autophosphorylation (*20*, *21*). Forced expression of Dok-7 induced an intense tyrosine phosphorylation of MuSK but not the kinase-inactive mutant with a Lys/Ala substitution (MuSK-KA), indicating that Dok-7 induced the autophosphorylation of MuSK (Fig. 1G). This activity was unique to Dok-7; no other mammalian Dok-family proteins induced phosphorylation of MuSK (fig. S6). It was also conserved; Dok-7 from puffer fish was able to activate even mammalian MuSK. Also, in C2 myotubes, the forced expression of Dok-7 induced tyrosine phosphorylation of MuSK and the β subunit (AChRβ1) of the AChR complex, which is known to be tyrosine-phosphorylated upon activation of MuSK (*22*) (Fig. 1H). Furthermore, this forced expression induced numerous clusters of AChRs, and the number of AChR clusters correlated with the amount of Dok-7 expression plasmid (Fig. 1, I and J; fig. S7A and supporting online material). The exogenous Dok-7–induced AChR clusters were elaborately branched, and their complicated architecture resembled the differentiated "pretzel-like" AChR clusters formed in vivo (fig. S7, B and C). In addition, forced expression in myotubes of Dok-7 that had been fused with enhanced green fluorescent protein (EGFP) induced Dok-7 and AChR coclustering (fig. S7, D to I), as observed at postsynaptic areas in vivo (Fig. 1, C and F).

Because the regulatory interaction of Dok-7 with MuSK as described above implies their physical interaction, we examined whether Dok-7 binds to MuSK by way of the PTB domain in 293T cells. MuSK was coimmunoprecipitated with Dok-7 but not with Dok-7 carrying three Arg/Ala substitutions (Dok-7–RA) in the PTB domain (Fig. 2A). Consistently, the mutant MuSK carrying either a Tyr/Phe substitution at Tyr^{553} (MuSK-YF) or an Asn/Ala substitution at Asn^{550} (MuSK-NA) in the PTB target motif was not coimmunoprecipitated with Dok-7 (fig. S9 and Fig. 2B). The failure of the MuSK-KA kinase-inactive mutant to be coimmunoprecipitated with Dok-7 confirms the requirement of tyrosine phosphorylation for the binding of MuSK with Dok-7 via the PTB domain. These results indicate that Dok-7 binds to MuSK through the PTB domain in a manner dependent on the tyrosine phosphorylation of its target motif in MuSK.

Nevertheless, mutations in the PTB domain (Dok-7–RA) or PTB target motif (MuSK-NA or -YF) did not block activation of MuSK, at least in heterologous cells (Fig. 2, A and B). In addition, the N- and C-terminal deletion mutants of Dok-7 (Dok-7-ΔN and -ΔC) revealed that the C-terminal moiety, but not the PH domain, of Dok-7 is dispensable for MuSK activation in heterologous cells (fig. S10). Also, the forced expression of Dok-7–RA or Dok-7–ΔC induced MuSK activation even in C2 cells at day 3 of differentiation into myotubes (Fig. 2C), when very few myotubes have formed. Unexpectedly, however, the PTB domain and C-terminal portion were indispensable for Dok-7–induced MuSK activation and AChR clustering in fully differentiated C2 myotubes at days 6 and 7 of differentiation (Fig. 2, C and D). In addition, the PH domain, responsible for membrane localization in general, was indispensable for the activation of MuSK in fully differentiated myotubes (fig. S11), as was seen in heterologous cells (fig. S10). Together these findings suggest that a negative regulatory mechanism preventing MuSK activation is established upon differentiation into myotubes, which is accompanied by increased expression of MuSK and Dok-7 (fig. S12). Trace phosphorylation of MuSK in myotubes might allow physical interaction with Dok-7, in turn facilitating dimerization and/or conformational changes in MuSK that are necessary for its sustained activation.

MuSK-deficient myotubes do not form agrin-dependent or -independent clusters of AChRs unless MuSK is reintroduced (*18*, *19*, *23*). To confirm whether Dok-7–mediated AChR clustering is dependent on MuSK, we introduced Dok-7 into MuSK-deficient myotubes. Unlike its effect in C2 myotubes, forced expression of Dok-7 induced no AChR clustering in the MuSK-deficient myotubes; however, additional expression of wild-type MuSK resulted in robust clustering of AChRs in these cells (Fig. 3A). Furthermore, the MuSK-KA and MuSK-YF mutant each failed to complement the MuSK deficiency, regardless of exogenous Dok-7. These findings demonstrate that Dok-7–induced AChR clustering in myotubes depends on Dok-7 interaction with MuSK and subsequent activation of MuSK catalytic activity. Thus, we examined the regulatory interaction of Dok-7 with a MuSK mutant [MuSK-Val/Met (MuSK-VM)] that carries a Val^{790} to Met substitution. This mutation is causally associated with the congenital myasthenic syndrome by way of an as yet unclear mechanism (*24*). As observed with MuSK-YF (Fig. 2B), forced expression of Dok-7 in 293T cells induced the autophosphorylation of MuSK-VM, but its coimmunoprecipitation with Dok-7 was barely detectable in these heterologous cells (fig. S13). Forced expression of Dok-7 with MuSK-VM induced only very weak AChR clustering in MuSK-deficient myotubes (Fig. 3A). Therefore, the congenital myasthenic syndrome–associated Val^{790} to Met mutation impaired interaction of MuSK with Dok-7, suggesting a possible cause of neuromuscular junction dysfunction in these patients.

To examine the effects of Dok-7 down-regulation in myotubes, we used a small interfering RNA (siRNA) designed specifically to block its expression. Inhibition of Dok-7 suppressed the tyrosine phosphorylation of

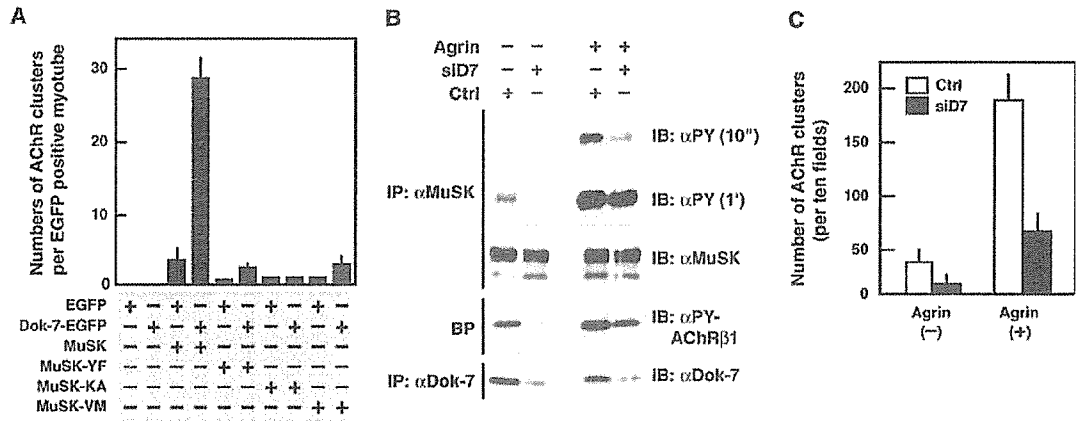

Fig. 3. Dok-7 is essential for activation of the MuSK-pathway to AChR clustering in myotubes. (**A**) MuSK is required for Dok-7–induced AChR clustering. MuSK-deficient myotubes were transfected with the indicated plasmids. The number of AChR clusters (mean ± SD) per EGFP-positive myotube is shown. MuSK-VM is a congenital myasthenic syndrome–associated mutant. (**B**) Activation of the MuSK pathway requires Dok-7. C2 myotubes transfected with Dok-7 siRNA (siD7) or the control (Ctrl) without (−) or with (+) agrin treatment for 15 min were studied as in Fig. 1H. Both short [10 s (10″)] and long [1 min (1′)] exposures are shown for the anti-PY IB of the anti-MuSK IP. (**C**) Dok-7 is essential for AChR clustering. C2 myotubes were transfected with Dok-7 siRNA (siD7) or the control (Ctrl) with or without agrin treatment for 12 hours. The number of AChR clusters (mean ± SD) is shown.

MuSK and AChRβ1 in C2 myotubes, demonstrating its essential role in the aneural, basal catalytic activity of MuSK (Fig. 3B). Indeed, MuSK-dependent spontaneous AChR clustering was suppressed by this siRNA-mediated inhibition (Fig. 3C). Moreover, the inhibition of Dok-7 impaired the agrin-dependent activation of MuSK, the phosphorylation of AChRβ1, and the subsequent formation of AChR clusters (Fig. 3, B and C). Thus, we conclude that Dok-7 is essential for aneural activation of MuSK and AChR clustering in myotubes and is also crucial for agrin-dependent activation of MuSK and AChR clustering. Nonetheless, our results do not exclude the possibility that Dok-7 might also play a role downstream of MuSK. Indeed, Dok-7 and MuSK were synchronously tyrosine phosphorylated upon treatment of myotubes with agrin (fig. S14).

We generated mice lacking Dok-7 to explore its role in vivo (fig. S15). Like mice lacking MuSK or agrin (*6*, *7*), all Dok-7–deficient (Dok-7$^{-/-}$) mice were immobile at birth and died shortly thereafter (26 homozygotes were observed among the first 137 pups), although their wild-type and heterozygous littermates appeared normal. Also, the alveoli of the mutant mice were not expanded at birth (fig. S15D), indicating a failure to breathe and suggesting a severe defect in neuromuscular transmission in the skeletal muscles. Consistently, there were no detectable AChR clusters in the endplate area of the diaphragm muscle in Dok-7$^{-/-}$ embryos at either E14.5 or E18.5 (Fig. 4, E and K). Because nascent AChR clusters are formed in a nerve- and agrin-independent manner at E13.5 to E16.5, whereas most neuromuscular junctions are formed in a nerve- and agrin-dependent manner at E18.5, our findings indicate a requirement for Dok-7 in both types of MuSK-dependent postsynaptic specialization, although we cannot exclude the possibility that nascent AChR clustering is a prerequisite for nerve- and agrin-dependent AChR clustering (*9*–*11*). Consistent with this finding, Dok-7 transcripts were expressed in the endplate area of the diaphragm muscle (fig. S5). In addition, axonal branches extending from the motor nerve trunk were aberrantly long in the endplate area of Dok-7$^{-/-}$ diaphragms at E18.5 and, unlike the controls, did not terminate near the nerve trunk (Fig. 4, G and J). Overall, these pre- and postsynaptic abnormalities are indistinguishable from those found in mice lacking MuSK (*7*), demonstrating an essential role in vivo for Dok-7 in neuromuscular synaptogenesis, a MuSK-dependent vital process.

MuSK-dependent postsynaptic specialization during neuromuscular synaptogenesis appears to be controlled by multiple regulatory mechanisms (*2*, *25*). We have shown that Dok-7 may be a muscle-intrinsic activator of MuSK by demonstrating its essential role in the aneural activation of MuSK and subsequent AChR clustering in cultured myotubes. This conclusion is further supported by our findings that mice lacking Dok-7 showed marked disruption of neuromuscular synaptogenesis that was indistinguishable from the disruption found in MuSK-deficient mice. Thus, neuromuscular synaptogenesis requires Dok-7 within the skeletal muscle. Dok-7 dysfunction may be involved in the pathogenesis of neuromuscular junction disorders.

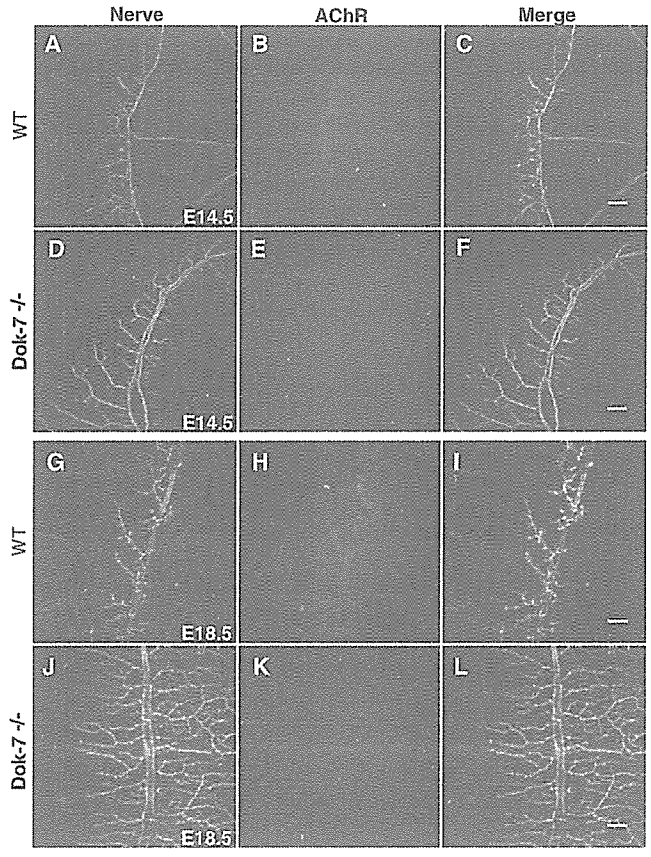

Fig. 4. Dok-7 is essential for neuromuscular synaptogenesis in vivo. Diaphragm muscles were prepared from the wild-type control (WT) or Dok-7$^{-/-}$ embryos at E14.5 (**A** to **F**) or E18.5 (**G** to **L**) and subjected to whole-mount anti-neurofilament and α-bungarotoxin staining, to visualize nerve and AChR, respectively. Scale bars, 100 μm.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/312/5781/1802/DC1
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Figs. S1 to S15
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Significant inhibition of TRAIL-mediated fibroblast-like synovial cell apoptosis by IFN-γ through JAK/STAT pathway by translational regulation

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The pathway of interferon-γ (IFN-γ)-induced suppression in tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-mediated apoptosis of fibroblast-like synovial cells (FLS) was investigated. rTRAIL triggered FLS apoptosis in a type II cell death manner, whereas IFN-γ pretreatment significantly inhibited TRAIL-mediated apoptosis. As disruption of mitochondrial transmembrane potential ($\Delta\Psi m$), Leu-Glu-His-Asp ase (IETD ase) activity, and the appearance of hypodiploid DNA + cells were markedly suppressed in IFN-γ-treated FLS in response to TRAIL, IFN-γ-induced suppression was supposed to achieve at upstream of caspase-8. IFN-γ rapidly phosphorylated signal transducers and activators of transcription 1 (STAT1), STAT3, and STAT6 as well as ERK, whereas enhanced neither phosphorylation of Akt nor nuclear translocation of nuclear factor κB (NF-κB) p65. Janus kinase (JAK)-induced phosphorylation of STAT1/3/6, which acts at translational regulation, seemed to be crucial because chemical inhibition of JAK as well as cycloheximide (CHX) abolished both the phosphorylation of STAT1/3/6 and the IFN-γ-induced inhibitory effect. Although ERK was phosphorylated through IFN-γ, chemical inhibition of ERK by PD98059 did not abolish the IFN-γ-induced inhibitory effect. The authors tried to determine the responsible molecules; however, expression of TRAIL receptors; pro-caspase-3/-8/-9; Fas-associated death domain protein (FADD); tumor necrosis factor receptor 1-associated death domain protein (TRADD); silencer of death domain (SODD); FLICE inhibitory protein (FLIP); and Bcl-2, Bcl-xL, and Bax in FLS was not modulated by IFN-γ. Although the authors have not yet clarified the precise mechanism, these data suggest that IFN-γ/JAK/STAT pathway, which is supposed to be activated in inflammatory rheumatoid arthritis (RA) synovial tissues, contributes to form apoptosis resistance phenotype of the cells *in situ*, leading to a marked increase in cellularity of synovial cells. (J Lab Clin Med 2006;147:182–190)

Abbreviations: Abs = antibodies; ATA = aurintricarboxylic acid; BSA = bovine serum albumin; cDNA = complementary DNA; CHX = cycloheximide; DcR = decoy receptor; DEVD = Asp-Glu-Val-Asp; DISC = death-inducing signaling complex; DR = death receptor; $\Delta\Psi m$ = disruption of mitochondrial transmembrane potential; ECL = enhanced chemiluminescence; EDTA = ethylenediaminetetraacetic acid; ERK = extracellular signal-regulated kinase; FADD = Fas-associated death domain protein; FITC = fluorescein isothiocyanate; FLIP = FLICE inhibitory protein; FLS = fibroblast-like synovial cells; IETD = Leu-Glu-His-Asp; IFN-γ = interferon γ; JAK = Janus kinase; LEHD = Leu-Glu-His-Asp; mAbs = monoclonal antibodies; MFI = mean fluorescence intensity; NF-κB = nuclear factor κB; OA = osteoarthritis; PE = phycoerythrin; PMSF = polymethylsulfonylfluoride; PVDF = polyvinylidene fluoride; RA = rheumatoid arthritis; rTRAIL = recombinant human TRAIL;

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SCID = severe combined immunodeficiency; SD = standard deviation; SDS-PAGE = sodium dodecyl sulfide-polyacrylamide gel electrophoresis; SODD = silencer of death domain; STAT = signal transducers and activators of transcription; TBS = Tris-buffered saline; TNF = tumor necrosis factor; TRAIL = tumor necrosis factor-related apoptosis inducing ligand; TRADD = tumor necrosis factor receptor 1-associated death domain protein

Gene expression signatures of rheumatoid synovial tissues defined by cDNA microarray are divided into at least two molecularly distinct forms: One strongly expresses gene clusters predominantly found in lymphoid-lineage cells and antigen-presenting cells, and the other expresses those involved in tissue remodeling and stromal cell differentiation.[1,2] Inflammatory response in the former RA patients group is higher than the latter RA patients group,[1] and the former gene clusters include the genes, expression of which are induced by the IFN-γ/JAK/ STAT pathway.[1,2] IFN-γ, which is crucial for the initiation of both innate and acquired immune systems, is a representative cytokine to stimulate the JAK/STAT signaling pathway.[3] IFN-γ stimulates synovial cells *in vitro* to induce the expression of HLA class II and costimulating molecules,[4,5] and T-cells in rheumatoid synovial tissues grafted in SCID mice produce large amounts of IFN-γ.[6] Immunohistochemical studies have found the reinforced expression of STAT proteins in rheumatoid synovial tissues as compared with OA synovial tissues,[2] which suggests the importance of the IFN-γ/JAK/STAT pathway to perpetuate rheumatoid synovial inflammatory responses.

Impaired apoptosis in synovial cells is closely associated with hyperplasia of synovial tissues found in patients with RA.[7-9] Synovial cells *in vitro* are committed to apoptotic cell death through varying stimuli; however, histochemical studies have shown the paucity of apoptosis of synovial cells *in situ* in the synovial tissues isolated from RA patients.[7-10] Various kinds of cytokines determined in rheumatoid synovial tissues inhibit apoptosis sensitivity of cultured synovial cells,[7] which may contribute to apoptosis resistance phenotype of rheumatoid synovial cells *in situ*.

Recent studies have demonstrated that blockage of TRAIL-mediated apoptosis accelerates the severity of collagen-induced arthritis in mice, indicating the critical role of the TRAIL death signal in inflammatory arthropathy.[11,12] Upon binding to death domain-containing TRAIL receptors, DR4 and DR5, TRAIL triggers apoptotic cell death in various cell types,[13,14] and the above data may imply that TRAIL is a negative regulator of synovial cell growth. In this regard, these authors as well as other investigators have recently shown that FLS are committed to TRAIL-mediated apoptosis *in vitro* in a type II cell death manner[15-17]; however, the role of IFN-γ toward TRAIL-mediated synovial cell apoptosis has not yet been investigated.

This study was designed to define the role of IFN-γ in TRAIL-mediated apoptosis of FLS. IFN-γ treatment did not change the expression of TRAIL receptors, whereas IFN-γ significantly suppressed TRAIL-mediated FLS apoptosis. STAT1, STAT3, and STAT6, activated by IFN-γ through JAKs, were considered crucial in the suppression of TRAIL-mediated apoptosis through translational regulation where the effect acts at upstream of caspase-8. These data suggest that IFN-γ, locally produced in rheumatoid synovial tissues from T-cells, promotes the survival of synovial cells *in vivo*, leading to synovial cell hyperplasia.

MATERIALS AND METHODS

Reagents. The following antibodies were used in the current study. Goat polyclonal Abs toward human DR4, DR5, DcR1, and DcR2 were purchased from R&D Systems Inc. (Minneapolis, Minn). Anti-phospho-STAT1 Ab (Ser727), anti-phospho-STAT3 Ab (Ser727), anti-phospho-STAT6 (Tyr641) Ab, anti-phospho-extracellular signal-regulated kinase1/2 (ERK1/2) Ab (Thr 202/Tyr 204), and anti-phospho-Akt Ab (Ser 473) were purchased from Cell Signaling Technology (Beverly, Mass). Mouse anti-human Bcl-2 Ab was obtained from Dako Japan (Kyoto, Japan) and rabbit anti-human Bax Ab from Santa Cruz Biotechnology (Santa Cruz, Calif). Mouse anti-human Bcl-xL Ab was purchased from Trevigen (Gaithersburg, Calif). Antibodies to caspases were purchased from MBL (Nagoya, Japan) (mouse monoclonal Abs toward casapse-3, caspase-8, and caspase-9, respectively). Antibodies toward FADD, TRADD, and FLIP were purchased from MBL. Anti-SODD was obtained from Upstate biotechnology Inc. (Lake Placid, NY). β-actin was obtained to see internal control protein expression (Sigma Chemical Co., St. Louis, Miss). rTRAIL was obtained from R&D Systems Inc., and ATA, an inhibitor of JAKs,[18] was purchased from Sigma Chemical Co. CHX, a inhibitor for the translation, was also obtained from Sigma Chemical Co.

Isolation of FLS. FLS were isolated from 14 patients with RA at the time of orthopedic surgery (total knee replacement) conducted in National Ureshino Hospital as previously described.[15,16] A signed consent was also obtained from each patient. Briefly, the synovial tissues were trimmed of fat and minced with scissors, and then added to a mixture of collagenase (Sigma Chemical Co., St. Louis, Miss) and dispase (Godo Shusei Co., Tokyo, Japan). The tissue mixture was digested over a 45-min period during gentle stirring at 37°C, and the harvested cells were allowed to adhere to Petri dishes (Falcon 3003; Becton Dickinson Co., Oxnard, Calif). The

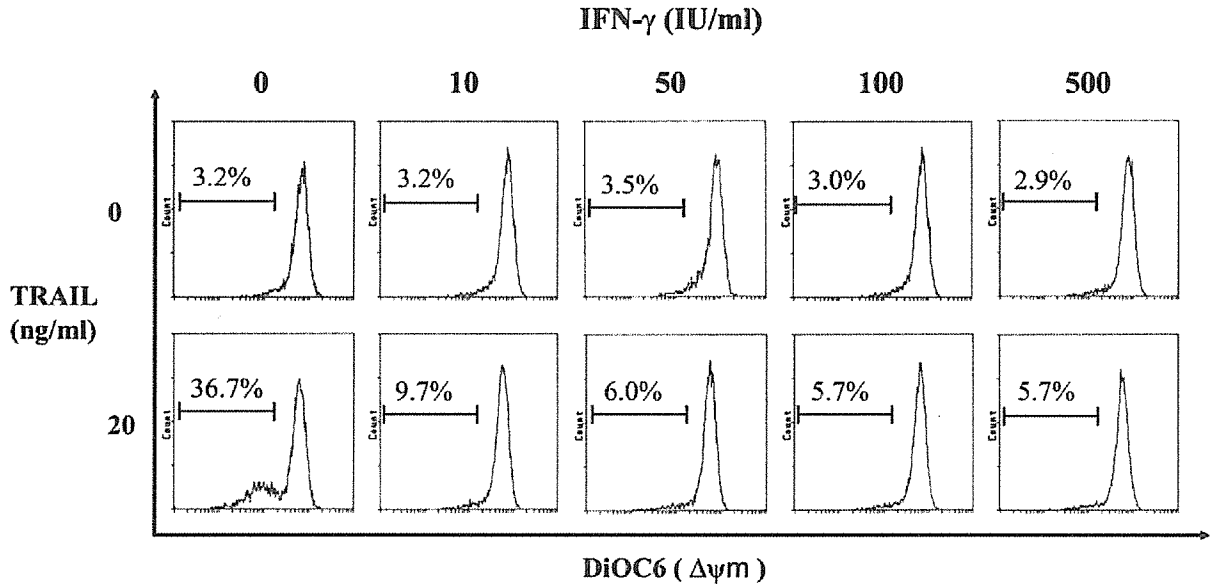

Fig 1. Significant inhibition of TRAIL-mediated FLS apoptosis by IFN-γ. FLS were cultured with or without various concentrations of rIFN-γ for 24 h and further incubated with rTRAIL (20 ng/mL) for another 2 h. After cultivation, apoptosis of FLS was quantified by ΔΨm as described in the Materials and Methods section. Note the dose-dependent inhibition of TRAIL-mediated apoptosis by IFN-γ, the effect of which was noted at 10 IU/mL with a peak effect at 50 IU/mL. Numbers indicate % of ΔΨm. Representative data from six patient samples.

Table I. IFN-γ-induced inhibition of the activation of caspase-3/-8/-9 in FLS in response to TRAIL

Stimuli		DEVDase + cells (%)	IETDase + cells (%)	LEHDase + cells (%)
IFN-γ(IU/mL)	TRAIL (ng/mL)			
0	0	2.5 ± 0.2	2.3 ± 0.2	2.2 ± 0.1
50	0	2.2 ± 0.2	2.1 ± 0.2	2.2 ± 0.1
0	20	25.2 ± 1.7 *	32.4 ± 1.6 *	24.4 ± 1.3 *
50	20	6.9 ± 0.5 *	7.2 ± 0.6 *	5.7 ± 0.4 *

Notes: FLS were cultured with or without 50 IU/mL of rIFN-γ for 24 h, and further incubated with rTRAIL (20 ng/mL) for another 2 h. After cultivation, the activation of caspase-3 (DEVDase), caspase-8 (IETDase), and caspase-9 (LEHDase) was quantified as described in the Materials and Methods section. Data are mean ± SD % of intercellular active caspase + cells from five patient's samples.
*$P < 0.01$, between two data.

adherent synovial cells used in this study at third to fifth passages were less than 1% reactive with various mAbs, including CD3, CD68, CD20, and von Willebrand factor, which define FLS.

Determination of TRAIL-mediated apoptosis in FLS. FLS were cultured in RPMI1640 containing 2% BSA in the presence or absence of rIFN-γ (Shionogi & Co., Ltd., Osaka, Japan) for 24 h, washed, and further incubated with rTRAIL for 2 h. After cultivation, TRAIL-mediated FLS apoptosis was examined by ΔΨm, activation of caspases, and the presence of hypodiploid DNA+ cells as previously described.[15,16] ΔΨm was examined by flowcytometric analysis. Treated FLS were detached by adding 0.265-mM EDTA, washed, and further incubated with a saturating amount of DiOC6 (3, 3'-dihexyloxacarbocyamine iodide; FLUORESZENZTECHNOLOGIE, Grottenhofstr, Austria) at 37°C for 15 min. After incubation, % of ΔΨm in FLS was quantified by flowcytometer (Epics XL; Beckman Coulter, Hialeah, Fl).

Activation of caspases in FLS during TRAIL-induced apoptosis was estimated by CaspGLOW active caspase-3, caspase-8, and caspase-9 staining kit (MBL). In brief, treated FLS were mixed with FITC-conjugated DEVDase for caspase-3, FITC-conjugated IETDase for caspase-8, or FITC-conjugated LEHDase for caspase-9 at 37°C for 60 min. After incubation, the activities of DEVDase, IETDase, and LEHDase were evaluated by a flowcytometer as % of intracellular each active caspase + cells (Epics XL).

DNA fragmentation was quantified by the percentage of cells with hypodiploid DNA. In brief, treated FLS were

Table II. IFN-γ-induced inhibition of the presence of hypodiploid DNA + cells in FLS in response to TRAIL

Stimuli		Hypodiploid DNA + cells (%)
IFN-γ (IU/ml)	TRAIL (ng/ml)	
0	0	2.4 ± 0.1
50	0	2.1 ± 0.1
0	20	32.5 ± 3.6 *
50	20	5.0 ± 0.5 *

Notes: FLS were cultured with or without 50 IU/mL of rIFN-γ for 24 h, and further incubated with rTRAIL (20 ng/mL) for another 2 h. After cultivation, the presence of hypodiploid DNA + cells was quantified as described in the Materials and Methods section. Data are mean ± SD % of hypodiploid DNA + cells from six patient's samples.
*$P < 0.01$, between two data.

fixed with 70% ethanol and treated with RNAase (100 μg/mL; Sigma Chemical Co.) and then stained with propidium iodide (100 μg/mL; Sigma Chemical Co.) for 30 min on ice. The stained cells were analyzed by a flowcytometer (Epics XL) to detect the presence of cells with hypodiploid DNA.

In some experiments, FLS were preincubated with ATA (10 μM) for 1 h before adding rIFN-γ to examine the functional role of JAK/STAT pathway during the process. Furthermore, CHX (Sigma Chemical Co.; 1-h preincubation with 2.5-μg/mL CHX) was used to determine the importance of the translational regulatory mechanism during the process.

Phosphorylation of STATs, ERK, and Akt in FLS by IFN-γ. Western blotting was performed to examine the phosphorylation of STATs, ERK, and Akt in FLS as described previously.[15] In brief, FLS were cultured in RPMI1640 containing 2% BSA in the presence or absence of rIFN-γ for an indicated time. After cultivation, the cells were collected and lysed by the addition of a lysis buffer (1%NP-40, 50-mM Tris, pH 7.5, 100-mM NaCl, 5-mM EDTA, and 1-mM PMSF) for 25 min on ice. Insoluble material was removed by centrifugation (14,000 rpm, 15 min, 4°C). Supernatants were collected, and protein concentration was determined using the Bio-Rad protein assay kit (Melville, NY). Identical amounts of protein (10 μg) for each lysate were subjected to 12.5% SDS-PAGE using Pagel (Bio-Rad, Tokyo). Proteins were transferred to a PVDF filter. The filter was blocked for 2 h using 5% nonfat dried milk in TBS (50-mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, washed with TBS, and incubated at 4°C overnight with each primary antibody (anti-phospho-STAT1/3/6, anti-phospho-ERK1/2, and anti-phospho-Akt, respectively). The filter was then washed with TBS and incubated with 1:1000 dilution of secondary antibodies, coupled with horseradish peroxidase. The ECL system (Amersham, Arlington Heights, Ill) was used for detection. The authors next examined the involvement of the JAK/STAT pathway in the phosphorylation of the above kinases by using ATA. Briefly, FLS were preincubated with 10 μM of ATA for 1 h and further cultured with rIFN-γ, and Western blotting was performed as described above.

Expression of TRAIL receptors, pro-caspase-3/-8/-9, FADD, TRADD, Bcl-2-related proteins and SODD in FLS. Expression of the above molecules in FLS was determined by Western blotting and flowcytometer. In brief, FLS treated with or without rIFN-γ for 24 h, lysed, and the expression of TRAIL receptors (DR4, DR5, DcR1, DcR2), pro-caspase-3/-8/-9, FADD, TRADD, FLIP, Bcl-2-related proteins (Bcl-2, Bcl-xL, Bax), and SODD in FLS was examined by Western blotting as described above. The expression of DR4, DR5, DcR1, and DcR2 on FLS was also examined by flowcytometry as previously described (% of positive cells and MFI of positive cells)Å[16] In brief, treated HUVECs were reacted with the above Abs for 30 min on ice, washed, and further incubated with PE-conjugated anti-gout IgG for 30 min on ice. After incubation, the expression was determined by a flowcytometer.

Statistical analysis. Data were expressed as mean ± SD. Differences between groups were tested for statistical significance using the Student *t*-test. A *P* value less than 0.05 was considered significant.

RESULTS

IFN-γ-induced inhibition of TRAIL-mediated FLS apoptosis. Figure 1 shows an inhibitory effect of IFN-γ toward TRAIL-mediated FLS apoptosis examined by ΔΨm. Short-time exposure of FLS to IFN-γ (~2 h) did not affect apoptosis sensitivity of the cells in response to TRAIL (data not shown); however, 24-h cultivation of FLS with IFN-γ markedly suppressed apoptosis in a dose-dependent manner, the effect of which was noted at 10 IU/mL (Fig 1). As described previously,[15] the activation of caspase-3, caspase-8, and caspase-9 was induced in FLS in response to TRAIL (Table I); however, as noted in ΔΨm of Fig 1, IFN-γ treatment markedly suppressed the activation of caspase-3/-8/-9 induced by TRAIL (Table I). The similar results were obtained by qualifying TRAIL-mediated FLS apoptosis by the presence of hypodiploid DNA (Table II). As the authors recently described,[15,16] the presence of hypodiploid DNA+ cells as well as ΔΨm induced by TRAIL was almost inhibited by adding IETD-FMK (50 μM of IETD-FMK, MBL, inhibitor for caspase-8) in FLS (data not shown). In addition, the activation of caspase-3 and caspase-9 in TRAIL-treated FLS was significantly suppressed by adding 50 μM of IETD-FMK (Fig 2).

Phosphorylation of STAT1, STAT3, and STAT6 by JAK is an important step in IFN-γ-induced suppression of TRAIL-mediated FLS apoptosis. The authors initially examined whether IFN-γ modulated TRAIL receptor expression on FLS. FLS expressed all four TRAIL receptors: DR4, DR5, DcR1, and DcR2, whereas 24-h incubation with IFN-γ did not affect the expression of TRAIL receptors (Fig 3 by Western blotting and Table III by flowcyto-

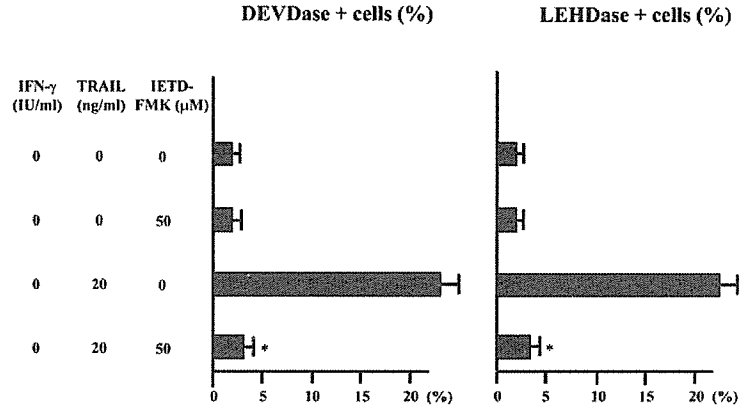

Fig 2. Inhibition of the activation of DEVDase and LEHDase in FLS in response to TRAIL by IETD-FMK. FLS were preincubated in the presence or absence of 50 μM of IETD-FMK for 1 h and further cultured with or without rTRAIL (20 ng/mL) for 2 h. After a total of 3 h of incubation, % of intracellular DEVDase + cells or LEHDase + cells was examined as described in the Materials and Methods section. Note that IETD-FMK significantly suppressed the activation of DEVDase (caspase-3) and LEHDase (caspase-9) in response to TRAIL. *$P < 0.01$, vs 20-ng/mL TRAIL only. Data are mean ± SD from five patient samples.

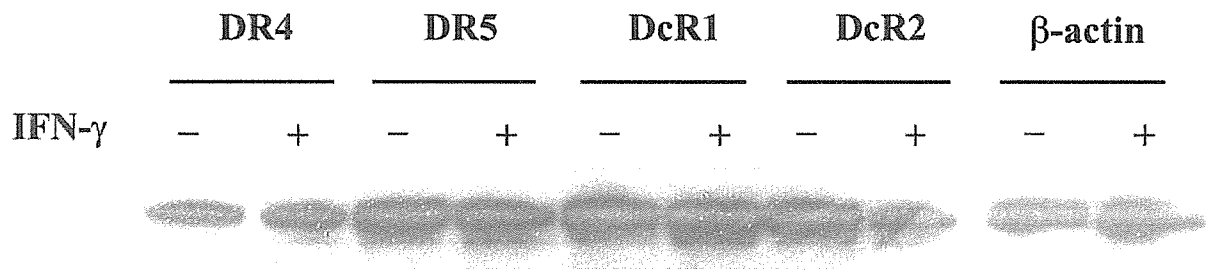

Fig 3. TRAIL receptor expression on FLS by Western blotting. FLS were cultured with or without 50 IU/mL of rIFN-γ for 24 h, and the expression of DR4, DR5, DcR1, and DcR2 was examined by Western blotting as described in the Materials and Methods sectionn. Note that the expression of DR4, DR5, DcR1, and DcR2 on FLS is not changed by IFN-γ. Representative data from five patient samples.

metric analysis, respectively) despite marked inhibition of TRAIL-mediated apoptosis (Fig 1 and Table II). These data indicate that IFN-γ modulates intracellular apoptosis-inducing signal and thus suppresses TRAIL-mediated apoptosis. As shown in Fig 4, *A*, phosphorylation of STAT1, STAT3, and STAT6 as well as ERK was clearly found in FLS in response to IFN-γ, whereas phosphorylation of Akt was not obviously accelerated by IFN-γ. In addition, TRAIL-induced NF-κB p65 nuclear translocation in FLS was not modulated by IFN-γ (data not shown; experiments were performed by microscopic immunofluorescence assay using anti-NF-κB p65; Santa Cruz Biotechnology). The role of activation of STAT1/3/6 during the process was further investigated by chemical inhibition, which showed that administration of JAK inhibitor, ATA, abolished both IFN-γ-induced phosphorylation of STAT1/3/6 (Fig 4, *B*) and IFN-γ-induced inhibitory effect (Fig 5). Interestingly, the use of CHX mimicked the action of ATA regarding TRAIL-mediated apoptosis (Fig 5). Although ERK was phosphorylated in FLS in response to IFN-γ, the use of ERK inhibitor, PD98059 (preincubation for 1 h at 50 μM, purchased from Calbiochem, La Jolla, Calif), did not modulate the IFN-γ-induced inhibitory effect (Fig 6). In addition, IFN-γ-induced phosphorylation of ERK in FLS was not affected by PD98059 (Fig 4, *B*). The authors also examined the expression of apoptosis-related molecules other than TRAIL receptors acting within DISC and downstream of DISC; however, these protein expressions that (FADD, TRADD, pro-caspase-8, FLIP, SODD) act within DISC, and pro-caspase-3, pro-caspase-9, Bcl-2, Bcl-xL, and Bax that act downstream of DISC were not changed by IFN-γ treatment (Fig 7).

Table III. TRAIL receptor expression on FLS was not modulated by IFN-γ treatment

Expression of TRAIL receptor (% of positive cells and MFI)	IFN-γ (IU/ml)			
	0		50	
	%	MFI	%	MFI
DR4	12.2%	2.05	12.4%	2.02
DR5	38.7%	3.01	36.8%	2.99
DcR1	39.4%	2.97	37.2%	2.97
DcR2	37.2%	2.96	36.5%	2.95

Notes: FLS were cultured with or without 50 IU/mL of rIFN-γ for 24 h. After incubation, expression of TRAIL receptors was examined by flowcytometer as described in the Materials and Methods section. Representative data from five patient's samples, demonstrating that expression of neither % of positive cells nor MFI of positive cells was modulated in the presence or absence of 50 IU/mL of IFN-γ. Data of the other four samples showed similar results.

DISCUSSION

The main features of the IFN-γ signal are transduced by activation of JAK1/2, leading to phosphorylation of STAT protein.[3] This study revealed that IFN-γ significantly suppressed TRAIL-mediated apoptosis of FLS, and the authors have shown here that STAT1, STAT3, and STAT6 were rapidly phosphorylated in FLS in response to IFN-γ. ERK was also rapidly phosphorylated in FLS by IFN-γ; however, the chemical inhibition of the process by PD98059 did not modulate the IFN-γ-induced inhibitory effect. Furthermore, ATA (chemical inhibitor for JAKs) did not suppress the phosphorylation of ERK in response to IFN-γ. These data indicate that ERK may not be the principle molecule to transmit the effect of IFN-γ regarding the inhibition of TRAIL-mediated FLS apoptosis. Neither Akt phosphorylation nor NF-κB p65 nuclear translocation, the molecules that interfere with pro-apoptotic stimuli in FLS,[10,15] was not stimulated by IFN-γ. IFN-γ-induced inhibition of TRAIL-mediated apoptosis and the phosphorylation of STAT1, STAT3, and STAT6 were abolished by chemical inhibition with ATA, which suggests that STAT1/3/6 are principal transducers that elicit IFN-γ-induced inhibition toward TRAIL-mediated apoptosis of FLS.

Receptor-mediated apoptosis is divided into type I and type II, based on the requirement of mitochondrial perturbation.[19,20] Type I cell death does not depend on the mitochondria, whereas the latter is critically involved in type II cell death. Recent work by the authors showed that FLS are classified into type II cells with regard to TRAIL-mediated apoptosis, and the essential caspase triggering the mitochondria is caspase-8.[15] The current data suggest that IFN-γ-induced inhibition occurred upstream of caspase-8 in DISC, which results in

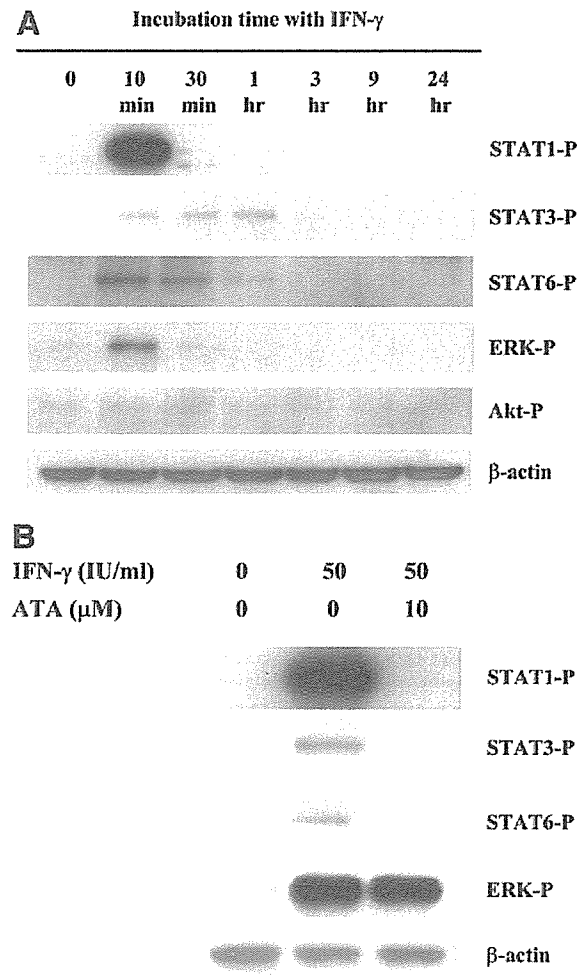

Fig 4. Phosphorylation of STAT1, STAT3, STAT6, ERK, and Akt in FLS in response to IFN-γ. (**A**) Time-kinetic study. FLS were cultured with 50 IU/mL of rIFN-γ for an indicated time (up to 24 h), and phosphorylation of STAT1, STAT3, STAT6, ERK, and Akt was examined as described in the Materials and Methods section. Note that phosphorylation of STAT1, STAT3, STAT6, and ERK in FLS was rapidly induced by IFN-γ, whereas that of Akt was not accelerated. β-actin was used as an internal control protein. Representative data from five patient samples. (**B**) Inhibiton by ATA. FLS were preincubated for 1 h with 10-μM ATA and further cultured with 50 IU/mL of rIFN-γ for 10 min. After incubation, phosphorylation of STAT1, STAT3, STAT6, and ERK was examined as described in the Materials and Methods section. Note that IFN-γ-induced phosphorylation of STAT1, STAT3, and STAT6 in FLS was clearly abolished by ATA, whereas IFN-γ-induced phosphorylation of ERK was not obviously suppressed by ATA. β-actin was used as an internal control protein. Representative data from five patient samples.

suppression of $\Delta\Psi m$ and DNA fragmentation. Up to 24 h is required to show an inhibitory effect of IFN-γ in TRAIL-mediated apoptosis. These observations are consistent with the data that, CHX, an inhibitor of the translation, abolished IFN-γ-mediated suppression.

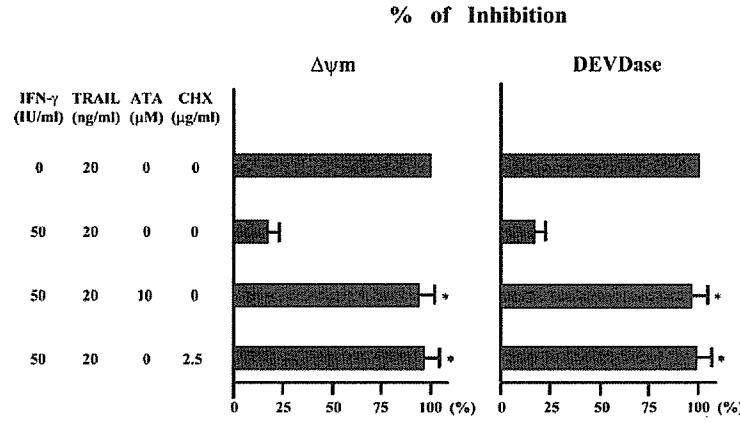

Fig 5. ATA and CHX almost completely abrogated IFN-γ-induced suppression of TRAIL-mediated FLS apoptosis. FLS were preincubated with ATA (10 μM) or CHX (2.5 ng/mL) for 1 h and further cultivated in the presence or absence of rIFN-γ (50 IU/mL) for another 24 h, washed, and incubated with rTRAIL (20 ng/mL) for an additional 2 h. After a total of 27 h of cultivation, ΔΨm and DEVDase activity were examined as described in theh Materials and Methods section. ΔΨm and DEVDase activity of TRAIL only were expressed as 100%, and the effect of ATA and CHX was expressed as % of inhibition relative to that of TRAIL only. Note that IFN-γ-induced inhibition of TRAIL-mediated apoptosis was abolished by ATA and CHX. $^*P < 0.01$ vs IFN-γ-treated FLS with 20-ng/mL TRAIL. Data are mean ± SD of % inhibition from four patient samples.

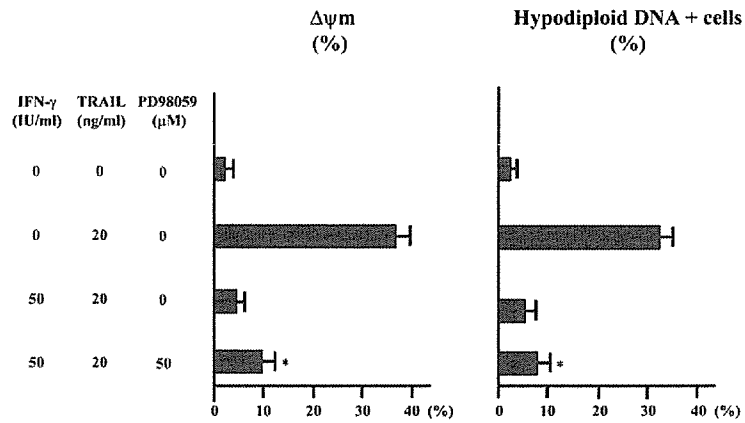

Fig 6. PD98059 did not abolish the IFN-γ-induced suppression of TRAIL-mediated apoptosis in FLS. FLS were preincubated with PD98059 (50 μM) for 1 h and further cultivated in the presence or absence of rIFN-γ (50 IU/mL) for another 24 h. FLS were cultured with rTRAIL (20 ng/mL) for an additional 2 h. After a total of 27 h of cultivation, ΔΨm and hypodiploid DNA + cells were examined as described in the Materials and Methods section. Note that the IFN-γ-induced inhibitory effect was not abolished by PD98059. $^*P < 0.01$ vs FLS cultured with 50-IU/mL IFN-γ and 20-ng/mL TRAIL. Data are mean ± SD of five patient samples.

Thus, it is possible to speculate that the IFN-γ/JAK/STAT-regulated molecule is sensitive to CHX, which acts at the proximity of caspase-8 to inhibit TRAIL-mediated FLS apoptosis. The DISC of the TRAIL pathway is composed from several molecules, including TRAIL receptors, FADD, TRADD, and caspase-8[21]; however, IFN-γ did not change the expression of the above molecules. FLIP and SODD, which are inhibitors of caspase-8 activation, act within DISC,[21,22] whereas the expression was not modulated by IFN-γ. The authors also examined the expression of other apoptosis-related molecules such as Bcl-2, Bcl-xL, and Bax, neither of which expression was changed by IFN-γ. Therefore, other approaches such as the cDNA microarray technique may be indispensable to explore candidate molecules acting downstream of STAT.

Other unsolved questions are which STATs are required for eliciting the protective effect. To date, seven distinct but homologous members of the mammalian STAT family have been identified (designated as

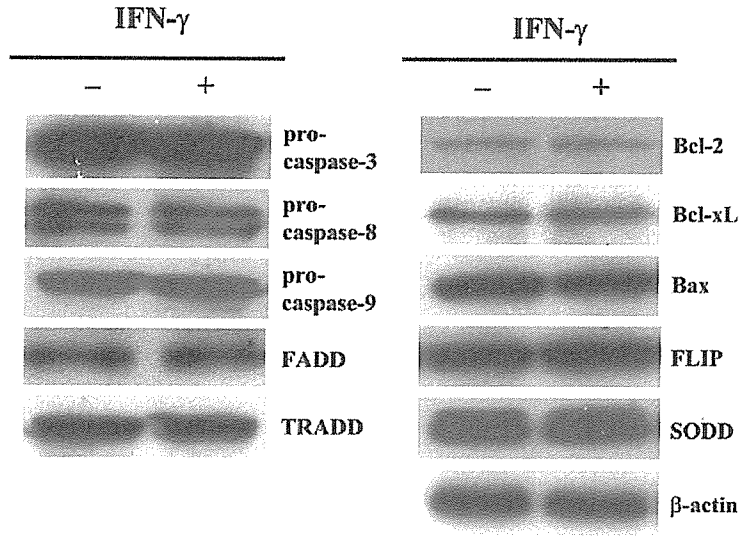

Fig 7. Expression of apoptosis-related molecules in FLS. FLS were cultured with or without rIFN-γ (50 IU/mL) for 24 h, and the expression of each molecule was examined by Western blotting. Note that the expression of pro-caspase-3/-8/-9, FLIP, FADD, TRADD, Bcl-2, Bcl-xL, Bax, and SODD was not modulated by IFN-γ treatment. Data are representative experiments from six patient samples.

STAT1 through STAT6; STAT5A and STAT5B are encoded by different genes),[2,3] and previous studies have reported the elevated and/or activated expression of STAT1, STAT3, and STAT6 in rheumatoid synovial tissues.[2] In general, IFN-γ preferentially activates STAT1, whereas these data showed that STAT3 as well as STAT6 were rapidly phosphorylated in FLS in response to IFN-γ. STAT1, STAT3, and STAT6 are required for the expression of inflammatory genes and the perpetuation of autoimmunity[2,3,23,24]; however, their roles in apoptosis regulation remain controversial.[2,25–28] As STATs are pleiotropic proteins that can have different, and even opposite, activities under different conditions or in different cell types,[2,3] further experiments including the RNA interference study are crucial for functional characterization of each STAT in FLS.

Recent work from the authors has pointed out that Akt works as an anti-apoptotic kinase through post-transcriptional regulatory mechanism in TRAIL-mediated FLS apoptosis,[15] and this study shows that the IFN-γ/JAK/STAT pathway possesses an anti-apoptotic effect through translational regulation. IFN-γ/JAK/STATs are important mediators of inflammatory and immune reactions; thus, identification of the precise molecular mechanisms may assist the development of new therapies that specifically act against synovial cell proliferation by inhibiting JAK/STAT pathways.

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Characterization of peripheral natural killer cells in primary Sjögren's syndrome: Impaired NK cell activity and low NK cell number

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The aim of this study was to compare the number of peripheral blood natural killer (NK) cells, NK cell activity, expression of NK cell activating receptors, and serum cytokine levels in patients with primary Sjögren's syndrome (SS) vs normal controls. The authors found that NK cell number, NK cell killing activity, and the expression of activating receptors CD2 and NKG2D were significantly decreased, and the expression of NKp46, as well as the percentage of apoptotic NK cells, were significantly increased in primary SS patients compared with healthy controls. NK cell killing activity on a per-cell basis was similar in primary SS patients and healthy controls. Moreover, the levels of IL-18 and TNF-α, cytokines that have been shown to promote NK cell death, were significantly increased in sera from patients with primary SS compared with controls. These data suggest that reduced NK cell numbers, probably a result of apoptotic death, may contribute to impaired NK cell activity in patients with primary SS. (J Lab Clin Med 2006;147:242-249)

Abbreviations: AICD = activation induced cell death; $DiOC_6(3)$ = 3,3'-dihexyloxacarbocyanine iodide; ELISA = enzyme-linked immunosorbent assay; ET = effector target; FCS = fetal calf serum; FITC = fluorescein isothiocyanate; GaM = goat anti-mouse; GLCD = granzyme B leakage-induced cell death; HLH = hemophagocytic lymphohistiocytosis; IL = interleukin; ITIM = immunoreceptor tyrosine-based inhibitory motif; IU = international unit; $\Delta\psi m$ = loss of mitochondrial membrane potential; LU = Lytic unit; mAb = monoclonal antibody; MFI = mean fluorescence intensity; MHC = major histocompatibility complex; NK = natural killer; PBS = phosphate buffered saline; PE = phycoerythrin; SD = standard deviation; SHP = Src-homology domain-bearing tyrosine phosphatase; SS = Sjögren's syndrome; TNF-α = tumor necrosis factor-α

NK cells are an important cytolytic component of the innate immune system.[1,2] By monitoring the level of class I MHC molecules expressed on the surface of cells, NK cells can recognize and eliminate transformed and virus-infected autologous cells.[3-5] Target cells expressing reduced levels of class I MHC molecules deliver reduced levels of inhibitory signals to NK cells, resulting in the release of cytotoxic

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Table I. Patient profiles

	Primary SS	Healthy Controls
Number	15	16
Sex (M/F)	0/15	1/15
Age	56.7 ± 15.8	51.6 ± 14.7
White cell number (/μL)	4291 ± 930†	5834 ± 1392
Lymphocyte number (/μL) (percentage)	1464 ± 402 (34.2 ± 7.5)	1659 ± 350 (30.1 ± 9.2)
T cell number (/μL) (percentage)	827.3 ± 352.2 (56.2 ± 16.9)	1016.1 ± 323.7 (60.2 ± 9.5)
NK cell number (/μL) (percentage)	108.2 ± 78.6† (7.6 ± 5.0)*	198.7 ± 106.9 (12.1 ± 6.3)
NKT cell number (/μL) (percentage)	164.1 ± 87.8 (11.5 ± 5.5)	192.1 ± 60.1 (11.6 3.0)
Serum cytokine		
IL-18 (pg/mL)	250.6 ± 154.2*	110.7 ± 68.5
IL-15 (pg/mL)	4.1 ± 1.2	4.5 ± 0.9
TNF-α (pg/mL)	22.1 ± 11.8*	11.9 ± 6.4
IFN-γ (IU/mL)	0.16 ± 0.9	0.09 ± 0.5
IL-6 (pg/mL)	2.6 ± 2.1	1.9 ± 1.4

Notes: Values are the mean ± SD. Serum levels of cytokines were measured by enzyme-linked immunosorbent assay. *Abbreviations:* IFN-γ, interferon γ; IL, interleukin; TNF-α, tumor necrosis factor α.
*$P < 0.05$ versus controls.
†$P < 0.01$ versus controls.

granules containing effector molecules (ie, perforin and granzymes) that bring about target cell death.[6] In addition, NK cells can participate in immune regulation by eliminating autoreactive T cells and B cells.[7–10] Reduced NK cell number or impaired NK cell function might permit the persistence of viral infection, malignant disease, or autoimmune disease.

The authors previously reported that the number of NK cells was significantly decreased in the peripheral blood of patients with systemic autoimmune diseases compared with normal controls.[11] Serum concentrations of IL-18, IL-15, and TNF-α were inversely related to the number of NK cells in both patients and healthy controls. Moreover, IL-18 + IL-15, IL-18 + IL-12, or TNF-α-induced NK cell death *in vitro*, which suggests that high levels of IL-18, IL-15, and TNF-α are associated with the decreased number of NK cells in patients with systemic autoimmune diseases.[11] In addition to low NK cell number, NK cell dysfunction is well known in autoimmune diseases.[12–14] The molecular mechanism of the NK dysfunction in patients with autoimmune disease is poorly understood.

Primary SS is an autoimmune disease that is characterized by symptoms of dry mouth (xerostomia) and dry eyes (kerato-conjunctivitis sicca). Many investigators have reported NK cell cytolytic impairment in SS patients.[15–17] Impaired NK cell activity has been postulated to contribute to the increased incidence of lymphoid malignancy in patients with primary SS. For these reasons, it is very important to identify the mechanisms of NK cell dysfunction in SS patients. In this study, the authors show that NK cell activity is significantly decreased in patients with primary SS and that this decrease is accompanied by reduced numbers of NK cells. Moreover, the percentage of apoptotic NK cells is significantly increased in primary SS compared with controls.

METHODS

Patients and controls. Fifteen patients who presented to the Nagasaki University Arthritis Clinic for evaluation of a primary SS were included in this study. Sixteen healthy control subjects were recruited from the medical staff. Profiles of patients and healthy controls are presented in Table I. White cells (number) and NK cells (percentage and number) were significantly decreased in primary SS patients compared with healthy subjects, whereas lymphocytes (percentage and number), T cells (percentage and number), and NKT cells (CD3+CD161+, percentage and number) were not significantly different between the two groups. Neither steroids nor immunosuppressive drugs were used in any patients with primary SS. Before inclusion in the study, informed consent was signed by each patient approved by the ethical committee of the Nagasaki University.

mAb and flow cytometry. FITC-conjugated anti-human CD3 (UCHT-1; IgG1), PC5-conjugated anti-human CD56 (NKH1; IgG1), PE-conjugated anti-human CD161 (191B8; IgG2a), anti-human CD244 (C1.7.1; IgG1), anti-human CD2 (T11; IgG1), anti-human CD16 (3G8; IgG1), anti-human NKp46 (BAB281; IgG1), anti-human NKp30 (Z25; IgG1), and anti-human NKG2D (ON72; IgG1) mAbs were purchased from Beckman Coulter (Hialeah, Fl). Control mAbs

(FITC; IgG1, PE; IgG1 and IgG2a, and PC5; IgG1) were purchased from Beckman Coulter. Peripheral lymphocytes derived from patients and healthy persons were purified by centrifugation over Ficoll-Hypaque (Amersham Biosciences Corp., Piscataway, NJ). The triple immunofluorescence analysis method has been described in detail elsewhere.[18] Briefly, cell pellets (1×10^6 cells) were incubated with the indicated mAbs for 30 minutes at 4°C. After incubation, the cells were washed twice with PBS supplemented with 2% FCS. The triple-immunofluorescence experiments were analyzed with a flow cytometer (Epics XL; Coulter Electronics, Hialeah, Fl). In the apoptosis experiments, anti-CD2 mAb (T11.3, IgG3) was kindly provided by Dr. Ellis Reinherz (Dana-Farber Cancer Institute, Boston, Mass).[19] Anti-CD16 mAb (3G8, IgG1), anti-CD56 mAb (C218, IgG1), and anti-CD244 mAb (C1.7.1; IgG1) were purchased from Beckman Coulter. Anti-NKG2D mAb (149810, IgG1) was purchased from R&D (Minneapolis, Minn). Goat anti-mouse IgG (GaMIgG) was purchased from Jackson Immunoresearch laboratories Inc. (West Grove, Penn). PE-conjugated AnnexinV was purchased from BD Biosciences (San Jose, Calif).

Isolation of NK cells. NK cells were purified from buffy coat cells (kindly provided by Nagasaki Red Cross Blood Center [Nagasaki, Japan]) using magnetic bead depletion as previously described.[11] Briefly, PBMCs were separated from whole blood by centrifugation over Ficoll-Hypaque. For NK cell isolation, cells were depleted of T lymphocytes, B lymphocytes, and macrophages/monocytes using an NK cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) that includes beads coupled to mAb against CD3, CD14, CD19, CD36, and IgE. Using a MACS magnetic separator (Miltenyi Biotec GmbH), NK cells were enriched to obtain populations containing >95% CD3–CD56+, as determined by flow cytometry (EPICS XL, Coulter Electronics).[18] Enriched NK cells were cultured in final medium (RPMI 1640 with 10% FCS and pen/strep) with or without recombinant human IL-2 [150 IU/mL; which was kindly provided by Takeda Pharmaceutical Co. (Osaka, Japan)] in 250 cm^2 flask (Costar, Cambridge, Mass).

Cytotoxicity assay. Cytotoxicity was measured by means of a 4-h ^{51}Cr release assay previously described.[20] Briefly, target K562 cells (2×10^6) were radiolabeled with 3700-kBq $Na_2{}^{51}CrO_4$ at 37°C for 1 h with occasional shaking. They then were washed three times with PBS containing 2% FCS and finally resuspended at 2×10^5 cells/mL in final medium (RPMI1640 with 10% FCS and penicillin/streptomycin). Chromium-labeled target cells (1×10^4/50 μL) were dispensed into the wells of 96 well U-bottomed microtiter plates (Costar), after which graded numbers of effector cells (peripheral lymphocytes) in 100-μL final medium were added to give effector cell to target cell ratios (E:T ratios) of 50:1, 20:1, and 10:1. Each assay was performed in triplicate. The plates were centrifuged for 5 min at 1000 rpm, and then incubated for 4 h at 37°C at 5% CO_2. After incubation and recentrifugation, aliquots (100 μL) of the supernatant were removed from each well, and their radioactivity was determined in a γ-counter. The data were expressed as the percentage of ^{51}Cr release, calculated according to the following formula: % cytotoxicity = (cpm experimental release- cpm spontaneous release/ cpm total release- cpm spontaneous release) × 100. A lytic unit was defined as the number of effector cells required to produce a specified percentage of target cell lysis.[14,21] The computations can be expressed in a formula: number of lytic units (LU)/10^7 effectors = 10^7/ TXp. T is the number of target cells (eg, 1×10^4), p is the reference lysis level (eg, 20%), and Xp is the E:T ratio required to lyse p% of the targets. LU per NK cell means the cytotoxic power in one NK cell and can be expressed in a formula: 10^7/TXp/number of NK cells (in 10^7 effectors).

Cytokine measurements. Sera from the study subjects were obtained at the same time of day as the peripheral blood, and samples were collected and stored at −20°C. IL-18, IL-15, and TNF-α were measured using cytokine-specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions.[11] The IL-18, IL-15, TNF-α, IFN-γ, and IL-6 ELISA kits were purchased from MBL (Nagoya, Japan), R&D Systems, Inc., Biosource International, Inc. (Camarillo, Calif), Biosource Europe (Nivelles, Belgium), and Fujirebio (Hachioji, Japan), respectively. The detection limits of the assays were as follows: 12.5 pg/mL for IL-18, 2 pg/mL for IL-15, 3 pg/mL for TNF-α, 0.1 IU/mL for IFN-γ, and 0.2 pg/mL for IL-6.

Apoptosis assays. Enriched NK cells (CD3–CD56+>95%) were incubated with 10%FCS RPMI1640 medium supplemented with recombinant IL-2 for 3 days. IL-2-stimulated NK cells were suspended in final medium containing recombinant IL-2 at a concentration of 1×10^6 cells/mL in 24-well plates (Costar). Cells were then warmed to 37°C before adding the indicated antibodies. Apoptosis was analyzed by staining cells with the mitochondria-potential sensitive fluorescent dye $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide) from Lambda Co.(Graz, Austria) to evaluate $\Delta\psi m$ as previously reported.[22-25] Briefly, cells were stained for 15 min at 37°C with 40 nM of $DiOC_6(3)$. After washing with PBS once, the decrement of $\Delta\psi m$ was measured by flow cytometry.

Annexin V expression was quantified using a modi-

Table II. NK activity in primary SS patients compared to normal controls

	NK activity (% specific lysis) at E:T Ratio of			NK activity (Lytic units)	
	50:1	20:1	10:1	L/U	LU/NK
Primary SS	24.7 ± 14.0	10.6 ± 7.4	6.1 ± 4.2	28.2 ± 23.4	0.314 ± 0.148
Controls	39.4 ± 15.3	20.9 ± 11.9	12.4 ± 7.2	90.8 ± 128.0	0.465 ± 0.631
P value (primary SS vs controls)	0.005	0.002	0.0004	0.0027	0.6926

Notes: A lytic unit that has been defined as 10^7 effector cells required to lyse a specified percentage (20%) of target cells in each person. Values are the mean ± SD. *Abbreviation:* LU, lytic unit.

fication of the manufacturers' instructions. Briefly, peripheral lymphocytes derived from patients or healthy controls were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL. One hundred microliters of the solution (1×10^5 cells) was transferred to a fisher tube, then incubated with 5 μL of Annexin V-PE and 7 μL of FITC-conjugated anti-human CD3 and PC5-conjugated anti-human CD56 mAbs for 15 min at room temperature in the dark. Four hundred microliters of 1× binding buffer was gently added to each tube. Stained cells were analyzed by flow cytometry.

Statistical analysis. Data were expressed as the mean ± SD. Differences between groups were tested for statistical significance using Mann-Whitney's *U*-test. *P* values less than 0.05 were considered significant.

RESULTS

NK cell activity in patients with primary SS. To investigate the cytolytic function of NK cells, the authors examined NK cell killing activity by performing a ^{51}Cr release assay. As shown in Table II, NK cell activity was significantly decreased in primary SS patients compared with healthy controls at all E:T ratios. Moreover, the authors calculated a lytic unit that has been defined as the number of effector cells required to lyse 20% of target cells in each person, and then they compared the lytic units produced by NK cells derived from primary SS patients and healthy controls. This comparison revealed that the lytic units produced by NK cells from primary SS patients are significantly less than the lytic units produced by NK cells from healthy controls (Table II), which demonstrates that NK activity is impaired in patients with primary SS. However, lytic units per NK cell, which measures the cytotoxic power on a per cell basis, was similar in primary SS patients and healthy controls (Table II).

Expression of NK cell activating receptors on NK cells in patients with primary SS. As NK cell cytotoxicity is triggered by activating receptors on NK cells, the authors selected seven surface molecules that could activate NK cells, and they examined the expression of each

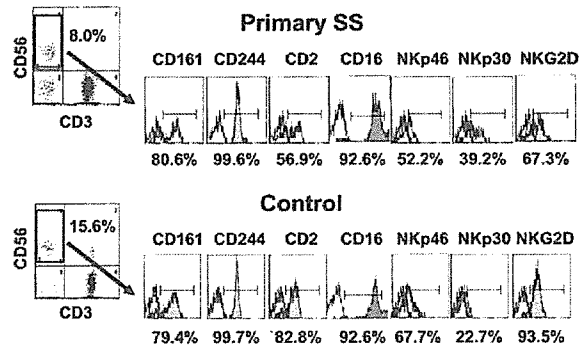

Fig 1. Triple-color flow cytometric analysis of activating receptor expression on peripheral lymphocytes from primary SS and healthy controls. Freshly isolated peripheral lymphocytes were analyzed for expression of CD161, CD244, CD2, CD16, NKp46, NKp30, and NKG2D on the CD3–CD56+ population. In the panel at the left, the CD3–CD56+ region shows quadrilateral line (% = percentage of NK cells). In each histogram, background fluorescence is recorded with a line, and the gray-filled histogram quantifies the expression of the indicated molecules (% = percentage of positive expression of each molecules). The results of the flow cytometric analysis are presented in Table III.

antigen using flow cytometry (Fig 1). Triple staining with mAbs reactive with CD3, CD56, and the indicated antigens allowed quantification of the expression of activation receptors on CD3–CD56+ NK cells. Table III shows that the percentage of CD2 and NKG2D expression on NK cells is significantly decreased in primary SS patients compared with normal controls, whereas the percentage of CD161, CD244, CD16, NKp46, and NKp30 expression is similar in the two groups. The analysis of MFI of each antigen revealed that expression of NKG2D was decreased, whereas expression of NKp46 was increased in primary SS patients compared with normal controls. In contrast, in the T cell population (CD3 positive region), the expression and MFI of all antigens are similar in peripheral lymphocytes derived from both primary SS patients and normal controls (data not shown).

Serum cytokine levels in patients with primary SS. The authors previously reported that high levels of certain

Table III. Surface expression of activating receptors on NK cells in primary SS patients and normal controls

	Expression % (MFI)						
	CD161	CD244	CD2	CD16	NKp46	NKp30	NKG2D
Primary SS	77.4 ± 15.2	99.1 ± 0.7	65.0 ± 13.7*	92.0 ± 5.5	64.3 ± 14.6	29.2 ± 11.5	76.5 ± 13.7*
	(11.4 ± 3.2)	(10.0 ± 2.2)	(5.3 ± 1.7)	(105.5 ± 46.8)	(5.5 ± 1.6)*	(2.7 ± 0.5)	(4.8 ± 1.4)*
Controls	78.4 ± 9.0	99.4 ± 0.5	75.5 ± 11.6	93.9 ± 3.3	62.9 ± 13.2	27.5 ± 6.2	85.6 ± 9.0
	(9.8 ± 2.1)	(11.7 ± 2.1)	(6.7 ± 1.8)	(176.2 ± 73.9)	(4.2 ± 0.9)	(2.5 ± 0.3)	(5.7 ± 1.3)

Notes: Values are the mean ± SD. NK cells means the CD3-CD56+ population. T cells means the CD3+ population. The data show the percentage of expression of each indicated molecule on NK cells detected by flow cytometry. *Abbreviations:* MFI, mean fluorescence intensity; ND, not detected.
*$P < 0.05$ versus controls.

cytokines are found in the serum of patients with systemic autoimmune diseases.[11] Cytokines, including IL-18, IL-15, TNF-α , IFN-γ, and IL-6, which could influence NK cell function, were evaluated in this study. IL-18 and TNF-α (but not IL-15, IFN-γ, and IL-6) levels were significantly increased in sera from patients with primary SS patients compared with normal controls (Table I).

Increment of apoptotic NK cells in patients with primary SS. Finally, to examine whether peripheral NK cells derived from patients with primary SS undergo apoptosis, blood samples were newly collected from 31 primary SS patients (1 man and 30 women; age 57.0 ± 15.9 years) and 21 normal controls (1 men and 20 women; age 52.7 ± 11.4 years). The authors then isolated lymphocytes to evaluate apoptosis (Annexin V positive cells). Three-color analysis was used to quantify early NK cell death in peripheral blood lymphocytes derived from primary SS patients and normal controls. Analysis of apoptosis in specific lymphocyte subsets revealed that the percentage of lymphocyte apoptosis in CD3–CD56+ (NK) cells was significantly increased in patients with primary SS compared with controls, whereas a few cells undergo apoptosis in CD3+ (T) cells in both groups (Fig 2).

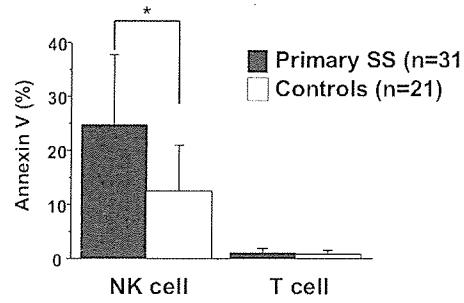

Fig 2. Apoptosis in CD3–CD56+ NK cells from primary SS patients. Apoptosis was determined in CD3–CD56+ NK cells using Annexin V labeling in 31 primary SS patients and 21 normal controls. Results from donors are shown as the percentage of Annexin V positive staining. Bars show the mean and SD. *$P < 0.01$.

DISCUSSION

In this study, the authors compared the number of peripheral NK cells, NK cell activity, and the expression of NK cell activating receptors in patients with primary SS vs normal controls. NK activity was significantly impaired, and NK cell number and expression of CD2 and NKG2D were significantly decreased in primary SS patients compared with healthy controls. NK cell lytic activity on a per-cell basis was similar between the two groups, which suggests that low NK cell number might contribute to impaired NK cell activity in patients with primary SS. The levels of IL-18 and TNF-α, cytokines that have been shown to promote NK cell death following reduced NK cell number, were significantly increased in sera from patients with primary SS compared with controls. Moreover, the percentage of apoptotic NK cells was significantly increased in primary SS compared with controls. These data suggest that an apoptotic process may contribute to low NK cell number resulting in impaired NK cell activity in patients with primary SS.

The authors previously reported that NK cell number is decreased in patients with autoimmune diseases, including SS, and that cytokine (IL-18, IL-15, and TNF-α)- induced NK cell death is one of the explanations for low NK cell number in these patients.[11] Significant increased levels of IL-18 and TNF-α in sera from primary SS patients may contribute to the NK cell reduction. It is possible that other cytokines (ie, IL-10) contribute to the depletion of NK cells in these patients.[26-28] Grom et al[29] reported on the NK cell dysfunction in patients with systemic-onset juvenile rheumatoid arthritis, macrophage activation syndrome, and HLH. Like primary SS, virus-associated HLHs have very low or absent cytolytic NK cell activity, which will be related to decreased numbers of NK cells.[29] Serum levels of TNF-α in patients with virus-associ-

ated HLH were very high in a previous report.[30] Serum levels of cytokines in patients are much lower than the cytokine concentrations used in previous *in vitro* experiments.[11] However, high concentrations of IL-18 and TNF-α will be produced by macrophages in the local tissues and bone marrow, which suggests that these cytokines may induce apoptosis in NK cells leading to a low NK cell number in the peripheral blood.

Another possibility is that activation-induced NK cell death triggered by activating receptor stimulation contributes to the depletion of NK cells in these patients. The authors recently reported on a novel type of NK cell death, named GLCD.[31] GLCD is a rapid form of NK cell death (loss of mitochondrial membrane potential starts within 30 min) that results from leakage of granzyme B from cytotoxic granules. When cytosolic granzyme B accumulates to a level exceeding that of its cytosolic inhibitor (PI-9), it cleaves bid to initiate mitochondrial dysfunction and apoptotic cell death.[31] GLCD is a new type of activation-induced NK cell death, which is triggered by CD2 stimulation.[32] The authors and others demonstrated that CD94 and CD16 also trigger NK cell death.[33] The rapid kinetics of this process suggests that it may be GLCD. As it was difficult to purify sufficient NK cells from primary SS patients to determine whether peripheral NK cells undergo activation-induced apoptosis, the authors used buffy coat cells derived from healthy donors in these assays. NK cell death was induced by CD2 or CD16, but not by NKG2D, CD56, or CD244 stimulation (data not shown). Activation-induced NK cell death triggered by CD2 or CD16 stimulation might be one reason for depletion of NK cells, although examination of NK cells derived from SS patients will be needed to prove this hypothesis. Peripheral NK cells purified from patients with primary SS have already started to undergo apoptosis, which was supported by experiments showing that the percentage of lymphocyte apoptosis in CD3–CD56+ (NK) cells was significantly increased in patients with primary SS compared with controls (Fig 2). Moreover, apoptotic stimulation may have already triggered apoptosis in NK cells, resulting in elimination from the peripheral blood. It is therefore possible that the residual NK cells found in the peripheral blood of SS patients may be intrinsically resistant to apoptosis. This result would make comparisons of AICD in SS and normal controls difficult to interpret.

Human NK receptors can be separated into distinct families: the immunoglobulin-like NK receptors; killer inhibitory receptors, natural cytotoxicity receptors (including NKp46, NKp30, and NKp44), CD244 (2B4), CD226 (DNAM1), and the C-type lectin-like NK receptors; and CD94/NKG2, NKG2D, NKp80, and CD161 (NKRP1).[34–36] Inhibitory receptors are characterized ITIMs present in their cytoplasmic tail. After receptor ligation, tyrosine phosphorylation of the ITIMs allows the recruitment of Src-homology domain-bearing tyrosine phosphatases (SHP-1, −2) that inhibit cellular activation. NK cells lacking inhibitor receptors efficiently lyse autologous cells, a consequence of the unregulated stimulation of activation receptors. In this study, the authors quantified the expression of five activating NK receptors that have been implicated in the regulation of NK cell cytolytic activity and cytokine production in primary SS patients. In addition, the expression of CD2 and CD16, the classic activating molecules, were also quantified. These receptors use different signaling pathways to bring about NK cell activation. For example, NKp46 and NKp30 transduce to Zap 70 or Syk via FcϵR1γ-CD3ζ and CD3ζ-CD3ζ, respectively.[4,35] In contrast, NKG2D uses the associated DAP10 polypeptide to trigger via the PI-3 kinase pathway.[35] Whereas the MFI of NKp46 on NK cells was significantly increased, the MFI of NKG2D was significantly decreased in patients with primary SS compared with normal controls. Increased expression of one activating receptor (ie, NKp46) may compensate for the reduced expression of another activating receptor (ie, NKG2D, CD2) on NK cells derived from primary SS patients. This finding may explain why NK activity on a per-cell basis is similar between the two groups.

The human NKG2D receptor binds to the stress-inducible proteins MHC class I chain-related molecule (MIC)-A and –B , as well as UL16-binding proteins that are generally not expressed on normal cells but can be induced by stress and upregulated on tumors and virally infected cells. The ubiquitous expression of NKG2D on NK cells and the inducible expression of its ligands on other cells provides an efficient mechanism for the rapid activation of NK cells that does not require downregulation of MHC class I.[37] Further examination will be needed to determine the mechanisms responsible for reduced expression of NKG2D.

Recently, many researchers have paid attention to NK cell subsets, (eg, CD56 dim and CD56 bright NK cells) possessing different functional properties.[38] CD56 dim NK cells function as cytotoxic cells, whereas CD56 bright NK cells work as cytokine producing cells. In this study, the authors examined the percentage of CD56 dim and CD56 bright NK cells in patients with SS and normal controls. As the percentage of CD56 bright NK cells is very small in both groups (5.1% and 3.3% in patients with primary SS and normal persons, respectively), the authors analyzed the total NK cells (CD3–CD56 +) in this experiment.

The incidence of lymphoma is increased in patients with SS. Lymphomagenesis is thought to be a multistep

process.[39,40] As NK cells provide a first line of protection against cellular transformation, reduced NK cell activity could contribute to this process. Moreover, NK cells are involved in immune regulation by eliminating autoreactive T cells and B cells that induce autoimmune diseases, including SS.[7-10] For these reasons, it is important to determine the mechanisms responsible for reduced activity of NK cells in patients with SS. This study demonstrates that low NK cell number is likely to contribute to reduced NK cell activity.

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INTERNAL MEDICINE

□ CASE REPORT □

Examination of IgM Rheumatoid Factor (IgM-RF) and Anti-cyclic Citrullinated Peptide Antibody (Anti-CCP Ab) in Japanese Patients with Palindromic Rheumatism

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Abstract

We have studied the serology of 6 patients with palindromic rheumatism. None of the patients fulfilled the classification criteria for rheumatoid arthritis at the entry; however, 4 out of the 6 patients were seropositive for IgM rheumatoid factor (IgM-RF) at entry. Sequential serological study was performed in 4 patients; IgM-RF changed from seronegative to seropositive in one patient, and the titer increased in another patient. Anti-cyclic citrullinated peptide antibody (anti-CCP Ab) at the entry was detected in only one of the 6 patients; that patient later developed RA. Although follow-up is necessary, the present study may suggest the importance of serological examination, especially anti-CCP Ab, in patients with palindromic rheumatism.

Key words: palindromic rheumatism, IgM-RF, anti-CCP Ab

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Introduction

Palindromic rheumatism is characterized by recurrent attacks of acute arthritis of short duration. In the long term, some of these patients develop a connective tissue disease, usually rheumatoid arthritis (RA) (1, 2). Previous studies in Europe and North America showed that the presence of IgM rheumatoid factor (IgM-RF) in palindromic rheumatism patients indicates a future risk for RA (1-4), and anti-cyclic citrullinated peptide antibody (anti-CCP Ab) was recently reported in palindromic rheumatism patients (5). Thus, we measured IgM-RF and anti-CCP Ab in the sera of Japanese palindromic rheumatism patients.

Case Presentation

We encountered 6 patients with palindromic rheumatism, as diagnosed by the criteria described by Gonzalez-Lopez et al (1). Informed consent was obtained from all of the patients.

Table 1 summarizes the profiles of the 6 palindromic rheumatism patients. All patients were prospectively followed-up, and the disease duration was estimated from the time of first attack until the last consultation. Seroposivitities of IgM-RF and anti-CCP Ab shown in Table 1, were obtained from the data of the latest serological examinations. Joint involvement in Table 1 shows the total joints affected during the follow-up period. Serology was serially examined in 4 patients; IgM-RF turned to be positive in one patient and its titer increased in one case (Fig. 1). Among the 6 patients, one patient developed RA later. IgM-RF was seropositive in 4 out of 6 patients; however, only the one patient who developed RA was positive for anti-CCP Ab. Anti-CCP Ab in this case was consistently positive; however, IgM-RF was negative during the follow-up period

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