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*J Immunol* 2007; 178:4926-4936; doi: 10.4049/jimmunol.178.8.4926

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Schnurri-2 Controls Memory Th1 and Th2 Cell Numbers In Vivo

Motoko Y. Kimura,* Chiaki Iwamura,* Akane Suzuki,* Takako Miki,* Akihiro Hasegawa,* Kaoru Sugaya,* Masakatsu Yamashita,* Shunsuke Ishii, † and Toshinori Nakayama2*

Schnurri-2 (Shn-2) is a large zinc-finger-containing protein, and it plays a critical role in cell growth, signal transduction and lymphocyte development. In Shn-2-deficient CD4 T cells, the activation of NF-κB was up-regulated and their ability to differentiate into Th2 cells was enhanced. We herein demonstrate that Th1 and Th2 memory cells are not properly generated from Shn-2-deficient effector Th1/Th2 cells. Even a week after the transfer of effector Th1/Th2 cells into syngeneic mice, a dramatic decrease in the number of Shn-2-deficient donor T cells was detected particularly in the lymphoid organs. The transferred Shn-2-deficient Th1/Th2 cells express higher levels of the activation marker CD69. No significant defect in the BrdU incorporation in the Shn-2-deficient transferred CD4 T cells was observed. The numbers of apoptotic cells were selectively higher in Shn-2-deficient donor Th1/Th2 cell population. Moreover, Shn-2-deficient effector Th1 and Th2 cells showed an increased susceptibility to cell death in in vitro cultures with increased expression of FasL. Transfer of Th2 effector cells over-expressing the p65 subunit of NF-κB resulted in a decreased number of p65-expressing cells in the lymphoid organs. As expected, T cell-dependent Ab responses after in vivo immunization of Shn-2-deficient mice were significantly reduced. Thus, Shn-2 appears to control the generation of memory Th1/Th2 cells through a change in their susceptibility to cell death. The Journal of Immunology, 2007, 178: 4926–4936.

After Ag recognition by TCR, naive CD4 T cells undergo clonal expansion followed by differentiation into functional effector T cell subsets; Th1 and Th2 cells (1). Th1 and Th2 cells exert effector functions by secreting a large amount of characteristic cytokines, IFN-γ and IL-4/IL-5/IL-13, respectively. The majority of activated effector Th1/Th2 cells undergo apoptotic cell death during the contraction phase, while a subpopulation that avoids cell death are thought to be differentiated and maintained for long time in vivo as memory Th1/Th2 cells (2–5).

IL-7 has been reported to play a critical role in the survival and homeostasis of memory CD4 T cells (6–10). Bcl2 and McI-1 are shown to be the downstream target molecules of the IL-7R that mediate the survival signals in T cells (11–14). The role of TCR/MHC interaction for the maintenance of memory CD4 T cells is still under debate. Initial studies suggested that TCR signaling is not required for the generation of CD4 memory T cells (15, 16), while others have suggested that it is critical for the turnover and preservation of the CD4 memory function rather than for simple survival (17). In another study, the contribution of TCR signaling for the homeostatic cell division of CD4 memory cells was demonstrated, but then only in the absence of IL-7 signaling (8).

The expression of GATA3 is critical for the maintenance of the Th2 cytokine expression (18–20) and chromatin remodeling of the Th2 cytokine gene loci (19). Memory Th2 cells maintain their Th2 identities, such as selective Th2 cytokine production, a high-level expression of GATA3 mRNA and open chromatin of the Th2 cytokine gene loci in an IL-4-independent manner (21). The characteristic features of memory T cells are thought to be acquired through specific differentiation processes during memory cell generation.

In addition to the quality of memory Th1/Th2 cells, the clone size of Ag-specific memory Th cells is also critical for the establishment of efficient immunological memory. Regarding the cell death pathways operating during the contraction phase, Fas-mediated activation-induced cell death (AICD) was initially reported to be important for the elimination of activated effector CD4 T cells (22). In addition, so called “cell autonomous death” induced by the abrogation of essential cytokines for T cell survival including IL-2 and IL-7 is considered to be another important mechanism for cell death of activated T cells (5). However, the TCR-mediated active cell death pathway may still play an important role in the cell death of activated CD4 effector T cells during the contraction phase (23, 24). The activated effector CD4 T cells may have a good chance to interact with self-Ags/MHC class II expressing APCs in vivo.

Drosophila Schnurri (Shn) is a large zinc-finger-containing protein, and the molecular mass of Shn is about 270 kDa. Vertebrates have at least three orthologs of Shn: namely Shn-1, Shn-2, and Shn-3 (25). We recently demonstrated that Shn-2 binds to the NF-κB

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Received for publication May 30, 2006. Accepted for publication February 7, 2007.

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1 This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid for Scientific Research in Priority Areas 17016010 and 17047007; Scientific Research B 17390317 and 17390318; and Special Coordination Funds for Promoting Science and Technology); the Ministry of Health, Labor and Welfare (Japan); The Japan Health Science Foundation; Kanoe Foundation; Uehara Memorial Foundation; Mochida Foundation; Yasuda Medical Foundation; Astellas Foundation; and Sagawa Foundation.

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motif directly and represses the transcriptional activity of NF-κB through the competition of NF-κB binding in T cells (26). Shn-2-deficient CD4 T cells show an increased capability to differentiate into Th2 cells, due to the constitutive activation of NF-κB and the subsequent up-regulation of GATA3 expression (26). However, the precise physiological roles of these Shn family members in immune responses in vivo still remain largely unknown.

We herein investigate the role of Shn-2 in memory Th1 and Th2 cell generation using Shn-2-deficient (Shn-2–/–) mice. Our results suggest that Shn-2 plays an important role in the regulation of memory Th1/Th2 cell numbers through the control of cell death.

Materials and Methods

Mice

Shn-2-deficient (Shn-2–/–) mice have been described previously (26, 27). The animals used in this study were backcrossed to BALB/c >12 times and were 6–8 wk old. Anti-OVA-specific TCRαβ (DO11.10) transgenic (Tg) mice were provided by Dr. Dennis Loh (Washington University School of Medicine, St. Louis, MO) (28). The Shn-2–/– xDO11.10 Tg mice were used at 10–12 wk of age. BALB/c and BALB/c nu/nu mice were purchased from Clea Inc. All mice used in this study were maintained under specific-pathogen-free conditions. All animal care was conducted in accordance with the guidelines of Chiba University.

Immunofluorescent staining and flow cytometry analysis

In general, one million cells were incubated on ice for 30 min. with the appropriate staining reagents, according to a standard method (29). For the intracellular staining, the cells were fixed, permeabilized, and then incubated with the appropriate staining reagents. The reagents used in this study were as follows: anti-CD4-biotin (RM4-5), anti-CD4-allophycocyanin (RM4-5), anti-CD44-FITC (IM7), anti-CD69-PE (H1.2F3), anti-CD62L-PE (MEL-14), anti-CD62L-allophycocyanin (MEL-14), anti-Fas-FITC, anti-KJ1-26-PE, anti-hNGFR-PE (C40–1457), anti-hNGFR-biotin (C40–1457), anti-Bcl2-FITC (3F11), and sAvdine-PerCP-Cy5.5, and sAvdine-allophycocyanin were purchased from BD Pharmingen. Flow cytometry analysis was performed on FACSCalibur (BD Biosciences), and the results were analyzed using the CELLQUEST software (BD Biosciences). For the detection of apoptotic cells, we used the Annexin V-FITC apoptosis detection kit II (BD Biosciences) according to the manufacturer’s protocol.

BrdU incorporation

Mice were injected with BrdU (1 mg) daily for the last 5 consecutive days before the analysis. For the detection of cells that incorporated BrdU, we used a FITC BrdU flow kit (BD Biosciences) according to the manufacturer’s protocol.

In vitro Th1/Th2 cell differentiation culture and adoptive cell transfer

Effector and memory Th1/Th2 cells were generated as previously described (21). In brief, splenic CD4 T cells purified from Shn-2+/+ and Shn-2–/– DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide (Loh15, 3 μM) plus APCs under Th1 or Th2 culture conditions for 6 days in vitro. These effector Th1/Th2 cells (2.5 × 106) were transferred i.v. into either BALB/c or BALB/c recipient mice. The recipient mice were not irradiated. One month later, the KJ1-26–transferred cells were sorted and used as memory T cells. For non-TCR Tg Shn-2+/+ and Shn-2–/– mice, splenic CD4 T cells were purified by magnetic-activated cell sorting (MACS) then cultured with immobilized anti-CD3 mAb (H57–597; 3 μg/ml) under Th1 or Th2 culture conditions for 6 days. For retrovirus infection, CD4 T cells were infected with pMXs-p65-IRES-GFP, pMXs-mock-IRES-GFP or pMXs-mock-IRES-hNGFR on day 0 as described previously (30). The infected cells were purified by cell sorting on day 2, and then were cultured for another 4 days in the presence of cytokines in the original Th1/Th2 culture conditions.
A representative CD62L/CD44 profile of the cells in the spleen, lymph node, liver, and PBMC of three individual animals from three independent experiments. Effector Th2 (A) and Th1 (B) cells were used for transfer. B and E, A representative CD62L/CD44 profile of the CD4 \(^+\) KJ1-26 cells in the spleen, lymph node, liver, and PBMC of three individual animals from three independent experiments. Effector Th2 (B) and Th1 (E) cells were used for transfer. C. BrdU was injected daily into Th2 memory mouse over the last 5 consecutive days before analysis. Memory Th2 cells in various tissues were prepared and examined for BrdU incorporation. The percentages of positive cells are shown in each panel.

**Quantitative PCR analysis**

Total RNA was isolated from cultured cells using the TRIzol reagent. Reverse transcription was conducted with Superscript II RT (Invitrogen Life Technologies). Samples were then subjected to real-time PCR analysis on an ABI PRISM 7300 sequence detection system (Applied Biosystems) under standard conditions. The primers and TaqMan probes for the detection of Shn-2, Fasl, Fas, Bim, Bcl-xL, Bcl2, Mcl1, and hprt were purchased from Applied Biosystems. mRNA expression was normalized using the hprt signal.

**In vivo immunization and Ab secretion**

The mice were immunized i.p. with OVA (100 \(\mu\)g) in CFA (100 \(\mu\)l) on day 0, and re-immunized 3 wk later. Serum samples were collected before immunization, and 2, 5 and 6 wk after the first immunization. For the analysis of T cell-independent responses, the mice were injected with DO11.10 Tg Shn-2\(^{+/+}\) or Shn-2\(^{-/-}\) mice were transferred into BALB/c nu/nu mice, and mice were sacrificed 1 month later. A and D, A representative CD4/KJ1-26 profile of the cells in the spleen, lymph node, liver, lung, and PBMC of three individual animals from three independent experiments. Effector Th2 (A) and Th1 (D) cells were used for transfer. B and E, A representative CD62L/CD44 profile of the CD4\(^+\) KJ1-26 cells in the spleen, lymph node, liver, and PBMC of three individual animals from three independent experiments. Effector Th2 (B) and Th1 (E) cells were used for transfer.

**Results**

**The decreased generation of memory Th2 cells in the absence of Shn-2 expression**

The aim of this study was to clarify the role of Shn-2 in the generation of Ag-specific memory CD4\(^+\) Th1 and Th2 cells. We first assessed the mRNA expression of Shn-2 in freshly prepared splenic CD4 T cells, in vitro generated effector Th1/Th2 cells, and in vivo generated memory Th1/Th2 cells using OVA-specific TCR\(\alpha\beta\) transgenic (DO11.10 Tg) mice. The expression levels of Shn-2 were decreased in effector Th1 and Th2 cells, and they were increased again in memory Th1 and Th2 cells, particularly in Th2 cells (Fig. 1A). Shn-2 expression in effector Th2 cells was restored three days after cell transfer into the syngeneic host mice (Fig. 1B). These results prompted us to examine the capability of Shn-2\(^{-/-}\) effector Th1/Th2 cells to become memory CD4 T cells in vivo. We used an adoptive transfer system (memory Th1 and Th2 mouse) as described previously (21). The memory Th1/Th2 cells generated in this system showed typical memory cell functions, including a quick and robust Th1/Th2 cytokine production after Ag restimulation. Shn-2\(^{-/-}\) xDO11.10 Tg effector and wild-type Th2 cells were differentiated in vitro, and then equivalent numbers of these cells were transferred into either recipient BALB/c nu/nu mice (Fig. 1, C and D) or normal BALB/c mice (Fig. 1, E and F). The expression profiles of CD4/KJ1-26 of the wild-type and Shn-2\(^{-/-}\) effector Th1/Th2 cells transferred were similar (data not shown). The number of Shn-2\(^{-/-}\) KJ1-26\(^+\) cells in the spleen of recipient nude mice 90 days after cell transfer was significantly lower when compared with wild-type cells (Fig. 1C). The number of Shn-2\(^{-/-}\) KJ1-26\(^+\) cells remained low for at least 7 days after cell transfer in PBMC (Fig. 1D). Similar results were obtained in normal non-lymphopenic BALB/c recipient mice transferred with Shn-2\(^{-/-}\) effector Th2 cells (Fig. 1, E and F). The reduction in the number of transferred KJ1-26\(^+\) cells was not observed in the Shn-2\(^{-/-}\) recipients transferred with wild-type Shn-2\(^{+/+}\) effector Th2 cells.
thus indicating the affect on the generation of memory Th2 cells could be due to an intrinsic defect in Shn-2/H11002/H11002 T cells (data not shown). Taken together, these results suggest that the generation of memory Th2 cells was impaired in the absence of Shn-2.

The generation of both memory Th1 and Th2 cells was impaired in the absence of Shn-2 expression

Consequently, we assessed the numbers of memory Th1 and Th2 cells in various tissues, Shn-2-/- DO11.10 Tg effector Th1/Th2 cells were prepared in vitro, and transferred into recipient BALB/c nu/nu mice. One month after cell transfer, we assessed the percentage of CD4+KJ1-26+ memory T cells and their expression profiles of CD62L/CD44 in the spleen, lymph node, liver, lung and PBMC. The numbers of CD4+KJ1-26+ memory cells were found to be substantially lower in every tissue from the memory mice with Shn-2-/- effector Th2 cell transfer (Fig. 2A) and Th1 cell transfer as compared with wild-type (Fig. 2D). A impaired generation of Shn-2-/- memory Th2 cells was also observed when we transferred the effector Th2 cells into nonlymphopenic normal BALB/c mice (data not shown). With regards to the phenotype of memory Th1 and Th2 cells, most of the donor CD4+KJ1-26+ T cells expressed CD44, and the percentages of CD62L cells were reduced in the Shn-2-/- groups (Fig. 2, B and E). The expression levels of IL-7Rα, Common-γ, IL-4Rα, IL-2Rβ, IL-2Rα and CD69 on memory Th1 and Th2 cells in the spleen and the lymph node were comparable between the Shn-2+/+ and Shn-2-/- memory T cells (data not shown).

To examine the steady-state proliferation of memory Th2 cells, BrdU incorporation was examined using Th2 memory mice. As shown in Fig. 2C, no detectable change was observed in all tissues examined, and rather a marginal increase was observed in the spleen, lymph node, and PBMC. Thus, Shn-2-/- memory Th2 cells appear to possess sufficient capability for steady-state proliferation.
The donor Shn-2−/− effector Th1 and Th2 cells were selectively reduced in lymphoid tissues a week after cell transfer.

We then sought to explore the molecular events operating in the transferred Th1/Th2 cells at the contraction phase. Seven days after effector Th1/Th2 cell transfer into recipient BALB/c nu/nu mice, we examined the number of CD4+ KJ1-26+ donor T cells and their expression profiles of CD62L/CD44 in the spleen, lymph node, liver, lung, and PBMC. A reduction in the number of CD4+ KJ1-26+ donor Th2 cells in Shn-2−/− cell transfer groups was detected in the spleen, lymph nodes, and PBMC (Fig. 3A). No obvious reduction was observed in the liver or lung. The percentages of CD62L+ cells were decreased in all tissues (Fig. 3B). Similar results were obtained in the experiments using BALB/c mice as recipient mice (data not shown). As for Th1 cells, a reduction of CD4+ KJ1-26+ donor T cells was also evident in the spleen, lymph nodes, PBMC, and liver (Fig. 3C). The percentages of CD62L+ cells were decreased in all tissues (Fig. 3D). These results indicate that the numbers of donor Shn-2−/− T cells in the lymphoid organs were decreased one week after cell transfer.

The expression levels of CD69, an activation marker Ag, on donor derived Th2 and Th1 cells were assessed a week after cell transfer, and increased levels were observed in Shn-2−/− Th1 and Th2 cells (Fig. 3, E and F). These results suggest that Shn-2−/− Th1/Th2 cells preserved an activation phenotype even after being transferred into syngeneic host mice. In addition, the levels of proliferation, as assessed by the injection of BrdU everyday from days 1 to 5 after cell transfer, were also moderately increased in the spleen and lymph nodes of Shn-2−/− Th2 cells (Fig. 3G).

The ability to produce Ag-specific Abs was decreased in mice transferred with Shn-2−/− effector Th1/Th2 cells.

The immune responses induced in the mice transferred with Shn-2−/− effector Th1/Th2 cells were then investigated. The mice were injected with Ag (OVA) 7 days after cell transfer, and then the level of OVA-specific Abs in the serum was assessed 7 days later. The levels of OVA-specific Abs, Th2-dependent IgG1 and IgE were dramatically reduced in mice transferred with Shn-2−/− Th2 cells as compared with wild-type cells (Fig. 3H). Similarly, the
levels of OVA-specific IgG2a (Th1 dependent) Abs were reduced in the mice transferred with Shn-2−/− Th1 cells (Fig. 3f). These results indicate that Th1/Th2 cell-dependent immune responses are markedly impaired in the mice transferred with Shn-2−/− effector Th1/Th2 cells.

**Increased numbers of apoptotic cells in the transferred Shn-2−/− Th1/Th2 cells in vivo**

We then assessed the levels of dying cells in the transferred Shn-2−/− effector Th1/Th2 cells using Annexin V and propidium iodide (PI) staining of the CD4+ KJ1-26+ donor T cell population. As shown in Fig. 4A, higher numbers of Annexin V−PI− and Annexin V−PI+ cells were detected in the Shn-2−/− CD4+ KJ1-26+ donor cells in the spleen (Annexin V−PI−: Shn-2−/−, 4.6% vs Shn-2+/−, 8.8%, and Annexin V−PI+: Shn-2−/−, 1.3% vs Shn-2+/−, 3.5%) and the lymph node (Annexin V−PI−: Shn-2−/−, 17.7% vs Shn-2+/−, 30.1%, and Annexin V−PI+: Shn-2−/−, 3.0% vs Shn-2+/−, 18.6%). In the same animals, no obvious difference was observed in the number of Annexin V−PI− and Annexin V−PI+ cells in the CD4+ KJ1-26+ host cells (Fig. 4A, right panels). Similar results were obtained when the effector Th1 cells were transferred BALB/c nu/nu mice (Fig. 4B). In the nonlymphopenic normal BALB/c mice transferred with Th2 cells, an increased number of Annexin V−PI− cells was prominent in the spleen (Shn-2−/−, 10.4% vs Shn-2+/−, 30.3%), lymph nodes (Shn-2−/−, 19.8% vs Shn-2+/−, 47.7%) and PBMC (Shn-2−/−, 7.0% vs Shn-2+/−, 41.2%) in the Shn-2−/− group (Fig. 4C, left panel). In contrast, the levels of cell death of the host CD4 T cells (CD4+ KJ1-26+ cells) in the same mice did not reflect any obvious differences (Fig. 4C, right panel). These results indicate that Shn-2−/− Th1/Th2 cells were more susceptible to cell death in the host lymphoid organs as compared with wild-type Th1/Th2 cells. An increased number of Annexin V−PI− and Annexin V−PI+ cells in the CD4+ KJ1-26+ cell population in the lymph nodes was detected even 1 month after the cell transfer of effector Th1 or Th2 cells (data not shown).

To address the molecular mechanisms underlying the increased cell death in the transferred Shn-2−/− Th1/Th2 cells in vivo, we purified KJ1-26+ cells from the recipient mice 3 days after cell transfer and examined the expression levels of Fas, FasL, Bik, Bcl-xL, Bcl2, and McI1. Inefficient Th2 cells contained a higher number of Annexin V−PI− and Annexin V−PI+ cells (Shn-2−/−, 47.7%) and PBMC (Shn-2−/−, 41.2%) in the Shn-2−/− group (Fig. 4C, left panel). In contrast, the levels of cell death of the host CD4 T cells (CD4+ KJ1-26+ cells) in the same mice did not reflect any obvious differences (Fig. 4C, right panel). These results indicate that Shn-2−/− Th1/Th2 cells were more susceptible to cell death in the host lymphoid organs as compared with wild-type Th1/Th2 cells. An increased number of Annexin V−PI− and Annexin V−PI+ cells in the CD4+ KJ1-26+ cell population in the lymph nodes was detected even 1 month after the cell transfer of effector Th1 or Th2 cells (data not shown).

Next we assessed the susceptibility to cell death of Shn-2−/− T cells in vitro to gain some additional insights. Effector Th2 cells were cultured in vitro with medium or immobilized anti-TCR-mAb for 16 h, or with IL-7 (10 U/ml) for three days, and Annexin V/PI staining profiles of the cultured cells were analyzed. Increased Annexin V−PI− cells (Shn-2−/−, 14.3% vs Shn-2+/−, 22.7%) and Annexin V−PI+ cells (Shn-2−/−, 9.2% vs Shn-2+/−, 24.2%) were detected in the Shn-2−/− T cell cultures with medium alone (Fig. 5A). Re-stimulation with immobilized anti-TCR mAb for 16 h induced activation-induced cell death (AICD) in T cells, and the levels of Annexin V−PI+ cells were thus significantly enhanced, and the values were obviously higher in Shn-2−/− cells.

![Figure 5](http://www.jimmunol.org/content/184/11/4931/F5)

**FIGURE 5.** Shn-2−/− Th2 cells are susceptible to dying in vitro. A. In vitro differentiated effector Shn-2−/− Th2 cells were cultured with medium alone for 16 h, anti-TCR stimulation for 16 h or IL-7 (10 U/ml) for 3 days. Then each sample was subjected to Annexin V and PI staining. Three independent experiments were performed with similar results. B. The expression of CD69, Fas, CD62L, and Bcl2 was examined with the same samples as in A. Solid line, Shn-2−/− cells; dotted line, Shn-2+/− cells; and the hatched area shows background staining. Two independent experiments were conducted with similar results. The values of MFI’s shown are for the whole population. C. The mRNA expression of FasL, Fas, Bik, Bcl-xL, Bcl2, and McI1 was assessed in the cultured cells under indicated conditions. □, Shn-2−/− cells; ■, Shn-2+/− cells. Quantitative real-time PCR analysis was done. Three independent experiments were performed with similar results.
Even in the cells cultured with IL-7, increased Annexin V/PI cells in the Shn-2/H11002 cell culture were observed (Shn-2/H11002, 21.9% vs Shn-2/H11002, 35.5%).

Concurrently, we assessed the expression of CD69, Fas, CD62L, and Bcl2 by flow cytometry. The expression levels of CD69 and Fas were increased in Shn-2/H11002 effector T cells compared with wild-type cells, while those of CD62L were decreased.

No significant difference in the expression of Bcl2 was detected between wild-type and Shn-2−/− effector Th2 cells (Fig. 5B, upper panels). The increased expression of CD69 and the decreased expression of CD62L in Shn-2−/− cells were consistently detected after cultivation in the medium, immobilized anti-TCR mAb or in the presence of IL-7, thus suggesting that the activation of T cells was sustained in Shn-2−/− T cells. With regards to the expression

No significant difference in the expression of Bcl2 was detected between wild-type and Shn-2−/− effector Th2 cells (Fig. 5B, upper panels). The increased expression of CD69 and the decreased expression of CD62L in Shn-2−/− cells were consistently detected after cultivation in the medium, immobilized anti-TCR mAb or in the presence of IL-7, thus suggesting that the activation of T cells was sustained in Shn-2−/− T cells. With regards to the expression

FIGURE 6. Expression of CD69, Fas, and CD62L in NF-κB p65-overexpressed Th2 cells. Splenic CD4 T cells were infected with pMxs-mock-IRES-hNGFR or pMxs-p65-IRES-hNGFR, and cultured under Th2 conditions for 5 days (p65-overexpressed Th2 cells). A. The expression levels of CD69, Fas, and CD62L expression were examined by flow cytometry. B. p65-overexpressed Th2 cells were cultured with medium alone for 16 h or IL-7 for 3 days, and then the expression levels of CD69, Fas, and CD62L expression were examined. The level of hNGFR expression was used for an indicator of the efficiency of infection. Three independent experiments were performed with similar results.

FIGURE 7. NF-κB p65-overexpressed Th2 cells showed a reduced cell number in the spleen and lymph nodes. CD4+ KJ1-26+ Th2 cells were infected with pMxs-IRES-hNGFR-mock (pMxs-IN-mock), pMxs-IRES-GFP-mock (pMxs-IG-mock), or pMxs-IRES-GFP-p65 (pMxs-IG-p65) viruses. A cell mixture of pMxs-IG-mock infected cells and pMxs-IN-mock infected cells or a mixture of pMxs-IG-p65 infected cells and pMxs-IN-mock infected cells were transferred into BALB/c nu/nu mice. A. The expression profiles of GFP/hNGFR in transferred cells are shown. B. The expression profiles of GFP/hNGFR on CD4+ KJ1-26+ cells in various tissues 7 days after cell transfer (left). Right graph indicates the ratio of GFP-positive cells to hNGFR-positive cells. Two independent experiments were done with similar results.
level of Fas, no dramatic difference was observed in the cultured Th2 cells. The expression levels of Bcl2 were not decreased in these cultures.

Moreover, an assessment of the mRNA expression levels of FasL, Fas, Bim, Bcl-xL, Bcl2 and Mcl1 in Shn-2−/− effector Th2 cells cultured under various conditions was conducted (Fig. 5C). Increased expression of FasL was observed in most of the Shn-2−/− cultured cells tested. Increased expression of Fas and Bim was observed only in Shn-2−/− effector Th2 cells. In contrast, no significant difference was observed in the mRNA expression of Bcl-xL, Bcl2 or Mcl1 between Shn-2−/− and Shn-2+/+ cultured cells.

Overexpression of NF-κB p65 resulted in the induction of CD69 and Fas in Th2 cells

Shn-2−/− CD4 T cells showed constitutive activation of NF-κB (26), and NF-κB activation is known to enhance apoptotic cell death under some conditions (31–34). In particular, the activation of NF-κB up-regulates the expression of Fas, FasL, and TRAIL (35). Therefore, we prepared the effector Th2 cells with an increased activation level of NF-κB and examined the changes in cell surface phenotype. In vitro differentiated effector Th2 cells were introduced with pMxs-mock-IRES-hNGFR (pMxs-IN-mock), and pMxs-p65-IRES-hNGFR (pMxs-IN-p65) using a retrovirus vector.
system. The activation levels of NF-κB assessed by an EMSA assay were enhanced in Th2 cells that were treated with pMxs-p65-IRESGFP (data not shown). As shown in Fig. 6A, p65-overexpressing Th2 cell population (hNGFR<sup>high</sup>) showed an increased expression of CD69 and Fas, and a decreased expression of CD62L as compared with noninfected population (hNGFR<sup>neg</sup>). These phenotypes are similar to those of Shn-2-<sup>−/−</sup> effectorTh2 cells (Fig. 5B, upper panels). To examine whether these phenotypes are sustained in a resting culture, we cultured p65-overexpressed Th2 cells in medium alone for 16 h or with IL-7 for 3 days. Interestingly, the expression levels of CD69 and Fas remained at increased levels, and those of CD62L remained decreased (Fig. 6B).

Transfer of NF-κB p65-overexpressed Th2 cells resulted in a reduced number of Th2 cells in the lymph nodes

Consequently, we transferred the effector Th2 cells with overexpression of NF-κB p65 to assess the fate off these cells in vivo. In vitro differentiated effector Th2 cells were treated with pMxs-p65-IRESGFP (pMxs-IG-p65), pMxs-mock-IRESGFP (pMxs-IG-mock), or pMxs-mock-IRESGFP (pMxs-IN-mock) as an internal control using a retrovirus vector system. A cell mixture of pMxs-IG-mock infected cells and pMxs-IN-mock infected cells or a mixture of pMxs-IG-p65 infected cells and pMxs-IN-mock infected cells (Fig. 7A) was prepared and transferred into BALB/c nu/nu mice. Seven days later, the numbers of GFP-positive cells and hNGFR-positive cells in the various tissues were assessed by flow cytometry (Fig. 7B). The ratio (GFP<sup>/</sup>/hNGFR<sup>/</sup> cells) in each group is shown in the Fig. 7B, right panel. The numbers of p65-introduced cells were preferentially decreased in the lymph nodes. We made attempts to look for increased levels of apoptosis in the p65-overexpressed cells in vivo, but failed to obtain reproducible results, perhaps because of the paucity of p65-overexpressed cells detected (data not shown). Minimally, however, these results suggest that the overexpression of NF-κB p65 in effector Th2 cells results in a reduced number of Th2 cells in the lymph nodes.

Decreased secondary Ab responses in Shn-2-deficient mice

Finally, we assessed the Th1/Th2 cell-dependent immune responses in Shn-2-<sup>−/−</sup> mice by an in vivo immunization method. When we immunized Shn-2-<sup>−/−</sup> mice with OVA and CFA twice, a severely depressed serum Ab response was seen (Fig. 8A). The effect was observed in both Th2-dependent isotypes (IgG1 and IgE) and Th1-dependent isotype (IgG2a). Both Ag-specific and total levels of IgG Abs were reduced particularly at 5 and 6 wk after immunization. In addition, we observed increased levels in the total IgM in Shn-2-<sup>−/−</sup> mice. In contrast, when Shn-2-<sup>−/−</sup> mice were immunized with TNP-Ficoll which is a T-independent Ag, we observed no difference in the production of TNP-specific Abs in the serum among wild-type, Shn-2-<sup>−/−</sup> and Shn-2-<sup>−/−</sup> mice (Fig. 8B). These results indicate that T cell-dependent immune responses, particularly secondary immune responses are impaired in Shn-2-<sup>−/−</sup> mice.

We examined the levels of apoptotic cell death in Shn-2-<sup>−/−</sup> CD4<sup>+</sup> T cells after in vivo OVA stimulation using DO11.10 OVA-specific TCR Tg mice (Fig. 8C). Three days after soluble OVA (100 μg) injection, we detected a significant reduction in the percentages of CD4<sup>+</sup> T cells and a significant increase in the percentages of Annexin V<sup>+</sup> cells among CD4<sup>+</sup> T cells in the lymph node of Shn-2-<sup>−/−</sup>×DO11.10 Tg mice. These results suggest that Shn-2-<sup>−/−</sup> CD4<sup>+</sup> T cells activated in vivo show a similar phenotype to the transferred Shn-2-deficient effector Th1/Th2 cells in the lymph nodes.

Discussion

In this study, we demonstrate that the generation of memory Th1/Th2 cells is impaired in the absence of Shn-2 expression. Shn-2 appears to control the susceptibility to cell death of effector Th1/Th2 cells at the contraction phase in vivo. The involvement of down-regulation of Fas/FasL expression is suggested.

In analogy to two CD8 memory T cell subsets, two types of memory CD4<sup>+</sup> T cells have been proposed; namely central memory T (TCM) cells expressing CD44<sup>+</sup>CD62L<sup>+</sup> phenotype and effector memory T (TEM) cells with CD44<sup>−</sup>CD62L<sup>−</sup> phenotype (36). TEM cells preferentially migrate into the inflammatory regions and interact with tissue macrophages and immature dendritic cells, and then they are activated to exert their effector functions. In contrast, T<sub>CM</sub> cells that migrate into the lymph nodes are re-primed by resident APCs, and acquire an ability to help B cells (37, 38). In this report, we found that the numbers of T<sub>CM</sub> type CD44<sup>+</sup>CD62L<sup>+</sup> Th1 and Th2 cells were substantially lower in the number in the mice transferred with Shn-2<sup>−/−</sup>/Th1 and Th2 cells, respectively, as compared with wild-type cells (Figs. 2 and 3). This is consistent with the observation that the Ab production was dramatically lower in the recipient mice with Shn-2<sup>−/−</sup> effector cell transfer (Fig. 3, H and I). We also observed that the levels of Th1- and Th2-dependent IgG Ab production induced by OVA/CFA immunization were significantly reduced in Shn-2<sup>−/−</sup> mice as compared with those in wild-type mice (Fig. 8).

Because CD62L is known to be a homing receptor for the lymph node through with high endothelial venule (39), we first thought that the reduced number of cells in the lymph nodes was due to the low expression of CD62L on the Shn-2<sup>−/−</sup> effector cells. However, the forced expression of CD62L on Shn-2<sup>−/−</sup> effector cells failed to rescue the numbers of donor Shn-2<sup>−/−</sup> effector cells in the lymph nodes (M. Y. Kimura and T. Nakayama, unpublished observation), and thus, we conclude that the involvement of CD62L expression itself is unlikely to explain the reduction in Shn-2<sup>−/−</sup> T cells numbers in the lymph node.

The low numbers of memory Th1 and Th2 cells observed in Shn-2<sup>−/−</sup> memory mice could be the result of increased cell death and/or the lack of survival and homeostasis. The latter possibility appears to be supported by the fact that Shn-2<sup>−/−</sup> T cells do not expand very well even several weeks after cell transfer (Fig. 1, D and F). However, we detected no obvious change in the incorporation of BrdU in Shn-2<sup>−/−</sup> memory T cells (Fig. 2C), suggesting that the levels of steady-state proliferation in vivo are not affected in the absence of Shn-2. In contrast, we saw more impressive evidence supporting the increased cell death in Shn-2<sup>−/−</sup> T cells in vivo. We detected a selective increase in the apoptotic cells in the donor derived Shn-2<sup>−/−</sup>/Th1/Th2 cells in vivo (Fig. 4) and also increased apoptosis in the Shn-2<sup>−/−</sup> effector T cells cultured under various conditions (Fig. 5).

With regards to the cell death pathways, Fas/FasL-mediated cell death appears to be most important. Shn-2<sup>−/−</sup> effector Th2 cells showed increased levels of Fas expression (Fig. 5, B and C). In addition, Shn-2<sup>−/−</sup> effector Th2 cells cultured in medium or with IL-7 showed significantly increased levels of FasL mRNA (Fig. 5C). We also detected a high level expression of FasL in Shn-2<sup>−/−</sup>/Th2 cells freshly prepared from Th2 memory mice (Fig. 4D). Another cell death pathway, which may be involved, is the passive autonomous cell death induced by the withdrawal of growth/survival factors through BH3-only protein Bim (40, 41). We did assessed the mRNA levels regarding various factors involved in the induction of autonomous cell death and found that among them, the mRNA expression level of Bim was slightly increased in Shn-2<sup>−/−</sup> Th2 effector cells, whereas mRNA levels of Bcl-X<sub>L</sub>, Bcl2,
and McI1 were not significantly affected (Fig. 5C). In fact, we observed an increased cell death in the Shn-2−/− Th2 cell cultures with medium alone and with IL-7 (Fig. 5A). Therefore, although further investigation is in order, we suspect that the Bim-dependent autonomous cell death pathway may be involved in the enhanced cell death in Shn-2−/− T cells. A slight decrease in the expression of Bcl2 in Shn-2−/− Th2 cells one week after cell transfer was detected (Fig. 4D), but the difference in the levels was not so dramatic and thus this is unlikely to be responsible for the increased cell death in the Shn-2−/− T cells.

The numbers of CD4 and CD8 T cells were significantly low in the spleen and lymph nodes of Shn-2−/− mice without any treatment (26). We observed significantly high percentages of Annexin V+ cells among CD4 T cells in the spleen and lymph nodes of Shn-2−/− mice, and this was also observed when DO11.10 OVA-specific Shn-2−/− mice were injected with soluble OVA (Fig. 8C). Thus, Shn-2−/− T cells seem prone to die regardless of the presence or absence of the stimulation through TCR.

Another interesting observation is that total IgM levels after the OVA/CFA immunization were higher in the Shn-2−/− mice as compared with wild-type although the levels of total IgG (IgG2a, IgG1) and IgE were markedly reduced in Shn-2−/− mice (Fig. 8A). This could be the result of a compensatory change induced by the immunization with CFA; however, it is also possible that changes in the B cells secreting IgGs are taking place. T cell-independent Ag-induced Ab responses were within normal range in Shn-2−/− mice (Fig. 8B), and no defect was observed in the IL-4/LPS- or IL-4/soluble CD40L-induced Ig class switch in B cells in vitro (M. Y. Kimura and T. Nakayama, unpublished observation). Thus, no major defect in the function of B cells was apparent; however, further investigation is necessary to clarify the nature of B cells in Shn-2−/− mice.

As we previously reported, a constitutive activation of NF-xB is observed in Shn-2−/− CD4 T cells (26). To address the relationship between the constitutive activation of NF-xB and the generation of memory CD4 T cells, we performed careful cell transfer experiments with p65-overexpressed Th2 cells and several control Th2 cells (Fig. 7). The numbers of p65-overexpressed Th2 cells were found to be reduced selectively in the lymph nodes (Fig. 7B). However, these p65-overexpressed Th2 cells did not show significantly greater susceptibility to cell death in vitro (M. Y. Kimura and T. Nakayama, unpublished observation). This could be due to the fact that NF-xB also possesses anti-apoptotic activities under some conditions (42, 43). Thus, we are not able to definitively conclude that the impaired generation of Shn-2−/− memory CD4 T cells is due to the constitutive activation of NF-xB at this time. However, it is interesting that the p65-overexpressed Th2 cells showed increased CD69 expression and Fas expression (Fig. 6, A and B), which is reminiscent of the unique phenotype of Shn-2−/− effector Th2 cells (Fig. 5B). In any event, the investigation to address the possible link between NF-xB activation and the generation of memory CD4 T cells will be of interest.

In summary, our results indicate that Shn-2 plays an important role in the regulation of memory Th1/Th2 cell numbers through the control of cell death.

Acknowledgment

We are grateful to Dr. Ralph T. Kubo for helpful comments and constructive criticism in the preparation of the manuscript.

Disclosures

The authors have no financial conflict of interest.