DR. KEVIN C CHUNG (Orcid ID : 0000-0002-2822-3164)

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Citrullinated Inhibitor of DNA Binding 1 is a Novel Autoantigen in Rheumatoid Arthritis

Ray A. Ohara, B.S.,[1] Gautam Edhayan, M.S.,[1] Stephanie M. Rasmussen, B.S.,[1] Takeo Isozaki, M.D., Ph.D.,[2] Henriette A. Remmer, Ph.D.,[3] Thomas M. Lanigan, Ph.D.,[1] Phillip L. Campbell, B.S.,[1] Andrew G. Urquhart, M.D.,[4] Jeffrey N. Lawton, M.D.,[4] Kevin C. Chung, M.D., M.S.,[4] David A. Fox, M.D.,[1] and Jeffrey H. Ruth, Ph.D.[1]

[1]Division of Rheumatology, Clinical Autoimmunity Center of Excellence, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA; [2]Division of Rheumatology, Department of Medicine, Showa University School of Medicine, Tokyo, Japan; [3]Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI, USA; and [4]Department of Orthopaedic Surgery, University of Michigan Health System, A. Alfred Taubman Health Care Center, Ann Arbor, MI, USA

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Correspondence address: Jeffrey H. Ruth, Ph.D.

University of Michigan Medical School
Department of Medicine, Division of Rheumatology
109 Zina Pitcher Drive, 4023 BSRB
Ann Arbor, MI 48109-2200
Phone: 734-615-9374; Fax: 734-615-2506
Email: jhruth@med.umich.edu

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ABSTRACT

Objective. We explored the intrinsic role of inhibitor of DNA binding 1 (ID1) in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) and investigated whether ID1 is citrullinated and autoantigenic in RA.

Methods. RA patient serum ID1 levels were measured before and after infliximab treatment. RA FLS were transfected with a CRISPR/Cas9 construct targeting ID1 to examine the effects of ID1 deletion. RA synovial fluids (SFs) and homogenized synovial tissues (STs) were immunoprecipitated for ID1 and measured for citrullinated residues using ELISA and Western blot. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on *in vitro* citrullinated recombinant human ID1 (citID1) to localize the sites of citrullination. Normal (NL) and RA sera and SFs were analyzed by immunodot blot (IDB) for anti-citrullinated protein antibodies (ACPAs) to citID1.

Results. RA patient serum ID1 levels positively correlated with several disease parameters and were reduced after infliximab treatment. RA FLS displayed reduced growth and robust increase in interleukin-6 (IL-6) and IL-8 production upon deletion of ID1. ID1 immunodepletion significantly reduced the levels of citrullinated residues in RA SFs, and citrullinated ID1 was detected in homogenized RA STs. IDB analyses revealed ACPAs to citID1, but not to native ID1, in RA PB sera and SFs, but not in NL PB sera. Following analyses of LC-MS/MS for citrullination sites and corresponding reactivity in IDB assays, we determined the critical arginines in ID1 for autoantigenicity: R33, R52 and R121.

Conclusions. Novel roles of ID1 in RA include regulation of FLS proliferation and cytokine secretion and autoantigenicity following citrullination.

INTRODUCTION

Inhibitor of DNA binding 1 (ID1) is a nuclear transcription factor containing a helix-loop-helix (HLH) domain that it utilizes to regulate cell growth and differentiation via selective binding and sequestering of distinct transcription factors. By this method, ID1 controls transcriptional activation of target genes. ID1 is also known to be actively transcribed in cells exhibiting hyperproliferative responses and is regarded as a marker of cellular self-renewal. Rheumatoid arthritis (RA) synovial fluid (SF) contains abundant amounts of ID1, and the primary source is activated RA fibroblast-like synoviocytes (FLS). Once released, ID1 acts as a potent inducer of angiogenesis and also exhibits endothelial progenitor cell (EPC) chemotactic activity (1), suggesting that ID1 may contribute to angiogenesis and vasculogenesis by independent mechanisms. ID1 is packaged into exosomes which are released from FLS, and potentially delivered to other inflammatory cells within RA synovium (2). Although the concept of a secreted nuclear protein may be unconventional, a similar phenomenon occurs in the inflamed joint with DEK, a nuclear protein that functions as a regulator of transcription involved in chromatin architecture and mRNA processing. DEK is secreted by macrophages, found in exosomes, can be detected in the SFs of juvenile arthritis patients, and is both an autoantigen and a potent neutrophil chemotactic factor (3, 4).

The current study investigates three key potential roles for ID1 in RA: first, as a secreted nuclear protein that correlates to several disease parameters; second, as a regulator of cell proliferation and inflammatory cytokine production by FLS; and third, as a citrullinated autoantigen. We assessed the roles of ID1 in FLS by use of CRISPR/Cas9 targeting of ID1. Immunoassays were employed to measure citrullinated ID1 (citID1) in synovial tissues (STs). Mass spectrometry was employed to define the specific arginines within ID1 that are converted to citrulline, as well as the identity of specific citrulline residues which render ID1 autoantigenic. The results point to multi-dimensional contributions of ID1 and citID1 to the pathogenesis of RA.

PATIENTS, MATERIALS, AND METHODS

Patient samples. Data were collected from a cohort of RA patients (2009-2012), who were diagnosed by the 1987 American College of Rheumatology (ACR) classification criteria for RA. Serum samples (n = 27, median age 49 [range 21-76]) were collected from the patients before the initial treatment with infliximab. All RA patients were receiving methotrexate and 14 were receiving additional disease-modifying antirheumatic drugs (sulfasalazine or bucillamine). Fourteen age and sex-matched healthy subjects (n = 14, median age 42.5 [range 29-55]) were recruited on a voluntary basis as controls. All the specimens were obtained with written informed consent and collected following approval from the Showa University Institutional Review Board (IRB). SF samples were obtained from RA patients during arthrocentesis and stored at -80ºC in aliquots after centrifugation to remove SF cells. ST samples were obtained from RA patients undergoing total joint replacement and were snap frozen (liquid nitrogen) in 10% DMSO in FBS and stored at -80ºC. All the samples were obtained with IRB approval from RA patients who met the ACR criteria for RA.

Enzyme-linked Immunosorbent Assay (ELISA). Patient samples and normal (NL) controls were measured by ELISA kits for human ID1 (MyBioSource) and rheumatoid factor (RF, Alpha Diagnostic International). For the ID1 targeting experiments, cell culture supernatants were measured by ELISA kits for human interleukin-13 (IL-13), epithelial-derived neutrophil-activating peptide-78 (ENA-78/CXCL5), IL-6 and IL-8 (R&D Systems). For detection of citrulline residues by ELISA, see Supplementary Method 1.

CRISPR/Cas9 targeting of ID1. CRISPR U6gRNA-pCMV-Cas9-2A-RFP plasmid (Sigma-Aldrich) containing the guide RNA (gRNA) 5'-GAATCATGAAAGTCGCCAGTGG-3' was designed to target the human ID1 gene. CRISPR human EMX1-s4 positive control plasmid (Sigma-Aldrich, #CRISPR11-1EA) and CRISPR plasmid targeting an irrelevant gene (e.g. THBS1) were used as controls. All transfections were performed by electroporation using Amaxa Nucleofector Technology (Lonza Group). Transfected cells were sorted for fluorescent marker (red fluorescent protein [RFP] or green fluorescent protein [GFP]) via fluorescence-activated cell sorting (FACS) and analyzed for CRISPR/Cas9 activity by Tracking of Indels by Decomposition (TIDE) using web tool by Desktop Genetics (5). For verifying ID1 depletion, transfected cells were lysed in RIPA buffer supplemented with EDTA-free protease inhibitor

cocktail (Thermo Fisher Scientific) and analyzed by WB. For assays of cell proliferation and cytokine expressions, sorted cells were plated in 96-well plates (6,000-10,000 cells/well) in complete cell culture medium and imaged hourly for 120 hours using the IncuCyte® S3 Live-Cell Analysis System (Essen Biosciences). Cell culture supernatants were collected at 24 hours post-seeding for analysis by ELISA.

Immunoprecipitation (IP). For ID1 pulldown experiments, Direct IP kit (Thermo Fisher Scientific) was used with polyclonal rabbit anti-ID1 antibodies (Abcam, #ab170511, #ab192303), or rabbit IgG isotype control (Thermo Fisher Scientific). RA ST (~0.5 cm^3) homogenates were prepared in ice cold RIPA buffer supplemented with EDTA-free protease inhibitor cocktail (Thermo Fisher Scientific) using electric homogenizer and were centrifuged and filtered (45 µm) to collect the supernatant. For all IP experiments, manufacturer's kit protocol was followed. Samples were eluted with low pH elution buffer supplied in the kit and prepared in Laemmli sample buffer for Western blotting; flow-throughs were retained for ELISA. All sera, SFs, and ST homogenates were incubated with polyclonal goat anti-human IgM (µ-chain specific)-conjugated Agarose (Sigma-Aldrich) at 4°C overnight for removal of RF prior to all immunoassays.

Citrullination of recombinant human ID1 protein. Recombinant human (rh) ID1 protein (OriGene Technologies) was citrullinated *in vitro* using rh peptidyl arginine deiminase 4 (PAD4) enzyme (Cayman Chemical) or rabbit PAD enzyme (Sigma-Aldrich) as previously described (6). The preparation of noncitrullinated ID1 (noncitID1) was performed similarly with cell culture grade water (Sigma-Aldrich) in place of PAD enzymes.

Western blotting. Citrullinated and noncitrullinated proteins (100 ng each) and IP eluted samples from RA ST homogenates (30 µL) were prepared in Laemmli sample buffer and were resolved on SDS-PAGE gels before transfer to nitrocellulose membranes (NCMs) using semi-dry transfer cell (Bio-Rad). Blots were washed with 0.05% Tween-20 in TBS (TBST) between each subsequent step. After blocking in 5% non-fat dry milk in TBST, the blots were probed with monoclonal rabbit anti-ID1 (OriGene Technologies, #TA310605, clone EPR7098, 1:1000) and then probed with HRP-linked anti-rabbit IgG (Cell Signaling Technology, #7074, 1:1000).

SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) was used for detection prior to image acquisition with Amersham Imager 600 (GE Healthcare Life Sciences). For detection of citrulline residues by Western blotting, see Supplementary Method 2.

In-gel Digestion and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). LC-MS/MS was performed by Proteomics and Peptide Synthesis Core at the University of Michigan as described in Supplementary Method 3.

Immunodot blotting (IDB). Samples were dotted onto NCMs at 10 ng/dot and blocked in 5% goat serum (Sigma-Aldrich) in TBS. Blots were incubated in either samples (RA SF or PB serum, NL PB serum, 1:10,000), normal human IgG control (R&D Systems, #1001A, 1 μg/mL), or control antibodies, including monoclonal mouse anti-ID1 (Abcam, #ab66495, clone 2456C1a, 1 μg/mL) and polyclonal rabbit anti-PAD4 (Abcam, #ab50247, 1 μg/mL), then probed with peroxidase AffiniPure goat anti-human IgG (Jackson ImmunoResearch, #109035003, 1:5,000). To verify specificity for citID1 reactivity of RA specimens, additional control blots were included on the same NCMs using various proteins, including noncitID1, BSA, citBSA, ENA-78, citENA-78 and rhPAD4. Densitometry analysis was performed using ImageJ.

Statistics. GraphPad Prism was used for all statistical analyses. ELISAs for serum ID1 levels were evaluated with unpaired, nonparametric Mann-Whitney U test or paired, nonparametric Wilcoxon signed-rank test. Correlations between serum ID1 levels and clinical parameters were evaluated with nonparametric Spearman's rank correlation. Cell proliferation assay and ELISAs for cytokine expression were evaluated with unpaired, parametric t test. ELISA on RA SFs depleted of ID1 and measured for total citrullinated antigens were evaluated with paired, nonparametric Wilcoxon signed-rank test. Results are expressed as the mean ± SEM. (Two-tailed P values < 0.05 were considered significant.)

RESULTS

Correlations between serum ID1 levels and various disease parameters, and effect of infliximab treatment in a cohort of RA patients. Serum ID1 levels were measured in a Japanese cohort of RA patients before and after infliximab treatment. We found that serum ID1

levels were significantly elevated compared to age- and sex-matched normal controls (Fig. 1A). Serum ID1 levels were compared before and 12 weeks after the initiation of infliximab. We observed a significant decrease in serum ID1 levels after infliximab treatment (Fig. 1B). Additionally, several clinical/laboratory parameters were measured, including matrix metallopeptidase 3 (MMP3), erythrocyte sedimentation rate (ESR), Disease Activity Score-28 (DAS28-ESR), and Simple Disease Activity Index (SDAI), with significant improvements observed after infliximab treatment (Fig. 1C). Next, baseline serum ID1 levels were analyzed for correlation with disease parameters including RF, ESR, C-reactive proteins (CRP), MMP-3, SDAI, Clinical Disease Activity Index (CDAI), DAS28-CRP and DAS28-ESR. We found positive correlations between serum ID1 levels and all parameters except RF (Fig. 1D). Analysis of responders and non-responders using change in SDAI after infliximab treatment showed that serum ID1 level correlated significantly with reduction in disease activity in the responders (24 of 27 patients) (Fig. 1E).

***In vitro* targeting of ID1 in RA FLS by the CRISPR/Cas9 system.** We have previously shown that ID1 is upregulated in RA synovium and soluble ID1 exhibits inflammatory and angiogenic properties (1, 2). To examine the effects of ID1 targeting in RA FLS by CRISPR/Cas9, cell proliferation assays were performed using the IncuCyte® S3 Live-Cell Analysis System. Results of ID1 gene targeting are compared to the targeting of an irrelevant gene, THBS1. We achieved a maximum efficiency of 24.8% (Fig. 2A) which, given an ample starting number of cells, sufficed for our downstream experiments which used only the sorted RFP-positive cells. We found significant reductions in FLS growth beginning 13 hours post-plating in culture (Fig. 2B). To fully verify active and accurate genome editing by Cas9, transfected cells were sorted via FACS and genomic DNA was isolated and sequenced. We then examined for aberrant sequences at the target site caused by insertions and deletions (indels) of non-homologous end joining (NHEJ) using TIDE analysis (Fig. 2C). We found aberrant sequences and corresponding indel frequencies with overall efficiency of 4.4% at the *P* value threshold of 0.001 with R^2 of 0.99. To verify ID1 depletion, Western blot analysis was performed on the sorted cells (Supplementary Fig. S1).

Deletion of ID1 in RA FLS results in decreased cellular proliferation and increased IL-6 and IL-8 production. Sorted cells were cultured for 24 hours following overnight serum starvation and supernatants were analyzed for cytokine expression (IL-13, ENA-78/CXCL5, IL-6 and IL-8) by ELISA (Fig 2D). RA FLS transfected with GFP control plasmid served as an experimental control. We found significantly increased production of IL-6 and IL-8 as compared to the controls, with no significant changes to IL-13 or ENA-78/CXCL5. RA FLS do not secrete appreciable amounts of IL-13 and it was measured as a control cytokine in these experiments. RA FLS do spontaneously secrete ENA-78/CXCL5, as well as IL-6 and IL-8, but no substantial changes to ENA-78/CXCL5 levels were seen, indicating that the increases in IL-6 and IL-8 are specific effects of CRISPR/Cas9 targeting of the ID1 gene.

Detection of the citrullinated form of ID1 in RA SFs and STs. In our 2-step strategy to discover citID1, we first performed IP on RA SFs using polyclonal antibodies independently to pull out both native and citrullinated forms of ID1. Prior to proceeding to the next step, we verified effective depletion of ID1 from RA SFs using ELISA (Fig. 3A). We then performed an indirect ELISA on the depleted RA SFs to detect total citrullinated antigens using the anti-modified citrulline (MC) antibody (Ab) as described (Supplementary Method 1). Depletion of ID1 significantly reduced the level of total citrullinated antigens in RA SFs (Figs. 3B). This suggests that the anti-ID1 Ab that we used recognized both forms of ID1, and that citID1 is present in RA SFs. The data also shows that total citrullinated protein concentration was significantly higher in isotype control *vs.* anti-ID1 Ab-treated SF samples (Fig. 3C). Next, ID1 immunoprecipitated from RA ST homogenates (using the same anti-ID1 Ab) was analyzed by Western blotting using the anti-MC Ab as described (Supplementary Method 2). We detected a single prominent band (or complex of adjacent bands) when probing for either ID1 directly with an anti-ID1 antibody or for citID1 indirectly with the anti-MC antibody. Two out of the 3 samples tested contained citrullinated forms of ID1 (Fig. 3D).

***In vitro* citrullination of rhID1 protein.** We incubated rhID1 with rhPAD4 at various enzyme-substrate molar ratios in appropriate reaction buffer. To control for the potential confounding effects of reaction buffer and conditions, parallel aliquots of rhID1 were subjected to the same condition except rhPAD4 was replaced with sterile water. To verify whether rhID1 was

citrullinated, we performed Western blots and found that citID1 was recognized by the anti-MC Ab, while noncitID1 was not recognized as expected (Fig. 4A, *right*). As a control, we also probed the samples using anti-ID1 Ab and confirmed that noncitID1 was recognized (Fig. 4A, *left*); citID1 was also recognized by this antibody, suggesting that citrullination did not alter its epitope and that both forms can be recognized by a single antibody. In addition, we performed ELISAs using the anti-MC Ab on citID1 for further confirmation of citrullination. We observed a markedly higher absorbance from the citID1 sample as compared to the noncitID1 (Fig. 4B). Citrullinated BSA (citBSA) was used as a positive control for citrullination and as a relative standard for this ELISA.

Identification of specific citrullines in the *in vitro* citrullinated rhID1 by LC-MS/MS. Human ID1 contains 10 arginines (Fig. 5A), all of which are potential candidates for citrullination by PAD enzymes. We found that citID1 runs visibly higher than noncitID1 on a gel (Fig. 5B), likely due to the increase in hydrophobicity and the change in charge from positive to neutral from the citrullination reaction. Subsequently, the samples were analyzed by LC-MS/MS to detect the site(s) of citrullination based on the tandem-MS fragmentation pattern and the expected neutral loss of isocyanic acid that is diagnostic for citrullination in MS (7). We found multiple arginines citrullinated, with the number varying, depending on the batch of rhID1 as well as the experimental conditions of *in vitro* citrullination. Arginine R121 was consistently citrullinated by rhPAD4, as shown in a representative spectrum (Fig. 5C). Interestingly, MS analysis identified native citrulline residues in our noncitID1 samples, expected from the production of the rhID1 in HEK293T cells. However, WB analysis using anti-MC Ab was not able to recognize these citrulline residues (Fig. 4A), suggesting that MS analysis is a more sensitive technique to identify low-level constitutive citrullination.

Measurement of reactivity to citID1 with ACPAs in NL and RA PB sera and RA SFs. We found positive ACPA reactivity against citID1, but not against noncitID1, in RA PB sera and SFs (Fig. 6A). All antigens, including control antigens (non-citID1, BSA, citBSA, ENA-78, citENA-78 as well as rhPAD4 (not shown)), were dotted in triplicate on the same blot and probed together in the same patient sample to ensure citrulline-specific binding by the ACPAs. All control blots showed no significant evidence of antibody binding but did show positivity for cit-

ID1 (Supplementary Fig. S2). NL sera were used as controls and no reactivity was observed. To control for non-specific serum antibody binding, human IgG was used at a relatively high dilution (1 µg/mL). Furthermore, all additional control antibodies were negative (images not shown). By densitometry analysis of IDB, we found that 4/10 RA sera recognized citID1 versus 0/4 control sera. 1/6 RA patients tested positive for anti-citID1 in their SFs (Fig. 6B).

Identification of the critical arginines conferring autoantigenicity to citID1. IDB analysis for ACPA reactivity showed that citID1 displayed unique autoantigenicity and autoantibody reactivity depending on the citrullination pattern. Specifically, samples that showed reactivity for batch 1 and 2 did not shown any reactivity to batch 3, likely due to the lack of key epitopes. Thus, a batch was designated ACPA positive, if it showed reactivity to any of our limited patient samples. We did not observe any ACPA binding to the baseline modified arginines that were present in rhID1 obtained from the vendor. Using a series of LC-MS/MS and IDB analyses, we identified key arginines in rhID1 that may be autoantigenic targets for ACPA development in RA. Of the 10 available arginines in rhID1, the critical arginines for ACPA reactivity were located at positions R33, R52 and R121 (Fig. 6C). To further study the role of these arginines, citrullinated peptides (12 amino acids in length) spanning these regions were tested but were negative for ACPA binding (data not shown). Moreover, denaturing citID1 before IDB negated its reactivity (Fig. 6A). Thus, the critical arginines at R33, R52 and R121 may control conformational epitopes that render citID1 antigenic *in vivo*. This data corroborates our observation that multiple forms of ID1 are expressed *in vitro* and *in vivo*, but only certain modified forms bind ACPAs (Fig. 4A).

DISCUSSION

As a nuclear protein that alters the activity of many transcription factors, ID1 appears to affect multiple cellular properties including proinflammatory cytokine expression. This would place ID1 in a strategic position to regulate chronic inflammatory responses directly by inhibition of cytokine production at the transcriptional level. Such regulatory activity could profoundly influence the severity and progression of inflammatory outcomes in chronic diseases such as RA. There is mounting evidence that permanently altered FLS function is the result of somatic mutations in key genes that regulate the FLS cell cycle, proliferation and apoptosis (8,

9). It has also been proposed that RA synoviocytes possess characteristics similar to tumor cells, as a number of oncogenes involved in cell cycle regulation, or that act as transcription factors such as c-fos, ras, raf, myc and myb, are expressed at high levels in RA FLS (9). We explored the possibility that another nuclear regulatory protein, namely ID1, plays a central role in RA pathogenesis, independent of TNF-α, both by regulating cytokine secretion, and as an inflammatory protein that can undergo post-translational modifications.

We successfully transfected primary RA FLS with a plasmid containing a CRISPR/Cas9 construct, demonstrated successful ID1 gene targeting using a TIDE algorithm and output analysis (5), and confirmed these findings by FACS and Western blotting analysis. We found substantial increases in IL-6 (30 fold) and IL-8 (50 fold), but not ENA-78/CXCL5, in supernatants from transfected FLS compared to sham transfected control FLS. We also found that FLS deleted of ID1 showed, using a cell imaging system, a greater than 20% sustained reduction in proliferation. This may be due in part to elevated production of IL-6, a cytokine known to inhibit fibroblast proliferation (10). More likely, a permanent mutation in an important nuclear regulatory protein, critical for cell proliferation, may have altered the FLS population into a phenotype capable of elevated proinflammatory cytokine secretion. Notably, it has been shown that ID1 antisense RNA prevents early passage fibroblasts from entering the S phase of the cell cycle (11). Firestein *et al.* put forth a model suggesting a duality of FLS populations labeled “passive responders” and “transformed aggressors” (9); that partly arise from the combination of a highly inflamed environment of the RA joint and somatic mutations. Thus, both our new data and previously reported findings suggest that FLS proliferation and heightened FLS secretion of IL-6 represent distinct stages of the contributions of FLS to the pathogenesis of RA. Moreover, the current findings indicate that ID1 can mediate transition of FLS between these two important pathogenic phases.

Analysis of clinical specimens revealed that soluble ID1 is present and upregulated in the serum of RA patients and shows a significant positive correlation with a number of disease parameters including ESR, CRP, MMP-3, SDAI, CDAI, DAS28-CRP and DAS28-ESR. This indicates that circulating serum ID1 levels could be a potential biomarker for RA severity. Additionally, because serum ID1 concentration does not correlate with RF titer, it is possible to identify severe RA patients who are seronegative for RF by measurement of elevated ID1. Furthermore, after twelve-weeks of infliximab, serum levels of ID1 showed significant

reductions. We found that 24 out of 27 responded to infliximab using SDAI, and that reduction in the level of ID1 significantly correlated with reduction in disease activity in the responders. Because of the elevated circulating concentrations of ID1, we surmised that citrullinated forms of ID1 could be present and immunogenic in RA patients, as autoantibodies to citrullinated proteins are well-known disease-associated phenomena in RA (12-16).

ACPAs are implicated in RA pathogenesis in synergy with smoking, the environmental risk factor for RA, and the "shared epitope MHC allele" (17). However, the full range of citrullinated autoantigens in RA is not yet defined. We show that ID1 can be citrullinated *in vitro*. Moreover, by depleting ID1 from RA SFs we reduced the total amount of citrullinated proteins detected by as much as 64% (mean 33%) as measured by cit-ELISA. We further found by immunoblotting that a subset of RA patients has high titer autoantibodies to citID1. Therefore, citID1 in RA SF may account for a significant portion of citrullinated proteins that are the targets of the ACPA response in some RA patients.

We next performed IDBs with RA and NL PB sera, as well as RA SFs, against various citrullinated and noncitrullinated proteins. We were able to confirm the presence of ACPAs to citID1 in both RA sera and SF, but not in the PB from normal (healthy) individuals; supporting our hypothesis that citrullination of ID1 increases its autoantigenicity in RA. Experiments using IDBs to ID1 and citID1 revealed that antibodies to citID1 can be detected in RA sera and SF. In SF, the presence of large amounts of citID1 could sequester anti-citID1 in immune complexes, which could then incorporate RF, and therefore be undetectable in our cit-ELISAs. Such mechanisms could account for the relatively low frequency of SFs positive for anti-citID1.

Various citrullinated forms of ID1 may function differently as agonists or autoantigens, complicating the analysis of the activity of citID1. This is because citrullination reactions using PAD enzyme are notoriously inconsistent, resulting in differences in the numbers and patterns of arginines actually converted to citrullines. These inconsistencies make it difficult to determine the extent of how citrullination of ID1 leads to alterations in its activity. Indeed, we have found that the numbers and locations of arginines that are citrullinated in ID1 can change by simply altering the source of PAD enzyme used in the reaction mix. Moreover, many of the mechanistic roles of ACPAs remain unknown. Interestingly, Schett *et al.* reported that autoantibody to citrullinated vimentin directly induces bone loss, providing a mechanism for osteopenia in early or pre-clinical RA (18, 19). In the case of the chemokine IL-8/CXCL8, only one of three

arginines was citrullinated, yet this resulted in alteration of function (20). In stromal cell-derived factor 1 (SDF-1)/CXCL12, a single arginine citrullinated by these methods, again resulted in functional changes (21).

We found that some forms of citID1 can be highly reactive with ACPAs formed in RA, but that less deiminated forms do not retain autoantigenicity. It appears that the degree of citrullination of ID1 may alter the folding pattern and immune properties of ID1, leading to autoantigenicity. We have previously investigated the autoantigenicity of citrullinated ENA-78/CXCL5, which is also highly upregulated in RA. Similar to citID1, citrullination of ENA-78/CXCL5 at arginine R48 enabled binding to ACPA in RA sera and SFs in our assays. Furthermore, we previously showed that citrullination of ENA-78/CXCL5 induced a functional change of the protein from a neutrophil chemoattractant to a monocyte chemoattractant (6). Since citrullination of a protein with only two arginines such as ENA-78/CXCL5 can cause a profound increase in autoantigenicity and change in function, the impact citrullination can have on ID1, which contains 10 arginines, could be very substantial.

By detailed MS analysis, we observed conversions of ID1 in all but 1 arginine at R103, perhaps explained by structural unavailability or PAD preference for certain arginines. The annotated fragmentation spectrum (Fig. 5) shows the ions corresponding to the partial sequence of ID1 around arginine 121, as an example. The ions highlighted in red in the spectrum show the neutral loss of isocyanic acid resulting from the fragmentation of the ureido group in citrullines, which is a marker for citrullination, confirming the modification of ID1. Through a series of citrullination reactions followed by MS, we identified that the modifications at arginines R33, R52, and R121 in citID1 can bind to and perhaps induce ACPAs. However, small peptides spanning these regions were not reactive with RA serum, and boiling citID1 before IDB assays negated binding by RA sera. Overall, the data is most consistent with a model that involves recognition by ACPA of conformational, but not linear autoantigen epitopes on citID1.

We previously reported that ID1 is secreted by inflammatory FLS and is an angiogenic mediator (1, 2). It is possible that free ID1 is citrullinated either in FLS and/or extracellularly in the inflamed RA joint to render it autoantigenic in RA tissues. We found a potential role for ID1 in transforming FLS into a pathogenic phenotype, and identified citID1 as a novel candidate autoantigen in RA. Further investigation is needed to determine whether and how citrullination

of ID1 may alter its functions. Potentially, ID1, citID1, and/or ACPAs to citID1 may serve as promising therapeutic targets or biomarkers in RA.

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FIGURE LEGENDS

Figure 1. Effect of infliximab treatment on serum ID1 levels in patients with RA and clinical correlations. (A) ID1 is elevated in RA serum (n = 27) compared to age- and sex-matched normal controls (n = 14) (**P* < 0.05), and (B) serum ID1 levels decrease following infliximab treatment at 12-week time point (n = 27, ***P* < 0.01). The red dashed line indicates the sensitivity of the ID1 ELISA (7.81pg/mL). (C_{1-5}) ID1 and clinical parameters decrease following treatment with Infliximab. Each individual colored line represents a single patient. (D_{1-8}, shaded region) Baseline serum ID1 levels correlate with various clinical and laboratory parameters. Spearman's rank correlation coefficient and two-tailed P values are indicated. Symbols represent individual patients. (E) Baseline values minus post treatment values for SDAI

and ID1 correlate, showing improved SDAI scores with decreased ID1 levels (n = 24, *P < 0.05).

Figure 2. Transfection of RA FLS with CRISPR/Cas9 plasmid with gRNA targeting the ID1 gene. (A) FACS of RA FLS transfected with ID1 CRISPR/Cas9 plasmid or control THBS1 CRISPR/Cas9 plasmid (*y-axis*: side scatter area, *x-axis:* fluorescent signal). (B) RA FLS transfected with ID1 CRISPR/Cas9 plasmid or THBS1 plasmid were assayed for cell proliferation using the IncuCyte® S3 Live-Cell Analysis System. (*P < 0.05; THBS1 control, n = 19; ID1 CRISPR plasmid, n = 9). (C) Due to imperfect repair by Cas9 nuclease, DNA in the cell pool contains a mixture of indels, yielding a composite sequence trace after the break site (blue dotted line). Shown is an overview of TIDE algorithm and output, consisting of visualization of aberrant sequence signal in control (black) and treated sample (green). The chart indicates that the Cas9 nuclease cut the genome and the cell repaired the damage by NHEJ resulting in aberrant sequences. (D) RA FLS transfected with either sham control, GFP control, or ID1 CRISPR/Cas9 plasmid were sorted and cultured for 24 hours for cytokine expression analysis using ELISAs. (*P < 0.05; shams, n = 17+; GFPs, n = 5; CRISPRs, n = 5+).

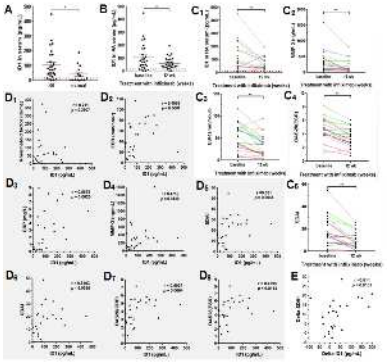
Figure 3. Immunodepletion of ID1 and detection of citrullinated ID1 in RA SFs and STs. (A) Immunodepletion of ID1 in RA SFs using αID1 Ab. RA SFs (n = 5) immunodepleted of ID1 were verified for depletion via ELISA. ID1 can be effectively depleted from RA SFs. (B) Measurement of total citrullinated antigens in ID1-depleted RA SFs. RA SFs (n = 5) immunodepleted of ID1 were measured for total citrullinated antigens. Depletion of ID1 resulted in a significant reduction in total citrullinated antigens as compared to isotype control, suggesting that ID1 is present in citrullinated forms in RA SFs (n = 5, *P < 0.05). (C) Total citrullinated protein concentration in isotype control versus anti-ID1 Ab-treated RA SFs. (n = 5, *P < 0.05) (D) Detection of citrullinated forms of ID1 in RA STs. IP was performed on RA ST homogenates (n = 3) using anti-ID1 Ab and probed for ID1 or total citrullinated antigens. CitID1 was detected in 2 out of 3 RA ST homogenates, indicating that citID1 is present in RA STs.

Figure 4. In vitro citrullination of rhID1 by rhPAD4. (A) Verifying citrullination of rhID1 by rhPAD4 via Western blot. Representative blots of citID1 (lane 1) and noncitID1 (lane 2) probed

by αID1 Ab or αMC Ab. CitID1 exhibits multiple forms corresponding to the degree of modification and runs noticeably higher than noncitID1, likely due to the increased hydrophobicity and the change in charge from the citrullination reaction. (B) Verifying citrullination of rhID1 by rhPAD4 via ELISA. Standard indirect ELISA protocol has been modified to incorporate an acidic modification step for the αMC Ab as described. CitID1 and noncitID1 can be distinguished by the αMC Ab. CitID1 produces an OD equivalent to ~300 ng/mL of citBSA. CitBSA is used as a relative standard due to its abundant modifiable arginines.

Figure 5. MS analysis of in vitro citrullinated rhID1. (A) Sequence of human ID1 isoform 1. Human ID1 contains 10 arginines (R) (highlighted red). RhID1 is citrullinated in vitro by rhPAD4 and analyzed via MS. (B) CitID1 (left lane) and noncitID1 (right lane) were run on SDS-PAGE and stained with "blue-silver" Coomassie for in-gel digestion prior to MS. (C) The annotated mass spectrum of tryptic peptide confirms citrullination of R121 in rhID1. All detected ions of the peptide are shown in black in the sequence and are annotated in the spectrum. The lower-case r in the sequence (highlighted in red) denotes citrulline. The ions highlighted in red in the spectrum show the neutral loss of isocyanic acid (HNCO; ~43Da), diagnostic marker ions for citrullination.

Figure 6. Detection of ACPAs to citID1 in RA specimens. (A) RA SFs RA sera and NL sera were assayed for autoantibodies to citID1 by IDB. We found ACPA reactivity for citID1, but not native ID1, in RA SF and PB, but not in NL PB. Human IgG (1 μg/mL) was used as a control and showed no reactivity. Additionally, ACPAs from RA PB did not bind when citID1 was boiled (denatured). Control dots showed no significant evidence of ACPAs or antibody binding reactivity in RA SF, RA PB or in NL PB. (B) Analysis of IDBs via densitometry shows higher reactivity to citID1 than noncitID1 in RA PBs (n = 30, 10 patients, ***$P < 0.001$, samples run in triplicate) and in RA SFs (n = 18, 6 patients, *$P < 0.05$, samples run in triplicate), but NL PBs (n = 12, 4 patients, samples run in triplicate). (C) Analysis of the roles of individual citrulline residues in ACPA reactivity of *in vitro* citrullinated rhID1. Arginines R33, R52, and R121 were critically necessary for ACPA reactivity (C denotes citrullines; # denotes native citrullines from the production of rhID1 in HEK293 cells).



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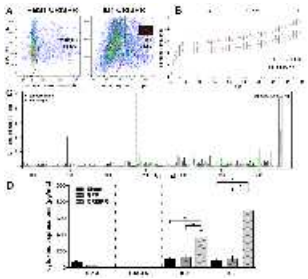

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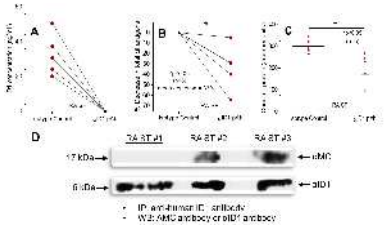

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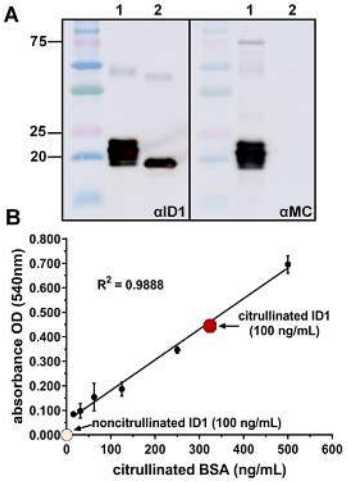

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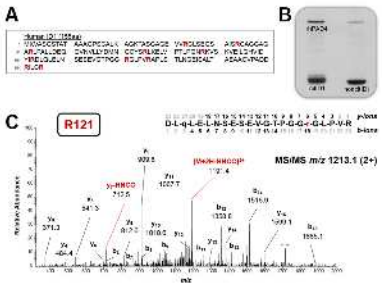

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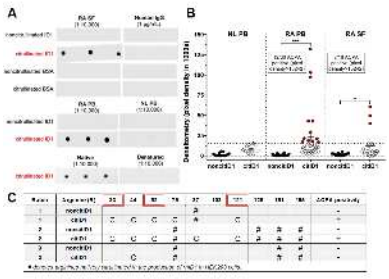

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