

RHEUMATOID ARTHRITIS

Technical comment on “Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis”

A. J. Salinger^{1,2}, M. L. Dubuke^{1,3}, C. Carmona-Rivera⁴, A. J. Maurais, S. A. Shaffer^{1,3},
E. Weerapana², P. R. Thompson^{1*}, M. J. Kaplan^{4*}

Copyright © 2020
The Authors, some
rights reserved;
exclusive licensee
American Association
for the Advancement
of Science. No claim
to original U.S.
Government Works

Reassessment of citrullinome cargo in neutrophil extracellular traps confirms the presence of citrullinated peptides.

In an article previously published in *Science Immunology* (1), we reported a previously unidentified mechanism of induction of pathogenic adaptive immunity against citrullinated antigens in rheumatoid arthritis (RA). This mechanism was mediated by an interaction between neutrophil extracellular traps (NETs) and synovial fibroblasts (FLS) that promote the presentation of citrullinated peptides identified in NETs by the FLS to the adaptive immune system. The identification of citrullinated peptides in NETs was performed by a variety of methods described in this and previous manuscripts and included the use of citrulline-specific probes, antibodies, and mass spectrometry (MS) (1, 2).

Following this publication, we were contacted by an investigator with concerns about our MS analysis. Given the magnitude of the mass shift expected between a citrulline and an arginine residue (+0.984 Da), misannotation of citrulline-containing peptides can occur because of fragmentation of misassigned monoisotopic mass peaks. In addition, the +0.984-Da mass increase upon citrullination is identical to the mass increase observed upon protein deamination of asparagine and glutamine residues within a peptide, thereby confounding the correct assignment of citrullinated species. Last, most of the previously identified peptides contained a C-terminal citrulline. Whereas several endogenous proteases, including cathepsin B, cleave after citrulline, trypsin, the protease used in sample preparation, does not (3). These discrepancies prompted us to reevaluate the initial proteomic data more stringently. Upon reevaluation, the spectra were either not consistent with citrullination or not definitive enough to be consistent with a citrullinated residue. Therefore, we generated new samples for analysis. Herein, we report the identification of 58 sites of citrullination on 32 proteins that are present in NETs using a highly stringent data analysis workflow.

In the analysis, healthy neutrophils were purified as described (1), resuspended in RPMI, seeded in 12-well plates, and incubated with 2.5 μ M calcium ionophore (A23187, Sigma-Aldrich) or 100 μ g of rheumatoid factor for 4 hours at 37°C. Supernatants were carefully removed, and NETs were harvested with micrococcal nuclease (MNase) (10 U/ml) in RPMI for 15 min at 37°C. Supernatant-containing NETs were isolated via centrifugation and stored at –80°C until analysis. Note that the proteomic analysis was performed on NETs and not from cells

treated with ionophore or rheumatoid factor; hence, it is not possible to generate control proteomes.

NETs (50 μ g) were precipitated with 20% trichloroacetic acid. The pellets were washed with acetone and then resuspended in urea (8 M). Ammonium bicarbonate (100 mM) was then added to the solution. Samples were reduced with dithiothreitol (15 mM) and alkylated with iodoacetamide (12.5 mM). Next, samples were diluted to 1.8 M urea with phosphate buffer (pH 7.4). Samples were then digested overnight at 37°C with Trypsin Gold (2 μ g, Promega) and 1 mM CaCl₂. Tryptic peptides were separated on a ZORBAX extended C18 column (Agilent) over a 1-hour, biphasic gradient from 0% buffer A (10 mM ammonium bicarbonate plus 5% acetonitrile) to 100% buffer B (10 mM ammonium bicarbonate and 90% acetonitrile). The resulting 96 fractions were pooled to yield 6 fractions for liquid chromatography–tandem MS (MS/MS) analysis.

Fractions were dried and resuspended in 25 μ l of 5% acetonitrile/0.1% trifluoroacetic acid. Peptides were eluted from a gravity-pulled analytical column packed with 3- μ m (100 Å) Magic C18AQ particles using a biphasic linear gradient from 5 to 60% of B (acetonitrile and 0.1% formic acid) in mobile phase A (water and 0.1% formic acid) over 120 min. Ions were introduced by positive electrospray ionization at 1.4 kV into a Thermo Scientific Q Exactive hybrid mass spectrometer. Raw data files were peak processed by MaxQuant 1.6.3.4, and identifications were searched by Andromeda against the Human SwissProt database. First-search mass tolerance was 20 parts per million (ppm) for precursors and 20 ppm for fragment ions, and main search tolerance after precursor mass recalibration was 4.5 ppm for precursors and 20 ppm for fragments. All positive citrullination sites were verified manually.

To unambiguously detect and verify the presence of citrullinated arginines, we followed a recently proposed workflow (4). First, we filtered the data to remove C-terminal citrullines. Next, we compared the observed MS isotopic envelopes to predicted envelopes for arginine- and citrulline-containing (+0.984 Da) peptides. If the MS spectra demonstrated a mass shift correlating with citrulline, then we searched for a neutral loss of isocyanic acid (–CNOH, –43.0058 Da) in the MS/MS fragmentation spectra. Neutral loss of isocyanic acid, which is unique to citrulline-containing ion fragments, is frequently observed during high-energy collision dissociation (4). It is important to note that, although deamination of glutamine and asparagine results in a similar mass shift, it does not yield the neutral loss. Using this algorithm, we identified 58 sites of citrullination on 32 proteins (Table 1; spectra data available upon request).

Notably, three of the proteins we found as being citrullinated, actin, heat shock protein 90 (HSP90), and plastin-2 (PLSL), were also identified as being citrullinated in RA patient samples (5). In addition,

¹Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605, USA. ²Department of Chemistry, Boston College, Chestnut Hill, MA 02467, USA. ³Mass Spectrometry Facility, University of Massachusetts Medical School, Shrewsbury, MA 01545, USA. ⁴Systemic Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

*Corresponding author. Email: Paul.Thompson@umassmed.edu (P.R.T.); mariana.kaplan@nih.gov (M.J.K.)

Table 1. Identified sites of citrullination. r, citrulline; c, alkylated cysteine; m, oxidized methionine; n or q, deamination; m/z, mass/charge ratio; IO, ionophore; RF, rheumatoid factor.

Protein	Modified arginine	Peptide sequence	Charge	m/z	Pred. m/z	NET ind.
ACTB	183	(R)LDLAGDLTDYLMK(I)	2	812.9162	812.9162	IO
	196	(K)ILTEGYSFITTAER(E)	2	873.4393	873.4389	RF
	210	(R)GYSFTTAAFEVR(D)	3	544.2738	544.2739	RF
ACTC	256	(R)FrCPeALFQPSFLGMEScGHETTFNSIMK(C)	3	1179.2135	1179.2100	IO
	312	(K)DLYANTVLSGGTTMYPGIADrmQK(E)	3	868.4185	868.4209	IO/RF
	30	(K)AGFAGDDAPAVFPSIVGRPR(H)	3	719.7115	719.7115	RF
ARPC3	39	(R)AVFPSIVGrPRHQGMVMGMGQK(D)	4	588.8162	588.8161	RF
	256	(K)SYELPDGQVTIGNEFRC	3	1048.0225	1048.0264	RF
	34	(K)GPAPEtKTDIVDEAIYYFK(A)	2	699.0182	699.0200	RF
CDD	5	(K)hPACTLKPEcVOQLLcSQAQ(K)	3	873.7701	873.7696	IO
	12	(K)FHVFGQPAKADQcYEDVR(V)	4	581.7795	581.7774	IO
	416	(R)hAAPEASGTPSSDAVSRLEEMR(K)	3	816.3864	816.3871	IO/RF
CZIB	453	(K)RLDLLEETVQAK(-)	2	729.8989	729.8992	IO
	58	(R)LMDSVALKGGcGSASMVQK(C)	3	646.0028	646.0045	IO
	405	(R)IVLENSFEDKHEcPFGR(S)	4	544.2626	544.2628	RF
ELMO1	96 and 97	(K)IYVLLrQAQAGK(-)	2	823.4727	823.4728	IO
	393	(R)AQEEAEERLEADrMAALR(A)	3	653.9898	653.9899	IO
	49	(K)SIQEIQLDKDESrLK(Y)	3	683.0105	683.0112	IO
HMGBl/2	24	(K)MSSYAFFVQTcREEHK(K)	3	716.9998	716.9996	IO
	347	(K)MSSYAFFVQTcREEHK(K)	3	722.3307	722.3312	RF
	347	(K)VTEKIPVHLVTK(D)	4	396.9828	396.9832	IO
HNRPU	558	(K)NLTLLQrAPQcLGK(F)	2	806.9442	806.9456	IO
	601	(K)MclFAGFQrK(A)	2	629.8071	629.8072	IO
	269	(K)LEDVLPAAFTLcEPK(E)	3	634.6734	634.6729	IO
HSP90A	293 and 297	(K)LrVDirPQLL(N)	2	676.9167	676.9166	IO
	355	(R)APDFLENrK(K)	2	619.3152	619.3142	IO
	106/107	(R)QEGESrLNLVQR(N)	2	779.4013	779.4026	IO
LDHA/B	587	(K)DLAAFDKSHDQAVrTYQEHK(A)	4	590.7869	590.7871	IO

Protein	Modified arginine	Peptide sequence	Charge	m/z	Pred. m/z	NET ind.
MOES	P26038	(R)QEAEKALLQASrDQK(K)	3	682.3433	682.3430	IO
		(K)AQMVOEDLEKTAELK(T)	3	630.6595	630.6594	RF
		(K)ALTSELANADSKK(T)	3	545.2847	545.2846	IO
MPO H14	P05164	(K)hNGFPVALAR(A)	2	551.3118	551.3118	IO
		(K)LrGDILPFVVPR(R)	3	476.2770	476.2770	RF
MYH9	P35579	(K)TLEEAATHEAQIQEMrQK(H)	3	767.3764	767.3778	IO
		(R)IAQLEEELEEQGNTELINDLrK(K)	3	948.1448	948.1487	IO
NCF1		(K)IAQLEEQLDNETKErQAACK(Q)	3	792.3874	792.3884	IO
		(R)IEADATETADAMNfEVSSLK(N)	3	737.3418	737.3425	IO
	P14598	(K)WFDGQrAAEnRQGLTEYcSTLMSLPTK(I)	3	1088.1752	1088.1763	IO
	(K)WFDGQrAAEnrQGLTEYcSTLMSLPTK(I)	3	1088.1761	1088.1763	IO	
		(R)RqArPGQSPGSPLEEEER(Q)	3	664.9952	664.9960	IO
PGM1	P36871	(K)DLEALMFDfSVGK(Q)	3	543.6045	543.6045	RF
		(M)ac-ARGSVSDEEMMELR(E)	2	826.8567	825.8664	IO
PLSL	P13796	(R)SrGFgFVYATVEEVDAAMNARPHK(V)	4	689.3417	689.3381	IO
ROA1	P09651	(K)TLETVPLErK(K)	3	396.2277	396.2274	IO
ROA2	P22626	(K)ILETEcPQYrK(G)	3	560.2991	560.2993	IO
S100-A8	P05109	(K)ILETEcPQYrK(K)	2	775.8961	775.8978	IO
		(R)ELPFLGKtDEAAAFK(L)	3	646.6691	646.6707	IO
S10A4	P26447	(R)YVSTVtGSrEIKSQSEVTR(I)	3	781.7313	781.7311	IO
SH3L3	Q9H299	(R)YVSTVtGSrEIKSQSEVTR(I)	3	781.7313	781.7311	IO
		(R)YVSTVtGSrEIK(S)	2	714.3721	714.3725	IO
		(R)IQYQLVDSQDNALRDEMfALAGNPK(A)	3	987.1703	987.1696	IO
SPTB2	Q01082	(K)MLTAQDMsYDEArNLHSK(W)	3	709.6548	709.6542	IO
TBA4A	P68366	(K)EIIDPVLDrHrK(L)	2	734.9221	734.9221	IO
TBB4B/5	P68371	(K)IREEYDfHMNTfSVVPSPK(V)	3	793.3715	793.7386	IO/RF
TLN1	Q9Y490	(K)FLPSELrDEH(-)	2	622.3012	622.3013	RF
		(R)ALAVNPrDPPSwVLGHrRT	3	711.0381	711.0461	IO
VIME	P08670	(K)SfLGDLYEEEMREL(R)	3	500.2326	500.2329	IO
		(K)ILETEcPQYrK(K)	2	948.9588	948.9596	IO
		(K)FADLSEAAfNNDALR(Q)	2	889.4271	889.4268	RF

over half of the proteins found in the NETs were also previously found in the proteome of RA synovial fluid and neutrophils treated with ionophores (6). Together, our new data uncovered 15 previously unreported citrullinated proteins present in NETs.

The identified proteins include vimentin and myeloperoxidase (MPO), the latter also identified in the original report (1). Notably, citrullinated vimentin, α/β -tubulin, β -actin, and NCF1 are known RA antigens (1, 4, 7, 8). In addition, we identified S100-A8, plastin, coronin, moesin, and ezrin, which are all linked to RA (9). Several inflammation-associated proteins were also observed (S100A8, MPO, LKHA, and HMGB1). Furthermore, our list of citrullinated proteins includes numerous cytoskeletal and cell motility proteins such as PLSL, myosin, vimentin, tubulin, and actin. S100A8 is interesting because several targets of the iNOS-S100A8 transnitrosylase complex (i.e., EZR1, MOES, and VIME) are also present as citrullinated proteins in these NETs. Last, linking our analysis to NETosis is our identification of several citrullinated chromatin binding proteins (HMGB1, HP1B3, and HNRPU) and NCF1, which is a component of the nicotinamide adenine dinucleotide phosphate oxidase complex.

In summary, these findings continue to support the conclusions of our previous publications (1, 2), mainly, that NETs are a source of citrullinated autoantigens. We have identified 58 sites of citrullination in 32 proteins from purified NET samples induced via rheumatoid factor or calcium ionophore. Because citrullination is a modification in such low abundance, generating a comprehensive database of citrulline-containing peptides found in any complex proteome is challenging (10). In addition, the need to verify each identified peptide via manual inspection of MS and MS/MS spectra has hampered the growing field exploring this specific modification. Methods to enrich for citrulline-containing peptides and/or computationally automate the process of picking out true citrulline peptides by neutral loss MS/MS analysis would further facilitate the process of decoding the citrullinome.

REFERENCES AND NOTES

1. C. Carmona-Rivera, P. M. Carlucci, E. Moore, N. Lingampalli, H. Uchtenhagen, E. James, Y. Liu, K. L. Bicker, H. Wahamaa, V. Hoffmann, A. I. Catrina, P. R. Thompson, J. H. Buckner, W. H. Robinson, D. A. Fox, M. J. Kaplan, Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. *Sci. Immunol.* **2**, eaag3358 (2017).
2. R. Khandpur, C. Carmona-Rivera, A. Vivekanandan-Giri, A. Gizinski, S. Yalavarthi, J. S. Knight, S. Friday, S. Li, R. M. Patel, V. Subramanian, P. Thompson, P. Chen, D. A. Fox, S. Pennathur, M. J. Kaplan, NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci. Transl. Med.* **5**, 178ra140 (2013).
3. Y. Anami, C. M. Yamazaki, W. Xiong, X. Gui, N. Zhang, Z. An, K. Tsuchikama, Glutamic acid-valine-citrulline linkers ensure stability and efficacy of antibody-drug conjugates in mice. *Nat. Commun.* **9**, 2512 (2018).
4. C. Y. Lee, D. Wang, M. Wilhelm, D. P. Zolg, T. Schmidt, K. Schnatbaum, U. Reimer, F. Pontén, M. Uhlén, H. Hahne, B. Kuster, Mining the human tissue proteome for protein citrullination. *Mol. Cell. Proteomics* **17**, 1378–1391 (2018).
5. R. Tilwala, S. H. Nguyen, A. J. Maurais, V. V. Nemmara, M. Nagar, A. J. Salinger, S. Nagpal, E. Weerapana, P. R. Thompson, The rheumatoid arthritis-associated citrullinome. *Cell Chem. Biol.* **25**, 691–704.e6 (2018).
6. V. Romero, J. Fert-Bober, P. A. Nigrovic, E. Darrah, U. J. Haque, D. M. Lee, J. van Eyk, A. Rosen, F. Andrade, Immune-mediated pore-forming pathways induce cellular hypercitrullination and generate citrullinated autoantigens in rheumatoid arthritis. *Sci. Transl. Med.* **5**, 209ra150 (2013).
7. M. A. Badillo-Soto, M. Rodríguez-Rodríguez, M. E. Pérez-Pérez, L. Daza-Benitez, J. J. Bollain-Y-Goytia, M. A. Carrillo-Jiménez, E. Avalos-Díaz, R. Herrera-Esparza, Potential protein targets of the peptidylarginine deiminase 2 and peptidylarginine deiminase 4 enzymes in rheumatoid synovial tissue and its possible meaning. *Eur. J. Rheumatol.* **3**, 44–49 (2016).
8. Y. Zhou, L.-L. An, R. Chaerkady, N. Mittereder, L. Clarke, T. S. Cohen, B. Chen, S. Hess, G. P. Sims, T. Mustelin, Evidence for a direct link between PAD4-mediated citrullination and the oxidative burst in human neutrophils. *Sci. Rep.* **8**, 15228 (2018).
9. M. Wagatsuma, M. Kimura, R. Suzuki, F. Takeuchi, K. Matsuta, H. Watanabe, Ezrin, radixin and moesin are possible auto-immune antigens in rheumatoid arthritis. *Mol. Immunol.* **33**, 1171–1176 (1996).
10. R. Tilwala, P. R. Thompson, Peptidyl arginine deiminases: Detection and functional analysis of protein citrullination. *Curr. Opin. Struct. Biol.* **59**, 205, –215 (2019).

Funding: This work was supported by the Intramural Research Program at the National Institute of Arthritis and Musculoskeletal and Skin Disease/NIH (ZIAAR041199). This work was also supported in part by NIH grants R35GM118112 (P.R.T.), F32GM128231 (A.J.S.), 1R01GM117004 (E.W.), and 1R01GM118431-01A1 (E.W.).

Submitted 9 April 2019
Accepted 6 January 2020
Published 31 January 2020
10.1126/sciimmunol.aax5672

Citation: A. J. Salinger, M. L. Dubuke, C. Carmona-Rivera, A. J. Maurais, S. A. Shaffer, E. Weerapana, P. R. Thompson, M. J. Kaplan, Technical comment on “Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis”. *Sci. Immunol.* **5**, eaax5672 (2020).

Technical comment on "Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis"

A. J. Salinger, M. L. Dubuke, C. Carmona-Rivera, A. J. Maurais, S. A. Shaffer, E. Weerapana, P. R. Thompson and M. J. Kaplan

Sci. Immunol. **5**, eaax5672.
DOI: 10.1126/sciimmunol.aax5672

ARTICLE TOOLS <http://immunology.sciencemag.org/content/5/43/eaax5672>

REFERENCES This article cites 9 articles, 3 of which you can access for free
<http://immunology.sciencemag.org/content/5/43/eaax5672#BIBL>

PERMISSIONS <http://www.sciencemag.org/help/reprints-and-permissions>

Use of this article is subject to the [Terms of Service](#)

Science Immunology (ISSN 2470-9468) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science Immunology* is a registered trademark of AAAS.

Copyright © 2020 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works