


Research Article

Schnurri 3 promotes Th2 cytokine production during the late phase of T-cell antigen stimulation

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Th1 and Th2 polarization is determined by the coordination of numerous factors including the affinity and strength of the antigen-receptor interaction, predominant cytokine environment, and costimulatory molecules present. Here, we show that Schnurri (SHN) proteins have distinct roles in Th1 and Th2 polarization. SHN2 was previously found to block the induction of GATA3 and Th2 differentiation. We found that, in contrast to SHN2, SHN3 is critical for IL-4 production and Th2 polarization. Strength of stimulation controls SHN2 and SHN3 expression patterns, where higher doses of antigen receptor stimulation promoted SHN3 expression and IL-4 production, along with repression of SHN2 expression. SHN3-deficient T cells showed a substantial defect in IL-4 production and expression of AP-1 components, particularly c-Jun and Jun B. This loss of early IL-4 production led to reduced GATA3 expression and impaired Th2 differentiation. Together, these findings uncover SHN3 as a novel, critical regulator of Th2 development.

Keywords: CD4 T cells · cell differentiation · IL-4 · signal transduction · Schnurri (SHN) proteins



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

CD4⁺ T lymphocytes are critical members of the adaptive immune response that are required for effective cell-mediated immunity. CD4⁺ T cells promote tolerance to commensals and self-antigens, direct inflammatory responses to pathogens, and orchestrate tumor immunity. To direct these various immune processes, naïve CD4⁺ T cells differentiate into functionally distinct CD4⁺ Th subsets including Th1, Th2 and Th17 lineages [1, 2]. Th1

cells produce the proinflammatory cytokines TNF and IFN- γ , and are critical for the clearance of intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13 and are required for both antibody production by B cells and clearance of extracellular microbes [3, 4]. Exaggerated Th2 responses can lead to allergic responses and asthma [5, 6]. Th17 cells produce IL-17A, IL-17F, IL-22, and IL-21, which play a critical role in recruitment of proinflammatory leukocytes and tissue inflammation, including autoimmunity [7, 8].

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Due to the diverse and critical nature of these distinct T-cell subsets, differentiation is tightly controlled by cytokines in the environment and master transcription factors induced after antigen activation [9]. Very low doses of antigen peptide induced IL-4 expression and Th2 phenotypes, while midrange peptide doses (0.3–0.6 μM) direct the development of Th1 cells [10]. Interestingly, higher doses of antigenic peptide ($>10 \mu\text{M}$) skew differentiation to Th2. This low dose-IL-4/medium dose-IFN- γ shift has been also reported by other groups [11–14]. In vivo observations using vaccination or infection models also show that lower and higher doses of antigen stimulate Th1 and Th2 responses, respectively [15, 16]. The transcription factor T-bet is involved in the commitment of Th1 cells by inducing IFN- γ synthesis and repressing IL-4 and IL-5 production [17, 18]. During Th2-cell differentiation, IL-4 induces the expression of GATA3, an essential transcription factor for Th2 cells [19]. IL-2 supports early Th2 differentiation by inducing expression of the IL-4 receptor α -chain and expression of GATA3 [14, 20]. NF- κB and JunB are other transcription factors critical for IL-4 expression and commitment to the Th2 lineage. NF- κB is required for the induction of GATA3 and JunB [21, 22]. Both GATA3 and JunB are upregulated in Th2, but not in Th1 cells [23, 24].

Schnurri (SHN), first identified in *Drosophila*, is a protein that interacts with Mad-Medea, an orthologue of the SMAD family of transcription factors to mediate decapentaplegic (DPP) signaling during disk development [25]. DPP is an orthologue of the mammalian TGF- β /activin/MBP superfamily [26, 27]. Three mammalian counterparts of SHN proteins have been identified: SHN1 (also known as HIVEP1, MBP-1, and ZAS1), SHN2 (also known as HIV-EP2, MBP-2, and ZAS2), and SHN3 (also known as HIVEP3, KRC, and ZAS3). Since its discovery in *Drosophila*, SHN proteins have been found to play a significant role in BMP/TGF- β signaling in adipocytes, epithelial cells, and during bone formation and remodeling [28–31].

In T cells, SHN2 plays a critical role in T-cell development, thymic positive selection, and memory T cell numbers in vivo [32–34]. SHN2 also inhibits Th2 differentiation and is a negative regulator of airway inflammation [35]. SHN2-deficient CD4 T cells have an increased propensity to differentiate into Th2 cells, in part due to constitutive activation of NF- κB and the subsequent upregulation of GATA3 expression [36]. SHN3 forms a complex with TNF receptor-associated factor (TRAF) 2 to repress nuclear translocation of NF- κB [37], and blocks the functions of NF- κB by competitively binding to the NF- κB consensus sequence [38]. SHN3 also physically associates with the transcription factor c-Jun, and serves as a coactivator to augment AP-1-dependent IL-2 gene transcription in CD4 $^+$ T cells [39].

Previously, we demonstrated that Shc and sustained ERK signaling is required for the expression of IL-2 by activated T cells [40]. In this work, we assessed the role of SHN3 in primary CD4 T-cell activation and functional maturation. The data show that SHN3 promotes Th2 development by IL-4 expression during the early stage of T-cell activation.

Results

Kinetics of SHN3 and SHN2 expression by naïve CD4 $^+$ T cells

We previously showed that expression of IL-2 by T cells requires sustained ERK signaling and that Shc is required for this process [41, 40]. Based on these data, we hypothesized that sustained ERK activation induces de novo expression of factors that are required for IL-2 production [42]. We isolated genes turned on in Shc and ERK-dependent manners in activated T cells. cDNA from activated Jurkat T cells was subtracted using mRNA from activated J.SL1 cells, which lack expression of Shc and do not produce IL-2. From our screen, we identified Schnurri-3 (SHN3) as a candidate gene induced in a Shc-dependent manner, critical for IL-2 expression (Supporting information Fig. S1). Others have also reported that SHN3 is critical for IL-2 expression in T cells [39].

To decipher the role of SHN3 in T-cell activation, we examined the expression of SHN3 in primary CD4 $^+$ T cells. Expression of *SHN3* mRNA was low in resting CD4 $^+$ T cells (Fig. 1A). Stimulation with anti-CD3 and anti-CD28 antibodies induced expression of *SHN3*. The level of *SHN3* mRNA peaked 1 day after stimulation, followed by a rapid decrease after 2 days. This expression pattern was clearly distinct from that of *SHN2*, which was expressed at its highest level by unstimulated CD4 T cells and declined substantially after CD3 stimulation (Fig. 1A). A similar time course of *SHN2/SHN3* expression was observed in CD4 $^+$ T cells from p25 TCR Tg mice activated by cognate antigen [43]. This expression pattern of *SHN2/SHN3* indicates that these two genes are regulated in an opposing manner during T-cell activation.

To further decipher the regulation of *SHN2* and *SHN3* expression in T-cell activation, we determined the threshold of stimulation required for expression. CD4 $^+$ T cells from WT C57BL/6 mice were stimulated with increasing concentrations of anti-CD3 antibody (0.25–10 $\mu\text{g}/\text{mL}$). After 1 or 3 days of stimulation, cells were harvested, and the relative level of *SHN2* and *SHN3* mRNA was determined (Fig. 1B). After 1 day of stimulation, expression of *SHN3* peaked when T cells were stimulated by 2.5 $\mu\text{g}/\text{mL}$ or higher concentrations of anti-CD3. Lower concentrations of anti-CD3 (up to 0.5 $\mu\text{g}/\text{mL}$) did not upregulate the expression of *SHN3*. In contrast, expression of *SHN2* was high at low concentrations of antibody stimulation ($\leq 2.5 \mu\text{g}/\text{mL}$), and expression of *SHN2* decreased substantially at higher concentrations. On day 3, *SHN3* was expressed at basal levels regardless of the antibody concentration used. *SHN2* expression followed the same trend as on day 1 and 3, and was markedly reduced by stimulation with antibody concentrations greater than 1 $\mu\text{g}/\text{mL}$. Together, these data show that the kinetics and threshold of stimulation required for *SHN2* and *SHN3* expression are inversely correlated and require similar levels of antigen-receptor engagement.

Since SHN2 inhibits Th2 differentiation [36], we investigated the role of SHN3 in Th differentiation. We first examined if the strength of TCR stimulation that regulates the expression of *SHN3* correlates with IFN- γ (Th1) and IL-4 (Th2) production by CD4 $^+$ T cells. CD4 $^+$ T cells were stimulated by graded doses of

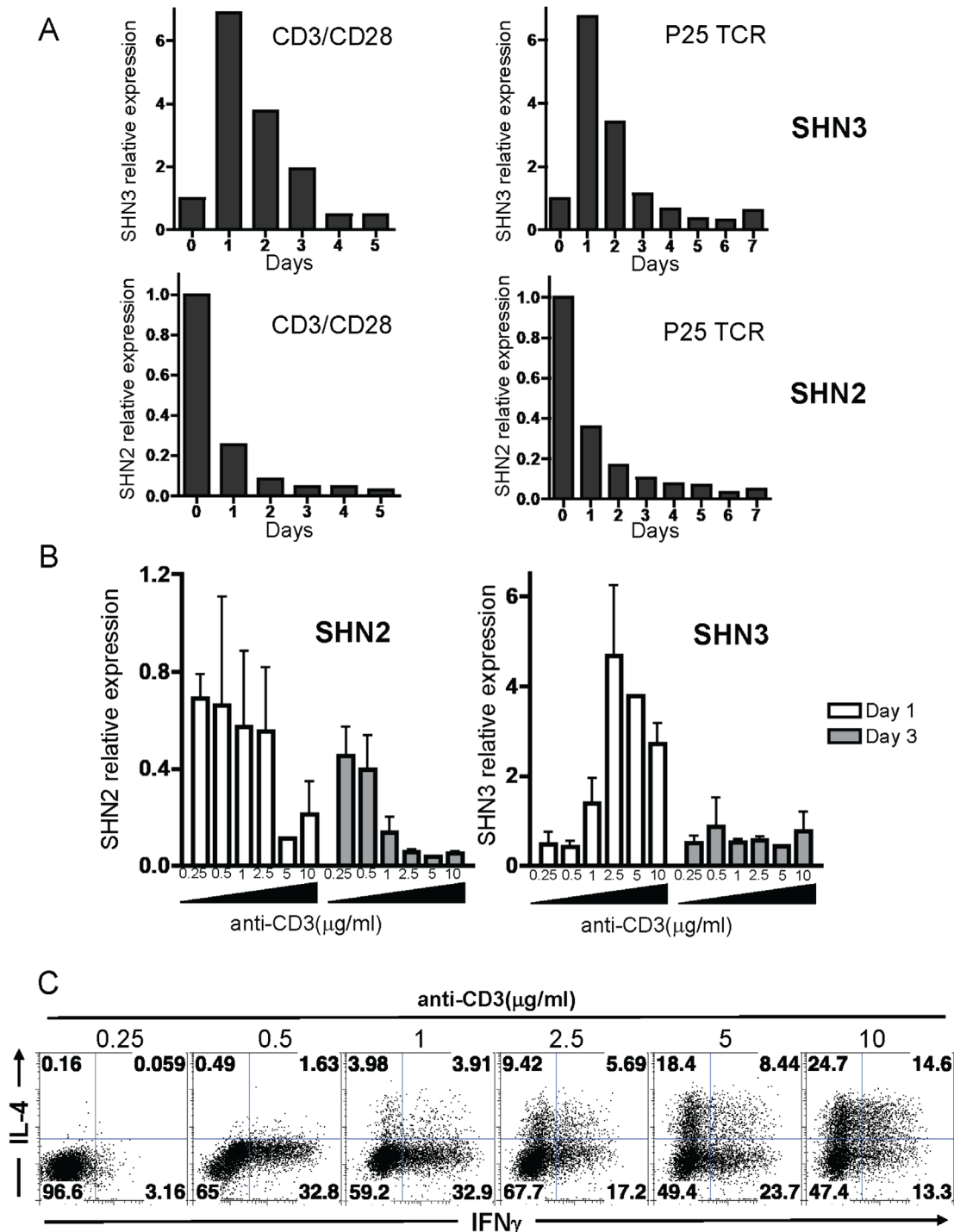


Figure 1. Expression of SHN2 and SHN3 by CD4⁺ T cells. (A) Time course of SHN2 and SHN3 mRNA expression by activated naïve CD4⁺ T cells. CD4⁺CD62L^{hi} naïve T cells from C57BL/6 mice (left column) or P25 TCR transgenic mice (right column) were stimulated by plate-bound anti-CD3 (5 μg/mL) plus soluble anti-CD28 (2 μg/mL) antibodies (for C57BL/6) or by antigen peptide (5 μg/mL) pulsed syngeneic irradiated APCs (P25). Cells were harvested 1–5 days after stimulation. Relative mRNA levels of SHN2 and SHN3 were determined by real-time PCR using day 0 sample as a reference point. (B) Anti-CD3 dose-dependent responses of SHN2 and SHN3 expression by CD4⁺ T cells. Cells were stimulated by graded doses of plate-coated anti-CD3 antibody (0.25–10 μg/mL) plus soluble anti-CD28 (2 μg/mL), then harvested 1 or 3 days later. SHN2 and SHN3 relative mRNA levels were examined by real-time PCR. Relative expression levels against the control gene expression (*gapdh*) are shown (n = 3). (C) Anti-CD3 dose-dependent responses of IL-4 and IFN-γ expression by purified CD4⁺ T cells. Cells were stimulated by graded doses of anti-CD3 antibody (0.25–10 μg/mL) plus soluble anti-CD28 antibody (2 μg/mL) for 3 days, then cultured for 5 days with IL-2 (no CD3/CD28 stimulation). Expanded cells were restimulated by PMA and ionomycin for 4 h and expression of IL-4 and IFN-γ was determined by intracellular cytokine staining. (A and B) Data are shown as ±SD and are from one experiment (n = 3) representative of at least three independent experiments. Students C also represents one set of data from three independent experiments. No gating since purified cells were stimulated by the antibody-coated beads.

anti-CD3 antibody for 3 days. After 5 days, cells were restimulated, and expression of IFN- γ and IL-4 was measured (Fig. 1C). Low doses of antibody stimulation (0.5 or 1 $\mu\text{g}/\text{mL}$) induced T cells to express predominantly IFN- γ , but not IL-4. Higher concentrations of anti-CD3 (2.5 $\mu\text{g}/\text{mL}$ or higher) induced T cells to express IL-4, while IFN- γ producing T cells were reduced. Stimulation by 2.5 $\mu\text{g}/\text{mL}$ anti-CD3 antibody corresponds to the threshold required for *SHN3* expression. Together, these data indicate a potential link between expression of *SHN2/SHN3* genes and *IL-4* gene expression.

Generation of T-cell-specific SHN3-deficient mice

To determine the *in vivo* functions of SHN3 in T cells, we generated T-cell-specific SHN3-deficient-mice using the *Cre-loxP* system (Supporting information Fig. S2A). A targeting vector for the *SHN3* gene was constructed to delete exon 4. The targeted allele was detected by Southern blot analysis and PCR (Supporting information Fig. S2B and C). cDNA encoding for EGFP was inserted with an internal ribosome entry site (IRES) at the 5' end of the *SHN3* gene to monitor expression of SHN3. To delete *SHN3* in a T-cell-specific manner, *SHN3^{loxP/loxP}* mice were bred with *CD4^{Cre}* mice (termed herein SHN3-deficient mice). Expression of *SHN3* mRNA was detected in the thymus from WT and *SHN3^{+/loxP}* heterozygous mice, but not from *SHN3^{loxP/loxP}* mice (Supporting information Fig. S2D). Unexpectedly, we could not detect EGFP-positive cells (thymocytes and activated T cells) that are known to express *SHN3* by flow cytometry (Supporting information Fig. S2E), suggesting that the level of EGFP expression was below the level of detection. Total mononuclear cell numbers in lymphoid organs (thymus and spleen) in SHN3-deficient mice were comparable to those in control mice (WT and heterozygous mice carrying the *CD4^{Cre}* gene) (not shown). Expression of CD4 and CD8 in thymocytes was similar in SHN3-deficient and WT littermates (Supporting information Fig. S2F). We observed a mild reduction in the relative frequency of splenic CD4⁺ cells in SHN3-deficient mice (data not shown). Surface expression of CD25, CD44, CD69, and CD62L by thymocytes and splenic CD4⁺T cells was comparable to that of control T cells (Supporting information Fig. S2G and data not shown).

iTreg differentiation does not depend on SHN3

tTregs and iTregs require TGF- β signaling and expression of the transcription factor Foxp3 for their differentiation and maintenance [44–47]. Since SHN3 acts downstream of members of the TGF- β /BMP family in other cell types, we asked if SHN3 is critical for Treg development. The frequency of tTregs in SHN3-deficient and control mice was comparable, as evident by similar expression of Foxp3 and CD25 in splenic T cells (Supporting information Fig. S2H). To test the differentiation of iTregs, SHN3-deficient or control CD4⁺CD62L^{hi} naive T cells were stimulated with plate-bound CD3 plus soluble anti-CD28 antibodies in the presence of TGF- β and IL-2. After 3 days, cells were analyzed for expression

of Foxp3 by intracellular staining (Supporting information Fig. S2I). Under these conditions, SHN3-deficient and sufficient T cells developed into Foxp3⁺ cells at comparable frequencies (upper panels, >80% of T cells). Similar results were observed when T cells were stimulated with anti-CD3 and T cell-depleted splenocytes (APCs, lower panels). Together, these data indicate SHN3 is not critical for the development and maintenance of tTregs and iTregs.

SHN3 controls IL-4 production and Th2 differentiation by CD4⁺ T cells

To determine the effect of SHN3 deficiency on cell proliferation, we stimulated SHN3-deficient and sufficient CD4⁺ T cell with graded doses of plate-bound anti-CD3 antibody in the presence or absence of soluble anti-CD28 antibody. Proliferation of stimulated T cells was determined by incorporation of [³H] thymidine after 48 h of stimulation (Fig. 2A). Under these conditions, SHN3-deficient CD4⁺ T cells proliferated significantly less than the control cells, both with and without the addition of anti-CD28. As previously reported [39], we also found that IL-2 production by SHN3-deficient CD4⁺CD62L^{hi} naive T cells was markedly lower than control cells (Fig. 2B). This, in part, may contribute to the reduction in proliferation.

In addition to the defect in IL-2, SHN3-deficient naive T cells showed a profound defect in IL-4 production, while they produced similar levels of IFN- γ as WT T cells (Fig. 2B and Supporting information Fig. S3). To further assess the T-cell defect caused by SHN3 deficiency, we determined if SHN3-deficient naive CD4⁺ T cells have a reduced propensity to differentiate into Th2 cells compared to SHN3-sufficient T cells. Naive SHN3-deficient or control T cells were stimulated by plate-bound anti-CD3 plus soluble anti-CD28, or by soluble anti-CD3 with APCs, without the addition of exogenous cytokines. After 7 days of culture, T cells were harvested, restimulated, and IL-4 and IFN- γ expression was examined (Fig. 2C). Consistent with the early IL-4 deficiency, SHN3-deficient cells showed a reduced frequency of IL-4-producing cells compared to control cells. The frequency of IFN- γ producing SHN3-deficient T cells was mildly increased over the control. Analysis of culture supernatants confirmed these results. SHN3-deficient CD4⁺ T cells produced significantly lower levels of Th2-type cytokines (IL-4, IL-5, IL-10, and IL-13) compared to control cells, while production of Th1 cytokines (IFN- γ and TNF- α) by SHN3-deficient CD4⁺ T cells was significantly elevated compared to the controls (Fig. 2D). These results suggest that SHN3-deficient naive CD4 T cells have a defect in Th2 differentiation *in vitro*.

SHN3 is critical for Th2 cytokines and antigen-specific IgG formation *in vivo*

IL-4 expression is critical for the differentiation of B cells including class-switching and the production of antibodies [48, 49].

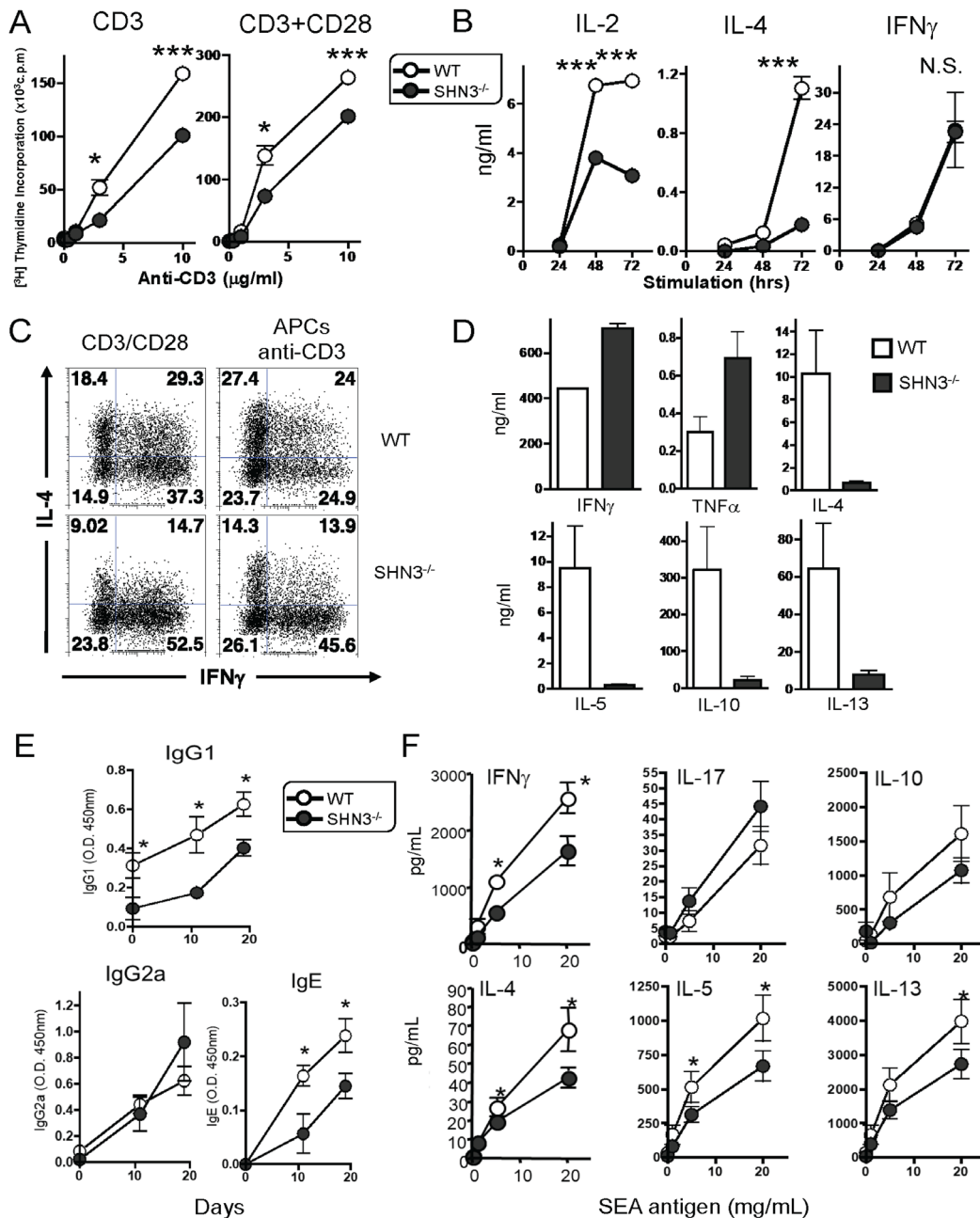


Figure 2. Impaired Th2 responses by T-cell-specific SHN3-deficient mice. (A) SHN3-sufficient (○) or -deficient (●) CD4⁺T cells were stimulated by various concentrations of plate-bound anti-CD3 antibody with or without anti-CD28 (1 μ g/mL) for 36 h. Proliferation of stimulated T cells was measured by [³H] thymidine incorporation during the last 8 h of culture. (B) SHN3-sufficient (○) or -deficient (●) CD4⁺ CD62L^{hi} naive T cells were stimulated by anti-CD3 (5 μ g/mL) plus anti-CD28 (2 μ g/mL) Abs for 24, 48, and 72 h. Culture supernatants were subjected to analysis by ELISA to determine the cytokine levels (IL-2, IL-4, IFN- γ). (A–B) Data shown are representative results from three independent experiments. Each experiment was performed with triplicate. Student t-test was performed to determine the p-value. **p* < 0.05, ****p* < 0.001. (C) SHN3-sufficient (WT) or SHN3^{-/-} CD4⁺CD62L^{hi} naive T cells were stimulated by plate-bound anti-CD3 (5 μ g/mL) plus soluble CD28 (2 μ g/mL) antibodies (left panels) or by soluble anti-CD3 (1 μ g/mL) plus APCs (T-cell depleted irradiated splenocytes, right panels). After 7 days, cells were restimulated by PMA and ionomycin for 4 h and the expression of IL-4 and IFN- γ was determined by intracellular cytokine staining. Representative data from three independent experiments are shown. (D) Cytokine production by SHN3-deficient naive CD4 T cells. CD4⁺CD62L^{hi} naive T cells from WT or SHN3-deficient mice were stimulated by plate-bound anti-CD3 (5 μ g/mL) plus soluble anti-CD28 (2 μ g/mL) Abs. After 7 days, CD4⁺T cells were restimulated by plate-bound anti-CD3 (1 μ g/mL) for 24 h. The concentration of cytokines produced was determined by ELISA: Th1 (IFN- γ , TNF- α) and Th2 (IL-4, IL-5, IL-13). Data shown are representative results from three independent experiments (*n* = 3). (E) Antigen-specific IgG1, IgG2a, and IgE in the serum from WT (○) or SHN3-deficient (●) mice immunized with OVA in alum (day 0). Mice were reimmunized with OVA in PBS on day 16. Serum from each mouse was obtained and examined at 0, 11, and 19 days after immunization by ELISA. (F) In vitro recall response by CD4⁺ T cells from WT (○) or SHN3-deficient (●) mice immunized with inactivated *S. mansoni* eggs. Splenocytes were collected 6 weeks after immunization. Splenocytes were then restimulated for 3 days with soluble SEA (0, 5, or 20 mg/mL) and the concentration of cytokines secreted was determined by ELISA using culture supernatants. (E and F) Data shown are pooled from at least (E) four or (F) eight independent experiments, \pm SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (Student's t-test).

Because T cells from SHN3-deficient mice showed a defect in IL-4 expression, we hypothesized that antibody production against an immunized antigen would be also impaired. SHN3-sufficient and -deficient mice were immunized with ovalbumin (OVA) and the OVA-specific antibody titer in serum was determined by ELISA for three Ig isoforms: IgG1, IgG2b, and IgE (Fig. 2E). IgE is a prototypic Th2-type isotype. Among IgGs, IgG1 is considered as a Th2-type antibody, while IgG2a and IgG2b are Th1-type antibodies, since IFN- γ enhances their production. SHN3-deficient mice produced a significantly lower level of OVA-specific IgG1 and IgE. In contrast, the levels of IgG2a were comparable between control and SHN3-deficient mice. Although SHN3-deficient mice showed a defect in IgG1 and IgE, the frequency of B-cell populations and accessory immune cells were comparable in SHN3-sufficient and -deficient mice (Supporting information Fig. S4). These data suggest that SHN3 expression in T cells is critical for the Th2-type generation of antibodies in vivo.

Th2 cells and the cytokines they produce play a critical role in immunity against parasitic infection [50]. To further determine the role of SHN3 in type 2 immunity, we injected SHN3-deficient and -sufficient mice intraperitoneally with *Schistosoma mansoni* eggs, which are known to elicit strong Th2 responses [51]. Six weeks after immunization, splenic T cells were isolated and restimulated in vitro by soluble egg antigen (SEA) (Fig. 2F). SHN3-deficient T cells produced significantly lower levels of IL-4, 5, and 13 in response to SEA stimulation ex vivo compared to control T cells. Production of the Th1 cytokine IFN- γ was mildly enhanced in control T cells. Though there was no statistical significance, SHN3-deficient T cells produced a slightly higher level of IL-17 compared to control mice, while IL-10 was similar. Together, these data indicate that SHN3-deficient mice have a specific defect in Th2 type immune response, but not in Th1 and Th17 responses.

Loss of Shn-3 exacerbates an autoimmune inflammatory bowel disease model

To investigate the in vivo effect of *Shn-3* deficiency on disease, we chose a mouse model system for T-cell-induced inflammatory bowel disease (IBD) [52]. The development of IBD in this model is worsened by T_H1 cytokines while T_H2 cytokines ameliorate the disease [53]. This model enables us to examine the function of *Shn-3*-deficient CD4 T cells in the WT environment where other CD4 expressing cells (e.g. NK T cells) are not affected by the loss of Shn3.

Regulatory T cell depleted CD4⁺CD25⁻CD45RB^{hi} T cells were purified from control or *Shn-3*-deficient mice by a cell sorter and were adoptively transferred into RAG1^{deficient} mice. After transfer, the body weight of the recipient mice was measured to monitor the conditions of the recipient (Fig. 3). After the adoptive transfer, all animals continued to gain weight at comparable rates up to 5 weeks. Thereafter, the weight of mice that had received the control mouse CD4 T cells started to drop substantially and after 60 days, the weight loss reached 85% of the peak weight (95% of the starting weight). Compared to the controls, mice

receiving *Shn-3*-deficient CD45RB^{hi} T cells showed more rapid weight loss and their weight reached 85% of the starting weight by 48 days after transfer (Fig. 3A). Histological examination also showed the development of more severe colitis in mice receiving *Shn-3*-deficient T cells than that of mice receiving control T cells (Fig. 3B).

To determine the effector subset of the donor T cells, we recovered CD4⁺T cells from spleen and mesenteric LNs of the recipient animals on day 60. Both control- and *Shn-3*-deficient donor T cells expressed comparable levels of activation- or memory- associated surface antigens. However, cytokine production by these transferred T cells showed substantial differences from control samples (Fig. 3C). In both spleen and mesenteric LNs, *Shn-3*-deficient T cells expressed markedly higher level of T_H1-type cytokines (IFN- γ and TNF- α) than T cells from control mice. IFN- γ and TNF- α double producers are particularly more prevalent in *Shn-3*-deficient T cells than in control (42.9 vs. 23.1%). Furthermore, 9% of control T cells expressed IL-4 while 5% of *Shn-3* deficient donor cells expressed IL-4. (Fig. 3C). These results indicated that deficiency of *Shn-3* in T cells exacerbated T_H1 effector T-cell differentiation or expansion while reducing T_H2 effector T-cell development in this autoimmune IBD model.

Exogenous IL-4 restores the Th2 defect in SHN3-deficient T cells

During naive CD4 T-cell differentiation, cytokines present in the surrounding environment greatly influence polarization into distinct Th subsets [54]. IL-4 and IL-2 are required for Th2 differentiation [55]. IL-4 induced-STAT6 activation is critical for a positive feedback loop that promotes IL-4 production by T cells [56, 57], and IL-2 induces GATA3 expression and IL-4 production [14, 58, 59]. As SHN3-deficient T cells have a defect in IL-4 and IL-2 production, we next questioned if SHN3 was critical for signaling downstream of cytokine receptors that induce Th2 differentiation.

To test this, we determined if SHN3-deficient T cells could differentiate into Th1 or Th2 subsets in the presence of exogenous cytokines (Fig. 4A). When cultured under the conditions that promote development of Th1 (IL-12 and anti-IL-4) or Th2 (IL-4 with anti-IL-12 and anti-IFN- γ), SHN3-deficient naive CD4 T cells expressed similar levels of IFN- γ (Th1) and IL-4 (Th2) as control mice. Therefore, SHN3-deficient naive T cells differentiated as effectively as control T cells into Th1 and Th2-subtypes. These data demonstrate that SHN3-deficient mice do not have a defect in reactivity and subsequent signaling in response to Th1- or Th2-inducing cytokines.

We next sought to determine the individual contributions of IL-4 and IL-2 to Th2 differentiation in SHN3-deficient mice. SHN3-deficient or control naive CD4 T cells were stimulated in the presence or absence of exogenous IL-4 or IL-2, and the frequency of IL-4- and IFN- γ -producing cells were determined by intracellular cytokine staining. Addition of exogenous IL-4 (≥ 1 ng/mL) effectively restored Th2 development by SHN3-deficient T cells (Fig. 4B and C). IL-4 also reduced the level of IFN- γ production

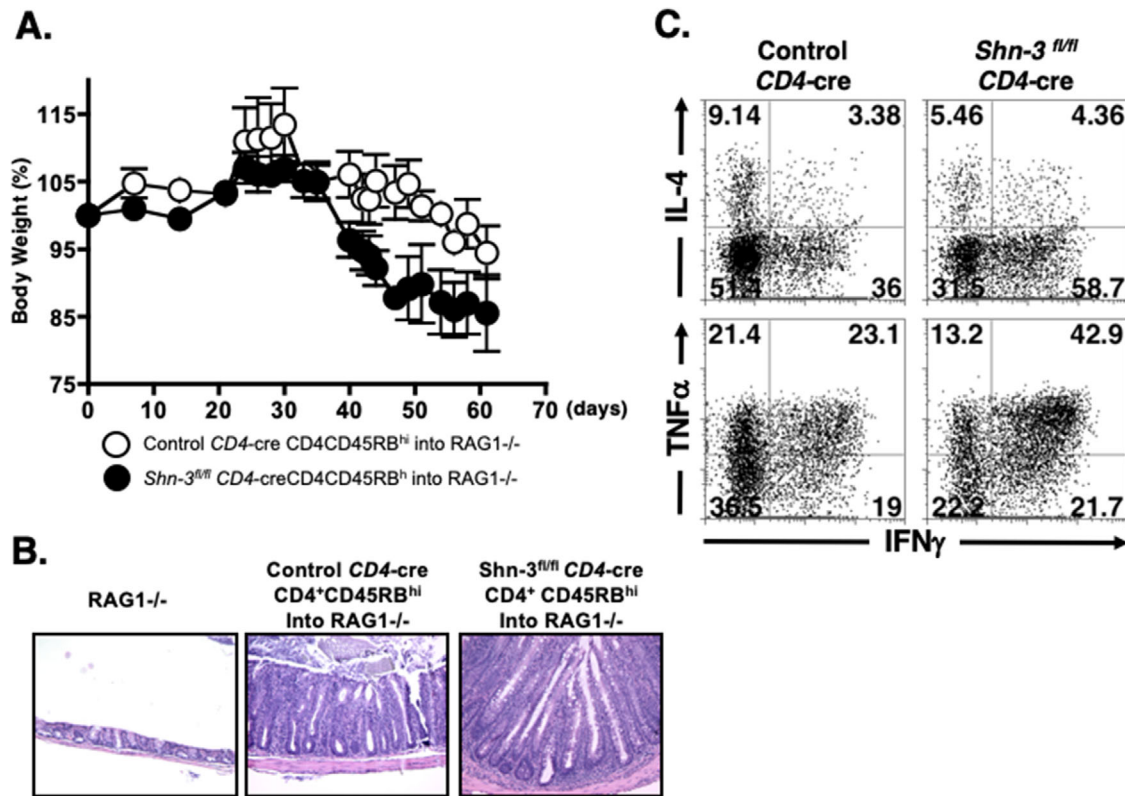


Figure 3. Responses of $Shn-3$ -deficient $CD4^+CD45RB^{hi}$ T cells in an experimental IBD model. (A) $RAG1$ -deficient mice were injected with 4×10^5 control (○) or $Shn-3$ -deficient (●) $CD4^+CD45RB^{high}CD25^{-}$ T cells. After injection, body weight of the host $RAG1^{deficient}$ mice was monitored as indicated (x-axis). $N = 3$. Data are shown as \pm SD and are representative of three independent experiments. (B) Eight weeks after cell transfer, the host $RAG1^{deficient}$ mice were sacrificed and the large intestines were removed. Cross-sections of fixed large intestines were stained with H & E. The size of each field corresponds to 0.6×0.6 mm of the tissue. (C) Spleen cells were isolated from the host $RAG1$ -deficient mice, and $CD4$ T cells were purified. Isolated $CD4$ T cells were restimulated in vitro with PMA ($50 \mu\text{g/mL}$) plus ionomycin ($1 \mu\text{M}$). After 4 h, IFN- γ , IL-4, and TNF- α levels were analyzed by intracellular cytokine staining. The data shown are representative of three independent experiments with three mice per group. Total $CD4$ T cells were used for the analysis.

by SHN3-deficient and control T cells (Fig. 4C). In contrast, addition of exogenous IL-2 failed to enhance IL-4 production by SHN3-deficient T cells, but increased IFN- γ production. Blocking IL-4 prevented the development of IL-4 $^+$ but not IFN- γ $^+$ cells, whereas blocking IL-2 prevented the development of IL-4 $^+$ and IFN- γ $^+$ cells in WT and SHN3-deficient T cells. As reported previously, IL-2 increased IL-4 production in control $CD4^+$ T cells [58, 60]. These data show that abrogated Th2 differentiation by SHN3-deficient T cells is caused by a reduction in early IL-4 production.

SHN3 controls transcription factors required for Th2 differentiation

To determine the mechanism by which SHN3 functions in early IL-4 expression, we examined transcription factors involved in $CD4^+$ T-cell differentiation: SHN2, T-bet, and GATA3. SHN2 inhibits Th2 development, in part by suppressing NF- κ B activation [36]. T-bet and GATA3 are critical transcription factors for Th1 and Th2 differentiation, respectively [17, 19]. We examined the mRNA levels of these transcription factors expressed by SHN3-deficient and -

sufficient $CD4^+$ naive T cells, with and without stimulation over 3 days (Fig. 5A). Expression of *SHN2* and *T-bet* by SHN3-deficient and -sufficient $CD4^+$ T cells was relatively comparable. In contrast, SHN3-deficient $CD4^+$ T cells showed a marked decrease in the expression of *GATA3* mRNA. Expression of *GATA3* mRNA by control cells started to increase after 2 days of stimulation and continued to increase on day 3. Expression of *GATA3* in SHN3-deficient T cells was substantially less than control on days 2 and 3. *GATA3* protein expression by stimulated SHN3-deficient T cells was also markedly reduced after stimulation, while *T-bet* expression was comparable (Fig. 5B).

IL-4 promotes expression of *GATA3* during the early stage of Th2 differentiation [61]. We hypothesized that the deficiency in *GATA3* expression was due to the lack of IL-4 production in SHN3-deficient mice. When we stimulated SHN3-deficient cells under Th2-polarizing conditions with the addition of IL-4, we found that IL-4 restored the defect in *GATA3* expression in SHN3-deficient mice (Fig. 5C). Together, these data suggested that SHN3-deficient T cells have a defect in early IL-4 expression, which causes a reduction in *GATA3* and subsequent Th2 differentiation.

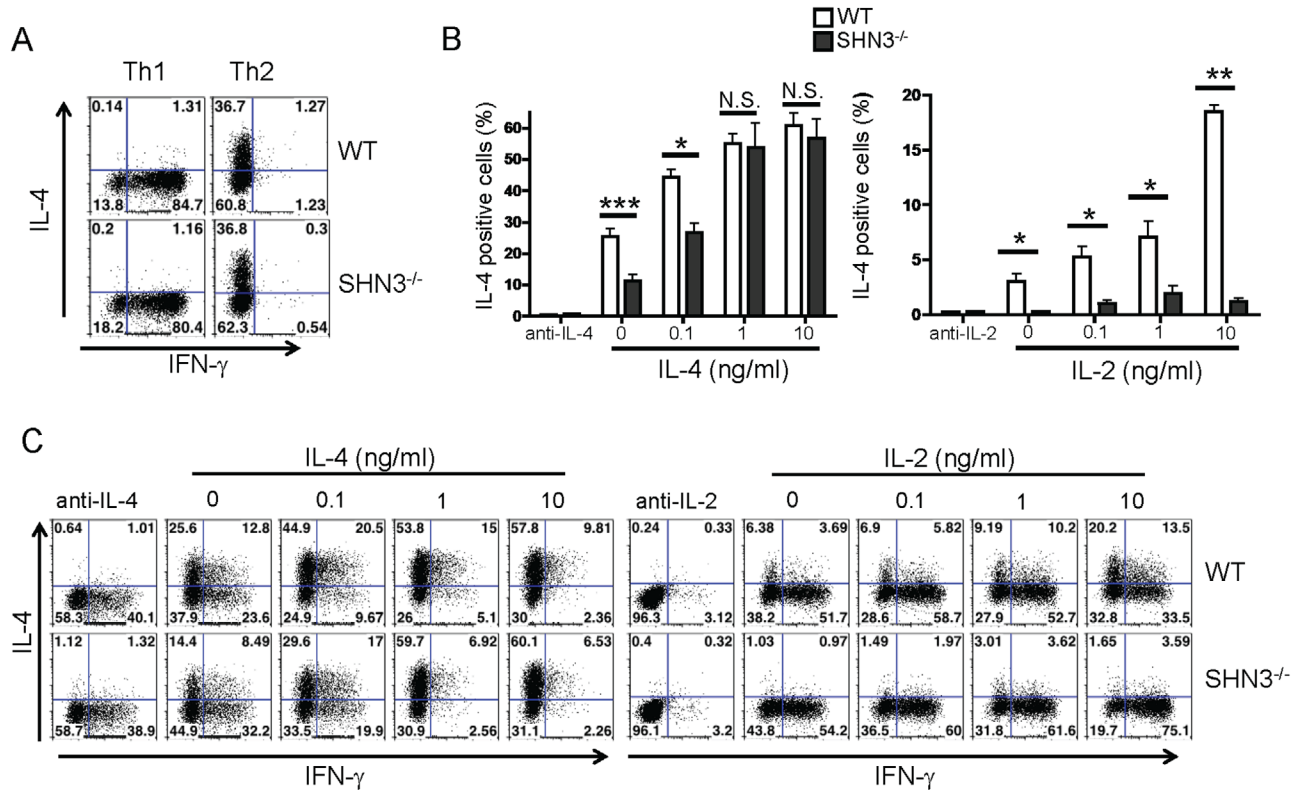


Figure 4. Exogenous IL-4 restores Th2 phenotype and IL-4 production in SHN3-deficient T cells. (A) IL-4 and IFN- γ production by WT (upper panels) or SHN3-deficient (lower panels) T cells under Th1- or Th2-inducing conditions. Naïve CD4⁺ T cells were stimulated by plate-bound anti-CD3 (5 μ g/mL) plus soluble anti-CD28 (2 μ g/mL) for 7 days. Cells were then restimulated by PMA and ionomycin for 4 h and the expression of IL-4 and IFN- γ was determined by intracellular cytokine staining. Results are representative of three independent experiments. (B, C) IL-4 and IFN- γ production by SHN3-deficient or WT T cells in the presence of exogenous IL-4 or IL-2. CD4⁺CD62L^{hi} naïve T cells were stimulated by anti-CD3 plus anti-CD28 Abs, in the presence of recombinant IL-4 (B, left panel) or IL-2 (B, right panel) at the concentrations shown for 7 days. Cells were then restimulated by PMA plus ionomycin for 4 h and the expression of IL-4 and IFN- γ was determined by intracellular cytokine staining. Experiments were performed three times. (B) Data are shown as \pm SD. * p < 0.05, ** p < 0.01, *** p < 0.001. N.S., not significant (Student's t -test).

SHN3 deficiency impairs activation or expression of Jun family transcription factors

Previous studies showed that NF- κ B and AP-1 are downstream targets of SHN3 [37–39]. Early IL-4 production and Th2 differentiation by T cells critically depends on these transcription factors [62, 63]. To determine if SHN3-deficient T cells have impaired NF- κ B and AP-1 activation, we determined the nuclear levels of NF- κ B and AP-1 in resting and activated T cells using a DNA gel shift assay (Fig. 6A). Unstimulated SHN3-deficient and WT samples showed similar levels of NF- κ B and AP-1. In activated CD4⁺ T cells, the levels of the nuclear NF- κ B complex were similar between activated SHN3-deficient and -sufficient CD4⁺ T cells. In contrast, the nuclear AP-1 complex in SHN3-deficient T-cell extracts was significantly reduced compared to control.

AP-1 is a complex formed by a homodimer of Jun proteins or a heterodimer between Fos and Jun proteins [64, 65]. To determine the specific members of AP-1 that are affected by the loss of SHN3, we performed gel-supershift assays using anti-c-Jun, JunB, JunD, or c-Fos antibodies (Fig. 6B). In control CD4⁺ T cells, the addition of anti-JunB, anti-JunD, and anti-c-Fos antibodies

showed a gel shift, while the addition of anti-c-Jun showed little, if any effect. This suggests that JunB, JunD, and c-Fos are the major components of AP-1 in activated CD4⁺ T cells. Compared to control T cells, SHN3-deficient T-cell nuclear extracts showed markedly reduced detection of AP-1 components. Anti-JunB and JunD antibodies induced a supershift of the remaining AP-1 complex. Together, these data show that nuclear AP-1 complex, particularly Jun proteins, is reduced in SHN3-deficient T cells.

To confirm the deficiency of AP-1 complex proteins in SHN3-deficient T cells, we performed western blot analysis of AP-1 components with and without stimulation (Fig. 6C). In resting T cells, c-Jun, JunB, JunD, and c-Fos were not detectable in the cytoplasm or nucleus of SHN3-deficient and control cells. By 72 h of stimulation, these proteins were clearly detectable in the cytoplasm and nuclei of the control cells. While SHN3-deficient and -sufficient T cells expressed comparable levels of Fos and Jun proteins (c-Jun, JunB, JunD) in the cytoplasm, SHN3-deficient T cells expressed lower levels of c-Jun and JunB, but not JunD in the nucleus. To determine if SHN3 controls Jun proteins transcriptionally, we determined the levels of *c-jun* and *junB* mRNA using quantitative PCR. There were no significant

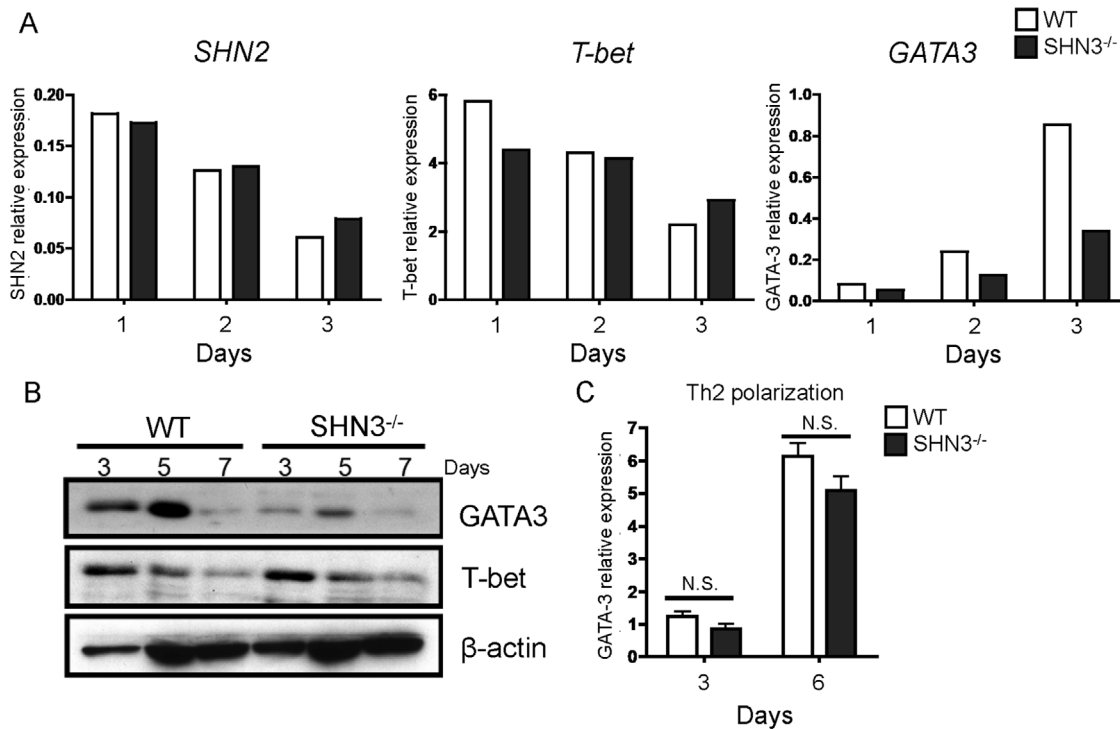


Figure 5. Expression of IL-4-promoting transcription factors by SHN3-deficient CD4⁺T cells. (A) CD4⁺CD62L^{hi} naive T cells from SHN3-sufficient (open bars) or -deficient mice (closed bars) were stimulated by plate-bound anti-CD3 (5 μg/mL) plus anti-CD28 (2 μg/mL) Abs. After 1–3 days, mRNA was isolated and real-time PCR was performed to determine the expression of SHN2, T-bet, and GATA3 mRNA. Relative expression levels against the control gene expression (*gapdh*) are shown. (B) Western blot analysis of GATA3 and T-bet. SHN3-sufficient or -deficient cells were stimulated as in (A), then harvested 3, 5, or 7 days after stimulation. Total cell lysates were used to determine the level of GATA3 (top), T-bet (middle), or β-actin (bottom) by Western blot. (C) GATA3 expression by cells stimulated under Th2-inducing conditions. WT (open bars) or SHN3-deficient (closed bars) CD4⁺ T cells were stimulated as in (A) except for the presence of cytokines and Abs that promote Th2 polarization (IL-4, anti-IFN-γ, anti-IL-12). Cells were stimulated for 3 or 6 days then harvested to isolate mRNA. Expression of GATA3 was determined by real-time PCR. Relative expression levels against the control gene expression (*gapdh*) are shown. (A–C) Data are shown as ±SD and are representative of three independent experiments. N.S.: not significant (Student's t-test).

differences in the expression levels of *c-jun* and *junB* mRNA between stimulated SHN3-deficient and control T cells (Fig. 6D). These results suggest that SHN3 is required for the nuclear localization of c-Jun and JunB proteins in activated T cells.

SHN3 synergizes with CD28 signaling to enhance AP-1 activity

Our data suggest that SHN3 plays a critical role in c-Jun and JunB nuclear localization in activated T cells. This model predicts that an increase in SHN3 expression would enhance AP-1 activity. To test this hypothesis, we overexpressed SHN2 or SHN3 in Jurkat human leukemic T cells and determined the effect on the AP-1 activity (Fig. 7A and B). Overexpression of SHN3 resulted in approximately a 10-fold increase in AP-1 activity in PMA/ionomycin (P + I)-activated Jurkat cells (Fig. 7A), while SHN2 overexpression induced less than a twofold increase (Fig. 7B). This confirmed that SHN3 enhances the activity of AP-1. CD28 plays a pivotal role in Th2 development and activates AP-1 [66–68]. We, therefore, sought to determine if SHN3 overexpression enhances CD28-mediated AP-1 activation (Fig. 7C

and D). In the absence of CD28 stimulation, SHN3 overexpression increased AP-1 activity more than 10-fold (Fig. 7C). Importantly, CD28 engagement and SHN3 overexpression further amplified AP1 activity more than twofold. In contrast, SHN2 overexpression with CD28 stimulation only mildly increased AP-1 activity (Fig. 7D). Together, these data show that SHN3 promotes AP-1 activity in activated T cells and can enhance CD28-mediated AP-1 activation.

SHN3 controls expression of the CARMA1-Bcl10 complex, upstream of JNK/AP-1, and NF-κB activation

Our results have demonstrated that SHN3-deficient T cells have decreased activity of the AP-1 components c-Jun and JunB, and decreased expression of GATA3. GATA3, and JunB directly promote IL-4 production and Th2 differentiation [23, 24]. We aimed to determine the signaling processes upstream of AP-1 and GATA3 that cause the subsequent defect in IL-4 and Th2 differentiation in SHN3-deficient T cells. The CARMA1-BCL10-MALT1 (CBM) complex is required for the expression and activation of c-Jun, JunB, and GATA3 [69, 70]. T-cell receptor stimulation and CD28

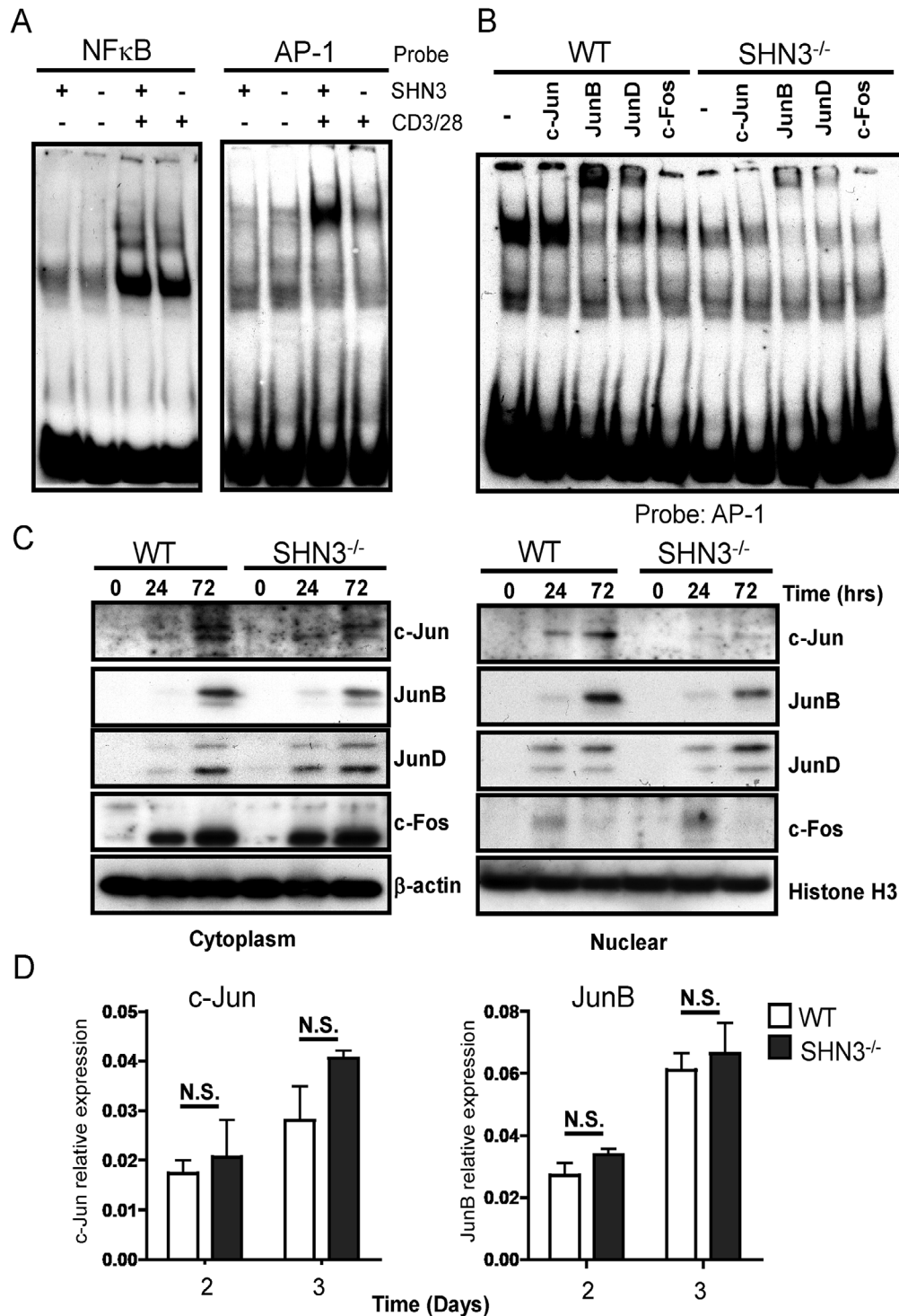


Figure 6. Reduced nuclear localization of AP-1 proteins by activated SHN3-deficient CD4⁺ T cells. (A, B) Gel-shift assay for NF-κB and AP-1. (A) SHN3-sufficient (indicated by +) or -deficient (indicated by -) CD4⁺ T cells were left unstimulated (indicated by -) or stimulated by plate-bound anti-CD3 (5 μg/mL) plus anti-CD28 (2 μg/mL) (indicated by +) for 72 h. Nuclear extracts were harvested after stimulation and were mixed with the oligonucleotide probes encoding the bindings sites for NF-κB (left panel) or AP-1 (right panel). DNA-protein complexes were subjected to EMSA analysis for each sample. (B) Stimulation conditions and extracts were harvested as in (A), then a supershift assay was performed using AP-1 component-specific antibodies (as indicated above each lane). (C) Cytoplasmic and nuclear levels of AP-1 components in activated WT or SHN3-deficient T cells. Cells were unstimulated or stimulated by plate-bound anti-CD3 (5 μg/mL) plus anti-CD28 (2 μg/mL) for 0, 24, or 72 h. Cytosolic (left panel) and nuclear extracts (right panel) were subjected to immunoblotting using anti-c-Jun, JunB, JunD, c-Fos, β-actin, or Histone H3 antibodies as indicated. (D) Expression of *c-jun* and *junB* mRNA by WT or SHN3-deficient CD4⁺ T cells. WT (open bars) or SHN3-deficient (closed bars) CD4 T cells were stimulated as in (C) and mRNA was isolated after 2 or 3 days of stimulation. mRNA levels of *c-jun* and *junB* were determined by real-time PCR. (A–D) Data are representative of three independent experiments. (D) Data are shown as ±SD. N.S., not significant (Student's t-test).

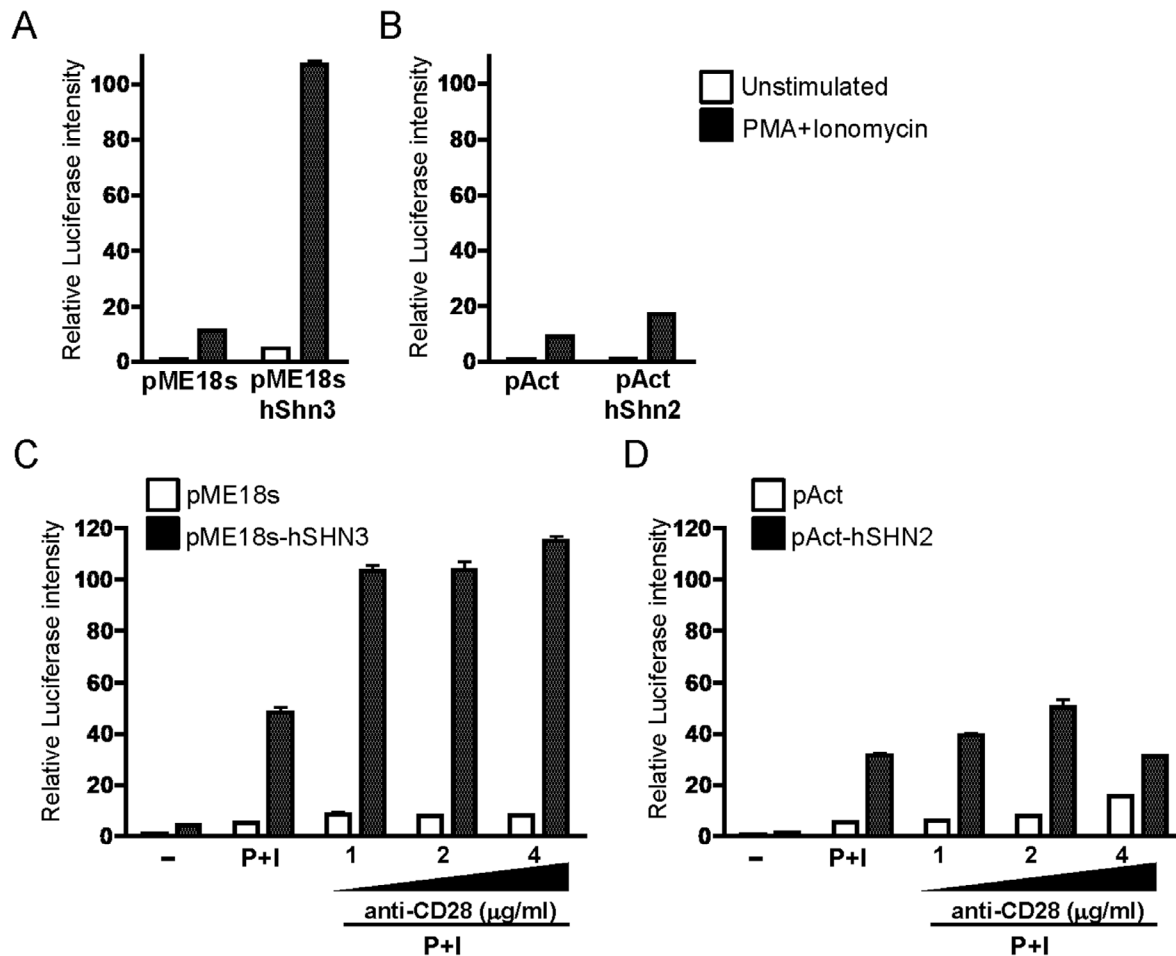


Figure 7. Synergistic effect of SHN3 overexpression and CD28 engagement on AP-1 activity (A, B) Jurkat T cells were transfected with AP-1 Luciferase reporter plasmid together with expression vectors for (A) SHN3, or (B) SHN2. Empty vector for each construct (pME18s, pAct, respectively) was used as the control. Transfection efficiency was normalized using Renilla luciferase expression by cotransfected pRL-TK vector. Twenty-four hours after transfection, cells were stimulated by PMA plus ionomycin. Luciferase activity was determined by triplicates for each sample. (C, D) Cells were transfected as in (A), then stimulated by PMA + ionomycin in the absence or presence of anti-human CD28 antibodies (1–4 μg/mL) for 20 h, then Luciferase activity was determined as in (A). (A–D) Data are shown as ±SD. Experiments were performed three times with similar results.

costimulation causes activation of PKC θ and phosphorylation and activation of CARMA1 [71, 72]. Once activated, conformational changes in CARMA1 cause recruitment of Bcl10 and MALT1 to form the CBM complex [73, 74]. CBM complex formation leads to the activation of the NF- κ B and MAPK/JNK pathways [75, 76].

CARMA1-deficient T cells have a defect in IL-4 production, GATA3 and JunB expression levels, and Th2 differentiation [77]. SHN3-deficient T cells have a similar phenotype to CARMA1-deficient T cells. Therefore, we asked whether SHN3 affects the expression of proteins in the CBM complex. We examined unstimulated or stimulated naïve CD4⁺ T cells from SHN3-deficient or control mice and assessed protein levels of the CBM complex (Fig. 8A and B). WT and SHN3-deficient naïve CD4⁺ T cells have comparable levels of CARMA1 protein expression prior to stimulation (day 0). One day after stimulation, SHN3-deficient T cells have decreased CARMA1 expression compared to WT littermate

controls. Bcl10 protein expression was similar in WT and SHN3-deficient T cells for both day 0 and 1. Together, these data show that SHN3 promotes CARMA1 expression in stimulated naïve CD4⁺ T cells, while SHN3 does not control expression of Bcl10. Since CARMA1 plays an essential role in JNK activation, we examined the levels of JNK and p-JNK (Thr183/Tyr185) in nuclear fraction of SHN3-deficient and WT T cells on day 0 and 1 day after stimulation. When JNK is phosphorylated at Thr183/Tyr185, this activates JNK to promote phosphorylation and activation of c-jun and JunB. The level of phosphorylated JNK (primarily the top band, isoform p54) was decreased in the nuclear fraction of SHN3-deficient T cells 1 day after stimulation compared to WT T cells (Fig. 8C). Total nuclear JNK protein level was not significantly reduced in SHN3-deficient T cells across multiple experiments (Fig. 8D). These data suggest that SHN3 controls the JNK/Jun pathway by promoting the expression of CARMA1, which leads to the phosphorylation and activation of JNK.

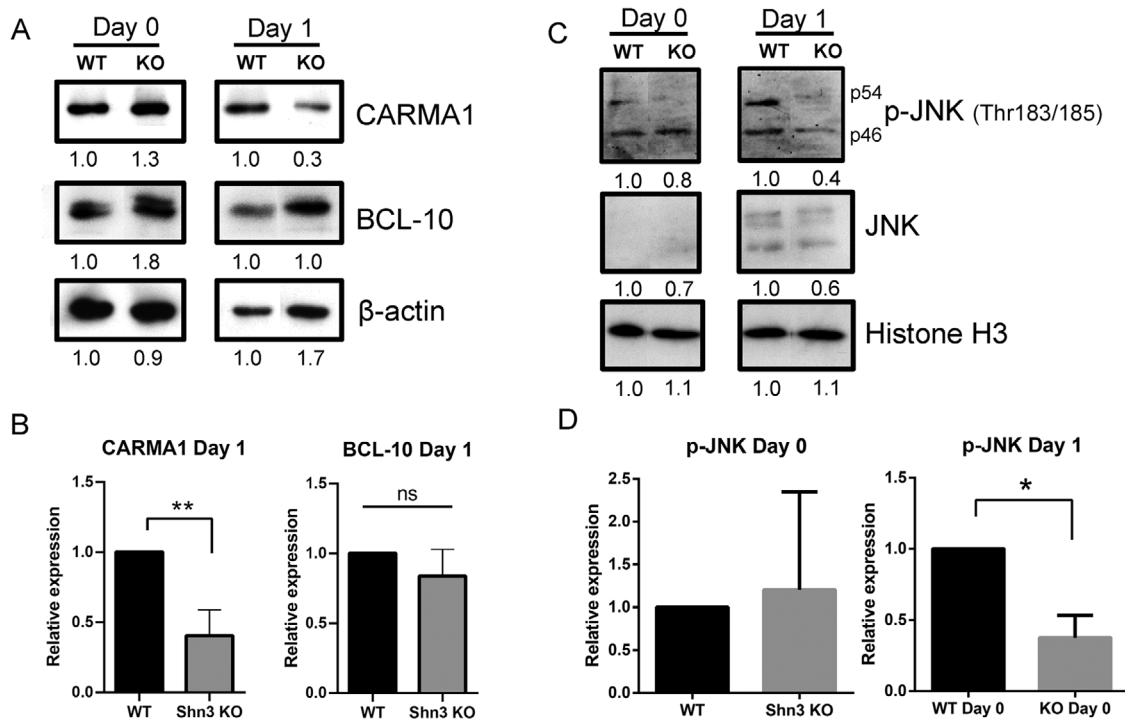


Figure 8. SHN3-deficient mice show reduced expression of CARMA1 and downstream p-JNK (A–D) Naïve CD4⁺ T cells were isolated from the spleens of SHN3-deficient and WT littermate control mice. Lysates were made from unstimulated naïve CD4⁺ T cells or 1 day after stimulation with anti-CD3/CD28. (A, B) Total cell lysates were used to determine the level of CARMA1 (top), BCL-10 (middle), or β-actin (bottom) by Western blot. (C, D) Nuclear extracts were prepared to determine p-JNK (top), total JNK (middle), and histone H3 by western blot. Data are shown as ±SD and representative of at least three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (Student's paired *t*-test).

Discussion

Our data show that SHN3 is required for the expression of IL-4 during early stages of CD4⁺ T-cell activation. Early IL-4 production by naïve CD4⁺ T cells is a critical process in Th2 differentiation. During the first 3 days of stimulation, SHN3-deficient T cells expressed significantly lower levels of IL-4. When IL-4 was added to SHN3-deficient T cells during the early phase of T-cell activation, GATA3 expression became comparable to SHN3-sufficient T cells. Together, these data suggest that loss of SHN3 primarily impaired early IL-4 production, which subsequently led to reduced GATA3 expression.

A reduction in early IL-4 production may be caused by the impairment of AP-1 activation. SHN3-deficient T cells expressed reduced levels of the AP-1 complex after TCR activation. Nuclear JunB and c-Jun were substantially reduced by the loss of SHN3. CARMA1, which couples TCR stimulation to c-Jun/JunB activation, was significantly reduced in SHN3-deficient T cells. Since SHN3-deficient and -sufficient T cells expressed comparable mRNA levels of *junB* and *c-jun*, we conclude that SHN3 controls JunB/c-Jun activation in a post-transcriptional manner via CARMA1.

JNK plays a critical role in post-translational regulation of Jun family proteins. Under resting conditions, c-Jun and JunB form a complex with JNK2, which phosphorylates Jun proteins to induce

ubiquitination and degradation. JunD is not subject to the degradation caused by JNK2 phosphorylation and expression. When T cells are activated by TCR stimulation, activated MEKK1 releases c-Jun and JunB from JNK2 and enables phosphorylation by JNK1, which stabilizes the proteins.

Data presented here demonstrate that JunD is not affected by loss of SHN3 and suggest that SHN3 may inhibit JNK2-dependent degradation. A previous study showed that SHN3 did not affect JNK kinase activity, but directly interacted with c-Jun and enhanced its function [39]. Our data show that SHN3 is critical for p-JNK expression in the nucleus of activated naïve CD4 T cells. The CBM complex is upstream of TAK1, MKK7, and JNK activation. Our data show that SHN3 is critical for CARMA1 expression one day after activation. Loss of CARMA1 is a likely cause of impaired Jun activation and production of IL-4. We currently do not know how SHN3 controls expression of CARMA1. SHN3 may act as a transcription factor to directly regulate expression of CARMA1 or upstream T-cell signaling molecules, which is currently under investigation.

Contrasting phenotypes of SHN2- and SHN3-deficient mice inevitably prompts us to consider the possibility that these two proteins mediate a yet identified mechanism that controls Th2-cell differentiation. Th2 development was highly elevated in SHN2-deficient mice [36]. In contrast, SHN3 deficiency caused impairment in Th2 development. The time course and strength

of stimulation required for SHN2 and SHN3 expression during T-cell activation also suggests that these two proteins may be functionally connected. While SHN2 is expressed by resting T cells, SHN3 is rapidly induced after activation. Although SHN2 and SHN3 share structural similarities, they carry opposite functions in Th2 development. SHN2 suppresses Th2 differentiation in part by NF- κ B repression by binding to the consensus NF- κ B sequence and competing off the binding of NF- κ B. Loss of SHN2 led to hyperactivation of the NF- κ B complex and caused elevated expression of NF- κ B target genes [36]. Loss of SHN3 caused little, if any, effect on NF- κ B nuclear localization, based on our gel shift assays. Rather, our data show that SHN3 mainly enhances AP-1 via activation of c-Jun/JunB. Importantly, CARMA1 is involved in both NF- κ B and c-Jun/JunB activation [78]. How loss of CARMA1 protein in SHN3-deficient T cells causes impaired JNK, but not NF- κ B activation is currently under investigation.

The data presented here suggest a potential link between the SHN2/SHN3 axis and the antigen-receptor signal-dependent regulation of the early phase of IL-4 expression. Previous studies showed that the strength and/or duration of antigen-receptor stimulation can dictate Th2 differentiation by early IL-4 production [79]. The threshold of TCR stimulation required for SHN2 repression and SHN3 expression was similar. Importantly, higher doses of anti-CD3 antibody were required to induce SHN3 and IL-4 and to reduce SHN2 expression. Lower concentrations of anti-CD3 antibody did not reduce SHN2 expression and IL-4 as well as expression of SHN3 was not observed. Based on these observations, we propose a model that the expression of SHN3 and repression of SHN2 sets the threshold for expression of “early IL-4” by naive T cells and a skewing toward Th2 differentiation.

While this model explains how a medium dose of antigen induces IFN- γ expression and higher dose peptide induces IL-4, it does not match earlier observations that says low affinity peptide induces IL-4 more efficiently than higher affinity peptide [80]. We think this is due to the sustained signal initiated by the low affinity peptide. It was shown that high affinity peptide, but not low affinity peptide, induces rapid internalization of surface TCRs [81]. Similarly, it was found that the high antigen dose that reduces surface TCR clearly correlates with the dose that inhibits IL-4 production [13]. This downregulation likely causes a reduction in sustained TCR signaling. It has been well established that intermediate affinity antigen peptide promotes positive selection of thymocytes via sustained TCR signaling [82–84]. High affinity peptides induce a robust signaling in T cells, such as ERK signaling, but the activation decreases very rapidly. In contrast, lower affinity peptide induces milder, but much more sustained signaling, presumably due to the continuous triggering of TCR since the surface expression of TCR is not reduced by the antigen stimulation.

In our experimental setting, T cells are stimulated by anti-CD3 antibody, which reduces the speed of receptor internalization. This would lead to a dose-dependent increase of signal duration in T cells. It should be noted that we have previously demonstrated that expression of IL-2 requires sustained ERK signaling and proposed a model for how sustained signaling controls the expression

of cytokine genes [42, 40]. SHN3 is a target of sustained ERK signaling in T cells. The fact that lower affinity antigen peptide as well as lower dose antigen peptide induces IL-4 better than higher dose antigen may be in part due to the sustained signaling that promotes expression of SHN3 and repression of SHN2. Together, these data illustrate the SHN2/SHN3 axis as a new mechanism controlling Th1 or Th2 differentiation.

Materials and methods

Generation of T-cell-specific SHN3-deficient Mice

Mouse genomic DNA for the SHN3 gene was isolated from a mouse 129-strain genomic λ phage library (a kind gift from Dr. Nancy Manley, University of Georgia). We constructed a targeting vector by cloning the genomic DNA fragment into the pBluescriptII-KS(+) plasmid vector (Stratagene, CA). A Herpes simplex virus thymidine kinase cassette and a PGK-Neo cassette flanked by FRT sequences (PGK-neo/FRT cassette) were inserted at the 5'- and 3'- flanking region of exon 4 as indicated in Supporting information Figure S2A. An EGFP gene followed by a loxP sequence and an IRES was inserted in the 5' end of the translation initiation site of SHN3. The targeting construct was linearized and transfected into ES cells from 129/C57BL/6 F1 mice (performed by ES core facility, Medical College of Georgia, Augusta, GA). Homologous recombinants were identified by Southern-blot analysis. Clones carrying the targeted allele (SHN3^{fl}) were injected into blastocytes from C57BL/6 and were implanted into foster mothers. Chimeric mice were bred back to C57BL/6 mice, and the F1 generation was screened for germline transmission of the targeting allele. The Neomycin selection marker was depleted by backcross with FLPeR transgenic mice (purchased from Jackson Laboratories, Bar Harbor, Maine). CD4-Cre mice expressing Cre recombinase under control of the mouse *CD4* gene regulatory region were purchased from Jackson Laboratories and were bred with SHN3 flox/flox mice to generate mice to delete SHN3 gene in a T-cell-specific manner.

We used the following primers (their locations are shown in Supporting information Fig. S2) to detect the SHN-3 WT allele and SHN3^{fl} allele: a, 5'- TCTCCGACCTTCAGCTGTTC -3'; b, 5'- GGACTTGAAGAAGTCGTGCT -3'; c, 5'- ACTTCCATCAGCCTTCTTGG -3'.

All mice were backcrossed to C57BL/6 mice for over eight generations and were maintained under specific pathogen-free conditions. All animal experiments were conducted in accordance with the protocol approved by Institutional Animal Care and Use Committee (IACUC).

Flow cytometry analysis

Single-cell suspensions were prepared from the thymus, spleen, and LN by mechanical disruption of small fragments of organ

between frosted glass slides followed by depletion of RBCs with ACK lysis buffer (Invitrogen, NY). Cells were then washed twice with 0.5% FCS in RPMI 1640 (Thermo Scientific, NH) and filtered through nylon mesh to make single-cell suspensions. Cells were pretreated with anti-Fc receptor antibody (2.4G2; anti-CD16/CD32; provided by Dr. M. Kubo, RCAI, Riken, Japan) at room temperature for 15 min. Cells were then incubated on ice for 30 min with specific Abs to CD4 (GK1.5), CD8 (53.6.7), CD25 (PC61), CD69 (H1.2F3), CD44 (IM7), CD62L (MEL-14), B220 (RA3-6B2), IgM (RMM-1), IgD (11-26c.2a), CD80 (16-10A1), CD86 (GL-1), CD11b (M1/70), and CD11c (N418). Antibodies were purchased from BioLegend (San Diego, CA). Cells were analyzed on a FACSCanto (BD Biosciences, San Jose, CA) with Flowjo software (Ashland, OR).

T-cell activation and RNA isolation

Splenic naive CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells from C57BL/6 or P25 TCR transgenic mice were isolated using magnetic-beads (Miltenyi Biotec, or BioLegend) following the manufacturer's directions. Purified T cells were then stimulated with plate-bound anti-CD3 antibody (5 µg/mL; 2C11, eBioscience) and soluble anti-CD28 (2 µg/mL; 37.51; eBioscience), or with antigen peptide (amino acids residues 240–254 [FQDAYNAAGGHNAVF] of Ag85B, 5 µg/mL) pulsed irradiated T-cell-depleted splenocytes from syngeneic mice (P25), as published previously [43]. After 1 to 5 days of stimulation, T cells were purified using a CD4 T-cell purification kit (BioLegend). Purified T cells were subject to total RNA isolation and cDNA synthesis using commercially available kits (RNAeasy from Qiagen, high-capacity cDNA reverse transcription kit, Thermo Fisher Scientific). For intracellular cytokine analysis, naive CD4 T cells were labeled with CFSE and stimulated by plate-bound anti-CD3 (5 µg/mL) plus anti-CD28 (2 µg/mL) Abs. After culturing for the indicated times, cells were restimulated (P + I) and subjected to intracellular staining with anti-IL-4 mAb.

CD4 T-cell differentiation

Naive CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells were isolated using magnetic beads (Miltenyi Biotec, or Biolegend), following the manufacturer's directions and were activated with plate-bound anti-CD3 antibody (5 µg/mL; 2C11, eBioscience) and soluble anti-CD28 (2 µg/mL; 37.51; eBioscience) in the presence of polarizing cytokines or/and blocking antibodies. For T_H1 polarization, anti-IL-4 (10 µg/mL; 11B11; eBioscience) and IL-12 (10 ng/mL; Peprotech) were used. For T_H2 polarization, anti-IFN-γ antibody (10 µg/mL; XMG1.2; eBioscience) and recombinant IL-4 (10 ng/mL or doses indicated in figures; Peprotech) were added to the stimulated T cells. For T_H-17 polarization, cells were stimulated in the presence of recombinant IL-6 (20 ng/mL; R&D) and TGF-β (2.5 ng/mL; R&D) with or without anti-IFN-γ (2 µg/mL; XMG1.2; eBioscience) and anti-IL-4 (2 µg/mL; 11B11, eBioscience) antibodies. For iT_{reg} differentiation, cells were stimu-

lated with TGF-β (2.5 ng/mL; R&D), IL-2 (10 ng/mL, Peprotech), anti-IFN-γ antibody (2 µg/mL; XMG1.2), and anti-IL-4 antibody (2 µg/mL; 11B11). After 7 days, cells were restimulated by P + I for 4 h and the expression of IL-4-, IL-17, IFN-γ, and TNFα-producing cells was analyzed by intracellular cytokine staining.

T-cell proliferation assay

CD4⁺T cells were stimulated with various concentrations of plate-bound anti-CD3 antibody with or without soluble anti-CD28 antibody (1 µg/mL) for 36 h and pulse labeled with 1 µCi of [³H]thymidine for an additional 8 h. The proliferative responses were measured as [³H]thymidine incorporation.

Quantitative real-time PCR

Gene expression was examined with a Bio-Rad iCycler Optical system with an iQ SYBR Green Real-Time PCR kit (Bio-Rad Laboratories, CA). Data were normalized to expression of mouse *Hypoxanthine-guanine phosphoribosyltransferase* (HPRT, a reference gene). Primers were mouse T-bet forward, 5'- AACCAGTATCCTGTTCCCAGC -3', and reverse, 5'- TGTCGCCACTGGAAGGATAG -3'; mouse GATA3 forward, 5'- CTCCTTTTGTCTCTCTTTTC -3', and reverse, 5'- AAGAGATGAGGACTGGAGTG -3'. Mouse HPRT, mouse *SHN2*, and mouse *SHN3* primers and probes were from ABI (GenBank accession code Mm00446968_m1, Mm00468908_m1, and Mm00495184_m1).

ELISA

Freshly isolated CD4⁺T cells or differentiated helper T cells were stimulated as indicated. Cytokines were quantified by sandwich ELISA using the following pairs of mAbs: anti-IL-2 (JES6-1A12)/biotin-anti-IL-2 (JES6-5H4), anti-IL-4 (11B11)/biotin-anti-IL-4 (BVD6-24G2), anti-IL-5 (TRFK5)/biotin-anti-IL-5 (TRFK4), anti-IL-10 (JES5-16E3)/biotin-anti-IL-10 (JES5-2A5), anti-IL-13 (eBio13A)/biotin-anti-IL-13 (eBio16H), and anti-IFN-γ (R4-6A2)/biotin-anti-IFN-γ (XMG1.2), anti-TNF-α (1F3F3D4)/biotin-anti-TNF-α (TN3-19).

Cell lysis and western blot

For western blot analysis, cells were lysed in the SDS sample buffer (containing 2% SDS, 5% beta-mercaptoethanol, 5% glycerol, and 62 mM Tris, pH 6.5), boiled, and frozen at -80°C until analysis. Lysates from an equal number of cells were loaded in each well, followed by electrophoresis and blotting onto PVDF membrane. Membranes were hybridized with antibodies specific for T-bet, GATA3, CARMA1, BCL-10, p-JNK, JNK, Histone H3, or β-actin, followed by HRP-conjugated secondary antibodies (Cell

Signaling). Bands were detected by the enhanced chemiluminescence system (GE Health Care).

Preparation of nuclear extracts

CD4⁺ T cells were stimulated with plate-bound anti-CD3 (5 µg/mL) and/or soluble anti-CD28 (2 µg/mL) for 24–72 h, as described above. After washing in cold PBS, cell pellets were resuspended in 200 mL buffer A (10 mM HEPES-KOH [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSE, 10 µg/mL aprotinin, 50 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM NaF, and 1 mM NaVO₄) and incubated on ice for 10 min. Then, 12.5 mL of 10% Nonidet P-40 solution was added and mixed carefully. Cells were immediately centrifuged at 15 000 rpm for 1 min at 4°C. The nuclear pellet was washed twice with 200 µL of buffer A and centrifuged again at 15 000 rpm for 1 min at 4°C. Pelleted nuclei were resuspended in 50 µL of buffer C (20 mM HEPES-KOH [pH 7.9], 1.5 mM MgCl₂, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM DTT, 1 mM PMSE, 10 µg/mL aprotinin, 50 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM NaF, and 1 mM NaVO₄) and incubate at 4°C for 15 min on shaking platform. The suspension was vortexed briefly and centrifuged at 15 000 rpm for 5 min at 4°C. Supernatants were stored at –80°C.

Electric mobility gel shift assay (EMSA)

Electric mobility gel shift assay (EMSAs) were done with the DIG Gel Shift kit (Roche Diagnostics) following the manufacturer's suggested protocol. Probe DNA fragments were digoxigenin (DIG)-labeled. EMSAs for NF-κB and AP-1 were performed with 2 µg of nuclear extract incubated with 1 µg of poly(dI:dC) in 20 mL of 1× binding buffer (10 mM Hepes [pH 7.6], 60 mM KCl, 0.1 mM EDTA, 1 mM DTT, 4% glycerol, and 0.2 µg/mL BSA) with DIG-labeled probe for 20 min at room temperature. Complexes were resolved on 5% nondenaturing PAGE with 0.5-fold TBE running buffer. DNA–protein complexes were electroblotted to nylon membrane for 1 h at 280 mA and the bands shift were visualized according to the user's manual for the DIG Gel Shift kit. For the supershift assay, we incubated protein–DNA complexes with 4 µg of antibodies specific for c-Jun (sc-45x), Jun B (sc-46x), Jun D (sc-74x), and c-Fos (sc-52x) (all from Santa Cruz Biotech) for 15 min on ice before electrophoresis. Sequence for the probes NF-κB: 5'-TCGAAGTTGAGGGGACTTCCAGGC-3'. AP-1: 5'-TCGACGCTTGATGAGTCAGCCGGAA-3'.

OVA immunization

Mice (eight to nine per group) were immunized intraperitoneally with OVA (1 mg/mL) in alum (day 0), reimmunized with OVA (in PBS) on day 16. OVA-specific serum IgG1, IgG2a, and IgE in SHN3 control and SHN3-deficient were determined on days 0, 11, and 19 after immunization by ELISA.

Schistosoma mansoni egg challenge

Mice were intraperitoneally injected with *S. mansoni* eggs (a gift from D. Jankovic, NIH, Bethesda, MD) inactivated by repeated cycles of freezing and thawing (5×10^3 eggs per mouse). Six weeks later, splenocytes from immunized mice were harvested and restimulated *ex vivo* for 3 days with SEA (from D. Jankovic) (0, 1, 5, 20 mg/mL). Cytokine expression by splenocytes was determined by ELISA.

T-cell transfer model of Colitis

Purified CD4⁺CD25[–]CD45RB^{hi} T cells were isolated from control- or T-cell-specific SHN3-deficient mice. Rag1^{deficient} mice were injected intraperitoneally with 4×10^5 CD4⁺CD25[–]CD45RB^{hi} T cells from Shn3-sufficient or -deficient mice. The body weight of the host RAG-deficient mice was monitored several days after the injection. Eight to ten weeks after cell transfer, mice were sacrificed, and the large intestines were removed. The large intestines of the host animals were fixed with 10% formalin and paraffin embedded, and the cross-sections were stained with H & E.

Transfection and luciferase assays

For each transfection, 5×10^6 Jurkat cells were incubated with AP-1–Luc reporter (addgene, Cambridge, MA) and pME18s empty vector, pME 18s-hSHN3 expression vector, pAct empty vector, or pAct hSHN2 (provided by Dr. T. Nakayama, Chiba University, Japan). pRL-null Renilla-Luc vector was used for normalization. Cells were transfected by electroporation (260 V, 800 µF). Transfected cells were cultured at 37°C for 20 h in RPMI 1640 medium (Gibco BRL) supplemented with 10% FCS, stimulated by 50 ng/mL PMA, 1 µM ionomycin in the presence or absence of the graded doses of anti-human CD28 antibodies (1–4 mg/mL) for 20 h, and applied to the dual-luciferase reporter assay (Promega).

Statistical analysis

A two-tailed Student's *t*-test was used for statistical analysis. Differences with a *p* value of less than 0.05 were considered significant.

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Abbreviations: **CBM:** CARMA1-BCL10-MALT1 · **DPP:** decapentaplegic · **EMSAs:** Electric mobility gel shift assay · **HPRT:** Hypoxanthine-guanine phosphoribosyltransferase · **IACUC:** Institutional Animal Care and Use Committee · **IBD:** inflammatory bowel disease · **IRES:** internal ribosome entry site · **OVA:** ovalbumin · **P + I:** PMA/ionomycin · **SEA:** soluble egg antigen · **SHN:** Schnurri · **SHN3:** Schnurri-3 · **TRAF:** TNF receptor-associated factor

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