

Inhibition of notch signalling ameliorates experimental inflammatory arthritis

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Supplementary materials

MATERIAL AND METHODS

Mice

Mice overexpressing Notch antisense (NAS) under the control of a mouse mammary tumor virus long terminal repeat promoter were generated, backcrossed to C57BL/6 mice and characterized as previously described.[1] NAS and non transgenic control C57BL/6 mice were maintained under a 12 h light/12 h dark cycle with continuous access to food and water. Male non transgenic C57BL/6 and male NAS mice of 3 months old were used in the experiments. This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Sungkyunkwan University School of Medicine (SUSM). SUSM is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) accredited facility and abide by the Institute of Laboratory Animal Resources (ILAR) guide.

γ -secretase inhibitors.

We have used very potent and selective γ -secretase inhibitors including DAPT (N-[N-(3,5-difluorophenacetyl-t-alanyl)]-S-phenyl-glycine t-butyl ester) (Calbiochem), and L-685,458 ((5S)-(tert-Butoxy-carbonylamino)-6-phenyl-(4R)-hydroxy-(2R)-benzylhexanoyl)-L-leucyl-L-phenylalanine (Tocris). DAPT is a widely used cell permeable dipeptide inhibiting γ -secretase (IC_{50} = 200 nM). L-685,458, an aspartyl protease transition state mimic, is a more potent and selective small molecule inhibitor of γ -secretase (IC_{50} = 17 nM) that displays > 50-fold selectivity over a range of aspartyl, serine and cysteine proteases.

Collagen-induced arthritis (CIA)

The collagen induced arthritis (CIA) was induced in C57BL/6 or NAS mice as previously described.[2] Briefly, mice were injected intradermally at two sites at base of the tail with 400 µg of chicken type II collagen (CII) (2 mg/ml, Chondrex, Redmond, WA ,USA) emulsified in 200 µl of Complete Freund's Adjuvant (4 mg/ml, Chondrex). This was followed by a booster immunization 21 days later with CII emulsified in Incomplete Freund's Adjuvant (Chondrex). Mice were monitored for the induction of arthritis by clinical scoring or by measuring hind paw swelling using callipers. Paw scores were allocated as follows; 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced swelling, 3 = ankylosis. These paw scores were summed for each mouse, giving maximum possible score of 12/mouse. Mice were treated with 10 or 20 mg/kg DAPT and vehicle (5% DMSO, 95% corn oil) intraperitoneally daily starting on the day of the booster injection.

To induce CIA, DBA/1J mice were injected intradermally at the base of the tail with 200 µg of bovine type II collagen (CII) (2 mg/ml) emulsified in 100 µl of Complete Freund's Adjuvant (4 mg/ml). This was followed by a booster immunization 21 days later. Mice were treated with 20mg/kg DAPT, 1 mg/kg L-685,458 and vehicle (10% DMSO) daily starting on the 32day of the booster injection. DBA/1J mice were monitored for the induction of arthritis by clinical scoring. Paws were scored individually as follows; 0 = normal, 1 = mild swelling and erythema confined to the midfoot and ankle joint, 2 = mild swelling and erythema extending to the midfoot and ankle joint, 3 = moderate swelling and erythema extending from the metatarsal joints to the ankle, 4 = severe swelling and erythema encompassing the foot, ankle and digits. These paw scores were summed for each mouse, giving maximum possible score of 16/mouse.

Histologic examination of joints

Knee joints were fixed in 10% neutral buffered formalin, decalcified using Decalcified Solution-Lite (Sigma, St. Louis, MO, USA) for 6 h, embedded in paraffin, and sectioned. Tissue sections were stained with H&E, and evaluated by investigators unaware of experimental details. Extents of synovitis and bone/cartilage destruction were evaluated using on a five-point scale: grade 0 (no sign of inflammation), grade 1 (mild inflammation with minimal hyperplasia of the synovial lining layer without cartilage destruction), and grades 2 through 4 (increasing degrees of inflammatory cell infiltration or cartilage and bone destruction).

Synoviocyte culture

Fibroblast-like synoviocytes were isolated from synovial tissues obtained during joint replacement surgery from patients with RA, defined according to the criteria of American College of Rheumatology.[3] Cells were passaged by replating at a 1:5 dilution when the cultures reached confluence. Fibroblast-like synoviocytes at the passage between 3 and 8 were used for the experiments.

Quantitative RT-PCR analysis

Total RNAs were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) after 2 μ M DAPT or 1 μ M L-685,458 treatment of human FLS for 24 h. The concentration of RNA was determined using UV-Vis spectrophotometer (NanoDrop2000, Thermo Fisher Scientific Inc., Wilmington, DE, USA). RNAs (1 μ g) were reverse-transcribed with iSCRIPTTM cDNA Synthesis kit (Bio-rad, California, WC, USA), after which real-time PCR amplification with SYBR[®] Premix Ex TaqTM II (Takara, Otsu, Shiga, Japan.) was performed using a

Mastercycler realplex thermalcycler (Eppendorf, Hamburg, Germany) or Line-Gene K (Bioer, Hangzhou, China). The primer sequences are listed in Supplementary Table 1. Data were analyzed according to the comparative Ct method and were normalized to GAPDH expression.

Non-invasive near-infrared fluorescence (NIRF) imaging

For optical imaging of inflammation in arthritic joints, hydrophobically modified glycol chitosan (HGC) nanoparticles were used. Because of the leaky nature of the vasculatures in tissues affected by arthritis, fluorescent HGC nanoparticles are trapped and retained in tissues by the enhanced permeation and retention (EPR) effect.[4, 5] HGC nanoparticles were prepared by conjugating glycol chitosan with hydrophobic 5 β -cholanic acid in the presence of EDAC and NHS, as previously described.[6] HGC was chemically labelled with Cy5.5 (a NIRF dye) and visualized using the eXplore Optix system (Advanced Research Technologies, Macungie, PA, USA).[7]

***In vivo* NIRF imaging of MMP3-activity**

The MMP-3-specific polymeric probe provided a clear early diagnosis and visualization of the arthritis progression using an NIRF imaging system.[4] An MMP-3-specific polymeric probe was developed by conjugating NIRF dye (FPR-675, excitation and emission maxima at 675nm and 695 nm, respectively), MMP-3 substrate peptide (*Gly-Val-Pro-Leu-Ser-Leu-Thr-Met-Gly-Lys-Gly-Gly*, substrate site is italicized and cleavage site is between *Ser* and *Leu*), and dark quencher (black hole quencher-3, BHQ-3, absorbance maximum 650 nm), to self-assembled chitosan nanoparticles (CNPs).[4] At 30 days after first immunization, 100 μ l of the MMP-3-specific polymeric probe (1 mg/ml) was intravenously injected into control or

CIA mice to evaluate MMP-3 activity. NIRF tomographic images were captured with a FPR-675 band pass emission filter (680-720 nm; Omega Optical), and the total NIRF intensity in hind limbs (360 mm²) was calculated using an eXplore Optix system (Advanced Research Technologies) at 1 h after injecting the polymeric probes.

Micro-computed tomography (Micro-CT)

At 35 days after first immunization of CII, CT images of the knee joints and hind limbs of control and CIA mice were obtained using a micro-CT (Skyscan 1076; SKYSCAN N.V., Kontich, Belgium) at a resolution of 18 µm using the following parameters: 60 kV X-ray voltage, 170 mA current, 2360 milliseconds exposure time, and rotation steps of 0.7°. For verification of bone destruction, three-dimensional models of knee joints were reconstructed using CT-AN 1.8 (SKYSCAN N.V.). The beam hardening errors were corrected to improve the quality of the micro-CT images. Plus, flat-field correction before scanning and beam hardening correction during reconstruction were performed to improve the quality of micro-CT images.

Bead-based cytokine assay

Serum samples were obtained from 4 weeks after immunization. Serum levels of TNF-α, INF-γ, IL-6, -10, -12, -17, MCP-1 were determined using a Milliplex Mouse Cytokine panel 1 (Millipore, Billerica, MA, USA) and the Luminex 100 system (Luminex, Georgetown, TX, USA), according to the manufacturer's instructions.

Measurement of TNF-α and IL-6

To determinate TNF-α and IL-6 levels in vitro, culture media was analyzed using ELISA kits

(Komabiotech, Seoul, Korea) for Human TNF- α and IL-6.

Measurement of CII specific IgG1 and IgG2a

To determinate collagen specific autoantibody levels *in vivo*, serum samples were analyzed using ELISA kits (Chondrex) for CII-specific IgG1 and IgG2a antibody levels.

Immunoblotting

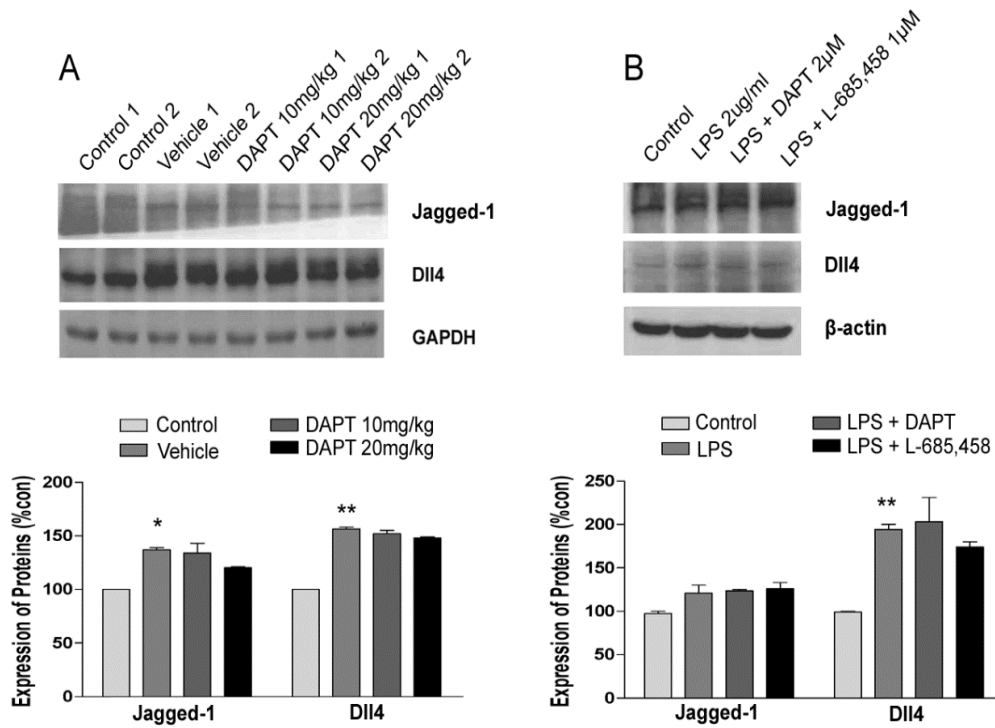
Tissue protein was extracted using T-PER tissue protein extraction buffer and a protease inhibitor cocktail (Roche, Basel, Switzerland). Briefly, 20 μ g of proteins were separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were blocked in 5 % non-fat milk for 1 h at room temperature, and incubated overnight at 4 °C and analysed with antibodies raised against Notch1 (upstate, Lake Placid, NY, USA), activated Notch1 (NICD) (Abcam, Cambridge, UK), Hes-1 (Santacruz, Santa Cruz, CA, USA), ICAM-1 (Santacruz), Jagged-1 (santacruz), Dll4 (santacruz), phospho-p65 (Cell Signaling, Danvers, MA, USA), MMP-3 (R&D systems, Minneapolis, MN, USA) or GAPDH (Thermo, Walteham, WA, USA).

γ -secretase activity assay

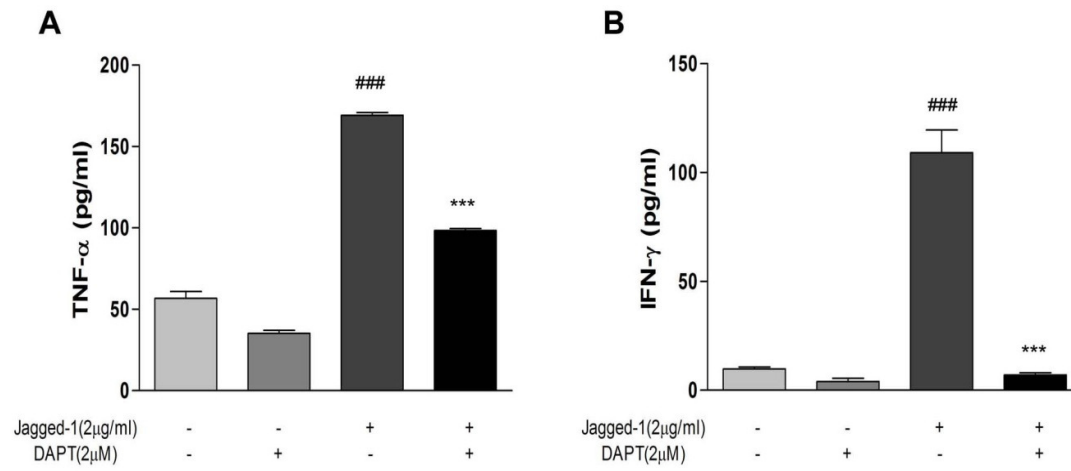
The fluorometric detection of activity of γ -secretase in mouse joint tissues was performed as previously described.[8] The method is based on the γ -secretase-dependent cleavage of a γ -secretase-specific peptide conjugated to the fluorescent reporter molecules EDANS and DABCYL, which can be detected using a fluorescent microplate reader with excitation wavelength 355 nm and emission at 510 nm. Data are expressed as fold increases in fluorescence over background controls.

Gene	Primer Sequences		Annealing Temperature (°C)	PCR product size (bp)
	Foward	Reverse		
Hes-1	cacggccttgctctgtttt	cggaggtgcttcactgtcat	60	200
ICAM-1	cggaggtgcttcactgtcat	ggggtctctatgcccaacaa	60	112
IL-6	aatcggtacatcctcgacgg	ttggaaggttcaggtgtttct	60	113
IL-10	gtgatccccaagctgaga	cacggccttgctctgtttt	60	138
IL-12	tggagtgccaggaggacagt	tcttgggtgggtcaggtttg	60	147
IFN- γ	tcagctctgcatcgttttgg	gttcattatccgctacatcgaa	60	120
GAPDH	caacaattcctggcgatacct	ggcatggactgtggatcatgag	60	87
TNF- α	cagaggcctgtacatcatc	ggaagaccctcccagatag	55	219
MCP-1	gtcacctgctgttataacttc	tgctgctggtgattcttcta	55	79
IL-17A	actacaaccgatccacacac	actttgctcccagatcacag	55	84
GAPDH	gacatcaagaaggtggtgaa	tgtcataccaggaaatgagc	55	178

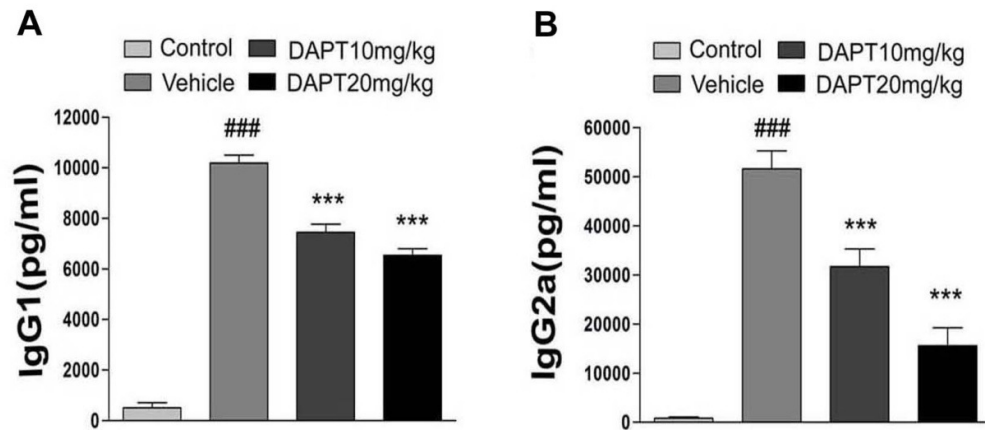
Supplementary Table 1 Sequence of PCR primers used in quantitative RT-PCR.



Supplementary Figure 1 Expression of Jagged-1 and DLL-4 in the joints of CIA and synoviocytes of RA patients. (A) Proteins in joint tissue samples (20 μg/lane) from control or CIA mice treated with vehicle or DAPT were immunoblotted using antibodies against Jagged-1, DLL4 and GAPDH. Values are means \pm SEM (n = 3), *p < 0.05, **p < 0.01. (B) RA synoviocytes pretreated with 2μM DAPT or 1μM L-685,458 for 2h and stimulated with 2μg/ml LPS for 24h. The expression of DLL4 was increased in RA synoviocyte treated with LPS. Values are means \pm SEM (n = 3), **p < 0.01.



Supplementary Figure 2 Notch ligand-mediated inductions of TNF- α and IFN- γ were blocked by DAPT. Splenocytes from naïve DBA1/J mice were stimulated with Jagged-1 in the presence or absence of DAPT. After 48 hr, culture supernatants were collected and analysed for TNF- α and IFN- γ by ELISA. TNF- α and IFN- γ levels were stimulated by Jagged-1 in the absence of DAPT but not in its presence. Values are means \pm SEM (n = 3).
^{###} $P < 0.001$ versus controls. ^{***} $P < 0.001$ versus Jagged-1-treated controls.



Supplementary Figure 3 DAPT decreases autoantibody production. Serum samples were collected from CIA mice at 46 days after primary immunization. Circulating serum anti-CII antibody levels (IgG1 and IgG2a) were markedly elevated in CIA mice versus controls. Furthermore, the productions of anti-CII IgG1 and IgG2a were dose-dependently inhibited by DAPT. Concentrations of CII-specific antibodies (IgG1 and IgG2a) were measured by specific ELISA. Values are means \pm SEM (n = 3). ^{###} $P < 0.001$ versus controls; ^{***} $P < 0.001$ versus vehicle-treated controls.

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