We read the letter by Shi and Andrade in response to our publication in Science Immunology (1). In their letter, Shi and Andrade focus their comments on the role of NETosis in the generation of citrullinated autoantigens and, thus, the ability of neutrophil extracellular traps (NETs) to “initiate an anticitrullinated protein antibody (ACPA) response.” To clarify, our hypothesis and the conclusions from our manuscript are that citrullinated autoantigens contained and externalized in NETs can be taken up by rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) and, in the context of human leukocyte antigen class II (shared epitope), are effectively presented in an antigen-specific manner to the adaptive immune system to generate pathogenic immune responses characteristic of RA.

Although NETosis by itself without the right genetic predisposition may not promote aberrant adaptive immunity, exacerbated NET formation characteristic of RA (2) can lead to the externalization of citrullinated proteins that, because they are bound to nucleic acids, HMGBl (high mobility group box 1), and other immunostimulatory molecules, display the ability to be internalized by antigen-presenting cells (1). We do not propose that NETs are the only source of citrullinated autoantigens, and several other mechanisms (tobacco and microbes) likely play important roles. Some of these environmental stimuli may also contribute to citrullination through their ability to induce NETs (3, 4). In contrast, the in vivo relevance of leukotoxic “hypercitrullination” in RA with regard to activation of pathogenic immunity and organ damage remains to be determined and is, at this state, primarily an in vitro observation, as recently discussed (5). Whether a threshold of hypercitrullination needs to be achieved to break tolerance and initiate ACPA responses has not been systematically demonstrated to be an important in vivo phenomenon in the context of RA.

We would like to directly respond to several points raised by Shi and Andrade:

(1) They dispute our finding that citrullinated proteins are generated during NETosis. However, the conditions they use differ considerably from those used in our manuscript, making comparisons difficult. We reported the pattern of citrullination in purified NETs that have been induced by rheumatoid factor (RF) (1), whereas Shi and Andrade use whole-cell protein lysates from neutrophils exposed to phorbol 12-myristate 13-acetate (PMA). Compared to many other stimuli, as recently reported, PMA does not induce histone H3 citrullination, and peptidylarginine deiminase (PAD)–dependent pathways do not seem to significantly affect the systematic ability of PMA to induce NETs in human neutrophils (6). In contrast, ionophore and RF induce significant histone citrullination (1, 6). Hence, we consider PMA, although effective at inducing NETs in vitro, to be a somewhat contrived system that does not represent the NETosis induced by many physiologic and/or pathologic stimuli, including the ones used in our studies. Another important point to clarify is that in contrast to the observations that RF induces NET formation, control immunoglobulin M (IgM) does not (2). Therefore, IgM stimulation was not included as a control for the pattern of citrullinated proteins in RF-induced NETosis because NETs are not generated by the control immunoglobulin (2). To further support our findings, we now show additional controls to emphasize that, depending on the type of NETs and the disease state, the pattern of citrullination displayed in NETs varies (Fig. 1A). Spontaneously formed lupus low-density granulocyte NETs have a citrullination profile that differs from NETs induced by RF in control neutrophils. Furthermore, when we compare the spontaneously formed NETs from lupus low-density granulocytes to the spontaneously formed NETs from low-density granulocytes previously exposed to type I interferons (IFNs), the intensity of the citrullination pattern is altered (Fig. 1B). These results indicate that the pattern of citrullination may be specific to the disease state or the stimulus used to induce NETs. In addition, we had previously reported that the NET protein cargo varies depending on the stimulus to induce NETs (2). Using unstimulated neutrophils to compare NETs does not represent, in our view, a good control for extracellular exposure of citrullinated autoantigens because control unstimulated neutrophils do not form NETs (6). The use of neutrophil lysates proposed by Shi and Andrade is also, in our view, not adequate as a NET control because it does not distinguish intracellular from extracellular citrullinated autoantigens.

(2) Shi and Andrade used calcium ionophores to induce in vitro hypercitrullination; this stimulus is also a very potent inducer of NETs but is nonspecific (6). Hence, differentiating citrullination patterns between NETosis induced by ionophore and leukotoxic hypercitrullination may be complicated when evaluating these experiments.

(3) Similar points are raised with regard to comparing Fig. 1B in our manuscript (1) with Fig. 1B in the technical comment written by Shi and Andrade. In their letter, Shi and Andrade focus their comments on the role of NETosis in the generation of citrullinated autoantigens and, thus, the ability of neutrophil extracellular traps (NETs) to “initiate an anticitrullinated protein antibody (ACPA) response.” To clarify, our hypothesis and the conclusions from our manuscript are that citrullinated autoantigens contained and externalized in NETs can be taken up by rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) and, in the context of human leukocyte antigen class II (shared epitope), are effectively presented in an antigen-specific manner to the adaptive immune system to generate pathogenic immune responses characteristic of RA.

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Proteins were transferred onto a nitrocellulose membrane and probed with high-
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Fig. 1. (A and B) Citrullinated protein patterns present in NETs vary depending
on the disease state and stimulus. (A) NETs were induced in control (Ctrl) neutrophils by RF or spontaneously generated
in lupus low-density granulocytes (LDGs). NETs were purified, and citrullinated pro-
teins were detected using rhodamine-phenylglyoxal probes. The citrullination
pattern varies when comparing the two samples. (B) Spontaneously formed NETs
were purified from lupus low-density granulo
cytes isolated from a different patient
as in (A), incubated in the presence or
absence of 1000 IU of recombinant IFN-
alpha for 2 hours. Citrullinated proteins were
detected using rhodamine-phenylglyoxal. Total histone H3 (Abcam) and MPO (Dako)
were used as loading controls. Increased citrullination of proteins is evident after
IFN treatment. (C) Serum from RA patients preferentially recognizes citrullinated over
native MPO. Human recombinant MPO (Lee BioSolutions) was citrullinated in vitro
with purified PAD4. Purified PAD4, native MPO, and citrullinated MPO (cit-MPO)
were resolved in a gradient 4 to 12% SDS–polyacrylamide gel electrophoresis gel.
Proteins were transferred onto a nitrocellulose membrane and probed with high-
titer ACPA* serum from a subject with RA (1:250 dilution). Anti-human IgG coupled
to horseradish peroxidase was used to develop the membrane. Same samples were
probed against MPO to demonstrate the presence of recombinant MPO. RA
serum can differentially recognize the citrullinated version of MPO over the native
one. Results are representative of five RA subjects.

Shi and Andrade. Again, we are using purified NETs, whereas Shi and
Andrade report whole-cell lysates under PMA-treated conditions,
making comparisons difficult. An additional concern here is that Shi
and Andrade use whole serum from RA patients with high levels of
ACPAs, which may affect specificity and intensity of these signals,
whereas we use ACPA antibodies purified from RA patients’ plasma
using a previously validated approach (7). Furthermore, in our man-
uscript, using IgG control does not lead to detection of citrullinated proteins in NETs above background intensity [Fig. 1B in (J)].

(4) We dispute the assertion by Shi and Andrade that NETs are
merely redistributors of the steady-state citrullinome. When we com-
pare the proteomic analysis of citrullinated proteins identified in
NETs induced by RF in our manuscript [Fig. 1E in (J)] to data pub-
lished by Romero et al. on the steady-state neutrophil lysates [fig. S2 in
(8)], we find a very different composition of citrullinated proteins.
Among the proteins we find citrullinated in RF-induced NETs, only
three are also citrullinated in steady-state neutrophil lysates, whereas
the other 17 proteins are not. Furthermore, previous studies by
Romero et al. also showed that myeloperoxidase (MPO) is distinct-
ly citrullinated in RA synovial fluid samples but not in steady-state
neutrophils (8), similar to the citrullinated MPO that we detected in
NETs and in agreement with our findings of anticitrullinated
MPO responses in the synovial fluid of RA patients (1). We consider
that a comprehensive analysis of the citrullinated proteome of NETs
under several other types of stimulation is warranted to better un-
derstand the potential variability among various patients or types of
stimulation; this may further our understanding of ACPA response
specificities in association with specific sources of NETs.

(5) It is possible that the initial ACPA responses target a limited
number of citrullinated proteins present in NETs with further expan-
son and epitope spreading as disease progresses. This is supported
by previous compelling studies in both human RA and animal models
of arthritis (9). With regard to ACPA specificity, we have demonstrated
both in vitro and in vivo that citrullinated NET peptides, when pre-
vented in a major histocompatibility complex class II–dependent man-
ner to antigen-specific T cells, will elicit T cell activation. Furthermore,
we have shown significantly enhanced ACPA production in the ani-
mals exposed to FLS with internalized NETs by three different methods:
(i) results using commercially available anti–cyclic citrullinated pep-
tide 2 (CCP2) test [this assay represents the second generation of anti-
CCP tests and is described to be highly specific for RA (95 to 99%)
and to provide excellent positive predictive value for RA diagnosis];
(ii) showing by dot blot that the serum from mice exposed to FLS
plus NETs recognizes citrullinated proteins at different levels of in-
ensity depending on the protein, whereas minimal detection is ob-
served when using serum from mice that received FLS without NETs,
thereby showing specificity to the NET effect; and (iii) finally, by means
of the protein array used in our study showing specificity of the anti-
bodies to certain citrullinated epitopes that are considered highly rel-
levant to RA (including histone, vimentin, and fibrinogen) and the target
of ACPAs. This is in agreement with the recent work by Corsiero et al. (10)
showing that B cells within the RA patients’ synovium target citrul-
inated proteins generated during NETosis of RA synovial neutrophils,
including citrullinated histones, fibrinogen, and vimentin, and that
these antibodies selectively recognize NETs from RA neutrophils. To
further support the specificity of our findings, we now show in this
response (Fig. 1C) that RA serum preferentially recognizes the citrul-
linated but not the native form of MPO. In contrast, commercial anti-
MPO antibody will have the ability to equally recognize both native
and citrullinated forms (Fig. 1C). Consistent with this, in our previous
work, there is a subset of autoantibodies in ACPA* RA that bind native
epitopes present in citrullinated antigens. Such autoantibodies target-
ing native epitopes in ACPA* RA may arise from epitope spreading of
the B cell response from citrullinated epitopes, but we do not consider
such autoantibodies to be ACPAs (9).

Overall, we consider that on the basis of the extensive work from
various groups including ours, there is now ample and compelling ev-
idence, both in vitro and in vivo in human and animal systems, that
NETs represent one of the sources of citrullinated autoantigens and
that these structures have the ability to activate innate and adaptive
immune responses in RA in a pathogenic manner.
REFERENCES AND NOTES


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

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