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The Novel Role of IL-7 Ligation to IL-7 Receptor in Myeloid Cells of Rheumatoid Arthritis and Collagen-Induced Arthritis

Zhenlong Chen,*1 Seung-jae Kim,*1 Nathan D. Chamberlain,* Sarah R. Pickens,* Michael V. Volin,† Suncica Volkov,* Shiva Arami,* John W. Christman,‡ Bellur S. Prabhakar,§ William Swedler,* Anjali Mehta,* Nadera Sweiss,* and Shiva Shahrara*

Although the role of IL-7 and IL-7R has been implicated in the pathogenesis of rheumatoid arthritis (RA), the majority of the studies have focused on the effect of IL-7/IL-7R in T cell development and function. Our novel data, however, document that patients with RA and greater disease activity have higher levels of IL-7, IL-7R, and TNF-α in RA monocytes, suggesting a feedback regulation between IL-7/IL-7R and TNF-α cascades in myeloid cells that is linked to chronic disease progression. Investigations into the involved mechanism showed that IL-7 is a novel and potent chemoattractant that attracts IL-7R+ monocytes through activation of the PI3K/AKT1 and ERK pathways at similar concentrations of IL-7 detected in RA synovial fluid. To determine whether ligation of IL-7 to IL-7R is a potential target for RA treatment and to identify their mechanism of action, collagen-induced arthritis (CIA) was therapeutically treated with anti–IL-7 Ab or IgG control. Anti–IL-7 Ab treatment significantly reduces CIA monocyte recruitment and osteoclast differentiation as well as potent joint monocyte chemoattractants and bone erosion markers, suggesting that both direct and indirect pathways might contribute to the observed effect. We also demonstrate that reduction in joint MIP-2 levels is responsible for suppressed vascularization detected in mice treated with anti–IL-7 Ab compared with the control group. To our knowledge, we show for the first time that expression of IL-7/IL-7R in myeloid cells is strongly correlated with RA disease activity and that ligation of IL-7 to IL-7R contributes to monocyte homing, differentiation of osteoclasts, and vascularization in the CIA effector phase.

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studies suggest that IL-7/IL-7R–mediated osteoclastogenesis in RA could extend beyond their role in T cells and have other critical implications in myeloid cells (16, 17).

Based on the significant elevation of IL-7 and IL-7R in RA ST and fluid macrophages (5), the ability of IL-7 to induce potent proinflammatory cytokines from myeloid cells (15), and the role of IL-7 in modulating differentiation of RA synovial fluid myeloid cells to mature osteoclasts (16, 17), we examined the significance of IL-7 ligation to IL-7R on myeloid cells. We also investigated whether expression of IL-7/IL-7R in RA blood myeloid cells is linked to TNF-α and disease activity levels.

In this study, we found that in RA blood monocyes, concentrations of IL-7, IL-7R, and TNF-α are closely correlated with each other and disease activity, suggesting that activation of IL-7/IL-7R cascade plays a crucial role in myeloid cell–mediated RA pathogenesis. The pathogenic role of IL-7R ligation was demonstrated by showing that IL-7 is a novel and potent monocyte chemoattractant that attracts IL-7R+ blood CD14+CD16+ monocyes via PI3K/AKT1 and ERK pathways. In collagen-induced arthritis (CIA), a chronic model of RA, we demonstrate that blockade of endogenous IL-7 relieves arthritis by markedly reducing joint monocyte homing, bone erosion, and vascularization either directly or indirectly through decreasing proinflammatory monocye chemoattractants (TNF-α and CCL2/MCP-1), markers of bone erosion (RANKL and Cathepsin K), and proangiogenic factor (MIP-2/CCL8). To our knowledge, these results suggest for the first time that ligation of IL-7 to IL-7R can affect myeloid cell migration and function, as well as have a positive feedback regulation with TNF-α production from these cells, leading to it being a potential therapeutic target in RA.

Materials and Methods

Monocyte chemotaxis

Experiments were performed to determine the direct effect of IL-7 on monocye chemotaxis. Mononuclear cells were isolated by Histopaque (Sigma-Aldrich) gradient centrifugation. Subsequently, monocyes were isolated from normal (NL) or RA peripheral blood (PB) using negative selection kit for CD14+CD16+CD16- (StemCell Technology, Vancouver, Canada; cat #19035; used for all monocyte chemotaxis experiments) and CD14+CD16- (StemCell Technology; cat #19019 used only in Fig. 2C) according to the manufacturer’s instructions (5, 20). Chemotaxis was performed in a Boyden chamber (Neuroprobe; Gaithersburg, MD) using NL monocyes for 2 h with IL-7 (R&D Systems, Minneapolis, MN) concentrations varying from 0.0001 to 100 ng/ml; FMLF (1 μM; Sigma Aldrich) was used as positive control, and PBS was used as negative control (21, 22).

To determine whether blockade of IL-7R on monocyes would inhibit IL-7–induced monocye chemotaxis, monocyes were blocked with anti-IL-7R Ab (10 μg/ml; R&D Systems), or isotype control for 1 h prior to performing the monocye chemotaxis in response to 10 and 50 ng/ml IL-7 for 2 h.

To determine the contribution of monocye subtypes (CD16+ versus CD14+CD16+CD16-; StemCell Technology) to IL-7– and 50 ng/ml IL-7 for 2 h–mediated monocye cell infiltration, in vitro chemotaxis was compared between CD14+CD16+CD16- and CD14+CD16- cells.

To determine signaling pathways associated with IL-7–induced monocye chemotaxis, monocyes were treated with 1 and 5 μM inhibitors to ERK (U0126) and PI3K/AKT (LY249024) and STAT3 (WP1066) and 10 and 50 μM for STAT5 (735108 STAT5 inhibitor; EMD Millpore; Billerica, MA) for 1 h. Subsequently, monocye chemotaxis was performed in response to 10 ng/ml of IL-7 for 2 h.

To demonstrate that inhibition of PI3K/AKT1 is specific to monocye migration mediated by IL-7, extravasation of monocyes pretreated with DMSO or PI3K inhibitor (LY249024; 5 μM) was examined in response to potent monocye chemoattractant such as FMLF (1 μM) as well as CCL2 (0.9 nm; R&D Systems), CCL5 (1.01 nm; R&D Systems), IL-17 (0.667 nM; R&D Systems) or IL-7 (0.58 nM). To validate that phosphorylated MAPK promotes CCL2, CCL5, and IL-17 but not IL-7–induced monocye cell infiltration, an inhibitor to p38 (SB203580; 5 μM) was included in these experiments.

To show that RA synovial fluid–mediated monocye chemotaxis is in part due to IL-7 function, 12 synovial fluids were diluted (1:20) and neutralized with anti-IL-7 Ab (10 μg/ml; R&D Systems) or control IgG. To demonstrate that RA synovial fluid monocye trafficking is mediated through IL-7 ligation to myeloid IL-7R, cells were incubated with Ab to IL-7R (10 μg/ml; R&D Systems) or IgG control for 1 h prior to performing monocye chemotaxis in response to 6 RA synovial fluids (1:20 dilution) for 2 h.

To validate that IL-7–mediated monocye chemotaxis is promoted through activation of the AKT pathway, NL monocyes were transfected with control (Ctl) or dominant negative (DN)-AKT plasmid (a gift from Dr. B.S. Prabhakar’s laboratory) (23) at 2.5 μg for 48 h. Cells were either untreated or stimulated with 100 ng/ml of IL-7 for 30 and 60 min prior to Western blotting. After demonstrating that DN-AKT significantly suppresses IL-7–mediated AKT1 phosphorylation, chemotaxis of NL monocyes transfected with Ctl or DN-AKT was examined in response to 10 ng/ml IL-7.

To confirm that ERK activation contributes to IL-7–mediated monocye cell infiltration, THP-1 cells (American Type Culture Collection, Manassas, VA) were transfected with 100 nM scrambled or ERK siRNA (Dharmacon, Thermo Scientific, Waltham, MA) for 48 h according to the manufacturer’s instructions. Thereafter, transfected THP-1 cells were probed for ERK and actin. Next, chemotaxis of control or ERK knockdown THP-1 cells was examined in response to 10 ng/ml IL-7.

RA patient population

RA specimens were obtained from patients with RA, diagnosed according to the 1987 revised criteria of the American College of Rheumatology (24). PB was obtained from 76 patients, 71 women, and 5 men (mean age, 48.2 ± 15.3 y). At the time of evaluation, patients received no treatment (n = 7, all women; mean age, 53.1 ± 19.5 y); treatment with nonbiological disease-modifying antirheumatic drugs (DMARDs; methotrexate, leflunomide, sulfasalazine azathoprine, hydroxychloroquine, or minocycline) alone (n = 29; 26 women and 3 men; mean age, 52.0 ± 16.0 y; of which two received hydroxychloroquine only [both women, 62 y old]; treatment with DMARDs plus prednisone (n = 7, all women; mean age, 54.7 ± 12.7 y), DMARDs plus rituximab (n = 1 woman, age 52 y); TNF-α inhibitor alone (n = 7; 6 women, 1 man; mean age, 38.3 ± 8.9 y), with prednisone (n = 1 woman, age 48 y), with DMARDs (n = 17, all women; mean age, 45.6 ± 12.6 y), with a DMARD plus prednisone (n = 5, 4 women, and 1 man, average age 37 ± 18.1; or anti–T cell therapy (Abatacept) with a DMARD (n = 1 woman, age 41 y) or with DMARD and prednisone (n = 1 woman, age 28 y).

These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors gave informed written consent. The number of patients was 76, but refer to the figure legends for the exact number of patients in each experiment.

Cytokine quantification

Human IL-7 (R&D Systems) was quantified by ELISA in STs from patients with RA or osteoarthritis (OA) or from NL samples, as well as synovial fluids (SFs) from patients with RA or OA according to the manufacturers’ instructions. Mouse IL-6, IL-1β, IL-17, TNF-α, CCL2/MCP-1, CCL5/RANTES, CXCL1, Ang-1, bFGF, VEGF, and MIP-2 ELISA Kit (R&D Systems) was used according to the manufacturer’s instructions, and the expression level of each factor was normalized to the ankle protein concentration.

IL-7 signaling in human peripheral blood monocyes

Monocyes were untreated or treated with IL-7 (10 ng/ml) for 15–65 min. Cell lysates were examined by Western blot analysis as described previously (22, 25). Blots were probed with p-ERK, p-AKT1, p-STAT1, p-STAT3, or p-STAT5 (Cell Signaling; 1:1000 dilution) overnight and after stripping, were probed with ERK, AKT, STAT3, or actin (Cell Signaling or Sigma-Aldrich; 1:3000 dilution).

Study protocol for CIA and anti–IL-7 treatment

The animal studies were approved by the Institutional Animal Care and Use Committee. DBA/1J mice (7–8 wk old; Jackson Laboratories, Bar Harbor, ME) were immunized with collagen on days 0 and 21. Bovine collagen type II (2 mg/ml; Chondrex, Redmond, WA) was emulsified in equal volumes of CFA (2 mg/ml; Chondrex). The DBA/1J mice were immunized s.c. in the tail with 100 μl emulsion. On day 21, mice were injected intraperitoneally with 100 μl collagen type II (2 mg/ml) emulsified in equal volumes of IFA (25). CIA mice were treated with IgG or anti–IL-7 (100 μg/injection; R&D Systems) Ab i.p. on days 26, 29, 33, 36, 40, and 42. Changes in ankle circumference (in millimeters) were recorded (n = 10–11

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mice). Mice were sacrificed on day 43, and ankles were harvested for protein and mRNA extraction or histologic studies. Serum was saved for laboratory tests. In a separate study, CIA ankle joints were harvested on day 45 and were compared with control mice that were not induced by CIA for IL-7R immunostaining and quantifying joint IL-7 by ELISA.

**Clinical assessments**

Ankle circumferences were determined by measuring two perpendicular diameters, including the laterolateral diameter and the anteroposterior diameter, as measured with a caliper (Lange Caliper; Cambridge Scientific Industries). Circumference was determined using the following formula: 

\[ C = 2\pi \times \sqrt{\frac{a^2 + b^2}{2}} \]

where \( C \) is the circumference and \( a \) and \( b \) represent the diameters (25). Ankle circumference evaluations were performed on days 3, 7, 10, 14, 17, 20, 23, 26, 28, 30, 33, 35, 37, 40, 41, and 42.

**Abs and immunohistochemistry**

The ankles of mice were fixed in formalin, decalcified, embedded in paraffin, and sectioned. Inflammation, synovial lining, and bone erosion (based on a score of 0–5) were determined using H&E-stained sections by two masked observers. Mouse ankles were stained with immunoperoxidase using Vector Elite ABC Kits (Vector Laboratories), with diaminobenzidine (Vector Laboratories) as a chromogen. Slides were deparaffinized in xylene for 15 min at room temperature, followed by rehydration by transfer through graded alcohols. Ags were unmasked by incubating slides in proteinase K digestion buffer (0.05 M; Dako) for 5 min at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% \( \text{H}_2\text{O}_2 \) for 5 min. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Dako). Nonspecific binding of Abs to the tissues was blocked by pretreatment of tissues with Protein Block (Dako). Sham injection or CIA tissues were immunostained with IL-7R (1:100 dilution; Santa Cruz Biotechnology). Additional CIA mice treated with IgG or anti–IL-7 were stained with F480 (1:100 dilution; Serotec), von Willebrand factor (1:1000 dilution; Dako), CD3 (1:100 dilution; Abcam), or control IgG Ab (Beckman Coulter). Positive immunostaining was scored on a 0–5 scale; score data were pooled, and the mean ± SEM was calculated in each data group. Each slide was evaluated by two masked observers.

To exclude the chance of ambiguous cells migrating in response to IL-7, cells attracted to chemotaxis polycarbonate membrane were stained with anti-CD68 Ab. For this purpose, membranes harvested from NL monocytes migrated in response to PBS, fMLF (1 μM) and IL-7 (10 ng/ml) were fixed, stained with mouse anti-human CD68 (1:100 dilution; Dako), and visualized with goat anti-mouse Ab labeled with Alexa 594 (1:500 dilution; Molecular Probes, Eugene, OR). The number of cells migrated was determined in 15 high-powered fields based on double-positive DAPI (blue nuclear staining) and CD68 (red) staining.

**Quantification of hemoglobin in the ankles of mice**

Using methemoglobin (Sigma-Aldrich), serial dilutions were prepared to generate a standard curve from 70 to 1.1 g/dl (25–28). Fifty microliters of the ankles of mice or standard were added to a 96-well plate in duplicate, and 50 μl tetramethylbenzidine (Sigma-Aldrich) was added to each sample. The absorbance at 650 nm was measured.

**FIGURE 1.** IL-7 and IL-7R expression correlates with DAS28 and TNF-\( \alpha \) levels in RA monocytes, and ligation of RA synovial fluid IL-7 to myeloid IL-7R promotes chemotaxis. Linear regression analysis was used to compare expression of TNF-\( \alpha \) with IL-7 (\( n = 76 \)) (A), IL-7R (\( n = 76 \)) (B), mRNA levels, and DAS28 (\( n = 76 \)) (C) in RA monocytes. Correlation was also calculated for DAS28 score (\( n = 76 \)) and expression levels of IL-7 (D) and IL-7R (E) in RA monocytes. The mRNA expression levels in RA monocytes are shown as fold increase above NL PB monocytes and are normalized to GAPDH. (F) Concentration of IL-7 was quantified by ELISA in RA, OA, and NL STs (\( n = 10 \)) as well as in OA SF (\( n = 18 \)). (G) Twelve RA SFs were preincubated with anti–IL-7 Ab (10 μg/ml) or control IgG for 1 h prior to performing monocyte chemotaxis in response to the RA SFs. (I) Monocytes were incubated with Ab to IL-7R (10 μg/ml) and IgG control for 1 h prior to performing monocyte chemotaxis in response to six RA SFs. Values demonstrate mean ± SE. *\( p < 0.05 \).
ple. The plate was allowed to develop at room temperature for 15–20 min with gentle shaking, and the reaction was terminated with 50 μl 2 N H2SO4 for 3–5 min. Absorbance was read with an ELISA plate reader at 540 nm. To calculate hemoglobin concentrations in the mice ankles, the values (grams per deciliter) were normalized to the weights of the ankles (milligrams per milliliter).

Flow cytometry analysis of CD3, CD4, TH-1, and TH-17 cells in CIA splenocytes

To quantify the percent CD3+ and CD4+ cells in CIA splenocytes, cells were washed, blocked using anti-mouse CD16/CD32, and stained with allophycocyanin-conjugated anti-CD3 or anti-CD4 Abs (eBioscience, San Diego, CA). To determine whether the percentage of TH-1 or TH-17 cells were effected by anti-IL-7 Ab treatment in CIA mice, splenocytes were stimulated with PMA (5 ng/ml) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of Brefeldin A (3 μg/ml; Sigma-Aldrich) for 4 h. Cells were then washed, blocked using anti-mouse CD16/CD32, and stained with APC-conjugated anti-CD4 Abs (eBioscience, San Diego, CA). Following CD4 staining, cells were blocked, fixed, and permeabilized using an IC-Flow Kit according to the manufacturer’s instructions (Imgenex, San Diego, CA). The percentages of TH-1– and TH-17–producing cells were determined by staining the splenocytes with FITC- or PE-labeled anti–IFN-γ or PE-labeled anti–IL-17 Abs (eBioscience), and the data were analyzed by flow cytometry (Beckman Coulter Cyan ADP).

Real-time RT-PCR

Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from the ankle homogenates. Subsequently, reverse transcription and real-time RT-PCR were performed to determine the expression of IL-7, IL-7R, and TNF-α levels in RA monocytes as well as RANKL and cathepsin K expression levels in CIA ankle joints as described previously (21, 22, 25). Relative gene expression was determined using the ΔΔCt method, and results were expressed as fold increase above conditions indicated in the figure legends.

**FIGURE 2.** IL-7 induces monocyte migration through ligation to IL-7R and activation of AKT1/PI3K and ERK pathways. (A) IL-7 monocyte chemotaxis was performed in a Boyden chemotaxis chamber with varying concentrations of IL-7 (n = 3). (B) Monocytes were incubated with anti–IL-7R Ab (10 μg/ml) or control IgG for 1 h; thereafter, chemotaxis was performed in response to IL-7 (10 and 50 ng/ml; n = 3). (C) Chemotaxis was performed using monocytes negatively selected for CD14+CD16−/− and CD14+CD16+ in response to IL-7 (1 and 50 ng/ml; n = 3). (D) To determine the mechanism of IL-7 in monocytes, cells were stimulated with IL-7 (100 ng/ml) for 0–65 min, and the cell lysates were probed for p-ERK, p-AKT1 and p-STAT1, and p-STAT3 and STAT5 (n = 3). (E) To examine signaling pathways associated with IL-7 monocyte migration, cells were preincubated with 1 and 5 μM of the identified chemical inhibitors for ERK (U0126), PI3K (LY294002; LY), and STAT3 (WP1066; WP) or 10 and 50 μM of STAT5 inhibitor (S5I) prior to performing chemotaxis in the Boyden chamber (2 h; n = 3). (F) Monocytes were treated with DMSO or inhibitors for PI3K/AKT (LY294002; 5 μM) and p38 (SB203580; 5 μM) to examine the impact of these pathways on fMLF (1 μM) as well as of CCL2 (0.9 nM), CCL5 (1.01 nM), IL-17 (0.667 nM), or IL-7 (0.58 nM)–induced monocyte migration (n = 3–5). Values demonstrate mean ± SE.*p < 0.05.

Tartrate-resistant acid phosphatase staining

Tartrate-resistant acid phosphatase (TRAP) staining was performed using an Acid Phosphatase Leukocyte Kit (Sigma-Aldrich) in paraffin-embedded mice ankles according to manufacturer’s instructions. Next, TRAP+ cells stained in CIA mouse ankles were scored on a scale of 0–5: 0 = no staining, 1 = few or rare positive cells, 2 = scattered staining, 3 = multiple foci of positive cells, 4 = clusters of positive cells, and 5 = diffuse staining (29).

**Statistical analysis**

The data were analyzed using one-way ANOVA followed by a post hoc two-tailed Student t test for paired and unpaired samples using GraphPad and Microsoft Excel. In RA monocytes, expression levels of IL-7, IL-7R, and TNF-α were correlated with each other and disease activity score based on 28 defined joints (DAS28) score using linear regression analysis in Excel. The p values < 0.05 were considered significant.

**Results**

**In RA monocytes, IL-7 and IL-7R correlate with DAS28 score and TNF-α levels**

Because expression of IL-7 and IL-7R is elevated in RA compared with NL monocytes (5), and because IL-7–activated monocytes can produce TNF-α (15), we asked whether there is a relationship between IL-7/IL-7R cascade with TNF-α and DAS28 score in RA monocytes. We found that the levels of IL-7 (R2 = 0.51) and IL-7R (R2 = 0.85) are closely correlated with TNF-α in RA monocytes (Fig. 1A, 1B). Furthermore, patients with greater levels of DAS28 had increased expression of IL-7 (R2 = 0.55), IL-7R (R2 = 0.56; Fig. 1D, 1E), and TNF-α (R2 = 0.57; Fig. 1C) in RA monocytes. We noticed that there were two groups of RA patients, in regard to myeloid IL-7 expression, that included groups below and above the 50-fold range. Therefore, in addition to linear regression...
analysis, the data were reevaluated using nonlinear curve analysis. Interestingly the nonlinear regression analyses for TNF and IL-7 ($R^2 = 0.60$; $p = 1.53 \times 10^{-16}$), DAS28 and IL-7 ($R^2 = 0.60$; $p = 1.61 \times 10^{-16}$) and DAS28 and IL-7R ($R^2 = 0.61$; $p = 1.01 \times 10^{-16}$) were still able to demonstrate a strong correlation between these factors. These results suggest that elevated levels of IL-7/IL-7R can predict higher RA disease activity and that there is a positive feedback regulation between IL-7/IL-7R and TNF-α pathways by producing and responding to TNF-α. 

IL-7 and IL-7R play an important role in RA synovial fluid–mediated monocyte trafficking

Because RA ST and fluid expressed significantly higher levels of IL-7 compared with OA or NL ST and fluid (Fig. 1F, 1G), experiments were performed to determine whether the IL-7 identified in RA synovial fluid was chemotactic for monocytes. Neutralization of IL-7 significantly reduced (40%; $p < 0.05$) monocyte chemotaxis compared with control IgG-treated RA synovial fluids (Fig. 1H). In addition, blockade of IL-7R on monocytes was effective in suppressing RA synovial fluid–mediated monocyte migration (Fig. 1I). These results indicate that IL-7 present in RA synovial fluid attracts circulating IL-7R+ monocytes into the joints.

Ligation of IL-7 to IL-7R contributes to monocyte trafficking through activation of PI3K/AKT1 and ERK pathways

IL-7 contributes to myeloid cell trafficking into the RA joints; therefore, the involved mechanism was examined next. IL-7 was chemotactic for monocytes at concentration as low as 0.1 ng/ml ($n = 3$; Fig. 2A). The mean concentration of IL-7 in the 18 RA synovial fluids analyzed was $138 \pm 19$ pg/ml (up to 414 pg/ml; Fig. 1G), a value that was highly chemotactic (Fig. 2A). Furthermore, blockade of IL-7R on monocytes suppressed IL-7–mediated chemotaxis (Fig. 2B), suggesting that monocyte recruitment occurs through direct ligation of IL-7 to IL-7R on these cells. To further demonstrate which subtypes of monocyte participate in IL-7–mediated monocyte trafficking into the RA joints, we examined the chemotactic effect of IL-7 on different monocyte subsets. We found that ERK, AKT1, STAT3, and STAT5 but not STAT1 were phosphorylated by IL-7 activation.

**FIGURE 3.** Inhibition of AKT and ERK cascades suppresses IL-7–mediated monocyte homing. As with RA, IL-7R is expressed in CIA ST lining and sublining macrophages and sublining endothelial cells. (A) NL monocytes were transfected with Ctl or DN-AKT plasmid at 2.5 μg for 48 h. Cells were then either untreated or stimulated with 100 ng/ml of IL-7 for 30 and 60 min and probed for p-AKT, AKT, and actin ($n = 3$). (B) Migration of Ctl or DN-AKT–transfected NL monocytes was examined in response to 10 ng/ml IL-7 or PBS, and response to fMLF was tested only in Ctl-transfected monocytes ($n = 3$). (C) THP-1 cells were transfected with 100 nM scrambled (ctl si) or ERK siRNA (ERK si) for 48 h. Subsequently, transfected cells were probed for ERK and actin ($n = 3$). (D) Migration of THP-1 Ctl or ERK knockdown cells was examined in response to 10 ng/ml IL-7 or PBS and the ability of fMLF to attract cells was examined only in control knockdown cells ($n = 3$). (E) Polycarbonate membranes harvested from NL monocytes that migrated in response to PBS, fMLF (1 μM), or IL-7 (10 ng/ml) were fixed, stained with anti-CD68 Ab (1:100 dilution; original magnification ×400), and visualized with a secondary Ab labeled with Alexa 594 (1:500 dilution; original magnification ×400). (F) The total number of migrated cells was quantified in 15 high-powered fields based on double-positive DAPI (blue) and CD68 (red) staining. (G) Ankles harvested from PBS Ctl or CIA-induced mice were stained with anti–IL-7R Ab (original magnification ×200). (H) IL-7R positive immunostaining was scored on a 0–5 scale on ST lining and sublining macrophages (mac) as well as sublining endothelial cells (Endo; $n = 5$–7; original magnification ×200). (I) IL-7 levels were determined in ankles harvested from PBS-injected control or CIA mice by ELISA ($n = 5$). Values demonstrate mean ± SE. *$p < 0.05$. 

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in monocytes (Fig. 2D). To determine which of these pathways might contribute to IL-7–mediated chemotaxis, monocytes were preincubated with inhibitors of the ERK, PI3K/AKT1, STAT3, and STAT5 before performing the chemotaxis. Although inhibition of STAT3 and STAT5 pathways was ineffective, inhibition of the PI3K/AKT1 and ERK cascades significantly reduced IL-7–induced monocyte recruitment (Fig. 2E). We also demonstrate that inhibition of PI3K/AKT1 is specific to monocyte migration mediated by IL-7, because suppression of this pathway did not affect extravasation induced by other potent monocyte chemoattractants including CCL2/MCP-1, CCL5/RANTES, IL-17, or fMLF (Fig. 2F). We also show that when monocyte chemoattractants are used at the similar concentration, IL-7 is at least as potent as CCL2/MCP-1 in attracting monocytes (Fig. 2F). Although CCL2-, CCL5-, and IL-17–induced (21) myeloid cell migration is promoted through p38 (Fig. 2F), this pathway is not activated by IL-7 in myeloid cells, suggesting that the IL-7 mechanism of action does not overlap with other monocyte chemoattractants.

To eliminate the possible nonspecific effect of kinase inhibitors, significance of AKT and ERK activation was assessed on IL-7 monocyte recruitment using DN-AKT vector or ERK siRNA. We demonstrate that expression of DN-AKT in normal myeloid cells was capable of markedly reducing AKT1 phosphorylation and monocyte infiltration mediated by IL-7, whereas the control had no effect on these two functions (Fig. 3A, 3B). Next, we confirm that IL-7–induced THP-1 chemotaxis is significantly suppressed by ERK compared with the control knockdown (Fig. 3C, 3D).

To document that cells migrating in response to IL-7 are monocytes rather than other ambiguous cells, we demonstrate that IL-7 can strongly attract THP-1 cells, a human monocytic cell line (Fig. 3D). We also show that similar to fMLF, cells attracted to IL-7 on the chemotaxis membrane are 99.02% positive for CD68 (based on colocalization of CD68+ cells with DAPI nucleus staining; Fig. 3E, 3F). Collectively, our results demonstrate that IL-7 can strongly recruit monocytes through ligation of IL-7R and activation of PI3K/AKT1 and ERK but not STAT pathways.

IL-7 and IL-7R expression levels are greatly elevated in CIA ankle joints and anti–IL-7 therapy reduces joint inflammation and bone destruction

To evaluate the mechanism by which IL-7/IL-7R induces RA pathogenesis, an experimental arthritis model was used. We show that, as in RA, IL-7R is significantly elevated in the lining and sublining macrophages as well as sublining endothelial cells in CIA compared with PBS-treated ankles (Fig. 3G, 3H). In addition, CIA mice produced 3-fold higher joint IL-7 levels compared with the control group (Fig. 3I). Therefore, to examine the role of IL-7/IL-7R in CIA pathology, CIA mice were treated therapeutically with anti–IL-7 Ab or IgG control (Fig. 4A, 4B) starting on day 26 after CIA induction. These studies demonstrated that anti–IL-7 Ab treatment significantly reduced joint inflammation on days 33, 37, 40, 41, and 42 after CIA induction compared with the control group; however, there were no differences detected between the two treatment groups on days 28 and 30 (Fig. 4A). We document that blockade of IL-7 was also capable of reducing CIA synovial inflammation (40%), joint lining thickness (45%), and erosion (40%) compared with the control treated mice (Fig. 4B, 4C). Consistent with the histologic studies, we demonstrate that TRAP+...
cells are markedly higher in control mice (45%) compared with anti–IL-7 Ab treatment group (Fig. 4D, 4E). We next show that bone loss detected in anti–IL-7 Ab-treated CIA mice is due to significant decrease in key bone erosion markers including RANKL (3-fold) and cathepsin K (10-fold) expression (Fig. 4F) compared with control mice. These results indicated that IL-7 has a crucial role in CIA disease progression and bone erosion; therefore, subsequent studies were performed to identify the cell types and the mechanism by which IL-7 promotes disease.

Anti–IL-7 Ab therapy reduces joint TNF-α and CCL2/MCP-1 levels and monocyte trafficking in CIA mice

Based on the IL-7/IL-7R feedback regulation with TNF-α shown by us (5) and others (13) and the ability of IL-7 to induce monocyte migration, we asked whether joint TNF-α or other potent monocyte chemoattractants were affected by anti–IL-7 therapy in CIA. We found that joint TNF-α (Fig. 5A) as well as ankle and serum levels of CCL2/MCP-1 (Fig. 5B) were 2-fold higher in the IgG group compared with anti–IL-7 Ab-treated CIA mice; however, concentration of joint CCL5/RANTES (Fig. 5C) was not significantly different in the two treatment groups. Because of these findings, we next examined the role of IL-7 in CIA-mediated monocyte migration. We found that anti–IL-7 Ab treatment significantly reduced monocyte joint recruitment compared with control group (Fig. 5D, 5E). This finding may be due to disruption in joint IL-7 ligation to IL-7R on monocytes and their homing into the inflammatory site or indirectly because of reduced potent monocyte chemoattractant such as TNF-α and CCL2. It is also possible that both mechanisms of action contribute to this detected effect. Collectively, these results suggest that like in RA, IL-7 plays a key role in myeloid cell trafficking and function in CIA joint.

**FIGURE 5.** Neutralization of IL-7 reduces potent monocyte chemoattractants and CIA monocyte homing. Changes in TNF-α (A), CCL2 (B), and CCL5 (C) expression levels in ankle homogenates (n = 7) and sera (n = 10) from CIA mice treated with IgG control or anti–IL-7 Ab were determined by ELISA. (D) STs from CIA mice treated with IgG or anti–IL-7 Ab were harvested on day 43 and immunostained with anti-F480 Ab (original magnification × 200). Arrows demonstrate F480+ cells. (E) Macrophage staining was quantified on a 0–5 scale (n = 5–7). Values are mean ± SE. *p < 0.05.

Anti–IL-7 treatment reduced CIA vascularization, joint MIP-2, and hemoglobin levels compared with control treatment

Because we have demonstrated previously that macrophages and endothelial cells stimulated with IL-7 could produce a number of proangiogenic factors (5), the effect of anti–IL-7 therapy was examined on CIA vascularization and joint proangiogenic factors. We show that anti–IL-7 Ab treatment in CIA ankles specifically reduces MIP-2 levels while having no effect on CXCL1, Ang-1, bFGF, and VEGF concentration compared with the control treatment (Fig. 6A–E). Interestingly, anti–IL-7 Ab treatment was capable of reducing CIA ankle hemoglobin levels by 6-fold (Fig. 6F) and joint vascularization (Fig. 6G, 6H), suggesting that IL-7 can contribute to angiogenesis in CIA.

**Joint TH-17 promoting cytokines, TH-17 polarization, and T cell trafficking was unaffected by anti–IL-7 Ab treatment in CIA mice**

Previous studies had demonstrated the importance of IL-7 in TH-1 (30) and TH-17 differentiation (11); therefore, we asked whether blockade of IL-7 could affect the percentage of CD3, CD4, TH-1, and TH-17–positive cells in splenocytes. We show that the percentage of CD3, CD4, TH-1, and TH-17–positive cells was similar in anti–IL-7 and IgG treatment groups (Fig. 7A). Consistent with these findings, joint IL-6 levels (Fig. 7B) were unaffected by anti–IL-7 therapy, but there was an insignificant trend toward lower levels of joint IL-1β and IL-17 (Fig. 7C, 7D). Therefore, the effect...
of anti–IL-7 Ab treatment was determined on joint T cell migration. We found that although joint T cells were not significantly reduced, there was a trend toward a lower number of T cells in the anti–IL-7 Ab-treated CIA mice compared with the control group (Fig. 7E, 7F).

**Discussion**

This study identifies a novel mechanism for IL-7/IL-7R in CD14+CD16+ cell trafficking through activation of the AKT/PI3K and ERK cascades that is distinct from the effect of IL-7 on T cells, which is mediated through the JAK/STAT pathway (31). In addition, we show that expression of IL-7 and IL-7R in RA monocytes is linked to increased TNF-α and DAS28 levels, suggesting the importance of IL-7/IL-7R function in myeloid cells to RA progression. To demonstrate the effect of IL-7R ligation to IL-7 in disease pathogenesis, we document that therapeutic anti–IL-7 treatment in CIA relieves arthritis by reducing monocyte extravasation, osteoclast differentiation, and joint vascularization (Fig. 7G). Although the role of IL-7 and IL-7R in T cell differentiation is well described, their significance in the effector phase of RA and myeloid cell function is unknown.

Based on our recent studies demonstrating that IL-7 and IL-7R are highly elevated in RA synovial fluid, tissue, and blood myeloid cells (5), we asked whether expression of these factors has a significant role in myeloid cell function. Previous studies had shown that cell-to-cell contact of macrophages with T cells was required for IL-7–mediated TNF-α production (6); however, IL-7–activated PB monocytes did not require T cell interaction and were capable of producing TNF-α (15). Concentration of IL-7 correlated with levels of TNF-α and number of CD68+ cells in RA tissue, but there was no correlation found between IL-7 levels and numbers of RA ST CD3+, CD8+, or CD8+ T cells, suggesting that myeloid cells are the main source of IL-7 production (13). Furthermore, it has been shown that TNF-α is the common factor that induces expression of IL-7 and IL-7R in RA myeloid cells and endothelial cells (5), suggesting that there is a positive feedback regulation between TNF-α and the IL-7/IL-7R cascade. As such, anti–TNF-α responders (13) show significantly reduced circulating IL-7 levels, reflecting our results that demonstrate a strong association between IL-7/IL-7R and TNF-α and RA disease severity.

Macrophages in RA synovial fluid expressed 45 fold higher IL-7R compared with control cells (5); therefore, we postulated that IL-7 expressed in RA synovial fluid could be important for attracting myeloid cells from blood into the joint. We show that blockade of IL-7R and neutralization of IL-7 can significantly reduce synovial fluid–mediated monocyte migration, justifying elevated levels of IL-7R on RA joint macrophages. Interestingly, the mechanism by which IL-7 mediates monocyte recruitment is not shared by other potent monocyte chemoattractant and is distinct from signaling pathways associated with IL-7–induced T cell differentiation (31).

Expression of IL-7R in CIA lining and sublining myeloid cells as well as upregulation of IL-7 in CIA ankle joints justified the use of this experimental arthritis model to examine whether the IL-7/IL-7R cascade could be used as a target for RA therapy. We show that therapeutic treatment of CIA mice markedly reduces joint inflammation, limiting thickness, and bone degradation. We demonstrate that downregulation of TRAP+ cells in anti–IL-7 treated mice
CIA mice is due to decreased RANKL and cathepsin K levels. Interestingly, others have shown that osteoblasts activated with TNF-α and IL-1β produce IL-7, which can contribute to RANKL-dependent or independent osteoclastogenesis (32). In RA, synovial tissue fibroblasts or synovial fluid T cells (33) are responsible for RANKL secretion. Because anti–IL-7 treatment was unable to markedly reduce joint T cell migration, our results might suggest that IL-7 mainly affects RANKL transcription from joint fibroblasts. Consistent with this conclusion, we (5) and others (34) demonstrate that RA fibroblasts express both IL-7 and IL-7R and that levels of this ligand and receptor are modulated by TNF-α, indicating the responsiveness of fibroblasts to IL-7 stimulation.

Our results also highlight that fold reduction of cathepsin K (10-fold decrease), a collagen degrading protease, present in myeloid cells and mature osteoclasts (35) is greater than RANKL (3-fold decrease), which is in agreement with the effect of anti–IL-7 treatment on CIA myeloid cell homing.

We also show that potent monocyte chemoattractants, such as TNF-α and CCL2 but not CCL5, were significantly reduced in the ankles of mice with CIA that were anti–IL-Ab treated compared with controls. This finding suggests that reduction in joint myeloid cell extravasation following anti–IL-7 Ab treatment could be due to the disrupted ligation of IL-7 to IL-7R on monocytes or indirectly due to the lower levels of joint TNF-α and CCL2. Our results corroborate earlier studies demonstrating that preventative anti–IL-7R treatment could reduce CIA front paw TNF-α production levels, despite their study showing a lack of an effect in number of spleen myeloid cells and that joint macrophage immunostaining was not performed (36). Our in vivo results in CIA correlate with findings of in vitro studies demonstrating that in myeloid cells, concentrations of TNF-α (5) and IL-7 (15) are in cross regulation, which is consistent with the strong relation noted between CD68⁺ cells and levels of IL-7 and TNF-α in RA ST as well as reduction in circulating IL-7 levels detected in TNF-α responders compared with nonresponders (13). However, we were unable to demonstrate that IL-7 is capable of inducing CCL2 from myeloid cells, RA fibroblasts, and endothelial cells; therefore, reduction of CCL2 levels in anti–IL-7 Ab therapy may be due to reduced joint monocyte differentiated macrophages rather than a direct effect of IL-7 on the joint. In addition, the lack of effect observed following anti–IL-7 Ab treatment on joint CCL5 levels may be the result of the inefficient reduction of joint T cells, because CCL5 is primarily secreted from T cells and myeloid cells (37).

One of the mechanisms by which IL-7 induces pathogenesis is its ability to induce potent proangiogenic factors such as IL-8 and Ang-1 from macrophages, endothelial cells, or both (5). In CIA, neutralization of IL-7 was capable of reducing joint MIP-2 (homolog of human IL-8); however, regarding Ang-1, bFGF, CXCL1, and VEGF, although lower, their concentration was not significantly reduced compared with the control group. Consistently, others have shown that despite the inefficiency of anti–IL-7R Ab treatment in reducing CIA joint bFGF concentrations, von
Willebrand factor (VWF) expression levels were significantly reduced compared with the control group (36), suggesting that IL-7/IL-7R-mediated angiogenesis in CIA is distinct from VEGF or bFGF pathways. Although IL-7R expression is elevated in endothelial cells both in RA (5) and CIA ankles, we were unable to demonstrate that ligation of IL-7 to endothelial IL-7R could promote endothelial cell migration, suggesting that reduced vasculogenesis in CIA is indirectly due to modulation of joint MIP-2 levels.

We show that anti–IL-7 Ab treatment in CIA mice had no effect on the percentage of spleen CD3, CD4, TH1, and TH-17–positive cells compared with control treatment. In agreement with these results, although there was a trend toward lower joint CD3 immunostaining in the anti–IL-7 Ab treatment group, CD3+ cells were not markedly reduced compared with the control group. Similarly, levels of joint IL-6, IL-17, and IL-17 were unaffected by the treatment. In contrast with our results, preventative treatment of CIA with anti–IL-7R Ab demonstrated a significant reduction in front paw IL-17 and IL-1β levels as well as the percentage of CD4+, CD8+, naïve, and memory T cells (36). The contrasting data may be due to treatment time point, indicating that ligation of IL-7 to IL-7R before disease onset could be important in maintaining and restoring T cell homeostasis, whereas subsequent to disease onset these factors could have a role in myeloid cell migration and function. Previous studies demonstrate that IL-7 contributes to TH-17 cell differentiation by increasing IL-1R1 expression on CD4+ cells, thereby making these CD4+IL-1R1+ cells more responsive to IL-1β stimulation (38). Therefore, anti–IL-7 Ab treatment may have failed to reduce the percentage of TH-17 cell development because of its lack of effect on IL-1R1 expression in CD4+ cells owing to treatment after onset. Similar to our findings in CIA, others have shown that TH-1 cell population is unaffected by IL-7R antagonist treatment in autoimmune encephalomyelitis (11).

Our findings in the effector phase of CIA suggest that ligation of IL-7 to IL-7R contributes to increased monocyte homing and angiogenesis, which reflects the expression pattern of IL-7 and IL-7R in RA ST (5). Therefore, association of IL-7 and IL-7R with angiogenesis, which reflects the expression pattern of IL-7 and IL-7R in synovial fluids of rheumatoid arthritis and osteoarthritis patients, Rheumatology (Oxford) 43: 435–441.


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