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α9β1 Integrin-Mediated Signaling Serves as an Intrinsic Regulator of Pathogenic Th17 Cell Generation

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The interaction between matricellular proteins such as tenascin-C (TN-C) and osteopontin (OPN) and integrins has been implicated in the pathology of rheumatoid arthritis in which Th17 cells are recognized as primary pathogenic cells. The differentiation of Th17 cells is tightly regulated by cytokines derived from APCs, receiving various signals including TLR stimuli. In this study, we used a collagen-induced arthritis model and found that increased numbers of α9 integrin-positive conventional dendritic cells and macrophages were detectable in the draining lymph node (dLN) shortly following first immunization, and these cells produced both TN-C and OPN, ligands for α9 integrin. α9 integrin-mediated signaling, induced by TN-C and OPN, promoted the production of Th17-related cytokines by conventional dendritic cells and macrophages in synergy with TLR2 and 4 signaling. This led to the Th17 cell differentiation and arthritis development. Moreover, Th17 cells generated under blocking of α9 integrin-mediated signaling showed low level of CCR6 expression and impaired migration ability toward CCL20. Thus, we have identified α9 integrin-mediated signaling by TN-C and OPN as a novel intrinsic regulator of pathogenic Th17 cell generation that contributes to the development of rheumatoid arthritis. The Journal of Immunology, 2011, 187: 5851–5864.

During inflammatory responses, APCs at the draining lymph node (dLN) play a critical role in the differentiation of naive CD4+ T cells into several types of Th subset cells by producing cytokines. It has been well known that several cytokines, including IL-6 and IL-23, drive the production of IFN-γ-producing CD4+ Th cells (Th1) (1, 2).

The production of Th1- or Th17-polarizing cytokines by dendritic cells (DCs) and macrophages at the dLN is triggered and regulated by TLR-, CD40-, and C5a receptor-mediated signaling (3–7). Following recognition of different types of pathogen-associated molecular patterns through specific TLRs, APCs produce several types of cytokine, which results in the differentiation from naive CD4+ T cells into distinct Th cell subset such as Th1 or Th17 (1, 8, 9). Although recent study has revealed that a specific pathogen such as segmented filamentous bacteria can induce the generation of Th17 cells (10), APCs often produce both Th1- and Th17-polarizing cytokines at the same time following stimulation with certain TLR ligand (11–14), suggesting that other complex mechanisms might be involved for the decision of Th1 and/or Th17 development in vivo.

It has been demonstrated that matricellular proteins such as tenascin-C (TN-C) and osteopontin (OPN) are upregulated at various pathological foci, including autoimmunee arthritis, experimental allergic encephalomyelitis, and inflammatory bowel diseases (15–19). Rheumatoid arthritis (RA) is an autoimmune disease associated with synovial inflammation and hyperplasia (20, 21). By using a collagen Ab-induced arthritis (CAIA) model, which better serves for an analysis of effector phase of arthritis (22), we have previously found that synovial macrophages and fibroblasts at pathological foci express α9 integrin, which is a common receptor for TN-C and OPN, and demonstrated that the inhibition of the interaction between α9 integrin and TN-C/OPN by the blocking Abs against either OPN or α9 integrin reduced the production of inflammatory cytokines at arthritic joints, leading to the attenuation of arthritis (16, 23). We also found that signaling through α9 integrin induced IL-6 production, which is well known as a signature cytokine for Th17 cell development, by both synovial macrophages and fibroblasts. Interestingly, a recent study strongly suggests that TN-C acts as an endogenous activator for TLR4 and results in the production of proinflammatory cytokines and development of RA (24). Thus, the evidences imply that there might be tissue microenvironment where α9 integrin-mediated signaling regulates Th17 cell development in cooperation with TLR signaling in the context of inflammation.

In this study, we have used the collagen-induced arthritis (CIA) model, in which both recognition and effector phase of arthritis can be analyzed (25), to examine whether α9 integrin-mediated signaling affects the development of Th17 cells. We report in this study that α9 integrin-mediated signaling induces Th17-related cytokine productions such as IL-6 and IL-23 by conventional DCs (cDCs) and macrophages in synergy with TLR2 and 4 signaling at the dLN and results in the promotion of functional Th17.
cell generation and development of arthritis. In addition, Th17 cells, generated under blocking of $\alpha_\beta$ integrin function, showed impaired expression of chemokine receptor, critical for the migration of Th17 cells to target organs. The results highlight the importance of $\alpha_\beta$ integrin-mediated signaling as an intrinsic regulator for pathogenic Th17 cell generation and migration into target organs.

**Materials and Methods**

**Abs**  
Abs specific for CD3, CD4, Foxp3, IFN-\(\gamma\), CD11c, CD80, CD86, CD62L, F4/80, CCR6, and CCR7 were purchased from BioLegend. Abs specific for Mac-1, CD11b, class II, CD40, and CD28 were purchased from BD Pharmingen. mAb specific for $\alpha_\beta$ integrin (181RD) was used for flow cytometry analysis (16).

**Mice**  
Eight-week-old DBA/1J mice were obtained from Charles River River (Yokohama, Japan). Seven- to 8-week-old C57BL/6 mice were purchased from Japan SLC. These animals were maintained in specific pathogen-free condition. All animal experiments were in accordance with the guidelines of an institutional committee at Hokkaido University.

**Induction of CIA**  
Immunization grade bovine type II collagen (CII) dissolved in 0.05% acetic acid was purchased from Chondrex (Redmond, WA). DBA/1J or C57BL/6 mice were immunized by s.c. injection with 100 \(\mu\)g CII emulsified with equal volume of CFA containing 4 mg/ml heat-killed Mycobacterium tuberculosis strain H37RA (BD Biosciences) into the tail base. At 21 d after primary immunization, mice were boosted with the same preparation of CII/CFA. The clinical severity of arthritis was graded in each of the four paws on a 0–4 scale. The disease severity was recorded for each limb, as described previously (16). In brief: 0, normal; 1, focal slight swelling and/or thickening in one digit; 2, moderate swelling and erythema of \(\geq 2\) digits; 3, marked swelling and erythema of the limb; and 4, maximal swelling, erythema, deformity, and/or ankylosis. In some experiments, 400 \(\mu\)g anti-$\alpha_\beta$ integrin Ab (55A2C) was administered at days 1 and 2 or days 9 and 10.

**Production of mutated forms of human OPN and TN-C**  
An N-terminal fragment of human OPN (T17-R109), in which RGD was mutated to RAA (designated as OPN N half/RAA) (26), was cloned into pGEX6P-1 vector from the full-length OPN cDNA as a template using a pair of primers, as follows: OPN-5′-GACATCATACAGTAAAACAGCTGCTGAT-3′ and OPN-RAA-5′-GTCTGGTCAAGTCCTGTTCTCAGCTGTC-3′ and OPN-RAA-3′-GGCCTGCTACGGCATCAAAGACG-5′. A third fibronectin type III repeat domain of human TN-C (R297-P279) in which RGD was mutated to RAA (designated as FNIII) (27) was cloned into pGEX6P-1 from cDNA derived from HT-1080 cells using a pair of primers, as follows: TN-C-5′-TCTGGATCCATACCAGTGTTCTCT-3′ and TN-C-RAA-5′-GCTCTTTGATGCCGTAGGTGG-3′ and TN-C-RAA-3′-GCTTTGGCTGGTTGCTTGACATGGAGCTCT-5′. A mutated form of FNIII (designated as ΔFNIII) was cloned into pGEX6P-1 vector from TN-C construct as a template using a pair of primers, as follows: TN-CΔ-5′-GCCTGTACAGGCTGACACCAACCAGCTGGTCTGCTG-3′ and TN-C(RAA)Δ-5′-TCTGGATCCATACCAGTAAAACAGCTGCTGAT-3′ and TN-CΔ-RAA-3′-GCTAGTTGTTCTGTTCTGATCGTACGGCATCAAAGACGGCTCGTC-5′ and TN-CΔ-RAA-3′-GCTTTGGCTGGTTGCTTGACATGGAGCTCT-5′. A mutated form of FNIII (designated as ΔFNIII) was cloned into pGEX6P-1 vector from TN-C construct as a template using a pair of primers, as follows: TN-CΔ-5′-GCCTGTACAGGCTGACACCAACCAGCTGGTCTGCTG-3′ and TN-C(RAA)Δ-5′-TCTGGATCCATACCAGTAAAACAGCTGCTGAT-3′ and TN-CΔ-RAA-3′-GCTAGTTGTTCTGTTCTGATCGTACGGCATCAAAGACGGCTCGTC-5′ and TN-CΔ-RAA-3′-GCTTTGGCTGGTTGCTTGACATGGAGCTCT-5′. Amplification of specific primers using in this study are shown in Supplemental Table I. The expression level of mRNA was calculated by calibration curve method using LightCycler Software, version 3. Data were standardized by G3PDH.

**Measurement of protein level in LN s**  
Inguinal LNs obtained from normal or immunized mice were homogenized in PBS containing protease inhibitor mixture (Roche Diagnostics; 8.5 \(\mu\)l/mg tissue). The suspension of LNs mixed with 10% Triton X-100 (1 \(\mu\)l/mg tissue) and 10% Nonidet P-40 (0.5 \(\mu\)l/mg tissue) was vortexed and incubated on ice for 30 min. Then, the suspensions were centrifuged at 15,000 rpm, 30 min, and the supernatants were collected. OPN, TN-C, and OPN N half concentrations were measured by ELISA.

**T cell proliferation assay**  
LN cells were collected from mice treated with 55A2C or control IgG at day 20. Cells were isolated from LNs and labeled with CFSE (5 nM; Dojindo, Kumamoto, Japan). Then, cells were plated on 96-well plates and cultured in the presence of stimulation with CII (100 \(\mu\)g/ml). After 72 h, cells were stained for CD3 and CD4. In some samples, cells were additionally cultured in the presence of PMA (20 ng/ml), ionomycin (250 ng/ml), and brefeldin A (1 \(\mu\)l/ml) or MEK/ERK inhibition (Y-27632, 10 \(\mu\)m). Flow cytometry analysis was performed, as described previously (29). Briefly, the migration of T cells to CCL20 was evaluated by using 24-well, 5-\(\mu\)m pore-size Transwell system (Costar). CD4+ T cells (1 \(\times\)105) were isolated from inguinal LNs of DBA/1J mice 7 d after first immunization and placed on
the upper well in RPMI 1640. CCL20 (50 ng/ml) was added into lower wells in RPMI 1640 containing 1% FCS. After 12 h of incubation at 37°C, the percentage of CD4+IL-17+ T cells in cells migrated into lower well was analyzed by flow cytometric analysis. The specific migration was calculated by the following formula: (the ratio of IL-17+ T cells to CD4+ in lower well after migration/the ratio of IL-17+ T cells to CD4+ before migration) × 100.

Histology

Ankle joints were harvested at day 35 after first immunization. All specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut and stained with H&E or fast green/safranin-O. Light microscopy was performed to assess synovial inflammation and bone erosion. The number of cells infiltrating into joints was counted in at least three different fields per section under microscopic observation with original magnification ×400.

In vitro Th17 induction

CD62Lhigh naive CD4+ T cells and cDCs were isolated from the spleen of normal mice and the dLN 7 d after immunization, respectively, by using FACSAria. These cells were cocultured and stimulated with full TN-C or BSA (20 μg/ml) in the presence or absence of control IgG or 55A2C (10 μg/ml) or anti-IL-6 Ab (BioLegend; 10 μg/ml) for 72 h. Then, cells were restimulated with PMA (20 ng/ml) and ionomycin (250 ng/ml) in the presence of brefeldin A (1 μM) for 5.5 h. The percentage of IL-17+ cells and the expression of CCR6 on Th17 were evaluated by flow cytometric analysis.

Immunofluorescence

Tissue-frozen sections (8 μm) were blocked in 10% normal goat serum (Jackson ImmunoResearch Laboratories) in PBS for 30 min at room temperature. Next, endogenous biotin/avidin of the sections was further blocked by using Histofine Avidin/Biotin blocking kit (Nichirei Bioscience, Tokyo, Japan). Then, sections were incubated with primary Ab for 2 h at room temperature. Rat mAb specific for mouse TN-C (Abcam) were diluted at 1 μg/ml in 10% goat serum in PBS. Hamster mAb specific for CD11c (BD Pharmingen) were diluted 1:100 in 10% goat serum in PBS. After incubation, sections were washed three times with PBS. For TN-C detection, sections were then incubated for 1.5 h at room temperature with biotinylated anti-hamster IgG, which was diluted 1:100. After three times washing with PBS, sections were incubated for 30 min at room temperature with FITC-conjugated streptavidin (diluted 1:100). For CD11c detection, AlexaFluor546 conjugated anti-rat IgG (diluted 1:200). After incubation, sections were washed three times with PBS and embedded.

Microcomputed tomography

The hind paws were obtained at day 35 and fixed with 10% formalin. Samples were analyzed by using LCF-200 (ALOKA, Tokyo, Japan) at 48 μm pixel size and 96 μm slice thickness by 360˚ scan. Tomographic images of ankle joints (∼200 slices) were used for bone density evaluation with LaTheta software (ALOKA).

Western blot analysis

M-CSF–induced macrophages were generated, as previously described (30). In short, bone marrow cells obtained from C57BL/6 mice were cultured with RPMI 1640 media containing 30 ng/ml M-CSF (Wako, Osaka, Japan) for 6 d. M-CSF macrophages (1 × 107) were cultured with 10% FCS containing media for 12 h, and then cultured without FCS for additional 3 h. After incubation with FNIII or BSA for 0, 15, 30, 45, or 60 min at 37°C, cells were harvested and lysed with TNE (10mM Tris-HCl; pH7.5, 0.1M Nacl, 1mM EDTA) buffer containing protease inhibitor (Roche Diagnostics). The supernatants were subjected to immunoblotting analysis. Immunoblotting analysis was performed, as described previously (31). The short samples were applied to SDS-PAGE, transferred to membrane, and immunoblotted with either anti-ERK1/2 or anti-phosphorylated ERK1/2 Ab (Cell Signaling Technology). The intensity of the bands was evaluated by using Image J.

Statistical analysis

Statistical evaluation was performed based on the Student t test to compare the differences between groups. For the calculation of appearance ratio of arthritic joint, χ² statistics were derived using the CHIDIST function of Microsoft Excel. Correlation factor was analyzed by using CORREL function of Microsoft Excel, and the significance of correlation was assessed by using TDIST function of Microsoft Excel. Asterisks (*, **, ***) indicate that values are significantly different between control and experimental group, with p values <0.05, <0.01, or <0.001, respectively. N.S. represents that the difference was not significant.

Results

The administration of anti-α9 integrin Ab at early phase can reduce the severity of CIA

Previously, we have generated an inhibitory anti-α9 integrin Ab (55A2C) and demonstrated that treatment with 55A2C reduces the production of proinflammatory cytokines and ameliorates ongoing arthritis in CAIA model (16). However, T cell involvement in the pathogenesis of autoimmune arthritis in CAIA model is very limited, if any (32, 33). Therefore, to analyze the involvement of α9 integrin-mediated signaling in T cell-dependent autoimmune arthritis, we have used CIA, which is a well-known T cell-dependent arthritis model (25, 34). To induce CIA, mice were first immunized with CII emulsified with CFA (CII/CFA), and 21 d later, mice were boosted with CII/CFA. We first examined whether inhibition of α9 integrin function affects the development of CIA. To address this question, 55A2C was given 1 d before and 2 d after first immunization (Fig. 1A). We found that 55A2C-treated mice showed delayed onset and attenuated clinical score compared with control mice, which received control IgG (Fig. 1B, 1C). In control mice, marked inflammatory cell infiltration, synovial hyperplasia, pannus formation, and bone destruction were evident at day 35. However, in the arthritic joints of 55A2C-treated mice, inflammatory cell infiltration was significantly milder than that of control mice (Fig. 1D). In addition, the pannus formation and bone destruction as judged by H&E-stained section (marked by arrows in Fig. 1D) and cartilage degeneration as judged by fast green/safranin-O–stained sections (data not shown) of arthritic joint of 55A2C-treated mice were also milder. To determine the degree of bone destruction, the bone density of ankle joints of arthritic mice treated with 55A2C or control IgG was analyzed by microcomputed tomography at day 35. The bone density of 55A2C-treated mice was significantly higher than that of control IgG-treated mice (Fig. 1E). The data indicate that inhibition of α9 integrin function at early phase (within 1 wk after first immunization) of arthritis induction ameliorates synovial inflammation and joint destruction.

cDCs and macrophages in the dLNs express TN-C and OPN and their common receptor, α9 integrin, following immunization

The finding that inhibition of α9 integrin function at early phase of CIA induction reduced the severity of arthritis prompted us to further examine its role in T cell response and development. Naive CD4+ T cells recognize Ag presented by APCs in the dLNs and expand to generate large number of Ag-specific T cells. During T cell activation, T cells received several signaling through TCR, costimulatory molecules, and cytokine receptors, results in the differentiation into Th1, Th2, and Th17 cells (2, 4, 35, 36). We first examined whether APCs in the dLN express α9 integrin following s.c. immunization with CII/CFA. The expression of α9 integrin was detectable by cDCs (B220−CD11c+CD44low) and macrophages (CD11b+CD11c+ F4/80−), but not pDCs (B220+CD11clow) (Fig. 2A, 2B). We then investigated whether the ligands of α9 integrin, TN-C and OPN, are induced in the dLN. The levels of TN-C and OPN were significantly increased in the dLNs following immunization (Fig. 2C). N-terminal fragments of OPN (OPN N half), which can interact with α9 integrin (26), were also induced in the dLN (Fig. 2C).

Previous studies have demonstrated that several types of APCs, including synovial macrophages, can produce TN-C and OPN (16,
Inhibition of α9 integrin function leads to the reduction of Th17 cell generation following first immunization and the accumulation of Th17 cells in the dLNs postsecondary immunization

Th17 cells have been recognized as primary cause of pathology of RA and autoimmune arthritis in rodent models (40–43). It has been reported that, in CIA model, IL-6–dependent Th17 generation occurs in the dLNs within 7 d after immunization, and these Th17 cells further expand following secondary immunization, results in the development of arthritis in CIA model (44, 45). Therefore, we next examined whether the attenuation of arthritis by treatment with 55A2C at days −1 and 2 after the first immunization (early-phase treatment, as depicted in Fig. 1A) was due to the reduction of Th17 cell generation. We found that 55A2C-treated mice showed reduced number and frequency of Th17 cells in the dLN at day 7 following first immunization (Fig. 3A–C). At day 20 (after secondary immunization), there was no difference in the number of Th17 cells in the dLNs between two groups (Fig. 3A–C). However, in contrast to what we expected, 55A2C-treated mice contained more Th17 cells in the dLNs at day 24 (after secondary immunization) in comparison with control mice (Fig. 3A–C). This result suggests that the inhibition of α9 integrin function does not affect Ag processing and presentation by APCs. In fact, cDCs from the dLNs of 55A2C-treated mice at day 4 showed similar levels of CD80, CD86, CD40, and MHC
class II expression compared with cells from control IgG-treated mice (data not shown).

When the distribution of Th17 cells was examined in the spleens and joints following secondary immunization at days 24 and 28, respectively, it was apparent that the frequency of Th17 cells was markedly reduced in both tissues of 55A2C-treated mice compared with control mice (Fig. 3E,3F). Moreover, Th17 cells obtained from the dLNs of control IgG- and 55A2C-treated mice at day 20 showed similar proliferative ability, as evidenced by dilution of CFSE after 3 d of in vitro stimulation with CII (Fig. 3G), suggesting that the accumulation of Th17 cells in the dLN of 55A2C-treated mice at day 24 is not due to enhanced secondary expansion of Th17 cells generated in 55A2C-treated mice. Thus, our data suggest that the functional blockade of α9 integrin at early phase of CIA induction leads to the generation of Th17 cells that have impaired trafficking ability at later phase (after second immunization).

We have previously reported that the interaction of α9 integrin with its ligands induces the activation of synovial fibroblasts and macrophages at arthritic joints (16) in CAIA model in which involvement of T cell is very limited (32, 33). Therefore, we thought that the attenuation of arthritis by treatment with 55A2C at early phase (days 21 and 2 after first immunization) was also due to the suppression of synoviocytte activation at joint. To explore this possibility, we gave mice 55A2C on days 9 and 10 after first immunization, when synovial inflammation had not yet appeared (data not shown), and analyzed disease scores after secondary immunization. The 55A2C-treated mice showed comparable severity and incidence with control IgG-treated mice (data not shown), indicating that 55A2C treatment at intermediate phase does not influence the synovial inflammation at joints. Similar to our observation, it has been reported that the suppressive effect by anti–IL-6R Ab treatment on CIA was only observed when this mAb was injected on day 0 or 3, but not on days 7, 14, and 21 following first immunization, consistent with the data that Th17 cell generation was induced within 7 d after immunization (44, 45). Collectively, our data indicate that α9 integrin-mediated signaling at early phase of CIA induction regulates the generation of Th17 cells at the dLN that can subsequently migrate into the joints following secondary immunization.

α9 integrin-mediated signaling can induce IL-6 production by cDCs

We demonstrated that α9 integrin-mediated signaling contributes to the generation of Th17 cells in the dLN. It has been well known that IL-6 is a key cytokine to induce Th17 cell development (2, 46). IL-6 and IL-23, which are important cytokines for Th17 cell survival and expansion (47), are produced by APCs in response to several TLR stimuli (9). We previously reported that α9 integrin-mediated signaling induced proinflammatory cytokines, including IL-6, by synovial macrophages (16). However, it is unknown whether α9 integrin-mediated signaling can induce
Th17 cell-related cytokines by cDC and macrophages in lymphoid tissues. TN-C contains several domains, including fibronectin-like type III repeat domain, which interacts with α9 integrin, epidermal growth factor-like repeats domain, and fibrinogen-like globule (FBG) domain (24, 37). A recent report demonstrated that FBG domain induced IL-6 synthesis in human macrophages through TLR4 signaling (24). Therefore, we first analyzed IL-6 production by cDCs, obtained from the dLN at day 7, following stimulation with full TN-C. Full TN-C stimulation induced IL-6 production by cDCs in a dose-dependent manner (Fig. 4A), consistent with a previous report (24). In contrast, full TN-C stimulation showed much less effect on IL-12p40 production by cDCs (Fig. 4B), consistent with our data that TN-C production was correlated with IL-6 production by cDCs obtained from the dLN at day 7, but not with IL-12p40 (Fig. 4C). We next tried to understand how full TN-C stimuli can contribute to IL-6 production by cDCs. Unexpectedly, we found that IL-6 production was significantly reduced when cDCs were stimulated with full TN-C in the presence of 55A2C (Fig. 4D), suggesting that α9 integrin-mediated signaling also contributes to IL-6 production by cDCs. It should be reminded that under these conditions a FBG domain of full TN-C should interact with TLR4 (24).
expressed by cDC, thus also contributing to the production of IL-6.

To confirm that \( \alpha_9 \) integrin-mediated signaling can induce IL-6 production, we synthesized recombinant FNIII protein, which specifically interacts with \( \alpha_9 \) integrin (27). We purified cDCs from the spleen of naive mice and stimulated them with either FNIII or mutant FNIII (\( \Delta FNIII \)), which lacks \( \alpha_9 \) integrin-binding sequence, and cytokine productions were analyzed. We found that FNIII, but not \( \Delta FNIII \), induced the production of IL-6, but not IL-12p40 (Fig. 4E). Moreover, as compared with cDCs from naive mice, cDCs (Fig. 4F, left panel) purified from the dLN at day 7 following immunization produced significantly higher amounts of IL-6 in response to FNIII stimulation. Macrophages purified from the dLN at day 7 also produced a small amount of IL-6 in an \( \alpha_9 \) integrin-dependent manner (Fig. 4F, right panel). Similarly, cDC purified from the dLN at day 7 produced IL-6 in response to the stimulation with OPN N half/RAA (Fig. 4G). IL-23p19 gene expression by cDCs was also induced by FNIII stimulation (Fig. 4H). Augmented IL-6 production in cDCs induced by FNIII stimulation was reduced when cDCs were cultured with 55A2C (Fig. 4I).

Collectively, our data indicate that \( \alpha_9 \) integrin-mediated signaling can induce IL-6 and IL-23, but not IL-12p40, by both quiescent and activated APCs.

### FIGURE 4.
\( \alpha_9 \) integrin-mediated signaling enhances the expression of IL-6 and IL-23. cDCs obtained from the dLN at day 7 were cultured in the presence of indicated dose of full TN-C for 48 h, and the supernatants were subjected to ELISA analysis for the production of IL-6 (A) and IL-12p40 (B). *p < 0.05, **p < 0.01, ***p < 0.001 versus nonstimulation. C, Scatter diagrams showing correlation between amounts of IL-6 or IL-12p40 production and TN-C production by cDCs obtained from the dLN at day 7. D, cDCs obtained from the dLN at day 7 were stimulated with full TN-C (5 \( \mu g/ml \)) in the presence of 55A2C (10 \( \mu g/ml \)) for 48 h. The supernatants were subjected to ELISA analysis. n = 3 per group. E and F, SP cDC obtained from normal mice (E) or cDCs (left panel) and macrophages (right panel) obtained from the dLN at day 7 (F) were stimulated with BSA, FNIII, or \( \Delta FNIII \) (10 \( \mu g/ml \)), which lack \( \alpha_9 \) integrin-binding domain for 48 h. The production of IL-6 or IL-12p40 was assessed by ELISA analysis. n = 3 per group. G, cDCs obtained from the dLN at day 7 were stimulated with BSA or OPN N half/RAA (10 \( \mu g/ml \)), and the IL-6 production was measured by ELISA analysis. n = 3. H, The induction of IL-23 expression by the stimulation with FNIII. Total RNA was extracted from cDCs obtained from the dLN at day 7 after 24-h culture in the presence of the stimulation with BSA or FNIII (10 \( \mu g/ml \)), and was subjected to real-time PCR analysis. I, The inhibition of IL-6 production from cDCs obtained from the dLN at day 7 by addition of 55A2C. cDCs were cultured on the plates coated by BSA or FNIII (2.5 \( \mu g/ml \)) in the presence of 55A2C or control IgG (10 \( \mu g/ml \)) for 48 h. The supernatants were used for the measurement of IL-6 by ELISA analysis. n = 3 per group. All data are representative of at least two experiments. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
obtained from naive mice (Fig. 5A) and cDCs (Fig. 5B, left panel) and macrophages (Fig. 5B, right panel) purified from the dLN at day 7 following immunization were stimulated with FNIII in the presence of LPS or LM, which are ligands for TLR4 and TLR2, respectively, the production of IL-6 was synergistically augmented compared with either α9 integrin stimuli (FNIII) (Fig. 4E, 4F) or TLR stimuli alone (Fig. 5A, 5B). This synergistic augmentation of IL-6 production was specifically dependent on α9 integrin stimuli because this effect was canceled when we used ΔFNIII, lacking α9 integrin-binding sequence (Fig. 5A, 5B). OPN N half/RAA stimulation also could enhance IL-6 production in synergy with TLR2 signaling (Fig. 5C). In contrast, the synergistic effect of α9 integrin stimuli and TLR stimuli on IL-12p40 or TNF-α production was not detectable (Fig. 5D, data not shown). Interestingly, we found that IL-12p40 was barely produced by CD11b+, CD11c+, F4/80+ macrophages obtained from the dLN stimulated with LPS or LM and/or FNIII or OPN N half/RAA (data not shown). In addition, the stimulation with FNIII could not affect the production of IL-27p28, which is a Th17-suppressive cytokine (50) (data not shown). The synergistic effect of α9 integrin-mediated signaling and TLR signaling on IL-6 production in both cDC and macrophage seems specific for TLR2 and 4 signaling (Fig. 5A, 5B), because the combination of FNIII stimulation (α9 integrin-mediated signaling) and CpG (TLR9 signaling) only slightly augmented the production of IL-6 by those cells (Fig. 5E).

Importantly, observed in vitro data were consistent with in vivo data that 55A2C-treated mice showed lower levels of IL-6 and IL-23 expression in the dLNs at day 3 following immunization compared with control IgG-treated mice (Fig. 5F). However, the gene expression of TGF-β was not affected by 55A2C treatment (data not shown). These data suggest that α9 integrin-mediated signaling can strongly drive the generation of Th17-polarizing microenvironment in corporation with TLR2 and 4 signaling.

α9 integrin-mediated cytokine synthesis is dependent on MEK/ERK pathway

It has been reported that α9 integrin signaling stimulates the phosphorylation of focal adhesion kinase, Akt, ERK, and NF-κB in neutrophil (51–53). Although it has been thought that both IL-6 and IL-12p40 production are regulated by NF-κB activation (54), our finding in which α9 integrin-mediated signaling induced IL-6, but not IL-12p40, by cDCs, suggests that NF-κB–independent pathway may be involved in IL-6 production induced via α9 integrin signaling. Therefore, we cultured cDCs, obtained from

**FIGURE 5.** α9 integrin-mediated signaling induces IL-6 production in synergy with TLR signaling. SP cDCs obtained from normal mice (A) or cDCs (left panel) or macrophages (right panel) obtained from the dLN at day 7 (B, D) (7 × 10^5/ml) were stimulated by BSA, FNIII, or ΔFNIII (10 μg/ml), which lacks α9 integrin-binding sequence, in the presence of LPS (2 ng/ml) or LM (1 μg/ml) for 48 h. The supernatants were used for the measurement of IL-6 (A, B) and TNF-α (D) production. Error bars represent mean ± SEM. n = 3 per group. C, cDCs isolated from the dLN at day 7 were stimulated with BSA or OPN N half/RAA (10 μg/ml) in the presence of LM (1 μg/ml) for 48 h. The production of IL-6 was measured by ELISA analysis. Error bars represent mean ± SEM. n = 3 per group. E, cDCs (left panel) or macrophages (right panel) obtained from the dLN at day 7 were stimulated with BSA or FNIII (10 μg/ml) in the presence of indicated dose of CpG for 48 h. The production of IL-6 was assessed by ELISA analysis. Error bars represent mean ± SEM. n = 3 per group. F, Gene expression of IL-6 and IL-23 at the dLN. Mice were treated with control IgG or 55A2C and immunized by CII/CFA, as depicted in Fig. 1A. The dLN was collected at day 3, and the gene expression of IL-6 and IL-23 was analyzed by using quantitative real-time PCR. Error bars represent mean ± SEM (normal, n = 4; control IgG and 55A2C, n = 9). *p < 0.05, **p < 0.01, ***p < 0.001. All data are representative of at least two independent experiments.
the dLN at day 7 after immunization, with FNIII, which can interact with α9 integrin, but not with TLR4, in the presence or absence of NF-κB inhibitor or MEK inhibitor (U0126) for 48 h and analyzed IL-6 production. We found that U0126 strongly inhibited the enhancement of IL-6 production induced by FNIII stimulation alone, whereas NF-κB inhibitor partially inhibited the IL-6 production (Fig. 6A). IL-6 production induced by the combination of FNIII and LM (signaling through α9 integrin and TLR2) was significantly inhibited when either MEK inhibitor, U0126, or NF-κB inhibitor was added in the culture (Fig. 6A). So, we next examined whether the stimulation with FNIII can induce the phosphorylation of ERK. We could find that the phosphorylation of ERK1/2 was induced when we stimulated M-CSF–inhibited α9 integrin-positive macrophages (30) with FNIII (Fig. 6B, 6C). These data indicate that not only NF-κB pathway, but also MEK/ERK pathway is critically involved in α9 integrin-mediated IL-6 production by cDCs.

The lack of α9 integrin-mediated signaling leads to the generation of defective Th17 cells, which express low level of chemokine receptor CCR6

It has been reported that CCR6 plays an important role in the migration of Th17 cells from lymphoid tissues into the peripheral tissues, such as inflamed joint and Peyer’s patch (29, 55, 56). Therefore, we analyzed whether the accumulation of Th17 cells found in the dLN of 55A2C-treated mice following secondary inflammation is due to the impairment of CCR6 expression by Th17 cells. We first stimulated naive CD4+ T cells with immobilized anti-CD3 mAb, cDCs, and full TN-C, which contains α9 integrin-binding domain, for 72 h, and analyzed the frequency of Th17 cells and CCR6 expression. We found that TN-C stimulation induced more Th17 cell generation compared with BSA stimulation. The frequency of Th17 cells was significantly reduced when 55A2C was added in the culture, but not control IgG (Fig. 7A). Moreover, Th17 cells generated in the presence of TN-C showed higher level of CCR6 expression compared with the cells generated in the presence of BSA, and the increased CCR6 expression was significantly reduced by 55A2C treatment (Fig. 7B). These results are consistent with the data that the increased IL-6 production by cDCs following the stimulation with FNIII was inhibited by 55A2C treatment (Fig. 7C). In fact, the neutralizing of IL-6 by using a specific Ab strongly reduced the differentiation of Th17 cells induced by the stimulation with full TN-C (Fig. 7D).

To directly clarify the role of α9 integrin-mediated signaling on the differentiation of functional Th17 in vivo, we analyzed CCR6 expression by Th17 cells in the dLN of 55A2C-treated mice. We first examined CCR6 expression by Th17 cells. As we expected, CCR6 expression was induced by immunization (Fig. 7E). We found that Th17 cells generated in 55A2C-treated mice showed lower level of CCR6 expression compared with cells in control mice at day 7 following immunization (Fig. 7F), and this reduction was still detectable at day 24 (Fig. 7G). In contrast, the expression of CCR7, which is essential for the localization of T cells in lymphoid organs (57), was not affected by 55A2C treatment (data not shown). To test the functional relevance of the decreased CCR6 expression by Th17 cells, we examined the migration ability of Th17 cells in response to CCL20, which is the ligand of CCR6 and highly expressed in arthritic joints (29). Th17 cells from 55A2C-treated mice showed the reduced migration against CCL20 compared with cells from control IgG-treated mice. We did not detect any specific migration of Th1 cells, which does not express CCR6, in response to CCL20 (Fig. 7H). Collectively, our data suggest that α9 integrin-mediated signaling in APCs is responsible for the generation of functional Th17 cells in the dLN by regulating the production of IL-6 (Fig. 8).

Discussion

During T cell activation, naive CD4+ T cells received several stimuli through TCRs, costimulatory molecules, and cytokine receptors (4, 35, 36). Mounting evidence strongly suggests that cytokine milieu is a critical factor for Th17 cell differentiation. Several cytokines, including IL-6, IL-23, and TGF-β, promote Th17 cell differentiation (1, 8, 9), and these Th17 cell-promoting

![FIGURE 6. α9 integrin-mediated signal induces the production of IL-6 in MEK/ERK-dependent manner. A, cDCs obtained from the dLN at day 7 were cultured on the BSA- or FNIII-coated plates (10 μg/ml) in the presence of DMSO, NF-κB inhibitor (10 μM), or MEK/ERK inhibitor, U0126 (10 μM), for 48 h. The supernatants were used for measurement of IL-6 by ELISA analysis. Error bars represent mean ± SEM. n = 3 per group. *p < 0.05, **p < 0.01, ***p < 0.001. B, Immunoblotting of p-ERK and total ERK. M-CSF–induced α9 integrin-positive macrophages were incubated in the presence of FNIII or BSA for indicated time points. After the incubation, cells were harvested and lysed, and the supernatants were subjected to immunoblotting analysis. The supernatants were applied to SDS-PAGE, transferred to a membrane, and immunoblotted with anti-ERK or anti-phosphorylated ERK Ab. The intensity of the bands was evaluated by using Image J (C). All data are representative of at least two experiments.](http://www.jimmunol.org/10.1172/JIMM.2016.186908.60)
cytokines are mainly produced by DCs and macrophages by stimulation, including TLRs and C-type lectin (9, 58, 59). However, TLR stimuli also induce the production of Th1 cell-promoting cytokine such as IL-12 and Th17-suppressive cytokine such as IL-27 by APCs (11–14). Therefore, during infection/inflammation, it is likely that there is another pathway, collaborating with TLR signaling to promote Th17-related cytokine productions by APCs. In the current study, we have used CIA model and demonstrated that \( \alpha_9 \) integrin-mediated signaling, which is induced by matricellular proteins such as TN-C and FIGURE 7. \( \alpha_9 \) integrin-mediated signaling is required for the generation of functional Th17 cells, which highly express CCR6. CD62L\(^{high} \) naive CD4\(^+ \) T cells were cultured with cDCs, which were obtained from the dLN at day 7, in the presence of anti-CD3 mAb (1 \( \mu \)g/ml), TGF-\( \beta \) (2 ng/ml), and full TN-C (20 ng/ml) for 72 h. In some wells, 55A2C (10 \( \mu \)g/ml) (A–C) or anti–IL-6 Ab (D) was added. The generation of Th17 cells was analyzed (A, D), and the expression of CCR6 on Th17 cells was assessed by flow cytometry analysis (B). Numbers in B indicate the mean fluorescence intensity (MFI) of CCR6 expression. After 72 h of culture, the supernatants were used for the measurement of IL-6 by ELISA analysis (C). BSA and control IgG (Cont-IgG), \( n = 4; \) 55A2C, \( n = 3. \) E, Representative histogram plots for CCR6 expressed on Th17 cells in the dLN. The dLN cells were harvested from C57BL/6 mice before or 7 d after immunization with CII. Normal, represents nonimmunized mice. Representative histogram plots (left panel) and MFI (right panels) for CCR6 expressed on Th17 in the dLN, which were obtained from control IgG- or 55A2C-treated DBA/1J mice at day 7 (F) or day 24 (G). Normal, \( n = 3; \) control IgG and 55A2C, \( n = 6. \) H, Selective migration of Th17 cells toward CCL20. CD4\(^+ \) T cells (1 \( \times \) 10\(^6 \)) sorted from the dLN of control IgG- or 55A2C-treated DBA/1J mice at day 7 were put on the upper well, and CCL20 (50 ng/ml) was added to lower well in RPMI 1640 containing 1% FCS. After 12 h of incubation, cell migration was evaluated by using flow cytometry analysis. The statistical results were shown at the right. \( \cdot \) \( p \) per group. All data are representative of two independent experiments. Error bars represent mean \( \pm \) SEM. * \( p < 0.05, ** p < 0.01, *** p < 0.001. \)
OPN, regulates Th17 cell-promoting cytokine production by cDCs and macrophages in synergy with TLR4 and 2 signaling. This led to the generation of functional Th17 cells and the development of arthritis.

Shortly following immunization with CII/CFA, the upregulation of TN-C and OPN proteins was detectable in the dLN. This could reflect increased numbers of cDCs and macrophages in the dLN, because these cells produced both TN-C and OPN (Fig. 2D). However, we do not exclude the possibility that other cells contribute to the production of TN-C and OPN. In fact, TN-C was produced not only by cDC and macrophages, but also by pDC (Fig. 2D) and other CD11c-negative cells (Fig. 2E). Moreover, it has been reported that activated T cells express OPN (60). The finding that upregulation of TN-C and OPN was detectable in the dLN at day 1 postimmunization suggests that there might be tissue microenvironments in which quiescent cDCs and macrophages can be stimulated by these proteins even without TLR stimuli. In fact, we detected that α9 integrin-mediated signaling alone or in synergy with TLR signaling was capable of inducing IL-6 production by quiescent cDCs (Figs. 4E, 5A). Moreover, such interaction between α9 integrin and its ligands at early phase after initial immunization should be critical for the generation of Th17 cells at the dLN and subsequent arthritis development. In fact, mice treated with 55A2C until day 2 showed decreased number of Th17 cells in the dLN at day 7 (Fig. 3A–C). This might be due to the reduced IL-6 production by cDCs and macrophages in the dLN (Fig. 5F).

It is not likely that reduced number of Th17 cells is due to the inability of Ag processing and presentation by APCs in 55A2C-treated mice, because APCs derived from 55A2C-treated mice showed comparable expression of class II and costimulatory molecules as compared with those from control IgG-treated mice (data not shown). Moreover, the numbers of Th1 and Treg were comparable between 55A2C-treated and control mice (Supplemental Fig. 2).

The expression of orphan nuclear receptor, retinoic acid receptor-related orphan receptor (ROR) γt, and RORα, which promote Th17 generation, is induced by IL-6 in a STAT3-dependent manner (61–63). It has been reported that T cells defective in both RORα and RORγt show reduced CCR6 mRNA expression (64). Furthermore, the transfection of RORγt gene into CCR6–/– CD4+ T cells induces CCR6 expression (29). In addition, IL-23 also drives the CCR6 expression in synergy with PGE2 (65). Thus, our data that Th17 cells generated under blocking of α9 integrin-mediated signaling by 55A2C expressed low level of CCR6 (Fig. 7) might reflect decreased IL-6 and/or IL-23 production by cDCs and macrophages. Additionally, we detected Th17 cell accumulation in the dLN of 55A2C-treated mice after secondary immunization, leading to the reduced number of Th17 cells in joint, which resulted in the attenuation of arthritis development (Figs. 1, 3). It has been reported that CCR6 is critical for emigration of pathogenic Th17 cells from the dLN into the circulation, thus target organ (29), and CCL20, which is a ligand for CCR6, is produced by synovial fibroblasts in arthritic joints (29). It has been also reported that lymphatic endothelial cells activated by IL-1β or TNF-α also produce CCL20 (66), and functional blockade of CCR6 with an antagonistic peptide results in the accumulation of dividing CD4+ T cell in the LN (67), suggesting that CCR6 expression might be required for the egress of Th17 cells from the dLN. It should be pointed out that the number of Th17 cells in the dLN at day 20 was comparable between 55A2C-treated mice and control mice, despite the fact that Th17 cell number was reduced in 55A2C-treated mice at day 7. Possible explanation for this observation is that Th17 cells, generated in 55A2C-treated mice, are somehow hampered to egress from the dLNs into circulation, as compared with the cells generated in control mice, because Th17 cells in 55A2C-treated mice at day 7 already showed lower level of CCR6 expression (Fig. 7F).

In the animal models for RA, including CIA, it has been known that the severity of arthritis is significantly attenuated by the inhibition of Th17 cell recruitment into joints with anti-CCR6 Ab or anti–TNF-α Ab (29, 68). However, it should be noted that the requirement of Th17 cells in synovial tissues for RA progression is debatable. A previous report demonstrated that the infiltration of Th17 cells into synovial tissues of RA patients is marginal in comparison with Th1 cells (69). In contrast, there are reports showing that the production of IL-17 is significantly upregulated at synovial tissues or synovial fluids of RA patients (70–72). This discrepancy may be due to the difference in the stage of RA, because the significantly elevated expression of IL-17 is detectable in synovial fluid only at early stage of RA (73).

In the current study, we found that cDCs and macrophages in the dLN contribute to CIA development through α9 integrin-dependent cytokine production. The importance of myeloid cells in the development of CIA has been reported by several studies. The Ag recognition by APCs, including DCs and macrophages, is essential for the initiation of CIA, because the haplotype of MHC class II affects the susceptibility for the CIA (74). Moreover, M-CSF–deficient mice, which show the severe defects of macrophage function, are resistant to CIA (75) and the deficiency of dendritic cell immunoreceptor, which negatively regulates the expansion of DCs, results in the enhancement of sensitivity for CIA (76).

Recent reports have demonstrated that complement activation contributes to triggering Th17 cell-mediated autoimmune arthritis in synergy with TLR or GM-CSF signaling (5, 6). In addition, Lm, a component of M. tuberculosis within CFA, which we have used in this study as an adjuvant, can activate DCs through TLR2 (48, 49). Moreover, TN-C acts as an endogenous activator for TLR4–mediating immunity through its FBG domain (24), suggesting the possibility that α9 integrin-mediated signaling can cooperate with TLR signaling. Our data that α9 integrin-mediated signaling in synergy with TLR4 and TLR2 signaling, but not with TLR9 signaling, leads to the generation of Th17 cells via IL-6 production, but not Th1, are consistent with previous reports that TLR9 induces a Th1 response by promoting IL-12 production by APCs (77–80), whereas TLR2 signaling favors Th17 response (77, 81, 82). In this study, α9 integrin-mediated signaling did not significantly induce IL-12p40 production by cDCs and macrophages in synergy with TLR2 and TLR4 signaling (data not shown), consistent with the data in which generation of Th1 cells was not affected by blocking of α9 integrin function (Supplemental Fig. 2). In contrast, α9 integrin-mediated signaling could not affect the production of IL-27p28 (data not shown). These data strongly support the idea that α9 integrin can positively regulate the Th17 cell generation. It has been reported that IL-6 regulates Treg/Th17 balance (83). However, Treg differentiation was not affected by blockade of α9 integrin-mediated signaling, whereas IL-6 expression was reduced in the dLN of 55A2C-treated mice (Fig. 5F).

The reason for the discrepancy between our data and the previous report on the role of IL-6 in Treg generation is not known. However, consistent with our data, recent reports showed that the lack of IL-6 signaling or blockade of IL-6R barely inhibited the differentiation of Treg in vivo (44, 84).

Another important issue that should be addressed in this work is that Midwood et al. (24) previously demonstrated that a FBG domain of TN-C induces a significant amount of IL-6 production via TLR4 signaling. In our study, TLR4 signaling alone (LPS) or α9 integrin signaling (FNIII) alone is able to induce significant,
but small, amounts of IL-6 production by normal cDCs or cDCs or macrophages derived from immunized mice. Nevertheless, synergistic augmented production of IL-6 was detected when FNIII (αβ integrin signaling) and LPS (TLR4 signaling) or LM (TLR2 signaling) were added together (Fig. 5A, 5B). The molecular basis for the discrepancy between previous and our present data is currently not known; however, we used freshly isolated cDCs or macrophages from normal or immunized mice, whereas M-CSF–stimulated human monocyte, M-CSF–stimulated murine bone marrow cell-derived macrophages, or human RA-derived synoviocytes were used in a previous study, thus indicating that species difference or cell culture conditions may explain the difference.

The role of TNF-α in the development of RA has been well studied. Recent study has revealed that although TNF-α blockade increased numbers of both Th1 and Th17 cells in LNs, it inhibited accumulation of these pathogenic T cells in the joint, thereby resulting in the amelioration of RA development (68). We found that expression level of TNF-α in the dLN at day 3 following immunization was comparable between 55A2C-treated and control mice (data not shown). These data suggested that observed impairment of functional Th17 cell generation by blocking of αβ integrin function is not due to the reduction of TNF-α production.

Finally, we in this study clarified the molecular basis of αβ integrin signaling-induced augmentation of IL-6 production by cDCs. This process is ERK dependent (Fig. 6A). It has been reported that mechanical stress leads to the production of IL-6, but not IL-12p40, in pERK1/2-dependent manner in smooth muscle cells (85). In addition, the inhibition of ERK pathway suppresses the production of IL-23 and IL-1, but not IL-12p40, by DCs, which results in the attenuation of experimental autoimmune encephalomyelitis development through the regulation of Th17 cell generation (86). Thus, ERK pathway may play an important role in the differentiation of Th17 cell through inducing the expression of IL-6 and IL-23, but not IL-12p40, by cDCs and macrophages.

In conclusion, our proposed model for the role of αβ integrin in the generation of pathogenic Th17 cells is illustrated in Fig. 8. Matricellular proteins such as TN-C and OPN are rapidly induced by activated cDCs and macrophages (also by other cells) following immunization, and these proteins interact with its receptor, αβ integrin, expressed by cDCs and macrophages. The autocrine and paracrine signaling through αβ integrin amplifies the production of IL-6 and IL-23, but not IL-12, by cDCs and macrophages in synergy with TLR2 and 4 signaling. Therefore, the blockade of αβ integrin-mediated signaling by 55A2C treatment results in the reduction of IL-6 and IL-23 production, leading to the generation of dysfunctional Th17 cells that express low level of CCR6. CCL20, which is a ligand for CCR6, produced by activated synovial cells, induces the recruitment of Th17 cells into joints. However, dysfunctional Th17 cells, which express reduced level of CCR6, are unable to migrate into joints, resulting in attenuation of the development of arthritis. Our data suggest that the interaction between matricellular proteins such as TN-C and OPN and αβ integrin is critical for the generation of pathogenic Th17 cells.

**FIGURE 8.** Schematic illustration of lymphoid tissue microenvironment. A, cDC and macrophages express TN-C and OPN at the dLN within 1 wk after immunization. These cells express αβ integrin, a common receptor for TN-C/OPN. The stimulation of those cells with TN-C/OPN and TLR ligands synergistically acts to produce IL-6 and IL-23, which favors the generation of pathogenic Th17 cells. B, Abrogation of αβ integrin-mediated signaling by anti-αβ integrin Ab results in the reduction of Th17 cell generation in the dLN at early phase and accumulation of Th17 cells in the dLN at late phase due to the reduction of CCR6 expression, which is important for migration of Th17 cells from the dLN to target organs, joint tissues. This leads to the amelioration of arthritis.
in lymphoid tissues. Thus, α9 integrin may serve as a potential therapeutic target for Th17 cell–related disorders.

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Disclosures

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