AD_____

Award Number: W81XWH-10-1-0102

TITLE: The Role of IL-17 in the Angiogenesis of Rheumatoid Arthritis

PRINCIPAL INVESTIGATOR: Shiva Shahrara, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois
Chicago, IL 60612

REPORT DATE: July 2011

TYPE OF REPORT: Revised Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2011	Revised Annual	1 JUL 2010 - 30 JUN 2011

4. TITLE AND SUBTITLE

The Role of IL-17 in the Angiogenesis of Rheumatoid Arthritis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0102

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Shiva Shahrara, Ph.D.

E-Mail: shahrara@uic.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Illinois
Chicago, IL 60612

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

None provided.

15. SUBJECT TERMS

None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	36	19b. TELEPHONE NUMBER *(include area code)*

PROJECT TASKS

TASK 1. To examine the mechanism by which IL-17 induces angiogenesis. We will: **(time frame, months 1-13)**

1a. determine whether IL-17 contributes to RA synovial fluid (SF)-induced angiogenesis employing HMVEC chemotaxis *in vitro* as well as in matrigel plug assay *in vivo*. (time frame, months 1-5). As indicated in the progress report we have examined the contribution of IL-17 to RA SF-mediated *in vitro* studies as well as role of IL-17 in matrigel plug assay *in vivo*.

1b. examine which IL-17 receptor is involved in RA SF-induced angiogenesis *in vitro*. (time frame, months 6-9). We have demonstrated that IL-17 RC plays an important role in RA synovial fluid mediated endothelial cells. We may need to confirm our results using IL-17RA-/- mice. In that event we will require 20 mice per treatment group in three different groups (IL-17, PBS and bFGF). Hence we will require 60 C57BL/6 mice.

1c. investigate whether PI3K pathway is required for IL-17-induced chemotaxis *in vitro* and whether AKT-/- mice are deficient in IL-17-mediated blood vessel formation in matrigel plug assay *in vivo*. (time frame, months 9-13). We have shown that AKT is important in IL-17 mediated endothelial migration. We will continue to work on the animal experiments. In the first set up we will require (6 treatment groups x20 mice) 120 C57BL/6 mice, and for the second set up we will require (3 treatment groups x20 mice) 60 C57BL/6 mice. This aim will totally require 180 mice.

TASK 2. To determine whether IL-17 plays a role in endothelial cell homing in RA ST severe combined immunodeficient (SCID) chimera model. We will: **(time frame, months 14-24)**

2a. examine whether IL-17 injected into RA ST engrafted into SCID mice plays a role in HMVEC cell homing. (time frame, months 14-18). In aim 2a we will require 10 mice in three treatment groups (IL-17, PBS and bFGF), therefore we will need 30 SCID mice for this subaim. As an alternative approach we may need to use an adenoviral vector (Ad) expressing IL-17 and or CMV control. In that event we will require 10 mice in 4 treatment groups (Ad-IL-17, Ad-CMV, PBS and bFGF). Hence 40 SCID mice will be utilized. This subaim may require 70 SCID mice in total. 10 different RA synovial tissues will be implanted into the SCID mice employed in each treatment group.

2b. investigate whether neutralization of IL-17 and/or specific IL-17 receptor could reduce HMVEC cell migration into RA ST engrafted into SCID mice. (time frame, months 18-21). For aim 2b we will require 10 mice per 7 treatment groups (anti-IL-17RA, anti-IL-17RC, anti-IL-17RA and RC, anti-IL-17, IgG control, PBS and bFGF) therefore we will require 70 SCID mice. 10 different RA synovial tissues will be grafted into the SCID mice utilized in each treatment group.

2c. determine whether IL-17 synergizes with TNF-α in mediating endothelial cell homing into RA ST engrafted into SCID mice. (time frame, months 21-24). For aim 2c we will require 10 mice per 6 treatment groups (IL-17, TNF-α, IL-17 plus TNF-α, IgG control, PBS and bFGF), therefore we will require 60 SCID mice. 10 different RA synovial tissues will be implanted into the SCID mice used in each treatment group.

TASK 3: to examine the indirect role of IL-17 in mediating angiogenesis and arthritis. We will:
(time frame, months 24-36)

3a. examine whether IL-17-induced angiogenesis *in vitro* will be suppressed by blocking VEGF.
(time frame, months 24-26). For aim 3a we will require 3 to 5 different HMVEC lines which will be purchased from Lonza.

3b. investigate whether IL-17 angiogenesis in matrigel plug assay *in vivo* is due to VEGF.
(time frame, months 26-30). In aim 3b we will require 20 mice in four treatment groups (IL-17 plus IgG, IL-17 plus anti-VEGF, PBS and bFGF), therefore we will need 80 C57BL/6 mice for this subaim. As a potential pitfall we may need to use 80 CXCR2-/- mice (20 mice in four treatment groups) in order to determine whether IL-17-induced angiogenesis is partially through corresponding ligands to CXCR2.

3c. determine whether neutralization of VEGF ameliorates IL-17-induced arthritis in mice.
(**time frame, months 30-36).** In aim 3c we will require 20 mice in four treatment groups (Ad-IL-17 plus IgG, Ad-IL-17 plus anti-VEGF, Ad-CMV control plus IgG and Ad-CMV control plus anti-VEGF), therefore we will need 80 C57BL/6 mice for this subaim. Alternatively we may need to neutralize joint CXCL1 and CXCL5 in this experimental arthritis model to determine whether IL-17-induced arthritis and angiogenesis is partially mediated through these chemokines. In that event we will require 20 mice in four treatment groups (IgG control, anti-CXCL1, anti-CXCL5 and anti-CXCL1 and 5) therefore we will need 80 mice. This subaim may require 160 C57BL/6 mice in total.

TABLE OF CONTENTS

The specific aims of this proposal will remain unchanged and will be performed as initially proposed.

INTRODUCTION

TH-17 cells are a newly discovered CD4 helper T-cells that produce interleukin-17A (also known as IL-17). IL-17 is found in Rheumatoid Arthritis (RA) synovial tissue and fluid, and the percentage of TH-17 cells is significantly higher in RA synovial fluid compared to RA or normal peripheral blood (1, 2). IL-17 has been shown to have a profound effect in experimental models of arthritis however its role in Rheumatoid Arthritis is undefined. Angiogenesis is an early and a critical event in the pathogenesis of RA. Since our preliminary data suggests that IL-17 plays an important role in RA angiogenesis, this grant was proposed to determine the mechanism by which IL-17 induces neovascularization.

Our overriding hypothesis is that IL-17 mediates angiogenesis in RA through activation of the PI3K pathway, and this effect may be dependent or independent of other proangiogenic factors. To test this hypothesis we will investigate the contribution of IL-17 and its receptors to RA synovial fluid-mediated endothelial migration and blood vessel growth. We will also identify signaling pathways that are involved in IL-17-mediated neovascularization and examine whether mice deficient in a particular signaling pathway are unable to mediate neovascularization through IL-17. To further examine the role of IL-17 and its synergistic effect with TNF-α in inducing HMVEC migration *in vivo,* we will employ a RA synovial tissue (ST) severe combined immunodeficient (SCID) chimera mouse model. Additionally, the indirect role of IL-17 in induction of angiogenesis and arthritis will be examined by blocking the effect of VEGF in *in vitro* and *in vivo* models of angiogenesis and experimental arthritis. The unmet need is to elucidate the mechanisms by which IL-17 mediates angiogenesis in RA and to determine whether targeting IL-17 and/or its intermediary molecules will provide a therapeutic intervention for RA patients.

BODY

This report is written demonstrating our annual progress and research results are demonstrated according to the tasks outlined in the statement of work.

In **task 1**, we proposed to examine the mechanism by which IL-17 induces angiogenesis, (time frame, months 1-13).

1a. determine whether IL-17 contributes to RA synovial fluid (SF)-induced angiogenesis employing HMVEC chemotaxis *in vitro* as well as in matrigel plug assay *in vivo*. (time frame, months 1-5).

We asked whether the IL-17 identified in human RA synovial fluid is chemotactic for human microvascular endothelial cells (HMVECs). In these experiments, we demonstrated that human RA synovial fluid is chemotactic for HMVECs, similar to positive control VEGF **(Figure 1A)**. Furthermore, human RA synovial fluid immunodepleted with anti-IL-17 significantly reduced HMVEC chemotaxis ($p<0.05$), compared with control IgG-treated fluids **(Figure 1A)**. Interestingly, angiogenic factors present in human RA synovial fluid are mostly produced by RA synovial tissue fibroblasts (VEGF, bFGF, VCAM1, IL-6 and ELR+ CXC chemokines) or macrophages (TNF-α, IL-8 and IL-1β) (3). IL-17 is the only lymphokine that contributes to human RA synovial fluid-mediated angiogenesis, suggesting that T cells may also be important in this process.

A recent publication demonstrated that tumor growth in subcutaneous and lung metastases are enhanced in IL-17-/- mice compared to the wild type controls, suggesting that IL-17 may suppress tumor development (4). Conversely, others have shown that IL-17 markedly increases

neovascularization in rat cornea (5) and vascularization in tumors (6), indicating that IL-17 may promote angiogenesis. However, there is also evidence demonstrating that IL-17 induces production of proangiogenic factors including nitric oxide (NO), hepatocyte growth factor (HGF), CXCL1/KC, CXCL2/MIP-2, prostaglandin (PGE)1, PGE2 and VEGF from RA synovial fibroblasts, and the production of some of these factors is further enhanced by TNF-α (7).

Since production of potent proangiogenic factors such as VEGF is induced by IL-17 in RA synovial tissue fibroblasts and HMVECs (Figure 2) we therefore asked whether neutralization of both VEGF and IL-17 may have a stronger effect in reducing RA synovial fluid-induced endothelial migration. Our results demonstrate that immunodepletion of IL-17 and VEGF in RA synovial fluids does not have an additive or synergistic effect in reduction of HMVEC migration beyond the effect noted with neutralization of one factor alone (Figure 1B). As shown with IL-17(8), VEGF-induced HMVEC chemotaxis is mediated through PI3K (9, 10). Therefore, the lack of synergy between IL-17 and VEGF in inducing HMVEC chemotaxis may be due to both mediators using the same signaling pathway or that as shown previously HMVEC cell migration is within a bell shaped curve (11, 12); therefore, the synergistic effect could not be detected.

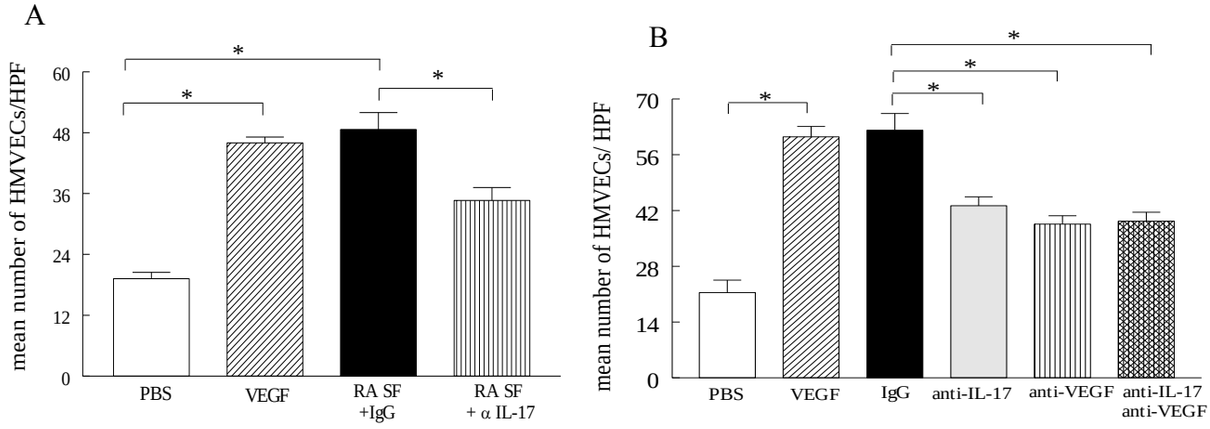


Figure 1. RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 and IL-17 does not synergize with VEGF in RA synovial fluid induced HMVEC migration. **A.** anti-IL-17 (10 μg/ml) or control IgG was added to RA synovial fluids from 8 patients (1:20 dilution) (1h at 37ºC) prior to performing HMVEC chemotaxis in response to human RA synovial fluids. **B.** RA synovial fluids from 8 patients (1:20 dilution) were incubated with antibodies to IL-17 (10 μg/ml), VEGF (10 μg/ml), or both as well as isotype control, PBS or VEGF for 1h prior to performing HMVEC chemotaxis in response to RA synovial fluids. The values represent the mean ± SE. * represents $p < 0.05$.

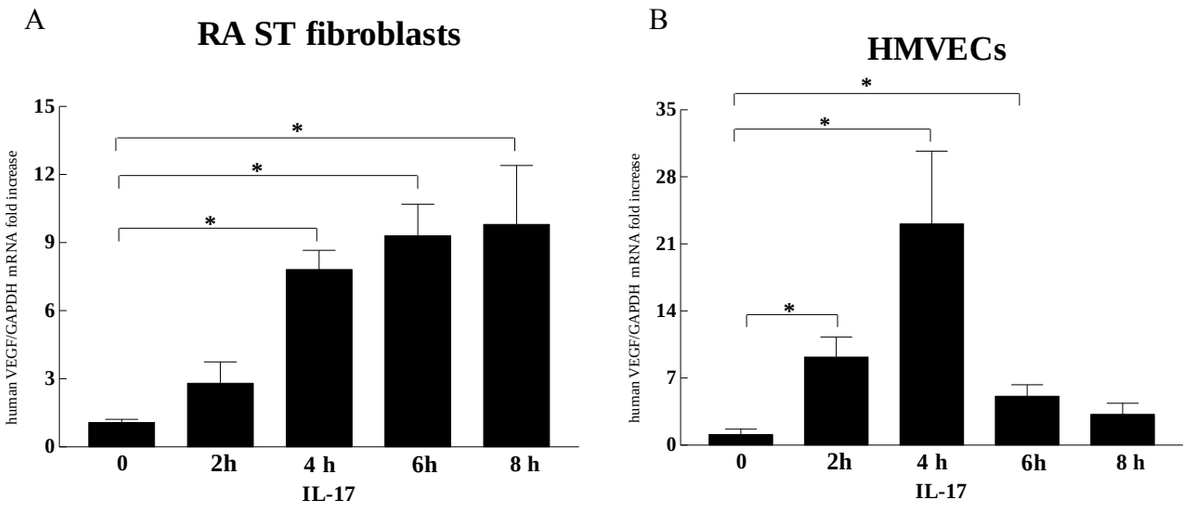


Figure 2. IL-17 induces expression of VEGF from RA synovial tissue fibroblasts and HMVECs. RA synovial tissue fibroblasts or HMVECs were either untreated or treated with IL-17 (50ng/ml) for various time points. Thereafter, the mRNA was harvested and used to measure VEGF mRNA levels in RA synovial tissue fibroblasts **(A)** and HMVECs **(B)** by real-time RT-PCR. The data are shown as fold increase above untreated cells and are normalized to GAPDH**.** Values are the mean ± SE, n=4.

1b. examine which IL-17 receptor is involved in RA SF-induced angiogenesis *in vitro*. (time frame, months 6-8).

We have previously shown that HMVECs express both IL-17 receptors (8). These experiments were performed to determine which IL-17 receptor is involved in HMVEC tube formation and chemotaxis. IL-17 induced HMVEC tube formation in matrigel was examined employing concentrations of IL-17 ranging from 0.001 ng/ml to 100 ng/ml, the optimal concentration of IL-17 to induce HMVEC tube formation was 50 ng/ml ($p<0.05$) (data not shown). Neutralization of IL-17RC (30%) or both IL-17 receptors (45%) on HMVECs significantly decreased ($p<0.05$) IL-17-mediated HMVEC tube formation (Fig. 3A-G). Blockade of IL-17RA alone was inefficient in this process. Consistent with the HMVEC tube formation data, some reduction of HMVEC chemotaxis was noted with an anti-IL-17RA antibody, although it was not significant. Inhibition of chemotaxis was significant when IL-17RC (40%) or both receptors (47%) were neutralized (Fig. 3H). **Collectively, our data suggest that IL-17RC plays a more important role in IL-17-mediated HMVEC tube formation and chemotaxis compared to IL-17RA.**

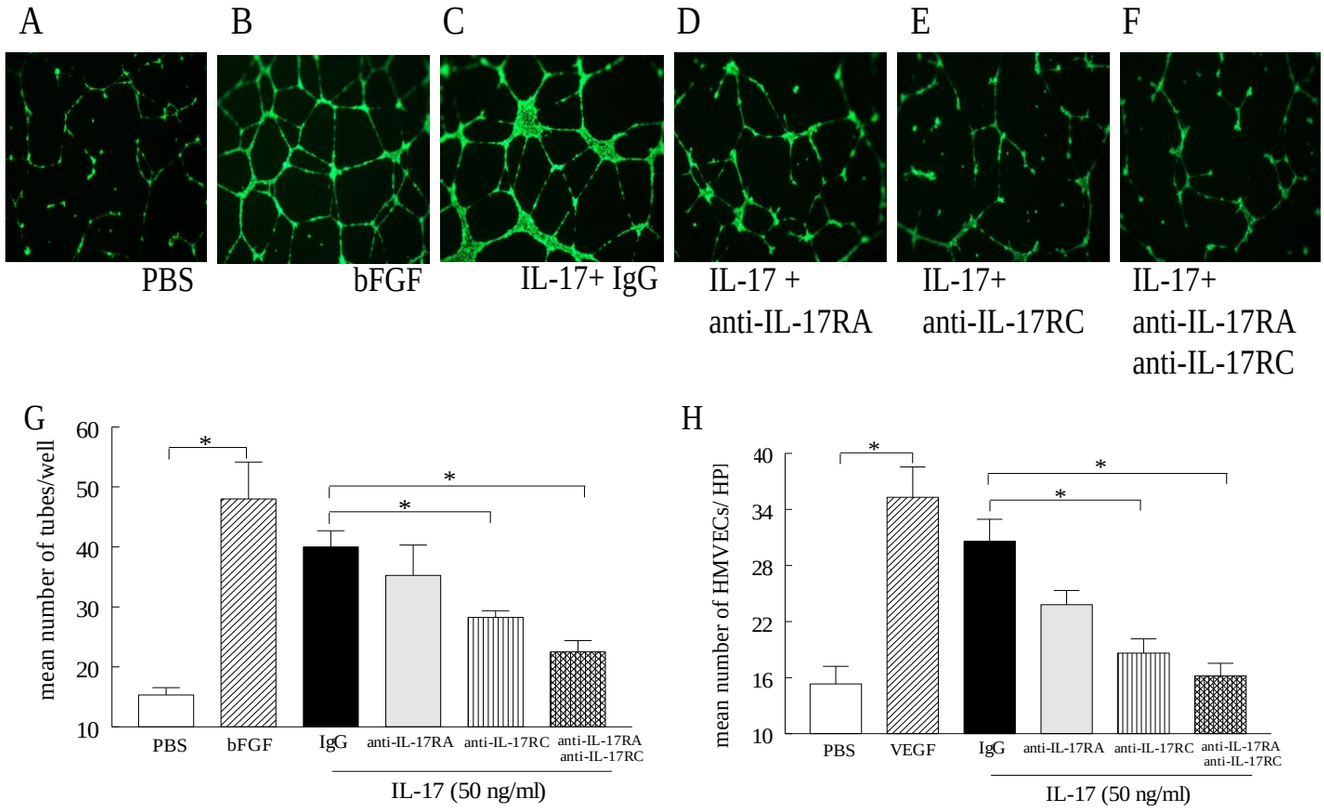


Figure 3. IL-17-mediated HMVEC tube formation and chemotaxis is mediated primarily through IL-17RC. HMVECs were incubated with antibodies to IL-17RA, IL-17RC, both IL-17RA and RC or IgG for 45 minutes at 37°C. Cells were then added to polymerized matrigel, IL-17 (50ng/ml) was placed in the wells, and the plate was incubated for 16h at 37ºC (in triplicate). Photomicrographs taken of representative wells treated with PBS **(A)**, FGF (20 ng/ml) **(B)**, IL-17 (50 ng/ml) plus IgG **(C)**, IL-17 (50 ng/ml) plus anti-IL-17RA (10μg/ml) **(D)**, IL-17 (50ng/ml) plus anti-IL-17RC (10μg/ml) **(E)** and IL-17 (50ng/ml) plus anti-IL-17RA and RC (10μg/ml) **(F)** in which IL-17-induced tube formation is significantly reduced by the neutralization of IL-17RC or both receptors ($p<0.05$). **(G.)** Data presented

demonstrates mean number of branch points/tubes in each treatment group from three experiments. **(H.)** HMVECs were incubated with mouse anti-human IL-17RA and IL-17RC antibodies (10μg/ml) or control IgG (10μg/ml) for 1h. Thereafter HMVEC chemotaxis was performed in response to IL-17 (50 ng/ml) for 2h. PBS was used as a negative control, and VEGF (60 nM) as a positive control. Values demonstrate mean ± SE, n=3. * denotes $p<0.05$.

IL-17 RC is involved in RA synovial fluid-mediated HMVEC chemotaxis. Neutralization of IL-17RA and RC on HMVECs showed that only IL-17RC was effective in suppressing human RA synovial fluid-mediated HMVEC migration (Fig. 4). Although IL-17RC plays a major role in IL-17-mediated HMVEC chemotaxis and tube formation, neutralization of both receptors is more effective in this process compared to IL-17RC alone. Like monocytes, HMVECs express both IL-17RA and IL-17RC (13). However, in contrast to HMVEC chemotaxis, IL-17-mediated monocyte migration is induced through both IL-17RA and RC (13). Interestingly, a novel IL-17 receptor-like protein has been identified in HUVEC that interacts with fibroblast growth factor receptor 1 and inhibits activation of the ERK pathway and production of FGF, indicating that various IL-17 receptors may modulate angiogenesis differently (14).

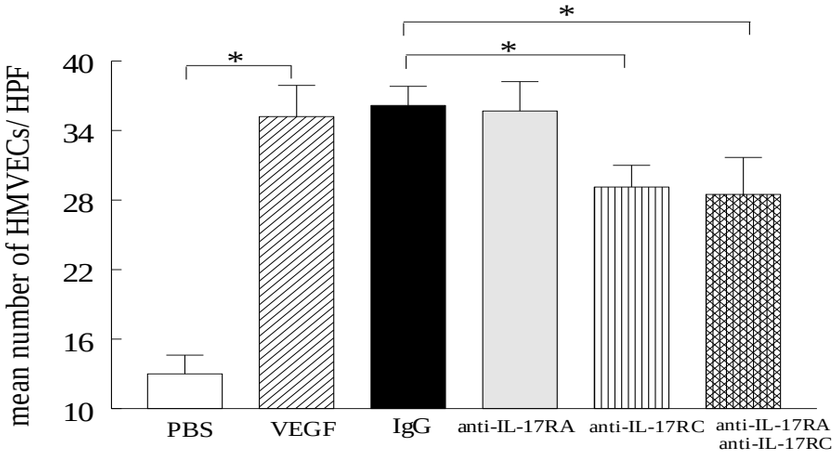


Figure 4. RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 through ligation to IL-17RC. HMVECs were incubated with antibodies to IL-17 RA and RC (10 μg/ml), as well as isotype control for 1h prior to performing HMVEC chemotaxis in response to 8 human RA synovial fluids. The values represent the mean ± SE. * represents $p<0.05$.

1c. investigate whether PI3K pathway is required for IL-17-induced chemotaxis *in vitro* and whether AKT-/- mice are deficient in IL-17-mediated blood vessel formation in matrigel plug assay *in vivo*. (time frame, months 9-13).

IL-17 activates ERK, JNK and PI3K pathways in HMVECs. To determine which signaling pathways in HMVECs were activated by IL-17, cells were untreated or treated with IL-17 (50 ng/ml) for 15-75min. Cell lysates were examined by Western blot analysis, and probed for pERK, pJNK, p-p38 and pAKT1. Our preliminary data indicate that IL-17 phosphorylates ERK, JNK and AKT1 as early as 15 min. While the activation of ERK and JNK is gradually reduced at 75 or 65 min (Fig. 5A and 5B), respectively, AKT1 is still strongly phosphorylated at 75min (Fig 5C). p38 pathway was not activated by IL-17 in HMVECs (data not shown). These results collectively suggest that ERK, JNK or PI3K pathway may be potentially important for IL-17-induced HMVEC migration.

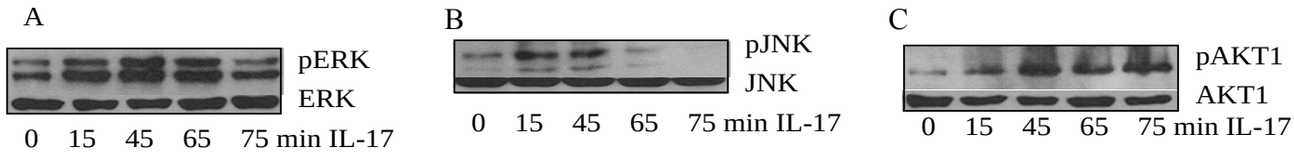


Figure 5. IL-17 stimulates phosphorylation of ERK, JNK and AKT1 in HMVECs. HMVECs were stimulated with IL-17 (50ng/ml) for various time points. Cell lysates were probed with p-ERK **(A)**, pJNK **(B)** and pAKT1 **(C)** or pan antibodies. Results are representative of three experiments.

Inhibition of PI3K suppresses IL-17-induced HMVEC tube formation and chemotaxis. To determine which signaling pathways mediate HMVEC tube formation, chemical inhibitors at concentrations of 1 and 5 μM were utilized. Inhibition of ERK and JNK was ineffective in suppressing IL-17-induced HMVEC tube formation, while inhibition of PI3K reduced tube formation by 30-40% ($p<0.05$) starting at 1 μM (Fig. 6A-G). Similarly, while inhibition of PI3K (starting at 1μM) reduced IL-17 induced HMVEC migration ($p<0.05$) (Fig. 6H), suppression of ERK and JNK had no effect on this process. These results suggest that IL-17-induced HMVEC tube formation and chemotaxis are mediated through the PI3K/AKT1 pathway. Consistently, CCL2/MCP-1-mediated endothelial chemotaxis is through activation of PI3K, as well as the ERK pathway (15). Further, others have shown that PI3K signaling plays an important role in regulation of VEGF production as well as VEGF-mediated endothelial migration (9, 16), suggesting that PI3K is involved in the mediation of angiogenesis by various proinflammatory factors. A recently published paper shows that increased expression of IL-17 in IFNγ -/- mice mediates tumor growth and angiogenesis through STAT3 phosphorylation (17). In contrast to IL-17, which promotes angiogenesis, IL-17F inhibits HUVEC tube formation, indicating that different IL-17 isoforms may modulate angiogenesis differently (18).

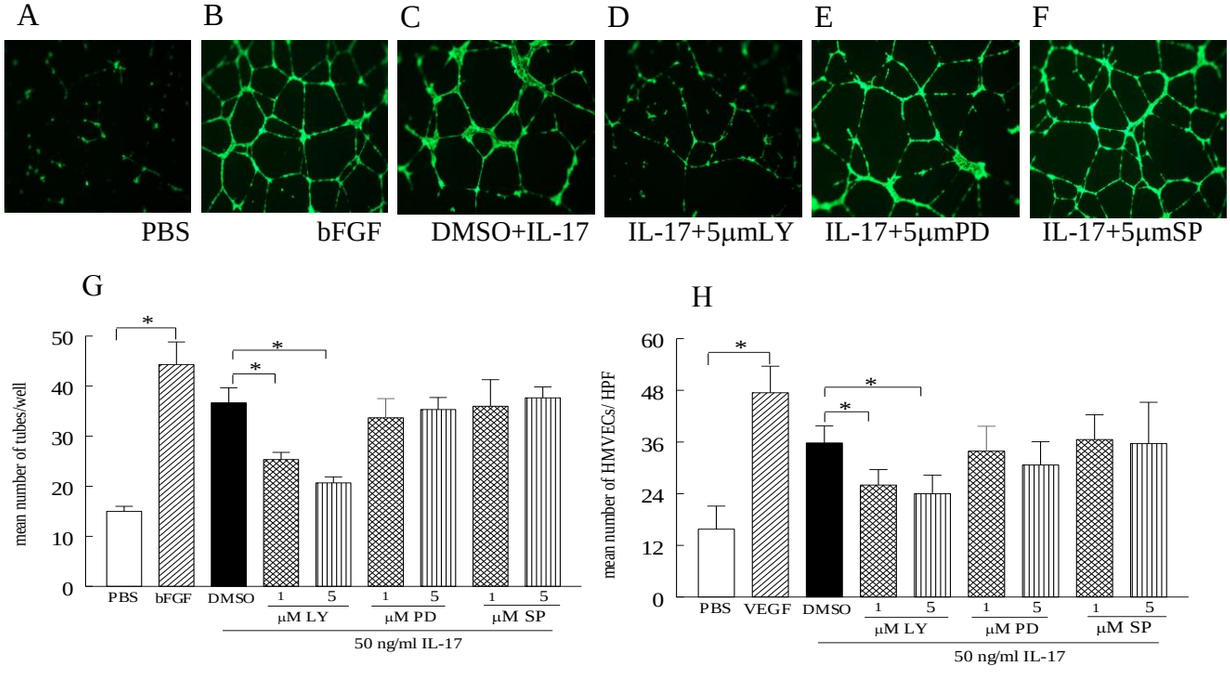


Figure 6. IL-17 mediates HMVEC tube formation and migration through PI3K activation. HMVECs were incubated with inhibitors to PI3K (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), JNK (SP600125; 1 and 5 μM) or DMSO for 45 minutes at 37°C prior adding to polymerized matrigel. IL-17 (50ng/ml) was then added to the wells and the plate was incubated for 16h at 37ºC (in triplicate). Photomicrographs taken of representative wells treated with PBS **(A)**, FGF (20 ng/ml) **(B)**,

IL-17 (50ng/ml) plus DMSO **(C)**, IL-17 (50ng/ml) plus LY294002 (5 μM) **(D)**, IL-17 (50ng/ml) plus PD98059 (5 μM)**(E)** and IL-17 (50ng/ml) plus SP600125 (5 μM) **(F)** in which IL-17-induced tube formation is significantly reduced by the inhibition of PI3K/AKT1 pathway ($p<0.05$). **G.** demonstrates mean number of tubes/well where LY294002 (1 and 5 μM; PI3K/AKT1 inhibitor) significantly reduces the number of branch points induced by IL-17 activation in matrigel tube formation assay whereas ERK (PD98059; 1 and 5 μM) and JNK inhibitors (SP600125; 1 and 5 μM) were ineffective. **H.** To determine signaling pathways associated with IL-17 mediated HMVEC migration, cells were treated with the chemical inhibitors for PI3K (LY294002; 1 and 5μM) or ERK (PD98059; 1 and 5 μM) as well as JNK (SP600125; 1, 5μM) 2h in the Boyden chamber. Only inhibition of PI3K downregulated IL-17 induced HMVEC migration. Values demonstrate mean ± SE of three experiments in triplicate. * represents $p<0.05$.

TASK 2. To determine whether IL-17 plays a role in endothelial cell homing in RA ST severe combined immunodeficient (SCID) chimera model. We will: **(time frame, months 14-24).**

Task 2 has not been accomplished.

TASK 3. to examine the indirect role of IL-17 in mediating angiogenesis and arthritis. We will:
(time frame, months 24-36).

Task 3 has not been achieved.

KEY RESEARCH ACCOMPLISHMENTS

Within this one year time frame we have shown that:

- RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 and IL-17 does not synergize with VEGF in RA synovial fluid induced HMVEC migration.

- Neutralization of IL-17RC but not IL-17RA was involved with suppressing RA synovial fluid-mediated HMVEC migration.

- Activation of PI3K is responsible for IL-17 mediated HMVEC tube formation and migration.

REPORTABLE OUTCOMES

Publications:

1. Pickens SR, Volin MV, Mandelin II AM, Kolls JK, Pope RM, **Shahrara S**. IL-17 Contributes to Angiogenesis in Rheumatoid Arthritis. J. Immunol. 184; 6 :3233-3241, 2010.

2. **Shahrara S**, Pickens SR, Mandelin AM, Huang Q, Karpus W, Kolls JK, Pope RM. IL-17-mediated monocyte migration is partially through CCL2/MCP-1 induction. J. Immunol. 184; 8:4479-4487, 2010.

3. Ruth RH, Park CC, Amin MA, Lesch C, Marotte H, **Shahrara S** and Koch AE. IL-18 as an *in vivo* Mediator of Monocyte Recruitment in Rodent Models of Rheumatoid Arthritis. Arthritis Res. Ther. 16; 12: R118, 2010.

4. Thumbikat P, **Shahrara S**, Sobkoviak R, Done J, Pope RM and Schaeffer AJ. Prostate secretions from men with CP/CPPS inhibits proinflammatory mediators. J. Urol. 184:1536-42, 2010.

5. Pickens SR, Chamberlain ND, Volin MV, Pope RM, Mandelin II AM, ***Shahrara S**: Characterization of CCL19 and CCL21 in rheumatoid arthritis: Arthritis Rheum 63: 914–922, 2011.

6. Pickens SR, Chamberlain ND, Volin MV, Mandelin AM 2nd, Agrawal H, Matsui M, Yoshimoto T, **Shahrara S**. Local expression of IL-27 ameliorate collagen induced arthritis: Arthritis Rheum 2011, In press. Mar 7. [Epub ahead of print].

7**.** Picken SR, Chamberlain ND, Volin MV, Pope RM, Talarico NE, Mandelin II AM and **Shahrara S.** Characterization of IL-7 and IL-7R in the pathogenesis of Rheumatoid Arthritis. Arthritis Rheum 2011 In press.

8**. Shahrara S**. Reply. Arthritis Rheum. 2011 Jun 23. doi: 10.1002/art.30496. [Epub ahead of print].

9. Michael V. Volin, and **Shiva Shahrara**. Role of TH-17 cells in human disease. Invited review from Journal of Molecular Cell Biology.

Invited Lectures:

2010	University of Illinois in Chicago, Rheumatology rounds
2010	American College of Rheumatology Research and Education Foundation *Within Our Reach* meeting in Dallas
2010	Northwestern University, Feinberg School of Medicine, Pathology and inflammation group
2010	American College of Rheumatology 2010 annual meeting held in Atlanta
2011	Department of Microbiology and Immunology lectures in University of Illinois at Chicago
2011	The Institute for Personalized Respiratory Medicine lectures in University of Illinois at Chicago
2011	Department of Rheumatology Grand Rounds in University of Illinois at Chicago

Abstracts and/or oral presentations presented at American College of Rheumatology (ACR) 2010:

1. Pickens SR, Volin MV, Mandelin II AM, **Shahrara S**. Local expression of IL-27 ameliorate collagen induced arthritis. Arthritis Rheum 714: S298, 2010. **(Oral presentation)**

2. Pickens SR, Volin MV, Pope RM, Mandelin II AM, **Shahrara S**. Characterization of CCR7 and its ligands CCL19 and CCL21 in rheumatoid arthritis. Arthritis Rheum 1506: S627, 2010.

3. Pickens SR, Volin MV, Mandelin II AM, Kolls JK, **Shahrara S**. Anti-CXCL5 therapy ameliorates IL-17-induced arthritis. Arthritis Rheum 270: S112, 2010.

4. Pickens SR, Volin MV, Pope RM, Mandelin II AM, **Shahrara S**. Downstream targets of IL-17 in Rheumatoid Arthritis. Arthritis Rheum 23: S9, 2010.

Submitted abstracts and/or oral presentations for American College of Rheumatology (ACR) 2011:

1. Nathan D. Chamberlain, Michael V. Volin and **Shiva Shahrara**. CXCL5 an Important IL-17 Mediated Proangiogenic Factor in Rheumatoid Arthritis and Experimental Arthritis Model. Submitted to ACR meeting 2011.

2. Nathan D. Chamberlain, Sarah R. Pickens, Richard M. Pope, Michael V. Volin and **Shiva Shahrara** IL-7 Contributes to Monocyte Migration in Rheumatoid Arthritis. Submitted to ACR meeting 2011.

3. Sarah R. Pickens, Nathan D. Chamberlain, Michael V. Volin, Arthur M. Mandelin II and **Shiva Shahrara.** CCL21 a Novel Proangiogenic Factor in Rheumatoid Arthritis. Submitted to ACR meeting 2011.

4. Nathan D. Chamberlain, Michael V. Volin, Richard M. Pope, Arthur M. Mandelin II and **Shiva Shahrara**. Characterization of TLR7 and TLR8 in Rheumatoid Arthritis. Submitted to ACR meeting 2011.

5. Nathan D. Chamberlain, Michael V. Volin, Richard M. Pope, Arthur M. Mandelin II and **Shiva Shahrara.** TLR5; a Novel and Unidentified Inflammatory Mediator in Rheumatoid Arthritis. Submitted to ACR meeting 2011.

Employment opportunities:

The funding provided to us by DOD enabled me to secure a tenure track position as an Associate Professor of Medicine in University of Illinois at Chicago (UIC). My position started at February 1st of 2011 and the funding provided by DOD was the basis of obtaining this opportunity. I am humbled and grateful for receiving this Investigator Initiated Award from the Department of Defense. This progress report is being submitted 6 months after our move to the new institution at UIC.

CONCLUSION

In RA patients percent TH-17 cells correlate with disease activity score suggesting that IL-17 can mediate inflammation at early disease stage and may also be involved in disease progression (19). Therefore to evaluate the role of IL-17 in RA angiogenesis, we examined which IL-17 receptors and signaling pathways are associated with rheumatoid arthritis synovial fluid mediated endothelial migration. We show that although HMVECs express both IL-17RA and RC, RA synovial fluid-mediated HMVEC chemotaxis is mediated by binding primarily to IL-17RC. We also report that while IL-17 activates ERK, JNK and PI3K pathways in HMVECs, only inhibition of PI3K reduces IL-17-induced HMVEC chemotaxis and tube formation. Finally, we show that IL-17 and VEGF neutralization in RA synovial fluid does not significantly reduce HMVEC migration beyond the effect of one factor alone. Hence, therapy directed against IL-17 may reduce leukocyte migration by inhibiting angiogenesis in RA.

REFERENCES

1. Stamp, L.K., M.J. James, and L.G. Cleland. 2004. Interleukin-17: the missing link between T-cell accumulation and effector cell actions in rheumatoid arthritis? *Immunology and cell biology* 82:1-9.
2. Shahrara, S., Q. Huang, A.M. Mandelin, 2nd, and R.M. Pope. 2008. TH-17 cells in rheumatoid arthritis. *Arthritis Res Ther* 10:R93.
3. Szekanecz, Z., and A.E. Koch. 2009. Angiogenesis and its targeting in rheumatoid arthritis. *Vascul Pharmacol* 51:1-7.
4. Kryczek, I., S. Wei, W. Szeliga, L. Vatan, and W. Zou. 2009. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood* 114:357-359.
5. Numasaki, M., J. Fukushi, M. Ono, S.K. Narula, P.J. Zavodny, T. Kudo, P.D. Robbins, H. Tahara, and M.T. Lotze. 2003. Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 101:2620-2627.
6. Numasaki, M., M. Watanabe, T. Suzuki, H. Takahashi, A. Nakamura, F. McAllister, T. Hishinuma, J. Goto, M.T. Lotze, J.K. Kolls, and H. Sasaki. 2005. IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. *J Immunol* 175:6177-6189.
7. Numasaki, M., M.T. Lotze, and H. Sasaki. 2004. Interleukin-17 augments tumor necrosis factor-alpha-induced elaboration of proangiogenic factors from fibroblasts. *Immunol Lett* 93:39-43.
8. Pickens, S.R., M.V. Volin, A.M. Mandelin, 2nd, J.K. Kolls, R.M. Pope, and S. Shahrara. IL-17 contributes to angiogenesis in rheumatoid arthritis. *J Immunol* 184:3233-3241.
9. Jiang, B.H., and L.Z. Liu. 2008. AKT signaling in regulating angiogenesis. *Curr Cancer Drug Targets* 8:19-26.
10. Nakashio, A., N. Fujita, and T. Tsuruo. 2002. Topotecan inhibits VEGF- and bFGF-induced vascular endothelial cell migration via downregulation of the PI3K-Akt signaling pathway. *Int J Cancer* 98:36-41.
11. Park, C.C., J.C. Morel, M.A. Amin, M.A. Connors, L.A. Harlow, and A.E. Koch. 2001. Evidence of IL-18 as a novel angiogenic mediator. *J. Immunol.* 167:1644-1653.
12. Volin, M.V., L.A. Harlow, J.M. Woods, P.L. Campbell, M.A. Amin, M. Tokuhira, and A.E. Koch. 1999. Treatment with sulfasalazine or sulfapyridine, but not 5-aminosalicyclic acid, inhibits basic fibroblast growth factor-induced endothelial cell chemotaxis. *Arthritis Rheum.* 42:1927-1935.
13. Shahrara, S., S.R. Pickens, A. Dorfleutner, and R.M. Pope. 2009. IL-17 induces monocyte migration in rheumatoid arthritis. *J Immunol* 182:3884-3891.
14. Yang, R.B., C.K. Ng, S.M. Wasserman, L.G. Komuves, M.E. Gerritsen, and J.N. Topper. 2003. A novel interleukin-17 receptor-like protein identified in human umbilical vein endothelial cells antagonizes basic fibroblast growth factor-induced signaling. *J. Biol. Chem.* 278:33232-33238.
15. Arefieva, T.I., N.B. Kukhtina, O.A. Antonova, and T.L. Krasnikova. 2005. MCP-1-stimulated chemotaxis of monocytic and endothelial cells is dependent on activation of different signaling cascades. *Cytokine* 31:439-446.
16. Hayashi, H., H. Nakagami, Y. Takami, H. Koriyama, M. Mori, K. Tamai, J. Sun, K. Nagao, R. Morishita, and Y. Kaneda. 2009. FHL-2 Suppresses VEGF-Induced Phosphatidylinositol 3-Kinase/Akt Activation via Interaction With Sphingosine Kinase-1. *Arterioscler Thromb Vasc Biol*
17. Wang, L., T. Yi, M. Kortylewski, D.M. Pardoll, D. Zeng, and H. Yu. 2009. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *J Exp Med* 206:1457-1464.
18. Starnes, T., M.J. Robertson, G. Sledge, S. Kelich, H. Nakshatri, H.E. Broxmeyer, and R. Hromas. 2001. Cutting edge: il-17f, a novel cytokine selectively expressed in activated t cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J. Immunol.* 167:4137-4140.

19. Leipe, J., M. Grunke, C. Dechant, C. Reindl, U. Kerzendorf, H. Schulze-Koops, and A. Skapenko. Role of Th17 cells in human autoimmune arthritis. *Arthritis Rheum* 62:2876-2885.

APPENDICES

1. Our paper published in J Immunol, 2010, 184:3233-3241, supports our finding presented in this progress report.

2. A recently published paper in Arthritis and Rheum Arthritis Rheum 2011, In press. Mar 7. [Epub ahead of print] also validates role of TH-17/IL-17 in experimental arthritis angiogenesis.

IL-17 Contributes to Angiogenesis in Rheumatoid Arthritis

Sarah R. Pickens,* Michael V. Volin,† Arthur M. Mandelin, II,* Jay K. Kolls,‡ Richard M. Pope,*,§ and Shiva Shahrara*

Angiogenesis is an early and a critical event in the pathogenesis of rheumatoid arthritis (RA). Neovascularization is dependent on endothelial cell activation, migration and proliferation, and inhibition of angiogenesis may provide a novel therapeutic approach in RA. In this study, we document a novel role of IL-17 in mediating angiogenesis. Local expression of IL-17 in mouse ankles increases vascularity. We further demonstrate that IL-17 is angiogenic by showing its ability to promote blood vessel growth in Matrigel plugs in vivo. Additionally, IL-17, in concentrations present in the RA joint, induces human lung microvascular endothelial cell (HMVEC) migration mediated through the PI3K/AKT1 pathway. Furthermore, suppression of the PI3K pathway markedly reduces IL-17–induced tube formation. We also show that both IL-17–induced HMVEC chemotaxis and tube formation are mediated primarily through IL-17 receptor C. Neutralization of either IL-17 in RA synovial fluids or IL-17 receptor C on HMVECs significantly reduces the induction of HMVEC migration by RA synovial fluid. Finally, RA synovial fluid immunoneutralized with anti–IL-17 and antivascular endothelial growth factor does not reduce HMVEC migration beyond the effect detected by immunodepleting each factor alone. These observations identify a novel function for IL-17 as an angiogenic mediator in RA, supporting IL-17 as a therapeutic target in RA. ***The Journal of Immunology*****, 2010, 184: 3233–3241.**

T helper-17 cells are a newly discovered $CD4^+$ helper T cell subset that produce IL-17A (also known as IL-17). IL-17 binds to transmembrane receptors, which exist as IL-17 receptor A (IL-17RA) homodimers or IL-17RA/IL-17 receptor C (IL-17RC) heterodimers (1). IL-17 RA and RC are widely expressed in endothelial cells, B and T cells, fibroblasts, and monocytes (2, 3). TGF-β, IL-6, IL-1β, and IL-21 have been shown to drive the differentiation of Th-17 cells (4–6), although variation between humans and mice has been described previously (2, 6–8). In humans, IL-1β is the most effective inducer of Th-17 cell differentiation, although this process is enhanced by IL-6 and IL-23 (6).

IL-17 has been shown to have a profound effect in experimental models of arthritis. Local expression of IL-17 in mouse joints results in acute inflammation and cartilage proteoglycan depletion (9). The incidence and severity of collagen-induced arthritis was markedly attenuated in IL-17–deficient mice (10). IL-17 also plays an important role downstream of IL-1 signaling and in response to TLR4 ligands (11, 12). Spontaneous IL-1 receptor antagonist knockout mice develop a polyarthritis, which is completely suppressed when these mice are crossed with IL-17–deficient mice (13), suggesting that Th-17 cell differentiation may be the reason for the arthritis.

IL-17 is found in rheumatoid arthritis (RA) synovial tissue and fluid, and the percentage of Th-17 cells is significantly higher in RA synovial fluid compared with RA or normal peripheral blood (3, 14). IL-17 may play an important role in the pathogenesis of RA by inducing the production of proinflammatory cytokines and chemokines from RA synovial tissue fibroblasts including IL-6, CXCL8/IL-8, CCL2/MIP-3α, CXCL1/growth-related oncogene-α, and CXCL2/growth-related oncogene-β (15–17). IL-17 is also capable of activating macrophages to expression of IL-1, TNF-α, cyclooxygenase 2, PGE_2, and matrix metalloproteinase-9 (18–20). We recently demonstrated that IL-17 is directly chemotactic for monocytes (21), and others have shown that IL-17 activates neutrophil recruitment through the stimulation of chemokines such as CXCL1 in rats and CXCL8/IL-8 in humans (22, 23).

Angiogenesis, the development of new capillaries, is involved in leukocyte ingress into the synovium during the development and progression of RA (24, 25). However, the role of IL-17 in angiogenesis is undefined. There are contradictory results regarding the role of IL-17 on tumor development and angiogenesis. In mice, tumors transfected with IL-17 demonstrated markedly less growth compared with control tumors (26). Additionally, tumor growth and lung metastasis were increased in IL-17–deficient mice (27), suggesting that IL-17 inhibits tumor development and neovascularization. Others have shown that IL-17 increases blood vessel development in rat cornea and tumor vascularity in animal models, indicating that IL-17 may be important for angiogenesis (28). However, there are also data to suggest that IL-17 alone is unable to induce angiogenesis but can indirectly mediate human lung microvascular endothelial cell (HMVEC) growth by promoting the mitogenic activity of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (29, 30). Therefore, on the basis of the existing data, it is unclear whether IL-17 can promote angiogenesis in RA and whether IL-17 acts directly on endothelial cells through

*Division of Rheumatology, Department of Medicine, Feinberg School of Medicine, Northwestern University; §Jesse Brown Veterans Affairs Hospitals, Chicago Healthcare System, Chicago, IL 60611; †Department of Microbiology and Immunology, Chicago College of Osteopathic Medicine, Midwestern University, Downers Grove, IL 60515; and ‡Department of Genetics, Louisiana State University Health Sciences Center, New Orleans, LA 70112

Received for publication October 6, 2009. Accepted for publication January 13, 2010.

This work was supported in part by grants from the National Institutes of Health (AR056099, AR055240, AR048269, and NS34510), the Arthritis National Research Foundation, and Within Our Reach from The American College of Rheumatology.

Address correspondence and reprint requests to Dr. Shiva Shahrara, Division of Rheumatology, Department of Medicine, Northwestern University Feinberg School of Medicine, McGaw Pavilion, 240 East Huron Street, Suite M300, Chicago, IL 60611. E-mail address: s-shahrara@northwestern.edu

Abbreviations used in this paper: Ad, adenovirus; bFGF, basic fibroblast growth factor; EBM, endothelial basal medium; Endo, endothelial cell; FGF, fibroblast growth factor; HEK, human embryonic kidney; HMVEC, human lung microvascular endothelial cell; IL-17RA, IL-17 receptor A; IL-17RC, IL-17 receptor C; p, phospho; RA, rheumatoid arthritis; VEGF, vascular endothelial growth factor.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0903271

binding to its receptors or indirectly by inducing proangiogenic factors from cells present in the RA synovium.

In the current study, we have therefore evaluated the role of IL-17 in HMVEC migration and tube formation as well as blood vessel development. Our results demonstrate that local expression of IL-17 increases vascularity in mouse ankle joints. Using a Matrigel plug assay, we demonstrate that IL-17 enhances blood vessel formation in vivo. Although HMVECs express both IL-17RA and RC, IL-17–induced HMVEC chemotaxis and tube formation are mediated by binding primarily to IL-17RC and activation of the PI3K pathway. We report that RA synovial fluid-mediated endothelial migration is significantly reduced by IL-17 and/or IL-17RC neutralization. Finally, we show that IL-17 and VEGF neutralization in RA synovial fluid does not significantly reduce HMVEC migration beyond the effect of one factor alone. Hence, therapy directed against IL-17 may reduce leukocyte migration by inhibiting angiogenesis in RA.

Materials and Methods

Study protocol for local expression of IL-17 in mouse ankle joints

The animal studies were approved by the Northwestern University Institutional Review Board. Adenovirus (Ad) constructed as reported previously to contain an IL-17 payload was provided by J. K. Kolls (Louisiana State University Health Science Center, New Orleans, LA) (31). Four- to six-week-old C57BL/6 mice were injected intra-articularly with 10^7 PFU Ad-IL-17 or Ad-CMV control. Ankle circumference and articular index score were determined on days 4 and 10 post–Ad-IL-17 injection and compared with the control group (data not shown). Ankles were harvested on day 10 post–Ad-IL-17 injection for histological studies. Levels of IL-17 were quantified by ELISA on days 4 and 10 from ankles treated with Ad-IL-17 or Ad-CMV control.

Abs and immunohistochemistry

Mouse ankles were decalcified, formalin fixed and paraffin embedded, and sectioned in the pathology core facility of Northwestern University. Mouse ankles were immunoperoxidase stained using Vector Elite ABC Kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen. Slides were deparaffinized in xylene for 20 min at room temperature, followed by rehydration by transfer through graded alcohols. Ags were unmasked by first incubating slides in boiling citrate buffer for 15 min, followed by type II trypsin digestion for 30 min at 37˚C. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 5 min. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories). Nonspecific binding of Abs to the tissues was blocked by pretreatment of tissues with diluted normal goat serum. Tissues were incubated with rabbit polyclonal Ab to von Willebrand factor (1/100 dilution; Serotec, Oxford, U.K.) or a rabbit IgG control Ab (Beckman Coulter, Fullerton, CA). Slides were counterstained with Harris hematoxylin and treated with lithium carbonate for bluing. Each slide was evaluated by a blinded observer (32–35) (M.V.V.). Tissue sections were assigned a vascular score representing the number of blood vessels in each section. A semiquantitative score of 1 represented a tissue with few blood vessels, whereas a score of 4 represented a highly vascularized tissue. Scored data were pooled, and the mean ± SEM was calculated in each data group (n = 5).

Matrigel plug assay in vivo

To examine the effect of IL-17 on angiogenesis in vivo, we used a Matrigel plug assay. Four- to six-week-old C57BL/6 mice were injected s.c. in the dorsal area with 500 μl Matrigel. Matrigel plus PBS served as negative control, Matrigel containing bFGF (20 ng/ml) served as positive control, and experimental conditions included Matrigel with mouse recombinant IL-17 (2 μg). After 10 d, mice were sacrificed, Matrigel plugs were carefully dissected out, surrounding connective tissue was removed, and plugs were analyzed for vascularity by hemoglobin measurement or by histology. For hemoglobin measurement, plugs were weighed by placing them into 1 ml preweighed double-distilled H_2O and then homogenized for 5–10 min on ice and spun. Using methemoglobin, serial dilutions were prepared to generate a standard curve from 70 to 1.1 g/dl (36, 37). Fifty microliters of supernatant or standard was added to a 96-well plate in duplicate, and 50 μl tetramethylbenzidine was added to each sample. The plate was allowed to develop at room temperature for 15–20 min with gentle shaking, and the reaction was terminated with 150 μl 2 N H_2SO_4 for 3–5 min. Absorbance was read with an ELISA plate reader at 450 nm. To calculate hemoglobin concentrations, the values (grams per deciliter) were normalized to the weights of the plugs (grams) (36, 37). On day 10, IL-17 concentrations were quantified in Matrigel plugs harvested from the IL-17–treated group using ELISA.

For histology, plugs were embedded in paraffin and sectioned in the pathology core facility of Northwestern University. Histology slides from different groups were examined by H&E and Masson's trichrome staining (37, 38) and scored by a blinded observer (A.M.M.) based on a scale of 0–4, where 0 = no tubules, 1 = tubules only, 2 = tubules containing RBCs, 3 = tubules with multilayer wall, and 4 = tubules with multilayer walls with connective tissues surrounding them.

HMVEC chemotaxis

To examine chemotaxis, HMVECs were incubated in endothelial basal medium (EBM) with 0% FBS and no growth factors for 2 h before use. HMVECs (1.25×10^4 cells/25 μl EBM with 0.1% FBS) were then placed in the bottom wells of a 48-well Boyden chemotaxis chamber (NeuroProbe, Cabin John, MD) with gelatin-coated polycarbonate membranes (8-μm pore size; Nucleopore, Pleasant, CA) (37, 39). The chambers were inverted and incubated at 37˚C for 2 h, allowing endothelial cell attachment to the membrane. The chamber was reinverted, and PBS, positive control VEGF (10 ng/ml; R&D Systems, Minneapolis, MN), or IL-17 at varying concentrations from 0.001 to 100 ng/ml (R&D Systems) was added to the upper wells, and the chamber was further incubated for 2 h at 37˚C. The membranes were then removed, fixed, and stained with the Protocol HEMA 3 stain set (122-911; Fisher Scientific, Waltham, MA). Readings represent the number of cells migrating through the membrane (the average of three high-power ×40 fields/well, averaged for each triplicate of wells). To test specificity of IL-17–induced HMVEC migration, HMVEC chemotaxis was examined with heat-inactivated IL-17 (10 and 50 ng/ml incubated at 100˚C for 15 min) or IL-17 neutralized by anti–IL-17 Ab or IgG control (10 μg/ml for 1 h in 37˚C) (R&D Systems) (21). To examine for chemokinesis, a series of checkerboard experiments were performed by placing increasing concentrations of IL-17 (0, 0.01, 1, and 10 ng/ml) together with HMVECs in the bottom wells with different concentrations of IL-17 in the top wells. To determine which IL-17 receptors are important for IL-17 HMVEC migration, Abs to IL-17RA (R&D MAB177; 10 μg/ml), IL-17RC (R&D AF2269; 10 μg/ml), both Abs, or IgG control were added to HMVECs, incubated for 1 h at 37˚C, and further incubated for 2 h in the Boyden chamber with IL-17 (50 ng/ml). To define which signaling pathway(s) mediated IL-17–induced HMVEC chemotaxis, HMVECs were incubated with inhibitors to PI3K (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), JNK (SP600125; 1 and 5 μM), or DMSO for 2 h in the Boyden chamber with IL-17 (50 ng/ml). HMVEC chemotaxis induced by RA synovial fluids was examined following 1-h incubation (37˚C) of fluids with control IgG or neutralizing anti–IL-17 Ab (10 μg/ml). The fluids were diluted 1/20 before addition to the top wells (21). To examine whether IL-17 receptors are involved in RA synovial fluid-induced HMVEC chemotaxis, HMVECs were incubated with Abs to IL-17RA and RC (10 μg/ml) or both Abs, as well as IgG control (1 h at 37˚C), before adding the RA synovial fluid to the top wells (21). HMVEC chemotaxis was performed to examine the synergistic effect of IL-17 and/or VEGF in RA synovial fluid induced HMVEC migration. Chemotaxis induced by RA synovial fluids was examined following incubation of fluids (diluted 1/20) (n = 8 fluids) with control IgG, anti–IL-17, anti-VEGF, or both Abs (10 μg/ml) for 1 h prior to performing the assay.

Quantification of IL-17RA and RC expression on HMVECs

Skin and lung HMVECs, HUVECs, and human embryonic kidney (HEK) 293 were cultured in EGM-2, EGM, and 10% DMEM, respectively. Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from all different cell types. Subsequently, reverse transcription and real-time RT-PCR were performed to determine IL-17RA and IL-17RC expression level as described previously (14, 21, 40). Relative gene expression was determined by the $\Delta\Delta C_t$ method, and results were expressed as fold increase above levels detected in HEK 293 cells.

Characterization of IL-17 signaling pathways in HMVECs

HMVECs (passages 3–8) were grown to 80% confluence in EGM-2 MV bullet kit (Lonza, Walkersville, MD). Cells were then switched to EGM-MV (EBM plus SingleQuots of growth supplements) for 2 d and were incubated in EBM with 0% FBS for 2 h prior to treatment. Cells were then untreated or treated with IL-17 (50 ng/ml) for 15–75 min. Cell lysates were examined by Western blot analysis, as described previously (21, 40, 41). Blots were probed with phospho (p)-AKT, pERK, and pJNK (1/1000 dilution; Cell Signaling Technology, Beverly, MA) overnight and after stripping were probed with AKT, ERK, and JNK (1/3000 dilution; Cell Signaling Technology) overnight.

HMVEC tube formation assay

To perform the Matrigel tube formation assay, BD Matrigel Matrix (BD BioSciences, Bedford, MA; 50 μl) was polymerized for 30 min at 37°C in a 96-well plate. To determine which IL-17 receptors play a role in IL-17–induced HMVEC tube formation, trypsinized HMVECs were resuspended (4×10^5 cells/ml) in EBM without growth factors with 2% FBS and incubated with Abs to IL-17RA, IL-17RC, both IL-17RA and RC or IgG for 45 min at 37°C. Cells were then added to polymerized Matrigel, IL-17 (50 ng/ml) was added to the wells, and the plate was incubated for 16 h at 37°C. To examine which signaling pathways contribute to IL-17–mediated HMVEC tube formation, cells were incubated with inhibitors to PI3K (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), JNK (SP600125; 1 and 5 μM), or DMSO for 45 min at 37°C prior to adding to polymerized Matrigel. As above, IL-17 (50 ng/ml) was then added to the wells, and the plate was incubated for 16 h at 37°C. Each condition was performed in triplicate, fibroblast growth factor (FGF) (20 ng/ml) was used as a positive control, and PBS was used as a negative control. Following incubation, culture medium was removed, and cells were washed with HBSS. Thereafter, tube formation was quantified using calcein-AM fluorescent dye (BD BioSciences) diluted with HBSS and DMSO, according to the manufacturer's instructions, for 30 min. Subsequently, the plates were washed with HBSS, and the number of branch points/tubes was quantified as described previously (36, 37).

Statistical analysis

The data were analyzed using two-tailed Student *t* tests for paired and unpaired samples. Values of $p < 0.05$ were considered significant.

Results

Local expression of IL-17 in mouse ankles induces joint inflammation and vascularity

Local expression of IL-17 using an adenoviral vector (10^7 PFU) resulted in increased inflammation, synovial lining thickness, and bone erosion in the ankles of C57/BL6 mice, compared with Ad-CMV–infected controls (10^7 PFU) (data not shown). The Ad-IL-17–treated group demonstrated significantly greater ankle circumference (data not shown) on days 4 and 10 postinjection compared with the control group. Von Willebrand factor staining of ankles harvested from day 10 postinjection demonstrated that Ad-IL-17–treated mice have significantly higher endothelial staining compared with the control group (Fig. 1). The concentration of joint IL-17 in the IL-17–induced arthritis model was 1200 and 400 pg/mg compared with 47 and 31 pg/mg detected in the Ad-CMV control group on days 4 and 10 post-Ad injection. These results suggest that IL-17 may be important for angiogenesis in vivo.

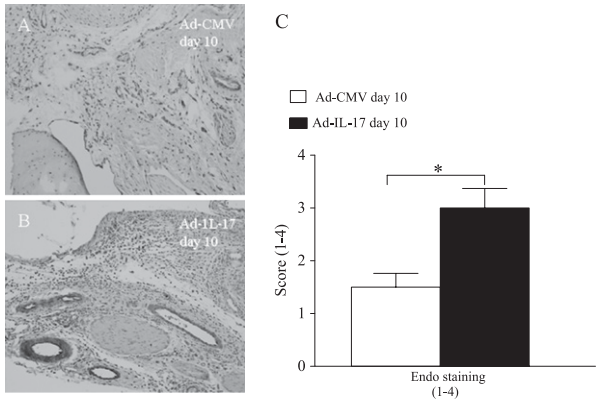


FIGURE 1. Local expression of IL-17 increases vascularity in mouse ankles. Ad-IL-17 or Ad-CMV control was injected intra-articularly into the ankle joints of 4–6-wk-old C57BL/6 mice. Ankles from day 10 post-Ad injection were harvested, paraffin embedded and decalcified. Ankles were stained with Von Willebrand factor for identifying endothelial cells (Endo) and scored on a 1–4 scale. Control ankles (*A*) had significantly lower endothelial staining compared with ankles locally expressing IL-17 (original magnification ×200) (*B*). *C*, Quantification of each of the parameters in the Ad-CMV control and Ad-IL-17 groups. Values demonstrate mean ± SE; $n = 5$. $*p < 0.05$.

IL-17 induces angiogenesis in vivo in Matrigel plugs

The role of IL-17 on angiogenesis in vivo was assessed by determining its effect on blood vessel formation in Matrigel plugs in mice by using hemoglobin quantification, as well as Masson's trichrome staining. The hemoglobin content of the IL-17–treated group was 10 times greater ($p < 0.05$) than the PBS control (Fig. 2*A*). Matrigel blood vessel formation was also examined histologically by using H&E (Fig. 2*C*, 2*E*, 2*G*) and Masson's trichrome staining (Fig. 2*D*, 2*F*, 2*H*). The histological analysis demonstrated that IL-17 markedly enhances ($p < 0.05$) blood vessel growth compared with the control group (Fig. 2*B*). The levels of IL-17 detected in Matrigel plugs harvested on day 10 were 198 ± 35 pg/ml, which is within the range detected in IL-17–induced arthritis model as well as in the RA synovial fluid (mean was 233 pg/ml). These results support the role of IL-17 in angiogenesis in vivo.

IL-17 induces endothelial (HMVEC) migration

Experiments were performed to determine whether IL-17 is directly chemotactic for endothelial cells. For this purpose, chemotaxis was performed in a Boyden chamber with varying concentrations of IL-17, as well as positive (VEGF; 10 ng/ml) and negative (PBS) controls. IL-17 was chemotactic for HMVECs at concentrations ranging from 0.01 ng/ml ($p < 0.05$) to 100 ng/ml ($p < 0.05$) ($n = 5$) (Fig. 3*A*). The mean concentration of IL-17 in the 30 RA synovial fluids analyzed was 233 ± 64 pg/ml (21), a concentration that was highly chemotactic for HMVEC migration. IL-17's endotoxin levels were quantified by using *Limulus* amebocyte cell lysate assay. At a concentration of IL-17 10-fold higher than that used in the HMVEC chemotaxis assay (500 ng/ml), the level of endotoxin was below the detection limit for this assay (<0.01 ng/ml LPS) (data not shown), suggesting that our results are specifically due to IL-17 and not endotoxin contamination. Supporting this interpretation, heat inactivation of IL-17 or incubation of IL-17 (50 ng/ml) with neutralizing Ab to IL-17 suppressed HMVEC migration (Fig. 3*B*).

We next determined the effect of IL-17 on chemokinesis. In the absence of IL-17 in the lower chamber, IL-17 in the upper chamber was chemotactic for HMVECs. When equivalent or higher concentrations of IL-17 were present in the lower compartment, no enhanced migration of HMVECs occurred (Fig. 3*C*). Taken together, our results suggest that IL-17 is chemotactic, not chemokinetic, for endothelial cells.

IL-17 receptors are involved in IL-17–induced HMVEC chemotaxis and HMVEC tube formation

Our results demonstrate that skin and lung HMVECs as well as HUVECs express significantly higher levels of IL-17RA and IL-17RC compared with HEK 293 cells (Fig. 4*A*, 4*B*). These experiments were performed to determine which IL-17 receptor is involved in HMVEC chemotaxis and tube formation. Although some reduction of HMVEC chemotaxis was noted with an anti–IL-17RA Ab, it was not significant. Inhibition of chemotaxis was significant when IL-17RC (40%) or both receptors (47%) were neutralized (Fig. 5*A*). IL-17 also induced HMVEC tube formation in Matrigel. Using concentrations of IL-17 ranging from 0.001 to 100 ng/ml, the optimal concentration of IL-17 to induce HMVEC tube formation was 50 ng/ml ($p < 0.05$) (data not shown). Consistent with the HMVEC chemotaxis data, neutralization of IL-17RC (30%) or both IL-17 receptors (45%) on HMVECs significantly decreased ($p < 0.05$) IL-17–mediated HMVEC tube formation (Fig. 5*B–H*). Blockade of IL-17RA alone was inefficient in this process. Collectively, our data suggest that IL-17RC plays a more important role

in IL-17–mediated HMVEC chemotaxis and tube formation compared with IL-17RA.

IL-17 activates ERK, JNK, and PI3K pathways in HMVECs; however only inhibition of PI3K reduces IL-17-induced HMVEC chemotaxis and tube formation

To determine which signaling pathways in HMVECs are activated by IL-17, phosphorylation of the MAPK and AKT pathways was determined by Western blot analysis. Our data demonstrate that IL-17 phosphorylates ERK, JNK, and AKT1 as early as 15 min. Although the activation of ERK and JNK is gradually reduced at 75 and 65 min (Fig. 6*B*, 6*C*), respectively, AKT1 is still strongly phosphorylated at 75min (Fig. 6*A*). The p38 pathway was not activated by IL-17 in HMVECs (data not shown). To determine which signaling pathways mediate HMVEC migration, chemical inhibitors at concentrations of 1 and 5 μM were used, whereas 10 μM was toxic and resulted in cell death, as determined by trypan blue staining (data not shown). Inhibition of ERK and JNK was ineffective in suppressing IL-17–induced HMVEC chemotaxis, whereas inhibition of PI3K reduced ($p < 0.05$) chemotaxis starting at 1 μM (Fig. 6*D*). Similarly, although inhibition of PI3K (starting at 1 μM) reduced IL-17–mediated tube formation by 30–40% ($p < 0.05$) (Fig. 7), suppression of ERK and JNK had no effect on this process. These results suggest that IL-17–induced HMVEC chemotaxis and tube formation are mediated through the PI3K/AKT1 pathway.

IL-17 and its receptor are involved in RA synovial fluid-mediated HMVEC chemotaxis

Next, we asked whether the IL-17 identified in human RA synovial fluid is chemotactic for HMVECs. In these experiments, we demonstrated that human RA synovial fluid is chemotactic for HMVECs, similar to positive control VEGF (Fig. 8*A*). Furthermore, human RA synovial fluid immunodepleted with anti–IL-17 significantly reduced HMVEC chemotaxis ($p < 0.05$), compared with control IgG-treated fluids. Neutralization of IL-17RA and RC on HMVECs showed that only IL-17RC was effective in suppressing human RA synovial fluid-mediated HMVEC migration (Fig. 8*B*). These results suggest that IL-17 and its receptor IL-17RC may play an important role in angiogenesis in RA. Furthermore, we demonstrate that immunodepletion of IL-17 and VEGF in RA synovial fluids does not have an additive or synergistic effect in reduction of HMVEC migration beyond the effect noted with neutralization of one factor alone (Fig. 8*C*), suggesting that both IL-17 and VEGF may be mediating HMVEC migration through the same signaling pathway.

Discussion

In this study, we show that IL-17 increases vascularity in experimental arthritis and induces blood vessel development in Matrigel plugs in vivo. Therefore, studies were performed to determine whether IL-17 might directly mediate angiogenesis and whether IL-17 contributes to neovascularization in RA. Our data demonstrate that IL-17 induces HMVEC chemotaxis at concentrations present in human RA synovial fluid. This effect is directly mediated by IL-17, because heat inactivation and neutralizing Abs to IL-17 and/or IL-17 receptors abrogate IL-17–induced HMVEC chemotaxis. We further demonstrate that IL-17–induced HMVEC chemotaxis and tube formation are mediated primarily through ligation to IL-17RC on HMVECs and activation of PI3K. We show that human RA synovial fluid-mediated HMVEC chemotaxis is markedly reduced by neutralization of IL-17 in the synovial fluids or blocking of IL-17RC on HMVECs. Last, we demonstrate that IL-17 and VEGF-immunodepleted RA synovial fluid does not reduce HMVEC chemotaxis any further than neutralization of each factor by itself.

In this paper, we confirm the results of others demonstrating that the local expression of IL-17 in mouse ankle joints induces arthritis (9). Histological analysis of mice receiving intra-articular injections of IL-17 demonstrated that IL-17 plays an important role in joint neutrophil migration (42). We demonstrate that IL-17–induced arthritis is associated with increased vascularity. Others have shown that IL-17 can promote tumor growth by upregulating proangiogenic factors such as VEGF and matrix metalloproteinase-9 from tumor cells, suggesting that IL-17 is indirectly associated with angiogenesis (43). We have also shown that forced ectopic expression of IL-17 induces expression of proangiogenic CXC (ELR^+) chemokines in mouse ankles (S. Pickens and S. Shahrara, unpublished data).

On the basis of our results from the IL-17–induced arthritis model, we hypothesized that IL-17 may be important for angiogenesis in RA. Because there is some evidence demonstrating that

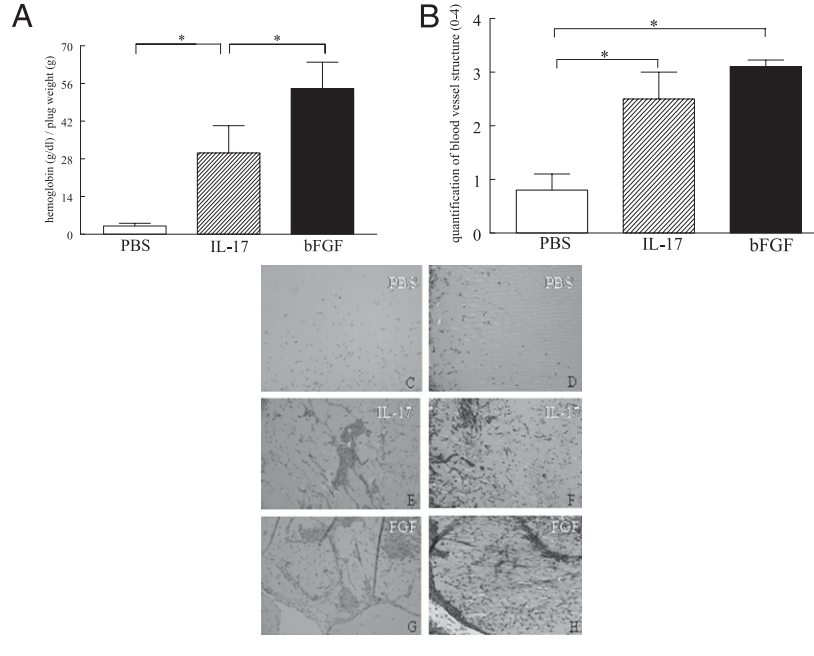
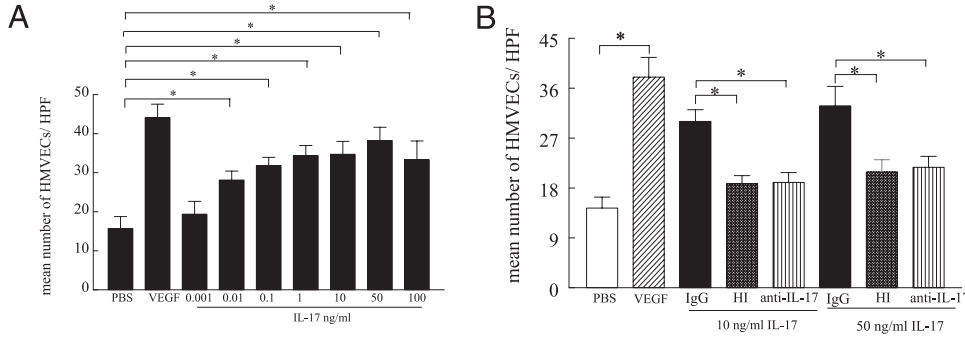


FIGURE 2. IL-17 enhances blood vessel growth in Matrigel plugs in vivo. *A*, IL-17 (2 μg) induced angiogenesis in the Matrigel plugs to a significantly greater degree compared with PBS control. Matrigel containing bFGF (20 ng/ml) served as positive control. The values represent the concentration of hemoglobin (grams per deciliter)/plug weight (grams) ± SE, with $n = 10$. *B*, The histology was quantified on a score of 0–4 scale in Matrigel plugs that contained PBS, IL-17, or bFGF using H&E and Masson's trichrome staining. A representative assay shows H&E (*C*, *E*, *G*) and Masson's trichrome (*D*, *F*, *H*) staining of blood vessels in paraffin sections of Matrigel plugs containing PBS (*C*, *D*), IL-17 (*E*, *F*), and FGF (*G*, *H*) that was histologically scored in *B* (original magnification ×200). Values demonstrate mean ± SE, with $n = 5$. $*p < 0.05$.



C

Lower Chamber/HMVECs \ Upper chamber	0 ng/ml IL-17	0.01ng/ml IL-17	0.1 ng/ml IL-17	1 ng/ml IL-17	10 ng/ml IL-17
0 ng/ml IL-17	14±1.14	24.69±1.9	23.67±1.76	26.11±2.45	29.22±2.78
0.01 ng/ml IL-17	19±1.24	20.89±1.44	24.56±1.42	24.22±2.79	27.22±1.78
1 ng/ml IL-17	15±1.7	19.8±1.07	18.7±2	19.33±2.69	25±1.68
10 ng/ml IL-17	18±1.68	18.3±1.46	18.2±2.12	18.2±1.72	18.56±1.65

FIGURE 3. IL-17 induces HMVEC migration and not chemokinesis. *A*, Dose-response curve of IL-17–induced HMVEC chemotaxis. IL-17 HMVEC chemotaxis was performed in a Boyden chemotaxis chamber with varying concentrations of IL-17. Values demonstrate mean ± SE from five different experiments. *B*, IL-17–induced HMVEC chemotaxis was suppressed by heat inactivating IL-17 (both 10 and 50 ng/ml incubated in 100°C for 15 min) or neutralization of IL-17 (10 and 50 ng/ml) by anti–IL-17 Ab but not by IgG control (10 μg/ml 1 h in 37°C) for 2 h. Values are the mean ± SE from three different experiments. $*p < 0.05$. *C*, A series of checkerboard experiments was performed by placing increasing doses of IL-17 together with HMVECs in the lower well in addition to placing different concentrations of IL-17 in the top well of the chemotaxis chamber. The experiment was read at 2 h. Results are representative of three different experiments.

IL-17 alone is unable to induce angiogenesis but can indirectly promote HMVEC chemotaxis by producing proangiogenic factors (29, 30) from RA synovial tissue fibroblasts, we investigated the role of IL-17 on HMVEC migration and tube formation. Our results demonstrate that IL-17 induces HMVEC chemotaxis at concentrations available in the human RA joint, which is mostly due to its ligation to IL-17RC. Although IL-17RC plays a major role in IL-17–mediated HMVEC chemotaxis and tube formation, neutralization of both receptors is more effective in this process compared with IL-17RC alone. Like monocytes, HMVECs express both IL-17RA and IL-17RC (21). However, in contrast to HMVEC chemotaxis, IL-17–mediated monocyte migration is induced through both IL-17RA and RC (21). Interestingly, a novel IL-17 receptor-like protein has been identified in HUVECs that interacts with FGF1 and inhibits activation of the ERK pathway and production of FGF, indicating that various IL-17 receptors may modulate angiogenesis differently (44).

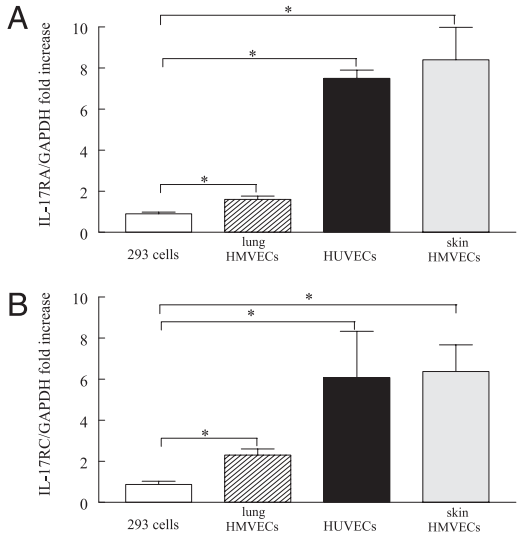


FIGURE 4. HMVECs express IL-17RA (*A*) and RC (*B*). Skin and lung HMVECs, HUVECs, and HEK 293 were cultured and real-time RT-PCR was performed to determine IL-17RA and IL-17RC expression level. The relative gene expression levels were normalized by GAPDH and determined by the $\Delta\Delta C_t$ method, and results were expressed as fold increase above levels detected in HEK 293 cells. Values are the mean ± SE, $n = 3–6$. $*p < 0.05$.

Next, experiments were performed to investigate signaling pathways that were associated with IL-17–induced HMVEC chemotaxis and tube formation. Inhibition of the IL-17–activated pathways in HMVECs demonstrated that only activation of PI3K significantly reduces IL-17–mediated chemotaxis and tube formation, and suppression of ERK and JNK pathways was ineffective in this process. Consistently, CCL2/MCP-1–mediated endothelial chemotaxis is through activation of PI3K, as well as the ERK pathway (45). Furthermore, others have shown that PI3K signaling plays an important role in regulation of VEGF production as well as VEGF-mediated endothelial migration (46, 47), suggesting that PI3K is involved in the mediation of angiogenesis by various proinflammatory factors. A recently published article shows that increased expression of IL-17 in IFN$\gamma^{-/-}$ mice mediates tumor growth and angiogenesis through STAT3 phosphorylation (43). In contrast to IL-17, which promotes angiogenesis, IL-17F inhibits HUVEC tube formation, indicating that different IL-17 isoforms may modulate angiogenesis differently (48).

A recent publication demonstrated that tumor growth in s.c. and lung metastases are enhanced in IL-17$^{-/-}$ mice compared with the wild-type controls, suggesting that IL-17 may suppress tumor

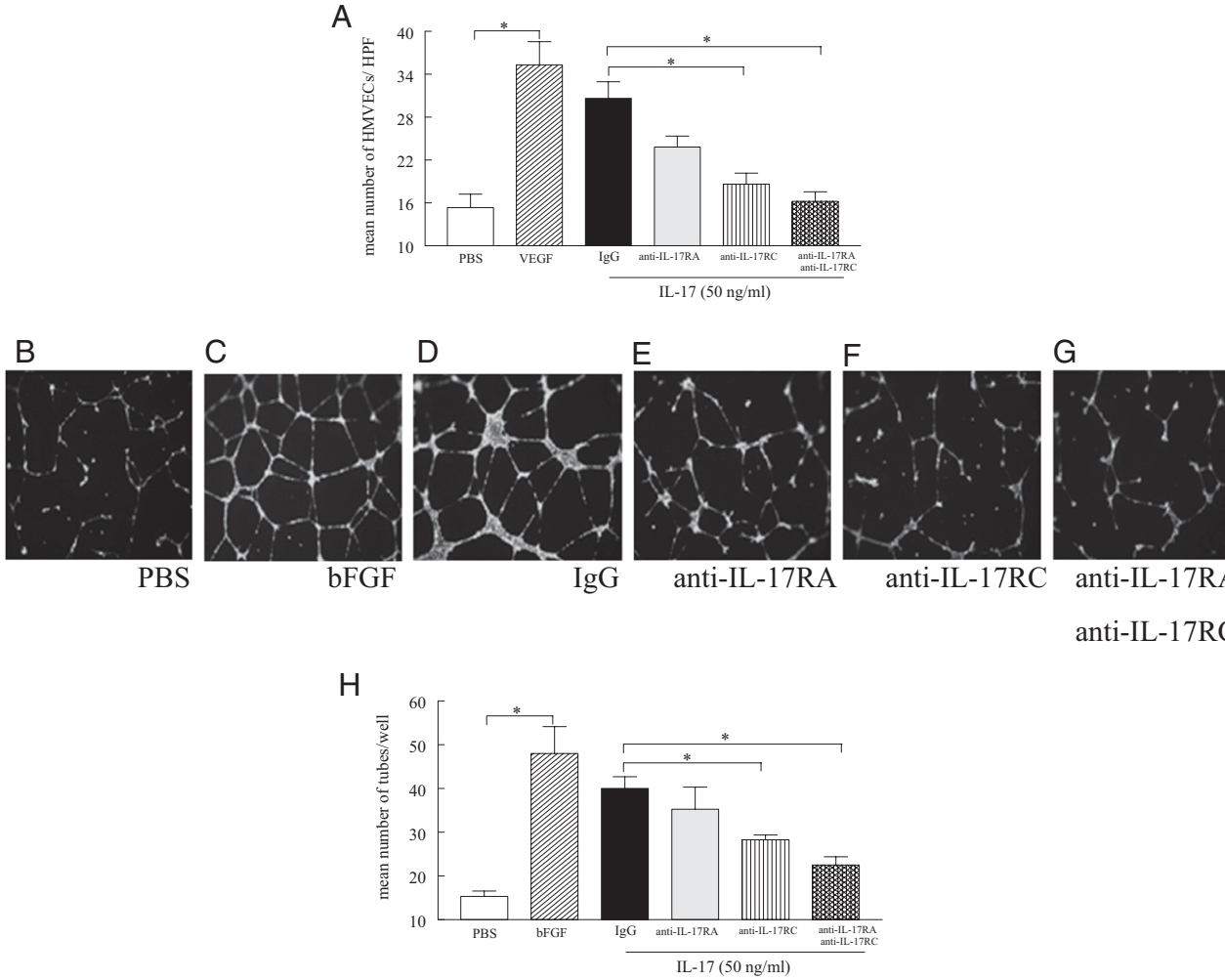


FIGURE 5. IL-17–mediated HMVEC chemotaxis and tube formation are regulated through both IL-17RA and IL-17RC. *A*, HMVECs were incubated with mouse anti-human IL-17RA and IL-17 RC Abs (10 μg/ml) or control IgG (10 μg/ml) for 1 h. Thereafter, HMVEC chemotaxis was performed in response to IL-17 (50 ng/ml) for 2 h. PBS was used as a negative control and VEGF (60 nM) as a positive control. HMVECs were incubated with Abs to IL-17RA, IL-17RC, both IL-17RA and RC, or IgG for 45 min at 37˚C. Cells were then added to polymerized Matrigel, IL-17 (50 ng/ml), placed in the wells, and the plate was incubated for 16 h at 37˚C (in triplicate). Photomicrographs (original magnification ×100) taken of representative wells treated with PBS (*B*), FGF (20 ng/ml) (*C*), IL-17 (50 ng/ml) plus IgG (*D*), IL-17 (50 ng/ml) plus anti–IL-17RA (10 μg/ml) (*E*), IL-17 (50 ng/ml) plus anti–IL-17RC (10 μg/ml) (*F*), and IL-17 (50 ng/ml) plus anti–IL-17RA and RC (10 μg/ml) (*G*) in which IL-17–induced tube formation is significantly reduced by the neutralization of IL-17RC or both receptors ($p < 0.05$). *H*, Data presented demonstrates mean number of branch points/tubes in each treatment group. Values are the mean ± SE, $n = 3$. $*p < 0.05$.

development (27). Conversely, others have shown that IL-17 markedly increases neovascularization in rat cornea (28) and vascularization in tumors (49), indicating that IL-17 may promote angiogenesis. However, there is also evidence demonstrating that IL-17 induces production of proangiogenic factors including NO, hepatocyte growth factor, CXCL1/KC, CXCL2/MIP-2, PGE_1,

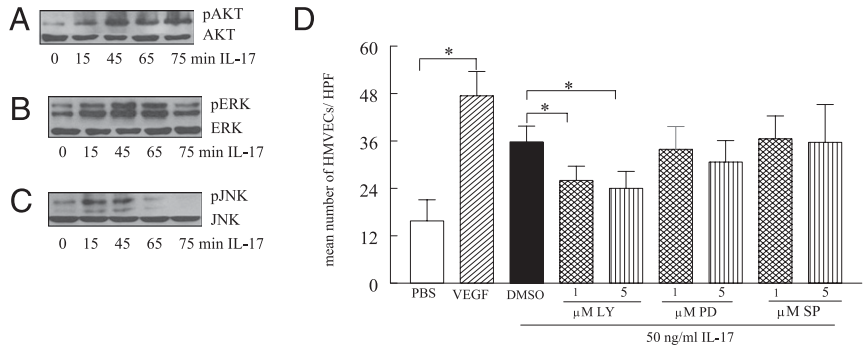


FIGURE 6. IL-17–induced HMVEC migration is suppressed by PI3K inhibition. To determine the mechanism of IL-17 in HMVECs, cells were stimulated with IL-17 (50 ng/ml) for 0–75 min, and the cell lysates were probed for p-AKT (*A*), pERK (*B*), or pJNK (*C*). These results are representative of three experiments. *D*, To determine signaling pathways associated with IL-17 HMVEC migration, cells were treated with the identified chemical inhibitors for PI3K (LY294002; 1 and 5 μM) or ERK (PD98059; 1 and 5 μM) as well as JNK (SP600125; 1, 5 μM) 2 h in the Boyden chamber. Only inhibition of PI3K downregulated IL-17–induced HMVEC migration. Values demonstrate mean ± SE of three experiments in triplicate. $*p < 0.05$.

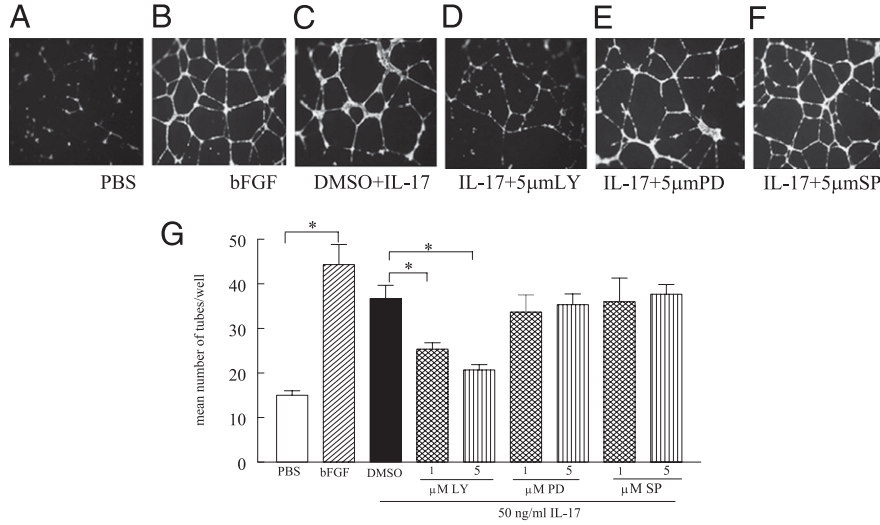


FIGURE 7. IL-17–mediated tube formation is reduced by inhibition of PI3K. HMVECs were incubated with inhibitors to PI3K (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), JNK (SP600125; 1 and 5 μM), or DMSO for 45 min at 37°C prior adding to polymerized Matrigel. IL-17 (50 ng/ml) was then added to the wells, and the plate was incubated for 16 h at 37°C (in triplicate). Photomicrographs (original magnification ×100) taken of representative wells treated with PBS (*A*), FGF (20 ng/ml) (*B*), IL-17 (50 ng/ml) plus DMSO (*C*), IL-17 (50 ng/ml) plus LY294002 (5 μM) (*D*), IL-17 (50 ng/ml) plus PD98059 (5 μM) (*E*), and IL-17 (50 ng/ml) plus SP600125 (5 μM) (*F*) in which IL-17–induced tube formation is significantly reduced by the inhibition of PI3K/AKT1 pathway ($p < 0.05$). *G* demonstrates mean number of tubes per well where LY294002 (1 and 5 μM; PI3K/AKT1 inhibitor) significantly reduces the number of branch points induced by IL-17 activation in Matrigel tube formation assay, whereas ERK (PD98059; 1 and 5 μM) and JNK inhibitors (SP600125; 1 and 5 μM) were ineffective. Values are the mean ± SE, $n = 3$. *$p < 0.05$.

PGE_2, and VEGF from RA synovial fibroblasts, and the production of some of these factors is further enhanced by TNF-α (50). Therefore, we investigated the contribution of IL-17 to human RA synovial fluid-mediated HMVEC chemotaxis. Neutralization of IL-17 in RA synovial fluid partially reduced RA synovial fluid-mediated HMVEC chemotaxis. RA synovial fluid-mediated HMVEC chemotaxis was mediated through IL-17RC, confirming the importance of this receptor in IL-17–mediated angiogenesis. Interestingly, angiogenic factors present in human RA synovial fluid are mostly produced by RA synovial tissue fibroblasts (VEGF, bFGF, VCAM1, IL-6, and ELR^+ CXC chemokines) or macrophages (TNF-α, IL-8, and IL-1β) (25). IL-17 is the only

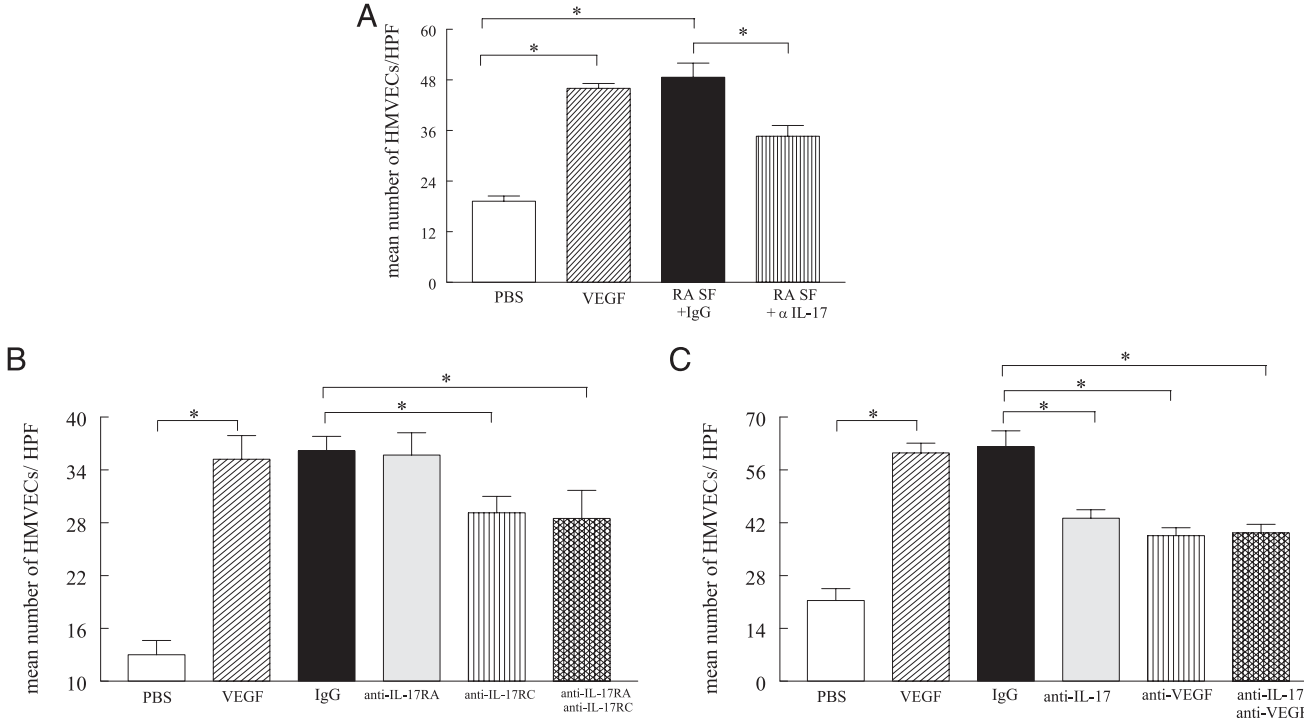


FIGURE 8. RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 through ligation to IL-17RC, and IL-17 does not synergize with VEGF in RA synovial fluid-induced HMVEC migration. *A*, Anti–IL-17 (10 μg/ml) or control IgG was added to RA synovial fluids from eight patients (1/20 dilution) (1 h at 37°C) prior to performing HMVEC chemotaxis in response to human RA synovial fluids. *B*, HMVECs were incubated with Abs to IL-17 RA and RC (10 μg/ml), as well as isotype control for 1 h prior to performing HMVEC chemotaxis in response to eight human RA synovial fluids. *C*, RA synovial fluids from eight patients (1/20 dilution) were incubated with Abs to IL-17 (10 μg/ml), VEGF (10 μg/ml), or both as well as isotype control or PBS or VEGF for 1 h prior to performing HMVEC chemotaxis in response to RA synovial fluids. The values represent the mean ± SE. *$p < 0.05$.

lymphokine that contributes to human RA synovial fluid-mediated angiogenesis, suggesting that T cells may also be important in this process. The data presented in this study, together with our previously reported evidence demonstrating that IL-17 is important for monocyte migration (21) in RA synovial fluids, highlight the importance of IL-17 in RA pathogenesis.

Interestingly, our results show that neutralization of IL-17 and VEGF do not synergize in reducing RA synovial fluid-induced HMVEC migration beyond the effect detected with one factor alone. As shown with IL-17, VEGF-induced HMVEC chemotaxis is mediated through PI3K (46, 51). Therefore, the lack of synergy between IL-17 and VEGF in inducing HMVEC chemotaxis may be due to that both mediators are using the same signaling pathway or that as shown previously HMVEC cell migration is within a bell shaped curve (37, 52); therefore, the synergistic effect could not be detected.

In conclusion, endothelial migration and tube formation induced by IL-17 were mediated through activation of the PI3K pathway and ligation to both IL-17 receptors. However, neutralization of only IL-17 or IL-17RC significantly downregulated human RA synovial fluid-mediated endothelial migration, suggesting that IL-17 plays an important role in RA angiogenesis.

Disclosures

The authors have no financial conflicts of interest.

References

1. Dong, C. 2008. TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat. Rev. Immunol.* 8: 337–348.
2. Nistala, K., and L. R. Wedderburn. 2009. Th17 and regulatory T cells: rebalancing pro- and anti-inflammatory forces in autoimmune arthritis. *Rheumatology (Oxford)* 48: 602–606.
3. Stamp, L. K., M. J. James, and L. G. Cleland. 2004. Interleukin-17: the missing link between T-cell accumulation and effector cell actions in rheumatoid arthritis? *Immunol. Cell Biol.* 82: 1–9.
4. Manel, N., D. Unutmaz, and D. R. Littman. 2008. The differentiation of human T(H)-17 cells requires transforming growth factor-β and induction of the nuclear receptor RORγt. *Nat. Immunol.* 9: 641–649.
5. Volpe, E., N. Servant, R. Zollinger, S. I. Bogiatzi, P. Hupé, E. Barillot, and V. Soumelis. 2008. A critical function for transforming growth factor-β, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat. Immunol.* 9: 650–657.
6. Annunziato, F., L. Cosmi, F. Liotta, E. Maggi, and S. Romagnani. 2009. Type 17 T helper cells—origins, features and possible roles in rheumatic disease. *Nat. Rev. Rheumatol.* 5: 325–331.
7. Gabay, C., and I. B. McInnes. 2009. The biological and clinical importance of the 'new generation' cytokines in rheumatic diseases. *Arthritis Res. Ther.* 11: 230.
8. Pernis, A. B. 2009. Th17 cells in rheumatoid arthritis and systemic lupus erythematosus. *J. Intern. Med.* 265: 644–652.
9. Koenders, M. I., E. Lubberts, F. A. van de Loo, B. Oppers-Walgreen, L. van den Bersselaar, M. M. Helsen, J. K. Kolls, F. E. Di Padova, L. A. Joosten, and W. B. van den Berg. 2006. Interleukin-17 acts independently of TNF-α under arthritic conditions. *J. Immunol.* 176: 6262–6269.
10. Nakae, S., A. Nambu, K. Sudo, and Y. Iwakura. 2003. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J. Immunol.* 171: 6173–6177.
11. Abdollahi-Roodsaz, S., L. A. Joosten, M. M. Helsen, B. Walgreen, P. L. van Lent, L. A. van den Bersselaar, M. I. Koenders, and W. B. van den Berg. 2008. Shift from toll-like receptor 2 (TLR-2) toward TLR-4 dependency in the erosive stage of chronic streptococcal cell wall arthritis coincident with TLR-4–mediated interleukin-17 production. *Arthritis Rheum.* 58: 3753–3764.
12. Koenders, M. I., I. Devesa, R. J. Marijnissen, S. Abdollahi-Roodsaz, A. M. Boots, B. Walgreen, F. E. di Padova, M. J. Nicklin, L. A. Joosten, and W. B. van den Berg. 2008. Interleukin-1 drives pathogenic Th17 cells during spontaneous arthritis in interleukin-1 receptor antagonist-deficient mice. *Arthritis Rheum.* 58: 3461–3470.
13. Nakae, S., S. Saijo, R. Horai, K. Sudo, S. Mori, and Y. Iwakura. 2003. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc. Natl. Acad. Sci. USA* 100: 5986–5990.
14. Shahrara, S., Q. Huang, A. M. Mandelin, II, and R. M. Pope. 2008. TH-17 cells in rheumatoid arthritis. *Arthritis Res. Ther.* 10: R93.
15. Katz, Y., O. Nadiv, and Y. Beer. 2001. Interleukin-17 enhances tumor necrosis factor α-induced synthesis of interleukins 1,6, and 8 in skin and synovial fibroblasts: a possible role as a "fine-tuning cytokine" in inflammation processes. *Arthritis Rheum.* 44: 2176–2184.
16. Chabaud, M., G. Page, and P. Miossec. 2001. Enhancing effect of IL-1, IL-17, and TNF-α on macrophage inflammatory protein-3α production in rheumatoid arthritis: regulation by soluble receptors and Th2 cytokines. *J. Immunol.* 167: 6015–6020.
17. Kehlen, A., K. Thiele, D. Riemann, and J. Langner. 2002. Expression, modulation and signalling of IL-17 receptor in fibroblast-like synoviocytes of patients with rheumatoid arthritis. *Clin. Exp. Immunol.* 127: 539–546.
18. Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicoeur, Y. He, M. Zhang, F. Mineau, and J. P. Pelletier. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-β and TNF-α, by human macrophages. *J. Immunol.* 160: 3513–3521.
19. Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, P. Reboul, Y. He, F. C. Jolicoeur, and J. P. Pelletier. 2001. Modulation of TIMP-1 synthesis by antiinflammatory cytokines and prostaglandin E_2 in interleukin 17 stimulated human monocytes/macrophages. *J. Rheumatol.* 28: 712–718.
20. Jovanovic, D. V., J. Martel-Pelletier, J. A. Di Battista, F. Mineau, F. C. Jolicoeur, M. Benderdour, and J. P. Pelletier. 2000. Stimulation of 92-kd gelatinase (matrix metalloproteinase 9) production by interleukin-17 in human monocyte/macrophages: a possible role in rheumatoid arthritis. *Arthritis Rheum.* 43: 1134–1144.
21. Shahrara, S., S. R. Pickens, A. Dorfleutner, and R. M. Pope. 2009. IL-17 induces monocyte migration in rheumatoid arthritis. *J. Immunol.* 182: 3884–3891.
22. Laan, M., Z. H. Cui, H. Hoshino, J. Lötvall, M. Sjöstrand, D. C. Gruenert, B. E. Skoogh, and A. Lindén. 1999. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J. Immunol.* 162: 2347–2352.
23. Luzza, F., T. Parrello, G. Monteleone, L. Sebkova, M. Romano, R. Zarrilli, M. Imeneo, and F. Pallone. 2000. Up-regulation of IL-17 is associated with bioactive IL-8 expression in *Helicobacter pylori*-infected human gastric mucosa. *J. Immunol.* 165: 5332–5337.
24. Szekanecz, Z., and A. E. Koch. 2007. Mechanisms of disease: angiogenesis in inflammatory diseases. *Nat. Clin. Pract. Rheumatol.* 3: 635–643.
25. Szekanecz, Z., and A. E. Koch. 2009. Angiogenesis and its targeting in rheumatoid arthritis. *Vascul. Pharmacol.* 51: 1–7.
26. Benchetrit, F., A. Ciree, V. Vives, G. Warnier, A. Gey, C. Sautès-Fridman, F. Fossiez, N. Haicheur, W. H. Fridman, and E. Tartour. 2002. Interleukin-17 inhibits tumor cell growth by means of a T-cell–dependent mechanism. *Blood* 99: 2114–2121.
27. Kryczek, I., S. Wei, W. Szeliga, L. Vatan, and W. Zou. 2009. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood* 114: 357–359.
28. Numasaki, M., J. Fukushi, M. Ono, S. K. Narula, P. J. Zavodny, T. Kudo, P. D. Robbins, H. Tahara, and M. T. Lotze. 2003. Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 101: 2620–2627.
29. Ryu, S., J. H. Lee, and S. I. Kim. 2006. IL-17 increased the production of vascular endothelial growth factor in rheumatoid arthritis synoviocytes. *Clin. Rheumatol.* 25: 16–20.
30. Honorati, M. C., S. Neri, L. Cattini, and A. Facchini. 2006. Interleukin-17, a regulator of angiogenic factor release by synovial fibroblasts. *Osteoarthritis Cartilage* 14: 345–352.
31. Schwarzenberger, P., V. La Russa, A. Miller, P. Ye, W. Huang, A. Zieske, S. Nelson, G. J. Bagby, D. Stoltz, R. L. Mynatt, et al. 1998. IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J. Immunol.* 161: 6383–6389.
32. Ruth, J. H., M. V. Volin, G. K. Haines 3rd, D. C. Woodruff, K. J. Katschke, Jr., J. M. Woods, C. C. Park, J. C. M. Morel, and A. E. Koch. 2001. Fractalkine, a novel chemokine in rheumatoid arthritis and in rat adjuvant-induced arthritis. *Arthritis Rheum.* 44: 1568–1581.
33. Koch, A. E., B. J. Nickoloff, J. Holgersson, B. Seed, G. K. Haines, J. C. Burrows, and S. J. Leibovich. 1994. 4A11, a monoclonal antibody recognizing a novel antigen expressed on aberrant vascular endothelium. Upregulation in an in vivo model of contact dermatitis. *Am. J. Pathol.* 144: 244–259.
34. Shahrara, S., A. E. Proudfoot, J. M. Woods, J. H. Ruth, M. A. Amin, C. C. Park, C. S. Haas, R. M. Pope, G. K. Haines, Y. Y. Zha, and A. E. Koch. 2005. Amelioration of rat adjuvant-induced arthritis by Met-RANTES. *Arthritis Rheum.* 52: 1907–1919.
35. Shahrara, S., A. E. Proudfoot, C. C. Park, M. V. Volin, G. K. Haines, J. M. Woods, C. H. Aikens, T. M. Handel, and R. M. Pope. 2008. Inhibition of monocyte chemoattractant protein-1 ameliorates rat adjuvant-induced arthritis. *J. Immunol.* 180: 3447–3456.
36. Haas, C. S., M. A. Amin, J. H. Ruth, B. L. Allen, S. Ahmed, A. Pakozdi, J. M. Woods, S. Shahrara, and A. E. Koch. 2007. In vivo inhibition of angiogenesis by interleukin-13 gene therapy in a rat model of rheumatoid arthritis. *Arthritis Rheum.* 56: 2535–2548.
37. Park, C. C., J. C. Morel, M. A. Amin, M. A. Connors, L. A. Harlow, and A. E. Koch. 2001. Evidence of IL-18 as a novel angiogenic mediator. *J. Immunol.* 167: 1644–1653.
38. Kumar, P., M. A. Amin, L. A. Harlow, P. J. Polverini, and A. E. Koch. 2003. Src and phosphatidylinositol 3-kinase mediate soluble E-selectin-induced angiogenesis. *Blood* 101: 3960–3968.
39. Koch, A. E., M. M. Halloran, C. J. Haskell, M. R. Shah, and P. J. Polverini. 1995. Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. *Nature* 376: 517–519.
40. Shahrara, S., C. C. Park, V. Temkin, J. W. Jarvis, M. V. Volin, and R. M. Pope. 2006. RANTES modulates TLR4-induced cytokine secretion in human peripheral blood monocytes. *J. Immunol.* 177: 5077–5087.
41. Shahrara, S., H. P. Castro-Rueda, G. K. Haines, and A. E. Koch. 2007. Differential expression of the FAK family kinases in rheumatoid arthritis and osteoarthritis synovial tissues. *Arthritis Res. Ther.* 9: R112.
42. Lubberts, E., L. A. Joosten, B. Oppers, L. van den Bersselaar, C. J. Coenen-de Roo, J. K. Kolls, P. Schwarzenberger, F. A. van de Loo, and W. B. van den Berg. 2001. IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis. *J. Immunol.* 167: 1004–1013.

43. Wang, L., T. Yi, M. Kortylewski, D. M. Pardoll, D. Zeng, and H. Yu. 2009. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *J. Exp. Med.* 206: 1457–1464.
44. Yang, R. B., C. K. Ng, S. M. Wasserman, L. G. Kömüves, M. E. Gerritsen, and J. N. Topper. 2003. A novel interleukin-17 receptor-like protein identified in human umbilical vein endothelial cells antagonizes basic fibroblast growth factor-induced signaling. *J. Biol. Chem.* 278: 33232–33238.
45. Arefieva, T. I., N. B. Kukhtina, O. A. Antonova, and T. L. Krasnikova. 2005. MCP-1-stimulated chemotaxis of monocytic and endothelial cells is dependent on activation of different signaling cascades. *Cytokine* 31: 439–446.
46. Jiang, B. H., and L. Z. Liu. 2008. AKT signaling in regulating angiogenesis. *Curr. Cancer Drug Targets* 8: 19–26.
47. Hayashi, H., H. Nakagami, Y. Takami, H. Koriyama, M. Mori, K. Tamai, J. Sun, K. Nagao, R. Morishita, and Y. Kaneda. 2009. FHL-2 suppresses VEGF-induced phosphatidylinositol 3-kinase/Akt activation via interaction with sphingosine kinase-1. [Published erratum appears in 2009 *Arterioscler. Thromb. Vasc. Biol.* 29: e132.] *Arterioscler. Thromb. Vasc. Biol.* 29: 909–914.
48. Starnes, T., M. J. Robertson, G. Sledge, S. Kelich, H. Nakshatri, H. E. Broxmeyer, and R. Hromas. 2001. Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J. Immunol.* 167: 4137–4140.
49. Numasaki, M., M. Watanabe, T. Suzuki, H. Takahashi, A. Nakamura, F. McAllister, T. Hishinuma, J. Goto, M. T. Lotze, J. K. Kolls, and H. Sasaki. 2005. IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. *J. Immunol.* 175: 6177–6189.
50. Numasaki, M., M. T. Lotze, and H. Sasaki. 2004. Interleukin-17 augments tumor necrosis factor-α-induced elaboration of proangiogenic factors from fibroblasts. *Immunol. Lett.* 93: 39–43.
51. Nakashio, A., N. Fujita, and T. Tsuruo. 2002. Topotecan inhibits VEGF- and bFGF-induced vascular endothelial cell migration via downregulation of the PI3K-Akt signaling pathway. *Int. J. Cancer* 98: 36–41.
52. Volin, M. V., L. A. Harlow, J. M. Woods, P. L. Campbell, M. A. Amin, M. Tokuhira, and A. E. Koch. 1999. Treatment with sulfasalazine or sulfapyridine, but not 5-aminosalicyclic acid, inhibits basic fibroblast growth factor-induced endothelial cell chemotaxis. *Arthritis Rheum.* 42: 1927–1935.

ARTHRITIS & RHEUMATISM
Vol. 63, No. •, Month 2011, pp 000–000
DOI 10.1002/art.•

AQ: 1
AQ: 2 ok.

Local Expression of Interleukin-27 Ameliorates Collagen-Induced Arthritis

Sarah R. Pickens,[1] Nathan D. Chamberlain,[1] Michael V. Volin,[2] Arthur M. Mandelin II,[1] Hemant Agrawal,[1] Masanori Matsui,[3] Takayuki Yoshimoto,[4] and Shiva Shahrara[1]

AQ: 3 ok. ***Objective.*** **To determine the mechanism of action of interleukin-27 (IL-27) against rheumatoid arthritis (RA).**

Methods. **Adenovirus containing IL-27 transcript was constructed and was locally delivered into the ankles of mice with collagen-induced arthritis (CIA). Progression of arthritis was determined in treated and untreated mice by measuring ankle circumference and through histologic analysis. IL-17 and its downstream targets as well as cytokines promoting Th17 cell differentiation were quantified by enzyme-linked immunosorbent assay in CIA mouse ankles locally expressing adenoviral IL-27 as well as in control-treated mouse**
AQ: 4 ok **ankles. Ankles from both treatment groups were immunostained for neutrophil and monocyte migration. Finally, vascularization was quantified by histology and by determining ankle hemoglobin levels.**

AQ: 5 ok. ***Results.*** **Ectopic expression of IL-27 in CIA mice ameliorated inflammation, lining hypertrophy, and bone erosion compared with that in control-treated CIA mice. Serum and joint levels of IL-17 were significantly reduced in the IL-27–treated group compared with the control-treated group. Two of the main cytokines that induce Th17 cell differentiation and IL-17 downstream target molecules were greatly down-regulated in CIA mouse ankles receiving forced expression of IL-27. The control mice had higher levels of vascularization and monocyte trafficking than did mice ectopically expressing IL-27.**

Conclusion. **Our results suggest that increased levels of IL-27 relieve arthritis in CIA mouse ankles. This amelioration of arthritis involves a reduction in CIA mouse serum and joint levels of IL-17 and results in decreased IL-17–mediated monocyte recruitment and angiogenesis. Hence, IL-27 may be a therapeutic target in RA.** yes

Interleukin-17 (IL-17) is found in rheumatoid arthritis (RA) synovial fluid and in the T cell–rich areas of RA synovial tissue (1,2). Th17 cells, which are derived from RA synovial tissue, are significantly increased in RA synovial fluid compared with RA or normal peripheral blood (3). Our recent studies have shown that IL-17 mediates angiogenesis in RA synovial fluid through ligation to IL-17 receptor C (4). IL-17 can also contribute to the pathogenesis of RA by inducing monocyte migration into the inflamed synovial tissue (5, 6).

IL-17 plays a profound role in the pathogenesis of experimental arthritis. Collagen-induced arthritis (CIA) is markedly reduced in IL-17$^{-/-}$ mice (7), and treatment of the experimental CIA model with anti–IL-17 antibody decreases the severity of inflammation and bone destruction (8). Further, local expression of IL-17 increases inflammation and synovial lining thickness (9), which we have shown to be associated with increased vascularity and monocyte recruitment (3,6).

IL-27 is a heterodimeric cytokine produced by macrophages and dendritic cells; it belongs to the IL-12 cytokine family, which includes IL-23 and IL-35 (10).

Supported by the Arthritis National Research Foundation, the NIH (grant AR-056099), the American College of Rheumatology Research and Education Foundation (Within Our Reach grant to Dr. Shahrara), and the US Department of Defense.

[1]Sarah R. Pickens, MA (current address: University of Illinois at Chicago), Nathan D. Chamberlain, BBmE (current address: University of Illinois at Chicago), Arthur M. Mandelin II, MD, PhD, Hemant Agrawal, PhD, Shiva Shahrara, PhD (current address: University of Illinois at Chicago): Northwestern University Feinberg School of Medicine, Chicago, Illinois; [2]Michael V. Volin, PhD: Midwestern University, Chicago College of Osteopathic Medicine, Downers Grove, Illinois; [3]Masanori Matsui, PhD: Saitama Medical University, Saitama, Japan; [4]Takayuki Yoshimoto, PhD: Tokyo Medical University, Tokyo, Japan.

Dr. Mandelin has received honoraria for Speakers' Bureau service from Abbott and UCB (less than $10,000 each).

Address correspondence to Shiva Shahrara, PhD, University of Illinois at Chicago, Department of Medicine, Division of Rheumatology, E807 MSB, 835S Wolcott Avenue, Chicago, IL 60612. E-mail: shahrara@uic.edu.

Submitted for publication July 6, 2010; accepted in revised form February 22, 2011.

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931

AQ: 10 IL-27 is composed of 2 subunits, Epstein-Barr virus–induced gene 3 and p28, whose transcriptions are regulated independently. As such, dissociation of the expression of the 2 subunits may occur (11). Dendritic cells produce IL-27 when stimulated by pathogen-associated molecular patterns through Toll-like receptors (TLRs) (12). We have shown that macrophages from RA synovial fluid have significantly higher levels of IL-27 production compared with control cells; however, both groups of cells produced similar levels of IL-27 in the presence of TLR-2 ligation (3). Consistently, others have shown that IL-27 is expressed in RA synovium (13).

IL-27 mediates its proinflammatory effect by modulating the initial step of Th1 cell differentiation through the induction of IL-12 receptor $\beta 2$ (IL-12R$\beta 2$) expression, which can lead to interferon-γ (IFNγ) production (14,15). Concordant with these results, IL-27R$^{-/-}$ mice demonstrated reduced inflammation in the proteoglycan-induced arthritis model by down-regulating IFNγ–producing T cells (16). In contrast, IL-27 can also suppress inflammation by inhibiting murine Th17 cell differentiation mediated by IL-6 and transforming growth factor β (TGFβ). It was shown that the absence of IL-27 increased the severity of experimental autoimmune encephalomyelitis (EAE) by promoting T cell proliferation and Th17 cell differentiation (17,18). Further, EAE in IL-27R$\alpha^{-/-}$ mice was ameliorated by employing antibody against IL-17 (17). The suppressive effect of IL-27 was distinct from that of IFNγ, since EAE induced in double knockouts of IFNγ and IL-27Rα was more severe than in each single knockout alone. It was further shown that IL-27 is a potent suppressor of Th17 cell development in a STAT-1–dependent and IFNγ-independent way (17,18). Others have shown that the antiinflammatory properties of IL-27 may also be due to induction of IL-10 by CD4+ cells through a STAT-1– and STAT-3–dependent pathway (19).

Experiments were performed to examine the mechanism by which IL-27 affects the pathogenesis of CIA. Our results demonstrate that 2 of the cytokines promoting Th17 cell differentiation and downstream targets of IL-17 in macrophages and fibroblasts were significantly reduced in ankles adenovirally expressing IL-27 compared with ankles injected with control phosphate buffered saline (PBS). Ectopic expression of IL-27 in the ankles down-regulated CIA vascularization and monocyte migration into synovial tissue compared with the control group. Using RA memory T cells we demonstrated that while IL-27 treatment significantly reduced the percentage of Th17 cells, it had no effect on Th1 cells. These results suggest that inhibition of Th17 cell polarization through IL-27 may be a useful RA treatment.

MATERIALS AND METHODS

Construction of AdIL-27. Mouse IL-27 complementary DNA (cDNA) was obtained from p3xFLAG-IL-27 plasmid described previously by Matsui et al (20), and AdIL-27 was constructed by Welgen. Briefly, IL-27 cDNA was cloned into pCR-TOPO vector (Invitrogen) employing polymerase chain reaction. Thereafter, IL-27 cDNA were released with *Bgl* II and ligated to pENT-CMV predigested with the same enzyme, and the positive clones were screened by digesting with *Bam* HI and sequenced. The pENT-IL-27 cDNA was treated with LR Clonase II enzyme (Invitrogen) and ligated to a pAdREP plasmid that contains the remaining adenovirus genome. The recombination products were transformed into *Escherichia coli* cells, and after overnight incubation the positive clones were selected and cosmid DNA was purified. The purified cosmid DNA (2 μg) was digested with *Pac* I and then transfected into 293 cells with Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). The 293 cells were grown in Dulbecco's modified Eagle's medium. The adenovirus plaques were seen 7 days after transfection. The concentration of the AdIL-27 was 3×10^{10} plaque-forming units (PFU) as determined by plaque assay. The Adcontrol employed in this study was an empty pEntCMV shuttle vector with no insert (adenovirus purchased from Welgen).

Transfection of AdIL-27 in 293 cells and detection of AdIL-27 in mouse ankles. We cultured 293 cells in a 6-well plate to 50–75% confluence. The next day, cells were infected at 0, 5, 10, or 25 multiplicities of infection (MOI) of AdIL-27. Following 48-hour incubation, conditioned medium and cells were collected. The conditioned medium was concentrated using 30-kd columns (VWR Scientific Products); AdIL-27 was detected in the conditioned medium and cell lysates by probing for FLAG (1:3,000 dilution), and the equal loading was determined by actin (1:3,000 dilution) or stained with Coomassie blue. Mice were injected intraarticularly (IA) with 10^5, 10^6, or 10^7 PFU AdIL-27 (injected into both ankles) or with control PBS, and ankles were harvested after 5 days. Ankles were then homogenized in a 50-ml conical centrifuge tube containing 1 ml of Complete Mini-protease inhibitor cocktail (Roche Molecular Biochemicals) homogenization buffer. Ankle homogenization was completed on ice using a motorized homogenizer, followed by 30 seconds of sonication. Homogenates were centrifuged at 2,000*g* for 10 minutes and filtered through a 0.45 μm–pore size Millipore filter (21–23). AdIL-27 expression was examined in ankle homogenates through Western blot probing of FLAG (1:3,000 dilution), and equal loading was examined by actin (1:3,000 dilution).

Study protocol for CIA and AdIL-27 treatment. DBA/1J mice (age 7–8 weeks) were immunized with collagen on days 0 and 21. Bovine type II collagen (2 mg/ml; Chondrex) was emulsified in an equal volume of Freund's complete adjuvant (2 mg/ml of *Mycobacterium tuberculosis* H37Ra; Difco or Chondrex). The DBA/1J mice were immunized subcutaneously in the tail with 100 μl of emulsion. On day 21, mice were injected intradermally with 100 μl of type II collagen (2 mg/ml)

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931

emulsified in an equal volume of Freund's incomplete adjuvant. AdIL-27 (10^7 PFU, n = 15) or Adcontrol (10^7 PFU, n = 15) was injected IA on day 23 after CIA induction. Mice were killed on day 42; ankles were harvested for protein and messenger RNA extraction as well as histologic studies, and serum was saved for laboratory tests.

Clinical assessments. Ankle circumferences were determined by measurement of 2 perpendicular diameters, including the laterolateral diameter and the anteroposterior diameter, using a caliper (Lange Caliper; Cambridge Scientific). Circumference was determined using the following formula: circumference = $2B\sqrt{(a^2 + b^2/2)}$, where a and b represent the diameters. Ankle circumference evaluations were performed on days 21, 23, 26, 28, 30, 33, 35, 36, and 41.

Flow cytometry. RA peripheral blood mononuclear cells were isolated by Histopaque gradient centrifugation, and memory CD4+ T cells were isolated employing a negative selection kit (StemCell Technologies) according to the manufacturer's instructions. RA memory CD4+ T cells were cultured and treated with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (1 μg/ml) with or without IL-27 treatment (100 ng/ml) for 48 hours. The cells were supplemented with brefeldin A (10 μg/ml) 18 hours prior to performing flow cytometry. Cells were then stained with anti-CD4 (RPA-T4; BD PharMingen), anti–IL-17 (eBio64DEC17; eBioscience), anti-IFNγ (4S.B3; BD PharMingen), or isotype control antibodies. Th17 or Th1 cells were identified as CD4+IL-17+ or CD4+IFNγ+, respectively.

Antibodies and immunohistochemistry. Mouse ankles were decalcified, fixed in formalin, and embedded in paraffin, and were sectioned in the pathology core facility of Northwestern University. Inflammation, synovial lining, and bone erosion (scored on a 0–5 scale) were determined by a blinded observer (AMM) viewing hematoxylin and eosin–stained sections. Mouse ankles were stained with immunoperoxidase using Vector Elite ABC Kits (Vector), with diaminobenzidine (Vector) as a chromogen, at the pathology core facility of Northwestern University.

Briefly, slides were deparaffinized in xylene for 15 minutes at room temperature, followed by rehydration by transfer through graded alcohols. Antigens were unmasked by incubating slides in proteinase K digestion buffer (Dako) for 5 minutes at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 5 minutes. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Dako). Nonspecific binding of antibodies to the tissues was blocked by pretreatment of tissues with Protein block (Dako). Tissues were incubated with GR1 (1:200 dilution; Novus Biologicals), MAC 387 (1:200 dilution; Serotec together with animal research kit [ARK; Dako]), von Willebrand factor (1:1,000 dilution; Dako), or control IgG antibody (Beckman Coulter). Slides were counterstained with Mayer's hematoxylin and treated with lithium carbonate for bluing. Neutrophil and macrophage staining were scored on a 0–5 scale. Vascularity was quantified as number of blood vessels per 5 random high-power fields at 10× magnification (24). The data were pooled, and the mean ± SEM was calculated in each data group. Each slide was evaluated by a blinded observer (AMM) (22,23,25,26).

Quantification of proinflammatory factors. Mouse ankle and/or serum IL-17, IL-1β, IL-6, tumor necrosis factor α (TNFα), CXCL1, CXCL5, CCL20, and CCL2 were quantified by enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (R&D Systems). The sensitivity of the ELISAs performed to quantify mouse IL-17, IL-1β, IL-6, CXCL1, and CXCL5 was 7.8 pg/ml, whereas for TNFα and CCL20 it was 15.6 pg/ml. The expression level of each factor was normalized to the ankle protein concentration and shown as pg/mg, and serum levels are shown as pg/ml.

Quantification of hemoglobin in mouse ankles. Employing methemoglobin, serial dilutions were prepared to generate a standard curve from 70 gm/dl to 1.1 gm/dl (4,27,28). Fifty microliters of homogenized mouse ankles or standard was added to a 96-well plate in duplicate, and 50 μl tetramethylbenzidine was added to each sample. The plate was allowed to develop at room temperature for 15–20 minutes with gentle shaking, and the reaction was terminated with 150 μl 2*N* H_2SO_4 for 3–5 minutes. Absorbance was read with an ELISA plate reader at 450 nm. To calculate hemoglobin concentrations in the mouse ankles, the values (gm/dl) were normalized to the weights of the ankles (mg/ml) (4,27,28).

Statistical analysis. The data were analyzed using Student's 2-tailed *t*-tests for paired and unpaired samples. *P* values less than 0.05 were considered significant.

RESULTS

Expression of AdIL-27 in transfected 293 cells and in mouse ankles. To verify that AdIL-27 was capable of expressing IL-27, 293 cells were transfected with 0, 5, 10, and 25 MOI of AdIL-27. Following a 48-hour incubation, protein expression was determined by Western blotting of both cell lysates and conditioned medium. Using anti-FLAG antibody we were able to detect AdIL-27 construct at 5, 10, and 25 MOI from both 293 cell lysates and conditioned medium (Figure 1A). To validate the expression of IL-27 in vivo, mouse ankles were bilaterally injected with 10^5, 10^6, or 10^7 PFU of AdIL-27 or PBS control. After 5 days, ankles were homogenized and AdIL-27 construct was detected employing anti-FLAG antibody in Western blotting analysis. AdIL-27 construct was detectable only in ankles injected IA with 10^6 or 10^7 PFU AdIL-27, and not in ankles injected IA with 10^5 PFU AdIL-27 or PBS control (Figure 1B). Since both mouse ankles injected with 10^7 PFU AdIL-27 strongly expressed IL-27 compared with only 1 mouse ankle injected with 10^6 PFU AdIL-27, the dose of 10^7 PFU was selected for performing these experiments.

Effect of local IL-27 expression in CIA. To determine the effect of IL-27 administration into the arthritic joint, AdIL-27 or Adcontrol (10^7 PFU each) was injected IA into DBA/1J mouse ankles 23 days after CIA induction. In the Adcontrol-injected mice, disease activity determined by ankle circumference began on

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931

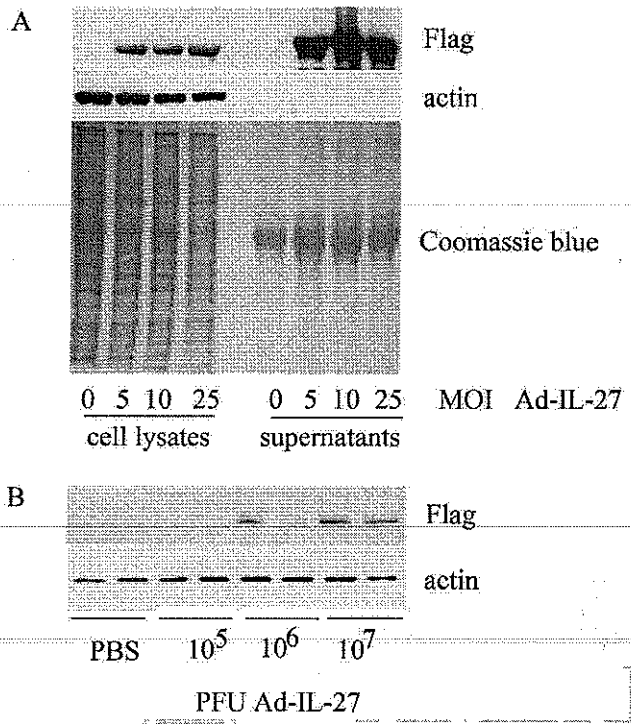


Figure 1. Western blotting analysis of AdIL-27 construct. **A,** Western blotting analysis using anti-FLAG, antiactin, or Coomassie blue protein staining of lysates and conditioned medium obtained from 293 cells transfected with 0, 5, 10, or 25 multiplicities of infection (MOI) of AdIL-27. **B,** Western blotting analysis using anti-FLAG or antiactin antibody on homogenates of mouse ankles injected with phosphate-buffered saline (PBS) control or with 10^5, 10^6, or 10^7 plaque-forming units (PFU) of AdIL-27.

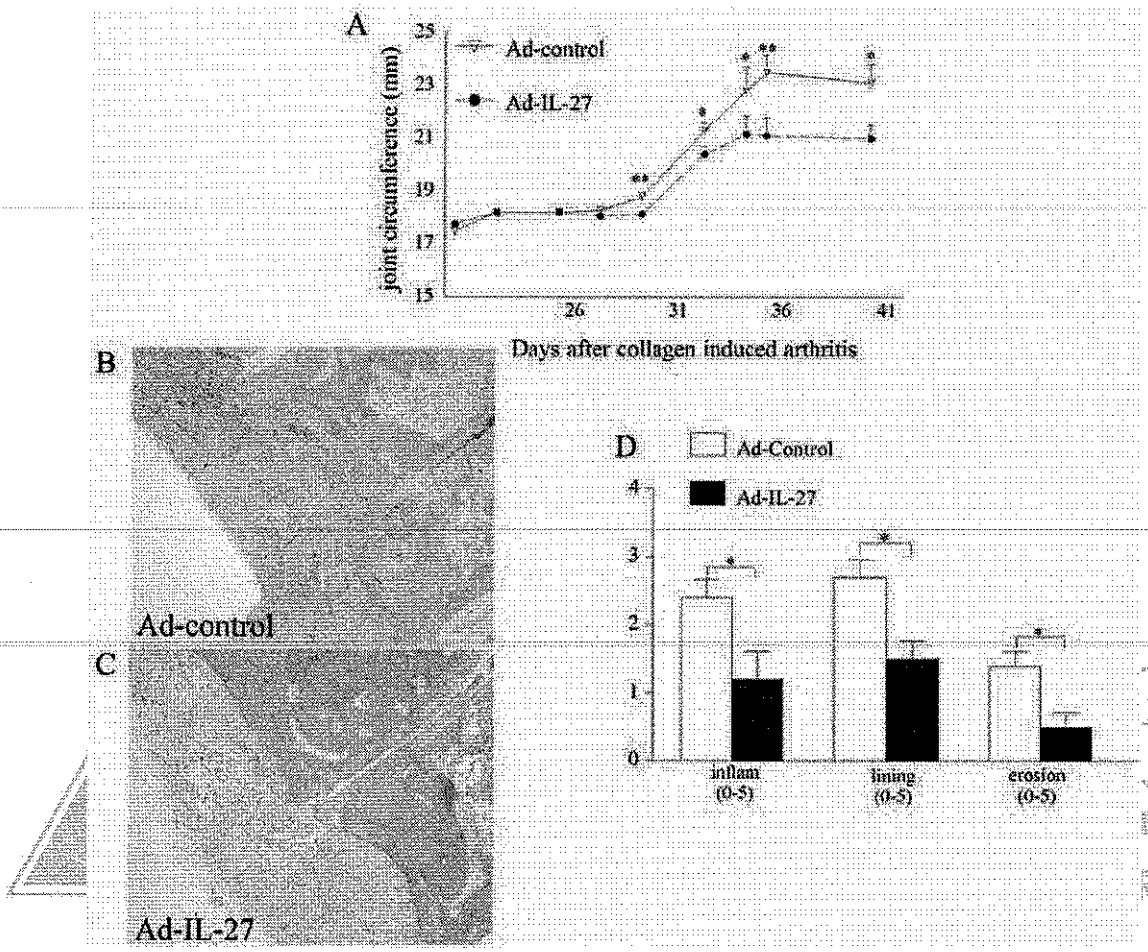
day 30 and progressed through day 36, plateauing thereafter until the termination of the experiments on day 42 (Figure 2A). Mice treated with AdIL-27 demonstrated significantly reduced joint circumference compared with control animals ($P < 0.05$ and $P < 0.01$). Next, histologic examination of the joints was performed to determine the effect of treatment on inflammation and joint destruction. Histologic analysis of ankles from day 42 confirmed that mice treated with AdIL-27 had significantly less inflammation (50% decrease), synovial lining thickness (45% decrease), and bone erosion (65% decrease) compared with control mice (Figures 2B–D). These results suggest that local expression of IL-27 can reduce CIA joint inflammation, synovial lining thickness, and bone destruction.

Effect of AdIL-27 treatment on expression of proinflammatory factors in CIA mouse ankles. IL-27 is known to suppress inflammation by inhibiting Th17 cell differentiation; therefore, IL-17 expression levels were determined in serum and ankles of CIA mice receiving AdIL-27 or Adcontrol. Our results demonstrate that IL-17 expression was significantly lowered in sera and ankle homogenates (by 35% and 55%, respectively) from AdIL-27–treated animals compared with those from control animals (Figure 3A). Interestingly, we demonstrated that 2 of the cytokines that drive Th17 cell differentiation, namely, IL-1β and IL-6, were significantly reduced (by 55%) in mouse ankles, and levels of IL-1β were also decreased (by 45%) in sera from CIA mice locally expressing IL-27 compared with sera from mice receiving control treatment (Figures 3B and C). We have shown that CXCL1, CXCL5, and CCL2 are neutrophil and monocyte chemokines that are induced by IL-17 in RA synovial tissue fibroblasts and macrophages as well as in a model of IL-17–induced arthritis (unpublished observations) (see ref. 6). We found that ectopic expression of IL-27 significantly decreased joint levels of CXCL1 (by 65%), CXCL5 (by 70%), and CCL2 (by 55%) compared with Adcontrol treatment in CIA (Figures 3D–F). These results suggest that local expression of IL-27 could suppress Th17 cell polarization as well as IL-17 downstream target genes.

Treatment with IL-27 significantly reduces RA Th17 cells without affecting Th1 cells. Since splenocytes and T cells are difficult to transfect due to low expression of adenovirus receptor, in order to demonstrate that IL-27 can directly reduce CD4+IL-17+ cells, RA peripheral blood memory T cells were isolated by negative selection and were treated with PMA and ionomycin with or without IL-27. Results from these experiments demonstrate that while IL-27 treatment significantly reduced Th17 cells from 3% to 1%, it had no effect on the Th1 cell population (Figures 4A and B). Consistently, when IL-17 levels in conditioned medium were quantified by ELISA (after 48 and 72 hours), cells treated with IL-27 had lower secretion of IL-17 compared with cells in the control treatment group (data not shown).

Effect of AdIL-27 treatment on leukocyte recruitment into CIA mouse joints. We have shown thus far that local expression of IL-27 in CIA mouse ankles significantly reduces inflammation as well as Th17 cell polarizing cytokines and IL-17–induced downstream factors. We next examined the effect of AdIL-27 on leukocyte recruitment into inflamed CIA mouse ankle joints. Consistent with the clinical data, local expression of IL-27 greatly suppressed neutrophil ingression (by 60%) (Figures 5A–C) and monocyte ingression (by 35%) (Figures 5D–F) into CIA mouse joints compared with

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931



COLOR

Figure 2. Local expression of interleukin-27 (IL-27) ameliorates pathology of collagen-induced arthritis (CIA). **A,** Changes in joint circumference. * = $P < 0.05$; ** = $P < 0.01$ versus AdIL-27–treated mice. **B** and **C,** Hematoxylin and eosin staining of CIA mouse ankles injected with Adcontrol (**B**) or AdIL-27 (**C**). Original magnification × 200. **D,** Effect of local expression of IL-27 on inflammation, lining thickness, and bone erosion. * = $P < 0.05$. Values in **A** and **D** are the mean ± SEM (n = 10).

AQ: 46

control mouse joints. Our results suggest that reduction of joint IL-17 levels can down-regulate trafficking of neutrophils and monocytes into CIA mouse ankles.

Effect of AdIL-27 treatment on vascularization in CIA. Since angiogenesis is critical for leukocyte ingress, the effect of local IL-27 expression on blood vessel formation in CIA mice was studied. Vascularization in CIA mice was quantified by measuring ankle hemoglobin levels and blood vessel staining. AdIL-27–treated CIA mice had markedly lower hemoglobin levels compared with control mice (Figure 6A). Consistently, there were 40% fewer blood vessels in CIA mouse ankles that locally expressed IL-27 than in control mouse ankles (Figures 6B–D). Our results suggest that IL-27 treatment inhibits IL-17–mediated angiogenesis in CIA.

F6,AQ:41

OK.

DISCUSSION

In this study, we show that local expression of IL-27 in CIA mouse ankles ameliorates joint inflammation and bone destruction. We further demonstrate that IL-27 modulates arthritis through reducing 2 important Th17 cell polarizing cytokines as well as IL-17–activated factors in the CIA mouse joint. In RA peripheral blood, IL-27 treatment directly reduced the percentage of Th17 cells; however, Th1 cells were unaffected. Consequently,

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931

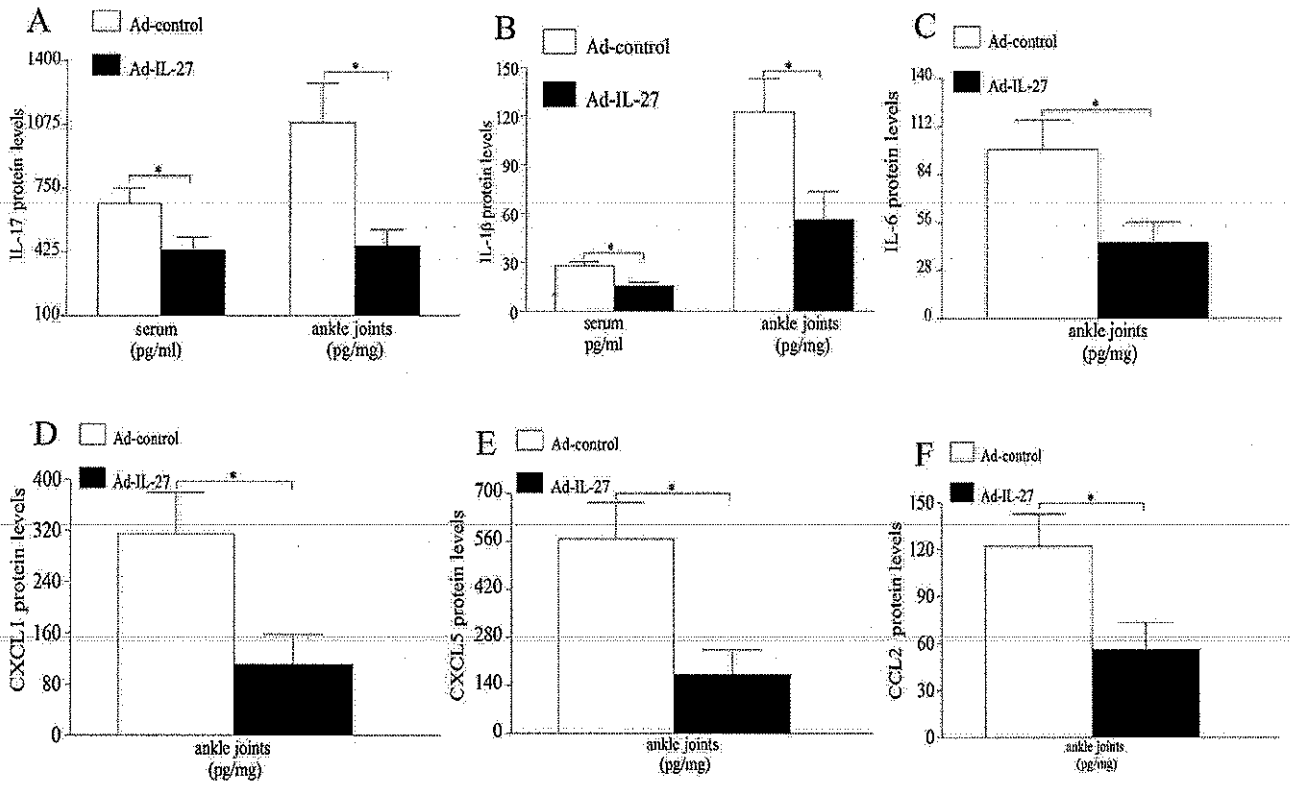


Figure 3. Forced expression of IL-27 reduces joint levels of IL-17 and proinflammatory factors. **A** and **B**, Changes in expression of IL-17 (**A**) or IL-1β (**B**) in sera and ankle homogenates from CIA mice treated with Adcontrol or AdIL-27 were determined by enzyme-linked immunosorbent assay (ELISA). Levels of expression of IL-6 (**C**), CXCL1 (**D**), CXCL5 (**E**), or CCL2 (**F**) in ankle homogenates from both treatment groups were quantified by ELISA. Values are the mean ± SEM (n = 9–10). * = $P < 0.05$. See Figure 2 for other definitions.

local expression of IL-27 in CIA mouse ankles suppresses IL-17–mediated neutrophil and monocyte trafficking as well as vascularization. These results suggest that IL-27 can inhibit IL-17–induced acute (neutrophil migration) and chronic (monocyte recruitment) inflammation by affecting leukocyte ingress, controlled in part by the reduction in angiogenesis.

Early neutralization of IL-17 using an IL-17 receptor IgG Fc fusion protein in CIA suppresses the onset of the disease (29). Treatment of CIA after disease onset using anti–IL-17 antibody decreases the severity of inflammation and bone destruction in CIA (8). These studies demonstrate that IL-17 plays an important role in the initiation and progression of CIA. Hence, we investigated whether inhibition of Th17 cell differentiation could reduce joint inflammation in CIA. The effect of local expression of IL-27 on Th17 cells was observed both systemically and in the ankle joints, since IL-17 levels in the sera and ankle homogenates were markedly decreased compared with those in the control group. Experiments were performed in RA peripheral blood in order to demonstrate that IL-27 treatment could directly inhibit Th17 cell differentiation and that reduction in IL-17 levels was distinct from IFNγ–mediated Th17 cell suppression. Consistent with previous findings (17,18), we showed that Th17 cell differentiation was suppressed 3-fold while Th1 cell polarization was unaffected by IL-27 treatment in RA peripheral blood.

We found that local expression of IL-27 could alleviate clinical signs of CIA. Consistently, histologic analysis demonstrated reduced inflammation, synovial lining thickness, and bone erosion, which may be due to suppressed joint levels of IL-17. It has been shown that IL-17 is involved in bone degradation through elevating expression of RANKL in CIA mouse ankles (30) as well as synergizing with TNFα and IL-6 in this process (31,32).

TGFβ, IL-6, IL-1β, and IL-21 drive the differentiation of Th17 cells (33–35). However, some variation between humans and mice has been described. Levels of

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931

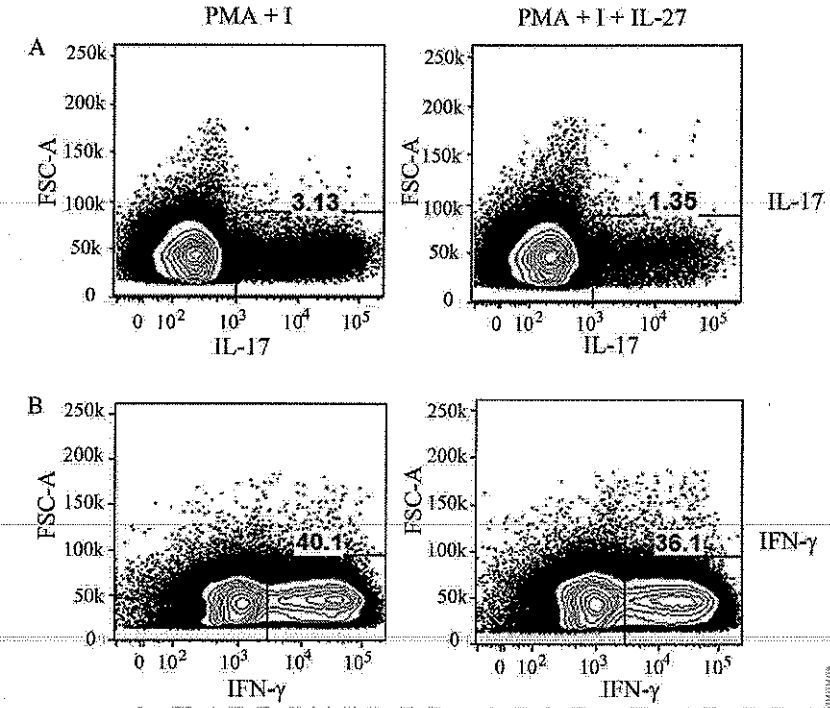


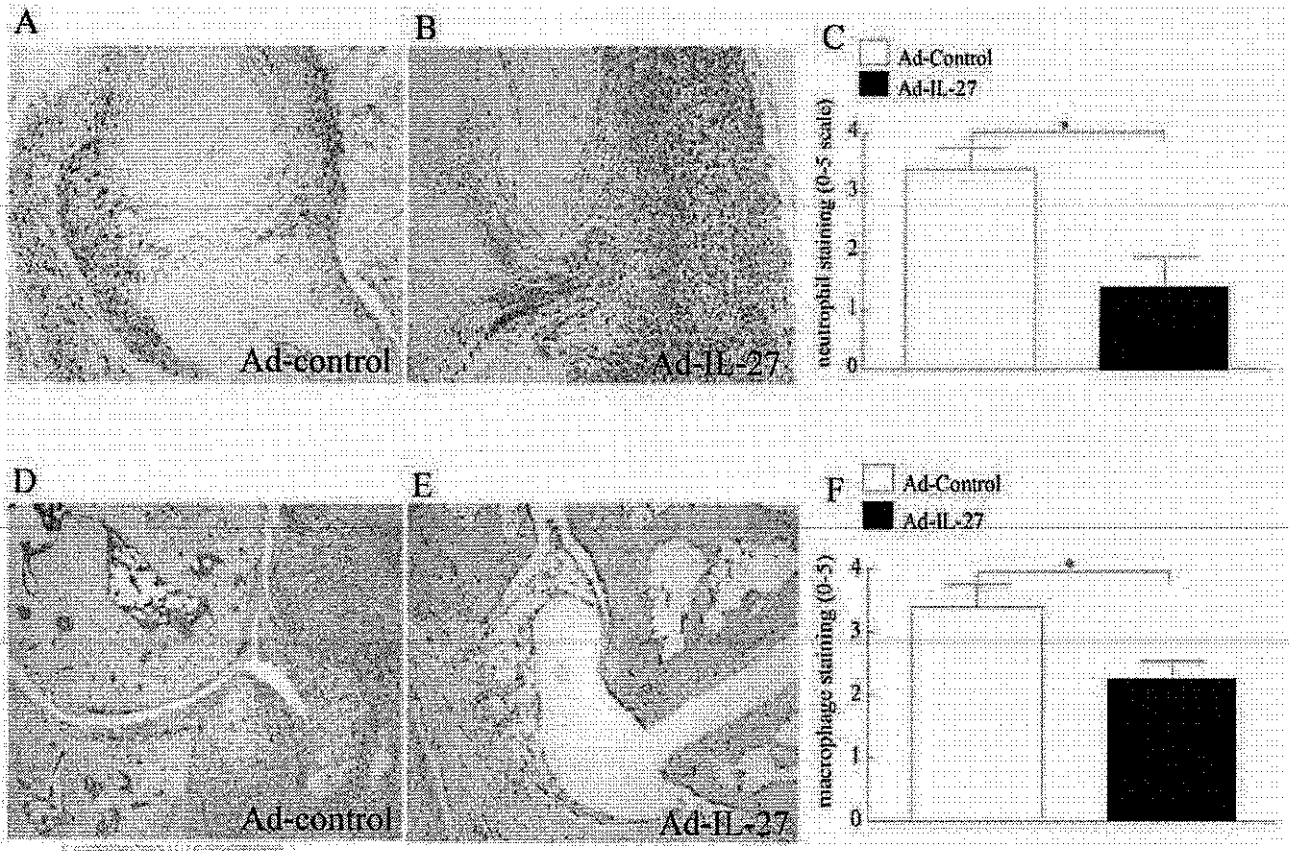
Figure 4. Interleukin-27 (IL-27) treatment reduces percentage of Th17 cells but not of Th1 cells in rheumatoid arthritis (RA) peripheral blood. **A** and **B**, Percentages of CD4+IL-17+ cells (**A**) or CD4+ interferon-γ (IFNγ)–positive cells (**B**) were quantified in RA peripheral blood memory CD4+ T cells treated with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (I; 1 μg/ml) or with PMA, ionomycin, and IL-27 (100 ng/ml) for 48 hours. The cells were supplemented with brefeldin A (10 μg/ml) 18 hours prior to performing flow cytometry. Results demonstrate that IL-27 treatment reduces Th17 cells without affecting Th1 cells.
FSC = forward scatter.

IL-1β and IL-6 but not TNFα were markedly reduced in CIA mouse ankles locally expressing IL-27 compared with control mouse ankles. Others have shown that in IL-1Ra$^{-/-}$ mice, elevated levels of IL-1β are responsible for an increase in the number of Th17 cells (36). In CIA, IL-6 is essential for Th17 cell differentiation, since anti–IL-6R antibody markedly suppresses induction of Th17 cells and arthritis development (37). Consistent with our data, a previous study demonstrated that systemic administration of recombinant IL-27 could reduce serum levels of IL-6 (13). Interestingly, in CIA mouse synoviocytes, neutralization of IL-1β and IL-6 significantly reduces IL-17–mediated expression of TLRs 2, 4, and 9 (38). The results from our laboratory and those of others suggest that IL-1β and IL-6 are 2 of the cytokines that play an important role in Th17 cell differentiation in CIA. Therefore, IL-27 can suppress polarization of Th17 cells by modulating joint levels of IL-1β and IL-6.

Our unpublished studies demonstrate that IL-17 can induce CXCL1 expression from RA synovial tissue fibroblasts, macrophages, and human microvascular endothelial cells. CXCL5 is also produced from IL-17–activated RA synovial tissue fibroblasts and macrophages (: unpublished observations). Previous studies have shown that neutrophil migration mediated by IA injection of IL-17 is dependent on CXCL1 and CXCL5, suggesting that both neutrophil chemokines are produced by cells in the ankle joints and play an essential role in IL-17–mediated neutrophil ingress (39). Neutrophil chemotaxis caused by conditioned media from IL-17–stimulated gastric epithelial cells was inhibited by neutralizing antibodies to IL-8, suggesting that in human cells IL-8 is responsible for IL-17–induced neutrophil trafficking (40). Similar to IL-8, CXCL1 and CXCL5 bind to CXCR2 and therefore may induce neutrophil migration through activation of the same pathway. Col-

AQ: 44

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931



COLOR

Figure 5. Local expression of IL-27 down-regulates CIA-mediated neutrophil and monocyte ingression. Synovial tissue harvested from Adcontrol- or AdIL-27–injected CIA mouse ankles on day 42 was immunostained with GR1 (neutrophil marker) (A and B) or MAC-387 (macrophage marker) (D and E). Original magnification × 200. C and F, Shown is quantification of neutrophil staining (C) and macrophage staining (F) from CIA mouse ankles harvested on day 42. Values are the mean ± SEM (n = 10). * = $P < 0.05$. See Figure 2 for definitions.

AQ: 47

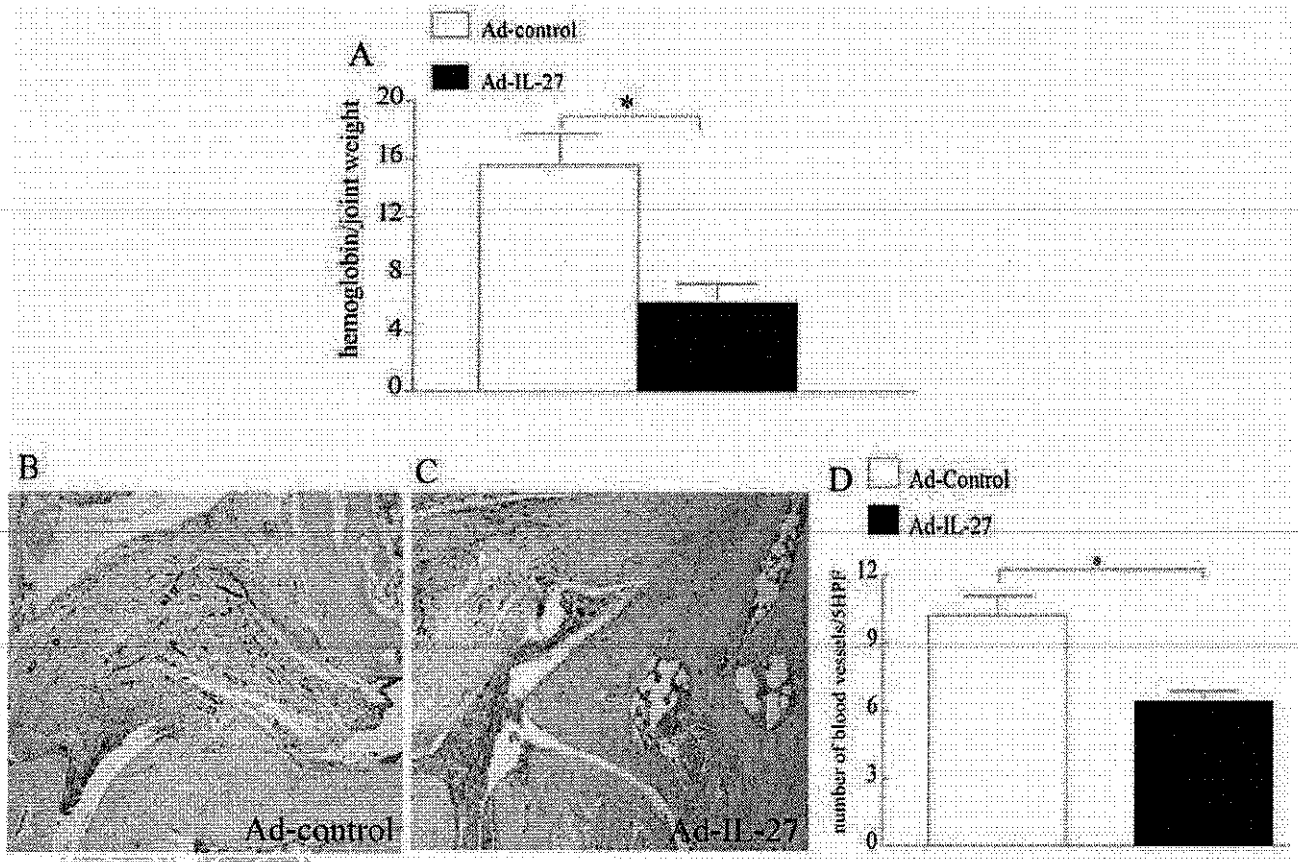
n=10 mice

lectively, the data suggest that reduction of IL-17 by AdIL-27 suppresses neutrophil migration through modulating CXCL1 and CXCL5 in CIA mouse ankle joints.

We have shown that IL-17 plays an important role in monocyte migration in RA, since neutralization of IL-17 in RA synovial fluid or its receptors on monocytes significantly reduces monocyte migration mediated by RA synovial fluid (5). Further, IL-17 promotes monocyte migration through activation of p38 MAPK (5). We also found that IL-17 activates CCL2 production from macrophages and RA synovial tissue fibroblasts and in experimental arthritis models (6). In addition to the direct effect of IL-17 on monocyte chemotaxis, we demonstrated that IL-17–mediated monocyte recruitment into the peritoneal cavity was due in part to CCL2 production (6). Despite the ability of IL-17 to induce the production of other monocyte chemokines such as CCL20 from cells present in the synovial lining (6), forced expression of IL-27 in CIA mouse ankles did not affect the expression levels of this chemokine. Based on our previous studies, inhibition of monocyte recruitment into CIA mouse ankles locally expressing IL-27 may be directly due to reduction of IL-17 levels or indirectly due to lower expression of IL-17–induced CCL2, or perhaps both mechanisms are essential for this process.

Angiogenesis is an early and critical event in the pathogenesis of RA which is triggered by the inflammatory process mediated by cytokines, chemokines, and hypoxia (41). Previous studies demonstrate that angiogenesis is essential for CIA progression (42). In the current study we show that local expression of IL-27 significantly reduced synovial vascularity in CIA mice compared with that in control animals. This effect may be due to down-regulation of joint levels of IL-17 in ankles with forced IL-27 expression. We previously observed that IL-17, in concentrations present in the RA

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931



COLOR

Figure 6. Reduced vascularization detected in CIA mouse ankles locally expressing IL-27. **A,** Shown are quantified hemoglobin levels in CIA mouse ankles harvested from different treatment groups on day 42. Results are shown as hemoglobin (gm/dl)/joint weight (mg/ml). **B** and **C,** Synovial tissue harvested from Adcontrol-injected (**B**) or AdIL-27–injected (**C**) CIA mouse ankles on day 42 was immunostained with von Willebrand factor (endothelial marker). Original magnification × 200. **D,** Vascularization was quantified as the number of blood vessels per 5 random high-power fields (hpf) at 10× magnification in each CIA mouse ankle harvested on day 42. Values in **A** and **D** are the mean ± SEM (n = 10). * = $P < 0.05$. See Figure 2 for other definitions.

joint, induces endothelial migration through the phosphatidylinositol 3-kinase/Akt-1 pathway (4). Further, we have demonstrated that IL-17 is angiogenic, determined by its ability to promote blood vessel growth in Matrigel plugs in vivo (4). However, reduced levels of the proangiogenic chemokines CXCL1 and CXCL5 may also be responsible for the decreased vascularity in the AdIL-27 treatment group compared with that in the control group (43). Given that angiogenesis promotes ingress of leukocytes, reduction in new blood vessel formation can affect neutrophil and monocyte trafficking.

In summary, local expression of IL-27 in CIA results in reduced disease severity quantified by joint swelling, synovial lining thickness, bone erosion, and leukocyte migration. In CIA, AdIL-27 treatment leads to reduced IL-1β and IL-6 production, resulting in a depressed Th17 cell response characterized by decreased joint levels of IL-17. This leads to decreased synovial production of the neutrophil and monocyte chemokines CXCL1, CXCL5, and CCL2, ultimately resulting in fewer infiltrating leukocytes and lessened blood vessel formation.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Shahrara had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Pickens, Shahrara.

Acquisition of data. Pickens, Chamberlain, Volin, Mandelin, Agrawal, Shahrara.

Analysis and interpretation of data. Pickens, Chamberlain, Volin, Matsui, Yoshimoto, Shahrara.

Providing reagents. Matsui, Yoshimoto.

REFERENCES

1. Stamp LK, James MJ, Cleland LG. Interleukin-17: the missing link between T-cell accumulation and effector cell actions in rheumatoid arthritis? Immunol Cell Biol 2004;82:1–9.
2. Lubberts E, Koenders MI, van den Berg WB. The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. Arthritis Res Ther 2005;7:29–37.
3. Shahrara S, Huang Q, Mandelin AM II, Pope RM. TH-17 cells in rheumatoid arthritis. Arthritis Res Ther 2008;10:R93.
4. Pickens SR, Volin MV, Mandelin AM II, Kolls JK, Pope RM, Shahrara S. IL-17 contributes to angiogenesis in rheumatoid arthritis. J Immunol 2010;184:3233–41.
5. Shahrara S, Pickens SR, Dorfleutner A, Pope RM. IL-17 induces monocyte migration in rheumatoid arthritis. J Immunol 2009;182: 3884–91.
6. Shahrara S, Pickens SR, Mandelin AM II, Karpus WJ, Huang Q, Kolls JK, et al. IL-17–mediated monocyte migration occurs partially through CC chemokine ligand 2/monocyte chemoattractant protein-1 induction. J Immunol 2010;184:4479–87.
7. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 2003;171:6173–7.
8. Lubberts E, Koenders MI, Oppers-Walgreen B, van den Bersselaar L, Coenen-de Roo CJ, Joosten LA, et al. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. Arthritis Rheum 2004;50:650–9.
9. Koenders MI, Lubberts E, van de Loo FA, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, et al. Interleukin-17 acts independently of TNF-α under arthritic conditions. J Immunol 2006;176:6262–9.
10. Pflanz S, Timans JC, Cheung J, Rosales R, Kanzler H, Gilbert J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells. Immunity 2002;16:779–90.
11. Li J, Gran B, Zhang GX, Rostami A, Kamoun M. IL-27 subunits and its receptor (WSX-1) mRNAs are markedly up-regulated in inflammatory cells in the CNS during experimental autoimmune encephalomyelitis. J Neurol Sci 2005;232:3–9.
12. Yoshida H, Nakaya M, Miyazaki Y. Interleukin 27: a double-edged sword for offense and defense. J Leukoc Biol 2009;86: 1295–303.
13. Niedbala W, Cai B, Wei X, Patakas A, Leung BP, McInnes IB, et al. Interleukin 27 attenuates collagen-induced arthritis. Ann Rheum Dis 2008;67:1474–9.
14. Yoshida H, Hamano S, Senaldi G, Covey T, Faggioni R, Mu S, et al. WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection. Immunity 2001;15:569–78.
15. Chen Q, Ghilardi N, Wang H, Baker T, Xie MH, Gurney A, et al. Development of Th1-type immune responses requires the type I cytokine receptor TCCR. Nature 2000;407:916–20.
16. Cao Y, Doodes PD, Glant TT, Finnegan A. IL-27 induces a Th1 immune response and susceptibility to experimental arthritis. J Immunol 2008;180:922–30.
17. Batten M, Li J, Yi S, Kljavin NM, Danilenko DM, Lucas S, et al. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. Nat Immunol 2006;7:929–36.
18. Stumhofer JS, Laurence A, Wilson EH, Huang E, Tato CM, Johnson LM, et al. Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. Nat Immunol 2006; 7:937–45.
19. Stumhofer JS, Silver JS, Laurence A, Porrett PM, Harris TH, Turka LA, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. Nat Immunol 2007;8:1363–71.
20. Matsui M, Moriya O, Belladonna ML, Kamiya S, Lemonnier FA, Yoshimoto T, et al. Adjuvant activities of novel cytokines, interleukin-23 (IL-23) and IL-27, for induction of hepatitis C virus-specific cytotoxic T lymphocytes in HLA-A*0201 transgenic mice. J Virol 2004;78:9093–104.
21. Shahrara S, Amin MA, Woods JM, Haines GK, Koch AE. Chemokine receptor expression and in vivo signaling pathways in the joints of rats with adjuvant-induced arthritis. Arthritis Rheum 2003;48:3568–83.
22. Shahrara S, Proudfoot AE, Woods JM, Ruth JH, Amin MA, Park CC, et al. Amelioration of rat adjuvant-induced arthritis by Met-RANTES. Arthritis Rheum 2005;52:1907–19.
23. Shahrara S, Proudfoot AE, Park CC, Volin MV, Haines GK, Woods JM, et al. Inhibition of monocyte chemoattractant protein-1 ameliorates rat adjuvant-induced arthritis. J Immunol 2008;180:3447–56.
24. Kennedy A, Ng CT, Biniecka M, Saber T, Taylor C, O'Sullivan J, et al. Angiogenesis and blood vessel stability in inflammatory arthritis. Arthritis Rheum 2010;62:711–21.
25. Ruth JH, Volin MV, Haines GK III, Woodruff DC, Katschke KJ Jr, Woods JM, et al. Fractalkine, a novel chemokine in rheumatoid arthritis and in rat adjuvant-induced arthritis. Arthritis Rheum 2001;44:1568–81.
26. Koch AE, Nickoloff BJ, Holgersson J, Seed B, Haines GK, Burrows JC, et al. 4A11, a monoclonal antibody recognizing a novel antigen expressed on aberrant vascular endothelium: up-regulation in an in vivo model of contact dermatitis. Am J Pathol 1994;144:244–59.
27. Haas CS, Amin MA, Ruth JH, Allen BL, Ahmed S, Pakozdi A, et al. In vivo inhibition of angiogenesis by interleukin-13 gene therapy in a rat model of rheumatoid arthritis. Arthritis Rheum 2007;56:2535–48.
28. Park CC, Morel JC, Amin MA, Connors MA, Harlow LA, Koch AE. Evidence of IL-18 as a novel angiogenic mediator. J Immunol 2001;167:1644–53.
29. Bush KA, Farmer KM, Walker JS, Kirkham BW. Reduction of joint inflammation and bone erosion in rat adjuvant arthritis by treatment with interleukin-17 receptor IgG1 Fc fusion protein. Arthritis Rheum 2002;46:802–5.
30. Lubberts E, van den Bersselaar L, Oppers-Walgreen B, Schwarzenberger P, Coenen-de Roo CJ, Kolls JK, et al. IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-κB ligand/osteoprotegerin balance. J Immunol 2003;170:2655–62.
31. Kotake S, Sato K, Kim KJ, Takahashi N, Udagawa N, Nakamura I, et al. Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. J Bone Miner Res 1996;11: 88–95.
32. Romas E, Gillespie MT, Martin TJ. Involvement of receptor activator of NFκB ligand and tumor necrosis factor-α in bone destruction in rheumatoid arthritis. Bone 2002;30:340–6.
33. Manel N, Unutmaz D, Littman DR. The differentiation of human T_H-17 cells requires transforming growth factor-β and induction of the nuclear receptor RORγt. Nat Immunol 2008;9:641–9.
34. Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupe P, Barillot E, et al. A critical function for transforming growth factor-β, interleukin 23 and proinflammatory cytokines in driving and modulating human T_H-17 responses. Nat Immunol 2008;9:650–7.
35. Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. Type 17 T helper cells—origins, features and possible roles in rheumatic disease. Nat Rev Rheumatol 2009;5:325–31.
36. Koenders MI, Devesa I, Marijnissen RJ, Abdollahi-Roodsaz S, Boots AM, Walgreen B, et al. Interleukin-1 drives pathogenic Th17 cells during spontaneous arthritis in interleukin-1 receptor antagonist–deficient mice. Arthritis Rheum 2008;58:3461–70.

rich3/zar-anr/zar-anr/zar00811/zar9291d11g | springj | S=6 | 5/10/11 | 10:58 | 4/Color Figure(s): 2,5,6 | Art: 10-0931

37. Fujimoto M, Serada S, Mihara M, Uchiyama Y, Yoshida H, Koike N, et al. Interleukin-6 blockade suppresses autoimmune arthritis in mice by the inhibition of inflammatory Th17 responses. Arthritis Rheum 2008;58:3710–9.
38. Lee JH, Cho ML, Kim JI, Moon YM, Oh HJ, Kim GT, et al. Interleukin 17 (IL-17) increases the expression of Toll-like receptor-2, 4, and 9 by increasing IL-1β and IL-6 production in autoimmune arthritis. J Rheumatol 2009;36:684–92.
39. Lemos HP, Grespan R, Vieira SM, Cunha TM, Verri WA Jr, Fernandes KS, et al. Prostaglandin mediates IL-23/IL-17-induced neutrophil migration in inflammation by inhibiting IL-12 and IFNγ production. Proc Natl Acad Sci U S A 2009;106:5954–9.
40. Luzza F, Parrello T, Monteleone G, Sebkova L, Romano M, Zarrilli R, et al. Up-regulation of IL-17 is associated with bioactive IL-8 expression in Helicobacter pylori-infected human gastric mucosa. J Immunol 2000;165:5332–7.
41. Szekanecz Z, Koch AE. Angiogenesis and its targeting in rheumatoid arthritis. Vascul Pharmacol 2009;51:1–7.
42. Mould AW, Tonks ID, Cahill MM, Pettit AR, Thomas R, Hayward NK, et al. Vegfb gene knockout mice display reduced pathology and synovial angiogenesis in both antigen-induced and collagen-induced models of arthritis. Arthritis Rheum 2003;48:2660–9.
43. Numasaki M, Watanabe M, Suzuki T, Takahashi H, Nakamura A, McAllister F, et al. IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. J Immunol 2005;175:6177–89.