+/+}* cultures and 0.6% (24 h), 5.4% (48 h), 36.9% (72 h), and 37.9% (96 h) in *Shn-2^{-/-}* cells. Two independent experiments were performed with similar results.

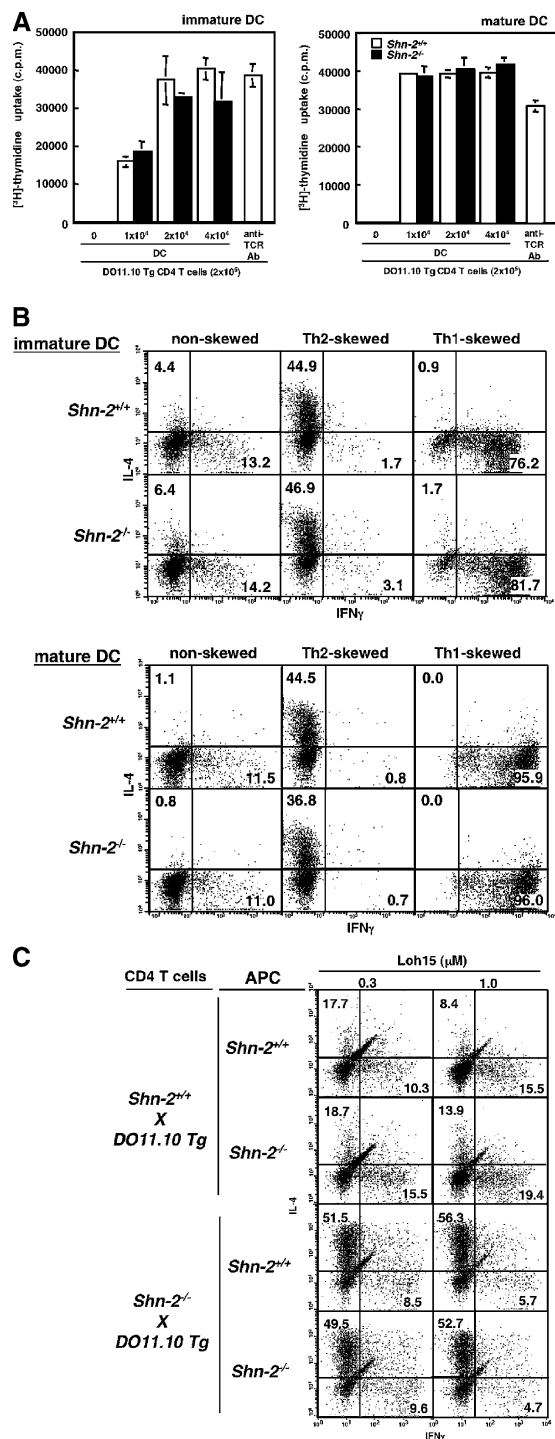


Figure 3. The function of *Shn-2*^{-/-} APCs. (A) OVA-pulsed immature or mature BMDCs were cocultured with DO11.10 Tg CD4 T cells for 72 h. [³H]thymidine (37 kBq/well) was added to the stimulation culture for the last 16 h. The results (mean and standard deviation) of [³H]thymidine incorporation are shown. Three independent experiments were performed with similar results. (B) OVA-pulsed immature or mature BMDCs (2×10^4 cells) were cocultured with DO11.10 Tg naive (CD44^{low}) CD4 T cells (2×10^5 cells) under nonskewed, Th2 cell-skewed, and Th1 cell-skewed conditions for 5 d. Intracellular staining profiles (IFN- γ /IL-4) of the cultured cells are shown with percentages of the cells in each quadrant. Three independent experiments were performed with similar results. (C) Naive (CD44^{low}) CD4 T

Moderate increases in the generation of Th1 cells were observed in groups with no exogenous IL-12 or a low dose of IL-12 (1 U/ml). However, no further increases but rather a slight decrease in the number of Th1 cells generated was observed at higher doses of IL-12 (10 or 100 U/ml). A similar pattern was obtained in STAT6-deficient Th1 cell cultures (not depicted). We observed similar enhancement in the generation of Th2 cells in *Shn-2*^{-/-} mice with either a B6 background or a B6 \times BALB/c background (not depicted).

Anti-TCR-induced cell division of *Shn-2*^{-/-} CD4 T cells

Because some cycles of cell divisions are reported to be required for the generation of IL-4-producing Th2 cells (34), we examined the anti-TCR-induced cell division of *Shn-2*^{-/-} CD4 T cells. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled naive CD4 T cells were stimulated with anti-TCR- β mAb in the presence of IL-2 and IL-4 (Th2 cell-skewed condition), and 24, 48, 72, and 96 h later, IL-4 production in the developing Th2 cells was assessed (Fig. 2 C). 24 h after the stimulation, most cells had not undergone cell division (cell division no. 0), and IL-4-producing Th2 cells were not detected. At 48 h, cells had undergone up to three cell divisions, and there was a slight increase in the numbers of cells with three cell divisions detected in *Shn-2*^{-/-} CD4 T cells. The percentages of IL-4-producing T cells generated were not apparently different between *Shn-2*^{-/-} and *Shn-2*^{+/+} CD4 T cell cultures at this time point. After 72 and 96 h in culture, the rate of cell division appeared to be slightly increased in *Shn-2*^{-/-} CD4 T cells, and the generation of IL-4-producing T cells was higher at all numbers of cell division. These results suggest that although *Shn-2*^{-/-} CD4 T cells show a slightly increased rate in cell division, the enhanced Th2 cell generation in *Shn-2*^{-/-} CD4 T cells could not be simply explained by the difference in the number of cell divisions that the developing Th2 cells had undergone.

We reported previously that CD28 costimulation induced increased Th2 cell differentiation (35) and induced NF- κ B activation and enhanced GATA3 expression in developing Th2 cells to facilitate chromatin remodeling of the IL-5 gene loci (36). *Shn-2*^{-/-} T cells were stimulated with anti-TCR plus anti-CD28 mAb under Th2 cell-skewed conditions (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20040733/DC1>). *Shn-2*^{-/-} T cells showed increased levels of Th2 cell differentiation without anti-CD28 mAb as compared with wild-type (46.2 vs. 32.4%). No obvious additional enhancement of Th2 cell differentiation was detected in the presence of CD28 costimulation under the conditions where the CD28-mediated enhancing effect was observed in *Shn-2*^{+/+} T cell cultures.

cells (1.5×10^4 cells) were purified from *Shn-2*^{-/-} \times DO11.10 Tg mice by cell sorting and stimulated with indicated doses of specific OVA peptides (Loh15: 0.1 μ M) and irradiated CD4⁻ spleen cells (10^5 cells) from non-DO11.10 Tg *Shn-2*^{+/+} or *Shn-2*^{-/-} mice. Two independent experiments were performed with similar results.

No functional defect was observed in DCs from *Shn-2*^{-/-} mice

The results thus far suggested that the observed enhancement of Th2 cell differentiation is due to an alteration in T cells because we used purified T cells (Figs. 1 and 2) and APCs from normal mice (Fig. 1). However, *Shn-2* mRNA is expressed in both CD4 T cells and APCs (not depicted), and thus, we wished to evaluate the effect of *Shn-2* deficiency on APC function using bone marrow-derived DCs (BMDCs). The expression levels of CD11c, I-A^d, H-2K^d, CD40, B7-1, B7-2, and Fas were comparable between *Shn-2*^{+/+} and *Shn-2*^{-/-} BMDCs (not depicted). OVA-pulsed BMDCs from *Shn-2*^{+/+} or *Shn-2*^{-/-} mice were cocultured with DO11.10 Tg CD4 T cells, and proliferative responses of T cells were assessed by [³H]thymidine uptake. Under these conditions, the APC function of both immature and mature BMDCs was found to be comparable between *Shn-2*^{-/-} or *Shn-2*^{+/+} mice (Fig. 3 A). We then examined the ability of OVA-pulsed BMDCs as APCs to induce Th1/Th2 cell differentiation. Stimulation with either immature or mature BMDCs resulted in similar levels of Th1/Th2 cell differentiation in the cultures with *Shn-2*^{+/+} and *Shn-2*^{-/-} BMDCs (Fig. 3 B).

To further evaluate the APC function of *Shn-2*^{-/-} mice, spleen cells were irradiated and tested for their ability to induce Th1/Th2 cells. The numbers of Th2 cells generated were found to be comparable between cultures with *Shn-2*^{-/-} and *Shn-2*^{+/+} APCs (Fig. 3 C). Taken together, the enhanced Th2 cell generation in *Shn-2*^{-/-} T cell cultures appears to be due to an alteration in CD4 T cells themselves and not to any change in APC function.

Hyperactivation of NF-κB in *Shn-2*^{-/-} CD4 T cells

We examined the NF-κB activation in *Shn-2*^{-/-} CD4 T cells by electrophoretic mobility shift assays (EMSA). CD4 T cells from *Shn-2*^{+/+} and *Shn-2*^{-/-} mice were stimulated with anti-TCR and anti-CD28 for 3 h, and subsequently, nuclear extracts were prepared. To facilitate the stimulation of the TCR complex on naive T cells, we used both anti-TCR and anti-CD28 mAbs. The NF-κB DNA binding activity was found to be increased after TCR stimulation in *Shn-2*^{+/+} CD4 T cells (Fig. 4 A, lanes 2 and 3, and B, lanes 2 and 14). In contrast, in *Shn-2*^{-/-} CD4 T cells, substantial levels of binding activity could already be detected even in nonstimulated T cells (Fig. 4 A, lane 4, and B, lanes 7 and 17), and an only

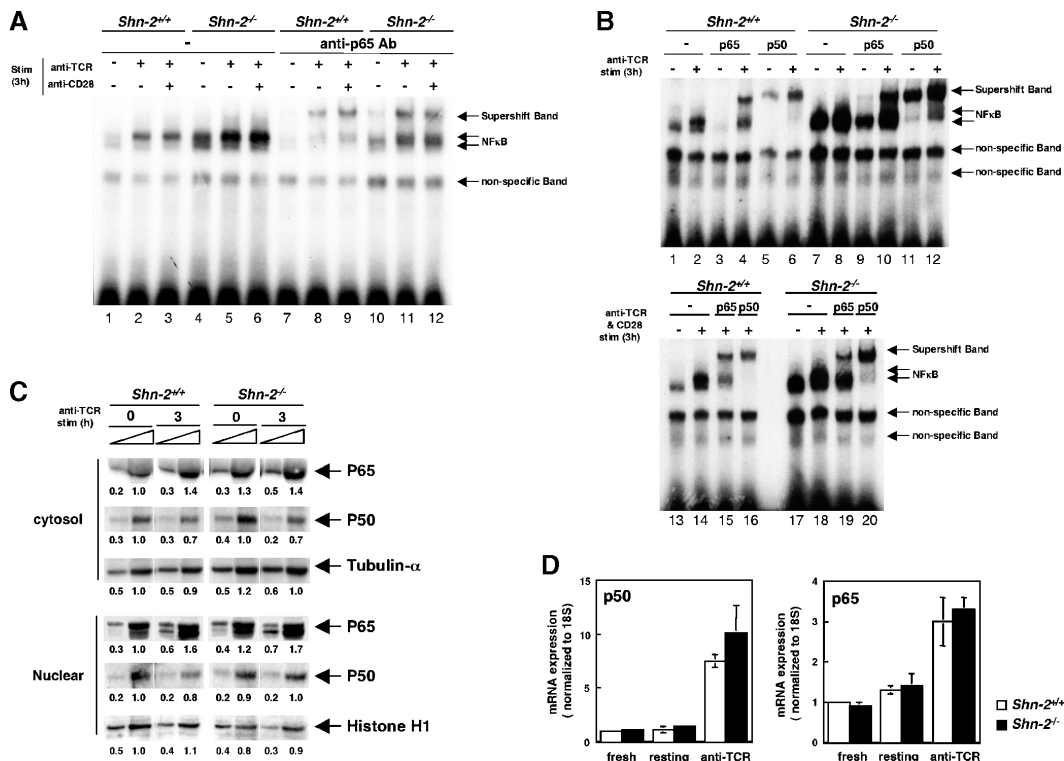
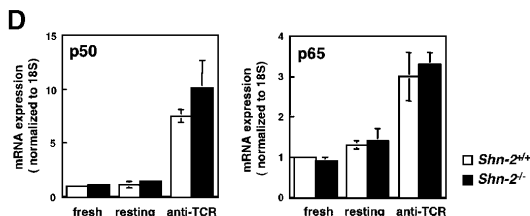


Figure 4. Hyperactivation of NF-κB in *Shn-2*^{-/-} CD4 T cells.

(A and B) Splenic CD4 T cells were incubated with medium alone overnight and then stimulated with immobilized anti-TCR-αβ mAb in the presence or absence of agonistic anti-CD28 antibody for 3 h. Nuclear extracts of the cultured cells were prepared and subjected to EMSAs with NF-κB probes. The supershift assays were performed with antibodies specific for NF-κB p50 and p65 subunit detection. Three independent experiments were performed with similar results. (C) Splenic CD4 T cells were incubated with medium alone overnight and then stimulated with immobilized anti-TCR-β



mAb for 3 h. Subsequently, both cytosol and nuclear extracts were prepared and these were subjected to immunoblotting using anti-p50, anti-p65, anti-tubulin-α, and anti-histone H1 antibodies. Arbitrary densitometric units are shown under each band. (D) RNAs were prepared from fresh splenic CD4 T cells, resting CD4 T cells that were incubated with medium alone overnight, and anti-TCR-stimulated CD4 T cells, and then quantitative PCR assay was performed. The expression levels of p50 and p65 were normalized with 18S expression. Two independent experiments were performed with similar results.

moderate elevation in activity was observed after TCR stimulation (Fig. 4 A, lanes 5 and 6, and B, lanes 8 and 18). We performed a supershift assay to determine the specificity for NF- κ B subunits and observed a substantial supershift with the anti-p65 antibody (Fig. 4 A, lanes 8 and 9, and B, lanes 4 and 15). A small, but reproducible shift was detected in *Shn-2*^{-/-} nonstimulated samples (Fig. 4 A, lane 10, and B, lane 9). Increased amounts of p65 supershift band were detected after anti-TCR stimulation in *Shn-2*^{-/-} T cells. Furthermore, an EMSA with anti-p50 antibody revealed that most of the NF- κ B EMSA band detected in nonstimulated *Shn-2*^{-/-} T cells contained the p50 subunit (Fig. 4 B, lane 11). There was a substantial increase in the level of the p50 band after anti-TCR stimulation (Fig. 4 B, lane 12). These results suggest that NF- κ B is constitutively activated in *Shn-2*^{-/-} CD4 T cells and it is hyperactivated after anti-TCR stimulation. We did not observe any obvious disappearance of specific bands in *Shn-2*^{-/-} groups. This could be due to an insufficient amount of Shn-2 for visualization in the EMSA assay.

An assessment of the protein expression levels of NF- κ B p50 and p65 subunits in the cytosol and in nuclear fractions revealed that no obvious difference was detected between *Shn-2*^{+/+} and *Shn-2*^{-/-} T cells before or after anti-TCR stimulation, suggesting that the nuclear translocation of the NF- κ B p50 and p65 subunits is not altered in the absence of Shn-2 (Fig. 4 C). Furthermore, transcriptional levels of p50 and p65 were compared by quantitative RT-PCR assay using freshly prepared CD4 T cells, resting CD4 T cells, and those after anti-TCR stimulation for 3 h (Fig. 4 D). In all

cases, basically no difference was detected between *Shn-2*^{+/+} and *Shn-2*^{-/-} groups.

We also examined the activation of AP-1, CREB, and IL-4 NFAT in *Shn-2*^{-/-} CD4 T cells by EMSA (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20040733/DC1>). The DNA binding ability of AP-1 was slightly, but reproducibly, enhanced in *Shn-2*^{-/-} T cells, whereas the DNA binding activity of CREB and IL-4 NFAT appeared to be similar in *Shn-2*^{+/+} and *Shn-2*^{-/-} T cells.

Enhanced expression of GATA3 in anti-TCR-activated *Shn-2*^{-/-} CD4 T cells

Because the levels of GATA3 expression are critical for Th2 cell differentiation (14) and because GATA3 expression is regulated by NF- κ B (30), we next assessed the protein expression of GATA3 in cultured *Shn-2*^{-/-} T cells under Th2 cell-skewed condition. Splenic CD4 T cells from *Shn-2*^{-/-} mice were stimulated with anti-TCR- β mAb and IL-4, and 16 h later, the expression of GATA3 was assessed by immunoblotting with specific antibodies. The levels of GATA3 protein were found to be reproducibly increased in *Shn-2*^{-/-} T cells (Fig. 5 A). No detectable GATA3 protein was observed in freshly prepared *Shn-2*^{-/-} CD4 T cells (not depicted). Concurrently, the mRNA levels were assessed by semiquantitative RT-PCR analysis, and increased GATA3 mRNA levels were detected (Fig. 5 B). These results suggest that enhanced induction of GATA3 takes place in early developing *Shn-2*^{-/-} Th2 cells as compared with that of control *Shn-2*^{+/+} Th2 cells.

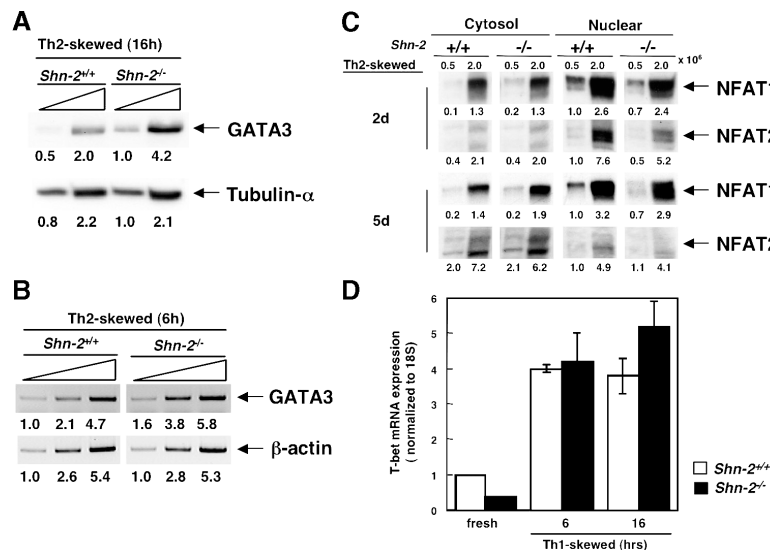


Figure 5. Increased GATA3 expression in *Shn-2*^{-/-} developing Th2 cells. (A) Splenic CD4 T cells were cultured under Th2 cell-skewed conditions for 16 h. The cells were lysed, and cell lysates with threefold serial dilutions of cell lysates were subjected to immunoblotting using anti-GATA3 or anti-tubulin- α antibodies. Arbitrary densitometric units are shown under each band. (B) Splenic CD4 T cells were cultured under Th2 cell-skewed conditions for 6 h, and the expression levels of GATA3 mRNA were determined by RT-PCR. Arbitrary densitometric units are shown under each band. (C) Expression of NFAT1 and NFAT2 in *Shn-2*^{-/-} Th2 cells. Splenic

CD4 T cells were stimulated under Th2 cell-skewed conditions for 2 or 5 d, and then both cytosol and nuclear cell lysates were prepared. Immunoblotting was performed with anti-NFAT1 or anti-NFAT2 antibodies. Arbitrary densitometric units are shown under each band. (D) Splenic CD4 T cells were cultured under Th1 cell-skewed conditions for 6 or 16 h, and the expression levels of T-bet mRNA were determined by quantitative PCR assay. The expression was normalized with 18S expression. Two independent experiments were performed with similar results.

We examined the cytosolic or nuclear protein expression levels of NFAT1 and NFAT2 in developing Th2 cells (Fig. 5 C). In either cytosolic or nuclear lysate, there was no clear difference in the expression of NFAT1 detected between *Shn-2^{+/+}* and *Shn-2^{-/-}* T cells, suggesting normal nuclear translocation of NFAT1 occurs. The nuclear translocation of NFAT2 was, however, slightly impaired in *Shn-2^{-/-}* T cells. An assessment of the expression of other molecules that may regulate Th2 cell differentiation directly or indirectly, such as JunB, cMaf, Bcl-6, and TRAF2, revealed no clear difference in mRNA expression levels in *Shn-2^{-/-}* developing Th2 cells (not depicted). In addition, mRNA levels of several NF- κ B target genes (IL-6, IL-1 β , IL-15, and IL-17) are increased in *Shn-2*-deficient T cells (not depicted). The levels of T-bet in the *Shn-2^{+/+}* and *Shn-2^{-/-}* cells cultured under Th1 cell-skewed conditions are shown in Fig. 5 D. No significant difference was observed in the levels of T-bet mRNA.

Shn-2 inhibits an NF- κ B-dependent transcriptional activity

The ability of Shn-2 protein to bind to the consensus NF- κ B binding motif was assessed. 293 T cells were transfected with a pAct-Flag-hShn-2 vector, and 2 d later, nuclear extracts were prepared. Biotinylated NF- κ B and control AP-1 oligonucleotides were absorbed on to streptavidin-agarose beads

and incubated with the nuclear extracts. The amount of Shn-2 protein in the precipitates of agarose beads was assessed by immunoblotting with anti-Flag mAb. Total nuclear extracts were also run in parallel as loading controls. As can be seen in Fig. 6 A, a substantial binding of Flag-Shn-2 protein to NF- κ B oligo was observed. This result suggests that Shn-2 protein is able to bind to a consensus NF- κ B binding motif.

Next, we wished to examine whether Shn2 competes effectively with p50 NF- κ B for binding to a consensus NF- κ B binding motif. Nuclear extracts were prepared from 293 T cells that were transfected with pAct-Flag-hShn2 or pCMX-p50 vectors. The extracts were mixed in specific ratios and then incubated with NF- κ B oligonucleotides absorbed with streptavidin-agarose beads. The bound protein was detected by immunoblotting with an anti-Flag mAb for Shn-2 and an anti-p50 mAb for p50 protein. As shown in Fig. 6 B, left, the binding of p50 to NF- κ B oligonucleotides was inhibited by Shn-2-containing extracts in a dose-dependent manner. In addition, the binding of Shn-2 to NF- κ B oligonucleotides was inhibited by the presence of p50 (Fig. 6 B, right). These results clearly suggest that Shn-2 competes with p50 for binding to a consensus NF- κ B motif.

Consequently, to determine whether Shn-2 protein plays a functional role in NF- κ B-dependent transcription, NF-

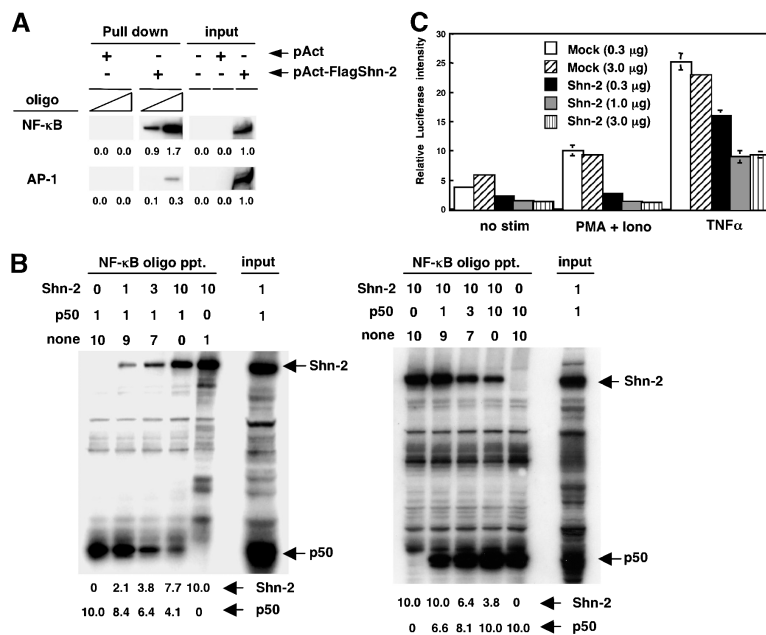


Figure 6. Inhibition of NF- κ B-dependent transcriptional activity by Shn-2. (A) 293 T cells were transfected with a control pAct or a pAct-Flag-hShn-2 vector, and 2 d later, their cell lysates were prepared. Biotinylated NF- κ B and control AP-1 oligonucleotides were absorbed by streptavidin-agarose beads, and then the beads were incubated with cell lysates. The amount of Shn-2 protein in the precipitates was assessed by immunoblotting with anti-Flag mAb. Total cell lysates (10⁶ equivalent/lane) were also run as controls (input). Three independent experiments were performed with similar results. (B) Nuclear extracts were prepared from 293 T cells transfected with pAct-Flag-hShn2 or pCMX-p50 vectors. The extracts were mixed in a certain ratio and then incubated with NF- κ B oligonucleotides absorbed with streptavidin-agarose beads. The numbers

represent the volume of cell lysates (μ l; 1 μ l = 5 \times 10⁵ cell equivalent). none, nontransfected 293 T lysates. The bound protein was detected by immunoblotting with an anti-Flag mAb for Shn-2 and an anti-p50 mAb for p50 protein. Total cell lysates were also run as controls (input). The position of Shn-2 and p50 are indicated. Three experiments were performed with similar results. (C) 293 T cells were transfected with 25 ng of the 5 \times NF- κ B reporter constructs with the indicated doses of a pAct control vector (Mock) or a pAct-Flag-hShn-2 vector (Shn-2). Additionally, 1 ng pRL-TK vector was added into each transfection as an internal control. 24 h after the transfection, cells were stimulated with 50 ng/ml PMA and 500 nM ionomycin or 10 ng/ml TNF- α for 12 h and then assayed for luciferase activity.

κ B-induced luciferase assay was performed with 293 T cells transfected with the 5 \times NF- κ B reporter constructs along with graded doses of pAct-Flag-hShn-2 vector after stimulation with PMA plus ionomycin or TNF- α (Fig. 6 C). Some level of luciferase activity was detected without stimulation, and a significant inhibition was observed in the presence of Shn-2. After PMA plus ionomycin stimulation, the luciferase activity was increased and this was efficiently inhibited by Shn-2 (Fig. 6 C, middle). The luciferase activity was increased dramatically by stimulation with TNF- α , and this was moderately inhibited in the presence of Shn-2 protein (Fig. 6 C, right). These results suggest that Shn-2 protein is able to inhibit the NF- κ B-dependent transcriptional activity.

DISCUSSION

In this report, we demonstrated a crucial role for *Shn-2* in Th2 cell differentiation. Shn-2 appears to control NF- κ B DNA binding activity and subsequent GATA3 induction in early developing Th2 cells.

The DNA binding of NF- κ B was enhanced in *Shn-2*-deficient CD4 T cells (Fig. 4, A and B). The enhancement was already apparent in nonstimulated *Shn-2*-deficient CD4 T cells. However, we found the NF- κ B protein equally expressed in the nucleus and cytoplasm of nonstimulated and stimulated *Shn-2*-deficient CD4 T cells (Fig. 4 C). Thus, the enhanced DNA binding of NF- κ B might be regulated by nuclear events such as those involved in the DNA binding process. A previous report suggested that Shn-3 binds to a NF- κ B motif and represses transactivation of NF- κ B-dependent genes (33). We show here that Shn-2 protein is able to bind to a consensus NF- κ B motif (Fig. 6 A) and compete with p50 for DNA binding in a dose-dependent manner (Fig. 6 B). In addition, we detected significant inhibition of the NF- κ B-dependent transcriptional activity in 293 T cells. Thus, it is conceivable that Shn-2 is able to bind to NF- κ B DNA binding motifs and thus compete with the binding of NF- κ B complexes or to repress directly the transactivation of the NF- κ B-dependent genes in early developing Th2 cells. This might be a mechanism to account for the elevated NF- κ B DNA binding activity in nonstimulated *Shn-2*-deficient CD4 T cells (Fig. 4, A and B).

In addition, we detected a further enhancement of DNA binding of NF- κ B after TCR stimulation (Fig. 4, A and B). This could explain the enhanced induction of GATA3 in early developing *Shn-2*^{-/-} Th2 cells (Fig. 5, A and B). A similar regulation was demonstrated in an experimental system using p50^{-/-} mice (30). In our preliminary results, the generation of IL-4-producing Th2 cells in wild-type BALB/c CD4 T cell cultures was increased by the ectopic expression of both p50 and p65 (unpublished data). Thus, it appears probable that the enhanced induction of GATA3 by NF- κ B hyperactivation is a mechanism that is responsible for the enhanced Th2 cell differentiation observed in *Shn-2*-deficient T cells.

Another interesting possibility for the molecular targets of Shn-2 is the involvement of TRAF2-mediated NF- κ B activation. A previous report suggested that there is a physi-

cal association of Shn-3 with TRAF2 (31). Our preliminary results suggest that a physical association of Shn-2 with TRAF2 in 293 T cells can be demonstrated (unpublished data). Thus, Shn proteins may bind to TRAF2 and inhibit the TNFR-induced NF- κ B activation and nuclear translocation in T cells. However, this appeared to be unlikely because no obvious difference in the protein expression of p50 and p65 was observed in the cytosol or nucleus before or after anti-TCR stimulation between *Shn-2*^{+/+} and *Shn-2*^{-/-} T cells (Fig. 4 C). Lieberson et al. (37) have reported that Th2 cell responses are enhanced in dominant-negative TRAF2 Tg CD4 T cells. This result would appear to be contradictory to the above hypothesis. However, the dominant-negative TRAF2 used in the study by Lieberson et al. inhibits JNK activity efficiently, but it does not affect NF- κ B activity very significantly (38). Thus, it is still possible that the Shn-2-TRAF complex is formed in T cells and that it is involved in the control of Th2 cell differentiation by regulating NF- κ B activation. A more precise investigation is needed to clarify this issue.

As for the signaling pathways in which Shn-2 is involved, Shn-2 may regulate outcomes of both the TCR- and TNF-dependent pathways if the major function of Shn-2 is mediated by a mechanism proposed above, namely, competition with NF- κ B for DNA binding in the nucleus. In this paper, we focused our analysis mostly on T cells where the TNF-dependent signaling pathway is not activated efficiently, and thus, further investigation will be required to address this issue. In addition, we did not observe CD28 costimulation-mediated enhancement of Th2 cell differentiation in *Shn-2*^{-/-} cells (Fig. S4). CD28 costimulation induced NF- κ B activation and enhanced GATA3 expression in developing Th2 cells (36). Thus, it is possible that the effect of CD28 costimulation was masked by hyperactivation of NF- κ B in *Shn-2*^{-/-} T cells.

It has been established that the expression of GATA3 is crucial for the efficient Th2 cell differentiation (16). GATA3 plays an essential role in chromatin-remodeling processes, such as acetylation of histone H3 and H4 of the Th2 cell cytokine gene loci (36). The activation of STAT6 is most critical for the induction of GATA3 transcription (16, 39). More recently, several regulatory molecules for GATA3 transcription have emerged. One is the NF- κ B activation discussed above (30). We reported previously that a polycomb group gene product, mel-18, controls Th2 cell differentiation by regulating GATA3 transcription (40). Shn-2 is another example of a protein that regulates GATA3 transcription and expression in developing Th2 cells. Further investigation of the regulation of GATA3 expression is likely to provide a clearer molecular view of the initiation of Th2 cell differentiation.

Recombinant TGF- β inhibited Th2 cell differentiation of normal and *Shn-2*-deficient T cells very efficiently (unpublished data). In addition, Smad7 expression was not changed in *Shn-2*-deficient T cells (unpublished data). Thus, Shn-2 may not be involved in the TGF- β -mediated signal-

ing pathway in T cells, at least not in the TGF- β -mediated inhibition of Th2 cell differentiation. However, because mammalian Shn has three orthologues (Shn-1, Shn-2, and Shn-3), it is still possible that Shn-1 and/or Shn-3 could substitute for the function of Shn-2 in *Shn-2*-deficient T cells.

As for the effects of Shn-2 deficiency on Th1 cell differentiation, Shn-2 may not play critical roles in Th1 cell differentiation. No effect was observed in typical Th1 cell-skewed cultures containing excess amounts of IL-12 (Fig. 1 A). However, we observed some enhancement in Th1 cell differentiation when IL-12 is limiting (Fig. 2 B). Also, IFN- γ -producing cells generated under Th2 cell-skewed conditions were increased consistently (Fig. 1 C). Thus, it is still possible that Shn-2 may act as a negative regulator of the generation of IFN- γ -producing cells.

In summary, the results of this study indicate that Shn-2 plays crucial roles in the control of Th2 cell differentiation by regulating the activation of NF- κ B and subsequent GATA3 induction in early developing Th2 cells.

MATERIALS AND METHODS

Mice. *Shn-2*-deficient (*Shn-2*^{-/-}) mice were described previously (25). Animals used in this study were backcrossed to BALB/c more than 12 times and were 6–8 wk old. Anti-OVA-specific TCR- $\alpha\beta$ (DO11.10) Tg mice were provided by D. Loh (Washington University School of Medicine, St. Louis, MO; reference 41). *Shn-2*^{-/-} \times DO11.10 Tg mice were used at 10–12 wk of age. STAT6-deficient (*STAT6*^{-/-}) mice were provided by S. Akira (Osaka University, Osaka, Japan; reference 39). All mice used in this study were maintained under specific pathogen-free conditions. Animal care was in accordance with the guidelines of Chiba University.

Immunofluorescent staining and flow cytometry analysis. In general, 10⁶ cells were incubated on ice for 30 min with the appropriate staining reagents according to a standard method (42). The reagents used in this study were as follows: anti-CD4-PE (RM4-1-PE), anti-CD4-FITC (RM4-1-FITC), anti-CD44-FITC (IM7-FITC), anti-CD44-PE (IM7-PE), anti-CD69-FITC (H1.2F3), anti-CD62L-FITC (MEL-14), anti-CD25-FITC (7D4), anti-IL-4R α antibody, and anti- γ C antibody-PE were purchased from BD Biosciences. Anti-rat Ig-FITC was purchased from CAPPEL. Anti-TCR- β -FITC (H57-FITC), anti-CD3-FITC (2C11-FITC), and anti-CD8-Cy5 were prepared in our laboratory. Flow cytometry analysis was performed on a FACSCalibur (Becton Dickinson), and results were analyzed with CELLQuest software (Becton Dickinson). Intracellular staining of IL-4 and IFN- γ was performed as described previously (11). FITC-conjugated anti-IFN- γ antibody (XMG1.2), APC-conjugated anti-IL-5 antibody, and PE-conjugated anti-IL-4 antibody (11B11; all from BD Biosciences) were used for detection.

Cell purification. Splenic CD4 T cells were purified by using magnetic beads and an Auto-MACS Sorter (Miltenyi Biotec) yielding a purity of >98%. Where indicated, CD4 T cells with naive phenotype (CD44^{low}) were isolated from spleens on a FACSVantage cell sorter (Becton Dickinson) yielding a purity of >98% as described previously (11).

Bone marrow DC cultures. A modified method of Inaba et al. (43) and Chen-Woan et al. (44) was used for bone marrow DC cultures. In brief, bone marrow cells depleted of FeR⁺ cells (0.5–1.0 \times 10⁶ cells) were cultured in 1 ml medium in the presence of 10 ng/ml GM-CSF. The culture medium was changed every other day by gently swirling the plates, aspirating ~75% of the medium, and adding back fresh medium with cytokines. On day 6, the cells were harvested and then CD11c⁺ cells (immature DCs) were purified using anti-CD11c mAb-conjugated micro beads and a MACS

LS column (Miltenyi Biotec). The purified immature CD11c⁺ DCs were cultured for an additional 2 d with 10 ng/ml GM-CSF and 1 μ g/ml LPS to allow further maturation and were used as mature DCs.

Proliferation assay. 2 \times 10⁵ splenic CD4 T cells were stimulated in 200- μ l cultures for 40 h with immobilized anti-TCR- β mAb (H57-597) and anti-CD3 ϵ mAb (2C11). Immature or mature DCs were pulsed with 1 μ M OVA peptide for 2 h at 37°C and then cocultured with CD4 T cells from DO11.10 Tg mice for 72 h. [³H]thymidine (37 kBq/well) was added to the stimulation culture for the last 16 h, and the incorporated radioactivity was measured by using a β plate (40).

ELISA for the measurement of cytokine concentration. The production of IL-2, IL-4, IL-5, IL-13, and IFN- γ was measured by ELISA as described previously (40).

In vitro Th1/Th2 cell differentiation cultures. Naive splenic CD4 T cells were stimulated with 3 μ g/ml of immobilized anti-TCR- β mAb (H57-597) in the presence of 30 U/ml IL-2 and 1–100 U/ml IL-4 as described previously (10). Where indicated, naive splenic CD4 T cells were labeled with CFSE (Molecular Probes) using the same procedure as described previously (40). For Th1 cell differentiation, naive splenic CD4 T cells were stimulated with 3 μ g/ml of immobilized anti-TCR- β mAb (H57-597) in the presence of 30 U/ml IL-2, IL-12, and anti-IL-4 mAb. 1.5 \times 10⁴ sorted DO11.10 Tg CD44^{low} CD4 T cells were stimulated with antigenic OVA peptide (Loh15, OVA; 323–339) and 10⁵ irradiated (3,000 rad) normal BALB/c splenocytes. For stimulation with purified DCs, 2 \times 10⁵ sorted DO11.10 Tg CD44^{low} CD4 T cells were cocultured with 2 \times 10⁴ OVA-pulsed immature or mature DCs. Appropriate cytokines and anti-cytokine antibodies were added in Th1/Th2 cell differentiation cultures as described previously (11). In typical DO11.10 Tg T cell cultures, Th2 cell-skewed (IL-4 with anti-IL-12 mAb and anti-IFN- γ mAb), Th1 cell-skewed (IL-12 with anti-IL-4 mAb), and non-skewed (IL-2 with anti-IL-4 mAb, anti-IL-12 mAb, and anti-IFN- γ mAb) conditions were used.

Immunoblotting. The Immunoblotting for the detection of GATA3, tubulin- α , NFAT1, and NFAT2 was performed as described previously (45). For NF- κ B detection, anti-p65 (F-6), anti-p50 (E-10), and anti-histone H1 (AE-4, all from Santa Cruz Biotechnology, Inc.) antibodies were used.

EMSA. EMSAs were performed using Gel Shift Assay Systems (Promega) according to the manufacturer's instructions. In brief, nuclear extracts were incubated at 4°C with ³²P-labeled double-stranded oligo (NF- κ B oligo; AGTTGAGGGGACTTTCCCAGGC, AP-1 oligo; CGCTTGATGAGTCAGCCGGAA, CREB oligo; AGAGATTGCTGACGTCAGAGAGCTAG, IL-4 NFAT; oligo; and GTAATAAAATTTTCCAATGTAAA) in DNA binding buffer (Promega). For supershift assays, nuclear extracts were incubated with anti-p50 (C-19; Santa Cruz Biotechnology, Inc.) or anti-p65 (F-6; Santa Cruz Biotechnology, Inc.) antibody at 4°C for 60 min and then incubated with ³²P-labeled NF- κ B double-stranded oligo at 4°C. Electrophoresis was performed on a 4% native polyacrylamide gel (0.5 \times TBE; acrylamide/bisacrylamide, 29:1), and the radioactivity was visualized by autoradiography.

Pull-down assay. A detailed protocol was described elsewhere (46). In brief, 293 T cells were transfected with 1.0 μ g pAct or pAct-Flag-hShn-2, and 2 d later, the transfected cells were lysed for immunoblotting with anti-Flag mAb (M2; Sigma-Aldrich). Cell lysates were incubated with biotinylated oligonucleotides of NF- κ B (AGTTGAGGGGACTTTCCCAGGC) and AP-1 (CGCTTGATGAGTCAGCCGGAA). The bound protein were eluted and separated on an SDS polyacrylamide gel and then subjected to immunoblot analysis using specific antibodies (anti-Flag Tag; M2; Sigma-Aldrich). For the competition assay shown in Fig. 6 B, 293 T cells were transfected with pAct-Flag-hShn-2 or pCMX-p50, and their cell extracts were mixed in certain ratios and then the mixed extracts were incubated

with NF- κ B oligonucleotides as described above. pCMX-p50 was provided by J. Inoue (University of Tokyo, Tokyo, Japan). Anti-Flag Tag (M2; Sigma-Aldrich) mAb and anti-p50 (E-10; Santa Cruz Biotechnology, Inc.) mAb were used for detection.

Transfection and luciferase assays. 293 T cells were transfected with 5 \times NF- κ B luciferase reporter plasmid together with pAct vector or pAct-Flag-hShn-2 vector and pRL-TK as normalization control. Transfected cells were stimulated with or without 50 ng/ml PMA and 500 nM ionomycin or 10 ng/ml TNF- α for 12 h before luciferase assays (Promega).

PCR analysis. Total RNA was isolated from cultured cells using the TRIzol reagent. Reverse transcription was performed with Superscript II RT (Invitrogen). Threefold serial dilutions of template cDNA were performed. The PCR reaction was performed as described previously (46). The primers used are as follows: β actin forward, GAGAGGGAAATCGTGCGTGA-3'; β actin reverse, 5'-ACATCTGCTGGAAGGTGGAC; GATA3 forward, gAAGgCATCCAgACCCgAAAC-3'; GATA3 reverse, 5'-ACCCATgg-CggTgACCATgC; Shn-2 forward, ggAAAgAgggAAAgAgAgATTCA-CggAgAT-3'; and Shn-2 reverse, 5'-ATCTgAgTgTCATCACAAgAgT-CACTgggT.

For quantitative PCR assay, first-strand cDNA was synthesized using random primers and Superscript II RT (Invitrogen). Samples were then subjected to real-time PCR analysis on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) under standard conditions. The primers and TaqMan probes for the detection of p50, p65, T-bet, and 18S were purchased from Applied Biosystems. p50, p65, and T-bet expression were normalized using the 18S signal.

Online supplemental material. Fig. S1 shows the phenotypic feature of splenic CD4 T cells from *Shn-2*^{-/-} mice. (A) The yields of thymocytes and splenocytes are shown in boxed numbers. (B) Each histogram depicts the expression of the indicated marker antigens on electronically gated splenic CD4 T cells. Background staining is shown as hatched areas. (C) The histogram shows the percentages of indicated V β cells among splenic CD4 T cells. Fig. S2 shows the proliferative responses and Erk phosphorylation status after TCR stimulation. Arbitrary densitometric units are shown under each band in B. Fig. S3 shows the expression of Shn-2 mRNA in developing Th1 and Th2 cells. The mRNA levels were determined by RT-PCR with threefold serial dilutions of template cDNA. Fig. S4 shows the effects of CD28 stimulation on Th2 cell differentiation. Gel shift assay for AP-1, CREB, or IL-4 NFAT are shown in Fig. S5. Figs. S1–S5 are available at <http://www.jem.org/cgi/content/full/jem.20040733/DC1>.

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