

AWARD NUMBER: W81XWH-13-1-0210

TITLE: Citrullinated Chemokines in Rheumatoid Arthritis

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REPORT DATE: December 2016

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
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<b>REPORT DOCUMENTATION PAGE</b>				<i>Form Approved</i> <i>OMB No. 0704-0188</i>	
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<b>1. REPORT DATE</b> December 2016		<b>2. REPORT TYPE</b> Final		<b>3. DATES COVERED</b> 30 Sep 2013 - 29 Sep 2016	
<b>4. TITLE AND SUBTITLE</b>  Citruillinated Chemokines in Rheumatoid Arthritis				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-13-1-0210	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b>  David A. Fox, MD  E-Mail: <a href="mailto:dfox@umich.edu">dfox@umich.edu</a>				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Regents of The University of Michigan 503 Thompson St. Ann Arbor, MI 48109-1340				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Citruillination, catalysed by peptidylarginine deiminase (PAD), is a post-translational modification of arginine to citrulline, which contributes to the pathogenesis of rheumatoid arthritis (RA). We show that citruillinated epithelial-derived neutrophil-activating peptide 78/CXCL5 (cit-ENA-78/CXCL5) is significantly higher in RA synovial fluids (SFs) compared to osteoarthritis (OA) and other inflammatory rheumatic diseases (OD) SFs, and its concentration correlates with RA disease activity. Citruillinated chemokine concentrations were measured by enzyme linked immunosorbent assay (ELISA) in RA and normal (NL) sera and in RA, osteoarthritis (OA), and other inflammatory rheumatic disease (OD) synovial fluids (SFs). The correlation between the citruillinated chemokine levels and clinical data was analyzed. A strong correlation was found between the amounts of citENA-78/CXCL5 and C-reactive protein or erythrocyte sedimentation rate (ESR) in RA SFs. These results indicate that citENA-78/CXCL5 may have novel inflammatory properties in RA pathogenesis.					
<b>15. SUBJECT TERMS</b> Citruillination, chemokines, chemotaxis, rheumatoid arthritis, immunology					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>  Unclassified	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b>  Unclassified	<b>b. ABSTRACT</b>  Unclassified	<b>c. THIS PAGE</b>  Unclassified			<b>19b. TELEPHONE NUMBER</b> (include area code)

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## 1. Introduction

Citrullination, catalysed by peptidyl arginine deiminase (PAD) enzymes, is a post-translational modification of arginine to citrulline that contributes to the pathogenesis of rheumatoid arthritis (RA). Chemokines, which play an important role in the development of RA, can be citrullinated *in vitro*. We are examining whether citrullinated chemokines and nuclear growth factors secreted into the RA joint are detected in RA biological fluids, and if so, what their biological activities are. We posit that modification of seminal chemokines and inflammatory mediators in the RA synovial fluid (SF) confers unique properties that significantly modify the inflammatory environment of the RA joint. Chemokines, including epithelial-derived neutrophil-activating peptide 78 (ENA-78/CXCL5), play a critical role in the development of RA, and are involved in inflammatory leukocyte migration into rheumatoid synovial tissue (ST). Recent studies have shown that several chemokines, ENA-78/CXCL5, interleukin-8 (IL-8/CXCL8), interferon gamma-induced protein 10 (IP-10/CXCL10), interferon-inducible T-cell alpha chemoattractant (I-TAC/CXCL11), and stromal derived factor-1 $\alpha$  (SDF-1 $\alpha$ /CXCL12), can be citrullinated by PAD *in vitro*. We also found that a regulatory nuclear protein, inhibitor of DNA binding-1 protein (Id1) can regulate fibroblast chemokine expression and can also be found in citrullinated form in RA tissues. In this study, we investigated whether ENA-78/CXCL5, macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ /CCL3), and monocyte chemoattractant protein-1 (MCP-1/CCL2), which are representative chemokines in RA, are citrullinated in RA, osteoarthritis (OA), and normal (NL) control subjects. We also examined the regulatory angiogenic inflammatory protein Inhibitor of DNA binding-1 (Id1) protein and its role in citrullination and ACPA binding in RA. The goal was to identify the inflammatory properties of these modified proteins to explore their novel functions that will offer additional insight into their function in chronic diseases such as RA.

## 2. Keywords

Rheumatoid arthritis (RA)  
Osteoarthritis (OA)  
Normal (NL)  
Other diseases (OD)  
Synovial Fluid (SF)  
Synovial Tissue (ST)  
Serum  
Chemokines  
Chemotaxis  
Anti-cyclic-citrullinated peptide antibodies (anti-CCP or ACPA)  
Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)  
Citrullination  
Enzyme Linked Immunosorbent Assay (ELISA)  
Epithelial Neutrophil Chemoattractant Peptide-78 (ENA-78/CXCL5)  
Monocyte Chemoattractant Protein-1 (MCP-1/CCL2)  
Macrophage Inflammatory Protein-1 alpha (MIP-1 $\alpha$ /CCL3)  
Peptidyl Arginine Deiminase (PAD)  
Neutrophils (PMNs)  
Monocytes (MNs)  
Arginine  
Inhibitor of DNA binding-1 (Id1)  
Recombinant human (rh)  
Adjuvant induced arthritis (AIA)  
anti-modified citrulline antibody (AMC antibody)  
Rheumatoid factor (RF)

### 3. Overall Project Summary

**Current Objectives: Specific Aim 1: To determine how citrullinated chemokines compare in RA, vs. OA, vs. other rheumatic diseases (OD), vs. NLs.**

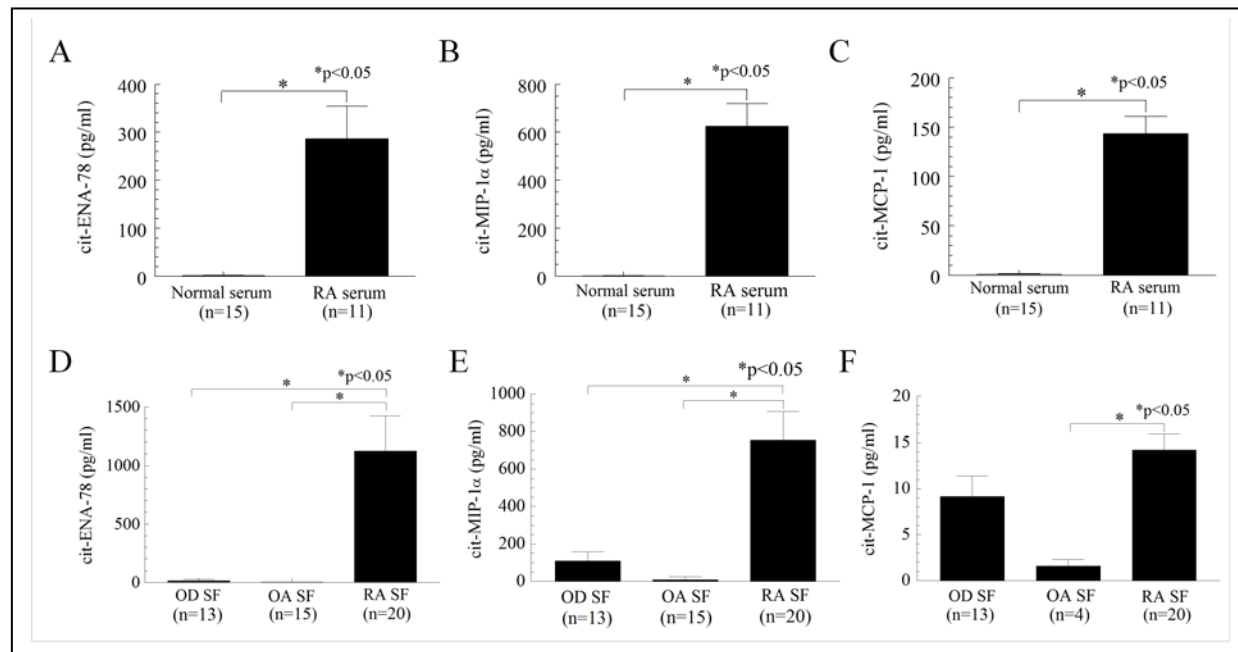
**Major Task 1:** Characterize citrullinated chemokines in RA.

Subtask 1: SF and sera will be obtained from patients with RA, OA or ODs and assayed for key citrullinated and non-citrullinated chemokines thought to be important in the pathogenesis of RA.

Subtask 2: ST will be obtained from RA, OA, or NL joints, homogenized and assayed for citrullinated and non-citrullinated chemokines.

**Summary of Results:** We have successfully recruited the necessary patients for this study. We obtained enough patient fluids and tissues to perform all assays proposed in this grant. We have generated data to show that ENA-78/CXCL5, MIP-1 $\alpha$ /CCL3 and MCP-1/CCL2 can all be detected in RA SFs. We have made tissue homogenates obtained from patients with RA and OA, and from NL ST obtained from tissue repositories. Using these homogenates, we have shown that RA ST joint homogenates contain citrullinated (cit) ENA-78/CXCL5 and that this can be detected by Western blotting analysis. Furthermore, through mass spectrometry (LC-MS/MS) and immune-dot blot analysis, we have been able to identify the critical arginine(s) in citENA-78/CXCL5 and citId1 that are responsible for the auto-antigenic activity of these modified proteins in RA

**Progress and Accomplishments:** Citrullination is a post-translational modification mediated by PAD. Chemokines, which play an important role in the development of RA, can be citrullinated *in vitro*. We undertook this study to examine whether the citrullinated chemokines ENA-78/CXCL5, MIP-1 $\alpha$ /CCL3, and MCP-1/CCL2 are detected in RA biological fluids, and if so, what their biological activities are.



**Figure 1.** Citrullinated chemokines are highly expressed in RA sera and SFs. Citrullinated chemokines were measured using an ELISA in which chemokines were captured on an ELISA plate followed by detection with anti-modified citrulline after chemical modification of citrulline residues. RF (rheumatoid factor) was depleted from RA SFs and sera prior to the ELISA assay. A), B), and C), CitENA-78/CXCL5, MIP-1 $\alpha$ /CCL3, and MCP-1/CCL2 concentrations in RA and normal sera. These citrullinated chemokines were all greater in RA compared to normal sera. D), E), and F) CitENA-78/CXCL5, MIP-1 $\alpha$ /CCL3, and MCP-1/CCL2 concentrations in RA, OD, and OA SFs. CitENA-78/CXCL5 and MIP-1 $\alpha$ /CCL3 were significantly greater in RA SFs than in OA and OD SFs. CitMCP-1/CCL2 was higher in RA compared to OA SFs. Bars show the mean  $\pm$  SEM (n = the number of patients). \*p < 0.05 was considered significant. cit-ENA-78 = citrullinated ENA-78/CXCL5; cit-MIP-1 $\alpha$  = citrullinated MIP-1 $\alpha$ /CCL3; cit-MCP-1 = citrullinated MCP-1/CCL2.

Recombinant human (rh) chemokines were citrullinated by PAD. Citrullinated chemokine concentrations were measured by enzyme linked immunosorbent assay in RA and normal sera and in RA, OA, and OD SFs. The correlation between the citrullinated chemokine levels and clinical data was analyzed. We found that citENA-78/CXCL5 was significantly higher in RA sera and SFs than normal sera and OD including OA SFs, respectively (figure 1). A strong correlation was found between the amount of citENA-78/CXCL5 and C-reactive protein or erythrocyte sedimentation rate in RA SFs. Our conclusions for Aim 1 are that citENA-78/CXCL5 can be detected in RA tissues and is highly correlated with RA disease activity.

Taken together, evidence is strong for citrullination being integral to inflammation and the pathogenesis of RA. Inflammatory cytokines are known to be perpetrators of the disease process, but they may also be more than that. Citrullinated chemokines are present and appear to be pathogenic early in the course of RA. These preliminary studies should establish whether this is the case for key chemokines involved in RA pathogenesis. The results of these studies will also help us determine if citrullinated chemokines should be pursued as potential biomarkers for RA. We have recently published a manuscript supporting our hypothesis<sup>1</sup>, and have shown that citrullination can effect chemokine activity and be associated with markers of disease severity.

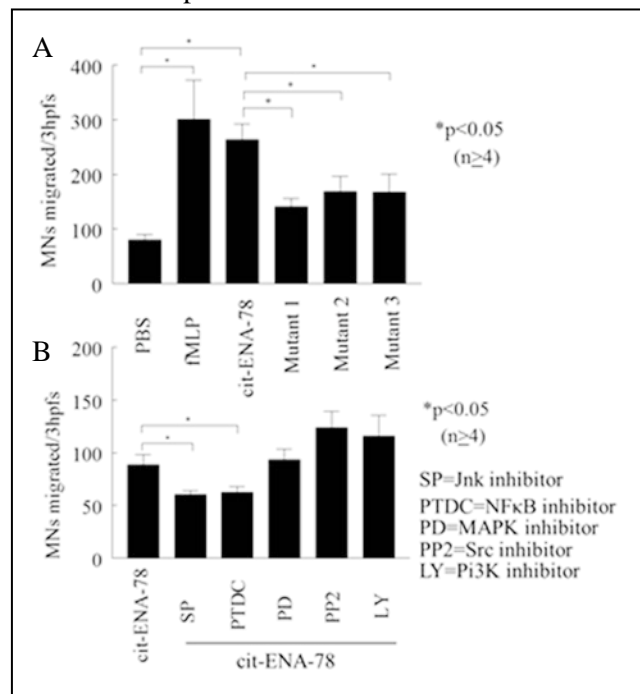
***Current Objectives: Specific Aim 2: To determine the mechanisms of citrullination of chemokines leading to altered chemokine function in RA.***

**Major task II:** To determine the cellular signaling pathways induced by citrullinated chemokines.

Subtask 1: Leukocyte chemotaxis assays, modifying chemokines containing ELR motif and analysis, site directed mutagenesis of chemokines.

Subtask 2: Signal transduction analysis of endothelial cells and/or leukocytes.

***Summary of Results:*** LC-MS/MS analysis confirmed citrullination of citENA78/CXCL5. CitENA78/CXCL5 induced monocyte (MN) migration was significantly reduced by Jnk and NFkB inhibitors, but not by inhibitors of Src, p38, and Erk<sub>1/2</sub>. We found that Jnk siRNA-transfected MNs displayed decreased citENA78/CXCL5 induced MN migration compared to control transfected MNs and that citR45K and citR48K mutations significantly reduced MN chemotaxis, suggesting that citENA-78/CXCL5 induced MN migration is dependent, at least partially, on both citrulline residues (figure 2). We also tested these findings in vivo using the air pouch model. The mouse air pouch model showed marked MN recruitment in response to citENA-78/CXCL5



**Figure 2. MN chemotaxis and signaling inhibition to citENA-78/CXCL5:** MN migration assays were performed by placing cells in a 48-well Neuroprobe microchemotaxis chamber. As shown, MNs are chemotactic for citENA-78/CXCL5. A. To determine the mechanism involved, MN chemotaxis assay was performed with citENA78/CXCL5 in modified Boyden chambers to mutants (R45K, R48K and R45/48K) of rhENA-78/CXCL5. Mutants were generated by transmutation of arginines to lysines and were used in MN chemotaxis assays. CitR45K and citR48K mutations significantly reduced MN chemotaxis, suggesting that citENA78/CXCL5-induced MN migration is dependent, at least partially, on both citrulline residues. B. To determine the kinases required for citENA-78 mediated MN chemotaxis to citENA-78/CXCL5, cells were incubated with chemical signaling inhibitors. MNs were preincubated with chemical signaling inhibitors for 1 h prior to the assay, and the inhibitors were present in the upper chamber with the MNs during the assay. The following inhibitors were purchased from and used at concentrations recommended by Calbiochem (La Jolla, CA): PD98059 (10μM Erk1/2 inhibitor), PDTC (100μM NFkB inhibitor), LY294002 (10μM PI<sub>3</sub>K inhibitor), SP (Jnk inhibitor) and PP2 (1μM Src inhibitor). We found that SP (Jnk inhibitor) and PDTC (NFkB inhibitor) significantly reduced MN migration towards citENA-78/CXCL5. The other inhibitors used had no significant effect upon citENA-78/CXCL5 mediated MN chemotaxis (n = number of independent experimental replicates).

compared to the controls, suggesting that citENA-78/CXCL5 increases MN ingress in vivo. CitENA-78/CXCL5 stimulated MNs showed increased expression of phosphorylated Jnk and phosphorylated NFκB (p-NFκB) which was inhibited by signaling inhibitors. We discovered that a Jnk inhibitor reduced the level of p-NFκB, suggesting that Jnk is upstream of NFκB in citENA78/CXCL5-stimulated MNs. We also found that citENA78/CXCL5 induces MN migration via Jnk and NFκB signaling pathways. Therefore, we could conclude that MN chemotaxis induced by citENA-78/CXCL5 is partially dependent on both citrulline residues. This study is in preparation to be submitted as a full length manuscript.

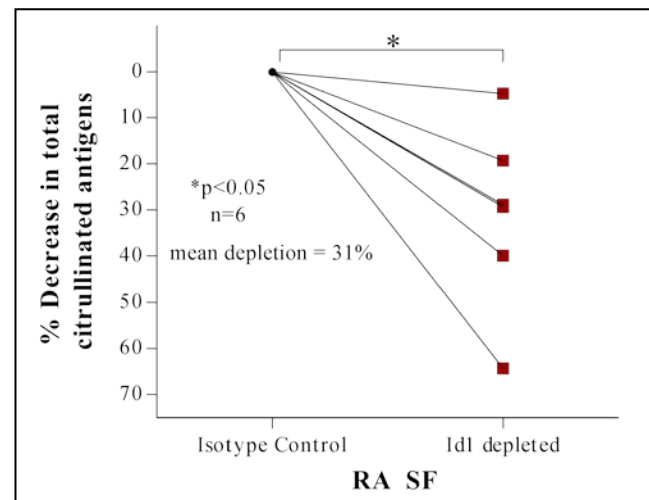
**Progress and Accomplishments:** Previously, we have shown that citrullination converts ENA-78/CXCL5 from a neutrophil recruiter to a MN recruiter. In this study, we investigated the signaling pathways involved in MN recruitment by citENA-78/CXCL5. Rh ENA-78/CXCL5 was citrullinated by incubation with rhPAD4 enzyme. To confirm the citrullination sites, citENA-78/CXCL5 was analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). To determine the signaling pathways involved, MN chemotaxis assay was performed with citENA-78/CXCL5 in modified Boyden chambers with chemical signaling inhibitors. MNs that were also transfected with JNK siRNA. Three mutants (R45K, R48K and R45/48K) of rhENA-78/CXCL5 were generated by transmutation of arginines to lysines and were used in MN chemotaxis assays. To assess citENA-78/CXCL5 induced MN migration in vivo, a mouse subcutaneous air pouch model was used. The exudates from the air pouch were collected after 24 hours and immunofluorescence was performed to detect MNs/macrophages using anti-F4/80 antibody. MNs were stimulated with citENA-78/CXCL5 for 15 minutes and Western blots were performed to evaluate phosphorylation of signaling molecules. We also assessed the crosstalk between the signaling pathways after incubating with chemical signaling inhibitors before citENA-78/CXCL5 stimulation.

We found that citENA-78/CXCL5 plays an important role in MN migration in vivo in the air pouch inflammatory model. Our data suggest that citENA-78/CXCL5 induces JNK and NFκB signaling pathways with JNK upstream of NFκB. Therefore, targeting citENA-78/CXCL5 and its signaling pathways may be a novel approach to treat MN dependent diseases in which citENA-78/CXCL5 is expressed.

**Current Objectives: Specific Aim 3: To identify if patients with RA produce antibodies to citrullinated vs. noncitrullinated chemokines.**

**Major task III:** To examine autoantibody formation to citrullinated chemotactic factors in RA and rat adjuvant induced arthritis.

Subtask 1: Sera will be obtained from RA, OA, or NL individuals and assayed against citrullinated vs. noncitrullinated chemokines to determine if autoantibodies are made.

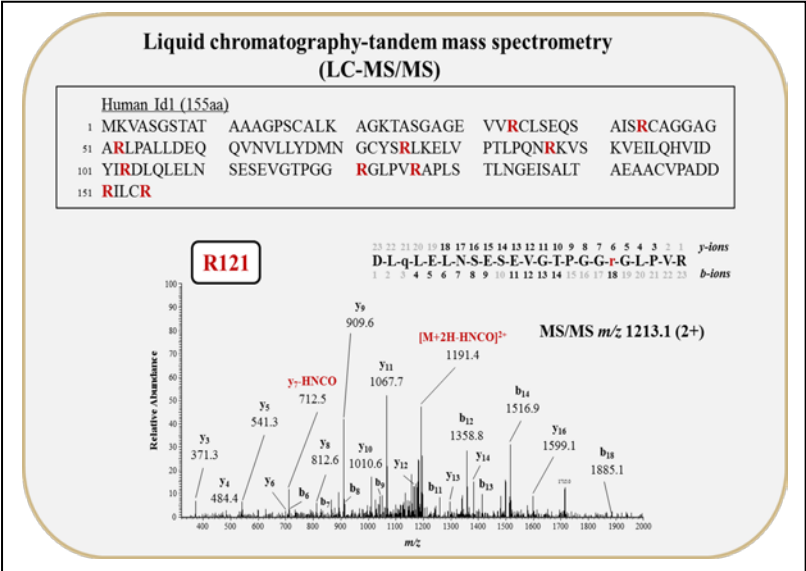


**Figure 3.** RA SFs immunodepleted with either Id1 or sham depleted (isotype control antibody) were measured by cit-ELISA to determine the total amount of citrullinated protein contained in RA SFs before and after Id1 immunodepletion. We found that by removing Id1 could significantly reduce the total amount of citrullinated protein in RA SF. Although the total amount of citrullinated protein contained in all 6 patient specimens significantly declined with immunodepletion of Id1, we cannot rule out that co-immunoprecipitation of id1-associated proteins that might also be citrullinated is a possible contributing factor to the observations (\* $p < 0.05$ ,  $n =$  no. of samples; note that two of the samples were very close in value at 29% and appear as a single line).

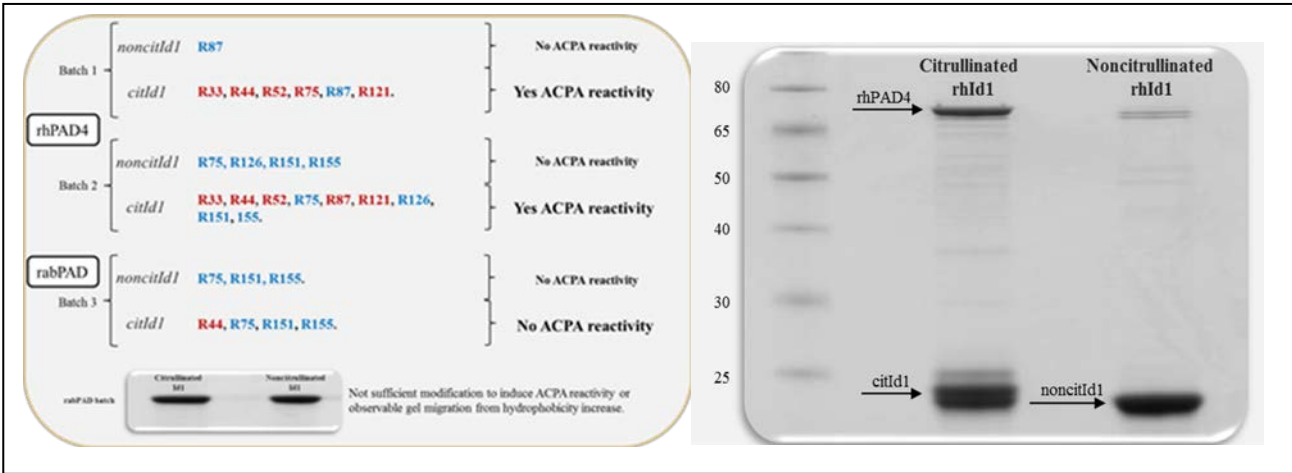


Subtask 2: Citrullinated chemokines will be adsorbed out of RA serum to determine how much reactivity for citrullinated antigens remains.

**Summary of Results:** Our data suggested that citrullination of nuclear proteins may account for much of the citrullination seen in RA SF and RA PB. To investigate this further, we examined the possibility that Id1 (a potential regulator of chemokine expression from RA fibroblasts) may be a significant contributor of citrullination in RA tissues. Therefore, we performed many of our immunoassays to citrullinated chemokines with citrullinated Id1 for comparison. ELISA analysis of RA SFs showed that the levels of total citrullinated antigens were significantly reduced upon immunodepletion of Id1 (figure 3). Western blot (WB) analysis of immunoprecipitated Id1 from homogenized RA STs showed that a significant portion of the total Id1 was in the modified form. Most importantly, we were able to detect citId1 via the cit-ELISA. To verify by another method that we could citrullinate Id1 successfully, we performed liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS) on modified Id1. We could identify modified arginines after incubation of recombinant Id1 with PAD enzyme by detecting the neutral loss of isocyanic acid (HNCO; -43Da), much like we observed with citENA-78/CXCL5. These are diagnostic marker ions for citrullination and confirm that Id1 has been modified by PAD enzyme. Immunodot blot (IDB) analysis of mouse anti-human Id1 antibody (previously used in immunodepletion and immunoprecipitation) showed positive signals from



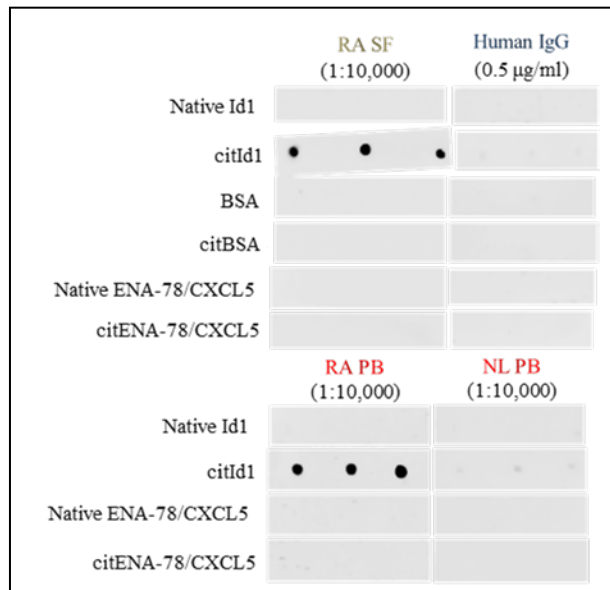
**Figure 4.** Mass spectrometry of citrullinated human Id1. All detected ions are shown in black in the sequence and are annotated in the spectrum as space permits. The lower case r in the sequence (highlighted in red) denotes deiminated Arginine (Citrulline). The ions highlighted in red in the spectrum show the neutral loss of isocyanic acid (HNCO; -43Da). These are diagnostic marker ions for citrullination (Hao et al 2009, J. American Society for Mass Spectrometry).



**Figure 5.** Left panel: Citrullination reaction showing the conversion of arginine to citrulline catalyzed by PAD enzyme resulting in ACPA binding activity for citId1. Right panel: SDS-PAGE gel stained with silver stain shows both noncitId1 and citId1 at approximately 15-25 kDa range (see white boxes). Note that citId1 runs slightly higher on the gel compared to native Id1 due to the change in charge of the modified protein. The thick dark band in the upper region of the citId1 lane is PAD enzyme.



both citId1 and noncitId1, suggesting that this binding epitope was not modified by citrullination. IDB analysis of the patient sera showed robust signals for citId1, and weak but significant signals for citENA-78/CXCL5 (not shown in figure because the signal compared to citId1 is very low), from multiple RA patient PB sera, displaying a four-fold increase in average reactivity for citId1 as compared to NL patient PB sera. We show for the first time the presence of ACPAs with specificity to citId1, and weak specificity for citENA-78/CXCL5 in RA patient PB sera, and propose citId1, and to a lesser extent citENA-78/CXCL5 as novel autoantigen candidates in RA. The results of the IDB and ACPA binding studies helped determine the critical arginines involved in the conversion of a native protein into a potential autoantigen (figures 5 and 6).



**Figure 6.** Analysis of ACPAs to citId1. RA SFs and sera obtained from NL individuals and RA patients were assayed for autoantibodies to citrullinated Id1 (citId1) by immune dot blot analysis. Before performing the dot blots, all sera and SFs were immunodepleted of rheumatoid factor (RF) by incubating specimens with anti-IgM coupled beads. Immunodepletion of RF was confirmed by measuring the amounts of RF before and after treatment with the anti-IgM coupled beads. Antigens were blotted onto nitrocellulose membrane (NCM) and blocked with 5% goat serum in TBST buffer. NCM was incubated overnight with RA SF or sera diluted 1:10,000 in TBST. Blots were washed and incubated with a secondary antibody [horseradish peroxidase conjugated goat anti-human IgG (Millipore)] diluted 1:10,000 in TBST for detection of ACPAs by digital image analysis. Imaging was done using electrochemiluminescence (ECL) reagent after 5 minute incubation. Exposure time was 1 minute for all blots. As shown in the representative blots, we found positive results for ACPA reactivity for citId1, but not native Id1, in RA SF and RA PB, but not in the PB of NL (healthy) subjects. To control for non-specific antibody binding, control antigens including native Id1, BSA, citBSA, native ENA-78/CXCL5 and citENA-78/CXCL5 were tested on the same NCM as citId1. Human IgG served as a control for non-specific serum antibody binding. Control blots showed no significant evidence of ACPAs or antibody binding reactivity in RA SF, RA PB or in NL PB. We found 4/9 RA PBs and 0/5 NL PBs that were significantly immunopositive for citId1 (all samples were run in triplicate).

We discovered by running a series of experiments that the type of PAD enzyme used in the reaction mix, and the conditions (length of incubation, amount of antigen, etc.) that we would achieve different citrullination patterns for the same protein. We subsequently discovered however, that only certain critical arginines need to be modified in order to achieve properties of proteins in their modified forms. For example, ENA-78/CXCL5 would not display the folding patterns and hydrophobicity, as well as its ACPA binding activity, if arginine 48 was not modified by PAD. Additionally, citId1 would not bind ACPAs sufficiently in RA SF unless arginines 33, 52 and 121 were modified (out of ten arginines available; figures 5 and 6). These important findings help explain how native proteins can become autoantigens, and identify those essential modifications that can lead to significant pathology in inflammatory disease. But most importantly, these studies opened new avenues of discovery for potential therapeutic development. For example, we are currently investigating how

the ACPAs binding citId1 are formed and how pathogenic they may be in RA. We have shown that depletion of Id1 can reduce the citrullinated antigen load in RA SFs by approximately 31% (figure 3), suggesting that citId1 is readily present in the RA SF and potentially autoantigenic. It will be exciting to isolate and test such autoantibodies. There is also the potential to aggravate arthritis in established rodent models of RA using these antibodies.

**Current Objectives: Specific Aim 4: To determine the role of citrullinated chemokines in rodent arthritis development.**

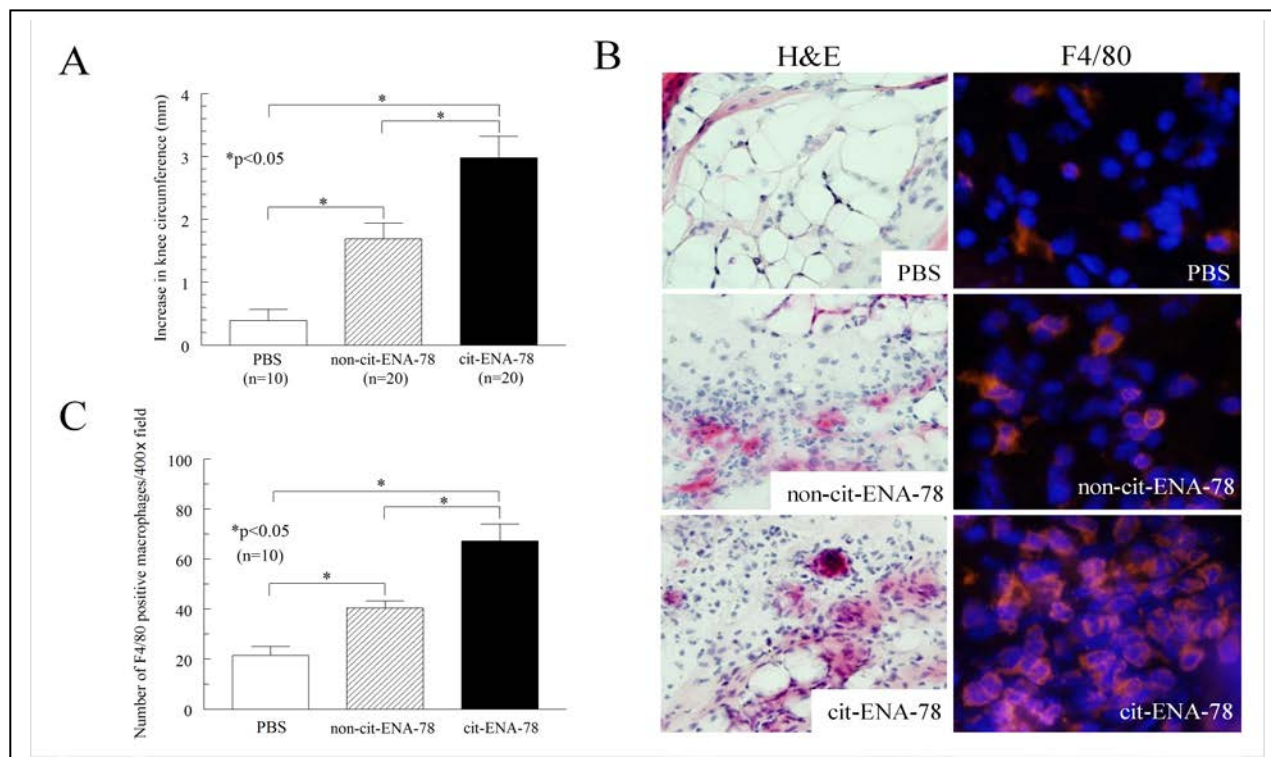
**Major task IV: To determine the role of citrullinated ENA-78/CXCL5 in rodent arthritis development**

Subtask 1: We will examine the inflammatory effect of injecting citrullinated chemokine vs. non-citrullinated chemokine into rodent joints.

Subtask 2: We will perform rat AIA, a rodent RA model, and determine whether citrullinated chemokines and antibodies to these chemokines are present before development of arthritis and during the course of disease development.

**Summary of Results:** Since the aim of our studies is to determine the biological role of citrullinated chemokines in arthritis development, a process that involves leukocyte recruitment and angiogenesis in the joint, we employed rodent models to examine these processes *in vivo*. Specifically, we have shown that citrullination of ENA-78/CXCL5 converted a normally neutrophil recruiting chemokine into a chemokine that recruits monocytes. We now know that this conversion is critically related to the conversion of arginine 48 much like the conversion of Id1 is dependent upon the citrullination of arginines 33, 52 and 121 (figures 5 and 6).

**Progress and Accomplishments:** Id1 is a nuclear transcription factor actively transcribed in endothelial progenitor cells and in cells that exhibit hyperproliferative responses such as synovial fibroblasts (2). Previously, we identified Id1 as an angiogenic and chemotactic factor expressed in RA STs and upregulated in RA SFs. Although it is a relatively small protein of approximately 16 kDA, Id1 contains 10 modifiable arginines. As a variety of citrullinated proteins are known to bind to anti-citrullinated protein antibodies (ACPAs), we investigated citrullinated Id1 (citId1) as a potential autoantigen in RA and compared its potential ACPA producing activity compared to citENA-78/CXCL5, known to be expressed in RA (figure 1). Previously, RA SFs were immunodepleted of ENA-78/CXCL5 and measured



**Figure 7.** Citrullinated ENA-78/CXCL5 induces more severe articular inflammation and F4/80 positive macrophage recruitment *in vivo* compared to non-citrullinated ENA-78/CXCL5. C57BL/6 female mice were injected intraarticularly with 20  $\mu$ l/knee of PBS, non-citrullinated (16 ng), or citrullinated ENA-78/CXCL5 (16 ng) to induce inflammation on day 0. A) The difference between the circumference on day 0 and 24 hours after the injection was defined as the increase in knee circumference. Increase in knee circumference was significantly higher in the group injected with citrullinated ENA-78/CXCL5 compared to the group injected with PBS and non-citrullinated ENA-78/CXCL5. Increases in knee circumference also were significantly higher in the group injected with non-citrullinated ENA-78/CXCL5 compared to PBS. B) H&E staining shows inflammatory cell infiltration in each group (original magnification,  $\times 400$ ). B) and C) Immunofluorescence staining shows that citrullinated ENA-78/CXCL5 recruited more F4/80 positive monocytes/macrophages into synovial tissues compared to PBS and non-citrullinated ENA-78/CXCL5 (original magnification,  $\times 400$ ). Fluorescent red and blue staining show F4/80 positive monocytes/macrophages and cell nuclei, respectively. Bars show the mean  $\pm$  SEM (n = the number of joints). \* denotes  $p < 0.05$ .

by ELISA using anti-modified citrulline (AMC) antibody for total citrullinated antigens pre and post depletion. ENA-78/CXCL5 was also immunoprecipitated from homogenized RA STs and analyzed by WB using anti-human ENA-78/CXCL5 or AMC antibodies (2). In this study, we performed similar assays upon Id1 as this protein is a nuclear protein and can be deiminated similar to other nuclear proteins (i.e. histones), making it a suitable molecule for comparison to ENA-78/CXCL5. CitId1 was prepared in vitro by incubation of rhId1 with PAD4 enzyme (noncitrullinated Id1 (noncitId1) was prepared identically without rhPAD4). To confirm the citrullination sites, citId1 and noncitId1 were analyzed much like we did previously for ENA-78/CXCL5 by using LC-MS/MS. To test the presence of ACPAs to citENA-78/CXCL5 and citId1, NL and RA patient PB sera immunodepleted of rheumatoid factor (RF) were analyzed using IDB analysis. CitENA-78/CXCL5 and citId1 as well as noncitENA-78/CXCL5 and noncitId1 were dotted onto nitrocellulose membranes, blocked, and incubated in the patient sera. Bovine serum albumin (BSA) and citBSA were used as antigen controls; anti-human Id1 antibody and rh IgG were used as sera controls (figure 6). We found that citENA-78/CXCL5 only weakly bound ACPAs in RA SF and RA PB, whereas citId1 reacted much more strongly to ACPAs in the RA SF and especially RA PB, suggesting that the modified forms of these proteins may serve as potential autoantigens in RA.

#### References:

1. Yoshida Y, Korchynskyi O, Tak PP, Isozaki T, Ruth JH, Campbell PL, Baeten DL, Gerlag DM, Amin MA, Koch AE: Citrullination of ENA-78/CXCL5 results in conversion from a non-monocyte recruiting to a monocyte recruiting chemokine. *Arthritis Rheumatol.* 66:2716-27, 2014.
2. Edhayan G, Ha CM, Isozaki T, Amin MA, Ohara RA, Morgan R, Campbell PL, Haines GK III, Arbab S, Friday S, Stinson WA, Fox DA, Ruth JH: Inflammatory Properties of Inhibitor of DNA Binding 1 as a Unique Fibroblast Derived Nuclear Protein. *Arthritis Res Ther.* 18:87, 2016.

#### **4. Key Research Accomplishments**

- CitENA-78/CXCL5 was significantly higher in RA sera and SFs than NL sera and OD including OA SFs, respectively.
- A strong correlation was found between the amount of citENA-78/CXCL5 and C-reactive protein or erythrocyte sedimentation rate (ESR) in RA SFs.
- Our conclusions for the first year of experiments is that citENA-78/CXCL5 can be detected in RA tissues and is highly correlated with RA disease activity.
- We have generated mutants for our project (as proposed) and now have constructs of ENA-78/CXCL5 that are non-mutated, and two others that have the arginine replaced by a lysine residue (Origene). These mutant forms were tested against non-mutated ENA-78/CXCL5 and used to confirm the monocyte recruitment ability of citENA-78/CXCL5 and the critical arginines involved in this process.
- Mice have been immunized with citENA-78/CXCL5 to produce a monoclonal antibody to the modified form of this pro-inflammatory chemokine. We are isolating and using these antibodies to examine the expression and function of citENA-78/CXCL5 in RA tissues.
- CitENA-78/CXCL5 plays an important role in MN migration in vivo in the air pouch inflammatory model. Our data suggest that citENA-78/CXCL5 induces JNK and NFκB signaling pathways with JNK upstream of NFκB. Targeting citENA-78/CXCL5 and its signaling pathways may be a novel approach to treat MN dependent diseases.

- CitR45K and citR48K mutations in citENA-78/CXCL5 significantly reduced MN chemotaxis, suggesting that citENA78/CXCL5-induced MN migration is dependent, at least partially, on both citrulline residues.
- IDB analysis of the patient sera showed robust signals for citId1, and weak but significant signals for citENA-78/CXCL5 (not shown in figure because the signal compared to citId1 is very low), from multiple RA patient PB sera, displaying a four-fold increase in average reactivity for citId1 as compared to NL patient PB sera.
- We show that cit-ENA-78/CXCL5 and citId1 can bind ACPAs in human RA SF if citrullinated at specific arginines unique to each protein. In other words, we have identified the specific arginines in these modified proteins that are attributable to them becoming autoantigenic (arginine 48 for citENA-78/CXCL5 and arginines 33, 52 and 121 for citId1).
- We show for the first time the presence of ACPAs with weak specificity to citENA-78/CXCL5 and robust binding activity for citId1 in RA patient PB sera, and propose ENA-78/CXCL5 and especially citId1 as novel autoantigen candidates in RA.

## 5. Conclusion

Citrullinated chemokine concentrations were measured by enzyme linked immunosorbent assay in RA and NL sera and in RA, OA, and OD SFs. The correlation between the citrullinated chemokine levels and clinical data was analyzed. We found that citENA-78/CXCL5 was significantly higher in RA sera and SFs than NL sera and OD including OA SFs, respectively. A strong correlation was found between the amount of citENA-78/CXCL5 and C-reactive protein or erythrocyte sedimentation rate in RA SFs. Our conclusion are that citENA-78/CXCL5 can be detected in RA tissues and is highly correlated with RA disease activity. Also, citENA78/CXCL5 plays an important role in MN migration in vitro and in vivo and signals via the JNK and NFκB signaling pathways. We also show for the first time the presence of ACPAs with specificity to citId1, and with weak specificity for citENA-78/CXCL5 in RA patient PB sera. We also show some reactivity for these antigens in rat serum from rats induced to develop AIA. Finally, through a series of mass spectrometry experiments, we now have the ability to identify the critical arginines that can change the hydrophobicity and autoantigenicity of a protein (e.g. ENA-78/CXCL5 and/or Id1). Protein modification by citrullination can dramatically change the function of the protein, such as when a single arginine conversion in ENA-78/CXCL5 can turn a normally neutrophil chemotactic factor into one that recruits monocytes. This process exemplifies the complexity of cell migration in the RA joint. Taken together, evidence is strong for citrullination being integral to inflammation and the pathogenesis of RA.

## 6. Publications, Abstracts and Presentations

*Peer-reviewed manuscripts:*

1. Yoshida Y, Korchynskyi O, Tak PP, Isozaki T, Ruth JH, Campbell PL, Baeten DL, Gerlag DM, Amin MA, Koch AE: Citrullination of ENA-78/CXCL5 results in conversion from a non-monocyte recruiting to a monocyte recruiting chemokine. *Arthritis Rheumatol.* 66:2716-27, 2014.
2. Edhayan G, Ha CM, Isozaki T, Amin MA, Ohara RA, Morgan R, Campbell PL, Haines GK III, Arbab S, Friday S, Stinson WA, Fox DA, Ruth JH: Inflammatory Properties of Inhibitor of DNA Binding 1 as a Unique Fibroblast Derived Nuclear Protein. *Arthritis Res Ther.* 18:87, 2016.

3. Ohara RA, Lepore N, W. Stinson WA, Edhayan G, Campbell PL, Arwani S, Morgan RL, Ruth JH, Koch AE, Fox DA, Amin MA: Citrullinated ENA78/CXCL5 Induces Monocyte Migration via JNK and NFκB Signaling Pathways. *In preparation*.
4. Ohara RA, Remmer HA, Campbell PL, Fox DA, Ruth JH: Citrullination of Inhibitor of DNA Binding-1 Leads to Autoantigenicity in Rheumatoid Arthritis. *In preparation*.

*Abstracts accepted to national meetings:*

1. Yoshida K, Campbell PL, Amin MA, Koch AE, Fox DA, Ruth JH: Citrullination of ENA-78/CXCL5 changes its receptor affinity from CXCR2 to CXCR1 and induces monocyte migration. *Arthritis Rheum*. 65 S10:327, 2013.
2. Edhayan G, Ha CM, Ohara RA, Isozaki T, Amin MA, Arbab AS, Tsou PS, Campbell PL, Schiopu E, Khanna D, Morgan R, Friday SC, Fox DA, Ruth JH: Inflammatory Properties of Inhibitor of DNA Binding 1 as a Unique Fibroblast Derived Nuclear Protein. *Arthritis Rheumatol*. 66 S10:863, 2014.
3. Ohara RA, Edhayan G, Ha CM, Amin MA, Arbab AS, Campbell PL, Fox DA, Ruth JH: Evidence for citrullination of the nuclear transcription factor Inhibitor of DNA Binding 1 (Id1) in Rheumatoid Arthritis. *Arthritis Rheumatol*: 66 S10:189, 2014.
4. Ohara RA, Edhayan G, Stinson WA, Campbell PL, Remmer HA, Amin MA, Fox DA, Ruth JH: Evidence for Citrullinated Inhibitor of DNA Binding 1 (Id1) as a Novel Autoantigen Candidate in Rheumatoid Arthritis. *Arthritis Rheumatol* In press.
5. Edhayan G, Ha CM, Ohara RA, Isozaki T, Amin MA, Arbab AS, Campbell PL, Morgan R, Stinson WA, Friday SC, Fox DA, Ruth JH: Inhibitor of DNA Binding 1 as a Fibroblast Derived Inflammatory Angiogenic Agonist in Rheumatoid Arthritis. *Arthritis Rheumatol* In press.
6. Ohara RA, Lepore N, W. Stinson WA, Edhayan G, Campbell PL, Arwani S, Morgan RL, Ruth JH, Koch AE, Fox DA, Amin MA: Citrullinated ENA78/CXCL5 Induces Monocyte Migration via JNK and NFκB Signaling Pathways. *Arthritis Rheumatol* In press.
7. Ohara RA, Remmer HA, Campbell PL, Fox DA, Ruth JH: Citrullination of Inhibitor of DNA Binding-1 at Specific Locations Leads to Autoantigenicity in Rheumatoid Arthritis. *Arthritis Rheumatol*. 68 S10, 2016.
8. Ohara RA, Edhayan G, Saunders TL, Lanigan TM, Morgan R, Stinson WA, Campbell PL, Graham J, Fox DA, Ruth JH: Regulation of Rheumatoid Arthritis Synovial Fibroblast Cytokine Production By Inhibitor of DNA Binding-1 Via Crispr/Cas9 Transfection. *Arthritis Rheumatol*. 68 S10, 2016.

## **7. Inventions, Patents and Licenses**

Nothing to report.

## **8. Reportable outcomes**

- We developed an ELISA that can successfully detect citrullinated chemokines in RA tissues.
- We developed a Western blotting technique that can successfully detect citrullinated chemokines in RA tissues.
- We can correlate RA disease severity with citENA-78/CXCL5 concentrations in RA SF.

- We found that citENA78/CXCL5 plays an important role in MN migration in vivo in the air pouch inflammatory model. Our data suggest that citENA-78/CXCL5 induces JNK and NFκB signaling pathways with JNK upstream of NFκB. Targeting citENA-78/CXCL5 and its signaling pathways may be a novel approach to treat MN dependent diseases.
- We discovered that alteration of arginine residues R45K and R48K (by replacing them with lysine residues) results in a significant reduction in citENA-78/CXCL5 MN chemotaxis. This suggests that both arginine residues are responsible for conversion of ENA-78/CXCL5 from a normally neutrophil recruiting chemokine into a monocyte chemotactic factor after deimination.
- ACPAs can be detected in RA PB for ENA-78/CXCL5 and especially for citId1 in RA PB and SF, suggesting that ENA-78/CXCL5 and especially citId1 may serve as autoantigens in RA.
- By mass spectrometry, we can now determine the critical arginines responsible for converting native proteins such as ENA-78/CXCL5 or Id1 into potential autoantigens in RA, capable of binding ACPAs in RA patient effusions.

## 9. Other Achievements

- We found that a nuclear regulatory factor Id1 protein can be deiminated and has robust binding for ACPAs in RA SF and especially RA PB. We also have preliminary evidence that Id1 may regulate chemokine expression from RA fibroblasts.
- We found ACPAs in rat serum of rat AIA (day 7) but with high background. We are currently working with this assay to achieve meaningful results that can be compared with what was observed with the patient RA SFs.
- By mass spectrometry, we can determine which amino acids in a protein (eg. ENA-78/CXCL5 and Id1) are the critical arginines involved in converting a natively expressed protein into one that exhibits autoantigenic activity by binding ACPAs in RA patient effusions.

## 10. References

1. Yoshida Y, Korchynskyi O, Tak PP, Isozaki T, Ruth JH, Campbell PL, Baeten DL, Gerlag DM, Amin MA, Koch AE: Citrullination of ENA-78/CXCL5 results in conversion from a non-monocyte recruiting to a monocyte recruiting chemokine. *Arthritis Rheumatol.* 66:2716-27, 2014.
2. Edhayan G, Ha CM, Isozaki T, Amin MA, Ohara RA, Morgan R, Campbell PL, Haines GK III, Arbab S, Friday S, Stinson WA, Fox DA, Ruth JH: Inflammatory Properties of Inhibitor of DNA Binding 1 as a Unique Fibroblast Derived Nuclear Protein. *Arthritis Res Ther.* 18:87, 2016.

## 11. Appendices

Please see manuscripts (references 1 and 2) enclosed.

## Quad/Gantt chart

TIMETABLE FOR RESEARCH PROGRAM			
	YEAR 01	YEAR 02	YEAR 03
<b>SPECIFIC AIM #1</b>	COMPLETED: Synovial fluids and sera will be obtained from patients with RA, OA or other diseases and assayed for key citrullinated and non-citrullinated chemokines thought to be important in the pathogenesis of RA.	COMPLETED: Synovial tissue will be obtained from RA, OA, or NL joints, homogenized and assayed for citrullinated and non-citrullinated chemokines.	
<b>SPECIFIC AIM #2</b>	COMPLETED: Leukocyte chemotaxis assays, modifying chemokines containing ELR motif and mass spec analysis, site directed mutagenesis of chemokines.	COMPLETED: Signal transduction analysis of endothelial cells and leukocytes.	COMPLETED: <i>In vitro</i> Angiogenesis assays and Matrigel plug assay (if needed)
<b>SPECIFIC AIM #3</b>		COMPLETED: Sera obtained from RA, OA, or NL individuals will be assayed against citrullinated vs. non-citrullinated chemokines to determine if autoantibodies are made.	COMPLETED: Citrullinated chemokines will be adsorbed out of RA serum to determine how much reactivity for citrullinated antigens remains.
<b>SPECIFIC AIM #4</b>		COMPLETED: Determine the inflammatory effect of injecting citrullinated chemokines vs. native chemokines into normal rodent knees.	COMPLETED: Perform rat adjuvant induced arthritis and determine whether citrullinated chemokines and antibodies to these chemokines are present before and during development of arthritis.



# Citrullination of Epithelial Neutrophil–Activating Peptide 78/CXCL5 Results in Conversion From a Non–Monocyte-Recruiting Chemokine to a Monocyte-Recruiting Chemokine

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**Objective.** To examine whether the citrullinated chemokines epithelial neutrophil–activating peptide 78 (ENA-78)/CXCL5, macrophage inflammatory protein 1 $\alpha$ /CCL3, and monocyte chemotactic protein 1/CCL2 are detected in the biologic fluid of patients with rheumatoid arthritis (RA), and if so, to determine the biologic activities of these chemokines.

**Methods.** Recombinant human chemokines were citrullinated by peptidylarginine deiminase. Enzyme-linked immunosorbent assays were performed to measure the concentrations of citrullinated chemokines in sera from patients with rheumatoid arthritis (RA) and normal individuals and in synovial fluid from patients with RA, patients with osteoarthritis (OA), and patients with other inflammatory rheumatic diseases. The correlation between the citrullinated chemokine levels and clinical data was analyzed. Monocyte and neutrophil chemotaxis assays were performed, and native (non-

citrullinated) or citrullinated ENA-78/CXCL5 was injected into mouse knees to evaluate the biologic activities of these chemokines.

**Results.** The concentration of citrullinated ENA-78/CXCL5 was significantly higher in RA sera and SF than in normal sera and in SF from patients with other rheumatic diseases including OA. In RA SF, a strong correlation between the amount of citrullinated ENA-78/CXCL5 and the C-reactive protein level or the erythrocyte sedimentation rate was observed. Citrullinated ENA-78/CXCL5 induced monocyte chemotaxis via CXCR1 and CXCR2, while noncitrullinated ENA-78/CXCL5 did not. In a mouse model of inflammatory arthritis, citrullinated ENA-78/CXCL5 induced more severe inflammation and recruited more monocytes than did noncitrullinated ENA-78/CXCL5.

**Conclusion.** Citrullinated ENA-78/CXCL5 is highly correlated with RA disease activity and, unlike noncitrullinated ENA-78/CXCL5, recruits monocytes. These results indicate that citrullinated ENA-78/CXCL5 may exert previously unrecognized inflammatory properties in RA by recruiting monocytes to inflamed joint tissue.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by the infiltration of inflammatory cells into synovial tissue and synovial fluid (SF) (1). Anti-cyclic citrullinated peptide (anti-CCP) antibodies are present in the sera of nearly 70% of patients with RA and have become key diagnostic markers for this disease (2). Citrulline residues in target antigens are essential in the formation of anti-citrullinated peptide/protein antibody epitopes (3,4). These epitopes result from citrullination, a posttransla-

Supported by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs and by the Frederick G. L. Huetwell and William D. Robinson, MD, Professorship in Rheumatology at the University of Michigan (awarded to Dr. Koch).

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Submitted for publication June 20, 2013; accepted in revised form June 12, 2014.

tional modification of arginine. Peptidylarginine deiminase (PAD) enzymes are calcium-dependent enzymes that catalyze citrullination of target proteins by converting arginine to citrulline (5). Five PAD family members, PAD type 1 (PAD1), PAD2, PAD3, PAD4, and PAD6, have been identified in many types of tissue, including synovial tissue, and in human leukocytes (6).

Chemokines play an important role as monocyte and polymorphonuclear neutrophil (PMN) recruiters in the setting of RA synovitis and tissue destruction (7,8). Chemokines are classified into 4 subfamilies based on the number and spacing of their first cysteine residues in the primary amino acid sequence (9). These chemokine families are designated as CXC, CC, C, and CX<sub>3</sub>C. The CXC chemokines are further divided into 2 subgroups based on whether the Glu-Leu-Arg (ELR) motif precedes the first cysteine residue. Epithelial neutrophil-activating peptide 78 (ENA-78)/CXCL5, a CXC chemokine, is an 8.3-kD protein with 78 amino acids containing 4 cysteine residues positioned identically to those of interleukin-8 (IL-8/CXCL8) (10). We previously observed that the concentration of ENA-78/CXCL5, which is associated with neutrophil recruitment, is significantly higher in RA SF compared with osteoarthritis (OA) SF or SF from patients with other forms of arthritis (11). In addition, we demonstrated that neutralization of ENA-78/CXCL5 ameliorated the severity of adjuvant-induced arthritis in a rat model (12). These findings support the notion that ENA-78/CXCL5 is important in the pathogenesis of RA.

Although the presence of citrullinated proteins such as fibrinogen,  $\alpha$ -enolase, and vimentin in RA has been reported (4,13–15), citrullinated chemokines have not yet been detected in RA. We chose to examine chemokines, because they are biologically relevant joint proteins. Several chemokines are highly expressed in the joints of patients with RA, and PAD2 and PAD4 are mainly present in synovial tissue. The expression levels of these PADs are correlated with inflammation, thickness of the synovial lining layer, and vascularity (16). These observations support the hypothesis that citrullinated chemokines may be present in rheumatic joints.

We compared the presence of citrullinated ENA-78/CXCL5, citrullinated macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ )/CCL3, and citrullinated monocyte chemoattractant protein 1 (MCP-1)/CCL2 in the biologic fluid of patients with RA with that in the biologic fluid of patients with other arthritic diseases, using a newly developed enzyme-linked immunosorbent assay (ELISA) system, and examined the biologic activity of these chemokines in vitro and that of ENA-78/CXCL5 in vivo.

## PATIENTS AND METHODS

**Patients and normal control subjects.** Serum samples were obtained from 11 patients with RA and 15 normal control subjects, and SF samples were obtained from 20 patients with RA, 15 patients with OA, and 13 patients with other inflammatory rheumatic diseases, including gout ( $n = 4$ ), pseudogout ( $n = 2$ ), psoriatic arthritis ( $n = 1$ ), spondyloarthritis ( $n = 3$ ), Behçet's disease ( $n = 1$ ), Lyme disease ( $n = 1$ ), and unclassified arthritis ( $n = 1$ ). Rheumatoid factor (RF) was immunodepleted from RA sera and SF using goat anti-human IgM ( $\mu$ -chain specific) agarose (Sigma-Aldrich) prior to measuring citrullinated chemokines by ELISA. All samples were obtained after approval by the Institutional Review Board and provision of informed consent by the subjects.

**Chemokine DNA cloning.** Full-length ENA-78/CXCL5 complementary DNA (cDNA) (NCBI accession no. NM\_002994) was obtained from Open Biosystems. We generated cDNA libraries for MCP-1/CCL2 and MIP-1 $\alpha$ /CCL3 from primary synovial fibroblasts derived from patients with RA. The cDNA fragments encoding mature proteins were amplified by polymerase chain reaction (PCR) using primers with an incorporated *Nco* I restriction site in the forward primers and with 6 codons encoding histidines followed by a stop codon and the *Eco* RI restriction site in the reverse primers (Primer Express software; Applied Biosystems). The primers used are as follows (forward and reverse, respectively): for ENA-78/CXCL5, 5'-TAATCCATGGGAGCTGGTCCCTGCCGCTGCTGT-3' and 5'-TAAGAATTCTCAGTGATGTGTGATGGTGTGTTTTCCTTGTTCACCGT-3'; for MIP-1 $\alpha$ /CCL3, 5'-TAATCCATGGGAGCTGACACGCCGACCGCCTG-3' and 5'-TAAGAATTCTCAGTGATGGTGATGGTGTGATGGGCACTCAGTCCAGGTCGC-3'; for MCP-1/CCL2, 5'-TAATCCATGGGACAGCCAGATGCAATCAATGCC-3' and 5'-TAAGAATTCTCAGTGATGGTGATGGTGTGATGAGTCTTCGGAGTTTGGGTTTG-3'. *Nco* I/*Eco* RI-flanked PCR fragments containing a C-terminus 6 $\times$ His tag were initially cloned into a pQE-TriSystem Vector (Qiagen). All sequences were verified using BigDye Terminator sequencing (Life Technologies). For optimization of mammalian expression, inserts containing a C-terminus 6 $\times$ His tag were further cloned into the mammalian expression vector pcDEF (17).

**Transfection of HEK 293T cells and purification of recombinant human chemokines.** All mammalian expression vectors were transfected into HEK 293T cells using polyethylenimine (Polysciences) to collect 6 $\times$ His-tagged chemokines from cellular lysates prepared with 1% Triton X-100. Chemokines (6 $\times$ His tagged) from HEK 293T cell lysates were purified with ProBond Nickel beads (Life Technologies), rinsed extensively with 10 mM imidazole, and eluted gradually with 50–200 mM imidazole. The quality and quantity of expressed recombinant proteins were assessed with appropriate DuoSet ELISA kits (R&D Systems) specific for these proteins and with colloidal Coomassie staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis resolution.

**In vitro citrullination of chemokines.** After the concentration of purified chemokines was measured using DuoSet ELISA kits, 100 microliters of purified recombinant human chemokine ( $\sim 100$  ng/ml) was incubated with 0.5 units of rabbit

skeletal muscle PAD (Sigma-Aldrich) in 40 mM Tris HCl, pH 7.6, 10 mM CaCl<sub>2</sub>, and 2.5 mM dithiothreitol for 2 hours at 37°C. Deimination was stopped by chelating the calcium with 25 mM EDTA. These citrullinated chemokines were subsequently used as standards for ELISA of citrullinated chemokines. Alternatively, 5  $\mu$ M recombinant human ENA-78 (rhENA-78)/CXCL5, recombinant human MIP-1 $\alpha$ /CCL3, or recombinant human MCP-1/CCL2 (OriGene) was incubated with rabbit skeletal PAD (250 nM) at enzyme:substrate molar ratios of 1:20 in 40 mM Tris HCl with 2 mM CaCl<sub>2</sub> (pH 7.4) for 1.5 hours at 37°C. Deimination was stopped with 0.1% trifluoroacetic acid (TFA). These citrullinated chemokines were used in both in vitro chemotaxis assays and in vivo experiments. Citrullination of rhENA-78/CXCL5 was confirmed by Western blotting (18). For detection of citrullinated protein, citrulline residues were chemically modified to form a ureido group adduct before addition of an anti-modified citrulline antibody (Millipore) according to the manufacturer's protocol, which enables detection of citrulline residues independent of neighboring amino acid sequences (19). The diagnostic +1Da mass shift occurring upon citrullination was identified by liquid chromatography tandem mass spectrometry (MS Bioworks) (20).

**Sandwich ELISA for citrullinated chemokines.** An ELISA was designed to determine the concentrations of citrullinated chemokines in biologic fluid. Ninety-six-well plates (Thermo Fisher Scientific) were coated overnight at room temperature with mouse anti-human ENA-78/CXCL5, mouse anti-human MCP-1/CCL2, or goat anti-human MIP-1 $\alpha$ /CCL3 (R&D Systems). Between each step, the plates were washed with wash buffer (0.05% Tween 20 in phosphate buffered saline [PBS]). The plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature and incubated with serum, SF, or standards for 2 hours at room temperature. Citrullinated recombinant human chemokines and native (noncitrullinated) recombinant human chemokines purified from the transfected HEK 293T cells were used as standards and negative controls, respectively, for the ELISA. The samples were crosslinked onto the plates with 1% glutaraldehyde in PBS for 30 minutes at room temperature.

The plates were then incubated with 0.2M Tris HCl (pH 7.8) for 30 minutes at room temperature to block the crosslinking. The plates were then incubated overnight at 37°C in a citrulline-modification solution consisting of 2 parts solution A (0.025% [weight/volume] FeCl<sub>3</sub>, 4.6M H<sub>2</sub>SO<sub>4</sub>, and 3.0M H<sub>3</sub>PO<sub>4</sub>), 1 part solution B (1% diacetylmonoxime, 0.5% antipyrine, and 1M acetic acid), and 1 part H<sub>2</sub>O (18). The plates were incubated for 2 hours at room temperature with rabbit anti-modified citrulline (Millipore), diluted 1:2,500 in PBS containing 1% BSA. The plates were incubated for 2 hours at room temperature with horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (Dako), diluted 1:1,000 in PBS containing 1% BSA. Biotin-Tyramide Reagent (PerkinElmer), diluted 1:1,000 in 0.05M Tris (base pH 8.5), was added, followed by HRP-conjugated streptavidin. The plates were developed using tetramethylbenzidine, development was stopped with 2N H<sub>2</sub>SO<sub>4</sub>, and the plates were read on a microplate reader at 450 nm. Serum and SF chemokine concentrations were also measured using DuoSet ELISA kits.

**In vitro monocyte and polymorphonuclear neutrophil (PMN) chemotaxis assays.** Monocytes and PMNs were isolated from normal human peripheral blood. Monocyte and PMN chemotaxis assays were performed using 48-well modified Boyden chambers (Neuro Probe) as described previously (21,22). Noncitrullinated chemokines (OriGene), citrullinated chemokines, and reaction buffer consisting of 40 mM Tris HCl, 2 mM CaCl<sub>2</sub>, 250 nM rabbit skeletal PAD, and 0.1% TFA were diluted by PBS with calcium and magnesium in the same manner and tested for chemotaxis. The composition of the reaction buffer that contained PAD enzymes was the same as the composition of the stock solution of citrullinated chemokines. PBS with calcium and magnesium and fMLP (100 nM) (Sigma-Aldrich) were used as negative and positive stimuli, respectively. For the inhibitor studies, monocytes (2.5  $\times$  10<sup>6</sup>/ml) were pretreated with 500 ng/ml pertussis toxin (Sigma-Aldrich), which is a G protein-coupled receptor antagonist, 10  $\mu$ g/ml anti-CXCR1, anti-CXCR2, or isotype control IgG2a (R&D Systems) in PBS with calcium and magnesium for 1 hour to examine whether G protein-coupled receptors are used by citrullinated ENA-78/CXCL5 to induce monocyte chemotaxis.

**Mouse model of inflammatory arthritis.** Female C57BL/6 mice (8–10 weeks old) were purchased from the National Cancer Institute. The mice were divided into the following 3 treatment groups: PBS, noncitrullinated ENA-78/CXCL5 (OriGene), and citrullinated ENA-78/CXCL5. The mice were anesthetized, and the knee circumference was determined by caliper measurements before intraarticular injection and calculated using the following formula: circumference =  $\pi(a + b)/2$ , where  $a$  is the laterolateral diameter and  $b$  is the anteroposterior diameter. The anesthetized mice received 20  $\mu$ l/knee joint of PBS, noncitrullinated ENA-78/CXCL5 (16 ng), or citrullinated ENA-78/CXCL5 (16 ng). In all of the mice, circumference measurements were obtained in a blinded manner 24 hours after the intraarticular injection.

**Hematoxylin and eosin (H&E) and immunofluorescence staining.** Mouse knee joints embedded in OCT compound were frozen and cut (10  $\mu$ m). H&E staining was performed as described previously (23). Immunofluorescence staining was performed on cryosections from mouse knee joints to determine monocyte/macrophages, using rat anti-mouse F4/80 antibodies (GeneTex) at 1  $\mu$ g/ml as primary antibody and Alexa Fluor 555-conjugated goat anti-rat IgG (Life Technologies) at a dilution of 1:200 as secondary antibody. The method of immunofluorescence staining has been described previously (21). The number of F4/80-positive monocyte/macrophages was calculated as the average of the number of cells in 3 fields (400 $\times$ ) that showed the most remarkable infiltrates in the joint space where the injections were administered.

**Statistical analysis.** Statistical differences between experimental groups were determined by Student's  $t$ -test or one-way analysis of variance followed by Tukey's multiple comparison test for post hoc analysis. The clinical correlation between chemokine levels and clinical data was assessed by Pearson's correlation coefficient. Statistical analysis was performed with assistance from personnel at the Center for Statistical Consultation and Research at the University of Michigan. Results are expressed as the mean  $\pm$  SEM.  $P$  values less than 0.05 were considered significant.

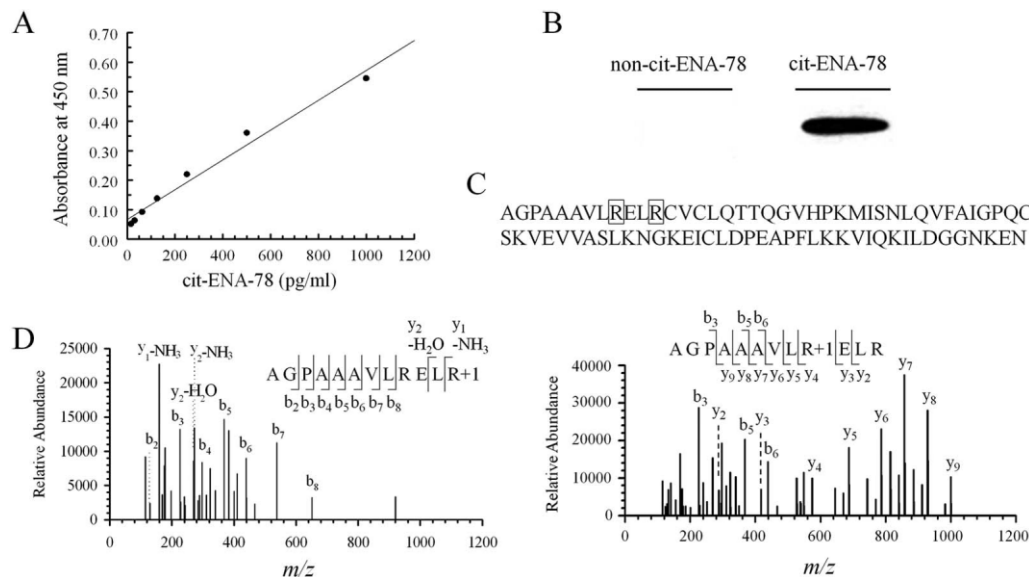


# RESULTS

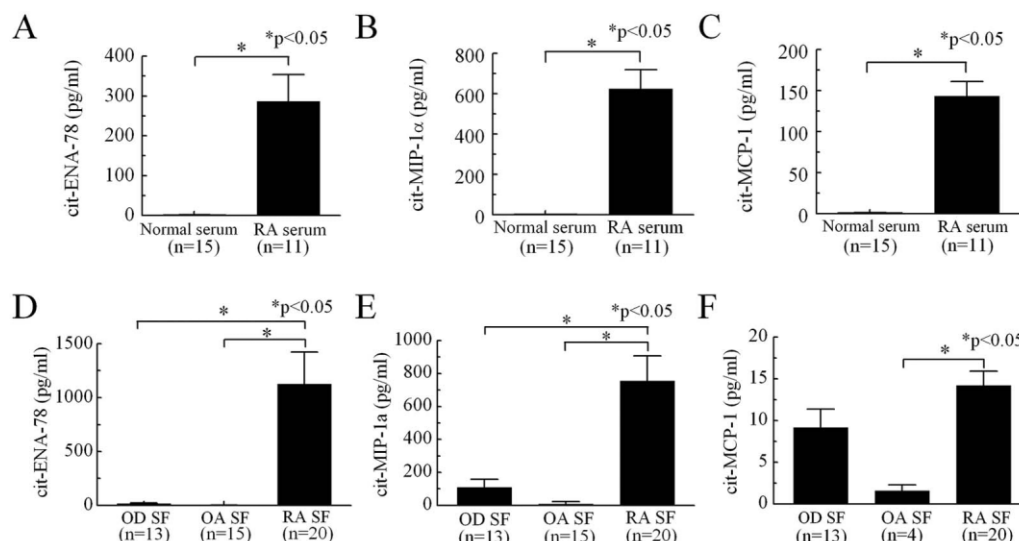
**Detection of citrullinated rhENA-78/CXCL5 and verification of in vitro citrullination of ENA-78/CXCL5.** As shown in Figure 1A, the standard curve of citrullinated rhENA-78/CXCL5, as determined by ELISA, demonstrated high correlation between the concentration of citrullinated rhENA-78/CXCL5 and absorbance (Figure 1A). Noncitrullinated ENA-78/CXCL5 (1000 pg/ml) was not detected by this ELISA. We then performed Western blotting to confirm that rhENA-78/CXCL5 was citrullinated in vitro. Citrullinated ENA-78/CXCL5 was recognized by anti-modified citrulline antibody, while noncitrullinated ENA-78/CXCL5 was not (Figure 1B). The diagnostic +1Da mass shift occurring upon citrullination of ENA-78/CXCL5, which has 2 arginine residues, was identified by mass spectrometry (20). The mass spectrometry data indicated that both arginine residues were detected and citrullinated (Figures 1C and D). Citrullination of MCP-1/CCL2 and MIP-1 $\alpha$ /CCL3, which have 4 and 3 arginine residues,

respectively, was also identified by mass spectrometry (data not shown).

**Citrullinated chemokine concentrations in RA sera and normal control sera.** To ascertain whether citrullinated chemokines were detectable in biologic fluids, we assayed serum samples obtained from patients with RA and normal control subjects, using a citrullinated chemokine sandwich ELISA. In normal control subjects and patients with RA, the mean  $\pm$  SEM levels of citrullinated ENA-78/CXCL5 were  $1.2 \pm 0.7$  pg/ml and  $286 \pm 68$  pg/ml, respectively (Figure 2A). The levels of citrullinated MIP-1 $\alpha$ /CCL3 in normal and RA sera were  $1.2 \pm 1.2$  pg/ml and  $624 \pm 96$  pg/ml, respectively (Figure 2B), and the levels of citrullinated MCP-1/CCL2 in normal and RA sera were  $0.9 \pm 0.5$  pg/ml and  $143 \pm 18$  pg/ml, respectively (Figure 2C). The levels of citrullinated ENA-78/CXCL5, MIP-1 $\alpha$ /CCL3, and MCP-1/CCL2 in patients with RA were all significantly higher than the levels in normal control subjects ( $P < 0.05$ ). The mean  $\pm$  SEM concentrations of ENA-78/CXCL5



**Figure 1.** Detection of citrullinated recombinant human epithelial neutrophil-activating peptide 78 (ENA-78)/CXCL5 and verification of successful in vitro citrullination of ENA-78/CXCL5. **A**, Standard curve of citrullinated recombinant human ENA-78/CXCL5 as determined by enzyme-linked immunosorbent assay. Each point was determined using duplicate assays. **B**, Immunoblots showing that citrullinated recombinant human ENA-78/CXCL5 was recognized by anti-modified citrulline antibody (right) while noncitrullinated ENA-78/CXCL5 was not (left). The blot used to detect citrullinated protein was chemically modified prior to immunostaining. **C**, Amino acid sequence of ENA-78/CXCL5 (mature form 37–114). The boxed areas show the citrullinated arginines that were detected. **D**, Annotated tandem mass spectrometry (MS/MS) fragmentation spectra for the citrullinated ENA-78/CXCL5 peptides, showing the citrullinated arginine residues. Left, MS/MS spectrum confirming the presence of C-terminal citrullination at position 12 (R48) from the  $y_1$  and  $y_2$  ions present in the spectrum. Right, The presence of ions  $y_4$  to  $y_9$  in the spectrum confirms the presence of the “middle” R+1 (citrullination) at position 9 (R45). The MS/MS fragmentation data were annotated using Expert System (Max-Planck Institute of Biochemistry). The precursor ion was observed with a mass error of  $<1$  parts per million, and the error for the fragment ions was  $\leq 0.02$  daltons.



**Figure 2.** Expression of citrullinated chemokines in rheumatoid arthritis (RA) sera and synovial fluid (SF). Citrullinated chemokines were measured using an enzyme-linked immunosorbent assay (ELISA) in which chemokines were captured on an ELISA plate, followed by detection with anti-modified citrulline after chemical modification of citrulline residues. Rheumatoid factor was depleted from RA SF and RA sera prior to the ELISA. **A–C**, Concentrations of citrullinated epithelial neutrophil-activating peptide 78 (ENA-78)/CXCL5 (**A**), macrophage inflammatory protein 1α (MIP-1α)/CCL3 (**B**), and monocyte chemotactic protein 1 (MCP-1)/CCL2 (**C**) in normal sera and sera from patients with RA. **D–F**, Concentrations of citrullinated ENA-78/CXCL5, citrullinated MIP-1α/CCL3, and citrullinated MCP-1/CCL2 in SF from patients with other inflammatory rheumatic diseases (OD), patients with osteoarthritis (OA), and patients with RA. Bars show the mean ± SEM.

were  $3,165 \pm 825$  pg/ml in normal sera and  $3,942 \pm 1,139$  pg/ml in RA sera, the levels of MIP-1α/CCL3 were  $230 \pm 176$  pg/ml and  $727 \pm 420$  pg/ml, respectively, and the levels of MCP-1/CCL2 were  $43 \pm 22$  pg/ml and  $289 \pm 271$  pg/ml, respectively; none of the differences between groups were statistically significant (results not shown).

**Citrullinated chemokine concentrations in SF from patients with RA, patients with OA, and patients with other rheumatic diseases.** The concentrations of citrullinated chemokines in SF samples obtained from patients with RA, patients with OA, and patients with other inflammatory rheumatic diseases were measured by sandwich ELISA. The mean ± SEM concentration of citrullinated ENA-78/CXCL5 was significantly higher in SF from patients with RA ( $1,126 \pm 297$  pg/ml) than in SF from patients with OA ( $2.3 \pm 1.0$  pg/ml) and patients with other inflammatory rheumatic diseases ( $15 \pm 9$  pg/ml) ( $P < 0.05$ ) (Figure 2D). The mean ± SEM concentration of citrullinated MIP-1α/CCL3 was also significantly higher in the SF of patients with RA ( $755 \pm 152$  pg/ml) compared with that in the SF of patients with other inflammatory rheumatic diseases ( $109 \pm 49$  pg/ml) and patients with OA ( $9 \pm 15$  pg/ml) ( $P < 0.05$ ) (Figure 2E). Citrullinated MCP-1/CCL2 concentrations were

significantly higher in RA SF ( $14 \pm 2$  pg/ml) compared with OA SF ( $1.6 \pm 0.7$  pg/ml) ( $P < 0.05$ ) (Figure 2F).

We also used ELISA kits to measure chemokine concentrations. The mean ± SEM concentrations of ENA-78/CXCL5 were  $163 \pm 107$  pg/ml in RA SF,  $0 \pm 0$  pg/ml in the SF of patients with other diseases, and  $19 \pm 13$  pg/ml in OA SF. The concentrations of MIP-1α/CCL3 were  $460 \pm 171$  pg/ml in RA SF,  $31 \pm 10$  pg/ml in SF from patients with other rheumatic diseases, and  $75 \pm 52$  pg/ml in OA SF. The concentrations of MCP-1/CCL2 were  $1,085 \pm 398$  pg/ml in RA SF,  $288 \pm 42$  pg/ml in SF from patients with other rheumatic diseases, and  $390 \pm 80$  pg/ml in OA SF. There were no significant differences in SF chemokine concentrations between these groups.

**Positive correlation between citrullinated ENA-78/CXCL5 concentrations and the C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR) in RA SF.** We analyzed the relationship between the citrullinated chemokine concentrations in SF and clinical data in patients with RA. As shown in Table 1, the clinical characteristics of these patients were as follows: mean ± SEM age  $52.7 \pm 3.4$  years, disease duration  $145.7 \pm 39.2$  months, ESR  $38.1 \pm 4.3$  mm/hour, CRP level  $40.5 \pm 9.9$  mg/liter, Disease Activity Score in 28

**Table 1.** Clinical characteristics of the patients with rheumatoid arthritis whose synovial fluid was studied\*

Male/female, % (n = 20)	20/80
Age, years (n = 20)	52.7 ± 3.4
Disease duration, months (n = 20)	145.7 ± 39.2
Tender joint count in 28 joints (n = 13)	5.7 ± 2.1
Swollen joint count in 28 joints (n = 13)	4.5 ± 1.9
Bone erosion, yes/no, % (n = 20)	40/60
ESR, mm/hour (n = 15)	38.1 ± 4.3
CRP, mg/liter (n = 14)	40.5 ± 9.9
DAS28 (n = 12)	5.1 ± 0.4
Rheumatoid factor, units/ml (n = 10)	74.8 ± 43.2
Anti-CCP, AU/ml (n = 10)	2,296.1 ± 1,559.1

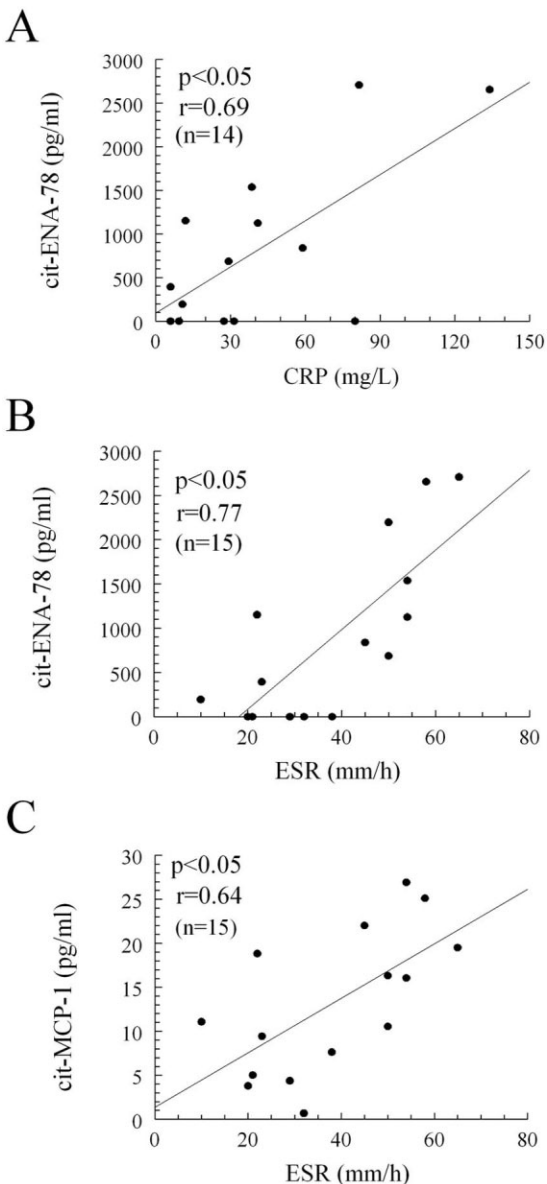
\* Except where indicated otherwise, values are the mean ± SEM. ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints; anti-CCP = anti-cyclic citrullinated peptide.

joints (24)  $5.1 \pm 0.4$ , tender joint count  $5.7 \pm 2.1$ , swollen joint count  $4.5 \pm 1.9$ , RF level  $74.8 \pm 43.2$  units/ml, and anti-CCP antibody level  $2,296.1 \pm 1,559.1$  AU/ml. As shown in Figure 3, citrullinated ENA-78/CXCL5 concentrations were significantly correlated with CRP levels ( $r = 0.69$ ,  $P < 0.05$ ) and the ESR ( $r = 0.77$ ,  $P < 0.05$ ), and citrullinated MCP-1/CCL2 concentrations were significantly correlated with the ESR ( $r = 0.64$ ,  $P < 0.05$ ). There was no significant correlation between the concentration of citrullinated MIP-1 $\alpha$ /CCL3 in RA SF with the other clinical parameters examined.

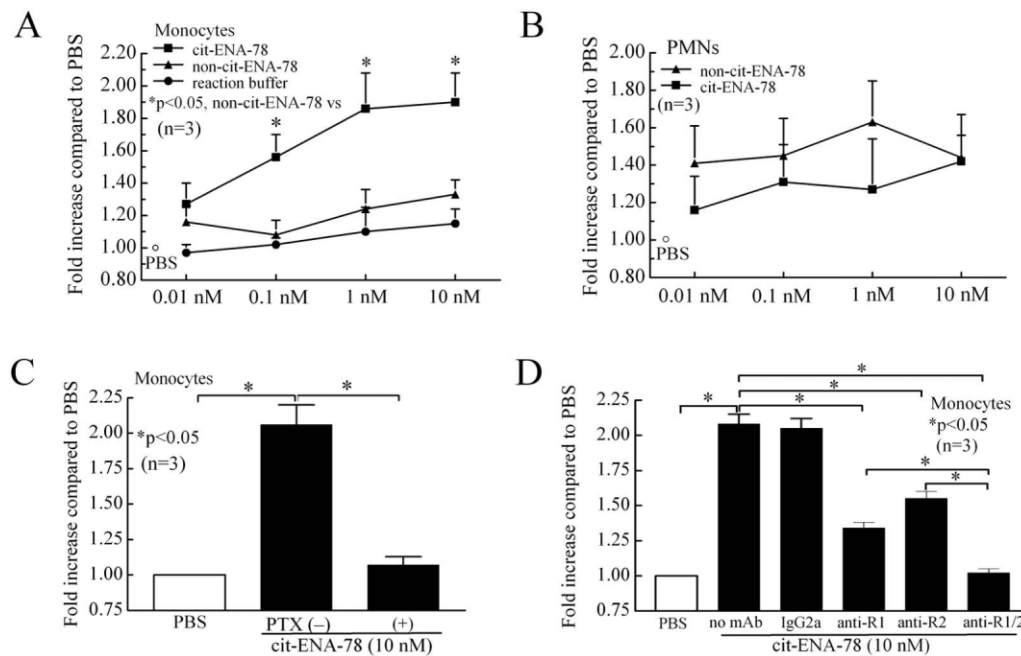
**Citrullinated ENA-78/CXCL5-induced monocyte migratory activity via CXCR1 and CXCR2.** Under normal conditions, ENA-78/CXCL5 induces PMN migration, but it does not induce monocyte migration (10,25,26). We performed monocyte and PMN chemotaxis assays using a modified Boyden chamber, and the results showed that the fold increase in monocyte migration was significantly higher in response to citrullinated ENA-78/CXCL5 at 0.1 nM, 1 nM, and 10 nM (mean ± SEM  $1.6 \pm 0.1$ ,  $1.9 \pm 0.2$ , and  $1.9 \pm 0.2$ , respectively) than in response to noncitrullinated ENA-78/CXCL5 and PBS (Figure 4A). We also tested various concentrations of reaction buffer in citrullinated ENA-78/CXCL5 stock solution for chemotaxis of monocytes. The reaction buffer including PAD enzymes did not induce monocyte migration at the concentrations tested. In contrast to citrullinated ENA-78/CXCL5, citrullinated MCP-1/CCL2 recruited significantly fewer monocytes at 10 nM and 100 nM than did noncitrullinated MCP-1/CCL2 (data not shown), and citrullinated MIP-1 $\alpha$ /CCL3 tended to have the same level of monocyte-recruiting activity as noncitrullinated MIP-1 $\alpha$ /CCL3 at 0.01–100 nM (data not shown).

PMN chemotaxis assays showed that noncitrulli-

nated ENA-78/CXCL5 had significantly higher PMN chemotactic activity at 0.1 nM ( $1.5 \pm 0.2$ ) and 1 nM ( $1.6 \pm 0.2$ ) compared with PBS ( $P < 0.05$ ), while citrullinated ENA-78/CXCL5 had significantly higher activity at 10 nM ( $1.4 \pm 0.1$ ) compared with PBS ( $P < 0.05$ ) (Figure 4B). Thus, the fold increase in PMN



**Figure 3.** Correlation of clinical data with citrullinated chemokine levels in RA SF. Positive correlations between citrullinated ENA-78/CXCL5 and the C-reactive protein (CRP) level (A), citrullinated ENA-78/CXCL5 and the erythrocyte sedimentation rate (ESR) (B), and citrullinated MCP-1/CCL2 and the ESR (C) were observed. See Figure 2 for other definitions.



**Figure 4.** Monocyte recruitment by citrullinated epithelial neutrophil-activating peptide 78 (ENA-78)/CXCL5. **A**, Fold increase in monocyte migration in response to citrullinated ENA-78/CXCL5 and noncitrullinated ENA-78/CXCL5, as determined by monocyte chemotaxis assay. At 0.1 nM, 1 nM, and 10 nM, the response to citrullinated ENA-78/CXCL5 was significantly higher than the response to noncitrullinated ENA-78/CXCL5 and phosphate buffered saline (PBS). As controls, various concentrations of reaction buffer including peptidylarginine deiminase enzymes were tested for chemotaxis of monocytes. There was no significant difference between the response to reaction buffer and the response to PBS. The mean  $\pm$  SEM fold increase in monocyte migration in response to fMLP (100 nM) was  $4.4 \pm 0.5$ . **B**, Fold increase in polymorphonuclear neutrophil (PMN) migration in response to citrullinated ENA-78/CXCL5 and noncitrullinated ENA-78/CXCL5, as determined by chemotaxis assay. At either 0.1 nM or 1 nM, the response to noncitrullinated ENA-78/CXCL5 was significantly higher than the response to PBS ( $P < 0.05$ ). The response to citrullinated ENA-78/CXCL5 was lower than the response to noncitrullinated ENA-78/CXCL5, but the difference was not significant. The mean  $\pm$  SEM fold increase in PMN migration in response to fMLP (100 nM) was  $7.5 \pm 2.5$ . **C**, Effect of pertussis toxin (PTX) on the increased monocyte migration in response to citrullinated ENA-78/CXCL5. **D**, Effect of anti-CXCR1 (anti-R1) and anti-CXCR2 (anti-R2) on the increased monocyte migration in response to citrullinated ENA-78/CXCL5. All assays were performed in quadruplicate, with 3 high-power fields (400 $\times$ ) counted in each replicate well. The fold increase was determined by dividing the number of cells migrated by the number of cells migrated to the negative control PBS. Values are the mean  $\pm$  SEM.

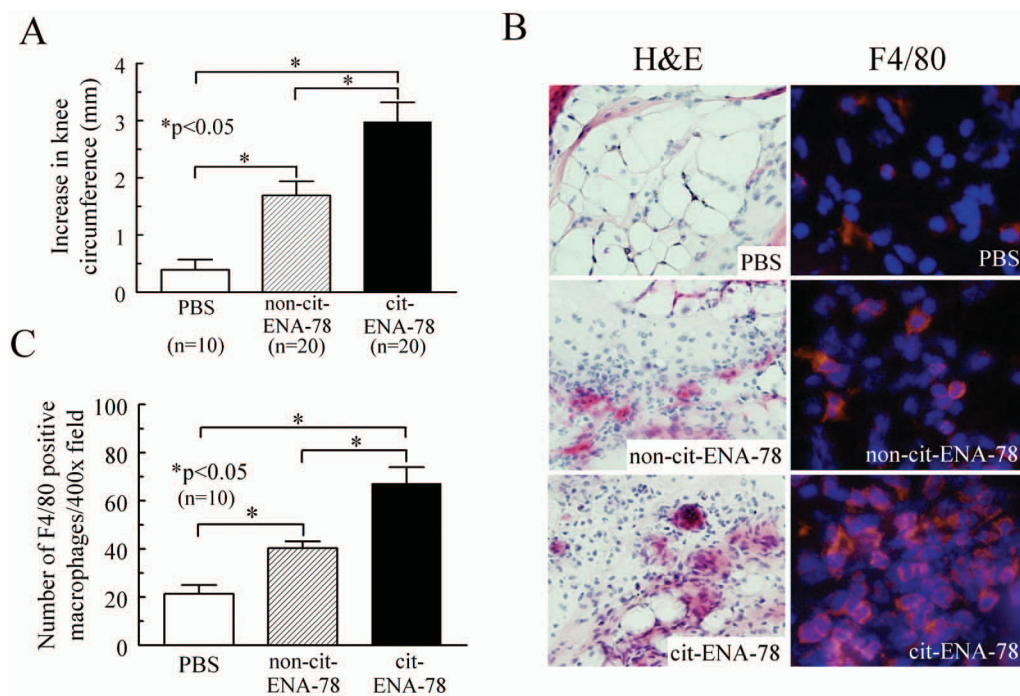
migration in response to citrullinated ENA-78/CXCL5 tended to be lower than that in response to noncitrullinated ENA-78/CXCL5. However, there was no significant difference in PMN chemotaxis between noncitrullinated and citrullinated ENA-78/CXCL5.

In monocyte chemotaxis assays using pertussis toxin (a G protein-coupled receptor antagonist), anti-CXCR1, and anti-CXCR2 antibodies, the fold increase in the response to citrullinated ENA-78/CXCL5 with pertussis toxin was significantly lower (mean  $\pm$  SEM  $1.07 \pm 0.06$ ) compared with that without pertussis toxin ( $2.06 \pm 0.14$ ;  $P < 0.05$ ) (Figure 4C). We then examined whether monocyte migration is mediated via the G protein-coupled receptors CXCR1 and CXCR2. The fold increases in the response to citrullinated ENA-78/CXCL5 with anti-CXCR1 or anti-CXCR2 antibodies

were significantly lower ( $1.34 \pm 0.04$  and  $1.55 \pm 0.05$ , respectively) compared with that without the antibodies ( $2.05 \pm 0.07$ ), and the fold increase in the response to citrullinated ENA-78/CXCL5 with a combination of anti-CXCR1 and anti-CXCR2 antibodies was significantly lower ( $1.02 \pm 0.03$ ) compared with that without the antibodies, with anti-CXCR1, or with anti-CXCR2 ( $P < 0.05$ ). IgG2a was used as an isotype-matched control monoclonal antibody that did not inhibit monocyte migration (Figure 4D). These results indicated that citrullinated ENA-78/CXCL5 induced monocyte migration via CXCR1 and CXCR2.

**Citrullinated ENA-78/CXCL5-enhanced induction of inflammatory arthritis and recruitment of monocyte/macrophages in vivo.** To test the inflammatory activity of citrullinated ENA-78/CXCL5 in vivo, we





**Figure 5.** Effect of citrullinated ENA-78/CXCL5 in vivo. The knees of female C57BL/6 mice were injected intraarticularly with PBS, noncitrullinated ENA-78/CXCL5, or citrullinated ENA-78/CXCL5 to induce inflammation. **A**, Circumference of the mouse knees 24 hours after administration of the intraarticular injections. The increases in knee circumference were significantly greater in mice that received citrullinated ENA-78/CXCL5 compared with those that received PBS or noncitrullinated ENA-78/CXCL5 and were also significantly greater in mice that received noncitrullinated ENA-78/CXCL5 compared with those that received PBS. **B**, Left, Hematoxylin and eosin (H&E)-stained mouse knee joint sections, showing inflammatory cell infiltration in all groups and severe inflammation in mice treated with citrullinated ENA-78/CXCL5. Right, Immunofluorescence-stained mouse knee joint sections, showing a greater number of F4/80-positive monocyte/macrophages in mice treated with citrullinated ENA-78/CXCL5. Fluorescent red and blue staining show F4/80-positive monocyte/macrophages and cell nuclei, respectively. Original magnification  $\times 400$ . **C**, Numbers of F4/80-positive monocyte/macrophages in mice treated with PBS, noncitrullinated ENA-78/CXCL5, and citrullinated ENA-78/CXCL5. Values are the mean  $\pm$  SEM (n values are the number of joints). See Figure 4 for other definitions.

injected PBS, noncitrullinated ENA-78/CXCL5, or citrullinated ENA-78/CXCL5 into mouse knee joints. Joints injected with citrullinated ENA-78/CXCL5 had a significantly greater increase in circumference (mean  $\pm$  SEM  $3.0 \pm 0.3$  mm), and hence joint swelling, compared with knees injected with PBS ( $0.4 \pm 0.2$  mm;  $P < 0.05$ ) or noncitrullinated ENA-78/CXCL5 ( $1.7 \pm 0.3$  mm;  $P < 0.05$ ). Joints injected with noncitrullinated ENA-78/CXCL5 had significantly increased joint swelling compared with joints injected with PBS ( $P < 0.05$ ) (Figure 5A). H&E staining showed inflammatory cell infiltration in each treatment group, and inflammation was especially severe in the group that received citrullinated ENA-78/CXCL5 (Figure 5B). Immunofluorescence staining revealed that the number of F4/80-positive monocyte/macrophages was significantly higher in mouse knee joints injected with citrullinated ENA-78/CXCL5 (mean  $\pm$  SEM  $67 \pm 7$  macrophages) compared with joints injected with noncitrullinated ENA-78/

CXCL5 ( $40 \pm 3$  macrophages) or PBS ( $21 \pm 4$  macrophages). In addition, the number of F4/80-positive monocyte/macrophages was significantly higher in the mice treated with noncitrullinated ENA-78/CXCL5 compared with those that received PBS ( $P < 0.05$ ) (Figures 5B and C).

## DISCUSSION

We observed the presence of citrullinated ENA-78/CXCL5, MIP-1 $\alpha$ /CCL3, and MCP-1/CCL2 in RA sera and RA SF. There is evidence that some chemokines can be citrullinated in vitro (27–30); however, not all cytokines can be citrullinated. Proost et al showed that PAD efficiently and site-specifically citrullinated ENA-78/CXCL5 and IL-8/CXCL8 but not IL-1 $\beta$  (27). Struyf et al demonstrated that stromal cell-derived factor 1 (SDF-1)/CXCL12 can be citrullinated in vitro, and that SDF-1/CXCL12 and PAD were coexpressed in

the colon tissue of patients with Crohn's disease (30). However, those investigators did not directly demonstrate the presence of citrullinated SDF-1/CXCL12 in diseased colon tissue.

In the current study, we demonstrated that the concentrations of citrullinated ENA-78/CXCL5, MIP-1 $\alpha$ /CCL3, and MCP-1/CCL2 were all significantly higher in RA sera than in normal control sera. Furthermore, citrullinated ENA-78/CXCL5 and MIP-1 $\alpha$ /CCL3 concentrations were significantly higher in RA SF than in SF from patients with OA and patients with other rheumatic diseases, and the level of citrullinated MCP-1/CCL2 was significantly higher in RA SF than in OA SF. In particular, the concentration of citrullinated ENA-78/CXCL5 was high in RA SF but was barely detected in SF from patients with OA or other rheumatic diseases. Additionally, the PAD4 level measured by ELISA is significantly elevated in RA SF compared with SF from patients with OA and patients with ankylosing spondylitis (31). This fact supports the notion that chemokines, including ENA-78/CXCL5, are citrullinated more efficiently in RA joints than in the inflamed joints of patients with other diseases. In our study, the concentrations of ENA-78/CXCL5 and MIP-1 $\alpha$ /CCL3 in SF as measured by standard ELISA kits (R&D Systems) were lower than the concentrations of citrullinated ENA-78/CXCL5 and MIP-1 $\alpha$ /CCL3 in SF as measured by the new ELISA system developed by our group. We observed that after citrullination, purified chemokines were not detected by standard ELISAs (data not shown). It is possible that these ELISAs may not completely recognize citrullinated chemokines in RA SF.

We showed that among 3 citrullinated chemokines tested in RA SF, the concentrations of citrullinated ENA-78/CXCL5 correlated most closely with clinical data (the CRP level and the ESR). Chemokines are readily detectable in RA SF (11,32–36), and the concentrations of MIP-1 $\alpha$ /CCL3 and IL-8/CXCL8 in SF correlate with CRP levels in serum (35,36). In our study, we observed significant positive correlations between citrullinated ENA-78/CXCL5 and not only the CRP level but also the ESR, whereas the ENA-78/CXCL5 concentration did not correlate with any clinical parameters examined (data not shown). These results suggest that compared with the other chemokines that we examined, citrullinated ENA-78/CXCL5 is more closely related to disease activity in RA.

Citrullination has been reported to decrease the activities of certain chemokines in some instances (37). In vitro, ENA-78/CXCL5 (0.1–10 nM) induces PMN

migration in a dose-responsive manner (10,25). However, citrullination of ENA-78/CXCL5 has been reported to reduce in vitro and in vivo PMN chemotaxis, intracellular calcium signaling, phosphorylation of extracellular signal-regulated kinase, and internalization of CXCR2 compared with noncitrullinated ENA-78/CXCL5 (38). We showed that citrullination of ENA-78/CXCL5 directly supports monocyte migration but not PMN migration. These results indicate the possibility that citrullination of ENA-78/CXCL5 does not increase the function of ENA-78/CXCL5 as a PMN recruiter but instead dampens it.

Surprisingly, we observed that citrullinated ENA-78/CXCL5 acquired a monocyte-recruiting function that noncitrullinated ENA-78/CXCL5 did not have. Normally, ENA-78/CXCL5 is not a potent chemotactic factor for monocytes but rather for PMNs and endothelial cells and also possesses angiogenic properties (10,25,39,40). Monocyte chemotaxis assays showed that citrullinated ENA-78/CXCL5 recruited monocytes in a dose-dependent manner, while noncitrullinated ENA-78/CXCL5 did not. These results suggest that citrullination of ENA-78/CXCL5 results in its conversion from a non-monocyte-recruiting chemokine to a monocyte-recruiting chemokine.

We then investigated whether citrullinated ENA-78/CXCL5 recruited monocytes through the G protein-coupled receptors CXCR2, which is the primary ENA-78/CXCL5 receptor, and CXCR1, which has 78% homology with CXCR2 at the amino acid level (41,42). Citrullinated ENA-78-induced monocyte migration was completely inhibited by pertussis toxin (a G protein-coupled receptor antagonist) or by a combination of anti-CXCR1 and anti-CXCR2 antibodies. These results suggest that citrullinated ENA-78/CXCL5 recruits monocytes via both CXCR1 and CXCR2. ENA-78/CXCL5 elicits PMN chemotaxis by interacting with the chemokine receptor CXCR2 present on the PMN cell surface (43,44). This receptor is expressed on many different cells including PMNs and monocytes (45). CXCR1 is also present on the surface of human monocytes (26,46). CXCR1 and CXCR2 are expressed on 23–90% and 22–93% of human monocytes, respectively (26). IL-8/CXCL8, neutrophil-activating peptide/CXCL7, growth-related oncogene  $\alpha$ /CXCL1, and ENA-78/CXCL5 bind to CXCR2 with high affinity, whereas IL-8/CXCL8 exhibits high affinity for CXCR1 as well (47,48). Our results suggest that citrullination may increase the affinity of ENA-78/CXCL5 for CXCR1 (38).

We clearly show that at physiologically relevant concentrations, citrullinated ENA-78/CXCL5 recruits

monocytes. We also show data confirming that citrullinated ENA-78/CXCL5 preferentially binds to the CXCR1 receptor instead of the normal receptor for ENA-78/CXCL5, namely CXCR2. This indicates that modification of ENA-78/CXCL5 changes its receptor-binding affinity (and likely its conformation). This also explains, at least in part, the ability of citrullinated ENA-78/CXCL5 to recruit monocytes. Nonetheless, assuming that both citrullinated ENA-78/CXCL5 and non-citrullinated ENA-78/CXCL5 are present in SF and are binding to the same receptor, the modified or unmodified forms of ENA-78/CXCL5 that are present in greater quantities would be the forms that are more competitive for binding the available receptor. However, we now present evidence that citrullinated ENA-78/CXCL5 utilizes an alternative receptor (CXCR1), indicating that the concentration of citrullinated ENA-78/CXCL5 is likely more important when considering the monocyte-recruiting activity of citrullinated ENA-78/CXCL5. We believe that the conversion of ENA-78/CXCL5, a potent neutrophil recruitment factor to citrullinated ENA-78/CXCL5, an active monocyte recruitment factor, represents a change to a more chronic inflammatory response in RA-affected joints.

Last, we examined whether citrullination of ENA-78/CXCL5 results in increased inflammation and monocyte migration *in vivo* compared with noncitrullinated ENA-78/CXCL5. To test this hypothesis, we injected noncitrullinated or citrullinated ENA-78/CXCL5 into mouse knee joints and evaluated joint inflammation and monocyte migration. Citrullinated ENA-78/CXCL5 increased the knee circumference more than noncitrullinated ENA-78/CXCL5. Immunofluorescence staining showed that citrullinated ENA-78/CXCL5 induced more F4/80-positive monocyte/macrophage ingress into the synovial tissue of mouse knees compared with noncitrullinated ENA-78/CXCL5. These results suggest that citrullination of ENA-78/CXCL5 induces more severe inflammation in mouse knee joints through monocyte recruitment.

In the current study, although RA sera and RA SF were obtained from different groups of patients, the concentration of citrullinated ENA-78/CXCL5 in RA SF was higher than that in RA sera. These results suggest that citrullinated ENA-78/CXCL5 may be produced mainly in the joints and functions to recruit monocytes to the joints. Other citrullinated proteins (e.g., citrullinated vimentin) stimulate proinflammatory cytokine production from fibroblast-like synoviocytes or peripheral blood mononuclear cells in RA (49,50). It is possible that citrullinated ENA-78/CXCL5 may also stimulate

cells other than monocytes in mouse knee joints to release inflammatory mediators and aggravate the inflammatory response.

In addition, our study demonstrated that non-citrullinated ENA-78/CXCL5 recruited more monocyte/macrophages *in vivo* compared with PBS. In general, ENA-78/CXCL5 recruits neutrophils *in vitro* and *in vivo*. However, it is possible that other types of cells besides neutrophils respond to ENA-78/CXCL5 *in vivo*. It has been reported that macrophages, which are either inflammatory monocyte-derived cells or reemerging tissue-resident macrophages, increase in number during acute inflammatory responses (51). It is quite possible that the number of monocyte/macrophages increases *in vivo* during ENA-78/CXCL5-induced acute inflammation.

In conclusion, citrullinated ENA-78/CXCL5 was detected in RA SF and sera. The concentrations were significantly higher in SF from patients with RA than in SF from patients with OA or other inflammatory rheumatic diseases and correlated with the CRP level and the ESR. Citrullinated ENA-78/CXCL5 induced monocyte migration via CXCR1 and CXCR2 *in vitro*, while non-citrullinated ENA-78/CXCL5 did not. Mouse knees injected with citrullinated ENA-78/CXCL5 had more inflammation and monocyte/macrophages than did those injected with noncitrullinated ENA-78/CXCL5, suggesting that citrullination enhances the proinflammatory activity of ENA-78/CXCL5 and accelerates disease progression in inflammatory arthritis.

## ACKNOWLEDGMENTS

We thank Dr. David A. Fox (Division of Rheumatology, University of Michigan Medical School) for helpful comments and interpretation of the results; Dr. Henriette A. Remmer (Proteomics & Peptide Synthesis Core, University of Michigan Medical School) and Dr. Michael Ford (MS Bio-works) for helpful comments on the mass spectrometry data and for providing the spectra; and Ray A. Ohara (University of Michigan) for assistance performing the experiments.

## 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. Yoshida 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.** Yoshida, Korchynskyi, Tak, Ruth, Campbell, Amin, Koch.

**Acquisition of data.** Yoshida, Korchynskyi, Isozaki, Baeten, Gerlag, Amin.

**Analysis and interpretation of data.** Yoshida, Korchynskyi, Ruth, Campbell, Koch.



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RESEARCH ARTICLE

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# Inflammatory properties of inhibitor of DNA binding 1 secreted by synovial fibroblasts in rheumatoid arthritis

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## Abstract

**Background:** Inhibitor of DNA binding 1 (Id1) is a nuclear protein containing a basic helix-loop-helix (bHLH) domain that regulates cell growth by selective binding and prevention of gene transcription. Sources of Id1 production in rheumatoid arthritis synovial tissue (RA ST) and its range of functional effects in RA remain to be clarified.

**Methods:** We analyzed Id1 produced from synovial fibroblasts and endothelial cells (ECs) with histology and real-time polymerase chain reaction (RT-PCR). Fibroblast supernatants subjected to differential centrifugation to isolate and purify exosomes were measured for Id1 by enzyme-linked immunosorbent assay (ELISA). Western blotting of Id1-stimulated ECs was performed to determine the kinetics of intracellular protein phosphorylation. EC intracellular signaling pathways induced by Id1 were subsequently targeted with silencing RNA (siRNA) for angiogenesis inhibition.

**Results:** By PCR and histologic analysis, we found that the primary source of Id1 in STs is from activated fibroblasts that correlate with inflammatory scores in human RA ST and in joints from K/BxN serum-induced mice. Normal (NL) and RA synovial fibroblasts increase Id1 production with stimulation by transforming growth factor beta (TGF- $\beta$ ). Most of the Id1 released by RA synovial fibroblasts is contained within exosomes. Endothelial progenitor cells (EPCs) and human dermal microvascular ECs (HMVECs) activate the Jnk signaling pathway in response to Id1, and Jnk siRNA reverses Id1-induced HMVEC vessel formation in Matrigel plugs in vivo.

**Conclusions:** Id1 is a pleiotropic molecule affecting angiogenesis, vasculogenesis, and fibrosis. Our data shows that Id1 is not only an important nuclear protein, but also can be released from fibroblasts via exosomes. The ability of extracellular Id1 to activate signaling pathways expands the role of Id1 in the orchestration of tissue inflammation.

**Keywords:** Inhibitor of DNA binding-1 protein (Id1), Inflammation, Rheumatoid arthritis, Fibroblasts, Angiogenesis

## Background

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by inflammation and joint destruction. Angiogenesis is important in a variety of vasculoproliferative states such as wound repair and RA synovitis. Many inflammatory mediators found in RA synovial tissues (STs) and synovial fluids (SFs) display angiogenic properties. Inhibitor of DNA binding 1 (Id1) is a member of the

helix-loop-helix (HLH) family of transcription factors and a marker of cellular self-renewal. Inhibition of Id1 in the bone marrow (BM) results in significant decreases in endothelial progenitor cell (EPC)-linked tumor-associated vasculogenesis [1]. Gao et al. showed that it was possible to identify, track, and target BM-derived endothelial EPCs in vivo using a mouse model of pulmonary metastasis by measurement of Id1 expression [1, 2].

Id1 lacks DNA binding activity, but instead forms heterodimers with members of the basic helix-loop-helix (bHLH) family of transcription factors, allowing Id1 to inhibit DNA binding and transcriptional activation of

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proteins with which it interacts. bHLH proteins such as BMAL1-Clock (circadian locomotor output cycles kaput) [3], which is a core transcription complex in the molecular circadian clock, bind Id1. Id1 also interacts with various genes, such as *c-Myc* [4] and hypoxia-inducible factor-1 (HIF-1) [5], that have been linked to cancer due to their effects on cell growth and metabolism. Id1 binds tightly to ubiquitously expressed E proteins, that heterodimerize with tissue-restricted bHLH proteins to form active transcription complexes. By sequestering E proteins, Id1 inhibits tissue-restricted gene expression in multiple cell lineages using the same biochemical mechanism [6, 7].

The human *Id1* gene has been cloned and characterized by Hara et al. [8], who also cloned a related gene, *Id2*. A splice variant (*Id1*-prime) that does not show the growth-regulated expression normally seen with *Id1* has also been described [9]. The *Id1*-related proteins *Id2* and *Id3* [10] are also negative regulators of pluripotent stem cell maturation [11]. As a protein affecting the activity of many transcription factors, *Id1* can affect multiple cellular properties. Targeting of *Id1* transcription blocks EPC mobilization, causes angiogenesis inhibition, impairs the spread of metastasis, and increases the survival of tumor-bearing mice [1, 2]. Interestingly, human *Id1* mRNA is barely detectable in quiescent early-passage fibroblasts, but its expression can be induced by serum. Moreover, *Id1* antisense RNA prevents early-passage fibroblasts from entering the S phase of the cell cycle [8].

Histologic analysis of ST revealed that *Id1* is highly expressed in the vasculature of RA ST [12], and also in synovocytes (SNCs). Sakurai et al. also showed substantial expression of *Id1* and *Id3* in RA compared to osteoarthritis (OA) synovium at the protein and transcriptional levels [13]. Our group was the first to report that RA synovial fluid (SF) contains abundant amounts of *Id1*, and we now show that the primary source is not from EPCs or endothelial cells (ECs), but from RA ST fibroblasts. We also show that *Id1* is packaged within extracellular vesicles (EVs) and released from fibroblasts via exosomes. Lastly, we provide evidence that *Id1* activates EC signaling pathways, inducing angiogenic responses [12], which can be targeted to reduce blood vessel growth.

## Methods

### Ethical use of animals

Procedures involving animals in this study were approved by The University Committee for the Use and Care of Animals (UCUCA) at the University of Michigan. Mice were housed in sterile rodent micro-isolator caging with filtered cage tops in a specific pathogen-free environment. Severe combined immunodeficient (SCID) mice were obtained from the National Cancer Institute (NCI). C57/BL6 wildtype (Wt) mice were bred in house. All efforts were

made to reduce stress or discomfort in the animals used in these studies.

### Patient samples

STs were obtained from RA patients undergoing total joint replacement who met the American College of Rheumatology criteria for RA. Prior to surgery unrelated to the proposed research, patients were asked whether they were willing to contribute ST to the study. ST specimens were stored at -80 °C. All human specimens were consented for use in this study by the Institutional Review Boards of the University of Michigan Medical School (IRBMED).

### K/BxN serum-induced arthritis model

To generate arthritic K/BxN mice, K/B-positive mice were crossed with NOD/LTj mice as previously described [14]. Naïve C57BL/6 mice at the age of 5–7 weeks were injected with 150 µL of K/BxN serum intraperitoneally, and this was considered day 0 of arthritis. Another injection of 150 µL of K/BxN serum followed on day 2. Robust arthritis with severe swelling of the joints typically developed on day 5. Articular index (AI) scores and joint circumferences were determined starting on day 0 and scored at least every other day up to day 23 after induction of arthritis, as described previously for rat adjuvant-induced arthritis [15]. Clinical scoring for arthritis was performed using a 0–4 AI scale, where 0 = no swelling or erythema, 1 = slight swelling and/or erythema, 2 = low-to-moderate edema, 3 = pronounced edema with limited use of the joint, and 4 = excessive edema with joint rigidity. All measurements were taken by observers blinded to the experimental conditions. Mouse ankles were harvested for histology.

### Immunohistochemistry (IHC)

RA, OA, and normal (NL) (no arthritis) ST cryosections as well as ankle sections of Wt mice induced with K/BxN serum were fixed in cold acetone for 30 min at 4 °C. The tissue sections were blocked with 5 % donkey serum and 20 % fetal bovine serum (FBS) in phosphate-buffered saline (PBS) at 37 °C for 1 h. The sections were then incubated with either mouse anti-human *Id1* antibody (Abcam, Cambridge, MA, USA, 10 µg/mL), rabbit anti-mouse *Id1* antibody (CalBioReagents, San Mateo, CA, USA, 10 µg/mL), or purified nonspecific mouse and rabbit immunoglobulin G (IgG) (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at 37 °C in blocking buffer. After washing, tissues were incubated with a biotinylated anti-mouse or anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA, 10 µg/mL) for 1 h at 37 °C in blocking buffer. Vectastain ABC kit (Vector Laboratories) was used to detect the antibodies on the tissues, following manufacturer's protocols. Sections were



mounted with Cytoseal 60 (Thermo Fisher Scientific), visualized under an Olympus microscope (Olympus, Tokyo, Japan) and scored by a pathologist.

#### **Immunofluorescence histology**

RA, OA, and NL ST sections were fixed in cold acetone for 30 min. The STs were blocked with 5 % donkey serum and 20 % FBS in PBS at 37 °C for 1 h, and then incubated with rabbit anti-human Id1 antibody (Abcam, 10 µg/ml) or purified nonspecific rabbit IgG for 1 h at 37 °C in blocking buffer. The synovial tissue samples were washed with PBS, and a 1:200 dilution in blocking buffer of fluorescent-conjugated donkey anti-rabbit antibody was added, and incubated for an additional 1 h at 37 °C. Finally, slides were washed, dried, coverslipped and viewed under a fluorescence microscope (×400).

#### **Cell culture**

EPCs (CD34+ cells from cord blood) were isolated from cord blood from granulocyte-colony stimulating factor (G-CSF)-mobilized leukopheresis samples on the basis of CD133 expression, using an antibody-coupled magnetic bead cell isolation system (Stemcell Technologies, Vancouver, BC, Canada) as previously described [16]. Human umbilical cord blood was collected by the method of Moore et al. [17], as previously described [16]. To confirm purity of the EPCs, isolated cell populations were subjected to flow cytometry analysis as described previously [18, 19]. EPCs with appropriate cell markers (CD34+, CD133+, CD14-) were used in cell signaling studies.

Human dermal microvascular ECs (HMVECs) as well as all fibroblasts were collected from human tissues, which were digested in a cell culture media supplemented with FBS, collagenase, and hyaluronidase as done previously [20–22]. HMVECs were isolated from skin biopsies while the fibroblasts were isolated from synovial tissues of arthritis patients obtained at arthroplasty or synovectomy. HMVECs were isolated and purified using CD31 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). HMVECs were grown in EBM-2 (Lonza, Walkersville, MD, USA) and were placed in reduced-serum EBM-1 before stimulation. EBM-1 without serum was used for HMVEC stimulations. EPCs were grown and stimulated in StemSpan SFEM (Stemcell Technologies) with no added supplement. Fibroblasts were grown in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10 % FBS and were stimulated in serum free RPMI-1640.

#### **Id1 enzyme-linked immunosorbent assay (ELISA)**

HMVECs, EPCs, monocytes, and RA, OA, and NL synovial fibroblasts were plated in 6-well plates at 200,000 cells/well and serum starved overnight. Media was exchanged and collected after 24 h. The collected media

was analyzed by ELISA for Id1 (MyBioSource, San Diego, CA, USA). Manufacturer protocols were followed. SFs of RA, OA, and several other diseases were also analyzed by this ELISA. Both synovial and dermal fibroblasts were stimulated with varying cytokines and concentrations. Cytokines used were tumor necrosis factor alpha (TNF-α, Thermo Fisher Scientific), chemokine (C-X-C motif) ligand 16 (CXCL16, R&D Systems, Minneapolis, MN, USA), interleukin 17 (IL-17, R&D Systems), and TGF-β (R&D Systems). These cytokines were chosen because they are known to be upregulated in RA ST [23, 24]. The supernatant and exosome fractions were collected after 24 h and analyzed by this ELISA.

#### **RNA extraction and quantitative real-time polymerase chain reaction (RT-PCR)**

Total RNA was isolated from synovial fibroblasts and HMVECs using RNeasy mini RNA isolation kits in conjunction with QIAshredders (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Following isolation, RNA was quantified and checked for purity using a Nanodrop spectrophotometer (Thermo Fisher Scientific). cDNA was then prepared using a Verso cDNA kit (Thermo Fisher Scientific) as per the manufacturer's protocol. Quantitative PCR (qPCR) was performed using Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher Scientific) following the manufacturer's protocol. The primer pairs used were based on published sequences. Diluted cDNA was mixed with Platinum SYBR green qPCR SuperMix-UDG, forward and reverse primers specific for each gene (10 µM final concentrations), and incubated at the following cycles; 50 °C for 2 min, 95 °C for 2 min and 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 68 °C for 30 s using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The primers for human Id1 [25], are forward: AGAACC GCAAGGTGAGCAA and reverse: CCAACT GAAGGTCCCTGATGTAG. The primers used for β-actin were used previously, [12, 26] and are forward: GCTAGGCAGCTCGTAGCTCT and reverse: GCCATG TACGTTGCTATCCA. All samples were run in duplicate.

#### **Exosome purification**

Exosomes were isolated from cell culture supernatants by differential centrifugation. RA synovial fibroblasts were plated at 200,000 cells/well in a 6-well tissue culture plate in serum-free media. Media was exchanged and incubated for 24 h after which the supernatant was collected. An aliquot of the supernatant was taken for later analysis and the remaining amount was subjected to several steps of differential centrifugation. All centrifugation was conducted at 4 °C. First, the supernatant was centrifuged at 300 × g for 10 min to remove free cells. The remaining supernatant was centrifuged at

10,000 × g to remove cellular debris, then at 30,000 × g to remove smaller debris. The supernatant was then transferred to an ultracentrifuge and spun at 110,000 × g overnight to pellet the exosomes. The supernatant was stored and the pellet was separated on an Optiprep density gradient (Sigma-Aldrich, St. Louis, MO, USA). The fractions containing exosomes were then isolated. Notably, exosomes isolated from RA and OA SFs were isolated similarly from rheumatoid factor-depleted SFs. The original whole supernatant, exosome and cellular debris-depleted fractions, exosome fractions, exosomes isolated from SFs as well as exosome fractions lysed with 0.5 % Triton X-100 (Sigma-Aldrich) were individually analyzed using the Id1 ELISA.

### Western blotting

HMVECs or EPCs were plated in 6-well plates at 500,000 cells/well and serum starved overnight. The wells were then stimulated with Id1 (Abnova, Taipei, Taiwan, 10 nM) over a 45-min time course. Cell lysates were prepared in reducing conditions and western blot analysis was done. Proteins were transferred from the gel to nitrocellulose membranes which were then blocked in 5 % nonfat dry milk in Tris-buffered saline + Tween 20 (TBST, pH 7.6). Membranes were probed with rabbit anti-human antibodies to both the phosphorylated (\*p) and nonphosphorylated forms of Jnk, Erk<sub>1/2</sub>, PI3k, and P38 (Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution) in blocking buffer overnight at 4 °C. Membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody (Cell Signaling Technology, 1:1000 dilution) for 1 h at 25 °C. Membranes were washed with TBST and visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). X-ray film was used to visualize the blots after ECL. These films were scanned and bands were quantified by UN-SCAN-IT (Silk Scientific, Orem, UT, USA). Gel loading was accounted for by adjusting all phosphorylated data by the total amount of signaling protein, and fold change of the phosphorylated signal molecules was calculated with respect to the unstimulated lysate.

### Severe combined immunodeficiency (SCID) Matrigel plug angiogenesis assay

To examine the effects of the Jnk pathway in angiogenesis with Id1 stimulation *in vivo*, SCID mice were injected subcutaneously with sterile Matrigel (Corning Life Sciences, Tewksbury, MA, USA, 500 µL/injection) containing Id1 (10 nM) and Jnk-silenced HMVECs. HMVECs were silenced using silencing RNA (siRNA) inhibiting the Jnk signaling molecule (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Either Jnk siRNA or nonspecific siRNA was transfected into the HMVECs using the TransIT-

TKO Transfection Reagent (Mirus Bio, Madison, WI, USA). Manufacturer's protocols were followed for the transfection. Twenty-four hours after transfection, some of the cells were lysed to confirm Jnk knockdown and the rest were injected into mice together with the Matrigel ( $2.5 \times 10^5$  cells/injection). The Matrigel plugs were removed 5 days later and weighed. Hemoglobin (Hb) analysis was conducted on the plug homogenates as a measure of angiogenesis. Hb levels were measured by adding 25 µL of homogenate mixed with 25 µL of 3, 3', 5, 5'-tetramethylbenzidine (TMB) reagent to 96-well plates. Finally, samples were incubated at room temperature for 5 min. Absorbance was read with a microplate reader at 450 nm. Hb concentration was determined by comparison with a standard curve in mg/mL. Hb concentration, after normalization with plug weight, is a reflection of the number of blood vessels in the tissue [27].

### Statistics

#### Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Data were analyzed using a Student's *t* test. *P* values less than 0.05 were considered significant. For histology data on cryosections from human and rodent synovial tissues, all cells were considered for positive or negative Id1 staining by a board-certified pathologist blinded to the experimental setup. The results are presented as the percentage of positive cells in each section. At least three sections were evaluated (for both mouse and human specimens) and the results were averaged, then pooled in the respective groups for all tissues evaluated.

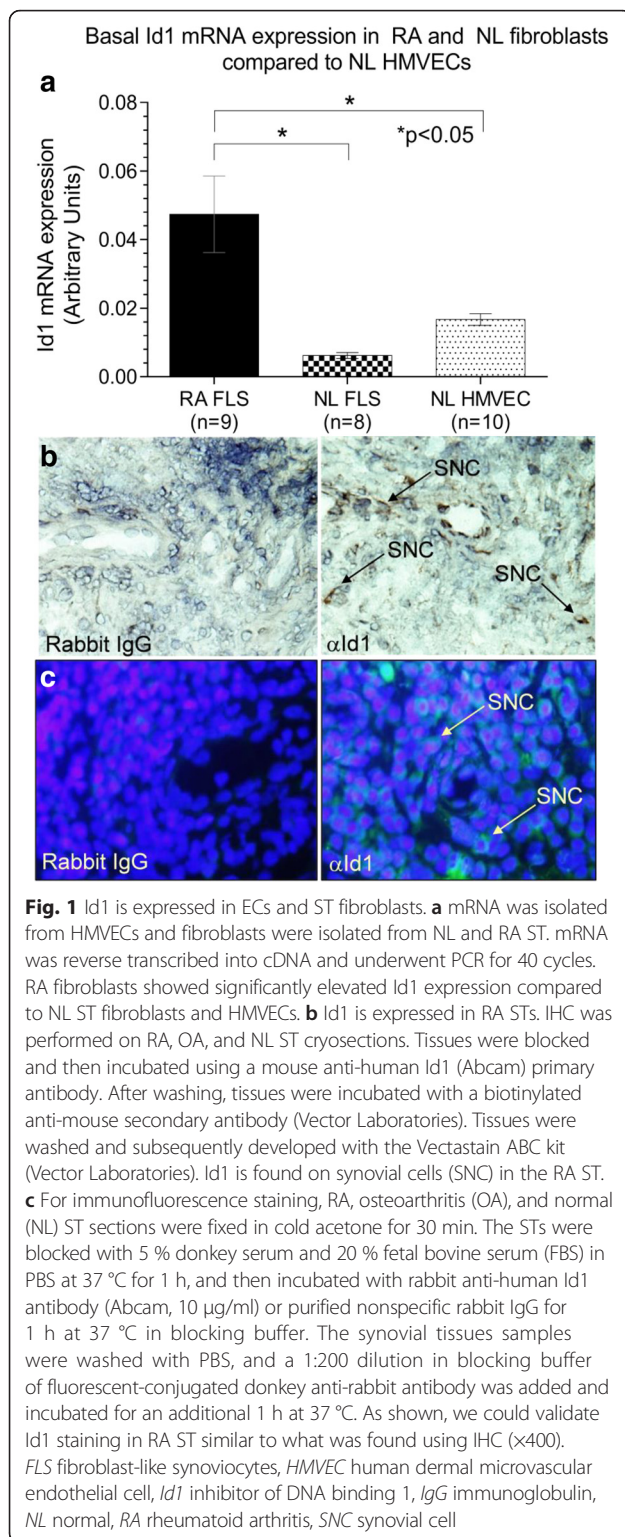
### Results

#### RA ST fibroblasts express Id1

Total RNA was isolated from nonstimulated ST fibroblasts and HMVECs. There was a significantly elevated Id1 mRNA expression in RA compared to NL ST fibroblasts and HMVECs, showing that Id1 production is upregulated in activated fibroblasts compared to NL ST fibroblasts or ECs (Fig. 1a).

#### Immunohistochemical analysis of Id1 expression in mouse and human synovium

IHC staining for Id1 on RA, OA, and NL STs and K/BxN serum-induced mouse ankles indicated the presence of Id1 in these tissues. Id1 was highly expressed on SNCs of RA ST as well as in the K/BxN serum-induced mouse ankles (Figs. 1b, c and 2a–c). Immunofluorescence histology further validated the Id1 staining pattern seen with IHC (Fig. 1c). Analysis of these tissues by a pathologist revealed that RA STs had a significantly higher percentage of SNCs positive for Id1 than did OA and NL STs (Fig. 2a). Similarly, the day 12 K/BxN serum-induced mouse ankles had



more Id1-positive SNCs than day 0 mouse ankles; although the difference was less striking (Fig. 2b and c). All images were taken at  $\times 400$ .

### Effects of cytokines on fibroblast expression of Id1

Analysis of supernatants of unstimulated cells of several types found in the RA synovium showed that synovial fibroblasts are the primary producer of Id1, and that RA fibroblasts produce more basal Id1 compared to NL and OA fibroblasts (Fig. 3a). Monocytes also produce some Id1, however much less compared to synovial fibroblasts. The ECs produced an undetectable amount of Id1. ELISA analysis of cell culture supernatants of NL synovial fibroblasts stimulated with various cytokines showed that TGF- $\beta$  (at 10 and 50 ng/mL) and to a lesser extent IL-17 (at 50 ng/mL) increased Id1 production (Fig. 3b). TNF- $\alpha$  and CXCL16 had only minor effects on the secretion of Id1 by these cells. ELISA analysis of RA, OA, and NL synovial fibroblast cell culture supernatants stimulated with TGF- $\beta$  at 10 and 50 ng/mL showed that TGF- $\beta$  had a large effect on Id1 production by synovial fibroblasts (Fig. 3c). RA fibroblasts were significantly more sensitive to TGF- $\beta$  stimulation with respect to Id1 production compared to OA and NL synovial fibroblasts.

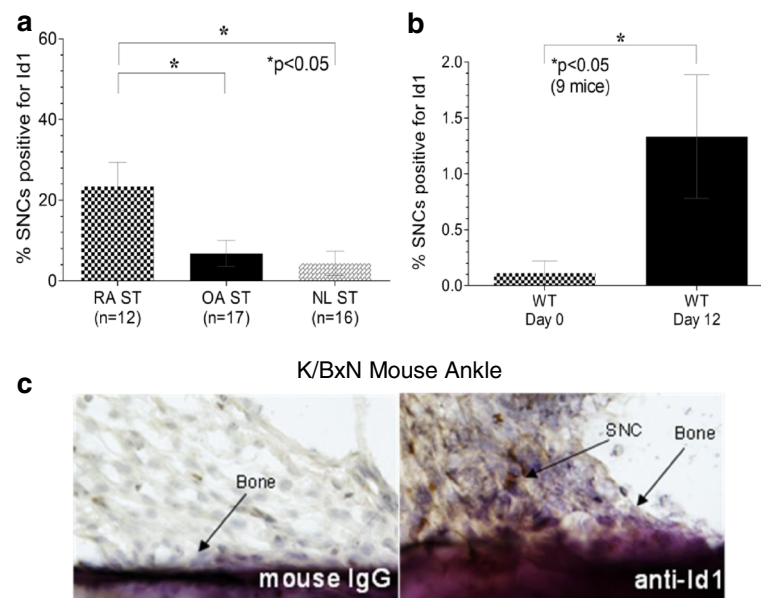
### Identification of Id1 in fibroblast exosomes

Analysis of fractions taken during a successive series of steps to isolate exosomes using differential centrifugation from RA synovial cell supernatants showed that the majority of the Id1 is found within exosomes when compared to the other fractions (Fig. 4). A small amount of Id1 was found in the whole supernatant and exosome-depleted fractions, and no Id1 was found in the unlysed exosome fraction. Triton X-100 at 0.5 % was used to lyse the exosomes and the majority of Id1 was found in this fraction. Notably, exosomes isolated from RA and OA SFs showed very low levels of Id1 unless the exosomes were disrupted by treatment with Triton X-100, confirming that Id1 is largely packaged within cellular exosomes and distributed outside of the cell (Fig. 2e).

### Western blot analysis of Id1-mediated signaling in ECs

Western blot analysis of  $\text{pJnk}$  in HMVECs and EPCs following stimulation with Id1 showed that  $\text{pJnk}$  was upregulated after 1 min of stimulation with Id1, an effect that persisted for at least 45 min in EPCs. However, in HMVECs upregulation was delayed by 15 min and was less robust, but continued to 45 min (Fig. 5a and b). Analysis of  $\text{pP38}$  in EPCs showed that  $\text{pP38}$  was upregulated almost immediately after stimulation with Id1 and this effect persisted at 45 min of stimulation (Fig. 5c).  $\text{pErk}$  and  $\text{pPI3k}$  did not show any upregulation after Id1 stimulation in either EPCs or HMVECs and  $\text{pP38}$  did not show any upregulation in HMVECs after Id1 stimulation (Fig. 5e). Results are shown as fold increase from nonstimulated (NS) cells arbitrarily set at 1. Upper bands represent phosphorylated signaling molecules (p)





**Fig. 2** SNC Id1 expression is significantly higher in inflamed ST and in the ankles of K/BxN serum-induced mice. **a** Id1 expression was visualized on SNCs in ST by IHC. A significantly greater percentage of SNCs were positive for Id1 in RA compared to OA and NL ST. **b** Percentages of cells expressing Id1 were also evaluated on SNCs from joint tissues taken from K/BxN serum-induced mice. A significantly greater percentage of SNCs positive for Id1 compared to Wt (noninduced mice) was found. **c** Murine tissues were blocked and then incubated using a rabbit anti-mouse Id1 antibody (CalBioReagents). After washing, tissues were incubated with a biotinylated anti-rabbit secondary antibody (Vector Laboratories). Tissues were washed and subsequently developed with the Vectastain ABC kit (Vector Laboratories). Id1 is found on SNCs near the bone in the K/BxN mouse ankles. *Id1* inhibitor of DNA binding 1, *IgG* immunoglobulin, *NL* normal, *OA* osteoarthritis, *RA* rheumatoid arthritis, *SNC* synovial cell, *ST* synovial tissue, *Wt* wildtype

and the bottom bands represent the total signaling molecule for each respective protein. Independent lysates were run for both phosphorylated and total amounts of signaling proteins, and a representative blot is shown above each graph in Fig. 5. Using these bands from each respective blot, the amount of phosphorylated signaling molecule was quantified and the results were pooled.

#### Id1 induces angiogenesis in vivo

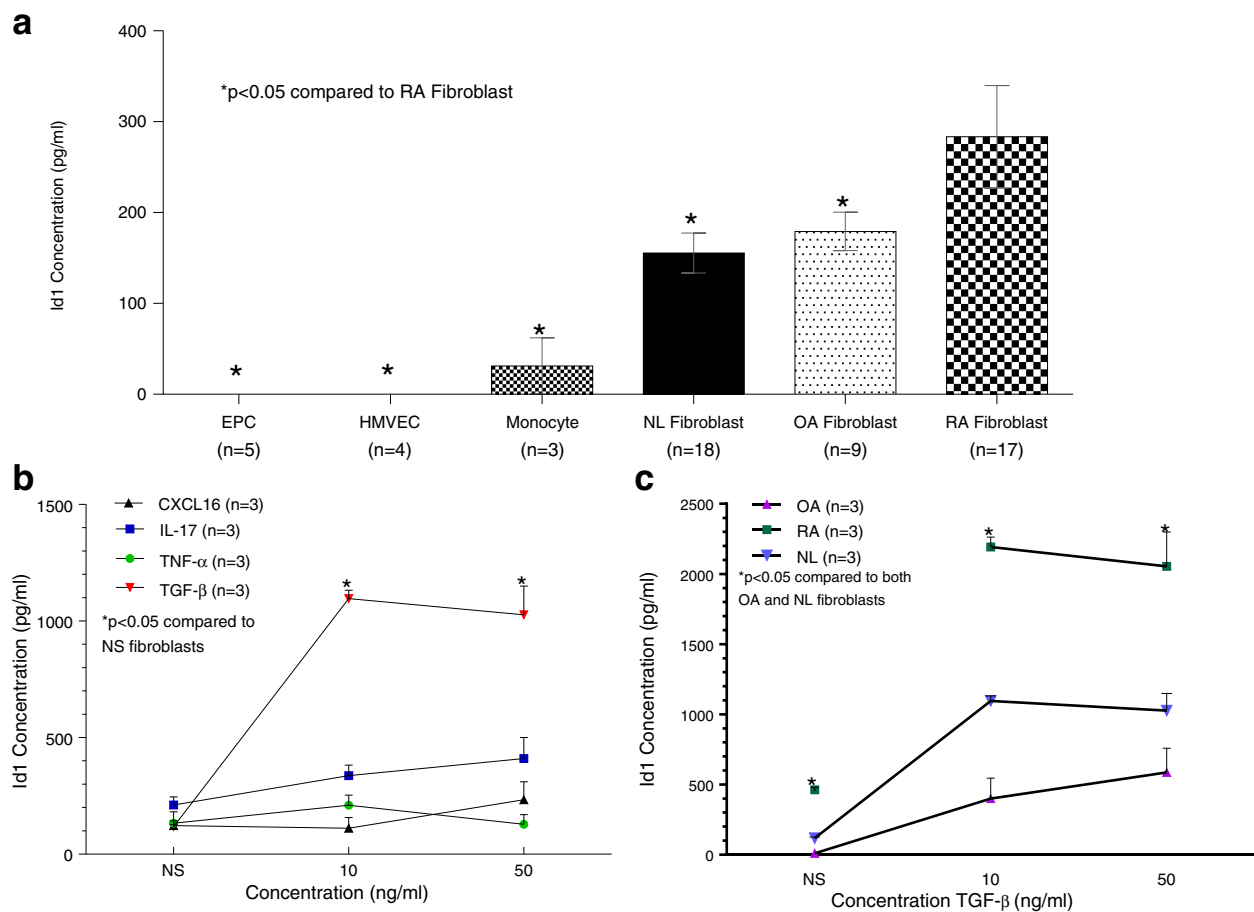
Hemoglobin analysis of Matrigel plugs injected into SCID mice with HMVECs and 10 nM Id1 showed less hemoglobin when the HMVECs were pretreated with Jnk siRNA compared to the control siRNA (Fig. 6). Hemoglobin concentration is representative of vascularization of the Matrigel plug. The results indicate that Id1 signaling through Jnk mediates the angiogenic effect of extracellular Id1.

#### Discussion

Id1 is known to be a nuclear transcription factor characteristic of EPCs and cells that display a hyperproliferative phenotype. Our group has previously shown that Id1 is detected in RA synovium and SFs, and displays pro-angiogenic activity as an extracellular agonist [12]. Levels of Id1 are elevated in RA SFs and correlate with expression of the angiogenic chemokine CXCL16 (the ligand

for the CXCR6 receptor) [12]. Correspondingly, CXCR6 knockout mice develop attenuated angiogenesis associated with profound decreases in arthritis progression and inflammatory cell recruitment to arthritic joints in K/BxN serum-induced mice. We now present evidence that synovial fibroblasts are largely responsible for the elevated amounts of Id1 in RA SF, and that approximately 80 % of the Id1 released by fibroblasts is packaged within exosomes. Moreover, pro-inflammatory cytokines known to be elevated in RA patients, such as IL-17 and especially TGF- $\beta$ , induce significantly more fibroblast-derived Id1 in RA compared to OA or NL fibroblasts in vitro. Of special note, RA fibroblasts are significantly more sensitive to TGF- $\beta$  stimulation than OA or NL fibroblasts with respect to Id1 production. These findings indicate that RA fibroblasts are primed to release Id1 in response to pro-fibrotic and angiogenic mediators, potentially due to epigenetic alteration of these cells in vivo.

Histologic analysis of ST revealed that Id1 is highly expressed on synovial SNCs and in the vasculature of the RA ST [12] in complete agreement with Sakurai et al., who showed Id1 and Id3 mRNA and protein are elevated in RA synovium [13]. Studies of knockout mice lacking expression of Id1 have shown its involvement in vasculogenesis and neuroblast differentiation in various cancer

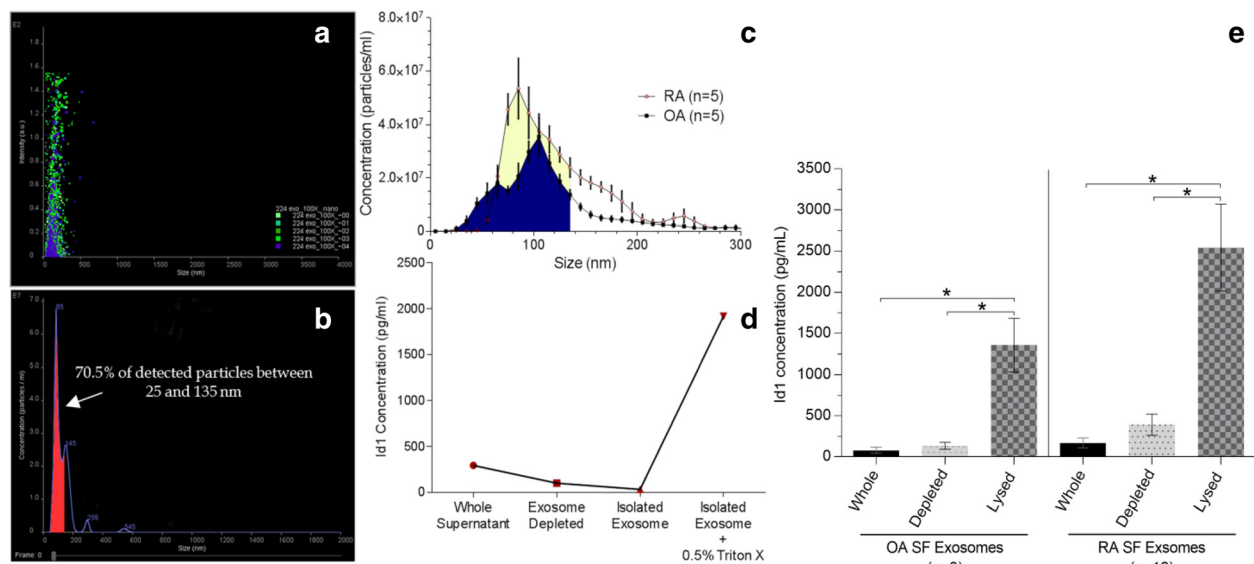


**Fig. 3** RA ST fibroblasts secrete Id1 and upregulate Id1 expression upon stimulation with TGFβ. **a** EPCs, HMVECs, monocytes, NL, RA, and OA fibroblasts were plated and serum starved. Cell culture media was replaced and the supernatants were collected 24 h later. The cell culture supernatants were examined for Id1 expression by ELISA (MyBioSource). EPCs, HMVECs as well as all fibroblasts were collected from human tissues, which were digested in a mix of cell culture media supplemented with FBS, collagenase, and hyaluronidase. HMVECs were isolated from skin biopsies while the fibroblasts were isolated from STs of patients with either RA, OA, or from NL patients. HMVECs were isolated and purified using CD31 microbeads (Milenyi Biotec). All cell lines were between passages 3 and 6. No cytokine stimulation was used. **b** NL fibroblasts were plated and serum starved under the same conditions as panel a. Cell culture media with the respective cytokine was exchanged and collected 24 h later. CXCL16 (R&D Systems), IL-17 (R&D Systems), TNF-α (Life Technologies) and TGF-β (R&D Systems) were used at 10 ng/mL and 50 ng/mL concentrations. A not stimulated (NS) well was also used with no added cytokine (n = number of experimental replicates). **c** Synovial fibroblasts were plated under the same conditions as panel a. Cell culture media with TGF-β was exchanged and collected 24 h later. CXCL16 chemokine (C-X-C motif) ligand 16, EPC endothelial progenitor cell, HMVEC human dermal microvascular endothelial cell, Id1 inhibitor of DNA binding 1, IL-17 interleukin 17, NL normal, NS nonstimulated, OA osteoarthritis, RA rheumatoid arthritis, TGF-β transforming growth factor beta, TNF-α tumor necrosis factor alpha

models normally characterized by extensive vascularization [28]. Tumors transplanted into these mice failed to grow and metastasize due to lack of proper tumor angiogenesis. Branching and sprouting of blood vessels in the neuroectoderm is also defective in double knockout mice lacking expression of Id1 and the related Id3, as these animals show premature differentiation of neuroblasts [28]. Thus it appears that Id1 expression is critical for angiogenic processes found in the tumor microenvironment or the RA joint, further suggesting that dysregulation of Id1 can lead to unwanted inflammatory outcomes. Furthermore, our findings provide additional evidence that Id1

may be used to assess or grade the severity of pathologic inflammatory conditions, as has been done previously for some cancers [29].

One of the many interesting features of Id1 is its ability to repress inhibitors of angiogenesis. Previous studies showed that Id1 indirectly regulates angiogenesis through transcriptional repression of thrombospondin-1 (TSP-1) [30]. Interestingly, a recent study showed that TSP-1 is strongly expressed by RA ST fibroblasts induced by TGF-β [31], both at the transcriptional and translational levels. This finding could raise questions about the role for Id1 repression of TSP-1. One possible



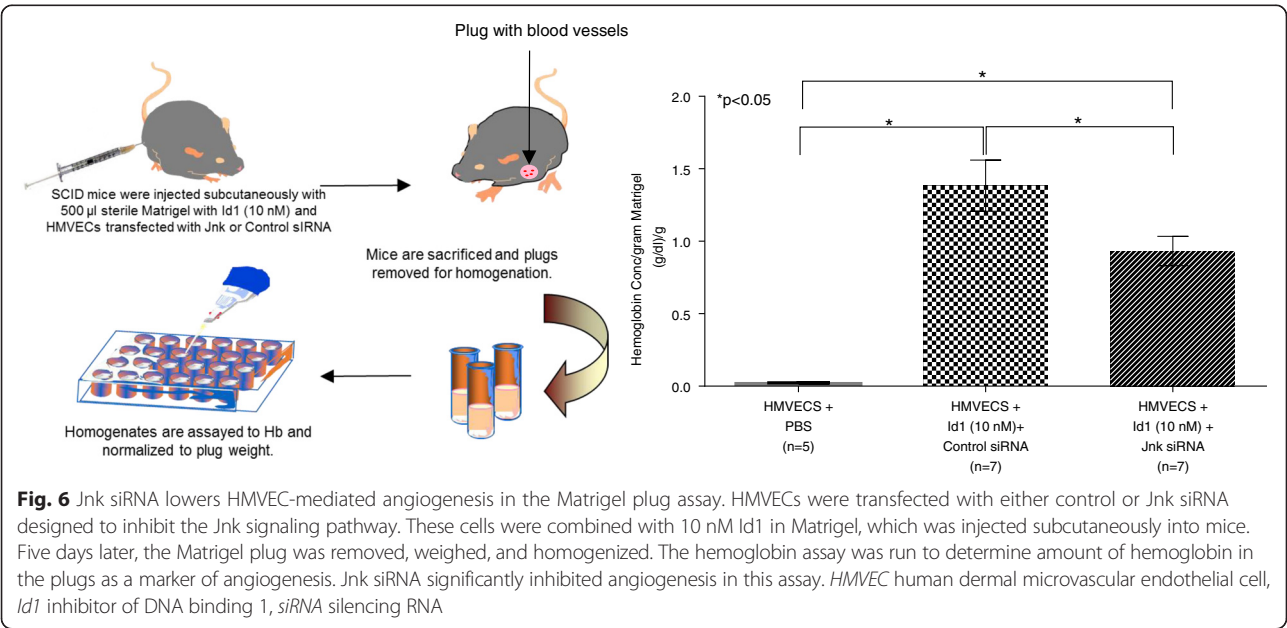
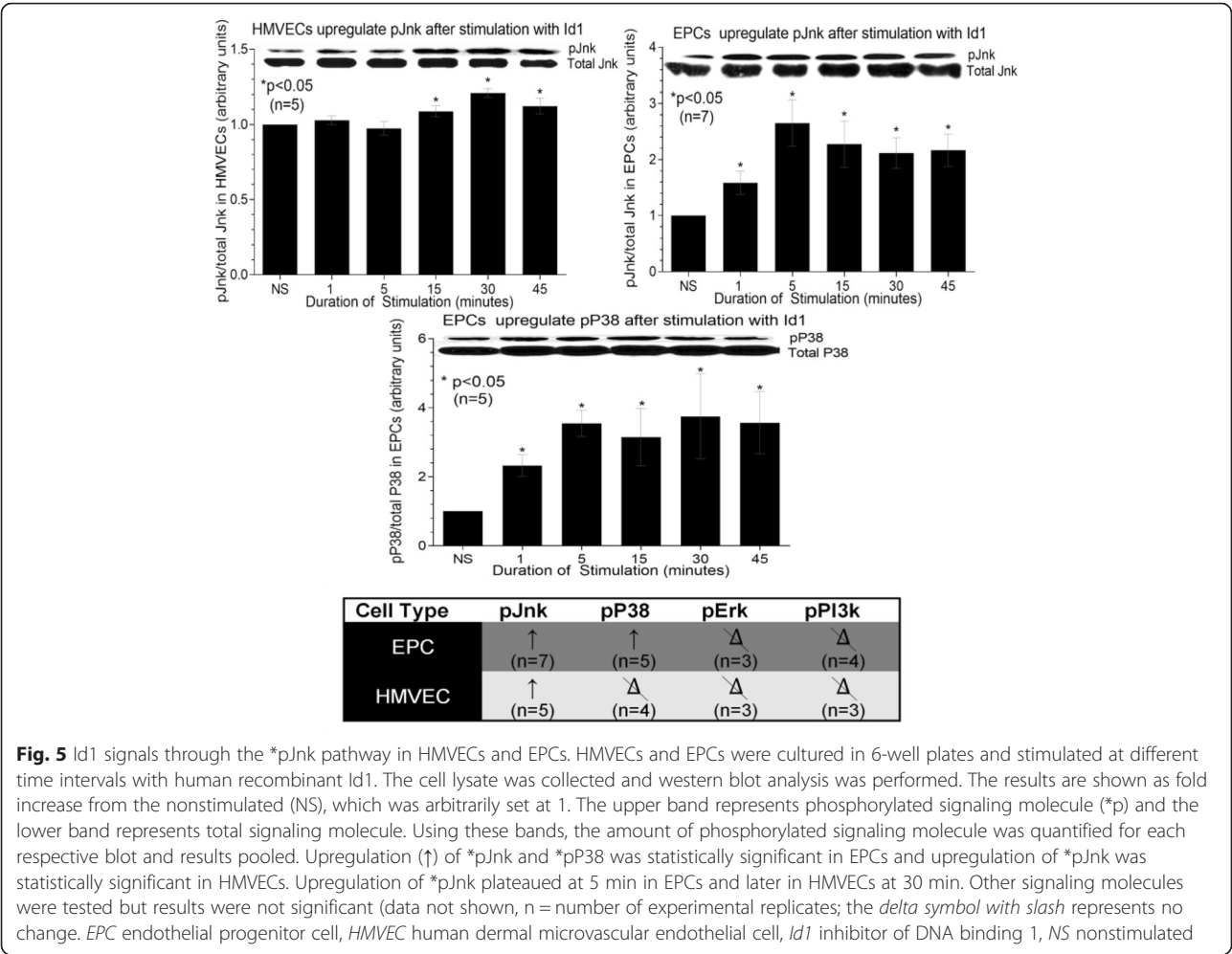
**Fig. 4** Id1 is contained in fibroblast exosomes. Fibroblasts from RA, osteoarthritis (OA), and normal (NL) STs were plated and maintained in CMRL medium supplemented with 20 % FBS, 2 mM L-glutamine, and 1 % penicillin/streptomycin in T175cm<sup>2</sup> flasks and were used between passages 4 and 10. Cultures were moved to serum-free media DMEM/F-12 with Pepsigrow serum replacement (PeproTech) for 2 days before harvesting. Fibroblast culture supernatants were concentrated to 1 mL (per flask) by centrifugation through an Amicon Ultracel 30 K filter (EMD Millipore, Billerica, MA, USA). Exosomes from cell supernatants were isolated and purified by serial ultracentrifugation. Nanoparticle Tracking Analysis was used to quantify the size and concentration of particles within the exosome fractions. **a** and **b** Cells were spun out at 1500 rpm for 5 min. Then the supernatants were cleared of heavier debris by spins at 10,000 × g for 30 min and 30,000 × g for 1 h. Exosomes were then spun down at 110,000 × g for 4–20 h. Exosome pellets were washed in PBS at 110,000 × g for 1.5 h – overnight. Some exosomes were further purified using a density gradient Optiprep (Sigma-Aldrich). Optiprep was diluted with PBS to produce the following layers: 5, 10, 15, 20, 30, 40, and 50 % w/v (densities of 1.031, 1.050, 1.084, 1.110, 1.163, 1.215, and 1.268 g/mL). The expected size of exosomes is between 25 and 135 nm and >70 % of the EVs isolated fall into this range (red area). **c** Representative diagram showing that we find more exosomes in RA (yellow and blue areas) compared to OA (blue area only) in fibroblast supernatants. **d** and **e** Whole and lysed (0.5 % Triton X-100) exosome fractions were then measured for Id1. We found that >80 % of the detected Id1 is contained within RA fibroblast cell supernatant exosomes. We similarly found that OA and RA SF have exosomes containing Id1 (n = number of sample wells measured for exosomes isolated from SF specimens from two separate patients for OA; and three separate patients for RA). All data was pooled in the respective OA and RA SF groups. Id1 inhibitor of DNA binding 1, OA osteoarthritis, RA rheumatoid arthritis, SF synovial fluid

explanation for this discrepancy may be that TGF- $\beta$  could directly induce TSP-1 expression in RA, potentially overriding any transcriptional control exerted by Id1. Moreover, TSP-1 has also been shown to upregulate TGF- $\beta$  in experimental inflammatory glomerular disease. Thus it appears that TGF- $\beta$  and TSP-1 may upregulate expression of the other, making it tempting to speculate that upregulated Id1 serves as a negative control mechanism for TSP-1 and TGF- $\beta$  expression [32].

The expression of diverse genes involved in remodeling of the extracellular matrix, angiogenesis, and intracellular signaling are also affected by Id1 [30]. Furthermore, Id1 represses p21 expression [4] to control EPC growth and maturation in the BM. Because of the ability of Id1 to downregulate expression of these potent repressors, it was reported that Id1 can function as an effective pro-angiogenic mediator produced by EPCs and pluripotent stem cells [33]. This idea was reinforced by reports identifying Id1 and Id3 as negative regulators of pluripotent stem cell maturation [11]. Because of its potent regulatory control of angiogenic and vasculogenic processes, it was

not surprising to find that Id1 acts as a negative transcriptional regulator for proteins associated with malignant melanoma development [34, 35].

It is well documented that Id1 is a regulatory nuclear protein, however, we and others have previously shown that Id1 can be detected on the ECs in the RA synovium and in soluble form in synovial effusions [12, 13]. This suggests that Id1 travels outside the nucleus, but the functionality and method of this transfer has not been previously described. One possibility is that Id1 may be packaged into EVs such as cellular exosomes that can be released into the joint space or directly into adjacent cells. To test this possibility, we isolated EVs from RA synovial fibroblast supernatants known to contain Id1, and measured Id1 in the EVs and soluble protein fractions. We used differential ultracentrifugation to isolate EVs specifically around the size of exosomes. We visually confirmed a band at the density gradient between fractions 4 and 5, where exosomes would be expected (density between 1.084 g/mL and 1.163 g/mL). To more clearly define where Id1 is located, we further subdivided





the EV fraction over a discontinuous Optiprep density gradient with seven fractions from 1.268 g/mL to 1.031 g/mL and collected the fractions containing exosomes. Id1 was barely detected on the surface of exosomes or on other EVs of similar density. However, addition of Triton X-100 (which lyses exosomes) revealed that >80 % of the detected Id1 is contained within exosomes, indicating that fibroblasts likely utilize exosomal mechanisms for Id1 export from the nucleus to the cytoplasm and out of the cell.

Id1 is also expressed at high levels in pro-B cells, but is downregulated in pre-B cells and mature B cells [36, 37]. Constitutive expression of Id1 in transgenic mice shows that these animals display severe defects in the development of B cells, demonstrating that cells in early development express Id1 and subsequently downregulate it as they acquire a mature phenotype [36]. It is possible that mature cells in the RA joint may take up fibroblast-derived Id1 (released within exosomes) to regulate cell proliferation in the inflammatory milieu of the RA synovium. Cellular crosstalk mediated by Id1 could thus transfer information from one cell to another despite inability of many inflammatory cells in the RA synovium to make Id1. Similarly, it was recently reported by Bourdonnay et al. that alveolar macrophages secrete the STAT-induced STAT signaling inhibitors SOCS1 and SOCS3 in exosomes and microparticles, respectively, for uptake by alveolar epithelial cells and subsequent inhibition of STAT activation *in vitro* and *in vivo* [38].

Kim et al. have generated transgenic mice in which Id1 is expressed specifically in T cells with the total number of thymocytes in these mice observed to be less than 4 % of that in Wt mice [39], again demonstrating the repressive nature of Id1 when overexpressed. Most cells were CD4-/CD8- double-negative cells bearing cell surface markers of multipotent progenitor cells, with apoptotic cells constituting about 50 % of the total thymocytes. Also of note, Tanaka et al. reported that the expression of Id1 in cardiac myocytes leads to the induction of apoptosis through a redox-dependent mechanism [40].

As previously noted, Id1 is upregulated in RA SF, and we show herein that Id1 initiates cell signaling events via an as yet unknown receptor. We did find that Id1 stimulates EPC signaling through \*pP38 and \*pJnk at almost all times measured, but not through Erk<sub>1/2</sub> or PI3k. Peak upregulation of \*pP38 and \*pJnk was found at a stimulation time of approximately 5 min and persisted for at least 45 min. This is in contrast to HMVECs that displayed a more delayed and subtle response to Id1, showing significance in \*pJnk expression after 15 min, then plateauing at 30 min. The kinetics of HMVEC and EPC signaling in response to Id1 with respect to \*pJnk reveals differences in these cells, with the mature ECs showing a weaker response to Id1 than EPCs. Nonetheless, this

experiment does show that it is possible for \*pJnk to be a common signaling molecule in both EPCs and HMVECs when these cells are stimulated with Id1. This finding also identifies EPCs and ECs as cells able to bind Id1, further characterizing Id1 as both a vasculogenic and angiogenic mediator in the RA joint [12].

Locating where cell signaling events intersect in mature or progenitor ECs could be used to identify targets for Id1 inhibition and unwanted angiogenic activity. We have performed similar signaling experiments on RA synovial fibroblasts as shown here for ECs and found that they signal through \*pP38, \*pJnk, \*pJak2 and \*pNF- $\kappa$ B in response to Id1 (data not shown), thus providing further evidence that Id1 may induce similar signaling events/pathways in various cell types. Of special note is the data (presented in Fig. 6) showing that inhibition of Jnk in HMVECs with siRNA significantly reduces the amount of Id1-induced Hb in the mouse Matrigel plug angiogenesis assay. Hb is a direct measure of angiogenesis, and we show that Jnk inhibition significantly reduced Id1-driven blood vessel formation *in vivo*. This finding demonstrates the feasibility of using signaling inhibition to disrupt essential pro-inflammatory functions (e.g., angiogenesis) initiated by Id1. Finally, it should be noted that the signaling and angiogenesis activity was shown using recombinant Id1, and not Id1 isolated from exosomes taken from RA SF or from fibroblast cell culture supernatants. In future studies, it would be interesting to examine the activity of exosome-derived Id1 for potential epigenetic alterations of the molecule, and if found, how they might affect Id1 activity.

The current standard of care for RA patients includes inflammatory cytokine inhibition. Hence, blockade of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) has resulted in improved pain control and decreased structural deformity in this disease. However, it is well documented that nearly one third of RA patients do not benefit from anti-TNF- $\alpha$  therapy. We posit that Id1 plays a central role in RA pathogenesis, independent of TNF- $\alpha$ , by expanding the vascular network. Overall, our data is suggestive that fibroblast-derived Id1 may contribute to vasculogenesis as well as angiogenesis by independent mechanisms, and that soluble Id1 can serve as either a biomarker or therapeutic target for angiogenesis in RA tissues.

## Conclusions

We show that Id1 is a pleiotropic nuclear protein exhibiting multiple functions including roles in angiogenesis, vasculogenesis, cell growth, and cellular self-renewal. We show that Id1 is released from fibroblasts and initiates cell activation, angiogenic and pro-inflammatory properties. We identify Id1 as a fibroblast-derived inflammatory protein capable of functioning as a signaling agonist, regulatory molecule, and angiogenic mediator in RA tissues.

## Abbreviations

AI: articular index; bHLH: basic helix-loop-helix; BM: bone marrow; CXCL16: chemokine (C-X-C motif) ligand 16; EC: endothelial cell; ELISA: enzyme-linked immunosorbent assay; EPC: endothelial progenitor cell; EV: extracellular vesicle; Hb: hemoglobin; HMVEC: human dermal microvascular endothelial cell; Id1: inhibitor of DNA binding 1; IgG: immunoglobulin G; IHC: immunohistochemistry; IL-17: interleukin 17; NL: normal; OA: osteoarthritis; RA: rheumatoid arthritis; RT-PCR: real-time polymerase chain reaction; SF: synovial fluid; siRNA: silencing RNA; SNC: synovial cell; ST: synovial tissue; TGF- $\beta$ : transforming growth factor beta; TNF- $\alpha$ : tumor necrosis factor alpha; TSP-1: thrombospondin-1; Wt: wildtype.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

GE performed ELISA and histology assays and revised the manuscript. RAO performed the ELISA and histology assays and revised the manuscript. WAS performed in vivo and molecular studies and revised the manuscript. MAA performed the in vivo studies, assisted with study design, and revised the manuscript. TI performed the in vivo and histology studies and revised the manuscript. CMH performed ELISA and histology assays and revised the manuscript. GKH III performed histological analysis and data interpretation and revised the manuscript. RM performed the exosome studies, assisted with study design, and revised the manuscript. PLC assisted with the histology, ELISA and in vivo assays, and revised the manuscript. ASA provided EPCs and assisted in the design of experiments using EPCs and revised the manuscript. SCF assisted with the ELISA assays, fibroblast isolations, and revised the manuscript. DAF assisted with the design of the study, analysis of data, and drafting of the manuscript. JHR conceived and designed all aspects of the study and drafted the final manuscript. All authors have read and given final approval of the manuscript.

## Sources of funding

This work was supported by a grant from the Department of Defense (PR120641). Additional support included the Frederick G.L. Huetwell and William D. Robinson, M.D. Professorship in Rheumatology.

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Received: 11 February 2016 Accepted: 29 March 2016

Published online: 12 April 2016

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