RHEUMATOID ARTHRITIS

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

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Discrepancies on the role of citrullination in the induction of experimental arthritis by neutrophils extracellular traps.

In a recent publication in *Science Immunology*, Carmona-Rivera *et al.* (*1*) report that HLA-DRB1*04:01 transgenic mice immunized with fibroblast-like synoviocytes (FLSs) loaded with neutrophil extracellular traps (NETs) developed antibodies specific to citrullinated rheumatoid arthritis (RA) autoantigens. This work adds support for the role of citrullination in NETs as a critical component of RA pathogenesis. In the context of these findings, this manuscript offers an opportunity to revisit the nuances of defining citrullination during NETosis and to reexamine the definition of antibodies specific for citrullinated RA autoantigens.

The major foundation for this work is supported by the idea that citrullination in NETs provides the antigens that initiate an anti-citrullinated protein antibody (ACPA) response (*1*, *2*). To strengthen this critical component, Carmona-Rivera *et al.* showed in Fig. 1A of the manuscript that citrullinated proteins are generated during NETosis. To induce NETs, the authors used soluble immunoglobulin M (IgM) purified from non-RA patients with monoclonal IgM cryoglobulinemia with rheumatoid factor reactivity. Using a new method to detect citrullination (i.e., rhodamine-phenylglyoxal), Carmona-Rivera *et al.* showed patterns of citrullination that have not been described in NETs elsewhere (*3*, *4*). Because we and others have questioned the extent of citrullination in NETs (except for some citrullination of histones) (*4*, *5*), we addressed whether the findings by Carmona-Rivera *et al.* may result from the use of rhodamine-phenylglyoxal to detect citrullinated proteins in NETs. Using a canonical inducer of NETosis [phorbol 12-myristate 13-acetate (PMA)] and rhodamine-phenylglyoxal, we replicated the patterns of citrullination depicted by Carmona-Rivera *et al.* (Fig. 1A, lane 1). However, the inclusion of controls demonstrated that, except for one protein of ~12 kDa more prominent in NETs (likely a histone), citrullinated proteins were similarly detected in control (unstimulated) neutrophils (Fig. 1A, lane 2). The limited extent of citrullination found in NETs was highlighted when compared to mechanisms that induce robust hypercitrullination, such as ionomycin from *Streptomyces conglobatus*, which saturated the detection assay in the time required to detect citrullination in NETs and control cells (Fig. 1A, lane 3). These findings are consistent with patterns of hypercitrullination induced by bacterial and host pore-forming proteins (*3*, *4*, *6*), which result from a process termed leukotoxic hypercitrullination (LTH) (*4*). Because Carmona-Rivera *et al.* did not include controls in Fig. 1A of the manuscript, it is impossible to define the significance and magnitude of their results and whether citrullination detected in cryoglobulin-induced NETosis may only correspond to background found in control unstimulated neutrophils.

The importance of including informative controls is similarly underscored in additional figures in the manuscript of Carmona-Rivera *et al.* In Fig. 1B of the manuscript, Carmona-Rivera *et al.* demonstrated that ACPAs target several proteins in NETs. Although the detection of antigens in NETs by RA autoantibodies is reproducible using PMA (Fig. 1B, lane 1), the inclusion of unstimulated neutrophils demonstrates that the same bands are also found (some even more prominently) in control cells (Fig. 1B, lane 2). This is far less than the prominent detection of autoantigens in hypercitrullinated neutrophils (Fig. 1B, lane 3). Nevertheless, the absence of controls in Fig. 1B of the manuscript makes it impossible to confirm whether citrullinated autoantigens are generated during NETosis. Because dying cells redistribute their intracellular proteins, it is not surprising that citrullinated proteins found in control neutrophils may be redistributed during NETosis (or any other form of cell death) and detected with ACPAs by immunofluorescence, as observed in Fig. 1C of the manuscript. ACPA binding is also observed in cells that are not NETting (Carmona-Rivera *et al.*, fig. S1), supporting the idea that NETosis is not a generator of hypercitrullination but rather a redistributor of an existent steady-state citrullinome in neutrophils.

The identification of citrullinated proteins in unstimulated neutrophils is a finding that we have confirmed by mass spectrometry (MS) [see table S2 in (*3*)]. Stimuli with the potential to enhance citrullination therefore require inclusion of proper controls to determine whether citrullination is generated (both qualitatively and quantitatively) above the background of nonstimulated cells. Otherwise, hundreds of stimuli can be proposed to induce citrullination (which can be confirmed by rhodamine-phenylglyoxal and MS) simply by detecting the background citrullinome that is found in neutrophils. The absence of controls in Fig. 1E and table S1 of the manuscript questions whether the limited number of citrullinated proteins detected in NETs [which are not representative of the entire known RA citrullinome (*6*, *7*)] were generated during NETosis or merely represent redistribution of the steady-state citrullinome found in neutrophils.

Last, although it is certain that antibodies targeting the same antigen both as native and citrullinated can be found in RA (*8*, *9*), this finding has been overexploited to justify caveats in the study of ACPAs in experimental models of arthritis and in NETs. Because antibodies to native sequences can bind any region of the antigen that is not affected by citrullination, as illustrated in Fig. 1 (C and D), these antibodies can target both native and citrullinated small peptides and proteins (Fig. 1, C and D, respectively). However, that does not mean that they should be defined as ACPAs. To prove this simple idea, the experiment shown in Fig. 6C of the manuscript (used to demonstrate

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ACPAs in mice immunized with FLS loaded with NETs) was reproduced using anti-native protein antibodies from commercial sources (Fig. 1E). As expected, antibodies to native proteins, but not irrelevant mouse IgG, strongly reacted with the citrullinated proteins. Because ACPAs are not necessary in mice to develop arthritis (*10*), the production of antibodies to native proteins can explain the induction of arthritis by FLS loaded with NETs. The epitope chip analysis shown in Fig. 6H and fig. S13 of the manuscript demonstrates that the antibody response in these mice is predominant to native sequences. Nevertheless, in the absence of controls, these antibodies can be erroneously defined as specific to citrullinated antigens. In this context, antibodies to native and citrullinated myeloperoxidase and neutrophil elastase in Fig. 1 (G and I) of the manuscript were determined by comparing titers in RA versus osteoarthritis. Although this analysis can define whether these antibodies are significantly associated with RA, it provides no evidence that the antibodies are specific to citrullinated sequences. Defining an antibody as an ACPA requires demonstrating its preferential binding to the citrullinated versus native version of an antigen.

In summary, although differences in experimental conditions may be used to justify discrepancies regarding the role of NETosis in generating citrullinated autoantigens, the purpose of this letter is to underscore the importance of including strict controls to study citrullination in NETs. The data from Carmona-Rivera *et al*., in combination with our work, suggest that NETosis is not a generator of citrullinated autoantigens, except for some citrullination of histones that is not exclusive to NETosis (*3*). Therefore, the role of NETosis as a trigger of the ACPA response in mice and in RA should be taken with caution.

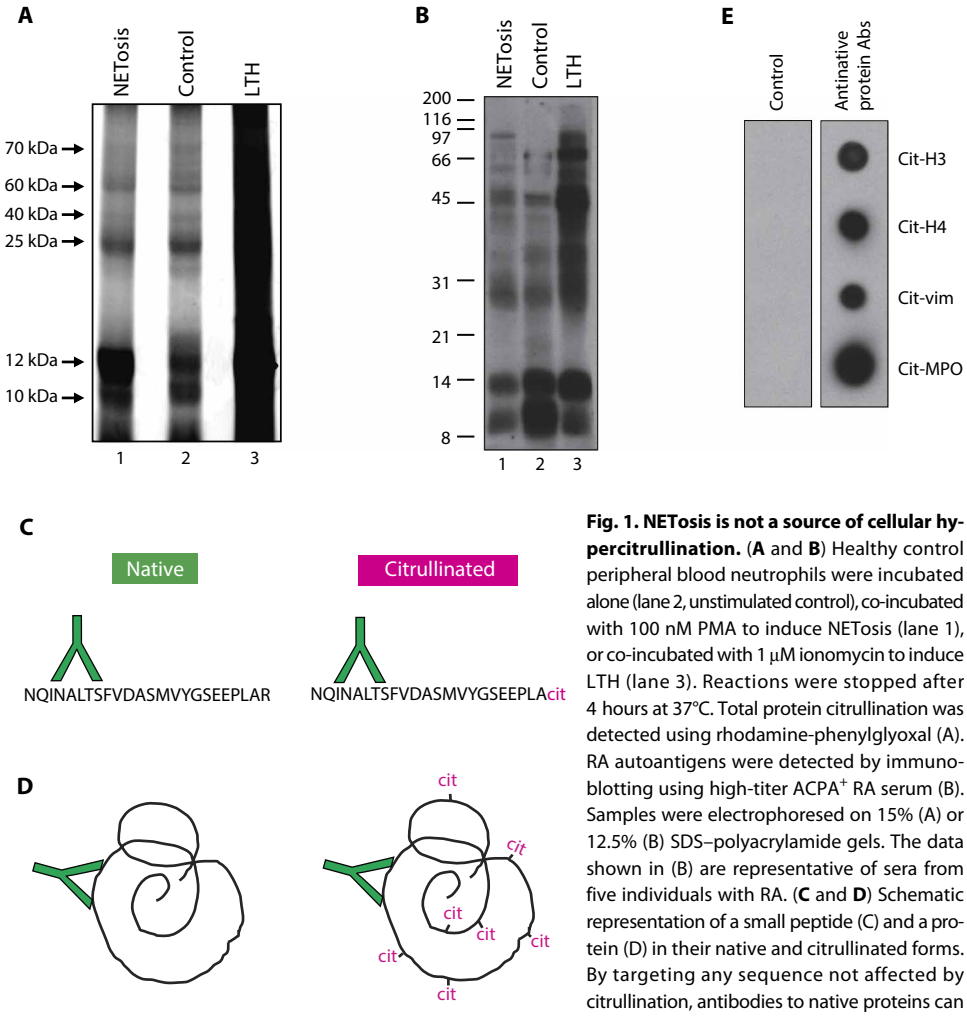

Fig. 1. NETosis is not a source of cellular hypercitrullination. (**A** and **B**) Healthy control peripheral blood neutrophils were incubated alone (lane 2, unstimulated control), co-incubated with 100 nM PMA to induce NETosis (lane 1), or co-incubated with 1 μM ionomycin to induce LTH (lane 3). Reactions were stopped after 4 hours at 37°C. Total protein citrullination was detected using rhodamine-phenylglyoxal (A). RA autoantigens were detected by immunoblotting using high-titer ACPA$^+$ RA serum (B). Samples were electrophoresed on 15% (A) or 12.5% (B) SDS–polyacrylamide gels. The data shown in (B) are representative of sera from five individuals with RA. (**C** and **D**) Schematic representation of a small peptide (C) and a protein (D) in their native and citrullinated forms. By targeting any sequence not affected by citrullination, antibodies to native proteins can similarly recognize both native and citrullinated peptides and proteins. The peptide sequence in (C) is from myeloperoxidase (MPO) and was obtained from Fig. 1F in the study of Carmona-Rivera *et al*. (*1*). (**E**) Purified histone H3 (H3), histone H4 (H4) (New England Biolabs), vimentin (vim) (PeproTech), and MPO (Millipore) were citrullinated in vitro with purified recombinant human PAD4 (peptidylarginine deiminase 4). The citrullinated (cit) proteins were detected by dot blot using irrelevant mouse IgG (catalog no. M5409, Sigma-Aldrich) (control) or commercial antibodies (Abs) to native histone H3, histone H4, vimentin (clone nos. 96C10, L64C1, and 5G3F10, respectively, Cell Signaling), and MPO (clone no. 392105, R&D Systems).

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