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Response to comment on "*Aggregatibacter actinomycetemcomitans*-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis."

Maximilian F. Konig[1], **Jon T. Giles**[2], **Ricardo P. Teles**[3,4,‡], **Niki M. Moutsopoulos**[5], and **Felipe Andrade**[6,*]

[1]Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

[2]Division of Rheumatology, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA

[3]Department of Periodontology, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[4]Department of Applied Oral Sciences, Forsyth Institute, Cambridge, MA 02142, USA

[5]National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA

[6]Division of Rheumatology, The Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA

We thank Dr. Volkov and colleagues for their interest in our study and attempt to replicate part of our findings. In our study, we provided mechanistic and clinical evidence that implicates the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (*Aa*) as a candidate trigger of autoimmunity in rheumatoid arthritis (RA) (1). We showed that among different periodontal pathogens, *Aa* has the ability to dysregulate peptidylarginine deiminases in neutrophils and induce patterns of hypercitrullination found in periodontitis and the rheumatoid joint. We demonstrated that *Aa*-induced hypercitrullination is mediated by its major virulence factor leukotoxin A (LtxA). Using antibodies to LtxA as surrogate markers of *Aa* infection, we found that exposure to leukotoxic *Aa* strains was highly prevalent both in periodontitis and in RA when compared with individuals without periodontitis. Exposure to *Aa* was associated with distinct anti-citrullinated protein antibody (ACPA) specificities in RA, strengthening the hypothesis that *Aa*-induced hypercitrullination may be relevant to RA pathogenesis by inducing ACPAs. Moreover, a significant association of HLA-DRB1 shared epitope (SE) alleles with ACPA and rheumatoid factor (RF) positivity was only observed in RA patients exposed to *Aa* [*P* values for interaction: $P = 0.022$ for

*Corresponding author: andrade@jhmi.edu.
‡Deceased.

Competing interests: F.A. is an inventor on issued patent no. 8,975,033 held by The Johns Hopkins University that covers "Human autoantibodies specific for PAD3 which are cross-reactive with PAD4 and their use in the diagnosis and treatment of rheumatoid arthritis and related diseases." F.A. received a grant from MedImmune and has served as consultant for Bristol-Myers Squibb Company and Pfizer.

ACPA positivity, $P = 0.022$ for anti–cyclic citrullinated peptide 2 (CCP2), and $P = 0.012$ for RF] (1). Together, these studies provide a mechanism to explain the tantalizing hypothesis that periodontitis is a driver of RA (1).

In their comment, Volkov et al. (2) only addressed the clinical associations reported in our study. First, they asked whether exposure to *Aa* as measured by anti-LtxA antibodies is specific for RA. Second, the authors attempted to replicate associations between *Aa* exposure, SE alleles, and ACPAs in a group of 594 arthritis patients selected from the Leiden Early Arthritis Clinic (EAC) cohort. Their analysis highlights important differences in data interpretation and cohorts studied. Despite this, we believe that their data are consistent with our findings.

Different from the authors' assumption, our study did not suggest specificity of *Aa* exposure to RA. *Aa* has been firmly established as a pathogen associated with periodontitis (3–5). Indeed, a considerable portion of our manuscript is dedicated to characterizing anti-LtxA antibodies as markers of periodontal disease status (Fig. 6B, fig. S4 A–B, and table S4) (1), and we showed that anti-LtxA concentrations correlate with periodontitis severity in patients without RA ($\beta = 1.12$; $P < 0.0001$; fig. S4B) (1). The idea that antibodies to LtxA may be restricted to patients with RA is therefore rejected by our data and misinterpreted by Volkov et al. (2). Periodontitis is a chronic inflammatory disease that is highly prevalent in the general population. The presence of antibodies against LtxA in any individual with periodontitis exposed to *Aa* (with or without any chronic illness) is therefore expected. Regardless of these data, Volkov et al. initially demonstrated that antibodies to LtxA are associated with *Aa* infection in periodontitis patients without RA (Fig. IA) (2), which confirmed our data. Having again demonstrated that anti-LtxA antibodies are markers of *Aa*-associated periodontitis in individuals without RA, the authors tested whether these antibodies are exclusively found in RA compared to healthy individuals or patients with other arthritides of unknown periodontal status.

Different from our study, Volkov et al. did not include healthy controls without periodontitis to define cut-offs for anti-LtxA positivity (2). In the absence of clinically relevant cut-offs to define exposure to leukotoxic strains of *Aa*, it is impossible to identify individuals who are truly positive for anti-LtxA antibodies. Using an arbitrary cut-off instead ("the lowest point of the linear part of the standard curve" of mixed RA patient sera), Volkov et al. reported that 58% of patients with RA have antibodies against LtxA (2). Although this exceeds the prevalence of anti-LtxA positivity in our cohort (43%) (1), it supports the high prevalence of *Aa* exposure in patients with RA. The authors further showed that anti-LtxA antibodies were significantly enriched in RA as compared to healthy controls ($P < 0.0001$) and patients with other inflammatory arthritides ($P < 0.001$ for psoriatic arthritis and $P < 0.05$ for spondyloarthritis with peripheral arthritis). Moreover, anti-LtxA antibody concentrations in RA were markedly higher than in any other form of non-RA arthritis, although sample size appeared prohibitive to reach statistical significance in some groups (Fig. 1B). The presence of anti-LtxA positivity reported by Volkov et al. in healthy controls and patients with non-RA arthritides likely reflects that some of these individuals have *Aa* and periodontitis, which is an oral pathology that is not exclusively found in RA. Regarding the consequences of *Aa* exposure, however, our model proposes that only individuals at risk for RA (through the

carriage of genetic susceptibility genes) will develop autoimmune sequelae from *Aa*-induced hypercitrullination (1). It is therefore expected that not everybody who is infected with *Aa* will develop RA.

Although absolute numbers of positivity for anti-LtxA reported by the authors in Fig. 1B are impossible to ascertain in the absence of non-periodontitis controls, a more stringent cut-off for positivity may have been more consistent with our own validated data set. Regardless of these limitations, the association of anti-LtxA antibodies with RA in the EAC cohort is compelling. Yet, the implications of these results in the context of our work were unfortunately not discussed by the authors.

Altogether, we believe that these data strongly support our findings by a) validating anti-LtxA antibodies as powerful tools to identify individuals exposed to *Aa*, b) reemphasizing the association of anti-LtxA antibodies with chronic periodontitis, and c) confirming a high prevalence and significant enrichment of *Aa* exposure in early RA as compared to controls and other inflammatory arthritides. The authors' assertion that patients with RA may be "more frequently anti-LtxA-positive simply because they have more periodontitis" is epidemiologically correct, but in no way negates an etiologic role of *Aa* in the genetically susceptible host.

Volkov et al. next analyzed the association of anti-LtxA antibodies with anti-CCP positivity (referred to as "ACPA" in their comment) and SE alleles in RA (2). The authors showed that anti-LtxA antibody titers do not differ by either anti-CCP or SE status in RA. Similar to their findings, we reported that anti-LtxA positivity was not associated with anti-CCP antibodies (table S5) (1). In this respect, their findings are consistent with our own data. In our study, anti-LtxA positivity was only significantly associated with distinct ACPA fine specificities measured by Bio-Plex bead-based assays [citrullinated antigens: apolipoprotein A1, $P < 0.001$; apolipoprotein E, $P = 0.004$; histone H2B, $P = 0.020$; vimentin, $P = 0.018$; Apolipoprotein E (277–296, cyclic peptide), $P = 0.032$; clusterin (231–250, cyclic peptide), $P = 0.020$; hnRNP B1b (RA33), $P = 0.038$; Table 1] (1), but not with anti-CCP positivity in general (table S5) (1). More importantly, we showed that the known association of SE alleles and autoantibody positivity (anti-CCP, Bio-Plex bead-based ACPA, or RF) was restricted to RA patients with *Aa* exposure (Table 2). In their analysis (which only included anti-CCP), Volkov et al. found only a small increase in the interaction of anti-LtxA and SE alleles for anti-CCP positivity in the Leiden cohort.

Several important differences in cohorts and methodology may account for this difference in effect size: 1) The authors' cohort is known to differ substantially from our cohort in anti-CCP positivity (only 58% in Leiden vs. 77% in Baltimore) (1). These differences are not easily explained by ethnicity, because the authors reported ACPA positivity in 75% of Dutch patients with established RA as part of the IMPROVED cohort (these patients fulfilled the ACR 1987 criteria for RA at the time of inclusion) (6). Compared with other cohorts, there is a marked discrepancy between the expected and reported anti-CCP positivity in the EAC cohort. This was acknowledged in a recent study, in which the authors reported the frequency of anti-CCP2 positive RA as only 51% in the EAC (total cohort) as compared with 65% in IMPROVED. Both frequencies are lower than in our cohort (77% in ESCAPE

RA), and this was suggested to be due to differences in inclusion criteria by the authors. Notably, the Leiden EAC includes patients with recent onset of arthritis, in whom definitive diagnoses are established after 1 year of follow-up (6). The large subset of anti-CCP-negative RA in the EAC cohort may therefore be comprised of early arthritis patients who were either misclassified as having RA (7, 8), or who will have milder forms of disease. Notably, anti-peptidylcitrulline reactivities in the Leiden EAC were observed in a larger subset of RA patients by multiplex array (64 %) when compared with anti-CCP2 (51%) (6), suggesting that in this early cohort, CCP2 may indeed not be sensitive enough to capture the entirety of the evolving ACPA repertoire. Moreover, given the range of disease durations in our cohort (from early disease to many decades), which is wider than that of the Leiden EAC cohort, a potential scenario for interpreting our finding is that the context of *Aa* may be required to observe the evolution of the ACPA repertoire, and other autoantibodies, among those with SE over the longer course of clinical disease, and not just in the earliest phase. Certainly, further studies are required to confirm this hypothesis. 2) The authors' definitions of anti-LtxA positivity differ considerably from our cohort (cut-offs were selected arbitrarily by Volkov et al., but experimentally established using non-periodontitis healthy controls in our study). Despite not affecting comparisons of median antibody concentrations between groups, uncertainty in cut-off selection can alter any interaction analyses of anti-CCP, SE, and anti-LtxA status. 3) Differences in racial and ethnical composition of our cohort (Baltimore) as compared with Europe (Leiden), may have influenced the effect of *Aa* in inducing ACPAs, although this is less likely.

In summary, our study provides mechanistic insights into how the periodontal pathogen *Aa* promotes hypercitrullination and neutrophil cell death, suggesting a biologically plausible link between chronic periodontal infection and the promotion of autoimmunity directed against citrullinated proteins. Virulent strains of *Aa* are strongly associated with chronic and aggressive periodontitis, and may explain the intriguing association of RA and periodontitis in a subgroup of patients with RA. Of note, *Aa* is not a pathogen exclusive to RA. Indeed, the identification of a single bacterial species in RA that can fulfill Henle and Koch's postulates of causation (9, 10), as implied by Volkov and colleagues (2), is highly unlikely. Instead, *Aa*-induced hypercitrullination provides a model to understand how pathobionts may influence the development of autoimmune diseases in susceptible individuals. It is certain that further studies are necessary to confirm our model. However, we believe that the interpretation of the data and conclusions presented by Volkov *et al.* cannot exclude a biologically relevant role for *Aa* in the etiopathogenesis of RA.

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