Kitakanto Med J
2008 ; 58 : 133～139

Impaired Proliferation and Th1 Differentiation of CD4+ T Cells of SHPS-1 Mutant Mice

Yuka Kaneko,[1,2] Yoriaki Kaneko,[3] Hiroshi Ohnishi,[1]
Takeshi Tomizawa,[3] Jun Okajo,[3] Yasuyuki Saito,[3]
Chie Okuzawa,[3] Yoji Murata,[1] Hideki Okazawa,[1]
Yoshihisa Nojima,[3] Koichi Okamoto,[2] and Takashi Matozaki [1]

Background & Aims : SHPS-1 is a transmembrane protein that binds the protein tyrosine phosphatases SHP-1 and SHP-2 through its cytoplasmic region. It is highly expressed on the surface of CD11c+ dendritic cells (DCs) and macrophages. We have recently shown that priming of CD4+ T cells by DCs is markedly impaired in mice that express a mutant form of SHPS-1 lacking most of the cytoplasmic region. We have now evaluated further the functions of CD4+ T cells derived from SHPS-1 mutant mice. **Methods :** The expression of cell surface molecules on CD4+ T cells was examined by flow cytometry. The proliferation of CD4+ T cells was measured by [^{3}H] thymidine incorporation. Cytokine production by CD4+ T cells was measured by ELISA. **Results :** SHPS-1 is expressed at low level on CD4+ T cells of wild-type mice. The T cell receptor (TCR)-stimulated proliferation of CD4+ T cells from SHPS-1 mutant mice was markedly decreased, whereas the TCR-stimulated production of IL-2 and IFN-γ by these cells was markedly increased, compared with those apparent with wild-type cells. Differentiation of CD4+ T cells from SHPS-1 mutant mice into Th1 cells was also impaired. **Conclusions :** Present results suggest that SHPS-1 is essential for proper regulation of CD4+ T cell functions.
(Kitakanto Med J 2008 ; 58 : 133～139)

Key Words : Th1/Th2 cells, autoimmunity, cell surface molecules, transgenic/knockout mice

Introduction

Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1),[1] also known as signal-regulatory protein α[2,3] or BIT,[4] is a transmembrane protein whose extracellular region comprises three immunoglobulin (Ig)-like domains and whose cytoplasmic region contains four tyrosine phosphorylation sites that mediate the binding of the protein tyrosine phosphatases SHP-1 and SHP-2. Tyrosine phosphorylation of SHPS-1 is regulated by various growth factors and cytokines as well as by integrin-mediated cell adhesion to extracellular matrix proteins.[5,6] SHPS-1 thus functions as a docking protein to recruit and activate SHP-1 or SHP-2 at the cell membrane in response to extracellular stimuli. CD47 is a ligand for the extracellular region of SHPS-1.[7,8] This protein, which was originally identified in association with $\alpha v\beta 3$ integrin, is also a member of the Ig superfamily, possessing an Ig-V-like extracellular domain, five putative membrane-spanning segments, and a short cytoplasmic tail.[9]

Among hematopoietic cells, SHPS-1 is especially abundant in dendritic cells (DCs), macrophages, and neutrophils, being barely detectable in T or B lymphocytes.[8,10−13] In contrast, CD47 is expressed in a variety of hematopoietic cells including red blood cells (RBCs) and T cells.[9] Indeed, the interaction of CD47 on RBCs with SHPS-1 on macrophages is thought to prevent phagocytosis of the former cells by the latter through activation of SHP-1, which forms a complex with SHPS-1.[14−16] Similarly, SHPS-1, through its

1 Laboratory of Biosignal Sciences, Institute for Molecular and Cellular Regulation, Gunma University 2 Department of Neurology, Gunma University Graduate School of Medicine 3 Department of Medicine and Clinical Science, Gunma University Graduate School of Medicine
Received : January 21, 2008
Accepted : March 4, 2008
Address : TAKASHI MATOZAKI Laboratory of Biosignal Sciences, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-Machi, Maebashi, Gunma 371-8512, Japan.

interaction with CD47, is thought to play a negative role in the immune system.[12] Ligation of SHPS-1 by CD47-Fc fusion proteins suppressed the phenotypic and functional maturation of immature DCs and inhibited cytokine production by mature DCs,[12] suggesting that SHPS-1 (on DCs), through its interaction with CD47 (on T cells), prevents activation of DCs. In contrast, interaction of CD47 with SHPS-1 promotes the proliferation of T cells as well as contributes to the activation of antigen-specific cytotoxic T cells by DCs in vitro.[13] Ligation of SHPS-1 stimulates nitric oxide production by macrophages,[17] suggesting that SHPS-1 might also play a positive role in the immune system.

We previously generated mice that express a mutant version of SHPS-1 that lacks most of the cytoplasmic region of the protein.[16,18] This mutant protein does not undergo tyrosine phosphorylation or form a complex with SHP-1 or SHP-2. Furthermore, the cellular abundance of the mutant protein is markedly reduced compared with that of the full-length protein in wild-type (WT) mice.[16,18] We have recently found that SHPS-1 mutant mice fail to develop experimental autoimmune encephalomyelitis (EAE).[19] Several lines of evidence indicated that DCs derived from SHPS-1 mutant mice are defective in the ability to prime $CD4^+$ T cells, suggesting that resistance to EAE in these animals is attributable to this impairment of DC function. However, it was possible that $CD4^+$ T cells also might be functionally defective in SHPS-1 mutant mice, given that these animals are totally resistant to EAE. With the use of SHPS-1 mutant mice, we have now examined the possible role of SHPS-1 in regulation of $CD4^+$ T cell functions.

Materials and Methods

Antibodies and reagents

Hybridoma cells producing the rat P84 monoclonal antibody (mAb) to SHPS-1 and those producing a rat mAb to mouse CD47 (miap301) were kindly provided by C.F.Lagenaur (University of Pittsburgh) and P.-A.Oldenborg (Umeå University, Sweden), respectively; the mAbs were purified from culture supernatants and conjugated to sulfo-NHS-LC biotin (Pierce). A hamster mAb to mouse CD3 (2C11) and a rat mAb to mouse IL-4 (11B11) were also prepared from the culture supernatants of hybridoma cells (kindly provided by K.Okumura, Juntendo University, Japan). An FITC-conjugated rat mAb to mouse CD4 (L3T4), a biotin-conjugated rat IgG to trinitrophenol (isotype control), and PE-conjugated streptavidin were from BD PharMingen. A biotin-conjugated rat mAb to mouse CD25 (PC61.5) and an FITC-conjugated rat IgG to trinitrophenol (isotype control) were from eBioscience. Recombinant murine IL-12 was from Peprotech. RPMI 1640 medium (Sigma-Aldrich) was supplemented with 10% heat-inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES-NaOH (pH 7.4), penicillin (100U/ml), streptomycin (100 μg/ml), and 1 mM sodium pyruvate to yield complete medium.

Mice

Mice that express a mutant version of SHPS-1 that lacks most of the cytoplasmic region were described previously[16,18] and were backcrossed onto the C57BL/6 background for nine generations. Mice were bred and maintained at the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions and were handled in accordance with the animal care guidelines of Gunma University.

Preparation of splenic $CD4^+$ T cells, T cell receptor (TCR) stimulation, and Th1 cell differentiation

For preparation of splenic $CD4^+$ T cells,[20,21] the spleen was gently ground with autoclaved frosted glass slides in phosphate buffered saline (PBS), and the released cells were exposed to Gey's solution, washed twice with PBS, and filtered through nylon wool. Cells in the filtrate were then subjected to purification with the use of magnetic beads coated with mAbs to mouse CD4 and a MACS column (Miltenyi Biotech). The purity of the isolated $CD4^+$ T cells was $>95\%$ as determined by flow cytometry.

Purified $CD4^+$ T cells from WT or SHPS-1 mutant mice were cultured for 48 h in 96-well, flat-bottomed plates coated with various concentrations of a mAb to CD3. For measurement of cell proliferation, the cells were exposed to [^{3}H] thymidine ([^{3}H] TdR) (1 μCi per well; 2 Ci/mmol) during the final 14 h of culture and the cell-associated radioactivity was subsequently measured with a scintillation spectrometer. Culture supernatants of the stimulated cells were also collected for measurement of the concentrations of IL-2, IFN-γ, and IL-4 with ELISA kits (BD PharMingen). For assay of Th1 cell differentiation,[20,21] purified $CD4^+$ T cells were cultured for 6 days with IL-12 (10 ng/ml) and a mAb to IL-4 (10 μg/ml) in culture plates coated with a mAb to CD3 (2 μg/ml). The cells were then harvested, washed twice with PBS, and cultured for 24 h in RPMI 1640 complete medium at a density of 2×10^6/ml in culture plates coated with a mAb to CD3 (1 μg/ml). Culture supernatants were then collected for measurement of IFN-γ.

Flow cytometric analysis

For examination of the surface expression of

SHPS-1 or CD47, purified splenic CD4+ T cells (1×10^6) were incubated with a biotin-conjugated mAb to SHPS-1 (1 μg/ml) or a biotin-conjugated mAb to CD47 (1 μg/ml), washed, and then incubated with PE-conjugated streptavidin (0.2 μg/ml). The cells were also labeled with an FITC-conjugated mAb to mouse CD4 (0.5 μg/ml) to confirm the surface expression of CD4. All stained cells were analyzed by flow cytometry with a FACS Calibur instrument and Cell-Quest software (Becton Dickinson). The expression of CD25 on splenic CD4+ T cells was determined by incubation of the cells first with a biotin-conjugated mAb to CD25 (0.5 μg/ml) and then with streptavidin-PE before flow cytometric analysis.

Statistical analysis

Data are presented as means ± standard error (SE) and were analyzed by Student's t test with the use of Stat View 5.0 software (SAS Institute). A p value of <0.05 was considered statistically significant.

Results

Expression of SHPS-1 was previously found to be virtually undetectable in T or B lymphocytes.[8,10,12] However, we prepared a biotin-conjugated mAb to SHPS-1 to increase the immunofluorescence signal of SHPS-1 expressed on the surface of CD4+ T cells. Flow cytometric analysis with this mAb revealed a low level of SHPS-1 expression on splenic CD4+ T cells of WT mice (Fig. 1A). The expression of SHPS-1 was also detected on CD4+ T cells of SHPS-1 mutant mice. In contrast, the abundance of CD47 on the surface of CD4+ T cells from SHPS-1 mutant mice was almost identical to that apparent on WT cells (Fig. 1A). We found that the expression of SHPS-1 on CD4+ T cells from WT mice was reduced by cross-linking of the TCR with a mAb to CD3 (Fig. 1B). In contrast, the expression of SHPS-1 on CD4+ T cells from SHPS-1 mutant mice was increased by TCR cross-linking (Fig. 1B).

The expression of SHPS-1 on CD4+ T cells suggested that SHPS-1 might regulate the functions of these cells. We therefore next examined the proliferation of and cytokine production by CD4+ T cells derived from WT or SHPS-1 mutant mice. Cell proliferation in response to TCR stimulation with a mAb to CD3 was markedly reduced in CD4+ T cells from the mutant animals compared with that apparent with WT cells (Fig. 2A). In contrast, the TCR-dependent expression of CD25 [IL-2 receptor (IL-2R) α chain] was not impaired in CD4+ T cells from SHPS-1 mutant mice (Fig. 2B), suggesting that these cells are defective in the proliferative response to IL-2.

We next examined the production of IL-2 in CD4+ T cells in response to TCR cross-linking. The production of IL-2 was enhanced in CD4+ T cells from SHPS-1 mutant mice compared with that apparent with WT cells (Fig. 3A). Moreover, the production of both IFN-γ (Fig. 3B) and IL-4 (Fig. 3C) in response to TCR cross-linking was also enhanced in CD4+ T cells from SHPS-1 mutant mice.

We also examined the Th1 differentiation of CD4+ T cells from SHPS-1 mutant mice by incubation of the cells with mAbs to IL-4 and to CD3 as well as with IL-12. The production of IFN-γ by such treated cells from SHPS-1 mutant mice in response to TCR cross-linking was markedly reduced compared with that apparent with WT cells (Fig. 4). These results thus suggest that both the proliferation and Th1 differentiation of CD4+ T cells are markedly impaired in SHPS-1 mutant mice.

Discussion

Expression of SHPS-1 was previously found to be virtually undetectable in T cells.[8,10,11] However, we have now detected a low level of expression of SHPS-1 on the surface of splenic CD4+ T cells from WT mice. Furthermore, this expression of SHPS-1 was down-regulated in response to cross-linking of CD3. SHPS-1 is thus a new member of the group of surface molecules on T cells that are regulated by TCR activation.[22] In contrast, the expression of mutant SHPS-1 on CD4+ T cells from SHPS-1 mutant mice was increased as a result of TCR activation. The molecular mechanism for such regulation is unknown at present, but we also found that the abundance of SHPS-1 mRNA encoding the extracellular region of this protein was also increased by TCR activation in the SHPS-1 mutant cells (data not shown).

The proliferation of CD4+ T cells from SHPS-1 mutant mice in response to CD3 cross-linking was found to be markedly impaired compared with that of WT cells. The observations that the production of IL-2 and the expression of CD25 (IL-2R α chain) induced by such stimulation were not reduced in the mutant cells suggest that signaling downstream of IL-2R may be defective in CD4+ T cells of SHPS-1 mutant mice. In addition, IL-12-promoted Th1 differentiation of CD4+ T cells from SHPS-1 mutant mice was also impaired. SHPS-1 mutant mice manifest minimal susceptibility to EAE, and such resistance is attributable, at least in part, to impaired priming of CD4+ T cells by DCs.[19] The present study now indicates that impaired differentiation of CD4+ T cells into Th1 cells might also contribute to the resistance of SHPS-1 mutant mice to EAE. IL-17-producing Th cells (Th17 cells), rather than Th1 cells, were recently suggested to play an essential role in the development

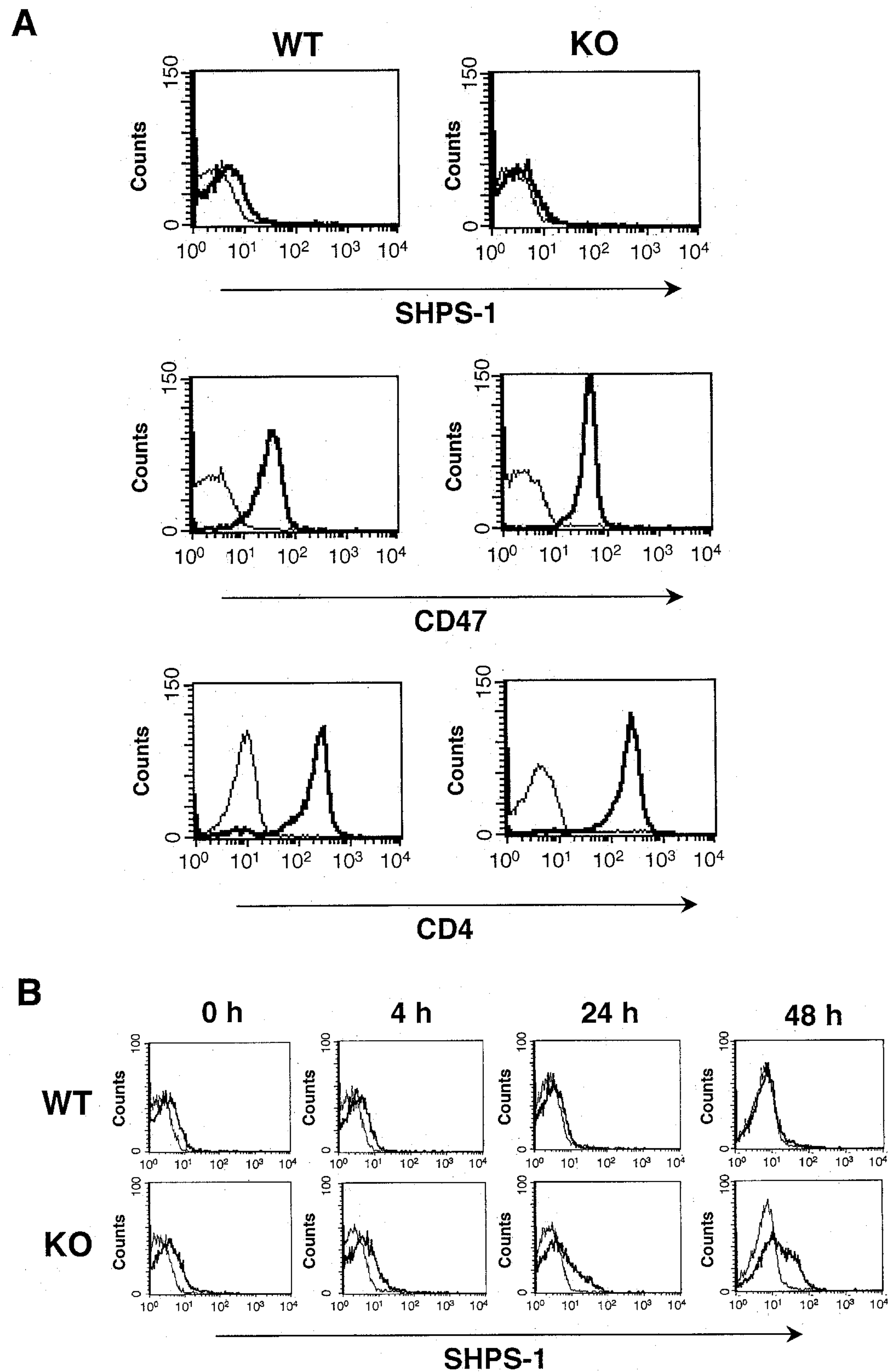

Fig. 1 Flow cytometric analysis of the surface expression of SHPS-1 and CD47 in $CD4^+$ T cells. (A) Freshly purified $CD4^+$ T cells from WT or SHPS-1 mutant (KO) mice were stained with a biotin-conjugated mAb to SHPS-1 (thick trace), a biotin-conjugated mAb to mouse CD47 (thick trace), or a biotin-conjugated control rat IgG (thin trace), as indicated. The cells were then incubated with PE-conjugated streptavidin. Cells were also labeled with an FITC-conjugated mAb to mouse CD4 (thick trace) or an FITC-conjugated control rat IgG (thin trace). All stained cells were analyzed by flow cytometry. (B) Freshly purified $CD4^+$ T cells from WT or SHPS-1 mutant mice were cultured for the indicated times on plates coated with a mAb to CD3 ($10\mu g/ml$), after which the cells were stained first with a biotin-conjugated mAb to SHPS-1 (thick trace) or a biotin-conjugated control rat IgG (thin trace) and then with PE-conjugated streptavidin. The stained cells were analyzed by flow cytometry. All results are representative of three separate experiments.

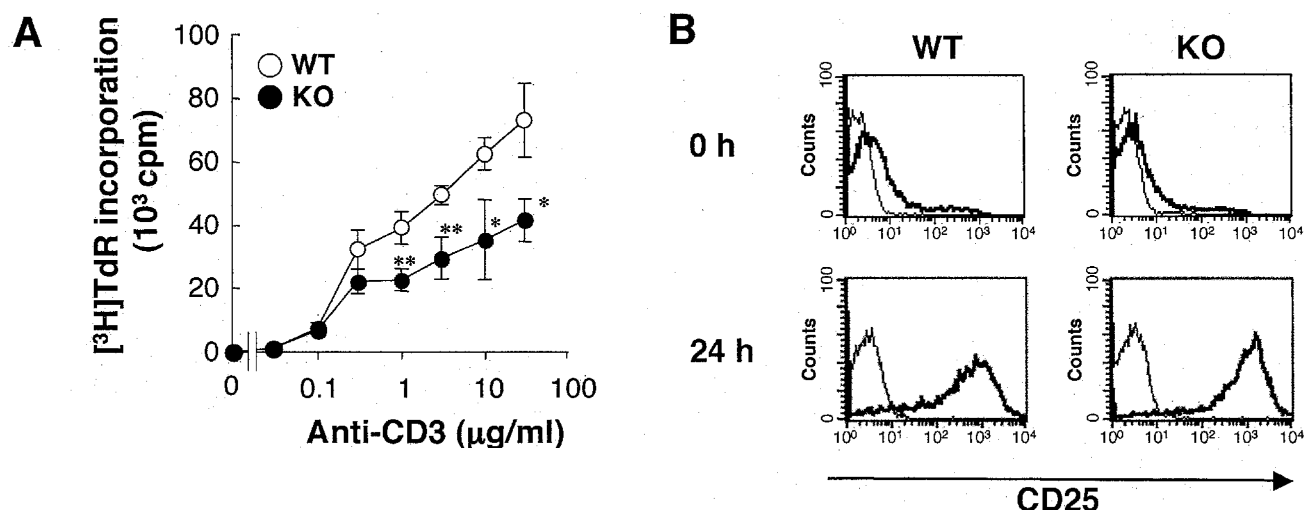

Fig. 2 Impaired proliferation of CD4$^+$ T cells from SHPS-1 mutant mice. (A) Purified CD4$^+$ T cells from WT or SHPS-1 mutant mice were cultured for 48 h on plates coated with various concentrations of a mAb to CD3. The cells were exposed to [^{3}H] TdR during the final 14 h of culture, and the cell-associated radioactivity was subsequently measured with a scintillation spectrometer. Data are means ± SE of values from triplicate determinations and are representative of three separate experiments. *$p<0.05$, **$p<0.01$ versus corresponding value for WT cells (Student's t test). (B) The expression level of CD25 on the surface of purified CD4$^+$ T cells from WT or SHPS-1 mutant mice was determined by flow cytometry at 0 and 24 h after stimulation by TCR cross-linking with a mAb to CD3 (10μg/ml). Data are representative of three separate experiments.

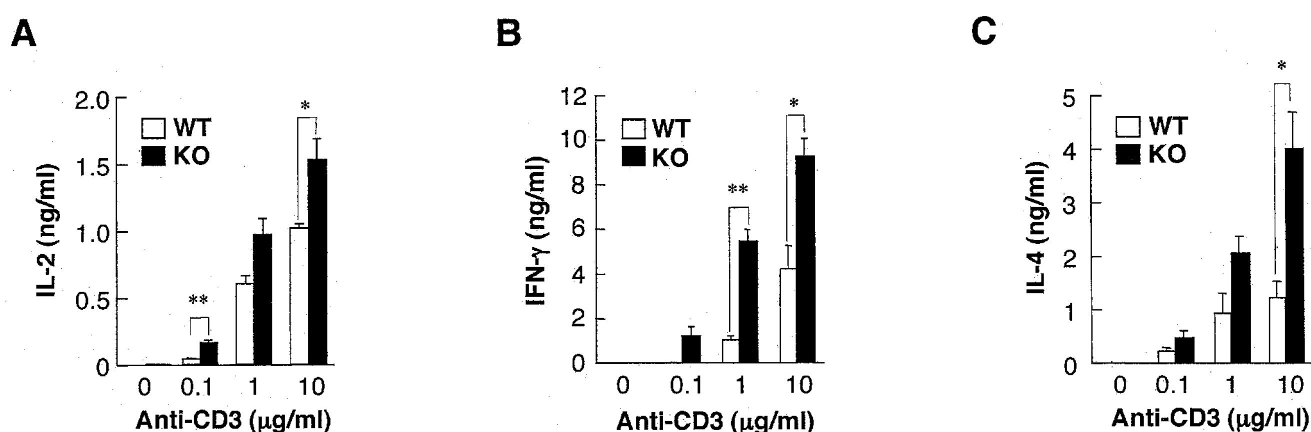

Fig. 3 Enhanced cytokine production by CD4$^+$ T cells from SHPS-1 mutant mice. Purified CD4$^+$ T cells from WT or SHPS-1 mutant mice were cultured for 48 h on plates coated with various concentrations of a mAb to CD3, after which the concentrations of IL-2 (A), IFN-γ (B), and IL-4 (C) in culture supernatants were determined. Data are means ± SE of values from triplicate determinations and are representative of three separate experiments. *$p<0.05$, **$p<0.01$ for the indicated comparisons (Student's t test).

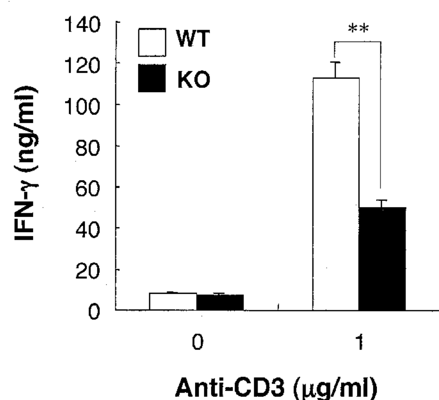

Fig. 4 Impaired Th1 differentiation of CD4$^+$ T cells from SHPS-1 mutant mice. Purified CD4$^+$ T cells from WT or SHPS-1 mutant mice were cultured for 6 days in medium containing IL-12 (10ng/ml) and a mAb to IL-4 (10μg/ml) and on culture plates coated with a mAb to CD3 (2μg/ml). The cells were then stimulated with a mAb to CD3 (1μg/ml) for 24 h, after which culture supernatants were assayed for IFN-γ. Data are means ± SE of values from triplicate determinations and are representative of three separate experiments. **$p<0.01$ (Student's t test).

of EAE as well as in that of other experimental autoimmune diseases.[23,24] SHPS-1 might thus also play a regulatory role in development of Th17 cells, and a defect in this function also might contribute to the resistance of SHPS-1 mutant mice to EAE.

The molecular mechanisms by which SHPS-1 positively regulates the effect of IL-2 on T cell proliferation and that of IL-12 on Th1 differentiation remain unknown. Signaling by JAK1 or JAK3 and by STAT5 is thought to mediate responses to IL-2.[25,26] Similarly, the JAK2-STAT4 signaling pathway and the p38 isoform of MAPK are implicated in this effect of IL-12.[27,28] Given that SHP-2 positively regulates activation of the JAK-STAT pathway and MAPK signaling[25] and that association of SHP-2 with SHPS-1 is specifically defective in SHPS-1 mutant mice, it is possible that the SHPS-1-SHP-2 complex positively regulates activation of the JAK-STAT pathway or p38 MAPK by IL-2 or IL-12 in $CD4^+$ T cells. We have also found that the IL-12-induced production of IFN-γ by DCs of SHPS-1 mutant mice is impaired.[19] It is thus likely that SHPS-1, presumably through complex formation with SHP-2, positively regulates JAK-STAT signaling in general.

In contrast to the impaired responses to IL-2 or IL-12, the production of IL-2, IFN-γ, and IL-4 by $CD4^+$ T cells of SHPS-1 mutant mice in response to TCR activation was increased, compared with that apparent in WT cells. The production of IFN-γ in response to TCR activation is markedly enhanced in $CD4^+$ splenocytes of motheaten viable mice,[29] which harbor a mutation in the SHP-1 gene, suggesting that SHP-1 plays a negative role in regulation of this process. SHPS-1 may therefore negatively regulate the TCR-stimulated production of IFN-γ through its formation of a complex with SHP-1. Fcγ receptor (FcγR)-mediated phagocytosis is enhanced in macrophages from SHPS-1 mutant mice, and it is thought that the SHPS-1-SHP-1 complex negatively regulates such phagocytosis.[15,16] The signaling pathway downstream of FcγR is similar to that downstream of the TCR ; the former includes the Src family kinase Lyn, which activates Syk, whereas the latter includes the Src family kinase Lck, which activates ZAP-70, a kinase structurally similar to Syk.[30,31] It is thus possible that the SHPS-1-SHP-1 complex negatively regulates processes mediated by Lyn/Lck and Syk/ZAP-70 signaling in immune and other hematopoietic cells.

In conclusion, our results indicate that SHPS-1 is essential for regulation of the proliferation and Th1 differentiation of $CD4^+$ T cells. Further studies are required to characterize the molecular mechanisms of such regulation.

Acknowledgments

We thank C.F. Lagenaur for the mAb to SHPS-1 ; P.-A. Oldenborg for the mAb to CD47 ; K.Okumura for the mAbs to CD3 and IL-4 ; and A.Morita, Y. Niwayama, Y.Hayashi, and R.Koitabashi for technical assistance. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas Cancer, a Grant-in-Aid for Scientific Research (B) and (C), a grant of Initiatives for Attractive Education in Graduate Schools, and a grant of the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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