Tolerance and immune regulation in rheumatoid arthritis

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Chapter 8

Periodontal infection and induction of autoimmunity in rheumatoid arthritis: *Aggregatibacter actinomycetemcomitans*-induced hypercitrullination

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Adapted from: Comment on "Aggregatibacter actinomycetemcomitans–induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis"

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Periodontal disease is a chronic inflammatory condition affecting the tissues surrounding the teeth and is caused by dysbiosis of the oral microbiota. Periodontal disease and RA are closely linked as both diseases are characterized by localized chronic inflammatory reactions. Clinical studies have shown that periodontitis is more prevalent in patients witch active RA than in health individuals; conversely, the prevalence of RA is also higher in individuals with periodontitis than in those without periodontitis. (1)

The periodontal pathogen *Aggregatibacter actinomycetemcomitans* (Aa) seems to represent a link between periodontal infection and autoimmunity. Recently, the group of Konig et al. describe a potential explanation for the link between periodontal infection and rheumatoid arthritis (RA) (2). They identify a specific periodontitis-associated bacterium: *Aggregatibacter actinomycetemcomitans* (Aa), which via its lytic toxin (leukotoxin A: LtxA) can dysregulate the activity of citrullinating enzymes in neutrophils. The thus-generated citrullinated autoantigens are the target of a key autoantibody system in RA: anti-citrullinated protein antibodies (ACPA). Furthermore, the authors report that the effect of the most important genetic risk factor for RA: the human leukocyte antigen-DRB1 shared epitope (HLA SE) alleles, was limited to RA patients who had been exposed to Aa as determined by seropositivity to LtxA in a set of 194 RA patients. Based on these findings, the authors hypothesize that LtxA may be a key factor in the initiation of the RA-specific anti-citrullinated protein immune response in genetically predisposed individuals. In light of the crucial implications of this novel hypothesis for the current thinking regarding RA development, we aimed to replicate these findings.

To this end, we focused on two main questions: 1) is the increased exposure to Aa as measured by the presence of anti-LtxA-antibodies specific for RA, or also present in other forms of inflammatory arthritis? 2) can we replicate the finding that the association between HLA SE alleles and ACPA-positive RA is limited to the anti-LtxA-positive subset?

To answer these questions, we established an enzyme-linked immunosorbent assay (ELISA) against purified LtxA. The purification of LtxA was performed according to the method described by Reinholdt et al. (3), which is the same method carried out from the same clone of the strain of Aa (HK921; JP2 positive) in the same laboratory as in the manuscript by Konig et al. The activity of LtxA was confirmed in a haemolytic assay, to correspond to the normal range of freshly purified LtxA. To confirm that the presence of antibodies against LtxA was indeed a reflection of exposure to the bacterium Aa, we first tested serum samples from periodontitis-patients (free from any chronic diseases such as RA) whose subgingival lesions had been tested for the presence of Aa by culturing techniques (4). As depicted in Figure 1A, the levels of anti-LtxA-antibodies were clearly elevated in Aa-positive patients (median level (interquartile range (IQR): 8853 (5544; 14147)) versus Aa-negative patients (median level (IQR): 764 (523; 4636)) confirming the specificity of the ELISA (Mann-Whitney U-test p-value < 0.001).

Thus, we subsequently tested sera from 594 patients participating in the Leiden Early Arthritis Clinic with various different diagnoses, including RA according to the 1987 ACR criteria (5)**.** Furthermore, we also measured anti-LtxA-levels and positivity in a group of 156 healthy controls (without chronic illnesses) from the Leiden area. Figure 1B depicts the anti-LtxA-levels in these various groups and illustrates that anti-LtxA antibodies could be found in a substantial proportion of RA patients, but also in patients with other forms of arthritis. Serial dilutions of a mix of 3 strongly positive RA patients were used as a standard, and the lowest point of the linear part of the standard curve (2000 AU/ml) was defined as the cut-off. Next, we investigate whether within RA patients, anti-LtxA-antibodies were preferentially present within the ACPA-positive group, and whether there was an association with the HLA SE-alleles. As depicted in Figure 1C, neither of these associations could be found in our cohort. Furthermore, as can be seen in Table 1, the association between HLA DRB1 SE alleles and anti-CCP-positive RA was similar among RA patients positive and negative for anti-LtxA. Therefore, the effect of the HLA SE alleles appears not to be confined to the patient group positive for anti-LtxA-antibodies.

Table 1 The association of SE alleles with anti-CCP based on exposure to LtxA in patients with RA. Anti-LtxA, anti-leukotoxin antibodies as determined by ELISA; Anti-CCP, anti-cyclic citrullinated peptide antibody, cut-off for positivity >20 U; SE, HLA-DRB1 shared epitope allele

	Anti-LtxA-negative RA (n = 143)				**Anti-LtxA-positive (n = 189)**			
	SE-negative (n = 44)	**SE-positive (n = 99)**	**OR**	**P**	**SE-negative (n = 66)**	**SE-positive (n = 123)**	**OR**	**P**
Anti-CCP positivity, %	34	70	4,45	0.0001	29	68	5,33	<0.0001

In summary, in this large cohort of arthritis patients, anti-LtxA-antibodies were not specifically associated with RA, and within RA patients, there was no association with the presence of ACPA or HLA SE alleles, in contrast to the findings of Konig et al. A possible explanation for these divergent results could be that the anti-LtxA-assay as used here, may have differed from the assay in the previous publication. However, the essential constituent (LtxA) and set-up of the assay was derived from the same source. Furthermore, we performed various controls e.g. in periodontitis patients, the results of which confirmed the validity of our assay.

It is possible that differences in proportions of positive patients reported in the original study by Konig et al and in our study could be due to differences in patient population between the United States and the Netherlands. It seems likely that differences in living environment, genetic background and referral strategy exist between these two countries. However, given the fact that Aa is a prevalent micro-organism causing periodontitis in both countries, it appears unlikely that population differences can explain the contrasting findings regarding the possible role of Aa as found by Konig versus us.

Although microbial influences may well be important in the development of RA, our results do not support a key role of exposure to LtxA originating from the periodontal pathogen Aa in linking the effect of the HLA SE alleles and periodontal disease to anti-citrullinated protein autoimmunity in RA.

Whilst the hypothesis of a causal link between periodontitis/periodontitis-associated microorganisms and RA seems appealing, it is also possible that RA patients are more frequently anti-LtxA-positive simply because they have more periodontitis as is known from epidemiological studies. We hope our findings can contribute to the discussion about the origins of autoimmunity in RA, and look forward to other replication experiments regarding these intriguing observations.

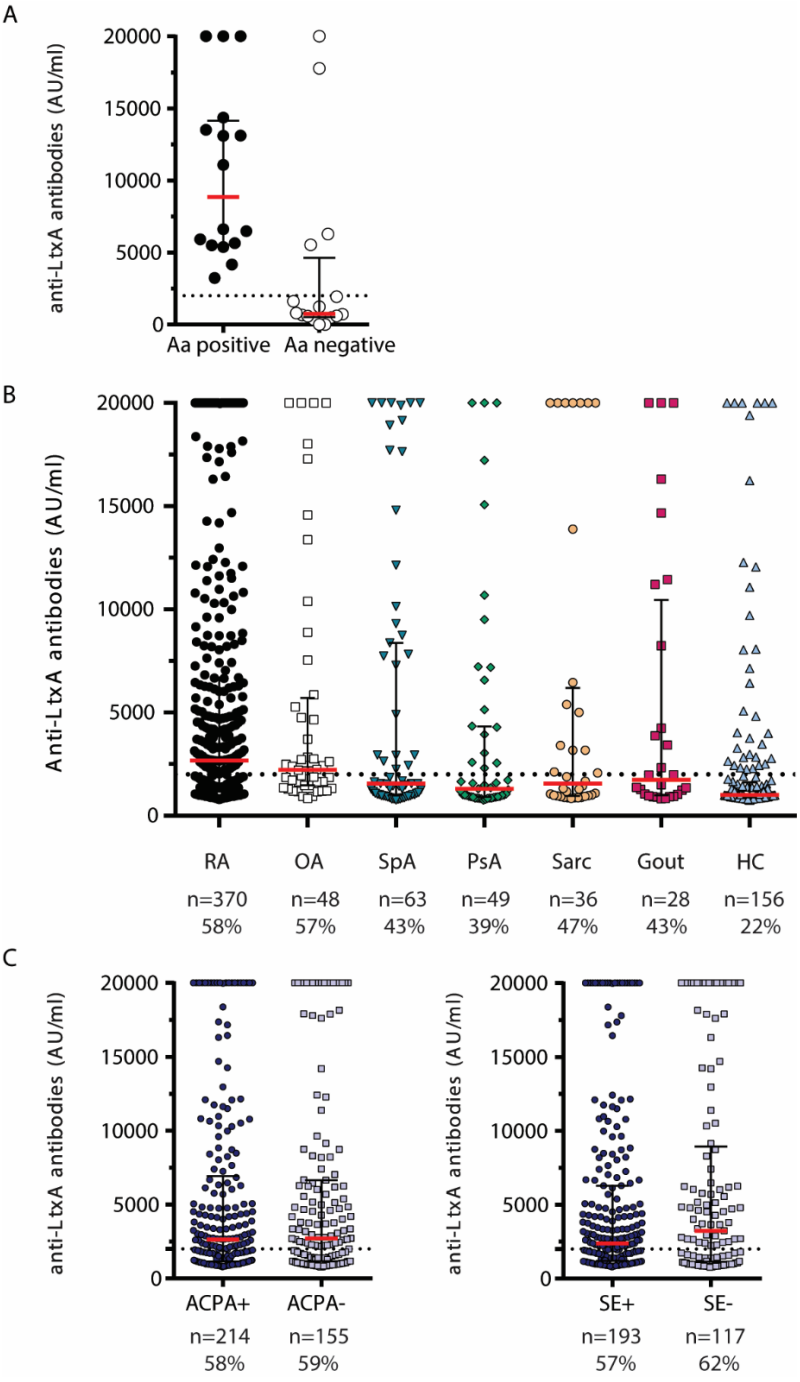
A
anti-LtxA antibodies (AU/ml)
20000
15000
10000
5000
0
Aa positive
Aa negative
B
Anti-LtxA antibodies (AU/ml)
20000
15000
10000
5000
0
RA
OA
SpA
PsA
Sarc
Gout
HC
n=370
58%
n=48
57%
n=63
43%
n=49
39%
n=36
47%
n=28
43%
n=156
22%
C
anti-LtxA antibodies (AU/ml)
20000
15000
10000
5000
0
ACPA+
ACPA-
n=214
58%
n=155
59%
anti-LtxA antibodies (AU/ml)
20000
15000
10000
5000
0
SE+
SE-
n=193
57%
n=117
62%

Figure 1. Serum antibodies to leukotoxin A. (A) Serum antibodies to LtxA were measured in periodontitis patients with and without Aa infection (n=16 per group) confirmed by culturing. (B) Distribution of anti-LtxA antibodies in sera of 594 patients suffering from early arthritis and 156 controls. Levels of anti-LtxA antibodies in the serum of each individual are shown. (C) Serum antibodies to LtxA were measured in RA patients stratified for the presence of anti-CCP2 antibodies, ACPA positive (n=214) and ACPA negative (n=155) RA patients (left panel). Presence of anti-LtxA antibodies in sera of RA patients carrying HLA-DRB1 shared epitope alleles, SE positive (n=243) and SE negative (n=117) RA patients (right panel).
LtxA Leukotxin A, RA Rheumatoid arthritis, Aa *Aggregatibacter actinomycetemcomitans*, OA Inflammatory osteoarthritis, SpA Spondylarthritis with peripheral arthritis, PsA Psoriatic arthritis, Sarc Sarcoidosis, ACPA anti-citrullinated protein antibodies, SE shared epitope. Red lines indicate the median level per group. The dashed line indicates the cut-off. The number of patients per group and percentage of patients positive according to the cut-off are shown underneath.

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