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Constitutive Activation of Integrin α9 Augments Self-Directed Hyperplastic and Proinflammatory Properties of Fibroblast-like Synoviocytes of Rheumatoid Arthritis

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Despite advances in the treatment of rheumatoid arthritis (RA), currently approved medications can have significant side effects due to their direct immunosuppressive activities. Additionally, current therapies do not address residual synovial inflammation. In this study, we evaluated the role of integrin α9 and its ligand, tenascin-C (Tn-C), on the proliferative and inflammatory response of fibroblast-like synoviocytes (FLSs) from RA patients grown in three-dimensional (3D)-micromass culture. FLSs from osteoarthritis patients, when grown in the 3D-culture system, formed self-directed lining-like structures, whereas FLSs from RA tissues (RA-FLSs) developed an abnormal structure of condensed cellular accumulation reflective of the pathogenic features of RA synovial tissues. Additionally, RA-FLSs grown in 3D culture showed autonomous production of proinflammatory mediators. Predominant expression of α9 and Tn-C was observed in the condensed lining, and knockdown of these molecules abrogated the abnormal lining-like structure formation and suppressed the spontaneous expression of matrix metalloproteinases, IL-6, TNFSF11/RANKL, and cadherin-11. Disruption of α9 also inhibited expression of Tn-C, suggesting existence of a positive feedback loop in which the engagement of α9 with Tn-C self-amplifies its own signaling and promotes progression of synovial hyperplasia. Depletion of α9 also suppressed the platelet-derived growth factor–induced hyperplastic response of RA-FLSs and blunted the TNF-α–induced expression of matrix metalloproteinases and IL-6. Finally, α9-blocking Ab also suppressed the formation of the condensed cellular lining by RA-FLSs in 3D cultures in a concentration-related manner. This study demonstrates the central role of α9 in pathogenic behaviors of RA-FLSs and highlights the potential of α9-blocking agents as a nonimmunosuppressive treatment for RA-associated synovitis. The Journal of Immunology, 2017, 199: 000–000.

Rheumatoid arthritis (RA) is a pleiotropic autoimmune disease characterized by formation of hyperplastic synovial pannus tissue, which mediates joint destruction. Various agents, such as anti–TNF-α and anti–IL-6 receptor Abs, that are effective in suppressing active inflammation are currently available and have significantly advanced the treatment of RA. However, one shortcoming of these agents is that they raise the risk of infection by suppressing systemic immunity (1). Furthermore, examinations with advanced imaging techniques such as ultrasonography and magnetic resonance imaging have revealed persistent synovitis and tenosynovitis in more than a half of RA patients, even in patients in clinical remission with the above agents (2–4). Therefore, there remains an unmet medical need to prevent the joint damage by nonimmune mechanisms.

Fibroblast-like synoviocyte (FLSs) are the major component of synovial membrane and the predominant cell type of the aberrant pannus tissue. It has been suggested that inhibition of the hyperplastic response of FLSs in RA may present a complementary therapy without deleterious effect on immune responses (1). Under normal conditions, FLSs form a thin intimal layer lining of the synovium and protect joint by providing structural support and controlling the composition of the synovial fluid and extracellular matrix (ECM) (5). In the presence of the proinflammatory milieu that occurs in RA, FLSs undergo phenotypic conversion to FLSs from RA tissues (RA-FLSs), adopting various abnormal characteristics such as increased survival, adhesion, and invasiveness (1, 6). These RA-FLSs aggressively and autonomously condense to form a hyperplastic lining and, together with infiltrating inflammatory cells form an expansive synovial tissue, pannus. Cadherin-11, which is known to be responsible for cell–cell interactions between FLSs under physiological conditions also plays a crucial role in establishment of the hyperplastic cell condensation of RA-FLSs.
(7–9). RA-FLSs not only produce their own proinflammatory mediators such as matrix metalloproteinase (MMP)-1, MMP-3, MMP-14, and TNFSF11 (also known as RANKL) (5, 10, 11), but they also respond to proinflammatory mediators in the inflammatory synovium, particularly to TNF-α, resulting in the production of proinflammatory mediators, including IL-6, platelet-derived growth factor (PDGF), TGF-β, and various MMPs, leading to establishment of chronic synovitis. Thus, RA-FLSs play a fundamental role in the pathogenesis of joint inflammation (1, 6, 9).

The cadherin-11–mediated cell–cell adhesion of FLSs and the integrin-mediated FLS adhesion to ECM have been reported to contribute to formation of the synovial lining and the hyperplasia in the synovial membrane (9, 12, 13), although the exact role of RA-FLS interaction with ECM in RA pathology remains to be fully elucidated. Integrins are composed of two subunits, α and β, and bind to ECM proteins such as fibronectin, laminins, collagens, and tenascins (14). Engagement of integrins with corresponding ECM ligand proteins activates intracellular signaling molecules, including focal adhesion kinase (FAK), Src, ERK, and PI3K, and regulates a wide range of cellular responses such as migration, survival, and proliferation (14). FAK is one of the first signaling molecules activated upon integrin engagement through autophosphorylation at Tyr397 and triggers outside-in integrin signaling (14). Src and PI3K act cooperatively with FAK in this pathway but also activate FAK-independent pathways (15, 16). Among various integrins expressed in FLSs, α9 is unique in that it does not bind to abundant ECM proteins such as fibronectin, laminins, vitronectin, or collagens but functions as a receptor for tenascin-C (Tn-C) (17), protease-cleaved osteopontin (18), VEGF-C (19), and thrombospondin-1 (20). Notably, expression of both α9 and Tn-C is augmented in synovial lining in RA (21), and pharmacological blockade of α9 has been shown to prevent synovial inflammation and joint destruction in the mouse model of arthritis (22). These findings suggest involvement of α9 in synovial inflammation. However, the role of α9 in the pathogenic behavior of RA-FLSs is poorly understood.

Previous studies have demonstrated that isolated RA-FLSs do not retain the intrinsic aggressiveness they show in primary tissues (23, 24). Recently, a unique three-dimensional (3D) culture system of RA-FLSs has been reported. In this system, isolated RA-FLSs not only establish hyperplastic lining-like aggressive cell assembly but also respond to TNF-α to produce proinflammatory cytokines, chemokines, and MMPs (25). The 3D-culture system thus appears to provide an in vitro model relevant to synovial hyperplasia in vivo and may be a powerful tool to analyze the abnormal behaviors intrinsic to RA-FLSs.

In the present study, using the 3D culture of patient-derived FLSs, we have shown that abnormally increased expression of α9 confers the abilities to RA-FLSs to form hyperplastic lining layers and enhances proinflammatory gene expression. Additionally, we provide evidence for involvement of α9 in the augmented response of RA-FLSs to TNF-α and PDGF. Our results have thus revealed the central role of α9 in the abnormal characteristics of RA-FLSs and highlight the therapeutic potential of α9 blockers to treat persistent synovitis in RA.

Materials and Methods

Inhibitor and blocking Abs

The FAK inhibitor (FAKi) PF-573228 was purchased from Sigma-Aldrich (St. Louis, MO). Etaonecept (Enbrel) and natalizumab (Tysabri) were obtained from Pfizer (New York, NY) and Biogen (Cambridge, MA), respectively. Y9A2, a mouse IgG, Ab to human α9β1, was purified from the culture supernatants of hybridoma producing the Ab, provided by the University of California, San Francisco under a licensing agreement. ASP5094, a humanized Ab of Y9A2 with some beneficial mutations, was prepared as follows: DNA sequences encoding V domain of H chain (VH) and L chain (VL) for improved blocking Ab to human α9 (26) were chemically synthesized and subcloned into pMK and pMA-T cloning vectors (Invitrogen, Waltham, MA), respectively, with attachment of HindIII (5′ end) and EcoRI (3′ end) sites. After confirmation of the entire sequences of the inserts, the DNA fragments were digested with HindIII and EcoRI, gel purified, and ligated into cassette expression vectors employing genes for C region of human IgG1 (pEE4 for VH and pEE12.4 for VL, respectively) at the corresponding enzyme sites. Both vectors were licensed from Lonza Biologics (Basel, Switzerland). The resultant plasmids encoding complete amino acids for VH and VL of human IgG1 were linearized by digestion with NotI and PvuI and ligated to each other to facilitate dual expression of both H and L chains from one plasmid according to the manufacturer’s recommendation. Master cell bank was developed from Chinese hamster ovary cells stably transfected with the plasmid. ASP5094 was purified from the culture supernatants of the master cell bank and used in this study.

Patient-derived tissues

RA and osteoarthritides (OA) were diagnosed according to the criteria of the American College of Rheumatology (27, 28). Synovial tissues were obtained from OA and RA patients undergoing orthopedic surgery.

Isolation of FLSs

FLSs were isolated from synovial tissues as described previously (29). Briefly, the tissues were minced, digested, and cultured in DMEM (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FBS (HyClone; GE Healthcare, Buckinghamshire, U.K.) and 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (Nacalai Tesque, Kyoto, Japan) in an incubator containing 5% CO2 at 37°C. The adherent cells (which usually became observed after 7–14 d of culture) were cultivated to confluence. The cells were detached from dishes by treatment with 0.05% trypsin solution containing 1 mM EDTA (Nacalai Tesque), split at a 1:3 ratio, and continuously cultured under identical conditions. The adherent cells, which had been passaged for 4–10 times after start of the culture, were used as isolated FLSs.

3D-micromass culture system

FLSs were resuspended in Matrigel (BD Biosciences, San Jose, CA) at a density of 4 × 105 cells/ml. Droplets of the cell suspension (35–45 μl) were placed onto 12-well culture plates coated with poly(2-hydroxyl methacrylate) (Sigma-Aldrich) for 1 h. After incubation at 37°C for 1 h to gelatinize the Matrigel, 1.5 ml of the culture medium was overlaid on the wells and the gelatinized droplets were floated by pipetting to start 3D-micromass culture. The floating cells in the Matrigel were maintained for 3 wk. Half of the culture medium was replaced twice a week throughout the culture period. In several experiments, the culture medium was supplemented with human TNF-α (20 ng/ml; R&D Systems, Minneapolis, MN) or human PDGF-BB (50 ng/ml; R&D Systems).

Immunofluorescent staining

Tissues or micromass architectures were embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan), freeze-fixed, and sliced by cryostat. The thickness of the sections was set at 8 and 20 μm for tissues and micromass architectures, respectively. For immunofluorescence staining, the sections were fixed in PBS-buffered 4% paraformaldehyde for 10 min followed by permeabilization in ice-cold acetone for 5–10 min. The slides were blocked in PBS-buffered 1% BSA (Nacalai Tesque) and incubated with primary Abs overnight at 4°C, followed by incubation with fluorescent-labeled secondary Ab for 1 h. The primary and secondary Abs were diluted in PBS-buffered 1% BSA. In several experiments, DAPI (Dojindo, Kumamoto, Japan) was added to the diluent for secondary Ab at 1 μg/ml. For phallolidin staining, the slides were incubated with Alexa Fluor 488–labeled phallolidin (Life Technologies, Carlsbad, CA) diluted in PBS-buffered 1% BSA for 1 h. In several experiments, DAPI was added to the diluent. Acquistion of microscopic images was performed by an Axio Imager upright microscope with an HBO 100 microscope illuminating system (Carl Zeiss Microscopy, Oberkochen, Germany). Polyclonal Ab to human α9 was generated in rabbit by immunizing carrier protein–conjugated synthetic peptide corresponding to aa 674–685 (NRRKENDSWSDWVKQNQ] in human α9 (NP_002198) and purified from the sera of the immunized rabbits (Cambridge, MA). Abs used in this study listed below were purchased from the indicated venders: anti–Tn-C (mouse mAb; clone 3-C62, Wako Pure Chemical Industries, Tokyo, Japan) and anti-pFAK (pY397) (rabbit polyclonal Ab; ab39967, Abcam, Cambridge, U.K.).
Quantification of lining-like structure

Thickness of lining-like structure was measured by image analysis of phalloidin-stained sections of 3D-micromass cultures. Immunofluorescent micrographs were obtained with an LSM-780 (Zeiss) confocal microscope. Quantification of the network-like structure with phalloidin-stained cells was performed as follows: thickness of the lining-like structure = area of lining – area of structure/area of perimeter of the lining-like structure.

Blocking activity of ASP5094 on binding of α9-expressing cells to Tn-C-derived fragment

The blocking activity of ASP5094 on human α9 binding to the ligand was evaluated in previously reported adhesion assay of α9-expressing cells to Tn-C-derived peptide (21). Expression plasmid for human integrin α9 was constructed by inserting PCR-amplified ITGα9 from human heart cDNA library into pcDNA3.1(−) mammalian expression vector (Invitrogen). To obtain cell clones stably expressing human α9, the plasmid was transfected to SW480 cells (American Type Culture Collection, Manassas, VA) with Lipofectamine (Invitrogen) and cultured in medium supplemented with Geneticin (1 mg/ml; Life Technologies) for a month. The enriched cell clones were isolated by standard FACS. A single-cell clone was further isolated from the population by standard cloning method and used for cell adhesion assay. Purified recombinant Tn-C peptide in which RGD sequence was mutated to RAA (Tn-C3/RAA) was prepared as described elsewhere (17) and used as a ligand for α9-expressing cells. Blocking activity of ASP5094 on adhesion of α9-expressing cells to Tn-C3/RAA was measured as follows: 96-well plates (Maxisorp Immunoplate; Nalgé Nunc, Rochester, NY) were coated with Tn-C3/RAA (10 μg/ml) for 1 h at 37˚C. Wells similarly coated with PBS-buffered 1% BSA were also prepared to generate background value. After washing the plate with PBS, the wells were blocked with PBS supplemented with 0.5% BSA for 1 h, washed with PBS, and provided to adhesion assay to examine the blocking activity of ASP5094 on α9-expressing cells. Well-bound cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated with antibodies against α9 integrin (clone Y9A2; BioLegend, San Diego, CA) and Alexa Fluor 488-labeled secondary antibody followed by staining with DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes, Eugene, OR) for 1 h. Cells were washed and mounted on glass slides with aqueous mounting media (Aquamount; Biomeda, Foster City, CA). Alexa Fluor 488-integrin positive cells were counted with a confocal microscope (Leica TCS SP5; Leica Microsystems, Wetzlar, Germany) and mean fluorescence intensity per cell was measured. The blocking activity of ASP5094 was calculated as follows: blocking activity (%) = 100 × (1 – mean fluorescence intensity of ASP5094-treated cells/mean fluorescence intensity of control cells).

Real-time PCR

Total RNA was extracted by Isogen (Nippon Gene, Tokyo, Japan) and further purified by an RNeasy Plus micro kit (QIAGEN, Hilden, Germany) (for 3D-cultured cells and tissues) or directly purified by an RNeasy Plus micro kit (for plate-cultured cells) according to the manufacturers’ instructions. cDNA was synthesized by the RT-PCR system for first-strand cDNA synthesis (First-Strand cDNA synthesis kit, Life Technologies) using 100–500 ng total RNA. Real-time PCR was performed using Thunderbird probe quantitative PCR mix (ToyoBio, Osaka, Japan) with gene-specific TaqMan probes in a Viia7 real-time PCR system (Applied Biosystems, Foster City, CA). PCR reaction was performed as follows: denaturation at 94˚C for 1 min followed by 45 cycles at 95˚C for 15 s, 55˚C for 45 s. Expression levels of each gene were normalized by those of GAPDH in vi in vitro experiments. Expression level of each gene, Hprt, was used in the normalizing gene for the normalization of expression using 100–500 ng total RNA. Expression of each gene was calculated as 2(−ΔΔCt).

Lentivirus infection

Lentiviruses were produced by 293T cells (Takara Bio, Shiga, Japan) in six-well plates. On the following day, the culture media were completely replaced to fresh media, followed by incubation for 48 h to allow production of recombinant lentivirus. After incubation at 20˚C for 2 h, 2 ml of fresh medium was added to each well. After incubation at 37˚C for 72 h, the cells were detached, counted, and provided to further experiments.

ELISA

Amounts of secreted proteins in the culture supernatants were determined by ELISA assays using commercially available kits as follows: MMP-1 (human gotal MMP-1 Duoset ELISA; R&D Systems), MMP-3 (human MMP-3 Duoset ELISA; R&D Systems), IL-6 (human IL-6 DuoSet ELISA; R&D Systems), Tn-C (Tn-C large [FNIII-B] ELISA; Immuno-Biological Laboratories). Mouse collagen Ab-induced arthritis model

Mouse IgG1 mAb for keyhole limpet hemocyanin (KLH) was obtained from hybridoma expressing anti-KLH IgG1 in our laboratory and used as a control. MA9-413, a mouse α9-blocking single-chain variable fragment of Ig g fused to mouse IgG1, was prepared as described above. Male DBA/1J mice (6–7 wk of age) were purchased from Charles River (Yokohama, Japan) and housed in a specific pathogen-free environment in an animal facility of Kyoto University. Mice were divided into three groups (n = 8 per group) on day −1, and arthritis was induced by injection of anti-collagen Ab mixture (1.5 mg per mouse; Chondrex, Redmond, WA) on day 0, followed by injection of LPS (50 μg per mouse; Chondrex) on day 3. Control mouse IgG3 (30 mg/kg) and M9-413 (10 and 30 mg/kg) were administered on days 0, 2, 4, 6, 8, 10, and 12 and the study was ended on day 13. Injection of anti-collagen Ab, LPS, control IgG1, and MA9-413 was performed via a subcutaneous injection. Joint swelling was scored on days 0, 3, 5, 7, 10, and 13 as described elsewhere (31). On day 13, paws were fixed in 10% neutral buffered formalin and processed for histologic examination.

Western blotting

Cells cultured in either a 3D-micromass system or tissue culture plate were lysed in TNEU buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 4 mM urea, 1% SDS [pH 7.8]) and clarified by centrifugation. Protein concentration of the lysates was determined by a micro BCA protein assay kit (Pierce, Rockford, IL) using BSA as a standard. Ten to fifty micrograms of the lysates was separated in SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with bovine serum albumin (Nacalai Tesque) and incubated with primary Ab overnight at 4˚C, followed by incubation with HRP-conjugated secondary Ab for 1 h. The primary and secondary Abs were diluted in Can Get Signal immunoreaction enhancer solution (Toyobo). The membranes were developed with Super-Signal West Dura extended duration substrate (Pierce) and imaged on an LAS-4000 mini luminescent image analyzer system (Fuji Film, Tokyo, Japan). The Abs used in the experiments were as follows: anti-human α9 (rabbit mAb; ab140599; Abcam), anti-human Cadherin-11 (rabbit polyclonal; LS-B2308; LSBio, Seattle, WA), anti-Tn-C (mouse mAb; clone 4F10TT; Immuno-Biological Laboratories, Gunma, Japan), anti-total FAK (rabbit mAb; 3285; Cell Signaling Technology, Danvers, MA), anti-pFAK (pY397) (rabbit mAb; 8536; Cell Signaling Technology), anti-MMP-14 (goat pAb; AP918; R&D Systems), anti-TNFSF11 (goat pAb; APB626; R&D Systems), HRP-conjugated anti-mouse IgG (NA934V; GE Healthcare), and HRP-conjugated anti-rabbit IgG (NA934V; GE Healthcare).

Knockdown experiments with lentivirus-introduced short hairpin RNA

Lentiviruses were produced by 293T cells (Takara Bio, Shiga, Japan) in six-well plates. On the following day, the culture media were completely replaced to fresh media, followed by incubation for 48 h to allow production of recombinant lentivirus in the supernatants. After removal of debris by centrifugation, the supernatants were concentrated by Lenti-X concentrator (Takara Bio) for 10-fold and used in infection experiments. For infection of the lentiviruses to RA-FLSs, the cells were seeded at 2 × 104 in six-well plates. On the following day, the culture media were completely removed and 1 ml of lentivirus containing 12 μg/ml hexadimethrine bromide (also termed Polybrene, Sigma-Aldrich) was added to the wells. After incubation at 20˚C for 2 h, 2 ml of fresh medium was added to each well. After incubation at 37˚C for 72 h, the cells were detached, counted, and provided to further experiments.

ELISA

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formalin (Nacalai Tesque), decalcified in EDTA-based neutral decalcifying solution (Wako pure chemical), and provided to histological analysis (left hindlimbs). The right hindlimbs were provided to real-time PCR after removal of skins.

Pathological analyses and immunohistochemistry

Paraffin-embedded joint tissues from collagen Ab-induced arthritis (CAIA) mice were sliced at 3-μm thickness and stained with H&E for pathological analysis. The severity of synovial hyperplasia and bone resorption was scored by five-grade evaluation (0–4) according to the previously reported criteria (22) with slight modifications as follows: for hyperplasia: 0, normal; 1, minimal infiltration of pannus in cartilage and subchondral bone of marginal zone; 2, mild infiltration of marginal zone with minor cortical and medullary bone destruction; 3, moderate infiltration with moderate hard tissue destruction; 4, severe infiltration associated with total or near total destruction of joint architecture; for bone resorption: 0, normal; 1, small areas of marginal zone/periosteal resorption, not readily apparent on low magnification; 2, more numerous areas of marginal zone/periosteal resorption, readily apparent on low magnification, minor overall cortical and medullary bone loss; 3, obvious resorption of medullary trabecular and cortical bone without thickness defects in entire cortex, loss of some medullary trabeculae, lesion apparent on low magnification; 4, full thickness defects in cortical bone and destruction of joint architecture.

Immunohistostaining was performed according to standard procedures. Briefly, the sections were incubated in 0.3% H2O2 for 20 min, blocked with PBS-buffered 1% BSA for 1 h, and incubated overnight at 4°C with rabbit anti-pFAK (pY397) Ab (10 μg/ml; ab39967; Abcam). The sections were then incubated with HRP-conjugated anti-rabbit IgG for 1 h at room temperature and visualized with dianaminobenzidine.

Study approval

Ethical approval for this study was granted by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine and Astellas Research Ethics Committee. Informed consent was obtained from all the patients prior to sample collection. Animal studies were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Statistical analysis

Statistical analyses were performed by GraphPad Prism 5 software (version 5.04; GraphPad software, Figs. 1, 2, 3A, 4A, 4C, 5) and SAS software (SAS Institute, Figs. 3D, 4B, 6, 7C; 7D, Supplemental Figs. 1, 2). A p value <0.05 was considered statistically significant.

Results

The 3D-micromass culture system reproduces disease-related aggressive behavior of RA-FLSs

To examine whether the ability to condense into the lining is intrinsic and specific to RA-FLSs or shared by FLSs in other arthritic diseases, RA-FLSs and FLSs from OA tissues (OA-FLSs) were isolated and cultured in a 3D-micromass system. The resultant cell architectures on micromass were freeze-fixed, sectioned, and stained with DAPI. Both RA- and OA-FLSs assembled at the interface between the matrix and the fluid phase in 3D-culture, but their architectures were markedly different. The lining-like architecture formed by RA-FLSs frequently showed regions of condensed cellular accumulation several cells deep, which was reminiscent of the synovial lining architecture in RA patients (Fig. 1A, right). In contrast, OA-FLSs formed mostly single-cell layered lining, and areas of condensed cellular accumulation were rarely seen (Fig. 1A, left). To analyze the lining-like structure, sections were stained with fluorescent-labeled phalloidin. Consistent with a previous report (7), the condensed cells displayed a layered lining, and areas of condensed cellular accumulation were significantly thicker than that by OA-FLSs (Fig. 1C). These results indicate that RA-FLSs have the intrinsic ability to form condensed synovial architecture that may drive the formation of hyperplastic synovial linings in vivo.

Given that the 3D-culture system of FLSs reproduced the structural features of synovial tissues, we next examined whether the 3D culture of FLSs could restore their ability to produce proinflammatory mediators in vitro. To address this question, real-time PCR was used to determine the expression levels of MMP1, MMP3, MMP14, IL6, and TNFSF11 (genes encoding MMP-1, MMP-3, MMP-14, IL-6, and TNFSF11, respectively) in FLSs. The expression of these proinflammatory genes in the 3D culture of RA- and OA-FLSs was markedly enhanced compared with that observed in the same cell types grown in two-dimensional (2D) culture (Fig. 1D). Additionally, the expression levels of MMP1, MMP3, and TNFSF11 in 3D culture were significantly higher in RA-FLSs than in OA-FLSs (Fig. 1D). Although not reaching a level of statistical significance, the expression levels of IL6 also tended to be higher in RA-FLSs (Fig. 1D). These findings indicate that RA-FLSs grown in 3D culture retain elevated proinflammatory gene expression seen in primary synovial tissues. Thus, the 3D-culture system recapitulates the self-directed aggressive behavior of RA-FLSs displayed in the synovial lining in vivo.

FIGURE 1. Aggressive behavior of RA-FLSs reproduced in the 3D-micromass system. (A) Typical microscopic images of arthritic synovial lining layer and in vitro established lining-like structure. Frozen sections of synovial tissues from OA and RA (upper), or 3D-cultured micromass architecture formed by OA- and RA-FLSs (lower), were stained with DAPI. Both RA- and OA-FLSs assembled at the interface between the matrix and the fluid phase in 3D-culture, but their architectures were markedly different. The lining-like architecture formed by RA-FLSs frequently showed regions of condensed cellular accumulation several cells deep, which was reminiscent of the synovial lining architecture in RA patients (Fig. 1A, right). In contrast, OA-FLSs formed mostly single-cell layered lining, and areas of condensed cellular accumulation were rarely seen (Fig. 1A, left). To analyze the lining-like structure, sections were stained with fluorescent-labeled phalloidin. Consistent with a previous report (7), the condensed cells displayed a layered lining, and areas of condensed cellular accumulation were significantly thicker than that by OA-FLSs (Fig. 1C). These results indicate that RA-FLSs have the intrinsic ability to form condensed synovial architecture that may drive the formation of hyperplastic synovial linings in vivo.

$p < 0.05$, $**p < 0.01$, ns, not significant.
Activation of FAK-mediated signaling causes the aggressive behavior of RA-FLSs in 3D culture

To address the signaling mechanism responsible for the above pathological behavior of RA-FLSs in 3D culture, we first examined the phosphorylation level of FAK in RA tissues and compared it with that in OA tissues. Frozen sections from the synovial tissues from RA and OA stained with Ab to pFAK were examined by fluorescence microscopy. Phosphorylation of FAK was marked in the lining of RA synovium, whereas pFAK was minimally seen in OA tissues (Fig. 2A). Phosphorylation of FAK in RA-FLSs in 2D or 3D culture was then assessed by Western blotting. RA-FLSs cultured under 3D but not under 2D conditions showed phosphorylation of FAK (Fig. 2B), indicating that the increased FAK-mediated signaling seen in synovial lining cells in vivo were maintained in the 3D-culture system.

Because these results indicated that RA-FLSs have an activated FAK pathway relative to OA-FLSs, we next examined the contribution of FAK activation to the proinflammatory character of RA-FLSs. RA-FLSs maintained in 3D culture in the presence of a FAKi (10 μM PF-573228) showed marked decreases in proinflammatory mediator gene expression compared with RA-FLSs cultured in the absence of PF-573228 (86.9, 98.3, and 55.4% inhibition of the control for MMP1, MMP3, and IL6, respectively) (Fig. 2C). Because FAK phosphorylation is induced by integrin activation, these results indicate that FAK-mediated integrin signaling plays a crucial role in the intrinsic production of proinflammatory mediators by RA-FLSs.

Depletion of α9 or Tn-C suppresses hyperplastic and proinflammatory phenotype of RA-FLSs in 3D culture

To identify molecules that trigger constitutive activation of the integrin signaling in 3D-cultured RA-FLSs, we examined expression of genes for various integrins and their ligands in 3D-cultured RA- and OA-FLSs. Notably, the expression of ITGA9 and TNC (encoding α9 and Tn-C, respectively) were significantly higher in RA-FLSs than in OA-FLSs (Fig. 3A, left, middle), whereas no significant differences were observed in expression of ITGAI, 2, 4, 5, 6, V, and ITGB1 (encoding integrin α1, α2, α4, α5, α6, αV, and β1, respectively, Supplemental Fig. 1A). Additionally, the expression of LAMA1 and FN1 (encoding laminin A1 and fibronectin, respectively) by RA- and OA-FLSs were not significantly different (Supplemental Fig. 1B). Furthermore, immunofluorescence staining revealed colocalization of α9 and Tn-C proteins in the lining-like structure formed by 3D culture of RA-FLSs (Fig. 3A, right). The clinical relevance of this finding was validated in primary synovial tissues from RA patients where deposition of Tn-C was observed around α9-positive cells (Fig. 3A, right). These results suggest that α9 is significantly upregulated in 3D-cultured RA-FLSs and that it binds to its ligand, Tn-C, which is produced by RA-FLSs themselves and also upregulated in 3D culture. These results suggest that interaction of α9 on RA-FLSs with Tn-C activates the integrin signaling pathway in the synovial lining.

To examine the role of α9 and Tn-C in hyperplastic and proinflammatory phenotype in 3D culture, RA-FLSs were transfected with shRNA for ITGA9, TNC, or control scrambled shRNA by lentivirus vector and subjected to 3D-micromass culture. Western blotting of the cell lysates and ELISA of the culture supernatants confirmed successful knockdown of ITGA9 and TNC in the experimental period (Supplemental Fig. 2). Despite depletion of α9 and Tn-C, RA-FLSs moved to the interface between the matrix and the fluid phase. However, the appearance of cell assembly analyzed by phalloidin staining was dramatically altered. Whereas the cells treated with the control shRNA formed condensed structural assembly with densely packed actin bundles (Fig. 3B), the cells treated with the shRNA either for ITGA9 or TNC displayed loose cell aggregates with much less phalloidin staining (Fig. 3C). These observations indicate that depletion of either α9 or Tn-C disrupted the ability of RA-FLSs to form condensed cell assembly.

We next examined the effect of α9 and Tn-C depletion on activation of FAK-mediated integrin signaling. Knockdown of either

![Figure 2](image-url)
**FIGURE 3.** Involvement of α9 and Tn-C in the self-directed abnormal behavior of RA-FLSs. (A) Gene expression levels of ITGA9 (left) and TNC (middle) in OA- (n = 5) and RA-FLSs (n = 5) were determined by real-time PCR and expressed as mean ± SE. Distributions of α9 and Tn-C in 3D-cultured RA-FLSs and RA synovial tissue was determined by costaining with Alexa Fluor 594-labeled Ab to α9 (red) and Ab to Tn-C with Alexa Fluor 488-labeled secondary Ab (green) (right). Scale bars, 100 μm. (B-E) RA-FLSs treated with shRNA either for ITGA9, TNC, or the control (Con) cultured in 3D micromass were analyzed the phenotypes. (B) Sections from the resultant architectures were costained with Alexa Fluor 488–phalloidin (green) and DAPI (blue). Representative data from three independent RA-FLSs are shown. Scale bars, 50 μm. (C) Cell lysates from 3D-cultured RA-FLSs were analyzed by Western blotting with Abs to pFAK (upper) and total FAK (lower). Representative data from five independent blots are shown. (D) Protein amounts of MMP-1, MMP-3, and IL-6 in the culture supernatants were determined by ELISA and indicated as mean ± SE (n = 5), (E) Western blotting of the cell lysates with Abs to MMP-14 (left), TNFSF11 (right) and GAPDH. A representative data from five independent blots was shown. Statistical analyses were performed by a Student t test (A) and a Dunnett test using within-subject error (D). *p < 0.05, **p < 0.01, ns, not significant.

ITGA9 or TNC in RA-FLSs in 3D culture blocked phosphorylation of FAK (Fig. 3C). Furthermore, similar to the findings of the FAKI-treated RA-FLSs in 3D culture, knockdown of either ITGA9 or TNC in RA-FLSs markedly decreased the amount of MMP-1, MMP-3, and IL-6 in the culture supernatants by determined by ELISA and indicated as mean ± SE (n = 5), (Fig. 3D). Protein expression of MMP-14 and TNFSF11 was also suppressed by knockdown of either ITGA9 or TNC (Fig. 3E). The suppression of MMP-1, MMP-3, and IL-6 production was more prominent in ITGA9-deficient cells than in TNC-deficient cells. Several α9 ligands produced by RA-FLSs, such as thrombospondin-1 (33) and VEGF-C (34), might complement the lack of Tn-C by treatment with shRNA at least in part. These results indicate that depletion of α9 and Tn-C induces the same phenotypes as that seen by blocking FAK activation, and they suggest that Tn-C acts as a ligand of α9 in 3D-cultured RA-FLSs and this α9/Tn-C axis triggers FAK-mediated integrin signaling that is involved in the self-directed cellular assembly and proinflammatory mediator release for development of RA pathology.

In addition to the above alterations of intrinsic proinflammatory properties, we found that depletion of α9 resulted in decreased gene and protein expression of Tn-C in 3D-cultured RA-FLSs (Fig. 4A). Similarly, we found suppressed TNC expression by disruption of FAK signaling by treatment with FAKi (Fig. 4C, left). These observations indicate that production of Tn-C is dependent on α9-dependent FAK activation in integrin signaling, suggesting that engagement of α9 with Tn-C leads to further expression of Tn-C and continuous activation of α9. Such a self-amplifying positive feedback loop accelerates pathogenic behavior of RA-FLSs and promotes sustained inflammation.

As described above, depletion of either α9 or Tn-C by shRNA almost completely abolished the formation of actin bundles in 3D-cultured RA-FLSs. These observations led us to examine expression of cadherin-11, which is responsible for cell–cell adhesion between FLSs and stimulates actin remodeling in RA-FLSs (7). Strikingly, RA-FLSs treated with either of the shRNAs significantly decreased the expression of cadherin-11 in both mRNA and protein levels (Fig. 4B), indicating that α9/Tn-C signaling is involved in the expression of cadherin-11. The expression of CDH11 (encoding cadherin-11) was not inhibited by treatment with FAKi in 3D-cultured RA-FLSs (Fig. 4C, right), suggesting the possible involvement of FAK-independent pathways in regulation of cadherin-11 expression beyond α9 engagement. FAK-independent PI3K signaling triggered by α9 activation (15, 16) might play an important role in increasing cadherin-11 expression in FLSs under inflammatory conditions (35).

**Role of α9 is indispensable for RA-FLS responses to inflammatory stimuli**

Although α9 plays a key role in the autonomous aggressive behavior of RA-FLSs, it remains uncertain whether it also contributes to exogenous inflammatory stimuli, often called the “passive” inflammatory responses of RA-FLSs. Hence, the roles of α9 in responses of RA-FLSs to exogenously applied stimuli (PDGF and TNF-α) were determined in 3D culture. RA-FLSs transfected with shRNA for ITGA9 or scrambled control shRNA were grown in 3D culture with medium supplemented with PDGF or TNF-α. Incubation of the control shRNA-transfected RA-FLSs with PDGF resulted in thickening of the cellular structure as determined by phalloidin staining (Fig. 5A, upper). Control shRNA-transfected
RA-FLSs incubated in 3D culture with TNF-α did not show this response (Fig. 5A, upper). These findings were consistent with reports in the literature (25, 29) and validated the assay response. Conversely, RA-FLSs transfected with shRNA for ITGA9 did not condense into lining-like architecture nor did it exhibit a hyperplastic response to PDGF (Fig. 5A, lower). Alternatively, RA-FLSs transfected with control shRNA and grown in 3D cultures with TNF-α showed significantly enhanced production of MMP-1, MMP-3, and IL-6. The release of these proinflammatory mediators in response to exogenous TNF-α was almost completely absent in 3D culture of RA-FLSs transfected with shRNA for ITGA9 (Fig. 5B, 99.4, 97.2, and 91.0% inhibition of the control, respectively). These results indicate that α9-mediated signaling in 3D-cultured RA-FLSs is required not only for the hyperplastic response to PDGF but also for the production of proinflammatory mediators in response to TNF-α. These data indicate that α9 plays a pivotal role in the passive inflammatory responses of 3D-cultured RA-FLSs to exogenous inflammatory mediators.

Pharmacological blockade of α9 signaling suppresses development of synovitis in mouse arthritis model

The above results show that α9 signaling mediates the expression of Tn-C, cadherin-11, MMPs, and IL-6 and elicits the two pathogenic features in 3D-cultured RA-FLSs, intrinsic hyperplastic and aggressive behavior and passive inflammatory responses. We therefore addressed whether such α9-mediated mechanisms could also play a role in synovial hyperplasia observed in a mouse arthritis model. A mouse α9-blocking single-chain variable fragment of Ig fused to mouse IgG1, termed MA9-413 (36), was generated and used in this experiment. The isotype control for this study was mouse IgG1 mAb to KLH. The CAIA model was chosen to test the effects of MA9-413 because it results in the development of severe arthritis with synovial lining hyperplasia.
ROLE OF INTEGRIN α9 IN RHEUMATOID ARTHRITIS

To examine the effect of MA9-413 on α9-mediated gene expression in the joints further, Mmp1, Mmp3, Il6, and Cdh11 expression levels (genes encoding mouse MMP-1, MMP-3, IL-6, and cadherin-11, respectively) were determined by real-time PCR. The expression of Mmp3, Il6, and Cdh11 was upregulated in the arthritic joint from mice treated with the control IgG1 (Fig. 6E) although the expression of Mmp1 was not detected. Administration of MA9-413 significantly suppressed expression levels of these genes (Fig. 6E), which is in accordance with the results of depleting α9 in vitro (Figs. 3D, 4A, 4B). These results indicate that α9 mediates enhanced expression of MMP-3, IL-6, and cadherin-11 in the CAIA joints.

Taken together, the data suggest that activation of α9 plays an important role in the development of synovial lining hyperplasia and production of inflammatory mediators in vivo.

Humanized anti-α9 Ab, ASP5094, suppresses self-directed hyperplastic behavior of RA-FLSs

The above results taken together suggest that α9 overexpressed in RA tissue may function as a driver of chronic synovitis in vivo. We therefore generated a humanized anti-human α9 Ab, termed ASP5094, and examined its pharmacological potential. ASP5094 inhibited the adhesion of SW480 cells stably expressing human α9 to Tn-C–derived peptide (Tn-Cfn3/RAA) in a concentration-dependent manner (IC50 of 3.87 ng/ml, Fig. 7A). Notably, ASP5094 suppressed the self-directed formation of multiple-layered assembly of RA-FLSs and converted these cells to single cell–layered lining such as OA-FLSs (Figs. 1A, 7B). ASP5094 resulted in a concentration-dependent reduction in the thickness of the lining-like structure of RA-FLSs in 3D culture (Fig. 7C, 7D). Neither etanercept, a TNF-α blocking agent, nor natalizumab, an integrin α4-blocking agent, exerted such effects at concentrations up to 10 and 100 μg/ml, respectively (Fig. 7C, 7D). These results indicate that the blockade of human α9 by ASP5094 can suppress development of a thickened synovial lining-like structure in 3D culture strongly suggest a possible pharmacological potential of ASP5094 in the treatment of RA-associated synovitis.

Discussion

RA is a severe debilitating autoimmune disease. Although there are several effective therapies for RA, these treatments all are limited by their immunosuppressive effects and the associated risks for infection and cancer. As such, there is a significant unmet medical need for effective RA therapies that are not by themselves immunosuppressive. RA-FLSs comprise the cellular lining of the joint, which becomes chronically inflamed and hyperplastic in RA. Therefore, treatments targeting RA-FLSs hyperplasia may represent a significant improvement in the treatment of RA without suppressing immune function.

The present study showed that RA-FLSs when maintained in 3D culture retained their disease-associated aggressive behavior, that is, self-directed formation of condensed lining architecture and autonomous production of proinflammatory mediators. In the 3D-culture system, RA-FLSs form thickened lining-like architecture that is not present in OA-FLSs grown under similar conditions (Fig. 1A, 1C). This thickened lining-like structure was comparable to that seen in synovial tissues from RA patients. In addition to these structural changes, the RA-FLSs grown in 3D culture showed an increased expression of ITGA9 and TNC (Fig. 3A), Elevated expression and functional involvement of α9 and Tn-C in the development of ankle joint swelling was also confirmed in the CAIA model, where administration of mouse α9-blocking Ab suppressed synovial lining hyperplasia, bone resorption, and proinflammatory gene expression (Fig. 6A–D). It is therefore
MMP3 not only phosphorylation of FAK but also upregulation of.

axis is also crucial in their autonomous induction of proin-

of structural assembly of RA-FLSs, we have found that the Tn-C/

Chd11 blocking Ab inhibited joint induction of

that depletion of either α9 or Tn-C (Fig. 3C, 3D). Administration of an

α9-blocking Ab in the CAIA model also inhibited phosphorylation

of FAK as well as induction of Mmp3 and Il6 in the arthritic joints

(Fig. 6D, 6E). Given the abundant expression of α9 and Tn-C in

3D-cultured RA-FLSs, these findings suggest that the Tn-C/α9 axis

enhances activation of integrin signaling and upregulates proin-

flammatory gene expression in the synovial lining layer in RA.

Activation of α9 signaling in RA-FLSs also appears to sensitize

these cells to external inflammatory stimuli. Evidence supporting

this conclusion is based on the observation that shRNA that

inhibited the synthesis of α9 abrogated PDGF-induced hyper-

plasia of RA-FLSs and blunted the TNF-α–mediated increase in

proinflammatory mediator (MMP-1, MMP-3, and IL-6) produc-

tion (Fig. 5). This suggests the involvement of α9-mediated integrin signaling in the cellular response to PDGF and TNF-α. One of the key players linking these pathways might be FAK. Previous reports described a critical role of FAK in cellular response of fibroblasts to PDGF and TNF-α. For example, pharmacological inhibition of FAK inhibits PDGF-induced proliferation of lung fibroblasts (38) and TNF-α–induced production of proinflammatory mediators in periodontal ligament fibroblasts (39). In this context, it is thought that α9-dependent autonomous activation of FAK in self-directed cell assembly potentiates the passive response of RA-FLSs to upstream stimuli, cytokines, and growth factors, leading to an amplified inflammatory response.

Collectively, our data thus clearly show that blockade of α9 is a promising approach to treat synovial inflammation in RA through suppression of the pathogenic behavior of RA-FLSs. ASP5094 may provide an innovative therapeutic approach for the unmet medical need of treatments for synovitis associated with RA.

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Disclosures

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References


Supplementary Figure 1 Gene expression levels of various integrins (A) and ECM components (B) in OA- (n=5) and RA-FLSs (n=5) grown in 3D-culture were determined by real-time PCR and expressed as mean±SE. Statistical analyses were performed by Student’s t-test. **; P<0.01, ns; not significant.
**Supplementary Figure 2** (A) Western blot analysis of the cell lysates from 3D -cultured RA-FLSs treated with shRNA for ITGA9 or the control prepared in Figure 3C with antibody to α9. (B) Protein amounts of Tn-C in the supernatants of 3D -cultured RA-FLSs treated with shRNA for TNC or the control prepared in Figure 3D were determined by ELISA and expressed as mean±SE (n=5). Statistical analysis was performed by paired t-test. **; P<0.01.