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α2β1 Integrin Regulates Th17 Cell Activity and Its Neutralization Decreases the Severity of Collagen-Induced Arthritis

Mohammed-Amine El Azreq,*† Marc Boisvert,*† Annabelle Cesaro,*† Nathalie Page,*† Lionel Loubaki,‡§ Isabelle Allaeyes,*† Jamila Chakir,‡§ Patrice E. Poubelle,‡§ Philippe A. Tessier,*† and Fawzi Aoudjit,*†

Th17 cells play a critical role in the pathogenesis of rheumatoid arthritis (RA), but the mechanisms by which these cells regulate the development of RA are not fully understood. We have recently shown that α2β1 integrin, the receptor of type I collagen, is the major collagen-binding integrin expressed by human Th17 cells. In this study, we examined the role of α2β1 integrin in Th17-mediated destructive arthritis in the murine model of collagen-induced arthritis (CIA). We found that α2β1 integrin is expressed on synovial Th17 cells from CIA mice and its neutralization with a specific mAb significantly reduced inflammation and cartilage degradation, and protected the mice from bone erosion. Blockade of α2β1 integrin led to a decrease in the number of Th17 cells in the joints and to a reduction of IL-17 levels in CIA mice. This was associated with an inhibition of receptor activator of NF-κB ligand levels and osteoclast numbers, and reduction of bone loss. We further show that α2β1 integrin is expressed on synovial Th17 cells from RA patients, and that its ligation with collagen costimulated the production of IL-17 by polarized human Th17 cells by enhancing the expression of retinoic acid receptor–related orphan receptor C through ERK and PI3K/AKT. Our findings provide the first evidence, to our knowledge, that α2β1 integrin is an important pathway in Th17 cell activation in the pathogenesis of CIA, suggesting that its blockade can be beneficial for the treatment of RA and other Th17-associated autoimmune diseases.


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cell interactions with collagen via α2β1 integrin could represent an important pathway in the pathogenesis of RA. In this study, we found that α2β1 integrin is expressed on synovial Th17 cells and its neutralization decreases the severity of collagen-induced arthritis (CIA; Th17 cell–driven RA animal model) by reducing synovial Th17 cell numbers and IL-17 levels. We further show that α2β1 integrin is expressed on synovial Th17 cells from RA patients and its ligation with collagen costimulated the production of IL-17 by polarized human Th17 cells by enhancing ERK and PI3K/AKT via the expression of retinoic acid receptor–related orphan receptor C (RORc), the master regulator of IL-17 gene transcription and Th17 differentiation. These data indicate for the first time, to our knowledge, that α2β1 integrin is an important pathway of Th17 cell activation in the pathogenesis of CIA, suggesting that its blockade can be beneficial for the treatment of RA and other Th17-associated autoimmune diseases.

Materials and Methods

Abs and reagents

Hamster anti-mouse α2β1 integrin blocking mAb (clone Ha1/29) and its isotypic control hamster IgG (clone Ha1/8), anti-mouse CD4-FITC, CD8-PE (clone RM4-5), anti-mouse IL-17–Alexa 647 (clone TC11-18H10), anti-human α2 integrin-PE (12F1), anti-mouse α2 integrin-PE (clone HM), and control isotypic Abs were from BD Bioscience (San Diego, CA). CD3/CD28 Dynabeads were from Invitrogen Dynal AS (Oslo, Norway). Chicken collagen II, LPS from E. coli (serotype 0111:B4), and CFA were purchased from Chondrex. Cytokines (TNF-α, IL-2, IL-6, IL-23) and ELISA kits (IL-17, RANKL) were from R&D Systems.

BSA, collagenase D, PMA, ionomycin, and Cof I were from Sigma (St. Louis, MO). The MEK1/2 inhibitor U0126, the JNK/MAPK inhibitor SP600125, the p38/MAPK inhibitor SB203580, and PI3K/AKT inhibitor LY294002 were from Calbiochem (San Diego, CA). The anti–phospho-ERK1/2 mAb (E-4), anti–ERK2 Ab (C-14), and anti–actin (C-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti–phospho-JNK1/2 Ab (G9), anti–phospho-p38 (9216) Ab, anti–phospho-AKT (#9271) Ab, and anti-AKT (#9272) were from Cell Signaling Technologies (Beverly, MA).

Patients, isolation of arthritic synovial T cells, and human Th17 cell differentiation

Synovial fluids from five RA patients with active disease based on the American College of Rheumatology criteria (3) (three had established RA and two were diagnosed as early RA) were collected by arthrocentesis during a clinical procedure. The three patients with established RA were between 31 and 52 y old with disease duration between 1 and 7 y. Two patients were treated with metotrexate, Plaquenil, and Salazopyrin, and one patient received abatacept and prednisone. The two patients with early RA were 21 and 26 y old with disease duration between 3 and 6 mo and were without treatment.

Neutrophils were depleted from the synovial fluids by positive selection using an EasySep Neutrophil Enrichment Kit (StemCell Technologies). CD4 T cells were then isolated by negative selection using magnetic beads (StemCell Technologies, Vancouver, BC) according to the manufacturer’s instructions. To obtain polarized human Th17 cells, we isolated naive CD4 T cells from peripheral blood of healthy adult volunteers as we previously described (26). To determine the association of α2 integrin with RA, we isolated CD4 T cells from RA patients and matched healthy controls.

Flow cytometry of cell-surface Ag and IL-17 expression by joint and spleen cells

Histopathological quantifications were performed by two observers in a blinded manner from images of three fields representing the distal interphalangeal joint, the proximal interphalangeal joint, and the carpal region.

ELISA assays of IL-17 and RANKL in sera and joint cell lysates

Blood was collected by cardiac puncture in heparinized tubes and centrifuged to recover the serum fractions. Hind paws were removed and homogenized on ice in a tissue grinder (Polytron, Kinematica AG) in PBS containing a mixture of protease inhibitors (Roche). Samples were then sonicated (Branson Sonifier 450) on ice for 2 min at power level 4. The supernatants were recovered and protein levels were assessed by Bradford protein assay. Lysates with equal amounts of protein were subjected to ELISA assay for the detection of IL-17 and RANKL using specific ELISA kits (R&D Systems).

Mice

Eight-week-old female DBA/1J mice (Jackson Laboratories) were used. Water and food (Purina Mouse Chow 5015) were provided ad libitum. All procedures involving animals were conducted according to the requirements of and with the approval of the Laval University animal protection committee.

Induction, treatment, clinical assessment, and histopathology of CIA

Mice were immunized s.c. (day 0) at the base of the tail with chicken Coll II (100 µg/mouse) emulsified in CFA. On day 28 after initial immunization, and to synchronize disease onset, animals were injected i.p. with LPS (25 µg/mouse) (25). Animals start to develop arthritis within 24 h after LPS injection. The anti–α2β1 integrin blocking mAb (Ha1/29) and isotypic control mAb (Ha4/8; 100 µg/mouse) were injected i.p. 24 h before LPS challenge (day 27) and at days 30, 32, and 34 thereafter (2, 4, and 6 d after LPS injection, respectively). Mice were closely monitored and scored daily for clinical symptoms of arthritis in a blinded manner by two observers until the animals were sacrificed at day 36 (8 d after LPS injection). A scoring scale from 0 to 4 per paw (for a maximum disease score of 16 per mouse) was used as previously described (26, 27): 0, normal; 1, mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; 2, moderate redness and swelling of ankle or wrist; 3, severe redness and swelling of the entire paw including digits; and 4, maximally inflamed limb with involvement of multiple joints. At day 36, mice were sacrificed, and blood samples and paws were recovered for analysis.

Forepaws were rapidly fixed in 4% paraformaldehyde, decalcified in TBD-2 Thermo-Shandon solution (Thermo Fisher Scientific) for 10 d, and embedded in paraffin. Sections (5 µm thickness) were prepared and stained with H&E (Thermo Fisher Scientific) to assess cell infiltration and bone degradation as previously described (26). Cell infiltration was evaluated following a 0 to 2 scale (0, normal; 1, mild infiltration; 2, severe infiltration), and bone degradation was scored using the following 0 to 3 scale: 0, no bone erosion; 1, mild surface erosion; 2, moderate surface erosion; and 3, strong surface erosion. Paw sections were also stained with safranin/fast green (SF/G; VWR International) to assess cartilage degradation by evaluating the presence and the intensity of red staining (corresponding to cartilage proteoglycans) using the following 0 to 2 scale: 0, normal; 1, mild loss; and 2, complete loss (26). Histopathological quantifications were made by two observers in a blinded manner from images of three fields representing the distal interphalangeal joint, the proximal interphalangeal joint, and joints in the carpal region.
Microcomputerized tomography analysis and tartrate-resistant acid phosphatase assay

Femurs were fixed in 4% paraformaldehyde and scanned with high-resolution microcomputerized tomography (micro-CT) scanner (SkyScan 1072; Antwerp, Belgium). In brief, image acquisition was performed at 45 kV/222 μA at resolution of 5.63 μm per pixel with step rotation of 0.9 degree for 180 degrees and step exposure of 2.87 s. Three-dimensional reconstructions were enabled by the three-dimensional creator software (NRecon v1.6.1.3) and CT-Analyzer [v18.1.2] supplied with the instrument. Trabecular bone indices such as bone volume/tissue volume (BV/TV), trabecular number ( Tb.N), and trabecular pattern factor (Tb.pf) were measured in the distal femur, 2.25 mm starting from growth plate.

Femurs were then decalcified in 10% EDTA and were embedded in paraffin and sliced at equivalent sections through the center of the bone. Sections were prepared and stained for tartrate-resistant acid phosphatase (TRAP) activity using the Sigma-Aldrich kit. The sections were counterstained with methyl green and examined histologically. Ten fields from each distal femur area were randomly chosen and observed microscopically at ×400 magnification, and the related pictures were analyzed by two observers in a blinded manner. Osteoclasts were identified by the red staining corresponding to TRAP activity. The number of TRAP+ cells per section was calculated by counting the osteoclasts present in the 10 fields analyzed for each femur. The mean values of TRAP+ cells per section were then calculated for the control IgG and anti-α2β1-treated groups. These experiments (micro-CT and TRAP) were carried out in the McGill Bone Center platform (McGill University, Montréal, QC).

Assessment of human IL-17 and RORc levels

These experiments were carried out as previously described (23). Human Th17 cells (2 × 10⁵ in 100 μl X-Vivo 15 medium) were activated in wells coated with anti-CD3 mAb and Coll I. The wells were first coated with 1 μg/ml anti-CD3 mAb overnight at 4°C. The wells were then washed three times with PBS and coated with 2 μg/ml Coll I or BSA (used as a control) for 2 h at 37°C, after which time they were washed three times with PBS. After activation, IL-17 production was measured by ELISA in cell-free supernatants from activated cells using specific ELISA kit (R&D Systems) according to the manufacturer’s instructions. IL-17 and RORc mRNA levels expressed by activated Th17 cells were evaluated by quantitative RT-PCR (qRT-PCR) as we previously described (23).

Immunoblotting and activation of MAPKs and AKT

Human Th17 cells (2 × 10⁵ in 100 μl X-Vivo 15 medium) were stimulated in wells coated with anti-CD3 mAb and Coll I. The wells were first coated with 1 μg/ml anti-CD3 mAb overnight at 4°C. The wells were then washed three times with PBS and coated with 2 μg/ml Coll I or BSA (used as a control) for 2 h at 37°C, after which time they were washed three times with PBS. After activation, IL-17 production was measured by ELISA in cell-free supernatants from activated cells using specific ELISA kit (R&D Systems) according to the manufacturer’s instructions. IL-17 and RORc mRNA levels expressed by activated Th17 cells were evaluated by quantitative RT-PCR (qRT-PCR) as we previously described (23).

Statistical analysis

Statistical analysis was performed by the Student t test (two-tailed, two-sample equal variance). Results with p values <0.05 were considered significant.

Results

α2β1 integrin blockade inhibits the development of CIA

To examine the role of α2β1 integrin in Th17 cells and in experimental RA, we first evaluated the effect of anti-α2β1 mAb targeting the α2 integrin chain (Hα1/29) on CIA in mice, a model of RA known to be driven by Th17 cells (11). The Hα1/29 mAb has previously been shown to have potent blocking activity in vivo (29, 30). Mice treated with the anti-α2β1 mAb showed a marked decrease in arthritis severity compared with the control mAb (IgG)-treated mice (Fig. 1A). By day 36, the arthritic clinical score was 64.2% inferior in the anti-α2β1 mAb-treated group compared with the control IgG-treated group (Fig. 1A).

H&E staining revealed a significant decrease in cellular infiltration and bone destruction in paws of anti-α2β1 mAb–treated animals in comparison with the control mAb-treated group (Fig. 1B, upper panel). Similarly, S/FG staining of joint tissues indicated that anti-α2β1 integrin mAb also reduced cartilage destruction, because the red staining is more intense in anti-α2β1 mAb–treated animals (Fig. 1B, lower panel). Quantification analysis indicated that anti-α2β1 integrin mAb treatment reduced inflammation by 62%, cartilage degradation by 56%, and bone destruction by 74% (Fig. 1C). Taken together, these results indicate that neutralization of α2β1 integrin with a specific mAb decreases arthritis severity in the CIA murine model.

α2β1 integrin is associated with Th17 cells from CIA mice, and its neutralization reduces Th17 cell numbers and activity in the joints

We have previously shown that α2β1 integrin is the major collagen-binding integrin expressed by human Th17 cells (23). To examine...
whether the protective effect of α2β1 integrin neutralization on the severity of CIA occurs via Th17 cells, we first evaluated whether α2 integrin can be associated with pathogenic Th17 cells in the CIA model. As shown in Fig. 2A and 2B, ∼40% of all CD4 T cells from the joints of CIA mice express α2 integrin. Furthermore, a significant proportion of Th17 cells (60%) also express α2 integrin (Fig. 2A, 2B). This is in contrast with CD4 T cells isolated from the spleen where only 10% of the total CD4 T cell population expressed α2 integrin (Fig. 2A, 2B). In addition, the proportion of IL-17+ cells expressing α2 integrin is higher on those isolated from the joints than from the spleen (Fig. 2B, right panel). The Th17 levels detected in the joints in our model are comparable with those reported by other investigators (27, 31). These results indicate that α2 integrin is associated with CD4 T cells from CIA mice and that the CD4/α2 integrin cell population is enriched in the joint tissue. This is in agreement with previous studies showing that collagen-binding integrins are expressed at higher levels in effector CD4 T cells found in extralymphoid tissues rather than those found in lymphoid organs and blood circulation (15, 16, 18).

We then examined the effect of anti-α2β1 mAb on Th17 cell activity in CIA mice. To this end, total cell suspensions (total cells) from joint tissues of control IgG and anti-α2β1 mAb-treated animals were characterized by flow cytometry. Double-staining analysis indicates that anti-α2β1 mAb treatment reduces the number of Th17 cells (CD4/IL-17+) in the joints of CIA mice (Fig. 3A). Treatment with anti-α2β1 mAb reduced by ∼40% the number of CD4 T cells, by 50% the number of IL-17+ cells, and by 39% the number of CD4/IL-17+ cells present in the joints (Fig. 3B).

We then tested whether anti-α2β1 mAb affected IL-17 protein levels. As expected, IL-17 production was reduced by 51% in the serum and by 65% in the paw joints of CIA mice treated with anti-α2β1 mAb in comparison with CIA mice treated with a control IgG (Fig. 3C). Together, these results show that α2β1 integrin neutralization inhibits Th17 cell numbers in the joints and reduces IL-17 levels in CIA mice.

α2β1 integrin blockade reduces bone damage in CIA mice

The results in Fig. 1 indicated that α2β1 integrin regulated bone destruction associated with CIA. Th17 cells have a major role in bone degradation associated with RA. IL-17 stimulates the development of osteoclasts, the cells responsible for bone resorption, by increasing the levels of RANKL by osteoblasts and other stromal cells (13, 32). Thus, we reasoned that if α2β1 integrin neutralization affected Th17 cell activity, it would also protect CIA mice from bone damage. Accordingly, we found that treatment of CIA mice with anti-α2β1 mAb inhibited by ∼45 and 65% the production of RANKL in sera and in the paw joints, respectively, in comparison with CIA mice treated with control IgG (Fig. 4A). This is consistent with the inhibitory effect of anti-α2β1 mAb on IL-17 levels (Fig. 3C). In addition, TRAP staining of distal femur sections indicated that CIA mice treated with anti-
The expression of α2β1 in autoimmune diseases and association with Th17 cells has not been previously addressed. Thus, to further examine the importance of α2β1 integrin in Th17 cells and in RA, we determined whether it is expressed on human RA synovial Th17 cells because of the limited quantities of synovial fluid, and thus because of the limited numbers of Th17 cells. We therefore used the model of human Th17 cells polarized from peripheral blood T cells in which we have shown that α2β1 integrin is the major collagen-binding integrin expressed and where its engagement with collagen enhanced TCR/CD3-mediated IL-17 production (23).

Integrin signaling is known to activate the MAPK pathways; therefore, we tested the implication of ERK, p38, and JNK in collagen co-stimulation of IL-17. To this end, we used specific inhibitors for each MAPK (U0126 for ERK; SB203580 for p38 and SP600125 for JNK). Treatment of Th17 cells with U0126 and SP600125 almost completely abolished TCR/CD3-(anti-CD3)-induced increase of IL-17 mRNA (Fig. 6A) and protein levels (Fig. 6B). Similarly, both U0126 and SP600125 inhibitors abolished the production of IL-17 in cells activated with anti-CD3+collagen. However, treatment of cells with the p38 inhibitor SB203580 had no effect on anti-CD3–induced IL-17 production but significantly reduced the costimulatory effect of collagen (Fig. 6A, 6B). No differences in cell viability and cell proliferation were noticed among the different samples after 24 h of treatment (data not shown). Immunoblot analysis showed that collagen alone increased ERK phosphorylation by 2.7-fold and also increased ERK phosphorylation in anti-CD3–activated Th17 cells by almost 2-fold (Fig. 6C, upper left panel). In contrast, collagen had no effect on JNK activation and did not costimulate anti-CD3–induced JNK phosphorylation (Fig. 6C, lower left panel). In summary, only the p38 pathway was costimulated by collagen.

α2β1 integrin costimulates IL-17 via ERK and p38 MAPKs and PI3K/AKT pathways

To gain more insights into how α2β1 integrin regulates Th17 cell activation, we investigated the signaling pathways involved in IL-17 production downstream of α2β1 integrin. We could not use human RA synovial Th17 cells because of the limited quantities of synovial fluid, and thus because of the limited numbers of Th17 cells. We therefore used the model of human Th17 cells polarized from peripheral blood T cells in which we have shown that α2β1 integrin is the major collagen-binding integrin expressed and where its engagement with collagen enhanced TCR/CD3-mediated IL-17 production (23).

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phosphorylation (Fig. 6C, upper right panel). Finally, we found that the TCR/CD3 complex is a weak inducer of p38 phosphorylation, and although collagen by itself did not stimulate p38 phosphorylation, it did increase by 2-fold its activity in anti-CD3–activated Th17 cells (Fig. 6C, lower panel). Together, these results indicate that JNK and ERK are essential for anti-CD3–induced IL-17 production, and that \( \alpha_2 \beta_1 \) integrin costimulates IL-17 production by increasing the ERK and p38 activities.

Similarly, we evaluated the implication of the PI3K/AKT pathway in the production of human IL-17. We found that the specific inhibitor LY294002 completely blocks the expression and production of IL-17 elicited by anti-CD3 and by anti-CD3+collagen (Fig. 7A, 7B). Collagen did not activate AKT but increased its phosphorylation in anti-CD3–activated Th17 cells (Fig. 7C), indicating that collagen could also costimulate IL-17 production via the PI3K/AKT pathway.

Collagen increases the levels of RORc in Th17 cells via ERK and PI3K/AKT pathways

RORc is the master regulator of IL-17 gene transcription and Th17 differentiation (33). Activation of Th17 cells increases RORc expression, which then enhances IL-17 gene transcription. We therefore sought to examine the regulation of RORc in human Th17 cells by collagen/\( \alpha_2 \beta_1 \) integrin signaling. A significant 1.5-fold increase is observed after 2 h of treatment and reaches a 2-fold increase between 4 and 8 h of treatment with anti-CD3 mAb (Fig. 8A). Collagen had no effect on RORc mRNA levels but significantly costimulated the anti-CD3 effect. The strongest effect of collagen (2-fold) is observed at 2–4 h and to a lesser extent at 8 h of anti-CD3 treatment (Fig. 8A). The costimulatory effect of collagen is blocked by an anti-\( \alpha_2 \beta_1 \) integrin mAb (data not shown), which is consistent with our previous study showing that the blocking anti-\( \alpha_2 \beta_1 \) integrin mAb inhibits the costimulatory effect of collagen on IL-17 production (23). The ERK and PI3K/AKT inhibitors abolished the expression of RORc elicited by anti-CD3 and anti-CD3+collagen (Fig. 8B). However, the JNK inhibitor, which also blocked IL-17 expression, and the p38 inhibitor, which blocked the costimulatory

**FIGURE 4.** Blockade of \( \alpha_2 \beta_1 \) integrin inhibits RANKL production, the number of osteoclasts, and trabecular bone loss. (A) RANKL levels in the sera and hind-paw lysates (joints) of control IgG and anti-\( \alpha_2 \beta_1 \)-treated CIA mice (n = 8) were measured by ELISA. (B) Representative TRAP staining (corresponding to osteoclasts) of distal femur sections from control IgG- and anti-\( \alpha_2 \beta_1 \)-treated CIA mice (n = 5) (upper panel). Quantification of osteoclast numbers in control IgG- and anti-\( \alpha_2 \beta_1 \)-treated CIA mice (n = 5) (lower panel). (C) Representative threedimensional reconstruction of micro-CT images of trabecular BV from control IgG- and anti-\( \alpha_2 \beta_1 \) mAb-treated CIA mice (upper panel). Histomorphometric analysis of trabecular BV from control IgG- and anti-\( \alpha_2 \beta_1 \)-treated mice (n = 5). BV/TV, Tb.N, and Tb.pf (lower panels). Data are the mean ± SEM. *p < 0.05.

**FIGURE 5.** \( \alpha_2 \beta_1 \) integrin is expressed on RA synovial Th17 cells. CD4 synovial T cells were activated with PMA+ionomycin for 6 h in the presence of brefeldin and stained with control isotypic Abs or with anti-\( \alpha_2 \beta_1 \) integrin mAb followed with intracellular staining with anti-IL-17 mAb as described in Materials and Methods. The cells were then analyzed by flow cytometry, and the percentage of cells in each quadrant is noted. (A) Flow cytometry analysis of a representative sample. (B) Numbers of \( \alpha_2 \beta_1 \) integrin/IL-17+ cells (%) from five different RA samples.
effect of collagen on IL-17 expression, had no effect (Fig. 8B).

Together, these results show that the ERK/MAPK and PI3K/AKT pathways are essential for RORc expression, and that collagen enhances RORc mRNA levels via the ERK and AKT pathways and independently from the p38 MAPK.

Discussion

Th17 cells are major effector cells in the pathogenesis of RA and other autoimmune diseases. However, the mechanisms regulating their activity are not fully understood. Growing evidence suggests that cell–ECM interactions could be an important factor in the regulation of T cell–mediated immunity and inflammation (15, 16). In this study, we have shown that targeting the type I collagen receptor, α2β1 integrin, reduces the severity of arthritis in the CIA murine model of RA by affecting Th17 cell activity. Our results demonstrate that α2β1 integrin is expressed on synovial Th17 cells, and collagen costimulation of IL-17 involves the PI3K/AKT pathway. Th17 cells were stimulated with 1 μg/ml coated anti-CD3 mAb in the presence or absence of 2 μg/ml coated collagen (Coll I). Specific inhibitors of MAPKs (U0126 [U] for the ERK inhibitor; SP600125 [SP] for the JNK inhibitor and SB203580 [SB] for the p38 inhibitor) were added at 10 μM for 1 h before cell stimulation. (A) IL-17 mRNA levels were determined after 6 h of activation by qRT-PCR, and the results are presented as fold increase normalized to β-actin levels. (B) The IL-17 protein levels were determined after 24 h of activation by ELISA assay. The results in (A) and (B) represent mean values (± SD) from five different experiments performed in triplicate with T cells isolated from five different blood donors. (C) Collagen activation of ERK, JNK, and p38 MAPKs. The cells were activated with coated anti-CD3 mAb, collagen I (Coll I), or anti-CD3+collagen for 1 h. The cells were lyzed, and activation of ERK (left upper panel), JNK (right upper panel), and p38 (lower panel) was determined by immunoblot analysis using specific Abs recognizing the phosphorylated forms. The blots were stripped and reprobed with anti–ERK-2 and anti–β-actin Abs as indicated to ensure equal loading. Representative immunoblots from three independent experiments are shown. The numbers under the bands represent fold increase of the ratio between total phospho-AKT and total AKT. *p < 0.05. NT, Nontreated cells.
cells from CIA mice, and its neutralization with a specific mAb reduces the number of Th17 cells infiltrating the joints and the levels of IL-17 in CIA mice. Blockade of α2β1 integrin led to reduction of synovial inflammation, cartilage destruction, and a reduction of RANKL levels, osteoclast numbers, and bone loss. Th17 cells play a central role in orchestrating the inflammatory response and tissue destruction in the joints, and the model of CIA is a Th17-driven disease (6, 10, 11, 13). Therefore, our results suggest that the observed inhibition of Th17 cell activity is likely to represent a major mechanism underlying the protective effect of anti-α2β1 mAb treatment on the severity of CIA.

We have recently reported that α2β1 integrin is the major collagen-binding integrin expressed on human Th17 cells polarized from naïve CD4 T cells, and that its ligation with collagen enhanced the production of IL-17 (23). A previous study using immunoblot analysis has reported that α2β1 can be expressed on human RA synovial T cells (34). In this study, and although we were limited in RA samples and synovial fluid quantities, we further show that α2β1 integrin is expressed on human RA synovial Th17 cells. This study also provides evidence for a pathophysiological role of α2β1 integrin in Th17 cell activity associated with a murine model of RA. Taken together, these results strongly support an important role for α2β1 integrin in Th17 cell activation and suggest that it can represent an important pathogenic pathway in RA.

Our results showed that α2β1 integrin controls both the number of Th17 cells in the joints and the levels of IL-17 in CIA mice. This could be explained by three nonexclusive mechanisms. First, α2β1 integrin can be important for the migration and retention of Th17 cells in the joint tissue. In the influenza infection animal model, α2β1 integrin was not found to be important for effector CD4 T cell migration to the lungs (35). However, α2β1 integrin seems to be necessary for the homing of CD4 effector/memory T cells to the bone marrow (36). These studies suggest that α2β1 integrin can regulate the migration of effector CD4 T cells to the tissues that are rich in collagen such as the bone marrow and the synovium. Accordingly, inhibition of Th17 cell migration to the joints of CIA mice is likely to represent one mechanism contributing to the function of α2β1 integrin in Th17 cells and in CIA pathogenesis. Inhibition of apoptosis is a hallmark in autoimmune disease and RA. We and others have previously demonstrated that α2β1 integrin engagement with collagen inhibits Fas-induced apoptosis of effector T cells (19, 22), suggesting the possibility that α2β1 integrin could promote Th17 cell survival in the arthritic joint tissue. Finally, α2β1 integrin could also costimulate Th17 cells; thus, its blockade could lead to the reduction of IL-17 levels observed in anti-α2β1 mAb-treated animals. This is supported by the fact that α2β1 integrin ligation with collagens, which are abundant in the synovium, significantly enhances the production of IL-17 by human Th17 cells (23). The fact that the anti-α1 integrin blocking Ab also reduced serum IL-17 levels in CIA mice is likely to be the consequence of the observed reduction of IL-17 in the joints.

A recent study using the α2β1 integrin knockout mice has shown that α2β1 integrin also regulates synovial fibroblast production of MMP-3 and subsequent cartilage destruction in RA animal models (37). IL-17 is known to promote cartilage degradation by enhancing the production by synovial fibroblasts and chondrocytes of various metalloproteinases involved in matrix breakdown (38, 39). Thus, α2β1 integrin is likely to contribute to cartilage destruction associated with arthritis by enhancing Th17 cell activity (this study) and through activation of synovial fibroblasts, further emphasizing the importance of the α2β1 integrin pathway in RA pathogenesis. In addition to cartilage degradation, our study showed that α2β1 integrin promotes bone loss associated with RA, which is a major factor in increased fractures observed in RA patients. Neutralization of α2β1 integrin reduced RANKL levels and osteoclast numbers, and protected the CIA mice from trabecular bone loss. Th17 cells are critical in the development of osteoclasts and bone erosion (13, 32). IL-17 increases the levels of RANKL, a crucial cytokine involved in the development of osteoclasts, which are responsible for bone erosion. Therefore, it is likely that the function of α2β1 in bone degradation occurs, at least in part, at the level of Th17 cell activation. In support, α2β1 integrin expression is not associated with neutrophils or the monocyctic/macrophage lineage (40–42), which constitutes the main source of osteoclast precursors. In addition, previous studies and recent evidence from the α2β1 integrin–deficient mice indicated that although osteoclasts may express α2β1 integrin, it seems that their function depends more on αvβ3 integrin rather than on α2β1 and other integrins (43, 44). In contrast, α2β1 integrin has been shown to regulate mast cell activation (45, 46), and mast cells have been associated with the pathogenesis of arthritis (47). Although mast cells are not required in the model of CIA used in this study (48), the earlier findings suggest that α2 integrin blockade can also affect mast cell activity during the course of arthritis and, therefore, contribute to the reduction of arthritis severity. Thus, although α2β1 integrin can contribute to RA by regulating other cell types such as fibroblasts but given the critical role of Th17 cells in RA and in the CIA model, our results indicate that the functional association of α2β1 integrin with Th17 cells is a major pathway.
involved in inflammation and tissue destruction associated with arthritis.

Blockade of α2β1 integrin using the same Ab as we did in this study also reduced the symptoms of experimental autoimmune encephalomyelitis (EAE) (29), an animal model of multiple sclerosis. However, that study did not investigate the mechanisms underlying the protective effect of anti-α2β1 mAb treatment. Because EAE and multiple sclerosis in humans is Th17-based diseases, it is possible that blockade of α2β1 integrin could have blocked EAE symptoms by blocking Th17 cell activity as in the CIA model (this study).

Finally, to further understand the function of α2β1 integrin in Th17 cells, we undertook in vitro experiments to decipher the signaling pathways activated by α2β1 integrin. We found that ligation of α2β1 integrin with collagen promotes IL-17 production by enhancing the activation of ERK and p38 MAPKs and PI3K/AKT pathways. Collagen also increased the expression of RORα, which occurred through ERK and AKT pathways independently from the p38 MAPK.

Interestingly, ERK, p38, and AKT have been involved with the development of Th17 cells in mice, and inhibition of p38 and ERK pathways attenuated autoimmune diseases such as EAE and colitis, respectively (49–53). Together, these studies further support our findings in human Th17 cells and reinforce the importance of α2β1 integrin in Th17 cell activation and in the development of CIA. The fact that α2β1 integrin has been shown to regulate the development of EAE (29) suggests that it can do so by increasing the production of IL-17 through ERK, p38, and AKT pathways, which may also operate downstream of α2β1 integrin in CIA. Although additional work is needed to understand how these signaling pathways regulate Th17 cells and autoimmune diseases, our findings with human polarized Th17 cells argue in favor of targeting these pathways along with α2β1 integrin for the treatment of RA and other Th17-dependent diseases. However, although our study shows the importance of α2β1 integrin in the development of arthritis, it is unclear whether its neutralization will provide clinical benefit in arthritis and other inflammatory diseases. Given the complexity of ECM and their receptors, it is possible that additional β1 or β3 integrin pathways might compensate for its inhibition.

In summary, to our knowledge, our study provides the first evidence that α2β1 integrin is expressed on human RA synovial Th17 cells and that it regulates the development of inflammatory diseases (CIA) by targeting Th17 cells, suggesting that α2β1 integrin can represent an important pathogenic pathway in RA. Further understanding of the mechanisms by which α2β1 integrin regulates Th17 cell activation and RA is likely to lead to new insights in autoimmunity and RA pathogenesis, and to the development of new therapeutic avenues.

Disclosures

The authors have no financial conflicts of interest.

References


