ARTHRITIS & RHEUMATISM
Vol. 63, No. 1, January 2011, pp 53–62
DOI 10.1002/art.30081

Immune Complexes Containing Citrullinated Fibrinogen Costimulate Macrophages via Toll-like Receptor 4 and Fcγ Receptor

Jeremy Sokolove, Xiaoyan Zhao, Piyanka E. Chandra, and William H. Robinson

Objective. **Rheumatoid arthritis (RA) is associated with the presence of anti–citrullinated protein antibodies (ACPAs). Nearly two-thirds of patients with ACPA-positive RA have immune complexes that contain citrullinated fibrinogen, and these citrullinated fibrinogen–containing immune complexes (cFb-IC) can exacerbate disease in murine models of RA; however, the exact role of such ACPA ICs in RA pathogenesis has remained elusive. We undertook the present study to investigate a novel mechanism by which ACPAs specifically targeting citrullinated fibrinogen may directly stimulate macrophage tumor necrosis factor (TNF) production.**

Methods. **Murine or human macrophages were stimulated with native fibrinogen (nFb), cFb, or in vitro–generated nFb-IC or cFb-IC, and TNF production was measured by enzyme-linked immunosorbent assay. ICs were generated with either polyclonal anti-Fb antibodies or pooled IgG from patients with ACPA-positive RA. To evaluate the role of the Toll-like receptor 4 (TLR-4)/myeloid differentiation protein (MyD88) pathway and the Fcγ receptor (FcγR) pathway in the induction of TNF by Fb and Fb-IC, parallel experiments were performed using 1) TLR-4–deficient or MyD88-deficient macrophages, and 2) inhibitors of TLR-4 or FcγR.**

Results. **Citrullinated Fb stimulated macrophage TNF production more potently than did native Fb. Incorporation of cFb into ICs augmented its ability to stimulate TNF production by macrophages. Stimulation of TNF by cFb was dependent on TLR-4 and MyD88, while stimulation by cFb-IC was dependent on both TLR-4/MyD88 and FcγR.**

Conclusion. **We demonstrated that cFb-IC can costimulate macrophages via dual engagement of TLR-4 and FcγR, resulting in the synergistic induction of TNF production. Our findings suggest a potential role of citrullination in increasing the potency of an endogenous innate immune ligand and provide insight into the mechanism by which anticitrulline autoimmunity may contribute to the onset and propagation of inflammation in RA.**

Rheumatoid arthritis (RA) is an autoimmune disease characterized by the presence of autoantibodies, specifically those targeting proteins that have undergone posttranslational conversion of peptidyl arginine to citrulline, a process known as citrullination. Although citrullination occurs at sites of inflammation in diverse inflammatory conditions (1,2), the presence of anti–citrullinated protein antibodies (ACPAs) is specific to RA. The physiologic targets of ACPAs are many and diverse (3); nevertheless, nearly two-thirds of ACPA-positive RA patients have immune complexes containing citrullinated fibrinogen (cFb-IC), both circulating and deposited in the synovium (4). Although transfer of ACPAs that target cFb has been shown to exacerbate disease in a murine model of RA (5), the role of citrullination in inflammation and the mechanism by which ACPAs may contribute to RA pathogenesis has not been elucidated.

A role of fibrinogen in the pathogenesis of auto-

Supported by NIH/National Institute of Arthritis and Musculoskeletal and Skin Diseases grants RC1-AR-058713 and R01-AR-054822, an American College of Rheumatology Research and Education Foundation Within Our Reach Award, and VA Health Care System funding to Dr. Robinson. Dr. Sokolove received salary support from an American College of Rheumatology Research and Education Foundation Physician Scientist Development Award.

Jeremy Sokolove, MD, Xiaoyan Zhao, PhD, Piyanka E. Chandra, BA, William H. Robinson, MD, PhD: Stanford University School of Medicine, Stanford, California and the VA Palo Alto Health Care System, Palo Alto, California.

Address correspondence to Jeremy Sokolove, MD, VA Palo Alto Health Care System, Mail Stop 154R, 3801 Miranda Avenue, Palo Alto, CA 94304. E-mail: sokolove@stanford.edu.

Submitted for publication May 10, 2010; accepted in revised form September 30, 2010.

immune arthritis has been suggested by the finding that Fb-deficient mice are protected against the development of collagen-induced arthritis (CIA) (6). In addition, impaired resolution of synovitis during antigen-induced arthritis in urokinase-deficient mice is associated with excessive deposition of fibrin in the synovium and can be reversed by treatment with a fibrinolytic agent (7). Mice expressing a mutant form of Fb that is unable to interact with monocytes and macrophages, while maintaining full clotting function, were also found to be relatively resistant to CIA (6), suggesting that interaction with monocyte/macrophages is important in the pathogenicity of Fb.

Macrophages accumulate in the synovial lining and sublining in RA, where they produce cytokines and chemokines that mediate inflammation, as well as destruction of cartilage and bone (8). The most prominent of the macrophage-produced cytokines is tumor necrosis factor (TNF), a molecule that mediates much of the articular and extraarticular pathology associated with RA (9). Interestingly, Fb deficiency attenuated TNF expression in the joints of mice with CIA but could not protect against inflammatory arthritis in transgenic mice overexpressing TNF, suggesting that Fb acts upstream of TNF in the promotion of synovitis (6).

Macrophages can be activated by T cell contact or T cell–released cytokines such as interferon-γ or interleukin-17 (IL-17), by ICs which trigger Fcγ receptor (FcγR) signaling, and by innate immune receptors, such as Toll-like receptors (TLRs). TLRs are pattern-recognition receptors that recognize conserved microbial products known as pathogen-associated molecular patterns (PAMPs), as well as endogenous ligands known as damage-associated molecular patterns (DAMPs) (8).

Both TLR-2 and TLR-4 are highly expressed in RA synovial tissue (10,11), and macrophages isolated from the joints of RA patients exhibit increased levels of TLR-2 and TLR-4 and are more responsive to PAMPs (12). Furthermore, findings in animal models have implicated TLR-4 in the pathogenesis of RA (13–15). Although bacterial TLR ligands have been observed in RA synovial tissue (16,17), endogenous TLR ligands, such as Hsp96 (18) and tenascin-C (19), have also been implicated in RA pathogenesis. Fb, too, has been shown to serve as an endogenous activator of TLR-4 (20,21) and is highly expressed in RA synovium (22).

Thus, Fb can induce TLR-4 signaling, and cFb-IC can exacerbate murine arthritis. What remains unclear, however, is the extent to which these processes are interlinked and the role played by citrullination and ACPA in RA. In this study, we investigated one mechanism by which cFb-IC can stimulate macrophage TNF production. We demonstrated that 1) citrullination renders fibrinogen a more potent stimulator of macrophage cytokine production; 2) this stimulation is TLR-4 dependent; and 3) cFb-IC can costimulate macrophages via TLR-4 and FcγR, resulting in synergistic induction of TNF. These findings suggest that citrullination boosts the local inflammatory response at sites of damage or inflammation and provide the first explanation to date for the pathogenic specificity of an ACPA target.

MATERIALS AND METHODS

Sample collection. Serum was obtained from peripheral blood collected from patients with RA or osteoarthritis (OA). All RA patients met the American College of Rheumatology criteria for the disease (23). The patients were recruited from the Palo Alto Veterans Administration Medical Center. Informed consent was obtained from all participating patients, and the studies were approved by the Stanford University Institutional Review Board. Cell-free plasma was isolated by centrifugation and stored at –80°C.

Cell isolation and culture. Peritoneal exudate macrophages (PEMs) from wild-type C57BL/6 mice (The Jackson Laboratory) and from *TLR-4*$^{Lps\text{-}n}$ C57BL/6 mice and *MyD88*$^{-/-}$ C57BL/6 mice (both a generous gift from Dr. Lawrence Steinman, Stanford University) were obtained by peritoneal lavage with 8 ml of cold Dulbecco's modified Eagle's medium (DMEM). PEMs were allowed to adhere to plastic wells for 1 hour, washed with DMEM, and cultured overnight in DMEM supplemented with 10 ng/ml murine macrophage colony-stimulating factor (M-CSF) (PeproTech). For costimulation experiments, PEMs were pretreated for 12 hours with 100 units/ml murine interferon-γ (PeproTech). The RAW 264.7 murine macrophage cell line was purchased from American Tissue Culture Center and used in experiments within 15 passages. RAW 264.7 macrophages were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 units of penicillin, 100 μg/ml streptomycin, and 100 μ*M* glutamine.

For generation of monocyte-derived macrophages, peripheral blood mononuclear cells (PBMCs) were obtained by density-gradient centrifugation of buffy coats (Stanford Blood Center) over Ficoll (Invitrogen). Human monocytes were purified from PBMCs by negative selection as recommended by the manufacturer (Miltenyi Biotec) and differentiated into macrophages by culture for 7 days in RPMI containing 10% FCS and 50 ng/ml human M-CSF. Alternatively, monocytes were purified by adhesion for 2 hours, washed, and then cultured as described above. M-CSF–containing media were replaced after 3 days. After 7 days in M-CSF culture, macrophage purity obtained with monocyte negative selection was observed to be similar to that obtained with selection by adhesion. The experiments using animal or human materials were approved by the Stanford University Institutional Review Board.

Antibodies and reagents. Lipopolysaccharide (LPS) was from Sigma-Aldrich, and CpG-containing oligodeoxy-

nucleotides (ODNs) and the TLR-4 inhibitor CLI-095 were from InvivoGen. Murine FcγRII/III-blocking antibody (2.4G2) was from eBioscience. Anti-human CD32 (FcγRIIa) antibody (clone IV.3) was from Stem Cell Technologies. Purified human fibrinogen depleted of von Willebrand factor and fibronectin (Enzyme Research Laboratories) were used in either unmodified native form or citrullinated form. In vitro citrullination of fibrinogen was performed as previously described (4) and confirmed by mobility shift on sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis and by dot blot analysis using human ACPA-positive RA sera, antifibrinogen antibodies (DakoCytomation), and anti–modified citrulline antibodies (Millipore). Native Fb (nFb) was subjected to sham citrullination, in which it was processed in an identical manner to cFb but without the addition of the peptidyl arginine deiminase (PAD) enzyme. PAD enzyme incubated with citrullination buffer and dithiothreitol but without Fb served as a control to assure no contribution or contamination from the small amount of enzyme remaining in cFb. For some experiments, cFb and nFb were dialyzed against phosphate buffered saline (PBS) with a Slidalyzer (Pierce); macrophage stimulation with dialyzed or nondialyzed Fb produced identical results (data not shown), confirming that the citrullination buffer was not a confounding factor.

Macrophage stimulation. Murine macrophages (1×10^5) were incubated with nFb, cFb, nFb-IC, or cFb-IC for 16–18 hours, after which TNF levels in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA; PeproTech). The TLR-4 ligand LPS (100 ng/ml) and the TLR-9 ligand CpG ODN (1 μg/ml) were used as controls for TNF induction and TLR-4 specificity. ICs were generated in vitro by incubation of nFb or cFb with a polyclonal rabbit antibody against human fibrinogen (DakoCytomation) or, as a control, with normal polyclonal rabbit IgG (DakoCytomation) at 37°C for 45 minutes. Cross-titration of antibody and antigen yielded an optimal ratio for formation of ICs: final concentrations of 10 μg/ml of Fb and 50 μg/ml of antibody were used for IC stimulation of RAW 267.4 cells, while 50 μg/ml of Fb and 100 μg/ml of antibody were used for IC stimulation of PEMs and human monocyte–derived macrophages. At final dilutions, all reagents used in the stimulation assays were tested for endotoxin contamination by application of the *Limulus* amebocyte assay according to the instructions of the manufacturer (Associates of Cape Cod), and were shown to possess endotoxin levels below the detectable range (<0.03 endotoxin units/ml). For further confirmation that endotoxin contamination was not a confounding factor, in some experiments fibrinogen was treated with Detoxigel endotoxin-removal resin (Pierce) or stimulations were performed in the presence of 10 μg/ml of polymyxin B (Sigma Aldrich).

For IC stimulation of human macrophages, human IgG derived from patients with ACPA-positive RA was used to generate platebound human cFb-IC. IgG from 3 pooled plasma samples shown by ELISA to contain high levels of anti-cFb antibodies was purified by affinity chromatography on protein G columns, according to the instructions of the manufacturer (Pierce). The eluted IgG fractions were concentrated by centrifugation with buffer exchange to PBS (Amicon Ultra; Millipore) and were depleted of endotoxin by filtration through a polymyxin B column (Detoxigel). IgG concentrations were estimated according to optical density at 280 nm; IgG was aliquoted, and stored at −80°C. For generation of cFb-IC, flat-bottomed 96-well culture plates were coated overnight at 4°C with 50 μl of cFb (20 μg/ml), washed in PBS containing 0.05% Tween 20, and then incubated for 2 hours at 4°C with 100 μl of anti-cFb–positive IgG (2.5 mg/ml) or, as a control, with citrullination buffer alone. Wells were again washed in PBS containing 0.05% Tween 20, and macrophages (50–75,000/well) in 200 μl RMPI containing 5% FCS were then added to the wells.

Detection of antibodies to nFb or cFb. For detection of antibodies to nFb or cFb, we used a direct ELISA that we developed previously (4). Briefly, microtiter plates (Nunc) were coated with 50 μl of nFb or cFb in carbonate buffer, at a concentration of 20 μg/ml, and incubated at 4°C overnight. After blocking in PBS containing 1% bovine serum albumin (BSA), plasma samples were diluted 1:10 in dilution buffer (PBS containing 0.1% BSA and 0.1% Tween-20), added to the plate in duplicate at 100 μl/well, and incubated for 2 hours at room temperature. The samples were then incubated for 1 hour at room temperature with a 1:5,000 dilution of a monoclonal, horseradish peroxidase–labeled goat anti-human antibody. The reaction was developed by application of 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich) for 15 minutes and stopped by addition of 50 μl of 2*N* H_2SO_4. Relative quantification of antibodies against nFb or cFb was performed by optical densitometry.

Statistical analysis. Student's unpaired *t*-test (GraphPad Software) was used to compare cytokine production between groups. *P* values less than 0.05 were considered significant.

RESULTS

Citrullinated Fb stimulates macrophage TNF production. Native Fb has been shown to stimulate the production of cytokines by monocytes and macrophages (20,21). To determine the effect of citrullination on the proinflammatory properties of fibrinogen, we compared the effect of nFb and cFb on macrophage TNF production. Native Fb induced the production of TNF by RAW 274.6 macrophages in a dose-dependent manner (Figure 1A), in accordance with previous findings (20,21). Compared to nFb, cFb was 10 times more potent an inducer of macrophage TNF production (Figure 1A). Similar results were obtained with primary murine PEMs (Figure 1B). To rule out a significant contribution of endotoxin contaminant to the induction of TNF, we performed parallel stimulation experiments in the presence of polymyxin B, an antibiotic that binds and inactivates endotoxin. Polymyxin B markedly suppressed TNF induction by LPS but did not affect TNF induction by nFb, cFb, or CpG ODN (Figure 1C), confirming that endotoxin contamination was not a significant confounding factor in these assays. These findings demonstrate that citrullination renders fibrinogen a more potent inducer of macrophage TNF production.

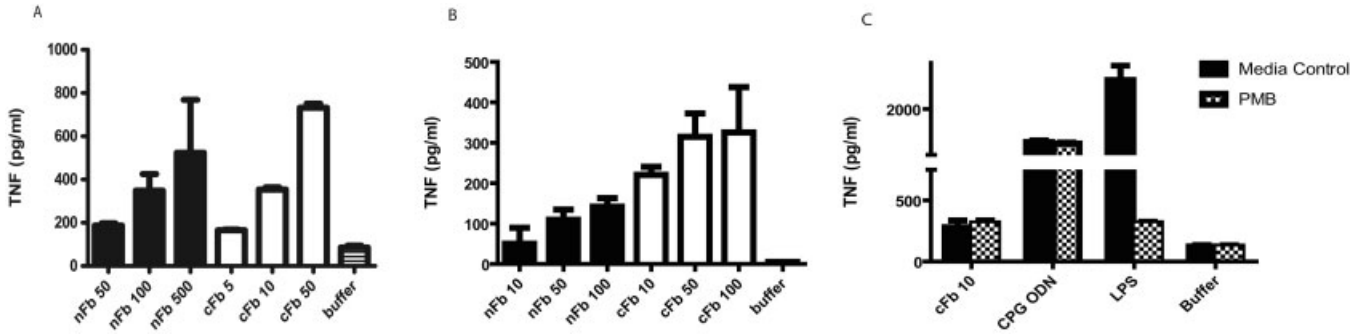

Figure 1. Citrullination renders fibrinogen a more potent inducer of macrophage tumor necrosis factor (TNF) production. **A** and **B,** RAW 267.4 macrophages (**A**) and murine peritoneal macrophages (**B**) were stimulated with native fibrinogen (nFb) or citrullinated fibrinogen (cFb) at the indicated concentrations (μg/ml). **C**, RAW 267.4 macrophages were pretreated for 30 minutes with the endotoxin inhibitor polymyxin B (PMB) (10 μg/ml) and then stimulated with cFb, CpG-containing oligodeoxynucleotide (ODN) (1 μg/ml), or lipopolysaccharide (LPS) (100 ng/ml). TNF levels in culture supernatants after 16–18 hours of stimulation were measured by enzyme-linked immunosorbent assay. Results are representative of experiments performed at least twice. Values are the mean ± SEM from triplicate cultures.

Citrullinated Fb induces TNF production via TLR-4. Native Fb has been shown to activate monocytes and macrophages via ligation of TLR-4 (20,21). To determine whether cFb activation of macrophages is also mediated by TLR-4, we examined cFb-induced TNF production in PEMs from wild-type mice and from TLR-4–deficient mice. TLR-4 deficiency abrogated TNF production in response to cFb (Figure 2A). Likewise, the TLR-4–specific inhibitor CLI-095 abrogated the production of TNF in response to cFb and LPS, but it did not affect TNF production in response to the TLR-9 agonist CpG ODN (Figure 2B).

Citrullinated Fb–containing immune complexes costimulate via TLR-4 and FcγR. To examine whether incorporation of cFb into ICs enhances its ability to stimulate macrophage TNF production, we stimulated macrophages with in vitro–generated cFb-IC or with cFb alone. Macrophages produced significantly greater amounts of TNF in response to cFb-IC than in response to cFb alone. Induction of TNF by cFb-IC was significantly greater than induction by nFb-IC (Figure 3B), suggesting that the proinflammatory response to ICs is influenced by the specific autoantigen incorporated in the IC. Furthermore, ICs containing substimulatory concentrations of cFb (i.e., that do not themselves increase TNF production) were also able to induce substantial TNF production, whereas nFb-IC in similar concentrations induced only minimal TNF production (Figure 3B). Treatment with anti-Fb antibody alone did not significantly stimulate TNF production. Unlike nFb-IC or cFb-IC, a mixture of cFb and normal polyclonal rabbit IgG (which is unable to form ICs) did not increase TNF production above that achieved with cFb

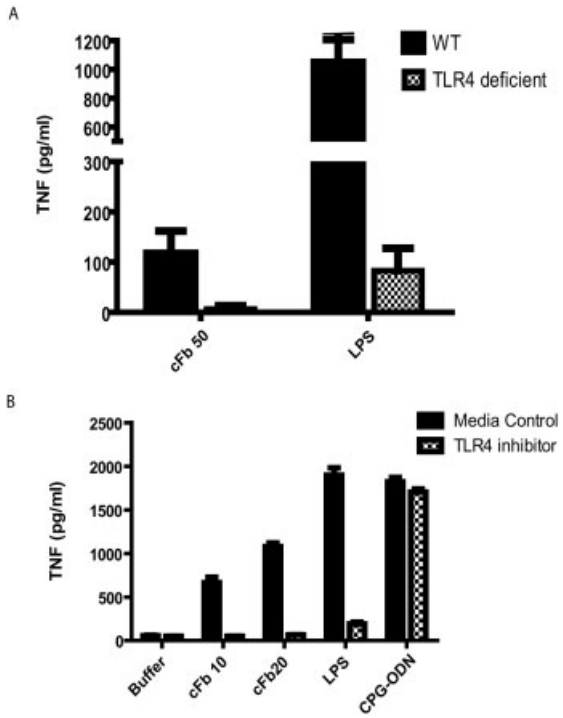

Figure 2. Citrullinated fibrinogen induces macrophage TNF production via Toll-like receptor 4 (TLR-4). **A**, Peritoneal macrophages from TLR-4–deficient or wild-type (WT) B6 mice were stimulated with cFb (50 μg/ml) or LPS (100 ng/ml). **B**, RAW 267.4 macrophages were pretreated with the TLR-4 inhibitor CLI-095 (0.5 μg/ml) or vehicle and then stimulated with increasing concentrations of cFb (10 or 20 μg/ml), with the TLR-4 agonist LPS (100 ng/ml), or with the TLR-9 agonist CPG ODN (1 μg/ml). TNF levels in culture supernatants after 16–18 hours of stimulation were measured by enzyme-linked immunosorbent assay. Results are representative of experiments performed at least twice. Values are the mean ± SEM from triplicate cultures. See Figure 1 for other definitions.

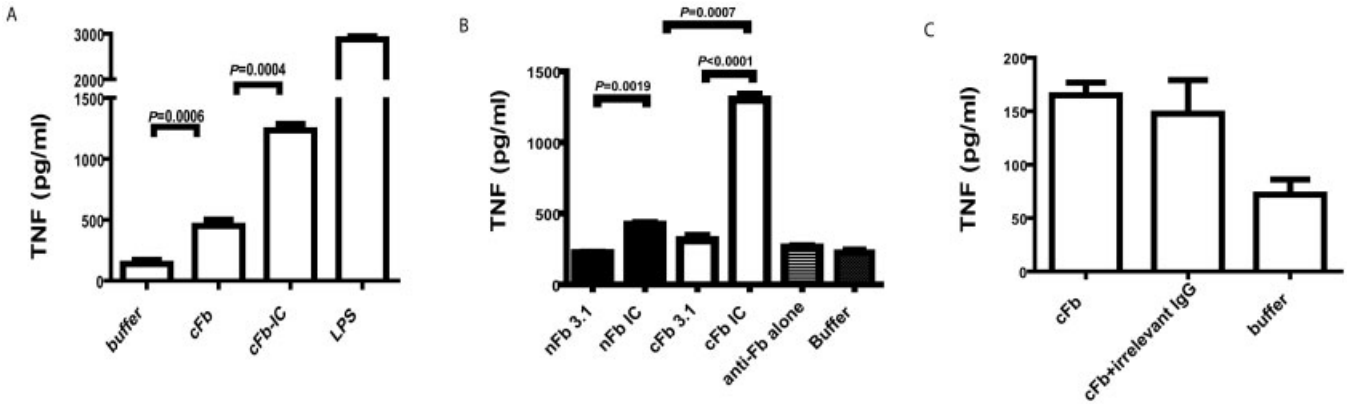

Figure 3. Citrullinated Fb–containing immune complexes (cFb-IC) costimulate macrophage TNF production. **A**, RAW 267.4 macrophages were stimulated with cFb (10 μg/ml) alone or with cFb-IC generated by incubating cFb (10 μg/ml) with polyclonal anti-Fb antibody (50 μg/ml). Citrullination buffer alone and LPS (100 ng/ml) were used as negative and positive controls, respectively. **B**, RAW 267.4 macrophages were stimulated with Fb-IC generated using nFb or cFb at a concentration that alone elicits negligible macrophage TNF production (3.1 μg/ml), with nFb or cFb (3.12 μg/ml) alone, or with anti-Fb antibody alone. **C**, RAW 267.4 cells were stimulated with cFb (10 μg/ml) alone or incubated with normal polyclonal IgG (50 μg/ml). TNF levels in culture supernatants after 16–18 hours of stimulation were measured by enzyme-linked immunosorbent assay. Results are representative of experiments performed at least twice. Values are the mean ± SEM from triplicate cultures. See Figure 1 for other definitions.

alone (Figure 3C). Taken together, these findings suggest that incorporation of cFb into ICs enhances its ability to induce macrophage TNF production. Interestingly, measurement of several other inflammatory cytokines, including IL-1, IL-6, and IL-12, in the same culture supernatants did not demonstrate a similar pattern of cFb-IC–induced costimulation (data not shown).

To determine whether TLR-4 is required for cFb-IC–mediated TNF production, we examined cFb-IC–induced TNF production in PEMs from wild-type, TLR-4–deficient, and MyD88-deficient mice. TLR-4 deficiency abrogated both cFb- and cFb-IC–induced TNF production (Figure 4A). Signaling downstream of TLR-4 bifurcates into a TRIF-dependent pathway and an MyD88-dependent pathway (24). We found that cFb- and cFb-IC–induced TNF production was abrogated in MyD88-deficient PEMs, indicating that cFb-induced TNF production is dependent on the TLR-4/MyD88 signaling pathway. The small-molecule TLR-4 inhibitor CLI-095 also abrogated the macrophage response

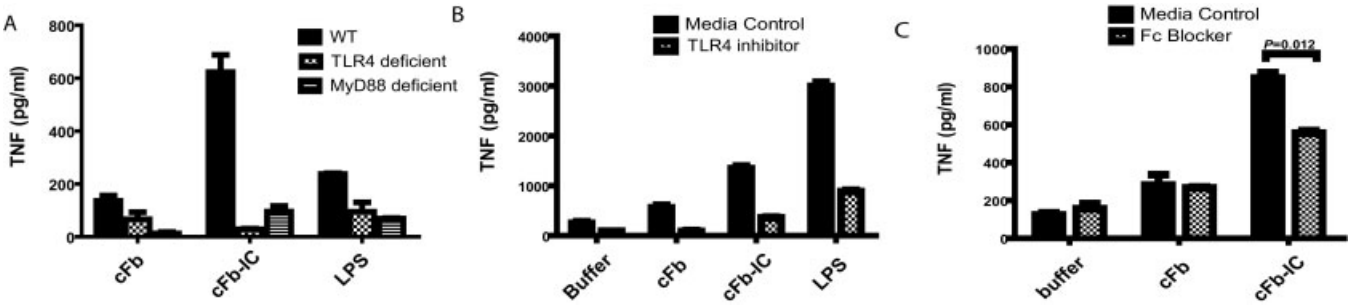

Figure 4. Costimulation of macrophage TNF production by cFb-containing immune complexes (cFb-IC) is dependent on Toll-like receptor 4 (TLR-4) and Fcγ receptor (FcγR). **A**, Peritoneal macrophages from wild-type (WT), TLR-4–deficient, or myeloid differentiation factor 88 (MyD88)–deficient mice were stimulated with cFb (50 μg/ml) alone or cFb-IC (50 μg/ml of cFb incubated with 75 μg/ml of polyclonal anti-Fb antibody). The TLR-4 agonist LPS (100 ng/ml) was used as a positive control. **B**, RAW 267.4 macrophages were pretreated for 30 minutes with the TLR-4 inhibitor CLI-095 (0.5 μg/ml) or media control and then stimulated with cFb (10 μg/ml) alone or cFb-IC (10 μg/ml of cFb incubated with 50 μg/ml of polyclonal anti-Fb antibody). **C**, RAW 267.4 macrophages were pretreated for 30 minutes with FcγR-blocking antibody (10 μg/ml), and then pretreated wells and untreated control wells were washed twice with serum-free media and stimulated with cFb alone or cFb-IC. TNF levels in culture supernatants after 16–18 hours of stimulation were measured by enzyme-linked immunosorbent assay. Results are representative of experiments performed at least twice. Values are the mean ± SEM from triplicate cultures. See Figure 1 for other definitions.

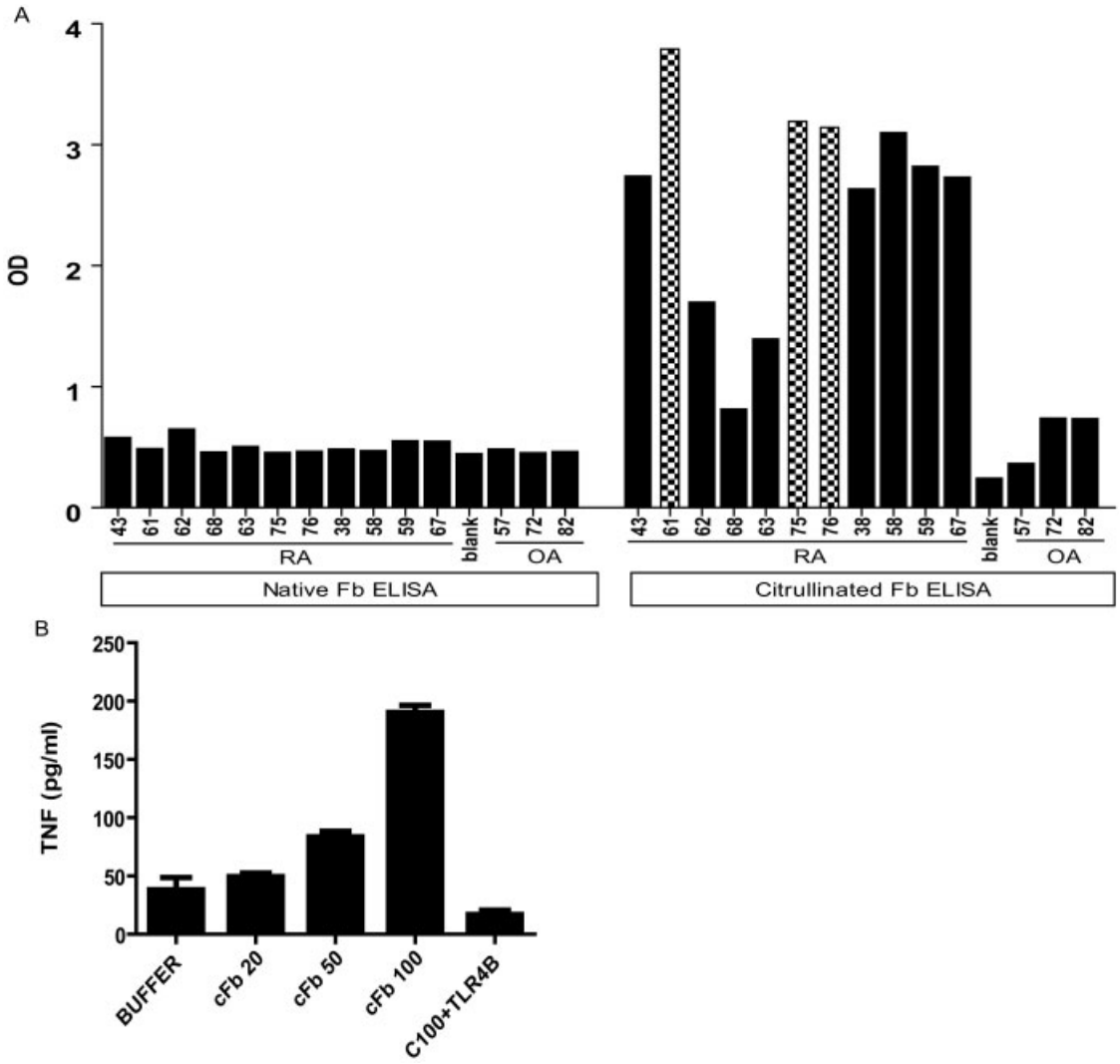

Figure 5. Citrullinated Fb stimulates TNF induction by human macrophages. **A,** Plasma samples from patients with anti–citrullinated protein antibody–positive rheumatoid arthritis (RA) or from patients with osteoarthritis (OA) were assayed by enzyme-linked immunosorbent assay (ELISA) for reactivity against nFb (left) and cFb (right). The highest levels of cFb reactivity were found in patients 61, 75, and 76 (checkered bars). **B,** Human monocyte–derived macrophages were stimulated for 18 hours with increasing concentrations (20, 50, or 100 μg/ml) of cFb in solution. For assessment of Toll-like receptor 4 (TLR-4) dependence, cells were pretreated with the TLR-4 inhibitor CLI-095 (TLR4B; 1.0 μg/ml) before addition of cFb at 100 μg/ml (C100). Results are representative of experiments performed at least twice. Values are the mean ± SEM from duplicate cultures. OD = optical density (see Figure 1 for other definitions).

tocFb-IC (Figure 4B), confirming the dependence of the response on the TLR-4 signaling pathway.

ICs activate cell signaling by binding to FcγR on the surface of innate immune effector cells (25). To determine whether cFb-IC stimulate macrophages through costimulation of both FcγR and TLR-4, we pretreated macrophages with an FcγR-blocking antibody. Blockade of FcγR significantly reduced TNF production in response to cFb-IC (Figure 4C). As expected, FcγR blockade had no effect on TNF production induced by cFb alone. Taken together, these results suggest that cFb-IC serve as potent inducers of macrophage TNF production by coligating TLR-4 and the FcγR.

Citrullinated Fb is targeted by autoantibodies in most ACPA-positive RA patients, but nFb is not. Citrullinated Fb has been described as a target of ACPA (26), and we previously detected the presence of cFb-

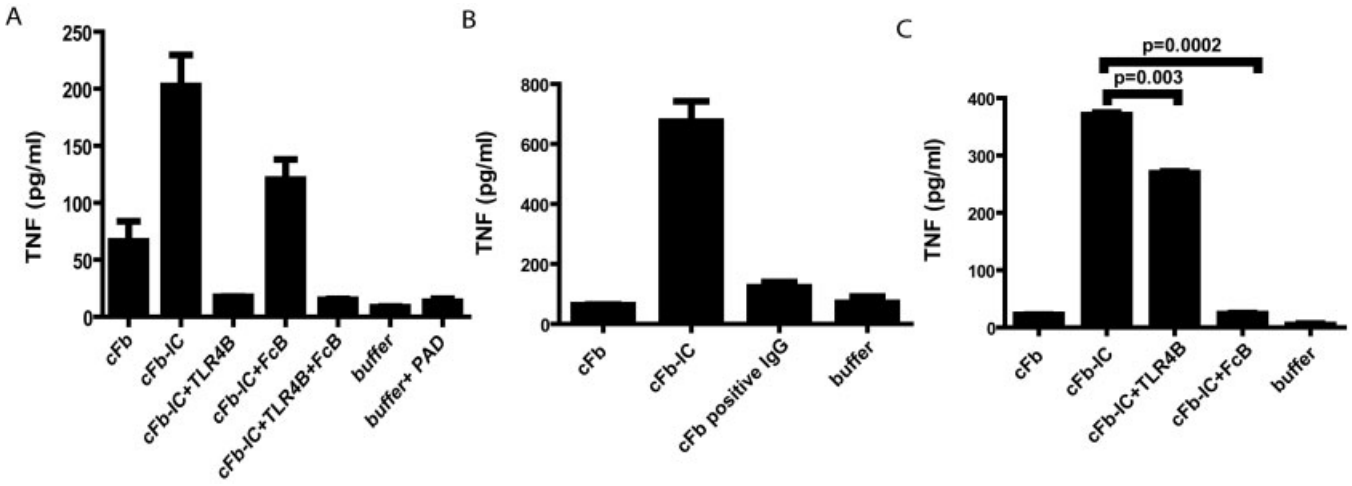

Figure 6. Citrullinated Fb immune complexes (cFb-IC) formed with polyclonal anti-Fb antibodies or IgG from rheumatoid arthritis (RA) patients costimulate human macrophages to produce TNF. **A,** Human monocyte–derived macrophages were pretreated for 30 minutes with the Toll-like receptor 4 (TLR-4) inhibitor CLI-095 (TLR4B; 1.0 μg/ml), Fcγ receptor IIa (FcγRIIa)–blocking antibody (FcB; 10 μg/ml), both inhibitors, or media alone and then stimulated with cFb (50 μg/ml) or cFb-IC (50 μg/ml of cFb incubated with 75 μg/ml of polyclonal anti-Fb antibody). Plates coated with buffer alone or with buffer and peptidyl arginine deiminase (PAD) were used as negative controls. **B,** Human monocyte–derived macrophages were added to plates precoated with cFb-IC generated using pooled IgG from 3 RA plasma specimens with high levels of anti-cFb antibodies (patients 61, 75, and 76 in Figure 5A). Wells coated with nFb incubated with IgG containing anti-cFb antibodies and plates coated with buffer alone were used as negative controls. **C,** For assessment of TLR-4 dependence and/or FcγR dependence, an identical aliquot of monocyte-derived macrophages was preincubated with CLI-095 (1.0 μg/ml) or FcγRIIa-blocking antibody before being added to the cFb-IC–coated plates. Results are representative of experiments performed at least twice. Values are the mean ± SEM from triplicate cultures. See Figure 1 for other definitions.

containing ICs both in the circulation and in the synovium of patients with ACPA-positive RA (4). To confirm this finding in an additional cohort and to obtain purified IgG for further experimentation, we measured the levels of antibodies against nFb or cFb in plasma from 11 patients with ACPA-positive RA and 3 patients with OA. Antibodies targeting cFb, but not nFb, were found in a majority (73%) of the ACPA-positive RA patients tested (Figure 5A). In contrast, control sera from OA patients targeted neither nFb nor cFb, consistent with prior observations (26).

RA patient–derived IgG can form cFb-IC and costimulate TNF production from human monocyte–derived macrophages. To determine the relevance of our findings in a human system, we evaluated the ability of cFb to stimulate TNF production from human monocyte–derived macrophages. We demonstrated a clear dose response to cFb (though at concentrations slightly higher than that needed to stimulate murine RAW cells), and this effect was TLR-4 dependent (Figure 5B). PAD enzyme at a concentration identical to that obtained with cFb 100 μg/ml displayed negligible stimulatory capacity for macrophage TNF release (Figure 6A). We then used in vitro–generated cFb-IC formed with polyclonal antifibrinogen antibody (as above) to again demonstrate TLR-4– and FcγR-dependent costimulation of human macrophages (Figure 6A).

To further demonstrate the potential importance of cFb-IC–mediated macrophage costimulation in human RA, we stimulated human monocyte–derived macrophages with cFb-IC generated by incubating platebound cFb with pooled IgG from 3 ACPA-positive RA patients (patients 61, 75, and 76 in Figure 5A). Platebound cFb showed a nonsignificant trend toward stimulation of TNF production. Significantly higher levels of TNF were induced with cFb-IC than with cFb alone, and in plates coated with nFb incubated with an identical concentration of IgG containing anti-cFb antibodies there was no stimulation of macrophage TNF production (Figure 6B), ruling out endotoxin contamination from human IgG. Interestingly, though both TLR-4 and FcγR inhibition abrogated macrophage TNF production induced by stimulation with platebound cFb-IC, the role of FcγR seemed more significant and TLR-4 less significant (Figure 6C). TLR-4 blockade alone resulted in nearly complete abrogation of macrophage TNF release in response to soluble ICs, while FcγR blockade resulted in nearly complete abrogation in response to platebound ICs. Thus, concomitant blockade of both TLR-4 and FcγR resulted in minimal additional abrogation of TNF release in our assay (Figure 6A).

DISCUSSION

Neither the mechanisms contributing to the persistent activation of macrophages in RA synovium nor the role of ACPAs in RA pathogenesis have been fully elucidated. In the present study we showed that two interlinked processes can augment the proinflammatory properties of fibrinogen: 1) citrullination of Fb increases its potency as a TLR-4 agonist and hence an inducer of TNF production, and 2) incorporation of cFb into ICs further increases its potency as an inducer of TNF production by enabling costimulation of macrophages via simultaneous ligation of TLR-4 and FcγR. Thus, a scenario can be envisioned in which local inflammation in the RA synovium induces citrullination of Fb, resulting in the generation of cFb-specific ACPAs and the consequent formation of cFb-IC, which in turn amplify local inflammation and lead to further citrullination of Fb. In this way, cFb-IC could potentially participate in a feed-forward amplification loop that propagates the synovial inflammation characteristic of RA.

In addition to Fb, several other innate immune ligands of TLR-4, including Hsp22 (27), Hsp60 (28), Hsp70 (29), biglycan (30), fibronectin (31), and oligosaccharides of hyaluronic acid (32), have been identified and, in some cases (18,19), implicated in the pathogenesis of RA. Notably, tenascin-C was shown to induce TLR-4–mediated inflammation in synovial joints via its fibrinogen globe region (19), a region with significant homology to the globular domain of the β- and γ-chains of fibrinogen (33). Several lines of evidence suggest that, among the many targets of the ACPA immune response, cFb is an important contributor to RA pathogenesis: immunization of mice with citrulline-containing Fb can induce murine arthritis (34), transfer of anti-cFb antibodies can exacerbate murine arthritis (5), and the majority of patients with ACPA-positive RA have cFb-IC (4). Our current observations provide mechanistic evidence in support of a key role of cFb-IC. Nevertheless, not all ACPA-positive RA patients have cFb-IC. It is therefore notable that several other DAMPs have also been identified as targets of the RA-associated autoantibody response (35,36). Thus, the paradigm of autoantigen/IC-mediated inflammation via innate immune receptors may extend beyond cFb to other targets of the immune response in RA.

The findings described herein provide the first reported example of an inflammation-associated posttranslational modification that directly increases the potency of the DAMP-induced inflammatory response. Because citrullination is a widespread phenomenon during inflammation, its ability to modify local DAMPs could have far-reaching implications regarding the innate immune response in a variety of inflammatory disorders. Citrullinated Fb itself could also potentially play a role in a variety of inflammatory disorders. Although normally confined to the vascular space, during inflammation Fb typically exudes into extravascular tissue—the same sites of inflammation to which neutrophils and monocytes migrate and release the PAD enzymes that mediate protein citrullination (37).

DNA-containing ICs have been shown to costimulate TLR-9 and B cell receptor (38) or TLR-9 and FcγR (39,40), and are thought to play an important role in the pathogenesis of systemic lupus erythematosus (41). Herein we show that, in an analogous manner, an RA-associated IC can coligate TLR-4 and FcγR, and that such an IC can elicit a potent inflammatory response from the macrophage, through the synergistic action of an innate immune receptor and an adaptive immune receptor.

A limitation of this study was the use of a commercial polyclonal anti-Fb antibody for the generation of ICs. We screened several polyclonal and monoclonal antibodies, and all demonstrated some preference for cFb or nFb, as assessed by dot-blot analysis (results not shown). The antibody used in this study was chosen on the basis of its ability to bind to nFb and cFb with equal affinity, thereby enabling us to demonstrate the importance of the cFb antigen in the synergistic cFb-IC–induced stimulation of macrophage TNF production. We further demonstrated similar costimulation by cFb-IC derived from ACPA-positive IgG, thus reinforcing the validity of results obtained using the polyclonal anti-Fb antibody. Antibodies isolated from ACPA-positive RA patients have been shown to bind cFb and stimulate macrophage TNF production, in a study that was limited to the effects mediated through FcγRIIa (42).

Although TNF production in response to cFb alone was not detected in the above-mentioned study (42), only platebound cFb was examined. We too found that, whereas addition of cFb to the culture media could induce TNF production, platebound cFb alone could not. This could be due to the relatively low concentrations of bound cFb remaining after washing or, alternatively, to the constrained structure of platebound cFb, such that it can no longer bind TLR-4 or accessory receptors. This may additionally explain why FcγR blockade was proportionally more inhibitory in the setting of platebound ICs, while TLR-4 blockade was more inhibitory in the setting of soluble ICs. Which

formation is more relevant in vivo remains unclear. However, previous work in our laboratory clearly demonstrated the presence of cFb-IC both circulating in blood and in RA pannus tissue (4). Given that citrullination of Fb has been shown to inhibit fibrin polymerization (43), it is possible that citrullination may contribute to the ability of Fb to remain soluble and thus available for both innate immune signaling and IC formation. Finally, as with most studies on citrullination and the ACPA immune response, in vitro citrullination of an antigen may not accurately reflect the true physiologic process that occurs in vivo.

Our data support the notion that ACPA, and specifically cFb-IC, has a role in RA pathogenesis. Not only is cFb capable of inducing robust cytokine production by macrophages, but cFb-IC can further enhance macrophage TNF production by costimulating TLR-4 and FcγR. Exudation and citrullination of Fb at sites of inflammation (such as the RA joint) may provide a potent inflammatory signal for macrophage cytokine production, and the ability of cFb-IC to costimulate macrophage TNF production suggests that citrullination of autoantigens, which are themselves self adjuvants, may provide a mechanism for the amplification and perpetuation of the inflammatory response. TLR-4 activation is increased in RA synovial fluid macrophages (12), and findings from both murine models (13,14) and early-phase clinical trials (44) suggest that TLR-4 blockade may have potential as a therapeutic approach in RA. These observations, taken together with the present findings, suggest several potential therapeutic targets, including the citrullination of innate inflammatory proteins, the production of anti–citrullinated protein antibodies, and the activation of the TLR-4 pathway.

ACKNOWLEDGMENTS

We thank Drs. Peggy Ho and Lawrence Steinman for providing TLR-4– and MyD88-deficient mice. We are grateful to the members of Dr. Robinson's laboratory for their insight into this work and Dr. Tamsin Lindstrom for critical review of the manuscript.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sokolove had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sokolove, Zhao, Robinson.

Acquisition of data. Sokolove, Zhao, Chandra.

Analysis and interpretation of data. Sokolove, Zhao, Chandra, Robinson.

REFERENCES

1. Makrygiannakis D, af Klint E, Lundberg IE, Lofberg R, Ulfgren AK, Klareskog L, et al. Citrullination is an inflammation-dependent process. Ann Rheum Dis 2006;65:1219–22.
2. Vossenaar ER, Smeets TJ, Kraan MC, Raats JM, van Venrooij WJ, Tak PP. The presence of citrullinated proteins is not specific for rheumatoid synovial tissue. Arthritis Rheum 2004;50:3485–94.
3. Hueber W, Kidd BA, Tomooka BH, Lee BJ, Bruce B, Fries JF, et al. Antigen microarray profiling of autoantibodies in rheumatoid arthritis. Arthritis Rheum 2005;52:2645–55.
4. Zhao X, Okeke NL, Sharpe O, Batliwalla FM, Lee AT, Ho PP, et al. Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis. Arthritis Res Ther 2008;10:R94.
5. Kuhn KA, Kulik L, Tomooka B, Braschler KJ, Arend WP, Robinson WH, et al. Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. J Clin Invest 2006;116:961–73.
6. Flick MJ, LaJeunesse CM, Talmage KE, Witte DP, Palumbo JS, Pinkerton MD, et al. Fibrin(ogen) exacerbates inflammatory joint disease through a mechanism linked to the integrin $\alpha_M\beta_2$ binding motif. J Clin Invest 2007;117:3224–35.
7. Busso N, Peclat V, van Ness K, Kolodziesczyk E, Degen J, Bugge T, et al. Exacerbation of antigen-induced arthritis in urokinase-deficient mice. J Clin Invest 1998;102:41–50.
8. Drexler SK, Kong PL, Wales J, Foxwell BM. Cell signalling in macrophages, the principal innate immune effector cells of rheumatoid arthritis. Arthritis Res Ther 2008;10:216.
9. Feldmann M, Brennan FM, Williams RO, Woody JN, Maini RN. The transfer of a laboratory based hypothesis to a clinically useful therapy: the development of anti-TNF therapy of rheumatoid arthritis. Best Pract Res Clin Rheumatol 2004;18:59–80.
10. Iwahashi M, Yamamura M, Aita T, Okamoto A, Ueno A, Ogawa N, et al. Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis. Arthritis Rheum 2004;50:1457–67.
11. Radstake TR, Roelofs MF, Jenniskens YM, Oppers-Walgreen B, van Riel PL, Barrera P, et al. Expression of Toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-γ. Arthritis Rheum 2004;50:3856–65.
12. Huang Q, Ma Y, Adebayo A, Pope RM. Increased macrophage activation mediated through Toll-like receptors in rheumatoid arthritis. Arthritis Rheum 2007;56:2192–201.
13. Abdollahi-Roodsaz S, Joosten LA, Roelofs MF, Radstake TR, Matera G, Popa C, et al. Inhibition of Toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis. Arthritis Rheum 2007;56:2957–67.
14. Choe JY, Crain B, Wu SR, Corr M. Interleukin 1 receptor dependence of serum transferred arthritis can be circumvented by Toll-like receptor 4 signaling. J Exp Med 2003;197:537–42.
15. Lee EK, Kang SM, Paik DJ, Kim JM, Youn J. Essential roles of Toll-like receptor-4 signaling in arthritis induced by type II collagen antibody and LPS. Int Immunol 2005;17:325–33.
16. Schrijver IA, Melief MJ, Tak PP, Hazenberg MP, Laman JD. Antigen-presenting cells containing bacterial peptidoglycan in synovial tissues of rheumatoid arthritis patients coexpress costimulatory molecules and cytokines. Arthritis Rheum 2000;43:2160–8.
17. Van der Heijden IM, Wilbrink B, Tchetverikov I, Schrijver IA, Schouls LM, Hazenberg MP, et al. Presence of bacterial DNA and bacterial peptidoglycans in joints of patients with rheumatoid arthritis and other arthritides. Arthritis Rheum 2000;43:593–8.
18. Huang QQ, Sobkoviak R, Jockheck-Clark AR, Shi B, Mandelin AM II, Tak PP, et al. Heat shock protein 96 is elevated in rheumatoid arthritis and activates macrophages primarily via TLR2 signaling. J Immunol 2009;182:4965–73.
19. Midwood K, Sacre S, Piccinini AM, Inglis J, Trebaul A, Chan E, et

al. Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. Nat Med 2009;15:774–80.
20. Kuhns DB, Priel DA, Gallin JI. Induction of human monocyte interleukin (IL)-8 by fibrinogen through the Toll-like receptor pathway. Inflammation 2007;30:178–88.
21. Smiley ST, King JA, Hancock WW. Fibrinogen stimulates macrophage chemokine secretion through Toll-like receptor 4. J Immunol 2001;167:2887–94.
22. Clemmensen I, Andersen RB. Properties of fibrinogen-antigenic material on the rheumatoid synovial membrane and in the rheumatoid synovial fluid. J Lab Clin Med 1978;92:678–89.
23. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
24. Akira S, Hoshino K, Kaisho T. The role of Toll-like receptors and MyD88 in innate immune responses. J Endotoxin Res 2000;6: 383–7.
25. Nimmerjahn F, Ravetch JV. Fc-receptors as regulators of immunity. Adv Immunol 2007;96:179–204.
26. Masson-Bessiere C, Sebbag M, Girbal-Neuhauser E, Nogueira L, Vincent C, Senshu T, et al. The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the α- and β-chains of fibrin. J Immunol 2001;166:4177–84.
27. Roelofs MF, Boelens WC, Joosten LA, Abdollahi-Roodsaz S, Geurts J, Wunderink LU, et al. Identification of small heat shock protein B8 (HSP22) as a novel TLR4 ligand and potential involvement in the pathogenesis of rheumatoid arthritis. J Immunol 2006;176:7021–7.
28. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the Toll-like receptor-4 complex. J Immunol 2000;164:558–61.
29. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, et al. Novel signal transduction pathway utilized by extracellular HSP70: role of Toll-like receptor (TLR) 2 and TLR4. J Biol Chem 2002;277:15028–34.
30. Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, et al. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. J Clin Invest 2005;115:2223–33.
31. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, et al. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem 2001;276:10229–33.
32. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, et al. Oligosaccharides of hyaluronan activate dendritic cells via Toll-like receptor 4. J Exp Med 2002;195:99–111.
33. Gulcher JR, Nies DE, Alexakos MJ, Ravikant NA, Sturgill ME, Marton LS, et al. Structure of the human hexabrachion (tenascin) gene. Proc Natl Acad Sci U S A 1991;88:9438–42.
34. Ho PP, Lee LY, Zhao X, Tomooka BH, Paniagua RT, Sharpe O, et al. Autoimmunity against fibrinogen mediates inflammatory arthritis in mice. J Immunol 2010;184:379–90.
35. Tishler M, Shoenfeld Y. Anti-heat-shock protein antibodies in rheumatic and autoimmune diseases. Semin Arthritis Rheum 1996;26:558–63.
36. Monach PA, Hueber W, Kessler B, Tomooka BH, BenBarak M, Simmons BP, et al. A broad screen for targets of immune complexes decorating arthritic joints highlights deposition of nucleosomes in rheumatoid arthritis. Proc Natl Acad Sci U S A 2009;106:15867–72.
37. Vossenaar ER, van Venrooij WJ. Citrullinated proteins: sparks that may ignite the fire in rheumatoid arthritis. Arthritis Res Ther 2004;6:107–11.
38. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 2002;416:603–7.
39. Boule MW, Broughton C, Mackay F, Akira S, Marshak-Rothstein A, Rifkin IR. Toll-like receptor 9–dependent and –independent dendritic cell activation by chromatin–immunoglobulin G complexes. J Exp Med 2004;199:1631–40.
40. Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. J Clin Invest 2005;115: 407–17.
41. Marshak-Rothstein A, Rifkin IR. Immunologically active autoantigens: the role of Toll-like receptors in the development of chronic inflammatory disease. Annu Rev Immunol 2007;25: 419–41.
42. Clavel C, Nogueira L, Laurent L, Iobagiu C, Vincent C, Sebbag M, et al. Induction of macrophage secretion of tumor necrosis factor α through Fcγ receptor IIa engagement by rheumatoid arthritis–specific autoantibodies to citrullinated proteins complexed with fibrinogen. Arthritis Rheum 2008;58:678–88.
43. Okumura N, Haneishi A, Terawasa F. Citrullinated fibrinogen shows defects in FPA and FPB release and fibrin polymerization catalyzed by thrombin. Clin Chim Acta 2009;401:119–23.
44. Vanags D, Williams B, Johnson B, Hall S, Nash P, Taylor A, et al. Therapeutic efficacy and safety of chaperonin 10 in patients with rheumatoid arthritis: a double-blind randomised trial. Lancet 2006;368:855–63.