

## RHEUMATOID ARTHRITIS

## Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis

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Rheumatoid arthritis (RA) is characterized by synovial joint inflammation and by development of pathogenic humoral and cellular autoimmunity to citrullinated proteins. Neutrophil extracellular traps (NETs) are a source of citrullinated autoantigens and activate RA synovial fibroblast-like synoviocytes (FLS), cells crucial in joint damage. We investigated the molecular mechanisms by which NETs promote proinflammatory phenotypes in FLS and whether these interactions generate pathogenic anti-citrulline adaptive immune responses. NETs containing citrullinated peptides are internalized by FLS through a RAGE-TLR9 pathway, promoting FLS inflammatory phenotype and their up-regulation of major histocompatibility complex (MHC) class II. Once internalized, arthritogenic NET peptides are loaded into FLS MHC class II and presented to antigen-specific T cells. HLA-DRB1\*04:01 transgenic mice immunized with mouse FLS loaded with NETs develop antibodies specific to citrullinated forms of relevant autoantigens implicated in RA pathogenesis as well as cartilage damage. These results implicate FLS as notable mediators in RA pathogenesis, through the internalization and presentation of NET citrullinated peptides to the adaptive immune system, leading to pathogenic autoimmunity and cartilage damage.

## INTRODUCTION

Rheumatoid arthritis (RA) is the second most prevalent autoimmune condition, affecting 1% of the world population. It is a chronic, systemic inflammatory disease that affects the peripheral synovial joints and is associated to high morbidity and enhanced mortality. A significant proportion of RA patients exhibit RA-related autoantibodies, which include rheumatoid factor and antibodies to citrullinated protein antigens (ACPAs) (1). RA is characterized by a prolonged (3 to 5 years) subclinical phase where ACPAs are detected before the onset of clinically apparent disease (2–5). ACPA reactivity is directed against various citrullinated intracellular and extracellular antigens, including vimentin, histones, fibrinogen, and enolase. T cell responses to citrullinated peptides also develop in RA. Reactivity to citrullinated antigens correlates with the presence of the HLA-DRB1\*04:01 shared epitope, which includes HLA-DRB1\*04:01, HLA-DRB1\*04:04, and HLA-DRB1\*01:01, haplotypes associated with risk of developing RA (6, 7). Citrullination of specific anchor residues enhances the ability of peptides to bind and be presented by the major histocompatibility complex class II (MHC II)-shared epitope alleles, allowing the activation and expansion of citrulline-specific CD4<sup>+</sup> T cells, and the subsequent promotion of ACPA generation (8–12).

In early stages of RA, neutrophils are abundant in both synovial tissue and fluid, supporting an important role for these cells in the initial events contributing to the pathogenesis of this disease (13). Recent work from our group and others indicates that RA synovial and peripheral blood neutrophils display an enhanced capacity to form neutrophil extracellular traps (NETs) (14, 15). During NET formation, there is intracellular activation of peptidylarginine deiminase-4 (PAD4), a myeloid-specific PAD involved in citrullination, and neutrophils extrude a meshwork of nuclear material coupled to cytoplasmic and granular proteins. Because of PAD activation, proteins externalized in NETs become citrullinated, and several of them have been characterized as important RA autoantigens (14). Hence, NET formation may represent an important process leading to the citrullination of autoantigens that, in a genetically predisposed host, could promote activation of innate and adaptive immune responses and contribute to RA development.

One important cellular participant in RA is the fibroblast-like synoviocyte (FLS). These cells are major effectors in cartilage damage and participate in synovial inflammation in the rheumatoid joint. FLS express a variety of Toll-like receptors (TLRs) and have the capacity to act as antigen-presenting cells (APCs) in the synovium (16–19). Recent evidence demonstrates that FLS are activated by NETs, leading to up-regulation of inflammatory cytokine and adhesion molecule synthesis (14). However, the mechanisms by which NETs activate FLS remain to be fully characterized. We hypothesized that specific citrullinated autoantigens contained in NETs can be taken up by FLS and presented to T cells in an MHC II-dependent manner, leading to antigen-specific enhanced T and B cell responses relevant to disease pathogenesis.

## RESULTS

## ACPAs induce NETosis and recognize multiple citrullinated autoantigens exposed in NETs

Putative arthritogenic peptides such as histones H3 and H4 and vimentin have been reported to be citrullinated in NETs (14, 20, 21). Because

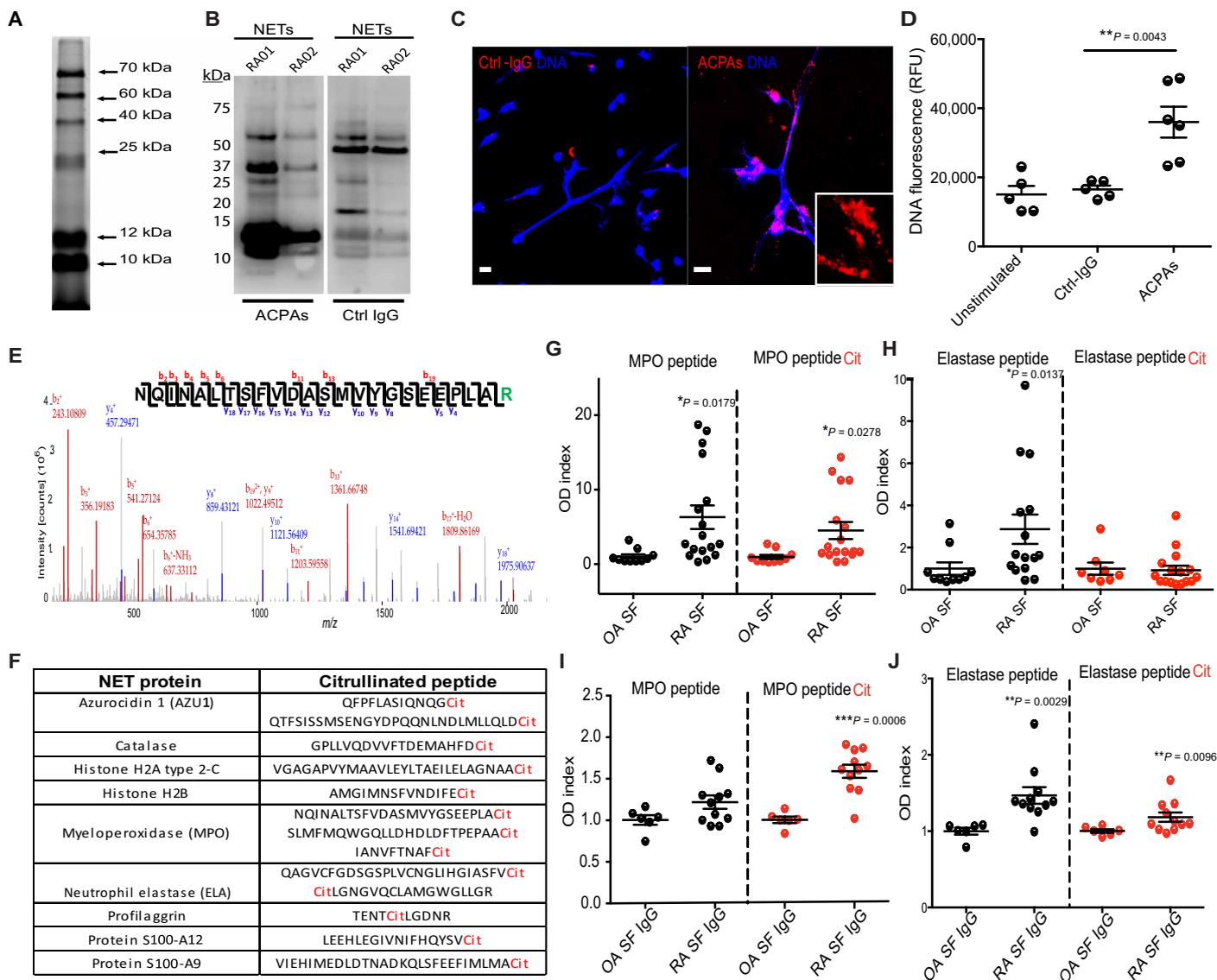
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citrullination and response to citrullinated antigens are considered key in RA pathogenesis, we investigated whether other peptides are citrullinated in NETs and could function as autoantigens in this disease. Using a rhodamine phenylglyoxal (Rh-PG)-based probe to quantify citrullination, multiple citrullinated proteins were detected in NETs that were induced in control neutrophils by rheumatoid factor stimulation, a known inducer of NETosis (14) (Fig. 1A). Western blot and immunofluorescence analyses demonstrated that ACPAs isolated from RA patients differentially recognized multiple citrullinated proteins in spontaneously

generated RA-NETs when compared with control immunoglobulin Gs (IgGs), suggesting that RA-specific autoantigens are contained within these structures (Fig. 1, B and C, and fig. S1). In addition, purified RA ACPAs enhanced NETosis in control neutrophils when compared with control IgG (Fig. 1D). These results confirm the hypothesis that RA-NETs or NETs induced by RA-relevant stimuli externalize multiple citrullinated autoantigens; in turn, RA autoantibodies enhance NET formation.

To identify in more detail the proteins that are citrullinated in NETs, we performed proteomic analysis in NETs similarly induced



**Fig. 1. ACPAs recognize multiple citrullinated peptides in NETs and induce NETosis.** (A) Rh-PG probe against citrulline was used to detect specific citrullinated proteins in purified NETs generated by stimulating control neutrophils with IgM rheumatoid factor. (B) ACPAs differentially recognize citrullinated autoantigens in NETs when compared with control IgG. Spontaneously generated NETs from peripheral blood neutrophils from two RA patients (RA01 and RA02) were isolated and resolved in SDS-PAGE. Western blot was performed using ACPAs or control IgG. (C) ACPAs bind to NETs and (D) enhance NETosis. Red, control IgG (ctrl-IgG) or ACPAs; blue, Hoechst stain. (E) Representative histogram of citrullinated peptides detected in NETs using PEAKS<sup>3</sup> software (Thermo-Fisher Scientific). (F) Mass spectrometry analysis demonstrates multiple citrullinated peptides in NETs. (G and H) ELISA analysis of synovial fluid from OA ( $n = 10$ ) and RA ( $n = 17$ ) patients to detect autoantibodies recognizing citrullinated or noncitrullinated forms of MPO and neutrophil elastase, respectively. (I and J) ELISA analysis of IgGs isolated from synovial fluid (SF) of OA ( $n = 6$ ) and RA ( $n = 11$ ) patients showing recognition of native or cit-MPO or elastase. (A) to (C) are representative of three independent experiments. Scale bars, 10  $\mu$ m. Results are the means  $\pm$  SEM of  $n = 5$  to 6. For statistical analyses, Mann-Whitney  $U$  test was used.  $m/z$ , mass/charge ratio; OD, optical density.

in peripheral blood control neutrophils by rheumatoid factor stimulation [control IgM does not induce NETs (14)]. This analysis detected several citrullinated proteins in these structures (Fig. 1, E and F, and table S1), including azurocidin, catalase, histone H2B, myeloperoxidase (MPO), neutrophil elastase, profilaggrin, S100-A12, and S100-A9. Because MPO and neutrophil elastase play important roles in NET formation (22) and are abundant and citrullinated in NETs, we investigated whether RA patients develop autoantibodies against citrullinated forms of these proteins. We selected two epitopes, IANVFTNAFR (citrullinated in MPO) and RLGNGVQCLAMGWLLGR (citrullinated in neutrophil elastase), and generated synthetic peptides with or without citrullination sites. We identified the presence of autoantibodies against the citrullinated MPO peptide (cit-MPO) and against the native-form elastase peptide in RA synovial fluid but not in the synovial fluid from patients with osteoarthritis (OA) (Fig. 1, G and H). Confirming these findings, IgGs purified from RA synovial fluid recognized cit-MPO and cit-elastase (Fig. 1, I and J). These observations confirm and expand the repertoire of citrullinated molecules present in NETs to which RA patients develop autoantibody responses. These results also support previous observations that, in RA, antibody responses to both native and citrullinated version of various proteins are detected (23).

### Internalization of NET components by FLS promotes their inflammatory phenotype

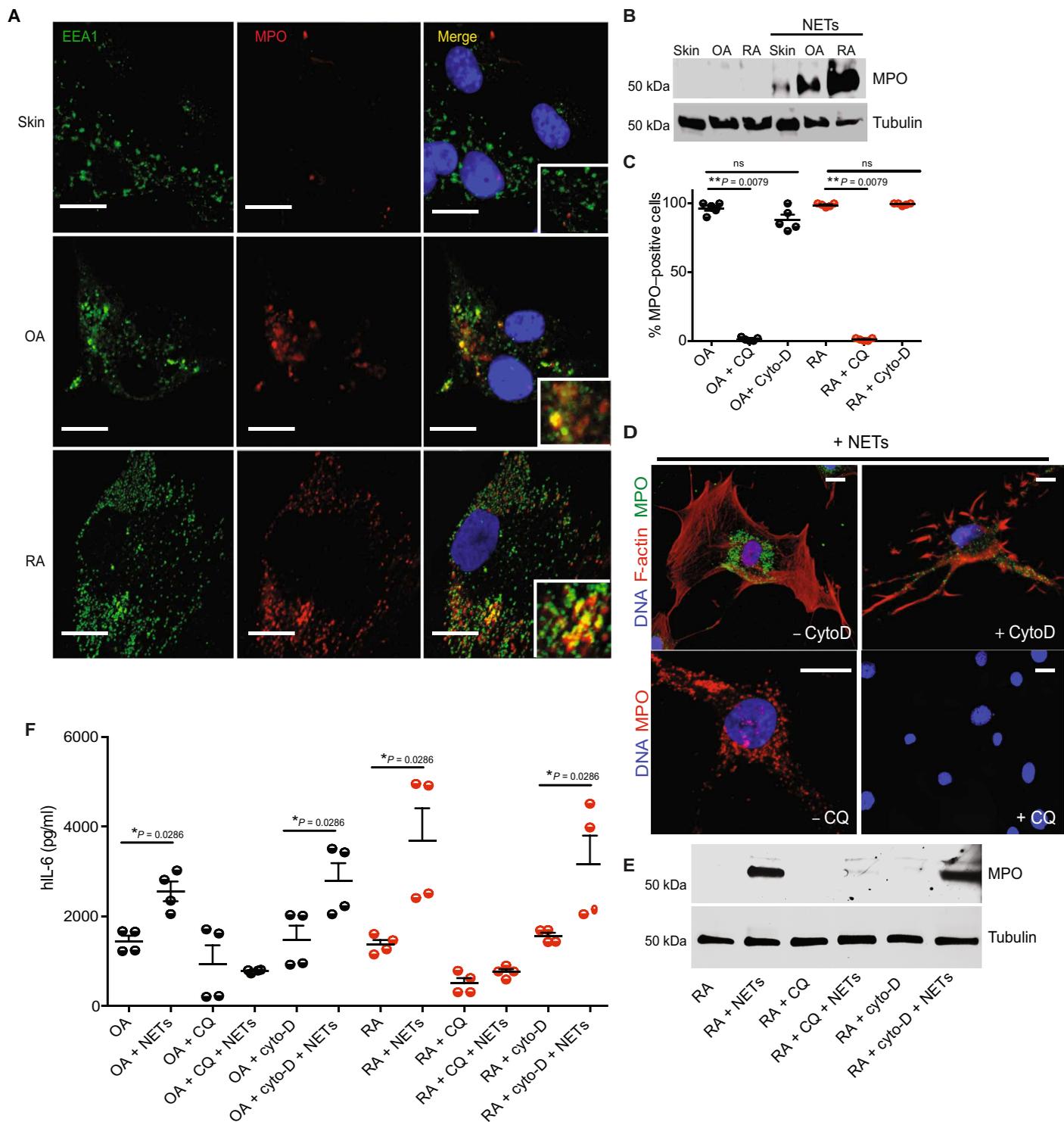
We previously showed that both RA and OA FLS exposed to NETs become proinflammatory and synthesize significantly increased levels of interleukin-6 (IL-6) (14). We then assessed the putative mechanisms involved in NET-induced FLS activation. OA and RA FLS and control dermal fibroblasts were incubated in the presence or absence of spontaneously formed peripheral blood RA-NETs for 2 hours. By confocal microscopy, we demonstrated that NETs are internalized by both OA and RA FLS, whereas skin fibroblasts showed minimal internalization (Fig. 2, A and B). To determine whether the activation status of the cell has an influence in NETs' internalization, we also incubated skin fibroblasts harvested from psoriasis patients with NETs from peripheral blood RA neutrophils. Psoriatic skin fibroblasts were also capable of internalizing NETs, suggesting that the activation status of the fibroblast has an impact on its ability to internalize NETs (fig. S2). Internalized NET components colocalized with the early endosome antigen-1 (EEA1) (24), implicating the endocytic pathway in NET internalization by FLS (Fig. 2A). To characterize the intracellular trafficking of NETs, we preincubated FLS with chloroquine (CQ), a lysosomotropic agent that prevents endosomal acidification, or with cytochalasin D (cyto D), an inhibitor of actin polymerization. FLS internalized NETs when exposed to cyto D but not when cultured with CQ (Fig. 2, C and D). By fluorescence microscopy visualization, we demonstrated significant decreases in the number of FLS positive for intracellular MPO, and Western blot analysis showed that MPO is not internalized by FLS treated with CQ (Fig. 2, C and E). These observations indicate that NET internalization was impaired by antimetabolites. Moreover, the levels of IL-6 synthesized by FLS significantly associate with the capacity to internalize NETs (Fig. 2F). These findings suggest that the internalization of NETs by FLS is independent of actin filaments and uses the endocytic pathway, most likely clathrin-coated vesicles. Furthermore, the release of proinflammatory cytokines by FLS triggered by NETs requires the internalization of these structures.

### The RAGE-TLR9 signaling pathway mediates NET internalization by FLS

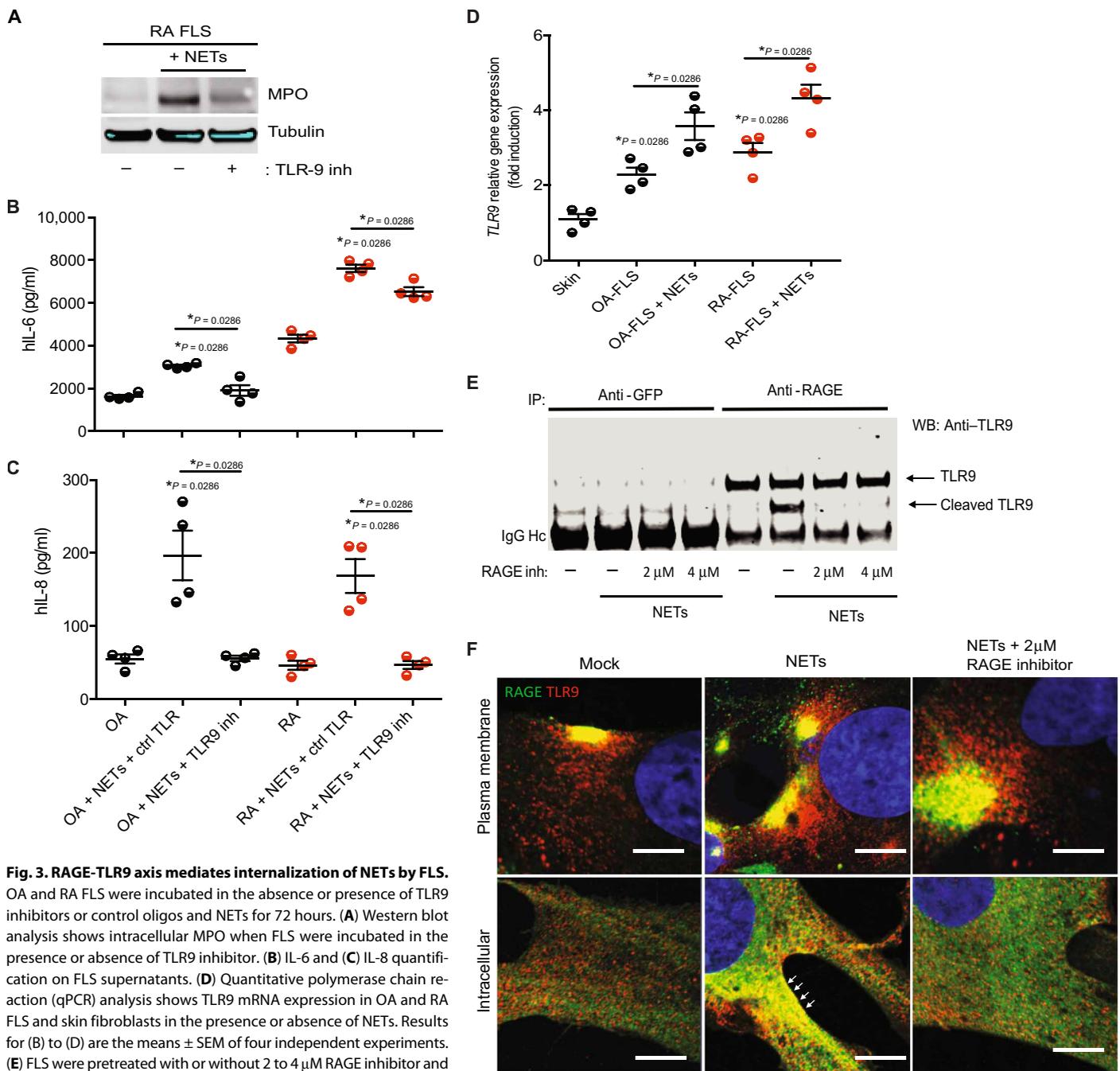
NETs contain DNA and nuclear and granule proteins (25). Given that unmethylated CpG sequences are recognized by TLR9, we tested whether this receptor was involved in the internalization of NETs by FLS. Western blot analysis demonstrated that OA and RA FLS preincubated with a TLR9 antagonist and then exposed to RA-NETs are impaired in their ability to internalize MPO, a molecule present in NETs (Fig. 3A). In addition, IL-6 and IL-8 synthesis by FLS was significantly reduced in the presence of a TLR9 antagonist, with the effect being more predominant on IL-8 expression (Fig. 3, B and C). The expression levels of TLR9 were significantly higher in OA and RA FLS than in control skin fibroblasts. Furthermore, TLR9 expression was significantly up-regulated when FLS were incubated with NETs, suggesting a positive regulatory feedback loop (Fig. 3D). Given the role of TLR9 in the internalization of NETs and the fact that this molecule is involved in type I interferon (IFN) synthesis in certain cell types, we quantified type I IFN genes in FLS after incubation with NETs for 24 hours. IFN- $\alpha$  was significantly up-regulated after NETs incubation (fig. S3). Because TLR9 resides in endosomes, we hypothesized that a receptor present on the plasma membrane of FLS would mediate internalization of NETs. The receptor for advanced glycation end products (RAGE) was previously reported to recognize HMGB1 (a molecule present in NETs) (26, 27), to promote DNA uptake into endosomes, and to lower the immune recognition threshold for TLR9 activation (28). FLS were incubated with 2 to 4  $\mu$ M RAGE peptide inhibitor for 30 min, followed by exposure to NETs for 1 hour. Coimmunoprecipitation analysis demonstrated that RAGE interacts with the active form of TLR9 (cleaved TLR9) to mediate NET internalization, and this interaction was abolished in the presence of a RAGE inhibitor (Fig. 3E and fig. S4). We also detected a constitutive interaction of RAGE with the inactive form of TLR9, but this interaction was independent of NETs (Fig. 3E). We hypothesized that RAGE and the inactive form of TLR9 interact in the plasma membrane of FLS and that the functional interaction with the active form of TLR9 occurs intracellularly. Immunofluorescence of nonpermeabilized cells demonstrated interaction of RAGE with TLR9 in the plasma membrane of FLS, and this interaction was not perturbed by the presence of a RAGE inhibitor (Fig. 3F). Intracellularly, however, RAGE and TLR9 interactions were blocked with a RAGE inhibitor (Fig. 3F). These results suggest that NETs are internalized by FLS via a RAGE-TLR9 axis and that the proinflammatory profile induced in FLS is dependent on NET internalization.

### NET internalization induces MHC II up-regulation in FLS in an IL-17B-dependent manner

FLS have the ability to acquire APC capabilities in the inflamed synovium (18), including MHC II up-regulation. We tested the hypothesis that the uptake of NETs by FLS would lead to presentation of NET citrullinated peptides to the adaptive immune system in an MHC II-dependent manner. We observed that, upon NET internalization, MHC II was up-regulated in FLS (Fig. 4A and fig. S5) when compared with cells not exposed to NETs or isotype control. Flow cytometry analysis confirmed up-regulation of MHC II intracellularly and in the plasma membrane of FLS incubated with NETs (Fig. 4B). This suggests that a molecule present in NETs may promote up-regulation of FLS MHC II. IFN- $\gamma$  is a known inducer of MHC II up-regulation in APCs, including FLS (19). However, NETs showed no detectable levels of this cytokine by Western blot and enzyme-linked immunosorbent



**Fig. 2. NETs are internalized by FLS into EEA1-positive compartments.** RA-NETs were incubated with OA or RA FLS or skin fibroblasts for 2 hours. **(A)** Internalized NETs colocalize with EEA1 compartments in FLS. Red, MPO; green, EEA1; blue, DNA. Results are representative of three independent experiments performed with a confocal microscope, Scale bars, 10  $\mu$ m. **(B)** Western blot analysis of fibroblasts incubated in the presence or absence of RA-NETs shows that MPO bound to NETs is internalized by OA and RA FLS but minimally by healthy control skin fibroblasts. Results are representative of three independent experiments. **(C)** Percentage of MPO-positive cells (FLS that internalized NETs) decreased after CQ but not cyto D exposure. **(D)** Representative confocal images after treatment with cyto D and CQ. Red, MPO or F-actin; green, MPO; blue, DNA. Scale bars, 10  $\mu$ m. **(E)** Western blot analysis confirms that MPO internalization is impaired in FLS preincubated with CQ but not in FLS preincubated with cyto D. **(F)** IL-6 release by FLS is dependent on NET internalization. Results are the means  $\pm$  SEM of  $n = 4$ . For statistical analyses, Mann-Whitney  $U$  test was used. ns, not significant; hIL, human IL.

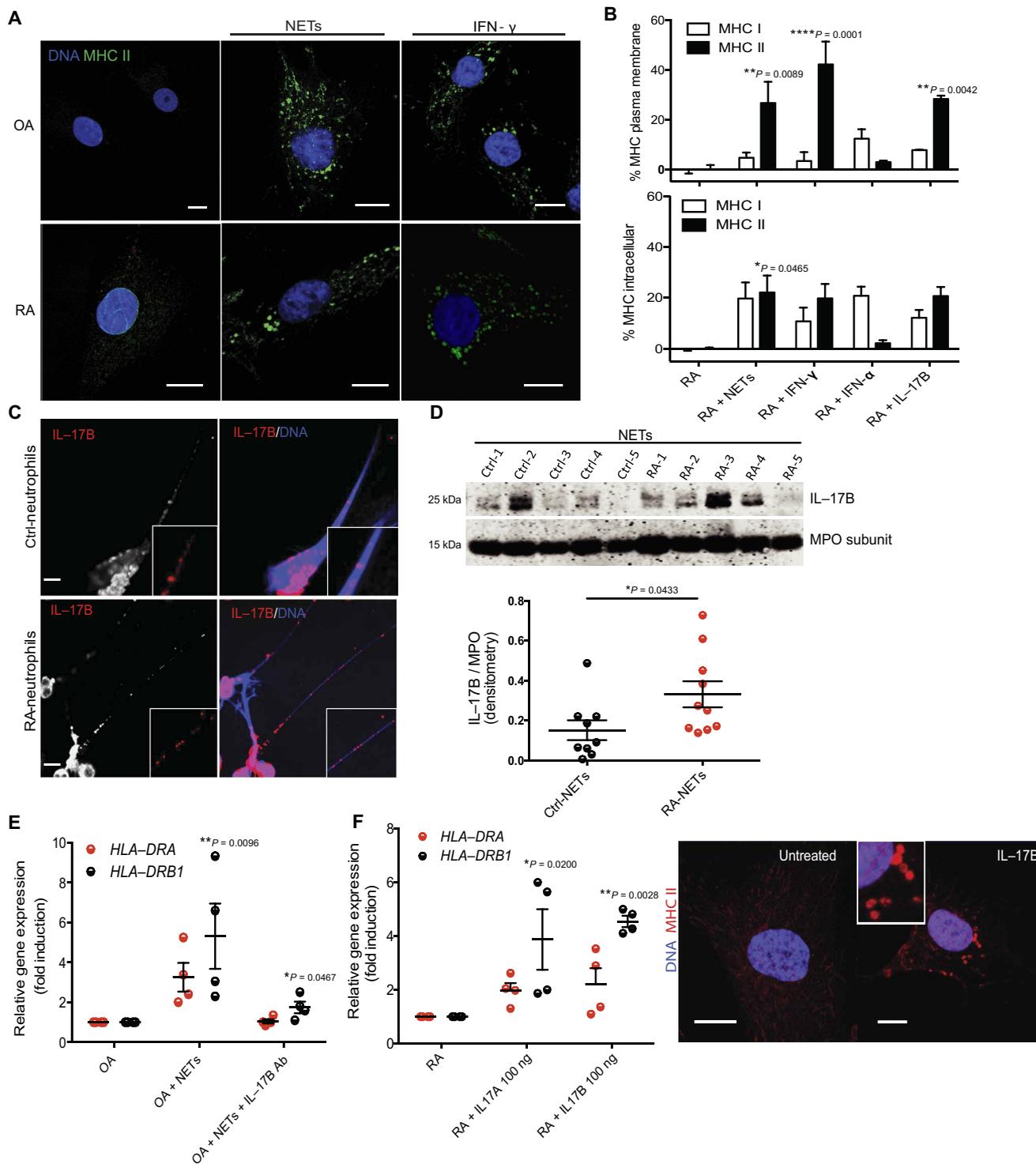


**Fig. 3. RAGE-TLR9 axis mediates internalization of NETs by FLS.**

OA and RA FLS were incubated in the absence or presence of TLR9 inhibitors or control oligos and NETs for 72 hours. (A) Western blot analysis shows intracellular MPO when FLS were incubated in the presence or absence of TLR9 inhibitor. (B) IL-6 and (C) IL-8 quantification on FLS supernatants. (D) Quantitative polymerase chain reaction (qPCR) analysis shows TLR9 mRNA expression in OA and RA FLS and skin fibroblasts in the presence or absence of NETs. Results for (B) to (D) are the means ± SEM of four independent experiments. (E) FLS were pretreated with or without 2 to 4 μM RAGE inhibitor and incubated with NETs for 1 hour. Coimmunoprecipitation (IP) was performed against RAGE, and TLR9 was detected by Western blot. Anti-green fluorescent protein (GFP) was used as negative control. (F) Plasma membrane (top) and intracellular (bottom) detection of RAGE and TLR9 were performed on FLS pretreated with or without RAGE inhibitor. Red, TLR9; green, RAGE; blue, DNA of three independent experiments; white arrows highlight areas of colocalization of RAGE and TLR9. Scale bars, 5 μm. Mann-Whitney U test was used.

assay (ELISA) (fig. S6, A and B). IFN-γ was also quantified by ELISA in supernatants of FLS incubated for up to 12 days with RA-NETs and was found to be undetectable (fig. S6C). This ruled out the possibility that FLS were induced to produce IFN-γ in response to NETs. Members of the IL-17 family play important roles in inflammatory responses in RA (29). Although most studies have focused on IL-17A, IL-17B synthesized by neutrophils was recently described as the most abundant IL-17 isoform in the RA synovium (30). Immunofluorescence and

Western blot analyses demonstrated that RA-NETs were decorated with significantly increased amounts of IL-17B but not IL-17A (Fig. 4, C and D, and fig. S7). Neutralization of IL-17B in spontaneously generated RA-NETs induced significant decreases in expression of HLA-DRA and HLA-DRB MHC II molecules (Fig. 4E). Furthermore, incubation of RA FLS with IL-17B or IL-17A led to up-regulation of HLA-DRA and HLA-DRB mRNA (Fig. 4F). Mature MHC II compartments were detected in FLS incubated with recombinant IL-17B (Fig. 4F). Inhibition of



**Fig. 4. IL-17B present in NETs up-regulates MHC II in OA and RA FLS.** OA and RA FLS were incubated in the presence or absence of spontaneously generated RA-NETs or IFN- $\gamma$  (1000 U/ml). **(A)** Detection of MHC II in FLS by immunofluorescence. Green, MHC II; blue, DNA. Results are representative of three independent experiments. Scale bars, 10  $\mu$ m. **(B)** Plasma membrane and intracellular MHC I and II were quantified by flow cytometry in RA FLS treated with NETs, IFN- $\gamma$ , or IFN- $\alpha$  for 5 days. **(C)** IL-17B (red) is externalized in control (Ctrl) NETs generated with LPS (1  $\mu$ g/ml) and in spontaneously generated RA-NETs. **(D)** IL-17B is detected in isolated NETs by Western blot analysis. Each lane depicts independent NET isolation per group (Ctrl and RA). Mann-Whitney  $U$  test was used. Results are the means  $\pm$  SEM of 10 independent experiments, \* $P$  < 0.05. **(E)** OA FLS were incubated with RA-NETs in the presence or absence of IL-17B (1  $\mu$ g/ml) neutralizing antibodies for 48 hours. Quantification of HLA-DRA and HLA-DRB1 mRNA was performed by real-time PCR. Bars are the means  $\pm$  SEM of four independent experiments. **(F)** RA FLS were incubated with 100 ng of human recombinant IL-17A or IL-17B for 72 hours. qPCR and immunofluorescence analyses assessed MHC II mRNA expression and protein localization, respectively. ANOVA with Bonferroni's test was performed for (B), (E), and (F). Results are the means  $\pm$  SEM of four independent experiments.

IL-17R using a neutralizing antibody did not impair NET internalization (fig. S8), suggesting that RAGE-TLR9 is the main pathway of NET internalization and that internalization is independent of IL-17R. Overall, these results indicate that IL-17B externalized in NETs can induce up-regulation of MHC II in FLS.

### Arthritogenic NET peptides internalized by FLS colocalize with MHC II and are presented to antigen-specific CD4<sup>+</sup> T cells

Because FLS internalize molecules present in NETs and up-regulate MHC II after this internalization, we tested whether these NET peptides were loaded onto the FLS MHC II compartment. After a 5-day incubation of FLS in the presence or absence of NETs, immunofluorescence confocal microscopy analysis demonstrated that MPO colocalizes with MHC II in both OA and RA FLS, suggesting that peptides derived from NETs are loaded onto MHC II compartments intracellularly (Fig. 5A). In addition, colocalization of NET peptides and MHC II was detected in the plasma membrane of nonpermeabilized FLS (Fig. 5B and fig. S9), suggesting that the complex traffics to the plasma membrane to expose the peptide.

In addition to ACPAs, autoantibodies targeting native proteins have been described in RA. In particular, a subset of RA patients develops autoantibodies to human cartilage-glycoprotein 39 (HC-gp39) (31). To investigate whether noncitrullinated arthritogenic peptides are also contained within the NETs, we performed Western blot analysis, which revealed that HC-gp39 is synthesized by neutrophils and externalized in NETs (fig. S6A). To determine the functionality of the peptide-MHC II complex with regard to the ability to activate antigen-specific T cells, we incubated FLS loaded with NETs with murine T cell hybridomas specific for immunodominant portions of HC-gp39 263 to 275 (RSFTLASSETGVG) and quantified IL-2 synthesis by the T cells. HC-gp39 T cell hybridomas synthesized significantly higher levels of IL-2 when incubated with FLS loaded with NETs than when exposed to FLS alone (Fig. 5C).

Because NETs contain citrullinated vimentin (cit-vimentin), among other citrullinated peptides, and to corroborate that FLS can present citrullinated peptides to antigen-specific T cells, DRB1\*04:04 RA FLS loaded with NETs were incubated with DRB1\*04:04 cit-vimentin-specific CD4<sup>+</sup> T cells isolated and expanded from RA patients (fig. S10). After 5 days of incubation, cit-vimentin-specific CD4<sup>+</sup> T cells displayed significant increases in secretion of IFN- $\gamma$ , TNF- $\alpha$  (tumor necrosis factor- $\alpha$ ), IL-10, and IL-1ra (Fig. 5D) when compared with T cells exposed to FLS alone. The release of these cytokines was reduced when cells were incubated with neutralizing antibodies against MHC II or against CD28 (Fig. 5D), indicating that costimulation is required. To test specificity of FLS toward citrullinated peptides, we cocultured CD4<sup>+</sup> T cells against citrullinated Aggrecan 225 with FLS. FLS were unable to activate Aggrecan 225 CD4<sup>+</sup> T cells (fig. S11), suggesting that specific citrullinated peptides are presented by FLS. Results indicate that FLS that internalize NET components can present arthritogenic peptides to antigen-specific T cells and activate adaptive immunity.

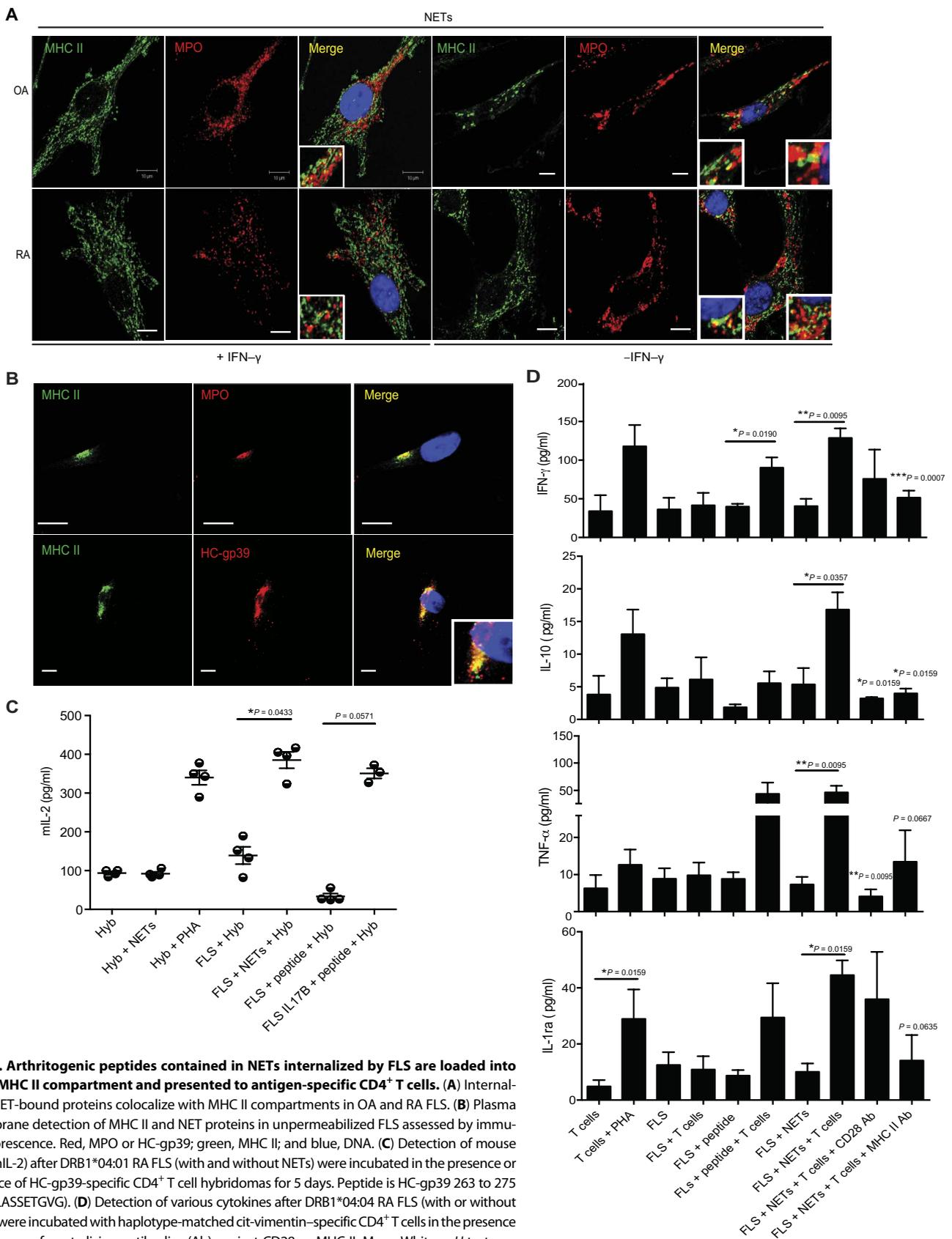
### Humanized HLA-DRB1\*04:01 transgenic mice develop ACPAs in response to immunization with FLS loaded with NETs

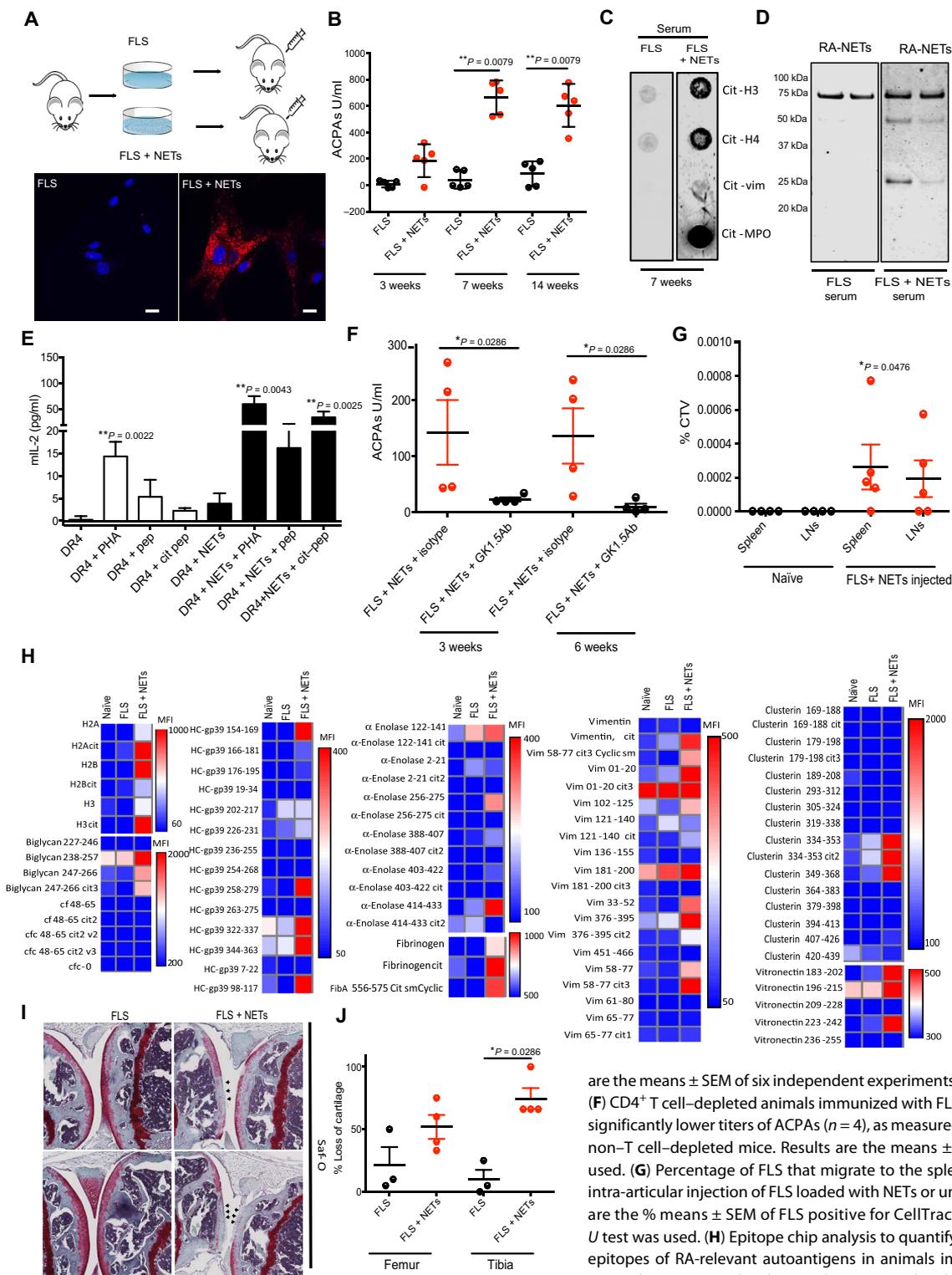
To confirm that NETs are an important source of citrullinated peptides and that FLS that internalize NETs can induce adaptive immune responses characteristic of RA in vivo, we used the humanized HLA-DRB1\*04:01 transgenic mouse model (32). These mice are DRB1\*04:01.

AEo; therefore, they lack endogenous class II molecules (both I-A and I-E chains). In this model, transgenic expression of this DR1 allele confers susceptibility to inflammatory arthritis induced by immunization with various stimuli including citrullinated fibrinogen (cit-fibrinogen) (33). Synovial tissue was harvested from healthy HLA-DRB1\*04:01 mice to isolate and expand FLS in culture (Fig. 6A). Mouse FLS were incubated for 3 days in the presence or absence of NETs isolated from peripheral blood RA neutrophils. Internalization of NETs by mouse FLS was confirmed by intracellular immunofluorescence against human MPO (Fig. 6A). A total of 100,000 FLS with or without internalized NETs were injected in one knee of each HLA-DRB1\*04:01 mouse (Fig. 6A). After seven rounds of injections performed every other week, serum ACPA levels were quantified using a commercial assay [anti-cyclic citrullinated peptide (CCP), Wuhan Huamei Biotech Co.]. High titers of anti-CCP antibodies were detected in the sera of mice that received intra-articular injections of FLS loaded with NETs when compared with animals that received FLS alone (Fig. 6B). Dot blot analysis demonstrated that sera from animals immunized with FLS loaded with NETs developed antibodies recognizing cit-histone H3 (cit-H3), cit-histone H4 (cit-H4), and cit-MPO as well as antibodies recognizing NET proteins (Fig. 6C). In addition, serum samples from mice immunized with FLS loaded with NETs differentially recognized proteins contained in RA-NETs, when compared with serum from animals that were immunized with FLS alone (Fig. 6D). Splenocytes from mice injected with FLS loaded with NETs displayed a significant response to citrullinated peptides (a cocktail of cit-H3, cit-H4, cit-MPO, and cit-vimentin) when compared with a cocktail of native peptides, as assessed by IL-2 synthesis (Fig. 6E). ACPA generation in these animals was dependent on CD4<sup>+</sup> T cells because CD4<sup>+</sup> T cell-depleted animals (using an antibody approach) injected with FLS loaded with NETs displayed significantly decreased levels of ACPAs (Fig. 6F and fig. S12).

Given that we detected systemic ACPAs in animals immunized with FLS loaded with NETs, we hypothesized that FLS can migrate to the spleen and/or lymph nodes to interact with T cells. Labeled FLS loaded with NETs were detected in spleen and lymph nodes of animals immunized with FLS when compared with naïve mice (Fig. 6G). These results indicate that induction of antigen-specific T cell responses and ACPA synthesis by FLS loaded with NETs likely occurs both intra-articularly and outside the joint.

An antigen array was used to identify the repertoire of autoantibodies generated after immunization. Animals immunized with FLS loaded with NETs displayed elevated levels of antibodies recognizing citrullinated histones cit-H2A, cit-H2B, and cit-H3 (Fig. 6H). Supporting our observation that NETs activate HC-gp39 T cell hybridomas, mice immunized with FLS-NETs also displayed elevated levels of antibodies against HC-gp39 (HC-gp39 154 to 169, HC-gp39 258 to 279, HC-gp39 322 to 337, and HC-gp39 344 to 363 epitopes) (Figs. 5C and 6H). Antibodies recognizing  $\alpha$ -enolase ( $\alpha$ -enolase 414 to 433), cit-fibrinogen, and cit-vimentin (vim 58 to 77 cit3) were also present in animals immunized with FLS-NET, when compared with those immunized with FLS alone (Fig. 6H and fig. S13). Other antibodies identified included those recognizing cartilage components, such as biglycan (epitopes 238 to 257, 247 to 266, and 247 to 266 cit3), suggesting that NETs may also modify FLS behavior, potentially promoting cartilage damage. Although animals immunized with FLS alone or FLS loaded with NETs did not develop overt arthritis, safranin O staining of murine synovial cartilage showed disruption in cartilage integrity, significantly increased cartilage loss and cartilage irregularity, and higher prevalence of pannus in those animals immunized with FLS-NETs





**Fig. 6. DRB1\*04:01 humanized mice that receive intra-articular injections of mouse FLS loaded with RA-NETs develop ACPAs and cartilage damage.** DRB1\*04:01 FLS were isolated and incubated with human RA-NETs for 3 days before intra-articular injection. (A) Internalization of NETs by FLS was assessed by immunofluorescence using an antibody against MPO (red). (B) Serum ACPA levels at various time points in animals immunized with FLS alone or FLS loaded with NETs ( $n = 5$  per group). Results are the means  $\pm$  SEM. Mann-Whitney  $U$  test was used. (C) Sera from three mice immunized for 7 weeks with FLS or with FLS loaded with NETs were analyzed by dot blot against citrullinated proteins. (D) Western blot analysis to detect serum autoantibodies recognizing human NET proteins (arrows) in DRB1\*04:01 animals that received FLS alone or FLS-NETs; each lane depicts two independent NET isolations. (E) IL-2 synthesis by DRB1\*04:01 mouse splenocytes incubated with a cocktail of native (pep; H3, H4, MPO, and vimentin) or citrullinated peptides (cit-pep; a cocktail of cit-H3, cit-H4, cit-MPO, and cit-vimentin), when comparing animals immunized with FLS loaded with NETs (DR4 + NETs) with animals immunized with FLS alone (DR4). PHA is used as positive control. Results are the means  $\pm$  SEM of six independent experiments. Mann-Whitney  $U$  test was used. (F) CD4<sup>+</sup> T cell-depleted animals immunized with FLS loaded with NETs demonstrate significantly lower titers of ACPAs ( $n = 4$ ), as measured by ELISA, when compared with non-T cell-depleted mice. Results are the means  $\pm$  SEM. Mann-Whitney  $U$  test was used. (G) Percentage of FLS that migrate to the spleen and lymph nodes (LNs) after intra-articular injection of FLS loaded with NETs or untreated mice ( $n = 4$  to 5). Results are the % means  $\pm$  SEM of FLS positive for CellTraceViolet (% CTV). Mann-Whitney  $U$  test was used. (H) Epitope chip analysis to quantify antibodies recognizing specific epitopes of RA-relevant autoantigens in animals immunized with FLS loaded with NETs when compared with animals immunized with FLS alone. Heat map represents the average of mean fluorescent units of five animals per group. (I) Sagittal sections of cartilage of the injected tibiofemoral compartment were stained with safranin O demonstrating impaired cartilage integrity (arrowheads) in animals immunized with FLS loaded with NETs when compared with animals immunized with FLS alone. (J) Percentage of cartilage loss of the femurs and tibias of animals immunized with FLS or FLS loaded with NETs. Results are the means  $\pm$  SEM of two to three independent experiments. Mann-Whitney  $U$  test was used.

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when compared with those immunized with FLS alone (Fig. 6, I and J). Overall, these results suggest that, in the presence of the shared epitope, FLS that have internalized citrullinated peptides present in NETs can induce adaptive immunity *in vivo*, effectively activating citrullinated antigen-specific T cells, which could, in turn, promote ACPA generation, as well as the promotion of synovial cartilage degradation.

## DISCUSSION

Increasing experimental evidence suggests that FLS in the RA synovium can act as immune sentinels. Their interaction with various leukocytes may promote intra-articular and peripheral inflammation and immune responses characteristic of this disease (34, 35). However, how the presentation of citrullinated autoantigens (key targets of the immune system in RA) to the adaptive immune system occurs in the synovium and the role of FLS in this process have been unclear. We now describe how neutrophil-FLS interactions in the RA synovium may play crucial roles in the promotion of joint damage and in the development of systemic dysregulated innate and adaptive immunity against citrullinated intracellular autoantigens. We found that NETs containing citrullinated and arthritogenic peptides are internalized by FLS through a RAGE-TLR9 endocytic pathway, leading to a proinflammatory phenotype in these cells. NET internalization promotes up-regulation of MHC II in the FLS, loading of NET peptides into the MHC II, trafficking to the FLS cell membrane, and presentation to antigen-specific T cells. This promotes T cell activation and modulation of B cell responses, leading to the generation of ACPAs and the propagation of inflammatory responses and cartilage damage.

A key histologic finding of the RA joint is a hyperplastic synovial lining and an invasive, inflammatory pannus across the surface of synovial joints. In addition to FLS, a distinct structural cell, the RA synovium contains macrophages, lymphocytes, and neutrophils and appears to function as a tertiary lymphoid structure (36). FLS display many proinflammatory properties that contribute to RA pathogenesis, including their ability to function as APCs through up-regulation of MHC II after exposure to inflammatory stimuli and their expression of costimulatory molecules that can provide second signals leading to T cell activation (18). During active phases of disease and in early disease, large numbers of activated neutrophils are found in the synovial fluid of RA patients as well as at the cartilage pannus interface, where they may interact with FLS (37, 38). In addition, T cell-FLS interactions are readily observed in the inflamed synovium and promote T cell recruitment and T helper cell 1 (T<sub>H</sub>1) and T<sub>H</sub>17 differentiation (39).

We previously showed that the RA synovium, characterized by ACPA and rheumatoid factor generation and increased proinflammatory cytokines, is highly conducive to NET formation. In turn, NETs may provide the immune system with access to enhanced sources of citrullinated proteins and thereby may represent an early event preceding epitope spreading. We now add additional evidence on the role of NETs as a source of immunogenic peptides by identifying several specific citrullinated antigens present in these structures that are recognized by ACPAs. In turn, ACPAs induce NET production, potentially creating a vicious inflammatory cycle in the synovium and in the periphery. This loop may promote disease by allowing an expansion of the citrulline specificities in RA. Our observations could help explain the broad array of citrullinated antigens seen by the antibody and T cell repertoire of RA patients. Whether citrullination of these proteins in-

involved in NET formation alters not only immunogenicity but also their physiologic function remains to be determined and was not explored in this manuscript.

NETs externalize substantial amounts of DNA bound to granule proteins, and this could be a mechanism by which FLS internalize NET components. RAGE promotes uptake of alarmin/DNA complexes into endosomes and lowers immune recognition threshold for TLR9 activation in other cell types (28). This pathway is operational in FLS and mediates NET internalization. This is also in accordance with recent observations that some NET proteins (e.g., LL37 and HMGB1) promote APC activation by facilitating antigen uptake, interaction with endosomal TLRs, and inflammatory cytokine release (40). Beyond nucleic acids, it is possible that other molecules present in NETs could bind to RAGE, but this was not explored in this study.

In the case of the RA synovium, we propose that NET internalization augments cytokine synthesis by FLS and leads to their up-regulation of MHC II, thereby enhancing APC capabilities. Specifically, we showed that IL-17B is externalized by NETs and promotes MHC II up-regulation in the FLS and that this phenomenon is IFN- $\gamma$ -independent. Our results support and expand previous observations that IL-17B induces neutrophilia, is expressed by synovial neutrophils, and is the predominant IL-17 cytokine in the RA synovium (41, 42), and that FLS express the IL-17RB receptor (30). Overall, our observations suggest that proteins present in NETs can significantly alter the FLS phenotype and endow these cells with APC capabilities.

Citrullinated peptides are preferentially recognized by the HLA-DRB1\*04:01/04 alleles, leading to their presentation to autoreactive T cells, which then have the ability to promote ACPA generation, a feature of severe erosive RA (10, 43, 44). In this context, we found that HLA-DRB1\*04:01/04-positive RA FLS that internalized NETs efficiently stimulated haplotype-matched antigen-specific T cells *in vitro*, and that HLA-DRB1\*04:01 transgenic mice developed ACPAs and enhanced T cell responses and cartilage damage when immunized with syngeneic FLS loaded with RA-NETs. These findings support a crucial interplay between genetic susceptibility factors and environmental stimuli (e.g., microbes known to promote NETosis) in promoting and amplifying local and systemic inflammatory responses. Although the administration of NET-loaded FLS to these mice did not lead to overt arthritis, they displayed disruptions in cartilage integrity that suggest a pathogenic local effect in the joint. It is likely that additional inflammatory triggers may be needed to promote full-blown disease, and this is supported not only by other animal models of arthritis (44) but also by the observation that ACPA development and immune dysregulation precede overt RA by many years (4).

B cells isolated from RA synovial tissue produce antibodies to NET targets and selectively recognize NETs synthesized by RA neutrophils (45). It is then conceivable that the activation of antigen-specific T cells by FLS loaded with NETs promotes ACPA production by B cells present in the synovium and in the periphery, and this is supported by our observation that *in vivo* T cell depletion abrogates ACPA production induced by NET-loaded FLS transfer. Our results support the concept that T<sub>H</sub>s with T cell receptors specific for processed NET peptides (including citrullinated and noncitrullinated peptides derived from proarthritogenic proteins) are present in the RA synovium and respond to local APCs, such as FLS, loaded with citrullinated NET antigens. Our findings are also consistent with previous evidence that NET products can be taken up by professional APCs. Myeloid dendritic cells that internalize NET components induce autoimmunity when injected into naïve mice (46).

Neutrophils may play additional roles in the resolution of inflammation in RA (47). Although FLS that internalized NETs activated T cells and induced proinflammatory cytokine synthesis, they also induced higher synthesis of the anti-inflammatory IL-1 receptor antagonist, and it will be important to assess whether these interactions also mediate resolution of inflammatory responses in RA. Furthermore, it is important to emphasize that other mechanisms inducing citrullination likely play important roles in subsets of RA patients (48).

Our observations highlight a novel mechanism that promotes immune dysregulation and pathogenic autoimmunity in RA and further supports the rationale for testing NETosis inhibitors and strategies that disrupt specific cell-cell interactions in the synovial joint in future clinical trials in RA and, potentially, other chronic inflammatory conditions. Antimalarials have been widely used in RA for many years, although their exact mechanism of action to explain efficacy remains to be determined. Because the internalization of NETs is decreased in the presence of antimalarials, our observations may provide an additional mechanism of action for this group of drugs and suggest that further exploring strategies that limit the interactions of NETs with FLS and other target cells may have potential therapeutic benefit.

## MATERIALS AND METHODS

### Study design

The study investigated the interactions between RA FLS and NETs and their role in driving adaptive immunity in this disease. Observations using human cells in vitro were also corroborated in animal models that recapitulate the genetic predisposition found in humans. Mice sample size and the number of in vitro experiments using murine and human samples were chosen on the basis of previous publications, and no randomization was performed to select groups (14, 26). The number of samples used per experiment is explained in each figure legend.

### Patient recruitment, cell isolation, and culture and generation of T cell hybridomas and antigen-specific T cells

#### Patient selection

Patients recruited fulfilled the 1987 American College of Rheumatology criteria for RA or were diagnosed with OA based on clinical and radiographic features and confirmed by pathological findings at joint surgery (49). Healthy controls were recruited by advertisement. All individuals gave written informed consent and enrolled in a protocol approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Diabetes and Digestive and Kidney Diseases (NIAMS/NIDDK) Institutional Review Board (IRB; 01-AR-0227), the University of Michigan IRB (HUM00043667 or HUM00045058), the Benaroya Research Institute IRB (07109-139), or the Karolinska Institute IRB (2003-138, 2010/935-31/3, and 2011/583-32). Peripheral blood was obtained by venipuncture, collected in EDTA-containing tubes, and fractionated via Ficoll Paque Plus (GE Healthcare) gradient. Neutrophils were isolated by dextran sedimentation and hypotonic salt solution, as previously described (14).

#### Isolation of FLS and dermal fibroblasts

Human OA and RA FLS were obtained, as previously described (18). In brief, FLS were obtained by collagenase (Worthington Biochemical) digestion of human synovial tissue obtained at arthroplasty or synovectomy from RA or OA joints. Cells were maintained in CMRL medium (Invitrogen Life Technologies) and used after passage 4 from primary cultures. Healthy control human dermal fibroblasts [a gift from E. Romm,

National Institutes of Health (NIH)] and psoriasis human dermal fibroblasts (a gift from J. T. Elder, University of Michigan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) and grown in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

#### Generation of HC-gp39 T cell hybridomas

The generation and characterization of murine MHC II-restricted T cell hybridomas specific for a 13-amino acid peptide of the arthritogenic HC-gp39 were previously described (50). These cells were cultured in RPMI 1640 medium (Invitrogen) with 0.6 mM sodium pyruvate, 1 mM HEPES, and 0.055 mM β-mercaptoethanol. All cell cultures were supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) and grown in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

#### Cloning of citrulline-specific T cells

T cell clones specific for cit-vimentin restricted by HLA-DRB1\*04:04 were generated by staining peripheral blood mononuclear cell (PBMC) samples directly ex vivo, as previously described (51). Briefly, tetramer-positive CD4<sup>+</sup> cells were sorted on a FACSaria II at single-cell purity. Clones were expanded in 96-well plates in the presence of 1.0 × 10<sup>5</sup> irradiated PBMCs and phytohemagglutinin (PHA) (2 µg/ml; Remel Inc.) and screened by restaining with tetramers. Antigen-specific T cell proliferation was assessed by stimulating 1.0 × 10<sup>4</sup> T cells in wells with 1 × 10<sup>7</sup> irradiated allogenic PBMCs in the presence of peptide (10 µg/ml). After 72 hours, cells were pulsed with H3-thymidine for an additional 24 hours and thymidine uptake measured by liquid scintillation counting. Figure S3 shows the specificity of the response to citrullinated versus native forms of vimentin, validation of immunogenicity, and the proliferative response of the clone. Aggrecan-specific T cell clones were used as negative control because aggrecan is not expressed in NETs.

#### Quantification of NET formation by plate assay

NET quantification was performed, as previously described (14). Sytox green is a nonpermeable DNA dye that will bind to DNA present in NETs generated during neutrophil culture. Briefly, neutrophils were resuspended in RPMI without phenol red containing 0.2 µM Sytox green (Invitrogen) for 2 to 3 hours. Neutrophils (2 × 10<sup>5</sup>) were incubated in the presence or absence of control IgG or ACPAs in 96-well black plates for 1 hour at 37°C. Fluorescence was measured in a BioTek Synergy H1 Hybrid Reader. Results were reported as DNA relative fluorescence units.

#### Isolation and purification of ACPAs

Plasma samples were obtained from RA patients (*n* = 38) with high anti-CCP2 antibody levels (more than 3× the cutoff levels). IgGs were purified from plasma samples on Protein G columns followed by anti-CCP2 IgG affinity purification on CCP2 columns, as previously described (52). IgGs from OA and from ACPA-seropositive RA synovial fluids were purified using the Melon Gel IgG purification kit (Thermo Fisher) according to the manufacturer's protocol.

#### NET isolation

NETs were isolated, as previously described (14). Briefly, RA neutrophils were purified and seeded in 24-well tissue culture plates in RPMI without phenol or stimuli and incubated for 2 hours at 37°C. Supernatants were harvested, and NETs were digested with micrococcal nuclease (10 U/ml; Thermo Fisher) for 15 min at 37°C. Supernatants were

collected and centrifuged at 300g for 5 min at 4°C. NET supernatants were transferred to a fresh tube and stored at -80°C until used.

### Detection of citrullinated proteins using Rh-PG probe

Isolated NETs from control neutrophils stimulated with rheumatoid factor (14, 53) were analyzed using the Rh-PG probe, as previously described (54). Briefly, a 30- $\mu$ l aliquot of each sample was prepared in 50 mM Hepes and treated with 20% trichloroacetic acid and 0.1 mM Rh-PG for 30 min at 37°C. Samples were quenched with citrulline (Sigma), cooled on ice for 30 min, and centrifuged at 14,000 revolutions per minute for 15 min at 4°C. The supernatant was removed, and samples were washed with cold acetone and dried at 100°C for 5 min. After resuspending in 50 mM Hepes, samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12%; 170 V for 50 min) and imaged on a Typhoon Imager (excitation, 532 nm; emission, 580 nm).

### Assessment of FLS-T cell interactions

FLS were cultured in the presence or absence of spontaneously generated RA-NETs for 3 days, washed twice with phosphate-buffered saline (PBS) to remove noninternalized NETs, and then plated at a density of 100,000 cells per well in T cell hybridoma medium or human T cell medium. HC-gp39-specific T cell hybridomas or human cit-vimentin-specific T cells were added to FLS cultures at a ratio of 1:2 (FLS/T cell) and incubated in the presence or absence of neutralizing antibodies (20  $\mu$ g/ml) against CD28 or MHC II for 5 days. Supernatants were then collected and centrifuged at 300g for 5 min at room temperature. Mouse IL-2 was quantified using ELISA Ready-SET-Go! (eBioscience) for the hybridoma experiments, and the Bio-Plex pro-human cytokine assay (Bio-Rad) was performed for the human cit-vimentin T cell experiments, according to the manufacturers' recommendations. Aggrecan-specific T cell clones were used as negative control.

### Generation of mouse FLS and in vivo administration of FLS with and without NETs

Breeding pairs of DR4 transgenic mice were a gift from C. David (Mayo Clinic) and were housed and bred at the NIH animal facility. These mice are DRB1\*04:01.AEo and lack endogenous class II molecules (both I-A and I-E chains) (33). All animal studies were performed according to the guidelines established by the NIAMS Laboratory Animal Care and Use Section and following approved protocol (A013-08-05). Eight-week-old DRB1\*04:01 female mice were euthanized, and synovium from the tibiofemoral compartment was isolated and dissected. A piece of synovial tissue was incubated in a 12-well plate with CMRL 1066 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. DRB1\*04:01 FLS were used at passage 4 to ensure purity of the cells. FLS were cultured in the presence or absence of 50  $\mu$ g of human RA-NETs, isolated as above, for 3 days before injection. FLS were washed with PBS, detached with trypsin, centrifuged at 300g for 5 min at room temperature, washed, and resuspended in 1 $\times$  Hanks' balanced salt solution. Uptake of NETs by FLS was confirmed by immunofluorescence microscopy. Twelve-week-old DRB1\*04:01 female mice were anesthetized using isoflurane vaporization, and the left hind-leg was shaved to expose the knee joint. A total of 1  $\times$  10<sup>5</sup> DRB1\*04:01 FLS with or without uploaded NETs were injected into the synovial space using a 27-gauge needle. This procedure was performed every 14 days for a total of 14 weeks (seven injections), and mice were sacrificed at 24 weeks of age.

### Epitope mapping by antigen array

Antigens were diluted to PBS (0.2 mg/ml) or water and robotically spotted onto SuperEpoxy 2 Microarray Substrate Slides (ArrayIt), as previously described (55). A total of 330 RA-associated autoantigens were used, of which 52 are citrullinated, 263 are native, and 9 are control. Arrays were circumscribed using a hydrophobic Aqua-Hold Pap Pen 2 (Fisher Scientific). Chip was blocked overnight with PBS containing 3% fetal calf serum and 0.05% Tween 20. Arrays were probed with 1:300 diluted mouse sera, washed, and incubated in a 1:2000 dilution of Cy3-conjugated goat anti-mouse IgG + IgM secondary antibody (Jackson ImmunoResearch). Arrays were scanned using the GenePix 4000 scanner at a wavelength of 532 nm, and the median pixel intensities of the features and background values were determined using GenePix Pro version 3.0 software (Molecular Devices). Results were expressed as median fluorescence units, representing the median values from four to eight identical replicates of an antigen on each array after subtraction of the median values of both intraslide negative control bovine serum albumin and interslide negative control (blank well features). The investigator who performed the array experiments was blinded to the experimental conditions used for each sample.

### Statistical analysis

Sample size for experiments using human samples was determined using similar patient numbers per experimental condition as in our previous publications assessing inhibition of NET responses (14). No samples, mice, or data points were excluded from the reported analysis once obtained. Data were analyzed using GraphPad Prism software. For samples with non-Gaussian distribution, we used Mann-Whitney *U* test. For multiple comparisons, we used analysis of variance (ANOVA) with Bonferroni's test. Results are presented as the means  $\pm$  SEM.

See the Supplementary Materials for additional materials and methods.

### SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. ACPAs recognize NET peptides.

Fig. S2. Skin fibroblasts from patients with psoriasis internalize NETs.

Fig. S3. NETs up-regulate type I IFN-inducible genes in FLS.

Fig. S4. Endogenous expression of RAGE and TLR9 in FLS.

Fig. S5. Up-regulation of MHC II in OA FLS incubated with NETs.

Fig. S6. IFN- $\gamma$  is not detected in NETs or FLS supernatants.

Fig. S7. Detection of IL-17A and IL-17B in neutrophil lysate and NETs.

Fig. S8. NETs are internalized by a TLR9-dependent and IL-17R-independent mechanism.

Fig. S9. FLS do not express HC-gp39 or MPO.

Fig. S10. Characterization of vimentin epitope binding to HLA-DRB1\*04:04 and T cell clone specificity.

Fig. S11. Cytokine profile of cocultures of Aggrecan 225 CD4<sup>+</sup> T cells and haplotype-matched FLS.

Fig. S12. GK1.5 antibody depletes specifically CD4 T cells but not CD8 T cells.

Fig. S13. DRB1\*04:01 transgenic mice immunized with FLS loaded with NETs develop in vivo antibody responses to citrullinated peptides.

Table S1. Mass spectrometry analysis demonstrates multiple citrullinated peptides in NETs induced with IgM rheumatoid factor.

Source data Excel file

Source data blots

Reference (56)

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## Abstract

**One-sentence summary:** Neutrophil extracellular traps containing citrullinated peptides promote inflammation in synovial fibroblasts.

**Editor's Summary:**  
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Neutrophil extracellular traps (NETs) activate synovial fibroblast-like synoviocytes (FLS) in joints of individuals with rheumatoid arthritis. Now, Carmona-Rivera *et al.* investigate the mechanism behind this activation. They found that NETs containing citrullinated peptides could be internalized by FLS through the RAGE-TLR9 pathway and then loaded onto major histocompatibility complex class II and presented to antigen-specific T cells, which contribute to joint inflammation. NET-loaded FLS induced autoantibody production and joint disease in mice. These data suggest that cross-talk between NETs and FLS may contribute to rheumatoid arthritis.

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