RHEUMATOID ARTHRITIS

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

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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 μM calcium ionophore (A23187, Sigma-Aldrich) or 100 μg 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 μg) 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 μg, Promega) and 1 mM $CaCl_2$. 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 μl of 5% acetonitrile/0.1% trifluoroacetic acid. Peptides were eluted from a gravity-pulled analytical column packed with 3-μm (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,

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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	P60709	183	(R)LDLAGrDLTDYLMK(I)	2	812.9162	812.9162	IO
		196	(K)ILTErGYSFTTTAER(E)	2	873.4393	873.4389	RF
		210	(R)GYSFTTTAErEIVR(D)	3	544.2738	544.2739	RF
				2	815.9070	815.9072	RF
		256	(R)FrcPEALFQPSFLGMEScGIHETTFNSIMK(C)	3	1179.2135	1179.2100	IO
		312	(K)DLYANTVLSGGTTMYPGIADrmQK(E)	3	868.4185	868.4209	IO/RF
ACTC	P68032	30	(K)AGFAGDDAPrAVFPSIVGRPR(H)	3	719.7115	719.7115	RF
		39	(R)AVFPSIVGrPRHQGVMVGMGQK(D)	4	588.8162	588.8161	RF
		256	(K)SYELPDGQVITIGNErFR(C)	3	1048.0225	1048.0264	RF
				2	699.0182	699.0200	RF
ARPC3	O15145	34	(K)GPAPrETKDTDIVDEAIYYFK(A)	3	810.4000	810.4006	IO
CDD	P32320	5	(K)rPAcTLKPEcVQQLLVcSQEAK(K)	3	873.7701	873.7696	IO
				2	1308.6492	1308.6508	IO
COR1A	P31146	12	(K)FrHVFGQPAKADQcYEDVR(V)	4	581.7795	581.7774	IO
		416	(R)rAAPEASGTPSSDAVSRLEEEMR(K)	3	816.3864	816.3871	IO/RF
		453	(K)RLDrLEETVQAK(–)	2	729.8989	729.8992	IO
CZIB	Q9NWV4	58	(R)LMDSVALKGGrGSASMVQK(C)	3	646.0028	646.0045	IO
ELMO1	Q92556	405	(R)IVLENSSrEDKHEcPFGR(S)	4	544.2626	544.2628	RF
ERH	P84090	96 and 97	(K)IYVLLrrQAQQAGK(–)	2	823.4727	823.4728	IO
EZR1	P15311	393	(R)AQEEAERLEADrMAALR(A)	3	653.9898	653.9899	IO
GDIR1	P52565	49	(K)SIQEIQELDKDDESLrK(Y)	3	683.0105	683.0112	IO
HMGB1/2	P09429	24	(K)MSSYAFFVQTcrEEHKK(K)	3	716.9998	716.9996	IO
			(K)mSSYAFFVQTcrEEHKK(K)	3	722.3307	722.3312	RF
HNRPU	Q00839	347	(K)VTEKIPVrHLYTK(D)	4	396.9828	396.9832	IO
		558	(K)LNTLLQrAPQcLGK(F)	2	806.9442	806.9456	IO
		601	(K)McLFAGFQrK(A)	2	629.8071	629.8072	IO
HP1B3	Q5SSJ5	269	(K)LEDVLPLAFTrLcEPK(E)	3	634.6734	634.6729	IO
		293 and 297	(K)LrVDIrPQLLK(N)	2	676.9167	676.9166	IO
HSP90A	P07900	355	(R)APFDLFENrK(K)	2	619.3152	619.3142	IO
LDHA/B	P00338	106/107	(R)QQEGESrLNLVQR(N)	2	779.4013	779.4026	IO
LKHA	P09960	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	408	(R)QEAEEAKEALLQASrDQK(K)	3	682.3433	682.3430	IO
		460	(K)AQMVQEDLEKTrAELK(T)	3	630.6595	630.6594	RF
		533	(K)ALTSELANArDESKK(T)	3	545.2847	545.2846	IO
MPO H14	P05164	219	(K)rNGFPVALAR(A)	2	551.3118	551.3118	IO
MYH9	P35579	1922 and 1923	(K)LrrGDLPFVVPR(R)	3	476.2770	476.2770	RF
		1191	(K)TLEEEAKTHEAQIQEMrQK(H)	3	767.3764	767.3778	IO
		1751	(R)IAQLEEELEEEQGNTELINDrLK(K)	3	948.1448	948.1487	IO
		1830	(K)IAQLEEQLDNETKErQAAcK(Q)	3	792.3874	792.3884	IO
		1912	(R)ELEDATETADAMNrEVSSLK(N)	3	737.3418	737.3425	IO
NCF1	P14598	85	(K)WFDGQrAAEnRQGTLTEYcSTLMSLPTK(I)	3	1088.1752	1088.1763	IO
		85 and 90	(K)WFDGQrAAENrQGTLTEYcSTLMSLPTK(I)	3	1088.1761	1088.1763	IO
		340	(R)RqArPGPQSPGSPLEEER(Q)	3	664.9952	664.9960	IO
PGM1	P36871	452	(K)DLEALMFDrSFVGK(Q)	3	543.6045	543.6045	RF
				2	814.9048	814.9031	IO
PLSL	P13796	3	(M)ac-ArGSVSDEEMMELR(E)	2	826.8567	825.8664	IO
ROA1	P09651	232	(R)SrGFGFVTYATVEEVDAAMNARPHK(V)	4	689.3417	689.3381	IO
ROA2	P22626	12	(K)TLETVPLErK(K)	3	396.2277	396.2274	IO
S100-A8	P05109	47	(K)LLETEcPQYIrKK(G)	3	560.2991	560.2993	IO
			(K)LLETEcPQYIrK(K)	2	775.8961	775.8978	IO
S10A4	P26447	49	(R)ELPSFLGKrTDEAAFQK(L)	3	646. 6691	646.6707	IO
SH3L3	Q9H299	15	(R)VYSTSVTGSrEIKSQQSEVTR(I)	3	781.7313	781.7311	IO
			(R)VYSTSVTGSrEIK(S)	2	714.3721	714.3725	IO
		51	(R)IQYQLVDISQDNALRDEMrALAGNPK(A)	3	987.1703	987.1696	IO
SPTB2	Q01082	1307	(K)MLTAQDmSYDEArNLHSK(W)	3	709.6548	709.6542	IO
TBA4A	P68366	21 and 23	(K)EIIDPVLDrIrK(L)	2	734.9221	734.9221	IO
TBB4B/5	P68371	162	(K)IREEYPDrIMNTFSVVPSPK(V)	3	793.3715	793.7386	IO/RF
TLN1	Q9Y490	2538	(K)FLPSELrDEH(–)	2	622.3012	622.3013	RF
		1625	(R)ALAVNPrDPPSWSVLAGHSR(T)	3	711.0381	711.0461	IO
VIME	P08670	145	(K)SrLGDLYEEEMRELR(R)	3	500.2326	500.2329	IO
				2	948.9588	948.9596	IO
		304	(K)FADLSEAANrNNDALR(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 erzin, 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.

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