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Supplementary Materials for

Neutrophil-mediated carbamylation promotes articular damage in rheumatoid arthritis

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This PDF file includes:

Supplementary Material

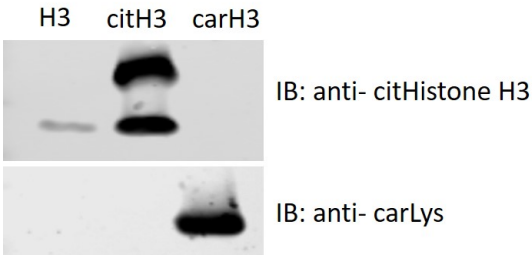

Figure S1. **Antibody specificity.** To test specificity of the antibodies used to discriminate between citrullination and carbamylation, 1 ug of recombinant histone H3 was incubated either in the absence or presence of recombinant PAD2 (for citrullination) or cyanate (for carbamylation) overnight. Western blot analysis was performed for citrullinated H3 (ab5103) or carbamylated lysine (STA-078, Cell biolabs). Representative blot of two independent experiments.

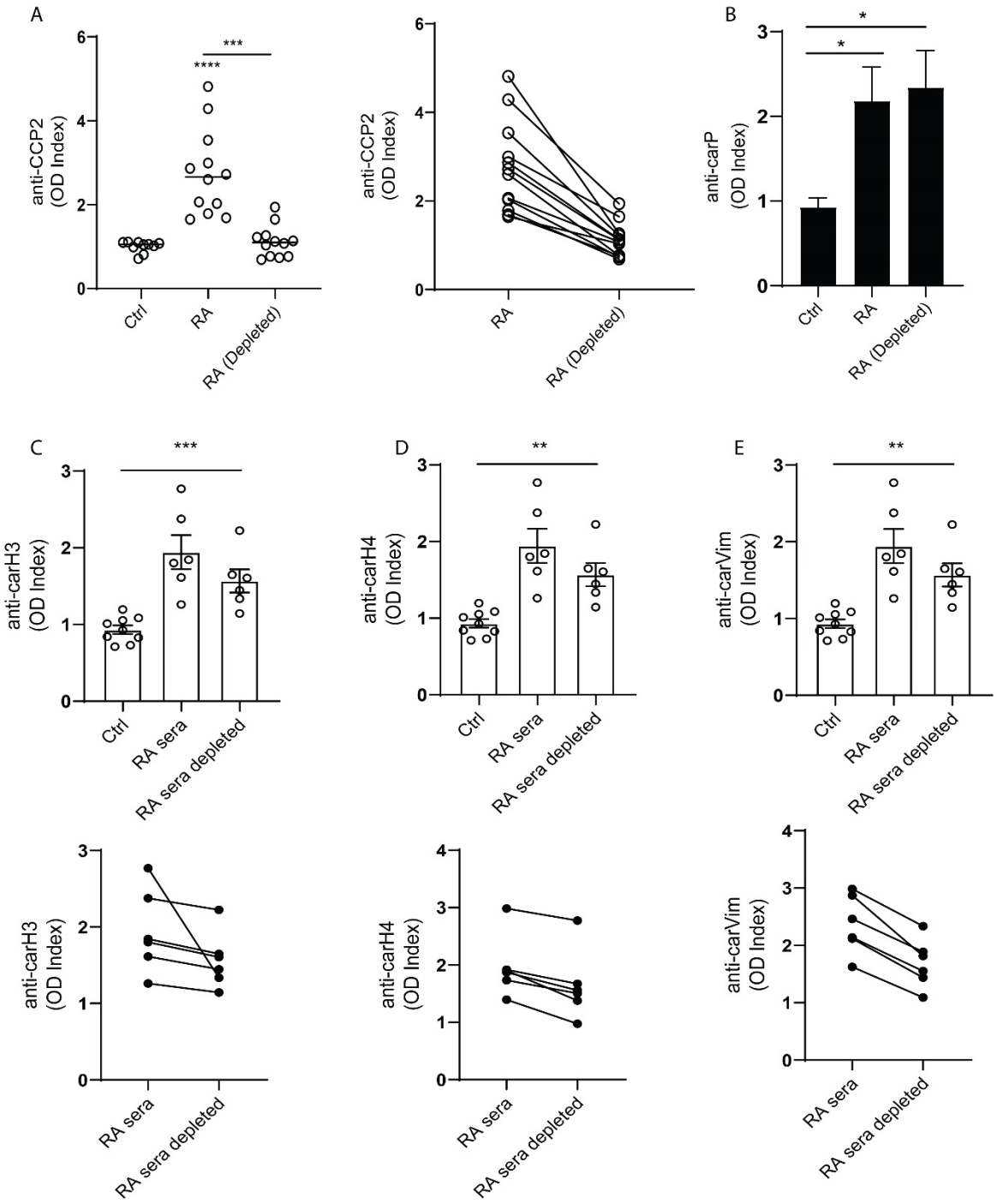

Figure S2. **Detection of carbamylated antibodies in ACPA-depleted sera from RA patients.** ACPAs were depleted from RA serum as described in Materials and Methods section. **A.** Anti-CCP2 Elisa kit was used to corroborate depletion of ACPAs in RA sera. **B** The presence of anti-CarP Abs was assessed in ACPA-depleted samples. Control (n= 9), RA (n=6) and ACPA-depleted RA (n=6) sera were used to detect the presence of anti-carbamylated histone H3 (**C**), anti-carbamylated histone H4(**D**) and anti-carbamylated

vimentin (**E**). Results are the mean +/- SEM. Mann-Whitney U- test was used. *p<0.05,**p<0.01, ***p<0.001.

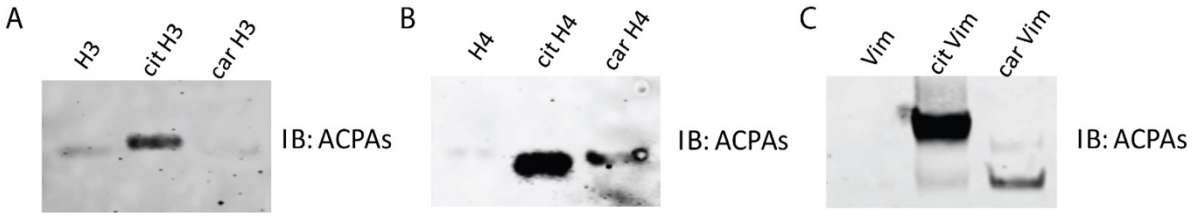

Figure S3. **Purified ACPA reactivity.** ACPAs were purified from RA sera as described in Materials and Methods and used to perform Western blot. One microgram of unmodified or *in vitro* citrullinated (cit) or carbamylated (car) (**A**) histone H3, (**B**) histone H4 and (**C**) vimentin were resolved on an SDS-PAGE gel. Western blot analysis was performed using ACPAs as primary antibodies in a dilution of 1:250 overnight at 4C. Blot was developed after 1-hour incubation with anti-human IgG secondary antibody at room temperature.

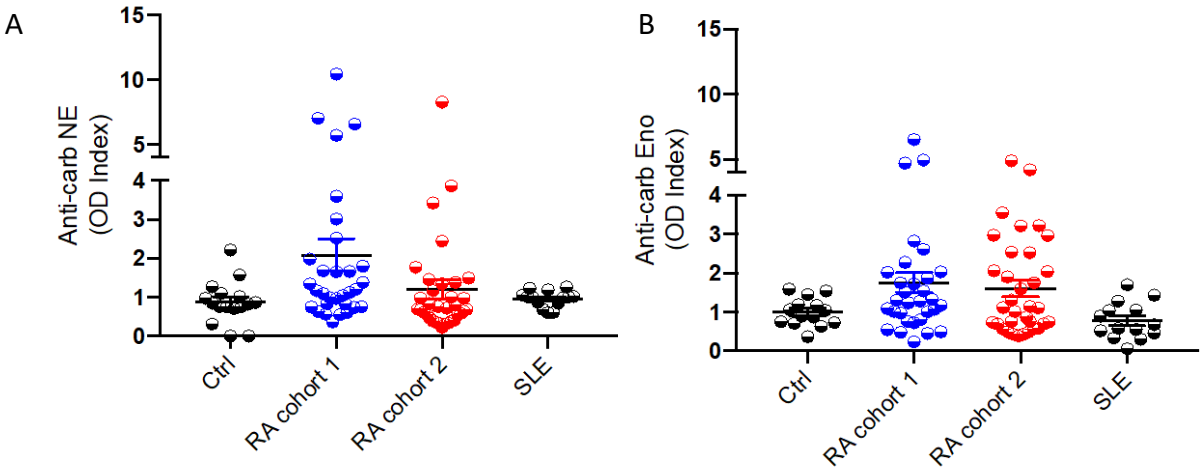

Figure S4. **Autoantibodies against carbamylated NET proteins are increased in RA**. Serum samples from healthy controls (n= 14), RA cohort #1 (blue, n=35), RA cohort #2 (red, n=34) and SLE (n=12) were analyzed for the presence of antibodies against carbamylated (**A**) neutrophil elastase (NE) and (**B**) alpha enolase (Eno). Results are the mean +/- SEM. Kruskal-Wallis test was used.

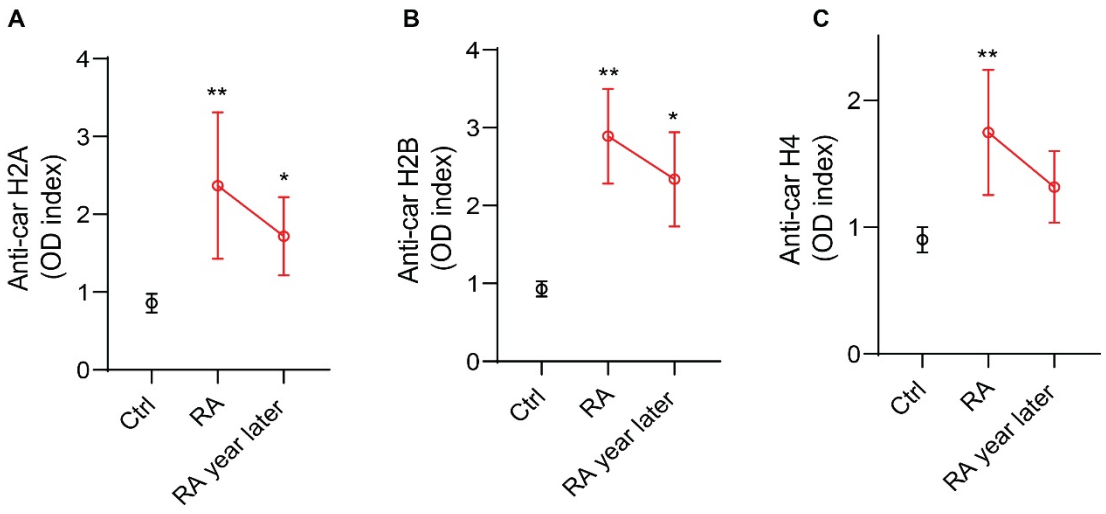

Figure S5. **Longitudinal analysis of anti-carbamylated histones in RA patients.** Serum samples from healthy controls (n= 5) and RA subjects (n=5) were analyzed longitudinally for the presence of antibodies against carbamylated (**A**) histone H2A, (**B**) histone H2B and (**C**) histone H4. Results are the mean +/- SEM. Kruskal-Wallis test was used. *p<0.05,**p<0.01.

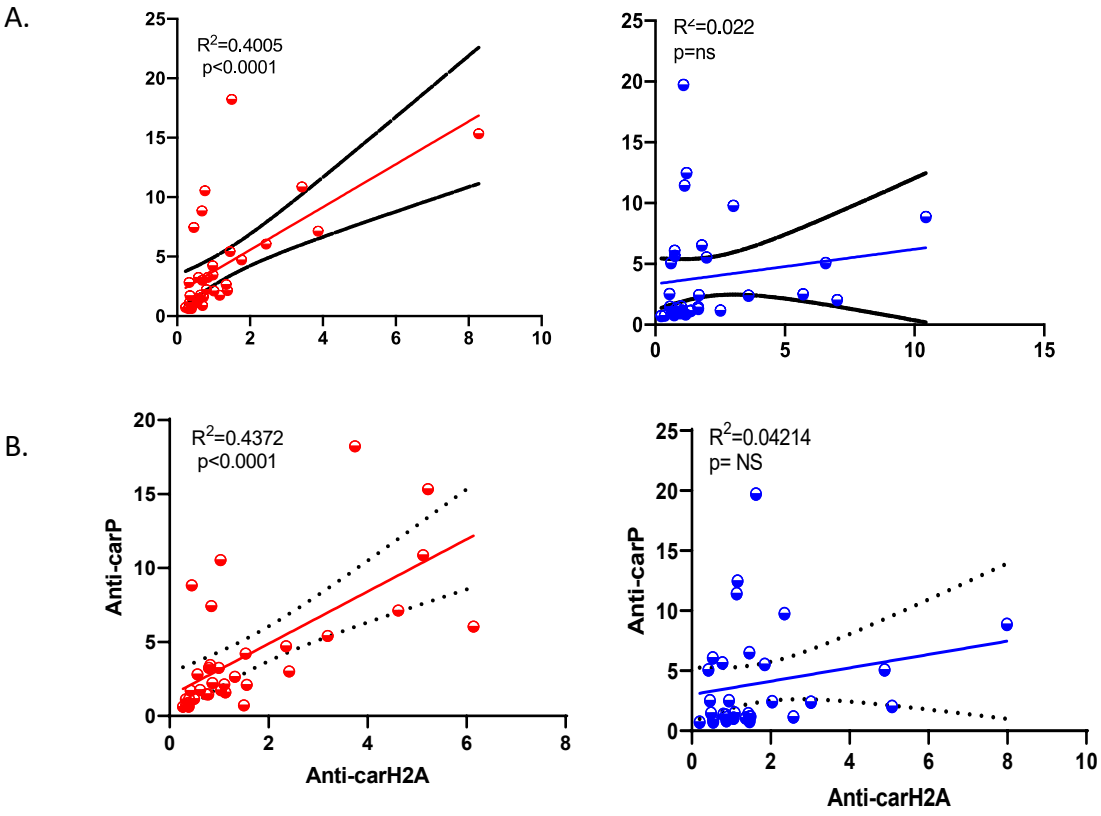

Figure S6. **Linear regression analysis of anti-CarP antibodies and carbamylated NET protein antibodies**. Correlation of anti-CarP antibodies with antibodies against carbamylated (**A**)neutrophil elastase (NE) and (**B**) histone H2A in two RA cohorts (red and blue).

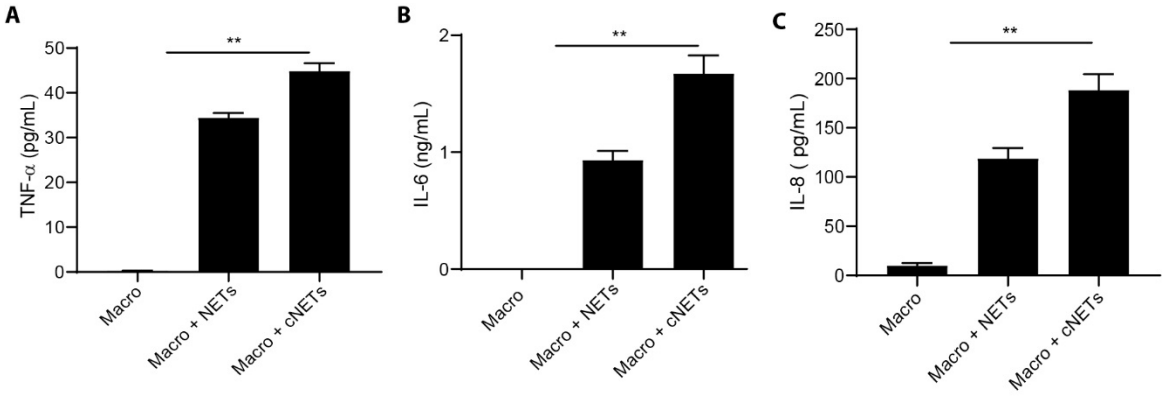

Figure S7. **Carbamylated NETs increase release of pro-inflammatory cytokines in M1 macrophages.** NETs and carbamylated NETs (cNETs) were incubated with M1 macrophages (Macro) for 48 hours. ELISA analysis was performed for the quantification of (**A**) TNF-alpha, (**B**) IL-6, (**C**) IL-8 in supernatants of M1 macrophages in the presence of NET and cNETs. Results are the mean +/- SEM of four independent experiments. Kruskal-Wallis test was used. **p<0.01.

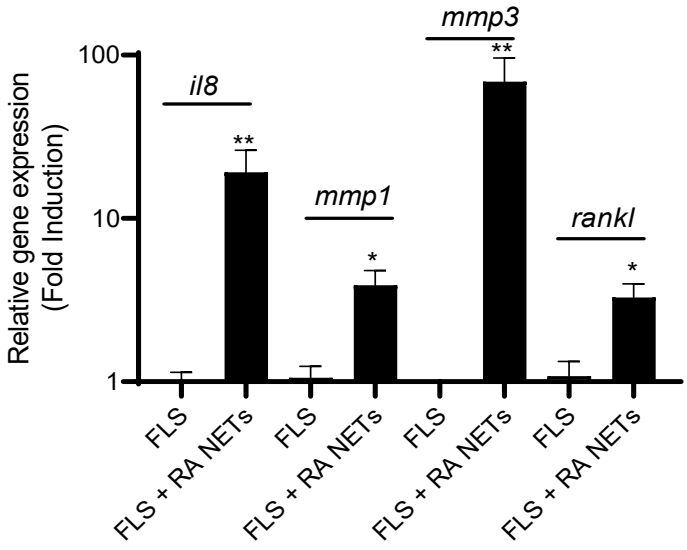

Figure S8. **Spontaneously generated RA-NETs upregulate pro-inflammatory cytokines and enzymes in FLS.** Purified RA NETs were incubated with FLS for 24 hours. Real time PCR analysis was performed to quantify the gene expression of *il8, mmp1, mmp3 and rankl*. Results are the mean +/- SEM of four independent experiments. Mann-Whitney U- test was used. *p<0.05**p<0.01.

	Cohort 1, n = 35 (IQR)	Cohort 2, n = 34 (IQR)
Age	44 (13)	47.5 (15.3)
Female	91.4%	85.3%
BMI	25.9 (4.7)	26.3 (5.9)
Disease Duration	9 (6)	5 (9.3)
RF +	85.7%	91.1%
ACPA +	91.4%	91.1%
DAS28-CRP	4.2 (1.0)	2.0 (2.3)
Smoker	2.9%	35.3%
Methotrexate	48.6%	67.7%
Prednisone > 10 mg daily	40.0%	0.0%
Biologic	25.7%	3.2%
Radiographic Erosions	71.4%	50.0%
SENS range	-	0 to 37

Table S1: **Demographic and clinical characteristics of 2 Rheumatoid Arthritis cohorts.** Continuous variables are listed as median with interquartile range (IQR). Dichotomized variables are listed by % affirmative. DAS28: Disease Activity Score. Biologic is considered active use at the time of visit. RF: Rheumatoid Factor. ACPA: Anti-citrullinated protein antibody. Radiographic erosion score defined using Simple erosion narrowing score (SENS) for cohort 2.