Autoantibodies against unmodified and citrullinated human endogenous retrovirus K envelope protein in rheumatoid arthritis patients

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Running title: Anti-citrullinated HERV-K in RA

Abstract

Objective. Autoantibodies against proteins encoded by human endogenous retrovirus K (HERV-K) have been reported in patients with rheumatoid arthritis (RA), but their relevance, if any, has remained unresolved. We revisited this question and tested if such autoantibodies may react with citrullinated epitopes on the envelope (Env) protein of HERV-K.

Methods. Immunoblotting and ELISAs were conducted with unmodified Env protein and with Env citrullinated by protein arginine deiminase (PAD) 4. Sera from 100 RA patients, plasma from 32 juvenile idiopathic arthritis (JIA) patients, and healthy adult and pediatric controls were included. Antibody reactivity was evaluated for correlations with clinical and laboratory parameters of the patients.

Results. We replicated and expanded upon published data that patients with RA or JIA have autoantibodies against HERV-K Env, some with high titers. Anti-HERV-K antibodies correlated with cigarette smoking and with circulating DNA-myeloperoxidase complexes indicative of non-apoptotic neutrophil cell death. Furthermore, most of the RA patients, but not JIA patients, had autoantibodies that reacted more strongly with Env that was citrullinated by PAD4. These anti-citrullinated Env autoantibodies correlated with seropositivity and tended to be higher in patients with erosive disease.

Conclusions. Our data suggest that anti-HERV-K immunity is elevated in RA and JIA and may have a connection with pathogenic protein citrullination in RA.

Background

A well-documented, but, at the time, puzzling discovery in the 1990s was that serum immunoglobulins from patients with RA or other autoimmune diseases (1-4) often reacted with Human Immunodeficiency Virus (HIV) proteins, *e.g.*, p24 of the HIV capsid, even if these patients had never encountered the virus. Such HIV-reactive antibodies were found in exceedingly few healthy subjects, but reportedly in up to 60% of RA patients. A likely answer to this conundrum was provided by the subsequent discovery (5) that members of a family of HIV-related endogenous retroviruses in the human genome, particularly HERV-K (6), are transcriptionally activated in some RA patients (7, 8). This raised the possibility that HIV-reactive antibodies in patients are, in fact, antibodies against HERV-K proteins that have a sufficient degree of sequence homology with HIV proteins. Indeed, two papers (9, 10) reported that 19% of RA patients have antibodies against an epitope in the HERV-K envelope protein (amino acids 19-37) and HERV-K gag, respectively.

A DNA copy of the RNA genome of HERV-K first entered our ancestral early hominid genome 32-44 million years ago (11) and represents the only HERV family that has continued to infect our germline until as recently as 150,000 years ago (12), perhaps even more recently, resulting in over 120 HERV-K provirus loci, some of which show insertional polymorphisms (*i.e.* only some people have them)(13-15) as well as polymorphic deletions (16). The most recent human insertions of the HERV-K subfamily termed HML-2 (Human MMTV-like 2), *e.g.*, HERV-K113 on chromosome 19p12b (17), are also intact enough to produce virions (18), albeit with poor infectivity. Another seemingly intact and young HERV-K provirus is located at Xq21.33 in approximately 2% of people, most of whom are of African ancestry (14). These youngest loci are transcriptionally silent in healthy individuals, but can be activated under certain circumstances, *e.g.*, during very early embryonic development (19), in malignancies of the breast (20) and prostate (21), and in HIV

infected individuals (22-26). Increased levels of HERV-K transcripts have also been detected in RA blood and synovial tissue (8, 27).

We sought to test the notion that reactivated HERV-K might contribute to the pathogenesis of RA by making two tentative assumptions: First, that reactivation of the youngest and most intact HERV-K loci would be more likely to cause immune pathology than expression of older and more 'domesticated' loci with frame-shifts, point-mutations, and stop codons. Second, that 'parasitic' genomic elements like HERV-K proviruses that are suppressed by DNA methylation and other epigenetic mechanisms, likely are silent during the development of T and B cell antigen receptor repertoires in early life, resulting in weak immunological tolerance against the proteins that they encode. If so, aberrant expression of these proteins later in life would likely provoke both cellular and humoral immunity (28). We report that patients with RA indeed have autoantibodies reacting with Env and, particularly well, with citrullinated Env.

Methods

Proteins and antibodies

The extracellular portion of the HERV-K108 Env (Fig. 1A) was purchased from Abcam (ab238358). cDNAs encoding the SU and TM portions of the Env protein of HERV-K Xq21.33 (Fig. 1A), both with an N-terminal 6xHis-tag, were designed with codons optimized for prokaryotic expression and synthesized by Twist Biosciences in the pET28 expression plasmid. The proteins were expressed in transformed *E. coli* and purified by Ni-agarose (Olympic Protein Technologies LLC, Seattle, WA). The SU protein was expressed and purified on a larger scale.

Human subjects

Sera from RA patients (n=100) and healthy controls (n=40) were obtained from the UW Rheumatology Biorepository and kept at -20°C until use. All patients met American College of Rheumatology criteria for RA. The patient characteristics and clinical and serological parameters of this cohort were described recently (29). The average age was 52.6 ± 14.0 years (females 52.1 ± 14.3 years; n=61, males 54.6 ± 13.4 years; n=18, not recorded n=21). Twelve of the patients had high disease activity (CDAI >22), 15 were classified as moderate (CDAI >10–22), and 10 had mild disease (CDAI >2.8–10). Fifty-nine patients were positive for anti-citrullinated protein antibodies (ACPA). Seropositivity was defined as positivity for both ACPA and rheumatoid factor. Healthy individuals (n=40) of similar average age were used as controls. Approval for this study was obtained from the University of Washington Institutional Review Board (STUDY00006196) and informed written consent was obtained from all participants according to the Declaration of Helsinki.

Plasma from a cohort of juvenile idiopathic arthritis (JIA) patients (n=32) were from Seattle Children's Hospital. The average age of this cohort was 13.9 ± 2.2 years. Twenty-four of them had active disease with an average of 3.3 involved joints and 13 patients had oligoarticular, 19 polyarticular JIA; 2 patients in the latter category were ACPA-positive. Healthy children (n=18) with of similar average age (12.3 ± 4.4 years) were used as controls. The study was approved by The Seattle Children's Research Hospital Human Subjects Committee (PIROSTUDY14045). Informed written consent was obtained from the parents or guardians of all participants according to the Declaration of Helsinki.

Immunoblotting

100 ng of HERV-K108 Env protein per lane was resolved by SDS gel electrophoresis, transferred to nitrocellulose membranes, and cut into 12-15 strips. The membrane strips were immunoblotted

with 1:100 diluted serum from patients or healthy controls and developed by horse radish peroxidase-conjugated anti-human IgG and enhanced chemiluminescence.

Enzyme-Linked Immunosorbent Assay (ELISA)

Purified HERV-K108 Env or HERV-K_Xq21.33 Env-SU protein was adsorbed onto 96-well polystyrene plates at 50 ng/well in 0.1 M carbonate (pH 9.6) buffer overnight, washed in phosphate-buffered saline with Tween, and blocked in 2% bovine serum albumin (BSA) in phosphate-buffered saline for 2 h. Control wells without Env-SU were also included for each patient. Patient, or healthy control, serum was added at 0.03% in blocking buffer for overnight incubation at 4°C, washed extensively and then incubated with 1:2,000 dilution of horse radish peroxidase-conjugated anti-human IgG. The reaction was then washed, and developed with 3,3',5,5'-tetramethylbenzidine (TMB, BioLegend), with the color reaction terminated with 2N sulfuric acid, and the absorbance measured at 450 nm using a plate reader (Synergy, BioTek). Background values of BSA-blocked wells without Env-SU were subtracted from values obtained in the presence of immobilized Env-SU. A dilution series of a specifically selected and highly reactive RA patient was included on every ELISA plate and used to normalize all data points. Measurements were made in triplicates, which were within 3.3 % (average 1.8%) of each other. Independent repeat ELISA data points were at most 19.4% different and generally much less.

Competition ELISAs

To compare the autoantibodies against Env-SU reported here with the autoantibodies recognizing a dominant epitope peptide (VWVPGPTDDRCPAKPEEEG) from Env reported by Mameli and co-workers (9), we performed two types of competition ELISAs: first, we coated wells with 50 ng of Env-SU, as described above, but added 1 μM of the peptide to half the replicates of the patient

sera 30 min before adding them to the ELISA plates. Second, we coated wells with the peptide and added 50ng Env-SU to half the serum samples 30 min before adding them to the plates.

ELISA for citrullinated HERV-K Env

Autoantibodies reactive with citrullinated HERV-K_Xq21.33 Env-SU protein were measured by immobilizing Env on ELISA plates as above, washing, and blocking the plates with 1.5 µg/ml of poly(Glu, Lys, Tyr) (6:3:1), which lacks arginine residues and therefore cannot be citrullinated. We have previously shown that this protein can block non-specific binding as well as BSA (29). 250 ng of recombinant PAD4 in 100µl Tris-buffered saline, pH 7.7, 5 mM $CaCl_2$ and 1 mM DTT was added and incubated at 37°C for 1 h. After extensive washing, the plates were incubated with patient sera, washed, and developed as described above. Data were normalized using the same selected RA patient as in the ELISA for anti-Env. Controls included wells without PAD4, but treated the same way, as well as wells without Env-SU with or without PAD4 treatment. Wells without Env-SU, but treated with PAD4, gave as low background as untreated wells, indicating that residual PAD4 recognized by anti-PAD4 autoantibodies did not contribute to our results. As positive controls, fibrinogen and histone H3 were included in separate wells on the same ELISA plates. Reactivity against these proteins was low before and high after treatment with PAD4 (not shown). RA patient-derived monoclonal ACPA 1325:04C03 (30) was used to demonstrate that Env was citrullinated in our ELISAs.

ELISA for circulating DNA-neutrophil myeloperoxidase complexes

DNA-myeloperoxidase (MPO) complexes (including neutrophil extracellular traps) were quantitated as before (31, 32). Briefly, microtiter plates were coated with a mouse monoclonal anti-MPO antibody (4 µg/ml, Biorad, clone 4A4), and then blocked with BSA. Plasma or serum (10%) was added and incubated overnight at 4°C. Anti-dsDNA-HRP (diluted 1:100, Roche

Diagnostic) was added for 2 hours. The reaction was developed and absorbance was measured at 450 nm. Data were normalized using a standard curve with 1 U/ml equaling neutrophil extracellular traps released by 10,000 neutrophils.

Statistics

For non-paired sample sets with non-Gaussian distribution, Mann-Whitney U test and Spearman's correlation test were used, as applicable. For paired sample sets, Wilcoxon matched-pairs signed rank test was used. In some analyses, logistic regression analysis was used for dichotomized variables. As a cut-off for positivity, the mean plus 2 standard deviations of the healthy controls was used. GraphPad Prism and IBM SPSS were used for the analyses. All analyses were considered statistically significant at $p<0.05$.

Results

Recognition of HERV-K Env by RA patient autoantibodies.

The *env* gene of HERV-K108 encodes an 80-kDa type I transmembrane protein with a long extracellular N-terminal portion, a transmembrane α-helix, and a short cytoplasmic domain (**Fig. 1A**). We first used a commercially available Env protein from HERV-K108 consisting of its first 543 amino acid residues (the entire extracellular portion) to determine if RA patient serum contains IgG autoantibodies reactive with this protein. The protein was resolved on SDS gels and immunoblotted with a 1:100 dilutions of sera from 73 RA patients, which revealed that 47 patients indeed had such autoantibodies, some very strongly reacting (n=16) and some modestly reactive (n=31) (**Fig. 1B**). In contrast, most healthy volunteers had no such antibodies, or, at most, weak reactivity (**Fig. 1B**, lanes 11-14).

The purified HERV-K108 Env protein was also used to optimize ELISA conditions to allow for a better quantitation of anti-Env autoantibodies, which were found to be higher in RA patients (n=100) than in healthy controls (n=40), albeit with a considerable overlap (**Fig. 1C**). The difference was statistically significant by the Mann-Whitney U test (p=0.001). Using the mean plus 2 standard deviations (128U/ml) as a cutoff, 18% of the RA patients were positive.

HERV-K proviruses are classified based on the presence (type 1) or absence (type 2) of a 292-bp deletion, which affects primary transcript splicing resulting in a different N-terminus of the respective Env proteins (**Fig. 2A**). During the assembly of HERV-K virions, the Env protein undergoes a proteolytic cleavage step, resulting in two parts, termed SU (for surface) and TM (for transmembrane), which remain associated via a disulfide bond. To determine which of the two proteins patient antibodies recognize, we expressed the sequence of the SU protein shared by both type 1 and type 2 Env proteins (calculated molecular weight 42 kDa) and the 20-kDa extracellular portion of the TM protein (**Fig. 2B**) with 6xHis tags in *E. coli* and immunoblotted bacterial lysates with patient serum, which revealed a strong reactivity with the SU protein (**Fig. 2C, lower panel**), but none with the TM protein (**Fig. 2D, lower panel**). Based on this result, we focused on the SU protein and purified milligrams of it via Ni-agarose affinity chromatography. The purified Env-SU protein was strongly detected by RA serum (**Fig. 2E**).

Anti-Env-SU autoantibodies in adult RA patients and in children with arthritis.

ELISAs with the Env-SU protein showed that RA patients had elevated IgG antibodies against this part of the Env protein (p=0.0018; **Fig. 3A**). with a similar pattern as seen with the whole extracellular part of Env (Fig. 1). Although the normalized values cannot be directly compared, the overall pattern appeared a bit lower, perhaps due to differences in accessibility of some epitopes between less denatured plastic-bound and fully denatured nitrocellulose-bound proteins. Another

possibility is that the 5 amino acid residues that differ between the commercial HERV-K108 and our SU construct based on HERV-K_Xq21.33 result in some differences in autoantibody reactivity. There may also have been a small number of patients with autoantibodies principally against the TM portion of Env. Indeed, a minority of patients had autoantibodies that recognized Env-TM as assessed by immunoblots (data not shown).

A cohort of 32 patients diagnosed with JIA were also assessed for anti-Env-SU autoantibodies (**Fig. 2A**). The JIA patients had marginally elevated anti-Env reactivity, which was not statistically different from the pediatric healthy controls. With the mean plus 2 standard deviations of the pediatric controls as a cut-off, only 6 of the JIA patients were positive. Together, these data indicate that a subset of patients with RA or JIA have IgG autoantibodies that can recognize denatured, non-glycosylated HERV-K Env-SU.

Anti-Env correlate with patient gender, ACPA status, smoking, and *in vivo* neutrophil cell death.

Anti-Env reactivity was higher in female than in male RA patients (p=0.0075)(**Fig. 3B**), in ACPA-positive patients, albeit not statistically significant (not shown), in active cigarette smokers (p=0.0004) (**Fig. 3C**), and in patients with higher amounts of serum DNA-myeloperoxidase complexes (p<0.0001) (**Fig. 3D**), a sign of ongoing death of neutrophils by extrusion of extracellular traps (NETs). There were also trends towards association with disease activity index, presence of rheumatoid factor, and erythrocyte sedimentation rate, but these did not reach statistical significance.

Comparison with previously reported anti-Env autoantibodies

Mameli and co-workers (9) reported that 19% of RA patients have IgG autoantibodies that recognize a synthetic peptide representing a computationally predicted epitope in the SU portion of the HERV-K Env protein. We used this peptide in competition experiments and found that it was able to reduce reactivity of RA serum IgG autoantibodies with Env-SU by 0-20% in a subset of 16 patients (**Fig. 3E**). Conversely, antibodies reactive with the immobilized peptide were competed out by Env-SU (which contains this sequence with only one amino acid difference) to 80% in one and over 90% in the other patients (**Fig. 3F**). These results support the notion that some anti-Env autoantibodies indeed recognize this epitope, but that they represent a minority of the anti-Env autoantibody repertoire.

RA patients have higher autoantibody reactivity against citrullinated HERV-K Env.

Since RA autoantibodies often recognize citrullinated epitopes, we tested whether citrullinated Env-SU would be differentially recognized by RA patient autoantibodies. To this end, we immobilized Env-SU on the ELISA plates, blocked all remaining protein-binding capacity with a protein that cannot be citrullinated, the 20-50 kDa poly(Glu, Lys, Tyr) (6:3:1), and treated the plates with or without PAD4 for 60 min, followed by extensive washing. To directly demonstrate that immobilized Env was indeed citrullinated by soluble PAD4, we used a broadly reactive RA patient monoclonal ACPA (30), which recognized Env in ELISA wells treated with PAD4, but not untreated Env, and not blocked wells without Env treated with PAD4 (**Fig. 4F**). The subsequent ELISA revealed that patient IgG autoantibodies reacted considerably better with citrullinated Env-SU than with untreated Env-SU in most patients (**Fig. 4A**). Interestingly, serum from healthy donors also contained IgGs that reacted better with citrullinated Env-SU than with untreated Env-SU, but the titers were mostly much lower (**Fig. 4B**). There were two patterns among the patients: those with clearly higher anti-cit-Env reactivity compared to their anti-Env-SU reactivity,

suggesting that most of their autoantibodies only recognized citrullinated epitopes, and patients with similar reactivity against unmodified and citrullinated Env-SU (**Fig. 4A and 4C**), suggesting that the antibodies mostly recognized epitopes that were not citrullinated. Stratifying the patients by seropositivity versus seronegativity, revealed that the titers were increased in both subgroups, but that the difference became statistically significant (p=0.03) only in the citrullinated Env-SU group (**Fig. 4D**).

In contrast to the adult RA patients, only two children with JIA showed any increase in reactivity against Env when it was citrullinated (**Fig. 4E**). In most of them, the reactivity was essentially unchanged and in two it was decreased. As in the adults, the low reactivity in healthy children also did not change significantly upon citrullination of Env (not shown).

As positive controls to ensure that PAD4 was catalytically active, we carried out ELISAs in which wells were coated with histone H3 (**Fig. 4F**) or fibrinogen (not shown) instead of Env. Serum IgGs from four ACPA-positive RA patients recognized these well-known autoantibody targets particularly well after treatment with PAD4.

Clinical correlations in the full RA cohort.

Based on the data described above, we decided to run ELISAs on the entire RA cohort for which clinical parameters, laboratory measures, and treatment history exists (29). As shown in **Fig. 5A**, the difference between healthy subjects and RA patients was statistically significant ($p<0.0001$ by the Mann-Whitney U test). Using the average plus 2 standard deviations of the HC data set (66.7 U/ml) as a cut-off, 55% of the RA patients were positive. Furthermore, the difference between ACPA-positive and -negative patients was significant (p=0.0007) (**Fig. 5B**), as was the differences between rheumatoid factor-positive and -negative (p=0.03) (**Fig. 5C**). **Fig. 5D** shows a weak

correlation between anti-cit-Env titers and ACPA titers (CCP test) reported in patients' medical records, suggesting that anti-cit-Env reactivity does not solely represent cross-reacting non-selective ACPA. Lastly, anti-cit-Env reactivity was also somewhat higher in patients with joint erosions than in those without, but this did not quite reach statistical significance (**Fig. 5E**).

Discussion

The molecular mechanisms that underpin the pathogenesis of RA remain incompletely understood. The presence of ACPA in 70-80% of patients (33) is a unique feature of RA (34, 35) and commonly used in its diagnosis. A pathogenic role of autoimmunity against citrullinated epitopes is also suggested by the genetic association of RA with polymorphisms in the genes for two of the citrullinating enzymes, protein arginine deiminase (PAD) 2 and 4 (36-39). However, it still remains unclear how and when these two enzymes cross the line between physiological citrullination and the quantitatively or qualitatively abnormal modification of self-proteins to create immunoreactive epitopes that drive an autoimmune response (40-42).

Our finding that many RA patients have IgG autoantibodies that recognize the envelope protein of HERV-K suggests that one or several of the HERV-K loci that encode this protein are, or recently were, transcriptionally active and translated into immunogenic protein in the antibody-positive patients. In this context, it is interesting to note that children with JIA had only marginally elevated anti-Env reactivity compared to adult RA patients. However, nearly all but two of the JIA patients were ACPA-negative and none of them had higher reactivity against citrullinated Env. These findings suggest that anti-HERV-K immunity may be an early feature of autoimmune arthritis, while anti-cit-Env autoantibodies are more closely associated with ACPA-positive RA.

Clearly, humoral and cellular autoimmunity against HERV-K proteins, as it occurs in HIV infected individuals and in patients with breast cancer (26, 43, 44), is not sufficient by itself to cause RA. At a minimum, other events and factors must participate. For example, the right MHC alleles may be needed for such autoimmunity to result in ACPA-positive RA. Many other predisposing genetic variants, *e.g.,* of *PTPN22* (45), may also need to be present for anti-HERV-K immunity to tip the balance towards RA pathogenesis. The magnitude of HERV-K expression, its duration and tissue location(s), and the cell surface exposure of HERV-K proteins likely affect the nature of this immunity. Arguably, autoimmunity against HERV-K expressed in a breast cancer cell, or in an HIV-infected T lymphocyte, is likely to be beneficial to the patient. In contrast, autoimmunity against HERV-K derived proteins in other tissues, for example the synovium, are more likely to be detrimental.

In our study, autoantibodies that specifically recognize citrullinated Env correlated more clearly with ACPA and seropositivity, and showed a trend towards correlation with more aggressive diseases. However, ACPA titers did not correlate closely with anti-cit-Env autoantibodies and some patients with the latter were ACPA-negative, suggesting that anti-cit-Env may not simply be broadly reactive ACPA, although we cannot rule out this possibility. A comparison of known citrullination sites with the sequence surrounding the 21 arginine residues in Env-SU only revealed one instance of Arg-Gly and one of Gly-Arg, which are seen in some other citrullinated proteins. While the latter would be predicted to be recognized by RA patient-derived monoclonal ACPA 1325:04C03 (30), future work will determine if this Arg residue is indeed citrullinated. At present, it also remains unclear if and how transmembrane Env may become citrullinated in RA patients, but a plausible mechanism may involve the surface exposure of catalytically active PAD4 and secretion of active PAD2 by human neutrophils (46). Another viral protein, Epstein-Barr Virus

Nuclear Antigen 1, is also known to be citrullinated and then recognized by ACPA in RA patients (47).

An intriguing feature of HERV-K as a potential player in RA, which is a female-dominated disease with a 4:1 female/male ratio, is the strong impact of estrogen and progesterone on its expression, particularly in breast cancer cells (48, 49). Indeed, we observed somewhat higher anti-Env reactivity in female RA patients compared to males (**Fig. 3B**). The correlation between anti-Env autoantibodies and smoking (**Fig. 3D**) could also reflect the increased expression of HERV-K by cigarette smoking (50, 51). Smoking is also a well-recognized risk factor for RA.

The presence of autoantibodies against citrullinated Env may implicate another potential mechanism by which autoimmunity against cit-Env may promote RA development, namely molecular mimicry. In this 'modified molecular mimicry' case, citrullinated epitopes in Env may have sufficient sequence similarity to citrullinated epitopes in other proteins for anti-cit-Env autoantibodies to cross-react with these self-proteins. This possibility should be explored.

We also note that autoantibodies against HERV-K envelope, either unmodified or citrullinated, could serve as a biomarker for more aggressive disease. However, demonstrating their utility in this respect will require testing in larger, and ideally longitudinal, studies. We also believe that a deeper understanding of the molecular events that lead to these autoantibodies and their possible involvement in initiating, perpetuating, or shaping RA will be required to uncover their true value as biomarkers in clinical practice.

Conclusions

In this paper we extend prior knowledge of autoantibodies in arthritis patients against the envelope protein of the HERV-K (HML-2) endogenous retrovirus family in several important ways: using

proteins encompassing most of the Env protein, rather than selected peptides, and both immunoblots and ELISA, we find that a portion of RA patients are positive, but that no more than 20% of the anti-Env autoantibodies recognize the previously reported epitope. Even more importantly, we find that many RA patients have higher titers of autoantibodies for Env when it is first citrullinated. Together, these findings raise the possibility that expression of endogenous retroviral proteins provoke a citrullination-dependent immune response that may be important in RA pathogenesis.

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FIGURE LEGENDS

Fig. 1. RA patient autoantibodies recognize HERV-K envelope protein. A, Schematic representation of the HERV-K Env protein and the used extracellular portion of Env from HERV-K108. **B,** representative strip blots of HERV-K108 Env protein with 1:100 dilutions of sera from 10 RA patient and 4 healthy volunteers. **C,** Quantitation of IgG autoantibodies against HERV-K108 Env (FL-Env) by ELISA in RA patients (n=100) and healthy volunteers (HC; n=40). The horizontal line in the graphs represents the median of each data set. Statistical significance was determined by the Mann-Whitney U test.

Fig. 2. RA patient autoantibodies recognize the SU portion of HERV-K Env. A, Schematic representation of type 1 and type 2 Env proteins. **B,** The used recombinant SU and extracellular portion of TM (TMext) proteins from HERV-K_Xq21.33. **C,** *E. coli* lysates expressing the SU portion of HERV-K Env protein immunoblotted with anti-6xHis tag antibody (upper panel) or RA patient serum (lower panel). **D,** *E. coli* lysates expressing the extracellular region of the TM portion of HERV-K Env immunoblotted with anti-6xHis tag antibody (upper panel) or RA patient serum (lower panel). **E,** Immunoblot of 1 µg (lane 1), 500 ng (lane 2) or 0 ng (lane 3) of purified HERV-K Env-SU with RA patient serum. The weaker lower band in lane 1 is a fragment of SU.

Fig. 3. ELISA for RA patient autoantibodies against the SU portion of HERV-K Env. A, Quantitation of IgG autoantibodies against Env-SU by ELISA in HC, adult RA patients (n=53), pediatric healthy controls (pHC; n=18), and JIA patients (n=32). **B,** Distribution of anti-Env-SU autoantibodies in RA patients (n=84) by gender (males n=23, females n=61). **C,** current smokers (n=6) have higher anti-Env-SU autoantibodies than RA patients who are not currently smokers (n=32). **D,** correlation between anti-Env-SU autoantibodies and circulating DNA-MPO complexes

indicative of recent non-apoptotic neutrophil death (n=38). **E,** Anti-Env-SU reactivity in the absence or presence of 1 μM of the Env peptide VWVPGPTDDRCPAKPEEEG (n=16). **F,** IgG antibodies reactive with the same peptide in the absence or presence of 50 ng of Env-SU (n=16). Statistical significance was determined by the Mann-Whitney U test and Spearman's correlation test (panel D), and the Wilcoxon matched-pairs signed rank test (panels E and F).

Fig. 4. Many RA patients have higher titers of autoantibodies recognizing citrullinated Env protein compared to unmodified Env. A, Titers in RA patients (n=40) against HERV-K Env treated with buffer alone (PAD-) or with PAD4 for 1 h at 37°C (PAD+). Each individual patient is connected with a line between the two data points. **B,** Titers in healthy subjects (n=7) against HERV-K Env treated with buffer alone (PAD-) or with PAD4 for 1 h at 37°C (PAD+). Each individual subject is connected with a line between the two data points. **C,** Relation between autoantibodies against unmodified (x-axis) and citrullinated (y-axis) Env in each RA patient. **D,** segregation by seronegativity (n=5) versus -positivity (n=24) of autoantibodies against unmodified (PAD-) or citrullinated (PAD+) Env. The horizontal line in the graphs represents the median of each data set. **E,** Titers in JIA patients (n=32) against Env treated with buffer alone (PAD-) or with PAD4 (PAD+). **F,** ELISA with detection by the patient-derived monoclonal ACPA 1325:04C03 of wells coated with Env, or only blocked, and treated with PAD4 (black bars) or citrullination buffer alone (gray bars). Statistical significance was calculated with the Wilcoxon matched-pairs signed rank test (panels A and B), Spearman's correlation test (panel C), and Mann-Whitney U test (D).

Fig. 5. Autoantibodies against citrullinated Env in the full RA cohort. A, Titers of autoantibodies against citrullinated Env in HC (n=40) and the full cohort of RA patients (n=100). The average plus two standard deviations of the HC data set is indicated by a dotted line. **B,**

Segregation of anti-citrullinated Env titers by ACPA status (positive n=58, negative n=18). **C,** Correlation of anti-citrullinated Env titers with rheumatoid factor (RF) status (positive n=56, negative n=21). **D,** Correlation between anti-citrullinated Env titers (y-axis) and ACPA titer reported in patient records. **E,** Anti-cit-Env antibody titers in patients with (n=38) or without (n=37) radiographic erosions. Note that the difference does not reach statistical significance. The horizontal line in the graphs represents the median. Statistical significance was calculated using the Mann-Whitney U test.

Fig. 1

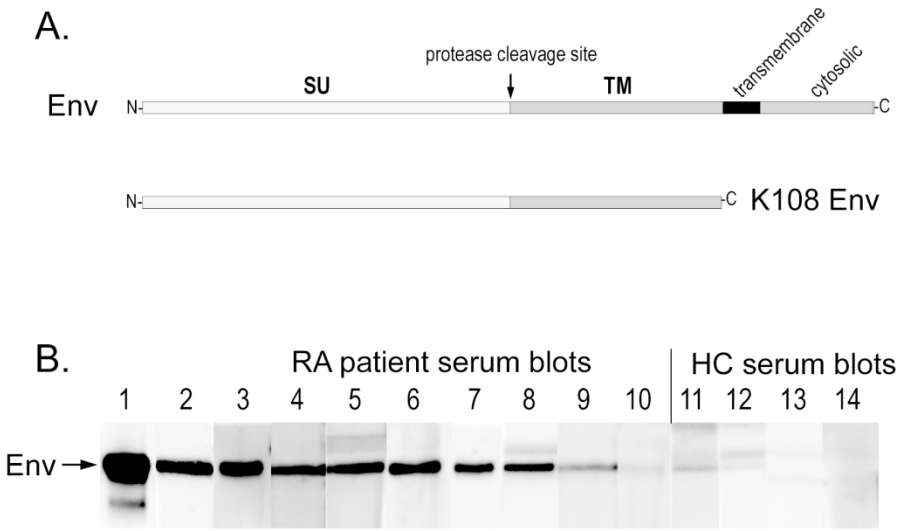

Figure 1

Fig. 2

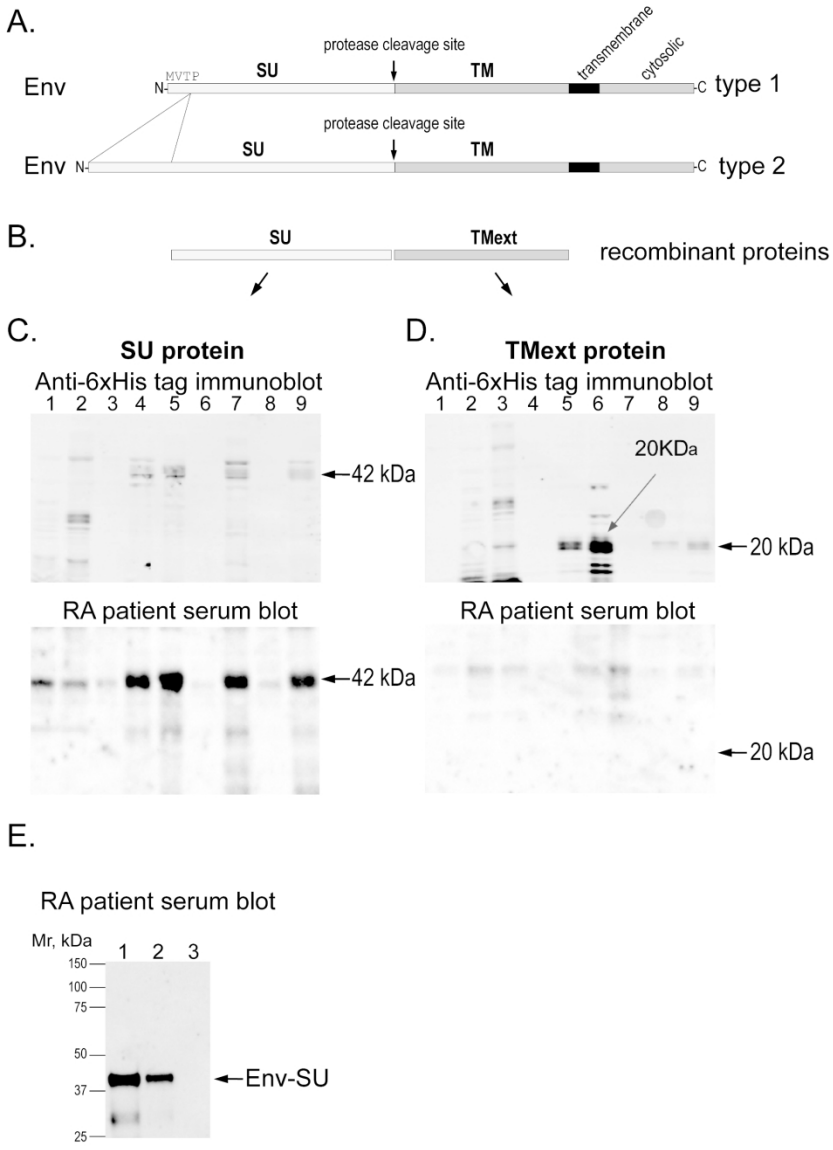

Figure 2

Fig. 3

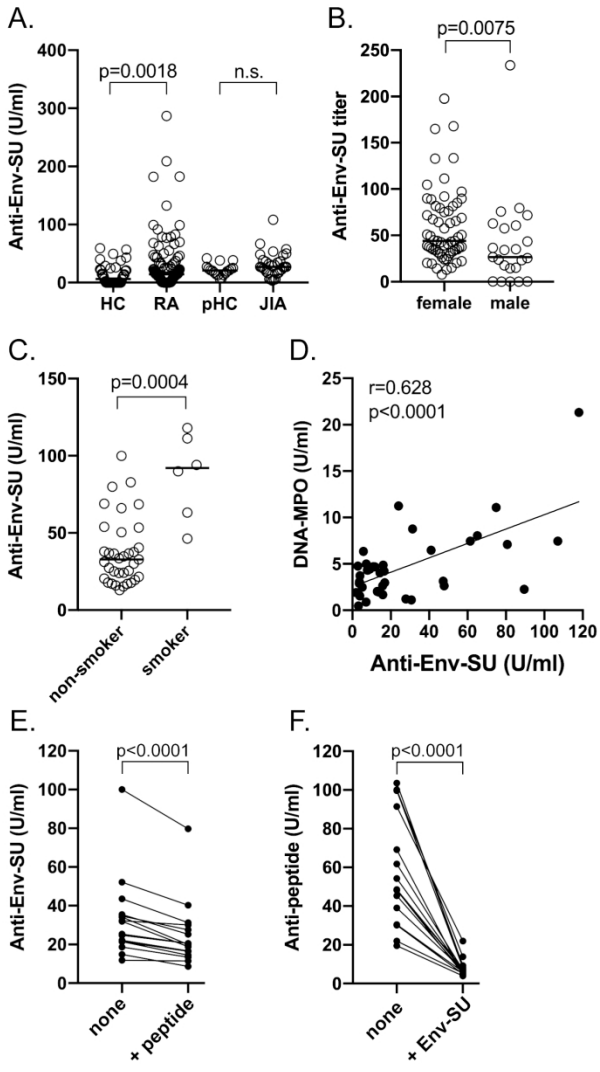

Figure 3

Fig. 4

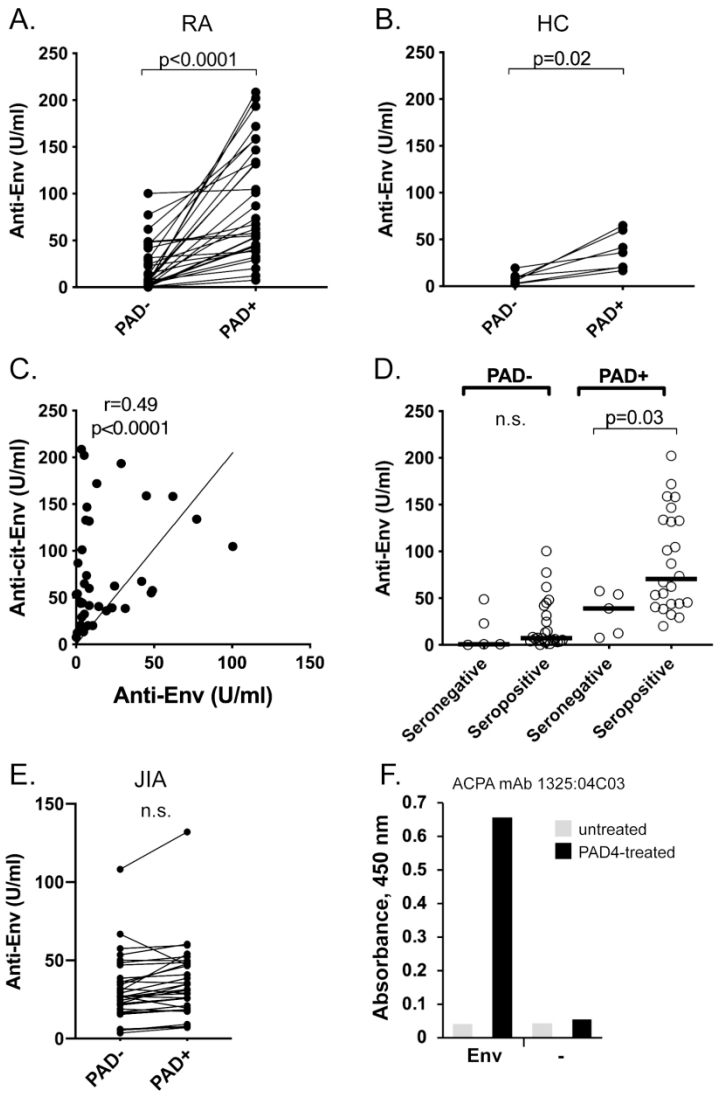

Fig. 5

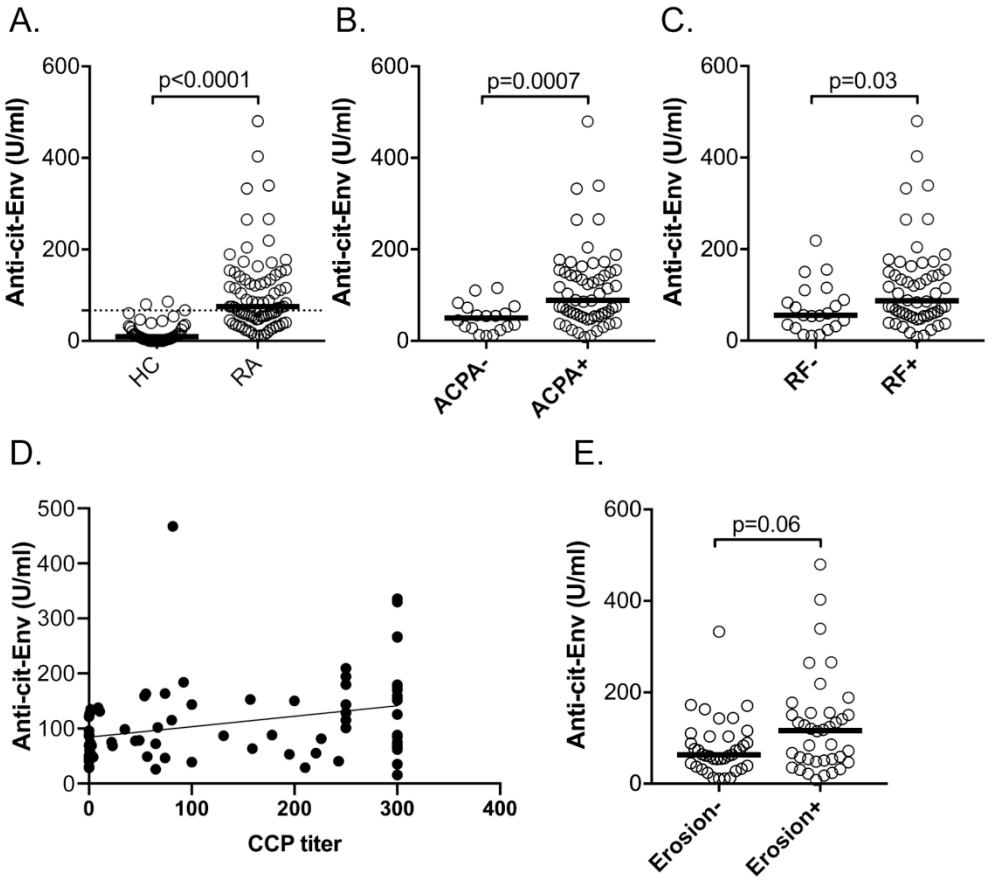

Figure 5